Antioxidant activity of extract of *Rhus oxyacantha* root cortex

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In the present study, the root extract of *Rhus oxyacantha* contained 25.33 mg of catechin equivalent per mg of fresh wt and was found rich in proanthocyanidins compared to vine shoot, grape pips and leaves. The chromatographic analysis of the extract suggested the presence of (+) catechin, (-) epicatechin -3-O-gallate as well as proanthocyanidinic oligomers and polymers. Root cortex inhibited the ascorbic acid oxidation by dioxygen. It also prevented DDT-induced thymocytes death in a dose-dependent manner. The results suggested antioxidant property of root extract of *Rhus oxyacantha* which could be ascribed to its free radical scavenging nature.

**Keywords**: DDT, Oxidative stress, Proanthocyanidins, *Rhus oxyacantha*

Flavanic or catechic tannins (500-3000 mol wt) have the property to form soluble and insoluble associations with proteins and polyols1. Plants containing condensed tannins (dimeric and oligomeric proanthocyanidins) have medicinal use in toothpaste, diarrhoea and haemorrhage treatment, dyeing2, cosmetic and therapeutic formulae3. Proanthocyanidins protect against cardiovascular diseases4, have anti-inflammatory5, antibacterial6 and antcarcinogenic effects7 and react as excellent free-radical scavengers8. Many plants, like raspberry and caneberry9, red rice10 and grape fruit and seed7-11, are particularly rich in having antioxidant, proanthocyanidins.

*Rhus oxyacantha* is a mediterranean plant that has been used for a long-time in traditional medicine, in Tunisia, as an anti-ulcer plant12.

In the present study, an attempt has been made to search proanthocyanidins in cortex of *Rhus oxyacantha* and to examine the antioxidant activity against ascorbic acid oxidation and inhibition of toxicity induced by an organochlorine pesticide, dichloro-diphenyl-trichloroethane (DDT) in rat thymocytes.

**Materials and Methods**

*Chemicals*—Vanillin was of commercial grade (Merck Co.), while 4-dimethylamino-cinnamaldehyde (DMACA) was purchased from Fluka. DDT (98%) and all other chemicals were purchased from Sigma Chemical Co (St Louis Missouri, USA).

*Animals*—Wistar male rats used in this study, were obtained from the Society of Pharmaceutic Industries of Tunisia (SIPHAT) and bred in our laboratory. Rats were housed under controlled conditions at $22^\circ\pm 1^\circ$C, with 14 h light/dark cycle. Food and water were provided *ad libitum*. Rats were sacrificed by decapitation at the age of 4 weeks.

Thymocytes were extracted from 6 rats and the incubated. Each result was expressed as mean ± SE of 6 determinations in duplicate.

*Proanthocyanidin extraction*—Acetone-water mixture was used to extract oligomeric proanthocyanidins13. Hundred ml of acetone-water mixture (90/10 v/v) was added to 10 g of *Rhus oxyacantha* root cortex. After three consecutive extractions for 2 h under continuous magnetic stirring at room temperature, the resulting suspension was filtered through a buchner funnel and concentrated in a rotatory evaporator until the acetone was removed.

*Proanthocyanidin analysis*—Analysis was carried out by condensation of vanillin onto phloroglucinol nucleus catalysed by $\text{H}_2\text{SO}_4$ (ref. 14). Extract (1 ml) of *Rhus oxyacantha* root cortex was added to 2 ml of fresh solution of vanillin (0.01 g,l$^{-1}$) in $\text{H}_2\text{SO}_4/\text{H}_2\text{O}$ (70% v/v). After 15 min of reaction in a water bath at 35$^\circ$C, maximum absorbance was read at 505 nm. The standard curve was calculated using an aqueous solution of catechin as reference. It follows the
equation: \( y = 2.2876x - 0.0342 \); where, \( x \) varying between 0.25 and 1 mg.ml\(^{-1}\); \( y \) is the absorbance measured at 505 nm. The correlation coefficient of this equation is equal to 0.997. An optimisation of the operative conditions allowed us to avoid the errors caused by the use of a relatively high concentration of the extract of \( Rhus oxyacantha \) root cortex. The choice of reaction time is essential concerning the dosage of proanthocyanidins. A kinetic study of the condensation reaction of proanthocyanidins with vanillin shows that initial rate of the reaction is faster that decreases with time. The efficiency of this reaction also increases with temperature for the time interval chosen for the study\(^{15}\).

