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Alleviate Effect of Pomegranate Peel Extract in Ameliorating Fluoride-Induced Cytotoxicity, Oxidative Stress in *Tetrahymena pyriformis* Model

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Abstract: Fluoride is a major oligo element found in nature, at excessive amounts can cause enormous harm in mammalian cells. Fruits peel, considered most often as a waste of juice processing, could play an important role in attenuating metal cytotoxicity. The present study evaluated the effect of pomegranate peel (*Punica granatum. L*) methanolic extract (PPE) on the Fluoride-induced toxicity and redox status in the protozoa *Tetrahymena pyriformis*. Polyphenols of peel extract were extracted using methanol and characterized by spectrophotometric methods, total phenolic content (TPC), total flavonoids content (TFC), and *in vitro,* antioxidant properties were assessed using the Folin-Ciocalteu method and DPPH, ABTS, and FRAP. Pomegranate peel is a rich source of phenolic compounds TP (223.21 ± 15 mg GAE/g dw), TF (52.12 ± 1.36 mg Qu/g dw) and showed high antioxidant properties DPPH (EC_{50} 0.043 ± 0.06 mg/ml), ABTS (EC_{50} 0.06 ± 0.01 mg/ml) and FRAP (1.47 ± 0.01 mg AA equivalents/g dw). Cells were incubated with fluoride alone and in combination with PPE. NaF (0.8 mM) significantly decreased the cell viability, induced oxidative stress by decreasing antioxidants enzyme activities, and increased intracellular fluoride content. Treatment with NaF in combination with PPE decreases CAT, SOD, and GPx activities and alleviates GSH content. These findings suggest that pomegranate peel biomolecules may have a protective effect against fluoride induced-toxicity.

Keywords: fluoride; *Punica granatum*; punicalagins**;** antioxidant.

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1. Introduction

Fluorine (F) represents 0.06-0.09% of earth's crust, found in nature in its inorganic and organic forms as Fluoride [1]. Due to the high electronegativity of this halogen, fluoride can interact with many cations, including hydrogen and a wide variety of metals, and therefore exists in acidic environments as its protonated form (HF). The main source of fluoride administration is drinking water in rural areas, especially areas surrounding phosphate production and aluminum manufacture [2-3]. Its deleterious impacts on health are evident in large areas of the world. Morocco is the leading producer and exporter of phosphate, and its derivate from the world, sharing about 30% of the global market [4].

Phosphate fertilizers synthesized from phosphate rocks contain different fluoride rocks as fluorite (CaF_2) or fluorapatite ($Ca_2(PO_4)_3F$) [5], which are suggested to be the main source

of contamination of groundwater by fluoride [6,7]. A study by Jaudi *et al*. showed that more than half (53%) of the wells exceeded the upper limit set by WHO (1.5 mg / l) [8]. The groundwater is supposedly the main source through which people are highly exposed to fluoride, compared with other sources of exposure. At low doses, Fluoride is necessary to maintain the structure and physiological function of bones and teeth [9-11]. However, excessive fluoride is toxic to soft tissues [12,13] and may cause health problems, including dental fluorosis, osteoporosis, and bone sclerosis [6-7]. Nevertheless, long exposure to fluoride can be harmful to mammalian nervous, reproductive, and immune systems [14-16], and may cause cell death and alteration of the antioxidant defense system and influence metabolism of lipids [17-18]. The primary mechanism involved in fluoride toxicity can be attributed to the oxidative stress caused by this halogen [19]. Commonly confirmed in previous studies, fluoride exposure induces the generation of free radicals, consequently causing the oxidation of macromolecules to which can lead to fragile membrane phospholipids, mitochondrial membrane depolarization, and apoptosis [20-21]. In addition, the accumulation of fluoride can inhibit the activity of antioxidant enzymes, such as SOD, CAT, and GPx [22,23].

In recent years, an increase of interest in search of fruits bioactive molecules, pomegranate (*Punica granutum L.*) is accepted as a unique fruit having considerably high antioxidant activity. Different parts contain valuable compounds, such as peel, arils, and seeds [24-25]. This valuable fruit is a rich source of polyphenols as hydrolyzable tannins, condensed tannins, flavonols, anthocyanins, and phenolic and organic acids [26], peel as the non-edible part of the fruit represents 50% and is characterized by antioxidant capacity compared to the edible part of the fruit consisted of 10% seeds and 40% arils [27]. The antioxidant capacity of the pomegranate has been attributed to its two major compounds: punicalagin and ellagic acid. These isomers constitute over 85% of tannins present in the peel [28,29]. The biological properties of pomegranates promoted us to evaluate their effect on NaF-induced toxicity in a protozoa model. *Tetrahymena pyriformis*, ciliated protozoa that could be found in freshwater, is a eukaryotic cell well establish as a suitable model of eco-toxicological studies and especially in aquatic ecosystems [30]. Therefore, our study aims to determine the dose-dependant of fluoride and evaluate whether an extract from pomegranate peel can exhibit a protective effect against the acute toxicity of fluoride.

