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GENTAMYCIN-INDUCED NEPHROTOXICITY IN CHICKENS: MODULATORY ROLE OF MORINGA OLEIFERA

MOHAMED FAHMY ABOU ELAZAB[*] and NAGWAN EL-HABASHI[**]

[*]Department of Clinical Pathology, Faculty of Veterinary Medicine, Kafr Elsheikh University, Kafr Elsheikh, Egypt.
[**]Department of Pathology, Faculty of Veterinary Medicine, Kafr Elsheikh University, Kafr Elsheikh Egypt.
[**]Corresponding author**:** Nagwan M. El-Habashi, Department of Pathology, Faculty of Veterinary Medicine, Kafr Elsheikh University, Kafr Elsheikh, Egypt.
Tel, Fax number: +20-473231311
Email: nagwan_hab@yahoo.com

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ABSTRACT

The aim of the present study was to determine whether *Moringa oleifera* (MOR) leaf powder, have a modulatory effect against gentamicin (GEN)-induced nephrotoxicity in chickens or not. Twenty laying hens were equally divided into four groups: First group (CON) received standard basal diet only. Second group (GEN) was injected with GEN (50mg/kg body weight, intramuscularly (*i.m.*), twice daily, for 5 consecutive days). Third group (GEN+MOR) was supplemented with MOR leaf powder (10gm/kg diet) daily for 12 consecutive days and injected with GEN (50mg/kg body weight, *i.m.*, twice daily, for 5 consecutive days) starting from the day 8. Fourth group (MOR) was supplemented with MOR leaf powder (10gm/kg diet) daily for 12 consecutive days. GEN administration increased serum creatinine (Cr), blood urea nitrogen (BUN), and malonaldehyde (MDA) and decreased total protein, albumin, reduced glutathione (GSH) content as well as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) activities indicating nephrotoxicity. Furthermore, histopathological findings revealed marked tubular vacuolar degeneration, focal interstitial mononuclear cells infiltrations, congestion, focal cystic change and tubular necrosis in GEN-treated kidneys. However, MOR leaf powder supplementation with GEN administration caused significant decrease of serum Cr, BUN, and MDA and increase of total protein, albumin, GSH, SOD, GPx, and CAT compared with GEN alone. Moreover, GEN+MOR treated group showed slight focal interstitial mononuclear cells infiltrations in renal tissues. These findings suggested that dietary supplementation of MOR leaf powder may prevent or reduce the GEN-induced nephrotoxicity.

Keywords: Chicken; Gentamicin; Moringa oleifera; Nephrotoxicity

INTRODUCTION

Currently, antibiotics are used on a large scale to treat or prevent infections in poultry industry. Gentamicin (GEN), bactericidal aminoglycoside antibiotic, is an important therapeutic agent that commonly used in poultry farms. GEN is effective against many gram-negative and gram-positive bacteria. However, it resulted in nephrotoxicity even when birds are treated with low doses (Flammer, 1994). Gentamycin exhibits concentration dependent microbial killing where the high drug concentration is the best way to obtain rapid bactericidal effects (Hagen and Oymar, 2009). The higher doses are more preferable than low doses; however, there is no accurate data about the corresponding toxicities. GEN is commonly used in birds; but resulted in kidney enlargement and changes resembling other causes of renal failure (Schmidt *et al.,* 2003). GEN is an effective and economical drug used to control infectious diseases in poultry but is highly toxic and had slow clearance from the body (Islam *et al.,* 2011). Experimental GEN toxicity has been reported in growing cockerels (Khan *et al.,* 2008), day old broiler chicks (Saleemi *et al.,* 2009), in commercial layers (Islam *et al.,* 2011) and growing broilers (Javed *et al.,* 2013). GEN is extensively used in combination with other antibiotics in poultry practice in Egypt. Gentamycin is used repeatedly for treatment of different bacterial infections in laying hens, its continuous use lead to severe nephrotoxicity that heavily influences their welfare and eventually may even lead to high mortality (Islam *et al.,* 2011).

