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Identification of Bioactive Phytochemicals using GC–Mass and TLC to the Estimation of Antimicrobial susceptibility of Plant Extracts

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Abstract. This study aims to evaluate the constituent compounds of plant extracts and their antimicrobial activity. Four different ethnomedicinal plant extracts including *Piper nigrum*, *Nigella sativa*, *Cinnamomum zeylanicum*, and *Elettaria cardamomum* were tested for antimicrobial susceptibility profile identified using GC-Mass and demonstrated TLC analysis. We found the combined action of ethanol plant extracts (alone) against oral isolates showed a synergistic effect profile up to 32.20% when combination A (Ci/Ca) was added. The stearic and palmitic acids were the major constituent compounds of plant extracts which was exhibited high antimicrobial susceptibility against the bacterial isolates. We conclude that the stearic and palmitic acids were major constituent compounds of plant extracts.

Keyword: Plants Extracts, GC-Mass, Antimicrobial susceptibility, TLC

1. Introduction

Challenges of resistance to synthetic antimicrobials have opened new vistas in the search for natural products. Since plants are the sources of more than 25% of prescription and over the counter drugs, conventional medicine is increasingly becoming receptive to the use of antimicrobials derived from plants and other natural products. The rise in the use of herbal medicines has renewed interest in the effects of plant extracts to control plaque and other oral diseases (Lobo et al., 2014). Medicinal plants have been used as traditional treatments for numerous human diseases for thousands of years. Plant extracts, phytochemical compounds, and essential oils were investigated for their ability to treat or prevent adhesion of oral bacteria to various surfaces (Al Qurashi et al., 2015). Medicinal plants have attracted increasing interest, because of their antimicrobial activity against pathogenic oral microorganisms. Plants that are used for traditional medicine contain a wide range of substances that can be used to treat chronic and



infectious diseases (Koo et al., 2011). The World Health Organization (Who) estimates that 80% of the population of certain Asian and African countries presently uses medicinal plants in several aspects of primary health care. Studies in the United States and Europe have shown that the use of these plants is less common in clinical settings but has increased in recent years, as scientific evidence regarding the effectiveness of herbal medicine has become more widely available (Who, 2002). Medicinal plants are important sources for pharmaceutical manufacturing and account for a significant percentage of the pharmaceutical market. For example, in Malaysia, the market for traditional medicine is estimated to be at 1 billion Malaysian ringgit annually (Aziz and Tey, 2009). The present research was planned to Identify of bioactive phytochemicals using GC–MS and TLC to estimation of the antimicrobial activity of medicinal plant extracts.

2. Materials and Methods

2.1. Inoculum preparation

All oral isolates were collected from different 50 selected individuals at dental clinics in Gambang area, Pahang State, Malaysia. Collected samples were transferred to the laboratory at University Malaysia Pahang. All oral samples were cultured onto nutrient agar (Merck) plates and incubated at 37 °C for 24 h, and then purified, cultured on agar slants, and kept in a chiller until use.

2.2. Medicinal plant used

Preparation of extracts: The selected four commercial plants and parts used in the study, namely, *Cinnamomum zeylanicum* (Ci) (bark), *Elettaria cardamomum* (Ca) (fruit), *Piper nigrum* (P) (fruit), and *Nigella sativa* (N) (seed), were purchased from the market at Kuantan area, Pahang, Malaysia.

2.3 Combined Action of Ethnomedicinal Plants

The combination of plants extracts obtained by using different solvents was studied at a ratio of (1:1). Three different combination groups A, B, and C, were prepared by using the combination of two plants (P/Ci, P/N, P/Ca, Ci/N, Ci/Ca, N/Ca), three plants (P/Ci/N, P/Ci/Ca, P/N/Ca, Ci/N/Ca), and four plants (P/Ci/N/Ca), respectively.

2.4 Antimicrobial Susceptibility Test

Muller–Hinton agar medium [CM0337, Oxoid] was used for antimicrobial activity. solvents were allowed to set onto the inoculated agar surface and incubated at 37 °C for 24 h. After incubation period, each plate was observed, and the inhibition zone of all isolates were recorded in millimeter (mm) (Ali et al , 2015b). All samples prepared triplicate and control discs for different solvents were used.