2. Materials and Methods

2.1. Chemicals.

All chemicals and reagents were purchased from Sigma-Aldrich Chemistry (Germany).

2.2. Fruit samples and extraction procedure.

Mature pomegranate fruit was purchased from a local market in old Abdellah, Beni Mellal province of Morocco (32°29'32.4"N 6°27'05.6"W). Fruits were manually peeled, and collected peels were then rinsed with distilled water. Peel and seed were dried in an oven (Binder, BD 56 Germany) with air circulation at 40 °C, and they were finely ground in a laboratory grinder. The dried sample was then stored at 20°C until further use. The peel and seed powder (10 g) was extracted with 60 mL of methanol by magnetic stirring at room temperature for 24 h. The extracts were filtered through Whatman no. 41 filter paper for the removal of particles. The residue was re-extracted with 50 mL of methanol and filtered. The pool of extracts was concentrated under a vacuum at 40 °C (Rotary evaporator Buchi R-210)

to obtain the final extracts: Pomegranate Peel Extract (PPE) and Pomegranate Seed Extract (PSE) [31].

2.3. Determination of total phenols and total flavonoids.

The total phenolic content was determined by the Folin-Ciocalteu method Singleton & Rossi. Briefly, pomegranate extracts (0.2 mg) were mixed with 1.0 mL of 10-fold diluted Folin-Ciocalteu reagent. After 3 to 5 min, 0.8 mL of a 7.5% (w/v) sodium carbonate solution were added, and the mixture was left to stand for 30 min at 30°C, then the absorbance was measured at 765 nm using a Genesys-5 UV-visible spectrophotometer, and results were expressed as mg of Gallic acid equivalents (GAE) per gram of dry extract [33]. Determination of flavonoid content is based on the formation of the complex flavonoid and aluminum. Briefly, 0.5 ml of both peel and seed extract were mixed 0.5 ml aluminum chloride 2%, then 3 ml of potassium acetate 5% (Merck, Germany) was added. After 40 min at room temperature and the absorbance was measured at 415 nm, results are expressed as rutin equivalents in mg/g [33].

2.4. Antioxidants properties.

2.4.1. DPPH free radical scavenging assay.

Radical DPPH scavenging capacity was estimated from the difference in absorbance with or without antioxidants and expressed as percent DPPH disappearance due to the sample concentration [34]. Briefly, different concentrations (50 and 100 ppm) of pomegranate peel were taken in different test tubes. The volume was adjusted to 100 µL, by adding methanol. Then 5 mL of a 0.1 mM methanolic solution of DPPH was added to these tubes and shaken vigorously [32]. After incubation at 27°C for 20 min, absorbance was measured at 517 nm. The radical scavenging activity is expressed as the following formula: RSA (%) = (control OD - sample OD/control OD) × 100. The antioxidant activity was calculated as the effective concentration at 50% (EC_{50}) of a sample required to decrease the absorbance by 50%.

2.4.2. ABTS cation radical assay.

ABTS assay was based on the method of Ramos *et al*. [35] with slight modifications. Briefly, the radical ABTS solution was prepared by mixing an equal volume of ABTS stock solution 7 mM with 2.45 mM of potassium persulfate. The mixture was allowed to stand for 16 hours at room temperature in the dark. Before been used, different concentrations of this solution in acetate buffer (pH 4.6 50 mM) were prepared to obtain an absorbance of 0.700 ± 0.02 at 734 nm. The assay was performed in a 96-well microplate. Hence 150 µL of the diluted sample was added to the plate, followed by adding 150 µL of the ABTS solution. Absorbance was measured after 6 min by spectrophotometer (Synergy HT, Bio-Tek Instruments, Winooski, VT, USA). ABTS scavenging percentage was calculated using the following equation: ABTS scavenging (%) = (control OD – sample OD/control OD) × 100. The EC_{50} values were calculated from standard curves.

2.5. Chelating activity.

2.5.1. Ferric reducing power assay (FRAP).

Determination of the ferric reducing antioxidant power (FRAP) was based on the method by Oyaizu *et al*. 500 µL of peel and seed were added to 1.25 mL of phosphate buffer (0.2 M, pH 6.6), 1.25 mL of ferricyanide potassium is added to each tube, followed by 1.25 mL of trichloroacetic acid (10% v/v). The mixture is incubated at 50°C for 15 min, the mixture was diluted in distilled water (1/2), and 0.25 mL of ferric chloride 1% was added, absorbance was measured at 700 nm, and results are expressed as equivalent mg ascorbic acid (AAE) per 100 g of dw [36].

