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Profile Analysis of Metabolite Fractions from Chamber Bitter Herb (*Phyllanthusniruri* L.) Ethanol Extract Using Gas Chromatography (GC-MS) Method with Derivatization

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Abstract:
Analysis of the secondary metabolite profile of chamberbitterherb was conducted by using derivatizationmethod of GC-MS. The purpose of this derivatization is to increase the detection limit and the form of chromatograms, as well as to increase the volatility of non-volatile compounds so the compounds detected in GC-MS are more various. Profiling secondary metabolite using methanol solvents, with RxiTM-1MS capillary columns (30 m x 0.25 mm, outer layer thickness 0.25 µm). The 70 Ev ionization system was used to detectGC-MS, Helium carrier gas (0.03 Mpa) with a flow rate of 1 mL/min, injector temperature of 250°C, column temperature of 280°C, detector temperature of 110oC and injection volume of 1 µL.The results show that derivatization can increase or improve volatility and stability so it can improve compound detection and improve chromatogram shape. The compounds obtained in the analysis were 31 compounds detected in the ethanol fraction, 25 compounds in the ethyl acetate fraction, and 25 compounds in n-hexane, whereas in previous studies metabolite analysis was carried out without derivatization, the results obtained were 2 compounds detected in the polar fraction, 4 compounds in the semipolar fraction, and 11 components in nonpolar. Compounds obtained included carboxylic acids, amides, amines, phenolic, terpenoids and alcohol.

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I. INTRODUCTION

Phyllanthusniruri Linn. (chamberbitter) is reported beneficial as medicines for asthma, bronchitis, excessive dehydration, anemia, jaundice, and tuberculosis (Kumar et al., 2012). Chamberbitterhas been used in medicine for more than 2000 years and has a number of traditional uses for jaundice, gonorrhea, and diabetes. In Malaysia, chamberbitterherbs are used internally for diarrhea, kidney disorders, gonorrhea and cough. Chamberbitter is the most commonly found family of Phyllanthaceae. This plant is traditionally used as a cure for several diseases including diarrhea, hepatitis, and stomach ache (Mahdi et al., 2011).

Research conducted by Widowati (2008) shows that chamberbitterleaves contain several chemical compounds derived from flavonoids,

including quercetin, quercetrin, isokuersetrin, astragalin, routine kamferol-4-ramnopiranoside, eridiocytol-7-ramnopiranoside, fisetin-4-O-glycosides, and irurin. The root parts of chamberbitter contain 3,5,7-trihydroxyplavone-4-O-α-ramnoside which is a flavonoid glycoside compound with camferol as aglycone, and ramnoside as glycone. In addition, there are also lignin-derived compounds such as, norsecurinin, securinin, allosecurinin, lignans, nirfilin, isolintetrain, hypophilanthine, nirtetralin, nirantin, filantin, hinikinin, ligtetralin, philanthostatine, transfitol, and alkaloids ethnosekurinin compounds.

Gas chromatography mass spectrometry (GC-MS) based on profiling metabolites biological sample is one of the key technologies for metabolite profiles and substantially contributes to understanding of metabolomics. Increasing the number of metabolites identified in the existing profile, the platform is a prerequisite for a scope that is substantially increased in profiling studies. This is conducted for the reproduction of identification metabolites and the exchange of identification between laboratories that will facilitate further developments, such as the extension of technological profiling of metabolic signals and the technical demands of analysis of other trace compounds (Kopka, 2005).

Ethanol extract of chamberbitterleaves shows the results that chamberbitter herbs have antioxidant activity. For profiles of the fractionation content of chamberbitter herb (Phyllantusniruri L.) ethanol extract using GC-MS method, there were 7 components detected in the polar fraction, 7 components in the semipolar fraction, and 19 components in nonpolar with the results of TLC (Thin Layer Chromatography) all fractions show the presence of phenol and flavonoid compounds, whereas only the nonpolar fraction shows the presence of terpenoid compounds (Saraswati, 2012).

Previous research by Saraswati (2012) on the analysis of secondary metabolite profiles of chamberbitter herb ethanol extract using GC-MS without derivatization results in poor chromatogram results in which chromatograms are still overlapping, so further action is needed to improve these results. According to Grob(1995) with derivatization in GC-MS analysis can increase the volatility and stability of the sample so that it can improve the reading of the detection limit and the shape of the chromatogram. Therefore, with the explanation and background, this research was carried out by analyzing using GC-MS with derivatization and finding its metabolite profile.

