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Evaluation of Antioxidant Activity and Cytotoxic Potential of *Thymus vulgaris* Leaf Extracts

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ABSTRACT

Thymus vulgaris is a most important medicinal plant, it is rich in bioactive secondary metabolites. The leaves are used for the treatment of several diseases. In the present study, the antioxidant activity and cytotoxic potential of *T. vulgaris* leaf extracts were evaluated. Leaf extracts were prepared using cold and hot procedures with varied polarity solvents (chloroform, acetone, ethanol, and aqueous methanol [50% v/v]). The total phenol content (TPC) and antioxidant potential of the extracts were determined. The leaf extracts were fractionated using Gas Chromatography/Mass Spectroscopy (GC/MS), and the acetone extract's cytotoxic effects on breast cancer cell lines were assessed. The results indicated that the aqueous methanol extract (hot method) had the highest TPC (76.45 ± 2.88 mg/g of dry plant content of gallic acid equivalence [mgGAEg^{-1}]), while the lowest TPC was obtained in the chloroform extract (cold method; 37.36 ± 2.45 mgGAEg^{-1}). The maximum antioxidant activity was observed in the ethanol extract (hot method; 83.9% ± 0.96), while a minimum value was recorded for the acetone extract (hot; 66.3% ± 0.71) at a concentration of 100 µg/mL. GC/MS analysis revealed the presence of phenolic compounds such as *p*-Cymen-3-ol, *p*-Cymen-2-ol, 2,5-diethylphenol, 6-ethyl-3, 4-dimethylphenol, 7,11-dimethyldodeca-2,6,10-trien-1-ol thymol and some flavonoids. The acetone extract showed an anticancer effect on the MCF-7 and Cal-51 breast cancer cell lines, as well as the HBL-100 normal cell line. The results of this study provide scientific justification for the medicinal use of *T. vulgaris* leaf extracts that contained various polyphenol components; it has strong antioxidant and antitumor activity.

***Keywords*:** Antioxidant capacity, Cytotoxic activity, GC-MS analysis, *Thymus vulgaris*.

Introduction

Medicinal plants have been used in clinical trials for a long time with notable benefits of a curative effect, fewer side effects, and the potential to be used to manage various symptoms and diseases.[1] *Thymus* is a genus containing the largest flowering plant species in the Lamiaceae family, which also includes flavoring compounds. These herbs have been used as a traditional medicine for centuries because they are a natural source of bioactive compounds.[2,3] *Thymus vulgaris L.* is mainly recognized for its antibacterial and medicinal properties. Its extract contains phenolic compounds, which are mainly responsible for its antibacterial properties. *Thymus* extracts are also used to treat wounds, tonsillitis, and laryngitis.[4,5] Phenolic compounds are biosynthesized compounds, contain one or more aromatic rings and a hydroxyl group in their structure. About 8,000 of the phenolic compounds have been differentiated according to their aromatic ring contents. The chemical structure of phenolic compounds determines their antioxidant activity. It can chelate transition metals and confine free radicals.[6]

Medicinal plants are being studied extensively as anticancer agents.[7,8] Recently, *T. vulgaris* (Thyme) has been shown to have anti-cancer properties in human colorectal cancer cells. The finding revealed that concentrations and exposure time affect growth inhibition.[9]

Some studies have investigated the improvement of stage II colon cancer in patients who used herbal/botanic supplements for treatment and found that herbal/botanic supplement users fared better than non-users. This observation is due to a high intake of fruits and vegetables, as well as avoiding foods high in refined sugar. As a result of all of these factors, colon cancer will become less common.[10] Other researchers looked into the influence of extract from thyme leaves on the rate of colon cancer in obese rats.[11] The antitumor effects of *T. vulgaris* L. were studied using MCF-7 and MDA-MB-231 mammary carcinoma models *in vivo* and *in vitro*.[12] In the present study, the antioxidant activity and cytotoxic potential of *Thymus vulgaris* leaf extracts were evaluated.

Materials and Methods

Sources of chemicals and reagents

Gallic acid, ascorbic acid, DPPH (2,2-diphenyl-1-picrylhydrazyl), folin ciocalteu's phenol reagent, and sodium phosphate dibasic were obtained from Sigma-Aldrich, Acetone, chloroform, ethanol, and methanol were purchased from Merck.

Source and preparation of plant sample

Dried leaves of *T. vulgaris* (grown locally) were purchased from a market in Amman, Jordan in October 2017 (local popular market Voucher number P/M/014). The leaves were ground to powder before being used for testing to prevent decomposition of constituents or loss of volatile materials.

