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Research Paper:

Synthesis, Characterization of Pyrano-[2,3-c]-Pyrazoles Derivatives and Determination of Their Antioxidant Activities

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ABSTRACT

Background: Antioxidants are developed to assist the immune system and overcome oxidative stress, the aggression of cellular constituents due to imbalance between reactive oxygen species and the inner antioxidant system. The main objective of this study was to search for new and potent antioxidants to protect humans against diseases associated with oxidative stress.

Methods: In this study, three pyrano-[2,3-c]-pyrazole derivatives were synthesized via Multicomponent Reaction (MCR) approach and were characterized, using a melting point, High-Performance Liquid Chromatography (HPLC), and spectroscopic analyses (IR; ^{1}H-NMR; ^{13}C-NMR). All of the generated compounds were screened for their antioxidant properties *in vivo*, using ciliate "Tetrahymena" as a model organism exposed to oxidative and nitrative stress. They were then studied *in vitro* by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assays.

Results: The results demonstrated that the three compounds (5a, b, c) are biologically active and possess potent antioxidant activities, especially the 5a and 5b derivatives. On the other hand, the *in vitro* bioassays revealed that the 5a derivative possessed a significant antioxidant activity much greater than ascorbic acid. Accordingly, the in silico data are consistent with the experimental data.

Conclusion: These findings confirmed the potent antioxidant property of the synthesized compounds, providing us with new inspiration and challenges to design a library of pharmaceutical compounds with strong activity and low toxicity in the future.

Keywords: Nitrosative, Oxidative stress, *In vitro*, Antioxidant, Tetrahymena

Introduction

Nowadays, a variety of antioxidant supplements have been developed to support the immune system and overcome oxidative stressors that are defined as the aggression of cellular constituents associated with an imbalance between reactive oxygen species and the body's immune system [1]. When human cells are encountered with reactive oxygen and nitrogen species, they suffer significant impairments, including damages to nucleic acids (DNA, RNA), proteins and lipids, which play pivotal roles in the pathogenesis of various diseases [2]. A variety of antioxidants control the over-

production of free radicals within the human cells by inhibiting the oxidative stressors via various mechanisms. Antioxidants assist with scavenging the oxidative species that induce peroxidation, break the auto-oxidative chain reaction, quench oxygen singlets (O_{2}), and inhibit peroxidase formation [3]. Chief among powerful enzymatic and non-enzymatic antioxidants, there are Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GPx), and Glutathione-S-Transferase (GST) [4]. These enzymes maintain the balance between the pro- and anti-oxidant processes within the human cells. Superoxide dismutase catalyzes the conversion of superoxide anion (O_{2}) into hydrogen peroxide (H_2O_2) and molecular oxygen [5]. The CAT protects the cells from the harmful effects of hydrogen peroxide by degrading H_2O_2 into two molecules, water and oxygen singlets [6]. Also, glutathione reductase together with GPx protect the cells from highly toxic hydrogen peroxides, by converting this product into water and oxygen singlets [6].

Over the last decades, pharmaceutical industry began to consider the idea of treating several free radical-related diseases, such as cancer, diabetes, kidney failure, Alzheimer's, and Parkinson's by catalyzing the synthesis of certain bioactive compounds through Multicomponent Reactions (MCRs) [7]. This is an approach in which three or more reactants are combined into a single reaction that leads to the formation of a desired compound. The MCRs cover such topics as sustainability, atom economy, and eco-efficiency. Consequently, we can reduce the required time and energy, which is regarded as step efficiency. Among the compounds produced by using the one-pot reaction we cited, such as pyrano-[2,3-c]-pyrazoles class, these valuable synthetic products are aberrantly exploited as efficient antioxidant drugs and their mimetic effect have renewed much interests within the scientific community. They are considered the cornerstone of a new concept in drug discovery and the pharmaceutical attention has quickly turned towards the "drug-like molecules" subject [8].

The pyrano-[2,3-c]-pyrazoles are the subject of modern biochemistry that is growing rapidly and has stimulated extensive applications. Chief among these are multiple pharmacological and biological properties, such as antioxidant, antibacterial, anti-molluscicidal, analgesic, antiviral potential, and inhibitors of human checkpoint kinase-1 [9]. Upon an extensive literature review, it was confirmed that the wide-range of pyrano-[2,3-c]-pyrazoles activities are linked to the structural base core of the condensed pyrane and pyrazole [10]. In this regard, researchers expended efforts on the development of a single structural framework by associating both motifs (pyrane and pyrazole) in the hope of enhancing the biological activity and pharmacological properties of this compound [11].

The current study aimed to synthesize pyrano-pyrazoles derivatives and examine their antioxidant activities. We first synthesized three derivatives, i.e., 5a, b, c, via rapid, simple, and efficient "one-pot reaction", catalyzed by the $Na_2CaP_2O_7$. Next, we characterized these compounds via spectroscopic analysis (IR; ^{1}H-NMR; ^{13}C-NMR) and the melting point. As part of an ongoing work, the synthesized derivatives were subjected to *in vivo* antioxidant screening assays for the first time, using a eukaryotic unicellular freshwater ciliate "Tetrahymena", a free-living unicellular eukaryote. This species appeared to be a suitable experimental model [12], which has been used widely in pharmacological and environmental studies, because it can easily be sub-cultured and maintained at optimal laboratory conditions [13]. The *in vivo* antioxidant activity of pyrano-pyrazoles derivatives is accompanied by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) test, confirming their protective potential. Specifically, the protective effects of pantoprazole on *Tetrahymena thermophila* have not been evaluated to date. This fact encouraged us to examine and discuss their effects, for the first time, on the physiological and kinetic parameters of *T. thermophila* cells under oxidative and nitrative stress conditions.

Previously, studies have extensively investigated the physiological features of this free-living protozoan [14]. the current study was designed to investigate *T. thermophila* at the behavioral, biochemical and molecular levels, using cellular, enzymatic and protein expression assays, respectively [15]. Based on our current data, we have made exciting findings and progress on this subject; however, some challenges still remain regarding the identification of the cellular and molecular pathways that are activated in Tetrahymena cells after exposure to certain stressors in the long-run.

Materials and Methods

Chemistry

Preparation of pyrano-[2,3-c]-pyrazoles: The three pyrano-pyrazole derivatives were synthesized by a 4-part reaction between aromatic aldehyde 1, ethyl acetoacetate 2, hydrazine hydrate 3, and malononitrile 4. The synthesis occurred in the presence of the pyrophosphate $Na_2CaP_2O_7$ as a catalyst according to a previously described experimental procedure (Figure 1S). Please refer to the "Supplemental Materials" at the end of the

article. The choice of this catalyst was made based on the satisfactory results from the application in certain organic syntheses [16]. The reactions and purification of pyrano-pyrazole 5 were monitored by Thin-Layer Chromatography (TLC) (Table 1). Thereafter, the synthesized products were characterized by melting point and spectroscopic analyses (IR, ^{1}H NMR, ^{13}C NMR) (Figure S2, S3, S4, S5, S6, S7, S8, S9, S10) [17]. The purity of the products was confirmed, using High-Performance Liquid Chromatography (HPLC) method (Figure S11 in supplementary material).

Biological & experimental data

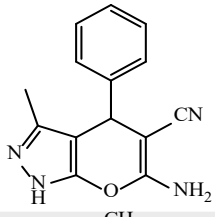
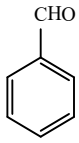
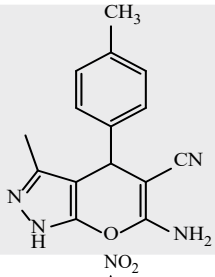
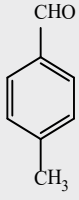
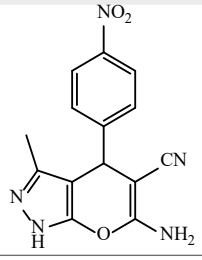
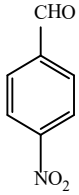
Strains and growth conditions: The toxicological assays were performed by *T. thermophila* strain "SB 1969". The cells were cultured to exponential phase ($1.5x10^5$ cells/mL) in the PPYE medium and maintained at a room temperature of 32°C. This liquid medium consisted of 1.5% (w/v) of peptone and 0.25% (w/v) of yeast extract. Before each experiment, the viability of Tetrahymena cells were examined under light microscopy.

Toxicity test of the molecules: To evaluate the toxicity of pyranopyrazoles on the ciliates, the synthesized compounds (5a, b, c) were taken at a final concentration of 10mg/mL in dimethyl sulfoxide (0.1%, v/v). Next, a panel of nominal concentrations was performed (Table 2). The cells were supplemented with 5µL of each dilution and maintained in continuous culture as described earlier, and samples were taken to check for the toxic effect of the specific compounds (pyranopyrazoles). The number of surviving protozoans was counted after 72hr of exposure to the toxin, and we used the non-lethal concentration of pyranopyrazoles that preserved the normal shape of *T. thermophila*.

Treatment with pyranopyrazoles derivatives: To examine the protective effect of each derivative (5a, b, c), we conducted our experiments under a series of time-course conditions. The experimental and control samples were kept in continuous culture for 96hr at 32°C. To measure the protozoan density, we used 1mL of the culture media hourly for 3hr, and the optical density was determined at 600nm in triplicate, using a Jenway 7315 UV-visible spectrophotometer. Simultaneously, the shape and motility of Tetrahymena cells were examined, using a KRÜSS Optronic light microscope.

