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Variation in phytochemical constituents and allelopathic potential of *Nigella sativa* with developmental stages

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ABSTRACT

This study was conducted to evaluate the phytochemical content and allelopathic potential of two *Nigella sativa* varieties, having a Tunisian and Indian origin. Aqueous extracts of seeds and aerial parts harvested at three developmental stages (vegetative, flowering and fruiting) were evaluated on lettuce germination and seedling growth. The total phenolics, flavonoids, flavonols and flavones, alkaloids and proanthocyanidins contents in the aqueous extracts were highest in the vegetative stage. For allelopathic activity, all aqueous extracts significantly delayed germination, reduced its rate and affected seedling growth, while seeds aqueous extracts affect only seedling growth. In addition, the analysis using the Whole-range assessment method (WESIA software) showed a stronger inhibition index of the Tunisian variety aqueous extracts of aerial parts harvested at flowering stage compared to the two other stages. While, Indian variety was most phytotoxic at the vegetative stage. Seeds of the two varieties showed similar toxicity for lettuce and they are less toxic than aerial parts. Results showed that it would be advisable to identify the development stage of a plant that has the greatest level of allelochemicals to assist harvest time and to maximize efficiency of the allelopathic potential of a given plant.

1. Introduction

Due to increasing numbers of herbicide-resistant weed biotypes and environmental concerns about the safety of synthetic herbicides, considerable effort has been put into designing alternative weed-management strategies and reducing dependence on synthetic herbicides. Towards these ends, the use of plants with strong allelopathic properties for weed control has shown promising approach (Jamil et al., 2009). A number of plant species have been reported to exhibit allelopathic activity on the growth of other plant species (Narwal, 1999; Duke et al., 2000; Haouala et al., 2008). Chemicals with allelopathic activity are present in many higher plants and in many plant organs (Inderjit, 1996; Duke et al., 2000; Ladhari et al., 2013) such as alkaloids, phenolics, terpenoids, flavonoids, coumarines, tannins, steroids and quinines (Xuan et al., 2005). These compounds are released into the environment, either as exudates from living plants or by decomposition of plant residues in sufficient quantities to affect neighboring or succession plants (Rice, 1984; Dayan et al., 2000). Active substances of medicinal plants have strong allelopathic properties (Fujii et al., 2003; Khan et al., 2009) that could be used safely in agro-ecosystems. Indeed, medicinal plants are inhibitory to selected weeds (Khan et al., 2011; Islam and Kato-Noguchi, 2012) and some of their herbicidal allelochemicals have been identified (Silva et al., 2012; Imatomi et al., 2013). It is easier to screen the allelopathic plants from medicinal plants because they are rich in metabolic compounds (Fujii et al., 2003). Many researchers have reported that the kind and concentration of secondary metabolites varied among plant species and they may even vary within the different parts of the same plant. These secondary metabolites are, particularly, prone to qualitative and quantitative variations depending on genetic drift, physiological conditions, season, harvesting time and analytical method sample preparation (Çirak et al., 2008).

Numerous investigations on the influence of the aforementioned factors have been conducted (Couceiro et al., 2006; Tatsis et al., 2007). The developmental stage is another source of variability that considerably influences the secondary metabolite concentration (Çirak et al., 2008; Omezzinea and Haouala, 2013). Therefore, the determination of the optimum harvest time is important to obtain maximum natural product production and to assess the viability of a medicinal plant as a potential crop (Taylor and van Staden, 2001).

Nigella (*Nigella sativa* L) is an annual herbaceous plant belonging to the Ranuculacea family, commonly grown in the Mediterranean

Abbreviations: NT, *Nigella sativa* Tunisian variety; NI, *Nigella sativa* Indian variety.

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countries, Eastern Europe, the Middle East, and Western Asia (Gad et al., 1963). Mature seeds commonly known as Black Cumin or "Habbatul Barakah" are consumed for edible and medical purposes. According to common Islamic and Arabic belief, Habbatul Barakah is a remedy for all ailments (Tariq, 2008). The composition and properties of this species have been fairly well investigated. The multiple uses of *N. sativa* seeds in folk medicine encouraged many investigators to isolate its active components, including: thymoquinone thymohydroquinone, dithymoquinone, thymol, carvacrol, nigellicine, nigellidine and alpha-hederin (Randhawa and Alghamdi, 2002; Gali-Muhtasib et al., 2006; Al-Saleh et al., 2006). A large number of in vitro and in vivo studies have been conducted on laboratory animals and humans in order to investigate its pharmacological properties, like immunostimulation, anti-inflammatory (El-Dakhakhny et al., 2002; Hajhashemi et al., 2004), hypoglycemic, antioxidant (Meral et al., 2001), antihypertensive, antiasthmatic, antimicrobial, antiparasitic (Randhawa and Alghamdi, 2002), hepatoprotective (Mahmoud et al., 2002; Kanter et al., 2005), as well as anticancer properties (Randhawa and Alghamdi, 2011). Also, it was reported that this species showed an insect repellent effects (Fisher, 2002) and allelopathic potential (Al-Charchafchi et al., 2007).

The present study aimed to check if secondary metabolites (polyphenols, flavonoids, flavonols and flavones, alkaloids and condensed tannins) production varied with developmental stages, and if there are correlations between them and allelopathic potentialities of Tunisian (NT) and Indian *N. sativa* (NI) varieties. This study was carried out by spectrophotometric method, for allelochemicals determination, and through the effects of aqueous extracts of aerial parts harvested at three developmental stages, on lettuce (*Lactuca sativa* L.), for allelopathic potential.

