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ORIGINAL ARTICLE

Pretreatment and Optimization of Processing Conditions for Extraction of Oleuropein from Olive Leaves using Central Composite Design

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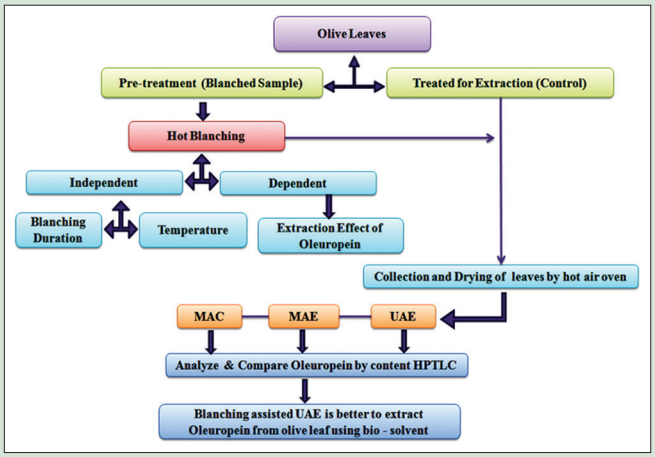
ABSTRACT

Background: The extraction methods used for isolation of biomolecule from Olive leaves show risk of residual solvent and less extraction efficiency. Hence, there is a need to develop novel techniques to encapsulate the risks headed with extraction process. **Objective:** The goal was to unravel the effect of novel extraction techniques on the extraction efficiency of Oleuropein from *Olea europaea*, a major secoiridoid resided in Olive leaf. **Materials and Methods:** Olive leaves were collected, authenticated, and subjected to proximate, phytochemical analysis to contemplate the source of active moiety. The précised solvent, i.e., water: glycerol (3:1%v/v) was functionalized to depict the influence of independent variable on response using central composite design. For hot blanching, the independent factors selected were treatment temperature (50°C–70°C) and duration of blanching (10–30 min) whereas the observed response is percentage extraction efficiency of Oleuropein. The hot blanched leaves were subjected to extraction by cold maceration, microwave-assisted extraction (MAE), and ultrasound-assisted extraction (UAE). The content of Oleuropein was analyzed by high-performance thin-layer liquid chromatography. **Results:** From the design space, the model is stable at a range of 0.002–0.80 which indicates lack of fit is very less and more curvature effects are clearly visualized with P = 5% level of significance. Maximum response was attained at a temperature of 60°C–65°C and duration of 15–20 min. Microstructural changes in leaf were observed through scanning electron microscopy studies. From the study, pretreated leaves followed by UAE result in higher yield of Oleuropein compared to MAE and maceration. **Conclusion:** Hot blanching technique shows a significant linear upswing in the concentration of Oleuropein when compared to direct extraction techniques. Blanching of Olive leaves causes deactivation of enzymes, and further exposure to ultrasonic waves enhances mass transfer of solvent and promotes the release of Oleuropein.

Key words: Central composite design, extraction efficiency, hot blanching, *Olea europaea*, Oleuropein, Olive leaf

SUMMARY

- The leaves were processed and subjected to proximate analysis, phytochemical screening, and quantification of polyphenols by Folin–Ciocalteu method. The results unfolded the presence of various secondary metabolites such as phenols, alkaloids, glycosides, and terpenoids in all the extracts, and the aqueous glycerol extracts contain the richest estimate of phenolic compounds followed by water: ethanol and water, respectively. The presence of glycerol increases the polarizability of solvent, hence dipole moment increases, which results in concomitant rise in the leaching of phenolic compounds from Olive leaf. From the experimentation, it was summarized that the factor duration of blanching and temperature shows a linear effect on the responses, and the results obtained are validated using correlation plot. In a nutshell, it was summarized that hot blanching technique followed by UAE using biosolvent at optimized conditions significantly increases the extraction efficiency of Oleuropein from Olive leaves.



Abbreviations Used: MAC: Cold maceration, MAE: Microwave-assisted extraction, UAE: Ultrasound-assisted extraction, RSM: Relative standard deviation, CCD: Central composite design, TPC: Total polyphenolic content, FC: Folin–Ciocalteu, GAE: Gallic equivalents, GA: Gallic acid, HPTLC: High-performance thin-layer liquid chromatography, TLC: Thin-layer liquid chromatography, SEM: Scanning electron microscopy.

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INTRODUCTION

Medical plants possess a treasure of potent drugs which are provided for the mankind to alleviate various ailments in spite of advancements in synthetic drugs. Due to different outcomes on herbal medicine, the importance of plant biomolecules has attained a commanding role in healthcare system. Consequently, the belief of people on herbal drugs was sustainable, and to compensate the therapeutic needs with

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traditional medicine, it is time to increase our emphasis on invention of new biomolecules for emerging diseases like meningitis.[1]

Olea europaea (OE), a versatile plant contains secondary metabolites such as polyphenols and secoiridoids, for example, oleuropein and hydroxytyrosol. Among all "Oleuropein" is claimed to have various biological activities such as antioxidative, anti-ischemic, hypolipidemic, cardioprotective effects, inhibition of oxidative stress and it regulates the expression of tumor necrosis factor-β which is due to the presence of o-diphenol system in oleuropein.

A research done by Javid[2] summarized that olive-enriched diet throughout the life may produce significant changes in the hippocampus and brain cortex, which will direct the research toward an concrete investigation and also it addresses the global crisis of central nervous system disorders.