II. RESEARCH METHOD

Instruments and Materials

Tools

Analytical balance (AND COMP GR. 202), sonicator (BRANSON 2510), glassware, micropipette, and analytical balance, a set of Shimadzu-GC 2010 chromatography equipped with mass selective detectors with columns RxiTM-1MS, ovens, syringes.

Materials

Materials used arechamberbitter herb ethanol fraction, ethyl acetate and n-hexane obtained from Saraswati (2013), ethanol *p.a*., distilled water, filter paper, aluminum foil, BSTFA solution in 1% TMCs.

Procedure of the Research

Collection of materials

The materials used are chamberbitterherbethanol fractions, ethyl acetate and n-hexane obtained from Saraswati (2013)

GC-MS optimization and derivatization

Chamberbitter herb fraction sample weighed 10 mg, then chamberbitterherbfraction sample was dissolved in 5mlp.a methanol and then sonication was conducted. From the solution, 100 µL was taken and then evaporated to dry in a vial tube. After drying the vial containing the remaining chamberbitter herb fraction was given a 100 µL BSTFA reagent and then heated in a 70^{o}C oven for 10 minutes. After heating, 1 µL was taken using a syringe for GC-MS analysis.

Analyzes were performed using Shimadzu-GC 2010 supplemented with Shimadzu-GCMS 2010S mass selective detector and capillary columns RxiTM–1MS (30m x 0.25mm, layer thickness 0.25µm). The ionization electron system with ionization energy of 70 eV was used for the detection of GC-MS, Helium carrier gas (0.03MPa, flow rate 1mL/min), column temperature was programmed from 50 to 280^{o}C with an increase of 5^{o}C/min. The detector temperature used was 70^{o}C. Manually injected 1µL in splitless mode. Components were identified by comparingmass spectra of samples with internal (Widyana, 2013).

III. RESULTS AND DISCUSSION

This research was conducted to observe the secondary metabolite profile of non-derivatizationchamberbitterherb extracts that have been conducted by previous researchers compared to studies conducted with the derivatization treatment using BSTFA.

Derivatization of the chamberbitterherbfraction was carried out by the silylation derivatization method using BTSFA. Silylation derivatization is derivatization in which the derivative process is the substitution of silyl groups into molecules. This silyl derivative is used to replace alkyl ethers for the analysis of polar samples, so that this derivatization can increase several readings of compounds such as carboxylic acids, phenols, steroids, amine alkaloids, and alcohols.

The reaction mechanism of a silylation derivatization using BSTFA is a nucleophilic substitution reaction in the trimethylsilyl group as a target to be attacked by nucleophilic groups (-OH). From the attack of the nucleophilic group there is an electron transfer which will form a compound containing the trimethylsilyl group (TMS) (Söderholmet al., 2010).

The results obtained in this study in the form of chromatograms and some compounds were detected in reading Willey Library 7. The results were then observed including qualitative aspects (chromatogram image and retention time) as well as quantitative aspects (number of peaks and detection compounds).

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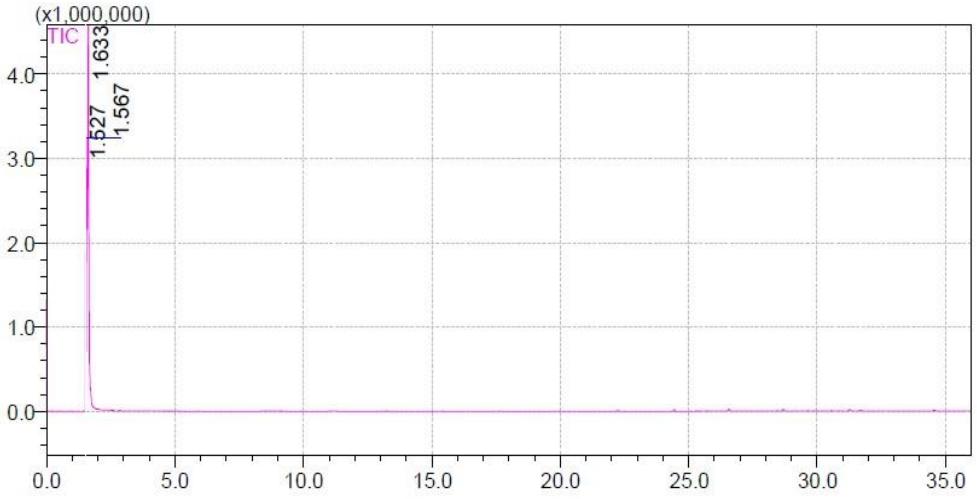

Figure 1. GC-MS Chromatogram of ChamberbitterHerb (Phyllanthusniruri L.) Ethanol Fraction before derivatization.

