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Cryoprotective Effect of Pyrano-[2,3-C]-Pyrazoles on H_2O_2 Induced Damage in *Tetrahymena thermophila*

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Abstract: Antioxidants are developed to assist the immune system and overcome oxidative stress, break the auto-oxidative chain reaction, quench oxygen singlets (O2-), and inhibit peroxidase formation. The main objective of this study was to search for potent antioxidant supplements to protect our cells against diseases associated with oxidative stress. We chose four pyrano-[2,3-c]-pyrazole derivatives to test their antioxidant properties in vivo using "*Tetrahymena* spp" as a cellular model. Then, we measured the activity of some antioxidant and biochemical biomarkers such as CAT, SOD, GAPDH, SDH, Gr, and MDA. Also, the fragmentation of DNA has been investigated. The current study demonstrated that the three compounds (5a, b, and c) have potent antioxidant activity, characterized by increased activity of some antioxidant enzymes, inhibiting lipid peroxidation and DNA damage. These findings suggest for the first time that Pyrano-[2,3-c]-Pyrazoles is a promising source of synthetic antioxidants that could offer protection against H_2O_2-induced- stress and provide us with a new challenge to design a library of pharmaceutical compounds with high activity and low toxicity.

Keywords: antioxidant; *Tetrahymena spp*; pyrano-[2,3-c]-pyrazoles; dosage; biomarker; damage.

1. Introduction

Oxygen is an essential element for our survival and development; the role of oxygen is not limited to the respiration and metabolism of our cells; it can also generate radical oxygen species (ROS), acidity, alterations, and cellular aging [1]. We can distinguish different types of reactive oxygen radicals, which vary according to their degree of toxicity and their mode of action [2,3].

The overproduction of ROS can cause "oxidative stress", which is defined as the aggression of cellular constituents, including DNA, proteins, and lipids, as a consequence of the appearance of several consequences the injuries such as diabetes, atherosclerosis, high blood pressure, neurodegenerative diseases such as Parkinson's and Alzheimer's, also some types of cancer [3–6].

To remedy this pathological situation, many antioxidants are developed to attenuate oxidative stress by direct quenching of these species or indirectly via the biosynthesis of a battery of antioxidant enzymes such as catalase, superoxide dismutase, glutathione reductase [7–9].

The main purpose of this paper is to describe the biological interactions between stressors reagents and a cellular model that is recognized as a bioindicator of water pollution "*Tetrahymena spp* (Protozoa, Ciliata, Oligohymenophorea)". This microorganism appeared to be a suitable experimental model, which has been used widely in pharmacological and toxicological research because of its biological system (nucleus, mitochondrion, cytoskeleton) [10–13]. Also, its response to toxic elements can be similar to those of multicellular organisms[14]; it can be easily sub-cultured and maintained at optimal laboratory conditions. All of these motivated us to use this genus as biological material in experimental bioassays to explain the toxic effects of some chemical compounds, study the impact of xenobiotics as well as the assessment of health risks and propose the mode of action of many antioxidants [15–19].

In general, eukaryotic organisms have evolved a comprehensive range of proteins to detoxify ROS and repair oxidative damage to DNA, lipids, and proteins. Chief among powerful enzymatic and non-enzymatic antioxidants, there are Superoxide Dismutase (SOD), Catalase (CAT), Glutathione reductase (Gr), Succinate dehydrogenase (SDH), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). These enzymes maintain the balance between the pro-oxidant and antioxidant systems in *T. thermophila* spp cells [11, 20–22].

Superoxide dismutase is implicated in the detoxification of ROS by catalyzing the conversion of superoxide anion (O^{2-}) into hydrogen peroxide and molecular oxygen [23]. The CAT protects human cells from the harmful effects of reactive species by degrading the H_2O_2 into water and oxygen singlets [24]. Also, glutathione reductase and Glutathione peroxidase (GPx) protects the cells from highly toxic hydrogen peroxides by converting this product into water and oxygen singlets [11]. The SDH and GAPDH played a pivotal role in the carbon metabolic route and participated in the transfer of energy in *Tetrahymena* spp. cells [25–27].

To achieve the purpose of this study, we evaluated the toxicity of hydrogen peroxide "H_2O_2" on cells of *Tetrahymena* spp. We investigated the protective effect of four Pyrano-[2,3-c]-Pyrazoles derivatives by monitoring the evolution of certain specific biomarkers such as the bioindicator of lipid peroxidation (Malonaldehyde or MDA), antioxidant enzymes (CAT, SOD, Gr) and some metabolic enzymes (GAPDH, SDH), as well as DNA fragmentation.

