Artemisia arborescens **L. leaf litter: phytotoxic activity and phytochemical characterization.**

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Abstract

Artemisia arborescens L. is a perennial fast-growing Mediterranean shrub, which releases abundant leaf litter upon soil surface throughout the year. The paper aimed to both evaluate the phytotoxic potential and identify major compounds occurring in the plant leaf litter. Following methanolic maceration of the leaf litter, the crude extract was then sequentially extracted with hexane, chloroform and ethyl acetate through a bio-guided fractionation method. The phytotoxic potential of the methanolic extract and its solvent fractions was assessed *in vitro* on germination and root growth of two sensitive (*Lactuca sativa* L., *Raphanus sativus* L.) and native (*Amaranthus retroflexus* L., *Cynodon dactylon* (L.) Pers.) species. Moreover, the most active fractions were chemically characterized by GC-MS and HPTLC analysis. In all species, either of the physiological processes were highly inhibited by both the methanolic extract and its solvent fractions. Several classes of biologically active phytochemicals such as terpenoids, fatty acids, lignans and phenolic compounds were identified in all fractions. *A. arborescens* leaf litter could be considered an important source of biologically active phytochemicals, which may have a significant allelopathic impact towards neighbouring species once released into the environment.

Keywords: *Artemisia* spp; leaf litter; root growth; seed germination; phenols; flavonoids.

Introduction

Artemisia arborescens L., an endemism of the Mediterranean area, is a pioneer defoliant aromatic species belonging to the *Asteraceae* family, characterized by fast growth and high colonization potential (Pignatti 1982). Since an increasing number of natural phytochemicals have been identified in plant tissues of the genus *Artemisia* (Ferchichi et al. 2006; Gouveia and Castilho 2011; Han et al. 2008; Ma et al. 2008), these species are receiving a growing attention in nutrition, pharmacology and agronomy fields (Carvalho et al. 2011). *Artemisia* spp have been traditionally used in medicine for their therapeutic potential (Ballero et al. 2001; Erdogrul 2002) and their antiviral, antimicrobial, antimalarial together with insecticidal properties have been largely

demonstrated (Jelodar et al. 2014; Sinico et al. 2005; Saddi et al. 2007; WHO 2010). Coumarins, terpenoids, phenolic acids, flavonoids, sterols, caffeoylquinic acids, and acetylenes constitute major classes of phytochemicals of this genus (Bora and Sharma 2011), characterized by high biological activity. Moreover, some of these are considered allelochemicals able to affect neighboring plants once released into the environment (Schenk et al. 1999). This phenomenon, well known as allelopathy, is crucial in defining plant community composition in natural and agro-ecosystems (Rice 1984; Torres et al., 1996). In particular, terpenoid allelochemicals were found in *A. californica* Less. (Muller 1966), *A. absinthium* L. (Funke, 1943), *A. princeps* var. *orientalis* (Yun and Kil, 1992), *A. tridentate* Nutt. ssp. *vaseyana* (Weaver and Klarich, 1977) and *A. vulgaris* (Barney et al., 2005). For this reason, *Artemisia* spp have been reported to exhibit phytotoxic activity towards several plants (Lydon et al. 1997; Escudero et al. 2000; Barney et al. 2005). It has been reported by Kil and Yun (1992) that the aqueous extracts obtained from stems, roots and mature leaves of *Artemisia princeps* var. *orientalis* (wormwood) severely inhibited both seed germination and seedling growth of target species, whereas this effect was not observed with juvenile leaves. Chon et al. (2003) demonstrated the phytotoxic potential of a number of allelochemicals, mainly phenolics, released by several *Artemisia* spp. The phytotoxicity of extracts of *Artemisia annua* on seed germination and seedlings of Isabgol (*Plantago ovate*) have also been reported (Moussavi-Nik et al. 2011). The volatiles released by *Artemisia ordosica* inhibited both growth and photosynthesis of *Palmellococcus miniatus* (Yang et al. 2012). The aqueous extracts of *Artemisia monosperma* aerial parts pointed out a strong inhibitory effect on seeds germination and seedlings growth of *Phaseolus vulgaris* L. (Al-Watban and Salama 2012). Recently, a potential phytotoxic activity of plant extracts from *A. arborescens* has been reported, together with the identification of major compounds occurring in plant tissues (Araniti et al. 2013). However, despite a lot of research on allelochemical activity or phytotoxic potential of aqueous extracts from *Artemisia* species, there are still few studies on the phytotoxic potential of plant litter produced by *A. arborescens*.

The plant litter accumulation may cause indirect chemical effects mediated by the release of allelochemicals into the environment after its decomposition (Xiong and Nilsson 1997; Bonanomi et al. 2006). This process could be considered an allelopathic strategy for pioneer species in controlling intra- and interspecific competition and structuring plant communities (Evans and Young 1970; Facelli and Picket 1991). Litter of *A. tridentate* retarded germination and growth of several perennial grasses (Schlatterer and Tisdale, 1969). Yun and Kil (1992) described the differential phytotoxic effects of *Artemisia princeps* var. *orientalis* (wormwood) residues on seedling growth and dry weight of various plant species in both field and laboratory experiments, suggesting that the decaying wormwood litter releases an amount of growth inhibitors into the soil. In the present study the phytotoxic potential of *A. arborescens* leaf litter was assessed for the first time by bio-guided fractionation on germination and root growth of two sensitive species (*Raphanus sativus* L. and *Lactuca sativa* L.) and two wild species (*Amaranthus retroflexus* L. and *Cynodon dactylon* (L) Pers.) sharing the same habitat with *A. arborescens.* The total phenolics and flavonoids content was also evaluated. The bioactive molecules in the solvent fractions from plant litter were chemically characterized by gas chromatography coupled to mass spectrometry (GC–MS) and through high-performance thin layer chromatography (HPTLC).