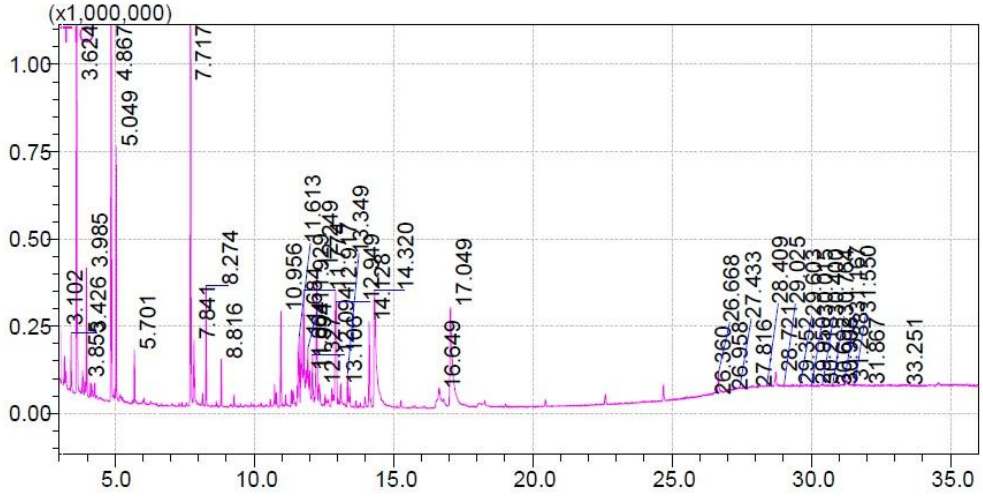

Figure 2. GC-MS Chromatogram of ChamberbitterHerb (Phyllanthusniruri L.) Ethanol Fraction after derivatization.

Comparison of the results of the non-derivatization and derivatization ethanol fraction chromatogram (figure 1 and 2) shows that the peak chromatogram appearing in the derivatization treatment was better than the non-derivatization treatment. This happened due to the lack of sensitivity of the non-derivatization treatment so the peak results on the chromatogram that appearedwere not good.

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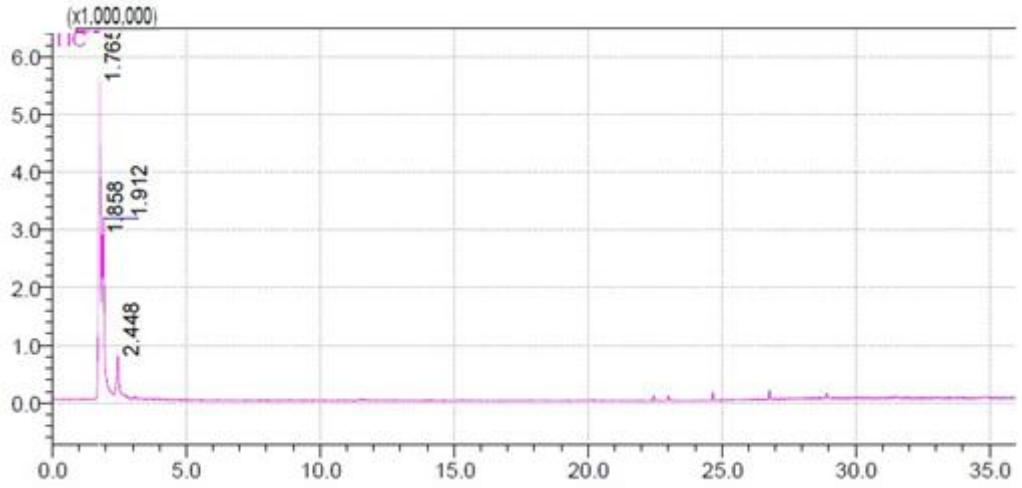

Figure 3. GC-MS Chromatogram of ChamberbitterHerb (Phyllanthusniruri L.) Ethyl Acetate Fraction before derivatization.

