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ENHANCING THE EXTRACTION OF POLYPHENOLS FROM *ACACIA LEUCOPHLOEA* BARK BY MICROWAVE-ASSISTED ENZYMATIC EXTRACTION METHOD AND *IN-VITRO* STUDY OF ITS ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY

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Keywords:

Microwave-assisted enzymatic extraction, Polyphenols, *Acacia leucophloea*, Antioxidant activity, Antimicrobial activity, medicinal plant

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ABSTRACT: This study aims to identify the suitable method for extraction of polyphenols from *Acacia leucophloea* bark using enzymes and to optimize the processing condition for extraction using Response Surface Methodology. A microwave-assisted enzymatic extraction (MAEE) method was developed and optimized to augment the polyphenol yield. The optimal extraction conditions were as follows: the amount of cellulase 1%, agitation speed 108 rpm, incubation time 1.5 h, and irradiation time 6 min. Under these conditions, polyphenol yield could reach 6.31%, which was higher than other extraction methods studied. The antioxidant activity of the crude extract and the encapsulated extract was evaluated by DPPH radical and reducing power assay. The antibacterial activities of crude extract and the encapsulated extract were studied using the disc diffusion technique. Among the tested bacteria, *Klebsiella pneumoniae* was most susceptible to polyphenol extract with the highest inhibition zone of 18.6 ± 0.5 mm at the concentration of 8 mg/mL. The outcome of the study indicates that the MAEE method was efficient and eco-friendly, and the polyphenols have remarkable antioxidant and antibacterial activities.

INTRODUCTION: Free radicals and reactive oxygen species (ROS) are generated in the body as byproducts of cellular metabolism. It was reported that free radicals and ROS might cause oxidative damage to living cells, which leads to the development of chronic diseases such as aging, heart diseases, stroke, arthritis, and cancers. Our body has a natural defense mechanism consisting of glutathione peroxidase, catalase, and superoxide dismutase against free radicals.

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However, these endogenous antioxidants are not able to combat efficiently and therefore, supply of antioxidants through diet is required to reduce the oxidative damage due to ROS in our system [1, 2].

Antioxidants are not only needed to diminish ROS in the body but also needed for use as food additives. Thus more attention has been focussed toward finding natural antioxidants, which could clear free radicals efficiently. Antioxidants act by inhibiting the initiation and propagation steps and thereby delaying the oxidation process. They also act as scavengers of free radicals [3]. Antioxidants used as food additives can be either natural or synthetic. The toxicity and carcinogenic activity of synthetic antioxidants direct the research towards natural and safer antioxidants for food applications [4].

Plant sources are rich in natural antioxidants such as tocopherol, vitamin C, carotenoids, phenolic acids, and flavonoids [2]. Natural antioxidants have anti-viral, anti-inflammatory, anti-cancer, anti-mutagenic, anti-tumour, and hepatoprotective properties [1, 5]. The change in consumer demand for naturally processed, additive-free, and safe products has occurred [6]. So, the replacement of synthetic antioxidants by natural ones interests food technologists.

Acacia leucophloea (Roxb.) Willd. (Syn. *Mimosa leucophloea*) (Mimosoideae), native to south and southeast Asia, is a non-contiguous large thorny tree attaining height of 35 m and diameters at breast height of 100 cm. It is distributed in arid India, Sri Lanka, Bangladesh, Burma, and much of Thailand [2]. It has a white to yellowish-gray bark, which exfoliates in long strips. The bark of the plant has been used in Indian traditional medicine.

It is used in the treatment of fever, dysentery, gum bleeding, diabetes, dry cough, mouth ulcers, skin diseases, and ulcers, as it has antimicrobial, anthelmintic, expectorant, and blood purifying activity. The gum and bark decoction is used as an anti contraceptive and menstrual complaint regulator. The chemical constituents found in the tree are n-hexacosanol, beta-amyrin, beta-sitosterol, and tannins [7]. The extracts from the bark were found to have phenolics and flavanoids [8].

Enzyme-assisted extraction can be used to increase bioactive components release from plant material, which reduces extraction time and usage of solvents and provide increased yield and quality of the product. Various enzymes such as cellulases, pectinases, and hemicellulase are often utilized to disrupt the structural integrity of the plant cell wall, thereby enhancing the extraction of bioactives from plants [9]. These enzymes have been studied to be efficient for extraction of polyphenols from plant sources such as kinnow peel, orange peel, Grape pomace, *Pinus taiwanensis* and apple pulp [10-14].

