Efficiency of propolis extract against mitochondrial stress induced by antineoplastic agents (doxorubicin and vinblastin) in rats

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To assess the oxidative stress and mitochondrial dysfunction associated with disease, toxic process and aging, in vivo and in vitro preventive effect of propolis extract against mitochondrial oxidative stress induced by two anticancer drugs (doxorubicin and vinblastin) have been investigated in female wistar rat using liver and heart mitochondria. The results show that doxorubicin and vinblastin altered mitochondrial functions as observed by a decrease in respiratory control value, an activation of swelling and overproduction of superoxide anion. Myocardial tissue from doxorubicin treated rats showed a marked increase in malondialdehyde production, a depletion of reduced glutathione contents and an inhibition of catalase and superoxide dismutase activities. Similar results were also observed in liver tissue. Pretreatment of rats with propolis extract (100 mg/kg/day po) (10^-4 M ip) administered 4 days prior to doxorubicin (20 mg/kg) and/or vinblastin (2 mg/kg) injection, substantially reduced the peroxidative damage in myocardium and hepatic tissues and markedly restored the tissues catalase and SOD activities. The results strongly suggest that propolis extract protects heart and liver tissues from oxidative stress by protecting the mitochondria.

Keywords: Doxorubicin, Heart, Liver, Lipid peroxidation, Propolis extract, Vinblastin

Doxorubicin and vinblastin are important and effective anticancer drugs widely used for the treatment of various types of cancer but their clinical uses are limited by dose-dependent cardiotoxicity and hepatotoxicity. Propolis extract protects, in vivo, the liver and blood tissues against cyclophosphamid and doxorubicin toxicity1,2 suggesting that polyphenols can help in preventing the oxidative stress induced by chemotherapeutics agents.

Oxidative stress and mitochondrial dysfunction are associated with disease and aging3. Oxidative stress results from an over production of reactive oxygen species (ROS), often leading to peroxidation of membrane phospholipids and production of reactive aldehydes. Moreover, inner mitochondria membrane is considered as targets of apoptotic process, owing to alterations of potential membrane4,7 and to ROS production.

Polyphenolic compounds, including flavonoids, tannins and phenolic acids are widely distributed in plant and foods of plant origin8. The chemical properties of phenolic acids or flavonoids, in terms of availability of the phenolic hydrogens as hydrogen donating radical scavengers, predict their antioxidant properties9.

Considerable interest in these compounds extracted from propolis has arisen with the recognition for their antibacterial and antioxidant effects10-12. However despite antioxidant and antibacterial data on the benefical effects of propolis extracts, the molecular mechanisms underlying interactions with isolated mitochondria have not yet been reported. Some of their protective effect can be attributed to direct scavenging properties13,14. Mitochondrial target was evoked to explain the protecting effect of polyphenols15-17. Some polyphenols with antioxidant and free radical scavenging properties were shown to inhibit also lipid peroxidation (Resveratrol, Propolis, Ginkgo biloba)1,18,19. This protective effect could be due to a direct action on mitochondrial functions.

With a view to elucidate the mechanism of interaction of polyphenols present in propolis on heart and liver mitochondria, the present study reports mitochondrial functions following chemotherapeutic drugs toxicity in female rats.

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Materials and Methods

Chemicals—Sucrose, EGTA, rotenone, malonate, antimycin A, oligomycin, succinate, malate, glutamate, NADH, ADP, Tris base and KH2PO4, cyclosporine A, albumin, pyruvate, succinate, nitroblue tetrazolium (NBT), vitamin E, quercetin, 1,1-diphenyl-2-picrylhydrazyl hydrate (DPPH) and dithiobisnitrobenzoic acid (DTNB) were purchased from Sigma Chemical Co. KCl, MgCl2 and KCN were obtained from Prolabo. All drugs were solubilized in distilled water in order to obtain a stock solution of 10⁻³ M. N,N,N’, N’-tetramethyl-p-phenylenediamine and oligomycin were solubilized in dimethylformamide (DMF).

The free radical-scavenging activity was calculated by the formula:

\[ I = \frac{(A_B - A_A)/A_B}{100} \]

where: \( I = \) DPPH⁺ inhibition, (%); \( A_B = \) absorption of blank sample (at t = 0 min) and \( A_A = \) absorption of tested compounds.

