Potential Antioxidant Effect of Pumpkin Seed Oil against Oxidative Stress induced by Nicotine in Mice

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Abstract
Nicotine (Nc) is a highly toxic chemical compound found in many plants such as tobacco. Exposure of living cells to nicotine is a reason in some biological effects like changes in the activity of antioxidant enzymes, the oxidative stress markers along with genes expression variations in kidney and liver tissues, DNA damaged and apoptosis. It is also known as a cancer-causing agent. Pumpkin seed oil (PSO) is numerously studied and its health benefits are reported. PSO is rich in minerals and healthy fats that reflect its positive effects against oxidative stress and toxicity. Our experiments applied on mice which have been divided into four groups; control, fed with PSO (4 mL/Kg) for two weeks, exposure to nicotine (16 mg/Kg) for ten days and the last one treated with PSO for 5 days pre- and along with nicotine-treatment for the next ten days. Data indicated the genotoxic and cytotoxic activity induced by nicotine in renal and hepatic tissues in vivo. Additionally, the protection and treatment properties of PSO on these tissues have observed. PSO increased intracellular glutathione and up-regulated the antioxidant genes expression like catalase (CAT), Glutathion peroxidase (GPx) and superoxide dismutase (SOD) in liver and kidney. We can conclude that our results proved the safety and effectivity of PSO in protect and treatment mice liver and kidney tissues against oxidative stress induced by nicotine.

Keywords: Pumpkin seed oil, Nicotine, Oxidative stress, Gene expression, Glutathione.

Introduction

Reactive oxygen species (ROS) i.e. •OH, $\bullet^{-}O_2$, and H_2O_2 are generated through normal metabolism or exogenous factors[49]. The exceeding generation of ROS molecules in the cells may destroy some components such as DNA, membrane lipids and protein molecules which caused many diseases and/ or induce cell oxidative stress[50]. Nevertheless, the cells can fight this damage by increasing their antioxidant protection mechanisms that lead to the glutathione (GSH) activation as well as catalase (CAT) (antioxidant enzymes) and superoxide dismutase (SOD). The aforementioned enzymes are extremely important in converting the free radicals to harmless compounds.[20,25] Nicotine is known as one of the oxidative stress producers that belongs to a large biologically active group of natural products produced by plant alkaloids. Despite, it is the major compound present in *Nicotiana tabacum*, it is also found in low levels in some vegetables such as tomatoes, potatoes and eggplants.[27] Literature survey revealed that Nc is responsible for the disordered balance between the generation of ROS and antioxidant proteins availability leading to cell damage.[26]

Additionally, it has been shown that Nc exposure can suppress immune system response and modulate its genes expression[15,53] as well as diminishing the GSH and SOD genes expression in rat.[24,44,49,52] Consequently, genes expression is reported to increase in caspase signaling (Casp8) and p53-apoptosis signaling (Perp) pathways[43].

Human genome can be affected by Nc through induction of somatic mutations and increase cancer incident which makes it a carcinogenic and mutagenic substance[9]. Recent investigations demonstrated that maternal Nc exposure increases renal oxidative stress and has injurious impacts on the embryos[35]. It has been reported that Nc enhanced the deposition of lipids, free fatty acid, sublethal injury in the kidneys and enhanced oxidative stress in mice model[7].

Nowadays, labs are looking for natural products components to fight diseases with harmless side effects. One of these products is pumpkin (*Telferia occidentalis*) seed oil (PSO). Pumpkin is a perennial plant belonging to the Cucurbitaceous family. Its oil contains essential antioxidants and nutritional supplements including lutein, gamma and *p*-tocopherols, vitamin A, carotenes, and tannins, as well as interesting organic acids such as oleic, linoleic and palmitic acid. The plant was also rich in omega 3, 6 and 9, phytosterols, alkaloids and chlorophyll.[1,23,47]

Additionally, pumpkin seeds are known as a source for some minerals like zinc (Zn), manganese (Mn), magnesium (Mg), phosphorus (P), copper (Cu), sodium (Na), calcium (Ca) and iron (Fe).[2,29] Furthermore, PSO has a prophylaxis effect in avoiding the risk of aflatoxin poisoning[19] and its mixture with vitamin E reduced the testicular toxicity in male rats by its effect on 5-α-reductase which converts testosterone into dihedrontestosterone[4,47].

