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CHEMICAL CHARACTERIZATION AND EVALUATION OF ANTIOXIDANT, ANTI-INFLAMMATORY AND ANTICOAGULANT ACTIVITY OF AQUEOUS EXTRACT AND ORGANIC FRACTIONS OF *THYMUS ZYGIS L. SUB SP. GRACILIS*

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ABSTRACT: This study was undertaken to evaluate the antioxidant, anti-inflammatory and anticoagulant activities of aqueous extract and organic fractions of *Thymus zygis*. Water, dichloromethane, ethyl acetate and methanol were used as extractant solvents. The fractions were analyzed by HPLC and screened for their antioxidant activity in vitro using different assay. *In vivo*, the fractions were evaluated by use of the oil croton-induced ear edema and carrageenan-induced paw edema model in mice and rat, respectively. Prothrombin Time and Partial Thromboplastin Time were used to determine the effect of aqueous extract, methanol, aqueous fractions and coumarone-rich extract on intrinsic, extrinsic and/or common pathway of the coagulation cascade. All fractions were found to possess considerable antioxidant activity and the rosmarinic acid is the major polyphenol compound in majority fractions. In comparison with the indometacin, the hydrophobic fractions presented stronger anti-inflammatory activity in the croton oil induced edema. Methanol and acetate ethyl fractions (50 mg/kg) significantly reduced the edema induced by carrageenan and their effect was comparable to the reference drug indometacin (10 mg/kg). In partial thromboplastin time and prothrombin time tests, all fractions tested showed the strongest anticoagulant activity.

INTRODUCTION: The genus Thymus (Labiatae), comprises more than 215 species, are perennial herbs and sub- shrubs adapted to the hot and dry climate of the Mediterranean region. In traditional medicine, thyme is used in the herbalist sector and as spice in several foods [1].

Generally, the genus Thymus is chemically characterized by its high content of polyphenol, which leads to it being regarded as an aromatic herb with potential health benefits [2].

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Thymus has become one of the most commercial genuses of plants for some countries because of the economical importance of its essential oil and its use as a condiment by herb-shops and the food industries [3]. In Morocco, the rate of endemism in this genus is 57%, representing 13 species [4]. Among them, *Thymus zygis*, also known as red thyme, is a small aromatic widespread endemic plant in the Mediterranean. Red thyme is commonly used in Erracidia area (south east of Morocco) as spice in several foods and traditionally as remedy for whooping cough, bronchitis, rheumatism and, generally, for its anti-inflammatory properties after topical or oral administration [5]. Physiopathologically, the crucial role was played of inflammation, coagulation and oxidant-antioxidant pathways in the pathogenesis of multiple chronic inflammatory disorders.

Growing researchers shows that coagulation system is in relationship with inflammatory process and the role of free radical to mediate inflammatory reaction [6, 7]. The excessive production of ROS by cells of the immune system during inflammation can propagate inflammation by stimulating release of cytokine [8] and may result in oxidative damage to many large biomolecules, such as lipids, DNA, and proteins [9].

Indeed, the current treatments anti-inflammatory used present many limitations. Side effects related to Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) have been extensively documented by numerous clinical trials. Long-term therapy with these medicines is often associated with gastrointestinal (GI), renal and haematological effects which reduce patient compliance [10].

For these reasons, the search for new antioxidant compounds, inhibitors of inflammation and coagulation, is of great medical importance due to their potential as potent medicament of inflammatory disease. Recently, the aqueous extract from the three thyme (*Thymus atlanticus, Thymus satureioides and Thymus zygis*) were evaluated for their antioxidant, anti-inflammatory and anticoagulant activities by our group Khouya et *al*., and the results showed that aqueous extract of *T. zygis* have a greet anti-inflammatory and anticoagulant activities [11]. The aim of this work was to identify the active fraction (s) of *T. zygis* using different polar and non-polar organic solvents as extractants and to determine the antioxidant capacities and the phenolic compounds for all fractions.

MATERIALS AND METHODS:

Plant material:

Aerial parts of *Thymus zygis* were collected in April-May 2015 in the Tafilalet region, Morocco. Voucher specimen was deposited at the herbarium of the Scientific Institute, University Mohammed V. Rabat, Morocco. *T. zygis L.* subsp. *gracilis* (Boiss.) R. Morales (No: RAB 77494). This plant was identified by Dr. Ibn Tatou.

