The Renoprotective Effect of Honey on Paracetamol - Induced Nephrotoxicity in Adult Male Albino Rats

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Abstract: Objectives: Oxidative stress plays a crucial role in the development of drug-induced nephrotoxicity. Honey has been known to be effective against oxidative stress-induced diseases. The study aims to investigate the antioxidant and ameliorative protective impact of clover flowers honey against paracetamol- induced nephrotoxicity in rats.
Material and methods: Forty adult male albino rats (120-180 gram b.wt.) were divided into four groups (n= 10 in each group). The animals in the control group (group I) did not receive any treatment, while those in group II received clover flowers honey (2 g/kg/day , p.o) for 4 weeks. The animals in group III received paracetamol (640 mg/kg, p.o), while in group IV, rats were pretreated with clover flowers honey (2 g/kg/day, p.o) for 4 weeks before paracetamol administration. At the end of the experiment, 24hrs urine was collected for glucose and protein determination, while blood was sampled for serum GSH, urea and creatinine determination. The kidneys were removed for assessment of tissue SOD, CAT and tumor necrosis factor (TNF-alpha).
Results: Exposure of rats with a nephrotoxic dose of paracetamol disturbed the kidney function tests; blood urea nitrogen (BUN) and serum creatinine (SC) levels, decreased the antioxidant capacity of GSH and SOD and elevated renal TNF-alpha. The protective use of clover flowers honey before paracetamol-induced nephrotoxicity resulted in a significant improvement in all evaluated parameters, rendering most of the disturbed parameters to their normal levels.
Conclusion: Results of the present study suggest that the protective activity of clover flowers honey may be related to its anti-inflammatory and antioxidant properties.
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1. Introduction

Prevention is better than cure, a prophylactic concept that should be applied in cases of drug induced nephrotoxicity which is a serious and common danger. The use of nephrotoxic drugs has been implicated as a causative factor in up to 25% of all cases of severe acute renal failure in critically ill patients ***(Pannu and Nadim, 2008)***. This is probably because the kidney is supplied with a large volume of blood accounting for 20% of total cardiac output. Therefore, the kidney is likely to be affected by secondary effects of drugs and their metabolites that are accumulated through the urine concentrating mechanism ***(Marieb, 2006).***

Paracetamol, PCM, or Acetaminophen, is a drug of para-aminophenol group which is considered one of the commonly used and safe over-the-counter antipyretic and analgesic drugs, when administered at recommended doses ***(Ozkaya et al., 2010)***. The main problem with this medication remains its misuse through intentional or unintentional ingestion of supra-therapeutic dosages which usually lead to hepatic necrosis. When administered at normal doses, PCM is primarily metabolized by conjugation with sulfate and glucuronic acid. A minor pathway through CYP450 has also been reported to yield a highly reactive metabolite, *N*-acetyl-p-benzoquinonimine (NAPQI). This metabolite is generally stabilized through conjugation with glutathione (GSH) and eliminated *via* the kidney. However, when an overdose of PCM is administered, the production of NAPQI exceeds the capacity of GSH to detoxify it. The excess NAPQI then causes liver damage associated with oxidative stress ***(Roberts and Bukley, 2007)***. PCM overdose is also known to be associated with inflammation, marked by an increase in the inflammatory cytokines; tumor necrosis-*a* (TNF-*a*), interleukins, as well as the upregulation of nitrogen oxide (NO) from serum, macrophages and hepatocytes (***Jaeschke et al., 2003***). Even in sensitive individuals, such as persons with renal insufficiency, therapeutic doses of paracetamol have also been implicated in kidney damage ***(Stern et al., 2005).***

Oxidative stress is reported to constitute a major mechanism in the pathogenesis of PCM-induced liver and renal damage in experimental animals. Because toxic overdoses of APAP were reported to have life-threatening impacts on the kidney, e.g. hepatic necrosis and renal failure in both human and experimental animals, early protection from APAP-induced nephrotoxicity has life-saving importance ***(Ghosh and Sil, 2007, Demirbag et al., 2010).*** Therefore, supplementation with antioxidants is very crucial to

delay, prevent or remove oxidative damage. There are numerous reports indicating that PCM-mediated oxidative stress or hepato-renotoxicity is attenuated by use of naturally occurring antioxidants and/or free radical scavengers such as vitamins, medicinal plants and flavonoids. Recently, the flavonoids have aroused considerable interest because of their potential beneficial effects on human health. The antioxidant capacity of these molecules seems to be responsible for many of their beneficial effects and confers a therapeutic potential in diseases such as cardiovascular diseases, gastric or duodenal ulcers, cancer and hepatic pathologies.

