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Antioxydant Capacity And Allelopathic Potential Of Roots Extracts Of *Pulicariaodoral*. (Compositae).

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ABSTRACT

The organic and aqueous roots extracts of Pulicariaodora L. a Moroccan medicinal plant, were examined for their antioxidant and allelopathic activities. Phytochemical screening of plant extracts revealed the presence of alkaloids, steroids, terpenoids and glycosides. Total phenols, flavonoids and tannins contents were also investigated. The highest flavonoid and phenol contents were found in the methanol, ethyl acetate, butanol and aqueous extracts of the plant. The highest concentration of tannins was found in the aqueous extract of P. odora.The antioxidant activity was determined by the DPPH assay. Results showed that the methanol, ethyl acetate and butanol extracts have a higher antiradicalaire capacity with IC_{50} values of 4, 5 and 4 µg/mL respectively, better than BHT and quercetin (IC_{50} values of 40 and 32.2µg/mL respectively). These same extracts showed a strong allelopathic effect on the germination of seeds and the growth of roots and hypocotyls of two target plant species, Medicagosativa and Medicagosativasubspfalcata. This is the first report on the antiradical capacity and allelochemicals constituents of P. odora.

Keywords: Pulicariaodora, Polyphenols, flavonoids, antiradical capacity, allelopathic effect.

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INTRODUCTION

The researches on bioactive secondary metabolites from medicinal and aromatic plants became a challenge for the scientific community. Thus, several studies have been carried out in order to find alternatives to the use of chemical products as antimicrobials, antioxydants, biocides or allelochemicals in agriculture, food, health and others industrial fields.

The genus Pulicaria belonging to the tribe Inuleae of the family Asteraceae consists of about 100 species with a distribution from Europe to North Africa and Asia, particularly around the Mediterranean [1].

The previous chemical investigations on Pulicaria species showed the occurrence of secondary metabolites such as triterpenoids and sterols [2], diterpenoids [3], monoterpenoids, sesquiterpenoids, essential oils [4-14], flavonoids and phenolic compounds [15-18].

The Pulicariaspecies are used in traditional systems of medicine as tonic, antispasmodic, antihypoglycemic and as ingredients of perfumes [13, 19,20]. Several studies have been published on the biological activities of some species of Pulicaria, such as antifungal, antibacterial [4, 13], antioxidant [4, 7, 18, 21,22], antihistaminic [23], antispasmodic [24] and anticancer [25] properties.

PulicariaodoraL. (Compositae) is a Moroccan medicinal plant. Its roots were widely used in traditional medicine to treat back-pain, intestinal disorders and menstrual cramps [13]. Relatively few studies have been done on PulicariaodoraL., and the two previous researches on this specie have focused especially on its essential oils and their antimicrobial activity [13, 14]. To the best of our knowledge, no previous phytochemical work has been done on other extracts of this specie. In addition, no information is available on its antioxidant capacity and its role in allelopathy. The aim of the present work is to evaluate the DPPH radical scavenging and the allelopathic effect of Pulicariaodora extracts on Medicago sativa and Medicago sativa subspfalcata seed germination and seedling growth. It is also of interest to find whether there is any correlation between phenolic contents of these plant extracts and the studied activities.

MATERIALS AND METHODS

Plant Material

P. odora was collected in April from Ourika, at 30 km on the east of Marrakech. The plant was identified by Professor M. Fennane from the Scientific Institute (Rabat, Morocco). A voucher specimen was deposited in the national herbarium of the Scientific Institute.

Plant extraction

A fine-powdered, air-dried roots of P. odora (100 g) was extracted under reflux three times with water. On the other hand, 500 g of powdered air-dried roots of P. odorawere extracted by Soxhlet with methanol. The extracts were evaporated under reduced pressure to give water and methanol crude extracts. The methanol crude extract was solubilized in water and extracted successively with equal volumes of four organic solvents of increasing polarity to give four fractions: hexane, dichloromethane, ethyl acetate and butanol. Each fraction was evaporated under vacuum and the final residues were stored at +4°C until tested after determining the weight and the yield.