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 [12]. 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.

Biochemical analysis of aqueous thyme extracts:

Chemicals and drugs:

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

Preparation of the crude aqueous *Thymus zygis* extract:

The aqueous extract from *T. zygis* was prepared by the same method as used by patients in folk medicine with some improvements. The dried powder from aerial parts (60 g) of the plant was epuised in a Soxhlet extractor for 4h, filtered and the solution obtained concentrated in a rotatory evaporator under vacuum at 60 °C. The yield of extract in terms of starting dried plant material was of 18% (w/w). The resulting aqueous extract was suspended in distilled water and the aliquots were stored at 20 °C before use.

Preparation of organic solvent extracts:

The 60 g of dried powder from aerial parts of *T. zygis* was extracted in a Soxhlet with n-hexane (C6H14) extractor for to degrease the plant material. The marc obtained was completely exhausted in increasing polarity order with deferent solvents: (CH2Cl2; polarity index P’ = 3.1), ethyl acetate (C4H8O2; P’ = 4.4), methanol (CH3OH; P’ = 5.1), and at the end, the marc was infused in distilled water (P’ = 10.2). Indeed, the obtained marc was air-dried and extracted with appropriate solvent for 16 h. Dichloromethane exhaustion and water infusion give the lipophilic and hydrophilic extracts respectively. Rotary evaporator was used to separate the solvent from the extract. The yields of extraction were 6, 6.5,10 and 4% for dichloromethane, ethyl acetate, methanol and aqueous fractions, respectively.

Preparation of coumarone-rich extracts:

Powdered dried leaves of *T. zygis* (1 g) were mixed with ethanol: water (1:1; v/v, 10 mL) and macerated under sonication, (water bath, room temperature, 30 min). The material was filtered and the crude extract obtained was suspended in distilled water at appropriate concentration. This procedure was repeated in triplicate. The comparison among the different extraction methods of coumarin by quantitative analysis by HPLC-UV showed that maceration under sonication had the best results, mainly considering the time/yield ratio [13].

High-performance liquid chromatography (HPLC):

The aqueous extract and organic fractions were 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 mm thermostated at 28 °C. Extract (100 mL) 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 aqueous extract and fractions.

Determination of *Thymus zygis* total polyphenol contents:

The polyphenol contents were determined according to the Folin Ciocalteu colorimetric method [14]; Caffeic acid was used to generate a calibration curve.

Antioxidant study of *Thymus zygis*:

Radical-scavenging activity (RSA) assay

The free radical-scavenging activity was evaluated by 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) according to the method reported by Barros [15]. 100 µl of diluted extract was mixed with 1.9 ml of DPPH methanolic solution (150 mM). The mixture was shaken vigorously and incubated for 30 min at 35°C. The absorbance of the samples was measured spectrophotometrically at 517nm. Trolox as DPPH-scavenging compound was used as positive control. The antiradical capacity of the studied extract was calculated using the following formula:

DPPH scavenging effect (%) =

$$[(A_0 - A_1)] \times 100\ A_0.$$

Where: A_0 is the absorbance of the control at 30 min, and A_1 is the absorbance of the sample at 30 min.

The antiradical activity was expressed as IC_{50}, which is the antiradical concentration required to cause 50% of inhibition. The IC_{50} was calculated by plot-ting inhibition percentages against concentrations of the sample. The experiment was repeated three times and the results were expressed as mean ± SD.

Ferric Reducing-Antioxidant Power (FRAP) assay:

To obtain the antioxidant capacity, the FRAP method was used as previously described [16]. FRAP solution consists of acetate buffer (300 mM) at pH = 3.6, TPTZ (tripyridyltriazine) and $FeCl_3$, 6 H_2O (20 mM) (10v/1v/1v). Briefly, a 2 mL aliquot of FRAP reagent was combined with 10 µL of each *T. zygis* extracts (0, 5 %), allowed to stand for 15 min and the absorbance measured at 593 nm by a spectrophotometer. The result of antioxidant power was expressed as Trolox equivalents (µg/mg extract, mean ± S.D. of three determinations).

Hemolytic activity and protection against AAPH-induced hemolysis.