2.5.2. Fe^{2+} Chelating Activity Assay.

Pomegranate peel and seed extracts of ferrous ions Fe^{2+} were measured using the method described by Dinis *et al*. [37]. Briefly, 0.5 mL of extract at different concentrations were added to 1.6 mL of distilled water and 0.05 mL of $FeCl_2$ (2 mM) were added, followed by adding 0.1 mL Ferrozine (5 mM). The mixture is incubated at room temperature for 10 min, a complex of Fe^{2+} and Ferrozine complex was measured at 562 nm. The chelating activity of the extract was calculated using the following equation: Metal chelating activity (%) = (OD control - OD sample/ OD control) × 100. The results were expressed as µmol of EDTA equivalent/g of dry weight.

2.6. Protective effect of pomegranate peel extract (PPE) on NaF–induced cytotoxicity.

2.6.1. Cell culture of tetrahymena pyriformis.

Tetrahymenna pyriformis, strain E, ref ATCC has grown aerobically without shaking to exponential phase at 28°C for 72 h in protease peptone yeast extract (PPY) medium containing (1.5 % protease-peptose, 0.25% yeast extract), at pH=7.4 [38]. For all experiments, the density of cells was adjusted to 5 x 10^3/ml.

2.6.2. Cell viability assays.

To study the inhibitory effect of fluoride, pomegranate peel extract, and ascorbic acid, the medium was supplemented with 0,2% (w/v) D-glucose (PPYG). Cells were supplemented with different concentrations of sodium fluoride (NaF) (100.200….1000 µmol) to determine the sub-lethal concentration, or pomegranate peel methanolic extract (PPE) (10, 25,50,75,100 µg/ml), and ascorbic acid as a positive control (10,25,50,75,100 µg/ml) alone. To study the protective effect of PPE on NaF induced toxicity, cells were incubated with IC_{50} of NaF combined with different PPE concentrations. After 24 h of incubation, the viability of cells was measured using mitochondrial succinate dehydrogenase activity. The purple color formazan product was measured at 570 nm. The percentage of inhibition was calculated compared to untreated cells, and the Inhibitory concentration IC_{50} of NaF, PPE, and ascorbic acid was defined by linear regression analysis.

2.7. Measurement of intracellular fluoride level.

Cells (5 × 10^3 cells/ml) were exposed to IC_{50} of NaF, and different concentrations of PPE and ascorbic acid below their respective IC_{50}. At the end of treatment, cells were collected

by centrifugation (12000 g, 15 min) and lysed using the cell lysis buffer (100 mM Tris–HCl, 50 mM EDTA and 1% Triton X-100) [39]. The level of Fluoride in the cell lysate was determined with an Orion fluoride ion analyzer (Thermo Fisher Scientific, Waltham, Massachusetts, USA), model 9609, and expressed in mg/L.

2.8. Measurement of antioxidants enzymes activity, and glutathione assay.

To evaluate the protective effect of PPE to an oxidative stress induced by fluoride, cells were exposed to (IC_{50}) of NaF and different concentrations of PPE and ascorbic acid below their respective IC_{50}. After 24 hours of incubation, the cells were harvested by centrifugation at 12000 g for 15 min at 4°C, and washed three times with phosphate buffer pH=7.4. The mixture is sonicated with a sonicator (Heidolf Diax 600, Germany) (30s, 2 cycles, 40 %), and responded in phosphate buffer. The supernatant was collected and used for enzyme activity. Determination of protein was carried out using the method of Lowry *et al*. [40]. The total activity of superoxide dismutase (SOD) was measured using the method of Beyer & Fridovich [41]. SOD activity was determined by measuring its ability to inhibit the photo-reduction of nitroblue tetrazolium (NBT); activity is expressed as UI/mg of protein. The activity of catalase (CAT) was measured according to the method of Sinha *et al*. [42]. The decomposition of H_2O_2 was determined by its absorbance decrease at 240 nm ($\varepsilon = 39.4\ mM^{-1}\ cm^{-1}$). The total activity of glutathione peroxidase (GSH-Px), according to the method of Battenberg *et al*. [43], absorbance was recorded at 420 nm. The activity was expressed as µmol of GSH/min/mg of protein. The level of glutathione (GSH) was measured using Ellman's method. Reduced thiol contents were expressed in nmol of GSH/mg of protein [44].

2.9. Statistical analysis.

Statistical analysis was performed using SPSS 23. Results were expressed as the mean ± SEM. Comparison between groups was made by one-way ANOVA followed by a Student post hoc test. Differences with values of $p < 0.05$ were considered statistically significant.

3. Results and Discussion

3.1. Total phenolic, total flavonoid contents.

The yield of extraction was found to be 13.24 ± 1.23 % and 8.23 ± 0.98 % in peel and seed, respectively total polyphenols ranged from 67.85 ± 07 to 223.51 ± 15 mg GAE/g dw. The total flavonoids ranged from 02.11 ± 0,2 to 52.12 ± 1.36 mg rutin/g dw in seed and peeled respectively (Table 1).

