OPTIMIZATION OF FORMULATION INGREDIENTS OF ANTICANCER SUPPLEMENT FROM *Annona Muricata* LEAVES EXTRACT USING D-OPTIMAL MIXTURE DESIGN

Nik Nurul Najihah N.M. Daud[1,3],H. Yaakob[2,*] ,N. Zainol[2] ,Siti Norazlina A.Rashid[2] ,Norfarahiyah, A. Nor[2] ,N. Sadek[2] ,Auni Salma S. Amri[1,2,3] ,Iylia Arina M.Zaki[1,2,3]

[1] Faculty of Chemical and Energy Engineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia
[2]Institute of Bioproduct Development, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia
[3]Department of Bioprocess Engineering, Universiti Teknologi Malaysia, 81310 Johor, Malaysia
[*]For correspondence; Tel. + (60) 75532502, E-mail: harisun@ibd.utm.my

ABSTRACT: *Annona muricata L. is a type of fruit that has antioxidant and anticancer properties which can help in treating cancer. However, no study has been done on the formulation ingredients that affect the quality and safety of the product. Therefore, this study was done to optimize the formulation ingredients of anticancer supplement containing A.muricata leaves extract. The formulation ingredients were optimized using D-optimal mixture design consist of extract, vitamin D_3, silicon dioxide, and maltodextrine. Spray dryer was used to obtain the formulation powder. The D-optimal design allowed selecting the optimal proportions of the excipients: extract (90.18 %), vitamin D_3 (2.00 %), silicone dioxide (1.25 %), and maltodextrine (6.58 %) with yield of powder (6.87 %), moisture content (6.80 %), EE for TPC (32.30 %) and TFC (24.89 %), and DR (1.07 mg/sec) The optimum formulation ingredients containing A.muricata leaves extract was safe to be used since the optimum formulation ingredients did not cause harm on normal cell while showed some inhibition on colon cancer cell.*

Keywords: Mixture design, supplement, *Annona muricata*, desirablity

1. INTRODUCTION

Annona muricata L. or graviola, as commonly known, is a medium-sized tree, which belongs to the Annonacea family and contain high antioxidant and anticancer compounds. The leaves are rich with *annonaceous acetogenin*, the most potent anticancer compounds. It is a series of polyketide-derived fatty acid with tetrahydrofuran rings and methylated gamma-lactone bonded together and composed of a large family of fatty acid derived-natural products with unique structures like saturated and unsaturated aliphatic compounds with oxygenated functional groups [1][2]. They displayed good reaction in antioxidant, antitumoral, cytotoxicity, antiparasitic, and pesticidal assays [3]. The native Brazilian Amazon used the leaf for treating the liver problem [4] meanwhile in Malaysia, the leaf was prepared as a decoction for treating high blood pressure and diarrhea [5]. Nowadays, it has become well-known globally for its anticancer properties due to the several studies done on few cells like pancreatic cancer [6], cervical cancer [7], breast cancer [8] and prostate cancer. Due to its high market demand as anticancer supplement product, it has been seen among the potential herbs that can be cultivated commercially with its fruit, root, barks and leaf. The ethanolic extracts of graviola leaves has a cytotoxic effect on breast and cervical cancer cell lines with a high IC_{50} value which were 17.1µg/mL [8] and 97µg/mL [7]. Meanwhile, the aqueous extract of graviola leaves also showed beneficial effect on pancreatic cancer where it has the ability to inhibit pancreatic oxidative damage introduced by Streptozomycin [6]. Another study performed by Torres [9] agreed that there is a progressive decrease in cell viability with the successive increase in the concentration of the capsule with raw crude powder after 48 hour of treatment on pancreatic cancer cells lines with high IC_{50} value which were 200 and 73µg/mL, respectively. On top of that, the leaves have been extracted with hexane and commercialized extract was also being tested on the pancreatic cancer, CAPAN-1 cell lines showed a high value of IC_{50} with 7.8-8µg/ml and 0.9-1.0µg/ml demonstrated that the extract induced mild cytotoxicity in pancreatic cancer cell lines [10].The leaves of this plant has a broad spectrum of anticancer activity, because of they are potential source of new classes of phenolic acids that could be useful for infectious diseases due to its broad *acetogenin* contents. It is a novel set of phytochemicals and found predominantly in the *A.muricata* leaves. Detail studies on the leaves of *A.muricata* had resulted in the isolation of eight cytotoxic *acetogenin*s. These *acetogenins* in the leaves have the potential to be developed into an anticancer herbal supplement product as it has the ability to inhibit multiple drug resistant (MDR) tumor cell lines. The development of a new formulation of capsule supplement is a very complex work that depends on both formulation factors and manufacturing process [11]. The complete knowledge of the factors affecting the development process of pharmaceutical capsule supplement is very important for the development of a new formulation. Once these factors are identified, they could be mathematically modelled and allowed to predict the behaviour of most important properties of the product. The development of any pharmaceutical form necessitate the complete study of different levels of excipients used in various formulations, in order to identify the exact proportion of what maximizes quality and stability of the final product. The aim of this work was to optimize a new capsule anticancer supplement formulation from the crude optimum extract of the leaves of *A.muricata*, by using a D-optimal mixture design.

