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Research Article

Determination of the active chemical compounds in the stem bark of *Vitex trifolia* Linn.

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Abstract: *Vitex trifolia* Linn. is known as a plant with compounds having antioxidant and anticancer activity. The current repot was conducted to isolate the stem bark of this plant in ethanol fraction. Ethanol extract concentrated and dissolved in ethyl acetate, extracted with 5% $NaHCO_3$, washed with NaOH three times, and then concentrated. Ethyl acetate extract fraction that had been further concentrated and separated by chromatography column, and purified by recrystallization. From the results obtained recrystallized fraction 12^{th} and 13^{th}. Examination of this fraction by proton ^{1}H-NMR, ^{13}C-NMR, IR Spectrometer, and mass spectometer and comparison with existing literature, it can be concluded that this study succeeded in obtaining the β-sitosterol crystals and crystal mixture of aromatic compounds and β-sitosterol.
Keywords: *Vitex trifolia Linn.,* , β-sitosterol, stem bark

INTRODUCTION

Legundi (Indonesian) plants, *Vitex trifolia* L., empirically used as a cure for stomach cramps, coughs, wounds, tonsillitis, puerperal fever,and typhoid fever [1]. . Several studies have reported the chemical content of the fruit and leaves of Legundi containing the flavonoid compounds (casticin; 3,6,7-trimethyl quersetagetin; vitexin; artemetin; 5-methyl artemetin; 7-desmethyl artemetin; luteolin; luteolin-7-O-β-D-glucuronide; luteolin-3-O-β-D-glucuronide and isoorientin) [2], in which flavonoids are secondary metabolites that have potential as antiatheroschlerosis agents, anti-inflammatory, antioxidant, anti-thrombogenik, antitumor, antiosteoporosis, and antiviral [3]. Yoshioka *et al.* [4] reported five phenolic compounds, 4-hydroxybenzoic acid methyl ester (**1**, vanillic acid methyl ester (**2**), 4-hydroxy benzaldehyde (**3**), 4-hydroxybenzoic acid (4) and ferulic acid (**5**), and four flavonoids, 5,5_-dihydroxy-4_,6,7-trimethoxyflavanone (6), luteolin (**7**), vitexicarpin (8) and artemetin (9), were isolated from fruits and leaves of *Vitex rotundifolia* L.

Present study aims to isolate and determine the molecular structure of chemical compounds contained in ethyl acetate fraction (neutral) in the stem bark of *Vitex trifolia* L. Isolation was done by maceration bark powder in the solvent ethanol. The separation of the components of the ethyl acetate fraction using chromatography column with silica gel as stationary phase and solvent mixture n-hexane and ethyl acetate as mobile phase. The results of separation was tested with thin layer chromatography. Purification of the components of the isolation by recrystallization. Chemical structure is determined by using IR – spectrophotometer, ^{1}H-NMR, ^{13}C-NMR and Mass Spectrometer and compared with existing literature data.

MATERIALS AND METHODS

Pretreatment

a. Soaking.

Legundi's bark used in this study obtained from Karawang beach area, West Java, Indonesia. Obtained bark as much as 10 kg cleaned from the dirt, was cut into pieces and dried in the open for 40 days. Once dried, ground bark to bark powder obtained as much as 710 grams. Next powdered bark soaked in the solvent ethanol for 7 days. This processing was repeated twice, then filtered and immersion results obtained green solution. The solution was then evaporated using vacuum distillation, obtained condensed green extract mixed with greenish-yellow oil. This crude extract had 16.40 grams.

b. Test spots. To determine the number of components in the fraction of ethanol, conducted the test patches with a thin layer chromatograpy methods. Best mobile phase using n-hexane and ethyl acetate with the ratio 9:3.

Isolation and extraction

a. Washing crude extract

Crude extracts of 16,40 g dissolved in 400 mL of ethyl acetate. Then washed three times with sodium bicarbonate 5%, every time washing used 300 ml sodium bicarbonate 5%. These washing results obtained two fractions, namely fraction of ethyl acetate and $NaHCO_3$ fractions. After being washed with $NaHCO_3$ solution, the fraction of ethyl acetate was washed with 5% NaOH solution three times, each time washing used

300 ml 5% NaOH. At this point the two fractions obtained i.e, fraction of ethyl acetate and NaOH fractions. The NaOH fraction was neutralized by added 5% HCl solution until pH 7. Further extracted with ethyl acetate for three times, each time the extraction used 400 ml ethyl acetate. Then the extract was washed with water. $NaHCO_3$ fraction was obtained early was neutralized with 5% HCl solution until pH 7. Next extracted with ethyl acetate three times, each time 400 ml, then washed with water. Three fractions were obtained, the fraction of ethylacetate, the fraction of $NaHCO_3$ and NaOH fraction. The solvent was evaporated at low pressure conditions. Results were obtained as follows: faction ethylacetate (12.30 g), fraction $NaHCO_3$ = 0.60 g , fraction NaOH (0.45 g).

