POLITÉCNICO DE COIMBRA
ESCOLA SUPERIOR AGRÁRIA

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Bioherbicidal effect of plant aqueous extracts and essential oils

Internal Supervisor: Prof. Doutora Cristina Galhano

Versão final

Coimbra, 2019

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Dissertação apresentada à Escola Superior Agrária de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de mestre em Agricultura Biológica

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Epigraph

"O génio, o crime e a loucura, provêm, por igual, de uma anormalidade; representam, de diferentes maneiras, uma inadaptabilidade ao meio." *Fernando Pessoa*

Acknowledgments

To Escola Superior Agrária de Coimbra and in particular to VALOREN laboratory for hosting me and making available all the equipment necessary to carry out this work.

To my Supervisor Professor Cristina Galhano for the availability and kindness she had in welcoming me and orienting me.

To Eng. Filipe Melo, for the friendliness and availability by providing me with the necessary plants for the preparation of the aqueous extracts.

To all my friends and family, who have always encouraged me to work to achieve my goals.

To all my thanks, without you all this work would not be possible.

Resumo

A População Mundial está a aumentar, prevendo-se 10 biliões de pessoas na terra em 2050. Para obter o maior rendimento de culturas, é crucial controlar as infestantes, pois causam uma perda da produção total anual de aproximadamente 9,7%. Durante muitos anos os herbicidas controlaram eficazmente as infestantes e sem estes a Revolução Verde não aconteceria, mas o seu uso abusivo a nível mundial, levou à emergência de infestantes resistentes a herbicidas e a elevados níveis de resíduos no solo, água e alimentos. Os Bioherbicidas podem ser uma alternativa natural aos herbicidas sintéticos, evitando impactos negativos no meio ambiente e na saúde humana, pois os ingredientes ativos são compostos naturais já presentes no ambiente, espectando-se serem mais ecológicos. Este trabalho teve como objetivo estudar a atividade herbicida pré e pós-emergente de seis extratos aquosos e oito óleos essenciais de plantas PAM, no dente-de-leão, Taraxacum officinale. Extratos Aquosos mostraram maior inibição da germinação no ensaio pré-germinativo, enquanto os Óleos essenciais maiores níveis de lesão no ensaio pós-emergente. Para avaliar o potencial e os mecanismos de ação destes extratos vegetais e óleos essenciais como bioherbicidas, e garantir o não comprometimento da biodiversidade e do desenvolvimento das culturas, mais estudos deverão ser realizados.

Abstract

World Population is increasing and 10 billion people are expected by the year 2050. To obtain the largest crop yields, it is crucial to control weeds, as they cause about 9.7% loss of total crop production every year. Herbicides have effectively controlled weeds for many years. Nonetheless, their worldwide overuse led to herbicide-resistant weed and high levels of herbicide residues in soil, water and food. Therefore, bioherbicides can be a natural alternative to synthetic herbicides, avoiding such negative impacts on environment and human health. Since bioherbicides have natural compounds as active ingredients already present in the environment, they are expected to be more ecologically friendly. Thus, this work aimed to study the pre and post emergent herbicidal activity of six Aromatic and Medicinal Plants (AMP), plant aqueous extracts and eight AMP essential oils, on dandelion, Taraxacum officinale. Aqueous extracts showed better seed germination inhibition on pre-emergency bioassay while essential oils had a higher injury level on post-emergency bioassay. More detailed studies should be carried out to better evaluate the potential and mechanisms of action of these plant extracts and essential oils as bioherbicide, in order to ensure that possible side effects will not neither compromise biodiversity nor crop development.

Summary

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1. Introduction

Every year, all over the world, a significant amount of money is spent on weed control. This control can be made through several methods, such as mechanical, chemical, biological, and cultural. Nevertheless, among all these methods, synthetic herbicides are the most frequently used. Despite its effectiveness, there are several reports about weed resistance to synthetic herbicides, besides the negative effect it causes to environment and human health (Batish *et al.*, 2004; Cordeau *et al.*, 2016).

According to Marshall *et al.* (2003), weed control is the most expensive input in crop production. Based on the statistical data of Food and Agriculture Organization of the United Nations (n.d.$_b$), and Fundação Francisco Manuel dos Santos (2018), the countries France, Germany, Greece, Italy, Portugal, Spain and United Kingdom were analysed for the year 2016. The countries that use more total pesticides per hectare of arable land are Italy and Germany. Regarding the use of herbicides, Italy remains the country with the highest amount of active ingredient used per hectare, followed by Spain (Fig. 1). From 1990 until 2016, the specific use of herbicides has undergone considerable variation for most of the countries, with a large reduction in UK since 2005 (Fig. 2) (Food and Agriculture Organization of the United Nations, n.d.$_b$).

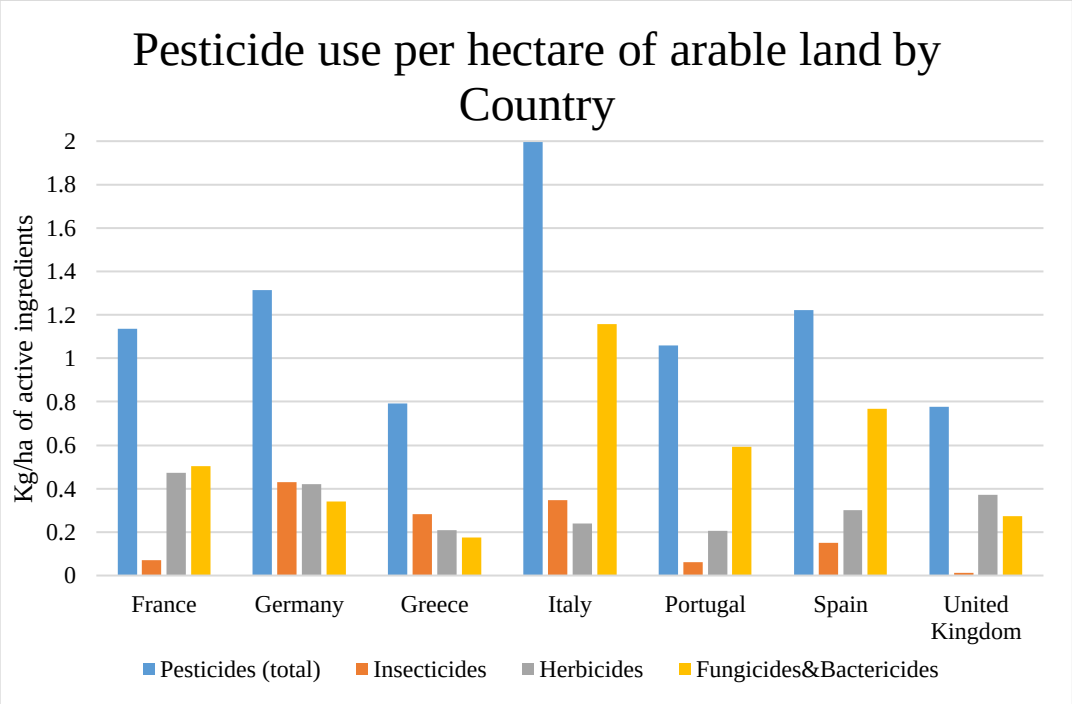

Figure 1 Pesticides, insecticides, herbicides, fungicides and bactericides (Kg/ha) uses in 2016, in France, Germany, Greece, Italy, Portugal, Spain and United Kingdom (data obtained from Food and Agriculture Organization of the United Nations (n.d.$_b$) and Fundação Francisco Manuel dos Santos (2018)).

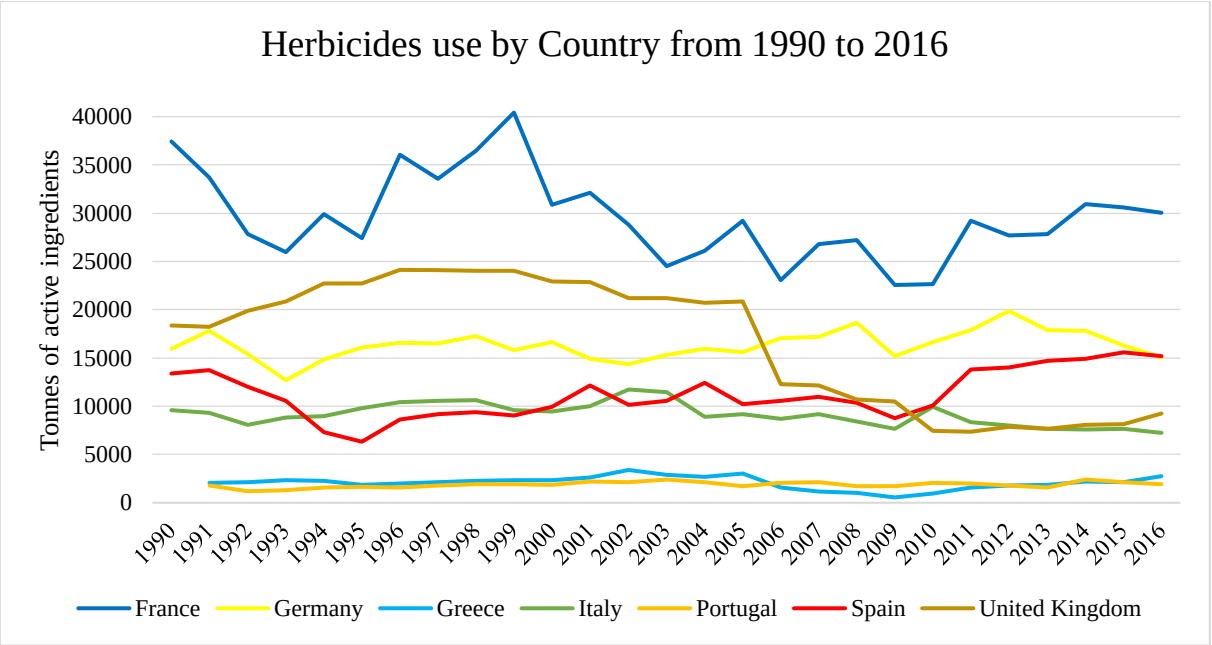

Figure 2 Total amount of herbicides used per Country between 1990 and 2016 in France, Germany, Greece, Italy, Portugal, Spain and United Kingdom (data from Food and Agriculture Organization of the United Nations (n.d.$_b$)).

The reduction of herbicide use in United Kingdom is not directly linked to an increase of the certified organic agriculture area. As a matter of fact, in the period under review, the organic agriculture area only increased from 2006 to 2010, decreasing since then. On the contrary, France and Italy showed a significant increase in certified organic agriculture area, while Greece, Portugal and Spain showed a more modest increase (Fig. 3).

