Manuscript Details

Manuscript number	SAJB_2019_887_R1
Title	Screening of Tunisian plant extracts for herbicidal activity and formulation of bioherbicide based on Cynara cardunculus
Article type	Research Paper

Abstract

The need to reduce the use of chemical pesticides currently fosters great interest in eco-friendly biological control agents. In addition, the isolation of plant allelopathic substances and the evaluation of their phytotoxic effects can lead to the discovery of new natural herbicides. In this context, our study aimed to assess the herbicidal activity of ten crude extracts obtained from aerial parts of Tunisian spontaneous plants against Trifolium incarnatum, Silybum marianum and Phalaris minor. It confirmed that the Cynara cardunculus methanolic extract best inhibited weed germination and seedling growth, and caused necrosis or chlorosis. Following a bioassay-guided fractionation, five main phenolic compounds, (1) syringic acid, (2) p-coumaric acid, (3) myricitrin, (4) quercetin and (5) naringenin were identified in the most active crude methanolic extract. Then, only 3 of the flavonoids contained in the most active fraction were tested on Trifolium incarnatum. The 3 compounds had a significant phytotoxic effect and therefore could be employed in a new composition of botanical herbicides to control crop weeds. Besides, a novel herbicide composition was designed to improve the post-emergence activity of the methanolic extract. The formulation containing the C. cardunculus crude methanolic extract showed the same herbicidal activity as the standard industrial bioherbicide containing pelargonic acid. These results make C. cardunculus a suitable source of natural compounds potentially usable as natural herbicides.

Keywords	Herbicidal activity; plant extracts; formulation; phenolic compound; bioherbicide

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Suggested reviewers	Chamroon Laosinwattana, Kamel Msaada, Hanen Falleh

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Highlights

- *C. cardunculus* extract had the best herbicidal activity among 10 plant extracts.
- Five phenolic compounds were identified in *C. cardunculus*.
- Myricitrin, naringenin and quercetin were the main active compounds in *C cardunculus*
- Fraction 2 of *C cardunculus* had the same herbicidal effect as biological herbicide.

Screening of Tunisian plant extracts for herbicidal activity and formulation of a bioherbicide based on *Cynara cardunculus*

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ABSTRACT

The need to reduce the use of chemical pesticides currently fosters great interest in eco-friendly biological control agents. In addition, the isolation of plant allelopathic substances and the evaluation of their phytotoxic effects can lead to the discovery of new natural herbicides. In this context, our study aimed to assess the herbicidal activity of ten crude extracts obtained from aerial parts of Tunisian spontaneous plants against *Trifolium incarnatum*, *Silybum marianum* and *Phalaris minor*. It confirmed that the *Cynara cardunculus* methanolic extract best inhibited weed germination and seedling growth, and caused necrosis or chlorosis. Following a bioassay-guided fractionation, five main phenolic compounds, (1) syringic acid, (2) *p*-coumaric acid, (3) myricitrin, (4) quercetin and (5) naringenin were identified in the most active crude methanolic extract. Then, only 3 of the flavonoids contained in the most active fraction were tested on *Trifolium incarnatum*. The 3 compounds had a significant phytotoxic effect and therefore could be employed in a new composition of botanical herbicides to control crop weeds. Besides, a novel herbicide composition was designed to improve the post-emergence activity of the methanolic extract. The formulation containing the *C. cardunculus* crude methanolic extract showed the same herbicidal activity as the standard industrial bioherbicide containing pelargonic acid. These results make *C. cardunculus* a suitable source of natural compounds potentially usable as natural herbicides.

***Key words*:** herbicidal activity, extracts, formulation, phenolic compound, bioherbicide

1. Introduction

Weeds are registered as harmful plant pests posing a serious problem in agriculture worldwide (Suksungworn et al., 2016). They cause huge economic losses which can rise up to 34% in major crops by affecting yields and competing with crops for nutrients, light, and water (Araniti et al., 2015; Jabran et al., 2015).

The most efficient weed control methods currently include mechanical or hand weeding, and application of chemical herbicides. The latter has been proven to have negative impacts on environmental, animal, and human health (Böcker et al, 2019). Besides, it can increase weed resistance to phytochemicals (Jabran et al., 2015; Ahmed et al., 2017). For these reasons, scientists are working on the identification of a biological solution that can minimize the impacts of synthetic herbicides in agricultural production (Morra et al., 2018; Sbai et al., 2016; Chengxu et al., 2011). This solution could offer a number of benefits such as increased target specificity and rapid degradation of the active substance (Cordeau et al., 2016).

Allelopathic compounds released by plant organs in interaction with the environment exhibit several biological activities, and some of them could be integrated in weed management (Schleiden et al., 2019). They are generally derived from secondary pathways and have been investigated as allelochemicals with allelopathic effects on plants. For these reasons, a huge interest has been focused on plant extracts as sources of allelochemicals used for weed management (Cordeau et al., 2016). For example, Li et al. (2010), Javaid et al. (2010), Yan et al. (2014), Araniti et al. (2014), Nebo et al. (2014), Ben El Hadj Ali et al. (2014), Ribeiro et al. (2015), Araniti et al. (2015), and Lim et al. (2017) showed that these plant extracts inhibited weed germination and seedling growth. Nevertheless, few studies have shown an herbicidal effect of these compounds in post emergence by direct spraying on weeds. This inhibitory effect has often been related to the presence of phenolic compounds (Omezzine et al., 2011; Sbai et al., 2016; Jelassi et al., 2016). These are the most represented secondary metabolites implied in

plant allelopathic compounds (Vyvyan, 2002; Dayan et al., 2012). They are among the most important groups of antioxidant substances, are produced by plants for protection against UV light, insects, and plant pathogens such as viruses (Medini et al., 2014), bacteria (Trabelsi et al., 2013) and fungi (Ksouri et al., 2012; Heleno et al., 2015; Ben Kaab et al., 2019).

The identification of these phenolic compounds and the study of their toxic effect on plants can lead to the development of new nature-based herbicides (Flamini, 2012; Dayan et al., 2012; Cordeau et al., 2016). In this context, our study aims to assess the herbicidal effect of different Tunisian plant extracts and identify the most active one, and to find out the bioactive compounds contained in that extract, so as to design a formulation based on this plant extract.

2. Materials and methods

2.1. Plant material, extraction and fractionation procedures

Aerial parts of ten wild Tunisian spontaneous plants were collected in their vegetative stage on February 2015 from different Tunisian regions that belong to various bioclimatic stages (Table 1 and Figure 1). The identification of the harvested plants was confirmed by Pr. Abderrazak Smaoui affiliated to the Biotechnology Center of Borj-Cédria (CBBC). All the selected plants were shade-dried for 15 days at 30°C. Seeds of *Phalaris minor* (*P. minor*) were collected in Tunisia from wheat fields. Seeds of *Trifolium incarnatum* (*T. incarnatum*), and *Sylibum marianum (S. marianum)* were obtained from ECOSEM Co. (Belgium).