Recent studies demonstrated that PSO phonophoresis can be used successfully in management and treatment of chronic nonbacterial prostatitis (CNBP)[46]. Moreover, POS showed protective effects against lead acetate toxicity in male mice[17] and against aspartame toxicity on the liver and kidney *in vivo*[3]. In addition, PSO supplementation improved arterial function and reduced blood pressure in postmenopausal women[51]. The present study reveals the curative and

antioxidant potential of pumpkin seed oil and nicotine-induced genotoxicity. The effect of PSO was also elaborated on the hepatic and renal oxidative stress of adult male mice.

Material and Methods

Material used: Nicotine was purchased from Merck with a purity of > 99% (CAS-No 54-11-5, Merck, Darmstadt, Germany, > 99% pure) and applied to the animal by oral gavages at 16 mg/Kg dose[8] for ten consecutive days. Pumpkin seed oil, manufactured by EL Captin Company (AlObour City, Cairo, Egypt) was administered in the animal by oral gavages with 4 ml/Kg volume[16] for fifteen consecutive days before and along with nicotine.

Animals Model: Swiss albino mice (40 adult male) were used in the experiment, MFI strain, 25–30 g weight and age ranging from 10-12 weeks obtained from the animal house of King Fahd Medical Research Center, King Abdel Aziz University. The polyplastic steel wire tops cages were used for the animal's caring kept in an air-conditioned room (25°C ± 2). The animals were kept in 12 h light and 12 h complete dark condition by providing 45–75% humidity condition and laboratory standard nourishment *ad libitum.*

Experimental design: All the animals were classified randomly into four groups. Each group contains ten animals. The first group was fed only with distilled water and this is the control group. The second group was fed with PSO (4 mL/Kg) for two weeks, and the third group was administered orally with nicotine (16 mg/Kg each day) for ten consecutive days. The fourth group received PSO for 5 consecutive days before and along with nicotine-treatment in the same way for the next 10 days. After 24 h treatment, animals were euthanized by cervical dislocation and samples of bone marrow, liver and kidney were sampled for the micronucleus assay and oxidative stress determination as well as genes expression analysis.

Micronucleus assay: Salamone and coworker`s[39] protocol for the micronucleus test on bone marrow was followed. Femurs were removed from the animals and a drop of bone marrow was placed on another drop of fetal calf serum on one end of a clean dry glass slide. On a dry and clean glass slide a drop of bone marrow (from the removed femurs of animal) was placed at one end and the fetal calf serum (one drop) was allocated on the other end of the slide. Cells were spread through another clean slide. After that, both slides were dried and as a result we got two slides for each animal. Each slide was stained with 5% Giemsa stain. The microscopic analysis indicated a total of 2000 polychromatic erythrocytes (PCEs) per mouse, after that the micro-nucleated polychromatic erythrocytes (Mn-PCEs) frequency was investigated. The cytotoxicity in bone marrow was calculated from the ratio between PCEs and NCEs.

Oxidative stress parameters

a. Determination of liver and kidney lipid peroxidation: Malondialdehyde (MDA) the product of lipid peroxidation in the kidney and liver was monitored spectrophotometrically with extinction coefficient of 156 mM/cm at 532 nm as reported by Ohkawa and his team[34] and the modified form of Silber and colleagues[42].

b. Measurement of reduced glutathione in kidney and liver: Reduced glutathione content in hepatic and renal tissues homogenate was determined by following Ellman procedure[18]. In this protocol, 5,5-dithiobis-(2- nitrobenzoic acid, DTNB) was reduced by thiol group (SH) of glutathione which results in the formation of 2-nitro-S-mercaptobenzoic acid for one mole of glutathione. The product appeared at λ_{max} = 412 nm with extinction coefficient of 13.7 mM/cm.

