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Purification of Antibacterial Compounds from *Spathiphyllum cannifolium* Leaf

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ABSTRACT

Spathiphyllum cannifolium is an 'araceae' species which can be found in Malaysia for landscaping. Previous study carried out in this lab found that this plant possesses the highest antibacterial activity among 19 flowering plants screened. In the present study, purification of antibacterial compound of the plant was conducted. For purification of antibacterial compound, open column chromatography was conducted and ethyl acetate, hexane and ethanol are used as the mobile phase. The results showed that the elution of column with 80% ethyl acetate in ethanol provide fractions with the highest antibacterial activity. The fractions were analyzed with TLC plates and revealed a single spot under UV light at 0.85 Rf value. Further purification of the single spot using HPLC showed two isolated compounds at retention time of 5.53 minutes and 8.26 minutes were obtained. The compounds were suspected as flavanoids as they can be detected at wavelength 360 nm and 400 nm.

Keywords: *S. cannifolium*, antibacterial activity, chromatography, HPLC.

INTRODUCTION

In tropical regions and in immunocompromised or immunodeficient patients, many infectious diseases were caused by microorganisms such as fungi and bacteria [1]. It was predicted that bacteria will become the major cause of infectious disease in the developed world in the next coming years [2]. Bacterial infections constitute up to 70% of the acquired immuno-deficiency syndrome (AIDS) disease caused by the human immunodeficiency virus (HIV) [3]. HIV infected patients were discovered to be much risky towards diseases like bacterial pneumonia by *Klebsiella pneumonia* [4,5]. The most common pathogens associated with infectious diseases and other severe respiratory tract infections includes *Streptococcus pneumoniae, Haemophilus influenza, Escherichia coli, Salmonella spp., Shigella spp., Pseudomonas aeruginosa,* and *Staphylococcus aureus* [6].

Good hygienic practices, appropriate preservation techniques of foods, and antibacterial/antimicrobial substances implementation are the various methods used to control and heal the infectious diseases in developed countries [7]. Though, the uses of antibiotics are widely employed in hospitals and specialized areas as their consumption are painless and give fast recovery effects, the increasing incidence of antibacterial resistance as well as the numbers of side effects reported from antibiotics encourage scientists to search for alternative medicine which are safe and inexpensive. Given the recent scenario, natural sources are outstanding candidates for new antibacterial agents. The investigation of natural sources for their bioactive components is one of a continuing process in order to control the widespread of microbial infections.

The active compounds which can either inhibit the growth of pathogens or kill them and have little or no toxicity effect to host cells are considered potential for the development of new antibiotics [8]. Several studies have been conducted to investigate the potential of antibacterial substances isolated from plants. Flavanoids are known to possess antibacterial effect, in which it has the ability to form complex with extracellular, soluble proteins and

bacterial cell walls [9]. In the same manner, purified alkaloids as well as their synthetic derivatives are used as remedies for their various biological effects such as analgesic, antispasmodic and bactericidal [10]. Other sub-classes of compounds such as tannins, saponins, terpenes, and etc are abundantly reported to be significant for the treatment of various infectious diseases.

Previous study had revealed the isolated lipid from the aerial parts of *S. cannifolium* is stigmasterol [11]. Stigmasterol is classified under phytosterol in which is known to be effective in reducing the risk of cancer such as colon, breast and prostate cancer [12]. However, the fact remains to be unknown whether stigmasterol found in *S. cannifolium* has anticancer property. Previous study carried out in this lab demonstrated that *S. cannifolium* possesses antibacterial activity against gram positive bacteria. However, the exact compound has not been purified and identified yet. In the present study, preliminary purification and identification of bioactive compounds responsible for the antibacterial activity was conducted.

MATERIALS AND METHODS

Sample preparation and Pre-treatment

S. cannifolium leaves collected from Shah Alam Botanical Park were cleaned and dried in an oven at 45°C. The sample was then ground into powder form using electrical blender. The extraction of *S. cannifolium* leaves was performed by dissolving the powdered sample into ethyl acetate at 0.1 g/ml. The mixture was agitated at 300 rpm for 9.6 hours under incubated condition of 27°C [13]. The extract was collected by filtration and centrifuge at 4000 rpm for 10 minutes to separate sediments from the extract. The crude extract was obtained by evaporating the supernatant in water bath at 50°C.