Both extraction and fractionation of plant materials were carried out according to the optimised method of Falleh et al. (2013). Phenolic extracts were obtained by stirring 10 g of dry powder with 100 ml of methanol (Emplura EMD Millipore Corporation, a subsidiary of Merck KGaA) for 30 min. Methanol was eliminated using a rotavapor in vacuum at 45°C, and residues were re-dissolved in Tween 1%. Extracts were then kept at 4 °C for 24 h, filtered through a Whatman No 4 filter paper and stored at 4 °C until analysis. The extraction yields varied between 5.29 and 29.71 %.

Methanol filtrates were combined, concentrated under vacuum, and fractionated using a reverse-phase silica gel (Sigma-Aldrich, a subsidiary of Merck KGaA) to remove sugars and other polar compounds. The samples were loaded on a column containing 10 g of C18 resin per gram of dry extract, followed by 80 ml of water. The phenolic fractions adsorbed onto the resin were then eluted with 30 ml of increasing MeOH percentages (successively 20, 40, 60, 80 and 100% methanol). The fractions were evaporated separately.

2.2. Formulation

A formulation was designed to mix the plant extracts with vegetable oil so as to render the penetration of active molecules through epicuticular waxes easier. It contained amphiphilic substances in order to mix together a hydrophobic vegetable oil (for the product to stick to the leaves) and our hydrophilic extract. The formulations varied among the phenolic extracts and fractions; they are presented in Table 2.

2.3. HPLC analysis

An HPLC system was used to identify the phenolic compounds. It was composed of a vacuum degasser, an autosampler, and a binary pump with a maximum pressure of 400 bar (Agilent 1260, Agilent technologies, Germany) provided with a reverse-phase C18 analytical column of 4.6 x 100 mm and 3.5 µm particle size (Zorbax Eclipse XDB C18). The DAD detector was set to a scanning range of 200-400 nm. The column temperature was fixed at 25°C. Mobile phase B contained milli-Q water with 0.1% formic acid, whereas mobile phase A consisted of 0.2% methanol. The flow rate was kept at 0.4 ml/min. The optimised elution gradient was as follows: 0-5 min, 10-20% A; 5-10 min, 20-30% A; 10-15 min, 30-50% A; 15-20 min, 50-70% A; 20-25 min, 70-90% A; 25-30 min, 90-50% A; 30-35 min, return to initial conditions.

A 2-µl volume was injected for each sample, and peaks were monitored at 280 nm. Phenolic compounds were identified based on retention times and the UV spectra of the phenolics

chromatogram. The pure standards were myricitrin, quercetin, *p*-coumaric acid, naringenin and syringic acid. Identification was performed by comparing the retention times of the standards with those of the extracts. For the quantitative analysis, a calibration curve was obtained by plotting the peak areas against different concentrations for each identified compound at 280 nm: all the curves showed a strong linearity (with an average $r^2 = 0.99$): y= 38,976x + 4,1296 for syringic acid; y= 32,266x + 17,439 for *p*-coumaric acid; y= 6,7915x - 35,235 for myricitrin; y=9,5824x - 7,4659 for quercetin, and y=23,691x - 88,898 for naringenin. The amount of each compound was expressed in milligrams per gram of residue.

2.4. Pre-emergence activity under laboratory conditions

Seeds of *T. incarnatum S. marianum* and *P. minor* were sterilised using 0.5 % sodium hypochlorite for 2 min. Each crude plant extract was first solubilised in Tween 1% and then diluted with distilled water to the desired concentration. Filter paper was moistened with 2 ml of Tween 1 % solution (which did not interfere with the assays) as a control, or with the crude methanolic extract solution prepared from different Tunisian plants at 5 g/L for the treatments. Ten seeds of *T. incarnatum*, *S. marianum* or *P. minor* were then immediately placed in Petri dishes, and three replicates (3 Petri dishes) were prepared for each extract and for each plant species. All Petri dishes were randomly placed in a growth chamber at 23 ± 1°C, in darkness. The number of germinated seedlings was counted, and their hypocotyl and root lengths were measured after 7 days. The inhibition rate of the root and hypocotyl lengths was calculated based on Equation (1):

$$\text{Inhibition rate (\%)} = {}^{T}/_{C} * 100, \quad (1)$$

Where *T* is the length of the roots or hypocotyls of the treated seedlings, and *C* represents the length of the roots or hypocotyls of the control seedlings.

132 *2.5. Post-emergence activity under greenhouse conditions*

133 A first post-emergence experiment was conducted to study the effects of the methanolic
134 extracts on 2-to-3-week-old plantlets of *T. incarnatum*, *S. marianum,* and *P. minor* under
135 controlled conditions. Seeds of *P. minor* were sown in boxes, whereas seeds of *T. incarnatum*
136 and *S. marianum* were sown in pots. The plants were watered daily. When the weeds reached
137 the 2-to-3-leaf stage, they were sprayed with 10 ml of one of the following solutions: a
138 methanolic plant extract at 7.5, 20 or 34 g/L, or formulated plant extracts, or formulated
139 fractions, or adjuvants only (as controls), or distilled water, or finally a commercial biological
140 herbicide containing 34 g/L of pelargonic acid (as a positive control).

141 A second experiment was carried out on the herbicidal activity of the pure phenolic
142 compounds identified in the most active fraction. Myricitrin, quercetin, naringenin are
143 commercially available (Sigma, Belgium) and were sprayed only on *T. incarnatum* leaves for
144 its strong resistance to pesticides (Mccurdy et al., 2013).

145 Whatever the post-emergence experiment, three replications were conducted for each treatment
146 in a completely randomised manner. Seven days after spraying, the treated weed plants were
147 examined to study wilting, necrosis, and chlorosis. The efficacy percentage was calculated
148 using Equation (2):

149 $$\text{Percentage of efficacy (\%)} = {}^{N}/_{T} * 100, \qquad (2)$$

150 Where *N* refers to the number of necrotic or withered leaves, and *T* represents the total number
151 of leaves.

152 *2.6. Statistical analysis*

153 The results were analysed using Minitab 17 Statistical Software (Minitab Inc., State
154 College, PA, USA), using one-way analysis of variance (ANOVA) followed by Tukey's

multiple range tests. The differences between individual means were considered significant only if $p < 0.05$. Therefore, values in a column followed by the same letter are not significantly different at $p < 0.05$.

3. Results

3.1. Effect of the plant extracts on weed germination and seedling growth

The phytotoxic effect of the different crude methanolic extracts obtained from aerial parts of Tunisian spontaneous plants is summarised in Figures 2 (germination) and 3 (seedling growth). The allelopathic influence on *T. incarnatum*, *S. marianum* and *P. minor* at 5 g/L on germination and seedling growth varied significantly depending on the plant extracts. A significant inhibitory effect on *T. incarnatum* seed germination was found only with the *Cynara cardunculus* (*C. cardunculus*) extract. In addition, all plant extracts significantly inhibited *S. marianum* germination as compared to the control. However, *C. cardunculus*, *Artemisia herba-alba* (*A. herba-alba*) and *Tamarix gallica* (*T. gallica*) best inhibited germination. *P. minor* was the most sensitive weed whatever the plant extract tested.