Table 1. Phenolic compounds in pomegranate extracts.

	% Yield	Total phenolic (mg GAE/g)	Total flavonoids (mg RE/g)
Peel	13,24 ± 1,23[a]	223.21 ± 15[a]	52.12 ± 1.36[a]
Seed	8,23 ± 0,98[b]	67,85 ± 0,7[b]	2,11 ± 0,02[b]

Different letters within the same column are indicating significant differences at $p < 0.05$. All data are expressed as mean ± SD of at least three replicates of each sample. (GAE: gallic acid equivalents. RE: rutin equivalents).

3.2. Antioxidants properties and chelating activity.

DPPH is an artificial radical widely use to determined antioxidant properties of various plant materiel [45]. As shown in (Table 2), scavenging 50% of the radical is found at a concentration of $EC_{50} = 0.04$ mg/ml in pomegranate peel while $EC_{50} = 0.88 \pm 0.02$ mg/ml in seeds. The antioxidant activity of pomegranate peel using ABTS was found 6.5-fold higher than the one found in seed extracts, thus $EC_{50} =$ 0,06 ± 0.01 mg/ml and $EC_{50} =$ 0,06 ± 0.01 mg/ml in peel and seed respectively (Table 2).

The reducing power was measured using the FRAP assay and Fe^{2+} Chelating Activity Assay, values expressed as mg ascorbic acid equivalent (AAE)/g of dw of peel or seed are shown in (Table 2). The values varied from 185.56−251.02 mg AAE/100 g for peel, whereas seed showed valued between 0.12−0.20 mg AAE/100 g, whereas Fe^{2+} Chelating Activity Assay revealed that PPE possesses a 2.29 ± 0.00 µmol EDTA equiv/ g dw, confirming the higher antioxidant properties of the pomegranate peel.

Table 2. Antioxidants activity of pomegranate extracts.

	DPPH assay EC_{50} (mg/mL)	**ABTS assay EC_{50} (mg/mL)**	**FRAP assay mg AA/ 100 g dw**	**Fe^{2+} chelating activity (µmol EDTA equiv/g dw)**
Peel	0.04 ± 0.06[a]	0,06 ± 0.01[a]	1.47 ± 0.01[a]	2.29 ± 0.00
Seed	0.88 ± 0.02[b]	0,40 ± 0.02[b]	0.02 ± 0.00[b]	ND

Different letters within the same column are indicating significant differences at $p < 0.05$. All data are expressed as mean ± SD of at least three replicates of each sample. (dw: Dry weight, AA : Ascorbic Acid)

3.3. Cytotoxicity of NaF on T.Pyroformis.

To evaluate the cytotoxicity of NaF on *T.pyriformis*, a range of concentrations of NaF as mentioned in material and methods were prepared and incubated. Results shown in (Figure.1) demonstrated an increase of cell proliferation by 8% ($p < 0.05$) at a concentration of 0.3 mmol. However, treatment with concentrations of NaF above 0.3 mmol induced a significant decrease in cell viability. Moreover, a concentration of 0.8 mmol produced a decrease of 54% ($p < 0.05$) of cell viability after 24 of treatment. Hence, this concentration was chosen for further studies to induce oxidative stress.

3.4. Effect of pomegranate peel extract on NaF-induced toxicity.

To establish the non-lethal concentrations of PPE and Vit C on *T.pyriformis.* Cells were incubated with PPE and Vit C alone for 24H. As shown in (Figure 2). Extracts of PPE were not toxic to a concentration of 75 µg/ml as well as Vit C. In order to evaluate the protective effect of PPE on NaF-induced cytotoxicity, cells were exposed to IC_{50} NaF, PPE, and Vit C simultaneously. When applied to cells, PPE could significantly restore cell viability to 80.17% at a concentration of 10 µg/ml and to 67.95% at 25 µg/ml. However, when treated with concentrations above 25 µg/ml, PPE had no significant effect on cell viability.