Phytochemical screening

The fresh water and methanolic crude extracts were qualitatively screened for the following secondary metabolites: flavonoids, coumarines, tannins, alkaloids, terpenes, glycoside and saponins according to Harborne[26]. The qualitative results have been rated from (+) for faint to (+++) for dense turbidity or color.

Total phenolic content

The content of total soluble phenolics in the extracts was determined spectrophotometrically according to the Folin–Ciocalteu method [27]. The reaction mixture was prepared by mixing 0.1 mL of a 1

mg/mL water solution of the extract with 7.9 mL of distilled water, 0.5 mL of Folin–Ciocalteu's reagent and 1.5 mL of 20% sodium carbonate. After 2 h, the absorbance at 760 nm was read against the control that was prepared in a similar way but with distilled water instead of the extract. Standard calibration curve for gallic acid in the range of 0-0.14mg/mL was prepared in the same manner and results were expressed as mg gallic acidequivalents (GAE) per gram of dry extract. Data presented are average of three measurements.

Total flavonoids content

Total flavonoids were determined using the colorimetric method developed by Dewanto et al. [28]. An aliquot of diluted extract or standard solution of quercetin was added to 75 µL of $NaNO_2$ solution (7%), and mixed for 6 min, before adding 0.15 ml $AlCl_3$ (10%). After 5 min, 0.5 mL of 1 M NaOH solution was added. The final volume was adjusted to 2.5 mL. The solution was well mixed and the absorbance was measured at 510 nm. Total flavonoids were expressed as mg quercetin equivalents (QE)/g dry extract, through the calibration curve of quercetin (0–400 µg/mL range). All extracts were analyzed in three replications.

Total tannins content

Condensed tannins were measured using the modified vanillin assay described by Sun et al. [29]. 3 mL of 4% methanol vanillin solution and 1.5 mL of concentrated H_2SO_4 were added to 50 µLof diluted extract. The mixture was allowed to stand for 15 min, and the absorbance was measured at 500 nm against methanol as a blank. A calibration curve of catechine was prepared in the range of 0–0.15 mg/mL and results were expressed as mg catechine equivalents (CAE)/g dry extract. All samples were analyzed in three replications.

DPPH radical assay

The radical-scavenging activity (RSA) of Pulicariaodora extracts was evaluated by 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) method [30]: 2 mL of (40µg/mL) DPPH methanolic solution was mixed with 50µL of diluted extract. The mixture was shaken vigorously and incubated for 30 min in the dark at room temperature. Butylatedhydroxytoluene (BHT) and quercetin were used as positive controls.

The absorbance of the control (Ac) and samples (As) was measured spectrophotometrically at 517 nm, and the RSA of the tested extracts expressed in percentage was calculated as follow:

$$\text{RSA (\%)} = \frac{(\text{Ac} - \text{As}) \times 100}{\text{Ac}}$$

The antiradical capacity was expressed as IC_{50} (the antiradical concentration required to cause 50% of inhibition). A lower value of IC_{50} (µg extract/mL) corresponds to a higher antioxidant capacity of sample [31]. All assays were carried in triplicates and results expressed as means ± standard deviation. Statistical comparisons were done with Student's test. Differences were considered to be highly significant at $P < 0.01$ and significant at $P< 0.05$.

Allelopathic effect

The allelopathic activity of Pulicariaodora extracts was tested on Medicago sativa and Medicagos. falcata seeds. These test plants were selected as test crops because these are important crops worldwide as food/feed and are reliable and sensitive species in bioassays. The M. sativa and M. s.falcata seeds were provided by Vita Maroc, development partner of Moroccan agriculture.

The germination tests were done in Petri dishes (9 cm diameter) in a germination chamber for seven days in dark at 25°C[32]. From each extract of P. odora, five concentrations (62.5, 125, 250 and 500 µg/mL) were prepared in methanol for the organic extracts or distilled water for the aqueous extract. Each Petri dish contained twenty five seeds of each Medicago species placed on two layers of filter paper (Whatman No.1). 4 mL of P. odora extracts were added per Petri-dish. The control dishes were wetted with 4 mL methanol or distilled water. Experiments were repeated in three replicates. Allelopathic behavior was appraised by

counting the number of daily germinated seeds until the control stabilized, reaching the maximum germination. Seeds were considered to be germinated when their emergent radical length was nearly 2 mm [33]. Relative germination was calculated as germination (%) of the treatment compared to controls [34]. The final germination percentage was calculated by the following formula: Germination percentage = Number of germinated seeds/Total number of seeds x 100.

