EFFECT OF ETHANOLIC RED CABBAGE EXTRACT ON
OXIDATIVE STRESS IN HYPERTHYROID RABBITS INDUCED BY L-
THYROXINE

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Abstract

The protective effect of red cabbage extracts against hyperthyroid induced by L-thyroxine in male rabbits was studied. Forty animals were divided randomly into four groups. Group A: Healthy rabbits, Group B: Healthy rabbits received (100 mg/kg.b.w) red cabbage extract, Group C: hyperthyroid rabbits were given an oral dose of (20µg/kg b.w) L-thyroxine once daily for four weeks to induce hyperthyroidism and group D: hyperthyroid rabbits received red cabbage extract. At the end of the experiment, blood samples were taken for biochemical analysis. The obtained results revealed that hyperthyroid rabbits induced by L-thyroxine had significant increase (P<0.01) in serum levels of triodothyronine (T3), Thyroxine (T4), lipid peroxidation marker malondialdehyde (MDA), as well as significant decrease in reduced glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) enzymes, compared to the normal control group. Oral administration red cabbage extract caused significant reduction in serum levels of T3, T4 as well as MDA and produced significant increase in thyroid stimulating hormone (TSH), GSH, GPx, SOD and CAT, compared to the positive group. Red cabbage treatment suppresses the hyperthyroidism-induced oxidative damage. These results suggest that experimental hyperthyroidism is accompanied with increased oxidative stress and with the consumption of antioxidant enzymes in induced oxidative aggressions. A protective effect of red cabbage extract on oxidative stress induced by excessive administration of thyroid hormones were detected and for the first time anti hyperthyroid activity was observed.
Introduction
Oxidative stress accompanying hyperthyroidism is caused by increased synthesis of reactive oxygen species (ROS) and changes in the antioxidant defence system (1-3). ROS have a high reactivity potential, therefore they are toxic and can lead to oxidative damage in cellular macromolecules such as proteins, lipids and DNA (4-5). In fact, the cell contains a variety of substances capable of scavenging the free radicals, protecting them from harmful effects. Among the enzymatic antioxidants, are GRx, GPx, CAT, SOD while the non-enzymatic antioxidants are GSH, vitamin E, vitamin C, β-carotene, and flavonoids (6). When ROS generation exceeds the antioxidant capacity of cells, oxidative stress develops (2, 7). This phenomenon has been related to many pathological conditions and it has also been suggested that some complications of hyperthyroidism are due to T3 induced oxidative stress in target tissues (8-10). Thyroid hormones are the most important factors involved in the regulation of the basal metabolic state, as well as in the oxidative metabolism (11). Experimental studies and epidemiological data suggest that hyperthyroidism is associated with a general increase in tissue oxidative stress (12-15). Great controversy exists as to whether hyperthyroidism is associated with an increase or a decrease in the activities of antioxidant enzymes (16). Vitamin E is a potent lipid soluble antioxidant in biological systems with the ability to directly quench free radicals and function as membrane stabilizer (17). Antioxidants treatments might be helpful in reducing the oxidative damage due to hyperthyroidism. Several studies have demonstrated that various agents, including vitamin E (15) melatonin (18), and Selenium (18) can prevent oxidative damage in hyperthyroidism. The antioxidant protection of natural plants is a promising therapeutic remedy for free radical pathologies (19-20). Among myriad natural plants, red cabbage (Brassica oleracea var capitata) and other Brassica vegetables, have been found to have antioxidant, antihyperglycemic (20), anticancer (21-23), hypolipidmic (24). The principle constituents of RC are isothiocyanates (glucosinolate), vitamin A, B, C and Anthocyanins (27-29). Anthocyanins, a group of phenolic natural pigments present in RC, were found to have the strongest antioxidizing power of 150 flavonoids (29). There are some review articles on the general biochemical, cellular and medicinal properties of anthocyanins (30-31), but no detailed mechanisms of its action has yet been published. To date there is no study on the protective effect of RCE, a known potent antioxidant and free radical scavenger on hyperthyroidism. This study aims to investigate oxidative stress parameters, antioxidant status markers, in hyperthyroid rabbits induced by thyroxine and their response to RCE supplementation.