Persia *et al.*[3] chalked out their findings in a journal called "Phytomedicine" which summarizes that major phenolic compounds in Olive leaves inhibit mast cell degranulation induced by both immune and nonimmune pathways. Olive biophenols can alleviate oxidative stress and oxidative damage including chain reaction.

As per the literature, recovery of Oleuropein from Olive leaves varied massively from 5.6 to 108.6 mg/g dry weight. Drying plays a subtle role in the recovery of Oleuropein from Olive leaf because drying of plant tissues damages cellular structures which facilitate percolation of the solvent into parenchymal cells. Hence, Olive leaves were dried immediately after harvesting to minimize the quantity loses and to reduce the moisture content degradation during storage.[4,5]

In the recent years, researchers have highlighted the various applications of extraction process and usage of various solvents in the extraction of Oleuropein from Olive leaves. However, the main disadvantages of those methods are the toxicity of solvents used, need of residual solvent removal, and reutilization of solvent and cost. Hence, in this research, "Glycerol" an environmental friendly solvent was used in conjunction with water which offers an advantage of changing dielectric constant of solvent water, thereby it supports for effective extraction.

Apostolakis *et al.*[6] highlighted the effect of glycerol as a biosolvent, and it was demonstrated that the use of heated aqueous glycerol was more efficient compared to hydroalcohols for the extraction of polyphenols. For potential industrial applications, this proposed method of extraction paves a way, and it would be highly desirable to combat the cons resulted with conventional technique. Henceforth, an attempt was made to escalate the process of extraction of Oleuropein from Olive leaf by inculcating various pretreatment techniques prior extraction.

In recent years, a promising change was developed in the methods of extraction of bioactive from the plants, to reduce extraction time, enhance extraction efficiency, and reduce solvent consumption and process specificity. The methods include ionic solvent-based microwave-assisted extraction (MAE) and ionic solvent-based ultrasound-assisted extraction (UAE). Hence, in this research, pretreated leaves were subjected to MAE and UAE techniques, and the Oleuropein extraction efficiency was compared with control samples (nonblanched MAE and UAE).

Pretreatment techniques

Pretreatment of Olive leaves before extraction process was done majorly to minimize the matrix effects on extractability of Oleuropein. A pretreatment technique "Blanching" was chosen in this research where leaves are soaked in the solvent for a specified period of time, which inactivates enzymes that cause browning and deterioration of the quality of leaves. In blanching, thermal treatment of Olive leaves exhibits structural changes in plant tissue which loosens the cellulosic networks; thereby, it promotes the leaching of contents from Olive leaves. Extraction and isolation of the active moiety from the plant can be easily affected by the processing methods. Hence, suitable conditions are required to maximize the extraction efficiency and to minimize the processing loss of phenolics.

Hot blanching technique

Leaves were prewashed in deionized water and dried with dry towel. The leaves were blanched in hot water at a temperature of 50°C–70°C and duration of about 10–30 min in a thermostatic water bath. The blanched leaves were notified as pretreatment leaves, while other samples without blanching or pretreatment were called control samples. After blanching, leaves were dried in oven dryer at a temperature of 60°C for about 10 min until the moisture content reduces below 10%. The dried leaves were subjected to size reduction, and the powder was then sieved manually using sieve shaker to select derived particle size of powder.[7-10]

Optimization of process variables in hot blanching technique

To determine the potential method for the extraction of Oleuropein from Olive leaves and to check the efficiency of the method for promoting the leaching of active metabolite, the leaves were subjected to optimization process.

In this protocol, Response surface methodology was used as tool to optimize the extraction of Olive leaves, which showcases information about influence of various parameters on the response of the design. DOE is able to predict interactions between parameters and to identify the extraction criteria using least number of experiments.[11-20]

In this experimental design, central composite design (CCD) was functionalized to depict the effect of temperature and process time on the extraction of Oleuropein, and the corresponding mathematical models were developed. The focal point of this study was majorly on optimization of hot blanching technique and to predict its ability to leach the phenolic compounds into the solvent used for extraction process.

MATERIALS AND METHODS

Olive leaves were collected from Rajasthan Olive Cultivation Limited, India. Solvents such as ethanol, methanol, glycerol, hydrochloric acid, sulfuric acid, and chloroform were purchased from Merck, India. All other materials and solvents used are of analytical reagent grade.

Methodology

Collection and identification of Olive leaf

According to the literature, the Oleuropein concentration was high in Olive leaves compared to the fruits. Hence, Olive leaves were chosen for this current research. The young and fresh leaves were harvested in the spring season which approximately contains 5.6–9.2 mg/g of Oleuropein; indeed, it is very high compared to autumn season. Good, fresh, and disease-free mature leaves of OE (Voucher Specimen: SVU/2017/1260) were collected from the natural and manmade forests of Rajasthan Olive Cultivation Limited. After hot blanching, leaves were dried using oven dryer at a temperature of 60°C for about 10 min and finally grounded in electrical grinder (Bajaj GX 11), stored in airtight amber-colored plastic containers with proper labeling until use.[7]

Proximate analysis

Plant species of OE was exhaustively processed for various parameters of proximate analysis (carbohydrates, fats, crude protein, moisture, dry matter, crude fiber, and ash) according to the Association of Official Analytical Chemists methods (AOAC, 1990) and other standard literatures.[8]

Extractive values were estimated by mixing about 2 g of dried leaf powder with 50 mL of 90% ethanol in a closed flask, occasionally shaking for 6 h

and allowed to stand still for 18 h. The mixture was filtered, and 25 mL of the filtrate were evaporated to dryness. The residue was dried at 105°C for few minutes and then weighed. Ash values were determined by heating 2 g of the powdered sample in a silica crucible to red hot for 30 min and cooled down to record its weight. Loss on drying was calculated by heating about 5 g of sample in tarred china dish of known weight and kept in hot oven at 100°C–105°C for 1 h. The weight of the powder was noted to estimate the percentage loss on drying with reference to air-dried specimen. The moisture content was calculated by drying the sample at 105°C in an oven until it attains a sustainable weight.

