

Impact of *Physalis angulata* on Methotrexate-Induced Neurotoxicity

Faiza Abdu

Department of Biological Science, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia.

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Oxidative stress contributes in the pathogenesis of methotrexate (MTX)-induced damage in the various organs. Many agents have been tested experimentally to reduce or inhibit the oxidative stress. However, the possible protective effect of *Physalis angulata* extract (PAE) on MTX-induced neurotoxicity in male rats and the mechanisms by which this plant exerts its beneficial effects is unknown. Male rats were allocated to eight groups, ten rats each. The groups of rats divided to control rats, MTX group (4 mg/kg BW, 4 times per week for one month), PAE groups (10, 20 and 30 mg/kg daily for one month, respectively) and PAE+MTX groups (10, 20 and 30 mg/kg+4mg/kg, respectively). At the end of the experiment, levels of a monoamine neurotransmitter, serotonin, immune protein, interferon gamma (IFN- γ) were determined in rat cerebellum. In addition, activities of several antioxidant enzymes and lipids oxidation analyses in rat cerebellum were assayed. The results revealed that the oxidative damage increasing with MTX in the cerebellum was prevented by PAE treatment. PAE treatment inhibited the effect of MTX on serotonin, immune protein IFN- γ , antioxidant enzymes and lipid oxidation while having no effect on control rats. These results suggested that PAE scavenges free radicals that are produced by MTX, increases the activity of antioxidant-defense system.

Key words: *Physalis angulata*, Methotrexate, Neurotoxicity, Antioxidants.

Neurotoxicity is a common dose-limiting complication of chemotherapy treatment¹. It's associated by clinical neurologic alterations ranging from headache, altered mental status, seizures, and vision loss, to loss of consciousness². However, the intensive efforts on the management of the neurologic side effects of chemotherapy in patients and the development of chemoprotective agents still unknown³. Therefore, several studies have been focused to investigate the molecular and cellular mechanisms of neurotoxicity against such toxicities^{4,5}.

Methotrexate (MTX) is one of the main chemotherapy agents which implicated in toxic effects in the central nervous system (CNS)². MTX has significant activity against leukemia and remains an integral component of treatment for acute lymphoblastic leukemia⁶. Intravenous (IV) and intrathecal (IT) MTX have mainly replaced cranial irradiation for treatment and prophylaxis of CNS leukemia leading to neuropsychological problems, growth retardation, and second malignancies⁷. The typical side effects of high-dose MTX chemotherapy on the CNS range from asymptomatic white matter changes to severe CNS demyelination⁸. Oxidative stress can be defined as an imbalance between the free radicals and the antioxidants levels⁹. Oxidative stress modulates levels and activities of serotonin and IFN- γ which are reflected brain dysfunction¹⁰.

* To whom all correspondence should be addressed.
Tel.: +966-55-555-0468; Fax: +966-2-695-3199;
E-mail: faiza.b.abdu@gmail.com

Serotonin is an important signaling molecule in the GI tract and plays a role in numerous functional systems in CNS. Therapeutic agents as MTX may induce neurotoxic brain damage¹¹ and alter serotonin signaling¹² or may lead to a reduction in serotonin¹³.

Interferon gamma (IFN- γ) is the most important trigger for the formation and release of reactive oxygen species (ROS). Anti-inflammatory and immunosuppressive treatment may contribute to a slow-down of the adverse effects of IFN- γ ¹⁴. IFN- γ is also considered as prime stimulators of NO production by a number of cells¹⁵. Sioka and Kyritsis¹⁶ suggested that MTX might cause interferons toxicity.

Superoxide dismutase (SOD) and catalase (CAT) are the most frequently examined enzymes, since their activities quenched by the antioxidant defense system¹⁷. Increasing levels of SOD and CAT lead to eliminating of ROS¹.

Thiobarbituric acid reactive substances (TBARS) are the end products of lipid peroxidation¹⁷. Lipid peroxidation, measured as TBARS, has been frequently used as a marker of oxidative stress in response to different environmental pollutants in a number of studies⁹.

Physalis angulata (Solanaceae) is a widespread indigenous herb found in tropical areas of Africa, Asia, and America, including the Amazon¹⁸. *Physalis sp.* contains biologically active components such as physalins, withanolides, phytosterols and polyunsaturated fatty acids e.g., linoleic acid and oleic acid. It contains high amounts of Vitamins A, B and C and polyphenols which give the herb the antioxidant property¹⁹.

Physalis angulata has been used in traditional medicine as analgesic, antirheumatic, to treat sore throat and abdominal pain. It is considered as antipyretic, antinociceptive, antidiuretic, and anti-inflammatory for hepatitis and cervicitis²⁰ and antimicrobial and anticancer²¹.

