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The Chemical Constituents of Tenggulun (*Protium javanicum*, Burm, F) Leaf

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ABSTRACT: *Protium javanicum*, Burm, F, locally known as Tenggulun, have been used in Balinese traditional culinary and medicines. This research aimed to investigate the chemical constituents of *Protium javanicum*, Burm leaves extract. The Leaves of Tenggulun collected at Bukit Jimbaran Bali, were air dried, blended to yield a powder and then extracted with ethanol. The crude ethanol extract was then shaken with ether to give two fractions, the ether soluble fraction A, and the ether insoluble fraction B. The fraction A was fisrt subjected to GC-MS to analyse any volatile compound present and subsequently separated on silica gel column chromatography with gradient elution by increasing polarity using n-hexane, dichloromethane, ethyl acetate, and methanol respectively. While the fraction B was hydrolysed with 1 M HCl. The major components of volatile compounds obtained from Tenggulun leaves were sesquiterpenes i.e: caryophyllene, caryophyllene oxide, and spathulenol. The non-volatile compounds that have been isolated were a series of long chain aliphatic alcohol, a binary mixture of α and ß-amyrine, and ß-sitosterol. In addition, the compound obtained from hydrolysis of insoluble material was tentatively deduced as a long chain aliphatic diol.

Keywords: chemical constituents, Tenggulun, *Protium javanicum* Burm. F., sesquiterpenes, α and ß-amyrin

INTRODUCTION

Natural substances isolated from plants are used for many purposes: for example, as medicinal agents in drug development, as cosmetics, pesticides, and as food flavours. The use of herbal and other traditionally based preparations for healing diseases or maintaining health and or as food spices has been long recognized in the Indonesian community.

In Indonesia, many plants that give a specific aroma (flavour) in food spices are also used in folk medicines. *Protium javanicum*, is one of the examples of which was collected for the present investigation. The leaves of this plant have been used as spices in Balinese culinary[1] and as traditional medicines elsewhere in Indonesia, while the woody parts are used for fine carving. Although this plant has not received much attention for medicinal use in Indonesia, the literature on the genera *Protium,* shows they have worldwide importance as sources of natural medicines.[2] Despite much previous phytochemical research on the *Protium,* genera, little attention has been paid thus far to the chemical constituents and biological activities of this plant. As a phytochemical study on the Indonesian plants, it would be of some interest to investigate the chemical constituents of Tenggulun (*Protium javanicum*).

The family Burseraceae is a well-known source of exudates and resins rich in aromatic substances that are used for many medicinal purposes as well as in the perfumery industry, production of varnish, and other uses. *Protium* is the principal genus in the family Burseraceae, which is spread widely in the tropical parts of South America. Leaves of species of Burseraceae, mainly from the genus *Protium*, are considered aromatic and medicinal[2].

In folk medicines, gum and oleoresin from *Protium* species are used traditionally for healing ulcers. The resin is also used as a tonic and stimulant as well as an anti-inflammatory agent.[3,4] While the essential oil from leaves and fruit of *Protium heptaphyllum* have been reported to possess acaricidal activity against *Shistoma mansoni*, as well as antiurcerognate[4], antinoceptic and anti-neoplastic activities,[3] the ether extract of the oleoresin possessed an effective non-opioid analgesic.[3] Other genera are reported as possessing antiseptic properties on skin diseases, anti-tumour and acaricidal activity.[2]

Chemical analysis of resin from species of the Burseraceae family has led to the isolation and identification of mainly terpenoid compounds. The resin, stems, roots, and leaves are reported as a source of essential oil,[5,6] steroid,[7] terpenes,[7] phenylpropanoid, lignans,[8] quercetin, coumarin, and other types of compounds.[2]

Protium javanicum (*Amyris protium* Linn) known locally in Bali as Tanggulun, Tranggulun, Gulun, or Tenggulun, is a tree (10-25 m) which grows in dry land 1-200 m above sea level.[9]

