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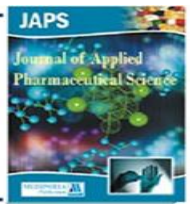

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Antioxidant response of *Camellia sinensis* and *Rosmarinus officinalis* aqueous extracts toward H_2O_2 stressed mice

Lahlou Fatima Azzahra , Hmimid Fouzia, Loutfi Mohammed and Bourhim Noureddine

ABSTRACT

Oxidative stress takes place when the balance between the antioxidant defenses and the generation of reactive oxygen species (ROS) is tipped in favor of the latter. Thus, hydrogen peroxide (H_2O_2) is directly involved in the production of ROS due to his high redox level. However aqueous extracts of green tea and rosemary have been investigated to be evaluated for their antioxidant properties. And the metabolic enzymes and antioxidant defense biomarkers were assessed. The results obtained revealed that in green tea and rosemary extracts groups, superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) activities in liver decreased significantly compared to groups treated with H_2O_2, as well as malondialdehyde in groups treated with both extracts. This study showed that green tea and rosemary extracts has an excellent antioxidant activities which is confirmed by determining the a,a-diphenyl-b-pricrylhydrazyl (DPPH) radical scavenging activities.

Keywords: oxidative stress, hydrogen peroxide, antioxidant, green tea, rosemary.

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INTRODUCTION

A homeostasis between rate of formation of reactive oxygen species (ROS) and the rate of their neutralization, if not maintained oxidative damage accumulates known as oxidative stress (Sies, 1991). ROS are products of regular cell metabolism ($^1O_2$: singlet oxygen, H_2O_2: hydrogen peroxide, $OH^{\cdot}$: hydroxyl radical, $O_2^{\cdot}$: superoxide radical). They participate in many cellular events including signal transduction and antibacterial defense (Droge, 2002). They are also capable of oxidizing cellular proteins, nucleic acids and lipids (Droge, 2002). They contribute to cellular aging (Finkel and Holbrook, 2000), mutagenesis (Takabe *et al.*, 2001) carcinogenesis (Kawanishi *et al.*, 2001) Alzheimer's disease (Ischiropoulos and Beckman, 2003), atherosclerosis (Steinberg, 2002) and coronary heart disease (Tian *et al.*, 1999) possibly through destabilization of membranes (Mora *et al.*, 1990), DNA damage (Takabe *et al.*, 2001), and oxidation of low-density lipoprotein (Kondo *et al.*, 1996).

To protect from these highly reactive intermediates, living organisms possess a defense system consisting of enzymatic and non-enzymatic antioxidants that scavenge them. It is well established that the most important antioxidant enzymes are superoxide dismutase (SOD), which ensures the dismutation of superoxide (O_2•) into a molecule of O_2 , catalase which catalyzes the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen, glutathione peroxidase (GPX), which reduces both H_2O_2 and organic peroxides by a glutathione-dependent reaction, and glutathione reductase (GR), which catalyzes the NADPH-dependent regeneration of glutathione (GSH) from the oxidized form (GSSG) generated by GPX. There is more and more evidence that glucose-6-phosphate dehydrogenase (G6PDH), the rate-limiting enzyme of the pentose phosphate pathway, plays a crucial role in modulating antioxidant defenses both in humans and mammals, as the NADPH generated in the reaction catalyzed by this enzyme is considered to be essential for the activity of the two H_2O_2 scavenging pathways of cells (Kirkman and Gaetani., 1984; Gaetani *et al.,* 1989; Scott *et al.,* 1991; Pandolfi *et al.,* 1995; Kirkman *et al.,* 1999; Tian *et al.,* 1999; Leopold and Loscalzo, 2000).

The non-enzymatic antioxidant system composed by scavenger molecules as vitamin E (tocopherol), vitamin C (ascorbic acid), and coenzyme Q (ubiquinone), plus selenium which is a trace element that functions as cofactor for reduction of antioxidant enzymes, such as glutathione peroxidases. They act by scavenging free radicals molecules with one or more unpaired electrons and transform them to stable molecules (Kinsky, 1989). Many others food compounds can ensure the same behavior such as: polyphenols, alkaloids… (Bors *et al.,* 1990).

