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Research article

Moringa oleifera inhibited the toxicity induced by administration of Tamoxifen in rats: using ultraviolet-visible and fluorescence spectroscopy investigations

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Key words: Tamoxifen, *Moringa oleifera,* blood serum, Ultraviolet-visible and Fluorescence Spectroscopy.

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Abstract

Tamoxifen (TMX) has treated different stages of the breast cancer; although it has several side effects. This study aimed to investigate the influence of *Moringa oleifera* aqueous extract (MOE) on the blood serum of rats injected with TMX through the ultraviolet-visible and fluorescence spectroscopy techniques, for optimizing the TMX therapy. The absorption spectra of blood serum were measured using a UV-VIS double beam spectrophotometer over wavelength range 200–500 nm. The fluorescence spectra were measured over wavelength range of 200-700 nm and with wavelength excitations at 278, 539, and 576 nm. The absorbance peak of TMX was found at 278 nm, which was higher compared to the healthy control, and the MOE extract supplementation decreased the absorbance peak to be nearly close to the healthy control. The fluorescent excitations of the blood serum were measured at 278, 539, and 576 nm; the fluorescence intensity of the TMX was significantly higher compared with the healthy control, however, the administration of MOE extract in combination with TMX restored the fluorescent peak of TMX closely to the healthy control. This study suggests that the administration of MOE extract in combination with TMX in the rats may promote the antioxidant defense mechanism, which in turn inhibits the generation of different reactive oxygen species, ameliorated the TMX-induced blood serum constituent's deterioration.

Introduction

Blood is a significant bio-fluid, and indicates any pathological changes in the body. The blood analysis helps to provide early diagnosis and treatment for diseases [1]. Blood serum has mainly proteins including albumin and globulin. Serum albumin constitutes 55% of blood proteins, and maintained the osmotic pressure of plasma which helps in the transport of lipids and steroid hormones. Globulins represent 38% of blood proteins and transport ions, hormones, and lipids assisting in immune function [2]. The fluorescence of albumin produced from tryptophan (Trp) residues, with a minor tyrosine residues depending on the excitation wavelength selected. Trp is a strongly sensitive to its local environment, and can be used to reveal changes in the fluorescence emission spectra due to protein conformational changes, binding to substrates, and denaturation [3].
Tamoxifen (TMX) is defined as a synthetic non-steroidal antiestrogen most applied in the chemotherapy treatment of breast cancer [4]. It has been reported that tamoxifen produced toxicity to the red blood cells [5], and caused endometrial cancer in some patients [6]. In addition, Tamoxifen produced toxicity in human serum albumin of blood when it bound to it [7].
Absorption spectroscopy can be used for following the conformational transitions in the proteins and nucleic acids. UV-visible absorption and fluorescence spectroscopies are very simple methods to probe the structural change and to define the complex formation in solution [8]. The spectroscopic measurements are strongly responsive, nondestructive, and require only small amounts from the material for the analysis.
Moringa oleifera leaves were utilized in treatment of different diseases [9]. Also, Moringa leaves can be employed for humans and animals treatment [10]; can be taken as food supplementary [11]. Moringa leaves include about 27% protein, vitamins and beneficial phytoactives [12].
Therefore, this study was aimed to investigate the influence of *Moringa oleifera* aqueous extract (MOE) on the blood serum of rats injected with TMX through the ultraviolet-visible and fluorescence spectroscopy techniques, for optimizing the TMX therapy.

Materials and methods

Herb extraction

Moringa (*Moringa oleifera*) herb obtained from a local supplier (Abd El-Rahman Harraz; Bab El-Khalk zone,

Cairo, Egypt), and identified by a special botanist at faculty of pharmacy, Cairo University. The taxonomic serial number (TSN) of *Moringa oleifera* is 503874.

