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The ameliorative effect of Saussurea costus root extract supplementation against cyclophosphamide-induced anemia in albino rats

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ABSTRACT

The current investigation sought to determine the impact of Saussurea costus (S. costus) root extract (SCRE) supplementation on cyclophosphamide (CTX)-induced anemia. Forty male albino rats were separated into four groups (10 rats each): Group I (control), Group II (SCRE), Group III (CTX), and Group IV (CTX+SCRE). Blood and femoral bones were collected after the end of the experiment. The following parameters were measured; hematological parameters, erythrocyte oxidative biomarkers, erythrocyte osmotic fragility, and plasma and erythrocyte Na^+, K^+ and Mg^{2+} ions levels. Moreover, the histopathological investigation was conducted. The results revealed that the CTX significantly reduced all hematological parameters and bone marrow cellularity. CTX also altered erythrocyte oxidative biomarkers, shifting the osmotic fragility curve to the right and disrupting plasma and erythrocyte Na^+, K^+ and Mg^{2+} ions levels. With the supplementation of SCRE, all these parameters improved. So, it could be concluded that CTX-induced anemia resulting from myelosuppression and erythrocyte damage caused by oxidative stress could be mitigated by Saussurea costus root extract (SCRE).

1. INTRODUCTION

Chemotherapy is a potent cancer treatment that kills fast-growing cancer cells (Skverchinskaya et al., 2023). Cyclophosphamide (CTX) is a common, non-specific chemotherapeutic drug. It can't distinguish cancer cells from other highly proliferating cells, such as bone marrow hematopoietic cells (Nawa-Nishigaki et al., 2018). Nevertheless, the most frequent side effects of CTX include hematological damage (Liu et al., 2021), bone marrow suppression (Zhu et al., 2016), and immunosuppression (Chen et al., 2012). Cyclophosphamide is a nitrogen-alkylating agent that is utilized in the treatment of a range of human malignancies and as immunosuppressive medication following organ transplantation. It's also used to treat autoimmune diseases such as nephritic syndrome in children, Wegener's granulomatosis, and rheumatoid arthritis (Patra et al., 2012; Meotti et al., 2013). CTX must be converted into phosphoramide mustard (PM) and acrolein by the liver's cytochrome P450 enzyme (Dixit et al., 2022). PM causes CTX cytotoxicity (Khan et al., 2014). Acrolein impairs DNA transcription and generates ROS which exacerbate oxidative DNA destruction (Oboh et al., 2012). Thus, acrolein causes myelosuppression and hematotoxicity (Kawabata et al., 1990). Myelosuppression manifests via direct injury to hematopoietic stem cells. Consequently, erythropoiesis is suppressed, causing anemia as well as leukopenia and thrombocytopenia were also observed (Livshits et al., 2014). Additionally, CTX aggravates erythrocyte damage via oxidative stress (Akamo et al., 2021)**.** That is why 70% of chemotherapy patients develop anemia (Bryer and Henry, 2018)**.** Whereas the etiopathogenetic mechanisms of chemotherapy-induced anemia (CIA) are deficient red blood cell generation (impaired erythropoiesis) and raised destruction (hemolysis) (Madeddu et al., 2021). The CIA reduces a person's quality of life by causing fatigue. As a result, to limit these adverse effects, chemotherapy is delayed or reduced in dosage, which hinders cancer treatment (Bryer and Henry, 2018)**.**

Phytochemicals serve as potent antioxidants via scavenging free radicals, boosting the intracellular antioxidant system, and inhibiting of the proapoptotic signal pathway; consequently, they possess considerable capacity for protecting against chemotherapeutic drug- and irradiation-induced oxidative damage and associated adverse reactions (Liu et al., 2021). Saussurea costus (Falc.) Lipschitz is synonymous with Saussurea lappa. C.B. Clarke (Nadda et al., 2020). It is one of the medicinal herbs abundant in antioxidants (Saleem et al., 2013). In traditional medicine, dried S. costus roots have been used in folk medicine to treat a wide range of diseases and ailments, including asthma, cough, throat infections, tuberculosis, dyspepsia, diarrhea, ulcers, and rheumatism (Hassan and Masoodi, 2020). The roots contain many chemical components, mainly sesquiterpene lactones like costunolide and dehydrocostus lactone, which have numerous biological functions, involving immunostimulant, anti-inflammatory, anti-tumor, anti-ulcer, and antioxidant activities (Ali and Venkatesalu, 2022). In addition to sesquiterpene lactones, its polyphenols, flavonoids, triterpenes, and steroids improve its antioxidant defenses (Singh et al., 2017). Hence, it prevents oxidative damage from toxicants such as triamcinolone (Abd El-Rahman et al., 2020) and chlorpyrifos ethyl (Deabes et al., 2021). Thereby, this work was conducted to explore the outcome of SCRE

