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ORGINAL ARTICLE

Process optimization and characterization of ultrasonic-assisted bee bread extract as functional ingredient

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ABSTRACT

The composition and components of bee bread vary based on geographic location and flora. Therefore, utilizing different extraction methods and characterizing the extract is essential to understand the potential of bee bread from various locations. This study focuses on the ultrasonic-assisted extraction of bee bread. In this research, bee bread was extracted using the ultrasonic-assisted method, and various evaluations were conducted, including ultrasonic extraction optimization, physicochemical analysis, functional group identification, total antioxidant content, and microbial and antimicrobial activity. The methodologies used for evaluation included Total Phenolic Content (TPC), Total Flavonoid Content (TFC), the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, and the (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) assay. The optimum ultrasonic extraction conditions determined using response surface methodology were an ultrasonic power of 65W, a temperature of 52℃, and a sonication time of 35 minutes. The experimentally obtained TPC, TFC, and extraction yield at these optimal values were 11,998 ± 96.19 µg of gallic acid equivalent (GAE)/g dry extract, 5,127.7 ± 112.81 µg of quercetin equivalent (QE)/g dry extract, and 6.45 ± 1.07%, respectively. The DPPH IC_{50} and ABTS IC_{50} radical scavenging activities of the bee bread extract were 586.823 µg/ml and 438.296 µg/ml, respectively. FTIR spectra for both bee bread powder and extract exhibited similar peaks at 1026.7 cm^{-1} and 3267.96 cm^{-1}. The predominant minerals in the bee bread sample were potassium (580 mg/100 g of bee bread) and calcium (113 mg/100 g of bee bread). The bacteria that showed the greatest susceptibility to the extract were Staphylococcus aureus and Escherichia coli. This study suggests that ultrasonic-assisted extraction of bee bread yields higher antioxidant content and extraction efficiency compared to maceration extraction. Additionally, the antioxidant activity, antibacterial properties, and nutritional content of Ethiopian bee bread align with findings from bee bread sourced from different locations reported in the literature.

Keywords: *- Antioxidant, Antimicrobial, Bee bread, Optimization, Ultrasonic assisted extraction.*

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1. Introduction

Bee bread is highly nutritious as it contains a combination of proteins, carbohydrates, fats, vitamins, minerals, and antioxidants Bee bread offers a lot of health benefits. (Bakour et al., 2022) (Khalifa et al., 2020). Some uses of bee bread include: antimicrobial, antioxidant, anticancer, and anti-inflammatory activities (Ćirić et al., 2022). The wide variety of phenolic compounds found in bee breads contribute to their biological and medicinal properties (Kolayli et al., 2024). Ethiopia have hives 6,018,223 in 2018 according to Global Beekeeping Industry Data (MERDAN, 2021). This show as the country has great potential in different bee products. In Ethiopia bee bread is underutilized and unexplored bee products. The novel technique known as Ultrasound-Assisted Extraction (UAE) uses high-frequency ultrasonic waves to improve the extraction of bioactive chemicals from solid matrices, including plant materials and food products (Oroian et al., 2020). Cavitation bubbles in the solvent created by ultrasound-assisted extraction swiftly form and burst, rupturing the material cell walls with shock waves and micro-jets (Kumar et al., 2021). Temperature, time, power, frequency, and solvent type are the primary extraction parameters that affect extraction performance (Yusoff et al., 2022). Maceration is the process of soaking powdered plant material in a solvent (usually water or alcohol) in a closed container at room temperature for an extended amount of time while shaking frequently to dissolve the soluble content. The mixture is then strained, and the residual solid (marc) is compressed to extract more liquid, which is subsequently clarified by decantation or filtration (Naviglio et al., 2019). The most significant disadvantage of maceration is that it can take several hours to several weeks to complete. The extraction method used for bee bread extraction have effect on composition and yield. Ultrasonic-assisted extraction (UAE) offers several advantages, such as improved solvent penetration into the food material, high yields of extracted products, excellent reproducibility, reduced solvent usage, and the ability to extract temperature-sensitive compounds. It can also be easily integrated with other extraction methods (Kutlu et al., 2022). Analyzing the quality and safety of bee bread is crucial, and green technologies like ultra- sonication have been effective in extracting bioactive compounds. As a functional food, bee bread offers potential as a natural ingredient, providing beneficial effects on physiological functions and overall well-being. This research aims to optimize and characterize bee bread which extracted by using ultrasonic assisted methodology.

2. Methodology

2.1 Sample Collection

In October and November of 2024, researchers obtained a sample of bee bread from the Holeta bee research site in the Oromia area of Ethiopia. The sample was then refrigerated and prepared for physicochemical analysis and extractio.

2.2 Sample Preparation for Extraction

Bee bread collected was dry and clean from different impurities and honey comb. The clean bee bread was store in refrigerate at -4 °C. Using ethanol bee bread extract extracted from dried bee bread.

Figure 1: preparation of bee bread for extraction: Honey comb (A), Bee bread separated from honey comb (B), Grounded bee bread (C). The Box-Behnken experimental design is being employed to optimize the ultrasonic extraction method. This design utilizes three factors, each with three levels. The independent variables chosen for this study are extraction temperature (X1), sonication time (X2), and ultrasonic power (X3) as given in Table 1.

Table 1: Factors and their coded levels used for the Box-Behnken design

Factor	Name	Level		
		-1	0	+1
A	Power (W)	65	60	70
B	Temperature (°C)	52	42	62
C	Time (min)	35	25	45

2.3 Optimization

The main objective of this study was to optimize ultrasonic extraction of bioactive component from bee bread. To achieve this, BBD involved the optimization of ultrasonic power, sonication time and temperature. Using Response Surface Methodology (RSM), a three-variable, three-level Box-Behnken Design (BBD) was used to model and optimize the responses (TPC, TFC and extraction yield). Using an appropriate experimental design, analysis of variance (ANOVA) was performed on the ultrasonic extraction data. A second-order polynomial equation was used to ascertain the link between the independent variable and the response.

$$Y = \beta o + \sum_{i=1}^{k} \beta i x i + \sum_{i=1}^{k} \beta i i x^2 i i + \sum_{i=1}^{k} \sum_{j=i+1}^{k} \beta i j x i x j \qquad \text{Eq- 1}$$

where Y represents the response, k is the number of components, xi and xj are the coded factors, and the regression coefficients for the intercept, linear, quadratic, and interaction terms are, respectively, β0, βi, βii, and βij.

A 3 g of bee bread was mixed with 50 mL of 96% ethanol to prepare sample for extraction and OVAT analysis as shown in Figure 2. Bioactive components were extracted using an ultrasonic extraction unit of the probe type. An ultrasonic probe is made up of a horn or probe that is attached to a transducer. With minimal energy loss, the probe transmits ultrasound in the medium while submerged in the extraction vessel. Because the probe's tip has a higher ultrasonic intensity than bath systems, probe-based systems are typically favored

Figure 2: Ultrasonic probe used for extraction

2.5 Physicochemical Analysis of Bee Bread

pH

The sample's free acidity was assessed by mixing 2 g of the bee bread sample with 5 mL of distill water, homogenizing the mixture, and then titrating the mixture against NaOH (0.05 M) using the procedure outlined by Costa et al. (2019). The pH was measured using a pH meter (WTW inoLab, pH 7110, Germany).

Water activity

Water activity of bee bread and bee bread extract was measured by water activity meter (Aqualab 4TE dew point water activity meter, USA).

2.6 Bee Bread Proximate Composition Analysis

Determination of Moisture Content

The moisture content was determined using the oven drying method per AOAC (2016). Aluminum dishes were cleaned initially with distilled water and dried at 105 °C for 20 minutes. After cooling in a desiccator for 15 minutes, the balance was calibrated, and the weight of the empty dishes was recorded. Then, 15 g of bee bread was transferred into the dishes, which were shaken to spread the sample evenly. The dishes were placed in a hot air oven at 105 °C for 3 hours, then cooled in a desiccator for 30 minutes. Finally, the dried sample was weighed after cooling.