Tea (*Camellia sinensis*) is the second most common beverage in the world next to water (Wei *et al.,* 1999). Although both green and black teas are derived from *Camellia. sinensis*, it is the production process which differentiates the two types of teas. Green tea contains polyphenols which include flavanols, flavandiols, flavonoids and phenolic acids. Most of the green tea polyphenols are flavanols, commonly known as catechins. The primary catechins in green tea are (-)-epicatechin (EC), (-)-epicatechin- 3-gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin- 3-gallate (EGCG), (+)-gallocatechin and (+)-catechin; they are comprised of aromatic rings with hydroxyl groups that possess strong antioxidant properties (Peterson and Totlani, 2005). It is believed that much of the anticancer effects of green tea are mediated by its polyphenolic constituents (Ahmad *et al*., 1998; Katiyar and Mukhtar, 1996). Moreover there is increasing interest in green tea catechins (GTCs) as protective agents against free radicals and cardiovascular disease. It has been demonstrated that increased consumption of green tea is associated with decreased serum total cholesterol and triacylglycerol and inversely related to risk of eventual coronary heart disease (Imai and Nakachi, 1995; Kono and Shinchi, 1992). Antioxidative activities have been demonstrated for the four major epicatechin derivatives in the following order EGC > EGCG > EC > ECG (Chen and Chan, 1996). Rosemary (*Rosmarinus officinalis*) a member of the mint family *Lamiaceaem*, is a woody, perennial herb, It's a common household plant grown in many parts of the world. It is used for flavoring food, in cosmetics and in traditional medicine for its chorereticS, hepatoprotective and antitumorigenic activity (Slamenov *et al.,* 2002). Rosemary is also known to exhibits antioxidant (Tena *et al.,* 1997; Bicchi *et al.,* 2000; Elgayyar *et al*., 2001; Ibanez *et al*., 2003), and antimicrobial activities (Pandit and Shelef, 1994; v *et al.,* 1998; Pintore *et al.,* 2002; Santoyo *et al.,* 2005). The potent antioxidant and antibacterial properties of rosemary extracts have been mainly attributed to its major diterpene, carnosic acid (Cuvelier *et al.,* 1994; Aruoma *et al.,* 1992; Frankel[a] *et al.,* 1996; Frankel[b] *et al.,* 1996) and some compounds of the essential oil. Carnosic acid is quite unstable and, usually, is converted to carnosol upon heating. Carnosol can degrade further to produce other compounds such as rosmanol, epirosmanol and metoxyepirosmanol which still possess antioxidant activity.

The objective of the present research was therefore to determine the effect of green tea and rosemary aqueous extracts, on oxidative cell damages, in liver induced by H_2O_2 and to analyze their free radical scavenging activity.

MATERIALS AND METHODS

Preparation of green tea and rosemary aqueous extracts:

Green tea (SULTAN AL ANBAR®) and rosemary leaves were blended in a blender.

Fifteen grams of ground tea leaves were then mixed with 200 ml of distilled water in a 500 ml round-bottom flask, equipped with a reflux condenser. The round-bottom flask is immediately placed in a water bath at 85°C for 20 mn (Record and Lane, 2001). And the aqueous extract was filtered through Whatman No. 1 filter paper. One hundred grams of rosemary leaves were mixed with 300 ml of distilled water in a 500 ml round-bottom flask, equipped with a reflux condenser. The round-bottom flask is immediately placed in a water bath 80°C for 60 mn (Aeschbacch and Rossi, 2003) then the aqueous extract was filtered through Whatman No. 1 filter paper.

Tests

The test concerned 70 males adult Swiss albino mice weighting 25-30 grams. They were acclimatized to laboratory conditions before the test and fed *ad libitum.* They were fasted 16 hours prior to the treatment (Zentella *et al.,* 2006).