Microwave-assisted enzymatic extraction (MAEE) is a novel and green extraction technique that can offer high reproducibility in a shorter time, simplified manipulation, reduced solvent consumption, and lower energy input without decreasing the extraction yield of the target species [15]. As a new-type extraction technique, MAEE is known as a more environmental-friendly process with economic advantages than the traditional extraction methods. In recent times, this technique has been commonly used for sample preparation [16]. There is limited work on the study of polyphenol extraction and antioxidant activity of *Acacia leucophloea* bark.

Therefore, the objective of this work is to study the feasibility of microwave-assisted enzymatic extraction (MAEE) for the polyphenols from *Acacia leucophloea* bark and to evaluate their antioxidant and antibacterial activities *in-vitro*. Box-Behnken design (BBD) combined with response surface methodology (RSM) was applied to study the interaction among MAEE operating parameters. Moreover, the effect of micro-encapsulation on antioxidant and antibacterial activities of *Acacia leucophloea* bark extract from MAEE was also studied.

MATERIALS AND METHODS:

Plant Material and Reagents: The bark of *Acacia leucophloea* was collected from different places of Tirupur and Erode districts, Tamil Nadu, India. Freshly collected bark of *Acacia leucophloea* was dried at 40℃ in a tray dryer (CM Envirosystems-Humidry-TD-12-S-E) and pulverized using a ball mill (Everflow) to a coarse powder and passed through 1.4 mm mesh sieve.

2,2-diphenyl-1-picrylhydrazyl (DPPH) and tert-Butylhydroquinone (TBHQ) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Gallic acid was obtained from Himedia, India, and cellulase was obtained from Sisco Research Laboratory Pvt., Ltd., Mumbai, India. All other chemicals used in the study were of analytical grade.

Methods of Extraction:

Non-Enzymatic Extraction: Powder of 1.0 g dried *Acacia leucophloea* bark was weighed and added into a round bottom flask with 50 mL 70% (v/v) ethanol. The flask was placed in a heating mantle and connected with a condenser, and then allowed to reflux for 3.0 h at 65 °C.

Enzymatic Extraction: Powder of 1.0 g dried *Acacia leucophloea* bark was weighed and added into a conical flask. To the flask, 50 mL of the enzymatic solution was prepared by dispersing 1.0

wt. % (with respect to solid sample) cellulase in distilled water was added, and then pH was adjusted to 5.5 using 0.1N HCl or 0.1N NaOH solution. The flask was kept in an orbital shaker (Remi CIS-24BL) and then allowed to incubate at 55°C and agitated at 100 rpm for 2 h.

Microwave-Assisted Enzymatic Extraction (MAEE): Powder of 1.0 g dried *Acacia leucophloea* bark was weighed and added into a conical flask with 25 mL double distilled water. The flask was sealed and microwave irradiated at 160W for a defined time (4.0-8.0 min). After microwave irradiation, an enzymatic solution prepared by dispersing (0.5-1.5 wt. % with respect to solid sample) cellulase in distilled water was added to the samples, and then pH was adjusted to 5.5 using 0.1N HCl or 0.1N NaOH solution. The flask was kept in an orbital shaker (Remi CIS-24BL) and then allowed to incubate at 55 °C and agitated at a different speed (80-120 rpm) for 1.0-2.0 h. The residues were then filtered, and total polyphenols in the filtrates were determined. All experiments were conducted in triplicate.

Concentration of the Extract: The filtrate was concentrated by drying in tray dryer at 50 °C and stored until needed for the bioassays at -4 °C.

Experimental Design: A four-variable, three-level Box–Behnken design (BBD) was applied to determine the best combination of extraction variables for the polyphenol yield from the bark of *Acacia leucophloea*. The four independent variables were the amount of cellulase (%, X_1), agitation speed (rpm, X_2), incubation time (h, X_3), and irradiation time (min, X_4), and each variable was set at three levels. The uncoded (actual) levels of the independent variables are given in **Table 1**.

TABLE 1: CODE AND LEVEL OF FACTORS CHOSEN FOR THE STUDY

Factors	Lower value	Middle value	Higher value
Amount of cellulase (%, X_1)	0.5	1.0	1.5
Agitation speed (rpm, X_2)	80	100	120
Incubation time (h, X_3)	1	1.5	2
Irradiation time (min, X_4)	4	6	8

Twenty-nine experiments, augmented at the center points five times, were carried out. Regression analysis was performed for the experiment data and was fitted into the empirical second-order polynomial model, as shown in the following equation:

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} X_i X_j \qquad (1)$$

Where β_o is the offset term, β_i is the i^{th} linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the ij^{th} interaction coefficient, X_i and X_j are input variables, real values converted from coded values, which influence the response variable Y.

