



## ***In-vitro* Evaluation of Antimicrobial and Antioxidant Efficacy of Thyme (*Thymus vulgaris* L.) Essential Oil**

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### **ABSTRACT**

The present study was designed to investigate *in-vitro* antimicrobial and antioxidant efficacy of Thyme essential oil (TEO) for its potential encapsulation in nano delivery systems and further application in meat products. For computation of antimicrobial efficacy, zone of inhibition assays and Minimum inhibitory concentration (MIC) method against Gram-positive (*Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Enterococcus faecalis*) and Gram-negative (*Salmonella enterica* serovar Typhi, *Escherichia coli*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Klebsiella pneumoniae*) organisms was done. In general, there was a strong inhibitory effect with zone size ranging from 27.5 mm to 45 mm against tested organisms, however, the effect was slighter higher against Gram positive bacteria than Gram negative bacteria. The antioxidant activity for various oil concentrations as determined by 1 diphenyl-2picrylhydrazyl (DPPH) and 2-2-azinobis-3ethylbenthiazoline-6-sulphonic acid (ABTS) methods revealed that radical scavenging activity ranged between 8.14% to 78.73% and 6% to 67.75% for DPPH and ABTS assay, respectively. It can be concluded that thyme essential oil possesses significant antimicrobial and antioxidant activity and may be encapsulated in nano delivery systems for potential application in any meat matrices.

**Keywords:** Thyme essential oil, antimicrobial, antioxidant, ABTS, DPPH

Meat has expanded its vital role in human evolution and is an important component of well-balanced diet attributing to its nutritional richness. Therefore, safety of meat and meat products is of greater concern in modern society (Mehta *et al.*, 2013, 2015; Pereira and Vicente, 2013). The major issue with meat quality is deterioration caused by microbial spoilage and lipid oxidation which makes it unacceptable by consumers. To combat this issue, meat industry is widely using synthetic GRAS preservatives. However, with growing concern on the adverse health impact of these synthetic preservatives, demand for natural additives with equally good antimicrobial and antioxidant properties has increased multifold (Alves-silva *et al.*, 2013; Govaries *et al.*, 2010). This has prompted meat industry to look forward to antimicrobials and antioxidants

of plant origin. Since antiquity, herbs and spices have been used in local medicines and to boost flavor, aroma, and appearance of foods. In recent years, essential oils and their components obtained from these herbs and spices have played an important role, owing to their safe and versatile functional properties (Bounatirou *et al.*, 2007; Sawamura, 2000). Essential oils are aromatic oily liquids obtained as a secondary metabolite from plants and are a valuable source of bioactive molecules which possess strong antioxidant and antimicrobial activity against many foodborne bacteria (Ahmad and Viljoen, 2015). Components of these oils are hydrophobic in nature, which makes them pass easily through bacterial cell membrane, interfering with molecular transport mechanism leading to cell death (Goni *et al.*, 2009; Burt, 2004) and has

proven to be potent natural food additives by extending shelf-life of foodstuffs (Chouliara *et al.*, 2007). Thyme essential oil obtained from Thyme (*Thymus vulgaris* L) of *Lamiaceae* family, is characterized by the presence of thymol and p-cymene as its major constituents (Grigore *et al.*, 2010; Rota *et al.*, 2008). Recent works show that *Thymus* species possess strong antibacterial, antifungal, antiviral, carminative, spasmolytic and antioxidant activities (Baranauskiene *et al.*, 2003; Stahl-Biskup and Saez, 2002). The aim of this study is to investigate and evaluate *in vitro* antioxidant and antimicrobial potential of thyme essential oil for its effective application in meat products substituting synthetic additives.

## MATERIALS AND METHODS

### Thyme essential oil: Source and composition

Thyme essential was procured from Moksha Lifestyles Products, New Delhi, India. The detailed composition as per GC-MS analysis is presented in Table 1. The oil had yellow red to dark red clear appearance with characteristic spicy odour. The specific gravity was 0.880 @ 20°C and refractive index was 1.485 to 1.510 @ 20°C. All the reagents and chemicals used in study were of analytical grade and procured from reputed firms.