The mechanism of GEN-induced nephrotoxicity is still unknown. However, reactive oxygen species (ROS) appear to be involved in the pathophysiology of GEN-induced nephrotoxicity (Yang *et al.,* 1995; Walker *et al.,* 1999; Martı´nez-Salgado *et al.,* 2002 and Morales *et al.,* 2010). Further more, several recent studies had shown that co-treatment with different antioxidants is useful for prevention or amelioration of GEN-induced nephrotoxicity (Kadkhodaee *et al.,* 2007; Karadeniz *et al.,* 2008; Koyner *et al.,* 2008; Yaman and Balikci, 2010 and Kang *et al.,* 2013).

Moringa oleifera (MOR) is a highly valued medicinal plant where its various parts have been used as food and medicine from a long time. The nutritional and medicinal benefits of MOR have been attributed to its roots, bark, leaves, flowers, fruits, and seeds (Ramachandran *et al.,* 1980; Anwar *et al.,* 2007 and Kumar *et al.,* 2010). Phytochemical analyses have shown that MOR leaves are particularly rich in essential minerals, vitamins A and D, essential amino acids, as well as potent antioxidants such as β-carotene, vitamin C, and flavonoids (Bennett *et al.,* 2003; Aslam *et al.,* 2005; Manguro and Lemmen, 2007; Amaglo *et al.,* 2010 and Gowrishankar *et al.,* 2010).

MOR relatively contains high antioxidant activity in its leaves, flowers, and seeds (Chumark *et al.,* 2008; Sreelatha and Padma, 2009; Verma *et al.,* 2009 and Atawodi *et al.,* 2010). The extracts of MOR both mature and tender leaves have potent antioxidant activity against free radicals, prevent oxidative damage to major biomolecules and afford significant protection against oxidative damage (Sreelatha and Padma, 2009). Therefore, the present study was designed to investigate the possible modulatory effect of MOR leaf powder against GEN-induced nephrotoxicity in chickens.

MATERIALS and METHODS

1. Animals

Twenty laying hens (Dokki-4 strain; age, 15 months) were obtained from animal production center, Kafr Elshiekh, Egypt. Chickens maintained under standardized environmental conditions on 12 h light/dark cycle under a temperature of 25±2°C and provided with commercial balanced diet and tap water, *adlibitum* throughout the experiment. Animals were acclimatized for two weeks before starting the experiment. All chicken's related procedures were carried out in accordance with appropriate methods, and an approval was obtained from the ethical committee of Kafr Elshiekh University.

2. Experimental design

Chickens were randomly divided into four groups of five birds each. The first group (CON) received standard basal diet for 12 days. The second group (GEN), received GEN (50 mg/kg body weight, *i.m.*, twice daily for 5 consecutive days. Gentamicin sulfate was purchased from Bremer Pharma, GmbH, Germany. The third group (GEN+MOR) was given MOR leaf powder (10gm/kg diet/day) for 12 consecutive days and injected with GEN (50mg/kg body weight, *i.m.*, twice daily, for 5 consecutive days) starting from the day 8. The fourth group (MOR) was supplemented with MOR leaf powder (10gm/kg diet/day) for 12 consecutive days.

3. Blood collection

Blood samples were collected from the wing veins of all laying hens 12h from the last dose of gentamycin. Approximately 4 ml of blood were collected per hen. Serum was separated from clotted blood by centrifugation at 3,000 x g for 20 minutes and stored at -20 ºC until use.

4. Biochemical meausurment:

4.1. Estimation of kidney markers:

Creatinine (Cr); blood urea nitrogen (BUN), total protein and albumin were determined by using commercially available diagnostic kits (Biorex Diagnostics, Ltd, United Kingdom®).

