Preventive Effect of Ethanolic Extract of Cactus (Opuntia Ficus-Indica) Cladodes on Methotrexate Induced Oxidative Damage of the Small Intestine in Wistar Rats

Introduction

Methotrexate is a cytotoxic chemotherapeutic agent used for leukemia and other malignancies [1,2]. However its efficacy is often limited by severe side effects and toxic conditions [3]. The chemical and morphological changes in the small intestine may possibly be triggered by crypt cells damage [4]. Recently, special interest has been focused on the cactus to Opuntia species. Indeed prickly pear fruits are recommended for their beneficial and therapeutic properties [5,6]. Indeed, according to several

Abstract

Context: Methotrexate (MTX) is a cytotoxic chemotherapeutic element for various inflammatory diseases. The cytotoxic effect of MTX is also seen in normal tissues having a high proliferation rate, including gastrointestinal and bone marrow.

Aims: The aim of the present study is to find out whether oxidative damage could be relevant for MTX-induced toxicity in vivo using Wistar rats, and to investigate the preventive potential of cactus cladodes.

Methods and Material: Adult, healthy male Wistar rats (200-250 g) were pre-treated by ethanol fraction of cactus cladode. Following a single dose of methotrexate (20 mg/kg) given intraperitoneally, either vehicle (saline) or ethanolic fraction (400 mg/kg) was administered intraperitoneally. All animals were killed 24 hours after the intraperitoneal injection of MTX. Small intestine samples were collected for MDA level, protein carbonyl generation and Peroxidase and Catalase activity measurement. Small intestine was also collected for histopathology analysis.

Statistical analysis used: Each experiment was conducted in triplicate separately. Values were presented as means ± Standard Deviation. Differences were considered significant at P<0.05.

Results: Our results showed that MTX induced significant alterations in oxidative stress markers noticed in the form of intestinal tissues damage, MDA level increase and protein carbonyls generation. Catalase and Peroxidase activities decreased with MTX administration. The combined treatment of MTX with cactus extracts showed a reduction of MTX induced oxidative damage.

Conclusions: It could be concluded that cactus cladodes extract was effective in the protection of the small intestine against MTX-induced damage.

Keywords: Methotrexate-Cactus cladodes-Oxidative stress-MDA induction-Proteins carbonyls-Catalase activity, Peroxidase
studies have reported, both cactus its efficiency in the treatment of several diseases [7-9]: cactus extract exhibit anti-tumoral [10] and anti-viral [11], anti-inflammatory [12], anti-oxidant effects [13], in addition to anti-hyperlipidemic properties [14] and an analgesic action [15]. These data have made cactus pear fruits and cladodes perfect candidates for cytoprotective investigations.

Materials and Methods

Chemicals

Methotrexate, Triobarbituric acid (TBA); Trichloroacetic acid (TCA) and Butyl hydroxyl toluene (BHT) were obtained from Sigma Chemical Co. (St. Louis, Mo), 2,4-dinitro-phenylhydrazine (2,4-DNPH) and guanidine were purchased from Prolabo (France).

Preparation of the extracts of cactus cladodes

Young cactus cladodes of Opuntia ficus-indica (2-3 weeks of age) collected from the local area were washed with water, chopped into small pieces and dried. Air-dried cladodes were ground to fine powder and successively extracted with solvents of increasing polarity (hexane, dichloromethane, ethyl acetate, ethanol and then methanol). Thus, 20 g of cladodes powder were placed in hexane (200 ml) for 18 h under frequent agitation at ambient pressure and temperature. The mixture was filtered using Wattman paper (GF/A, 110 mm) and the experience was repeated twice. The solvent was evaporated using a rotary evaporator under vacuum at 35°C. and the experience was repeated twice. The solvent was evaporated using a rotary evaporator under vacuum at 35°C. Then, the firstly extracted powder was extracted again with dichloromethane under the same conditions as with hexane. The same procedure was applied for the following solvents: ethyl acetate, ethanol and methanol.

