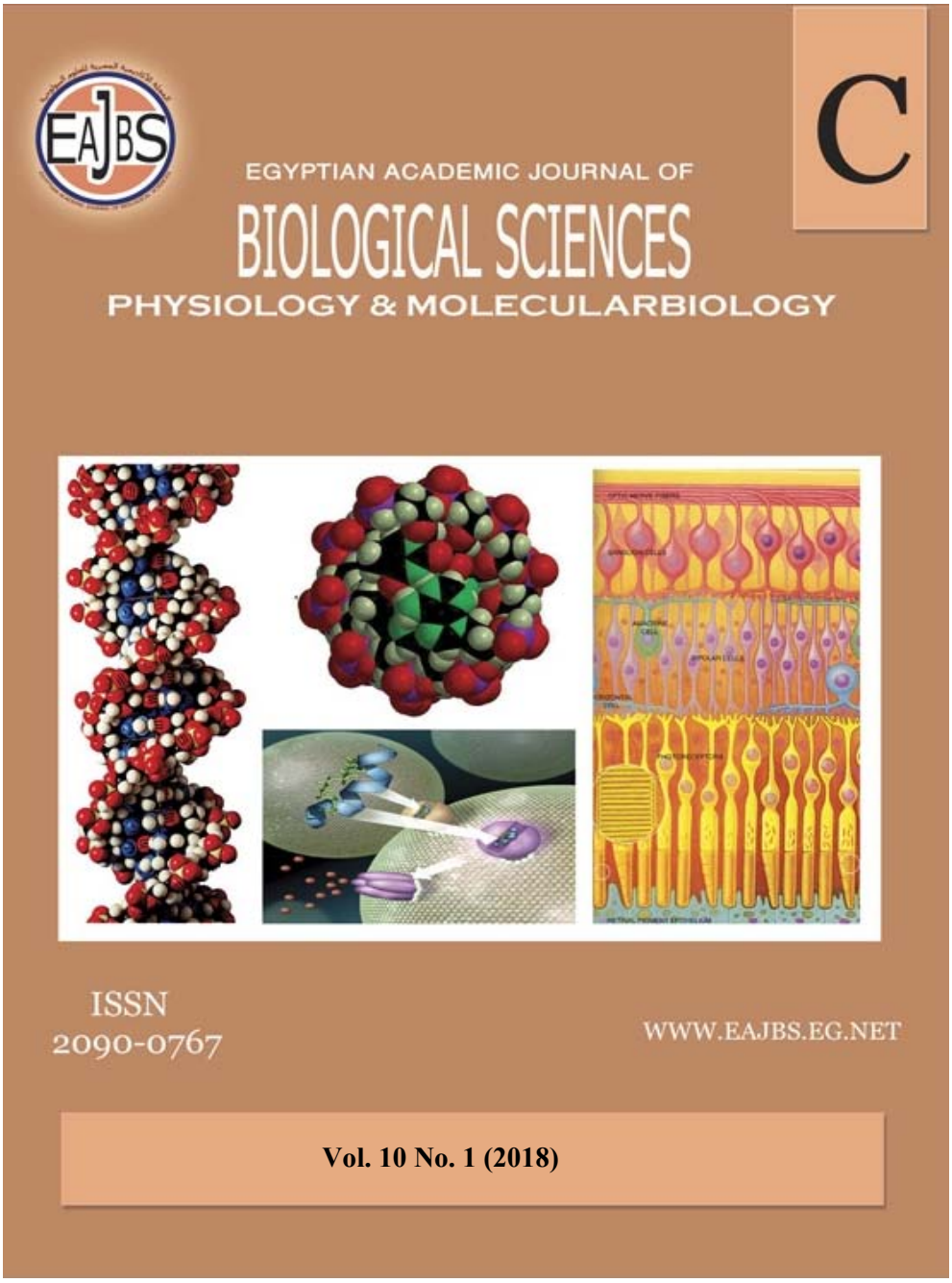

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Ameliorative And Erythrocytes Membrane Stabilizing Effects of *Mentha piperita* on Experimentally Induced Nephrotoxicity by Gentamicin

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ABSTRACT

Background: The clinical usefulness of gentamicin (GM) is limited due to the development of nephrotoxicity. Potential therapeutic approaches to protect (or) reverse GM damage would have been of very important clinical consequences in increasing the safety of the drug. Several natural agents have been used to ameliorate drugs toxicity. The survey of literature reveals that the *Mentha piperita* Linn. are found to be used in the traditional system of medicine. However, ameliorative and membrane stabilizing activity of *M.piperita* have not been scientifically investigated. So, the present study was design to evaluate effects of *M.piperita* L. on nephrotoxicity in rat model.

Methodology: Kidney damage was induced in rats by administrating Gentamicin (GM) intraperitonially at the dose of 100mg/kg body weight for 6 consecutive days. Freshly prepared ethanolic and aqueous extracts of *M.piperita* (EMPet and AMPet) were orally administered to rats.