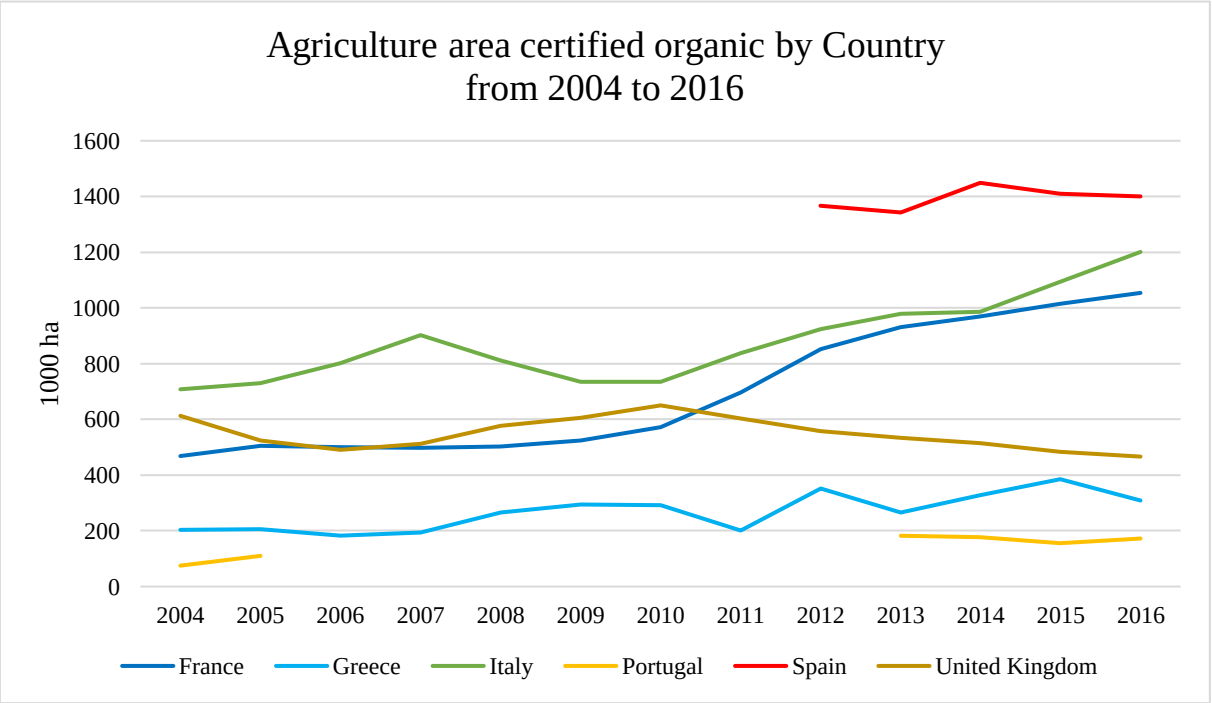

Figure 3 Area of certified organic land in France, Greece, Italy, Portugal, Spain and United Kingdom from 2004 to 2016 (data from Food and Agriculture Organization of the United Nations (n.d.$_b$))

If the certified organic agriculture area and world population continue to increase, would today's agricultural land be able to produce enough food to feed the world?

According to Food and Agriculture Organization of United Nations (n.d.$_b$), the world population will continue to increase, and almost 10 billion people will inhabit the earth. There are several authors who support that organic agriculture is able to produce as many food as conventional agriculture, as well as feed the current and future world population without increasing the agricultural area (Badgley *et al.*, 2007; Muller *et al.*, 2017; Pretty *et al.*, 2003; Reganold & Wachter, 2016; Seufert *et al.*, 2012). On the other hand, Connor (2008, 2013 and 2018) and Kirchmann *et al.* (2016) have a contrary opinion. Schrama *et al.* (2018) argues that with a right management and farm planning, the yield gap between conventional and organic farming can be reduced. While Meemken & Qaim (2018) refers that the possibility of organic farming feeding the world is an unrealistic future scenario for the estimated world population.

Notwithstanding the referred above, IFOAM Organics International (n.d.$_a$) and Food and Agriculture Organization of the United Nations (n.d.$_c$) states that the main obstacles to world food security are not the production methods themselves but social, economic and political conditions.

According to Food and Agriculture Organization of the United Nations, (2018): "Organic agriculture is a holistic production management system which promotes and enhances agro-ecosystem health, including biodiversity, biological cycles, and soil biological activity. It emphasises the use of management practices in preference to the use of off-farm inputs, taking into account that regional conditions require locally adapted systems. This is accomplished by using, where possible, agronomic, biological, and mechanical methods, as opposed to using synthetic materials, to fulfil any specific function within the system." Slightly different is the definition by IFOAM Organics International (n.d.$_b$): "Organic Agriculture is a production system that sustains the health of soils, ecosystems and people. It relies on ecological processes, biodiversity and cycles adapted to local conditions, rather than the use of inputs with adverse effects. Organic Agriculture combines tradition, innovation and science to benefit the shared environment and promote fair relationships and a good quality of life for all involved.", this definition reflects the four principles on which Organic Agriculture is based: health, ecology, fairness and care. They express how organic agriculture can contribute to the world and a vision to improve all agriculture in a global context as well as to inspire the organic movement in its full diversity (IFOAM Head Office, n.d.).

The principle of health advocates supporting and improving the health of the Earth as a whole, which includes the soil, plants, animals and humans as one and indivisible. Healthy soils

generate healthy yields leading to healthy beings. Individual and communitarian health cannot be disconnected from ecosystems health (IFOAM Head Office, n.d.).

The principle of ecology promotes agricultural systems that fit nature cycles, work with them and find an ecological balance. The Organic Agriculture protects the established habitats and promotes genetic and agricultural diversity, having a positive impact on landscapes, climate, habitats, biodiversity, air and water (IFOAM Head Office, n.d.).

The principle of fairness provides equity, respect and justice among people and other living beings. Organic Agriculture foments a good quality of life, contributing to food sovereignty and poverty reduction for everyone involved. The management of natural and environmental resources should be socially and ecologically fair, taking into account the future generations (IFOAM Head Office, n.d.).

The principle of care states that organic agriculture should have precaution and responsibility in order to ensure the health and well-being of current and future generations and the environment. Risks should be prevented by the adoption of appropriate and safe technologies and by rejecting all the unpredictable ones (IFOAM Head Office, n.d.).

These four principles serve as guide to IFOAM positions, programs and standards.

Organic agriculture in European Union is an agricultural production system regulated by Commission Regulation (EC) No. 889/2008 of 5th September 2008 that lay down detailed rules for the implementation of for Council Regulation (EC) No. 834/2007, and amended by many Commission Implementing Regulations (EU).

In May 2018, Regulation (EU) 2018/848 of the European Parliament and of the Council of the European Union was approved and repealed Council Regulation (EC) No. 834/2007. Regulation (EU) 2018/848 has entered into force since 17th June 2018, but it is only applicable from the 1st January 2021, according to the article 61 of the respective regulation, in order to allow a timely recognition of control authorities and control bodies. Until then, the rules established by regulation 834/2007 should be followed.

"On the 25th September of 2015, the 193 Member States of United Nations adopted the 17 Goals of Sustainable Development (SDG) of 2030 Agenda for Sustainable Development.", (Food and Agriculture Organization of the United Nations, n.d.$_d$). Until 2030, SDGs will shape the national development plans of Member States. The Agenda includes 17 goals, since it ends poverty, hunger, climatic changes, resources sustainability, food and agriculture. Two of the goals, Zero Hunger (goal 2) and Life on Land (goal 15), can be directly related with Organic Agriculture and indirectly with bioherbicides. In fact, they can help accomplish three of the targets of goal 2: i) by 2030, agricultural productivity and incomes of small-scale food producers should be

doubled; ii) by 2030, sustainable food production systems that increase productivity and help maintain ecosystems and improve land and soil quality should be established; c) by 2030, the genetic diversity of seeds, cultivated plants and farmed or domesticated animals should be maintained. On the other hand, Organic Agriculture can help accomplish two of the targets of goal 15: i) take action to reduce degradation of natural habitats and stop the loss of biodiversity and, by 2030, protect and prevent the extinction of threatened species; ii) by 2030, introduce measures to prevent the introduction of invasive species on land and water ecosystems, reduce de impact, control and/or eradicate the priority species(Food and Agriculture Organization of the United Nations, n.d.$_d$).

The principles and the regulations of organic agriculture, promote a minimal use of inputs, closing nutrient loops and valorisation of agri-food wastes, which is the basis of the circular economy applied to the agronomic sector (Agrocycle, 2017). Circular Economy aims to reduce or eliminate waste like nature does, and turns waste into a resource. The concept of circular economy is to add value to waste, create new job positions and generate profit, ending the accumulation of waste. The natural cycles do not generate waste, all matter is reused and recycled, being only generated by the humankind. (Circular Economy Portugal, n.d.; Ellen MacArthur Foundation ANB, 2017).

Biological diversity included in agroecosystem provides many biological functions like nutrient cycling and pest control. The reasons for biodiversity conservation biodiversity are moral, aesthetic, social and economic (Marshall *et al*., 2003).

Fortunately, the awareness of the need to a more sustainable society, respecting Environment and all the ecosystem services, is increasing (Environmental Science.org, 2018).

1.1 Weed control management

According to Radhakrishnan *et al*. (2018), weeds are plants considered undesirable in a particular situation. Weeds are a real problem in crop production however, they can be a major factor in agroecosystems. Weed flora have changed in the last century, with the abundance declining of some species and the increasing of others (Marshall *et al.*, 2003).

In the last years, some farmland insect and bird species have shown a substantial decline in their populations. Some studies correlate changes in agricultural practices with those declines, since many arable weed species support an insect diversity (Marshall *et al.*, 2003).

According to Lampkin (1990), as quoted by Ferreira *et al*. (2012), weeds are the biggest concern in organic agriculture constituting the major obstacle to the conversion from traditional to

organic farming. Sustainable weed control is one of the main challenges for both organic and conventional farming (Cordeau *et al.*, 2016).

Weed control refers to the actions used to eliminate weeds. Weed management is the prevention of weed reproduction, reduction of weed emergence after crop planting, and minimization of weed competition with crops. The ideal weed control management must take into account the optimization of competitive relationship between weeds and crop, identifying the suppression factors and adopting adequate rotations that have limiting action on weed development and on tillage reduction. It is also important to consider crop interventions, biodiversity promotion and plant interaction (Buhler, 2002; Ferreira *et al.*, 2012; Pannacci *et al.*, 2017).

Based on their life cycle, weeds can be classified into annuals and perennials (Ferreira *et al.*, 2012).

Annual plants produce seeds and die in only one year. They are well adapted to unstable and degraded ecosystems. Their survival strategy is the preparation for multiplication before environment changes to unfavourable conditions. They spend most of their life cycle and energy in seed production, producing a large quantity of seeds per plant (Ferreira *et al.*, 2012).

Perennial plants prefer stable and less disturbed ecosystems, being more frequent in non tilled soils. Their survival strategy consists in the preservation of mother plant and a small seed production as a next generation warranty. Beyond seed propagation, most of perennial plants produce stolons, bulbs, rhizomes and tubers as another form of propagation and as a food storage for the plant (Ferreira *et al.*, 2012).

Several methods can be used on weed control management, and some of them can only be used in conventional agriculture nevertheless, they will be addressed here, too. Weed management strategies are constituted by non-chemical and chemical methods. Although none of these tools alone offers completely control of weed, the combination of them is quite effective (Bajwa, 2014; Buhler, 2002; Pannacci *et al.*, 2017; Radhakrishnan *et al.*, 2018).

1.1.1 Non-chemical methods

Non-chemical methods are subcategorized in cultural, mechanical and physical methods. Some bibliography includes biological methods as a non-chemical when a living being, such an insect, fungus or bacteria, is used. However, when a bioderived substance is used, it is considered a chemical method. (Pannacci *et al.*, 2017; Uludag *et al.*, 2018). In this work, biological methods will be described on chemical methods chapter.

Prevention, crop rotation, cover corps, intercropping, tillage, stale seedbed, varieties selection, crop establishment, irrigation and fertilization can be considered as examples of cultural

methods. For mechanical methods examples, the hoe, rush-weeder, split-hoe, finger-weeder, flex-tine harrow and manual weeding can be referred. Mulching, solarization, flaming and steaming are considered good examples of physical methods (Pannacci *et al.*, 2017).

1.1.2 Chemical methods

Chemical methods include all synthetic and natural phytopharmaceutical products, which in the case of weed control management are synthetic herbicides or bioherbicides.

Synthetic herbicides and bioherbicides can be classified in many ways according to the growth phase and their translocation capacity (Food and Agriculture Organization of the United Nations, n.d.).

According to plant growth phase, they can be considered as pre or post emergent. Pre-emergent herbicides are applied to the soil and act before emergency, inhibiting seed germination while post-emergent herbicides act after the emergency and can have different mechanisms of action, that are described below (Food and Agriculture Organization of the United Nations, n.d.$_a$; Teicher, 2017).

According to their translocation capacity, herbicides can also be classified in systemic or contact herbicide.

