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Research Article

In vitro investigations of biological activities of *Thymus willdenowii* and *Thymus atlanticus* polyphenol-rich extracts

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Abstract: Thyme species produce a wide variety of phenolic compounds including tannins, phenolic acids, and flavonoids. *Thymus atlanticus* (*T. atlanticus*) and *Thymus willdenowii* (*T. willdenowii*) are important thyme species in the southeast of Morocco, with numerous biological properties. The polyphenolic extracts of these two thyme species were obtained using ethanol through Soxhlet apparatus. Antioxidant (DPPH, FRAP, and TAC methods), antihemolytic (2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) induced hemolysis test), hypolipidemic (3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity inhibition test), and anti-inflammatory (protein denaturation inhibition) effects of extracts were carried out using *in vitro* methods. The results showed that the polyphenolic extracts of these two species revealed important amounts of phenolic compounds. The contents of flavonoids were significant in the two species, while the contents of tannins and anthocyanin were very low. *T. atlanticus* showed an important antioxidant activity and a considerable antihemolytic effect in AAPH-induced hemolysis test (IC_{50} = 0.29 mg/mL), while *T. willdenowii* showed an important anti-inflammatory activity in heat-induced protein denaturation test (IC_{50} = 1.61 mg/mL). Moreover, both extracts at a dose of 20 µg/mL showed an important *in vitro* hypolipidemic activity by inhibiting HMG-CoA reductase activity (*T. willdenowii*: 51.16 %; *T. atlanticus*: 62.83 %). In conclusion, *T. willdenowii* and *T. atlanticus* extracts have considerable antioxidant, antihemolytic, hypolipidemic, and anti-inflammatory effects. The richness of these species in polyphenols gives them a large specter of biological properties, making them a valuable source of natural bioactive compounds that could prevent or treat various diseases.

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1. INTRODUCTION

Reactive nitrogen and oxygen species (RNS, ROS) are chemical intermediates with high reactivity, which have built-in chemical properties that confer reactivity to different biological targets (Li *et al.*, 2016; Schieber & Chandel, 2014). The over-production of these chemical elements can oxidize or modify biomolecules, causing several human disorders (Chatterjee, 2016). Antioxidant ability can be defined as the restriction of the oxidation process of bio-molecules such as lipids, deoxyribonucleic acid, proteins, or other bio-molecules. There are two

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types of antioxidants with two different mechanisms. Primary antioxidants have the ability to scavenge the effects of harmful free radicals in a direct manner. In contrast, secondary antioxidants indirectly prevent the generation of free radicals (Atasoy *et al.*, 2019). However, polyphenols can repress the harmful effects of ROS and RNS. These bioactive compounds are puissant antioxidants that provide many health advantages by various mechanisms, including free radical elimination, chelation of pro-oxidant metals, and regeneration and protection of other dietary antioxidant agents (Lima *et al.*, 2014). In addition, polyphenols have attracted more and more attention as powerful antioxidants for treating and preventing the development of pathologies associated with oxidative stress (Li *et al.*, 2014).

Thymus (Lamiaceae family) is considered one of the most heterogeneous genera of the flora of the Mediterranean area (Bartolucci & Domina, 2015). This genus has numerous species and varieties (Rota *et al.*, 2008). It contains more than 336 aromatic species with valuable medicinal properties (Soorni *et al.*, 2019). These species are considered important traditional herbs, widely used in the folk medicine of the Mediterranean area. Several species of thyme are popularly used as condiments, culinary herbs, and ingredients in medicinal products, teas, or syrups, according to their organoleptic, preservative, and medicinal properties (Taghouti *et al.*, 2020). Moreover, thyme herbs are used to treat various pathologies, including upper respiratory infections, coughs, acute and chronic bronchitis, whooping cough, and catarrh (Nabavi *et al.*, 2015).

