



## Synthesis, Characterization of Some $\alpha$ -aminophosphonate Derivatives and Comparative Assessment of their Antioxidant Potential *in-vivo* and *in-vitro*

Boutaina Addoum<sup>1</sup>, Bouchra El khalfi<sup>1</sup>, Papa Daouda Mar<sup>1</sup>, Najoie Filali.Ansari<sup>2</sup>, Abdelhakim Elmakssoudi<sup>3</sup> and Abdelaziz Soukri<sup>1\*</sup>

<sup>1</sup>Laboratory of Physiopathology, Genetics, Molecular and Biotechnology (PGMB), Faculty of Sciences Ain Chock, Research Center of Health and Biotechnology, University Hassan II of Casablanca, Morocco

<sup>2</sup>Laboratory of Biochemistry, Nutrition and Valorization of Natural Resources, Department of Biology, Faculty of Sciences, University of Chouaib Doukkali, Morocco

<sup>3</sup>Laboratory of Organic Synthesis, Extraction and Valorization, Department of chemistry, Faculty of Sciences Ain Chock, University Hassan II of Casablanca, Morocco

\*Corresponding Author: Abdelaziz Soukri, Department of Biology, Laboratory of (PGMB), Faculty of Sciences Ain Chock, Research Center of Health and Biotechnology, University Hassan II of Casablanca, Morocco. E-mail: ab.soukri@gmail.com

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### Abstract

Since their discovery, essential oils are known as a hugely valuable resource to treat a variety of infections due to their multiple biological functions, especially their antimicrobial, and antioxidant activities. Unfortunately, these volatile compounds have also certain limits such as the processes for extracting of these oils, the purification of their bioactive molecules and their minimal amount. For that reason, several scientists have been oriented their research towards synthetic molecules as a new tool to overcome the progression of many chronic diseases (cancer, fibrosis, cirrhosis, arthritis).

The prime focus of this work was the synthesis and characterization of four  $\alpha$ -aminophosphonate derivatives 4 (a-d). Then we evaluated their antioxidant activity against oxidative and nitrosative stress. The *in-vivo* experiments are conducted using Tetrahymena protozoan treated with IC<sub>50</sub> of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) or sodium nitroprusside (SNP); finally, we checked their antioxidant behavior *in-vitro* through DPPH and DC assay. The results of this paper confirmed that under the both conditions: *in-vivo* and *in-vitro*, two derivatives among the four synthesized possess an antioxidant activity; it is the 4a and 4c products. This project is an attempt to obtain new organic compounds that have the potential to be developed as new therapeutic agents.

**Keywords:**  $\alpha$ -aminophosphonates; Stress; Tetrahymena; *In-vivo*; *In-vitro*

### Introduction

Nowadays  $\alpha$ -aminophosphonate and their derivatives are used broadly in several applications: from agriculture to biomedical research. These organophorus compounds are well-known by their multiple biological properties including antifungal, antiviral, herbicidal, antitumorale, antibacterial effect [1,2] eventually they use in biomedicine as synthesis inhibitors, due to their mimetic potential to substitute natural amino acids. Moreover, these synthetic products regulate some pathophysiological phenomena by inhibiting and perturbing the growth of some organisms such as: human immunodeficiency virus (HIV) and tobacco mosaic virus [3,4]. Besides these biological activities discovered half a century ago, they

possess also a great antioxidant activity, and an impressive ability to quench the overproduction of ROS and RNS [5,6]. These free radicals play an essential role in our body, especially to maintain respiration, homeostasis process and regulate the autophagy in some organisms such as yeast, nematodes and eukaryotes [7,8] however, their accumulation undergo histological lesions (necrosis or apoptosis), cellular injury, and mitochondrial damage [9].

In this work, we tested for the first time the antioxidant activity of these compounds *in-vivo* using Tetrahymena cells as an experimental model. This protozoan is ubiquitous in various fields such as biochemistry, biomedicine, and ecotoxicology [10].

This freshwater protozoan is considered among the protists who occupy an important place in scientific researches because it can be easily grown in a small volume of culture medium [11,12]. It is defined as a biological indicator of water quality (Nicolau, *et al.* 1999). It is a eukaryotic system that offers to us a good chance to understand several cellular, physiological and biochemical processes: cell division, phagocytosis, signaling pathway [13,14]. Otherwise, this protozoan is used as a new tool to screen novel biological activity and to evaluate the toxic effect of some xenobiotic drugs. The toxic effect of these substances exhibits the changes in morphology, metabolism, growth and other toxicological biomarkers detected in Tetrahymena cells [15].

