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Antimicrobial and antioxidant activities of crude extracts and essential oils from two thyme species: *Thymus vulgaris* and *Thymus hyemalis* from northern Morocco

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Abstract

We investigated antioxidant and antimicrobial activities of extracts and essential oils from two species of thyme: *Thymus vulgaris* and *Thymus hyemalis*. Plant extracts were obtained using two techniques (maceration and sonication) combined with two solvents (methanol and ethyl acetate). Antimicrobial activities were tested against four strains namely: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC29213, *Bacillus subtilis* ATCC 3366, and *Candida albicans* ATCC 10231. DPPH was used to assess antioxidant activities. Statistical analyses performed demonstrated that plant extracts and essential oils yields were impacted significantly by plant species, extraction technique, solvent and their interactions. Extraction using sonication combined with methanol allowed a better recovery of plant extracts as compared to classic maceration. Moreover, *Thymus vulgaris* was higher in terms of yield, phenolics content, antimicrobial and antioxidant activities. Plant extracts and essential oils showed potential inhibitory effects on the majority of microbial strains involved in this study. The best records of inhibitory zone diameters of extracts were found in *Escherichia coli*. However for essential oils, *Staphylococcus aureus* had the most important record of inhibitor effect. These effects could be attributed to phenolic and flavonoid compounds. Further investigations are needed toward to their use for medicinal and therapeutic applications.

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Introduction

A history as ancient as that of human civilization, that of aromatic and medicinal plants, their therapeutic and aromatic virtues have been the subject of much attention since the dawn of time. Among all this rich and varied procession, thyme is one of the most popular.

Thyme *(Thymus*) is an important plant in the pharmacopoeia, and especially in the Mediterranean region where it grows naturally. Besides its aromatic use as flavoring agents (condiment and spice), its various virtues are able to relieve a wide variety of respiratory and intestinal ailments. It is thus a broad-spectrum anti-infective and a stimulant of immunity. In addition, *Thymus* species have been reported to possess a wide range of biological activities, and a wide diversity of phytochemicals. Between these phytochemicals, essential oils, hydroxycinnamic acid, and flavonoids were found as the major bioactive constituents of the most common Lamiaceae species, such as *Thymus vulgaris* (Vladimir *et. al.*, 2014). Botanically, genus *Thymus* accounts for about 215 species. In the literature, many thyme species were investigated for their antimicrobial and antioxidant activities including those reported by Asbaghian *et al.* (2011) and Zandi-Sohani (2011). In addition, Gonçalves *et al.* (2017) investigated the essential oil of *Thymus vulgaris* for its chemical composition and tested the antimicrobial activity against some microbial strains with the objective to design a particle essential oil thyme-based through complex coacervation.

In morocco, and especially in the north which is characterized by Mediterranean climate allowing development of many species of *Thymus* genus locally known “Zaatar” or “Zaitra”. It is widely used in the Moroccan folk medicine for its expectorant, antibroncholitic, antispasmodic, anthelimintic, carminative and diuretic properties plants (Jensen *et al.*, 2010). To the best of our knowledge, despite the richness of Morocco in terms of thyme species, limited studied have been carried out to investigate their potential regarding antimicrobial and antioxidant activities. Hence, our study focuses on the screening of two species from genus *Thymus* for their antimicrobial and antioxidant activities with the following objectives: (i) determine the antimicrobial and antioxidant activities of *Thymus vulgaris* and *Thymus hyemalis* in terms of extracts using two organic solvents and two extraction techniques, and (ii) compare these two species.

Material and methods

Plant material

Two aromatic and medicinal plants were involved in this work: *Thymus vulgaris* and *Thymus hyemalis*. The aerial parts of these two plants were collected from the National Institute of Medicinal and aromatic Plants-Taounate (34°32′9″N 4°38′24″W, Morocco) on 20 February 2014. Plants were identified and deposited in the herbarium of the institute. Then the collected material was dried at ambient temperature and ground using an electrical grinder.

Chemical reagents

DPPH (1, 1-diphenyl-2-picrylhydrazyl), DMSO (dimethyl sulfoxide), gallic acid, Folin Ciocalteau, Methanol, and ethyl acetate. These chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO). All solvents and reagents used were of the highest purity.

Extracts preparation

The extraction was achieved using two kinds of techniques namely: Maceration and Ultrasonic extraction.

