

1           **Biochemical composition of Tunisian *Nigella sativa* L. at different growth stages**  
2                           **and assessment of the phytotoxic potential of its organic fractions**

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14  
15 **Abstract**

16 The present study was conducted to study some biochemical characteristics of Tunisian  
17 *Nigella sativa* at different developmental stages of plant growth (vegetative, flowering and  
18 fruiting stages) and to screen the chemical constituents and the phytotoxic activity of their  
19 organic extracts on lettuce (*Lactuca sativa* L.). The GC-MS analysis of petroleum ether  
20 fractions revealed that *N. sativa* seeds were rich in linoleic acid (58% of total fatty acids),  
21 oleic acid (22% of total fatty acids) and palmitic acid (12% of total fatty acids). The fatty acid  
22 composition of aerial parts showed an **increase in the level** of saturated fatty acids  
23 accompanied by a concomitant decrease of polyunsaturated fatty acids levels during the  
24 developmental stage. The phytochemical investigation showed that among the organic  
25 extracts, **the** methanolic extract from aerial parts harvested at **the** fruiting stage contained the

26 highest amounts of phenolic and flavonoid compounds. The phytotoxic study revealed that *N.*  
27 *sativa* negatively affected the growth of lettuce plants. This effect was largely dependent on  
28 the developmental stage at which material was collected and the nature of extracting solvent.  
29 The methanolic extract of aerial parts harvested at the vegetative stage was the most active on  
30 seedling growth of lettuce.

31

32 **Key Words:** Tunisian *Nigella sativa*, developmental stages, biochemical characteristics,  
33 phytochemicals, phytotoxicity.

34

### 35 **Introduction**

36 Flowering plants undergo several distinct transitions during their development, including  
37 germination, vegetative growth to reproductive development and eventually seed set and  
38 senescence (Huijser and Schmid, 2011). The transitions between these phases are regulated by  
39 complex interactions between endogenous cues that include hormones and carbohydrate  
40 assimilates and environmental cues, such as temperature, light and nutrients (Huijser and  
41 Schmid, 2011; Yu, Lian, and Wang, 2015). According to Naghiloo et al. (2012), the  
42 knowledge of the factors that determine the chemical variability and yield for each species is  
43 important to optimize the time of collection and to obtain higher yields of phytochemicals  
44 compounds in particular for medicinal plants. In fact, it has been documented that  
45 environmental factors and developmental stage can have profound effects on yield,  
46 phytochemical constituents and biological activities of plant species (Thapliyal and Nene,  
47 1970; Naghiloo et al., 2012; Çirak, Radusiene, and Camass, 2008 and Cirak, Radusiene,  
48 Janulis, and Ivanauskas, 2007). On the other hand, successful determination of biologically  
49 active compounds from plant material is largely dependent on the nature of the solvent used in  
50 the extraction procedure, time of extraction and temperature.

51 *Nigella sativa* L. is an annual herbaceous plant belonging to the **Ranunculacea** family,  
52 commonly known as black seed (Yoruk, Tatar, Keles, and Cakir, 2017). The seeds are widely  
53 used for culinary and medicinal purposes. The phytochemicals reported in *N. sativa* seeds  
54 include alkaloids, such as Nigellicin, Nigellimine and Nigellidine, flavonoids and terpenoids  
55 (Atta-ur-Rahman, Cun-heng, and Clardy, 1985; Atta-ur-Rahman and Zaman, 1992; Atta-ur-  
56 Rahman et al., 1995; Merfort et al., 1997; Bourgou, Bettaieb, Hamrouni, and Marzouk, 2012).  
57 Several phenolic compounds have been identified in leaves and roots such as gallic acid,  
58 chlorogénic acid, *p*-dihydroxybenzoic acid, quercetin, epicatechin and catechin (Bourgou et  
59 al., 2008). *N. sativa* has been extensively studied for its biological activities and shown to  
60 possess wide spectrum of activities such as antidiabetic, anticancer and immunomodulatory,  
61 analgesic, antimicrobial, **anti-helminthic**, antiinflammatory, gastroprotective, hepatoprotective,  
62 and antioxidant properties (Burits and Bucar, 2000; Ahmad et al., 2013). For several years,  
63 scientists focused their attention on plant secondary chemicals to develop bio-herbicides as an  
64 alternative strategy for weed control in order to reduce the negative impact of synthetic  
65 herbicides on the environment and human health. In our previous study, we found that seeds  
66 and aerial parts aqueous extracts exerted significant phytotoxic potential on lettuce (Zribi,  
67 Omezzine, and Haouala, 2014). This investigation will evaluate the effects of **three** different  
68 solvents for their relative capacity to extract phytochemicals (such as phenolic compounds)  
69 from aerial parts of *N. sativa* and to determine the active ingredients responsible for the  
70 phytotoxic activity.

