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Phytochemical Screening of Antimicrobial Compounds in Unnamed Plant from Borneo by Using Percolation and GC-MS

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GRAPHICAL ABSTRACT

Leaf percolation extracts

ABSTRACT

The present study was carried out to identify the presence of potential antibacterial and antifungal agents in the leaves of an unnamed plant extracts by using GC-MS (Agilent 7820A GC System with 5977E MSD) subjected to cold percolation technique. Qualitatively phytochemical screening of leaves ethanolic extracts revealed the existence for both major and minor phytoconstituents. The identified bioactive compounds were matched with National Institute of Standards and Technology (NIST) library yield the selected compounds of 2-phenylbenzothiazole, bamethan, lysergamide, oxazepam, thimerosal, 2,4-di-tert-butylphenol, 2'-hydroxychalcone, 2,5-dihydroxybenzoic acid, thymol, and phytol. These different active compounds were classified according to their nature and its roles of biological activities as antibacterial and antifungal. 2-phenylbenzothiazole is potential as antibacterial properties, meanwhile 2,4-di-tert-butylphenol and phytol are potential as antimicrobial properties. Thymol and thimerosal revealed the existence as antibacterial and antifungal agent. The finding justifies its use in traditional medicine to treat various diseases.

Keywords: Plant, percolation, GC-MS, bioactive, antibacterial, antifungal

1. INTRODUCTION

Natural products are the chemical substances that produce naturally by living organisms. Some of the natural product possess particular activities that are beneficial to the human body. Asian countries especially Malaysia, Indonesia, China, India, and Philippines have great biodiversity and consists of various types of plants with high potential to be utilized in the field of food, cosmetics, and pharmaceuticals. Herbal plants are widely used in traditional medicine to prevent or treat diseases [1]. Occasionally, terms of herbal medicine can be related to animals, insects, plants and the microorganisms. These active compounds can be found in the form of flowers, barks, rhizomes, leaves, and fruits. This paper indicates how plants are extracted to obtain the bioactive compounds by using an appropriate extraction method.

Extraction is the process of separating medicinally active portions of plants substances when it is mixed with others. The sole purpose of general extraction for herbal plants is to obtain desirable chemical compound from the plant materials using selective solvents through standard procedures. The way of extraction are varied in terms of temperature, pressure, time, the solvent used based on the types of herbal plants used. The obtained plants extracts are then processed for medication in different types of products such as tea, syrups, essential oil, capsule, tablets or in powdered form.

The process of getting active compounds starts with the extraction techniques which can be divided into conventional and modern methods. Among the conventional methods of extraction including maceration, infusion, percolation, decoction, digestion, and hot continuous extraction method. Meanwhile, modern extractions method includes counter-current extraction, microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE) or sonication extraction, acceleration solvent extraction (ASE) and supercritical fluid extraction (SFE). Some simple extraction techniques may be employed for extraction of aromatic plants such as hydrodistillation and steam distillation methods [2]. The extracted active compounds can have wider range of biological activities that have its own beneficial effects.

Among the biological activities of these active compounds have great potential as antioxidant, antimicrobial and anticancer which valuable in curing cardiovascular, diabetes, cancer, eczema, and neurodegenerative diseases. Herbal plants have always been the main point of traditional remedies for both physical and spirituals because of producing phytochemical compound. Garlic has been used by ancient Egyptians as the treatment of cardiac and circulatory disorders [3]. The content of herbs medicine has active ingredients that come from the parts of the plants or combination of other plant materials. Pharmaceutical development nowadays has been proved that herbal plants can be isolated and used directly as drugs or by getting their active compounds and design them with or without additional synthetic into it. Crude extract and herbal medicine may expose to the humans on the significance of natural products. Several plants can be found its medicinal properties based on their family species. Hence, the discovery of new potential plants can be done using percolation techniques.

2. EXPERIMENTAL

2.1. Preparation of extracts

1.0 g of plant sample was moistened with small amount of 70% (v/v) ethanol for 6 hrs at room temperature. 25 mL of ethanol with similar percentage concentration was added and the mixture was left overnight. The plant extraction was done in triplicate using the same sample and filtered by using filter paper. Another fresh new 25 mL of similar solvent was decanted and allowed slow descent of solvent through plant sample. The collected extracts were subjected to rotary evaporator at temperature 65 oC for solvent evaporation to concentrate the extracts. The extracts was then syringe filtered using 0.45 μm and consequently, 0.22 μm polytetrafluoroethylene filter obtained from Sigma-Aldrich (United States). 2 mL of the extracts were sent for analysis with GC-MS (Agilent 7820A GC System with 5977E MSD) using ethanol MS grade solvent.