Thyme plants have several biological effects, including antioxidant, anti-inflammatory, cardio-protective, coagulant, antimicrobial, neuro-protective, anti-carcinogenic, and hypoglycemic properties (El Yaagoubi *et al.*, 2021; Elbouny *et al.*, 2022). The species of this genus are known to possess great quantities of phenolic acids and flavonoids and exhibit potent antioxidant capacity (Ramchoun *et al.*, 2015). Moreover, the phenolic-rich fractions extracted from thyme species are an important source for screening bioactive phytochemicals for possible uses in various areas, including, pharmaceutics, cosmetics, or food industries (Afonso *et al.*, 2020).

In this work, we studied the antioxidant, hemato-protective, anti-inflammatory, and hypolipidemic capacities of polyphenolic extracts of *T. atlanticus* and *T. willdenowii,* to evaluate their bioactivity and to justify their traditional use.

2. MATERIAL and METHODS

2.1. Plant Material and Extraction

Areal parts of *T. atlanticus* and *T. willdenowii* were collected in Errachidia region in April 2021 (32° 15′ N, 5° 25’ E, 1991-2055 m). The species were identified in the National Institute of Agronomic Research of Errachidia. Voucher specimens (Ta HerbFST # 92) and (Tw HerbFST # 93) were deposited in the Herbarium of Faculty of Sciences and Techniques of Errachidia. The plant material was ear-dried in the shade for 7 days before extraction.

Polyphenol extracts were obtained from *T. atlanticus* and *T. willdenowii* aerial parts, as described by Ramchoun *et al.* (2020). Fifty grams of air-dried plant powder was put in a Soxhlet apparatus and defatted with *n*-hexane until the eluent become colorless. Thereafter, *n*-hexane was discarded and the residue was dried. Then, a second Soxhlet extraction was carried out using an ethanolic solution 80% at 45 °C for 4 hours. The obtained hydro-ethanolic extract was dissolved in water and saponins were separated using *n*-butanol. Then, the polyphenolic extract (aqueous fraction) was recovered and concentrated to dryness.

2.2. Determination of Total Polyphenolic Content (TPC)

Polyphenols quantity was estimated using the Folin – Ciocalteu method, described by Singleton and Rossi (1965). A standard phenolic compound (gallic acid) was used to generate the calibration curve (Singleton & Rossi, 1965).

2.3. Determination of Total Flavonoid Content (TFC)

The total flavonoid content of extracts was determined by the aluminum trichloride colorimetric method as described by Khouya *et al*., (2022). To generate the calibration curve, rutin was used a standard flavonoid compound (Khouya *et al.*, 2022).

2.4. Determination of Total Condensed Tannins Content (TTC)

The analysis of condensed tannins was carried out according to the procedure of Heimler *et al.,* (2006). Catechin was used to generate the calibration curve (Heimler *et al*., 2006).

2.5. Determination of Total Anthocyanin Content (TA)

The amounts of anthocyanins in the polyphenol extract was estimated using the pH differential technique (Elisia *et al*., 2007). In brief, 200 microliters of each extract was separately dissolved in KCl (0.025 M, pH 1.0) and sodium acetate (0.4 M, pH 4.5) buffers. Afterwards, the optical densities values of samples were determined at 510 and 700 nm against a blank (water). The absorbance (A) of the sample was determined using the following equation:

$$A = (A_{\lambda vis\text{-}max} - A_{700\ nm})_{pH\ 1.0} - (A_{\lambda vis\text{-}max} - A_{700\ nm})_{pH\ 4.5}.$$

The amounts of anthocyanins were determined using the following formula:

$$\text{Total anthocyanins (mg/l)} = A \times M \times 1000/(Ma \times C)$$

- M: Molecular weight (449.2 g/mole) of cyanidin-3-glucoside
- Ma: Molar absorptivity (26900)
- C : The concentration of extract in the buffer in mg/mL

2.6. Antioxidant activity

2.6.1. *DPPH radical scavenging activity assay (DPPH)*

DPPH$^{\cdot}$ (1,1-diphenyl-2-picrylhydrazil) radical scavenging antioxidant capacity of extracts was evaluated as described by Elbouny *et al*., (2022). One hundred microliters of each concentration (0,1-1 g/L) of extracts were added to 1 mL of DPPH solution (0.1 mM in methanol). The mixture was incubated for 20 minutes. Then, the optical density was determined at 517 nm versus methanol (Elbouny *et al*., 2022). BHT (butylated hydroxytoluene) was used as an antioxidant reference compound. The following equation was used to determine the percentage of inhibition:

$$I\% = \frac{Ac - At}{Ac} \times 100$$

Where, at is the optical density of test samples, while Ac is that of control.

