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Biochemical Study of the Protective Effect of *Salvia officinalis* Essential Oil against the Oxidative Stress Induced by Hydrogen Peroxide in *Tetrahymena thermophila*

Papa D Mar, Hatim Otto, Bouchra El khalfi, Abdelaziz Soukri*

Department of Biology, Faculty of Sciences Ain Chock, Laboratory of Physiopathology, Molecular Genetics and Biotechnology (PGMB), University Hassan II Casablanca, Morocco
***Corresponding author:** Abdelaziz Soukri, Department of Biology, Faculty of Sciences Ain Chock, Laboratory of Physiopathology, Molecular Genetics and Biotechnology (PGMB), University Hassan II Casablanca, Morocco, Tel: +21266109549; Fax: 0522 23 06 74, E-mail: ab.soukri@gmail.com*

ABSTRACT

In our previous studies, we have illustrated that sage essential oil has a good protective effect against oxidative stress that is induced by hydrogen peroxide on the phenotypic parameters of the protozoan Tetrahymena, given that this protection is characterized by a significant improvement in cell growth and normal cell morphology.

On the whole, this work includes studying the intracellular effect of the essential oil of sage on the specific activity of certain antioxidant enzymes such as Catalase (CAT) and Superoxide Dismutase (SOD), lipid peroxidation rate (MDA) and specific activity of the GAPDH biomarker in the model organism Tetrahymena thermophila.

As a result, our studies have shown that H_2O_2 significantly decreases the activities of CAT SOD, GAPDH and significantly increases the rate of MDA. On the other hand, in the presence of sage oil, there is a very significant increase in the activities of the CAT and the GAPDH, a restoration of the activity of the SOD and the rate of MDA. These results confirm that sage oil has a protective effect against oxidative stress by trapping Reactive Oxygen Species (ROS) released by hydrogen peroxide and enhancing the activity of intracellular antioxidant enzymes. Therefore, this oil can be a good alternative for the prevention of oxidative stress-related diseases.

Keywords: Oxidative stress, Sage, *Tetrahymena*, Phenotypic parameters, Biochemical parameters

INTRODUCTION

Oxidative stress results from an imbalance between free radicals and antioxidant defense systems. Oxidative stress is a process that is involved in the deterioration of several cellular components such as structure, nucleic acids, proteins and lipids [1,2] that can abate health and cell viability or induce a variety of cell response by generating reactive species secondary causes cell death by necrosis or apoptosis [3,4]. Consequently, this phenomenon is involved in several diseases such as cancer [5] and neurodegenerative diseases [6]. In this context, the search for antioxidant supplements is an area of growing interest for the prevention of chronic diseases related to oxidative stress [7].

As previously illustrated, the essential oils of sage (*Salvia officinalis*) and oregano (*Oregano vulgare*) protect the growth and form of model organisms *Tetrahymena thermophila* and *Tetrahymena pyriformis* against oxidative stress induced by peroxide hydrogen (H_2O_2) [8]. Assuredly, these essential oils are depicted in literature as having antioxidant properties due to their phenolic compounds which are capable of scavenging reactive oxygen species (ROS), released by hydrogen peroxide to improve the activity of intracellular antioxidant enzymes [9,10].

The main purpose of this work is to study the effect of these essential oils in *T. thermophila* on the specific activity of certain antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD), the rate of lipid peroxidation and the specific activity of a biomarker of cellular metabolism, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Moreover, The choice of the model organism *T. thermophila* is based on several advantages: eukaryotic unicellular organism, ease of culture in axenic medium, short life cycle; ideal dimensions to studies under optical and electronic microscope; commonly used in the physiological, toxicological and genetic studies [11,12]; the intracellular system is comparable to more advanced animals while maintaining ease of manipulation.

MATERIAL AND METHODS

Strain and culture condition

The strain *Tetrahymena thermophila* SB 1969 is used in this study. The cells are maintaining in an axenic medium, the PPYE medium consisting of 1.5% (w/v) peptone protein and 0.25% (w/v) yeast extract [13]. The growth medium is inoculated with 1% (v/v) of a *T. thermophila* preculture. Incubation is carried out without shaking at 32°C.

