Universidade de Aveiro
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Eco-toxicologia e Análise de Risco, realizada sob a orientação científica da Doutora Helena Cristina Correia de Oliveira, Investigadora de Pós-doutoramento do Departamento de Biologia e do Centro de Estudos do Ambiente e do Mar (CESAM), da Universidade de Aveiro, e da Doutora Ana Marta dos Santos Mendes Gonçalves, Investigadora do Centro de Ciências do Mar e do Ambiente (MARE), Departamento das Ciências da Vida, Universidade de Coimbra e do Departamento de Biologia & CESAM, da Universidade de Aveiro.

Dedico este trabalho à minha sobrinha Aurora. Que nunca pare de sonhar e que tenha força e sabedoria para realizar os seus sonhos.

o júri

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palavras-chave

Toxicidade, Herbicida, Primextra® Gold TZ, S-metolacloro, linhas celulares humanas, HaCaT, HepG2.

resumo

Com o aumento da população humana surgiu a necessidade de otimizar e intensificar a produção agrícola aumentando a utilização de pesticidas e fertilizantes. O uso de pesticidas tem sido muito importante no combate a pragas que, se não fossem interrompidas, impossibilitariam o cumprimento da demanda alimentar. No entanto, o uso destes compostos tem consequências nefastas. Está comprovado que os pesticidas podem ter efeitos prejudiciais na saúde pública e ocupacional, pela contaminação do abastecimento de água potável, pelo depósito de resíduos nos alimentos, através da contaminação ao longo da cadeia alimentar ou simplesmente através do manuseamento dos referidos produtos químicos.

Neste estudo, avaliamos os efeitos do herbicida Primextra® Gold TZ e do seu principal ingrediente ativo, o S-metolacloro, em modelos de células da pele e do fígado humano. Primextra® Gold TZ é um dos herbicidas mais vendidos em Portugal, sendo referenciado pelas cooperativas do Vale do Mondego como o herbicida mais utilizado na cultura do milho. Os modelos celulares usados para avaliar o perigo potencial dos produtos químicos na pele e no fígado foram a linha celular de queratinócitos humanos, HaCaT, e a linha celular de hepatoma humano, HepG2, respetivamente. Primeiro, os efeitos citotóxicos da formulação comercial e do ingrediente ativo foram testados usando um ensaio colorimétrico do brometo de 3- (4,5-dimetil-2-tiazolil) -2,5-difenil tetrazólio (MTT). Depois de calcular os valores de IC30 e IC50 do ensaio de viabilidade celular MTT, procedeu-se à análise do ciclo celular para determinar se algum dos produtos químicos induziu alterações na dinâmica do ciclo celular. Finalmente, com base em dados da literatura apontando que Primextra® Gold TZ e S-metolacloro podem induzir alterações no perfil lipídico e polissacarídeo de organismos não alvo, avaliaram-se os efeitos bioquímicos nos modelos de células humanas indicados anteriormente.

Os resultados de MTT mostraram uma maior sensibilidade da linha celular HaCaT para o Primextra® Gold TZ e S-metolacloro do que a linha de células HepG2. Em HepG2, obtivemos um IC50 de 22,48 µg/ml para o Primextra® Gold TZ e um IC50 de 83,90 µg/ml para o S-metolacloro no ensaio de MTT de 48h, evidenciando uma toxicidade muito maior a formulação comercial nesta linha celular. Na linha celular HaCaT, tanto a formulação comercial quanto o ingrediente ativo apresentaram resultados semelhantes, com IC50 de 12,37 µg/ml e 12,28 µg/ml para Primextra® Gold TZ e S-metolacloro, respetivamente. Na análise do ciclo celular, Primextra® Gold TZ e S-metolacloro mostraram induzir paragem do ciclo celular na fase G2 na linha celular HepG2. Enquanto que, a linha celular HaCaT não mostrou nenhuma indicação de paragem do ciclo celular quando exposta aos produtos químicos. Quanto à análise do perfil de ácidos gordos, os resultados não mostraram alteração significativa na composição de ácidos gordos na exposição de células HepG2 aos químicos. A linha celular HaCat, no entanto, mostrou uma redução > 15% na percentagem de ácidos gordos saturados e um aumento proporcional nos ácidos gordos monoinsaturados. Os resultados da análise de polissacarídeos foram inconclusivos.

palavras-chave Toxicidade, Herbicida, Primextra® Gold TZ, S-metolacloro, linhas celulares humanas, HaCaT, HepG2.

resumo (cont.) Este estudo demonstrou que as linhas de queratinócitos humanos, HaCaT, são mais sensíveis a citotóxidade por exposição ao Primextra® Gold TZ e ao S-metolacloro, do que as linhas celulares do fígado, HepG2, apesar destas últimas serem mais suscetíveis a dano no DNA. A disparidade de resultados entre as linhas celulares pode ser devida às suas diferenças metabólicas intrínsecas, bem como às diferenças nos seus mecanismos de defesa inerentes. Assim sendo, para uma melhor compreensão das propriedades que conferem maior resistência da linha celular HepG2 à citotoxicidade por parte do Primextra® Gold TZ e do S-metolacloro, bem como se poder entender melhor os mecanismos de ação destes últimos, é importante a realização de mais estudos.

Keywords

Toxicity, Herbicide, Primextra® Gold TZ, S-metolachlor, human cell lines, HaCaT, HepG2.

Abstract

With the increase in the human population, came the need to optimize and intensify agricultural production by increasing the use of pesticides and fertilizers. The use of pesticides has been very important in combating pests that, if not stopped, would make it impossible to meet food demand. However, the use of these compounds has harmful consequences. It has been proven that pesticides can have deleterious effects on public and occupational health, by contaminating the drinking water supply, by depositing residues in food, through contamination along the food chain or simply by handling these chemicals.

In this study, we evaluated the effects of the herbicide Primextra® Gold TZ and its main active ingredient, S-metolachlor, in models of human skin and liver cells. Primextra® Gold TZ is one of the bestselling herbicides in Portugal, being referenced by the Mondego Valley cooperatives as the most-used herbicide in corn fields. The cell models used to evaluate the potential hazard of the chemicals on skin and liver were human keratinocyte cell line, HaCaT, and human hepatoma cell line, HepG2, respectively. First, the cytotoxic effects of both commercial formulation and active ingredient were tested using a colorimetric 3-(4,5- dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) assay. After calculating IC30 and IC50 values from MTT assay we proceeded to cell cycle analysis to determine if any of the chemicals induced cell cycle arrest. based on data from the literature indicating that Primextra® Gold TZ and S-metolachlor can induce changes in the lipid and carbohydrate profile of non-target organisms, the biochemical effects on the human cell models indicated above were evaluated. MTT results showed a higher sensitivity from HaCaT cell line to both Primextra® Gold TZ and S-metolachlor then HepG2 cell line. In HepG2 we obtained an IC50 of 22.48 µg/ml for Primextra® Gold TZ and an IC50 of 83.90 µg/ml for S-metolachlor in the 48h MTT assay. Thus, showing a much bigger toxicity of the commercial formulation in this cell line. In HaCaT both the commercial formulation and active ingredient showed similar results, with an IC50 of 12.37 µg/ml and 12.28 µg/ml for Primextra® Gold TZ and S-metolachlor, respectively. Regarding the effects on the cell cycle dynamics, Primextra® Gold TZ and S-metolachlor were shown to induce cell cycle arrest at G2 phase in the HepG2 cell line. Whereas the HaCaT cell line showed no indication of cell cycle arrest when exposed to the chemicals. Regarding the analysis of the fatty acid profile, the results showed no significant alteration in the composition of fatty acids in the exposure of HepG2 cells to chemicals. The HaCaT cell line, however, showed a >15% reduction in the percentage of saturated fatty acids and a proportional increase in monounsaturated fatty acids. Carbohydrate analysis results were inconclusive.

Keywords Toxicity, Herbicide, Primextra® Gold TZ, S-metolachlor, human cell lines, HaCaT, HepG2.

Abstract (cont.) This study demonstrated that human keratinocyte lines, HaCaT, are more sensitive to cytotoxicity by exposure to Primextra® Gold TZ and S-metolachlor, than liver cell lines, HepG2, although the latter are more susceptible to DNA damage. The disparity in results between cell lines may be due to their intrinsic metabolic differences, as well as differences in their inherent defence mechanisms. Therefore, for a better understanding of the properties that give the HepG2 cell line greater resistance to cytotoxicity by Primextra® Gold TZ and S-metolachlor, as well as to be able to better understand the mechanisms of action of the latter, it is important to carry out more studies.

Index

1. Introduction

1.1. Historical Overview

Having started about 11,500 years ago, agriculture has triggered such a change in society and the way in which people lived that its development has been dubbed the "Neolithic Revolution". The Neolithic Revolution was a process of transition from a nomadic lifestyle of hunter-gatherer to one of agriculture and pastoralism (Sadowski, 2017; Mazoyer & Rou, 2006), that allowed people to settle in communities and potentiated population growth. Since then, human beings have been in a never-ending battle with plant pests and diseases. Even with today's methods there is an average loss of 35 to 40 % of all potential food and fiber crops due to pests and diseases.

The need to improve agricultural results led to the development of plague control chemicals. The first documented records of measures used for control pests date back to 2500 BC, with the Sumerians using sulphur to protect them from insects. The oldest medical document of the ancient Egypt "Ebers papyrus" which dates to 1550 BC contains about 800 recipes, which include various substances acting as poisons for various plagues. Homer also recorded in 1000 BC the burning of sulphur as a fumigating procedure against insects. At that time, the use of arsenic, mercury, lime and insecticides of plant origin was already recorded in China (Banaszkiewicz, 2010; Fishel, 2013). Therefore, the use of chemicals is not new. However, it was only after the "green revolution" that the Humankind gave the biggest step in the fight against plagues. With the development of sophisticated machinery and irrigation methods, the extensive use of fertilizers and, the most important of all, the development of chemical pesticides, humankind could finally fight food shortages due to population growth (Ameen et al. 2018).

1.2. Human exposure to Pesticides

A pesticide is any substance used to eliminate organisms that are harmful and undesirable. Pesticides are usually divided according to their target organism, e.g., insecticides, herbicides, fungicides, rodenticides and play an important role in agricultural production, by preventing unwanted plagues to invade crops (Kaur et al. 2019). However, when applied to crops they can enter the soil, as well as surface and groundwaters via leaching and run-off thus affecting habitats, contributing to biodiversity loss, leading to the deterioration of ecosystem services and affecting the provision of clean drinking water(Ewald et al., 2015). By affecting the provision of drinking water, leaving residues in food or by affecting organisms throughout the food-chain, these chemicals pose a major threat to the Human health (Bjørling-Poulsen et al., 2008). Exposure through food-chain and by drinking contaminated waters is the most common route of exposure to the general population.

However, there are reports that living or working next to pesticide treated zones can substantially contribute to exposure to these chemicals. Accidental poisoning with pesticides from use around the house and garden has also been reported (Jaga et al., 2003 ; Davis et al.,1992).

Exposure of farmers, when mixing and applying the pesticide or working in treated fields, also poses a threat as the routine use of these chemicals can cause short and long-term health problems. In developing countries, there is even a bigger risk of exposure due to the use of banned or restricted pesticides, incorrect application techniques, inadequate storage practices, poor maintenance, use of inappropriate equipment, and often the reuse of old pesticide containers for food and water storage (Van Der Werf, 1996; Damalas et al., 2011 ; Ye et al., 2013).

Some of the most worrying toxic effects are due to the neurotoxic and endocrine-disruptive properties of certain chemicals (Bjørling-Poulsen et al., 2008). One of the best examples of a neurotoxic and endocrine-disruptive pesticide is DDT.

The case of DDT

DDT or 1,1,1,-trichlor-2,2,-bis (p-chlorophenyl) ethane, is an insecticide that was discovered in 1939 and was initially used during the World War II to fight diseases carried by insects, like typhus and malaria. Its production at a large scale began around 1945 and it was one of the most utilized pesticides worldwide for about 25 to 30 years. In fact it was such a major pesticides that in 1950 it was estimated that each North American citizen had ingested 0.28 mg per day (D'Amato et al., 2002). In 1962, Rachel Carson suggested in her book "Silent Spring" that the wide use of DDT was the main cause of the population reduction of various several bird species such as the peregrine falcon and the bald eagle (Carson, 1962). It was shown that DDT causes a deficiency in eggshell calcification of said species, which prevented the development of new offspring due to the action of DDT metabolites, such as dichlorodiphenyldichloroethane (DDE). This metabolite has the greatest impact on ecosystems, given its propensity to bioaccumulate, leading to endocrine disruptions that can affect embryo development (Colborn et al., 1993). The biomagnification of DDE in the trophic webs led to contamination of predators. In humans, contamination may occur by direct exposure (inhalation) or by contaminated food consumption. Within the organism DDT and DDT metabolites act over the sodium/potassium equilibrium in the axons membranes leading to neurotoxic effects such as behavioural alterations, sensorial disturbances, muscular contractions, convulsions, paralysis, respiratory insufficiency and death (D'Amato et al., 2002). In terms of endocrine disruption DDT can lower semen quality, induce spontaneous abortions and increase developing children's risk for autism (Beard 2006).

Pesticide exposure pathways

Pesticides, as other xenobiotics can enter the human body by three main pathways: percutaneous or skin absorption, oral ingestion, or inhalation (Figure 1). Pesticides enter the body by penetrating the initial cellular barrier (skin, intestinal mucosa, or the lining of respiratory tract) and from the interstitial fluid it penetrates the capillaries and enters the blood stream. The bloodstream ultimately distributes the pesticide throughout the body (Zakrzewski, 2002).

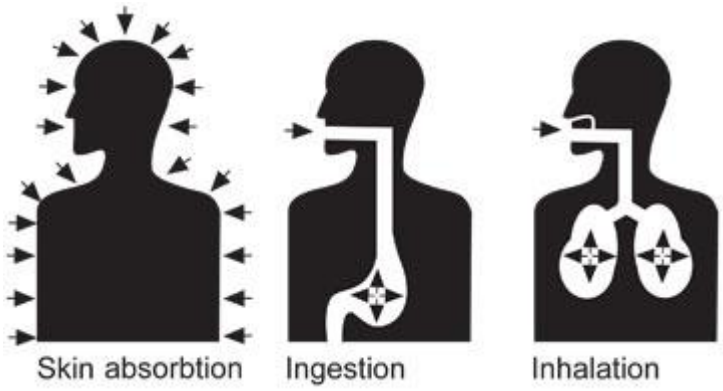

Figure 1 – Main routes of pesticide exposure.

Skin absorption

For a pesticide to pass through the skin, there are three possible routes: entry through sweat ducts, entry along the hair-follicle orifices and diffusion through the epidermis into the dermis. The latter is thought to be the major route of entry of toxins, because of its large surface area. The main obstacle to percutaneous penetration of pesticides is the outermost membrane of the epidermis, called the stratum corneum. Pesticides penetrate the stratum corneum by passive diffusion and continue passively through to the dermis where the blood vessels will transport them to the systemic circulation. The locus of entry by passive diffusion varies, depending on the chemical properties of the chemical. Polar substances are believed to penetrate cell membranes through the protein filaments, nonpolar ones enter through the lipid matrix. Hydration of the stratum corneum increases its permeability for polar substances. In general, gases penetrate skin more readily than liquids and solutes, while solids do not penetrate as much. Percutaneous absorption is a time-dependent process, with the passage through the stratum corneum being the rate-limiting reaction. Therefore, duration of exposure to a pesticide is critical (Zakrzewski, 2002 ; Holmgaard et al., 2009).

Pesticides can enter through the skin with relative ease, as they are usually mixed with different surfactants to change solubility characteristics and often to increase the penetration into plant leaves. These surfactants affect the barrier function of the skin by reducing the surface tension on hydrophilic solutions and allowing lipophilic substances to

mix with hydrophilic solutions and letting hydrophilic substances to penetrate lipophilic membranes (Holmgaard et al., 2009). Herbicides atrazine, alachlor, and trifluralin, for example, have been shown to have a penetration index inferior than that of their commercial formulations Aatrex, Lasso, and Treflan, at the same concentration, due to the effect of their surfactants (Brand & Mueller, 2002).

Dermal exposure to some pesticides, like glyphosate based herbicides, have been shown to induce various symptoms, from light symptoms like irritation or dermatitis to severe ones like skin burns, porphyria cutanea tarda and even skin cancer and neurological impairments, among others (Bradberry et al., 2004; Mariager et al., 2013; Bakre & Kaliwal, 2017).

Inhalation

The respiratory system consists of three regions: nasopharyngeal, tracheobronchial, and pulmonary. The nasopharyngeal canal has the role to remove large, inhaled particles and to increase the humidity and temperature of inhaled air. The tracheobronchial region consists of the trachea, bronchi, and bronchioles and its role is to propel foreign particles from the deep parts of the lungs to the oral cavity, where they can be either expelled with the sputum or swallowed. The pulmonary region consists of respiratory bronchioles, alveolar ducts, and clusters of alveoli in which the exchange of gases between the environment and the blood takes place. Blood capillaries are in contact with the alveolar lining cells, present in the alveolar region, so that gases as well as solutes can easily diffuse between them. Inhaled pesticides can exert their harmful action either by damaging respiratory tissue or by entering the circulation and causing systemic toxicity (Zakrzewski, 2002).

Within the problems associated with exposure of the respiratory tract to herbicides are inflammation, chronic obstructive pulmonary disease (COPD), bronchitis, pulmonary fibrosis among other breathing problems (Rocco et al., 2004 ; Choi et al., 2013 ; Alif et al., 2017).

Ingestion

Absorption of ingested compounds begins in the mouth and oesophagus. When compounds arrive the stomach, they are mixed with food, acid, gastric enzymes, and bacteria, which can alter the toxicity of the chemical, either by influencing absorption or by modifying the compound. Most food is absorbed in the small intestine. The gastrointestinal tract possesses specialized carrier systems for certain nutrients such as carbohydrates and amino acids. Some pesticides use these routes of passage through the cells; others enter through passive diffusion. Lipid-soluble organic acids and bases, in nonionized form, are absorbed by passive diffusion. Particles with several nanometres in diameter can be absorbed from the gastrointestinal tract by pinocytosis and enter the circulation via the

lymphatic system (Zakrzewski, 2002). A percentage of pesticides absorbed in the gastrointestinal cells may be biotransformed by the gut microbiota before entering the circulatory system (Koppel et al., 2017). The absorbed compounds may enter the circulation either via the lymphatic system, which will eventually drain into the bloodstream, or via the portal circulation, which transports them to the liver (Zakrzewski, 2002).
Ingestion of pesticides, leads to an extremely high mortality rate, and can cause local irritation of the mouth, oropharynx and oesophagus and multiple organ failure (heart, brain, kidney, liver,…) (Chao & Fang, 2016).

Liver Role

After entering the bloodstream, a pesticide can affect any organ in the human body (brain, kidney, liver). However, the entry of a pesticide into the bloodstream does not necessarily ensure that it will arrive unchanged at its specific receptor. Pesticides absorbed from the gastrointestinal tract are carried by the portal vein to the liver, which has a very active xenobiotic-metabolizing system in which compounds can be altered before their release through the hepatic veins into the general circulation. Alternatively, they can be excreted into the bile and returned to the gastrointestinal tract. From there they may be completely or in partially excreted or reabsorbed and carried back to the liver. This process is referred to as enterohepatic circulation (Zakrzewski, 2002).
Due to the liver being the initial site of contact for many types of orally ingested chemicals, it is particularly susceptible to chemical-induced injury. Among the chemical induced liver diseases are acute and chronic hepatitis, granulomatous hepatitis, cholestasis with bile duct injury, cholestasis with or without hepatitis, steatohepatitis, vascular disorders and tumours. The severity of chemical-induced liver injury varies from minor nonspecific changes in hepatic structure and function to acute liver failure, cirrhosis and liver cancer (Gu et al., 2012).

1.3. Pesticide Directives and Regulations in EU

Due to the toxicological and ecotoxicological hazards that pesticides in general present, European Union has adopted directives that aimed at regulating the use of these substances and thus minimizing their impact on ecosystems.

One of the most important ones is the Regulation on plant protection products ((EC) No 1107/2009, 2009), in which the European Commission is required to identify active ingredients with certain properties as candidates for substitution. Member States posteriorly evaluate whether these active ingredients might be replaced by others that are less harmful. The objective of this regulation is to promote the use of less harmful pesticides.

