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ULTRASONIC - ASSISTED EXTRACTION AND MICROENCAPSULATION OF BIOACTIVE COMPOUND FROM PIGEON PEA SEED

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The global COVID-19 pandemic that began at the end of 2019 has not finished until now. So it is necessary to use local food that contains antioxidant compounds that can improve the immune system. Pigeon pea is one of the indigenous foods that can be utilized as a functional food because it contains bioactive compounds—the objective of this work is to obtain the optimal condition for extracting pigeon pea seed based on total phenol content (TPC) and antioxidant activity and then microencapsulation it. It was demonstrated that the best extraction conditions were 30 min, a temperature of 60 °C, and 90% methanol with TPC 36.0219 mg GAE/30 g dry sample and antioxidant activity 19.6091 mg AAE/30 g dry sample. Pigeon pea seed extract was entrapped by Freeze-drying using gum arabic and maltodextrin as wall material. Microcapsules contained 8.85% water with a solubility of 87.35%. Microcapsules have a color of greenish-yellow chromaticity (GY) and irregular shape with an average size of 23.03 μm. The best bioactive compounds in pigeon pea seed extract showed the most compounds: mome inositol (42,76%) and isoflavones (13,85%). In addition, there are several other compounds showing bioactivity as antioxidants.

Keywords: *Pigeon Pea, Ultrasonic-assisted extraction, Response Surface Methodology, Microencapsulation.*

INTRODUCTION

The global COVID-19 pandemic that began at the end of 2019 has not finished until now. There have been multiple waves of COVID-19 in Indonesia, with the number of cases increasing daily. During the transition from pandemic to endemic, strengthening the community's immune system is crucial for controlling COVID-19 cases. Because of that, it is vital to develop functional foods with immunomodulatory properties to enhance people's immune systems.

Immunomodulators are chemicals that alter the immune system and have the capacity to stimulate the immunological response[1]. Functional foods with immunomodulatory qualities can play a significant role in combating the present COVID-19 pandemic by modifying the host immune system, creating antiviral activity in the host, and manufacturing biologically active molecules that are effective against the virus[2]. Local foods that are unfamiliar to most people can provide dietary benefits. Utilizing local food as a functional food source will enhance the local food's value. Pigeon pea is one of the indigenous foods that can be utilized as a functional food.

Pigeon pea *(Cajanus Cajan L.)* is one of South Sulawesi's native foods. Pigeon pea is also referred to as *bintutoeng* in some places and *kence* in Bugis. Flavonoids and isoflavones such daidzein, genistein, chrysin, apigenin, quercetin, and kaempferol are found in pigeon pea[3]. Isoflavones are a type of flavonoid with antioxidant, anticancer, antibacterial, and anti-inflammatory effects[4]. Additionally, daidzein and genistein molecules are predicted to be bioactive inhibitors of COVID-19 replication[5]. Bioactive chemicals can be obtained from

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isoflavones found in pigeon pea seeds by extracting them.

The Ultrasonic-assisted extraction (UAE) method is advantageous because it is simple to use, safe, performed at atmospheric pressure and room temperature, and the extraction process is quick, resulting in greater effectiveness and efficiency[6]. In addition to the extraction technique, optimization of extraction must be performed utilizing Response Surface Methodology (RSM). By constructing suitable mathematical equations, RSM can determine the interaction of several variables. Optimization utilizing RSM does not require a large amount of data. Therefore, optimal response conditions can be produced fast and cheaply.

The extracted bioactive molecules with antioxidant capabilities are unstable and susceptible to degradation by environmental factors. Therefore, microencpsulation is used. Microencapsulation is a technique that encases a core substance in a polymer wall layer, converting it into microparticles. By encapsulating extracts of natural substances in microcapsules, phytoconstituents are protected, their stability is increased, and activity loss is prevented[7].

Pigeon peas can be a functional food that acts as an immunomodulator. Ultrasonic-assisted extraction(UAE) and microencapsulation of pigeon pea extract have never been investigated. It is necessary to microencapsulate the bioactive compounds found in pigeon pea extract in order to protect them from environmental factors. During the COVID-19 pandemic, the microcapsules produced may be advantageous for the body's immune system.