**Table 1:** Chemical composition (%) of Thyme essential oil (GC-MS analysis)

Sl. No.	Name of active compound	%age
1	γterpinene	32.60
2	Thymol	29.10
3	p-cymene	23.56
4	Phenol derivatives	2.35
5	βPinene	1.96
6	2,6,6- Trimethylbicyclo[3.1.1] hept-2-ene	1.53
7	D-Limonene	1.12
8	Apiol	0.52
9	Camphene	0.20
10	Miscellaneous minor constituents	7.06

### Bacterial strains

Pure freeze dried cultures of common food borne pathogens were procured from Institute of Microbiology

Technology (IMTECH), Chandigarh, India. Four Gram positive organisms viz. *Bacillus cereus* (MTCC 1272), *Enterococcus faecalis* (MTCC 890), *Listeria monocytogenes* (MTCC 1143) and *Staphylococcus aureus* (MTCC 96) and six Gram negative organism viz. *Escherichia coli* (MTCC 723), *Salmonella enterica serovar typhi* (MTCC 733), *Shigella flexneri* (MTCC 1457), *Pseudomonas aeruginosa* (MTCC 74), *Proteus mirabilis* (MTCC 425) and *Klebsiella pneumoniae* (MTCC 109) were used in study. These cultures were revived and stock cultures were prepared and maintained at -80°C by regular passaging.

### Antimicrobial Activity: Inhibition effect by agar well diffusion Assay

The antimicrobial potential of thyme essential oil against common foodborne spoilage and pathogenic organisms listed above was determined by agar well diffusion method as per method followed by Kalemba and Kunicka (2003), with slight modifications. Bacterial cultures were rejuvenated in sterile BHI broth by incubating overnight for 37°C. Later, the density of each bacterial suspension was adjusted equal to 0.5 McFarland standard. BHI agar plates were prepared and kept undisturbed for 24 hours. 100µl of each inoculum was spread on agar surface and well of 7mm diameter were bored using sterile cork borer. 100µl of Thyme essential oil was poured in each well and to hasten diffusion of oil, plates were pre-incubated at 4°C for 1 hour followed by overnight incubation at 37°C. The antibacterial activity as indicated by Zone of Inhibition (ZoI) surrounding the well containing essential oil was measured using a zone scale (Hi-Media) and expressed in millimetre. All the tests were performed in duplicate, and the mean values of the diameter of inhibition zones were recorded.

### MIC by micro-well method

The minimal inhibition concentration values of thyme oil were estimated against tested bacterial strains using the method described by Gulluce *et al.* (2003). The inoculum of microorganisms was prepared using 12-hour broth cultures and density of suspension was adjusted to 0.5 McFarland standard. Thyme oil was dissolved in 10% dimethylsulfoxide (DMSO) and then serial two-fold dilutions were made in a concentration range from

0.025% to 2% using nutrient broth. 100µl of thyme oil was added to 96 well microtiter plate containing 100µl of each inoculum and nutrient broth and negative control was made by dispensing inoculum in nutrient broth. The suspension was mixed using micro-pipette and incubated at 37°C for 24 hours. After incubation, 100 µl of each sample was taken from the respective wells and spread on nutrient agar plates to check the visible growth of bacteria on overnight incubation.

#### Antioxidant activity of thyme essential oil

The antioxidant activity of thyme essential oil was assessed spectrophotometrically in terms of radical scavenging activity or hydrogen donating ability using 2, 2'-azinobis-3-ethylbenzthiazoline-6-sulphonate (ABTS) radical and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. Ability of essential oil to donate hydrogen atoms or electrons was measured from bleaching of coloured methanolic solutions.