Materials and Methods
Preparation of Red Cabbage Extract
Red cabbage leaves were sliced into small pieces and oven-dried at 50°C. Dried plants (800 g) were extracted in 8000 ml of 70% aqueous ethanol using ultrasonic treatment at an intensity of 70 W/cm² and oscillation frequency at 20 kHz for 5 min. The use of an ultrasound extraction method has been shown to diminish the danger of thermal degradation. Ultrasound also provides a greater penetration of solvent
into cellular materials, via cavitations, and improves the release of cell contents into the bulk medium (32). Ultrasonic irradiation was applied by means of a Branson 450 digital sonifier (20 kHz, 450 W) equipped with a cylindrical titanium alloy probe (12.70 mm in diameter). The use of dry plants can be effective to minimize enzymatic degradation of phonetic compounds inside plant tissues. After overnight maceration, the extract was filtered through gauze and ethanol was evaporated under reduced pressure at 50°C by using a rotary evaporator. The remaining water extract was dried using a freeze dry system under reduced pressure. The dried extract was dissolved in distilled water to a concentration of 1 g/ml before administration to normal and diabetic rats. The extraction yield for RC was about 12%.

**Animals & Experimental design**

Male New Zealand rabbits (1400–1500 g) were obtained from the Animal House at the biological control center, Ministry of Health. Rabbits were maintained on standard pellet diet and tap water *ad libitum*. They were kept in plastic cages under a 12 h light/dark cycle and room temperature 22–24°C and were acclimatized to the environment for 2 weeks prior to experimental use. Procedures for the care and use of research animals at Iraq meet or exceed all applicable local, national and international laws and regulations. The animals were randomly divided into four groups of ten animals each group. The control rabbit's group one, received only vehicle. The animals in the experimental group two received RCE orally at a dose of 100 mg/kg per day for 4 weeks. The animals in the experimental group three were orally administered L-thyroxine (20 µg/kg b.w) in 0.5 ml sterile physiological saline. The animals in the experimental group four, received L-thyroxine (20µg/kg b.w) and RCE (100 mg /kg) for 4 weeks. RCE was administered at the same time each day. At the end of the experimental period, the blood samples were collected through heart puncture after overnight fasting in test tubes without anticoagulant and serum separated by centrifugation at 2500 g for 5 minutes at room temperature to estimate thyroid function tests: TSH, T3, and T4, oxidative stress markers MDA, GSH, SOD, GPx, and CAT.

**Assay of thyroid function tests.**

Quantitative determination of rabbit T3 hormone was performed using rabbit thyroxine hormone, T3 ELISA Kit Catalog No: E0433Rb from ELA Ab, company. determination of rabbit thyroxine hormone was performed using rabbit thyroxine hormone, T4 ELISA Kit Catalogue Number: E04T0040 from Shang Hai Blue Gene Biotech CO., LTD. Quantitative determination of rabbit thyroid stimulating hormone was performed using rabbit thyroid stimulating hormone, TSH ELISA Kit Catalog No: E0463Rb from ELA Ab. The principle depending on the microtiter plate provided in this kit has been pre-coated with an antibody specific to T3. The principle depending on the microtiter plate provided in this kit has been pre-coated with an antibody specific to T3. Standards or samples are then added to the appropriate microtiter plate wells with abiotin-conjugated polyclonal antibody preparation specific for T3 and Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB substrate solution is added to each well. Only those wells that contain T3, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of T3 in the samples is then determined by comparing the O.D. of the samples to the standard curve.

