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Chemical and biological studies of Thyme based on different solvents extraction

Yasser A. Selim *, Hany Helmy, Mennat-allah E. Hamed & Nehal Abdel-latif

Faculty of Specific Education, Zagazig University, Zagazig, 44519, Egypt.

Abstract

Thyme was employed in this work to analyze the overall composition using its methanolic and ethanolic extracts. protocatechuic acid, p-hydroxybenzoic acid, rosmarinic acid, cateachin, caffeic acid, apigenin, kaempferol, ferulic acid, and rutin were the most potant bioactive compounds of thyme extracts. These substances have antimicrobial properties against G+ bacteria and G- bacteria. It is also necessary to test thyme and its extracts for their efficacy *in vivo* in order to confirm the findings of the current investigation. With respect to G+ bacteria (*Bacillus cereus* and *Staphylococcus aureus*), G- bacteria (*Escherichia coli and Pseudomonas aeruginosa*), and fungi (*Candida albicans*), the ethanolic and methanolic extracts have anti-microbial activity (Ampicillin as antibacterial agent and Amphotericin B as antifungal agent).

Keywords: Thymus; polyphenolic; antimicrobial activity; HPLC analysis.

1. Introduction

Lamiaceae plants mainly thyme have long been traditionally used as medication against infections and commercialized due to their biological activities.[1], [2] Thyme is indigenous in the Mediterranean specially Northern Africa, widely cultivated in Egypt.[3] Thyme is a perennial flowering aromatic plant. The Greek word of ‘thyme’ means ‘to fumigate’, for it is one of sweet-smelling herbs.[4] The genus Thymus is composed of around 215 species has been utilized as a food supplement or as an herbal remedy for medicinal properties, such as antispasmodic, digestive, carminative, antiseptic, antitussive, and expectorant.[5]–[7]

The thyme plant is used in the treatment of several intestinal infections, uni- and multi-cellular fungi, pathogenic Gram-positive and Gram-negative bacteria.[3], [8], [9] The polyphenols have been examined far less than the essential oils of thyme. In many thyme species, rosmarinic acid is the main component. In addition, derivatives of flavones such as apigenin and luteolin were identified.[10]

The antibacterial potential of apigenin has been tested against many bacteria species and various strains within the same species. Apigenin has been shown to possess antibacterial, antiviral, antifungal, and antiparasitic activities.[11]

Beside gallic acid, its derivatives are also widely used in the food, chemical, pharmaceutical and cosmetic industries. Some studies show that these substances present therapeutic potential, especially as antimicrobial, in addition to acting as reactive oxygen species sequesters.[12]

Protocatechuic (PCA) displays broad-spectrum antibacterial activity against species such as Escherichia coli, Pseudomonas ceruminous, Staphylococcus aureus, Bacillus cereus, Streptococcus pneumoniae, Acinetobacter barramundi and Helicobacter pylori.

Caffeic (CA), coumaric and ferulic acids, the major representative of phenolic acids, are present in many natural plants including thyme. The antibacterial activity of CA against S. aureus strains was observed. It has been shown that among many polyphenolic compounds as caffeic acid could be considered as one of the most potent and promising antimicrobial agents.[13-15]

Rosmarinic acid (RA) is an ester of caffeic acid. The antibacterial activity of rosmarinic acid have been reported.[16-19]

Therefore, the present study is carried out with the aim of Estimating the effect of phytochemical composition, the nutritional value, the preference to use thyme as extract, and investigating the antimicrobial activities of biological active thyme extracts.

*Corresponding author e-mail: y2selem@yahoo.com

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Materials and Methods

General.

All chemicals of high grade of purity were obtained from Sigma-Aldrich. Analyses, qualitative and quantitative measurements involved the use of the following tools and systems: IKA® RV 10B rotary evaporator, Agilent Technologies 1100 series liquid chromatograph equipped with an auto sampler and a diode-array detector. The analytical column was an Eclipse XDB-C18 (150 X 4.6 µm; 5 µm) with a C18 guard column (Phenomenex, Torrance, CA), ICP & microwave devices. Spectronic (Milton Roy) spectrophotometer.

Plant collection.

