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Oxidative profile in patients with colon cancer: effects of *Ruta chalepensis* L.

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Abstract. – *Aim:* **To verify the involvement of free radicals in tumor progression and to investigate the effects of an ethanolic extract of *Ruta Chalepensis* L. and of rutin in blood of patients with colon cancer.**

Materials and Methods: **Leaves of *Ruta Chalepensis* L. were collected in the area around Catania (Italy). For the preparation of the ethanol extract of leaves, an exhaustive extraction of 100 g of the drug was carried out in Soxhlet with 800 ml of 95% ethanol.**

Fifty-six patients with colorectal cancer were randomly selected for this study; among these, 34 were affected by an early stage (T1 N0 M0 according to scale), while 22 were affected by an advanced stage (T4, N1-2, M0) of cancer. Data obtained from these patients were compared with those of a control group consisting of 20 healthy subjects. Plasma of each sample was used for determining non-proteic antioxidant capacity, thiol groups, lipid hydroperoxides and nitrite/nitrate levels, evaluated by spectophotometric tests. In addition, percentage of haemolysis was evaluated incubating (for 2 hours at 37° C) erythrocyte suspension with a free radical donor (50 mM 2,2'-azobis-amidino propane chloridrate), in the presence or absence of ethanolic extract of *Ruta Chalepensis* L. (250 µg/ml) or rutin (1 mM).

Results: **Non-proteic antioxidant capacity was significantly lower in cancerous patients than in healthy subjects ($p<0.001$). This decrease was stage-related. In fact, non-proteic antioxidant capacity resulted lower in advanced than in early colorectal cancer ($p<0.001$). The same significant stage-related decrease was observed in plasma thiol groups ($p<0.001$). Coherently with the decrease in non-proteic antioxidant capacity and thiol groups, higher levels of lipid hydroperoxides and nitrite/nitrate were observed in patients with colorectal cancer with respect to healthy subjects ($p<0.001$) and the increase in these markers of oxidative stress was related to the cancer stadiation. Neoplastic patients also showed an increased percentage of oxidative hemolysis respect to controls and the haemolytic damage was correlated with the stage of colon cancer.**

Both the extract of *Ruta Chalepensis* L. and rutin were able to protect erythrocytes from oxidative stress induced by the free radical donor, but the extract of *Ruta Chalepensis* L. was more effective than rutin. This protective effect was significant only in erythrocytes from patients with early colorectal group, whereas no significant modification was induced by *Ruta Chalepensis* L. or rutin in red blood cells from advanced colorectal cancer patients exposed to the same experimental conditions.

Conclusion: **Oxidative stress correlates with colon cancer stadiation and both the extract of *Ruta chalepensis* and rutin are able to protect red blood cells from radical-induced damage. However, their effects are significant in early stages of cancer. So these natural antioxidants might be usefull to prevent carcinogenesis and/or tumor progression.**

Key Words:

Colon cancer, Oxidative stress, Reactive oxygen species, Hemolysis, *Ruta chalepensis*, Rutin.

Introduction

Colon cancer is the second leading cause of death in both men and women in the Western World. Worldwide, there are an estimated 450.000 new cases each year. The Surveillance,

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Epidemiology and End Results Program (SEER) data predicts a five-year relative survival rate of 63% for the US white population and 53% for black patients with colon cancer.

European and Indian tumor registries report a significantly lower 5 year survival of 41% and 42% respectively[1,2].

Several factors have been implicated in this carcinogenic transformation such as oxidative stress, immunity, chronic inflammation and carcinogenesis[3-7].

In fact, it has been demonstrated that inflammatory cells are particularly effective in generating oxygen-derived oxidants[8]. The possibility that chronic inflammation poses a risk for cancer in men is inferred from considerable clinical experience indicating human malignancies often occur at sites of ongoing chronic inflammation as well as from a number of recent experimental observations[9].

A large body of evidence suggests important roles for reactive oxygen species (ROS) in the expansion of tumor clones and the acquisition of malignant properties[4,10,11].

Overproduction of ROS could cause an imbalance between oxidative and antioxidative processes. ROS can damage lipids, proteins, and nucleic acids thereby initiating apoptosis or necrosis. Several studies reported that free radicals are potentially the cause of hundreds of different types of chemical changes in DNA which could potentially be mutagenic and involved in the etiology of cancer[12].

Evidence indicates that DNA damaging hydroxyl radicals ($OH^{\cdot}$) are produced through the interaction of hydrogen peroxide (H_2O_2) and superoxide radical with transition metals. In fact, metal chelators blocking the formation of $OH^{\cdot}$ can inhibit DNA damage and malignant transformations induced by ROS in cell-free and cellular systems. Thus, in biological systems, a diversity of antioxidant defense systems operate to control excessive levels of ROS[13-15]. The colon is constantly subject to a wide variety of chemical and biological insults and has evolved complex and varied systems of defense. Xenobiotic compounds are essentially detoxified by highly aerobic mature colonocytes at the crypt surface. Free radical generating mechanisms in the mature colonocyte may yield oxygen radicals which permeabilize capillaries and allow genotoxins to reach stem cells[16].