Counting cells: The Tetrahymena cells were fixed in 2% formaldehyde and Phosphate Buffered Saline (PBS). Then 5µL of each sample (treated and control) was placed on the hemocytometer plate (Malassez) to count the cells. The cell counting was repeated three times.

Table 1. The synthesis of pyrano-pyrazoles and their physical data

Entry	Compound	Structure	Aldehyde	Yield (%)	Time (min)	Aspect	M.p. (°C)	Lit. M.p. (°C) [Reference]
1	5a			95	60	White, solid	245-246	245-246 [26]
2	5b			96	60	White, solid	205-207	207-209 [27]
3	5c			93	60	Yellow, solid	249-250	248-250 [28]

***In vitro* antioxidant activity:** We used DPPH, a stable radical, to measure the free radical scavenging activity *in vitro*. At this experimental condition, the derivatives 5a, b, c served as the antioxidants and inhibited DPPH, by causing a color change, from deep violet to yellow. The degree of discoloration that occurred represented the radical-scavenging potential for each of the designated compounds. The scavenging activities of 5a, b, c and ascorbic acid were determined at concentrations ranging from 2–10 mg/mL with the DPPH solution 0.004 %.These were left for 30 minutes at room temperature and the absorbance was read again in a UV-spectrophotometer at 517nm [18, 19].

Results

Chemistry

Characterization of the synthesized pyranopyrazoles: The analyses of the results shown in Table 1 demonstrated that the pyrano-[2,3-c]-pyrazoles were produced in good yields (93-95%), influenced by the nature of the substituent at the para position of the phenyl group. The yield decreased when the aromatic aldehyde rich in electron, substituted by a methyl (CH_3) group in the para position, was replaced by an aromatic aldehyde, which was poor in electrons and substituted by a nitro group ($-NO_2$). This result is in agreement with the experimental results obtained previously by Maddila et al. [20]. This indicated that the reactivity of aromatic aldehydes can be accentuated by the introduction of an electron donor group on the benzene nucleus.

Screening of biological activities

Stress effect on tetrahymena cells: As shown in Figure 1A, the oxidative and nitrative stress induced a change in the growth of *T. thermophila*. Notably, the stress caused a lag phase elongation compared to the normal growth conditions. Furthermore, the toxic effect of stress was more pronounced when we used the hydrogen peroxide (H_2O_2) compared to Sodium Nitroprusside (SNP) as a stressor. Under this experimental condition, several cellular parameters were affected, including the morphology; growth rate and ciliary motility.

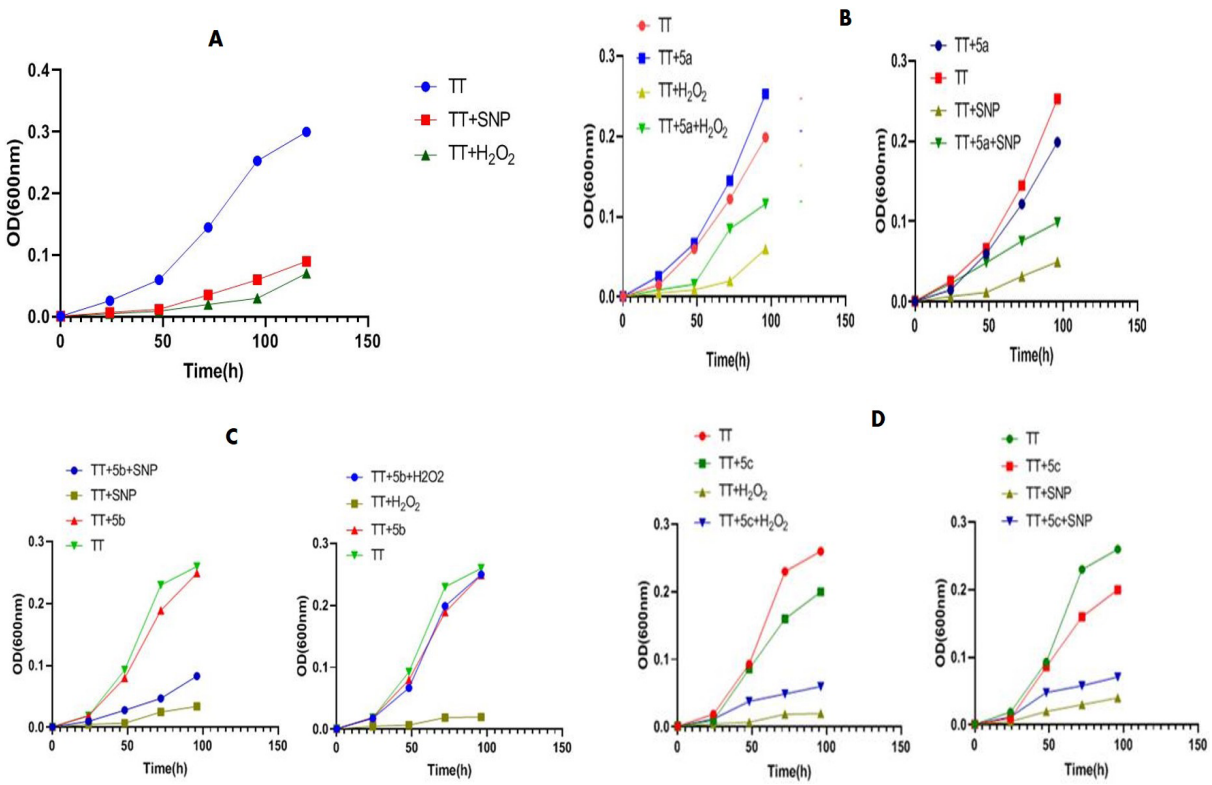


Figure 1. Growth curves of *Tetrahymena thermophila* at different conditions

The stress effect on *T. thermophila* growth (Panel A); The growth curves of *T. thermophila* cultures supplemented with 5a derivative (Panel B); The protective effect of 5b derivative after induction of oxidative and nitrosative stress (Panel C); Protective effect of 5c compound on stressed *T. thermophila* protozoan (Panel D).

Table 2. The evaluation of Tetrahymena response after treatment with pyrano-pyrazoles

Toxicological Data	Compound	10 mg/ml	5 mg/ml	2.5 mg/ml	1.25 mg/ml	0.625 mg/ml	0 mg/ml
Turbidity	5a	+++	++++	++++	+++++	+++++	++++++
	5b	++	+++++	+++++	+++++	+++++	++++++
	5c	+	+++	+++++	+++++	+++++	+++++
Density	5a	$0.8x10^5$	$1x10^5$	$1.4x10^5$	$1.6x10^5$	$2 x10^5$	$2x10^5$
	5b	$1.6x10^5$	$1.8x10^5$	$1.9x10^5$	$2x10^5$	$2.2x10^5$	-
	5c	$0.7x10^5$	$1x10^5$	$1.3x10^5$	$1.6x10^5$	$2x10^5$	-

[+] The growth of Tetrahymena in the PPYE medium; [-] 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.

