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Evaluation of Antioxidants Effect of *Citrus reticulata* in *Schistosoma mansoni* Infected Mice

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Abstract: The antioxidant activity of flavonoid contents of *Citrus reticulata*. Baladi roots cultivated in Egypt, Family Rutaceae has been evaluated in six groups of healthy and infected mice with *Schistosoma mansoni*. Evaluation of the results was accomplished using a standard drug, Mirazid. Several antioxidant parameters were tested: lipid peroxide, glutathione (GSH), vitamin C (Vit C) and E (Vit E), catalase enzyme and liver function enzymes. *Schistosoma mansoni* infection showed a drastic changes of all the parameters under investigation. Treatment with *Citrus reticulata* and Mirazid showed amelioration in the hepatic antioxidant parameters; LP, GSH, Vit. C, Vit. E, Catalase enzyme as well as liver function enzymes; aspartate and alanine aminotransferases (AST and ALT) and alkaline phosphatase (ALP).

Key words: *Citrus reticulata*, roots, flavonoids, antioxidant parameters, liver function enzymes

INTRODUCTION

Schistosomiasis is considered a wide-spread problem that affects Egyptians at different ages (El-Sayed *et al*., 1995). The chronic nature of the disease and its endemic property in Egypt affect both the patient and the society with regards to the cost of the treatment, especially in complicated cases (Mostafa *et al*., 1998).

Adult worms that usnally reside in portal and mesenteric venules of the host lay large number of eggs that are trapped in hepatic and portal venules causing granulomatous inflammatory reactions followed by a characteristic pattern of hepatic fibrosis (Von Brand, 1979). Free radicals have been implicated in a number of diseases, such as cardiovascular and neurodegenerative diseases, cancer, viral infections (AIDS) and parasitic disease (Gharieb *et al*., 1999; Sanchez *et al*., 2000). In various reports concerning *Schistosoma mansoni* (Pascal *et al*., 2000), it was postulated that lipid peroxidation was elevated in both serum and liver of man and mice infected with *S. mansoni* which revealed an increase in free radicals. Several studies showed changes in enzymatic and non enzymatic antioxidants in the liver and serum of human and mice infected with *S. mansoni* (Sheweita *et al*., 1998; Yousif and El-Regal, 2004). Also several natural extracts were reported to possess antioxidant properties and the antioxidant activity of plants is responsible for their therapeutic effect against cancer, cardiovascular disease and diabetes (Anderson *et al*., 2004). Polymethoxylated flavonoids and Nobiletin, specifically those occurring in citrus, showed antiinflammatory and antitumor effects (Gharieb *et al*., 1999), while citrus antioxidant auraptene, isolated from citrus fruits, was proved to be a potential chemopreventive agent against N, N-diethylnitrosamine-induced hepatocarcinogenesis in rats (Sakata *et al*., 2004). Chalcones, which are a rare class of substances isolated from fruit exudates of *Myrica gale* L., showed inhibition of the initiation of lipid peroxide by inhibited superoxide on ion production (Sheweita *et al*., 1998).

Orange and grape fruit juice are known to increase protein oxidation biomarker 2-aminoadipic semialdehyde and hepatic quinine reductase activity. Interruption of the orange-rich diets, for a

month led to the disappearance of the abnormal coloration of the skin and serum levels of carotene and vitamin A became normal (Pascal *et al*., 2000). Grape fruit juice was reported to possess nutritive value as well as antigenotoxic and antioxidant effects by reducing lipid peroxide in mice liver (Gonzalez *et al*., 2004; McCord, 1986).

This study was undertaken to evaluate liver function enzymes and the antioxidant activity of the flavonoid content isolated from *Citrus reticulata* roots.

MATERIALS AND METHODS

Chemicals

All chemicals used are of high analytical grade, Sigma (USA), Merck and Reidel (Germany) and BDH (England). Mirazid (the oleo-resin extract from Myrrh of *Commiphora molmol* tree, Family: Burseraceae) was donated by Pharco Pharmaceutical Company, Egypt.

The dosages of the administered agents were: Mirazid: Two oral doses (600 mg kg^{-1}) purified commiphora extract for 3 consecutive days on empty stomach, at least one hour before eating (Haridy *et al*., 2003).