The germination percentage, radical length, and hypocotyl length were recorded for successive seven days. Statistical analysis was carried out by one-way analysis of variance (ANOVA) test using a statistical package program (SPSS version 23.0) and the significance of the difference between means was followed by the Tukey test, using $p < 0.05$ as the level of significance. Data were expressed with mean ± standard error of three parallel measurements.

RESULTS AND DISCUSSIONS

Extraction and solvent fractionation

The yields of the crude aqueous extract, crude methanolic extract of P. odora roots and its respective fractions (n-hexane, dichloromethane, ethyl acetate, n-butanol) are presented in Table 1. The maximum yield was obtained for crude extract of methanol (4.22%) followed by the crude aqueous extract (1.87%). The dichloromethane fraction is negligible.

Table 1: Percentage yields of P. odora roots crude extracts and various fractions.

	Water	Methanol	n-Hexane	Dichloromethane	Ethyl acetate	n-Butanol
P. odora roots	1.87	4.22	0.41	0.12	0.63	0.52

Phytochemical screening

Methanol and water extracts are phytochemically similar except in the presence of flavonoids, anthocyanins-catechols and glycosides (Table 2). The phytochemical constituents of P. odora roots methanol extract proved to be rich in alkaloids, flavonoids, terpenoids, steroids, tannins and anthocyanins-catechols. Aqueous extract was poor in flavonoids, saponins and anthocyanins-catechols while methanolic extract was poor in saponins and glycosides. These results are in agreement with previous studies on Pulicaria genus which report the occurrence of significant secondary metabolites with typical biological activities [4, 13, 18,21,34,].

Table 2: Phytochemical screening of the methanol and the water crude extracts of P. odora roots.

Phytochemicals	Methanol	Water
Alkaloids	**+++**	**+++**
Flavonoids	**+++**	**+**
Terpenoids	**+++**	**+++**
Steroids	**+++**	**+++**
Anthocyanins-catechols	**+++**	**+**
Tannins	**+++**	**+++**
Saponins	**+**	**+**
Glycosides	**+**	**+++**

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Contents of total phenols, flavonoids and tannins

The total phenols, flavonoids and tannins contents in P. odora extracts are given in Table 3. The aqueous extract showed higher tannins content (131.75 mg CAE/g dry extract) than the methanol (79.25 mg CAE/g dry extract) or the other extracts. The highest amount of total phenols and flavonoids contents were found in the methanol, ethyl acetate and butanol extracts. While dichloromethane extract contains relatively a very low quantity of polyphenols (24.28 mg GAE/g dry extract) and flavonoids (2.82 mg QE/ g per dry extract).

Table 3: Total phenols, total flavonoids and total condensed tannins contents of P. odoraroots extracts

Extracts	Phenols content (mg GAE/ g)	Flavonoids (mg QE/g)	Tannins (mg CAE/g)
Water	274.02±0.30	155.62±0.35	131.75±0.02
Methanol	385.09±0.04	173.41±0.25	79.25±0.05
Hexane	nd	nd	nd
Dichloromethane	24.28±0.10	2.82±0.10	9.25±0.02
Ethyl acetate	387.02±0.02	220±0.05	6.43±0.02
Butanol	362.51±0.20	224.28±0.02	nd

mg GAE/g: Milligram-gallic acid equivalents per gram of dry extract
mg QE/g : Milligram-quercetin equivalents per gram of dry extract
mg CAE/g Milligram-catechin equivalents per gram of dry extract
nd: Not determined

The total phenol and flavonoids content of plants belonging to the Pulicaria genus were described by several previous works: ethyl acetate extract of P. mauritanica growing in Morocco contain 72.88 mgGAE/g dry extract of total phenolic and 38.95 mgQE/g dry extract of flavonoids, while methanol extract contain 39.37 mg GAE/g of total polyphenols and 25.79 mg QE/g of flavonoids [21]. Other previous studies report that methanol extract of P.dysentericacontain 374.39 mg GAE / g per dry extract of total phenolic compounds and 52.00 mgQE/ g per dry extract of flavonoids, while water extract of this species contain 114.23 mg GAE/g and 49.14 mg QE/g per dry extract of total phenolic and total flavonoids respectively [36].