MATERIAL AND METHODS

Materials

Dried Pigeon Pea Seeds (Fig. 1) were purchased from the local market. Technical methanol, methanol p.a from Merck, distilled water, Ascorbic Acid p.a, gallic acid from Merck, Folin-Ceocalteu reagent, sodium carbonate (Na_2CO_3), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) from sigma Aldrich, gum arabic and maltodextrin.

Fig. 1: Pigeon Pea Seed (*Cajanus Cajan L.*).

Pigeon Pea Seed Preparation

Dried pigeon peas are sorted from dirt and sand. Then grind it using a grinder. The mashed sample has a range of 60-120 mesh. The output of the sample preparation is pigeon pea powder which will be extracted.

Ultrasonic assisted extraction (UAE) with Box-Behnken Design (BBD)

Each sample of 30 grams was extracted using 90 ml methanol solvent by sonication based on the Box-Behnken experimental design. The extraction result is then concentrated with a rotary evaporator.

Total Phenol Content (TPC) Determination

A sample of 0.2 ml was combined with 0.5 ml of 10 % (v/v) Folin-Ciocalteu and let stand for 3 min. After adding 1 ml of 10% (w/v) Na_2CO_3, the mixture was homogenized and left to stand for 30 min at room temperature. A spectrophotometer was then used to measure the absorbance. As the standard curve, gallic acid was used. This analysis results in the total phenol content concentration in the pigeon pea extract.

DPPH radical scavenging ability assay (Modification Belwal et al.[8])

Pipetting 1 ml of the sample solution and 3 ml of 50 ppm DPPH was added to complete the sample testing. The mixture was then homogenized and incubated in a dark chamber at room temperature for 30 min. At 520 nm in wavelength, the absorbance was measured. The blank and ascorbic acid comparison tests were handled the same way. The outcome of this analysis is the amount of ascorbic acid present in the sample as a DPPH inhibitor.

Table 2: Box-Behnken experimental design.

Run	Code			Variable Independent		
	X_1	X_2	X_3	Time (Min)	Temperature (°C)	MeOH (%)
1	-1	-1	0	30	40	70
2	1	-1	0	50	40	70
3	-1	1	0	30	60	70
4	1	1	0	50	60	70
5	-1	0	-1	30	50	50
6	1	0	-1	50	50	50
7	-1	0	1	30	50	90
8	1	0	1	50	50	90
9	0	-1	-1	40	40	50
10	0	1	-1	40	60	50
11	0	-1	1	40	40	90
12	0	1	1	40	60	90
13	0	0	0	40	50	70
14	0	0	0	40	50	70
15	0	0	0	40	50	70
16	0	0	0	40	50	70
17	0	0	0	40	50	70

Optimization and Confirmation with Response Surface Methodology (RSM)

The gathered data were then examined using version 11 of the Design Expert software. Three repetitions of the optimal operating conditions were also performed for confirmation. The result is the optimal extraction conditions for pigeon peas. Response analysis using ANOVA with a significance p-value < 0.05.

Microencapsulation Extract Pigeon Pea Seed

Microcapsules containing extract of pigeon pea seed were created by combining gum arabic and maltodextrin in a ratio of 1:1. The ratio of pigeon pea seed extract to coating material is 3:7, and the concentration for each coating is 20%. The coating material is combined. The pigeon pea seed extract and coating mixture were combined for 30 min. The mixture was freeze-dried, then crushed and sieved through a 100-mesh screen. The output is microcapsule powder of pigeon pea seed extract.

Microcapsule Moisture Analysis

The difference between the initial weight and the weight of samples dried in an oven is then determined. The water content analysis output is the water proportion affecting the sample's quality and shelf life.

Solubility Test

The test was carried out by dissolving 1 gram of the sample into 20 mL of distilled water. Then filtered with filter paper and weighed the filter paper with the dried residue. The output is the percentage of microcapsule solubility.

Microcapsule Color Analysis

A Colour test was carried out with a chromameter. The output of this analysis is the color of the microcapsules.

Morphological Analysis

Morphological characteristics of microcapsules using a Scanning Electron Microscope (SEM). Microcapsules powder samples were placed in the specimen holder. Images at 100 x and 200 x magnification were recorded at 15 kV.