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supplementation as antioxidant agent against CTX-induced anemia that resulting from myelosuppression and erythrocyte oxidative damage.

2. MATERIAL AND METHODS

2.1. Drugs:
Cyclophosphamide, CTX (Endoxan®), was acquired from Baxter Oncology, Halle, Germany.

2.2. Extraction procedures:
S. costus roots were purchased from Haraz, Cairo, Egypt. The air-dried roots were pulverized and stored in sachets. 1 kg of the powder was mixed with 2300 ml ethanol 70% and sonicated for 30 minutes. filtrating after one day of maceration. This was done twice more. The filtrate was gathered and dehydrated beneath vacuum at 50 °C using a rotary evaporator. Dark brown residues weighing 273.1g were produced and stored at 4 °C until use (Guccione et al., 2017).

2.3. Gas Chromatography–Mass Spectrometry Analysis.
According to El-Kareem *et al.* (2016) the SCRE 's phytochemical constituents were analyzed.

2.4. Phytochemical analysis:
SCRE 's phytochemical analysis followed procedures developed by Attard (2013) for total phenolic content (TPC), Kiranmai, et al. (2011) for total flavonoids content (TFC), Boly et al. (2016) for the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity, Arnao et al. (2001) for the ABTS+ (2,2'azinobis-(3-ethylbenzo-thiazoline-6-sulfonate) radical scavenging activity, and Benzie and Strain (1996) for ferric reducing activity power (FRAP).

2.5. Animals:
Forty male albino rats, 190±10 g in weight, 8–9 weeks old, were used in this work. The rats were procured from Egyptian holding company for biological products and vaccines (VACSERA in Giza, Egypt). Rats were acclimatized for 7 days before starting the experiment under standard housing conditions. They resided in separate, clean stainless-steel cages and were stayed at temperature (25 ± 1 °C) and relative humidity (50 ± 5%) under 12 h light/dark cycles in the animal house at the faculty of veterinary medicine, Benha University. Rats were granted unlimited access to typical pellet food and water. The current study's experimental design was ethically authorized by the Ethics Review Committee of the Faculty of Veterinary Medicine, Benha University, Egypt. (Approval number: BUFVTM15-03-23).

2.6. Experimental design:
Within current experiment subsequently the acclimation period, forty male albino rats were categorized into four groups, (10 animals per group) as follows: Group I (control); rats received distilled water orally once each day for 30 days. Group II (SCRE); SCRE (600 mg/kg b.wt.) was supplemented orally daily for 30 days (Abd El-Rahman et al., 2020). Group III (CTX); CTX was administered intraperitoneally (40 mg/kg b.wt. dissolved in saline) on day 22, 25, and 28 of the experiment (Bao et al., 2021; Zhu et al., 2021). Group IV (CTX+SCRE): SCRE (600 mg/kg b.wt.) was supplemented orally once a day for 30 days, and on day 22, 25, and 28 of the experiment, CTX (40 mg/kg b.wt.) was administered intraperitoneally.

2.7. Sampling:
After the experiment, blood samples and femoral bones were collected. Rats were anaesthetized by inhaled isoflurane 100% after an overnight fast, and blood was taken from the retro-orbital venous plexus. The blood was drawn into EDTA vials for hematological parameters. Other blood samples were collected into heparinized vials for osmotic fragility, oxidative stress biomarkers, and plasma and erythrocyte Na^+, K^+, and Mg^{2+} ions levels determination. Once the blood was sampled, all animals were sacrificed via decapitation. Femoral bones were taken for bone marrow histopathology.