Thin Layer Chromatography

Thin layer chromatography (TLC) was performed in order to select the best solvent which efficiently isolate and separate the antibacterial compounds from *S. cannifolium* extracts. In this study, five solvent systems were chosen as the mobile phase for thin layer chromatography analysis. The solvent systems involved are ethyl acetate with distilled water, acetone with ethyl acetate, ethanol with ethyl acetate, methanol with distilled water, and methanol with ethyl acetate. Each solvent system was prepared in gradient of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9. Single solvent system of each solvent was used as well in TLC analysis. The spots form on the TLC plates were visualized under ultra violet (UV) irradiation light, and retardation factor, Rf value, of each spot was measured.

Open Column Chromatography

In open column chromatography, ethyl acetate, hexane and ethanol are used as the mobile phase. The reverse phase chromatography is applied for the isolation of antibacterial compounds from 0.48 g of *S. cannifolium* extracts. Minimum volume of 50 percent hexane in ethyl acetate was used to dissolve the extracts. The 2.0 cm diameter column is packed with 15 cm silica gel, which was first suspended in hexane. The packed column is then introduced with the extracts at the front layer followed by small layer of cotton wool at the top to avoid the disruption of layer as well as mixing up with different eluent.

The solvent systems for mobile phase were prepared in gradient, from the single solvent of hexane, 60% hexane in ethyl acetate, 50% hexane in ethyl acetate, 40% hexane in ethyl acetate, 30% hexane in ethyl acetate, 20% hexane in ethyl acetate, 10% hexane in ethyl acetate, ethyl acetate only and 80% ethyl acetate in ethanol. Each solution was prepared in 100 ml to be used for elution in the column. The purification step is started with the elution in the column by 100ml of hexane, followed by 100ml of different ratio of hexane in ethyl acetate as mentioned above, and completed by 100ml of 80% ethyl acetate in ethanol.

The eluents were collected in series of 10 fractions for each solvent system. However, the final elution with 80% ethyl acetate in ethanol was collected into 20 fractions to provide a better separation in each fraction. The series of fractions were then concentrated by evaporation in water bath at 50°C. The crude of each fraction was dissolved in its solvent system at concentration of 0.1g/ml and followed by TLC analysis using ethyl acetate as the mobile phase.

After TLC analysis, fractions of each solvent system with similar retardation factor (Rf) value were combined as it assumed the same compound present in each collection. The antibacterial effect of the fractions/mixtures against the *Bacillus subtilis* growth was evaluated using disc diffusion assay.

High Performance Liquid Chromatography

To prepare sample for analysis using high performance liquid chromatography (HPLC), fraction that was identified with the highest antibacterial effect from open column chromatography were spotted on TLC plates and placed into the chamber containing ethyl acetate as the mobile phase. The spots developed were scrapped from the TLC plates

and dissolved in ethanol at 0.5 g/ml. The mixture was then centrifuged at 5000 rpm for 10 minutes and the supernatant was filtered using Whatmann no. 1 filter paper. The filtrate was then collected for HPLC analysis.

HPLC analysis was performed on reverse phase using Alltech Alltima HP, C18 (2.1 x 150mm). The injection volume was 10 µl, and the flow rate was set to 0.2 ml/min. The mobile phase used consists of two solvent mixtures which were 0.5% formic acid with 99.5% ethyl acetate and 0.5% formic acid, 94.5% ethyl acetate with 5% distilled water. The sample was run to be detected at UV wavelength of 280nm, 310 nm, 360 nm and 400nm.