Experiment 1

It concerned 6 groups which received either 0.9 % NaCl (control) or 1.5 g/kg of H2O2, each group consisted of 5 mice. After 5 mn, 30 mn, 1 h 30 mn, 4 h and 24 h from 1.5 g /kg H_2O_2 intraperitoneal injection; animals were sacrificed and liver was removed examined and processed for enzyme assays.

Experiment 2

Just before the experiment, a solution at 500 mg/kg (Shahidi and Wanasundara, 1992) was prepared of green tea, and

rosemary. After intraperitoneal injection, animals were scarified and liver was removed examined and processed for enzyme assays. All experiments were in accordance with the guidelines provided by the CPCSEA.

Tissue preparation for analytic procedures

Livers were rapidly thawed and manually homogenized, using a Potter homogenizer with a glass pestle, in 3 volumes of ice-cold 10 mM HEPES, 1 mM EDTA, 0.25 M Sucrose and 10 mM 2-mercaptoethanol, pH 7.4. All procedures were performed on ice. Homogenates were centrifuged at 20000 g for 60 mn at 4°C (sigma 2-16K) and the resultant supernatants were aliquoted and stored at – 20°C for later enzyme assays.

Chemicals

All biochemicals were obtained from Sigma (St. Louis, MO), Roche Diagnostics (Mannheim, Germany), or Bio-Rad Laboratories (Hercules, CA). All the other chemicals were purchased from Merck (Darmstadt, Germany) and all other chemicals were of analytical grade.

Biochemical assays

All assays were conducted at 25°C using a spectrophotometer (Thermo electron corporation, Biomate 3).

Catalase

The consumption of 7.5 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7) was monitored at 240 nm as indicated in (Aebi, 1984).

Glutathione reductase

The assay of (Di Ilio *et al.*, 1983) was used. The assay mixture contained 0.5 mM oxidized glutathione, 1 mM EDTA, 0.1 mM NADPH and 50 mM potassium phosphate buffer (pH 7.4) and NADPH consumption was monitored at 340 nm.

Superoxide dismutase

The enzyme was assayed according to ((Paoletti *et al.*, 1986) with assay conditions: 5 mM EDTA, 2.5 mM MnCl2, 0.27mM NADH, 3.9 mM 2-mercaptoethanol in 50 mM potassium phosphate buffer (pH 7), monitored at 340 nm. The decrease in absorbance is measured after the addition of NADH to 0.27 mM as final concentration.

Thiobarbutiric acid reactive substances

Lipid peroxidation was estimated by the formation of thiobarbituric acid reactive substances (TBARS) and quantified in terms of malondialdehyde (MDA) equivalents according to the method described by (Samokyszyn and Marnett, 1990): 1 ml of samples was added to 1 ml solution (0.375% thiobarbituric acid and 15% trichloracetic acid in 0.25 M hydrochloric acid). The tubes were heated at 100°C during 15 min and they were cooled in the ice to stop the reaction. One then carries out a centrifugation with 1000 x g during 10 min. The reading of supernatant was made to 535 nm.

Protein assay

Protein content was measured according to the Bradford procedure (Bradford, 1976) by using bovine serum albumin (BSA) as standard. Protein reagent [Coomassie brilliant blue G-250 0.01% (w/v) (Sigma Chemical Co., Bornem, Belgium], ethanol [(95%) 4.7% (w/v), phosphoric acid (85%) 8.5% (w/v)] was added to protein solutions. The absorbance was measured at 595 nm after 10 mn of incubation in the dark.