Other important directives include: Water framework Directive, which manages the pesticide contamination of surface waters (Directive 2000/60/EC, 2000) with the aim to reduce emissions, discharges and losses of substances under the Priority Substances Directive (The European Parlament and the Council of the European Union, 2013); The Groundwater Directive which sets a maximum concentration of pesticides in groundwater; the Drinking Water Directive (UNION, 1998) which stipulates a maximum concentration of 0.1 µg/l for any single pesticide and its relevant metabolites in potable water; the Regulation (EU) No 396/2005 which sets the maximum residue levels of pesticides allowed in or on food and feed of plant and animal origin and the Directive on the Sustainable Use of Pesticides.

These directives and regulations seem however, to have little effect on the overall use of pesticides. As we can see in figure 2 the reduction in sales in some countries is balanced by the increase of sales in the others.

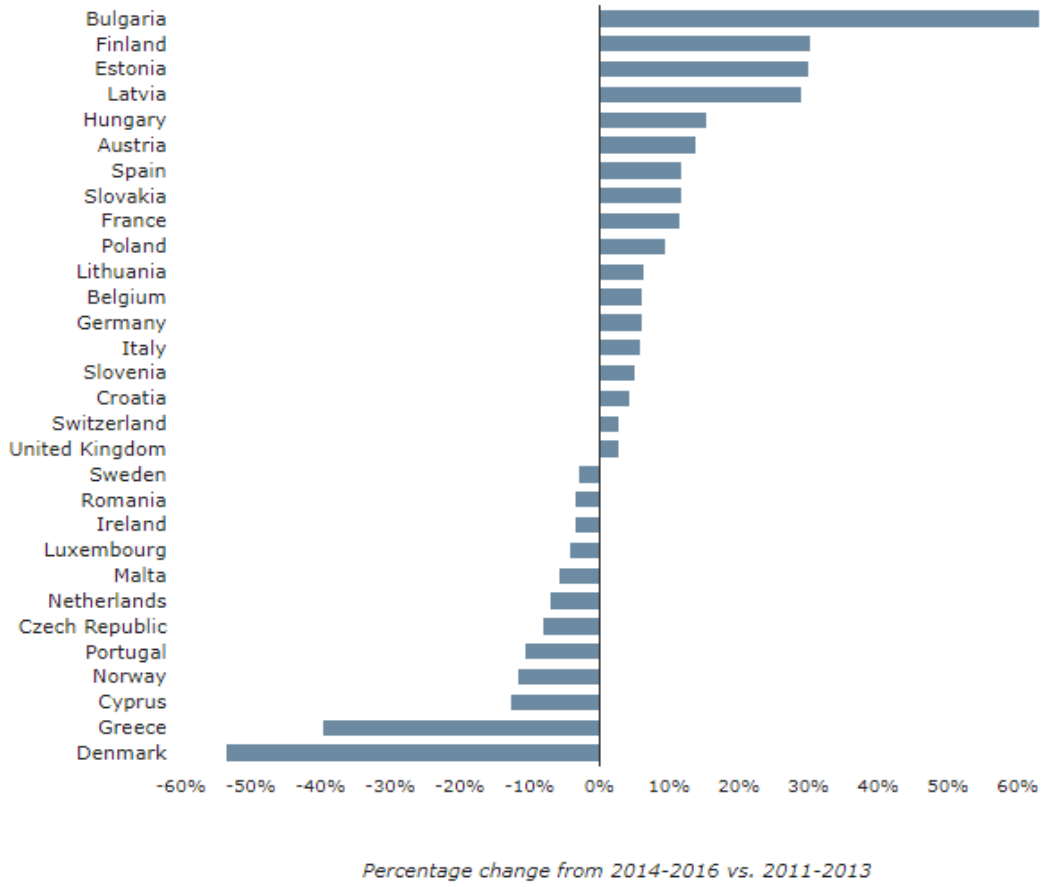

Figure 2 - Pesticide sales changes in EU countries from 2011 to 2016. From: European Environmental Agency (https://www.eea.europa.eu/ accessed in 14/12/2019)

The sales declining trend shown in Portugal seems to be a good indicator of a mentality change on the use of pesticides. Nevertheless, if we compare Portugal to, for example, Denmark, which had a bigger decline on sales than Portugal, regarding the pesticide sales records until 2018 (table 1) and cereal production expected in 2019 (Figure 3), we observe that Denmark was expected to produce 10 times more cereals than Portugal with ¼ of pesticide sales. Putting this into perspective, it is still a long way to go for Portugal and other European countries to reach a sustainable agriculture.

Table1: Overall Pesticide sales (in kilogram of herbicides) in EU member states. Adapted from Eurostat (https://ec.europa.eu/eurostat accessed in 17/12/19).

	2013	*2014*	*2015*	*2016*	*2017*	*2018*
France	66 757 420	74 873 617	66 629 333	72 076 247	70 458 871	84 969 518
Germany	43 525 028	46 078 470	47 850 856	46 889 567	48 302 682	44 953 791
Spain	71 454 156	78 818 308	77 216 754	76 940 641	71 987 324	61 343 224
Poland	22 191 786	23 550 583	23 996 280	24 451 662	25 073 556	23 156 567
Turkey	…	…	…	49 961 210	53 874 955	59 921 959
United Kingdom	16 590 705	22 662 719	21 325 235	15 488 157	15 870 120	15 516 296
…	…	…	…	…	…	…
Denmark	4 197 102	1 821 895	2 583 747	2 586 881	2 706 105	2 646 128
Portugal	10 124 633	12 889 226	10 003 046	9 772 274	8 121 275	8 057 311
…	…	…	…	…	…	…
Switzerland	2 276 935	2 232 525	2 207 505	2 139 911	2 006 768	2 019 618
Norway	759 737	859 791	631 551	674 927	667 884	620 322

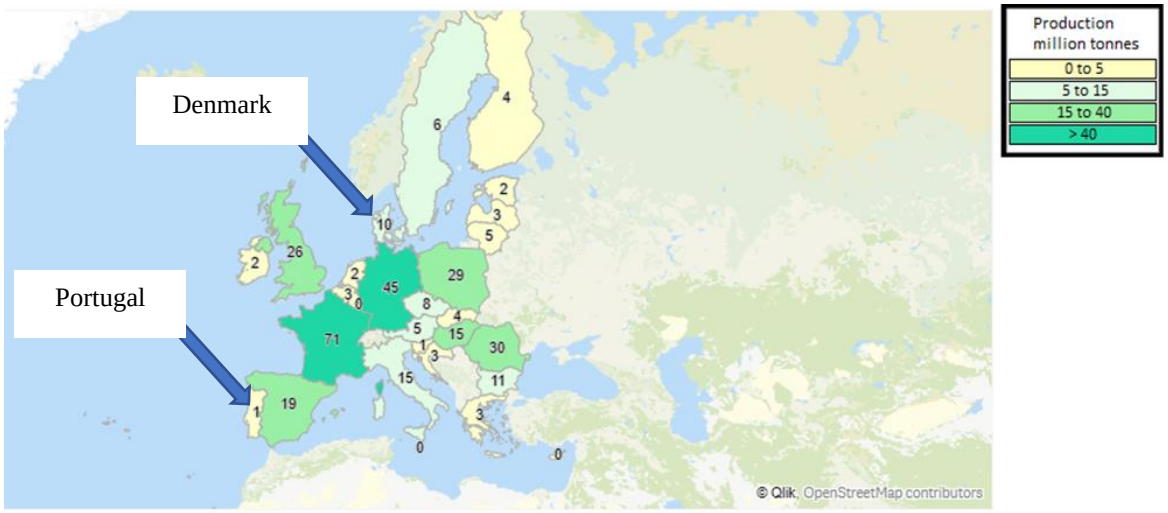

Figure 3 - Cereal production expected in European countries in 2019. From: Agri-food Data Portal of European commission Website (https://agridata.ec.europa.eu/extensions/DashboardCereals/CerealsProduction.html, accessed in 17/12/19).

1.4. Herbicides

Herbicides are pesticides that kill weeds or other undesirable plants. Weeds reduce the quality and quantity of agricultural production and can be a health threat as they produce allergens, that may induce contact dermatitis. Therefore, weeds are treated as a major plague in agriculture. Herbicides represent the most effective and economical mean of control of these pests. Without the use of herbicides, it would have been impossible to mechanize the full production of crops like cotton, sugar beets, grains, potatoes, or corn. Today herbicide sales represent about 30% of all pesticides sales. This fact reveals the importance of these chemical to food production.
Herbicides can be classified into multiple categories, according to the following criteria:

- Application timing;
- Method of Application;
- Selectivity;
- Mobility;
- Mode of action.

Application timing

Herbicides can be classified according to the application timing. There are three major categories of application timing: preplant, preemergence, and post emergence.

Preplant herbicides, as the name indicates, are herbicides applied to soil before planting. In some cases, the herbicide is applied in the fall to suppress early weed growth in the next spring before the planting. Preplant herbicides will get incorporated into the soil and kill weeds as they grow through the herbicide treated zone. Metam-sodium, Treflan and Dazomet are examples of preplant herbicides. And crops grown in soil treated with these herbicides include corn, soybeans and tomatoes.

Preemergence herbicides are applied after planting but before the crop and weeds emerge through the soil surface. These herbicides affect the cell division of emerging weeds thus killing them as they grow through the treated zone. Preemergence herbicides do not affect weeds that have emerged before the application. Examples of pre-emergence herbicides are Lasso, Dual, Lorox.

Postemergence herbicides are applied after weeds emerge from the soil. Generally, multiple applications of the formulation are needed. An example of post-emergence herbicide is 2,4-D (Vats, 2015).

Method of application

Regarding the application method, herbicides can be soil or foliar applied. Soil applied herbicides are usually taken up by the root or shoot of emerging seedlings and used as preplant and preemergence treatment. Effectiveness of this type of herbicides is usually dependent on the positioning of the herbicide in the correct layer of soil, on the adsorption to soil elements, and on the processes to which they may be subjected. Photolysis and volatility are two of the processes that reduce the availability of herbicides. Examples of soil applied herbicides are EPTC and Trifluralin.

Foliar applied herbicides are applied above the ground and are absorbed by the exposed tissues. After being absorbed they can either act on site or be translocated throughout the plant. These types of herbicides are postemergence. Glyphosate and 2,4D are examples of foliar applied herbicides (Vats, 2015).

Selectivity

A selective herbicide is the one that kills or slows the growth of weeds while other plants are tolerant and manage to grow under the same treatment. Selectivity of the herbicide is based on absorption, morphological and physiological differences between plants. Examples of selective herbicides are 2,4-D and Dicamba (Vats, 2015 ; Blanco et al. 2015).

Nonselective herbicides kill all plant material they come in contact with. This type of herbicides is usually used to clear industrial sites, waste grounds and railways. Examples of non-selective herbicides include Paraquat and Glyphosate (Vats, 2015).

Mobility

Herbicides can be classified according to their mobility within the plant. Contact/non systemic herbicides kill the plant parts where the chemical is applied. They are fast acting herbicides and are the most effective against annuals, weeds that germinate from seeds and grow to maturity each year, and less effective on perennial plants, that can regrow from rhizomes, roots, or tubers. Uniform spray coverage and particle size are essential for adequate application of these type of herbicides and repeated application is

essential to prevent the regrowth from hidden parts. Bromoxynil and Bentazon are examples of contact herbicides (Vats, 2015).

Translocated/systemic herbicides are herbicides that are extensively translocated in the plant through its vascular system along with water, nutrients, and other materials from sites of absorption, like roots or some above-ground parts, to sites of action. Even though this kind of herbicides may be effective against all weed types, they are more effective in the control of established perennials. Systemic herbicides require longer time than contact herbicides to kill weeds. Examples of systemic herbicides are Glyphosate and Dicamba (Vats, 2015).

Mode of action

Due to an increase in the resistance of weeds to certain herbicides, a rotation of the herbicides based on the mechanisms of action (MOA) became imperative. To facilitate growers into choosing the right herbicide to select during rotation and devise an appropriate crop-management strategy, a MOA classification system was created. In fact, 3 MOA classification systems were created: The Weed Science Society of America (WSSA) classification system, the Australian classification system and the Herbicide Resistance Action Committee (HRAC) classification system (Beffa et al. 2019).
The classification system used in Europe and in most countries around the world is the HRAC classification system. This system organizes herbicides in groups by letters, according to their site of action and is comprised of 25 groups from A to Z. There are 4 Z groups in the HRAC system because even though the sites of action of the herbicides in this group are not well defined, they appear to act differently (see figure 4 and table 2 to understand the sites of action of the various groups presented on the HRAC classification system). Herbicides belonging to a specific group have the same mode of action. Groups like K1, K2 and K3 compromise the same biological processes by acting over different sites of action. Some chemical families appear in more than one group because depending on the active ingredient they may have different MOAs. The HRAC classification system is continuingly changing as the discover of new groups introduces slight variations on the classification.

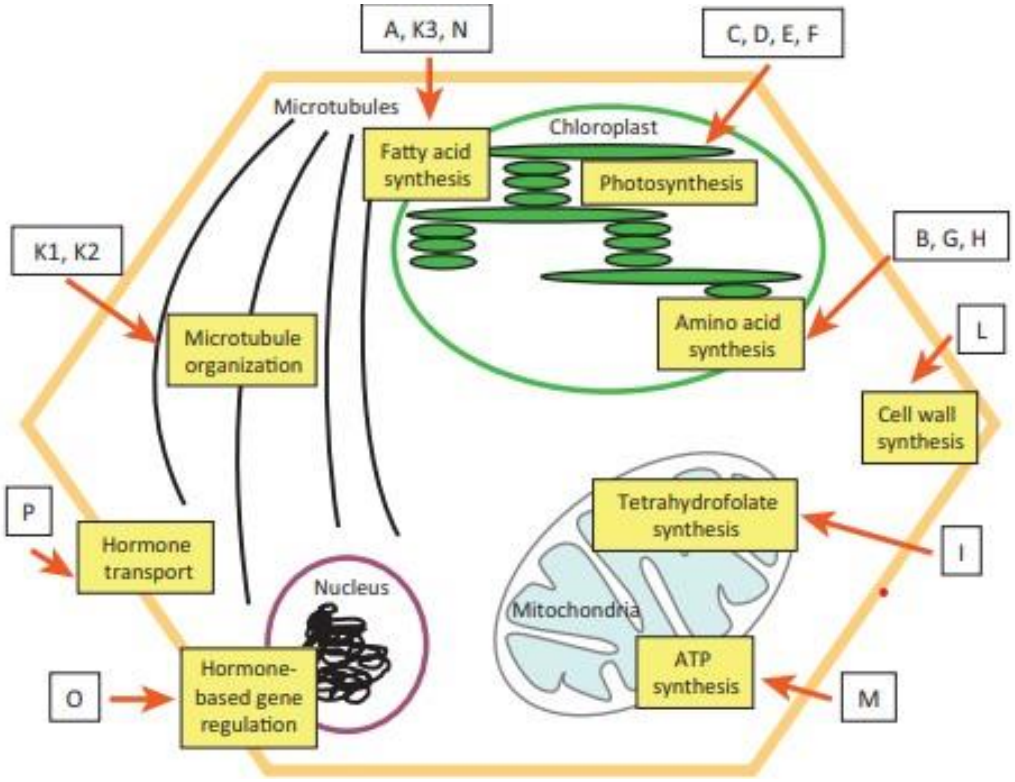

Figure 4 - Cellular targets of herbicide action and herbicide classification by mode of action according to the Herbicide Resistance Action Committee (HRAC). From: (Délye et al. 2013)

HRAC classification by mode of action:

Fatty acid synthesis inhibitors:

Group A: This group is comprised by aryloxyphenoxypropionate (FOPs), cyclohexanedione (DIMs) and Phenylpyrazoline (DEN). These herbicides inhibit the enzyme acetylCoA carboxylase (ACCase), thus blocking the first step in fatty acid synthesis and consequently preventing the formation of lipid and secondary metabolites in susceptible plants. This results in loss of cell membrane integrity, metabolite leakage, and cell death (Kaundun, 2014; Takano et al. 2019).

Group K3: This group is formed by acetamide, chloroacetamide, oxyacetamide, and tetrazolinone herbicides. These families of herbicides inhibit very long chain fatty acid (VLCFA) synthesis. These compounds are effective to susceptible weeds preemergence, but do not inhibit seed germination. Group K3 affects plant growth like the rest of Groups K1 and K2 but by different means (Sherwani et al., 2015).

Group N: The chemical families of herbicides within the group N are Benzofuranes, chlorocarbonic acids, phosphorodithioates, and thiocarbamates. These herbicides are known to block very long chain fatty acid elongases (VCLFAE) thus compromising the synthesis of waxes and cutins, as well as sphingolipids. Such disruption leaves plants open to external stresses resulting various lethal effects on plants in their initial developmental stages (Böger, 2003; Busi, 2014).

Photosynthesis inhibitors

Group C: There are 3 C ggroups. Phenylcarbamates, pyridazinones, triazines, triazinones and uracils form Group C1, amides and ureas form Group C2 and benzothiadiazinones, nitriles, and phenylpyridazines form group C3. These herbicide´s families inhibit the QB-binding site of the D1 protein in the photosystem II complex in chloroplast thylakoid membranes. This inhibition results in a disruption of the chloroplastic electron transport chain, resulting in the build-up of reactive oxygen species (Murphy et al. 2019). The formation of Groups is due to the binding to the D1 protein being different.

Group D: This group is formed by 2 herbicides of the Bipyridylium family, Paraquat and Diquat. These herbicides accept electrons from photosystem I (PSI) and reduce them to form an herbicide radical. This radical then reduces other molecules to form reactive oxidative species that overwhelm the plant antioxidant defences resulting in DNA damage, and cell and tissue death (Lascano et al., 2012).

Group E: This group is represented by Diphenylethers, N-phenylphthalimides, oxadiazoles, oxazolidinediones, phenylpyrazoles, pyrimidindiones, thiadiazoles, and triazolinones. These families of herbicides inhibit the protoporphyrinogen oxidase (PPO or Protox). Inhibition of the plant enzyme causes accumulation of the protoporphyrinogen-IX substrate, which is exported to the cytoplasm, where it is oxidized by O_2 in the mitochondrion and chloroplast. This produces a photosensitive protoporphyrin IX. With exposure to light, this protoporphyrin IX generates singlet oxygen molecules that causes lipid peroxidation and cell death (Hao et al. 2011; Dayan & Duke, 2010).

Group F: There are 3 F groups. The group F1 is formed by Pyridazinone, Pyridinecarboxamide, group F2 by Triketone, Isoxazole, Pyrazole and group F3 by Triazole, Isoxazolidinone, Urea, Diphenylether. These groups of herbicides interfere with carotenoid biosynthesis by different means. The compounds belonging to F1 inhibit phytoene desaturase, which is an enzyme that plays a major role in carotenoid biosynthesis. F2 herbicides inhibit phydroxyphenyl pyruvate dioxygenase (HPPD), which plays an important role in plastoquinone biosynthesis. As a cofactor of phytoene desaturase, inhibition of plastoquinone will interfere with carotenoid biosynthesis. F3 herbicides don’t have a well determined site of action but plants exposed to these herbicides have been shown to

accumulate phytoene, phytofluene, carotenes, and lycopene, all precursors of carotenoid synthesis, suggesting that phytoene desaturase, lycopene cyclase, imidazole glycerol phosphate dehydratase, nitrate reductase, or catalase may be inhibited (Rao, 2014).

Amino acid synthesis inhibitors:

Group B: This group is composed by Imidazolinones, pyrimidinylthiobenzoates, sulfonylaminocarbonyltriazolinones, sulfonylureas and triazolopyrimidines. These kinds of herbicides inhibit acetolactate synthase (ALS or AHAS) which is a key enzyme in the biosynthesis of the branched-chain amino acids isoleucine, leucine, and valine. Plant death results from events occurring in response to ALS inhibition and low branched-chain amino acid production (Shaner, 1999; Monqueiro et al. 2001).

Group G: This group is comprised of glycines. Glycines are herbicides that inhibit 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase. This enzyme is found in the aromatic biosynthesis pathway, also known as the shikimate pathway and catalyzes the transfer of a carboxyvinyl group from phosphoenolpyruvate (PEP) to shikimate-3-phosphate. EPSP synthase inhibition leads to depletion of the aromatic amino acids tryptophan, tyrosine, and phenylalanine, all needed for protein synthesis or for biosynthetic pathways leading to growth. The events occurring in response to EPSP synthase inhibition results in plant death (Siehl, 1997; Dong et al., 2019).

