Chemical and biological study of the plant species *Tithonia diversifolia*

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ABSTRACT

With the advance of agriculture and the growing need for ways to control plagues, the large-scale use of synthetic herbicides has been causing some problems, such as soil pollution by persistent herbicide molecules, causing the emergence of resistant weed populations to control methods. Therefore, a lot of studies has been done aiming to find new compounds less toxic and persistent on the environment. The vegetable species Tithonia diversifolia is widely known for its invasive behavior. It is a plant native to Central America that spread across South and North America and across the world as an ornamental plant. A lot of reports on literature discourse about its alelopatic effect on many plant species, causing inhibition of growing and germination of seeds. Such effects are associated to sesquiterpene lactones, characteristic secondary metabolites from Asteraceae family, in which T. diversifolia belongs. In the present work, the extract of leaf washing of T. diversifolia using dichloromethane as solvent, from which the majoritarian compound was isolated and identified. The crude extract of leaf washing was submitted to in vitro phytotoxicity essay with Lactuca sativa seeds. The majoritarian compound was isolated though fractionation of the crude extract on silica flash column followed by a series of dilutions of fractions 1 and 2, and identified though nuclear magnetic resonance as being Tagitinin C. The signals of hydrogen and carbon were confirmed by comparison with data found on the literature. It was obtained a yield of 47,26% from the crude extract. The phytotoxicity essay with the crude extract of leaf washing reduced the growth of seedlings of L. sativa, however, exhibit less activity when compared with the herbicide Flumyzin®.

Keywords: Herbicide, Asteraceae, Natural products, Sesquiterpene lactones, Phitotoxicity.

1 INTRODUCTION

With the increasing demand for mass food production, agriculture has become one of the most important human activities. Several techniques have been developed throughout history, with the aim of producing more and more food to supply a population in constant ascendancy. That said, there is a lot of investment in studies that seek to optimize crop production yields and minimize the costs associated with them. Among the adversities faced by producers, weeds stand out as the main cause of crop losses, exceeding all other forms of pests, such as insects, nematodes, diseases and rodents (ABOUZIENA; HAGGAG, 2016). It is estimated that about 20 to 30% of crop expenditures are allocated to weed control (ARANTES, 2007; SANTOS, 2009). Weeds reduce yields, hinder harvesting, and reduce food quality due to contamination with seeds and impurities (ARANTES, 2007), which is why herbicides are the most consumed type of pesticide in Brazil, representing more than 50% of this total (SANTOS, 2009; OFOSU *et al*., 2023).

Research dedicated to the study of herbicides between the 1950s and 2000s focused only on synthetic herbicides (VYVYAN, 2001). However, the large-scale use of synthetic herbicides has presented a number of problems for both human health and the environment. Among these problems, the effect of the herbicide on non-target plants, residues in soil and water, toxicity to non-target organisms, emergence of resistant plant populations, and harm to human health and safety can be highlighted (ABOUZIENA; HAGGAG, 2016). Among these problems, the emergence of resistant plants stands out, which has become the target of research related to the study of synthetic herbicides. The emergence of resistant weeds is closely linked to the accumulation of synthetic herbicide residues that are difficult to degrade. These substances exert a selection pressure on plant populations, selecting biotypes that are resistant.

Herbicide resistance is a natural and heritable characteristic of some biotypes present in a given population, which allows individuals who have such biotypes to be able to grow and reproduce after exposure to a dose of a herbicide that would be lethal to a normal population (CHRISTOFFOLETI; LÓPEZ-OVEJERO, 2003). There are five primary mechanisms of herbicide resistance: 1) Target-site resistance, the result of a mutation that alters the herbicide's binding site, often in an enzyme, preventing or reducing the herbicide's ability to bind; 2) Metabolism enhancement, so as to increase the plant's ability to metabolize the herbicide compound; 3) Decreased uptake and/or translocation, which may result in restriction of the herbicide's movement towards the site of action; 4) Sequester of the herbicide compound into the cell wall or vacuole, reducing the concentration of herbicide that reaches the site of action; 5) Amplification or overexpression of the gene for the production of the target enzyme, which generates the need for higher concentrations of herbicide to cause the death of the plant (HEAP, 2014). According to Heap (2014), the classes of herbicides that most selected resistant biotypes are acetolactate synthase inhibitors (ALS), photosystem II inhibitors, acetyl-CoA carboxylase

inhibitors (ACCase), synthetic auxins, bipyridilliums, and glyphosate. On the other hand, Ofosu *et al*. (2023) reports cases of resistance to 5-enol pyruvilxikimate-3-phosphate synthase (EPSP) inhibitors and fatty acid synthesis inhibitors, in addition to those mentioned above. ALS inhibitors are especially resistant in weed species. They have an active site in the enzyme acetolactate synthase, which is an enzyme vulnerable to mutations that confer resistance by the mechanism of alteration of the target site (EBERLEIN *et al*., 1999; HEAP, 2014).

In addition to the mechanism of action, factors related to the bioecological characteristics of the plant species can also define the potential for the development of herbicide resistance. Such factors are a short life cycle, high seed production, low seed dormancy, several reproductive generations per year, extreme susceptibility to a particular herbicide, and great genetic variety (CHRISTOFFOLETI; LÓPEZ-OVEJERO, 2003).

Many synthetic herbicides, like pharmaceuticals, are based on natural compounds. About 70% of new pesticides are registered with ingredients that originate from natural product research. However, only 8% of conventional herbicides are derived from natural compounds and 7% are natural compounds (DAYAN; DUKE, 2014). Such compounds originate from the secondary metabolism of plants, which produce them to protect against herbivory, fungi, bacteria, viruses, nematodes and give a competitive advantage to the species, increasing the chance of survival (BALBINOT-JÚNIOR, 2004).

Allelopathy is defined as the effects, usually negative, exerted by one species of plant on the development of other species. Such effects are caused by secondary metabolites produced by the invasive species and released into the environment. Allelochemicals, as they are called, can be released by the plant by the processes of volatilization, root exudation, leaching, and decomposition of the plant body (MIRANDA *et al*., 2015). The understanding of allelopathic relationships between plant species is a great ally in determining strategies to combat invasive plants (LOPES, 2016).

There is a growing concern for new herbicide formulations that are safer, both for human health and the environment, and allelochemicals may represent good substitutes as synthetic herbicides. In general, compounds of natural origin are less aggressive due to easy chemical and biological degradation (BALBINOT-JUNIOR, 2004; DAYAN; DUKE, 2014). Not to mention that working with natural compounds has the advantage that their biological activity is already established and, generally, they have a diversity of biosynthesized carbon skeletons that exhibit a range of biological activities associated with them (DAYAN; ROMAGNI; DUKE, 2000). For this reason, research on the isolation and phytotoxicity testing of allelopathic compounds from invasive plants has gained ground, with the objective of obtaining new compounds as potential natural herbicides and in the elucidation of new mechanisms of action (DAYAN; DUKE, 2014; MIRANDA *et al*., 2015).

The plant species *Tithonia diversifolia*, belonging to the Asteraceae family, tribe Heliantheae, is an invasive plant that has a high growth rate (ORSOMANDO *et al*., 2016). This species has great allelopathic potential and is capable of dominating hectares of agricultural and non-agricultural land, becoming a weed and disturbing native plant communities. *T. diversifolia* has allelopathic potential in the germination and growth of several plant species (KATO-NOGUCHI, 2020) and, as an invasive plant, it is a possible source of allelochemicals with phytotoxic potential (ALVES *et al*., 2011). The present work discusses the potential of natural products derived from the plant species *T. diversifolia* as phytotoxic agents.

