Anti-inflammatory, anticoagulant and antioxidant effects of aqueous extracts from Moroccan thyme varieties

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ABSTRACT

Objective: To evaluate the anti-inflammatory, anticoagulant and antioxidant effects of aqueous extracts of thyme varieties from Moroccan.
Methods: The aqueous extracts of tree medicinal plants [*Thymus atlanticus* (*T. atlanticus*), *Thymus satureioides* and *Thymus zygis* (*T. zygis*)] were screened for their antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl radical-scavenging, ferric reducing antioxidant power assay, radical scavenging activity method, the inhibition of 2,2'-azobis (2-amidinopropane) dihydrochloride that induces oxidative erythrocyte hemolysis and thiobarbituric acid reactive substances assay. The anti-inflammatory activity of aqueous extracts was evaluated *in vivo* using croton oil-induced ear edema and carrageenan-induced paw edema in mice and rats, respectively. This extracts were evaluated *in vitro* for their anticoagulant activity at the different concentrations by partial thromboplastin time and prothrombin time activated.
Results: All thyme varieties were found to possess considerable antioxidant activity and potent anti-inflammatory activity in the croton oil-induced edema. Administration of aqueous extracts of two varieties (50 mg/kg) (*T. zygis* and *T. atlanticus*) reduced significantly the carrageenan-induced paw edema similar to non-steroidal anti-inflammatory drug (indomethacin, 10 mg/kg). In partial thromboplastin time and prothrombin time tests, *T. atlanticus* and *T. zygis* extracts showed the strongest anticoagulant activity. In contrast, *Thymus satureioides* did not show the anticoagulant activity in these tests.
Conclusions: All aqueous extracts possess considerable antioxidant activity and are rich in total polyphenol and flavonoid but they act differently in the process of inflammatory and coagulation studied. This study shows great variability of biological activities in thyme varieties.

1. Introduction

Inflammation is closely linked with other physiological systems including the coagulation-fibronolytic system and oxidant-antioxidant pathways [1]. Physiologically, the activation of inflammation and the coagulation system in acute trauma is recognized as a physiologic reaction to initiate healing and to act as a barrier to injury propagation and infection [2]. Systemic inflammation will invariably lead to activation of the coagulation system, but vice versa, components of the coagulation system can markedly modulate the inflammatory response [3]. Pathophysiologically, inflammation and the coagulation system play crucial roles in the pathogenesis of multiple chronic inflammatory disorders [4].

Physiological concentrations of free radicals are required to mediate physiological processes such as inflammatory reaction [5]. Indeed, during inflammation, cells of the immune system are recruited to the site of damage. This results in respiratory burst, an overproduction of reactive oxygen species that can propagate inflammation by stimulating release of cytokines [6] and cause

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oxidative damage to bimolecular constituents in the body which eventually leads to chronic diseases, such as atherosclerosis, cancer, diabetes, aging and other degenerative diseases [7,8].

Despite their incontestable efficacy in the treatment of venous thromboembolic and inflammatory diseases, the current treatments of anticoagulants and the anti-inflammatory used for several decades, present many limitations [9,10]. Side effects related to non-steroidal anti-inflammatory drugs and heparins, administered only by injection, have been extensively documented by numerous clinical trials [10,11]. There is a need therefore to discover new anti-inflammatory and anticoagulants which will present no undesirable effects [9,12]. Medicinal plants are believed to be an important source for the discovery of potential anti-inflammatory and anti-coagulant substances. Several plant extracts and different classes of phytochemicals have been investigated and shown potential anti-inflammatory and anticoagulant activity [9,12].

The genus *Thymus* L. (Lamiaceae), aromatic plants of the Mediterranean flora, economically important due to their use in folk medicine for their numerous medicinal and aromatic properties, have been reported to possess various biological effects including antioxidant and antimicrobial activities [13]. Many works showed that some *Thymus* species possess anti-inflammatory activity but to our knowledge, no study has investigated the effect of these plants on the coagulation.

Thymus satureioides (*T. satureioides*), *Thymus atlanticus* (*T. atlanticus*) and *Thymus zygis* (*T. zygis*) are an endemic species of Morocco [14] used in the folk medicine in form of infuse and decoctions to treat whooping cough, bronchitis and rheumatism and, generally, for its anti-inflammatory properties after topical or oral administration [15]. Recently, Ramchoun *et al.*, analyzed the aqueous extracts of these plants for their hypocholesterolemic and antioxidant activities and concluded that all extracts possess considerable antioxidant activities but only the *T. atlanticus* extract exhibits a potent hypolipidemic capacity [14]. Indeed in the present work we confirm, for the first time, previous established works by our group Ramchoun *et al.*, on the antioxidative stress activities of such plant extracts by lipid peroxidation based on the thiobarbituric acid reactive substances (TBARS) assay [14]. Moreover, the extracts were evaluated *in vivo* for their anti-inflammatory activity in two animal models according to Tubaro *et al.* [16] and Winter *et al.* [17] in mice and rats, respectively and *in vitro* for their anticoagulant activity at the different concentrations by activated partial thromboplastin time (APTT) and prothrombin time (PT).