In Indonesian traditional medicines, the leaf juice is taken with lemon and sugar (honey) for coughs and diarrhoea, while poultices are applied to affected areas of the body, for example the stomach. The fruit is given to increase appetite.[10] However, hardly any reports are found in the literature about the chemical constituents of this species. Most of the research that has been reported on *Protium* species is mainly from the point of view of the composition of their essential oil, which is believed to be responsible for their biological activity. Therefore, the aim of this research is to isolate and identify the chemical constituents of *Protium Javanicum* Burm, F leaf.

METHODS AND EXPERIMENTAL DETAILS

Materials and Apparatus

Plant material was collected at Bukit Jimbaran Bali, Indonesia, and identified at the Biology Department of the Faculty of Mathematics and Natural Sciences, Udayana University *as Protium javanicum* Burm, F.

Extraction

The leaves of *P. javanicum* were air dried and blended to yield a powder. The sample (750 g) was extracted with ethanol (3 L) at room temperature for 24 hours. The solvent was reduced under vacuum until the volume of the extract was 100 mL. The crude extract was then shaken with ether to give two fractions, the ether soluble fraction A, and the ether insoluble fraction B.

The ether extract of Fraction A was washed with water, dried over anhydrous Na_2SO_4 and concentrated in vacuo to yield a dark green gum (15.32 g). This extract was subjected to GC-MS for analysis of volatile constituents. The solid, non ether-soluble fraction B (48.32 g) was hydrolysed by stirring with 1 M HCl (500 mL) at room temperature overnight. The mixture was extracted with ether, the extract was dried over anhydrous Na_2SO_4 and concentrated to yield a dark green oil (0.97 g) and a brown solid.

Column Chromatography of Fraction A

A sample of the ether extract above (5 g) was subjected to silica gel column chromatography with gradient elution of increasing polarity using light petroleum: dichloromethane (DCM) (250 mL each fraction). Eleven (11) fractions were collected. Each was concentrated *in vacuo* and then subjected to ^{1}H NMR and GC-MS analysis. Fractions 8, 9 and 10 (70, 80, 90% DCM respectively), were rechromatographed over silica gel using light petroleum ether:ethyl acetate as eluent (gradient elution/increasing polarity from P.E:EtOAc 100:0 to 50:50).

Identification

Melting points were determined on a reichert hot stage microscope and are uncorrected.

Spectroscopic characterization. Infrared spectra were recorded on a Perkin Elmer 1600 Fourier-Transform spectrometer using fused sodium chloride cells. GC-MS analyses were performed on a Varian Saturn 4D instrument, with a ZB-55% phenylmethylpolysiloxane column (30m x 0.25mm I.D and 0.25 µM thickness).

^{1}H and ^{13}C NMR were recorded with a Varian Gemini spectrometer in $CDCl_3$ at 200 and 50.28 MHz respectively. Tetramethylsilane (TMS) was used as an internal standard with chemical shifts given in parts permillion (ppm). Mass Spectra and high-resolution mass spectra were recorded on a Bruker BioApex II 47e FTMS fitted with an analytica electrospray source (EI or ESI).

Identification of non volatile components.

Compound 1 (long chain aliphatic alcohol):

^{1}H NMR)($CDCl_3$) δ 0.88 ppm, t, J 6.4 Hz, 3H, CH_3; 1.25 ppm, s, CH_2; 1.56 ppm, bs, 1H, OH; 3.64 ppm, t, J 6.6 Hz, 2H, CH_2.

^{13}C NMR ($CDCl_3$) δ14.3, CH_3; 22.9, CH_2; 26.0, CH_2, 30.0, CH_2; 63.3, CH_2OH.