Determination of Total Polyphenolic Content: The total polyphenols content of the extract was obtained by following the Folin-Ciocalteau method [17]. 0.1 mL aliquots of dilute extracts were adjusted to 3 mL by adding distilled water and shaken with 0.5 mL of Folin-Ciocalteau reagent. After 3 min, 2 mL of 20% (w/v) sodium carbonate was added, and the mixture was shaken well and kept at 50 °C for 1 min. After incubation, the absorbance was measured at 650 nm on a spectrophotometer (Elico SL244). The results are expressed as a percentage of polyphenol content (gallic acid equivalents) in the raw material studied.

Antioxidant Activities:

DPPH Radical Scavenging Activity Assay: The DPPH radical-scavenging assay was used to determine the antioxidant activity of the polyphenols extract following the method of Zhang *et al*. [18] The 0.2 mmol/L solution of DPPH in methanol was freshly prepared before use. Serial dilutions of the extracts were carried out. Solutions (2 mL each) were then mixed vigorously with methanolic solution DPPH (2 mL) and allowed to stand in the dark for 30 min at ambient temperature for any reaction to occur, and the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity, which was analyzed from the graph plotted of inhibition percentage against compound concentration. TBHQ (tert-butyl hydroquinone) was used as a positive control. All tests were performed in triplicate and averaged. The scavenging activity was estimated based on the percentage of DPPH radical scavenged as the following equation:

$$\%\ inhibiton = \left[\frac{1-(Ai-Aj)}{Ac}\right] x\ 100 \qquad (2)$$

Where Ac is the absorbance of DPPH solution without sample, Ai is the absorbance of the sample with DPPH solution, and Aj is the absorbance of the sample without DPPH solution.

Reducing Power: The reducing power of the extract was determined according to the method of Vaquero *et al*. [19] Extract aliquots of varying concentration was mixed with phosphate buffer (0.2 M, pH 6.6) and 1% (w/v) potassium ferricyanide. After the mixture was incubated at 50 ºC for 25 min, 10% (w/v) trichloroacetic acid was added, and the mixture was centrifuged at 3000 rpm for 10 min. The upper layer of solution was mixed with distilled water and 0.1% ferric chloride for 10 min, and then the absorbance was measured at 700 nm against a blank. Increasing absorbance of the reaction mixture indicates increasing reducing power [18]. TBHQ was used as a positive control. All tests were performed in triplicate and averaged.

Encapsulation of the Extract: As a large number of polyphenolic compounds show limited water solubility and unpleasant taste, it insists the formulation of a finished protecting product, which is able to maintain the structural integrity, mask its taste, increase its water solubility and bioavailability, and convey it precisely towards a physiological target. Among the existing stabilization methods, encapsulation is an interesting means. The extract obtained from the MAEE method at optimized conditions was then mixed with 10% maltodextrin, 0.3% tween 80, and mixed using a beater until foam was retained. The foam obtained was scattered on the pan and dried at 60 °C for 2h in tray dryer (CM Envirosystems-Humidry-TD-12-S-E). The dried powder was blended and sifted using a sieve 100 mesh [20].

Antibacterial Activity:

Microbial Strains and Inoculum Preparation: The *in-vitro* antibacterial activity of the polyphenol extract was evaluated against four pathogenic microorganisms *viz. Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus vulgaris* and *Staphylococcus aureus.* All strains stock cultures were maintained at 4 °C on nutrient agar medium. Active cultures were prepared by inoculating fresh nutrient broth medium with a loopful of cells from the stock cultures at 37 °C for overnight. To get desirable cell counts for bioassays, overnight grown bacterial cells were subcultured in fresh nutrient broth at 37 °C [21].

Disc Diffusion Method: The antimicrobial activity of extract of *Acacia leucophloea* bark was screened using the disc diffusion technique. Inoculum of 0.1 mL from microorganism suspensions (10^6 CFU/ml) was spread on the solid agar plates and was allowed to stand for 15 min. Autoclaved filter paper discs of 6mm were loaded with 250 µL of polyphenol extracts of different concentrations (2, 4, 6, 8 mg/mL). Cefotaxime (1 mg/mL) was used as a positive control to determine the sensitivity of bacterial species tested. The loaded disc was placed on the surface of the medium and incubated at 37 °C for 24 h. The diameters of the inhibition zones were measured in millimeters. These studies were performed in triplicate.

Determination of MIC by Visual Analysis: The minimal inhibitory concentration (MIC) of polyphenol extract was determined by serial dilution technique [22]. The tested concentrations of polyphenol extract ranged from 0.025 to 5 mg/mL [18].

Statistical Analysis: All results of twenty nine experiments in Box-Behnken design were analyzed using Design Expert® software version 8.0.7.1 from Statease Inc., Minneapolis. The significance of the intercept, linear, squared, and interaction coefficients of the factors was evaluated by analysis of variance (ANOVA).