2.2. GC-MS Analysis

GC-MS analysis was performed using gas chromatography equipped with an Elite-5 capillary column (5% phenyl 95% dimethyl polysiloxane) (30 m x 0.25 mm i.d., x 0.25 μm film thickness) and mass detector operated in electron impact (EI) mode at 70eV. Helium was used as carrier gas at flow rate of 1 mL/min. The injector and detector were operated at 260°C and 300°C and the oven temperature was programmed at 40°C with an increasing temperature of 6°C per min until reached 290°C. Mass range between 40 to 600 amu. The injection volume to GC-MS was 1 μL. Interpretation on mass spectrum of GC-MS was done using database of National Institute Standard and Technology (NIST).

3. RESULTS AND DISCUSSION

3.1. GC-MS analysis of plant extracts

The compounds obtained from leaf extracts are shown in Figure 1, with each of the peak represents signal created when a compound elutes from the GC column. Retention time and percentage area were obtained together for each individual compound. GC-MS identifies the active compounds present in the extracts.

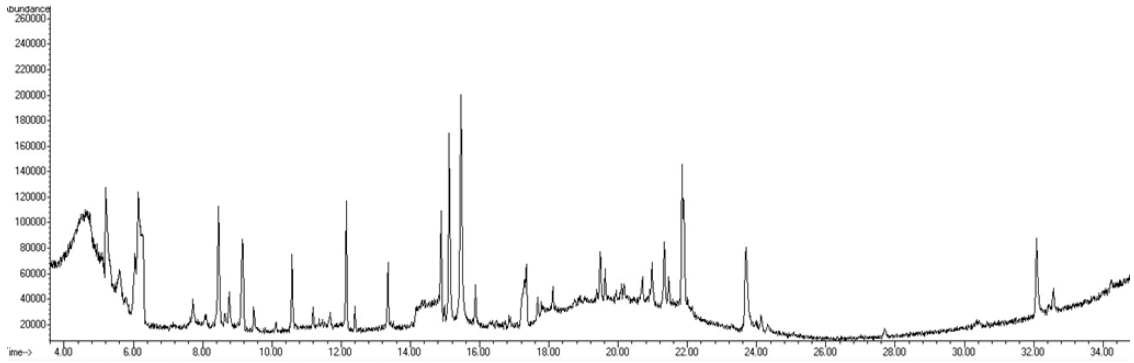

Figure 1. GC-MS total ion chromatogram (TIC) of phytochemical constituents of ethanol leaves extract. GC-MS condition: fused silica capillary column (30 m x 0.25 mm i.d., x 0.25 μm film thickness) was employed (Agilent Technologies, USA) with GC-MS detection of ionization energy 70 eV.

In this study, only one type of sample used subjected to percolation using 70% (v/v) ethanol. Leaves part of the plant was chosen to extract its active compounds which believe the most bioactive constituents obtained from the extracts. Semi-polar solvent like alcohol act as intermediate solvent which frequently employed for percolation due to the abilities of extracting both polar and intermediate polarities of the compounds. Aqueous mixture containing ethanol has been chosen as an extractant since its known as good solvent for polyphenol extraction and safe for human consumption. Ethanol MS grade was used as mobile phase which it carries the mixture of leaf extracts to the stationary phase and selectively attract the compounds in the sample mixture. Solvent delay was set at 3.5 min in GC-MS system to prevent high concentration of solvent vapour in the sources.

Compound detected at retention time of 15.460 min, could possibly be the highest compounds obtained in the leaves ethanol extract corresponding to its abundance. The compound identified was 2'-hydroxychalcone. However, this compound detected at the highest peak, 2'-hydroxychalcone was not necessary potential as antibacterial or antifungal

agents. Meanwhile, the lowest peak of the compounds were 2,6-dichloro-4-(9H-fluoren-9-ylideneamino)-phenol and N-(2-methyl-4-{[1,3]oxazolo[4,5-b] pyridin-2-yl}phenyl)acetamide detected at 10.122 min and 14.980 min of retention time respectively with no biological activities (Figure 1).

