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Original Research

Total Phenols Compounds, Ferric Reducing Antioxidant Power and Scavenging Activities of *Thymus Vulgaris* L. Crop Extracts: Analgesic, Anti-Inflammatory, Anxiolytic, Antidepressant Responses in Swiss Strain Male Albino Mice, and Anti-Bacterial Effects

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Abstract

The purpose of this study was to identify the most important analgesic, anti-inflammatory, anxiolytic, antidepressant, antioxidant and anti-bacterial effects of *Thymus vulgaris* L. crop extracts obtained after optimization of extract time, temperature and speed conditions. Thyme crop extraction was established through magnetic stirring (MST) using ethanol after optimization of conditions using response surface methodology (RSM). Total phenols content (TPC), ferric reducing antioxidant power (FRAP), and scavenging activity (DPPH) were evaluated and then animals were subjected to Thyme extracts used for neuropharmacological and Antinociceptive properties determination by different experiments. DPPH, FRAP and TPC of Thyme leaves were about 3652.69 mg TE/100g DW; 5835.96 mg TE/100g DW and 1243.73 mg GAE/100g DW, respectively, for a temperature extraction (60ºC), time extraction (15 min) and speed extraction (600 rpm). The predicted values of DPPH, FRAP and TPC represent 92%, 89% and 90%, respectively compared with experiment values. Extraction temperature, extraction time and extraction speed affect positively on the DPPH, FRAP and TPC levels ($p<0.05$). Animals treated with Thyme extract (250 mg/kg) presented potential neuropharmacological and antinociceptive effects varied according to time of exposure. The promising neuropharmacological and antinociceptive effects obtained from Thyme extracts can be enhanced. The effects of the plant extracts analyzed vary widely not only from the plant species but also especially from the extraction

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conditions. The neuropharmacological and antinociceptive effects depend significantly on the treatment time.

Keywords: anti-bacterial, antioxidant, antinociceptive, hotplatee analysis, neuropharmacological, swimming test

Introduction

Medicinal and aromatic plants contain chemical compounds that have a special effect on prevention and treatment of human diseases. These plants contain as herbal remedy continued chemical compounds. Plants can be used medicinally. They contains essential oils. Among the most important contents of medicinal and aromatic plants are alkaline compounds, volatile oils, tannins and resins. The secondary metabolites of plants are used as medicinal compounds. The plant volatile oils are one of the most important of these products. Thyme contains the medically important 22 terpenes compounds such as digitoxigenin, tetrahydrocannabinol and salvinorin. Plant active chemical components have therapeutic effect for many diseases [1-4]. Since 1950, more than 1600 documents deal with medicinal and aromatic plants around the world. The Thyme was among the best-known plants from the scientific committee for its therapeutic and aromatic properties. The genus *Thymus* (Lamiaceae) comprises more than 250 species subdivided into eight sections: *Mastichina*, *Micantes*, *Piperella*, *Pseudothymbra*, *Thymus*, *Teucrioides*, *Hyphodromi*, and *Serpyllum*. This subshrub is native to Mediterranean regions, where it presents a high chemical variability. Several traditional uses of Thyme from different regions were previously described. It is known from literature that the Thyme species were used for their antispasmodic, sedative, antioxidant, antimicrobial, and antifungal properties. Moreover, Thyme were used to treat diarrhea, digestive and respiratory system disorders [5, 6]. Also, they were employed for their tonic, anti-inflammatory, antiparasitic, antitussive, and carminative properties. In addition, essential oils from these species were used for perfumery, cosmetic, and medicinal applications. Many biological activities were deduced such as antioxidant, hepatoprotective, anti-cancer, antimicrobial, antinociceptive, and analgesic activities. Some biological properties of Thyme were investigated around the world, as in Saudi Arabia [7, 8]. Oxidative stress is a metabolic disorder or an imbalance between free radicals and antioxidants. The compound or species known free radicals are oxygen-containing molecules with an uneven number of electrons allows them to easily react with other molecules. Free radicals in animal and plant cells can cause large chain chemical reactions called oxidation. They can be beneficial or harmful. When it is oxidative stress, the free radicals caused damage to fatty tissue, DNA, and proteins. They are also responsible for a vast number of diseases include diabetes complications, atherosclerosis, inflammatory conditions, hypertension, heart disease, neurodegenerative diseases, and cancer [9, 10]. Cells produce free radicals through processes like metabolism, inflammation or exposed to free radicals from environment [11]. Some sources of environmentally oxygen-containing molecules include ozone, pesticides and cleaners, cigarette, radiation, and pollution [12, 13]. Cells exposed to oxidative stress lead to increased ROS production [14]. Cells cannot completely avoid free radical exposure and oxidative stress. However, it can minimize the effects of oxidative stress through increase in levels of antioxidants and decrease in free radicals damages. To prevent oxidative stress, cells need to maintain a certain balance of free radicals and antioxidants. Antioxidants are from various sources like endogenous antioxidant present and produced in the body and exogenous antioxidant source, such as polyphenols [15]. There are the most important natural antioxidants coming from consuming vegetables and fruits or by consuming synthetic antioxidants generated by natural vegetable sources [16]. Antioxidants from various sources have some biological properties such as anticarcinogenicity, antimutagenicity, antiallergenicity, and antiaging activity [17-19]. They have been reported for natural and synthetic antioxidants. Plants contain a large variety of phytochemicals such as vitamin C, vitamin E, carotenoids, and phenolic compounds, which they are the contributors to the antioxidant activity in all higher plants, and in all parts (wood, bark, stems, pods, leaves, fruit, roots, flowers, pollen, and seeds). The screening of inexpensive raw materials for identifying new antioxidants has fostered interest. *In vitro* and *in vivo* test systems have been previously documented to assess the free radical scavenging activity of phytochemicals. The compounds are classified into strong, moderate and weak antioxidants, based on the efficiency of free radical scavenging. Several naturally occurring antioxidants have demonstrated protective role against free radical scavenging damages [20]. The cytoprotective effect of the commonly used antioxidant vitamins remains controversial. One of the modern technique we used in the optimization of three independent variables of antioxidants from Thyme leaves using response surface methodology (RSM) and it was used in a previous study from basil leaves [21].