Due to their free radical scavenging properties, micronutrient antioxidants are potential chemopreventive agents against colon cancer, yet little is known about the real concentration of these antioxidants in colonic mucosa. Antioxidants such as tocopherols and carotenoids, effective scavengers of free radicals present in colon mucosa, have been considered natural chemopreventive agents against colon cancer[17-19].

Antioxidants may directly scavenge some radical species, may suppress lipid peroxidation by recycling other antioxidants such as α-tocopherol, by donating a hydrogen atom to the tocopherol molecule or may chelate metal ions such as iron and copper, thereby preventing free radical formation from these pro-oxidants[20-22].

A variety of antioxidants can be introduced through diet (high levels are found in certain food plants) and a large number of polyphenols are effective antioxidants against free radicals that initiate lipid peroxidation in human red blood cells and in rat liver microsomes[23].

It is known that plants may be used both as a medicine and a food and it is difficult to draw a line between these two categories: food may be medicine, and *vice versa*. For example, many studies on the potential health benefits of traditional foods show that such plants have specific pharmacological effects. Today, the pharmacological properties of these plants and of the constituents isolated from them are of particular interest.

It has been reported that the herb *Ruta chalepensis* L. (Rutaceae), a perennial herb that usually grows on rocky slopes, is characterized by glabrous, alternate bi-pennatisect leaves with narrow oblong-lanceolate or obovate segments and cymose inflorescence.

In Saudi Arabia, a decoction of the aerial parts of the plant is used as an analgesic and antipyretic and for the treatment of rheumatism and mental disorders. The plant is prescribed in the Indian system of medicine for the treatment of dropsy, neuralgia, rheumatism and menstrual and other bleeding disorders. In China, a decoction of the roots of the plant is used as an anti-venom. This hints at a putative anti-inflammatory property of *Ruta chalepensis* L[24].

Phytochemical screening of *Ruta* species has characterized the presence of alkaloids, flavonoids, coumarins, tannins, volatile oils, glycosides, sterols and triterpenes as possible active constituents[25-29].

In particular, several pharmacological properties of *Ruta chalepensis* L. are attributed to the high content of alkaloids, flavonoids, phenols, amino acids, furocoumarins and saponins found in the leaves and young stems of the plant[30-33].

These show a broad range of biological activity and a number of them are used in medicine[28]. In fact, flavonoids, glycosides and tannins are considered potent inhibitors of pro-inflammatory signaling molecules[29-30]. *Ruta chalepensis* L. contains rutin, a flavonoid, diglycoside of quercetin that can be hydrolyzed to quercetin in the gastrointestinal tract.

Recently, several studies demonstrated that rutin and quercetin have a nitric oxide scavenging activity[34] and antioxidant properties with involvement in heme oxygenase 1 (HO-1) up-regulation and NO-mediated pathways[15,34,35-37]. In fact, recent *in vivo* studies reported that rutin decreased nitric oxide (NO) production by inhibiting inducible nitric oxide synthase (iNOS) protein expression and without altering cytokine levels[30,38].

Moreover, our previous study reported the protective *in vivo* effect of rutin against hepatic ischemic/reperfusion injury and suggested that it might be due both to its scavenger capacity and to its ability to induce HO-1 expression and inhibit iNOS expression[37].

The present study evaluates non-proteic antioxidant capacity, thiol groups, lipid hydroperoxide, nitrite/nitrate levels in plasma of human subjects affected by colon cancer. In addition, given that *Ruta chalepensis* L. is used in traditional medicine to treat many inflammation diseases and that chronic inflammation poses a risk for cancer in men, in the present study the effects of the ethanolic extract of *Ruta chalepensis* L. and rutin against free radical-initiated hemolysis of human red blood cells (RBCs) induced by 2,2'-azobis-amidino propane chloridrate (AAPH) was also evaluated[39,40].

Materials and Methods

Chemicals

Rutin, nicotinamide-adenine dinucleotide reduced form (NADH), and xylenol orange were obtained from Sigma Aldrich Co. (St. Louis, MO, USA). 2,2'-azobis-amidino propane chloridrate (AAPH) was obtained from Trimital (Milano, Italy). All other chemichals were from Merck (Frankfurt, Germany).

Plant Material

Leaves of *Ruta chalepensis* L. were collected in the area around Catania (Italy) in April 2009. The fresh material was lyophilized and powdered.

Ruta chalepensis L. specimens were obtained thanks to the Regional Forest Corps Detachment of Catania-Nicolosi and authenticated by botanist Prof. S. Ragusa, Department of Pharmacobiological Sciences, University of Catanzaro, Catanzaro, Italy. The fresh material was air-dried and powdered. A voucher specimen of the plant was deposited in the Herbarium of the Pharmaco-biological Department of the University of Messina.

