Phytochemical screening and antioxidant activity of four Moroccan *Thymus* species: *T. leptobotrys* Murb. , *T. pallidus* Batt. , *T. broussonetti* Boiss. *and T. maroccanus* Ball.

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Abstract: In this study, four species of *Thym*us (*T. leptobotrys* Murb. , *T. pallidus* Batt. , *T. broussonetti* Boiss. and *T. maroccanus* Ball.) were studied. The phytochemical screening revealed that leaves and steams of the four species, in general, contain tannins, flavonoids, coumarins, saponins, carotenoids, reducing sugars, quinones, terpenoids, steroids and triterpenoids. The antioxidant effects of methanolic extracts from Moroccan *Thymus* species were studied using two experiments having different mechanisms. The antioxidant activity was measured by 1,1-diphenyl-2- picrylhydrazyl (DPPH) reduction spectrophotometric method and ferric reducing antioxidant power (FRAP). The results of antioxidant activity were different for each species depending on the applied test. The highest activity is presented by DPPH in the stems methanolic extract of *T. broussonetti* Boiss. (IC_{50}=0.132±0.034 mg/mL). However, the test of FRAP showed that *T. pallidus* Batt. has the highest antioxidant activity (EC_{50}=0.095±0.001 mg/mL).

Keywords: *Thymus*, Phytochemical screening, antioxidant activity, DPPH, IC_{50}, FRAP, EC_{50}

1. INTRODUCTION

Generally, plants are very rich sources of secondary metabolites. These are the classes of compounds which are known for their curative activity against several ailments in Man. This fact could, therefore, explain the traditional use of medicinal plants for the treatment of some diseases. Polyphenols, one of these secondary metabolites, constitute one of the most common groups of substances in plants. The biological properties of polyphenols include antioxidant, anticancer and anti-inflammatory effects (Ferranzzano et al., 2001). A great number of medicinal plants have been investigated for their antioxidant properties. They are very effective in preventing the destructive processes caused by oxidative stress (Pisoschi et al., 2015).

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Recently, natural antioxidants, such as vitamins and phenolic phytochemicals, have received a growing interest both in the industry and in scientific research because they are known to have a role as chemopreventive agents against oxidative damage (Yen et al., 1994) and used in pharmaceutical, cosmetic and perfume industry, also for flavoring and preservation of several food products (Bauer et al., 1997) . Consequently, many studies have been carried out with the aim of develop this traditional medicine to encourage the use of plants and to search for new bioactive compounds.

Moroccan traditional medicine is a cultural heritage. It stills a popular practice because of its medical benefits such as therapy, disease prevention and health care (Bellakhdar, 1989). The genus *Thymus* belongs to the *Lamiaceae* family, locally called ''Za-itra'', and it is represented by 21 species, 12 of which are endemic in Morocco (Benabid, 2000). The species of this genus are well known for their aromatic and medicinal character. It is largely used in popular medicine in the form of powder, infusions and decoctions to treat diarrhea, fever, coughs, cols… (Bellakhdar, 1997, Hmamouchi, 1999).

In previous studies, the essential oils of thymus species are generally rich in phenolic compounds such as thymol and carvacrol (El ouariachi et al., 2014). For example, *T. broussonetti* Boiss. essential oil is rich of borneol, p-cymène, carvacrol and thymol (El ouariachi et al., 2014; Belaqziz et al., 2010; Tantaoui et al., 1993). The major components of *T. maroccanus* Ball. Essential oil are carvacrol, camphre, α-pinene and linalool (Belaqziz et al., 2010). For *T. pallidus* Batt essential oil the main components are: thymol, p-cymène and borneol (Ghalbane et al., 2011).

In the present study, preliminary phytochemical screening has been conducted in order to determine the presence of the main classes of phytoconstituents in the methanolic and aqueous extracts from leaves and stems of different *Thymus* species collected from different localities. In addition, the *in vitro* antioxidant activity of the tested extracts has been evaluated using two complementary methods: 2,2- diphenyl-1-picrylhydrazyl (DPPH) radical activity method and ferric reduction antioxidant power method (FRAP).

2. MATERIAL AND METHODS

2.1. Plant material

2.2. Four local Moroccan species of *Thymus* (*T. leptobotrys* Murb., *T. pallidus* Batt. , *T. broussonetti* Boiss. and *T. maroccanus* Ball.) have been studied. Voucher specimens have been deposited in Natural History Museum of Marrakech – Cadi Ayyad University.