In search of finding new resources and potent antioxidant to protect human tissues and cells exposed to external aggressions (environmental stress), the present study aimed to: (1) Chemical synthesis and characterization of some  $\alpha$ -aminophosphonate derivatives 4 (a-d); (2) evaluate the antioxidant potential of these products *in-vivo* by using Tetrahymena cells treated respectively with  $H_2O_2$  and SNP. (2) Investigate the antioxidant behavior of the 4 a and 4 c product *in-vitro* using DPPH and Dienes conjugated assay.

## Materials and Methods

### Experimental Section

The melting point of the  $\alpha$ -aminophosphonate derivatives were detected on Buchi 510 Melting point apparatus B510K. The NMR spectra of  $^1H$  and  $^{13}C$  were detected and recorded on Bruker TM 300 MHz by using the internal standard  $CDCl_3$ . The chemical shifts ( $\delta$ ) are expressed in ppm and coupling constant (J) expressed in Hertz units. IR spectra were performed as KBr pellets on FTIR (IR Affinity - 1S, Fourier Transform Infrared Spectrophotometer, SHIMADZU). The purification was performed on silica gel used as adsorbent in the column chromatography.

### Synthesis of $\alpha$ -aminophosphonate derivatives 4(a-d)

The  $\alpha$ -aminophosphonate products were synthesized via the method reported in literature [16]: an equimolar mixture of aromatic aldehyde (1mmol), aniline (1mmol), and diethyl phosphate (1mmol), was prepared in presence of  $Na_2CaP_2O_7$  catalyst (0.3 g). The completion of this reaction was performed by thin layer chromatography on the silica gel after 7h, then visualized on UV at 254 nm. After the process of filtration using the hexane/ EtOAc (1/4), the crude product was obtained with high yields: 80-91 %.

### Protozoan culture

The two protozoa are grown axenically at a temperature of 28°C for *Tetrahymena pyriformis* and 30 °C for *Tetrahymena thermophila* without stirring for 72 hours in the liquid medium (PPYE), this medium contains 1.5% (w/v) of peptone and 0.25% (w/v) of yeast extract [17].

### Kinetics of Tetrahymena growth

To follow the growth of Tetrahymena, we measured absorbance at 600 nm each 24 hours using the UV-Visible Spectrophotometer.

### Microscopic observation

In order to study the morphology and the mobility of Tetrahymena, an aliquot of 1 ml was taken immediately, then samples were fixed with neutral buffered formalin which is prepared at 2% in phosphate buffer saline: PBS [18], finally 6  $\mu$ l of suspension were taken to make the visualization under light microscope: A. KRÜSS Optronic optical microscope (objective x 10).

All the experiments shown in this paper were carried out at least in triplicate or more.

### Stress condition

To evaluate the effects of SNP and  $H_2O_2$  on the growth of Tetrahymena, erlenmeyers flasks containing the medium culture (PPYE) are inoculated with 1% (v/v) of Tetrahymena protozoan (*thermophila* or *pyriformis*), while cultures were supplemented with  $IC_{50}$  of stress agents after 24 h of cells growth. Then Protozoan growth was monitored by sterile sampling every 24 h, and the absorbance of Tetrahymena was measured at 600 nm for 120 h [18].

Simultaneously, a negative control was prepared in the same condition without stress in order to follow the normal growth of Tetrahymena [19]. The  $IC_{50}$  used in this experiment are determined in table 1.

### Toxicity test of the molecule

The products 4 (a-d) are prepared at the finale concentration of 25 mg/ml in DMSO (0, 1% v/v), and a concentration range is performed via serial dilution of 10<sup>-1</sup> to 10<sup>-4</sup>. After 3 days of cells culture in the PPYE medium supplemented with 5 $\mu$ l of each dilution, we detected the concentration that did not affect the growth or the morphology of Tetrahymena cells; it's the non-lethal concentration (Table 2).

Tetrahymena species	IC50	
	H <sub>2</sub> O <sub>2</sub>	SNP
<i>Tetrahymena pyriformis</i>	0.3 mM	1.8 mM
<i>Tetrahymena thermophila</i>	0.7 mM	1.8 mM

**Table 1:** The concentration of H<sub>2</sub>O<sub>2</sub> and SNP used to induce stress.

**Note:** IC<sub>50</sub>: is the half inhibitory concentration; H<sub>2</sub>O<sub>2</sub> is the hydrogen peroxide and the SNP is the sodium nitroprusside.