Preparation of Plant Extract

For the preparation of the ethanol extract of leaves, an exhaustive extraction of 100 g of the drug was carried out in Soxhlet with 800 ml of ethanol 95%. The extract was then dried under vacuum. The residue of the ethanol extract of the leaves was 26 g. One gram of ethanol extract of *Ruta chalepensis* L. was solubilized in sterile 2 ml polyethylene glycol and 3 ml olive oil.

Analysis of Rutin in the Extract by Reverse Phase HPLC

Forty micrograms per milliliters of rutin (Sigma) dissolved in absolute methanol (HPLC grade) was scanned at 200-900 nm (U2000 Hitachi spectrophotometer Tokyo, Japan) and the λ_{max} of rutin was determined at 207 nm. The same wavelength was used to analyze the extract by reverse phase HPLC (Schimadzu LC4A with UV detector and C-18 ODS column, 15 cm long with 4.6 mm diameter, Kyoto Japan) using absolute methanol as eluent. The retention time for rutin at l= 207 nm was 4.3 min and a peak at the similar retention time was observed in the extract. Rutin was added to the test extract as an internal standard to confirm that the peak observed in the text extract corresponds to rutin. The percent composition of rutin in the plant extract was calculated by comparing the area covered under the peak of interest with the area observed for known concentrations of standard rutin. Peaks for the other compounds were also observed but they were not analyzed in the present study.

Patient Selection

This study was approved by Ethical Committee of the University of Catania, Italy. All subjects gave their written consent before inclusion in the study.

Fifty-six patients (30 males and 26 females) with colorectal cancer, having an average age of 60 years (range 36-84), were randomly selected for this study. Of these, 34 were affected by an early stage (T1 N0 M0, cancer has begun to

spread, but is still in the inner lining) while 22 were affected by an advanced stage (T4, N1-2, M0, cancer has spread to lymph nodes, but has not been carried to distant parts of the body). Results obtained were compared with those of a control group consisting by 20 healthy subjects (similar age).

Sample Preparation

Heparinized venous blood was collected after overnight fasting. Plasma was separated by centrifugation at 800 g for 10 min. The packed erythrocytes were then washed three times with cold phosphate buffered saline (PBS). The washed and packed erythrocytes were resuspended in appropriate buffer to yield a suspension with a final concentration of 2.5% and employed for hemolytic assay, as described below.

Plasma samples were immediately analyzed for total thiol groups, lipid hydroperoxide and nitrite and nitrate levels. Aliquots of plasma destined for non-proteic antioxidant capacity were frozen at –80°C and analyzed within 3 days.

Non-Proteic Antioxidant Capacity

Non-proteic antioxidant capacity (NPAC) of human plasma was evaluated measuring its free-radical scavenging ability. Superoxide anion was generated *in vitro* as described by Russo et al[35]. The assay mixture contained, in a total volume of 1 ml: 100 mM triethanolamine-diethanolamine buffer (pH 7.4), 3 mM NADH, 25 mM/12.5 mM EDTA/$MnCl_2$, 10 mM β-mercapto-ethanol and ethanolic extract of plasma. After 20 min incubation at 25°C, the decrease in absorbance at λ=340 nm was measured spectrophotometrically. Results are expressed as percentage of inhibition of NADH oxidation.

Thiol Group Determination

Thiol groups were measured, in 200 µl of plasma, using a spectophotometric assay based on the reaction of thiols with 2,2-dithio-bis-nitrobenzoic acid (DTNB) at λ=412 nm[41]. Results were calculated using an aborptivity of 13,600 cm^{-1} M^{-1} and expressed as µmol/ml plasma.

Determination of Lipid Hydroperoxide Levels

Lipid hydroperoxide levels were evaluated by oxidation of Fe^{+2} to Fe^{+3} in the presence of xylenol orange at λ= 560 nm[43]. The assay mixture contained, in a total volume of 1 ml: 100 µl of plasma, 100 µM xylenol orange, 250 µM ammonium ferrous sulfate, 90% methanol, 4 mM butylated hydroxytoluene, 25 mM H_2SO_4. After 30 min incubation at room temperature and removal of any flocculated material by centrifugation, the absorbance at λ=560 nm was measured using a U2000 Hitachi spectrophotometer. Calibration was obtained using hydrogen peroxide (0.2-20 µM). Results are expressed as nmol/ml plasma.

Determination of Nitrite and Nitrate Levels

Plasmatic nitrite and nitrate (NO_2^-/NO_3^-) concentrations were determined with Griess reagent at λ=540 nm[42]. Plasma was filtered through a centrifugal-driven 10,000 molecular weight cut-off cellulose membrane filter (Ultrafree-MC 10,000, Millipore, Bedford, MA, USA) at 10,000 g for 1h at room temperature to remove protein and hemoglobin. Calibration was obtained using known amounts of KNO_2/KNO_3.

