

Full Length Research Paper

Polyphenolic fractions of Algerian propolis reverses doxorubicin induced acute renal oxidative stress

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The most important pharmacologically active constituents in propolis are flavanoids with a broad spectrum of biological activities varying with their chemical composition. Propolis chemical composition depends on the floral and geographical origin present at the site of collection and thus in the climatic characteristics. However, until now, no mitochondrial functions in relation to stress and apoptotic process were determined. We hypothesized that propolis effects could be due to a direct action on mitochondrial functions. We evaluated whether polyphenols compounds had preventive properties against renal oxidative stress induced by doxorubicin. We present here an analytical and pharmacological study of the eastern Algerian propolis using Thin layer Chrommatography (TLC), Ultra Violet-High Phase Liquid Chromatography (UV-HPLC) and Gas Chromatography-Mass Spectrometry (GC-MS). The pharmacological study was carried out *in vivo* on wistar rat pre-treated with propolis extract 100 mg/kg/day for 7 days. Doxorubicin at 10 mg/kg of body weight was administered intravenously on day 7th. Serum creatinine concentration, scavenging effect of flavonoids, lipid peroxydation (MDA) and glutathione (GSH) concentration were measured. Chemical analysis allowed identification and quantification of the phenolic compounds including pinostrombin chalcone(38.91%), galangin(18.95), naringenin(14.27%), tectochrysin(25.09%), methoxychrysin(1.14%) and a prenylated coumarin compound suberosin (1.65%). The total flavonoid concentration in the propolis extract determined by aluminum chloride colorimetric method was 370 mg (quercetin equivalents QE) /g dry weight of propolis extract (QE/g DWPE). Data suggest protective effects of an Algerian propolis extract against doxorubicin-induced oxidative stresses. It restored the renal functions and clearly reduced the toxic effect of the drug.

Key words: Algerian propolis, chemical analysis, flavonoids, renal oxidative stress.

INTRODUCTION

Propolis is a complex resinous hive product, and a

mixture of wax, sugars and plant exudates collected by bees from plants (Bankova et al., 2002; Kartal et al., 2002). More than 300 constituents have been identified in propolis (Velikova et al., 2000; Bankova et al., 2002). Propolis chemical composition depends on its floral origin with constituents varying widely due to climate and geographical conditions (Kosalec et al., 2004; Seidel et al., 2008). Flavonoids, aromatic acids, diterpenic acids and phenolic compounds appear to be the principal components responsible for the biological activities of propolis (Kosalec et al., 2004). Nevertheless, the antioxidant activity of honey varies greatly depending

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Abbreviations: **AlCl₃**, aluminum chloride; **DWPE**, dry weight of propolis extract; **EEP**, ethanolic propolis extract; **GC-MS**, gas chromatography-mass spectrometry; **QE**, quercetin equivalents; **RP-HPLC-DAD**, reversed phase high performance liquid chromatography-diode array detection; **TLC**, thin layer chromatography; **UV-HPLC**, ultra violet high phase liquid chromatography.

on the floral source (Gardjeva et al., 2007), and external factors such as season, environment and its processing (Velikova et al., 2000). Oxidative stress is now known as the main cause of disturbance in the balance between cellular metabolic antioxidant and oxidant (Temple, 2000). Many situations can cause the production of these oxidants in the body. For example, some treatment as doxorubicin, produce reactive oxygen species (ROS) probably responsible for the renal or cardiac toxicities (Boutabet, 2007; Alyane et al., 2008; Benguedouar et al., 2008; Han, 2008). However, the mechanism of renal toxicity of doxorubicin is not clearly determined. The administration of doxorubicin led to the formation of reactive metabolites (e.g radical's superoxydes O₂⁻, hydrogen peroxides H₂O₂...). The latter will attack the cell membranes which lead to the peroxidation of polyunsaturated fatty acids and the massive release of MDA and depletion of GSH. Recently we have shown that propolis extract protect in vivo the liver and the heart tissues against cyclophosphamid and doxorubicin toxicity (Lahouel et al., 2004; Alyane et al., 2008). The purpose of this research is in order to investigate the protective effect of propolis extract against doxorubicin nephrotoxicity and further elucidated whether propolis flavonoids can protect kidney cells by analysing in vivo their scavenging ability and their effects on lipid peroxydation and on glutathione reserve.