Honey is a natural popular sweetener and is being used to treat a variety of illnesses due to its pleiotropic medicinal properties such as its antibacterial, hepatoprotective, hypoglycemic, reproductive, antihypertensive and antioxidant effects. The therapeutic role of honey in the treatment of various ailments has been receiving considerable attention recently, and its therapeutic value has been partly attributed to its antioxidant properties because it contains both aqueous and lipophilic antioxidants. These properties enable honey to act at different cellular levels as an ideal natural antioxidant ***(Aljadi and Kamaruddin, 2004).*** Honey with higher water content and with darker color proved to have a higher antioxidant activity. The antioxidant compounds present in honey include vitamin C, monophenolics, flavonoids, and polyphenolics ***(Schramm et al., 2003).***

The aim of the present study is to shed further light on the possible nephroprotective ameliorative effects of clover flowers honey on PCM-induced acute nephrotoxicity in albino rats.

2. Material and Methods

Animals:

The experimental protocol and animal handling were approved and performed according to the guidelines of animal use of the Ethical Committee, El-Azhar University. In this study, 40 healthy adult local strain male albino rats, weighing 120-180 g were utilized. They were housed in room temperature and regular light: dark cycle and fed rodent chow and water *ad libitum*. They were fasted 17 hours (4: 00 p.m- 9: 00 a.m) before the experiments, but were allowed free access to water.

Experimental design

Rats were kept in plastic cages during the experimental period. One week acclimatization period was allowed before initiation of the experiment. On the start of the 2nd week, rats were divided into four equal groups:

Group I: Control group (C) was supplied with access water and ordinary rat chow.

Group II: Control group receiving 2 gm/kg/day clover flowers honey (***Omotayo et al., 2010***) by oral route using a gastric gavage tube for 4 weeks (C/H).

Group III: Acute nephrotoxic group (N) were induced by single oral dose (640mg/kg b.wt.) of paracetamol using gastric gavage tube (***Deviet al., 2005***).The animals were treated between 9:00 and 10.00 A.M to minimize the possible diurnal effects in tissue glutathione concentration influencing the experimental results.

Group IV: Rats administrated clover flowers honey (2 gm/kg b.wt./day) for 4 weeks by gastric gavage tube and at the end of the fourth week single oral dose of paracetamol (640 mg/kg b.w) was administered by the same route (H/N).

Blood and tissue samples were collected 72 hours after paracetamol administration in groups treated by paracetamol;group III (acute paracetamol-induced nephrotoxic group, N) and group IV; (H/N).

Blood sampling:

Animals were anesthetized and blood samples were withdrawn from retro – orbital sinus by heparinized capillary tubes under light ether anesthesia after 12-14 hours fasting period. The withdrawn blood was collected in centrifuge tubes for serum separation, by allowing the blood to clot for an hour at room temperature and centrifugation at 3000 rpm for 15 minutes. Sera were separated and stored at – 20 C^{o} until used for estimation of serum urea (BUN), serum creatinine (SC) and glutathione content (GSH). Assessment of serum glutathione (GSH) content was performed using Cayman's GSH assay kit, serum urea was according to modified ***Berthelot – Searcy*** method ***(Henry, 1991),*** while serum creatinine activity was determined using the application of Jafe reaction ***(Wilson and Walkes, 2000).***

Determination of cytosolic catalase (CAT) activity was according to the method of ***Claiborne (1985),*** while the cytosolic superoxide dismutase (SOD) activity was according to ***Marklund (1985)***.

Kidney tissue sampling and preparation of its homogenate

Under light ether anesthesia, the animals were subjected to a dorsal midline incision by dissection of the muscle to reach renal bed and expose the kidneys. Careful dissection was done to the renal pedicle which was rapidly cut. The kidneys were removed and dissected free from the fat and connective tissue. The left kidney was longitudinally sectioned, and renal cortex was homogenized in cold potassium phosphate buffer (0.05 M, pH 7.4). The renal cortical homogenates were centrifuged at 5000 rpm for 10 min at 4 ˚C. The resulting supernatant was used for the

determination of the activities of SOD and catalase (CAT) using colorimetric assay.

