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ORTHOGONALITY OF SEPARATION IN TWO DIMENSIONAL CHROMATOGRAPHY OF *PLECTRANTHUS AMBOINICUS L.* EXTRACT

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ABSTRACT

Plectranthus amboinicus which from family Lamiacae and genus Plectranthus is traditionally used for the treatment of coughs, sore throat, asthma and disease affected by virus and bacteria. This plant is reported to have many biological activity such antiepileptic, anti-mutagenic, anti-inflammatory, anti-fungal and anti-tumor activity. Major chemical of this plants are carvacrol, thymol, a-terpineol, caryophylene oxide and B-seline. Although the plant showed an interesting bioassay, on the other hand, the extract was extremely complex for further purification. Thus the purpose of this research is to develop method to purify each of the active components. Two dimensional chromatography which refers to different selectivity between separation can improved the purification throughput complex mixtures. The plant was first extracted and then proceeds with column chromatography. By using column chromatography this plant was fractionated into 10 fractions by using methanol; methanol:chloroform and chloroform as mobile phase. Then, antioxidant activity was carried out for each of the fractions and extract using 1,1-diphenyl-2-picrylhydrazyl (DPPH) reagent. Finally all the fractions and extract were screening using Thin Layer Chromatography (TLC). The antioxidant activity of some fractions showed greater than the antioxidant activity of the extract. From the thin layer chromatography and HPLC, we found

that they were possibly seven compounds. Three of them in the area of highly polar, two in the area of moderate polar and two of them in the area of low polar.

KEYWORDS: *Plectranthus amboinicus*, two dimensional chromatography, fractionation, antioxidant activity, DPPH, methanol extract, column chromatography and thin layer chromatography.

INTRODUCTION

Historically, new drugs are derived from microorganism, chemical synthesis and also from plants. Plants are naturally gifted at the synthesis of medicinal compound because of presence of the phytochemical constituent.[1] Phytochemicals are chemical compounds that occur naturally in plants. There are many different phytochemical in plants that have pharmacological effect to treat diseases. New drugs with high therapeutic value can be produced by extraction of active compound in medicinal plants.[2]

Plectranthus amboinicus or its commercial name Indian Borage is from family *Lamiacae* and genus *Plectranthus*. It is a tender fleshy perennial plant with an oregano-like flavor and odor. The stem is very fleshy with long rigid hairs or soft, short and erect hairs. The leaves are undivided, broad, oval shaped with a tapering tip. The taste of this leaf is pleasantly aromatic with refreshing odour. Traditionally, this leaves are used for the treatment of coughs, sore throat, used to cure asthma and diseases affected by virus and bacteria.[3] Many biological activity such as antiepileptic, antimutagenic[4], antifungal activity[5], anti-inflammatory and anti-tumor activity [6] and also anti-malarial activities[7] have been reported for the leaves of *P.amboinicus.* Praveena Bhatt (2013) state that *P.ambonicus* contain phenolics, flavonoid, proanthocyanidins, phenolic acids have been associated with antioxidant, antibacterial, anticancerous and antiplatelet activities. While, major chemical compound of this plant which are carvacrol, thymol, a-terpineol, caryophylene oxide and B-selinene are believe contribute to against malarial vector Anopheles stephensi.[8]

Phytochemical analysis have important role in searching raw materials and resources for pharmaceutical industry. This techniques are helpful to find and locate chemical constituent which are source of pharmacological active principles. Phytochemical analysis is use to identify, separate, isolate and purify varies active compound presents in plants.[9] Two dimensional chromatography is combining two independent chromatographic separation. Two dimensional separation is an effective method for simplifying complex samples. By

using this separation the highest possible peak capacity can be achieved for chromatography separation. Two dimensional liquid chromatography performance depends on the peak capacity in both chromatographic and separation orthogonality.[10]

METHODS

Study design

The objective of this research was to develop a method for fractionation of *Plectranthus amboinicus* by using two dimensional chromatography.

In this research *Plectranthus amboinicus* plant was selected and extracted. Then, the extraction was fractionated into ten fractions by using open column chromatography. Next, the fractions were further applied to thin layer chromatography for screening. The research was carried out in six phases.