MATERIALS AND METHODS

Animals

The study was performed on 3-month old male albinos Wistar rats (Pasteur Institute of Algiers, Algeria) each weighing approximately 250 g. The animals were divided to 4 groups, with free access to water and food. The ambient temperature was set at 22°C, with a relative humidity of 60% and a light/dark period of 12 h, with the light period beginning at 06:00 am.

Treatment of animals

The animals were divided into four groups of:

- 1) 8 control rats receiving saline solution 0.9% daily for 7 days,
- 2) 8 rats receiving an oral administration of propolis extract (100 mg/kg/day) for 7 days,
- 3) 5 rats receiving an oral administration of propolis extract (100 mg/kg/day) for 7 days before the intravenous injection of 10 mg/kg doxorubicin on the 7th day,
- 4) 5 rats receiving a single intravenous injection of 10 mg/kg doxorubicin alone.

Collection of biological samples

The blood was collected at different times 24 h, 7 days and 14 days in the retro-orbital sinus in tubes and centrifuged at 3300 rpm for 10

min. The serum was collected and frozen for biochemical and enzymatic analyses. The urine was collected for measuring the activity of γ -glutamyl transpeptidase at times 24 h, 7 days and 14 days. The rats were sacrificed after ether anaesthesia at times 24 h, 7 days and 14 days after administration of the anticancer drug doxorubicin. Then, kidneys were quickly removed and frozen (-20°C) until biochemically assayed.

Methods

Propolis

Crude propolis (beeswax and resins collected by the honeybee from plants) was obtained from beekeepers of "Cooperative Apicole of Kaous, Jijel (Eastern Algeria)" in May of 2008. Samples, once received, were stored at 4°C in airtight /dark plastic containers until analysis.

Extraction and sample preparation

The bioactive substances of propolis were extracted by ethanol and methanol. One hundred grams of raw propolis were cut in small pieces, added with 9 volumes (900 ml) of 95% ethanol, the mixture was let for steeping for 15 days with agitation. After filtration on cotton, the filtrate was evaporated at 80°C using a rotary evaporator (Evaporator E100, Heidolph-instruments, Germany). The residual was retaken using 70% methanol and was let for steeping over night. After evaporation of the solvent, the raw extract or ethanolic propolis extract (EEP) was obtained. The extraction was continued by passage in various solvents to obtain aglycones and heterosides flavonic. Flavonoïds rate was determined by reactivity with aluminium chloride (AlCl₃) as described by Benguedouar et al. (2008).

Thin-layer chromatography (TLC)

TLC analyses of EEP were performed on silica gel plates 60-GF254 (Merck). The EEP samples were diluted with 95% ethanol (1/10 vol/vol) and 5 μ l of the propolis solution were applied to the plates. The mobile phase used is pure chloroform. The TLC chamber was saturated with the mobile phase at least 1h before analysis. After developing, plates were dried in the air, and propolis components were visualized under ultraviolet light (336 nm) after spraying with 1% ethanolic solutions of aluminum chloride. The TLC of EEP were used to identify the components of propolis.

High phase liquid chromatography (HPLC) analysis

Analyses of flavonoids and other phenolic acids in ethanolic extract of propolis (EEP) were carried out using reversed phase high performance liquid chromatography–diode array detection (RP-HPLC-DAD). The HPLC system was from Varian; DAD was a Varian Prostar monitoring from 200 to 700 nm wavelengths (Middelburg, The Netherlands). The phenolic compounds in EEP at concentration of 10 mg/ml were separated on a Nucleodur analytical column, 250 x 4.6 mm i.d (Machery-Nagel, Duren, Germany) packed with C18 stationary phase, particle size 5 μ m.

The linear binary gradient was used. The time of HPLC run was over 70 min. Binary mobile phase consisted of solvent A (trifluoroacetic acid 0.01%) and solvent B (acetonitrile). The

separation was obtained by using a gradient starting at 10% of B till 10 min; 10-50 min B increased to 50% and kept constant till 15 min; 65-70 min B increased at 100% and kept constant till 10 min; successively, the solvent B reached back 10% to reequilibrated the column (washing).