Enzyme activity expression

The specific activity of each enzyme was calculated using the following formula:

$AS = (\Delta Abs/mn \times 1000) / (\varepsilon \times [P] \times Ve)$

$\Delta Abs/mn$: absorbance variation/minute

ε (extinction coefficient) :

- $\varepsilon (H_2O_2) = 40\ M^{-1}.cm^{-1}$, for CAT
- ε (NADH) = $6220\ M^{-1}.cm^{-1}$, for SOD and GR
- ε (MDA-TBA complex) = $153000\ mM^{-1}.cm^{-1}$, for MDA

[P]: protein concentration

V_e: assay volume

Antioxidant activity assay

The free radical scavenging capacity of both green tea and rosemary aqueous extracts was determined and compared to vitamin C used as a standard according to the method described by (Hsu *et al.*, 2006). The extract (100 µl), was mixed with 1.9 ml of 0.1 mM DPPH methanolic solution. The mixture was shaken vigorously and left to stand for 30 min at room temperature, and the absorbance was then measured at 517 nm against a blank. The percentage scavenging effect was calculated as:

Scavenging rate (%) = $[(A_1-A_2)/A_0] \times 100$, where A_o was the absorbance of the control (without extract) and A1 was the absorbance in the presence of the extract, A_2 was the absorbance without DPPH.

STATISTICAL DATA ANALYSIS

The experimental data represent the mean of five independent assays ± standards deviations. ANOVA test (XL stat pro 7.01); a p-value lower than 0.05 was considered significant.

RESULTS AND DISCUSSION

Figures 1, 2 and 3 show a fast response of protective enzymes upon H_2O_2 treatment. After 5 mn catalase, superoxide dismutase, glutathione reductase activity and peroxidized lipid level (TBARS or malondialdehyde) increased significantly compared to control. Therefore, this response is maintained high until 30 mn then returned approximately to control values. It shows that H_2O_2 lead to changes or damage to the liver by generating ROS.

SODs catalyze the rapid removal of superoxide radicals. In mammals there are several types of SODs, which differ with respect to their location in the cells and the metal ions they require for their function. For example, a copper-zinc SOD is present in

the fluid filling the cell (i.e., the cytosol) and in the space between the two membranes surrounding the mitochondria. Furthermore, a manganese-containing SOD is present in the mitochondrial interior (i.e., matrix). Both of these enzymes are critical for prevention of ROS-induced toxicity (Fridovich, 1997).

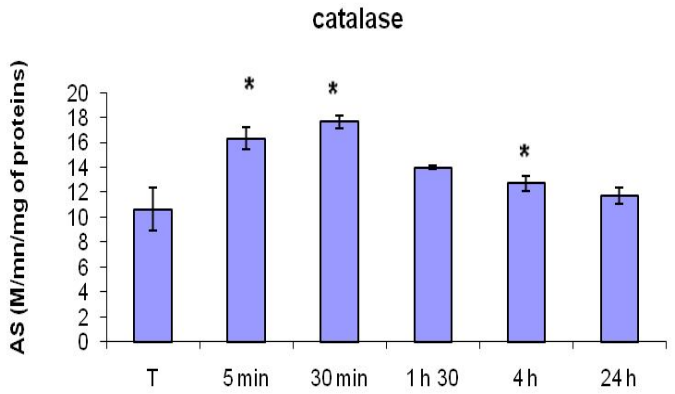

Fig. 1: Oxidative stress evaluated by catalase activity at different times (5mn, 30 mn, 1h 30, 4 hours, 24 hours) + 1.5 g/kg H_2O_2, T: control, * significantly different from control at P<0.05. The number of mice used in each group was 5.

