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EXTRACTION OF CRUDE ESSENTIAL FROM SENNA ALATA (POKOK GELENGGANG)

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Abstract

*Sennaalata*was one of the herbs plants that can be used to treat the ringworm in traditional method. In this study, the *sennaalata* was scientifically tested to prove the traditional medicinal used. Six forms of extract were extracted by using soxhlet apparatus and continued with the appropriate methods that can extract the chemical compound of anthraquinone. The six forms of extraction included anthraquinoneaglycone, anthraquinone glycoside, anthraquinoneaglycone from glycoside, anthraquinone from crude absolute ethanol, anthraqunone from crude *n*-hexane and anthraquinone from crude ethanol 70%. The extraction of six forms of anthraquinone extract as antifungal was observed by tested with two types of fungi; *Tricophytonrubrum* and *Microsporumgypseum*. The extraction from crude absolute ethanol and crude *n*-hexane to inhibit these fungi were compared to determine which type of solvent was the best to use for inhibition. This test was implemented by using well-diffusion and broth microdilution method to determine the minimum inhibitory concentration (MIC). The present of six forms of anthraquinone was examined by using Thin-Layer Chromatography (TLC).

Keywords: *Anthraquinone; Dermatophytes fungi; TLC (Thin Layer Chromathography); Sennaalata; well diffusion method; broth micro-dilution method.*

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1.0 Introduction

Sennaalata (L.)Roxb or cassia alataL. was a medicinal plant in the family Fabaceae which has been known in Thai language as Chumhetthet. The English names were Candelabra bush and Ringworm bush. At South Africa it was native plant and can be found widely in tropical region, up to 1500 m. In Indonesia, Philippines and Thailand, this plant can be found all over the countries, sometimes cultivated for medicinal purposes [1].Sennaalata has a flower which the flower can spikes look like golden candles when covered with unopened flower buds. The tree can reach a height of 10 to 15 feet [2]. The leaves of sennaalata contain a lot of chemical component such as phenols, tannis, saponins, alkaloids, steroids, flavonoids and carbohydrates. These chemical compounds were efficient in antimicrobial and antifungal activity. Sennaalata or Cassia alataalso has been reported to contain anthraquinone[3].Other part of Sennaalatasuch as roots, fruits and stem also consists of anthraquinone [4].Sennaalata is one of the plenty of plants that can treat ringworm which is easily spread to others just by touching [5]. Previous research showed that there were plenty of chemical compound presences in Sennaalatasuch tannis, flavonoid, phenol, alkaloids and steroid. From the phytochemical screening most of the active chromatography compound is flavonoid. However, there is no evidence that flavonoid is a main constituents of leaf extract [3]. Meanwhile, another previous research was determined the chemical compound that presence in the leaves that can react as antifungal is anthraquinone[6].

In previous research crude extract of ethanol was better than methanol use in extracting the chemical compound presence in the leaves and n-hexane was a good solvent in order to extract the chemical compound of leaves plants too [7]. Hence, in this experiment two types of solvents; absolute ethanol and n-hexane were used to determine which type of solvent was the best solvent can be used to extract anthraquinone

In this study, the main objectives of this research were to study the activity of antifungal by difference forms of anthraquinones extraction through well diffusion method and broth micro-dilution method. Besides to compare the activity of antifungal extracted from crude n-hexane and crude absolute ethanol.

2.0 Materials and methods

2.1 Preparations of plants

Sennaalata was obtained at the resident area KangarPulai, Johor Bahru. The leaves part was used in order to extract the chemical compound. The leaves were dried under the sun for about 5 days. Then the leaves were blended until it can be passing through to the sieve mesh with size 0.5 mm. The smaller the size of the leaves, the higher the amount of chemical compound can be obtained due to have large surface area and increased solubility throughout the extraction process [8].After that, 20 g leaves of Sennaalata were weight to be extracted in each form of extract.

2.2 Inoculum preparation

Pure isolated strains of test organisms were sub-cultured on SDA until full growth with proper characteristics [6]. Cultural characteristic was observed after incubated at 26oC- 30oC for 7 to 14 days. The two types of fungi that were sub-culture wasTricophytonrubrum and Microsporium gypseum.

2.3 Extraction of anthraquinoneaglycone and anthraquinone glycoside extracts

The extraction was done from 8 hour reflux of the aqueous part of 20 grams of S. alataleaf powder and 300 mL water; the aqueous extract was adjusted to pH 4 with 2M hydrochloric acid (0.3 mL), and later extracted with 200 mL of chloroform. The collected extracts from chloroform layer was evaporated to yield anthraquinoneaglycone extract while the aqueous layer was added with 1.392 g of sodium bicarbonate to adjust to neutral pH. The solution was centrifuged at 4000 rpm for 20 min.

The supernatant which contained anthraquinone glycoside was evaporated to dryness to give anthraquinone glycosides extract. It was evaporated by using rotary evaporator at 45oC with 60 rpm.