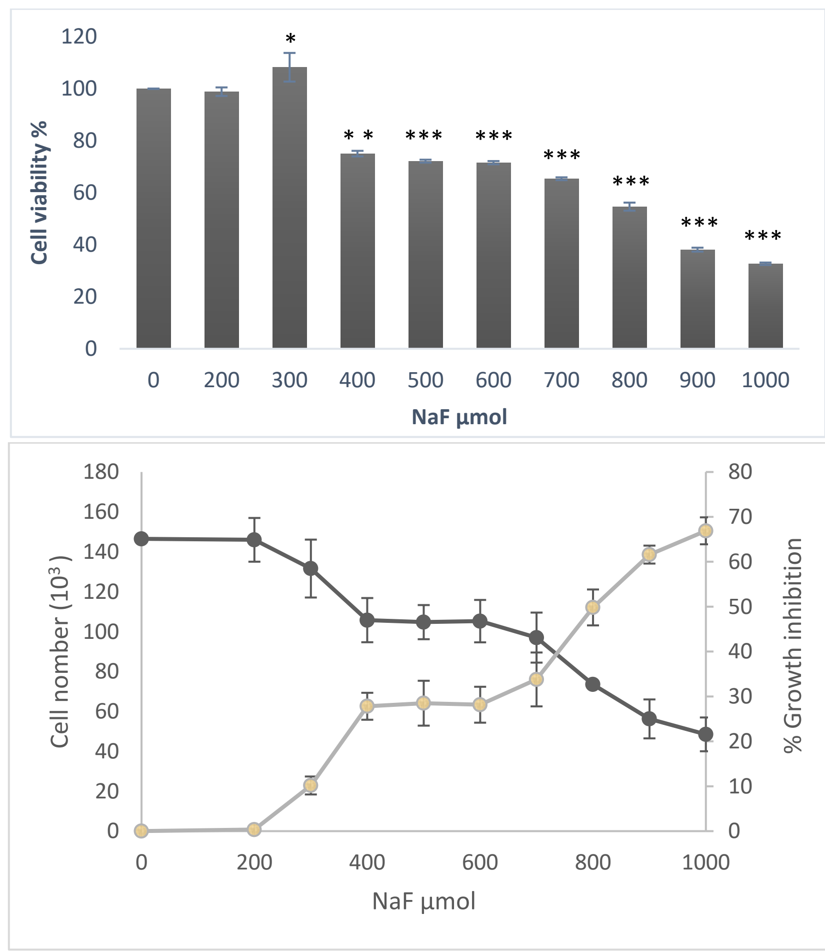

Figure 1. *T. pyriformis* was cultivated in a PPYG medium in the presence of different concentrations of NaF (100-1000 µM). **(a)** Results were obtained by the MTT method. **(b)** Effect of NaF on T. pyriformis growth. The results shown are the mean ± SD of the three independent experiments. Values were normalized to the control and are given as percent of the control. The significance following the Student-t-test are shown as: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$..

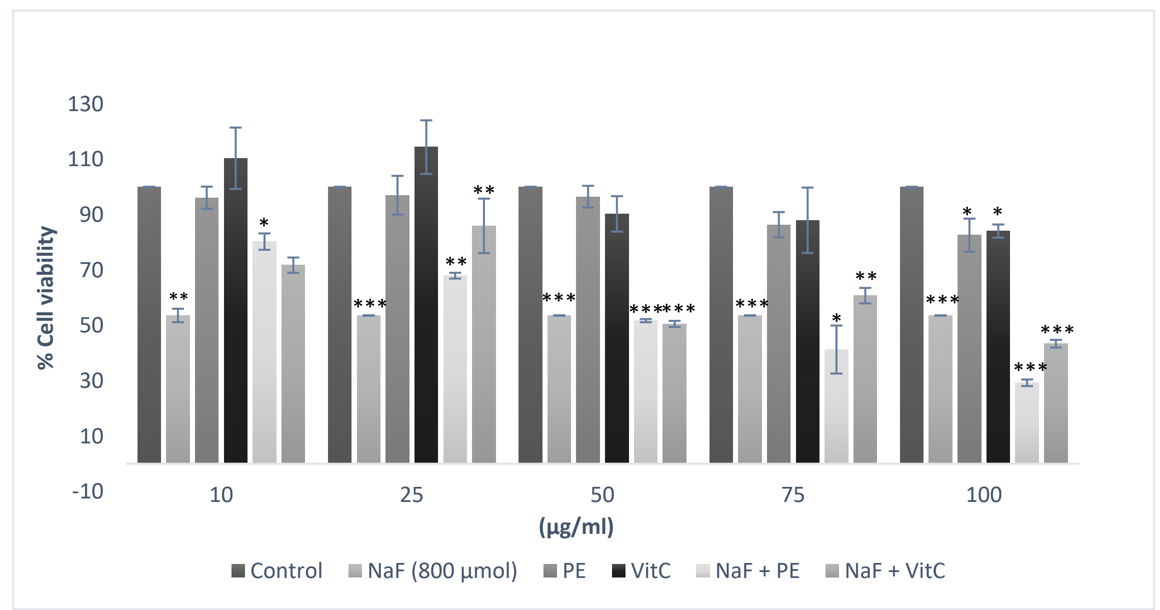

Figure 2. Protective effect of peel extract against NaF induced oxidative stress condition in *T. pyriformis* promoted by NaF at 800 µM after 24 hours of incubation. Cell viability was determined via the MTT method. The values in each column represent the mean ± standard deviation (S.D). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ represent statistically significant differences compared to control.

