CARDIOPROTECTIVE EFFECTS AND MECHANISM OF ACTION OF POLYPHENOLS EXTRACTED FROM PROPOLIS AGAINST DOXORUBICIN TOXICITY

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ABSTRACT
Propolis is one of the major hive products of bees and is rich in flavonoids, which are known for antioxidant activities. It is well known that the chemical properties of phenolic acids or flavonoids, in terms of the availability of the phenolic hydrogens as hydrogen donating radical scavengers, predict their antioxidant properties.

In this study, the flavonoids scavenging activity of propolis has been exploited to obtain protection against the peroxidative damage in rat heart mitochondria which was induced by the administration of an acute dose of doxorubicin (20mg kg⁻¹, i.p). The peroxidative lesions were evaluated biochemically and biophysically, 24H after DXR administration.

Abnormal biochemical changes in heart mitochondria from DXR treated rats including a marked increase in both malondialdehyde (MDA) and anion superoxide production; decrease both of respiratory chain ratio (RCR= V₃/V₄) and P/O.

Pretreatment of rats with propolis extract, given per os (100mg/kg/day) during four days prior to DXR injection, substantially reduced the peroxidative damage in the heart mitochondria: we showed significant reducing both of mitochondrial MDA formation and production of superoxide anion, restoration of RCR and P/O and reducing of rate and the amplitude of mitochondrial swelling. The data demonstrate that antioxidants from natural sources may be useful in the protection of cardiotoxicity in patients who receive doxorubicin and as reported for its claimed beneficial effect on human health by biomedical literature.

Keywords: Propolis; antioxidant; flavonoids; lipid peroxidation; swelling.

INTRODUCTION
Doxorubicin (Adriamycin) is the most widely used anthracycline. It is an analogue of daunorubicin. The major toxicity with use of this compound is an insidious dose-related cardiotoxicity, the precise mechanism of which is unclear (Ratin and Ewesuedo 1999). However, several hypotheses have been put forward foremost of which is that, oxygen free radical formation by doxorubicin (Chopra et al., 1995; Van Acker et al., 1996) enhance the susceptibility of cardiac tissue to lipid peroxidation leading to a progressive dose-related irreversible loss of myofibrils, dilation of the sarcoplasmic reticulum, cytoplasmic vacuolization, swelling of mitochondria, increased number of lysosomes and myocyte necrosis in response to the doxorubicin (Minotti et al., 2004).

One of the reasons why cardiomyocytes would be more susceptible than other tissues to apoptosis induced by doxorubicin is that cardiomyocytes exhibit low levels of antioxidants defences (Andrieu-Abadie, 1999; Minotti et al., 2004).

Propolis, or bee glue, is a dark-coloured resinous substance collected by honeybees from leaf buds and cracks in the bark of various tree species (Gómez-Caravaca et al., 2006). The chemical composition of propolis is quite complicated and over 150 components have been identified (Marcucci, 1995). Among these compounds flavonoids were suggested to be responsible for the biological activities (Chang et al., 2002).

A cardioprotective effect of propolis extract has been reported (Chopra et al., 1995; Horakova et al., 2001; Siess et al., 2000). However, the mechanism of action of these flavonoids is not well-defined but it is evident that some of their protective effect can be attributed to direct scavenging properties (Van Acker et al., 1996).

Mitochondria, intrinsic pathway of apoptosis, was evoked to explain the protecting effect of flavonoids (Shen and Zhou, 1995; Morin et al., 2001a; Brooks et al., 2002) because several studies have shown that on mitochondria, doxorubicin via reactive oxygen species (ROS) formation, induces apoptosis by favoring cytochrome c release and consequent formation of the apoptosome complex through
up-regulation of Bax or down-regulation of Bcl-XL (Minotti et al., 2004).

Recently, we have reported that flavonoids of propolis extract, an effective free radical scavenger, protect liver and the blood tissues in rats treated with anticancer drugs (Lahouel et al., 2004) and we hypothesized that the protective effect of flavonoids could be due to a direct action on mitochondrial functions.