1.1 FAMILY ASTERACEAE

The Asteraceae family is the largest family of angiosperms, comprising 1,600 genera present on the planet. In Brazil, there are about 180 genera and 1,900 species distributed across different biomes (ROQUE; BAUTISTA, 2008; BROOK; SILVA; CASTRO, 2010). It represents 10% of the world's angiosperm flora and has been intensively studied in terms of anatomy, morphology and ecology, as well as in terms of its phytochemistry, metabolomics and macromolecular structure (NAKAJIMA; SEMIR, 2001). The family is represented by plants with very varied characteristics, which occupy a wide range of habitats, prevailing in areas with a tropical climate. They are represented by small plants, which can be herbaceous or shrubby, rarely arboreal (VERDI; BRICHENTE; PIZZOLATTI, 2005). A striking feature of the family is the capitulated inflorescences, whose flowers are inserted in a wide and rounded receptacle with a discoid shape containing bracts (SANTOS, 2019). Chapter cover can contain from one to more than 500 flowers whose ovary is inside the receptacle and is uniovulated (ROQUE; BAUTISTA, 2008; SANTOS, 2019).

Species of the Asteraceae family are widely studied from a phytochemical point of view because they present a diverse range of biologically active secondary metabolites, already presenting some compounds that provide the development of drugs, insecticides and other natural products (VERDI; BRICHENTE; PIZZOLATTI, 2005). The ethnobotanical use of Asteraceae species is widely reported in various parts of the globe for various purposes (HEINRICH *et al*., 1998; MARTUCCI, 2016). In addition to their therapeutic use, they are also explored in food, cosmetic production and as ornamental plants (ROQUE; BAUTISTA, 2008).

Of the therapeutic compounds associated with plants of the Asteraceae family, flavonoids, polyacetylenes, coumarins, terpenoids and sesquiterpene lactones (ROQUE; BAUTISTA, 2008; MARTUCCI, 2016; KATO-NOGUCHI, 2020). Sesquiterpene lactones are compounds that have a diversity of biological activities, among them they have high phytotoxic potential. They are one of the classes of compounds associated with the evolutionary success and invasiveness of species, which have developed a unique chemical defense system (ROQUE; BAUTISTA, 2008). Therefore,

sesquiterpene lactones may be good candidates for the study of new herbicides based on natural products (ARANTES, 2007; SANTOS, 2009; MIRANDA *et al*., 2015).

1.2 TRIBO HELIANTHEAE

The tribe Heliantheae is the most diverse and largest tribe of the family Asteraceae, considered by many taxonomists to be one of the most primitive taxa of Asteraceae (CHRISTENSEN; LAM, 1990; OLIVE TREE; SILVA; BARROS, 2007). The species that make up the tribe are characterized by having alternate or opposite leaves, usually trinervated, with a terminal inflorescence that can be paniculiform, corymbiform or in a capitulum with bisexual flowers (ALVES; ROQUE, 2016). They are found in regions with tropical and subtropical climates, with few representatives in regions with a temperate climate (OLIVEIRA; SILVA; BARROS, 2007). The tribe is composed of 113 genera comprising 1,460 species spread throughout Central and South America, and in Brazil there are about 60 genera and 374 species (OLIVEIRA; SILVA; BARROS, 2007; ALVES; ROQUE, 2016).

The relationships between Heliantheae species, as well as the relationships of the tribe with other closely related taxonomic groups, were proposed from chemotaxonomic studies, which revealed different classes of compounds found in the tribe, such as flavonoids, acetylenes and sesquiterpene lactones (CHRISTENSEN; LAM, 1990). The sesquiterpene lactones characteristic of this tribe are produced and stored in the glandular trichomes present in the aerial parts of plants (ROCHA, 2009, SILVA *et al*., 2017). Such compounds occur in species of the tribe Heliantheae with an enormous variety of carbon skeletons (STEFANI, 2006), which opens space for several studies on natural products that the species of this tribe can provide, in view of the diversity of biological activities presented by sesquiterpene lactones (SCHMIDT, 2006). Among the most well-studied species of the tribe Heliantheae, the present work deals with the species *T. diversifolia.*

1.3 *TITHONIA DIVERSIFOLIA*

The plant species *T. diversifolia* is a shrubby plant that can reach up to 4 meters in height, has erect and very branched branches, alternate, petiolate leaves, usually divided into 3 to 5 lobes. The flowers, 12 to 14, are inserted peripherally in a capitul inflorescence that is surrounded by the corolla whose sepals resemble petals of a simple flower of bright yellow color (PÉREZ *et al*., 2009). The inflorescence and leaves of *T. diversifolia* are shown in figure 1.

It is the best-studied species in the genus *Tithonia*. It exhibits highly invasive behavior and has a high rate of biomass production (ORSOMANDO et al., 2016). This plant can produce 80,000 to 160,000 seeds per m2, with germination rates between 18 and 56% at 25ºC (AJAO; MOTEETEE, 2017). However, there are several reports of the use of *T. diversifolia* for agricultural and therapeutic purposes. The ability of this species to adapt to nutrient-poor soils and to recover these soils is reported

in the literature (OLABODE *et al*., 2007), in addition to being able to grow and colonize soils polluted by heavy metals, being able to remove metals from the environment and store them in their tissues (AYESA; CHUKWUKA; ODEYEMI, 2018). The species also has a high content of nutrients such as nitrogen, potassium and phosphorus, making it a good fertilizer alternative for soil management (OLABODE et al., 2007). In addition, the high nutrient content makes the species good to be used as fodder for animals (ORSOMANDO *et al*., 2016; AJAO; MOTEETEE, 2017).

Figure 1 – Specimen of *T. diversifolia*

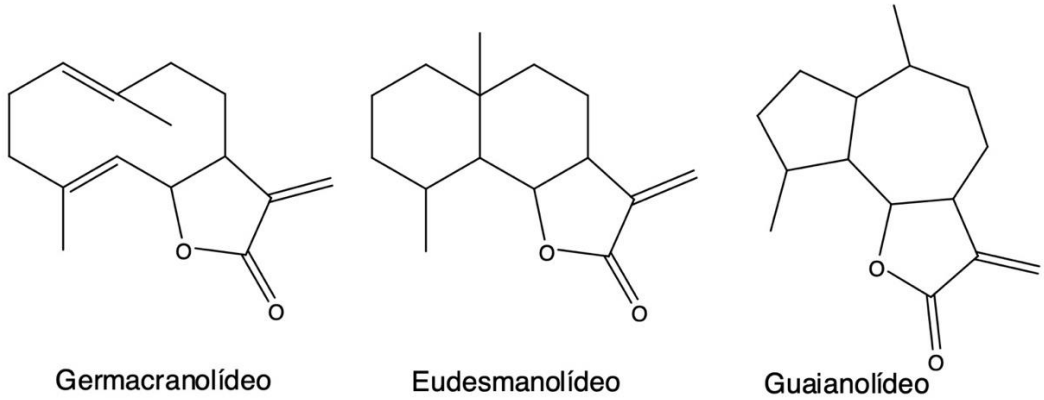
Source: The Authors (2023)