The protein content in each supernatant sample was estimated according to **Lowry *et al.*, 1951).**

The right kidney was homogenized in 175 µl of lysis buffer for 10 minutes and the tissue was centrifuged for 20 minutes at 15,000 rpm. 350 µl of SV RNA dilution buffer was added to 175 of tissue homogenate, the mixture was placed in a water bath at 70˚C for 3 minutes. The mixture was centrifuged at 12,000-14,000rpm for 10 minutes at 20-25˚C.

For detection of TNF-α gene expression, RNA was extracted, reversely transcribed into cDNA and amplified by PCR and then detected using agarose gel electrophoresis ***(Williams, 1989)***. RNA was extracted from the kidney tissue using SV total RNA isolation system (Promega, Madison, WI, USA).

Statistical Analysis:

Data input and analysis were done using SPSS computer program. All results were expressed as the mean ± standard error. Mean values of the different groups were compared using a one way analysis of variance (ANOVA). Least significant difference (LSD) post hoc analysis was used to identify significantly different mean values. *P* value < 0.05, 0.0005 was accepted to denote a significant difference**.**

Results

Effect of paracetamol-induced nephrotoxicity and their pretreatment with clover flowers honey on the renal functions

Table 1 depicts the paracetamol-induced nephrotoxicity as reflected by the disturbed kidney functions. The applied model of paracetamol-induced nephrotoxicity in group II(N) caused a significant elevation of serum levels BUN (2.8 times, *P*< 0.0005) and SC (2.2 times, *P*< 0.0005), compared to the normal control group. The disturbance of the kidney functions was further reflected on its defective reabsorptive capacity of albumin and protein, which led to their appearance in high quantities in the urine.

Pretreatment with clover flowers honey (2 gm/kg b.wt./day) for 4 weeks prior to paracetamol administration (Group IV, H/N) showed significant depression of the levels of BUN(-52.7%), SC (-62%); both reaching their normal levels, when compared to nephrotoxic group II (N), at *P*<0.0005. The significant depletion of urinary albumin (-98.8%, P <0.0005) and urinary glucose (-97.6%, *P*<0.0005), which were rendered non-traceable in the urine, indicated a successful renoprotective impact of clover flowers honey.

Table 1: **The serum profile of kidney functions and GSH level in the experimental groups**

	Group I (C)	Group II (C/H)	Group III (N)	Group IV (H/N)
BUN (mg/dl)	**25.75 ±1.1**	**26.92± 3.7**	**59.75±3.2 ***	**28.25±0.5 *#**
SC(mg/dl)	**0.31 ±0.01**	**0.30 ± 0.3**	**0.84±0.05 ***	**0.32±0.02#**
Urinary albumin (mg/dl)	**0**	**0**	**43.6±0.1***	**0.5±0.1*#**
Urinary glucose (mg/dl)	**0**	**0**	**125±5***	**3±0.05*#**
Serum GSH (mg%)	**37.69±0.55**	**37.01±0.1**	**24.94±0.73***	**36.91±0.68 #**

Values shown are mean ± SE(n=10 rats per each group). The experimental groups include control group; C, control group+ honey; C/H, paracetamol-induced nephrotoxic group; N, honey pretreatment to nephrotoxic group; H/N. As compared with control group (*), N group ($^{\#}$) (one-way ANOVA) followed by LSD test), *P*< 0.05, *P*< 0.0005.

Effect of paracetamol-induced nephrotoxicity and their pretreatment with clover flowershoney on the assessed oxidative stress indicators

72 hours after induction of acute kidney failure by paracetamol in group III(N), the serum level of GSH was significantly reduced (*P*<0.05) to reach nearly 33% of its normal level in the control animals (Table 1). This highlights the hampered antioxidant system, which was further extended to the renal tissues.

Renal activities of SOD and CAT were significantly depressed (-72%, -52.5%, respectively at *P*<0.0005) in group III, compared to the control group.

Pretreatment with clover flowers honey for 4 weeks succeeded to restore the total antioxidant capacity at the blood as well as the renal tissues. It prevented the GSH depletion by causing a significant increase in its level (62%, *P*<0.0005), when compared to the nephrotoxic group III (N).

The amelioration of the renal SOD and CAT activities in group III, due to the prophylactic use of clover flowers honey, was reflected on a 2.7 fold increase in renal SOD activity and a 1.9 fold increase in renal CAT activity in group IV (H/N), at *P*< 0.0005 (Figure 1).