RESULTS AND DISCUSSION

Thin Layer Chromatography of *S. cannifolium* extract

Before purification of antibacterial compounds from *S. cannifolium* was conducted using open column chromatography, investigation for the most appropriate solvent to be used as the mobile phase in the chromatography was carried out. The selection of a suitable solvent is essential for any reaction because it affects both chemical reactivity and reaction rates [14]. TLC plates were used to analyze several solvents to separate and isolate the antibacterial compounds from *S. cannifolium* extract. Numerous studies on antibacterial effect have also used TLC method to identify appropriate solvents for separation and isolation of major phytoconstituents. Hexane, ethyl acetate and ethanol were combined in increasing polarity to serve as mobile phase in TLC analysis of hexane and ethanol extract of *Parkia Biglobosa* and *Parkia Bicolor* leaves [15]. In other study, Kumar et al., (2010) [16] combined chloroform and methanol at ratio of (95:5) to perform TLC analysis of root and aerial parts of *Andrographis serpyllifolia* extract.

The present study had considered methanol, ethanol, ethyl acetate, acetone and distilled water developed in gradient as the mobile phase. Combination of these solvent mixtures with different ratio to change their polarity index result in numbers of spots presents on TLC plate with various Rf value (Table 1-5). Rf value is a measure of ratio of the distance moved by the compound from its origin to the movement of the solvent from the origin. A particular solvent is used as mobile phase to develop the movement of compound on a stationary phase such as silica on TLC plates. The polarity difference between compounds and solvent systems, make the compounds that have relatively high Rf values in polar solvents will have low Rf values in non-polar solvents [17].

The TLC spots were visualized by ultra violet (UV) irradiation at 254nm and 366 nm. The Rf value obtained are the representation of an average of three developed spots on TLC plates. In comparison with other solvents and solvent mixtures as mobile phase, only ethyl acetate had produced maximum of seven spots developed on the TLC plates (Table 1-3 and 5). Solvent mixture of 90% ethyl acetate in distilled water (Table 1), 90% ethyl acetate in acetone and 80% ethyl acetate in acetone (Table 2) were able to exhibit five spots on the developed TLC plate. While, four identical spots were obtained by developing the TLC plate in 80% ethyl acetate in distilled water (Table 1), 70% ethyl acetate in acetone, 60% ethyl acetate in acetone (Table 2), and 40 % ethyl acetate in ethanol (Table 3). The rest of the solvent and solvent mixtures were resulted to less than four spots visualized at different Rf values (Table 1-5). From the result obtained, it can be suggested that single solution of ethyl acetate is efficient to separate as many compositions or compounds from *S. cannifolium* extract.

Different polarity of solvent and solvent mixtures is responsible for the different spots adsorbed at different levels on TLC plates. Table 1-5 showed the variation of polarity index resulted from solvent and solvent mixtures of particular polarity differences. Polarity index (PI) is defined as the overall solvation capability for reactants and activated complexes as well as for molecules in the ground and excited states, which in turn depends on the action of all possible, specific and nonspecific, intermolecular forces between solvent and solute molecules, including Coulomb interactions between ions, directional interactions between dipoles, and inductive, dispersion, hydrogen-bonding, and charge transfer forces, as well as solvophobic interactions [18].

The maximum numbers of spots appeared on the developed TLC plates was obtained from the moderately polar solvent as the mobile phase (ethyl acetate, PI=4.4). Fewer spots were observed from other solvents of varying polarities. While in solvent mixtures with PI=9.04 and PI=9.62 (Table 1), the TLC plates showed no visible spots at all. Thus the mobile phase of combined methanol and distilled water with PI>9.0 were not conducted for developing the TLC plates (Table 5).

Through literature surveys, it was observed that different eluents of varying polarity exhibited varying fractions or spots for certain species of plants tested. It may be due to reason that several antibacterial compounds present in a specific plant might dissolved in a particular solvent at different levels and polarity. As reported in Dhiman et al., [19], the TLC plates developed with benzene: ethanol: ammonium hydroxide (BEA) (36:4:0.4), ethyl acetate: methanol: water (EMW) (40:5.4:4), chloroform: ethyl acetate: formic acid (CEF) (20:16:4) for methanolic extract

leaf of *Psidium guajava* L. had resulted to different bands separated. In non-polar solvent system BEA, there were 17 bands separated compared to 11 bands using CEF as an intermediate polar solvent system and 9 bands by more polar EMW solvent system. The present study revealed that ethyl acetate was the most effective to separate maximum spots compared to other solvent system, thus it was chosen to be employed in open column chromatography for the purification of antibacterial compounds from *S. cannifolium* extract.

