PROTECTIVE EFFECT OF *MORINGA OLEIFERA* LEAVES EXTRACTS AND SILIYMARIN ON HEPATIC TOXICITY INDUCED BY CYCLOPHOSPHAMIDE

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**ABSTRACT**

The leaves of *Moringa oleifera* plant are documented as active pharmaceutically agent with high antioxidant activity. In this study the protective effect of ethanolic leaves extract (ELE) and aqueous leaves extract (AQLE) of *Moringa oleifera* and Silymarin (SIL) were investigated against cyclophosphamide (CYP) injury on liver of rats. Fifty males of albino rats were divided into five groups, as follows: 1) control group - received distilled water orally; the second group was CYP group which injected with cyclophosphamide at dose of 150 mg/kg through i.p. to rats twice; one dose on the 15th day and another on the 30th day. Other 3 groups basically injected with CYP as a group 2 Moreover the 3rd group i.e. (ELE + CYP) group the rats were administered with ELE at dose 500 mg/kg/day orally through an intra-gastric tube for consecutive 14 days, before and after CYP injection. The 4th i.e. (AQLE+CYP group) the rats were given AQLE at dose 500 mg/kg/day orally for consecutive 14 days, before and after CYP injection. 5) SIL+CYP group - Rats were administrated with SIL at dose of 100 mg/kg/day by gavage for consecutive 14 days, before and after CYP injection. One day after the last treatment, blood samples and livers werewc . collected for histopathological investigations and biochemical determinations. The level of lipid peroxidation, thiobarbituric acid-reactive substances (TBARS), serum albumin, total protein, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) values were evaluated. TBARS, AST, ALT, ALP, and LDH in serum were elevated significantly increased (P<0.05) in CYP group, compared to control group, while total protein and albumin were decreased compared to control group and other groups. Histopathologically, it was observed hepatocytes damage in CYP induced group compared with the control group and other groups. In conclusion, *Moringa* leaves extracts exhibited antioxidant activity by the presence of free radical quenching scavenger constituents which protect liver from cyclophosphamide injury.

**Keywords**: Albion rats; Cyclophosphamide; Leaves extracts; Liver toxicity; *Moringa oleifera*; Oxidative stress; Silymarin

**INTRODUCTION**

Cyclophosphamide (CYP), is one of the most important and widely drug used in the past until the present time as mentioned by Jurado-Garcia et al., 2008.it is a potent anticancer agent. As well as it is effective against a wide spectrum of malignancies, such as leukemia, lymphoma, breast, lung, prostate, and ovarian cancers (Khan et al 2004). The usage of CYP is severely limited by its physiologic side effects, such as hepatotoxicity, nephrotoxicity, urotoxicity, cardiotoxicity and myelosuppresion (Motawi et al 2010, Lameire et al 2011 and Newton, 2012).

Many anticancer drugs are known for the generation of Reactive Oxygen Species (ROS) in cancer cells (Hanane et al 2012) and these ROS generated lead to oxidative damage in the cell (Maiti, 2012). During bioactivation of CYP, reactive oxygen species are also formed, which can modify the components of both healthy and neoplastic cells...
leading to decreased antioxidative capacity (Stankiewicz and Skrzylewska 2003). CYP’s antineoplastic effects are associated with phosphamide mustard, while acrolein is linked with its toxic side effects (Kern and Kehrer, 2002). The cytotoxic effects of CYP and other chemotherapeutic drugs result in part from their interaction with DNA leading to defective DNA, abnormal cell function and cell death (Lee and Schmitt, 2009). Several studies suggest that antioxidant supplementation can influence the response to chemotherapy as well as the development of adverse side effects that result from treatment with antineoplastic agents (Weijl et al 1997).

Silymarin (SIL) is a C25 containing flavonoid mixture, extracted from the Silybum marianum (milk thistle) plant. Silymarin extract contains approximately 65% to 80% flavonolignans (silybin A and B, isosilybin A, isosilybin B, silydianin and silychristin), with small amounts of flavonoids, and approximately 20% to 35% of fatty acids and polyphenolic compounds possessing a range of metabolic regulatory effects (Comelli et al 2007). SIL is a drug used to treat liver disorders. It is well-known for its antioxidant and chemoprotective effects on the liver (Post-White et al 2007 and Stiuso et al 2014) and it is often prescribed as a complementary and alternative hepatoprotective medicine (Testino et al 2013).

