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IN VITRO EVALUATION OF ANTIMICROBIAL, ANTI-INFLAMMATORY AND CYTOTOXIC PROPERTY AGAINST HEPG2 CELL LINE OF ESSENTIAL OIL EXTRACTED FROM *THYMUS VULGARIS* LEAVES

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ABSTRACT

Thymus vulgaris, also known as thyme, is being used as a versatile herb with both culinary and medicinal applications. It also incorporated into skincare and haircare products due to its antimicrobial and soothing properties. Recent studies highlighted its potential therapeutic benefits. Thyme essential oil was extracted via the hydro-distillation method. Gas chromatography (GC-MS) analysis revealed bioactive substances found in the essential oil. Additionally, leaf extracts were evaluated for various biological functions, including antioxidant, anti-inflammatory, and antimicrobial properties. Total phenol content was examined through the Folin-Ciocalteau's method, and Antioxidant activity was measured through DPPH assay. Antimicrobial activity against various bacterial and fungal pathogens was tested using the disc diffusion method. Furthermore, cytotoxicity activity was evaluated by assessing the cell viability of the HEPG2 cell line. These findings underscored the potential of thyme as a provider of therapeutically useful bioactive properties.

Keywords: *Thymus vulgaris*, GC-MS analysis, Antioxidant, Antimicrobial, Cytotoxicity

INTRODUCTION

T.vulgaris, is a growing plant that is found all over the world and is native to Southern Europe. It was possessed by *Lamiaceae* family. The plant is found in Mediterranean Basin, its neighbours, North Africa, and some parts of Asia and a few of the African nations where the plant is known to grow. Thyme has long been used by humans as a spice, herb, and medication. The herb is used as an infusion to treat coughs, diabetes, and colds. Thyme also cure sore throat and infection because it has antibacterial, antifungal, and antiviral properties **(Kuete., 2017)**. One of the most difficult medical issues of now a days is cancer, which calls for novel approaches for successful treatment. Exploring natural chemical's potential as anti-inflammatory agents has garnered attention in recent times. Thymus vulgaris is a contender among them **(Niksic *et al*., 2021)**.
Thyme has culinary and aromatic properties **(Hammoudi Halat *et al*., 2022)** and is rich in bioactive compounds appreciated for its many activities, including anti-inflammatory properties. Thyme plant contains essential oils where the main components are Monoterpene hydrocarbons and oxygenated monoterpenes. Thymol, carvacrol, borneol, trans-caryophyllene, p-cymene, and cis-sabinene hydrate particularly have greatest ratios **(Kubatka *et al*., 2019)**. To reveal its chemical composition, GC-MS was employed **(Al Hashmi *et al*., 2013)**, which provides a unique insight into the various compounds that make the plant so useful. The primary constituents of thyme essential oil are oxygenated monoterpenes and monoterpene hydrocarbons. In this oil, the most prevalent components are the phenol isomer carvacrol and the naturally occurring terpene thymol. Based on the summary of the analysis it is clear that *T. vulgaris* could be a chemotherapeutic agent derived from plants. Numerous studies have shown that they have important anti-apoptotic and free radical scavenging properties on the human HepG2 cell line **(Kozics *et al*.,2013)**. Investigation of pro-apoptotic and anti-proliferative properties of thyme essential oil have been demonstrated by its cytotoxic activity. T. vulgaris is among the phytonutrients that has the strongest antibacterial properties. T. vulgaris has been shown in numerous preclinical trials to have anti-inflammatory capabilities **(Nagoor Meeran *et al*., 2017)**.

MATERIALS AND METHODS

Sample extraction

The Thymus vulgaris leaves were collected from different locations in Zamin Uthukuli, Pollachi, Coimbatore, Tamil Nadu (10.6519° N, 76.9795° E). The leaves are subjected to air dry in a closed condition. Once dried, the leaves were milled into powder (using a milling machine) and kept in a dry, cold place until the essential oil could be extracted. Further 180 grams of powdered sample is added to 400 ml of distilled water, which is added to Clevenger apparatus in order to extract essential oil by the hydro-distillation at 100°C for a 3 hours. Hydro-Distillation is continued until the oil volume readings remain consistent. Then the extracted oil is drained, dried and filtered through filter paper using anhydrous sodium sulfate and stored at 4°C.