4.2. Estimation of oxidative stress markers

Measurement of MDA:

MDA (an indicator of lipid peroxidation) was calorimetrically determined according to the method adapted by Esterbauer *et al.* (1982). This method is based on the measurement of malondialdehyde (MDA) as one of the main end products of lipid peroxidation by the thiobarbituric acid test. Thiobarbituric acid reacts with malondialdehyde in acidic medium at 95 ºC for 30 minute to form thiobarbituric acid reactive product. The absorbance of the resultant color product measured at 534 nm.

Measurement of GSH:

GSH was determined according to the methods of Beutler *et al.* (1963). This method is based on spectrophotometrically measurement of the yellow color of 2-nitro-5-thiobenzoic acid which was produced from the following reaction: Glutathione + 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) 2-nitro-5-thiobenzoic acid + glutathione disulfide (GSSG).

Measurement of SOD:

SOD activity was determined spectrophotometrically according to the reported method (Paoletti and Mocali, 1990). The method is based on the ability of SOD to inhibit the oxidation of NADH, mediated by superoxide radical. The addition of SOD to the reaction mixture causes proportional inhibition of the rate of NADH oxidation. The unit of activity is defined as the amount of enzyme causing 50 percent inhibition of the rate of the superoxide-driven NADH oxidation.

Measurement of GPx:
GPx activity was assayed according to the method of Gross *et al*. (1967). The activity of GPx was measured directly by determining the amount of unconsumed reduced glutathione (GSH) remaining at specific time intervals in the presence of small amounts of peroxide.

Measurement of CAT:
CAT activity was determined by the method described by Sinha, (1972). The dichromate/ acetic acid reagent can be thought of as a "stop bath" for catalase activity. As soon as enzyme reaction mixture hits the acetic acid, its activity is inhibited, any hydrogen peroxide, which has not been split by catalase will react with dichromate to give a blue precipitate of perchromic acid. This unstable precipitate was then decomposed by heating to give the green color solution which was measured spectrophotometery at 570 nm.

All chemicals used in this study were of analytical grade.

5. Histopathological examination
Animals from each group were sacrificed on the day of bleeding and the kidneys were isolated. Tissue specimens were fixed immediately in 10% formalin and processed for histopathological studies, using routine paraffin embedding method. Sections of 4µm thick were cut and stained using hematoxylin and eosin as mentioned by Bancroft and Gamble, (2007) and examined microscopically. For each slide, minimum of 20 microscopic field (20X)/slide were examined and evaluated. The severity of changes is scored according to the following scale: no change (-), mild, <10% of tubules showed changes (+), moderate changes affecting 10-25% of tubules (++), severe damage affecting 25-50% of tubules (+++).

6. Statistical analysis
Statistical analysis was carried out by using the SPSS for windows software, version16 (SPSS Inc., Chicago, IL, USA). Groups data were compared by one-way analysis of variance (ANOVA), followed by LSD test. The statistical significance was accepted at a level of $P<0.05$.

RESULTS

1. Effect of MOR leaf powder supplementation on Cr, BUN, total protein and albumin levels in GEN-treated chickens; Firstly, serum Cr and BUN levels were measured. Chickens treated with GEN alone showed significant increase in the serum levels of Cr and BUN compared to control group and MOR only-treated group ($p<0.05$). However, Supplementation of MOR leaf powder in the GEN+MOR-treated group significantly reduced the serum levels of Cr and BUN compared to GEN-treated group ($p<0.05$) (table 1). Also, chickens treated with GEN alone showed significant decrease in the serum levels of total protein and albumin compared to control group and MOR only-treated group ($p<0.05$). However, Supplementation of MOR leaf powder in the GEN+MOR-treated group significantly increase the serum levels of total protein and albumin compared to GEN-treated group ($p<0.05$) (table 1).

2. Effect of MOR leaf powder supplementation on oxidative stress markers in GEN-treated chickens: MDA, GSH, SOD, GPx, and CAT were measured in the serum of all chickens as markers for oxidative stress. Data presented in table 2 showed that chickens treated with GEN alone showed significant increase of MDA concentration, in addition to, significant decrease in GSH concentration as well as SOD, GPX, and CAT activities compared to normal control group and MOR-only treated group ($p<0.05$). In contrast, MOR+GEN-treated group showed significant lower concentrations of MDA and higher GSH concentrations as well as serum activities of SOD, GPX, and CAT as compared to GEN- treated group ($p<0.05$).