These fractions then tested with a thin layer chromatograpy with mobile phase of n-hexane: ethyl acetate = 9:3. Based on observation, the fraction of ethyl acetate was given separation a better spot and the amount at most, so that this fraction followed the process.

b. Chemical separation of ethyl acetate fraction.

To separate the chemical components of ethyl acetate, separation by column chromatography was applied. The column used had a length of 45 cm and 3 cm in diameter. Stationary phase was silica gel 60 G (E. Merck Art 7731) of 80 g while eluent used was a mixture of n-hexane - ethyl acetate. A total of 8.00 g ethyl acetate extract mixed with a little choloroform plus 2 g of silica gel and stirred to mix and allowed to dry. Then the mixture was put into the column previously filled stationary phase. The mixture was then eluted with a solvent mixture n-heksan-ethyl acetate with its polarity increased gradually, by making some fraction of the comparison as shown in the table. 1. Fraction collected every 100 ml.

Table 1. Comparison mobile phase on Chromatography column

n-hexane (ml)	ethyl acetate (ml)
100	0
95	5
90	10
85	15
80	20
75	25
70	30
65	35
60	40
55	45
50	50
45	55
40	60
35	65
30	70
25	75
20	80
15	85
10	90
5	95
0	100

The first color fraction was out of the column at mobile phase ratio n- hexane-ethyl acetate (85:15). Later observations showed that in the first fraction to 12^{th} of green oil. Fraction 13^{th} after 5 days to produce fine crystal fibers but still mixed with green oil. Next to the fraction 13^{th} was purification by recrystallization using solvent chloroform and n-hexane. After stored for one day re-formed white crystals with a weight of 9 mg. Next tested by TLC.

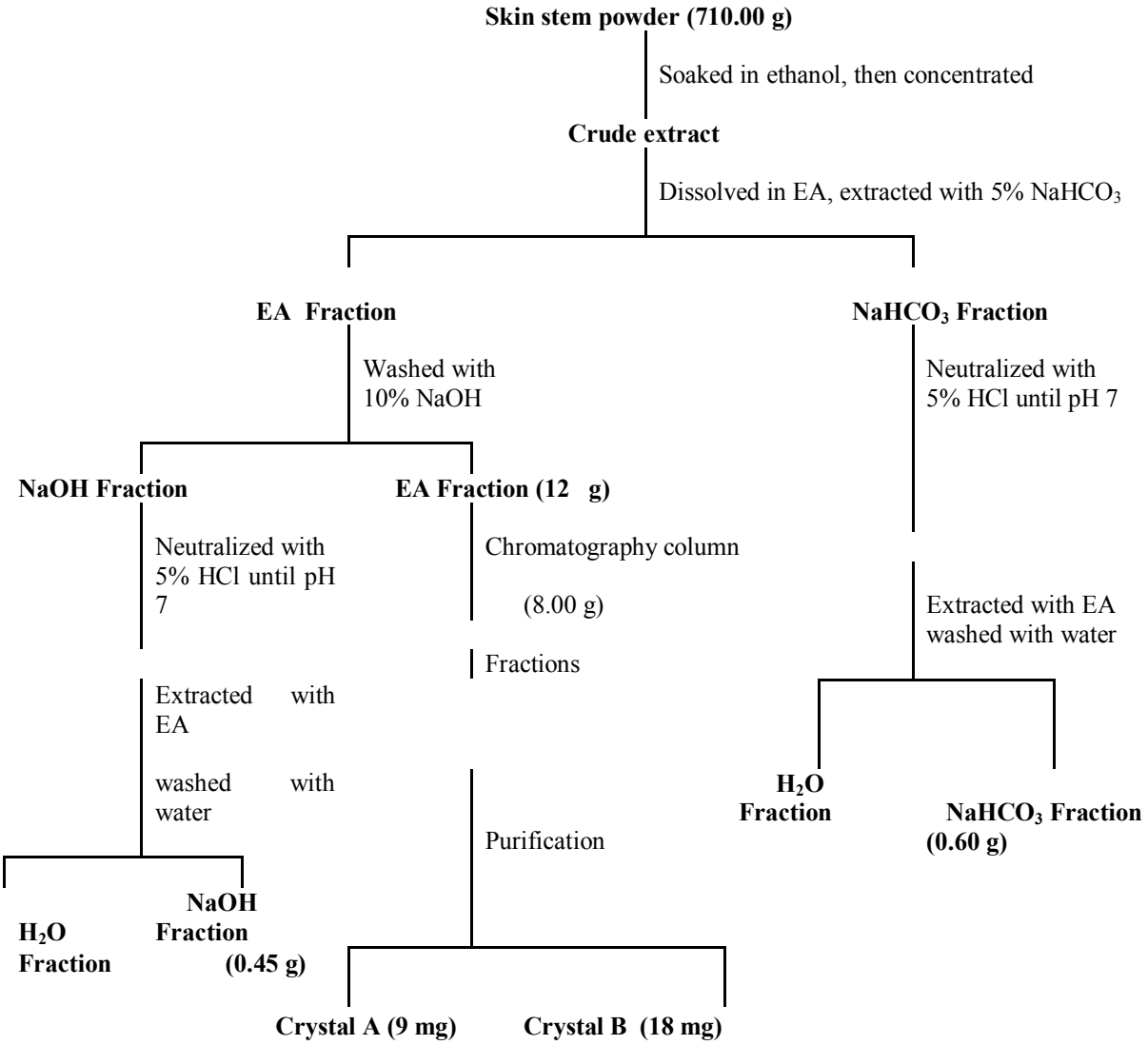
After elucidation using a solvent mixture n-hexane-ethyl acetat (9:3) seen that the top of the column there was samples not yet elucidated. Further , this samples elucidated back by using a solvent mixture of ethyl acetate with ethanol, by the same work as before.