3.5. Measurement of fluoride level.

The intracellular fluoride content in *T. pyriformis* treated with 0.8 mmol increased significantly by 411% (p <0.05) compared to non-treated cells. When cells are treated both with NaF and PPE, we observed a significant decrease of intracellular fluoride concentration by 79% (p <0.05) compared to cells treated with NaF only (Figure 3). The effect of PPE can be compared to those of the positive control (ascorbic acid).

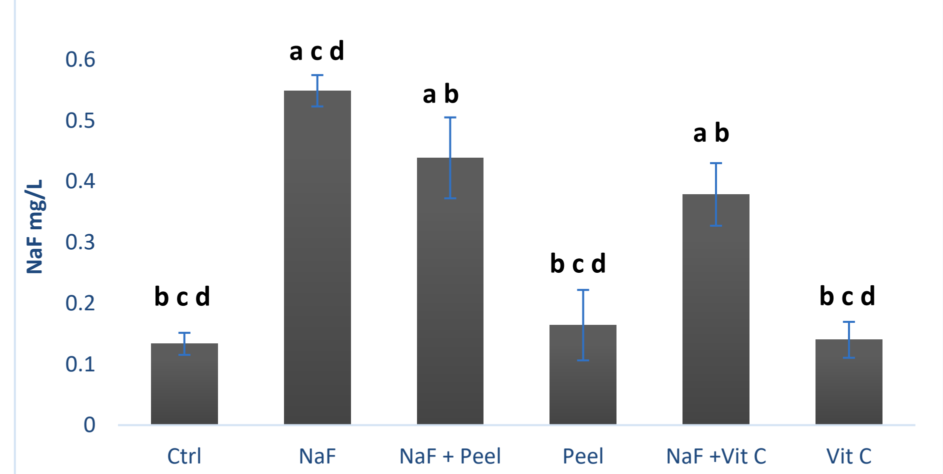

Figure 3. Level of NaF in cell lysate after 24 h of treatment, data showed are the mean of three triplicates. Different letters discriminate statistical differences between groups of parameters: a: compared to Ctrl, b: compared to NaF; c: compared to NaF + peel; d: compared to NaF+Vit C.

3.6. Effect of PPE on antioxidant enzyme activities.

The present study revealed that treatment with 0.8 mmol lead to a significant decline of SOD activity by 32.64% (p <0.05) in the group receiving NaF alone.

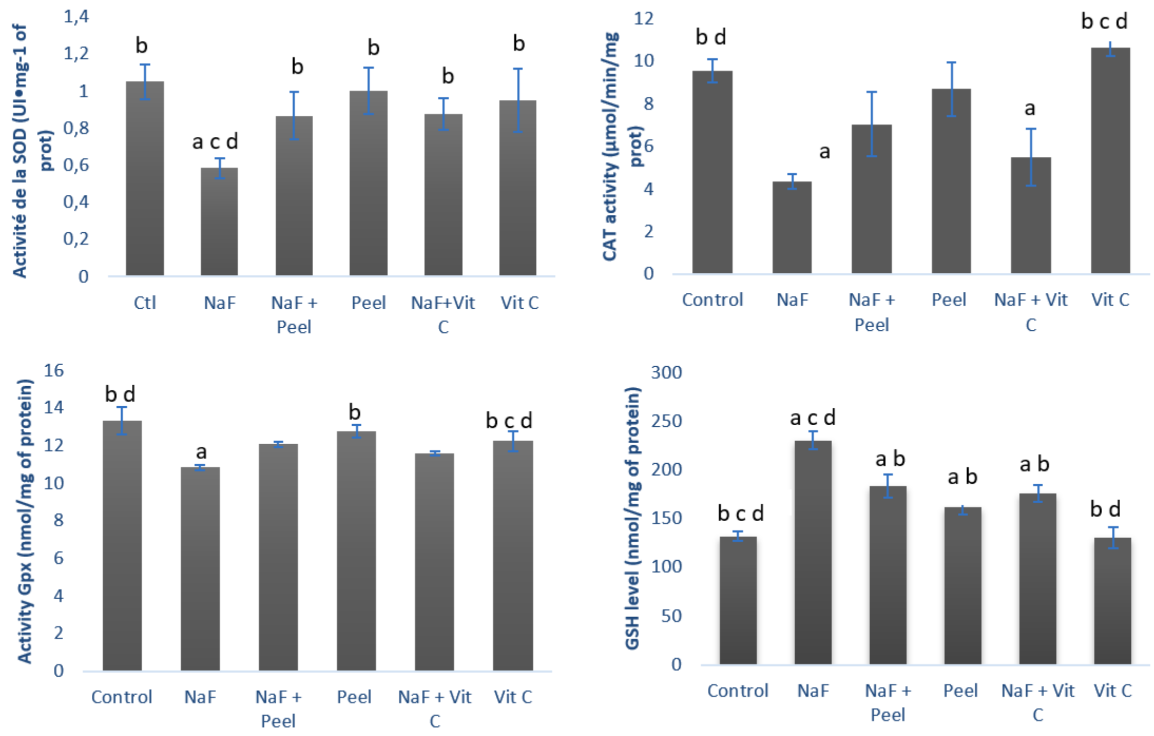

Figure 4. Protective effect of PPE on antioxidant enzyme activities. Different letters discriminate statistical differences between groups of parameters: a: compared to Ctrl, b: compared to NaF; c: compared to NaF + peel; d: compared to NaF+Vit C.