In the present study, we collected Thyme from Qassim province that are widely consumed in the kingdom of Saudi Arabia, and analyzed antioxidant activities of ethanoic extracts prepared from this plant. Three responses of the plant extracts were evaluated including total phenols content (TPC, mg GAE/100g DW),

Ferric reducing antioxidant power (FRAP, mg TE/100 g DW), and scavenging activity (DPPH, mg TE/100 g DW). The effect of the temperature, time, and speed extraction on the antioxidant activity of the Thyme extracts were also investigated. Therefore, the purpose of this study is to establish a high throughput screening of the Thyme antioxidants for their analgesic, antibacterial, anti-inflammation and muscle relaxant effects.

The present study was designed to test the following hypotheses (1) antioxidants of Thyme crops were optimally extracted under non-control conditions, (2) Thyme antioxidants quickly extracted under high temperature, (3) its maximum antioxidants extraction occurs at high speed conditions, (4) extraction yield decreases under long time of extraction, (5) the combination of extraction conditions (temperature, speed and time of extraction) synergistically inhibits extraction yield in comparison to factors affecting independently and (6) the quality of the antioxidant could act on the pharmaceutical and medicinal effects of these plant extracts.

Material and Methods

Folin-Ciocalteu phenol reagent, ferric chloride ($FeCl_3$ $6H_2O$), and HCl were obtained from Merck, (Darmstadt, Germany). The 2,2-Diphenyl-1-picrylhydrazyl (DPPH), TPTZ (2,4,6-tris(2-pyridyl)-s-triazine), and Trolox (6-hydroxy-2,5,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma (St Louis, MO, USA). Sodium carbonate was purchased from RDH (Seelze, Germany) and glacial acetic acid from Mallinckrodt Baker (Phillipsburg, USA). All chemicals and reagents used in this study were of analytical grade.

Dried Thyme leaves, not ground, were purchased from a local supplier in Almethnab city, Qassim region, during September 2021 in the kingdom of Saudi Arabia (KSA). The sample leaves were checked and examined before the experiments by Prof. Abdulrahman Alsoqeer from plant production and protection department. Samples were then ground using a blender to ensure that the sample was free from any additives or impurities. The powder sample was then subjected to extraction.

For extraction and isolation of the active constituents the ground plant material (1 g) was extracted by an aqueous solution of ethanol (20 mL). Extraction was carried out by mixing sample for different time durations (h), different extraction speeds (rpm), and different extraction temperatures, using a magnetic stirrer (IKA, Germany). All extracted samples were centrifuged using tabletop centrifuge (Thermoline, China) for 10 min at 10000 rpm. The extract was evaporated and then redissolved in 5 mL methanol [21].

Antioxidant extraction from Thyme was produced through magnetic stirring (MST) using ethanol. A protocol for the extraction of bioactive components from Thyme leaves was established by response surface methodology (RSM) which was employed to determine the best combination of variables for optimum extraction yield and antioxidant activity and previously described [22]. Total phenols content (TPC), Ferric reducing antioxidant power (FRAP), and scavenging activity (DPPH) were evaluated for different temperature, time, and speed extraction conditions. Experimental data were analyzed by RSM to fit the second-order polynomial model.

The content of total phenolics (TPC) was estimated using the modified Folin-Ciocalteu method [23] using gallic acid as the standard. A (100 µL) aliquot of plant extract was oxidized with diluted Folin-Ciocalteu reagent (500 µL) in Eppendorf 96-deep well platee (Germany). After 5min, the mixture was neutralized with 1 ml sodium carbonate (7.5%, w/v), and incubated for 120 min before reading absorbance at 765 nm using a SPECTROstar Nano absorbance reader.

The Ferric Reducing Antioxidant Power (FRAP) was assayed using method of Benzie and Devaki [24]. Antioxidant activity was determined based on reduction of a colorless Fe^{3+}-TPTZ complex into intense blue Fe^{2+}-TPTZ once it interacts with a potential antioxidant. The FRAP reagent was prepared using 300 mM acetate, pH 3.6 (3.1 g sodium acetate trihydrate, as well as 16 ml of glacial acetic acid made from 1 liter with distilled water). The acetate buffer was mixed with 10 mm TPTZ (2,4,6-tri (2-pyridyl) -s-triazazine) in 40 mM HCl and 20 mM $FeCl_3$ $6H_2O$ at the ratio of 10:1:1 to produce the working reagent. For assays, 1900 µL freshly warmed prepared FRAP reagent was mixed with 100 µL sample, standard, or blank in wells of Eppendorf 96-deep well plate (Germany) and incubated for 30 min before reading absorbance at 595 nm.

2,2-diphenyl-1-picrylhydrazyl radical (DPPH) assay was carried out to measure the free radical scavenging activity as described previously [22]. The percentage inhibition of $DPPH^{\bullet}$ was calculated according to the formula: percentage inhibition = $[(A_B - A_A)/AB] * 100$, where A_B and A_A are the absorbance values of the test and of the blank sample, respectively, after 15 min.

The antioxidant activity was performed by DPPH radical scavenging assay according to standard protocol described by Ndezo et al. [25]. The positive control used was vitamin C. The solution was allowed to stand for 30min, in dark the absorbance was measured at 517 nm. Decreasing of the DPPH solution absorbance indicates an increase of the DPPH radical scavenging activity. Scavenging of free radicals by DPPH as percent radical scavenging activities (%RSA) was calculated as follows: % DPPH = (OD control – OD sample) × 100 / OD control.

The antibacterial potential of *Thymus vulgaris* L. whole plant was determined against four different bacteria (*E. coli*, *S. boydi*, *B. subtilis*, and *S. aureus*) by the standard disk diffusion method [26]. Standard antibiotic, Tetracycline at 5 µg/disk was taken as positive controls, while 5% DMSO was used as the negative control.

A Swiss strain male albino mice (18-22 g) were handle in these experiments. The mice were housed in polypropylene cages with 6 animals each under a standard circadian cycle, and maintained at 25±2ºC. The animals were allowed free access of pellet feed supplied from a local animal feed vendor for rat and mice, and water was provided ad libitum.

