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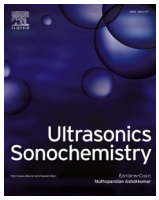

Ultrasound assisted extraction of phytochemicals from *Piper betel L.*

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ABSTRACT

Piper betel contains phytochemicals with diverse pharmacological effects. The objective of this study was to enhance the extraction efficiency of phytochemicals and the chlorophyll content using ultrasonication. The Box-Behnken design was employed to optimize the time (10, 20, 30 min), temperature (20, 30, and 40 °C), and solid-solvent ratio (1:10, 1:20, 1:30) by utilizing response surface methods with three independent variables. Multiple parameters, including extract yield, total phenol, total flavonoid, antioxidant activity, and chlorophyll content were used to optimize the conditions. The linear relationship between power intensity and responses was determined to be statistically significant, with a p-value less than 0.01. The interaction effect of temperature, time, and ratio of solid solvent was shown to be statistically significant ($p < 0.05$) for all the obtained results. The optimal parameters for achieving the highest extract yield were as follows: a temperature of 40 °C, a sonication time of 30 min, and a solid solvent ratio of 1:10. These conditions result in an extract yield of 21.99 %, a total flavonoid content of 44.97 mg/GAE, a total phenolic content of 185.05 mg/GAE, a DPPH scavenging activity of 99.1 %, and a chlorophyll content of 49.95 mg/ml. This study highlights the significance of customized extraction methodologies for optimizing the bioactive capacity of phytochemicals derived from betel leaves. The elucidation of extraction parameters and the resultant phytochemical profiles serves as a fundamental framework for the advancement of innovative pharmaceuticals and nutraceuticals, capitalizing on the therapeutic attributes of this traditional medicinal botanical.

1. Introduction

Betel leaf belongs to the *Piperaceae* family and has the scientific name *Piper Betel L.* Betel leaf, an aromatic perennial vine, has a heart-shaped appearance. Betel leaf is referred to as "Green Gold" because of its leaf color. This plant is highly sought after in India, Malaysia, Thailand, Sri Lanka, Taiwan, and other Southeast Asian countries because of its positive effects on oral health [1]. Piper betel has been the subject of numerous studies, which have consistently identified several significant chemical constituents. These include chavibetol, chavibetol acetate, caryophyllene, allyl pyrocatechol diacetate, camphene, chavibetol methyl ether, eugenol, a-pinene, f-pinene, u-Limonene, saprobe, 1–8-cineol, and allyl pyrocatechol monoacetate. These components are highly regarded for their medical characteristics including their ability to stimulate the body. They have been found to have anti-platelet and anti-inflammatory actions, as well as the ability to modulate the immune system, protect the stomach, and function as antidiabetic agents [2]. An experiment was conducted using a methanolic extract of red betel leaf to evaluate its cytotoxic and anti-migratory effects on metastatic breast cancer [3]. These plants can be utilized in the search for a more organic, environmentally friendly, and cost-effective supply of medication in the primary healthcare system owing to the extensive range of their bioactive components [4]. The essential oils found in betel leaves are responsible for their flavor and aroma, which are vital for creating their unique taste. Furthermore, these essential oils are used in the production of perfumes and mouth fresheners [5].

Ultrasound extraction has gained popularity over Soxhlet extraction in the pharmaceutical industry because it is a viable alternative to

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traditional extraction procedures. This is mostly due to its superior efficiency, low solvent consumption, low energy demand, and quick extraction time. Ultrasound-assisted oil extraction is frequently employed because of its ecological safety and ease of integration with other extraction processes. Ultrasound-aided extraction requires less solvent than the traditional extraction methods [6]. Environmentally friendly extraction methods such as microwave-assisted extraction, ultrasound-assisted extraction, supercritical fluid extraction, pulse electric field extraction, and pressurized liquid extraction are used for the extraction [7,8]. The UAE method, characterized by reduced solvent amounts, shortened process and residence times, and accelerated heat and mass transfers, is widely regarded as the most ecologically sound process among these procedures [9]. UAE functions based on the cavitation hypothesis, which leads to the generation of localized high pressures, extremely high temperatures, and shear forces inside the medium. The production of macroturbulence caused by the implosion of cavitation bubbles on a solid surface leads to cell disintegration and the release of phenolics into the solvent. This approach proved to be efficient in isolating bioactive compounds with higher extraction yields compared to conventional methods, while also maintaining the desired biological properties of the extracts [10] and [11]. UAE has been widely used for the retrieval of polyphenols, flavonoids, and antioxidants owing to its exceptional capability in terms of recovery efficiency and extraction speed [12].

The response surface methodology facilitates the evaluation of the quadratic, linear, and interaction effects of independent factors on the response variables. RSM promotes the construction of models, experimental design, and simultaneous detection of the linear and interaction effects of several parameters to obtain optimal conditions while minimizing the number of experiments [13]. The current study aimed to determine the most effective extraction conditions for the ultrasonic-assisted extraction (UAE) method using ethanol as a solvent. This will be achieved through the application of a multivariate technique known as the Box-Behnken design in combination with response surface methodology. Subsequently, this study assessed the efficiency of UAE on betel leaf powder and analyzed the presence of bioactive compounds, including total phenol, total flavonoid, antioxidant activity, and total chlorophyll content. Response surface methodology (RSM) was employed to optimize the process variables, including sonication temperature, sonication time, and solid-to-solvent ratio. This study contributes to the existing body of knowledge on phytochemical extraction from betel leaves by introducing innovative approaches to maximize the yield of bioactive compounds. Our research explores novel extraction techniques and systematically analyzes the effects of various parameters to provide a comprehensive understanding of the extraction process. Specifically, we investigated the effects of various solvents, extraction durations, and temperatures on the extraction efficiency and composition of the phytochemicals. Additionally, we will assess the antioxidant and chlorophyll contents of the extracted compounds to provide valuable insights into their potential applications in the pharmaceutical and food industries.