2. Materials and methods

2.1. Plant material and aqueous extract preparation

Two varieties of mature black cumin (*N. sativa* L.) seeds were purchased from an herbal market in Sousse (Tunisia). One sample was reported to be imported from India (NI), and the other one was a Tunisian variety (NT). Nigella seeds were sown in greenhouse in December 2012. Aerial parts were harvested at vegetative [plants with 7 leaves], flowering [50% of flowers open] and fruiting stages [50% of the pods have reached a typical length]. Fresh plants were washed with tap water, then oven-dried at 60 °C for 72 h, powdered and used for extraction. One hundred grams of each type of dried plant material was soaked in 1000 ml distilled water at room temperature for 24 h to give a concentration of 100 g/l dry tissue i.e. 10% (*w*/*v*) (Samedani et al., 2013) and the extracts were filtered through a filter paper.

2.2. Phytochemical screening

2.2.1. Total phenolic (TP) content

The TP was measured using the modified Folin–Ciocalteau method (Velioglu et al., 1998). Sample extract (100 μl) was mixed with 500 μl of 1/10 diluted (in Milli-Q water) Folin–Ciocalteau phenol reagent and allowed to react for 5 min in the dark at room temperature. Then 400 μl of sodium bicarbonate (7.5%) was added to the mixture. After 90 min of incubation in the dark at 30 °C, the absorbance was read at 765 nm. TP was expressed as mg gallic acid equivalent/g dry matter (mg GAE/g dw) using gallic acid calibration curve ($R^2 = 0.984$).

2.2.2. Total flavonoid (TFd) content

The TFd content was determined spectrophotometrically according to standard method (Quettier et al., 2000). Briefly, 0.5 ml of 2% solution of $AlCl_3$ in methanol was mixed with the same volume of extract. Absorption readings at 430 nm were taken after 30 min against a blank. TFd content was expressed as mg quercetine equivalent/g dry weight (mg QE/g dw) using quercetine calibration curve ($R^2 = 0.970$).

2.2.3. Total flavonol and flavone (TFl) content

The TFl content was determined using the method of Kumaran and Karunakaran (2007). To 2 ml of sample, 2 ml of 2% $AlCl_3$ in methanol and 3 ml (50 g/L) sodium acetate solutions were added. The absorption at 440 nm was read after 2.5 h of incubation at 20 °C. TFl content was expressed as mg quercetine equivalent/g dry weight (mg QE/g dw) using quercetine calibration curve ($R^2 = 0.986$).

2.2.4. Total precipitable alkaloid (TA) content

The TA content was determined by spectrophotometric method with Dragendorff reagent (Stumpf, 1984). Principally, 300 μl of plant extract was mixed with 100 μl of Dragendorff reagent. After centrifugation at 7000 *g* for 1 min, the supernatant was removed and dissolved in 1 ml of 2.45 M NaI. An aliquot of 10 μl of each tube was added to 1 ml of 0.49 M NaI, after which the absorbance was read at 467 nm. TA content was expressed as mg papaverine hydrochloride equivalent/g dry weight (mg PAHE/g dw) using papaverine hydrochloride calibration curve ($R^2 = 0.990$).

2.2.5. Total proanthocyanidin (TPA) (condensed tannin) content

The TPA content determination was based on the procedure reported by Sun et al. (1998). A volume of 0.5 ml of extract was mixed with 3 ml of 4% vanillin–methanol (*w*/*v*) and 1.5 ml hydrochloric acid. The mixture was allowed to stand for 15 min, and then the absorbance was measured at 500 nm. TPA content was expressed as mg catechin equivalent/g dry weight (mg CE/g dw) using catechin calibration curve ($R^2 = 0.999$).

2.3. Laboratory bioassays

2.3.1. Tests with aqueous extracts

Each extract was diluted with sterile distilled-water to give final concentrations between 5 and 100 g/l. They were tested on lettuce (*L. sativa* L.), a species known to be very sensitive to allelochemicals (Ervin and Wetzel, 2003). Seeds were surface-sterilized with 0.525 g/l of sodium hypochlorite for 15 min and then rinsed four times with deionized water (Chon et al., 2005). Twenty imbibed seeds of lettuce were separately placed on the filter paper in sterilized Petri dishes (9 cm) and 5 ml of each extract was applied for each treatment. 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 with 400 mol photons m^{-2} s^{-1} photosynthetically active radiation (PAR) at 24/22 °C for 14/10 h light and dark periods, respectively (Omezzine et al., 2014).