3. Effect of MOR leaf powder supplementation on renal tissue damage induced by GEN: Histopathological findings of kidney sections of control and MOR-treated chickens showed normal morphological appearances except mild vacuolar degeneration of renal tubular cells was observed in MOR-treated chicken (Fig. 1), whereas GEN-treated group revealed marked enlargement, congestion and greenish discoloration grossly and microscopically, marked vacuolar degeneration of the proximal convoluted tubular epithelium (Fig. 2A) as well as focal interstitial mononuclear cells infiltrations (Fig. 2B) sometimes admixed with heterophils were observed. Marked congestion was also observed together with sloughing of the degenerated renal tubular cells in the lumen of the affected tubules. Brown circular golden crystals were also observed either free in the lumen (Fig. 2C) or in the proximal convoluted epithelial cells or engulfed by heterophils. Focal cystic change as well as focal tubular necrosis was also observed. Kidney of GEN+MOR-treated chicken demonstrated focal interstitial mononuclear cells infiltrations (Fig. 3) as well as vacuolar degeneration of the renal tubules. The severity degrees of histopathological changes in different treatment groups were summarized in table 3.

Table 1: Effect of *Moringa oleifera* leaf powder supplementation on creatinine, blood urea nitrogen, total protein and albumin in gentamycin-treated chickens.

Groups	Creatinine (mg/dl)	Blood urea nitrogen (mg/dl)	Total protein (gm/dl)	Albumin (gm/dl)
CON	0.57± 0.02	5.90 ± 0.13	4.04± 0.03	2.72 ± 0.01
GEN	0.78± 0.02*	10.93 ± 0.78*	2.32± 0.01*	0.86 ± 0.01*
GEN+MOR	0.45± 0.01	5.84 ± 0.55	4.14± 0.17	1.36 ± 0.03
MOR	0.57± 0.01	6.08 ± 0.10	4.10± 0.04	2.60 ± 0.17

a) Data are expressed as means ± SD.
b)*: Significantly different from control ($p<0.05$).

Table 2: Effect of *Moringa oleifera* leaf powder supplementation on oxidative stress markers in gentamicin-treated chickens.

Groups	MDA (nmol/ml)	GSH (µmol/ml)	SOD (U/ml)	GPx (U/ml)	CAT (nmol/ml)
CON	7.95±0.49	1.70±0.16	0.75±0.03	1.28±0.03	1.40±0.06
GEN	11.64±0.26*	0.87±0.13*	0.63±0.01*	1.20±0.00*	1.22±0.01*
GEN+MOR	8.95±1.10	2.10±0.33	0.65±0.09	1.28±0.04	1.30±0.06
MOR	7.75±0.02	6.00±0.96	0.73±0.01	1.47±0.10	1.49±0.02

a) Data are expressed as means ± SD.
b)*: Significantly different from control ($p<0.05$).

Table 3: The severity of histopathological changes observed in the kidneys of chicken treated with gentamycin with or without MOLP.

Chicken No.	Vacuolar and hydropic degeneration	Interstitial mononuclear cells infiltrations	Tubular necrosis	Congestion	Cystic tubular change	Brown circular crystal	Cellular casts
CON1	-	-	-	-	-	-	-
CON2	-	-	-	-	-	-	-
CON3	-	-	-	-	-	-	-
CON4	-	-	-	-	-	-	-
CON5	-	-	-	-	-	-	-
MOR1	-	-	-	-	-	-	-
MOR2	-	-	-	-	-	-	-
MOR3	+	-	-	-	-	-	-
MOR4	+	-	-	-	-	-	-
MOR5	+	-	-	-	-	-	-
GEN1	+++	+	++	+++	+	-	-
GEN2	+++	+++	+	+++	+++	+++	+++
GEN3	+++	+++	+++	+++	+++	+++	+++
GEN4	+++	+++	+++	+++	+++	+++	+++
GEN5	+++	++	++	+++	+	-	++
GEN+MOR1	-	-	-	-	-	-	-
GEN+MOR2	+++	+++	-	+	+	-	+
GEN+MOR3	+	+	-	+	-	-	-
GEN+MOR4	++	++	-	+	-	-	+
GEN+MOR5	+	+	-	+	-	-	-