Maceration

About one hundred grams of dried and crushed aerial part of each plant was macerated in a first time using 1000 ml of ethyl acetate during 72 hours at room temperature. Then, maceration was achieved with methanol instead of ethyl acetate. Then, extracts were filtered through Whatman paper n°1. After filtration, crude extracts were obtained through evaporation under vacuum.

Ultrasonic Extraction

Extraction was performed using methanol and ethyl acetate independently in an ultrasonic bath (Elma–Transsonic TI-H-15, USA). Power and temperature of this apparatus were fixed at: 100W, 30°C respectively). Briefly, flasks containing 50g of air-dried and crushed plant material with 200ml of methanol were immersed in the ultrasonic bath.

Sonication was run with ultrasound frequency of 35 KHz for 30 min (three repetitions were performed). After filtration each mixture was evaporated under vacuum to obtain crude extracts.

Essential oils extraction

The same plant material described above was used for the isolation of the essential oil. One hundred grams from each one of two plants, fine powder were firstly subjected to hydrodistillation using Clevenger-type (Clevenger 1928) apparatus for 3h according to the European Pharmacopoeia (1980). Essential oil was dried over sodium sulphate anhydrous and the samples obtained were stored at 4°C until uses (Pina-vaz *et al.* 2004).

Microbial strains

Both essential oils and Extracts (obtained by Methanol and ethyl acetate) were tested for antibacterial activities against the following microbial strains: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC29213, *Bacillus subtilis* ATCC 3366, and *Candida albicans* ATCC 10231.

Total phenolics determination

Total phenolic contents were determined thrice in the extracts following the Folin-Ciocalteu method (Benzie *et. al.*, 1999). Briefly, an aliquot of the extracts was mixed with 5ml Folin- Ciocalteu reagent and 10 ml of saturated sodium carbonate in test tubes. The tubes were vortexed for 15s and allowed to stand in the dark for 30min. Absorbance was then measured against a blank at a wavelength of 760nm using a JASCO UV-VIS spectrophotometer. A standard curve was prepared using Gallic acid. Total phenolic content was expressed as mg Gallic acid /g dry extract (mg GAE/g DM).

Antimicrobial activity

Antibacterial activities of the extracts (methanolic and ethyl acetate) was examined by disk-diffusion method (Balouiri *et al.*, 2016) with slight modifications. Briefly, bacterial strains were cultured overnight at 37°C and 30°C for fungal strains on Luria-Bertani broth, then inoculum consisting of 0.5 McFarland was prepared in physiologic saline.

The bacterial inoculum (100µl) was inoculated in Petri dishes containing a sterile Luria-Bertani Agar medium. Sterile filter paper discs (5mm diameter) were deposited on medium and impregnated with 10µl of extract solution (500mg/ml of dimethyl sulfoxide DMSO to 2%). The control was performed with discs containing 10 µl of DMSO to 2%. Each experiment was performed in triplicate.

Essential oils antimicrobial activities were assessed using the agar disc diffusion method. A suspension of the test microorganisms (adjusted with Mac Farland 0.5) was spread on the solid media plates. Filter paper discs which account for 6mm in diameter were soaked with 10µl of the oils and placed on the inoculated plates and kept at 2°C during 24h. Then, the inhibition zones diameters were measured in millimeters (Azza *et al.*, 2003).

Antioxidant activities assessment

The free radical-scavenging activity assessment of extracts was based on the measurement of the reducing ability of antioxidants toward the DPPH radical. The method described by Brand-Williams *et al.* (1995). was used with slight modifications. 20µl of different extracts (at different concentrations) was added to 980µl of the methanolic DPPH solution (90µM). The reaction was allowed to stand at room temperature in the dark for 30min and the absorbance was recorded at 517nm against a blank consisting in a methanol solution using a UV–vis spectrophotometer. The measurements were performed in triplicate. The radical-scavenging activity was calculated using the following equation:

$$\% \text{ inhibition (I)} = [(A_B - A_A)/A_B] \times 100$$

Whereas the DPPH inhibition, %; A_B and A_A are the absorbance values of the control and of the test sample, respectively.

DPPH scavenging activity is presented by IC_{50} value, defined as the concentration of the antioxidant needed to scavenge 50% of DPPH present in the test solution. All tests were carried out in triplicate. DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek

Instruments, Inc; Winooski, USA), and calculated as a percentage of DPPH discoloration using the formula: [(ADPPH– AS)/ADPPH] × 100, where AS is the absorbance of the solution containing the sample at 517nm, and ADPPH is the absorbance of the DPPH solution (Reis *et al.*, 2012).