Germination was determined by counting the number of seeds that had germinated at 24 h intervals over 6 days. Germination percentage (G %) was calculated by dividing the total number of seeds that germinated on the seventh day in each treatment by the number of seeds sown 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 index of germination (GI) was calculated using the following formula (Chiapuso et al., 1997):

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

where $N_1, N_2, N_3 \ldots N_n$ = proportion 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. The

following formula was used to calculate the % inhibition/stimulation (Chung et al., 2001):

$$Inhibition(-)/Stimulation(+)(\%) = \left[\frac{extract-control}{Control}\right] \times 100$$

2.3.2. Inhibition index (I)

The allelopathic dose–response effects of aqueous 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. (1), used by Liu et al. (2007), where concentrations tested ranged from 0 to D_n (D_n was dose–concentration tested from 0, D_1, $D_2 \ldots D_n$), D_c was the threshold dose at which response equalled 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, in the presence of *N. sativa* different extracts were used to calculate inhibition indices (I) using the WESIA (Whole-range Evaluation of the Strength of Inhibition in Allelopathic-bioassay) software (Liu et al., 2007):

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

2.4. Statistical analysis

All data were reported as means ± standard deviation (S.D.) of three replicates and analyzed using IBM SPSS Statistics 20.0. Differences between the means were established, using a general linear model (GLM) procedure ($P < 0.05$) related to the two variables: extraction type and phenological stage. Differences at the 5% level ($P < 0.05$) were considered statistically significant.

3. Results

3.1. Phytochemical screening

The content of total phenolics (TP), flavonoids (TFd), flavonols and flavones (TFl), precipitable alkaloids (TA) and proanthocyanidins (TPA) in aerial parts and seeds of the two varieties of *N. sativa* is given in Table 1. Results showed that the two varieties produced the highest amounts of all compounds during the vegetative stage and the smallest amounts during the fruiting one, except for TP and TFl in Indian variety for which the aerial parts were richer at the fruiting stage than the flowering stage (116.91 mg of GAE/g DW and 26.88 mg of QE/g DW respectively). Results showed also that aerial parts from the two varieties were richer in TP, TFd, TFl, and TPA than seeds. At vegetative stage, TP presented the highest amount in NI and NT aerial parts with 178.14 mg of GAE/g DW and 124.17 mg of GAE/g DW respectively. At the flowering stage, overall, the compounds have intermediate contents compared to those recorded at the two other stages. In all cases, aerial parts contained remarkable content of phenols followed by flavonoids.

3.2. Aqueous extract effect on germination and growth

The results showed that *N. sativa* aqueous extract effect varied significantly with concentration and with developmental stage (Table 2). Aqueous extracts of aerial parts and seeds severely inhibited germination of lettuce at 100 g/l. Furthermore, at 50 g/l, all aqueous extract delayed the start of lettuce germination but did not affect final germination percentage. In fact the lowest germination index was recorded with NT aerial parts harvested at the vegetative stage (GI = 50.54%) (Table 2). Regarding the aqueous extracts of NI aerial parts, the lowest Germination index was recorded at 50 g/l with plant material harvested at the fruiting stage (GI = 65.82%) followed by plant material harvested at vegetative stage (GI = 66.86%). The aqueous seed-extracts of both varieties didn't affect the final germination percentage of lettuce but caused a delay in the starting of germination at 100 g/l.

To estimate the sensitivity of lettuce against *N. sativa* extracts, the IC50 (concentration extract inducing 50% germination inhibition) and MIC (minimum inhibitory concentration) values were simply estimated by means of an analysis of the dose–response relationship (Table 3). The IC50 values for seed germination were 74, 76 and 77 g/l in the presence of aqueous extracts of NT material harvested at vegetative, flowering and fruiting stages, respectively, and the MIC value of these extracts was 100 g/l (Table 3). Lettuce seeds were less sensitive to NI aqueous extracts than NT aqueous extracts. In fact, the IC50 values for seed germination were 86 and 77 g/l in the presence of NI aqueous extracts of material harvested at vegetative and fruiting stages and higher than 100 g/l with material harvested at flowering stage.

For lettuce growth, the aqueous extracts of the two varieties of *N. sativa* aerial parts were toxic at all concentrations, especially for roots which were more sensitive than shoots and their growth was inhibited at the lowest concentration used (5 g/l) (Fig. 1). In the presence of NT aqueous extract, the greater significant toxicity was recorded with extracts of material harvested at vegetative stage ($IC_{50} < 5$ g/l), and at flowering and fruiting stages IC_{50} values were 8 and 8.5 g/L, for the root growth. For the shoot growth, IC_{50} values were 70, 21 and 64 g/L for aqueous extracts of material harvested at vegetative, flowering and fruiting stages respectively. The MIC value for seedling growth was 100 g/l at the three stages. (Table 3, Fig. 1). At low doses (<25 g/l), a stimulating effect on shoot growth was observed with most extracts. The highest stimulation of shoot growth (41%) was recorded at the vegetative stage (Fig. 1). With NI extracts, lettuce seedling growth was sensitive to those of plant material harvested at the vegetative stage, which toxicity was the strongest, followed by plant material harvested at fruiting, then flowering stages. For, the root growth, IC_{50} values were 7, 12 and 9 g/l for aqueous extracts of material harvested at vegetative, flowering and fruiting stages, respectively, while the respective values for shoot growth were 71, 83 and 80 g/L (Fig. 1, Table 3). The seeds aqueous extracts of the two varieties were less toxic than those of aerial parts. They provoked a significant inhibition at higher concentrations

Table 1
Total phenolic (TP), total flavonoid (TFd), total flavonol and flavone (TFl), total precipitable alkaloid (TA) and total proanthocyanidin (TPA) (condensed tannin) contents in Tunisian and Indian varieties of *N. sativa* seeds and aerial parts harvested at vegetative, flowering and fruiting stages.