Systemic herbicides tend to be hydrophilic and, depending on chemical characteristics of active ingredient, translocation take place via phloem or xylem. Contact herbicides tend to be lipophilic, being absorbed by the plant waxy cuticle resisting to wash-off by rain (Teicher, 2017).

The formulation of a bioherbicide and a synthetic commercial herbicide product include the active ingredient as well as adjuvants, adjuvant activators, surfactants, stabilizers and conserving agents (Ash, 2010; Teicher, 2017).

The adjuvant improves formulation biological efficiency or safety while increase spray droplets retention. Adjuvant activator optimizes spreading and penetrating properties, having a critical importance for herbicides, as it can improve phytotoxicity for the crop. Surfactants improve spray coverage on leaf surfaces by reducing droplets superficial tension, allowing the spreading beyond their initial contact area (Teicher, 2017).

The phytopharmaceutical registration process in the EU is more complex in comparison with other countries. EU plant protection Regulation 1107/2009 does not recognize biopesticides as a regulatory category, which may hinder biopesticide registration. Although, in August of 2017, Regulation 2017/1432 made changes in Regulation 1107/2009 and introduced two new categories, basic substances and low risk substances (European Commission, Directorate-

General for Health and Food Safety, 2017; European Parliament, Council of the European Union, 2009; Teicher, 2017).

1.1.2.1 Synthetic herbicides

Synthetic herbicides have been quite effective in reducing yield losses, stabilizing weed populations at acceptable levels and contributing to food security. Although their intensive use have silent impacts on surface and groundwater contamination by leaching and leading to adverse effects to humans and other living organisms (Popp *et al.*, 2012, Radhakrishnan *et al.*, 2018; Vasileiadis *et al.*, 2015; Zhang *et al.*, 2015).

Herbicide modes of action can be distinguished in two slightly different concepts, mode of action and mechanism of action. Mode of action is related with plant symptoms developed after herbicide application, while mechanism of action is considered the biochemical target of herbicide (Teicher, 2017).

Most common synthetic herbicides affect photosynthesis, by inhibiting important photosynthetic metabolic chains, and others non-photosynthesis related herbicides mimic plant hormones or inhibit cell division (Teicher, 2017).

Common inhibition targets of a photosynthesis related synthetic herbicides are the Photosystem I or II, EPSPS (5-endopyruvylshikimate-3 phosphate synthase), HPPD (hydroxyphenylpyruvate dioxygenase), PDS (phytoene desaturase), ACCase (acetyl-CoA carboxylase), ALS (acetolactate synthase) and Glutamine synthetase. The non-photosynthetic related synthetic herbicides have as active ingredient synthetic auxins or cell division inhibitors (Teicher, 2017).

Depending on active ingredients and their use, it can be necessary a hydrophilic element to allow uptake and deliver inside the plant, and a lipophilic element to allow the cross through biological membranes. The active ingredient solubility is determined by their lipophilicity and their dissociation constant (Teicher, 2017).

The chemical nature of active ingredient is crucial to phytopharmaceutical product formulation. In fact, a lipophilic active ingredient may resist to solubilization in water and usually requires a formulation with organic solvents or oils, while an hydrophilic active ingredient is easily solubilized in aqueous spray solutions. The uptake of this molecules by lipophilic foliar barriers requires the presence of a surfactant in the final product. Lipophilicity allows a quick uptake through a waxy, cuticular and chitinous lipophilic barrier, facilitating the passage of cellular membranes and walls. When the active ingredient uptake has a delayed or an insufficient

absorption, it can lose effectiveness, as it can be leached by rain or degraded by UV (Teicher, 2017).

Systemic herbicides tend to be hydrophilic and, depending on chemical characteristics of active ingredient, translocation take place via phloem or xylem. Contact herbicides tend to be lipophilic, being absorbed by the plant waxy cuticle resisting to wash-off by rain (Teicher, 2017).

1.1.2.2 Bioherbicides (pre and post emergent)

Bioherbicides is a subgroup of biopesticides or biocontrol agents. Biocontrol is often used in pest control, where the balance between pest population and profit is found, instead of chasing their eradication. Four groups of biocontrolers are usually considered: macroorganisms (insects or nematodes), microorganisms (bacteria, fungus or virus), chemical mediators (pheromones), and natural extracted substances (plant or animal) (Ash, 2010; Cordeau *et al*., 2016).

Biocontrol for weed management is less developed comparing with the widespread biocontrol for pests and diseases. Biopesticides are the product of extraction or formulation of various natural compounds already present in the environment, that are expected to be biodegradable, environmentally friendly, and leave few (or non) harmful residues (easier residue management) and are less likely to harm non-target species (Cordeau *et al*., 2016; Teicher, 2017).

Bioherbicides can be subdivided in Microbial bioherbicides and Bio-derived (biochemical) bioherbicides. Microbial herbicides are made of bacteria, fungus or virus, being in their active form (liquid formulation) or in dormant form (dry formulation). Bio-derived bioherbicides have as active ingredients natural molecules extracted in most cases from plants (Teicher, 2017).

The most researched types of bioherbicides are parasitic fungus, followed by bacteria and essential oils. Aqueous plant extracts as bioherbicides are not so widely studied and the extraction methodology is quite diverse, despite their good potential in pre and post emergency bioassays. Nevertheless, a few researches were conducted to understand the mechanism of action and physiological effect of bioherbicides. Therefore, it is very important to carry out new researches to disclose the interaction mechanisms of these biomolecules in weed control.

If the bioherbicide research success is defined as the approval and commercialization of a phytopharmaceutical product, the success index is quite low. In 2010, bioherbicides corresponded less of 10% of market quota of biopesticides (Cordeau *et al*., 2016; Ash, 2010).

The first bioherbicide reached the market in 1980, and since then many researches were made in this field. Many studies evidence that fungus, bacteria and plant extracts efficiently control weed germination and growth (Cordeau *et al*., 2016; Radhakrishnan *et al*., 2018).

Only a few countries have approved bioherbicides, being the major part microherbicides. In Europe a few commercial products are approved, while in the USA, Canada and China more bioherbicides are approved and available at market (Teicher, 2017).

In Portugal only one, Beloukha®, is approved since 2016 for weed control, suppression of vine wild shots and desiccant of the potato branch. The active ingredient, pelargonic acid, is extracted from rapeseed oil. This product is exclusive for professional users and it is not approved for organic agriculture (BELOUKHA, 2018).

The term bioherbicide can sometimes give the idea that it can be applied in organic agriculture, but it is not true. In European Union organic agriculture, the only phytopharmaceutical product allowed for application are the ones allowed by the regulations and which have sale authorization on origin countries. Commission Regulation (EC) No. 889/2008 and Commission Implementing Regulation (EU) 2018/1584 forbade the use of vegetable oil as herbicide, being this rule maintained on Regulation (EU) 2018/848 that only would be applicable from the 1st January 2021. Even if essential oils show bioherbicidal effect, it cannot be guaranteed that the rules will change.

In USA one Bioherbicide is approved for organic agriculture, Avenger®, whose active ingredient is d-limonene extracted from orange peel. The company and some on-line pages reveal many positive results with this product. However, no scientific articles were published in renown magazines or conference journals since 2010, the year that this commercial product was approved by United States Environmental Protection Agency (Avenger Products, 2017).

Bioherbicide mechanisms of action are not so different from some of the synthetic herbicides. Microbial bioherbicides produce plant cell wall degrading, photobleaching and phytotoxic molecules. On the other hand the target of bioderived bioherbicides are many of the same plant metabolic processes targeted by synthetic herbicides, although binding in a different site or they may have multiple modes of action (Radhakrishnan *et al.*, 2018; Teicher, 2017).

Microbial and bio-derived (molecules extracted from plant, animal, bacterial or fungal material) bioherbicides have distinct formulation requirements, as microherbicides have live organisms which need a medium for growth and proliferation, while bio-derived bioherbicides need chemical stabilization (Teicher, 2017).

Delivery technology is a very important issue in phytopharmaceutical efficacy and few research were applied to bioherbicides. Spray droplet retention is affected by weeds, chemical composition, droplet size, and travel speed. The size of the droplets that give best herbicidal effect depends on the type of active ingredient. Therefore it is very important the study of the best type of nozzles used in application (Ash, 2010; Teicher, 2017).

1.2 Essential Oils

Known by their antiseptic, bactericide, viricide, fungicide and medicinal properties, essential oils (EOs) are extracted and used since middle ages. They are usually obtained by hydrodestilation or distillation by steam of most plants, and cold expression for citrus. (Bakkali *et al*., 2008).

Essential oils are a very complex mixture of natural volatile compounds and may contain between 20 to 60 different compounds at different concentrations. They have two or three major compounds, usually monoterpenes, and others in small quantities and molecular weight, usually aromatic or aliphatic. Major compounds usually determine the biological properties of essential oils (Bakkali *et al*., 2008).

Allelopathy is the direct or indirect effect that a plant causes to another through the release of chemicals into the environment with a beneficial or harmful outcome. Allelochemicals are mostly extracted from plant material because their ability to synthetize aromatic secondary metabolites as phenolic acids, phenols, flavones, flavonoids, flavonols, saponins and coumarins, which accumulate in the cells of the epidermis of plant organs, such as flowers, leaves, stems, roots, seeds and fruits in small quantities (Cowan, 1999; Dornbos & Spencer, 1990; Inderjit, 1996; Torti *et al.*, 1995; Sakihama *et al.*, 2002).

Essential oils and their volatile compounds are in the spotlight due to their phytotoxicity and allelopathy combined with a quick degradation in the environment. Many essential oils have shown potential as bioherbicide, having the surfactants as challenge because they are a key for application and a good dispersion (Dayan *et al.*, 2009; Batish *et al.*, 2004). Alipour & Saharkhiz (2016) and Hazrati *et al*. (2018) referred that rosemary essential oil containing α-pinene as the major compound, inhibit germination percentage and root and shoot lengths. According to Hazrati *et al*. (2018), *Satureja hortensis* essential oil, that contains near 56% of carvacrol, decreased significantly seed germination rate. Some *Eucaliptus* spp. essential oil has citronellal as one of the major compound and its incorporation in a commercial bioherbicide may also bring desirable results, according to Kohli *et al.* (1998).

Diallyl disulphide and diallyl trisulphide are volatile organosulfur compounds extracted from garlic, *Allium sativum* L., and other species from *Allium* genus. The strong allelopathic potential of garlic is due to the presence of these compounds, inhibiting seed germination, seedling size and root elongation. The effect is concentration related, that is having inhibitory effect at high concentration and stimulator at low concentration (Cheng *et al.*, 2016; Ren *et al.*, 2018).

Acordding to Fagodia *et al.* (2017), *Citrus aurantiifolia* whose major compounds, limonene (40.92%) and citral (27.46%) have strong inhibitory potential on seed germination. Nevertheless limonene has shown no significant effect inhibiting seed germination.

Despite essential oil application seams to be very promising, their performance is very fast but their efficiency very limited, probably due to volatility compounds. Formulations need to be improved and the technology of application need to be widely researched (Dayan *et al.*, 2009; Teicher, 2017).

1.3 Plant aqueous extract

A plant extract is a preparation of a plant material containing the biologically active substance without its cellular residue. The extraction methods for allelochemicals use organic or inorganic solvents according to the compounds of interest which will be extracted. Preliminary trials of plant properties usually begin with alcoholic and aqueous extractions, followed by various organic solvent extraction methods (Cowan, 1999; Farlex, n.d.).

For alcoholic extracts, parts of dried and ground plants are usually used, which are soaked in methanol or ethanol for an extended period. While on aqueous extracts parts of plants are washed and blotted with paper towel, blended and filtered. Both extracts can be centrifuged multiple times for clarification (Cowan, 1999).

In addition to the solvent, agitation or an increase of temperature can be part of the extraction method. While Carvalho *et al.* (2019) used a hot ethanolic extraction method, Yonli *et al.* (2010) used a methanolic and ethanolic extraction, Gholamnezhad (2019) used methanolic extraction, El-Kenany & El-Darier (2013) used cold and hot aqueous extraction, Hayat *et al.* (2018) and Ting-ting *et al.*(2011) used aqueous cold extraction.