The erythrocytes hemolysis was measured according to the method described by Prost modified [17]. The blood were obtained from a rabbit and diluted with 10 mM of heparinized phosphate-buffered saline (PBS) solution at pH 7.4. Briefly, an erythrocytes suspension was incubated with PBS (control), or pre-incubated with Trolox (used as antioxidant standard) and *T. zygis* extracts diluted with (PBS) at 37 °C, AAPH in PBS was then added to the mixture; the absorbance was read at 540 nm every 5 min. The protection of the erythrocytes by the extract and fractions was deduced from the time

required for half- hemolysis (50% reduction of A540 nm) compared to control values (PBS, pH 7.4).

Anti-inflammatory activity:

2Croton oil-induced mice ear edema:

According to the procedure established by Tubaro et *al.*, with minor modifications [18], the anti-inflammatory activity was measured as inhibition of the croton oil-induced ear edema in mice. 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) (crude and fraction 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 inflammatory drug (NSAID) indomethacin (500µg/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.

Carrageenan-induced rat paw edema:

For this study, standard drug used was indomethacin at 10 mg/kg, p.o. The rats were treated orally by gastric gavage. Rats in negative control group received 1 mL of DW while rats in treated groups received 50 mg/kg of aqueous extract and organic fractions.

After 60 min of extract administration, rats were injected with 1% carrageenan [carrageenan (1%) suspended in Phosphate Buffered Saline (PBS), pH: 7.4 into the plantar tissue of the right hind paw [19]. Paw volume was measured using a plethysmometer (Ugo Basile n°37140, Italy), 1 h after carrageenan injection, at hourly intervals up to 24 h.

The edema rate of each group was calculated as follows:

$$\text{Edema rate (E) \%} = V_t - V_0/V_0$$

Where V_t is the paw volume of the rat after carrageenan injection and V_0 is the paw volume of the rat before carrageenan injection at 't' h.

Anticoagulant activity:

Plasma sampling:

Blood samples were collected from healthy rats in tubes containing 3.8% tri-sodium citrate in a polypropylene container (9 parts of blood to 1 part of tri-sodium 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.

Partial Thromboplastin Time (PTT):

For Partial Thromboplastin Time (PTT) assay, citrated normal rat plasma (50 µl) was mixed with a solution of a plants extract (25 µl) and incubated for 10 min at 37 °C, then PTT reagent (C.K.PREST® provided by Diagnostica Stago) (50 µl) was added to the mixture and incubated for 5 min at 37 °C.[20] Thereafter clotting was induced by adding 0.025 mol/L CaCl2 (50 µl) and clotting time was recorded. The anticoagulant activity of the series of the tested aqueous, coumarin-rich extracts and fractions (aqueous and methanol fractions) of thymus in the different concentrations was expressed in seconds. The following concentrations of extracts were used in the clotting mixtures: 11.428, 5.714, 2.857, 1.428, 0.714, 0.357 and 0,178 mg/mL.

Prothrombin Time (PT):

In Prothrombin Time (PT) assay, citrated normal rat plasma (50 µl) was mixed with a solution of plants extract (25 µl) and incubated for 10 min. Then, prothrombin time reagent (NEOPLASTINE ® Cl provided by Diagnostica Stago) (100 µl), pre-incubated for 10 min at 37°C was added and clotting time was recorded [20]. The anticoagulant activity of the series of the tested aqueous, coumarin-rich extracts and aqueous, methanol fractions of plant in the different concentrations were expressed in seconds. The following concentrations of extracts were used in the clotting mixtures: 11.428, 5.714, 2.857, 1.428, 0.714 and 0.357 mg/mL. All experiments were carried out six times, and were measured automatically using coagulometer (Stago, star 4).

Statistical analysis:

Statistical evaluation was carried out with Stat View. Data are expressed as the mean ± S.D. Statistical differences were evaluated by One-way

analysis of variance (ANOVA) and the Student's t-test. P values less than 0.05 were considered statistically significant.