FT-IR (*Fourier Transform Infra Red)* Analysis

Tests were conducted to analyze the functional groups on the coating and microcapsules using Shimadshu's FTIR-8400s.

Identification of Bioactive Compounds with GC-MS

This was carried out using Shimadzu 2010 GC-MS and RTX-5 capillary column (30 mm x 0.25 mm x 0.25 µm) with a split ratio of 40:1 and a temperature of 70ºC, the heating rate of 10ºC min^{-1}, up to 300ºC, maintained for 5 min with a total analysis time of 25 min. Helium was used as a carrier gas, constantly flowing at 1.0 ml/min, and the temperature of the inlet was 280ºC, pre-column pressure was 80 kPa, and ionization voltage of 70 eV. Using GC-MS, the number of bioactive chemicals included in the optimal extraction condition of pigeon pea seed extract and microcapsules derived from pigeon pea seed extract sonicated for 10 and 30 min was determined.

RESULTS AND DISCUSSION

Box-Behnken Design Result

The responses indicate a correlation between independent variables or operating parameters that can influence the extraction results.

Effect of Operating Conditions on The TPC Response

The p-value for the analysis of total phenol in the quadratic model was below 0.0001, indicating that the model was significant. The response of the total phenol content is influenced by time, temperature, and methanol concentration, with p-values less than 0.05. The p-value for the lack of fit being greater than 0.05, which is 0.0983, indicates that it is not significant, indicating that the model is suitable for describing the extraction procedure.

The 3D surface plot displays a red color representing the highest total phenol concentration, which is 90% after 30 min. However, decreasing the concentration will diminish the total amount of phenol. The presence of water in the solvent will cause polyphenols to oxidize, hence diminishing their antioxidant capacity[9]. An increase in temperature will increase phenol compounds. However, phenolic compounds in contact with high temperatures for a long time will degrade these phenolic compounds[10–13].

Table 3: The results of the analysis (response) and independent variables using the Box-Behnken design (BBD).

Run	Independent Variable			Respons	
	Time (Min)	Temperature (ºC)	MeOH (%)	TPC (mg GAE/ 30 g dry sample)	Antioxidant Activity (mg AAE/ 30 g dry sample)
1	30	40	70	19.49	10.55
2	50	40	70	12.38	18.25
3	30	60	70	17.25	13.18
4	50	60	70	17.64	13.84
5	30	50	50	21.46	10.21
6	50	50	50	17.91	14.43
7	30	50	90	33.96	20.96
8	50	50	90	26.99	23.39
9	40	40	50	13.81	9.98
10	40	60	50	19.49	8.44
11	40	40	90	22.12	21.94
12	40	60	90	27.25	19.59
13	40	50	70	8.56	13.47
14	40	50	70	9.22	11.09
15	40	50	70	10.67	13.43
16	40	50	70	8.56	11.81
17	40	50	70	8.04	13.69

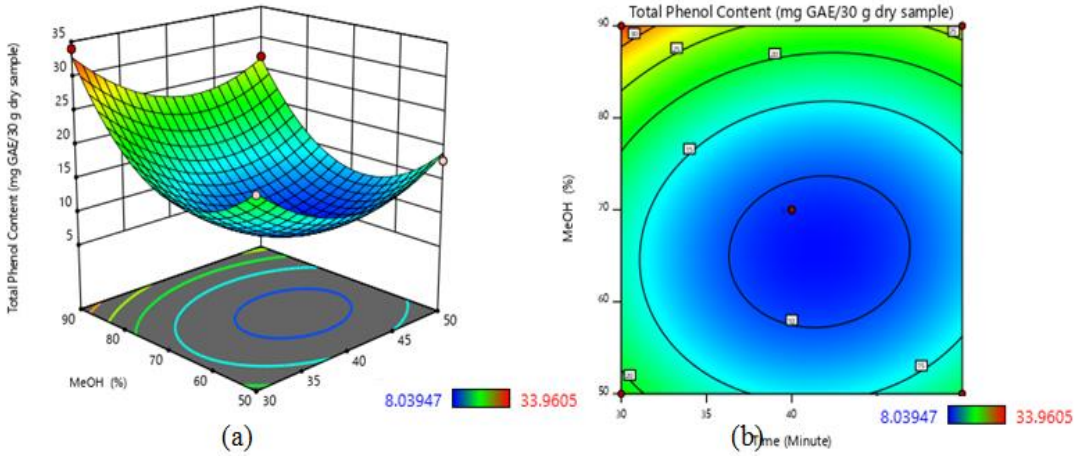


Fig. 2: The effect of time (min) and concentration (%) at 50 °C on the TPC response (a) 3D surface plot and (b) contour plot.