On germination bioassays, some plant extracts have shown pre-emergent bioherbicidal proprieties with higher concentrations, and biostimulating proprieties with lower concentrations.

1.4 Objectives

Having into account that authorized products for organic agriculture with herbicidal effect are still not available in Europe, it should be a priority to search for ecologically sustainable alternatives to synthetic herbicides. It also should respect the principles of organic agriculture and provide new data on efficacy and safety so that regulations could change and allow the use of this plant extracts as a bioherbicide and thus bringing farmers a new method to control weeds.

Therefore, the main goal of this work was to study the bioherbicidal potential of some essential oils and plant extracts obtained from Aromatic and Medicinal Plants (AMP).

In this trials, pre and post-emergent bioherbicidal proprieties of *Allium sativum*, *Artemisia dracunculus*, *Cinnamomum camphora*, *Citrus limonum*, *Eucaliptus citriodora*, *Origanum vulgare*, *Rosmarinus officinalis*, and *Thymus mastichina* essential oils and aqueous extract were studied on *Medicago minima*, *Rumex crispus* and *Taraxacum officinale*.

2. Material and methods

In this work, it was studied the effect of extracts and essential oils of some plants on seed germination, and seedling development, pre-emergency and post-emergency effects, of three weed species: *Medicago minima*, *Rumex crispus*, *Taraxacum officinale*, abundant in a certified field for Organic Production in Coimbra College of Agriculture of the Polytechnic Institute of Coimbra. All the used plant aqueous extracts and essential oils and respective concentrations are summarized in Tab. 1.

Table 1 Plant aqueous extracts and essential oils concentrations used in pre and post-emergency bioassays

Plants			**Pre-emergency bioassay**				**Post-emergency bioassay**		
Scientific Name	**Common name**	**Family**	**Aqueous extract**	**Essential oil**			**Aqueous extract**	**Essential oil**	
Allium sativum	Garlic	Amaryllidaceae	1:5 w/v	0.3 mg/L	0.6 mg/L	1.2 mg/L	1:5 w/v	5%	7,5%
Artemisia dracunculus	Tarragon	Asteraceae	-	0.3 mg/L	0.6 mg/L	1.2 mg/L	-	5%	7,5%
Cinnamomum camphora	Camphor	Lauraceae	1:5 w/v	0.3 mg/L	0.6 mg/L	1.2 mg/L	1:5 w/v	5%	7,5%
Citrus limonum	Lemon	Rutaceae	1:5 w/v	0.3 mg/L	0.6 mg/L	1.2 mg/L	1:5 w/v	5%	7,5%
Eucaliptus citriodora	Lemon Scented Eucalyptus	Myrtaceae	-	0.3 mg/L	0.6 mg/L	1.2 mg/L	-	5%	7,5%
Origanum vulgare	Oregano	Lamiaceae	1:5 w/v	0.3 mg/L	0.6 mg/L	1.2 mg/L	1:5 w/v	5%	7,5%
Rosmarinus officinalis	Rosemary	Lamiaceae	1:5 w/v	0.3 mg/L	0.6 mg/L	1.2 mg/L	1:5 w/v	5%	7,5%
Thymus mastichina	Mastic Thyme	Lamiaceae	1:5 w/v	0.3 mg/L	0.6 mg/L	1.2 mg/L	1:5 w/v	5%	7,5%

After being harvested in April 2017, the three weed seeds were dried under natural air circulation conditions and stored in paper bags till their use.

2.1 Essential oil solution

All the essential oils (EOs) used in this study were purchased in Aromazone online store, an international online seller recognized for the quality of their EOs and other nonsynthetic products (Tab. 2). The company is registered in France having a physical store in Paris.

Table 2 Plant species and respective parts used in essential oil solution preparation.

Specie	Plant part	Type of extraction
Allium sativum	bulb	complete distillation by steam
Artemisia dracunculus	flowery aerial parts	complete distillation by steam
Cinnamomum camphora	decamphorated bark	complete distillation by steam
Citrus limonum	peel	cold expression
Eucalyptus citriodora	leaves	complete distillation by steam
Origanum vulgare	leaves	complete distillation by steam
Rosmarinus officinalis	branches	complete distillation by steam
Thymus mastichina	leaves and branches	complete distillation by steam

Aromazone provides the characterization information for each batch of their essential oils. Chemical compilation of constituents is on Appendix 6.1.

For pre-emergency assay, three EOs concentrations, 0.3, 0.6, and 1.2 mg/L, were tested, while in post-emergency assay the two tested concentrations were 5 and 7.5% (w/v), based on Batish *et al*. (2004) research.

Firstly, 100 mL of a 5 g/L concentration stock solution was prepared, using Tween20® at 5000 ppm (v/v) as emulsifier, as described below. A 100 mL Erlenmeyer with 500 mg of EO, 0.5 mL of Tween20®, and 50 mL of sterilized distilled water was placed on a magnetic stirrer. After being stirred for some seconds, the solution was poured into a graduated cylinder and sterilized distilled water was added up to 100 mL.

To prepare 0.3, 0.6, and 1.2 mg/L solutions, 3, 6, and 12 µL of stock solution respectively, and 0.25 mL Tween20®, were added to three 50 mL Falcon tubes. Then, distilled water was added to make up 50 mL of each solution (Fig. 4).

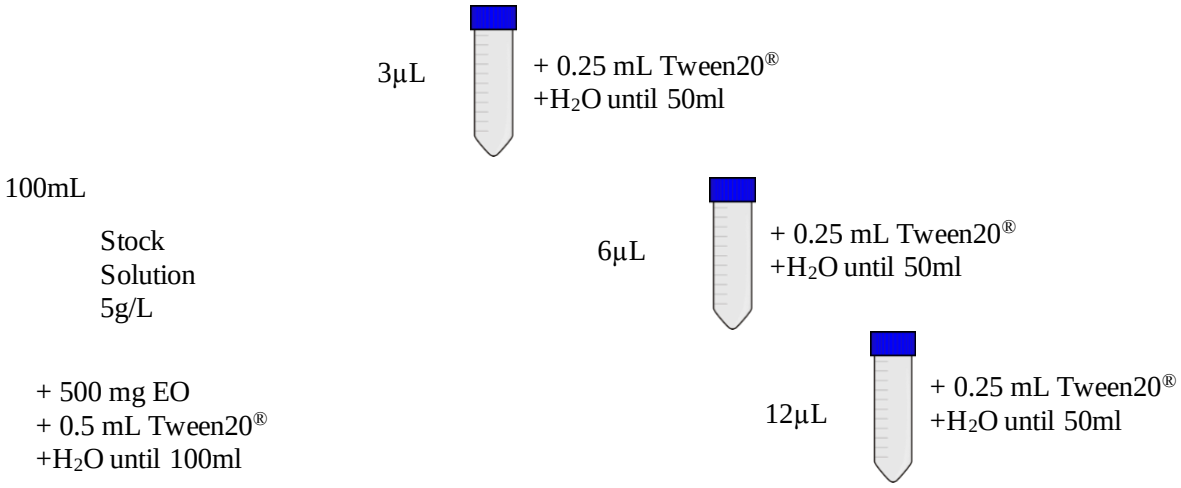

Figure 4 Essential oil solution preparation main steps at 0.3, 0.6 and 1.2mg/L

The 5 and 7.5% EO solutions were prepared in a 100 mL Erlenmeyer, weighing 5 and 7.5 g of each essential oil, respectively, and then adding 0.5 mL of Tween20®, and sterilized distilled water up to 100 mL. To ensure solutions homogeneity, a magnetic stirrer plate was used.

2.2 Plant aqueous extract preparation

All plant extracts tested in this study were obtained using fresh or dry plant parts from different AMP: *Allium sativum* bulb, *Cinnamomum camphora* leaves, *Citrus limonum* peel, *Origanum vulgare* leaves and branches, *Rosmarinus officinalis* leaves and branches, and *Thymus mastichina* leaves and branches (Tab. 3).

Table 3 Plant species and respective parts used in aqueous extract preparation.

Specie	Plant part	Hydration state of plant material
Allium sativum	Bulb	Fresh
Cinnamomum camphora	leaves	Fresh
Citrus limonum	peel	Fresh
Origanum vulgare	leaves	Fresh
Rosmarinus officinalis	leaves and branches	Fresh
Thymus mastichina	leaves and branches	Fresh

All the extracts were prepared at 1:5 (w/v) concentration, as suggested by Ferreira *et al*. (1998). For each plant referred above, 20 g of healthy looking material were selected and smashed in a

porcelain mortar with 100 mL of distilled water and, after 24 hours maceration at room temperature, the extracts were blended using a RUSSELL HOBBS Nutri Boost® blender (Fig. 5).

Figure 5 Smashing Origanum vulgare leaves in a porcelain mortar with 100 mL destilled water

All extracts were centrifuged for 10 minutes at 13.000 rpm, using the Rotanta Hettich Zentrifugen R-460 centrifuge. The supernatant liquid was filtrated twice through a Whatman® nº 1 filter paper to a 100 mL Erlenmeyer (Fig.6). Then, the extracts were sterilized using a 0.2 µm Minisart® syringe filters to sterilized 50 mL Falcon tubes, in a vertical laminar flow chamber.

Figure 6 Aqueous plant extracts before microfiltration From left to right: Artemisia dracunculus, Origanun vulgare, Rosmarinus officinalis, Thymus mastichina, Cinnamomum camphora, Citrus limonum and Allium sativum aqueous extracts.

2.3 Seed sterilization

Seeds surface sterilization is a crucial step in seeds germination tests in order to avoid contamination from potential external agents present in the seed surface that can compromise this kind of studies.

Seeds sterilization was made following the procedure described by Kaur *et al.* (2010).

All the three weed species seeds used in this study were placed in a 150 mL beaker with 100 mL of 0.1% sodium hypochlorite. This solution was stirred, for 2 minutes, using a magnetic stirrer plate. Then, seeds were washed three times with sterilized distilled water and dried with a sterilized paper towel inside a laminar flow chamber.

2.4 Bioassay

As previously referred, pre and post-emergency assays were conducted in order to study the bioherbicidal potential of some aqueous plant extracts and essential oils in relation to three weed species, *Medicago minima*, *Rumex crispus*, and *Taraxacum officinale*.

2.4.1 Pre-emergency bioassay

In the pre-emergency bioassay, it was followed the methodology referred by Batish *et al.* (2004).

In these studies, 9 cm Petri dishes with filter paper circles were used. They were previously sterilized in a Memmert model UM400® dry heat oven, covering the bottom. The bioassays were conducted under aseptic conditions, using a laminar flow chamber.

For each aqueous plant extract, the circle paper was moistened with 3 mL 1:5 (w/v) of extract. For each plant essential oil, 0.3, 0.6, and 1.2 mg/L solutions were tested. For each concentration, the filter paper circle was also moistened with 3 mL.

Ten sterilized weed seeds were placed in each Petri dish which was posteriorly sealed with Parafilm®. Sterilized distilled water served as control. Five replicates were made for each treatment and control (tab. 4). Since the time between the first and the last Petri dishes prepared to start the bioassay was longer than initially expected, 2 controls were made with 5 replicates each, one at the beginning and other at the end of transferring seeds to wet filter paper, in order to warrant that conditions are equal to the first and the last Petri dishes. Petri dishies were then placed on a laboratory bench, in dark conditions, under room temperature.

After three weeks, germinated seeds were counted and the germination percentages were calculated.

