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Efficacy of Different Solvents in the Extraction of Bioactive Compounds and Anti-cancer Activities of *Thymus vulgaris* Leaves and Twigs

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ABSTRACT

Thyme contains phytochemicals that exhibit cytotoxic and cytogenic activities. Differ solvents have different abilities in dissolving plant bioactive compounds. This study aims to determine the anti-cancer properties of thyme leaves and twigs, including a profile of bioactive compounds, cytotoxicity, the ability of apoptosis induction, and caspase-3 activation of different solvent extracts on the T47D breast cancer cell line. An experimental study was conducted in eight groups: the T47D breast cancer cell line was treated with thyme extracts as the treatment group and doxorubicin as a positive control. In contrast, untreated T47D cells were used as a negative control. Thyme was extracted by maceration using six different solvents: methanol, ethanol, chloroform, ethyl acetate, dichloromethane, and n-hexane. Bioactive compounds of all extracts were analyzed using GC-MS. Cytotoxicity was investigated through an MTT assay on the T47D breast cancer cell line. The percentage of apoptosis and caspase-3 were detected by flow cytometry using Annexin V-PI and BD Cytofix/Cytoperm, respectively. Thymol, the main bioactive compound in thyme, was found in all extracts. MTT results revealed that six solvent extracts had moderate to weak cytotoxicity, with dichloromethane having the lowest IC_{50} values at 120.23 µg/ml. All thyme extracts promoted apoptosis in T47D cells with apoptosis percentages of methanol, chloroform, ethyl acetate, ethanol, dichloromethane, n-Hexane, and doxorubicin were 82.67; 80.98; 73.13; 72.28; 60.62; 27.74 and 98.43%, respectively. Among all extracts, methanol extract showed the highest apoptosis and caspase-3 activation percentage. We conclude that all thyme extracts had anti-cancer properties in the T47D breast cancer cell line, although the efficacy of each solvent extract has differed.

Keywords: *Thymus vulgaris* L.; cytotoxicity; apoptosis; caspase-3; breast cancer

INTRODUCTION

Cancer is one of the leading causes of death worldwide, and breast cancer is ranked as the most common cancer globally (World Health Organization, 2021). The breast cancer burden has risen rapidly in Indonesia. An estimated 65,858 new cases were diagnosed in 2020, and it is predicted that by 2030, new cases will increase by 25.7% (Global Cancer Observatory, 2020). Unfortunately, current anti-cancer agents and therapeutic strategies have adverse side effects (Islam & Lam, 2020; Prša et al., 2020). Therefore, developing improved pharmaceutical agents, particularly those from natural compounds, is critical.

Thyme (*Thymus vulgaris* L.) is a commonly used herb for culinary and medicinal purposes as it contains bioactive compounds with antioxidant, anti-inflammatory, anti-helminthic, anti-microbial, and anti-cancer activity. Thyme extracts, essential oil, and their purified or synthesized constituents have been shown to treat a broad range of diseases, including cough, diabetes, infection, dermatitis, eczema, and many more (Salehi et al., 2018). Lately, extensive studies have been conducted to evaluate the anti-cancer activity of thyme bioactive compounds as well as their mechanism of action.

Thyme has been suggested as an anti-cancer agent. Thymol can inhibit CRC cell proliferation and induce apoptosis (Zeng et al., 2020). Thyme

extracts exhibited significant cytotoxicity and cytogenic effect, as well as inducing cell cycle arrest on a number of cancers (Adham et al., 2020; Al-seragy et al., 2019; Fathima et al., 2017; Tuţă-sas et al., 2019). Furthermore, thyme methanol extract has been able to prevent DNA damage due to chemotherapy agents (Salmani et al., 2015).

Since plant bioactive compounds usually exist in very low concentrations, extraction as an initial step in bioactive compound profiling plays a considerable role (Brahmi et al., 2012). The solvents-based extraction method is preferable to the other extraction methods of plant bioactive compounds since its simplicity with lower cost are needed (Sung et al., 2015). The extraction of bioactive compounds from plant material is highly dependent on the solvent polarity. Considering the wide chemical structure of plant bioactive compounds, it is necessary to choose an appropriate solvent for the extraction process (Nur Syukriah et al., 2014). The previous study showed that thymol is very soluble in ethanol (Villanueva Bermejo et al., 2015), whereas another study reported that methanol exhibited the best solvent for extracting thyme phenolic compound (Rababah et al., 2010; Roby et al., 2013). Moreover, Hossain et al. (2013) reported the highest antioxidant content of thyme extract was with ethyl acetate solvent, and the lowest antioxidant content was chloroform extract as in the order of value was ethyl acetate>methanol >butanol>hexane> chloroform. These facts reveal the importance of choosing the appropriate solvent.