Aqueous extract

The MO aqueous extract was prepared according to the method of Berkovich *et al.,* [13]. Firstly, the dry leaves of MO were powdered, and kept dry in an air-tight container prior to the extraction. Secondly, the aqueous extract of the powdered herb was prepared in the laboratory by mixing 50 g of the dry-powdered leaves with 500 ml boiling distilled water for 15 minutes. The mixture then filtered through sterile Whitman filter paper number 42 (Whatman International Ltd, Maidstone, England) using a Buchner funnel. The filtrate was subjected to lyophilization process through freeze drier (Snijders-Scientific-tilburg, Holland) under pressure, 0.1- 0.5 mBar and temperature -35-41°C conditions. The dry extract was stored in a dark bottle at -40°C until usage.

Phytochemical analysis

Table 1 shows quantitative phytochemical analysis of the Moringa leaf aqueous extract. The analysis was carried out for the presence of flavonoids, anthraquinone, alkaloids, saponins, steroids, terpenoids, cardiac glycoside, anthocyanin, tannins and carotenoids using the standard procedures as described by Edeoga *et al.*, and Oluduro [14,15].

Table 1. Phytochemical bioactive compounds of *Moringa oleifera* aqueous extract.

Phytochemical	%
Flavonoid	3.738
Anthraquinone	12.264
Tannins	9.7344
Terpenoids	5.1788
Saponins	1.5622
Carotenoids	1.2876
Alkaloids	3.1314
Anthocyanin	0.0612

Animal and experimental design

Adult male Wistar albino rats (*Rattus norvegicus)* weighting 120-150g were obtained from Animal House, National Research Centre, Giza, Egypt. The animals were housed in suitable plastic cages for one week for acclimation with the new room condition. Excess tap water and standard rodent food pellets (proteins, lipid, fibers, NaCl, lysine, methionine, vitamins, salts and wheat; obtained from Meladco Company, El-Obour City, Cairo, Egypt) were always available. All animals were received human care in compliance with the standard institutions criteria for the care and use of experimental animals as cited by animal ethical committee number FWA00014747. After animals being acclimatized with experimental room conditions, they were randomly divided into three groups (10 animals each) as following:

Group (I): normal rats fed daily normal diet and acting as control.

Group (II): normal rats fed normal diet and subjected to daily oral administration of aqueous solution of (3mg/Kg/3days) anticancer chemotherapy drug (Tamoxifen -20mg; obtained from AMRIYA Pharmaceutical Industries, Amriya, Alexandria City, Egypt) for consecutive six weeks to evaluate the drug-toxic effects [16].

Group (III): normal rats were subjected to daily oral administration of aqueous extract of *Moringa oleifera* (300mg/kg/day) in combination with Tamoxifen (3mg/kg/3days) for consecutive six weeks. Moringa extract and Tamoxifen solutions were prepared in sterilized deionized water

Blood sampling

At the end of the study period, animals were fasted overnight, and anesthetized, blood specimens (3-7 ml) were drawn from the retro-orbital plexus using capillary sterile glass and heparinized tube (single draw vacutainer needle) into open vacutainer collecting tubes [17]. Blood specimens were left to clot then centrifuged at 3000 rpm for 20 minutes using centrifuge (IEC centra-4R, International Equipment Co., USA). The sera were separated at once by micropipette, divided into aliquots and stored at -70°C until spectroscopic measurements performed as soon as possible.

UV-Visible spectroscopy

Ultraviolet and visible spectroscopy of blood serum samples were measured using a UV-VIS double beam spectrophotometer (UV-1601 PC, Shimadzu, Japan; H14 grating UV) through shortwave NIR with optical resolution of 0.4 nm. Absorbance measurements were obtained over 200–800 nm at room temperature using quartz cuvettes (1 cm path length). The cuvettes were cleaned before each use by sonicating for 5 min in deionized water followed by rinsing with deionized water [18].