Several studies seek to elucidate the chemical constituents of *T. diversifolia.* The presence of flavonoids, sesquiterpenoids, diterpenoids and, in smaller amounts, phytosterols, xanthans, coumarins, ceramides, chromones and chromenes has been reported (CHAGAS-PAULA *et al*., 2012). There are also reports of the presence of tannins, alkaloids and saponins (HERRERA; VERDECIA; RAMÍREZ, 2020). Orsomando *et al*. (2016) identified 161 volatile compounds in *T. diversifolia,* with monoterpenes standing out, which accounted for 46.9% of the volatile oil composition. The most present compounds in volatile oil are -αpinene, limonene, and cis-chrysantenol (ORSOMANDO *et al*., 2016). The most representative class of secondary metabolites of the species is sesquiterpene lactones (CHAGAS-PAULA *et al*., 2012; PASSONI *et al*., 2013; SILVA *et al*., 2017). Such compounds give *T. diversifolia* a high allelopathic potential, which influences the germination and growth of several plant species (CHAGAS-PAULA *et al*., 2012). Many secondary metabolites of *T. diversifolia* have been studied regarding their biological activities and applicability. Due to the high production of biomass and biologically active metabolites, T. diversifolia has the potential to be exploited as a source of natural products for therapeutic and agricultural purposes (CHAGAS-PAULA *et al*., 2012). Tagitynin C is a secondary metabolite of *T. diversifolia*, of the sesquiterpene lactone class, produced in the glandular trichomes of leaves (SILVA et al., 2017).

1.3.1 Sesquiterpene Lactones

Sesquiterpene lactones are highly distributed secondary metabolites of plants, which have a great diversity of structures and metabolic activities (SCHMIDT, 2006). This class of compounds is a striking characteristic of the Asteraceae family and its diversity of structures is used as markers for chemotaxonomic studies of the groups belonging to this family (CHAGAS-PAULA *et al*., 2012). This class of molecules is characterized by having a 15-carbon skeleton that is classified according to its arrangement. The main groups of STLs are the germacranoids, eudesmanolides, and guaianolids, and the most representative minor subgroups are the heliangolides and pseudoguayanollids (PADILLA-GONZALEZ; SAINTS; DA COSTA, 2016). Figure 2 represents the carbon skeletons of the major classes of STLs.

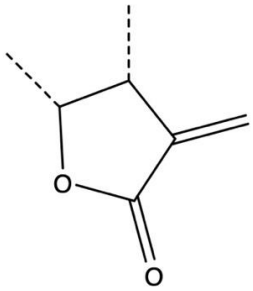
Figure 2 – Carbon skeletons of the main classes of sesquiterpene lactones



Fonte: Santos (2009)

The γ -lactone ring is present in the vast majority of STLs, containing an exocyclic methylene conjugated with a carbonyl (figure 3), and this part of the molecule is responsible for the diversity of biological activities (PADILLA-GONZALEZ; SAINTS; DA COSTA, 2016). The biological activities associated with STLs often occur through a mechanism of action based on interference in the function of cellular macromolecules through covalent bonds of the Michael addition type between the electrophilic part of the lactone and the nucleophilic center of the biological targets, which leads to the alkylation of the biological molecules (SCHMIDT, 2006). Figure 3 shows a common structure of the lacton ring. One of the activities presented by STLs is phytotoxicity, many of which affect the germination and development of some plant species, so they can be explored as models for new herbicides (ARANTES, 2007).

Figure 3 – General Chemical Structure of the Lactone Ring



Fonte: Santos (2009)

The plant species *T. diversifolia* produces large amounts of the sesquiterpene lactones Tagitynins A and C in its glandular trichomes (STEFANI, 2006). It has been reported that such compounds reduce seed germination and seedling growth, suggesting the possibility that these compounds are responsible for the invasive behavior and high allelopathic potential of the species (MIRANDA et al., 2015; KATO-NOGUCHI, 2020). The present work aims to explore the phytotoxic activity of extracts and compounds of *T. diversifolia*, and to evaluate the potential of this plant species as a source of natural products for agricultural purposes.

1.4 SESQUITERPENE LACTONES DERIVED FROM *TITHONIA DIVERSIFOLIA* AS AN ALTERNATIVE TO SYNTHETIC HERBICIDES

Herbicides are the most consumed class of pesticides among farmers. In 2019, the global pesticide market reached values close to $84.5 billion, and accounts for 51.9% of agricultural product sales (OFOSU *et al*., 2023). However, the large-scale use of synthetic herbicides is leading to a problem faced by rural producers, which is the emergence of weed populations resistant to commonly used products.

Heap (2014) conducted a data survey through the website called "The International Survey of Herbicide-Resistant Weeds", in which registered users and scientists from all over the world can register cases of pesticide resistance occurring in agriculture. According to the author, in 2013, there were 404 unique cases of a relationship between plant species and site of action of the herbicide. 220 plant species showed resistance to one or more herbicide mechanisms, 130 dicots and 90 monocots. The site presented data on resistant populations in 61 countries, with the countries with the highest records of occurrence being the United States, Canada, Australia, and France, respectively. In the year of publication of the article, Heap (2014) found that Brazil ranked eighth in the ranking of countries with the most cases of herbicide resistance, with 31 cases.

In a new search on the website's page carried out by the author in 2023, a decade after the one reported by Heap (2014), the page had 523 unique cases of species x herbicide action site relationship, with 269 plant species that showed resistance to one or more herbicide mechanisms of action, 154

dicotyledonous and 115 monocot. In 2023, the site presented cases of resistant populations in 72 countries and, in addition, Brazil came to occupy fourth place among the countries with the highest occurrence of resistant populations with 49 cases, behind only the United States, Canada, and Australia (http://www.weedscience.org). Comparing the data presented by Heap (2014) and those presented by the page after a decade, it is possible to observe that the problem of resistant populations is something that occurs all over the world, and there is a notable increase in the number of species that are becoming resistant to commercial synthetic herbicides. This poses a major problem for food production in the world because weeds compete for space and nutrients with cultivated plants, which reduces yield and crop quality. According to Hussein (quoted by ABOUZIENA; HAGGAG, 2016), 0.19 kg of weed dry matter results in 1 kg of loss of onion bulb yield, and allowing weeds to grow close to harvest can lead to the removal of 36.9; 9.6 and 57.0 kg per acre of soil nitrogen, phosphorus, and potassium, respectively.

That said, there is a great incentive to the search for natural products, extracted from plants, which can be as effective as synthetic herbicides, but which present less damage to environmental health (BALBINOT-JUNIOR, 2004). Allelochemicals originating from plants are a promising source in the search for new herbicidal compounds or new mechanisms of action (ARANTES, 2009). The invasive behavior and allelopathic potential of *T. diversifolia* makes the species the target of research regarding its allelochemicals and its potential as a source of herbicidal compounds.

2 METHODOLOGY

2.1 COLLECTION AND DRYING OF PLANT MATERIAL

The leaves of *Tithonia diversifolia* were collected in the municipality of Alegre – ES, Latitude -20.7633 Longitude -42.5339 (20º 45' 48" South and 41º 32' 2" West), and identified by researchers from the Federal University of Espírito Santo, *Alegre campus*. A total of 1,905 kg of plant material were collected. The material was dried in an oven at 50ºC for 48 hours. After drying the plant material, the leaves were taken to the Laboratory of Organic Chemistry and Pharmacognosy, located at UFES *Alegre campus*, to obtain the dichloromethane extract.