Crude extracts contain many compounds which act synergistically or antagonistically. In this study, we used different polarity solvents in the thyme extraction process to evaluate the impact of solvents type on the bioactivity compounds dissolved and their mechanism. Hence, this study aims to examine the cytotoxicity, apoptosis, and caspase-3 induction ability of thyme extracts using six different solvents to determine the most effective solvent having anti-cancer properties.

MATERIALS AND METHODS

Plant Sample Extraction and Bioactive Compound Profiling

Thyme leaves and twigs were collected from Research Center for Pharmaceutical Ingredient and Traditional Medicine, Research and Innovation Agency, Central Java province, Indonesia. Thyme is a herb whose small leaves grow on clusters of thin stems. Picking the leaves off one by one from its twig is difficult, and we get only a small amount of biomass to be used as raw material. Hence, we used two parts: leaves and twigs. The sample was cleaned thoroughly and shade-dried until constant weight. The following solvents were used for the extraction process: methanol, ethanol, chloroform, ethyl acetate, dichloromethane, and n-hexane. All the solvents used in this study were analytical grade by Emsure. The concentration of solvents was mostly ≥ 99.5%, except n-hexane was ≥ 96%. The dry leaves and twigs were crushed into powder. Dry powdered (each 180 g) was mixed with 100 mL of each solvent for 24 hours and then filtered. All the filtrate was evaporated at room temperature. The plant residue was re-extracted twice. The clear glass bottles, sealed with parafilm, were used to store all crude thyme extracts. Bioactive compound profiling of thyme extracts was evaluated using GC-MS (GC 2010 Shimadzu) at 70-300^{0}C with a rate of temperature rise of 5^{0}C/min. The GC column used was a semipolar HP 5 or Rtx 5 MS type with a length of 30 m, a diameter of 0.25 mm, and a thickness of 0.25 µm. Helium gas was used as the carrier gas with a 28 ml/minute flow rate. Each sample was injected with as much as 3 µL. The GC-MS detector used was EI 70 Ev. Compounds were identified by comparing MS spectra samples with WILEY229 Library and NIST62 database.

Cell Culture

T47D cell lines were maintained in RPMI 1640 medium (Gibco) supplemented with 10% FBS (Gibco), 2% penicillin-streptomycin (Gibco), and 0.5% fungizone (Gibco), then incubated in a culture flask with humidified atmosphere 5% CO_2 at 37 °C. Cells were harvested after reaching approximately 80% confluence using 0.25% Trypsin-EDTA (Gibco).

Cytotoxicity Assay

The MTT assay was performed to determine the cytotoxicity of the extracts. An amount of 2×10^4 cells/well were seeded onto 96-well microplates in 100 µL RPMI and incubated at 37°C and 5% CO_2 overnight. Then, the old RPMI medium was replaced by 100 µL extracts at different concentrations (7.85, 15.7, 31.3, 250, and 1000 µg/mL), followed by incubation at 37°C for 24 hours. Doxorubicin (5 µg/mL) was used as the positive control, whereas T47D cells cultured in RPMI were used as the negative control. Cells cultured in 0.5% DMSO (Invitrogen) were used as the solvent blank. Five percent of MTT (Invitrogen) solution was added to each well (both the treated

and non-treated cells) and incubated for 4 hours. Then, the supernatant was discarded, and a stopper reagent (10% SDS in 0.01N HCl) was added to the wells. The amount of purple formazan was measured at 550 nm using ELISA Reader (BIO-RAD 680XR). The T47D cell's viability was calculated as follows:

$$\% \text{ Cell viability} = \frac{\text{absorbance of treated well}}{\text{absorbance of cell control}} \text{x } 100\%$$

The Inhibitory Concentration 50% (IC_{50}) value was determined by probit analysis using value among cell viability and log concentration of thyme extracts.

Apoptosis and Caspase-3 Assay

T47D cells were cultured in 6-well microplates with density $1x10^6$ cells/2000µL RPMI in each well overnight. Then, the old media was replaced with thyme extracts at IC_{50} concentration or doxorubicin for 24 hours. The effect of thyme extracts on T47D cell apoptosis was determined by flow cytometer (BD FACSCalibur™) with Annexin V/PI Biolegend double staining, whereas BD Cytofix/Cytoperm™ was used for caspase-3 activation assay. Flow cytometry analysis output was shown in dot plot quadrants. The first quadrant represented viable cells (FITC-/PI-), the second quadrant showed early apoptotic cells (FITC+/PI-), the third quadrant was considered late apoptotic cells (FITC+/PI+), and the fourth quadrant contained necrotic cells (FITC-/PI+).

Statistical Analysis

The statistical difference among the various extracts was determined by one-way ANOVA and Tukey post-test employing SPSS 26.0 software. Values are expressed as the mean ± standard deviation. P <0.05 was considered statistically significant.