Table 2. Comparison mobile phase on Chromatography column

Ethyl acetate (ml)	Ethanol (ml)
100	0
95	5
90	10
85	15
80	20
75	25
70	30
65	35
60	40
55	45
50	50
45	55
40	60
35	65
30	70
25	75
20	80
15	85
10	90
5	95
0	100

All fractions other than 40^{th}, the shape of a green oil. Fraction 40^{th} after five days to produce a greenish white crystal weighing 18 mg. Crystal was then recrystallized by the use of chloroform and n-hexane. To determine its purity, it was analyzed by TLC with mobile phase $CHCl_3$: MeOH = 4: 1

Fig. 1:Diagram of above chemical compounds separation of stem bark *Vitex trifolia* Linn is as follows



RESULT S AND DISCUSSION

Determination of the molecular structure of component isolation

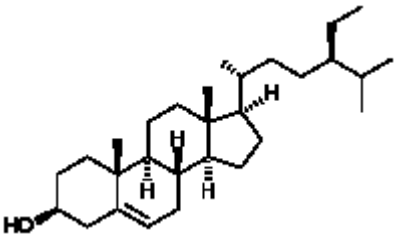
Component A

Component A which was the result of recrystallization of the fraction 13th was a white crystalline form of fine fibers. Based on measurements with a Mass Spectrometer, this component provides a molecular ion with m / e = 414 (22%) and base peak with m / e = 43 (100%). Other molecular ion peak is located in the region m / as follows: 396.4 (36.0%); 381,4 (15.0%); 329.4 (16.0%); 303.3 (14%); 255.2 (27.0%); 231.2 (30.0%); 159.1 (28.5%); 145.1 (45.5.0%); 107.0 (49.0%); 95.0 (54.0%); 81.4 (63.5%); 69.0 (60.0%); 57.0 (84.0%); 43.0 (99.0%), 32.0 (84.0%); By comparing the MS data of samples with MS compounds β-sitosterol, obtained the following data[5,6,7].