Phase 1: Collection and Identification of Plant

The plant were collected from MAHA (Malaysia Agriculture, Horticulture Association) exhibition in Serdang (Figure 1). Then, the plant was sent for identification to the Institute of Bioscience, University Putra Malaysia.

Figure 1: Collection of the *Plectranthus amboinicus* plant

Phase 2: Preparation of plant for extraction

The leaves of *Plectranthus amboinicus* were first washed under running tap water followed by sterile distilled water. Then the leaves were dried in the oven at 50% until all the water molecules evaporated and all the leaves became well dried for grinding. The leaves were grinded using mechanical blender into fine powder and transferred into airtight container.[11] Refer Figure 2.

Phase 3: **Extraction of the leaves**

The dried finely powdered leaves was macerated in 450mL methanol for 24 hours. Then, the soaked powdered leaves were filtered and poured into round bottomed flask and put in the rotary evaporator until the solvent get evaporated.[12] A pure quality of semi-solid, plant extract was obtained by using this process. After that, the extraction was placed in a beaker and weighed. The dried extract will be keep in refrigerator at 4'C for future use (Figure 3).

(a) Drying of the leaves by an oven at 50°C (b) The dried leaves ready for grinding

Figure 2: Preparation of the plant (a) & (b)

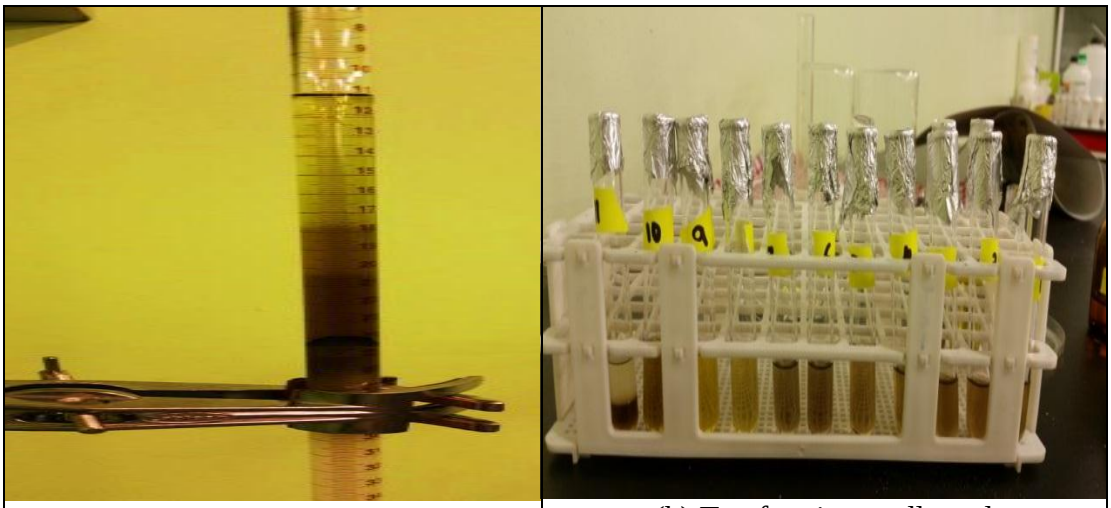
(a) Evaporation using rotary evaporator (b) The pure dried extract obtained

Figure 3: Extraction of the leaves (a) & (b)

Phase 4: **Fractionation using column chromatography**

The crude extract of *Plectranthus amboinicus* was dissolved in DMSO and diluted in distilled water. The dissolved extract was applied on a column (50mL) packed with silica gel and methanol using the wet slurry method.[13] The solution of silica gel with methanol were prepared in a beaker subsequently adding onto the column till it is about half-filled. The side of the column was tapped for a few times to make sure the silica gel was packed in the column and to prevent trapping of air bubbles. Then, the pinch clamp was opened to drain the excess methanol until the level of methanol was just above the top layer of silica gel. 1 cm of sand was added to the top of the silica gel to maintain the silica gel as stationary phase. The

extract was added onto the column on top of the sand. A substantial amount of methanol : chloroform were poured continuously onto the column and allowed to drain but prevented from reaching below the sand level. The elute was collected based on the colour bands. Ten fractions were collected in the test tubes and placed in freezer for further use[14] (Refer Figure 4).