Gene expression analysis

a. RNA extraction from kidney and liver: Trizol reagent (Invitrogen, Paisley, UK) was employed for the extraction of total RNA from renal and hepatic tissues. RNA samples were employed to treat DNase in order to eliminate any contamination in the DNA genome in RNase inhibitor presence. Agarose gel electrophoresis and spectrophotometric methods were applied for the integrity and purity of total RNA[40].

b. **Reverse transcription (RT) reaction:** 5 µg of total RNA using Fermentas kits (Sigma, St. Louis, MO) was used for the preparation of first-strand cDNA. During the RT reaction procedure, 60 min at 42ºC (cDNA synthesis) and 5 min at 94ºC (denaturation) program were used. After this, the RT provisions were employed for the amplification of DNA through polymerase chain reaction (PCR)[10]**.**

c. **Gene Amplification:** The first-strand cDNA isolated from various models served as a template for PCR amplification with the following definite pairs of primers as depicted in table 1. Thermal cycler (Thermo, PxE 0.5) is employed for PCR amplification following the protocol of preliminary denaturing step at 95 °C for 5 minutes and then at the same temperature 30 cycles for 30 sec (denaturing), annealing for 60 sec at 55°C and extension for 1 min and extended to 7 mins at 72°C. Agarose gel (1.5%) in Tris-borate-EDTA buffer was used to run PCR product and envisaged in a UV Trans-illuminator. Computerized Gel-Pro (version 3.1 for window 3) used for signal intensities and of the bands of ethidium bromide-stained gel. The levels of β-actin (internal control) and the target gene amplification product were envisaged to normalize for initial variation in sample concentration as a control for reaction efficiency[36].

Statistical Analysis: Analysis of Variance (ANOVA) for the statistically analysis and the variation in the results of various groups were performed through Waller–Duncan k-ratio[48]. The significance of the results was based on probability of $P \leq 0.05$.

Results

The current results showing the treatment of mice with the nicotine displayed significant increase in the frequency of

Mn-PCEs compared with the control group (Table 2 and fig. 1). We observed that PSO was effective in lessening the clastogenic impact of nicotine as evaluated by inhibiting the micronuclei induction which is correlated with the improvement in the polychromatic erythrocytes to normochromatic erythrocytes (PCE/NCE) ratio of bone marrow cells. The PSO was orally administered. The oral administration is the most suitable way just like human exposure. It is worth mentioning that the dose of PSO did not cause a significant increase in the percentage of Mn-PCEs or a decrease in the PCE/NCE ratio compared with the respective control group.

The combined treatments of administering PSO before simultaneous injection with nicotine dose showed a significant ($p < 0.05$) decrease in the frequency of Mn-PCEs and increase in the PCE/NCE ratio when compared with nicotine treated group. The data obtained show that the PSO dose has anticlastogenic and anticytotoxic effects against the nicotine-induced increase in the Mn-PCEs and decreases the ratio of PCE/NCE in bone marrow cells. However, the Mn-PCEs and PCE/NCE ratio in the animals treated with the combination of PSO and nicotine did not reach the control level of PSO-treated group.

Oxidative stress parameters: The alterations in the intracellular glutathione and lipid peroxidation concentration in the renal and hepatic tissues induced by nicotine as well as the improving effects of PSO are recorded in table 3. A significant nicotine depletion ($P < 0.05$) in the renal and hepatic tissues GSH content after treatment (9.52 ± 0.692 and 12.727 ± 0.504 respectively) when compared to that of the control values (13.440 ± 1.95 and 16.125 ± 0.875) was observed. Pumpkin seed oil treatment significantly enhanced the liver GSH content (27.125 ± 0.852) as compared to control group. Moreover, PSO pre and along with Nc significantly improved the GSH content in both tissues when compared to that of the 16 mg/Kg dose of nicotine subjected group.

Table 1
The primer sequences used to amplify the studied genes.

Genes	Primer sequences	Fragment size
β-Actin	5'-CGTGACATTAAGGAGAAGCTGTGC-3' 5'-CTCAGGAGGAGCAATGATCTTGAT-3'[37]	374bp
CAT	5'-GCAGATACCTGTGAACTGTC-3' 5'-GTAGAATGTCCGCACCTGAG-3'[41]	229bp
SOD	5'-GTGCTGAAGGGCGACG-3' 5'-TTTCCACCTTTGCCCAAG-3'[30]	370bp
GPx	5'-GGGCTCCCTGCGGGGCAAGGT-3' 5'-ATGTACTTGGGGTCGGTCATG-3'[45]	354 bp