Moringa oleifera is called "Miracle Vegetable" because it is both a medicinal and a portion of functional food (Verma-Arti et al 2009). Moringa oleifera Lam is a highly valued plant in tropic and subtropical countries (Khalafalla et al 2010). The leaves are highly nutritious. It is a good source of amino acids, vitamins A, B, C and E, minerals, β-carotene, riboflavin, folic acid, nicotinic acid, pyridoxine and various phenolic compounds (Anwar et al 2007 and Khalafalla et al 2010), it is being a significant source of glycosides, sterols, and alkaloids (Siddharaju and Becker, 2003 and Anwar et al 2007). Moringa oleifera has been regarded as a food substance since ancient times and has also been used as a treatment for many diseases. Recently, various therapeutic effects of M. oleifera such as antimicrobial, anticancer, anti-inflammatory, antidiabetic, and antioxidant effects have been investigated (Lae Jung 2014). Phytochemical screening is of paramount importance in identifying new source of therapeutically and industrially valuable compounds which have medicinal significance, to make the best and judicious use of available natural wealth. Consequently, the present study aimed to investigate the antioxidant and chemoprotective effect of Moringa oleifera leaves extracts and Silymarin (SIL) against CYP-induced oxidative stress in rats.

MATERIALS AND METHODS

Plant Material

The leaves of Moringa oleifera (MO) Lam were collected from Moringa trees cultivated in private farm located at Al-Shobak Al-Garby, Shebin Al-qanater El-khayria, Qaliubiya governorate, Egypt. They were transferred immediately to the biochemistry laboratory, (Biochemistry Department, Faculty of Agriculture, Ain Shams University). Washing was done in tap water then spread and dried under the shade for five days. The dried leaves of Moringa oleifera Lam. (MO) were crushed and powered by the blinder, then stored in the deep freezer at -10 °C until analysis.

Experimental animals

Age matched healthy males Wistar Albino rats of similar weight about 180-200g were obtained from Animal house, Agriculture Research Center, Giza, Egypt.

Chemicals and Reagent methodology kits

All chemicals, silymarin (SIL), and reagent methodology kits were purchased from El Gomhoureya Company for Drugs Trade & Medical Supplies, Medical Supply Store in Cairo, Egypt.

Methods

Preparation of Moringa ethanolic extract

The ethanolic leaves extract (ELE) of Moringa was prepared as follows in brief, powder of Moringa oleifera leaves (500 g) was saturated with 70% ethanol(1:3w/v) and shaken well for one hour, then soaked overnight. After that supernatant was collected and filtered. The residual Moringa powder was re-extracted with ethanol for three times, further, all the filtrates were collected and evaporated to dryness under vacuum. The residue was stored in the deep freezer at -10 °C until uses.

Preparation of Aqueous Moringa extract

The aqueous leaves extract (AQLE) of Moringa was prepared in the same way of ethanolic extract but by using distilled water instead of ethanol.
Experimental Rats management

Age matched healthy males albino rats of similar weight about 180-200g were used for this study. They were kept for two weeks for acclimatization period before starting the experiment. Rats had been housed in cages and received normal basal diet and tap water ad- libitum in constant environment (room temperature (25 ± 2 °C) with 12 h light and 12 h dark cycle.

After acclimatization period, the experimental animals were divided randomly into five groups, each one had ten animals. They were received the food diet for 30 days as follows:

- **Group I (control)**: Rats received distilled water orally (untreated check).
- **Group II (CYP)**: Cyclophosphamid at the dose of 150 mg/kg was given through its intraperitoneal (i.p) according to Kim et al (2013) and Sadashiviah Jnaneshwar et al (2013).
- **Group III (ELE+CYP)**: Rats were administrated orally with ethanolic leaves extract 500 mg/kg/day according to Nevine et al (2015) through an intragastric tube for consecutive 14 days, before and after CYP injection with a dose of 150 mg/kg which was injected twice; one dose on the 15th day and the other on the 30th day.
- **Group IV (AQLE+CYP)**: received aqueous leaf extract 500 mg/kg/day for consecutive 14 days, before and after CYP injection at a dose of 150 mg/kg was injected twice; one dose on the 15th day and the other on the 30th day.
- **Group V (SIL+CYP)**: Rats were administrated with aqueous solution of silymarin at dose of 100 mg/kg/day by gavage according to Avci et al (2017) for consecutive 14 days, before and after CYP injection at a dose of 150 mg/kg that was injected twice; one dose on the 15th day and the other on the 30th day.

Blood Samples collection and rats dissection

After the last treatment, rats were unfed for 24 h. and Diethyl ether was used to anesthetize animals and then dissected the rats from the ventral side of the body. Blood was collected by piercing heart in two types of tubes; the first type was, for serum analysis and stored in small falcon tubes then was centrifuged to obtain serum. The second type was collected in EDTA containing tubes for whole blood analysis. From the dissected animal, livers were removed, weighed and placed in saline solution. Liver was stored in 10% neutral buffered formalin solution for histological study.

Biochemical analysis

Serum samples were analyzed for total protein by the Biuret method according to Gornall et al (1949). Albumin was determined using Bromocresol green method according to the method described by Doumas et al (1971). Serum alanine aminotransferase (ALT; EC 2.6.1.2) and aspartate aminotransferase (AST; EC 2.6.1.1) activities were determined using commercial kits. The principle reaction of the colorimetric determination of AST or ALT activities is based on the reaction of aspartate or alanine with α ketoglutarate to form oxaloacetate, which is spontaneously decarboxylated to pyruvate. Pyruvate can be determined as its concentration in alkaline reaction with 2, 4-dinitrophenyl hydrazine leading to the formation of a pink colored compound of pyruvate hydrazone that can be measured at 546 nm in spectrophotometer (Young, 1990). Serum Alkaline phosphatase (ALP; EC 3.1.3.1) activity was measured at 405 nm by the formation of para-nitrophenol from para-nitrophenyl phosphate as a substrate (Rosalki et al 1993). Serum lactate dehydrogenase (LDH; EC 1.1.1.27) was determined according to the method of (Dito, 1979). Lipid peroxidation was assayed by the measurement of MDA levels produced from the oxidative degradation of polyunsaturated fatty acids. MDA reacts with thiobarbituric acid and the procedure colour was measured at 532 nm, according to the method of Buege and Aust (1978).

Histological investigations

Autopsy samples were taken from the liver of rats in different groups and fixed in 10% formal saline for twenty-four hours. Washing was done in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56°C degrees in hot air oven for twenty-four hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by slide microtome. The obtained tissue sections were collected on glass slides, deparaffinized, and stained by hematoxylin and eosin stain for routine examination through the light electric microscope (Banchroft et al 1996).

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Statistical analysis

All results calculated were presented as means ± SE from six to seven replicates and subjected to one-way analysis of variance ANOVA test. The means of different treatments were compared using Duncan’s multiple range tests at p ≤ 0.05. Statistical analyses were performed using repeated analysis of variance (SAS system), as described by Snedecor and Chochran, (1980).

RESULTS AND DISCUSSION

The present experiment was carried out to determine the protective role of Moringa leaves extracts (MLE) and silymarin (SIL) against CYP induced liver toxicity in rats.

Estimation of cyclophosphamide toxicity on serum protein and albumin

Cyclophosphamide treatment of rats caused significant changes in the serum protein profile. The fluctuations in serum total protein and albumin were illustrated in Table 1. A significant difference was observed in CYP treated group in comparison to control group. MLE treatments and SIL treatment showed a protective role, since it decreased significantly (P<0.05) its level in the serum. The reduction in serum protein and albumin can be attributed to changes in protein and free amino acids metabolism and their synthesis in the liver (Attia and Nasr, 2009). Also, the protein level suppression may be due to loss of protein either by reducing protein synthesis or increased proteolytic activity or degradation (Yeragi et al 2003). Besides, the observed decrease in serum proteins could be attributed in part to the damaging effect of CYP on liver cells, as confirmed by the increase in serum AST and ALT activities. Natural antioxidant (MLE) mitigated the changes in the protein by regulating the metabolic activities and protein synthesis. It is evident from the results that ELE of Moringa had similar results to Silymarin (SIL) and they possess the highest protective action against liver injuries attributed to CYP. The extract induced oxidative stress higher than the aqueous extract.