(a) Open column chromatography | (b) Ten fractions collected

Figure 4: Fractionation using column chromatography (a) & (b)

Phase 5: **Antioxidant test of the crude extract and the fractions**

Each of the crude extract and the dried fractions were weighed dissolved to make up a stock solutions of 1g of extract to 10mL of methanol. The DPPH solution was also prepared by dissolving 6mg of DPPH in 100mL of methanol. After that, 1mL of each extract was added into the test tubes containing 2mL of freshly prepared DPPH solution (Figure 5). The mixtures were shaken vigorously and were left to stand in the dark for 30 minutes. The absorbance of the resulting solution was measures by using a spectrophotometer at absorbance of 517nm. The scavenging activity of each extract on DPPH radical was calculated using the follow equation.[15]

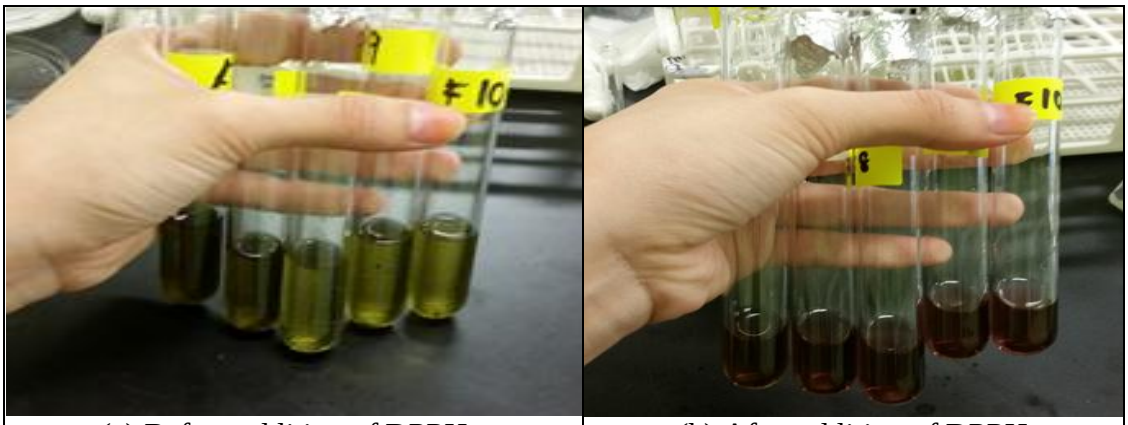
$$\text{Scavenging activity (\%)} = \left(\frac{1 - absorbance\ sample}{absorbance\ control}\right) \text{x } 100$$

Phase 6: **Screening using TLC**

Each fraction of *Plectranthus amboinicus* was dropped on TLC paper and the plant extract was used as standard. The TLC plate were then place in beakers with series percentage of methanol:chloroform were used as mobile phase. The components of the sample were

separated according to their partitioning between the mobile and stationary phases. Each of the coloured spots were marked and Rf value was calculated using the follow equation.

$$Rf = \frac{\text{distance moved by the compound}}{\text{distance moved by the solvent}}$$ [19]



(a) Before addition of DPPH (b) After addition of DPPH

Figure 5: Antioxidant activity (a) & (b)

RESULTS & DISCUSSION

1. Plant identification

The identification of the plant was sent to Institute Biosains, University Putra Malaysia, and the sample was confirmed and identified. The identification of the plant is shows in the table1.

Table 1: Identification of the plant

Scientific Name	Local Name	Family Name
Plectranthus amboinicus	*Bangun-bangun*	Lamiaceae

2. Extraction percentage yield of the plant

The plant was extracted with methanol and later evaporated by rotary evaporator. The calculation of the extraction yield is the weight percentage of the crude extract to the raw material. The percentage yield is calculated using the following formula.[16]

$$\text{Percentage of the yield \%} = \left(\frac{\text{Amount of extract yield (g)}}{\text{Amount of dried plant used (g)}}\right) \times 100$$

The result was summarized in table 2 that shows percentage yield of Plectranthus amboinicus extraction is 20%.