71 The purpose of the present work was to assess carbohydrates, major mineral (P, K, Ca), lipids  
72 contents and the quantitative analysis of phenolic compounds in different organic extracts **of**  
73 Tunisian *N. sativa* at different developmental stages of plant growth (vegetative, flowering  
74 and fruiting stages) and to screen the phytotoxic activity of their organic extracts on *Lactuca*  
75 *sativa* L. a plant model known to be very sensitive to allelochemicals.

## 76 **Material and methods**

### 77 **Plant material**

78 Tunisian *Nigella sativa* seeds were **obtained** from an herbal market in Sousse (Tunisia).  
79 The plants were sown in January 2013 (temperature 13/15°C), under standard greenhouse  
80 condition in the experimental station of the Higher Institute of Agronomy of Chott Mariem,  
81 University of Sousse (latitude 35°56'45.6''N, longitude 10°33'57.6 ''E, coastal region, East  
82 of Tunisia with a **sub-humid** climate); photoperiod light-dark cycle LD 10:14; Irrigation:  
83 every 2-3 days. Samplings were carried out during the vegetative [plants with 8-9 leaves (60  
84 days old)], flowering [50% of flowers open (105 days old)] and fruiting stages [50% of the  
85 pods have reached a typical length (125 days old)].

### 86 **Total water soluble carbohydrates**

87 Total soluble sugar content was determined by phenol sulfuric acid method (Dubois, Gilles,  
88 Hamilton, Ruberg, and Smith, 1956) using glucose (Sigma chemicals) as standard. Fresh plant  
89 material (0.1 g) was extracted with 2 ml of 80% ethanol for 48 h. After evaporation of ethanol  
90 on a water-bath at 70°C, 20 ml of distilled water were added, and the mixture was shaken  
91 vigorously. To 1 ml of sample, 1 ml of 5% phenol, and 2 ml of H<sub>2</sub>SO<sub>4</sub> were added, and the  
92 mixture was stirred. After cooling in an ice bath for 25 min, the absorbance of the sample was  
93 recorded at 490 nm ( $R^2= 0.994$ ).

### 94 **Calcium, phosphorus and potassium contents**

95 After drying and grinding, 1 g of seeds or aerial part at different growth stages were  
96 dry-ashed at 220°C for 2 hours, then at 550°C for 6 hours. Ash was put in solution with 2 ml  
97 of concentrated **hydrochloric** acid (HCl) and heated on a hot plate until evaporation. **Five** ml  
98 of N/10 HCl (8.24 mL of concentrated HCl 36% in 500 mL distilled water) were added and

99 the mixture was kept for 10min then the residue was filtered and brought up to a 100 ml with  
100 distilled water. Calcium (Ca) and potassium (K) contents were determined by atomic  
101 adsorption methods (Martin-Prével, Gonard, and Gautier, 1984). The phosphorus (P) content  
102 was estimated using the Nitrovanadomolibdate method described by Fleury and Leclerc  
103 (1943).

#### 104 **Phytochemical screening**

105 Seeds and dried aerial parts were extracted successively with petroleum ether, chloroform and  
106 methanol in their increasing order of polarity. The aerial parts were dried in shade, and  
107 powdered in a mechanical grinder. Fifty grams of seeds and dried plant material were kept in  
108 petroleum ether for 7 days at room temperature and then extracted with chloroform followed  
109 by methanol (Omezzine, Bouaziz, Simmonds, and Haouala, 2014). The organic extracts were  
110 evaporated to dryness under reduced pressure at 40-45°C, using a Rotavapor R-114 (Buchi,  
111 France). For each sample, the residue was weighed and the extraction yield was determined.  
112 Dry fractions were stored at 4°C until use. All organic solvents were analytical reagent grade.

