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COMPARATIVE STUDY BETWEEN TOXIC EFFECTS OF NEWLY SYNTHESIZED AMINOPHOSPHATES (AP) AND GLYPHOSATE ON FRESH-WATER CILIATES MODEL; *PARAMECIUM TETRAURELIA*

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NEWLY SYNTHETIZED AMINOPHOSPHATES
ORGANOPHOSPHATES
CYTOTOXICITY
RESPONSE PERCENTAGE
ANTIOXIDANTS

ABSTRACT. – Synthetic aminophosphates are a class of organophosphorus compounds commonly used for protecting crops, however, the toxic effects on terrestrial and aquatic non-target organisms limit their uses. Freshwater protozoan ciliate, *Paramecium tetraurelia* was used as an alternative cellular model to evaluate the cytotoxic effects of aminophosphates. The present study was therefore, aimed to compare the toxic effects of two newly synthesized aminophosphates (Ap1 at 30 & 60mM and Ap2 at 40 & 60mM) and glyphosate (0.66mM), a commercialized pesticide in Algeria, on growth and major cellular antioxidant makers (GSH, GST, and CAT) in *Paramecium tetraurelia*. The median lethal concentration (LC_{50}) values of Ap1, Ap2, and glyphosate were preliminarily determined. The test chemicals caused marked disruption in *Paramecium* kinetic growth and the cellular behavior including the trajectory and motility velocity and decreased percentage of response. In addition, the GSH level, and the enzymatic activity of GAT and GST were significantly decreased in treated cells. The observed adverse effects were less pronounced in Ap1 and Ap2 as compared with glyphosate-treated cells. Conclusively, the Ap1 and Ap2 proved to be promising lesser toxic and safer compounds than glyphosate on protozoan ciliate, *Paramecium tetraurelia*.

INTRODUCTION

Organophosphorus compounds are world-widely used in agriculture, medicine, and the chemical industry due to their low persistence and effective insecticidal activity, as well as nutritional benefits (Upadhyay & Dutt 2017), and antibacterial properties (Jones & Daly 1993). Further, aminophosphate are effective organophosphorus compounds and structural analogs to their corresponding aminoacids, in which a phosphonic, $P(O)(OH)_2$, or phosphinic acid group, P(O)(OH)R replaces carboxylic acid group (Hellal *et al*. 2016). In addition, they have typical biological and biochemical properties and act as herbicides, enzyme inhibitors, and antitumor agents (Tajti & Keglevich 2018). Hence and due to their low toxicity to the environment, several researchers have been worked on developing new methodologies for the synthesis of aminophosphonate derivatives (Dake *et al*. 2011, Devineni *et al*. 2013, Bálint & Keglevich 2016). However, these compounds may be toxic at non-negligible concentrations and may contaminate the aquatic environment through waterways and runoffs, putting thus, a wide variety of marine organisms at considerable risk (Miyoshi *et al*. 2003). Accordingly, aminophosphonates have been reported to induce hemolysis of erythrocytes (Trela *et al*. 2001), cytotoxicity (Kraicheva *et al*. 2010), and genotoxicity (Kraicheva *et al*. 2012). Interestingly, the toxicological monitoring of aquatic environment contamination by pesticides using non-target organisms as alternative models is a valuable tool for protecting and preserving environmental integrity (Twardowska *et al*. 2007, Zhang *et al*. 2015). Protozoan cells were reported to be an ideal-typical model as bioindicators of chemicals contaminated aquatic environments due to their sensitivity to environmental alterations (Nalecz-Jawecki *et al*. 1993, Tushmalova *et al*. 2014). Additionally, their use to assess the toxic effects of pesticides contaminated waste waters was relatively recent throughout the world (Rao *et al*. 2008). Consequently, *Paramecium tetraurelia* was commonly used in several studies investigating the cytotoxicity of various compounds, causing physiological, morphological, and behavioral alterations (Minogue & Thomas 2004, Prokop'ev & Kan 2020). Thus, to our knowledge, this study is the first to compare the toxic effects of the commonly used herbicide (glyphosate) and two newly synthesized aminophosphonates on behavior and biochemical markers in *Paramecium tetraurelia*.

MATERIALS AND METHODS

Chemicals: Two aminophosphates (AP) namely, diethylphenyl-ureido-methyl-phosphonate ($C_{12}H_{19}N_2O_4P$), and diethyl-4-oxo-16-methyl-phenyl-ureido-methylphosphonate

($C_{13}H_{21}N_2O_5P$) synthesized at the Laboratory of Applied Organic Chemistry of Annaba University, Algeria, and provided in powder form were used in this study as test compounds. They have a structural analogy with glyphosate, the most worldwide used herbicide, and act against both broadleaf plants and grasses (Fig. 1). Moreover, the chemical structure and the level of the theoretical toxicity risk of the synthesized aminophosphates were determined by using Osiris Property Explorer (Table I) (Sander 2001).