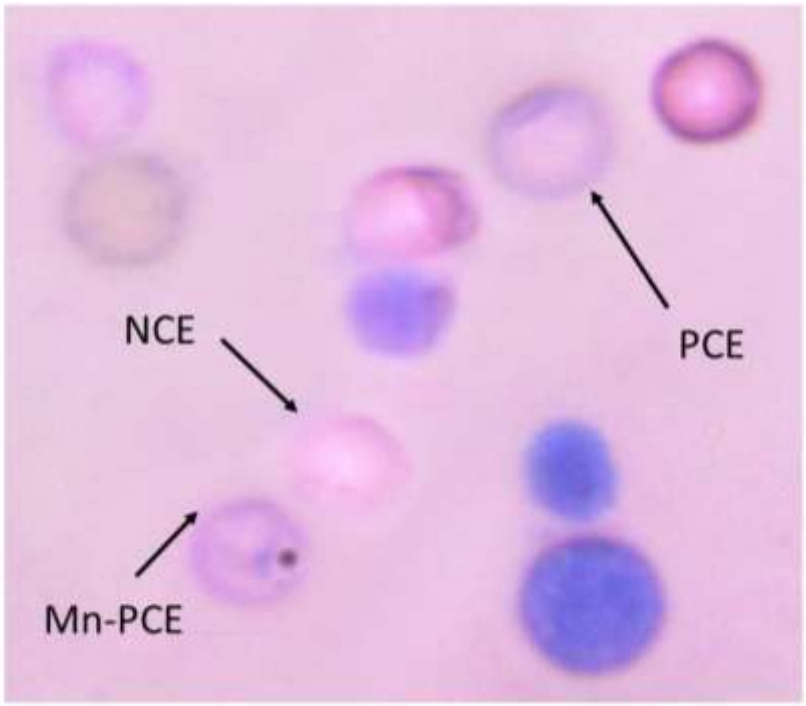

Fig. 1: A smear of bone marrow stained with 5% Giemsa; showing polychromatic erythrocyte (PCE), micronucleated polychromatic erythrocyte (Mn- PCE) and normo chromatic erythrocyte (NCE)

The results of MDA in renal and hepatic tissues (Table 3) exhibited a significant rise in the animals treated with a dose of 16 mg/Kg nicotine for ten days (77.688 ± 0.685 and 45.708 ± 0.751 respectively) compared with control untreated group (42.276 ± 1.314 and 21.727± 0.504 respectively). On the other hand, PSO, when treated pre and along with nicotine, succeeded to decrease MDA in both tissues.

Assessment of genes expression: The gene expression was assessed from the signal intensities generated from amplifying cDNA of CAT, SOD, GPx, and β-actin used as control in this experiment. The ratio among maximum optical density (max OD) and the corresponding max OD of β-actin was obtained for each band of the target amplification product. Expression of CAT, SOD and GPx mRNA in the renal and hepatic tissues of the different treated groups of mice is shortened in table 4 and figures 2-4.

A significant decline was observed in expression level of the inspected genes in both tissues of nicotine-treated group by correlating with the other groups. PSO treated groups indicated insignificant changes in the expression level of CAT, SOD and GPx mRNA contrasted with the control group. PSO treatment with and along with nicotine meaningfully ($P < 0.05$) upregulated the expression of all target genes.