#### 113 **Determination of Total phenolics (TPC), flavonoids ((TFC), flavonols and flavones** 114 **(TFIC) and proanthocyanidins (TPAC) (condensed tannins) contents**

115 The phenolics content was measured using the modified Folin-Ciocalteu method (Velioglu,  
116 Mazza, Gao, and Oomah, 1998). Gallic acid was used as a standard to produce the calibration  
117 curve. Total phenol content was expressed as mg gallic acid equivalent/g dry matter (mg  
118 GAE/g dw) ( $R^2= 0.996$ ). The flavonoids (*TFd*) content was determined  
119 spectrophotometrically according to the method described by Omezzine and Haouala (2013)  
120 and expressed as mg quercetine equivalent/g dry weight (mg QE/g dw) using **quercetin**  
121 calibration curve ( $R^2= 0.993$ ). Total flavonols and flavones content was determined using the  
122 method described by Omezzine and Haouala (2013) and expressed as mg **quercetin**  
123 equivalent/g dry weight (mg QE/g dw) using **quercetin** calibration curve ( $R^2= 0.932$ ). The

124 proanthocyanidins content was performed using the method described by Broadhurst and  
125 Jones (1978) and expressed as mg catechin equivalent/g dry weight (mg CE/g dw) using  
126 catechin calibration curve ( $R^2 = 0.995$ ).

### 127 **Identification of fatty acids in petroleum ether extracts using GC-FID Analysis**

128 To determine the fatty acid composition, approximately 10 mg of petroleum ether seeds  
129 and aerial parts extracts were dissolved in 0.2 ml of hexane , followed by the addition of  
130 0.5 ml of Boron trifluoride (BF<sub>3</sub>) reagent (methanol / BF<sub>3</sub>-Methanol (14% Boron  
131 trifluoride in methanol) / hexane (55:25:20). Samples were placed in a water-bath at 70°C  
132 for 1.5 h in tightly closed tubes, then 0.5 ml of saturated NaCl solution, 0.2 ml of 10%  
133 H<sub>2</sub>SO<sub>4</sub>, and 7 to 8 ml of hexane were added to the tubes. The samples were shaken, and  
134 0.5µl of the organic layer was taken to determine the fatty acid composition by gas  
135 chromatography (GC). GC analyses were performed using a Hewlett-Packard 6890 Series  
136 gas chromatograph equipped with a flame ionization detector (FID) and an electronic  
137 pressure control (EPC) injector. An apolar column VF-WAX ms (Agilent J&W cp9205)  
138 (30 m, 0.25 mm id, 0.25µm film thickness) was used. The carrier gas was N<sub>2</sub> with a flow  
139 rate of 1.7 ml/min. The injection was performed in on-column mode. The analyses were  
140 performed using the following temperature program: raise from 55°C to 150°C (at  
141 30°C/min), then up to 250 °C at 5°C/ min, and finally maintained at 250°C for 10 min.  
142 Analyses were performed in triplicate. Fatty acid methyl esters were identified by  
143 comparison of their retention times with those of pure reference standards (external  
144 standards) purchased from sigma-aldrich (Diegem, Belgium). Individual fatty acids were  
145 expressed as percentage of the total fatty acids in the considered sample (Toma et al.,  
146 2013).