TPC (mg GAE/30 g dry sample) = 9,01316 – 2,15461X_1 + 1,72687X_2 + 4,70383X_3 + 1,87499X_1X_2 – 0,855257X_1X_3 – 0,131261X_2X_3 + 6,04256X^2_1 + 1,63497 X^2_2 + 10,0231X^2_3 (1)

The time, temperature, and concentration coefficients are -2.15461, 1.72687, and 4.70383. The coefficient values for the temperature and concentration factors show positive values so that the increase in temperature and time can increase the number of phenolic compounds obtained. In contrast, the negative time coefficient values indicate that a long time will produce lower compounds.

Effect of Operating Conditions on The Antioxidant Activity Response

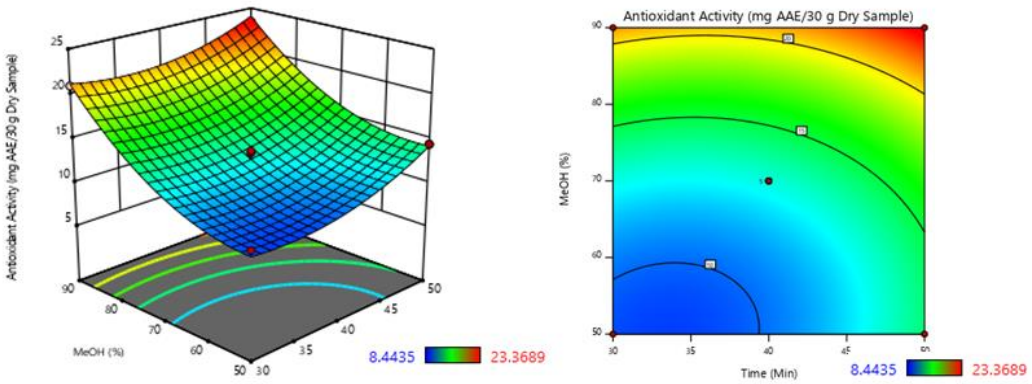
One of the most often used techniques to analyze antioxidant activity is the DPPH scavenging assay. When the DPPH reagent solution is combined with antioxidant molecules, which can donate hydrogen atoms, the solution's purple color generally disappears[14&15]. The initial absorbance of DPPH and the absorbance after potential antioxidants were added were measured. Then convert the sample DPPH inhibitory absorbance into an Ascorbic Acid Equivalent (AAE). Ascorbic acid, commonly called vitamin C, is one of the antioxidants that can donate one equivalent of a reducing agent to form monodehydroascorbates. Also, ascorbic acid reacts better with radical compounds than non-radical compounds[16].

The quadratic model for antioxidant activity response has a p-value <0.05, so the model is significant and appropriate to show the response of the antioxidant activity of the sample. The time and concentration factors showed p-value <0.05, that is 0.0016 and <0.0001. Thus, time and concentration significantly affect the antioxidant activity of the sample. The p-value for the lack of fit being greater than 0.05, which is 0.6590, indicates that it is insignificant.

The highest antioxidant activity value was at a solvent concentration of 90% and a time of 50 min. However, time-varying at a concentration of 90% still showed a high value of the antioxidant activity. The decrease in the concentration of the methanol solvent will reduce the antioxidant compounds extracted.

Antioxidant Activity (mg AAE/30 g dry sample) = 12,6994 + 1,87366X_1 – 0,708952X_2 + 5,34915X_3 – 1,75907X_1X_2 – 0,453095X_1X_3 – 0,202559X_2X_3 + 1,75534X^2_1 – 0,499465 X^2_2 + 2,78944X^2_3 (2)

Equation 2 shows the quadratic equation of the antioxidant activity of the extract. The time, temperature, and concentration coefficients are 1.87366, respectively; -0.708952; and 5.3491. The coefficient values for time and concentration factors show positive values so that increasing concentration and time can increase the number of antioxidant compounds obtained, while negative temperature coefficient values indicate that a long time will produce lower antioxidant compounds. These equations can predict the extraction process conditions to produce optimal antioxidant compounds for extracting pigeon pea seeds.