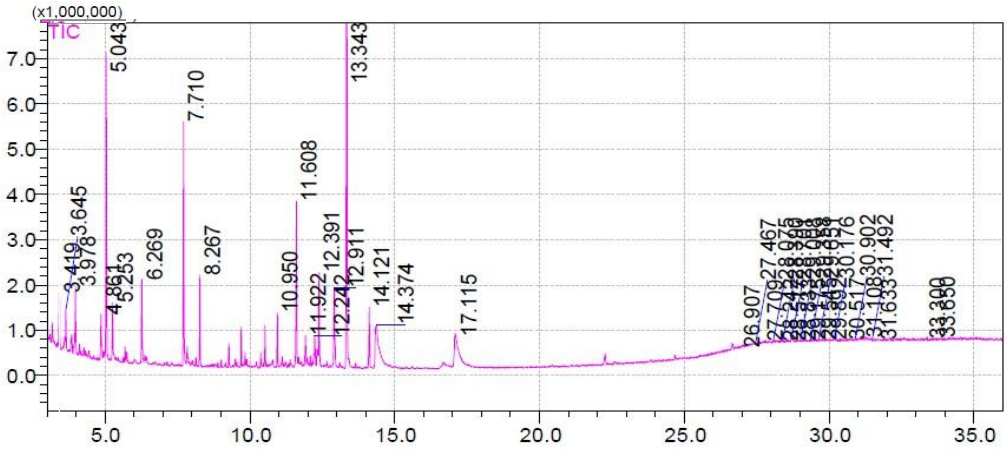

Figure 4. GC-MS Chromatogram of ChamberbitterHerb (Phyllanthusniruri L.) Ethyl Acetate fraction after derivatization.

The comparison of the results of the chromatogram the ethyl acetate fraction non-derivatization and derivatization (figure 3 and 4) shows that the peaks that appeared in the non-derivatization treatment showed that the peaks in the chromatogram reading wasfewer, it is shown by the number of peaks that existed, moreover in the results of semi-polar derivatization chromatograms, where the peak appeared more frequently.

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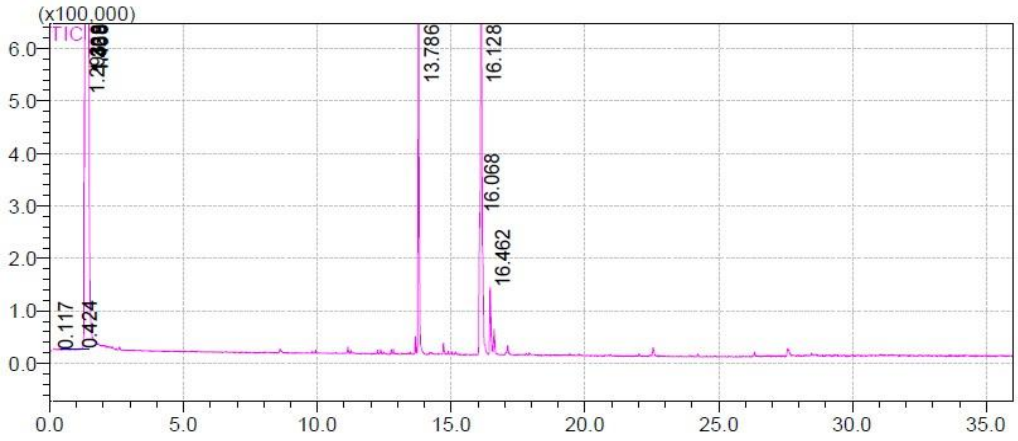

Figure 5. GC-MS Chromatogram of ChamberbitterHerb (Phyllanthusniruri L.) N-hexane Fraction before derivatization.