Crude lipid content of the sample was determined using Soxhlet type of direct solvent extraction method using petroleum ether as solvent. Carbohydrate content was determined by subtracting the sum of the percentage of moisture, ash, crude protein, and fat from 100. Proximate analysis for pretreated leaves was done using same procedure, and the results are tabulated.

Preliminary phytochemical tests (qualitative and quantitative tests)

Phytochemical analysis of the major bioactive compounds of interest of the Olive species was performed using the methods of Harbone, 1984; Trease and Evans, 1989; and other literature methods.[9,10]

Procedure for extraction

5 g of powder is extracted with 50 mL of various solvents such as water, water: glycerol (3:1%v/v), and ethanol: water (1:3%v/v) using cold maceration technique of initial 12 h of dynamic maceration and 48 h of static maceration. Extracts were concentrated in the rotary flash evaporator at a temperature of 40°C for 10 min, collect the extracts and stored in refrigerator at a temp of 2°C–8°C until further use. Extracts were further used for preliminary phytochemical screening.

Phytochemical tests

The extracts obtained were subjected to test for alkaloids using Wagner reagent, test for flavonoids using dilute sodium hydroxide, test for glycosides, test for saponins was confirmed by formation of a persistent foam layer of 1 cm above the solution, test for steroids was done by adding 1 mL of the extract in 10 mL of chloroform, and equal volume of concentrated Sulfuric acid was added down the side of the test tube. Red upper layer and a yellow sulfuric acid layer with a greenish fluorescence indicated the presence of steroids, test for phenols was done by using ferric chloride reagent, and test for terpenoids was done by adding 1 mL of chloroform followed by addition of few drops of concentrated sulfuric acid. A reddish brown precipitate was obtained which indicates the presence of terpenoids.

Quantitative analysis of extract

Total polyphenolic content of the extracts was determined using Folin–Ciocalteu (FC) reagent using the literature methods with slight modifications. The concentrations of the total polyphenols were determined in terms of gallic equivalents (GAEs) per gram of the extract.[11-13]

Standard curve for gallic acid

Standard curve for gallic acid (GA) was constructed by dissolving 8 mg of GA in 10 mL of distilled water in calibrated flask. Aliquots of 1 mL were withdrawn and further diluted to 10 mL in another calibrated flask. Further dilutions were made by taking 1, 2, 3, and 4 mL of this solution separately in calibrated flasks and diluting up to 10 mL in volumetric flasks. From these solutions, 1 mL was mixed with 1 mL of FC reagent, 10 mL distilled water, and filled up to 25 mL calibrated flask with Na2CO3 solution. The absorbance of this GA solution was measured after 30 min of incubation at 760 nm. The phenolic content was calculated as follows:[14,15]

$T = C \times V/M$…

T = Total phenolic content, C = Concentration of gallic acid established from the calibration curve, V = Volume of the extract in solution in mL, M = Weight of the extract.

Estimation of polyphenolic content

Briefly, 5 mL of the extract was taken in calibrated flasks and volume made up to 25 mL with distilled water. 2 mL of this diluted extract was drawn out to which 1 mL of FC reagent and 10 mL of distilled water were added and volume was made up to 25 mL with Na_2CO_3 solution (290 g/L). After incubating the sample for 30 min in darkness, the absorbance of this sample was determined at 760 nm by UV spectrophotometer (Agilent 8453 UV-Vis spectrometer). The blank determination was done in the same manner using pure water instead of the extract. The polyphenol content was calculated from the standard calibration curve and calculated in terms of GAE per gram of the extract.

Experimental design and statistical analysis

All experimental runs were figured out with the use of the free trial version of Design Expert 11.0 (Stat Ease, Inc., Minneapolis, MN, USA) aiming an objective to study the influence of various independent variable like, treatment temp (50°C–70°C) (X1) and duration of blanching (10–30 min) (X2) at various levels on response i.e, Extraction Efficiency. CCD was chosen as a statistical model to identify and optimize the process parameters to achieve a maximum extraction efficiency of Oleuropein. The independent and dependent variables selected are mentioned in Table 1. Experimental design was analytically presented in Table 2. The design consists of replicated center points, and the set of points lying at the midpoint of each edge of the multidimensional cube defines region of interest. In total, thirteen batches were designed including five center points, and the concomitant responses are measured. The data projected from the experimental runs were subjected to stepwise regression analysis, to derive the equation which reveals the correlation between the response and the independent variables. Analysis of variance was useful to analyze the statistical significance of the model.[16-21]

Comparative assessment of extraction process

Procedure for microwave-assisted extraction

Extraction process was done using domestic microwave oven (Samsung MW718). A measured weight of 3 g of powdered sample having particle size of 250 μm was mixed with 70% (v/v) aqueous glycerol at solvent ratio of 50 ml/g in a closed Duran bottle. The mixture was irradiated at 150 W, 300 W, and 450 W for about 2–10 min. The extract is not allowed to super boil. On irradiation, the extract was screened using fine cloth and filter using 0.45 μ syringe filter and analyzed the drug concentration by high-performance thin-layer liquid chromatography (HPTLC).[22-25]