Table 4 Description of pre-emergency bioassay and seed species used. Essential oils, aqueous extracts trials and controls with number of replicates, seeds and Petri dishes used

Weed	Pre-emergency bioassay														Control	Total
	Essential Oils								Extracts							
	Canfor	Eucalypt	Garlic	Lemon	Oregano	Rosemary	Tarragon	Thyme	Canfor	Garlic	Lemon	Oregano	Rosemary	Thyme		
Medicago minima	3 [ ] x 5 replicates x 10 seeds	3 [ ] x 5 replicates x 10 seeds	3 [ ] x 5 replicates x 10 seeds	3 [ ] x 5 replicates x 10 seeds	3 [ ] x 5 replicates x 10 seeds	3 [ ] x 5 replicates x 10 seeds	3 [ ] x 5 replicates x 10 seeds	3 [ ] x 5 replicates x 10 seeds	5 replicates x 10 seeds	5 replicates x 10 seeds	5 replicates x 10 seeds	5 replicates x 10 seeds	5 replicates x 10 seeds	5 replicates x 10 seeds	2 controls x 5 replicates x 10 seeds	1600 seeds
Rumex crispus	3 [ ] x 5 replicates x 10 seeds	3 [ ] x 5 replicates x 10 seeds	3 [ ] x 5 replicates x 10 seeds	3 [ ] x 5 replicates x 10 seeds	3 [ ] x 5 replicates x 10 seeds	3 [ ] x 5 replicates x 10 seeds	3 [ ] x 5 replicates x 10 seeds	3 [ ] x 5 replicates x 10 seeds	5 replicates x 10 seeds	5 replicates x 10 seeds	5 replicates x 10 seeds	5 replicates x 10 seeds	5 replicates x 10 seeds	5 replicates x 10 seeds	2 controls x 5 replicates x 10 seeds	1600 seeds
Taraxacum officinale	3 [ ] x 5 replicates x 10 seeds	3 [ ] x 5 replicates x 10 seeds	3 [ ] x 5 replicates x 10 seeds	3 [ ] x 5 replicates x 10 seeds	3 [ ] x 5 replicates x 10 seeds	3 [ ] x 5 replicates x 10 seeds	3 [ ] x 5 replicates x 10 seeds	3 [ ] x 5 replicates x 10 seeds	5 replicates x 10 seeds	5 replicates x 10 seeds	5 replicates x 10 seeds	5 replicates x 10 seeds	5 replicates x 10 seeds	5 replicates x 10 seeds	2 controls x 5 replicates x 10 seeds	1600 seeds
															Total 480 Petri dishes	

Table 5 Description of post-emergency bioassay and seed species used. Essential oils, aqueous extracts trials and controls with number of replicates, seeds and Petri dishes used

Weed	Post-emergency bioassay														Control	Total
	Essential Oils								Extracts							
	Camphor	Eucalypt	Garlic	Lemon	Oregano	Rosemary	Tarragon	Thyme	Camphor	Garlic	Lemon	Oregano	Rosemary	Thyme		
Medicago minima	2 [ ] x 5 replicates x 5 seeds	2 [ ] x 5 replicates x 5 seeds	2 [ ] x 5 replicates x 5 seeds	2 [ ] x 5 replicates x 5 seeds	2 [ ] x 5 replicates x 5 seeds	2 [ ] x 5 replicates x 5 seeds	2 [ ] x 5 replicates x 5 seeds	2 [ ] x 5 replicates x 5 seeds	5 replicates x 5 seeds	5 replicates x 5 seeds	5 replicates x 5 seeds	5 replicates x 5 seeds	5 replicates x 5 seeds	5 replicates x 5 seeds	5 replicates x 5 seeds	575 seeds
Rumex crispus	2 [ ] x 5 replicates x 5 seeds	2 [ ] x 5 replicates x 5 seeds	2 [ ] x 5 replicates x 5 seeds	2 [ ] x 5 replicates x 5 seeds	2 [ ] x 5 replicates x 5 seeds	2 [ ] x 5 replicates x 5 seeds	2 [ ] x 5 replicates x 5 seeds	2 [ ] x 5 replicates x 5 seeds	5 replicates x 5 seeds	5 replicates x 5 seeds	5 replicates x 5 seeds	5 replicates x 5 seeds	5 replicates x 5 seeds	5 replicates x 5 seeds	5 replicates x 5 seeds	575 seeds
Taraxacum officinale	2 [ ] x 5 replicates x 5 seeds	2 [ ] x 5 replicates x 5 seeds	2 [ ] x 5 replicates x 5 seeds	2 [ ] x 5 replicates x 5 seeds	2 [ ] x 5 replicates x 5 seeds	2 [ ] x 5 replicates x 5 seeds	2 [ ] x 5 replicates x 5 seeds	2 [ ] x 5 replicates x 5 seeds	5 replicates x 5 seeds	5 replicates x 5 seeds	5 replicates x 5 seeds	5 replicates x 5 seeds	5 replicates x 5 seeds	5 replicates x 5 seeds	5 replicates x 5 seeds	575 seeds
															Total 345 cups	

2.4.2 Post-emergency bioassay

In the post-emergency bioassay, 200 mL disposable plastic pots filled with Siro Germinação Bio®, an authorized substrate for Organic Agriculture by the Regulation nº 834/2007, were used. Five seeds were sown in each pot (tab. 5). Pots were placed on a table outdoors covered with a bird net protecting pots from birds, cats, and wind. The pots were watered twice a day, using tab clock connected to a 16 mm diameter black hose with microdiffusers.

The aqueous plant extracts, at 1:5 (w/v) concentration, and essential oils, at 5.0 and 7.5% concentrations according to Batish *et al*. (2004), were applied once, four weeks after weed seedlings emergence.

Every plant was sprayed with 2 mL of treatment solution both for extracts and for EOs. Water served as control. There were five replicates for each treatment and for control.

Twenty-four hours after treatment, weed injury was carefully and visually analysed. The observed damages were classified using a scale between 0 (with no injury) and 5 (dead seedling). When no injury was observed, chlorophyll content was determined (Batish *et al*., 2004).

2.4.2.1 Chlorophyll analysis

To determine chlorophyll content of weed seedling leaves which did not show significant signs of injury after being treated with aqueous extracts 30 mg of leaves from each replicate were weighed and then placed in a Falcon tube with 5 mL of methanol and centrifuged at 2500 rpm, using the Rotanta Hettich Zentrifugen R-460 centrifuge (Fig. 7) (Rydzyński *et al.*, 2017).

The chlorophyll extract was 5-fold diluted in methanol and then analysed by spectrophotometry, using a PG instruments T80+ UV/VIS spectrophotometer. The absorption spectra was measured using a 550-750nm spectrum (Rydzyński *et al.*, 2017). The measured absorbance at a wave-length of 665 nm was used to calculate chlorophyll concentration, based on Lambert-Beer Law ($A = \varepsilon \iota C$), where A is the measured absorbance, ε the molar extinction coefficient of chlorophyll in methanol, ι length of solution the light passes through, and C the chlorophyll concentration. For this calculation it was assumed that $\varepsilon = 66600 M^{-1}\ cm^{-1}$ (Rydzyński *et al.*, 2017).

As the methanol extracts were five fold diluted, the absorbance value obtained at 665nm was multiplied 5 times before the Lambert-Beer Law formula was used.

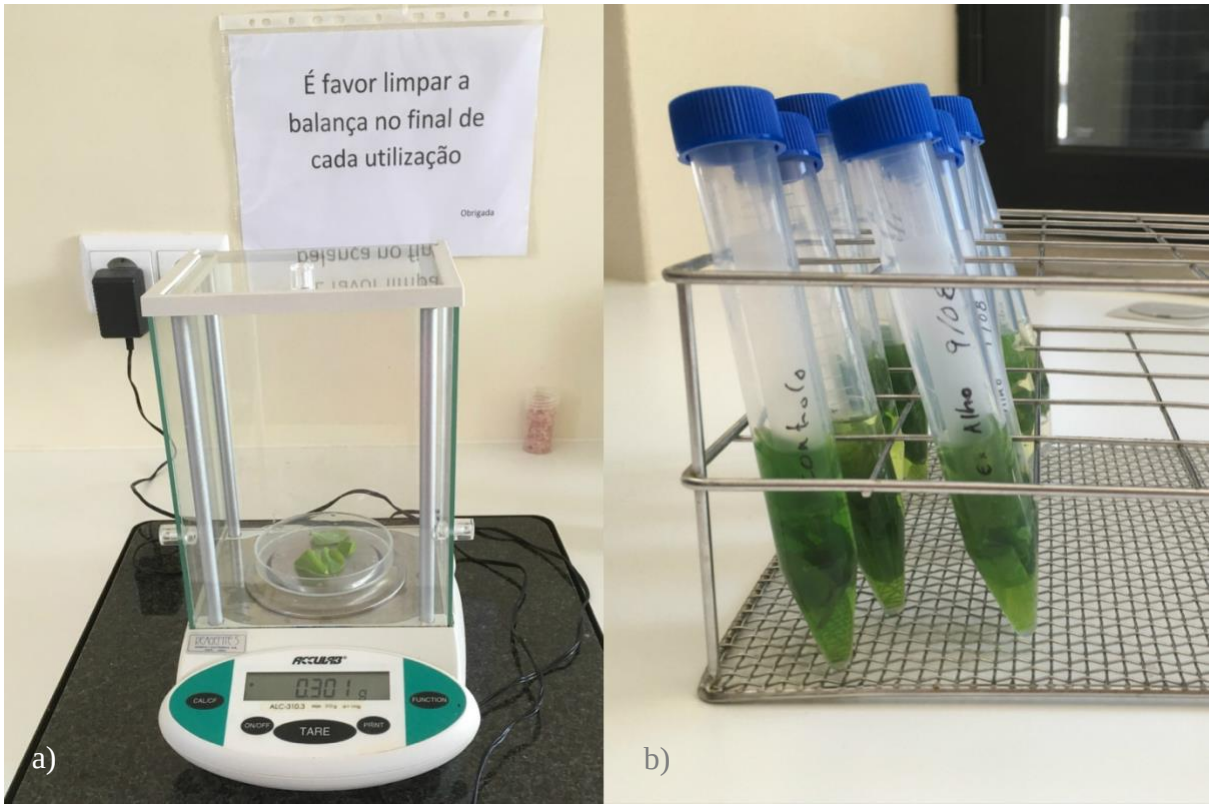

Figure 7 Chlorophyll extraction process. a) Weighing leaves for chlorophyll extraction; b) Falcon tubes with leaves immersed in methanol.

2.5 Statistical analysis

SPSS® and Microsoft Excel® were the software used to analyse the obtained results.

To perform a one-way ANOVA, the pre-emergency bioassay data need to pass the assumptions: sampled randomly and independently of each other as well as normality, and homogeneity of variance. Normality was tested with Shapiro-Wilk test and homogeneity of variance with Levene`s test. One-way ANOVA was performed followed by a post hoc test, the Tukey test, to test if there were significant differences between groups and in which groups there were differences.

Post-emergency bioassay results were treated similarly to those of the pre-emergency results. For Chlorophyll analysis data, as the assumption of homogeneity of variance fail in Levene's test, one-way ANOVA could not be performed. Instead it was performed a Kruskal-Wallis test to test if there were statistically significant differences between groups.

All the tests were carried out with 0.05 level of significance.

3. Results and Discussion

In this section, taking into account that the seeds of both weed species, *Medicago minima* and *Rumex crispus*, did not germinate in the control, only the results obtained with species *Taraxacum officinale* will be presented and discussed.

It should be highlighted that *Medicago minima* germinates in early fall when the temperature drops and the first rain comes while *Rumex crispus* needs temperature and light variation to germinate. The bioassays were carried out under dark conditions and at room temperature. Most of the days, the temperature was above 25ºC, being too hot for seed germination of *Medicago minima* and to stable for *Rumex crispus* (Klos, 1999; Taylorson & Hendricks, 1972). Thus, the pre-emergency and post-emergency effects of extracts and EOs on *Medicago minima and on Rumex crispus* should be studied later on.

3.1 Pre-emergency bioassay

The results obtained in the bioassay that evaluated the effect of the aqueous plant extracts and essential oil solutions on *Taraxacum officinale* seed germination are shown in Fig. 8.