Concerning the inhibition of root and hypocotyl growth, the *C. cardunculus* crude methanolic extract had the highest inhibitory effect on *T. incarnatum* (Figure 3), which reached 87.57% for hypocotyl length. Altogether, the plant extracts had a higher inhibitory effect on hypocotyl rather than radicle growth. Moreover, the statistical analysis showed that the *C. cardunculus* and *A. herba-alba* plant extracts had the highest phytotoxic effect on *S. marianum* seedling growth, ranging between 80.87 and 100.00 %.

The phytotoxic effect of the plant extracts was higher on *P. minor* than on *T. incarnatum* and *S. marianum.* In fact, the majority of plant extracts including *C. cardunculus* and *A. herba-alba* completely inhibited *P. minor* seedling growth.

178 *3.2. Effect of the plant extracts on post-emerged weeds under greenhouse conditions*

179 Different methanolic extracts were sprayed on *T. incarnatum*, *S. marianum,* and *P.*
180 *minor* at 7.5, 20, or 34 g/L (Table 3). Only the *C. cardunculus* extract at 7.5 g/L and 20 g/L
181 caused some necrosis and chlorosis on the leaves of all three weeds. Its herbicidal activity
182 reached up to 37 %. At 34 g/L, several plant extracts had a phytotoxic effect against these
183 weeds. *C. cardunculus* seemed to have again the best herbicidal activity, which reached
184 62.76%. That was why it was selected for the identification of bioactive compounds and the
185 formulation of crude extracts and of the active fraction.

186 *3.3. Effect of C. cardunculus fractions on seed germination and seedling growth*

187 The *C. cardunculus* crude methanolic extract was the most phytotoxic one among all
188 the extracts tested. For this reason, a bioguided fractionation of the *C. cardunculus* crude
189 methanolic extract was performed to identify the bioactive compounds that inhibited
190 germination and seedling growth. Five fractions (named F1 to F5) were obtained and tested on
191 *T. incarnatum*. The extract was chromatographed on silica gel, and the fractions were tested on
192 *T. incarnatum* germination and seedling growth at 6 g/L. F1 and F2 seemed to be phytotoxic
193 fractions in terms of inhibition of germination (Figures 4, 5), i.e., 30% and 60% inhibition after
194 5 days, respectively. However, the statistical analysis showed that the germination rate of *T.*
195 *incarnatum* treated with F3, F4, and F5 was similar to that of the untreated seeds. F1 and F2
196 nearly completely inhibited *T. incarnatum* seedling growth (Figure 6). Conversely, the two less
197 polar fractions (F4 and F5) did not have any significant effect on *T. incarnatum* seedling
198 growth, while F3 had an intermediary effect.

199 *3.4. Phytochemical investigation of the C. cardunculus crude methanolic extract and its*
200 *fractions*

201 HPLC analysis was carried out to identify the phenolic compounds of the *C.*
202 *cardunculus* crude methanolic extract and its fractions. The chemical profile showed the

presence of 5 phenolic compounds in common in the first polar fractions (F1, F2, and F3). These compounds were syringic acid, *p*-coumaric acid, myricitrin, quercetin and naringenin (Table 4). No phenolic compound was identified in fractions 4 and 5, which had no effect on *T. incarnatum* germination or seedling growth. The amount of these compounds was low in the crude *C. cardunculus* extract, with 0.108, 0.487, 0.755, 0.383, and 0.359 mg/g dry weight (DW), respectively, as compared to the fractions. *p*-coumaric acid, quercetin and myricitrin were concentrated in F1, F2 and F3 (41.209, 17.427, and 64.764 mg/g DW, respectively).

3.5. Effect of the formulated crude C. cardunculus extract and active fraction on post-emerged weeds under greenhouse conditions

The spraying of formulated *C. cardunculus* crude methanolic extract burnt *T. incarnatum* down. Six hours after spraying, *T. incarnatum* leaves wilted rapidly, followed by the stems 3 days later, demonstrating that the formulation allowed active compounds to pass into the stems. Fraction 2 from the *C. cardunculus* crude methanolic extract was also selected for formulating because it was the most efficient one on pre-emergence activity. At 20 g/L, it had the same effect on *T. incarnatum* as pelargonic acid at 34 g/L (Figure 7). It caused total leaf drying followed by plant death, as shown in Figure 8.

3.6 Effect of the identified phenolic compounds on post-emerged weeds under greenhouse conditions

In that last trial, quercetin, naringenin or myricitrin, which had been identified in the most active fraction, were sprayed on *T. incarnatum* at 250 µg/ml, 90 µg/ml and 60 µg/ml, respectively, i.e., the concentrations recorded in the most active fraction (F2) when used at 20 g/L. These compounds (formulated like F2) had a significant phytotoxic effect as compared to the compound-free formulation (Figure 9). Efficacy reached 52.33% with formulated myricitrin.

4. Discussion

We determined the herbicidal activity of ten Tunisian spontaneous plants in pre- and post-emergence to select the most promising plant(s) for potential use as a bioherbicide. The greatest herbicidal activity was obtained using extracts from two *Asteraceae* plants (*A. herba-alba* and *C. cardunculus*), which inhibited seed germination and seedling growth of three weeds. The *Asteraceae* family has been found to harbour the most prominent biocidal substances for agriculture and to be a good source for isolating and purifying allelopathic secondary metabolites (Bessada et al., 2015; Watanabe et al., 2014). In addition, the *C. cardunculus* crude methanolic extract had the best herbicidal activity in post-emergence under greenhouse conditions. *C. cardunculus* leaves are known to be a good source of polyphenols (Omezzine et al., 2011; Falleh et al., 2008) and could inhibit the development of other invasive weeds such as barnyard grass (*Echinochloa crus-galli*) and *Brachiaria* sp. (Rial et al., 2014). The herbicidal activity of phenolic extracts in pre-emergence is widely documented (Javaid et al., 2010; Araniti et al., 2015; Watanabe et al., 2014; Nekonam et al., 2014; Bessada et al., 2015; Uddin et al., 2014; Ma et al., 2018; Schleiden et al., 2019), but to our knowledge no study had yet focused on their effect in post-emergence by direct spraying on weed leaves. *C. cardunculus*, which is well known for its coagulant properties (Prados & Pino, 2007) and phytotoxic effect in pre-emergence (Rial et al., 2014; Rial et al., 2016; Scavo et al., 2019), is unveiled for the first time for its herbicidal effect in post-emergence. A formulation was used to improve its efficiency, containing a vegetable oil and nonionic surfactants. The latter can increase the adsorption rate of polar molecules, dissolve cuticular fatty acids and therefore improve the penetration of the hydrophilic active substance (Batish et al., 2007; Hazrati et al., 2017). Based on these data, a recent study showed that a formulation based on palm oil, Tween 20 and Span 80 improved the herbicidal activity of *Phoma* sp. metabolites (Todero et al., 2018).