Hemolytic Assay

Erythrocyte suspension was incubated with 50 mM 2,2'-azobis-amidino propane chloridrate (AAPH), in presence or absence of ethanolic extract *Ruta chalepensis* L. (250 µg/ml) (10 µl/ml) or rutin (1 mM) for 2 hours at 37°C. After centrifugation at 200 rpm for 2 min, the concentration of hemoglobin released in the supernatant was measured at λ=408 nm[40]. The hemolytic effect was expressed as percentage of hemolysis. Reference samples were obtained by adding 10 µl of non-azide buffer solution, or 10 µl of absolute ethanol or 10 µl bidistilled water to 990 µl of 1.5% erythrocyte suspension (0% and 100% hemolysis, respectively).

Statistical Analysis

One-way analysis of variance (ANOVA) followed by Bonferroni's *t* test was performed in order to estimate significant differences among groups. Data were reported as mean values ± S.D. and differences between groups were considered to be significant at $p<0.001$.

Results

Patients with Early and Advanced Colon Cancer

Data obtained in this study demonstrated that NPAC (non-proteic antioxidant capacity) was significantly lower in subjects affected by colon

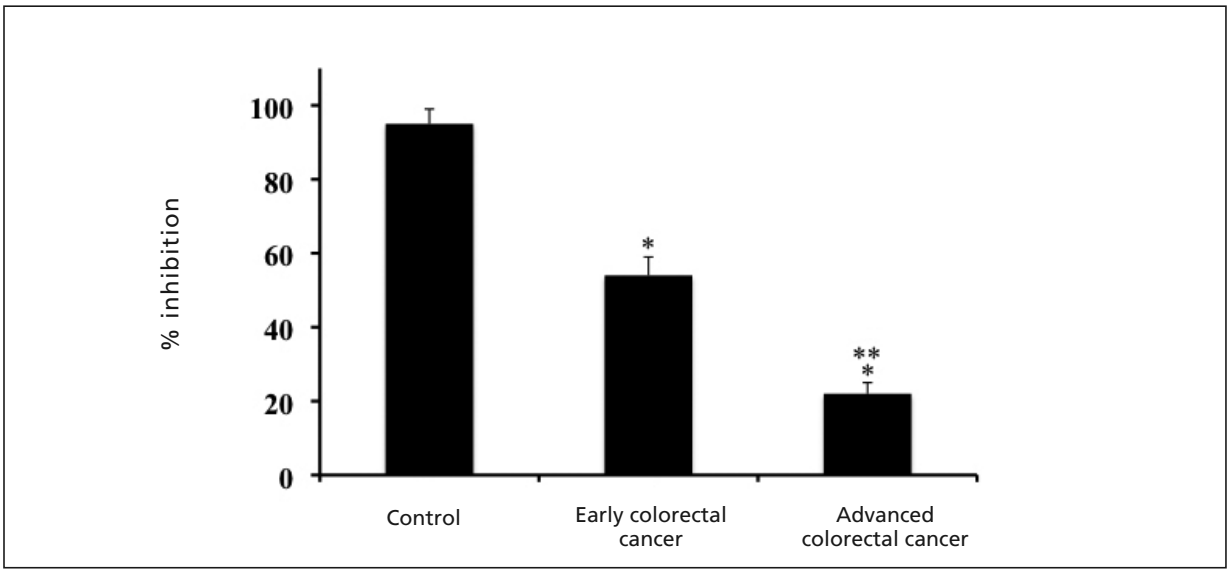

Figure 1. NPAC in plasma of healthy subjects (control) and of colon cancer patients (early and advanced colorectal cancer). Values are means ± SD of 4 determinations for each subject. *Statistically significant vs control, $p<0.001$; **Statistically significant vs early colorectal cancer, $p<0.001$).

cancer when compared to healthy subjects. In particular, NPAC is correlated with the stage of colon cancer, in fact it was significantly lower in T4, N1-2, M0 colorectal cancer patients than in T1, N0, M0 group (Figure 1). In patients with colon cancer the decrease in NPAC was accompanied by a significant decrease in thiol groups (Figure 2) respect to control group. Also this parameter was stage-dependent; in fact, thiol groups resulted lower in patients with advanced rather than in early colorectal cancer.