Animals treatment—Female Wistar rats (purchased from Pasteur Institute, Algiers, Algeria) weighing 250-280 g were housed in stainless steel cages in room with a controlled temperature (22°C) and light cycle light on at 1200 hr) during the experimental period. They had free access to food (Groupe Avicole, Medea, Algeria) and water. The rats were divided into three subgroups:

Pretreated group (n = 24), rats were treated with propolis extract (100 mg/kg/day, po) for 4 days prior to anticancer drug administration. After 4 days, they were injected, ip, with adriamycin (20 mg/kg), vinblastin (2 mg/kg) or adriamycin (20 mg/kg) + vinblastin (2 mg/kg).

Untreated group (n = 24), rats were injected, ip, with adriamycin (20 mg/kg), vinblastin (2 mg/kg) or adriamycin (20 mg/kg) + vinblastin (2 mg/kg).

Control group (n = 8), rats were injected, ip, with 0.9 % saline solution.

After 24 hr of injections, the rats were sacrificed by decapitation and their livers and hearts were rapidly excised, weighed and placed in ice-bath.

Isolation of rat liver and heart mitochondria—Mitochondria were extracted from a homogenate of rat heart and liver by differential centrifugations²¹. Briefly, samples of freshly excised hearts (approximately 0.5 g) and livers (approximately 5 g) were respectively homogenized in ice-cold heart isolation medium (mannitol 225 mM, sucrose 75 mM, Hepes 5 mM, EGTA 1 mM and albumin 1 mg/ml, pH 7.4 at 37°C) and liver isolation medium (Tris 20 mM, sucrose 250 mM, EGTA 2 mM and MgCl2 5 mM, pH 7.2 at 4°C) using a Potter-Elvejhem homogenizer. The homogenates were centrifuged at 2000 g for 10 min at 4°C to remove cell debris and nuclei and supernatants were collected. Mitochondria were separated from the supernatant by centrifugation at 12000 g for 10 min. The pellet (mitochondria) was washed and resuspended in a respiratory buffer (KH2PO4 1 mM, sucrose 50 mM, KCl 100 mM, pH 7.4 at 37°C) or (KH2PO4 10 mM, sucrose 200 mM, pH 7.2 at 25°C) for measuring heart and liver mitochondrial enzymatic and respiratory activities respectively. Protein concentration of the mitochondrial suspension was assayed by Lowry’s method.

Measurement of mitochondrial respiration—Oxygen uptake was determined with a Clark-type microelectrode (Hansatech). Each experiment was carried out as follows: aliquot of mitochondria suspension equivalent to 1mg/ml were incubated with
(or without) the tested drug for 1 min at 37°C or 25°C in 1000 µl of the respiratory buffer with or without the inhibitors, then the substrate (pyruvate/malate 6 mM for heart mitochondria) or (succinate 6 mM for liver mitochondria) was added and oxygen consumption was checked (State 2). To initiate state 3 respiratory activity, 200 µM ADP was added to the cuvette. When all ADP was converted to ATP, the state 4 was measured. The following parameters were determined: the respiratory rates calculated as nanomoles of O₂/min/ mg of mitochondrial protein and respiratory chain ratio (RCR) expressed as the ratio of state 3/state 4 oxygen consumption.

Mitochondrial superoxide anion (O₂⁻) assay—
Generation of O₂⁻ was achieved using rat heart and liver mitochondria. Reaction mixture contained mitochondria (0.2 mg/ml), 1 µM CA and NBT (100 µM) in 1.2 ml of respiration buffer at 37°C and 25°C. The reaction was started by adding malate/pyruvate (6 mM) or succinate (6 mM) for heart and liver respectively in the assay cuvette and the rate of NBT reduction was measured at 560 nm. In absence of mitochondrial substrate (malate/pyruvate) or (succinate), there was no O₂⁻ production.

Mitochondrial swelling measurement—
Mitochondrial swelling was assessed by measuring the change in absorbance of the suspension at 540 nm (A540) using a Hitachi model UV-3000 spectrophotometer. Mitochondria (1 mg/ml) were incubated in a total volume of 1.8 ml of heart mitochondria buffer (KH₂PO₄ 1 mM, Sucrose 50 mM, KCl 100 mM, pH 7.4 at 37°C) in the presence of malate/pyruvate 6 mM at 37°C or in ice-cold isolation liver mitochondria medium (Tris 20 mM, sucrose 250 mM, EGTA 2 mM and MgCl₂ 5 mM, pH 7.2 at 4°C). 100 µM CaCl₂ was added to induce swelling before addition of increasing concentrations of the propolis extract.