2. EXPERIMENTAL DETAILS

Extract and Excipients

The *A.muricata* leaves extract was obtained from the optimization process using soxhlet extractor. The marker compound from the optimum extract was screened by HPLC and identified using NMR-FTIR. The capsule from Halagel Malaysia (size 1, vegetable capsules VEGECAPS) was used in this formulation study. The four ingredients which were involved in this formulation are graviola leaf extract, vitamin D_3, silicon dioxide, and maltodextrine.

Experimental Design

The excipients proportion restrictions used for capsule supplement formulation are listed in table (1). The mixtures of active ingredient and excipient were made according to the 18-run D-optimal mixture design. This kind of design is particularly useful in a restricted region because of maximization of the volume of each ingredient in a k-dimensional space. In this way, you can use only the interest excipients, keeping constant the quantities of the rest of the formulation ingredients [12]. The total amount of the mixture (100%) was held constant. The relative amounts of the different excipient varied according to the mixture design shown in figure (1). The spray dryer was used to obtain the powder extract and the drying process was carried out in a Separation Lab at Fakulti Kejuruteraan Kimia UTM Skudai with a co-current flow. The conditions of spray drying process were set constant based on the previous findings related to spray drying process of *A.muricata* leaf [13].

Table (1) Constraints of each variables of anti-cancer capsule formulation containing graviola leaf

Causal Factor Variables[1,2]		Coded Level of Variables (%)	
		Lower limit	Upper limit
A	Extract	79	92
B	Vitamin D_3	2	4
C	Silicon Dioxide	1	2
D	Maltodextrine	5	15

Yield of Powder

$$\text{Yield of powder (\% w/w)} = \frac{\text{mass of solid content (g)}}{\text{mass of raw material (g)}} \text{ x100}$$

Moisture Content

Moisture content was measured using the moisture analyzer (KERN MLS, Germany). 2g of the studied sample was dried at the temperature 100-105 °C until a constant weight was reached. The result was the loss of weight through drying (%). The moisture content of the sample was calculated using the equation below:

$$\text{Moisture content (\% w/w)} = \frac{\text{weight of wet sample (g)}}{\text{weight of dry sample (g)}} \text{ x10}$$

Dissolution Rate

The dissolution was defined as the transmission of molecules, or ions from a solid state into a solution state [14]. The rate of dissolution was given by Noyes-Whitney equation below that took into account the two processes necessary for dissolution to occur which were saturation of the diffusion layer and diffusion of drug molecules into solvent:

$dm/dt=KS(Cs-Ct)$

Encapsulation efficiency of total phenolic and flavonoid content

Encapsulation efficiency (EE %) was calculated as the amount of TPC and TFC encapsulated in spray dried powder (fp) (divided by the TPC and TFC of the solution used for the preparation of spray dried powder (fs)), as shown in equation below:

EE % = fp / fs (100)

Cell Lines and Culture

Trypan Blue exclusion method was a cell-based assay used in this study to detect the mechanism of toxicity in cells. HT-29 cells, colorectal cancer cells were obtained from Kuliyyah of Allied Health Science and preodontal ligament, PDL normal cells from Integrated Centre for Research Animal, Care & Use, IIUM Kuantan. Cells were grown in Dulbecco's modified eagle medium (DMEM) with 10 %(v/v) fetal bovine serum & 1% (v/v) of penicillin streptomycin. Cells were cultured at 37◦ C in 5% CO_2 humidified atmosphere. Cells were sub cultured when it reached >80% confluency.