Materials and methods

Collection of leaf litter

A. arborescens leaf litter was collected in Southern Italy (Calabria) in September 2013. The amount of plant litter was ~ 47 g per square meter of soil/floor mass. After collection, the plant material was immediately chopped and air dried and powdered using a Wiley Mill (0.2 mm mesh). Plant powders were stored at room temperature in the dark until use. Main chemical properties of the leaf litter are: pH_{H2O} 5.6, $EC_{1:10}$ 3.47 dS m^{-1}, C 35.20 $\pm$ 0.08 %, N 0.90 $\pm$ 0.07 %, C to N ratio 39.1.

Extraction and fractionation of leaf litter *A. arborescens*

Leaf litter (500 g) was macerated in a methanol:water (9:1, by vol) solution (48 h x 3 times) at room temperature in dark condition. The combined crude extract was then solubilized in methanol:water (9:1) and then sequentially fractionated with hexane, chloroform and ethyl acetate as described by Araniti et al. (2013). The solvent fractions were then dried through rotary evaporator and the extraction yields were measured. Yields of methanolic extract and its solvent fractions were the following: 22.3% methanolic extract, 3.02% hexane, 6.82% chloroform and 1.64% ethyl acetate fractions.

Total phenolic content

Total phenolic content was evaluated in the methanolic extract and chloroform and ethyl acetate fractions, using the Folin-Ciocalteu method as reported by Araniti et al. (2014b). The absorption was measured at 726 nm wavelength (Perkin-Elmer Lambda 35 UV/VIS spectrophotometer) and the content in phenolics was expressed as chlorogenic acid in mg g^{-1} of dry weight extract.

Total flavonoid content

Total flavonoid content in the methanolic extract and chloroform and ethyl acetate fractions was determined as reported by Araniti et al. (2014a). The absorption was measured at 430 nm wavelength (Perkin-Elmer Lambda 35 UV/VIS spectrophotometer) and the content in flavonoids was expressed as quercetin in mg g^{-1} of dry weight extract.

GC-MS analysis

The hexane and chloroformic fractions were analyzed through GC-MS using a Hewlett-Packard mod. 6890 gas chromatography equipped with a SE-30 capillary column (30 m length x 0.25 mm diameter x 0.25 μm film thickness) and coupled to a selective Hewlett-Packard mod. 5973 mass detector EI (Electron Impact mode, 70 eV). The GC operating conditions were the following: 3 min at 60°C, from 60 to 280°C at 16°C min^{-1} rate, 30 min at 280°C. Temperature settings of injector and

detector were 250 and 280°C, respectively. Elium was used as carrier gas (1 mL min^{-1} flow rate). Spectra analysis was conducted using the library "Wiley 138"(Hewlett-Packard Co.).

HPTLC analysis

Phenolic compounds were identified and quantified through High-Performance Thin-Layer Chromatography (HPTLC). This technique has widely been used for medicinal drugs analyses allowing an excellent separation of complex mixtures of molecules and their qualitative and quantitative characterization (Nicoletti, 2011; Rashmin et al., 2011).

For the experiments a Linomat 5 sample applicator and a TLC Visualizer (CAMAG, Muttenz, Switzerland) linked to winCATS software were used. Normal phase HPTLC plates 20 x 10 cm (VWR International s.r.l., Milano, Italy) with glass-backed layer silica gel 60 (2 µm thickness) were pre-washed with methanol, carefully dried for 3 min at 100°C and used for the analysis. The operating conditions were previously described by Araniti et al. (2014a). The mobile phase used for the identification of rutin, quercitrin, chlorogenic acid, caffeic acid, catechin and luteolin was: ethyl acetate:dichloromethane:acetic acid:formic acid:water (100:25:10:10:11, by vol). For the identification of quercetin, kaempferol, cinnamic acid, ferulic acid, sinapic acid, *p*-coumaric acid and gallic acid was used instead a mobile phase composed by ethyl acetate:dichloromethane:acetic acid:formic acid:water (80:25:1:1:1, by vol). The derivatization of the developed layers was carried out using the Natural Product Reagent (NPR) (1 g diphenylborinic acid aminoethylester in 200 mL of ethyl acetate) and anhysaldehyde (1.5 mL *p*-anisaldehyde, 2.5 mL sulphoric acid, 1 mL acetic acid in 37 mL ethanol). Treated plates were overseen under an ultraviolet light (254 and/or 366 nm) or white visible light upper and lower (WRT).

For the chemical identification, standards were prepared at 3 mg mL^{-1} in methanolic or ethanolic solution, whereas the ethyl acetate fraction was solubilized in methanol at the concentration of 50 mg mL^{-1} and applied in triplicate on the TLC plates.

The quantification of the identified compounds was carried out using standard solutions at known concentrations (0.5, 1, 2, 3, 4, 6, 8, 10 mg mL^{-1}). Standard compounds were diluted in methanol (rutin, chlorogenic acid, quercitrin and catechin) or ethanol (caffeic acid, luteolin and *p*-coumaric acid) and distributed on HPTLC plates in order to have an absolute amount, for each standard, of 0.5, 1, 2, 3, 4, 6, 8, 10 µg band^{-1}. In the calibration curve, the linear relationship between the standard concentration and the peak areas was confirmed using the absolute amount (µg band^{-1}) as independent variable (X axis) and the peak area of standards as dependent variable (Y axis). Quantification of molecules was performed using linear regression equations (correlation coefficient $R^2 > 0.98$). Data were analysed using the software GraphPad Prism (San Diego, CA, USA).