2.8. Hematological parameters:
Erythrocyte count (RBC), total leukocyte count (WBC), platelet count, hemoglobin concentration (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were determined using an auto hematology analyzer (Genrui KT-6400 Shenzhen, China).

2.9. Erythrocyte oxidative biomarkers determination:
Catalase (CAT) activity, superoxide dismutase (SOD) activity, total antioxidant capacity (TAC), and malondialdehyde (MDA) levels were measured according to Aebi (1984), Nishikimi et al. (1972), Koracevic et al. (2001), and Ohkawa et al. (1979) respectively in hemolysate. Working with diagnostic kits in accordance with the manufacturer's instructions (Bio-diagnostic Company, Dokki, Giza, Egypt).

2.10. Erythrocyte osmotic fragility test:
Osmotic fragility of erythrocyte was detected using the method outlined by Faulkner and King (1970) and adjusted by Oyewale (1991).

2.11. Plasma and erythrocytes Na^+, K^+ and Mg^{2+} ions level measurement:
Plasma Na^+ and K^+ ions levels were measured according to Tietz (1976), while plasma Mg^{2+} ion level according to Thomas (1998). Erythrocyte Na^+, K^+ and Mg^{2+} ions levels were determined according to Malon and Maj-Zurawska, (2005). Working with diagnostic kits in accordance with the manufacturer's instructions (Spectrum Diagnostics Company, Cairo, Egypt)

2.12. Histopathological study:
Femoral bones were sliced to 3–4 mm thick, fixed in 10% neutral buffered formalin, decalcified with 10% formic acid, dehydrated in ascending concentrations of ethanol, cleared in xylene, and embedded in paraffin. To analyze general tissue structure, paraffin blocks were microtome-sectioned at 4–6 µm thickness and stained with H&E by Bancroft and Stevens, (2013).

2.13. Statistical analysis:
The statistical analyses were done using SPSS (Version 26, SPSS Inc., Chicago, USA). Using one-way ANOVA followed by the Duncan test as a post hoc to determine the statistically significant difference between groups. The data are presented as means ± SE, with significance set at $P< 0.05$.

3. RESULTS

3.1. Gas Chromatography–Mass Spectrometry Analysis.
GC/MS analysis showed the main components of SCRE. According to the obtained result, the extract had 22

components, the most notable of which were; Dehydrocostuslactone (45.53%), 2(3H)-Benzofuranone,6-ethenylhexahydro-6-methyl-3-methylene-7-(1 methyl ethenyl)-, [3aS-(3aà,6à,7á,7aá)])-(17.86%), β-Costol (7.29%), 1, 8, 11, 14 Heptadecatetraene, (Z, Z, Z)-(6.47%), Dihydro dehydrocostus lactone (5.31%) and Costunolide (2.56%) as shown in Table 1.

Table 1. Gas Chromatography–Mass Spectrometry Analysis of Saussurea costus root extract.

Compound	Retention time (min)	Peak Area %	Molecular Formula	Molecular Weight
Elemene	14.60	0.22	C15H24	204
Caryophyllene	15.20	0.25	C15H24	204
Longipinene	16.87	0.25	C15H24	204
Alloaromadendrene	17.31	0.27	C15H24	204
Caryophyllene oxide	21.46	0.45	C15H24O	220
1,8,11,14-Heptadecatetraene, (Z,Z,Z)-	21.28	6.47	C17H28	232
Bergamotol, Z-à-trans-	22.22	0.13	C15H24O	220
γ-costol	23.0	0.25	C15H24O	220
β-Costol	23.63	7.29	C15H24O	220
Aromadendrene oxide-(2)	24.35	0.43	C15H24O	220
10-Heptadecen-8-ynoic acid, methyl ester, (E)-	24.50	0.40	C18H30O2	278
2(3H)-Benzofuranone, 6-ethenylhexahydro-6-methyl-3-met hylene-7-(1-methylethenyl)-, [3aS-(3aà,6à,7á,7aá)]	25.49	17.86	C15H20O2	232
HEXADECANOIC ACID, METHYL	26.54	1.22	C17H34O2	270
4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)-	26.81	0.84	C23H34O2	342
Dihydro dehydrocostus lactone	27.17	5.31	C15H20O2	232
Dehydrocostus lactone	29.36	45.53	C15H18O2	230
Reynosin	29.57	0.23	C15H20O3	248
Costunolide	30.20	2.56	C15H20O2	232
OCTADECANOIC ACID, METHYL ESTER	30.40	0.35	C19H38O2	298
5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)-	30.92	0.47	C21H34O2	318
9,12-Octadecadienoyl chloride, (Z,Z)-	31.48	7.94	C18H31Clo	298