Gas chromatography-mass spectrometry (GC-MS) analysis

Gas chromatography-mass spectrometry was carried out on a Fisons GC 2010 gas chromatograph coupled to a Fisons MSQP_2010 mass detector under electron impact ionization (70 eV) at a maximum temperature of 350°C. The chromatographic column for the analysis was non-polar (FS-FE 30 CB) capillary column (25m x 0.25mm i.d). The carrier gas used was helium, and the samples volumes (0.5µl) of EEP solutions were injected. Two programs were tested, the first in EEP, with the initial temperature 150°C, the final temperature 280°C and a stage 10°C/min, the second in different fractions (that is, the phase ether diethyl or aglycone flavonic), with the initial temperature 100°C, the final temperature 280°C and a stage 3°C/min.

Free radical scavenging assay

The free radical scavenging activity of test compounds (propolis extract, and quercetin) was evaluated using the stable radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH°). 15 µl of each compounds test was mixed with 1.5 ml (1mg) DPPH° in ethanol. The absorbance of the remaining DPPH° was determined each 15 second during 5 minute at 515 nm. Blank sample contained the same amount of ethanol and DPPH°. The measurements were performed in triplicate. The radical scavenging activity was calculated by the formula: $I = [(Aa - Ab) * 100]$; where I = DPPH° inhibition, %;

Ab = absorption of blank sample (t = 0 min);

Aa = absorption of tested compounds.

Determination of malondialdehyde (MDA)

Homogenates of the cortical and outer medullar zones of kidney were made prepared at 10% (w/v) in 0.1 mol/L Tris-HCl buffer, pH 7.4, and malondialdehyde (MDA) steady-state levels were determined. MDA was measured according to the method described by (Sastre et al., 2000). Thiobarbituric acid 0.67% (w/v) was added to aliquots of the homogenate previously precipitated with 10% trichloroacetic acid (w/v). Then the mixture was centrifuged, and the supernatant was heated (100°C) for 15 min in a boiling water bath. After cooling, n-butanol was added to neutralize the mixture, and the absorbance was measured at 532 nm. The results were expressed as nmol of MDA/g tissue.

Determination of glutathione

Cellular glutathione content was measured as described by Ellman (Ellman, 1959). 0.5g of fresh kidney was homogenized with three volumes of trichloroacetic acid TCA (5%) using a grinder DOUNCE. Homogenized and centrifuged at 2000 rpm, then 50 µl of the supernatant were diluted in 10 ml of phosphate buffer (0.1M, pH = 8). Consequently, were added 20 µl of DTNB 0.01 M (acid 5, 5'-dithiobis 2-nitrobenzoic acid) to 3 ml of the mixture dilution. The measurement of the optic density was performed at 412 nm against

a control prepared in the same conditions using TCA 5%. The concentrations are expressed in mmoles of glutathione /g of kidney. They are deducted from a range of glutathione, which was prepared with the same conditions as dosage did.

Determination of serum creatinine

Serum creatinine is measured by the colorimetric method of Jaffe (Bonsnes and Tausky, 1945) through the formation of a colored product in the presence of an alkaline picrate. The measurement is made using a spectrophotometer at 492 nm. The serum creatinine levels are expressed as µmoles/L.

Measurement of the enzymatic activity of gamma-glutamyl transpeptidase (GGT)

The enzymatic activity of GGT in urine was measured in 0.1 mol/L Tris-HCl buffer (1 M Tris, 100 mM MgCl₂), pH 9.0, following the method of Beck and Thomson (1977). 50 µl were incubated at 25°C in the presence of 5 mmol/L L-gamma-glutamyl-p-nitroanilide. The enzymatic reaction is stopped by the addition of 1 ml of 1 N acetic acid and the rate of p-nitroanilide release was determined spectrophotometrically at 405 nm. One unit of GGT activity is defined as the amount of enzyme releasing µmol of p-nitroanilide per minute. The specific activity of the enzyme was expressed as units/mg protein.

