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2,4-D herbicide induced cytotoxicity on HEp-2 Cells and Vitamin C reverse effect

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Abstract – 2,4-Dichlorophenoxyacetic acid (2,4-D) and its derivatives are herbicides widely used to control the growth of broadleaf and woody plants. Although 2,4-D is well known to be moderately toxic, little information is available on its cytotoxicity and mechanism of its toxicity. The aim of the present study was to investigate the cytotoxic effects of different forms of 2,4-D on a laryngeal carcinoma cell line: HEp-2 cells. Cells were treated with 2, 4, 8 and 16 mM of 2,4-D; 2,4-D Na and "Désormone lourd"; cell viability and induction of apoptosis were determined. Apoptosis was analyzed by cell nuclear staining with Hoechst 33342 dye and DNA fragmentation**.** The second purpose of our study was to compare the cytotoxicity of 2,4-D alone or modulated by the cytoprotective effects of additional antioxidants such as vitamin-C. Our results indicated that (i) 2,4-D can be responsible for oxidative damage to HEp-2 cells (ii) 2,4-D is cytotoxic for HEp-2 cells in a concentration and time-dependant manner (iii) 2,4-D exerts its cytotoxic effects by the induction of apoptosis (iiii) antioxidant compounds should be associated to herbicide formulations to decrease their deleterious effects on cells.

Keywords: pesticide; cytotoxicity; HEp- 2 cells; IC_{50}; apoptosis.

1. Introduction

Throughout the world, pesticides are widely used in agriculture and herbicides represent the most prominent class. Phenoxyacetic herbicides constitute one of the largest groups of herbicides. Among them and since 1940, 2,4- Dichlorophenoxyacetic acid (2,4-D) has been introduced (Zeljezic and Graj-Vhovac 2004). 2,4-D is one of the oldest herbicides used in the United States. It was first developed during World War II and became famous as a component of the controversial Agent Orange used during the Vietnam War. It is a chlorine–substituted phenoxy acetic acid herbicide. 2,4-D is sold as acid, salt (mostly amine), or ester formulations under many different trade names. Formulations include liquids, water-soluble powders, dusts, granules, or pellets of 2,4-D alone or in mixtures with other herbicides. Soluble 2,4-D derivatives are used as agricultural herbicides against broad-leaf weeds in cereal crops as well as on pastures, lawns, golf courses, and in parks; whereas granular formulations of 2,4-D are used as aquatic herbicides in ponds, lakes, and in or along irrigation canals (Tomlin 2003). Its herbicidal activity is mediated by an auxin like capacity to alter normal protein synthesis and cell division in plant meristems and leaves. Although, it has been suggested that its herbicidal activity may also be due to an increase in the production of oxygen reactive species leading to the generation of oxidative stress in the weed (Romero-Puertas et al. 2004).

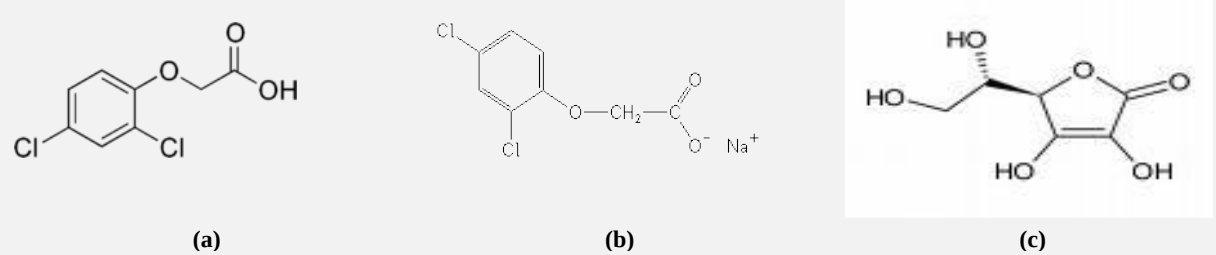
2,4-D is one of the most widely used herbicides due to it its relatively moderate toxicity and to its biodegradability in the soil. Despite its short half- life in soil and aquatic environnement, it appears to be persistent in ground water supplies, so its extensive use poses a health risk (Hayes and Laws 1991). Thus it can cause low growth rates, reproductive problems, change in appearance or behaviour, or death in non-target species, including plants, animals and micro organisms. Current knowledge about the biological effects of 2,4-D elicits a number of adverse biological effects in humans and animals that range from embryotoxicity and teratogenicity to neuro, immuno and hepatotoxicity (Rosso et al.