Results: The altered renal markers (urea, uric acid, creatinine, and BUN) after GM administered were normalized in extracts treated animals. The membrane stabilizing effects were confirmed by erythrocytes osmotic fragility and to RBC morphology. The ameliorative effects were also confirmed by histopathology of renal tissues.

Conclusion: The results of the present study indicate that *Mentha piperita* may emerge as a ameliorative and membrane-stabilizing agent against nephrotoxicity. Further studies need to be undertaken in order to confirm these findings and its extrapolation in humans.

INTRODUCTION

Nephrotoxicity is one of the most common kidney problems due to the body exposure to a drug or toxin (Porter and Bennett, 1981). Toxic chemical-induced nephrotoxicity tends to be more common among certain patients and in specific clinical situations. Acute kidney injury (AKI) is generally defined as a decline in

kidney function resulting in accumulation of waste products in the bloodstream. The main causes of AKI are nephrotoxins, aminoglycosides, oxytetracycline, and nonsteroidal anti-inflammatory drugs (NSAIDs) (Geor and Papich MG, 2003). Gentamicin (GM) is probably one of the most commonly used aminoglycoside antibiotics for the treatment of serious and life-threatening infections caused by Gram-negative aerobes (Negrette-Guzman *et al.*, 2013).

GM nephrotoxicity accounts for 10–15% of all cases of acute renal failure and about 30% of patients show signs of nephrotoxicity (Mattew 1992; Shifow *et al.*, 2000). GM-induced nephrotoxicity is characterized by morphological alterations including destruction, necrosis, and apoptosis of kidney cells which eventually lead to AKI and dysfunction (Selby *et al.*, 2009). The renal toxicity is due to its selective accumulation in the renal proximal convoluted tubules and its long term stay subsequently leading to loss of brush border integrity (Raju S *et al.*, 2011; Pedraza-Chaverri *et al.*, 2000). The nephrotoxicity exerts toxic effects by one or more common pathogenic mechanisms (Fig, 1). These include altered intraglomerular hemodynamics, tubular cell toxicity, inflammation, crystal nephropathy, rhabdomyolysis, and thrombotic microangiopathy (Zager 1997; Schetz *et al.*, 2005).

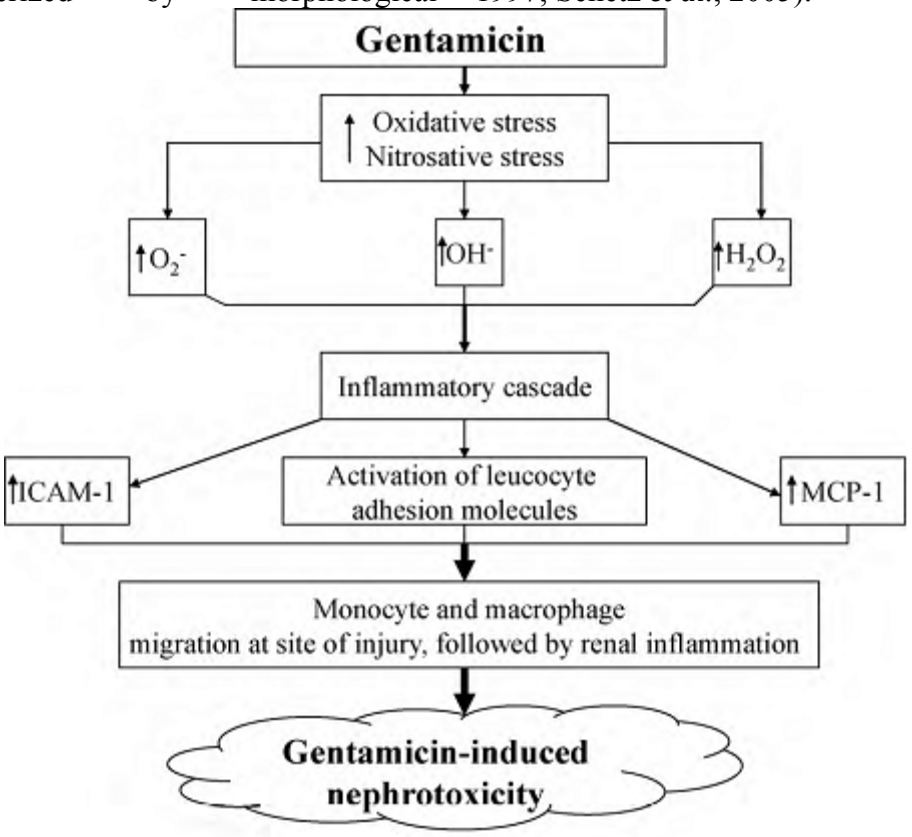


Fig. 1: Shows the pathological role of reactive oxygen species in the induction of gentamicin-nephrotoxicity (ICAM-1-Intercellular Cellular Adhesion Molecule-1; MCP-1-Monocyte Chemoattractant Protein-1; O_2^-- superoxide radical; OH^-hydroxyl radical; H_2O_2 - hydrogen peroxide. (Pitchai B *et al.*, 2010)

Therefore, the clinical usefulness of GM is limited due to the development of nephrotoxicity (Cuzzocrea *et al.*, 2002). Thus, a therapeutic approach to protect or reverse renal damage would have very important clinical consequences. To date, there is no specific agent used to protect against GM-induced nephrotoxicity. In this regard, various medications have been used concomitantly with GM to prevent AKI in laboratory animal models (Stojiljkovic *et al.*, 2012; Ye *et al.*, 2010; Kalayarasan *et al.*, 2009; Hozayen *et al.*, 2011; Randjelovic *et al.*, 2012). Several natural agents have been used to ameliorate some toxic, carcinogenic and drugs toxicity.