According toAlgabr et al. [22], the ethyl acetate extract of P. jaubertii from Yemen had the highest content of polyphenols (322.98 mg GAE/g extract) and flavonoids (159.80 mg QE/g extract) if compared to n-butanol extract of the same species (77.83 GAE/g extract and 19.52 mg QE/g extract respectively).

Our results are also in agreement with several previous phytochemical studies on Pulicaria species, which report the isolation of flavonoids and phenolics from P. arabica [37], P. incisa [38] and P. undulata [18, 39].

Scavenging effect on DPPH radical

The direct antioxidant assay using DPPH revealed the potency of the various extracts from roots of P. odora to scavenge free radicals. The inhibition percentage of DPPH radical for all extracts increases with increasing concentration (Figure 1) and a significant concentration-effect was obtained with all the extracts ($p<0.05$). These data suggested that methanol, ethyl acetate and butanol extracts of P. odora have a similar and remarkable ability to scavenge radicals. Their chemical antiradical activity with IC_{50} values of 4 µg/mL, 5µg/mL and 4 µg/mL(Table 4) respectively, thus is better than that of BHT (40µg/mL) or quercetin (32.2µg/mL).

Table 4: IC_{50}values of DPPH scavenging byP.odora extracts

Extracts/standards	IC_{50} (µg/mL)
Water	22.14±0.05
Methanol	4±0.03
Dichloromethane	36.4±0.02
Ethyl acetate	5±0.05
Butanol	4±0.02

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BHT	40±0.03
Quercetin	32.2±0.03

IC_{50} =50%inhibitory concentration.Results arereported as the means ± SD(n=3), P<0.05.

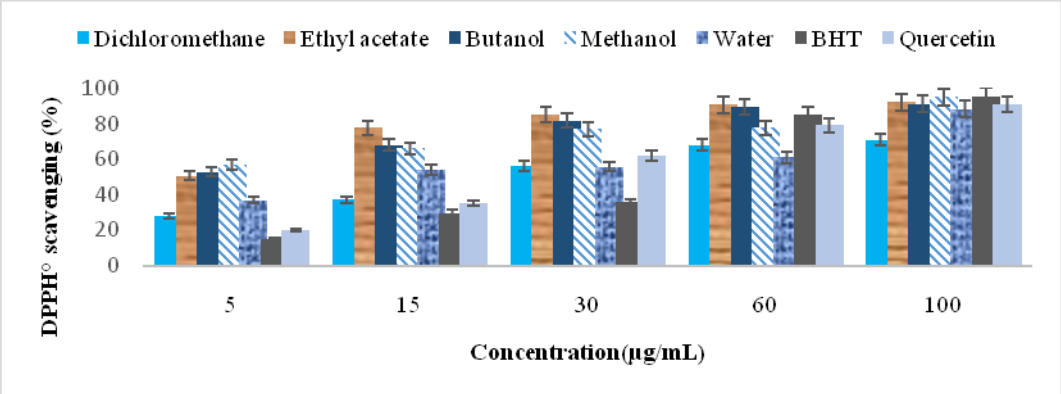

Figure 1: DPPH radical scavenging activities of different extracts of P. odora. Each value represents a mean ± SD (n=3), P<0.05

The IC_{50} of water and dichloromethane extracts were calculated to be 22.1µg/mL and 36.4 µg/mL respectively (Table 4).