Statistical analyses

Each determination was achieved in triplicates. The combined analyses of variance (ANOVA) were calculated to assess magnitude effects of plant species, extraction technique, and solvent on yield and total phenolics. Quantitative differences were assessed by general linear procedure followed by Duncan's test. Data statistical analyses were performed using the SPSS package version 13.0 for Windows. Values were expressed as means ± standard deviations (SD). Differences were considered significant at $p < 0.05$. Antioxidant activities of the two plant species were regressed on essential oil concentration.

Results and discussion

Analyses of variance (ANOVA)

Table 1 shows the mean squares from the combined analyses of variance for yield (%) and total phenolics (mg GAE/g DM). From these results, it has been shown that plant species, extraction technique, solvent, and their interactions affected significantly the yield and total phenolics. Regarding yield, solvent was the main source of variability and accounted for about 67% of the total variance, however the effects of extraction technique and plant species were of lesser magnitude and explained only 19 and 7% of the total variance respectively. Concerning the total phenolics, the plant species effect was the most important and explained around 40% of the variability, meanwhile the effects of extraction technique and solvent were of lesser extent and explained respectively 24 and 23% of the total variance. Finally, for both yield and total phenolics, the following interactions were also significant but of lesser extent : plant species× extraction technique, plant species × solvent, extraction technique × solvent, plant species × extraction technique × solvent.

Table 1. Mean squares from the combined analyses of variances for yield (%) and total phenolics (mg GAE/g DM). * Significant at 0.05 probability level; ** Significant at 0.01 probability level; *** Significant at 0.001 probability level.

Source of variation	Df	Yield (%)	Total phenolics (mg GAE/g DM)
Plant species	1	54.843***	44.55***
Extraction technique	1	154.534***	26.80***
Solvent	1	550.467***	25.50***
Rep (Solvent)	4	0.003	0.032*
Plant species ×Extraction technique	1	3.888***	9.83***
Plant species ×Solvent	1	9.959***	0.43***
Extraction technique×Solvent	1	26.376***	0.90***
Plant species×Extraction technique×Solvent	1	12.615***	1.73***
Residual	12	0.002	0.01
Total (corrected)	23		

Mean comparison of yield and total phenolics between plant species, solvent, and extraction technique

Yield mean values (%) for plant species (*Thymus vulgaris* and *Thymus hyemalis*), extraction technique (sonication and maceration), and solvent used (methanol and ethyl acetate) are presented in Fig. 1. Between the two studies plant species, *Thymus vulgaris* was found to be higher than *Thymus hyemalis*.

As regards of extraction techniques used, sonication was found to possess the highest yield which was 15.38% as compared to maceration (10.31%). Between solvents used, yield was reported to be higher in methanol (17.63%) than in ethyl acetate (8.06%). These results are similar to those reported by Hernandez-Hernandez *et al.* (2009). These two solvents are widely used for the extraction of polyphenols from plants (Jain and *al.* 2013). Methanol was used in this research because these phenolic compounds are generally more soluble in polar solvents (Lachkar *et al.*, 2016) Moreover, methanol is known that is the most polar solvent, allowing it to extract a wide range of metabolites from plant material. On the other hand, ethyl acetate with its moderate polarity resulting in the extraction of a less wide range of metabolites. This difference explains the difference in yields obtained with methanol and ethyl acetate (Fig. 1).

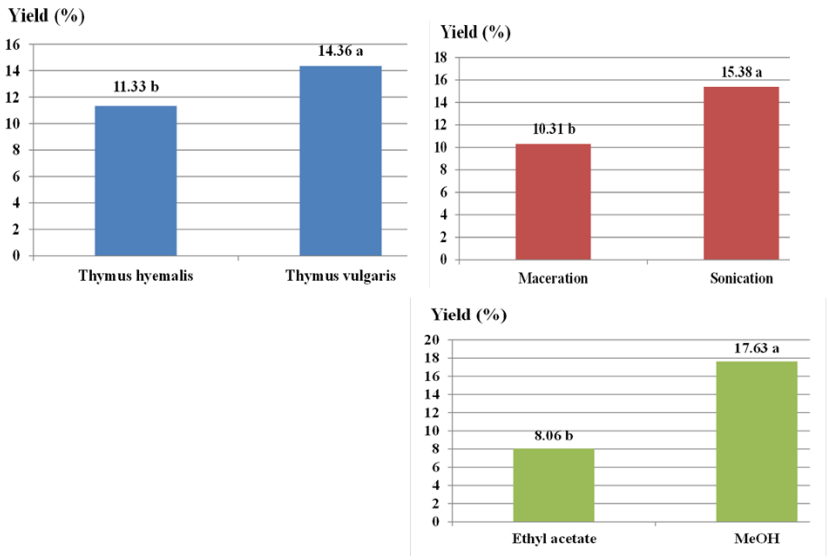

Fig. 1. Yield mean values in % for plant species (*Thymus vulgaris* and *Thymus hyemalis*), extraction technique (Maceration and sonication), and solvent used (Methanol: MeOH and ethyl acetate).