2000; Grabrant and Philbert 2002; Tayeb et al. 2010; Tayeb et al. 2011; Qurratu and reehan 2016). To our knowledge, there are no published reports on the Cytotoxicity of 2,4-D herbicide in Tunisia.
2,4-D has been classified as possibly carcinogenic to humans (IARC, group 2B) (Persson et al. 1993). It was associated with soft tissues sarcoma, non-Hodgkin lymphoma and Hodgkin disease in epidemiological studies (Munro et al. 1992). The observations of an increased incidence of malignant lymphoma in dogs, lymphosarcoma in rats, hemangiosarcoma of the spleen in mice as well as various tumors at other sites in rats and mice supported the evaluation of a high-level concern with respect to the carcinogenicity of 2,4-D and its salts and esters (Alvanja et al. 2003).
Both in vitro and in vivo reports have studied the cellular and molecular mechanisms of 2,4-D toxicity (Bongiovanni et al. 2012). *In vitro*, cytotoxicity of 2,4-D was evaluated within the frame of the Scandinavian MEIC Study (Multicenter Evaluation of In Vitro Cytotoxicity), and 2,4-D was chosen among 50 substances known for their *in vivo* toxicity. For 2,4-D, an IC_{50} ranging between 4 and 8 mM was calculated using neutral red and MTT assays in mousse 3T3 cells (Clemedson et *al. 1996*).
One area of increasing interest is the study of the capability of some elements to modulate the effects of environmental toxicants. In that respect, numerous studies showed that antioxidant substances protect cells against deleterious effects of environnemental agents (as radiations or chemical products) (Savini et al. 1999; Gehin et al. 2005). Vitamin C (Vit C) has been identified as nutrient, it protects against oxidative damage caused by certain pesticide through two separate mechanisms (i) it recycles tocopherol, the major antioxidant of biological membranes, from tocopheroxyl free radical and (ii) it acts directly as a scavenger of reactive oxygen and radical species. Vit C is the major water- soluble antioxidant found in the aqueous compartments of cells and extra-cellular fluids (Rumsey and Levine 1998 reviewed in Fetoui et *al*. 2008).
The purpose of this study was to investigate and compare the cytotoxicity of different forms of 2,4-D alone or modulated by the cytoprotective effects of additional antioxidant such as Vit C, on the laryngeal carcinoma cell line: HEp-2 cells. Also, to study if apoptose is induced as cytotoxic effect of 2,4-D herbicide.

2. Materials and methods

2.1. Materials

The culture medium used is Dubelco's Modified Eagle's Minium Essential Medium (DMEM; Gibco BRL). The other products utilized were Fetal Bovine serum (FBS) (Gibco BRL; France), Trypsin (0.25%), 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide(MTT), dimethylsulfoxide (DMSO), Phosphate- buffered saline (PBS), Ascorbic acid (Vitamin C); 2,4-Dichlorophenoxyacetic acid (2,4-D) $C_8H_5Cl_2O_3$ (98% purity), 2,4-D Sodium salt (2,4-D Na) $C_8H_5Cl_2NaO_3$ (98% purity), and they were purchased from Sigma-ALDRICH. Also, 2,4-D commercial formulation (Désormone Lourd) consisting of 600 g/l 2,4-D Ester butylglycol, register number *H.96064* (SEPCM), available in Tunisia, was used in the experiments. The chemical structures of 2,4-D; 2,4-D Na and vitamin C were given in Fig 1.



(a) **(b)** **(c)**

Figure 1: Chemical structures of 2,4-D (a); 2,4-D Na (b) and Vit C (c)

2.2. Cell culture

HEp-2 cells, a laryngeal carcinoma cell line, were grown in DMEM medium supplemented with 10% (v/v) FBS. Cells were cultivated as monolayers in plastic culture flasks (Nunc, Denmark) at 37°C under a humified atmosphere of 5% CO_2 in air.