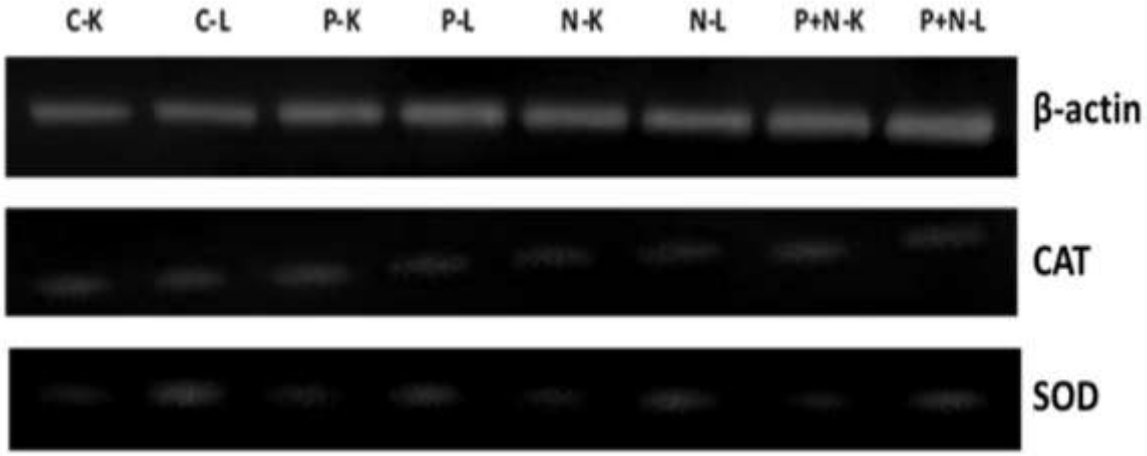

Fig. 2: Agarose gel electrophoresis of CAT, SOD and β-actin RT-PCR products of different groups. C-K: Control kidney, Group C-L: Control liver, Group P-K: Pumpkin treated kidney, Group P-L: Pumpkin treated liver, Group N-K: Nicotine treated kidney, Group N-L: Nicotine treated liver, Group P+N-K: Pumpkin + Nicotine treated kidney and Group P+N-L: Pumpkin + Nicotine treated liver

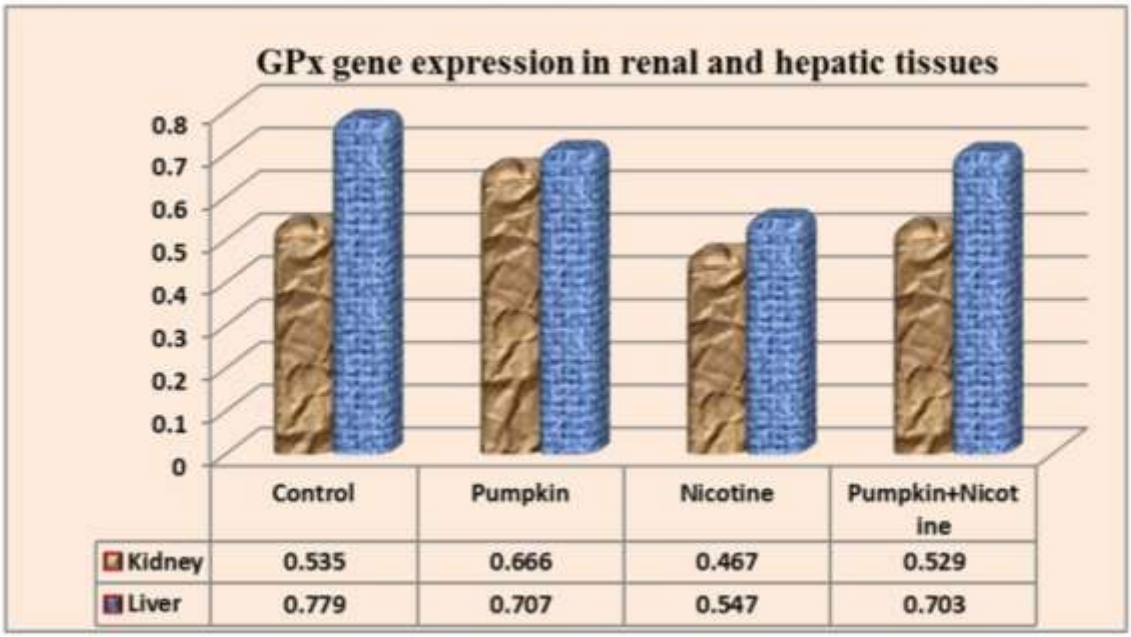

Fig. 3: RNA expression of GPx gene, in the kidney and liver of control and treated mice. The results indicated normalized levels of β-actin gene. The ratios of gene intensity divided β-actin was given by mean ± S.E.

Table 2
Effect of pumpkin seed oil and nicotine on micronuclei induction and polychromatic to normochromatic erythrocytes ratio from femoral bone marrow of mice.

Treatments	Mn-PCEs (Mean ± S.E.)	PCEs % (Mean ± S.E.)	NCEs % (Mean ± S.E.)	PCEs / NCEs (Mean ± S.E.)
Control	3.0 ± 0.49 ^{c}	54.3 ± 2.56 ^{a}	45.7 ± 2.55 ^{c}	1.24 ± 0.12 ^{a}
Pumpkin	2.7 ± 0.51 ^{c}	56.8 ± 1.25 ^{a}	49.6 ± 1.25 ^{c}	1.11 ± 0.05 ^{a}
Nicotine	20.3 ± 1.52 ^{a}	37.8 ± 1.46 ^{c}	62.2 ± 1.46 ^{a}	0.61 ± 0.04 ^{c}
Pumpkin+Nicotine	9.0 ± 0.32 ^{b}	47.2 ± 1.16 ^{ab}	52.8 ± 1.15 ^{bc}	0.90 ± 0.04 ^{ab}

Ten mice per group. The means values are designated with superscripts a, b and c by different group in the same column t at $P < 0.05$ significance difference.