2.2 EXTRACT PRODUCTION AND COMPOUND ISOLATION

The extract was prepared from the washing of the leaf surface of *T. diversifolia*, since in the literature it is reported that the substances of interest, the sesquiterpene lactones, are produced and stored in the glandular trichomes of species of the tribe Heliantheae (ROCHA, 2009; PAULA *et al*., 2018). The solvent used was dichloromethane, chemical formula CH2Cl2. The choice of solvent was based on polarity studies of sesquiterpene lactones, which have good solubility in nonpolar solvents, such as acetone, ether and dichloromethane (SCHMIDT, 2006; SILVA *et al*., 2016). The extract was

obtained by washing the leaf surface using 6L of solvent for a period of 3 minutes, until the glandular trichomes disappeared, and then it was filtered and concentrated in a rotaevaporator. After all the solvent has dried, the crude extract was ready.

The isolation of the compounds occurred through several chromatographic techniques in stationary phases of silica and a series of dilutions in solvents with extreme polarities. Initially, the crude extract was analyzed by thin layer chromatography (CCD) in order to analyze the best combination of organic solvents that could be used to conduct the fractionation of the extract. The solvent combination selected was Methanol:Ethyl Acetate:Dichloromethane acidified with acetic acid. After analysis by CCD, a column of normal-phase flash silica gel was performed at moderate pressure, following the concentrations described in Table 1. The fractions obtained were analyzed by CCD, in order to identify whether the fraction was a mixture or a pure compound.

Table 1 – Concentrations used in the fractionation of the crude extract of dichloromethane from *T. diversifolia*

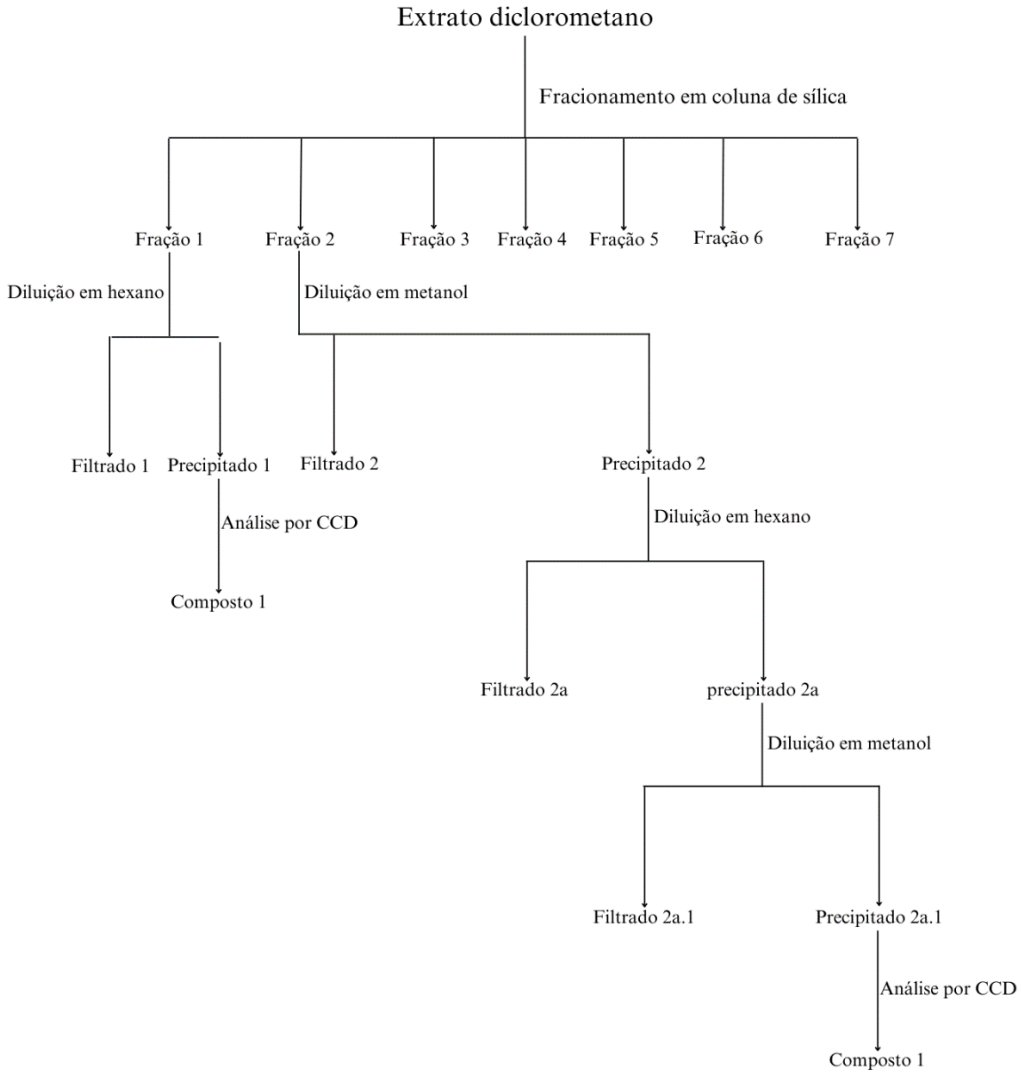
Methanol	**AcOEt**	**CH2Cl2**
*5%	*0%	*95%
5%	0%	95%

Note: * Fraction not acidified with acetic acid (0.01%).
Source: The Authors (2023)

The first fraction, Methanol:CH2Cl2 (5%:95% - without acetic acid) was diluted in Hexane forming a precipitate. The liquid phase was separated from the precipitate by filtration with a glass and cotton pipette. The precipitate was analyzed by CCD revealing the pattern of only one stain, thus being a pure compound.

The second fraction, Methanol:CH2Cl2 (5%:95% - acidified with acetic acid) formed a precipitate when diluted in Methanol for analysis. The liquid phase was separated from the precipitate with a glass and cotton pipette, and the precipitate was subjected to dilution in hexane, forming a new precipitate. The new precipitate was again diluted in Methanol, generating a third precipitate. This was analysed in CCD revealing only one stain. In a comparative analysis by CCD, it was possible to observe the same pattern of the compound obtained from the first fraction, being called compound **(1).** The flowchart shown in figure 4 shows the path taken to obtain the compost (1). Therefore, the fractions of compound 1 were mixed and taken to be identified by physical methods of compound identification.

Figure 4 – Flowchart of the path taken to obtain the compost (1)



Source: The Authors (2023)

2.3 IDENTIFICATION OF THE MAJOR COMPOUND

The identification of the isolated majority compound through the technique described in the previous item took place at the UNIFAL campus, in the city of Alfenas – MG, Brazil. The equipment used was a Tesla Bruker 7.05 spectrometer, model AC-300 located in the Nuclear Magnetic Resonance Laboratory, operated at 300 MHz in the hydrogen frequency and 75 MHz in the carbon frequency. The solvent used was deuterated chloroform.

2.4 PHYTOTOXICITY BIOASSAY

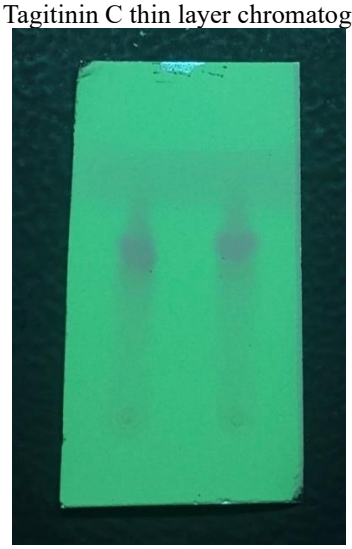
The phytotoxicity test was carried out at the Chemistry Laboratory of the Federal Institute of Espírito Santo, located in Rive, municipality of Alegre. Five solutions made at 70:30, water:dichloromethane, with concentrations of T. diversifolia crude extract µ equal to 3000, 1500, 750, 375 and 187.5 ppm ($^{g.mL-1}$) in vitro *seeds were tested*. A solution of distilled water with the solvent

dichloromethane (70:30) was used as a negative control (C-), and the herbicide Flumyzin® was used as a positive control (C+) at the concentration of use indicated by the manufacturer. Each treatment was performed on *L. sativa* seeds arranged in Petri dishes lined with filter paper moistened with the crude extract solutions. The Petri dishes were arranged in a completely randomized design and deposited in a BOD incubator at 24ºC throughout the experiment. The percentage of germinated seeds was observed after 8, 16, 24, 32, 40 and 48 hours of exposure to the treatments. Root and shoot growth were determined after 48 and 96 hours, respectively, of exposure to the concentrations used, with the aid of a digital caliper. From the data obtained, the following variables were evaluated: percentage of germination after 48 hours (%F), germination speed index (IVG) calculated according to Maguire (1962): IVG = G1/N1 + G2/N2 + ... + Gn/Nn, where G1, G2, Gn= number of seedlings germinated from the first to the last count and N1, N2, Nn= number of days from the first to the last count.