**Measurement of MDA**

Lipid peroxidation (LPO) is frequently investigated in biomedical research, and the assays for thiobarbituric acid-reactive substances (TBARSS) are more widely used than any other index of LPO in biological samples. Thiobarbituric acid reacts with LPO aldehydes, such as malondialdehyde (MDA). Therefore, assessment of TBARS is a useful index of oxidative deterioration and LPO determination in body fluids. MDA levels were determined at 532 nm by the method of Ohkowa (33). MDA formed a colored complex in the presence of thiobarbituric acid, which was detectable by measurement of absorbance at 532 nm. Absorbance was measured with Shimadzu UV-160 spectrophotometer. 1, 1, 3, 3 tetraethoxypropane was used as a standard. Levels were calculated as nmol/ml.
Measurement of GSH.
Total glutathione content was measured according to the method of Tietze (34). In brief, 0.5mL sample or standard solution was mixed with 0.25 mL of 1mol/L sodium phosphate buffer (pH 6.8) and 0.5 mL 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB, 0.8 g/l in the phosphate buffer) for 5 minutes. Then, the absorbance was measured at 412 nm using a Shimadzu UV-160 spectrophotometer. The GSH concentration was determined using standard aqueous solutions of GSH. Results were expressed as mg/dl.

Measurement of GPx
Glutathione peroxidase activity was determined by a minor modification of the method of Paglia and Valentine (35). A 50 µl supernatant was transferred to a 1mL quartz cuvet, containing 950 µL of the reaction mixtures (Tris buffer, 50 mmol/L, pH 7.6, containing per liter, 1 mmol of Na2EDTA, 2 mmol of reduced glutathione, 0.2 mmol NADPH, 4mmol sodium Azide, and 1000U of glutathione reductase). The mixture was incubated 5 minutes in 37°C. Then, the reaction was initiated by adding 25 µL of H₂O₂, 8.8 mmol/L (% 30). The decrease in NADPH absorbance at 340 nm was followed for 3 minutes. The non enzymic reaction rate (blank) was determined by substituting water for the supernatant. The decrease in NADPH absorbance was recorded.

Determination of (SOD) activity:
Superoxide Dismutase (SOD), which catalyzes the dismutation of the superoxide anion (O₂ -) into hydrogen peroxide and molecular oxygen, is one of the most important antioxidative enzymes. In order to determine the SOD activity, several direct and indirect methods have been developed. Among these methods, an indirect method using nitroblue tetrazolium (NBT) is commonly used due to its convenience and ease of use. However, there are several disadvantages to the NBT method, such as poor water solubility of the formazan dye and the interaction with the reduced form of xanthine oxidase. The K-ASSAY SOD Assay kit Cat. No. KT-019 allows very convenient SOD assaying by utilizing KAMIYABIOMEDICALCOMPANY’s highly water soluble tetrazolium salt, 1(2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt)that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with O₂- are linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD. Therefore, the IC50 (50% inhibition activity of SOD or SOD-like materials) can be determined by a colorimetric method.

Determination of (GPx) assay:
Serum glutathione peroxidase was determined according a sandwich enzyme immunoassay method for the in vitro quantitative measurement of rabbit GPX in serum, plasma and other biological fluids (ELISA Kit for Rabbit Glutathione Peroxidase (GPX) Cat. No.: E0295Rb Us cn Life Science Inc. Wuhan Company.

Determination of (CAT) assay:
Serum catalase was determined according a sandwich enzyme immunoassay method for the in vitro quantitative measurement of rabbit CAT in serum, plasma and other biological fluids (ELISA kit for Rabbit Catalase ELISA kit Catalogue Number: E04C0086 Uscn Life Science Inc. Wuhan Company.

Statistical analysis.
The data analysis was performed with SPSS soft ware package (SPSS, Chicago, IL, USA). Data are presented as mean values (Mean±S.E.M, (n=10). Data were statistically analyzed using a one-way analysis of variance followed by a Student-t test to compare the groups. Linear regression analysis was used with thyroid function as the dependent variable. A value of P<0.05 was accepted as significant.