Statistical analysis

Results are given as averages and standard deviations. The averages of the groups treated with drugs were compared with those of control and flavonoid-treated groups using one-way analysis of variance (ANOVA) followed by a t-test and P-values less than 0.05 were considered significant.

RESULTS

Identification of compounds

The different phenolic compounds were identified by their UV spectra, which had been recorded with a DAD coupled to the HPLC, bathochromic movement of band I (320 - 380 nm) and band II (240 - 280 nm). HPLC-UV chromatograms of the phenolic fractions of Propolis indicate the presence of 3 classes of polyphenols: Flavonoids (maximal absorption at 280 and 370nm); Chalcones: dihydrochalcone (λ_{max} = 280 - 290 nm) and Phenolic acids. The identification was accomplished using computer searches on commercial libraries (Figure 1). The components of EEP were determined by considering their areas percentage of the total ion current (Table 1). The major flavonoids detected in propolis extract are pinostrobin chalcone, pinocembrin, tectechrysin, chrysin and naringinin.

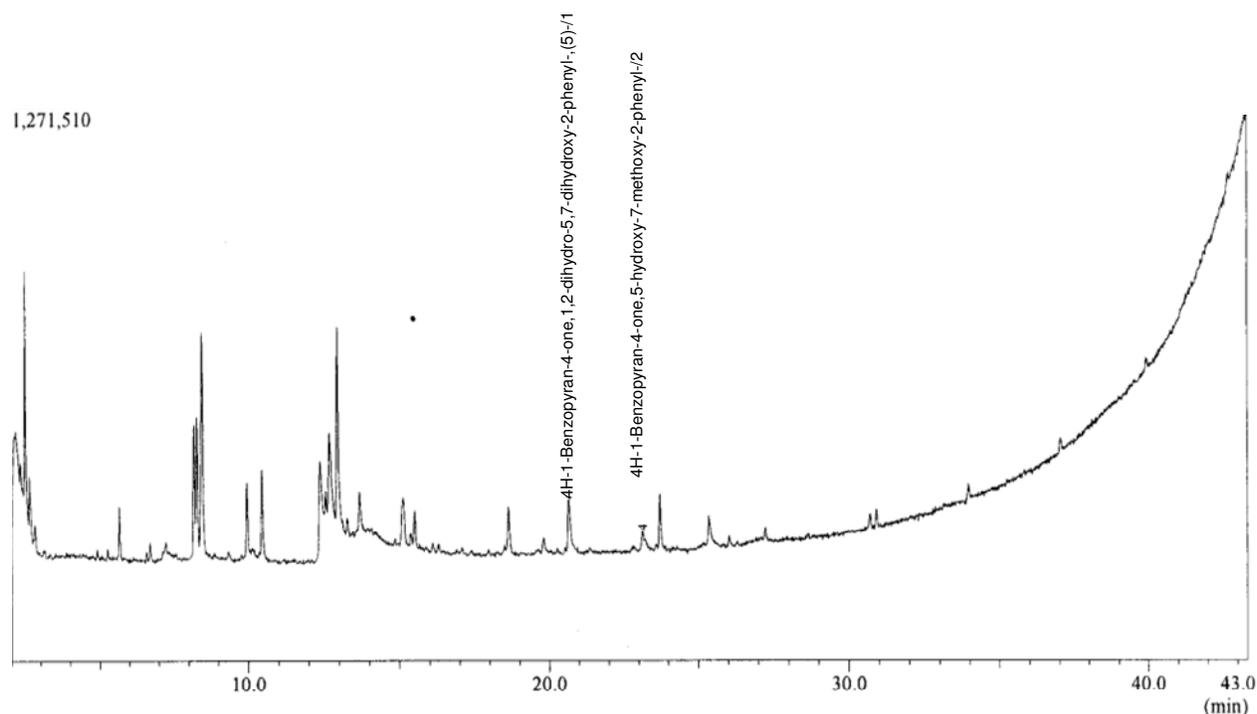


Figure 1. Gas Chromatography Chromatogram of ethanolic extract of propolis (EEP).

Table 1. Main chemicals of the Algeria propolis.