Compound 2 (binary mixtures of α- amyrine and β-amyrine):

ν_{max} 3450; 2919; 1377 cm^{-1}

^{1}H NMR ($CDCl_3$) δ 0.79 ppm, s, 6H, 2 x CH_3; 0.88 ppm, s, CH_3; 0.95 ppm, s, CH_3; 1.00 ppm, s, 3H, CH_3; 1.03 ppm, s, 6H, 2 x CH_3; 1.07 ppm, s, 3H, CH_3; 1.25-1.97 ppm, m, 20H, 10 x CH_2; 3.64 ppm, t, J 5.8 Hz, 1H, CHOH; 3.64 ppm, t, J 5.8 Hz, 1H, CHOH; 5.12 ppm, t, J 3.4 Hz, 1H, CH=; 5,38 ppm, t, J 3.4 Hz, 1H, -CH=.

^{13}C NMR (a):($CDCl_3$) δ 15.6 ppm; 15.6 ppm; 16.9 ppm; 17.6 ppm; 18.4 ppm; 21.4 ppm; 23.3 ppm; 23.4 ppm; 26.6 ppm; 27.3 ppm; 28.1 ppm; 28.4 ppm; 28.7 ppm; 32.9 ppm; 31.3 ppm; 33.6 ppm ;36.9 ppm; 38.6 ppm; 38.8 ppm; 39.6 ppm;

39.6 ppm; 40.0 ppm; 41.5 ppm; 42.1 ppm; 47.7 ppm; 55.2 ppm; 59.1 ppm; 79.1 ppm; 124.1 ppm; 139.3 ppm.

^{13}C NMR (b):($CDCl_3$) δ15.6 ppm; 15.6 ppm; 16.9 ppm; 18.4 ppm; 23.5 ppm; 23.7; 25.9 ppm; 26.2 ppm; 26.9 ppm; 27.3 ppm; 28.1 ppm; 28.4 ppm 31.3 ppm; 32.3 ppm; 32.5 ppm; 32.7 ppm; 34..7 ppm; 37.2 ppm; 38.6 ppm; 38.8. ppm; 39.7 ppm; 39.9 ppm; 41.5 ppm; 46.8 ppm; 47.3 ppm; 47.7 ppm; 55.2 ppm; 79.1 ppm; 121.8 ppm; 145.2 ppm.

Mass spectrum of peak 1 (β-amyrine): m/z 426 (M^+, 5%); 257 (5); 218 (100), 203 (84), 189 (37), 175(16); 107 (18); 95 (24); 81 (18); 57 (20), 41 (34).

Mass spectrum of peak 2 (α-amyrine): m/z 426 (M^+, 6%); 286 (1); 257 (5); 218 (100),

203 (25), 189 (28), 175 (10); 161(18); 135 (16); 122 (20); 95 (19); 81 (18); 57 (14), 41 (28).

Compound 3 (β-sitosterol):

ν_{max} 3424; 2932; 1377; 1463; 1455;1382; 1361; 1054 cm^{-1}

^{1}H NMR ($CDCl_3$) δ 0.67 ppm, s, 3H, CH_3; 0.80 ppm, d, J 6.6 Hz, 6H, 2 x CH_3; 0.84 ppm, d, 6.6 Hz, 3H, CH_3; 0.92 ppm, d, J 6.3 Hz, 3H, CH_3; 1.06 ppm, s, 3H, CH_3; 1.12-1.30 ppm, m, 6H, 3x CH_2; 1.44 ppm, 6H, 3 x CH_2; 1.48 ppm, 6H, 3 x CH_2; 1.55 ppm, s, 3H, 3 x CH; 1.62 ppm, m, 6H, 3 x CH_2; 1.82 ppm, 2H, 2 x CH; 1.98 ppm, 1H, CH; 2.27 ppm, 1H, CH; 3.52 ppm, m, 1H, CHOH; 5.35 ppm, d, J 5.3 Hz, 1H, CH=.