2.5 Gas chromatography – mass spectrometry (GC - MS) analysis

The method of GC-MS analysis of seven different plant extracts (4 plants and 3 combinations) described here was developed by Wang et al. (2005). GC was performed using a Varian GC/MS 4000 equipped with a VF-5ms MS capillary column (30 m x 0.25 mm i.d., 0.25 μ m) (Agilent Technologies 7890A GC System, G3171A, USA). For GC-MS detection, an electron ionisation system with ionization energy of 70 eV was used. It was the carrier gas at the flow rate of 1 mL min⁻¹. Injector and MS transfer line temperatures were set at 220 and 280 °C, respectively. The programmer used was 60–180 °C at a rate of 8 °C min⁻¹, held isothermal for 10 min and finally raised to 300 °C at 10 °C min⁻¹. Diluted samples (1 / 10 000, V/V, in ethanolic) of 1.0 μ L were injected manually and in the splitless mode. Identification of the major components of plant extracts were confirmed by comparison with their relative retention time and mass spectra with the standard compounds.

2.6 Qualitative Analysis by Thin Layer Chromatography

Extract was to begin with, checked by Thin Layer Chromatography (TLC) on analytical plates over silica gel. TLC was carried out to isolate the principle antimicrobial components that were present in most effective extracts of the plants. The different solvent systems of different polarities were prepared and TLC studies were carried out. The above prepared plant extracts were applied on pre-coated TLC plates (TLC Silica gel 60 F₂₅₄, Merck) by using capillary tubes and developed in a TLC chamber using suitable mobile phase (15% Hexane/Ethanol). The developed TLC plates were air dried and observed under UV light at both short 254 nm and long 365 nm. They were later sprayed with different spraying reagents and some were placed in hot plate air oven for 1 min for the development of color in separated bands. The movement of the analyze was expressed by its retention factor (R_f) values were calculated for different samples as shown in equation (S. Gupta1) (Harborne, 1998).

$$R_f = \frac{\text{Distance traveled by solute or sample}}{\text{Distance traveled by solvent front}}$$

3. Statistical Analysis

Statistical analyses were performed using ANOVA for data on disc agar diffusion assays, to test the antimicrobial susceptibility profile of isolates within medicinal plant extracts on the zone of inhibition using software Minitab 17. All antimicrobial susceptibility data were determined in diameter (mm).

4. Results and Discussion

4.1. Ethnomedicinal Plants Combined Action

The results of antimicrobial activity testing of 11 different combinations using different solvents were recorded in (Table 1). Among 95% ethanol, hot water, and cold-water extracts, the 95% ethanolic extract of (Ci/Ca) (combination A) showed antimicrobial activity against 59 oral isolates with a higher synergistic effect up to 32.20% than other combinations, followed by combination B [95% ethanolic extract of (P/Ci/Ca)], with 30.50%. The lowest antimicrobial activity against oral isolate (25.42%) was observed in synergistic effect of group C [95% ethanolic extract of (P/Ci/N/Ca)].

The combination of aqueous extracts of medicinal plants did not exhibit an inhibitory effect against the tested oral isolates. In this study, the ethanolic extract preparation shows better antimicrobial activity, in accordance with the results obtained by (Aneja and Joshi, 2009). Our results agreed with (Aneja and Joshi, 2009) which reported that ethanol is capable of extracting tannin, flavonoid, polyphenols, terpenoids, alkaloids, and essential oils in low amounts. Flavonoids, alkaloids, and phenolics able to inhibit the synthesis of bacterial cell walls. Phenolics and tannin coagulate protein in the cell wall and cut cross-linked peptide bonds within peptidoglycans, resulting in the weakening of the bacterial cell wall structure in bacteria that may affect cell permeability. Alkaloids disrupt the formation of cross-linked peptide bonds in the peptidoglycan structure and compromise the cell's ability to defend itself from external attacks. Despite the development of antimicrobial activity in products, oral bacterial infections remain a major health problem because of environmental changes, as well as microorganism behaviors. Therefore, developing new antimicrobial compounds with high activity and low toxicity and side effects is urgently needed. Our study revealed that all of the medicinal plant combinations exhibited high antimicrobial activity against oral isolates, with different synergistic or antagonistic effects.