2.6.2 *Total antioxidant capacity assay (TAC)*

This assay was evaluated using the reduction capacity of molybdenum by extracts at low pH (Prieto *et al*. 1999). Results of TAC assay were represented as mg of ascorbic acid equivalent per gram of extract (AAE mg/gE mean ± STD of three replicates) (Prieto *et al.*, 1999).

2.6.3 *Ferric reducing antioxidant power assay (FRAP)*

This technique was developed to measure the capacity of antioxidants to reduce ferric iron (Fe^{+3}) to ferrous iron (Fe^{+2}), based on the method of Benzie and Strain (1999). Ferrous sulfate

(FeSO4, 0.1-1 mM) was used as a reference to generate the calibration curve. Results were represented as Fe^{2+} equivalent (mmol) per gram of extract (Fe^{2+} E mM/gE mean ± STD of three replicates) (Benzie & Strain, 1999).

2.7. Hematoprotective Activity Against (AAPH)-Induced Red Cells Oxidative Hemolysis

The anti-hemolytic effects of thymes was effectuated as described by Kandikattu *et al.,* (2015) with slight modifications (Kandikattu *et al.*, 2015). Blood was collected from male Wistar albino rats in heparin tubes and centrifuged at 4000 rpm for 6 minutes. The lysed erythrocytes were eliminated by repeated phosphate-buffered saline (PBS) wash. Afterwards, 2 % v/v of hematocyte solution was prepared in PBS (pH=7.4). AAPH (2,2'-azo-bis(2-amidinopropane) hydrochloride) which is a peroxyl radicals initiator was used to induce oxidative hemolysis. In clean test tubes, eight hundred microliters of red hematocyte suspension were mixed with 500 µL of extract or ascorbic acid as an antioxidant standard in PBS and 300 µL AAPH (400 mM) were added. Thereafter, the solution was incubated at 37ºC for 3 hours, and centrifuged at 4000 rpm for 6 minutes. Then, the optical density of the supernatant was determined at 540 nm. A control was made by adding 300 µL AAPH and PBS instead of extracts or standard and expressed as 100% hemolysis. The following formula was used to determine the percentage of inhibition:

$$\%\text{Hemolysis inhibition (\%HI)} = \frac{(\text{Ac} - \text{As})}{\text{Ac}} \times 100$$

A_C is the optical density of control and A_S is that of samples.

2.8. *In vitro* Anti-Inflammatory Assay

Thyme extracts' anti-inflammatory effect was effectuated using the method of protein denaturation inhibition as described by Chandra *et al.* (2012) with slight modifications (Chandra *et al.,* 2012). The following solutions were introduced into test tubes: 100 µL of 25% egg albumin in PBS buffer (pH 7), 700 µL of PBS, and 500 µL of varying concentrations of extracts (0.1-1.5 mg/mL). A control was made using distilled water 500 µL. Afterwards, the solutions were incubated (15 minutes at 37°C) and heated (5 minutes at 70°C). After cooling, the optical density at 660 nm was determined. Indomethacin was used as a reference drug in the same conditions as extracts. The following formula was used to determine the percentage of inhibition of protein denaturation:

$$\text{Percentage of inhibition } (\%) = \frac{(\text{Ac} - \text{As})}{\text{Ac}} \times 100$$

A_S is the optical density of test sample and A_C is that of control.