RESULTS AND DISCUSSION:

Comparison of MAEE with other Extraction Methods: Polysaccharides are the major barrier to the release of polyphenolic compounds. A recent study has revealed that the formation of the complex polysaccharide–phenol entities might be due to two mechanisms of association, *viz*., (1) hydrogen bonds and (2) hydrophobic interactions[23]. Cellulase can catalyze the breakdown of the rigid skeleton of cellulose and degrade the plant cell wall constituents to improve the release of polyphenolic compounds [24]. Microwave-assisted extraction (MAE) is a fast extraction process where microwave energy is delivered efficiently to materials through molecular interaction with the electromagnetic field and offers a rapid transfer of energy to the extraction solvent and raw plant materials [25, 26]. Furthermore, the direct interaction of microwave

with solvent also results in the rupture of the plant cells and release of intracellular products into the solvent quickly [27, 28]. As both cellulase and MAE are independent cases and are effective in rupturing the cell, they must be combined together to check whether the enzyme and MAE can work together to improve polyphenol extraction yield.

The efficiency of extracting polyphenols from *Acacia leucophloea* bark by different methods, including non-enzymatic extraction, enzymatic extraction, and microwave-assisted enzymatic extraction (MAEE) was studied. The extraction conditions and yields are shown in **Table 2**.

TABLE 2: COMPARISON OF POLYPHENOL YIELD BY DIFFERENT EXTRACTION METHODS

Method	Extraction time (h)	Temperature (°C)	Solvent used	Microwave irradiation time (min)	Polyphenol yield (%)[a,b]
Non-Enzymatic	3	65	70% ethanol	-	1.9 ± 0.10
Enzymatic	2	55	Water	-	4.3 ± 0.15
MAEE	2	55	Water	6 min	5.7 ±0.16

[a] The values of polyphenol yield are expressed as mean and standard deviation (SD) calculated from three independent experiments.
[b]Polyphenol yield is a percentage of extracted polyphenol content in the raw material studied

Table 2 shows that non-enzymatic extraction yield (about 1.9 ± 0.1%) was inferior compared with those of enzymatic extraction and MAEE. It can be also established that the extraction yield (5.7 ± 0.16%) obtained by MAEE was higher than that (4.3 ± 0.15%) by enzymatic extraction. The highest polyphenol yield using MAEE indicated that MAEE was more efficient than the other two methods. This higher efficiency could be recognized as microwave irradiation, which produces the disruptions of tissues and cell walls, leading to a greater contact area between solid and liquid phase, better access of solvent to valuable components [29].

Optimization of MAEE Conditions using Box–Behnken Design:

Fitting the Model:

TABLE 3: BOX-BEHNKEN DESIGN WITH EXPERIMENTAL RESPONSES

Run	Independent variable				Response
	X_1 Amount of cellulase (%)	X_2 Agitation speed (rpm)	X_3 Incubation time (h)	X_4 Irradiation time (min)	Polyphenol yield (%)
1	0.5	100	1	6	3.86
2	1	100	1.5	6	6.12
3	1	80	1	6	3.79
4	1	120	1.5	8	4.34
5	1	100	2	8	4.05
6	1	100	1	8	4.17
7	1.5	100	1.5	4	5.21
8	0.5	80	1.5	6	2.79
9	1.5	120	1.5	6	5.29
10	1	100	1	4	3.79
11	1	80	2	6	4.69
12	1	100	1.5	6	6.16
13	1	100	1.5	6	6.19
14	1	120	1	6	5.11
15	1	80	1.5	8	4.09
16	0.5	100	2	6	3.56
17	1	100	2	4	5.11
18	0.5	100	1.5	4	3.56
19	1.5	100	1	6	4.72
20	1.5	100	2	6	5.79
21	1.5	80	1.5	6	5.59
22	0.5	100	1.5	8	3.23
23	1.5	100	1.5	8	4.72
24	0.5	120	1.5	6	5.09
25	1	100	1.5	6	6.14