RESULTS AND DISCUSSIONS

Thyme Bioactive Compounds Profile

In this study, extraction was performed using six different solvents (methanol, ethanol, chloroform, ethyl acetate, dichloromethane, and n-hexane) based on their polarity to evaluate the impact of the solvents' polarity on bioactivity compounds dissolved. Methanol had the highest extract yield (5.62%), while the lowest yield of thyme crude extract was obtained by dichloromethane (1.35%) (data was not shown). The chromatograms of each extract (Figure 1) which shows that the detected peaks correspond to the bioactive compounds present in the extract. Ethanol extract of thyme showed the presence of 71 peaks, which was the highest peak detected among other extracts, followed by dichloromethane (55), n-hexane (47), chloroform (46), ethyl acetate (36), and methanol (25). Taken together, in this study, we conclude that ethanol was the most efficient solvent to recover thyme bioactive compounds.

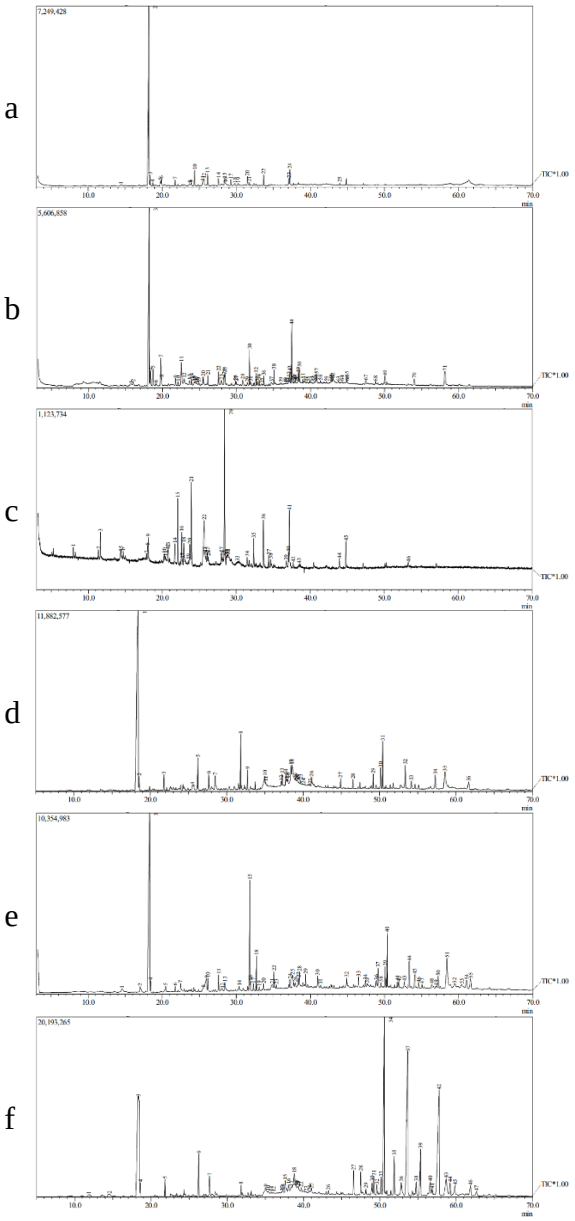


Figure 1. Total Ion Gas chromatography-mass spectrometry (GC-MS) chromatograms of thyme extracted by methanol (A); ethanol (B); chloroform (C); ethyl acetate (D); dichloromethane (E); and n-hexane (F).

Table I. Tentative Identified compounds of thyme extracts of possess anti-cancer activity