2. Materials and methods

2.1. Plant material

Thyme varieties were collected in April–May 2014 in the Errachidia Region, Morocco. They were identified by Dr. Ibn Tatou and voucher specimens were deposited at the herbarium of the Scientific Institute, University Mohammed V, Rabat, Morocco. *T. satureioides* Cosson (No: RAB 77497), *T. zygis* subsp. *gracilis* (Boiss.) R. Morales (No: RAB 77494) and *T. atlanticus* (Ball) Roussine (No: RAB 77496) were screened for this study.

2.2. Animals

Male Wistar rats weighing 150–200 g and male wistar mice weighing 20–30 g were used in this study. They were obtained from the animal facility of the Biology Department (Faculty of Sciences, Errachidia, Morocco) in accordance with international guidelines [18]. They were allowed free access to standard dry pellet diet and given water *ad labium*. The animals were grouped and housed in appropriate cages at room temperature of (22 ± 2) °C.

2.3. Biochemical analysis of aqueous thyme extracts

2.3.1. Chemicals and drugs

All solvents used were obtained from Sigma Chemical Co.: croton oil, indomethacin, carrageenan, Folin-Ciocalteu, caffeic acid, rutin, 1,1-diphenyl-2-picrylhydrazil (DPPH), trolox, tri-pyridyltriazine (TPTZ), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and triton WR-1339.

2.3.2. Preparation of thyme extracts for biochemical and antioxidant analysis

The plant material was dried at room temperature and powdered (60 g) and extracted with 400 mL of distilled water in a Soxhlet extractor for 16 h. The extracts were filtered and evaporated by rotary vapor. The yields of extraction were 10%, 10% and 7.6% for *T. satureioides*, *T. zygis* and *T. atlanticus* respectively.

2.3.3. High-performance liquid chromatography (HPLC)

The aqueous thyme extracts was analyzed by HPLC using a Reprosil Pur C18 column equipped with a photodiode array detector. Analysis was performed on a C18 analytical column (250 mm × 3 mm) with a particle size of 5 μm thermostated at 28 °C. Extract (100 μL) was separated at 28 °C. The flow rate was 0.5 mL/min and the absorbance changes were monitored at 215, 250 and 280 nm. The solvents for chromatographic analysis were: (A) methanol/water (20/80) + 0.2% glacial acetic acid and (B) methanol/water (80/20) + 0.2% glacial acetic acid [100% (A) and 0% (B) at 0 min, 50% (A) and 50% (B) during 10 min, 17% (A) and 83% (B) during 20 min, which was changed to 100% (A) and 0% (B) in 5 min (35 min, total time)]. The retention time of standards and the corresponding UV spectra were used for identification of the compounds in thyme extracts.

2.3.4. Determination of total polyphenol content

The aqueous extracts of thyme was analyzed for its phenolic content according to the Folin-Ciocalteu colorimetric method [19]. Aqueous extracts (30 μL, three replicates), 4.22 mL of distilled water, 250 μL of the Folin-Ciocalteau reagent and 500 μL of 10% sodium carbonate were introduced into the test tubes. The tubes were mixed and allowed to stand for 30 min. The absorbance was measured at 725 nm in a spectrophotometer and result of phenolic content was expressed as caffeic acid equivalents (μg/mg extract, mean ± SEM of three determinations).

2.3.5. Determination of flavonoid contents

The flavonoid contents of the extracts were determined spectrophotometrically according to the procedure established by Jay *et al.* [20]. This method is based on the formation of a flavonoid–aluminum complex, having its maximum absorption at 430 nm. The reagent of $AlCl_3$ is prepared by dissolving 133 mg of $AlCl_3$ in 100 mL of a mixture solvent of methanol: water: acetic acid (70:25:5, v/v/v). Aqueous extract (1 mL)

and reagent $AlCl_3$ (0.5 mL) were introduced in the test tube. The tube was mixed, allowed to stand for 30 min and measured at 430 nm in a spectrophotometer. The result of flavonoid contents were expressed in mg per g of rutin equivalents.

2.4. Antioxidant study of thyme extracts

2.4.1. DPPH radical scavenging activity assay

The method widely used for a relatively rapid evaluation of antioxidant activities was based on the principle of reduction of a stable radical. DPPH is an unstable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule having a specific absorption at 517 nm which it gives a violet color [21]. Reducing DPPH by an antioxidant is accompanied by the transition from purple to yellow color of the solution, measured by spectrophotometer. Trolox as DPPH-scavenging compound was used as positive control. The antioxidant activity was estimated by percentage inhibition or percentage of antioxidant activity according to the following formula:

$$\text{Antioxidant activity}(\%) = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{test}}}{\text{Abs}_{\text{control}} \times 100}$$

where Abs is the absorbance at a wavelength of 517 nm.

