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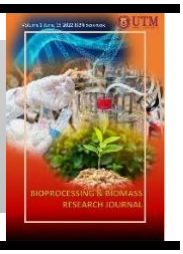

Research Article

Optimizing Wall Material Ratios for Enhanced Bioactive Compound in *Ficus deltoidea* using Freeze Drying

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ABSTRACT

Ficus deltoidea (*FD*) is well documented for its pharmacological properties, including anticancer, antibacterial, anti-inflammatory, antiviral and anti-aging properties. However, the stability and adsorption of many bioactive compounds in herbs are poor, limiting their bioavailability. This study aimed to investigate the optimal formulation ratios and different wall materials (maltodextrin (MD) and gum Arabic (GA)) for encapsulating FD extract using freeze drying. Physicochemical characterizations of the formulated *FD* powders included evaluation of marker compound (isovitexin), phenolics (TPC) and flavonoids (TFC) content. The safety of the optimized encapsulated FD (OEFD) powder was assessed through microbiological assay. The optimal conditions, determined through response surface methodology (RSM), were a solid ratio of 20% w/v, a 75% MD: 25%GA and 3:1 of core to wall ratio, which resulted in the highest retention of isovitexin at 1.96 g/g, phenolic content of 1473 GAE mg/ 10 g extract and flavonoids content of 185 CE mg/ 10 g extract. Optimizing the freeze -drying process for FD extract improved its quality by retaining the marker compound. This optimization ensures that the bioactive compounds remain stable and effective, enhancing the therapeutic potential of the extract.

INTRODUCTION

Ficus deltoidea (FD), commonly known as Mas Cotek, exhibits various health benefits, including antioxidant, antimicrobial, anti-diabetic, anti-inflammatory, and anti-cancer properties. Several studies have reported that different parts of FD such as leaves, stems, roots, and fruits demonstrate significant antioxidant activities due to their rich phenolic and flavonoid content (Misbah *et al*., 2013). Markers of *FD* such as isovitexin and vitexin are responsible for enzyme inhibitory of α-glucosidase and α-amylase (Farsi et al., 2011) (Choo *et al*., 2012). Research has reported promising anticancer properties of FD extracts against various cancer cell lines, including breast, colon, lung, prostate, liver cancers, and leukemia. Notably, aqueous FD extracts and those obtained using solvents of varying polarities, such as ethyl acetate, methanol, and ethanol, have demonstrated positive effects in anticancer studies (Abolmaesoomi *et al*., 2019).

However, many plants bioactive compounds exhibit low bioavailability due to poor solubility in aqueous solutions. Encapsulation techniques have been shown to improve the bioavailability of these compounds and reduce drug toxicity (Munin & Edwards-Lévy, 2011). Encapsulated extracts serve as effective drug carriers, enhancing aqueous solubility and stability. This encapsulation protects the molecules from environmental degradation, provides the desired pharmacokinetic profile, allows controlled release, and facilitates oral administration. Several studies have demonstrated that encapsulating FD extracts improves skin penetration efficacy using nanostructured lipid carriers such as liposomes, niosomes, and virgin coconut oil (VCO) (Azmi, 2018; Tran *et al*., 2022).

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An important step in the encapsulation process is the careful selection of wall materials based on the active ingredient, the system in which it will be applied, and the desired release mechanism. One simple and effective encapsulation technique is freeze drying, which dehydrates emulsions at low temperatures under vacuum conditions. Commonly, maltodextrin (MD) and gum arabic (GA) are used as carrier agents for encapsulating juice powders from whole fruits and herbs. Several studies have optimized the ratio of MD to GA to achieve stable and safe product yields (Heredia *et al*., 2024; Shishir & Chen, 2017). Antimicrobial analysis is vital in the production and quality assurance of encapsulated freeze-dry formulations. It not only ensures the safety and efficacy of the product but also helps in complying with regulatory standards and maintaining consumer trust. Robust pathogen detection protocols are essential for the production of high-quality, safe products in various industries.

Most FD products available today are typically not extracted but ground into powder form, which can present issues related to quality and safety. To our knowledge, this is the first study to investigate the optimisation of aqueous FD leaf extracts using a combination of maltodextrin (MD) and gum arabic (GA) with a freeze dryer. This study aims to optimize the blend of MD/GA to stabilize the bioactive compounds.