2.9. HMG-CoA Reductase Inhibition Assay

This test was conducted to evaluate the inhibition capacity of the two thymes on HMG-CoA reductase activity. Simvastatin (5 µg/mL) was used as a reference drug. HMG-CoA reductase enzyme assay kit (Sigma-Aldrich) was used.

In an ELISA 96 well plate, 181 µL of 1x assay buffer was mixed with 1 µL of different extracts (20 µg/mL) or simvastatin (5 µg/mL), four µL NADPH solution, 12 µL HMG-CoA substrate, and 2 µL enzyme. Theabsorbance of wells was then measured at 37 °C with a microplate spectrophotometer (Epoch TM 2 microplate spectrophotometer, EPOCH2, BioTek Instruments, USA) at 340 nm wavelength and the absorbance was read every 20 seconds for 10 minutes. For HMG-CoA reductase activity wells, 1µL of 1x assay buffer was added instead of inhibitors. For the blank well, 3 µL of 1x assay buffer was added instead of inhibitors and HMG-CoA reductase solution. The assay was carried out in triplicate for each test well. The activity of HMG-CoA reductase was determined using the following formula as described by the manufacturer.

$$Units/mgP = \frac{(\Delta\text{Absorbance}) \times \text{Tv}}{12.44 \times \text{V} \times 0.6 \times \text{LP}}$$

Where:

ΔAbsorbance: ΔA340/minsample - ΔA340/minblank

Tv: Total volume of the reaction mixture in mL

12.44: Extinction coefficient of NADPH ($2 \times 6.22 mM^{-1}cm^{-1}$) (340 nm).

0.6 = Enzyme concentration in mg-protein/mL (0.6 mg P/mL)

V = Volume of enzyme used (0.002 mL)

LP = Light path in cm (0.55 cm).

2.10. Determination of Inhibitory Concentration 50 Values (IC_{50})

Results were expressed as IC_{50} (50% inhibition) for DPPH, hematoprotective, and anti-inflammatory tests. The concentration that decreases the absorbance of control sample by 50% is defined as IC_{50}. It was determined using the linear regression equation from the inhibition values.

2.11. Statistical Analysis

Data with three groups were analyzed using one-way ANOVA test and those with two groups were analyzed using unpaired t-test. Differences were considered significant when P value is less than (0.05). Results are represented as means± SD.

3. RESULTS

3.1. Determination of Different Polyphenolic Compounds Contents

Table 1 summarizes the findings of the estimation of the total contents of different groups of polyphenolic compounds. The results demonstrated that the polyphenolic fractions of *T.willdenowii* and *T. atlanticus* have an important amounts of phenolic compounds according to Folin Ciocalteu estimation method, *T. atlanticus* had the most significant amounts of these phenolic compounds (p<0.01). Total polyphenols and flavonoids are the most abundant in the extracts of both thymes, whereas tannins and anthocyanins are present in very low quantities.

Table 1. The results of TPC, TFC, TTE, and TA assays .

	TPC (GAE mg/gE)	TFC (RE mg/gE)	TTC (CE µg/gE)	TA (µg/mL)
T. atlanticus	200.91 (±1.78)	76.12 (±0.92)	0.42 (±0.04)	0.36 (±0.09)
T. willdenowii	141.82 (±1.27)	41.7 (±0.78)	0.04 (±0.02)	0.17 (±0.01)
P value	<0.0001	<0.0001	0.0073	0.0024

Values in the same column were analyzed using unpaired t-test. Data are represented as the mean of 3 replicates ± SD.

3.2. Antioxidant Activity

The results of antioxidant assays are represented in Table 2. The two thyme species showed an important antioxidant effect. However, *T. atlanticus* had the strongest antioxidant potential, demonstrated by radical scavenging ability (DPPH), ferric reducing ability (FRAP), and total antioxidant capacity (TAC) assays (DPPH IC_{50} = 0.33 mg/mL, 2.3 Fe^{2+}E mM, and 0.31 AAE mg/gE, respectively) (p<0.01).

Table 2. Result of antioxidant activity.

