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Research Article
Pumpkin and Vitamin E as Potent Modulators of Apoptosis in Gentamicin-induced Rat Nephrotoxicity

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Abstract

Background and Objective: Translocation of cytochrome c into the cytosol is a main mechanism of gentamicin induced renal apoptosis even through its direct or indirect effects on mitochondrial membrane. The current study aimed to investigate the possible protective effect of pumpkin in relation to vitamin E as a common natural antioxidant on a nephrotoxic model induced by gentamicin. **Materials and Methods:** About 40 male rats were divided into four groups, of 10 each: Control group, G-treated group (120 mg kg^{-1}/day i.p., for 15 days), G+Vit. E-treated group (200 mg kg^{-1} orally for 45 days) and G+PK-treated group (20% of the diet for 45 days). Gentamicin injection was started on the 30th day for both last groups. All data were statistically evaluated by one-way analysis of variance (ANOVA) using SPSS software, version 15 (Chicago, USA) followed by the Tukey's *post hoc* test for comparisons. **Results:** This study revealed that pumpkin significantly decreased BAX/BCL-2 ratio and attenuated gentamicin induced apoptosis. It also exhibited powerful antioxidant effects as indicated by a significant decrement in MDA level and a significant increment in total antioxidant capacity (TAC) level and catalase (CAT) activity. Furthermore, pumpkin successfully improved the kidney function of rats which was manifested by amelioration of deteriorated serum creatinine, urea, sodium and potassium levels and this effect is thought to be as a result of the anti-apoptotic and anti-oxidant properties of pumpkin. **Conclusion:** The performance of pumpkin as anti-apoptotic and antioxidant was to some extent similar to that of vitamin E and it successfully protected against the toxic direct and indirect effects of gentamicin on kidney through down regulation of BAX and up regulation of BCL-2 gene expression and further inhibition of mitochondrial cytochrome c translocation into cytosol.

Key words: Pumpkin, gentamicin, nephrotoxicity, malondialdehyde, apoptosis, BAX/BCL-2 ratio

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Kidneys are vital tissues that clean the blood from toxins and metabolic wastes and maintain homeostasis. Various environmental agents, including definite drugs, may impact kidney functions[1]. Some chemical compounds and drugs disrupt the function and structure of different tissues and produce adverse effects within the kidney, liver, intestine and heart[2]. There are many factors rendering any organ vulnerable to the toxicity of xenobiotics including tissue-specific distribution of this substance and its selective accumulation, its tissue-specific biochemistry or metabolic activation to a reactive toxicant in addition to the organ's ability to repair the characteristic damage[3].

Gentamicin, an aminoglycoside antibiotic, is the most commonly prescribed antibiotic for the treatment of Gram-negative bacterial infections. The drug has been the only effective therapeutic intervention in many cases against bacterial strains resistant to other different antibiotics[4]. Many factors were responsible for their success and continued use, including their rapid bactericidal effect, a low incidence of bacterial resistance, chemical stability, interaction with beta-lactam antibiotics and low cost[5]. The main reason for its cautious therapeutic use is its side effects. These side effects are nephrotoxicity and auditory ototoxicity. They complicate therapy with this antibiotic. An attempt to diminish gentamicin toxicity has been done by using a single dailydose, but its long-term use can increase the severity of those side effects and the inadequacy of its plasma concentrations can lead to treatment failure[6].

Extensive academic interest has been focused on the participation of reactive oxygen species (ROS) in kidney diseases. Oxidative stress is induced by a diversity of factors, including xenobiotics and drugs. The gentamicin toxicity is supposed to be interrelated to the generation of ROS in the kidney[7]. The ROS induce vasoconstriction and reduce glomerular filtration rate. Additionally, ROS induce cellular damage and necrosis via protein modification and lipid peroxidation[5]. Gentamicin nephrotoxicity is characterized by tubular injury arising from tubular epithelial cell cytotoxicity. Treatment of rats with gentamicin induces apoptosis Li *et al.*[8] of tubular epithelial cells in vivo and also in cultured cells[9]. One of the great interests of researchers is to look for solution of drug toxicity including nephrotoxicity. They focus on the use of medicinal plants and natural products such as herbs, fruits and vegetables that are rich in antioxidants that can scavenge the harmful ROS responsible for organ toxicity.