### 147 **Phytotoxic bioassays**

148 **Tests with organic extracts**

149 The organic residues, obtained with petroleum ether, chloroform and methanol, were  
150 dissolved in an appropriate organic solvent (the same solvent used for the extraction) at 1, 3  
151 and 6 mg/ml to prepare the test solutions. Organic extracts were tested on the plant model  
152 *Lactuca sativa* L, a species known to be very sensitive to allelochemicals (Ervin and Wetzel,  
153 2003). Four controls were used: distilled water, petroleum ether, chloroform and methanol to  
154 eliminate the organic solvent effect. Filter paper, placed in each Petri dish, was wetted with  
155 distilled water or various organic extracts. Solvents were evaporated at 24 °C for 24 h, then 5  
156 ml of distilled water were added and 20 soaked seeds/pre-germinated seeds were placed in the  
157 Petri dishes (Omezzine et al., 2014). Two sets of Petri plates were prepared. In the first set,  
158 imbibed seeds were used to evaluate the effect of extracts on germination. The second set of  
159 pre-germinated seeds, with 1 mm root length, was used to evaluate the effect of extracts on  
160 root and shoot growth. The Petri dishes were placed in a growth chamber at 24/22 °C for  
161 14/10 h light and dark periods, respectively. Germination was determined by counting the  
162 number of seeds that had germinated at 24 h intervals over 6 days. Germination percentage (G  
163 %) was determined using the following formulae on the seventh bioassay day (Eq. 1):

164 
$$G\% = \frac{\text{Total number of germinated seeds}}{\text{Total number of seeds}} \times 100 \quad (\text{Eq. 1})$$

165 The index of germination (GI) was calculated using the following formula (Eq. 2) (Chiapuso,  
166 Sanchez, Reigosa, Gonzaiez, and Pellissier, 1997):

167 
$$GI = (N_1) \cdot 1 + (N_2 - N_1) \cdot 1/2 + (N_3 - N_2) \cdot 1/3 + \dots + (N_n - N_{n-1}) \cdot 1/n \quad (\text{Eq. 2})$$

168 where  $N_1, N_2, N_3, \dots, N_n$  = Number of germinated seeds observed after 1, 2, 3, . . . n days. This  
169 index represents the delay in germination induced by extract (Ahmed and Wardle, 1994); GI  
170 (% of control) was obtained by dividing GI of extract by GI of control and multiplied by 100.

171 Shoot and root lengths were measured 7 days after placing the pre-germinated seeds in each  
 172 Petri dish. Data were transformed to percent of control for analysis. The following formula  
 173 (Eq. 3) was used to calculate the % inhibition/stimulation (Chung, Ahn, and Yun, 2001):

$$\frac{\text{Inhibition (-)}}{\text{Stimulation (+)}} (\%) = \left[ \frac{\text{extract} - \text{control}}{\text{Control}} \right] \times 100 \quad (\text{Eq. 3})$$

174 *Inhibition index (I)*

175 The concentration –response effects of organic extracts of *N. sativa* on lettuce germination,  
 176 root and shoot length were assessed by the Whole-range assessment method. Inhibition index  
 177 was calculated by Eq. 4, used by Liu, An, and Wu (2007), where concentrations tested ranged  
 178 from 0 to  $D_n$  ( $D_n$  was dose–concentration tested from 0,  $D_1$ ,  $D_2$ ... $D_n$ ),  $D_c$  was the threshold  
 179 dose at which response equaled the value of control and above which the responses were  
 180 inhibitory,  $R(0)$  was the response at 0 extract concentration (control) and  $f(D)$  represented the  
 181 response function. Inhibition of germination and reduction of root and shoot growth, caused  
 182 by *N. sativa* extracts, were used to calculate inhibition index (I) using the WESIA (Whole-  
 183 range Evaluation of the Strength of Inhibition in Allelopathic-bioassay) software (Liu et al.,  
 184 2007):

$$I = \frac{\int_{D_c}^{D_n} [R(0) - f(D)] dD}{\int_0^{D_n} R(0) dD} = 1 - \left[ \frac{D_c}{D_n} + \frac{1}{R(0)D_n} \int_{D_c}^{D_n} f(D) dD \right] \quad (\text{Eq. 4})$$

## 185 **Statistical analysis**

186 All data were reported as means  $\pm$  standard deviation (S.D.) of three replicates and analyzed  
 187 using IBM SPSS Statistics 20.0. Experimental data were subjected to one-way analysis of  
 188 variance (ANOVA) followed by Duncan’s Multiple Range Test (DMRT) to determine  
 189 significance differences among mean values at the probability level of 0.05.