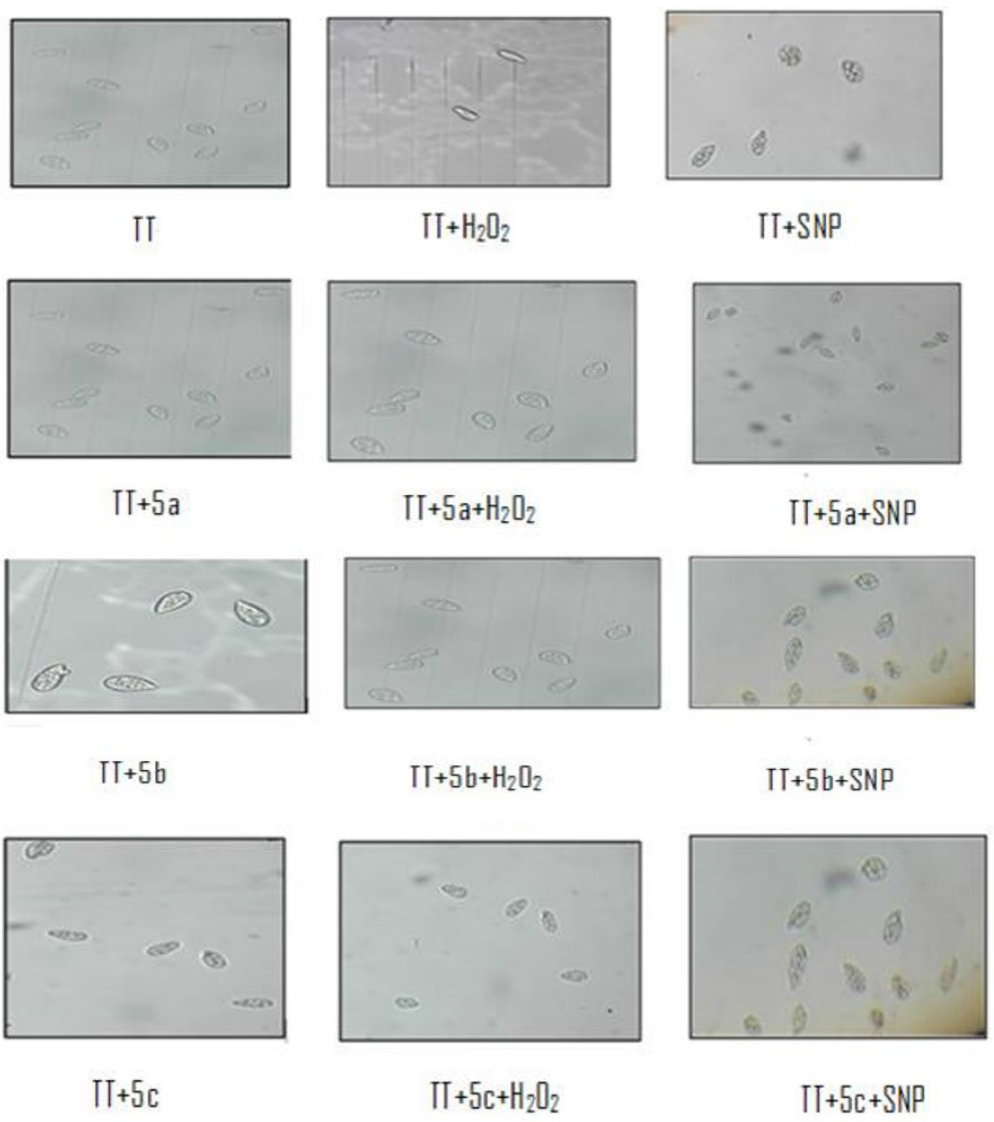


Figure 2. The morphological changes of *Tetrahymena thermophila* (stressed, treated and control)

Microscopic images of Tetrahymena were captured at the objective (x10).

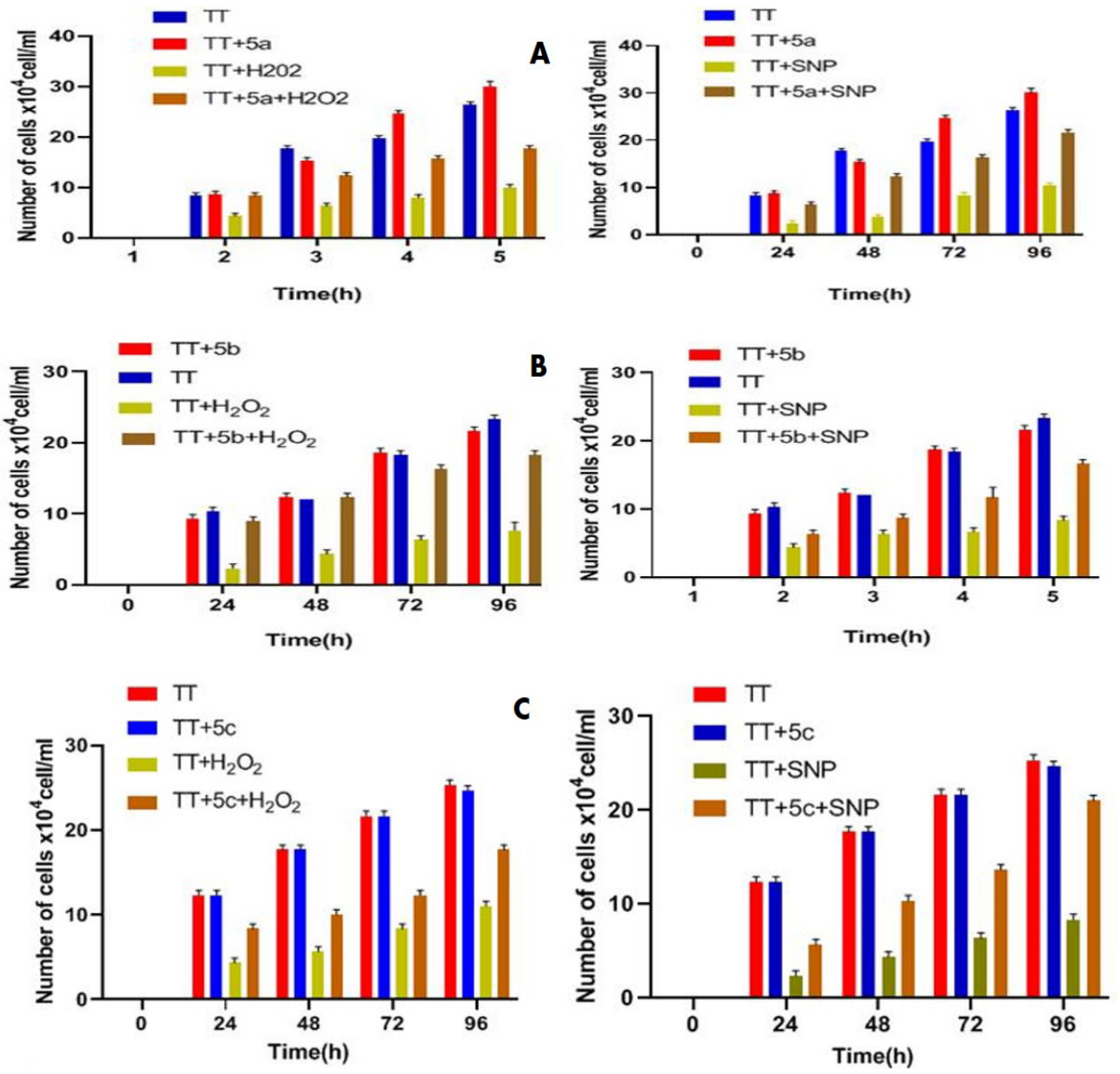


Figure 3. The cell densities of *T. thermophila* samples treated with 5A (Panel A), 5B (Panel B) and 5C (Panel C) derivatives

All studied groups were compared with the Negative Control (NC) and stressed cells. For significant comparisons between all studied groups, a student test t has occurred (*significant at P<0.05 and ** highly significant at P<0.01).

Anti-oxidative and anti-nitrative stress of 5a compound

Effect on the growth of the protozoan: As seen in Figure 1B, the obvious finding confirmed that the compounds (5a) had an impressive effect on the oxidative and nitrative stresses. Also, we noticed that this derivative stimulated the proliferation of Tetrahymena (TT+5a), thus exceeded the normal growth rate of *T. thermophila*.

Effect on the shape and motility of the protozoan: The analysis of the shape of *T. thermophila* cells under stress conditions showed an alteration in their shape, by getting elongated and round (Figure 2). We also observed a decrease in the cell number (Figure 3A). Conversely, the results in Table S2 confirmed that the supplementation with the 5a compound improved the main swimming speed of the ciliated protozoans (Table S3 in ESI).

Effect on cell density: During the growth of Tetrahymena, we counted the cells at several time points up to 96hr. As seen in Figure 3A, the results illustrate that H_2O_2 and SNP significantly reduced the growth rate of the *T. thermophila* population by 50%, while the addition of the 5a derivative to the culture media protected a large number of cells against the stress. Therefore, this derivative increasingly enhanced the cell densities to levels that exceeded the normal rate.

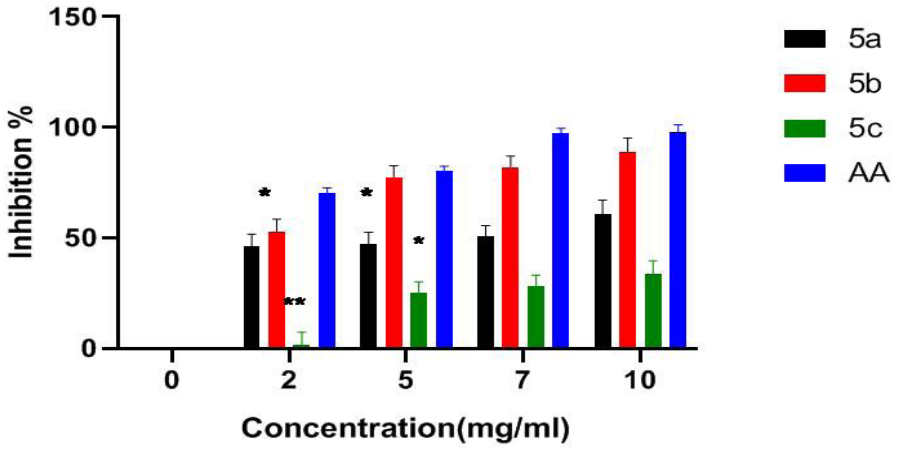


Figure 4. The inhibition of DPPH by the pyrano-pyrazoles derivatives 5A, B, C

The obtained values are expressed in Mean±SD of three independent experiments analyzed via the one ANOVA variance test. Accordingly, significance was calculated by using the student t-test (P<0.05 is considered a significant difference).

Protective effect of 5b compound

Effect on the growth of the protozoan: The above results (Figure 1C) indicated the protective effect of 5b against oxidative stress (S). Under these experimental conditions, the protozoan growth (TT+5b) increased to a level that approached the normal rate for *T. thermophila* (TT).

Effect on the shape of the protozoans: The investigation of the stress effect on *T. thermophila* kinetics led to an alteration in their pear-shaped form, the cells transformed into elongated and spherical shapes. While the supplementation with the 5b compound caused the cells to maintain their normal shape despite the toxicity of both stressors (Figure 2).

