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

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Abstract

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

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

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

Introduction

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

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

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

Material and methods

Plant material

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

Total water soluble carbohydrates

Total soluble sugar content was determined by phenol sulfuric acid method (Dubois, Gilles, Hamilton, Ruberg, and Smith, 1956) using glucose (Sigma chemicals) as standard. Fresh plant material (0.1 g) was extracted with 2 ml of 80% ethanol for 48 h. After evaporation of ethanol on a water-bath at 70°C, 20 ml of distilled water were added, and the mixture was shaken vigorously. To 1 ml of sample, 1 ml of 5% phenol, and 2 ml of H_2SO_4 were added, and the mixture was stirred. After cooling in an ice bath for 25 min, the absorbance of the sample was recorded at 490 nm (R^2= 0.994).

Calcium, phosphorus and potassium contents

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

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

Phytochemical screening

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

Determination of Total phenolics (TPC), flavonoids ((TFC), flavonols and flavones (TFlC) and proanthocyanidins (TPAC) (condensed tannins) contents

The phenolics content was measured using the modified Folin-Ciocalteau method (Velioglu, Mazza, Gao, and Oomah, 1998). Gallic acid was used as a standard to produce the calibration curve. Total phenol content was expressed as mg gallic acid equivalent/g dry matter (mg GAE/g dw) (R^2= 0.996). The flavonoids *(TFd)* content was determined spectrophotometrically according to the method described by Omezzine and Haouala (2013) and expressed as mg quercetine equivalent/g dry weight (mg QE/g dw) using quercetin calibration curve (R2= 0.993). Total flavonols and flavones content was determined using the method described by Omezzine and Haouala (2013) and expressed as mg quercetin equivalent/g dry weight (mg QE/g dw) using quercetin calibration curve (R^2= 0.932). The

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

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

To determine the fatty acid composition, approximately 10 mg of petroleum ether seeds and aerial parts extracts were dissolved in 0.2 ml of hexane , followed by the addition of 0.5 ml of Boron trifluoride (BF_3) reagent (methanol / BF_3-Methanol (14% Boron trifluoride in methanol) / hexane (55:25:20). Samples were placed in a water-bath at 70℃ for 1.5 h in tightly closed tubes, then 0.5 ml of saturated NaCl solution, 0.2 ml of 10% H_2SO_4, and 7 to 8 ml of hexane were added to the tubes. The samples were shacked, and 0.5µl of the organic layer was taken to determine the fatty acid composition by gas chromatography (GC). GC analyses were performed using a Hewlett-Packard 6890 Series gas chromatograph equipped with a flame ionization detector (FID) and an electronic pressure control (EPC) injector. An apolar column VF-WAX ms (Agilent J&W cp9205) (30 m, 0.25 mm id, 0.25µm film thickness) was used. The carrier gas was N2 with a flow rate of 1.7 ml/min. The injection was performed in on-column mode. The analyses were performed using the following temperature program: raise from 55°C to 150°C (at 30°C/min), then up to 250 °C at 5°C/ min, and finally maintained at 250°C for 10 min. Analyses were performed in triplicate. Fatty acid methyl esters were identified by comparison of their retention times with those of pure reference standards (external standards) purchased from sigma-aldrich (Diegem, Belgium). Individual fatty acids were expressed as percentage of the total fatty acids in the considered sample (Toma et al., 2013).

Phytotoxic bioassays

Tests with organic extracts

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

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

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

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

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

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

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

Inhibition index (I)

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

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

Statistical analysis

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

Results and discussion

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.

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

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

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

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

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

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

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

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

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

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

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

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

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

Conclusion

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

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