MATERIALS AND METHOD

Materials

FD var. *kunstleri* leaves was procured from Moro Seri Utama Enterprise, Batu Pahat, Malaysia. HPLC- grade methanol, formic acid, ethanol, Folin–Ciocalteu reagent purchased from Merck® (Germany). Isovitexin standard was purchased from Sigma Aldrich® (USA).

Preparation of FD Leaves Aqueous Extract

The dried leaves of FD var. *kunstleri* were grounded into powdered using waring blender followed by the extraction with sterile distilled water at the ratio of 1:42 (w/v). The mixture was then heated and stirred on hotplate for 1 h and 50 min at 90 °C followed by cooling and filtration using filter paper and filter funnel. The sample then concentrated under reduced temperature (40 ℃) using rotary evaporator (IKA, USA) and subjected to lyophilization by a freeze-dryer (Labogene,Denmark) to produce a dark brown color powdered form of the extract.

Wall Materials and Encapsulation of FD Freeze-Dried Extract

Box Behnken Design (BBD) acquired from Design Expert 12 was employed to study the interaction effects between processing parameters (independent variables) and its responses (dependent variables) in order to predict the optimum wall materials ratio for the encapsulation active compound. A series of formulation was developed using factorial design by combining a different ratio of wall material such as maltodextrin and Arabic gum. The BBD will be used to study the interaction of significant process variables after screening to predict the optimum process condition for production of encapsulated extract by applying Response surface methodology (RSM). Statistical tool for the analysis variance (ANOVA) is used to analyze the data from experiments and to make decision about whether a given factor has a significant optimum on the response variable. The concentrated extract was mixed with different wall materials, water, and a combination of wall material at different ratio based on design of experiment (DOE) suggestion. The mixture was stirred gently with the mixer to dissolve solid particles prior to freeze drying. Then the samples were freeze dried using pilot scale freeze dryer. The entire freeze-drying process will be carried out in 48 h. The encapsulated powders will be collected, packed in polythene bags, and stored in a desiccator for further characterization.

Determination of Phenolics and Flavonoids Content

Total phenolic content (TPC) of encapsulated extract was carried out using Folin-Ciocalteu assay following Husin *et al.*(2024) using gallic acid as standard (ranging from 0.02 - 0.10 mg/ml). In brief, 5 ml of Folin-Ciocalteu reagent (10 %) was added into 1 ml of the sample or standard and mixed for 5 min. After that, 4 ml of sodium carbonate (5%) solution was added and left for 60 min. The absorbance was read at 760 nm and the results of total phenolic content expressed as gram of garlic acid equivalents (GAE) per 10 g of extract. Following Soib *et al.* (2015) for total flavonoid content (TFC), 4 ml of distilled water was added into 1 ml of sample or standard stock solution, followed by 0.3 ml of sodium nitrate, $NaNO_2$ (5%). After 5 min, 0.3 ml of aluminium chloride, $AlCl_3$ (10 %) was added into the volumetric flask and the mixtures were left for another 6 min. Next, 2 ml of sodium hydroxide, NaOH (1 M) was added followed by distilled water to make up to a 10 mL of volumetric flask. The absorbance was read at 510 nm using microplate reader (Biobase, China) and the result was expressed as a gram of catechin equivalents (CE) per 10 g of extract.

Determination of Isovitexin Content by HPLC

The amount of marker compounds (isovitexin) was identified using a High-Performance Liquid Chromatography (Millford,USA). A reversed-phase Nucleosil C18 column was maintained at 25 °C, while mobile phase consisting of methanol (absolute): formic acid (1%) was used. The elution programed at flow rate of 1 ml/min. The stock solution of isovitexin (2 mg) was prepared in 2 ml of ethanol. A standard concentration was further diluted to obtain concentration of 100 µg/ml to 1 µg/ml. The stock solution and samples are filtered through 0.45 µm nylon filter. An amount of 10 µl of the samples was injected into the column and monitored using UV detector at 330 nm. The separation time estimated was 30 min.

Microbial Analysis

Total Plate Count (Pour Plate Method)

One gram of sample was dissolved in 9 ml of buffered sodium chloride-peptone solution pH 7.0, and 9 mL of sterile diluents were prepared in universal bottles. 1 ml of the first dilution was transferred into next dilution blank. Dilutions up to 10^{-5} were prepared accordingly. One milliliter of the dilution was transferred into a petri dish and 20 ml of soybean casein digest agar medium that was previously melted and cooled to approximately 45 °C was added to each petri dish. The plates containing agar were left to solidify at room temperature. Positive control was performed by inoculating with reference culture and was

performed as per test procedure. Blank media was treated as negative control. Then, the plates were incubated for 5 days at 30-35 °C and the results were recorded.