The motility of Tetrahymena cells: The results shown in Table S3 (see supplementary material ESI) confirmed that the cells exposed to H_2O_2 and SNP had low motility subsequent to the shape transformation to spherical shape. Nonetheless, the addition of the 5b compound to the culture media led to a relative improvement of the ciliary motility.

Effect on cell density: As shown in Figure 3B, the addition of 5b derivative to the culture media reduced the toxic effects of the stressor. We also documented a substantial effect against oxidative stress compared to a slight effect after the nitrative stress.

Protective effect of 5c derivative

The cell growth: The induced stress led to perturbation of the *T. thermophila* growth rate (Figure 1D), hence after the supplementation with the 5c compound, the protozoans regained their normal growth rate, consistent with the expected antioxidant property of this molecule.

The shape and motility of Tetrahymena: Prompted by our finding, we confirmed once again that cells exposed to H_2O_2 and SNP were less motile with the acquisition of a peculiar shape (Figure 2). Nevertheless, the supplementation with the 5c molecule caused an improvement in the swimming speed of this species (Table S4).

The cell number of Tetrahymena: The results in Figure 3C illustrate that the stress restricted the growth rate of Tetrahymena. The harmful effects of stress were less pronounced when we supplemented the culture media with the 5c molecule, which protected against the stress caused under nitrative conditions. The results correlated with our previous data, confirming the property of this product in preserving the normal shape of the protozoans.

Antioxidant activity *in vitro*: Inhibition of DPPH Radical: The scavenging activity of 5a,b,c derivatives compared to ascorbic acid is presented in Figure S15, where the Ascorbic Acid (AA) represents the absorbance of the control (in a methanolic solution of DPPH) and a sample represents the absorbance of the tested compound in a methanolic solution of DPPH. The calculated inhibition levels are summarized in Figure 4, indicating that 5a and 5b compounds showed significant scavenging effects while the 5c caused a very low effect, spanning from 1.83 to 33.5%.

Discussion

The current study investigated *T. thermophila* at the behavioral, biochemical and molecular levels and has pro-

duced exciting findings on the subject. However, there remain challenges on the mechanisms that are activated in Tetrahymena cells after exposure to chronic stressors.

This paper reports the toxic effects of oxidative and nitrative stresses on this species under the selected experimental conditions, which affected the cellular morphology, growth and ciliary motility after treatment of the protozoans with H_2O_2. The oxidative stress inhibited or slowed down the growth rate of *T. thermophila* in a dose-dependent manner. The lethal effect of hydrogen peroxide has been well studied by Errafiy et al. [21]. This stressor has also inhibited the growth of other organisms, such as yeasts (*Saccharomyces cervicea*)-protozoa (*amoeba*) and several other pathogenic bacteria (*Vibrio harveyi*) [21].

The SNP-induced stress had a harmful effect on this species similar to that of H_2O_2. This might be due to the fact that SNP is a source of NO gas, the overproduction of which can modify certain cellular and molecular mechanisms. For example, the expression profile of some proteins, such as Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) plays a key role in the carbon metabolic route in Tetrahymena cells [15, 18]. This is in agreement with the previous work, demonstrating that both stressors affected the target protein directly and reduced its activity; which in turn affected the Tetrahymena's growth rate [21].

The current study was devoted to the assessment of the protective effect of the synthesized products 5a,b,c. The results of our experiments demonstrated that the 5a and 5b compounds exhibited a prominent antioxidant effect as compared to 5c. These derivatives are likely to protect the cells' morphology and shape, while increasing the cell number and improving the motility. Consequently, we corrected the retarded growth rate that was likely related to the overexpression of ROS and RNS. Previous studies on Tetrahymena used such natural products, as sage essential oils as an antioxidant supplement, which improved the effect against stressors through activating such enzymes as catalase, SOD and GAPDH [22].

The protective effect of the compounds 5a,b,c may be explained by such hypotheses as their potential to scavenge RONS, which is consistent with the activation of the antioxidant defence. Meanwhile, we believe that their activity can also be linked to the structure of the pyrazoles scaffold. Previous studies have shown that H_2O_2 and SNP significantly decrease the activities of CAT, SOD, and GPx in Tetrahymena cells [22]. Nevertheless, the treatment with the pyrano-pyrazoles may preserve the activities of these enzymes through the restoration of SOD level and activation of GPx that block the glutathione oxidation, mediated by H_2O_2 [22]. These compounds also induce the activity of CAT, inhibit sodium nitroprusside [23] and H_2O_2-stimulated lipid peroxidation. Consequently, these cellular activities tend to maintain the oxidative balance [2] (Figure S18).

Pyrano-pyrazoles provide valuable tools for chemists and have a significant importance in the pharmaceutical field due to their multiple activities. These span their drug-like and mimetic proprieties, expanding the use of plants and the derivatives by designing the analog of these natural products [11]. We successfully designed useful heterocyclic compounds through the use of greener protocols, including $Na_2CaP_2O_7$ as an efficient heterogeneous catalyst [24, 25]. The main advantage of this procedure is obtaining the desired products at high purity, short reaction time, ample yields, and simple procedures, compared to the classic methods described in the literature [7].

In this context, the derivatives have considerable antioxidant potentials that are justified based on the structure and function studies. Our literature review confirmed that the substitution at N or C-3 position of 1H,4H-dihydropyrano-(2,3-c)-pyrazoles could potentially improve their antioxidant activities. These properties increased when the aromatic ring contained an electron-donating methoxy group (OCH_3). This finding is in agreement with our experimental results observed in the current study. The methyl group on the phenyl ring had an effective impact on the antioxidant activity of the pyrano-pyrazoles 5b compared to that of 5c, containing the electron-withdrawing nitro group [20] (Figure 4).

We can conclude that a single administration of this compound controlled the circulation of RONS in Tetrahymena cells, likely by suppressing the lipid peroxidase pathway and restoration of the enzymatic activities. Our observations are in agreement with those reported by a previous *in vitro* study on the bioactivity of pyrano-pyrazoles [26, 27].

In summary, this study demonstrated that the use of the pyrano-pyrazole derivatives had cytoprotective properties against the harmful effects of stressors, such as H_2O_2 and SNP, by quenching free radicals while improving the activities of antioxidant enzymes.

Conclusions

In the current study, we developed a simple protocol for the synthesis of pyrano-[2,3-c]- pyrazoles derivatives, 5a,b,c, via a 4-component condensation reaction

procedure. The desired compounds were obtained in ample yield and their structures were confirmed by spectral analysis. These compounds appear to be biologically active, especially the derivatives containing methyl (CH_3) group at the para positions, i.e., 5b. The protective mechanism of these compounds against cellular stressors is characterized by inducing antioxidant enzymes and diminishing lipid peroxidation. These findings are an excellent starting point to explore the cellular and molecular mechanisms of the novel 5a,b,c molecules toward the development of more efficient antioxidant drugs in the future.

Limitations of the study: Given the above facts, a single bioassay is not able to reflect the full picture of the derivatives' effects on an organism.

Recommendations for future studies: We recommend using a battery of representative *in vitro* and *in vivo* experiments. Morphological, structural, biochemical, and especially molecular assays provide broad information about the capacity of these compounds to counter the toxic effect of stressors.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

Funding

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Author's contributions

Conceptualization and supervision: Abdelaziz Soukri and Bouchra El Khalfi; Methodology: Abdelhakim Elmakssoudi and Ouafaa Aniq Filali; Investigation, writing – original draft, and writing – review & editing: All authors; English editing: Reda Derdak and Souraya Sakoui; Data analysis: Abdelhakim Elmakssoudi and Boutaina Addoum; Funding acquisition and resources: Abdelaziz Soukri.

Conflict of interest

The authors declared no conflict of interest.

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Supplemental Materials

1. General information

The crystalline structure of the $Na_2CaP_2O_7$ was identified by using X-Ray Diffraction (XRD) analysis (Bruker D8 Advance X-ray diffractometer with Cu-Kα radiation: λ= 1.5406 A°). The melting point of the pyrano-pyrazoles derivatives was determined using a Buchi 510 apparatus. The NMR spectra of ^{1}H and ^{13}C were recorded on a Bruker 300 MHz in DMSO-d_6. The chemical shifts (δ) are expressed in ppm. The IR spectra of the samples were acquired as KBr pellets on FTIR (IR Affinity - 1S, Fourier Transform Infrared Spectrophotometer, Shimadzu). Scanning Electron Microscope (SEM) studies were performed on a HIROX SH-4000M. Analytical thin-layer chromatography was performed with Silica on TLC Alu foils purchased from Sigma Aldrich. Visualization of the developed chromatogram was performed by UV light (254nm). HPLC analyses were carried out on a Shimadzu LC-20AT equipped with a C18, 5µm x 250 mm column, detection at λ=254nm.

Analysis of the pyrano-pyrazoles 5a, 5b, and 5c were carried out using an isocratic mobile phase of 2% acetonitrile-98% water (The flow rate was 1 $mL.min^{-1}$). The mobile phase was filtered through a 0.45µm nylon membrane filter before use. All reactions were carried out under air. Solvents and starting materials (Aldrich) were used without further purification.