(a) (b)

Fig. 3: The effect of time (min) and concentration (%) at 50 °C on the antioxidant activity response (a) 3D surface plot and (b) contour plot.

Similar results were also obtained in the study by Al-Saeedi and Hossain[17], where methanol solvent is one of the extraction solvents with a relatively high TPC value compared to butanol and water but a lower TPC value than hexane and chloroform. For antioxidant activity, methanol solvent also has a high result of antioxidant activity. The conventional method utilized in this study also obtained significant TPC and antioxidant activity results, although it requires considerable time to extract it. In addition, the influence of sample preparation and sample size are considered to produce maximum results.

A correlation analysis was carried out to determine the relationship between antioxidant activity and TPC in pigeon pea seed extract, namely the Pearson correlation analysis. The Pearson correlation coefficient can measure the linear relationship between two variables ranging from -1 to 1. In Fig. 4, the positive value of the Pearson correlation coefficient is 0.570. A positive value indicates that the increase in total phenol will be similar to the increase in antioxidant activity of the sample. The Pearson correlation coefficient with moderate correlation strength ranges from 0.4-0.59[18]. Thus, the relationship between TPC and antioxidant activity has a moderate correlation strength. Other compounds with antioxidant properties can contribute to the analysis of antioxidant activity[10].

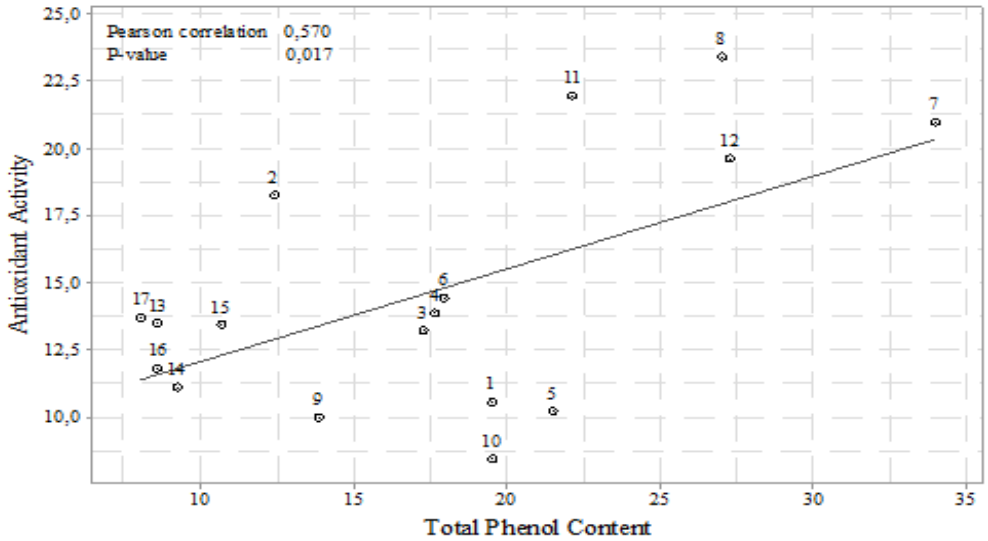


Fig. 4: Graph of correlation between antioxidant activity and TPC.

Optimization and Confirmation of Extraction Operating Conditions

The optimization process in the Design Expert 11 program recommends a process condition of 30 min, a temperature of 60 ºC, and 90% methanol as the optimal formula solution with a high desirability value of 0.840. The desirability value is a value to determine the degree of accuracy of the optimal solution. TPC response and antioxidant activity under optimal operating conditions were 34.147 mg GAE/30 g dry sample and 21.521 mg AAE/30 g dry sample, respectively. The average confirmation value for optimal operating conditions for total phenol and antioxidant activity was between 95% PI Low and 95% PI High, or 36.0219 mg GAE/30 g dry sample and 19.6091 mg AAE/30 g dry sample, respectively.

