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Effectiveness of Flower Extract of *Hibiscus sabdariffa* L. against Anticancer Drug Cyclophosphamide Induced Hepatotoxicity and Oxidative Stress

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Abstract

Cyclophosphamide (CP) is an alkylating agent that has been used extensively in medicine as an antineoplastic agent for the treatment of several tumors. Treatment with CP is associated with significant toxicity due to overproduction of reactive oxygen species (ROS) and free radicals resulting in increased levels of oxidative stress (OS) as well as hepatotoxicity. The *Hibiscus sabdariffa L.* Extract (HSE) was given to mice as an antineoplastic agent (CP) at a dose of 250 mg/kg body weight daily for 2 weeks. The degree of liver injury was analysed using several serum parameters including AST, ALT, GGT, total protein and albumin contents. Lipid peroxidation (LPO) which reflects oxidation stress was established by determining catalase and superoxide dismutase in liver homogenate. The biochemical results showed that the administration of CP induced hepatic damage was associated with a significant increase in serum marker enzymes (AST, ALT and GGT) and a decrease in total protein and albumin. In addition, oxidative stress in the liver was also increased, and this was evident from the increase in LPO. In this case MDA , GSH content activities of CAT and SOD were increased. Furthermore, the hepatic cytotoxicity induced by CP treatment was substantiated by the reduction of nucleic acids and protein levels in hepatic cells. Flower extract of HSE administration (250mg/kg) daily improved liver functions and prevented the CP induced hepatocellular injury and oxidative stress. These data presented herein showed that HSE had a protective property against the harmful effects of CP, suggesting that *H. sabdariffa* has the potential to be used as a new therapeutic approach for the treatment of hepatic disorders.

Keywords: *Hibiscus sabdariffa,* extract, cyclophosphamide, oxidative stress, cytotoxicity, hepatotoxicity.

1. Introduction

The increasing demand for nutraceuticals has enhanced attempts to develop new approaches for the treatment of human disease. *Hibiscus sabdariffa* that grows in many countries has many bioactive components as flavonoids (gossiping, sabdaretine, hibicitine, and anthocyanins), phenolic acids (e.g. protocatechuic acid) and rich in vitamin C content (Lin et al., 2011; Pietta, 2000). It was reported that this herb exhibits anticancer, antiseptic, antidepressant and antipyretic activities (Duke, 1985), anti-inflammatory (Dafallah and Al-Mustafa, 1996), anti-mutagenic effect (Farombi and Fakoya, 2005), antidiabetic, and also jaundice and ulcer treatment properties (Yeşilada et al., 1995). The plant was studied for its effect in hypolipidemia (Hirunpanich et al., 2006), immune protection, cytotoxicity (Okoko and Ere, 2012), atherosclerosis, cardiovascular diseases and diabetes (Farombi and Ige, 2007). HSE has a broad range of pharmacological activities, particularly used as free radical scavenging agent (Oboh and Okhai, 2012) and has antioxidant properties (Olusola et al., 2012) . The health benefits of HSE has attracted scientific interest, and this resulted in several papers published in the last few years. Antioxidants and free radical scavenging are often mentioned as the mechanism leading to the protective benefit of HSE. Antioxidants and anthocyanins were found to be effective hepatoprotective agents against cadmium-induced liver injury in rats (Al-Kubaisy et al., 2016). Acute liver damage can be induced by carbon tetrachloride (CCl4) (Liu et al., 2006). Liu et al. (2010) found that significant protection against acetaminophen-induced liver damage in rats can be achieved using HSE. An investigation conducted by Al-Groom and Al-kubaisy (2016) showed that cadimium chloride ($CdCl_2$) can induce hypochromic microcytic anemia and oxidative stress in rat red blood cells. It was also found that the flower extract of *H. sabdariffa* was proven to be an effective agent against $CdCl_2$-induced depletion of rat hepatic antioxidant system and increase in malondialdehyde (MDA) (Khaled et al., 2016). The supplementation of floral extract partly affects the toxic effect of $CdCl_2$ on oxidative stress and repairs liver tissue. The constituents of *H. sabdariffa* were found to be effective in immune modulation, cholesterol lowering and overall health assistance particularly in cancer treatment. (De Jong *et al.,*2003)

The objective of this study was to determine the effectiveness of flower extract of *Hibiscus sabdariffa* L. in alleviating the side effects of anticancer drug

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Cyclophosphamide (CP) in relation to hepatotoxicity and oxidative stress.