Synthesis of the aminophosphonates: The synthesis process of these aminophosphonates was performed by using three components; Kabachnik-Fields reaction of urea, triethylphosphite, and aldehyde, by one-pot synthetic strategy as previously described (Bouzina *et al*. 2016). The powdered chemicals were beforehand, dissolved in 2 % acetone and diluted in 10 ml aliquot solutions. Of note, glyphosate (Sigma-Aldrich (Saint Quentin Fallavier, France), was used in the experiments as a positive control (Fig. 2).

Cell culture: *P. tetraurelia* cells selected as test ciliated protists for the present study were obtained from the laboratory of cell toxicology of the Annaba University of Algeria. The cells were cultured in a medium prepared as previously described (Azzouz *et al*. 2011). In brief, 10 g of lettuce, 5 g of hay, 5 g of wheat, 5 g of cucumber, 5 g of potato, and 2 g of peanuts were boiled for 1 h in 1.5 L of distilled water. Once the mixture becomes cool, it is filtered and then sterilized by boiling at 100° C for 30 min in a heat-resistant bottle. The pH of the resulting mixture was adjusted to a value of 6.5, and the mixture was afterward kept away from light at a temperature close to 25° C. The prepared medium was incubated in an oven (Memmert UM 400) at temperature ranges between 28 and 16° C. The paramecia cells were subcultured in the culture medium every three days to get a yellowish film on the surface of the medium culture due to the presence of wheat grains, enabling effectively the growth of the *Paramecium* population to get their substantial size.

Treatment: A pilot study using a range of concentrations of test chemicals was performed to determine the toxic concentrations able to induce marked effects on *Paramecium tetraurelia*. As a result, 10 ml of the test compounds (0.66 mM glyphosate, 30 mM Ap1, and 60 mM Ap2) was added to the culture medium of *P. tetraurelia* grown in the logarithmic growth phase with an initial cell density of 203 cells ml^{-1} at a temperature about 30° C.

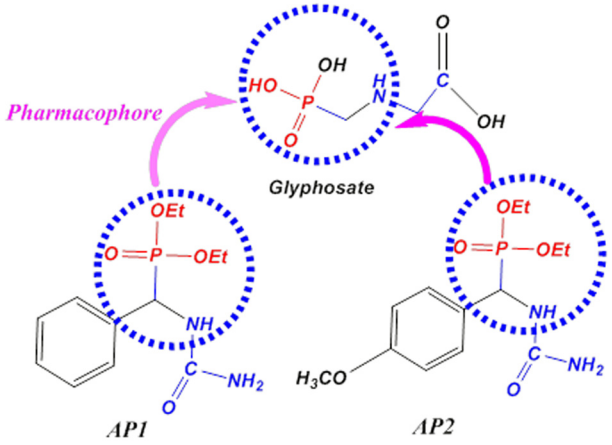

Fig. 1. – Structural analogy of the synthetized amino-phosphonates (Ap1 & Ap2) with glyphosate (positive control).

Calculation of median lethal concentrations (LC_{50}): The value of the median lethal concentration (LC_{50}) was estimated as previously described (Finney 1952) based on the mortality curve for different concentrations of the test chemicals. The corrected mortality rates were calculated using a Probit regression line which is in the function of doses into decimal logarithms.

Determination of percentage of response (PR): Chemicals-induced inhibition of protozoa cell growth after 72 h was evidenced by the determination of the cell response percentage (RP). Of note, the growth inhibition is indicated by positive values, and the growth stimulation is indicated by negative values of the percentage of response (Wong *et al*. 1999). The PR is calculated according to the following equation:

$$RP = 100 \times Cn - EnCn$$

where *RP* is the protozoa responses percentage (%), *Cn* is the cell control number (cell/mL), and *En* is the treated cells number (cell/ml).

Growth kinetic study: The growth kinetics of paramecia cells exposed to the test compounds was daily monitored under a light microscope (Leica ATC 2000 Microscope, Wetzlar, Germany) by counting the precise number of living cells in a known culture volume from the first contact with the toxicants (T = 0) up to 144 h.