2. Material method

2.1. Raw material collection

Fresh betel leaves were collected from the Paan Mandi Laxmi Bazaar, Naka Hindola, and Lucknow. Damaged leaves were separated from the collected leaves, and the dirt and residue were washed away with flowing water. These betel leaves were cut into tiny pieces and left to dry overnight in a hot air oven set at 40 °C. The sample size was decreased using a grinder, and after it had been ground into a powder, it was hand-screened to a 150-μm particle size. The chemicals 2,2 diphenyl-1-picrylhydrazyl (DPPH), Aluminum Chloride, Potassium Acetate, Quercetin, gallic acid, Folin-Ciocalteu reagent, ethanol, sodium carbonate, phenol reagent, and Nutrient Agar (HI Media) were purchased from HI Media.

2.2. Ultrasound-assisted extraction

In a 250-ml beaker, 10 g of betel leaf powder was transferred, and the solvent was added at different ratios (1:30, 1:10, and 1:20). Several extraction times and temperatures were used. The temperatures used for sonication were 20, 30, and 40 °C, with corresponding sonication periods of 10, 20, and 30 min. These values were determined based on the results of the Box-Behnken design presented in Table 1. The extract was filtered through Whatman filter paper with a pore size of 0.45 μm. Following filtration, the extract containing the green color was transferred into Petri dishes to allow the solvent to evaporate at ambient temperature. The ethanol was evaporated, and the sample was stored in a hermetically sealed container. During ultrasonication (Sonicator Pro 250), the probe transmits ultrasonic energy directly to the leaves for sonication treatment. The design was created using Design Expert 11.0, software developed by Stat Ease, Inc., Minneapolis, USA. The experiment had 17 trial runs, including five center points that approximately represented the sum of squares for the pure error.

2.3. Response surface methodology

The study examined the application of the response surface approach to simultaneously treat betel leaves with an ultrasound probe and extract phytochemicals, while also investigating the color qualities. A Box–Behnken design was used to determine the optimal conditions for extracting betel leaves using sonication. The three independent variables were time, solid solvent ratio, and temperature. Response Surface Methodology (RSM) combines statistical and mathematical techniques by utilizing polynomial equations to accurately model experimental data.

2.4. Analysis of phytochemicals

2.4.1. Extraction yield

The extraction yield (%) measures the amount of extract recovered in mass relative to the initial amount of the sample, which evaluates how well the solvent extracts particular components from the starting material. Each strategy examined was established. The extraction yield was determined by weighing the dried extract [14,15]. Yield was determined by measuring the dry weight of the extract (Eq. (1)).

$$\text{Extract yield}\,(\%) = \left(\frac{Dryweightofextractedproduct}{weightfplantmaterial}\right) \times 100 \quad (1)$$

2.4.2. Total phenolic content

According to [16], the total phenolic content of extracts was determined using a modified version of the Folin-Ciocalteu reagent (FC) technique. After 4 min, 800 ml of sodium carbonate (7.5 %) was added to 200 μL of the extract, and 1 ml of 10 % Folin-Ciocalteu reagent was added. Before evaluating the absorbance at 760 nm, the combination was prepared by a reaction at room temperature. The determinations were performed in triplicates. The absorbance was measured at 760 nm and compared to a blank consisting of Folin-Ciocalteu reagent, Na_2CO_3, water, and distilled water without extract or gallic acid.

2.4.3. Free radical scavenging activity by DPPH assay

The antioxidant activity of a compound was first evaluated using a procedure known as 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity; this procedure was performed by Brand-William et al. [17]. This method was used to determine antioxidant activity, with some minor modifications. To prepare the DPPH solution, use 0.2 mg) was added to 50 ml of 99.9 % methanol. The dark-purple solution was stored in a refrigerator at 20 °C. The sample was diluted to a concentration of 1 mg/mL in 1 mL, and then, 3 mL of DPPH solution and 3

Table 1
Combination of independent variables and responses obtained as per Box–Behnken Design (BBD).

Run	A: time Factor 1 minutes	B: Temperature Factor 2 celsius	C: solid-solvent ratio Factor 3 w/v	Yield Response 1 %	Chlorophyll Response 2 mg/ml	Phenol Response 3 GAE/gm	Flavonoid Response 4 QAE/gm	Antioxidant Response 5 %
1	30	30	10	18.86	58.78	153	37	82.8
2	30	40	20	16.51	40.12	174	44	74.8
3	30	30	30	11.5	37.82	168	42	42.01
4	20	40	10	14.4	51.7	156	38	80.66
5	20	30	20	6.5	48.6	141	33.2	43.3
6	20	30	20	6.5	48.6	141	33.2	43.3
7	20	20	10	10.81	66.25	154.3	37.7	79.7
8	20	40	30	15.33	30.09	192	50	52.66
9	20	30	20	8.22	48.71	147	35.1	45.6
10	10	20	20	8.78	56.23	150	36	58.67
11	20	20	30	6.5	48.5	174	43.9	56
12	20	30	20	6.5	48.6	141	33.2	43.3
13	30	20	20	11.52	55.55	111	28.9	80.33
14	10	40	20	13.14	41.6	105	31.8	66.53
15	10	30	10	10.81	59.2	128	29	59.33
16	20	30	20	6.5	47.23	141	33.2	43.3
17	10	30	30	13.59	36.43	171	43.6	41.23

mL of methanol were added. During the 30-minute incubation period, the reaction mixture was kept at room temperature in the dark. The same steps were used to prepare a control solution as a sample solution, but methanol was used instead of the sample, and ascorbic acid was used as a positive control. A spectrophotometer (V-760 UV–Visible Spectrophotometer) was used to measure absorbance at 517 nm [18]. Radical scavenging activity was determined using Eq. (2).

$$\text{Free radical scavenging rate} = \frac{\text{OD of Control} - \text{OD of Sample}}{\text{OD of Control}} \times 100 \quad (2)$$

2.4.4. Determination of total flavonoid content

The aluminum chloride colorimetric method, with a few changes, was used to determine the total flavonoid content [19]. Quercetin was used as a standard. Quercetin was diluted to different concentrations with distilled water (100–500 μg/mL), and the plant extracts were diluted to 1 mg/mL in ethanol. There were 3 ml of 99.9 % ethanol and 1 ml of plant extract combined, 0.2 ml of 1 M potassium acetate buffer, 0.2 ml of aluminum chloride (10 %), and 5.6 ml of distilled water in this reaction mixture. The mixture was incubated for 30 min at room temperature. After incubation, the absorbance was read at 415 nm against a blank. The blank did not contain quercetin or the plant extract. The total flavonoid content was determined using a quercetin standard curve.