Mentha piperita, the peppermint plant belongs to the genus Lamiaceae (Fig. 2). It is an aromatic and carminative herb cultivated throughout all regions of the world (Saharkhiz *et al.*, 2012), has traditionally been used in folk remedy or in complementary and alternative medical therapy. A literature search reveals that peppermint has been ascribed a variety of biological properties, *viz.* antibacterial (Shapiro *et al.*, 1994), antiallergenic (Inoue *et al.*, 2002), anti-inflammatory (Inoue *et al.*, 2002), antitumor (Ohara and Matsuhisa, 2002), antimycotic (Pattnaik *et al.*, 1996), gastrointestinal protective (Mahmood *et al.*, 2003), antiviral (Yamasaki *et al.*, 1998), hepatoprotective (Akdogan *et al.*, 2003), and chemopreventive (Samman *et al.*, 1998). *M.piperita* contains active ingredients, such as menthone, menthol, menthyl acetate flavonoids, polymerized polyphenols, tocopherols, carotenes, saponin, and choline (Saharkhiz *et al.*, 2012; Iwu *et al.*, 2009; Georgiev *et al.*, 2006; Cragg and Newman, 2001; Sharafi *et al.*, 2001) together with several other minor constituents, including pulegone, menthofuran and limonene (Nair, 2001). However, nephroprotective activity of *M.piperita* has not been scientifically investigated. So, the present study was design to evaluate nephroprotective effects of *Mentha piperita* Linn. (Mint) leaves extract on GM-induced nephrotoxicity.

MATERIALS AND METHODS

Preparation of Plant Extracts:

The separated leaves of *M.piperita* (Fig. 2) were washed with tap water to remove the dust and other foreign materials. The washed leaves were dried under shade for one week. Approximately about 500 g of air-dried whole leaves were pulverized into powdered form by using heavy duty commercial blender.

Preparation of Ethanolic *M.piperita* Extracts (EMPet):

The powder samples (50 g) were extracted with 95% ethanol (1:3 w/v) by using Soxhlet extractor at 37°C for two days. The total yield was 4.67 g (9.34% w/w) of dark greenish extract. Because of very low aqueous solubility, the EMPet from *M.piperita* was reconstituted to a final concentration of 5% (w/v) using aqueous solution of gum acacia (5%) for further treatments (Sasikumar *et al.*, 2016).

Preparation of Aqueous *M.piperita* Extracts (AMPet):

The aqueous extracts of *M.piperita* leaves were prepared according to the method of Hossain *et al.*, (1992). *M.piperita* leaves yielded 13% light greenish semisolid which was stored at 0–4°C until used.

Fig. 2: Photographs of *M.piperita* Linn.

Chemicals:

The antibiotics, GENTAM® were purchased from SPIMACO, Al–Qassim, Kingdom of Saudi Arabia and other chemicals and solvents used were of analar grade.

Animals:

Healthy, male albino Wister rats (*Rattus norvegicus albinus*) each weighing 150-200 g were used for this study. The rats were housed in polypropylene cages and maintained under standard conditions (12 h light and dark cycles, at 25±3°C and 35-60% humidity). Standard pelletized feed (Grain Silos & Flour Mills Organization, Riyadh, KSA) and tap water were provided *ad libitum*. The experimental design was approved by the Deanship of Research, University of Hail (Proposal No. CM4 2013, date 16-12-2014).

Induction of Kidney Damage:

Kidney damage was induced in rats by administrating gentamicin (GM) intraperitonially at the dose of 100mg/kg body weight for 6 consecutive days (Yaman and Balikci, 2010).

Experimental Design:

The animals were grouped as follows and each group contains 6 rats. A diagrammatic representation of experimental protocol is given in the Figure 3. **Group I:** Normal animals received standard feed and water *ad libitum*. No other treatment. **Group II:** GM-induced group received gentamicin (100mg/kg body weight, ip) for 6 consecutive days along with standard feed and water *ad libitum*. **Group III:** Treatment group received GM as Group II for 6 days followed by the treatment with EMPet orally (300 mg / kg b.wt per day for 10 days). **Group IV:** Treatment group received GM as Group II for 6 days followed by the treatment with AMPet orally (400 mg / kg b.wt per day for 10 days). **Group V:** Drug alone treated group received EMPet orally (300 mg / kg b.wt per day for 10 days). **Group VI:** Drug alone treated group received AMPet orally (400 mg / kg b.wt per day for 10 days).