3 RESULTS AND DISCUSSION

The major compound was isolated and identified as Tagitynin C, which corroborates data in the literature (ROCHA, 2009; PASSONI *et al*., 2013; MIRANDA *et al*., 2015; SILVA *et al*., 2017; KATO-NOGUCHI, 2020). Tagitinin C was supplied by the first and second fractions from the fractionation of the crude extract of *Tithonia diversifolia* in a flash silica column with moderate pressure, using a 95:5 solution of CH2Cl2:MeOH as eluent, the first fraction without the addition of acetic acid and the second with the addition of acetic acid. Tagitinin C was identified as a pure compound by thin layer chromatography analysis, which revealed only one stain. The compounds isolated from fractions 1 and 2 were identified as the same compound because they had the same retention factor (Figure 5). The eluent used was 50:30:20 hexane:ethyl acetate:dichloromethane with the addition of acetic acid.

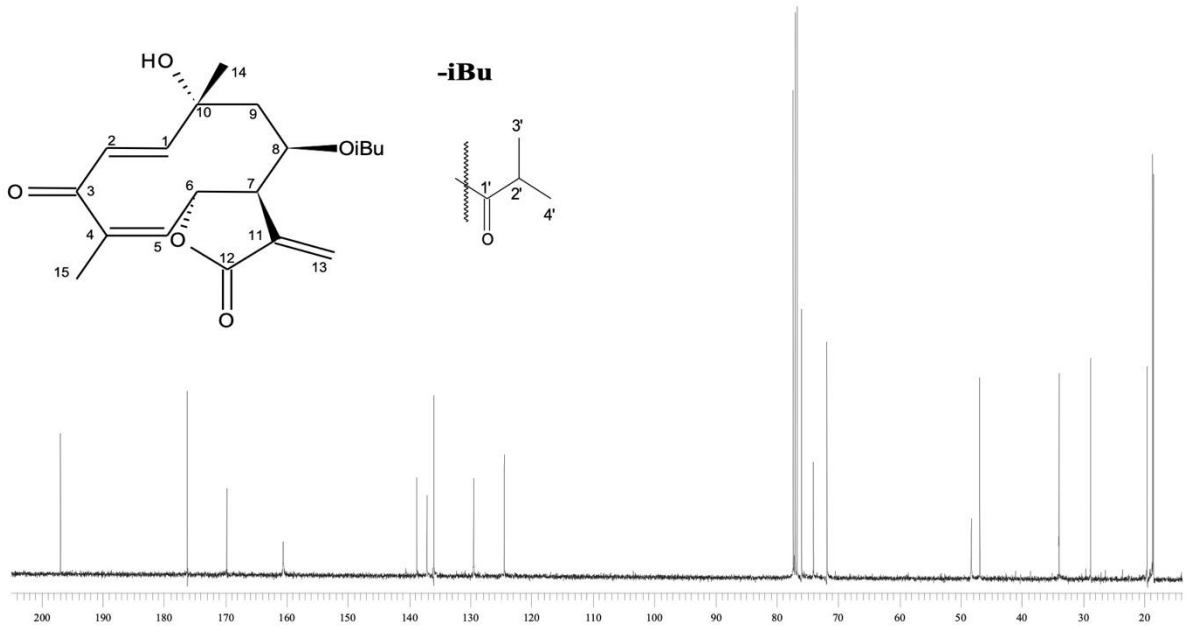
Figure 5 – Tagitinin C thin layer chromatography plate



Source: The Authors (2023)

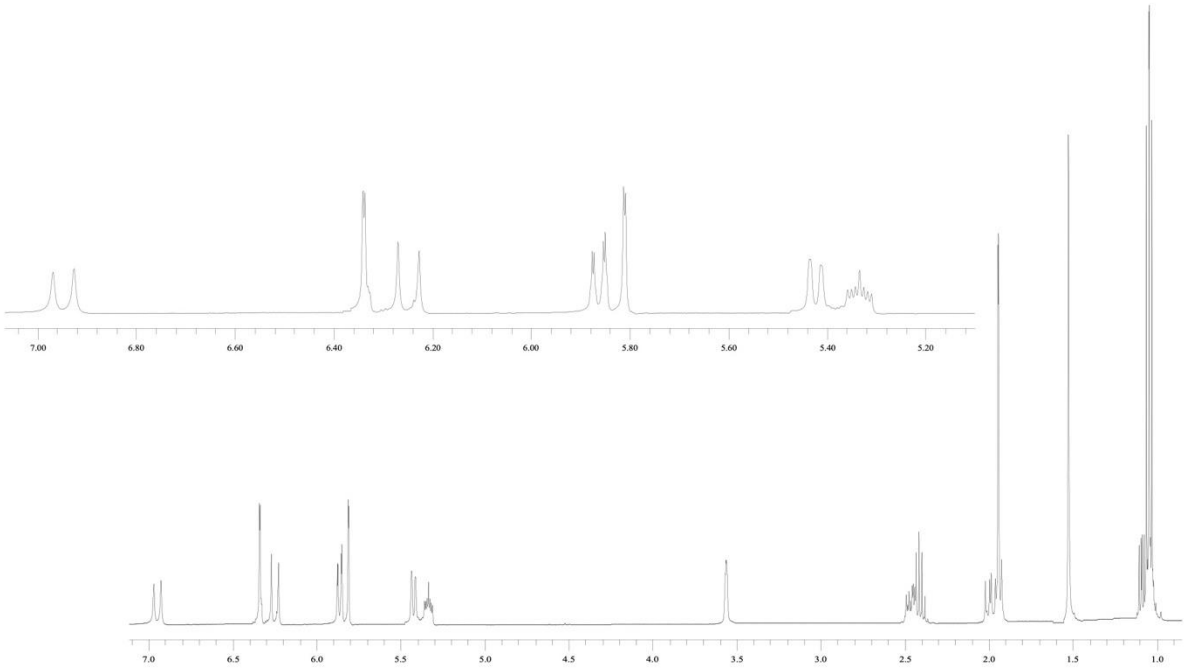
Confirmation of the chemical structure of Tagitinin C was achieved by nuclear magnetic resonance analysis of 13C and 1H. Figures 6 and 7 show the spectrum of 13C and 1H, respectively. The carbon spectrum showed the values of displacement of the carboxyl carbons in the conjugated ketoneδ, resonating at C 197.0 (C-3), in the ester group, resonating at C 176.2 (C-1') and in the lacton ring, resonating at C 170.0 (δ C-12 δ). In addition to them, it also showed the quaternary carbon attached to the methylene group in the lacton ring resonating at C 136.0 (C-11), another quaternary carbon resonating at C 72.0 attached to the hydroxyl group and a methyl δ (C-10), and another quaternary carbon δ in the double bond. The carbons and hydrogens of the double bonds showed displacement values equal to C 160.5/H 5.82 (C-1), C 129.5/H 6.25 (C-2) and C 137/δH 5.42 δ (C-5), C δ $_{139.0}$ δ (δδ δ $_{C-4}$). The spectra show the remaining carbons of the lacton ring resonating at C δ $_{76.0}$/H 6.99 for the oxygen-bound carbon (C-6) and C 48.3/H δ 3.58 for the tertiary δ_{carbon} (δC-7). The chiral carbon attached to the ester group has a carbon and hydrogen signal resonating at C $\delta_{74.0}$/H δ 5.35 (C-8). The chiral center is attached to a methylene whose hydrogens showed different displacement signals, resonating at C $\delta_{47.0}$/H δ 2.00 and 1.10 (C-9). The chiral center also influences the displacement values of methylene bound to the lacton ring, whose signs were present in C δ $_{129.5}$/H 6.35 δ and 5.80 (C-13). The spectra obtained for the compound (1) were compatible with the data presented in the literature for Tagitynin C (SANCHÉZ-MENDOZA *et al*., 2011). Table 2 shows all the 13C and 1H displacement signals presented by the Tagitinin C nuclear magnetic resonance spectrum.