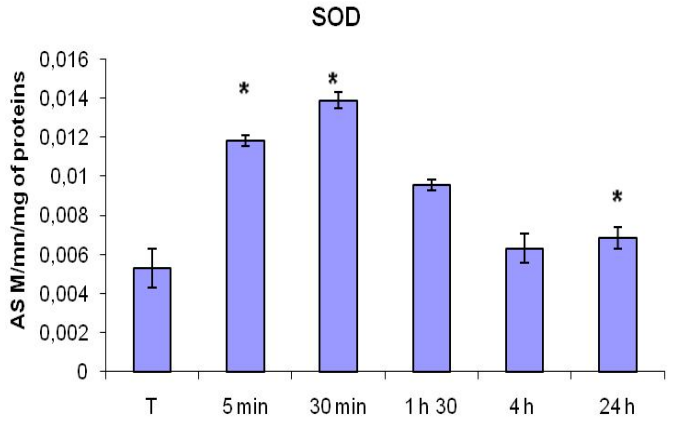

Fig. 2: Oxidative stress evaluated by superoxide dismutase activity at different times (5 mn, 30 mn, 1h 30, 4 hours , 24 hours) + 1.5 g/kg H_2O_2, T: control, * significantly different from control at P<0.05. The number of mice used in each group was 5.

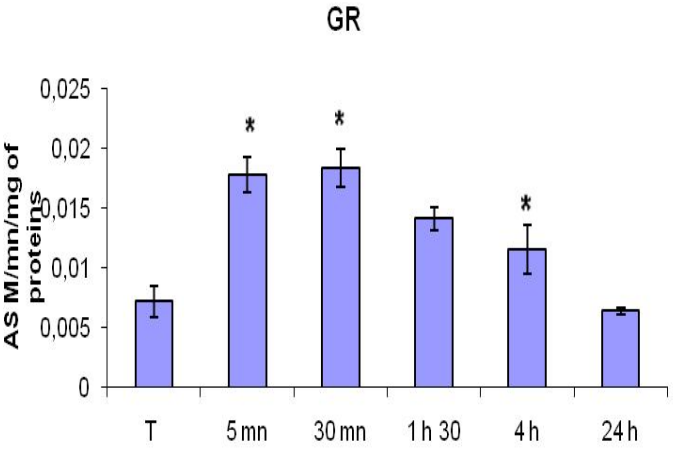

Fig. 3: Oxidative stress evaluated by glutathione reductase activity at different times (5mn, 30 mn, 1h 30, 4 hours, 24 hours) + 1.5 g/kg H_2O_2, T: control, * significantly different from control at P<0.05. The number of mice used in each group was 5.

Catalase and the glutathione peroxidase system both help to remove hydrogen peroxide. Catalase is an iron-containing enzyme found primarily in the small membrane-enclosed cell components called peroxisomes; it serves to detoxify hydrogen peroxide and various other molecules. One way that catalase eliminates hydrogen peroxide is by catalyzing a reaction between two hydrogen peroxide molecules, resulting in the formation of water and O_2. In addition, catalase can promote the interaction of hydrogen peroxide with compounds that can serve as hydrogen donors so that the hydrogen peroxide can be converted to one molecule of water, and the reduced donor becomes oxidized. While the glutathione peroxidase system consists of several components, including the enzymes glutathione peroxidase and glutathione reductase and the cofactors glutathione (GSH) and reduced nicotinamide adenosine dinucleotide phosphate (NADPH). Together, these molecules effectively remove hydrogen peroxide (Fig1). GSH, which consists of three amino acids, is an essential component of this system and serves as a cofactor for an enzyme called glutathione transferase, which helps remove certain drugs and chemicals as well as other reactive molecules from the cells. Moreover, GSH can interact directly with certain ROS (e.g., the hydroxyl radical) to detoxify them, as well as performing other critical activities in the cell.

Malondialdehyde is a terminal product of lipid breakdown due to peroxidation damage and this (and other aldehydes) can be detected by its reaction with thiobarbituric acid. MDA is one of the most frequently used indicators of lipid peroxidation (Nielsen *et al.*, 1997). The production of hydroxyl radical and other powerful radicals can initiate a chain reaction of lipid peroxidation in which polyunsaturated fatty acids are converted into lipid peroxides. Increased MDA can be interpreted as resulting from cellular membrane damage initially caused by increased formation of radicals (Niedernhofer *et al*., 2003).

