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The one-pot synthesis of some bioactive pyranopyrazoles and evaluation of their protective behavior against extracellular H_2O_2 and SNP in *T. Thermophila*.

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Abstract

This study aims to evaluate the antioxidant activity of pyranopyrazoles derivatives synthesized *via* multicomponent reaction and characterized by spectroscopic techniques (^{1}H-NMR; ^{13}C-NMR and IR). All the synthesized compounds were screened for their anti-stress properties *in vivo* by using as a test organism the eukaryotic cells "*Tetrahymena*" exposed to oxidative and nitrosative stress. Thereafter the antioxidant activity *in vitro* is performed by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assays.

Our results clearly showed the protective behavior of the compounds studied **(5a** and **5b)**, especially against H_2O_2-induced stress. On the other hand, the *in vitro* bioassays reveal that the **5a** derivative possesses significant antioxidant activity much greater than ascorbic acid (Vitamin C). All these findings confirmed the prominent antioxidant behavior of the synthesized compounds **5a** and **5b**. However, further studies are needed to describe the mechanism action and the effect of substitution on the activity of these compounds.

Keywords: Pyranopyrazoles, *Tetrahymena,* Stress, Antioxidant Activity, Synthesis.

1. Introduction

Oxidative stress is defined as the aggression of cellular constituents associated with an unbalance between reactive species and the antioxidant system (Kumar et *al*., 2015). The cells are damaged when they are emerged by ROS and RNS, it includes nucleic acids (DNA, RNA), proteins and lipids sequestration, which play an important role in the pathogenesis of several diseases (Fourrat *et al*., 2007).

Recently, a large variety of synthetic products were aberrantly exploited as efficient antioxidant drugs, which supports the inner antioxidant defense system (SOD, GLx, Glutathione, Vitamin C) and overcomes many free radical-related diseases including cancer, diabetes, kidney failure, Alzheimer's, and Parkinson's (Pisoschi and Pop, 2015).

Among these efficient and available synthetic products, we found the pyranopyrazoles derivatives (Dove P, 2016) that are considered as primeval measuring sticks for expansive preclinical and pharmacological investigations (Debasis, Banerjee, and Mitra, 2014) due to their wide-ranging activities such as antioxidant, antibacterial, antiviral, antifungal, anti molluscoidal and analgesic behavior (Mamaghani and Hossein Nia, 2019).

This study began with the synthesis of pyranopyrazoles derivatives *via* a multicomponent reaction (MCR) approach (Zahouily *et al*., 2005) starting from aromatic aldehyde, ethyacetoacetate, malononitrile and hydrazine hydrate in the presence of $Na_2CaP_2O_7$ (Tekale *et al*., 2013) as a catalyst in a green solvent (water) (Maleki *et al*., 2016). Then the bioactivity of these compounds was screened *in vivo* and *in vitro.* In this regard, the well-studied protozoan *Tetrahymena thermophila* (Mar *et al.*, 2017; Sugimoto *et al.*, 1960) was subjected to oxidative and nitrosative stress then was treated with these compounds. This protozoan can be easily cultured and maintained under laboratory conditions (Doerder and Brunk, 2012) and their sensitivity to stress (Zhang *et al.*, 2015) exposure encouraged several researchers to consider this freshwater ciliate as a standard test organism to understand many cellular and molecular processes (Huang *et al.*, 2016). On the other side, these compounds were subjected also to the DPPH test to confirm their protective potential *in vitro* compared to the ascorbic acid as a potent antioxidant.

To be specific, the protective effects of pyranopyrazoles on *T. thermophila* have not been evaluated so far. Hence, we tested, examined and discussed for the first time the effects of these compounds on the physiological and kinetic parameters of *T. thermophila* cells exposed to oxidative stress, however, further studies are needed such as the evaluation of their

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effect on the specific activity of some antioxidant enzymes like: catalase (CAT), superoxide dismutase (SOD) and GAPDH (Glyceraldehyde 3-phosphate dehydrogenase).

2. Experimental

2.1. Material and methods

2.1.1. Instruments

All the chemicals were purchased from Sigma Aldrich and used without any further purification. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 300 MHz in DMSO-d_6 as a solvent. Chemical shifts are given in parts per million (δ-scale). The X-ray diffractometer (XRD) pattern of the diphosphate (DIPH) $Na_2CaP_2O_7$ was recorded on a Bruker D8 Advance X-ray diffractometer with Cu-Kα radiation. The simples were analyzed by FT-IR spectroscopy (using a SCHIMADZU IRAffinity-1S in the range of 4000-400cm^{-1}). Analytical thin-layer chromatography was performed with Silica on TLC Alu foils. Visualization of the developed chromatogram was performed by UV light (254nm). The melting point of the pyranopyrazoles derivatives was determined using a Buchi 510 apparatus.