On the other hand, when cells were supplemented with PEE and NaF combined, we observed amelioration of SOD activity by 27% ($p < 0.05$) compared to the NaF group. A reduction of CAT activity of this enzyme was observed in the NaF group by 55.24% ($p < 0.05$), while treatment with PPE restored the CAT activity significantly by 27.70% (Figure 4). Gpx activity was similar to previous enzymes, with a reduction of activity in the NaF group of 18.91 % ($p < 0.05$) and a restoration of activity by 9.32 %. In our study, when IC50 NaF applied NaF-induced oxidative stress, the quantity of GSH in cells increased significantly by 74.27% ($p < 0.05$) in the NaF group compared to the untreated group (control) while when NaF+PPE treatment reduced the GSH content by 35.28% compared to NaF group.

3.7. Discussion.

Commonly known, pomegranate peel is a major source of phenolic compounds. The ellagitannins and anthocyanins are considered the most prevalent polyphenols in pomegranate peel as their content can reach 66% of the total polyphenols in the peel [46-47-48]. A previous study by our team using UHPLC performed the characterization of the phenolic compounds present in the methanolic pomegranate extracts. The results showed that the concentrations of punicalagin isomers, ellagic acid, and gallic acid were lower in the seed than peel [49]. In this present study, we choose to use methanol as a solvent of extraction, considering its superior extractability and possessing the highest phenolic content [50,51]. The obtained phenolics and flavonoids contents data confirm that the pomegranate peel fraction possesses the highest phenolic compounds compared to seed, which totally agrees with previous studies. Orak *et al*. compared the chemical and antioxidant properties in different parts of ‘Hicaznar’ pomegranate variety, widely grown in Turkey. The results demonstrated that peel is more effective than juice and seed extracts as a potential source of natural antioxidants [52]. Other studies by Derakhshan *et al*. compared pomegranates from three regions of Iran were analyzed for phenolic contents in the peel, seed and juice of their extracts. It was made clear that pomegranate peel showed the highest phenolics content [53]. The same results were found by Balli *et al*. in a recent study [54].

Two artificial radicals were chosen to evaluate the antioxidant capacity of pomegranate extracts (DPPH, ABTS). Data from DPPH assay were similar to data from previous works, a study by Liu *et al*. evaluating the antioxidant properties of pulp and peel showed that peel methanolic extract exhibits a lower value of scavenging activity: EC_{50}=12-25 mg/mL [55]. Another study by Amri et al. reported higher values EC50=0.84 mg/mL compared to edible and no edible pomegranates peel varieties in Tunisia [56]. Moreover, ABTS is another artificial radical widely used to estimate with higher currency the antioxidant capacity of food [45], results from the ABTS assay are in total agreement with those of literature, a study by Elfaleh *et al*. reported EC_{50} values of peel between 0.062-0.085 mg/mL, and 0.40-0.48 mg/mL for seed extracts [57]. In general, *In vitro* antioxidant activity demonstrated that PPE exhibits superior radical scavenging compared to PSE. Radicals scavenging activity of pomegranate peel is mainly attributed to the antioxidative compounds. Numerous studies showed a high correlation between phenolic content and antioxidant capacity [52-58]. Phenolic compounds possess the capacity of donating hydrogen from their hydroxyl group, thus neutralizing free radicals by forming stable complexes [59,60]. Additionally, we evaluated the antioxidant activity by FRAP The ferric reducing antioxidant power (FRAP), a colorimetric method based on reducing the complex Fe^{3+} and ferricyanide complex to the ferrous form. As shown in (Table 2), peel extract showed high chelating activity compared to seed, the same results were reported in the

literature [49-61]. The chelating capacity of PPE was also assessed using ferrous ions Fe^{2+} and reported as μmol EDTA equivalents per gram of dry weight; results as showed in (Table 2), demonstrated the good chelating ability of PPE, however, no identified chelating capacity of PSE was observed. Once they are deprotonated, phenolic compounds can play the role of chelators of various metals [62]. These results outline the potential effect of pomegranate peel extract on NaF toxicity in a tetrahymenna model. This protozoan has proven its efficiency in investigating the toxicity of different metals pollutants in aquatic environments [63,64].