#### 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical assay

Free radical scavenging activity of Thyme essential oil was measured using a methanolic solution of stable free radical (DPPH). The method of Blois (1958) was used to study the effect of various oil concentrations on DPPH radicals with slight modifications. A solution of DPPH (0.15mmol/L) in methanol was prepared. Oil concentrations ranging 0.01% to 2% were prepared in methanol and 200µl of each dilution was mixed with 50µl of DPPH solution in a 96-well microtiter plate. The mixture was incubated in dark for 30 min at room temperature and the decrease in absorbance was measured at 517 nm. Butylated hydroxytoluene (BHT) served as synthetic references. The radical scavenging activity was expressed as % radical Inhibition and was calculated using the following formula:

$$\% \text{ radical Inhibition} = \frac{\text{Ab control} - \text{Ab sample}}{\text{Ab control}} \times 100$$

where, Ab control: Absorbance of methanolic DPPH; Ab sample: Absorbance of DPPH +sample dilution.

#### 2, 2'-azinobis-3-ethylbenzthiazoline-6-sulphonate (ABTS) radical assay

The ABTS<sup>+</sup> decolourization activity of thyme essential oil

was determined as described by Yang *et al.* (2010) and Kanth *et al.* (2018) with slight modifications. The ABTS cation solution was prepared by mixing ABTS (14 mM) with an equal amount of (4.95 mM) potassium persulphate. Diluted ABTS radical cation with the absorbance of 0.7 at 732 nm was used for assay. Further, 50µl of various concentrations of thyme oil was mixed with 150µl of ABTS solution and after 1 min incubation at room temperature, absorbance values were measured at 732 nm. The cation scavenging activity was measured similar to DPPH assay

$$\% \text{ radical Inhibition} = \frac{\text{Ab control} - \text{Ab sample}}{\text{Ab control}} \times 100$$

#### STATISTICAL ANALYSIS

Data was analyzed statistically on 'SPSS-16.0' (SPSS Inc., Chicago, II USA) software package as per standard methods (Snedecor and Cochran, 1989). Whole set of experiment was repeated three times and the mean values were reported along with standard error.

#### RESULTS AND DISCUSSION

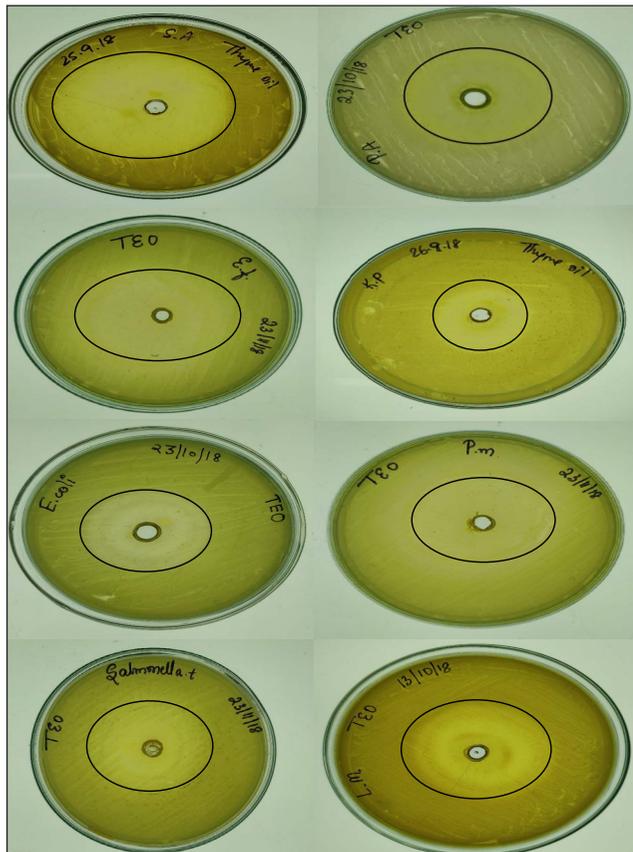
##### Composition of Thyme essential oil

The effect of any essential oil depends on concentration of its key components. In present study, the oil used was analysed by gas chromatography coupled with mass spectrophotometry. The percent composition of volatile compounds was calculated and qualitative analysis was carried out on basis of percent area of each peak of oil component. Mass spectrum of each component was compared with NIST 98 spectrum library. Nine components representing 92.94% of total detected constituents were identified. The major components in thyme essential oil under test were γ-terpinene (32.6%), thymol (29.1%) and p-cymene (23.56%) (Table 1). The composition is highly variable depending upon region, season and time of harvesting. Similar findings have been reported by Boruga *et al.* (2014) for *Thymus vulgaris* essential oil.