Citrus Reticulata

Ethanolic extracts of citrus plants were prepared by Natural product Dep. National Research Centre. Oral doses of 10 μg mL^{-1} mouse for 3 consecutive weeks were given daily eight weeks post infection (Nogata *et al*., 2001; Manthey and Guthrie, 2002).

Animals

Forty eight male mice provided by lab-bred colony of similar age and weight (18-20 g) were selected for this study. They were obtained from Schistosome Biological Supply Program (SBSP), Theodor Bilharz Institute, Cairo, Egypt. Animals were kept in a controlled environment and were allowed free access to diet and water during the study.

Plant Material

Citrus reticulata Blanco cuv. (Baladi Rutaceae roots) were collected from Modereyet El Tahrir, Behera, Egypt in December 2002. They were authenticated by Dr. Mohamed Abd El Ghaffar, Faculty of Agriculture, Al-Azhar University, Egypt. A voucher specimen is deposited at the Dept. of Natural P, NRC, Dokki, Cairo, Egypt.

Extraction and Isolation

Air-dried, powdered roots of *C. reticulata* (0.85 kg) were extracted with 80% EtOH. The ethanolic extract was evaporated and the aqueous residue extracted sequentially thrice with equal volumes of n-hexane, Et_2O, EtOAc and n-BuOH. The EtOAc extract was evaporated to dryness. The residue monitored by TLC using precoated silica gel 60 F254 aluminium sheets (0.2 mm tickness, Merck) and it was found to contain flavonoids. The phenolic residue was subjected to bioassay testing.

Experimental Design

Animals were divided into six groups, each of 8 animals. Group 1: served as normal healthy control. Group 2: Normal mice administrated citrus extract orally to show its side effect. Group 3: Normal mice administered Mirazid (Purified *Commiphora molmol* extract) orally to show its side effect. Group 4: *Schistosoma mansoni* infected mice with 100 cercariae by tail immersion method (Oliver and Stirewalt, 1952) and sacrificied after 2 months. Group 5: infected mice treated with *Citrus reticulata* extract. Group 6: infected mice treated with Mirazid.

Preparation of Tissue Homogenates

The livers were separated from the mice, plotted with a filter paper and weighed. 20% homogenate was prepared from the liver in bi-distilled water using Potter Elvehjem homogenizer with Teflon pestle.

Biological Estimation

- Estimation of liver total protein was carried out according to the method of Bradford (1976).
- *Lipid peroxide* was determined according to the method of Buege and Aust (1978).
- *Glutathione* (GSH) was estimated by Moron *et al.* (1979).
- *Vitamin C* was estimated by the method adapted by Jagata and Dani (1982).
- *Vitamin E* was measured by colorimetric assay (Angustin *et al.*, 1985).
- *Catalase activity* was assayed spectrophotometrically by Nelson and Kiesow (1972).
- Alanine and aspartate aminotransferases were determined according to the method of Bergmeyer *et al.* (1974).
- Alkaline phosphatase is determined according to the method of Belfield and Goldberg (1971).

Statistical Analysis

Data are expressed as mean±SD. Statistical significance values were determined by one way analysis of variance (ANOVA) accompanied by post- hoc (SPSS Computer Program).

RESULTS

Table 1 shows the levels of LP, GSH, Vit C and E and catalase enzyme activity in control (G1), infected (G4) and infected-mice-treated with *Citrus reticulata* roots extract (G5) and infected treated with Mirazid (G6). The levels of lipid peroxide showed a significant increase in G4 as compared to normal control group, while the other antioxidants showed a significant decrease. After treatment of the normal healthy mice with *Citrus reticulata* (G2) and Mirazid (G3), no change in the LP was shown but the extract of *Citrus reticulata* only showed significant change in both Vit E and catalase. Mirazid healthy control-treated group (G3) recorded significant increase in glutathione, Vit C and catalase, while it showed significant decrease in case of Vit E. Infected treated mice with *C. reticulata* (G5) showed significant increase in all antioxidant parameters except catalase which showed significant decrease. On