## 190 **Results and discussion**

### 191 **Phytochemical screening of *N. sativa***



192 Sugars, which are the first products of photosynthesis, are converted into starch, protein, oil,  
193 cellulose, lignin, and thousands of other chemical compounds. Soluble sugar content reached  
194 the maximum level at flowering stage (0.05 mg/mg FW) (Figure 1). According to Konow and  
195 Wang (2001), the changes in starch, sucrose, and glucose concentration in the leaves  
196 frequently coincide with mobilization of carbohydrates necessary for flower spike formation.  
197 Urban, Lu, and Thibaud (2004) reported that the soluble sugars needed to support flowering  
198 were produced through starch conversion in stems.

199 The mineral compositions of *N. sativa* (K, Ca and P) which were measured in seeds and aerial  
200 parts are shown in Figure 2. In seeds, P is the most abundant element followed by K. Our  
201 results are in agreement with previous studies reporting that the most abundant mineral in *N.*  
202 *sativa* seeds was K (Atta, 2003; Cheikh-Rouhou et al., 2007 and Sultan et al., 2009).  
203 Potassium and Ca levels were higher during the vegetative growth stage (Ca = 0.86%; K=  
204 2.93%) than during flowering and fruiting stages. While, no significant difference between  
205 stages was recorded for P (P = 0.2%). According to Bojović and Stojanović (2005), the  
206 greatest influence on development of plants in general and their leaf surface of  
207 macrometabolic elements is exerted by nitrogen, which effect is enhanced by P and to a lesser  
208 extent by K. P is involved in many metabolic processes essential for normal growth, such as  
209 photosynthesis. This element exerts influence on stability of the chlorophyll molecule. K is  
210 also essential for photosynthesis because it activates many enzymes involved in this process.  
211 Ca plays a very important role in plant growth and nutrition, as well as in cell wall deposition  
212 and increasing mechanical strength of the plant Karimi, Yari, and Ghasmpour (2012). Our  
213 results are in agreement with the results of Akporhonor, Egwaikhide, and Odilora (2005) who  
214 reported reduction in K levels in maize plants stem with age. Karimi et al. (2012) reported that  
215 K and Ca decreased markedly with increasing maturity of *Satureja hortensis*, while P did not  
216 greatly alter by stage of maturity.

217 The analyses of Fatty acid methyl esters (FAMES) were done by gas chromatography GC-FID  
218 on petroleum ether fraction of *N. sativa* seeds and aerial parts harvested at vegetative,  
219 flowering and fruiting stages (Table 2). Our results emphasize the significant role of growth  
220 stage governing lipid content and composition. The oil content of *N. sativa* seeds calculated  
221 from the petroleum ether extract on the basis of dry matter weight was of 24 %. This result  
222 was slightly lower than that obtained by Cheikh-Rouhou et al. (2007) who reported an oil  
223 content of 28.48% in *N. sativa* seeds from Tunisian location; however, they proceeded to the  
224 extraction of oil by Soxhlet apparatus during 8 h using the hexane solvent. As shown in Table  
225 1, linoleic (C18:2 = 58%), oleic (C18:1 = 21%) and palmitic acids (C16:0 = 14%) represents  
226 the major fatty acid of petroleum ether fraction of *N. sativa* seeds. Our results are in  
227 agreement with those reported by Cheikh-Rouhou et al. 2007 and Toma et al. (2013).

228 As it can be seen from Table 2, the fatty acids in petroleum ether fraction from aerials parts  
229 harvested at vegetative, flowering and fruiting stages are dominated by the common plant  
230 plasma membrane longer-chain fatty acids, such as C18 and C16, which are typical in higher  
231 plants (Millar, Smith, and Kunst, 2000). This study showed that fatty acids composition varies  
232 considerably with the growth stages. Aerials parts harvested at vegetative stage were  
233 characterised by a high proportion of linoleic acid representing 38,5 % of fatty acid methyl  
234 esters (FAMES), followed by palmitic and oleic acids. During the flowering stage, linolenic  
235 and palmitic acids were the major compounds representing 38 and 27 % of FAMES  
236 respectively, followed by linoleic acid. During the fruiting stage the level of palmitic acid was  
237 increased to 58 % of FAMES accompanied by a concomitant drastic decrease in the level of  
238 linoleic and linolenic acids to 3% of FAMES. Bourgou, Pichette, Lavoie, Marzouka, and  
239 Legault (2012) reported that linolenic, palmitic and linoleic acids were the major compounds  
240 in *N. sativa* fresh vegetative leaves cultured under hydroponic conditions. Several  
241 developmental processes during the life cycle of plants are characterized by changes in the