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Source of breast cancer cell lines
Some breast cancer cell lines were obtained from the Cellular Bank Unit in Cancer Research and Medical Genetics, Iraqi, Baghdad. The cell lines were the human breast adenocarcinoma (MCF-7), epithelial cell line (HBL-100), and malignant pleural effusion CAL-51.

Preparation of T. vulgaris leaf extracts
Plant extraction was done using both the hot and cold methods. In the hot method of extraction, 100 mL of various solvents were mixed separately with 10 g of leaves and poured into a Soxhlet extractor until the extraction was complete.[13] The solvents used (arranged in increasing order of polarity) include 50% methanol/water, ethanol, acetone, and chloroform. For the cold extraction method, 10 g of finely ground plant material was poured into a 250 mL conical flask and mixed with 100 mL of each solvent separately (50% methanol/water, ethanol, acetone, and chloroform). The suspension was covered by aluminum foil and left under stirring for 24 hours at room temperature. After the extraction by cold maceration method, the extracts were filtered, and the various solvents were evaporated by drying in an incubator at 48ºC for 24 hours. The dry extracts were collected and kept in a dark dried bottle at 4ºC for subsequent analyses.[14]

Determination of total phenol content (TPC) in T. vulgaris leaf extracts
The Folin-Ciocalteu colorimetric method was used to measure the total phenol content in the plant extracts.[15] Different concentrations (400, 200, 100, 50, and 25 mg/L) of gallic acid were used to construct the standard calibration curve.[16] The absorbance was measured at 750 nm and the total phenol content (mg/mL) was estimated from the calibration curve. Then, the content of phenols in the extracts was expressed in terms of gallic acid equivalent (mg of GA/g of extract).[17]

Determination of the antioxidant activity of T. vulgaris leaf extracts
To estimate the antioxidant activity of *T. vulgaris* leaf extracts, 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was used as described by Es-Safi *et al.*[18] The radical scavenging activity was calculated as the percentage inhibition (I%) of DPPH radical using the following equation:

Percentage Inhibition (I%) = $[(A_{control} - A_{sample})/A_{control}] \times 100$.
Where A_{sample}: absorbance of sample and $A_{control}$: absorbance of control.

Gas Chromatography-Mass Spectroscopy analysis of T. vulgaris leaf extracts
Gas Chromatography-Mass Spectroscopy (GC/MS) analysis of the various crude *T. vulgaris* leaf extracts was performed at the Ministry of Science and Technology, Department of Environmental and Water Pollution, Baghdad, Iraq using a Shimadzu AOC-20I GC/GCMS Injector/Autosampler. It was equipped with a VF-5 MS fused silica capillary column (30 m x 0.25 mm ID x 0.25 µm) for analysis. The injector was set up in a splitless mode (1 min hold) with 1µL injection volume at an inlet temperature of 280°C. Helium was pumped at a rate of 1 mL/min. The temperature of the oven was ramped from 60 to 300°C at a rate of 10°C per minute, and then constant for 4 minutes. The temperatures of the transfer line and the ion source were set at 280 and 200°C, respectively, and the scan range of 40-600 amu. The relative percent of chemical components in crude extracts of *T. vulgaris* leaves was represented by the normalization peak region. [19]

Cytotoxic analysis of T. vulgaris leaf extracts
The cytotoxic analysis of the *T. vulgaris* leaf extracts was performed in the Department of Molecular Biology, Iraqi Center for Cancer Research and Medical Genetics, Baghdad, Iraq. A DMEM culture medium (Biochrom, Germany) was used to maintain the cell lines. The cytotoxicity assay was used to evaluate the potential activity of phenolic compounds extracted from *T. vulgaris* against growth inhibition of some cancer cell lines stained with crystal violet. A 200 µL of cell suspension (triplicate) was poured on a 96-well microtiter plate and left for 24 hours. The cells were then exposed to 10, 20, 30, 40, and 50 µg/mL of diluted *T. vulgaris* leaf extracts for a period of 24, 48, and 72 hours. After each exposure time, the cells were removed from the medium and stained with crystal violet. A plate reader was used to measure the optical density (OD) at a wavelength of 492 nm. The percentage of tumor inhibitory rate (IR %) was determined using the equation below: [20]

$$\text{IR \%} = \frac{A - B}{A} \times 100$$

Where A: Absorbance of control (untreated cells); B: Absorbance of treated cells.

Statistical analysis
Each experiment was performed in triplicates and the results were expressed as mean ± standard error of the mean (SEM). The analysis was performed using the statistical program, SPSS (version 21). A Student t-test was used to evaluate the differences in the quantitative determination of phenols among the extracts. All data were analyzed by the statistical program, SPSS for Windows version 8.0. The Significant is when $p \leq 0.05$.

