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Extraction and Purification of Antifungal Compounds from *Piper betle*

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Piper betle **also known as 'sireh' has been traditionally used for medicinal purposes. It has been reported that *P. betle* leaves possess antifungal activity but no effort to optimize the extraction conditions to obtain high yield of antifungal compounds has been carried out yet. In this study, optimized extraction condition was determined. It was first conducted by screening parameters that significantly affect the extraction using Fractional Factorial Analysis. This is followed by identifying the levels for those parameters by employing under Response Surface Method (RSM) FFA. Linear effects of temperature and solvent to solvent ratio as well as interactive terms between temperature and incubation time, and between temperature and solvent to solvent ratio were found to significantly ($p<0.05$) affect the extraction. Temperature and incubation time were selected for optimization experiment and the best condition obtained was extraction for 15 hours at 50°C. In the present study, purification of the extracted antifungal compounds was also carried out. Using column chromatography, the resulting fractions from elution with mixture of hexane and ethyl acetate at ratio 8:2 successfully purified the antifungal compounds. Thin layer chromatography (TLC) showed two distinct spots at Rf value 0.82 and 0.86. Identity of the two spots will be carried out in future research.**

Key words: Extraction, Column chromatography, Fractional Factorial Analysis, *Piper betle*, Response Surface Method (RSM).

The problem of severe nosocomial fungal disease has become more seriously, especially in patients with severe immunological impairment. The development of medicine, surgery and transplantology in the last thirty years has caused a dramatic increase in the number of immunocompromised individuals who are more susceptible to fungal infection[1]. Patients with immunological impairment, HIV infection, leukopenia (haematological malignancy patients), after surgery, organ transplantation or cancer are at the risk of development mycoses. Well-known, the use of broad-spectrum antimicrobial agents, immunosuppressive agents and corticosteroid therapy are also risk factors.

Among all the fungi only few of them are pathogenic to humans. Nowadays, there are some effective antifungal agents, but, unfortunately, some of the pathogenic species show increasing resistance[1]. These fungi are ubiquitous and can be acquired from host surrounding or are components of normal endogenous flora such as *Candida albicans*. *C. albicans* is thought to be the major fungal pathogen of humans. It can enter the bloodstream by direct penetration from the epithelium after tissue damage, or by dissemination from biofilms formed on medical devices introduces

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into patient's body, through, e.g. catheters, dental implants, endoprostheses, artificial joints or central nervous system shunts[2,3]. Thus it easily cause infections like vaginal yeast infections, thrush (infection of tissues of the oral cavity) and diaper rash.

To treat the disease cause by fungal infections, commercial antifungal drugs are used. However, these drugs have negative side effects and some of them are very toxic like amphotericin B which causes chills, fever, headache, nausea, vomiting and may also lead to nephrotoxicity and hepatoxicity[4]. It has been reported that *P. betle* leaves possess antifungal activity[5] and may present less side effect as it is used daily for chewing purposes in some Asian countries. Thus, a large amount of its antifungal compounds is required for further verification before it can be developed as antifungal drug. In this study, optimized extraction condition for the compounds was determined. The purification of the antifungal compounds was conducted as well.

MATERIALS AND METHODS

Extraction of antifungal compound

P. betle leaves used for this study were collected from Gombak, Selangor. They were cleaned, dried and grind using mechanical grinder to obtain powder form. The powder was later mixed in ethyl acetate (1:10 ratio) to extract antifungal compounds[6] by agitation at various conditions. After the agitation, samples were filtered and spun to remove the debris from the resulting supernatant. The ethyl acetate was evaporated by incubation in water bath at 50°C to obtain the crude extract. The crude extract was later tested for antifungal assay.

Antifungal assay

Antifungal activity of the extract was determined using disk diffusion assay against *C. albicans*. *C. albicans* were obtained from Faculty of Health Sciences, Universiti Kebangsaan Malaysia. For the assay, fungal culture was prepared by transferring 5 to 10 colonies of *C. albicans* into sterile distilled water and adjusted to 0.5 McFarland (OD_{530nm} = 0.12 – 0.15) standard by measuring the OD at a wavelength of 530 nm[7]. The culture was plated on the potato dextrose agar (PDA) before disc containing the plant extract were transferred onto the plated PDA plates. After overnight culture, the antifungal activity was determined from the measurement of the inhibition zone (no fungal growth) diameter around the disc. Larger inhibition zones indirectly showed higher antifungal activity.

Screening significant parameter and optimization of extraction

To determine the best processing condition to extract antifungal compounds, screening experiments using Fractional Factorial Analysis was conducted (Table 1). This experiment identified parameters that significantly affect the process. Five parameters were screened which include temperature (°C), incubation time (hours), agitation speed (rpm), amount of solvent per weight of sample (v/w) and amount of solvent to solvent (v/v).