Table 1: Origin of the four species of *Thymus*

Species	Voucher specimens N°	Origin	Date
T. pallidus Batt.	MHNM-Ro-150	Ourika (Marrakech)	16/04/2014
T. leptobotrys Murb.	MHNM-Ro-68	Tiznit	26/05/2014
T. broussonetti Boiss.	MHNM-Ro-149	Ounarha (Essaouira)	22/04/2014
T. maroccanus Ball.	MHNM-Ro-53	Ait ourir (Marrakech)	20/04/2014

Each plant was dried at room temperature. The leaves and stems were separated, grinded and stored for further analysis.

Extract preparation

The powders of leaves and stems of different species of *Thymus* were used to prepare various extracts (methanolic and aqueous extracts). A quantity of 40 g of the powder was extracted using 250 mL of solvent. The mixture was left for 24 hours. The obtained extracts were filtered and concentrated under vacuum at 40°C on a rotary evaporator. The extracts were stored in sealed glass vials at 4 to 5°C prior to analysis. Each extraction was performed in triplicate.

2.3. Phytochemical screening

Phytochemical screening of the tested extracts was used to detect the eventual presence of different classes of constituents such as alkaloids, tannins, flavonoids, anthocyanins, leucoanthocyanins, coumarins, quinons, terpenoids, steroids, triterpenoids, carotenoids, saponononins and reducing sugars.

2.3.1. Test for Alkaloid

Extracts were dissolved individually in diluted hydrochloric acid and filtered. The filtrate was divided into two portions and Mayer's reagent was added to one portion, while Wagner's reagent was added to the other. The formation of a yellow colored precipitate (Mayer's test) or brown precipitate (Wagner's test) indicates the presence of alkaloids (Khanam et al., 2015).

2.3.2. Test for tannins

Catechin tannins: The Plant material was extracted with boiling water. The extract was added by reagent of Stiasny (10 mL of 40% formalin and 5 mL of concentrated HCl). The mixture was kept in a water bath at 90°C for 30 min. The observation of large precipitates flakes indicates the presence of catechin tannin compounds (Kouako Yéboué et al., 2014).

Gallic tannins: The solution containing the flakes is filtered and the collected filtrate was then saturated with sodium acetate. 1 mL of ferric chlorid 1% was added to the mixture. Blue-black coloration indicates the presence of gallic tannins (Kouako Yéboué et al., 2014).

2.3.3. Test for flavonoids

The alcohol extract was added to hydrochloric acid and magnesium. Red coloration indicates the presence of flavonoids (Sabri et al., 2012).

2.3.4. Test for anthocyanins

Aqueous extract was treated by hydrochloric acid. The appearance of red color indicates the presence of anthocyanins (Sabri et al., 2012).

2.3.5.Test for leucoanthocyanins

An aqueous extract was mixed with ethanol-HCl (50:50). The appearance of red color indicates the presence of leucoanthocyanins.

2.3.6.Test for coumarins

The ethanolic extract was evaporated; the residue was then dissolved in 1-2 mL of hot distilled water and 0.5 mL of NH_4OH (25%). The solution was exanimated under UV light. Intense fluorescence indicates the presence of coumarins.

2.3.7.Test for quinons

A few drops of NaOH 1/10 were added to the extract of petroleum ether. The color formation indicates the presence of quinon compounds (Isela et al., 2014).

2.3.8. Test for terpenoids

Methanolic extract was added to 2 mL of acetic anhydride and concentrated sulfuric acid, The formation of blue-green ring indicates the presence of terpenoids (Khanam et al., 2015).

2.3.9. Test for steroids and triterpenoids

Liebermann Burchard test: crude extract was mixed with few drops of acetic anhydride, boiled and cooled. Concentrated sulfuric acid was then added directly to the bottom of the test tube. The formation of a brown ring at the junction of two layers indicates a positive test for steroids and triterpenoids (Isela et al., 2014).

2.3.10.Test for carotenoids

The aqueous extract was acidified by HCl or H_2SO_4. The appearance of blue-green color indicates the presence of carotenoids (Isela et al., 2014).

2.3.11.Test for sapononins

The plant material was extracted using boiling water. After cooling, the extract was shaken vigorously to froth, and was then allowed to stand for 15-20 min. The formation of 1 cm of foam indicates the presence of saponins (Khanam et al., 2015).