Table 5: The inhibitory effects of P. odora extracts on Medicago sativa and MedicagoMedicago sativa Subsp.falcataseeds germination

Extracts	Concentration (µg/mL)	Medicago sativa	Medicago sativa subsp.falcata
Dichloromethane	0	100±0	100±0
	62.5	100±0	100±0
	125	98.2±016	100±0
	250	95.2±0.2	94.1±0.55
	500	90.15±0.27	87±0.2
Ethyl acetate	0	100±0	100±0
	62.5	70.2±0.53	90.2±0.42
	125	65.5±0.32	80.15±0.2
	250	4.25±0.1	60.83±0.78
	500	2.1±0.2	16.3±0.15
Butanol	0	100±0	100±0
	62.5	90.06±0.31	98±0.1
	125	.802±0.38	95.2±0.15
	250	77±0.62	94.15±0.32
	500	67.3±0.52	20.1±0.8
Methanol	0	100±0	100±0
	62.5	90.5±0.23	23.4±0.33
	125	80.25±0.15	23.2±0.2
	250	75.4±0.45	16.75±0.53
	500	4.2±0.2	2.1±0.5

Water	0	100±0	100±0
	62.5	100±0	100±0
	125	90.1±0.25	90.25±0.15
	250	80.5±0.31	90±0.1
	500	70.15±0.65	85.15±0.45

Hamdouch et al. [4] report that methanol extract of P. mauritanica showed a strong scavenging activity of DPPH (IC_{50}=0.027mg/mL), while petroleum ether extract exhibited low scavenging effect (IC_{50}=0.123 mg/mL). According to Senhaji et al.[21], the ethyl acetate extract of P. mauritanica had the highest inhibition percentage of free radical DPPH followed by chloroform extract and the methanol extract. Algabr et al. [22] studied the antiradical capacity of P. jaubertii leaves; they found that ethyl acetate and butanol extracts have a remarkable ability to scavenge radicals with IC_{50}values of 7.17 ± 0.82 µg/mL and 20.06 ± 1.86 µg/mL respectively.

Previous investigations reported that the beneficial effect of plants is mainly related to the antioxidant activity of their phenolic compounds [40]. As reported in Table 3, the methanol, ethyl acetate and butanol extracts of P. odora are markedly rich in polyphenols and flavonoids. Furthermore, the phenolic compounds and more particularly the flavonoids are recognized as substances potent antioxidants with the ability to trap the radical species and reactive forms of oxygen [41]. This supports the idea that the polar compounds (phenolic and flavonoids) [42] present in the methanol, butanol and ethyl acetate extracts of P. odora are mainly responsible for its antioxidant activity.

The coefficient of correlation between phenolic, flavonoids content and DPPH scavenging activity was studied using statistical program (SPSS, version 23).

Result show a high positive correlation coefficient between total phenolic content with flavonoids compounds (r= + 0.99) with ($p<0.01$). In addition, negative correlation appeared between IC_{50} for DPPH scavenging with total phenolic and flavonoid contents (r= - 0.99 and r = -1, respectively) ($p<0.01$). These results indicate that when the polyphenol and flavonoid contents increase the inhibitory concentration of 50% of the free radical DPPH decreases. However, the correlation coefficient between IC_{50} for DPPH scavenging and the condensed tannins contents was found to be very small (r = 0.26) suggesting there was no correlation ($p>0.01$).

Allelopathic potential of P. odora extracts

Allelochemicals are secondary metabolites produced and released by plant in their environment. They play an important role in shaping interactions and communities in agricultural and functional ecology [43, 44].Allelochemicals produced by weeds and invasive plants have detrimental effects on the growth crops. They can inhibit the growth of competing vegetation through direct or indirect means, and subsequently confer a competitive advantage to the invader [43]. In fact, the allelochemicals reduce the cell division and have several phytotoxic effects including the reduction of water use efficiency, inhibition of foliar expansion and root elongation in addition to the reduction of rate of photosynthesis and nutrient uptake [45]. Studding and developing natural products as environmentally friendly herbicides can eliminate damages to human health as well as to ecosystem [44]. In this work, we elucidate for the first time an allelopathic interaction of P. odora.

Seed germination: For finding out the phytotoxicity of the root extracts of P.odora, various concentrations were used to test their allelopathic activity against seed germination of two tested plants (Medicago sativa and Medicago s. falcata). Results showed that the inhibitory effects on seed germination depends on the type of extract, it concentration and the test plants (Table5) ($p<0.05$). In fact, the phytotoxicity response of the various extracts differed markedly among the two studied plants.