Many authors have demonstrated that the phytotoxic effect of plant extracts was related to the presence of phenolic compounds (Li et al., 2010; Lim et al., 2017; Yan et al., 2014; Ribeiro et al., 2015; Araniti et al., 2014; Jelassi et al., 2016; Ben El Hadj Ali et al., 2014; Araniti et al., 2015; Sbai et al., 2016; Javaid et al., 2010; Nebo et al., 2014). Methanol seems to be the best solvent to extract phenolics due to their good solubility in it (Ben El Hadj Ali et al., 2014). In this context, our study showed that the most bioactive fraction isolated by HPLC analysis from the *C. cardunculus* crude methanolic extract contained three flavonoids: myricitrin, naringenin and quercetin. Each of these flavonoids had a significant phytotoxic effect on *T. incarnatum*. In this context, De Martino et al. (2012) reported a significant phytotoxic effect of quercetin and naringenin on radish. To our knowledge, myricitrin was tested for the first time in our experiments. In the same line, Javaid et al. (2010) isolated flavonoids from mango (*Mangifera indica* L.) and showed that they caused yellowing of parthenium (*Parthenium hysterophorus* L.) seedlings. Moreover, flavonoids purified from a root extract of *Stellera chamaejasme* L. collected in China acted as potential major phytotoxins against *Arabidopsis thaliana* (Yan et al., 2014). Finally, fractionation of *Derris urucu* ethanol extracts permitted the identification of three new dihydroflavonols named urucuol A, urucuol B and isotirumalin, which showed potential herbicidal activity (Da Silva et al., 2013).

The mode of action of allelochemicals, including the flavonoids highlighted in our study, still remains unknown (Soltys et al., 2013; Vyvyan, 2002). In fact, these secondary metabolites act as toxins for the plant metabolism and affect several physiological functions of plant cells such as membrane integrity, photosynthesis and respiration, metabolic and proteomic activity, phytohormonal activity, and ion uptake (Yan et al., 2014; Ribeiro et al., 2015; Yan et al., 2015; Ahmed et al., 2017). They modify the expression of one or more genes, which then leads to plant death (Cordeau et al., 2016). Chalcone, an aromatic precursor of the synthesis of these flavonoids, induced programmed cell death in *Arabidopsis thaliana* roots (Tielas et al.,

2013). In the same line, phenolic acids act as phytotoxic agents by inducing overproduction of reactive oxygen species (ROS) that disturb respiration and photosynthesis (Lim et al., 2017; Ladhari et al., 2014; Franco et al., 2015; Araniti et al., 2014). These ROS play an important signalling role in the control of a number of essential processes like growth, development, the response to environmental constraints, pathogen defence and stomatal behaviour. They can generate mutations, react with DNA, proteins, and lipids, inducing tissue injury, membrane damage, and programmed cell death (PCD) processes (Yan et al., 2015). Flavonoids are known for their antioxidant properties (de Martino et al., 2012; Belmekki & Bendimerad, 2012; Sakihama et al., 2002), but under certain conditions (concentration, pH modification, solubility characteristics, potential metal-reducing chelating behaviour), they have potential prooxidant properties (Eghbaliferiz & Iranshahi, 2016). The phenolic compounds such as myricitrin, naringenin and quercetin – identified in our active fraction – are involved in diverse physiological effects such as mineral uptake alteration, disruption of membrane permeability, stomatal closure, induction of water stress, deleterious effects on photosynthesis and protein synthesis, and alteration of enzyme activities. Flavonoids cause overproduction of phenoxyl radicals, directly linked to lipid peroxidation and ROS accumulation in the cell, and this can damage DNA, lipids, and other biological molecules (Sakihama et al., 2002; de Martino et al., 2012; Ribeiro et al., 2015). Moreover, on a morphological scale, we noticed that root inhibition in response to different methanolic plant extracts was related to the development of thin secondary roots. This can be caused by oxidative stress (Franco et al., 2015), potentially due to the interaction of phenolic compounds with auxin, cytokinin, and gibberellin transport (Ribeiro et al., 2015). In accordance with our study, Franco et al. (2015) found that exogenous flavonoids, such as those identified in our *C. cardunculus* extract, could delay primary root growth and improve the expansion of lateral roots. They modified the expression model of specific genes involved in root tissue differentiation.

5. Conclusions

Our results show that *C. cardunculus* is a promising plant with pre-emergence but also post-emergence herbicidal activity under greenhouse conditions. Its formulated active fraction at 20 g/L had the same herbicidal effect as commercial pelargonic acid at 34 g/L. This opens new perspectives on the application of Tunisian plant extracts as novel botanical herbicides for weed management. Finally, myricitrin was identified for the first time as one of the bioactive molecules contained in the active fraction. It can be employed in a new herbicidal formulation and offers new strategies and pathways for the biopesticide industry to create an eco-friendly alternative to chemical herbicides. To go one step further, it could be really interesting to determine the mode(s) of action of the *C. cardunculus* crude methanolic extract and of its active compounds such as myricitrin on weeds and to optimise the formulation to improve the effectiveness and stability of the emulsion.

Funding source

This work was supported by funding from the Integrated and Urban Plant Pathology Laboratory, University of Liège, Gembloux Agro-Bio Tech and Laboratory of Aromatic and Medicinal Plants, Biotechnology Center of Borj-Cedria (CBBC).

Conflict of interest

The authors declare no conflict of interest.

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Figures

Figure 1. *C. cardunculus* collected at vegetative stage in its natural environment of the Enifidha region located in Tunisia which showed the best herbicidal activity.