Compounds	%
Aliphatic acids	
9,12- Octadecadienoic acid (Z/Z)	1.89
2- Propenoic acid, 3-(4-hydroxyphenyl)	0.77
4-(Methylamino)butyric acid	0.92
Aromatic acids	
Benzene acetic acid, alpha- (hydroxymethyl)-, (+/-).	2.39
p-Hydroxycinnamic acid	5.02
5-O-Methyl-d-gluconic acid dimethylamide	0.03
Esters	
p-Hydroxycinnamic acid, ethyl ester	0.91
Hexanoic acid, 2-phenyl ester	0.62
Hexadecanoic acid, 15-methyl-,methylester	0.90
6-Octadecanoic acid, methyl ester	0.70
1,2-Benzenedicarboxylic acid, diisooctyl ester	0.41
4-Fluoro-1-methyl-5-carboxylic acid, ethyl ester	0.07
Chloromethyl 2-chlorododecanoate	0.15
Flavonoids	
Pinostrobin chalcone	0.67
Pinocembrin	0.33
Tectochrysin	1.00

Table 1. Conts.

Sesquiterpenes	
1.4-Methanoazulen-7(1H)-one, octahydro-4,8,8,9-tetramethyl	1.00
Alcohols	
6-Phenyl-n-hexanol	0.94
1,4-Benzenediol,2,5-bis(1,1-dimethylethyl)	0.83
1-Octadecanol	2.52
Cyclohexanol, 2,3,dimethyl	0.14
Others	
Cyclopropane,1,1,2-trimethyl-3-(2-methyl-1-propenyl)	2.05
Benzene, 3-cyclohexan-1-yl	1.28
3(2H)-Furanone, dihydro-2,2-dimethyl-5-phenyl	1.20
Benzene, 1,1'-(1,2-cyclobutanediyl)bis-,cis	1.07
1H-1,2,3-Triazole-1,5-diamine,3-nitro	0.35
3-[4-Cyano-1,2,3,4-tetrahydronaphthyl) propanenitrile	2.68
Chathine	0.29
2-Chloropropionamide	0.16
Cyclopentadecanone, 2-hydroxy	2.20
5-Iodohistidine	0.13
Acetamide, 2-cyano	0.13

The effect of propolis extract on renal oxidative stress

Scavenger effect of propolis extract

One of the more prominent properties of the flavonoids is their excellent radical scavenging ability. The scavenging activity of propolis extract is observed at concentrations up to 0.12 mg/ml showing it to be more stronger than that of the positive control, quercetin (Figure 2). Indeed, at 1 mg/ml propolis extract had a percentage reduction of 88.30% against 75.51% for the quercetin.

Malondialdehyde (MDA) of renal tissue

Propolis extract showed, a strong effect on lipid peroxidation of kidney cells as shown in the Figure 3. A significant reducing of malonedialdehyde (MDA) concentrations in kidney cells of animals pre-treated with 100 mg/kg propolis extract daily for seven days prior to doxorubicin 10mg/kg IV injection is observed (25.30 ± 1.13 nmol/g tissue against 75.4 ± 0.96 nmol/g tissue in rats treated with the drug alone on day seven). Propolis extract provide a protection against free radicals formed during the metabolism of doxorubicin. It prevents the binding of these reactive metabolites with the lipid membrane of the cell, which resulted in decreasing rates

of lipid peroxidation.

Glutathione (GSH) of renal tissue

Glutathione plays an important role in detoxification through the formation of reactive metabolites conjugates and protects cell membranes against oxidation. Rats treated with doxorubicin show a very significant ($p < 0.01$) decrease in renal GSH concentrations at day 14 of treatment (0.77 ± 0.55 mM / g tissue against 2.97 ± 0.32 mM/g tissue for controls) (Figure 4). The rats pretreated with propolis extract show a very significant increase of GSH levels compared to those receiving doxorubicin alone (3.47 ± 0.07 mM against 1.55 ± 0.09 mM) at time 14 days. The glutathione concentrations decreases in rats receiving doxorubicin alone compared to that of controls (0.77 ± 0.55 mM) and increased in the group of animals treated with propolis extract (3.47 ± 0.07 mM/g tissue) at the same time (day 14). This result suggests a recovery capacity of the synthesis of GSH after the renal elimination of doxorubicin.