2.4.2. Ferric reducing antioxidant power (FRAP) assay

The method is based on the reduction of the Fe^{3+}-TPTZ complex to the ferrous form at low pH. The antioxidant activity of the thyme extracts was measured by monitoring the change in absorption at 593 nm. FRAP solution consists of acetate buffer (300 mmol/L) at pH = 3.6, TPTZ and $FeCl_3 \times 6\ H_2O$ (20 mmol/L) (10v/1v/1v). According to Benzie and Strain's method, 10 μL of the plant extract (0.5%) was added to the FRAP solution (2 mL), and allowed to stand for 15 min and the absorbance was measured at 593 nm with a spectrophotometer [22]. The result of antioxidant power was expressed as trolox equivalents (μg/mg extract, mean ± SEM of three determinations).

2.4.3. TBARS assay

The malondialdehyde (MDA) or TBARS assay was used to measure the potential antioxidant capacity of thyme extracts, using a rich plasma lipid that is taken from mice treated with triton WR-1339 (600 mg/kg) according to the procedure established by Park et al. [23]. The TBARS assay quantifies oxidative stress by measuring the peroxidative damage to lipids that occurs with free radical generation. Free radical damage to lipids results in the production of MDA, which reacts with thiobarbituric acid generating a chromogen that can be measured spectrophotometrically. Rich lipid plasma was used to make different samples [control sample: rich lipid plasma + phosphate buffer saline (PBS); oxidation sample: rich lipid plasma + $CuSO_4$ (0.33 mg/mL); and plants extract sample: rich lipid plasma + $CuSO_4$ + plant extract (25, 50 or 100 mg/mL)].

The absorbance of the red colored phase was measured in a spectrophotometer at 532 nm. The quantity of TBARS was calculated from a standard range of 1, 1, 3, 3, tetramethoxypropane.

2.4.4. Hemolytic activity and protection against AAPH-induced hemolysis

The hemolysis of erythrocytes were extensively used as an *ex vivo* model for studying reactive oxygen species-induced disruption of cell membranes and the protective effect of antioxidants. One of the most frequently studied models is the water-soluble azo compound (AAPH) that used for evaluation of the antioxidant activity of the plant extract described by Prost with modified [24]. The incubation of erythrocytes with AAPH provoked the production of the peroxyl radical generated by decomposition of AAPH in the presence of molecule oxygen at 37 °C. A rabbit erythrocyte suspension in PBS was used to make different samples [control sample: erythrocyte suspension in PBS; hemolysis sample: erythrocyte suspension in PBS + AAPH; and plants extract sample: erythrocyte suspensions in PBS + AAPH + plant extract (0.143 mg/mL)]. The erythrocyte suspension was mixed with 1 mL of thyme extracts diluted with PBS. AAPH and PBS were then added to the mixture. The reaction mixture was gently shaken during incubation at 37 °C and the absorbance was read at 540 nm every 5 min. The protection of the erythrocytes of the extracts was deduced from the time required for half-hemolysis (50% reduction of A540 nm) compared to control values (PBS, pH 7.4).

2.5. Croton oil-induced ear edema in mice

The anti-inflammatory activity was measured as inhibition of the croton oil-induced ear edema in mice according to the procedure established by Tubaro et al. with minor modifications [16]. Skin inflammation was induced to the inner surface of the right ear of mice (surface: about 1 cm^2) by applying 240 μg of croton oil dissolved in the same volume of 42% acetone/ethanol (v/v) [aqueous extracts (900 μg/ear), and the relevant controls]. The left ear remained untreated. The substances under testing were applied together with the croton oil, except for control animals which received only the irritant. The evolution of right ear edema was determined at 2, 4 and 10 h. As a reference, the non-steroidal anti-inflammatory drug indomethacin (500 μg/ear) was used. The anti-inflammatory activity was expressed as ear edema rate and inhibition rate of edema in treated mice with regard to control mice was calculated. The ear edema rate and inhibition rate of each group were calculated as follows:

Ear edema rate (%) = $V_r - V_l/V_l$

Inhibition rate (%) = $E_c - E_t/E_c$

where V_r is the right ear volume (treated ear) and V_l is the volume of the left ear (untreated ear); E_c is the ear edema rate of the control group and E_t is the ear edema rate of the treated group.

2.6. Carrageenan-induced pleurisy in rats

According to the described method of Winter et al. [17], 30 rats equally were divided into five groups of six; they were fasted for 16 h for experimentation. The initial volume (V_0) of the right hind paw was measured for each rat. The rats were orally treated with indomethacin as standard anti-inflammatory drug (10 mg/kg) (reference group) with PBS (control) or aqueous thyme extracts (50 mg/kg). After 1 h of treatment, the inflammation was inducted in rats by a subplantar injection of 0.1 mL of carrageenan suspension (100 μg/paw). The paw volumes were determined hourly using a plethysmometer (Ugo

Basile, Italy) for 24 h. The paw edema rate and inhibition rate of each group were calculated as follows:

$$\text{Paw edema rate (\%)} = V_t - V_0/V_0$$

$$\text{Inhibition rate (\%)} = E_c - E_t/E_c$$

where V_t is the paw volume of the rat after carrageenan injection and V_0 is the paw volume of the rat before carrageenin injection; E_c is the edema rate of the control group and E_t is the edema rate of the treated group.

