Variation in Total polyphenols, Tannin and Catechin contents and Antioxidant activity among Moroccan thyme genotype

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Abstract:

The objective of this study was to evaluate the antioxidant activity, total polyphenols, tannins and catechins of the leaves extract of nine genotypes of three *Thymus* species (*Thymus hyemalis*, *Thymus satureioides* and *Thymus broussonetii*) obtained from *in vitro* culture. Firstly, the extracts were analyzed for the total polyphenols, tannins and catechins contents using spectrophotometry method. Furthermore, the antioxidant study was carried out using 2,2'-azinobis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS•+), 2,2-Diphenyl-1-picrylhydrazyl (DPPH•) and Ferric reducing antioxidant power (FRAP). The results show the presence of phytochemicals compounds in different genotypes of three species, with a considerable variation between species on one side and between genotypes of the same species on another side. Antioxidant activity was also present with varying levels depending on the species and genotypes.

Keywords: Antioxidant activities; Catechins; *in vitro* selection; Tannins; *Thymus*; Polyphenols

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Introduction

Among natural products of great interest, there are vegetable polyphenols. These ones have shown several applications in different fields, including the medicine, thanks to their proven biological, antiviral, antitumor and antibacterial activities. These activities are based on their characteristic reactions with metal ions and with amino acids present in proteins (Chang et al. 1995; Chung et al. 1998). The phenolic compounds may contribute directly to antioxidative action (Li et al. 2014; Vuolo et al. 2019). The flavonoids have particularly attracted attention because of their high antioxidant capacity. Catechin is a member of the flavonoid family, which has a variety of pharmacological effects such as cardioprotective, diuretic and hypotensive actions (Bors et al. 1990). Several plants extract like thyme extract have been used as source of this phenolic compounds (Sharma et al. 2020).

The species of *Thymus* genus are herbaceous perennial shrubs, commonly used as spices and/or medicinal herbs. Since ancient times, *Thymus* species were used in human nutrition as a spice and food flavor and as antioxidant for treatment of many diseases (Mathur 2003). They are also reported to possess some biological effects such as antifungal, antioxidant, insecticidal, and antibacterial activities (Zarzuelo and Crespo 2002; Nieto et al. 2010; Saad et al. 2010; Fadli et al. 2012; Ramchoun et al. 2012; Drioiche et al. 2022) and have many potentials uses in the food industry as a dietetic supplement and food preservative (Youdim and Deans 1999; Nieto et al. 2011; Li et al. 2019). In addition, thyme has become a major source of bioactive compounds for pharmaceutical industry.

The *Thymus* species are a rich source of phytochemicals, such as flavonoids and other phenolic compounds. Studies have indicated that these phytochemicals, especially polyphenols, have high free-radical scavenging activity, which helps to reduce the risk of chronic diseases, such as, cardiovascular disease, cancer, and age-related neuronal degeneration (Ames et al. 1993).

The harvest of Thyme species by diverse users occurs mainly at wild state in large quantities during the flowering period before seed formation. This causes the exhaustion of wild plant genetic resources, biotope destruction, and thus also threatens valuable incomes for rural households. Besides, abiotic environmental factors as well as biotic effects were proven to influence secondary metabolites such as essential oil and polyphenol production, leading to a

chemical variability (Sáez 2001). Therefore, it is impossible to find a chemically homogeneous and standardized raw material of *Thymus* species in the natural habitat. This imposes many disadvantages such as the heterogeneity of plant material, the difficulty of predicting supplies for industry and lack of control. Additionally, the interests of pharmaceutical, food, perfumes and cosmetics industries do not focus on all genotypes available in nature, but only on a few, namely thymol and/or carvacrol and linalool chemotypes. Generally, the chemical composition of the plant was affected by genetic and environmental factors.