Evaluation of serum creatinine

The concentrations of serum creatinine in rats treated with doxorubicin 10 mg/kg alone or combined with the

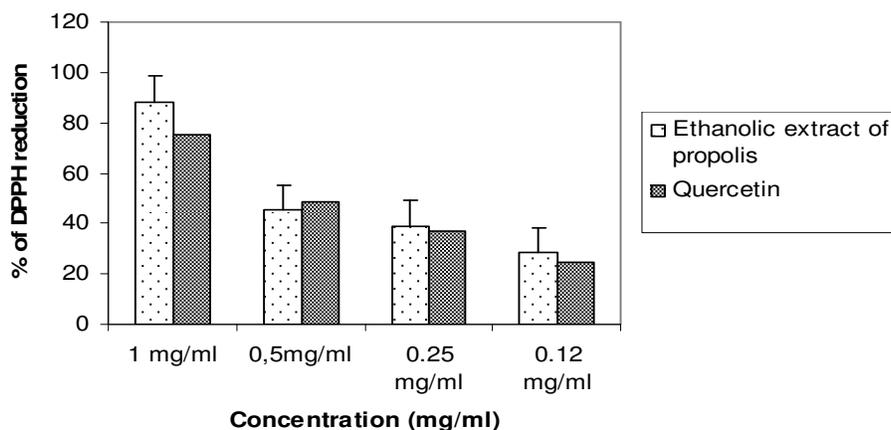


Figure 2. Scavenger effects of the ethanolic extract of propolis vs quercetin.

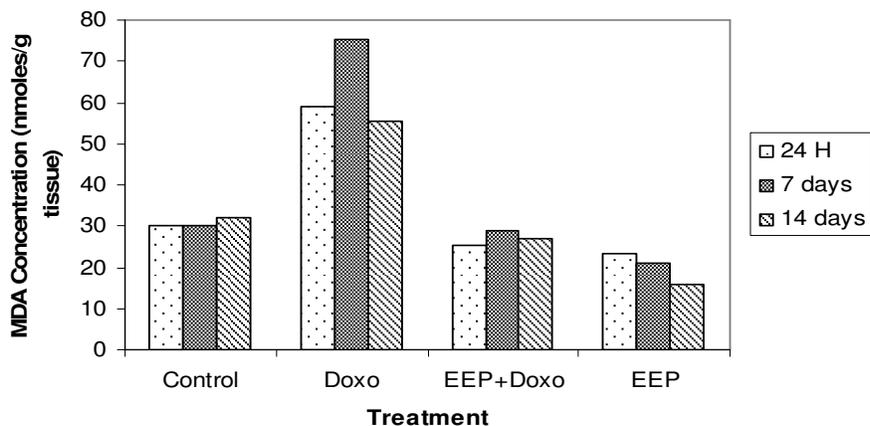


Figure 3. Changes in malonaldehyde levels of renal tissue after a single dose of doxorubicin 10 mg/kg alone or combined with the propolis extract 100 mg/kg. The results are expressed as mean \pm SEM.

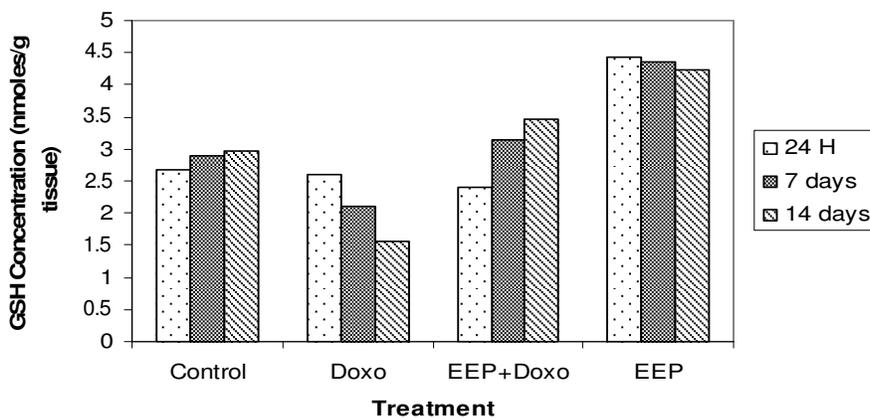


Figure 4. Changes in rates of renal tissue glutathione after administration of a single dose of doxorubicin 10 mg/kg alone or combined with the propolis extract 100 mg/kg. The results are expressed as mean \pm SEM.