The body weight of the animals was recorded throughout the experimental period starting from Day 0. After the experimental regimen, the rats were fasted overnight and were sacrificed by cervical dislocation under light ether anesthesia and the blood was collected on decapitation.

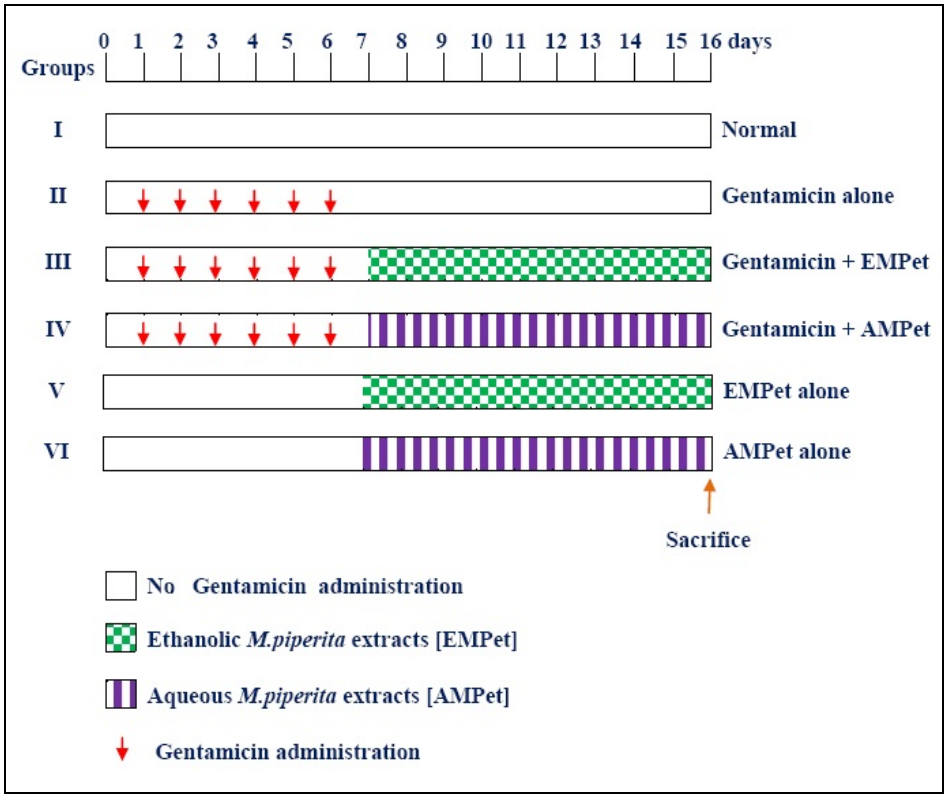


Fig. 3: Experimental protocol for ameliorative and membrane stabilizing studies.

Histopathology:

For histopathological studies, kidney tissues were fixed in 10% formalin and were routinely processed and paraffin embedded, 5µm sections were cut in a rotary microtome and were stained with hematoxylin and eosin.

Estimation of Kidney Markers:

Urea concentration in blood was estimated by NED Dye method (Lequang *et al.*, 1987). Concentration of serum creatinine was measured by alkaline picrate method (Bartels *et al.*, 1972). Blood urea nitrogen (BUN) was measured with the commercial kit developed by the Parsazmoon Company (Tehran, Iran) based on the method described by Talke and Schubert (1965). Serum uric acid was measured by using commercially available reagents (Smith, 1985). Total protein level was estimated by colorimetric assay with modified Biuret end point method using protein estimation kit (Gornall, 1949). Serum albumin level was estimated by BCG method using albumin estimation kit (Gornall, 1949).

Determination of Erythrocyte Osmotic Fragility:

The *in vitro* erythrocyte osmotic fragility was evaluated in all the rats in each group using the method described by Faulknet and King (1970)

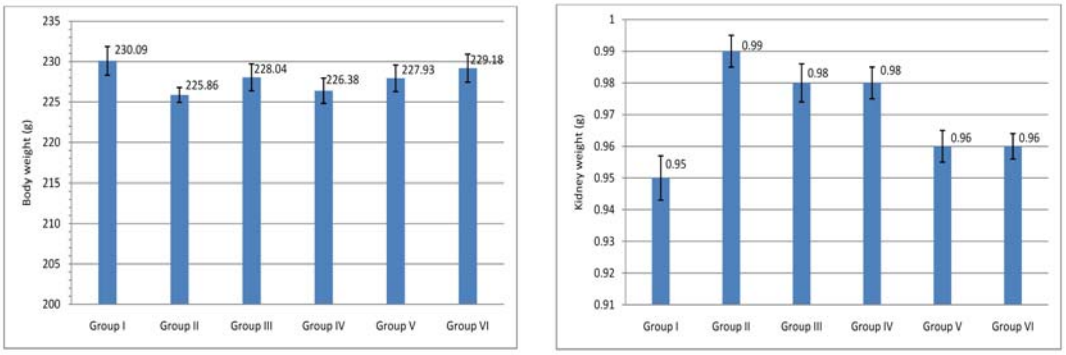
Statistical Analysis:

The values are expressed as mean ± SD. The statistical comparisons were performed by one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT), using SPSS version 16.0 for windows (SPSS Inc.Chicago; http://www.spss.com). The values are considered statistically significant if the *p* value was less than 0.05.