Surface Morphology Analysis

Morphology was observed using an electron microscope (FESEM, JSM-6701F) operating at 15kV. Prior to scanning, samples in a powder form were attached to double sided adhesive tape which was made electrically conductive (10 mm stubs). Digital images were obtained with an excitation voltage of 5kV and magnification varied from 2.50 to 25.00KX. FESEM images were prepared with a JEOL model 6330F FESEM (JEOL USA, Inc., Peabody, MA) using the following parameters: 5.0 kV accelerating voltage, 12.5 µamp emission current, 7.0 probe current (spot size), secondary electrons at 20,000x magnification and slow capture scan. Magnification accuracy for the FESEM was tested using an ASTM-certified magnification reference standard (MRS-4, Geller Microanalytical Laboratories, Topsfield, MA).

Data Analysis

All responses for anticancer capsule supplement characterization were treated using Design Expert software (Version 6.0.1, Stat-Ease, Inc. Minneapolis, USA). The best-fitted mathematical models for each response were selected. To select the model that best describes the variability of response depending on the factors used (quantities of excipients), the following criteria were taken into account; the highest adjusted R-squared value (R2) and the predicted R-squared (Q2). For the selection of the model was also considered the different minor between R2 and Q2, the minor value of the sum of squares of the predicted error (PRESS) and the test of lack of fit without statistical significance ($p> 0.05$). The number of experimental points (USP, 2012) was enough to adjust the response in this study.

3. RESULTS AND DISCUSSION

Characterization of anticancer capsule supplement

A D-optimal mixture design was carried out to evaluate the powder behaviour as a function of selected excipient proportion. Figure (1) shows the results of the experiments according to D-optimal mixture design for anticancer supplement formulation. The physicochemical properties has been proposed as an indirect measure of yield of powder, moisture content, encapsulation efficiency of total phenolic and flavonoid content, and dissolution rate of materials because all of these properties can influence on it [15]. The values were obtained and they show acceptable good flow properties. Normality in particle size distribution of powders in this formulation allows them to flow and encapsulated well. In all cases, powder humidity was minor than 10%, value reported as good [16]. No relationship of these responses (particle size and humidity) with the excipients proportion variability was observed. Maltodextrine (MD) was the excipient that makes the greatest variability in the formulation of anticancer supplement powder. High amounts of this excipient in formulations, improve the production of

powder during spray drying process. Silicone dioxide and vitamin D_3 showed little influence on this property. Yield of powder was increase when the amount of MD in the formulation increased. The normal distribution and appropriate residual moisture in powders improve the flow ability and fluidity of these powders [17].

Independent variables					Responses				
Sample	X_1 A	X_2 B	X_3 C	X_4 D	Y_1 YOP (%)	Y_2 MC (g/g)	Y_3 EEP (%)	Y_4 EEF (%)	Y_5 DR (mg/sec)
1	1.000	0.000	0.000	0.000	7.28	8.05	9.0	10.0	0.4238
2	0.769	0.154	0.077	0.000	4.72	5.3	5.18	1.26	0.2705
3	0.923	0.000	0.077	0.000	4.44	5.12	29.52	32.31	0.7672
4	0.462	0.077	0.077	0.385	13.63	9.24	21.64	6.14	0.2500
5	0.885	0.077	0.038	0.000	8.07	7.65	13.44	13.95	0.8100
6	0.577	0.000	0.038	0.385	12.03	11	28.08	4.77	0.3655
7	0.154	0.000	0.077	0.769	5.51	6.15	44.01	32.17	0.4800
8	0.846	0.154	0.000	0.000	7.37	9.96	6.59	0.24	0.3372
9	0.038	0.154	0.038	0.769	7.75	7.15	29.33	25.47	0.9805
10	0.154	0.077	0.000	0.769	10.68	6.85	5.35	9.92	0.8500
11	0.308	0.077	0.038	0.577	5.86	6.53	26.12	13.06	0.1288
12	0.750	0.038	0.019	0.192	5.73	6.59	44.65	26.95	0.6522
13	0.635	0.115	0.058	0.192	6.27	7.62	13.17	10.87	0.5305
14	0.462	0.154	0.000	0.385	11.49	8.62	15.99	12.77	0.3000
15	0.231	0.000	0.000	0.769	8.74	9.84	0.39	0.34	0.7878
16	0.923	0.000	0.077	0.000	7.24	7.65	15.7	13.02	1.3700
17	0.038	0.154	0.038	0.769	10.3	8.96	6.9	8.34	0.8555
18	1.000	0.000	0.000	0.000	13.73	9.55	33.29	10.54	0.3200

Fig (1) D-Optimal Mixture Design Arrangement and Responses Value for Formulation Process.