Coherently with the impairment of these antioxidant defenses, plasma LOOH (lipid hydroperoxide) levels were significantly higher in colon cancer patients than in healthy subjects and the higest levels were observed in advanced colorectal cancer (Figure 3). In patients with colon cancer a significant increase in NO_2^-/NO_3^- levels was observed (Figure

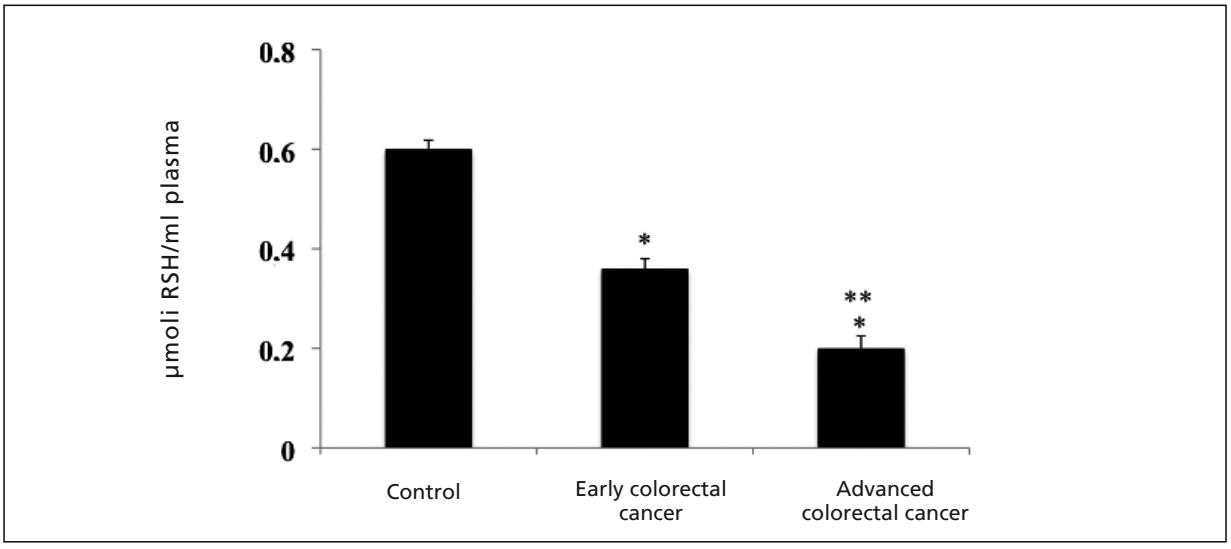

Figure 2. Total thiol groups (RSH) in plasma of healthy subjects (control) and of colon cancer patients (early and advanced colorectal cancer). Values are means ± SD of 4 determinations for each subject. *Statistically significant vs control, $p<0.001$; **statistically significant vs early colorectal cancer, $p<0.001$).

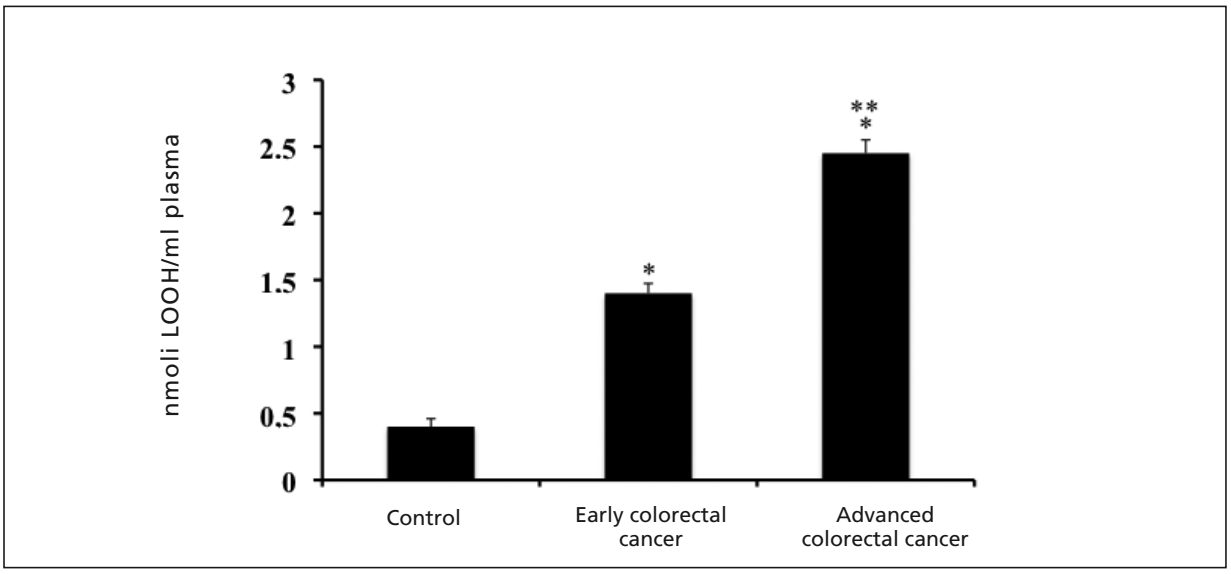

Figure 3. Lipid hydroperoxide in plasma of healthy subjects (control) and of colon cancer patients (early and advanced colorectal cancer). Values are means ± SD of 4 determinations for each subject. *Statistically significant vs control, $p<0.001$; **Statistically significant vs early colorectal cancer, $p<0.001$).