2. General procedure for preparation of pyranopyrazoles 5a-c

The catalyst $Na_2CaP_2O_7$ (20mol %) was added to a mixture of the aldehyde 1 (1 mmol), ethyl acetoacetate 2 (1mmol), hydrazine hydrate 3 (2 mmol), malononitrile 4 (1.2 mmol), and 1 ml of water in a 5 ml flask fitted with a reflux condenser. The resulting mixture was heated to reflux (an oil bath) with stirring for 60 min. Acetone (2 ml) was added and the mixture stirred for 2 min. The products and catalyst were isolated as described above and recrystallized from 96% ethanol (20 ml) to afford pyrano[2,3-c] pyrazoles 5a-c (Figure S1).

3. NMR and infrared spectral analysis

See Figures S2, S3, S4, S5, S6, S7, S8, S9, S10.

4. The HPLC analysis of pyranopyrazoles 5a,b,c

See Figure S11.

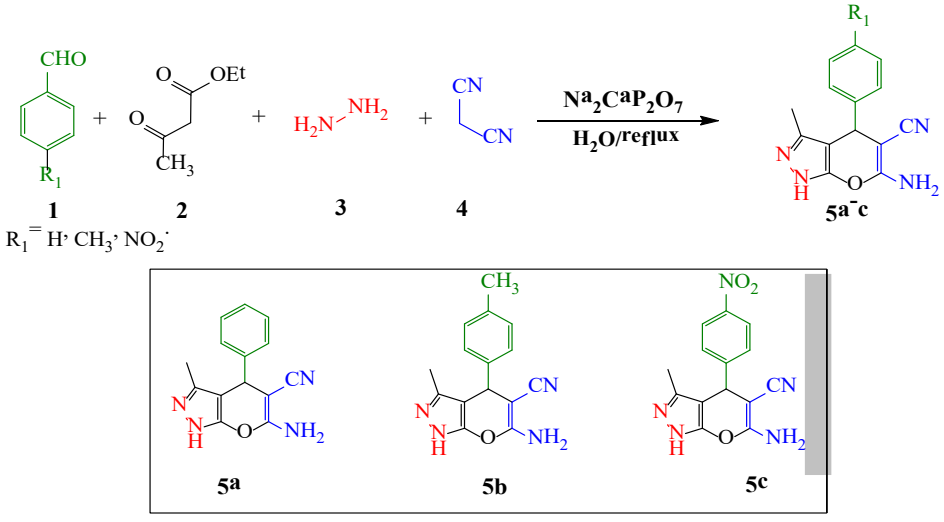

Figure S1. The synthesis of pyrano-pyrazoles catalyzed by $Na_2CaP_2O_7$

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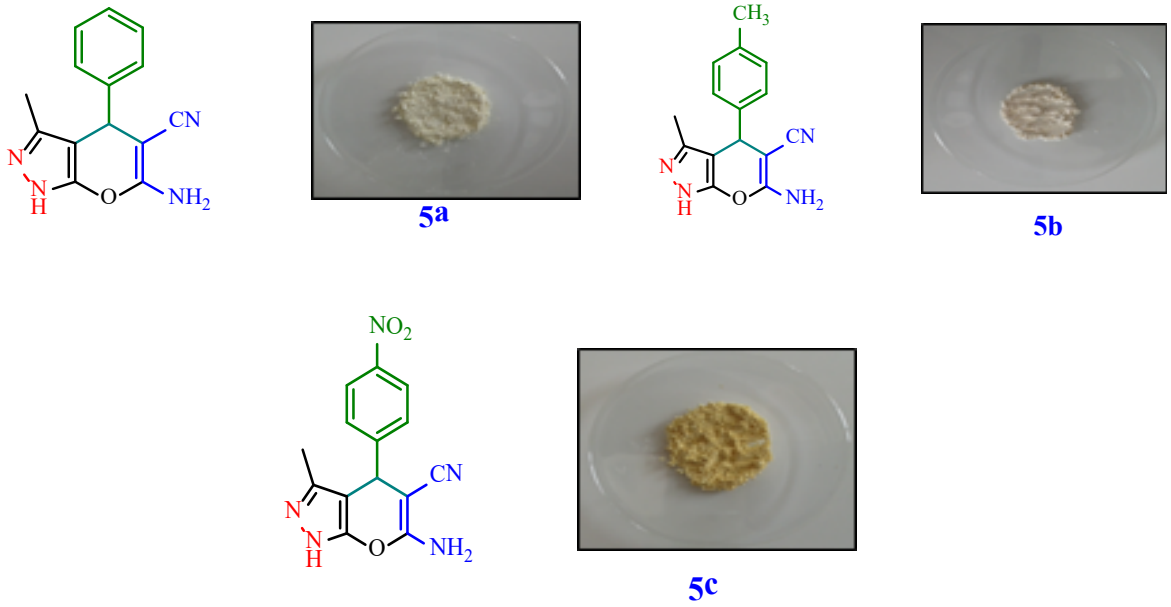

Figure 5A: 6-Amino-3-methyl-4-phenyl-1,4-dihydropyrano [2,3-c]pyrazole-5-carbonitrile; White solid; m.p. 245-246 °C (lit. 244-245 °C)(1) ; R_f (20% AcOEt/hexane) 0.61; (HPLC): (t_R = 3.63 min); ^{1}H NMR (300 MHz, DMSO-d_6, ppm): δ 1.73 (s, 3H), 4.54 (s, 1H), 6.83 (s, 2H), 7.11-7.45 (m, 5H), 12.11 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6, ppm): δ 9.7 (CH_3), 36.2 (pyran C_4), 57.2 (C_5-CN), 97.6 (C_8), 120.7 (CN), 126.7, 127.4, 127.5, 128.4, 128.5, 135.5 (aromatic carbons), 144.4 (C_3), 154.7 (C_7), 160.8 (C_6-NH_2); IR (KBr, cm^{-1}): 3473 (NH_2), 3170 (NH), 2191(CN), 1649 (C=N), 1604 (Ar).

5B: 6-Amino-3-methyl-4-(4-methylphenyl)-1,4-dihydropyrano [2,3-c]pyrazole-5-carbonitrile; White solid; m.p. 205-207 °C (lit. 206-207 °C)(2); R_f (20% AcOEt/hexane) 0.7; (HPLC): (t_R = 3.53 min); ^{1}H NMR (300 MHz, DMSO-d_6, ppm): δ 1.75 (s, 3H, CH_3), 2,22 (s, 3H, CH_3), 4.51 (s, 1H, C_4-H), 6.81 (s, 2H, NH_2), 7,03 (m, 4H, H_{Ar}), 12.06 (s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6, ppm): δ 9.7 (CH_3), 20.5 (CH_3), 35.8 (pyran C_4), 57.4 (C_5-CN), 97.7 (C_8), 120.7 (CN), 127.3-129.1, 136.7 (6 aromatic carbons), 141.4 (C_3), 154.7 (C_7), 160.7 (C_6-NH_2) ; IR (KBr, cm^{-1}): 3483 (NH_2), 3113 (NH), 2193 (CN), 1641 (C=N), 1602 (Ar).

5C: 6-Amino-3-methyl-4-(4-nitrophenyl)-1,4-dihydropyrano [2,3-c]pyrazole-5-carbonitrile; Wellow solid; m.p. 248-250 °C (lit. 249-250 °C) (3); R_f (20% AcOEt/hexane) 0.64; (HPLC): (t_R = 3.56 min); ^{1}H NMR (300 MHz, DMSO-d_6, ppm): δ 1.8 (s, 3H, CH_3),4.51 (s, 1H, C_4-H), 7.05 (s, 2H, NH_2), 7,46 (d, 2H, H_{Ar}), 8,21 (d, 2H, H_{Ar}), 12.20 (s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6, ppm): δ 9.6 (CH_3), 35.8 (pyran C_4), 55.9 (C_5-CN), 96.5 (C_8), 120.4 (CN), 123.8, 124.1, 128.8, 135.8 (5 aromatic carbons), 146.3 (C_3), 152.0 (C_7), 154.6 (CAr-NO_2), 161.1 (C6-NH_2); IR (KBr, cm^{-1}): 3477, 3228 (NH_2), 3118 (NH), 2196 (CN), 1651 (C=N), 1595 (Ar).