Figure 6 – Molecular structure of Tagitynin C and 13C nuclear magnetic resonance spectrum



Source: The Authors (2023)

Figure 7 – 1C nuclear magnetic resonance spectrum of Tagitynin C



Source: The Authors (2023)

Table 2 – 13C and 1H shift signals from Tagitinin C nuclear magnetic resonance spectra

Position	13C (75 Hz)	13C (Lit.)	1H (300 Hz)	1H (Lit.)
1	160,5	160,1	5.82 (dd, 15Hz)	6,94 (d, 17.1 Hz)
2	129,5	129,6	6,25 (d, 15Hz)	6,25 (d, 17.1 Hz)
3	197,0	196,7	-	-
4	139,0	138,8	-	-
5	137,0	137,2	5,42 (d, 8Hz)	5,87 (d, 9.0 Hz)
6	76,0	75,9	6,99 (d, 8Hz)	5,41 (d, 9.0 Hz)
7	48,3	47,0	3.58 (quartet, 3Hz)	3,54 (m)
8	74,0	73,9	5.35 (septeto, 3Hz)	5,30 (m)
9.a	47,0	48,3	2,00 (f, 16 e 8Hz)	2,42 (f,14.1 e 4.2 Hz),
9.b	47,0	48,3	1,10 (f, 2 e 8Hz)	2,02 (f, 14.1 e 4.2)
10	72,0	71,9	-	-
11	136,0	136,0	-	-
12	170,0	169,7	-	-
13.a	129,5	124,9	6,35 (f, 8Hz)	6,35 (d, 1.8
13.b	129,5	124,9	5,80 (f, 8Hz)	5,81 (d, 1.8)
14	19,8	19,0	1,51 (s)	1,54 (s,
15	29,0	29,0	1,92 (s)	1,95 (s
1’	176,2	176,2	-	-
2’	34,0	30,0	2,50 (m, 6Hz)	
3’	18,4	18,8	1,05 (d, 8Hz)	1,05 (d, 6.9
4’	18,6	18,6	1,05 (d, 8Hz)	1,07 (d, J 6.9 Hz

Source: The Authors (2023)

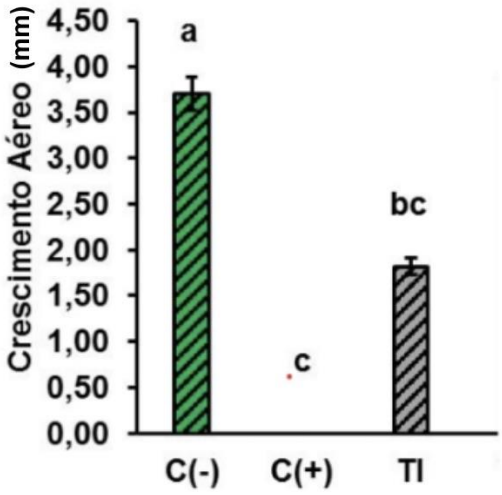
Tagitinin C is a sesquiterpene lactone of the heliangolide type (PAULA, 2018) whose molecule is lipophilic (ROCHA, 2009), which explains the large amount of this compound in extracts prepared from less polar solvents, such as dichloromethane. This is demonstrated in the study carried out by Passoni *et al*. (2013) by performing the identification and quantification of the classes of compounds

in different extracts *of T. diversifolia*, resulting in an extraction of a large amount of Tagitinin C in the extract of the plant's leaf wash with acetone and, on the other hand, there was no detection of this compound in the polar extract. A mass of 2.5 grams of the compound was obtained from 5.29 grams of crude extract, obtaining a yield of 47.26%, presenting a satisfactory yield.

For the phytotoxicity bioassay, the extract made from the washing of the leaf surface of *T. diversifolia* with dichloromethane was used. On the leaf surface is where the glandular trichomes are found, which are the structures that produce and store sesquiterpene lactones such as Tagitynin C (SILVA *et al., 2017), whose inhibitory activity on the development of several plant species is reported in the literature (MIRANDA et al., 2015*; KATO-NOGUSHI, 2020). In the present work, the germination of *Lactuca sativa* (lettuce) was used to demonstrate the phytotoxic effect of the extract. The percentage of germinated seeds was observed every 8 hours until the completion of the first 48 hours, and the measurement of roots and aerial parts occurred after 48 and 96 hours of exposure to the treatment. Figure 8 represents the result of the inhibition of germination and growth *of L. sativa* by the crude extract of *T. diversifolia* compared to the negative control (water:dichoromethane solution 70:30) and the positive control (Flumizyn® herbicide).

It is possible to observe that the crude extract *of T. diversifolia* showed inhibition of the development of *L. sativa seedlings*. However, the inhibition was about 50% lower when compared to the positive control, meaning that the extract has reasonable phytotoxic activity. The study was conducted during the initial development of the target plant, as they are more sensitive at this stage (LOPES, 2016).

Figure 8 – Result of the bioassay of phytotoxicity of *T. diversifolia* extract against the development of *L. sativa*



Source: The Authors (2023)

Sesquiterpene lactones, in general, are known for their potential as phytotoxic agents that can be exploited as models for new herbicides (ARANTES, 2007; RIAL *et al*., 2016; DE OLIVEIRA, 2020). Santos (2009) investigated the phytotoxic activity of several sesquiterpene lactones synthesized on the root growth of *Sorghum bicolor* (sorghum) and *Cucumis sativus (cucumber*), whose percentage of inhibition was higher than 50% for some lactones. In addition, it has been shown to increase the efficacy of inhibition when the compound is encapsulated with carriers, such as SO3Hcx6 and β-cyclodextrin, whose encapsulated lactone increased from an inhibition percentage of 24.7% to 62.2% (SANTOS, 2009). Martins (2022) observed a dose-dependent inhibition of root growth of *Bidens pilosa* by the lapidolid sesquiterpene lactone extracted from *Lapidia aplicifolia*, another plant species belonging to the Asteraceae family. Rial *et al*. (2016) tested the phytotoxicity of several sesquiterpene lactones, isolated from the genera *Decachaeta,* Salvia *and Podachaenium*, against the development of barnyardgrass and brachiara, and concluded that 4 of the 5 sesquiterpene lactones that showed better inhibition were of the heliangolid type.