2.4 Extraction of anthraquinoneaglycone from glycosidic fraction

The extraction was done similar to the process of anthraquinone glycosides extract except for evaporation to dryness. Ferric chloride hexahydrate solution (50 mL) was added to the anthraquinone glycosides extract, and then refluxed for 30 min. The solution was added with concentrated HCl (0.1 M), 15 mL and refluxed for another 30 min and filtered after cool down. The filtrate was shaken with 200 mL of chloroform in a separating funnel and separated the chloroform layer, combined and washed with 200 mL of distilled water, then evaporated to dryness to yield anthraquinoneaglycone from glycosidic fraction. In rotary evaporator the temperature used was 25oC with 45 rpm.

2.5 Extraction of anthraquinoneaglycone from crude ethanol 70% extract

S. alataleaf powder 20 g was extracted with 300 mL of 70% ethanol using a soxhlet apparatus. A portion of the crude ethanol extract was further extracted by adding 170 mL of water and 30 mL of 70% ethanol. After 10% of the solution was decrease ferric chloride hexahydrate solution which was about 50 mL was added, the mixture was refluxed for 30 min before adding 20 mL concentrated hydrochloric acid (0.1M) and refluxed for another 30 min. When the mixture was cooled down, it was filtered and the filtrate was extracted with 200 mL of chloroform.

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The chloroform layer was combined and washed with distilled water (200mL), then evaporated to dryness to yield anthraquinoneaglycone from crude ethanol 70% extract. The temperature used was 25oC with 45 rpm.

2.6 Extraction of crude absolute ethanol extract

S. alataleaf powder 20 g was extracted with 300 mL of absolute ethanol. Then, the solution was evaporated at rotary evaporator with the temperature 45oC- 50OC and 55 rpm.

2.7Extraction of crude *n*-hexane extract.

S. alataleaf powder 20 g was extracted with 300 mL of n-hexane. Then, the solution was evaporated at rotary evaporator with the temperature 45oC- 50OC and 55 rpm.

2.8Well diffusion method

Average zone diameter was done by using agar well diffusion. The size of the well use was 0.7 cm of the diameter. 1mL from each of six forms of the extracts was diluted with 9mL of Dimethyl Sulfoxide (DMSO4). The fungi that were growth with the characteristic were prepared using distilled water. 80 µl of inoculum was inoculated into each of saboraoud dextrose agar plate surface layer. The agar make up for three well diffusions for three different types of extracts concentration. Three different concentrations of extract were used which were 50 µl, 80 µl and 100 µl.

The agar was incubated for 24 hours at 26oC – 30oC. After 24 hours the results for the zone of inhibition area was taken. Ketoconozole was act as a positive control while distilled water used as a negative control. The way to calculate the zone of inhibition is by place the ruler under the dish and measure the diameter of the zone of inhibition. The zone of inhibition was measure to the nearest millimetre. If the fungal growth is not inhibited around the well area, the zone of inhibition is the diameter of the well area. If it is measured in 1 cm means it is equal with 10 mm.

2.9Broth micro-dilution method

In Minimum Inhibitory Concentration (MIC) six forms of extracts were serial dilution with Sabouraud Dextrose Broth prior inoculated into the microtiter plate wells. The dilutions that prepared were 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.0625 mg/mL, 0.03125 mg/mL and 0.0156 mg/mL. After that, 20 µl of suspended inoculum is inoculated into each of the dilution tubes. The control will be growth control (fungus and broth), control (broth only).

To identify either the solution of Dimethyl Sulfoxide, (DMSO4) contain of antifungal agent, it also tested in MIC reading which was diluted 1 mL of DMSO4 into 9 mL of SDB and inoculated with 20 µl of fungus. Then, all the solution prepared was inoculated into the wells with 200 µl. According to Kaya, et al., (2012) prior the plate is incubated, the reading of OD is taken to ease calculate the percentage of inhibition. After that, the plate was incubated for 24 hours then OD reading was taken again.

This formula is to obtain the percentage of inhibition is as followed [9]:

$$\left(1 - \frac{OD\ (\ test\ \ well\ )}{OD\ (Untreated\ \ \ control\ \ well\ )} \text{x } 100\right) \qquad \text{equation 1}$$

3.0 Results and Discussion

3.1 Determination of active compound

Determintion for active compound in sennaalatawas by using Thin Layer Chromatography (TLC) plate. Thin layer chromatography was used to detect the presence of anthraquinone in each of the extract form. The size of the thin layer aluminium plate was 7cm x 10 cm. The presence of anthraquinone in each form of the extract on the plate, was by spraying with 10% methanolic KOH. The presence of anthraquinone will turn the extract form to red-pink. From figure 10, the presence of anthraquinone compound was detected at the TLC plate. After spraying with methanolic 10% KOH the presence of chemical compound, anthraquinone can be determine by red-pink colour on the plate [10] [11].The yellow orange colour was also detected to attribute antraquinone formation[11]. It also showed that all form of extracts have the presence of antraquinone

The hRf values were obtained by using this formula [12] :

$$\text{hRf} = \frac{\text{Distance travelled by solute from origin}}{\text{Distance travelled by solvent from origin}} \qquad \text{equation 2}$$