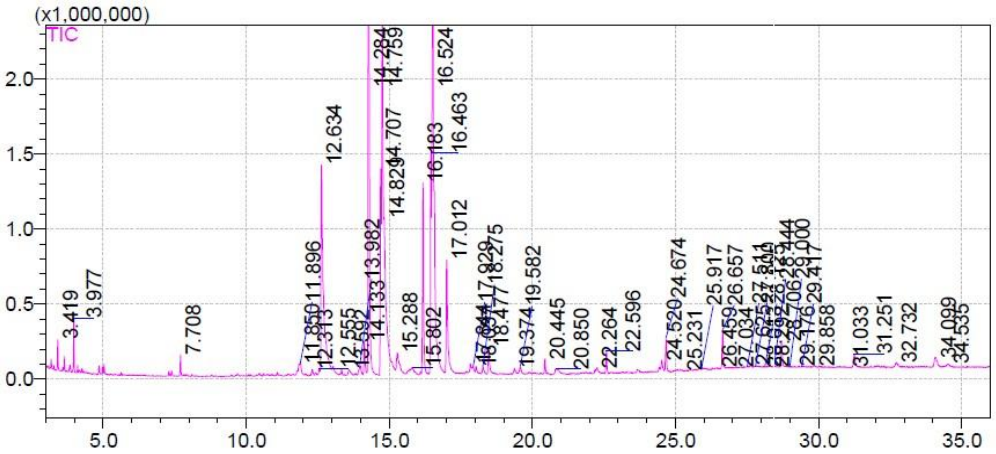

Figure 6. GC-MS Chromatogram of Chamberbitter Herb (Phyllanthusniruri L.) N-hexane Fraction after derivatization.

Comparison of the chromatogram results of the N-hexane fraction non-derivatization and derivatization treatment (figures 5 and 6) shows that the number of peaks that appeared in derivatization treatment was found more than the non-derivatization treatment.

The overall results of the existing chromatogram show that the derivatization treatment can improve the sensitivity and volatility of the compound in GC-MS, so that the results of the reading on the chromatogram on the derivatization treatment show better results than the non-derivatization treatment.

The results of the content of secondary metabolite profile compounds detected from GC-MS analysis were matched with Willey version 7 of the Library Database on Table 1, 2 and 3.

The results showed that secondary metabolites which appeared in the ethanol fraction of chamberbitterherbshowed 2 compounds in the non-derivatization treatment, while in the derivatization treatment 35 compounds appeared.

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Table 1. Secondary metabolite compounds in chamberbitterherb (Phyllanthusniruri L.) ethanol fraction

No.	Compound Name Before Derivatization (*RetentionTime*/min) (saraswati,2013)	No.	Compound Name after Derivatizatoin(*Retention Time*/min)
1	Ethane, fluor- (1.53)	1	**Carboxylic acid (17 Compounds)**
2	Propane-2-on (1.63)		Oxalate acid-DITMS(3.10); acetate acid-2TMS(3.43); phosphate acid-TRITMS(4.87); propanoates acid-2,3 DITMS(5.70); maloate acid-3TMS(7.17); pyroglutamatic acid-DITMS(7.84); tetranoate-tetrakis-acid OTMS(8.82); 1-cyclohexane-1-carboxylateacid-TRITMS(11.61); arabinonanoate acid-TRITMS(11.68); citrate acid-TETRATMS(11.77); manoate acid TRITMS(11.94); glucuronic acid PK B 5TMS(13.11); 3,4,5- trihydroxybenzoate acid-TETRATMS(13.35); palmitate-Mono acid TMS(14.32); trans-9-octadecanoate acid-1TMS(16.649); stearic acid MONOTMS(17.05); metoxymandelic acid-DITMS(31.87)
		2	**Amide (3 compounds)**
			Acetamide,-2,2,2-trifluoro-N,N-2TMS(3.86); Pentanamide(29.03); Butanamid(29.60)
		3	**Amine (3 compounds)**
			Metylisobutylenitrosamine(26.36);Ethanamine(29.32), Sinefrine(30.91)
		4	**Phenolic (3 compounds)**
			2-methyl-2-butanol(14.33);1-propanol(30.76); 2,2dimetyl propanol(33.51)
		5	**Steroid(1 compound)** Glycerol-tri-TMS(5.49)
		6	**Terpenoide (4 compounds)**
			Erythritol(8.27); Adonitol-5TMS(10.96); Trimethyl hydrazine(27.82); pyrano[3,4-b]indol-3(9H)-on, 1-(4pentinil)(31.12)
Compound	2 Compounds	Compound	31 Compounds
Peak	3 Peaks	Peak	52 Peaks

Secondary metabolites that appeared in the meniran herb ethyl acetate fraction showed 4 compounds in the non-derivatization treatment, while in the derivatization treatment 28 compounds appeared.