This is followed by identifying the levels of significant parameters obtained from the screening experiments using under Response Surface Method (RSM) (Table 3). Statistical analysis was carried out to determine which parameters have the greatest influence on the response and it involved analysis of variance (ANOVA), F-test and regression analysis.

Purification of antifungal compound

Using the identified optimized condition, the resulting crude extract with antifungal compounds was purified using an open column chromatography. The reverse phase chromatography is applied for the isolation of antifungal compounds from 0.5 g of *P. betle* extracts. Minimum volume of 50 percent hexane in ethyl acetate was used to dissolve the extracts. The 2 cm diameter column is packed with 15 cm silica gel, which was first dissolved 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, 90% hexane in ethyl acetate, 80% hexane in ethyl acetate, 70% hexane in ethyl acetate and 60% hexane in ethyl acetate. 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. The eluents were collected in series of 10 fractions for

each solvent system and were then concentrated by evaporation in water bath at 50°C. The crude of each fraction was dissolved in its solvent system with concentration of 0.1g/ml and analyzed by thin layer chromatography (TLC) as well as tested for anti-fungal assay.

RESULTS AND DISCUSSION

Based on previous studies, there are many methods of extraction which are steam distillation, soxhlet extraction, maceration and enfleurage that had been used to extract various compounds. In this study, solvent extraction with agitation is used to extract antifungal compounds. The method is used due its simplicity and reduced operational cost. As different conditions of agitation extraction may have different effect on target compounds, the present study determined the best condition to extract the compound using Design expert software. The parameters included are temperature, time, agitation speed, solvent to solid ratio and solvent to solvent ratio. These parameters were previously found to have differing effect on agitation extraction[8, 9, 10] of various compounds. In the present study, all of these parameters were screened for their significant effect on the antifungal compounds extraction. Later, the exact condition of the significant parameters was identified for efficient extraction of antifungal compounds.

The results of screening experiments (Table 1) were analyzed statistically by ANOVA and showed in Table 2. From Table 2, the parameters that were significantly affect the extraction were the linear effects of temperature and solvent to solvent ratio as well as interactive terms between temperature and incubation time, and between temperature and solvent to solvent ratio ($p<0.05$). Conclusively, parameters of temperature, solvent to solvent ratio and incubation time had an effect to the process. However, for optimization experiments, only temperature and incubation time were selected as solvent to solvent ratio more than 100% was not possible. 11 optimization experiments with various temperature and incubation time of extraction were designed by FCCD under Response Surface Method (RSM) (Table 3) and were carried out. The results of these experiments were fitted to a quadratic polynomial model, thus multiple regression, regression coefficient and ANOVA (Table 4) were obtained.

Unfortunately, optimization experiment conducted in the present study failed to generate significant statistical polynomial model ("Model F-value" of 2.25, $p>0.05$; Table 4). Thus, the regression equation developed by the model could not be used to determine the optimize condition to

Table 1. Screening experiments using factorial design (1/2 Fraction)

Run	Temperature (°C)	Time (hours)	Agitation Speed (rpm)	Solvent : Solid (w/v)	Solvent : Solvent (v/v)	Inhibition zone (mm)
1	30.00	5.00	200.00	10.00	100.00	23
2	30.00	15.00	200.00	15.00	100.00	29
3	30.00	15.00	300.00	10.00	100.00	33
4	50.00	15.00	200.00	10.00	100.00	25
5	50.00	5.00	300.00	15.00	70.00	19
6	30.00	15.00	200.00	10.00	70.00	27
7	30.00	15.00	300.00	15.00	70.00	28
8	30.00	5.00	300.00	15.00	100.00	27
9	50.00	15.00	200.00	15.00	70.00	15
10	50.00	5.00	200.00	10.00	70.00	17
11	50.00	5.00	300.00	10.00	100.00	26
12	30.00	5.00	200.00	15.00	70.00	29
13	50.00	15.00	300.00	15.00	100.00	26
14	30.00	5.00	300.00	10.00	70.00	23
15	50.00	5.00	200.00	15.00	100.00	26
16	50.00	15.00	300.00	10.00	70.00	17

Table 2 Analysis of variance (ANOVA) of screening experiments

Source	Sum of Square	DF	Mean Square	F value	Prob>F
Model	375.00	12	31.25	13.89	0.0262*
A	144.00	1	144.00	64.00	0.0041*
B	6.25	1	6.25	2.78	0.1942
C	4.00	1	4.00	1.78	0.2746
D	4.00	1	4.00	1.78	0.2746
E	100.00	1	100.00	44.44	0.0069*
AB	25.00	1	25.00	11.11	0.0446*
AC	0.25	1	0.25	0.11	0.7608
AE	56.25	1	56.25	25.00	0.0154*
BC	4.00	1	4.00	1.78	0.2746
BD	16.00	1	16.00	7.11	0.0759
BE	9.00	1	9.00	4.00	0.1393
CE	6.25	1	6.25	2.78	0.1942
Residual	6.75	3	2.25		
Cor total	381.75	15			