Table 2: Percentage of extraction of *Plectranthus amboinicus*

Amount of fresh leaves (g)	Amount of extract yield (g)	Percentage of yield (%)
1500	300	20%

3. Open Column Chromatography

Open column chromatography used to determine and identify number of component in the mixture and also separate and collect each of the component individually based on colour bands.[16] 250g of crude extract was fractionated into 10 fractions. 151g is the total fractions weight. The percentage of each fraction were summarized in table 3. Based on table 3 fraction 2 shows the highest percentage of fraction which is 10.80% while fraction 6 shows the lowest percentage with 2%.

4. Antioxidant activity using DPPH

Each of the fraction and extraction were test for antioxidant using DPPH. Vitamin E was used as the control. The result for the antioxidant shows in figure 6.

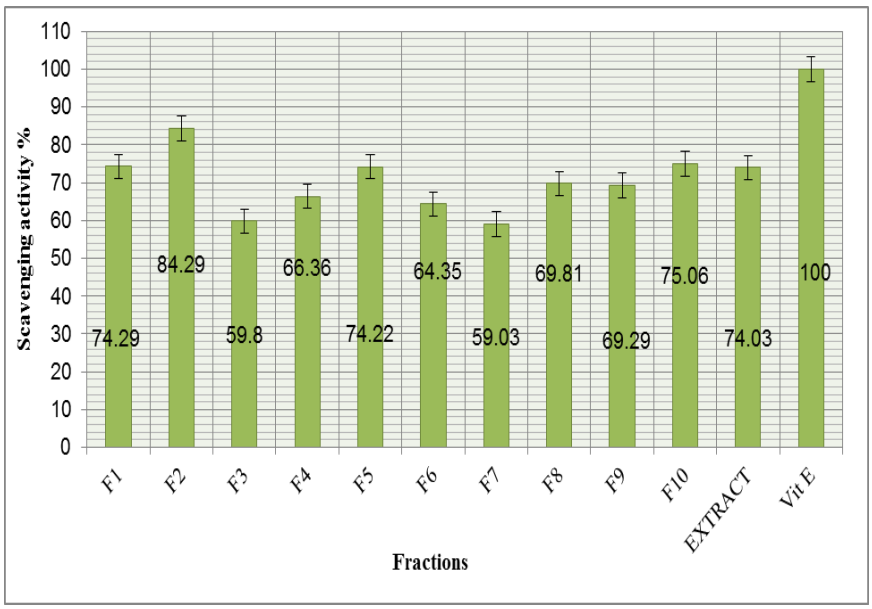


Figure 6: Antioxidant activity of extract and fractions

Table 3: Percentage of each fraction

Fraction	Weight (g)	% of the fraction
Fraction 1	15	9.93
Fraction 2	27	17.88
Fraction 3	11	7.28
Fraction 4	25	16.56
Fraction 5	14	9.27
Fraction 6	5	3.31
Fraction 7	11	7.28
Fraction 8	13	8.60
Fraction 9	11	7.28
Fraction 10	19	12.58

Based on figure 6, antioxidant activity of the crude extract is 74.03. However, some of the fractions showed better antioxidant activity than the crude extract such as fraction 1 with 74.29, fraction 5 with 74.22, fraction 10 with 75.06 and fraction 2 shows the highest antioxidant with 84.29.

5. Thin Layer Chromatography

Thin layer chromatography used to see the separation of the component in the extraction and the fractions. Each of the colour spot were marked and refractive index (rf) value were calculated and summarized in table 4.