Toxicity *in vitro* tests showed that concentrations of NaF above 0.3 mmol resulted in a significant decrease in cell viability. The present results are in total agreement with previous findings presented in the literature. A study by Ameermaja et al. shows that treatment of A549 human epithelial cells with 5 mM of NaF alone caused a decrease in cell viability by 54% [39]. Another study by Das *et al*. demonstrated that cell viability decreased by 50% when hepatocytes are treated with NaF at a dose of 100 mM [22]. Results from another study showed that NaF inhibits cell growth to 48% in mouse ameloblast-derived cell line (LS8) at a dose of 0.2 mM, after 24 hours of treatment [65]. Dose-dependent and effect of NaF on cellular metabolism vary remarkably according to cell type [1]. Previous studies demonstrated that fluoride at micromolar concentrations could be a good promoter of cell proliferation *in vitro*. Nevertheless, higher concentrations can induce a decreased viability of cells [66-67]. Such an effect is mainly related to the role of fluoride as a trigger of apoptosis and necrosis process in a large type of cells [68]. Furthermore, treatment with PPE alone has not shown any significant toxicity effect up to 100 µg/ml. Danesi *et al*. demonstrated that pomegranates fruit extract didn't show any toxicity up to 0.6 mg/ml when applied on HepG2 cells line [69].

Fluoride can penetrate the cytoplasmic membrane by simple diffusion, mainly by his non-ionic diffusion of HF, which can explain the significate increase of fluoride concentration in the NaF cells group. In addition, the activity of calcium and potassium can markedly increase the accumulation of fluoride in cells [21]. On the other hand, when PPE treatment was applied in combination with NaF, there was a significant decrease in intra-cellular fluoride content. Several studies have mentioned the beneficial effect of bioactive natural products on fluoride toxicity [70]. Sestili *et al*. reported that the preparations from part of Punica granatum showed a significant cytoprotective effect against H_2O_2 toxicity when applied in oxidatively injured mammalian cells [71]. Ghosh *et al*. reported that arjunolic acid exhibited a cytoprotective effect against NaF-cytotoxicity on hepatocytes [72]. Yet, the mechanism is still scarce. We suggest that the cytoprotective properties of a pomegranate can be a derivate of its antioxidants activities, especially from its polyphenolic constituents, especially its hydrolyzable tannins. A study by X.Li *et al*. demonstrated that pomegranate phenolic compounds:gallic acid, ellagic acid and punicalagin, were able to promote cell proliferation in human neuroblastoma cells (SH-SY5Y) [73]. Another study by Berköz & Krośniak mentioned that punicalagin derivated from pomegranate peel may induce apoptosis in A549 cell line through mitochondria-mediated pathway [74]. Data from the cytoprotective effect promoted us to investigate further the effect of fluoride and the treatment by the PPE on the properties of the antioxidant enzyme. Previous studies mentioned the role of fluoride in the generation of O^{2-} in a large mitochondrial cell, enhancing a cascade of reactions leading to excessive production of ROS both *in vitro* [75] or in-vivo [76-77]. It has been shown that fluoride intake at high doses can alter the antioxidants enzymes activity by forming a complex with the co-factors of metallo-enzymes, blocking the activity of these enzymes [12].

It is noteworthy that superoxide dismutase (SOD) is the first cellular barrier against excessive free radicals generation by catalyzing superoxide radicals to H_2O_2 [78]. In addition, Catalase (CAT) can break down H_2O_2 into a molecule of water and oxygen [42]. According to our results, a significant effect on antioxidant enzymes activities was observed between the group treated NaF and PPE compared to the control group. Thus, treatment with 0.8 mmol of sodium fluoride (NaF) revealed that (CAT, SOD, and Gpx) activities were decreased significantly ($p < 0.05$), however, the level of GSH increased instead. GSH is a physiological antioxidant compound under stressful attacks by heavy metals. Mammalian kidney cells secret GSH that forms inert bonds with metals, leading to forming a GSH-Metal complex transportable by the Na^+ amino-acid cotransporter [79,80]. After treatment with NaF and PEE simultaneously, enzyme activities were ascertained to be restored to normal levels. The same effect was observed in the NaF and Vit C combined group. Besides, no significant effect was observed when cells are treated with PPE or Vit C alone.

4. Conclusions

In summary, this study showed that Punicalagin and ellagic are the predominant phenolic compounds found in pomegranate peel and showed high antioxidant properties. Our data indicate that exposure to sodium fluoride led to an imbalance of the antioxidant enzyme activities of T. pyriformis and increased intracellular fluoride content. In addendum, PPE may play a good natural protective agent by restoring the oxidant/antioxidant hemostasis. Our findings are in total agreement with the survey literature. However, further studies should be undertaking to understand the mechanisms behind the beneficial effect. Considered as a waste of food processing, pomegranate peel represents a promising alternative to attenuate fluoride toxicity.

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Conflicts of Interest

The authors declared no conflict of interest.

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