Germination and root growth bioassays

Germination and root growth were assayed as reported by Araniti et al. (2014a). For the bioassays, the methanolic extract and its solvent fractions were diluted appropriately with methanol (MeOH) to give the following final concentrations: 0, 0.625, 1.25, 2.5, 5, 7.5, 10 mg mL^{-1}. Two mL of each test sample were poured on a double layer of filter paper in Petri dish (6 cm diameter) and allowed to dry at room temperature until complete solvent evaporation was reached. Radish (*Raphanus sativus* L.) and lettuce (*Lactuca sativa* L.) were selected as test crops for the *in vitro* phytotoxic assays because their inherent sensitivity to allelochemicals (Belz and Hurle 2005). Bermuda grass (*Cynodon dactylon* (L.) Pers.) and redroot amaranth (*Amaranthus retroflexus* L.) were chosen as reference of weeds because they colonize the same growing area of *Artemisia* spp. (Pignatti 1982). Ten sterilized seeds for each species and for each concentration were then uniformly distributed into Petri dishes (6 cm diameter) between a double layer of filter paper, which were moistened with 2 mL of sterile deionized water (0 mg mL^{-1}) and 2 mL of different concentrations of methanolic extract or its solvent fractions. Successively, Petri dishes were sealed with parafilm and transferred in a growth chamber settled with the following conditions: temperature 25 ± 1 °C, relative

humidity 70%, photoperiod 16:8 hours light:dark. The germinated seeds of *L. sativa*, *R. sativus* and *A. retroflexus* were counted after 48 hours, whereas those of *C. dactylon* after 96 hours. Total Germination Index [G_T (%)] was evaluated using the equation reported by Chiapusio et al. (1997). For root growth bioassay, five pre-germinated seeds of *L. sativa*, *R. sativus, A. retroflexus* and *C. dactylon* were distributed into Petri dishes and treated as previously reported for the germination bioassay. After 48 h of exposure to methanolic extract or its fractions, an image of root of each species was digitally captured by a scanner and Total Root Length (TRL, %) measured using the software WinRhizo Pro System v. 2002a (both scanner and software were produced by the Instruments Règent Inc., Quebec, Canada).

Experimental design and statistical analysis

The experiments were carried out adopting a completely randomized design with five replications. Both seed germination and root growth responses to increasing doses of methanolic extract and its solvent fractions were described through a nonlinear regression model using a log-logistic function, as previously reported by Araniti et al. (2013), to obtain the parameter ED_{50}, which is defined as the dose required to inhibit by 50% the total response. The ED_{50} data were firstly evaluated for deviation from normality (Kolmogorov-Smirnov test) and homogeneity of within-group variances (Levene's test). After running a one-way ANOVA to check any significant effect of the treatment on the variability of the data (the block effect in the experimental design was found to be not significant at *P* <0.05), multiple pairwise comparison of means was done by Tukey's HSD (Honestly Significant Difference) test at *P* <0.05 level of significance.

Results

Bioassays on seed germination and root growth

Seed germination process of *L. sativa*, *R. sativus*, *A. retroflexus* and *C. dactylon*, was strongly inhibited depending on dose, plant species and solvent fractions (Figure 1). In particular, the

methanolic extract completely inhibited seeds germination in lettuce already at 2.5 mg mL^{-1} and similar results were observed on radish seeds. Conversely, values around 5 mg mL^{-1} of the methanolic extract were necessary to cause similar inhibitory effect on *A. retroflexus* and *C. dactylon* seeds germination (Figure 1 A).

The hexane fraction did not affect *C. dactylon* (L.) seed germination whereas it was able to completely inhibit lettuce, radish and *A. retroflexus* L. seed germination at concentration close to 7.5 mg mL^{-1} (Figure 1 B). A sharp drop on germination process at values higher than 2.5 mg mL^{-1} concentration was observed in response to the chloroform fraction in all species (Figure 1 C) except for *A. retroflexus*, which showed a constant decreasing trend up to 7.5 mg mL^{-1} where a complete inhibition was reached. Conversely, seeds of all species exposed to ethyl acetate fraction were significantly inhibited already at 1.25 mg mL^{-1} (50% inhibition) before reaching a complete inhibition at 2.5 mg mL^{-1} (Figure 1 D).

The comparison of the ED$_{50}$ values confirmed the high inhibitory activity of *A. arborescens* methanolic extract and its solvent fractions on seed germination. In particular, the ethyl acetate fraction resulted extremely active on all species as suggested by the ED$_{50}$ values ranging from 1.19 to 1.46 mg mL^{-1}. Conversely, the hexane fraction was the least toxic one showing the ED$_{50}$ values between 5.31 and 6.25 mg mL^{-1} (Table 1).

In all species, root growth was strongly inhibited by all the extracts showing a sensitivity even greater than that of the germination process (Figure 2). In lettuce, methanolic extract and both the hexane and chloroform fractions showed a very similar pattern of inhibition, resulting in a root growth reduction by approximately 60% already at 5 mg mL^{-1} concentration (Figure 2 A-C). A complete inhibition was reached at the highest dose (10 mg mL^{-1}) (Figure 2 A-C). Conversely, the ethyl acetate fraction determined a greater inhibitory effect on lettuce, causing 50% of root growth reduction already at lower dose (1.25 mg mL^{-1}) (Figure 2 C). This inhibition was dose-dependent, reaching 85% inhibition at the highest concentration (Figure 2 C). In radish, the methanolic extract and both chloroform and ethyl acetate fractions reduced root growth by 55% already at 1.25 mg

mL^{-1} concentration (Figure 2 A, C, D), reaching approximately 97% of inhibition at the highest treatment (10 mg mL^{-1}) (Figure 2 A, C, D). The hexane fraction inhibition became appreciable at concentrations higher than 2.5 mg mL^{-1} (Figure 2 B).

A. arborescens litter extract and its solvent fractions resulted highly phytotoxic towards *A. retroflexus* and *C. dactylon* weeds reaching 90% inhibition at the highest dose (Figure 2 A-C). The comparison of ED$_{50}$ parameters pointed out that all the species appeared to be more sensitive to the ethyl acetate and chloroform fractions, which evidenced the ED$_{50}$ values lower than 2 mg mL^{-1}, except in lettuce where the chloroform fraction showed ED$_{50}$ values equal to 3.15 mg mL^{-1} (Table 2). In contrast, the hexane fraction appeared to be less phytotoxic than the other ones (Table 2).

GC-MS analysis

In the hexane fraction, six terpenes were identified by GC-MS analysis, and the most abundant ones were the monoterpene α-thujone (4.02%) and the sesquiterpene alcohol β-eudesmol (2.74 %). The endo-borneol and L-4-terpineol monoterpenene alcohols and the sesquiterpenes *trans*-caryophyllene and β-selinene were found only in traces (Table 3).