An increase in the demand for thyme of cultivated origin must be expected with standardized composition and yield of the chemical compounds and with uniform properties of the leaves. The propagation of selected plant species or clones in commercial scale is ideal to ensure the sustainable supply of thyme raw material. Plant Cell Tissue (PTC) and organ culture technologies have been proven to be efficient and useful tools for the breeding (selection) of high-quality Medicinal and Aromatic Plants (MAPs) and vulnerable endangered species preservation (El Meskaoui 2013). they enables the production of biomass, under controlled environments, by ensuring the supply of the raw material. This technique was successfully applied to many *Thymus* species such as *Thymus vulgaris* (Lê 1989; Furmanowa and Olszowska 1992; Ozudogruet al. 2011), *Thymus piperella* (Sáezet al. 1994), *Thymbra spicata* L. var. *spicata* L. (Daneshvar-Royandezagh 2009), *Thymus lotocephalus* (Coelhoet al. 2012), *Thymus hyemalis* (Nordine et al. 2013a, 2014a), *Thymus bleicherianus* (Nordine and El Meskaoui 2014), *Thymus broussonetii* (Nordine et al. 2014b) and *Thymus satureioides* (Nordine et al. 2013). *In vitro* clonal propagation techniques offer following benefits: source of mastered production of phytochemical products and novel products not found in nature, use of rare, vulnerable, endangered or protected plants, independence from climatic factors and seasonal constraints, more flexible production cycles in limited spaces within short periods throughout the year (El Meskaoui 2013).

Data on the intraspecific et interspecific variabilities of *Thymus* are basically restricted to the volatile components. Similarly, available literature data on the reactions of thyme to environmental factors mostly refer to the volatile compounds. It is in this context, that the main aim of the proposed work was to highlight the interspecific and intraspecific variations on the total polyphenols, tannins and catechin contents, and to evaluate the antioxidant activity, of leaves extracts of different genotypes and *Thymus* species cultured under *in vitro*

conditions. According to our knowledge, this is the first time that total polyphenolis, tannins and catechin contents and antioxidant activity were studied in the *Thymus hyemalis*, *Thymus broussonetii* and *Thymus satureioides* species under controlled conditions.

Material and methods

Plant material

The species of *Thymus hyemalis*, *Thymus broussonetii* and *Thymus satureioides* were previously *in vitro* established (Nordine et al. 2013; 2013a; 2014b). To homogenize the growing conditions and eliminate the effect of plant growth regulation (PGR), we used culture media constituted by Murashige and Skoog (MS) (Murashige and skoog 1962) (3 % sucrose, 0.4 % gellun gum and pH 5.8) without PGR. The cultures were kept at 23 ± 2 °C with a 16 light/8 h photoperiod at light intensity of 60 µ mol m– 2s– 1 provided by white fluorescent tubes (Sylvania Sylfast SSE T5, Germany). After the 8th subculture, the genotypes (Th1, Th3, and Th5) of *Thymus hyemalis*, (Tb8, Tb7 and Tb10) and (Ts17, Ts8 and Ts9) of *Thymus broussonetii* and *Thymus satureioides* respectively were harvested from *in vitro* culture (**Figure 1**) and used as plant materials (The number of plants used for each genotype varies between 40 and 50 plants depending on the species).