26	1	80	1.5	4	3.77
27	1	120	2	6	5.28
28	1	100	1.5	6	6.16
29	1	120	1.5	4	5.4

TABLE 4: ESTIMATED REGRESSION COEFFICIENTS AND ANALYSIS OF VARIANCE (ANOVA) FOR THE QUADRATIC POLYNOMIAL MODEL

Parameter	Estimated coefficients	Standard error	DF[a]	Sum of squares	*F*-value	Prob > *F*
Intercept						
β_o	-50.1663	0.024706	1	7.099408	636.581297	< 0.0001
Linear						
X_1	13.096	0.015948	1	2.793675	2326.131373	< 0.0001
X_2	0.484042	0.015948	1	0.770133	915.351640	< 0.0001
X_3	12.66567	0.015948	1	0.418133	252.335296	< 0.0001
X_4	4.665167	0.015948	1	1.69	137.001989	< 0.0001
Quadratic						
X_1^2	-0.065	0.027623	1	0.469225	553.730935	< 0.0001
X_2^2	1.37	0.027623	1	0.0064	153.742247	< 0.0001
X_3^2	-0.04	0.027623	1	0.133225	2.096969	0.1696
X_4^2	-0.01825	0.027623	1	0.4761	43.651363	< 0.0001
Interaction						
$X_1 X_2$	-0.00863	0.027623	1	0.5184	155.994851	< 0.0001
$X_1 X_3$	-0.36	0.027623	1	4.787184	169.854507	< 0.0001
$X_1 X_4$	-3.43633	0.021691	1	2.587443	1568.527681	< 0.0001
$X_2 X_3$	-0.00158	0.021691	1	4.10306	847.779522	< 0.0001
$X_2 X_4$	-3.18133	0.021691	1	7.924941	1344.373582	< 0.0001
$X_3 X_4$	-0.27633	0.021691	1		2596.618426	< 0.0001
Model			14	27.20006	636.581297	< 0.0001
Lack of fit			10	0.040008	5.883578	0.0512
Pure error			4	0.00272		
R^2	0.998			Adjusted R^2	0.996	
C.V. %	1.2					

[a] Degree of freedom

Table 3 presents the experiment design and corresponding response data for the polyphenols yield from *Acacia leucophloea* bark. The regression coefficients of the intercept, linear, quadratic, and interaction terms of the model were are shown in **Table 4**. It was evident that all linear and all the interaction parameters were significant at the level of $P < 0.01$, whereas one quadratic parameter was insignificant ($P > 0.05$). Neglecting the non-significant parameter, the final predictive equation obtained was as follows:

$$Y = - 49.9263 + 12.856 X_1 + 0.484042 X_2 + 12.66567 X_3 + 4.625167 X_4 -0.065 X_1 X_2 +1.37 X_1 X_3 - 0.01825 X_2 X_3 - 0.00863 X_2 X_4 - 0.36 X_3 X_4 - 3.43633 X_1^2 - 0.00158 X_2^2 - 3.18133 X_3^2 - 0.27633 X_4^2 \quad (3)$$

The analysis of variance (ANOVA) for the experimental results is given in **Table 4**. For the model fitted, the determination coefficient (R^2) was 0.996, which implied that the sample variations of 99.6% for the MAEE efficiency of *Acacia leucophloea* bark polyphenols were attributed to the independent variables, and only 0.4% of the total variations cannot be explained by the model. *F*-value for the lack of fit was insignificant ($P > 0.05$), thereby confirming the validity of the model. The value of the coefficient of variation (C.V.) was 1.2%, suggesting that the model was reliable and reproducible [30, 31]. The results indicated that the model could work well for the prediction of polyphenols extract from *Acacia leucophloea* bark.

Analysis of Response Surfaces: In order to investigate the interactive effects of operational parameters on polyphenols extraction, the three-dimensional response surface plots were generated by plotting the response on the *Z*-axis against two independent variables while keeping the other two independent variables at their middle value.

Phenols are more linked to the cell wall and are contained in vacuoles, and several factors can

affect phenols released from cell walls and vacuoles, including the amount of enzyme. The amount of enzyme is a critical variable, which can improve the extraction of phenols from the cell wall. Apparently, the enzyme assisting the release of phenols from the cell-wall matrix occurs via an enzyme catalysed hydrolytic degradation of the cell wall polysaccharides and breakage of the ether and/or ester linkages between the phenols and the plant cell-wall polymers [23]. However, it must be taken into account that the amount of enzyme is applied for extraction with two main aims: (1) to calculate the amount of enzyme that is required for complete extraction, and (2) to avoid the excess use of enzymes [24].