Total phenolics averaged values for plant species, extraction technique, and solvent are summarized in Fig. 2. The same image reported for yield was reflected for total phenolics. In fact, total phenolics were found to be higher in *Thymus vulgaris* than *Thymus hyemalis*. In addition, total phenolics obtained by sonication were also higher than those of maceration. The methanolic extracts were found to have the score of total phenolics as compared to ethyl acetate. Besides, phenolic compounds were able of scavenging reactive oxygen species without invoking further oxidative reactive (Al-Abd *et al.,* 2015). It has been shown that total phenolics takes on variable values depending on the Thymus species (Jabri-Karoui *et al.,* 2012). Total phenolics values reported in Safaei-Ghomi *et al.* (2009), for *Thymus caramanicus* were similar to those obtained for the two species investigated in the present study. The phenolic content of each plant, however, is a function of several factors such as the extraction method used and the phenological stage at which the plant material is collected (Gharibi *et al.,* 2015).

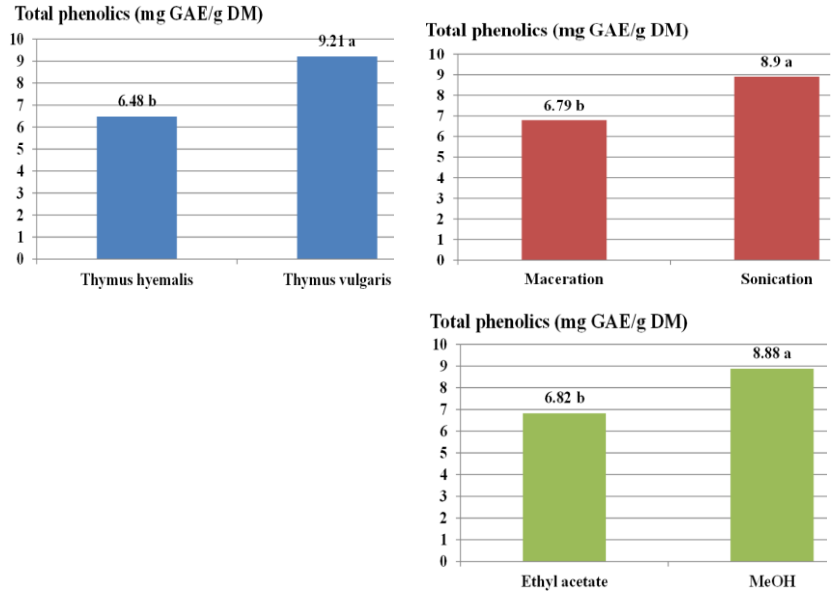

Fig. 2. Averaged values of total phenolics in mg GAE/g DM) for plant species (*Thymus vulgaris* and *Thymus hyemalis*), extraction technique (Maceration and sonication), and solvent used (Methanol and ethyl acetate).

Antimicrobial activities in the extracts and essential oils

Table 2 presents mean values inhibitory zone diameters (IZD) for areal parts extracts using two different techniques (maceration and sonication) and two solvents (methanol and ethyl acetate) against four microbial strains. A wide variability was detected between microbial strains, extraction techniques, and solvents. It is important to notice that *Thymus vulgaris* was found to possess higher values of IZD as compared to *Thymus hyemalis*. For extracts achieved by maceration using methanol *Escherchia coli* showed the highest IZD among microbial strains in both plant species. However any inhibitory activity was observed for *Candida albicans* with methanolic extracts. Regarding extracts obtained by maceration and ethyl acetate as solvent, *Candida albicans* was found to be higher in terms of IZD between microbial strains in both plant species. For results obtained with sonication, values of inhibitory zone diameters were higher than those of maceration for the two solvents used.