2. Material and Methods

2.1. Preparation of aqueous flower extract (HSE)

H. sabdariffa L. (Rossle) was obtained from a general herbal store in Amman. A botanist from the University of Jordan identified the sample of the plant specimen. The flower was removed and ground with an electric dry mill in to fine powder. An aliquot 100 g ground powder was soaked in 500 ml of distilled water while shaking for 24 hours at 40°C. Subsequently, the sample was filtered through Whatman filter paper No.1 in distilled water. The filtrate was boiled to produce concentration of 250mg/kg when given to the livestock.

2.2. Experimental animals

A combination of 18 healthy albino (20 to 25 g) were obtained from the Al-Ahlyyiah-Amman University and were used throughout this study. Free access to food, water and libitum was permitted for all. The animals were randomly split into three groups of six: A: Group1: Served as control that was treated with corn oil vehicle. B: Group2: Mice were injected with single dose of CP (75 mg/kg) and were used for comparison. C: Group3: Mice were treated with 250mg/kg body weight of (HSE) for two weeks daily and subsequently exposed to a single injection (75mg/kg) of CP, two hours after the last (HSE\vehicle) treatment (Srivastava et al., 2021). All animals were treated in accordance with the rules of the Animal Ethics Committee organizations. Analytical grade chemicals were used throughout this investigation.

2.3. Blood collection

At the end of the testing period animals were subject to etheric anesthesia. Cardiac puncture was conducted to collect the blood which was transferred to EDTA tubes. Then, the samples were centrifuged at 3500 rpm for 15 minutes.

2.4. Blood biochemistry

Serum enzymes: aspirate transaminase (AST), gamma glutamate transaminase (GT), alanine transaminase (ALT), complete protein and albumin were tested on the same day in compliance with the instructions given by the kits manufacture (Randox-UK).

2.5. Hepatic tissue biochemical testing

The mice were sacrificed and their livers were excised and then washed with ice-cold saline and blotted to dryness; samples of liver tissue were homogenized with ice-cold (0.25 M sucrose).

2.6. Assessment of cellular damage (Lipid peroxidation)

The level of lipid peroxides (LPO) served as an index of the intensity of oxidative stress (OS). Malondialdehyde (MDA) was determined in the liver homogenate as described by (Al-Kubaisy et al., 2016), after incubation at 95 °C with thiobarbituric acid to produce pink color. This color has an absorption maxima at 532 nm.

2.7. Estimation of oxidative stress

Estimation of total reduced glutathione (GSH) was determined in the liver homogenates by the method of Ellman (Ohkawa et al., 1979). Yellow color developed when 5.5 dithiol-bis 2 nitrobenzoic acids was added to the supernatant. The intensity of this color was measured by spectrophotometrically at a wavelength of 412 nm against reagent blank with no homogenate. Estimation of catalase (CAT) activity was performed by following the method described by (Beutler, 1963). Catalase activity was determined spectrophotometrically utilizing H_2O_2 as a substrate at the wavelength of 240 nm. The homogeneity of superoxide dismutase (SOD) was estimated by the method described by Johansson and Borg, (1988). This activity was measured under alkaline condition at 325 nm.

2.8. Quantification of nucleic acids and total protein in liver homogenate.

The hepatic homogenate was prepared in cold and hot perchloric acid ($HCLO_4$). The supernatant was used to determine the concentrations of RNA, DNA and total proteins after final extraction, incubation and centrifugation. The RNA was obtained after treating the nucleic acid extract with orcinol reagent. The resultant solution was green in color. The intensity of the color was measured spectrophotometrically at 660 nm. (Kakkar et al., 1984). The diphenylamine extract of hepatic homogenate with the developed blue color was determined using spectrophotometer at wavelength of 600 nm and this reading indicated DNA concentration (Patterson and Mura, 2013). Total protein was determined by the method using the folin-ciocalteu reagent at 750 nm spectrophotometrically (El-Nekeety et al., 2014; Lowry et al., 1951).

2.9. Statistical analysis

Data were expressed as mean ± standard deviation. One-way analysis variance (ANOVA) with the software SPSS version (SPSS version 20) was used. Values below 0.05 ($P<0.05$) were considered as significant.

3. Result

3.1. Hepatic biochemical markers

The activity of AST, ALT and GGT in the serum of the control and experimental animals are summarized in Table 1. The activity of these enzymes was significantly increased ($P<0.05$) after injection of Cyclophosphamide (CP) as compared to livestock's in group 1 and 3. Pretreatment of group 3 animals with HSE significantly ($P<0.05$) reduced the levels of these enzyme markers as compared to group 2. The mean values of total protein and albumin in the serum of control and experimental mice were given in Table 1. Decrease in the values of the biochemical markers was observed in the serum of mice after administration of CP ($P<0.05$). However, these values relatively increased in group 3, but never reached the value obtained in group 1.