Using the following equation, the moisture content is finally calculated.

$$Moisture\ \% = \frac{Ws-(Wds-Wbd)}{Ws} \times 100 \qquad \text{Eq- 2}$$

where Wds is the weight of the aluminum dish containing the dried sample, Wbd is the weight of the aluminum dish in its blank state, and Ws is the weight of the sample.

Ash Content Determination

The ash content was determined following AOAC (2016). Crucibles were cleaned with 6N HCl, rinsed with distilled water, dried for 20 minutes in an oven, and then cooled for 30 minutes in a desiccator. Then weight of the cooled crucibles (Wbc). After adding 2.5 g of the sample, the crucibles were heated to 550 °C for five hours in a muffle furnace (Nabertherm, Germany). The final weight (Wca), was noted following an hour of cooling in a desiccator until it was below 250 °C. Equation 3 was used to determine the ash content.

$$Ash\,\% = \frac{Wca - Wbc}{Ws} \times 100 \qquad \text{Eq- 3}$$

where Ws is the sample weight, Wca is the weight of the crucible after it has been filled with ash, and Wbc is the weight of the blank crucible.

Protein Content Determination

The protein content was determined using AOAC (2016), method number 976.05. A 2.5 g sample was mixed with 12 mL of H_2SO_4, 6 g of catalyst, 7 g of K_2SO_4, and 0.8 g of $CuSO_4$, then left overnight. The mixture was digested for 2 hours at 420 °C until clear. After cooling, the sample was prepared for distillation. In the Kjeltec analyzer, 25 mL of 40% NaOH and 25 mL of boric acid were added, along with a methyl red indicator. The distillate was titrated with 0.1 M HCl to a reddish color, and the results were recorded. Nitrogen was calculated using Equation 4.

$$Nitrogen\,\% = (Vs - Vb) \times 14.01\,Ws\ \times 10 \qquad \text{Eq- 4}$$

In this case, Vs and Vb represent the milliliter quantities of standard HCl used to titrate a sample and a blank, respectively. The atomic weight of nitrogen is 14.01, the weight (g) of the test portion or sample is Ws, the molarity of standard HCl is M, and the factor to convert mg/g to percent is 10. The nitrogen result was then multiplied by a specific factor to calculate the crude protein concentration. The formula N x 6.25 (1/0.16 = 6.25) was used to convert nitrogen content into crude protein content using Equation 5 because early research findings indicated that the average nitrogen (N) content of proteins would be approximately 16 percent.

$$Protein\,\% = Nitrogen \times 6.25 \qquad \text{Eq- 5}$$

Determination of Fat Content

According to the official procedure, the Soxhlet technique was used to determine the fat content (AOAC, 2016). The extraction flask was cleaned completely with hot distilled water and then dried in a drying oven (BINDER, Germany) for 30 minutes at 103±2 °C. After that, it was moved to a desiccator to reach room temperature. The Soxhlet machine (auto solvent extractor, VELP scientifica, Italy) was then filled with 50 mL of petroleum ether, and the mass of the dried flask designated as Wf was measured by weighing it. A 2 cm layer of fat-free cotton was placed on the bottom of the extraction thimble to prepare it for extraction. Prior to being put into the thimble, a test sample weighing two grams was weighed and recorded as Ws. The thimble was then connected to the condenser, which was then fitted to the extraction flask's mouth, and the sample's top was covered with another piece of defatted cotton. For four hours,

the extraction was carried out at 70 °C. The extraction flasks were taken out of the extractor after the extraction procedure was finished. To get rid of the solvent, the flask with the extracted fat was dried in an oven set at 105 °C for another half hour. The weight of the dried flask with fat (Wff) was determined by weighing the extraction flask once more after it had cooled in the desiccator for thirty minutes. Equation 6 was used to determine the crude fat value, and the analysis was carried out three times to get an average value.

$$Crude\ fat\ \% = \frac{Wff - Wf}{Ws} \times 100 \qquad \text{Eq- 6}$$

Where:

Wff = weight of dried flask with fat,

Ws = sample weight,

Wf = mass of dried flask,

Crude Fiber

Crude fiber content of bee bread sample was quantified by using (AOAC, 2016). Each 250 mL beaker was filled with 150 mL of 1M sulfuric acid and 1g of grounded bee bread sample. The sample was vacuum-filtered after boiling for 30 minutes at 92 °C. The residue was recovered after being washed twice with hot distilled water and acetone. By using a light vacuum, acetone was recovered and then transferred proportionally to the beaker. The beaker was poured with 150 mL of 1M potassium hydroxide, heated for 30 minutes, and then filtered.

After digestion, the sample passes through drying and incarnation, which involves placing it in a desiccator and drying it for three hours at 103 ℃. The crucible is then weighed. After two hours of 550 °C heating in a muffle furnace, the sample was next chilled in a desiccator. Finally, crude fiber calculated using Equation 7.

$$Crude\ Fiber\ \% = \frac{W2 - W3}{W1} \times 100 \qquad \text{Eq- 7}$$

where W1 represents the weight of the sample, W2 represents the crucible and any leftover weight following drying, and W3 represents the weight of the crucible plus any leftover weight following incineration.

Determination of Total Carbohydrate

The total amount of carbohydrates was determined using the difference approach (Organization, 2003). The result was given as a percentage by adding up the other components of the sample, such as protein, fat, moisture, and ash, which was each determined separately. The percentage of total carbohydrate was given by Equation 8.

$$Total\ carbohydrate\ (\%) = 100 - (\ Moisture + Ash + Protein + Fat) \qquad \text{Eq- 8}$$

2.6 Determination of Antioxidant Properties of Bee Bread Extract

Determination of Total Phenolic Content (TPC)

To ascertain the total phenolic content in the bee bread extracts, the Folin-Ciocalteu technique, modified by Didaras et al. (2021), was employed. One milliliter of each bee bread extract or standard was added to each test tube as part of the protocol. Next, each test tube received 1 mL of the Folin-Ciocalteu phenol reagent (Sigma Aldrich) at a 1:1 dilution ratio. After being well combined, the mixture was left to stand for three minutes. The liquid was then left to stand in the dark for ninety minutes after three milliliters of 10% sodium carbonate had been added to each test tube. Using pure water, the volume was adjusted to 15 mL. Following the incubation period, a spectrophotometer (SHIMADZU UV-1800, Japan) set to 760 nm was used to measure the absorbance of the resultant combination. Gallic acid concentrations ranging from 25 to 350 µg/ml were used to create the standard curve. Three duplicates of the experiment were carried out. The total phenolic content, measured in micrograms of gallic acid equivalent per gram of extract (µg GAE g^{-1}), was determined using the absorbance values that were obtained.

$$\text{TPC} = C + \frac{V}{M} \qquad \text{Eq- 9}$$

where TPCc is the total phenol content (µg of GAE/g dry extract), V is the volume of the stock solution (mL), M is the dry weight of the extract present in the stock solution (g), and C is the gallic acid equivalent concentration (mg/mL) determined from the calibration curve.

Determination of Total Flavonoid Content (TFC)

The total flavonoid content of the functional beer was ascertained using the aluminum chloride technique. The total flavonoid content with slight modification (TFC) of the bee bread extract was ascertained using the methodology outlined by El Hariri et al. (1991) and Ezeigbo et al. (2017). Using distilled water to get the volume to 10 mL, 500 µL of sodium nitrite (5% w/v), 500 µL of aluminum chloride (10% w/v), 1 mL of bee bread ethanol extract, and 2 mL of 1 M sodium hydroxide were used to prepare the reaction mixture. Following the appropriate vortexing of the combination, a spectrophotometer (SHIMADZU UV-1800, Japan) was used to measure the absorbance at a wavelength of 415 nm. Quercetin concentrations ranging from 50 to 300 µg/mL were used to create the standard curve.