Herbal products are commonly considered safe and their medical usages have been increased in developing countries[10]. Free radical scavengers have verified to be useful in lowering the progress of renal damage induced by gentamicin administration[11]. Previous studies stated that dietary fruits and vegetables may protect against various degenerative diseases[12]. Pumpkin is a leafy green vegetable. It belongs to the Cucurbitaceae family. Several pharmacological properties have been reported for these plants including, antioxidant, hepatoprotective, lipid lowering[13], anti-carcinogenic[14], anti-microbial[15], analgesic, anti-ulcer role, nephroprotection[16] and anti-diabetic activity[17].

The antioxidant efficacy of *Cucurbitaceae* seeds was correlated with total phenolic compounds[18]. There are 5-8 novel phenolic glycoside derivatives of 4-Hydroxy benzyl alcohol were identified from different varieties of *Cucurbitaceae* seeds[19]. Pumpkin (*Cucurbitapepo* L.) was widely studied for uses in herbal medicine[20]. It is considered as a good supply of bioactive dietary[21]. Phytosterols had been documented within the *Cucurbitaceae* as biologically active components. Less well-studied *Cucurbitaceae* is now receiving attention owing to both food and medicinal applications[18]. Pumpkin has many health benefits because it is rich in antioxidant and vitamins, such as tocopherols and carotenoids[22].

Therefor the current study aimed to assess the possible protective effect of pumpkin on a rat nephrotoxic model induced by gentamicin compared to vitamin E as a common natural antioxidant as a trial study to share in finding a solution of gentamicin-induced nephrotoxicity.

MATERIALS AND METHODS

Chemicals: Gentamicin-sulphate (Garamycin) was obtained from Schering-Plough (USA). Vitamin E® capsules (400 mg) were obtained from Safe Pharma. Malondialdehyde (MDA), catalase (CAT), total antioxidant capacity (TAC), sodium and potassium commercial kits were purchased from Biodiagnostic Company for research kits, Egypt. Commercial diagnostic kits of serum creatinine and urea were obtained from Spinreact and Diamond companies, respectively. Other non-mentioned chemicals used in the current study were the highest analytical grade and obtained from Sigma, USA.

Natural substance: Pumpkin fruits were obtained from local market. They were peeled and homogenized, then divided into aliquots that were kept frozen at -20°C till the time of use.

Animals and experimental design: The study involved the use of 40 adult male sprague dawley rats, whose weights were 120-150 g at the start of the experiment. The animals were obtained from the Egyptian Organization for Biological Products and Vaccines. Rats were kept in standard cages at room temperature (25±2°C) with humidity (70%) and exposed to natural daily light-dark cycles. Rats have nourished *ad libitum* and clean water was continuously available. The experiment was carried out in Biochemistry Department, Faculty of Veterinary Medicine, Beni-Suef University in the period from the beginning of March to the beginning of last week of April, 2016. All experimental measures were performed in accordance with the guidelines for the care and use of laboratory animals and were approved by the local Animal Care and Use Committee at Beni-Suef University.

One week after acclimatization, the rats were allocated into 4 groups, consisting of a control and three experimental groups, each contains 10 rats.

Animals in the control group served as control and were received normal saline daily by intraperitoneal injection. Rats in the G-treated group were administered normal saline by intraperitoneal injection for 30 days and then followed by intraperitoneal injection of gentamicin for 15 days at a dose of 120 mg kg^{-1}/day[23]. Rats in G+Vit., E-treated group were administered vitamin E orally for 45 days at a dose of 200 mg kg^{-1}/day[24], the gentamicin "120 mg kg^{-1}/day" intraperitoneal injection was started on the 30th day until the end of the experiment. Rats in G+PK-treated group were received pumpkin at a dose of 6% of the diet for 45 days[25], the gentamicin "120 mg kg^{-1}/day" intraperitoneal injection was started on the 30th day until the end of the experiment.