Table 1: Rf value and polarity for mixture of ethyl acetate (EA) and distilled water (dH_2O)

	Retardation factor (Rf) value										
Solvents ratio	EA	9:1	8:2	7:3	6:4	1:1	4:6	3:7	2:8	1:9	dH_2O
	0.183	0.467	0.583	-	0.367	-	-	-	-	-	n.d
	0.333	0.567	0.700		0.533						
(EA:dH_2O)	0.400	0.700	0.800		0.583						
	0.500	0.883	0.967								
	0.617	0.967									
	0.850										
	0.967										
Polarity Index	4.4	4.98	5.56	6.14	6.72	7.3	6.12	8.46	9.04	9.62	10.2

Table 2: Rf value and polarity for mixture of acetone (Ace) and ethyl acetate (EA)

	Retardation factor (Rf) value										
Solvents ratio	Ace	9:1	8:2	7:3	6:4	1:1	4:6	3:7	2:8	1:9	EA
	0.350	0.533	0.550	0.608	0.600	0.717	0.400	0.333	0.317	0.233	0.183
(Ace: EA)	0.567	0.750	0.767	0.833		0.767	0.683	0.667	0.583	0.500	0.333
	0.833					0.933	0.750	0.750	0.700	0.667	0.400
							0.917	0.850	0.867	0.850	0.500
									0.933	0.933	0.617
											0.850
											0.967
Polarity Index	5.1	5.03	4.96	4.89	4.82	4.75	4.68	4.61	4.54	4.47	4.4

Table 3: Rf value and polarity for mixture of ethanol (EtOH) and ethyl acetate (EA)

	Retardation factor (Rf) value										
Solvents ratio	EtOH	9:1	8:2	7:3	6:4	1:1	4:6	3:7	2:8	1:9	EA
	n.d	0.150	0.150	0.667	0.133	-	-	0.700	0.817	0.717	0.183
(EtOH: EA)		0.683	0.650	0.750	0.667			0.933	0.950	0.800	0.333
		0.800	0.767	0.900	0.733					0.933	0.400
					0.850						0.500
											0.617
											0.850
											0.967
Polarity Index	4.3	4.31	4.32	4.33	4.34	4.35	4.36	4.37	4.38	4.39	4.4

Table 4: Rf value and polarity for mixture of methanol (Me) and distilled water (dH_2O)

	Retardation factor (Rf) value										
Solvents ratio	Me	9:1	8:2	7:3	6:4	1:1	4:6	3:7	2:8	1:9	dH_2O
(Me: dH_2O)	-	-		0.433					n.d	n.d	n.d
			0.583								
			0.733				0.667	0.667			
			0.858	0.850	0.800		0.800	0.783			
					0.908	0.950		0.867			
Polarity Index	5.1	5.61	6.12	6.63	7.14	7.65	8.16	8.67	9.18	9.69	10.2

Table 5: Rf value and polarity for mixture of methanol (Me) and ethyl acetate (EA)

	Retardation factor (Rf) value										
Solvents ratio	Me	9:1	8:2	7:3	6:4	1:1	4:6	3:7	2:8	1:9	EA
	n.d	0.500	0.500	0.483	0.583	0.633	0.583	0.600	0.567	0.483	0.183
(Me: EA)						0.775	0.650		0.667	0.600	0.333
							0.750		0.850	0.850	0.400
											0.500
											0.617
											0.850
											0.967
Polarity Index	5.1	5.03	4.96	4.89	4.82	4.75	4.68	4.61	4.54	4.47	4.4

Column Chromatography Analysis

Open column chromatography was carried out using glass column with dimension 20 x 200 mm. The type of chromatography employed in this study is liquid-solid chromatography where the solute adsorption is depends on silica gel as polar adsorbent [20]. In a study conducted by Espinoza et al., [21] column chromatography was employed by using the hexane-ethyl acetate gradient as the eluents for purification of *Idriella sp*. extract. A similar technique was implemented in the present study with a slight modification where ethanol was introduced in combination with ethyl acetate in the final elution in order to investigate the compounds present in a slightly more polar solvent system (Table 6).