The TFC assay was conducted in triplicates. The obtained absorbance values were utilized to calculate the TFC, which was expressed in micrograms of quercetin equivalent per gram of extract (µg QE g^{-1}).

$$TFC = C + \frac{V}{M} \qquad \text{Eq- 10}$$

where TFC stands for total flavonoid content (µg QE/g sample), C for calibration curve concentration (mg/mL), V for stock solution volume (mL), and M for sample weight (g) in the stock solution.

DPPH Radical Scavenging Activity

The 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) test was used to assess the extract samples' antioxidant capacity. With some slight adjustments, the Oršolić et al. (2012) method was utilized to assess the extracts' capacity to scavenge DPPH radicals. A stock solution of bee bread extract containing 1 mg/mL was made in ethanol. Samples were generated at concentrations ranging from 20 to 100 µg/mL. One milliliter of the sample was combined with three milliliters of DPPH solution, and the absorbance was adjusted to 0.976 using ethanol.The stock solution should be serially diluted to yield solutions with concentrations of 20 µg/mL, 40 µg/mL, 60 µg/mL, 80 µg/mL, and 100 µg/mL, respectively. The solutions should then be incubated for 30 minutes at room temperature in the dark. At 517 nm, the mixture's absorbance was then determined. As a positive control, ascorbic acid was utilized at concentrations ranging from 20 to 100 µg/mL. The radical scavenging activity (RSA) is computed using the absorbance values that were obtained. The sample concentration needed to scavenge 50% of the DPPH free radical is known as the IC50 value (Phuyal et al., 2020). A graphic representation of the extract's and standards' IC50 was produced. Equation 11 was used to calculate the findings of the triple DPPH radical scavenging activity.

$$\%I = [(ODc - ODs)/\ ODc] \times 100 \qquad \text{Eq- 11}$$

In this case, ODc stands for absorbance of DPPH with ethanol (control), ODs for absorbance of DPPH with extract or standard, I% for antioxidant activity percentage inhibition of DPPH, and IC50 values for the extracts' radical scavenging capabilities.

ABTS Radical Scavenging Activity

Water was used to dissolve the ABTS reagent. After reacting with 2.45 mM potassium persulfate, the ABTS radical cation was allowed to sit at room temperature in the dark for 12 to 16 hours before being used. The samples were tested by equilibrating the ABTS solution at 30 °C after diluting it with ethanol until its absorbance at 734 nm was 0.70. Initially, 1 milliliter of the sample and 3 milliliters of ABTS solution were used to dilute the extracts. After the tubes were sealed and left to stand at room temperature for six minutes, the absorbance of the samples was measured at 734 nm (Re et al., 1999). The radical scavenging activities are expressed using the IC50 values from Equation 12.

$$\%I = \frac{AC - AS}{AC} \times 100 \qquad \text{Eq- 12}$$

Where: AC = Absorbance of the control (1 mL ethanol + 3mL ABTS solution),

AS = Absorbance of the sample solution, and

I% = Antioxidant activity % inhibition of ABTS.

2.7 FTIR Analysis

Samples were placed onto the flat diamond crystal surface of a machine (Nicolet iS50, Thermo Scientific, Germany) that had an ATR (attenuated total reflection) detector in order to produce FT-IR spectra. Plotting transmittance versus wavenumber in the 4000 cm-1 to 400 cm-1 range with 64 consecutive scans at a resolution of 4 cm^{-1} allowed researchers to examine the functional groups present in the samples (Svečnjak et al., 2023).

Determination of Microbiological Properties of Bee Bread Extract

Total Coliform Count

The following process was used to calculate a sample total coliform count using ISO 4832:2006. In order to create the culture media, 38.5g of VRBL agar was first suspended in 1000 mL of distilled water and heated until it dissolves completely. After autoclaving at 121 °C for 15 minutes and cooling to 45–50 °C, 40.01 g of BGBB was suspended in 1000 ml of distilled water. 15g of casein digested by enzyme was suspended in one thousand milliliters of distilled water, to which 8.5 grams of sodium chloride was added. The mixture was then autoclaved at 121°C for fifteen minutes, and stored at 4°C. Sample preparation involves dilution by adding 1 mL of the sample to 9 mL of peptone salt solution. The sample was prepared by dilute it by a factor of ten by adding 1mL of the sample to 9mL of peptone salt solution.

Next, 1ml of the initial sample was pipetted into petri dishes with labels, and 1 mL of the diluted sample was moved to different petri dishes with labels already on them. Each dish was get about 15 mL of VRBL agar media, which was left to harden before 4mL of agar media is put on top. For a full day, the dishes were incubated at 35°C. Following incubation, a gas absorption test was performed to confirm the presence of coliform bacteria in the dishes, and they were examined under a digital colony counter. Lastly, the sample total coliform count per gram was ascertained.

Yeasts

ISO 4832, (2006) was used for the determination of yeasts. 90 mL of peptone water solvent were used to homogenize 10 grams of each sample. The same solvent was used to make decimal dilutions. Yeast and molds were counted using DG18 (Dichloran 18% glycerol), and the mixture was incubated at 25 °C for five days. Colony-forming units per gram (cfu/g) of the sample were used to report the microbial counts.

2.8 Determination of Antimicrobial Properties of Bee Bread Extract

Antimicrobial effect of the bee bread sample analysis against gram positive and gram negative bacteria were assessed (A. Urcan et al., 2018). The test organisms S. aureus (ATCC25923), B. cereus (ATCC27895), P. aeruginosa (ATCC27853) and E. coli (ATCC 25922) were sub cultured onto fresh plates of Mueller Hinton agar for 24 h at 37 °C. Mueller-Hinton broth was used to suspend the colonies on these plates until their turbidity reached the 0.5 McFarland standard, which is equivalent to 108 colony forming units (cfu)/mL. The antimicrobial test medium used was Mueller-Hinton agar. The different test microorganisms were evenly seeded onto labeled media plates using a sterile brush that had been rolled in the suspension and streaked across the agar surfaces. The extract concentrations that were utilized were 1, 0.5, and 0.25 mg/mL. Ethanol was used as the solvent to create dilutions. After allowing the extract to diffuse for one hour at 4 °C in the refrigerator, each plate was incubated for twenty-four hours at 37 °C.

Statistical Analysis

The process of extracting bee bread was modeled using a three-level, three-factor Box-Behnken architecture. Design Expert 13 software was used to model the parameters using a second-order polynomial response. Variance analysis

The validity of the model was evaluated using ANOVA, the coefficient of determination (R2), and the adjusted coefficient of determination (Adj-R2). Each experiment was conducted three times, and the mean ± SD was used to express the results. Tukey's test and one-way analysis of variance (ANOVA) were used to assess the significance of mean value differences at the 0.05 level of significance using the statistical program Minitab 2019.

3. Results and Discussions

3.1 Proximate Composition Analysis of Bee Bread

The proximate composition of bee bread is illustrated in Table 2 which show the moisture content 18.1± 1.10%, ash content 5.90 ± 2.09%, fat content 10.60 ± 2.41%, fiber content 7.57 ± 1.54% protein content 18.75 ± 2.57% and carbohydrate content 46.65 ± 8.17% respectively. According to Kaplan et al. (2019) proximate composition of five bee bread from Adana and Mersin part of Turkey were reported as moisture content 11.0 -16.4 %, ash 1.86-2.4 %, fat 7.0-13.4 %, and protein between 18.6-21.6 %. Moisture content 11.81 ± 0.90, Ash 2.11 ± 0.15, fat 11.40 ± 0.84 and protein 18.50 ± 1.40 were demonstrated in study of Fallah et al. (2022) from three beekeeping farms at Shiraz city (Fars, Iran). Moisture content between 11.4 and 15.9 %, ash between 1.9 and 2.54 %, fat between 5.9 and 11.5 %, and protein between 14.8 and 24.3 % was reported by Kaplan et al. (2016). A study by Bakour et al. (2017) found that the Moroccan bee bread samples had a moisture content of 9.85%, lipid content of 2.31%, carbohydrate content of 28.46%, protein content of 12.81%, and fiber content of 8.30%, as determined by chemical analysis. According to a study by Beykaya et al. (2021), the contents of protein, fat, and carbohydrates were 19%, 9.8%, and 43%, respectively. The value obtained in this study was closer with slight variation to the previous study done by different scholars. This variation could be due to location and season of collection because the origin of sample the most determinant factor for characterize bee bread.