4); according to the other measured parameters, also the increase in NO_2^-/NO_3^- levels were stage-dependent; in fact, their values were significantly higher in patients with T4, N1-2, M0 colorectal cancer respect to T1, N0, M0.

Results regarding the percentage of hemolysis are reported in Figure 5. In patients affected by colon cancer the percentage of hemolysis induced by the radical donor AAPH (2,2'-azobis-amidino propane chloridrate) resulted significantly higher than in red blood cells from control group; the susceptibility to oxidative hemolysis was higest in T4, N1-2, M0 group (Figure 5).

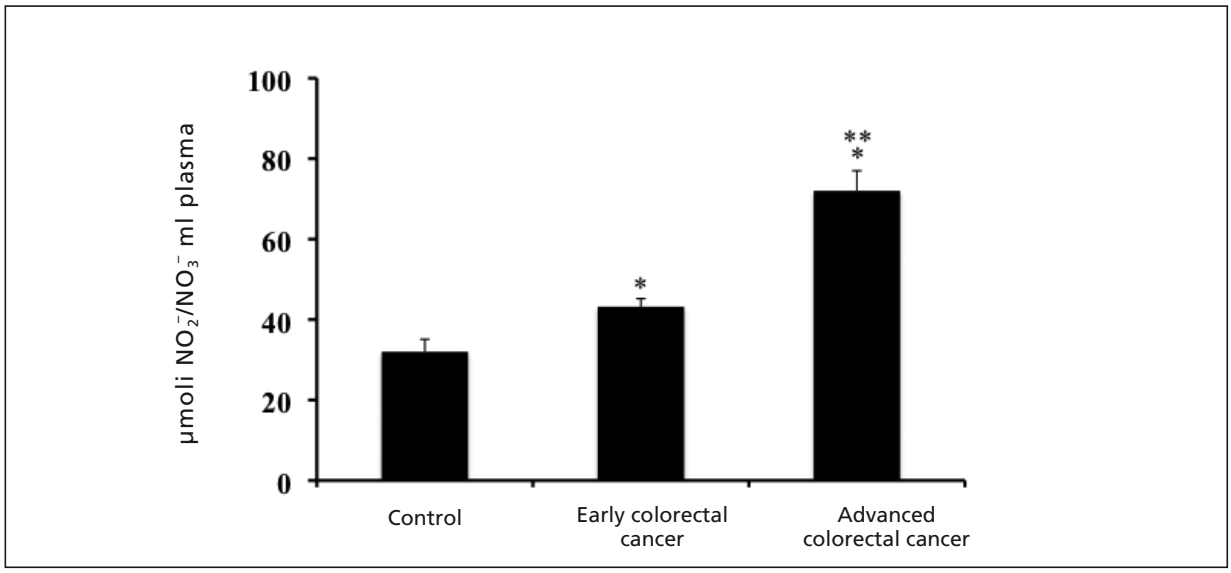

Figure 4. Nitrite and nitrate in plasma of healthy subjects (control) and of colon cancer patients. (Early and advanced colorectal cancer). Values are means ± SD of 4 determinations for each subject. *Statistically significant vs control, $p<0.001$; **statistically significant vs early colorectal cancer, $p<0.001$).