Behavioral study: The inhibition by test compounds of protozoa motility was used as a toxicity endpoint, determined by the swimming trajectory and speed of *P. tetraurelia* using an auto-

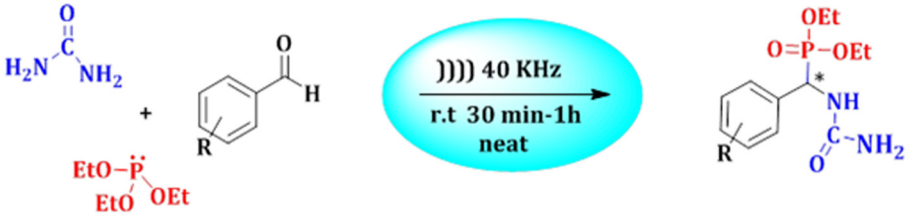

Fig. 2. – Synthesis process of the studied amidophosphonates (AP1 and AP2).

Table I. – Osiris calculations of toxicity risks of the studies aminophosphate and glyphosate. Highly toxic: (---), Not toxic (+++). [a] MUT: Mutagenic, TUM: Tumorigenic, IRRIT: Irritant, RE: Reproductive effective. [b] cLogP: Solubility, DL: Drug-likness, DS: Drug-Score. MW: Molecular weight.

Compounds	MW	Toxicity risks[a]				Osiris calculations[b]			
		MUT	TUM	IRRI	REP	cLogP	cLogS	DL	DS
Ap1	286	+++	+++	+++	+++	0.98	–2.63	–33.5	0.64
Ap2	316	+++	+++	+++	+++	0.91	–2.65	–33.1	0.54
Gly	253	---	---	+++	---	0.44	–3.01	2.77	0.88

Table II. – Median Lethal Concentration and 95 % fiducial limits of Ap1, Ap2 and glyphosate (72 h LC_{50}) to a freshwater protozoan ciliate, *Paramecium tetraurelia*.

Test compounds	Exposure time (hour)	Acute toxicity range (LC_{50} mM)		Regression equation (Y = b + ax)	95% confidence limit (mM)
		Upper	*Lower*		
Ap1	72	80.30	29.51	Y = +3.217 + 1.196x	33.09 ± 0.67
Ap2	72	76.44	15.37	Y = +0.94 + 2.461x	41.36 ± 0.73
Glyphosate	72	50.21	4.93	Y = –1.124 + 3.057x	111.07 ± 0.4

mated video-tracking system (Kinovea video analysis software 0.8.15).

Antioxidant response of *P. tetraurelia*: The enzymatic activity of catalase (CAT), glutathione S transferase (GST), and the content of glutathione (GSH) are the main indicators of a defensive mechanism fighting against chemicals–induced oxidative stress (Sara *et al*. 2016). Accordingly, the activity of CAT was determined as reported elsewhere (Aebi 1984), where the absorbance decrease is spectrophotometrically recorded for three minutes at 240 nm, using the extinction coefficient as ε = 39400 $L.\mu M\text{-}11{\cdot}cm^{-1}$. The activity of glutathione S-transferase (GST) was assayed as previously described (Habig *et al*. 1974). The reaction is based on GST and the substrate CDNB (1-Chloro2, 4 dinitrobenzenes), resulting in the formation of 1-S-glutathionyl 2-4 Di nitrobenzene. Whilst, the content of glutathione (GSH) was determined according to a method previously reported (Weckbecker & Cory 1988), where the reaction between acid5,5'-dithiobis-2-nitrobenzoic acid (reagent Elleman) and SH groups of glutathione results in 2-nitro-5-mercapturic acid whose absorbance can be read at 412 nm.

Statistical analysis: Experiments were conducted three times, and results are expressed as mean ± SE of the mean. Comparisons of multiple groups were tested by one-way ANOVA using GraphPad Prism (Version 5. 01) where $p < 0.05$ was considered significant.

RESULTS

Median lethal concentration (LC_{50})

The LC_{50} value of the test compounds in *Paramecium* for 72 h was higher in Ap1 and Ap2; 33.09 mM and 41.36, respectively than that of glyphosate (0.66 mM) treated cells (Table II).

Response percentage

Fig. 3 shows a significant decrease in the response percentage (RP) in cell culture treated with Ap1 30 mM ($P < 0.01$), Ap1 60 mM ($P < 0.01$), Ap2 40 mM ($P < 0.01$), and Ap2 60 mM ($P < 0.001$) as compared with glyphosate.