GC-MS analysis

This analysis conducted using a Shimadzu TQ8040 GC system equipped with Rxi-5MS and HP-INNOWAX capillary columns (30 m in length, 0.25 mm in diameter internally, 0.25 µm film thickness). The GC system was linked to an MSD via an appropriate interface for mass spectrum detection. Initially set for 3 minutes at 40°C, the column temperature was raised to 90°C at a pace of 3°C per minute and maintained for 4 minutes. A further increase was made to 115°C at 3°C/min, which was maintained for 10 minutes. After that, it was held for 8 minutes at 140°C at 2°C/min, then for 5 minutes at 210°C at 3°C/min. Thus, the parameter settings and system management of the GC-MS were set up with a scan time of 70 eV as the ionisation energy of 0.3 sec and a mass range of 45–500 AMU **(Fan *et al*., 2018)**. Helium was used as the medium gas and its flow rate was 1.2mL/min, which moves at 1.2 millilitres per minute. Data from chromatography and mass spectra were obtained throughout the process.

Thin layer chromatography technique

The pre-coated TLC plate had a pencil start line drawn on it and the dimensions of the TLC plate was 10×20 cm. Subsequently, the essential oil (sample) was deposited, drop by drop (up to 20–40 drops based on requirement), onto the TLC plate that had been previously coated and left to dry. Then, submerge the plate in the system of solvents: (Toluene:ethyl acetate:formic acid, 5:4:0.2) . Upon

reaching the mobile phase to maximum height of the TLC plate, remove the TLC plate and let it to dry for a minute at room temperature, kept in an iodine chamber for a few minutes **(Musa *et al*., 2017)** and then measure the retention factors (Rf values) and examined the TLC plate employing the UV trans-illuminator **(Awaley *et al*., 2020)**.

RF value = Distance migrated by the solute/ Distance migrated by the solvent

Antioxidant activity

Free radical scavenging activity (DPPH assay): Two separate tubes were prepared, labelled as "Blank" and "Sample" respectively, blank and sample preparation was shown in table 1

Table 1 Preparation of sample and blank

Chemical constituents	Test tube 1 (Blank)	Test tube 2 (Sample)
Water	0.5mL	-
Essential oil	-	0.5mL
0.1N DPPH	0.2 mL (incubate for 5 min)	0.2mL(incubate for 5 min)
Tris-HCL	0.4 mL	0.4mL

The both tubes were allowed about half an hour to sit at room temperature. At 517 nm, the optical density was then measured using a spectrophotometer. Using Ascorbic acid as a standard the antioxidant level was calculated in mg/g **(pranav *et al*., 2024)**.

Estimation of Total Phenol Content

Two tubes were prepared, one for the sample containing 1 mL of essential oil, and the other for the blank containing 1 mL distilled water. Then, 0.2 mL of folin's reagent added to both tubes,1 mL of both tubes received 20% sodium carbonate, and they had been raised in a water bath at 45° over 45 minutes. Finally, the optical density (OD) values for both tubes were measured at 765 nm **(proestos *et al*., 2013)**. The concentration of TPC was measured in mg/g using gallic acid as a standard.

Anti-inflammatory activity

Protein denaturation assay: Two separate tubes were prepared and labelled as sample and blank. In the blank tube, 0.5 mL of distilled water was added. While in the sample tube, 0.5 ml thyme essential oil was included. 0.25 mL of Diclofenac was added to both tubes. Subsequently, 1.5 mL of PBS and 0.5 mL of 1% egg albumin solution were placed in the sample and blank tubes. Incubation at 37°C for 20 minutes ensued, then denaturation at 90°C for 2-3 minutes. Finally, optical density at 660 nm **(Ashallangwa *et al*., 2017)** was measured after cooling.

$$\frac{\text{Absorbance of control - Absorbance of Test}}{\text{Absorbance of control}} \times 100$$

Antibacterial activity

The disc diffusion method was performed by placing a 5 mm diameter disc, loaded with 10µL of essential oil, onto a plate. Then the disc was inoculated for duration of 24 hours. After inoculation the disc was taken 15 minutes before to the experiment. Preparation for antibiotic susceptibility testing involved autoclaving four Petri plates and media. 15 mL of nutrient agar was poured into each Petri plate, 60mL agar, and allowed to solidify. A separate cotton swab **(Mr *et al*., 2005, Karuppusamy *et al*., 2024)** was prepared for each plate for inoculation. 90 µL of culture (*Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa*) added onto each plate and spread evenly with the swab. In each Petri plate, the sample disc (which had been prepared) and Azithromycin AZM 15 mcg antibiotic disc (+ve control) were placed. A cork borer was used to create a well and DMSO (-ve control) was enhanced that effectively. For a whole day, At 37°C, the plates were kept in the incubator, and the zone of inhibition was calculated **(Bachir and Benal, 2012)**.