RESULTS:

The chemical composition:

The chemical composition analyses of extracts and organic fractions of *T. zygis* were carried out by HPLC. The results revealed the presence of various compounds in *T. zygis* extracts (**Table 1**). Rosmarinic acid was the compound identified in aqueous and all fractions except dichloromethane fraction. The hydrophobic fractions (dichloromethane and ethyl acetate fractions) were particularly rich in quercetin; the luteolin-7-*O*-glucoside was detected only in aqueous extract and hydrophilic fractions (methanol and aqueous fractions).

The total phenolic was determined by using Folin-Ciocalteu reagent in different extracts and fractions (**Table 2).** Folin-Ciocalteu reagent reacts nonspecifically with phenolic compounds; it can also be reduced by a number of non-phenolic compounds, e.g., vitamin C, Cu (II), etc. Although the exact reaction of the reagent with the reducing species is not known, it is considered that a complex is formed between phosphomolybdic tungstate and the reducing species, phenolate ion, changing colour from yellow to blue, where absorbance at 755 nm is measured [21].

Results in **Table 2** showed that ethyl acetate was the best solvent for extracting phenolic compounds, followed by methanol then water, where 340.46± 0.5, 270.09± 1.04 and 220.88± 0.34 µg eq caffeic acid/mg of dry residue were obtained, respectively. The lower polarity solvent: dichloromethane showed much lower ability in extracting the phenolic compounds as compared to the polar solvents. However, the aqueous extract showed a large polyphenol content compared to fractions with a content of 482.92± 5.60 and 400± 1.01 µg eq caffeic acid/mg of dry residue for aqueous and rich-coumarin extracts respectively.

TABLE 1: IDENTIFIED COMPOUNDS AND THEIR RETENTION TIMES

Extract	Retention time (min)	Identified compound
Aqueous extract	20.970	Cafeic acid
	29.889	Luteolin-7-O-glucoside
	32.916	Rosmarinic acid
Dichloromehatne fraction	40.294	Quercetin
Ethyl acetate fraction	20.970	Cafeic acid
	32.916	Rosmarinic acid
	40.294	Quercetin
Methanol fraction	29.795	Hyperoside
	29.889	Luteolin-7-*O*-glucoside
	32.916	Rosmarinic acid
Aqueous fraction	28.982	Rutin
	29.889	Luteolin-7-O-glucoside
	32.916	Rosmarinic acid

Antioxidant activity:

As shown in **Table 2**, extract and fractions, except dichloromethane fraction, displayed the most favourable activity against DPPH and were most active compared to reference compound, Trolox ($p>0.05$). The IC_{50} values ranged from 0.27± 0.01 to 6.8± 0.02 mg/mL dry residue. The antioxidant activity was also evaluated by means of reducing power test. The reductive potential measures the ability of a compound to act as electron donor. The electron donor reacts with free radicals, converts them to more stable products, and finally terminates radical chain reactions. The FRAP assay shows that the antioxidant activity was between 10.34± 0.19 and 90.68± 1.59 mmol Trolox/g of extract. Results (**Table 2**) clearly indicate that all extracts exhibited antioxidant activity as flows: ethyl acetate fraction > methanol fraction>aqueous extract> coumarin-rich extract> aqueous fraction > dichloromethane fraction. **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 hemolysis half-time from 180.00± 4.47 min to 73.33± 5.16 min (-59.26%, p<0.01). Application of the aqueous and fractions from *T. zygis* to the erythrocytes suspension with AAPH induced an increase of the hemolysis half-time by 222.74 ((p<0.001), 159.10 (p<0.001), 218.20 (p<0.001), 154.56 (p<0.001), 154.56 (p<0.001) from aqueous extract, dichloromethane fraction, ethyl acetate fraction, methanol fraction, aqueous fraction respectively. Trolox was used as standard antioxidant showed an increase of the hemolysis half-time by 354.57 % (p<0.001). These results indicated that the aqueous extract and organic fractions of *T. zygis* had a greater protective effect against hemolysis of erythrocytes.

TABLE 2: POLYPHENOL CONTENT AND ANTIOXIDANT ACTIVITY OF EXTRACTS AND FRACTIONS OF *T. ZYGIS*.