Growth kinetics of **P. tetraurelia**

As shown in Fig. 4, the cell growth was similar between control and glyphosate cells and those exposed to Ap1 (30 and 60 mM) between 12 and 48 h. Also, cells exposed to Ap2 (40 and 60 mM) have almost close cell growth to controls, but higher than that of glyphosate in the same exposure time. In addition, the growth evolution measured during the exponential growth phase of

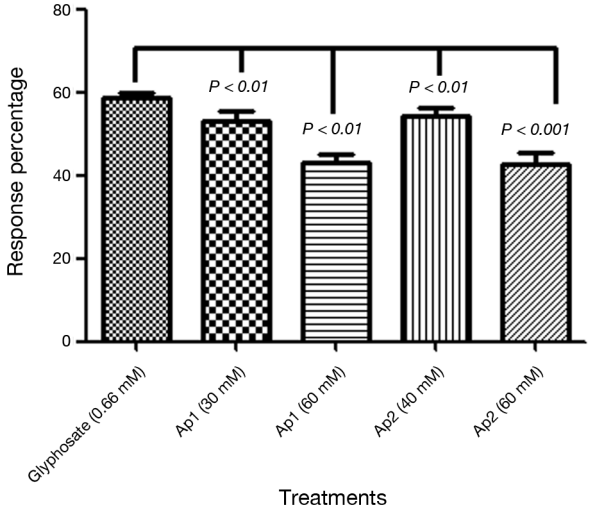

Fig. 3. – Evolution of the response percentage of *P. tetraurelia* treated with glyphosate (positive control), Ap1 (30 mM & 60 mM) and Ap2 (40 and 60 mM). Data are given as mean ± SD. Values with superscripts are statistically different p value.