3.2. Screening and identification of active compounds by GC-MS

Qualitative analysis was done using GC-MS (Agilent 7820A GC System with 5977E MSD) with electron energy 70eV and identification of the compounds were matched with database of National Institute for Standards and Technology (NIST) library. The active compounds of plants were sorted in the table according to their name with its area and retention time of the compounds being eluted.

Table 1. Phytochemical screening of active compounds by GC-MS

Name of compound	Retention time (min)	Area (%)
2-phenylbenzothiazole	5.189	7.83
Benzoic acid, 4-methyl-2-trimethyl silyloxy-, trimethylsilyl ester	6.042	2.86
Octamethyl-cyclotetrasiloxane	6.145	6.18
Benz(a)acridine, 9,10,12-trimethyl	7.718	1.29
Bamethan	8.456	5.91
Lysergamide	8.657	0.38
2-(1-hydroxynaphthyl-2)quinoline	8.765	1.67
Decamethylcyclopentasiloxane	9.143	3.19
Bisphenol A	9.475	1.01
2,6-dichloro-4-(9H-fluoren-9-ylideneamino)-phenol	10.122	0.36
N-adamantan-2-yl-3,4,5- trimethoxy-benzamide	10.579	2.60
2-[2-(2-aminobenzylidene) aminobenzylideneamino]-benzaldehyde	11.186	0.64
Naphthalen-1-yl(1-pentyl-1H-indol-3-yl) methanone	11.689	0.54
Oxazepam	12.147	4.57
Thimerosal	12.393	0.64
1-tripropylsilyloxytetradecane	13.349	2.01
Dodecamethylpentasiloxane	14.882	3.68
N-(2-methyl-4-{[1,3]oxazolo[4,5-b]pyridin-2-yl}phenyl)acetamide	14.980	0.36
2,4-di-*tert*-butylphenol	15.123	7.96
2'-hydroxychalcone	15.460	11.56
Alternariol monomethyl ether	15.884	1.25
2,5-dihydroxybenzoic acid	17.297	4.06
2,5-dihydroxybenzoic acid	17.349	1.97
1-dimethyl(phenyl)silyloxydodecane	17.680	0.91
Oxime-cholestan-3-one	18.115	0.75
Octadecmethylcyclononasiloxane	19.489	1.89
2-Octen-1-ol	19.620	1.03
Benzenepropanoic acid, 3,5-bis(1,1 -dimethylethyl)-4-hydroxy-, methylester	20.976	1.81
N-(3-allyl-2-oxo-2,3-dihydro-1,3-benzothiazol-6-yl)acetamide	21.331	2.49
p-menthane-1,3-diol	21.457	0.83
Thymol	21.846	6.07
Phytol	23.689	5.91
Perhydro-htx-2-one, 2-depentyl-, acetate ester	24.123	0.67
Piperazine, 1-(2-fluorophenyl)-4-[4-(4-fluorophenyl)thiazol-2-yl]-	27.694	0.45
Phthalic acid, 2-methoxyethyl undecyl ester	32.083	4.65

As listed in the table, different components were identified in the leaves extract. First compound eluted was 2-phenyl-benzothiazole with retention time 5.189 min meanwhile the last compound eluted was phthalic acid, 2-methoxyethyl undecyl ester with extended retention time of 32.083 min. The major components of ethanol extracts were 2'-hydroxychalcone (11.56%), 2,4-di-tert-butylphenol (7.96%), 2-phenyl-benzothiazole (7.83%),

octamethylcyclotetrasiloxane (6.18%) and thymol (6.07%). The minor components were bamethan (5.91%), phytol (5.91%), phthalic acid, 2-methoxyethyl undecyl ester (4.65%), oxazepam (4.57%), 2,5-dihydroxybenzoic acid (4.06%), dodecamethylpentasiloxane (3.68%), decamethylcyclopentasiloxane (3.19%), benzoic acid, 4-methyl-2-trimethyl silyloxy-, trimethylsilyl ester (2.86%), N-adamantan-2-yl-3,4,5- trimethoxy-benzamide (2.60%), N-(3-allyl-2-oxo-2,3-dihydro-1,3-benzothiazol-6-yl)acetamide (2.49%), and 1-tripropylsilyloxytetradecane (2.01%). Other components constituted less than 2.0% of the total yield (Table 1).