Extracts	plyphenol (mg equivalent caffeic acid/g DR)	Radical scavenging activity [IC_{50} (mg/mL DR)]	FRAP (mmol trolox/g DR)
Aqueous extract	482.92 ± 5.60	0.44 ± 0.02*	60.5 ± 0.94
Dichloromethane fraction	170.244 ± 0.33	6,8 ± 0.2**	10.34 ± 0.19
Ethyl acetate fraction	340.46 ± 0.5	0.27 ± 0.01*	90,68 ± 1,59
Methanol fraction	270.09 ± 1.04	0,4 ± 0.02*	60,81 ±0.15
Aqueous fraction	220.88 ± 0.34	0.35 ± 0.01*	30.85 ± 0.28
Coumarin-rich extract	400 ± 1.01	0.50 ± 0,03	50 .5 ± 0.4
Trolox	-	0.51 ± 0.02	44.33 ± 7.55

*: P < 0.01; **: P < 0.001; –: Absent; DR: dry residue.

TABLE 3: ANTIHEMOLYTIC ACTIVITY OF AQUEOUS EXTRACT AND ORGANIC FRACTIONS OF *T. ZYGIS*.

Plant samples	Hemolysis half-time	% Diviation[a]	% Diviation[b]
Control	180.00± 4.47	-	-
AAPH sample	73.33± 5.16**	-59.26	-59.26
AAPH+AE 2%	236.67±25.82a^{ns} b***	+31.48	+222.74
AAPH+DF 2%	190.00±15.49a^{ns} b***	+5.56	+159.10
AAPH+EF 2%	233.33±20.66a* b***	+29.63	+218.20
AAPH+MF 2%	186.67±10.33a^{ns} b***	+3.70	+154.56
AAPH+AF 2%	186.67±18.62a^{ns} b***	+3.70	+154.56
AAPH+Trolox 1%	166.67± 10.33a^{ns} b***	+85.19	+354.57

AE: Aqueous extract, DF: Dichloromethane fraction; EF: Ethyl acetate fraction; MF: Methanol fraction; AF: Aqueous fraction; * P<0.05; **P<0.01; ***P<0.05; [ns]: not significant; Control *vs* AAPH; [a]: AE, DF, EF, MF, AF and Trolox vs Control; [b]: AE, DF, EF, MF, AF and Trolox *vs* AAPH sample.

Anti-inflammatory activity:

The anti-inflammatory activity of aqueous extract and fractions was evaluated as the inhibition of the croton oil-induced ear edema in mice a topical inflammation and was also evaluated using the carrageenan-induced edema, an acute inflammation model, in the rat hind paw.

The topical anti-inflammatory activity of extract and fractions of plant are reported in **Table 4**, topical application of croton oil induced cutaneous inflammation at the ears of mice, which caused significant increase in right ear volume when compared to the vehicle-treated left ear. As a positive control, indomethacin (500µg/ear) gave rise to a significant inhibition of 57 % in ear edema volume. When dichloromethane and ethyl acetate fractions were topically applied at 900µg/ear, they provided inhibition of 75 and 65 %, respectively, in ear edema volume, 8 h later of treatment. In contrast, topical treatment of methanol and aqueous fractions did not reduce ear edema compared to the negative control. However, the aqueous extract has the most activity and its tropical application at 900µg/ear significantly inhibited edema by 70 %, 8h later of treatment.

With the aim of proving the anti-inflammatory property of aqueous extract and fractions, we evaluated their effects on the carrageenan-induced paw edema in rat. The anti-inflammatory response obtained by the administration of the extract and fractions of *T. zygis*, indometacin and vehicle on the carrageenan-induced hind paw edema in rats is shown in **Table 5.** Treatment of animals with aqueous extract, ethyl acetate and methanol fractions (50 mg/kg, p.o.) 1 h before injection of carrageenan significantly inhibited the edema

formation at1–24 h when compared to control group. After 5 h of treatment, compared to the control (27.95% ± 0.06%), aqueous extract, methanol and ethyl acetate fractions (50 mg/kg) significantly reduced the paw edema volume (3.74% ± 0.01%, 4.73±0.13% and 2.44±0.07% for aqueous extract, ethyl acetate and methanol fractions, respectively ($P < 0.01$) and their effect was comparable to the reference drug indometacin (10 mg/kg) (10.92% ± 0.06%) ($P > 0.05$) (**Table 5**). In contrast, oral treatment with the aqueous and dichloromethane fractions (50 mg/kg) did not inhibit carrageenan-induced paw edema ($p > 0.05$).