In the first experiment, for the control and the general trend of activity was highest levels 30 mn followed by lower levels. Elevated SOD activity indicates the production of higher levels of ROS generated by H_2O_2 treatment during the incubation time, which in turn triggered the elevated SOD expression in the cells. Control showed comparatively lower activity.

Elevated CAT indicates the active role of CAT in modulating the harmful effects of H_2O_2; this is also directly correlated to high GR activity. Lowest CAT and GR activity was observed in the case of unstressed control cells.

Under stressed H_2O_2 conditions higher MDA content was observed at 30 mn indicating higher levels of lipid peroxidation. However control showed the lowest MDA content indicating the normal membrane deterioration process. Given that MDA is considered as a valuable indicator of oxidative damage of cellular components, our results suggest that H_2O_2 treatment enhanced reactive oxygen species generation in the liver and that antioxidant defenses were not totally able to effectively scavenge them, thus leading to lipid peroxidation. That's why according to these results, 30 mn was investigated for treatment with green tea, rosemary and vitamin C + H_2O_2.

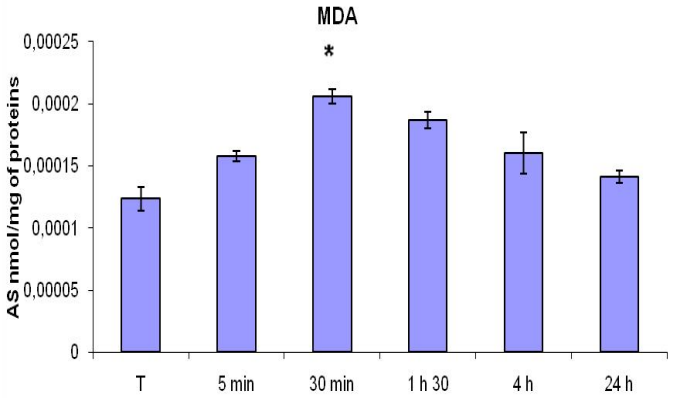

Fig. 4: Oxidative stress evaluated by malondialdehyde level at different times (5mn, 30 mn, 1h 30, 4 hours, 24 hours) + 1.5 g/kg H_2O_2, T: control, * significantly different from control at P<0.05. The number of mice used in each group was 5.

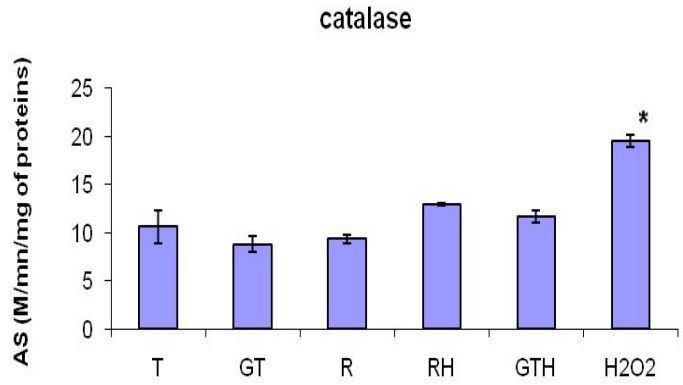

Fig. 5: Effect of green tea (GT) and rosemary (R) on catalase activity. T : control, GT : green tea, R : rosemary, RH : rosemary + H2O2 1.5 g/kg, GTH : green tea + H_2O_2 1.5 g/kg * significantly different from control at P<0.05. The number of mice used in each group was 5.