Table 2: Proximate composition of bee bread comparison with literature reports

Bee bread location	Moisture content (%)	Ash content (%)	Fat Content (%)	Fiber content (%)	Protein content (%)	Carbohydrate (%)	Reference
Ethiopia	18.1± 1.10	5.90 ± 2.09	10.60 ± 2.41	7.57 ± 1.54	18.75 ± 2.57	46.65 ± 8.17	From this study
Turkey	18.4±0.2	2.55±0.04	5.12±0.051	-	20.9±0.15	-	Mayda et al. (2020)
Iran	11.81±0.90	2.11±0.15	11.40±0.8	-	18.50±1.40	55.60±3.30	Fallah et al. (2021)
Thailand	16.1	2.30	7.4	-	15.5	58.7	Chuttong et al. (2018)
Mexico	15.5	2.5	1.1	-	20.49	33.10	Contreras-Oliva et al. (2018)

3.2 Variance (ANOVA) Model Equation and Analysis

It is possible to use the equation in terms of coded factors to predict the reaction for particular amounts of each element. The coded equation can be used to compare the factor coefficients and ascertain the factors' respective impacts.

$$TPC = 12904.24 + 180.08A - 233.05B + 95.34C + 656.78AB - 593.22AC - 487.29BC - 1250.00A^2 - 1313.56B^2 + 275.42C^2 \quad \text{Eq-13}$$

$$TFC = 4826.92 + 834.62A - 185.10B + 559.78C - 512.82AB + 407.69AC - 91.35BC + 293.11A^2 - 859.78B^2 - 522.60C^2 \quad \text{Eq-14}$$

$$Yield = 6.84 - 0.1825A - 0.1075B - 0.1950C + 0.1625AB + 0.2175AC + 0.9425BC - 1.14A^2 - 0.4717B^2 - 0.3017C^2 \quad \text{Eq-15}$$

Where:

TPC is total phenolic content

TFC is total flavonoid content

A, B, C coded symbol for ultrasonic power, temperature and time, respectively.

In Table 3 the result obtained from dependent variables, namely: ultrasonic power(W), extraction temperature (°C) and sonication time (min) are presented. Total phenolic content, total flavonoid content and extraction yield are the responses of this study.

Table 3: Box- Behnken design employed for optimize extraction.

A: Power (W)	B: Temperature (°C)	C: Time (min)	TPC µg of GAE/g dry extract	TFC µg of QE/g dry extract	Yield (%)
65	52	35	12904.2	4888.46	6.82
60	52	45	12395.8	3811.54	5.11
70	62	35	10870.3	4401.28	5.09
70	52	25	12650	4567.95	5.25
65	52	35	12904.2	5106.41	6.88
60	52	25	11039.8	3724.36	6.02
65	42	25	11463.6	2869.23	7.27
60	42	35	11124.6	3093.59	5.69
65	52	35	12989	4721.79	6.8
65	52	35	12904.2	4708.97	6.89

70	52	45	11633.1	6285.9	5.21
70	42	35	10107.6	5798.72	4.97
65	42	45	12650	4388.46	5.08
60	62	35	9260.17	3747.44	5.16
65	52	35	12819.5	4708.97	6.79
65	62	25	12056.8	2683.33	5.16
65	62	45	11294.1	3837.18	6.74

3.2.1 ANOVA for Total Phenolic Content

The ANOVA findings for total phenolic content are shown in Table 4, where a model F-value of 403.00 denotes statistical significance. With just a 0.01% possibility that such a high F-value may be the result of noise or random variation, this indicates that the observed link between the response variable and the predictor variables is unlikely to have happened by accident. If a term's P-value is less than 0.0500, it is deemed statistically significant in the model. The terms A, B, C, AB, AC, BC, A^2, B^2, and C^2 are recognized as important contributions in this analysis. P-values larger than 0.1000 indicate that a term is not significant. Furthermore, when compared to pure error, the difference between the model and the observed data is not significant, as indicated by the lack of fit F-value of 2.17. Since it indicates that the model correctly reflects the underlying patterns in the data, a non-significant lack of fit is preferred. The likelihood that a high lack of fit F-value is due to random noise is 23.46%. These results generally show that the model is significant, that a number of terms are statistically significant, and that the lack of fit is not significant, indicating that the model does a decent job of representing the data.

Table 4: ANOVA for TPC

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.954E+07	9	2.171E+06	403.00	< 0.0001	significant
A-Power	2.594E+05	1	2.594E+05	48.17	0.0002	
B-Temperature	4.345E+05	1	4.345E+05	80.67	< 0.0001	
C-Time	72716.17	1	72716.17	13.50	0.0079	
AB	1.725E+06	1	1.725E+06	320.33	< 0.0001	
AC	1.408E+06	1	1.408E+06	261.33	< 0.0001	
BC	9.498E+05	1	9.498E+05	176.33	< 0.0001	
A^2	6.579E+06	1	6.579E+06	1221.40	< 0.0001	

B^2	7.265E+06	1	7.265E+06	1348.77	< 0.0001	
C^2	3.194E+05	1	3.194E+05	59.30	0.0001	
Residual	37704.68	7	5386.38			
Lack of Fit	23340.99	3	7780.33	2.17	0.2346	Not significant
Pure Error	14363.69	4	3590.92			
Cor Total	1.957E+07	16				

3.2.2 NOVA for Total Flavonoid Content

As shown in Table 5, the ANOVA results for total flavonoid content show a model F-value of 53.41, which suggests that the model is statistically significant. Given that there is only a 0.01% possibility that such a high F-value might be the consequence of noise, it is not likely that the observed relationship between the component variables and the response variable is the product of chance or random f The P-values for the terms A, B, C, AB, AC, A^2, B, and C^2 in this analysis are less than 0.0500, suggesting that they significantly contribute to the model. On the other hand, P-values greater than 0.1000 indicate that a term is not significant. With a 46.48% likelihood that such a large lack of fit F-value might be caused by random noise, the lack of fit F-value of 1.04 further suggests that the lack of fit is not substantial when compared to the pure error. These results collectively imply that the response variable is highly influenced by the model terms.

Table 5: ANOVA for TFC

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.478E+07	9	1.642E+06	53.41	< 0.0001	significant
A-Power	5.573E+06	1	5.573E+06	181.30	< 0.0001	
B-Temperature	2.741E+05	1	2.741E+05	8.92	0.0203	
C-Time	2.507E+06	1	2.507E+06	81.56	< 0.0001	
AB	1.052E+06	1	1.052E+06	34.22	0.0006	
AC	6.649E+05	1	6.649E+05	21.63	0.0023	
BC	33376.48	1	33376.48	1.09	0.3320	
A^2	3.617E+05	1	3.617E+05	11.77	0.0110	
B^2	3.112E+06	1	3.112E+06	101.26	< 0.0001	
C^2	1.150E+06	1	1.150E+06	37.41	0.0005	

Residual	2.152E+05	7	30736.89			
Lack of Fit	94382.40	3	31460.80	1.04	0.4648	not significant
Pure Error	1.208E+05	4	30193.95			
Cor Total	1.499E+07	16				

2.2.3 ANOVA for Extraction Yield

Table 6 displays the results of the ANOVA for extraction yield. The association between the predictor or factor variables and the response variables is very unlikely to have happened by accident or random variation, as indicated by the model's statistical significance (F-value of 367.33). The likelihood of achieving such a high F-value because of noise is a mere 0.01%. When the individual model terms were examined, it was found that the terms A, B, C, AB, AC, BC, A2, B2, and C2 had P-values below 0.0500, indicating that they were significant model terms. The model terms are considered non-significant if their values exceed 0.1000. In comparison to the pure mistake, the lack of fit is not substantial, according to the lack of fit F-value of 2.57. A strong lack of fit F-value has a 19.16% probability of being caused by noise. The response variable is significantly impacted by the terms in the model.