Fig. 1: High performance liquid chromatogram of ethanolic extract of propolis (EEP); flavonoids: 280, 370nm; phenolic acids 325nm.

Fig. 2: Effect of doxorubicin 1micro-molar and the propolis extract on the mitochondrial production of superoxide anion in vitro.
MATERIALS AND METHODS

Materials
Crude propolis was collected from beekeepers of “Coopérative Apiocolle de Kaous, Jijel (Algérie)” in May-April, 2005. Samples, once received, were stored at 4°C in airtight /dark plastic containers until analysis.

Doxorubicin (Ebewe Untrerach, Autriche) was kindly offer by “Centre Anti Cancer, CHU de Constantine”. Sucrose, EGTA, rotenone, malonate, antimycin A, oligomycin, succinate, malate, glutamate, NADH, ADP were purchased from Sigma Chemical Co. (Algiers, Algeria). KCl, MgCl₂ and KCN were obtained from Prolabo. Tris base (Sigma) and KH₂PO₄ (Sigma) were used. All other chemicals and reagents used were of analytical grade.

Methods
Extraction of propolis compounds
Propolis extract was achieved by using ethanol and methanol. The propolis, is cut into small pieces; dived in alcohol 95° (1g/10ml) for 15 days, filtered and then evaporated to 80°C using a rotary evaporator (Evaporator).
E100). The residual was retaken using methanol 70° and was let for steeping for one night. After evaporation of the solvent, the produced extract is called; raw extract or propolis extract. Flavonoids rate was determined by reactivity with AlCl3 method as described by Bankova and Marcucci (2000). Then, the concentration of total flavonoid in propolis extract was expressed as milligrams of quercetin equivalents (QE) per gram dry weight of propolis extract (DWPE), using the following linear equation:

\[
\text{Flavonoid concentration} = \frac{\text{Absorbance} \times M \times V \times \frac{d}{W}}{\varepsilon}
\]

\(\varepsilon\): Molar extinction coefficient of quercetin; \(M\): Molar mass of quercetin; \(d\): dilution factor; \(W\): weight of propolis extract.

**Analyses of propolis compounds**

**High performance liquid chromatography of EEP**

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 purchased from Varian; DAD was a Varian Prostar, monitoring from 200 to 700 nm wavelengths (Middelburg, The Netherlands). The phenolic compounds in EEP at a concentration of 10 mg/ml were separated on a Nucleodur analytical column, 250 mm x 4.6 mm i.d (Machery-Nagel, Duren, Germany) packed with C\(_{18}\) stationary phase, with a particle size of 5\(\mu\)m.

A 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 reequilibrate the column (washing).

**Animals**

Female Wistar rats were purchased from Pasteur institute, Algiers. The animals were maintained at 22°C during the experimental period. They have free access for food and water. Animals were divided to three groups: pretreated, untreated, and control (n=8 in each group). Rats were pretreated with an oral administration of propolis extract (100 mg/kg/day) during four days before the injection of adriamycin. The pretreated rats were given an intraperitoneal injection of adriamycin (20 mg/kg) and were sacrificed after 24h. Likewise, rats received intraperitoneal injection of Adriamycin (untreated) or saline solution 0.9% (control).

**Isolation of heart mitochondria**

Mitochondria were extracted from a homogenate of rat heart by differential centrifugations (Rustin et al., 1994). Briefly, rats (weighing 280-300 g) were killed by decapitation and the heart removed and placed quickly in an ice-bath. The heart was then homogenized (6 ml/g of tissue) in ice-cold isolation medium (Mannitol 225 mM, Sucrose 75 mM, Heps 5 mM, EGTA 1 mM and albumin 1 mg/ml, pH 7.4 at 37°C) using a Potter-Elvejhem homogenizer. Mitochondria isolation was performed at 4°C without delay using differential centrifugation. The homogenate was centrifuged at 2000 g for 10 min. to remove cell debris and nuclei, and then mitochondria were separated from the supernatant by centrifugation at 12000 g for 5 min. The pellet (mitochondria) was washed and resuspended in a respiratory buffer (KH\(_2\)PO\(_4\) 1mM, Sucrose 50 mM, KCl 100 mM, pH 7.4 at 37°C) for measuring mitochondrial enzymatic and respiratory
activities. The total protein contents in mitochondrial suspension were determined by the by the Folin-Phenol procedure (Lowry et al., 1951).