Antifungal activity

The disc diffusion method **(Bansod and Rai, 2014)** was performed by placing a 5 mm diameter disc, loaded with 10µL of essential oil, onto a plate. Then the disc was inoculated for duration of 24 hours. After inoculation the disc was taken 15 minutes before to the experiment. The Petri plates and media were autoclaved and ampicillin was added to the malt agar before pouring 15 mL of malt agar into each plate, totalling 30 mL, allowing it to solidify. Separate cotton swabs were prepared for inoculation. Next, 90 µL of culture (*Aspergillus Niger, Fusarium oxysporum)* was added onto each plate and swabbed. Sample discs were placed in the Petri plates, and wells for fluconazole (+ve control) and DMSO (-ve control) were created using a cork borer, with the solution poured into them. The plates were maintained at room temperature for 2 - 3 days. after, zone of inhibition was measured.

Cytotoxicity assay

To analyze the effect of different sample volumes on HepG2 (liver cell line) growth, a 96-well plate was used. DMSO was employed as the blank control. Sample concentrations of 2µg/mL, 4Mg/mL, 6µg/mL, 8µg/mL, and 10µg/mL each containing 100µL of the cell line (control) were added to separate well. Furthermore, a CO2 incubator was used to incubate the 96-well plate for the entire day to facilitate cell growth. Afterward 50µL of trypsin and DMSO were added for washing solution and to dissolve the formazan crystals. Subsequently, 20 µL MTT dye was inserted, then the plate was cultivated for 2-4 hours. Optical density (OD) readings at 630 nm were obtained using an ELISA reader and microscopic images were taken for further analysis of the results **(Yousefzadi *et al*., 2013)**.

RESULT AND DISCUSSION

Qualitative analysis using gc-ms

The *T.vulgaris* oil analysis results indicated the existence of a number of chemical compositions, some of which are mentioned in Tables 2 and 3, and the chromatogram shown in Figure 1 as follows.

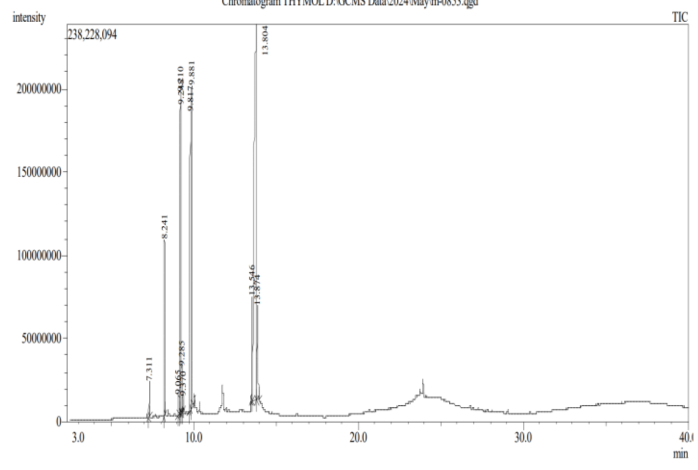

Figure 1 *Thymus vulgaris* oil GC-MS chromatogram

Table 2 Compounds recognised in *Thymus vulgaris* essential oil

S.NO	RETENTION TIME (In minutes)	AREA %	HEIGHT %	A\H	COMPOUND NAME	CHEMICAL FORMULA
1	7.311	0.68	1.65	1.82	.alpha.-Pinene	C10H16
2	8.241	4.17	8.32	2.23	Cyclohexane, 1-methylene-3-(1-meth ylethenyl)-,(R)-	C10H20
3	9.065	0.85	0.92	4.11	Benzene, 1-methyl-3-(1-methylethyl)-	C10H14
4	9.210	14.82	15.19	4.33	5-Allyl-4-[1-(p-aminophenyl)ethylidenehydrazono]-6-methyl-2-phenylpyrimidin	C14H14N2O
5	9.248	6.73	14.57	2.05	5-Allyl-4-[1-(p-aminophenyl)ethylidenehydrazono]-6-methyl-2-phenylpyrimid	C14H14N2O
6	9.285	0.97	2.19	1.97	D-Limonene	C10H16
7	9.370	0.52	0.69	3.37	Benzene, 1-methyl-3-(1-methylethyl)-	C10H14