Results and Discussion

Yield of T. vulgaris leaf extract
The extraction efficiency of the *T. vulgaris* leaf extracts in polar solvents using both the hot and cold methods was determined (Table 1). Four different polar solvents were used: chloroform, acetone, ethanol, and 50% methanol/water. In the cold extraction method, the 50% methanol/water solvent produced 1.97 g of extract per 10 g of dry *T. vulgaris* leaves which was higher than the other solvents. For the cold method of plant extraction, 0.38 g per 10 g of dry *T. vulgaris* was obtained for the chloroform solvent which was the lowest extract yield among all the test solvents. The results indicated that the hot method of plant extraction produced a better result. Also, the outcome of the extraction showed that a water-organic solvent combination system offered the best extraction solvent. The variation observed in the yield of extracts may be due to the difference in polarity of solvents used. As the polarity of solvent increases, the extraction efficiency decreases.[21]

Total phenol content in T. vulgaris leaf extracts
The total phenol content in *T. vulgaris* leaf extracts was represented using the Gallic acid equivalence. The equation of the standard curve for Gallic acid was represented as (y = 0.1845x - 0.0471, R2 = 0.9721) as presented in Figure 1 and the concentration of the total phenols was expressed as mg of GA/g of extract. The results (Table 2) revealed that all the leaf extracts contained phenolic compounds which varied from 37.36 to 76.45 mg based on the solvent and extraction procedure used. In the hot extraction method, the highest total phenol content and the best extraction yield were obtained by using aqueous methanol, while the lowest yield was obtained with chloroform. The yield of phenol content in plant extracts is influenced by the polarity of the solvent used.

Table 1: Yield of *Thymus vulgaris* leaf explants in different solvent systems

Solvent	Sample (g)	Obtained extract (g)	Extract percentage (w/w%)
Acetone (cold)	10	0.915	9.15
Acetone (hot)	10	1.424	14.24
Chloroform (cold)	10	0.379	3.79
Chloroform (hot)	10	0.776	7.74
Ethanol (cold)	10	0.684	6.84
Ethanol (hot)	10	1.862	18.62
50% Methanol/ water (cold)	10	1.971	19.71
50% Methanol/ water (hot)	10	1.124	11.24

This observation was in agreement with the previous report that the high solubility of phenols in polar solvents resulted in high concentrations of these compounds.[22,23]

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In this study, a mixture of organic solvents and water was used which created a moderately polar solvent that enhanced the extraction of the polyphenols.[24] Consequently, it can be deduced that the most effective solvent for the extraction of polyphenol is 50% aqueous methanol. Concerning the cold extraction method, the highest amount (61.27 ± 1.92 mg of GAE/g of extract) of phenolic compounds was obtained when aqueous methanol was used as a solvent, while the lowest amount (37.36 ± 2.45 mg of GAE/g of extract)was recovered for the chloroform solvent. However, when acetone was used instead of ethanol as a solvent, the phenolic content was higher. The solvents with the most effective extraction ability when the cold procedure was used are in the order of methanol > acetone > ethanol > chloroform. Furthermore, it was observed that high molecular weight phenolic compounds were more effectively extracted with acetone compared to the other solvents. The results of the present study agree with the previous findings that acetone had the lowest polarity of all the solvents tested. It does, however, have the highest value for total phenolic compounds.[25,26] The hot method of extraction resulted in the extraction of bioactive compounds from the leaves over some time. Because the Soxhlet apparatus can repeat the diffusion process of solvents into leaf cells, more soluble bioactive chemicals can be transferred to the bulk of the solvent over time.[27, 28]

Antioxidant activity of T. vulgaris leaf extracts by DPPH radical scavenging assay

The antioxidant activity of the crude extracts of *T. vulgaris* leaves in the test solvents using a hot method of extraction showed that the activity was in the range of 66-83% (Table 3). The highest antioxidant activity was obtained in the crude ethanol extract, while the lowest activity was in the acetone crude extract. The antioxidant activity was in the order of ethanol > 50% aqueous methanol > chloroform > acetone. In the cold extraction method, antioxidant activity ranged between 71 and 82%. Similarly, the highest activity was recorded in the ethanol crude extract, while the lowest was in the chloroform crude extract. The activity of the extracts is in the order of ethanol > acetone > 50% aqueous methanol > chloroform. Different concentrations of *T. vulgaris* extracts have different hydrogen donation abilities. The difference in inhibition percentages may be due to the efficiency of the extracted compounds. Several factors, including the location of the plant and the extraction conditions such as temperature, time of extraction, and solvent polarity determine the quality of extract.[29] Moreover, the selected group(s) of the antioxidant compounds and their activities could be influenced by a change in the polarity of solvents.[30,31] Studies have shown that extraction procedure with 40% v/v acetone was simple, less expensive, and more efficient method of recovering antioxidant phenolic acids from highland barley (BSG).[32] In another report, methanol showed the highest extraction capacity for such phenolic compounds from thyme, sage, and marjoram and also displayed the strongest antioxidant capacity.[33]