RESULTS

Figure 4 shows the effect of *M.piperita* on the physical parameters. In the present study, the body weight (Fig. 4a) of rats administered with GM was reduced significantly (230.09±1.78 vs 225.86±0.92) ($p<0.05$) in comparison to the normal control group. Although there was an increase in body weight of Group III and Group IV, the increase in body weight was not significant ($p<0.05$) compared to normal group. Rats treated with EMPet (Group V) and AMPet (Group VI) alone showed no significant difference in body weight status compared to control animals.

The levels of kidney weights of rats in control and all treated groups are showed in Figure 4b. GM treatment induced a significant increase in the relative weight of kidneys with respect to normal controls (0.93±0.005 vs 0.98±0.005) ($p < 0.05$). However, oral administration of EMPet (300 mg/kg b.wt) (Group III) and AMPet (400 mg/kg b.wt) (Group IV), reverted the weight of kidney to near normal range. Animals treated with EMPet (Group V) and AMPet (Group VI) alone showed no significant difference in kidney weight as compared to control animals.



(a) (b)

Fig. 4: Effects of *M.piperita* on body weight (a) and kidney weight (b) in normal and experimental rats.

Table 1 shows the status of kidney markers in serum of the control and experimental groups. The concentration of serum creatinine, urea, uric acid, and blood urea nitrogen were increased significantly in Group II (GM alone) as compared to control animals. Oral administration of EMPet (300 mg/kg b.wt) and AMPet (400 mg/kg b.wt) animals significantly decreased the levels of kidney markers. EMPet and AMPet (Groups VII and VIII) alone treated animals showed no significant difference in kidney markers as compared to control animals.

The levels of total protein were significantly increased in serum whereas decreased in albumin as compared to control animals. Oral administration of EMPet and AMPet at a dose of 300 mg/kg b.wt and 400 mg/kg b.wt to GM administered animals, respectively, revert back the status of total protein and albumin to near normal concentration. Rats treated with EMPet and AMPet alone showed no significant differences in serum total protein and albumin levels as compared to control animals.

Table 1. Effects of *M.piperita* on kidney markers in gentamicin induced nephrotoxicity.

Parameters	Group I Normal	Group II GM	Group III GM + EMPet	Group IV GM + AMPet	Group V EMPet	Group VI AMPet
Creatinine (mg/dL)	0.57±0.03^{a}	1.75±0.11^{b}	0.60±0.04^{a}	0.61±0.03^{a}	0.57±0.03^{a}	0.59±0.04^{a}
Urea (mg/dL)	44.52±2.42^{a}	97.16±5.29^{b}	65.11±3.55^{c}	70.18±3.82^{d}	55.02±3.00^{e}	57.13±3.11^{e}
Uric acid (µmol/L)	137.20±9.49^{a}	167.52±13.26^{b}	161.48±11.72^{bc}	144.34±9.49^{a}	161.05±13.12^{bc}	150.61±8.45^{ac}
BUN (mg/dL)	15.21±1.05^{a}	28.18±1.51^{b}	18.12±0.97^{cd}	19.12±1.03^{c}	18.6583±1.19^{c}	16.80±0.91^{d}
Total protein (g/dL)	53.29±3.19^{a}	95.81±5.74^{b}	62.10±3.72^{c}	71.14±4.26^{d}	61.27±3.67^{c}	64.73±3.88^{cd}
Albumin (g/dL)	4.57±0.32^{a}	3.03±0.16^{b}	4.36±0.30^{ac}	3.97±0.22^{d}	4.12±0.22^{cd}	3.95±0.18^{d}

Values are expressed as mean ± SD for 6 animals in each group.
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT).
EMPet-Ethanolic *M.piperita* extracts; AMPet-Aqueous *M.piperita* extracts

Table 2 shows the kidney histopathology of normal and experimental groups. In histopathological examination, control (Group I) samples of kidneys showed normal kidney morphology. Gentamicin administered animals caused significant changes in tubular epithelium like vacuolization; desquamation, atrophy and necrosis; interstitial edema and inflammation in general architecture. But administration of EMPet and AMPet showed a well improvement in the renal morphology. Tubular and glomerular structures were seen close to their normal structures in the EMPet treated group.

Table 2. Histopathological changes in kidney tissues of *M.piperita* leaf extracts treated and gentamicin administered animals.