Based on our current data, we have made exciting progress on this topic. However, some challenges remain in identifying the cellular and molecular pathways that are activated in *Tetrahymena* cells after long-term exposure to certain stressors [28]. Also, the prediction of the selected compounds' possible antioxidant mechanism of action.

2. Materials and Methods

2.1. Strain and culture conditions.

T. thermophila, the wild strain, was grown aerobically at 32°C without shaking to exponential phase (72h of growth) in a PPYE broth medium containing 1.5% Tryptone and 0.25% yeast extract [4].

2.1.1. Stress exposure.

Ciliate cultures previously grown in PPYE medium were exposed to different stress conditions after 24 h incubation. We used the peroxide hydrogen as an oxidative stress inducer (from Sigma Aldrich, Saint Louis, MO, USA) at a final concentration of 0. 5Mm.Negative control was carried out under the same conditions but without stressors. The cells were counted after 72 h of growth [4].

2.1.2. Preparation of *Tetrahymena* cell-free extracts.

T. thermophila cells were recollected by centrifugation at 7500 rpm for 15 min. The pellets were washed with PBS buffer (20 mM (pH 7.5) three times to eliminate the broth medium, then resuspended at a ratio of about 3 ml/g (wet weight) in the same buffer. Thereafter, the cells were discarded in the cold with a Bandelin Sonoplus HD 2070 sonicator (90 w, 180 s). The extract was centrifugated, and the crude extract was conserved at – 20°C until enzymatic bioassays[21].

2.2. Biochemical assays.

2.2.1. Antioxidant enzymes.

2.2.1.1. Catalase.

Following the consumption of peroxide hydrogen at 240 nm, catalase activity was measured. The reaction mixture contained 7.5 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.0) and 50 µl enzyme extract. CAT activity was calculated using the molar extinction coefficient of H_2O_2 (0.0394 mM−1 cm−1) and expressed as µmol H_2O_2 consumption/min/mg of protein [21].

2.2.1.2. Superoxide dismutase.

SOD activity was determined by measuring the ability of enzyme extracts to inhibit NADH oxidation caused by superoxide radicals produced in a chemical system at 340 nm. The SOD activity was investigated according to [29]with the flowing assay conditions: Our Final volume (1mL) of assay mixture contained 5 mM EDTA, 0.27 mM NADH, 2.5 mM MgCl2, 3.9 mM 2-mercaptoethanol in 50 mM potassium phosphate buffer (pH 7) and 50 µl enzyme extract. The SOD activity was initiated by adding NADH to a final concentration of 0.27 mm. The activity of SOD was expressed in the unit of activity/mg of protein.

2.2.1.3. Glutathione reductase.

The measure of Glutathione reductase (Gr) activity has been performed according to [30]by following the decrease in absorbance caused by the oxidation of NADPH at 340 nm with assay conditions of: 0.5 mM GSSG, 1 mM EDTA in 50 mM potassium phosphate buffer (pH 7.4) and 50 µl of enzyme extract. The activity started with adding 100 µl NADPH (0.1 mM as a final concentration in 1 mL of the reaction mixture). The Gr activity was expressed as µmol NADPH oxidized/min/mg of protein, using a molar extinction coefficient of NADPH (6.22 mM^{-1} cm^{-1}).

2.2.2. Biochemical biomarkers.

2.2.2.1. GAPDH.

GAPDH activity was determined by monitoring NADH generation at 340 nm [31]. The reaction mixture of 1 ml contained 50 mM Tricine–NaOH buffer (pH 8.5), 10 mM sodium arsenate, 1 mM NAD+ and 2 mM DG3P.The activity of the studied enzyme is expressed in unit of activity/mg of protein by using the molar extinction coefficient of NADH (6220 $M^{-1}cm^{-1}$).

2.2.2.2. Succinate dehydrogenase.

The enzyme was assayed according to[25]: a reaction mixture of 1 ml (final volume) contained 0.053 mM DCIP, 100 mM potassium phosphate buffer (pH 7.4), 0.3 mM EDTA, and 20 µl of protein extract was pre-incubated 10 min at 25°C before adding 50 ml of KCN-Succinate (containing 3.25 mg/ml of KCN in 0.5 M succinate). The measure of activity has been carried at 625 nm. The SDH activity was expressed as µmol DCIP reduced/min/mg protein, using a molar extinction coefficient of DCIP (19,1 M^{-1} cm^{-1}).