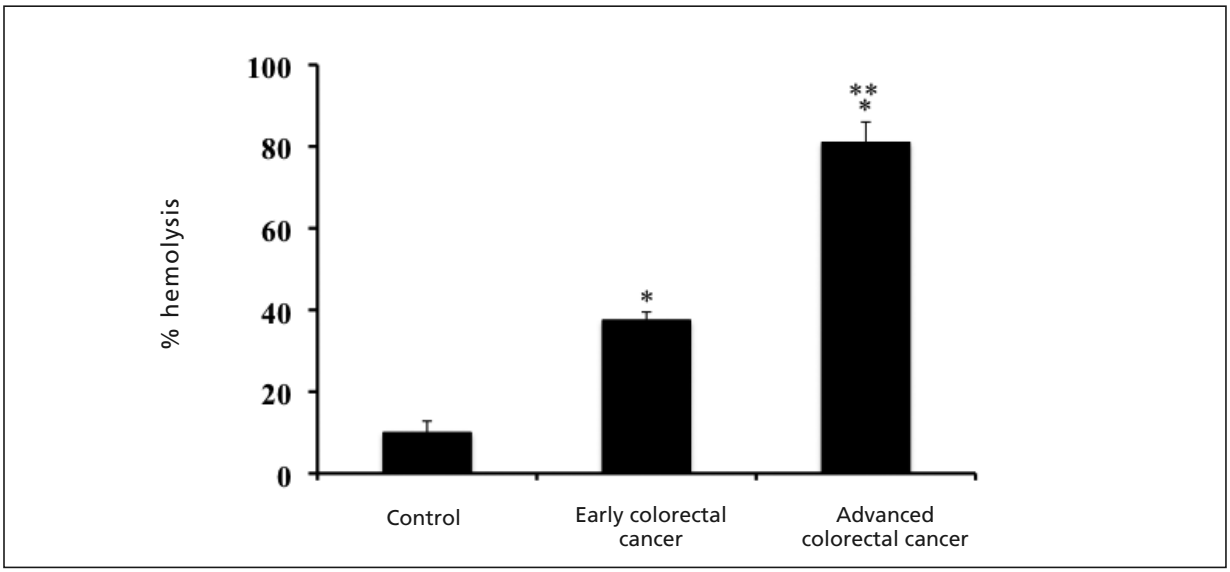

Figure 5. AAPH-induced hemolysis of human RBCs of healthy subjects (control) and of colon cancer patients (early and advanced colorectal cancer). Values are means ± SD of 4 determinations for each subject. *Statistically significant vs control, $p<0.001$; **Statistically significant vs early colorectal cancer, $p<0.001$).

Effect of rutin and the extract of Ruta chalepensis L.

Figure 6 reports how the extract of *Ruta chalepensis* L. and rutin are able to protect erythrocytes from oxidative stress induced by AAPH; in particular, the extract of *Ruta chalepensis* L. was more efficient than rutin. However it has to be noted that this effect is significantly evident only in T1, N0, M0 colorectal cancer patients, whereas neither *Ruta chalepensis* L. extract, nor rutin were able to protect erythrocytes from patients with advanced colorectal cancer.

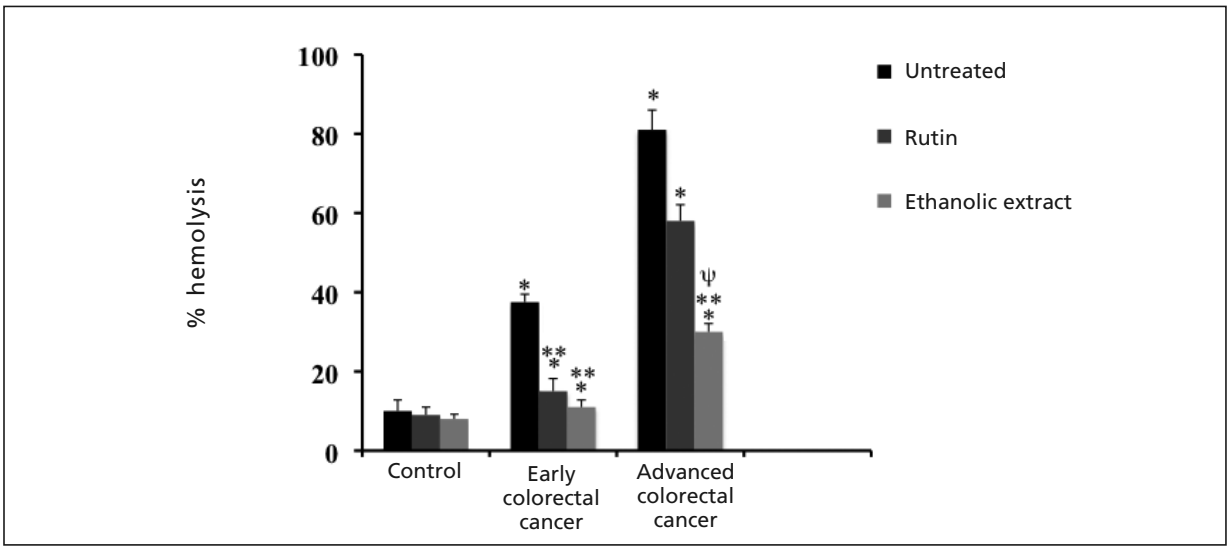

Figure 6. Effect of extract of *Ruta chalepensis* L. and of rutin against AAPH-induced hemolysis of human RBCs of healthy subjects (control) and of colon cancer patients (early and advanced colorectal cancer). Values are means ± SD of 4 determinations for each subject. *Statistically significant vs control group, $p<0.001$; **Statistically significant vs untretaed group; Ψ statistically significant vs rutin).

Discussion

In recent years there has been increasing interest in oxidative status and particularly in the pathologic role of free radicals which, mediating peroxidation of membrane lipids and oxidative damage to DNA, are associated with a variety of chronic health disorders, such as cancer, atherosclerosis, neurodegenerative diseases and aging[43,44]. Therefore, inhibition of oxidative damage by antioxidants became an attractive therapeutic strategy to reduce the risk of these diseases[45].

The inflammatory process has long been known to be a risk factor for human cancers, particularly those of the lung, bladder, colon and stomach[22]. The excessive production of nitric oxide (NO) by immune system cells is involved in the mechanism of carcinogenesis[46].