*2.2. Procedure for synthesis of pyranopyrazoles derivatives **5a** and **5b**.*

The catalyst $Na_2CaP_2O_7$ (20mol %) was added to a mixture of the aromatic aldehyde **1** (salicylaldehyde **1a**, *p*-anisaldehyde **1b**) (1 mmol), ethyacetoacetate **2** (1mmol), malononitrile **3** (1.2 mmol), hydrazine hydrate **4** (2 mmol), and 1 ml of water in a flask fitted with a reflux condenser. The resulting mixture was heated to reflux (an oil bath) with stirring for 20 min. Acetone (2 ml) was added and the mixture stirred for 2 min. The catalyst was removed by filtration and then the resulting crude reaction mixture was poured onto crushed ice and precipitated solid was collected and recrystallized using 96% ethanol to afford pyranopyrazoles **5a** and **5b** (Maleki *et al.*, 2016). The products were well characterized by their melting points ^{1}H, ^{13}C NMR, and IR spectroscopy.

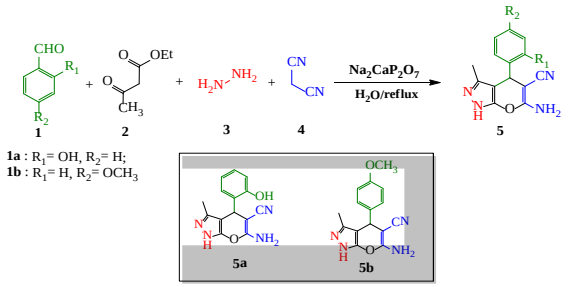


Figure 1. The synthesis reaction catalyzed by the $Na_2CaP_2O_7$.

2.3. Biological Evaluation: microorganisms' growth and toxicological bioassays

2.3.1. Reagents

Peroxide hydrogen and SNP were purchased from Himedia. The culture media such as yeast extract and Tryptone were obtained from Biokar, DPPH, and ascorbic acid were obtained from Sigma-Aldrich. The pyranopyrazoles were synthesized in the Laboratory of Organic Synthesis, Extraction and Valorization according to the references and their structures are summarized in figure 1.

2.3.2. Strains and growth conditions

Before the experiments, the strains *Tetrahymena Thermophila* (SB 1969) were grown axenically in the stock medium of PPYE (1.5% of protease peptone and 0.25% of yeast extract, autoclaved in high-pressure at 120° C for 30 min before use. The culture medium is inoculated with 1 % (v / v) of *Tetrahymena Thermophila* then incubated at 32°C (Addoum *et al.*, 2018; Mar, El Khalfi, and Soukri, 2018).

2.3.3. Cells exposed to stress

To evaluate the effect of stress on protozoan growth, the cultures of *T. thermophila* are treated respectively with 0.4 mM of H_2O_2 and 1.8mM of SNP after 24h of incubation as reported previously in our previous work (Addoum *et al.*, 2018). Each sample (treated and control) was maintained at 32°C for 72h and accompanied by an optical microscope display (A. KRÜSS Optronic) then a cell counting was performed to evaluate the time and number of generation (figure 2).

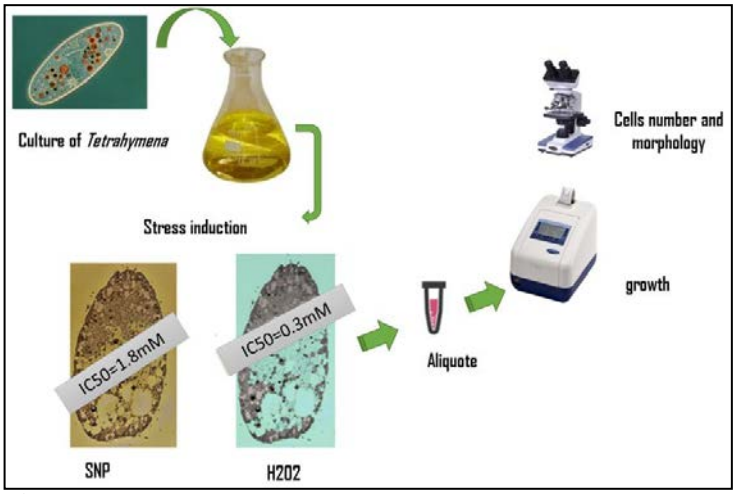


Figure 2. General protocol for stress induction. Cells were grown at 32°C in PPYE medium containing H_2O_2 reagent (0.3mM) or SNP reagent (1.8mM).