TABLE 4: ANTI-INFLAMMATORY ACTIVITY OF AQUEOUS THYME EXTRACTS IN CROTON OIL-INDUCED MOUSE EAR EDEMA

Group	Ear volume in mL (Mean ±SEM)					
	0 hr	2 hr	4 hr	6 hr	8 hr	10 hr
Control	0	28.89 ± 0.75	41.31± 0.79	41.30± 0.60	47.39± 0.50	37.32± 0.67
Aqueous extract (900 µg/ear)	0	9.673 ± 0.55	17.11± 0.54	24.85± 1.40**	13.99±0.54***	9.67± 0.55***
Dichloromethane fraction (900 µg/ear)	0	16.16± 1.69	29.60± 2.76	12.76±0.24***	11.78±0.83***	4.17±0.45***
Ethyl acetate fraction (900 µg/ear)	0	16.07±1.19***	5.90± 1.29	28.55±3.97***	16.54±1.57***	14.58± 1.4*
Methanol fraction (900 µg/ear)	0	14.12±1.96	31.60±1.65	24.07±2.67	15.28± 1.21	12.27± 0.56**
Aqueous fraction (900 µg/ear)	0	37.04± 1.96	48.84± 2.07	36.81± 1.69	52.55± 2.30	35.42± 2.54
Indometacin (500 µg/ear)	0	34.023±0.95*	33.33±1.36**	17.59± 0.72	15.51±0.64***	11.57± 0.07***

The animals were treated with aqueous and fractions from *T. zygis* 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 mean ±SEM, (n=6), where * corresponds to $p<0.05$, ** corresponds to $p<0.01$ and *** corresponds to $p<0.001$ compared to control.

TABLE 5: ANTI-INFLAMMATORY ACTIVITY OF AQUEOUS EXTRACT AND FRACTIONS IN CARRAGEENAN-INDUCED RAT PAW EDEMA

Group	Paw edema rate (E) in % (Mean ±SEM)									
	0 hr	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	8 hr	10 hr	24 hr
Control	**0**	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
Aqueous extract (50 mg/kg)	**0**	2.96± 0.01	4.14± 0.1**	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*
Dichloromethane fraction (50 mg/kg)	0	4.86±0.14	14.13±0.14	23.44±0.14	25.03±0.14	24.41±0.14	23.87±0.14	21.88±0.14	17.25±0.14	7.40±0.14
Ethyl acetate fraction (50 mg/kg)	0	3.2±0.13*	6.3±0.12***	7.18±0.13***	6.78±0.13**	4.73±0.13**	2.92±0.12**	2.69±0.12*	2.71±0.12*	1.19±0.13
Methanol fraction (50 mg/kg)	0	1.83±0.07***	3.52±0.06***	5.17±0.06***	4.55±0.06**	2.44±0.07**	2.25±0.06**	1.18±0.07*	0.42±0.06*	0*
Aqueous fraction (50 mg/kg)	0	7.18±0.04	16.5±0.04	26.99±0.04	26.92±0.04	26.47±0.04	25.99±0.07	23.85±0.04	19.71±0.04	8.84±0.04
Indometacin (10 mg/kg)	0	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 and fractions from T. zygis at 50 mg/kg. Indomethacin was used as a positive control (10 mg/kg). The differences between treatment and control were tested using ANOVA. Values are mean ±SEM, (n=6), where * corresponds to $p<0.05$, ** corresponds to $p<0.01$ and *** corresponds to $p<0.001$ compared to control

Anticoagulant activity:

All extracts and fractions were tested for blood coagulation effects in normal rat plasma and found to be significantly prolonged the Partial Thromboplastin Time (PTT) and Prothrombin Time (PT) of the normal rat plasma (**Table 6** and **7**).