RT, retention time

3.2. TPC, TFC, and the free radical scavenging capacity of Saussurea costus root extract.

TPC averaged 28.55 mg GAE/g and TFC 7.51 mg RE/g. The antioxidant capabilities of SCRE constituents were evaluated by DPPH•, ABTS+, and FRAP. From the obtained result, SCRE has antioxidant activity with average scavenging ability at 40.11 µM TE/mg, 217.36 µM TE/mg, and 62.85 µM TE/mg, respectively (Table 2).

Table 2 Total phenolics, flavonoids, and free radical scavenger activity of SCRE.

Parameter	Mean ± SE
TPC (mg GAE/g)	28.55 ±2.13
TFC (mg RE/g)	7.51± 0.34
DPPH (µMTE/mg)	40.11 ±1.13
ABTS (µMTE/mg)	217.36 ±5.65
FRAP (µMTE/mg)	62.85±3.33

TPC, total phenolics content; TFC, total flavonoids content; DPPH• , 1,1-diphenyl-2-picrylhydrazyl; ABTS+, 2, 2'azinobis-(3-ethylbenzothiazoline-6-sulfonate); FRAP, ferric reducing antioxidant power. Values are expressed as mean ± SE.

3.3. The effect of CTX and/or SCRE on hematological parameters

Compared to the control rats, CTX administration decisively (P<0.05) reduced RBC, WBC, PLT counts, Hb concentration, and PCV (Fig. 1). When compared to control rats, WBC count increased significantly (P<0.05). Conversely, SCRE supplementation caused an immense (P<0.05) rise in the hematological parameters of CTX-administrated rats, however, all parameters were still drastically (P<0.05) less than those of control rats except PLT count.

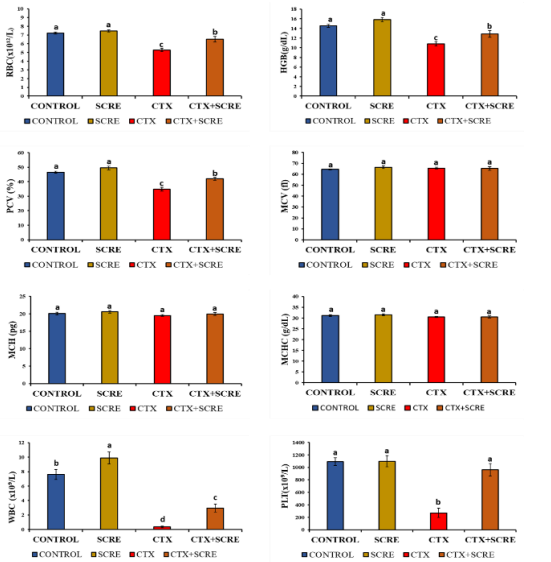

Figure 1. Effect of CTX and/or SCRE on hematological parameters. Data presented as a mean ± SE. different Superscript letters reveal a statistically significant difference (P<0.05).

3.4. The effect of CTX and/or SCRE on erythrocyte oxidative biomarkers

CTX administration considerably (P<0.05) decreased the activities of CAT, SOD, and TAC, while substantially (P<0.05) increased MDA levels, in comparison to the control group (Fig. 2). Contrary, SCRE supplementation markedly (P<0.05) boosted CAT, SOD, and TAC activities while tremendously (P<0.05) decline MDA levels of CTX-administrated rats. But all these values still dramatically (P<0.05) differed from the control group.