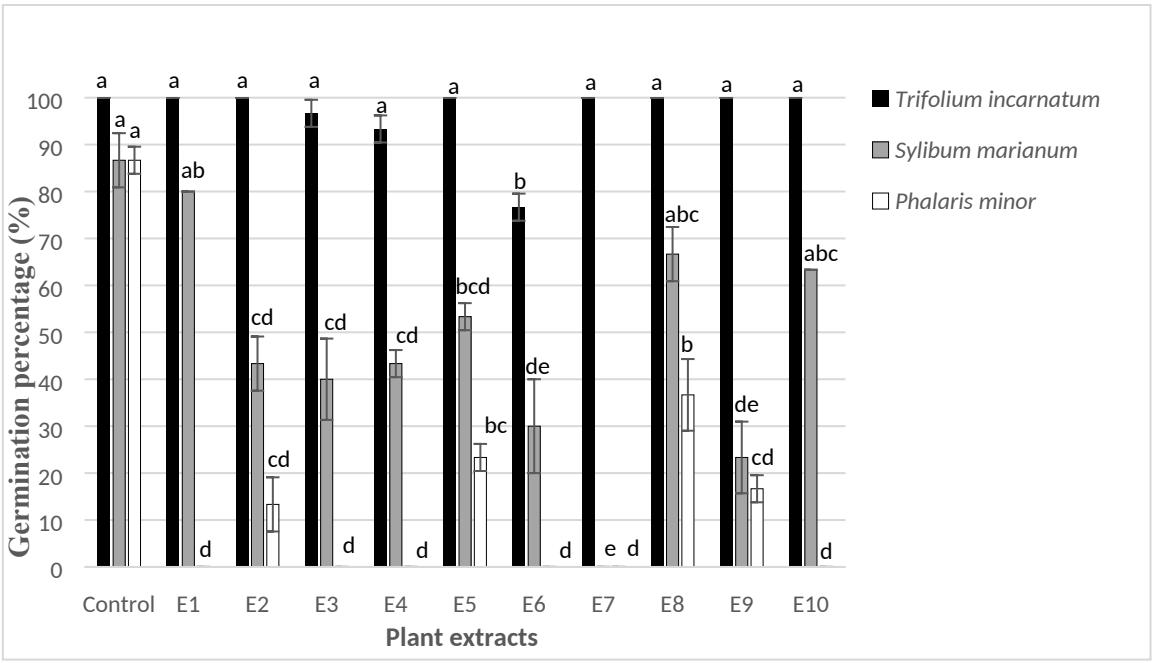

Fig. 2. Seed germination inhibition of weeds exposed for 7 days to 5g/L of different crude extracts obtained from the aerial parts of Tunisian spontaneous plants at the vegetative stage (E1: *L, guyonianum;* E2: *P, harmala;* E3: *R, chalepensis;* E4 *R, communis;* E5: *N, retusa;* E6: *C, cardunculus;* E7: *A, herba-alba;* E8: *M, edule;* E9: *T, gallica* E10: *D, stramonium*). For each weed, value in a column followed by the same letter are not significantly different at P <0.05, as established by Tukey's test.

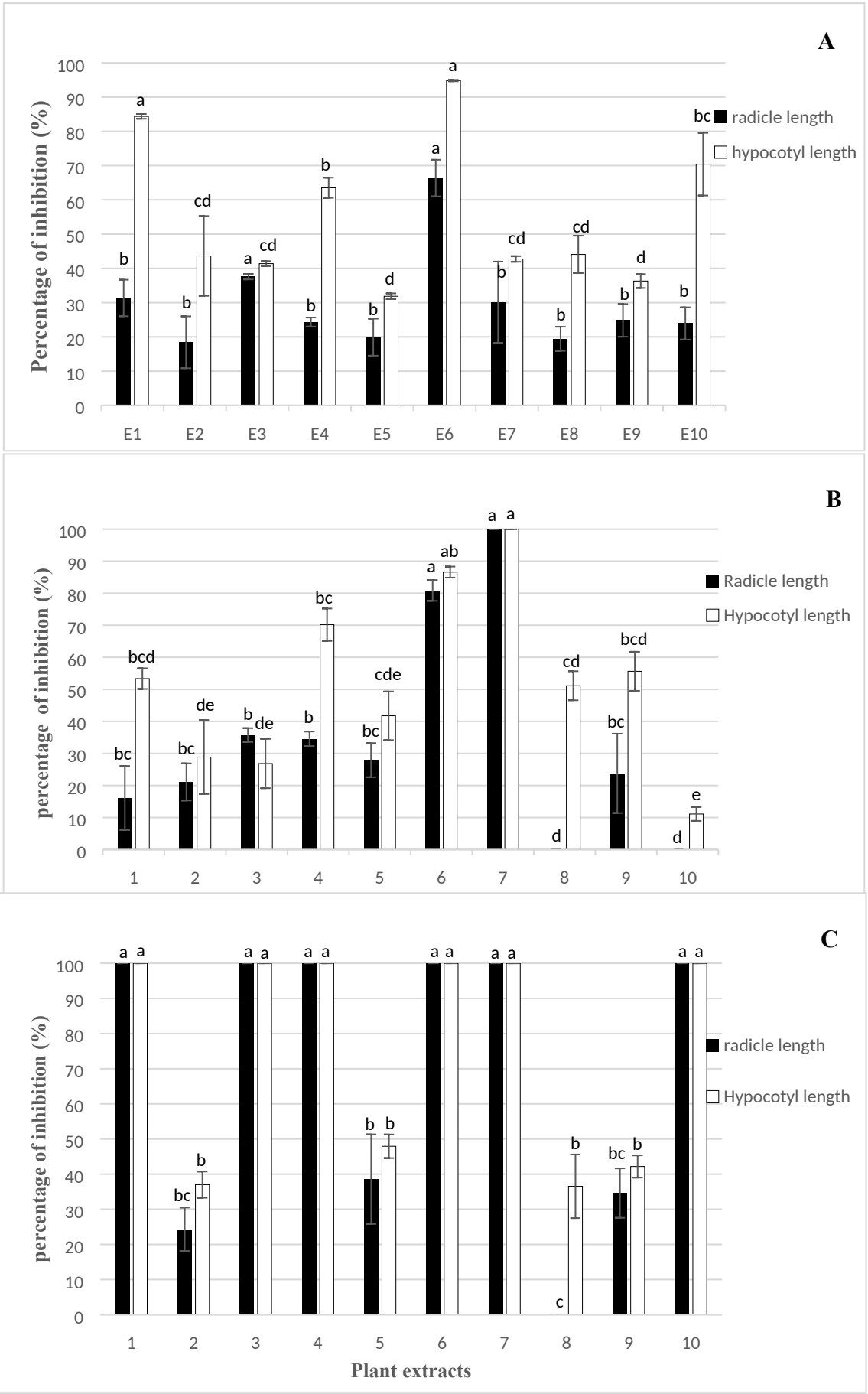

Fig. 3. Phytotoxic effect of different crude extracts obtained from of aerial parts of Tunisian spontaneous plants at the vegetative stage at 5 g/L (E1: *L. guyonianum;* E2: *P. harmala;* E3: *R. chalepensis;* E4 *R. communis;* E5: *N. retusa;* E6: *C. cardunculus;* E7: *A. herba-alba;* E8: *M. edule;* E9: *T.gallica* E10: *D. stramonium*) on *T. incanatum* (A), *S. marianum* (B) and *P. minor* (C) seedling growth. For each part (radicle or hypocotyl) of each weed, value in a column followed by the same letter are not significantly different at P <0.05, as established by Tukey's test