Tested compounds	Tetrahymena species	
	<i>Tetrahymena thermophila</i>	<i>Tetrahymena pyriformis</i>
4 a	++++	+++
4 b	+/-	+/-
4 c	++++	++++
4 d	-	-

**Table 2:** Inoculation of protozoan Tetrahymena in the presence of  $\alpha$ -aminophosphonate derivatives.

**Note:** + 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 nontoxic concentration of 10<sup>-4</sup>.

### DPPH radical scavenging activity

The antioxidant activity *in-vitro* was performed using the method reported previously in several studies, where 2.5 ml of DPPH or 1,1-diphenyl-2-picrylhydrazyl solution (0,1 mm) prepared in fresh methanol, was mixed quickly with each tested compound or with BHT (25  $\mu$ g/ml to 180  $\mu$ g/ml). Finally, the decrease of optical density was detected by a color change of this mixture from purple to yellow, which is monitored spectrophotometrically at 517nm [20]. The inhibition of free radical of DPPH by the  $\alpha$ -aminophosphonate derivatives was calculated and expressed in percent (RSA%) :

$$\text{RSA}\% = \frac{\text{Ac} - \text{As}}{\text{Ac}} \quad (1)$$

Where:

Ac: is the absorbance of the control

As: is the absorbance of the samples

### Dienes conjugated test: lipid oxidation

During the formation of hydroperoxydes throughout the peroxidation of lipids, conjugated dienes (CDs) are typically produced [21]. To follow the processes of CDs formation we measured the absorbance at 234 nm. The increase of optical density correlates with the appearance of these products. The progress of this biochemical reaction is carried out by preparing a mixture of linoleic acid (7,5 mM) with 10mM of tween 20 (0,1v/v%), this mixture is prepared in phosphate buffer (pH = 7) and incubated with each compound at a final concentration of 25 $\mu$ g/ml to 180  $\mu$ g/ml. Oxidation reaction is initiated by using 10  $\mu$ M of CuSO<sub>4</sub> freshly prepared; while to stop the oxidation we use an ice bath containing the solution of EDTA (100  $\mu$ M) and BHT (20  $\mu$ M). A positive control is carried out by using the BHT [22].

The inhibition of CDs is evaluated, calculated and expressed in percent as follows:

$$\text{DCs}\% = \frac{\text{Ac} - \text{As}}{\text{Ac}} \quad (2)$$

Where:

Ac: is the absorbance of the control

As: is the absorbance of the samples

## Results

### Characterization of synthesis products

Diethyl phenyl (phenylamino) methylphosphonate 4a, White solid. m.p. 92 - 94°C; R<sub>f</sub> (35% AcOEt/hexane) 0.38. <sup>1</sup>H-NMR (CDCl<sub>3</sub>)  $\delta$ : 1.2 (3H, JHH = 7.2 Hz, t, OCH<sub>2</sub>CH<sub>3</sub>); 1.4 (3H, JHH = 7.2 Hz, t, OCH<sub>2</sub>CH<sub>3</sub>); 3.73 - 4.3 (4H, m, OCH<sub>2</sub>CH<sub>3</sub>); 4.9 (1H, JHP = 24.6 Hz, d, CHP), 5 (<sup>1</sup>H, s, NH); 6.6 - 7.8 (10H, m, HAr). <sup>13</sup>C-NMR (CDCl<sub>3</sub>)  $\delta$ : 16.46 (d, 3JCP = 6.03 Hz, OCH<sub>2</sub>CH<sub>3</sub>); 16.7 (d, 3JCP = 6.03 Hz, OCH<sub>2</sub>CH<sub>3</sub>); 56.35 (d, 1JCP = 149 Hz, CHP); 63.5 (d, 2JCP = 6.79 Hz, OCH<sub>2</sub>CH<sub>3</sub>); 63.53 (d, 2JCP = 6.79 Hz, OCH<sub>2</sub>CH<sub>3</sub>); 114.13 (s); 118.64 (s); 128.1 (s); 128.85 (s); 129.43 (s); 136.2 (s); 146.6 (s). IR (KBr)  $\nu$  3304 (NH), 2985 (CH), 1605 (C=C), 1514 (C=C), 1240 (P=O), 1020 (P-O) cm<sup>-1</sup>.

Diethyl 4-methylphenyl(phenylamino)methylphosphonate 4b, White solid. m.p. 63 - 65°C; R<sub>f</sub> (35% AcOEt/hexane) 0.4, <sup>1</sup>H-NMR (CDCl<sub>3</sub>)  $\delta$ : 1.25 (3H, JHH = 7.2 Hz, t, OCH<sub>2</sub>CH<sub>3</sub>); 1.4 (3H, JHH = 7.2 Hz, t, OCH<sub>2</sub>CH<sub>3</sub>); 2.45 (3H, s, C<sub>6</sub>H<sub>4</sub>CH<sub>3</sub>); 3.79 - 4.31 (4H, m, OCH<sub>2</sub>CH<sub>3</sub>);