Dried leaves of Thymus (*Thymus vulgaris*, Lamiaceae) were bought in July 2021 from local market Haraz - Agricultural Seeds, Herbs and Medicinal Plants Company at 1 Ahmed Maher St, Al-Darb Al-Ahmar, Cairo Governorate, Egypt.

Plant preparation and extraction.

Dried leaves were finely ground into a fine powder. The alcoholic extracts were prepared by the cold maceration method. The plant powder (100 g) was soaked per 1 L of Ethanol absolute, kept at 25 °C for 72 hr. Then the extract was filtered and stored at 25 °C. Then the extract was evaporated by Rotary apparatus, then the powder extract was ready for further investigations. The plant powder (100 g) was soaked per L of Methanol absolute, kept at 25 °C for 72 hr. Then the extract was filtered and stored at 25 °C. Then the extract was evaporated by Rotary apparatus, then the powder extract was ready for further investigations.[6, 20, 21]

For HPLC, Sample (1g) was placed in quick fit conical flask and 20 ml of 2M NaOH was added and the flasks were flushed with N2 and the stopper was replaced. The samples were shacked for 4 h at room temperature. The pH was adjusted to 2 with 6 M HCl. The samples were centrifuged at 5000 rpm for 10 min and the supernatant was collected. Phenolic compounds were extracted twice with 50 ml ethyl ether and ethyl acetate 1:1. The organic phase was separated and evaporated at 45°C and the samples re-dissolved in 2ml methanol.[22]

Phytochemical screening.

Preliminary phytochemical screening of the extract was carried out to identify the active constituents, using standard methods. [23-27]

Chemical composition analysis.

Grinded fine powder of dried leaves, dried Ethanol extract and dried Methanol extract were used for analysis of total composition; total protein % [28], total fat % [29], Total fiber % [30], humidity % [31], and total ash % [32]. For digestion of Thyme, take 0.2 gm of dried thyme powder gm HNO_3 results in clear pale yellow solution. Method on device (microwave):

Step	Temperatur	Time (min)	Pressure (Ps
1	120	2	15
2	150	2	20
3	180	4	25

Then wait until vessels have chilled to room temperature carefully open the digested vessel in a fume hood waring hand, eye and body protection. Then take 0.2 gm into 50 mL deionized water. The sample is ready to do in the ICP device to measure the elements: Na, k, Mg, Se, and Zn. [33]

Determination of Polyphenolic compounds.

HPLC analysis was carried out using Agilent Technologies 1100 series liquid chromatograph equipped with an auto sampler and a diode-array detector. The analytical column was an Eclipse XDB-C18 (150 X 4.6 µm; 5 µm) with a C18 guard column (Phenomenex, Torrance, CA). The mobile phase consisted of acetonitrile (solvent A) and 2% acetic acid in water (v/v) (solvent B). The flow rate was kept at 0.8 ml/min for a total run time of 70 min and the gradient program was as follows: 100% B to 85% B in 30 min, 85% B to 50% B in 20 min, 50% B to 0% B in 5 min and 0% B to 100% B in 5 min. The injection volume was 50 µl and peaks were monitored simultaneously at 280 and 320 nm for the benzoic acid and cinnamic acid derivatives, respectively. All samples were filtered through a 0.45 µm Acro disc syringe filter (Gelman Laboratory, MI) before injection. Peaks were identified by congruent retention times and UV spectra and compared with those of the standards.[22]

Microbiological analysis.

Antimicrobial activity of the tested samples was determined using a modified Kirby-Bauer disc diffusion method[34]. Briefly, 100 µl of the test bacteria/fungi were grown in 10 ml of fresh media until they reached a count of approximately 108 cells/ml for bacteria or 105 cells/ml for fungi.[35]

100 µl of microbial suspension was spread onto agar plates corresponding to the broth in which they were maintained . Isolated colonies of each organism that might be playing a pathogenic role should be selected from primary agar plates and tested for susceptibility by disc diffusion method [36].

Plates inoculated with filamentous fungi as *Aspergillus flavus* at 25oC for 48 hours; Gram (+) bacteria as *Staphylococcus aureus, Bacillus cereus*; Gram (-) bacteria as *Escherichia coli, Pseudomonas aeruginosa* they were incubated at 35-37oC for 24-48 hours and yeast as *Candida albicans* incubated at 30oC for 24-48 hours and, then the diameters of the inhibition zones were measured in millimeters[6], [34], [37].