Histopathological changes	Group I Normal	Group II GM	Group III GM + EMPet	Group IV GM + AMPet	Group V EMPet	Group VI AMPet
Tubular necrosis	-	+	-	+	-	-
Tubular dilatation	-	+	-	-	-	-
Tubular epithelial desquamation	-	++	+	+	-	-
Tubular atrophy	-	+	+	+	-	-
Interstitial inflammation	-	+	-	-	-	-
Interstitial edema	-	++	+	+	-	-
Tubular casts	-	+	-	-	-	-

EMPet-Ethanolic *M.piperita* extracts; AMPet-Aqueous *M.piperita* extracts
Quantification scores (-): no meaningful histopathologic change (+): mild degree; (++): moderate degree; (+++): severe degree

Osmotic fragility curves for control and experimental animals in each group are shown in Figure 5. The fragility curve of GM administered animals (Group II) was shifted to the right for the control animals (Group I). Treatment of GM induced animals with EMPet (Group III) and AMPet (Group IV) shifted the curve to the left, those of nephrotoxicity animals. The fragility curve did not differ significantly in animals treated with the extracts of *M.piperita* (EMPet and AMPet) alone as compared to control animals.

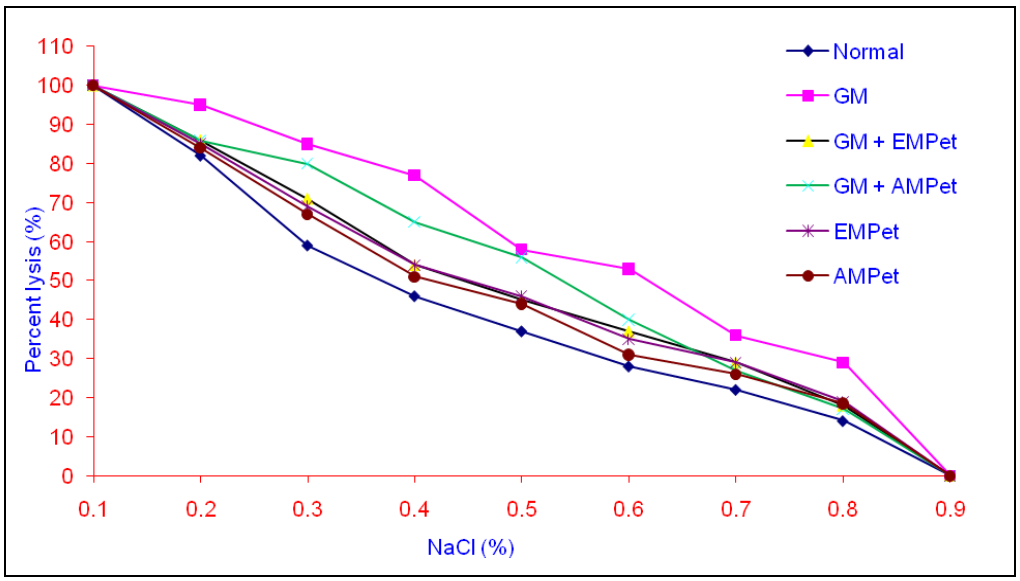


Fig. 5: Erythrocyte osmotic fragility curves for normal and experimental animals.

Figure 6 shows the photomicrography of Red Blood Cells smears from blood samples of normal and experimental animals. In Group I animal's erythrocytes is shows within normal limits. There were no morphological changes. But in Group II gentamicin administered animal's erythrocyte showed an altered cell structure and formation of acanthocytes. The structure of erythrocytes in EMPet and AMPet treated group of rats was found to be significantly normal. There are no morphological structural changes found in EMPet and AMPet alone treated group of animals.