Animals treatment

Adult and healthy male albinos rats (200-250 g) provided from an animal breeding centre (SEXAL St. Doulchard, France following the agreement of the Ethics Committee named National committee of Medical Ethics CNEM, BP 74-Pasteur Institute, Tunis 1002 TUNISIA) were used. The animals were kept one week for acclimatization under constant conditions of temperature and a 12 hours light/dark cycle. Animals had free access to standard granulated chow and drinking water. Following a single dose of methotrexate (20 mg/kg) given intraperitoneally, either vehicle (saline) or ethanol fraction of cactus cladode (400 mg/kg) was administered intraperitoneally. Treatment was continued daily for 10 consecutive days. In control rats, following a single dose of saline injection, either saline or ethanol fraction of cactus cladode was administered for 10 days. Each group consisted of 6 rats. After treatment, animals were sacrificed by decapitation. Blood and small intestine were dissected out.

Preparation of small intestine extract

The small intestine was homogenized with a Potter (glass-Teflon) in the presence of 10mM Tris-HCl, pH 7.4 at 4°C and centrifuged at 4000 rpm for 30 min at 4°C. The supernatant was collected for analysis and the protein concentration was determined in the intestinal extract using Protein BioRad assay [16].

Evaluation of lipid peroxidation status

Lipid peroxidation was determined indirectly by measuring the production of MDA in the intestinal extracts following the method of Buege and Aust [17]. Briefly, 200 µl of intestinal extracts were mixed with 150 µl of TBS (Tris 50 mM and NaCl 150 mM, pH 7.4) and 250 µl TCA-BHT (20% TCA and BHT 1%). The mixture was vigorously vortexed and centrifuged at 1500 rpm for 10 min. A volume of 400 µl of the supernatant was added to HCl 0.6 N and 320 µl Tris-TBA (Tris 26 mM and TBA 120 mM); the content was mixed and incubated for 10 min at 80°C. Absorbance was measured at 535 nm. The optic density corresponding to the complex formed with the TBA-MDA was proportional to MDA concentration of and lipid peroxide. The concentration of µmol of MDA/mg of proteins is calculated from the absorbance at 530 nm using the MDA molar extinction coefficient 1.56 .10^5 M^-1cm^-1.

Protein Carbonyl Assay

Protein carbonyls content was determined as described by Mercier et al. [18] in intestinal homogenates by measuring the carbonyl groups reactivity with 2,4-dinitrophenylhydrazine (2,4-DNPH). Thus, 200 µl of small intestine supernatant were placed in two glass tubes. A volume 800 µl of 10M DNPH in 2.5 M HCl was added. Tubes were left in the dark for the 1 h incubation at room temperature. Samples were vortexed every 15 min. Then 1ml of 20% TCA was added to samples, and the tubes were left in an ice bucker for 10 min and centrifuged for 5 min in a tabletop centrifuge to collect the protein precipitates, and the supernatants were discarded. Another wash was then performed using 1 ml of 10% TCA, and protein pellets were broken mechanically with the aid of a glass rod. Finally, the pellets were washed with 1 ml of ethanol-ethyl acetate (v/v) to remove the free DNPH. The final precipitates were dissolved in 500 µl of guanidine hydrochloride 6 M and left for 10 min at 37°C with general vortex mixing. Any insoluble materials were removed by additional centrifugation. Protein carbonyls concentration was determined from absorbance at 370 nm, applying the molar extinction coefficient of 22.0 M^-1cm^-1. A range of nmoles of carbonyls per ml is usually obtained for most proteins and is related to the protein content in the pellets.

Catalase activity determination

Catalase activity was measured in the intestinal extract at 240 nm, 25°C according to Clairbone [19]. Briefly, 20 µl of the extract was added in the quartz cuve containing 780 µl phosphate buffer and 200 µl of H₂O₂ 0.5 M. Catalase activity was calculated using the molar extinction coefficient (0.04 mM⁻¹cm⁻¹). The results were expressed as µmol of H₂O₂/min/mg of proteins.

Peroxidase activity determination

Peroxidase activity was measured at 25°C using guaiacol as hydrogen donor [20]. The reaction mixture contained 9 mM guaiacol, 19 mM H₂O₂ in 50 mM phosphate buffer pH 7 and 50 µl of enzyme extract in 1ml final volume. The reaction was initiated by the addition of H₂O₂ and monitored by measuring the increase in absorbance at 470 nm. Peroxidase activity was expressed in nmol of guaiacol oxidized per min with a molecular extinction coefficient of 26.2 M⁻¹cm⁻¹ for calculation.
**Histopathological analysis**

The animals were killed by decapitation after urethane anaesthesia. Three tissue samples of the small intestine were cut off at a distance of 5 cm from the proximal end of the small intestine of each animal, fixed with 10% neutral formalin, embedded in paraffin and cut with a microtome set at a thickness of 5-6 μm. The tissue sections were stained with haematoxylin and eosin for histopathological analysis and examined with a light microscope.