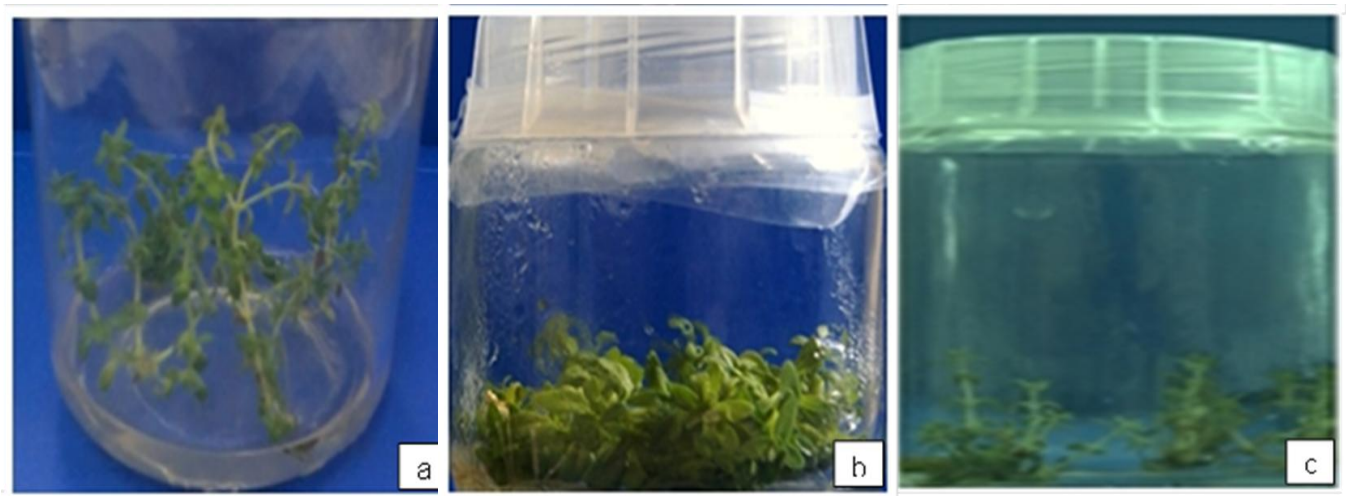

Figure 1: *In vitro* culture of *Thymus* species: a) *Thymus hyemalis*; b) *Thymus brousonetii*; c) *Thymus satureioides*

Sample preparation and extraction method

The leaves and stems of all genotypes of thyme cited above were dried at room temperature in the dark (The plant is considered dry when its weight remains constant). The dried plant materials were ground into a fine powder. Thus, the obtained powder was stored in glass jars, labeled and stored in darkness. The extraction of the various components of the plant (20 g of each genotype) is carried out by maceration with stirring at room temperature for 24h, using methanol and water (70:30 v:v) solvent. Samples were filtered through a Whatman No. 1 filter paper and the filtrate was evaporated using a rotary evaporator (RE300 Model, United Kingdom) at T= 40°C.

Evaluation of chemical compounds contents

The Total Polyphenolic contents (TP) of extracts of all samples were determined with the Folin-Ciocalteu (FC) reagent (Machado et al. 2013), with minor modifications. Indeed, a stock solution was prepared at a concentration of 4 mg of extract/1 ml of methanol. Various dilutions were then carried out to arrive at the following concentrations: 2; 1; 0.5; 0.25; 0.0125 mg/ml. After a series of measurements, the appropriate dilution for each sample was chosen. For the analysis, 0.5 ml of each sample was mixed with 0.5 ml of FC and 10 ml of sodium carbonate (75 g/l), the final volume is made up to 25 ml with distilled water. The mixture was incubated in dark, for 60 min at room temperature. The absorbance of extracts was measured at 750 nm and compared to the prepared blank. The TP was calculated on the basis of the calibration curve of gallic acid (GA) and the results were expressed as gallic acid equivalents (mg GAE)/g of extract, according to the correlation equation: (TP) (mg GAE/ g of extract) = 463.03 x A_{750} nm- 4.7419.

The determination of catechin content was done according to the method described by Kim et al. 2003. The principle of this assay is based on the ability of catechin condensation with carbonyl compounds in acidic media. For analysis, 1 ml of extract was added to 2 ml of the vanillin reagent (1%), the final volume is made up to 10 ml with HCL (70%). The mixture is allowed to stand for 25 min at room temperature before reading the absorbance at 500 nm. The results were expressed as (mg D-catechin equivalent/g of extract) according to the following equation: (mg D- catechin equivalent/ g of extract) = 156.95 x A_{500}- 0.25 (A_{500}=Absorbance at 500 nm).