At the highest concentration of ethyl acetate, methanol, butanol and water extracts, the inhibition (%) in germination of M. sativa grains was 98, 96, 33 and 30% less than control, respectively. However, the inhibition (%) in germination of M.falcata seeds with these extracts was 84, 98, 80 and 15% less than control, respectively (Table5). Thus, at the highest concentration, the ethyl acetate extract is the most effective for the

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germination inhibition in the case of M. sativa while the methanolic extract is the most active in the case of M.falcata.

Radical and hypocotyl length: The ethyl acetate, butanol and methanol extracts of P. odora significantly inhibited the seedling growth parameters (radical and hypocotyl length) compared to control ($p<0.05$).

The radical length of M.sativa was markedly inhibited under all studied concentrations of P.odora extracts compared to control(Figure 2). The maximum inhibition (%) in M. sativa radical length was at the highest concentration of ethyl acetate extract (99.6%) followed by methanol and butanol extracts (98% and 93.1% over control respectively). Generally, M.falcata radicals were less sensitive to the toxicity of extracts than M. sativa; the inhibition (%) of its radical length was 96.5% over control at the highest concentration of butanol extract, followed by methanol, ethyl acetate and dichloromethane extracts (93.1% over control).

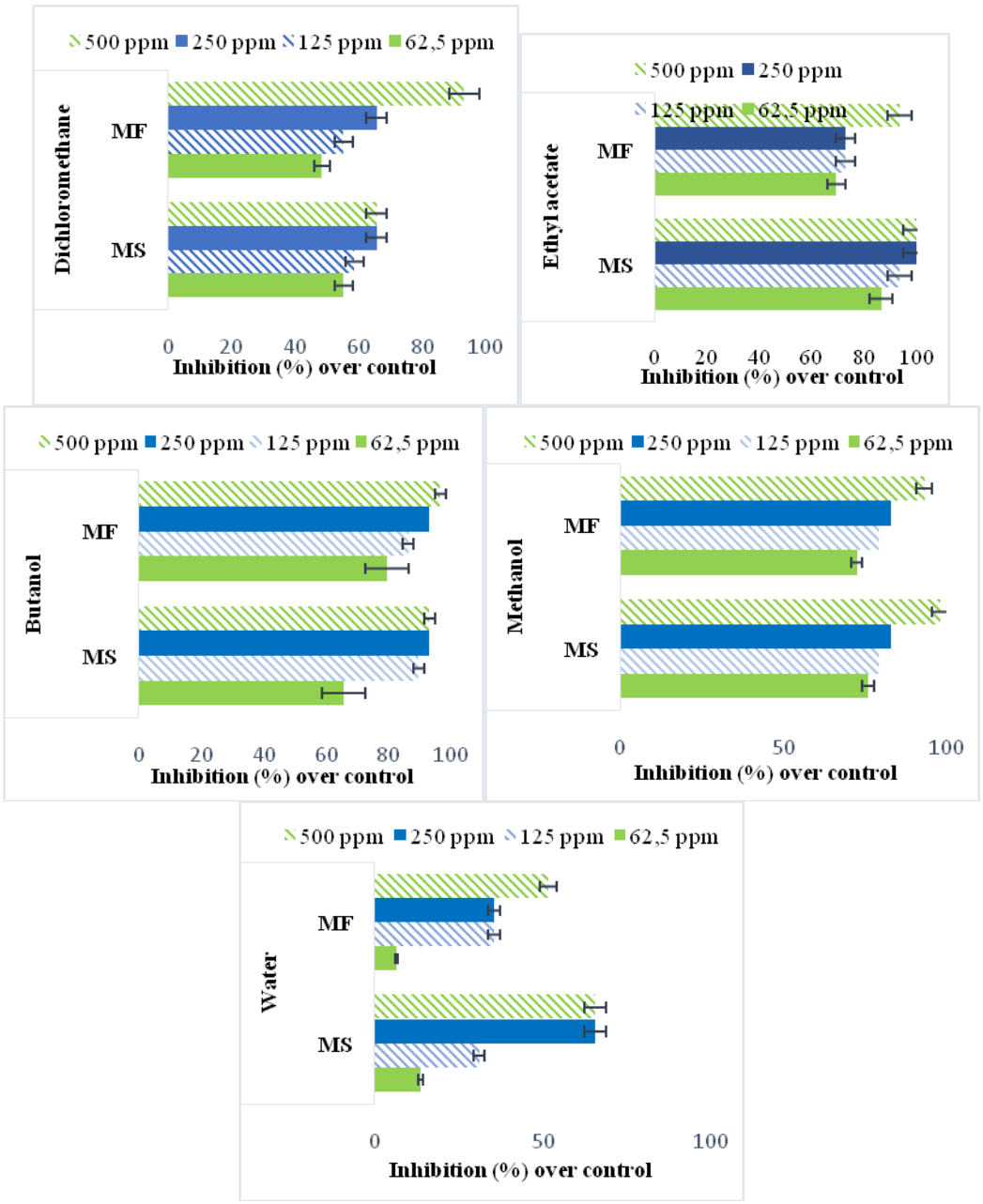

Figure 2: Allelopathic effect of P.odora extracts on roots growth of Medicago sativa (MS) and Medicago sativa subspfalcata (MF) seeds at different concentrations.

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The hypocotyl growth of M. falcata, as well as M. sativa was markedly inhibited under all studied concentrations of P.odora extracts (Figure 3). However, at the highest concentration (500µg/mL), M. sativa hypocotyls were less sensitive to the toxicity of extracts than M.falcata, except for ethyl acetate extract, which was more toxic (99.7%) to hypocotyl length in M. sativa. The maximum inhibition (%) in M. falcata hypocotyl length was 93.7, 78.1 and 75% at the highest concentration of butanol, dichloromethane and methanol extracts respectively. While in M. sativathe maximum inhibition (%) of hypocotyl growth was 99.7, 84.4 and 75.2% over control under the effect of ethyl acetate, butanol and methanol extracts respectively.

