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Extraction and purification of cytotoxic compounds from *Premna serratifolia* L. (bebuas) for human breast cancer treatment

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Abstract

Breast cancer is considered as one of the most common cancers all over the world. A huge effort has been made to create a safe and cost effective breast cancer treatment. All of these features exist in the plants sources. In this study, the effect of local vegetable salad, *Premna serratifolia* (Bebuas) against MCF-7 cells (human breast adenocarcinoma) was determined. The optimum condition to extract breast cancer cytotoxic compound from the plant was investigated and the exact cytotoxic compound was identified as well. To determine the plant cytotoxicity effect against MCF-7 cells, MTT assay was used. Two important parameters in the sonication extraction method which are duration of time and temperature were optimized by carrying out a series of experiments which were designed by Face Centered Central Composite Design (FCCCD). The extraction efficiency of each experiment was determined by measuring the yield of extract and the half maximal inhibitory concentration (IC_{50}) of the extract against MCF-7 cells. The results obtained from the experiments were fitted to the second order polynomial model to generate equation that was used to determine best extraction processing condition. Based on the generated equation, the best sonication processing condition to extract the cytotoxic compound is at 30°C for 67 min. Analysis of variance (ANOVA) showed that the duration of extraction time has great influence ($p<0.05$) on the yield of crude extract, while both parameters, time and temperature significantly ($p<0.05$) affected the IC_{50} value of the plant extract. Two purified compounds from the plant, acacetin and acacetin-7-O-glycoside were identified to be responsible for the cytotoxicity activity. Conclusively, in terms of pharmaceutical and medical field applications; the optimal extraction processing condition obtained from this study ensures that the extraction of cytotoxic compound from the plant is conducted at the low temperature and short duration of time. Further study on the identified compounds is required as it is a potential candidate for anticancer drug.

Introduction

Cancer is known as abnormal cell growth, and medically known as malignant neoplasia. In cancer, the cells are divided rapidly and grow uncontrollably that leads to malignant tumor. Cancer can attack any parts of the body, spread and rapidly invade other organs. The number of cancer cases has been increasing significantly, globally and it is expected to ascend by about 70 percent in the next two decades according to Steward and Wild (2014).

There are five predominant cancer types among men; prostate cancer, lung cancer, stomach cancer, colorectum cancer and liver cancer. As for women, breast cancer, cervix cancer, stomach cancer, colorectum cancer and lung cancer are predominant. Breast cancer comprises of 22.9 percent of all cancers excluding non-melanoma skin cancer (Boyle and Levin, 2008). Breast cancer can be classified according to different categories depending on the cancer classification approaches such as; histopathology, grade, receptor status and stage.

There are many approaches to remedy the breast cancers, most of them depend on the surgery, chemotherapy or radiation. All conventional cancer treatment approaches have drawbacks such as expensive and unsafe. In the last two decades, many studies have been carried out in the field of cancer treatment, most of these studies focused on the natural products from plants and functional food as anti-cancers. Since the plant is the prime source of many drugs of chronic diseases, one of the local vegetable salad from *Premna* species was investigated. *Premna* species is widely spread in Malaysia and considered as the researchers' target, due to their potential effectiveness against many types of cancers and

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many diseases. It has been reported that, *Premna* species have important medical properties such as antioxidant properties (Mustafa *et al*., 2010), hepatic disorder treatment and anti-cancers (Suresh *et al.,* 2011). In the present study, the particular *Premna* species that was studied is *Premna serratifolia*. *Premna serratifolia,* which previously known as *Premna cardifolia* belongs to Lamiaceae family and was first described by Linnaeus in 1771 (Kok, 2013). *P. serratifolia* is known as bebuas in Malaysia. This plant has many medical compounds such as phenolic and flavonoid; and has significant radical scavenging as well as antioxidant activities. According to Mustafa *et al*. (2010), the content of phenolic compounds that presents in this plant are considerable and has many flavonoids compounds such as catechin, quercetine and myricetin.