^{13}C NMR($CDCl_3$): δ11.9 ppm; 11.9 ppm; 18.8 ppm; 19.1 ppm; 19.4 ppm; 19.8 ppm; 21.1 ppm; 23.1 ppm; 24.3 ppm; 26.1 ppm; 28.2 ppm; 29.2 ppm; 31.7 ppm; 31.9 ppm; 31.9 ppm; 33.9 ppm; 36.1 ppm; 36.5 ppm; 37.3 ppm; 39.8 ppm; 42.3 ppm.; 42.3 ppm; 45.9 ppm; 50.2 ppm; 56.1 ppm; 56.8 ppm; 71.9 ppm; 121.7 ppm; 140.7 ppm.

Mass spectrum: m/z 414 (M^+, 35%); 396 (26); 381 (20); 329 (48); 303 (18), 273 (24), 255 (26), 231 (22); 213 (34); 199 (18); 161 (32); 105 (44), 67 (31); 43 (100).

Syhthesis of Caryophyllene Oxide

m-Chloroperoxybenzoic acid (2.1 g, 12.6 mmol) in dichloromethane (25 mL) was added slowly to a solution of caryophyllene (2.04 g, 10 mmol) at 25°C for 5 min. The mixture was stirred at room temperature for 1 h. Subsequently, sodium sulphite (0.2 g) was added until the solution was negative to iodine-starch test. The organic layer was collected, washed with 25% $NaHCO_3$, brine and water. The extract was dried over Na_2SO_4 and concentrated to yield thick pale yellow oil (1.98 g, 90%).

^{1}H NMR ($CDCl_3$) δ 0.98 ppm, s, 6H, 2 x CH_3; 1.20 ppm, s, 3H, CH_3; 1.32 ppm, m, 1H, CH, 1.42 ppm, s, 1H, CH; 1.67 ppm, m, 2H, CH_2; 1.67 ppm, 2H, CH2; 1.75 ppm, m, 1H, CH_2; 2.06 ppm, m, 1H, CH; 2.13 ppm, m, 2H, CH_2; 2.27 ppm , m, 2H, CH_2; 2.61 ppm, m, 1H, CH; 2.87 ppm, dd, 1H, CH; 4.85 ppm, d, J 1.5 Hz, 1H, CH; 4.97 ppm, d, J 1.5 Hz, 1H, CH.

Mass spectrum: m/z 221(M+H, 2%), 203 (100), 187 (20), 159 (35), 133 (28), 119 (40), 105 (44), 95 (55), 43 (58)

Hydrolysis of the Insoluble Material of Fraction B

Hydrolysis of the ether insoluble residue from the original extraction was carried out using 1M HCL. The reaction was stirred overnight at room temperature and then extracted with ether to yield a green oily product (0.98 g). ^{1}H NMR ($CDCl_3$) δ 0.87 ppm, t, J 6.6 Hz, 3H, CH_3; 1.24 ppm, bs, 22H, 11 x CH_2; 2.30 ppm, q, J 7.0 Hz, 2H, CH_2; 3.70, s, 2H, CH_2O; 4.29, bs, CH_2OH; 5.36, t, 5.8 Hz, 1H, -CH=.

RESULTS AND DISCUSSION

Analysis of the Volatile Components

The volatile components of crude extract were analysed using GC-MS spectrometry. The GC-MS spectra (Figure 1) showed 14 peaks but only seven peaks could be unambiguously identified, as summarised in Table 1.