Phytochemical constituents of T. vulgaris leaf extracts by GC/MS analysis

GC/MS analysis was used to identify and characterize the various organic crude extracts from the two extraction methods (Table 4). In the cold extraction method, the p-Cymen-3-ol (24.69%) and p-Cymen-2-ol (24.69%) in the acetone crude extract resulted most prominent constituents and most biologically active compounds (47.30%). Conversely, there were 16 different compounds in the acetone crude extract with the hot extraction method. Dimethyl ketone (5.09%), Diacetone alcohol (5.02%), 6-ethyl-3, 4-dimethylphenol (1.32%), 6-ethyl-3, 4-dimethylphenol (4.94%), and 5-methoxysterigmatocysin were the most common constituents (77.09%).

On the other hand, the chloroform crude extract obtained by the cold method of extraction contained 22 different compounds and the most prominent constituents were the tritetracontane (4.55%), 7-tetradecenol (2.07%) and thymol (0.59%). Moreover, the chloroform crude extract when the hot method of extraction was used had 21 different compounds.

Table 2: Total phenolic contents of *Thymus vulgaris* leaf extracts

Extraction solvent	Total phenolic content (mg of GAE/g of extract)
Acetone (cold)	53.8 ± 2.24
Acetone (hot)	63.29 ± 1.91
Chloroform (cold)	37.36 ± 2.45
Chloroform (hot)	55.25 ± 1.32
Ethanol (cold)	45.88 ± 2.74
Ethanol (hot)	65.19 ± 2.95
Methanol/water 50% (cold)	61.27 ± 1.92
Methanol/water 50% (hot)	76.45 ± 2.88

Table 3: Percentage scavenging activity of *Thymus vulgaris leaf extracts using* DPPH assay

Extract	Scavenging activity (%)
Ascorbic acid	93.1 ± 1.76
Acetone (cold)	81.2 ± 1.45
Acetone (hot)	66.3 ± 0.71
Chloroform (cold)	71.2 ± 0.97
Chloroform (hot)	78.6 ± 0.88
Ethanol (cold)	82.9 ± 0.91
Ethanol(hot)	83.9 ± 0.96
Methanol/water 50% (cold)	77.7 ± 1.17
Methanol/water50% (hot)	80.3 ± 0.88

DPPH: 2,2-diphenyl-1-picrylhydrazyl

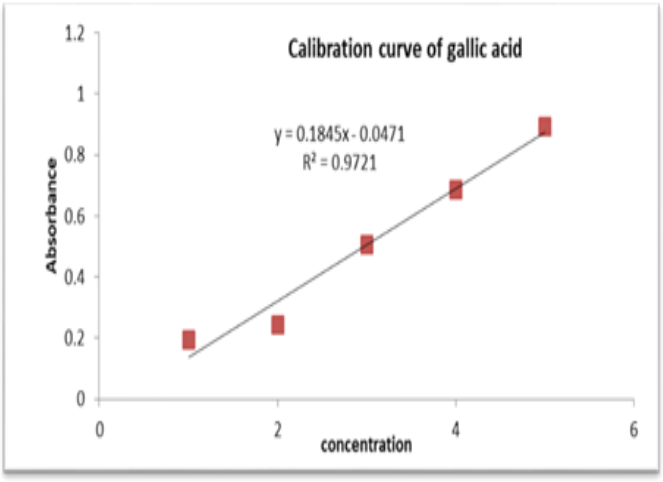

Figure 1: Gallic acid standard curve

The most prominent constituents were the 6-ethyl-3, 4-dimethylphenol (18.95%), 2,5-diethylphenol (39.29%), and p-cresol, 2,2'-methylenebis [6-tert-butyl] (1.00%). The ethanol crude extract profile with the cold extraction method revealed 33 different compounds. The most prominent constituents were the 6-ethyl-3,4-dimethylphenol (3.44%), 2,5-diethylphenol (8.92%), palmitic acid (5.87%), and 17-octadecynoic acid (37.82%). With the hot extraction method, the ethanol crude extract contained 15 different compounds. The most prominent constituents were the 6-ethyl-3,4-dimethylphenol (4.96%), 2,5-diethylphenol (17.09%) and pacamine (10.41).