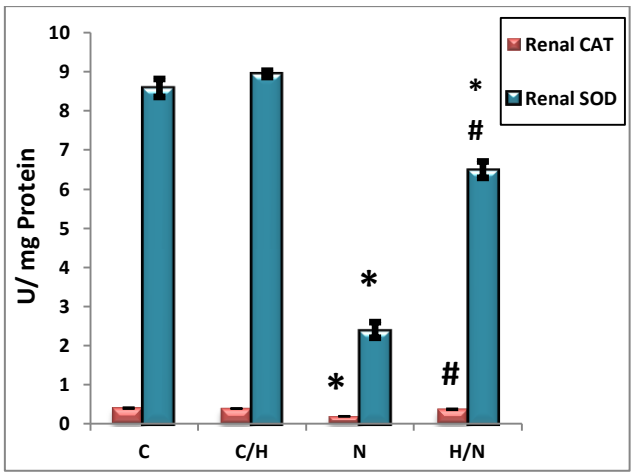

Fig. 1:Mean activities of renal SOD and CAT ± SE. The experimental groups include control group; C, control group + honey; C/H, paracetamol-induced nephrotoxic group; N, honey pretreatment to nephrotoxic group; H/N. As compared with control group (*), N group (#) (one-way ANOVA) followed by LSD test), *P*< 0.05, P< 0.0005.

Effect of paracetamol-induced nephrotoxicity and their pretreatment withclover flowershoney on renal TNF-α gene expression:

Figure 2 illustrates the impact of the nephrotoxic model on the renal tissue gene expression of the apoptotic indicator,TNF-α.The induced nephrotoxicity significantly elevated the renal tissue TNF-α gene expression (5.6 times , *P*< 0.003) when compared to normal. The anti-apoptotic effect of clover flowershoney was reflected by the significant amelioration of the renal tissue TNF-α gene expression (-31%, *P*< 0.0005), an effect that could be correlated to its antioxidant activity.

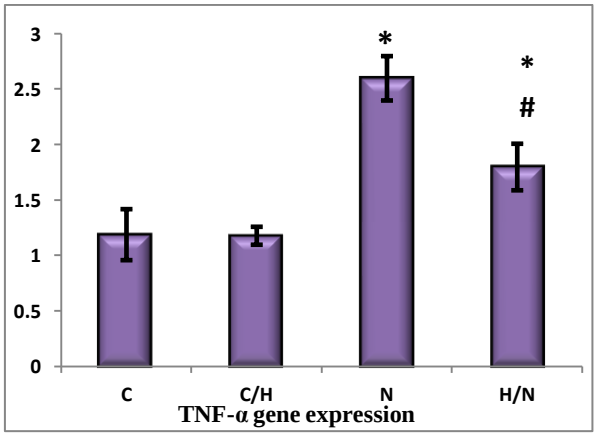

Fig.2:Mean TNF-α gene expression ± SE. The experimental groups include control group;C, control group+ honey; C/H, paracetamol-induced nephrotoxic group; N, honey pretreatment to nephrotoxic group; H/N. As compared with control group (*), N group(#) (one-way ANOVA) followed by LSD test), *P*< 0.05, *P*< 0.0005.

4. Discussion

Paracetamol toxic overdose is often linked to many metabolic disorders including serum electrolyte, urea and creatinine dearrangements. Serum urea and creatinine are considered nephrotoxicity markers, but serum urea concentration is often considered a more reliable renal function predictor than serum creatinine ***(Palani et al., 2009).***

In the present study, administration of nephrotoxic dose of paracetamol to rats resulted in a significant elevation of serum levels of urea and creatinine in paracetamol administrated group when compared to the normal control group. These results are in agreement with that observed by ***Isik et al. (2006)*** who noticed an elevation in serum urea and creatinine in rats after 1 g/kg b.w. of paracetamol administration. Moreover ***Satirapoj et al. (2009)*** reported an elevation in serum urea and creatinine in a woman following therapeutic dose of paracetamol three days before hospital admission.

Karadeniz et al. (2008) and ***Ajami et al. (2010)*** explained this elevation in the levels of urea and creatinine by the presence of strong correlation between nephrotoxicity and oxidative stress. The elevated H_2O_2 and O_2^- production alters the filtration surface area and modifies the filtration coefficient; both factors could decrease the glomerular filtration leading to accumulation of urea and creatinine in the blood .