Table 1. Summary of antimicrobial activity percentage of ethnomedicinal plants combinations using different solvents.

Group Code	Combined groups†	Combination effect of medicinal plants using different solvents (%)		
		95% Ethanol	Hot water	Cold water
A	P/Ci	22.03	3.38	1.69
	P/N	15.25	3.38	1.69
	P/Ca	15.25	1.69	1.69
	Ci/N	23.72	3.38	3.38
	Ci/Ca	32.20	6.77	5.08
	N/Ca	11.86	3.38	3.38
	F- value	43.36	29.03	24.91
	P- value	0.000	0.000	0.001
	P/Ci/N	28.81	8.64	18.64
	P/Ci/Ca	30.50	13.55	13.55
B	P/N/Ca	27.11	20.33	20.33
	Ci/N/Ca	25.42	6.94	20.33
	F- value	653.82	16.96	128.86
	P- value	0.000	0.006	0.000
C	P/ Ci/ N /Ca	25.42	10.16	5.08

† Combination group: P: *Piper nigrum*.; Ci: *Cinnamomum zeylanicum*; N: *Nigella sativa*; Ca: *Elettaria cardamomum**All percentage represented in the table explain the calculation of antimicrobial susceptibility percentage of all 59 oral isolate. The antimicrobial susceptibility of combination of plant extract using different solvents P-value > 0.05 significant different

4.2. GC-MS characterization of plants extracts combinations

Different classes of organic chemicals constituents were identified by GC-MS studies. The GC-MS analysis resulted in the identification of a number of organic compounds of ethanolic extracts of four medicinal plants under study exhibited the major selected constituent's compounds with a percentage of > 90% similarity and area percentage work at the cellular level and most have antibacterial and antifungal effects against oral isolates were selected. The results show medicinally valued phytochemicals are present in the different plant extracts as

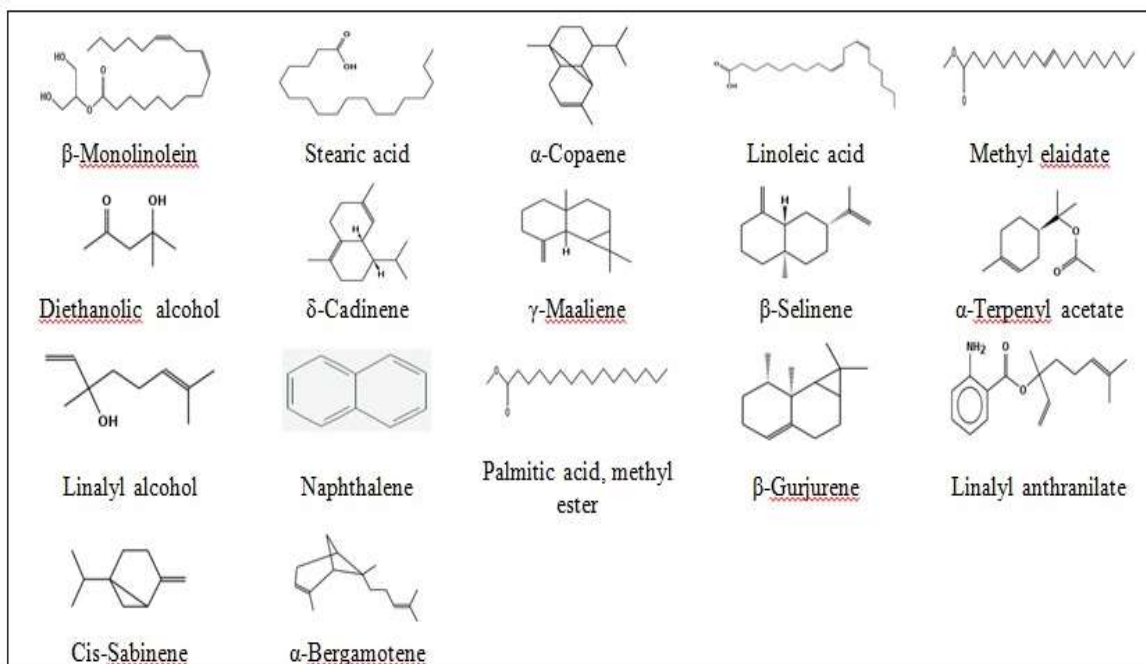


Figure These compounds are well known as plant-derived antimicrobial agents (Tables 2-5) (Figures 2a-d)