3.3. Selection of active compounds from GC-MS

In GC-MS chromatogram, various types of phytochemical present including both major and minor compounds. Bioactive compounds identified in leaves extract were selected, classified based on its nature and biological activities.

Table 2. Selected active compounds from GC-MS analysis with its pharmacological activities

Name of compound	Nature	Pharmacological Activities	Source
2-phenylbenzothiazole	Alkaloid	Antibacterial	PubChem
Bamethan	Phenolic	Peripheral vasodilator	PubChem Drugs.com
Lysergamide	Alkaloid	Psychedelic	ChEBI
Oxazepam	Benzodiazepine	Anxiolytic	ChEBI DRUGBANK
Thimerosal	Benzoic acid derivatives	Antibacterial, Antifungal	DRUGBANK
2,4-di-*tert*-butylphenol	Phenolic	Antibacterial, Antifungal	PubChem
2'-hydroxychalcone	Phenolic	Anti-inflammatory	ChEBI
2,5-dihydroxybenzoic acid	Phenolic acid	Anti-inflammatory, Antioxidant	PubChem
Thymol	Monoterpene	Antiseptic, Antibacterial, Antifungal	DRUGBANK Drugs.com
Phytol	Diterpene	Antibacterial Antifungal	PubChem

Bioactive compounds obtained from GC-MS possessed wider range of pharmacological activities including peripheral vasolidator, psychedelic, anxiolytic, antibacterial, antifungal, anti-inflammatory, antioxidant, and antiseptic. 2-phenylbenzothiazole is potential as antibacterial properties, meanwhile 2,4-di-*tert*-butylphenol and phytol are potential as antimicrobial properties. Among bioactive compounds that revealed the existence as antibacterial and antifungal agent are thymol and thimerosal (Table 2).

3.4. Antibacterial and antifungal compounds identified by GC-MS

Antimicrobial agent prevents microorganisms or stops their growth from spreading. The selected bioactive compounds from leaves extract were then specifically classified the potential of antibacterial and antifungal agent with infectious microorganisms that cause diseases.

Table 3. Bioactive compounds with antibacterial properties

Name of compound	Nature	Bacteria		Fungus	Source
		Negative	Positive		
Phytol	Diterpene	*E.coli, P. aeruginosa*		*C.albicans, A.niger*	PubChem [4],[5]
2,4-Di-*tert*-butylphenol	Phenolic		*S.aureus*	*F. oxysporum, P. chrysogenum, A. niger*	PubChem [6]
2-phenylbenzo thiazole	Alkaloid	*E. coli, Salmonella*	*Bacillus subtilis, S.aureus*		PubChem ChEBI [7]
Thymol	Monoterpene	*E.coli, Salmonella*	*S. mutans*	*C. albicans, C. tropicalis, Saccharomyces cerevisiae*	DRUGBANK Drugs.com
Thimerosal	Benzoic acid derivatives		*S.aureus*	*C.albicans Fusarium spp.*	DRUGBANK Sigma-Aldrich NCATS

Based on the results obtained in the table, phytol inhibited gram-negative bacteria meanwhile 2,4-di-*tert*-butylphenol inhibited gram-positive bacteria with antifungal properties. Thymol inhibited both gram-positive, gram-negative bacteria and fungi showing its antimicrobial properties. 2-phenylbenzothiazole only active towards bacteria while thimerosal active towards fungi respectively (Table 3). The structures of fungi and bacteria differ in very significant ways (such as the diploid nature of most fungi and the longer generation time of fungi compared to bacteria). The available antibacterial and antifungal agents target structures and functions most relevant to the organisms to be inhibited.

Antibacterial compounds has the abilities in inhibiting the growth of microorganism depending on the type of bacteria. Many antibacterial agents inhibit the formation of peptidoglycan, the essential component of the bacterial cell wall. The substance was firstly adsorbed on the bacterial cell wall. The deterioration of the cytoplasmic membrane and subsequent leakage of the cytoplasmic components leads to the death of the cell. This prevent from the reproducing and spreading of bacteria. The bacteria attack the cells by releasing toxins and antibacterial agent knocks out any nearby bacteria. In contrast, most antifungal compounds target either the formation or the function of ergosterol, an important component of the fungal cell membrane. Antifungal compounds attacks fungi cell wall of the microorganism causes the content of the cell to leak out and die. This prevent the fungal cell from growing and reproducing. Ergosterol, fungal lipids which were thought to be plasma membrane components were demonstrated to be extracellular components with key roles towards fungal virulence. Dynamic molecular complex of fungal surface has great potential to stimulate the host's immune response. Antifungal compounds or classes have potential to target ergosterol biosynthesis and interacts with immune system inducing pyroptosis. The cell death that occurs most frequently upon infection with intracellular pathogens and likely to form part of the antimicrobial response.