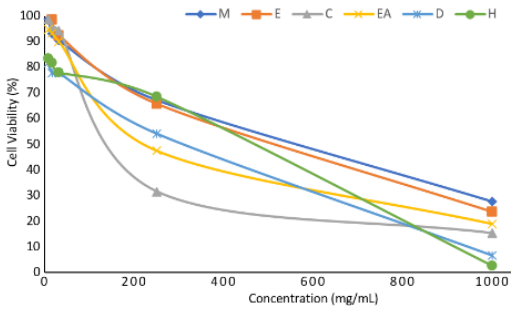
No	Compound	Group	Peak Area (%)					
			M	E	C	EA	D	H
1	Thymol	Phenol	64.3	27.35	1.39	39.87	34.24	16.52
2	Carvacrol	Monoterpene	2.17	1.53		0.89	0.71	
3	Caryophyllene	Sesquiterpene	1.2	0.8	2.33	0.97	0.39	0.57
4	Humulene	Sesquiterpene	0.54		7.24			
5	Lauric acid	Fatty Acid	2.15				3.62	
6	9,12,15-Octadecatrienal	Fatty Aldehyde	2.39	1.68	1.55	2.42	2.16	
7	Methyl tetradecanoate	Tetradecanoic Acid	1.34					
8	1-octadecyne	Alkene	0.57	2.83		3.34	7.67	
9	Methyl isopalmitate	Fatty Acid	2.1		4.29		0.41	
10	Methyl linolelaidate	Fatty Acid	1.38	1.31	1.59	3.62	0.72	1.04
11	Methyl linoleate	Fatty Acid	3.06	1.13		0.76		
12	Dihydroactinidiolide	Terpene		0.56				
13	α-terpineol	Monoterpenoid		0.51				
14	Phytol	Diterpene		6.79				1.61
15	Isopulegol	Monoterpenes		0.46				
16	Farnesol	Sesquiterpene Alcohol		0.57		1.07		0.39
17	Undecane	Alkanes			2.14			
18	Valeric acid	Fatty Acid			1.32			
19	Limonene	Monoterpenoid			0.63			
20	Steric acid	Fatty Acid				3.14	0.73	1.82
21	Geranyl Alcohol	Monoterpenoid				0.92		
22	Tridecane	Alkane				0.7		1.08
23	Eicosane	Alkane				2.12	4.16	14.48
24	Octadecanoic acid	Fatty Acid					1.41	
25	Nonadecanol	Fatty Alcohol					1.78	
26	17-Acetoxy-19-kauranal	Diterpene					8.23	
27	Linalool	Monoterpene Alcohol						0.37
28	Octadecyne	Alkene						0.38
29	Squalene	Terpene						0.95

M: methanol; E: ethanol; C: chloroform; EA: ethyl acetate; D: dichloromethane; H: n-hexane

The tentative identified and comparative bioactive compounds with the anti-cancer activity (Table I) and non-anticancer bioactive compounds (Supplementary Data). We identified some bioactive compounds with anti-cancer activity in all thyme extracts (Table I). Thymol, as the main bioactive compound of thyme, was detected in all extracts. The highest peak area of thymol was obtained in methanol solvent (64.3%). The crude extracts contain some compounds that can act synergistic or antagonistic. The cytotoxicity of thyme crude extract is probably caused by some compounds, not only due to the act of thymol. Anti-cancer bioactive compounds detected in three or more treatment groups were Thymol (Kubatka et al., 2019), Carvacrol (Khan et al., 2023), Caryophyllene (Lei et al.,2021) 9,12,15-Octadecatrienal (Zhang and Jiang, 2015), Methyl linolelaidate (Kumar et al., 2017), Methyl linoleate (Yang et al., 2018), Farnesol (Jung, 2018), Steric acid (Khan 2013) and Eicosane (Ali, 2021).

Our results showed that solvents significantly affect the yield of thyme crude extract and bioactive compounds solubility. Based on our study, the highest extraction yield and bioactive compound detected were found with polar solvents, indicating that most substances in thyme are hydrophilic. Thymol is a phenolic compound that highly soluble in polar protic solvents like methanol (Rezaie *et al.*, 2015).

A. Cell viability



B. The IC_{50} of thyme extracts

Thyme extract	IC_{50} (µg/mL)	Cytotoxic effect
Methanol	389.05±18.3	Weak
Ethanol	338.84±2.9	Weak
Chloroform	147.91±12.3	Moderate
Ethyl acetate	194.98±7	Moderate
Dichloromethane	120.23±15.1	Moderate
n-hexane	151.36±14	Moderate