The valuable medical compounds and the yield of extracts from medicinal plants are affected by the method of extraction. It is well known that, the main problem that associated with the classical methods of extraction process is time consuming. Ultra sonication methods could overcome this problem as many studies showed that it took 30 to 70 minutes to conduct extraction (Hromadkova *et al*., 2008). In the present study, the plant was extracted by ultra-sonication and the best processing condition to obtain the highest yield of extract was determined. The effect of the processing condition toward cytotoxicity property of the extract against breast cancer cells, MCF-7 was also investigated. In this study, the crude extract obtained from the sonication process was purified to identify which compounds responsible for the cytotoxic property.

Materials and methods

Plant materials

Fresh leaves with branches of *P. serratifolia* were collected from the local farms and were verified by by Dr. Rasahad Mat Ali (Forest Research Institute Malaysia, FRIM) with voucher number (SBID: 019/14). The leaves were immediately sorted from branches leaving out the yellow ones. Then, they were washed and cleaned from any impurities before they are cut into small pieces. The cut pieces were dried at 40^{o}C for 24 hours before grinded into powder form prior to extraction process.

Extraction

In this study, extraction of plant was carried out using ultra-sonication. Before the best sonication processing condition was determined, screening for the best solvent to extract cytotoxic compounds was carried out. Four types of solvent were screened namely methanol, ethanol, ethyl acetate and hexane to obtain the best extract yield and cytotoxicity activity. In this screening, 10 g of plant powder was sonicated in 100 ml solvent. After the sonication, the whole extracts were transferred into 50 ml centrifuge tube and centrifuged at 8000 rpm for 10 minutes. The extract was then filtrated by using Whatman No.1 filter paper using vacuum pump. Later, the solvent was removed from the extract using rotatory evaporator. The dried extract was measured, transferred into microtube and stored at 4°C prior to the cytotoxicity test (MTT assay).

MTT assay

MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide, a tetrazole) was applied as a cytotoxicity assay (Young *et al*., 2005) to investigate the cytotoxic effect of P. serratifolia extract against both cancer (MCF-7) and normal (VERO) cell lines. Firstly, all wells on 96-well plate were filled with 100 µl of media except the wells of column number 2 of the plate, followed by the addition of 5000 cells for each well. Then, the plate was incubated in a humidified incubator supplied with 5% CO_2 at 37°C for 24 hours for cell recovering and reattaching processes. For the extract preparation, the crude extract was dissolved in a media containing 10% (v/v) DMSO at the concentration of 2000 µg/ml. Then, the extract was filtered by using 0.22 µm syringe filter into the 9 cm petri dish and added to all wells in column number 2 and 3 of the plate (100 µl). To make a series of extract concentrations, 100 µl was transferred from the column number 3 to the column number 4 and so on until the column number 12. Then the plate was re-incubated for 44 hours before 20 µl of 5 mg/ml of MTT was added into each well and kept for 4 hours in the dark at the room temperature. After 4 hours, the well contents were discarded and 150 µl of DMSO was added to each well. Then the plate was analyzed using a spectrophotometer to record the absorbance or optical density (OD) at 570 nm. The cell viability was obtained by applying the Equation (1):

$$\text{Cell viability } 100\ \% = \frac{OD\ of\ treatedcell}{ODof\ untreated\ cells} \quad (1)$$

Using the calculated cell viability values above, a graph of cell viability (y axis) versus crude extract concentration (x axis) was created to detect the half maximum inhibitory concentration (IC_{50}) value of crude extract.