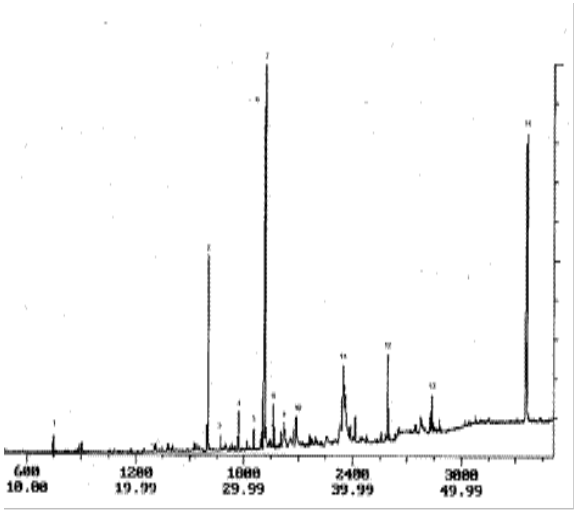

Figure 1. GC-MS spectra of the leaf extract *Protium javanicum* Burm, F.

Comparison with the mass spectral library suggested that major components obtained from the leaf extract were ß-caryophyllene (M^+=204), caryophyllene oxide (M+1=221), and spathulenol (M+1=221). This conclusion were confirmed by comparison their retention time to those of authentic samples of ß-caryophyllene and caryophyllene oxide. The retention time of spathulenol was reported in the literature to be very close to caryophyllene oxide. The minor products were tentatively identified as 3-carene (M^+=136), and α-humulene (M^+=204). Two additional compounds were present in the extracts that are not regarded as plants constituents. t-Butylated hydroxytoluene

Table 1. The volatile components of *Protium javanicum* Burm, F

Peak No.	M^+	Retention time	Assignment	% relative
1	136	12.43	3-carene	0.69
2	204	26.64	ß-caryophyllene	8.62
3	204	27.83	α-humulene	0.61
7	267	29.46	BHT	1.59
5	204	31.60	isocaryophyllene	0.98
6	221 (205) 221 (203)	31.83 31.89	spathulenol caryophyllene oxide	19.63 19.90
14	390	55	dioctyl pthalate	25.29

(BHT) is almost certainly an antioxidant for plastics, and dioctyl phthalate was also identified and could possibly arise from the plasticiser used in the container in which the extract was kept.

The non-volatile Components

A white solid with a sharp melting point of 76-78°C was isolated from fraction 8 after column chromatography of fraction A. This compound was not very soluble in chloroform, dichloromethane, acetone or methanol. Its ^{1}H NMR spectrum (Figure 2) showed resonances only in the region between δ 0.80 ppm and 3.65 ppm, suggesting that there were no double bonds. The resonance at δ 3.64 ppm (t, 2H, *J* 6.63 HZ) is ascribed to a proton CH-O, while the signal at δ 0.87ppm (t, 3H) corresponds to terminal methyl (-CH_3) and that at δ 1.24 ppm is due to methylene proton (-CH_2). These signals suggested that the compound was a long chain aliphatic alcohol or a closely related homologous mixture of primary alcohols. A series of straight chain alcohols (C14, C16, C18, C20, C24, C26) was synthesized by lithium aluminium hydride reduction of a standard mixture of long chain fatty acids, to help elucidate the structure of the alcohol isolated from *Protium javanicum*, Burm, F. The ^{1}H NMR spectra (Figure 3) of the synthetic alcohol mixture was very similar to that of the alcohol from the natural product, except for the ratio of the chain –CH_2) resonances compared to the CH_2OH. This confirms that the natural compound is a long chain aliphatic alcohol or a short homologous series, having a carbon chain, estimated from the ^{1}H NMR spectrum, as C32 to C40. The ^{13}C NMR spectrum showed a single C-OH at δ 63.3 ppm. Resonances at δ 32.9 ppm to δ 22.9 ppm correspond to methylene carbons, and δ 14.3 ppm for terminal –CH_3) but the exact number of carbons in the chain could not be determined.