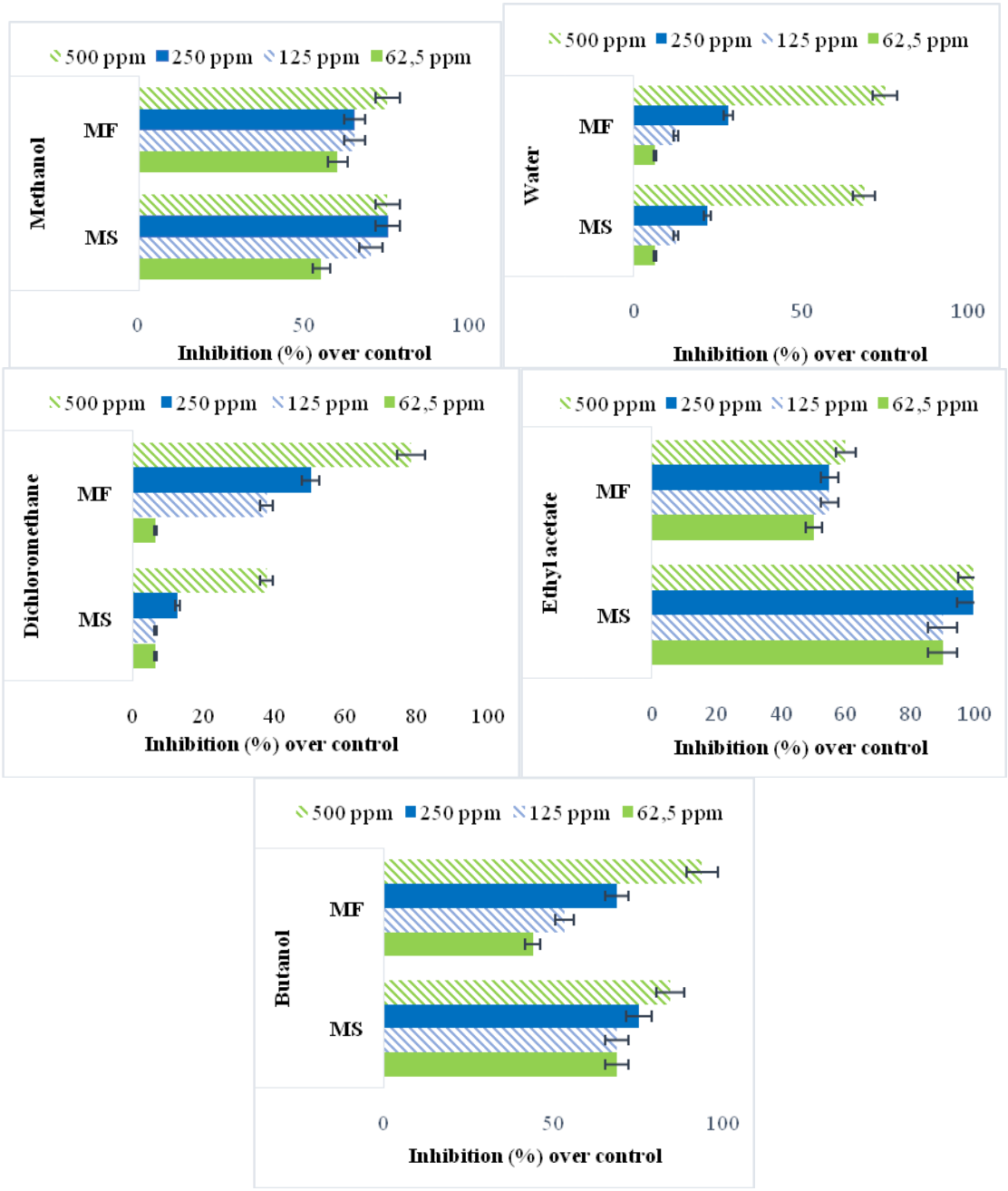

Figure 3: Allelopathic effect of P.odora extracts on hypocotyl growth of Medicago sativa (MS) and Medicago sativa subspfalcata (MF) seeds at different concentrations.

Allelochemicals can be classified based on their diverse chemical structures and properties into several groups: water-soluble organic acids, straight-chain alcohols, aliphatic aldehydes, and ketones; simple unsaturated lactones; long-chain fatty acids and polyacetylenes; simple and complex quinines); phenolics; cinnamic acid and its derivatives; coumarins; flavonoids; tannins; steroids, alkaloids and terpenoids [43, 46, 47]. The phytochemical constituents of P. odora roots extracts proved to be rich in alkaloids, flavonoids,

terpenoids, steroids, tannins and anthocyanins-catechols (Tables 2-3). The highest amount of total phenols and flavonoids contents were found in the methanol, ethyl acetate, butanol and water extracts. While dichloromethane extract contains relatively a very low amount of polyphenols and flavonoids.

Phenolic compounds are a class of the most important and common plant allelochemicals in the ecosystem. It was found that phenolic allelochemicals could inhibit plant root elongation, cell division, change cell ultra-structure, and then interfere with the normal growth and development of the whole plant [48]. The allelochemical potential of some phenolics has been implicated in plant competition, antagonism and species distribution [49]. In this study, phytochemicals screening results and the compositions of allelochemicals extracts are in accordance with seed germination, radical and hypocotyl growth findings, where methanol, butanol, and ethyl acetate extracts of P. odora roots