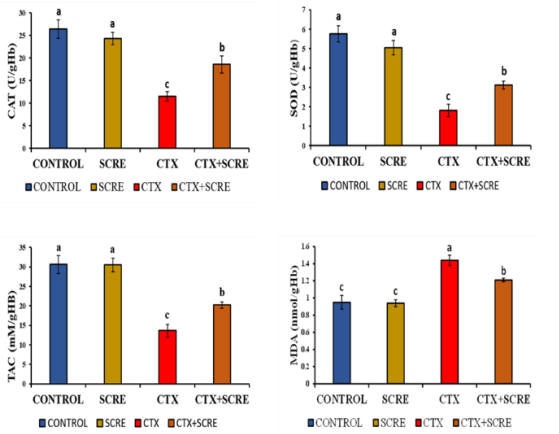

Figure 2. Effect of CTX and/or SCRE on erythrocyte oxidative biomarkers. Data reported as a mean ± SE. different Superscript letters reveal a statistically significant difference (P<0.05).

3.5. The effect of CTX and/or SCRE on erythrocyte osmotic fragility

The CTX group's osmotic fragility curve (OFC) shifted to the right, showing that erythrocyte hemolysis was considerably (P<0.05) greater than in the control, SCRE, and CTX+SCRE cotreated groups (Fig. 3). The CTX+SCRE cotreated group's erythrocyte osmotic fragility curve shifted to the left, showing a substantial (P<0.05) drop in erythrocyte hemolysis, compared to the CTX group but still drastically (P<0.05) higher than the controls.

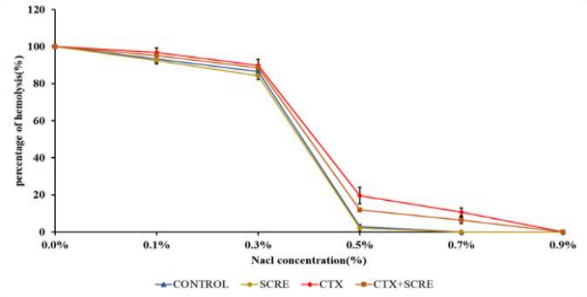

Figure 3. Osmotic fragility curve (OFC) of CTX and/or SCRE treated rats. Data reported as a mean ± SE. (P<0.05).

3.6. The effect of CTX and/or SCRE on plasma and erythrocyte Na^+, K^+ and Mg^{+2} ions levels

In the CTX-administrated group, PNa^+ was enormously (P<0.05) decreased, whilst PK^+ was considerably (P<0.05) increased, opposed to the control group. PNa^+ and PK^+ in the SCRE-supplemented group were not substantially (P>0.05) different from the control group (Fig. 4). Contrarily, compared to the CTX group, in the CTX+SCRE cotreated group, PNa+ was substantially (P<0.05) elevated whereas PK^+ was significantly (P<0.05) reduced. There was no discernible difference (P>0.05) in PMg^{+2} between control, SCRE, CTX, and CTX+SCRE groups.

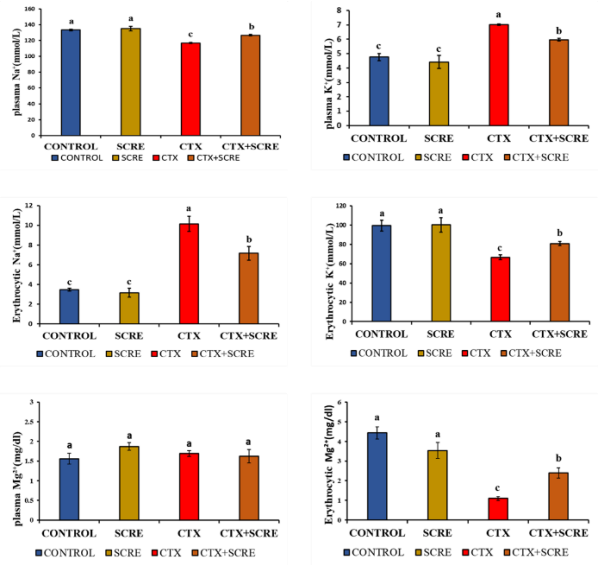

Figure 4. Effect of CTX and/or SCRE on plasma and erythrocyte Na^+, K^+ and Mg^{2+} ions levels. Data reported as a mean ± SE. different Superscript letters reveal a statistically significant difference. (P<0.05).