	DPPH (IC_{50} mg/mL)	FRAP (Fe^{2+}E mM)	TAC (AAE mg/gE)
T. atlanticus	0.33 (±0.01)	2.3 (±0.01)	0.31 (±0.01)
T.willdenowii	0.74 (±0.02)	1.88 (±0.01)	0.28 (±0.01)
Trolox	0.13 (±0.00)	-	-
P value	0.0013	<0.0001	0.0084

Values in the same column were analyzed using unpaired t-test. Data are represented as the mean of 3 replicates ± SD.

3.3. Hematoprotective Activity and Protein Denaturation Inhibition

Both extracts showed that they have an antihemolytic effect against AAPH-induced hemolysis (Table 3). *T. atlanticus* (IC_{50} = 0.29 ±0.01 mg/mL) had a higher hematoprotective effect than that of *T. willdenowii* (IC_{50} = 0.36 ±0.02 mg/ml) which corresponds with the results of antioxidant tests. However, the antihemolytic effect of ascorbic acid (IC_{50} = 0.06 ±0.01 mg/mL) was higher than that of both extracts.

Results of the protein denaturation inhibition assay showed that the extracts of both thymes showed anti-denaturation of protein effect in which *T. willdenowii* had the highest effect (IC_{50} = 1.61 ±0.05 mg/mL) compared to *T. atlanticus* (IC_{50} = 1.91 ±0.04 mg/mL) and indomethacin (IC_{50} = 1.85 ±0.04 mg/mL) (p<0.05).

Table 3. Effect of *Thymus* extracts and standard drugs on protein denaturation and red blood cells hemolysis. Results are represented in IC_{50} value in mg/mL.

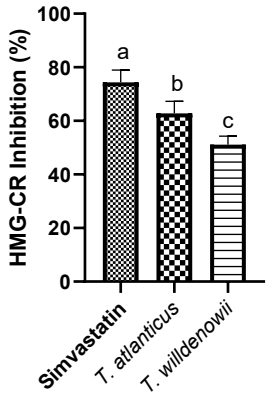
	Inhibition of protein denaturation	Antihemolytic effect
T. atlanticus	1.91 (±0.04)^{a}	0.29 (±0.01)^{a}
T.willdenowii	1.61 (±0.05)^{b}	0.36 (±0.02)^{b}
Indomethacin	1.85 (±0.04)^{a}	-
Ascorbic acid	-	0.06 (±0.01)^{c}

Values in the same column with the same letter group are not significantly different (p<0.05). Data are represented as the mean of 3 replicates ± standard deviation.

3.4. HMG-CoA Reductase Inhibition Assay

The results of HMG-CoA reductase inhibition are shown in Figure 1. All samples have inhibited the activity of the enzyme. However, simvastatin at 5 µg/mL showed the highest effect (74.34 %), whereas *T. atlanticus* exhibited a moderate effect (62.83 %), and *T. willdenowii* had the lowest effect (51.16 %). The three groups had significant different effects (p<0.05).

Figure 1. Values of HMG-CoA reductase inhibition (%) by *T. atlanticus* and *T. willdenowii* extracts or simvastatin. Data are represented as the mean of 3 replicates ± SD. Values of all groups are significantly different (p<0.05).



4. DISCUSSION

Plants synthetize numerous phenolic compounds as secondary metabolites. These bioactive phytochemicals have been known as one of the most widespread groups of plant secondary metabolites with important biological effects related with the prevention of several chronic diseases (Ziaullah & Rupasinghe, 2015). Moreover, several studies have demonstrated that *Thymus* plants are rich in polyphenolic compounds, especially flavonoids and phenolic acids (Boutaoui *et al.*, 2018; Gedikoğlu *et al.,* 2019). Moreover, the plants of this genus showed that they possess a broad variety of active compounds that exert strong antioxidant potential (Bistgani *et al.*, 2019; Labiad *et al.*, 2017; Tohidi *et al.,* 2017). The antioxidant properties of these bioactive compounds may be higher than those of some powerful antioxidant agents such as butylated hydroxytoluene and α-tocopherol antioxidants (Kuete, 2017). However, the antioxidant potential of the extracts of thyme plants can make them a promising source of antioxidant agents.