Group H: The herbicides that comprise this group are Glufosinate and Bialophos. These herbicides belong to a family known as Phosphinic acids. They inhibit glutamine synthase (GS) which is responsible for converting glutamate and ammonia to glutamine (Gln). A decrease in glutamate and glutamine leads to the accumulation of metabolites glyoxylate, phosphoglycolate, and glycolate in photorespiration. The accumulation of high levels of glyoxylate leads to the inhibition of ribulose-1,5-bisphosphate carboxylase/oxygenase activase which is a key enzyme in the Calvin–Benson cycle. All this results in the accumulation of reactive oxygen species, lipid peroxidation and cell death (Berlicki, 2008; Brunharo et al. 2019).

Cell wall synthesis inhibitors:

Group L: The families of herbicides that form this group are nitrile, benzamide, triazolocarboxamide, quinoline carboxylic acids. These are herbicides that affect cellulose synthase in susceptible plants. As the name indicates, cellulose synthase is the enzyme responsible for the synthesis of cellulose. Because cellulose plays an important structural role in cell wall reinforcement, the inhibition of cellulose biosynthesis in the cell wall causes

loss of directional cellular expansion, thus leading to cells becoming radially swollen and growth organs becoming dwarfed (Tateno et al., 2016).

Tetrahydrofolate synthase inhibitors:

Group I: This group is only formed by carbamate herbicide asulam. This herbicide appears to inhibit 7,8-dihydropteroate (DHP) synthase an enzyme involved in folic acid synthesis. The inhibition of folic acid synthesis will affect the synthesis of tetrahydrofolate which is a coenzyme that plays a big role in many reactions, especially in the metabolism of amino acids and nucleic acids (Illarionova et al., 2002).

ATP synthesis inhibitors:

Group M: The herbicide representative of this group is Dinitrophenol. This herbicide is a known oxidative phosphorylation uncoupler. Dinitrophenol has the ability to separate the flow of electrons and the pumping of H^+ ions for ATP synthesis. Low ATP levels are lethal (Fry et al. 1980).

Hormone based gene regulators:

Group O: Phenoxy-carboxylic-acid, Benzoic acid, Pyridine carboxylic acid, quinoline carboxylic acid are the herbicides that form group O. These herbicides act as natural auxin indole-3-acetic acid (IAA). They enter the cell by binding to an F-box protein called TIR1, a subunit of the SCFTIR1 (Skp-Cullin-F-box) ubiquitin ligase protein complex. TIR1 directly binds auxin, and this binding allows TIR1 to associate with Aux/IAA proteins. The Aux/IAA repressor proteins are ubiquitinated by the SCFTIR1 complex and degraded by the 26S proteasome. The removal of the Aux/IAA proteins relieves the repression of auxin responsive genes (Gleason et al. 2011). The herbicide then begins to make damage by causing a rapid increase in ethylene production and an increase in abscisic acid (ABA) biosynthesis. The increase of ABA levels inhibits plant growth by closure of the stomata, which subsequently limits carbon dioxide assimilation and leads to the accumulation of hydrogen peroxide in the plant. This accumulation of reactive oxygen species leads to tissue damage and cell death (Kraft et al. 2007).

Hormone transport inhibitors:

Group P: This group is comprised of phthalamate and semicarbazone herbicides. Although the mechanisms are not clear it has been shown that these herbicides inhibit polar auxin transport (Teale et al. 2018). Auxin is a crucial hormone in controlling various physiological

phenomena like cell elongation, apical dominance, and tropism. So inhibition of this hormone will ultimately affect plant growth and development (Saniewski et al., 2017).

Microtubule organization inhibitors:

Groups K1 and K2: These K groups affect microtubule polymerization. The K1 group is comprised by the dinitroaniline, Phosphoroamidate, Pyridine, Benzamide and Benzoic acid chemical families and the K2 group is comprised by the Carbamate herbicides, carbetamide, propham and chlorpropham. These groups of herbicides are known to bind to plant tubulin. This herbicide-tubulin complex inhibits polymerization of microtubules thus affecting all processes in which microtubules are essential, like alignment and separation of chromosomes during mitosis and cell wall formation (Quinlan et al. 1980; Alebrahim et al., 2017; Délye et al., 2004).

Unknown MOA:

Group Z: Even though the mechanisms of action of Group Z herbicides are not well known, they can be divided into at least four groups that act on different sites of action: the Organoarsenicals, known to be nucleic acid inhibitors; the Arylaminopropionic acids, known antimicrotubule mitotic disrupters; the Pyrazoliums, known cell elongation inhibitors; and the Uknown, herbicides who have not yet been classified.

Table 2: The 21 known groups of herbicides classified by mechanisms of action (MA) according to the global Herbicide Resistance Action. The absence of other groups in the table is due to lack of understanding of their mode of action. Adapted from Caverzan et al. 2019. See HRAC website (http://www.hracglobal.com) to check all modes of action as well as the corresponding active ingredients.

HRAC Group	Mode of Action (MA)	Biological Process Compromised	Herbicide Chemical Family
A	Inhibition of acetyl-CoA carboxylase (ACCase)	Fatty acid biosynthesis	Aryloxyphenoxy-propionate " FOPS" , Cyclohexanedione " DIMs" , Phenylpyrazoline " DEN"
B	Inhibition of acetohydroxyacid synthase (AHAS, ALS)	Amino acid biosynthesis (Leu, Ile, Val)	Sulfonylurea, Imidazolinone, Triazolopyrimidine, Pyrimidinyl(thio)benzoate
C1/C2/C3	Inhibition of photosystem II protein D1 (psbA)	Photosynthesis (electron transfer)	Triazine, Triazinone, Triazolinone, Uracil, Pyridazinone, Phenyl-carbamate / Urea, Amide / Nitrile, Benzothiadiazinone, Phenyl-pyridazine
D	Diversion of the electrons transferred by the photosystem I ferredoxin (Fd)	Photosynthesis (electron transfer)	Bipyridylium
E	Inhibition of protoporphyrinogen oxidase (PPO)	Photosynthesis (heme biosynthesis for chlorophyll)	Diphenylether, Phenylpyrazole, N-phenylphthalimide, Thiadiazole, Oxadiazole, Triazolinone, Oxazolidinedione, Pyrimidindione
F1/F2/F3	Inhibition of phytoene desaturase (PDS) or 4-hydroxyphenylpyruvate dioxygenase (4-HPPD) or of an unknown protein	Photosynthesis (carotenoid biosynthesis)	Pyridazinone, Pyridinecarboxamide / Triketone, Isoxazole, Pyrazole/ Triazole, Isoxazolidinone, Urea, Diphenylether
G	Inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)	Amino acid biosynthesis (Phe, Trp, Tyr)	Glycine
H	Inhibition of glutamine synthase	Amino acid biosynthesis (Gln)	Phosphinic acid

Table 2 continuation: The 21 known groups of herbicides classified by mechanisms of action (MA) according to the global Herbicide Resistance Action. The absence of other groups in the table is due to lack of understanding of their mode of action. Adapted from Caverzan et al. 2019. See HRAC website (http://www.hracglobal.com) to check all modes of action as well as the corresponding active ingredients.

HRAC Group	Mode of Action (MA)	Biological Process Compromised	Herbicide Chemical Family
I	Inhibition of dihydropteroate synthase	Tetrahydrofolate biosynthesis	Carbamate
K1/K2	Enhancement of tubulin depolymerization	Microtube polymerization	Dinitroaniline, Phosphoroamidate, Pyridine, Benzamide, Benzoic acid / Carbamate
K3	Inhibition of fatty acid synthase (FAS)	Fatty acid biosynthesis	Chloroacetamide, Acetamide, Oxyacetamide, Tetrazolinone
L	Inhibition of cellulose-synthase	Cell wall biosynthesis	Nitrile, Benzamide, Triazolocarboxamide, Quinoline carboxylic acid
M	Uncoupling of oxidative phosphorylation	ATP biosynthesis	Dinitrophenol
N	Inhibition of fatty acid elongase	Fatty acid biosynthesis	Thiocarbamate, Phosphorodithioate, Benzofuran, Chloro-Carbonic-acid
O	Stimulation of transport Inhibitor response protein 1 (TIR1)	Regulation of auxin-responsive genes	Phenoxy-carboxylic-acid, Benzoic acid, Pyridine carboxylic acid, Quinoline carboxylic acid
P	Inhibition of auxin transport	Long-range hormone signaling	Phthalamate, Semicarbazone

1.5. Case of study

Primextra® Gold TZ

Primextra® Gold TZ is a selective and systemic herbicide of root and leaf absorption indicated for pre (4 to 4.5 L/ha) and post-emergence application (3.5 to 4 L/ha) on corn and sunflower crops for the control of grass weeds and water grass (Syngenta, 2017). This commercial formulation is manufactured by Syngenta AG and is one of the bestselling herbicides in Portugal, being referenced by the Mondego Valley cooperatives as the most-used herbicide in corn fields (Gonçalves et al., 2016).
Primextra® Gold TZ has two main active ingredients (a.i.), S-metolachlor (30.2% (w.w.)) and therbuthylazine (17.75% (w/w)) plus coadjuvant substances that are supposedly inert but that can potentiate the effects of the isolated a.i. (Neves et al., 2015) (see table 3 for Primextra® Gold TZ composition).

Table 3: Primextra® Gold TZ composition adapted from safety data sheet - (Syngenta, 2018)

Chemical	**CAS number** **CE number** **Index number** **Registration number**	**Classification**	**Concentration (% w/w)**
S-metolachlor	87392-12-9 607-432-00-4	Skin Sens. 1; H317 Aquatic Acute 1; H400 Aquatic Chronic 1.; H410	>= 25 - < 30
Terbuthylazine	5915-41-3 227-637-9 613-323-00-2	Acute Tox. 4; H302 STOT RE 2; H373 Aquatic Acute 1; H400 Aquatic Chronic 1; H410	>= 10 - < 20
poly(oxy-1,2-ethanediyl), -[2,4,6- tris(1-phenylethyl)phenyl]- -hydroxy-	104376-75-2	Aquatic Chronic 2; H411	>= 2.5 - < 10
sodium; 1,2-bis-(2-ethylhexyloxycarbonyl)-ethane sulfonate	577-11-7 209-406-4 01-2119491296-29	Skin Irrit. 2; H315 Eye Dam. 1; H318	>= 1 - < 3
poly(oxy-1,2-ethanediyl), alphasulfo-omega-[tris(1-phenylethyl)phenoxy]-, ammonium salt	119432-41-6	Aquatic Chronic 3; H412	>= 1 - < 2.5
1,2-benzisotiazol-3(2H)-ona 2	2634-33-5 220-120-9 613-088-00-6	Acute Tox. 4; H302 Skin Irrit. 2; H315 Eye Dam. 1; H318 Skin Sens. 1; H317 Aquatic Acute 1; H400	< 0.05

Primextra® Gold TZ is considered hazardous to human health and according to Syngenta safety sheet the herbicide can cause severe ocular irritation; cutaneous allergic reaction; organ damage after prolonged or repeated exposure; extreme toxicity to aquatic organisms with long lasting effects. Main pathways of exposure are ingestion, inhalation, skin contact and eye contact. Safety data sheet of the herbicide Primextra® Gold TZ also mentions the toxicity values in various toxicity tests: In rats an acute toxicity test via oral exposure obtained a LD_{50} of > 3.000 mg/kg. In fish a LC_{50} of 8.32 mg/l was obtained after exposure of *Oncorhynchus mykiss* (rainbow trout) to the compound for 96 h. For invertebrates an EC_{50} of 35.2 mg/l was obtained by exposing *Daphnia magna* to Primextra® Gold TZ for 48 h. In algae an EC_{50} and NOEC (No observed effect concentration) of 0.131 mg/l and 0.013 mg/l was obtained after exposing *Raphidocelis subcapitata* (formerly *Pseudokirchneriella subcapitata)* (green algae) for 72 h to the compound (Syngenta, 2018). In *Daphnia longispina* exposed to the commercial product and to the a.i. S-metolachlor was obtained a chronic EC_{50} of 6.58 mg/l and 8.24 mg/l, respectively (Neves et al., 2015).

Because herbicides can contaminate the water bodies near agricultural areas through run-off drainage, spry drift and accidental spills, most of the literature is focused on determining if the compound represents danger to non-target aquatic species. Toxic effects observed in literature include cell growth inhibition and fatty acid changes in marine planktonic species (Filimonova et al., 2016), immobilization, reduction in fecundity, developmental delay and reduction of number of broods on the life of the freshwater species *Daphnia longispina* (Neves et al., 2015) and lethality and fatty acid changes in marine bivalves (Gonçalves et al., 2016) (See table 4 for additional toxicity data, found in literature, on Primextra® Gold TZ).

Table 4: Summary of toxicity data found in literature about the effects of Primextra® Gold TZ in non-targeted species. *s means to small size class; *b means to big size class.

Compound	Species	Effect	Concentrations	Duration	Reference
Primextra® Gold TZ	*Thalassiosira weissflogii*	EC_{10} EC_{20} EC_{50}	0.0025 mg/l 0.0038 mg/l 0.0078 mg/l	96 h	(Filimonova et al., 2016)
	Acartia tonsa	EC_{10} EC_{20} EC_{50}	0.145 mg/l 0.289 mg/l 0.925 mg/l	48 h	
	Artemia franciscana	EC_{10} EC_{20} EC_{50}	5.42 mg/l 10.54 mg/l 20.35 mg/l	48 h	
	Daphnia longispina	EC_{10} EC_{20} EC_{50}	15.69 mg/l 23.23 mg/l 37.65 mg/l	48 h	(Neves et al., 2015)
		EC_{10} EC_{20} EC_{50}	4.94 mg/l 6.34 mg/l 6.58 mg/l	21 d	
	Scrobicularia plana	LC_{10s*} LC_{20s*} LC_{50s*} LC_{10b*} LC_{20b*} LC_{50b*}	2.206 mg/l 3.351 mg/l 5.539 mg/l 6.338 mg/l 8.715 mg/l 13.263 mg/l	96h	(Gonçalves et al., 2016)
	Cerastoderma edule	LC_{10s*} LC_{20s*} LC_{50s*} LC_{10b*} LC_{20b*} LC_{50b*}	22.873 mg/l 24.376 mg/l 27.252 mg/l 21.298 mg/l 23.868 mg/l 28.784 mg/l	96h	

S-metolachlor

S-metolachlor(2-Chloro-N-(2-ethyl-6-methylphenyl)-N-[(1S)-2-methoxy-1-methylethyl] acetamide), is the isomer S of metolachlor (Figure 5).

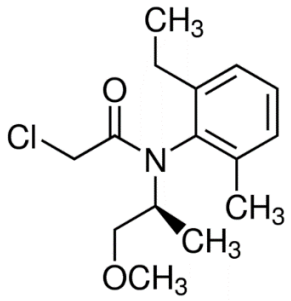

Figure 5 – S-Metolachlor molecular structure. From: https://www.sigmaaldrich.com/portugal.html - accessed in 29/01/2020.

Metolachlor was discovered in the decade of 1970 and when applied in terrain showed an excellent control over weeds and proved to be well tolerated by fundamental cultures, like corn. Sold for the first time in 1976 ended up being registered by over 90 countries. Only in 1980 thanks to the availability of new technologies of synthesis was possible to produce the active isomer.

S-Metolachlor is physically and chemically equivalent to metolachlor, but is more active at the site of action of susceptible plants, thus being considered more efficient and less harmful to the environment ("Regulamento (CEE) n.o 4064/89 do Conselho," 2004; Liu et al., 2006) as it reduces the risk of contamination to workers, consumers, and the environment (O'Connell et al., 1998). S-metolachlor is one of the most used chloroacetamide herbicides in agriculture for control of preemergent annual grasses and broadleaf weeds in a variety of crops, such as corn, sunflower, soybean and potato (Nikoloff et al, 2013). As a chloroacetamide it is known for inhibiting biosynthesis processes, namely lipids, fatty acids, leaf wax, terpenes flavonoids and protein synthesis, on meristematic zones of plants (Karam et al., 2003; Demailly et al., 2019). It also interferes with normal cell development and inhibits both cell division and cell enlargement (Liu & Xiong, 2009). In fact, S-metolachlor inhibits elongases involved in the elongation of highly unsaturated fatty acid (HUFA) and very long chain fatty acids (VLCFA) (Trenkamp et al., 2004). S-metolachlor is also responsible for the oxidation of cell components increasing the production of reactive oxygen species (ROS) and starting lipid peroxidation which explains its toxicity (Špoljarić Maronić et al., 2018). Even though this is considered potentially dangerous to environmental and aquatic systems, S-metolachlor's sales license was extended until 31 of July of 2021 (European Commission, 2020).

Toxicological information for S-metolachlor was documented in the 2004 “Review report for the active substance S-Metolachlor” by the Standing Committee on the Food Chain and Animal Health (*European Commission*, 2004). In this report the toxicology and metabolism of the compound is assessed through various tests: for the acute toxicity assay LD_{50} for toxicity through oral ingestion on rats was of 3267 mg/kg bw and 2577 mg/kg bw for males and females, respectively. For the acute toxicity through dermal contact a LD_{50} > 2000 mg/kg bw was obtained and finally, for the acute toxicity through inhalation a LC50 >2.91 mg/l air was calculated.

In terms of carcinogenicity, S-metolachlor was shown to trigger the emergence of eosinophilic foci and neoplastic nodules in rat liver. However, with a NOAEL of 14 mg/kg bw/d the compound could not be labelled as carcinogenic.

As for the genotoxicity assessment, *in vivo* assays showed increased DNA-synthesis at 500 mg/kg bw and CYP2B induction at 24.5 mg/kg bw in rats. Despite that, S-metolachlor is not considered to be genotoxic as the transient increase of DNA-synthesis *in vivo* was declared as a result of hepatotoxicity and possible stimulation of cell proliferation. Higher concentrations induced hepatotoxic but not genotoxic effects (*European Commission*, 2004).

Since 2004, further assays have been conducted as the herbicide’s ability to induce damage to water organisms has been shown consistently over the years. S-metolachlor has been shown to induce: immobilization, reduction in fecundity, developmental delay and reduction of number of broods on the life of *D. longispina* (Neves et al., 2015), growth inhibition of green algae (Liu & Xiong, 2009; Liu et al., 2012), bioluminescence inhibition on *Aliivibrio fischeri* (formerly *Vibrio fischeri*) *(Joly et al., 2013)*, mortality, malformations and inhibition of embryo hatching of zebrafish (*Danio rerio*) (Quintaneiro et al., 2017), mortality and fecundity reduction on *D. magna* (Liu et al., 2006). In 2009 it was also demonstrated that the herbicide affected the bioenergetics of rat liver and the respiratory activity of *Bacillus stearothermophilus* (Pereira et al., 2009) (See table 5 below for additional toxicity data on S-metolachlor).

In 2018, after a reassessment on the carcinogenicity of the compound, US. EPA declared S-metolachlor as “not likely to likely to be Carcinogenic to Humans” at doses that do not induce cellular proliferation in the liver (USEPA, 2018).

Table 5: Summary of toxicity data found in literature about the effects of S-metolachlor in non-targeted species.

Compound	Species	Effect	Concentrations	Duration	Reference
S-Metolachlor	*Daphnia longispina*	EC_{10}	12.73 mg/l	48h	(Neves et al., 2015)
		EC_{20}	14.78 mg/l		
		EC_{50}	18.71 mg/l		
		EC_{10}	3.84 mg/l	21d	
		EC_{20}	5.09 mg/l		
		EC_{50}	8.24 mg/l		
	Scenedesmus obliquus	EC_{50}	0.255 mg/l	24h	(Liu et al., 2012)
			0.177 mg/l	48h	
			0.131 mg/l	72h	
			0.130 mg/l	96h	
	Daphnia magna	LC_{50}	51.2 mg/l	24h	(Liu et al., 2006)
		NOEC	0.1 mg/l	21d	
		LOEC	0.5 mg/l		
	Aliivibrio fischeri	IC_{50}	178±22.8 mg/l		(Joly et al., 2013)
	Danio rerio	LC_{50}	46.26 mg/l	96h	(Quintaneiro et al., 2017)
		EC_{50}	29.4 mg/l		
		LOEC	29.0 mg/l		
	Chlorella pyrenoidosa	EC_{50}	0.116 mg/l	24h	(Liu & Xiong, 2009)
			0.106 mg/l	48h	
			0.081 mg/l	72h	
			0.068 mg/l	96h	

Terbuthylazine

Terbuthylazine (N2-tert-butyl-6-chloro-N4-ethyl-1,3,5-triazine- 2,4-diamine) is a pre- and post-emergence selective and systemic herbicide from the triazine family (Figure 6). This compound is mainly used in maize, sorghum, sunflower, legumes, vines, citrus, and cotton cultures to fight broad-leaf weeds and grasses.

