Antioxidant activity and protective effects of commercial propolis on gentamicin induced nephrotoxicity in rabbits - in vitro study

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ABSTRACT

Objective: the aim of this study is to investigate the in vitro antioxidant activity, the total phenol and flavonoid content and the possible protective effects of commercial propolis on gentamicin-induced nephrotoxicity in rabbits.

Methods: the in vitro antioxidant activity was measured by ferric reducing antioxidant power assay and cupric reducing antioxidant capacity assay, the total phenols content was measured by folin–ciocalteau assay, the flavonoids content by the alcl3 colorimetric method and the renoprotective effects of propolis methanol extract was evaluated in a rabbit model of gentamicin-induced nephrotoxicity. The protective effects of propolis on gentamicin-induced nephrotoxicity in rabbits were evaluated through biochemical parameter (measuring serum urea and creatinine) and histopathological alterations in kidneys

Results: methanol extract of propolis showed a strong antioxidant activity, which is attributed to its high phenolic and flavonoid contents. Oral administration of propolis extract to rabbits at a dose of 1 mg/kg body weight significantly protected against histopathological and biochemical alterations induced by gentamicin.

Conclusion: the present study demonstrated that commercial propolis is strong antioxidant and is effective for the prevention of gentamicin-induced renal damage in rabbits

Key Words: Bee glue, reducing power, renal damage, total phenol, histopathological alterations

Conflict of Interest: The authors declare that, there is no conflict of interest regarding this manuscript.

ÖZET

Amaç: Bu çalışmanın amacı tavşanlarda gentamicin kaynaklı nefrotoksisitede ticari arı reçinesinin invitro çalışmalar ile anti oksidan aktivitesinin, total fenol ve flavonoid içeriğinin ve muhtemel koruyucu etkinisinin araştırılmasıdır.

Metod: Metanol çözümlü arı reçinesinin böbrek koruyucu etkisi gentamicin kaynağı nefrotoksisisite modeli olan tavşanlar üzerinde değerlendirilmiş olup, invitro antioksidan aktivitesi ferrik indirgeme ve kuprik indirgeme antioksidan kapasitelerini, total fenol içeriği Folin–Ciocalteau, flavonoid içeriği ise AIClrenk ölçüm metodu ile belirlenmiştir. Tavşanlarda gentamicin kaynaklı nefrotoksisisite karşısında konuma etkisi biyokimyasal parametreler (serum ure ve keratin ölçümü) ve böreklerdeki histopatolojik değişiklikler ile değerlendirilmiştir.

Sonuç: Arı reçinesinin methanol ekstrakti kuvvetli antioksidan aktivitesi göstermiştir. Bu kuvvetli antioksidan altıftime yüksek fenol ve flavanoid içeriğine dayandırılmışmaktadır. Tavşanlara 1 mg/kg vücut ağırlığı dozunda arı reçinesinin efgzidend verilmiş sntamicin kaynaklı histopatolojik ve biyokimyasal değişiklikleri karşısında belirgin bir koruma sağlamıştır.

Yorum: Bu çalışma ticari arı reçinesinin kuvvetli bir antioksidan olduğunun ve tavşanlarda gentamicin kaynağı renal hasara karşı koruma sağladığını göstermiştir.

Anahtar Kelimeler: Arı reçinesi, tıbbi uygulamada, total fenol, histopatolojik değişiklikler

Çıkar Çatışması: Yayınların bu çalışma için bildirimcileri bir çıkış çatışması bulunmamaktadır.

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**Introduction**

Propolis or “bee-glue” is a yellow-green to dark brown resinous and rubbery like substance collected and transformed by honey bees, *Apis mellifera* from various plant sources to seal holes in their hives and to protect it from intruders [1]. Propolis has long been used in folk medicine in many parts of the world. Ancient Egyptians, used propolis for embalming their dead, and Greeks used it to cure external and internal sores and ulcers due to its anti-inflammatory and healing properties [2]. It exhibits a wide range of biological activities such as anti-oxidant [3], anti-bacterial [4], anti-viral [5], anti-fungal [6], anti-parasitic [7], anti-inflammatory activities [8] and as hepatoprotective agent against carbon tetrachloride induced hepatotoxicity [9]. These biological activities are based on its rich phenolic, flavonoid and trepenoid contents [10].

Gentamicin is an aminoglycoside antibiotic, used to treat bacterial infections, caused by Gram-negative bacteria. Because of its serious side effects: nephrotoxicity and ototoxicity, the use of gentamicin in treatment of gram negative bacterial infection is restricted. It is estimated that 5-35 % of all cases treated with gentamicin develop renal failure [11]. The exact mechanism of gentamicin induced toxicity is controversial, but reactive oxygen species (ROS) appear to be implicated in the toxic effects of gentamicin [12]. Several studies reported that the use of antioxidant such as vitamin C, reduced the nephrotoxicity associated with gentamicin [13].