were most inhibitory to for M. sativa and M. falcata seed germination and seedling growth (radical and hypocotyl), followed by the dichloromethane and or water extracts, that show less inhibitory effects. Inhibition in germination potential and seedling growth of both M. sativa and M. falcata may be due to synergistic effect of phytochemicals present in roots extracts of P. odora.

CONCLUSION

The results obtained in this study clearly showed that all the extracts from the roots of P.odora possess antioxidant activity. The methanol, ethyl acetate and butanol extracts exhibited a strong antioxidant activity and have most potent scavenging ability of DPPH radical, which may be caused by their high content of polyphenols and flavonoids. The very strong antioxidant activity of the P. odora suggests that the extracts obtained by polar solvents from the roots could be used as an effective natural source of antioxidant and food additives.

All the studied extracts of P. odora roots inhibited the seed germination, radical and hypocotyl length of M. sativa and M. falcata. The phytotoxicity response of the various extracts differed markedly among the two test plants. Thus, at the highest concentration, the ethyl acetate extract is the most effective for the germination inhibition in the case of M. sativa while the methanolic extract is the most active in the case of M.falcata.The ethyl acetate, butanol and methanol extracts of P. odora significantly inhibited the seedling growth parameters (radical and hypocotyl length) of both test plants. The above results provide plausible evidence that P. odora is potentially promising resource of naturally occurring herbicides.

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