Data recorded in **Table 2**, the GC-MS analysis of the plant extract using ethanolic was identified the presence of about 15 compounds which are Cyclohexene; α -Copaene; Caryophyllene; Humulene; Naphthalene; Cyclohexane, 1-methyl-2,4-bis(1-methylethenyl); Caryophyllene oxide; Ledene oxide-(II); 10,10-Dimethyl-2,6-dimethylenebicyclo[7.2.0]undecan-5 β ; Isoaromadendrene epoxide; Phthalic acid; Benzamide, N-(2,5-dimethoxyphenyl)-2-methoxy; Bicyclo[4.1.0]heptan-3-one, 4,7,7-trimethyl-, [1R-(1 α ,4 α ,6 α)]; 1H-Inden-1-one, octahydro-7a-methyl-, trans; and trans-1-Cinnamoylimidazole. Data recorded in **Table 3**, the GC-MS analysis of the plant extract using ethanolic was identified the presence of about 5 compounds which are 9-Octadecenoic acid, methyl ester, (E); linoleic acid; 9,12-Octadecadienoic acid, ethyl ester; Diisooctyl phthalate; and 9,12-Octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester. Data recorded in **Table 4**, the GC-MS analysis of the plant extract using ethanolic was identified the presence of about 4 compounds which are Cinnamaldehyde, (E)-; α -Copaene; Coumarin; and Diisooctyl phthalate. One of the major components with high percentage was palmitic and Cinnamaldehyde. This result was consistent with the investigation conducted by Wang et al. (2005), cinnamaldehyde was the major compound in the cinnamon oil. According to this study, cinnamaldehyde, stearic and palmitic may be a potential lead compounds for the development of antimicrobial activity. As the result reported by Singh et al. (1995); Wang et al. (2005); Bang et al., (2000) cinnamaldehyde is a strong antifungal agent against human

pathogens. According to Nattakarn et al., 2015, the phytochemical analysis of *Solanum torvum* Sw. demonstrated the presence of 32 chemical constituents which were mainly phenolic compounds, terpenoids, palmitic acid, palmitic acid ester, linoleic acid, linolenyl alcohol, linolenic acid ester and stearic acid. The antibacterial activity of the extract was tested against five different pathogenic bacteria and exhibited growth inhibition against all bacteria with minimum inhibitory concentration and minimum bactericidal concentration values that ranged between 1.95 and 31.25 mg.mL⁻¹. The extracted oil from the leaves of *Crotalaria pallida* was found the most abundant unsaturated and saturated fatty acids were linolenic acid (34.06 ± 0.23%) and palmitic acid (24.47 ± 0.22%), respectively. Other unsaturated fatty acids were linoleic acid (13.50 ± 0.12%), oleic acid (4.60 ± 0.11%), 7-hexadecanoic acid (1.00 ± 0.01%) and a very small amount of 7,10-hexadecadienoic acid (0.09 ± 0.01%). Among the saturated fatty acids other than palmitic acid, stearic acid (4.84 ± 0.05%), eicosanoic acid (3.61 ± 0.10%), behenic acid (3.67 ± 0.11%) and tetrasanoic acid (4.08 ± 0.08%) were present in considerable amounts. Nine other saturated fatty acids were present in minute amounts. These compounds showed antimicrobial activities against Gram-positive and Gram-negative bacteria, though more significantly on Gram-negative bacteria (Sushobhan et al., 2016). The GC-MS analysis of fatty acids isolated from *Spirulina platensis* showed the presence of eight compounds. Out of these compounds five are major and the other three are minor compounds. Stearic acid, gamma linolenic acid, linoleic acid, heptadecanoic acid and oleic acid were found to be major compounds. The results of testing the fatty acid mixture isolated from *Spirulina platensis* against four bacterial species might be due to the synergistic effect of all long chain fatty acids (Jubie and Dhanabal, 2012).