To our knowledge there is no evidence reporting the protective effects of propolis against gentamicin induced nephrotoxicity.

The aims of this study were to evaluate the in vitro antioxidant activity and the protective effects of commercial propolis on gentamicin induced nephrotoxicity in rabbits.

**Materials and Methods**

**Materials**

All chemicals used are of analytical grade and were obtained from Sigma Chemical Co. (St Louis, MO) and Scharlau, Germany. Propolis was purchased from local market in Sakaka City, Saudi Arabia.

**Methods**

**Preparation of propolis extract**

Propolis powder (10 g) was mixed with 100 ml of 80% (v/v) methanol and shaken at room temperature for 24 h. The mixture was filtered and centrifuged at 1400g for 5 minutes. The supernatant was concentrated using rotary evaporator. The dry extract was kept frozen at -20 °C in dark glass tubes.

**Preparation of test and standards solutions**

Propolis dry extract and standard antioxidants (gallic acid, rutin and ascorbic acid) were dissolved in 80% (v/v) methanol separately to obtain 1mg/ml stock solutions. The stock solutions were serially diluted to obtain lower concentrations and used for the in vitro antioxidant assays.

The propolis dry extract was dissolved in normal saline and used for animal study experiment.

**Estimation of total phenols**

Total phenol content of the methanol extract of propolis was estimated by using Folin-Ciocalteu reagent [14]. The method based on reaction between phenolic compounds with phosphomolybdic acid in Folins reagent to produce a blue-colored complex in alkaline medium, which can be estimated spectrophotometrically at 765nm.

The original procedure was modified to use with total volume of 1.5 ml instead of 5 ml used in the original method. Briefly 0.15ml of methanol extract (1mg/ml) was incubated with 0.75 ml of 10% FC reagent and 0.6 ml of 7.5% Na2CO3 for 30 min at room temperature. The absorbance of the color developed was measured at 765 nm against blank. The total phenolic content was expressed as gallic acid equivalents/g of dry extract (GAE/g dry extract) using a standard curve generated with gallic acid as standard.

**Total flavonoid content**

Total flavonoid content was determined by a colorimetric method described previously [15]. The original procedure was modified to use with total volume of 1.5 ml instead of 2.5 ml used in the original method. Briefly, 0.2 ml of the extract (1mg/ml) was diluted with 0.78 ml of distilled water. Then 60 µl of a 5% NaNO2 solution was added to the mixture. After 5 min, 60 µl of a 10% AlCl3 solution were added, and the mixture was allowed to stand for another 5 min at room temperature. 400 µl of 1 M NaOH was added. The solution was well mixed, and the absorbance was measured immediately against the prepared blank at 510 nm using a spectrophotometer in comparison with the standards prepared similarly with rutin. The results are expressed as milligrams of rutin equivalents/g of dry extract (mg RE/g dry extract).

**Ferric reducing antioxidant power assay (FRAP assay)**

The reducing ability of the methanol extract of propolis was determined according to the method of [16]. The method based on formation of blue colored complex due to reduction of ferric (Fe3+) form to ferrous (Fe2+) form by antioxidant. The original procedure was modified to use with total volume of 1.7 ml instead of 8 ml used in the original method. Briefly 0.2 ml of propolis extract or ascorbic acid (25-250 µg/ml) was mixed with 0.5 ml of Phosphate buffer (0.2 M, pH 6.6) and 0.5 ml of 1% potassium ferricyanide. The mixture was incubated for 20 minutes at 50°C. At the end of the incubation, 0.5 ml of 10% trichloroacetic acid was added to the mixture and
Animals

Adult male white New Zealand rabbits weighing 2.0 to 2.5 kg were obtained from the animal house, College of Applied Medical Sciences, Al-Jouf University and housed individually in stainless steel wire cages with controlled environment at 21 ± 2 °C, and relative humidity at 50 ± 5 under a 12 h dark/light cycle for one week of acclimatization. The animals were fed standard laboratory diets and allowed free access to tap water. All the experimental procedures were carried out in accordance with international guidelines for care and use of laboratory animals of [18], and were approved by the Bioethical Committee of the Al Jouf University.