2.2.3. Level of lipid peroxidation "TBARS"

Lipid peroxidation level was estimated by the formation of thiobarbituric acid reactive substances (TBARS) and quantified in terms of malondialdehyde (MDA) equivalents using the protocol described by [32]: 100 µl of crude extract was added to 900 µl of a mixture that contained: 0.375% thiobarbituric acid and 15% trichloracetic acid prepared in 0.25 M hydrochloric acid. Then, the preparation was heated at 100 °C for 15 min, and they were cooled in the ice to stop the reaction. After that, we performed centrifugation with 1000xg for 10 min. One then carries out the optical density of the obtained supernatant, have been read at 535 nm. The degradation product of polyunsaturated fatty acids was calculated using the molar extinction coefficient of the MDA (1.56 × 105 M−1 cm−1)[21]. Results were expressed as µmoles MDA per mg protein.

2.3. Statical analysis.

The results were expressed as the means ± standard deviation (SD). Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Tukey's posthoc test using the Prism 9 software for Windows (GraphPad Software Inc., San Diego, CA, USA). Values of $P < 0.05$ were considered statistically significant.

2.4. DNA extraction.

DNA fragmentation was analyzed quantitatively by detecting the laddering pattern of genomic DNA. We have extracted the DNA of *T. thermophila* flowing through these steps:

2.4.1. Pretreatment of *Tetrahymena* cells for DNA extraction.

Harvesting up to 3×10^5 cells by centrifugation; then, we resuspended the cell pellet in 300µl of lysis buffer. After that, we added 100µl of proteinase K (prepared at a final concentration of 25mg/mL) to the sample tube and incubated it for 20 min at 56°C.

2.4.2. Automated DNA extraction using Maxwell®RSC.

DNA was extracted using the automated Maxwell 16 ® RSC (Promega) configuration. Elution tubes containing 100µl nuclease-free water and cartridges containing the sample and buffers were placed within the instrument, and all subsequent steps were automated following the pre-programmed DNA extraction protocol reported in the manual of Maxwell ® RSC.

2.4.3. Electrophoresis gel.

After DNA extraction, RNase was added to each sample to remove RNA. The DNA samples (5 µl) were electrophoresed on a 1% agarose gel in an EDTA (pH 8.0) running buffer

at 50 V. A negative control was performed under the same conditions but without H_2O_2 or molecule. After electrophoresis, the gel was stained in0.5 mg/mL of ethidium bromide solution, and DNA laddering was visualized under UV light.

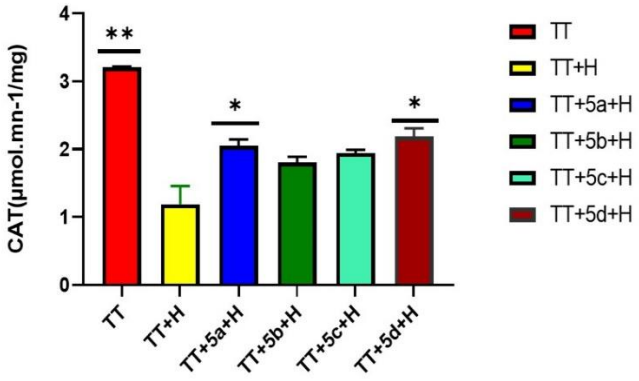

Figure 1. CAT activity in control and experimental groups of *Tetrahymena* treated with Pyrano-[2,3-c]-Pyrazoles derivatives. Values are expressed as means of three separate experiments +/- standard deviations.

3. Results

3.1. Effect of the selected compound on stress biomarkers.

3.1.1. CAT activity.

The enzymatic activity of catalase was measured as described previously. As illustrated in Figure 1, catalase activity was significantly (* $P<0.05$) decreased in H_2O_2 -treated cells. However, the pretreatment with Pyrano-[2,3-c]-Pyrazoles derivatives (Figure 1), increased significatively (* $P<0.05$) the specific activity in these cells in comparison with the stressed cells. We find that the derivatives (5a and 5d) exhibit the best antioxidant effects.

Catalase is an enzymatic antioxidant recognized as the first line of defense against reactive oxygen species. This enzyme work in synergy with SOD and GPx to eliminate ROS. Studies have demonstrated that the decrease of activity of this enzyme in stress conditions is caused mainly by the production of toxic free radicals such as the hydroxyl radical. These free radicals participated in the oxidation of the thiol groups of the proteins, the formation of the disulfide bridge, and as a consequence, the alteration of the structure and function, which induced a loss of enzyme activity [24,33].

3.1.2. SOD activity.

Superoxide dismutase (SOD), a mitochondrial enzyme, prevents oxidative damage and controls the excessive production of ROS in *T. thermophila* cells. Accordingly, our results presented in figure 2 indicate that oxidative stress generates a significant decrease (* $P<0.05$) in the activity of superoxide dismutase in the cells treated by H_2O_2 compared to the control (Figure 2). On the other hand, we see that the treatment with the 5a derivative can protect *T. thermophila spp* cells against H_2O_2-induced stress; a highly significant increase reflects this protective effect (** $P<0.01$) in intracellular SOD activity. While the derivatives 5c and 5d restored the normal activity of the SOD.