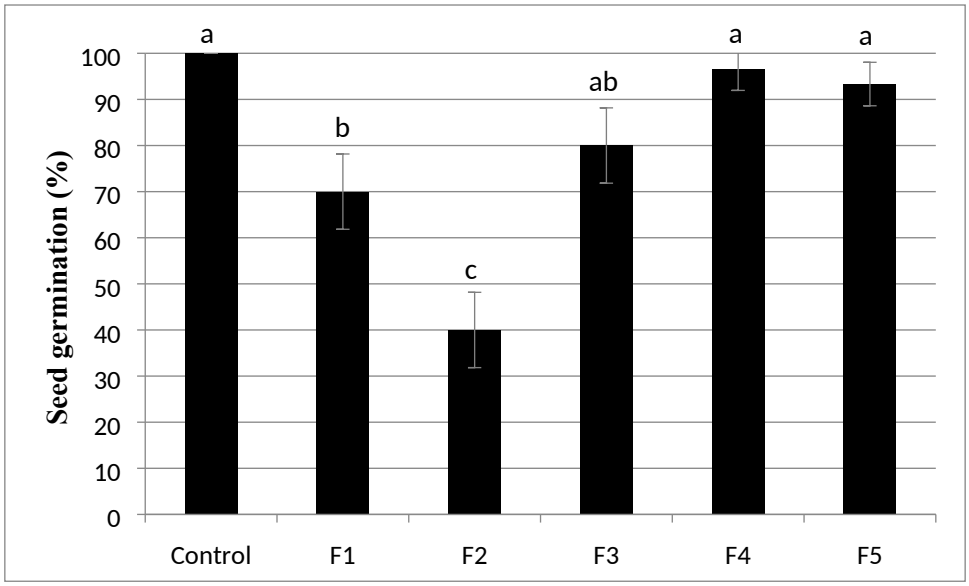

Fig. 4. Percentage of *T. incarnatum* germination after 5 days treated by one of the following fractions: F1: fraction 1 (20 % methanol); F2: fraction 2 (40% methanol); F3: fraction 3 (60% methanol); F4: fraction 4 (80% methanol); F5: fraction 5 (100 % methanol). Fractions were obtained by fractionation of *C.cardunculus* methanolic crude extract. Value in a column followed by the same letter are not significantly different at P <0.05, as established by Tukey's test.

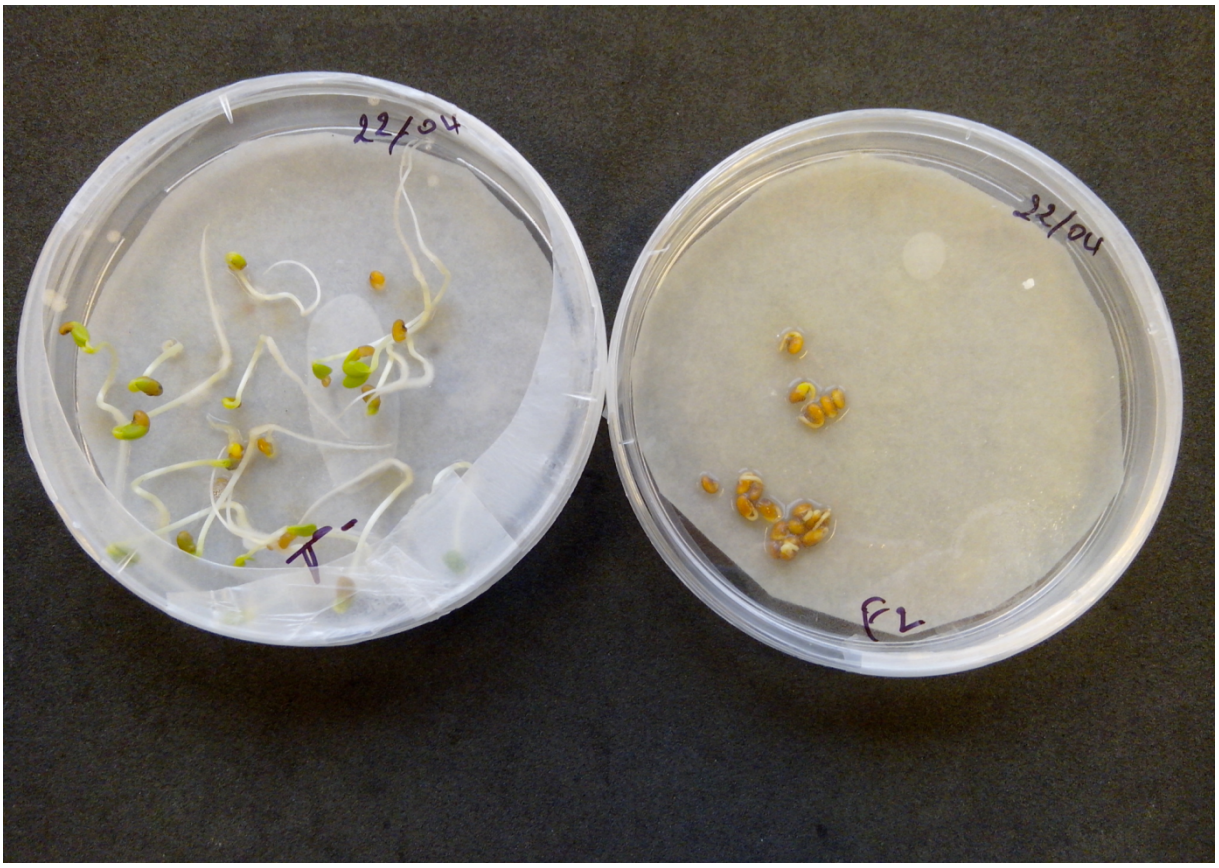

Figure 5 Inhibitory effects of *C. cardunculus* plant extract, obtained from aerial plant at vegetative stage, on the germination and seedling growth of *T. incarnatum* at 6 g/L after 5 days. Left: Untreated *T. incarnatum* Right: Treated *T. incarnatum* with *C. cardunculus* plant fraction at 6 g/L after 5 days.