Developmental stage	Vegetative	Flowering	Fruiting	Seeds	Vegetative	Flowering	Fruiting	Seeds
	Nigella sativa Tunisian *var*				*Nigella sativa* Indian *var*			
TP (mg GAE/g dw)	124.17 ± 3.53^{c}	116.29 ± 5.71^{b}	117.49 ± 1.83^{b}	56.32 ± 0.77^{a}	178.14 ± 2.32^{d}	76.07 ± 4.30^{b}	116.91 ± 1.34^{c}	35.07 ± 0.18^{a}
TFd (mg QE/g dw)	81.25 ± 7.32^{c}	79.95 ± 5.79^{c}	64.06 ± 4.49^{b}	21.42 ± 1.94^{a}	70.12 ± 3.26^{b}	69.79 ± 3.17^{b}	70.61 ± 4.13^{b}	22.53 ± 2.83^{a}
TFl (mg QE/g dw)	32.70 ± 2.62^{c}	30.27 ± 1.99^{c}	24.85 ± 1.81^{b}	2.82 ± 0.97^{a}	45.70 ± 2.86^{d}	23.66 ± 0.94^{b}	26.88 ± 1.26^{c}	4.77 ± 0.13^{a}
TA (mg PAHE/g dw)	0.56 ± 0.06^{c}	0.2 ± 0.02^{a}	0.19 ± 0.01^{a}	0.32 ± 0.05^{b}	0.48 ± 0.06^{c}	0.16 ± 0.02^{a}	0.18 ± 0.01^{a}	0.26 ± 0.26^{b}
TPA (mg CE/g dw)	49.83 ± 1.56^{d}	26.31 ± 1.00^{c}	22.83 ± 0.66^{b}	10.39 ± 0.81^{a}	21.40 ± 1.14^{c}	20.13 ± 0.62^{ab}	19.10 ± 0.86^{b}	12.13 ± 1.55^{a}

All analyses are the mean of five measurements ± standard deviation. Means followed by at least one same letter are not significantly different at $P < 0.05$.

Table 2
Germination index (GI), expressed in % of control, and germination percentage (G %) of *Lactuca sativa* in the presence of aqueous extracts, at different concentrations, of Tunisian and Indian varieties of *N. sativa* seeds and aerial parts harvested at vegetative, flowering and fruiting stages.

Aqueous extracts concentration (g/l)	Developmental stage							
	Vegetative		Flowering		Fruiting		Seeds	
	GI	G%	GI	G%	GI	G%	GI	G%
Nigella sativa Tunisian var								
Control	–	100 ^{c}	–	100 ^{b}	–	100 ^{b}	–	100 ^{a}
5	100 ^{c}	100 ^{c}	100 ^{c}	100 ^{b}	100.56 ^{d}	100 ^{b}	100.56 ^{b}	100 ^{a}
15	100 ^{c}	100 ^{c}	100 ^{c}	100 ^{b}	101.71 ^{d}	100 ^{b}	100 ^{b}	100 ^{a}
25	100 ^{c}	100 ^{c}	100.57 ^{c}	100 ^{b}	97.77 ^{c}	100 ^{b}	100.56 ^{b}	100 ^{a}
50	50.54 ^{b}	95 ^{b}	72.62^{b}	100 ^{b}	82.58 ^{b}	100 ^{b}	98.87 ^{b}	100 ^{a}
100	0^{a}	0^{a}	0^{a}	0^{a}	1.46 ^{a}	5^{a}	68.20 ^{a}	100 ^{a}
Nigella sativa Indian var								
Control	–	100 ^{b}	–	100 ^{a}	–	100 ^{b}	–	100 ^{a}
5	99.44 ^{e}	100 ^{b}	101.13 ^{c}	100 ^{a}	101.70 ^{d}	100 ^{b}	101.14 ^{b}	100 ^{a}
15	85.59 ^{d}	100 ^{b}	100.56 ^{c}	100 ^{a}	101.15 ^{d}	100 ^{b}	100 ^{b}	100 ^{a}
25	75.84 ^{c}	100 ^{b}	101.71 ^{c}	100 ^{a}	89.72 ^{c}	100 ^{b}	101.13 ^{b}	100 ^{a}
50	66.86 ^{b}	100 ^{b}	93.73 ^{b}	100 ^{a}	65.82 ^{b}	100 ^{b}	100.29 ^{b}	100 ^{a}
100	11.68 ^{a}	30 ^{a}	52.31 ^{a}	96.6 ^{a}	1.47 ^{a}	5^{a}	68.58 ^{a}	100 ^{a}

Means with the same letter in a column are not significantly different at $P < 0.05$ (LSD test). Values ($N = 3 \pm$ S.D.).

for roots and shoot growth and induced a significant stimulation at low dose.

Under the all aqueous extracts treatment, roots were fragile and suffered from necrosis and the degrees of necrotic roots increase with concentration (Fig. 2). At 50 and 100 g/l, all the extracts induced the formation of root curving, characterizing the seedlings as abnormal (Fig. 2).

3.3. Correlation analysis

Aqueous extracts of the two varieties of *N. sativa* contain different groups of allelochemicals. In order to determine the extent of the potential allelopathic contribution of each one of these compound groups, relationships between total phenolics, flavonoids, flavonols and flavones, total proanthocyanidins and precipitable alkaloid contents in aerial parts and seeds, and allelopathic activity of their aqueous extracts assessed on lettuce germination and growth were established. Table 4 shows a significant correlation between all groups of allelochemicals and lettuce germination and seedling growth.