**Statistical analysis**

Each experiment was conducted in triplicate separately. Values were presented as means ± Standard Deviation. Differences were considered significant at P<0.05.

**Results**

**Lipid peroxidation induction**

Results of MTX effect alone and jointly with ethanol fraction of cactus cladode on lipid peroxidation induction in intestinal extract as determined by MDA level are shown in Figure 1. A volume of 20 mg/kg b.w of MTX induced a significant increase in MDA formation as compared to control groups. Interestingly, when animals were treated with cladodes extract (400 mg/kg b.w.), 10 days prior to MTX treatment, a sharp decrease in MDA level was noticed in tissues. MDA level was found to be similar to that of control groups (ethanol fraction of cactus cladode and saline group).

**Protein carbonyl assay**

Protein carbonyls formation, indicative of severe protein oxidation, was assayed in the small intestine tissue homogenate and results are illustrated in Figure 2. MTX induced protein carbonyls formation in the small intestine compared to control groups. Ethanol fraction of cactus cladode remarkably decreased protein carbonyls formation induced by a fixed dose of MTX (20 mg/Kg b.w.).

**Catalase activity**

Figure 3 illustrates the effect of MTX and cladodes extract on Catalase activity. MTX induced a marked increase in Catalase activity in the intestinal extract. When animals were given cactus ethanol fraction of cactus cladode, 8 days prior to MTX treatment a striking decrease of this activity in tissue was noticed.

**Peroxidase activity determination**

MTX treatment significantly decreased GPX level in the intestinal tissue. However, the supplementation of ethanol fraction of cactus cladode to MTX-treated rats significantly decreased this antioxidant level in the tissue (Figure 4).

**Weight loss**

The weight of the animals in groups was compared; there was no significant change in the cactus-treated and control groups. When the body weight in the other group was compared, it was lower in the MTX-treated group than the cactus group (P<0.05) (Figure 5).

**Histological studies**

MTX treatment induced serious damage to the intestine. The results revealed that MTX-induced small intestinal injury was characterised by villous shortening, variable fusion rates and epithelial atrophy (Figures 6A-6C). Clear improvement in the intestine was noticed when the rats were treated with MTX and the ethanol fraction of cactus cladode. The histopathological findings of our study that ethanol fraction ameliorated mucosal destruction and preserved for the intestinal epithelium morphology (Figure 6D).

**Discussion**

Methotrexate, a drug widely used in antimetabolite cancer therapy or in various forms of arthritis, may have to serious unpredictable side effects causing important clinical problems. Cyclical high doses of methotrexate, used for leukemia [21] or severe psoriasis have been associated with organ toxicity including hepatic fibrosis, cirrhosis and renal failure [22]. Therefore, the cytotoxic effect of methotrexate does not select cancer cells, but it also affects normal tissues having a high proliferation rate, including the haematopoietic cells of the bone marrow and the actively dividing cells of the gut mucosa [23]. Enterocolitis used by intestinal damage is one of the most frequent and severe side effects of MTX for which no effective preventine method been defined so far. This can lead to forcing reduction of chemotherapy.

![Figure 1](image1.png) Lipid peroxidation as determined by MDA level in small intestine of Wistar rat. *P <0.05 compared with saline treated control group

![Figure 2](image2.png) Concentrations of protein carbonyls in small intestine of treated rats. *P ≤ 0.05 compared with saline-treated control group  
*P ≤ 0.05 compared with methotrexate -cactus-treated group
polyunsaturated fatty acids in the endoplasmic reticulum [26]. So, lipid peroxidation may be an important cause of destruction and damage to cell membranes and can contribute to the development of MTX-mediated tissue damage. MDA is frequently used in the measurement of lipid peroxide levels, but it does provide a good correlation with lipid peroxidation rate. It is increasingly apparent that lipid hydroperoxide may play an important role in mediating cellular and molecular events in degenerative pathophysiological processes that lead to intestinal disorders [27]. To further assess MTX induced oxidative damage to Wistar rats, protein carbonyls generation was monitored. Protein carbonylation is a sign of irreversible oxidative damage, often leading to a loss of protein function, which may have lasting detrimental effects on cells and tissues. Protein carbonyls formation is currently the most widely used marker of severe oxidative stress and a number of assays have been recently developed [28]. Our results clearly showed that MTX induced a marked increase in protein carbonyls generation in small intestine extracts which was totally annulled with ethanol fraction of cactus cladode in both organs.