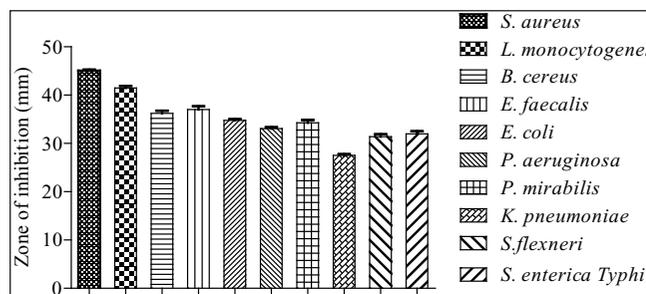
##### Antimicrobial activity and MIC of Thyme essential oil

*In-vitro* antimicrobial activity of thyme essential oil was studied against ten food borne bacterial strains using agar well diffusion technique and a significant degree of

inhibition was found against all tested strains with zone size ranging between 27.5 mm to 45 mm (Fig. 1 and 2).



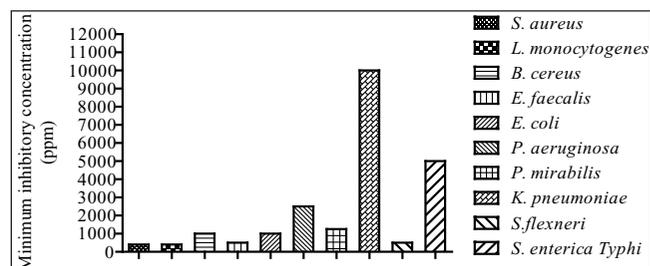
**Fig. 1:** Zone of inhibition assay (mm) of Thyme Essential Oil (TEO) against common food spoilage and pathogenic microorganisms



**Fig. 2:** Zone of inhibition assay (mm) of Thyme Essential Oil against common food spoilage and pathogenic microorganisms (Mean  $\pm$  S.E.), n=6

Maximum growth inhibition as evident by larger zone of inhibition diameter was reported against *Staphylococcus*

*aureus* followed by *Listeria monocytogenes* and *Enterococcus faecalis*. MIC is the lowest concentration at which visible growth of microorganisms is inhibited (Onawunmi, 1989) and for thyme oil it ranged from 400 ppm to 10000 ppm (Fig. 3). The results of zone inhibition assays corresponded to the MIC values and it was found that gram-positive organisms were comparatively more sensitive for thyme oil as compared to gram-negative organisms. It could be due to difference in their cell wall structure as gram positive bacterial cell wall is more permeable to hydrophobic molecules which can act on both wall structure as well as cytoplasm leading to destruction. The present findings were in agreement with Nezhadali *et al.* (2014) who reported that thyme species showed a better inhibitory effect against Gram-positive organisms than Gram-negative organisms. Sienkiewicz *et al.* (2012) investigated the antimicrobial activity of thyme essential oil against multidrug resistant clinical bacterial strains of *Staphylococcus*, *Enterococcus*, *Escherichia* and *Pseudomonas* genus using agar well diffusion technique and reported that oil possessed strong inhibitory activity against all tested strains.



**Fig. 3:** Minimal Inhibitory Concentration (ppm) of Thyme Essential Oil against common food spoilage and pathogenic microorganisms (Mean values), n=6