The tannins content was determined by spectrophotometer using the method described by Sarneckis et al. (2006). The protocol is carried out as follows: from a methanolic stock solution (4 mg of extract/1 ml of methanol), 1 ml of extract was mixed with 3 ml of a methylcellulose solution (0.04% w/v), using a vortex, and let stand 2-3 min. Then 2 ml of a saturated solution of ammonium sulphate and 4 ml of distilled water were added. The mixture was stirred again and allowed to stand for 10 min. Then, the mixture was centrifuged at 8000 rpm for 5 min, and the absorbance was read directly after centrifugation at 280 nm. All steps were performed at room temperature. The total tannin content was determined in Tannic Acid Equivalent (mg/l) based on the following relationship: Total Tanins (mg/l Tanic Acid /g of extract) = [(A_c280-A_S280) + 0.0065] / 0.0029 (Ac = control Absorbance; As = Absorbance sample).

Antioxidant Activity

Plant extracts are always characterized by a diversity and complexity of their phytochemical compounds. This requires the application of many methods to assess the antioxidant activity.

In this study, three methods have been used to measure the antioxidant activities in 9 genotypes of *Thymus hyemalis, Thymus broussonetii* and *Thymus satureioides.*

2,2'Azinobis (3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS) radical cation decolorization assay

This assay is based on decolorization that occurs when the radical cation $ABTS^{\bullet+}$ is reduced to $ABTS^{+}$ (Re et al. 1990; Rivero-Pérez et al. 2007). Briefly, a stock solution of ABTS•+ was prepared by mixing 7 mM of ABTS solution (0.0384 g of ABTS in 10 ml of pure water) with 2.45 mM of ammonium persulfate ($K_2O_8S_2$) (0.0066 g K_2O8S_2 in 10 ml pure water) (1:1 v/v). The reaction mixture was incubated in the dark and at room temperature for 16 h. This is the time needed to obtain stable absorbance value (0.7 ± 0.01) at 734 nm. The assay was made up with 980 µl of $ABTS^{\bullet+}$ and 20 µl of the sample. The absorbance (A_f) was measured at 734 nm after incubating the reaction in the dark for 45 min, the choice of this incubation period is based on a series of reactions which have been followed kinetically.

The results were expressed in mM of Trolox according to a linear model (**figure 2**) by applying the following relationship: mM Trolox = 0.0139 + 2.0968 × (A_0-A_f), and if the extract is diluted the final relationship becames: mM Trolox = 0.0139 + 2.0968 × (A_0-A_f) x dilution factor.

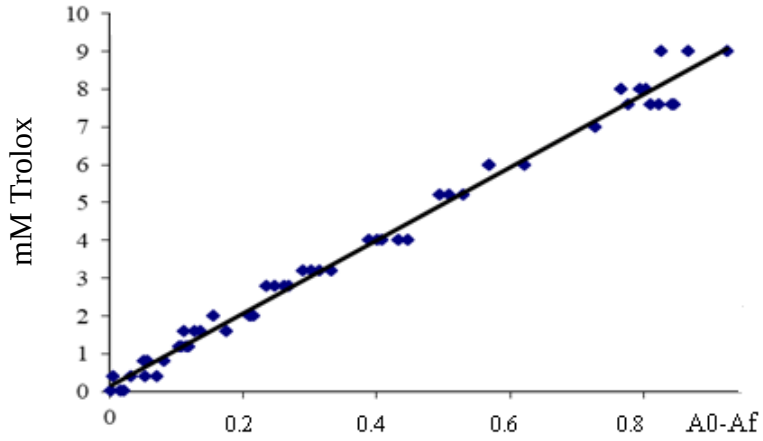

Figure 2: ABTS Calibration curve

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Method

The free radical scavenging capacity of all genotypes was measured using stable DPPH˙ radical as described previously (Rabah et al. 2004), with some modifications. The protocol is performed as follows: The DPPH solution (5.07 M) was prepared by dissolving 0.10 mg of DPPH in 50 ml of methanol, the solution is kept in the dark and at room temperature.