2.3. Treatment of cultures

At 60- 80% confluence, HEp- 2 cells were trypsinized, transfered in to 96- well cell culture plates at a density of 2.1 x 10^4 cells/well and incubated overnight for adherence. Then we exposed cells to

increasing concentrations of the three forms of 2,4-D (2- 4- 8 and 16 mM) for two incubation periods (24 and 48 h). In parallel we co-exposed cells to increasing concentrations of 2,4-D (2- 4- 8 and 16 mM) and Vit-C at 0.1 mM for two incubation periods (24 and 48 hours). Each experiment was done twice and each deterination was done in triplicate.

2.4. Cytotoxicity assay

After the exposure period, MTT was added to the culture medium to yield a final MTT concentration of 0.5 mg/ml. Cells were incubated with the MTT for 4 hours at 37°C in a CO_2 incubator. After this incubation, the MTT reaction medium was removed and formazan blue was solubilised by 100µl of DMSO. This assay was based on the reduction of the yellow tetrazolium salt MTT by the mitochondrial succinate dehydrogenase to form an insoluble formazan-blue product. Only viable cells with active mitochondria reduce significant amounts of MTT (Mossmann 1983) and formazan–blue formation was quantified with a spectrophotometer at 570 nm. Values of absorbance were converted into percentage of viability. A curve of percentage of viability was represented according to the herbicide concentration and we calculated the inhibition concentration 50% (IC_{50}) which is the toxic concentration resulting in 50% cell death.

2.5. Identification of apoptotic cells

Apoptosis was analyzed by staining the nuclear chromatin with Hoechst 33342 dye. Briefly, untreated and treated cells with 4 mM of the three forms of 2,4-D, for 24 hours, were collected, washed with PBS, stained with 1 mg/mL Hoechst 33342 (Sigma, USA) dye for 15 min at 37°C, mounted on glass slides, and observed by microscopy. The percentage of apoptotic cells (chromatin condensation and nuclear fragmentation) was determined by counting 300 cells in each sample.

2.6. Statistical analyses

The calculation of the IC_{50} was carried out by the probit method by using EPA program (improved by the Environnement Protection Agency) (**U.S EPA, 1993**).

3. Results

3.1. Measurement of IC_{50} of the three forms of 2,4-D after 24 h exposition

Our study indicated for the three forms of 2,4-D an IC_{50} between 1,86 and 3,71 mM calculated in HEp-2 cells (Table 1). In fact, the commercial product containing 600g /L 2,4-D appears to be more toxic than the two active ingredients of 2,4-D investigated.

Table 1 . Values of IC_{50} for the three forms of 2,4-D calculated after 24 h exposition **i**n HEp-2 cells

Treatment and parameter	**2,4-D**	**2,4-D Na**	**« Désormone lourd »**
IC $_{50}$ (mM)	3.71	3.24	1.86

3.2. Determination of cytotoxicity profiles of the three forms of 2,4-D co-incubated with vit-C after 24 h and 48 h exposition

We have noted a time and dose dependent cytotoxic effect of the three forms of 2,4-D (Figures 2, 3 and 4). When comparing cytotoxicity profiles of the three forms of 2,4-D co-incubated with vit-C after two periods of exposition, we have noted a time dependent cytoprotective effect of Vit C.