Data expressed as mean ± SD of triplicate

C. T47D cell morphology

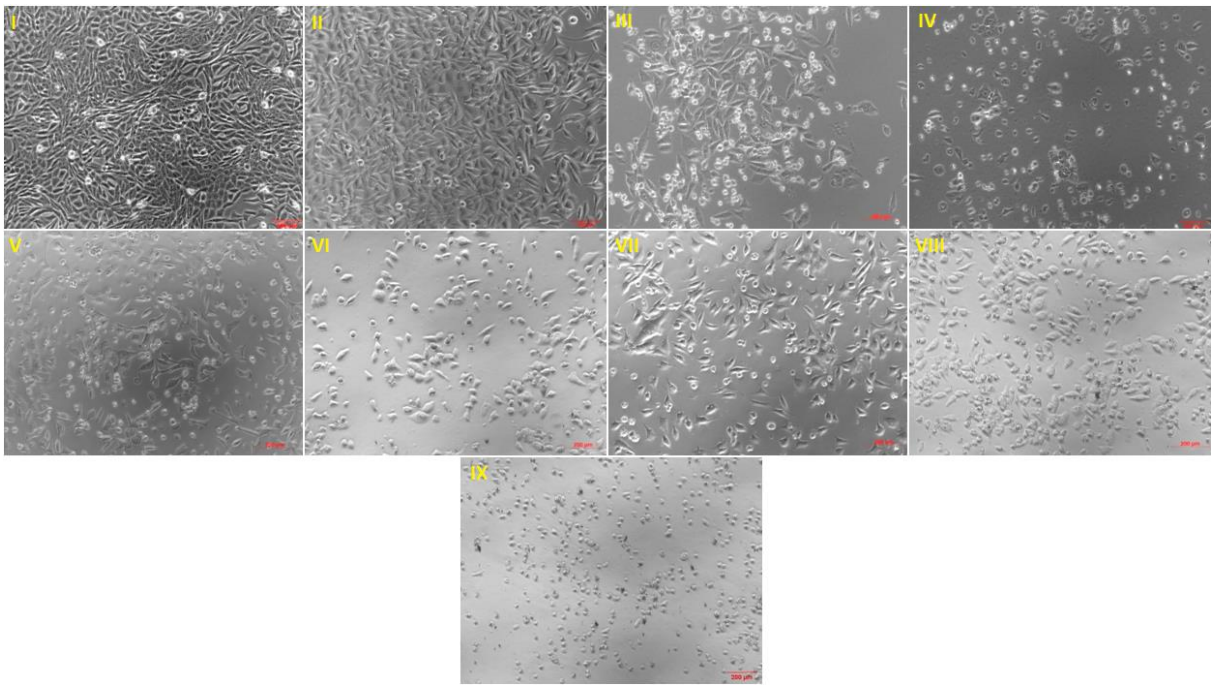


Figure 2. The effect of thyme extracts. A. T47D breast cancer cell line viability. Methanol (M), ethanol (E), chloroform (C), ethyl acetate (EA), dichloromethane (D), and n-hexane (H) extract. B. The IC_{50} of thyme extracts. C. T47D cell morphology after 24 hours of treatment with thyme extracts. (I) Control, (II) DMSO, (III) Methanol, (IV) Ethanol, (V) Chloroform, (VI) Ethyl acetate, (VII) Dichloromethane, (VIII) n-Hexane, (IX) Doxorubicin. The cell was observed using an inverted microscope Axio Vert.A1 Zeiss with 200x magnification.

Thus, the highest thymol percentage was obtained from methanol solvent. Methanol has been reported as the most suitable solvent for phenol extraction due to its ability to inhibit polyphenol oxidation by polyphenol oxidase (Brahmi et al., 2012). Although methanol and ethanol have nearly the same polarity, the percentage of thymol in ethanol extract differed from methanol extract, possibly because of the solubility of thymol in a solvent. According to Zhu et al. (2016), the solubility of thymol increases in six different solvents as follows: 1-butanol < 1-propanol < 2-propanol < ethanol < methanol < acetonitrile. This study supports our data that thymol is more soluble in methanol than ethanol.

Table II. Percentage of T47D cell population and caspase-3 after treatment with thyme extract

Treatment	Applied concentration (µg/mL)	Cell percentage (%)			Caspase-3 (%)
		Normal	Apoptosis	Necrosis	
Methanol	389.05	8.34*±0.65	82.67*±1.12	9.22*±0.47	16.86
Ethanol	338.84	5.7*±2.35	72.28±12.82	22.34*±10.59	14.66
Chloroform	147.91	1.33*±1.31	80.98± 3.40	17.84*±4.65	14.37
Ethyl acetate	194.98	4.53*±5.76	73.13*± 15.67	22.52*±10.03	14.62
Dichloromethane	120.23	2.42*±1.36	60.62*†±8.09	37.06*†±6.77	6.01
n-Hexane	151.36	38.98†±16.1	27.74†‡±3.73	33.58*†±12.3	11.76
Doxorubicin	5	1.57	98.43	0.01	79.06
Negative control	-	93.76‡±1.74	4.66‡±0.43	1.65*‡±1.36	0.68

Data of cell percentage are expressed as mean ± SD from two replication. Different symbols in different columns and rows showed significantly different values determined by one-way ANOVA and Tukey post-test P <0.05.

Cytotoxicity of thyme extracts

The present study determined the cytotoxic effect of six thyme crude extracts at a concentration range of 7.85-1000 µg/ mL on the T47D breast cancer cell line by MTT assay. Cell viability after 24h of treatment with each thyme extract (Figure 2A). All solvent extracts decreased the viability of T47D cells in a dose-dependent manner. The higher concentration of thyme extracts caused a lower percentage of cell viability.