On the other hand, octadecane was the most representative of alkanes (13.49%). Among other constituents, the ketone camphor, a product of borneol oxidation, was the major compound (26.85%). Two lignans, sesamin and fargesine, were also identified (7.88% and 1.93%, respectively). Minor components such as the fatty acid myristic acid, the phytosterol β-sitosterol and the two degradation products 3,4-dimethoxytoluene and 1,2-dimethoxy-4-(2-propenyl)-benzene were identified (Table 3).

In the chloroform fraction, three aromatic hydrocarbons were identified: 1,4,6-trimethylnaphthalene, 3-(1,1-dimethylethyl)-1,2-dihydro-naphthalene, 3,3'-dimethylbiphenyl (Table 4). Among other constituents, the flavonoid artemetin and the terpenoid chamazulene were the most abundant compounds (10.71% and 9.56%, respectively). Minor components, dimethyl 2,3-bis(1,3-dimethylindol-2-yl)fumarate, palmitic acid, 8-methoxy-5-(hydroxymethyl)chrysene, yangambin (a

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furofuran lignin), santoflavone (a flavonoid) and the sesquiterpene cadalene ranged from 3.52 to 0.14%. Linalool, a monoterpene alcohol, was found only in traces (Table 4).

Total phenolic content

Total phenolic content in the methanolic extract was 110.67 mg g^{-1} (equivalent to ca. 25 mg g^{-1} DW) (Table 5) whose flavonoids content represented approximately 40% (equivalent to ca. 10 mg g^{-1} DW) (Table 5). During the bio-guided fractionation, phenolics were preferentially partitioned into the chloroform and the ethyl acetate fractions (Table 5). In particular, in the chloroformic fraction, phenolic compounds and flavonoids content was approximately 71 (4.8 mgg^{-1} DW) and 58 (3.9 mg g^{-1} DW) mg g^{-1} of extract, respectively. On the other hand, in the ethyl acetate fraction both phenolics and flavonoids showed values of 475 and 180 mg g^{-1}respectively (equivalent to ca. 8 and 3 mg g^{-1} DW) (Table 5).

Chemical characterization and quantification of phenolic compounds by HPTLC

In the ethyl acetate fraction, seven out of thirteen phenolic compounds by HPTLC were identified: two flavonoid glycosides rutin and quercitrin, the phenolic acid chlorogenic acid, the flavonoids catechin (a flavanol) and luteolin (a flavone), caffeic and *p*-coumaric acids (cinnamic acids) (Table 6). Chromatographic profiles of the fraction and of the first six components rutin, quercitrin, chlorogenic acid, caffeic acid, catechin and luteolin, identified by comparison with corresponding standards, are reported (Figure 3). Further analysis allowed us also to identify *p*-coumaric acid, whose chromatographic profile is reported in Figure 4 (R_f = 0.91). Quantitative analyses using the regression equations were performed. Among molecules, chlorogenic acid, quercitrin and catechin (amount of 55.09 ± 1.45, 49.65 ± 1.70 and 41.76 ± 0.48 mg g^{-1} of extract, respectively) occurred in largest amount together with rutin (36.84 ± 3.77 mg g^{-1} of extract) (Table 6). Low concentrations of caffeic (28.83 ± 0.46 mg g^{-1}), *p*-coumaric (27.76 ± 0.77 mg g^{-1}) acids and luteolin (20.89 ± 0.44 mg g^{-1} of extract) were detected.

Discussion

Litter released upon soil surface may directly or indirectly affect plant community structure and their dynamics through light interception and the consequent shading of seeds and seedlings, decreasing water availability and/or releasing chemicals in the soil through leaching and degradation (Kimura et al., 2015). However, a significant impact on the environment could be achieved only when litter is being produced abundantly and constantly, as in the case of *Artemisia arborescens*. Leaves of this species are characterized by a short half-life and consequently plant litter deposition on soil surface is always renewed along time. In comparison with litter production values reported by Vogt et al. (1986), litter released by *Artemisia* was about 50 g per square meter/floor mass, representing a considerable amount for a shrubby species.

The *in vitro* bioassay of extracts from *A. arborescens* leaf litter evidenced a strong inhibitory action on either germination or root growth processes of both tested crops and weeds. These findings confirmed the high phytotoxicity of *A. arborescens* plant tissues reported by Araniti et al. (2013). Moreover, it was also observed that the inhibition caused by the extract was dose-dependent, and each plant species showed a different level of sensitivity. As observed by other authors (Chung and Miller 1995; Valera-Burgos et al. 2012), response to the methanolic extract and its solvent fractions was species-specific and dose-dependent for both physiological processes. Although with a slight variability, seed germination of all tested species was found to be highly sensitive to the differing extracts, except for *C. dactylon*, which appeared to be insensitive to the hexane fraction. These data were in agreement with Bell et al. (2009) who found a high resistance of this pest species towards the allelochemicals. The hexane fraction did not cause any effect on germination process of *Echinochloa crus-galli*, a weed considered highly resistant to allelochemicals (Araniti et al. 2013). The results also confirmed that root elongation was more sensitive to allelochemicals than seed germination process in all species as previously observed (Araniti et al. 2014a; Chung and Miller 1995; Turk and Tawaha 2003). The higher sensitivity of crops than weeds to methanolic extract and all its fractions was also established.

The comparative analysis of the ED_{50} values of any fraction, a specific index measuring the phytotoxic potential of a compound, clearly evidenced that phytochemicals with strongest phytotoxic activity were preferentially recovered in the ethyl acetate and chloroform fractions. Conversely, the hexane fraction yielded the lowest phytotoxic compounds. This latter result appeared to be rather contrasting with what previously observed by Araniti et al. (2013) in *A. arborescens* above-ground extract, sampled at full vegetative stage. The reduction of the potential phytotoxicity of the hexane fraction might be due to the loss of volatile compounds (terpenes, fatty acids, etc.) caused by: i) nature and amount of secondary metabolites occurring in plant tissues change during the growing stages as suggested by Barney et al. (2005); ii) volatilization and/or degradation of highly volatile compounds (i.e. terpenes, fatty acids, etc.) occurring during the decaying phase of litterfall or the extraction procedure.