A = temperature, B = time, C = agitation speed, D = solvent:solid ratio, E = solvent:solvent ratio.
* $p<0.05$ is significantly different

extract antifungal compounds. After taking into consideration of various aspects, we decided to select the extraction condition that produced extract with the highest antifungal activity from the optimization experiments (Table 3). The condition was extraction at 50°C for 15 hours. Using this processing condition, the plant was extracted in larger scale for purification of antifungal compounds using column chromatography. To purify the antifungal compounds, a mixture ratio for hexane and ethyl acetate from 9:1 to 6:4 ratios were used[11] (Table 5). The compounds were successfully purified using mixture of hexane and ethyl acetate at ratio 8:2 as the eluent. Thin layer chromatography (TLC) of the resulting fractions showed two distinct spots at Rf value 0.82 and 0.86 (Table 5). Identity of the two spots is not determined yet in the present study. But, there is a high possibility that the compound could be a secondary metabolite compounds. Plants secondary metabolites are known for their protective function against pathogenic attack thus fungus[12,13]. One of the subclasses of plant secondary metabolites which posses antimicrobial effects is phenolic compounds. Literature surveys had exhibited numerous phenolic derivatives which responsible for antimicrobial activities, including phenols, phenolic acids, quinines,

Table 3. Optimization of extraction experiments

Run	Temperature (°C)	Incubation Time (hours)	Inhibition zone (mm)	
			Actual value	Predicted value
1	40	10	25.7	25.17
2	40	15	25	25.82
3	40	20	25.7	25.41
4	50	10	26	26.82
5	50	15	28	27.47
6	50	15	27.3	27.47
7	50	15	28.3	27.47
8	50	20	26.7	27.06
9	60	10	26.7	26.41
10	60	15	26.7	27.06
11	60	20	26.7	26.64

flavones, flavanoids, flavanols, tannins and coumarins[14]. Other plant secondary metabolite compound which has similar activity is alkaloid. Indoquinoline alkaloids isolated from *Cryptolepsis sanguinolenta* had shown its bioactivity against several gram negative bacteria and yeast[15]. Terpenes are another group of plant secondary metabolites which had been found with antimicrobial activites[16]. Although, many studies above found that bioactive compounds responsible for antimicrobial activities in plants are secondary metabolites, this study might find otherwise. The exact identity of the purified compound will be determined using NMR and MALD-TOFF in the near future.

Table 4. Analysis of variance (ANOVA) of optimization experiments

Source	Sum of Square (SS)	Degree of Freedom (DF)	Mean Square (MS)	F-values (F-VAL)	Probability (P-VAL)	
Model	6.86	5	1.37	2.25	0.1975	not significant
A	2.28	1	2.28	3.74	0.1111	not significant
B	0.082	1	0.082	0.13	0.7296	not significant
A^2	2.71	1	2.71	4.44	0.0890	not significant
B^2	0.72	1	0.72	1.18	0.3263	not significant
AB	0.000	1	0.000	0.000	1.0000	not significant
Residual	3.05	5	0.61			
Lack of fit	2.53	3	0.84	3.20	0.2472	not significant
Pure error	0.53	2	0.26			
Cor total	9.92	10				

A = temperature, B = time R^2 = 69.60%, R^2 (adj) = 38.40%, R^2 (pred) = -93.88%
*Cor total = Total sum of squares correcter for the mean

Table 5. Antifungal activity of fractions from purification of *P. betel* extract using mixture of hexane and ethyl acetate solvent systems

Ratio hexane:ethyl acetate (ml/ml)	R_f value	Inhibition zone (mm)
100:0	0.8	-
	0.9	-
90:10	0.7	6
	0.8	6
80:20	0.8	16.33 ± 0.02
70:30	0.7	6
	0.9	6
60:40	0.7	6
	0.8	6
	0.9	6

CONCLUSIONS

Another study should be carried out in the future to obtain significant model to determine optimum processing condition to extract antifungal compound from *P. betle*. Wider range for parameters of temperature and incubation time should be considered in the future. The present study however, successfully purified antifungal compounds using mixture of hexane and ethyl acetate at ratio 8:2 as the eluent for column chromatography. The identity of the purified compounds will be determined in the future research. There is a high possibility that the compound is a secondary metabolite compounds as they has been repeatedly proven as protective agents against pathogenic attack, pests and environmental stress.

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