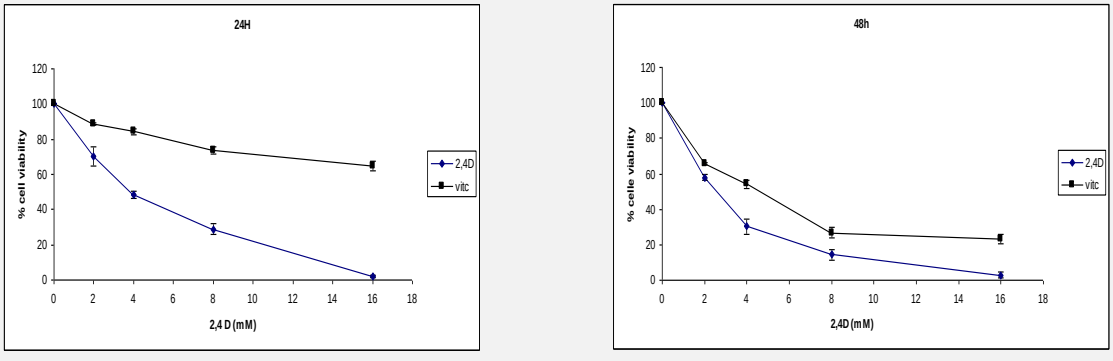


Figure 2: Cytotoxicity profiles of 2,4-D incubated alone or co-incubated with Vit-C in HEp-2 cells after 24 H and 48 H exposition

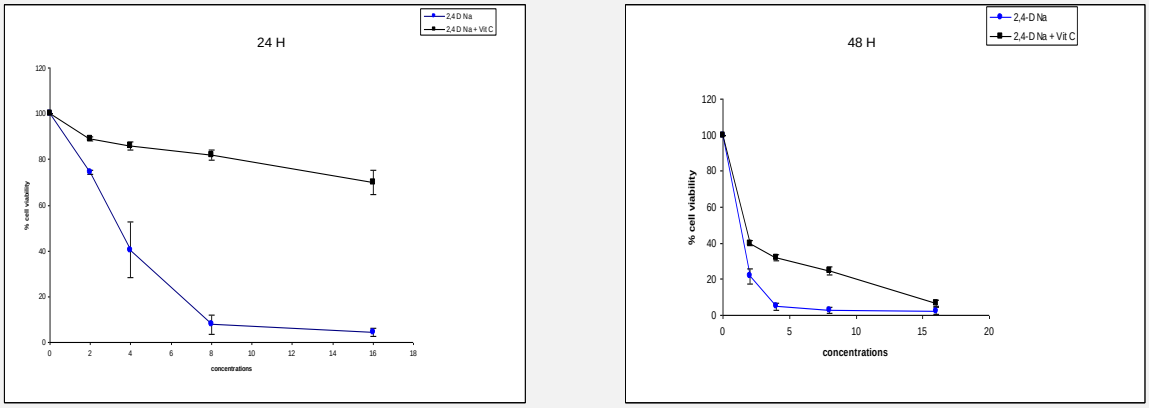


Figure 3: Cytotoxicity profiles of 2,4-D Na incubated alone or co-incubated with Vit-C in HEp-2 cells after 24 H and 48 H exposition

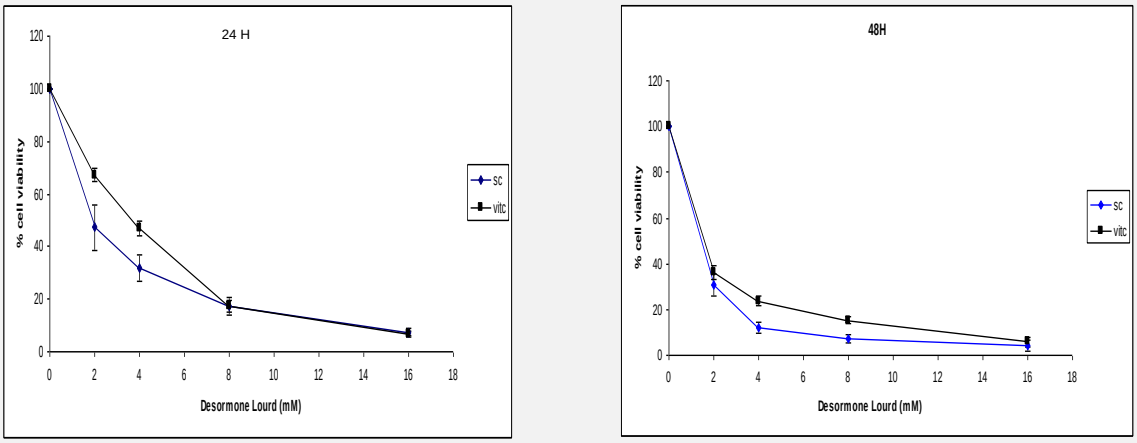


Figure 4: Cytotoxicity profiles of "Désormone lourd", a commercial formulation of 2,4-D, incubated alone or co-incubated with Vit-C in HEp-2 cells after 24 H and 48 H exposition