It is evident that all the tested aqueous plant extracts significantly inhibited *Taraxacum officinale* seed germination. Besides that, there were also some EOs that also significantly inhibited *T. officinale* seed germination compared to control: 1.2 mg/L *Citrus limonum*, 1.2 mg/L *Eucaliptus citriodora*, and 0.6 and 1.2 mg/L *Origanum vulgare*.

It should be highlighted that 100% inhibition was obtained with five of the thirty treatments: aqueous extracts of *Allium sativum* bulb, of *Cinnamomum camphora* leaves, of *Citrus limonum* peel, of *Rosmarinus officinalis* leaves and branches, and of *Thymus mastichina* leaves and branches.

No significant difference between control 1 and 2 was observed, so we can assume that conditions are equal from the beginning until the end of inoculation.

The extracts of *Allium sativum, Cinnamomum camphora, Citrus limonum*, *Origanum vulgare*, *Rosmarinus oficinalis*, *Thymus mastichina*, and 0.6 mg/L *Origanum vulgare* EO, caused the highest *Taraxacum officinale* seed germination inhibition. There are no significant differences among them but they all significantly different from the controls.

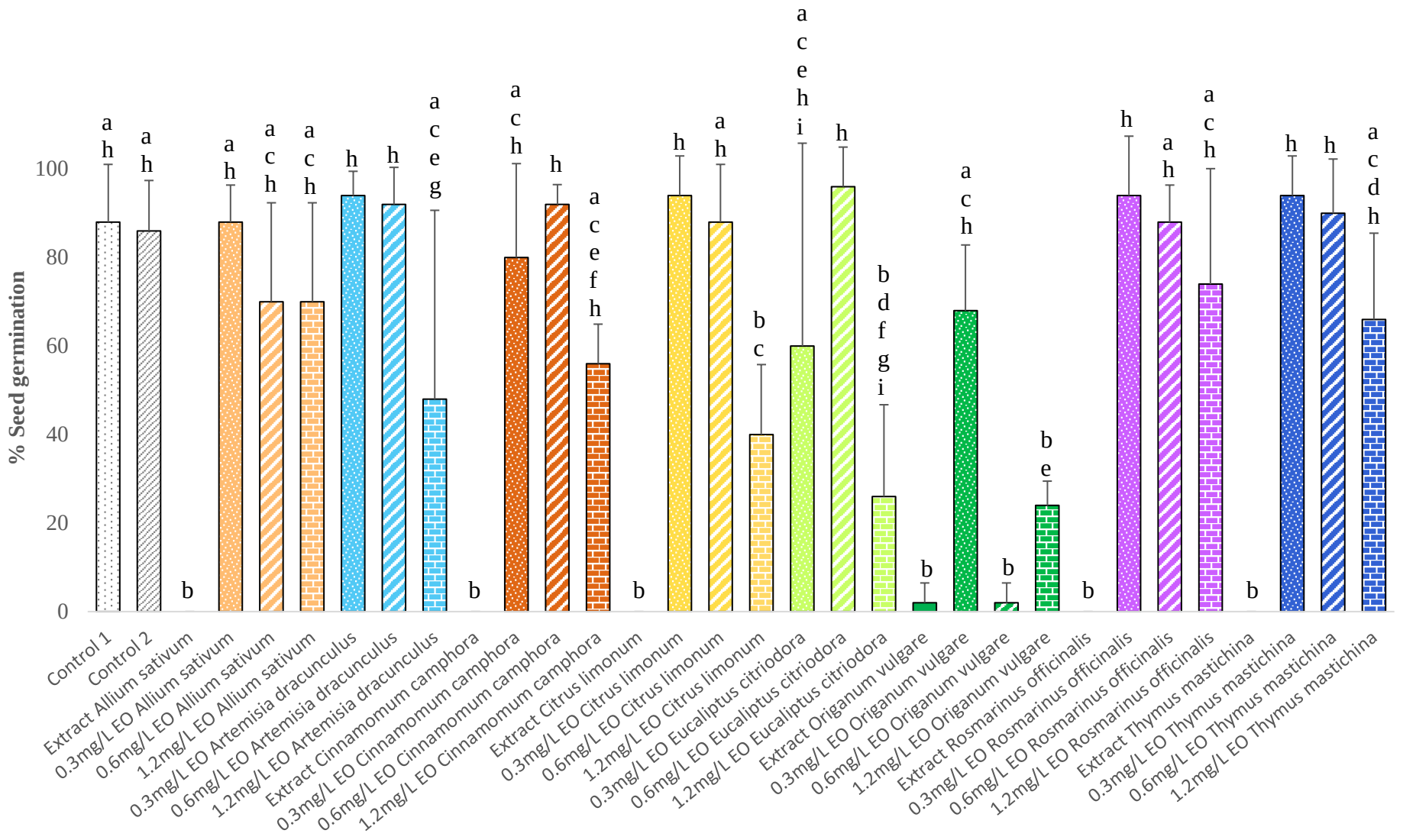

Figure 8 Effect of plant aqueous extracts on Taraxacum officinale seed germination (%). Results are means of 5 replicates with 10 seeds/replicate. Columns with different letters are significantly different at $p<0.05$ according to Tukey HSD test.

The EOs of *Citrus limonum*, *Eucaliptus citriodora*, and *Origanum vulgare* at 1.2 mg/L had some inhibitory effect. These results are significantly different from controls but they are not significantly different from treatments that are not significantly different from controls. Thus, they can be considered as having an intermediate inhibitory effect.

The EOs of *Eucaliptus citriodora*, *Origanum vulgare*, and *Cinnamomum camphora* at 0.3 mg/L and *Allium sativum* EO at 0.6 mg/L and 1.2 mg/L and also *Artemisia dracunculus*, *Cinnamomum camphora*, *Rosmarinus oficinalis* and *Thymus mastichina* EOs at 1.2mg/L did not inhibit *Taraxacum officinale* seed germination, since all treatments are not significantly different from controls. However, as they are not significantly different from treatments that are significantly different from controls, it can also be considered that they have intermediate results but without inhibition effect.

According to Alves *et al.*(2018), carvacrol showed phytotoxicity, cytotoxicity and genotoxicity at high concentrations being the major chemical compound of *Origanum vulgare* EO. This oil at 0.6 mg/L concentration showed the highest inhibition seed germination rate among EO treatments. Contrary to what was expected, 1.2 mg/L did not show a higher inhibition even at higher concentration.

According to Batish *et al.* 2004, the germination rate was significantly reduced by *Eucaliptus citriodora* EOs at 0.3 mg/L, 0.6 mg/L, and 1.2 mg/L. However, in this bioassay, 0.3 mg/L, 0.6 mg/L was statistically equal to controls. According to Singh *et al*. (2006) emergence, seedling and root growth are severely affected in response to citronellal, the major compound of *Eucaliptus citriodora* EOs.

Carvalho M. *et al.* (2019) refered that ethanolic *Amarathus* spp. extracts showed a significant inhibition on letuce seed germination, decreasing the mitotic index and chromosome binding, attributing the observed phytotoxic effects to coumarins compounds.

Hayat *et al.* (2018) emphasizes that garlic aqueous extract, at high concentrations, inhibited tomato seedlings growth, causing oxidative damage. At lower concentrations instead of cellular damage, the extract acts as stimulant and promotes seedling growth.

According to Singh *et al*. (2002), cineole, the major compound of *Cinnamomum camphora* and *Thymus mastichina* oils, inhibits seed germination and speed germination as well as inhibits chlorophyll through degradation or synthesis reduction, leading to pigment loss.

Singh *et al*. (2006) evaluated the bioherbicidal potencial of *Artemisia scoparia* oil, which is from the same genus as *Artemisia dracunculus*, but with a totally different chemical characterization. *Artemisia scoparia* has β-myrcene (29.27%) as the main constituent, followed

by (+)-limonene (13.3%), (Z)-β- ocimene (13.37), γ-terpinene (9.51%) and acenaphthene (17.8%), while *Artemisia dracunculus* has as the main constituent methyl-chavicol (78.36%), followed by methyl-eugenol (0.18%), (Z)-beta-ocimene (7.18%), (E)-beta-ocimene (7.37%) and limonene (4.46%).

Andrianjafinandrasana *et al.* (2013) reported that methyl-chavicol rich chemotypes of *Ravensara aromatica* Sonn has an inhibitory effect of greengram and rice seed germination.

According to Martino *et al.* (2010), d-limonene and citral, the major compounds of *Citrus limonum* EOs, are the monoterpenes that showed less seed germination inhibition effect.

Singh *et al*. (2006) suggests that the inhibition of germination on bioassays indicates that under natural conditions these plants emanate these chemical compounds, that enter the soil, and may be involved in suppression of associated vegetation.

Even that in many bibliographies it is referred that the above EOs showed pre-emergent bioherbicidal potential, in this bioassay EO are not the most effective at inhibiting seed germination, with exception of 1.2 mg/L *Citrus limonum*, 1.2 mg/L *Eucaliptus citriodora*, and 0.6 and 1.2 mg/L *Origanum vulgare*. The concentrations used were based on Batish *et al*. (2004) research, but knowing that chemical composition of plants can be different according to climate conditions, soil characteristics, development stage, harvesting season and that the extraction method can influence the chemical composition of the extract, in future studies an increase of concentration is advised (Khan *et al.*, 2018;Teixeira *et al*., 2013).

Since all aqueous extracts showed high inhibition of germination, future studies should be conducted in order to evaluate the optimum concentration and if at lower concentrations they will act as biostimulants.

Batish *et al.* (2004) refer that different plants respond differently to EOs. In future studies in addiction to change concentrations, other plant species seed germination should be tested.

3.2 Post-emergency bioassay

The results obtained in the bioassay that evaluated the effect of spraying aqueous plant extracts and essential oil on *Taraxacum officinale* four weeks after emergency are shown in Fig. 9.

It is evident that *Taraxacum officinale* seedlings were significantly injured when sprayed with 5 and 7.5% of *Allium sativum*, *Artemisia dracunculus*, *Eucaliptus citriodora*, *Origanum vulgare*, *Thymus mastichina* EOs and with 7.5% of *Citrus limonum* EO, compared to control. Among these treatments, 7.5% of *Eucaliptus citriodora* EO and 5 and 7.5%

Origanum vulgare EO had shown the highest injury level in *Taraxacum officinale* seedlings with comparable effects.

The treatments 7.5% of *Allium sativum* EO, 5 and 7.5% of *Artemisia dracunculus* EO, 7.5% of *Citrus limonum* EO, 5% of *Eucaliptus citriodora* EO and 7.5% of *Thymus mastichina* EO caused a significant injury level in *Taraxacum officinale* seedlings, being significantly different from control.

The treatments 5% of *Allium sativum* and *Thymus mastichina* EOs showed some inhibition effect since they are significantly different from control, but they are not significantly different from treatments which in turn are not significantly different from controls. So, the results were considered as intermediate with some inhibition effects.

All tested aqueous extracts did not cause visible significant injury on *Taraxacum officinale* seedlings, 24 hours after spraying.

Batish *et al*. (2004) reported that plants sprayed with *Eucaliptus citriodora* EOs exhibited varying levels of injury and that 21 DAS (day after spray) the treated and killed plants do not showed recovery or regrowth.

Gill and Holley (2006) cited by Alves, *et al.* (2018) referred that at 5.0 or 10 mmol L^{-1} concentration, carvacrol, major compound of *Origanum vulgare* caused adenosine triphosphatase inhibition.

According to Abrahim *et al*. (2003), α-pinene, the major compound of *Rosmarinus officinalis*, at lower concentrations stimulates mitochondrial respiration and at higher concentrations have inhibitory effect.

Cineole, present in *Thymus mastichina, Origanum vulgare* and *Cinnamomum camphora* are already used as an active ingredient in one commercial bioherbicide referred by Putnam (1988) cited by Kohli *et al*. (1998).