Miladi *et al.* (2013) studied antibacterial activity of essential oil of *Thymus vulgaris L.* and *Rosmarinus officinalis L.* against foodborne pathogens by a disc-diffusion method and microdilution method. They found that thyme oil had highest bactericidal activity than rosemary oil and similar to our findings, they reported highest inhibition diameter against Gram-positive organisms (26 to 41 mm) with MIC and MBC value between 0.78 to 3.12 mg/ml for all the test organisms. Alizadeh (2013) noticed a high degree of correlation between p-cymene and  $\gamma$ -terpinene with thymol components and reported that strong antimicrobial effect found against all tested organisms might be because of the

synergistic action of one or more components present in the TEO. A similar correlation between antimicrobial activity and chemical components of thyme oil were reported by Rota *et al.* (2008) and Boskovic *et al.* (2015). There is a slight variation in obtained values for zone diameters and MIC, when compared with work carried out by various workers that can be attributed to greater variability in the chemical composition of TEO under study. This variability in composition occurs mainly because of variation in plant type and origin, method of extraction of Essential oils and methods involved to assess antimicrobial activity (Kanth *et al.*, 2018; Kumar *et al.*, 2017).

### Antioxidant activity of thyme essential oil

*In vitro* antioxidant assays mimic the oxidation-reduction pathways commonly occurring in biological systems and are helpful in estimating antioxidant potential of various biomolecules. The results for antioxidant activity of TEO as determined by using two different radical scavenging assays has been presented in Table 2. DPPH, a stable free radical, produces violet colour in methanol and upon reaction with antioxidants (that donates hydrogen), changes its colour to yellow depending on number of electrons taken up by the system (Umamaheshwari and Chatterjee, 2008). In accordance to MIC values obtained in the study, seven different concentrations for assay were selected ranging from 100 ppm to 20000 ppm (Table 2). An incremental value of radical scavenging activity was reported with increasing concentration of the oil. Percent inhibition by DPPH assay was found in range of 8.14% to 78.73 %.

**Table 2:** DPPH and ABTS Radical Scavenging activity of Thyme essential oil (TEO) (Mean±S.E.), n=6

Sl. No.	Tested Concentrations (ppm)	DPPH Radical Scavenging Activity (%)	ABTS Radical Scavenging Activity (%)
1	100	8.14±0.27	6.00±0.22
2	500	18.80±0.30	12.73±0.11
3	1000	27.52±0.43	22.84±0.17
4	5000	53.12±0.44	42.23±0.30
5	10000	67.49±0.44	53.94±0.23
6	15000	72.48±0.22	61.03±0.39
7	20000	78.73±0.36	67.75±0.40

Although, ABTS radical assay displayed similar results when compared with DPPH radical scavenging assay, but slightly lower values were reported ranging from 6.0% to 67.75% at tested concentrations. Scavenging activity increased with increasing levels of thyme oil and highest inhibition was at 20000 ppm. However, antioxidant activity of thyme oil was slightly lower than the reference synthetic antioxidants (BHT). Grigore *et al.* (2010) determined antioxidant activity of *Thymus vulgaris* L. volatile oil obtained by two different methods. They reported that doses higher than 3 mg/ml showed over 50% inhibition on DPPH free radical corresponding to predominant compounds i.e. thymol (30.86%) and p-cymene (30.53%) along with other non-volatile compounds from lipophilic fraction. Similarly, Sacchetti *et al.* (2005) found that DPPH inhibition for TEO at 10µl concentration was 75.6%. Roby *et al.* (2013) compared radical scavenging activity of Thyme extract with that of sage and marjoram. They observed that thyme extract was comparatively better than other extracts for free radical inhibition and it is mainly related to higher concentration of its active components.

### CONCLUSION

*In-vitro* investigation of antimicrobial and antioxidant effect of thyme essential oil exhibits its promising role as a potent natural agent. A higher antimicrobial effect against common food spoilage and pathogenic microorganism with a broad spectrum of activity against both Gram positive and Gram-negative organisms along with significant radical scavenging activity can be owned to its active principles and synergistic effects amongst constituents. It can be concluded that thyme essential oil can serve as a key replacer of synthetic antimicrobials and antioxidants thereby minimizing their ill effects on health. Further, it can be used as green preservatives in foodstuffs, especially meat and meat products and can prevent lipid oxidation and retard microbial growth resulting in enhanced shelf life during refrigerated storage. Nevertheless, owing to strong aroma and flavour, it may be encapsulated in micro or nano delivery systems for potential application in any meat matrices.

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### CONFLICT OF INTEREST

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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