3.7. The effect of CTX and/or SCRE on bone marrow histopathology

The control group has bone marrow tissue with a normal histological structure with abundant densely packed bone marrow cells and a few adipocytes (Fig.5 A). The examined bone marrow of rats in SCRE-supplemented group showed a similar microscopic picture, like the control rat (Fig. 5 B). On the other hand, examined bone marrow in CTX-treated group was prominently hypocellular with an increase in adipose tissue more than cellular tissue compared with control group (Fig. 5C). Compared to the CTX-administrated group, the CTX+SCRE cotreated group exhibited significant improvement and a noticeable decrease in adipocytes, as well as a significant increase in bone marrow cells, this microscopic picture of bone marrow was nearly equivalent to the control group (Fig. 5D).

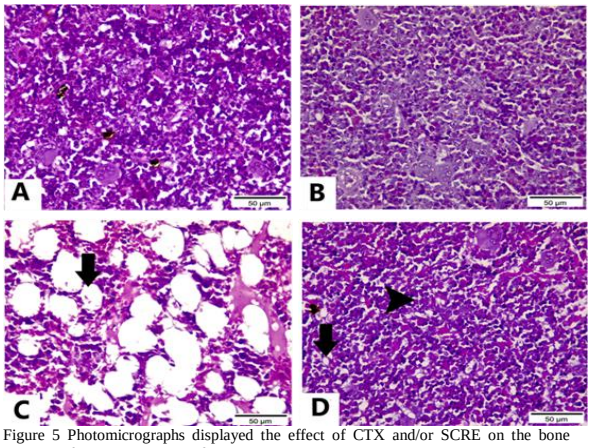

Figure 5 Photomicrographs displayed the effect of CTX and/or SCRE on the bone marrow of treated rats. (A) from the control group, the bone marrow has normal histological structure with densely packed cell distribution. (B) from the SCRE-supplemented group, exhibited the same assembly of normal structure as control group. (C) from the CTX- injected group, highlighted severe alterations, evidenced by the existence of large number of adipocyte (arrows) with obvious hypocellularity of bone marrow cells. (G) from the CTX+SCRE cotreated group showed great improvement and a discernible decline in the number of adipocytes (arrows), as well as a marked increase in bone marrow cells (arrowhead). Using H & E stain and Scale Bar= 50 µm for (A), (B), and (C), while Scale Bar= 200 µm for (D)

4. DISCUSSION

In this particular research, the GC-MS of SCRE revealed the existence of 22 components. These findings matched with Ali and Venkatesalu (2022). Along with its capacity to scavenge free radicals, the presence of the SCRE's total phenols (28.55 mg GAE/g) and total flavonoids (7.51 mg GAE/g) confirmed its antioxidant activity. That is due to phenolic compounds having antioxidant activity through their redox properties and playing a significant role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, and decomposing peroxides (Yingming et al., 2004). In addition, flavonoid compounds may contribute to this effect through scavenging or chelation processes (Tungmunnithum et al., 2018). This result agreed with Lee and Lim (2020), demonstrated that SCRE is rich in phenolic and flavonoid compounds and has excellent ABTS and DPPH radical scavenging capabilities, and ROS reduction potency.

Normal rats supplemented with SCRE showed higher WBC count compared to control rats this accord with Abd El-Rahman et al. (2020). This observation could be attributed to the stimulation of lymphoid tissue and increasing leukocytes by SCRE (Pandey, 2012).