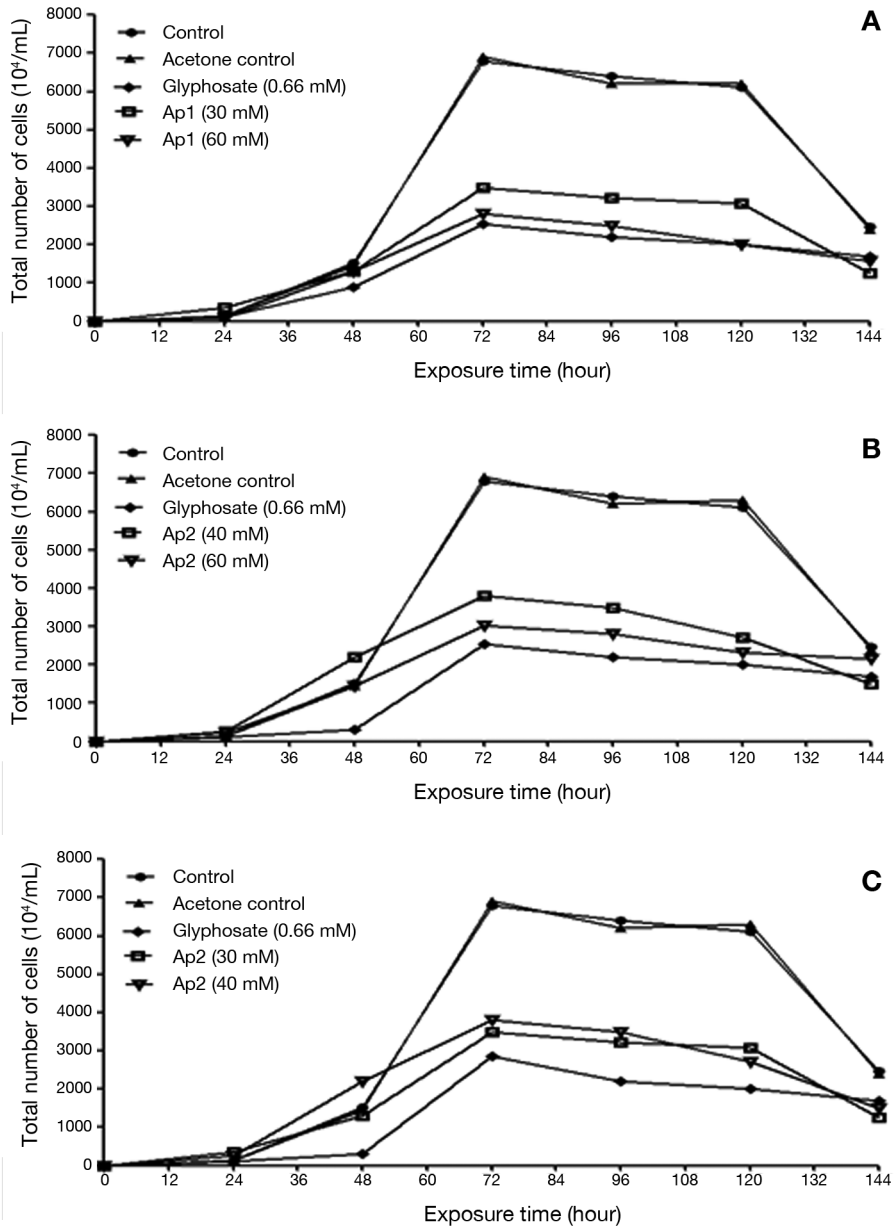

Fig. 4. – Kinetics of growth of the control paramecia, and those treated with 30 and 60 mM Ap1 (**A**), 40 and 60 mM Ap2 (**B**), and 30 mM Ap1 and 40 mM Ap2 (**C**) in comparison with paramecia received acetone and glyphosate.

paramecium showed normal growth of control cells with a cell number reaching 7000 cells/ml during the exponential phase (72-120 h), followed by decreased growth rate after 120 h. Similarly, the treated cells reached their maximum in 72 hours but with a lower number of cells than that of the controls (approximately 3000 cells/ml), and subsequently, the growth rate was markedly decreased after 120 h and reached nearly 2000 cells/ml.

Behavior response of Paramecia

The control organisms revealed motility with successive waves of contractions running from the surface of the body along the lines of cilia insertion, and a linear swimming trajectory (Fig. 5A). Additionally, a marked trajectory disturbance was noticed in paramecium cells treated with Ap1 30 & 60 mM (Fig. 5B, C), and Ap2 40 & 60 mM (Fig. 5D, E), and similarly those received glyphosate (Fig. 5G). Further, the swimming velocity was significantly decreased ($P < 0.01$) in Ap1, Ap2, and glyphosate-treated paramecia compared with that of controls. Hence, the exposure length (72 h) caused inhibition velocities ranging from 20 to almost 50 %. Hence, the motility velocity was decreased significantly ($P < 0.01$) in Ap1, glyphosate, and ($P < 0.01$) Ap2 40 mM and ($P < 0.001$) Ap2 60 mM when compared with control untreated cells. By comparison with glyphosate-treated cells, the motility velocity was significantly increased in Ap1 and Ap2 ($P < 0.01$) treated cells. Importantly, velocity disturbances are manifested by a sudden change in paramecia direction followed by a zigzag swim, and accordingly, the trajectories lose their linearity and become more circular