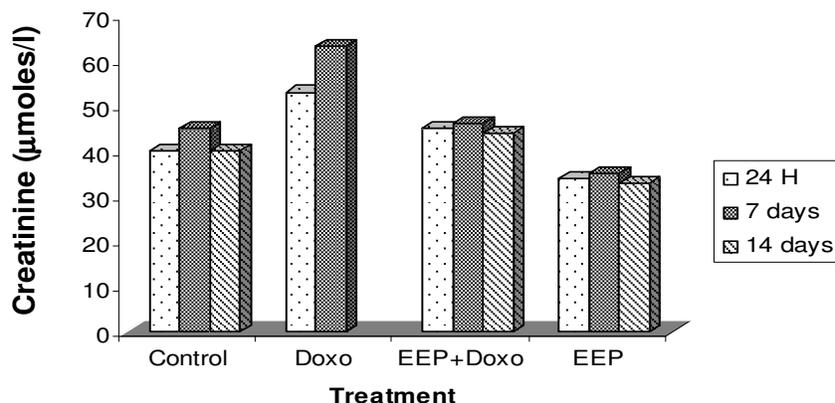


Figure 5. Changes in rates of serum creatinine during treatment with doxorubicin 10 mg/kg alone or combined with the propolis extract 100 mg/kg. The results are expressed as mean \pm SEM.

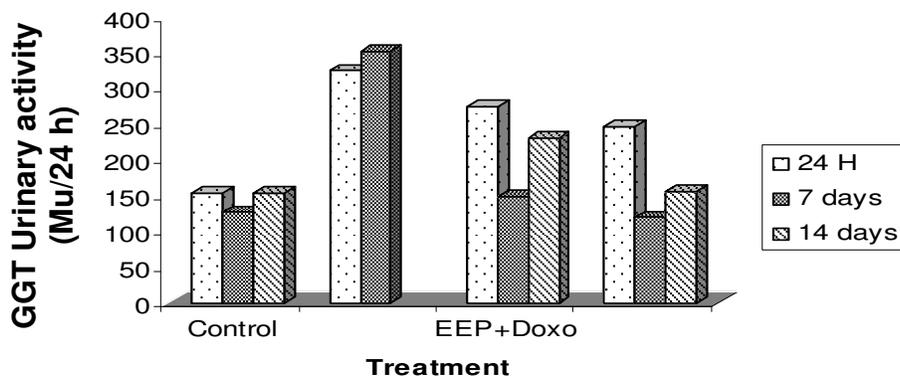


Figure 6. Variations of urinary enzymatic activity of gamma-glutamyl-transpeptidase after treatment with doxorubicin 10 mg/kg alone or combined with the propolis extract 100 mg/kg. The results are expressed as mean \pm SEM.

ethanolic extract of propolis administered for 7 days at 100 mg/kg are listed in Figure 5. In rats treated with doxorubicin 10 mg/kg alone, there was a significant increase in plasma creatinine. 24 h after injection of the drug, the creatinine concentration reached (53.69 ± 15.15 μ M/L in treated rats against 40.14 ± 8.14 μ M/L in controls) and increased progressively until day 7 ($p < 0.001$). However, the combination of doxorubicin to propolis extract shows a significant decrease in serum creatinine (46.8 ± 10.4 μ M/L against 63.72 ± 7.21 in rats treated with doxorubicin alone) at the same time.

Variation of the urinary activity of gamma-glutamyl transpeptidase (γ GT)

The doxorubicin treated rats showed a highly significant

increase of the urinary enzyme activity between the first and 7th day ($p < 0.001$). This activity is still increased compared to control (351.67 ± 7.32 mU in treated rats against 127.38 ± 2.89 mU in controls) at 7th day (Figure 6). However, a very important modification of the urinary enzyme activity recorded in rats treated with ethanolic extract of propolis at 100 mg/kg for 7 days followed by a single dose of doxorubicin 10 mg/kg (149.24 ± 3.47 mU/24 h).