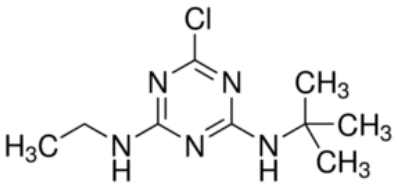

Figure 6 – Terbuthylazine molecular structure. From: https://www.sigmaaldrich.com/portugal.html - accessed in 29/01/2020.

Terbuthylazine has been used as a substitute of atrazine in countries in which the latter has been prohibited (Plhalova et al., 2012). This is due to the herbicide presenting a major adsorption coefficient than atrazine thus being considered less risky regarding contamination, even though its metabolites present high mobility and consequently contaminate subterranean waters (Barra Caracciolo et al., 2005).

Terbuthylazine is known to inhibit photosynthesis in photosystem II (PS II). This is due to the compound's capability of binding to the plastoquinone-binding protein in photosystem II, inhibiting electron transport (Cañero et al., 2011; Jerzykiewicz & Kłobus,. 2007).

Little is known about the specific effects of terbuthylazine in mammals as most recent literature found is directed towards understanding the toxicological effects of the herbicide to aquatic organisms. Toxicological effects found in the literature include: mortality and immobilization of *D. magna* (Pereira et al., 2017) growth retardation, developmental and histological changes (Plhalova et al., 2012) and mortality in *Cyprinus carpio* (Velisek et al., 2015), growth retardation, developmental and histological changes in *C. carpio* and mortality in *Carassius auratus, Oncorhynchus mykiss and Lepomis macrochirus* (Velisek et al., 2015) (See table 6 below for additional toxicity data on terbuthylazine).

Table 6: Summary of toxicity data found in literature about the effects of Terbuthylazine in non-targeted species.

Compound	Species	Effect	Concentrations	Duration	Reference
Terbuthylazine	*Daphnia magna*	EC_{50}	950 µg/l	48h	(Pereira et al., 2017)
	Cyprinus carpio	NOEC	160 µg/l	30d	(Plhalova et al., 2012)
		LOEC	520 µg/l		
		LC50	2.992 µg/l		(Velisek et al., 2015)
		LOEC	2.9 µg/l	35d	
	Carassius auratus	LC_{50}	9.4 mg/l	96h	
	Oncorhynchus mykiss	LC_{50}	3.8 mg/l	96h	
	Lepomis macrochirus	LC_{50}	4.6 mg/l	96h	
	Cyprinus carpio	LC_{50}	7.5 mg/l	96h	

According to USEPA (2018), Terbuthylazine is considered a Group D in terms of carcinogenicity classification as it is "Not Classifiable as to Human Carcinogenicity". This compound had its sales license extended on February of 2019 until 31 December of 2023 (*Commission Implementing Regulation (EU) 2019/291*, 2019).

1.6 *In vitro* assays

As the name indicates, a test performed "*in vitro*" is a test done outside of a living organism. These tests usually involve isolated tissues, organs, or cells. *In vitro* testing has risen for the past decades as there has been an increasing global commitment to the reduction of animal usage whenever possible, in accordance to the 3Rs principle (Refinement, Reduction, Replacement) (Bednarczuk et al., 2010). These kinds of tests also have the particularity of allowing human cells to be studied without extrapolation from an experimental animal's cellular response.

Methods for *in vitro* toxicity assessment

Numerous approaches can be made when assessing *in vitro* toxicity. The most common approach is tracking cell vitality, while others include genotoxicity, mutagenicity, immunogenicity, and oxidative stress assessment.

Cytotoxicity Assessment

Cytotoxicity is the capability of a chemical to be toxic to the cells. The selection of an assay for cytotoxicity analysis must take into consideration various factors: *in vitro* cell culture model, the chemical properties of the toxicant, culture platforms, the endpoint and the mechanism of cytotoxicity. The most frequently used endpoints in cellular toxicity testing are based on the breakdown of the cellular permeability barrier, changes in cell morphology and proliferation and mitochondrial function reduction (Eisenbrand et al., 2002). Based on these endpoints there are a battery of tests that can be performed to assess cytotoxicity of a compound.

Characterization and quantification of morphological indicators of toxicity in the cell, like the formation of vacuoles, apoptotic particles, and nuclear fragmentation can be obtained by the use of white light, fluorescence and electron microscopy methods (Poteser, 2017).

Cell proliferation can be measured by Sulforhodamine B assay. The SRB assay is based on binding of the dye to basic amino acids of cellular proteins, and colorimetric evaluation provides an estimate of total protein mass, which is related to cell number (Orellana & Kasinski, 2016).

Membrane integrity is usually measured by the capability of a dye to pass through the cellular membrane and bind to a specific target within the cell, e.g. intracellular proteins (Trypan Blue exclusion assay), lysosomes (Neutral Red uptake assay), DNA (Propidium Iodide uptake assay). Another way of measuring membrane integrity is by measuring the release of intracellular enzymes such as lactate dehydrogenase (LDH assay) (Boucher et al., 2014).

Mitochondrial activity can be measured by tetrazolium based colorimetric assays such as 3-(4,5- dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) assay, 3-(4,5-

dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium (MTS) assay, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1). The most used of these four assays is MTT assay , however all of these tests are based on the conversion of a tetrazolium salt into a coloured formazan by mitochondrial activity of living cells (Aslantürk, 2018; Bahuguna et al., 2017). AlamarBlue assay, also known as resazurin reduction assay, can also be used to measure mitochondrial activity. This fluorimetric assay is based on the conversion of the blue nonfluorescent dye resazurin, which is converted to the pink fluorescent resorufin by mitochondrial activity (Aslantürk, 2018 ; Springer et al., 1998).

Other cytotoxicity evaluation approaches include: ATP-based luminescent assay, which connects the levels of ATP within a sample to the number of viable cells present; Caspase-3/7 enzyme activity assays (apopain and CPP32 assays), fluorescence-based tests that measure the amount of these protease enzymes that play a crucial role in the apoptotic processes (Boucher et al., 2014); Cell cycle analysis, in which a fluorescent dye is used to stain DNA and analyse if and at what point cell cycle arrest occurs (Cobb, 2013).

Genotoxicity Assessment

Genotoxicity refers to the ability of a chemical to induce damage to the genetic material within a cell. Genotoxicity assays use the frequency and extent of DNA strand breaks to determine the amount of DNA damage. Within the various test methods approved by OECD, to measure the induction of mutations or the genotoxic potential of a chemical, we can highlight the comet assay and micronucleus assay as the most popular(Oecd, 2015).

Comet assay, also known as single-cell electrophoresis assay is based on supercoiled duplex DNA strand breakage. The name “Comet assay” was given due to the resulting image that is obtained, which resembles a "comet" with a distinct head and tail. The head of the comet represents the intact DNA, while the tail consists of damaged or broken pieces of DNA. In this assay individual cells are embedded in a thin agarose gel on a microscope slide. Cells then go through a lysing process. The DNA unwinds under alkaline/neutral conditions and undergoes electrophoresis which allows the broken DNA fragments or damaged DNA to migrate away from the nucleus. After staining with a DNA-specific fluorescent dye such as ethidium bromide or propidium iodide, the gel is read for amount of fluorescence in head and tail and length of tail. The extent of DNA liberated from the head of the comet is directly proportional to the amount of DNA damage (Jain et al., 2018).

Micronucleus is defined as the small nucleus that forms whenever a chromosome or its fragment is not incorporated into one of the daughter nuclei during cell division (Cole, 2019). The micronucleus assay allows to determine if a chemical induces DNA damage at a chromosome level. This kind of testing is of major importance to genotoxicity assessment since chromosomal mutation is a relevant event in carcinogenicity (Jain et al., 2018). In addition to the two referred assays, Chromosomal Aberration test (Registre et al., 2016),

Sister Chromatid exchange assay (Latt et al., 1980) and Gamma-H2AX assay (Ivashkevich et al., 2012) can also be used to determine the possible genotoxic potential of a chemical.

Mutagenicity assessment

A mutagenic toxicant is a chemical that induces transmissible changes on the genetic material of cells. One well known method for assessing mutagenicity is the Ames test. This is a very simple and fast method which uses genetically engineered strain of auxotrophic bacterial *Salmonella typhimurium.* This strain is mutant for the biosynthesis of histidine amino acid*,* and so incapable of synthesizing it. However, these bacterial cells cannot grow and form colonies in a medium lacking histidine. The bacteria is seeded on agar plates with growth medium lacking histidine and with the potential mutagen. Mutagenic chemicals cause a reversal of the mutation in bacterial cell, enabling the bacteria to grow and form colonies. The reverse-mutated colonies are then counted. The mutagenic potential of a compound is proportional to the number of cell forming colonies (Jain et al., 2018). Other mutagenicity test methods to be mentioned are: Forward Gene Mutation assay; HGPRT Gene Mutation assay; Mouse Lymphoma assay.

Immunogenicity Assessment

An immunogenic toxicant is a chemical that has the ability to induce unwanted immune responses within the cell. The most used tests in "*in vitro*" immunological evaluation are the enzyme-linked immunosorbent assay (ELISA) and the radio-allergosorbent test (RAST). ELISA is a plate-based technique that allows the detection and quantification of peptides, proteins, antibodies, and hormones by antigen binding. The antigen in fluid phase is immobilized. The antigen is then allowed to bind to a specific antibody, which is itself subsequently detected by a secondary, enzyme-coupled antibody. A chromogenic substrate for the enzyme produces a colour or fluorescence change which indicates the presence of antigen. Quantitative or qualitative measures can be assessed based on colorimetric reading (Boucher et al., 2014; Gan & Patel, 2013).

RAST test was the first immunoassay available for the measurement of IgE antibodies to specific allergens in human serum. In this type of test an allergen is bound to a solid phase, and then incubated with test serum. Allowing the allergen to react to the specific IgE in the sample. Finally, a radiolabelled anti-human IgE is added to detect bound IgE, if present. Binding of the anti-IgE antibody is measured with a special fluorometer (Makhija et al., 2012; Crameri, 2009; Wachholz et al., 2005).

Reactive oxygen species monitoring

Reactive oxygen species, namely Superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and singlet oxygen ($^1O_{2)}$, are normally generated as by-products of oxygen metabolism and play important roles as signalling molecules to regulate biological and physiological processes. Processes like protein phosphorylation transcriptional,

immunity and apoptosis are usually dependent on proper ROS production. However, ROS presence in a cell needs to be kept at low levels. Increases in ROS production provoked by environmental stressors (e.g. UV, pollutants, heavy metals,…) and xenobiotics causes imbalance, which in turn causes deleterious effects to cell structures including proteins, lipids and nucleic acids in a process called oxidative stress (Schieber & Chandel, 2014 ; Pizzino et al., 2017). Oxidative stress can be measured directly, with dyes or compounds that bind to reactive oxygen species (ROS) or indirectly by looking the expression levels of ROS scavenger proteins, like glutathione (GSH : GSSG ratio) and activities of glutathione peroxidase, superoxide dismutase, glutathione S-transferase and catalase, which comprise the cellular line of defence against rising oxidative stress (Poteser, 2017).

Omics Approach

Omics techniques are those that allow the detection of subtle changes in genes (genomics), transcriptome (transcriptomics), proteins (proteomics) and metabolites (metabolomics) even before cytotoxic are detectable. The use of these techniques in toxicology assessment allows a better understanding of the mechanism behind the toxicity of a chemical.

Genomics involves the study of structure, function, and expression of all genes in a cell, tissue or organism. To this effect DNA Microarrays are great tools as they can measure DNA sequence between individuals and can analyse the expression of thousands of genes simultaneously. They also reveal abnormalities in chromosomes in a process called comparative genomic hybridization (Horgan et al., 2011).

These studies provide valuable information on the structure of the genome.

Transcriptomics is the study of transcriptomes. Transcriptome is the complete set of RNA transcripts produced by the genome of a cell. Early transcriptomic approaches revolved around the use of gene expression microarrays that measured packaged mRNA. However, novel approach RNA sequencing allows the detection of all transcripts in a sample (Horgan et al., 2011 ; Blumenberg, 2019 ; Ouedraogo et al., 2012).

Proteomics is the study of all expressed proteins in a cell tissue or organism including proteins expression, structural status, functional states, and their interactions with other cellular components. Important proteomic methods include two-dimensional gel electrophoresis coupled to mass spectrometry, antibody microarrays and LC-MS-MS of protein fragments (Ouedraogo et al., 2012 ; Aslam et al., 2017).

Metabolomics refers to the systematic identification and quantification of the small molecule metabolic products (carbohydrates, lipids, amino acids and organic acids) of a cell, tissue or organism. Metabolomic methods include chromatography, such as High Performance Liquid Chromatography (HPLC), for separating metabolites and spectroscopy techniques such as nuclear magnetic resonance (NMR) and mass spectrometry (MS), for their identification (Horgan et al., 2011 ; Escudero et al., 2017).

In vitro techniques and biomarkers of interest

Cell viability assay – MTT

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay has been widely used and is considered as a gold standard for measuring cell viability and drug cytotoxicity. This assay determines cell viability through determination of mitochondrial function of cells by measuring activity of mitochondrial enzymes. In this assay cells are treated with MTT (yellow tetrazole) dye which is reduced to formazan (purple) by NADH, produced in the mitochondria of living cells (figure 7). The quantity of formazan, which is directly proportional to the number of viable cells, is measured by recording changes in absorbance in a wavelength between 500 and 600 nm using a plate reading spectrophotometer (Aslantürk, 2018 ; Salih Istifli et al., 2019).

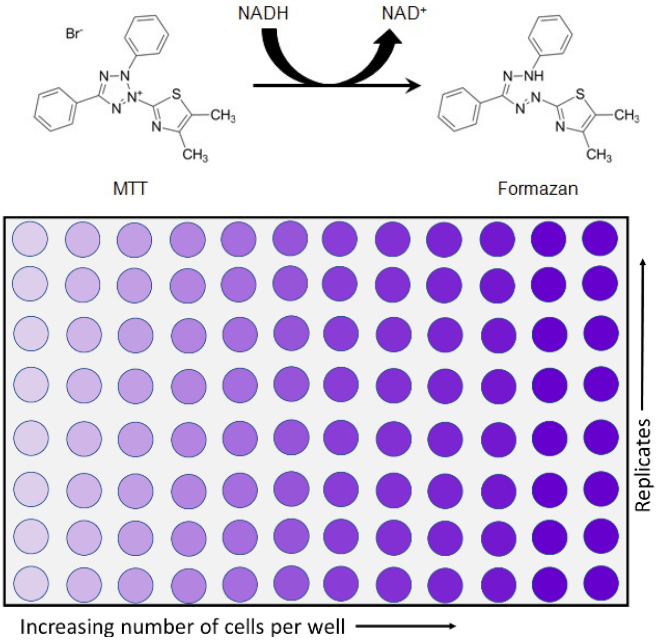

Figure 7 - Metabolism of MTT to a formazan salt by viable cells and in a 96-well plate. Adapted from Sigma Aldrich (https://www.sigmaaldrich.com accessed in 30/12/2021)

Cell cycle Analysis

The cell cycle is the process an eukaryotic cell will go through to replicate its material and divide itself from one cell into two identical cells. The cell cycle consists of two phases: Interphase, in which the cell grows and makes a copy of its DNA and the mitotic or M phase, in which the cell separates its DNA into two sets and divides its cytoplasm, forming two new cells.

The interphase can be divided in three sub-phases: G1, where cells increase in size and the cellular content gets duplicated (RNA and protein synthesis), S, in which DNA gets replicated and chromosomes duplicate and G2 where cells prepare for cell division (repair of any DNA damage reorganization of the DNA structure). There are also checkpoints during this phase of the cell cycle. These checkpoints, at the end of G1 and G2 exist to detect potential DNA damage and allow repair it before cell division occurs. If there is DNA damage and cannot be repaired, a cell becomes targeted for apoptosis. Cells can also reversibly stop dividing and temporarily enter a quiescent or senescent state G0 (see figure 8 for better understanding of cell cycle checkpoints) (Rabinovitch, 1994).
The mitotic phase involves two distinct processes: mitosis, during which parent cells chromosomes are divided between two sister cells, and cytokinesis, in which occurs the division of cytoplasm, leading to the formation of two distinct cells. During mitosis there is a checkpoint, between mitotic phases anaphase and telophase, where Spindle assembly is checked.

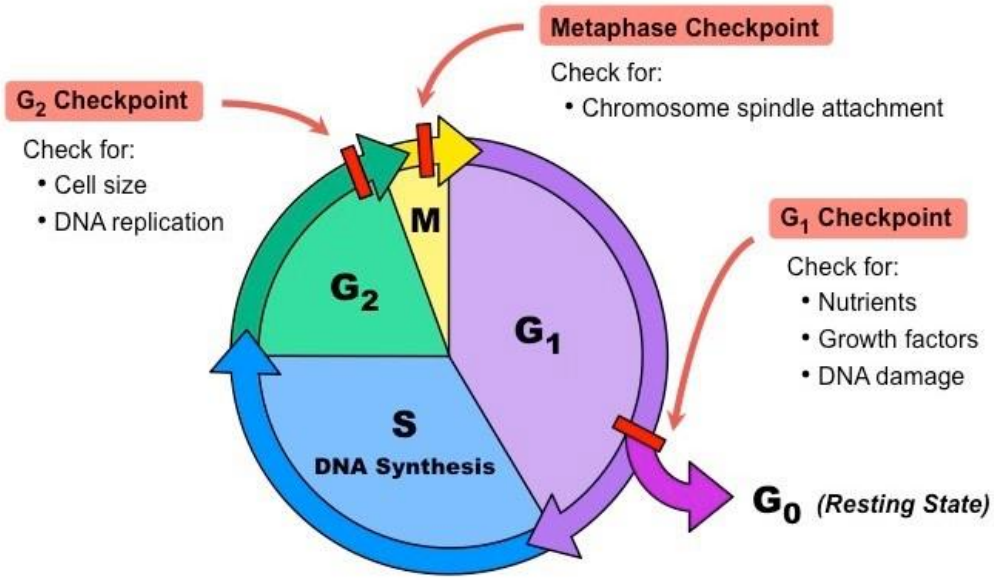

Figure 8 - Cell cycle checkpoints. From: https://ib.bioninja.com.au/standard-level/topic-1-cell-biology/16-cell-division/cell-checkpoints.html (accessed in 4/18/2020).

Flow cytometry allows DNA content measurement in single cells. This method consists in staining cells with a fluorescent dye that binds to DNA. After staining, samples will pass through a light beam. Each particle in suspension, when passing through the beam, will disperse the light in a certain way, and the fluorescent dye found in or attached to the particle can be excited by emitting longer wavelength than that of the light source. The amount of fluorescent signal is directly proportional to the amount of DNA. Because of the alterations that occur during the cell cycle, analysis of DNA content allows discrimination

between G1, S, G2 and M phases (Cobb, 2013) (see figure 9). Before staining, cells must be treated with RNase to ensure that only DNA is measured.

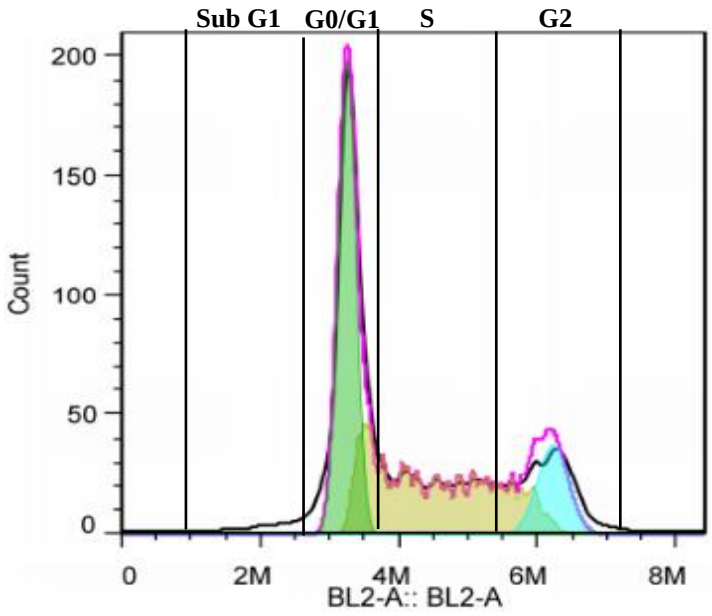

Figure 9 – Example of a cell cycle profile obtained by flow cytometry.