Table 6: ANOVA for Extraction Yield

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	11.79	9	1.31	367.33	< 0.0001	significant
A-Power	0.2665	1	0.2665	74.70	< 0.0001	
B-Temperature	0.0925	1	0.0925	25.92	0.0014	
C-Time	0.3042	1	0.3042	85.28	< 0.0001	
AB	0.1056	1	0.1056	29.61	0.0010	
AC	0.1892	1	0.1892	53.05	0.0002	
BC	3.55	1	3.55	996.10	< 0.0001	
A^2	5.44	1	5.44	1525.27	< 0.0001	
B^2	0.9370	1	0.9370	262.69	< 0.0001	
C^2	0.3834	1	0.3834	107.48	< 0.0001	
Residual	0.0250	7	0.0036			

Lack of Fit	0.0165	3	0.0055	2.57	0.1916	not significant
Pure Error	0.0085	4	0.0021			
Cor Total	11.82	16				

3.2.4 Fit Statistics for the Model

As the number of terms in the model increased, the adjusted R^2 value decreased. The Predicted Residual Error Sum of Squares (press) serves as a metric for evaluating how well the model fits each design point. Using the press value, one can calculate the predicted R^2 values, which represent the extent to which the model can explain variation in new data, referred to as R^2pred (Predicted R^2 Value). Ideally, there should be a tolerance of 0.20 between the adjusted R^2 and predicted R^2 values. In this context, the predicted R^2 values of 0.9798 for total phenolic content (TPC), 0.8867 for total flavonoid content (TFC), and 0.9766 for extraction yield demonstrate a strong correlation with the adjusted R^2 values, as the differences remain within the acceptable limit of less than 0.20, as shown in Table 7.

Table 7: ANOVA Analysis for Fit Model

Parameter	**TPC(µg of GAE/g dry extract)**	**TFC(µg of QE/g dry extract)**	**Extraction Yield(%)**
R^2	0.9981	0.9856	0.9979
Adjusted R^2	0.9956	0.9672	0.9952
Predicted R^2	0.9798	0.8867	0.9766
$_{Adeq}$ Precision	64.5503	26.8362	50.5922

The AdeqPrecision metric evaluates the ratio of signal to noise, with a preferred ratio exceeding 4. The study demonstrated adequate signal levels with ratios of 64.550 for total phenolic content (TPC), 26.836 for total flavonoid content (TFC), and 50.592 for extraction yield. These results indicate that the model is effective for exploring the design space.

Interaction Effects of Factors on TPC, TFC and Extraction Yield

Process variables are measurable quantities used to assess specific physical phenomena. They have a dual impact: they not only affect the outcome directly but also interact with one another. These interactions are crucial, as they determine the extent to which the desired outcome is achieved. In practical applications, various parameters influence processes, each contributing uniquely within the defined range of consideration. Bioactive extraction is closely linked to the levels of process variables, particularly focusing on power, temperature, and time. To explore these relationships, response surface analysis was conducted using 3D response surface plots, which illustrated the interactions among the independent variables and their effects on the response variables.

The interaction effect of the three independent variables on the TFC, TFC and extraction yield are illustrated with three-dimensional surface plot (Figure 3, Figure 4 and Figure 5). Using a 3D plot, the effects of the independent variables—ultrasonic power, temperature, and sonication time on the response (dependent variables), which include extraction yield, total phenolic content, and total flavonoid content, were examined.

Interaction Effect of Ultrasonic power, Temperature and sonication time on TPC

The response characteristics found in this study are in the form of a maximum response, as can be seen from the surface response curve of the total phenolic content in Figure 3.

As illustrated in Figure 3A, the total phenolic content of extract increases with a rise in power (60-68W) and temperature (42-52℃) and the value decreases with increasing in power and temperature above this value. Figure 3B shows that the total phenolic content increases with an increase in power from 60–70W and increase with an increase in sonication time from 25 to 45 min. As seen in Figure 3C Raising the temperature from 42 to 62℃ results in an increase in the total phenolic content.

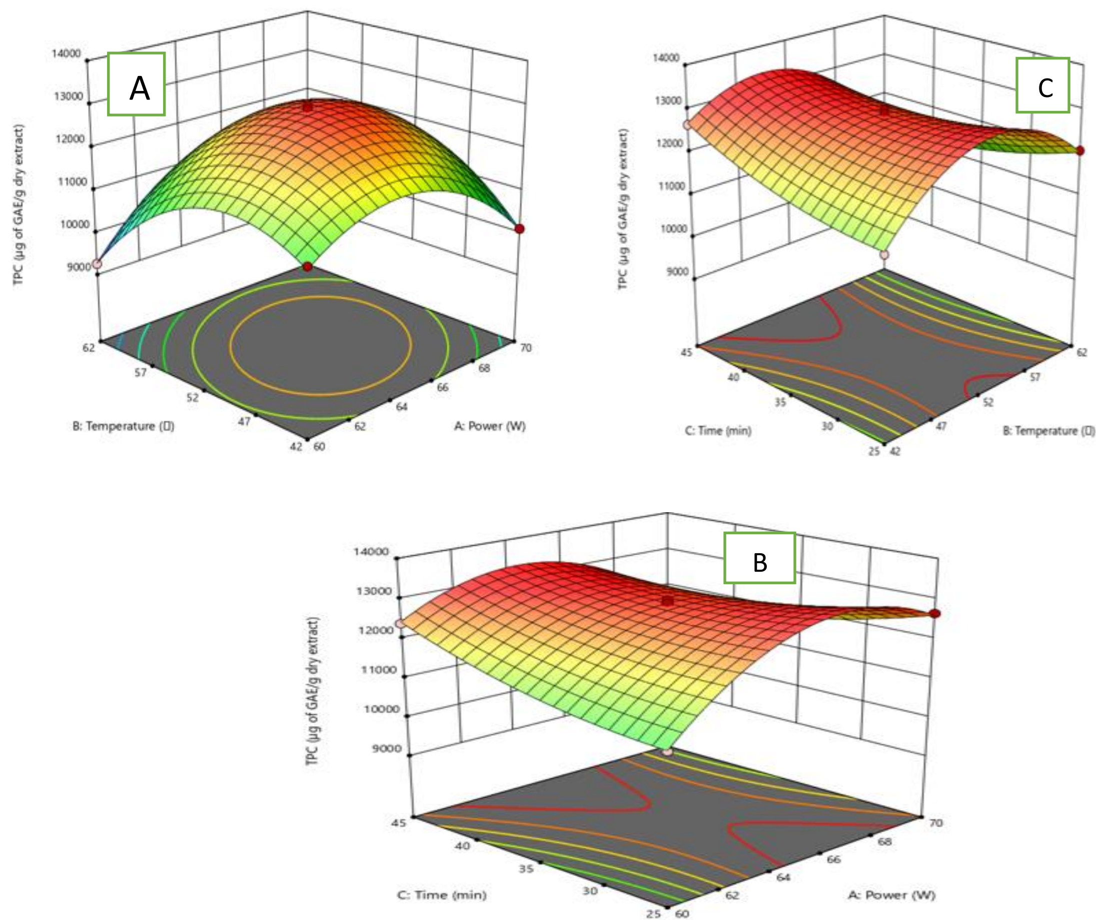

Figure 3: Response surface plots showing the interaction effects of process variables on TPC.