Total Yeast and Mold Count (Pour Plate Method)

One gram of sample to be examined was dissolved in 9 ml of buffered sodium chloride-peptone solution. If the product is known to have antimicrobial activity, an activating agent may be added to the diluent. pH of samples was adjusted to about pH 7. Several dilution bottles were prepared as needed, each containing 9 ml of sterile diluent. The solution was thoroughly mixed by using vortex over a period of about 12 sec. One milliliter of the first dilution was transferred into next dilution blank. Dilutions were prepared up to 10^{-6} and 1 ml was transferred for each dilution into petri dishes with 20 ml of Sabouraud Dextrose Agar Medium at not more than 45 ℃. The agar was allowed to solidify at room temperature and both positive and negative control was also prepared. The culture plates were then incubated at 20-25 ℃ for 5 days.

Detection of *Escherichia coli*

One gram of the product was used to inoculate the corresponding bacterial species in 9 ml of Casein Soya Bean Digest Broth and was incubated at 30 to 35 °C for 18 to 24 h. After incubation the Casein Soya Bean Digest Broth was shaken thoroughly. Then 1 ml from Casein Soya Bean Digest Broth was transferred into 100 ml of MacConkey Broth and incubated at 42-44 ℃ for 18–24 h. By mean of an inoculating loop, bacterial suspension was sub-cultured from the MacConkey Broth onto the MacConkey Agar and incubated at 35-37 ℃ for 18–72 h. Presence of *E. coli* was spotted with pink colonies pink-red colonies. Gram negative rod was observed through gram staining. Uninoculated media was treated as negative control.

Detection of Enterobacteria and Gram-negative bacteria

One gram of sample to be examined was dissolved in 10 ml of Casein Soya Bean Digest Broth solution pH 7.0, homogenized and incubated at 20- 25 ℃ for 2 to 5 h. From incubated broth, a one in ten dilution was prepared up to dilution of 10^{-3} with buffered sodium chloride-peptone in required number of universal bottles. After that 1 ml Casein Soya Bean Digest Broth of from each dilution of 10^{-1} to the 10^{-3}, were transferred to 9 ml of Enterobacteriaceae Enrichment Broth (Mossel) accordingly and incubated at 30-35 ℃ for 24 to 48 h. The culture of Enterobacteriaceae Enrichment Broth (Mossel) was then sub cultured onto the Violet-red Bile Agar (VRBG) and incubated at 35-37 °C for 18-24 h. Positive control was performed on VRBG and negative control was the uninoculated media. The growth of well-colonies was generally red or reddish of colonies hence it shows as positive result with gram-negative bacteria constitutes. Gram staining was performed as referred to standard operating procedure.

Detection of Salmonella species

Ten grams of sample to be examined was dissolved into 90 ml Casein Soybean Digest Broth, homogenized, and incubated at 30-35 ℃ for 18 to 24 h. After incubation, 0.1 ml of Casein Soybean Digest Broth culture was pipetted into 10 ml of Rappaport Vassiliadis Salmonella enrichment broth and incubated at 30-35 °C for 18 to 24 h. By means of an inoculating loop, enrichment culture was streaked from Rappaport Vassiliadis Salmonella enrichment broth on Xylose Lysine Deoxycholate Agar. The culture plates were then incubated at 30-35 ℃ for 18 to 48 h. Both positive and negative control was prepared according to test procedure. The probable presence of Salmonella is indicated by the growth of cultures having well developed red colonies, with or without black centers.

Detection of *Pseudomonas aeruginosa*

Ten milliliters of sample were inoculated onto 100 ml of soya-bean casein digest medium, homogenized, and incubated at 37 ℃ for 18 to 48 h. It was then sub cultured on cetrimide agar plate and incubated at 37 ℃ for 18 to 72 h. Positive control and negative control was prepared accordingly. The sample passes the test if there were no growth of microorganism on the plate. Vice versa, gram staining was done. The colonies were transferred to soya-bean casein digest medium and incubated at 43 ℃ for 18 to 24 h. The sample passes the test when there is no growth occurs at 43 ℃.