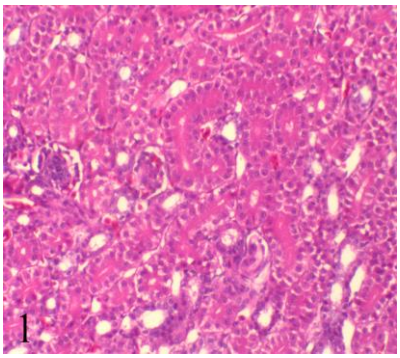
The degrees of severity are: no change (-), mild (+), moderate (++) and severe (+++)

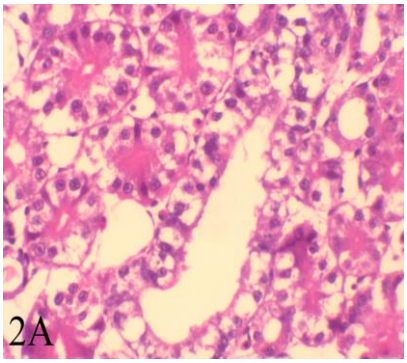
Figure captions

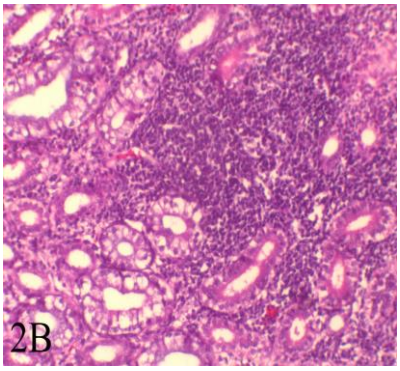
Fig. 1: Representative micrograph for the kidney lesions following treatment of chickens with MOR(10gm/kg diet for 12 consecutive days). The kidney showing normal renal tubular and glomeruli architecture with very mild vacuolar degeneration of renal tubular cells (H&E, X 200).

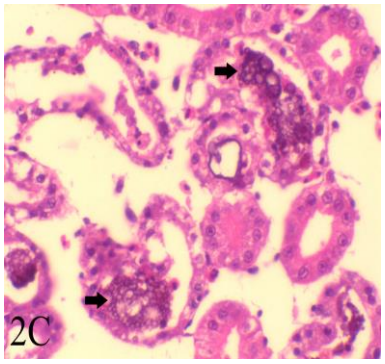
Fig. 2: Representative micrographs for the kidney lesions following treatment of chickens with GEN (50mg/kg body weight, i.m., twice daily, for 5 consecutive days). **(A)** The kidney showing marked vacuolar degeneration of the proximal convoluted tubular epithelium (H&E, X 400), **(B)** The kidney showing Focal interstitial mononuclear cells infiltrations as well as vacuolar degeneration of the proximal convoluted tubular epithelium (H&E, X 200), **(C)** The kidney showing Brown circular golden crystals (arrow) in the lumen of the proximal convoluted tubules together with sloughed necrotic epithelial cells (H&E, X 400).

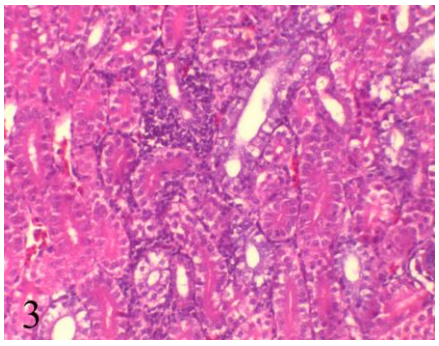
Fig. 3: Representative micrograph for the kidney lesions following treatment of chickens with GEN+MOR (MOR leaf powder, 10gm/kg diet/day for 12 consecutive days and injected with GEN, 50mg/kg body weight, i.m., twice daily for 5 consecutive days). The kidney showing focal interstitial mononuclear cells infiltrations as well as mild vacuolar degeneration of the proximal convoluted tubular epithelium (H&E, X 200).