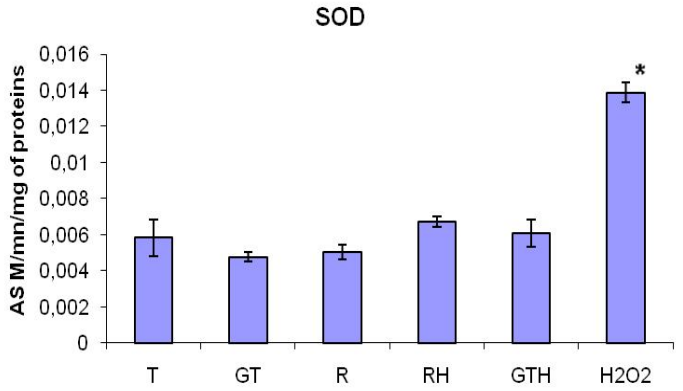

Fig. 6: Effect of green tea (GT) and rosemary (R) on superoxide dismutase activity. T : control, GT : green tea, R : rosemary, RH : rosemary + H_2O_2 1.5 g/kg, GTH : green tea + H_2O_2 1.5 g/kg * significantly different from control at P<0.05. The number of mice used in each group was 5.

Therefore the second experiment showed that green tea and rosemary concentration used in 500 mg/kg didn't lead to stress damage and didn't show toxic effect as we detect no death and increase of CAT, SOD, GR, plus a prevention of lipid oxidation. In the other hand we notice that catalase, superoxide dismutase, glutathione reductase activity and peroxidized lipid level (TBARS or malondialdehyde) + aqueous extracts not only decrease significantly but also returned approximately to control values (figures 7, 8, 9 and 10). These results have shown that green tea, rosemary and vitamin C aqueous extracts fortunately controlled free radicals formation. It's known that plant polyphenols are natural antioxidants. An antioxidant can be defined as any substance that when present at low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate (Percival, 1992; Young and Woodside, 2001). Trough this definition we can say that green tea and rosemary at 500mg/kg played a protective effect against free radical, therefore it's reported that green tea contain powerful antioxidants (Cabrera *et al.*, 2006): catechin-based flavonoids with epigallocatechin gallate (EGCG) being present in greatest amounts. Rosemary (Rosmarinus officinalis L.) extracts have a potent antioxidant activity (Tena *et al.*, 199; Ibanez *et al.*, 2003). The antioxidant activity of rosemary extracts has been associated with the presence of several phenolic diterpenes such as carnosic acid, carnosol, rosmanol, rosmariquinone and rosmaridiphenol (Cuvelier *et al.*, 1994; Frankel *et al.*, 1996), which break free radical chain reactions by hydrogen donation. This lead to confirm the ability of these herbs to stabilize, deactivate free radicals before the latter attack cells and biological targets. This is in accordance with our results shown by a similar level of antioxidant defense biomarkers to the control after adding both extracts; which is in accordance with our results shown by a similar level of antioxidant defense biomarkers to the control after adding both extracts.

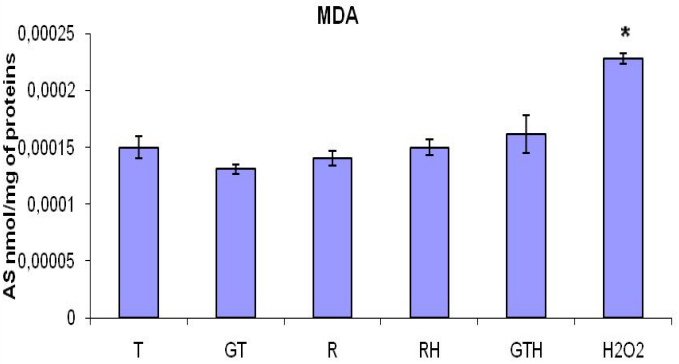

Fig. 7: Effect of green tea (GT) and rosemary (R) MDA level. T : control, GT : green tea, R : rosemary, RH : rosemary + H_2O_2 1.5 g/kg, GTH : green tea + H_2O_2 1.5 g/kg * significantly different from control at P<0.05. The number of mice used in each group was 5.