Results of Moisture Content and Solubility of Microcapsule

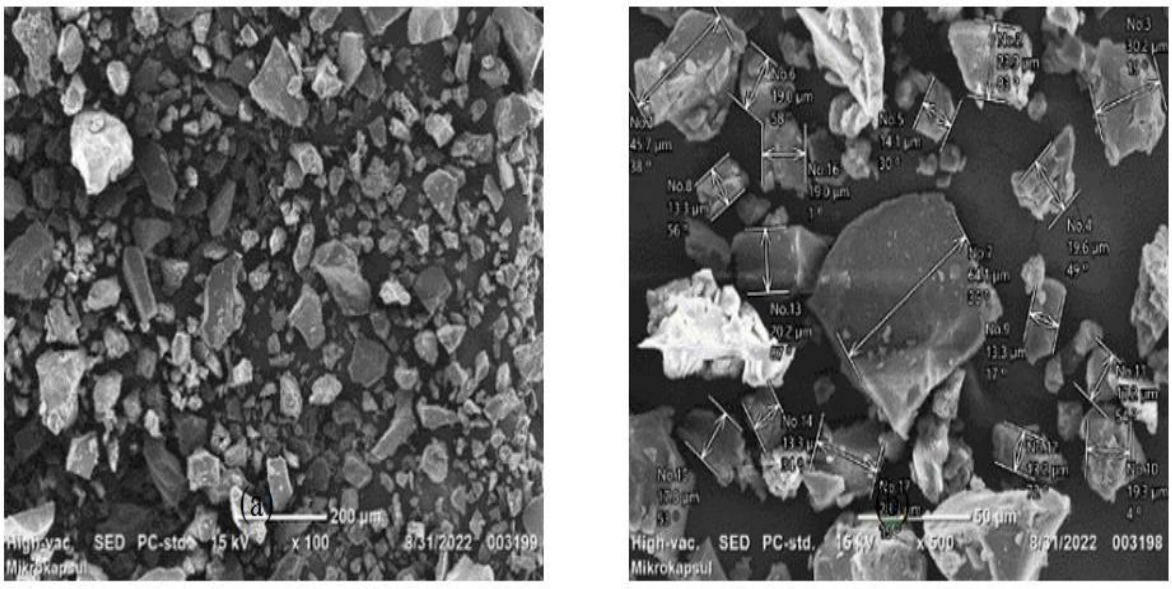
The results of the water content test showed that the microcapsules contained 8.85% water. In addition, the solubility of microcapsules in pigeon pea seed to water was 87.35%. The solubility of a product in water is related to the release of bioactive compounds in the application of microcapsules. High water solubility indicates a good release of bioactive compounds in microcapsules[19].

Colour Analysis Results

The microcapsules' color with a chromameter showed the values of L, a*, and b*, respectively, of 88.6, 0.53, and 11.69. Based on the calculation, the value of °HUE is 153, indicating the color of greenish-yellow chromaticity (GY).

Morphological Analysis Result

Fig. 5 shows the irregular shape of the microcapsule powder-like lumps with an average size of 23.03 μm. The microencapsulation method using freeze-drying has a particle size range of 20 μm to 5000 μm[20]. The difference in size and shape is due to the crushing of the microcapsules after the freeze-drying process. The same thing was also found in the research of Lauryna et al.[21] and Dadi et al.[22], which shows the magnification of microcapsules in the form of lumps.



(a) (b)

Fig. 5: Morphological structure of microcapsules by freeze-drying method with magnifications of (a) ×100 and (b) ×500.