Sampling and biochemical analysis

Blood samples collection: About 24 h after the last dose of treatment, blood samples were collected via retro-orbital bleeding. Blood samples were left at room temperature for 20 min to clot. The clotted blood samples were centrifuged at 1500 rpm for 15 min to obtain serum samples. The serum samples were kept at -20°C till use.

Specimen collection: Rats were then sacrificed and kidney tissues were quickly removed and then washed with physiological saline. The kidney samples were divided into two parts. The first part of the kidney (0.5 g) was suspended in 5 mL phosphate buffered saline (pH: 6.8) for homogenization (Teflon Homogenizer, India). The kidney tissue homogenate was centrifuged at 20,000 rpm for 10 min at 4°C using a high-speed cooling centrifuge. The supernatants were kept at -20°C till the time of determination of oxidative/antioxidant parameters[26]. The second part of the kidney was preserved at -80°C for measurement of BAX and BCL-2 gene expression by real time PCR (RT-PCR) after the addition of RNase inhibitors.

Detection of BAX and BCL-2 gene expression by real time-polymerase chain reaction (RT-PCR): Total RNA was extracted from kidney tissue homogenate using SV total RNA Isolation System (Promega, Madison, WI, USA) according to manufacturer's instruction. The RNA concentration was measured using a UV spectrophotometer.

Complementary DNA (cDNA) synthesis: About 1 µg of total RNA was reversely transcribed by oligonucleotide using a reverse transcription kit (Super Script III First-Strand Synthesis System) as described in the manufacturer's protocol (K1621, Fermentas, Waltham, MA, USA).

Real-time quantitative polymerase chain reaction (RT-PCR): Real-time PCR amplification was performed using an Applied Biosystems with software version 3.1 (StepOne™, USA). The PCR assay with the primer sets (the primers of BAX, BCL-2 and β-actin) were optimized at the annealing temperature. The primer sequence was shown in Table 1. The PCR amplification reactions were carried out at 50°C for 2 min, 95°C for 10 min and 40 cycles of denaturation for 15 sec and annealing/extension at 60°C for 10 min. The ABI Prism 7500 sequence detection system software was used for analysis of the data obtained from real-time assays. The data were calculated using the v1.7 Sequence detection software from PE Biosystems (Foster City, CA). Relative expressions of BAX and BCL-2 genes were calculated using the comparative Ct method. All values were normalized to the beta-actin gene and reported as fold change. All these steps were performed according to the method described by Livak and Schmittgen[27].

Table 1: Sequences of the primers used for amplification of mRNAs encoding BCL-2 and BAX by quantitative real-time PCR

mRNA	Sequences (5'→3')	Gene accession No.
BCL-2	Forward primer: CATGTGTGTGGAGAGCGTCAA	NM_016993
	Reverse primer: GCCGGTTCAGGTACTCAGTCA	
BAX	Forward primer: GGGGACGAACTGGACAGTAACAT	NM_017059
	Reverse primer: GGAGTCTCACCCAACCACCCT	
β-actin	Forward primer : ATGAGCCCCAGCCTTCTCCAT	NM_007393
	Reverse primer: CCAGCCGAGCCACATCGCTC	

Oxidative/antioxidant markers: The renal MDA concentration was determined according to the method of Satoh[28], which was based on the reaction of thiobarbituric acid with malondialdehyde in acidic medium. The determination of TAC was performed by the reaction of antioxidants in the sample with a defined amount of exogenously provided hydrogen peroxide (H_2O_2)[29]. The CAT activity measurement was based on the reaction of catalase with a known quantity of H_2O_2 according to the method defined by Aebi[30].

Estimation of kidney function tests: The measurement of serum creatinine concentration was based on the reaction of creatinine with sodium picrate forming a red complex according to the method described by Henry *et al.*[31]. The determination of serum urea concentration was based on enzymatic hydrolysis of urea according to the method described by Patton and Crouch[32]. The measurement of serum sodium concentration was dependent on the reaction of sodium ions with excess uranyl acetate and magnesium acetate according to the method described by Trinder[33]. The measurement of serum potassium concentration based on the reaction of potassium ions with sodium tetraphenyl boron forming a colloidal solution which could be measured colorimetrically[34]. All chemical reactions were measured by using Hitachi spectrophotometer, Model U -2000 (Hitachi Ltd. Tokyo, Japan).