TABLE 6: PARTIAL THROMBOPLASTIN TIME (PTT) MEASUREMENTS OF EXTRACTS AND FRACTIONS MADE *IN VITRO* EXPERIMENTS IN RAT POOLED PLASMA

Concentration of a sample in the clottiong mixture [mg/mL]	*In vitro* PTT measurements [s]			
	Aqueous extract	Methanol fraction	Aqueous fraction	Coumarin-rich extract
11.428	> 900***	> 900***	55.06± 1.2**	299.1± 5.72***
5.714	> 900***	200.47± 1.19***	40.2± 1.19**	65.87± 3.5**
2.857	300.1±5.72***	104.41± 0.55**	23.4± 1.19**	29.47± 1.19**
1,428	60.87± 3.5**	68.974± 1.61***	18.09± 1.19**	22.97± 0.25*
0.714	29.47± 1.19**	29.04± 0.6***	17.06± 1.19**	18.17± 0.4
0.357	20.97± 0.25**	19.64± 0.11***	16.11± 1.19**	16.07± 0.12
0.178	18.07± 0.4**	17.5± 0.26**	16.22± 1.19**	15.97± 0.05
Negative control	16.1± 0.15	16.1± 0.15	16.1± 0.15	16.1± 0.15

Values are expressed as mean of 6 measurements ± S.D. *p<0.05, **p<0.01, ***p<0.001. Aqueous extract, methanol fraction, aqueous fraction, coumarin-rich extract *vs* negative control.

In PTT test the aqueous and methanol extract completely inhibited the plasma clot formation in the concentration of 5.714 and 11.428 mg/mol in the clotting mixtures, respectively, and both strongly prolonged time of clotting still at the concentration of 0.178 g/mol in the clotting mixtures ($p<0.01$). In PT test aqueous extract and methanol fraction were also found as the most activity anticoagulant (**Table 7**). Aqueous extract completely inhibited clotting process in the

concentration of 11.428 mg/ml in clotting mixtures and strongly prolonged the process even at the concentration of 2.857 mg/ml in clotting mixtures for aqueous extract and methanol fraction. The coumari-rich extract show the significant anticoagulant activity in PTT and PT assay (**Table 6** and **7**). The PTT assay was used to determine the effects on intrinsic factors such as II, V, VIII, IX, XI and XII and/or common pathways.

TABLE 7: PROTHROMBIN TIME (PT) MEASUREMENTS OF EXTRACTS AND FRACTIONS MADE *IN VITRO* EXPERIMENTS IN RAT POOLED PLASMA.

Concentration of a sample in the clotting mixture [mg/mL]	*In vitro* PT measurements [s]			
	Aqueous extract	Methanol fraction	Aqueous fraction	Coumarin-rich extract
11.428	>300***	190.01±0.24	30.06±1.2**	100.1±5.72***
5.714	99.13±1.34***	111.34±0.07**	27.2±1.19**	80.87±3.5**
2.857	27.27±0.58***	54.40±0.62***	23.4±1.19**	20.47±1.19**
1.428	15.93±0.5	20.4±1*	18.09±1.19**	18.97±0.25**
0.714	13.63±0.34	15.67±0.35	17.06±1.19**	18.17±0.4**
0.357	14.17±0.12	14.94±0.15	16.11±1.19	15.07±0.12
Negative control	15,01±0,24	15,01±0,24	15.01±0.24	15.01±0.24

Values are expressed as mean of 6 measurements ± S.D. *p<0.05, **p<0.01, ***p<0.001. Aqueous extract, methanol fraction, aqueous fraction, coumarin-rich extract *vs* negative control.

DISCUSSION: Recently, the aqueous extract from the three thyme (*Thymus atlanticus, Thymus satureioides* and *Thymus zygis)* were evaluated for their antioxidant, anti-inflammatory and anticoagulant activities by our group Khouya et al., and the results showed that aqueous extract of *T. zygis* have a greet anti-inflammatory and anticoagulant activities . The aim of this work was to identify the active fraction (s) of *T. zygis* using different polar and non-polar organic solvents as extractants and to determine the antioxidant capacities and the phenoliccompounds for all fractions. The chemical composition analyses of extracts and organic fractions of *T. zygis* were carried out by HPLC. Cafeic acid was particularly present in aqueous extract and ethyl acetate fraction. Caffeic acid plays a central role in the biochemistry of Lamiaceae and occurs predominantly in the dimer form as rosmarinic acid [22]. Several studies have reported on the detection and quantification of rosmarinic and caffeic acids in thymus varieties preparations and have showed that the rosmarinic acid is the most abundant caffeic dimer in thyme species originating from the Errachidia [11, 23].