Swiss strain male albino mice of (8-12) weeks, weighing between (18-22 g), were segregated into groups of 6 animals. Group I was treated with saline (10 ml/kg), group II was treated with TramadolR (30 mg/kg i.p). Group III, IV and V were treated with 100, 200 and 300 mg/kg VBME, i.p. respectively.

Hot plate test BALB/c mice of either sex (n = 6) weighing (18-22 g) were acclimatized to laboratory conditions one hour before the start of experiment with food and water available ad libitum. Animals were then subjected to pre-testing on hot plate (Havard apparatus) maintained at 55±0.1ºC. Animals having latency time greater than 15s on hot platee during pre-testing were rejected (latency time) [17]. All the animals were divided in eight groups each of six mice. Group I was treated with saline (10 ml/kg), group II was treated with TramadolR (30 mg/kg i.p). Group III, IV and V were treated with 100, 200 and 300 mg/kg VBME, i.p. respectively. After 30 min of treatment the animals were placed on hot plate and the latency time (time for which mouse remains on the hot plate (55±0.1ºC) without licking or flicking of hind limb or jumping) was measured in seconds. In order to prevent the tissue damage a cut-off time of 30 s were imposed for all animals. To find out the opiodergic mechanism in the analgesic activity of VBME, Groups VI and VII were treated with naloxone (0.5 mg/kg s.c.) and after 10min these groups were treated with VBME (200 and 300 mg/kg, i.p), while group VIII was treated with TramadolR (30 mg/kg i.p.) after 10min of naloxone injection. The latency time for all groups was recorded at 0, 30, 60, 90 and 120 min. Percent analgesia was calculated using the following formula: % Analgesia = (Test latency – control latency)/ (Cut – off time – control latency) × 100.

BALB/c mice of either sex (n=6) weighing (18-22 g) were used. All animals were withdrawn from food 2 h before the start of experiment and were divided in five groups. Group I was injected with normal saline (10 ml/kg) as control, Group II received standard drug diclofenac sodium (10 mg/kg) while the remaining groups III, IV and V were injected with 100, 200 and 300 mg/kg i.p. of VBME respectively. After 30 min of saline, diclofenac sodium and plant extract injection, the animals were treated i.p. with 1% acetic acid. The number of abdominal constrictions (writhes) were counted after 5 min of acetic acid injection for the period of 10 min.

The staircase test was carried out following the available protocol, with slight modifications. A stair of five identical steps having dimensions 2.5 9 10 9 7.5 cm were placed on an elevated surface. The animals were divided into control, standard and treated groups, consisting of six mice in each group. The control group was treated with distilled water (10 ml/kg), the standard group was treated with diazepam (1 mg/kg) and the remaining groups were treated with HEVB 0.3, 0.4 and 0.5 g/kg body weight. After 30 min of treatment, the animals were placed on the first step of the elevated stairs and each animal was observed for the number of steps climbed up and the number of rearing over a period of 3 min. A step was considered to be climbed only if the mouse had placed all four paws on the step. The stairs were cleaned from feces and urine after each mouse performance.

Traction test in this procedure, a metal wire coated with rubber was used, both ends of which were rigidly supported with stands about 60 cm above the laboratory bench. Different groups (n = 6) were treated with diazepam (1 mg/kg), distilled water (10 ml/kg) and HEVB (0.3, 0.4 and 0.5 g/kg). The animals were exposed to the traction test after 30, 60 and 90 min of treatment. Each animal was hung by their hind legs from the wire and the time of hanging was recorded for 5 s. Failure to hang for less than 5 s was considered as the presence of muscle relaxant activity and vice versa.

The antidepressant activity of the extract was evaluated using the forced swimming test (FST). All the mice were trained for swimming in a bath with dimensions (42 9 19 9 19 cm). The bath was filled with water (25±2ºC) up to a depth of 15 cm. On the day of experiment, animals were acclimatised with the laboratory environment, i.e. dim red light and soundproofed. Animals were divided into five groups (n = 6); control group, standard group (fluoxetine) and the remaining groups were treated with HEVB (0.3, 0.4 and 0.5 g/kg). After the above treatment, the animals were allowed to swim for 6min, and the durations of immobility were recorded during the last 240 s of the swimming period.

The plane used in this procedure consisted of two plywood boards. Both boards were connected with each other in such a way that one board formed the base and the other is fixed with the base at an angle of 65. Different groups (n = 6) were treated with diazepam (1 mg/kg), distilled water (10 ml/kg) and HEVB (0.3, 0.4 and 0.5 g/kg). After 30, 60 and 90min of treatment, the animals were placed on the upper part of the inclined plane for 30 s to hang or fall.

Results are expressed as means with standard Error (SE) of three measurements. The significance of differences among mean values was evaluated by analysis of variance (ANOVA) using MINITAB® (17.1.0) software. Differences were considered significant at probability value of <0.05.

Results and Discussion

Interaction between extraction temperature, extraction time and extraction speed with the content of

DPPH, FRAP and TPC was done in Table 1. The tested extraction temperature range was obtained at 40ºC and 80ºC. The extraction time was used in the range of from 6 to 24 min. The extraction speed was used in the range of 400 to 800 rpm. Levels of independent variables along with the observed values for the response variables TPC, FRAP, and DPPH of antioxidant activity of Thyme leaves under different conditions of magnetic stirrer extraction based on a central composite design (CCD) for surface analysis were determined. Final equation of antioxidant prediction in terms of DPPH, FRAP and TPC were completed based on ANOVA for antioxidants fitted quadratic polynomial model of extraction parameter done in Tables 2, 3 and 4:

$$\text{DPPH mgTE/100gDW} = + 3652.69 + 347.25A + 321.80B + 32.05C + 187.12AB + 90.38AC - 229.62BC - 740.38A^2 - 1110.91B^2 - 870.84C^2 \quad (1)$$

$$\text{FRAP mgTE/100gDW} = 5835.96 - 6.83A + 1430.81B + 1069.08C - 476AB + 584AC + 535.75BC - 604.17A^2 - 793.67B^2 - 1210.16C^2 \quad (2)$$

$$\text{TPCmgTE/100gDW} = 1243.73 + 1340.97A + 430B + 218.36C + 177.88AB + 105.63AC + 299.62BC + 994.66A^2 + 219.96B^2 + 328.56C^2 \quad (3)$$

According to the variance analysis, all of the models were significant. The R^2 values for DPPH, FRAP and TPC were 0.9468, 0.9853, and 0.9884, respectively. The lack-of-fit tests were not significant for all DPPH, FRAP and TPC, which also showed a good fit between the experimental data and the model (Tables 5 and 6).