Procedure for ultrasound-assisted extraction

UAE was performed using Sonicator (Q Sonica Q800, 20 KHz). A measured weight of 3 g of powdered sample was mixed with 70% (v/v)

Table 1: Variables in central composite design

Factor	Level				
	−1.41421	−1	0	1	+1.4142
Temperature (°C)	50	55	60	65	70
Duration of blanching (min)	10	15	20	25	30
Response	Constraint				Importance
Extraction efficiency of Oleuropein (%)	Maximize				30%-50%

Table 2: Design layout for factors and responses

Run	Factor 1 temperature (coded value)	Factor 1 temperature (actual value)	Factor 2 duration of blanching (coded value)	Factor 2 duration of blanching (actual value)	Response 1 extraction efficiency of Oleuropein (%w/w)
13	−1	55	−1	10	25.3
12	1	65	−1	10	24.2
3	−1	55	1	25	25.3
7	1	65	1	25	58.9
1	−1.41421	50	0	20	25
4	1.41421	70	0	20	25.6
8	0	60	−1.41421	10	25.5
2	0	60	1.41421	30	27
10	0	60	0	20	26.1
5	0	60	0	20	26.2
11	0	60	0	20	25.8
9	0	60	0	20	26.2
6	0	60	0	20	26.1

Inference: The independent variables and dependent variables selected were shown along their high, medium, and low levels. Thirteen batches were formulated (including five-center points) and responses were measured

aqueous glycerol at a solvent ratio of 50 ml/g in a container. The mixture was sonicated at a power of 150W, 300W, and 450W for about 2–10 min under intermittency ratio, ∞ of 4/5 where, ∞-fraction of cycle; ∞ = ton/(ton + Toff). After sonication, the extract was screened using fine cloth and filter using 0.45 µ syringe filter and analyzed the drug concentration by HPTLC.[26-28]

Scanning electron microscopy analysis

The microstructural changes of the leaf samples before and after hot blanching were examined with Quanta™ 200 FESEM scanning electron microscope (FEI, USA) operated at 10 kV accelerating voltage under low vacuum mode. The structural change was notified through scanning electron microscopy (SEM) images.[29]

Estimation of Oleuropein content by high-performance thin-layer liquid chromatography

Preparation of standard stock solution of Oleuropein

A stock solution of Oleuropein was prepared by dissolving 10 mg of Oleuropein in methanol and making up the volume up to 10 ml with methanol. From this solution, 1 ml was pipetted out and diluted to 10 ml using methanol to get the final concentration of 100 µg/ml.

Preparation of sample solution with extract

Sample solution of Olive leaf extract was prepared by dissolving 10 mg of extract in methanol and making up the volume to 10 ml to get the concentration of 1000 µg/ml, and the solution was filtered through Whatman filter paper no. 41, diluted to a final concentration of 100 µg/ml for further chromatographic analysis.

Methodology

The extracts were dissolved in the solvent – acetone and it was spotted in the way of a band of width 80 mm with a Camag Microlitre Syringe on precoated silica gel Aluminum plate using a Camag Linomat V sample applicator. Sample volume applied was 250 µl for recording each track at an application rate of 1 µl/s, and the space between the bands was 5 mm. The split bandwidth was set at 20 nm, and each track was scanned at 5 nm using a solvent composition of chloroform: methanol (4:1 V/V).

Method development was carried out by lining the plate with filter paper for 30 min before the development, and for running a chromatogram, a linear ascending development was carried in 20 × 10 cm twin trough glass chamber (Camag), and the chamber saturation time for mobile phase was 20 min at room temperature. After the development, the chromatograms were dried and scanned by TLC scanner using WIN CATS Software 1.46 (Camag). Concentration of the compound Oleuropein in chromatogram was determined by the intensity of diffusely reflected light. The analysis and interpretation of chromatogram were done by comparing the peak areas of standard and extract. The peak purity was analyzed by comparing peak start, peak apex, and peak end (Heyden *et al*., 2008; Attimarad *et al*., 2011).

Statistical analysis

The data obtained were analyzed and interpreted by ANOVA and DMRT at 5% level at big using Duncan's multiple range test. All the measurements were performed in triplicate, and the values are expressed as mean ± standard deviation.

RESULTS AND DISCUSSION

The results of proximate analysis were presented in Table 3. From the data, it was showcased that moisture content in the Olive leaves was very less, and there was a significant uphill in the extraction values, which gives a preliminary idea about the effect of blanching on extraction. This shows that blanching helps in acceleration of drying process. Ash values indicate the presence of mineral content in the sample, and the presence of fat content also plays a pivotal role in the cellular metabolism.

The results of preliminary phytochemical studies were depicted in Table 4. In the preliminary phytochemical investigation, the presence of various secondary metabolites such as phenols, alkaloids, flavonoids, glycosides, and terpenoids in all the extracts was identified qualitatively. Among all the secondary metabolites, phenolic compounds play a dominant role in eliciting desired pharmacological action. The presence of phenolics in Olive leaves after blanching shows that there is no loss of phenolics after blanching of leaves.