The test therefore consists of mixing 980 µl of the DPPH• solution with 20 µl of extract. The absorbance (A_f) was measured at 515 nm after incubating the reaction in the dark and at room temperature for 2 h. A blank control (A_0) was also prepared by replacing 20 µl of the extract with 20 µl of methanol.

The results were expressed in mM of Trolox according to the following relationship:

mM Trolox = 1.6903× (A_0-A_f) – 0.0056 (linear model) (**Figure 3**), and if dilutions were made the final relationship would be as follows: mM Trolox = (1.6903× (A_0-A_f) – 0.0056) x dilution factor.

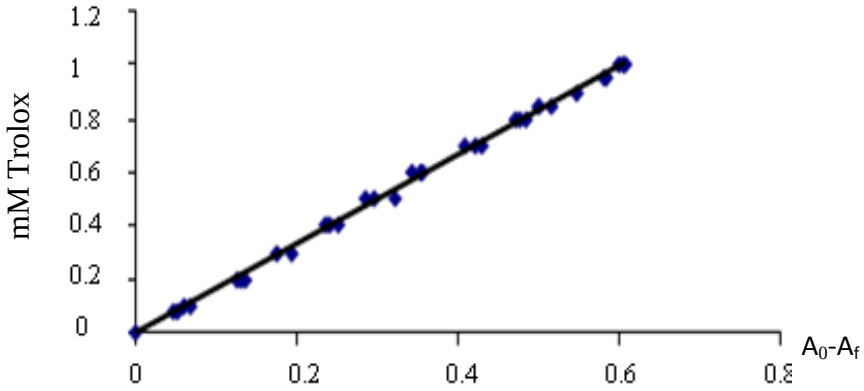

Figure 3: DPPH Calibration curve

Ferric reducing antioxidant power (FRAP) Method

This test is also used to measure the antioxidant activity. At low pH (<3) and 37°C, the tripyridyltriazine ferric complex (TPTZ-Fe 3^{+}) was added to the sample. The principle of this technique is based on antioxidants which reduce the complex present in the form of Fe2 $^{+}$ and the absorbance is read at 593 nm. This test is quick and gives repeatable results for biological solutions or for pure solutions of antioxidants (Katalinic et al. 2003). The reaction takes place by mixing 2.5 ml of TPTZ (10 mM), 2.5 ml of $FeCl_3$ (20 mM), 25 ml of sodium acetate buffer solution (300 mM) and 3 ml of distillated water. In Eppendorf tubes, 30 µl of sample was added to 970 µl of the latter reactive mixture and was incubated at 37°C for 30 min. The absorbance was measured at 593 nm. The results were expressed as (mM Fe (II)/g of extract), according the equation: Fe (II) (mM) = (1.4744 A + 0.0304) x dilution factor (A= Absorbance $_{extract}$ – Absorbance $_{blanc}$), using linear calibration curve **(Figure 4)** obtained with different concentration of $FeSO_4$.