Antioxidant activity is a complex procedure usually happening through several mechanisms. In the present work, three complementary tests were used to assess the antioxidant activity of extracts and fractions: DPPH free radical scavenging, ferric reducing power assays and protection against AAPH-induced hemolysis. The results indicated that the aqueous extract and fractions possess a high antioxidant and had a greater protective effect against hemolysis of erythrocytes. A correlation between the total phenolic content and the antioxidant activity was observed for the hemolytic activity, Frap and DPPH assays (r^2=0.722, r^2=0.71 and r^2= 0.62 respectively). The potent antioxidant activity of *T. zygis* extracts and fractions can be due to its high content of total phenolic. Caffeic acid and rosmarinic acid are common in many plants and all are strong radical scavengers.

The anti-inflammatory activity of aqueous extract and fractions was evaluated as the inhibition of the croton oil-induced ear edema in mice and was also evaluated using the carrageenan-induced edema in the rat hind paw. In topical anti-inflammatory, he organic extracts (less polar) appear to have an anti-inflammatory effect than the more polar aqueous and methanol fractions. This is explained by their greater lipophilicity than the latter. Indeed, the epidermal barrier is essentially formed by the stratum corneum which is especially a lipophilic barrier limiting [24]. Lipophilic compounds may dissolve in this bariere and diffuse into the lipidic matrix located and into the intercellular lipids, between the keratin filaments. In contrast, hydrophilic molecules, much less diffusible, slowly migrate in the water-rich regions. The difference in activity between these fractions (less polar and polar) can also be explained by the difference in chemical composition. Indeed flavonoid found

during photochemical screenings may explain this anti-inflammatory effect.

In fact, the topical application of quercetin present in the polar fractions month exerts a strong inhibition of the cyclooxygenase (COX) and lipoxygenase [25, 26]. Moreover, serval reachearchs identify the luteolin-7-*O*-glucoside, present in the aqueous extract, as the most powerful flavonoid tested in the inhibition of TNF-α, inhibition of cyclooxygenase, activation of NF-kB induced by lipopolysaccharide (LPS), and the activation of protein (AP)-1 [27].

The carrageenan-induced paw edema is a largely used test for screening of new anti-inflammatory drugs and evaluation of anti-inflammatory effect of natural products [28–29]. The inflammatory response involves three phases through sequential release of several mediators. The early phase (the first 90 min) involves the release of histamine and serotonin; the second phase (90–150 min) is mediated by kinin and the third phase (after 180 min) is mediated by prostaglandin [30]. The results from this study suggest that the methanol and ethyl acetate fractions of *T. zygis* possibly act by inhibiting the release or action of histamine, serotonin and kinin and prostaglandin of the edema development. Furthermore, rosmarinic acid is present in aqueous extract, ethyl acetate and methanol fractions at relatively high levels, this phenolic acid possesses the high inflammatory effect evaluated by different test *in vitro* and *in vivo* including carrageenan-induced paw edema model which explain the high activity of these fractions [29].

Considering the relationship between inflammation and the coagulation systems, the extracts (aqueous and coumarin-rich extracts) and fractions (methanol and aqueous fractions) of *T. zygis* were evaluated *in vitro* for their anticoagulant activity at the different concentrations by partial thromboplastin time and prothrombin time activated. In PT and PTT tests, all fractions tested showed the strongest anticoagulant activity. The prolongation of the PTT 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 PTT and PT assay showed that extracts and fractions of *T. zygis* had prolonged coagulation times compared with the control sample treated with PBS, suggesting that extracts inhibited the common pathways.

CONCLUSION: All fractions of *T. zygis* were found to possess considerable antioxidant activities and are rich in total polyphenol compounds and rosmarinic acid was the compound identified in majority fractions. These fractions act differently on the process of inflammatory and coagulation studied, the ethyl acetate fraction showed anti-inflammatory activity in the acute and chronic edema models. Methanol fraction prolonged the blood clotting time by causing a factor deficiency in the intrinsic and extrinsic pathways and is effective in reducing carrageenan-induced ear edema, although it was not active in the acute ear edema. Thus, the present studies scientifically validated the traditional use of extract from *T. zygis* in treatment of inflammatory diseases and identify that methanol and ethyl acetate fractions are the most active anti-inflammatory and anticoagulant fractions.

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