Lipid peroxidation assessment—The lipid peroxidation in heart and liver mitochondria was assessed using an incubation mixture, containing 800 µl of suspended mitochondria in 0.9 % NaCl, 100 µl of the oxidizing solution Fe²⁺/Fe³⁺ (50 µM / 50 µM). Incubation was carried out in a water bath at 37°C for 30 min. The reaction was stopped by the addition of 1 ml of 10 % TCA. The tubes were shaken well and 1.5 ml of thiobarbituric acid (1 % in 0.05 NaOH) reagent was added and the mixture was heated at 100°C for 30 min. Tubes were recooled in ice for 5 min, then centrifuged at 3000 rpm for 15 min and the colours developed in the supernatant were read at 530 nm. The reaction without Fe²⁺/Fe³⁺ was carried out for each mitochondria suspension as the blank. Results were expressed as µM MDA / mg of mitochondrial membrane proteins.

Dosage of glutathione (GSH)—Portions (approximately 1 g of liver or 0.5 g of heart freshly excised or frozen) were homogenized in 3 volumes of 5% TCA using Dounce homogenizer. The samples were centrifuged at 2000 rpm for 15 min. The supernatant (50µl) was diluted in 10 ml phosphate buffer (0.1 M, pH 8). Consequently, 20 µl of DTNB 0.01 M was added to 3 ml of the dilution mixture. The measurement was performed at 412 nm against a control prepared in the same conditions using 5% TCA. The concentrations are expressed in mmoles of GSH / g of liver. They are deducted from a range of GSH, which was prepared with the same conditions as dosage did.

Catalase and superoxide dismutase (SOD) measurement—Isolated mitochondria prepared as described above were sonicated for 1 min. The sonic extract was centrifuged at 12,000 rpm for 15 min. The supernatant was then assayed by spectrophotometry for catalase activity by the method of Clairborne using catalase (Sigma Chemical Co.) as a standard and the SOD activity by the method of Beauchamp using SOD (Sigma Chemical Co.) as a standard.

Statistical analysis—The data are presented as mean±SD of three experiments for each compound and different experimental group. The Student’s t test and/or the F test (one way analysis of variance [ANOVA]) were used to evaluate the probability of significant differences between groups and compounds. Significance was considered to have occurred at P <0.05.

Results

Doxorubicin has no effects on liver and vinblastin on heart mitochondria. Therefore, the data are not presented.

Antioxidant effects of propolis extract—There are many different antioxidant components in a natural sample, and it is relatively difficult to measure each one separately. Several methods have been employed to determine antioxidant activity of biological samples, and the results are compared with those of reference antioxidant standards.

Free radical-scavenging activity of the propolis extract using DPPH⁺ was investigated. The results were compared with those of reference antioxidants.
A higher extract concentration required to scavenge the radicals means a lower antioxidant activity. The decrease of absorbance at 515 nm with the presence of antioxidants indicates the consumption of free radicals in the reaction mixture. The propolis ethanol extract at $10^{-4}$ M exhibited the highest free radical scavenging activity when compared with the other two polyphenols, vitamin E and quercetin (Fig. 1).

**Effect of propolis extract on SO production**—In order to evaluate antioxidant role of the propolis extract in heart and liver mitochondria, SO anions production was measured *in vitro* and *in vivo* on isolated heart and liver mitochondria. Propolis extract reduced the production of anion at concentrations between $10^{-4} - 10^{-8}$ M *in vitro* either on mitochondria incubated with doxorubicin 1 µM or control (Fig. 2). *In vivo*, propolis extract (100 mg/kg) given orally, starting 4 days prior to the administration of the anticancer drugs (doxorubicin and vinblastin) also reduced significantly the heart and liver mitochondrial production of SO ($P < 0.01$) (Figs. 3 a and b).

**Effect of propolis extract on lipid peroxidation**—Propolis extract inhibits the lipid peroxidation of heart or liver mitochondrial and cellular membranes (Fig. 4). The inhibition of lipid peroxidation reached 90% at $10^{-6}$ M concentration. *In vitro*, the inhibitory effect of propolis extract on lipid peroxidation of heart and liver mitochondrial membranes was observed at $10^{-4}$ M showing it to be more than a ten stronger than that of the positive control, quercetin.

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**Fig. 1**—Flavonoids scavenger effects (DPPH• Reduction).

**Fig. 2**—Effect of different concentrations ($10^{-2} - 10^{-10}$ M) of propolis extract on the heart mitochondrial production of SO *in vitro*.