AAPH is a peroxyl radical initiator that creates free radicals by thermal decomposition. It attacks hematocytes to cause the chain oxidation of lipids and proteins, disturbing the membrane stability which leads eventually to hemolysis (Ramchoun *et al*., 2015). Moreover, AAPH induced hemolysis of red blood cells is an important experimental model for evaluating free radical-induced membrane damage and for measuring the antioxidative properties of several phytochemicals (Takebayashi *et al*., 2007). At the same time, the resistance of red cells to hemolysis exerted by both thyme extracts can be linked to their richness in polyphenols, including flavonoids.

Denaturation of proteins results from alterations in the chemical, biological, and physical characteristics of the protein by disruption of its structure (David Eckersall, 2008). Denaturation of protein leads to the production of autoantigens in conditions such as rheumatic arthritis, diabetes, and cancer which are inflammation conditions. Therefore, inhibition of protein denaturation can inhibit the inflammatory process (Dharmadeva *et al*., 2018). In the present study, *T. atlanticus* and *T. willdenowii* extracts showed anti-inflammatory activity by inhibiting protein denaturation induced by heat treatment. Moreover, several studies have reported that other thyme species exert important anti-inflammatory activities (Lunin & Novoselova, 2010; Shallangwa *et al*., 2016; Ustuner *et al.*, 2019), which makes the plants of this genus an important source of anti-inflammatory compounds.

T. atlanticus and *T. willdenowii* exhibited an important HMG-CoA reductase inhibiting activity. This enzyme is the rate-limiting factor of the mevalonate pathway, responsible for cholesterol biosynthesis. Furthermore, numerous lipid-lowering drugs are used to prevent coronary heart disease by inhibiting HMG-CoA reductase, such as statins. However, these chemical drugs can lead to serious side effects (Sultan *et al.*, 2019). Consequently, using natural extracts to treat and prevent cholesterol-related diseases is a better alternative. Moreover, *Thymus* species possess numerous pharmacological properties, including hypocholesterolemic effects. In a previous study, Ramchoun *et al.,* (2020) reported that *T. atlanticus* lowered cholesterol levels in high-fat diet-fed hamsters. This study showed that the extract of this thyme significantly lowered total cholesterol levels and HMG-CoA reductase gene expression in high-fat-diet fed animals (Ramchoun *et al*., 2020). This study and our study approve that this thyme species can lower cholesterol levels by decreasing the gene expression of HMG-CoA reductase and inhibiting its activity.

The richness of thyme species in phenolic acids and flavonoids can explain their potent biological properties. Flavonoids are important polyphenolic compounds with significant antioxidant properties (Heim *et al.,* 2002). These bioactive compounds have demonstrated in many studies that they have important *in vitro* anti-inflammatory (Rauf *et al*., 2015; Ruiz-Ruiz

et al., 2017; Tasleem & Imam, 2017) and antihemolytic (Naqinezhad *et al*., 2012; Ramchoun *et al*., 2015) activities.

5. CONCLUSION

In this present study, antioxidant, antihemolytic, anti-inflammatory, and hypolipidemic properties of polyphenolic extract of *T. willdenowii* and *T. atlanticus* were carried out using *in vitro* methods. The results showed that these two species have numerous phenolic compounds, including flavonoids. These thyme species had considerable antioxidant, anti-inflammatory, antihemolytic, and hypolipidemic properties. The findings of the present study, along with other previous scientific investigations, confirm that *Thymus* species are an important natural producer of bioactive phenolics with a wide variety of biological properties and applications.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Elbouny Hamza and **Ouahzizi Brahim**: Investigation, resources, visualization, software, formal analysis, and writing -original draft. **Sellam Khalid** and **Alem Chakib**: Supervision, validation, and editing.

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