Table 6 Polarity of eluents used in open column chromatography

Solvent	Polarity Index
Hexane and Ethyl acetate (3:7)	3.11
Hexane and Ethyl acetate (2:8)	3.54
Hexane and Ethyl acetate (1:9)	3.97
Ethyl acetate	4.4
Ethyl acetate and Ethanol (8:2)	4.38

TLC Analysis of Different Fractions Collected from Column Chromatography

Table 7 showed TLC analysis of different fractions (F) collected from open column chromatography which used different mobile phase (as shown in Table 6). The TLC plates were developed using only ethyl acetate as the mobile phase. It was shown that certain fractions (10ml per fraction) collected from different mobile phase demonstrated the presence of several identical compounds in the samples. The Rf value of those fractions are very close to each other, thus similar compounds are expected to present in all fractions isolated from (3:7), (2:8), (1:9) hexane: ethyl acetate, and single ethyl acetate as the mobile phase. Suleiman et al. [17] also suggested the same when they observed spots with almost similar Rf value from many fractions of column chromatography.

No spot was visualized on TLC plates developed by (3:7) hexane: ethyl acetate fractions, while three spots were presented by (2:8) hexane: ethyl acetate and two spots appeared from (1:9) hexane: ethyl acetate and single ethyl acetate. For combination of (8:2) ethyl acetate: ethanol, the collection of sample fraction was at 5ml per collection resulting to 20 fractions to be analyzed. This was carried out in order to increase the accuracy of sample separation and spots appearance on developed TLC plates. The observations of all fractions showed the presence of two spots for F1 to F10 and one spots for F11 to F20 under UV irradiation lights.

Table 7 TLC results for fractions (F) obtained from open column chromatograpy

Solvent Systems	Retardation factor (Rf) value									
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
Hexane and Ethyl acetate (3:7)	-	-	-	-	-	-	-	-	-	-
Hexane and Ethyl acetate (2:8)	0.583 0.708 0.883	0.583 0.708 0.867	0.567 0.675 0.833	0.583 0.733 0.892	0.567 0.683 0.850	0.567 0.700 0.867	0.467 0.700 0.892	0.500 0.667 0.867	0.567 0.708 0.867	0.517 0.700 0.875
Hexane and Ethyl acetate (1:9)	0.500 0.892	0.500 0.892	0.500 0.912	0.408 0.875	0.408 0.883	0.417 0.883	0.417 0.883	0.400 0.883	0.383 0.875	0.408 0.883
Ethyl acetate	0.500 0.883	0.500 0.867	0.412 0.883	0.400 0.875	0.392 0.858	0.400 0.883	0.392 0.858	0.383 0.833	0.400 0.875	0.383 0.867
Ethyl acetate and Ethanol (8:2)	F1 0.483 0.550	F2 0.550 0.600	F3 0.467 0.517	F4 0.517 0.600	F5 0.500 0.600	F6 0.500 0.600	F7 0.500 0.600	F8 0.500 0.600	F9 0.500 0.567	F10 0.500 0.600
	F11 0.550	F12 0.550	F13 0.550	F14 0.550	F15 0.550	F16 0.550	F17 0.550	F18 0.550	F19 0.533	F20 0.517

Evaluation of Antibacterial Activity of Isolated Fractions

Antibacterial disc diffusion assay was carried out to evaluate the antibacterial effect of fractions collected from open column separation technique. Table 8 showed average zone of inhibitions determined from selected sample fractions of different solvent or solvent mixtures systems. In Table 7, no spot were detected for all fractions of eluents, hexane: ethyl acetate (3:7), however, the antibacterial test of selected fractions (F4 and F5) showed an average of 11.0 mm zone of inhibition against *B. subtilis* growth. It is suspected that the fractions collected from hexane: ethyl acetate (3:7) eluent contain bioactive compounds which unable to be detected by UV irradiation light.