Experimental design

Response Surface Methodology (RSM) was

Table 1. Yield of extract and IC_{50} of *P. serratifolia* methanolic extract at various sonication extraction conditions

Run	Time (minute)	Temperature (°C)	Yield (g/g)	IC_{50} (µg/ml) against MCF-7 cell line	IC_{50} (µg/ml) against Vero cell line
1	60	45	2.234	58.84	912
2	30	30	1.406	58.40	905
3	90	45	2.021	62.03	1301
4	30	60	1.476	60.17	1290
5	60	45	2.229	58.98	915
6	60	45	2.221	58.98	922
7	60	60	2.139	60.07	1180
8	60	30	2.218	58.58	900
9	90	60	1.859	67.24	1321
10	30	45	1.479	57.24	902
11	90	30	1.757	56.50	895

used to determine the best sonication processing condition. The parameters investigated in RSM were temperature and duration of time of extraction. A total of 11 experiments with three replicates were designed by Face-centered central composite design FCCCD. Design Expert soft-ware (version 6.0.8) was used to analyze the experiments statistically. Table 1 shows the 11 designed experiments with each parameter at a certain range. The efficiency of extraction was determined by measuring the yield of extract and the IC_{50} of the extract against MCF-7 cells.

Purification and identification of cytotoxic compounds

The methanolic crude extract of *P. serratifolia* from the extraction process mentioned above was subjected to purification by open column chromatography. However, before the purification was conducted, the crude extract was fractionated and chlorophyll was removed using DIAION™ column. The purified compounds were identified chemically by Nuclear Magnetic Resonance (NMR) technique. Two NMR spectrums (13C and 1H) were used to detect both carbons and protons numbers, respectively. The data of NMR was analyzed and based on this analysis the chemical structures of cytotoxic compounds were drawn by Chemsketch software.

Results and discussion

Screening of cytotoxic effects of P. serratifolia

P. serratifolia crude extracts obtained from all four solvents were subjected to MTT assay to determine the cytotoxicity effect of each extract against breast cancer cells, MCF-7. The results revealed that both methanol and hexane crude extracts had high cytotoxic activity against MCF-7 cells with IC_{50} of 58.40 µg/ml and 53.81 µg/ml respectively. As for both ethyl acetate and ethanol crude extracts, they showed a weak activity with IC_{50} of 272 µg/ml and 820 µg/ml respectively. Taxol (Tamoxifen) which acted as a positive control in the MTT assay showed a low IC_{50} value of 1.25 µg/ml, indicating a very potent cytotoxic effect against MCF-7 cell line. Vero cell line was used in the present study to evaluate the cytotoxic effect of *P. serratifolia* crude extract on the normal cells. All four extracts showed negligible effect toward Vero cell line (IC_{50} of more than 886 µg/ml). However, Tamoxifen showed almost similar potent cytotoxic effect that it had against MCF-7 cell line (IC_{50} of 2.3 µg/ml). This indicates that Tamoxifen affected cancer cell as well normal cells.

According to US National Cancer Institute (NCI), the crude extract is considered safe if the IC_{50} is more than 20 µg/ml against mammalian cell lines (Boik, 2001). Thus, based on this guideline, Tamoxifen is considered unsafe to both cancer and normal cell. Based on this guideline as well, methanolic and hexane extracts had the IC_{50} value that was almost considered to be unsafe or harmful toward breast cancer cells (58.40 µg/ml and 53.81 µg/ml, respectively). Most probably, the IC_{50} or the cytotoxic effect of both extracts against cancer cells might be enhanced if the chlorophyll content of *P. serratifolia* was removed. De *et al.* (2005) found that chlorophyll content of many plants affect the antiproliferative effect against the cancer cells.

Optimization of sonication extraction

The aim of the optimization experiments conducted in this study was to determine the best processing sonication conditions using the statistical approach to obtain a higher yield of extract with a sustained bioactive compound for the purification process. Based on the results, the highest yield of extract was 22.34% under the condition of 45°C for 60 minutes and lowest yield of extract was 14.06% at 30°C for 30 minutes (Table 1). While the highest IC_{50} against MCF-7 cell line was 67.24 µg/ml under the condition of 60°C for 90 minutes and the lowest IC_{50} value was 56.5 µg/ml under the condition of 30°C for 90 minutes. All the crude extracts produced from