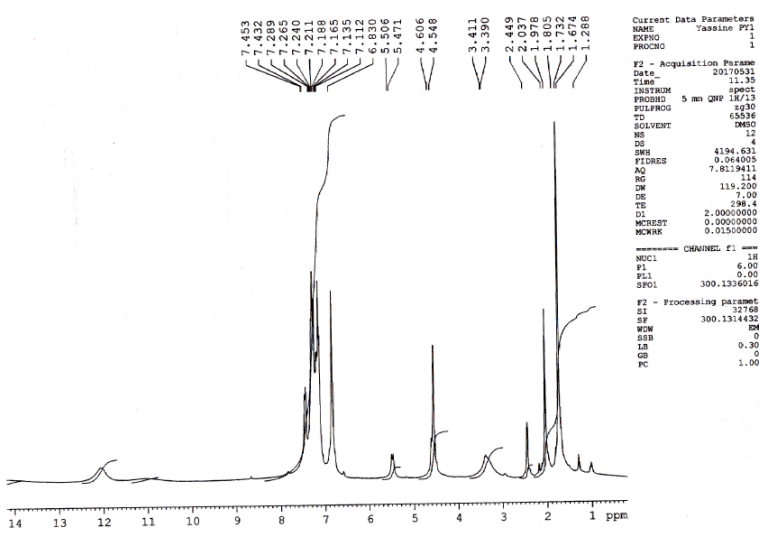

Figure S2. The ^{1}H NMR pattern of the 5A derivative

6-Amino-3-méthyl-4-phényl-2, 4-dihydropyrano [2, 3-c] pyrazole-5-carbonitrile (5A)

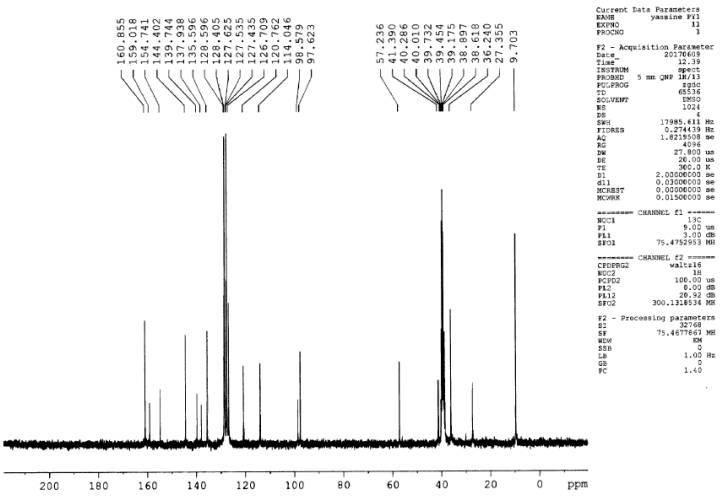

Figure S3. The ^{13}C NMR pattern of the 5a derivative

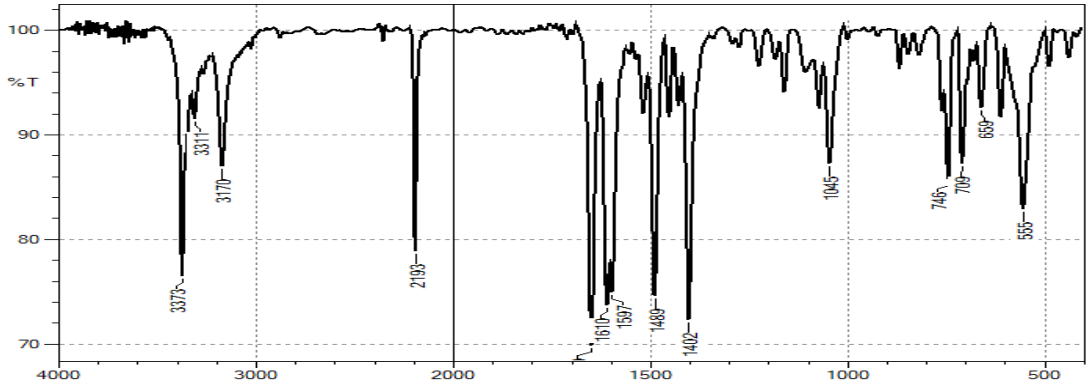

Figure S4. The infrared pattern of the 5a derivative

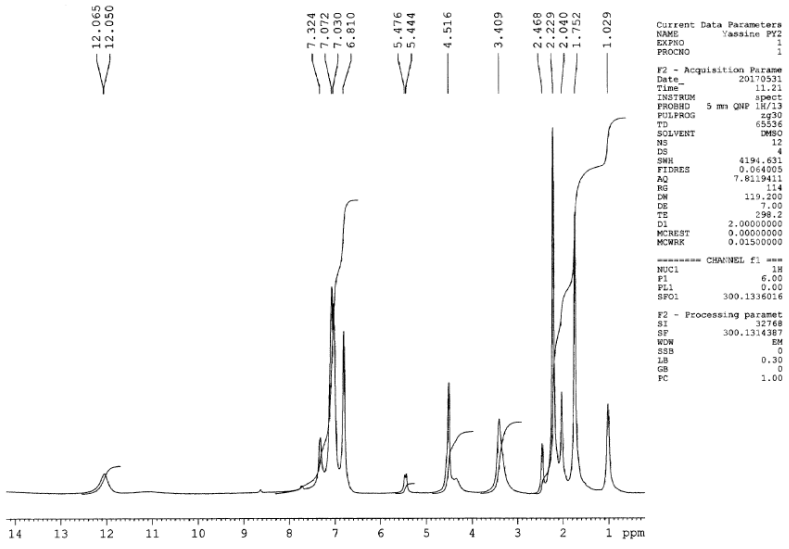

Figure S5. The ^{1}H NMR pattern of the 5B derivative

6-Amino-3-méthyl-4-(4-méthylphényl)-2,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (5B)

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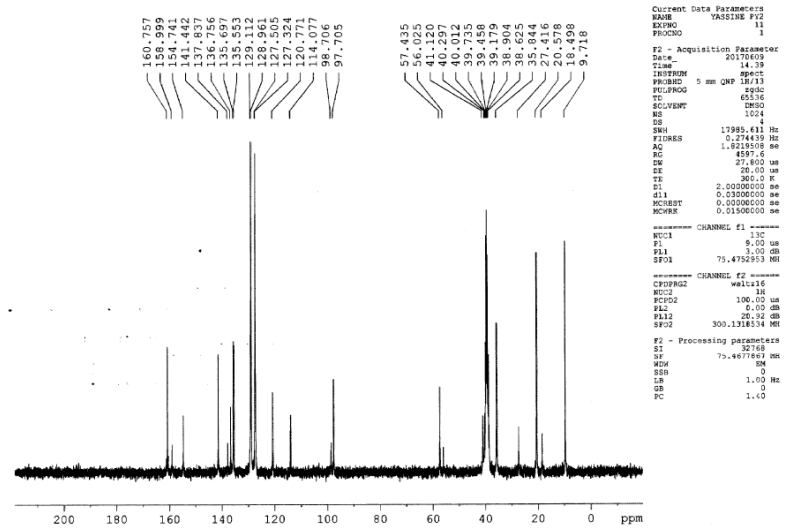


Figure S6. The ^{13}C NMR pattern of the 5B derivative

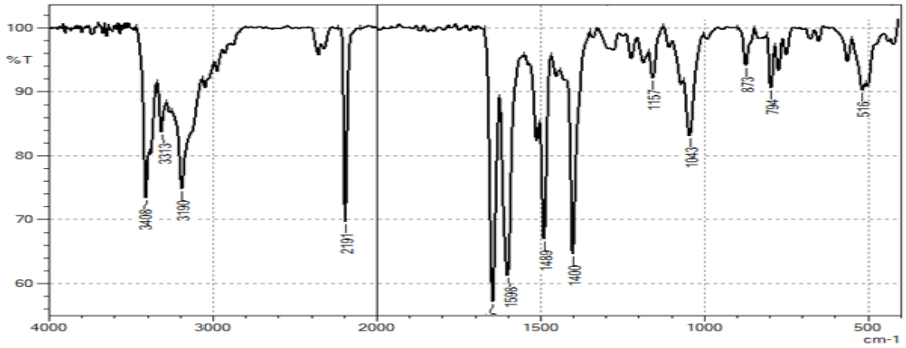


Figure S7. The infrared pattern of the 5B derivative

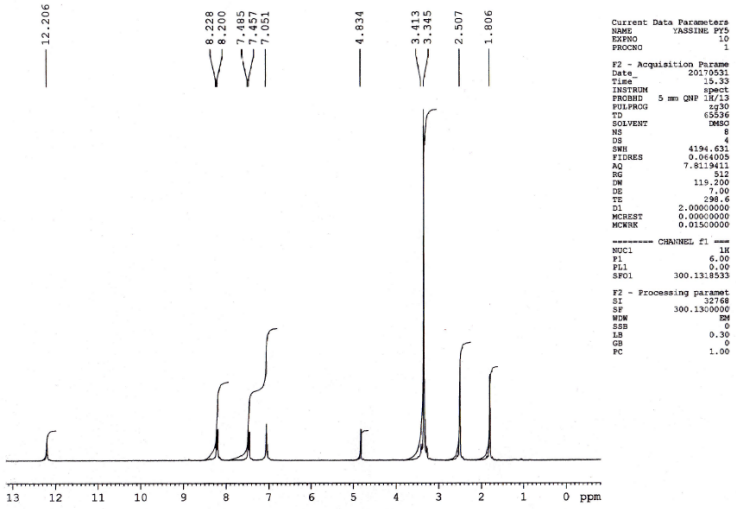


Figure S8. The ^{1}H NMR pattern of the 5C derivative

6-Amino-3-méthyl-4-(4-nitrophényl)-2, 4-dihydropyrano [2, 3-c] pyrazole-5-carbonitrile (5C)