The results shown in the present work were obtained through the guided experiment at the Federal Institute of Espírito Santo and represent preliminary results of a series of bioassays to be carried out, using new forms of extraction and also using isolated compounds, such as Tagitynins C, A and F. Through the results obtained it is possible to observe that the extract has phytotoxic potential, despite being smaller when compared to herbicide. However, in the literature, it is possible to verify the difference in the herbicide potential of different extracts, produced from different solvents and forms of extraction of *T. diversifolia.* Miranda *et al*. (2015) observed differences in the phytotoxic activity of extracts from different parts of the plant made from ethyl acetate and methanol, finding that the leaf extract produced with AcOEt showed higher phytotoxicity, which corroborates the fact that phytotoxic compounds are produced and stored in aerial parts. Kato-Noguchi (2020) conducted a survey in which phytotoxic activity of aqueous extracts of the leaves and shoots of *T. diversifolia* was reported against the growth and germination of several plants, such as rice*, B. pilosa*, corn, Amaranthus cruentus*, barley, cabbage, cucumber, onion, radish, tomato*, Sorghum bicolor*, among others.*

The target species selected for the phytotoxicity bioassay was *Lactuca sativa*, which is a standard target species for this type of experiment. Growth-inhibiting activity of *L. sativa* is widely reported by extracts of *T. diversifolia* (MIRANDA *et al*. 2015; KATO-NOGUSHI, 2020). Henzel (2022) found, through experiments, that aqueous extracts of *T. diversofolia* in different concentrations delayed the germination of *L. sativa seeds,* decreased the germination speed index, and caused morphological changes in germinated seedlings. On the other hand, Miranda *et al*. (2015) observed an inhibition rate equal to that of the herbicide Logran® against *L. sativa* for extracts produced with methanol and AcOEt of *T. diversifolia*, being the most active AcOEt extract. Therefore, it is possible

to say that the extracts of the plant species *T. diversifolia* have herbicidal potential and can be explored as candidates for natural products for agricultural purposes.

Several factors related to the procedures performed in the production of extracts and isolation of substances can interfere with the degree of biological activity of the natural product. The extraction process must be designed in order to select the metabolites of interest, taking into account the extraction efficiency, stability of the substances, availability of the media, process costs, and purpose of the extract (DE OLIVEIRA, 2020). Silva *et al*. (2017) conducted a comparative study of two forms of extraction of Tagitinin C taken from *T. diversifolia*, ultrasound-assisted extraction and dynamic maceration, and observed that ultrasound-assisted extraction was able to remove greater amounts of Tagitinin C. In addition, the solvent used to obtain the extract is also a key piece for determining the degree of activity of the extract, as can be seen in the work of Miranda *et al*. (2015). This relationship can also be observed in the work of Passoni *et al*. (2013), in which the extract made from acetone presented large amounts of the active ingredient Tagitinin C, while in the polar extract the compound was not detected. For the sesquiterpene lactones of *T. diversifolia,* the best solvents are those that are relatively nonpolar, such as chloroform, ethyl ether, benzene, acetone, dichloromethane, and ethyl acetate (SILVA *et al*., 2017; DE OLIVEIRA, 2020). That said, further research should be carried out in order to carry out more tests using other solvents and forms of extraction, in addition to the elucidation of the other active compounds and tests of their phytotoxic activity.

4 CONCLUSIONS

The extract of the leaf lavage of *Tithonia diversifolia* presented Tagitinin C as the major compound. Through the phytotoxic assay it was possible to observe that the extract made by washing the leaves of *T. diversifolia* showed inhibition of the development of *Lactuca sativa*, however, the action of the extract was lower than that of the herbicide Flumyzin® used as a positive control. Finally, the potential of *T. diversifolia* extracts as natural products for pest control purposes is evident, so the present study should be continued in order to carry out more tests with extracts obtained from different forms of extraction, under different conditions and using different solvents, and also to carry out a study on the compounds responsible for phytotoxicity.

REFERENCES

ABOUZIENA, H.F.; HAGGAG, W.M. Weed Control in Clean Agriculture: a review1. Planta Daninha, v. 34, n. 2, p. 377-392, jun. 2016.

AJAO, A.A.; MOTEETEE, A.N. Tithonia diversifolia (Hemsl) A. Gray. (Asteraceae: heliantheae), an invasive plant of significant ethnopharmacological importance. South African Journal Of Botany, [S.L.], v. 113, p. 396-403, nov. 2017.

ALVES, M.; ROQUE, N. Flora da Bahia: asteraceae ⁖ tribo heliantheae. Sitientibus Série Ciências Biológicas, v. 16, n. 1, p. 16-63, 25 nov. 2016.

ALVES, L. L. et al. Atividade alelopática de extratos aquosos de plantas medicinais na germinação de Lactuca sativa L. e Bidens pilosa L. Bras. Pl. Med., Botucatu, v. 13, n. 3, p. 328-336, fev. 2011.

ARANTES, F. F. P. Síntese e Avaliação da Fitotoxicidade de Novas Lactonas Sesquiterpênicas. 2007. 147 f. Dissertação (Mestrado) - Curso de Agroquímica, Universidade Federal de Viçosa, Viçosa, 2007.

AYESA, S.A.; CHUKWUKA, K.S.; ODEYEMI, O.O. Tolerance of Tithonia diversifolia and Chromolaena odorata in heavy metal simulated-polluted soils and three selected dumpsites. Toxicology Reports, v. 5, p. 1134-1139, 2018.

BALBINOT JUNIOR, A. A. Manejo das Plantas Daninhas pela Alelopatia. Agropec. Catarin., Canoinhas, v. 17, n. 1, p. 61-64, mar. 2004.

CHAGAS-PAULA, D. A. et al. Ethnobotany, Chemistry, and Biological Activities of the Genus Tithonia (Asteraceae). Chemistry And Biodiversity, Ribeirão Preto, v. 9, n. 01, p. 210-235, jul. 2012.

CHRISTENSEN, L. P.; LAM, J. Acetylenes and related compounds in heliantheae. Phytochemistry, v. 30, n. 1, p. 11-49, jan. 1991.

CHRISTOFFOLETI, P. J.; LÓPEZ-OVEJERO, R. Principais Aspectos da Resistência de Plantas Daninhas ao Herbicida Glyphosate. Planta Daninha, Viçosa, v. 21, n. 3, p. 507-515, dez. 2003.

DAYAN, F. E.; DUKE, S. O. Natural Compounds as Next-Generation Herbicides. Plant Physiology, v. 166, n. 3, p. 1090-1105, abr. 2014.

DAYAN, F. E.; ROMAGNI, J. G.; DUKE, S. O. Investigating the mode of action of natural phytotoxins. Journal Of Chemical Ecology, v. 26, n. 9, p. 2079-2094, jan. 2000.

EBERLEIN, C. V. et al. Physiological consequences of mutation for ALS-inhibitor resistance. Weed Science, v. 47, n. 4, p. 383-392, ago. 1999.

HEAP, I. Global perspective of herbicide-resistant weeds. Pest Management Science, v. 70, n. 9, p. 1306-1315, 15 jan. 2014.

HENZEL, A. B. D. Avaliação de extratos de Tithonia diversifolia (Hemsl.) A. Gray sobre a germinação e desenvolvimento inicial de Lactuca sativa L. e Cucumis sativus L. 2022. 113 f. Dissertação (Mestrado) - Curso de Agronomia, Universidade Federal de Pelotas, Pelotas, 2022.