From table 1 the hRf value for extract form of anthraquinoneaglycone was 24. For anthraquinoneaglycone from glycoside was 16. For anthraquinone glycoside was 16. For anthraquinone from crude ethanol 70% was 15. For anthraquinone from absolute ethanol were 33 and for anthraquinone from crude n-hexane were 28. The hRf values of main components were determine by comparing with the hRf value of anthraquinone standard [10]. The standard of hRf value for Rhein is 15 (hRf =15), Aloe-emodin (hRf = 23.3) and Emodin (hRf = 38.3). From the standard hRf value for anthraquinone, we can determine that the hRf value for all forms of extract were near to the standard anthraquinonehRf value.Anthraquinoneaglycone (hRf = 24) near to standard Aloe-emodin. Anthraquinoneaglycone from glycoside (hRf = 16), Anthraquinone glycoside (hRf = 14) and anthraquinone from crude ethano 70% (hRf = 15) near to standard Rhein (hRf = 15). For anthraquinone from absolute ethanol (hRf = 33) near to standard Emodin (hRf = 38.3) and for anthraquinone from crude n-hexane was (hRf = 28) near to standard Aloe-emodin (hRf = 23.3). From the comparison can be detected that the dot red-pink colour was exactly the anthraquinone chemical compound presence in the extraction form of antraquinone.

3.2 Zone of inhibition

Table 2 shows the zone of inhibition of two types of dermatophyte fungi which were Tricophytonrubrum and Microsporumgypseum. The effectiveness of each form of extract was tested by using well diffusion method. In well-diffusion method it can determine the average of zone of inhibition of each extracts. For extract A, which was anthraquinoneaglycone, Tricophytonrubrumshows better respond to this extract compare M.gypseumwith 1.47 cm zone of inhibitonat 80 µg/mL. For M.gypseum the best zone of inhibition for extracts A was at 100 µg/mL with 1.8 cm zone of inhibitions.

For extract B, which was anthraquinone glycoside, generally M.gypseum more respond to this extract. The large size of zone inhibition at extract B was 1.6 cm at 100 µg/mL. For T.rubrum the best size of zone of inhibiton at extract B was 1.2 cm at 100 µg/mL too. For extract C,which was anthraquinoneaglycone from glycoside, T.rubrum more respond to this extract compare M.gypseum. The size of zone of inhibition was large at 100 µg/mL for both of these fungi which were 1.5 cm and 1.43 cm.

In extract D, which was anthraquinone from absolute ethanol,T.rubrumalso shows better respond to this extract compare M.gypseum. The size of zone of inhibition was large at 80 µg/mL with 1.73 cm. While for M.gypseum the size of zone of inhibition was large at 100 µg/mL of the extract which was 1.06 cm. For extract E, which was anthraquinone from n-hexane, M.gypseum shows better response towards this extract. Both fungi show large size of inhibition at 100 µg/mL of this extract which was 1.43 cm from M.gypseum and 1.37 cm from T.rubrum.

In the extraction of anthraquinoneaglycone from crude ethanol 70%, with F labelled, T.rubrum shows the size of zone of inhibition was large at 100 µg/mL with 1.57 cm and M.gypseum was at 1.43 cm. Hence, from all forms of extract of antraquinone, the size of zone of inhibition was the best at 100 µg/mL except for antraquinone from crude absolute ethanol which show best zone of inhibition area at concentration 80 µg/mL. The largest size of zone of inhibition was extraction from anthraquinoneaglycone with 1.8 cm diameter at 100 µg/mL towards M.gypseum.

Table 3 shows the comparison activity of antifungal between crude absolute ethanol and crude n-hexane. At concentration 50 µg/mL the zone inhibition area crude n-hexane was larger than absolute ethanol which was 1.16 cm and 1.17 cm. At concentration 80 µg/mL the zone inhibition area for absolute ethanol was larger than crude n-hexane (1.73 cm) which for T.rubrum but for M.gypseumn-hexane shows greater size of inhibition area (1.23 cm). At 100 µg/mL the zone of inhibition area for n-hexane was larger than absolute ethanol which was 1.43 cm and 1.37 cm.

From this result, it can conclude that the activity of antifungal between absolute ethanol and crude n-hexane was dependent on the concentration extract used and currently seem that the extract from crude n-hexane show better antifungal activity at 50 µg/mL and 100 µg/mL compare to extract from crude absolute ethanol. At 80 µg/mL extract from crude absolute ethanol show great zone of inhibition area compare to 80 µg/mL which from crude n-hexane.

3.3Minimum Inhibitory Concentration

Table 4 and 5 show the percentages of inhibition of antifungal by different concentration. Table 4 show the percentage of inhibition for Tricophytonrubrum and 5 for Microsporumgypseum. From table 4 all form of extracts show the higher percentage of inhibition at 1 mg/mL which was 98.51%, 82.50%, 99.84%, 93.24%, 95.46%, and 97.14%. The other concentrations of extracts also show positive percentage of inhibition but in lower percentage. The percentage of positive control for Tricophytonrubrumwas 97.69%. The positive control used in this study was ketoconazole.

The highest percentage of inhibition of the extract for Tricophytonrubrumwas anthraquinoneaglycone from glycoside, 99.84%. The lowest percentage of inhibition was antraquinone glycoside with percentage 82.50%. From table 5 all form of extract show higher percentage of inhibition at 1 mg/mL which was 99.70%, 79.02%, 99.73%, 84.79%, 84.68% and 99.28%. The percentage of positive control for Microsporumgypseumwas 82.51%. The highest percentage of inhibition of the extract for Microsporumgypseum was anthraquinoneaglycone from glycoside which was 99.73%.