DPPH, FRAP and TPC were evaluated for different temperature, time, and speed extraction conditions and results were analyzed by RSM to fit the second-order polynomial model (Fig. 1). The interaction between extraction temperature (x1), extraction time (x2) and extraction speed (x3) is visualized. Time and temperature, as well as speed- extraction influenced

Table 1. Antioxidant activity of extract of *Thymus vulgaris* L. Under different conditions of magnatic stirrer extraction based on a central composite desighn (CCD) for surface analysis.

Run	Extraction Condition			Analytical results		
	A: Extraction Temperature (°C)	B: Extraction Time (min)	C: Extraction speed (rpm)	DPPH mg TE/100g DW	FRAP mg TE/100g DW	TPC mg GAE/100g DW
1	60.00	15.00	600.00	193	986	1118
2	60.00	15.00	600.00	451	1325	7560
3	60.00	1.00	600.00	1121	3877	1033
4	80.00	6.00	400.00	1983	2040	3715
5	40.00	15.00	600.00	485	1109	1248
6	80.00	24.00	800.00	960	3612	3418
7	60.00	15.00	600.00	350	5971	1890
8	60.00	15.00	400.00	1718	6742	5266
9	60.00	15.00	600.00	1062	4664	1914
10	60.00	30.00	600.00	2120	3612	6418
11	80.00	15.00	600.00	153	1211	780
12	60.00	15.00	800.00	933	5935	4954
13	80.00	6.00	800.00	1022	850	1282
14	60.00	15.00	600.00	1422	3998	1282
15	80.00	24.00	400.00	3365	5968	1478
16	60.00	15.00	600.00	3579	5624	1246
17	40.00	24.00	400.00	3800	5889	1246
18	40.00	6.00	800.00	4397	5870	2660
19	40.00	6.00	400.00	3326	6983	1977
20	40.00	24.00	800.00	3438	5830	1518

*All results are the mains ±SD (n = 3).

Table 2. ANOVA for DPPH fitted quadratic polynomial model of Extraction parameter.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3.467E+07	9	3.852E+06	38.56	<0.0001	Significant
A-extraction temperature	1.647E+06	1	1.647E+06	16.49	0.0023	
B-extraction time	1.414E+06	1	1.414E+06	14.16	0.0037	
C-speed of magnetic stirrer	14029.31	1	14029.31	0.1404	0.7157	
AB	2.801E+05	1	2.801E+05	2.80	0.1250	
AC	65341.13	1	65341.13	0,6541	0.4375	
BC	4.218E+05	1	4.218E+05	4.22	0.0670	
A^2	7.900E+06	1	7.900E+06	79.08	< 0.0001	
B^2	1.779E+07	1	1.779E+07	178.04	< 0.0001	
C^2	1.093E+07	1	1.093E+07	109.41	< 0.0001	
Residual	9.989E+05	10	99892.84			
Lack of Fit	1.822E+05	5	36447.51	0.2231	0.9373	Not significant
Pure Error	8.167E+05	5	1.633E+05			
Cor Total	3.567E+07	19				

*P≤0.05 indicate the the model terms are significant.

Table 3. ANOVA for FRAP fitted quadratic polynomial model of Extraction parameter.

Source	Sum of Squares	df	Mean Square	F-value	p-value	Significant
Model	8,074E+07	9	8,971E+06	74,55	<0.0001	Significant
A-extraction temperature	636,66	1	636,66	0,0053	0,9435	
B-extraction time	2,796E+07	1	2,796E+07	232,33	<0.0001	
C-speed of magnetic stirrer	1,561E+07	1	1,561E+07	129,71	<0.0001	
AB	1,813E+06	1	1,813E+06	15,06	0,0031	
AC	2,728E+06	1	2,728E+06	22,67	0,0008	
BC	2,296E+06	1	2,296E+06	19,08	0,0014	
A^2	5,260E+06	1	5,260E+06	43,71	<0.0001	
B^2	9,078E+06	1	9,078E+06	75,44	<0.0001	
C^2	2,111E+07	1	2,111E+07	175,38	<0.0001	
Residual	1,203E+06	10	1,203E+05			
Lack of Fit	1,114E+06	5	2,228E+05	12,48	0,0075	Significant
Pure Error	89299,33	5	17859,87			
Cor Total	8,194E+07	19				
R-Squared				0,9853		
Adj R-Squared				0,972		

*P ≤0.05 indicate the the model terms are significant.

significantly in the value of antioxidant capacity. Extraction temperature (x1), extraction time (x2) and speed (x3) affect positively on the DPPH, FRAP and TPC levels ($p<0.05$).

The experimental values of DPPH, FRAP and TPC of Thyme leaves were about 3652.69 mg TE/100 g DW; 5835.96 mg TE/100 g DW and 1243.73 mg GAE/100 g DW, respectively, for a temperature extraction

Table 4. ANOVA for TPC fitted quadratic polynomial model of Extraction parameter.