INTERNATIONAL Herbicide-Resistent Weed Database. 1993. Disponível em: http://www.weedscience.org/Home.aspx. Acesso em: 02 nov. 2023.

KATO-NOGUCHI, H. Involvement of Allelopathy in the Invasive Potential of Tithonia diversifolia. Plants, v. 9, n. 6, p. 766, 19 jun. 2020.

LOPES, P. G. Alelopatia em Lepidaploa aurea (Asteraceae) como Ferramenta de Restauração Ecológica: potencial para o controle de gramíneas exóticas invasoras no cerrado. 2016. 105 f. Dissertação (Mestrado) - Curso de Ecologia, Universidade de Brasília, Brasília, 2016.

MARTINS, M. L. Lactonas Sesquiterpênicas e Flavonas de Lapidia apicifolia Roque & S.C. Ferreira (Asteraceae): atividade fitotóxica e citotóxica. 2022. 39 f. Dissertação (Mestrado) - Curso de Botânica, Universidade de São Paulo, São Paulo, 2022.

MARTUCCI, M. E. P. Metabolômica e screening de interações ecoquímicas de plantas da subtribo Lychnophorinae (Asteraceae). 2016. 34 f. Tese (Doutorado) - Curso de Ciências Farmacêuticas, Universidade de São Paulo, Ribeirão Preto, 2016.

MIRANDA, M. A. F. M. et al. Phytotoxins from Tithonia diversifolia. Journal Of Natural Products, v. 78, n. 5, p. 1083-1092, 16 abr. 2015.

NAKAJIMA, J. N.; SEMIR, J. Asteraceae do Parque Nacional da Serra da Canastra, Minas Gerais, Brasil. Revta Brasil. Bot., São Paulo, v. 24, n. 4, p. 471-478, dez. 2001.

RIBEIRO, A. O.; SILVA, A. F.; CASTRO, A. H. F.. Identificação de espécies da família Asteraceae, revisão sobre usos e triagem fitoquímica do gênero Eremanthus da Reserva Boqueirão, Ingaí-MG. Rev. Bras. Pl. Med., Botucatu, v. 12, n. 4, p. 456-465, ago. 2010.

ROCHA, B. A. Transformações Microbianas da Lactona Sesquiterpênica Tagitinina C. 2009. 87 f. Dissertação (Mestrado) - Curso de Ciências Farmacêuticas, Universidade de São Paulo, Ribeirão Preto, 2009.

ROQUE, N; BAUTISTA, H. Asteraceae: caracterização e morfologia floral. Salvador: Edufba, 2008. 75 p.

OFOSU, R. et al. Herbicide Resistance: managing weeds in a changing world. Agronomy, v. 13, n. 6, p. 1595, 13 jun. 2023.

OLABODE, O.s. et al. Evaluation of Tithonia diversifolia (Hemsl.) A Gray for Soil Improvement. World Journal Of Agricultural Sciences, Ibadan, v. 3, n. 4, p. 503-507, fev. 2007.

OLIVEIRA, L. F. de. Eficiencia dos Métodos de Extração para Obtenção de Lactonas Sesquiterpênicas: uma revisão sistemática. 2020. 68 f. TCC (Graduação) - Curso de Engenharia Química, Unidade de Ensino Superior de Feira de Santana, Feira de Santana, 2020.

OLIVEIRA, L. dos S. D. de; SILVA, E. F. de; BARROS, R. F. M. de. Estudo taxonômico da tribo Heliantheae Cass. ocorrente em área de carrasco no município de Cocal, estado do Piauí, Brasil. Revista Brasileira de Biociências, Porto Alegre, v. 5, n. 2, p. 636-638, jul. 2007.

ORSOMANDO, G. et al. Mexican sunflower (Tithonia diversifolia, Asteraceae) volatile oil as a selective inhibitor of Staphylococcus aureus nicotinate mononucleotide adenylyltransferase (NadD). Industrial Crops And Products, v. 85, p. 181-189, jul. 2016.

PADILLA-GONZALEZ, G. F.; SANTOS, F. A. dos; COSTA, F. B. da. Sesquiterpene Lactones: more than protective plant compounds with high toxicity. Critical Reviews In Plant Sciences, v. 35, n. 1, p. 18-37, 2 jan. 2016.

PASSONI, F. D. et al. Repeated-dose toxicological studies of Tithonia diversifolia (Hemsl.) A. gray and identification of the toxic compounds. Journal Of Ethnopharmacology, v. 147, n. 2, p. 389-394, maio 2013.

PAULA, P. S. de. Extração e Isolamento de Lactonas Sesquiterpênicas Presentes nos Tricomas Glandulares de Tithonia diversifolia. In: CONGRESSO NACIONAL DE INICIAÇÃO CIENTÍFICA, 18., 2018, São Paulo. Anais do Conic-Semesp. São Paulo: Unip, 2018. p. 1-4.

PÉREZ, A. et al. Tithonia diversifolia (Hemsl.) A. Gray. Pastos y Forrajes, Matanzas, v. 32, n. 1, p. 1-15, mar. 2009.

RIAL, C. et al. Phytotoxicity evaluation of sesquiterpene lactones and diterpenes from species of the Decachaeta, Salvia and Podachaenium genera. Phytochemistry Letters, v. 18, p. 68-76, dez. 2016.

SÁNCHEZ-MENDOZA, M. E. et al. Bioassay-Guided Isolation of an Anti-Ulcer Compound, Tagitinin C, from Tithonia diversifolia: role of nitric oxide, prostaglandins and sulfhydryls. Molecules, v. 16, n. 1, p. 665-674, 17 jan. 2011.

SANTOS, F. A. dos. Metabolômica para Análise de Cultivares, Quimiotaxonomia e Triagem de Inibidores de Lactato desidrogenase-5 (LDH-5) em Espécies de Asteraceae. 2019. 30 f. Tese (Doutorado) - Curso de Ciências Farmacêuticas, Universidade de São Paulo, Ribeirão Preto, 2019.

SANTOS, J. de O. Síntese e Avaliação da Atividade Herbicida de Lactonas Sesquiterpênicas. 2009. 138 f. Dissertação (Mestrado) - Curso de Agroquímica, Universidade Federal de Viçosa, Viçosa, 2009.

SCHMIDT, T. J. Structure-Activity Relationships of Sesquiterpene Lactones. Studies In Natural Products Chemistry, Müster, v. 33, n. 1, p. 309-391, fev. 2006.

SILVA, A. R. et al. Comparison of ultrasound-assisted extraction and dynamic maceration over content of tagitinin C obtained from Tithonia diversifolia (Hemsl.) A. gray leaves using factorial design. Pharmacognosy Magazine, v. 13, n. 50, p. 270, 2017.

STEFANI, R. Quimiotaxonomia e Fitoquímica de Espécie da Tribo Heliantheae (Asteraceae) e uso de Quimioinformática em Elucidação Estrutural. 2006. 122 f. Tese (Doutorado) - Curso de Ciências, Universidade de São Paulo, Ribeirão Preto, 2006.

VERDI, L. G.; BRIGHENTE, I. M. C.; PIZZOLATTI, M. G. Gênero Baccharis (Asteraceae): aspectos químicos, econômicos e biológicos. Química Nova, v. 28, n. 1, p. 85-94, fev. 2005.

VYVYAN, J. R. Allelochemicals as leads for new herbicides and agrochemicals. Tetrahedron, v. 58, n. 599, p. 1631-1646, out. 2001.