Assay of mitochondrial oxygen consumption
Oxygen uptake was determined with a Clark type oxygen microelectrode (Hansatech). Each experiment was carried out as follows: a aliquot of mitochondria suspension equivalent to 1mg/ml were incubated with (or without) the tested drug, incubated 1min. at 37°C in 1000 µl of the respiratory buffer with or without the inhibitors, then the substrate (Pyruvate/malate 6 mM) was added and oxygen consumption was checked (State 2). To initiate state 3 respiratory activity, 200 µM ADP was added to the mixture. When all ADP was converted to ATP, the state 4 was measured. The following parameters were determined: the respiratory rates calculated as nmol O₂ /mg mitochondrial protein/min and the RCR as State3/State4 ratio.

Mitochondrial Superoxide anion (O₂⁻) assay
Generation of O₂⁻ was achieved using rat heart mitochondria. Reaction mixture contained mitochondria (0.2 mg/ml), 1µM Cyclosporin A and nitroblue tetrazolium (NBT, 100 µM) in 1.2 ml of respiration buffer at 37°C. The reaction was started by adding malate/pyruvate (6 mM) in the assay mixture and the rate of NBT reduction was measured at 560 nm. In absence of mitochondrial substrate (malate/pyruvate), there was no O₂⁻ production.

Free radical scavenging assay
The antioxidant activity of propolis extract was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl free radical) assay. Different dilutions of EEP dissolved in ethanol were added DPPH° ethanolic solution (100µM). The bleaching of DPPH° was monitored at 515 nm (Spectrophotometer Hitachi U-3000, Tokyo, Japan) against a blank constituted by the EEP solution containing all

Fig. 6a: Effect of doxorubicin 20mg/kg and the propolis extract on the mitochondrial respiration function and the respiratory control (CR= V3/V4); V3 and V4 are the respiratory rate of states 3 and 4 respectively. The values are mean of three experiments for each animal (n=8).

Fig. 6b: Effect of doxorubicin administration alone or in association with propolis extract on the the P/O ratio.
Cardioprotective effects and mechanism of action of polyphenols

reagents except DPPH°. The percentage of DPPH radical-scavenging activity was plotted against the EEP concentration to determine the amount of extract necessary to decrease DPPH radical concentration by 50% (called EC50).

Lipid peroxidation assessment
Heart Mitochondria were suspended in NaCl 0.9% (0.2mg/ml) and then incubated in a total volume of 1 ml in the presence of Fe²⁺/Fe³⁺ (50µM / 50µM) for 30 min. The reaction was stopped with 1 ml of 3% trichloracetic acid and 1.5 ml of 1 % thiobarbituric acid was added. The mixture was then heated to 100°C for 30 min and recooled on ice for 5 min. Then, the supernatant was read at 530 nm. Results were expressed as µM MDA / mg of mitochondrial membrane proteins.

STATISTICAL ANALYSIS
Data were expressed as means ± s.e.m. Statistical comparison between different groups was done using one way analysis of variance (ANOVA) followed by t-test. Significance was accepted at p<0.05.

RESULTS

Chemical of propolis
This study showed that total flavonoid concentration in the raw propolis sample determined by aluminium chloride colorimetric method is equal to 370 mg QE/g DWPE.

HPLC chromatograms of the phenolic fractions of Propolis (fig. 1) indicated the presence of 3 classes of polyphenols: Flavonoids (maximal absorption at 280 and 370nm); Chalcones: dihydrochalcone (λ<sub>max</sub> = 280-290 nm) and Phenolic acids: hydroxycinnamic acids derivates (λ<sub>max</sub> = 225 nm). However, our extracts appears to be rich in flavonoids because the 80% ethanolic extract showed highest absorption at 290 nm, which means that the highest concentration of flavonoids (especially of kaempferide, acacetin and isorhamnetin) (Gómez-Caravaca et al., 2006) which could explained the in vitro highest free radical scavenging activity of EEP observed in our study because studies by Hodnick et al. (1988) showed that the flavonoids with the most hydroxyl groups were most easily oxidized.