The solubility of the powder using the dissolution rate were evaluated. The best dissolution rate (0.1288 mg/sec) takes place when the amount of MD in the formulations was 5% which shown in Runs 11. Silicon dioxide had no significant effect on the variability of the flow properties. The higher flow rate occurs when the amount of silicon dioxide in the formulation is low (1%). For uncoated ingredients a time not more than 15 min is recommended. The statistical significance of the ANOVA test ($p<0.05$), the higher values of R2 and Q2, and the lower PRESS compared with the other considered models, demonstrated that the quadratic model is the better predictor of the dissolution time variability. It was observed that there was a statistical significance for interactions between ingredients ($p<0.05$). All interactions tend to reduce the dissolution time. Figure 2 (A), (B), (C), (D), and (E) presents a trace graph showing the influence of the excipients in the supplement. Based on the traces plotted graph, the line for the leaf extract (A) and MD (D) were seen as the longest compared than to the other two. The ranges of MD and the extract used in this formulation were high compared to other ingredients to increase the production powder obtained as well as to aid the spray drying process. the line for vitamin D_3 (B) and silicon dioxide (C) can be seen shorter than the line A and D due to the several reasons prior to the toxicity ranges of silicon dioxide that need to be followed in developing the anticancer formulation supplement product.

Formulation Optimization

For the optimization of this oral dosage form, a numerical method based on desirability function was used. This method takes into account various criteria for different response in only one mathematical equation. Table (2) shows the predicted solution of the optimized anticancer capsule supplement formulation. From the previous discussion, the maximum of all responses data were attained at desirability near to 1. A t-test was made to compare the observed physicochemical properties with the predicted values for the optimization model. There were no statistical different between observed and predicted values in this formulation (p-value>0.05). The results for optimum condition of the formulation were obtained from RSM using Mixture Design D-optimal Design was verified by carrying out the experiment using the optimum formulation condition. From table (2), optimized formulation for graviola leaf extract to obtain maximum yield of powder, minimum moisture content, maximum encapsulation efficiency for total phenolic content and total flavonoid content, and maximum dissolution rate was achieved at the maximized parameter range. The obtained results demonstrate that the D-optimal mixture design can be properly used for the optimization of the graviola extract capsule supplement. The rest of the technological properties for this formulation were satisfactory [15]. The D-optimal mixture design allowed the obtaining of optimal proportions of the excipients for the anticancer supplement formulation. MD was the excipient that showed more influence on the physicochemical properties. The obtained powder showed good technological properties. The proportions of the optimal mixture; expressed as percentage was MD 6.58%, silicon dioxide 1.25%, and vitamin D_3 meanwhile for graviola leaves extract was 90.18%.

Table (2) Predicted solution of the optimization of processing parameters of formulation

Extract (%)	Vit. D_3 (%)	SD (%)	MD (%)	YOP (%)	MC (%)	EE TPC (%)	EE TFC (%)	DR (mg/sec)
90.18	2.00	1.25	6.58	6.87	6.80	32.20	24.89	1.07

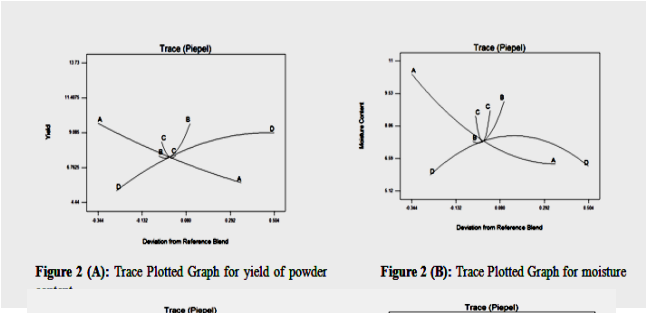

Fig (2) (A), (B), (C), (D) and (E) Trace Plotted Graphs of Expedients in Supplements Influences.