The extraction from anthraquinoneaglycone from glycoside also show the best extract for MIC compare to other extraction [6]. The other concentrations of the extract also show positive percentage of inhibition but in middle and lower percentage. The lowest percentage of inhibition was at extract anthraquinone glycoside with only 79.02%. Anthraquinoneaglycone extract from glycoside show the highest percentage of inhibition for both of fungi. The percentage of inhibition for positive control was higher at T. rubrum compare to M. gypseum. Each form of extractions showed positive inhibition towards both of dermatophytes fungi.

From table 6 the percentage of inhibition of Tricophytonrubrumfor crude n-hexane was higher than crude absolute ethanol which was 95.46%% at 1mg/mL. While at table 7 the percentage of inhibition of Microsporumgypseum was higher at extract crude absolute ethanol which was 84.79%, 1 mg/mL. It can be conclude that, the types of fungi were reacted with different types of extracted form used.

4.0 CONCLUSION

Sennaalata can be extracted by using soxhlet extraction. The same amount of sennaalata used at each of the extract forms can determine the best extract form produce anthraquinone in order to inhibit the fungi by average of zone inhibition and also minimum inhibitory concentration. The presence of anthraquinone can be detected by using TLC by spraying it with methanolic 10% KOH. From all forms of extract of antraquinone, the size of zone of inhibition was the best at 100 µg/mL except for antraquinone from crude absolute ethanol which show best zone of inhibition area at concentration 80 µg/mL. In minimum inhibitory concentration the best form of extraction to inhibit T.rubrum and M.gypseum was athraquinoneaglycone from glycoside. The percentage of inhibition of T.rubrum was 99.84% while for M.gypseum was 99.73%. The concentration used for the highest percentage of inhibition was at 1 mg/mL. For extracted from crude n-hexane and absolute ethanol it can be conclude that, the types of fungi were reacted with different types of extracted form used.

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Figure 1: Flower of *Sennaalata*

Figure 2: Leaves of *Sennaalata*

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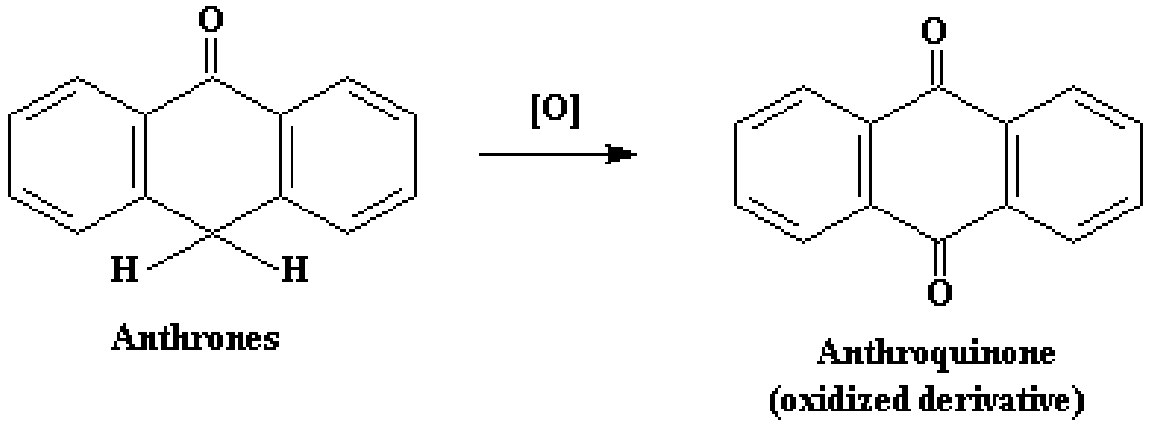