2.3.12.Test for reducing sugars

5-8 drops of boiling Fehling's solution was added to 2 mL of aqueous extract. A red-brick precipitate shows the presence of reducing sugars (Sabri et al., 2012).

2.4. Total phenols content

The total content of polyphenols was determined by using the Folin-Ciocalteu method. The extracts' solution was diluted (10 times). 100 µL of this dilution were mixed with 2.5 mL of Folin-Ciocalteu reagent and 2.5 mL of Na_2CO_3 solution. The mixture was incubated in darkness for 60 min, and then the absorbance was measured at 515 nm. Gallic acid was used as a standard for the calibration curve and the results were expressed as mg of gallic acid equivalent per gram of dry weight (mg GAE/g DW).

2.5. Total Flavonoids Content

The total content of flavonoids was determined by using the colorimetric method of aluminum trichloride (Arvouret-Grand et al., 1994). 100 µL of the extracts were mixed with 0.4 mL of distillated water, 0.003 mL of $NaNO_2$ solution (5%) and 0.02 mL of $AlCl_3$ solution (10%); after 5 min, 0.2 mL of Na_2CO_3 solution (1 M) and 0.25 mL of distilled water were added to the mixture. The absorbance was measured at 510 nm. Quercetin was used as a standard for the calibration curve and the results were expressed as mg of quercetin equivalent per gram of dry weight (mg QE/g DW).

2.6. Tannins content

Condensed tannins were determined by using vanillin method (Price et al., 1978). 500 µL of the extracts were added to 3 mL of vanillin/methanol solution (4%) and 1.5 mL of HCl. The absorbance was measured at 500 nm. Catechin was used as a standard for the calibration curve and the results were expressed as mg of catechin equivalent per gram of dry weight (mg CE/g DW).

2.7. Determination of antioxidant activity

The extracts of all the plants were subjected to screening for possible antioxidant activity by two known complementary methods namely DPPH free radical and Ferric reduction antioxidant power method (FRAP).

2.7.1. DPPH free radical-scavenging activity

The free radical scavenging activity was measured by DPPH radical (Burits et al., 2000). The extracts were diluted at various concentrations. 50 µL of each concentration was added to 1950 µL of DPPH solution in methanol at $6.34.10^{-5}$ M, after incubation in the dark for 30 min, the absorbance was measured at 515 nm.

The radical-scavenging activities of the tested extracts, expressed as percentage inhibition of DPPH, were calculated according to the formula (Burits et al., 2000):

$$IC_{50}\ (\%) = \left[\frac{A_{blanc} - A_{sample}}{A_{blanc}}\right] \times 100$$

Where A_{blanc} is the absorbance of the control reaction (containing all reagents except the test compound), A_{sample} is the absorbance of the test compound. Percent inhibition after 30 min at room temperature was plotted against concentration, and the equation for the line was used to obtain the IC_{50} value. Synthetic antioxidant reagent ascorbic acid was used as the positive control and all tests were carried out in triplicate. A lower IC_{50} value indicated greatest antioxidant activity.

The reduction of DPPH, in presence of the extracts, is followed by monitoring the decrease in its absorbance during the reaction (Brand-Williams et al., 1995).

2.7.2. Ferric reduction antioxidant power method (FRAP)

The FRAP method was carried out to evaluate the capacity of the extracts to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) and produce ferrous-tripyridyltriazine. The color of the samples changes depending on the reducing power of each extract (Oyaizu, 1986).

Various concentrations of the extracts in distilled water were mixed with 1.25 mL of $K_3Fe(CN)_6$ solution (1%). The mixture was centrifuged at 3000 rpm for 10 min. 1.25 mL of supernatant was mixed with 2.5 mL of distilled water and freshly prepared $FeCl_3$ solution (250 µL at 0.1%). The absorbance was measured at 700 nm and Ascorbic acid was used as a positive control. EC_{50} value (mg/ml) is the effective concentration giving an absorbance of 0.5 for reducing power and was obtained from linear regression analysis. A lower EC_{50} value indicated greater antioxidant activity.

2.8. Statistical analysis

The data were presented as mean ± standard deviation (SD) of at least three measurements. Statistical analyses were performed using Student's *t*-test. All computations were done by employing the statistical software (SPSS, version 20.0).