For germination index, a perfect significant negative relationship ($p < 0.01$) was found with TFl content of NI aerial parts harvested at vegetative stage ($r = -1$) followed by TPA content ($r = -0.999$), and then TA ($r = -0.972$) of aerial parts harvested at flowering stage. In addition, a strongest correlation ($p < 0.05$) was found with TFl content ($r = -0.908$) of NT aerial parts harvested at flowering stage. Regarding germination rate, the strongest relations were registered for TP content of NT aerial parts harvested at flowering stage ($r = -0.989$) followed by TPA content of NT seeds ($r = -0.887$). A significant negative relationship ($p < 0.05$) was found with TFd content of NI aerial parts harvested at flowering and fruiting stages ($r = -0.992$) and with TA content of NI aerial parts harvested at vegetative stage ($r = -0.972$). Concerning the other groups, correlation was slightly weaker (Table 4).

Regarding root growth, the strongest relations were registered for TFl, TA and TFd contents of NT aerial parts harvested at vegetative stage with respective *r* values of 0.985, 0.959 and 0.950. Furthermore, the highest positive relationship was recorded between TFd content of NI aerial parts harvested at vegetative stage and root growth ($r = 0.952$) (Table 4). Indeed for shoot growth, the strongest positive relations were recorded for TFd ($r = 0.989$) of NT seeds followed by TPA of NT aerial parts at vegetative stage ($r = 0.875$). Likewise, a significant positive relationship was recorded between TP of NI aerial parts harvested at vegetative and shoot growth ($r = 0.994$), followed by TA of NI seeds ($r = 0.892$).

Table 3
The half inhibitory concentrations (IC_{50}) (g/l) and the minimum inhibitory concentrations (MIC) (g/l) for *Lactuca sativa* germination and growth in the presence of different concentrations of aqueous extracts of Tunisian and Indian varieties of *N. sativa* seeds and aerial parts harvested at vegetative, flowering and fruiting stages.

		Vegetative	Flowering	Fruiting	Seeds
N. sativa Tunisian var					
Germination	IC_{50}	74.7^{a}	76^{b}	77.3^{c}	>100
	MIC	100	100	100	>100
Root growth	IC_{50}	<5	8^{a}	8.5^{b}	10.5^{c}
	MIC	100	100	100	100
Shoot growth	IC_{50}	70^{c}	21.3^{a}	64^{b}	>100
	MIC	100	100	100	100
N. sativa Indian var					
Germination	IC_{50}	86^{a}	> 100	77.5^{b}	>100
	MIC	100	100	100	>100
Root growth	IC_{50}	7^{a}	12^{c}	9^{b}	34^{d}
	MIC	100	100	100	100
Shoot growth	IC_{50}	71.5^{a}	83^{c}	80.4^{b}	>100
	MIC	100	100	100	100

Means with the same letter in a column are not significantly different at $P < 0.05$ (LSD test). Values ($N = 3 \pm$ S.D.).

3.4. N. sativa phytotoxicity assessed by inhibition index (I)

The Whole-range assessment can display a visual comparison between different biological parameters while the conventional analysis cannot provide any such portrayal as these growth parameters have different units and this affects the details of the grouping and ranking order; i.e. this analysis allowed us to group and to identify the most toxic extracts (or the phenological stages at which the plant could produce the most toxic material) (Omezzine et al., 2014) (Table 5).

For NT aqueous extracts, germination percentage and germination index were especially affected by the extract of plant material harvested at the vegetative stage, which was the most toxic, followed by that collected at flowering and then fruiting stages. In fact, the inhibition index (I) went from 24.5% to 21.9% for germination percentage and from 43.9% to 29.2% for germination index. However, root length and shoot length were especially affected by the extract of plant material harvested at the flowering stage followed by that collected at vegetative stage. Indeed, the inhibition index (I) went from 76.4% to 64.9% for root length and from 65.3% to 43.4% for shoot length