DISCUSSION

GEN is one of the main aminoglycosides antibiotics in use against gram-negative infection in animal and poultry medicine. However, nephrotoxicity represents the most common side effect of GEN administration (Tulkens, 1999). Scanty information is available about toxicopathological changes associated with GEN toxicity in avian species. Recently, administration of GEN at a dose of 50 mg/kg and above was found to be toxic to the birds (Khan *et al.,* 2008 and Saleemi *et al.,* 2009). Thus using the same dose in the present study was to evaluate GEN induced nephrotoxicity on the basis of biochemical estimation of renal damage markers, oxidative damage markers and histopathological examination of treated kidney as well as possible modulatory effect of MOR leaf powder against such toxicity. Also, changes in biochemical parameters were correlated with renal histopathological results.

The results of present study showed that administration of GEN alone produced kidney damage as indicated from the high levels of serum Cr and BUN. Similar observations had shown that GEN administration leaded to nephrotoxicity that was characterized by an increase in serum Cr and BUN levels in rats (Afzal *et al.,* 2004; Romero *et al.,* 2009; Tavafi and Ahmadvand, 2011) and in birds (Bird *et al.,* 1983, Itoh and okada, 1993, Khan *et al.,* 2008, Saleemi *et al.,* 2009 and Javed *et al.,* 2013). Also, serum total protein and albumin were measured. GEN-treated group showed a significant reduction in the total protein and albumin levels when compared to control group. Decreased serum total protein and albumin levels were previously reported in birds following GEN administration (Bird *et al.,* 1983, Itoh and okada, 1993, Khan *et al.,* 2008, Saleemi *et al.,* 2009 and Javed *et al.,* 2013). Hypoproteinemia and Hypoalbuminemia may be caused by decrease in synthesis of serum proteins associated with severe hepatocellular disease or excessive excretion/loss through damaged kidneys (Tennant and Center, 2008). But, MOR leaf powder supplementation impeded the decrease in total protein and albumin caused by gentamicin. This modulatory effect may be due to its high content of antioxidants and essential amino acids.

The mechanism of GEN-induced nephrotoxicity is not yet completely clear, however, several reports have been documented that ROS and free radicals play an important role in GEN-induced nephrotoxicity (Nakajima *et al.,* 1994; Yang *et al.,* 1995; Walker *et al.,* 1999; Martínez-Salgado *et al.,* 2002 and Morales *et al.,* 2010). ROS and free radicals are highly reactive molecules which lead to excessive oxidation of cellular macromolecules such as DNA, proteins, and lipids. All cells are variably capable of endogenous self protection against this stress through the actions of enzymes such as SOD, GPx, and CAT as well as through reducing molecules such as GSH. Cellular inability to reduce ROS leads to oxidative stress (Fulda *et al.,* 2010).

Evaluations of MDA, GSH concentrations and activities of SOD, GPx, and CAT have been used as markers of oxidative stress and tissue damage. GEN administration induced oxidative renal damage, as evidenced by a significant increase in serum MDA concentration and a significant decrease in GSH content, as well as significant decrease of SOD, GPx, and catalase activities. This result indicates an increase in the generation of free radicals and antioxidant enzymes depletion as a result of the process of combating oxidative stress. Similar studies have been shown that GEN-induced kidney damage is associated with lipid peroxidation and reduction of the kidney GSH content and reduction of the antioxidant enzyme activities such as GPx, SOD, and catalase (Polat *et al.,* 2006; Karadeniz *et al.,* 2008; Khan *et al.,* 2009; Lee *et al.,* 2012 and Kang *et al.,* 2013).