3.3. Induction of apoptosis by 2,4-D

Hoechst staining was used to determine the morphological changes and quantify the apoptotic HEp-2 cells obtained with 4 mM of the three forms of 2,4-D. Percentages of 33.75, 41 and 72 % of Apoptotic cells with condensed chromatin were observed in treated cells, respectively, with 2,4-D; 2,4-D Na and the commercial formulation "Désormone lourd" (Fig. 5).

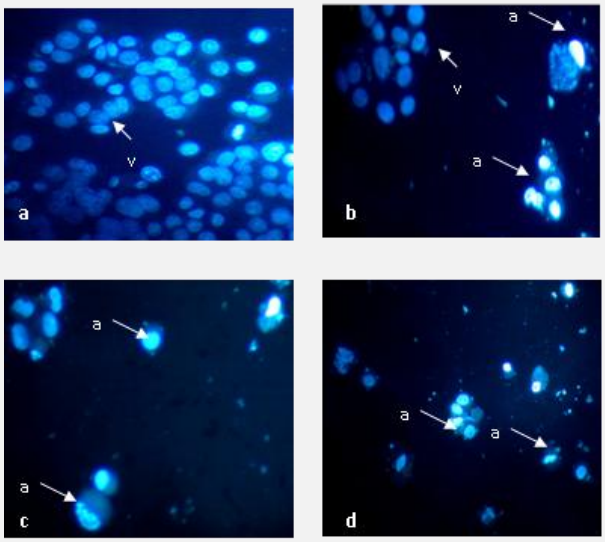


Figure 5: Hoechst 33342 staining of HEp-2 cells detected by fluorescent microscopy without (a) and after treatment with (b) 2,4-D (c) 2,4-D Na and (d) "Désormone Lourd" at 4 mM for 24 hours. Highly condensed or fragmented nuclear represent apoptotic cells. Intact nuclei represent viable cells. Arrows, examples of apoptotic (a) and viable (v) cells

4. Discussion

The aim of the present study was to evaluate the cytotoxicity of the 2,4-D herbicide. We were able to demonstrate a time- and dose- dependent effect of 2,4-D on HEp-2 cells, the induction of apoptosis in HEp-2 cells and the cytoprotective effect of Vit-C.

Although, 2,4-D is a widely used herbicide and various toxicities have been described for humans and animals, few studies were performed at the cellular level to elucidate the mechanisms of its toxicity. From our study, we have studied the cytotoxic effects of 2,4-D on Laryngeal carcinoma cells, especially that 2,4-D exposure has been linked to an increase risk of many cancers (Duffie et *al*., 2001; Bukowska 2006). In fact, some authors have mainly looked for the cytotoxic effects of 2,4-D on various cell lines including HepG2, Jurkat T MCF-7, cells and HEK293 (Lin and Garry 2000; Kaimouva et al. 2001; Tushl and Schwab 2003; Gunnes 2007).