Statistical analysis: All data were statistically evaluated by one-way analysis of variance (ANOVA) using SPSS software, version 15 (Chicago, USA) followed by the Tukey's *post hoc* test for comparisons. The results were expressed asa mean±standard error of the mean (SE). The difference was considered statistically significant at $p<0.05$.

RESULTS

Effects of pumpkin and vitamin E on BAX and BCL-2 gene expression levels: Present results revealed that the antiapoptotic properties of pumpkin were similar to that of vitamin E as it decreased BAX and increased BCL-2 gene expression levels (Fig. 1) and decreased BAX/BCL-2 ratio (Fig. 2) and became statistically non-significant as compared to those of the control group at $p<0.05$.

Effects of pumpkin and vitamin E on oxidative/antioxidant parameters: The antioxidant activity of pumpkin was parallel to that of vitamin E as it significantly decreased the elevated renal MDA level in comparison with G-treated group and it became statistically non-significant as compared to that of the control group at $p<0.05$ (Fig. 3). Pumpkin significantly increased renal TAC level and CAT activity as compared to those of the G-treated group and these results were similar to that of vitamin E (Fig. 4, 5).

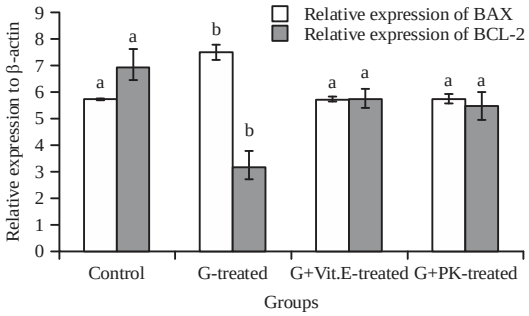

Fig. 1: Relative expression of BAX and BCL-2 in different studied groups
The different letters mean significance difference at $p<0.05$ between different groups

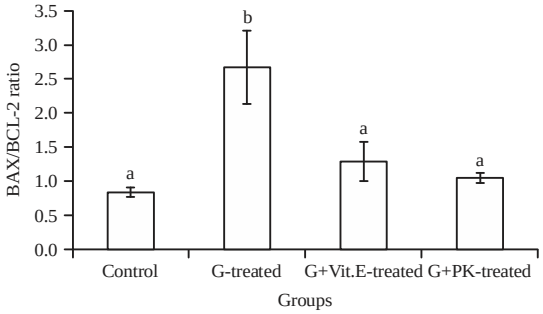

Fig. 2: BAX/BCL-2 ratio in the various studied groups
The different alphabets mean significance difference at $p<0.05$ between different groups

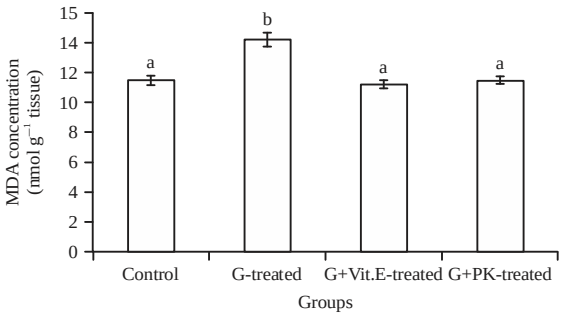

Fig. 3: Changes of renal MDA levels in different studied groups
The different letters mean significance difference at $p<0.05$ between different groups

Effects of pumpkin and vitamin E on kidney function tests: Pumpkin significantly ameliorated the elevated serum creatinine and urea levels as compared to G-treated group and these results were similar to that of vitamin E at $p<0.05$.