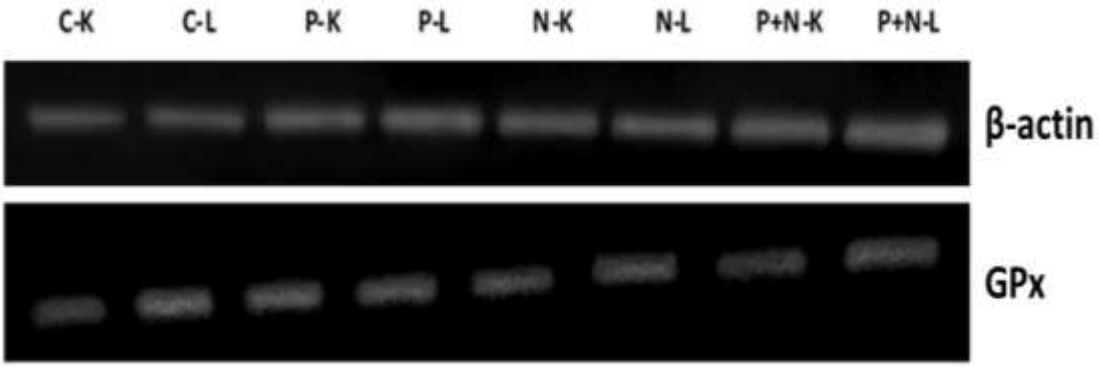

Fig. 4: Agarose gel electrophoresis of GPx and β-actin RT-PCR products of different groups. C-K: Control kidney, Group C-L: Control liver, Group P-K: Pumpkin treated kidney, Group P-L: Pumpkin treated liver, Group N-K: Nicotine treated kidney, Group N-L: Nicotine treated liver, Group P+N-K: Pumpkin + Nicotine treated kidney and Group P+N-L: Pumpkin + Nicotine treated liver

Table 3
Effect of pumpkin seed oil and nicotine on the intracellular glutathione and lipid peroxidation concentration in the renal and hepatic tissues of mice.

Treatment	Glutathione mM/cm		Lipid peroxidation mM/cm	
	Kidney	Liver	Kidney	Liver
Control	13.440 ± 1.95 ^{a}	16.125 ± 0.875 ^{c}	42.276 ± 1.314 ^{c}	21.528 ± 1.228 ^{c}
Pumpkin	13.686 ± 0.520 ^{a}	27.852 ± 1.339 ^{a}	40.601 ± 0.342 ^{c}	19.344 ± 0.776 ^{c}
Nicotine	9.521 ± 0.692 ^{b}	12.727 ± 0.504 ^{d}	77.688 ± 0.685 ^{a}	45.708 ± 0.751 ^{a}
Pumpkin + Nicotine	13.179 ± 1.258 ^{a}	19.934 ± 0.940 ^{b}	46.956 ± 0.898 ^{b}	28.010 ± 0.577 ^{b}

Ten mice per group was used in the experiment, the means values are designated with superscripts a, b and c by different group in the same column t at $P < 0.05$ significance difference.

Table 4
RNA expression of CAT and SOD in the kidney and liver of control and treated mice tissues.

Treatment	Relative transcript level of CAT gene		Relative transcript level of SOD gene	
	Kidney	Liver	Kidney	Liver
Control	0.282 ± 0.058 ^{a}	0.319 ± 0.057 ^{a}	0.157± 0.030 ^{a}	0.274 ± 0.058 ^{a}
Pumpkin	0.263 ± 0.036 ^{a}	0.271 ± 0.041 ^{ab}	0.154 ± 0.028 ^{a}	0.260 ± 0.035 ^{a}
Nicotine	0.133 ± 0.019 ^{c}	0.153 ± 0.031 ^{c}	0.124 ± 0.017 ^{b}	0.171 ± 0.060 ^{b}
Pumpkin + Nicotine	0.240 ± 0.023 ^{b}	0.264 ± 0.029 ^{b}	0.149 ± 0.028 ^{ab}	0.258 ± 0.030 ^{a}

The results indicated normalized levels of β-actin gene. The ratios of gene intensity divided β-actin was given by mean ± S.E. Ten mice per group was used in the experiment, the means values are designated with superscripts a, b and c by different group in the same column t at $P<0.05$ significance difference.