2.3.4. Growth measurement

The growth kinetics of *Tetrahymena* was established by daily monitoring of the optical density OD at a wavelength of 600 nm during 120h.

2.3.5. Morphological changes

To observe the morphology of *T. Thermophila* in different experimental conditions (treated and untreated), fresh samples were removed, examined and images were taken using the A. KRÜSS Optronic microscope (magnifications x10).

2.3.6. Cells densities

To determine the cell viability an aliquot of 1ml was taken immediately from the treated cultures and the control then supplemented with a suitable volume of formaldehyde (2-4%). After fixation, the relative cell number was determined by using a hemocytometer counting chamber (Malassez double cells).

2.3.7. Growth Curves

Growth and cell viability curves of *T. Thermophila* were produced based on time-OD and cell density-time. To produce growth curves, flasks of growth medium were inoculated with *Tetrahymena* cells and followed different treatment (stress, antioxidant treatment, and control). During the culture period, growth (OD) and cell number were measured every 24 hours up to 120h. Three biological replicates were included in this study. The obtained values were used to produce the *T. Thermophila* curves and analyzed via the GraphPad prism 8:0.

2.4. Chemicals

2.4.1. Stock solution

A stock solution of pyranopyrazoles (**10mg/mL**) was prepared in dimethylsulfoxide (**DMSO**) (0.01%) (Reddy et *al.*, 2019). Then we prepared a gamut of dilutions from 10-0.6 mg/ML. The prepared molecules were stored at ambient temperature in obscurity.

2.4.2. Toxicity assays

For each derivative two independent tests were performed; we started with the turbidity test that consists to add 5µl of each dilution separately to the culture medium, then we inoculate 1% (v/v) a calibrated inoculum ($1.5x10^5$ cells/mL) of protozoan *Tetrahymena*. Under this experimental condition, the protozoan is maintained at 32°C for 72h. Ultimately, we retain the minimum inhibitory concentration (MIC) to evaluate their protective effects against stress conditions. This concentration was referred to as the lowest concentration of the tested compound that yields a visible growth of the protozoan on the liquid medium tubes. On the other side, this test is confirmed by the investigation of total cells number via microscopic visualization (X10). Estimates of cell viability were reported as a percentage of cell numbers (individuals/ml).

2.5. Antioxidant treatment

To assess the effect of pyranopyrazoles on ROS production; the appropriate stock solution and the test dilutions were selected. Five nominal test concentrations were chosen (table1) and added to a ciliate-containing flask (100 ml of PPYE medium). The treatment with pyranopyrazoles was done at the beginning (t = 0) before the transplantation of *Tetrahymena* cells(105cell/ml). All experiments were performed with protozoan cells in the exponential growth phase. The optical densities of the protozoan are measured by a UV-visible spectrophotometer (Jenway 7315 UV) as described in the literature (Addoum *et al.*, 2018; Errafiy, Ammar, and Soukri, 2013).

Simultaneously the determination of absorbance was accompanied by microscopic imaging analysis and we calculated also the cell densities via hemocytometer technique as described previously in this paper.

In each bioassay, we used two controls: the negative control (Zero mg/mL concentration of pyranopyrazoles), and the positive control (living cells not exposed to stress but treated with pyranopyrazoles). Each concentration was conducted in triplicate.

2.6. DPPH scavenging activity

The detection of the anti-radical activity of pyranopyrazoles was performed by the method of DPPH (El-borai *et al.*, 2016). Various concentrations of selected compounds were mixed with about 1ml of methanol solution then placed in a tube which was added 4ml of DPPH prepared in methanolic solution (0.004%). Then, the tubes are maintained at obscurity for 30 min. The absorbance was screened at 517 nm *via* a UV spectrophotometer; this reduction was accompanied by the color change from purple to yellow. We used as standard various concentrations of the ascorbic acid prepared in distilled water (El-borai *et al.*, 2016).

The percentage inhibition of free radical DPPH (I%) was calculated by the following formula:

$$I\,\% = (A_{control} - A_{sample} / A_{control}) \times 100$$

Where $A_{control}$ reflected the absorbance of the ascorbic acid and A_{sample} is the absorbance of pyranopyrazoles products.

2.7. Statistical Analysis

The statistical data are expressed as mean +/- SD for three successive measurements by using Graph Pad Prism software 8.0. For statistical significance, we used Student's t-test and the P-value was fixed in ≤ 0.05 for a statistically significant difference.