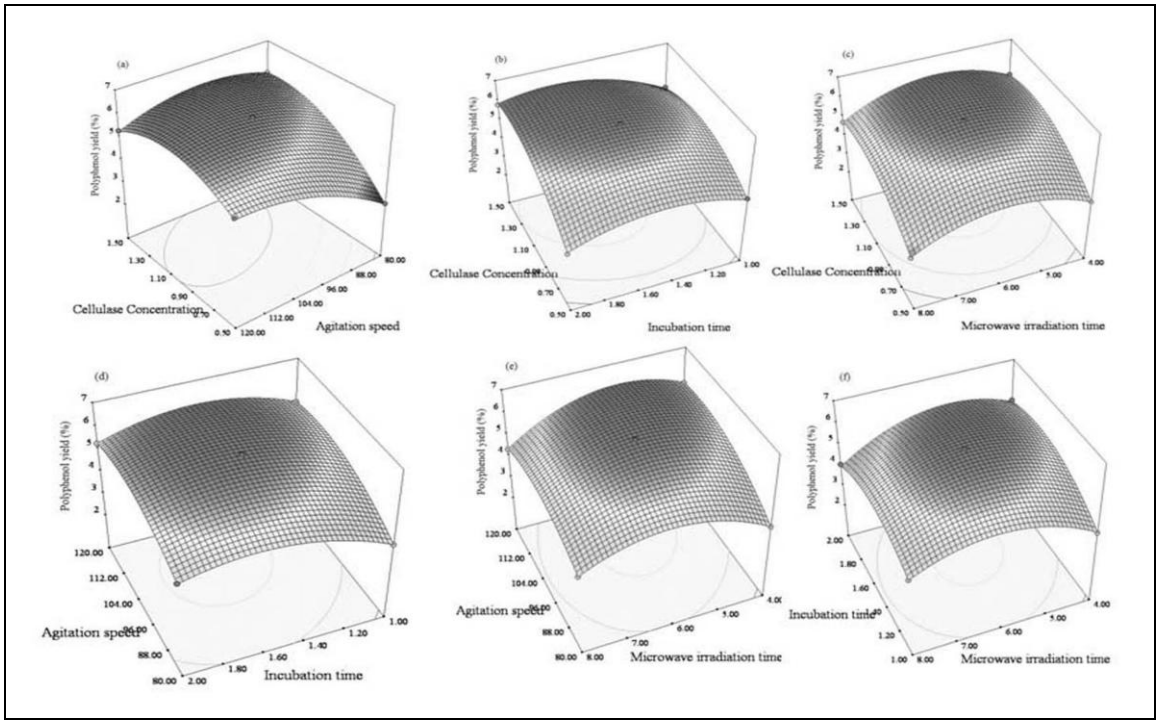

FIG. 1: RESPONSE SURFACE PLOTS SHOWING EFFECTS OF THE EXTRACTION PARAMETERS ON POLYPHENOL YIELD: (A) AT VARYING AMOUNT OF CELLULASE (%) AND AGITATION SPEED (RPM), (B) AT VARYING AMOUNT OF CELLULASE (%) AND INCUBATION TIME (H) (C) AT VARYING AMOUNT OF CELLULASE (%) AND IRRADIATION TIME (MIN) (D) AT VARYING AGITATION SPEED (RPM) AND INCUBATION TIME (H) (E) AT VARYING AGITATION SPEED (RPM) AND IRRADIATION TIME (MIN) (F) AT VARYING INCUBATION TIME (H) AND IRRADIATION TIME (MIN)

Fig. 1a-c depicts the interactions between the amount of cellulase and each of the three other factors (agitation speed, incubation time, and irradiation time) on the extraction yield of polyphenols. As can be seen from **Fig. 1a-c** that the extraction yield of polyphenols increased gradually with increasing amounts of cellulase from 0.5 to 1.2 wt.%, while increasing the amount of cellulase from 1.2% to 1.5%, the polyphenol yield was maintained at 6.2 %, indicating that 1.2% enzyme provided sufficient amounts of activity for the hydrolysis of the cell-wall.

The interactions of agitation speed with each of the three other factors on the extraction yield of polyphenols are shown in **Fig. 1a**, **d,** and **e**. In all three cases, the extraction yield increased rapidly with increasing agitation speed from 80 to 107 rpm, and then followed by a decline with the further increase. A similar influence of the agitation speed has been observed in various studies. Excessively high mixing speeds (200 rpm) lowered the extent of cellulose conversion, while moderate mixing speeds (100-200 rpm) provided a good combination of fast initial hydrolysis rates and high conversion yields in Avicel and paper pulp [32]. Cellulose hydrolysis by cellulase enzymes requires adequate mixing to ensure sufficient contact between the substrate and enzymes and to promote heat and mass transfer within the reaction vessel. Some agitation increases the hydrolysis rate and yields, but excessive mixing can deactivate the enzymes and reduce the conversion yield [33, 34].

The deactivation effect has been attributed to the shear force generated by the mixer and the entrapment of air bubbles into the medium at the air-liquid surface [35].

Fig. 1b, d, and **f** display the interactions between incubation time and each of the three other factors on the polyphenols extraction yield. The results showed that the polyphenols extraction yield increased with the extension of incubation time till 1.6 h.

The interaction of irradiation time with each of the three other operational parameters on the polyphenol yield is shown in **Fig. 1c, e,** and **f**. It can be observed from the figure that the polyphenol yield increased with increasing irradiation time and reached a maximum value in 6 min. After that, longer irradiation time caused negative effects due to degradation or conversion of the polyphenols[18].