Extraction process was carried out by cold maceration technique using various solvents, and the collected extracts were shown in Figure 1. The results for quantitative analysis were depicted in Table 5. From the data, it was observed that aqueous glycerol extract contains rich estimate of phenolic compounds followed by water:ethanol and water, respectively. Binary mixture of water and glycerol having more dielectric constant compared to other tested solvents. Hence, aqueous glycerol solvent has high potential to solubilize the polar solvents in extract, and this justifies the role of glycerol as potential solvent in the extraction of phenolic compounds.

The independent variables and factors selected for optimization of processing conditions for hot blanching were tabulated in Table 1. The design layout for studying the processing parameters was depicted in Table 2. From the results, it was observed that the entire batches showed extraction efficiency ranging from 24% to 53%. The results of optimization and correlation between the variables and responses are depicted in Table 6. Four models were considered, namely linear, linear + 2 factor interactions + quadratic, linear vs. 2 FI, and linear + quadratic + 2 FI + cubic. The model with less *P* value at 5% level of significance was suggested for this design, and the model with highest R^2 value was selected as best fit model to derive the relationship between factors and response.

The results of model summary statistics were mentioned in Table 7. From the design of experimentation, it was depicted that 2 FI vs. linear model was suggested and the lack of fit for this model was very less, i.e., 4; hence, the experimental design was perfectly matched to figure out a better conclusion between factors and responses. Model curvature effects are clearly visualized in the standard error of design of temperature and hot duration interaction plot. Hence, CCD model was the best choice of mathematical model to achieve a better conclusion between factors and response.

The fractional design space, line of linear fit, and interaction between the variables and responses are figured out in Figure 2. From the fraction design space, it was clearly observed that the model is stable at a range of 0.00–0.80; so, it gives an idea to select the desirable level for each factor to get maximum response. The difference between adjusted and predicted R^2 was found to be <0.2; henceforth, the model is significant and executed. From the perturbation plot, it was found that a two-way interaction effects play a vital role in maximizing the response. Henceforth, temperature and duration of blanching together show a significant effect on the extraction of Oleuropein from Olive leaves.

The response surface graph and contour graph were mentioned in Figure 3. While optimizing the hot blanching process parameters by CCD model, the process order fits to the linear model and prediction for function of desirability is 1.00. Response surface plots clearly represent the curvature effects, and the green zone indicates the design operable range for the factors to get maximum response without errors in processing. Contour plots reflect that extraction efficiency was increased by hot blanching technique within desirable limits of selected process parameters.

Figure 1: (a) Extraction of Oleuropein from Olive leaf; (b) concentrated extract of Olive leaf

Table 3: Proximate analysis of the pretreated and control samples of Olive leaf

Parameter	Before blanching	After hot blanching
	Results (% W/W)	
Moisture content	12.82±0.45	9.45±0.58
Carbohydrate	29.20±0.12	25.4±0.50
Protein	3.5±0.10	3.4±0.10
Fat content	3.1±0.13	2.9±0.30
Loss on drying	15.5±0.09	12.3±0.90
Extractive value	19.28±0.11	25.42±0.80
Ash value	13.42±0.10	9.58±0.20

Results are the mean of triplicate determination±SD. SD: Standard deviation

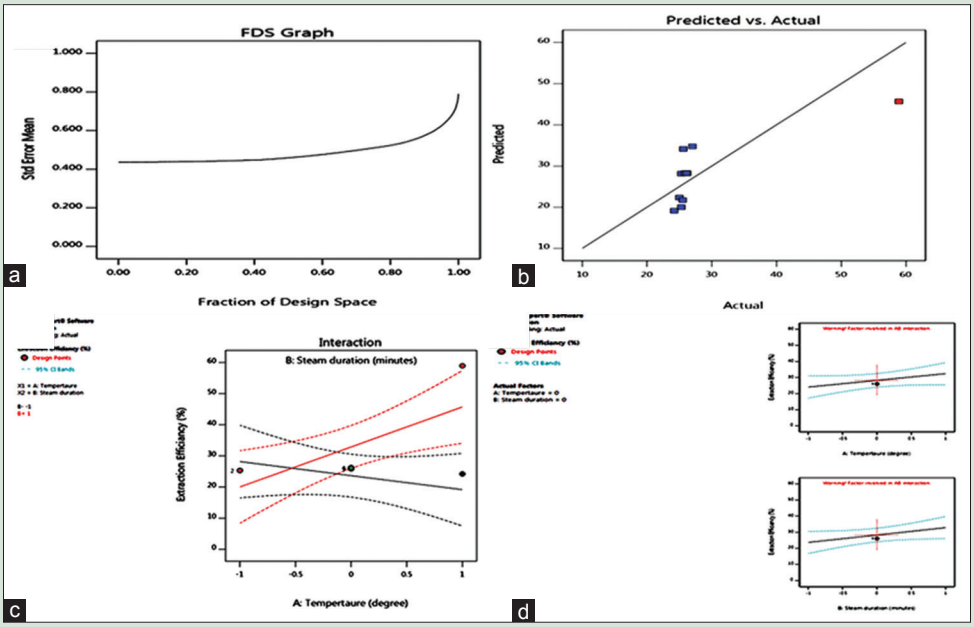

Figure 2: (a) Fractional design space graph; (b) residual line of fit which estimates the correlation between factors and responses; (c) prediction of interaction effects on the response; (d) prediction of main effects on the response