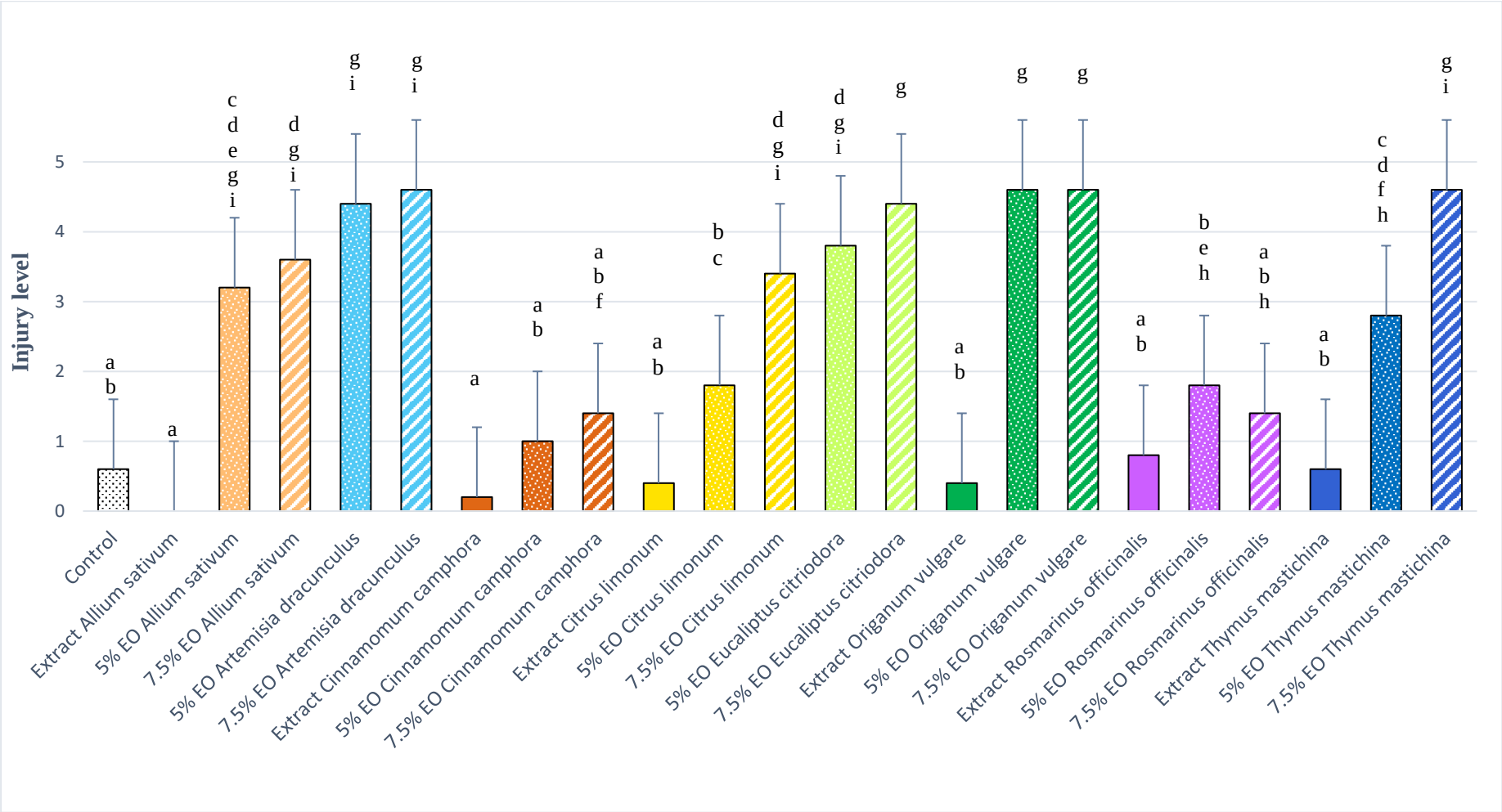

Figure 9 Injury level on Taraxacum officinale seedlings caused by aqueous extracts and essential oil solutions evaluated visually on a scale between 0 (no injury) and 5 (mortality). Results are means± SD of injury level of replicates. Columns with different letters are significantly different at $p<0.05$ according to Tukey HSD test.

The commercial bioherbicide Avenger® has d-limonene as active ingredient and reported good herbicidal results (Avenger Products, 2017).

As *Allium sativum*, *Artemisia dracunculus, Citrus limonum, Eucaliptus citriodora*, *Origanum vulgare,* and *Thymus mastichina* EOs had the highest post-emergency herbicide effect on *T. officinale. Though,* more detailed studies should be carried out.

In future studies, the injury level should be assessed for longer periods after spraying, taking into account that other authors observed injuries 7 days after spray (DAS) (Kaur *et al.*, 2010) or 21 DAS (Batish *et al.*, 2004).

In this work it was studied some aromatic plant aqueous extracts and essential oils. However, in the future the potential of agrofood waste as bioherbicides should be studied to value these by-products, giving them a new life through their reintroduction in an economic and ecological cycle. Thus being possible on future application the use of invasive plants, such as *Datura stramonium* leaves as referred by Ayoub & Niazi (2001) or agricultural waste like vine-shoot waste as refered by Sánchez-Gómez, et al. (2017).

3.2.1 Chlorophyll analysis

The chlorophyll content was determined in *Taraxacum officinale* seedlings used in the post-emergency bioassay that did not show significant injuries 24 hours after being sprayed with plant aqueous extracts.

The results of the absorption spectra of chlorophyll isolated from *Taraxacum officinale* leaves that did not show significant injuries 24 hours after being sprayed with plant aqueous extracts are shown in Fig.10.

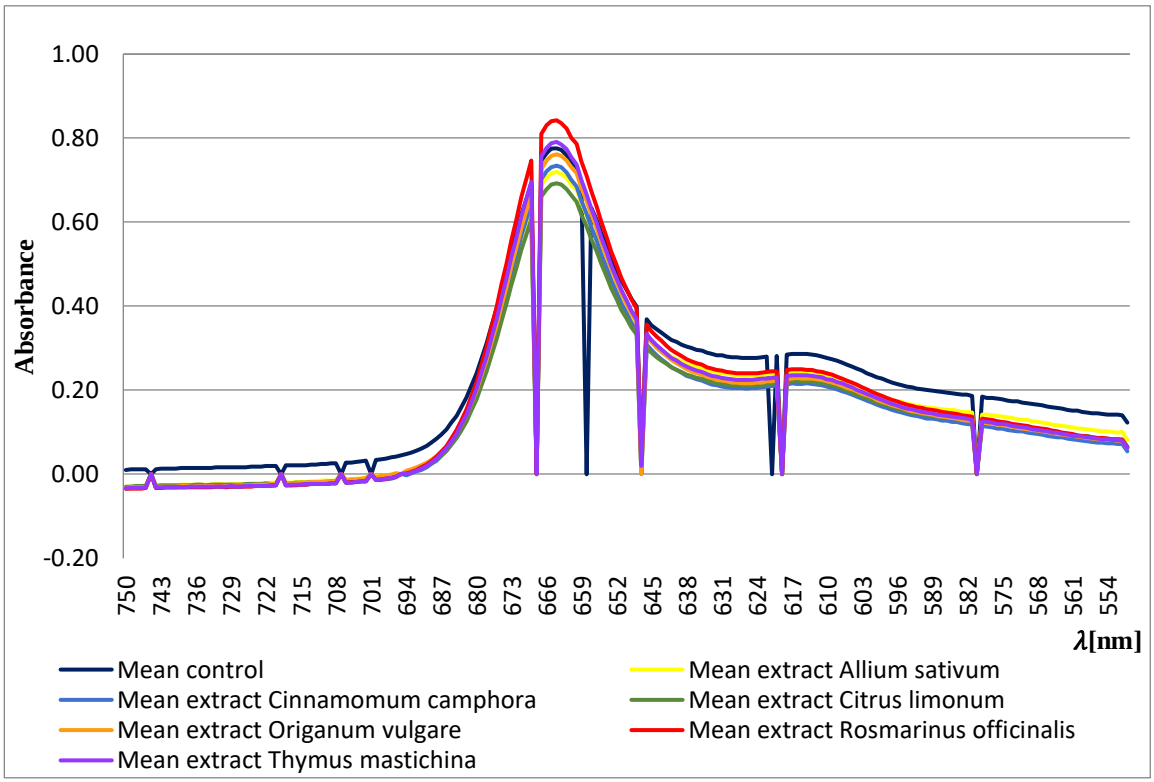

Figure 10 Absorption spectra (750 - 555nm) of chlorophyll isolated from Taraxacum officinale leaves 24 h after being sprayed with aqueous plant extracts/water (as control).

There were no statistically significant differences among different extracts including control. Therefore, the sprayed plant extracts did not cause significant changes in the chlorophyll content, 24 hours after application.

The chlorophyll concentration means and standard deviation were calculated through Lambert-Beer Law for each aqueous plant extract treatment and control which are shown in Tab. 6.

Table 6 Chlorophyll molar concentration using the spectrophotometer values and calculated through Lambert-Beer Law, assuming $\varepsilon = 66600\ M^{-1}cm^{-1}$

Treatments	Chlorophyll concentration
	[M] x 10^-5 ± SD
Control	5,81 ± 2,11
Extract *Allium sativum*	5,37 ± 2,77
Extract *Cinnamomum camphora*	5,49 ± 1,43
Extract *Citrus limonum*	5,17 ± 0,75
Extract *Origanum vulgare*	5,69 ± 0,62
Extract *Rosmarinus officinalis*	6,30 ± 1,89
Extract *Thymus mastichina*	5,91 ± 1,64

According to Rydzyński *et al.* (2017), chlorophyll is the most abundant pigment on earth. However, as a result of abiotic and biotic stresses, it undergoes a quick degradation.

Kaur *et al.* (2010) and Batish *et al.* (2004) also estimated the chlorophyll content of plant material after essential oil spraying, but utilizing a different extraction and quantification method. Their results showed that when cholorophyll content is lower on treatments than on control, a degradation or a decrease on sintesis of chlorophyl ocuurs, affecting the photosynthesis and leading to a loss of respiratory ability.

As in this bioassay chlorophyll content was determined only 24 hours after spraying, it cannot be predicted if treatments that do not show signs of injury would damage, dry or reduce the chlorophyll content if the damages were evaluated later.

The chlorophyll analysis confirms the no significant weed injury that was visually analysed on post-emergency bioassay.

4. Conclusion

In this preliminary study, all plants studied showed some bioherbicidal potential on dandelion. All aqueous extracts showed pre-emergent herbicidal potential and *Allium sativum, Artemisia dracunculus, Citrus limonum, Eucaliptus citriodora, Origanum vulgare,* and *Thymus mastichina* EOs had the highest post-emergency herbicide effect on *T. officinale*.

Aqueous extracts were more effective on pre-emergency, while essential oils were more effective on post-emergency bioassay.

The concentration used in this preliminary bioassay in all aqueous extracts was very high in order to test if any of the extracts inhibit seed germination. A sequential less concentrated extracts solutions should be studied.

The effect of aqueous extracts and essential oils should be compared to a commercial herbicide, as a positive control.

Following the results obtained in this work, it would be interesting the study of aqueous extracts as pre-emergent and essential oils as post emergent bioherbicides.