3. Results

3.1. Chemistry

3.1.1. Synthesis

The current study described the catalyzed synthesis of some biologically active pyranopyrazoles by using an ecofriendly catalyst, and these compounds are prepared *via* a one-pot reaction depicted in scheme 2. Primarily, we endeavored to the catalyst four-component commercially available to condense *in situ*; aromatic aldehyde **1**, ethylacetoacetate **2**, hydrazine hydrate **3,** and malononitrile **4** in the water at reflux temperature. The pyranopyrazoles **5a** and **5b** were obtained in high yields (87% and 98 %, respectively).

The structures of the synthesized compounds were confirmed by using spectroscopic techniques.

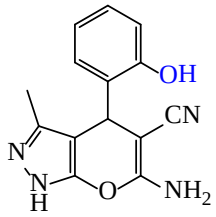
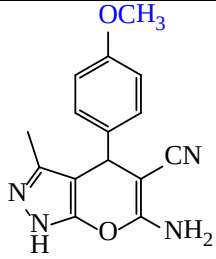
3.1.2. Characterization Spectroscopic data of synthesized products 5a and 5b

6-Amino-3-methyl-4-(2-hydroxyphenyl)-1,4-dihydropyrano[2,3-*c*] pyrazole-5-carbonitrile 5a: White solid; m.p. 215-218 °C; ^{1}H NMR (300 MHz, DMSO-d_6, ppm): δ 1.9 (s, 3H), 4.56 (s, 1H), 6.70 (2H), 6.94-7.18 (m, 5H), 11.00 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6, ppm): δ 9.85, 28.64, 55.06, 104.95, 115.1, 120.79, 123.53, 124.25, 127.55, 128.93, 136.52, 148.39, 159.09, 160.08; IR (KBr, cm^{-1}): 3448, 3419, 3352, 2189, 1660, 1610 (Kanagaraj and Pitchumani, 2010).

6-Amino-3-methyl-4-(4-methoxyphenyl)-1,4-dihydropyrano[2,3-*c*] pyrazole-5-carbonitrile 5b: White solid; m.p. 210-212 °C; ^{1}H NMR (300 MHz, DMSO-d_6, ppm): δ 1.78 (s, 3H), 3.72 (s, 3H), 4.54 (s, 1H), 6.83 (2H), 6.86-7.08 (m, 4H), 12.09 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6, ppm): δ 9.66, 35.51, 55.00, 57.9, 97.89, 113.79, 120.69, 128.44, 135.64, 136.43, 154.77, 158.01, 160.68; IR (KBr, cm^{-1}): 3483, 3255, 3113, 2193, 1643, 1602 (Maleki *et al* ., 2016) .

3.1.3. Physical data of synthesized pyranopyrazoles

Table 1. Physical analytic data of synthesized compounds **5a** and **5b**

Compound	Structural Formula	Aspect	Color	R_f	m.p.(°C)	Yield (%)
5a			White	0.36	215-218	87
5b			White	0.65	210-212	98

3.2. Toxicological assays

3.2.1. Strains and growth conditions

During this study, we screened the effect of the molecule on the protozoan viability to find the toxic lethal concentration. Accordingly, the results gathered in table 2 demonstrate that both products can be used at their non-toxic concentration (625µg/ml). At this concentration, the growth and viability of this protozoan are not affected.

Table 2. The evaluation of Tetrahymena response after treatment with pyranopyrazoles

Toxicological data	Compound	10mg/ml	5mg/ml	2.5mg/ml	1.25mg/ml	0.625mg/ml	0mg/ml
Turbidity	5a	+++	++++	++++	+++++	+++++	++++++
	5b	++	+++++	+++++	+++++	+++++	++++++
Density	5a	$0.8x10^5$	$1x10^5$	$1.4x10^5$	$1.6x10^5$	$1.8x10^5$	$2x10^5$
	5b	$1.6x10^5$	$1.8x10^5$	$1.9x10^5$	$2x10^5$	$2.2x10^5$	

* (+) means the growth of Tetrahymena in the PPYE medium; - means the absence of Tetrahymena growth in the PPYE medium. The four derivatives are added to the PPYE medium at a non-toxic concentration of 1.25mg/ml. The number of cells is expressed as a function of cells/ml.

3.2.2. Treatment with pyranopyrazoles

3.2.2.1. Effect on the growth of Tetrahymena

By comparing and analyzing the results reported in figure 3, we noticed that the supplementation with **5a** and **5b** derivative protected the normal growth of the protozoan especially against the oxidative stress; besides that, we found that the compound substituted with a hydroxyl group **5a** and **5b** are more active against oxidative stress (Figure 3). This effect is more pronounced and appreciated after 48 h of continuous culture.