Acute paracetamol intoxication induced significant appearance of glucose and protein in urine. These results are in agreement with that observed by ***Melo et al. (2006)*** who noticed an increase in the urinary excretion of albumin and glucose after paracetamol injection associated with augmented serum urea and creatinine, both indicating glomerular damage. ***Abdel-Raheem et al. (2009)*** reported that detection of small quantities of urinary albumin is one of the early biomarkers of altered glomerular permeability. The appearance of glucose and protein may be attributed to the dysfunction of the proximal convoluted tubule because glucose and proteins are completely absorbed from the proximal convoluted tubules under normal conditions. These results are supported by the findings of ***Abdel – Zaher et al. (2008),*** who stated that the nephrotoxicity induced by paracetamol overdose was characterized by damage and necrosis in the proximal tubule.

Abbate et al. (2006) reported that progressive renal damage by proteinuria may be a mechanism that exerts destructive effect on the kidney. Proteinuria may accelerate kidney disease progression to end stage renal failure and whenever proteinuria is decreased by treatment, progression to end stage renal dysfunction is reduced.

Several studies have clearly demonstrated that acute paracetamol overdose induces renal oxidative

stress as manifested by a decrease in antioxidant enzymes and an increase in lipid peroxidation product (malondialdehyde) ***(Abdel – Zaher et al., 2007 and Ghosh & Sil, 2007).***

The current study showed that the administration of paracetamol (APAP) in acute single toxic dose resulted in a significant decrease in renal SOD and CAT activities when compared to control group. The present results are in agreement with that observed by ***Palani et al. (2009)*** and ***Demirbag et al. (2010)*** who observe a significant decrease in levels of SOD and CAT after acute paracetamol overdose administration to rats when compared with normal control rats and explain these results on the base that paracetamol overdose enhances lipid peroxidation or inactivates the antioxidative enzymes. In addition, ***Linares et al. (2006)*** reported that, during kidney injury, superoxide radicals are generated at the site of damage and modulate SOD and CAT, resulting in the loss of activity and accumulation of superoxide radical, which damages kidney. SOD and CAT are the most important enzymes involved in ameliorating the effects of oxygen metabolism.

Current evidence suggests that intracellular GSH plays an essential role in detoxification of APAP and prevention of APAP-induced toxicity in the liver and kidney ***(Newton et al., 1996 and Richie et al., 1996).*** The generation of the reactive oxygen species appears as an early event which precedes intracellular GSH depletion and cell damage in paracetamol toxicity ***(Manov et al., 2003).***

In the present study, paracetamol overdose administration caused a significant decrease in serum GSH content. This decrease in GSH level could be considered another mechanism for the observed paracetamol nephrotoxicity and suggests that the administration of high dose of paracetamol saturates the metabolic pathway, decreases the liver clearance of APAP and allows higher amounts of the un-metabolized drug to come in contact with the kidney ***(Gu et al., 2005).*** Because the same enzyme system as the liver is also present in the kidney, it is most probable that N-acetyl-P-benzoquinone imine (NAPQI) will also be formed in the kidneys, giving rise to toxicity but much later than in the liver (***Roberts and Bukley, 2007).*** The major pathway of metabolism during toxicity is via CYP-450 due to saturation of glucurinidation and sulfation pathways forming the intermediate NAPQI in high amounts which in turn will be conjugated with GSH to detoxify this product with consequent exhaustion of cellular GSH reserve ***(Sener et al., 2005).*** At sufficiently high doses, GSH becomes depleted leaving NAPQI free ***(Dai et al., 2006).*** The free NAPQI is strongly electrophilic and binds covalently and irreversibly to critical cellular, protein causing cellular necrosis ***(James et al., 2003).***

Tumor necrosis factor-α is one of the most important proinflammatory cytokines that can cause cell death through binding to its specific receptor tumor necrosis factor receptor on the cell surface. Accordingly, it was described to be increased in many diseases in which apoptosis is included in its mechanism such as glomerular injury, acute renal failure and chronic renal affection ***(Locksley et al., 2001).***

The present study showed a significant increase in the renal TNF-α gene expression after acute paracetamol overdose administration. These results are supported by the study of ***Das et al. (2010)*** who reported that paracetamol administration at a single dose of 2g/kg body weight orally to male adult albino mice increased plasma level of blood urea, creatinine and TNF-alpha. This increase is explained in terms of the induction of renal damage through oxidative stress.