All thyme extracts induced a dose-dependent cytotoxic effect on the T47D cell line. This cytotoxicity effect was consistent with cell morphological change after 24h of treatment with thyme extracts (Figure 2C). The marked morphological change could be seen after 24h treatment, characterized by rounding of cells, and cells began detaching from the surface. Almost all cells in the control and DMSO-treated cells were live and had normal morphology. T47D cells in these groups form tightly cohesive mass structures displaying robust cell-cell adhesions. On the other hand, in doxorubicin-treated cells, most T47D cells undergo death. The cells became shrunken and showed signs of detachment from the surface of the wells. Cell morphology after thyme extract treatment varied. Furthermore, the IC_{50} of each extract was calculated based on the cell viability curve (Figure 2B). Compared with other extracts, dichloromethane presented the lowest IC_{50} value at 120.23 µg/mL and possessed a moderate cytotoxic effect.

Based on US National Cancer Institute, the cytotoxic effect was categorized into four groups by IC_{50} value: very toxic (≤ 20 µg/mL), moderate (21-200 µg/mL), weak (201-500 µg/mL), and non-toxic (≥ 500 µg/mL) (Widiyastuti et al., 2019). Two of the thyme extracts, methanol and ethanol, met the weak criteria with IC_{50} values of 389.05 and 338.84 µg/mL, respectively. In contrast, the other four extracts have moderate cytotoxic effects, with the crude dichloromethane extract of thyme being the most cytotoxic. The cytotoxicity effects differences were attributed to different bioactive compounds present in each extract which were mainly affected by the extraction solvent (Purnamasari et al., 2019). Our results are consistent with other studies demonstrating a weak cytotoxic activity of thyme ethanolic extract on THP-1, Caco-2, and HepG2 cell lines (Ayesh et al., 2014; Taghouti et al., 2020). In agreement with our study, another study shows that chloroform fraction of thyme extract demonstrated better inhibitory activity against the NCI-H929 cell line, compared with ethyl acetate, butanol, and n-hexane fraction (Adham et al., 2020). Since MTT assay only detected cell death, we further analyzed their mechanisms.

Apoptosis and caspase-3 Activation

One of the anti-cancer mechanisms is apoptosis induction. Apoptosis is a programmed cell death process regulated by an integrated signaling pathway in order to maintain cellular homeostasis (Fadholly et al., 2020). The apoptosis marker of T47D cells after being treated with thyme extracts for 24h was detected by flow cytometry. Compared to the negative control, all thyme extracts were able to increase the percentage of apoptotic cells. Meanwhile, among all thyme extracts, the highest total apoptosis cell percentage was reached with methanol extracts (82.67%), although it remained lower than doxo-rubicin as the positive control (98.43%) (Table II).

These results indicated that thyme extracts could induce apoptosis of the T47D breast cancer cell line.

Apoptotic cells undergo morphological changes, including cell shrinkage, pyknosis, membrane blebbing, karyorrhexis, and formation of apoptotic bodies (Majtnerová & Roušar, 2018). Those morphological changes were observed in the cells treated with thyme extracts. On the other hand, our results showed a significant increase in the total apoptosis (early and late apoptosis) cell percentage following 24h treatment of thyme extracts compared with untreated cells. Most cells in the untreated/negative control group were normal (93.76%), while necrosis cells were only 1.65% (Table II). Cell treated with methanol extract possessed the highest total apoptotic percentage with the lowest necrosis. Furthermore, the other solvents extract, such as chloroform, ethyl acetate, and ethanol, had high apoptotic cells, but the necrosis cells were more than 10%. Interestingly, although dichloromethane extract had the lowest IC_{50} values, it had the highest necrosis cell (37.06%). On the contrary, methanol extract had the highest IC_{50} values, but it had the lowest necrosis cell (9.22%). Most of the T47D cells undergo apoptosis after being treated with methanol extract (82.67%). These facts made methanol extract the safe anti-cancer agent among the other solvent extracts.

To examine the mechanism of thyme extracts-induced apoptosis, caspase-3 activity was detected by flow cytometry. All thyme extracts promoted caspase-3 activation in T47D cells compared to untreated cells (Table II and Figure 3). The highest percentage of caspase-3 was detected in methanol extract (16.86%), although it remained lower than doxorubicin as the positive control (79.06%).

The apoptosis induction mechanism is commonly initiated through a caspase-dependent pathway, which includes intrinsic (mitochondrial-mediated) or extrinsic (DR-mediated) pathways. Both pathways congregate at the execution phase as a final pathway, mediated by caspases (Jan & Chaudhry, 2019). Many studies have also examined the caspase-independent apoptotic pathway of thyme (Deb et al., 2011). However, caspase-3 is the most important effector caspase in the caspase-dependent apoptotic pathway, which executes apoptosis when activated by any of the initiator caspases (caspase-2, -8, -9, and -10) (Boice & Bouchier-Hayes, 2020; Jan & Chaudhry, 2019). Thus, we investigated whether the extracts could trigger the T47D apoptotic pathway via caspase-3 activation. All thyme extracts increased the caspase-3 activation compared with the control. Moreover, caspase-3 was activated by 16.86% after being treated with methanol extracts, being the highest among other extracts.