Discussion

PSO are not well scrutinized before to protect nicotine toxicity or its treatment. Long term health risks and acute toxicity are related to nicotine uses[8,13]. Several studies have been reported, which explored the nicotine metabolism and its effect on organisms[28]. We explored the antioxidant and antigenotoxic potential of PSO on the nicotine induced mice. We did not observe the genotoxic or mutagenic possessions of PSO in this study. Elfiky et al[16] reported that a dose of 4ml/Kg of PSO does not produced any genotoxic effect. Also, Das and Rao[14] demonstrated that polyunsaturated fatty acids like PSO, induced insignificant changes in the number of Mn-PCEs compared to those of the control and these results agreed with the present results.

The present data showed the clastogenic effect of nicotine which is the mean of micronucleated polychromatic erythrocytes (MnPCEs) and ratio of mean percentage of polychromatic to normochromatic erythrocytes (PCEs/NCEs). A significant and gradually increase in Mn-PCEs mean number has been found but a significant decrease in ratio of mean percentage of PCEs/ NCEs comparing with control indicating genotoxic and cytotoxic effects was also found.

We believed that DNA damaged due to nicotine might not be repaired[6,8,22]. This observation was based on the fact that micronuclei are biomarkers of chromosome which on breakage or loss could not be repaired[11,12,38]. An improvement in the ratio of mean percentage of PCEs/NCEs and a significant decrease in the increase of the mean number of Mn-PCEs induced by nicotine toxicity alone was observed by treating with nicotine and PSO. Our observation demonstrated that PSO did not pose toxic nor adverse events.

The antioxidant defense system of the cell such as enzymes, including SOD, CAT and GSH-Px, is responsible to convert the harmful free radicals into harmless products which are believed as the main indicators of oxidative stress[20,25]. Several reports have been reported in the literature regarding the oxidative stress-inducing potential of nicotine in animal species and its detoxifications[21,22,31]. In this study, we indicated the performance of PSO to exert its antioxidant effect *in vivo* through the antioxidant system. We propose that the oxidative stress was due to nicotine which is responsible for the exhaustion in glutathione peroxidase activity and glutathione (GSH) level[32].

An enhanced in lipid peroxidation (LPx) was observed in the MDA level of liver and kidney tissues after treating the mice with nicotine. No significant difference in the tissue MDA levels was found between PSO administered and control group, suggesting no adverse effect of PSO on the antioxidant defense system at specific dose and time period. Contrary, determining the enhanced hepatic GSH activity insignificantly changed renal GSH activity, with insignificant increase in the MDA levels of these tissues suggesting that PSO is safe and does not produce any adverse effect on the antioxidant system of the organism.

The change in the enzymatic activities is associated with PSO composition. Therefore, we believed that the PSO brought some physiological changes in the enzymatic activity as PSO helps in eliminating the dangerous free radicals generating in the system[16]. Also, the down-regulated mRNA of the hepatic and renal CAT, GPx and SOD in nicotine treated mice is alleviated by PSO treatments signifying the transcriptional control by pumpkin. In relevant researches[5,19,33] literature survey suggested that PS/extract/isolate/oil modified some antioxidant enzyme activity.

Conclusion

The study report indicated the exploration of nicotine at indicated dose and exposed time and resulted in genotoxic and cytotoxic effects as represented by induction of Mn-PCEs, decreased the ratio of PCEs/NCEs, changes in the activity of antioxidant enzymes, the oxidative stress markers along with genes expression variations in kidney and liver tissues which are characteristic to the reported data. PSO was safe and success to overwhelm the toxicity and defend mice against oxidative stress induced by nicotine.

The mechanism by which PSO induces its protection activity may be due to its ability to increase intracellular glutathione and up-regulation of antioxidant genes expression which seems to be attributed to the powerful antioxidant compounds in the pumpkin.

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