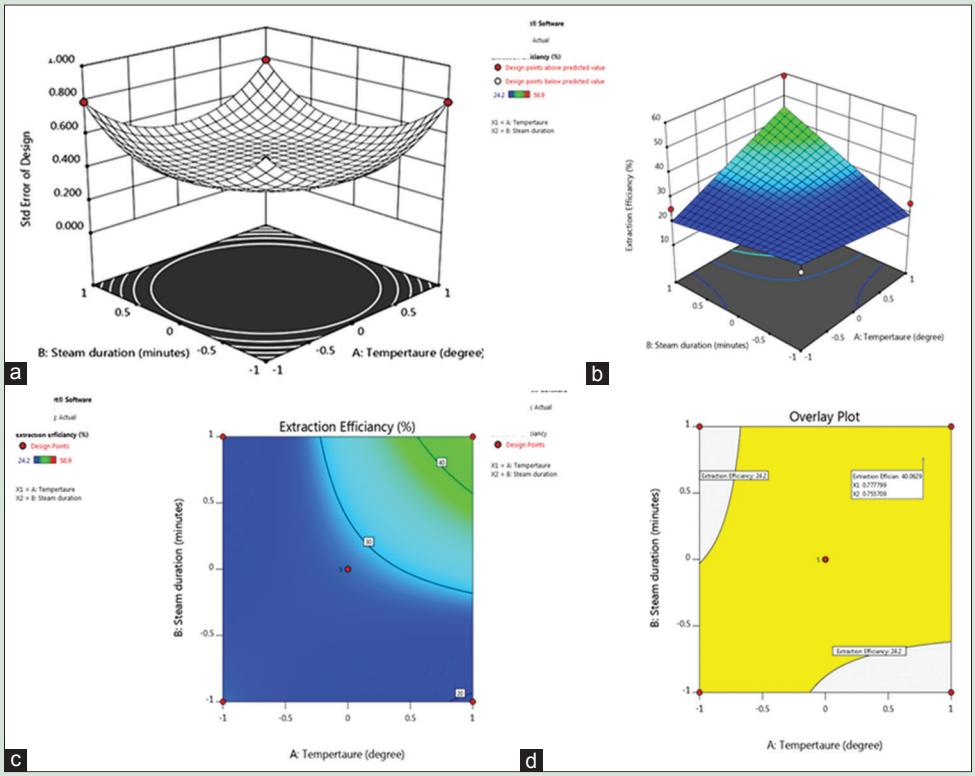

Figure 3: (a) Contour plot with standard error of design; (b) response surface plot three-dimension showing effect of factors temperature and steam duration on response extraction efficiency; (c) contour graph showing the design space to maximize the response; (d) factors overlay plot

Table 4: Preliminary phytochemical tests for pretreated and control samples of Olive leaf

Extract	Reagents/tests performed with the extracts before hot blanching					
	Phenols	Alkaloids	Flavonoids	Steroids	Glycosides	Terpenoids
Water	+	+	+	-	+	+
Ethanol:water (1:3%v/v)	+	+	+	-	+	+
Glycerol:water (1:3%v/v)	+	+	+	-	+	+
	Reagents/tests performed with the extracts after hot blanching					
Water	+	+	+	-	+	+
Ethanol:water (1:3%v/v)	+	+	+	-	+	+
Glycerol:water (1:3%v/v)	+	+	+	-	+	+

Inference: Preliminary Identification confirms the presence of phenolic compounds in the extract. No loss of active moiety during hot blanching. +: Presence; −: Absence

Table 5: Quantitative yield of phenolic bioactives from Olive leaf extracts

Extract	Polyphenol content (mg GAE g of dry extract)	
	Before hot blanching	After hot blanching
Water	75.60±2.12	85.60±2.12
Water:glycerol (3:1%V/V)	101.5±2.54	135.42±2.68
Ethanol:water (1:3)	60.42±1.38	72.80±2.54

Inference: Aqueous glycerol solvent possesses high ability to dissolve the phenolic compounds compared to other solvents, due to similar polarity between the solvent and phenolics. GAE: Gallic equivalents

From the pareto chart which depicted in Figure 4, it was inferred that on enhancement of temperature from 50°C to 70°C, there was a significant increase in extraction efficiency of Oleuropein from Olive leaves.

From the solutions observed from Table 8, it was confounded that aqueous glycerol solvent at a temperature of 60°C–65°C and duration of 20–25 min will turn around the process to achieve maximum extraction efficiency. In this case, no outliers have been detected for the dependent variable (extraction efficiency), and the model suggested attains $P = 0.95$ at 5% level of significance.

The effect of independent variables on extraction efficiency could be quantified using multiple linear regression equation which was depicted in Table 9.

The line equation is:

$Y = +28.25 + 4.17\ A + 4.60\ B + 8.68\ AB$

In terms of actual factors,

Extraction efficiency of Oleuropein = +28.25 + 4.17 temperature (T) +4.60 duration of blanching (S) +8.68 T and S.

The positive sign before a factor indicates that response is increased with factor and vice versa. It was observed that on increasing the temperature and duration of blanching, the extraction efficiency is also increasing. This effect could be attributed to the fact that during a rise in temperature and duration, the penetration of solvent to the parenchyma cells of powder extract is increasing; thereby, it increases the leaching of active moiety to the menstrum.

The validity of the model was concluded by checkpoint analysis from Table 10. The new batch of extract was formulated and responses were measured. The observed values show a close relation with predicted values, and percentage error was calculated to validate the method. It pings that percentage error between actual and predicted values is <2%. Hence, by this, the validity of the optimization procedure to maximize the response was proven.