Fluorescence spectroscopy

Fluorescence characterization of rat blood serum samples was performed using a FluoroMax-2 JOBAN YVON-SPEX, Instruments S.A., Inc., France. The fluorescent emission spectra were recorded and all solutions were prepared at temperature (25°C). The excitation wavelengths of blood serum samples were set at λex = 278, 539 and 576 nm respectively and the samples were scanned in the range of 200–700 nm. Emission maxima values were taken as a measure of fluorescence intensity of blood serum proteins. Measurements were performed on series of blood serum samples with a 10-mm light path cuvette [18].

Results

UV-absorption spectroscopy

Figure 1 shows the blood serum absorption spectra for control, TMX, and TMX plus MOE treated groups. The observed absorption maximum at wavelength of 278 nm was attributed to the blood serum proteins. This peak might be due to the aromatic amino acids (Trp, Tyr, and Phe). Figure 1 displayed that the absorbance intensity peak for TMX was higher when compared to control. While when MOE injected with TMX, the absorbance intensity peak decreased to become closed the control.

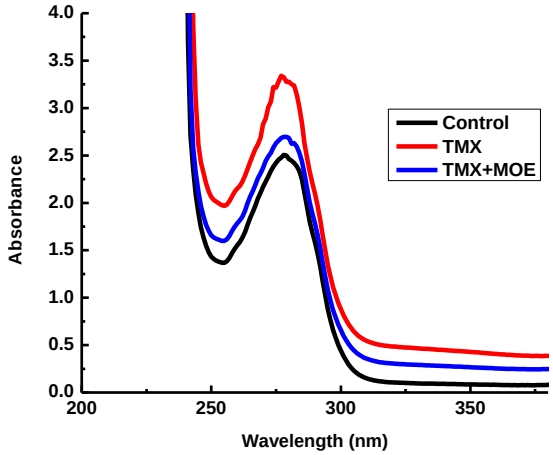

Figure 1. UV-visible absorption spectra of blood serum of rats injected with Tamoxifen and MO extract in the range 200 – 400 nm.

Fluorescence spectroscopy

The fluorescence emission spectra (counts) of blood serum of rats in the control, TMX, and TMX plus MOE treated groups were measured in the wavelength range 100-550nm with excitation wavelength λ_{ex} = 278 nm, (Figure 2). This excitation wavelength induced autofluorescence in the sample blood serum of all groups of rats, allowing the observation of tryptophanyl and tyrosyl residues of the blood serum proteins. It can be seen from figure 2 that the blood serum proteins have emission peak at 350 nm. Figure 2 demonstrated that the fluorescence intensity peak for TMX was slightly higher when compared to control. While when MOE injected with TMX, the fluorescence intensity peak tends to become closed the control.

To observe more details about the interactions of TMX and TMX+MOE with the serum proteins, figure 3 shows fluorescence intensity (counts) against wavelength (nm) for a sample of rat's blood serum taken from control, TMX, and TMX plus MOE treated groups at excitation wavelength λ_{ex} =539 nm. The excitation wavelength of 539 nm was used to induce auto fluorescence in a sample rat's blood serum of all groups. Figure 3 demonstrated that the fluorescence intensity peak for TMX was higher when compared to control. While when MOE injected with TMX, the fluorescence intensity peak decreased to become closed the control.

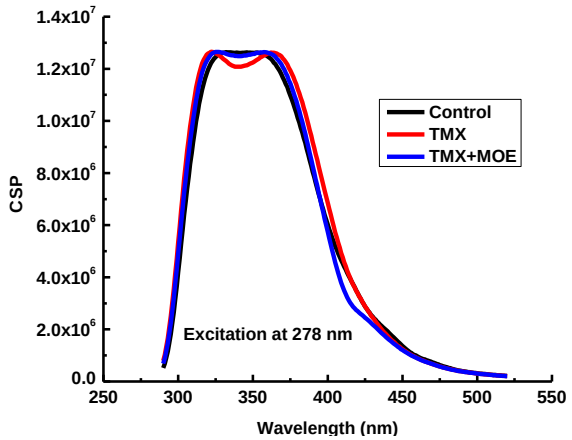

Figure 2. Fluorescence spectra of blood serum rats injected with Tamoxifen and MOE extract at excitation wavelength 278 nm.