2.7. Anticoagulant assays

All experiments were carried out six times, and were measured automatically using coagulometer (Stago, STart 4). Two separate assays measuring APTT and PT were carried out to investigate the stage, at which extrinsic and intrinsic blood clotting pathways [25]. The anticoagulant activity of the series of the tested aqueous thyme extracts at different concentrations was expressed in seconds, as clotting time measured in APTT and PT tests. The following concentrations of extracts were used in the clotting mixtures: 11.43, 5.71, 2.86, 1.43, 0.71, 0.36 and 0.18 mg/mL.

Blood samples were collected by cardiac puncture with syringe from healthy rats in tubes containing 3.8% trisodium citrate in a polypropylene container (9 parts of blood to 1 part of trisodium citrate solution). It was immediately centrifuged at 25 000 r/min for 10 min, and plasma was separated and pooled. The freshly prepared plasma was stored at 4 °C until its use.

For APTT assay, normal citrated rat plasma (50 μL) was mixed with a solution of a plant extracts (25 μL) and incubated for 10 min at 37 °C, then APTT reagent (C.K. Prest® provided by Diagnostica Stago) (50 μL) was added to the mixture and incubated for 5 min at 37 °C. Thereafter clotting was induced by adding 0.025 mol/L CaCl₂ (50 μL) and clotting time was recorded. In PT assay, normal citrated rat plasma (50 μL) was mixed with a solution of plant extracts (25 μL) and incubated for 10 min. Then, PT reagent (Neoplastine® Cl provided by Diagnostica Stago) (100 μL), pre-incubated for 10 min at 37 °C was added and clotting time was recorded.

2.8. Statistical analysis

Statistical evaluation was carried out with StatView. Data were expressed as mean ± SEM. Statistical differences were evaluated by One-way ANOVA and the student's t-test. P values <0.05 were considered statistically significant.

3. Results

3.1. Biochemical analysis and antioxidant activities of thyme extract

3.1.1. HPLC analysis of aqueous thyme extracts

The analysis of the typical HPLC chromatogram (Figure 1) depicted that rosmarinic acid was present in all extracts at relatively high levels and was the major phenolic acid. Quercetin was the major flavonoid especially in *T. atlanticus*. The extracts of *T. satureioides* was particularly rich in luteolin-7-glycoside and hesperetin was detected only in this plant. Rosmarinic acid was expected to represent the single most antioxidative constituent (Table 1).
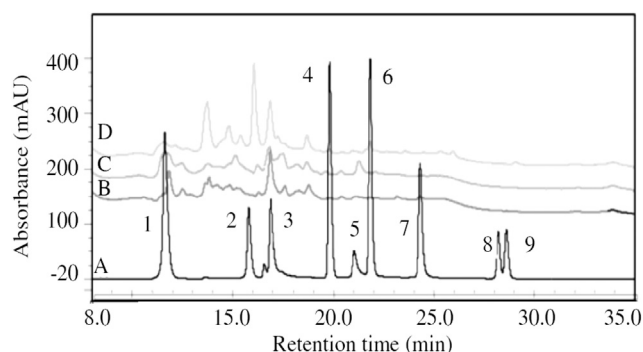

Figure 1. Comparison of the HPLC profiles from the tested *Thymus* extracts as detected at 280 nm.
A: Standards; B: *T. zygis*; C: *T. atlanticus*; D: *T. satureioides*; 1: Caffeic acid; 2: Luteolin-7-glycoside; 3: Rosmarinic acid; 4: Daidzein; 5: Quercetin; 6: Hesperetin; 7: Apigenin; 8: Thymol; 9: Carvacrol.

Table 1
Identified compounds and their retention times.

Plants	Identified compound	Retention time (min)
T. atlanticus	Caffeic acid	11.60
	Rosmarinic acid	17.00
	Quercetin	21.20
T. zygis	Caffeic acid	11.60
	Rosmarinic acid	17.00
T. satureioides	NI	13.70
	Luteolin-7-glycoside	15.96
	Rosmarinic acid	17.00
	Hesperetin	22.00

NI: No identified compound.

3.1.2. Determination of total phenols and flavonoid

Thyme aqueous extracts were analysed for its phenolic content by using Folin-Ciocalteu reagent. Folin-Ciocalteu reagent reacted nonspecifically with phenolic compounds; it also reduced a number of non-phenolic compounds. From the results summarized in Table 2, we can easily concluded that all three varieties are rich in polyphenol and flavonoid. The total polyphenol contents were between (475.00 ± 8.30) and (495.47 ± 6.10) mg equivalent caffeic acid/g of extract. The flavonoid contents were between (155.11 ± 3.90) and (208.13 ± 4.20) mg equivalent rutin/g of extract. Several aqueous extracts displayed potent activity against both DPPH and FRAP (Table 2). The IC_{50} values from three thyme varieties were between (0.12 ± 0.02) and (0.44 ± 0.02) mg/mL of extract. The FRAP assay showed that the antioxidant activity was between (40.02 ± 6.10) and (65.00 ± 9.40) mmol trolox/g of extract.