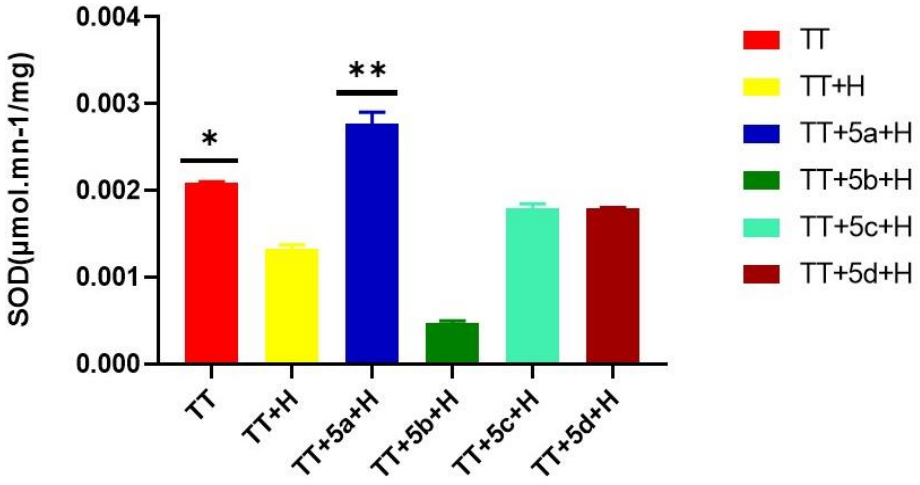

Figure 2. SOD activity in control and experimental groups of *T. thermophila*. Comparison of nontreated control cells and treated cells with Pyrano-[2,3-c]-Pyrazoles. Significantly different from the control value at $p < 0.05$ (Student t-test).

3.1.3. Glutathione reductase (GR)activity

Glutathione reductase (GR) plays a key role in the elimination of oxygen free radicals by a continuous supply of intermediate metabolites, such as reduced glutathione (GSH) used by several antioxidant enzymes (SOD and CAT). According to the results gathered in figure 3, the glutathione reductase activity increased after the induction of oxidative stress. Nevertheless, the treatment of *Tetrahymena* cells with the derivatives of Pyrano- [2,3-c]-Pyrazoles, had shown a decrease in the activity of this enzyme. The derivative (5a) seems to have the best activity against oxidative stress. However, the 5b, 5c, and 5d derivatives also recorded a great protective effect against the H_2O_2-induced stress (Figure 3).

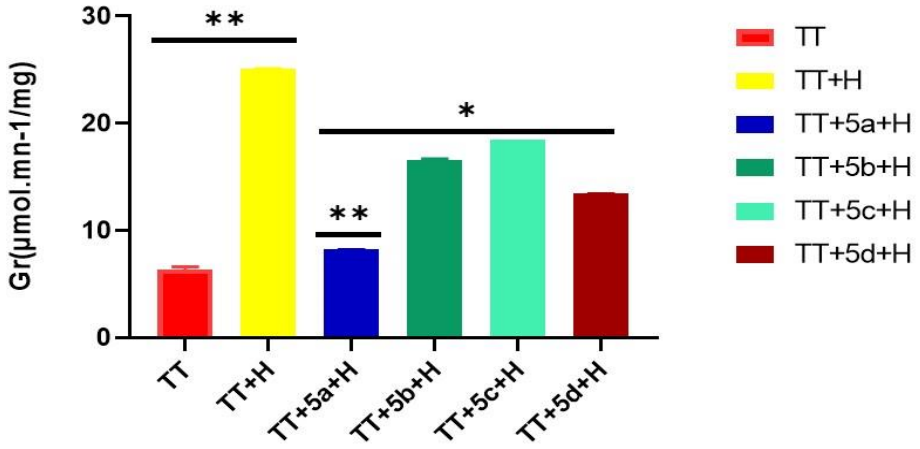

Figure 3. Antioxidant status of glutathione reductase in *Tetrahymena* spp cells treated with Pyrano-[2,3-c]-Pyrazoles derivatives and compared to control group (*Tetrahymena* spp treated with H_2O_2).Significantly different from the control value at $p < 0.05$ (Student t-test).

3.2. Effet of the Pyrano-[2,3-c]-Pyrazoles on metabolic biomarkers.

3.2.1. Succinate dehydrogenase (SDH).

Metabolic markers are also an ideal tool for monitoring the oxidative status of a biological system[34]. The expression of functional succinate dehydrogenase (SDH) is essential for energy production and metabolism for the growth or proliferation of *T. thermophila* cells.