Data recorded in **Table 5**, the GC-MS analysis of the plant extract using ethanolic was identified the presence of about 8 compounds which are Eucalyptol; Butanoic acid, 3-hexenyl ester, (Belavsky)-; Terpinyl acetate; 1-Methyl-4-(1-acetoxy-1-methylethyl)-cyclohex-2-enol; 1-Cyclohexene-1-carboxaldehyde, 4-(1-methylethyl); 2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)-,cis-;2,6-Dimethyl-3,5,7-octatriene-2-ol,Z,Z; and Phthalic acid.

Table 2: The GC-MS analysis of the *Piper nigrum* extract using ethanol

Peak No.	RT	Compound	Area %
1	12.002	Cyclohexene	1.56
2	12.688	α -Copaene	4.50
3	13.444	Caryophyllene	12.37
4	13.856	Humulene	1.52
5	14.348	Naphthalene	2.85
6	14.897	Cyclohexane, 1-methyl-2,4-bis(1-methylethenyl)	1.28
7	15.670	Caryophyllene oxide	3.15
8	16.322	Ledene oxide-(II)	4.18
9	16.471	10,10-Dimethyl-2,6-dimethylenebicyclo[7.2.0]undecan-5 β -ol	9.27
19	16.928	Isoaromadendrene epoxide	2.08
11	34.855	Phthalic acid	3.61
12	35.204	Benzamide, N-(2,5-dimethoxyphenyl)-2-methoxy	2.86
13	36.675	Bicyclo[4.1.0]heptan-3-one, 4,7,7-trimethyl-, [1R-(1 α ,4 α ,6 α)]	1.31
14	36.755	1H-Inden-1-one, octahydro-7a-methyl-, trans	1.91
15	37.871	trans-1-Cinnamoylimidazole	1.52

All compounds represented in the table were selected as major constituent according to the highest peak

Table 3: The GC-MS analysis of the *Nigella sativa* extract using ethanol

Peak No.	RT	Compound	Area %
1	29.190	9-Octadecenoic acid, methyl ester, (E) Palmitic acid	1.90
2	29.785	linoleic acid	1.02
3	32.938	9,12-Octadecadienoic acid, ethyl ester	2.39
4	34.872	Diisooctyl phthalate	2.05
5	36.034	9,12-Octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester	1.40

Table 4: The GC-MS analysis of the *Cinnamomum zeylanicum* extract using ethanol

Peak No.	RT	Compound	Area %
1	10.394	Cinnamaldehyde, (E)-	33.61
2	12.665	α -Copaene	2.36
3	12.963	Coumarin	19.24
4	34.872	Diisooctyl phthalate	11.99

Table 5: The GC-MS analysis of the *Elettaria cardamomum* extract using ethanol

Peak No.	RT	Compound	Area %
1	6.594	Eucalyptol	19.03
2	11.784	Butanoic acid, 3-hexenyl ester, (Belavsky)-	3.01
3	12.070	Terpinyl acetate	22.32
4	13.209	1-Methyl-4-(1-acetoxy-1-methylethyl)-cyclohex-2-enol	7.09
5	14.188	1-Cyclohexene-1-carboxaldehyde, 4-(1-methylethyl)	3.26
6	14.548	2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)-,cis-	2.62
7	14.737	2,6-Dimethyl-3,5,7-octatriene-2-ol,Z,Z	5.64
8	34.838	Phthalic acid, di(2-propylpentyl) ester	4.99