In previous finding, thymol inhibited the growth of Saccharomyces cerevisiae in vitro. Following a 90-minute incubation at 30 °C, the IC50 was 274 mg/l . Thymol was investigated with respect to its antimicrobial activity on oral bacteria of *Streptococcus mutans* with an incubation period of 24 to 72 hours at 37 °C, the lowest concentration necessary to prevent visible growth (minimum inhibitory concentration, MIC) was in the range from 125 to 500 µg/ml. The extremely rapid efflux of intracellular components (free, non-protein-bound amino acids, pentose, inorganic phosphate) suggested cell wall damage. Thymol, a group of monoterpene, was a hydrophobic, phenolic compound able to bind with bacterial proteins, which results in cell membrane disintegration and permeability, thus making it a potent, broad-spectrum antimicrobial [8].

Another study addressed the mechanism of thimerosal action towards *C. albicans*. The activity was determined by employed doubling dilutions of the tested agents and MIC thimerosal inhibited *C. albicans* were between 1.5 – 0.1 µg/mL (NCATS). Thimerosal also exhibited greatest activity towards *Fusarium spp*. in vitro activity with broth microdilution method was performed for 48 hr at 35ºC incubation period [9].

3.5. Mass Spectra bioactive compounds with antimicrobial properties

Mass spectra of the compounds obtained after the sample mixture being eluted from the column of gas chromatography, entered mass spectrometry and sorts the ions based on mass per charge ratio (m/z). Electrons from analyte

bombarded by electron impact ionization was at 70e to form ions. High energy required in the conversion of atoms into ions since electron impact ionization (EI) was hard ionization technique. Structural information provided by extensive fragmentations from EI was useful for interpreting unknown spectra.

Phytol is a diterpenoid with molecular formula C20H40O, hexadec-2-en-1-ol substituted by methyl groups at positions 3, 7, 11 and 15. Phytol has atomic mass of 296.54 but the molecular ion peak are diminish and likely disappear. This is due to unstable molecular ions peak makes them to break into small fragments. Base peak of phytol can be seen at m/z 71.0, the highest peak in the mass spectrum (Figure 2)

2,4-di-tert-butylphenol is a member of the class of phenols with molecular formula C14H22O carrying two tert-butyl substituents at positions 2 and 4. In the EI mass spectrum, a large peak is seen at m/z value 191.0 indicates the base peak. 2,4-di-tert-butylphenol has a weight of 206.33 atomic mass units, or Daltons, so the peak at m/z 206.0 represents the molecular ion. Molecular ion for this compound is small. Cleavage of the C-C bond next to the oxygen usually occurs. The tallest peak at at m/z 191.0 represents 2,4-di-tert-butylphenol molecule in which a CH3 is removed by fragmentation (Figure 3).

2-phenylbenzothiazole with molecular formula C13H9NS has nominal atomic mass of 211.28. The molecular ion peak can be seen as the base peak in this mass spectrum since it shows the highest peak and most abundance of the ion at m/z 211.0. The mass spectrum of 2-phenylbenzothiazole illustrates the presence of an odd molecular ion and even fragments. This rule is a result of nitrogen's unique property (Figure 4).

Thymol with molecular formula C10H14O is a natural monoterpene derivative of cymene which has nominal atomic mass of 222.0. Molecular ion represents by this compound is shown at the peak of m/z 222.0. Similar to 2,4-di-tert-butylphenol, cleavage of the C-C bond next to the oxygen usually occurs. The base peak of the compound is seen at m/z 207 which indicates the removal of CH3 by fragmentation. Thymol shows three significant fragment ions at m/z 222.0, 207.0 and 71.0 (Figure 5).