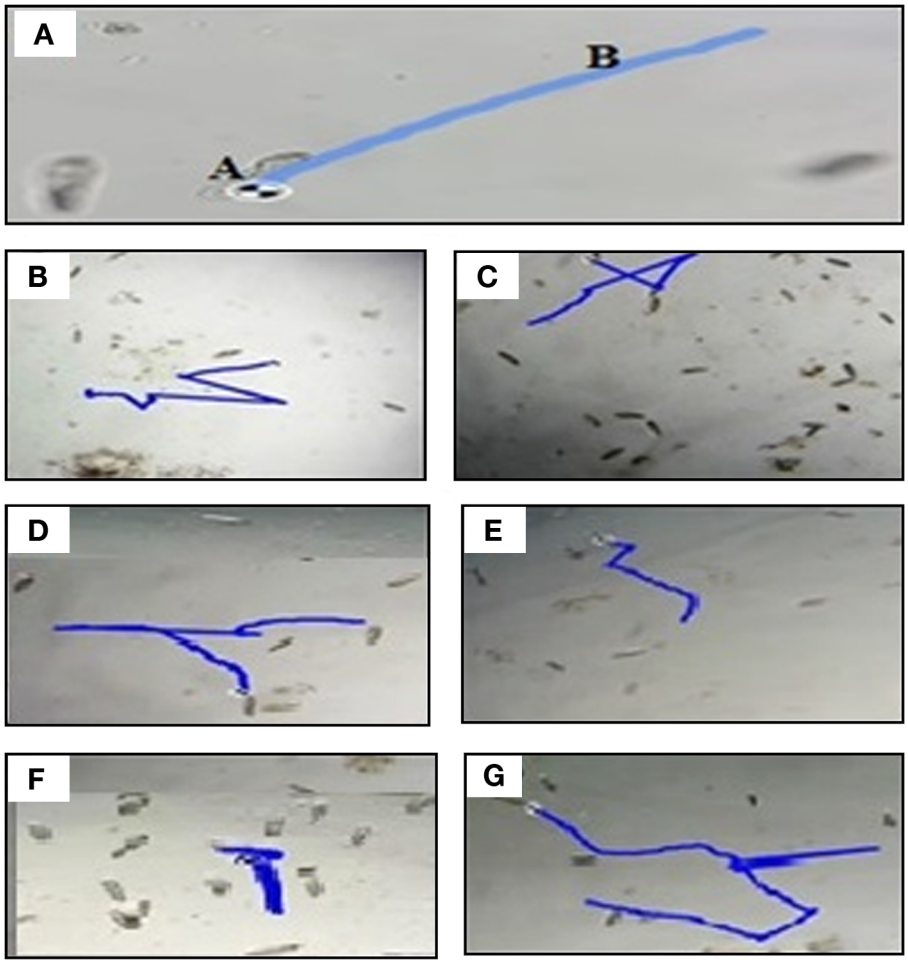

Fig. 5. – Light microscopic (Gr ×40) showing the swimming activity and trajectory motility of control paramecia (**A**), and those receiving Ap1 30 and 60 mM (**B**, **C**), Ap2 40 and 60 mM (**D**, **E**), and glyphosate 0.66 mM (**F**, **G**).

with more rotations on the spot at the highest concentrations of the two test compounds (Fig. 6).

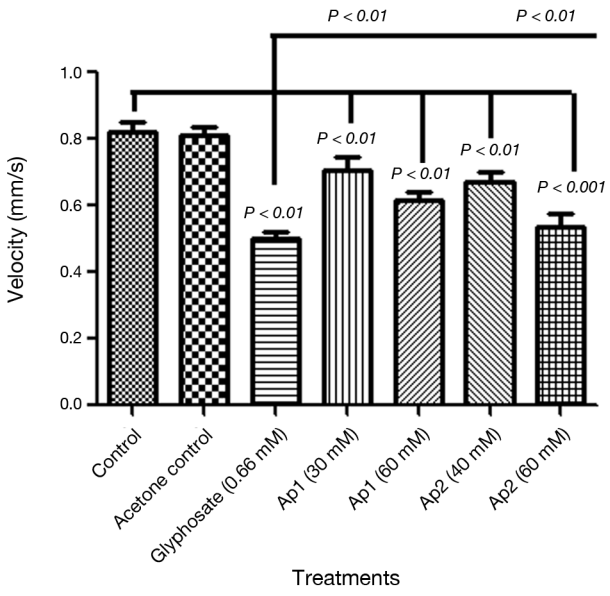

Fig. 6. – Velocity motility of control and treated paramecia recorded after 72 h.

Results of morphological abnormalities

The morphology of control paramecia showed a normal cell shape with an intact membrane providing it normal motility and swimming pattern (Fig. 7A). Whilst, different degrees of morphological abnormalities were observed in Ap1, Ap2, and glyphosate-treated paramecia compared with controls. These morphological changes are shown by swelling, round and oval-shaped cells, in addition to the formation of vesicles and budding in the cell membrane, darkening, and condensation of the cytoplasm, which consequently interrupt the normal swimming pattern of the paramecia (Fig. 7B, C, D, E, F). However, glyphosate-treated paramecia resulted in cell swelling associated with the destruction of the cell membrane leading to cell lysis and leakage of the cytoplasmic contents out of the cell (Fig. 7 G).

Antioxidant responses of paramecia

As shown in Fig. 8, the reduced glutathione (GSH) level decreased significantly ($P < 0.01$) in a concentration-dependent manner in Ap1 (30 and 60 mM), Ap2 (40 mM, 60 mM), and glyphosate when compared with