3.2. Determination of MDA by TBARS

The MDA levels determined by TBARS method for three concentrations of aqueous extracts (25, 50 and 100 mg/mL) were given in Figure 2. The quantity of TBARS was calculated from a standard range of 1, 1, 3, 3, tetramethoxypropane. The result of TBARS levels was expressed as MDA equivalents (MDA nmol/L, mean ± SEM of three determinations). The results showed that the aqueous extracts of thyme were very significantly inhibited the formation of MDA compared to positive control. The amount of MDA observed in the

Table 2

Polyphenol content and antioxidant activity of thyme aqueous extracts from the three thyme varieties.

Extracts	Polyphenols (mg equivalent caffeic acid/g TAE)	Flavonoid (mg equivalent rutin/g TAE)	Radical scavenging activity [IC_{50} (mg/mL TAE)]	FRAP (mmol trolox/g TAE)
T. zygis	482.92 ± 5.60	208.13 ± 4.20	0.44 ± 0.02[*]	65.00 ± 9.40
T. atlanticus	495.47 ± 6.10	155.11 ± 3.90	0.12 ± 0.02[**]	40.02 ± 6.10
T. satureioides	475.00 ± 8.30	182.79 ± 3.23	0.44 ± 0.01[*]	40.14 ± 4.55
Trolox	–	–	0.51 ± 0.01	44.33 ± 7.55

[*]: $P < 0.01$; [**]: $P < 0.001$; –: Absent; TAE: thyme aqueous extracts.
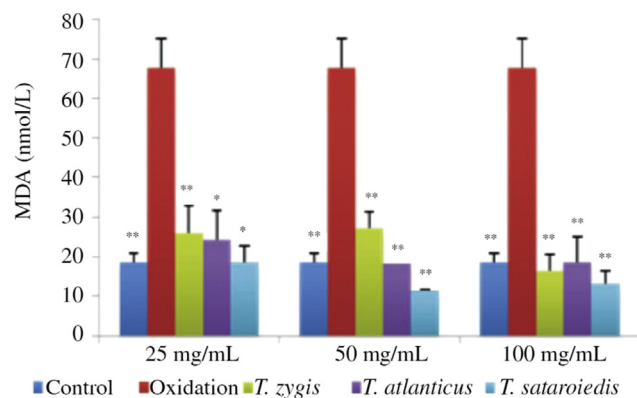
Figure 2. Effects of different concentrations of aqueous extracts of thyme on lipid peroxidation product (MDA).
Results are expressed as mean ± SEM of three separate experiments; [*]: $P < 0.01$; [**]: $P < 0.001$.

groups containing the aqueous extracts is similar to that observed in the negative control ($P > 0.05$). This indicated the significant lipid peroxidation inhibiting activity of thyme varieties.

3.3. Hemolytic activity and protection against AAPH-induced hemolysis

Table 3 shows the inhibition percentage of hemolysis, as a result of protection against the oxidative damage of cell membranes of erythrocytes from rat, induced by AAPH. Addition of AAPH induced a significant decrease in the half-time of hemolysis from (73.33 ± 2.88) min to (40.00 ± 0.05) min (45%, $P < 0.001$). Application of the aqueous extract from the three varieties to the erythrocytes suspension with AAPH induced an increase of half-time of hemolysis by 550% ($P < 0.001$), 470% ($P < 0.001$), and 305% ($P < 0.001$) from T. satureioides, T. atlanticus, and T. zygis, respectively. Trolox was used as standard antioxidant that showed an increase of half-time of hemolysis by 525% ($P < 0.001$). These results indicated that the

Table 3

Antihemolytic activity of aqueous extracts of thyme.

Groups	Hemolysis half-time (min)	Deviation (%)
Control	73.33 ± 2.88	
AAPH sample (%)	40.00 ± 0.05[*]	−45
AAPH + T. atlanticus	228.33 ± 11.54[*]	470
AAPH + T. zygis	162.00 ± 35.38[*]	305
AAPH + T. satureioides	260.00 ± 17.32[*]	550
AAPH + trolox	250.00 ± 13.26[*]	525

[*]: $P < 0.001$; AAPH sample vs control; T. atlanticus, T. zygis, T. satureioides and Trolox.

aqueous thyme extracts had a greater protective effect against hemolysis of erythrocytes.

3.4. Croton oil-induced mice ear edema

The anti-inflammatory activities of the all thyme aqueous extracts were also tested using the croton oil-induced mice ear edema model. Topical application of croton oil on the ears of mice caused a significant increase in the volume of the right ear compared with the left ear (47.39% ± 0.50% at 8 h after the treatment) (Table 4). In comparison with the non-steroidal anti-inflammatory drug indomethacin, aqueous extracts of three medicinal plants presented stronger and effective anti-inflammatory activity in the experimental animal model used. The ear edema was significantly reduced after indomethacin treatment (500 μg/ear) by 67.27%. Moreover, at 8 h after the treatment, aqueous extract significantly reduced the edema volume in comparison with control by 70.47%, 84.62% and 29.66% at 900 μg/ear for T. zygis, T. atlanticus and T. satureioides respectively.