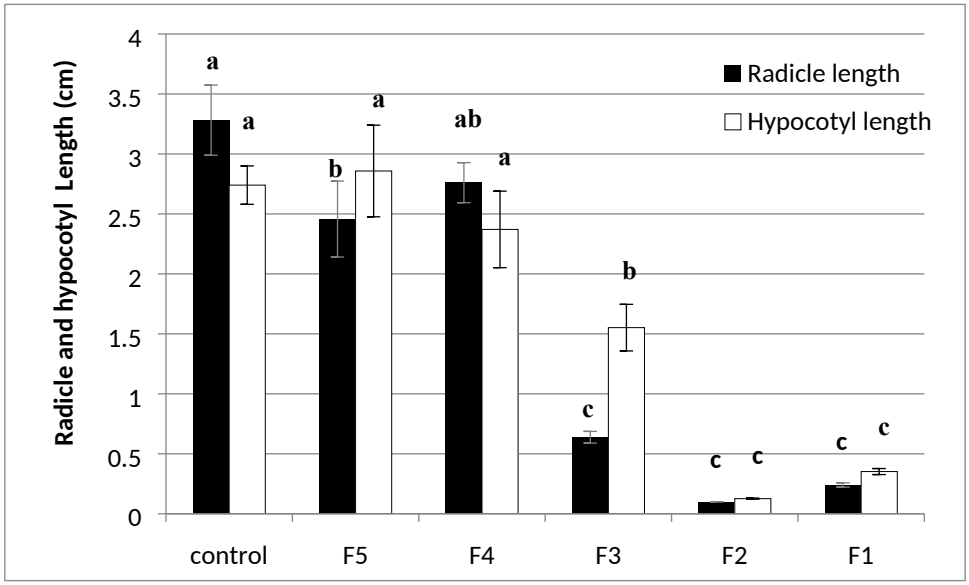

Fig. 6. Radicle and hypocotyl length of *T. incarnatum* after 5 days treated by 5 fractions F1: fraction 1 (20 % methanol); F2: fraction 2 (40% methanol); F3: fraction 3 (60% methanol); F4: fraction 4 (80% methanol); F5: fraction 5 (100 % methanol) obtained by fractionation of *C. cardunculus* methanolic crude extract. C. Value in a column followed by the same letter are not significantly different at P <0.05, as established by Tukey's test.

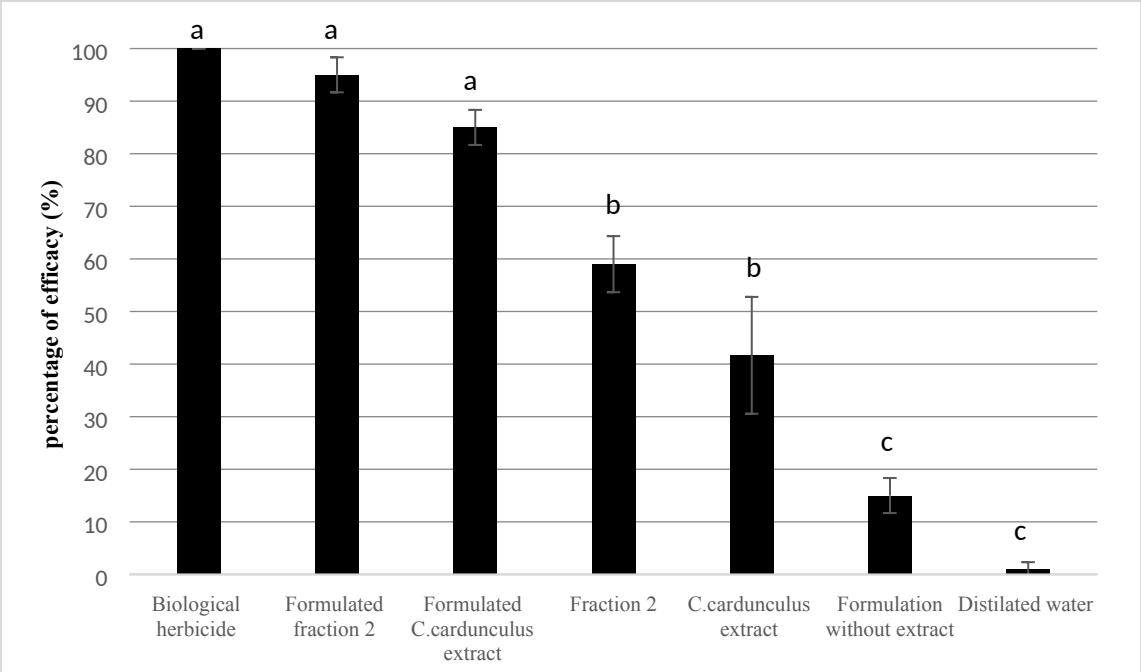

Fig. 7. Post-emergence activity of different herbicide compositions on *T. incarnatum* after 5 days of treatment: *C. cardunculus* crude methanolic extract with and without formulation at 34 g/L; fraction 2 with and without formulation at 20 g/L isolated from methanolic crude extract of *C. cardunculus* plant extract; Biological herbicide based on pelargonic acid at 34 g/L (the same concentration as in the market), formulation contained only vegetable oil and adjuvants. Value in a column followed by the same letter are not significantly different at P <0.05, as established by Tukey's test.

Figure 8. Herbicidal activity of formulated fraction obtained from *C. cardunculus* plant extract by fractionation on *T, incarnatum* after 7 days. Left: Treated *T. incarnatum* with formulated *C. cardunculus* plant fraction at 20 g/L. Right: Treated *T. incarnatum* with Formulation without plant extract.

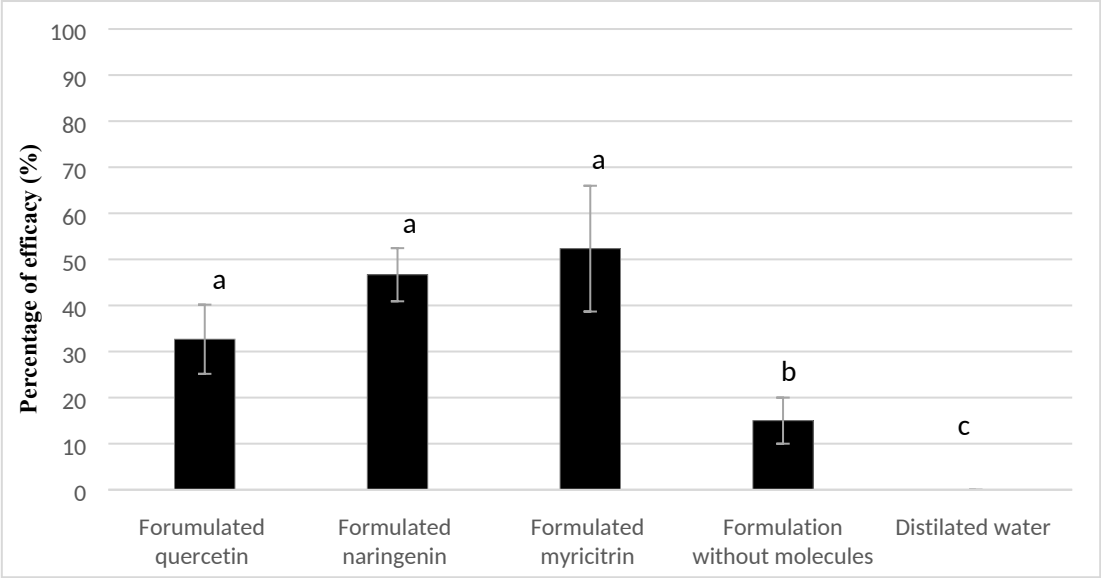

Fig.9. Post-emergence activity of formulated phenolic compounds on *T. incarnatum* after 5 days of treatment: Querectin, Naringenin, Myricitrin were sprayed respectively at 250 µg/ml, 90 µg/ml and 60 µg/ml which are the concentrations in the most active fraction (F2) at 20 g/L. The formulation contained vegetable oil and adjuvants. Value in a column followed by the same letter are not significantly different at P <0.05, as established by Tukey's test.