Interaction Effect of Ultrasonic power, Temperature and sonication time on TFC

The response characteristics found in this study take the form of a maximum response, as can be observed from the surface response curve of the TFC in Figure 4. As seen in figure 4A, raising the temperature from 42 to 62 ℃ and the power from 60 to 70W causes TFC to increase. Similarly Figure 4C shows that the TFC increases as power increases from 60 to 70W and sonication time 25 to 45 min. It can be shown from Figure 4B that TFC decrease with increasing temperature.

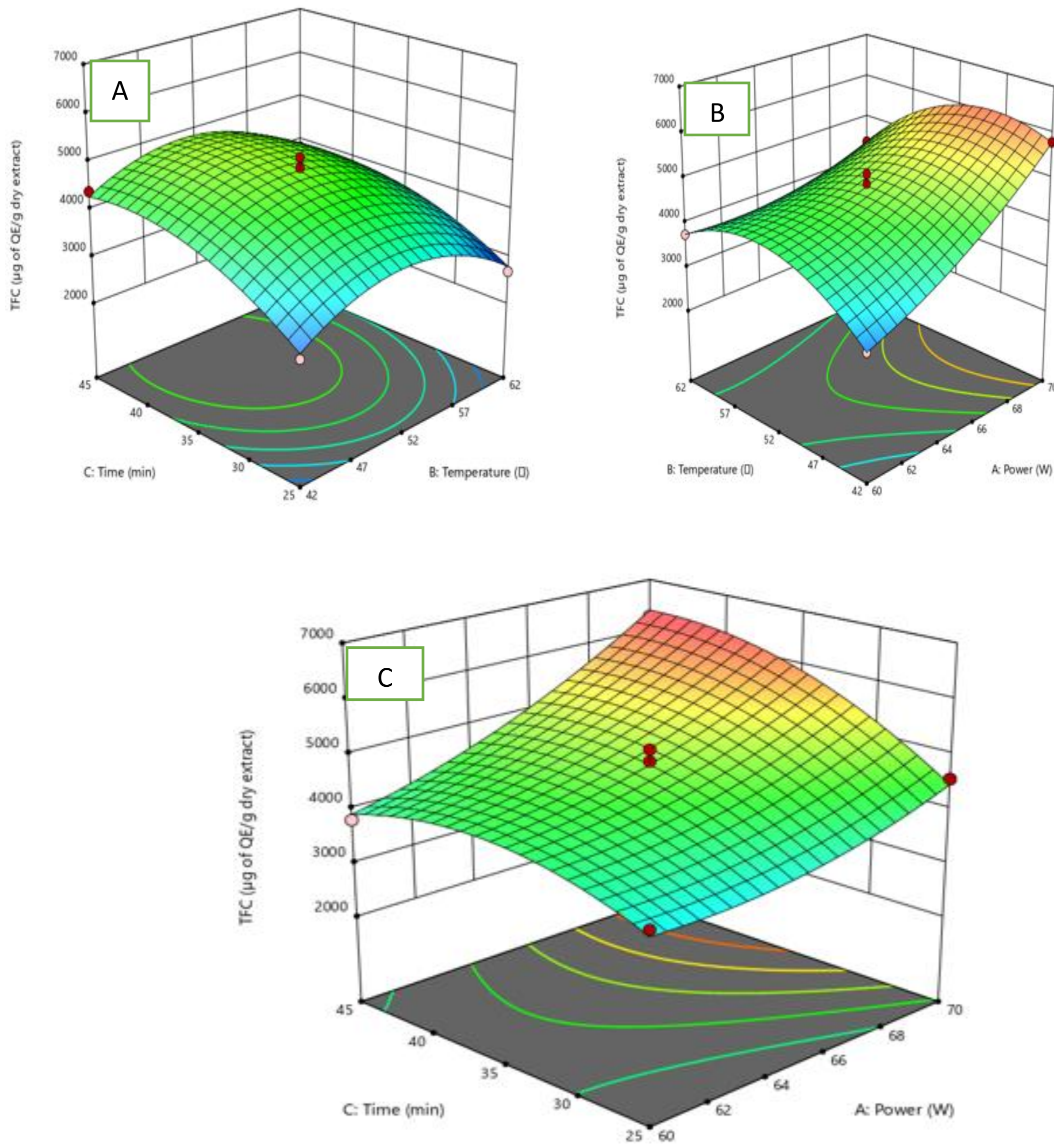

Figure 4: Response surface plots showing the interaction effects of process variables on TFC.

Interaction Effect of Ultrasonic power, Temperature and sonication time on Extraction Yield

The response characteristics identified in this study reveal a peak response, as evidenced by the surface response curve for extraction yield shown in Figure 5. As seen in figure 5A, raising the temperature from 42 to 52℃ and the power from 60 to 65W causes the extraction yield to increase. Similarly Figure 5B shows that the extraction yield increases as power increases from 60 to 65W and sonication time increase from 25 to 35 min. It can be shown from Figure 5C that extraction yield decreases with increasing temperature and sonication time.

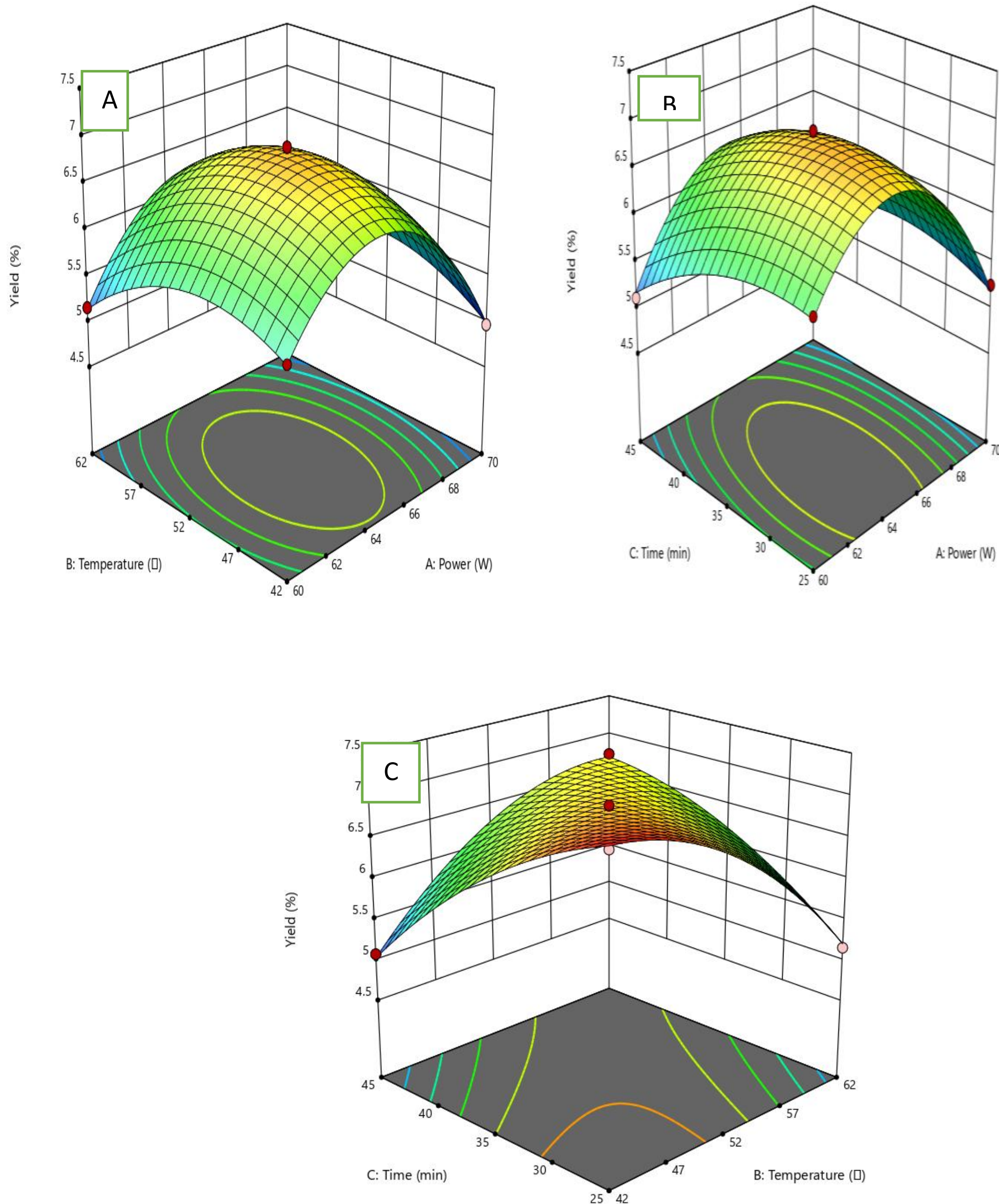

Figure 5: Response surface plots showing the interaction effects of process variables on extraction yield.