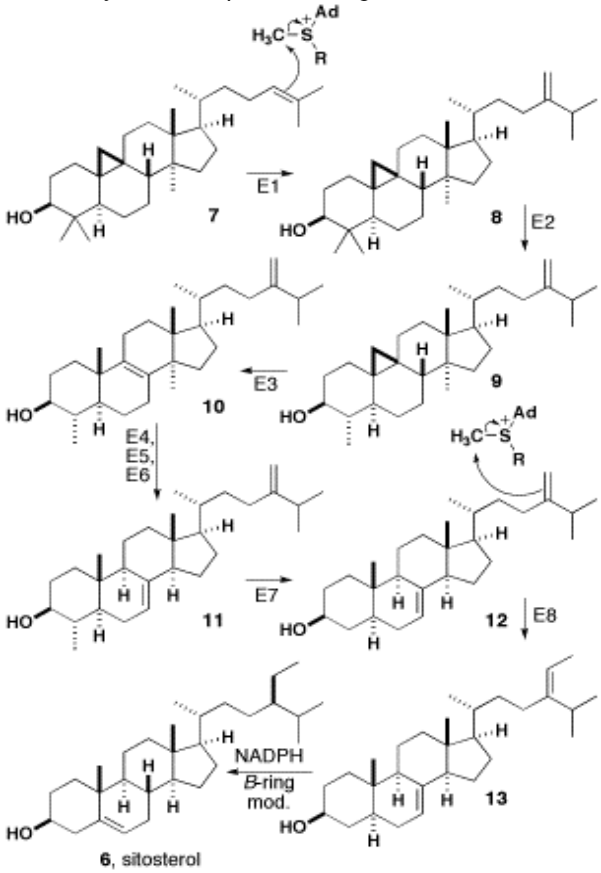
Table 3. MS data Coumpounds A and β-sitosterol

Compounds from isolation	β-sitosterol
414.2 (22.5%)	414.2 (20.0%)
396.4 (36.0%)	396.0 (25.0%)
213.2 (30.0%)	213.0 (17.0%)
145.1 (45.5%)	145.0 (20.0%)
107.0 (49.0%)	119.0 (35.0%)
95.0 (54.0%)	95.0 (60.0%)
81.4 (63.5%)	91.0 (100%)
57.0 (84.0%)	55.0 (85.0%)
43.0 (99.0%)	43.0 (80.0%)

Melting point component A 135 to 140 ^{o}C. Analysis using IR Spectrophotometer showed spectrum at 3373 cm^{-1} a prolonged of-OH bonds stretching; 2867 and 2958 cm^{-1} a prolonged aliphatic C-H stretching; 1641 cm^{-1} as C=C absorption peak; peak at 1457 cm^{-1} for CH_2 possisbly due to a bending frequency for cyclic $(CH2)_n$; By comparing with the data and existing literature, it can be assumed that the compound A is β-sitosterol with the molecular formula $C_{29}H_{50}O$ with the following formula:



A reference [8] suggested the below biosynthesis of β-sitosterol path:



Matsuoka *et.al.*[9] mentioned that alone and in combination with similar phytosterols, β-sitosterol reduces cholesterol levels, and is sometimes used in treating hypercholesterolemia. β-sitosterol inhibits cholesterol absorption in the intestine. In Europe, β-sitosterol is used in herbal therapy, especially for benign prostatic hyperplasia (BPH) [10]. At high levels of β-sitosterol concentrations in blood, however, have been correlated with increased severity of heart disease in men after suffering a heart attack[11]..

Component B

Component B was the result of recrystallization of the fraction 40th which was a white crystal. Melting point component B was from 137 to 139 ^{o}C.

Analysis using ^{1}H-NMR provides signal peaks at chemical shift region between δ= 0.6 ppm - 2.6 ppm which was a methyl group signal of-CH_3 and-CH_2-methylen. In the chemical shift around 3.5 ppm indicates the existence of a proton bound to -OH. whereas at 5.3 ppm indicating H atom attached to the bond C = C [7]. While the chemical shift region between δ = 7.5 - 7.8 ppm is the proton signal of benzene (aromatic) [9].. The chemical shift around 4.2 ppm showed the proton Methyne -CH2- bound electronegative atoms, whereas the other signals coming from remaining fat.

Analysis using Mass Spectrometer, produces molecular ion peak at m / e: 369.4; 329.4; 303.3; 255.3; 213.2; 145.1; 95.1; 57.1 and the base peak with m / e =

43 (100%). Measurements with MS showed similar results with compound A. The measurement with the IR results showed peak in 3000 - 3500 cm^{-1} i.e; a prolonged vibration of-OH bonds and in 2800 – 3000 cm^{-1} i.e, a prolonged vibration of-C-H bonds.

Based on the data collected, compared with the existing library, then in the crystal B allegedly containing aromatic compounds with impurities β-sitosterol; in other words crystal B was still a mixture of several chemical compounds. The difficulties of obtaining sitosterol in pure state had also been reported by other researchers[11-14].

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