3.5. Carrageenan-induced rat paw edema

The anti-inflammatory activities of aqueous extracts were further evaluated by the inhibition of carrageenan-induced hind paw edema in rats (Figure 3). After 5 h of treatment, compared to the control (27.95% ± 0.06%), T. zygis and T. atlanticus (50 mg/kg) significantly reduced the carrageenan–induced paw oedma volume (3.74% ± 0.01% and 9.52% ± 0.04% for T. zygis and T. atlanticus, respectively) ($P < 0.001$) and their effect was comparable to the reference drug indometacin (10 mg/kg) (10.92% ± 0.06%) ($P > 0.05$) (Table 5). The aqueous extract of T. zygis and T. atlanticus were found to possess potent anti-inflammatory activity in the carrageenin-induced edema.

3.6. Anticoagulant activity

In our experiment, APTT and PT were used to evaluate the anticoagulant effect of aqueous extract. Blood samples were collected by cardiac puncture with syringe from healthy rats. In APTT test, T. atlanticus and T. zygis extracts showed the strongest anticoagulant activity (Table 6). They completely inhibited the plasma clot formation in the concentrations of 2.86 and 5.72 mg/mL in the clotting mixtures, respectively, and both strongly prolonged time of clotting still was the concentration of 0.18 mg/mL in the clotting mixtures ($P < 0.01$).

In PT test, T. atlanticus and T. zygis showed the strongest anticoagulant activity (Table 7). These plants completely inhibited clotting process at the concentrations of 5.72 and 11.43 mg/mL in clotting mixtures for T. atlanticus and T. zygis

Table 4

Anti-inflammatory activity of aqueous extracts of thyme in croton oil-induced mice ear edema.

Groups	Ear edema rate (%)				
	2 h	4 h	6 h	8 h	10 h
Control	28.89 ± 0.75	41.31 ± 0.79	41.30 ± 0.60	47.39 ± 0.50	37.32 ± 0.67
T. zygis (900 µg/ear)	9.67 ± 0.55**	17.11 ± 0.54	24.85 ± 1.40***	13.99 ± 0.54***	9.67 ± 0.55***
T. atlanticus (900 µg/ear)	22.68 ± 1.68**	26.85 ± 0.98**	18.52 ± 0.71	7.29 ± 0.61***	4.17 ± 0.65
T. satureioide (900 µg/ear)	11.04 ± 0.27	23.05 ± 0.48	40.13 ± 1.28	33.33 ± 1.36**	41.86 ± 0.22**
Indometacin (500 µg/ear)	34.02 ± 0.95	33.33 ± 1.36**	17.59 ± 0.72***	15.51 ± 0.64***	11.57 ± 0.07***

The animals were treated with aqueous extract of *T. zygis*, *T. atlanticus* or *T. satureioides* at 900 µg/ear. Indomethacin was used as a positive control (500 µL/ear). The differences between treatment and control were tested using ANOVA. Values are expressed as mean ± SEM, ($n = 6$). **: $P < 0.01$ compared to control; ***: $P < 0.001$ compared to control.
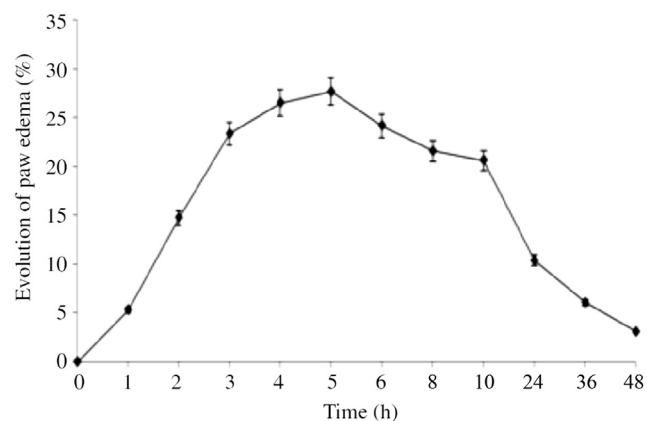

Figure 3. Evolution of edema formation in rat paws (control).
The animals ($n = 6$) were treated with distillated water. Data obtained from animal experiments were expressed as mean ± SEM.

Table 6

APTT measurements of aqueous extracts made *in vitro* experiments in rat pooled plasma.