3.4 Optimization of The Extraction Parameters

The objective of this study was to maximize the extraction yield, total flavonoid content (TFC), and total phenolic content (TPC). The Box–Behnken experimental design was used to experimentally establish the maximum and average values of these variables, which were then used to calculate the desirability function. The experimental confirmation of the ideal circumstances for bioactive extraction from bee bread is shown in Table 8. According to the optimization results, the optimal conditions—which included an ultrasonic power setting of 65 W, an extraction temperature of 52°C, and a sonication duration of 35 minutes produced the greatest results for TPC, TFC, and total yield.

Table 8: Results of experimental validation of the optimal conditions for ultrasonic extraction

	Factor			**Response**		
	Ultrasonic power(W)	**Temperature (°C)**	**Sonication time (min)**	**TPC (µg of GAE/g dry extract)**	**TFC (µg of QE/g dry extract)**	**Yield (%)**
Predicted value	65 W	52 °C	35	12904.2	4826.92	6.84
Experimental value	65 W	52 °C	35	11998 ± 96.19	5127.67 ±112.81	6.46 ± 1.07
Absolute residual error (%)	-	-	-	7.55	5.86	5.94
Control by (maceration)	65 W	52 °C	35	7064.67 ± 83.51	3091.33 ± 90.09	4.00 ± 0.59

The absolute residual error is equal to [(experimental value - predicted value)/experimental value] times 100, and all the results are means ± standard deviations.

The model produced an optimal 12904.2 µg of GAE/g dry extract TPC, 4826.92 µg of QE/g dry extract TFC and 6.84 % extraction yield at ultrasonic power of 65 w, temperature 52°C, and 35 min sonication time. The TPC, TFC and extraction yield experimental obtained were 11998 ± 96.19 µg of GAE/g dry extract, 5127.7 ± 112.81 µg of QE/g dry extract and 6.45±1.07% respectively. This shows the model can reasonably predict the optimum values.

In maceration extraction method used for comparation, the extracts obtained results 7064.67 ± 83.51, 3091.33 ± 90.09 and 4.00 ± 0.59 TPC,TFC and extraction yield respectively . This result illustaete the ultrasonic extraction is effective

than maceration. The TPC and TFC levels measured in maceration were in the same order of those reported in the literature, (Didaras et al., 2021) (Khalifa et al., 2020).

3.5 Optimum Bee Bread Extract Characterization

3.5.1 pH

pH indicates the acidity or alkalinity of a solution and is a crucial chemical property utilized in various applications. pH of bee bread and bee bread extract obtained in this study were 3.87±0.03 and 4.36±0.10 respectively. Jaya et al. (2020) stated that the pH of bee bread ranges from 3.8 to 4.3 based on honeybee species.

3.5.2 Water Activity

Understanding the shelf life and stability of food goods depends on water activity. Moisture content important to know shelf life but food with similar moisture content has different shelf life (preservation time) this is because of water molecules interact in different way with other food constituents thus, water activity is the better indicator. High Aw favours the microbial growth on food. Measuring water activity involves assessing the amount of free water available for chemical reactions and microbial growth (Barbosa-Cánovas et al., 2020). In this study, water activity of bee bread and bee bread extract was recorded 0.5445 and 0.6411 respectively

3.5.3 DPPH and ABTS Radical Scavenging Activity of Bee Bread Extract

Various assays have been developed to assess the antioxidant capacity of foods and biological samples. The idea of antioxidant capacity originated in chemistry before being applied to fields such as biology, medicine, epidemiology, and nutrition. The ABTS assay relies on the production of a blue-green ABTS·+ radical that can be reduced by antioxidants, while the DPPH assay measures the reduction of the purple DPPH· radical to 1,1-diphenyl-2-picryl hydrazine. Both assays are widely used due to their ease of application (Gulcin & Alwasel, 2023; Marinova & Batchvarov, 2011; Shah & Modi, 2015). IC_{50} values from the DPPH and ABTS•+ radical scavenging assays were used to evaluate the antioxidant activity of bee bread samples. A lower IC_{50} value indicates a greater antioxidant capacity. DPPH and ABTS radical scavenging activity of bee bread extract in this study were found to be 586.823 µg/ml and 438.296 µg/ml respectively. In the DPPH assay, IC_{50} values varied from 0.18 to 1.8 mg/mL. For the $ABTS^{\bullet+}$ assay, the IC_{50} values ranged from 0.38 to 1.80 mg/mL was recorded by Didaras et al. (2021). In other studies ABTS assay (IC_{50} = 0.50 ± 0.04 mg/mL), and DPPH assay (IC_{50} = 0.98 ± 0.06 mg/mL) was presented previously by Bakour et al. (2019).

3.5.4 Nutritional Composition of Bee Bread Extract

The pre-dominant minerals in bee bread sample were potassium (580 mg/100 g of BB) and followed by calcium (113 mg/100 g of BB). In study of Ciric et al. (2019) which is done on 12 Serbian bee bread sample from different geographic origin shown potassium was the major mineral followed by calcium. The mean range for potassium 5515±361.20 to 7487±381.50 and 1190±76.38 mg/kg to 1806±44.98 mg/kg for calcium. The value obtained in this study was in this range for potassium and calcium. According to the study done by Mayda et al. (2020) 42 minerals found in bee bread sample by ICP-MS in different concentration as detected on this study the main five element existed

in higher concentration were K, P, Mg, Ca and Si respectively. The range of third element magnesium was from 688.82 to 1399.43 mg/kg from the study. The amount magnesium in Table 9 is in this range with this study.

Table 9: mineral composition and sugar content of bee bread extract

Mineral content (mg/100g)		**Sugar content (% by mass)**	
Calcium	113 ± 1.42	Fructose	25 ± 1.14
Magnesium	79 ± 2.36	Glucose	15.80 ± 0.74
Potassium	580 ± 4.12	Sucrose	< 0.25
Iron	1.79 ± 0.57	Maltose	< 0.26
Zinc	2.48 ± 0.68	Turanose	< 0.20

The analysis of free sugars (Table 9) revealed that fructose (25 % by mass) is the representative one followed by glucose (15.80 % by mass) and sucrose (< 0.25 by mass), maltose (< 0.26 by mass) and turanose (< 0.20 by mass). The results obtained is comparable with the results present by A. C. Urcan et al. (2018) which show fructose 19.32 % , glucose 13.19%, maltose 0.89% and turanose 0.66% for Romania bee bread collect in July 2016. Sugar and mineral composition bee bread associated with geographic area and plant diversity around the apiaries.

Sugars are the main energy source for bees, with worker bees depend on available sugars for their survival. Among different types of sugars, fructose is overlooked, whereas glucose and maltose rank closely in significance. Sucrose is found in lesser quantities as a result of being broken down during lactic fermentation. During this process, bacteria use these sugars to produce lactic acid, which further reduces the availability of sucrose (Bakour et al., 2019).