3. RESULTS AND DISCUSSIONS

3.1. Phytochemical screening

The preliminary phytochemical screening of the four species (Table 2) revealed an abundance of tannins, flavonoids, reducing sugars, quinons, terpenoids, steroids and triterpenoids. The results showed, also, the absence of alkaloids, anthocyanins and leucoanthocyanins. However, coumarin compounds were detected only in leaves and stems of *T. broussonetti* Boiss. and *T. maroccanus* Ball. stems. The absence of carotenoids was shown in the *T. pallidus* Batt. *stems*; and the absence of saponins was detected in stems of *T. broussonetti* Boiss. and leaves of *T. maroccanus* Ball.

Table 2: Preliminary phytochemical screening of the four species of *Thymus*

Test		*T. pallidus* Batt.		*T. leptobotrys* Murb.		*T. broussonetti* Boiss.		*T. maroccanus* Ball.	
		Leave	Stems	Leave	Stems	Leave	Stems	Leave	Stems
Alkaloids	Mayer's test	-	-	-	-	-	-	-	-
	Wagner's test	-	-	-	-	-	-	-	-
Catechin tannins		+	+	+	+	+	+	+	+
Galic tannins		+	+	+	+	+	+	+	+
Flavonoids		+	+	+	+	+	+	+	+
Anthocyanins		-	-	-	-	-	-	-	-
Leucoanthocyanins		-	-	-	-	-	-	-	-
Coumarins		-	-	-	-	+	+	-	+
Quinones		+	+	+	+	+	+	+	+
Terpenoids		+	+	+	+	+	+	+	+
Steroids and triterpenoids		+	+	+	+	+	+	+	+
Carotenoids		+	-	+	+	+	+	+	+
Saponins		+	+	+	+	+	-	-	+
Reducing sugars		+	+	+	+	+	+	+	+

(+): presence (-): absence

These results show that the studied plants have quite a number of chemical constituents, which may be responsible for the many reported pharmacological actions. Furthermore, the presence or absence of different chemical groups depends essentially on the species and the part of the studied plants.

3.2. Total phenols content

The results of the total phenols are reported in Table 3. All extracts contain a considerable amount of phenolic metabolites. The extracts had high variability. The highest content of phenols was found in the methanolic extract of *T. broussonetti* Boiss. stems (219.875 mg GAE/gDW) and *T. maroccanus* Ball. (125.875 mg GAE/g DW). The extract with high content of flavonoids was the methanolic extract of *T. broussonetti* Boiss. stems.

About tannins, the aqueous extract of *T. maroccanus* Ball. stems and the methanolic extract of *T. leptobotrys* Murb. leaves showed the highest content (27.708 mg CE/g DW and 22.637 mg CE/g DW , respectively).

According to Mendez et *al. (*2015), the spanish *T. mastichina* contained 6.8 - 56.4 mg CAE / GDW of polyphenols, while the methanolic extracts of the studied *Thymus* spicies showed high values of polyphenols contents (12.352 - 219.875 mg GAE / GDW).

Table 3: Contents of polyphenols, flavonoids and tannins of the four species of *Thymus*

Species		Polyphenols (mg GAE /g)		Flavonoids (mg QE/g)		Tannins (mg CE/g)	
		Aqueous extract	Methanol extract	Aqueous extract	Methanol extract	Aqueous extract	Methanol extract
Leaves	*T.P*	53.661 ± 1.730	12.352 ± 0.970	1.273 ± 0.0986	0.489 ± 0.126	12.080 ± 0.7	10.816 ± 0.592
	T.L	21.063 ± 1.041	33.679 ± 0.963	10.308 ± 1.116	1.522 ± 0.386	4.601 ± 0.076	22.637 ± 0.512
	T.B	31.317 ± 0.422	26.400 ± 0.282	3.605 ± 0.116	5.331 ± 0.268	11.358 ± 0.283	1.716 ± 0.049
	T.M	25.187 ± 0.366	18.577 ± 0.425	4.300 ± 0.344	3.567 ± 0.267	13.083 ± 0.014	4.009 ± 1.039
Stems	*T.P*	60.817 ± 1.171	52.736 ± 0.867	5.170 ± 0.432	1.177 ± 0.386	4.886 ± 0.139	9.108 ± 0.223
	T.L	52.998 ± 1.101	23.492 ± 0.314	1.544 ± 0.251	0.255 ± 0.039	7.608 ± 0.431	9.017 ± 0.191
	T.B	48.717 ± 1.303	219.875 ± 0.932	4.177 ± 0.327	24.469 ± 0.502	13.304 ± 0.361	15.029 ± 1.035
	T.M	46.627 ± 0.350	125.875 ± 0.815	6.422 ± 0.075	8.044 ± 0.196	27.708 ± 0.530	7.336 ± 0.438