Figure 3: Chemical structure of anthraquinone

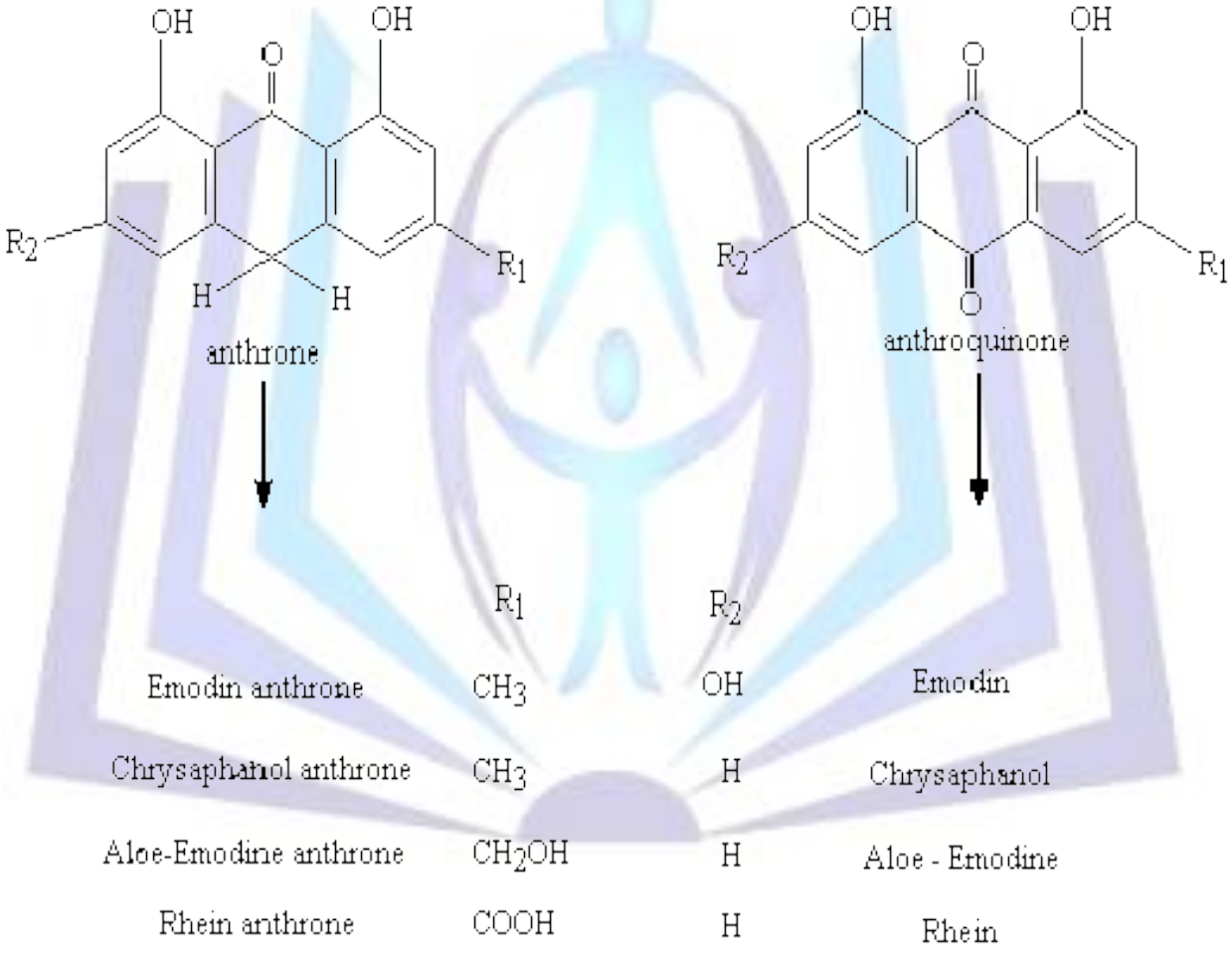

Figure 4: Chemical formulae and chemical structure of anthraquinone.

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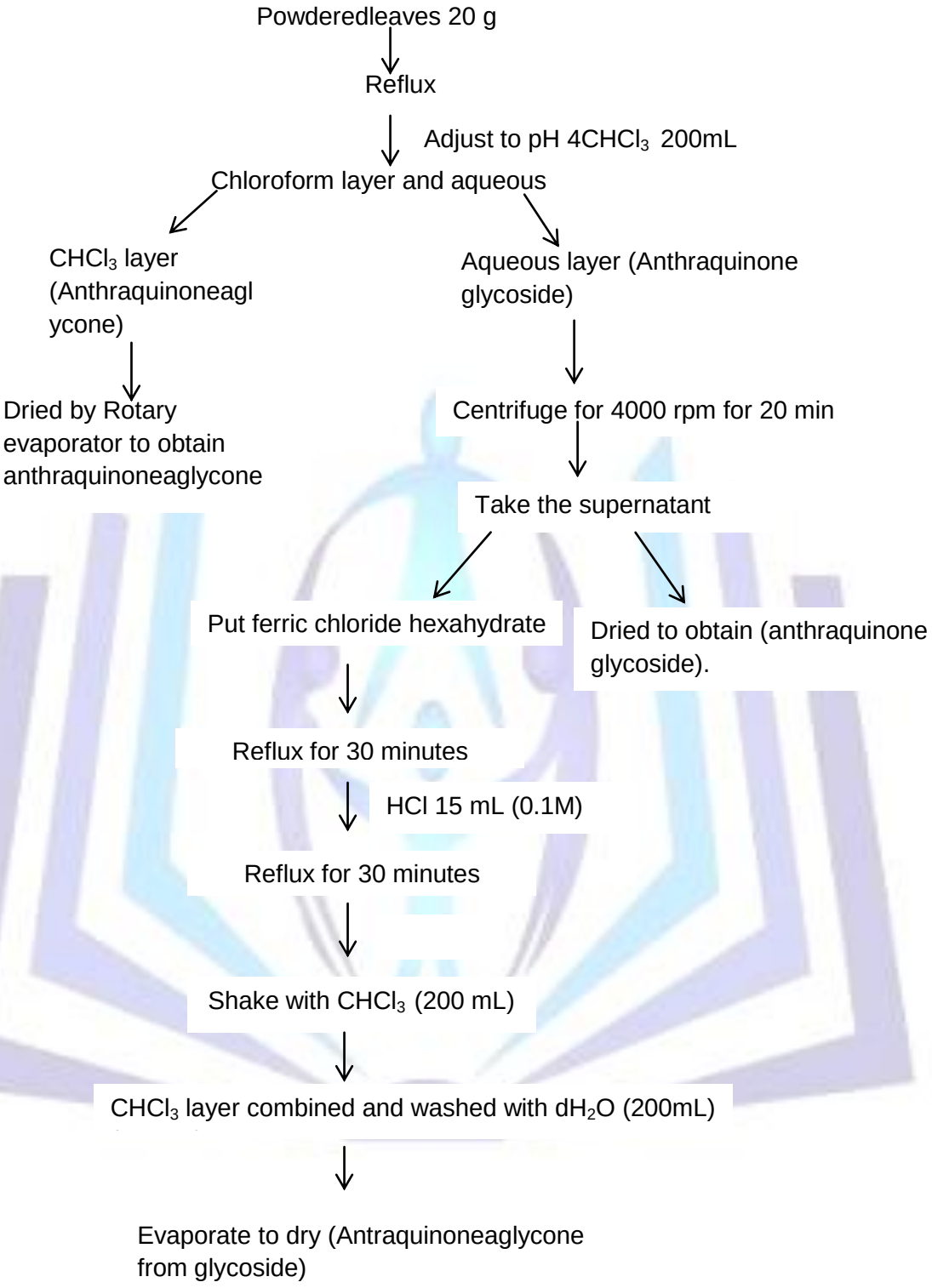