Tryphan Blue Dye Exclusion Viability Cell Count

Figure (3) shows the viability of cell of optimum spray dried formulation on HT-29 colorectal cancer cell. It was observed that the percentage of cell inhibition increases with the increase of samples's concentration. At lower concentration, optimum spray dried anticancer formulation stimulate the growth cell. At concentration from 25 µg/ml, the HT-29 cells started to suppressed significantly with p-value are less than 0.05. The cells density of treated cells reduced significantly compared to untreated cells. This showed that both extracts did cause suppression in proliferation on HT-29 cancer cell lines. The morphological cells was observed under the microscope before the initial seeding and continued to observe after given the treatment. In normal cell, there is no alteration or proliferation occurred even at the highest concentration of samples. Therefore, both samples caused no toxicity or harm on normal PDL cell lines.

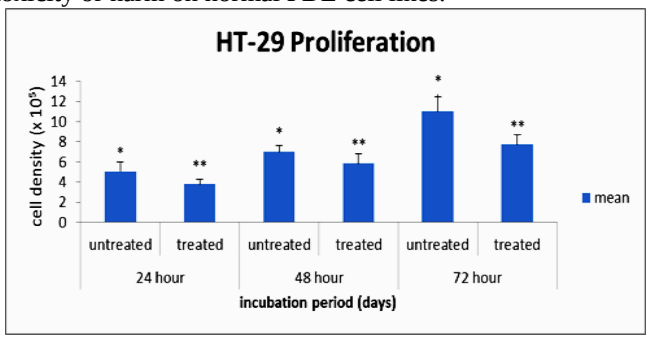

Fig (3) Cytotoxic study of optimum spray dried formulation on colon cancer cell, HT-29 cell lines. (*Results were expressed as mean for triplicate wells ± SE [*, $p < 0.05$; **, $p < 0.01$*]).

Field Emission Scanning Electron Microscopy (FESEM)

The electron micrographs showed that smooth spheres of spray-dried particles containing graviola leaf for anticancer formulation coated with maltodextrin and other excipients compared to the optimum crude extract were formed. The particle size of spray dried sample is acceptable within the range of 6.7µm to 7.3µm taken consideration of nozzle atomization used and addition of other excipients in the formulation. As can be seen in figure (4) (A), the morphological structure of the optimized spray dried formulation with coating material (MD) has smooth spherical shape compared to the crude optimum extract (B). The type and the amount of the excipients seem to affect greatly on the quality and performance of the formulation [18] especially on shape of the particles [19]. An addition of polymers (maltodextrine) was noted to increase surface roughness [20] whereas an increase in colloidal silicon dioxide was found increasing flowability properties, and stability [21]. The particle size of spray dried sample is depends on the numbers of factors.

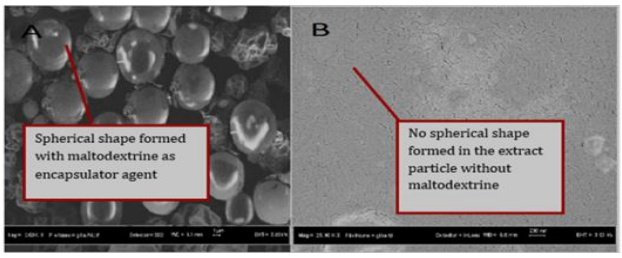

Fig (4) FESEM images of the spray dried particles of the optimized anticancer formulation (A) microcapsules and the optimized extract of *A.muricata leaf* (B) prepared with gold coating before scanning.

4. CONCLUSIONS

D-optimal mixture design was successfully applied for optimization of formulation ingredients of anticancer supplement from *A. muricata* leaves extract. The high regression coefficient of quadratic polynomial of the response showed that model fitted with the data well. After the optimization process, the optimal ingredients composition that fulfilled the requirement for yield of powder, moisture content, encapsulation efficiency of total phenolic and flavonoid content, and dissolution rate were found to be the leaf extract with 90.18%, vitamin D_3 with 2.00 %, silicon dioxide with 1.25%, and maltodextrine with 6.58%. Cytotoxicity study showed that there is no apparent effect of the optimized anticancer formulation cytotoxicity of the spray dried leaf on normal PDL cell lines since the graph trending was similar among the samples with the untreated cells. Meanwhile in treating the cancer cells (HT-29), it was observed that the percentage of cell inhibition increases with the increase of samples's concentration. FESEM results showed that smooth spheres of spray-dried particles containing *A.muricata* leaf for anticancer formulation coated with maltodextrin and other excipients were formed. Therefore, the optimized formulation was safe to be used for next step of investigations.

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