Concentrations of a sample in the clotting mixture (mg/mL)	*In vitro* APTT measurements (second)		
	T. zygis	*T. atlanticus*	*T. satureioides*
11.43	>900***	>900***	ND
5.71	>900***	>900***	ND
2.86	266.07 ± 7.98***	>900***	ND
1.43	47.87 ± 3.13**	68.97 ± 1.61***	ND
0.71	23.80 ± 0.56*	29.04 ± 0.60***	13.23 ± 0.49*
0.36	19.97 ± 0.23**	19.64 ± 0.11***	14.07 ± 0.19**
0.18	17.07 ± 0.36*	17.5 ± 0.26**	14.97 ± 0.05
Negative control	15.37 ± 0.14	15.37 ± 0.14	15.37 ± 0.14

Values are expressed as mean ± SEM of 6 measurements. ND: Not determined; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$. *T. zygis*, *T. atlanticus* and *T. satureioides* vs negative control.

Table 5

Anti-inflammatory activity of aqueous extracts of thyme in carrageenan-induced rats paw edema.

Groups	Paw edema rate (%)								
	1 h	2 h	3 h	4 h	5 h	6 h	8 h	10 h	24 h
Control	5.35 ± 0.01	14.86 ± 0.03	23.53 ± 0.04	26.75 ± 0.06	27.95 ± 0.06	24.39 ± 0.07	21.93 ± 0.08	20.89 ± 0.07	10.55 ± 0.04
T. zygis 50 mg/kg	2.96 ± 0.01	4.14 ± 0.10**	5.10 ± 0.01**	4.68 ± 0.02**	3.74 ± 0.01**	3.12 ± 0.02**	2.56 ± 0.02*	2.20 ± 0.03**	1.97 ± 0.02*
T. atlanticus 50 mg/kg	8.06 ± 0.03	9.10 ± 0.03	10.40 ± 0.03**	9.52 ± 0.04**	9.52 ± 0.04**	9.23 ± 0.04*	6.98 ± 0.03*	6.52 ± 0.03*	5.62 ± 0.03
T. satureioides 50 mg/kg	5.13 ± 0.01	11.54 ± 0.03	13.69 ± 0.03**	17.28 ± 0.03	22.26 ± 0.03	22.21 ± 0.03	21.29 ± 0.03	20.58 ± 0.03	5.14 ± 0.01
Indometacin 50 mg/kg	6.23 ± 0.02	10.90 ± 0.02	11.50 ± 0.02**	11.61 ± 0.03**	10.92 ± 0.06**	10.52 ± 0.05*	8.70 ± 0.05	7.45 ± 0.04*	4.87 ± 0.04

The animals were treated with aqueous extract of *T. zygis*, *T. atlanticus* or *T. satureioides* at 50 mg/kg/ear. Indomethacin was used as a positive control (10 mg/kg). The differences between treatment and control were tested using ANOVA. Values are expressed as mean ± SEM, ($n = 6$). *: $P < 0.05$ compared to control; **: $P < 0.01$ compared to control; ***: $P < 0.001$ compared to control.

Table 7

PT measurements of aqueous extracts made *in vitro* experiments in rat pooled plasma.

Concentrations of a sample in the clotting mixture (mg/mL)	*In vitro* PT measurements (second)		
	T. zygis	*T. atlanticus*	*T. satureioides*
11.43	>300[***]	>300[***]	ND
5.71	99.13 ± 1.47[***]	>300[***]	ND
2.86	27.27 ± 0.63[***]	55.73 ± 1.98[*]	ND
1.43	15.93 ± 0.55	20.40 ± 0.82[**]	13.27 ± 0.40
0.71	13.63 ± 0.37[*]	15.67 ± 0.31	13.87 ± 0.85
0.36	13.17 ± 0.14[*]	13.93 ± 0.14	14.57 ± 0.19
Negative control	14.67 ± 0.44	14.67 ± 0.44	14.67 ± 0.44

Values are expressed as mean ± SEM of 6 measurements. ND: Not determined; [*]: $P < 0.05$; [**]: $P < 0.01$; [***]: $P < 0.001$. *T. zygis*, *T. atlanticus* and *T. satureioides* vs negative control.

respectively and strongly prolonged the process even at the concentrations of 1.43 and 2.86 mg/mL in clotting mixtures for *T. atlanticus* ($P < 0.01$) and *T. zygis* ($P < 0.001$) respectively.

4. Discussion

The selection of the three species from the *Thymus* genus was based on their traditional use for its anti-inflammatory properties after topical or oral administration for the treatment of various inflammatory diseases such as bronchitis and rheumatism. These aromatic plants produce a large amount of secondary metabolites (*e.g.* phenol compounds) that are able to exert beneficial effects for human well-being [26]. Considering the involvement of free radicals in inflammation [27] and the relationship between inflammation and the coagulation systems [28], the current study aimed at analyzing the aqueous extracts of three endemic medicinal plants (*T. satureioides*, *T. zygis* and *T. atlanticus*) for their antioxidant, anti-inflammatory and anti-coagulant activities. The results demonstrated that the three varieties of thyme originating from the Errachidia area are rich in total polyphenol and flavonoid with rosmarinic acid which are the major polyphenol compounds. Evaluation of antioxidant activity for this extracts by different test shows that it possess a high antioxidant. AAPH is a peroxy radical initiator that generates free radicals by its thermal decomposition and will protect the erythrocytes to induce the chain oxidation of lipid and protein, disturb the membrane organization and eventually lead to hemolysis. In this study, the protective effect of thyme extracts on hemolysis by peroxyl radical scavenging activity was investigated. The results indicated that the aqueous thyme extracts had a greater protective effect against hemolysis of erythrocytes.