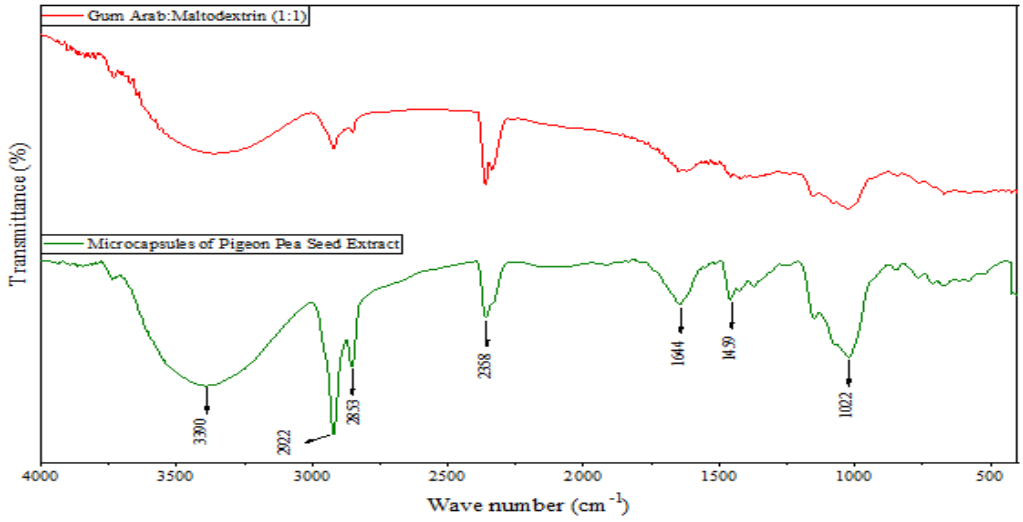


Fig. 6: FT-IR spectrum of gum arabic: maltodextrin (1:1) and microcapsules of pigeon pea seed extract**.**

FT-IR Analysis Result

The increase in peak intensity in the microcapsules compared to the coating indicated the presence of bioactive compounds in the microcapsules. This can be seen at peak 2922 cm^{-1}, the -CH group, and at peak 3390 cm^{-1}, which is the -OH group in phenol compounds. In addition, the absence of new peaks on the microcapsules indicated that the pigeon pea seed extract did not react with the coating material.

Identification of Bioactive Compounds

The GC-MS analysis of the optimal pigeon pea seed extract revealed 13.85 % of the isoflavone components. In addition to isoflavone, the extract contains mome inositol, which possesses anti-alopecic, anti-cirrhotic, lipotropic, chloesterolytic, anti-neuropathic, and anti-diabetic characteristics[23,24]. Other antioxidant substances as Hexadecanoic Acid by 7.6% and phenol compounds by 1.8%.

The sample of microcapsules that was sonicated for 10 min did not exhibit a bioactive chemical peak. Unlike the 30-min sonication duration, the GC-MS graph only displays mome inositol molecules. This demonstrates that the bioactive compounds contained within the microcapsules remain effectively protected.

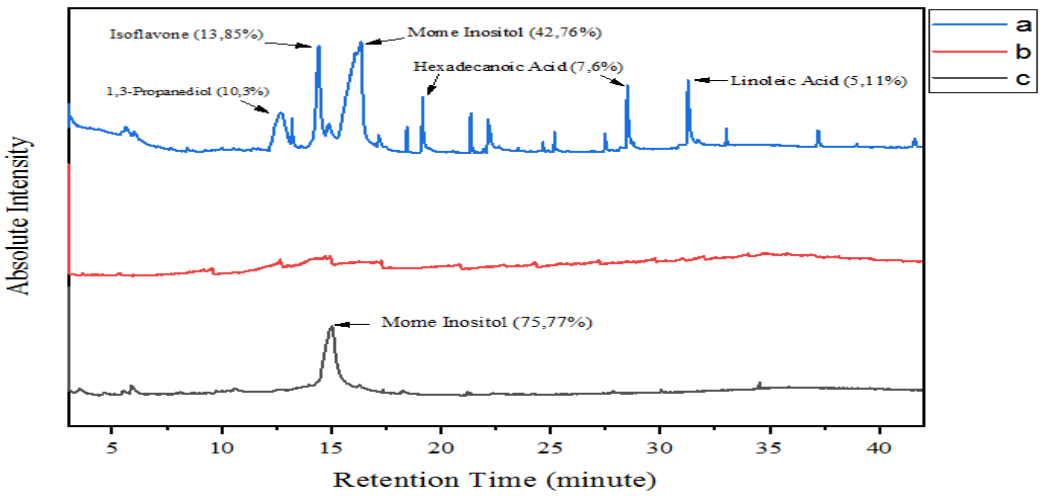


Fig. 7: GC-MS chromatography (a) Optimum pigeon pea seed extract, (b) Microcapsule Sonication for 10 min, and (c) Microcapsules Sonication for 30 min.