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Table 2. Secondary metabolites compounds in chamberbitterherb (*Phyllanthusniruri* L.) ethyl acetate fraction

No.	Compound Name Before Derivatization (*RetentionTime*/min) (saraswati, 2013)	No.	Compound Name after Derivatizatoin (*RetentionTime*/min)
1	1-chloro-1-fluoroethane(1.77)	1	**Carboxylate acid (12 compounds)**
2	Acetate acid (1.86) (Ac. Carboxylates)		Acetate acid TRITMS(3.42); malonate acid-DITMS(3.65); phosphor acid-TRITMS(4.86);
3	Silane, dimetoxiymetil-(1.91)		Succinic acid-DITMS(5.25); hexanoate acid
4	2-butoksietanol(2.45) (phenolic)		DITMS; butadenioate acid- TRITMS(5.25); 1siklohexane-1-carboxylate acid TMS(11.61); 3,4,5-trihydroxybenzoat acid – TETRATMS(13.34); palmitic acid-MONOTMS(14.37); stearic acid-MONOTMS(17.12); oxyrancarboxyilate acid-3,3-dimetyl(29.01); vannilactate acid-TRITMS(31.49); 4-metoxymandelic acid – DITMS(33.65)
		2	**Amide (3 compounds)**
			Acetamide(29.36); Propanediamide(29.55); Propanamide(31.11)
		3	**Amine (4 compounds)**
			1,3,4-thiadiazol-2-amine(26.91); tristrimethylsilyl-epinephrine(28.24); dimethylnitrosamine(29.82); 1H-purin-6amine(33.33)
		4	**Phenolic (2 compounds)**
			2-Metyl-2-butanol(27.71); Thymol-MONOTMS(28.08)
		5	**Steroid (1 compound)**
			Glycerol-TRITMS(5.04)
		6	**Terpenoide (3 compounds)**
			Erythritol(8.27); Adonitol 5TMS(10.95); Nitrososarcosin(31.63)
Compound	4 Compounds	Compound	25 Compounds
Peak	4 Peaks	Peak	42 Peaks

The secondary metabolite compounds that appeared in the N-hexane fraction of chamberbitterherbs showed 11 compounds in the non-derivatization treatment, while in the derivatization treatment 42 compounds appeared.

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Table 3. Secondary metabolite compounds in chamberbitter herb (*Phyllanthusniruri* L.) N-hexane fraction

No.	Compound Name Before Derivatization (*RetentionTime*/min) (saraswati, 2013)	No.	Compound Name after Derivatizatoin (*RetentionTime*/min)
1	3,3-dimetyl-2-phenyl-2-(1-oxo-1,2,3,4tetrahydronaphtalene-2-yl)azirane(0.12)	1	**Carboxylate acid (15 Compounds)**
	1,1'-bibicyclo(2.2.2)octyl-4-carboxylate acid (0.42)(carboxylate acid)		Acetate acid-TRITMS(3.42); Butanedioate acid – TRITMS(7.71);tetradekanoate acid-MONOTMS(11.85); 6- octadekanoate acid (12.56); palmitic acid 1TMS(13.98); 3-octaneoate acid-MONOTMS(14.13); hexadekanoate acid MONOTMS(12.63); octadeka-9,12-Dienoate acid (14.71); 9,12,15-octadekatrienoate acid (14.76); Octadekanoate acid(15.29); alpha-linoleate acid MONOTMS(16.52); stearic acid MONOTMS(17.01); pentakosanoateacidMONOTMS(18.04); 2-bromosebasiat acid-2TMS(19.58); pentakosanoate acid-MONOTMS(18.03);
2	1-chloro-1-fluoroethane (1.30)		
3	2-diacetone alcohol (1.33)(alcohol)		
4	2-propanone (1.37)		
5	Propanenitrile, 2-hidroxy-2-metyl(1.41)		
6	2,2-Dimetoxypropane(1.44)		
7	Heksadekanoate acid (13.79)(Carboxylate acid)		
8			
9	11,14- eicosadienoate acid (16,07)(carboxylate acid) 9,12,15-Octadekatrienoate acid(16.13)(		
10	carboxylate acid)		
		2	**Amide (2compounds)**
11	2-hexadekan-1-ol (16.46)(alcohol)		3-butenamide(25.23); 3-metyl-2propanamide(27.80)
		3	**Amine (2 compounds)**
			4H-imidazol-5-amine(28.44); 1H-purin-6amine(29.86)
		4	**Alcohol (1 compounds)**
			trans-3,4-dihydro-2,2-dimetyl-8-nitro-4isopropilamino-2H-1-benzopiran-3ol(29.42)
		5	**Phenolic (3 compounds)**
			Valerianol TMS(19.90); 1-propantiol; 2,3pentanediol(17.84); 2-heptanol(27.03)
		6	**Terpenoide (2 compounds)**
			Trans-phytol-TMS(16.18); N-1metylinosin(25.92); O,O,O-tris-trimetylsililepinefrin(27.51)
Compound	4 Compounds	Compound	25 Compounds
Peak	4 Peaks	Peak	55 Peaks