Induction of oxidative stress

Hydrogen peroxide (H_2O_2) is added at various concentrations that vary from 0.1 mM to 1 mM after 24 h of protozoan culture according to the method described by Errafiy et al. [14]. The concentration inhibiting half the number of cells after 72 h of culture is chosen for the rest of the tests. Protozoan growth is monitored by sterile sampling every 3 h and protozoan absorbance is measured at 600 nm using the Jenway 7315 UV-Visible Spectrophotometer. Accordingly the optical zero corresponds to the growth medium which does not contain protozoa. Furthermore, the shape and mobility of protozoa are also observed under an optical microscope. In the same vein, samples are collected sterilely every 24 h, prepared between slide and cover slip, and observed at the 10X objective of A.KRÜSS Optronic optical microscope.

Evaluation of the protective effect of the essential oil

The essential oil of sage (*Salvia officinalis*) is obtained directly from the leaves [8]. The non-lethal concentration used for the tests is the 10^{-3} dilution [8]. The protozoa are incubated in PPYE medium (1%, v/v) in the presence of the essential oil (0.1%, v/v). The stressor is added after 24 h of culture. Therefore, the growth and the form of the protozoa are monitored during 8 days of culture by measuring the optical density and by visualization of the cells under an optical microscope.

Evaluation of biochemical parameters

Protein extraction by sonication

The cells are harvested by centrifugation at 700 g for 10 min (Hettich Universal 320R) and are suspended in lysis buffer which contains 50 mM Tris-HCl pH 8; 1% Glycerol; 1 mM EDTA pH 8; 1 mM PMSF; 10 mM β-mercaptoethanol (with a ratio of 3 ml buffer /1 g of cells). The cells are lysed through the Bandelin Sonoplus HD 2070 sonicator, in 12 cycles with a breaking power of 90%, a break period of 20 s alternated with 1 min of rest in the ice. The obtained homogenates are centrifuged at 2000 g for 45 min to remove cell debris. The obtained supernatant is considered as crude extract.

Quantification of proteins by Nanodrop

The protein concentration of the crude extracts was measured using a NanoDrop® ND-1000 spectrophotometer. The Nanodrop assay has a number of advantages: 1 µl of sample is sufficient for assay performance, the assay does not require prior dilution, the manipulation is simple, there is no deposition in cuvette, and the cleaning is rapid. The process allows the measurement of the amount of nucleic acids and proteins.

Measurement of the activity of antioxidant enzymes

Catalase

The activity of catalase is measured according to the technique described by Aebi [15]. This technique is based on monitoring the decomposition of H_2O_2 into H_2O and O_2 following the catalytic activity of catalase. The crude extract is added to a quartz cuvette containing 50 mM phosphate buffer. Pre-incubation for 2 min is performed at 30°C. The cuvette is then introduced into a spectrophotometer. The reaction is initiated by adding 50 mM H_2O_2. The absorbance reading is performed at 240 nm. The molar extinction coefficient is 40 $M^{-1}cm^{-1}$. Yet, the activity of the enzyme is expressed in unit of activity/mg of protein.

Superoxide dismutase

The assay technique for superoxide dismutase (SOD) activity, described by Paoletti and his contributors, is based on inhibition of NADH oxidation by superoxide dismutase [16]. The reduction in the rate of oxidation of NADH is proportional to the concentration of the enzyme.
In the absence of SOD, the auto-oxidation of 2-β-mercaptoethanol in the presence of EDTA/$MnCl_2$ will generate superoxide anions in the reaction medium and causes the oxidation of NADH to NAD^+, which is translated by a decrease in absorbance at 340 nm. In the presence of SOD, there is a competition of generation reactions (2-β-mercaptoethanol + EDTA/$MnCl_2$) and a dismutation of superoxide anions. This tends to decrease the amount of superoxide anions in the medium, in this connection, causes the inhibition of NADH oxidation and, therefore, a less significant decrease in absorbance.

The reaction mixture contains 5 mM EDTA; 2.5 mM MnCl2; 0.27 mM NADH; 3.9 mM 2-β-mercaptoethanol in 50 mM phosphate buffer pH 7 with an adequate volume of the crude extract. A pre-incubation for 2 min is performed at 30°C. The reaction is initiated by 0.27 mM NADH addition. The measurement of the activity is determined spectrophotometrically at 340 nm. The molar extinction coefficient of NADH is 6220 $M^{-1}cm^{-1}$. Succinctly, the enzyme activity is expressed in unit of activity/mg of protein.