4.87 ( $^1\text{H}$ , JHP = 24.6 Hz, d, CHP), 5 (1H, s, NH); 6.71-7.5 (9H, m, HAR).  $^{13}\text{C}$ -NMR ( $\text{CDCl}_3$ )  $\delta$ : 16.5 (d, 3JCP = 5.8 Hz,  $\text{OCH}_2\text{CH}_3$ ); 16.71 (d, 3JCP = 5.8 Hz,  $\text{OCH}_2\text{CH}_3$ ); 21.4 (s,  $\text{C}_6\text{H}_4\text{CH}_3$ ); 56 (d, 1JCP = 150 Hz, CHP); 63.48 (d, 2JCP = 6.94 Hz,  $\text{OCH}_2\text{CH}_3$ ); 114.14 (s); 118.57 (s); 128 (s); 129.4 (s); 129.55 (s); 129.58 (s); 133 (s); 137.8 (s); 146.8 (s). IR (KBr)  $\nu$  3325 (NH), 2980 (CH), 1604 (C=C), 1498 (C=C), 1234 (P=O), 1016 (P-O)  $\text{cm}^{-1}$ .

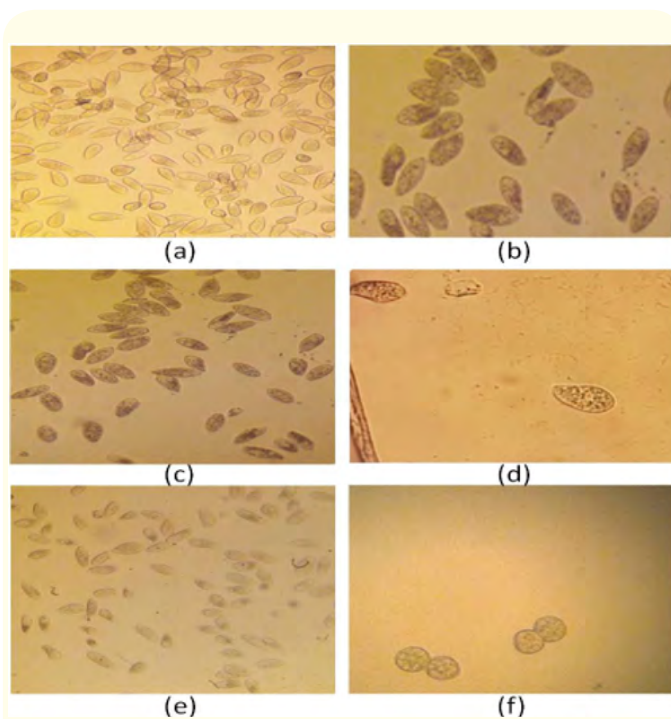
Diethyl 4-methoxyphenyl(phenylamino)methylphosphonate 4c, White solid. m.p; 102 - 103°C;  $R_f$  (35% AcOEt/hexane) 0.26;  $^1\text{H}$ -NMR ( $\text{CDCl}_3$ )  $\delta$ : 1.25 (3H, JHH = 7.2 Hz, t,  $\text{OCH}_2\text{CH}_3$ ); 1.39 (3H, JHH = 7.2 Hz, t,  $\text{OCH}_2\text{CH}_3$ ); 3.86 (3H, s,  $\text{C}_6\text{H}_4\text{OCH}_3$ ); 3.8 - 4.26 (4H, m,  $\text{OCH}_2\text{CH}_3$ ); 4.85 (1H, JHP = 23.1 Hz, d, CHP), 4.95 ( $^1\text{H}$ , s, NH); 6.7 - 7.53 (9H, m, HAR).  $^{13}\text{C}$ -NMR ( $\text{CDCl}_3$ )  $\delta$ : 16.53 (d, 3JCP = 5.7 Hz,  $\text{OCH}_2\text{CH}_3$ ); 16.72 (d, 3JCP = 5.7 Hz,  $\text{OCH}_2\text{CH}_3$ ); 55.44 (s,  $\text{C}_6\text{H}_4\text{OCH}_3$ ); 55.62 (d, 1JCP = 151.2 Hz, CHP); 63.43 (d, 2JCP = 6.9 Hz,  $\text{OCH}_2\text{CH}_3$ ); 63.47 (d, 2JCP = 6.9 Hz,  $\text{OCH}_2\text{CH}_3$ ); 114.15 (s); 114.32 (s); 118.57 (s); 128 (s); 129.2 (s); 129.33 (s); 146.67 (s); 159.56 (s). IR (KBr)  $\nu$  3300 (NH), 2983 (CH), 1600 (C=C), 1510 (C=C), 1232 (P=O), 1020 (P-O)  $\text{cm}^{-1}$ .