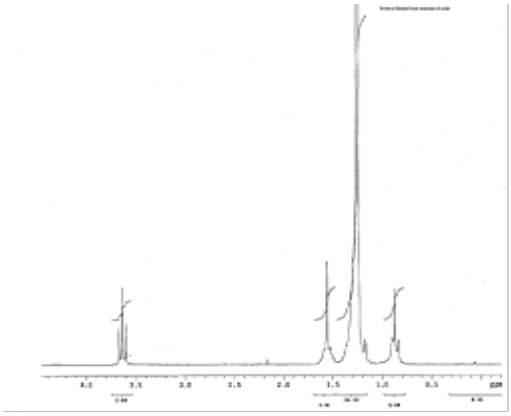

Figure 2. ^{1}HNMR spectra of natural aliphatic alcohol.

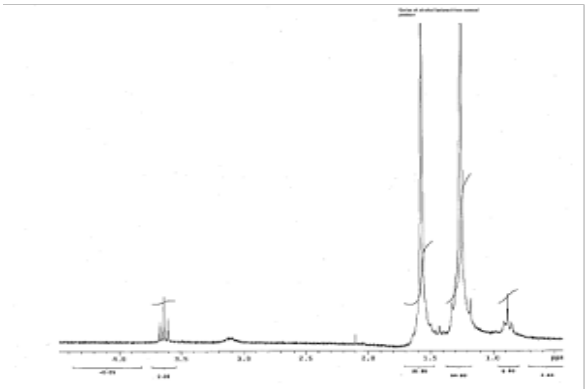

Figure 3. ^{1}HNMR spectra of synthetic aliphatic alcohol.

Unfortunately, the MS spectra did not show molecular ions but only fragment ions. Several attempts to obtain better GC-MS spectra of the mixture, by converting it into the corresponding, more volatile and more stable trimethylsilyl ethers, were still failed to give sharp peaks that consistent with the components of the mixture. Even the electrospray mass spectrum did not resolve the problem, and again a number of peaks, probably not corresponding to molecular ions, were obtained. This may due to the lack of solubility of the compound in most organic solvents. Hence, the material is believed to be a homologous series of naturally occurring aliphatic alcohols from $C_{32}H_{65}$OH-$C_{40}H_{81}$OH, but is not yet fully characterized.

The second solid compound, m.p. 168-170°C, was isolated from the mother liquors of the above alcohols, following further chromatography, and was identified as α- and β-amyrine (Figure 4). GC-MS spectra showed two peaks in the ratio of 2:1, with the same molecular ion peak at M^+=426, corresponding to the molecular formula $C_{30}H_{50}O$. The co-crystallisation suggests that the two compounds are very similar isomers. The IR spectrum revealed the presence of a hydroxyl group (ν_{max} 3450 cm^{-1}) and no peaks due to carbonyl groups. The ^{1}H NMR spectrum of this two component mixture showed the presence of protons of two trisubstituted double bonds at δ 5.12 ppm and 5.18 ppm, two secondary carbinol at δ 3.22 ppm and 3.64 ppm, and eight methyl groups in the region δ 0.79 ppm to δ 1.13 ppm. The ^{13}C NMR spectrum revealed doubly bonded carbon at δ 145.2 ppm and 139.3 ppm (quartenary carbon) and tertiary carbon at δ 124.3 ppm and 121.8 ppm. A resonanse at δ 71.9 ppm confirmed the presence of carbinol group. From the spectral data above and by comparison with ^{13}C NMR data found in literature,[11] the structure of the compound was deduced as a binary mixture of α -amyrine and β-amyrine. The two compounds are commonly found in plants.

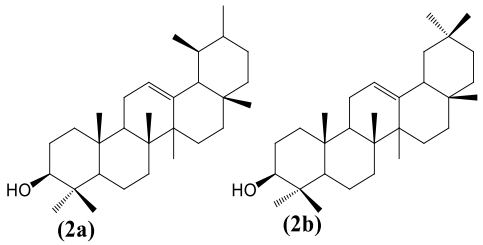

Figure 4. Structure of α -amyrine (2a) and β amyrine (2b).