Chromatographic proanthocyanidin characterization—Extract of \( Rhus oxyacantha \) root cortex was analysed by thin layer chromatography (TLC) that was conducted on precoated silica gel 60 F\(_{254}\) plates (0.2 mm, Merck) with acetone/toluene/formic acid (60/60/10, v/v/v)\(^{16}\). The proanthocyanidins spots were detected with 4-dimethylamino-cinnamaldehyde (DMACA) reagent spray.

Antioxidant effect of proanthocyanidins against ascorbic acid—Ten ml of ascorbic solution (17.6 g.l\(^{-1}\)) was exposed to a current of oxygen during 0 to 45 min in the presence or absence of different volumes of \( Rhus \) extract ranging from 5 to 40 ml. After 45 min, 25 ml of \( I_2 \) (0.05 \( M \)) was added that reacted with ascorbic acid, and the amount of unreacted \( I_2 \) was determined by a back titration with \( Na_2S_2O_3 \) solution (0.1 \( M \)). By knowing the amount of initial \( I_2 \) and determining the amount of \( I_2 \) that did not react with ascorbic acid, we determine the amount of ascorbic acid that did not oxidize \( O_2 \)\(^{17}\).

Isolation of thymocytes—Thymuses were removed, placed in ice cold phosphate buffer saline [PBS: 140 mM, NaCl; 2.68 mM, KCl; 8.1 mM, \( Na_3HPO_4 \)] and 1.47 mM, KH\(_2PO_4\). (\( pH \) 7.2)] dissected and dissociated using a potter homogeniser. The cell suspension was then filtered through Screen filter 70 mesh (Sigma) to remove debris. After washing twice with PBS, the cells were diluted to a final density of 10\(^7\) cells.ml\(^{-1}\) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 units.ml\(^{-1}\)) and streptomycin (100 \( \mu g.ml^{-1} \)). Viability, determined by trypan-blue exclusion, was greater than 95%. Isolated thymocytes were incubated for 4 and 6 h with or without DDT(2.10\(^{-5}\)M) in 95% \( O_2 \), 5% \( CO_2 \) atmosphere in a final volume of 1 ml/plate. To study the effect of \( Rhus oxyacantha \) root cortex extract, different concentrations i.e., 0.05, 0.12, 0.25, 0.63, 1.26 and 2.53 mg of catechin equivalent.ml\(^{-1}\) were used. DDT was dissolved in 50% of ethanol and added to the medium. The final concentration of ethanol was lower than 0.01% and had no detectable effect on cells.

Statistical analysis—Data was subjected to ANOVA test and analysed using Stat View 512+ software (Abacus Concept, Inc). Values were expressed as mean ± SE.

Results and Discussion
Results of the present study showed that \( Rhus \) root cortex was quantitatively rich in proanthocyanidins compared to vine shoot, grape pips and leaves. \( Rhus \) root cortex was found to contain 25.33 ± 0.07 mg of catechin equivalent (CE) per g of fresh weight (g.fresh wt), whereas grape pips, \( Rhus \) leaves and vine shoot were found to contain 10.75 ± 0.06, 6.12 ± 0.04 and 2.92 ± 0.03 mg of CE/g.fresh wt, respectively.

Proanthocyanidins could be analysed by chromatography\(^{18}\). On the basis of Rf, the chromatographical analysis suggested the presence of (+) catechin (-) epicatechin-3-O-gallate, as well as proantho-cyanidins dimers, trimers and polymers in root cortex extract (Table 1).