Diethyl 2-hydroxyphenyl(phenylamino) methylphosphonate 4d,  $R_f$  (35% AcOEt/hexane) 0.23,  $^1\text{H}$ -NMR ( $\text{CDCl}_3$ )  $\delta$ : 1.1 (3H, JHH = 7.2 Hz, t,  $\text{OCH}_2\text{CH}_3$ ); 1.25 (3H, JHH = 7.2 Hz, t,  $\text{OCH}_2\text{CH}_3$ ); 2.2 (1H, s,  $\text{C}_6\text{H}_4\text{OH}$ ); 3.84 - 4.18 (4H, m,  $\text{OCH}_2\text{CH}_3$ ); 5.01 (1H, JHP = 23.1 Hz, d, CHP), 5 (1H, s, NH); 6.59-7.19 (9H, m, HAR).  $^{13}\text{C}$ -NMR ( $\text{CDCl}_3$ )  $\delta$ : 16.52 (d, 3JCP = 5.7 Hz,  $\text{OCH}_2\text{CH}_3$ ); 16.68 (d, 3JCP = 5.7 Hz,  $\text{OCH}_2\text{CH}_3$ ); 54.25 (d, 1JCP = 151.6 Hz, CHP); 63.91 (d, 2JCP = 7 Hz,  $\text{OCH}_2\text{CH}_3$ ); 64.42 (d, 2JCP = 7.2 Hz,  $\text{OCH}_2\text{CH}_3$ ); 114.15 (s); 114.77 (s); 118.65 (s); 119.63 (s); 120.94 (s); 121.9 (s); 129.55 (s); 146.5 (s); 159 (s). IR (KBr)  $\nu$  3300 (NH), 2983 (CH), 1600 (C=C), 1510 (C=C), 1232 (P=O), 1020 (P-O)  $\text{cm}^{-1}$ .

## Study *in-vivo*

### Effect of stress on *Tetrahymena* morphology

The results of Figure 1 showed clearly that the two protozoa react differently depending on the types of stress (oxidative or nitrosative). The behavior of *Tetrahymena pyriformis* against this two stress reagent demonstrates that this organism is more sensitive to the SNP; nevertheless, the cells' sensitivity to hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) is appreciated with *Tetrahymena thermophila*. Otherwise, our data indicated that the stress manifested by a decrease of protists number with a notable increase of its size and its interior volume; we also spot the appearance of dark vacuoles.



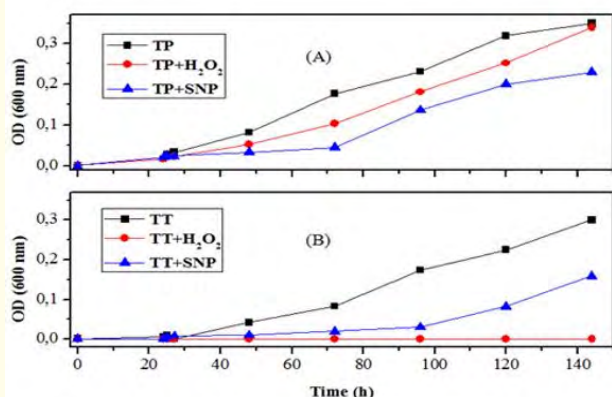
**Figure 1:** Microscopic analysis images of *Tetrahymena pyriformis* (TP) and *Tetrahymena thermophila* (TT) cells.

(a): TP control (b): TT control (c): TP+ $\text{H}_2\text{O}_2$  (d): TT+ $\text{H}_2\text{O}_2$  (e): TP+SNP (f): TT+SNP. Microscopic images were taken at objective ( $\times 10$ ) of cells grown at 32°C or 28°C in PPYE medium containing  $\text{H}_2\text{O}_2$  reagent (0.7mM for TT and 0.3mM for TP) or SNP reagent (1.8mM for TT or 0.5mM for TP).

### Effect of stress on the growth of *Tetrahymena*.

The results shown in Figure 2A reveal that the protozoan *Tetrahymena thermophila* is able to fight against nitrosative stress, but not against oxidative stress. SNP-treated cells could turn to their normal shape. However,  $\text{H}_2\text{O}_2$ -treated cells stills unable to overcome this oxidative stress.

As shown in Figure 2B, the characteristic growth curve of *Tetrahymena* was drastically modified after the induction of oxidative stress. However, *Tetrahymena pyriformis* cultivated with the  $\text{IC}_{50}$  of  $\text{H}_2\text{O}_2$  does not show a very strong decrease, after 72h we found that the curve shape is nearly similar to the controls.

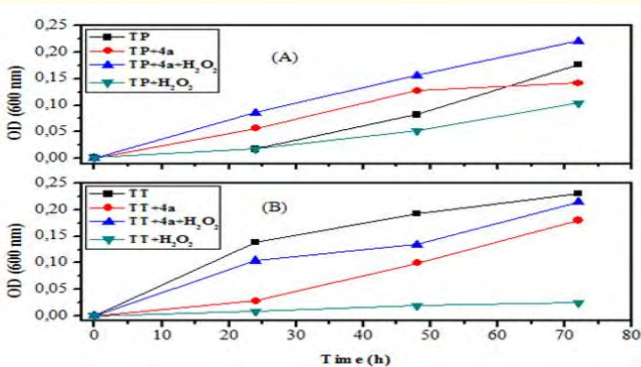


**Figure 2:** Growth curve of Tetrahymena in presence of stressors reagents.

A: Tetrahymena pyriformis; B: Tetrahymena thermophila. OD: is the optical density measured at 600 nm each 24 h of cells culture.