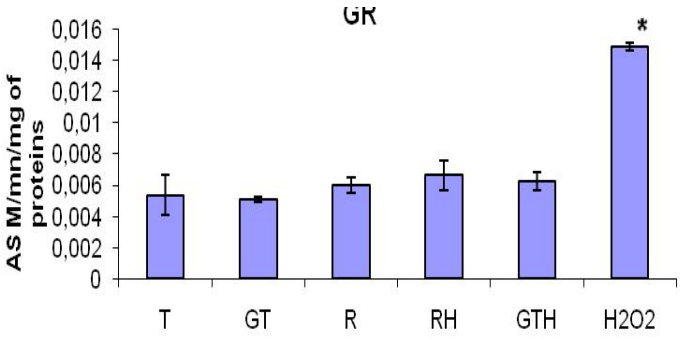

Fig. 8: Effect of green tea (GT) and rosemary (R) MDA level. T : control, GT : green tea, R : rosemary, RH : rosemary + H_2O_2 1.5 g/kg, GTH : green tea + H_2O_2 1.5 g/kg * significantly different from control at P<0.05. The number of mice used in each group was 5.

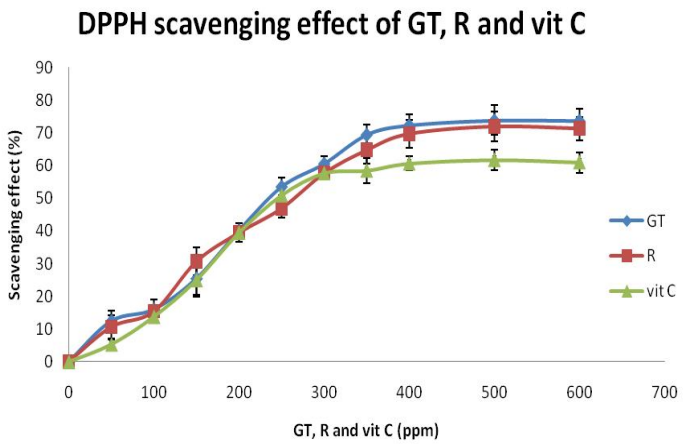

Fig. 9: DPPH scavenging effect of green tea, rosemary and vitamin C. The data presented are expressed as mean ± standard deviation (SD); Measurements were carried out in triplicate.

To confirm the antioxidant activities of rosemary and green tea, their antioxidant capacity was evaluated using assays for DPPH and hydrogen peroxide. DPPH· is a free radical which is capable of accepting an electron or a hydrogen radical to become a stable diamagnetic molecule (Soares *et al.*, 1997). DPPH· is typically used as a substrate for evaluating the free radical scavenging ability of an antioxidant (Oyaizu, 1986). We noticed that for low doses (0 to 150 ppm) the scavenging activity were similar for green tea, rosemary and vitamin C. furthermore the scavenging of DPPH radicals increased as the concentration of green tea and rosemary and vitamin C increased, then stabilized. The scavenging ability was greater for both rosemary and green tea aqueous extract (approximately 70 % for 400 ppm) compared to vitamin C (48 % for 400 ppm, 53 % for 500 ppm) as it is illustrated in Figure 9. These plants showed high antioxidant potential which explained their ability of scavenging ROS caused by H_2O_2 treatment leading to similar level to the control of antioxidant defense biomarkers. We demonstrated that both green tea and rosemary are highly efficient for preventing oxidative stress by neutralizing ROS.

In conclusion, the present study demonstrate the effectiveness of the antioxidant activities of green tea and rosemary extracts on preventing lipid oxidation and protecting against oxidative stress due to plant phenolics direct reactivity towards reactive oxygen species (ROS) caused by H_2O_2. Their stronger DPPH scavenging activity of tea antioxidant activity showed by DPPH test lead them to be a precious natural source of antioxidants, a bioactive phytochemicals for human health and the functional food industry. Therefore they should widely be used in order to prevent not only stress damages but also diseases that it contributes in.

Future studies with other medicinal plants will presumably give us further information of the implication of these plants in the antioxidation mechanism. One way to confirm these results at the molecular level, a necessary task is to examine the effects of these treatments on the gene transcription of all enzymes used in this study. These kind of studies are at the present in progress in our laboratory.

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