2.4.5. Total chlorophyll content

One milliliter of the plant extract (10 mg/ml) was mixed with 9.9 ml acetone (80 %), and the homogenizing mixture was centrifuged for 15 min at 2000 rpm. In this solution, 2 ml of solution was taken and diluted with 10 ml of acetone (acetone 80: water 20 v/v). In a spectrophotometer, the absorbance was measured at 645 nm and 663 nm to determine the amount of total chlorophyll present [20]. Total chlorophyll content was calculated using Eq. (3).

$$TotalChlorophyll = (20.2 \times Abs645) + (8.02 \times Abs663) \quad (3)$$

2.5. GC–MS (Gas chromatography–mass spectroscopy)

The phytochemical components in the extract were analyzed by GC–MS. The extract was analyzed using a Perkin Elmer GC Clarus 680 system with a gas chromatograph connected to a mass spectrometer. The analysis was conducted at the qualitative analysis report of the central instrumentation laboratory in Bathinda, using an Elite-1 fused silica capillary column. Sample injection in this experiment involved microextraction in the solid phase. A GC–MS detection system employing an electron ionization energy of 70 eV was used. A carrier gas consisting of 99.99 % helium gas was utilized at a constant flow rate of 1.1 ml/min. An injection volume of 1 μl was employed. The injector temperature was maintained at 280 °C and the column oven temperature was adjusted to 70 °C. The overall duration of the GC–MS process ranged from 5 to 63 min. By comparing the average peak area of each component to the total area, it was possible to ascertain the relative percentage quantity of each component. Mass spectra and chromatograms were managed using turbo-mass version 5.4.2 software. The analysis was conducted using a Turbo Mass Perkin Elmer device as the mass detector. The GC–MS chromatogram was elucidated using NIST, National Institute of Standards and Technology, and WILEY library databases. The chromatogram of the unidentified molecules was compared to the spectrum of compounds contained in the NIST and WILEY libraries, which were previously recognized. All characteristics of the molecules in the sample, including their structures, molecular weight, molecular formula, peak area percentage, and chemical name, were identified.

2.6. Scanning electron Microscope study

Surfaces are often characterized using spectroscopic and microscopic techniques. Microscopy techniques were employed to determine the average size of the dispersed particles. The Scanning Electron Microscope (SEM) provides comprehensive morphological information and enables a comparison of the metal surface with and without a corrosion inhibitor [21]. The morphology, encompassing the size, surface properties, and form, was assessed using SEM. Usually, statistical analysis software is employed to compute the mean particle size for samples within the range of 100–300 particles.

2.7. Statistical analysis of RSM (Response Surface Methodology)

The response surface methodology using Design Expert version 13.05 was used for model creation. The one-way analysis of variance (ANOVA) method was used in the RSM with 1 % and 5 % thresholds of significance to evaluate whether there was a significant difference between UAE process variables. The data were fitted to a comprehensive second-order model and the suitability of the model was evaluated. The effectiveness of the models that were created to explain how solid-solvent ratio, ultrasonication temperature, and time affected the responses (extract yield, chlorophyll content, DPPH antioxidant activity, total phenolic content, and total flavonoid content) was validated using the ideal conditions for each model that were predicted using the design expert software. Repetition trials under ideal conditions were used to calculate error percentages to assess the “fit of the model.”

3. Result and discussion

3.1. Extraction yield

Betel leaf powder was obtained by ultrasound-assisted extraction using 99.9 % ethanol. To assess the range of extract yield variation, which ranged from 6.5 % to 18.86 %, the maximum yield was achieved when both the duration of the process and the solid-to-solvent ratio were high. Ultrasound-induced cavitation bubbles during sonication facilitate penetration of the extraction solvent into the plant cell wall, potentially surpassing the efficiency of conventional techniques. This phenomenon allows for enhanced transport of the internal components [14,15]. According to the regression model, the effects of both linear and quadratic terms of all independent variables, as well as the interaction between ultrasound sonication temperature and time, and the relationship between the solid and liquid ratio and ultrasound sonication time, were found to be statistically significant ($p < 0.05$) for extract yield (Table 2). The effects of the independent variables on the yield of the extract were observed and analyzed using three-dimensional surface plots of the response data (Fig. 1a, b). Each 3D figure exhibits an interaction between any two independent variables while keeping the center value of one variable constant. The extract yield initially declined owing to improper interactions between the solid-to-solvent ratio. However, after attaining a temperature of 30 °C, a time of 20 min, and a solid to solvent ratio of 1:20, the yield of the extract increased, as depicted in the 3D plot. Prolonged sonication accelerates the propagation of ultrasonic waves inside liquid media, resulting in enhanced reactivity of the solvent and consequent rupture of the cell wall of the membrane. This rupture facilitates the release of a greater quantity of the extract into the solvent [22]. The enhanced solubility of bioactive compounds is a result of the interaction between time and the solid-to-solvent ratio, which accelerates their release and dissolution. Fig. 1(b) demonstrates a positive correlation between the increase in the solid–liquid ratio and sonication duration and the corresponding increase in the extract yield. The increased infiltration of solvent into plant cells was a result of a higher solid-to-solvent ratio and a longer duration of sonication [23].

The validity of the model was verified using ANOVA; a summary of the results is presented in Table 2. The analysis revealed that the model was statistically significant with an F-value of 180.27 ($p < 0.0001$). The model's validity was supported by the "Lack of Fit F-value" of 0.453, indicating that the lack of fit was not statistically significant compared to pure error ($p = 0.059$). The coefficient of determination (R^2) was calculated to be 0.985, indicating that 0.897 % of the variability in the data could be accounted for by the fitted model. The adjusted coefficient of determination (R^2 Adjusted), which was analogous to R^2, was 0.965 %, demonstrating a significant correlation between the observed and predicted values. Moreover, the low coefficient of variation (C.V. = 6.67 %) indicated that the experimental results were highly precise and reliable, with minimal variation in the mean value. These results demonstrate the model's precise representation of the relationship between the independent variables and outcomes. The model developed for extraction yield is shown in Eq. (4).