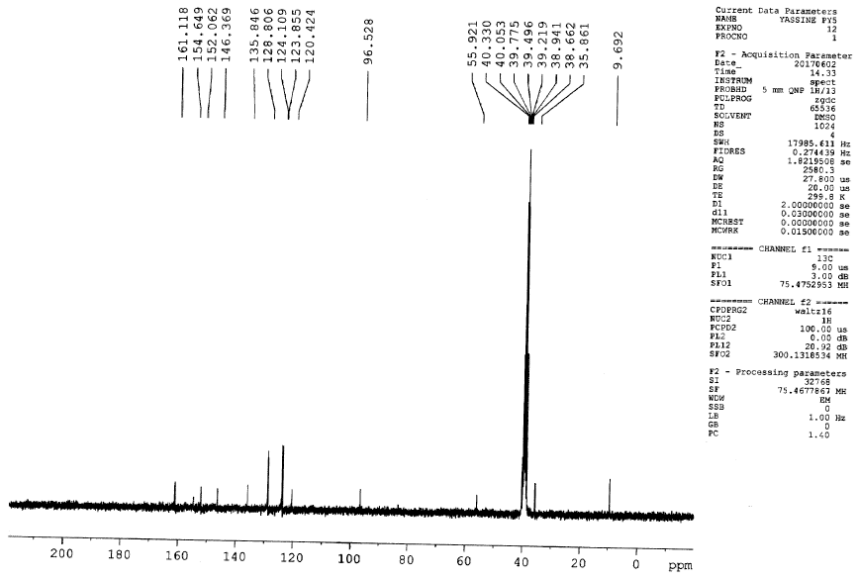


Figure S9. The ^{13}C NMR pattern of the 5C derivative

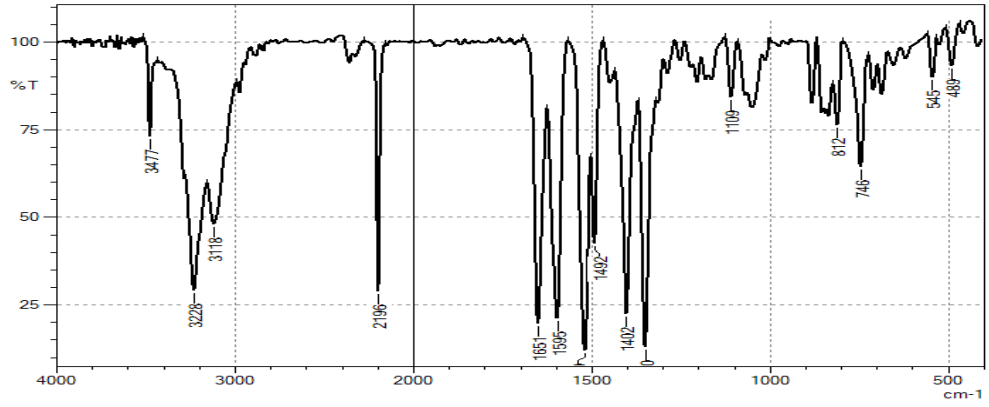
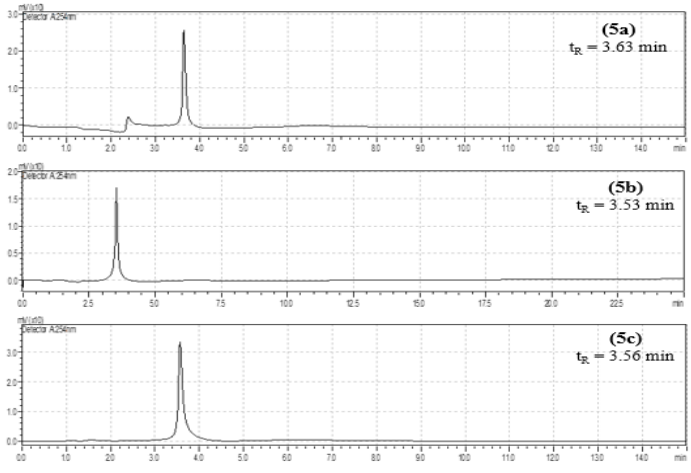


Figure S10. The infrared pattern of the 5C derivative



FigureS11. HPLC chromatograms of pyrano-pyrazoles 5 A, B, C

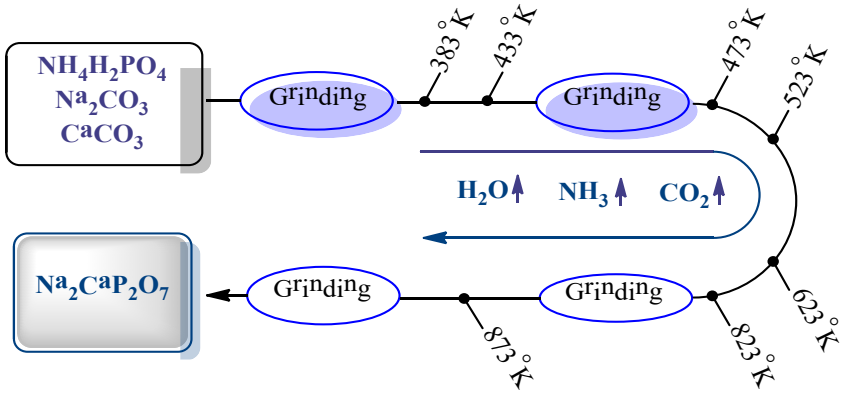


Figure S12. Steps of $Na_2CaP_2O_7$ synthesis

6. Method for the preparation of catalyst

The synthesis of the nano-structured diphosphate $Na_2CaP_2O_7$ has been carried by using the Na_2CO_3, $CaCO_3$, and $NH_4H_2PO_4$ in 1: 1: 2 proportions, respectively (purity of starting materials greater than 99%). These chemicals were mixed in an agate mortar and progressively heated in a porcelain crucible. The synthesis of $Na_2CaP_2O_7$ particles was confirmed by powder XRD, IR, SEM, and TEM studies. The steps of $Na_2CaP_2O_7$ synthesis are summarized in Figure S12.

7. XDR of the catalyst

The powder obtained was analyzed by X-ray diffractometer, the diffractogram was recorded using a Bruker D8 Advance diffractogram. The spectrum of the $Na_2CaP_2O_7$ diphosphate is reproduced in Figure S13 the crystallographic data obtained are gathered in Table S1. The diffractometer uses copper anticathode radiation Cu-Kα radiation: λ= 1.5406 A°. The crystalline parameters were refined on a computer using the least-squares method via the AFPAR program. The acquisition is carried out at ambient temperature with a scanning mode θ/2 (and a Bragg angle θspanning from10° to 50°.

8. The infrared spectrum of the catalyst

The infrared spectrum of the diphosphate $Na_2CaP_2O_7$ in powder form is shown in Figure S14. The appearance of symmetrical vibration bands of P-O-P at 720 cm^{-1} and anti-symmetric vibration bands at 893 cm^{-1} confirm the existence of

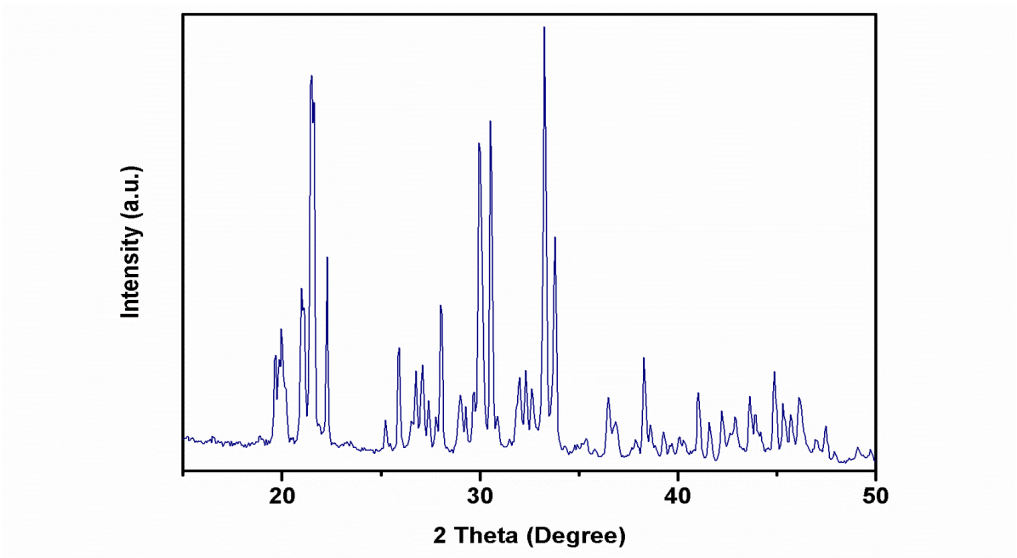


Figure S13. The XDR pattern of the diphosphate $Na_2CaP_2O_7$

Table S1. Allotment of the observed bands of $Na_2CaP_2O_7$ analyzed by X-ray diffractometer

Crystalloglaphic Data	
Space group	Triclinic,P1
M(g/mol)	260,0
a(Å)	5·361
b(Å)	7·029
c(Å)	8·743
α (°)	69·4
β (°)	89·02
ϒ (°)	88·78
V (Å^3)	308·5
Z	2