Results
Serum T₃, T₄ and TSH concentrations in the control and experimental groups are given in table 1. Serum T₃ and T₄ concentrations were significantly increased 1.99 ± .47 ng/ml and 16.5 ± 2.22 µg/dL respectively in rabbits with hyperthyroidism compared to controls 1.14 ± 0.37 ng/ml and 9.82 ± 2.33 µg/dL respectively. Administration of RCE alone for 4 weeks as show in group 2 in table 1 did not show significant changes in the thyroid hormones (P>0.05). Co-administration of RCE with L-thyroxine significantly decreased the elevated T₃ (P<0.01) to 1.24 ± 0.37ng/ml and 9.82 ± 2.33µg/dL respectively. Administration of RCE alone for 4 weeks as show in group 2 in table 1 did not show significant changes in the thyroid hormones (P>0.05). Co-administration of RCE with L-thyroxine significantly decreased the elevated T₃ (P<0.01) to 1.24 ± 0.37ng/ml and 9.82 ± 2.33µg/dL respectively.
1.04 ± 0.54 mIU/L compared with 0.66 ± 0.12 mIU/L before treatment. As expected, lipid peroxidation was significantly higher in the plasma of hyperthyroid group compared to the control group (4.62 ± 0.12 nmol/dL and 2.23 ± 0.08 mol/dL, respectively). It was decreased after treatment with RCE in the hyperthyroid group (P < 0.01). When the plasma were used to measure MDA concentrations as an index of lipid peroxidation, the result indicated a significant increase in plasma of hyperthyroid group compared to the control group. On the contrary, when rabbits were concurrently treated with RCE the result demonstrated a significant reduction in MDA concentrations (Table 2 and Figure 1).

Serum glutathione level was significantly lower (P < 0.01) in the hyperthyroid group (34.43 ± 0.19 μmol/dL) compared to the control group (44.32 ± 0.13 μmol/dL), while GSH level increased after treatment with RCE in the hyperthyroid group to 47.57 ± 0.16 μmol/dL. After treatment with RCE, it was also shown that the plasma status of oxidized and reduced glutathione in the hyperthyroid group was changed at the fourth week of treatment.

### Table 1: Effect of oral administration of red cabbage extract on serum levels of T3, T4 and TSH, levels (M±SE) in healthy and hyperthyroid rabbit's induced by T4

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Healthy</th>
<th>Healthy+RCE</th>
<th>Hyperthyroid</th>
<th>Hyperthyroid+RCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3, ng/ml</td>
<td>1.14 ± 0.37</td>
<td>1.03 ± 0.13</td>
<td>1.99 ± 0.47**</td>
<td>1.24 ± 0.37**</td>
</tr>
<tr>
<td>T4, μg/dl</td>
<td>9.82 ± 2.33</td>
<td>10.65 ± 3.75</td>
<td>16.5 ± 2.22**</td>
<td>11.2 ± 3.47**</td>
</tr>
<tr>
<td>TSH, mIU/L</td>
<td>2.04 ± 0.54</td>
<td>1.83 ± 0.63</td>
<td>0.66 ± 0.12**</td>
<td>1.04 ± 0.54*</td>
</tr>
</tbody>
</table>

*Significantly different (p<0.05), ** significantly different (p<0.01)

### Table 2: Effect of oral administration of red cabbage extract on serum levels of malondialdehyde (MDA) and reduced glutathione (GSH) (M±SE) in healthy and hyperthyroid rabbit's induced by thyroxine.

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA(nmol/ml)</th>
<th>GSH(µmol/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>2.23±0.08</td>
<td>44.32±0.13</td>
</tr>
<tr>
<td>Healthy+RCE</td>
<td>2.45±0.02</td>
<td>50.66±0.22</td>
</tr>
<tr>
<td>Hyperthyroid</td>
<td>4.62±0.12**</td>
<td>34.43±0.19**</td>
</tr>
<tr>
<td>Hyperthyroid + RCE</td>
<td>2.30±0.03*</td>
<td>47.57±0.16*</td>
</tr>
</tbody>
</table>

*Significantly different (p<0.05), ** significantly different (p<0.01)

### Table 3: Effect of oral administration of red cabbage extract on serum levels of antioxidant enzymes (M±SE) in healthy and hyperthyroid rabbit's induced by L- thyroxine.