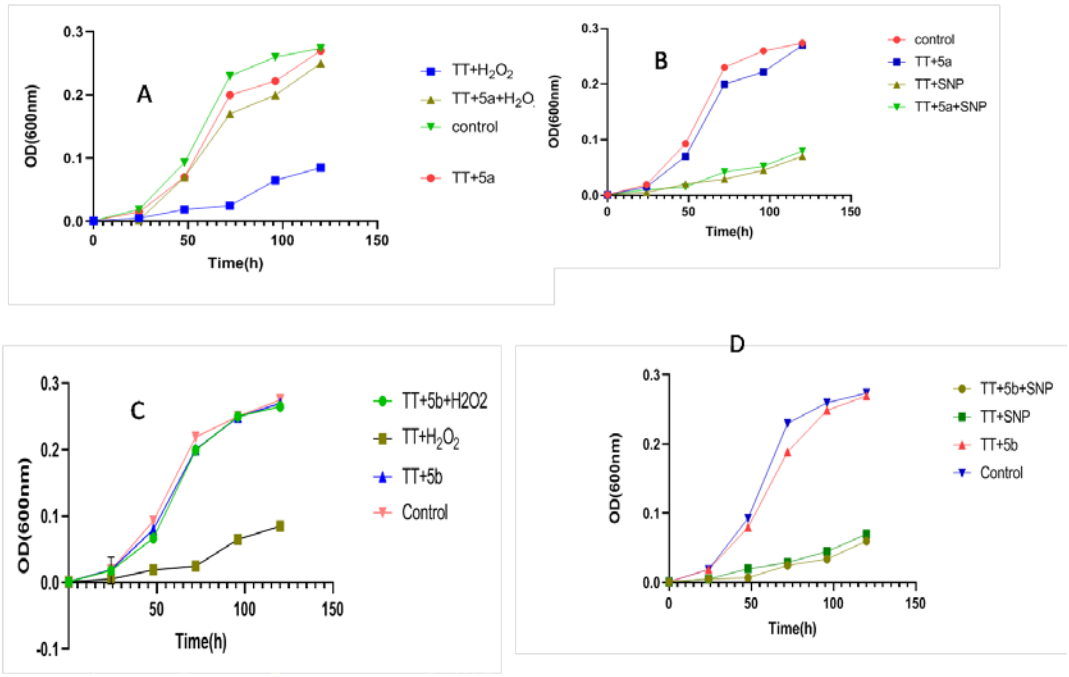


Figure 3. The anti-stress effect of pyranopyrazoles derivatives. Protozoan growth was determined by measuring the absorbance at 600 nm during 120 h. (A) reflected the protective effect of **5a** derivative against oxidative stress and (B) against nitrosative stress. The curve (C) represents the protective effect of **5b** derivative against oxidative stress and (D) against nitrosative stress.

3.2.2.2. Morphological behavior

Light microscopic analysis of treated cells in the PPYE medium showed clearly that *T. Thermophila* exposed to stress are characterized with morphological transformations such as elongated or spherical forms, the close observation demonstrates the presence of conjugation forms, as well as small dark vacuoles that appeared and increased in number with time. After the treatment of samples with **5a** and **5b** derivatives the protist keeps and preserves their normal shape (pear-shape) against stress damage (see figure 4).

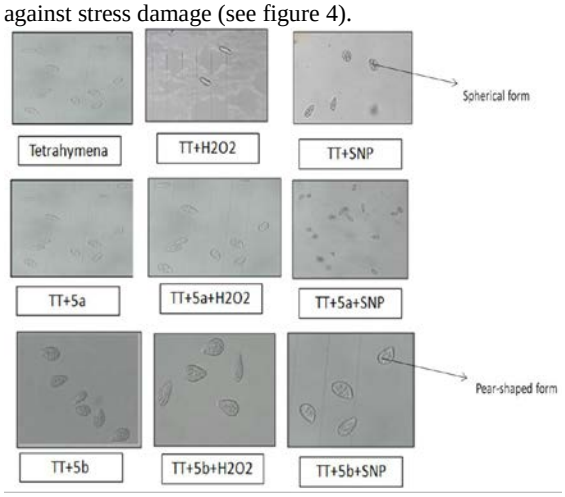


Figure 4. Microscopic analysis images of *Tetrahymena thermophila* (TT) cells. Microscopic images were taken at the objective (x10) of cells grown at 32°C in PPYE medium containing H_2O_2 or SNP as stressors. *Tetrahymena* cells cultivated without stress agents present negative control.