$$Y_E = 6.844 + 1.50875X_{st} + 2.72125\,X_T - 0.995\,X_r + 0.1575\,X_{st}X_T + -2.535\,X_{st}X_r + 1.31\,X_TX_r + 3.78675\,X_{st}^2 + 1.85675\,X_T^2 + 3.05925\,X_r^2 \quad (4)$$

3.2. Total phenol content (TPC)

The Folin-Ciocalteu technique was employed to detect the presence of phenolic compounds in each sample. Table 1 shows that the highest Total Phenolic Content (TPC) observed was 192 mg GAE/g, whereas the lowest TPC value obtained was 105 mg GAE/g. The value obtained was significantly higher than that of the conventional method and microwave-assisted TPC extraction from defatted flaxseed cake [14,15]. The TPC regression model demonstrated substantial linear effects of time, temperature, and solid-to-solvent ratio, as well as significant interactions between the three independent variables ($p < 0.05$). At quadratic, only the solid-solvent ratio and temperature had valuable effects (Table 2). The interaction between the time and temperature is shown in Fig. 2a. The total phenolic content (TPC) first increased with increasing time before beginning to degrade, and the effect of the solid-solvent ratio on increasing the solid-solvent ratio abbreviation decreased the total phenolic content, but over time, the total phenolic content increased faster than the TPC (Table 2). The TPC gradually increased and then initially decreased with an increase in the solid–liquid ratio. This shows that under the examined conditions, severe dilution of the plant material may not result in further improvement of the TPC. Fig. 2b shows the interaction between solid-to-solvent ratio and time. The cavitation bubbles created during the ultrasound-assisted extraction process shatter the plant cells, facilitating solvent penetration. Owing to solvent penetration, swelling and hydration promote the growth of cell wall pores, which accelerates the diffusion process and increases the movement of mass [24]. Thus, the duration of phenolic compound extraction from plant sources is a crucial factor in the UAE. Similarly, a larger solvent-liquid ratio increases TPC by creating a difference in concentration between the solvent and the plant cell wall, which increases the mass transfer rate and raises the TPC. However, a high solid–liquid ratio lengthened the distance of diffusion toward the interior tissues [25]. Fig. 2c shows the interaction between the solid-to-solvent ratio and temperature. Upon increasing the solid–solvent ratio with increasing temperature, the total phenolic content increased. The

Table 2
ANOVA for the dependent variables yield, chlorophyll, phenol, flavonoid, and antioxidant.

Source	Yield		Chlorophyll		Phenol		Flavonoid		Antioxidant	
	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value
Model	51	< 0.0001	165.93	< 0.0001	72.64	< 0.0001	436.47	< 0.0001	178.76	< 0.0001
X_{st}	34.2	0.0006	62.56	< 0.0001	19.7	0.003	0.1705	0.6864	146.78	< 0.0001
X_T	111.25	< 0.0001	32.88	0.0007	44.57	0.0003	478.11	< 0.0001	0.0001	0.9914
X_r	14.87	0.0062	299.07	< 0.0001	212.79	< 0.0001	831.13	< 0.0001	611.51	< 0.0001
$X_{st}X_T$	0.1863	0.679	539.68	< 0.0001	110.95	< 0.0001	–	–	17.93	0.0039
$X_{st}X_r$	48.27	0.0002	36.27	0.0005	27.45	0.0012	–	–	51.48	0.0002
X_TX_r	12.89	0.0089	12.29	0.0099	10.02	0.0158	–	–	1.85	0.2161
X_{st}^2	113.38	< 0.0001	88.18	< 0.0001	10.58	0.014	–	–	99.99	< 0.0001
X_T^2	27.26	0.0012	9.21	0.019	46.59	0.0002	–	–	583.77	< 0.0001
X_r^2	74	< 0.0001	428.06	< 0.0001	167.15	< 0.0001	–	–	40.07	0.0004
Lack of Fit	0.7667	0.5693	0.4177	0.7504	1.38	0.3701	3.37	0.1269	4.18	0.1004

X_{st} = time, X_T= temperature andX_r = solid to solvent ratio.

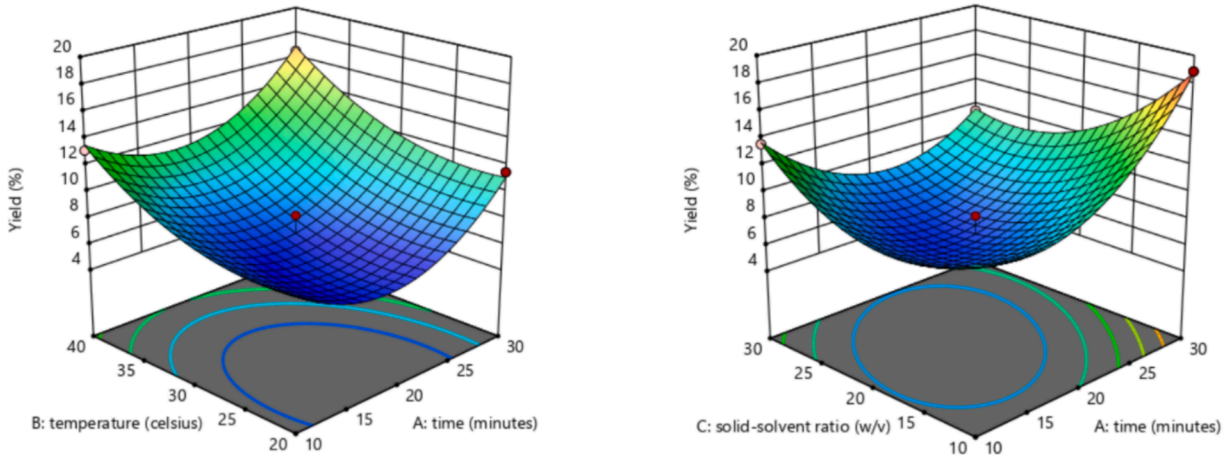

Fig. 1. Effects of independent factors on betel leaf extract yield (a) Effects of temperature and time on the betel leaf extract yield. (b) Effects of solid–liquid ratio and time on the betel leaf extract yield.