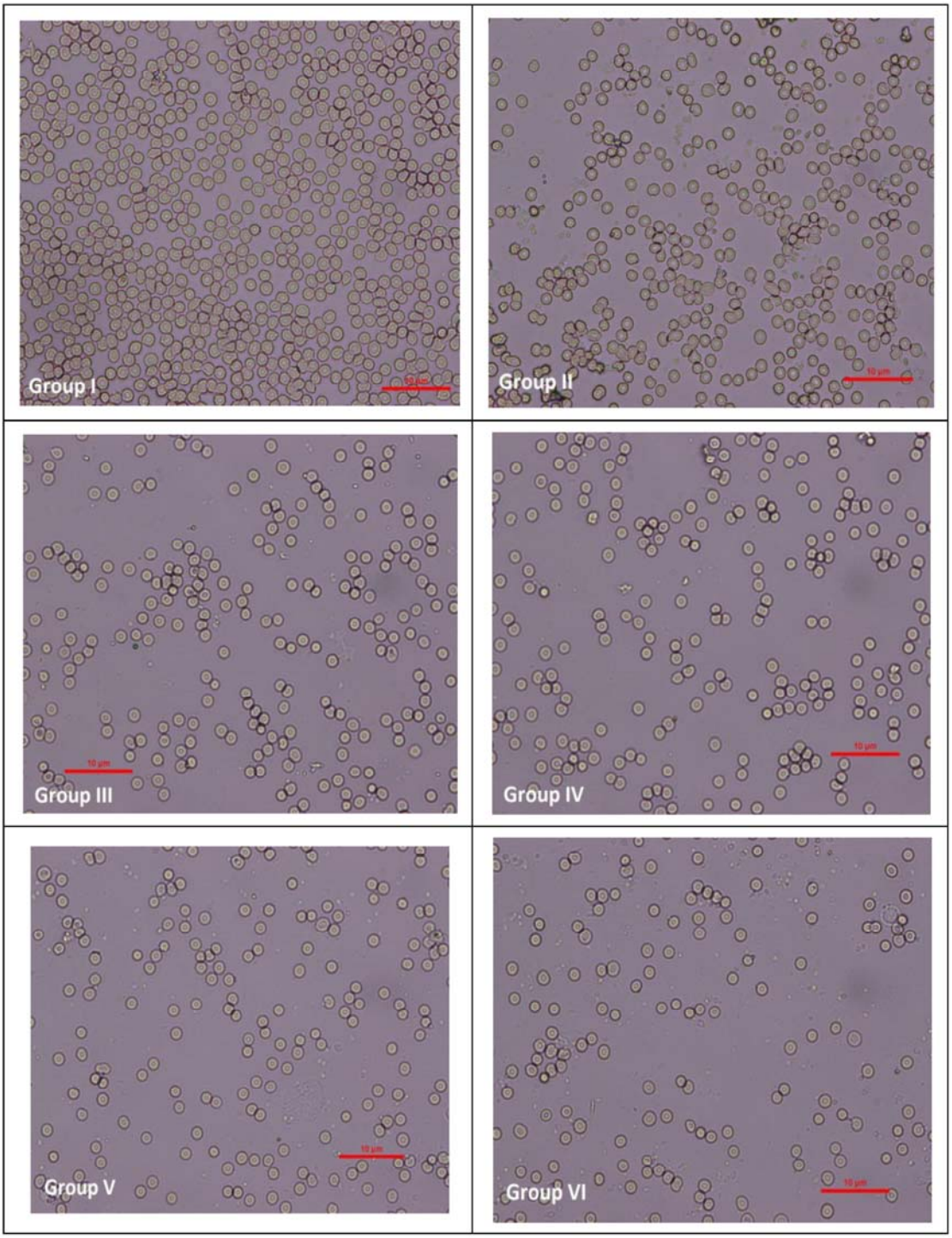


Fig. 6: Photomicrography of Red blood cells smears from blood samples of normal and experimental animals. Blood smears were prepared and dried. The morphology of red blood cells was evaluated under optical microscopy (40x) after image capture.

DISCUSSION

About 20% of hospital admissions due to acute kidney injury (AKI) are related to drug-induced nephrotoxicity. Nephrotoxic drugs can lead to alteration in intra glomerular hemodynamics, tubular epithelial cell damage, tubulointerstitial disease, glomerular disease, renal vasculitis, thrombosis, and obstructive nephropathy (Nolin and Himmelfarb J., 2010). The main causes of AKI are nephrotoxins, aminoglycosides, oxytetracycline, and nonsteroidal anti-inflammatory drugs (NSAIDs) (Geor and Papich MG, 2003). Gentamicin (GM) is probably one of the most commonly used aminoglycoside antibiotics for the treatment of serious and life-threatening infections caused by gram-negative aerobes (Negrette-Guzman *et al.*, 2013). Therefore, the clinical usefulness of this drug is limited due to the development of nephrotoxicity (Cuzzocrea *et al.*, 2002). Thus, a therapeutic approach to protect or reverse renal damage would have very important clinical consequences. Several natural agents have been used to ameliorate some toxic and carcinogenic and drugs toxicity.

As a measure of renal function status, blood urea and creatinine are often regarded as reliable markers (Gowda *et al.*, 2010). The gentamicin induced neprotoxicity were confirmed by an increase in serum creatinine, uric acid, urea, and blood urea nitrogen levels and severe proximal renal tubular necrosis, followed by deterioration and renal failure (Cuzzocrea *et al.*, 2002; Al-Majed *et al.*, 2002) in Group II animals (Table 1), are in agreement with a previous study done by Begum *et al.*, (2011). High values of blood urea and serum creatinine indicate renal damage (Adeneye and Benebo 2008; Khalid *et al.*, 2012) and this may be correlated with the significant and progressive body weight loss and kidney weight gain in the GM administered Group II (Fig. 5). These parameters were almost significantly normalized by oral administered *M.piperita* leaf extracts (Groups III and IV). This result is consistent with previous studies done using other traditional plants (El-Adawi *et al.*, 2011), and is strongly attributed to the scavenging free radicals and reduced lipid peroxidation mechanisms.

The GM administered animals caused severe histological damages, mainly tubular epithelial desquamation and interstitial edema in kidney tissues of Group II rats. Finally, it is clear that GM administration can cause an imbalance in oxidant/antioxidant system and histological damage in rat kidney tissue. Our results clearly showed that EMPet and AMPet treatment successfully prevent oxidative and histological damage caused by gentamicin. In the current study, the histological evaluations showed that no lesions were observed in the rats treated with EMPet and AMPet alone (Group V and VI).