Evaluation of lipid peroxidation

Lipid peroxidation is evaluated by measuring the malondialdehyde (MDA) level. The rate is evaluated by substances that react with thiobarbituric acid with Samokyszyn and Marnett's method [17]. 100 µl of the crude extract is added to 900 µl of a solution consisting of 0.375% thiobarbituric acid and 15% trichloroacetic acid. The mixture is then placed in a water bath at 100°C for 15 minutes and then cooled in ice to stop the reaction. Centrifugation at 1000 g for 10 min was performed, and the optical density measurement of the supernatant was performed at 535 nm. The degradation product of polyunsaturated fatty acids was calculated using the extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$.

Measurement of GAPDH activity

The enzymatic activity of NAD^+ dependent phosphorylation GAPDH is determined spectrophotometrically by measuring the onset of NADH at 340 nm [18]. The crude extract is added to the reaction mixture consisting of tricine buffer-NaOH 50 mM pH 8; 10 mM sodium arsenate; 0.4 mM NAD^+ and 0.4 mM D-G3P. The total volume of the reaction mixture is 1 ml. The molar extinction coefficient of NADH is 6220 $M^{-1}cm^{-1}$. The activity of the enzyme is expressed in unit of activity/mg of protein.

Statistical analysis

The results of our study are expressed on average with their standard deviation, represented on the diagrams by a vertical line (error bar). The comparison is estimated using the student-t-test between the control and treated groups, using the excel software. The difference is considered significant if p-value is less than 0.05 (*), highly significant if p value is less than 0.01 (**).

RESULTS AND DISCUSSION

Protective effect of sage on growth and form of *Tetrahymena thermophile*

The 0.4 mM H_2O_2 concentration is used to monitor growth and form of protozoa. Figure 1 shows that growth has decreased considerably in the presence of the stressor, while an improvement in growth is noted in the presence of sage oil. These results are confirmed during microscopic visualization in which the protozoa have a narrow (stressed) form in the presence of H_2O_2 and return to the normal form in the presence of sage oil (Figure 2). These results confirm the cellular damage generated by hydrogen peroxide and the protective effect of the essential oil against the effects of H_2O_2 [8].