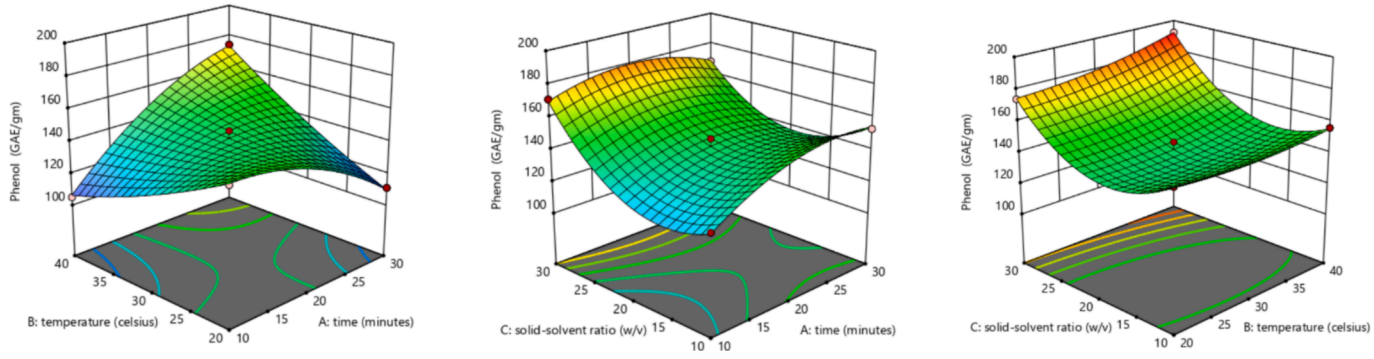

Fig. 2. Shown the effects of independent variables on the TPC from betel leaf extract a) effect of time and temperature on the TPC. (b) Shown the effects of time and solid: solvent ratio on the TPC. (c) Effects of temperature and solid: solvent ratio on the TPC.

model developed for extraction yield is shown in Eq. (5).

$$Y_{TPC} = 142.2 + 6.5X_{st} + 4.7125X_T - 14.2125X_r + 27\,X_{st}X_T + -7\,X_{st}X_r + 4.075\,X_TX_r - 10.6375\,X_{st}^2 + 3.4375\,X_T^2 + 23.4375\,X_r^2 \quad (5)$$

3.3. Total flavonoid content

Flavonoids are important natural bioactive substances. With the assumption that all flavonoids respond equally, the aluminum chloride colorimetric assay is frequently used to quantify the total flavonoid content. Flavonoids are important bioactive compounds. Quantitation of the total flavonoid content (TFC) is widely performed using the aluminum chloride colorimetric assay against a flavonoid standard, assuming equal responses from all flavonoids. When quercetin was used as a standard, TFC was determined at 415 nm. The highest flavonoid content was 50 QUE/mg and the lowest was 29 QUE/mg. In comparison to the Soxhlet extraction method utilizing a solvent in methanol and acetone, the total flavonoid content was found to be the highest in the extraction method that uses the ultrasound technique with ethanol as the solvent [26]. The regression in Table 2 of the total flavonoid content suggests that the model is significant. In this model, the p-value

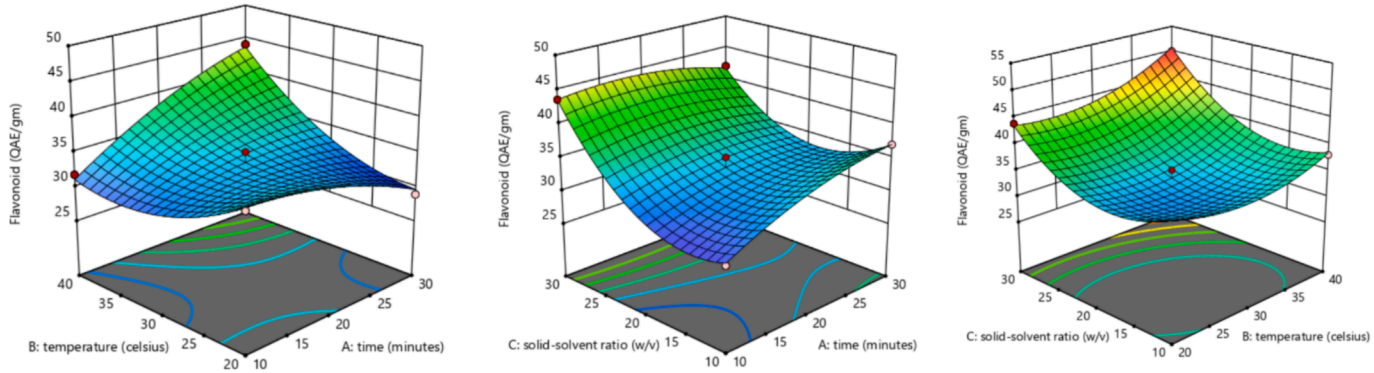

Fig. 3. Impact of independent variables on the TFC from betel leaf extract. (a) Impact of temperature and time on the TFC. (b) Effects of solid: solvent ratio and time on the TFC. (c) Impact of solid: solvent ratio and temperature on the TFC.

was <0.05, the F-value was 72.64, and the lack-of-fit was 1.38, which was not significant. The figures show the interaction between the time, solid-to-solvent ratio, and temperature. Upon increasing the solid-to-solvent ratio, the total flavonoid content initially decreases because the solubility of the material increases with increasing time and temperature; however, after a prolonged period of time, the total flavonoid content increases with increasing solid-to-solvent ratio. With increasing time, initially increased, but continued to increase, the TFC decreased. The temperature initially had a slight effect on the TFC, but after a prolonged period, the TFC increased with temperature. Fig. 3(a) shows the interaction between temperature and time. The total flavonoid content increased with time and temperature. Fig. 3(b) shows the interlinked solid-to-solvent ratio and time and shows that the TFC increases with increasing solid-to-solvent ratio and time. Fig. 3(c) shows the relationship between the solid-to-solvent ratio and temperature. Initially, the TFC content decreased, but with increasing s:s ratio with temperature, the TFC increased. Plants worldwide contain compounds called flavonoids, which are large groups of polyphenols with structural benzo-pyrone and antioxidant properties [27]. The relative stability of flavonols and flavones under various processing and storage conditions results in a decrease in the flavonoid concentration [28]. Flavonoids and phenolics were discovered in the ethyl acetate and ethanol fractions of the leaf extract, whereas secondary metabolites of steroids, phenolics, and flavonoids were found in the ethanol solvent fraction. Owing to the polar nature of flavonoids, polar or semi-polar solvents are necessary for their proper synthesis. Polar and semi-polar solvents were ethanol and ethyl acetate, respectively. Flavonoids have a wide range of biological effects, including antibacterial and anti-inflammatory effects (Indarti *et al.,* 2019). The differential phytochemical composition of plants is regulated by both internal and external factors, according to [29]. Genes are exterior factors, such as humidity, temperature, light, pH, and the soil's nutritional content, while the plant's genes are an internal factor. Phenolic substances, known as flavonoids, impair the permeability of bacterial cell walls and reduce their reproduction. The model developed for the total flavonoid content is presented in Eq. (6).