8	9.817	11.75	14.10	3.70	5-Methylene-1,3a,4,5,6,6a-hexahydropentalen-1-ol	C9H12O
9	9.881	12.72	15.24	3.71	5-Methylene-1,3a,4,5,6,6a-hexahydropentalen-1-ol	C9H12O
10	13.546	3.73	4.95	3.35	1-(2,4-Dimethylphenyl)ethanol	C10H14O
11	13.804	40.14	17.74	10.04	Thymol	C10H14O
12	13.874	2.92	4.45	2.91	(-)-Car-3-en-2-one	C10H14O

Table 3 Significant compounds present in *Thymus vulgaris's* essential oil.

S.NO	COMPOUND NAME	STRUCTURE	ACTIVITY
1	Alpha.-Pinene		Anti-inflammatory , hypoglycemic activities and wound healing activity **(Salas-Oropeza, 2021; özbek and Yılmaz , 2017)**
2	Cyclohexane, 1-methylene-3-(1-methylethenyl)-,(R)-		Potential Anti-microbial activity **(Shoaib *et al.*, 2019)**
3	Benzene, 1-methyl-3-(1-methylethyl)-		Anti-bacterial activity **(Kiki and Ibrahim, 2020)**
4	5-Allyl-4-[1-(p-aminophenyl)ethylidenehydrazono]-6-methyl-2-phenylpyrimid		Pharmaceutical and inhibitory activity **(Lu *et al.*, 2015)**
5	5-Allyl-4-[1-(p-aminophenyl)ethylidenehydrazono]-6-methyl-2-phenylpyrimid		Pharmaceutical and inhibitory activity **(Lu *et al.*, 2015)**
6	D-Limonene		Anti-oxidant activity **(Shah and Mehta, 2018)**
7	Benzene, 1-methyl-3-(1-methylethyl)-		Anti-bacterial activity **(Kiki and Ibrahim, 2020)**
8	5-Methylene-1,3a,4,5,6,6a-hexahydropentalen-1-ol		Anti-oxidant activity and Anti-microbial activity **(Odoom *et al.*, 2023)**
9	5-Methylene-1,3a,4,5,6,6a-hexahydropentalen-1-ol		Anti-oxidant activity and Anti-microbial activity **(Odoom *et al.*, 2023)**
10	1-(2,4-Dimethylphenyl)ethanol		No known activity
11	Thymol		Antioxidant and antimicrobial agents . **(Aldosary *et al.*,2023)**
12	(-)-Car-3-en-2-one		Anti-microbial activity, Anti-oxidant activity ,Analgesic activity **(Negi *et al.*, 2013; Griffin *et al.*, 2023)**

Thin layer chromatography

One method for separating substances in non-volatile mixtures was thin-layer chromatography (TLC) **(Tiwari and Talreja, 2022)**. Saturated iodine chambers assisted in the development of colour bands as shown in Figure 1. During TLC analysis, the essential oil spot travelled from the bottom to the top of the plate with a retention factor value of 0.65. The proportion of toluene, ethyl acetate, and formic acid in the solvent mixture was 5:4:0.2.

Figure 2 TLC results obtained from *Thymus Vulgaris* oil

Antioxidant activity

Free radical scavenging activity (DPPH assay): The assay of DPPH was frequently utilized to evaluate the sample's antioxidant qualities. The antioxidant content of sample oil was established in this investigation utilizing an ascorbic acid standard curve **(Aldosary *et al*., 2023)**. The assessment of the antioxidant activity through DPPH showed an IC50 of 76µg/mL. In Similar studies **(Shashank *et al*.,2021)**
it has been reported for thyme essential oil, the antioxidant activity through DPPH showed an IC50 of 94µg/mL. The variation can be due to the oil extraction method and plant species' geographical origin.