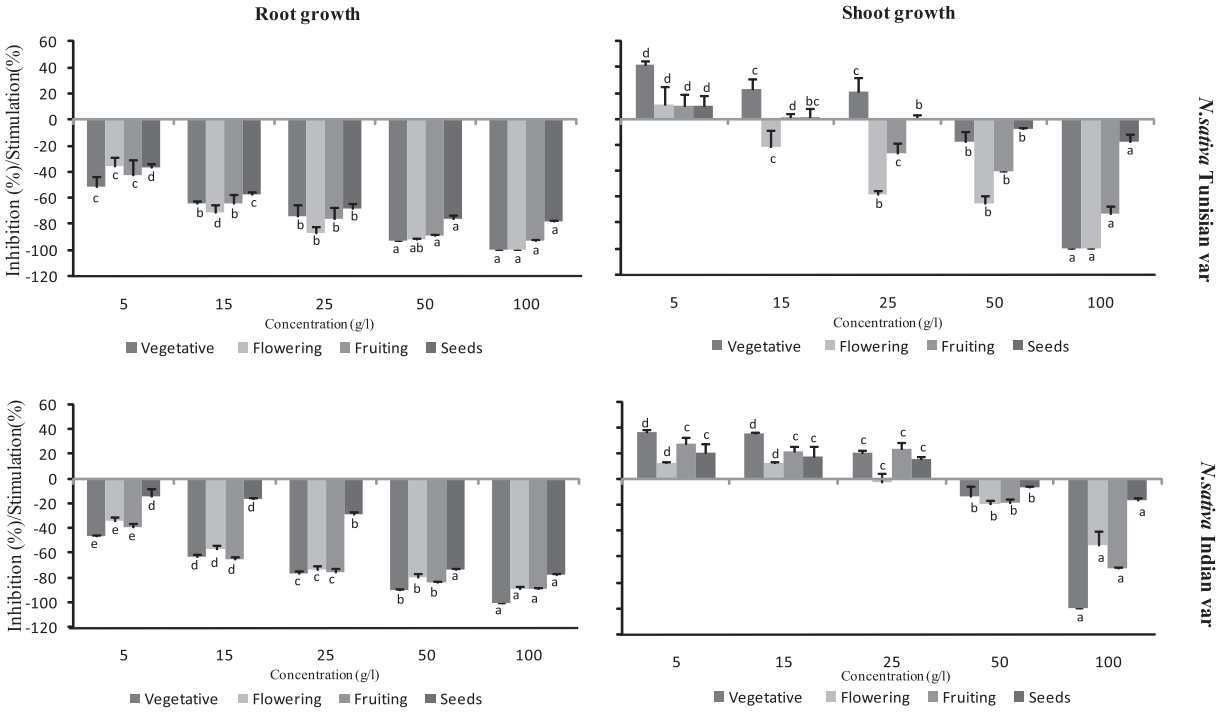

Fig. 1. Percentage inhibition (−) or stimulation (+) of lettuce root and shoot growth, in the presence of aqueous extracts (at different concentrations) of Tunisian and Indian varieties of *N. sativa* seeds and aerial parts harvested at vegetative, flowering and fruiting stages, 7 days after pre-germination. The bars on each column show standard error. Value (N = 3 ± S.E.). Different letters on columns indicate significant differences among concentrations at $P < 0.05$ (LSD test).

(Table 5). Furthermore, the results showed that NT seeds are less toxic than the aerial parts.

In the presence of NI aqueous extracts, Table 5 shows that material harvested at the vegetative stage was the most toxic for germination index (I = 40.2%), root length (I = 67.6%) and shoot length (I = 39.5%) followed by that collected at fruiting stage. However, germination percentage was especially affected by the extract of plant material harvested at the fruiting stage, which was the most toxic (I = 21.9%). NI seeds showed similar sensitivity to lettuce as NT seeds which are less toxic than the aerial parts.

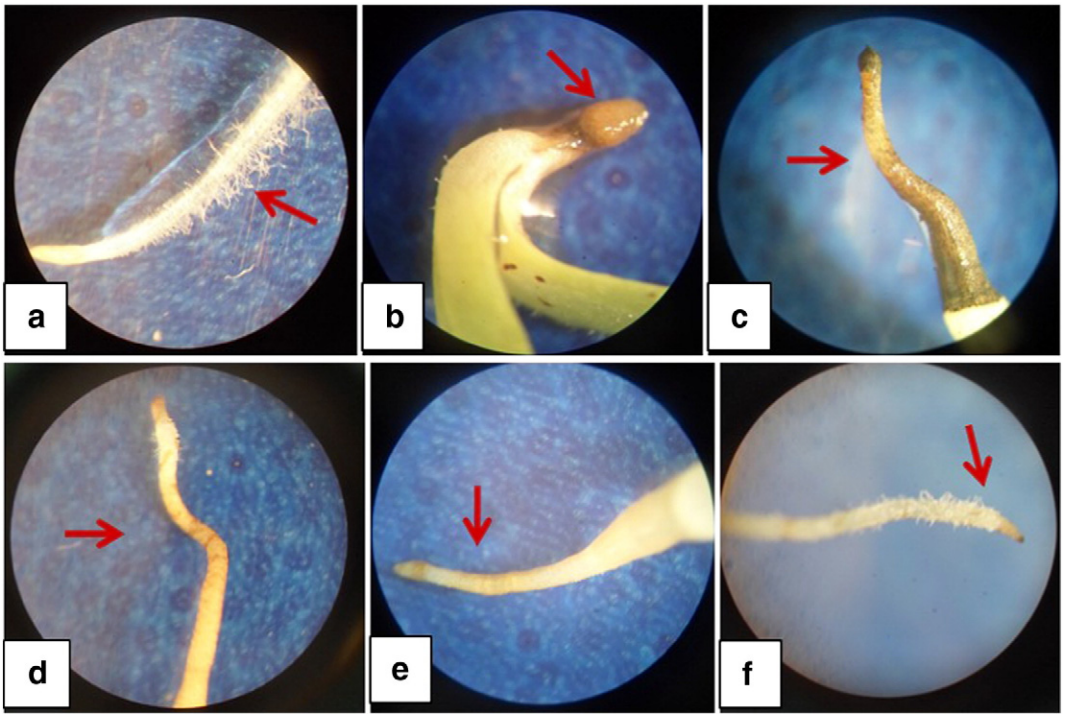

Fig. 2. Effect of aqueous extracts of Tunisian variety of *N. sativa* aerial parts harvested at flowering stage tested at five concentrations induced severe root necrosis on *Lactuca sativa*, in the seventh day after pre-germination. (a) Control, (b) 100 g/l, (c) 50 g/l, (d) 25 g/l, (e) 15 g/l and (f) 5 g/l (observation under a binocular microscope (G × 40).