Tables

Table 1: Place of collection, bioclimatic zone and extract yield of Tunisian spontaneous plants selected for the study

Samples	Tunisian spontaneous plants	Family	Place of collection	Bioclimatic zone	Latitude	Longitude	Plant crude extract yield (%)
E1	***L, guyonianum***	Plumbaginaceae	Gabes	LA	**34.109491 N**	**9.982843 E**	**17,86**
E2	***P, harmala***	Zygophyllacees	Sidi Bouzid	UA	**35.035066 N**	**9.496925 E**	**18,14**
E3	***R, chalepensis***	Rutaceae	zaghouane	SH	**36.388780 N**	**10.131152 E**	**14,57**
E4	***R, communis***	Euphorbiaceae	zaghouane	SH	**36.387816 N**	**10.130768 E**	**12,71**
E5	***N, retusa***	Nitrariaceae	Enfidha	LSA	**36.079476 N**	**10.347530 E**	**15,00**
E6	***C, cardunculus***	Asteracees	Enfidha	LSA	**36.101872 N**	**10.388882 E**	**9,71**
E7	***A, herba-alba***	Astéracees	Kairouan	UA	**34.873042 N**	**10.082678 E**	**5,29**
E8	***M, edule***	Aizoaceae	Ben arous	SH	**36.715148 N**	**10.412255 E**	**29,71**
E9	***T, gallica***	*Tamaricaceae*	Kairouan,	UA	**35.867603 N**	**10.207805 E**	**17,00**
E10	***D, stramonium***	Solanaceae	Ben arous	SH	**36.712268 N**	**10.436624 E**	**21.00**

SH: sub-humid; LSA: Lower semi-arid; UA: upper arid; La: Lower arid

Table 2: Composition of formulated herbicides

Composition	Extract 6 (%)	Fraction 2 (%)
Plant extract	3.40	-
Plant fraction	-	2.00
Vegetable oil of hazelnut	3.40	3.40
Ethoxylated (9) Castor oil[1]	0.67	0.67
Polyethylene glycol sorbitan monolaurate[2]	0.33	0.33
UEP-100	0.25	0.25
Ethanol	0.50	0.50
Water	91.45	92.85
Total	100.00	100.00

[1]CO_9 [2]Tween 20

Table 3: Post-emergence activity of crude different extracts obtained from aerial parts of Tunisian spontaneous plants at the vegetative stage on *T, incarnatum*, *S, marianum* and *P, minor*

Weeds	*T, incarnatum*			*S, marianum*			*P, minor*		
Concentrations (g/L)	**7,5**	**20**	**34**	**7,5**	**20**	**34**	**7,5**	**20**	**34**
L. guyonianum	0.0 ± 0.0^{B}	0.0 ± 0.0^{C}	0.0 ± 0.0^{D}	0.0±0.0	0.0 ± 0.0^{B}	0.0 ± 0.0^{C}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{D}
P. harmala	0.0 ± 0.0^{B}	0.0 ± 0.0^{C}	0.0 ± 0.0^{D}	0.0±0.0	0.0 ± 0.0^{B}	0.0 ± 0.0^{C}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{D}
R. chalepensis	0.0 ± 0.0^{B}	26.67 ± 2.22^{B}	30.0 ± 3.33^{C}	0.0±0.0	0.0 ± 0.0^{B}	16.67 ± 4.44^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	12.33 ± 1.78^{C}
R. communis	0.0 ± 0.0^{B}	0.0 ± 0.0^{C}	32.67 ± 1.78^{C}	0.0±0.0	0.0 ± 0.0^{B}	0.0 ± 0.0^{C}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{D}
N. retusa	0.0 ± 0.0^{B}	0.0 ± 0.0^{C}	32.67 ± 5.11^{C}	0.0±0.0	0.0 ± 0.0^{B}	0.0 ± 0.0^{C}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{D}
C. cardunculus	24.33 ± 3.77^{A}	37.0 ± 2.0^{A}	62.67 ± 4.88^{B}	0.0±0.0	17.0 ± 2.0^{A}	17.67 ± 1.78^{B}	17.0 ± 2.0^{B}	17.33 ± 2.22^{A}	16 ± 2.66^{B}
A.herba-alba	0.0 ± 0.0^{B}	0.0 ± 0.0^{C}	29.33 ± 2.88^{C}	0.0±0.0	0.0 ± 0.0^{B}	0.0 ± 0.0^{C}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{D}
M. edule	0.0 ± 0.0^{B}	0.0 ± 0.0^{C}	29.33 ± 3.78^{C}	0.0±0.0	0.0 ± 0.0^{B}	0.0 ± 0.0^{C}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	17.67 ± 1.78^{B}
T. gallica	0.0 ± 0.0^{B}	0.0 ± 0.0^{C}	28.33 ± 4.44^{C}	0.0±0.0	0.0 ± 0.0^{B}	0.0 ± 0.0^{C}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{D}
D.stramonium	0.0 ± 0.0^{B}	0.0 ± 0.0^{C}	31.33 ± 2.44^{C}	0.0±0.0	0.0 ± 0.0^{B}	0.0 ± 0.0^{C}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{D}
Biological herbicide[1]	-	-	100.0 ± 0.0^{A}	-	-	100.0 ± 0.0^{A}	-	-	100.0 ± 0.0^{A}

[1] Natural herbicide based on pelargonic acid was used as a positive control (at the same concentration in the market). [2] replicates were performed for each treatment. Value in a column followed by the same letter are not significantly different at P <0.05, as established by Tukey's test.

Table 4: Phenolic compounds identified and quantified in *C. cardunculus* crude methanolic extract and its obtained fractions

Identified phenolic compounds	Retention time (min)	Molecular formula	Concentrations in mg/ g of DW					
			Crude extract	F1[1]	F2	F3	F4	F5
Syringic acid	17.8	$C_9H_{10}O_5$	0,108	NI	NI	1,308	NI	NI
Myricitrin	20.7	$C_{21}H_{20}O_{12}$	0,755	NI	3,088	64,764	NI	NI
p-coumaric acid	20.09	$C_9H_8O_3$	0,487	41,209	NI	0,576	NI	NI
Quercetin	23.7	$C_{15}H_{10}O_7$	0,383	NI	17,427	1,430	NI	NI
Naringenin	24.3	$C_{15}H_{12}O_5$	0,359	NI	4,486	NI	NI	NI

NI: not identified %

[1]F1: fraction 1 (20 % methanol); F2: fraction 2 (40% methanol); F3: fraction 3 (60% methanol); F4: fraction 4 (80% methanol); F5: fraction 5 (100 % methanol).