Experimental design

Twenty four adult male white New Zealand rabbits were randomly divided into four equal groups. Gentamicin was administered intramuscular (i.m.) to animals from groups 1 and 2, in a two doses of 40 mg/kg body weight, for 5 days. Propolis was administered daily by gastrogavage to animals from group 2 and 3 in a single dose of 1 mg/kg body weight for 7 days. The treatment groups and experimental protocols are detailed below.

Group 1: gentamicin (40 mg/kg body weight, IM) twice a day for a period of 5 days.

Group 2: propolis (1 mg/kg body weight) once a day for 7 days (2 days before gentamicin and 5 days along with gentamicin) + gentamicin (40 mg/kg body weight, twice a day for a period of 5 days).

Group 3: propolis (1 mg/kg body weight), once a day for 7 days.

Group 4: normal control rabbits, received 2.5 ml normal saline (i.m.).

At the end of experiment, rabbits were sacrificed then blood samples and tissues specimens from kidneys were collected. Serum urea and creatinine levels were assayed using auto-analyzer (Mindray BS-300 chemistry analyzer). The tissue specimens were fixed in 10% neutral buffered formalin. After proper fixation, thin tissue-paraffin sections were routinely prepared from the kidneys and stained with H&E stain [19].

Statistical Analysis

Experimental analyses were performed in triplicate. Data were recorded as mean ± standard deviation and analyzed by GraphPad prism version 5. Statistical comparisons between groups were made using one way analysis of variance (ANOVA). Difference in mean values were considered significant when \( p \leq 0.05 \).

Results

Determination of total phenol & flavonoid content

The methanol extract of propolis showed high phenol content was 157.99 ± 12.3 mg GAE/g of dry extract. The total flavonoid content of propolis extract was 16.96 ± 1.48 mg/g of dry extract expressed as rutin equivalent. A high yield of phenolics and flavonoids in the extract indicated it is possess a high antioxidant activity.

Reducing Power assays

Propolis extract had effective reducing power determined by using the ferric reducing antioxidant power assay (FRAP assay) and copper ions (Cu^{2+}) reducing methods when compared to the standards.

Ferric reducing antioxidant power assay (FRAP assay)

The ability of propolis extract to reduce Fe^{3+} to Fe^{2+} was determined and compared with that ascorbic acid (Figure 1). All used concentration of propolis methanol extract showed some degree of electron donating capacity to reduce Fe^{3+} to Fe^{2+} in a dose dependent manner. According to the obtained result the methanol extract of propolis had high reducing power which was comparable to that of ascorbic acid.

Cupric reducing antioxidant capacity (CUP-RAC) assay

Further, the reducing ability of propolis methanol extract was confirmed by measuring its ability to reduce Cu^{2+} to Cu^{+} ions and result was compared with ascorbic acid as standard. All used concentration of propolis methanol extract showed some degree of electron donating capacity to reduce Cu^{2+} to Cu^{+} in a dose dependent manner. According to the obtained result the methanol extract of propolis had high reducing power (Figure 2). At low concentration (62.5 \( \mu \)g/ml), the reducing power of propolis extract exceeds that of vitamin C.
Effect of propolis on gentamicin-induced nephrotoxicity in rabbits

Propolis treatment (1mg/kg body weight) for 7 days did not change serum urea and creatinine level compared to untreated control. Gentamicin administration (40 mg/kg bodyweight every 12 h for 5 days) caused nephrotoxicity evident by increase serum levels of urea and creatinine. However propolis administration (1 mg/kg of body weight, orally) prevented urea and creatinine elevation induced by gentamicin (Table 1).

Gross and histopathological observations

PM examination of the rabbits in group I (gentamicin 40 mg/kg every 12 hours, for 5 days) revealed the presence of excessive amount of clear watery fluid in the abdomen with paleness of the kidneys (Figure 3A). The urinary bladders were distended with turbid urine (Figure 3B). Moreover, in some examined rabbits, the kidneys were enlarged, congested and their cut sections revealed minute red spots on both cortex and medulla. Histopathological examination of the kidneys of rabbits in group I showed that IM administration of gentamicin was accompanied with severe renal damages evidenced by necrosis of the renal tubular epithelium (Figure 3C). Multiple areas of degenerative changes in the form of cloudy swelling, fatty, hydropic and vacuolar degeneration of the lining epithelium of the renal tubules were also detected in some examined kidneys. Hyaline casts were seen in some dilated renal tubules. Congestion of the renal blood vessels and intertubular blood capillaries with perivascular edema was prevalent. Thrombosis of the renal blood vessels with degeneration of the muscular layer of renal blood vessels was observed (Figure 3D). Moreover; focal interstitial hemorrhages were seen in both cortex and medulla (Figure 3E). Propolis pretreatment significantly prevented gentamicin-induced nephrotoxicity. PM examination of the rabbits in group II (Propolis + gentamicin) revealed congestion of the kidneys (Figure 4A). However, in two rabbits, the kidneys were swollen and pale in color. Moreover, urinary bladders of all examined rabbits were free from turbid urine and showed only congested blood vessels (Figure 4B). Microscopically, no congestion of some renal blood vessels with more or less normal renal tubules and glomeruli was seen in the most examined kidneys (Figure 4C). However, focal areas of mild hydropic and vacuolar degeneration of the renal tubular epithelium and perivascular edema were noticed in two examined kidneys (Figure 4D).
Neither gross nor microscopic lesions were detected in the examined kidneys of rabbits received propolis alone (Group III) or saline (Group IV).