3.2. Effect on lipid peroxidation and oxidative stress in hepatic cells

Table 2 indicates that mice exposure to Cyclophosphamide resulted in hepatic oxidative stress. This study found a significant increase in hepatic tissue in case of (MDA) after CP exposure. HSE supplementation led to substantial lipid peroxidation (LPO) improvement in group 3. Furthermore, a significant decrease in total glutathione content in liver homogenate ($P<0.05$) was recorded throughout the period of treatment with CP. However, the supplementation with HSE (group3) resulted in a significant elevation in the level of GSH when compared to CP-treated (group 2). The antioxidant enzymes (CAT) and (SOD) in the liver were depleted

significantly when the livestock were treated with CP alone. The activity of these enzymes showed significant protection following treatment with HSE.

Table 1. Effect of pretreatment of HSE on serum biochemical's response of mice to CP

Parameter	Group 1	Group 2	Group 3
AST U/L	61.48 ± 1.81	108.72 ± 3.11 *	81.93 ± 2.98 *
ALT U/L	36.64 ± 1.27	74.75 ± 2.88	57.97 ± 3.77 *
GGT U/L	13.25 ± 1.61	19.23 ± 1.71 *	15.44 ± 1.66 *
T. protein mg/dL	6.69 ± 0.53	4.82 ± 0.66 *	5.59 ± 0.71 *
Albumin mg/dL	4.41 ± 0.78	2.18 ± 0.27*	3.32 ± 0.48*

Results expressed as Means ± SE. for six mice. Comparisons are made between livestock inoculated as follows:
Group 1: mouse treated with corn oil (normal control)
Group 2: mouse treated with corn oil + CP
Group 3: mouse treated with corn oil+ CP+ HSE
(*P <0.05).

Table 2. Effect of pretreatment of HSE on hepatic lipid peroxidation GSH, catalase and superoxide dismutase (SOD) response of mice to CP

Groups	MAD nmol/g liver	GSH nmol/g liver	CAT u/g liver	SOD u/g liver
Group 1	192.40 ± 4.33	71.82 ±2.78	81.52 ± 4.12	73.43 ± 4.87
Group 2	245.72 ± 6.18 *	52.44 ±.18 *	57.28 ± 3.30 *	43.19 ±3.72 *
Group 3	213.85 ± 3.78*	65.61 ±3.11*	68.82 ± 3.61*	61.57 ±4.17 *

Results are expressed as Means ± SE. Comparisons are made between livestock inoculated as follows:
Group 1: mouse treated with corn oil (normal control)
Group 2: mouse treated with corn oil + CP
Group 3: mouse treated with corn oil+ CP+ HSE
(*P <0.05).

3.3. Effect on nucleic acids and proteins levels in hepatic cell's DNA and RNA levels in hepatic cells

Treatment of the experimental animals with CP caused drop in the values of DNA, RNA, and protein. However, when the livestock was treated with CP and HSE the readings of preceding parameters were significantly increased (Table3), they never reached the values obtained for the control group.

Table 3. Effect of pretreatment of HSE on the protein and nucleic acid levels in hepatic cells response of mice to CP

Group	DNA mg/100mg	RNA mg/100mg	protein mg/100mg
Group 1	211.66 ±341	675.22 ±7.91	13.67 ±0.63
Group 2	188.72 ± 4.51 *	563.41 ±9.82*	11.27 ±.37*
Group 3	194.18 ± 5.71 *	639.51 ± 11.18 *	12.10 ± 0.43

Results are expressed as Means ± SE. Comparisons are made between livestock inoculated as follows:
Group 1: mouse treated with corn oil (normal control)
Group 2: mouse treated with corn oil + CP
Group 3: mouse treated with corn oil+ CP+ HSE
(*P <0.05).