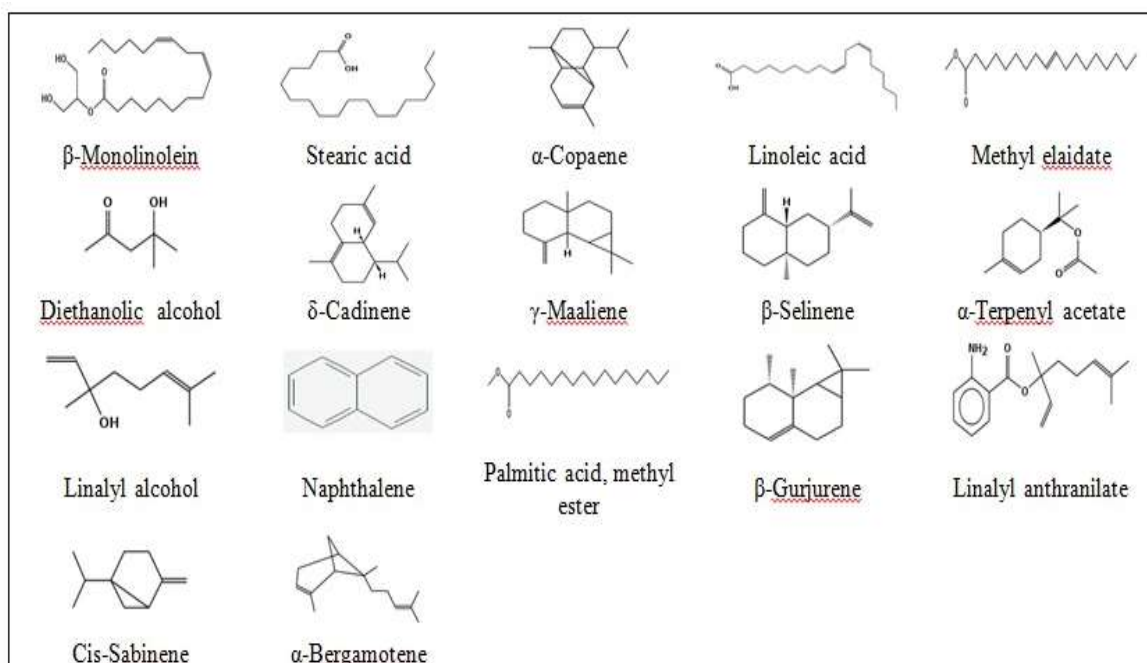


Figure 1: Most of the compounds structures of plant extracts determined by GC-MS analysis.