Thimerosal is an alkylmercury compound with molecular formula of C9H9HgO2S.Na which well-established as antiseptic and antifungal agent. Thimerosal has a weight of 456.00 atomic mass units, or Daltons, so the peak at m/z 456.0 represents the molecular ion which is the heaviest ion. In this mass spectrum, a large peak is seen at m/z value 225.0 indicates the base peak of the compound. Fragments appear due to bond cleavage next to C=O (alkoxy group loss, -OR) and hydrogen rearrangements. (Figure 6).

According to the mass spectra obtained, the most intense ion referred to the base peak which common fragment ions to be formed. The heaviest was molecular mass of the compounds which represents molecular ion. Some molecular ions would have very small peak tend to be unstable makes them broke into small fragments. Fragmentation of all ions derived the structural information of the compounds. The molecular ion is not necessarily the most abundant peak. The more stable an ion is, the more likely it is to form. The more of a particular sort of ion that's formed, the higher its peak height will be.

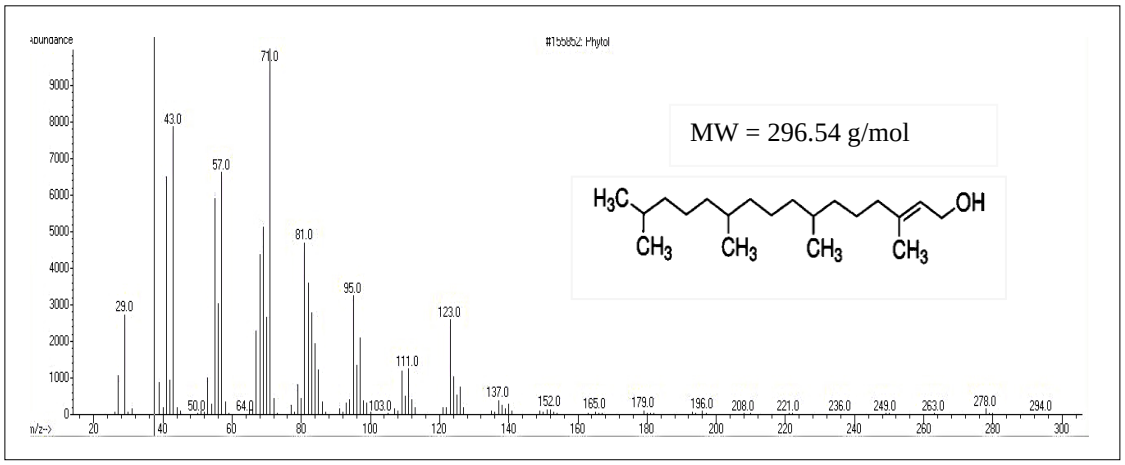

Figure 2. Mass spectrum of phytol

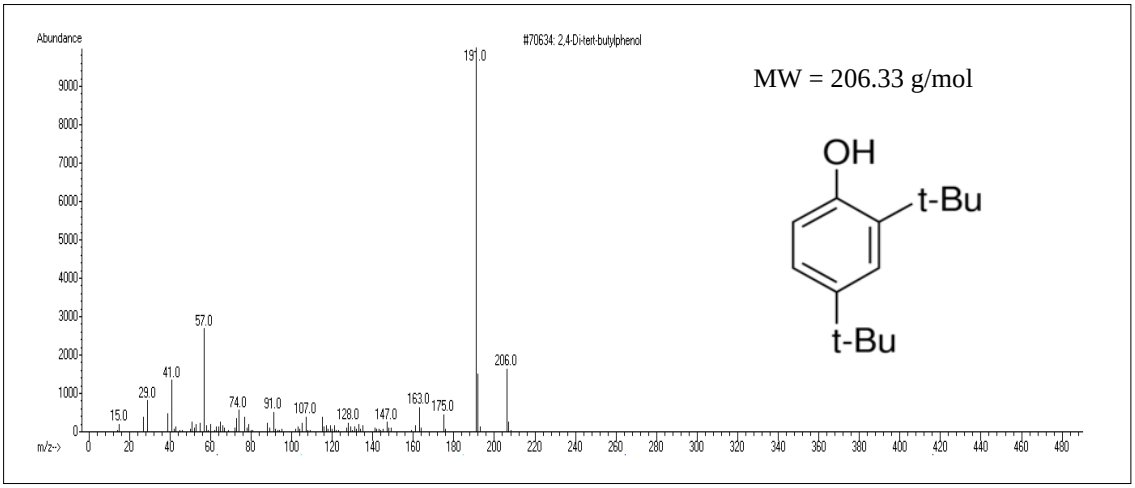

Figure 3. Mass spectrum of 2,4-di-*tert*-butylphenol

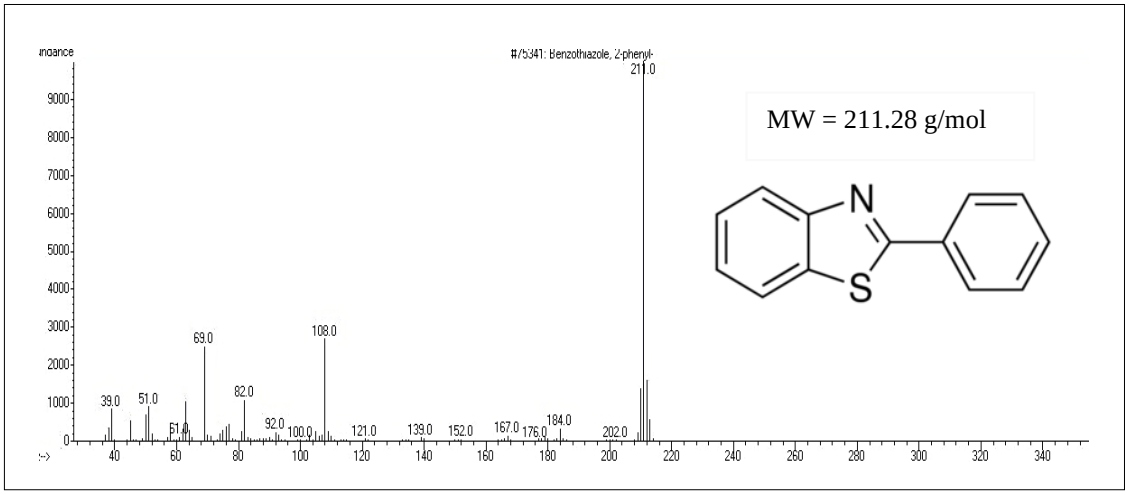

Figure 4. Mass spectrum of 2-phenylbenzothiazole

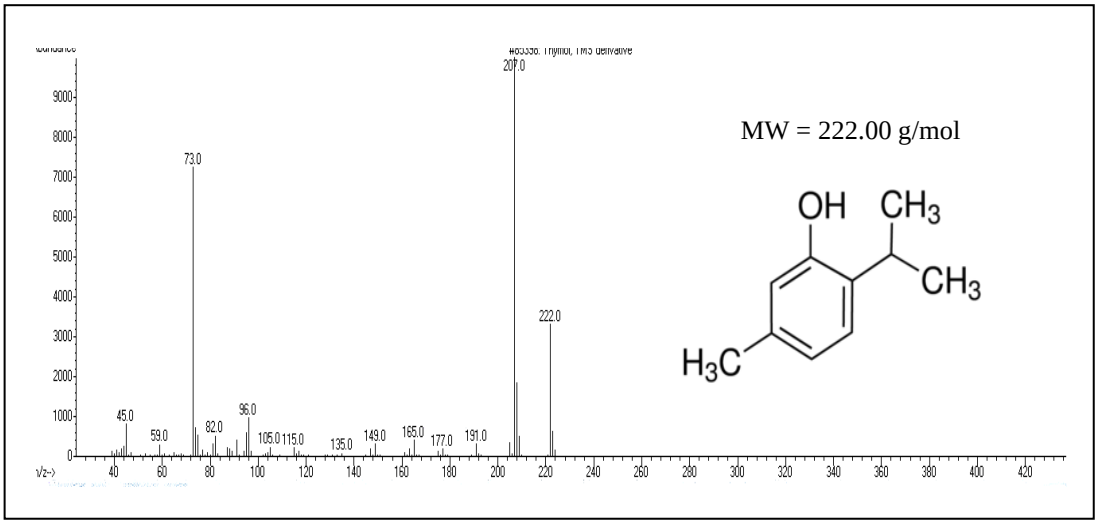