The potent apoptosis-induced effect of methanolic thyme extract may be attributed to bioactive compounds like terpenoids and phenolic in the proper concentrations and work synergistically since these compounds are reported to induce apoptosis in many cancer cells. In this study, thymol, as the main constituent of thyme, was highest extracted with a methanol solvent. The methanolic extract also recovered other anti-cancer bioactive compounds, including carvacrol, caryophyllene, humulene, lauric acid, eicosane, farnesol, phytol, and so on.

Late studies have assessed the mechanism behind the thyme bioactive compound's apoptotic effect. Thymol showed a significant increase in Bax protein expression, decreased Bcl2 protein expression, and induced activation of caspase-9, -8, and -3 as the hallmark of the caspase-dependent apoptotic pathway (Deb et al., 2011). Another study revealed that thymol increased ROS levels, leading to mitochondrial membrane potential loss as well as caspase-3 activation and DNA damage, resulting in cell cycle arrest (Kubatka et al., 2019). A high dose of carvacrol, an isomer of thymol, isolated from *Thymus spicata* may induce DNA damage (Aydin et al., 2005). The previous study shows that caryophyllene decreases following gene expression, which is responsible for cell proliferation, survival, metastasis, and angiogenesis: cyclin D1, bcl-2, bcl-xL, COX-2, VEGF, and IAP. Caryophyllene also inhibits the activation of PI3K/AKT/mTOR/S6K1 signaling cascade while increasing the activation of ERK, JNK, and p38 MAPKn in many tumor cells (Park et al., 2011). The apoptosis mechanism of ovarian cancer cells following caryophyllene treatment is mediated by caspase-3 activation and PARP cleavage (Arul et al., 2020). Limonene may inhibit lung cancer cell growth by enhancing the expression of apoptosis and autophagy-related genes (Yu et al., 2018), while other studies show that limonene-activated caspase-3 and caspase-9, as well as PARP cleavage in a dose-dependent manner in colon cancer cell line (Jia et al., 2013).