Discussion

The objectives of the current study were to determine the in vitro antioxidant activity of commercial propolis and to evaluate the protective effects of propolis on gentamicin induced nephrotoxicity in rabbits.

Commercial propolis is available in many parts of the world, and it is used for its antioxidant and other pharmacological properties [20]. Phenolic and flavonoid compounds have been shown to be responsible for the antioxidant activity of plant materials [21]. Therefore, the amount of total phenol & flavonoid in the methanol extract of propolis were determined by using Folin-Ciocalteu and AlCl$_3$ reagent respectively.

Phenolic and flavonoid contents of propolis extract reported in literature showed a great variation. This is mainly due to method of extraction used and its geographical location [22].

The highest phenolic content was found in Chinese propolis sample (302 ± 4.3 mg GAE/g) as reported by [23]; while the lowest (31.2 ± 4.3 mg GAE/g) was reported by [24].

The reducing power of a compound serve as a significant indicator of its potential antioxidant activity, because its associated with their ability to donate electron to free radical species, reducing them into more stable and un-reactive form [25]. The reducing power of propolis extract was determined using 2 methods; the FRAP and the CUPRAC assays. The antioxidant ability of propolis extract was compared with vitamin C as a standard. The obtained results showed that the reducing ability of commercial propolis methanol extract was comparable to that of the standard (vitamin C).

The obtained results were in agreement with that obtained by Moreira et al. [26].

Nephrotoxicity is the major factor limiting the use of gentamicin. Gentamicin-induced nephrotoxicity is a well-documented event involving some functional and cellular mechanisms such as glomerular lesions that interfere with glomerular hemodynamics, and altered tubular transport, with the injury ranging from solely functional lesions to the occurrence of necrosis [27]. The exact mechanism of gentamicin induced toxicity is controversial, but reactive oxygen species (ROS) appear to be implicated in the toxic effects of gentamicin [12].

In the present study, the administration of gentamicin caused severe renal damages evidenced by degeneration and even necrosis of the renal tubular epithelium together with the presence of hyaline casts in lumens of some tubules. In addition, vascular changes in the form of congestion, thrombosis with perivascular edema and interstitial hemorrhages were also detected. These microscopic changes were in agreement with results of previous investigations [28, 29].

Regarding to serum biochemical changes associated with administration of gentamicin, there are great increases in creatinine values reaching 166 mmol/l comparing with those recoded in control group where mean creatinine value was 53 mmol/L. Like creatinine, serum urea values were elevated up to 17.46 mmol/l in comparison with...
those observed in control where urea level which was 3.31 mmol/l. These results of serum biochemical analysis reflected the renal dysfunction caused by gentamicin and confirmed the histopathological findings of the examined kidneys. These findings were in agreement with results of previous investigations [30].

Concerning the effect of propolis supplementation on the gentamicin-induced nephrotoxicity, it was cleared that, propolis has a protective effect against nephrotoxicity induced by gentamicin, where no characteristic microscopic changes except congestion and perivascular edema with focal renal tubular degeneration were seen in the kidneys of rabbits administered with gentamicin plus propolis supplementation. Moreover, serum profile of these rabbits showed improvement where serum creatinine and urea levels became lower than those recorded in rabbit treated with gentamicin alone.

**Conclusion**

The obtained results showed that Commercial propolis methanol extract have high phenol and flavonoid content and possess strong antioxidant activity.

The study demonstrated that Gentamicin at a dose of 40 mg/kg/12hr IM for 5 successive days induced nephropathy in rabbits represented by nephrosis in the form of degeneration and necrosis of renal tubules associated with increases in serum creatinine and urea levels.

Propolis is effective in protecting against gentamicin induced nephrotoxicity in rabbits as it exerted an improvement on both functional and microscopic changes induced by gentamicin.

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**Conflict of Interest:** The authors declare that, there is no conflict of interest regarding this manuscript.

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