Figure 5: Preparation of anthraquinoneaglycone, anthraquinone glycoside and anthraquinoneaglyconefrom glycoside

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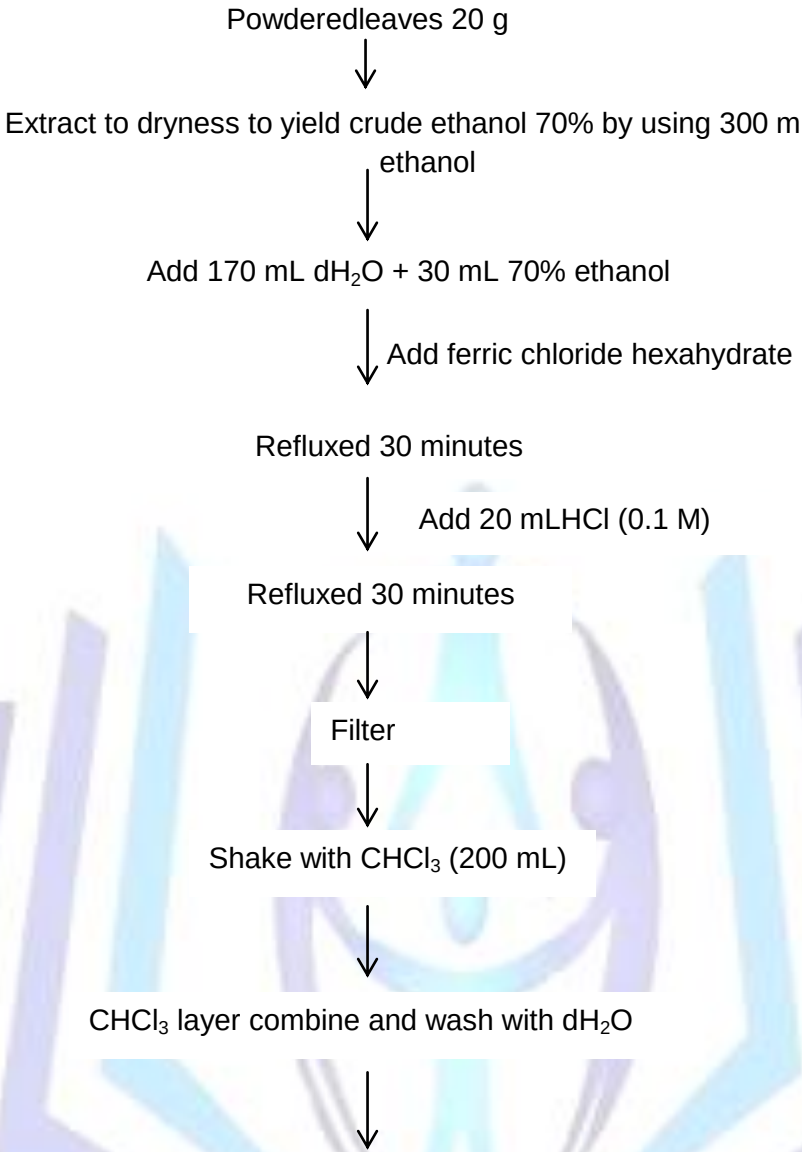