$$Y_E = 33.58 + 1.4375\,X_{st} + 2.1625\,X_T - 4.725\,X_r + 4.825\,X_{st}X_T + -2.47\,X_{st}X_r + 1.45\,X_TX_r - 1.4525\,X_{st}^2 + 3.0475\,X_T^2 + 5.7725\,X_r^2 \quad (6)$$

3.4. Total chlorophyll content

A spectrophotometer was used to determine the chlorophyll content of each sample. The betel leaf's rich concentration of chlorophyll *a* and chlorophyll *b* accounts for its dark-green hue. While chlorophyll *b* is green-yellow, chlorophyll *a* is blue-green [30]. Table 1 shows that the highest total chlorophyll value of 66.25 mg/ml was observed at 20 °C, 20 min, and a solid to solvent ratio of 10, whereas the lowest value of chlorophyll content (30.09) was found at 20 min, 40 °C, and 30 solids: solvent ratio. Time had no effect on chlorophyll content, but the degradation of chlorophyll content started with increasing temperature and solid-to-solvent ratio. Based on earlier research, the total chlorophyll content of betel leaves (66.25 mg/ml compared to cassava leaves (27.447 mg/g), kale leaves (16.767 mg/g), and spinach leaves (23.022 mg/g) [31]. The rate of photosynthesis increased because the betel leaves were more effective in absorbing solar radiation. Fig. 4 shows the relationship between time and temperature. Upon increasing the temperature, chlorophyll content decreased. There is an indirect relationship between the solid-solvent ratio and temperature. Thus, when parameters such as temperature and solid-to-solvent ratio increase, chlorophyll content decreases. According to earlier research, the destruction of cells caused by ultrasound shock considerably improves the effectiveness of organic solvents used to extract chlorophyll. The regression table demonstrates that, while the model is significant, the lack of fit is not. A quadratic model was fitted. The P-value was <0.05, and the F-value was 336.67.

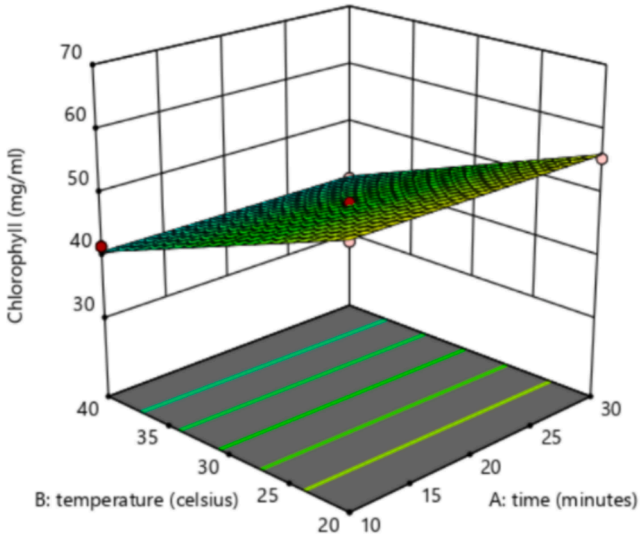

Fig. 4. Shows the impact of time and temperature on the total chlorophyll content.

3.5. DPPH antioxidants activity

The efficiency of the betel leaf antioxidant activity was evaluated using DPPH radical scavenging activity. The stable free radical is DPPH, and when DPPH reacts with an antioxidant compound (which can transfer hydrogen), it is converted to diphenyl picrylhydrazine. The strength of the absorption is reduced in the presence of antioxidants, and DPPH radicals change color from purple to light orange or golden hydrazine depending on the number of electrons caught. [14,15]. The percentage of antioxidants obtained by the study ranged from 82.8 % to 41.23 %. The maximum value of antioxidants was observed at 30 min, 30 °C, and a 1:10 solid-to-solvent ratio, and the minimum value was observed at 10 min, 30 °C, and a 1:30 solid-to-solvent ratio, as shown in Table 1. Fig. 5 shows 3D graphs with the impact of temperature, time, and solid-to-solvent ratio. On increasing the extraction time, the antioxidant activity increased. However, upon increasing the solid-to-solvent ratio, the temperature increased, the antioxidant activity initially decreased, and thereafter it declined. However, after prolongation of time, the antioxidant activity increased with increasing temperature. The antioxidant activity of the ultrasound-assisted extraction method was greater than that of Soxhlet extraction [19]. Fig. 5(a) shows the interaction between temperature and time. The antioxidant activity increased when the sonication period was increased; however, with the initial increase in temperature, the antioxidant activity decreased. Fig. 5 (b) shows the impact of time and solid solvent ratio on the antioxidant activity. The antioxidant activity decreased with an increase in the solid solvent ratio. Fig. 5(c) shows the effect of the solid-to-solvent ratio and temperature on antioxidant activity. Table 2 shows that the model was significant and the lack of fit was not significant. The p-value was <0.05, and the F-value was 178.