Physalis angulata extract (PAE) has an inhibitory action in macrophage culture and in rat's brain tumor cells¹⁸. However, the use of PAE for medical purposes is still limited. Therefore, the aim of the present work is to examine the effect of *Physalis angulata* extract on neurotoxicity prevention induced by MTX in male rats.

MATERIAL AND METHODS

Chemicals

Methotrexate and solvents were of analytical grade and purchased from Sigma-Aldrich Company (St. Louis, MO, USA).

Preparation of the Aqueous Extract of *Physalis angulata*

According to Abdel Moneim and El-Deib²¹, fresh fruits of *physalis* were purchased from local market in Saudi Arabia. The samples were identified in Botany Department, King Abdulaziz University. The fruits were washed, cleaned and minced, the juice and residue were used for the preparation of crude methanolic extract by percolation at room temperature with 70% methanol alcohol and kept in refrigerator for 24 hrs. Extract of *physalis* was concentrated under reduced pressure (bath temperature 50°C) and dried in a vacuum evaporator. The residue was dissolved in distilled water, filtered and used in experiments.

Experimental animals

Eighty adult albino male rats (140-160 g), purchased from the Animal provider, were maintained on standard laboratory diet (protein, 16.04%; fat, 3.63%; fiber, 4.1%; and metabolic energy, 0.012 MJ) and water *ad libitum* at the Animal House Laboratory, King Fahd Medical Research Center, King Abdulaziz University. After an acclimation period for a week, animals were divided into several groups (10 rats/ group) and housed individually in filter-top polycarbonate cages, housed in a temperature-controlled (23 \pm 1°C) and artificially illuminated (12 hr dark/light cycle) room free from any source of chemical contamination. All animals received humane care in compliance with the guidelines of the Animal Care and Use Committee of King Fahd Medical Research Center, King Abdulaziz University.

Experimental Design

Male rats were randomly divided to 8 groups (10 animals each) and treated for 30 days as follows: Group 1, control group: animals were treated intragastrically with solvent vehicle control (NaCl). Group 2, animals were given for 4 week intravenous injections of MTX (4 mg/kg BW)²². Groups 3-5, animals were treated intragastrically with 10, 20 and 30 mg/kg BW of PAE²⁰, respectively.

Groups 6-8, animals were treated with PAE (10, 20 and 30 mg/kg BW, respectively) then injected with MTX (4 mg/kg BW).

All animals were sacrificed by cervical dislocation after 24 hr of the last injection and the brain was immediately removed. The cerebellum was dissected for analyzing (a) the oxidative stress induced by MTX treatment and (b) the protective effect of *Physalis angulata* against MTX-induced toxicity. Levels of serotonin and immune protein such as Interferon gamma (IFN- γ) were determined in rat cerebellum. In addition, the activities of several antioxidant enzymes and lipids oxidation analyses in rat cerebellum were assayed. Thiobarbituric acid reactive substances (TBARS) levels were also measured as a marker of lipid peroxidation.

Serotonin Determination

Assessment of serotonin levels in cerebellum was carried out according to Hrycaj *et al.*²³. Serotonin was measured using an ELISA kit (Immuno-Biological Laboratories, Germany). The assay procedure followed the competitive ELISA protocols whereby competition takes place between biotinylated and non-biotinylated antigens for a fixed number of antibody binding sites. The amount of biotinylated antigens bound to the antibodies was inversely proportional to the N-acyl-serotonin concentration of the sample.

Assessment of IFN- γ

Level of IFN- γ was determined in cerebellum tissues according to Trotta *et al.*²⁴. Level of IFN- γ was measured using an ELISA kit (Thermo Scientific, USA) according to the manufacturer's instructions. This assay employs a quantitative sandwich enzyme immunoassay technique that measures IFN- γ in few hours. Level of rat IFN- γ less than 2 pg/mL was determined by this kit as minimum level.

Determination of Antioxidant Enzymes Activities

Determination of SOD Activity

Total SOD activity was assayed according to Becana *et al.*²⁵. The reaction products were measured at 560 nm. One unit of SOD (U) was defined as the amount of enzyme that produced a 50% inhibition of NBT reduction under assay condition.

Determination of CAT Activity

Catalase (CAT) activity was assayed by

the method of Aebi²⁶ using spectrophotometry. This method is based on the disappearance of H₂O₂ at 240 nm in a reaction medium containing 20 mM H₂O₂, 0.1% Triton X-100 and 10 mM potassium phosphate buffer (pH 7.0). CAT activity is represented as absorption change in time unit (1 min) per mg protein.