Table 4: *Rf* value for each components of the extract and fractions

Fractions	1	2	3	4	5	6	7
Extract	0.50	0.68	0.75	0.805	0.845	0.88	0.95
Fraction 1						0.88	0.95
Fraction 2						0.88	0.95
Fraction 3				0.805			0.95
Fraction 4							0.95
Fraction 5				0.805			0.95
Fraction 6				0.805			0.95
Fraction 7				0.805			
Fraction 8				0.805			
Fraction 9			0.75				
Fraction 10			0.75				

Based on table 4, the extract showed about seven colour spots in different fractions, this shows the extract possibly contains about seven compounds. Each of the fractions however showed only one to two compounds. The fractions with only one compound are pure compound while the fractions with two compounds are not very pure which may need another purification. About three compounds in this plants are in the area of highly polar, two components in the area of moderate polar and two of them are in the area low polar. This result was compared to HPLC chromatogram of crude extract of *Plectranthus amboinicus* from literature (Praveena Bhatt et al, 2013) and we found that it matched the result where it shows seven to eight peaks which possibly means they contain about seven to eight compounds. High peak was showed in the area of highly polar and lower peak was showed in the area of moderate polar and no peaks in the area of low polar as seen in figure 7.

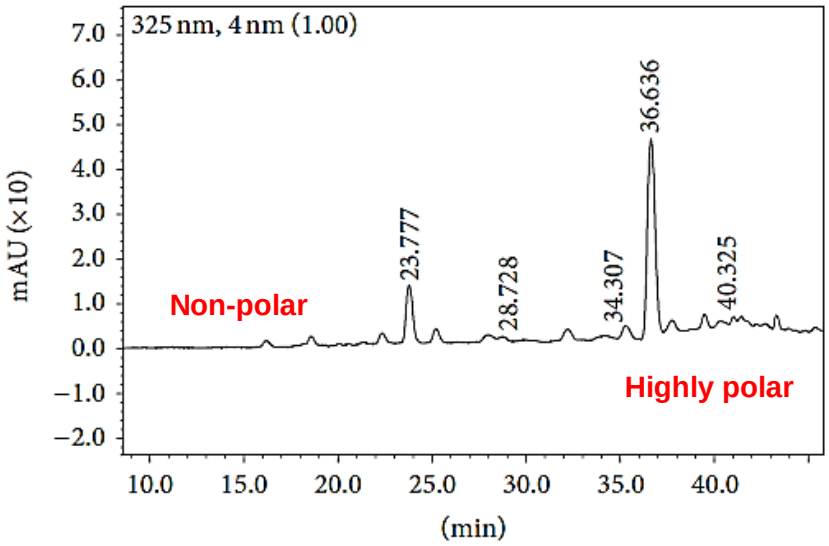


Figure 7: HPLC chromatogram of crude extract of *Plectranthus amboinicus*[4]

Phytochemical analysis of *Plectranthus amboinicus* from literature 'Analysis of Phytochemical Constituents and Anti-microbial activity of some medicinal plants in Tamilnadu, India' also support our finding that many phytochemicals of highly polar such as carbohydrate, phenol, sterol and flavonoid were presence and non-polar components such as steroid were absence. This matched the results obtained by using the thin layer chromatography where most of the fractions contain highly polar components and no non-polar components (Refer table 6).

Table 6: Phytochemical analysis of Plectrantus amboinicus crude extract[18]

NAME OF TEST	METHANOL EXTRACT	ACETONE EXTRACT
Saponins	+	+
Pholabatannins	+	+
Resins	+	+
Lipids or fat	+	+
Steroids	-	-
Glycosides	+	+
Acidic compounds	-	-
Terpenoids	+	+
Reducing sugars	+	+
Phenols	+	+
Carbohydrates	+	+
Antraquinione	+	+
Catachol	+	+
Sterols	+	+
Flavonoids	+	+
(+) = PRESENCE	**(-) = ABSENCE**	

CONCLUSION

Two dimensional chromatography refers to different selectivity between separations. Combining two dimensional separation modes in one single system can improves purification throughput complex mixtures. The extract fractionated into ten fractions and each of the fractions studied in much more detail. We found that the antioxidant activity of some fractions were greater than the antioxidant activity of the extract. From the thin layer chromatography and HPLC results, we found that they were possibly seven compounds.[20] Three of them were in the area of highly polar, two of them in the area of moderate polar and two of them in the area of low polar. Phytochemical analysis from the literature found to be matching with our results where there is no steroid found in this extract and acidic compound which made the extract highly non-polar.

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