Table 1: Antioxidant activity of flavonoids content of *Citrus reticulata* roots

Parameters	Negative control G1	Control treated with citrus reticulate roots G2	Control treated mirazid G3	Infected G4	Infected treated with citrus reticulata roots G5	Infected treated Mirazid G6	ANOVA
Lipid peroxide nmol/mg protein	0.68±0.02 (4,5,6)	0.70±0.04 (3,4,5,6)	0.65±0.07 (2,4,5,6)	2.01±0.11 (1,2,3,5,,6)	1.11±0.09 (1,2,3,4,6)	0.71±0.09 (1,2,3,4,5)	0.001
Glutathione µg/mg protein	48.71±1.49 (3,4,5,6)	51.56±2.88 (3,4,5,6)	58.15±3.43 (1,2,4,5,6)	19.42±1.75 (1,2,3,5,6)	72.82±0.87 (1,2,3,4,6)	29.15±3.05 (1,2,3,4,5)	0.001
Vitamin C µg/mg protein	9.18±0.13 (3,4,5)	8.96±0.42 (3,4,5)	11.20±0.71 (1,2,4,5,6)	7.46±0.67 (1,2,3,5,6)	10.22±0.64 (1,3,4,6)	8.71±0.82 (1,3,4,5)	0.001
Vitamin E µg/mg ptotein	2.53±0.14 (2,3,4,5,6)	3.72±0.13 (1,3,4,5,6)	1.80±0.10 (1,2,4,6)	1.45±0.07 (1,2,3,5,6)	5.77±0.20 (1,2,3,4,6)	1.75±0.19 (1,2,4,5)	0.001
Catalase µM/mg ptotein	9.53±0.27 (2,3,4,5,6)	7.24±0.19 (1,3,5,6)	11.68±0.24 (1,2,4,5,6)	7.45±0.39 (1,3,5,6)	7.49±0.27 (1,2,3,4,6)	8.44±0.34 (1,2,3,4,5)	0.001

Data are mean±SD, Analysis of data is carried out by one way (ANOVA) (Analysis of Variance) accompanied by post hoc (SPSS Computer Program)

Table 2: Effect of *C. reticulata* and mirazid on liver function enzymes in mice

Enzymes	Negative control G1	Control treated with citrus reticulata G2	Control treated mirazid G3	Infected G4	Infected treated with citrus reticulata G5	Infected treated mizard G6	ANOVA
Aspartate aminotransferase	40.21±2.66 (4,5,6)	38.50±2.62 (4,5)	39.40±2.14 (4,5)	26.11±1.98 (1,2,3,5,6)	36.50± 2.33 (1,2,3,4,6)	38.00±2.68 (1,4,5)	0.0001
Alanine aminotransferase	26.50±1.16 (2,4,5,6)	24.70 ±1.12 (1,3,4,5)	25.96±1.24 (2,4,5,6)	14.27±0,98 (1,2,3,5,6)	21.50±1.13 (1,2,3,4,6)	24.60±1.22 (1,3,4,5)	0.0001
Alkaline phosphatase	4.76±0.22 (4,5,6)	4.90±0.23 (4,5,6)	4.85±0.22 (4,5,6)	7.18±0.21 (5,6)	6.22±0.24 (6)	5.76±0.23 (4,5)	0.0001

Data are means±SD of eight mice in each group, All values are expressed as mol/min/mg protein, Statistics is carried out using ANOVA test and the difference between groups is analyzed by Post-Hoc (SPSS Computer Program)

the other hand, infected mice treated with Mirazid recorded significant decrease in all antioxidants except lipid peroxide which show significant increase.

Table 2 demonstrates significant reduction in AST and ALT, while a significant increase in ALP was recorded after bilharzial infection. *S. mansoni* infected mice treated with *C. reticulata* and Mirazid show enhancement levels in liver enzymes. Healthy control mice administered with both extracts recorded insignificant change.

DISCUSSION

The hepatic antioxidative defense system may be one of the protective mechanisms of the body against oxidative tissue damage caused by *Schistosoma mansoni* infection (Yousif and EL-Rigal, 2004). The data obtained in the present study showed that LP were elevated in the liver of the infected mice (G4). The complex mechanism of lipid peroxidation is known to require the participation of highly reactive oxygen and other reactive oxygen metabolites in the chain of biochemical reactions. Thus, in any part of the body where free radicals are produced, LP are in turn increased (Campbell *et al*., 1999). This is in agreement with the present data. Moreover, several studies reported that oxidative stress due to bilharziasis causes an elevation in lipid peroxides (Pascal *et al*., 2000; Cui *et al*., 2000).