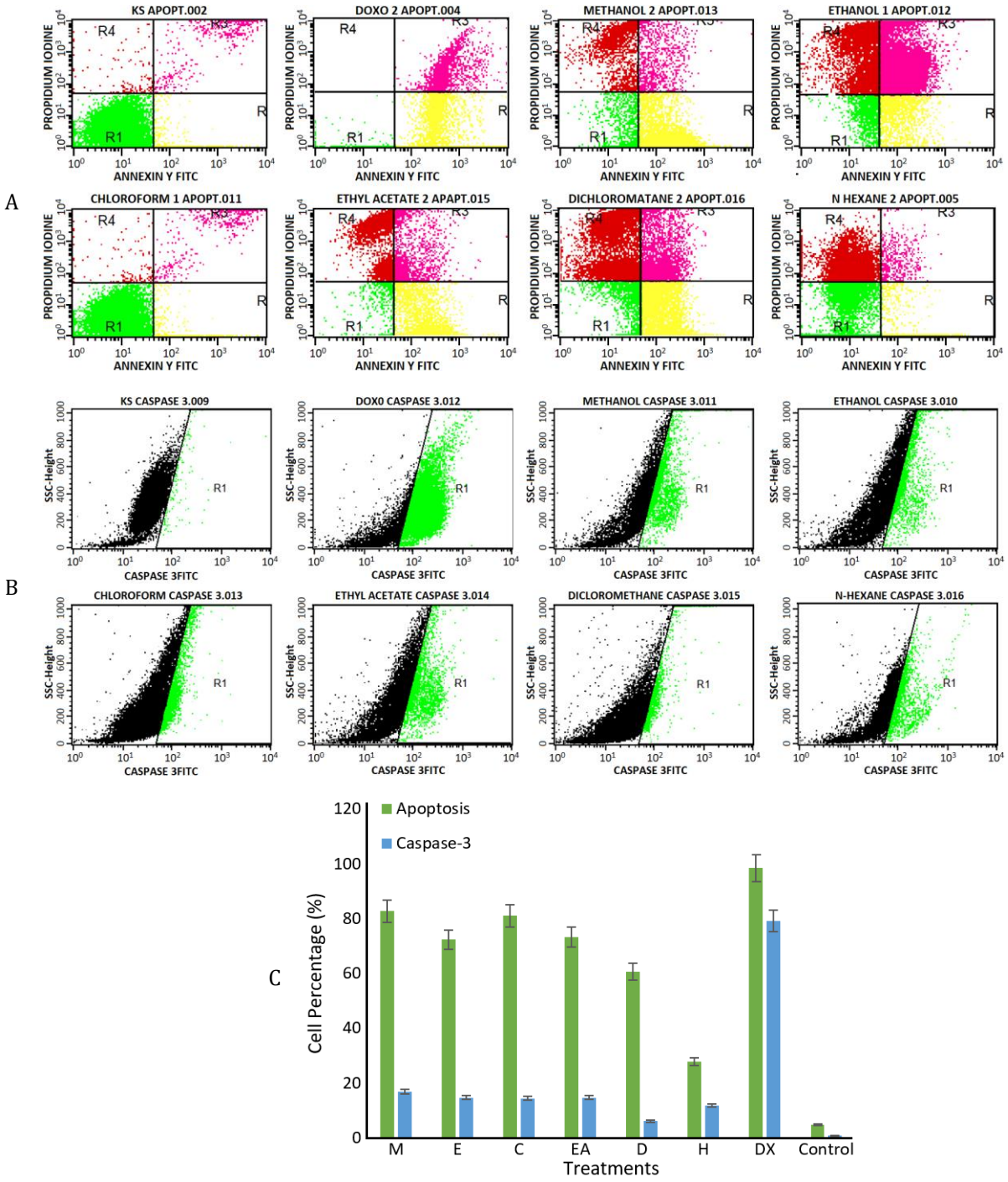


Figure 3. A. Apoptosis markers analysis of T47D cells after being treated with thyme extracts for 24h. Dot plot quadrants represent viable cells (R1), early apoptosis cells (R2), late apoptotic cells (R3), and necrosis cells (R4). B. Representative flow cytometric data of caspase-3 activation on T47D cells after treatment with thyme extracts. The black dot region represented control cells, while the green dot region (R1) showed caspase-3 activated cells. C. Correlation between percentage of apoptosis and caspase-3 activation in T47D breast cancer cell line after being treated with thyme extracts. (M) Methanol, (E) Ethanol, (C) Chloroform, (EA) Ethyl acetate, (D) Dichloromethane, (H) n-Hexane, (DX) Doxorubicin, and (Control) Negative control.

Apart from the caspase-dependent apoptotic pathway, thymol was reported to trigger apoptosis-inducing factor (AIF) release from mitochondria to cytosol and nucleus, indicating activation of the caspase-independent pathway. Translocated AIF to the nucleus initiates DNA fragmentation, leading to cell death (Deb et al., 2011). Thymol may also induce cell cycle arrest at the sub-G0/G1 phase and apoptotic cell death based on gDNA fragmentation in the HL-60 cell line (Salehi et al., 2018). A previous study demonstrated that eicosane suppressed cell survival protein expression and inhibited cell migration of glioma, cervical, and breast cancer cell lines (Mishra et al., 2019). Farnesol can enhance the THRβ1 expression, which plays a role in cell growth inhibition in breast cancer cell lines. Farnesol also activates the farnesoid X receptor to regulate gene transcription (Jung et al., 2018).

In this study, treatment by thyme extracts increased apoptosis and caspase-3 activation more than that in untreated cells. The apoptosis percentage of thyme extracts was nearly the value of doxorubicin. However, the percentage of caspase-3 from all treatments was very low compared to doxorubicin. This could possibly be due to the apoptosis mechanism of thyme extracts in T47D cells via signaling pathways other than caspase-3. It has been reported that the apoptosis mechanism of T47D cells is not only mediated by caspase-3 but also mediated by caspase-6 and -7 as effector caspase (Bandala et al., 2013; Yusuf et al., 2020).

This study has some strengths and limitations. To the best of our knowledge, this is the first study to investigate the effect of different solvents used in thyme extraction on anti-cancer properties of the T47D breast cancer cell line. Furthermore, this study is outstanding as we provide comprehensive results, comprising bioactive compound profiling in all extracts and the implication of each extract in mediating cytotoxicity effect on the T47D breast cancer cell line, including reducing cell viability, as well as increasing the apoptosis induction and caspase-3 activation. However, this study uses thyme crude extract. Therefore, identifying the anti-cancer mechanism of each bioactive compound cannot be performed. Importantly, the cytotoxicity effect of solvents is complex and affected by numerous factors, such as the type of cell line studied. A solvent may completely induce cytotoxicity of a cell line type but more or less tolerated in others (Ilieva et al., 2021). Further study is needed to investigate the exact bioactive compound constituent and concentration to eliminate cancer cells with minimum side effects effectively. A replication study in different cancer cell lines is also important to confirm the consistency of these findings in another cancer type.

CONCLUSIONS

All thyme extracts had anti-cancer properties in the T47D breast cancer cell line, although the efficacy of each solvent extract has differed. All thyme extracts have weak or moderate cytotoxicity in the T47D breast cancer cell line. The anti-cancer actions were through their ability to induce apoptosis via the caspase-3 activation pathway.

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