The column mobile phase of (2:8) hexane: ethyl acetate had demonstrated the presence of three spots for all fractions developed by TLC plates (Table 7). The spots are expected to be the same compounds in all the fractions because they have almost the same Rf value. Fractions (F4 and F5) of this eluent demonstrated an average inhibition zone of 11.25 mm. The mobile phase (1:9) hexane: ethyl acetate and single ethyl acetate fractions exhibited 2 spots of almost similar Rf values. The average zones of inhibitions for these 2 eluents are 8.5 and 8.0, respectively.

The sample collected from (2:8) ethyl acetate: ethanol showed different compositions (two spots) appeared in initial ten fractions (F1 to F10), while other component (one spot) in fractions F11 to F20. The antibacterial test was done separately according to number of spots presented. It was observed the fractions presenting only one spot had resulted to average inhibition zone of 11.0 mm, while the fractions containing two spots give inhibition zone of 13.0 mm. By comparing the average antibacterial effect of all sample collected, it was identified that the mobile phase (2:8) ethyl acetate: ethanol had potential to isolate the highest antibacterial compounds from *S. cannifolium* extract.

Table 8 Antibacterial activity of selected fractions from different eluents against *B.subtilis* growth

Solvent Systems	Fractions	No. of spots presented	Zone of Inhibition (mm)
Hexane and Ethyl acetate (3 :7)	F4 and F5	Cannot be observed	11.0±0.5
Hexane and Ethyl acetate (2 :8)	F4 and F5	3	11.5±0.5
Hexane and Ethyl acetate (1 :9)	F4 and F5	2	8.5±0.5
Ethyl acetate	F4 and F5	2	8.0±0.0
Ethyl acetate and Ethanol (8 :2)	F1 to F10	2	13.0±0.0
Ethyl acetate and Ethanol (8 :2)	F11 to F20	1	11.0±0.5

To confirm the efficacy of singular spot isolated from sample collection (F11 to F20) of mobile phase (8:2) ethyl acetate: ethanol, the antibacterial test was repeated using the spot sample adsorbed on the TLC plate. To isolate the compound contain in the spot from TLC plate, method according to Nalina & Rahim [22] with few modifications was carried out. The spots were scrapped out from the plate and dissolved in ethanol at concentration of 0.5g/ml. The sample was centrifuged at 4000 rpm for 10 minutes to remove the silica. The sample was filtered using Whatmann no. 1 filter paper before 10 µl of the supernatant tested for antibacterial effect. The result showed inhibition zone of 11.0 mm against *B. subtilis* growth (data is not shown).

In previous study, the purification of organic residue from buthanol extract of *Idriella sp.* by column chromatography was successful to isolate two compounds which were 5-hydroxymethyl-2-furaldehyde obtained from elution fraction of hexane: ethyl acetate (6:4) and 1-n-Butyl-ß-D-fructopyranoside from fractions eluted with ethyl acetate. Both compounds were effective towards plant pathogenic bacteria such as *X. axonopodis, P. carotovorum subs. atrosepticum, P. crhysanthemi* and *E. amylovora* [21]. For the present study, it was predicted that the most active compound present in fraction 11 to fraction 20 (F11-F20) of ethyl acetate: ethanol (8:2) eluent as a single spot showed zone of inhibition of 11.0 mm against *B. subtilis*. The fraction (F1-F10) of this solvent system which had better antibacterial effect was believed to possess synergistic effect between the compounds present in two different spots. An example of previous study on the isolation of two antibacterial compounds had revealed that the MIC value of combined compounds was much better than an individual compound against several bacteria such as *S. aureus, L. monocytogenes* and *E. coli* [23]. Therefore, the sample fraction (F11-F20) which expected to have a single isolated compound is selected to be analyzed using HPLC.

