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Ameliorative effect of pumpkin seed oil against emamectin induced toxicity in mice

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ABSTRACT

The current study was conducted to evaluate the toxic effects of emamectin insecticide in mice and the possible protective effect of pumpkin seed oil. Treated mice received emamectin benzoate in the diet at 75-ppm for 8 weeks, while another group of animals received emamectin in addition to pumpkin seed oil at a dose of 4 ml/kg. Biochemical analysis of MDA, DNA fragmentation, GSH, CAT and SOD was performed in liver, kidney and brain as oxidant/antioxidant biomarkers. In addition, gene expression of CYP2E1 and Mgst1 and histopathological alterations in these organs were evaluated. Emamectin administration induced oxidative stress in liver and kidney evidenced by elevated levels of MDA and percentage of DNA fragmentation with suppression of GSH level and CAT and SOD activities. Brain showed increase of MDA level with inhibition of SOD activity. Relative expressions of CYP2E1 and Mgst1 genes were significantly elevated in both liver and kidney. Emamectin produced several histopathological changes in liver, kidney and brain. Co-administration of pumpkin seed oil produced considerable protection of liver and kidney and complete protection of brain. In conclusion, pumpkin seed oil has valuable value in ameliorating the toxic insult produced by emamectin in mice.

1. Introduction

Emamectin is structurally similar to ivermectin and abamectin with high efficacy and beneficial selective toxicity in agriculture. It is derived from the avermectins series of natural products which are macrocyclic lactone products derived from the soil microorganism *Streptomyces avermitilis* [1–3]. It is developed for application on vegetables and fruits as a broad-spectrum lepidopteran insecticide [4,5].

Emamectin produces its insecticidal activity through activating γ-aminobutyric acid (GABA) receptors and glutamate-gated chloride channels leading to increase in membrane chloride-ion permeability, which decreases the excitability of neurons and kills insects in 3–4 days due to irreversible paralysis [6,7]. At first, emamectin was considered safe to human beings because GABA-reactive neurons are limited in human CNS [8], but the lipophilicity of emamectin makes it easy to penetrate cell membranes and produce considerable toxicity in human and animals [3].

Emamectin was found to cause elevation of serum transaminases, glucose, uric acid and TNF-α while reduced serum total protein, albumin, globulin and immunoglobulins in addition to production of some histopathological alterations in liver and spleen of rats [9,10]. Moreover, after lactation exposure, it caused neurotoxic effects in rat offspring [4].

Although the information about the effects of emamectin on antioxidant status is scanty, several studies have demonstrated that avermectins group of insecticides produced oxidative stress in intoxicated animals. Abamectin caused elevation of malondialdehyde and reduction of antioxidant enzymes in rats [11,12]. Similarly, it produced oxidative stress in liver and kidney of mothers and offspring after lactational exposure [13]. In addition, avermectin produced oxidative stress in pigeon and human HepG2 cells *in vitro* [14,15].

Apoptosis is a vital component of several processes occurring during development and is critical for the chemical induced cytotoxicity characterized by the cleavage of DNA into several fragments [16]. It can be initiated through one of two pathways; intrinsic or mitochondrial pathway and extrinsic pathway mediated by death receptors [17,18]. Both pathways activate caspases and induce cell death by degrading key structural and nuclear proteins [19].

Although no studies reported occurrence of apoptosis after emamectin intoxication *in vivo*, it has been reported that emamectin induced single and double-strand DNA breaks in human liver cells [15], and chromatin condensation with nuclear fragmentation in leukemia

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K562 and Molt-4 cells *in vitro* [20].

The cytochrome P450 (CYP450) proteins are monooxygenases which catalyze many reactions involved in breakdown of many toxic environmental chemicals and carcinogens that enter the body, drug metabolism and synthesis of cholesterol, steroids and other lipids. This enzyme includes subcategories CYP1, CYP2 and CYP3 in which the CYP2 subfamily is responsible for the majority of P450-mediated metabolism, specially the CYP2E1 subtype which metabolizes both endogenous and exogenous substances [21]. Changes in expression of CYP2E1 are found to be involved in various pathologies induced by toxic substances [22,23].

Microsomal glutathione S-transferase 1 (Mgst1) gene encodes a protein that is responsible for the reduction of lipid hydroperoxides and conjugation of electrophiles with glutathione. This protein is localized to the outer mitochondrial membrane and endoplasmic reticulum where it is thought to protect these membranes from oxidative stress [24,25]. Xenobiotic biotransformation is catalyzed by CYP2E1 and Mgst1 through two phases of metabolic reactions. In phase I, a polar reactive group is introduced into the molecule by oxidation in the presence of CYP450, then the resulting phase I metabolites are the target for a second type of phase II reactions through conjugation with glutathione by glutathione S-transferase to enhance xenobiotic solubility and facilitate its elimination outside the body [26]. This biotransformation results in formation of reactive intermediaries which may be more harmful than the original xenobiotic and are called bioactive compounds as they are the initial event for a lot of chemically induced toxicity [27].

Pumpkin (*Cucurbita pepo*) seeds are a popular snack in many countries including Egypt. They can be consumed roasted or raw and be used in baking cakes and bread. In addition, pumpkin seed oil has a wide acceptance as edible oil of good nutritional value [28,29]. One of the crucial therapeutic benefits of pumpkin seed oil is its use to treat benign prostate hyperplasia and symptomatic micturition disorders [30].