Determination of Lipid Oxidation Analysis by Determination of TBARS

Thiobarbituric acid-reactive substances (TBARS) were measured according to Ohkawa *et al.*²⁷. TBARS were determined by a spectrophotometer at 532 nm. A calibration curve was performed using 1,1,3,3-tetramethoxypropane as a standard. TBARS were represented as nmol TBARS/mg protein.

RESULTS

Effect of MTX and PAE on Serotonin Levels

Assessment of serotonin levels in cerebellum of male rats are summarized in Figure 1. The results revealed that MTX treatment reduced the level of serotonin in cerebellum of male rats by 42% compared to control group ($P < 0.01$). However, the level of serotonin in group of rats treated with all doses of PAE was relatively similar to that in control group. Treatment of MTX-treated rats with low (10 mg/kg) and medium (20 mg/kg) of PAE had no significant effect on serotonin level, however, using high dose of PAE (30 mg/kg) increased the level of serotonin in MTX-treated rats compared with rats treated with MTX alone ($P < 0.05$). This augmentation did not reach the normal level of serotonin in control ($P < 0.05$).

Effect of MTX and PAE on IFN- γ Levels

Determination of IFN- γ levels in the cerebellum of male rats are illustrated in Figure 2. The results revealed that MTX treatment elevated the level of IFN- γ significantly compared to control group ($P < 0.01$). However, the levels of IFN- γ in group of rats treated with all doses of PAE were similar to control group. Moreover, treatment of MTX-treated rats with low (10 mg/kg) and medium (20 mg/kg) doses of PAE significantly decreased IFN- γ level induced by MTX and control ($P < 0.05$). However, the high dose of PAE induced remarkable decrease of level of IFN- γ in MTX-treated rats compared with the rats treated with MTX alone ($P < 0.01$).

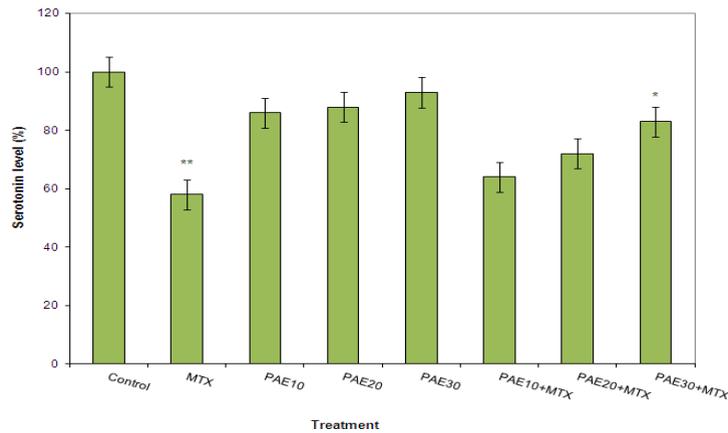


Fig. 1. Changes in serotonin levels (%) in cerebellum tissues of male rats treated with methotrexate (MTX) and *Physalisangulata* extract (PAE). Data are presented as mean \pm SEM. Values marked with an asterisk (*) are significantly different ($P < 0.05$) and (**) are significantly different ($P < 0.01$)

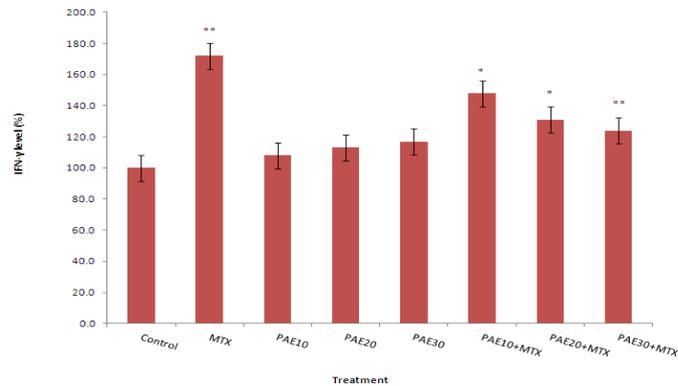


Fig. 2. Changes in IFN- γ levels (%) in cerebellum tissues of male rats treated with methotrexate (MTX) and *Physalisangulata* extract (PAE). Data are presented as mean \pm SEM. Values marked with an asterisk (*) are significantly different ($P < 0.05$) and (**) are significantly different ($P < 0.01$)