Table 2: Changes in renal function tests in the various studied groups

Groups	Creatinine (mg dL^{-1})	Urea (mg dL^{-1})	Sodium (nmol L^{-1})	Potassium (nmol L^{-1})
Control	0.75±0.02^{a}	36.25±0.75^{a}	146.50±0.5^{a}	5.87±0.04^{a}
G-treated	1.12±0.07^{b}	78.50±0.95^{b}	120.00±0.04^{b}	9.12±0.23^{b}
G+Vit. E-treated	0.85±0.06^{a}	35.00±.07^{a}	133.25±1.1^{c}	6.47±0.06^{c}
G+PK-treated	0.75±0.06^{a}	37.00±1.7^{a}	133.75±1.0^{c}	6.55±0.17^{c}

Values are represented as mean±standard error of mean. G: Gentamicin, G+Vit. E: Gentamicin+vitamin E, G+PK: Gentamicin+Pumpkin. The different superscript letters mean a significant difference at (p>0.05) between different groups in the same column

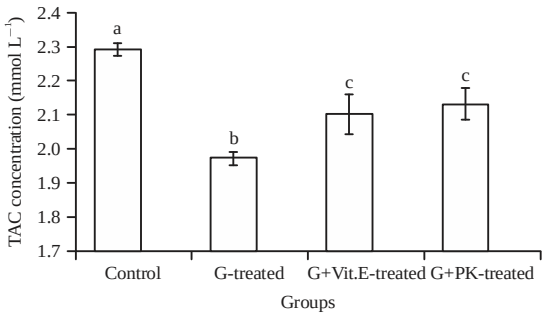

Fig. 4: Changes of renal TAC concentrations in the various studied groups
The different letters mean significance difference at p<0.05 between different groups

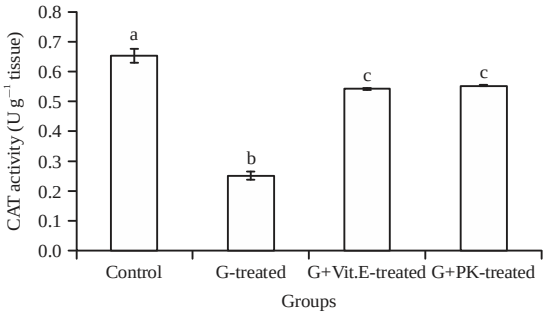

Fig. 5: Changes of renal CAT activities in the different studied groups
The different letters mean significance difference at p<0.05 between different groups

Moreover, pumpkin has a reno-protective effect similar to that of vitamin E as it significantly ameliorated changes of serum sodium and potassium levels as compared to G- treated group at p<0.05 as shown in Table 2.

DISCUSSION

The BCL-2 and BAX, which were two important members of the BCL-2 family, were known to perform anti-apoptotic and pro-apoptotic roles, respectively. The equilibrium between pro- and anti-apoptotic signals from this family had a crucial role in the liberation of cytochrome c and subsequent stimulation of caspases 9 and caspases 3 and finally causing cellular death[35].

In the intrinsic pathway, the apoptotic threshold was established by interactions between three structurally and functionally different subgroups of the BcL-2 protein family on the mitochondrial outer membrane. The first was BH3 (the BCL-2 homology 3) proteins which convey signals to stimulate apoptosis. The second was the pro-apoptotic effector proteins BAX (BcL-2-associated×protein) and BAK (BCL-2 antagonist/killer). The third was the pro-survival cell guardians consisting of BCL-2 itself[36].

Intrinsic apoptotic stimuli initiated BH3-only proteins leading to BAX and BAK activation. The BAX and BAK formed the oligomers which permeabilize the mitochondrial outer membrane leading to release of apoptogenic factors into the cytosol, principally cytochrome c[37]. The binding of cytochrome c to apoptotic protease-activating factor 1 (APAF1) induced its oligomerization and so forming an apoptosome that turned on an initiator caspase, caspase 9. The cleavage and activation of executioner caspases, caspase 3 and caspase 7 were initiated by caspase 9 and so leading to apoptosis. Anti-apoptotic BcL-2 proteins avoided the mitochondrial outer membrane permeability by way of binding BH3-only proteins and so inactivated BAX or BAK[36].

Gentamicin indirectly stimulated mitochondrial intrinsic pathway[38] which was demonstrated in this study by significant elevations in BAX and decrement in BCL-2 gene expression levels in a harmony with Quiros *et al.*[39]. Therefore, gentamicin enhanced the programmed cell death.