3.6. Variables optimization and validation

Utilizing RSM software in an optimized manner, the ultrasound-assisted extraction approach yielded the most desirable bioactive substances. The optimum UAE condition of betel leaf extract showed maximum extract yield, antioxidant activity, total chlorophyll content, total phenolic content, and total flavonoid content as compared to thermal processing. Process factors (independent variables), according to statistical analysis of data, that produced the intended results were temperature 40 °C, time 30 min, and solid to solvent ratio 1:10. The values of the low deviation or error deviation responses are listed in Table 3, as expected and experimental values. The optimized values

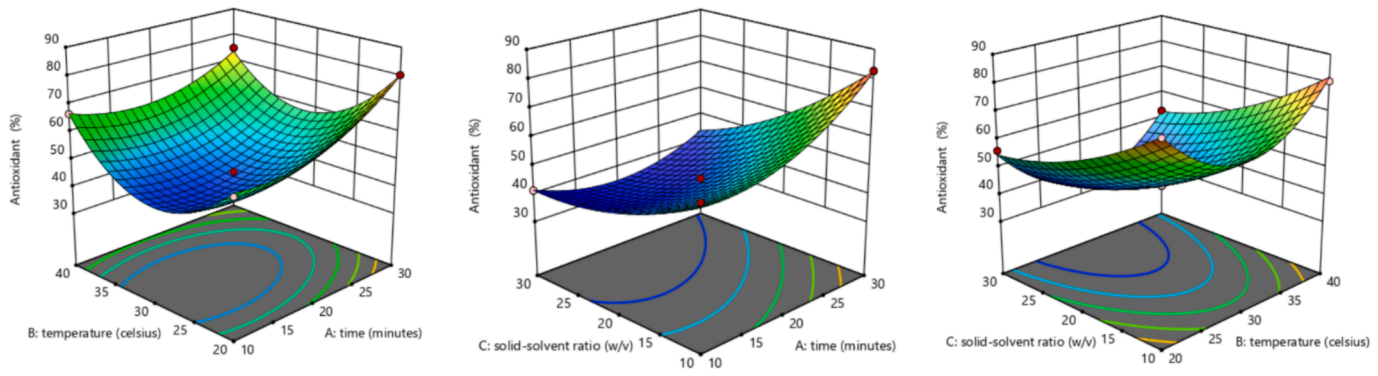

Fig. 5. Effects of independent variables on the antioxidant activity from betel leaf extract. (a) Effects of temperature and time on the Antioxidant activity. (b) Effects of solid: solvent ratio and time on the Antioxidant activity. (c) Effects of solid: solvent ratio and temperature on the Antioxidant activity.

Table 3
Statistical parameters for the models for dependent variables yield, chlorophyll, phenol, flavonoid, and antioxidant.

Statistical parameter	Yield	Chlorophyll	Phenol	Flavonoid	Antioxidant
R^2	0.985	0.9953	0.9894	0.9902	0.9957
Adjusted R^2	0.9657	0.9893	0.9758	0.9879	0.9901
Predicted R^2	0.8974	0.9766	0.9057	0.9806	0.9458
Adeq Precision	21.4317	48.1617	30.4554	73.9001	33.9684

Table 4
Responses from the optimized condition of UAE and observed values.

Analysis	Predicted data	Experimental data	Relative Deviation %
Yield (%)	22.1541	21.99	0.741
Chlorophyll(mg/ml)	50.8312	49.95	1.733
Phenol(mg/GAE)	185.362	185.05	0.168
Flavonoid(mg/QUE)	45.5974	44.59	2.209
Antioxidant %	98.9497	99.10	0.152

obtained for extract yield, total flavonoid content, Antioxidant activity, Total phenol content, and chlorophyll content were 21.99 %, 44.97 mg/QUE, 99.10 % 185.05 mg/GAE, and 49.95 mg/ml respectively (Table 4).

3.7. Scanning electron microscope analysis of betel leaf

The effect of ultrasonic-assisted extraction (UAE) on the extraction of bioactive compounds from betel leaves was investigated using scanning electron microscopy (SEM) to analyze the resulting morphological alterations. The Figure demonstrates that the efficiency of extraction is correlated with physical alterations in the sample. Fig. 6 illustrates the significant damage caused by ultrasound treatment to the surface of the sample, resulting in the fragmentation of its texture owing to the occurrence of cavitation phenomena that led to the bursting of plant cells. During the rupture process, chemical compounds were suddenly released from the cells into the surrounding fluid. Magnification at 1200 × g clearly showed many pores in the betel leaf extract residue derived by the UAE method. The swelling and enlargement of the pores in the material were clearly visible in the UAE sample, implying cell rupture and cell structural damage, which led to easier and more effective penetration of the solvent into the plant material. In a parallel study, researchers determined that ultrasound may induce cavitation events in green tea, resulting in structural modifications.

3.8. GC–MS (Gas chromatography–mass spectroscopy)

GC–MS is a technique used for the identification of phenolic compounds such as flavonoids, phenolic acids, and many more bioactive compounds. GC–MS of bioactive compounds is very effective and quick in analysis through enhanced resolution and separation of all compounds. In the present study, the optimized betel leaf extract had the best results for the responses that were selected for GC–MS analysis to characterize the walnut hull extract for the identification of bioactive compounds. GC–MS results showed the presence of various compounds with a wide range of applications, such as antioxidants, antimicrobials, flavors, and medicinal uses.

The GC–MS data presented the identified retention time, area %, molecular formula, height, and properties of the compound. The result from the GC–MS shows that betel leaf extract is mostly constituted of essential oil, and fatty acids such as 3-Allyl-6 methoxyphenol, 3-Allyl-6 methoxyphenyl, Phthalic acid, ethyl pentadecyl acetate, Hexadecanoic acid and many more compounds are found the betel leaves extract also have ester groups like 4-allyl-1,2-diacetoxybenzene. At a retention time of 23.178 min, the components 3-allyl – 6 methoxyphenyl acetate produced the highest peak with a maximum percentage area of 18.74 %, indicating the current extract composition. The GC–MS results showed the various medicinal properties of betel leaves, including anti-inflammatory, antibacterial, anti-cancer, anti-diabetic, anti-fungal, anti-microbial, antioxidant, and many properties.