<table>
<thead>
<tr>
<th>Groups</th>
<th>GPX(mmol/L)</th>
<th>SOD U/dL</th>
<th>CAT(mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>18.70±0.13</td>
<td>92.75±0.22</td>
<td>64.00±0.56</td>
</tr>
<tr>
<td>Healthy+RCE</td>
<td>19.95±0.88</td>
<td>89.66±0.26</td>
<td>66.60±0.76</td>
</tr>
<tr>
<td>Hyperthyroid</td>
<td>8.25±0.14**</td>
<td>55.45±0.19**</td>
<td>42.15±0.60**</td>
</tr>
<tr>
<td>Hyperthyroid + RCE</td>
<td>17.25±0.22**</td>
<td>88.61±0.18**</td>
<td>59.65±0.94**</td>
</tr>
</tbody>
</table>

*Significantly different (p<0.05), ** significantly different (p<0.01)
Figure 3: Superoxide dismutase (SOD) activity in healthy, healthy+RCE, hyperthyroid, and hyperthyroid +RCE treated groups of rabbits. Values are given as the mean ± SEM for 10 animals each. Hyperthyroid group was compared with healthy or RCE alone groups. Hyperthyroid treated group with RCE were compared with hyperthyroid untreated group.

Figure 4: Glutathione peroxidase (GPx) activity in healthy, healthy+RCE, hyperthyroid, and hyperthyroid +RCE treated groups of rabbits. Values are given as the mean ± SEM for 10 animals each. Hyperthyroid group was compared with healthy or RCE alone groups. Hyperthyroid treated group with RCE were compared with hyperthyroid untreated group.

There was an increase of reduced GSH levels in treated hyperthyroid animals with RCE as compared to the control (table 2 and figure 1). This increase was restored by the concurrent administration of RCE. The redox status (GSH/GSSG ratio), an oxidative stress indicator, was found to be significantly reduced ($P<0.05$). These decreases were restored to normal level by concurrent treatment with RCE (table 2). A significant decrease in the activity of Serum GPx was observed in the hyperthyroid group compared to the control group 8.25 ± 0.14 mmol/L and, 18.70 ± 0.13 respectively. After treatment with RCE, GPx activity in hyperthyroid group increased significantly and return to normal level 17.25 ± 0.22 mmol/L compare to the control group 18.70±0.13 mmol/L (Table 3 and Figure 4). The present finding revealed decrease in MDA level and increase in levels of GSH, GPx, SOD and CAT activity by the treatment of RCE, indicating reduction in oxidative stress in hyperthyroid state. Thus, it was concluded that the RCE had antioxidant property because of which there was reduction in oxidative stress. The CAT activity in normal control group treated with RCE (66.60 ± 0.76 mmol/L) showed insignificant increase when compared with control group, whereas in hyperthyroid groups, the CAT activity reduced significantly 42.15±0.60 mmol/L compared with control animal group (Figure 5), while treatment with RCE the activity increased to the mean
value near normal. The results are shown in Table 3. Thus, the marked increase in the oxidative stress was found in hyperthyroid groups as indicated by decrease in CAT activity, whereas treatment with RCE showed decrease in oxidative stress as indicated by the increased CAT activity as compared to hyperthyroid groups.

Figure 5: Catalase (CAT) activity in healthy, healthy+RCE, hyperthyroid, and hyperthyroid +RCE treated groups of rabbits. Values are given as the mean ± SEM for 10 animals each. Hyperthyroid group was compared with healthy or RCE alone groups. Hyperthyroid treated group with RCE were compared with hyperthyroid untreated group.