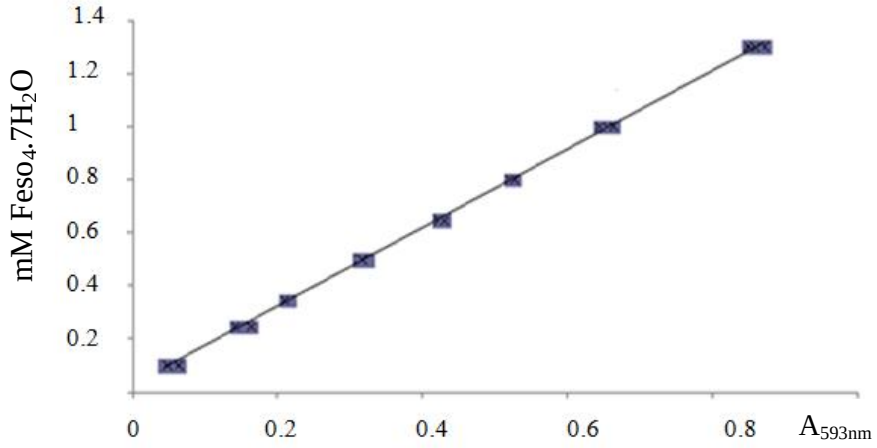

Figure 4: FRAP Calibration curve

Statistical analyses

All data are presented as the mean ± standard error (SE), calculated using replicate samples where n=4. Prior to any statistical analyses, data sets were tested for homogeneity of variance using Levene's test. Comparisons of means were performed by using analysis of variance (ANOVA) and the level of statistical significance was set at $p < 0.05$.

Results

In this study, the results showed that all species are rich in chemical compounds, but with different concentrations. The TP content was almost the same in the three species (all genotypes combined in the each species). However, it differs significantly between genotypes in the same species. This shows that all thyme species evaluated are characterized by intraspecific variation. Indeed, the Tb7 genotype is the richest in TP with 108.18±9.82 mg gallic acid/g of extract, followed by Th5 and Ts17 genotypes with 103.40±8.82 and 84.79±1.62 mg gallic acid/g of extract, respectively (**Table 1**).

Catechin contents were, also, identical with slight diffence (**Table 1**) among the three species, by showing that there is no interspecific variation between species. Furthermore, an intraspecific variation was also observed between genotypes. Indeed, the highest amount of catechin (mg D-catechin /g of simple) measured, between species, was found in *T. broussonetii* (1.61±0.56) followed by *T. hyemalis* (1.54±1.22) and *T. satureioides* (0.81±0.52). In genotypes level, the highest catechin content was 3.16±0.02 in the Th5 followed by 2.35±0.01 and 1.43 in Tb7 and Ts17 respectively (**Table 1**).

Table1. Determination of TP, catechins and tannins in the vitroplant in three species of thyme

Species	Genotypes	TP (mg GA/g of extract)	Catechins (mg D-catechin/g of extract)	Tannins (mg tannic acid/ g of extract)
T. hyemalis	Th1	27.50±5.17 **d**	0.91±0.01 **f**	3.99±0.11 **b**
	Th3	19.29±2.30 **de**	0.56±0.01 **h**	1.91±0.13 **c**
	Th5	103.40±8.82 **a**	3.16±0.02 **a**	5.26±2.18 **b**
	Total	50.06±40.50	1.54±1.22	3.72±1.83
T. broussonetii	Tb8	14.22±2.17 **e**	1.29±0.01 **d**	1.90±0.59 **c**
	Tb7	108.18±9.82 **a**	2.35±0.01 **b**	3.93±0.60 **b**
	Tb10	25.21±5.14 **d**	1.19±0.03 **e**	2.03±.32 **c**
	Total	49.20±25.35	1.61±0.56	2.62± 1.08
T. satureioides	Ts17	84.79±1.62 **b**	1.43±0.01 **c**	7.69±0.83 **a**
	Ts8	19.66±2.96 **de**	0.22±0.00 **i**	1.41±0.26 **c**
	Ts9	39.04±7.60 **c**	0.78±0.00 **g**	4.79±0.12 **b**
	Total	47.83±29.26	0.81±0.52	4.63±2.76

Data indicate mean ± SE. Values followed by the same letter within the same column are not significantly different at $p < 0.05$.

Like the other compounds, the tannin content does not show any interspecific variation between species, except a few minor differences. Indeed *T. satureioides* species had a highest amount of tannins (4.63±2.76) followed by *T. hyemalis* (3.72±1.83) and finally *T. broussonetii* with amount of tannins equal 2.62±1.08. At level of genotype, in the same species, the genotype Ts17 possessing 7.69±0.83 (**Table 1**) can be selected and cloned for the production of tannins that can be used in the food industry as condoms or medicine.