Table 2. Mean values of the zone inhibitory diameters (mm) for areal parts extracts using two different techniques (maceration and sonication) and two solvents (methanol and ethyl acetate) against four strains (*Escherichia coli, Candida albicans, Staphylococcus aureus, and Bacillus subtilis*). For the same plant species and within the same column, values followed by the same letter are not significantly different.

Plant species	Microbial strain	Maceration		Sonication	
		Methanol	Ethyl acetate	Methanol	Ethyl acetate
Thymus vulgaris	*Escherichia coli*	30.07 ± 3.27 a	33.90 ± 2.89 b	34.067 ± 1.76 b	39.97 ± 2.21 b
	Candida albicans	0.00 ± 0.00 d	37.90 ± 2.91 a	0.00 ± 0.00 d	56.67 ± 6.30 a
	Staphylococcus aureus	27.60 ± 4.47 b	11.10 ± 2.23 c	37.40 ± 0.89 a	17.47 ± 1.72 c
	Bacillus subtilis	9.37 ± 0.86 c	7.33 ± 0.76 d	13.70 ± 2.55 c	10.57 ± 0.59 d
Thymus hyemalis	*Escherichia coli*	23.40 ± 3.25 a	29.03 ± 1.50 b	30.27 ± 1.07 b	34.43 ± 2.18 b
	Candida albicans	0.00 ± 0.00 c	32.63 ± 4.08 a	0.00 ± 0.00 d	46.77 ± 5.83 a
	Staphylococcus aureus	23.17 ± 4.47 a	9.5 0± 1.50 c	33.13 ± 1.33 a	15.30 ± 1.54 c
	Bacillus subtilis	8.73 ± 0.87 b	7.63 ± 1.00 d	11.93 ± 2.00 c	9.20 ± 0.26 d

Antimicrobial activities of essential oils are shown in Fig. 3. The two plant species investigated here, were found to be different in terms their antimicrobial activities against four strains except for *Staphyloccus aureus* where there was no significant difference between these two plant species. The maximum of inhibitory zone diameter was obtained with *Staphyloccus aureus*, while the minimum was reached in fungal strain *Candida albicans*. Wide number of studies provides evidence that thyme has antibacterial effects under in vitro conditions (Asbaghia *et al.*, 2011, Zandi, 2011). The essential oil from the two species investigated here: *Thymus vulgaris* and *Thymus hyemalis* has been shown to possess a strong inhibitory potential against all the microbial strains tested. These antibacterial and antifungal activities of the essential oil are due to the presence of the highly active molecules, this is the case of thymol and the carvacrol belonging to the phenolic terpenes. This has been demonstrated by several studies such as Burt (2004) and Gonvaris *et al.* (2011).

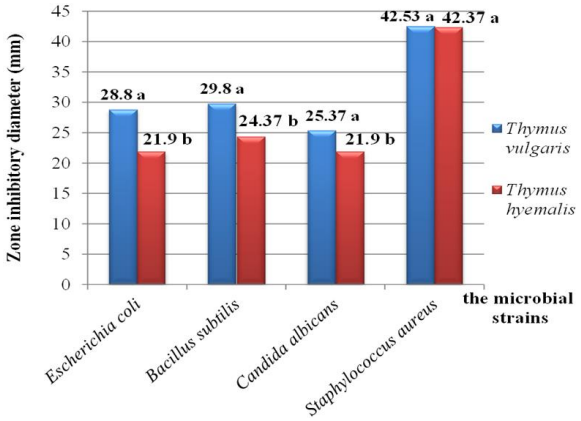

Fig. 3. Mean values of zone inhibitory diameter (mm) of essential oil from the studied plant species (*Thymus vulgaris* and *Thymus hyemalis*) against four microbial strains (*Escherichia coli*, *Candida albicans*, *Staphylococcus aureus*, and *Bacillus subtilis*). The same the microbial strain, values followed by the same letter, are not significantly different.

Antioxidant activities of essential oils

Essential oils yields were 1.46% and 1.38% for *Thymus vulgaris* and *Thymus hyemalis,* respectively. Mean comparisons of antioxidant activity for two investigated plants species as a function of essential oil concentration are summarized in Table 3.

Table 3. Averaged mean values of antioxidant activities (%) of *Thymus vulgaris* and *Thymus hyemalis* a function of concentration of essential oil (mg/ml). within the same line, values followed by the same letter are not significantly different.