Verification of the Predictive Model: By carrying out parameter optimization on the basis of the built mathematical models, the obtained experimental conditions for polyphenol yield were: the amount of cellulase 1.00wt%, agitation speed 108 rpm, incubation time 1.54 h, and irradiation time 6.28 min. Under the optimal parameters, the predictive value of polyphenol yield was 6.28%. Because the values of incubation time and irradiation time are difficult to operate in the actual extraction experiments, they were carried out with slight modifications: incubation time in 1.5 h and irradiation time in 6 min. The experimental polyphenol yield of 6.31% at optimized conditions was consistent with the predicted value. The strong correlation between the real and predicted results confirmed that the response model was satisfactory to reflect the expected optimization condition.

Antioxidant Activity: Phenolic compounds have exhibited strong antioxidant activities and are able to quickly reduce reactive oxygen species (ROS), including free radicals, thereby protecting biomolecules (*e.g.*, polyunsaturated fatty acids) against oxidation [36]. In this study, the antioxidant activities of the polyphenol extracts from *Acacia leucophloea* bark were evaluated by using in vitro antioxidant models, namely DPPH radical scavenging assay and reducing power.

DPPH Radical Scavenging Assay: The hydrogen atom or electron donation abilities of some pure compounds were measured by bleaching a purple-colored methanol solution of the stable DPPH radical. The DPPH solution has a deep violet colour, and radical scavenging activity of antioxidant compounds by the loss of absorbance as the pale yellow non-radical form (DPPH-H) is produced [2].

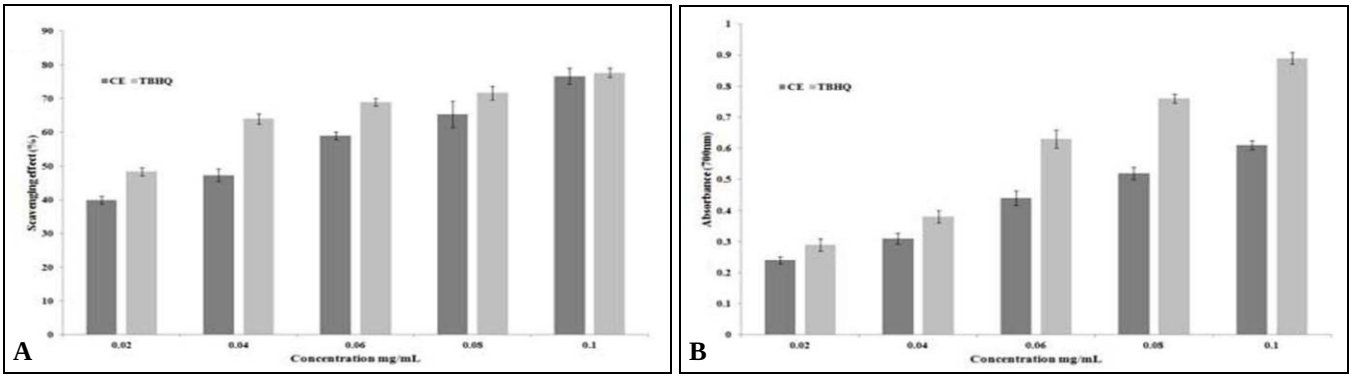

FIG. 2: ANTIOXIDANT ACTIVITIES OF THE POLYPHENOL EXTRACT FROM *ACACIA LEUCOPHLOEA* BARK BY MAEE METHOD: (A) DPPH RADICAL SCAVENGING ACTIVITY (B) REDUCING POWER. CE, CRUDE EXTRACT; TBHQ, TERT-BUTYLHYDROQUINONE (MEAN ± D, N=3)

Fig. 2a exhibits the radical scavenging capacities of the polyphenol extracts on DPPH (TBHQ as positive control). From **Fig. 2a**, a dose-response relationship was observed in the DPPH radical scavenging activity. The activity increased with increasing concentration of the polyphenol extract. Crude extract has shown DPPH radical scavenging activity of 80.33% higher than TBHQ (77.66%) in a concentration of 0.1mg/mL and lemon peel essential oil which showed 54.67% scavenging ability [37]. These results indicated that the scavenging effect of polyphenol extract on DPPH radical was remarkable.

Reducing power: In the reducing power assay, the presence of reductants (antioxidants) in the samples would result in the reduction of the Fe^{3+}/ ferricyanide complex to its ferrous form (Fe^{2+}) by donating an electron. Hence, the Fe^{2+} can then be monitored by measuring the formation of Perl's Prussian blue at 700 nm [38]. A higher absorbance value indicates higher reducing power.

Fig. 2b presented the reductive capabilities of polyphenol extracts and compared with TBHQ as control standards. As seen in **Fig. 2b**, the reducing power of all the samples increased gradually with the increase in concentration tested. The reducing power of the crude extract was found to be lower than TBHQ in the tested concentration range. The reducing power may increase with the purified extract of *Acacia leucophloea* bark, as shown by purified extract of polyphenols from peanut shells [18].