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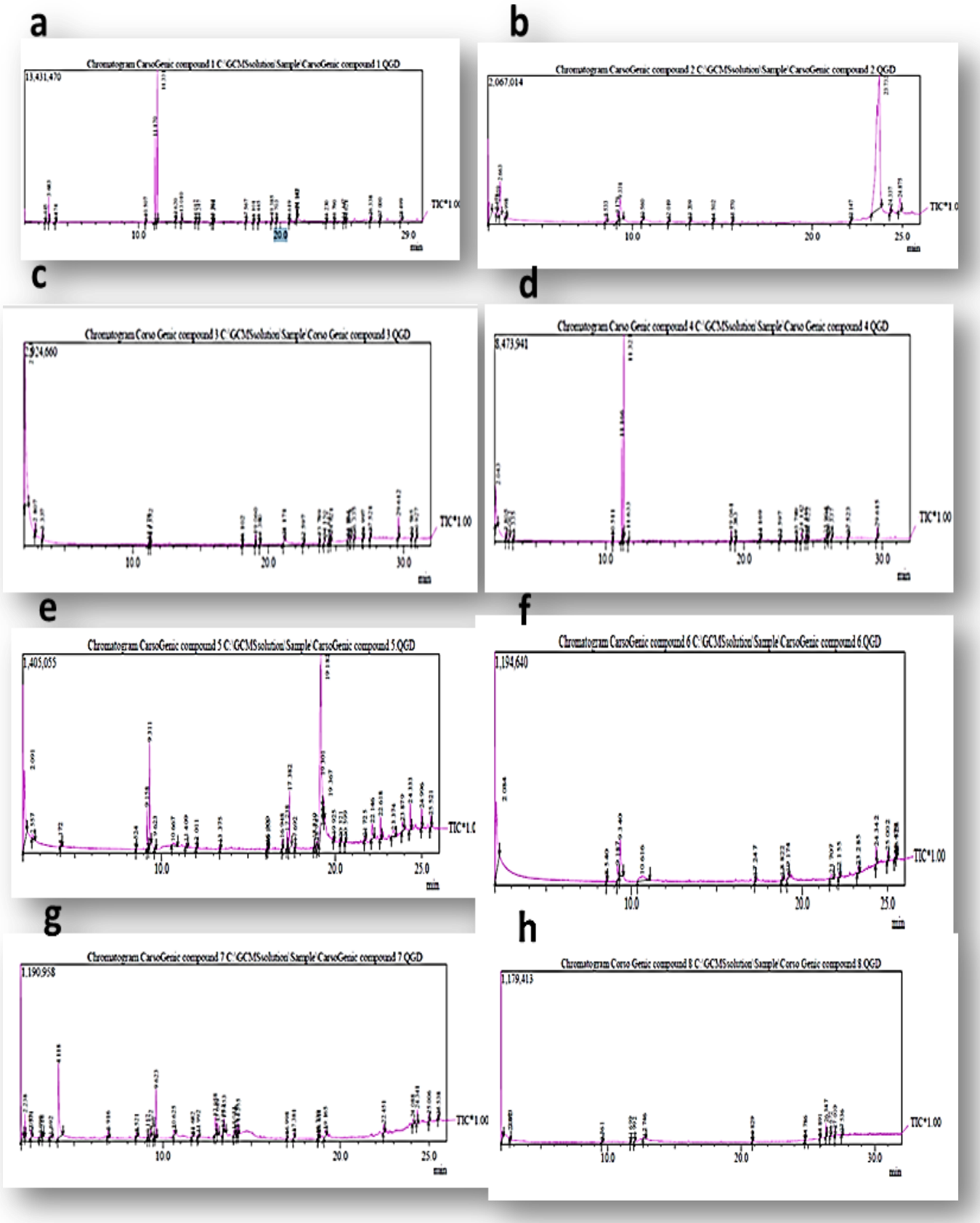

Figure 2: GC/MS analysis of *Thymus vulgaris* leaf extracts in different solvents and extraction methods (hot and cold).
A: Acetone cold; b: Acetone hot; c: Chloroform cold; d: Chloroform hot; e: Ethanol cold; f: Ethanol hot; g: 50% Methanol /water cold; h: 50% methanol /water hot.