Figure 6: Preparation of anthraquinoneaglycone from crude ethanol 70%

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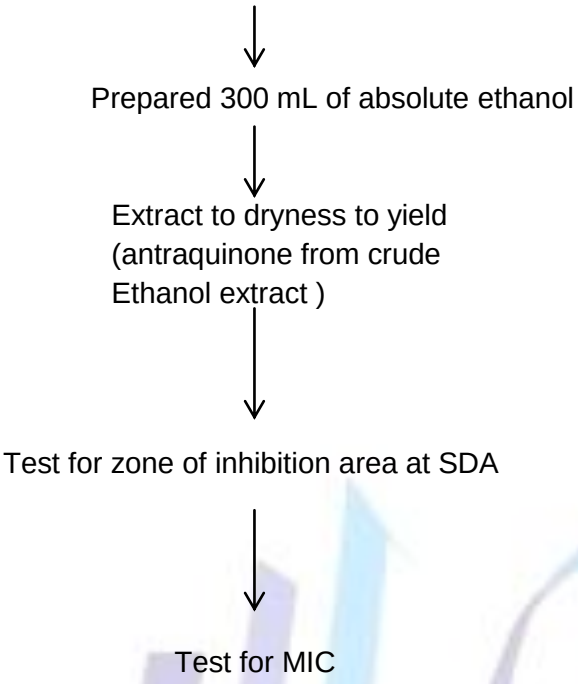

Figure 7: Preparation for anthraquinone from crude ethanol extract

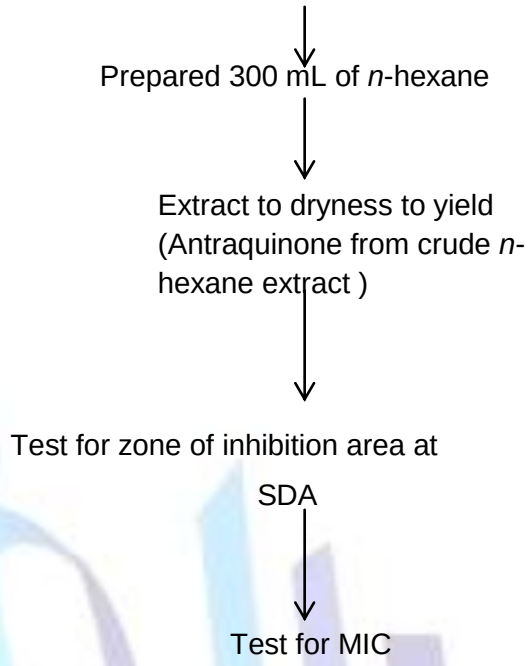

Figure 8: Preparation for anthraquinone from crude *n*-hexane extract

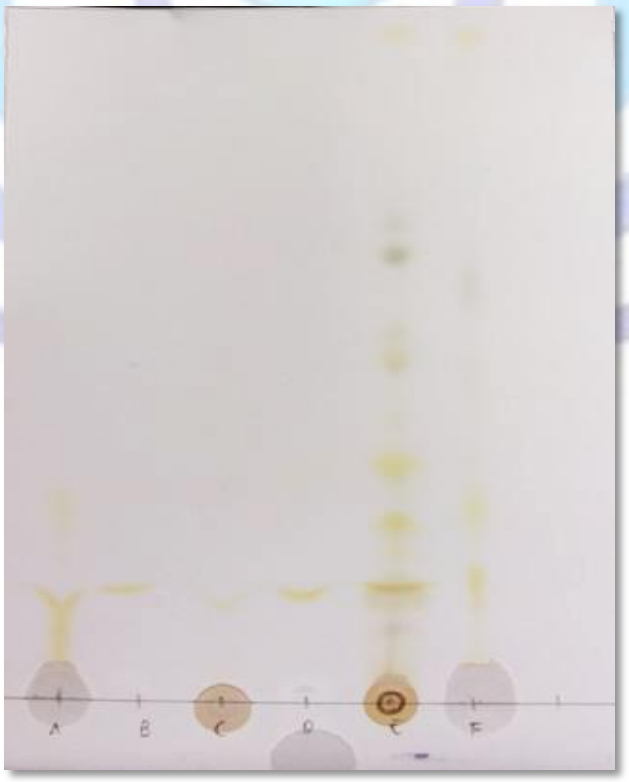

Figure 9: Show the present of chemical compound in each form of extract

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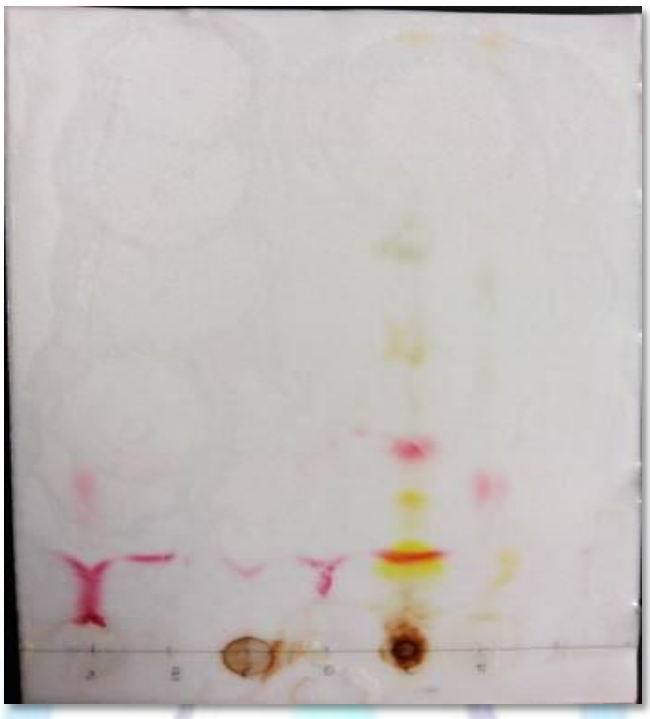

Figure 10: Detection of anthraquinone chemical compound

Lists of tables

Table 1: Show the hRf value for each of the extract form.