Both the ethyl acetate and chloroform fractions recovered a large amount of phenolic and flavonoid compounds such as chlorogenic, *p*-coumaric and caffeic acids, well known for their phytotoxic and allelopathic activities (Reigosa et al. 1999; An et al. 2000; Huang et al. 2000; Araniti et al. 2014). These phenolic compounds have been chosen for the chemical characterization because they were generally found in the Asteraceae family, especially in the *Artemisia* genus (Xiao and Gang; 2007; Mohamed et al., 2010; Lee et al., 2013). Phenols can interfere with a number of biochemical and physiological processes thus causing inhibition of plant growth (Weir et al. 2004). In forest ecosystems, for instance, they are considered as primary factors of allelopathy as they are able to influence plant succession and reforestation (Singh et al. 1999). Moreover, they are responsible for autotoxicity responses in many crop species such as asparagus (Miller et al. 1991) or coffee (Chou and Waller, 1980) due to caffeine molecules released during the litter decay process (Anaya et al. 2002). In addition, *trans*-cinnamic acid and some of its derivatives are capable of inhibiting root growth of several plant species (Abenavoli et al. 2004; Fujita and Kubo 2003) and interfering with water and nutrients uptake (Abenavoli et al. 2010; Booker et al. 1992; Yu and Matsui 1997; Lupini et al., 2015). In particular, potassium uptake was inhibited by ferulic acid in roots of *Avena sativa*

and *Lactuca sativa* (Jensén et al. 1993), while the *trans*-cinnamic acid, one of the most representative compound isolated from root exudates of *Cucumis sativus*, affects the sulfate and magnesium absorption (Yu and Matsui 1997). Besides physiological responses, phenols can also bring about root morphological alterations as observed in the root ultrastructure of *Sinapis alba* seedlings exposed to phenolic compounds (Kaur et al. 2010); or in *Rumex dentatus* and *Chenopodium album* seedlings treated with sunflower's extracts (Anjum and Bajwa 2010). Although flavonoids are not usually considered as allelopathic compounds exerting other roles in plants, some of them are largely known for their allelopathic potential. In particular, quercitrin (Inderjit and Dakshini 1995), catechin (Bais and Kaushik 2003; Chobot et al. 2009), luteolin (Beninger and Hall 2005) and rutin (Basile et al. 2000; Karabegovic et al., 2011), are able to interact with plant and bacterial soil communities, and they have been found in a considerable amount in *A. arborescens* leaf litter.

The inhibitory activity observed in seedlings treated with the hexane and chloroform fractions could be mainly due to non-polar and relatively polar compounds highly present and abundant. Several authors demonstrated that majority of terpenoids, fatty acids, phytosterols, lignans and flavonoids were dissolved in these fractions (Conforti et al. 2011; Marrelli et al. 2012). Furthermore, there are also many evidences regarding their biological activity and involvement on allelopathic phenomenon (Elakovich and Stevens 1985; Suzuki et al. 1996 ; Kpoviessi et al. 2006; Macías et al. 2007; Nakai et al. 2005). Others compounds such as chamazulene (chloroform fraction), camphor and thujone (hexane fraction) were also identified in leaf litter. Typically detected in essential oil of *A. arborescens* (Lai et al., 2007; Ornano et al., 2013), these compounds are the main responsible for the importance of this species in medicine and pharmacy.

Conclusions

A. arborescens leaf litter showed considerable phytotoxic activity due to a wide range of biologically active phytochemicals such as phenolic acids, flavonoids, terpenoids, fatty acids,

phytosterols, lignans, known for their phytotoxic and allelochemical activities. These compounds released during leaf litter decomposition at the soil surface may alter both physical and chemical properties thus affecting the dynamics and the organization of plant communities. It is also true that the biologically-active compounds entering the soil from decaying leaf litter might not be directly involved in allelopathic interactions as they can undergo further microbial transformation at the soil surface and possibly originate derivatives having an even stronger allelochemical potential. Release and characterization of potentially phytotoxic compounds from decaying *A. arborescens* leaf litter in soil environment deserve further investigation and constitutes the main aim of ongoing research. Finally, this study has confirmed the phytotoxic potential of this species and the importance of the bio-guided fractionation as a method to identify in a plant extract main classes of phytochemicals characterized by high biological activity.

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Table 1

	ED$_{50}$ (mg mL^{-1})			
	L. sativa	*R. sativus*	*A. retroflexus*	*C. dactylon*
MeOH	1.77 (0.30)[b]	1.61 (0.40)[c]	2.97 (0.28)[b]	3.05 (0.20)[a]
***n*-Hexane**	5.31 (0.12)[a]	5.35 (0.52)[a]	6.25 (0.64)[a]	ND
Chloroform	1.68 (0.18)[b]	2.80 (0.75)[b]	2.86 (0.99)[b]	1.38 (0.26)[b]
Ethyl acetate	1.29 (0.03)[c]	1.39 (0.71)[c]	1.46 (0.15)[c]	1.19 (0.02)[b]

Different letters along the column indicate statistical significative differences at $p<0.05$ (Tukey test). No Detectable (N.D.). Values within the brackets indicate Standard Deviation (SD); (N=5).

Table 1 Estimates of ED$_{50}$ (mean with standard deviation in brackets, *n=5*) relative to seeds germination process of *R. sativus, L. sativa, A. retroflexus* and *C. dactylon* exposed to increasing concentrations of the methanolic extract of *A. arborescens* and its solvent fractions. Values were estimated according to the non-linear log-logistic equation model (Araniti et al. 2013).