3.2.2.3. The speed swimming of Tetrahymena

The lowest mobility of *Tetrahymena* is recorded under the stress conditions described previously. In close observation, we noticed that some cells moved but not in straight light as the normal shapes. The protozoans exposed to stress are spinning around themselves. Accordingly, an improvement in speed swimming is observed after the addition of the derivative **5a** and **5b** product, which confirms their protective effect (table 3).

Table 3. The protective effect of pyranopyrazoles on physiological parameters of Tetrahymena.

*The density of *Tetrahymena* cells have been detected by using the light microscope and Malassez chamber.

3.2.2.4. The cells densities

Tetrahymena	Density*	Morphology	Speed swimming(motility)
TT	26.10^4 cell/ml	Pear-shaped	Rapid moving
TT+SNP	12.10^4cell/ml	Atypical form	Slight moving
TT+H202	10.10^4cell/ml	Weird form	Slight moving
TT+5a+SNP	13.10^4cell/ml	Atypical form	Slight moving
TT+5b+SNP	14.10^4cell/ml	Atypical form	Slight moving
TT+5a+H_2O_2	25.10^4cell/ml	Pear-shaped	Rapid moving
TT+5b+H_2O_2	20.10^4cell/ml	Pear-shaped	Rapid moving

The results reported in Figures 5 and 6 highlighted that the toxic effect of oxidative stress has reduced a significant number of cells, which confirms that *T. Thermophila* is more sensitive to this agent compared to SNP. The supplementation with pyranopyrazoles to the cultures has preserved a large number of cells. The highest activity was recorded after the addition of the **5a** derivative that protected a high number of *Tetrahymena* cells. As reported in Figures 5 and 6, the cell number of treated samples became similar to the control, which proved the protective potential of these compounds.

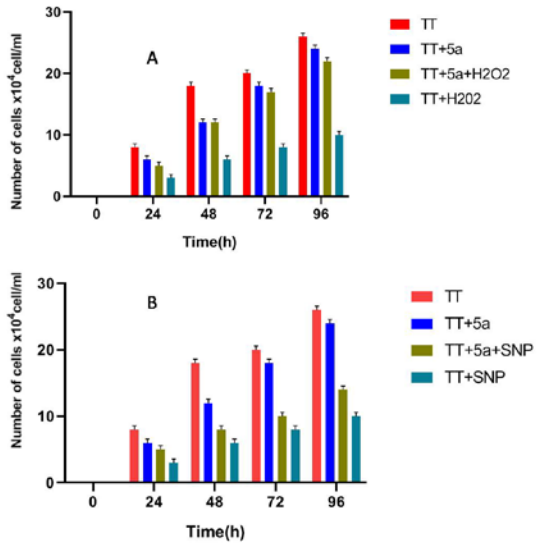


Figure 5. Relative survival of *T. Thermophila* based on cell densities. (A) reflected the protective effect of **5a** derivative against oxidative stress and nitrosative stress(B).

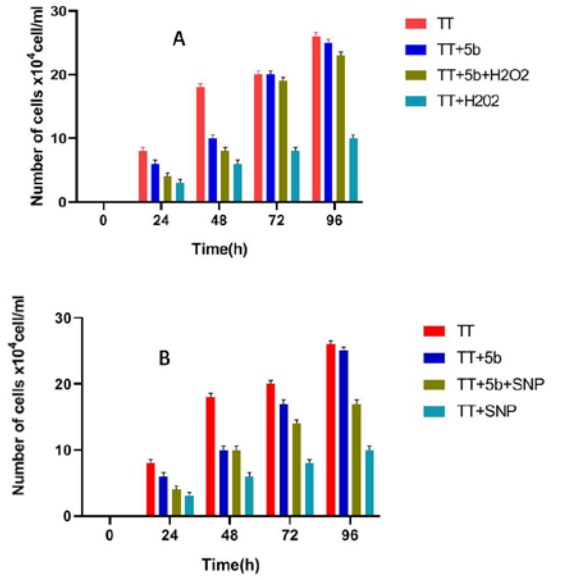


Figure 6. Relative survival of *T. Thermophila* based on cell densities. (A) The protective effect of **5b** derivative against oxidative stress and (B) against nitrosative stress. Error bars reflected the standard deviations for three independent biological replicates, with each experiment being comprised of three individual measurements.