FORM OF EXTRACT	hRf value
A: Anthraquinoneaglycone	24
B: Anthraquinoneaglycone from glycoside	16
C:Anthraquinone glycoside	14
D: Anthraquinone from crude ethanol 70%	15
E: Anthraquinone from absolute ethanol	33
F: Anthraquinone from crude *n*-hexane	28

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Table 2: Average zone of inhibition for different form of extracts

Dermatophytes	Average zone diameter (cm)								
	A			B			C		
	50 (µl)	80 (µl)	100 (µl)	50 (µl)	80 (µl)	100 (µl)	50 (µl)	80 (µl)	100 (µl)
M. gypseum	0.8 ±0.1	0.96 ±0.15	1.8 ±0.79	0.76 ±0.05	1.3 ±0.5	1.6 ±0.26	0.8 ±0.1	0.9 ±0	1.43 ±0.4
T. rubrum	1.13 ±0.25	1.47 ±0.45	1.23 ±0.32	0.8 ±0.1	1.0 ±0.36	1.2 ±0.1	1.17 ±0.15	1.3 ±0.1	1.5 ±0

Dermatophytes	Average zone diameter (cm)								
	D			E			F		
	50 (µl)	80 (µl)	100 (µl)	50 (µl)	80 (µl)	100 (µl)	50 (µl)	80 (µl)	100 (µl)
M. gypseum	0.7 ±1.36	1.0 ±0	1.06 ±0.11	1.16 ±0.42	1.23 ±0.37	1.43 ±0.6	1.17 ±0.35	1.33 ± 0.45	1.4 ± 0.55
T. rubrum	0.9 ±0.1	1.73 ±0.15	1.33 ±0.15	1.17 ±0.2	1.0 ±0.55	1.37 ±0.15	1.1 ±0.41	1.47 ±0.3	1.57 ±0.61

Table 3: Average of zone of inhibition for crude absolute ethanol and *n*-hexane

Dermatophytes	Extract from absolute ethanol			Extract from *n*-hexane		
	50 (µg/mL)	80 (µg/mL)	100 (µg/mL)	50 (µg/mL)	80 (µg/mL)	100 (µg/mL)
M.gypseum	0.7cm	1.0cm	1.06cm	1.16cm	1.23cm	1.43cm
T.rubrum	0.9cm	1.73cm	1.33cm	1.17cm	1.0cm	1.37cm

Table 4 :The percentage of inhibition of *T.rubrum* for each form of extracts

Extract form	Average percentage of inhibition (%)						
	Average dilution form of extraction (mg/mL)						
	1	0.5	0.25	0.125	0.0625	0.03125	0.0156
A	98.51	30.93	25.35	28.47	32.38	23.08	24.96
B	82.50	26.25	24.64	29.45	29.45	26.40	23.94
C	99.84	26.17	24.73	32.57	31.52	24.37	26.28
D	93.24	27.69	26.05	26.40	30.27	27.73	24.64
E	95.46	30.19	23.00	29.88	31.83	27.69	24.60
F	97.14	36.25	29.37	26.87	34.41	26.52	28.04
CONTROL	97.69						

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Table 5 : The percentage of inhibition of *M.gypseum* for each form of extracts

Extract form	Average percentage of inhibition (%)						
	Average dilution form of extraction (mg/mL)						
	1	0.5	0.25	0.125	0.0625	0.03125	0.0156
A	99.70	41.46	32.28	25.30	24.53	24.45	26.36
B	79.02	36.32	31.94	31.98	31.19	30.00	30.26
C	99.73	38.76	34.20	32.13	30.00	30.30	30.70
D	84.79	32.54	26.74	27.49	22.22	23.55	19.32
E	84.68	71.76	33.74	31.34	31.38	28.27	27.30
F	99.28	74.79	33.55	32.65	31.27	30.40	30.40
CONTROL				82.51			

Table 6: Percentage of inhibition of *T.rubrum* for crude absolute ethanol and *n*-hexane

Form of extract	Average percentage of inhibition (%)						
	Average dilution form of extract (mg/mL)						
	1	0.5	0.25	0.125	0.0625	0.03125	0.0156
Crude absolute ethanol	93.24	27.69	26.05	26.40	30.27	27.73	24.64
Crude *n*-hexane	95.46	30.19	23.00	29.88	31.83	27.69	24.60

Table 7: Percentage inhibition of *M.gypseum* for crude absolute ethanol and *n*-hexane

Form of extract	Percentage of inhibition (%)						
	Average dilution form of extract (mg/mL)						
	10.50.250.1250.06250.031250.0156						
Crude absolute ethanol	84.79	32.54	26.74	27.49	22.22	23.55	19.32
Crude *n*-hexane	84.68	71.76	33.74	31.34	31.38	28.27	27.30