Discussion

Hyperthyroidism accelerates ROS generation and produces changes in the antioxidant systems of various tissues (1-3). In the present study, increased plasma T3 and T4 levels and decreased in TSH levels were observed in the hyperthyroid animals induced by thyroxine. In this respect, the results of our study appear to be consistent with the findings of others (8,9,16,41). Also, the TSH level was significantly lower in the hyperthyroid group compared to the control group. Previous studies have suggested that hyperthyroidism increased free radical production and lipid peroxidation levels (6, 13, 36). The mechanisms behind the RCE-induced reduction in thyroid hormone are not clear. Possibilities include RCE induced modulation in deiodination system, which affects deiodinase activity through its antioxidant properties. Based on the results obtained, it can be concluded that the hyperthyroid group, which received RCE, shows a significantly different decrease of plasma T3 and T4 concentrations and significantly different increase of TSH levels. Pharmacological antioxidants may have an effect on the peripheral conversion of thyroid hormones by way of deiodination and/or mechanism of cell membrane defence, the integrity of which may have an effect on the activity of deiodinases (15). The cellular GSH plays an important role as biological antioxidant defence systems, which act as protective mechanisms against oxidative damage, therefore, the decreased level of GSH may be due to overproduction of free radicals and increased lipid peroxidation in hyperthyroidism (5, 16). In our study, serum GSH levels were decreased in hyperthyroid animals as compared to control animals, possibly secondary to increased ROS generation. RCE treatment caused normalization of GSH levels in hyperthyroid animals agree well with that reported by administration of L-thyroxine to rats’ results in decrease of GSH concentration in the heart (7, 37-39). The ratio of GSH to GSSG is considered an important marker of oxidative stress. The decrease in the ratio of GSH after hyperthyroid state suggests induction of oxidative stress in the animals. Furthermore, increase in the GSH levels of the RCE treated hyperthyroid rats suggests that RCE as a regulatory effect on the antioxidant system. Increased oxidative stress is a well-known phenomenon in the hyperthyroid state. Hyperthyroidism is believed to accelerate free radical generation that leads to oxidative damage of lipids (4).

In our study, the Serum MDA level was increased in the hyperthyroid rabbits as compared to the control rabbits. Our results are in accordance with Cetinkaya who reports a significant increase in MDA levels of hyperthyroidism (14). Increased plasma MDA concentrations in hyperthyroid rabbits suggest the role of free radicals in the pathogenesis of this lipid peroxidation and, thus, support the need for studies assessing the therapeutic role of antioxidants in hyperthyroidism. In support of the above statement, we found a highly significant decrease in the concentrations of serum MDA after treatment with RCE. The reduced MDA level by RCE likely indicates that RCE might be a novel agent to protect against oxidative damage induced by hyperthyroidism. It is also possible that the action of RCE is primarily a normalization of thyroid function and secondarily a normalization of the oxidative stress markers. Superoxide dismutase is an important intracellular oxygen radical-scavenging enzyme. It has been demonstrated that hyperthyroidism leads to accelerated free radical formation (2, 3, 9, 11, 16). Conversely, increased free radical formation enhances
intracellular scavenging enzymes like SOD in experimentally animals induced thyroxine treatment (16, 13).

Erythrocytes are perhaps the cells most exposed to peroxidative damage by ROS (40). During their relatively short life, in which no protein synthesis occurs, the cells are in close contact with free radicals from various sources. Oxidant/antioxidant response in erythrocytes to an agent might be a mirror for whole body response. In our study, erythrocyte SOD activity was found to be significantly higher in the hyperthyroid group, possibly as a response to increased superoxide anion radical formation. Our findings are consistent with those of Noor (35), who reported a positive correlation between SOD activity and the level of serum thyroxine, and Suchetha et al who noted that thyroid hormones increase SOD activity (16). We investigated the therapeutic value of RCE against oxidative stress in hyperthyroid animals. These plants, members of the Cruciferae and genus Brassica such as RC, broccoli, kale and Brussels sprouts, contain anthocyanin pigments that are described as free-radical scavenging and antioxidant agents (27). Anthocyanin isolates and anthocyanin-rich mixtures of bioflavonoid provide protection against myriad physiological failures such as LP, decreasing capillary permeability and fragility and membrane strengthening. RC extract contains vitamins A, B and C all of which have protective roles against oxidative damage (30, 31, 42). Our results indicate that RCE is beneficial as a protective agent against oxidative stress induced by hyperthyroidism in rabbits. The protection is probably due to multiple mechanisms involving free radical scavenger properties, attenuating lipid peroxidation and increasing the antioxidant status. However, further studies are essential to elucidate the exact mechanisms of protection by red cabbage extract.

References