T.P: *T. pallidus* Batt. , T.L: *T. leptobotrys* Murb. , T.B: *T. broussonetti* Boiss., T.M: *T. maroccanus*.Ball.*; Aq: aqueous; Meth: Methanolic; GAE: Equivalent of gallic acid ; QE:Equivalent of quercetin ; CE:Equivalent of catechin*

3.3Antioxidant activity

To compare the antioxidant capacity of a biological extract, one test seems to be not enough since various mechanisms are involved in the antioxidant action. For this reason, a multidimensional evaluation of antioxidant activity is required. Our samples were subjected to two antioxidant methods, representing two different antioxidant mechanisms. The antioxidant activity of all species was investigated by using free radical scavenging (DPPH) and reducing antioxidant power (FRAP).

As shown in the table 4, all the extracts showed very strong activity with low IC_{50} that varied between 0.132 ± 0.034 mg/mL and 6.48 ± 0.19 mg/mL. This activity is lower than the methanolic extract activity of Turkish *T. nummularius* (*Ertas* et *al.,* 2015). The highest activity was observed in methanolic extracts, in particular stems of methanolic extracts of *T. broussonetti* Boiss.*, T. pallidus* Batt. and *T. leptobotrys* Murb. , there IC_{50} are respectively 0.132 ± 0.034 mg/mL, 0.373 ± 0.070 mg/mL and 0.43 ± 0.401 mg/mL.

Concerning FRAP (Table 4), the highest activity was obtained by methanolic extracts, especially the stems of *T. broussonetti* Boiss. (EC_{50}=0.105 ± 0.021 mg/mL*)* and *T. leptobotrys* Murb. (EC_{50}=0.095 ± 0.001 mg/mL).

There were differences between free radical scavenging and reducing antioxidant power. Also, there is no simple correlation between phenol content and the antioxidant capacity.

In general, all the extracts had high content of polyphenols, high DPPH capacity and FRAP antioxidant capacity.

Table 4: Antioxidant activity

Control/ Species		IC_{50} (mg/mL)		EC_{50} (mg/mL)	
		Aqueous extract	Methanol extract	Aqueous extract	Methanol extract
Ascorbic Acid		1.105 ± 0.071		0.05 ± 0.002	
Leaves	*T.P*	1.210 ± 0.111	2.92 ± 0.630	0.301 ± 0.009	1.468 ± 0.01
	T.L	13.711 ± 1.141	1.95 ± 0.384	1.388 ± 0.003	0.332 ± 0.008
	T.B	22.61 ± 1.022	6.484 ± 0.190	0.597 ± 0.013	1.579 ± 0.014
	T.M	11.178 ± 0.350	5.123 ± 0.372	0.619 ± 0.033	1.038 ± 0.012
Stems	*T.P*	2.76 ± 1.036	0.373 ± 0.070	0.266 ± 0.007	0.543 ±0.005
	T.L	1.410 ± 0.150	0.43 ± 0.401	0.535 ± 0.035	0.095 ± 0.001
	T.B	7.665 ± 0.411	0.132 ± 0.034	0.489 ±0.011	0.105 ± 0.021
	T.M	2.422 ± 0.162	1.143 ± 0.174	0.869 ± 0.054	0.394 ± 0.006

3.4. Absorbance variation for DPPH

DPPH has the advantage of being unaffected by certain side reactions of polyphenols, such as metal ion chelation and enzyme inhibition. A freshly prepared DPPH solution displays a deep purple color at 517 nm which gradually vanishes in the presence of a good hydrogen donor. The decrease in absorbance of DPPH in the presence of ascorbic acid and *Thym*us extracts against reaction time at different dosages is shown in Figures 1 and 2.

With all extracts, the visible absorbance decreased quickly during the first 5 minutes as a result of the transfer of the most labile H atoms of the antioxidants (fast step). This step was followed by a much slower decrease of the visible absorbance. This might most probably be due to the residual H-donating ability of the antioxidant degradation products (slow step).