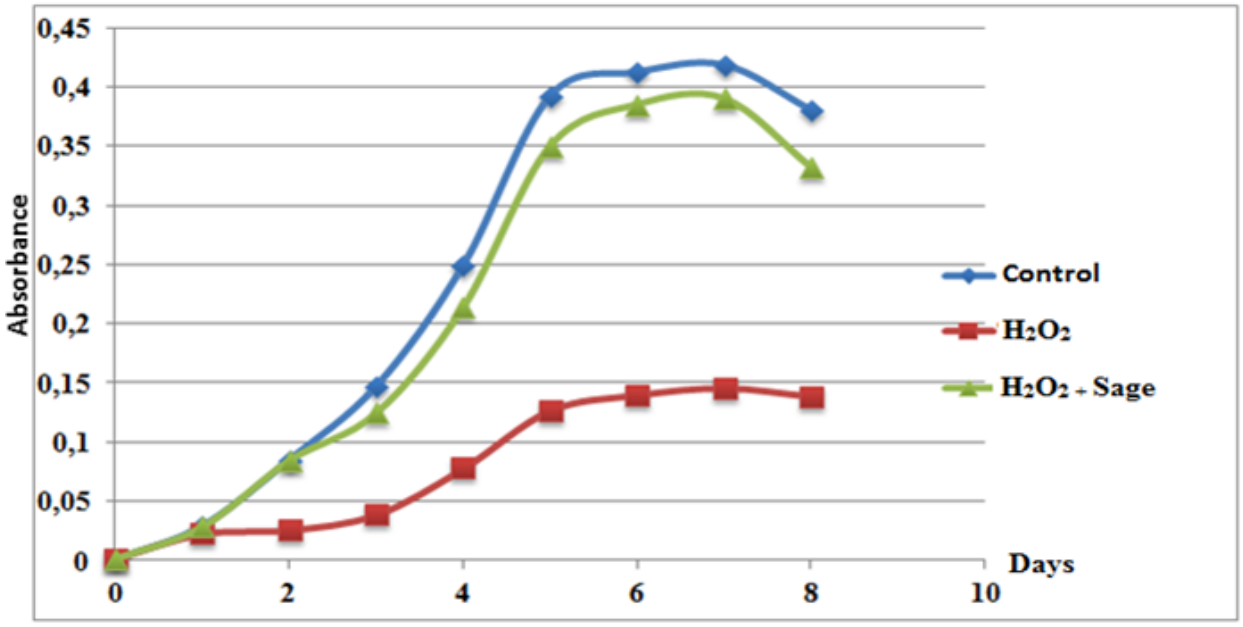
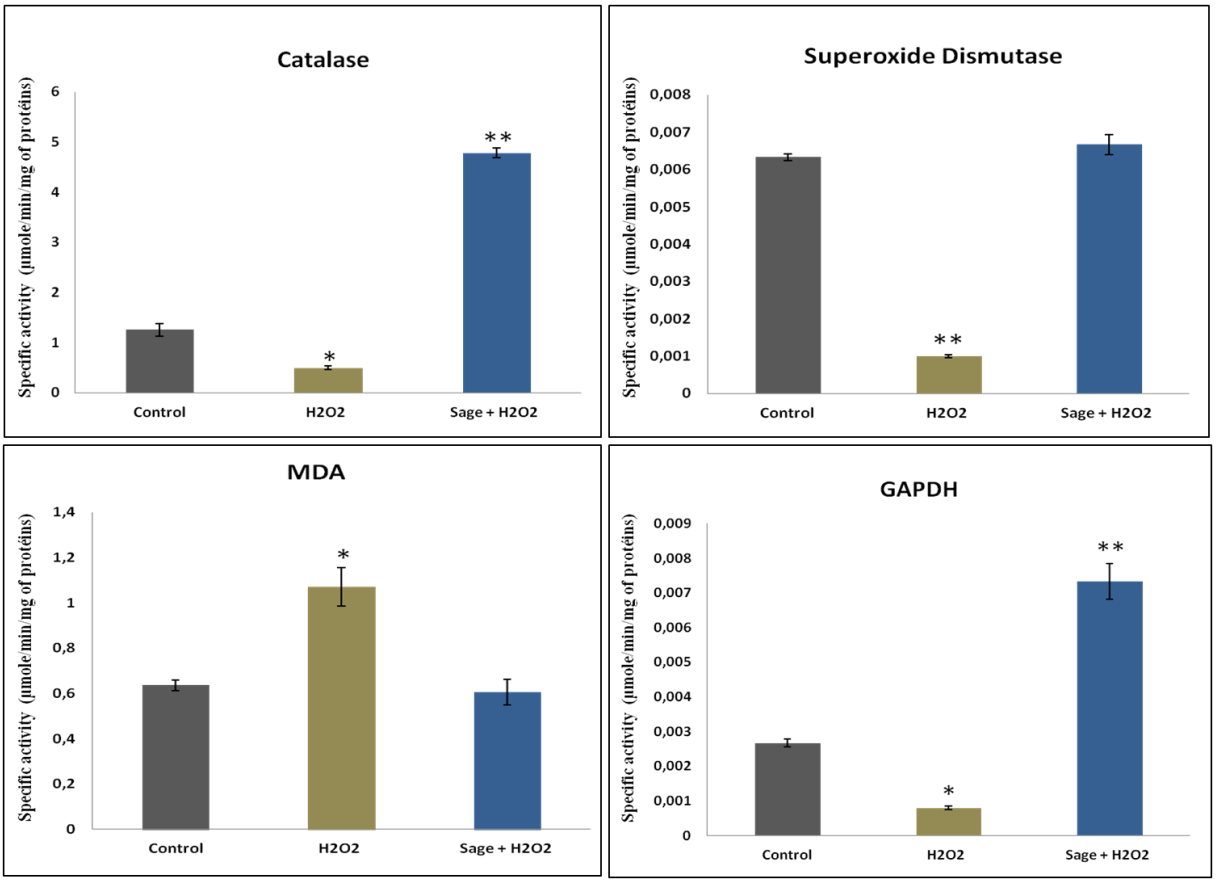


Figure 1: Effect of sage on the growth of *Tetrahymena thermophila* treated with H_2O_2