242 composition and turnover of intracellular lipids (Feussner, Kühen, and Wasternack, 2001).  
243 According to Zhang et al. (2005), to maintain membrane fluidity, plants increase the content  
244 of saturated and monounsaturated fatty acids, modulating their metabolism in response to  
245 increasing temperatures. Thus, increasing the saturation level of fatty acids appears to be  
246 critical for maintaining membrane stability and enhancing heat tolerance (Larkindale and  
247 Huang, 2004; Bitá and Gerats, 2013). Yang and Ohlrogge (2009) reported that during leaf  
248 senescence, macromolecule breakdown occurs and nutrients are translocated to support  
249 growth of new vegetative tissues, seeds, or other storage organs. The fatty acid levels in  
250 leaves began to decline at the onset of leaf senescence and progressively decreased as  
251 senescence advanced. In our study, a very small amount of C8 and C17 acids were also  
252 detected in aerial parts.

253 In the present study, the total phenolics (TPC), flavonoids (TFC), flavonols and flavones  
254 (TFIC) and proanthocyanidins (TPAC) contents of organic extracts of seeds and aerial parts of  
255 *N. sativa* were estimated by colorimetric methods (Table 3). Among the three organic  
256 fractions, petroleum ether fraction of seeds contained the highest content of TPC (6.5 mg  
257 GAE/g DW) and TPAC (3.6 mg CE/g DW). *N. sativa* seeds were found to be rich in  
258 polyphenols, while their content varies considerably depending upon the solvent used and the  
259 extraction method (Mariod, Ibrahim, Ismail, and Ismail, 2009). Regardless of the stage of  
260 development, the highest levels of TPC, TFC and TFIC were recorded in methanolic extracts  
261 of *N. sativa* aerial parts. Richness of methanolic extracts of stems and roots of *N. sativa* in  
262 phenolic compounds has also been reported by Bourgou et al. (2008). Our results showed also  
263 that the highest level of phenolic compounds was recorded at the fruiting stage.

264 **Phytotoxic activity of organic extracts of *N. sativa* on germination and seedling growth**  
265 **of lettuce**

266 The organics extract of *N. sativa* aerial parts showed phytotoxic effect on the germination of  
267 lettuce (Table 4). Speed of germination was strongly influenced by chloroform extract of  
268 aerial parts harvested at fruiting stage (Germination index = 44% at 6mg/ml) compared with  
269 the control. The same extract reduced also the final germination by 50%. Germination was  
270 slightly affected by petroleum ether extract of aerial parts harvested at fruiting and chloroform  
271 extract harvested at vegetative stage.

272 The data showed strong inhibition on root length in the presence of aerial parts methanolic  
273 extracts at whatever stage of development ranging from 25 % to 88 % and in the presence of  
274 petroleum ether extracts of plant material collected during vegetative stage (Inhibition ranging  
275 from 20% at 37%) (Figure 3). The inhibitory effects were increased with increasing  
276 concentrations. The methanol extract of aerial parts harvested at vegetative stage gave the  
277 highest inhibitory effect on root growth at 6 mg/ml (88 %). A slight stimulatory effect on root  
278 length ranging from 0.6 to 21% was recorded in presence of petroleum ether and chloroform  
279 extracts of seeds and aerial parts of *N. sativa*.

280 Overall, shoot length was near the control or slightly inhibited under the influence of the  
281 majority of the organic extracts of *N. sativa* (Figure 3). The highest inhibition effect was  
282 observed with methanolic extract of aerial parts harvested at vegetative stage with an average  
283 of 52% at 6 mg/ml followed by aerial parts collected at fruiting stage with an average of 28 %  
284 whatever the concentration used.