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Table 4: Phytochemical constituents of different *Thymus vulgaris leaf extracts*

Solvent	Retention time	Area%	Common name	Molecular formula
Acetone /cold	3.683	7.15	Diacetone alcohol	$C_6H_{12}O_2$
	11.170	24.69	p-Cymen-3-ol phenol	$C_{10}H_{14}O$
	11.331	47.30	p-Cymen-2-ol phenol	$C_{10}H_{14}O$
	13.010	3.34	Dithiodiglycol alcohol	$C_4H_{10}O_2$
	19.385	1.80	Palmitic acid	$C_{16}H_{32}O_2$
	21.124	1.62	11,14-Eicosadienoic acid	$C_{15}H_{28}O$
Acetone/hot	2.059	5.09	2-Propanone	C_3H_6O
	2.663	5.02	2-Propanone,4-hydroxy-4-methyl	$C_6H_{12}O_2$
	9.179	1.32	6-Ethyl-3,4-dimethylphenol	$C_{10}H_{14}O$
	9.331	4.94	2,5-Diethylphenol	$C_{10}H_{14}O$
	23.732	77.09	5-Methoxysterigmatocysin	$C_{19}H_{14}O_7$
Chloroform/cold	2.043	77.25	Chloroform	$CHCL_3$
	21.171	2.07	13-Tetradece-11-yn-1-ol	$C_{14}H_{24}O$
	24.621	1.24	10,12-Pentacosadiynoic acid 2,6,10,15-	$C_{25}H_{42}O_2$
	29.612	4.55	Tetramethylheptadecane	$C_{21}H_{44}$
Chloroform/hot	2.043	20.18	Chloroform	$CHCL_3$
	11.166	18.95	2,5-Diethylphenol	$C_{10}H_{14}O$
	11.323	39.29	2,5-Diethylphenol	$C_{11}H_{18}O_2$
	24.142	3.11	Palmitic acid. beta.-monoglyceride	$C_{14}H_{24}O$
	29.615	3.41	, 2, 6,10,15-tetramethyl heptadecane. (hydrocarbon lipid molecule)	$C_{21}H_{44}$
Ethanol /cold	2.091	17.47	Propanol, 2-(1-methylethoxy)	$C_6H_{14}O_2$
	9.158	3.44	Phenol, 2-ethyl-4,5-dimethyl	$C_{10}H_{14}O$
	9.311	8.92	2,5-Diethylphenol	$C_{10}H_{14}O$
	17.382	5.87	Pentadecanoic acid	$C_{15}H_{30}O_2$
	19.182	37.82	17-Octadecynoic acid	$C_{18}H_{32}O_2$
	22.146	2.38	Tricyclo[20.8.0.0(7,16)]triacontane, 1(22),7(16)-diepox cis	$C_{30}H_{52}O_2$
Ethanol/hot	2.048	46.78	1-Propanol, 2-isopropoxy-	$C_6H_{14}O_2$
	9.187	4.96	Phenol, 2-ethyl-4,5-dimethyl-	$C_{10}H_{14}O$
	9.340	17.09	2,5-Diethylphenol	$C_{10}H_{14}O$
	10.616	10.41	Dimethylamine, N-	$C_9H_{22}NP$
	19.147	2.53	(diisopropylphosphino)methyl Decahydro[1,7]naphthyridine	$C_8H_{16}N_2$
Methanol/water 50% cold	2.053	2.81	Glycidol	$C_3H_6O_2$
	2.238	4.05	2,3-Butanediol,	$C_4H_{10}O_2$
	2.571	3.11	dl-Glyceraldehyde	$C_3H_6O_3$
	4.118	27.83	Glycerin	$C_3H_8O_3$
	9.623	9.23	3-Cyclohexen-1-ol,	$C_{11}H_{18}O_2$
	12.958	8.11	Methyl .beta.-d-galactopyranoside	$C_7H_{14}O_6$
	123.453	4,69	1,2,3,5-Cyclohexanetetrol,	$C_6H_{12}O_4$
	19.165	3.41	cis-1,2-Cyclohexanedimethanol	$C_8H_{16}O_2$

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Methanol/hot	2.092	31.31	Acetyl-2,2,4-trimethyl-4-phenyl-	$C_7H_{16}O$
	2.673	5.01	1,2,3,4-tetrahydroquino	$C_6H_{14}O_2$
	9.561	3.60	2,2-Dimethoxybutane	$C_9H_{16}O_4$
	11.699	2.20	Oxalic acid, butyl propyl ester	C_3H_6O
	11.992	2.64	2-Propen-1-ol	$C_8H_{16}O$
	12.746	10.82	4-Heptanone, 3-methyl-	$C_5H_{14}N_2$
	26.347	20.11	1,2-Ethanediamine, N, N, N'-trimethyl-	$C_{27}H_{44}O_4$
	27.010	8.57	Terephthalic acid, 2-ethylhexyl undecyl ester	$C_{14}H_{24}O$
			7,11-Dimethyldodeca-2,6,10-trien-1-ol	
	27.536	5.64	3,7-Dimethylnonane	$C_{11}H_{24}$