Hepatic toxicity

Liver function tests commonly include measure activity levels of several enzymes, which are special proteins that help the body, break down and metabolize other substances. Enzymes that are often measured include AST; ALT and ALP. The protective effect of MLE and SIL on CYP – induced changes in serum ALT, AST and ALP in rats was tabulated in Table (1). ELE of Moringa had better results than SIL in decreasing the elevated enzyme activities. Treatment with CYP significantly (P<0.05) increased the activities of AST, ALT, ALP, and LDH in serum compared to control. The present study demonstrates that treatment with MLE and silymarin in combination with CYP elevated its harmful side effects and activities of the above-measured enzymes as shown in Table 1. The increased activity of ALP, ALT, AST activities, and level of LDH in the CYP–treated rats is a manifestation of induced hepatocellular damage. Increases in the ALP levels are generally associated with impairment of intrahepatic and extrahepatic bile flow, hepatobiliary injury, erythrocyte destruction or altered bilirubin metabolism (Singh et al., 2011). The increase of ALP activity in the serum is also a marker for kidney damage. Hepatic Chemotherapy-induced side effect lead to increase in ALT and AST which has been well documented by Ramadori and Cameron, (2010).

Table 1. Effect of Moringa leaves extracts and silymarin on some of serum biochemical parameters of cyclophosphamide-treated rats

| Treatments | Total Protein (g/dl) | Albumin (g/dl) | ALT (U/L) | AST (U/L) | ALP (U/L) | LDH (U/L) |
|------------|---------------------|----------------|-----------|-----------|-----------|-----------|
| Control    | 7.55±0.136^a        | 4.58±0.004^a   | 38.53±0.011^a | 107.11±0.073^a | 147.41±0.465^d | 1084.41±3.424^d |
| CYP        | 4.72±0.027^a        | 2.87±0.001^a   | 77.39±0.026^a | 281.36±0.043^a | 297.94±0.679^b | 1613.68±2.330^b |
| ELE + CYP  | 6.41±0.145^b        | 4.31±0.005^b   | 41.82±0.022^b | 136.69±0.064^b | 200.24±0.681^a | 1172.96±0.661^a |
| AQLE + CYP | 5.78±0.045^c        | 3.52±0.020^c   | 44.87±0.032^c | 168.83±0.320^c | 237.18±0.794^d | 1303.52±4.740^d |
| SIL + CYP  | 6.01±0.123^b        | 4.03±0.024^b   | 42.66±0.022^b | 144.82±0.022^b | 209.42±1.055^c | 1189.81±5.251^c |

Each value represents the mean ± SE calculated from six to seven replicates
Different letters refer to significant difference (p<0.05).
Lipid peroxidation estimation in serum

Serum lipid peroxidation contents are recorded in Table (2) and illustrated in Fig. (1). Serum MDA levels significantly (p≤0.05) increased to 11.676 nmoles/ml in cyclophosphamide conducted group compared to 5.748 nmoles/ml in the control group. Administration of different extracts of (MLE) and (SIL) led to significant decreases in MDA content (Table 2). Moreover, ELE of Moringa had similar results to Silymarin. Various studies have confirmed that free radicals are involved in various metabolic alterations and diseases. Radical reactions are mainly responsible for the in vivo toxic effects of CYP. Lipids confined in the cell membranes are very sensitive to oxidative stress. Lipid peroxidation converts polyunsaturated fatty acids into small and more reactive elements. CYP is a toxic chemical which produces various free radicals causing lipid peroxidation. An increase in lipid peroxidation is calculated in terms of thiobarbituric acid reacting substance (TBARS) which measures the damage caused to the membranes by free radicals (Attia and Nasr, 2009). So, TBARS content is supposed to be the main markers of CYP-induced oxidative stress (Stankiewicz et al 2002). In the current study, the elevation of TBARS due to CYP treatment was restored to normal level after treating with MLE. During the bioactivation of CYP, reactive oxygen species are also formed, which can modify the components of both healthy and neoplastic cell leading to decreased antioxidative capacity (Stankiewicz et al 2002). Free radicals produced by bioactivation of CYP increased lipid peroxidation and decrease the level of antioxidant enzymes resulting in damage to liver tissue. MLE restored the damage produced by CYP. (Chatto-padhyay et al 2011) found that administration of aqueous extract of M. oleifera prevent significantly the arsenic-induced alteration of hepatic function markers and lipid profile. This result coincided with Amien et al (2015) who reported that treatment of cyclophosphamide conducted rats with silymarin (150 mg/kg) significantly increased the level of malondialdehyde (MDA).