### Anti-oxidative and nitrosative potential of 4a product on Tetrahymena

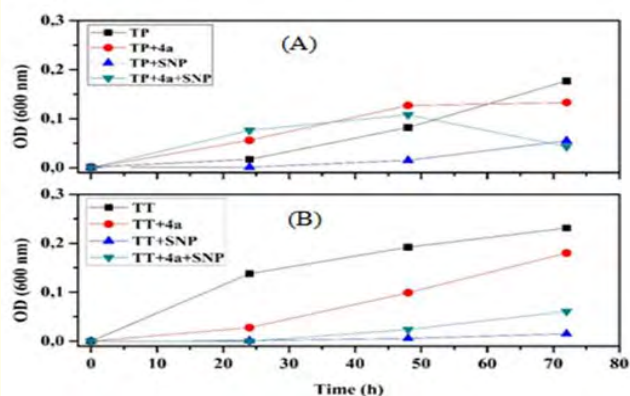
In this current study it was reported that 4a product has an impressive effect against oxidative stress. The addition of 4a product to the culture stimulates the growth of stressed protozoan and their growth curve becomes similar to the typical shape of normal Tetrahymena (Figure. 3).



**Figure 3:** Anti-oxidative effect of 4a compound on Tetrahymena protozoan.

Tetrahymena exposed to the  $H_2O_2$  as a function of OD (optical density) and treatment time A: Tetrahymena pyriformis treated with  $H_2O_2$ ; B: Tetrahymena thermophila treated with  $H_2O_2$ .

On the other hand, we find that the 4a product is enabled to protect against the nitrosative stress, because the addition of 4a product to the culture medium protects *Tetrahymena thermophila* against the RNS produced *in-vivo*. Furthermore, *Tetrahymena pyriformis* is influenced also by this compound; and sometimes it gains to exceed the growth curve of the normal protozoan (Figure 4).



**Figure 4:** Anti-nitrosative effect of 4a compound on Tetrahymena protozoan.

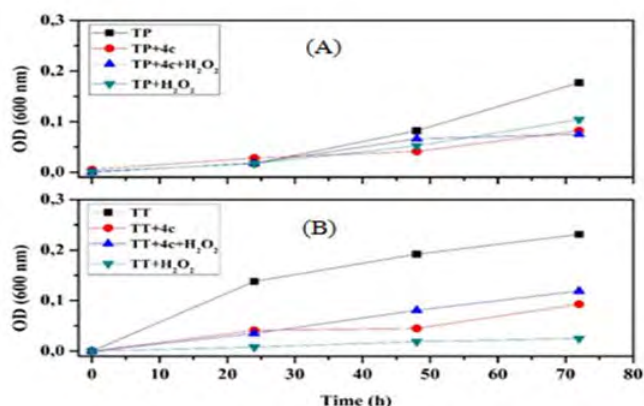
Tetrahymena exposed to the SNP as a function of OD (optical density) and treatment time.

A: Tetrahymena pyriformis; B: Tetrahymena thermophila.

### Anti-oxidative and nitrosative potential of 4c product on Tetrahymena

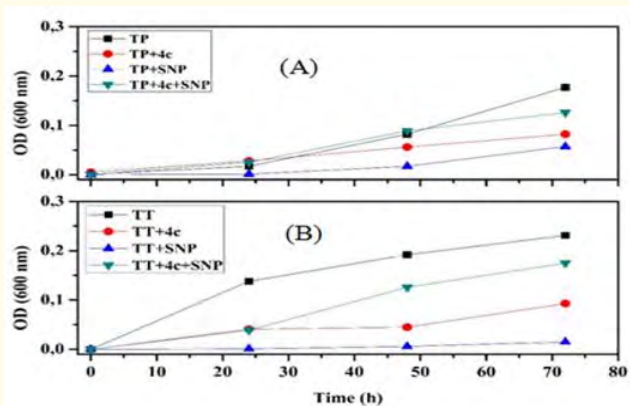
Our data confirmed that the 4c product has an impressive effect against nitrosative stress, and the addition of 4c derivative to the PPYE medium of Tetrahymena stimulates the growth of stressed protozoan. The protective effect of the 4c compound is more appreciated with *Tetrahymena pyriformis*. We observed that this 4c product controls the stress and activate the growth of this ciliated protozoan (Figure 5), especially in the presence of nitrosative stress (Figure 6).