Encapsulated *Acacia leucophloea* Bark: Natural polyphenols are valuable compounds possessing scavenging properties towards radical oxygen species and complexing properties towards proteins. Unfortunately, these properties are also responsible for a lack of long-term stability. Moreover, polyphenols often present a poor biodisponibility mainly due to low water solubility. Lastly, many of these molecules possess a very astringent and bitter taste, which limits their use in food or in oral medications. To circumvent these drawbacks, delivery systems have been developed, and among them, encapsulation would appear to be a promising approach [39].

The extract of *Acacia leucophloea* bark obtained at optimized conditions of MAEE method was encapsulated and its total polyphenol content, scavenging activity by DPPH assay, and reducing power were found to be 3.6 %, 59.19%, and 0.49 (1mg powder/mL). Desai and Park noted that maltodextrin could improve the stability of phenol compounds as maltodextrin can protect phenol compounds from oxidation effect, oxygen, water, and extreme temperature [40].

Antibacterial Activity: The results of antibacterial activity against four bacterial species are shown in **Table 5**. It can be observed that crude extract possessed an inhibitory effect on the bacterial species studied (*Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus vulgaris,* and *Staphylococcus aureus*). The inhibitory zones for the bacterial strains were in the range of 8.3 ± 0.5 to 10.3 ± 0.5 (2 mg/mL), 10.3 ± 0.2 to 12.1 ± 0.2 (4 mg/mL), 12.5 ± 0.5 to 15.0 ± 0.5 (6 mg/mL) and 13.8 ± 0.7 to 18.6 ± 0.5 (8 mg/mL), respectively. The zone of inhibition was increased with increasing the concentration of polyphenol extract. The composition and concentration of secondary metabolites determine the antimicrobial efficacy of plants. The bark extracts of *Acacia leucophloea* were found to have steroids, polyphenols, tannins, alkaloids, gums, and mucilages, which could be responsible for the medicinal properties of this plant [41]. Among the tested bacteria, *Klebsiella pneumoniae* was most susceptible to polyphenol extract with the highest inhibition zone of 18.6 ± 0.5 mm at the concentration of 8 mg/mL [42].

On the other hand, low antibacterial activity was exhibited against *Pseudomonas aeruginosa* and *Staphylococcus aureus* (14.8 ± 0.7 and 13.8 ± 0.7 mm, respectively). The encapsulated extract had been found to demonstrate remarkable antibacterial activity against the tested bacterial species. In addition, the minimal inhibitory concentration (MIC) of polyphenol extract was found in the 0.5-1.0 mg/mL concentration range. The lowest MIC value (0.5 mg/mL) was obtained for *Klebsiella pneumoniae.*

TABLE 5: ANTIBACTERIAL ACTIVITIES OF CRUDE EXTRACT (CE) ZONE OF INHIBITION AND MINIMUM INHIBITORY CONCENTRATIONS (MIC)

Bacteria	Zone of Inhibition [a]						MIC[b]
	CE (mg/mL)				Encapsulated extract	Cefotaxime	
	2	4	6	8			
Klebsiella pneumoniae	10.3±0.5	12.1±0.2	15.0±0.5	18.6±0.5	14.6±0.5	28.3±0.5	0.50
Pseudomonas aeruginosa	8.3±0.5	10.9±.05	13.1±0.5	14.8±0.7	9.3±0.5	26.2±0.4	1.0
Proteus vulgaris	8.5±0.5	10.3±0.2	14.5±0.5	16.5±0.5	11.8±0.2	23.1±0.2	1.0
Staphylococcus aureus	8.5±0.5	10.6±0.5	12.5±0.5	13.8±0.7	9.3±0.7	24.1±0.2	1.0

[a] Diameter of inhibition zones (mm) including the diameter of the disc (6 mm), values are expressed as the mean ± SD (n=3).
[b] Minimal inhibitory concentration (mg/mL) of CE.

CONCLUSION: In the present study, the extraction yield of total polyphenols using MAEE method was higher than other extraction methods. The operational parameters for enhanced extraction of polyphenols from *Acacia leucophloea* bark by MAEE process were optimized with a Box-Behnken design based on response surface methodology. The optimal extraction conditions of MAEE using response surface methodology obtained were as follows: the amount of cellulase 1%, agitation speed 108 rpm, incubation time 1.5 h, and irradiation time 6 min.

The predicted polyphenol yield was well consistent with the experimental value. The bark extract and encapsulated product exhibited DPPH radical scavenging activity, reducing power, and antimicrobial activity. The results of the present study may be helpful to further exploit and utilize this resource.

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