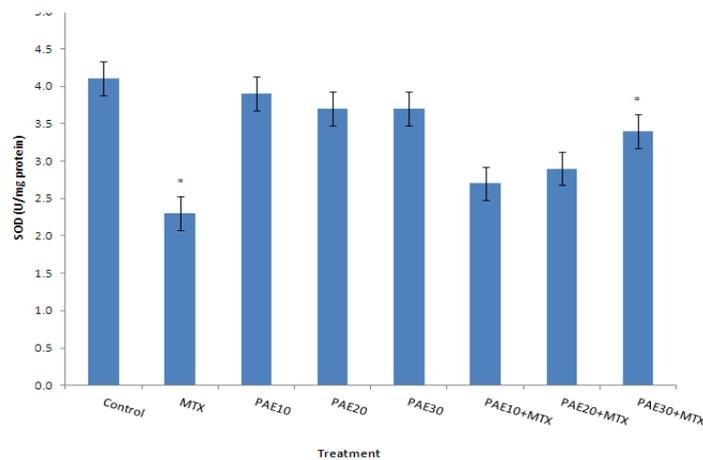


Fig. 3. Superoxide dismutase (SOD) activity in the cerebellum of male rats injected by methotrexate (MTX) and treated with *Physalisangulata* extract (PAE). Data are presented as mean \pm SEM. Values marked with an asterisk (*) are significantly different ($P < 0.05$)

Effect of MTX and PAE on SOD and CAT Activities

Figures 3 and 4 showed the activities of the antioxidant enzymes SOD and CAT in the cerebellum of male rats. The effect of MTX and PAE on SOD and CAT were nearly similar. The data revealed that male rats exposed to MTX decreased significantly the activity levels of SOD and CAT enzymes compared to control rats ($P < 0.05$). However, male rats treated with all doses of PAE did not alter the activity levels of SOD and CAT compared with control rats. The treatment of MTX-rats with low (10mg/kg) and medium (20mg/kg) doses of PAE increased slightly the activity levels of SOD and CAT compared with rats treated

with MTX alone, but using high dose (30mg/kg) of PAE significantly augmented the activity levels of SOD and CAT compared with rats treated with MTX alone ($P < 0.05$).

Effect of MTX and PAE on Lipids Oxidation Level

Levels of TBARS in the cerebellum tissues of male rats treated with MTX and PAE is summarized in Figure 5. The results indicated that TBARS content was significantly higher in the cerebellum tissues of male rats treated with MTX compared with those of control group ($P < 0.001$). In addition, the level of TBARS in the cerebellum tissues of male rats treated with all doses of PAE alone has no effect and was nearly similar to control

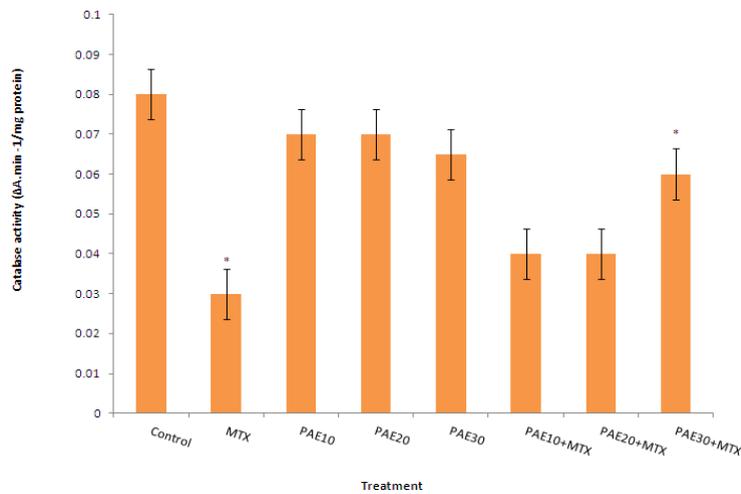


Fig. 4. Catalase activity in the cerebellum of male rats injected by methotrexate (MTX) and treated with *Physalisangulata* extract (PAE). Data are presented as mean ± SEM. Values marked with an asterisk (*) are significantly different ($P < 0.05$)

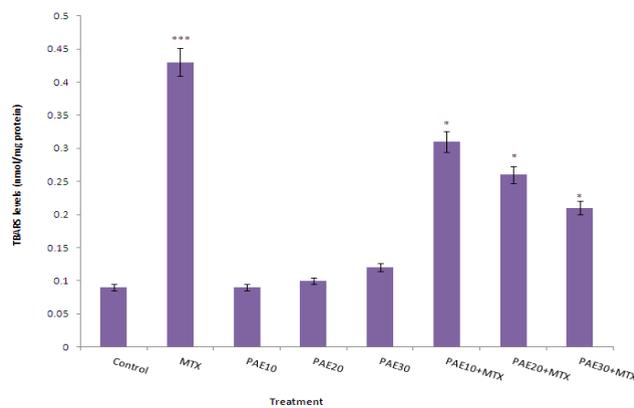


Fig. 5. TBARS level in the cerebellum of male rats injected by methotrexate (MTX) and treated with *Physalisangulata* extract (PAE). Data are presented as mean ± SEM. Values marked with an asterisk (*) are significantly different ($P < 0.05$) and (***) are significantly different ($P < 0.001$)

group. Treatment of MTX-treated rats with PAE inhibited the effect of MTX significantly ($P < 0.05$), but the most effect dose to reduce the hazardous effect of MTX was at (30mg/kg).