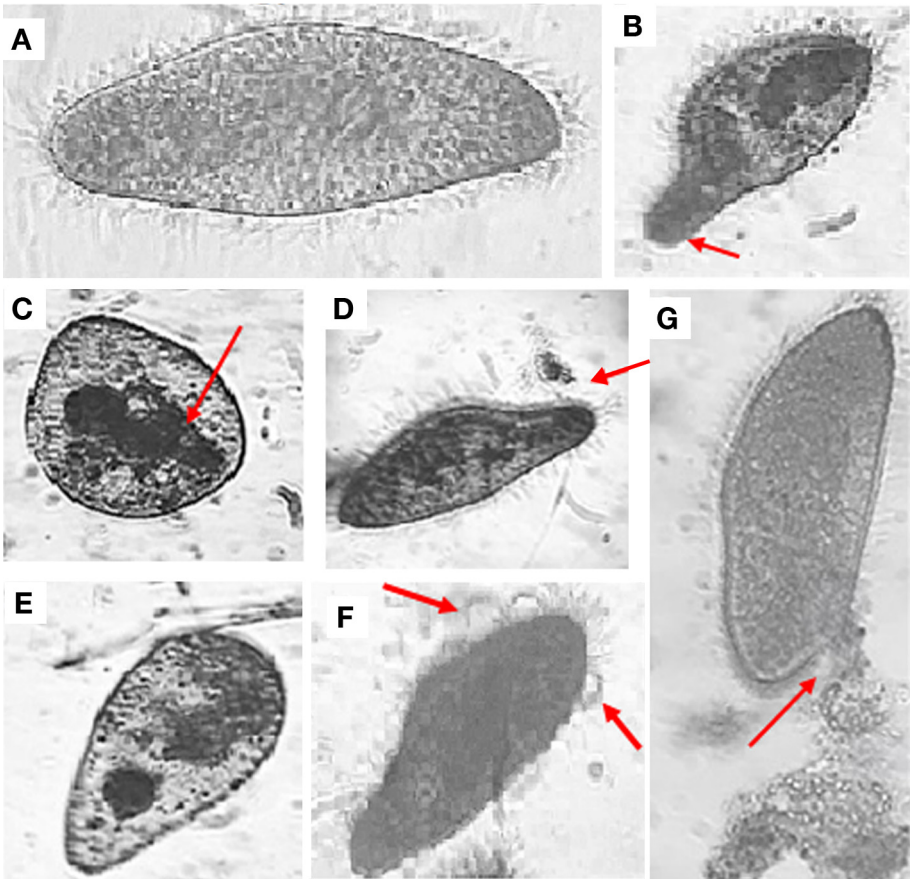

Fig. 7. – Light microscopic (Gr ×40) showing altered morphological features in control paramecia (**A**) and those exposed to Ap1 (30 mM) (**B**), Ap1 (60 mM) (**C**), Ap2 (40 mM) (**D**), Ap2 (60 mM) (**E**), and glyphosate (0.66 mM) (**F**).

control cells. However, the level of GSH increased significantly ($P < 0.01$) in treatment with Ap1 and ($P < 0.01$) Ap2 40 mM and ($P < 0.05$) Ap2 60 mM compared with glyphosate treatment. Similarly, the enzymatic activity of GST was significantly decreased ($p < 0.01$) in glyphosate, Ap1 (30 and 60 mM), Ap2 40 mM and ($P < 0.01$) Ap2 60 mM compared with controls, but significantly increased ($P < 0.001$) in Ap1, Ap240 mM and ($P < 0.05$) in Ap2 60 mM compared with glyphosate treated cells. Further, the enzymatic activity of CAT was decreased significantly ($P < 0.01$) in glyphosate, Ap1, and Ap2 as compared with controls, but significantly increased in ($P < 0.05$) Ap1 (30 mM), ($P < 0.01$) Ap1 (60 mM), Ap2 40 mM ($P < 0.01$), and Ap2 60 mM ($P < 0.001$) compared with glyphosate treated cells.

DISCUSSION

In this study, the toxic effects of the test compounds (Ap1 and Ap2) on *Paramecium tetraurelia*, a freshwater protozoan ciliate, was investigated based on the determined LC_{50} values, which were in the same range as those reported for other synthesized amino-phosphates (Saib *et al*. 2014). In addition, results showed an inhibitory cell growth kinetics effect in accordance with the negative evolution of response percentage induced by the test chemicals as compared with control and acetone control, showing no effect either on cell growth or response percentage. This is similarly reported in previous studies investigating the effect of organophosphorus on cell physiology and behavior (Amanchi & Hussain 2010, Mansano *et al*. 2020). The cell growth inhibition is somehow related to impairment in cell division and activation of cell death processes (Amanchi & Hussain 2010), and hence, It is evident from results that Ap1 and Ap2 act as potential genotoxic compounds at toxic concentrations to ciliate models. Furthermore, the motility velocity was significantly increased in Ap1 and Ap2 compared with the glyphosate-treated cells, but it is lower than those of controls, as well as the negative effect on the paramecia trajectory motility evidenced by a marked change in their motility direction (circular and zigzag movement). This is likely explained by the effect of test pesticide-induced disruption in cell metabolism and morphological deformities (Rao *et al*. 2006, Venkateswara *et al*. 2007). The toxicity of organophosphorus-induced alterations in morphology and motility velocity and direction of unicellular organisms have been well documented (Azzouz *et al*. 2011, Benbouzid *et al*. 2012). As a result, cells process an antioxidant defense acting effectively against oxidative stress-induced cellular damage (Di Giulio *et al*. 1989). In this regard, our findings revealed marked depletion in the level of GSH, an abundant peptide whose oxidation is