Regarding antioxidant activity, we used three evaluation tests: ABTS, DPPH and FRAP. **Figure 5** indicates that all genotypes and species have antioxidant activities. However, these activities vary from species to another and also between genotypes. Indeed, the Th5 genotype represents the highest antioxidant activity (1.03±0.06 and 0.64±0.04 mMTrolox/g of extract for ABTS and DPPH tests, 2.76±0.14 mM Fe (II)/g of extract for FRAP test), compared with all genotypes, and followed by the Tb7 genotype **(Figure 5a & 5b)**. *T. satureioides* genotypes are also characterized by an antioxidant activity according to the three tests used. The Ts17

genotype was the highest antioxidant activity of this species (**Figure 5c**). However, the Ts8 genotype was characterized by the lowest activity.

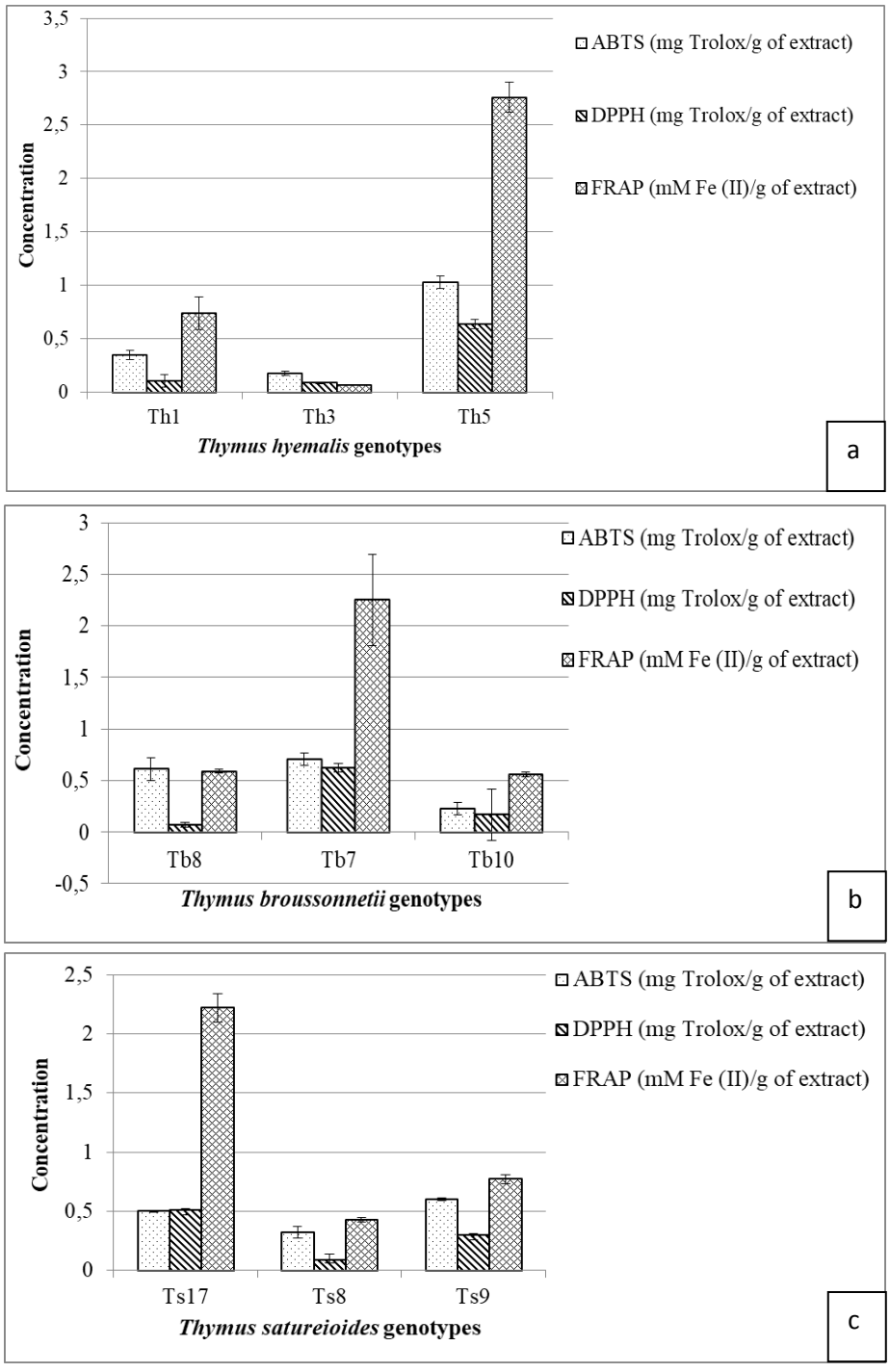

Figure 5: Antioxidant activities of three species of thyme. a) Antioxidant activity of *Thymus hyemalis.*; b) Antioxidant activity of *Thymus broussonetii;* c) Antioxidant activity of *Thymus satureioides*

Discussion

The chemical profile of several varieties of thyme and other plants, from different regions of the word is highly variable, the content and nature of their major compounds (essential oil, polyphenols, flavonoids, tannins…) vary considerably from one species to another depending on several factors. These variations in chemical composition are due to genetic profile of plant, geographical position, and harvest season, environmental and climatic conditions (Németh-Zámboriné et al. 2019; Drioiche et al. 2022). However, the plant material used in this study was grown *in vitro*, therefore under controlled and stable conditions and outside climatic and environmental variations. Despite, the species used always present interspecific and intraspecific variations. This confirms the strong impact of genetic material on chemical variation in thyme species (Dalir and Safarnejad 2017; Nordine et al. 2016).

The richness of the three species of thyme in phytocompounds is well documented previously in several studies (Ismaili et al. 2002, 2004; Elhabazi et al. 2008; Ramchoun et al. 2012; Radi et al. 2021). Intraspecific variation in phytocompounds is well, also, documented in thyme species (Jordán et al. 2009; Dalir and Safarnejad 2017).