Source	Sum of Squares	DF	Mean Square	F-(Ration) Value	p-value	Significant
Model	4,268E+07	9	4,742E+06	94,85	<0.0001	Significant
A-extraction temperature	2,326E+07	1	2,326E+07	465,18	<0.0001	
B-extraction time	2,537E+06	1	2,537E+06	50,74	<0.0001	
C-speed of magnetic stirrer	6,512E+05	1	6,512E+05	13,02	0,0048	
AB	2,531E+05	1	2,531E+05	5,06	0,0482	
AC	89253,13	1	89253,13	1,79	0,2111	
BC	7,182E+05	1	7,182E+05	14,37	0,0035	
A^2	1,426E+07	1	1,426E+07	285,18	< 0.0001	
B^2	6,909E+05	1	6,909E+05	13,82	0,0040	
C^2	1,556E+06	1	1,556E+06	31,12	0,0002	
Residual	5,000E+05	10	49995,62			
Lack of Fit	4,936E+05	5	98724,95	77,96	<0.0001	Significant
Pure Error	6331,50	5	1266,30			
Cor Total	4,318E+07	19				
R-Squared				0,9884		
Adj R-Squared				0,9780		

*P≤0.05 indicate the the model terms are significant

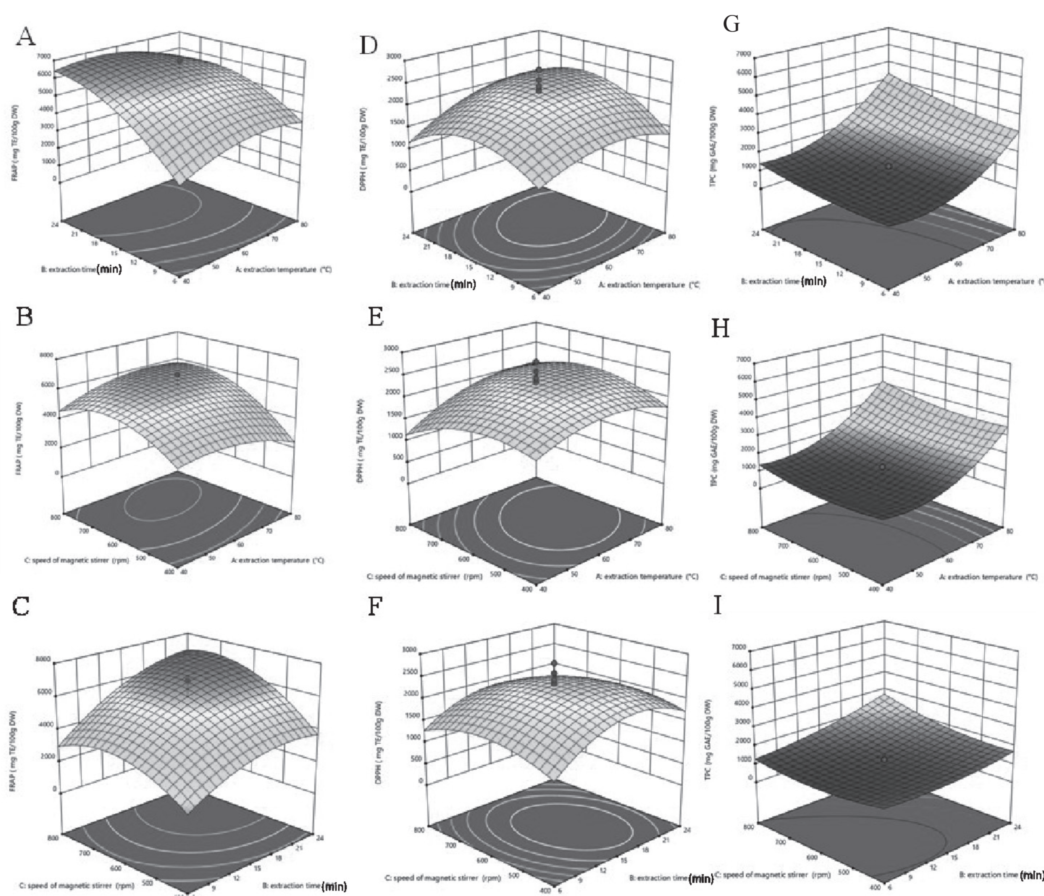

Fig. 1. Response surface plots showing the operating parameter effect on antioxidant capacity of *Thymus vulgaris* L. extracts. (A, B and C) the FRAP mgTE/100 g DW, (D, E and F) the DPPH mgTE/100 g DW and (G, H and I) the TPC mg GAE/100 g DW.

Table 5. Validation of the predicted values for antioxidant activity.

Factor	Name	Level	Low Level	High Level	Std. Dev.	Coding
A	temp	60	40	80	0	Actual
B	time	15	6	24	0	Actual
C	speed	600	400	800	0	Actual

Table 6. Prediction and experimental results of antioxidant activity of Thyme leaves.

Antioxidant Activity	Values	
	Prediction	Experimental
DPPH (mg TE/100 g)	1793,90	3652,69
FRAP (mg TE/100 g)	4055.10	5835.96
TPC (mg GAE/100 g)	2296,80	1243,73

(60ºC), time extraction (15 min) and speed extraction (600 rpm). The predicted values of DPPH, FRAP and TPC represent 92%, 89% and 90%, respectively compared with experiment values. It appears that the experimental results give values of DPPH, FRAP and TPC close to those of the theoretical values (Tables 5 and 6).

To analyse the possible analgesia effect of Thyme extracts, the animals were treated with saline (10 ml/kg) (DW), TramadolR (5 mg/kg i.p) (Tramadol) and thyme extract (250 mg/kg) (*Thymus vulgaris* L.). The results presented in Table 7 show animals' latency time on hot plate. The curve of the animals of the *Thymus vulgaris* L. group follow that of the TramadolR. The latency time from saline treated animals (DW) group was widely significantly lower than that of TramadolR (30 mg/kg i.p) and (*Thymus vulgaris* L.) groups during the first 30min. After this time, the latency time increased in *Thymus vulgaris* L. group and reached 85% of increase compared to DW group. It appears from those results that the thyme extract has potential analgesic effects.

The anti-inflammatory potential of Thyme crops was determined in animal treated with plant extract and then with 1% acetic acid. The results of anti-inflammatory effect of Thyme extracts were presented in Table 8. Thyme extract has an anti-inflammatory effect about 64% compared with non-treated animal (DW) injected with normal saline (10 ml/kg) as control. This anti-inflammatory potential represents 68% from that of Diclofenac.

Anxiolytics are known as minor tranquilizers and are prescribed to treat and prevent symptoms of anxiety or for the management of anxiety disorders. Anxiolytic potential of Thyme plant was determined using number of rearing and number of stairs up and down of treated animals. Anxiolytic effect of animals treated with 250 mg/kg of thyme extract was significantly higher than controls after 30min, and reached 16-21% after 60 and 90 min (Table 9). The higher number of stairs up and down was observed for (DW) treated group for 30, 60 and 90min (Table 10). It was twenty-two times higher than that of 250 mg/kg of thyme extract (*Thymus vulgaris* L.) after 30min.