The protective effect of the 4c compound is more appreciated with Tetrahymena pyriformis. We observed that this 4c product controls the stress and activate the growth of this ciliated protozoan (Figure 5), especially in the presence of nitrosative stress (Figure 6).



**Figure 5:** Anti-oxidative effect of 4c compound on Tetrahymena protozoan.

Tetrahymena exposed to the  $H_2O_2$  as a function of OD (optical density) and treatment time A: Tetrahymena pyriformis exposed to  $H_2O_2$ ; B: Tetrahymena thermophila exposed to  $H_2O_2$ .



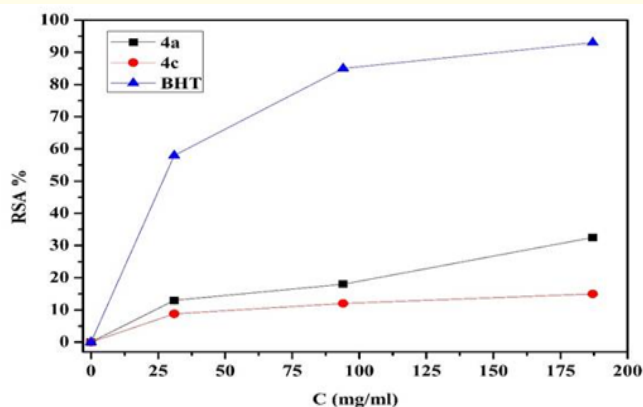
**Figure 6:** Anti-nitrosative effect of 4c compound on Tetrahymena protozoan.

Tetrahymena exposed to the SNP as a function of OD (optical density) and treatment time A: Tetrahymena pyriformis exposed to the SNP; B: Tetrahymena thermophila exposed to the SNP.

### Study *in-vivo*

#### DPPH Assay

As shown in Figure 7 all the tested compounds showed a significant ( $P < 0.01$ ) scavenging effect on the DPPH radical. At the maximal concentration (180  $\mu\text{g/ml}$ ), we notice that 4a compound showed the highest activity followed by 4c compound, but the BHT still the most potent antioxidant compared to the other samples.

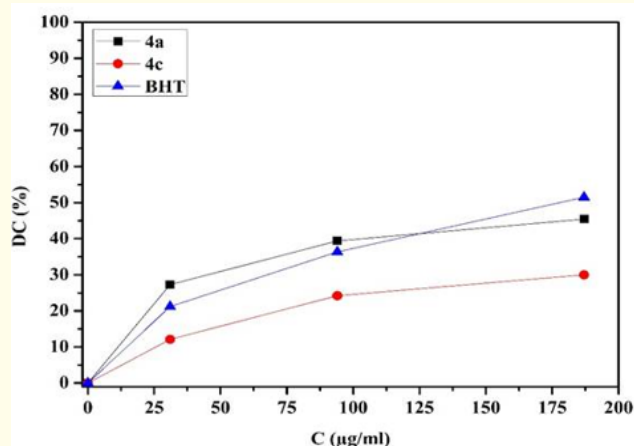


**Figure 7:** Scavenging activity of 4a and 4c product on DPPH free radical.

The radical scavenging activity (RSA %) of each substance was assessed by DPPH free radical method. When DPPH reacts with  $\alpha$ -aminophosphonate derivatives, it is reduced. The changes in color of the DPPH solution (from purple to yellow) were read at 517nm.

#### Conjugated Dienes (CDs) inhibition

In order to estimate the antioxidant potential of the isolated compounds during the primary oxidation of lipid, the inhibition of the linoleic acid peroxidation was quantified at various concentrations (25-180  $\mu\text{g/ml}$ ). As shown in Figure. 9 the two  $\alpha$ -aminophosphonate derivatives 4a and 4c showed significantly ( $P < 0.05$ ) inhibitory potential products on DCs production. The maximum inhibitory potential was obtained at concentration of 180 $\mu\text{g/ml}$  (Figure 8).



**Figure 8:** Evaluation of the inhibition rate of DC in the presence of  $\alpha$ -aminophosphonate 4 a and 4 c. Conjugated Dienes (CDs) are typically produced, due to the rearrangement of the double bonds. The resulting CDs evince the increase of absorption at 234 nm. In this experiment the BHT is considered as positive control, however the negative control is carried out by preparing this mixture without molecules.

## Discussion

Our results revealed that *Tetrahymena* exposed to oxidative or nitrosative stress induce the cell damage and perturbed some physiological and morphological parameters of this protozoan. Similar effects have been observed in *Tetrahymena pyriformis* cells treated with ethidium bromide [23], we notice that the morphology of the treated cells was markedly altered, the cortex was greatly distorted, and the cells assumed a variety of weird shapes. Besides that, the cells can't swim effectively, but turned erratically [19,20].