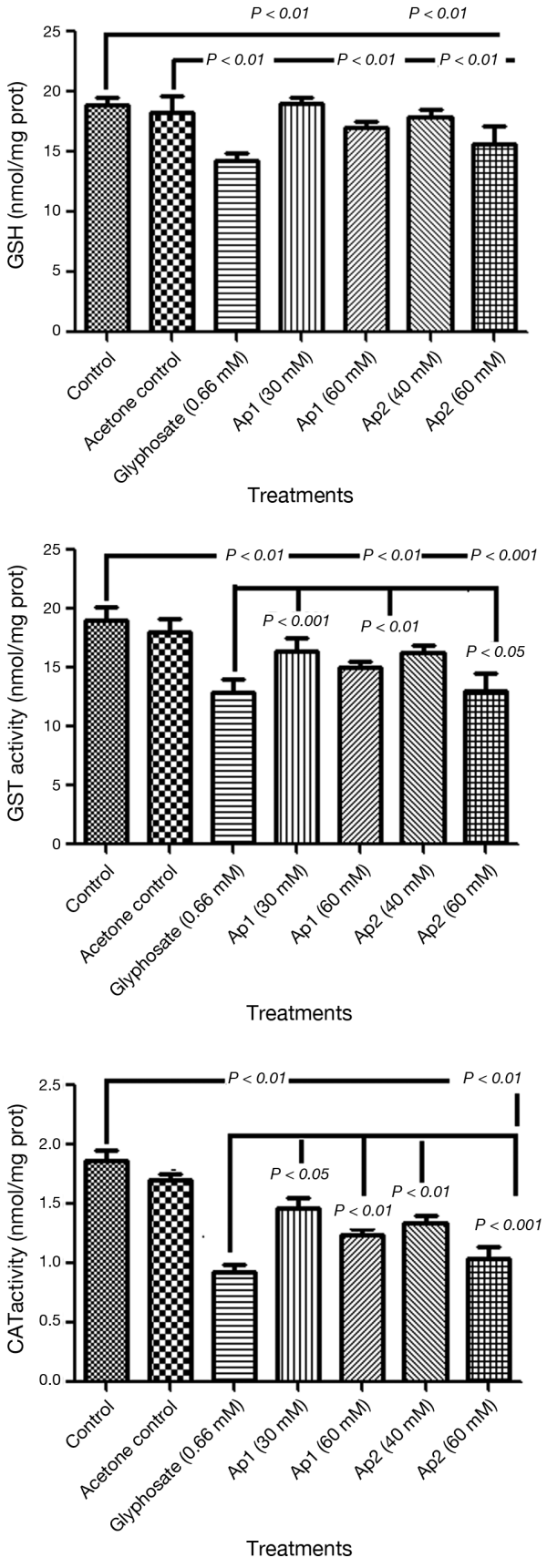

Fig. 8. – Level of reduced glutathione (GSH), and enzymatic activity of catalase (CAT) and glutathione S transferase (GST) in control and treated Paramecia.

ensured by glutathione peroxidase, in Ap1 and Ap2 treated cells as compared with controls, and this can confirm the involvement of GSH in chemicals-induced oxidative stress by scavenging free radicals causing cellular oxidative damages (Croute *et al*. 1985). However, this parameter increased significantly when compared with the positive control (glyphosate), and hence, the test compounds at the selected concentrations revealed a lesser toxic effect than glyphosate on the GSH level. Additionally, the enzymatic activity of GST and CAT was significantly lower in Ap1 and Ap2-treated paramecia than that in controls, but higher than that in glyphosate-treated cells. The decreased activity of these antioxidant markers explains the reduction of their synthesis, as well as consumption during the detoxifying process and their scavenging activity against reactive oxygen species (ROS) production in the cell (Ossana *et al*. 2019, Ramzan *et al*. 2022).

CONCLUSION

The toxicological evaluation of chemicals can be easily conducted by using paramecia, while the use of human and/or animal cell lines cannot be feasible. The performed study proved the importance of using *P. tetraurelia* as a unicellular model to determine the toxic effects of chemicals on physiological and biochemical markers. The tested compounds thereof, induced a reduction in kinetic growth and percentage response as compared with control cells, in addition to a marked decrease in the major antioxidant markers (GSH, GST, and CAT). Interestingly, Ap1 and Ap2 induced lesser physiological and biochemical changes as compared with glyphosate treatment, suggesting thus that Ap1 and Ap2 are toxic compounds on paramecia growth and antioxidant defense system, but they are less toxic compared with glyphosate.

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