Fatty Acid

Fatty acids (FAs) are essential components of lipids. They have important biological functions, structural and functional roles, and they represent a major source of energy. FA metabolism produces a huge quantity of ATP. The β-oxidation of FAs is a well-known process, mostly used by the heart and the muscular tissue to obtain energy (Nagy & Tiuca, 2017).

Fatty acids can be divided into two categories: Saturated Fatty Acids (SFA) and unsaturated Fatty acids. The difference in SFAs and unsaturated FAs resides in the fact the unsaturated FAs contain at least one double bond in their molecular structure while SFA are straight hydrocarbon chains (Filimonova et al., 2016 ; Rustan & Drevon, 2005).

Unsaturated FAs are divided into monounsaturated FAs (MUFA) that have a single double bond and polyunsaturated FAs (PUFA) with two or more double bonds. Within the PUFAs, there are highly unsaturated FAs (HUFA), with three or more double bonds, in which we can find essential FAs (EFA), important fatty acids that the human body cannot synthesize *de novo*. These EFAs are linoleic acid (LA, 18:2n-6) and alpha-linoleic acid (ALA, 18:3n-3) and they serve as precursors for omega-6 and omega-3 fatty acids, respectively (see figure 10).

Omega-6 fatty acids such as dihomo-γ-linolenic acid (DGLA; 20:3n-6) and arachidonic acid (AA; 20:4n-6), and omega-3 fatty acids, such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) play important roles in the biological activities of the

human body. Both omega-6 and omega-3 are important structural components of cell membranes that when incorporated into phospholipids, affect cell membrane properties, such as fluidity, flexibility, permeability, and the activity of membrane-bound enzymes and cell-signalling pathways (Jump et al., 2013 ; Stillwell & Wassall, 2003). Omega-6 and omega-3 fatty acids have been shown to play important roles in vision (SanGiovanni & Chew, 2005), central nervous system function (Innis, 2008), neuronal growth and synapse formation (Das, 2013) and regulation of gene expression (Sampath & Ntambi, 2004). AA, EPA and DHA can also produce Eicosanoids under the activity of cyclooxygenases, lipoxygenases and cytochromes P450. These metabolites play a critical role in inflammatory processes (Greene et al., 2011 ; Nieves & Moreno, 2006).

Fatty acids can be identified and quantified using various analytical methods, but the most widely used techniques are gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS), due to their selectivity, sensitivity and efficiency, with CS-MS being more sensitive than GC alone. Other analytical techniques used for the detection of fatty acids are: high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) (Nagy & Tiuca, 2017).

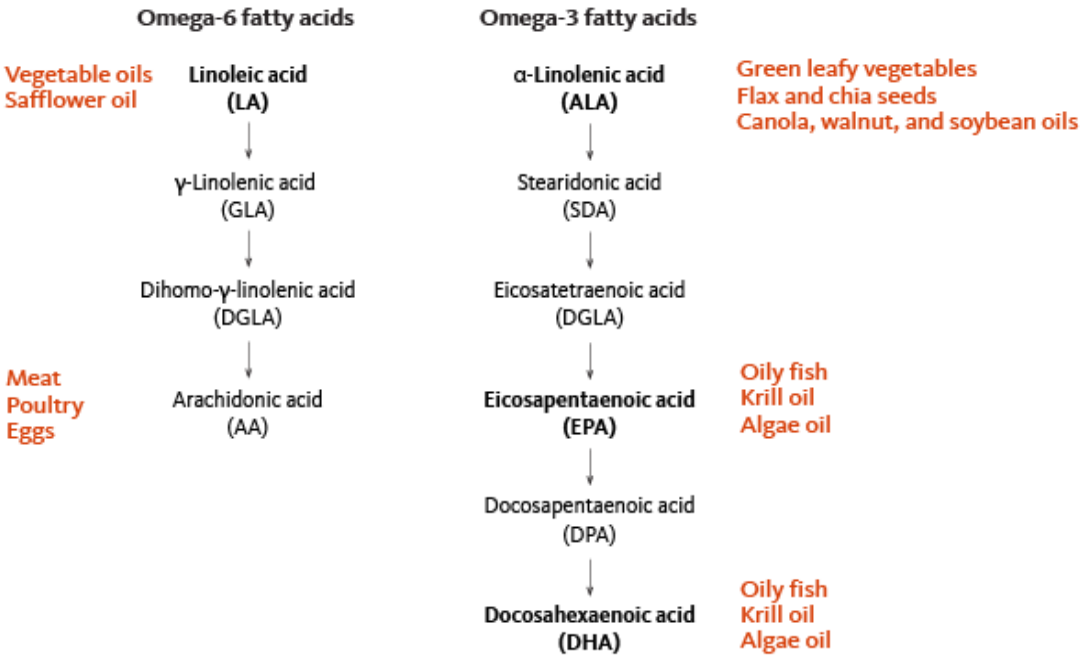

Figure 10: Classes of Essential fatty acids and their derivatives. Due to low efficiency of conversion of EFAs to their long chained PUFAs, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (AA) it is recommended to obtain them from additional dietary sources. Dietary sources are listed in red. From Oregon State University site: https://lpi.oregonstate.edu/mic/other-nutrients/essential-fatty-acids?fbclid=IwAR0-L-wLBZW5T-CS810nbV_eyLdjonH-AIKFUFzse3MjkQExsUBrhz8K_Aw#metabolism-bioavailability (accessed in 20/08/2020).

Carbohydrates

Carbohydrates are the main and immediate energy sources to cope with stress. During processes of immune defence carbohydrate metabolism may play an important role in the maintenance of cell homeostasis. Theoretically, the presence of organic pollutants may impose high energetic costs for immune regulation resulting in an enhance of carbohydrate metabolism including glycogenolysis, gluconeogenesis and glucose degradation(Lochmiller & Deerenberg, 2000). Hence, the quantification of the total carbohydrates content may offer precious information to determine the potential biochemical impacts of contaminants.

Polysaccharides are the most widespread carbohydrate polymers in nature. They are structures of monosaccharides linked together by glycosidic bonds that serve as source and storage of energy and give mechanical stability to the cell by forming the supporting tissue of plants and some animals (chitin in crustaceans and insects) (Van Dam et al., 2017). There are two classes of polysaccharides: homopolysaccharides, which consist of only one kind of monosaccharide, and heteropolysaccharides, consisting of two or more kinds of monosaccharide units (Robyt, 1998).

Cellulose and starch are the most widespread polysaccharides in nature. They are both homopolysaccharides and perform crucial functions on energy storage and cell wall structure of plants. In animals, glycogen is the homopolysaccharide responsible for energy storage. This polysaccharide is mainly found in the liver and muscles and it can be produced during a process called glycogenesis. Cellulose, starch and glycogen are the most important polysaccharides and are composed of glucose.

Complex polysaccharides may be composed of sugars other than glucose, such as galactose, fructose, mannose, xylose, and are often composed of branched chains, or contain substituents (esters) and functional chemical groups (e.g., carboxyl groups in pectin or amine groups in chitin) (Van Dam et al., 2017).

Even though cellulose, starch and glycogen are the most important polysaccharides, there are various other homopolysaccharides and heteropolysaccharides that are involved in vital functions of living organisms.

In the human body the following heteropolysaccharides can be found with important functions:

- Hyaluronic acid: provides lubrification in the synovial fluid of joints (Tamer, 2013);
- Chondroitin Sulfate: contributes to tensile strength and elasticity of cartilages, tendons, ligaments, and walls of the aorta (Scott, 2003);
- Dermatan Sulfate: found mainly in the skin, and also in vessels, heart and lungs. It may be related to coagulation and vascular diseases and other conditions (Trownbridge & Gallo, 2002);
- Keratan Sulfate: present in the cornea, cartilage bone and other structures such as nails and hair (Caterson & Melrose, 2018);
- Heparin: present in the blood, as an anticoagulant (Onishi et al., 2016);

The fact that polysaccharides are hydrophilic and sensitive to aqueous acid allows the determination of their sugar concentrations by measuring the monosaccharides liberated after acid hydrolysis (Van Dam et al., 2017 ; Borch & Kirchman, 1997).
Like fatty acids, carbohydrates can also be identified and quantified by GC, GC-MS, HPLC and capillary electrophoresis (Bradbury, 1990 ; Goubet et al., 2011).

Cell lines of interest

HaCaT

One of the most common ways of pesticide poisoning is through skin contact. Therefore, skin cell lines constitute appropriate *in vitro* models to study toxic effects of these chemicals. The innermost layer of the epidermis mainly consists of keratinocyte cells. Keratinocytes secrete keratin proteins which contribute to the skin's functional and mechanical properties (Karthik et al., 2019). Damage to Keratinocytes may compromise the integrity of the skin. HaCaT cell line is a nontumorigenic immortalized human keratinocyte cell line that can be keep in continuous proliferation for over 140 passages without affecting its capacity for normal differentiation, thus offering a suitable model to study regulatory mechanisms in the process of differentiation of human epidermal cells. (Boukamp, 1988).
HaCaT cells have been used in immunological and inflammatory response studies (Colombo et al., 2017), in studies involving vitamin D3 metabolism (Lehmann, 1997), gene expression studies (Seo et al., 2012), cytotoxicity studies (Hoh & Maier, 1993), skin sensitization tests (Chung et al., 2018), among others.

HepG2

The liver is the major responsible organ for the biotransformation of endogenous and exogenous xenobiotics, thus becoming a major target of these compounds. Therefore, there is a need of using cell lines that mimic the function of normal human liver cells in the assessment of xenobiotic toxicity.
HepG2 is a Human hepatoma cell line established from liver tumour biopsies obtained during extended lobectomy of a 15-year-old Caucasian male in Argentina in 1975. This cell line was isolated along with Hep3B by Aden et al., (1979). Because these cells express the majority of biotransformation/drug-metabolizing enzymes and secrete major plasma proteins found in the liver, like albumin, α2-macroglobulin, α1-antitrypsin, transferrin, and plasminogen, they are extensively used as cellular reference models in pharmaceutical and toxicological studies that aim to gain insight into drug metabolism (Bakre & Kaliwal, 2017 ; Knasmüller et al., 1998 ; Qiu et al., 2014).

In toxicology this cell line has been used to assess cytotoxicity and genotoxicity of xenobiotics (Juan-García et al., 2019), study liver disease mechanics (García-Ruiz et al., 2015), allow drug toxicity mechanisms understanding (Duthie et al., 1995 ; Usui et al., 2003) and give insights of transcriptional l regulation (Convertini et al., 2019) and gene expression (Costantini et al., 2013).
A study made by Nikoloff et al., (2013) used this cell line to compare cytotoxic and genotoxic effects induced by our target active ingredient S-metolachlor and the effects of one of its commercial formulations Twin Pack Gold. In this study S-metolachlor did not show reduction of mitochondrial or lysosomal activity nor it produced genotoxic effects.

1.7 Aims of study

The main aim of this thesis is to assess the hazardous effects of Primextra® Gold TZ and its main active ingredient S-metolachlor in liver and skin, by using cell lines that mimic these organs (HepG2 and HaCaT, respectively).
Therefore, this thesis can be divided in the following specific objectives:

- To evaluate the effects of the exposure to the herbicides on cell viability by assessing metabolic activity;
- To determine the effects of the herbicides on the cell cycle arrest induction by the herbicides;
- To characterize the fatty acid and carbohydrate profiles of both cell lines after exposure to the commercial compound and the a.i.;
- To assess potential biochemical changes in fatty acid and carbohydrate composition induced by the herbicides.

2. Materials and methods

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Pen/strep, fungizone, L-glutamine, sodium pyruvate, phosphate buffer saline (PBS, pH 7.4) and trypsin-ethylenedieminetetraacetic acid (trypsin-EDTA) were purchased from Life Technologies (Carlsbad, CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO), RNase and propidium iodide (PI) were obtained from Sigma-Aldrich (St. Louis, MO, USA).
HaCaT cell line was obtained from Cell Lines Services (Eppelheim, Germany) and HepG2 cell line was obtained from European Collection of Authenticated Cell Cultures and were also supplied by Sigma-Aldrich.

2.1 Cell culture maintenance

Both cell lines were grown in high glucose DMEM medium supplemented with FBS (10%), Fungizone (1 %), Pen/strep (1 %), Glutamine (1%) and Sodium Pyruvate 1 % in 75 cm2-flasks, at 37 ºC in 5% CO2 humidified atmosphere.
Subculture was performed when cells reached 80% confluence. For HepG2 cell line after removal of the culture medium, cells were washed with PBS (pH 7.4) and incubated with trypsin/EDTA at 37 ºC in 5% CO2 humidified atmosphere, for 5 min. Trypsin was then neutralized by adding complete DMEM in a volume two times higher the volume of trypsin/EDTA. Cells were counted and re-seeded in a ratio of 1:8. Cells were subcultured every 3 to 4 days. For HaCaT cells, similar procedure was followed, except that before adding the trypsin, cells were incubated with PBS/EDTA, at 37 ºC in 5% CO2 humidified atmosphere, for 10 min.

2.2 Cell viability assay - MTT

MTT assay protocol
Cell viability was determined by the colorimetric 3-(4,5- dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, HepG2 Cells were seeded at a density of $1.8x10^5$ cells/ml, in 96-well plates, for 24 hours assays and at a density of $1 x 10^5$ cells/ml for the 48 hours assays. HaCaT cells were seeded at a density of $6x10^4$ cells/ml, in 96-well plates, for 24 hours assay and at a density of $5 x 10^4$ cells/ml for 48 hours assay.
After 24 hours cells had completely adhered to the plate and the medium was replaced with control and treatments of Primextra® Gold TZ or S-metolachlor at a concentration range of 0.5 to 80 µL/ml (0.5, 0.1, 2.5, 5, 10, 20, 40 and 80). In control cells medium was replaced by fresh medium. After 24 or 48 h exposure, 50 µl of MTT were added and the plate was incubated at 37 ºC in 5% CO2 humidified atmosphere for an additional 4 h period.

Then, 150 µl of DMSO were added and the plate was shaken for 2 hours. After that, the absorbance at 570 nm was measured with a microplate reader (Synergy HT Multi-Mode, BioTeK, Winooski, VT). Results of MTT assay are expressed as the mean percentage of cell viability from three independent experiments performed in parallel with 5 replicates each.

Metabolic activity was calculated using the following formula:

$$\text{Metabolic activity \%} = \frac{\text{A570 of treatment}}{\text{A570 of control}} \times 100$$

Statistical Analysis

A bar chart was made using the results reported as mean ± standard deviation of the 5 replicates in each of the 3 independent experiments and the statistical significance between control and treatments was performed by one-way ANOVA, followed by Dunnett's test using Sigma Plot 12.5 software (Systat Software Inc.). Differences were considered significant when the p value was lower than 0.05. IC_{50} and IC_{30} concentrations were calculated based on the cell viability results for further experiments.

2.3 Cell cycle Assay

Cell cycle Protocol

Cell cycle arrest was assessed by flow cytometry. Briefly, Cells were plated at a density of $5x10^5$ cells/well in HepG2 and $2x10^5$ cells/well in HaCaT in 6-well plates with 3 replicates per concentration plus control and incubated 24 hours at 37ºC, 5 % CO2. Cells were exposed to Primextra® Gold TZ or S-metolachlor at IC_{30} and IC_{50} concentrations and then incubated at 37ºC, with 5 % CO_2 for 48h. Trypsinization was performed as described above. After that, cells were collected to eppendorfs and centrifuged at 700 *g* for 5 minutes. The supernatant was then discarded, and cells were fixed with 1 ml of cold ethanol at 85% and stored at -20ºC until analysis. At the time of analysis fixed cells were centrifuged at 3000 rpm for 6 min at 4ºC. The supernatant was then discarded, and the cells resuspended in 800 µl of PBS. After that 50 µl of RNase (1 mg/ml) and 50ul of PI (1 mg/mL) were added. Samples were incubated for 15 min in the dark at room temperature and then run through and Relative fluorescence intensity was measured in an Attune® Acoustic Focusing Cytometer (TermoFisher Scientific) equipped with an argon laser – 15 mW, 488 nm to measure its relative fluorescence intensity. For each sample, the number of events reached approximately 5000. Debris and doublets were excluded by the definition of the specific region (Area vs. FL peak height). For each sample approximately 5000 nuclei were analyzed. Cell cycle analysis was then conducted based on the histogram outputs.

Statistical Analysis

A bar chart was made using the results reported as mean ± standard deviation of the 3 replicates in each of the 3 independent experiments. The statistical significance between control and treatments was performed by one-way ANOVA, followed by Dunnett's test using Sigma Plot 12.5 software (Systat Software Inc.). Differences were considered significant when the p value was lower than 0.05.

2.4 Fatty acid and carbohydrate profile analysis

Preparation for lipid extraction

For this assay, cells were grown in 150 cm^2 Petri dishes for 2 to 3 days. When cells reached 80 % confluence, they were sub cultured at a ratio of 1:8 and incubated at 37ºC, with 5 % CO_2. After 24 hours the medium was removed and cells were exposed to IC_{30} concentrations of each compound, as well as fresh medium for control and further incubated again for 48 hours. Then, the medium was removed, and the plate was washed with PBS. Trypsinization was performed as mentioned above. After adding the medium, cells were collected to 50 ml eppendorfs and centrifuged at 300 g for 5 minutes at 4ºC. The supernatant was then discarded, and Neutral saline solution (NS) was added for another centrifugation at the exact same conditions previously mentioned. These last steps were repeated 3-4 times and after removing the supernatant for the last time, NS solution was added, and samples were stored at -80ºC.

Fatty acid extraction protocol

The total lipids of the samples were extracted and methylation to FA methyl esters (FAMEs) was conducted following the methodology described by Gonçalves et al., (2012). Methyl nonadecanoate (C19:0, Fluka 74208) was added to the samples as an internal standard for later fatty acid methyl esters (FAMEs) quantification.

Separation and quantification of FAMEs were performed through gas chromatography coupled with mass spectrometry (GC-MS), using an Agilent Technologies 6890 N Network (Santa Clara, CA) with a DB-FFAP column (30 m x 0.25 mm x 0.1 µm). The injector port was lined with a splitless glass liner of 4.0 mm i.d. . An Agilent 5973 Network Mass Selective Detector at 70 eV electron impact mode and scanning the m/z range of 40–500 in 1 s cycle in full scan mode acquisition was used. The initial oven temperature was 80 °C, following a linear temperature increase of 25 °C min^{-1} to 160 °C, followed by another temperature ramp of 2 °C min^{-1} to 190 °C and finally an increase of 40 °C min^{-1} until a final temperature of 230 °C which was maintained for 5 min. Helium was the carrier gas, at a flow rate of 4.4 mL min^{-1} and 2.66 psi of column head pressure. The detector starts operating 4 min after injection, corresponding to solvent delay. The injector and transfer line were maintained at 220 °C and 280 °C, respectively. The equipment's software output a chromatogram and allowed the integration of the peaks. Identification of each peak as a FAME was done by

retention time and mass spectrum of each FAME, in comparison with the Supelco® 37 component FAME mix (Sigma-Aldrich, Steinheim, Germany) and mass spectra from the library (Willey). The calculation of the FAMEs content was performed as described in Gonçalves et al. (2012).

Carbohydrate analysis

Carbohydrate analysis comprised the quantification of polysaccharides (analysed in the form of monosaccharides). For sugar extraction the lower layer after centrifugation in the above-mentioned protocol for fatty acid extraction was used. Samples were subjected to hydrolysis followed by reduction and acetylation, as described in Nunes et al., (2012). Samples were run through a Perkin-Elmer – Clarus 400 gas chromatography equipment equipped with a flame ionization detector (GC-FID). A DB-225 (30 m x 0.25 mm x 0.15 µm) GC column was used, and oven was programmed to an initial temperature of 200 °C, following a linear temperature increase at 40 °C min^{-1} to 220 °C, this temperature was maintained for 7 min, after which followed another linear increase of 20 °C min^{-1} until the final temperature of 230 °C, maintaining this temperature for 1 min. The carrier gas was hydrogen at a flow rate of 1.7 mL/min. The monosaccharides quantification was performed by comparison of the peaks from chromatogram to the peaks obtained for the internal standard used, 2-deoxyglucose.

Statistical analysis

Both Fatty acid and carbohydrate data were converted to proportional relative abundances. Bar charts were made using the results reported as mean ± standard deviation for both FA and sugar analysis. The statistical significance between control and treatments was performed by one-way ANOVA, followed by Dunnett's test using Sigma Plot 12.5 software (Systat Software Inc.). Differences were considered significant when the p value was lower than 0.05.