Table 2

	ED$_{50}$ (mg mL^{-1})			
	L. sativa	*R. sativus*	*A. retroflexus*	*C. dactylon*
MeOH	3.14 (0.29)[b]	1.22 (0.27)[b]	1.38 (0.17)[b]	2.15 (0.18)[b]
***n*-Hexane**	3.96 (0.25)[a]	2.06 (0.10)[a]	2.31 (0.21)[a]	3.98 (0.27)[a]
Chloroform	3.15 (0.32)[b]	1.04 (0.11)[b]	2.04 (0.24)[a]	1.63 (0.11)[c]
Ethyl acetate	1.68 (0.22)[c]	0.98 (0.12)[c]	0.92 (0.11)[c]	1.37 (0.05)[d]

Different letters along the column indicate statistical significative differences at p<0.05 (Tukey test). No Detectable (N.D.). Values within the brackets indicate Standard Deviation (SD); (N=5).

Table 2 Estimates of ED$_{50}$ (mean with standard deviation in brackets, *n=5*) relative to root growth of *R. sativus, L. sativa, A. retroflexus and C. dactylon* exposed to increasing concentrations of the methanolic extract of *A. arborescens* and its solvent fractions. Values were estimated according to the non-linear log-logistic equation model (Araniti et al. 2013).

Table 3

Terpenes[a]	RT[b]	RAP[c]
α-Thujone	10.140	4.02
Endo-Borneol	10.866	tr[d]
L-4-terpineol	11.003	tr
trans-Caryophyllene	13.918	tr
β-Selinene	14.598	tr
β-Eudesmol	16.147	2.74
Alkanes[a]	**RT**	**RAP**
Eicosane	22.131	tr
Docosane	23.571	7.91
Octadecane	25.538	13.49
Other constituents[a]	**RT**	**RAP**
Camphor	10.563	26.85
3,4-Dimethoxytoluene	11.741	tr
Benzene, 1,2-dimethoxy-4-(2-propenyl)	13.610	tr
Myristic acid	18.445	tr
Sesamin	29.784	7.88
Fargesin	31.299	1.93
β-sitosterol	33.528	tr

[a] Compounds listed in order of elution from SE30 MS column. [b] Retention time (as minutes). [c] Relative area percentage (peak area relative to total peak area %). [d] Compositional values less than 0.1% are denoted as traces.

Table 3 Major compounds occurring in the hexane fraction of *A. arborescens* leaf litter extract.

Table 4

Aromatic hydrocarbons [a]	RT[b]	RAP[c]
1,4,6-Trimethylnaphthalene	16.210	0.46
Naphthalene, 3-(1,1-dimethylethyl)-1,2-dihydro-	16.353	0.21
3,3'-Dimethylbiphenyl	17.445	1.03
Other constituents[a]	**RT**	**RAP**
Linalool	10.078	tr[d]
Chamazulene	17.050	9.56
Cadalene	18.210	0.14
Palmitic acid	18.622	1.20
8-methoxy-5-(hydroxymethyl)chrysene	20.559	0.71
Santoflavone	33.836	0.37
Artemetin	34.865	10.71
Yangambin	40.637	0.53
dimethyl 2,3-bis(1,3-dimethylindol-2-yl)fumarate	43.369	3.52

[a] Compounds listed in order of elution from SE30 MS column. [b] Retention time (as minutes). [c] Relative area percentage (peak area relative to total peak area %). [d] Compositional values less than 0.1% are denoted as traces.

Table 4 Major compounds occurring in the chloroform fraction of *A. arborescens* leaf litter extract.

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Table 5

	Phenolic compounds (mg g^{-1} extract)	Flavonoids (mg g^{-1} extract)
MeOH	110.67 (0.93)[b]	44.32 (0.68)[c]
Chloroform	70.50 (0.29)[c]	58.12 (0.16)[b]
Ethyl acetate	475.00 (0.58)[a]	180.40 (0.60)[a]

Different letters along the column indicate statistical significative differences at $p < 0.05$ (Tukey test). No Detectable (N.D.). Values within the brackets indicate Standard Deviation (SD); (N=4).

Table 5 Content of total phenolics and total flavonoids (mean with standard deviation in brackets, *n=4*) found in the methanolic extract, and in the chloroform and ethyl acetate fractions.

Table 6

Compound	Amount (mg g^{-1} of extract)
Rutin	36.84 (3.77)
Chlorogenic acid	55.09 (1.45)
Quercitrin	49.65 (1.70)
Catechin	41.76 (0.48)
Caffeic acid	28.83 (0.46)
Luteolin	20.89 (0.44)
p-Coumaric acid	27.76 (0.77)

Data are expressed as mean ± SD (N= 3).