Depression is considered as affective mood disorder, which is characterized by change in mood, lack of confidence. Muscle relaxant is a term usually used to refer to skeletal muscle relaxants (drugs), which act on the central nervous system to relax muscles. Animals were allowed to swim for 6 min, and the durations of immobility were recorded during the last 240 s of the swimming period (Table 11). The maximum effect was about 54%, shown after treatment with Fluoxetine (10 mg/kg). The muscle relaxant effect of Thyme extracts (*Thymus vulgaris* L.) was observed at 250 mg/kg (37%) and represent 68% from that of Fluoxetine drug.

The potential DPPH radical scavenging of Thyme extracts were assayed and results were given in Fig. 2. The electron donation abilities of the extracts follows that of the reducer used as a reference. The rate of reduction

Table 7. Analgesia effect (Latency time in seconds). Animals were divided in three groups each of six mice. Group (DW) was treated with saline (10 ml/kg), group (Tramadol) was treated with TramadolR (5 mg/kg i.p). Group (*Thymus vulgaris* L.) was treated with (250 mg/kg) of thyme extract. After 30 min of treatment, the animals were placed on hot plate and the latency time (time for which mouse remains on the hot platee (55±0.1ºC) without licking or flicking of hind limb or jumping) was measured in seconds. In order to prevent the tissue damage a cut-off time of 30 s were imposed for all animals.

Samples	Dose	30 min	60	90	120
DW	10 (ml/kg)	$5.65±0.22^{a**}$	$5.74±0.13^{a**}$	$4.99±0.16^{a**}$	$5.87±0.38^{a**}$
Tramadol	5 (mg/kg)	$22.54±0.79^{b**}$	$25.98±0.93^{b**}$	$26.98±0.88^{b**}$	$25.98±0.69^{b**}$
Thymus vulgaris L.	0.25 (ml/kg)	$10.5±0.3^{c**}$	$9.5±0.6^{c**}$	$9.2±0.2^{c**}$	$9.4±0.2^{c**}$

Asterisks indicate statistical significant. The values with different superscript letters are significantly different ($p<0.05$).

Table 8. Anti-inflammatory Effect (The number of abdominal constrictions; writhes). Animals were withdrawn from food 2 h before the start of experiment and were divided in three groups each of six mice. Group (DW) was treated normal saline (10 ml/kg) as control. Group (diclofenac) received standard drug diclofenac sodium (10 mg/kg). Group (*Thymus vulgaris* L.) was treated with (250 mg/kg) of thyme extract. Writhes were counted after 5 min of acetic acid injection for the period of 10 min.

Samples	Dose	Writhes
DW	10 (ml/kg)	80.76±5.4^{a**}
Diclofenac	10 (mg/kg)	5.87±0.74^{b**}
Thymus vulgaris L.	0.25 (ml/kg)	28.66±2.08^{c**}

Asterisks indicate statistical significant.
The values with different superscript letters are significantly different ($p<0.05$).

increased proportionally with the concentration of Thyme extracts. It reached approximatively 90-100% with the dose100 µg/ml for Thyme extracts compared with standards.

The Thyme extracts displayed antibacterial activity against two pathogenic bacteria, *B. subtilis* and *S. aureus*, as indicated by diameter of inhibition zones of 10-11 mm (Fig. 3). The standard antibiotic, Tetracycline, show inhibitory activity against *B. subtilis* and *S. aureus*. The Thyme extracts were more active against *B. subtilis* (50%-inhibition zone) than *S. aureus* (42%-inhibition zone) compared with Tetracycline.

In recent years, the interest in herbal medicinal products has risen enormously. There have been little systematic investigations available concerning the extraction conditions for phenolic substances from Thyme crops. In previous work, the extraction optimization of three independent variables of antioxidants from basil leaves was done using response surface methodology [21]. In this paper, we report the influence of three extraction parameters, extraction temperature, extraction time and extraction speed on the response variables on antioxidant activities. Our results suggest that an extract with good antioxidant activity, for a temperature extraction (60ºC), time extraction (15 min) and speed extraction (600 rpm). The R^2 values for DPPH, FRAP and TPC were 0.9720, 0.9853, and 0.9884, respectively. Moreover, the lack-of-fit tests were not significant for all DPPH, FRAP and TPC, which also showed a good fit between the experimental data and the model (Tables 5 and 6). Factors that influence extract quality during the maceration process are time and temperature extraction conditions. It has been found that raising the temperature from 23 to 55ºC has a positive effect on extract quality of active components in *Hypericum perforatum*. In the case of extracts from buckwheat (*Fagopyrum esculentum*) herb, increasing the extraction time is only relevant for the extraction at 25ºC. A longer extraction time does not influence the

Table 9. Anxiolytic effect (The number of rearing). The animals were divided into control, standard and treated groups, consisting of six mice in each group. The control group (DW) was treated with distilled water (10 ml/kg), the standard group (Diazepam) was treated with diazepam (1 mg/kg) and the remaining group (*Thymus vulgaris* L.) was treated with (250 mg/kg) of thyme extract. After 30 min of treatment, the animals were placed on the first step of the elevated stairs and each animal was observed for the number of rearings over a period of 3 min.

Samples	Dose	30 min	60	90
DW	10 (ml/kg)	2.5±0.3^{a**}	3.5±0.4^{b**}	3.5±0.2^{b**}
Diazepam	0.5 (mg/kg)	0.72±0.1^{c**}	0.97±0.1^{d**}	0.94±0.1^{d**}
Thymus vulgaris L.	0.25 (ml/kg)	2.09±0.5^{e**}	1.98±0.4^{e**}	2.98±0.2^{f**}

Asterisks indicate statistical significant. The values with different superscript letters are significantly different ($p<0.05$).