3.2.3. Antioxidant activity in-vitro: test DPPH

By analyzing and comparing the ongoing results we emphasize that the antioxidant activity of the selected pyranopyrazoles **5a** showed an activity that can exceed the antioxidant activity of the ascorbic acid (see figure 7). By analyzing the data reported in these graphs we noticed that the IC_{50} value of **5a** is detected at 6 µg/ml ±0.05 vs. 9 µg/ml ±0.05 for ascorbic acid. In parallel, the antioxidant activity of **5b** is important and shows also a significant activity compared to the antioxidant potential of the reference (see figure 8). Indeed, the IC_{50} value is recorded at 700 µg/ml ±0.05 for **5b** compared to 500 µg/ml ±0.05 for ascorbic acid.

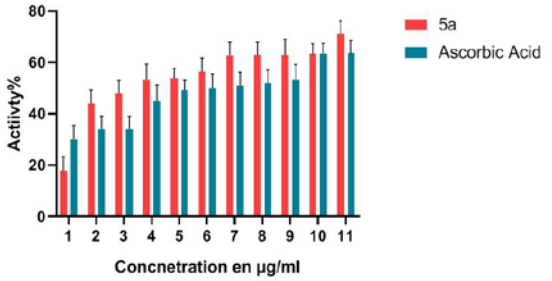


Figure 7. Antioxidant activity (% inhibition of DPPH) under different concentrations of **5a** derivative. Determination of IC50 of the pyranopyrazoles by DPPH method. Ascorbic acid was used as standard.

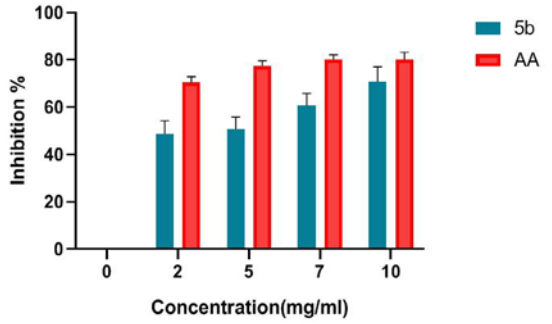


Figure 8. Evaluation of antioxidant properties of pyranopyrazoles **5b** by DPPH assay.

4. Discussion

This paper presents a preliminary study describing the protective effect of pyranopyrazoles products against oxidative and nitrosative stress on *Tetrahymena thermophile* growth, to investigate the possible use of these compounds as anti-stress agents. The evaluation of the acute toxicity of both stressors (H_2O_2 and SNP) on *Tetrahymena* cells will be considered as an important reference value for its further potential utilization to control chronic diseases. This protozoa *Tetrahymena* is characterized by three essential phases of growth (Errafiy and Soukri, 2012): a latent phase that lasts 24 h followed by an exponential phase and a stationary phase that approaches 160h (Mar, El khlafi and Soukri, 2018). When protozoa are treated with stressors, there is a disruption in protozoan growth that varies with the species and stress agent used (Mountassif *et al.*, 2007).

Our finding confirmed that Hydrogen peroxide completely inhibits the growth of *Tetrahymena Thermophila* however the nitroprusside sodium slows its growth (Mar, El khlafi and Soukri, 2018). Similar results have been reported previously by Errafy (Errafiy, Ammar, and Soukri, 2013). Some biological and molecular mechanisms in responses to stress have been examined by using many model organisms including protozoan, yeasts, and vertebrates (Errafiy and Soukri, 2012).

The Toxicities of stressors are routinely determined by analysis of some parameters such as the dynamic growth curves of T. Thermophila; the ciliary movement and the viability of the cells (Addoum *et al*., 2018). In this study,

the authors reported that the treated protozoan with peroxide hydrogen undergo some morphological and physiological transformations, and according to the literature surveys the Hydrogen peroxide acts in oxidative stress as a messenger molecule that diffuses through cells to initiate intermediate cellular effects (Ganjoor and Mehrabi, 2017) such as changes in the shape and recruitment of immune cells (Rahman et *al.,* 2012). Prompted by our finding, we confirmed that the cell densities of the Tetrahymena population were proportional to the IC50 concentration of stressors. At this lethal concentration, cell growth diminished to 50 %. Similar results were observed also by using other stressors such as ethanol (Nilsson, 1974), bromide ethidium (Ashour B, 1980), gelsemine (Ye *et al.*, 2019), and some xenobiotics. These stressors may influence a variety of physiological parameters such as the membrane fluidity, autophagic vacuolization, the rates of cell death and the main speed of *Tetrahymena* cells (Fryb and Henry, 1997)

Nitrosative stress, as well as oxidative stress, can activate a specific route of signalization via the overproduction of NO; this radical can react with ROS such as superoxide anion (O^{2-}) to form peroxynitrite ($ONOO^{-}$) (Sies, 2017). The peroxynitrite is recognized as a biological oxidant that influenced some mitochondrial functions and induced cell death by apoptosis (Sies, 2017).