With the cold method of extraction, methanol crude extract had 33 different compounds, notably lactic acid (4.05%), alpha-l-rhamnopyranose (4.69%), 4,4-dimethylpent-2-enal (9.23%), and butyl imidocarbamate (27.83%). There are 13 different compounds in the methanol crude extract when the hot method of extraction was used. The 2,3-dimethylpentanol (31.31%), 1,2-ethanediamine, N-ethyl- (10.82%), terephthalic acid, 2-ethylhexyl undecyl ester (20.11%), and 7,11-dimethyldodeca-2,6,10-trien-1-ol were the most common constituents (8.57%). All the major compounds from the different extracts are biologically active molecules. They are considered to be part of plants' defense systems, and as such have been included in a large group of protective molecules found in plants namely, phytoprotectants.[34] Thus, the identification of a good number of compounds from various *T. vulgaris* extracts might have some biological significance.

Cytotoxic effects of T. vulgaris extract on different cancer cell lines

GC/MS analysis revealed the presence of phenolic compounds such as p-Cymen-3-ol p-Cymen-2-ol, 2,5-diethylphenol, 6-ethyl-3,4-dimethylphenol, 7,11-dimethyldodeca-2,6, 10-trien-1-ol thymol, besides some flavonoids. All these phenolic compound derivatives inhibited the growth of the test cancer cell lines used in this research.

Cytotoxic effect of T. vulgaris leaf extract on MCF-7 cell line

The cytotoxic response of MCF-7 cells to acetone *T. vulgaris* leaf extracts was studied. There was no discernible inhibition in the first 24 h, but in the second 24 h, a concentration of 50 g/mL *T. vulgaris* leaf extract was found to cause substantial inhibition compared to other concentrations. Meanwhile, in the third 24 h, there was no significant inhibition observed in all the concentrations of test extracts (Figure 3). It was revealed in this study that the optimal time for the exposure of MCF-7 to the various concentrations of the extract was the second 24 h. Nevertheless, when studying the toxicity depending on the concentration, the maximum rate of inhibition was at 10 µg/mL (45%) after 24-h exposure time to extract and the low rate of inhibition was at 50 µg/mL (8%) after the third 24-h exposure to the extract. It was observed that the extract increased the percentage of dead cells by an increase in the concentration with time. Apoptotic bodies are formed by morphological changes associated with late stages of apoptosis, such as shrinkage, uneven shape, and condensed and fragmented chromatin and cytoplasm. The results in this study are in agreement with previous findings that showed the anti-tumor effects of *T. vulgaris* in *in vivo* and *in vitro* mammary carcinoma models having a strong anticancer activity[12]. It has been shown that *T. vulgaris* has a strong anti-cancer activity against colorectal cancer cell lines. Also, it has been demonstrated to possess anti-cancer activity against two different cell lines: colorectal and breast cancer.[35]

Cytotoxic effect of T. vulgaris leaf extract on CAL51 cell line

After 48 h of exposure, the acetone *T. vulgaris* leaf extract had the highest rate of inhibition (66%) on the CAL51 cell line at a concentration of 20 g/mL, while the lowest rate of inhibition was

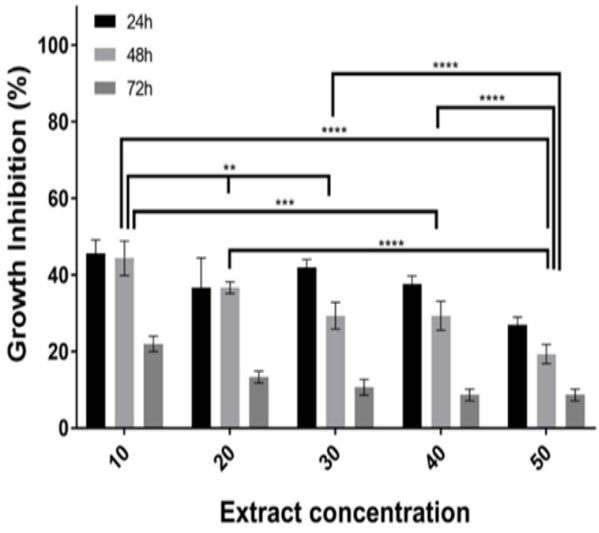

Figure 3: Effect of acetone *Thymus vulgaris* leaf extract on growth inhibition of breast cancer cell line, MCF7