The activity of SDH decreased significantly in samples treated with H_2O_2 compared with normal cells (*T. thermophila*), while after the treatment with molecules derived from Pyrano-[2,3-c]-Pyrazoles, we observed that they expressed their best behavior against oxidative stress (figure 4), especially the two derivatives (5a and 5b) which increased the intracellular activity of SDH. The derivatives (5c and 5d) also showed a significant ($p \leq 0.05$) increase when compared to the control.

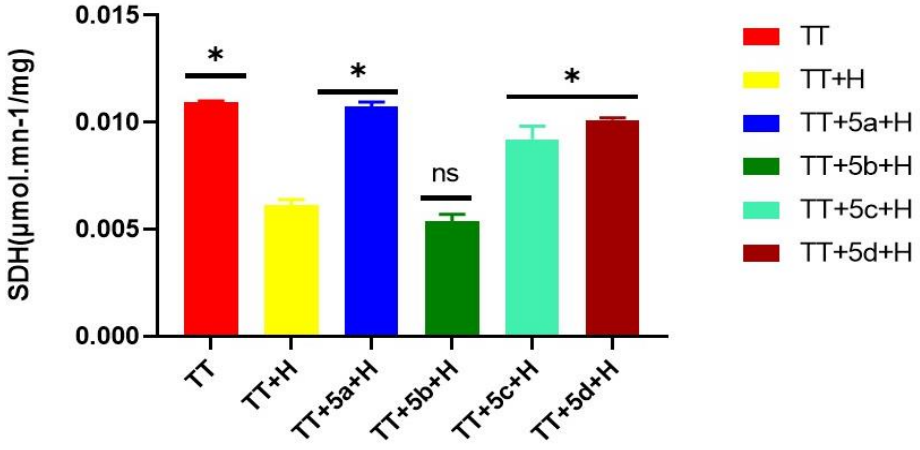

Figure 4. Antioxidant Status of SDH in *Tetrahymena spp* cells treated with Pyrano-[2,3-c]-Pyrazoles derivatives and compared to control group (*Tetrahymena spp* treated with H_2O_2).

3.2.2. The activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), in the *T. thermophila* system, is a promising antioxidative target. This glycolytic enzyme is an essential biomarker that actively contributes to the formation of cellular energy in the form of ATP. The measure of the specific activity of GAPDH reveals a significant ($p < 0.05$) decrease in the activity of this metabolic enzyme (Figure 5). However, the treatment with the derivatives of Pyrano- [2,3-c]-Pyrazoles strengthened the antioxidant system of *T. thermophila* cells. The derivatives5 a and 5b recorded a significant protective effect against oxidative stress (Figure 5). As a result, we can conclude that these derivatives neutralize free radicals by increasing the intracellular activity of GAPDH.

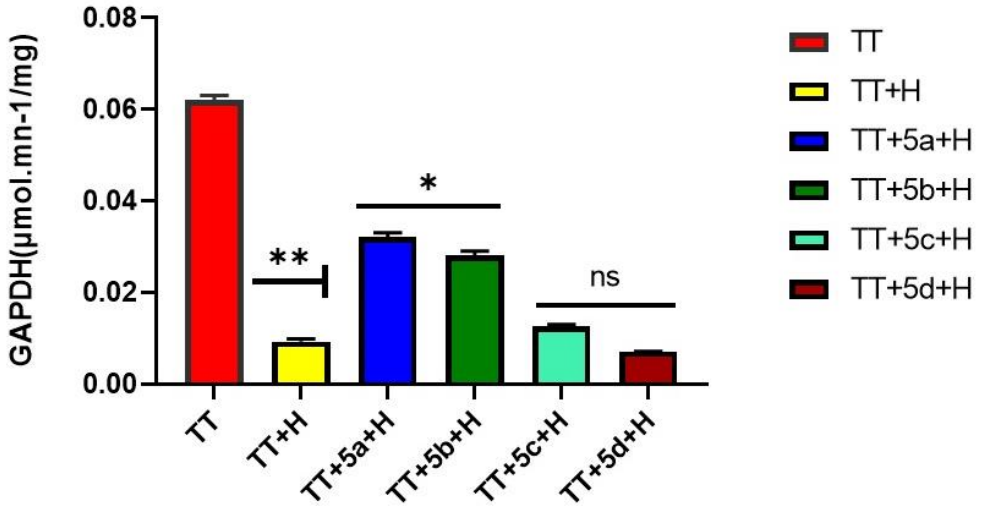

Figure 5. Specific Activity of GAPDH in *Tetrahymena* spp cells treated with Pyrano-[2,3-c]-Pyrazoles derivatives and compared to control group (*Tetrahymena* spp treated with H_2O_2). Each value is the average ± standard error of three replicates (* $P < 0.05$).