The cellular signaling pathways underlying the interaction of oxidative stress-induced cellular death and vitamin E function were currently the issue of active research[40]. The authors found that pre-treatment with vitamin E significantly modulated the effects of gentamicin on BAX and BCL-2 expression levels in agreement with Galal *et al.*[41], who reported that pre-treatment with vitamin E was shown to counteract the effects on members of the BCL-2 family.

Pumpkin supplementation attenuated the apoptosis by increasing BCL-2 and decreasing BAX gene expression levels. These results come in accordance with that obtained by Lopez-Novoa *et al.*[38], who reported that gentamicin-induced apoptosis was inhibited by using of the free radical scavengers.

In addition to changes in BAX/BCL-2 ratio, gentamicin directly initiated apoptosis by ROS generation. Free radicals might alter mitochondrial membrane potential by opening the mitochondrial permeability transition pores (mPTP), liberating cytochrome c with subsequent activation of caspases 9 and caspases 3 and finally leading to cell death[42].

This study revealed that gentamicin significantly elevated the renal MDA level and decreased both renal TAC level and CAT activity in agreement with a previous study[43]. This oxidative stress was coordinated with a reduction in the glomerular filtration rate[44] and direct tubular damage[43] which was recognized in the current study by a marked increase in the serum levels of creatinine, urea and potassium and a reduction in the serum level of sodium confirming kidney injury.

Lipid peroxidation altered the physical and chemical properties of cell membranes and their fluidity resulting in leakage of several degradative enzymes (e.g., proteases and phospholipases) which caused phospholipid and protein degradation and potentially leads to cytotoxicity[45]. Therefore, activation of these enzymes could be a major contributor to apoptosis.

The effect of vitamin E was conceivably attributed to the prevention of the progression of free radical reactions by acting as a peroxyl radical scavenger and guarding polyunsaturated fatty acids within membrane phospholipids and in plasma lipoproteins[46]. Storage of endogenous antioxidants such as vitamin E decreased gradually while reacting with free radicals and so vitamin E dietary supplementation had useful effects.

In this study, vitamin E significantly reduced the oxidative stress induced by gentamicin as manifested by the reduced level of MDA and the increased TAC level and CAT activity in accordance with Minamiyama *et al.*[47], who explained that vitamin E maintained the activities of membrane-bound enzymes at near normal values and thus preserving the integrity of mitochondrial membrane and protecting the enzyme activities from oxidation by free radicals. Subsequently, the alterations in serum levels of creatinine, urea, sodium and potassium detected in G-treated group were ameliorated in response to vitamin E supplementation.

The strong antioxidant activity of pumpkin efficiently protected the mitochondrial membrane as expressed by the considerable decrease in the level of MDA, the increase of TAC level and CAT activity with subsequent amelioration of kidney function biomarkers. These results were in harmony with Eraslan *et al.*[48], who stated that pumpkin caused physiological changes in the activity of antioxidant enzymes due to its potential of eliminating ROS generated under normal biological conditions.

Pumpkin is quite rich in vitamins E and β-carotene. It is well known that vitamins A and vitamin E bound to free radicals and prevented the oxidation of the cell membrane[49]. Their binding to free radicals generated by gentamicin prevented the lipid peroxidation. Additionally, the abundance of phenolic compounds in the pumpkin was taken into concern among its available mechanisms that prevented lipid peroxidation[50]. Furthermore, phytosterols found in the structure of pumpkin, were stated to have an antioxidant effect[51].

CONCLUSION

Present study concluded that pumpkin supplementation reduced cytochrome c translocation and protected kidney against gentamicin induced oxidative stress and apoptosis through its direct action as an antioxidant agent and its indirect anti-apoptotic action by decreasing BAX/BCL-2 ratio. The renoprotective influence of pumpkin was similar to that of vitamin E.

SIGNIFICANT STATEMENT

This study discovers the possible renoprotective effect of pumpkin against gentamicin-induced nephrotoxicity which was found to comparable to that of vitamin E. It can be beneficial for those exposed to drug-induced nephrotoxicity. This study will help the researchers to uncover the critical area of protection against drug-induced renal damage. Thus a new theory on the antiapoptotic and antioxidant of pumpkin and other new medicinal plants may be arrived at.

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