4. Conclusions

According to the findings of this study, UAE was effective in maximizing the high extraction yield, DPPH radical scavenging activity, total phenolic content, chlorophyll content, and total flavonoid content from the betel leaf extract time, temperature, and solid solvent ratio recovery of responses. It also proved to be a useful statistical strategy for the optimization of process variables. The optimized values obtained for extract yield, DPPH scavenging activity, Total phenolic Content, the chlorophyll content and total flavonoid content were 21.99 %, 99.10 %, 185.05 mg/GAE, 49.95 mg/ml, and 44.59 mg/QUE respectively. A persistent p-value <0.001. Importance of each model term in the evaluation of variance Additionally, it is clear from the outcome that UAE is an efficient extraction method with increased yields and shorter processing time. In addition to the solvent, ethanol provided a greater extraction yield of phytochemicals and antioxidants, as well as chlorophyll content. The SEM analysis revealed noticeable alterations in the structural composition of the optimized sample, which was attributed to cell breakage. This resulted in a notable increase in both the yield of the extract and its bioactive compounds, demonstrating its superior properties compared to alternative extraction methods. Furthermore, GC–MS analysis revealed the presence of specific compounds (3-allyl-6 methoxyphenol, hexadecane thiol, 4-allyl-1,2-diacetoxybenzene, 2-

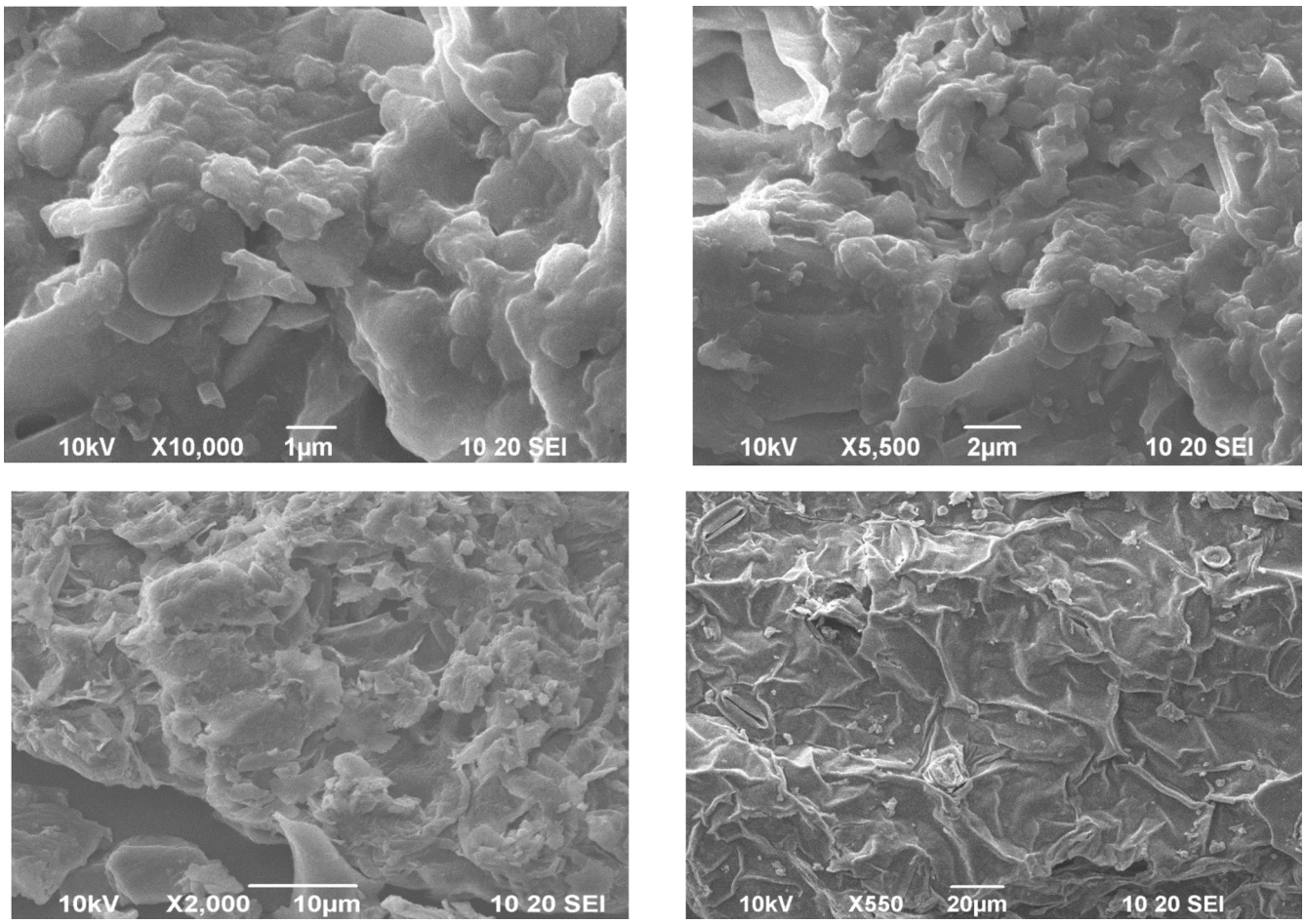

Fig. 6. SEM (Scanning Electron Microscope) analysis of betel leaf under optimization condition of Ultrasound-assisted extraction.

Hexadecen-1-ol) within the extract. These compounds exhibit a range of beneficial characteristics, such as aiding in processing and antifungal, anticancer, anti-inflammatory, antioxidant, and antimicrobial properties. This confirmed the suitability of the extract for diverse applications in the food industry. In essence, the SEM findings underline the enhanced yield and bioactive potential of the optimized extract, whereas the GC–MS results affirm its versatility for various food-related applications. Collectively, these results emphasize the promising prospects of this extraction method, suggesting its potential for further exploration and utilization in food processing and related industries. For the extraction of bioactive substances from betel leaves, ethanol is a secure, effective, environmentally friendly, and economically advantageous replacement for other organic solvents. Looking forward, the future prospects for this research are promising. The optimized UAE method, particularly when ethanol is used as a solvent, presents a secure, effective, environmentally friendly, and economically advantageous approach for extracting bioactive substances from betel leaves. Further studies should explore different extraction parameters to enhance specific compound yields or investigate the potential of the extract in novel product formulations. Moreover, the compounds identified from GC–MS analysis open avenues for targeted research into their individual effects and applications. This study lays a solid foundation for continued advancements in the utilization of betel leaf extracts for various beneficial purposes ranging from functional foods to pharmaceutical development, thereby contributing to the growing field of natural product research and application.

CRediT authorship contribution statement

Tripti Singh: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Vinay Kumar Pandey:** Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Rahul Singh:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Kshirod Kumar Dash:** Formal analysis, Data curation, Conceptualization, Validation, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision. **Béla Kovács:** Visualization, Validation, Software, Resources, Funding acquisition, Methodology, Project administration. **Shaikh Ayaz Mukarram:** Funding acquisition, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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