3.3. Effect of Pyrano-[2,3-c]-Pyrazoles on lipid peroxidation.

Lipid peroxidation was screened by measuring the level of the Malonaldehyde (MDA), as described previously[35]. The results of Figure 6 highlighted that the exposure of protozoan to stress causes a highly significant (*** P< 0.001) increase in the level of MDA. However, the pretreatment with derivatives of Pyrano- [2,3-c] -Pyrazoles decreased the level of MDA, and we observed that the derivative 5c presented a preventive effect against H_2O_2-induced stress. Likewise, in the presence of the derivatives (5a, 5b, and 5d), a highly significant (***P< 0.001) decrease in this parameter was observed.

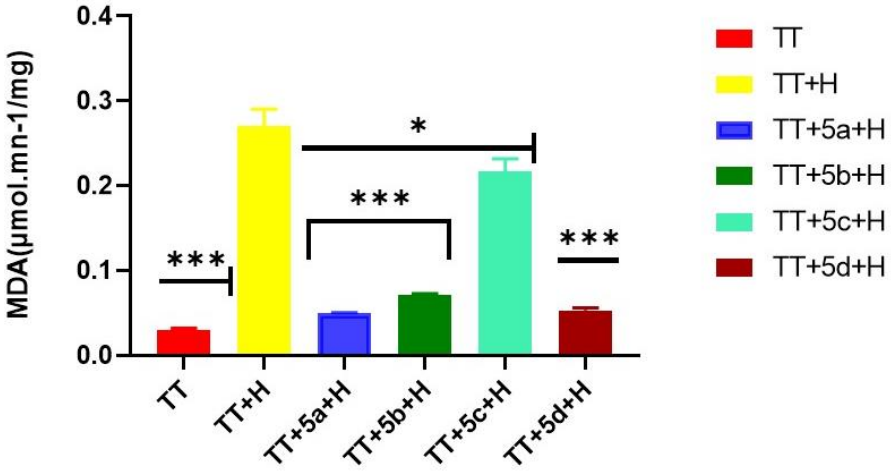

Figure 6. Effect of Pyrano- [2,3-c]-Pyrazoles on TBARS levelin *T. thermophila* cells. *Tetrahymena* cells were pretreated with Pyrano- [2,3-c]-Pyrazoles at a non-lethal concentration (625 µg/mL) for 24 h, followed by incubation with H_2O_2 for another 48 h. The data represent the mean of three independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001, compared with untreated control cells (control) and compared with cells treated with H_2O_2.

3.4. Effect of Pyrano- [2,3-c]-Pyrazoles on H_2O_2-induced DNA damage.

The cytoprotective activity of Pyrano-[2,3-c] pyrazoles against 0.5 mM H_2O_2 induced DNA damage in *T. thermophila* was demonstrated using a ladder assay, in which protozoal DNA was extracted and electrophoretically analyzed to determine the DNA profile (fragmentation).

Figure 7. Effect of 5c derivative on DNA fragmentation in *T. thermophila* cells treated with this compound at a non-lethal concentration (625 µg/mL) for 24 h, followed by incubation with Peroxide hydrogen. The data representedin this figure is the mean of three independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001, compared with untreated group of *Tetrahymena* spp. A: represents Tetrahymenaunder normal conditions, B: *Tetrahymena* group treated with H_2O_2, and C: Tetrahymena treated with 5c compound.

As shown in Figure 7, the DNA gel profile of untreated control cells was compact, indicating its integrity, whereas H_2O_2 treatment induces significant fragmentation, as confirmed by the laddering nature of the DNA band. Pretreatment with the 5c derivative reduces the smearing nature of DNA in a dose-dependent manner. This finding suggests that this selected compound may be able to protect DNA from oxidative damage.

4. Discussion

Hydrogen peroxide (H_2O_2) is a non-free radical essential for various physiological functions such as phagocyte killing, signaling receptor activation, and bacterial ingestion but it is toxic to living organisms when produced in excess. It has been established that excessive H_2O_2 production contributes to the pathogenesis of many diseases, including cancer, atherosclerosis, Alzheimer's diseases, cardiovascular diseases "CVDs," Parkinson's diseases, and sclerosis [36–40].

Several organisms are used in toxicological studies, such as bacteria, fungi, algae, and crustaceans [41]. Surprisingly, Freshwater protozoan ciliates like Tetrahymena spp; besides their implication in the regulation of microbial populations through the ingestion and digestion of bacteria, they are also considered excellent bioindicators of toxicity stress and environmental contaminants due to their high sensitivity to chemical materials [42]. This microbial eukaryotic system is involved in toxicological and pharmacological testing because it contains many genes conserved in several eukaryotes (including humans) [22]. It is a single-cell microorganism that does not possess a protective cell wall. Consequently, stressors reagents could penetrate easily in protozoan cells compared to most complex microorganisms and interact directly with the cellular structures and organelles [43,44]. For all these reasons, ciliates, especially *Tetrahymena spp*, have been well exploited as an ideal tool to screen the impacts of toxic compounds.