Our results indicated that when we exposed HEp-2 cells to different forms of 2,4-D at concentrations of 2, 4, 8 and 16 Mm for 24 and 48 h we have noted a time- and dose-dependent cytotoxic effect of this herbicide. The doses used in the present study are rather high in comparison with doses achieved *in vivo* by environmental contamination. The studied concentrations were chosen based on the results of MEIC study, a project initiated to establish in vitro methods for the determination of acute toxic effects of chemicals. From this study, Clemedson et *al*. (1996) indicated for 2,4-D an IC_{50} between 4 and 8 mM calculated in mouse 3T3 cells. In our investigation, for 2,4-D, 2,4-D Na and "Désormone lourd", an IC_{50} of 3.71, 3.24 mM and 1.24 (for 24 h, respectively) were calculated, using MTT assays and the probit method using EPA program, in Human laryngeal carcinoma cell line: HEp-2 cells.Thus, the commercial product "Désormone lourd" containing 600g /L 2,4-D appears to be more toxic than the two 2,4-D active ingredients investigated here. In fact, We used 2,4-D as a part of a commercial formulation commonly used by Tunisian farmers to determine its possible cytotoxic effects. The commercial grade of 2,4-D was utilized to include the impact of contaminants and inert materials that would be present in the actual exposure conditions. Yet, in most cases, the nature of the inert ingredients in a pesticide commercial formulation is unknown; so, the definition of their contribution to the induction of toxicity becomes an extremely difficult issue. Several reports have suggested that formulations may be more toxic than the active principle due to the different compounds that are included as inert ingredients (Mustonen et al. 1986; Clausen et al. 1990; Oakes and Pollack 1999; Lin and Garry 2000). Surfactants and detergents may increase pesticide bioavailability and enhance biological responses. For instance, Oakes and Pollak (1999) concluded that the surfactant in a commercial formulation of 2,4-D was implicated in the alteration of mitochondrial processes since the formulation induced changes at a lower dose than 2,4-D**.** Than, in 2000, Oakes and Pollack have evaluated the toxicities of three related herbicide formulations containing ester derivatives of 2,4,5-T and 2,4-D using sub-mitochondrial particles. The authors have established that the inert component i.e diesel fuel and surfactants contributed approximately 50% of the overall toxicity of the complete formulations.

In the reminder of our results, we confirmed the cytoprotective effect of Vit-C against the cytotoxicity of 2,4-D on HEp-2 cells. In deed, vit C, a non-enzymatic antioxidant, is the most important free radical scavenger in extracellular fluids. It trapping radicals in the aqueous phase and protecting biomembranes from peroxidative damage (Sulak et al. 2005). It may remove free radicals that are bound to vitamin E, thus serving to regenerate vitamin E (Fetoui et al. 2008). The transport mechanisms of Vit C have been well characterised in some cells (Rumsey and Levin 1998). However Savini et al.(1999) suggested the presence of an active transport system saturable at 1 mM Vit C; but , they also pointed out the rapid decomposition of Vit C in cell culture media (half-life: 0.9 h). In our work, the assayed concentration of Vit C is lower (0,1 mM). Gehin et al. (2005) have studied the cytotoxicity of glyphosate modulated by the cytoprotective effects of additional antioxidants such as Vitamin C and Vitamin E on the Human keratinocytes Cell line HaCaT. They have confirmed the cytoprotective effect of such antioxidant. But, surprisingly, the cytoprotective effect of associated Vit C+ Vit E was lower than Vit C or Vit E alone. Bongiovani et al. (2007) have confirmed the remarkable antioxidant properties of Melatonine to decrease the oxidative stress produced by 2,4-D in Rat Cerebellar Granule cells (CGC). Also, *in vivo*, the dietary supplementation of extra virgin olive oil counteracted the damage effect of 2,4-D in rat erythrocytes by the enhancement of antioxidant defence system and the decline of the lipid peroxidation (Nakbi et al. 2010).

So, from the current study we have noted the protective effect of a non-enzymatic antioxidant as Vit-C against the cytotoxicity of 2,4-D, this Confirms our previous results (Tayeb et al. 2010; Tayeb et al. 2011) showing that toxic effects of 2,4-D herbicide are attributed to the induction of oxidative stress

that arise as a result of excessive generation of ROS. These last can react with various biological molecules as nucleic acids, proteins, lipids leading to changes in the structure and activity of biologically relevant molecules generating cell damage (Mates et al. 1999). In addition, recent developments in molecular biology have demonstrated that free radicals play important roles at the cellular level. Thus, overproduction of these radicals would lead to significant changes in the redox state of the cell and to the inactivation of cell proliferation control systems. Knowing that, during cell proliferation, the p53 protein plays a key role in checking the integrity of DNA, then, when cellular damages are important, p53 triggers the death of the cell by apoptosis. Moreover, a state of oxidative stress may inhibit the activity of p53 which will lead to abnormal cell divisions later causing the development of tumors (Pincemail et al. 2001).