Table 4: Bioactive compounds and bioactivity from pigeon pea extract

Bioactive Compounds	Area (%)	Bioactivity	Reference
Mome Inositol	42.76	Anti-alopecic, anti-cirrhotic, anti-neuropathic, chloesterolytic, lipotropic property, and Anti-diabetic	[23], [24]
Isoflavone	13.85	Antioxidant, Anticancer, Antimicrobial, and Anti-inflammatory	[4]
1,3-Propanediol	10.3	Antimicrobial	[25]
Hexadecanoic Acid	7.6	Anti-inflammatory, Anticancer, and Antioxidant	[26], [27]
Phenolic	1.81	Antioxidant	[28]
Linoleic Acid	5.11	Anti-obesity effects, hypoglycaemic, and Alzheimer's dementia	[29]
Tocopherol	0.89	Alzheimer's, Cardiovascular disease, Anti-aging, and Antioxidant	[30]
9,12-Octadecadien-1-OL	4.75	Antifungal	[31]
9,12-Octadecadienoic Acid	6.32	Antioxidant and Anticancer	[32]
Benzedrex	0.64	*Nasal Decongestants*	[33]
Ergost-5-EN-3-OL	0.63	Antimicrobial and Anti-inflammatory	[34]
Uronat Acid	4.86	Antifungal	[35]

Conclusion

Ultrasonic-assisted extraction and microencapsulation of a bioactive compound of pigeon pea seed methanol extracted were investigated. It was demonstrated that the best extraction conditions were 30 min, a temperature of 60 °C, and 90% methanol. Pigeon pea seed extract was entrapped by Freeze-drying using gum arabic and maltodextrin as wall material. Microcapsules contained 8.85% water with a solubility of 87.35%. Microcapsules have a color of greenish-yellow chromaticity (GY) and irregular shape with an average size of 23.03 μm. The best bioactive compounds in pigeon pea seed extract showed the most compounds, namely mome inositol, and isoflavones. In addition, there are several other compounds showing bioactivity as antioxidants. Thus, microcapsules from pigeon pea seed extract have the potential as a functional food with immunomodulatory properties and anti-diabetic.

Competing Interests

The authors declare that there are no competing interests.

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استخلاص وكبسلة دقيقة للمركب النشط بيولوجيا من بذور بازلاء الحمام بواسطة الموجات فوق الصوتية

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انتشرت جائحة كوفيد-١٩ العالمية، منذ نهاية عام ٢٠١٩، ولم ينته الأمر بعد. لذلك، من الضروري تناول الأطعمة المحلية الغنية بمضادات الأكسدة لتعزيز جهاز المناعة. نظرًا لوفرة المكونات النشطة بيولوجيًا، تعد بازلاء الحمام أحد الأطعمة المحلية التي يمكن استخدامها كغذاء وظيفي. يهدف هذا العمل إلى تحديد الظروف المثلى لاستخراج بذور بازلاء الحمام بناءً على محتوى الفينول الكلي والنشاط المضاد للأكسدة ثم اجراء كبسلة دقيقة لها. تبين أن ظروف الاستخراج المثلى هي ٣٠ دقيقة، عند ٦٠ درجة مئوية، باستخدام ٩٠ ٪ من الميثانول، ذو محتوى فينول كلي يعادل ٣٦,٠٢١٩ مجم من مكافئ حمض الغاليك/ ٣٠ جم عينة جافة، ونشاط مضاد للأكسدة ١٩,٦٠٩١ مجم مكافئ حمض الأسكوربيك/ ٣٠ جرام عينة جافة. تم استخدام مالتوديكسترين والصمغ العربي كمواد جدارية أثناء تجفيف مستخلص بذور بازلاء الحمام بالتجميد. تحتوي الكبسولات الدقيقة على ٨,٨٥٪ محتوى مائي وتتمتع بقابلية ذوبان ٨٧,٣٥٪. تتميز الكبسولات الدقيقة بكونها غير منتظمة الشكل، يبلغ متوسط حجمها ٢٣,٠٣ مايكرومتر، وذات لون أصفر مخضر. كانت أعلى المكونات نشاطًا بيولوجيًا في مستخلص بذور البازلاء هي موم الإينوزيتول (٤٢,٧٦٪) والإيسوفلافون (١٣,٨٥٪). بالإضافة إلى ذلك، فإن العديد من المواد لها نشاط بيولوجي كمضادات للأكسدة.