GSH content in the liver of the infected mice showed a significant reduction; this is in agreement with several previous reports revealing that *Schistosoma mansoni* caused reduction in the content of GSH of the liver (Gharieb *et al*., 1999; Yousif and El-Rigal, 2004). Such depletion has been caused by increased cytotoxicity of H_2O_2 in endothelial cells, resulting from inhibition of GSH reductase and keeping GSH in its reduced state. Also, the present data showed a reduction in the content of both vit. C and E in the liver of the infected mice which occurred due to scavenging the free radicals formed, by *Schistosoma mansoni* (Rizk, 1998; Yousif and El- Rigal, 2004). Peroxyl radicals are effectively trapped by ascorbate (Frei *et al*., 1988; Goncalves *et al*., 2005). The data showed a reduction in the hepatic catalase of the infected mice, as reported in the studies with H_2O_2 (Pascal *et al*., 2000; Yousif and El-rigal, 2004). Treatment of the infected mice with *Citrus reticulata* extract and Mirazid ameliorated the levels of the hepatic antioxidants to a great extent. Lipid peroxides were greatly reduced. GSH, levels of Vit C, E and catalase activity were increased.

It is noteworthy to mention that the antioxidant activity of *Citrus reticulata* roots may be due to the presence of flavonoids, the potent antioxidants (Lopez-Revuelta, 2006). Chalcones showed inhibition to the initiation of lipid peroxide by inhibited superoxide anion production (Sheweita *et al*., 1998). It seems that the presence of 4'-hydroxyl group enhanced activity in chalcone (Chacha *et al*., 2005). The chemical structures of the isolated components included one or more aromatic rings bearing hydroxyl groups, these are phenolic which are easily oxidized to quinone and reduced back to phenols that are potentially able to act as reducing agents, as hydrogen donating antioxidants and as singlet oxygen quenchers (Goncalves *et al*., 2005). The polymethoxylated flavonoids are important bioactive compounds that show anti-inflammatory and antitumor activities (Roman *et al*., 2005).

It is concerned to study transaminases enzyme activities which showed a significant decrease after infection. El-Aasar *et al.* (1989) attributed the decrease of transaminase enzyme activities in mice livers to the decrease in hepatic cell population due to liver fibrosis or due to the release of the enzyme from the damaged livers into the circulation as a result of increased cell membrane permeability. The observed diminution of AST was more manifested than that of ALT denoting that, although the later is more specific for liver cells, yet it is less sensitive than AST in detecting liver cell damage (Awadalla *et al.*, 1975). Moreover, the presence of considerably more AST in human hepatic tissue indicated that the released ALT is too diluted in the extracellular compartment to cause significant increase in the ALT activity in *S. mansoni* patients. Therefore, variations in the release, destruction or excretion of the two enzymes or an unknown metabolism aberration are probably important contributory mechanisms (Salah *et al.*, 1976).

In the present study, ALP enzyme activity in infected mice showed a significant increase. Awadalla *et al.* (1975) and El-Aasar *et al.* (1989) observed an elevation in ALP activity in murine liver after *S. mansoni* infection. They attributed the increase in enzyme activity to the irritation of the liver cells by toxins or metabolic products of growing schistosomules, adult worms and eggs or due to increased loss of intracellular enzyme by diffusion through cell membranes which appears to act as a stimulus to the synthesis of more enzyme protein. Higher rates of formation would, in turn, increase the rate of diffusion and hence increase serum activity (Wilkinson, 1962). Abdel-Rahman *et al.* (1993) mentioned a significant rise in liver ALP isoenzyme in patients having hepatosplenic schistosomiasis. Mansour *et al.* (1982) added that the elevation of ALP enzyme activity in *S. mansoni* infected human is of intestinal origin especially since *S. mansoni* is a disease which primarily affected the intestine, while this elevation is not of hepatic origin as it is observed in both patients of *S. mansoni* and hepatosplenomegaly disease.

In conclusion, the ethanolic extract of citrus roots containing flavonoid showed amelioration in LP, GSH, Vit C and Vit E as well as liver function enzymes. These findings are confirmed by the previous results of El-Rigal and Hetta (2006) who found a significant reduction in ova count and worm burden of *Schistosoma mansoni* infected mice treated with *C. reticulata* extract, pointed out that toxic substances and free radicals elaborated from *S. mansoni* worms consume antioxidants and may affect the capacity of the liver to detoxify or neutralize the effect of the toxic endogenous and exogenous compounds, suggesting that *Citrus reticulata* roots possessed antioxidant activity.

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