High Performance Liquid Chromatography (HPLC)

The most commonly used technique for qualitative and quantitative determination and separation is known as HPLC. It is a non-destructive technique which can be applied for thermally labile compounds. Besides, it provides a high sensitivity technique as numerous method of detection can be implemented [23]. Reverse phase chromatography mode was chosen as it is an extremely versatile and highly capable of separating the wide range of compounds soluble in solvents from chloroform to water [24, 25]. The mobile phase can be developed by appropriately combined the solvent of which the sample is very soluble and another solvent in which the sample is less soluble. The retention time and separation can be determined at the equilibrium of the solutes in the mobile phase related to the bonded phase [26].

The mobile phase of choice for this study consists of two solvent mixtures which were 0.5% formic acid with 99.5% ethyl acetate and 0.5% formic acid, 94.5% ethyl acetate with 5% distilled water. The sample prepared from

individual spot of fraction (F11 to F20) by (8:2) ethyl acetate: ethanol eluent which showed potential antibacterial activity against *B. subtilis* was chosen for HPLC analyses. The 10 µl of sample injection in HPLC was detected at various wavelengths (280 nm, 310 nm, 380 nm and 400 nm) and the results are shown in the chromatogram below (Figure 1).

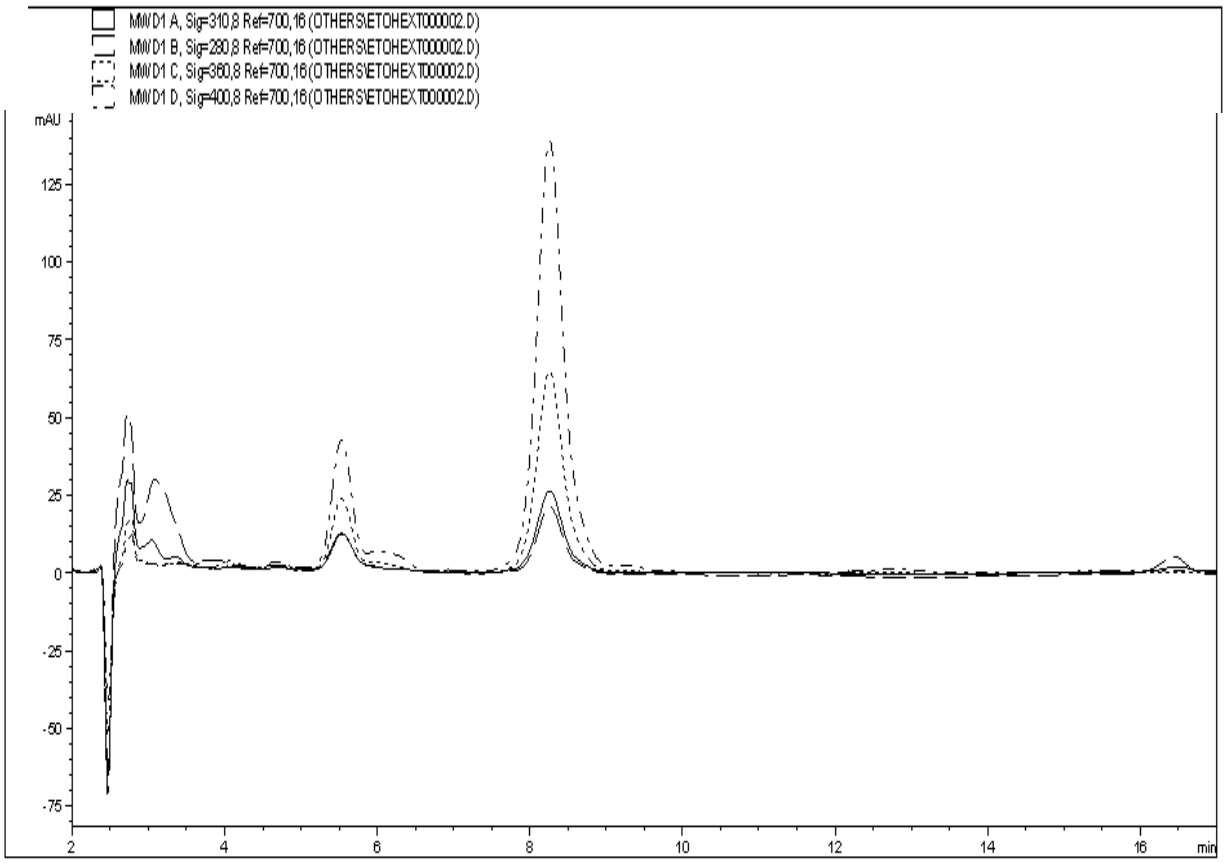

Figure 1: Chromatogram detected at various wavelength

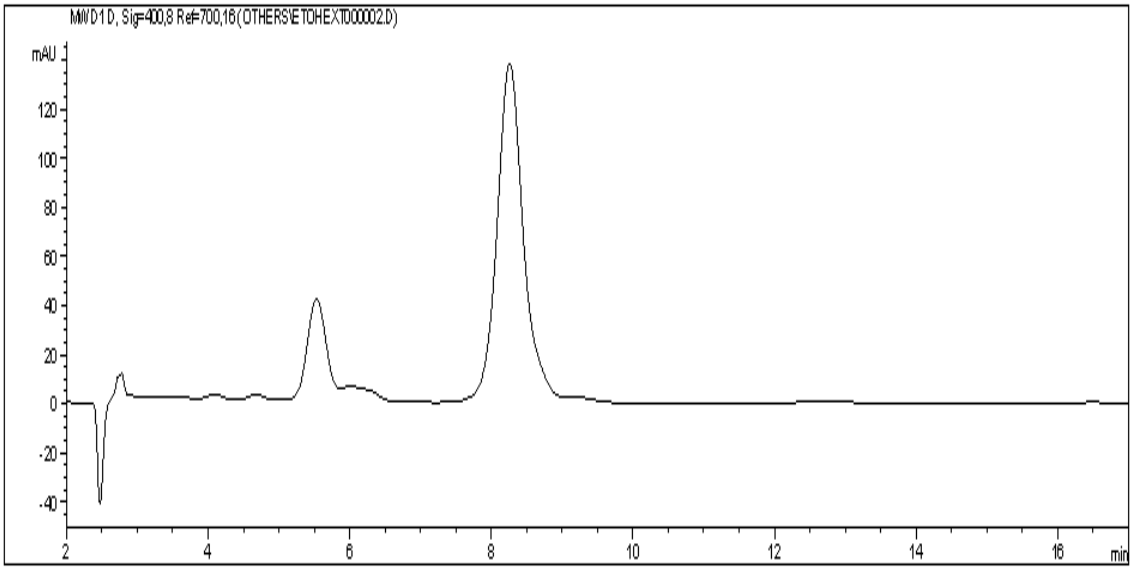

Figure 2: Chromatogram detected at 400nm wavelength