Antioxidants, including phenolic compounds (e.g., flavonoids, phenolic acids and tannins) have diverse biological effects such as anti-inflammatory, anti-carcinogenic and anti-atherosclerotic effects, as a result of their antioxidant activity (Chung et al. 1998). Most of the antioxidant activities of plant origin are due to phenolic compounds (Mansouri et al. 2005; Jouki et al. 2014). These compounds function as antioxidants by different mechanisms, such as the prevention of initiation of the chain, the peroxide decomposition, the prevention of hydrogen abstraction and the continuous trapping of radicals (Mao et al. 2006). Several analysis methods are used. The antioxidant activity of thyme species has been the subject of several studies (Bektas 2011; Alaoui et al. 2012; Pérez-Tortosa et al. 2012; El Bouzidi et al. 2013), and the genotype effect on the intraspecific variation of antioxidant activity in several species was well documented (Németh-Zámboriné et al. 2019).The same intraspecific essential oil variability was documented in *T. satureioides* species (Nordine et al. 2016).

Conclusion

The three species of thyme studied are rich in total polyphenols, tatnin and catechin. However, great variability was observed between genotypes within the same species. The variation in the chemical content induced a large variation in the antioxidant potential; even the effect of the environmental conditions was homogenized. Therefore, this variation was due to the genetic factor.

The chemical substances studied (PT, Tannins and catechin) could be, therefore, important basic criteria for the selection of genotypes for different industrial uses. This has confirmed the need for the selection of the starting plant material at level of genotype, to have a finished product quantitatively and qualitatively value. Our results show that clones produced, by PTC systems developed, could be TP, catechins, tannins great source, and antioxidant potential. There is a great interest in the production of specific metabolites under controlling environmental conditions that maintain a given phytochemical profile.

Selection of clone performances, i.e. with high level of these substances, and their clonal implementation on a large-scale cultivation could have significant scientific and economic benefits.

Conflicts of Interest

No potential conflict of interest was reported by the authors.

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