Table 4
Correlation between total phenolic (TP), total flavonoid (TFd), total flavonol and flavone (TFl), total proanthocyanidin (TPA) and total alkaloid (TA) contents of Tunisian and Indian varieties of *N. sativa* seeds and aerial parts harvested at different stages of development and allelopathic activity of their aqueous extracts assessed on lettuce.

		Nigella sativa Tunisian var				*Nigella sativa* Indian var			
		GI	G%	% inhibition of root growth	% inhibition of shoot growth	GI	G%	% inhibition of root growth	% inhibition of shoot growth
TP	Vegetative	−0.500	−0.500	−0.972	−0.476	−0.114	0.998*	0.345	0.994
	Flowering	0.989	−0.989	−0.477	0.419	−0.036	0.644	−0.885	−1**
	Fruiting	1**	0.500	−0.964	−0.996	0.855	−0.693	−0.792	−0.999*
	Seeds	−0.424	0.596	−0.862	−0.891	−0.658	0.866	−0.262	−0.481
TFd	Vegetative	0.569	0.569	0.950	0.547	0.713	0.559	0.952	0.694
	Flowering	0.847	−0.847	−0.791	0.018	−0.819	−0.992	0.091	0.546
	Fruiting	0.676	−0.300	−0.848	−0.608	0.926	−0.992	0.018	−0.629
	Seeds	0.691	−0.308	0.655	0.989	0.193	−0.5	0.71	−0.022
TFl	Vegetative	0.115	0.115	0.985	0.088	−1**	0.189	−0.89	0.016
	Flowering	−0.908	0.908	0.706	−0.145	0.196	0.803	−0.754	−0.973
	Fruiting	−0.500	0.5	0.712	0.421	0.115	−0.365	0.885	0.379
	Seeds	0.993	0.573	−0.207	0.713	−0.343	0.629	−0.592	−0.133
TPA	Vegetative	0.888	0.888	0.693	0.875	−0.999*	0.219	−0.876	0.046
	Flowering	−0.629	0.629	−0.514	−0.995	0.861	0.98	−0.014	−0.48
	Fruiting	−0.136	0.79	0.394	0.047	0.822	−0.649	−0.827	−0.995
	Seeds	−0.827	−0.887	0.637	−0.307	0.195	0.133	−0.924	0.401
TA	Vegetative	0	0	0.959	−0.027	−0.052	−0.972	−0.496	−0.998*
	Flowering	0.672	−0.672	−0.927	−0.253	−0.972	−0.878	−0.28	0.201
	Fruiting	−0.189	0.756	0.443	0.101	−0.262	0.5	−0.806	−0.237
	Seeds	−0.847	0.065	−0.45	−0.995	0.774	−0.527	−0.956	0.892

GI, germination index; G%, germination percentage. * Indicates significant difference between stages of development at $P < 0.05$. ** Indicates significant difference between stages of development at $P < 0.01$.

4. Discussion

The aim of this study was to evaluate the effect of the developmental stages (vegetative, flowering and fruiting) on the secondary metabolite production by two varieties of *N. sativa* (Tunisian and Indian varieties) and on its allelopathic potential. Results showed that *N. sativa* aqueous extracts were rich in phenols, flavonoids and tannins; they also possess high allelopathic activities. These parameters varied significantly with intrinsic parameters (such as growth stages and genetic) and with the season (James, 1950; Fratianni et al., 2007). Our results showed that *N. sativa* aerial parts aqueous extracts are richer in phytochemicals contents than seeds. Nevertheless, the levels of total polyphenols in NT and NI seeds were higher than those reported by Thippeswamy and Naidu (2005). The difference of phytochemical content between the organs of *N. sativa* was reported by Bourgou et al. (2008). The quantity of secondary metabolites found in the two varieties of *N. sativa* decreased with the increase of plant growth. In fact, phytochemical analysis of NT and NI aqueous extracts showed that aerial parts were richer in all groups of compounds at the vegetative stage. These findings are in agreement with those of Omezzinea and Haouala (2013) where the total phenolics, flavonoids, flavonols and flavones, alkaloids and proanthocyanidins contents in *Trigonella foenum–graecum* aqueous

Table 5
Phytotoxicity of aqueous extracts of Tunisian and Indian varieties of *N. sativa* seeds and aerial parts harvested at vegetative, flowering and fruiting stages, on lettuce germination and growth, assessed by Inhibition index (I) estimated from WESIA (Whole-range Evaluation of the Strength of Inhibition in Allelopathic-bioassay).