Figure 2: Effect of sage on the form of *Tetrahymena thermophila* treated with H_2O_2

Protective effect of sage on the specific activity of antioxidant enzymes

The enzymatic activities of catalase and superoxide dismutase were measured as described in material and methods. As illustrated in Figure 3, catalase and superoxide dismutase activities were significantly decreased in H_2O_2-treated cells. Conversely, in the presence of sage, there is a very significant increase in catalase activity compared to normal cells and a restoration of the activity of superoxide dismutase. Catalase and superoxide dismutase are enzymatic antioxidants and are the first line of defense against ROS. These two enzymes work in synergy to eliminate free radicals. Studies have demonstrated that the decrease of activity of these enzymes in the cells treated with H_2O_2 is caused mainly by the latter which allows the generation of toxic free radicals to recognize the hydroxyl radical. The latter causes the oxidation of the thiol groups of the proteins, the formation of Disulfide Bridge and the alteration of the structure and the enzyme function which result in a loss of activity [19-21]. Studies have also shown that acute and chronic treatments of HeLa cancer cells with H_2O_2 inhibit the activity of antioxidant enzymes CAT, SOD and glutathione peroxidase [22].



***significant at p<0.05 and ** highly significant at p<0.01**

Figure 3: Effect of sage on enzymatic activities of CAT, SOD and GAPDH, and MDA level

Protective effect of sage on lipid peroxidation rate

Malondialdehyde is a biomarker often used to study lipid oxidative damage and is a major product of lipid peroxidation. When the cells are treated with H_2O_2, it is found that the level of MDA has significantly increased, while the MDA level is restored in the presence of sage (Figure 3).

The increase of lipid peroxidation can be explained by the generation of ROS by H_2O_2, which attacks the polyunsaturated fatty acid residues of phospholipids leading to the excessive release of MDA. Similar studies have shown an increase in MDA levels in *T. thermophila* and *T. pyriformis* after induction of oxidative stress [14,23,24]. In addition; a rise of the level of MDA has also been reported in PC12 nerve cells that are treated with hydrogen peroxide [25].

In the presence of sage oil, the level of MDA in H_2O_2 - treated cells sharply decreased and returned to normal levels. This drop can be explained by the fact that the essential oil of the sage is able to trap the ROS in particular due to its phenolic compounds. A decrease in the level of peroxidation is found in the presence of argan oil in *T. pyriformis* cells which are treated with H_2O_2 [23]. Similar results have shown that *Salvia lavandulifolia Vahl* (spanish sage) oil decreases the lipid peroxidation rate of H_2O_2 - treated PC12 nerve cells [26].

Protective effect of sage on the specific activity of GAPDH

GAPDH is an essential biomarker of cell metabolism. When the protozoa were treated with H_2O_2, the enzymatic activity of GAPDH significantly decreased while in the presence of sage the activity was remarkably increased (Figure 3). Similar results showed that the decline in GAPDH activity is accompanied by a decline of growth and a change in the form of the protozoan *T. thermophila* treated with H_2O_2 [14]. Inactivation of GAPDH is caused by the stressor that releases the ROS that interfere with the thiol groups of the cysteine residues in the active site of the enzyme [26]. The oil of sage is characterized by its phenolic compounds with antioxidant power [27,28] that is able to trap the ROS released by H_2O_2 and thus protect the enzymatic activity of GAPDH.

GAPDH is an essential enzyme in glycolysis and actively participates in the production of cellular energy in the form of ATP. Studies have shown that *T. pyriformis* cells have the ability to form digestive vacuoles when exposed to metabolizable particles [29]. The formation of these vacuoles requires a low production of energy (in the form of ATP) to ensure the phagocytosis of these particles. Thus, the increase in GAPDH activity is observed in the presence of H_2O_2 and the essential oil of sage can correspond to a large production of energy in order to eliminate the stress agent probably neutralized by essential oils.

CONCLUSION

This study showed that oxidative stress affects the antioxidant defense systems of the model organism *T. thermophila* that leads to growth decline and a change in cell morphology. Consequently, the application of the essential oil of sage has presented cells protection against the harmful effects of oxidative stress by trapping free radicals and improving the activity of antioxidant enzymes. Therefore, this oil could be an excellent alternative prevention from diseases related to oxidative stress.

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