Total Phenol Content

Phenolic substances are necessary due to the scavenging activity of their hydroxyl group. The sample's optical density was measured using UV spectroscopy. Total phenol levels were calculated using a standard curve created using gallic acid as the standard **(Prakkash *et al*., 2019)**. The total phenol content was found to be ± 30 mg GAE/ g of the extracted thyme essential oil. In another study it is reported that, the total phenol content from thyme plant extract was reported to be 8.10 mg GAE/ g **(Roby *et al*., 2013)**. The significance of total phenol content lies in its powerful bioactive properties. Higher phenol content is a marker for antioxidant, anti-inflammatory, and antimicrobial potential, making it vital in disease prevention, food preservation, and product development in pharmaceuticals and cosmetics.

Anti-Inflammatory Activity

The protein denaturation was used to evaluate the effectiveness of anti-inflammatory thyme oil, with a 0.5 mL concentration of oil examined. The outcomes demonstrated that the essential oils considerably reduced the denaturation of proteins. Specifically, thymus oil showed a phenomenal 66.18% inhibition rate as a result. The results of this investigation demonstrated the thymus essential oil's promising anti-inflammatory qualities and indicated that it might be investigated further as a natural anti-inflammatory agent **(Suganya *et al*., 2015) (Ejaz *et al*., 2024)** Thus the potent anti-inflammatory property of the essential oil can contribute in managing inflammation-related diseases, reducing pain, and preventing long-term damage across various body systems.

Antibacterial activity

The thyme oil exhibited antibacterial action against bacterial pathogens, encompassing *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa*, and *Staphylococcus aureus*. The zone of inhibition of the bacterial species in combination with *T. vulgaris* oil was shown in Table 4 and Figure 3.

Table 4 Zone of inhibition (Diameter measured in millimeters) against *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *Staphylococcus aureus.*

Name of the organism	*Thymus Vulgaris* Essential oil (Sample)	Azithromycin disc (+ Control)	DMSO (- control)
Escherichia coli	17mm	14mm	Nil
Pseudomonas aeruginosa	16mm	17mm	Nil
Staphylococcus aureus	15mm	14mm	Nil
Klebsiella pneumoniae	14mm	15mm	Nil

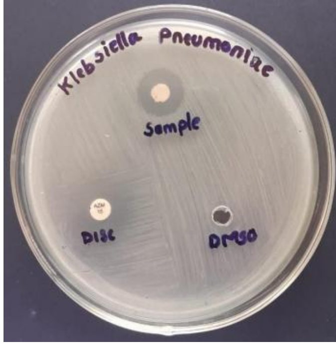

Figure 3 Antibacterial activity of *Thymus vulgaris* essential oil against *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Staphylococcus aureus*

Antifungal activity

Thyme oil demonstrated notable antifungal activity counter to the clinical isolates of *Aspergillus niger* and *Fusarium oxysporum*. The zones of inhibition were observed and recorded to as indicated in Table 5 and Figure 4, ascertain the essential oil's activity.

Table 5 Zone of inhibition (Diameters are measured in millimetres) against *Aspergillus niger* and *Fusarium oxysporum.*

Name of the fungi.	*Thymus vulgaris* essential oil (Sample)	Fluconazole (+ve control)	DMSO (-ve control)
Aspergillus niger	16mm	10mm	Nil
Fusarium oxysporum	13mm	-	Nil

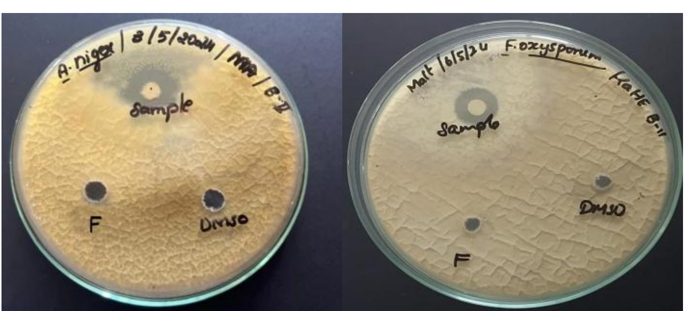

Figure 4 *Thymus vulgaris* essential oil's antifungal activity against *Fusarium oxysporum* and *Aspergillus niger*

Cytotoxicity

Thyme oil was selected then examined for its cytotoxicity using human HEPG2 liver cancer cell lines. An ELISA plate reader set at 570 nm was used to measure the properties **(Ganesh Moorthy *et al*., 2024)**. The results showed a promising potential to influence cell viability. A cytotoxic impact that depended on concentration was seen when the essential oil's concentration ranged from 2 to10 µg/mL. At 10 µg/mL of extract concentration, 53.80% of the cells were viable. These results demonstrated the ability of essential oil of *Thymus vulgaris* to inhibit the growth of HEPG2 cancer cells. These findings suggest that *Thymus vulgaris* oil may be helpful in inhibiting the proliferation of HEPG2 cells. Table 6 and Figure 5 provides the summary of the information from the study on thymus essential oil effect on HEPG2 cells.