In our study, we induced nitrosative stress by using the SNP, as a consequence we detected several morphological changes including the appearance of mostly elongated or spherical forms with wide gloomy vacuoles. All that, reflected the harmful effect of SNP that can damage the structure and alter some cellular constituents of the stressed protozoan (DNA; mitochondria; membrane…). On the other hand, excessive NO production can induce a reversible (or irreversible) modification of cellular proteins (Fourrat *et al.*, 2007). NO-induced stress inhibits several enzymes essential for metabolism, including the specific activity of GAPDH via S-nitrosylation correlated with a significant decrease in the growth of protozoan *Tetrahymena Thermophila*. By comparing the effect of both derivatives on the morphology and growth of protozoa in the presence of stressors, we can deduce that **5a** and **5b** compounds have a higher protective effect against the H_20_2-induced stress. The main speed of *Tetrahymena* (motility) is another physiological parameter which should be more studied to assess the cytotoxic effect of stress on the locomotion of this ciliate. The ciliary locomotion of treated protozoan is diminished, which can be an indicator of membrane toxicity; it can be also explained by an alteration of membrane potency. Several research studies indicate that toxicants alter the ion fluxes which have a direct effect on the swimming speed of *Tetrahymena* (Fryb and Henry, 1997)

To the best of our knowledge, the current study is devoted to examining the protective effects of pyranopyrazoles scaffold against stress-induced *Tetrahymena* cells. The supplementation with **5a** and **5b** compound has been improved the motility, the cell number, and the growth curve; which emphasizes the protective effect of both derivatives especially against oxidative damage (Mar *et al*., 2019). We also notified that the **5a** derivative is the most active compound that stimulates also the growth of *Tetrahymena*; this effect can be related to their basic pyranopyrazole skeleton of this compound and the nature of the substituent on the 4-phenyl ring. To remedy the damage caused by oxidative and nitrosative stress, we used two pyranopyrazoles derivatives. These compounds are characterized by several properties, such as antimicrobial, antitumoral, and antioxidant (Shahbazi *et al*. 2019). Nevertheless, these synthesized compounds own a toxic effect against the protozoa *Tetrahymena*. The non-lethal concentration of the two derivatives is used to evaluate their protective effect against stressors. The results reported herein showed clearly that **5a** and **5b** protect protozoa against extracellular H_2O_2, which can be related to the increase of SOD and glutathione peroxidase production (Mountassif et *al.*, 2007). According to the literature surveys, these synthesized products were reported to have significant antioxidant activity. For this purpose, we can say that both compounds used in our study can have a direct effect on the activity of ROS by trapping them or an indirect effect by increasing the production of intracellular antioxidant enzymes (Mar *et al*., 2019).

On the other hand, the study in vitro results reveal that **5a** derivative possesses the top of antioxidant activity compared to the antioxidant of reference. The IC 50 of this product is lower than the value of ascorbic acid (6 µg mL^{-1} for **5a** vs 9 µg mL^{-1} for Ascorbic acid). The second product **5b** besides also a good activity (IC_{50} value is recorded at 7mg/ml) compared to ascorbic (IC_{50} value is 5mg/ml). Overall, our finding established an interesting structure-activity relationship SAR. Likewise, the compound incorporating the hydroxyl group in the *meta*-position of the phenyl group (derivative **5a**) provided an impressive activity against oxidative stress. The most illustrative example has been reported by (Roghayeh, and Mahmoodi 2016), they indicated that the introduction of a hydroxyl group on the aromatic ring of pyranopyrazole can effectively modulate its mechanism of action and increase significantly their free radical scavenging activities (Jouha *et al*., 2017; Harshad G *et al*., 2012). These results are in harmony with our results reported herein.

In summary, the pyranopyrazoles are highly active compounds. Although its chemical structure and stereochemistry have been defined (Reddy *et al*., 2018), nevertheless its biological effects are poorly described.

5. Conclusion

We described herein a simple and efficient approach to synthesize some pyranopyrazoles derivatives. The results reported in this paper confirmed their antioxidant potenti*al.* We are convinced that the current study is a step to underlying the relationship between structure and biological function which will encourage to building-up a variety of motifs occurred via structural modifications onto existing bioactive pyranopyrazoles scaffolds, as a consequence we may enhance the inherent medicinal deed of these compounds or provide their drug-like proprieties.

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Conflict of interest

All the authors declared that there is no conflict of interest.

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