Multivariate statistical analyses were carried out using PRIMER 7 software to study the variation of FA profiles via non-metric multidimensional scaling (n-MDS) plots, using the data converted into similarity triangular matrices through Bray Curtis resemblance measures. A one-way analysis of similarity (ANOSIM) was performed for each chemical to assess the grade of differences of FA contents between samples. To analyse the contribution of individual FAs to similarities and dissimilarities within and between sample groups, a similarity percentage (SIMPER) analysis routine was performed for each compound. Ultimately, principal component analysis (PCA), was used to reduce the profiles to interpretable bidimensional plots that explain the highest proportion of variation in the data.

3. Results

3.1 Cell viability

MTT assay was used to evaluate cell viability upon exposure to Primextra® Gold TZ and S-metolachlor. MTT assay results are expressed in percentage of metabolic activity which is used as a measure of cell viability and proliferation.

Cell viability (figure 11) of the two cell lines when exposed to Primextra® Gold TZ was significantly decreased ($p \leq 0.001$) for both 24- and 48-hour assays for all concentrations tested.

As for cell viability upon exposure to S-metolachlor, HepG2 presented significant reduction for all concentrations tested for 24h exposure and for concentrations from 20 µg/ml to 80 µg/ml for 48-hour exposure. Regarding the cell viability of HaCaT cells, exposure to S-metolachlor induced a significant reduction for all concentrations tested.

Calculated values of Primextra® Gold TZ on HepG2 cells were of 136.26 µg/ml and 22.38 µg/ml for 24- and 48-hours assays, respectively. While for HaCaT cell line these values were of 44.26 µg/ml and 12.37µg/ml. In S-metolachlor this tendency appeared once more, as the LC_{50} values for this compound in HepG2 were of 739.30 µg/ml and 83.90 µg/ml and in HaCaT were of 23.74 µg/ml and 12.28 µg/ml for 24 and 48 hours, respectively (see table 7 for LC values summary).

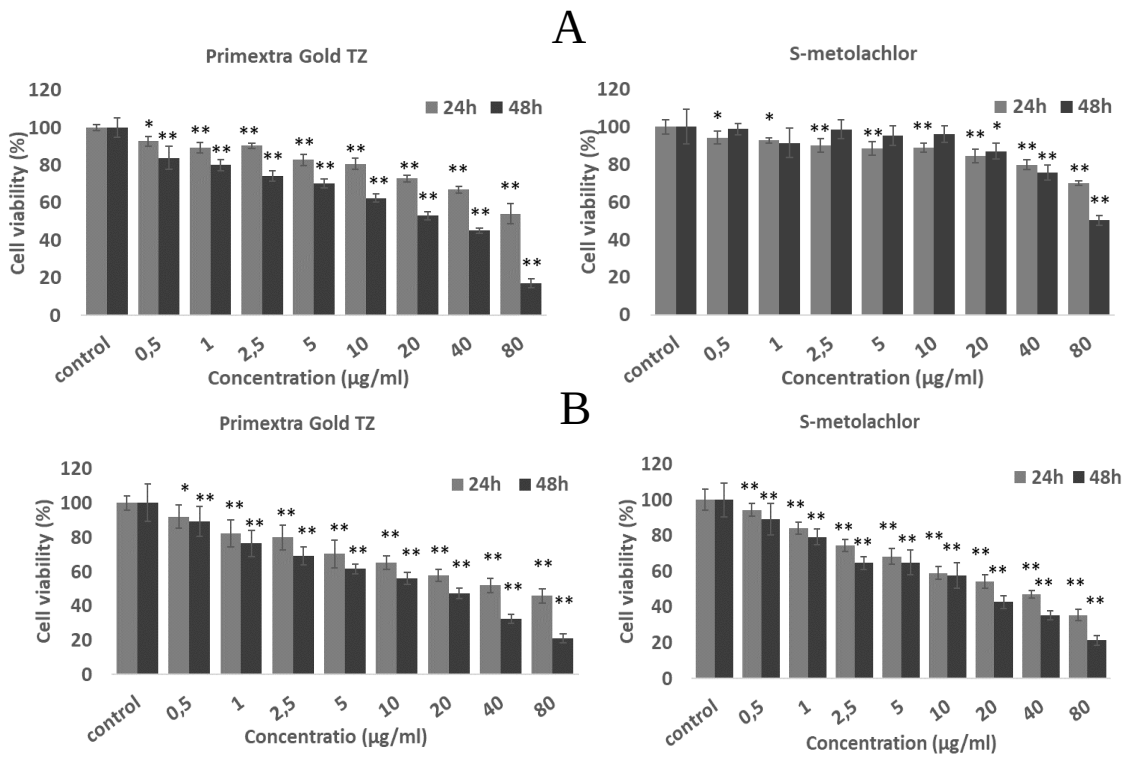

Figure 11 - Effects of different concentrations of the commercial formulation Primextra® Gold TZ and its active ingredient, S-metolachlor in cell viability of Hep G2 (A) and HaCaT (B) cell lines after 24/48h exposure. Results are expressed in mean and standard deviation. Concentrations showed significant differences compared to control group: *, P≤0.05 and **, P≤0.001.

Table.7 IC values obtained from cell viability assay.

	HepG2				**HaCaT**			
	Primextra® Gold TZ		S-metolachlor		Primextra® Gold TZ		S-metolachlor	
	24h	48h	24h	48h	24h	48h	24h	48h
IC_{50} (µg/ml)	136.26	22.38	739.30	83.90	44.26	12.37	23.74	12.28
IC_{30} (µg/ml)	28.7	6.20	99.25	49.89	5.91	2.86	4.53	2.83

3.2 Cell cycle Analysis

Figure 12 shows the effect of Primextra® Gold TZ and S-metolachlor on the cell cycle of both HepG2 and HaCaT cell lines after exposure to IC_{30} and IC_{50}.

In HepG2 cell line, it was shown that the exposure to Primextra® Gold TZ induced a decrease in the percentage of cells in sub-G1 phase in both treatments and an increase in the percentage of cells in G2 only in the LC_{50} treatment.

As for S-metolachlor, in addition to a decrease in the number of cells in sub-G1 on both treatments and an increase in cells at G2, there was a significant increase in the number of cells in G0/G1 phase. HaCaT cell line did not show any significant alterations in its cell cycle when exposed to each compound.

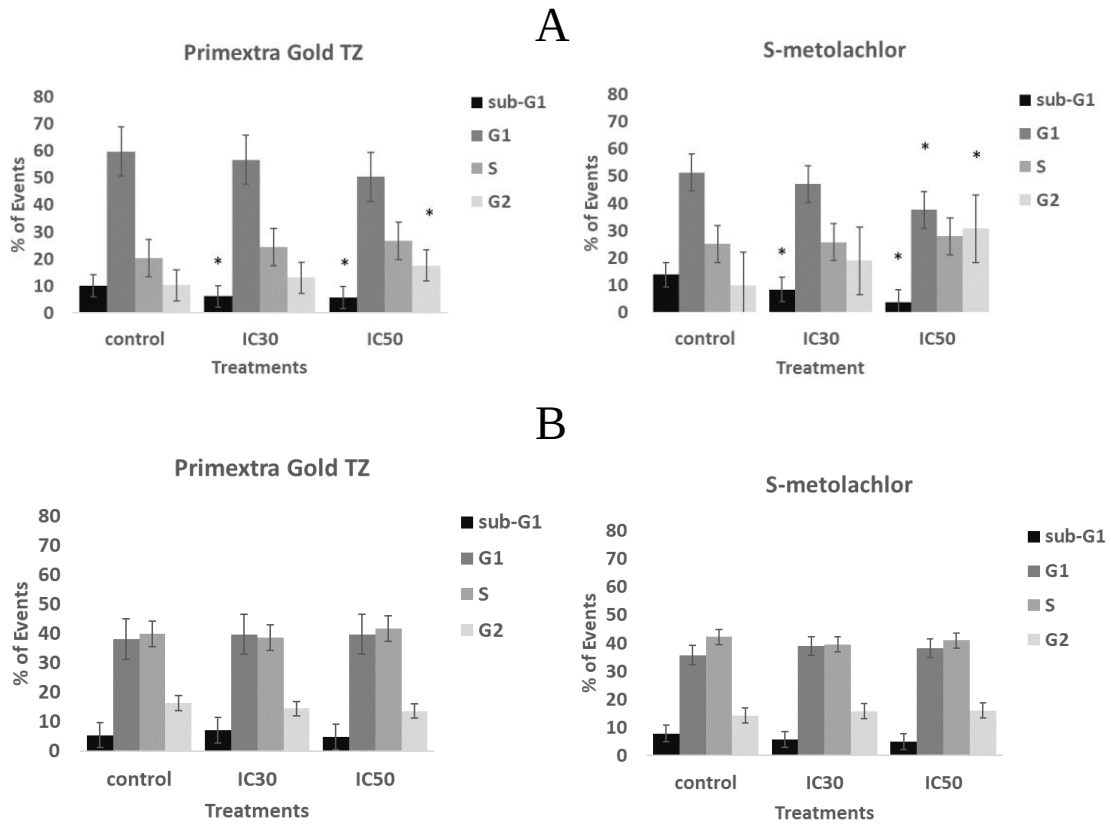

Figure 12 - Effects of the commercial formulation Primextra® Gold TZ and its active ingredient S-metolachlor on cell cycle dynamics of HepG2 (A) and HaCaT (B) cell lines, measured by flow cytometry, exposed to IC_{30} and IC_{50} concentrations. Results are expressed in mean and standard deviation. Concentrations showed significant differences compared to control group: *, P≤0.05 and **, P≤0.001.

Exposure of HaCaT to Primextra® Gold TZ and to S-metolachlor did not induce any significant changes to the coefficient of variation (CV) correspondent to cells in G0/G1 peak.

In HepG2 cell lines, only exposure to S-metolachlor showed significant differences (P≤0.05) (see table 8 for cv values).
Examples of cell cycle histograms can be seen in figure 13.

Table 8 - CV values of cells exposed to Primextra® Gold TZ and S-metolachlor. IC_{50} concentration of S-metolachlor showed significant differences compared to control (*P≤0.05).

	Primextra® Gold TZ			**S-metolachlor**		
	control	**IC_{30}**	**IC_{50}**	**Control**	**IC_{30}**	**IC_{50}**
HepG2	6.53 ± 1.12	6.81 ±1.04	6.41±0.83	6.56 ± 0.90	7.48±0.89	8.19±1.24*
HaCaT	5.71 ± 0.11	5.72±0.11	5.61±0.04	5.58±0.15	5.69±0.04	5.71±0.05

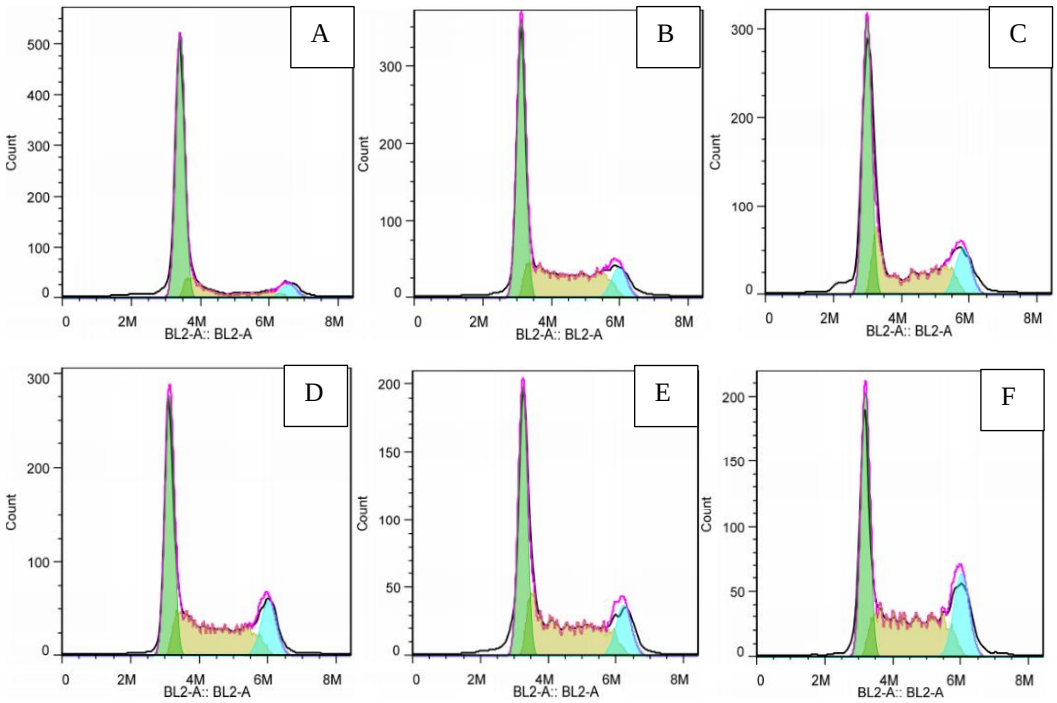

Figure 13 - Examples of cell cycle histograms obtained when exposing HepG2 and HaCaT to IC_{50} concentrations of Primextra® Gold TZ and S-metolachlor for 48 hours. A-HepG2 control; B-HepG2 when exposed to Primextra® Gold TZ; C-HepG2 after exposure to S-Metolachlor; D-HaCaT control; E-HaCaT when exposed to Primextra® Gold TZ; F-HaCaT when exposed to S-Metolachlor.

3.3 Fatty Acids

Fatty acid composition

In order to see if the compounds could affect the fatty acid (FA) composition of both cell lines, an analysis to assess FA profile of the total lipid extract from each cell line was performed by using GC-MS methodology (see table 9).

Table 9. Fatty acid profile of HepG2 and HaCaT cell lines when exposed to Primextra® Gold TZ and S-metolachlor IC30 concentrations. Results are expressed in relative percentage (%). SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

	Hep G2				**HaCaT**			
FA	**Control**	**S-metolachlor**	**Control**	**Primextra® Gold TZ**	**Control**	**S-metolachlor**	**Control**	**Primextra® Gold TZ**
C13:0	5.31	4.88	3.41	6.73	-	-	0.07	1.04
C16:0	33.35	30.65	30.43	30.52	39.14	30.23	33.90	36.92
C18:0	8.34	9.70	9.48	9.27	25.30	18.33	39.25	20.88
∑ SFA	47.00	45.22	43.32	46.52	64.44	48.56	73.21	58.84
C16:1n9	15.38	12.07	20.23	19.55	-	3.89	0.28	1.59
C18:1n9	21.30	26.50	21.28	19.65	31.8	40.21	26.09	32.35
C18:1n8	-	-	-	0.21	-	3.64	0.42	7.22
∑ MUFA	36.68	38.57	41.51	39.42	31.87	47.74	26.79	41.16
C18:2n6	16.31	16.21	15.17	14.06	3.69	3.70	-	-
∑ PUFA	16.31	16.21	15.17	14.06	3.69	3.70	-	-
N	6	6	6	7	4	6	6	6

ANOVA statistical analisis showed that there were no significant changes in overall fatty acid composition in HepG2 when exposed to each of the compounds.

In HaCaT, however, there is significant increase in Monounsaturated fatty acids (MUFA) ($P \leq 0.05$) and an equivalent decrease in Saturated fatty acid (SFA) when exposed to both compounds. In the increase of MUFA it is important to highlight the contribution of C18:1n9 and C18:1n8 whose values increased significantly in exposure to both compounds. And in the case of the SFA decrease the major responsible is the decrease in C16:0 and C18:0 content (see figure 14).

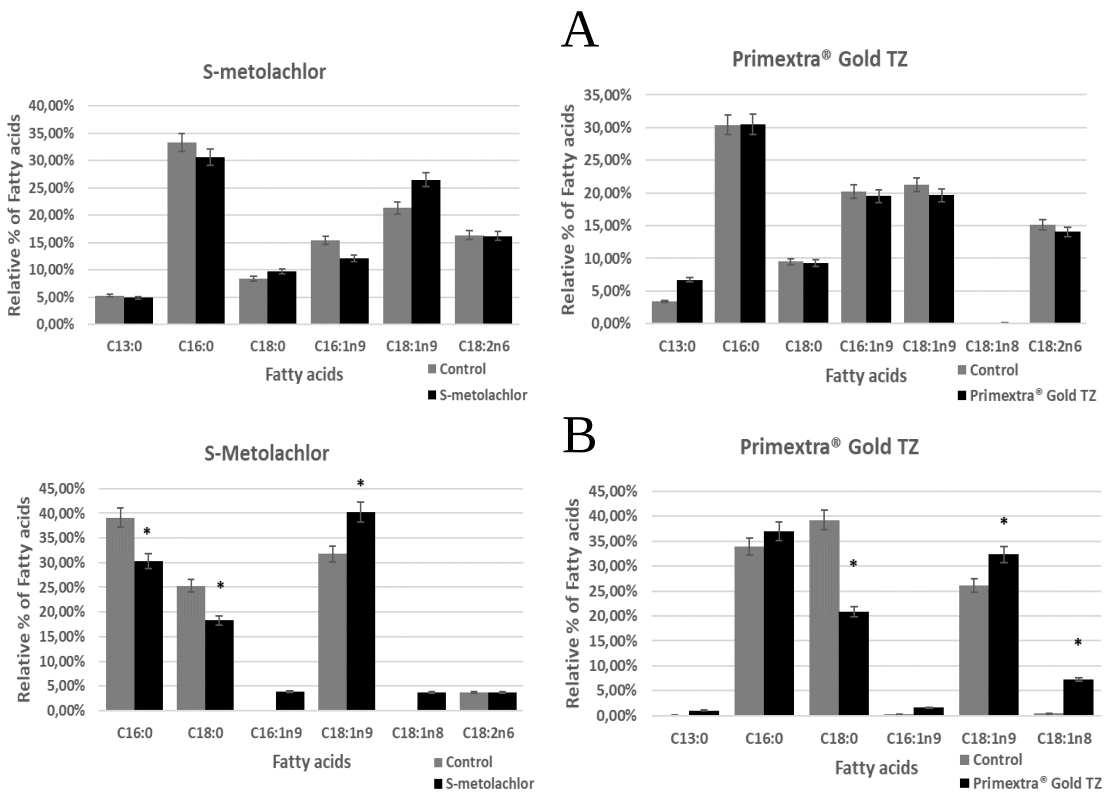

Figure 14 – Fatty acid profile of HepG2 (A) and HaCaT (B) cell lines when exposed to Primextra® Gold TZ and S-metolachlor. Results are expressed in relative percentage (%) of Fatty acids. Concentrations showed significant differences compared to control group: *, P≤0.05 and **, P≤0.001

Differences between cell lines were also detectable, as polyunsatured fatty acids (PUFA) have a bigger role in fatty acid composition of HepG2, with a percentage of 16%, than in HaCaT, with a maximum weight of 4% in its overall composition. Percentage by group of fatty acid can be observed in figures 15 and 16.

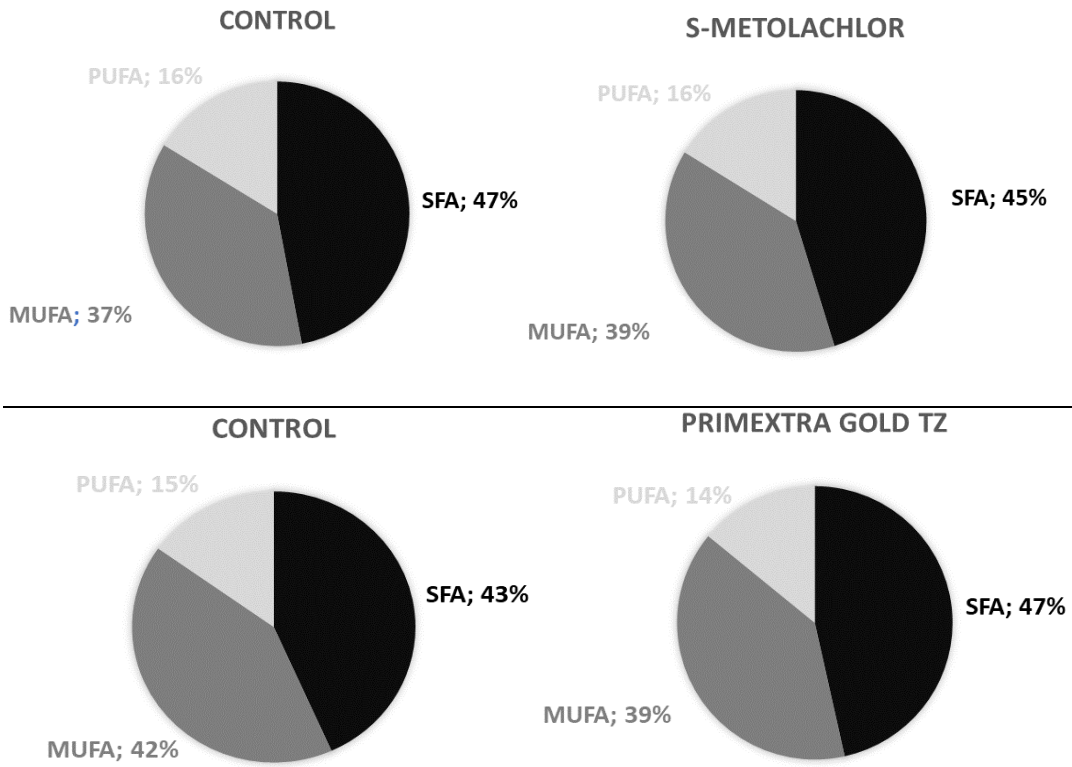

Figure 15 - Relative percentages of fatty acids by group of Fatty acid in HepG2 Cells when exposed to IC_{30} concentrations of S-metolachlor and Primextra® Gold TZ for 48h. PUFA: polyunsaturated fatty acids; MUFA: Monounsaturated fatty acids; SFA: Saturated fatty acids.