Antioxidant effects of polyphenols
The EEP at 10⁻⁴ dilution exhibited the highest free radical scavenging activity when compared with the other two polyphenols, vitamin E and quercetin. This result confirmed the in vivo effect of propolis as related in this study.

In order to evaluate the role of the antioxidant of the polyphenols in heart mitochondria, anion superoxide production was measured in vitro on isolated heart mitochondria. To verify also the active components of propolis in vivo we have compared the effect of propolis extract mixture treatment on anion superoxide production. Fig. 2 shows that in vitro, EEP reduces the production of superoxide anion at dilutions ranged between 10⁻⁴ and 10⁻⁸ on mitochondria incubated with doxorubicin 1µM or not (Control). In vivo, EEP (100 mg/kg) given by oral administration daily for 5 days, starting four days prior to the administration of doxorubicin 20mg/kg reduced also significantly the mitochondrial production of superoxide anion (p<0.01) as described by fig. 3.

By another way, lipid peroxides in rats treated by doxorubicin were assessed. These peroxides are formed

Fig. 7: One-electron redox cycling of anthracyclines (Minotti et al., 2004).
from oxidation of naturally occurring unsaturated fatty acids and rapidly decompose in vivo to yield a variety of products, the best characterized of which is malondialdehyde (Myers et al., 1977). Using the procedure outlined in fig. 5, an increased malondialdehyde production was readily detected 24 hours after the intraperitoneal injection of Adriamycin. This level of malondialdehyde formation did not occur in the cardiac tissue of animals treated with an oral administration of EEP (100mg/kg) 4 days to the injection of doxorubicin.

In vitro, the inhibitory effect of propolis extract on lipid peroxidation of heart mitochondrial membranes is observed at 10^-4 dilution showing it to be more than a ten stronger than that of the positive control, quercetin (fig. 4).

**The effect of polyphenols on mitochondrial functions**

There is few data on the effect of polyphenols on heart mitochondrial respiratory. So, we have studied different flavonoidal molecules like, quercetin, kaempferol and EEP to elucidate this effect on isolated heart mitochondria. We have also studied the in vivo protective effect of EEP on doxorubicin cardiotoxicity. We observed that 20 mg/kg doxorubicin increase the oxygen consumption (state3) and the inhibition of state 4 leads to a decrease of both respiratory chain ratio (RCR= V3/V4) (fig. 6) and P/O. Propolis extract inhibits the effect of doxorubicin showing a restoration of RCR and P/O in vitro and in vivo.

On the other hand, a massive mitochondrial swelling was observed in cardiac myocytes treated with doxorubicin (Minotti et al., 2004). For this reason, we have tested the capacity of EEP to inhibit mitochondrial swelling. Addition of EEP to a mixture of rat heart mitochondria with 25µM calcium inhibits slightly the rate and the amplitude of mitochondrial swelling at 10^-3 and 10^-6 dilutions affected by the doxorubicin addition to the medium at a concentration of 1µM (data not shown).

Modification of the mitochondrial membrane potential is a signal of apoptotic process. Progesterone and testosterone 0.5 mM have no effect on this parameter at concentrations. However the propolis extract seems to play an original role to restore the loss of the membrane potential induced by doxorubicin 1µM (data not shown).

**DISCUSSION**

Oxidative stress and mitochondrial dysfunction are associated with disease and toxic process. It results from over production of ROS, often leading to peroxidation of membrane phospholipids and production of reactive aldehydes (Hanasaki et al., 1994; Ferrari, 1996; Korshunov et al., 1997 and Morin et al., 2001a).

The chemical profiles analysis permitted to clear that the nature of the compounds of the propolis extracts is about flavonoids of type flavones and flavonols (Lahouel and Fillastre 2004). These last, have the capacity to capture and to deactivate the free radicals generated by doxorubicin (Hanasaki et al., 1994; Lahouel and Fillastre 2004) and/or by chelating iron (Minotti et al., 2004).