In the current study, we observed an enormous decline in RBC, WBC, PLT counts, Hb concentration, and PCV in the CTX-administrated animals compared to the control animals. That agrees with Mirazi et al. (2021).That could be attributed to CTX causing bone marrow cells' DNA adducts and oxidative damage, which prevent DNA replication, produce myelosuppression, and decrease peripheral blood cells (Deng et al., 2018). SCRE supplementation to CTX-administered rats significantly raised all hematological parameters as compared to the

CTX-administrated rats. This is consistent with Deabes et al., (2021). According to Minhas (2017)**,** SCRE can protect DNA from damage due to its antioxidant properties. Therefore, SCRE increases hematopoiesis and peripheral blood cell count by reducing oxidative stress, which harms DNA in hematopoietic stem cells. MCV, MCH, and MCHC were similar across groups revealing that CTX promotes normocytic normochromic anemia. This finding is verified by El-Sebaey et al. (2019)**.** This could be attributed to CTX interfering with DNA synthesis, which stops normal tissue proliferation, including bone marrow cells, causing pancytopenia (Neboh and Ufelle, 2015).

Regarding oxidative biomarkers, CTX administration significantly increased MDA levels, while substantially decreased the activities of CAT, SOD, and TAC, compared to the control. Subramaniam and Devi (1994) support this result. This could be explained by Liu et al. (2021), who proposed that the accumulation of CTX-generated ROS in the cell depletes natural antioxidant enzymes. Moreover, high OH• amounts damage the lipid membrane, causing lipid peroxidation, as shown by the high MDA levels**.** With the supplementation of SCRE to CTX-administrated animals, MDA levels were markedly reduced, while CAT, SOD, and TAC activities were significantly boosted, compared to the CTX group. That could be attributed to SCRE's radical scavenging ability, which reduces ROS generation, preserves natural antioxidant enzymes, and suppresses MDA levels (Lee and Kang, 2020)**.**

Concerning osmotic fragility, OFC shifted to the right in the CTX group, indicating a significant increase in erythrocyte hemolysis. That could be attributed to the increase in lipid peroxidation and oxidative damage to the erythrocyte membrane (Buffenstein et al., 2001). Moreover, in the present work, an abrupt surge in MDA levels and an apparent reduction in the antioxidant activities of erythrocytes confirmed this result. With supplementation of SCRE, the OFC shifted to the left in the CTX+SCRE cotreated group, indicating a substantial decrease in erythrocyte hemolysis. This is due to SCRE reducing lipid peroxidation and so it protects the erythrocyte membrane against hemolysis.

In this study, the administration of the CTX significantly decreased PNa^+, whereas considerably increased PK^+ in compared with the control rats. The supplementation of the SCRE to CTX-administrated rats substantially elevated PNa^+ , while significantly reduced PK^+ compared to the CTX-injected group. In the erythrocytes, CTX-administrated animals exhibited a dramatic increase in ENa^+ but a significant decrease in EK^+ and EMg^{+2}, compared to the control group. The supplementation of the SCRE to CTX-administrated rats substantially decreased ENa^+, whereas markedly increased EK^+ and EMg^{2+} compared to the CTX-treated group. This disturbance of ions in the CTX-administrated rats agrees with Du et al., (2020). Jyothi et al. (2010) and Chauhan et al. (2002) could explain this by oxidative damage causing limitation of Na^+, K^+, and Mg^{2+}-ATPase activity. The result of the improvement in the ions distribution with the supplementation of SCRE to the CTX-administrated rats is in keeping with the investigation of Abd El-Rahman et al. (2020). This could be explained by de Souza et al. (2004) who mentioned that, costus may enhance Na^+, K^+-ATPase activity due to its antioxidant capability and enhance the Mg^{2+}-ATPase activity.

The changes in hematological parameters were confirmed with bone marrow histopathology. Administration of CTX revealed significant damage to bone marrow with increases in adipocytes, compared to control group, this matches Huang et al. (2020)**.** The supplementation of SCRE to CTX-administrated animals exhibited significant improvement in the microscopic picture of bone marrow with decreases in the adipocytes compared with CTX group. This suggests that SCRE protects bone marrow cells against CTX adverse effect. This is attributed to SCRE's antioxidant activity, which was verified by the GC-MS and radical scavenging ability measurements.

5. CONCLUSION

In conclusion, SCRE supplementation alleviates the altered hematological parameters resulting from myelosuppression and erythrocyte damage induced by cyclophosphamide through its antioxidant activities.

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