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6. Appendix

6.1 Chemical characterisation of essential oils used in this bioassay (Aroma Zone, 2017)

Plant specie	Density	Flash point	Main biochemical compounds by gas chromatography
Allium sativum	1.080 - 1.095	54°C	**Sulphides:** **diallyl disulphide (46.18%),** **diallyl trisulphide (22.02%),** diallyl tetrasulphide (6.20%), diallyl sulphide (7.90%), methyl-allyl trisulphide (1.55%), methyl-allyl disulphide (1.98%)
Artemisia dracunculus	0.92 - 0.94	69°C	**Phenols methyl ether:** **methyl-chavicol (78.36%),** methyl-eugenol (0.18%) **Monoterpenes:** (Z)-beta-ocimene (7.18%), (E)-beta-ocimene (7.37%), limonene (4.46%)
Cinnamomum camphora	0.87 - 0.88	52°C	**Monoterpenic oxide**: **1,8-cineole (36.50%)** **Monoterpenes**: limonene (20.85%), alpha-pinene (14.79%), para-cymene (9.43%), alpha-terpinene (1.24%)
Citrus limonum	0.84 - 0.87	48°C	**Monoterpenes**: **limonene (65.67%**), beta-pinene (13.98%), gamma-terpinene (9.19%), sabinene (1.87%), alpha-pinene (2.05%), **Aldehydes**: geranial (1.55%)
Eucaliptus citriodora	0.860 - 0.882	77°C	**Monoterpenols**: citronellol (5.42%), isopulegol (7.81%), iso-isopulegol (7.81%),

			Terpenic aldehydes: **citronellal (75.50%)**
Origanum vulgare	0.905 - 0.950	66°C	**Phenols:** **carvacrol (61.24%),** thymol (3.17%) **Terpene alcohols:** linalool (2.18%) **Monoterpenes:** para-cymene (15.38%), gamma-terpinene (9.09%)
Rosmarinus officinalis	0.895 - 0.910	42°C	**Monoterpenes:** **alpha-pinene (23.33%)**, camphene (9.40%), limonene (4.03%) **Terpenic oxides:** **1,8- cineole (21.00%)** **Monoterpenones (ketones):** camphor (18.65%)
Thymus mastichina	0.900-0.920	55°C	**Terpenic oxides:** **1,8-cineole (56.64%)** **Monoterpenols:** linalool (15.43%), alpha-terpineol (3.26%) **Monoterpenes:** limonene (3.13%), beta-pinene (4.45%)

6.2 Statistical Analysis Tables

6.2.1 Pre-emergent bioassay

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
7,426	31	128	0,000

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2061,894	31	66,513	24,242	0,000
Within Groups	351,200	128	2,744		
Total	2413,094	159			

Pre-emergency bioassay multiple comparisons resume

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
control 1	1		=								=		=	=	=	=		=	=		=	=	=	=	=	=	=	=	=	=	=	=
control 2	2	=									=		=	=	=	=		=	=		=	=	=	=	=	=	=	=	=	=	=	=
Extract *Citrus limonum*	3				=	=	=	=	=	=		=					=			=													
Extract *Thymus mastichina*	4			=		=	=	=	=	=		=					=			=													
Extract *Origanum vulgare*	5			=	=		=	=	=	=		=					=			=													
Extract *Cinnamomum camphora*	6			=	=	=		=	=	=		=					=			=													
Extract *Allium sativum*	7			=	=	=	=		=	=		=					=			=													
Extract *Rosmarinus officinalis*	8			=	=	=	=	=		=		=					=			=													
1.2mg/L EO *Citrus limonum*	9			=	=	=	=	=	=		=	=	=	=	=	=	=			=		=						=	=				=
1.2mg/L EO *Thymus mastichina*	10	=	=							=			=	=	=	=	=	=	=		=	=	=	=	=	=	=	=	=	=	=	=	=
1.2mg/L EO *Origanum vulgare*	11			=	=	=	=	=	=	=			=			=	=			=													=
1.2mg/L EO *Cinnamomum camphora*	12	=	=							=	=	=		=	=	=	=	=	=		=	=	=	=	=	=	=	=	=	=	=	=	=
1.2mg/L EO *Allium sativum*	13	=	=							=	=		=		=	=		=	=		=	=	=	=	=	=	=	=	=	=	=	=	=
1.2mg/L EO *Rosmarinus officinalis*	14	=	=							=	=		=	=		=		=	=		=	=	=	=	=	=	=	=	=	=	=	=	=
1.2mg/L EO *Artemisia dracunculus*	15	=	=							=	=	=	=	=	=		=	=				=	=				=	=	=				=
1.2mg/L EO *Eucaliptus citriodora*	16			=	=	=	=	=	=	=	=	=	=			=				=														=
0.6mg/L EO *Citrus limonum*	17	=	=								=		=	=	=	=			=		=	=	=	=	=	=	=	=	=	=	=	=	=
0.6mg/L EO *Thymus mastichina*	18	=	=								=		=	=	=			=				=	=	=	=	=	=	=	=	=	=	=	=
0.6mg/L EO *Origanum vulgare*	19			=	=	=	=	=	=	=		=					=																	
0.6mg/L EO *Cinnamomum camphora*	20	=	=								=		=	=	=			=	=			=	=	=	=	=	=	=	=	=	=	=	=
0.6mg/L EO *Allium sativum*	21	=	=							=	=		=	=	=	=		=	=		=		=	=	=	=	=	=	=	=	=	=	=
0.6mg/L EO *Rosmarinus officinalis*	22	=	=								=		=	=	=	=		=	=		=	=		=	=	=	=	=	=	=	=	=	=
0.6mg/L EO *Artemisia dracunculus*	23	=	=								=		=	=	=			=	=		=	=	=		=	=	=	=	=	=	=	=	=
0.6mg/L EO *Eucaliptus citriodora*	24	=	=								=		=	=	=			=	=		=	=	=	=		=	=	=	=	=	=	=	=
0.3mg/L EO *Citrus limonum*	25	=	=								=		=	=	=			=	=		=	=	=	=	=		=	=	=	=	=	=	=
0.3mg/L EO *Thymus mastichina*	26	=	=								=		=	=	=			=	=		=	=	=	=	=	=		=	=	=	=	=	=
0.3mg/L EO *Origanum vulgare*	27	=	=							=	=		=	=	=	=		=	=		=	=	=	=	=	=	=		=	=	=	=	=
0.3mg/L EO *Cinnamomum camphora*	28	=	=							=	=		=	=	=	=		=	=		=	=	=	=	=	=	=	=		=	=	=	=
0.3mg/L EO *Allium sativum*	29	=	=								=		=	=	=	=		=	=		=	=	=	=	=	=	=	=	=		=	=	=
0.3mg/L EO *Rosmarinus officinalis*	30	=	=								=		=	=	=			=	=		=	=	=	=	=	=	=	=	=	=		=	=
0.3mg/L EO *Artemisia dracunculus*	31	=	=								=		=	=	=			=	=		=	=	=	=	=	=	=	=	=	=	=		=
0.3mg/L EO *Eucaliptus citriodora*	32	=	=							=	=	=	=	=	=	=	=	=	=		=	=	=	=	=	=	=	=	=	=	=	=	=	

6.2.2 Post-emergent bioassay

Injury level table

	Post-emergent trials															
	Essential Oils															
	Citrus limonum		*Allium sativum*		*Cinnamomum camphora*		*Eucaliptus citriodora*		*Origanum vulgare*		*Rosmarinus officinalis*		*Thymus mastichina*		*Artemisia dracunculus*	
treatment	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
	5%	7,5%	5%	7,5%	5%	7,5%	5%	7,5%	5%	7,5%	5%	7,5%	5%	7,5%	5%	7,5%
replicate 1	1	5	4	3	1	1	4	5	4	5	1	2	4	5	5	5
replicate 2	2	4	4	4	1	2	4	5	5	4	2	1	3	4	5	5
replicate 3	2	2	2	3	1	1	4	4	4	5	1	2	3	5	4	5
replicate 4	3	3	3	4	1	1	3	5	5	4	2	1	2	4	4	4
replicate 5	1	3	3	4	1	2	4	3	5	5	3	1	2	5	4	4
Mean	1,8	3,4	3,2	3,6	1	1,4	3,8	4,4	4,6	4,6	1,8	1,4	2,8	4,6	4,4	4,6

	Post-emergent trials						
	Extracts						**Controls**
	Citrus limonum	*Allium sativum*	*Cinnamomum camphora*	*Origanum vulgare*	*Rosmarinus officinalis*	*Thymus mastichina*	
treatment	2	3	4	5	6	7	**1**
replicate 1	1	1	0	1	1	2	1
replicate 2	0	0	0	0	1	1	1
replicate 3	1	0	0	0	0	0	1
replicate 4	1	0	0	0	0	1	0
replicate 5	0	1	0	0	0	0	0
Mean	0,6	0,4	0	0,2	0,4	0,8	0,6

Test of Homogeneity of Variances

injury

Levene Statistic	df1	df2	Sig.
2,615	22	92	0,001

ANOVA

injury

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	324,261	22	14,739	35,313	0,000
Within Groups	38,400	92	0,417		
Total	362,661	114			

Post-emergency bioassay multiple comparisons resume

Treatment		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Control	1									≠	≠	≠			≠	≠	≠	≠			≠	≠	≠	≠
Extract *Citrus limonum*	2									≠	≠	≠			≠	≠	≠	≠			≠	≠	≠	≠
Extract *Allium sativum*	3								≠	≠	≠	≠			≠	≠	≠	≠	≠		≠	≠	≠	≠
Extract *Cinnamomum camphora*	4								≠	≠	≠	≠			≠	≠	≠	≠	≠		≠	≠	≠	≠
Extract *Origanum vulgare*	5									≠	≠	≠			≠	≠	≠	≠			≠	≠	≠	≠
Extract *Rosmarinus officinalis*	6									≠	≠	≠			≠	≠	≠	≠			≠	≠	≠	≠
Extract *Thymus mastichina*	7									≠	≠	≠			≠	≠	≠	≠			≠	≠	≠	≠
5% EO *Citrus limonum*	8			≠	≠					≠		≠			≠	≠	≠	≠				≠	≠	≠
7.5% EO *Citrus limonum*	9	≠	≠	≠	≠	≠	≠	≠	≠				≠	≠					≠	≠				
5% EO *Allium sativum*	10	≠	≠	≠	≠	≠	≠	≠					≠	≠						≠				
7.5% EO *Allium sativum*	11	≠	≠	≠	≠	≠	≠	≠	≠				≠	≠					≠	≠				
5% EO *Cinnamomum camphora*	12									≠	≠	≠			≠	≠	≠	≠			≠	≠	≠	≠
7.5% EO *Cinnamomum camphora*	13									≠	≠	≠			≠	≠	≠	≠				≠	≠	≠
5% EO *Eucaliptus citriodora*	14	≠	≠	≠	≠	≠	≠	≠	≠				≠	≠					≠	≠				
7.5% EO *Eucaliptus citriodora*	15	≠	≠	≠	≠	≠	≠	≠	≠				≠	≠					≠	≠	≠			
5% EO *Origanum vulgare*	16	≠	≠	≠	≠	≠	≠	≠	≠				≠	≠					≠	≠	≠			
7.5% EO *Origanum vulgare*	17	≠	≠	≠	≠	≠	≠	≠	≠				≠	≠					≠	≠	≠			
5% EO *Rosmarinus officinalis*	18			≠	≠					≠		≠			≠	≠	≠	≠				≠	≠	≠
7.5% EO *Rosmarinus officinalis*	19									≠	≠	≠			≠	≠	≠	≠				≠	≠	≠
5% EO *Thymus mastichina*	20	≠	≠	≠	≠	≠	≠	≠					≠			≠	≠	≠				≠	≠	≠
7.5% EO *Thymus mastichina*	21	≠	≠	≠	≠	≠	≠	≠	≠				≠	≠					≠	≠	≠			
5% EO *Artemisia dracunculus*	22	≠	≠	≠	≠	≠	≠	≠	≠				≠	≠					≠	≠	≠			
7.5% EO *Artemisia dracunculus*	23	≠	≠	≠	≠	≠	≠	≠	≠				≠	≠					≠	≠	≠			

6.2.2.1 Chlorophyll analysis

Test of Homogeneity of Variances

chlorophyll

Levene Statistic	df1	df2	Sig.
1,551	6	28	0,198

Kruskal-Wallis test

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of VAR00002 is the same across categories of VAR00001.	Independent-Samples Kruskal-Wallis Test	.928	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.