Several studies have highlighted that mitochondria play an important role in the regulation of cell death. They contain many pro-apoptotic proteins such as Apoptosis Inducing Factor (AIF), Smac/DIABLO and cytochrome C. These factors are released from the mitochondria following the formation of a pore in the mitochondrial membrane. These pores are thought to form through the action of the pro-apoptotic members of the bcl-2 family of proteins, which in turn are activated by apoptotic signals such as cell stress, free radical damage or growth factor deprivation. In fact, the release of cytochrome C from the mitochondria is a particularly important event in the induction of apoptosis. Once cytochrome C has been released into the cytosol it is able to interact with a protein called Apaf-1. This leads to the recruitment of pro-caspase 9 into a multi-protein complex with cytochrome C and Apaf-1 called the apoptosome. Formation of the apoptosome leads to activation of caspase 9 and the induction of apoptosis (Dash 2012). Thus, in this axis, several studies indicate the role of apoptosis in the toxic effect of 2,4-D. Di Paolo et al. (2001) demonstrated the effect of 2,4-D on the induction of apoptosis following inhibition of mitochondrial function. Indeed, these authors demonstrated that 2,4-D can bind irreversibly to hepatic proteins in rat. Di Paolo et al. (2001) identified a 52-kDa protein modified selectively by 2,4-D in rat liver mitochondrial preparations both in vitro and in vivo. In addition, Sulik et al. (1998) observed histological changes in the ultrastructure of mitochondria and lysosomes from the livers of rats treated with 2,4-D. The most comprehensive study was performed by Kaimouva et al (2001), who tested the effect of the Dimethylammonium salt of 2,4-D on apoptosis in Jurkat T cells and human lymphocyte: these authors detected significant numbers of apoptotic cells at concentrations from 2 to 4 mM, in fact they were able to induce 50% apoptosis at concentrations of 3 mM 2,4-D after 24 h treatment. In fact, apoptotic effects of 2,4-D were described to occur in human lymphocytes and Jurkat cells by disruption of the mitochondrial transmembrane potential. Tuschl and Schwab (2003), demonstrated that 4 mM of 2,4-D were necessary to induce strand breaks in 50% of cells after 24 h of incubation. In contrast to the findings of kaimouva et al. (2001) and Tuschl and Schwab (2003), Lin and Carry (2000) could not detect any apoptosis in MCF-7 breast cancer cells. In fact, 2,4-D is classified as peroxisome proliferators, which would confirm the involvement of 2,4-D as apoptotic and tumor promoter (Reddy and Rao 1977; Beigel et al. 1993; De Moliner et al. 2002; Li et al. 2013). Therefore, it was necessary to study the cytotoxicity of 2,4-D on the HEp-2 cells. From our study, we were able to detect apoptotic cells at a percentage of 33.75; 41 % and 72 % after an exposition period of 24 hours to 4 Mm 2,4-D; 2,4-D Na and the commercial formulation of 2,4-D. Apoptosis was analyzed by cell nuclear staining with Hoechst 33342 dye and DNA fragmentation**.** The cell death occurred by apoptosis as identified by condensed chromatin and the formation of apoptotic bodies.

5. Conclusions and perspectives

In conclusion, although herbicide concentrations in the human environment are very low, our results from the first part of the study indicated: (i) a time and dose dependent cytotoxic effect of the three forms of 2,4-D (ii) an IC_{50} ranging between 1,86 and 3,71 mM calculated using Probit Analysis (iii) Induction of apoptosis by 2,4-D at a concentration of 4 mM. But it is still premature to draw definite conclusions on the role of apoptosis in 2,4-D induced toxicity so it will be interesting if we investigate in the specification of the cytotoxic effects of the test substance on the laryngeal carcinoma cell line (HEp-2 cells) by the use of other methods such as Annexin assay, gel electrophoresis, Nicoletti assay.

Also, the experimental evidence reported in the second part of the study supports the conclusion that Vit-C is an effective cytoprotective for HEp-2 *in vitro*. To what extent it could be also a protector *in vivo* deserves to be further explored. Thus, antioxidant compounds should be associated to herbicide formulations to decrease their deleterious effects on cells.

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