Increase in oxidation of ascorbic acid was seen with a function of paddling time of dioxygen current (Fig. 1). The root cortex extract inhibited this oxidation in a dose-dependent manner (Fig. 2). In the absence of the extract, 39.15% of ascorbic acid was oxidized by \( O_2 \), while the presence of 15 ml of root cortex extract in the reaction medium decreased oxidized ascorbic acid to 2.34%. The extract (40 ml) provided the total protection. This suggested the

<p>| Table 1—Proanthocyanidin retention factors (Rf) of ( Rhus oxyacantha ) root cortex. Proanthocyanidins were separated by chromatography on silica gel (Merck 60) with acetone-toluene-formic acid (60:60:10, v/v/v) |</p>
<table>
<thead>
<tr>
<th>Proanthocyanidin compounds</th>
<th>Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechin</td>
<td>0.65</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>0.53</td>
</tr>
<tr>
<td>Dimers</td>
<td>0.42</td>
</tr>
<tr>
<td>Trimmers</td>
<td>0.37</td>
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<tr>
<td>A</td>
<td>0.28</td>
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<tr>
<td>B</td>
<td>0.21</td>
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<tr>
<td>C</td>
<td>0.16</td>
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<tr>
<td>D</td>
<td>0.08</td>
</tr>
</tbody>
</table>

A, B, C, D—types of polymeric proanthocyanidins.
antioxidant potential of the extract of root cortex that might be due to the presence of proanthocyanidins. It may be mentioned that proanthocyanidins are efficient free radical scavengers and metal chelators\textsuperscript{19}. In fact, Hanasaki \textit{et al.}\textsuperscript{20} have demonstrated that (+) catechin and (-) epicatechin show OH scavenging effect superior (100-300 times) to that of mannitol, a potential OH scavenger. Recently, Jagan Mohan Rao \textit{et al.}\textsuperscript{21} have reported the existence of antioxidant and free-radical scavenging oligomeric proanthocyanidins in \textit{Mammea longifolia} buds.

In order to study the antioxidant potential of \textit{Rhus} root cortex extract in biological systems, we examined the viability of thymocytes incubated with or without DDT (2.10^{-5} M) in presence of different concentrations of proanthocyanidins. Treatment of cells with DDT resulted in time-dependent decrease in cell viability by 72.81\% after 6h of incubation, while spontaneous cell lethality in control cultures was less than 3\% under the same conditions. Addition of various concentrations of \textit{Rhus oxyacantha} root cortex extract prevented DDT-induced cell death in a dose-dependent fashion. Cell viability reached 85.69, 88.91, 90.49, 91.88, 92.11 and 93.79\%, respectively when 0.05, 0.12, 0.25, 0.63, 1.26 and 2.53 mg catechin equivalent ml\textsuperscript{-1} was supplemented in the media containing thymocytes (Fig. 3).

In a previous work, DDT has been shown to induce apoptosis in thymocytes\textsuperscript{22}. Therefore, increased viability of thymocytes could be due to inhibition of apoptosis induced by DDT. This result agrees with the finding of Chen \textit{et al.}\textsuperscript{23} who have shown that catechins extracted from tea leaves have protective effect on lead induced apoptosis in HepG\textsubscript{2} cells \textit{in vitro}. Joshi \textit{et al.}\textsuperscript{24} have also reported that cyanidin extract prevented apoptosis of hepatocytes by increasing Bcl\textsubscript{2} expression. Anti-apoptotic activity of proanthocyanidins may be due to their antioxidant potential because earlier studies have indicated that...
apoptosis induced by organochlorines is the result of oxidative alterations, which subsequently induce endonuclease activation leading to DNA fragmentation. In fact, recently, proanthocyanidins, like (+) epicatechin and (-) epicatechin-3-O- gallate, have been shown to be powerful antioxidants against lipid peroxidation in rats, that enhance cell antioxidant defence by increasing the expression of detoxifying enzymes.

In conclusion, this is the first report to identify and quantify proanthocyanidins in Rhus oxyacantha and to indicate that the plant root cortex is rich in antioxidant proanthocyanidins compared to vine shoot, grape pips and leaves.

Acknowledgement
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