Detection of *Staphylococcus aureus*

Ten milliliters of samples were dissolved in buffered sodium chloride-peptone solution pH 7.0 and were tenfold diluted. Then 10 ml of samples were inoculated into 100 ml of casein soybean digest broth, homogenized and incubated at 37 ℃ for 18 to 48 h. The medium was then subculture on the surface of Baird-Parker Agar (BPA). The agar was then incubated at 37 °C for 18 to 72 h together with its control. Upon examination, if there was a growth of black colonies surrounded by clear zone, Gram staining will be performed. If Gram-positive cocci were spotted, therefore the assay was proceeded with further confirmation tests. Both positive and negative control was performed as per test procedure.

RESULTS AND DISCUSSION

Optimization of Encapsulated FD with Wall Materials

The encapsulation of FD extract was optimized using Box-Behnken design. In order to optimize the encapsulation process of FD extract, 17 experimental runs were carried out using three factor variables which were solid (w/v), wall material (maltodextrin: Arabic gum) and core to crude extract. The response for encapsulation parameters which is TPC, TFC and isovitexin concentration were the response investigated for the variables selected. From **Table 1**, it can be deduced that the TPC value ranged from 179 - 1946 mg/10g GAE equivalent while TFC value ranged from 52 - 216 mg/10g CE equivalent, respectively. For isovitexin content, the value ranged observed was from 0 - 1.96 g/g. Maltodextrin is one of the common drying aids for drying, owing to its beneficial role as a carrier agent for encapsulating. The degree of protection is directly related to the dextrose equivalent (DE) of the hydrolyzed starch. Higher DE systems are less permeable to oxygen and result in powders with higher encapsulation efficiencies.

However, this ingredient lacks any emulsifying properties and typically result in poor retention of flavors during drying therefore Arabic gum is used in combination as a surface-active biopolymer.

Table 1: Box Behnken design and response variables for encapsulation of FD extract with selected wall materials.

Run	Independent Variables			Responses		
	X_1	X_2	X_3	Y_1	Y_2	Y
	Solid (%)	Wall material	Core to crude extract ratio	TPC	TFC	Iso-vitexin
1	10	25	1.625	1458	192	0.49
2	30	25	1.625	757	143	0.1
3	20	50	1.625	1011	132	0.24
4	10	75	1.625	1456	178	1.23
5	30	50	3	1168	141	0.49
6	20	25	3	1494	182	0.49
7	30	50	0.25	179	52	0.5
8	20	50	1.625	957	134	0.27
9	20	50	1.625	1005	133	0.29
10	20	25	0.25	271	75	0.39
11	20	50	1.625	1018	133	0.26
12	20	75	3	1473	185	1.96
13	10	50	0.25	508	61	0.29
14	30	75	1.625	741	143	0.39
15	10	50	3	1946	216	0.99
16	20	50	1.625	927	132	0.26
17	20	75	0.25	257	56	0.26

Influence of Solid, Wall Material and Core to Crude Extract Ratio on TPC, TFC and Isovitexin Content

The overall predictive capabilities and accuracy of these models were expressed by the coefficient of determination, R^2 and lack of fit test. The R^2 values of the models were 0.9954, 0.9998 and 0.9713 for TPC (**Table 2**), TFC (**Table 3**) and isovitexin (**Table 4**), respectively, indicating that the variations in the models were adequately explained by the regression model. The high R^2 values suggest that the models have strong ability to predict the responses accurately. There was no significant ($p > 0.05$) lack of fit for TPC and TFC responses, meaning that the models were accurately represent the data in the experimental regions except for isovitexin response ($P<0.05$). F-values and p-values demonstrate the significance of the experimental variables. The high F-value with a low probability p-value for the models ($p < 0.05$) indicated the significance of independent variables in the fitted models. This implies that the models effectively capture the relationship between the experimental factors and the responses, except for the isovitexin response, which requires further investigation to improve model fit.

Among all the experimental runs, run 12 showed the optimum condition namely optimum encapsulated FD (OEFD). The OEFD with formulation 20% w/v of solid ratio, 75% MD: 25% GA of wall ratio, and 3 to 1 of core to wall ratio exhibited highest retention of 1.96 g/g (%) of isovitexin during the freeze-drying process. This OEFD also demonstrated high phenolic content at 1473 GAE mg/ 10 g extract and flavonoids content at 185 CE mg/ 10 g extract.

Besides that, Table 1 depicted that increasing maltodextrin content at 50% and 75% in formulations resulted higher retention of phenolics, flavonoids and biomarker compound such as isovitexin. This may indicate that nature of wall material and combination of matrix composition had shown significant wall-core interactions. In which, its quadratic terms in **Table 2, 3** and **4** have shown strong impact of phenolics, flavonoids and isovitexin retention at increasing maltodextrin content.