Croton oil induced ear edema is widely accepted as a useful pharmacological model for the investigation of anti-inflammatory effects. Its application of croton oil can induce significant inflammatory responses, as characterized by edema, neutrophil infiltration, prostaglandins production and increases in vascular permeability [29]. In summary, aqueous extracts of these medicinal plants present a potent anti-inflammatory activity. 12-O-tetradecanoylphorbol-13-acetate and other phorbol esters are the primary irritants in croton oil. It has been reported that cyclo-oxygenase inhibitors and 5-lipoxygenase inhibitors are highly effective against inflammation caused by 12-O-

tetradecanoylphorbol-13-acetate [30]. Also, polyphenol and flavonoid such as caffeic acid, quercetin, luteolin, have been recognized as potent inhibitors of cyclo-oxygenase in different studies [31–33]. These reports, together with our results, suggest a possible relationship between the protective effects of aqueous extracts in an acute inflammatory animal model and the rich content of polyphenol in these extracts.

With the goal of proving the anti-inflammatory property of three thymus, we evaluated the effects of aqueous extracts of thyme on the carrageenan induced paw edema in rat. This method was chosen for this study since it is the most prominent experimental model in search for new anti-inflammatory drugs and evaluation of anti-inflammatory effect of natural products [34–36]. We found that the administration of aqueous extracts of two *Thymus* (*T. zygis* and *T. atlanticus*) significantly reduced the carrageenan-induced paw edema. The evolution of paw edema volume in control group is followed at 48 h; the maximum volume of the edema is at 5 h after treatment. The injection of carrageenan in rat produces a typical biphasic edema associated with the production of several inflammatory mediators such as histamine, serotonin, bradykinin, prostaglandins, nitric oxide and cytokines [17,35]. It has been demonstrated that a different profile of inflammatory mediators involved with the first and second phases of the carrageenan induce paw edema in mice [37] and demonstrated that treatment with the COX-1 inhibitor reduce the early phase of paw edema. Moreover, COX-2 is up-regulated only in the second phase [35]. According to the result of our study, aqueous extract of *T. zygis* and *T. atlanticus* was able to effectively inhibit the edema in both the earlier and in the later phases, suggesting that extracts inhibit different chemical mediators of inflammation.

The pre-clinical evaluation of the antithrombotic potential of novel molecules requires the use of reliable and reproducible experimental models. PT, APTT, fibrin polymerization and platelet aggregation are the most commonly used preparations to determine the efficacy of novel antithrombotic drugs [38]. In our experiment, APTT and PT using rat plasma were used to evaluate the anticoagulant effect of aqueous extract. The APTT assay was used to determine the effects of aqueous extract on intrinsic factors such as II, V, VIII, IX, XI and XII and/or common pathways. The prolongation of the APTT was indicative of the inhibition of the intrinsic factors and/or the common pathways. PT evaluates the extrinsic and/or common pathway of the coagulation cascade. The results of the APTT and PT assay showed that aqueous extract of *T. zygis* and *T. atlanticus* had prolonged coagulation times compared with the control sample treated with PBS, suggesting that extracts inhibited the common pathways. However, only *T. satureioides* did not have significant effect in these tests.

There is an evidence that inflammation and coagulation are intricately related processes that may considerably affect each other [28]. Systemic inflammation will invariably lead to activate the coagulation system, but vice versa, components of the coagulation system may markedly modulate the inflammatory response [3]. On the basis of our current experimental studies, it can be hypothesized that inhibitory modulation of coagulation by extracts could give promising anti-inflammatory mediators. Well-designed prospective studies are needed to prove this hypothesis. These anti-inflammatory and anticoagulant effects have been mostly attributed to the polyphenol and flavonoid compounds found in large quantities in these plants [29,34].

Rosmarinic acid is the most abundant caffeic dimer in this species thymus. Recently, Rocha *et al.* showed that acid rosmarinic possesses the high inflammatory effect evaluated by different test *in vitro* and *in vivo* including carrageenan-induced paw edema model [36]. All extracts possess the acid rosmarinic, but not shows the similar anti-inflammatory effect in different tests, suggesting the synergy between different compounds in these inflammatory processes.

This study shows great variability of biological activities in thyme varieties. All *Thymus* were found to possess considerable antioxidant activities and are rich in total polyphenol and flavonoid compounds but the three extracts act differently on the process of inflammatory and coagulation studied. The aqueous extracts of *T. atlanticus* and *T. zygis* exhibited significant anti-inflammatory activity at both early and late phases in acute and systemic inflammation. They act also on clotting and oxidative stress *in vitro* studied. The activity may be doing a cumulative of acid rosmarinic and other phytoconstituents. Thus, the present study scientifically validated the traditional use of aqueous extracts from some *Thymus* species in treatment of inflammatory diseases.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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