Numerous studies showed that the antineoplastic drugs induced some transformations in the liver by the system Cytochrome P450. These transformations succeed to the formation of highly reactive metabolites for the cell (Lemasters et al., 1998). The toxicity is function of the quantity of free radicals formed and of the cellular reserve in glutathione and the available glutation-s-transferases to assure the detoxification. Inner mitochondria membrane is considered as targets of toxic process during chemotherapy, leading to ROS production (Morin et al., 2001b; Green and Kroemer 2004). Indeed, most of chemotherapeutic agents (doxorubicin, cyclophosphamid...) are metabolized in the liver by the cytochrome P450 (Lemasters et al., 1998) and the reactive intermediates which are formed are responsible for apoptotic process and cell toxicity (Ichikawa et al., 2002).

The treatment of Wistar rats with a single dose of 20mg/kg/ip doxorubicin showed some effects in the heart mitochondria functions especially: the drug decreases the respiratory control rate and increases the superoxide anion and the MDA production. These biochemical abnormalities did not occur in heart mitochondria and cardiac tissue of animals treated with oral administration of EEP (100mg/kg/day) five days prior to the intraperitoneal injection of doxorubicin (20mg/kg), we observed: a restoration of the RCR, a decrease of superoxide anion production and an inhibition of the lipid peroxides formation. The same effects are observed in vitro on heart mitochondria setting in presence of increasing concentrations of propolis extract. These results suggested that lipid peroxidation, superoxide anion and decrease of RCR may play an important role in adriamycin toxicity. These results permitted also to clarify the prevention mechanism of propolis extract against doxorubicin cardiotoxicity.

Indeed, in cardiac tissue, addition of one-electron to the quinone moiety in ring C of doxorubicin and other anthracyclines has long been known to result in formation of a semiquinone that quickly regenerates its parent quinone by reducing oxygen to reactive oxygen species like superoxide anion (O2-) and hydrogen peroxide (H2O2) (Minotti et al., 2004). During this cycle the semiquinone can also oxidize with the bond between ring A and daunosamine, resulting in reductive deglycosidation and formation of 7-deoxyaglycone (fig.
7). Due to their increased lipid solubility, aglycones intercalate into biologic membranes and form ROS (O$_2^-$, H$_2$O$_2$) in the closest proximity to sensitive targets (Gille and Nohl 1997 and Licata et al., 2000). This ROS production has been proposed as a mechanism for the amplification of mitochondrial dysfunction by doxorubicin (Gille and Nohl 1997). For this reason, we have tested the impact of EEP on the suppression of superoxide anion production in cardiac tissue after adriamycin administration. From fig. 4, it is apparent that prior treatment of the animals with EEP diminishes the ability of adriamycin to induce ROS production.

Collapse of membrane potential and swelling are among mitochondrial perturbation observed in heart mitochondria isolated from animals treated with doxorubicin (Minotti et al., 2004). Koller and Haid (2005) have suggested that the restoration of the mitochondria transmembrane potential may be a way in the prevention of doxorubicin cardiotoxicity. Keeping this information in mind, we tested the capacity of EEP to restore potential membrane and to inhibit mitochondrial swelling. On both cases EEP, on the one hand, is able to restore the fall of membrane potential (unpublished data), which disruption has been an established indicator of mitochondrial damage in the progression of apoptosis (Ye et al., 1999); on the other hand, it inhibits slightly the rate and the amplitude of mitochondrial swelling at 10$^{-4}$ and 10$^{-6}$ dilutions. We have established also that the incubation of rabbit cardiomyocytes with propolis extract 10$^{-6}$M can induce a total protection from apoptosis induced by doxorubicin 1µM (results not shown). The results showed a significant decrease in the caspase 3 and 9 activities.

CONCLUSION

The mitochondria seems to play a fundamental role as well in the toxicity of the doxorubicin by the formation of ROS that a protective role when it is protected by the propolis polyphenols. It is evident from the present work that the Algerian propolis is a very interesting and might be able to solve the problem of the doxorubicin cardiotoxicity. As suggested by Samet et al (2007), propolis can open perspectives in the treatment of human health.

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