Table 6 HPTLC quantification of phenolic compounds (mean with standard deviation in brackets, *n=3*) found in the ethyl acetate fraction.
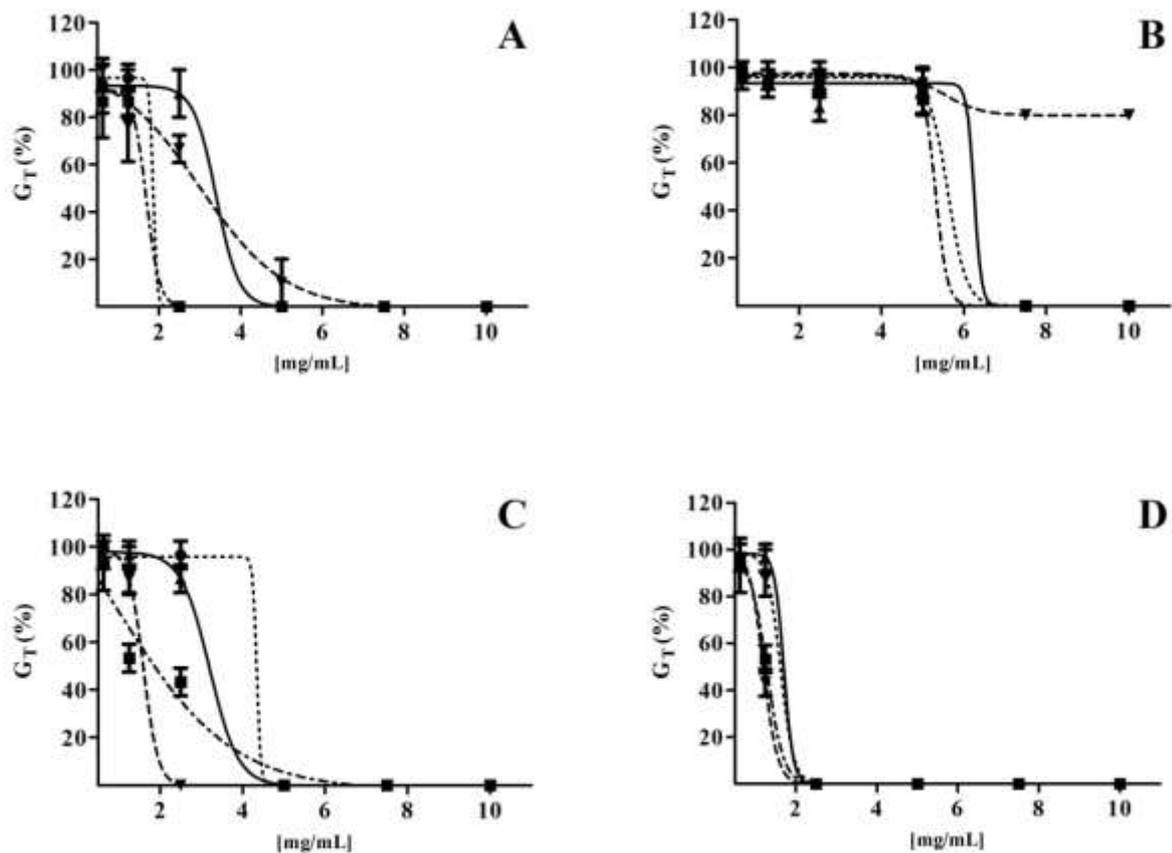
Fig. 1

Fig. 1 Dose response curves of Total Germination Index [G_T (%)] of *R. sativus* (●; •••), *L. sativa* (■; − • −), *A. retroflexus* (▲; ——) *and C. dactylon* (▼; − − −) exposed to the methanolic extract of *A. arborescens* (A) and its solvent fractions: hexane (B), chloroform (C), ethyl acetate (D). Fitting of the no-linear regression model to estimate the ED_{50} values reported in Table 2 was at a significance level of $P < 0.001$.
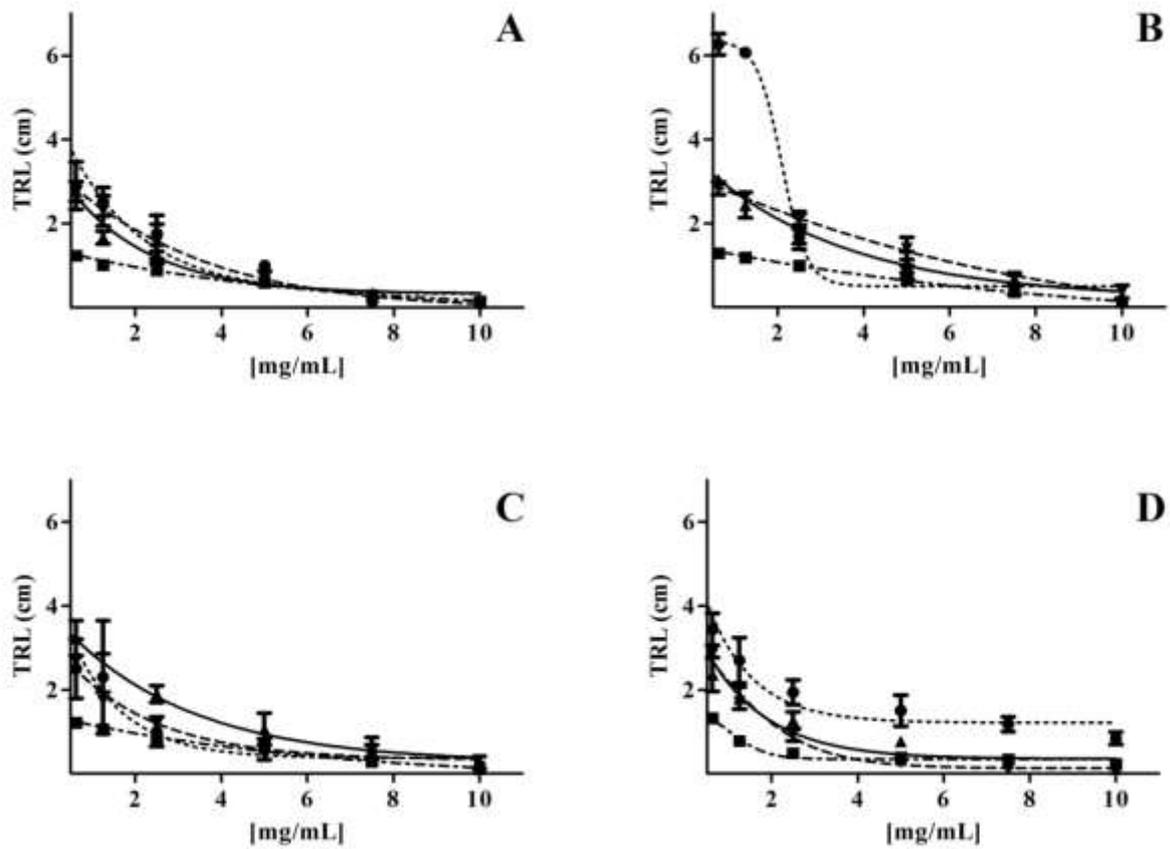
Fig. 2