Figure 5. Mass spectrum of thymol

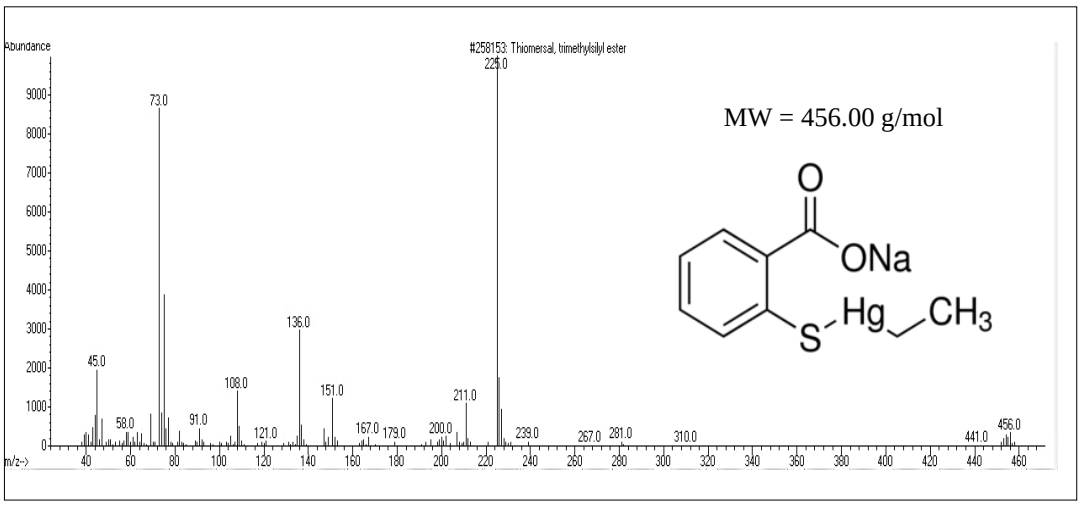

Figure 6. Mass spectrum of thimerosal

3.6. Lethal dose of antimicrobial agents

Pytochemical compoundss can have a wide range of effects on our health. Depending on how the chemical will be used, many kinds of toxicity tests may be required. Lehtal dose as indication of level toxicity given a substance. The median lethal dose, LD50 (abbreviation for "lethal dose, 50%"), LC50 (lethal concentration, 50%) or LCt50 (lethal concentration and time) of a toxin, radiation, or pathogen is the dose required to kill half the members of a tested population after a specified test duration. LD50 frequently used as a general indicator of a substance's acute toxicity. Dose descriptor used to identify the relationship between a specific effect of a chemical substance and the dose at which it takes place. A lower LD50 indicates of increased toxicity. Table shows the degree of lethal dose for both thymol and thimerosal compounds (NCBI).

Thymol can have higher amount of consumption to meet LD_{50} compared to thimerosal. For thymol, "LD_{50} (oral, rat) 980 mg/kg" means that 980 milligrams of thymol for every 1 kilogram body weight of the rat, when administered in one dose by mouth, causes the death of 50% of the test group, meanwhile mouse requires 640 milligrams of the thymol. Thimerosal has shown to have