Membrane lipids constitute about 50% of the mass of most animal cell plasma membranes. They play an important role in determining the various function and properties of red cells such as maintaining the integrity, permeability, fluidity, and function. Membrane fluidity is known to be dependent on the molar ratio of cholesterol to phospholipid (Yawata Y. *et al.*, 1984). Measurement of mean corpuscular fragility of erythrocyte membranes is useful to assess the alterations in the integrity of cell structure and function (Yawata Y. *et al.*, 1984). Alteration in membrane fragility (Fig. 5) has been documented in nephrotoxicity (Yawata Y. *et al.*, 1984) in gentamicin administered Group II animals.

Erythrocytes and erythrocyte membrane are more vulnerable to lipid peroxidation due to constant exposure to

high oxygen tension and richness in polyunsaturated fatty acids respectively (Eritsland J., 2000). Increased osmotic fragility in AKI animals (Group II) can be due to the increased oxidative stress in erythrocytes. Over production of reactive oxygen species has been implicated in the alterations of membrane structure and function. Increased lipid peroxidation observed in present study (results not showed) is therefore responsible for the increase in osmotic fragility (Habbel RP, 1986) in AKI animals (Fig. 5). Increased erythrocyte fragility and permeability in Group II animals are probably due to their altered lipids, lipid peroxidation and antioxidant status. Oral administration of *M.piperita* to these AKI animals prevented the alterations in red cell fragility and lipid profile, which indicates the role of *M.piperita* in maintaining the structural integrity of erythrocytes during AKI. The membrane stabilizing effect of ethanolic extract of *M.piperita* (EMPet) is more potent than that of the AMPet. During in the cholesterol accumulation process the structure of the membrane is slowly changed. At low concentrations spicules were formed on the membrane. With increase in cholesterol acquire an echinocytic appearance (Vatsala TM and Singh M., 1980) in gentamicin administered animals (Group II). The observed biochemical changes also resulted in significant morphological changes in the erythrocytes of gentamicin administered rats. Changes in membrane lipid composition lead to morphological changes, the prominent changes were the distortions in normal discocyte shape, appearance of central and peripheral protuberances and formation of acanthocytes (Sherman IW, 1979) in Group II animals. The drug treatment (*M.piperita* leaf extracts) had potent therapeutic efficacy in modulating erythrocyte function and structural abnormalities by this remarkable hypocholesterolaemic property.

CONCLUSIONS

This study revealed that the concurrent administration *M. piperita* successfully prevented renal damage associated with gentamicin, explored by various biochemical and histological examinations. Alteration in mean body weight, blood urea nitrogen, creatinine and uric acid associated with gentamicin were reduced by treating animals simultaneously with extract of *M. piperita*. In conclusion, the results of the present study indicate that *Mentha piperita* may emerge as a ameliorative and erythrocytes membrane-stabilizing agent against nephrotoxicity. Further studies need to be undertaken in order to confirm these findings and its extrapolation in humans.

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ARABIC SUMMARY

التأثيرات الإصلاحية واستقرار غشاء خلايا الدم الحمراء بواسطة النعناع الفلفلي في التسمم الكلوي المحدث مخبريا بواسطة الجنتاميسين.

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خلفية: إن الفائدة السريرية لعقار الجنتاميسين (GM) محدودة لتسببها في التسمم الكلوى. وسيكون للطرق العلاجية المحتملة للحماية (أو) الإصلاح للأضرار الناجمة عن الجنتاميسين نتائج سريرية مهمة جدا في زيادة سلامة الدواء. هذا وقد استخدمت العديد من المواد الطبيعية لاصلاح سمية الأدوية. ومن استطلاع البحوث السابقة وجد أن (النعناع *الفلفلي*) مستخدم في النظام الطبي التقليدي. بيد أنه لم تتم دراسة فعالية النعناع الفلفلي المحسِّنة والمثبتة للغشاء بصورة علمية. لذا تم تصميم الدراسة الحالية لتقييم آثار النعناع *الفلفلي* على تسمم الكلى في نموذج الفئران.

المنهجية: أحدث تلف كلوي للفئران باعطاء الجنتاميسين (GM) داخل الصفاق بجرعة ١٠٠ ملغم / كغم من وزن الجسم لمدة ٦ أيام متتالية. ثم أعطيت مستخلصات كحولية ومائية (EMPet and AMPet) للنعناع *الفلفلي* الطازج عن طريق الفم.

النتائج: تمت إعادة دلائل وظائف الكلى (البولينا وحمض البوليك والكرياتينين ونيتروجينا البولينا) المتغيرة بواسطة الجنتاميسين لمستوياتها الطبيعية في الفئران المعالَجة بالمستخلصات. كما تم التأكد من التأثيرات الإصلاحية بدراسة التغيرات النسيجية للكلي.

الخاتمة: تشير نتائج هذه الدراسة إلى أن النعناع الفلفلي قد يعمل كعامل إصلاحي ومثبت لاستقرار غشاء الخلية ضد تسمم الكلى. بيد أن هناك حوجة لإجراء مزيدا من الدراسات للتأكد من صحة هذه النتائج وجدوي تطبيقها في البشر.