However, MOR leaf powder supplementation induced a significant decrease in the MDA concentration and efficiently improved the GPx, SOD, and catalase activities. This effect might be due to its antioxidant properties. Recent findings have been demonstrated that the extracts of MOR leaves have potent antioxidant activity against free radicals, prevent oxidative damage to major biomolecules and afford significant protection against oxidative damage (Bajpai, 2005; Sreelatha and Padma, 2009 and Sharma and Singh, 2010). These properties may be mediated through scavenging of the free radicals (Verma *et al.,* 2009).

The above mentioned results were confirmed by the histopathological results, as evidenced by a decrease in the incidence and severity of GEN induced-renal histopathological lesions in GEN+MOR group. Vacuolar degeneration of the proximal convoluted tubular epithelium, interstitial mononuclear cells infiltrations in focal manner between the tubules in the corticomedullary junction, cystic luminal dilatation as well as focal tubular necrosis observed in the present study in GEN treated group were similar to those observed by previous reports in animals (Yoshiyama *et al.,* 1992, Shirwaikar *et al.,* 2003 and Abdel Raheem *et al.,* 2009) and birds (Khan *et al.,* 2008 and Saleemi *et al.,* 2009). Brown circular golden pigments or crystals either free in the lumen or in the renal epithelial cells of the proximal convoluted tubules observed in the present study may be GEN crystals or metabolites as previously mentioned that aminoglycosides throughout the endocytic pathway are taken up into the epithelial cells of the renal proximal tubules and stay there for a long time, which leads to nephrotoxicity (Nagai and

Takano, 2004 and Nagai, 2006). It is also evidenced that the renal accumulation of GEN is implicated in the induction of nephrotoxicity (Watanabe *et al.*, 2004). Moreover, acidic phospholipids, broadly distributed in the plasma membranes in various tissues, were considered to be the binding site of aminoglycosides in brush-border membrane of proximal tubular cells (Nagai and Takano, 2004, Nagai, 2006). The majority of administrated GEN enters specifically the proximal tubular epithelial cells, binds to anionic phospholipids in the target cells inducing lysosomal phospholipidosis leading to abnormalities in the function and metabolism of multiple intracellular membranes and organelles then developed injury in the proximal tubular epithelial cells of kidney that caused acute renal failure (Swan, 1997and Sandhu *et al.*, 2007).

In conclusion, MOR leaf may have protective effect against GEN-induced nephrotoxicity in chickens, possibly by inhibiting lipid peroxidation, enhancing renal glutathione content and activity of antioxidant enzymes.

CONFLICTS OF INTEREST

None declared.

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التسمم الكلوي بالجنتاميسين في الدجاج والتأثير الوقائي للمور ينجا

محمد فهمي ابو العزب ، نجوان محمود الحبشى

Email: nagwan_hab@yahoo.com

استخدمت عشرين دجاجة من الدجاج البياض في هذه الدراسة لتقييم دور المور ينجا في تحسين التأثير السمي للجنتاميسين من خلال دراسة وظائف الكلي ومضادات الأكسدة والنتائج الهستوباثولوجية للدجاج. قسم الدجاج إلى أربع مجموعات، مجموعة ضابطة بدون الجنتاميسين أو المور ينجا ، ومجموعة المور ينجا بدون جنتاميسين، ومجموعة الجنتاميسين بدون المور ينجا ، ومجموعة المور ينجا المعرضة للتسمم بالجنتاميسين. أشارت النتائج إلى زيادة الكرياتينين (CR)، اليوريا نيتروجين في الدم (BUN)، والمالون الدهيد malonaldehyde (MDA) وانخفض البروتين الكلي والألبومين ومضادات الأكسدة في مجموعة الجنتاميسين كما أن استخدام المور ينجا أدى إلى تحسين وظائف الكلي ومضادات الأكسدة وتقليل الاثار الهستوباثولوجية الضارة للجنتاميسين.