**Table 2.** Effect of Moringa leaves extracts and silymarin on the levels of serum MDA of cyclophosphamide treated rats

| Treatment                              | MDA (nmoles/ml) |
|----------------------------------------|-----------------|
| Control                                | 5.748 ± 0.087a  |
| Cyclophosphamide                       | 11.676 ± 0.230a |
| Ethanolic Leaf Extract + Cyclophosphamide | 6.499 ± 0.246d  |
| Aqueous Leaf Extract + Cyclophosphamide | 8.648 ± 0.085b  |
| Silymarin + Cyclophosphamide           | 6.868 ± 0.009c  |

Each value represents the mean ± SE calculated from six to seven replicates

**Fig. 1.** Effect of Moringa leaves extracts and silymarin on the levels of serum MDA of cyclophosphamide treated rats.
Fig. 2. Photomicrographs of the cyclophosphamide (CYP)-injected rats livers sections (with/without Moringa leaf extracts and silymarin). Sections were stained with hematoxylin and eosin. (A) Control rat; (B, C, D, E and F CYP-injected rats; (G) CYP + aqueous leaf extract (AQLE) of Moringa; (H) CYP + ethanolic leaf extract (ELE) of Moringa; (I) CYP + silymarin (SIL).

Microscopically, the hepatic sections of untreated rats (control group) stained with hematoxylin and eosin H&E revealed no histopathological alteration and the normal histological structure of the central vein and surrounding hepatocytes in the parenchyma as recorded in (Fig. 2A). However, CYP induced liver toxicity was observed in form of congestion in the central vein and sinusoids associated with diffuse kupffer cells proliferation in between the hepatocytes (Fig. 2B). There was fatty change in the hepatocytes surrounding the portal area (Fig. 2C&2D). The portal area showed oedema with periductal fibrosis surrounding the hyperplastic bile ducts (Fig. 2E). Focal haemorrhage was detected in the hepatic parenchyma (Fig. 2F).
Histopathological findings

The results illustrated a good correlation between cellular damage and the levels of serum marker enzymes. The levels of serum toxicity markers (AST and ALT) were increased in the CYP-injected group of rats and the levels were restored upon MEL treatment. Histopathological evaluation also supported the concept that MEL and SIL treatment had a protective effect on liver morphology.

Moreover, liver toxicity in AQLE + CYP group reported as congestion that was noticed in the central vein and sinusoids associated with diffuse kupffer cells proliferation in between the hepatocytes (Fig. 2G). These findings didn't report in case of rats treated with ELE + CYP, instead, there was no histopathological alteration observed (Fig. 2H).

Further, the group treated with SIL + CYP showed an obvious congested in the central and portal veins (Fig. 2I).

Conclusion

It can be concluded that both ethanolic and aqueous leaves extracts of Moringa and silymarin showed a protective effect against CYP-induced oxidative stress and inflammation leading to organ toxicity. The protective efficacy of Moringa oleifera can be attributed to its potent antioxidant and anti-inflammatory properties as it prevented the oxidative stress and increased the antioxidant effect in liver tissues of albino rats. It is hypothesized that MLE protects the tissues by scavenging the toxic metabolite, which is evidenced by the normalization of the clinical chemistry parameters. Our results demonstrate the MLE and SIL protective role against CYP generated free radicals damages and suggestions for further study to isolate the bioactive component in pure form of MLE.