Table 2: ANOVA for TPC response

Source	Sum of Square	df	Mean Square	F-value	p-value
Model	3.915E+06	9	4.350E+05	166.69	< 0.0001
A-solid (w/v)	7.958E+05	1	7.958E+05	304.93	< 0.0001
B-wall MD:GA (%)	366.66	1	366.66	0.1405	0.7189
C-core to crude ratio	2.959E+06	1	2.959E+06	1133.91	< 0.0001
AB	49.43	1	49.43	0.0189	0.8944
AC	50377.78	1	50377.78	19.30	0.0032
BC	11.46	1	11.46	0.0044	0.9490
A^2	40595.13	1	40595.13	15.56	0.0056
B^2	1947.69	1	1947.69	0.7463	0.4163
C^2	72485.24	1	72485.24	27.78	0.0012
Residual	18267.80	7	2609.69		
Lack of Fit	11977.88	3	3992.63	2.54	0.1949
Pure Error	6289.92	4	1572.48		
Cor total	3.933E+06	16			

Table 3: ANOVA for TFC response

Source	Sum of Square	df	Mean Square	F-value	p-value
Model	38775.45	9	4308.38	3572.37	< 0.0001
A-solid (w/v)	3511.95	1	3511.95	2911.99	< 0.0001
B-wall MD:GA (%)	104.83	1	104.83	86.92	< 0.0001
C-core to crude ratio	28913.95	1	28913.95	23974.49	< 0.0001
AB	41.91	1	41.91	34.75	0.0006
AC	1064.55	1	1064.55	882.69	< 0.0001
BC	117.42	1	117.42	97.36	< 0.0001
A^2	605.80	1	605.80	502.31	< 0.0001
B^2	1521.29	1	1521.29	1261.40	< 0.0001
C^2	3154.77	1	3154.77	2615.83	< 0.0001
Residual	8.44	7	1.21		
Lack of Fit	6.19	3	2.06	3.67	0.1208
Pure Error	2.25	4	0.5629		
Cor Total	38783.90	16			

Table 4: ANOVA for isovitexin response

Source	Sum of Square	df	Mean Square	F-value	p-value
Model	4.00	9	0.4443	26.32	0.0001
A-solid (w/v)	0.5100	1	0.5100	30.21	0.0009
B-wall MD:GA (%)	0.7812	1	0.7812	46.28	0.0003
C-core to crude ratio	1.22	1	1.22	72.08	< 0.0001
AB	0.0506	1	0.0506	3.00	0.1269
AC	0.0110	1	0.0110	0.6531	0.4456
BC	0.7482	1	0.7482	44.32	0.0003
A^2	0.0062	1	0.0062	0.3649	0.5648
B^2	0.4495	1	0.4495	26.63	0.0013
C^2	0.1978	1	0.1978	11.72	0.0111
Residual	0.1182	7	0.0169		
Lack of Fit	0.1168	3	0.0389	118.03	0.0002
Pure Error	0.0013	4	0.0003		
Cor Total	4.12	16			

Microbial Analysis

The results of microbiological analysis showed that OEFD from run 12 formulation was clear from pathogenic bacteria (**Table 5**). This product formulation was free from *Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella* sp. and *Escherichia coli* below the standard range reported.

Table 5: Microbiological analysis result of encapsulated FD extract

Parameter	Unit	Result
Total Viable Aerobic Count (Bacteria Count)	CFU/g	$6.8x10^2$
Total Viable Aerobic Count (Fungi Count)	CFU/g	$1.0x10^1$
Staphylococcus aureus	Absent/Present in 10 ml	Absent
Pseudomonas aeruginosa	Absent/Present in 10 ml	Absent
Salmonella	Absent/Present in 10 ml	Absent
Enterobacteria and certain other Gram-negative bacteria	MPN/ml	$< 10^3$
Escherichia coli	Absent/Present in 10 ml	Absent

CONCLUSION

From the results, a solid ratio of 20% w/v, a 75% MD: 25% GA and 3:1 of core to wall ratio effective in preserving the bioactive compounds in FD extract, specifically phenolics, flavonoids, and the biomarker isovitexin. Additionally, no microbial contamination was found in the optimized encapsulated FD. Therefore, the optimized formulation is safe for further investigations.

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