4. Discussion

Protective medicines like antioxidants have become increasingly attractive for hepatitis therapeutic illnesses. In this case, scavenging is the major antioxidant mechanism (Lowry et al., 1951). Antioxidant supplementation could affect chemotherapy reactions as well as negative side impacts resulting from antineoplastic agent therapy. These agents have improved toxicity which results from the overproduction of free radicals and reactive oxygen species (ROS) (Ferramosca et al., 2017). CP is an alkylating drug used commonly to treat cancer worldwide (Pramita et al., 2009). Increases peroxidation of the cell membrane and damages many cellular structures. Therefore, many scientists are engaged in finding natural compounds to minimize the negative impacts of CP-induced toxicity. Many crops obtained from natural products can be hepatoprotective to different chronic diseases of the liver. The free radicals and ROS include a range of reactive molecules capable of oxidizing the cell. Oxidative stress may be one of the manifestations of cellular damage in the toxicity of drugs and chemical toxins. Lipid is regarded as a membrane integrity-monitoring index. MAD is commonly used as a lipid peroxidation marker (LPO). The levels of MDA were significantly higher animals treated with CP as compared to the control group. The preventive impact of the HSE administration was noted and tabulated in Table 2. It can be seen from this table that readings were considerably lower and associated with decreased level of MDA. Previous studies demonstrated that HSE is an effective antioxidant and free radical scavenger *in vivo* and *in vitro* conditions. Reduced glutathione is the most abundant antioxidant in hepatic cells, and has a key function in defending tissue oxidation (Franco et al; 2008). In the present study, the depletion in the antioxidative status of the liver was moderately corrected by the use of HSE (Table 2). The reduction of CP in group 2 resulted in the reduction of glutathione by the oxidation of GSH to oxidised glutathione (GSSG). Treatment of the control group inoculated with CP and HSE resulted in an increase of liver GSH as compared to the control group given CP only. The main body defense employs antioxidant enzymes (CAT & SOD), which protects against cell-generated reactive oxygen species (ROS). The plant chemistry assessment of the Hibiscus floral extract revealed the presence of four significant flavonoids; sabdariffa, protocatechuic acid, anthocyanins and vitamin C (Liu et al., 2010). The antioxidant and hepatoprotective molecules activate certain plant-based flavonoids (Olusola et al., 2012;). The relative elevated concentration of polyphenol in floral crude extract of HSE indicates potential antioxidants (Al Groom & Al-Kubaisy, 2016). The DPPH radical scavenging action of HSE was more than vitamin C (Bansal & Simon, 2018). It is reported that the crude extract of HS flowers has total phenolic content of (77-87 mg\g) (Al-Hashimi, 2012). In the current study, CP treatments of the mice resulted in substantial liver damage. This damage is evident from the elevated serum marker activity (ALT, AST, GGT) reduced T. protein and albumin in the mice (Table 1). HSE dose of 250 mg enhanced antioxidant strength (Table 2). These markers were used to signify hepatocellular injury, because they

were situated in the cytoplasm and released into blood circulation after the cell membrane was ruptured. HSE therapy showed a significant protective action against CP-induced hepatotoxicity and modified complete protein and albumin concentration (Table 1).

Herein, the activity of these antioxidant enzymes in the liver tissue was lowered by CP administration. The reduction in the concentrations of these enzymes could be linked to the inactivation of the free radicals, which occurred while CP was metabolized. *H. sabdariffa* floral extract administration has considerably improved the operations of the enzyme by scavenging ROS, as well as the lipid peroxidation that inhibited HSE antioxidant characteristics. Flower extracted compounds showed antioxidant effect against oxidative stress in red blood cells (Al-Groom and Al-Kubaisy, 2016). This study revealed that HSE possesses a potent effect by reducing the resistance to oxidation stress. Damaged markers, and antioxidants including (enzymatic and non-enzymatic) showed that HSE could reduce CP-induced oxidative stress. Administration of free radical scavenging antioxidants can alleviate hepatotoxicity. Stimulation of ribosomal RNA polymerase and protein synthesis can lead to enhanced hepatocyte regeneration (Bhaargavi et al., 2014). HSE works effectively to scavenge ROS and detoxify free radicals produced during metabolic activities of CP. The activity of HSE was estimated to be equivalent to a dose of silymarin drug 20 mg/kg (Jain et al., 2012). Moreover, results obtained in the present study showed that CP-treatment produced a reduction in the DNA, RNA and protein content in hepatic cells. This indicated a significant cytotoxic effect. The treatment of mice with HSE significantly improved these biochemical parameters. One of the mechanisms that can explain the capacity of HSE to stimulate liver tissue regeneration is the increase DNA synthesis, ribosomal RNA, as well as protein synthesis in the injured hepatocytes. The available data suggest that flavonoid contents of HSE might be responsible for these activities. Our finding supports prior studies, particularly the work conducted by Al-Hashimi (2012).

5. Conclusion

Hibiscus sabdariffa L. Extract has significant inhibition of oxidative stress induced by CP. The use of herb resulted in the improvement of hepatocellular injury. Hence, it can be used as adjuvant therapy for preventing the side effects associated with chemotherapy.

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Conflict of Interest

the author declares that there is no conflict of interests.

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