3.5.5 Microbiological Properties

Bee bread is a complex substance that includes various microorganisms, such as bacteria and fungi, which contribute to its production and enhance its nutrient content. These microorganisms come from the saliva of bees, which is mixed with pollen, the primary ingredient in bee bread (Bakour et al., 2022). In the current study, the mean total viable count in bee bread extract was found to be 5.38 ± 0.03 log CFU/g, which is higher than standard microbiological count for pollen (the raw material of bee bread) (10^3 log CFU/g). The contaminants may arise from external factors such as wind, dew, rain-splash, sprinkler irrigation splash and drip, can also contribute to pollen contamination (Jaya et al., 2020)

Total yeast in bee bread

The yeast colony counts in bee bread was 6.03 ± 0.06 log CFU/g. This result was in line with the study done by Jaya et al. (2020) which range 5.25 ± 0.06 to 8.67 ± 0.03 for different species honeybee bee bread.

3.5.6 Antimicrobial Properties

Gram-positive and gram-negative bacteria were used to evaluate the bee bread sample's antibacterial properties, and the results showed that the tested bacterial strains were susceptible to the bee bread extract. After being subcultured onto brand-new Mueller Hinton agar plates, the organisms were incubated at 370C for 24 hours. Mueller-Hinton broth was used to suspend the colonies from these plates until the turbidity reached the 0.5 McFarland standard (108 colony forming units (cfu)/ml). Mueller-Hinton agar was used as the medium for the antimicrobial experiments, and each organism was incubated under the precise conditions needed. A sterile swab soaked in the mixture was streaked across the agar surface of labeled plates to evenly inoculate them with different test bacteria. 200 µl of the bee bread extract was applied to each of the 10 mm-diameter wells that were made using a sterile pipet tip. This procedure was repeated using ethanol as the solvent for dilutions and extract concentrations of 1, 0.5, and 0.25 mg/ml. As explained by Pretorius, each plate was incubated at 30°C for 24 hours after being refrigerated at 4°C for an hour to allow the extract to diffuse (Pretorius et al., 2003). Positive bioactivity was indicated by the millimeter-wide inhibitory zone surrounding each well for every extract concentration, as shown in Table 10. A ruler was used to measure the resulting zone diameters, and assessments were carried out for every test organism.

Table 10: Test bacteria and inhibition zone

Tested bacteria	**Inhibition zone (mm) for different concentration**		
	E1 (1mg/ml)	**E2 (0.5mg/ml)**	**E3 (0.25mg/ml)**
S. aureus (ATCC25923)	16.5 ± 2.121	9.5 ± 0.707	6 ± 1.414
B. cereus (ATCC27895)	12 ± 1.414	8 ± 1.414	5.5 ± 0.707
P. aeruginosa (ATCC27853)	8.5± 0.707	6± 1.414	4.5± 2.121
E. coli (ATCC 25922)	10.5± 2.121	7± 1.414	3.5± 0.707
Ethanol	ND	ND	ND
Ampicillin	22.5± 0.707	16.5± 0.707	9.5± 0.707

After a day, the zones of clearing showing the inhibition of bacteria were captured on camera. Both gram-positive and gram-negative bacteria were effectively inhibited by the extracts antibacterial properties. The disc-shaped filter paper presence of an inhibition zone suggested that the extract possessed an inhibitory effect on the bacteria. The reaction of the bacteria to the samples was higher in gram positive than gram negative bacteria. The bacteria that showed the

most susceptibility to the extract were Staphylococcus aureus and Escherichia coli, respectively. These findings clearly showed that bee bread extract have good antimicrobial action.

3.5.7 FT-IR Analysis

As shown in Figure 6, the FTIR spectra samples were measured between 4000 and 400 cm^{-1}. This FTIR spectrum has similarities to those described by Dranca et al. (2020) in a prior work. Additionally, samples of bee bread and bee bread extract had similar bands with only slight spectral variations. The impurity in bee bread is the cause of this slight variation. Because of this, bee bread has a higher absorbance than bee bread extract.

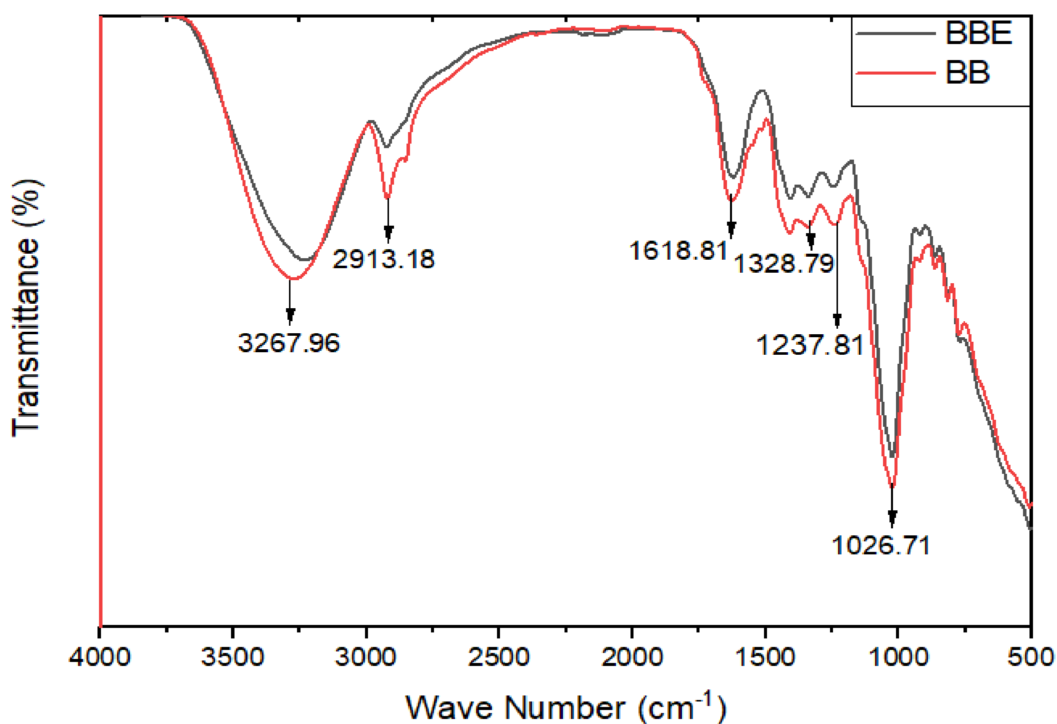

Figure 6: FTIR spectra for bee bread powder (BB) and bee bread extract (BBE)

In the 3600–3050 cm^{-1} range, one can notice the stretching vibrations of O–H and H bonds, which indicate the existence of water. Furthermore, this spectrum contains the functional groups of amides and amines I and II, which suggest the presence of proteins and amino acids. Between 3000 and 2800 cm^{-1}, the peaks linked to the symmetric and asymmetric stretching of C-H groups in the lipids and carbohydrates in bee bread samples were detected. Additionally, between 1790 and 1400 cm^{-1}, additional lipid-related peaks emerged, which were ascribed to the C=O stretching in ester bonds and the C-H deformation vibrations of lipids (Bleha et al., 2019). Furthermore, the existence of phenolic acids, flavonoids, and stilbenoids was shown by the C=O and C=C stretching vibrations. C-N stretching vibrations from amide II were found to be within the same range. C-OH groups from polyphenols and C-O vibrations from fatty acids and carbohydrates were associated with a notable peak in the 1390–900 cm^{-1} range (Ilie et al., 2024; Isopescu et al., 2020; Oroian et al., 2020).

4. Conclusion

In this study ultrasonic assisted extraction of bee bread was done which was effective than maceration extraction method. The total phenolic and flavonoid content of bee bread is higher during ultrasonic assisted extraction in optimum condition for power, temperature and sonication time. The optimum condition obtains in this study 65W ultrasonic power, 52°C temperature and 35 min sonication time. Bee bread extract was used as functional ingredients as an additional source of bioactive compounds and increase in antioxidant activity.

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