Table 2. ANOVA analysis of the yield extract of *P. serratifolia* response

Source	Sum of squares	F-value	p-value
Model	1.09	47.54	0.0003
A*	0.27	58.95	0.0006
B**	1.442	0.31	0.5999
A^2	0.68	147.66	< 0.0001
B^2	0.020	4.41	0.0898
AB	2.560	0.056	0.8229
Lack of fit	0.023	177.76	0.0056

*A = time.
**B = temperature

the experiments showed negligible effect against Vero cell line with minimum IC_{50} of 895 µg/ml under the condition of 30°C for 90 minutes and maximum IC_{50} of 1321 µg/ml under condition of 60°C for 90 minutes.

Analysis of variance (ANOVA) for the extract yield response

Based on the ANOVA for Response Surface Quadratic Model, F value of 47.54 and p-value of 0.0003 implies that model is significant (Table 2). Any models that possesses p-value less than 0.05 is significant (Li *et al*, 2008). The significance of each parameter can be specified by p-value which also points out the correlation between the variable. Based on the F values of each parameters involved in the sonication extraction, duration of time has great influence ($p<0.05$) on the yield of crude extract. The relation between p-value and the model significance is inverse; the model becomes more significant when the p-value gets smaller. The R^2 of the model is 0.9794 and Adjusted R-Squared is 0.9588. This indicated that the model is significant in term of convergence between the predicted and actual results. According to Reddy *et al.* (2008), the model gets fitter when the R^2 value is closer to 1.

A second order regression equation (2) was developed as shown below:

$$\text{Yield} = (+2.24+0.21^{*}A + 0.016^{*}B) - (0.52^{*}A2 + 0.090^{*}B2) + (8.000^{*}A^{*}B) \quad (2)$$

where A is time and B is temperature. Equation (2) was used to plot 3-D response graph (Figure 1a) which determine the best extraction processing condition to obtain the highest yield of extract from *P. serratifolia.*

The graphical model is also used for better understanding on the relation between the parameters and how they affect the yield of extraction. In general, both saddle and elliptical shapes indicate that the model is significant (Muralidhar *et al*., 2001). From the results observed in this study, duration of time affected the yield of crude extract. At duration of time of 60 minutes, the highest yield was achieved. Many previous studies proved that, the highest yield could be obtained when the duration of time of extraction in sonication method is 60 minutes. Abdullah *et al.* (2010) found that the highest yield of *Monopterus albus* oil extraction was achieved by when sonication was carried out for 60 minutes.

Table 3. ANOVA analysis of the *P. serratifolia* anti-cancer activity (IC_{50}) response

Source	Sum of squares	F-value	p-value
Model	69.32	11.29	0.0045
A*	16.53	8.08	0.0250
B**	32.67	15.96	0.0052
AB	20.12	9.83	0.0165
Lack of fit	14.31	438.13	0.0023

*A = time.
**B = temperature

Analysis of variance (ANOVA) for the anti-cancer activity (IC_{50}) response

Based on the ANOVA for Response Surface Quadratic Model, F value of 11.29 and p-value of 0.0045 implies that model is significant (Table 3). The F values of the parameters involved, both duration of time and temperature ($p<0.05$) significantly affected the IC_{50} value of crude extract. The R^2 of the model is 0.8287 while adjusted R^2 is 0.7553. This indicated that better resemblance between the predicted and obtained IC_{50} values. The lack of fit is sufficiently fit.

A second order regression equation (3) was developed as shown below:

$$IC_{50} = (+59.73 + 1.66\ ^{*}A) + (2.33\ ^{*}B) + (2.24^{*}A^{*}B) \quad (3)$$

where A is time and B is temperature. Equation (3) was used to determine the best extraction processing condition for cytotoxic activity of crude extract of *P. serratifolia*. Equation 3 was graphically interpreted into 3-D response surfaces as shown in Figure 1b.