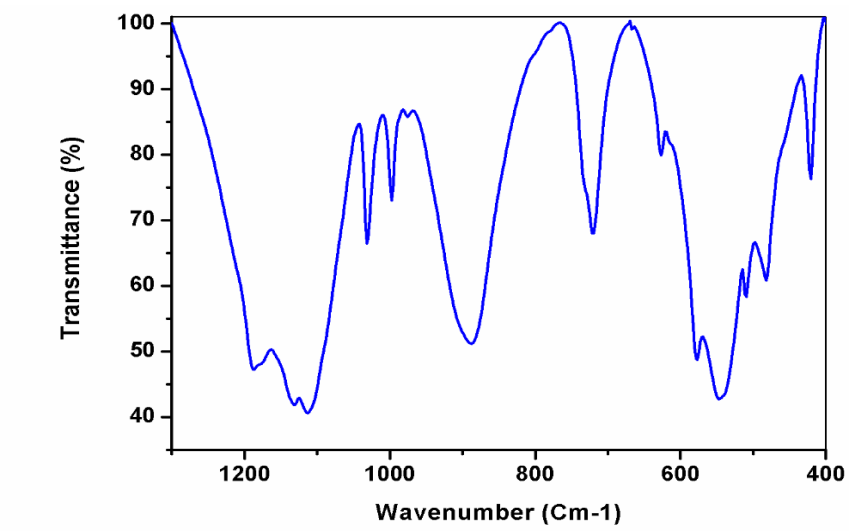


Figure S14. The infrared pattern of the catalyst was measured by using the FITR apparatus

Observed band (cm^{-1})	Awarding	
407	$\delta(P_2O_7)$	Distortion vibrations
419		
483		
511		
548		
577		
626		
720	$\upsilon_{sym}(P^-O^-P)$	Valence vibrations
893	$\upsilon_{antisym}(P^-O^-P)$	
996	$\upsilon_{sym}(PO_4)$	
1031		
1130		
1175	$\upsilon_{antisym}(PO_4)$	
1278		

Figure S15. Attribution of the observed bands of $Na_2CaP_2O_7$ analyzed by FTIR spectroscopy

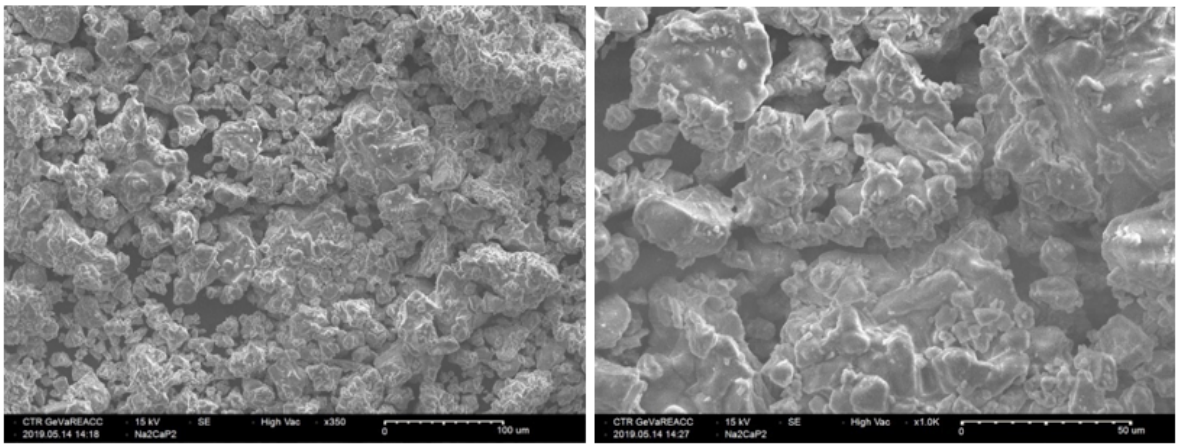


Figure S16. Scanning electron microscopic images of $Na_2CaP_2O_7$

P_2O_7 (Figure S14). Two vibration fields have proved the presence of the PO_4 group, the first at 996 cm_{-1} and 1031 cm_{-1} and the second going from 1130 to 1278 cm_{-1} (Figure S15).

9. Transmission and scanning electron microscopy

The morphological studies of the surface of $Na_2CaP_2O_7$ are performed by using the scanning electron microscope HIROX SH-4000M. To visualize the microstructure of the $Na_2CaP_2O_7$, Transmission Electronic Microscopy (TEM) was used on an FEI microscope operated at 120 kV (Figures S16 and S17).

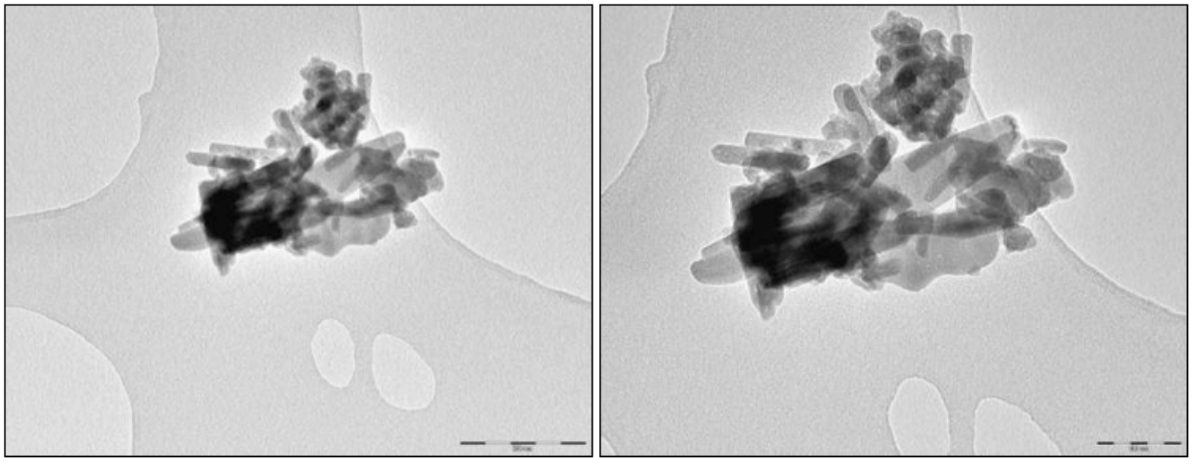


Figure S17. Transmission electronic microscopic images of $Na_2CaP_2O_7$

Table S2. The inhibition of DPPH par pyrano-pyrazoles (Activity+/- SD)

Concentration (mg/ml)	2	5	7	10
5a	46.18±5.40	47.40±5.13	50.55±5.00	60.87±5.00
5b	52.92±5,13	77.60±5.40	82.00±5.23	89.00±6.24
5c	1.83±0.005	25.00±1.24	28.16±2.12	33.50±5.13
AA	70.00±6.244	80.00±5.25	97.00±4.26	97.00±6.00

AA: Ascorbic Acid.

Table S3. The swimming speed of *Tetrahymena thermophila* (normal vs treated groups)

Protozoan	Swimming Speed
TT	++++
TT+5a	+++++
TT+H_2O_2	+-
TT+SNP	++
TT+5a+H_2O_2	++++
TT+5a+SNP	++++

Abbreviations: SNP: Sodium Nitroprusside; TT: *Tetrahymena thermophila*

10. Antioxidant activity in vitro: The DPPH test

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant assay based on electron-transfer that produces a purple solution in ethanol. This free radical, stable at room temperature, is reduced in the presence of an antioxidant which induces the coloration of ethanol solution. The use of the DPPH assay provides an easy and rapid way to evaluate antioxidants by spectrophotometry (Tables S2, S3, S4, S5).

Table S4. The swimming speed of *Tetrahymena thermophila* (normal & treated cells with 5b derivative)

Protozoan	Swimming Speed
TT	+++++
TT+5b	+++++
TT+H_2O_2	+/-
TT+SNP	++
TT+5b+H_2O_2	++++
TT+5b+SNP	++++

SNP: Sodium Nitroprusside; TT: *Tetrahymena thermophila*

Table S5. The effect of the 5c derivatives on the *Tetrahymena thermophila* mobility

Protozoan	Swimming Speed
TT	+++++
TT+5c	+++++
TT+H_2O_2	+/-
TT+SNP	++
TT+5c+H_2O_2	+++
TT+5c+SNP	+++

SNP: Sodium Nitroprusside; TT: *Tetrahymena thermophila*

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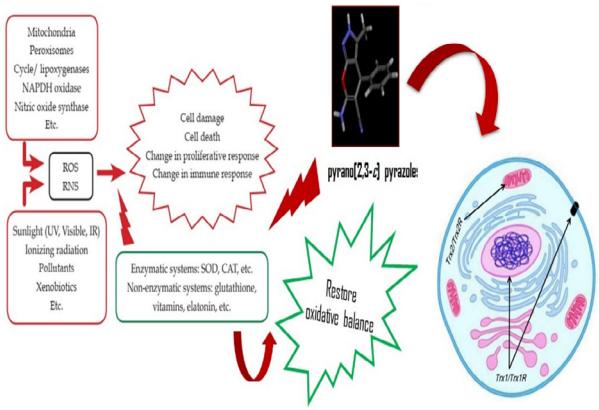

Figure S18. Summary of the cellular mechanism of based-pyrazoles derivatives to contour act of oxidative stress

11. Cellular mechanism of pyrazoles scaffolds

The main role of these synthetic compounds is the activation of the cellular antioxidants' enzymes. These enzymes controlled the Reactive Oxygen and Nitrogen Species (RNOS) via several reactions. Firstly the SOD converted O2− to H_2O_2, this hydrogen peroxide is also degraded to H_2O and O2 by the catalase, thereafter the peroxide is neutralized by the glutathione peroxidase (Figure S18).