Essential oil Concentration (mg/ml)	Antioxidant activity of *Thymus vulgaris* (%)	Antioxidant activity of *Thymus hyemalis* (%)
20	94.86 a	93.25 b
10	93.78 a	91.62 b
5	93.40 a	93.65 a
2.5	93.40 a	92.75 b
1.3	85.41 a	84.56 b
0.6	74.89 a	71.09 b
0.3	57.45 a	55.20 b

Antioxidants activities were higher in *Thymus vulgaris* than in *Thymus hyemalis* for almost concentrations. Antioxidant activities tended to decrease when essential oil concentrations decreased resulting in strong positive correlations between them as represented in Fig. 4 and 5. As rational function used to estimate antioxidant activities can be difficult to work with graphically, it can be transformed into a linear equation by taking the reciprocal of both sides of the equation resulting in the so called the Lineweaver-burk transformation.

A strong antioxidant activity of essential oils is obtained in *Thymus vulgaris* and *Thymus hyemalis*. Our results corroborate with other studies such as those conducted by Bouhdid *et al.* (2006) and Nathalia *et al.* (2017). All the bioactive components present in essential oils the *Thymus vulgaris* and *Thymus hyemalis* are extremely sensitive and naturally susceptible to degradation by exposure to light, heat or oxygen or due to the interaction with other compounds present in complex formulations, which could limit their biological action. The DPPH scavenging test is widely used to evaluate antioxidant activities (Baharfar *et al.,* 2015) Moreover, essential oils containing a wide range of phenolic monoterpenes and/or their terpenes have been recognized for their higher antioxidative capacity (Mancini and *al.* 2015) The IC_{50} values were found to be 51.39 and 134.37mg/ml for *Thymus vulgaris* and *Thymus hyemalis*, respectively. Results reported in our study were in line with those obtained by Ismaili *et al.*, (2017).

IC_{50} variation among the two species investigated here can be attributed to differences in their polyphenolic compounds as reported in Gharibi *et al.* (2015). The chemical structures of phenolics allow them to donate hydrogen to free radicals, the major factor contributing to the antioxidant activity of the species (Ang *et al.,* 2015)

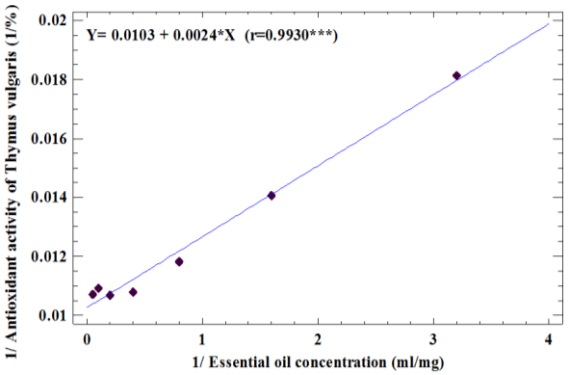

Fig. 4. Regression of 1/ antioxidant activity of *Thymus vulgaris* (1/%) on 1/ essential oil concentration (ml/mg). r = correlation coefficient. *** indicate significance at 0.001 levels of probability.

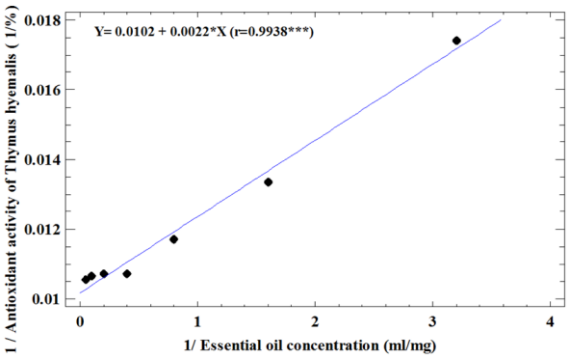

Fig. 5. Regression of 1/ antioxidant activity of *Thymus hyemalis* (1/%) on 1/ essential oil concentration (ml/mg). r = correlation coefficient. *** indicate significance at 0.001 levels of probability.

Conclusion

From our results, it can be concluded that the two plant species were promising sources of phenolic compounds. *Thymus vulgaris* had higher records in terms of yield of the extracts or essential oils and therefore presented greatest potential of antioxidant and antimicrobial activities. Extraction using sonication allowed a good recovery of plant extracts both with methanol or ethyl acetate but also essential oil yield.

Powerful potentials inhibitory against four microbial strains were demonstrated by plant extracts and essential oils. In this context, these two plant species could be used as an easily accessible source of natural antioxidants and antibiotics in commercial food products and drugs.

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