In this context, the main purpose of our study was to explore whether pyrano-[2,3-c]-pyrazoles possess a cytoprotective effect on H_2O_2-induced oxidative damage in *T. thermophila*. Pretreatment of these cells with selected molecules allowed this ciliate to be protected from the toxic effects of peroxide hydrogen.

In our preliminary study on the lethal effect of hydrogen peroxide, we also revealed a negative relationship between *T. thermophila* cell viability and stress reagent concentrations (cells treated with 0.4 mM of H_2O_2 significantly reduced the number of cells, and we observed an alteration in their morphology and a decrease in their mobility [4].

Over the last decades, several researchers began to classify H_2O_2 as an inhibitor of the growth of several organisms, including yeasts (*Saccharomyces cervicea*), protozoa (*amoeba*, *paramecium*, and *Tetrahymena spp*), and some pathogenic bacteria (*Vibrio harveyi*) [45–47]. It is known that the levels of proteins, lipids, and carbohydrates in Tetrahymena spp treated with H_2O_2 were significantly altered due to lipid peroxidation caused by ROS. This hypothesis is supported by the monitoring of enzymatic activities observed in this study.

Ciliates can respond to chemical stress very quickly due to their high metabolic ratio, small cell volume, and relatively high surface contact with their environment [48]. These protists can synthesize various proteins and specific enzymes, detoxify reactive oxygen species (ROS) and allow to maintain these ROS at a sufficiently low level. In this work, to study the effects of oxidative stress on Tetrahymena spp profoundly, we were interested in the basic metabolism of this genus of protists, especially the enzymatic system that may play a major role in controlling ROS generation and counteracting oxidative alterations [48,49].

Based on the results of our work, we confirmed that SOD and CAT were reduced in the H_2O_2-treated groups. SOD activity may have decreased due to SOD saturation during the process of converting O^{2-} to H_2O_2. Also, the decrease in CAT activity can be explained by either saturation of this enzyme during the processes of detoxification of free radicals or by proteolysis of this enzyme [49]. This observation is in agreement with the results obtained by [50], who recorded a decrease in SOD and CAT activity in Tetrahymena spp cells intoxicated with a high concentration of H_2O_2. Mar *et al*. also elucidated a significant decrease in SOD and CAT activity in *Tetrahymena* cells exposed to the peroxide of hydrogen [34].

Also, we revealed an increase in glutathione reductase (Gr) activity, which could directly result from depletion in GSH after exposure to stressors. It is well known that the redox cycle of glutathione (GSH) involves glutathione peroxidase and glutathione reductase, which plays an important role in the elimination of ROS via the synthesis of GSH, which protects cells from oxidative damage and cellular injury [33,51,52].

On the other side, producing energy and cellular metabolism requires the expression of functional enzymes such as succinate dehydrogenase (SDH) and Glyceraldehyde 3 phosphate dehydrogenase (GAPDH). Thus, the activity of SDH in *T. thermophila* H_2O_2-treated cells was found to be significantly lower than in untreated cells. This can be explained by the toxicity of H_2O_2 that can deactivate the mitochondrial SDH activity, which can disturb the Krebs cycle and participate in cell death of the studied protists, as shown in many works [21]. The toxic effect of H_2O_2 has also been detected by measuring the specific activity of GAPDH, which plays a key role in the glycolytic cycle of Tetrahymena spp. Thus, the inhibition of this enzyme may be related to post-translational modifications, including S-thiolation(–SNO) of cystine-150 and irreversible sulphonation (–SO_3H), which mediates cell death or dysfunction of this enzyme [26,34,46].

Instead, we demonstrated that the pretreatment of stressed cells with Pyrano-[2,3-c]-Pyrazoles at a non-lethal concentration (625 µg/mL) might prevent the H_2O_2-induced stress by increasing the activity of antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) or producing glutathione (GSH) by the consuming the glutathione reductase (Gr), which can reduce the intracellular activity of this enzymes. Indeed, the supplementation of PPYE medium with these compounds can increase the activity of GAPDH and SDH. These two metabolic enzymes exerted an essential role in the growth and motility of *Tetrahymena spp* [26].