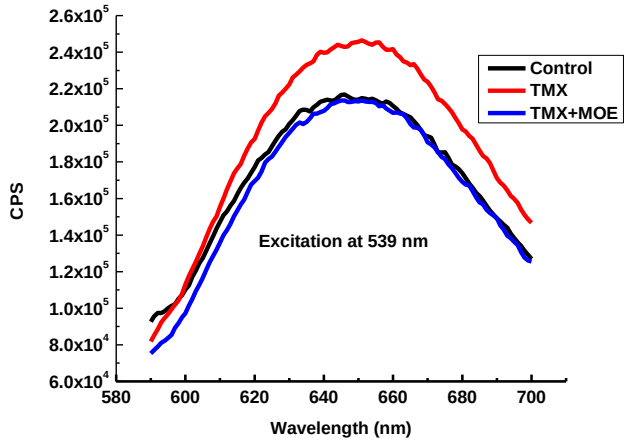

Figure 3. Fluorescence emission spectra of blood serum of rats injected with Tamoxifen and MO extract at excitation wavelength 539 nm.

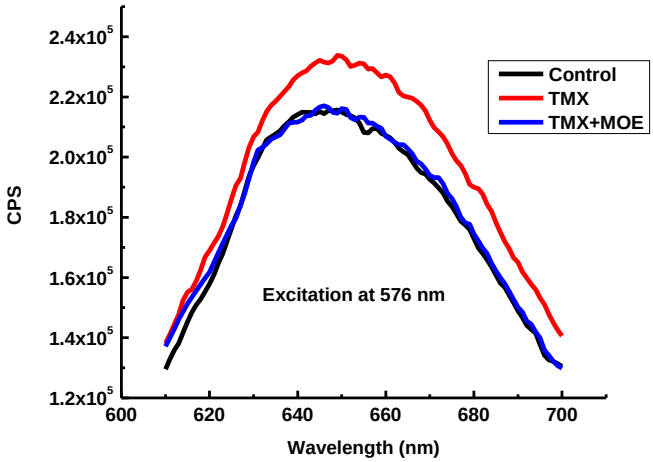

Figure 4. Fluorescence emission spectra of blood serum of rats injected with Tamoxifen and MO extract at excitation wavelength 576nm.

Figure 4 shows the fluorescence intensity (counts) against wavelength (nm) for a sample of rat's blood serum taken from control, TMX and TMX plus MOE treated groups at excitation wavelength λ_{ex} =576 nm, the maximum emission wavelength for unmodified blood serum proteins was at 650 nm. The excitation wavelength of 576 nm was used to induce auto fluorescence in a sample rat's blood serum of all groups. The fluorescence intensity peak for TMX was higher compared to control. Whereas when MOE injected with TMX, the fluorescence intensity peak decreased nearly to the control.

Discussion

Tamoxifen (TMX) is a hydrophobic structure assembled rapidly in phospholipid bilayers of membranes where it is assumed to stimulate oxidative stress [19]. It was reported that TMX administration producing a significant production of lipid peroxidation which might be assigned to hexose monophosphate shunt; rat liver is strongly suppressed by high dose of TMX, so that the NADPH levels inside cells is lowered [20, 21]. Our previous study (under publication) showed that intoxication with Tamoxifen only resulted in a significant elevation in ALAT and ASAT activities as well as urea and creatinine levels indicating marked TMX-toxicity; one or more mechanisms could illustrate the TMX-induced disorder; It was suggested that TMX caused mitochondrial dysfunction [22] and suppression of the electron transport chain leading to the formation of ROS which react with poly unsaturated fatty acid to produce lipid peroxidation products. These ROS can interact with either micro or macro bio-structures consequently produced disturbance in the cellular integrity as well as cellular permeability. Also, the oxidation process that happens as a result of TMX intoxication leads to release of iron ions that become more reactive and cooperate in production of hydroxyl radicals which are the most active reactive oxygen species (ROS) and they interact readily with most cellular components [23].