Effect of irradiation power on extraction efficiency of Oleuropein by microwave-assisted extraction and ultrasound-assisted extraction

The results for effect of irradiation power on Oleuropein content by cold maceration (MAC), MAE, and UAE were depicted in Figures 5-7 and Table 11. From the data, it was observed that when the microwave irradiation power was 300 W, the content of Oleuropein was very high compared to other power and starts decreasing at above 300 W.

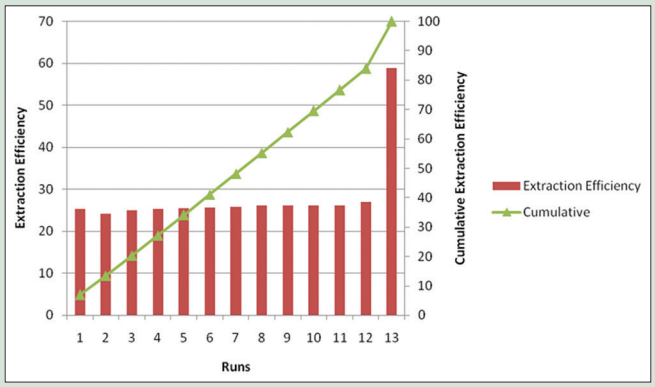

Figure 4: Pareto chart

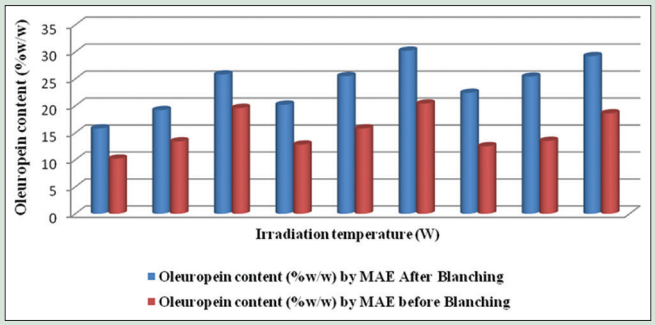

Figure 6: Comparative assessment of effect of irradiation temperature on extraction efficiency of Oleuropein content for pretreated and control samples by microwave-assisted extraction

At high irradiation power, there is a possibility of loss of bioactives from the extract. Hence, irradiation temperature of 200–300 W was selected as the microwave power range for extraction.

Effect of extraction time on extraction efficiency of Oleuropein by cold maceration, microwave-assisted extraction, and ultrasound-assisted extraction

The comparative assessment of effect of extraction time on Oleuropein content for pretreated and blanched leaves was depicted in Figure 8 and Table 11. The yield of Oleuropein gradually increases from 2 to 6 min and then decreases after 6 min. Hence, 2–6 min was selected

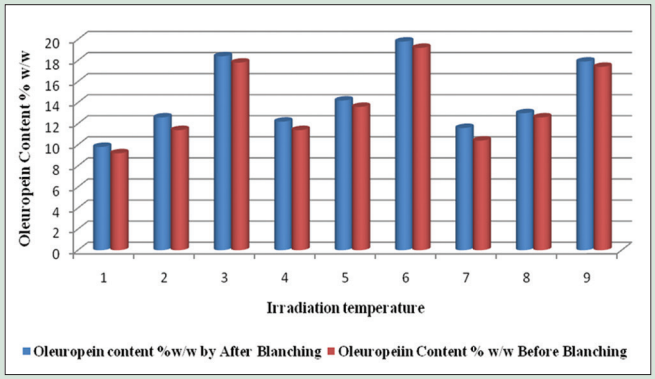

Figure 5: Comparative assessment of effect of irradiation temperature on extraction efficiency of Oleuropein content for pretreated and control samples by cold maceration

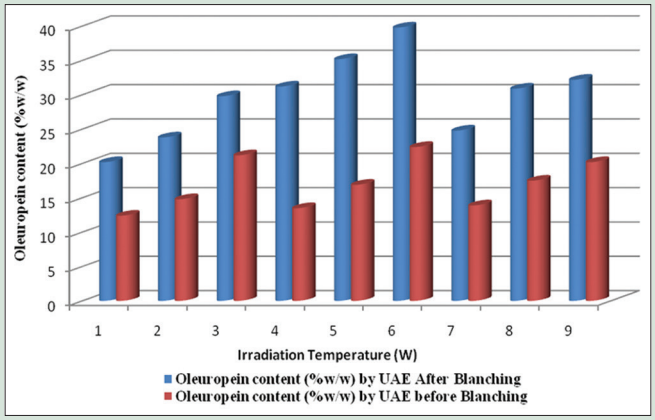

Figure 7: Comparative assessment of effect of irradiation temperature on extraction efficiency of Oleuropein content for pretreated and control samples by ultrasound-assisted extraction

Table 6: ANOVA for two-factor interaction model - extraction efficiency of Oleuropein (%w/w)

Source	Sum of squares	df	Mean square	*F*	*P*	Inference
Model	609.51	3	203.17	4.42	0.0360	significant
A - temperature	139.02	1	139.02	3.02	0.1162	
B - steam duration	169.48	1	169.48	3.68	0.0872	
AB	301.02	1	301.02	6.54	0.0308	
Residual	414.08	9	46.01			
Lack of fit	413.97	5	82.79	0.8427	0.6241	Not significant
Pure error	0.4620	4	0.0924			
Correlation total	1023.59	12				

Inference: Significance of the model was determined by incorporating the variables into a statistical model

as extraction time for extraction. There was a positive linear upswing between Oleuropein content and irradiation time. Exposing of sample to irradiation causes higher value of Oleuropein content. After a period of 6 min, there is a further decrease in Oleuropein content, which is due to degradation of Oleuropein to hydroxytyrosol. The increase in extraction efficiency was attributed due to interaction of microwaves and sonic waves with molecules by ionic conduction and dipole rotation. This interaction leads to rise in temperature and internal pressure inside the plant and also forms micropores. These changes will cause a rupture of cellular wall, leading to leaching of active compound into the solvent.