The stress is defined as the imbalance between the free radicals and antioxidant system (non enzymatic and enzymatic), it becomes a pathological situation when our antioxidant system is submerged by ROS and RNS; especially when this defense system is unable to quell these free radicals or to limit their reactivity by converting this reactive species to non-reactive one. Therefore, their overproduction affects cell metabolism and induces cellular, molecular and chemical modification of 3 basic biomolecules: proteins, DNA (chromatin) and lipids [24,25].

To evaluate the toxicity of oxygen and oxide nitric on *Tetrahymena* cells, we used two stress reagents:  $H_2O_2$  as donor of oxygen, and the SNP as a donor of nitric oxide [26,27]. Our data indicated that the two stressors acted differently depending on the type of protozoan: *Tetrahymena thermophila* or *Tetrahymena pyriformis*, but both inhibited the growth of this protozoan, and caused *Tetrahymena* mortality especially the SNP. Otherwise, our microscopic results have confirmed that stress conditions induce important changes in the cell structure as we show above in this work. Occasionally we observed cells, which had initiated division cycle but did not complete cytokinesis, and we noticed the appearance of some erratic cellular forms: elongated or rounded, these forms related to the activation of autophagy that manifested by damage of some organelles, sequestration of proteins, alteration of DNA and finally apoptosis [19]. Overall several studies were previously confirmed that the induction of oxidative stress via  $H_2O_2$  suppressed the growth of some organisms such as *Yarrowia lipolytica* and *Pichia pastoris* [28,29].

The broad use of plants and their essential oil in biomedical research is due to their biological properties and their therapeutic benefits, unfortunately their minimum amount and difficulty of extraction, have oriented the biologists to finding new complementary approaches to aromatherapy, for example the study of natural molecules generated by synthesis and hemisynthesis [30]. This paper presents a preliminary study describing the antioxidant potential of some  $\alpha$ -aminophosphonate derivatives against oxidative and nitrosative stress using as experimental model, the protozoan *Tetrahymena*. The aim of our work was to investigate the possibility to use these organothorium derivatives as new anti-stress compounds.

To remedy the cell damage induced by stress reagent [30], we worked under *in-vivo* and *in-vitro* conditions. Therefore, to enhance the protective behavior of these organophosphorus compounds against stressors reagent, we used a non-lethal concentration of this product. This test leads us to follow their antioxidant effect without damaging *Tetrahymena* cells or influencing the viability of this organism: it's the turbidity test. Our results indicated that 4 a product protect *Tetrahymena* cells against  $H_2O_2$ , by increasing protozoan growth and maintaining the normal form and mobility of this ciliated protozoan. However, the 4 c product behaves against the SNP by protecting the normal form of *Tetrahymena* cells.

The protective potential of 4 a product is related to the capacity of trapping ROS directly or indirectly by inducing the production of some enzymes that play a key role in cellular defense pathway such as catalase (CAT), Superoxide dismutase (SOD), glutathione reductase (GR) and glutathione-S-transferase (Gst); These antioxidant enzymes maintain our health by inducing the defense pathway [31,32].

The protective effect of 4 c compound is due to their capacity to regulate the nitric oxide metabolism, this NO reacts quickly with superoxide radical to produce peroxynitrite (ONOO<sup>-</sup>), the unstable form of peroxynitrite radical exhibit many cytotoxic effects *in-vivo* such as apoptosis and necrosis [33]. Indeed, the accumulation of NO induce the appearance of the redox form of this enzyme its: The S-nitroso glutathione (GSNO), this form inhibits the GAPDH protein by the S-nitrosylation. The negative regulation of this glycolytic enzyme can influence some physiological parameters in Tetrahymena cells such as: the morphology, mobility and growth kinetic of this protozoan [19,34].

The *in-vitro* study is carried out to clarify the protective effect of the  $\alpha$ -aminophosphonate against two types of stress: oxidative and nitrosative. Our data elucidate that the two compounds 4a and 4c are enabling to scavenge ROS/RNS and behaves as antioxidant during the primary lipid oxidation [35-37]. In this *in-vitro* study we used BHT as control to evaluate the antioxidant behavior of these products; This BHT is used in food industry as additive due to their potential to control the circulation of ROS and to suppresses oxidation process [35].

The biological results showed in this paper confirmed that two  $\alpha$ -aminophosphonate derivatives behave as antioxidant agents and could be commercialized as new low cost drugs.

## Conclusion

The  $\alpha$ -aminophosphonate derivatives play an important role in our body to remedy some chronic diseases and metabolic disorder; based on our data and other published by several authors we elucidate that these bioactive molecules have the antioxidant potential to be used in the pharmaceutical industry as new anti-stress agents.

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## Conflict of Interest

The authors declare no conflict of interest.

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