Standard discs of Ampicillin (Antibacterial agent), Amphotericin B (Antifungal agent) served as positive controls for antimicrobial activity but filter discs impregnated with 10 µl of solvent (distilled water,

chloroform, DMSO) were used as a negative control . Blank paper disks (Schleicher & Schuell, Spain) with a diameter of 8.0 mm were impregnated 10µ of tested concentration of the stock solutions . When a filter paper disc impregnated with a tested chemical is placed on agar, the chemical will diffuse from the disc into the agar. This diffusion will place the chemical in the agar only around the disc. The solubility of the chemical and its molecular size will determine the size of the area of chemical infiltration around the disc. If an organism is placed on the agar, it will not grow in the area around the disc if it is susceptible to the chemical. This area of no growth around the disc is known as a "Zone of inhibition" or" Clear zone." For the disc diffusion, the zone diameters were measured with slipping calipers of the National Committee for Clinical Laboratory Standards. [38]

Agar-based methods such as Etest and disk diffusion can be good alternatives because they are simpler and faster than broth-based methods.[39-41]

Results and Discussion

Phytochemical study.

All results of phytochemical analysis are showed in Table 1. In the present study, the powder and the extracts showed positive results for terpenoids and steroids as measured by the Liebermann-Burchard reaction. It was found that thyme extracts contain polyphenols and flavonoids, which may be responsible for the biological activities found.

Table 1 . Phytochemical screening of Thyme extract. Highly positive '+++', Moderate '++', Positive '+', Negative '−'

Terpe noid	Ster oid	Alkal oid	Sapo nin	Flavo noid	Coum arin
+	+++	+	++	+++	++

Chemical composition analysis.

Analysis of total composition; total protein %, total fat %, Total fiber %, humidity, and total ash % results of grinded fine powder of dried leaves, dried ethanol extract and dried Methanol extract are shown in Table 2. Results of elemental analysis (ICP) test showed the presence of Na (29.831), K, (151.636), Mg (40.710), Se (0.6295) and Zn (3.7022). these results evidence the high constituents of potassium and low amount of the sodium revealed that the hypotension effect of the tested plant and advise for the patients of cardiology with hypertensive

Table 2. Chemical composition of powder of dried leaves, ethanol extract, and methanol extract of thyme.

Sample	Total protein (%) ± SE	Total fat (%) ± SE	Total fiber (%) ± SE	Humidity (%) ± SE	Total ash (%) ± SE
Dried leaves powder	14.6 ± 0.56	2.47 ± 0.07	15.12 ± 1.13	11.49 ± 0.21	10.45 ± 0.10
Ethanol extract	3.9 ± 0.15	17.18 ± 0.49	15.9 ± 1.18	28.0 ± 0.50	2.76 ± 0.027
Methanol extract	4.3 ± 0.16	13.74 ± 0.39	4.17 ± 0.31	39.3 ± 0.71	7.54 ± 0.074

Determination of Polyphenolic compounds.

HPLC analysis indicates the presence of gallic acid, protocatechuic acid, p-hydroxybenzoic acid, gentisic acid, cateachin, chlorogenic acid, caffeic acid, syringic acid, vanillic acid, ferulic acid, sinapic acid, p-coumaric acid, rutin, rosmarinic acid, apigenin-7-glucoside, cinnamic acid, qurecetin, apigenin, kaempferol, and chrysin (Fig. 1& Table 3) that might have been responsible for their therapeutic potential. The amounts and structures of polyphenols are shown in Table 3 & Fig. 2.