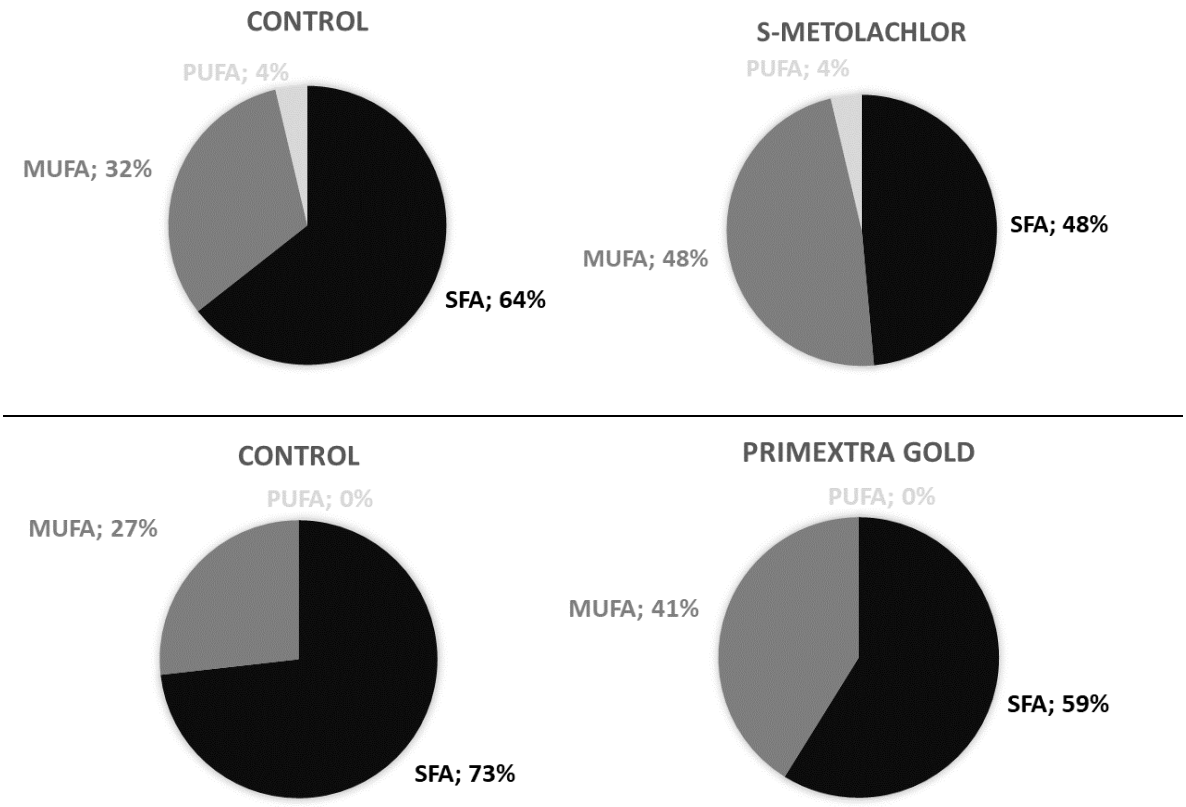

Figure 16 - Relative percentages of fatty acids by group of Fatty acid in HaCaT Cells when exposed to IC_{30} concentrations of S-metolachlor and Primextra® Gold TZ for 48h. PUFA: polyunsaturated fatty acids; MUFA: Monounsaturated fatty acids; SFA: Saturated fatty acids.

Multivariate analysis

Cluster analysis (figure 17) separated samples in 2 major groups (I and II) acording to the diversity and abundance of FAs. This division is clearly made by the type of cell line of the sample rather than the herbicide used, as HepG2 samples define the first group (I) and HaCaT samples define the second group (II). Group I is separated in two groups: Group Ia, which is comprised by control samples of S-metolachlor treatment and treated samples of S-metolachlor, and Group Ib, which is comprised of control and treatment of Primextra® Gold TZ. However, with a similarity >95 we can not say that the differences within group I are significant. Group II is also separated in two groups: Goup IIa, which is comprised by the treated samples of both compounds, and Group IIb, comprised by the control samples of both treatments. With a Similarity <85 we can say there is a clear separation of these last two groups.

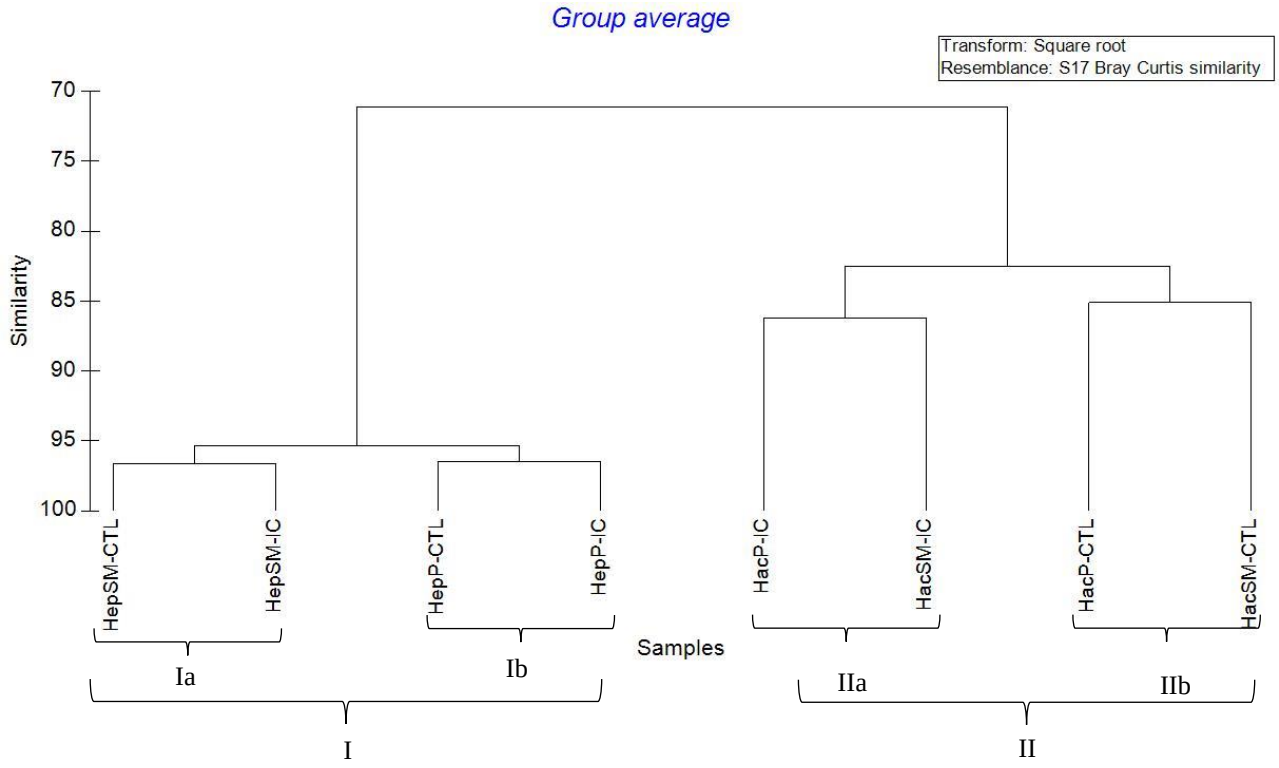

Figure 17 - Cluster analysis of Fatty acid profile. HepSM-IC: HepG2 cells when exposed to s-metolachlor treatment; HepSM-CTL: HepG2 cells when exposed to control of s-metolachlor; HepP-IC: HepG2 cells when exposed to Primextra® Gold TZ treatment; HepP-CTL: HepG2 cells when exposed to control of Pirmextra; HacP-IC: HaCaT cells when exposed to Primextra® Gold TZ treatment; HacP-CTL: HaCaT cells when exposed to control of Primextra® Gold TZ; HacSM-IC: HaCaT cells when exposed to S-metolachlor treatment; HacSM-CTL: HaCaT cells when exposed to control of Smetolachlor.

The n-DMS analysis showed a clear distribution by cell line based on FA abundance and composition (stress 0.06) (Figure 18). In Group I it can be seen all treatments and controls corresponding to HepG2 samples, and in Group II all treatments and controls corresponding to HaCaT samples are present. It is also shown a separation within Group II samples, as seen before in the cluster analysis, between control samples and treated samples. HaCaT cells treated with Primextra® Gold TZ and S-metolachlor form Group IIa and the corresponding controls form group IIb. This separation between cell lines was also revealed in the ANOSIM analysis (R=1, p=0.029).

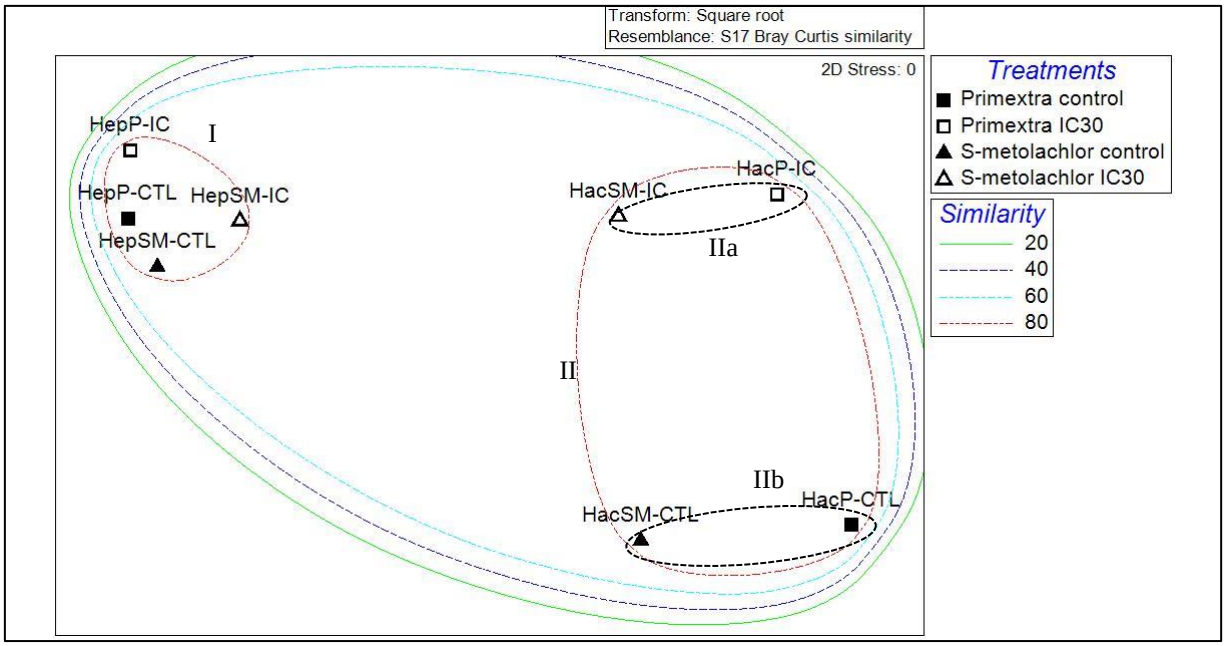

Figure 18 - Two-dimensional non-metric MDS plots of FA profiles. HepSM-IC: HepG2 cells when exposed to s-metolachlor treatment; HepSM-CTL: HepG2 cells when exposed to control of s-metolachlor; HepP-IC: HepG2 cells when exposed to Primextra® Gold TZ treatment; HepP-CTL: HepG2 cells when exposed to control of Pirmextra; HacP-IC: HaCaT cells when exposed to Primextra® Gold TZ treatment; HacP-CTL: HaCaT cells when exposed to control of Primextra® Gold TZ; HacSM-IC: HaCaT cells when exposed to S-metolachlor treatment; HacSM-CTL: HaCaT cells when exposed to control of Smetolachlor.

SIMPER analysis (table 10) shows that C16:0 and C18:1n9 are the two main FAs that contribute to the great similarity within each of the major group. Also according to SIMPER analysis the FAs responsible for the segregation of the two major groups based on the cell line are C16:1N9 (25.18%), C18:1n6 (23.61%), C18:0 (16.14%) and C13:0(15.22%). Within group II, the dissimilarity between Group IIa (treatments) and Group IIb (controls) are mainly due to FAs C18:1n8 (28.1 %), C16:1N9 (19.15 %), C18:0 (17.38 %) and C18:1n6 (13.67 %).

Table 10. Simper analysis of FA profiles. HacCTLs: control samples of HaCaT cell line; HacP-IC: HaCaT cells when exposed to Primextra® Gold TZ treatment; HacSM-IC: HaCaT cells when exposed to S-metolachlor treatment.

MDS group	Similarity	Fatty acid	Av.Abund.	Av.Sim.	Sim./SD	Contrib.%	Cum.%
HepG2 (I)	95.80	C16:0	5.59	23.32	116.17	24.34	24.34
		C18:1n9	4.70	19.10	33.02	19.94	44.28
		C18:1n6	3.93	16.24	26.80	16.95	61.23
		C16:1n9	4.08	15.96	10.22	16.66	77.89
		C18:0	3.03	12.55	32.16	13.10	90.99
HaCaT (II)	83.61	C16:0	5.91	28.33	13.04	33.89	33.89
		C18:1n9	5.70	26.69	22.58	31.92	65.81
		C18:0	5.04	22.40	9.09	26.79	92.60

Mds group	Dissimilarity	Fatty Acid	Av.Abund.		Av.Diss.	Diss./SD	Contrib.%	Cum.%
HepG2 /HaCaT (I/II)	28.72	C16:1n9	4.08	0.94	7.23	2.54	25.18	25.18
		C18:1n6	3.93	0.96	6.78	2.93	23.61	48.79
		C18:0	3.03	5.04	4.64	2.09	16.14	64.93
		C13:0	2.24	0.32	4.39	3.45	15.22	79.79
HacCTLs/HacP-IC + HacSM-IC (IIa/IIb)	17.43	C18:1n8	0.32	2.30	4.90	3.32	28.10	28.10
		C16:1n9	0.27	1.62	3.34	2.69	19.15	47.24
		C18:0	5.65	4.43	3.03	1.67	17.38	64.62
		C18:1n6	0.96	0.96	2.38	0.87	13.67	78.29

PCA analysis (Figure 19) highlights that HaCaT cells keep greater quantities of FA like C18:1n9, C18:1n8, C16:0 and C18:0 while HepG2 cells show greater quantities of FA such as C18:2n6, C16:1n9 and C13:0. With a contribution of 81.8 % from the first axes and a contribution of 10.7 % from second axes of total variation(see table 11). In table 12 we can see the the values of the variables in each axe in the PCA analysis. A positive value means that a variable correlates positively with the principal component. A negative value is indicative of a negative correlation.

In PC1 e.g. C18:0 has a strong negative value (-0.392) which means that higher values on PC 1 correspond to lower concentrations of this element. C16:1n9, on the other hand, has a strong positive value (0.588) which means that higher values on PC 1 corresponds to higher concentrations of this element.

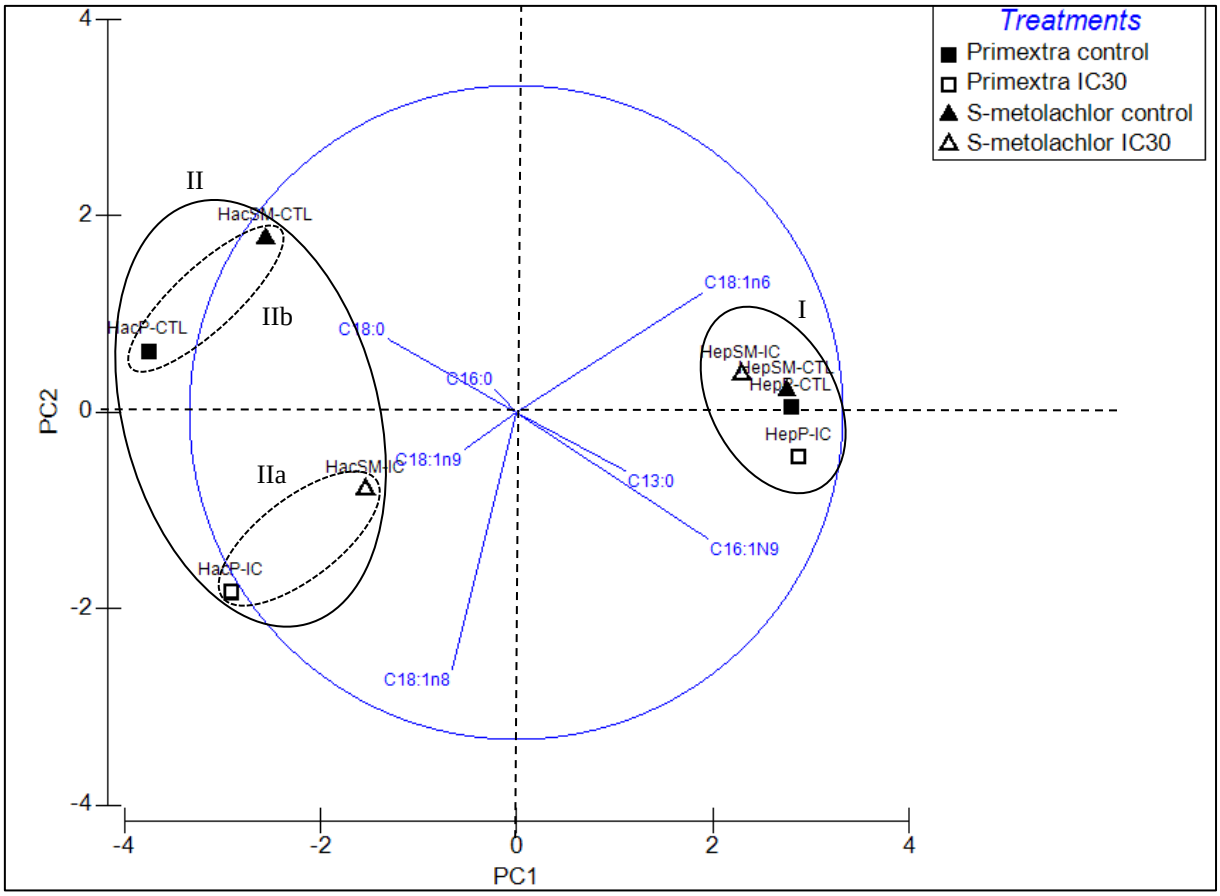

Figure 19 - Principal component analysis (PCA) representing samples from all treatments and their relations regarding FA contents. HepSM-IC: HepG2 cells when exposed to s-metolachlor treatment; HepSM-CTL: HepG2 cells when exposed to control of s-metolachlor; HepP-IC: HepG2 cells when exposed to Primextra® Gold TZ treatment; HepP-CTL: HepG2 cells when exposed to control of Primextra® Gold TZ; HacP-IC: HaCaT cells when exposed to Primextra® Gold TZ treatment; HacP-CTL: HaCaT cells when exposed to control of Primextra® Gold TZ; HacSM:IC: HaCaT cells when exposed to S-metolachlor treatment; HacSM-CTL: HaCaT cells when exposed to control of S-metolachlor.

Table 11 – Contribution of variation from the different principal components of the PCA analysis.

PC	Eigenvalues	% variation	Cum. % Variation
1	8.61	81.8	81.8
2	1.13	10.7	92.5
3	0.546	5.2	97.7
4	0.198	1.9	99.6
5	3.5E-2	0.3	99.9
6	5.7E-3	0.1	100.0
7	2.45E-5	0.0	100.0

Table 12 – values of the variables (fatty acid) for each axe of the PCA analysis.