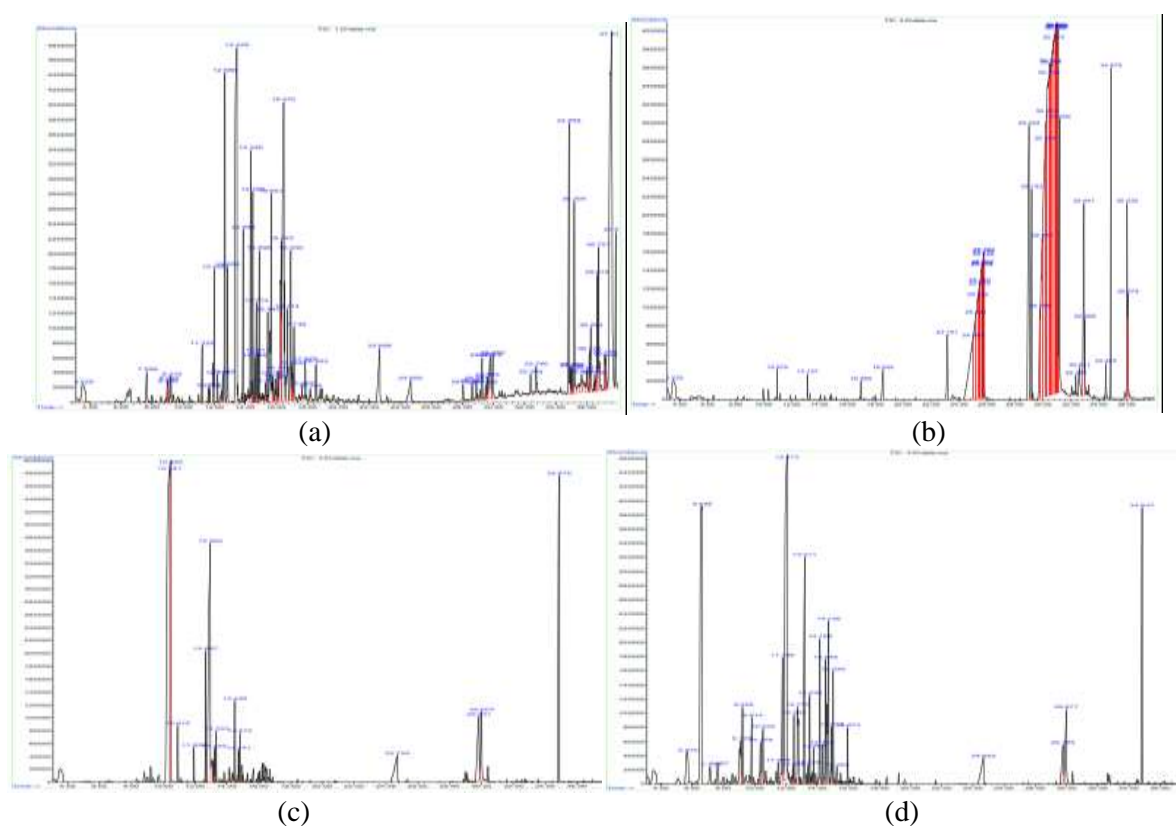


Figure 2: GC-MS chromatograms of different plant extracts of (a) *Piper nigrum* (b) *Nigella sativa*; (c) *Cinnamomum zeylanicum*; (d) *Elettaria cardamomum* using ethanol solvent.

4.3. Thin Layer Chromatography (TLC) Analysis

TLC of plant extracts and its mixtures revealed separated bands were dominated by one or two main constituents having R_f values of 0.74, 0.77 respectively when a solvent phase of n-Hexane : Ethanol (15%) was used (photo plate in Figure 3). These plant extract mixtures (Combinations A, B & C) proved the presence of stearic (St) and palmitic (Pl) acids got from GC-Mass results which may be responsible for the antimicrobial activity against oral pathogens. In previous reports, the antibacterial action of fatty acids is usually attributed as being a property of the long-chain unsaturated fatty acids, including oleic acid, linoleic acid, and linolenic acid, while long-chain saturated fatty acids, including palmitic acid and stearic acid, are reported as showing less antibacterial activity (Sun et al., 2003; Seidel and Taylor 2004). This tendency was prove the antimicrobial activity of these compounds were observed in the present study.

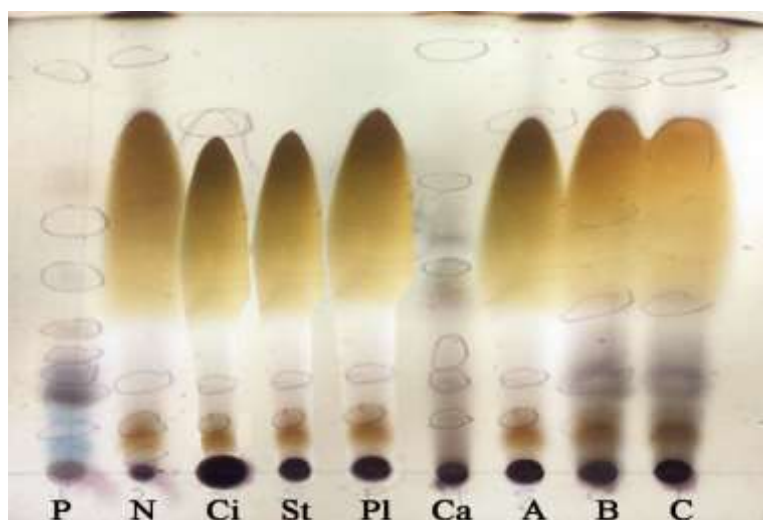


Figure. 3: TLC of the selected compounds demonstrated antimicrobial susceptibility.

The pencil circles indicate the visible different compounds bands under UV light chamber at short/long-wave (254/365 nm wavelength).

5. Conclusions

The ethanol extract for combination A (Ci/Ca), B (P/Ci/Ca) and C (P/Ci/N/Ca) showed antimicrobial susceptibility against bacterial isolates with a higher synergistic effect than other combinations. The stearic and palmitic acid were the major compounds of plant extracts identified using GC-MS and evidence by TLC, and exhibited high antimicrobial susceptibility against the isolates.

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