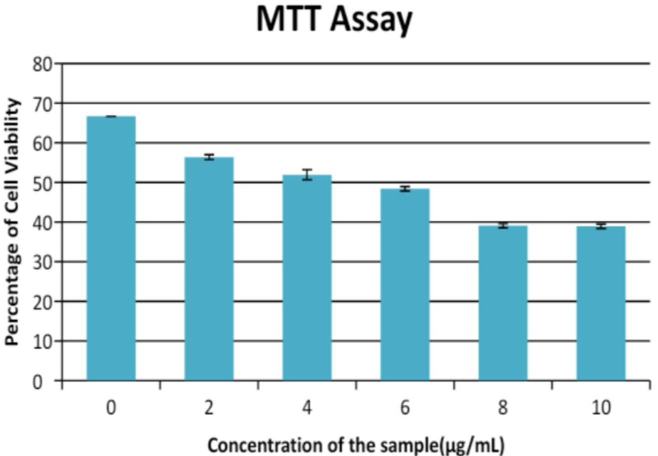

Figure 4 Cytotoxicity assay of *Thymus vulgaris* essential oil

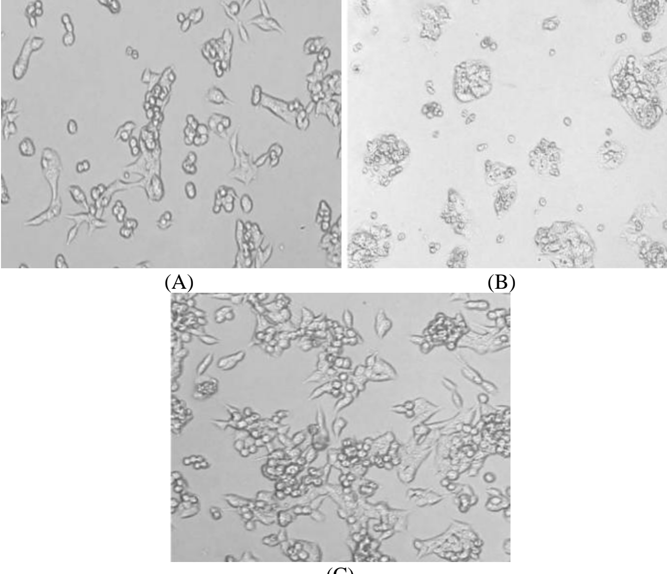

Figure 5 Microscopic picture of HEPG2 cells acted with *Thymus vulgaris* essential oil in the following ranges: (A) - 2µL, (B) - 10µL, and (C) - control

CONCLUSION

The essential oil isolated from *Thymus Vulgaris* leaves has been widely recommended since ancient times to heal and treat chest tightness and fluid production. In this study, we explored its anti-inflammatory, antioxidant, and antifungal characteristics. The antifungal effects of *T. vulgaris* oil were studied by comparing its efficacy against *Aspergillus niger* and *Fusarium oxysporum*. We also examined the antibacterial properties of thyme essential oil to assess its effectiveness against bacterial pathogens. GC-MS analysis of thyme oil revealed many chemical compounds, including D-limonene, alpha-pinene, and many more. For both qualitative and quantitative examination, thin layer chromatography (TLC), an affinity-based technique for separating chemicals in mixtures was utilized. These findings are effective in determining cell viability and the cytotoxic effects of compounds on hepatocellular carcinoma cells. Cytotoxicity analysis of *T. vulgaris* essential oil indicates its potential as a candidate for further investigation in the development of cancer therapy. HEPG2 cancer cell line is used to investigate the cytotoxic properties showed that cell viability was decreased by 53.80%. Overall, thyme has potential as a natural product to improve health.

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