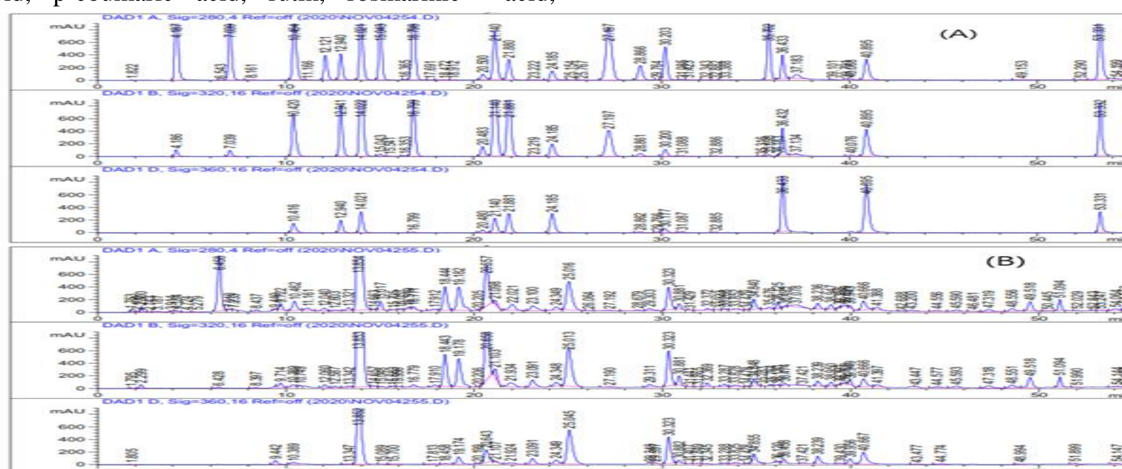

Figure 1. HPLC chromatogram: (A) Standard mixture of polyphenolic compounds; (B) wild thyme extract

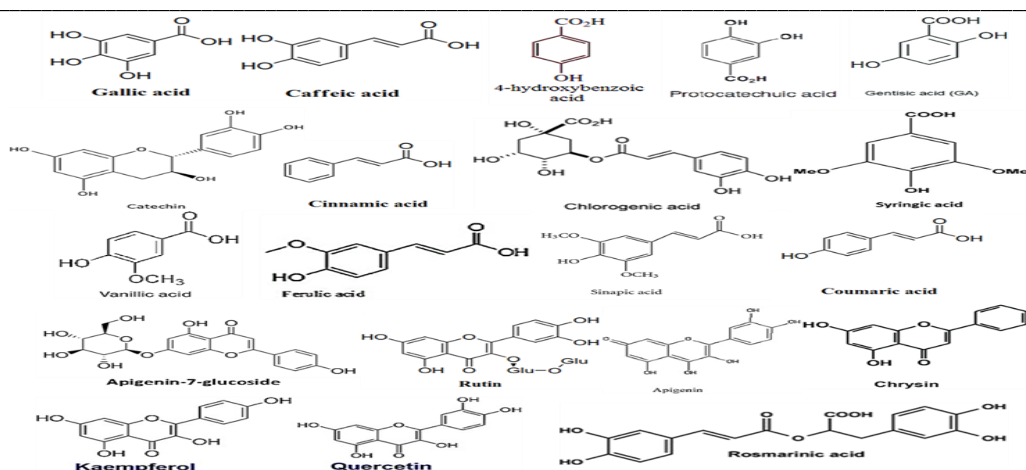

Figure 2. Chemical structures of polyphenolic compounds

Table 3. Polyphenolic compounds of wild thyme extract

Compound	Concentration (µg/g)	Compound	Concentration (µg/g)
Gallic acid	4.37	**Sinapic acid**	58.39
Protocatechuic acid	1331.91	***p*-coumaric acid**	21.93
***p*-hydroxybenzoic acid**	220.56	**Rutin**	182.26
Gentisic acid	0.00	**Rosmarinic acid**	143.47
Cateachin	209.70	**Apigenin-7-glucoside**	0.00
Chlorogenic acid	21.89	**Cinnamic acid**	20.07
Caffeic acid	2391.19	**Qurecetin**	38.05
Syringic acid	94.55	**Apigenin**	100.64
Vanillic acid	9.75	**Kaempferol**	186.63
Ferulic acid	559.51	**Chrysin**	0.00

Microbiological analysis.

As shown in table 4 and figure 3.

Table 4. Microbiological analysis results.