DISCUSSION

Oxidative stress results from an imbalance between the production of ROS and the system's ability to detoxify the reactive intermediates or repair the resulting damage¹⁰. In the present study, oxidative stress induced by MTX has been shown to modulate levels and activity of serotonin and IFN- γ in male rats. The obtained data showed that MTX caused multiple signs of brain toxicity as measured by reduction of serotonin and increase in IFN- γ as two biomarkers of brain dysfunction. Similar to the present findings, Aldbass *et al.*,¹⁰ found that oxidative stress have been shown to modulate levels and activity of serotonin, dopamine and glutamate, the principal neurotransmitters.

It's been known that ROS is accumulated in the body from the disequilibrium between oxidants and antioxidants²⁸. The increase of the antioxidant enzymes such as SOD and CAT are removed by ROS¹. Accumulation of ROS can cause chemical and functional modification of DNA, RNA, protein, lipid and carbohydrate moieties and leading to cellular dysfunction¹⁰. Our present study showed that MTX could induce significant redox imbalance through decrease the activity levels of SOD and CAT. Similar to this, Rajamani *et al.*²⁹ reported that marked reduction in the levels of antioxidant enzymes were observed in male rats treated with MTX. Bhalla and Dhawan³⁰ also added that MTX administration increases the level of lipid in the cerebellum and decreases the enzyme activities of SOD and CAT capacity.

The present study revealed that MTX treatment increased lipid oxidation in the form of TBARS level. This result is supported by Drafi *et al.*³¹ who noticed that lipids in brain tissues due to the excessive production of ROS in rats treated with MTX.

In the present study, the oxidative damage increasing with MTX in the cerebellum was prevented by PAE treatment. PAE treatment inhibited the changes of levels of the serotonin and immune protein IFN- γ , antioxidant enzymes and lipid oxidation where having no effect on control

rat. In consistent, Abdel Moneim and El-Deib²¹ found that *physalis* sp. exhibited a significant induction in the activities of SOD, CAT and conversely showed significant decrease in lipid and NO levels compared to carbon tetrachloride (CCl₄)-treated rats. They observed that *physalis* extract affords protection by impairing CCl₄ mediated lipid peroxidation, through decreased production of free radical derivatives.

Physalis sp. contain various chemical compounds like 28-hydroxywithanolide, withanolides, phygrine, kaempferol, and quercetin di- and tri-glycosides are reported to be present in *Physalis* sp.³². The antioxidant effect of flavonoids that was found in *Physalis* increase the process of regeneration and may be due to scavenge of free radicals, supplying a competitive substrate for unsaturated lipids in the membrane or accelerating the repair mechanism of damaged cell membrane³³.

The present results suggested the protective effects of *Physalis* sp. extract against MTX-induced oxidative stress, could be attributed to the high levels of polyphenols and other antioxidants like flavonoids since *Physalis* extract recovered the activities of the antioxidant enzymes such as SOD and CAT in MTX-treated rats.

In consistent with our justification Hassan *et al.*³⁴ and Abdel Moneim and El-Deib²¹ indicated that depletion of antioxidant enzymes and induction of membrane lipid peroxidation may prompt the extrinsic or intrinsic apoptotic pathways which suggest another possible mechanism of *physalis* extract that inhibited the oxidative stress regulating apoptosis which may attributes to the flavonoids. Therefore, we suggest that the induction of antioxidant enzymatic defense systems and suppression of lipid oxidation by *Physalis* extract could be effective in preventing apoptosis activation by caspase cascades triggered by MTX which might be supported by numerous studies^{34,35}.

In conclusion, the present results revealed that PAE attenuates the neurotoxicity induced by MTX in male rats. The protective effects of PAE are performed through multiple ways. PAE scavenges free radicals that are produced by MTX, increases the activity of antioxidant-defense system and a greater susceptibility of the brain to oxidant stress might be anticipated. Therefore, *Physalis* extract may be used as a potential dietary antioxidant to

preventing diseases caused by ROS or ameliorating oxidative damage in tissues.

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