The chromatogram showed two isolated compounds at retention time of 5.53 minutes and 8.26 minutes. Both two isolated compounds were detected at 360 nm and 400 nm (Figure 1 and 2) and barely detected at 310 nm and 280 nm. It is suspected the compounds present are flavanoids based on many studies which reveal the flavanoid group of compounds is detected within these range. In a study conducted by Gattuso et al., [27] the classes of flavanoids compounds were determined at wavelength ranged within 280 nm to 385 nm. In different study, flavanoids can be best detected at 350 nm [28]. The study also reported that 280 nm wavelength was appropriate for simultaneous vitamin-polyphenol-pigment detection, while 450 nm was suitable for carotenoids, chlorophylls and anthocyanins. The usage of HPLC in this study had improved the separation method adopted in column chromatography for antibacterial compounds of *S. cannifolium* extract as discussed above. Confirmation with mass spectrometry and NMR will identify the identity of the purified compounds which can be carried out in the future.

CONCLUSION

Purification of the most active compounds from *S. cannifolium* extracts was able to be developed using (8:2) ethyl acetate: ethanol as mobile phase in open column chromatography. The fractions showing identical antibacterial effects was subjected to HPLC analysis and resulted to isolation of two compounds at retention time of 5.53 minutes and 8.26 minutes. The compounds were suspected as flavanoids as they can be detected at wavelength 360 nm and 400 nm.

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