	Nigella sativa Tunisian var			*Nigella sativa* Indian var		
Growth parameter	Development stage	Inhibition index (I)	Phytotoxicity	Development stage	Inhibition index (I)	Phytotoxicity
GI	Vegetative	24.58	More toxic (+)	Fruiting	21.92	More toxic (+)
	Flowering	23.07		Vegetative	16.15	
	Fruiting	21.92		Flowering	0	
	Seeds	0	Less toxic (−)	Seeds	0	Less toxic (−)
G%	Vegetative	43.95	More toxic (+)	Vegetative	40.2	More toxic (+)
	Flowering	32.98		Fruiting	38.9	
	Fruiting	29.26		Flowering	13.36	
	Seeds	7.73	Less toxic (−)	Seeds	7.45	Less toxic (−)
Root length	Flowering	76.46	More toxic (+)	Vegetative	67.63	More toxic (+)
	Vegetative	70.40		Fruiting	61.90	
	Fruiting	64.93		Flowering	60.27	
	Seeds	50.63	Less toxic (−)	Seeds	45.35	Less toxic (−)
Shoot length	Flowering	65.30	More toxic (+)	Vegetative	39.54	More toxic (+)
	Fruiting	43.60		Fruiting	34.07	
	Vegetative	43.43		Flowering	27.59	
	Seeds	13.98	Less toxic (−)	Seeds	16.65	Less toxic (−)

GI, germination index; G%, germination percentage.

extracts were lower in the fruiting stage in comparison with the vegetative stage. In *Rhus*, *Euonymus* and *Acer* leaves, the total phenols increased rapidly at the early growth stages but thereafter the content was kept rather constant (Ishikura, 1976). Nevertheless, Ayan et al. (2007) reported that total phenol content reached the highest level during floral budding in *Hypericum hyssopifolium* and *Hypericum scabrum* and at full-flowering in *Hypericum pruinatum*.

Analysis by WESIA software, used to evaluate the strength of the allelopathic potential of different extracts, shows that the inhibitory effect of *N. sativa* extracts depends on its growth stage. The aqueous extract of NT aerial parts collected at the vegetative stage was the most toxic for lettuce germination. However, for lettuce growth, NT material harvested at flowering stage was the most toxic which might be attributed to polyphenols, flavonols and flavones. For NI aqueous extracts, material harvested at the fruiting stage was the most toxic for germination rate and material harvested at the vegetative stage was the most phytotoxic for seedling length which might be attributed to phenols and flavonoids. These findings are in agreement with those of Omezzine et al. (2014) and Omezzinea and Haouala (2013) who indicated that a stronger inhibition index in lettuce germination and growth was recorded in presence of *Trigonella foenum–graecum* aerial parts harvested at vegetative stage compared to the two other stages. These variations might be explained by several factors, from endogenous regulation of physiological processes to environmental characteristics. Plant tissue maturity affects the allelochemical contents and intensity. Thereby, the quantities of allelochemicals in soybean stubs were different at different growth stages (Wang et al., 2001; Hu and Kong, 2002). It is reported that phenolic, flavonoid, tannin and alkaloid compounds have been frequently implicated in allelopathic reactions, usually inhibiting seed germination and root growth (Rice, 1979; Li et al, 2010). Phenolic allelochemicals can lead to increased cell membrane permeability and increase lipid peroxidation followed by slow growth or death of plant tissue (Li et al, 2010). Einhellig (1995) stated that almost all cases of allelopathic inhibition in a plant community result from the combined effect of several compounds.

Our results indicate that seeds of the two varieties were less toxic than aerial parts. They provoked a significant inhibition at higher concentrations for seedling growth and induced a significant stimulation at low dose. Al-Charchafchi et al. (2007) showed that seeds extracts of *N. sativa* capable of inhibiting root length more than shoot in *Vigna radiata*, which is in agreement with our finding.

Allelochemicals from *N. sativa* affect germination at the highest concentrations and induce alterations in the germination distribution curve. This alteration could indicate interferences in the metabolic reactions that culminate in germination (Labouriau, 1983). Generally, observation verified a more pronounced allelopathic effect on the initial development of target plant seedlings when compared to germination, since the latter process uses the seeds' own reserves. According to Tigre et al. (2012), this dependency on the seed reserves means that germination is probably less susceptible to exogenous factors.

For lettuce growth, the aqueous extracts of the two varieties of *N. sativa* were toxic, especially to roots which were more sensitive than shoots. Chung et al. (2001) and Dana and Domingo (2006) attributed the more accentuated effect on the roots to their closer contact with the extract, particularly when maintained in filter paper. Javaid and Anjum (2006) showed that the reduction in seedlings length may be attributed to the reduced rate of cell division and cell elongation. In all cases, roots were fragile and suffered from necrosis which severity increased with concentration. Similar damages observed in root of lettuce are also described by Tigre et al. (2012). Darkening of the root coloration and fragility reveal damage, which indicates the action of toxic substances in the extracts (Maraschin-Silva and Aquila, 2006). With all aqueous extracts tested, stimulation effect of shoot length was observed when the concentration is less than 25 g/l; it might be attributed to allelochemicals substances like mineral nutrients, organic acids, carbohydrates and growth regulators (Tukey, 1969).

5. Conclusion

The present study reported that the phytochemicals content contributes significantly to the allelopathic activity of *N. sativa* aerial parts and seeds. The degree of inhibition was largely dependent on the developmental stage at which material was collected. The material harvested at vegetative stage had the highest total phenolics, flavonoids, flavonols and flavones and precipitable alkaloid contents. In addition, our results showed that the aqueous extracts of NT aerial parts were shown to be more toxic at flowering stage while those of NI were most phytotoxic at the vegetative stage. Seeds of the two varieties showed similar toxicity for lettuce and were less toxic than aerial parts. Finally, our study showed that it would be advisable to identify the development stage of a plant that has the greatest level of allelochemicals to assist harvest time and to maximize efficiency of the allelopathic potential of a given plant.

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