Fig. 2 Dose response curves of Total Root Lenght [TRL (cm)] of *R. sativus* (●; •••)*, L. sativa* (■; − • −)*, A. retroflexus* (▲; −−−) *and C. dactylon* (▼; − − −) exposed to the methanolic extract of *A. arborescens* (A) and its solvent fractions: hexane (B), chloroform (C), ethyl acetate (D). Fitting of the no-linear regression model to estimate the ED$_{50}$ values reported in Table 2 was at a significance level of $P < 0.001$.
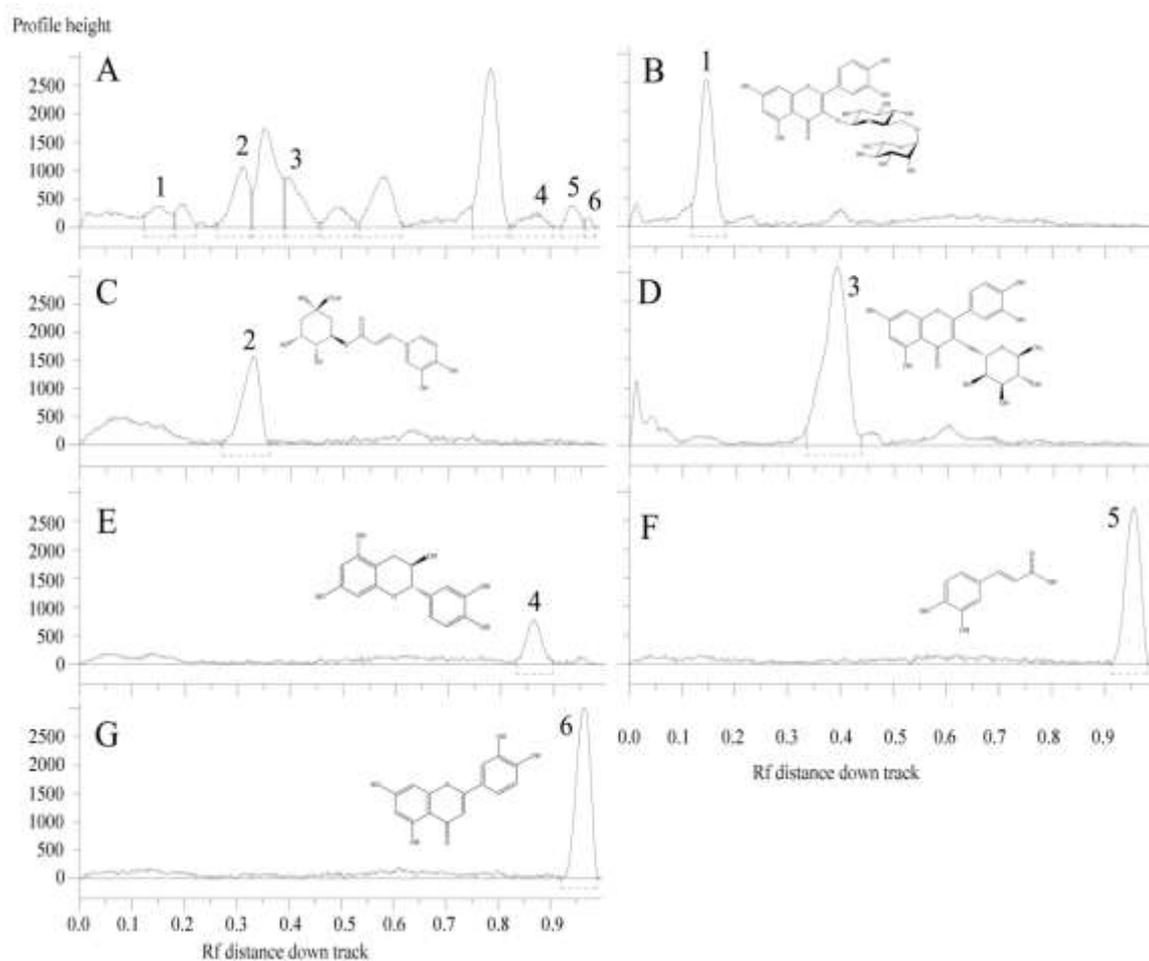
Fig. 3

Fig. 3 HPTLC chromatograms of the ethyl acetate fraction from *A. arborescens* leaf litter (A) and reference standards (B: rutin, $R_f=0.15$; C: chlorogenic acid, $R_f=0.32$; D: quercitrin, $R_f=0.40$; E: catechin, $R_f=0.87$; F: caffeic acid, $R_f=0.94$; G: luteolin, $R_f=0.97$). Chromatographic conditions are described in 2.6.
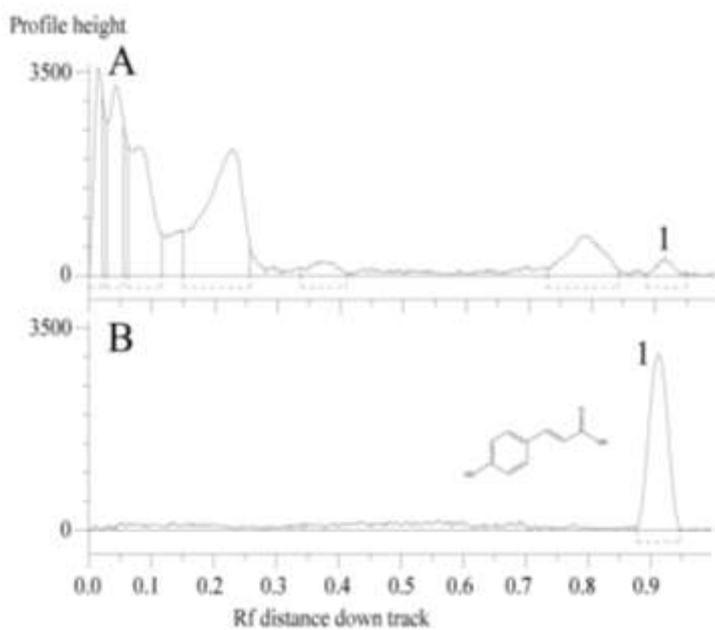
Fig. 4

Fig. 4 HPTLC chromatograms of the ethyl acetate fraction from *A. arborescens* leaf litter (A) and *p*-coumaric acid reference standard (B: *p*-coumaric acid, R_f=0.91). Chromatographic conditions are described in 2.6.