A third compound was isolated from the non volatile fraction by further chromatography, as white crystals, m.p. 132-136°C. The GC-MS spectra showed a molecular ion peak at M^+= 414 corresponding to the molecular formula $C_{29}H_{50}O$. The IR spectrum revealed the presence of a hydroxyl group (ν_{max} 3424 cm^{-1}) and there were no peaks arising from carbonyl groups. The ^{1}H NMR of the compound showed the presence of protons of a trisubstituted double bond at δ 5.36 ppm (d, J 5.1 Hz), a secondary carbinol at δ 3.49 ppm, and six methyl groups at the region δ 0.79 ppm up to 1.06 ppm. The ^{13}C NMR spectrum showed doubly bonded carbon at δ 140.7 ppm (quartenary carbon) and tertiary carbon at δ 121.7 ppm, while carbonyl group resonanced at δ 71.9 ppm. From the spectral data above and by the comparison with spectral data in the literature, the compound (3) was identified as β–sitosterol (stigma-5-en-3β-ol), as seen in Figure 5. This compound then was converted to its acetate derivative to confirm the structure. The acetate derivative of (3) melted at 122 °C, confirming that the compound isolated was β–sitosterol, which is commonly found in plants.

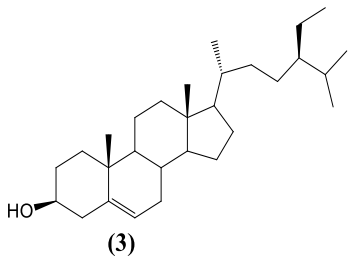

Figure 5. Structure of compound 3, stigma-5-en-3 β-ol (β-sitosterol).

Small amounts of further compounds were seen from fraction 10 of the non-volatile fraction following crystallisation. One was isolated as white crystals, m.p. 126-130 °C. The ^{1}H NMR of this material showed the presence of an aldehyde proton at δ 9.82 ppm, a proton of trisubstituted double bond at δ 5.43 ppm, and vinylic proton at δ 4.81 ppm and 4.33 ppm. In addition, resonances in the region δ 0.60 ppm to 2.20 ppm were also observed. Unfortunately, The GC-MS spectrum showed three major peaks, and it could not be adequately purified.

Hydrolysis of Insoluble Material

The ether insoluble residue from the original extraction was suspected of containing polysaccharide material, and accordingly was hydrolysed with 1M HCL. Green ether soluble oil was obtained, as compound 4. The ^{1}H NMR spectrum showed resonances at δ 0.87 ppm (t, 3H) corresponding to hydrogen from the -CH_3 terminal, δ 1.24 ppm (b, 22H) is due to methylene or methine hydrogens (CH_2/CH). The resonance at δ 2.30 ppm (2H) is assigned as an allylic methylene, δ 3.70 ppm (s, 2H) may be due to -CH_2 hydrogens of -CH_2OH, and δ 4.29 ppm may be due to (=C-CH_2-OH). The methine hydrogen from -CH attached to a trisubstituted double bond is indicated by the resonance at δ 5.36 ppm (t, 1H). The compound was tentatively deduced from ^{1}H NMR data as being a long chain aliphatic diol (Figure 6), but no further work has yet been carried out on this material.

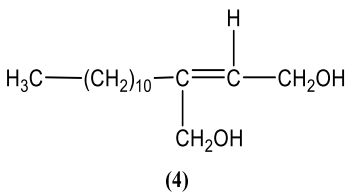

Figure 6. Proposed Structure of compound 4.

CONCLUSION

The major components of the volatile compounds obtained from Tenggulun (*Protium javanicum*, Burm F) leaf extract are sesquiterpenes i.*e*. caryophyllene, caryophyllene oxide, and spathulenol. The non volatile compounds that have been isolated are a series of long chain aliphatic alcohols, binary mixture of α-amyrine and β-amyrine, and β-sitosterol. In addition, a long chain aliphatic diol is tentatively deduced as components of insoluble material.

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