Sample	Inhibition zone diameter (mm/mg sample)					
	Bacterial species				Fungal species	
	G^+		G^-			
	Bacillus cereus	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Aspergillus flavus*	*Candida albicans*
ATCC	14579	6538	8739	9027	9643	10231
	--	--	25 ± 0.0	27 ± 0.0	--	--
Standard	28 ± 0.0	27 ± 0.0	--	--	--	--
	--	--	--	--	17	21 ± 0.0
Control: DMSO	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0	0.0 ± 0.0
Ethanol extract	18.33 ± 0.577	20.67 ± 1.155	18.67 ± 1.155	20.67 ± 0.577	0.0	12 ± 1.0
Methanol extract	21.33 ± 1.155	22.67 ± 3.06	20.67 ± 1.155	22 ± 0.0	0.0	12.33 ± 0.577
Thyme powder	15 ± 0.0	15.67 ± 1.155	15.67 ± 1.155	13 ± 0.0	0.0	0.0 ± 0.0

G: Gram reaction; Solvent: DMSO.

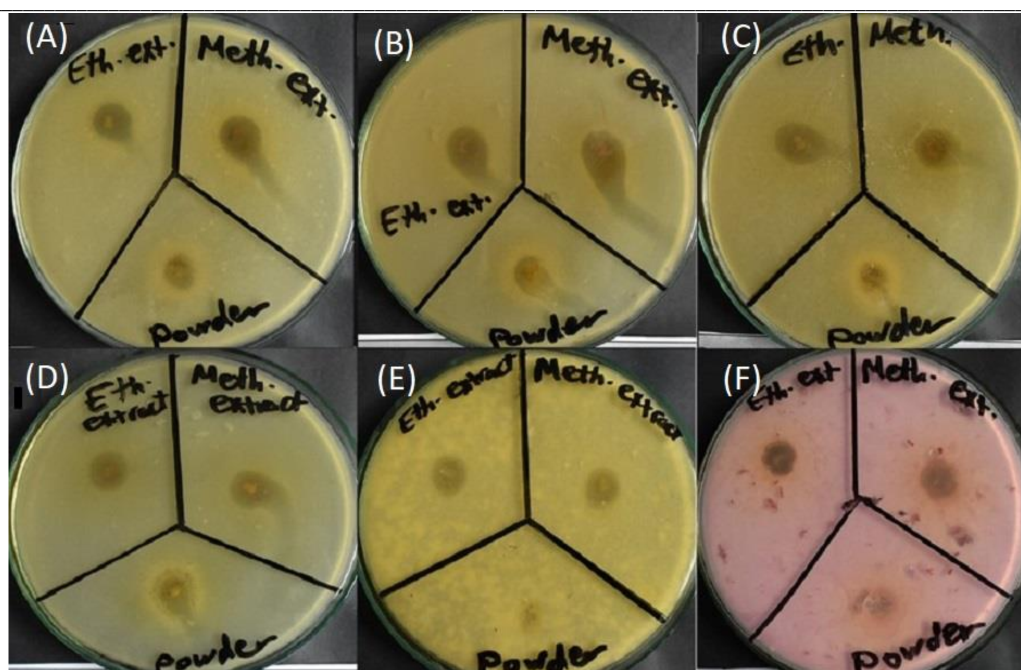

Figure 3. microbiological analysis inhibition zones; **A)** *Bacillus cereus*, **B)** *Staphylococcus aureus*, **C)** *Pseudomonas aeruginosa*, **D)** *Escherichia coli*, **E)** *Candida albicans* and **F)** *Aspergillus flavus*.

Conclusion

Thyme and its methanolic and ethanolic extracts were utilized in this study to analyze the herb's overall composition. According to the results of the phytochemical analysis of thyme extracts, protocatechuic acid, p-hydroxybenzoic acid, rosmarinic acid, cateachin, caffeic acid, apigenin, kaempferol, ferulic acid, and rutin are active components with a high polyphenolic content that have antimicrobial properties against G+ bacteria (*Bacillus cereus* and *Staphylococcus aureus*), G- bacteria (*Escherich coli*). Additionally, thyme and its extracts need to be tested for their in vivo application in order to confirm the findings of the current study.

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Authors contribution

Y.S. and H.H. conceived and designed the experiments; Y.S. and M.E. performed the experiments; H.H . analyzed the data; Y.S. and M.E. Contributed reagents/materials/analysis tools; Y.S., H.H., N.A. and M.E. wrote the paper. Y.S. and H.H. reviewed the paper.

Competing interests

The authors declare no competing interests.

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