Lipid peroxidation has been proposed as one of the molecular mechanisms involved in H_2O_2-induced toxicity (LPO). Several studies have shown that acute stress causes LPO in Tetrahymena and Paramecium spp cells. Thus, the overproduction of MDA is a key indicator of LPO. In the current study, the MDA level in Tetrahymena spp (stressed group) was increased. These results were confirmed by [49], who recorded increasing the MDA content in the cells of yeast (*Saccharomyces cerevisiae*) treated with cadmium [49,52]. Also, Mountassif *et al.* recorded an increase in the level of TBARS in the cells of freshwater *T. pyriformis* after their treatment with metal salts like cadmium chloride, iron sulfate, and Chromium nitrate [21].

It is interesting to note that the generation of the high level of reactive oxygens radical, especially hydroxyl radicals (OH·), can alter single or double-stranded DNA; as a result, several cytotoxic, mutagenic, and carcinogenic effects are detected [53]. As shown previously in this paper (Figure 7), DNA smearing was observed after the treatment of *T. thermophila* cells with hydrogen peroxide. However, this toxic effect of H_2O_2 can be reduced after the pretreatment of these eukaryotic cells with Pyrano-[2,3-c]-Pyrazole's derivatives. Numerous

studies have reported that antioxidants exert their cytoprotective effects against DNA damage by activating antioxidant systems or by preventing the oxidation of DNA[34,53]

In summary, this study demonstrated that the exposure of *Tetrahymena spp*. to a higher concentration of H_2O_2 inhibits cell growth, creates oxidative stress responsible for ROS synthesis, and alters the mitochondrial respiratory chain that is at the main source of respiratory metabolism, which can be caused by ATP-ase complex damage [21,48]. In response to this damage, *Tetrahymena spp*. triggers the SOD/CAT/Gr/ antioxidant system in cooperation with other metabolic enzymes to control the overproduction of intracellular ROS [26,37,54,55]. Furthermore, the use of Pyrano-[2,3-c]-Pyrazoles derivatives supported the inner defense of *Tetrahymena spp* and had a cytoprotective effect against the harmful effects of H_2O_2 by quenching free radicals while improving the activities of antioxidant enzymes.

A proposed mechanism of action of the selected compounds has been summarized in Figure 8.

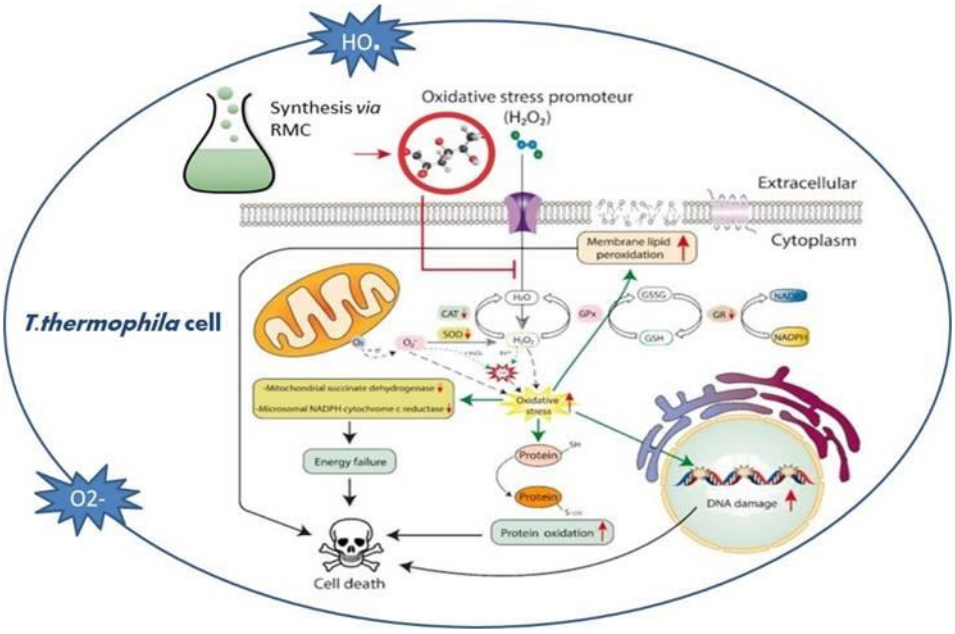

Figure 8. Schematic representation of the possible mechanism of action of Pyrano-[2,3-c]-Pyrazolesagainst oxidative stress-induced cell death.

5. Conclusions

The current study underlines the high sensitivity of *T. thermophila* spp. to Hydrogen peroxide toxicity, which leads to many cellular events such as the generation of free radicals that damage the inner antioxidant defense and the alteration of mitochondrial respiration, lipid peroxidation, DNA destruction, and cell mortality. Therefore, we found that the Pyrano-[2,3-c]-Pyrazoles compounds possess a preventive effect against all these negative consequences of oxidative stress.

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Conflicts of Interest

The authors declare no conflict of interest.

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