285 The strength of the interaction effects between three factors (organic extract type,  
286 concentration and plant development stage) on root and shoot growth was compared using  
287 General Linear Model Univariate procedure (followed by a post hoc test). Across all factors  
288 we found that the combination of organic extract type and plant development stage has a  
289 highly significant effect on root growth of lettuce ( $P < 0.0001$ ). Significant interaction

290 between the three factors was also recorded ( $P < 0.001$ ) on root growth. The results showed  
291 also significant interaction between the three 3 factors ( $P < 0.0001$ ) on shoot growth of  
292 lettuce. In conclusion, the aerial part harvested at vegetative stage and extracted in methanol  
293 was the most phytotoxic on lettuce at 6 mg /ml.

294 The Whole-range assessment can display a visual comparison between different biological  
295 parameters and allowed us to group and to identify the most toxic extracts (Omezzine et al.  
296 2014). Among all the organic extracts of *N. sativa*, the chloroform extract of aerial parts  
297 harvested at fruiting stage exhibited the most phytotoxic effect on lettuce germination  
298 (Inhibition index = 32%) (Table 5). While, chloroform and petroleum ether extracts of seeds  
299 had no effect on germination. Regarding seedling growth, methanolic extract of aerial parts  
300 harvested at vegetative stage was the most phytotoxic for root growth (I= 31%) followed by  
301 the methanolic extract of aerial parts collected at flowering stage (I= 24.3 %). Shoot length  
302 was especially affected by the extract of plant material harvested at vegetative stage (I= 26.9  
303 %).

304 The results of this study are different from our previous study, where we registered the  
305 highest toxicity on seedling growth for aqueous extract of material harvested at flowering  
306 stage (Zribi et al., 2014) and further studies are needed to explain the different behaviours.  
307 Similar observation was also reported by Omezzine and Haouala (2013). These authors  
308 reported that the difference in toxicity between aqueous and organic extracts could be  
309 attributed to the interactions between biologically active compounds that could act in synergy  
310 or antagonism. Despite their lower richness in TPC compared to the two other extracts, the  
311 high toxicity of methanolic extract from aerial parts harvested at vegetative stage and  
312 chloroform extract of those collected at fruiting stage could be explained by the presence  
313 highly active allelochemicals. The reduction in seedling growth may be attributed to  
314 interference of allelochemicals in major physiological processes of plant metabolism (Arora,

315 2013). Our study revealed that root length inhibition was more obvious than shoot length. The  
316 results of the present study revealed that *N. sativa* seeds and aerial part contain various types  
317 of phenols, flavonoids and proanthocyanidins. According to Li et al. (2010), phenolic  
318 allelochemicals can lead to increased cell membrane permeability. Consequently, cell  
319 contents spill and there is increased lipid peroxidation. Finally, there is slow growth or death  
320 of plant tissue. Phenolic allelochemicals can also inhibit plants from absorbing nutrients from  
321 surroundings and affect the normal growth of plants (Li, Wang, Ruan, Pan, and Jiang, 2010).  
322 Allelopathic effect could also be attributed to long-chain saturated fatty acids such as linoleic  
323 acid, palmitic and stearic acids. In fact these fatty acids are reported as showing allelopathic  
324 activity (Kakisawa et al., 1988; Inderjit and Keating, 1999; Quintana, El Kassis, Stermitz, and  
325 Vivanco, 2009).

326

## 327 **Conclusion**

328 Changes in some biochemical characteristics of Tunisian *N. sativa* were assessed during  
329 vegetative, flowering and fruiting stages. Results showed that total soluble sugars, chlorophyll  
330 (Chl (a + b)) content, and K, Ca and P content decreased with plant age. This study indicates  
331 also that the phytochemical composition (fatty acids, phenols, flavonoids and  
332 proanthocyanidins contents) and the phytotoxic activity of *N. sativa* vary considerably with  
333 the development stage of the plant and according to the nature of the extracting solvent used.  
334 The methanolic extracts of aerial parts harvested at the vegetative stage had a significant  
335 negative effect on seedling growth of lettuce. However, further studies are required to test the  
336 efficacy of extracts from this plant on weed control under field conditions and to isolate the  
337 chemical constituents responsible for the phytotoxic activity.

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