LD_{50} (oral, rat) 75 mg/kg which means every 1 kilogram body weight of the rat required 75 milligrams of thimerosal, Injection applied under the skin of rat, LD_{50} (subcutaneous, rat) 98 mg/kg required 98 milligrams of thimerosal per kilogram of rat's body weight causing 50% of the tested rats to die. To conclude, thymol has minimal potential toxicity and poses minimal risk rather than thimerosal (Table 4).

Table 4. Lethal dose of thymol and thimerosal compounds

Bioactive compounds	Trade Name	Lethal Dose	CAS number	Source
Thymol	Thymolum	Oral, mouse: LD50 = 640 mg/kg; Oral, rat: LD50 = 980 mg/kg;	89-83-8	Fischer Scientific
Thimerosal	Merthiolate	Orat, rat: LD50 = 75 mg/kg Subcutaneous, rat: LD50 = 98 mg/kg	54-64-8	USP SDS

4. CONCLUSION

In this study, phytochemical compounds were obtained by cold percolation technique. Phytochemical screening was done using GC-MS analysis to identify antibacterial and antifungal compounds found in the plant extracts. In this study, alkaloids, phenolics and terpenes active compounds were found to have antimicrobial properties. 2-phenylbenzothiazole is potential as antibacterial properties, meanwhile 2,4-di-tert-butylphenol and phytol are potential as antimicrobial properties. Thymol and thimerosal revealed the existence as antibacterial and antifungal agent which justifies its medicinal properties.

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