Table 10. Anxiolytic effect (The number of stairs up and down). The animals were divided into control, standard and treated groups, consisting of six mice in each group. The control group (DW) was treated with distilled water (10 ml/kg), the standard group (Diazepam) was treated with diazepam (1 mg/kg) and the remaining group (*Thymus vulgaris* L.) was treated with (250 mg/kg) of thyme extract. After 30 min of treatment, the animals were placed on the first step of the elevated stairs and each animal was observed for the number of steps climbed up over a period of 3 min.

Samples	Dose	30 min	60	90
DW	10 (ml/kg)	40.87±1.5^{a**}	44±1.8^{b**}	33±2^{c**}
Diazepam	0.5 (mg/kg)	10.13±0.9^{d**}	12.80±0.8^{e**}	9.50±0.5^{d**}
Thymus vulgaris L.	0.25 (ml /kg)	18.7±0.6^{e**}	28.7±0.6^{f**}	16.7±0.7^{g**}

Asterisks indicate statistical significant. The values with different superscript letters are significantly different ($p<0.05$).

Table 11. The antidepressant activity. Animals were divided in three groups each of six mice. Group (DW) was treated with distilled water (10 ml/kg) as control. Group (Fluoxetine) received standard fluoxetine (10 mg/kg). Group (*Thymus vulgaris* L.) was treated with (250 mg/kg) of thyme extract. After the above treatment, the animals were allowed to swim for 6 min, and the durations of immobility were recorded during the last 240 s of the swimming period.

Samples	Dose	Duration of immobility
DW	10 (ml/kg)	$114.54\pm8.6^{a**}$
Fluoxetine	10 (mg/kg)	$52.54\pm7.66^{b**}$
Thymus vulgaris L.	0.25 (ml/kg)	$71.27\pm8.01^{c**}$

Asterisks indicate statistical significant. The values with different superscript letters are significantly different ($p<0.05$).

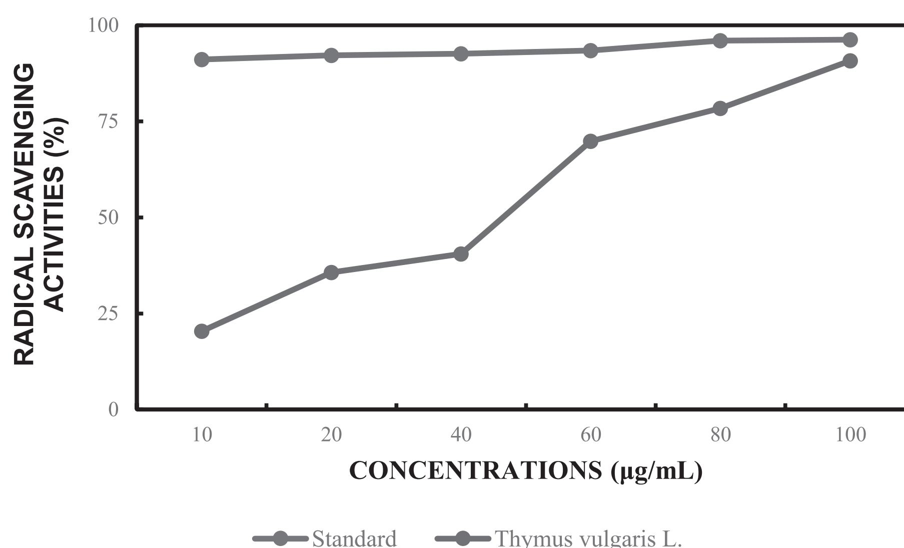

Fig. 2. DPPH radical scavenging assay. The electron donation abilities of the corresponding extracts/fractions (*Thymus vulgaris* L.) and Standards were measured from the bleaching of the purple-colored methanol solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH). Briefly, a 1 mM solution of DPPH radical solution in methanol was prepared and 1 ml of this solution was mixed with 3 ml of the sample (extracts/fractions) solutions in methanol (containing 10-100 µg) for various fraction while (containing 5-100 µg) for pure compounds and control (without sample).

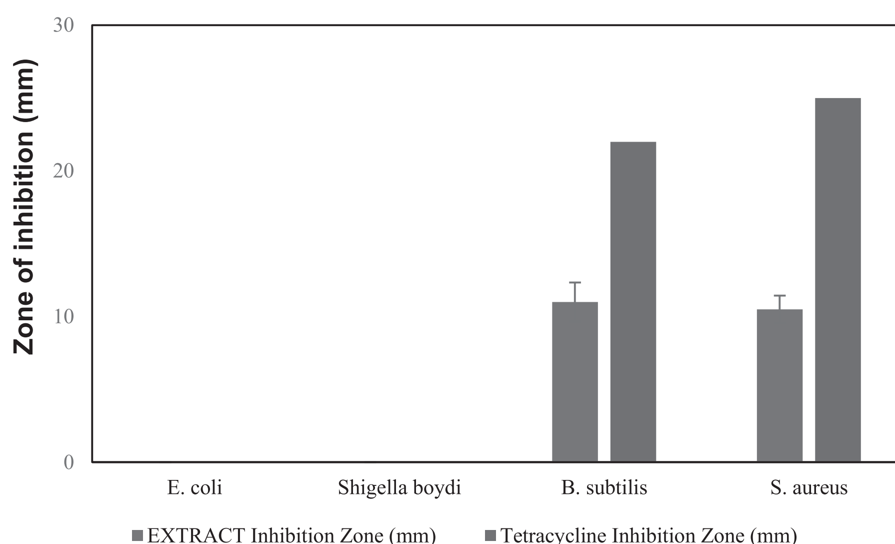

Fig. 3. Anti-bacterial activities The antibacterial potential of *Thymus vulgaris* L. whole plant was determined against four different bacteria (*E. coli*, *S. boydi*, *B. subtilis*, and *S. aureus*) by the standard disk diffusion method Standard antibiotic, Tetracycline at 5 µg/disk were taken as positive controls, while 5% DMSO was used as the negative control.

yield at 60ºC. Conversely, by keeping the temperature at a minimum level (60ºC) for a maximum extraction time period of 120 min produced the highest yields [27]. A prolonged extraction time at 80ºC decrease the extraction yield because the high temperature causes the oxidation and degradation of the desired compounds. At a fixed temperature of 70ºC, an increase in extraction time slightly decreased the yield [27]. Most phenolic compounds are heat-sensitive and easily oxidized [28]. At an extraction time of 120 min but with a minimum extraction temperature of 60ºC, DPPH radical scavenging activity was observed to be greater. A decrease in extraction time had little effect on the DPPH radical scavenging activity [27]. Our results show that extraction temperature, extraction time and speed affect positively on the DPPH, FRAP and TPC levels ($p<0.05$) (Fig. 1). Results were is in accordance with the study, which articulated that agitation increased the mass transfer coefficient, which reduces the size of the boundary layer surrounding the material and impairs the extraction process.