The results obtained from Table 1,2 and 3 show that there are peaks that appeared more than the compounds detected, in fact in the analysis of the compound data obtained in accordance with the peak that appeared, but in the detection of the compounds obtained there

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was the name of the same compound so the detection was only written with one compound with the consideration that the retention time appears faster. Neither the secondary metabolites nor the compounds that reacted with BSTFA in the results were displayed because they were not the compounds intended to find in this study.

Previous research by Saraswati (2013) metabolite analysis was carried out without derivatization, the results showed that with the integration area of 150000 the polar fraction showed 3 peaks, the semi-polar fraction appeared 4 peaks, and the non-polar fraction 11 peaks. The results of the compound obtained in Willey version 7 Library database of fractionation of chamberbitterherb (*Phyllantusniruri* L.) ethanol extract by GC-MS method found 2 components detected in the polar fraction, 4 components in the semipolar fraction, and 10 components.

After derivatization, the results showed that in 52 polar fractions appeared 52 peaks, there were 42 peaks in the semi polar fraction, and 55 peaks in non-polar. The results of the compounds obtained fractions by chamberbitter herb (*Phyllantusniruri* L.) ethanol extract with the GC-MS method contained 31 components detected in the polar fraction, 25 components in semipolar fraction, and 25 components in nonpolar.

The results of observations on derivatization and non-derivatization contained several compounds detected in the form of carboxylic acids (such as hexadecanoic acid), alcohol (2-hexadecone-1-ol), phenolic (2,3-pentanediol) and terpenoids (Erythritol). Carboxylic acid compounds, alcohols, phenolics, and terpenoids which were more likely detected in derivatization due to the presence of BSTFA as its derivatives. This occurred because BSTFA is able to increase the sensitivity of carboxylic compounds, alcohols, phenolics, and terpenoids so that the compounds detected in GC-MS are numerous (Figure 7).

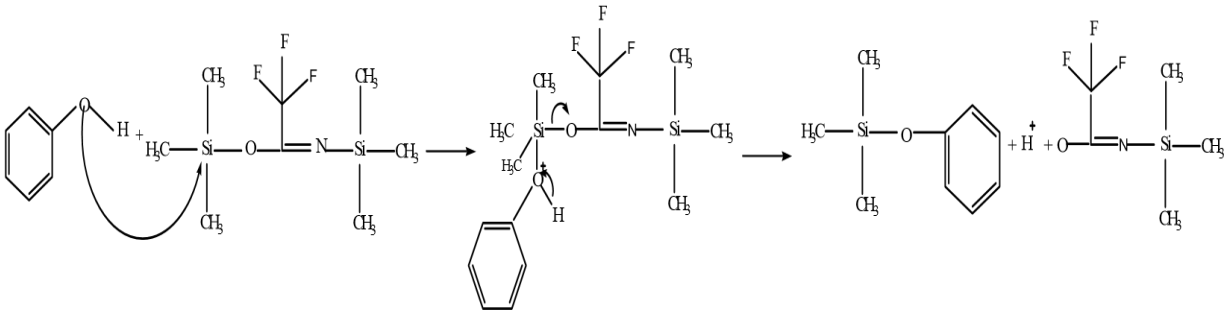

Figure 7. Reaction of nucleophilic substitution of phenol compounds by BSTFA silylation reagent

Derivatization in this study made the separation of compounds more clearly and the number of compounds detected was bigger. In this study, in the presence of derivatization, there were additional compounds that appeared previously without the derivatization without detecting the compounds contained in chamberbitterherb (*Phyllantusniruri* L.) ethanol extract.

IV. CONCLUSION

In the analysis of metabolite profiles in chamberbitter herb (Phyllanthusniruri L.) ethanol extract fractions produced compounds in the form of carboxylic acids, alcohols,phenolic and terpenoids.

Based on several compounds that appeared in each fraction, it necessary to do further research using other derivatization methods and other

optimization methods so that the utilization of thechamberbitter herb can develop.

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