The increased TNF-α gene expression could be attributed to activation of apoptosis pathways. Apoptosis can result from the activation of death receptors in response to ligand binding ***(Servais et al., 2008). Abeet al.*** **(2004)** reported another mechanism that increases TNF-α which is proteinurea that can result in complement activation, production of reactive oxygen species and cytokines synthesis e.g. TNF-α. ***Morais et al. (2005)*** reported that the exposure of proximal tubular cells to albumin produced strong upregulation in the expression of Fas and Fas ligand and apoptotic response. This finding correlates with the present results that showed proteinurea associated with an increase in some apoptotic markers as TNF-α.

Ghosh et al. (2010) stated that, exposure of rats with a nephro-toxic dose of APAP altered a number of biomarkers (like blood urea nitrogen and serum creatinine levels) and decreased the renal oxidative stress markers with elevation of renal tumor necrosis factor-alpha, these changes were occurred as a result to inactivation of the mitochondrial pathway during APAP-induced cell death .

The therapeutic role of honey in the treatment of various ailments has been receiving considerable attention recently, and its therapeutic value has been partly attributed to its anti-inflammatory, anti-apoptotic and antioxidant properties.

Interestingly, the present study revealed that prophylactic administration of clover flowers honey for 4 weeks before acute paracetamol overdose administration resulted in improvement of the kidney functions in the form of nearly complete absence of glucose and protein from urine, which could be attributed to the glomerular repair. These results are in accordance with ***Jaganathan and Mandal (2009)*** who reported an improvement in kidney functions after honey administration in rats with induced nephrotoxicity.

The current study showed that a significant elevation of the oxidative stress enzymes SOD, CAT and GSH enzyme activities results from honey administration. This increase coincides with ***Omotayo et al. (2010)*** who reported that honey supplementation ameliorates oxidative stress in kidneys of diabetic rats, which was proved by significant reduction of the activities of catalase (CAT) and glutathione. Also ***Cavuşoğlu et al. (2009)*** reported that the protective role of the honey in cases of cadmium induced genotoxicity in mice may be attributed to its anti-oxidant effect.

The present results are also consistent with those of ***Jaganathan and Mandal (2009)*** who reported that improvement of kidney functions and the correction of decreased level of oxidative stress enzymes in the form of SOD, CAT and Serum GSH by the use of honey are related to some minor constituents of honey, which is believed to have antioxidant properties. Among these constituents are flavonoids and phenolic acids, certain enzymes (glucose oxidase, catalase), ascorbic acid, carotenoid-like substances, organic acids, amino acids, and proteins.

Clover flowers honey supplementation for 4 weeks before paracetamol administration protected against paracetamol induced renal damage as indicated by reducing the elevated serum urea and creatinine. These results are ~~in~~consistent with ***Al-Waili et al. (2006)*** who noticed a significant decrease in serum urea and creatinine after honey treatment as it corrects the influence of hemorrhage and food restriction on renal functions.

The obtained results showed reduction in TNF-α gene expression in paracetamol induced nephrotoxicity after honey treatment which could be attributed to the anti-inflammatory, antiapoptotic and healing effect of honey.

These results are consistence with ***Majtan et al. (2010)*** who reported that, honey decreases the elevated TNF alpha and plays an important role in healing through degradation of type IV collagen in the basement membrane of tissues. So, honey may accelerate wound healing process.

The anti-inflammatory properties of honey were also proved by the histopathological evaluation of the inflamed eyes of rabbits treated by honey in the study of ***Öztürk et al. (2002).*** They found that the degree of inflammatory cell infiltration was significantly lower in the group treated with topical honey. This suppression of the inflammatory cells could be the cause of the decreased gene expression of TNF-α after honey treatment in APAP induced nephrotoxicity.

Similar findings were also recorded by ***Han et al. (2007)*** who reported that honey has immunomodulatory properties which could be useful in inhibiting the production of inflammatory cytokine and NO production in neurodegenerative diseases.

In conclusion, our study showed that honey has the ability to contribute to glomerular and tubular repair and recovery of the kidney from nephrotoxicity, which is proved by the improvements of the kidney function parameters and the decrease of the antioxidant and anti-inflammatory markers. Thus, honey is suggested to have a beneficial effect on renal tissue through potent paracrine anti-apoptotic and anti-inflammatory mechanisms.

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