Animal experiments demonstrated that pumpkin seed oil can be used for treatment of hypertension [31], hypercholesterolemia [32], arthritis [33] and diabetes by promoting hypoglycemic activity [34]. Diets rich in pumpkin seeds have been associated with lower levels of lung, gastric, breast and colorectal cancer [35]. Furthermore, the protective effect of pumpkin seed oil against the toxic insult induced by many toxicants was previously demonstrated [36–39].

This study was performed to explore the production of oxidative stress by emamectin and relating it to emamectin induced apoptosis. In addition, the impact of emamectin on CYP2E1 and Mgst1 gene expression in liver, kidney and brain of mice was studied. The possible ameliorative effect of pumpkin seed oil on emamectin induced toxicity was investigated.

2. Materials and methods

2.1. Chemicals

Speedo containing 5.7% emamectin benzoate was purchased from Starchem Egypt. Pumpkin seed oil was obtained from Al Tahhan Company, Alwadi Elgadid, Egypt. Malondialdehyde (MDA), glutathione reduced (GSH), catalase (CAT) and superoxide dismutase (SOD) test kits were obtained from Biodiagnostic (Egypt). Total protein kit, Tris base, EDTA, Triton x-100, NaCl and diphenylamine were purchased from sigma Aldrich.

2.2. Animals and experimental design

All methods were approved by the Ethical Committee of Animal Experiments of University of Sadat City, Egypt.

Sixty male albino mice, 6–9 week-old (20-25 g) were purchased from the animal care unit of Vacsera Pharmaceutical Company, Agouza, Egypt. Mice were kept under environmental standard conditions (a 12-h light/dark cycle; temperature maintained at 23 ± 2 °C) and provided with standard diet and water *ad libitum*.

Animals were allocated into 4 groups, 15 animals each. Control group; received no chemical treatment; Emamectin group; received emamectin benzoate in the diet at a dose level of 75-ppm (equivalent to 1/10th oral LD_{50} [3] for 8 weeks; Pumpkin seed oil group; received pumpkin seed oil daily by stomach tube at a dose of 4 ml/kg body weight [37] for 8 weeks; Emamectin + pumpkin seed oil group; received both emamectin benzoate in the diet at 75-ppm and pumpkin seed oil by stomach tube at 4 ml/kg/day for 8 weeks.

At the end of experiment, mice were sacrificed under ether anesthesia and liver, kidney and brain samples were collected and stored at −80 °C for assessment of oxidant/no space antioxidant biomarkers, DNA fragmentation and gene expression. Other portions of organ samples were fixed in 10% neutral buffered formalin and prepared for histopathological examination.

2.3. Assessment of oxidant/antioxidant biomarkers

Lipid peroxidation was investigated by measuring the level of malondialdehyde (MDA) as mentioned by Ohkawa et al. [40]. Glutathione reduced (GSH) was assessed according to Beutler et al. [41]. Catalase (CAT) activity was estimated as described by Aebi [42]. Superoxide dismutase (SOD) activity was measured according to Nishikimi et al. [43]. Total protein in tissue homogenate was determined according to Peterson [44].

2.4. DNA fragmentation assay for apoptosis evaluation

DNA fragmentation was determined according to the method described by Perandones et al. [45]. In brief, 10–20 mg were ground in 400-µl hypotonic lysis buffer (10 mM Tris base, 1 mM EDTA and 0.2% Triton X-100), centrifuged at 3,000 × *g* for 15 min at 4 °C. One-half of the supernatant was used for gel electrophoresis, and the other half together with the pellet were used for quantification of fragmented DNA by the diphenylamine. The developed blue color was quantified at 578-nm. Percentage of DNA fragmentation in each sample was expressed by the formula: %DNA fragmentation = (O.D Supernatant/O.D Supernatant + O.D Pellet) × 100.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR) of CYP2E1 and Mgst1 genes

Total RNA in different cell samples was extracted by using QIAmp RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's manual. The concentration and purity of the total RNA samples were obtained by using a nanodrop ND-1000 spectrophotometer. The isolated RNA was used for cDNA synthesis using reverse transcriptase (Fermentas, EU). Real-time PCR (qPCR) was carried out in a total volume of 20-µl using the reaction mixture of 1 µl cDNA, 0.5 mM of each primer (CYP2E1, forward: AGGCTGTCAAGGAGGTG CTA, reverse: GGAAGTGTGCCTCTCTTTGG; Mgst1, forward: CATCCC TTTGCTCTCTCCTG, reverse: GTCTTCTGGGTTGGCAAAAA and β-actin, forward: TGTTACCAACTGGGACGACA, reverse: GGGGTGTTGAAG GTCTCAAA), iQ SYBR Green Premix (Bio-Rad 170-880, U.S.A.). PCR amplification and analysis were achieved using Bio–Rad iCycler thermal cycler and the MyiQ realtime PCR detection system. The fast start polymerase was activated, and cDNA denatured by pre-incubation for 15 min at 95 °C, the template was amplified for 40 cycles of denaturation programmed for 45 s at 95 °C, annealing of primers at 62 °C programmed for 45 s and extension at 72 °C programmed for 10 min. Fluorescent data were acquired during each extension phase. Each assay includes triplicate samples for each tested cDNAs and no-template negative control, the expression relative to control is calculated using the equation $2^{-\Delta\Delta CT}$ [46].