From the graph, the IC_{50} of crude extract relatively increased by the rise in temperature. This might be ascribed to many bioactive compounds such as phenolic and flavonoids compounds which possess anti-cancer property; they are easily hydrolyzed or oxidized when exposed to high temperature for a long time. On the other hand, in this study the duration of time of sonication extraction method positively affected the IC_{50} value of methanolic crude extract of *P. serratifolia.* Long sonication time might enhance the cell disintegration causing solvent to reach the cell

Table 4. Validation of the experimental model

Run	Time (minute)	Temperature (°C)	Predicted yield (g/g)	Predicted IC_{50} (µg/ml)	Actual yield (g/g)	Actual IC_{50} (µg/ml)
1	67.18	30	2.1583	57.25	2.1604	56.60
2	66.76	30	2.1588	57.26	2.1600	56.81

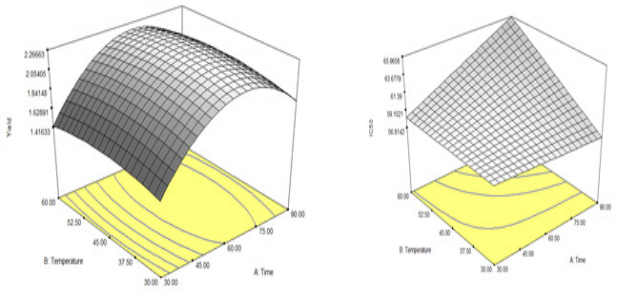

Figure 1. (a) The 3-D response surface of the effect of temperature and duration of time on the yield extract of *P. serratifolia.* (b) The 3-D response surface of the effect of temperature and duration of time on the IC_{50} of *P. serratifolia* extract.

matrix which result in better releasing of bioactive compounds. Saleh *et al*. (2015) demonstrated that the yield of silymarin was increased from Silybum marianum seeds by increasing the extraction time. Silymarin is a complex of compounds that has a significant cancer preventative effect (Ramasamy and Agarwal, 2008).

Validation analysis

From both generated second order regression equation above Equations (2) and (3), Design Expert software suggested the best processing condition of sonication extraction with high desirability (0.919) to obtain a high yield of extraction with a low cytotoxic activity (low IC_{50}) as shown in Table 4. Table 4 also lists the predicted and actual results of the suggested processing conditions (validation experiment).

From the results listed in Table 4, it can be seen that, both predicted and experimental results are almost close. This confirms that the condition of sonication process for *P. serratifolia* extraction that suggested by the software should be adopted to obtain higher yield of extract for the purification and bioactive compound identification process. The best sonication processing condition to extract the cytotoxic compound used was at 30°C and 67 minutes.

NMR analysis of purified cytotoxic compounds

After the sonication extraction using the processing condition mentioned in the validation analysis, the methanolic crude extract of *P. serratifolia* was subjected to various steps of purification. The identity of the purified compounds was determined by NMR analysis.

Two spectrums of NMR (13C and 1H), for both carbon and proton analyses were involved. Based on the spectrums used as well as previous literature (Liu *et al*., 2013), the analyzed compounds are acacetin and acacetin-7-O-glycoside, which is a type of flavonoid and sugar bound flavonoid, respectively. Previous studies have shown that acacetin exert anticancer efficacy against human colon carcinoma cells (Wang *et al*., 2004) as well as human liver and lung cancer cells, HepG2 and A549 cells, respectively (Hsu *et al*., 2004a; Hsu *et al*., 2004b). Other than our lab, only one previous study has shown that sugar-bound acacetin also possess anti-proliferative activity against cancer cells (Liu *et al*., 2012).

Conclusion

This study showed that *P. serratifolia* leaves extract is cytotoxic against breast cancer cells and the responsible bioactive compounds are acacetin and acacetin-7-O-glycoside. The optimal extraction processing condition obtained from this study ensures that the extraction of the cytotoxic compounds from the plant is conducted at the low temperature and short duration of time.

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