Animals treated with Thyme extract (250 mg/kg) presented potential analgesic, anti-inflammatory, anxiolytic, antidepressant, antioxidant and anti-bacterial effects varied according to time of exposure. Stable analgesia effect (85%) was observed only for Thyme extract after 120 min. When we compare data concerning Thyme plant extract in the scientific literature, variably of effects is dependent on a number of factors, such as the plant environment, geographical region and cultivation [29]. The observed analgesia effect in the present work compared with data concerning the chemical composition of Thyme crops in other work can explained in part the influence of extraction method on Thyme effect. Raskovic et al. [30] show that Thyme syrup and tincture exhibited effective analgesic activity in the hot plate pain model. Moreover, pre-treatment with Thyme formulations reduced analgesic activity of codeine, and potentiated the analgesic activity of paracetamol. Co-administration of Thyme formulations has led to potentiation of diazepam and pentobarbital depressive central nervous system effects. Authors suggested that Thyme formulations interacted with tested conventional drugs, probably through interference with their metabolic pathways and succeeding altered concentrations and pharmacological effects.

Results also show that Thyme extract induced significantly anti-inflammatory effects. Results were in agreement with those from Ocaña and Reglero [31], they showed changes on production and gene expressions of the proinflammatory mediators were dose dependent and according to the Thyme content of each species. Cytokines are considered to be key players in the inflammatory response. The action of Thyme fractions appears to involve the expression of the proinflammatory cytokines. Proinflammatory cytokines levels decreased in a dose-dependent manner with any Thyme fraction from *Thymus vulgaris, Thymus zygis,* or *Thymus hyemalis* used (after 24 or 48h of incubation).

Anxiolytics regulated by targeting key chemical messengers in the brain to help decrease disorder in excitability. Anxiolytic effect of Thyme extracts increased significantly with dose and time of treatment in rates. The anxiolytic activity dependent on the flavonoid, essential oil content, and other substances implicated in antioxidant activity [28]. Most of the essential oils that used in clinical research had been proved to be anxiolytic in animal models. Essential oils from medicinal plants have also been reported to be anxiolytic. However, the essential oils might vary greatly due to the species, origin place and extraction method and conditions [32].

The occurrence of depression has been found to be associated with the alterations in the levels of biogenic amines in the brain such as dopamine and epinephrine, indolamine, serotonin, 5-hydroxytryptamine (5-HT) and two catecholamines. Antidepressants aim to correct chemical imbalances of neurotransmitters in the brain that are believed to be responsible for changes in mood and behavior [33]. The antidepressants block the reuptake of both norepinephrine (NE) and serotonin (5HT). This phenomenon being the primary mechanism of actions of antidepressants brings changes in the physiological behaviour of neuro-receptors. Antidepressant effect was observed with Thyme extract. There has been an increase, worldwide, in the use of medicinal plants and herbs for developing nutraceuticals for treatment of depression [34]. At the dose of 200 mg/kg body weight, *O bracteatum* showed significant potential which was similar to that standard diazepam and fluoxetine (35).

The potential DPPH radical scavenging of Thyme extracts reached approximately 90-100% with the dose100 µg/ml for Thyme extract compared with standards. Despite demonstrated antioxidant activity, mediated through both direct free radical scavenging and activation of antioxidant activities, Thyme extraction conditions ameliorate antioxidant potential. Molecular mechanisms of diverse effects of Thyme preparations on pharmacological properties should be more discussed due to interaction with the chemical composition. Approximately 200 compounds have been extracted from plants with clarified molecular structures that were classified as glucosides, bibenzyls, phenanthrenes, quinones, biphenanthrenes, dihydrophenanthrenes, anthocyanins, steroids, triterpenoids, and phenolic acids. The published studies of the last 30 years discussed the interaction between chemical constituents, their pharmacologic activities, and clinical applications of medicinal plants [36]. Extraction conditions ameliorate antioxidant potential depending on plant species. These results were in agreement with those of results of effect of different

extraction conditions on the antioxidant potential of Baru almonds [37]. Values of antioxidant potential determined previously by various extraction methodologies with different varieties present high divergence due to extraction conditions. The antibacterial activity of Thyme extracts was tested against clinical bacterial strains of *E. coli, S. boydi, B. subtilis*, and *S. aureus*. The results of experiments showed that the Thyme extracts exhibited extremely strong activity against *B. subtilis* and *S. aureus*. Thyme oil demonstrated a good efficacy against antibiotics resistant strains of *Staphylococcus*, *Enterococcus*, *Escherichia* and *Pseudomonas* genera. Antibacterial effects of Thyme essential oils on some food-borne bacteria were determined by broth microdilution method [38].

Authors show that Thyme essential oils exhibited antibacterial activity against *Salmonella Enteritidis*, *Salmonella Thyphimurium*, *Staphylococcus aureus*, *methicillin resistant Staphylococcus aureus*, *Escherichia coli* and *Bacillus cereus*, and MICs).

Conclusions

The promising analgesic, anti-inflammatory, anxiolytic, antidepressant, antioxidant and anti-bacterial effects of *Thymus vulgaris* L. crop in relation with extraction conditions was analyzed. The obtained results present potential effects of the plant extracts but vary widely not only from the plant species but also especially from the extraction conditions and time of exposure of treated animals to plant extracts. The valorization of medicinal plants in the pharmaceutical field could be improved following an optimization of the extraction conditions, which increased the effectiveness of plant extracts and preserved the chemical composition of these extracts in terms of therapeutically active natural substances. Response surface methodology employed to determine the best combination of variables for optimum extraction yield might be reasonable.

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Disclosure of Interest

The author declares that he has no conflict of interest.

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