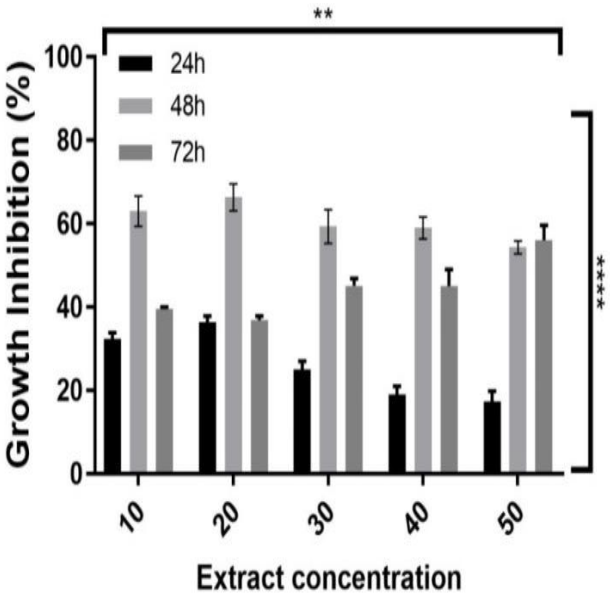

Figure 4: Effect of acetone *Thymus vulgaris* leaf extract on growth inhibition of breast cancer cell line, CAL

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obtained at 40 g/mL (18%) after 24 h (Figure 4). The inhibition was reduced to 17% when the plant extract concentration was increased to 50 g/mL. When compared to the other test concentrations, the 10 g/mL concentration displayed a higher significant inhibition ($p < 0.05$) after 48 hours. The rate of inhibition increased from 39 to 56% when the concentration was gradually increased to 50 g/mL. The morphological changes appeared to be similar to those observed in the MCF-7 cells. In this study, the outcome of the cytotoxicity assay revealed that *T. vulgaris* leaf extract had anti-proliferative and cytotoxic properties against breast carcinoma cell lines, especially the CAL51 breast cancer cells. Also, other researchers have found that *T. vulgaris* leaf extracts had a cytotoxic effect on MCF-7 and MDA-MB-231 cells. These findings suggest that *T. vulgaris* leaf extract selectively suppressed the proliferation of CAL51 triple-negative breast cancer cells.[12]

Cytotoxic effect of T. vulgaris leaf extract on HBL-100 cell line

When the HBL cell line was incubated in the 50 g/mL acetone *T. vulgaris* leaf extract for 72 hours, the maximum cytotoxicity was 14% (Figure 5). The inhibition of the normal cell line increased with a corresponding increase in the concentration of the plant extract, and the inhibition was more significant at high concentrations. It was observed that the inhibitions were 1.8 and 14% in concentrations of 10 and 50 µg/mL for incubation periods of 24 and 72 hours, respectively. Cytotoxicity of polyphenol has been studied in a number of cancer cells.[36] Polyphenols express anti-cancer properties by the mobilization of endogenous copper ions bounding with chromatin to induce apoptosis and DNA fragmentation.[37] Furthermore, polyphenols have also been reported to have anti-inflammatory effects, such as those observed in cancer cells.[38]

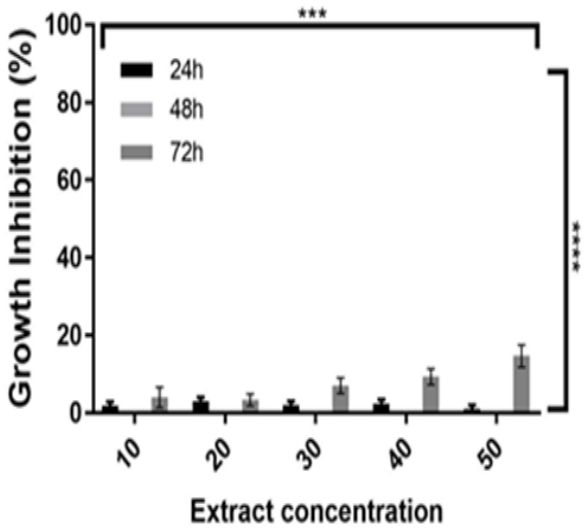

Figure 5: Effect of acetone *Thymus vulgaris* leaf extract on growth inhibition of breast cancer cell line, HBL-100

Conclusion

In this study, different solvents and methods have been employed to prepare plant extracts from *T. vulgaris*. The result of the GC/MS analysis revealed that the extracts contained various polyphenol components. *T. vulgaris* leaf extracts demonstrated strong antioxidant capacity and antitumor activity.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is their original work, and that they will be held liable for any claims relating to the content of this article.

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