Recent advances in medicine have demonstrated that oxygen radicals and hydrogen Peroxidase have been associated with MTX side effects [25]. Free radicals trigger cell damage through binding to cellular macromolecules, particularly membrane lipids and intensity, thereby potentially reducing the efficacy of anti-cancer treatment [24].
Catalase and Peroxidase activities were also studied in this paper. CAT is a haemeprotein that catalyses the reduction of $H_2O_2$ and protects the tissue from elevated ROS and hydroxyl radicals. In our study, we have shown that MTX treatment significantly reduced antioxidant enzyme capacity. Decreased CAT activities were probably due to the decrease in their synthesis [29]. Our results agree with the study of Vardi et al. [30] who reported that MTX caused a significant decrease in antioxidant enzyme parameters (CAT) in the rats small intestine. On the other hand, Uzar et al. [31] reported that MTX caused a significant increase in the antioxidant enzyme parameter (CAT) in the small intestine and cerebellum of rats. When cells are exposed to oxidative stress, they increase the activity and expression of antioxidant enzymes as a compensatory mechanism to be protected from the moderate level damage of toxic reactants inducing a rise in antioxidant enzymes [32]. The weight loss documented in the present experiments and noted by others [33] do emphasise the importance of evaluating nutritional factors in studying the response of the intestine to this drug. Gastrointestinal toxicity is one of the most serious side effects of MTX treatment. It has been known that methotrexate causes severe damage to the intestinal mucosa [34,35]. It increases the incidence of apoptosis in the area of rapidly dividing cells, including gastrointestinal tract mucosa [36]. The proliferation of small intestinal epithelial cells occurs in the crypts. The crypt cells, which rapidly regenerate, migrate to the villus tip, and replacement of intestinal epithelium is complete in about 2 days in rats and about 3 days in humans [37,38]. The histopathological changes in the small intestine occurring after MTX administration may be triggered by damage to the crypt cells [39,40,35]. The results of the present study have revealed that MTX-induced small intestinal injury is characterised by villous shortening, variable fusion degrees and epithelial atrophy. Our results are similar to other studies in which MTX was reported to cause severe damage in the small intestine [4,41]. The histopathological findings of our study showed that ethanol fraction of cactus cladode ameliorated the mucosal destruction and preserved the intestinal epithelium morphology. The results of this experimental study showed that cactus administration protected against the oxidative and morphological intestinal damage caused by MTX-treatment. The mechanism of this protection was probably related to the decrease in oxidative stress. Oxidative damage is the most potent threat facing any living organism. The intracellular accumulation of reactive oxygen species can arise from toxic insults and can perturb the cells natural antioxidant defence system resulting in damage to all major classes of macromolecules.

Over the last decades, oxidative stress has been shown to be a major component of several biological and pathological processes like aging, inflammation, carcinogenesis, wound healing activity [12] and several other diseases including Parkinson’s, Huntington’s [42,43]. Therefore, increasing attention is paid to the study of natural products, which may counteract the detrimental effects of oxidative stress and prevent multiple human diseases. In this line, different types of fruits and vegetables have been re-evaluated and recognized as valuable sources of nutraceuticals. Cactus should attract great interest because of its nutritional and antioxidant properties. According to several studies, cactus pear (Opuntia ssp.) yields high values of important nutrients and exhibits antioxidant functions, however hardly considered, most probably due to the scattered information available. Our study shows that ethanol fraction of cactus cladode has a protective effect against MTX-induced oxidative damage. This protection was evidenced in all tested oxidative damage endpoints. And it is certainly associated with the presence of several antioxidants such as ascorbic acid, vitamin E, carotenoids, reduced glutathione, flavonoids and phenolic acids actually detected in the fruits and vegetables of various cactus [9,15,44,45]. In addition, more recently, significant antioxidant properties of the most frequent cactus betalains have been revealed and numerous in vitro studies have demonstrated their ability to neutralize reactive oxygen species [46-48]. Lee et al. [49] investigated the antioxidant activity of cactus cladodes and concluded that this antioxidant property was due to several compounds, particularly flavonoids (quercetin, myricetin), and vitamins. Our results are in accordance with other published studies, which underlined the relevant preventive potential of cactus extracts [50,51].
References


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