DISCUSSION

Propolis has many anti-oxidant and antiapoptotic properties. Phenolic compounds (flavonoids and phenolic acid derivatives) are the most important pharmacologically active constituents in propolis. However, the constituents

of propolis vary widely with climate and location. The major flavonoids detected from extracts in our study were, pinostrobin chalcone, pinocembrin, galangin flavanon, naringenin (flavanon) and flavone's (chrysin and tectochrysin). So, we observe some similarities in the qualitative composition between Algerian and Turkish propolis (Kartal et al., 2002; Velikova et al., 2000) and also, the phenolic compounds were similar to those identified in propolis from American countries (Seidel et al., 2008) and Mediterranean region (Abd El Hady et al., 2002). Considerable interest in these compounds extracted from propolis has arisen with the recognition for their antibacterial and anticancer effects (Nirala and Bhadauria, 2008; Viuda-Martos et al., 2008). and also for their antioxidant effects (Benguedouar et al., 2008; Draganova-Filipova et al., 2008; Liu et al., 2008).

From a physiological stand point damages to the glomerular filter and tubular epithelium of the kidney leads to increased plasma concentrations of creatinine and urea. Impairment of renal function is also clearly reflected in this study by an increase proteinuria and enzymuria. The increase in urine gamma-glutamyl transpeptidase (γ GT) was used as a sensitive indicator of damage to proximal renal tubules (Pedraza-Chaverrí et al., 1995). Therefore, the increased γ GT activity in animals treated with doxorubicin 10 mg/kg observed in our study, would be due to the impairment of kidney brush border and this follows from the renal toxicity of the drug alone. These results were consistent with those Lahouel (1985) and Viotte et al. (1988). Our results are also consistent with those of Abo-Salem et al. (2009) and reinforce those of Viotte et al. (1988) and collaborators showing that injection of doxorubicin 10 mg/kg in rats increased plasma creatinine. However we observed a decrease in plasma creatinine in animals receiving the propolis extract before drug treatment.

Recently, we have reported that flavonoids of propolis extract protect liver and heart tissues in rats treated with anticancer drugs (Alyane et al., 2008) and we hypothesized that the protective effect of flavonoids could be due to their capacity to capture and to deactivate the free radicals. Adopting the method of DPPH^o (1,1, 2-Diphenyl hydrazyl), our study shows that ethanolic extract of propolis has a very strong scavenger effect; it is more active at concentrations up to 0.12 mg/ml (reduction of 88.30% against 75.51% with the quercetin at concentration 1 mg/ml). Lipid peroxidation is one of the major effects of the radical attack leading to the formation of malondialdehyde (MDA) (Li et al., 2008; Nirala and Bhadauria, 2008). Our study performed on the kidney, showed a significant increase of MDA in groups of animals receiving doxorubicin alone; this increase is an indicator of the toxicity of the drug. At the same time, the rates of glutathione recorded in rats treated with

doxorubicin alone were lower than in control rats. This decrease was probably mainly due to the toxic effect of reactive metabolites of the drug that can be fixed and neutralized by the detoxification system, resulting in decreased GSH concentrations, or degradation the GSH by the gamma-glutamyl transpeptidase. However, we observed a decrease of MDA in groups of animals receiving the flavonoids before doxorubicin treatment. We recorded also an increase in glutathione levels in groups of animals pretreated with flavonoids of propolis (100 mg/kg). Taken together these data demonstrated and may explain the preventive effect of flavonoids against renal oxidative stress induced by doxorubicin (Injac and Strukelj, 2008; Injac et al., 2008). The mechanism by which the natural product propolis extract prevent renal oxidative stress may include an increasing rate of GSH or by induction of its synthesis or by the effects of flavonoids scavenger ie instead of the toxic reactive metabolites bind to glutathione and consume, they will be captured by the flavonoids (naringenin, pinostrombin and galangin).

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