**Fig. 3**—a: Effect of (i) doxorubicin (20 mg/kg) and (ii) the propolis extract on the heart mitochondrial production of SO *in vivo*. b: Effect of (i) doxorubicin (20 mg/kg)+vinblastin (2 mg/kg) treatment and (ii) the propolis extract pre-treatment on the liver mitochondrial production of SO *in vivo*.

**Fig. 4**—Variations of heart and liver mitochondria MDA levels (nMole/mg protein) expressed as % inhibition of control in the presence of different concentrations (ranged between $10^{-2}$ and $10^{-10}$ molar) of propolis extract.
vivo, a significant reduction of MDA concentrations in heart and liver cells of animals pre-treated with 100 mg/kg propolis extract daily for five days prior to doxorubicin (20 mg/kg) or doxorubicin (2 mg/kg) + vinblastin (2 mg/kg) iv injection was observed (Table 1).

Effect of propolis extract on mitochondrial respiration and swelling—It was observed that 20 mg/kg doxorubicin and 20 mg/kg doxorubicin + 2 mg/kg vinblastin increased the oxygen consumption (state 3) and the inhibition of state 4 occurring a decrease of respiratory chain ratio (RCR = V3 / V4) and a decrease of P/O also (Figs 5a and b). Propolis extract inhibited the effect of drugs showing a restoration of RCR and P/O in vitro and in vivo (P<0.001). Additional propolis extract to a mixture of rat heart and liver mitochondria with 25 μM calcium, inhibited slightly the rate and amplitude of mitochondrial swelling at 10⁻⁵ and 10⁻⁶ M concentrations affected by the doxorubicin and doxorubicin + vinblastin addition to the medium at a concentration of 1 μM.

Effect of propolis extract on GSH reserve—The effect of chemotherapeutic agents alone or in combination with propolis extract on the tissue levels of GSH is presented in Table 2. An increase of GSH was observed in the group of rats receiving the extract of propolis, compared with controls, while the rats treated by doxorubicin or doxorubicin + vinblastin only showed a decrease of 60% in GSH levels (P<0.001).

Effect of propolis extract on enzymes activities; catalase and SOD—Modification of the mitochondrial enzymes is a signal of anticancer drugs (doxorubicin and vinblastin) oxidative stress. However the propolis extract seems to play an original role to restore the loss of the enzymes activities (catalase and SOD) induced by doxorubicin and vinblastin (Figs 6 and 7). This effect was in relation with the antioxidative effects of polyphenols. Indeed, this result show that polyphenols are able to deactivate quickly the ROS produced by mitochondria.

Discussion
Oxidative stress and mitochondrial dysfunction result from overproduction by complexes of the respiratory chain of ROS, often leading to peroxidation of membrane phospholipids and production of reactive aldehydes. On the other hand, considerable interest in the polyphenols present in plants and propolis has arisen with the recognition of their antioxidant effects.

A chemical analysis of propolis extracts revealed presence of flavones and flavonols, which act by exerting direct positive inotropism, but the prevailing belief is that they act by scavenging free radicals generated by doxorubicin and/or by chelating iron. They act by (i) fixing of the free radicals on the DNA, (ii) activation of the detoxification
The antineoplasic drugs induced some transformations in the liver by the system cytochrome P450. These transformations succeed to a highly toxic reactive metabolites formation for the cell that interact with mitochondria leading to ROS production. Most of chemotherapeutic agents like doxorubicin and cyclophosphamide are metabolized in the liver by the cytochrome P450 and the formed reactive intermediates are responsible for apoptotic process and cell toxicity. The toxicity is function of the quantity of free radicals formed, the cellular reserve in GSH and the available glutathione-s-transferases to assure the detoxification.

Doxorubicin and vinblastin altered mitochondrial functions as observed by a decrease in respiratory control value and the swelling activation, an SO overproduction and a lipid peroxidation either by heart and liver mitochondria. The data also showed mitochondrial functions restoration and polyphenols protective effect against the stress induced by chemotherapeutic agent. Polyphenols of propolis extract clearly reduced the drug toxic effect. Stress observed with the anticancer drug (decrease in lipid peroxidation, increase in GSH concentration and restoration of mitochondrial functions), was corrected entirely.