Variable	PC1	PC2	PC3	PC4	PC5	PC7	PC8
C13:0	0.334	-0.178	0.476	0.563	0.542	0.033	0.136
C16:0	-0.064	0.071	0.035	0.395	-0.561	0.036	0.720
C16:1n9	0.588	-0.386	0.176	-0.578	-0.128	0.203	0.288
C18:1n8	-0.196	-0.786	-0.230	0.092	-0.009	-0.529	0.039
C18:0	-0.392	0.224	0.334	-0.428	0.371	-0.379	0.469
C18:1n9	-0.158	-0.113	-0.594	-0.010	0.469	0.501	0.372
C18:1n6	0.568	0.364	-0.473	0.046	0.134	-0.530	0.144

3.4 Carbohydrate analysis

Carbohydrate composition

In order to see if the compounds could affect the carbohydrate composition of both cell lines, total sugar quantification from each cell line was performed by using GC-FID methodology (see table 12 and fig. 18).

Table 12. Carbohydrate composition of HepG2 and HaCaT cell lines when exposed to Primextra® Gold TZ and S-metolachlor. Results are expressed in relative percentage (%).

	HepG2				**HaCaT**			
Carbohydrate	**Control**	**Primextra® Gold TZ**	**Control**	**S-metolachlor**	**Control**	**Primextra® Gold TZ**	**Control**	**S-metolachlor**
Deoxyribose	22.73	5.48	1.44	8.21	6.42	10.64	21.25	17.76
Rhamnose	1.20	3.62	0.32	0.07	1.47	3.96	0.12	0.28
Fucose	0.57	12.56	20.75	0.58	1.12	0.74	1.43	1.66
Ribose	22.15	6.90	1.37	0.98	14.30	2.02	2.48	15.24
Arabinose	13.91	21.31	11.18	27.83	61.70	26.06	24.03	21.40
Xylose	6.03	3.69	4.56	5.32	4.31	13.17	11.65	4.51
Mannose	4.09	6.52	13.59	7.13	3.27	9.74	6.78	18.99
Galactose	5.14	8.65	7.48	8.42	3.23	19.07	12.92	7.71
Glucose	24.17	31.26	39.30	41.45	4.19	14.59	19.31	12.43

Carbohydrate composition analysis in HepG2 cell line, when exposed to Primextra® Gold TZ at concentration of 6.20 µg/ml for 48h showed a significant decrease in deoxyribose ($P \leq 0.001$), ribose ($P \leq 0.001$) and a significant increase in fucose($P \leq 0.001$), arabinose and glucose ($P \leq 0.05$) composition. When exposed to S-metolachlor, HepG2 cell line showed a significant decrease in fucose ($P \leq 0.001$), and mannose ($P \leq 0.05$) and a significant increase in deoxyribose ($P \leq 0.05$) and arabinose ($P \leq 0.001$) levels.

As for HaCaT, when exposed to Primextra® Gold TZ revealed a significant decrease in ribose and arabinose (P≤0.001) and a significant increase in xylose, mannose (P≤0.05), galactose and glucose (P≤0.001) composition. When exposed to S-metolachlor, HaCaT showed a significant decrease in xylose, galactose and glucose (P≤0.05) and a significant increase in ribose, mannose (P≤0.001) levels (see figure 20).

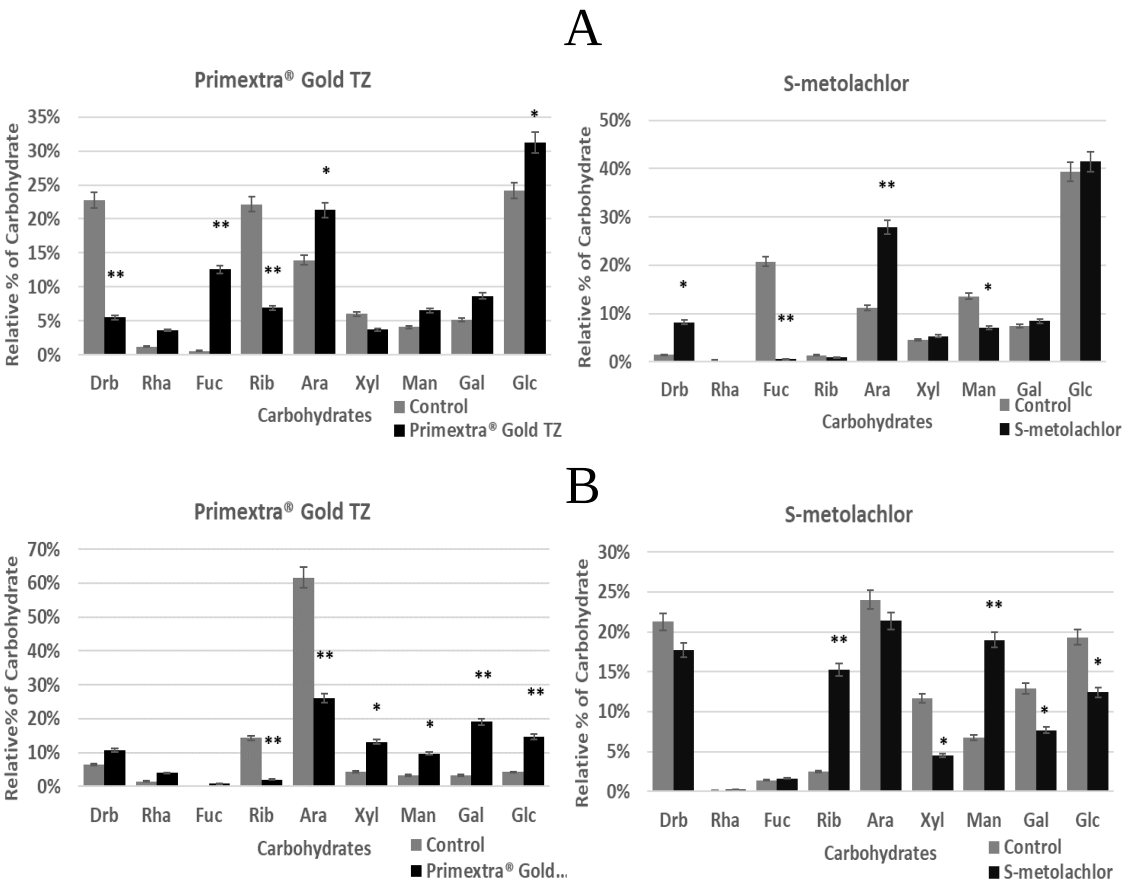

Figure 20 - Carbohydrate composition of HepG2 (A) and HaCaT (B) cell lines when exposed to Primextra® Gold TZ and S-metolachlor. Results are expressed in relative percentage (%) of Carbohydrate. Concentrations showed significant differences compared to control group: *, P≤0.05 and **, P≤0.001

Multivariate analysis

Cluster analysis (figure 21) separated samples in 3 major groups. With a maximum similarity <85 we can conclude that these 3 groups present a clear separation between them. Group I is made of HaCaT control samples from the Primextra® Gold TZ treatment. Group II is comprised of HepG2 samples treated with Primextra® Gold TZ and HepG2 control samples from S-metolachlor treatment. Group 3 can be divided in 3 sub-groups: sub-group IIIa, which is comprised of HepG2 control samples from Primextra® Gold TZ treatments and HaCaT samples treated with S-metolachlor, sub-group IIIb, represented by HepG2 samples treated with S-metolachlor, and sub-group IIIc, which is comprised by HaCaT samples treated with Primextra® Gold TZ and HaCaT control samples from S-

metolachlor treatment. With a similarity between these groups < 90 we can say that the separation between these groups is clear. However, division of the groups cannot be connected to neither cell type nor herbicide treatment factors.

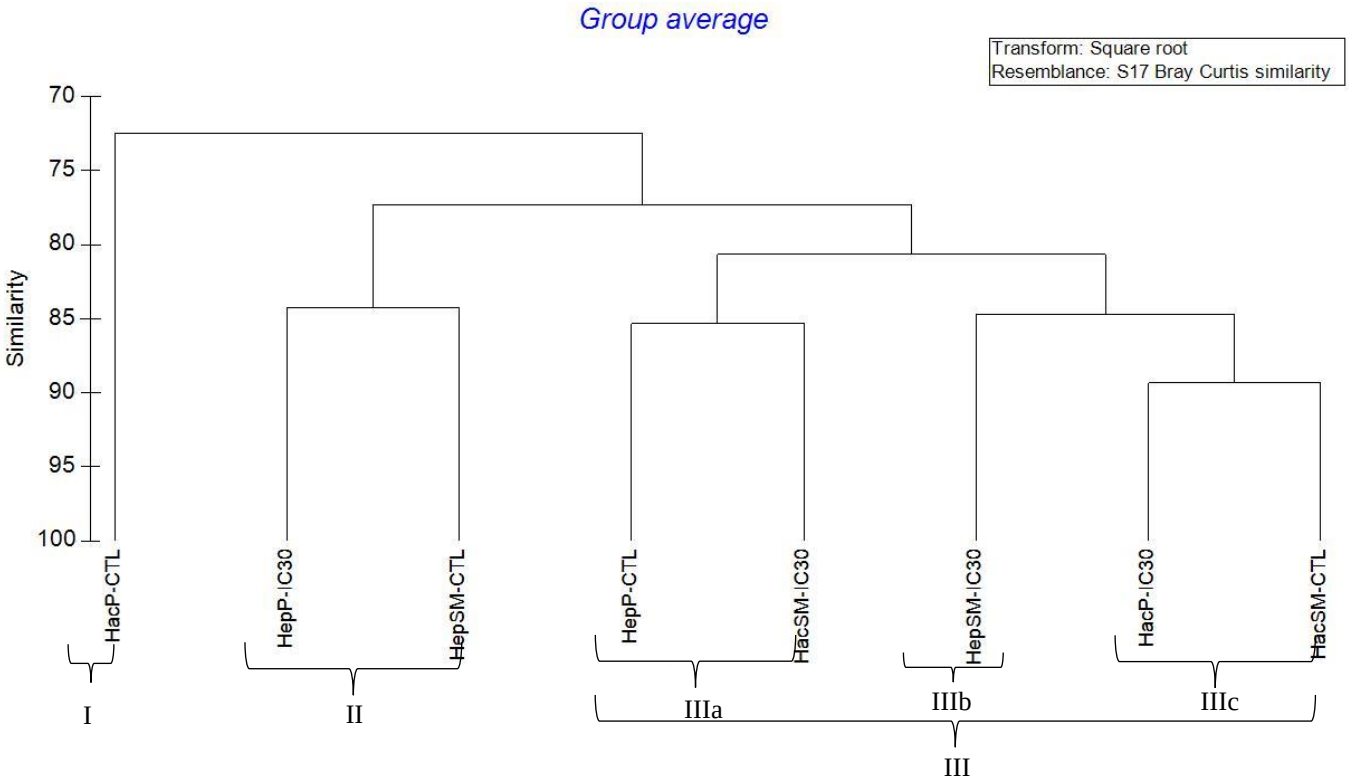

Figure 21 - Cluster analysis of Carbohydrate profile. HepSM-IC: HepG2 cells when exposed to s-metolachlor treatment; HepSM-CTL: HepG2 cells when exposed to control of s-metolachlor; HepP-IC: HepG2 cells when exposed to Primextra® Gold TZ treatment; HepP-CTL: HepG2 cells when exposed to control of Primextra® Gold TZ; HacP-IC: HaCaT cells when exposed to Primextra® Gold TZ treatment; HacP-CTL: HaCaT cells when exposed to control of Primextra® Gold TZ; HacSM-IC: HaCaT cells when exposed to S-metolachlor treatment; HacSM-CTL: HaCaT cells when exposed to control of Smetolachlor.

4. Discussion

The purpose of this study was to determine the cytotoxic potential of Primextra® Gold TZ and S-Metolachlor on human skin and liver tissue, motivated by the large use of such herbicide on Portuguese corn crops. For this purpose, we used MTT cell viability assay and cell cycle analysis by flow cytometry. Additionally, due to reports in literature of Primextra® Gold TZ having effect on the biochemical profile of other organisms, namely fatty acids and carbohydrate composition (Gutiérrez et al., 2019; Filimonova et al., 2016; Neves et al., 2015), it seemed pertinent to evaluate the effect over these biochemical biomarkers.

In the MTT cell viability assay, liver mimicking hepatoma cell line, HepG2, proved to be less sensitive to toxicity from both compounds, the herbicide Primextra® Gold TZ and the a.i. S-metolachlor than the nontumorigenic immortalized human keratinocyte cell line, HaCaT. HepG2 48 h MTT results pointed to an IC_{50} of 22.38 µg/ml and 83.90 µg/ml for Primextra® Gold TZ and S-metolachlor, respectively. HaCaT 48h MTT results pointed to an IC_{50} of 12.37 µg/ml and 12.28 µg/ml for Primextra® Gold TZ and S-metolachlor, respectively.

HepG2 results on cytotoxicity assay showed a higher toxicity of the commercial formulation over the active ingredient. This proves Primextra® Gold TZ to be much more toxic than S-metolachlor alone, which was also shown in literature (Neves et al., 2015; Pérez et al., 2011). This effect may be due to the synergic potential of both the active ingredients presented in the herbicide Primextra® Gold TZ, as the combinations of two or more active ingredients can be more lethal to non-target organisms than the active ingredient in isolation, despite the specificity of their mode of action. Also, it must be kept in mind that the adjuvants can contribute to the toxicity of an herbicide. These organic solvents, emulsifying and wetting agents which affect the pesticide penetration and performance, may synergize or antagonize the toxicity of the active ingredient, leading to the necessity to evaluate the toxic effect of a formulated pesticide not only by testing the a.i.'s in isolation or combined, but by testing the formulated pesticide (Nikoloff et al., 2013).

The concentrations used on the acute test were based on the article from Nikoloff et al., (2013). Nikoloff's study aimed to evaluate cytotoxicity and genotoxicity of S-metolachlor and its commercial formulation, Twin Pack Gold, on HepG2 cells. To assess the cytotoxicity, Nikoloff and colleagues performed a 24 hours MTT cell viability assay with a range of concentrations between 0 and 15 µg/mL of S-metolachlor and commercial formulation. The results for S-metolachlor were, however, different from the ones we obtained in our study. Our results pointed to a significant metabolic activity inhibition when HepG2 cells were exposed to any of the concentrations of S-metolachlor tested (from 0.5 to 80 µg/mL) on the 24 hours assay, while on Nikoloff's study none of the concentrations tested (0.5 to 15 µg/mL) showed to have significant effect on cell viability.

In the cell cycle assay using HepG2 cell line, Primextra® Gold TZ induced a decrease in the percentage of events of sub-G1 phase in both IC_{30} and IC_{50} treatments and an increase in

the percentage of events in G2 phase in the IC_{50} treatment. S-metolachlor exposure induced a decrease in percentage of events in sub-G1 at both treatments, a decrease of percentage of events at G0/G1 and an increase of percentage of events in G2. Sub-G1 is associated to apoptosis, or cell death. The fact that Primextra® Gold TZ and the a.i. decrease cell death could be contradictory to what MTT results suggested as in MTT when the dose increases, cell viability decreases. However, this could be explained by the mechanism the a.i. and the commercial formulation affect this cell line. In fact, what we see in MTT test could be a decrease of mitochondrial activity rather than cell death per se. The cell cycle arrest we see at G2 could be due to the DNA damage repair stop that occurs in this phase. The increase in G2 when exposed to S-metolachlor is contradictory to the information in literature that suggest S-metolachlor as non-genotoxic (*European Commission*, 2004). In fact, little is known about S-metolachlor mode of action on non-target organisms, especially in mammals, however studies using metolachlor showed that this compound could decrease the abundance of cyclin A transcript thus affecting the cell cycle progress of the S phase to the G2 phase (Hartnett et al., 2013). Taking into account that a complex, cyclin A2–cdk1, is also required for the progression from G2 to mitosis (Badie et al., 2000), this could somehow explain the mechanism behind S-metolachlor toxicity in this type of cell.

Results in HaCaT cell cycle were very different from those obtain in HepG2 cell line, as none of the compounds was shown to induce significant cell cycle alterations. That means that the mechanism behind the toxicity in this cell line may differ from the ones that affect HepG2.

As for the fatty acid profile analysis, results showed no significant alteration on fatty acid composition on exposure of HepG2 cells. HaCaT cell line, however, showed a >15% reduction on saturated fatty acid percentage and a proportional increase in monounsaturated fatty acids. The fatty acids affected were all long-chain fatty acids. This is due to the ability of S-metolachlor to inhibit Acyl-CoA elongation thus leading to the inhibition of long-chain and very-long-chain fatty acids synthesis (Jump, 2009 ; Schmalfu et al., 2000). Saturated fatty acids are of great importance for maintaining membrane integrity, as they provide rigidity and control the activities of membrane proteins through covalent bonding. They are also key factors in the formation of raft domains in membranes and in the barrier properties of ceramides located in the epidermis of the skin (Ehehalt et al., 2006). The replacement of saturated fatty acids for monounsaturated fatty acids in the membrane leads to a decreased rigidity of the membrane (Weijers, 2016). This can be an important factor in understanding the mechanism behind S-metolachlor and Primextra® Gold TZ toxicity in this cell line.

Carbohydrate analysis results were inconclusive as the controls of the experiment differ greatly in terms of carbohydrate composition. This may be due to handling and /or preparation throughout the carbohydrate process, as fatty acid results was not observed differences between the controls. We could expect, however, that both Primextra® Gold TZ and S-metolachlor would have an effect over carbohydrate biomarkers, as it was shown

in literature that both Primextra® Gold TZ active ingredients, S-metolachlor and Terbuthylazine, produced a decrease in glycogen (Gutiérrez et al., 2019). Because glycogen is mainly produced by the liver and muscles and is mainly comprised of glucose it would be expected a decrease in glucose. Since glycogen is produced in liver it would be expected to see a greater percentage of this polysaccharide in HepG2 cell line than in HaCaT.

Overall, results from HepG2 assays were very different from the results using HaCaT. Having in mind that test conditions were the same for both types of cells, the differences in toxicity could only be related to the intrinsic metabolic differences between them, as well as the differences in their inherent mechanisms, that counteract damage. The fact that HepG2 was less sensitive to cell viability assay but showed higher DNA damage than other cells could be comparable to what has been shown in a study by Želježić (2018) using terbuthylazine. In that study, using isolated lymphocytes and HepG2, Želježić showed that the highest tested concentration (8.00 ng/mL) reduced lymphocyte viability by 15 %, mostly due to apoptosis, while cytotoxic effects in HepG2 cells at the same concentration were negligible (Želježić 2018). However, comet assay proved that DNA instability was more pronounced in HepG2 cells.

5. Conclusion

The present study demonstrates that the largely used herbicide Primextra® Gold TZ and its main active ingredient, S-metolachlor, decreased cellular viability and provoked cell cycle arrest at G2 phase of HepG2. Thus, proving the cytotoxic potential of the compound to the human liver. In the case of HaCaT cell line both the herbicide Primextra® Gold TZ and the a.i. reduced cell viability and provoked alterations in overall fatty acid profile, leading to a reduction in saturated fatty acids and an increase in monounsaturated fatty acids, with no alterations in cell cycle.

We can also conclude that HaCaT proved to be more sensitive to cytotoxicity than HepG2, even though the latter was shown to be more susceptible to DNA damage. The differences between the results of both cell lines are proof of the intrinsic metabolic differences between them as well as the differences in their inherent mechanisms that counteract damage.

Overall, this study emphasizes the validity of performing pre-clinical tests using HepG2 and HaCaT cell lines, as models for liver and skin toxicity evaluation, respectively. The current study also underlines fatty acids to be a more sensitive biochemical biomarker than carbohydrates to be used as endpoint in ecotoxicological studies with HepG2 and HaCaT cell lines.

To conclude, in future works it would be important to study the effect of Primextra® Gold TZ on other cell lines or tissues. A study using pulmonary cell lines would be of great relevance, since Primextra® Gold TZ is applied by spraying method. Different toxicity assessments could also be made as a potential genotoxic effect of the commercial formulation and active ingredients is still a possibility. Also, the ability of S-metolachlor to increase the production of reactive oxidative stress, as described in literature, should be addressed. It would also be of great relevance to analyze the combined effect of S-metolachlor and terbuthylazine in the same proportions of the ones used in Primextra® Gold TZ, to assess the impact of the excipients on the overall toxicity of the commercial formulation.

6. References

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