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التأثير الوقائي لمستخلصات نبات المورينجا أويليفيرا ولسيلمران ضد سمية الكبد الناجمة عن السيكلوفوساميد في الفئران البيضاء

خديجة خالد على منتصر - همت عبد الفتاح إبراهيم - محمد أحمد طه عبد الرحيم - رضا كامل عطا الله

المجملز

أوراق نبات المورينجا أويليفيرا موثقة كعامل نشط دوائي ذو نشاط مضاد للأكسدة مرتفع. في هذه الدراسة تم فحص التأثير الوقائي لكل من المستخلصات الإيثانولية والمائية لأوراق نبات المورينجا والسيلمران ضد إصابة كبد الفئران الناجمة عن استخدم السيكلوفوساميد. تم تقسيم عدد خمسون من ذكور الفئران البيضاء في خمس مجموعات، على النحو التالي:

1. المجموعة الأولى (الضابطة) تم تلقيها الماء المقطر عن طريق الفم.
2. المجموعة الثانية (مجموعة السيكلوفوساميد) تم حقنها بالسيكلوفوساميد بجرعة 0.051 ملجم / كجم بالتجويف البيرونى: حيث تم حقنها مرة واحدة في اليوم الخامس عشر والأخرى في اليوم الثلاثين.
3. المجموعة الثالثة (CYP + SIL) تم إعطائها مركب السيلمران بجرعة 0.011 ملجم / كجم / يوم عن طريق الفم من خلال أنبوب المعدة لمدة 14 يومًا متتاليًا قبل وبعد حقن السيكلوفوساميد.
4. المجموعة الرابعة (AQLE + CYP) تم إعطائها المستخلص الإيثانولي أوراق المورينجا بجرعة 0.051 ملجم / كجم / يوم عن طريق الفم من خلال أنبوب المعدة لمدة 14 يومًا متتاليًا قبل وبعد حقن السيكلوفوساميد.

بعد يوم واحد من آخر معاملة، تم جمع عينات الدم والكبد من الفئران لإجراء فحوصات الأنسجة وبعض التقديرات البيوكيميائية، حيث تم تقدير مستويات كل من الأكسدة البيروكسيدية للليبيدات , البروتين الكلي , إنزيم ألانين أمينو ترانسفيراز وأسبارتيت أمينو ترانسفيراز وأنزيم الفوسفاتاز القاعدي وأنزيم دهيدروجينيز .

وقد أشارت النتائج إلى وجود زيادة معنوية في كافة الأorns و p < 0.05 في نشاط كل من إنزيم ألنين أمينو ترانسفيراز وأسبارتيت أمينو ترانسفيراز وأنزيم الفوسفاتاز القاعدي ود平稳ودرجهنرفيرز .

وبالإضافة إلى ذلك:

- تم إعطائها المجموعة الثالثة (CYP + ELE) موجملز من أوراق نبات المورينجا أويليفيرا بجرعة 500 ملجم / كجم يومًا عن طريق الفم من خلال أنبوب المعدة لمدة 14 يومًا متتاليًا قبل وبعد حقن السيكلوفوساميد.

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الفئران المعاملة بالسيكلوفوسفاميد مقارنة بمجموعة الكنترول والمجموعات الأخرى. فيما يخص فحص انسجة وخلايا الكبد، فقد تم ملاحظة العديد من مظاهر الضرر بالخلايا الكبدي عند الفئران المعالجة بالسيكلوفوسفاميد بفارق معنوي $P < 0.05$ مقارنة بمجموعة الكنترول والجماعات الأخرى.

في النهاية، أثبتت مستخلصات أوراق المورينجا فعاليتها كمضادات أكسدة بوجود مضادات للشوارد الحرة والتي تحمي الكبد من الأضرار الناتجة عن الإصابة بالسيكلوفوسفاميد.

الكلمات الدالة: سيكلوفوسفاميد، سمية الكبد، مستخلصات الأوراق، المورينجا أوليفيرا، الفئران البيضاء، الإجهاد التأكسدي، سيليمارين.