Treatment of Wistar rats with a single dose of 20 mg/kg iv doxorubicin and 2 mg/kg vinblastin showed some effects in the heart and liver mitochondria functions especially the drugs decreases the RCR and increases SO and MDA productions. The doxorubicin has a moderate effect on the hepatic functions. In the group of animal’s receiving 100 mg/kg propolis extract during 5 days before the treatment with chemotherapeutics drugs: restoration of the RCR, decrease of SO production and inhibition of the lipid peroxidation were observed. The same effects were observed in vitro on heart and liver mitochondria setting in presence of increasing concentrations of propolis extract. The results suggest prevention mechanism of propolis extract against doxorubicin cardiotoxicity and vinblastin hepatotoxicity. Doxorubicin and vinblastin are metabolized by the cytochrome P450.

### Table 2—Effect of doxorubicin (20 mg/kg), doxorubicin (20 mg/kg)+vinblastin (2 mg/kg) administrations alone or in association with the propolis extract on heart and liver GSH levels (μg/mg protein).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control (n = 8)</th>
<th>Doxorubicin (20 mg/kg) (n = 8)</th>
<th>Doxorubicin (20 mg/kg) + Vinblastin (2 mg/kg) (n = 5)</th>
<th>Propolis (100 mg/kg) + Doxorubicin (20 mg/kg) (n = 8)</th>
<th>Propolis (100 mg/kg) + Doxorubicin (20 mg/kg) + Vinblastin (2 mg/kg) (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH in heart cell mitochondria</td>
<td>0.78 ± 0.06</td>
<td>0.33 ± 0.02*</td>
<td>0.68 ± 0.06*</td>
<td>0.83 ± 0.06</td>
<td>0.92 ± 0.07</td>
</tr>
<tr>
<td>GSH in liver cell mitochondria</td>
<td>1.10 ± 0.09</td>
<td>1.02 ± 0.09</td>
<td>1.02 ± 0.09</td>
<td>1.21 ± 0.09</td>
<td>1.16 ± 0.10</td>
</tr>
</tbody>
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*P < 0.001
liver to free radicals intermediates, which act on heart and liver mitochondria by increasing SO production, activation of membrane lipid peroxidation and therefore the fragilisation of the cell. Besides, the propolis inhibits the production of anion oxide by mitochondria. This effect is observed in vivo in the group of rats receiving the propolis orally before the administration of the chemotherapeutics agents whereas the rate of anion was increased significantly among the animals treated by only the drugs. The present results confirm the anti-oxidizer role of the flavonoids\(^1\),\(^2\) which have the capacity to capture and deactivate the free radicals\(^3\),\(^4\),\(^3\),\(^5\).

A semiquinone radical is formed during liver doxorubicin metabolism\(^6\), suggesting that the mechanism of action of the propolis is similar in part to trans-resveratrol. The propolis polyphenols and trans-resveratrol, inhibit the O\(_2^+\) production in the cardiac and nervous cells, respectively\(^7\),\(^8\). This effect is due to the partial inhibition of the complex III (decylubiquinon cycle)\(^9\). The interaction of propolis polyphenols with the Q cycle inhibits the formation of radical ubisemiquinone; they can capture the first electron yielded by the cytochrome to the ubiquinol or can add an electron to the radical ubisemiquinone after departure of another of the ubiquinol in the Fe-S centre while inhibiting the formation of the same radical. The reduction of the radical O\(_2^+\) in the heart mitochondria can also be explained by the strong scavenger effect of the flavonoids as observed in the present study. Corroborating their antiradical properties, the flavonoids have direct actions against the radical OH• hydroxyles and O\(_2^+\) superoxides that are species implied in the starting of the lipid peroxidation\(^1\)\(^0\). The inhibition of antioxidant system (GSH levels, catalase and SOD enzymes activities) by the anticancer drugs and their restoration by propolis extract reinforce this hypothesis. Alternatively, it is also possible as suggested by Koller\(^1\)\(^1\) that the restoration of the mitochondrial membrane potential may be a way in the prevention of doxorubicin cardiotoxicity. A close relationship between the mitochondrial membrane potential and ROS production has been demonstrated\(^1\).

The present results indicate that propolis extract is able to restore the fall of membrane potential induced by doxorubicin and are able to protect the cell against the apoptosis of the drug.

**Conclusion**

The results confirmed the role of propolis extract in protecting heart and liver tissues from doxorubicin and vinblastin toxicity by protecting the mitochondria that should be given to minimise the damage membranes and enzymes. It is evident from the present work that the Algerian propolis is a very interesting antioxidant product that may be able to solve the problem of the anticancer drugs toxicity.

**References**