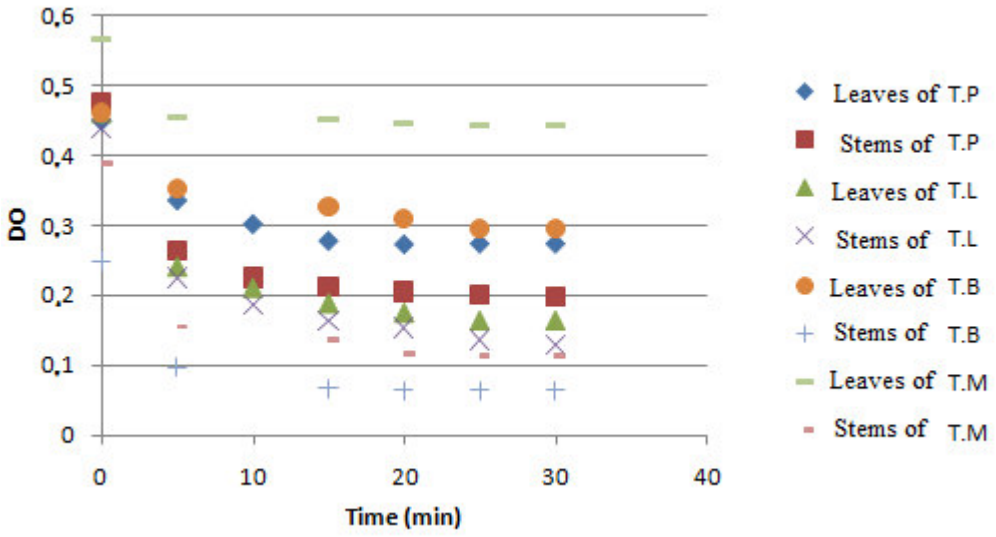

Figure 1: Absorbance variation for DPPH during 30 min in presence of aqueous extracts (IC_{50})

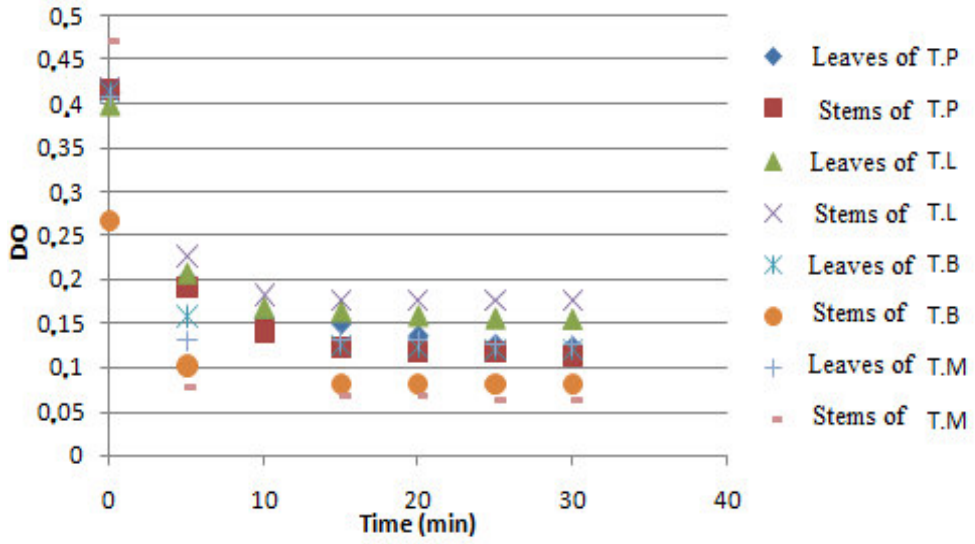

Figure 2: Absorbance variation for DPPH during 30min in presence of methanolic extracts (IC_{50})

4. CONCLUSION

In conclusion, our results provide evidence that Moroccan *Thym*us species (*T. pallidus* Batt. , *T. leptobotrys* Murb. , *T. broussonetti* Boiss. and *T. maroccanus* Ball.), possess active principles that exhibit marked therapeutic effects (tannins, flavonoids, coumarin, saponins, carotenoids, reducing sugars, quinones, terpenoids, steroid and triterpenoid), confirming and justifying the popular uses of these plants to treat some diseases in Morocco. All of the

studied species contained a considerable amount of polyphenols; there is no simple correlation between phenolic content and the antioxidant capacity. In the present study, analysis of free radical scavenging activity and reducing antioxidant power showed that mainly the *T. broussonetti* Boiss. and *T. pallidus* Batt. can be the potent source of natural antioxidants. It is very likely that the crude extracts contain some compounds which are more active.

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