Moringa oleifera-treated rats were evoked to increase reduced glutathione level (unpublished data). It was suggested that aqueous extract of dried *M. oleifera* leaves containing 2, 2-diphenyl-1-picrylhydrazyl with superoxide, hydroxyl radical scavenging activity favoring suppression of lipid peroxidation; as well as, phenol and flavonoids content [24].

As shown in figure 1; administration of TMX alone causes the highest absorbance peak at 278 nm; this effect could be attributed to unfolding of the serum proteins as a consequence to TMX-induced oxidative stress and production of reactive oxygen species. While the administration of the TMX and MOE together in the same time, decreased the absorbance peak observed at 278 nm to become close to that observed for the normal control; this could be attributed to the antioxidant and reducing activities of constituents present in MOE that succeeded in inhibition of oxidative radicals from reaching the cellular structures. Our results are in accordance with those reported by Depciuch *et al.*, Suárez *et al.,* [25- 27].

A fluorescence study was performed herein to explore the changes in conformational structure of serum proteins after administration of TMX; the results confirmed that TMX has a deteriorative potential on the conformational structure of serum proteins. Our results are agreed with the data reported previously [28, 29]. Additionally, the results of this study proved that the administration of TMX in combination with MOE lowered the fluorescent peak intensity close to that of control value. From the progressive quenching observed in the maximum fluorescence emission of blood serum proteins, it can be inferred that the conformational changes are induced by interactions of TMX with blood serum proteins [30]. The data revealed that the administration of TMX might induce the remarkable different degree of unfolding of serum protein under different degrees of oxidative stress, which confirmed by the increase in the protein absorbance. The study outcomes highlights the interaction of drugs with serum proteins should prove helpful for realizing the distribution and transportation of drugs in vivo, elucidating the action, mechanism and dynamics of a drug at the molecular level. Moreover, it will give better understand for pharmacokinetic and pharmacodynamics mechanisms of the drug [31]. These findings might promote the effective use of tamoxifen in the cancer therapy to eliminate its dose dependent side effects [32]. Combination of MOE with TMX significantly increased the antioxidant status, while decreasing lipid peroxides and oxidative stress (under publication). The results obtained suggest the importance of therapeutic co-administration of antioxidants along with conventional drug to breast cancer patients [33]. TMX is a molecule likely to assemble in membrane lipid and protein moieties in route to its target site [34]. The interaction forces between biomolecules and the drug may include hydrophobic forces, electrostatic interactions, van der Waals interactions, hydrogen bonds, etc. It was reported that the interaction process between TMX and serum albumin is spontaneous and the interaction force between them is hydrophobic force [35- 37]. In a previous study, curcumin shifted the TMX-albumin binding affinity consequently, a precaution is required in case of curcumin usage together with TMX [7]; while in the present study, MOE performed a protective role for the serum proteins and prevent toxicity caused by tamoxifen binding without influencing on the efficiency of TMX in treatment of breast cancer.

Conclusion

This study suggests that aqueous extract of Moringa is strongly antioxidant agent that performs its antioxidant potential through free radicals scavenging mechanism.

Also, it possesses a protective potential against TMX-induced toxicity. MOE returns the proteins to its native state without influencing on the efficiency of TMX in treatment of breast cancer. On the other hand, this study reported that Moringa neglect or cancels the toxicity caused by tamoxifen without interfering with tamoxifen binding site on the protein. Therefore, this study recommended the use of MOE together with TMX in breast cancer treatment as a dietary or food supplements.

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