Nowadays, colorectal cancer is still a major health problem, particularly because of the number of patients affected each year. It is, therefore, important to find new, reliable markers enabling an early diagnosis of this pathology.

Results obtained in this investigation beside to confirm the correlation between oxidative stress and colorectal cancer, also demonstrated that a relationship between cancer stadiation and plasmatic antioxidant/oxidative status really exists. In fact, a direct correlation was observed between colon cancer stage and reduction in NPAC and/or thiol groups levels. The decrease in these antioxidant defenses is associated with elevated levels of lipid hydroperoxide and NO_2^-/NO_3^- in cancerous patients and these levels are significantly higher in advanced colorectal cancer than in early stage. Previously we observed that plasma thiol groups progressively decrease during malignant transformation[41] and it has been suggested that the tumor co-opts GSH, which represents the most prevalent intracellular thiol tripeptide and the main component of plasma thiols.

In addition, a decrease in antioxidant defenses may result in hemolysis of red blood cells which are susceptible to oxidative damage because of the high polyunsaturated free fatty acid content in their membranes and high cellular concentrations of oxygen and hemoglobin, a potentially powerful promoter of oxidative processes[39]. Even under normal conditions, erythrocytes exposed to ROS continuously generated from either internal or external sources, may be targets for oxidative damage. Thus the reduction of plasmatic antioxidant defenses induces hemolysis.

In patients affected by advanced colorectal cancer, the decrease of NPAC and thiol groups associated with the significant increase of lipid hydroperoxides and NO_2^-/NO_3^- levels render their erythrocytes more susceptible to hemolysis.

In this study, hemolysis was induced by APPH (2,2'-azobis-amidino propane chloridrate)which offers a good approach for studying free radical-induced membrane damage[23]. In fact APPH is a water-soluble azo-compound which can decompose at physiological temperatures to generate alkyl radicals which then initiate lipid peroxidation. Since lipid peroxidation is a free radical chain reaction and one initiating radical could induce up to twenty propagation reactions[39,47], the RBCs membrane is rapidly damaged, leading to hemolysis. On the other hand, if antioxidants such as Vitamine E, C and flavonoids, are present or added to red blood cells (RBCs) they react with the peroxyl radicals to stop the peroxidation and hence inhibit hemolysis.

In neoplastic patients an increased percentage of hemolysis compared to controls was observed. Moreover, the haemolytic damage resulted correlated to the stage of colon cancer.

The extract of *Ruta chalepensis* L., contains about 3.5% of rutin. The addition of rutin to RBCs suspension significantly decreased hemolytic damage in neoplastic patiens with respect to health donors. In particular, in the advanced colorectal group, the extract was observed to be more effective in reducing hemolytic damage as compared to rutin. This may be due to the presence of various flavonoids along with other natural anti-oxidants such as phenolic compounds and their derivatives that may predictably contribute to the observed decrease in hemolytic damage.

The hemolytic event results in the liberation of iron which, participating in Fenton-type reactions, contributes to further formation of ROS and aggravates the oxidative stress state and leads to anemia, a common complication of tumors.

There is now increasing interest in the *in vivo* protective function of natural antioxidants contained in dietary plants, against oxidative damage caused by free radical species[48,49].

The antioxidant activity of phenolic phytochemicals, has been widely investigated in recent years[21,35,50,51]. Many fruits and vegetables contain compounds that may protect against mutations and cancer through various mechanisms. In particular, in previous studies it was

reported that *Ruta chalepensis* L. prophylaxis probably inhibited the function rather than the synthesis of tumor necrosis factor (TNF), and the synthesis/action of other type 1 proinflammatory cytokines involved in the pathogenesis of lethal endotoxemia such as interleukin 1 (IL-1) and interferon-γ (IFN-γ)[30,52,53]. Pharmacological investigations clearly indicated that antineoplastic and anti-inflammatory activities in many plants have been attributed to their flavonoid contents[27].

Various studies suggest that diets rich in polyphenols may have beneficial effects on health. In fact these compounds, acting as antioxidants and free radical scavengers, may play a significant role in different diseases[48,54-55].

In conclusion results obtained in this study showed that ethanolic extract of *Ruta chalepensis* L. and rutin protect human RBCs of neoplastic patients from oxidative hemolysis. This confirmed that the protective effect of ethanolic extract of *Ruta chalepensis* L. and of rutin is due to its antioxidant and scavenger capacities. The inhibition observed for the extract was significantly higher than that observed for rutin. These data indicate that the extract possess interesting antioxidant properties due to the synergetic effect of all the components present. These findings also indicate the potential beneficial effect of ethanolic extract on hemolytic damage.

In view of their antioxidant properties and their capacity to modulate important anti-inflammatory signaling pathways, rutin and ethanolic extract of *Ruta chalepensis* L., could be utilized in clinical practice as potential natural agents against oxidative damage and consequently in neoplastic diseases, such as colon cancer.

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