Table 7: Model summary statistics of response - extraction efficiency of Oleuropein (%w/w)

Source	Sequential (*P*)	Lack of fit (*P*)	Adjusted R^2	Predicted R^2	Model
Linear	0.1664	<0.0001	0.1617	−0.5183	
2FI	0.0308	<0.0001	0.8606	−0.6491	Suggested
Quadratic	0.7072	<0.0001	0.3719	−1.6050	
Cubic	0.0545	<0.0001	0.7253	−6.3184	Aliased

Inference: Linear with high regression model involving two-way factorial interactions was found to be the best fit for the response in comparison to all other models. 2FI: Two-factor interaction

Table 8: Factors at desired level to get maximum response

Factor	Name	Level (coded)	Level (actual)	Response
A	Temperature	0.778	62°C	Extraction efficiency of Oleuropein (%w/w)
B	Duration of blanching	0.7557	22 min	26.05

Inference: Predicting the desirable levels for an individual process variable to achieve maximum extraction efficiency

Table 9: Coefficient table which holds information about the interaction effects on response

	Intercept	*A*	*B*	AB
Extraction efficiency	28.2462	4.16857	4.60267	8.675
P		0.1162	0.0872	0.0308

Table 10: Comparative levels of predicted and the observed responses for the optimized formulation

Response	Actual value	Predicted value	Percentage error*
Extraction efficiency of Oleuropein (%)	26.05	25.58	1.837

Inference: The relative standard deviation between actual and predicted values was <2%. Hence, model is said to be significant within the design space. *Percentage Error= Actual Value Predicted value/Actual value ×100

Scanning electron microscopy studies

The extraction of bioactives from leaf was majorly relying on microstructure of plant, especially on glandular trichomes. The microstructural changes before and after hot blanching were mentioned in Figure 9. In this study, hot blanching of Olive leaf causes disruption of epidermal surface and forms micropores due to cavitations and

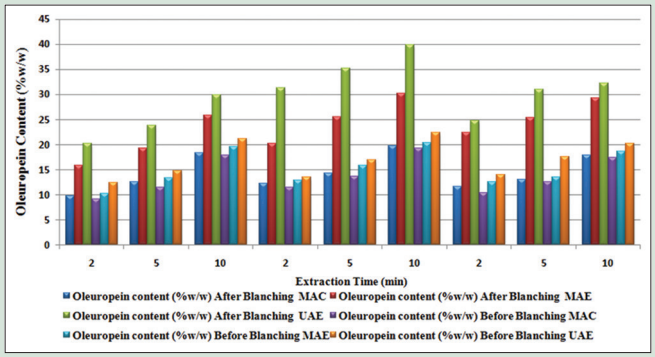

Figure 8: Comparative assessment of effect of extraction time on extraction efficiency of Oleuropein content for pretreated and control samples by cold maceration, microwave-assisted extraction, and ultrasound-assisted extraction

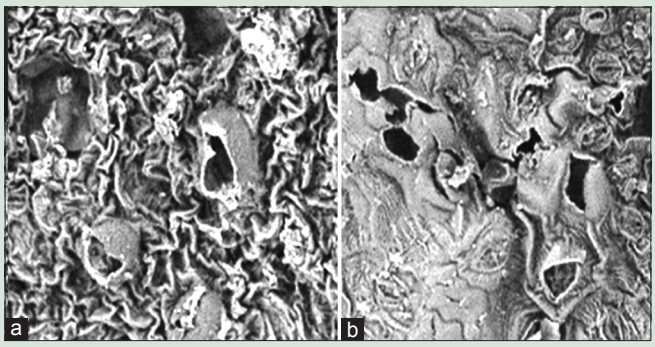

Figure 9: Scanning electron microscopy studies showing microstructural changes in Oleuropein leaf (a) surface of olive leaf before hot blanching; (b) surface of olive leaf after hot blanching

Table 11: Comparative assessment of extraction technique on extraction efficiency of Oleuropein content (%w/w)

Irradiation temperature (W)	Extraction time (min)	Oleuropein content (%w/w)					
		After hot blanching			Before hot blanching		
		MAC	MAE	UAE	MAC	MAE	UAE
150	2	9.8	15.8	20.2	9.2	10.2	12.4
	5	12.6	19.2	23.8	11.4	13.4	14.8
	10	18.4	25.8	29.8	17.8	19.6	21.2
300	2	12.2	20.2	31.2	11.4	12.8	13.5
	5	14.2	25.5	35.2	13.6	15.8	16.9
	10	25.5	30.2	39.8	19.2	20.4	22.4
450	2	11.6	22.4	24.8	10.4	12.5	13.9
	5	13.0	25.4	30.9	12.6	13.5	17.5
	10	17.5	29.2	32.2	17.4	18.6	20.2

MAC: Cold maceration; MAE: Microwave-assisted extraction; UAE: Ultrasound-assisted extraction

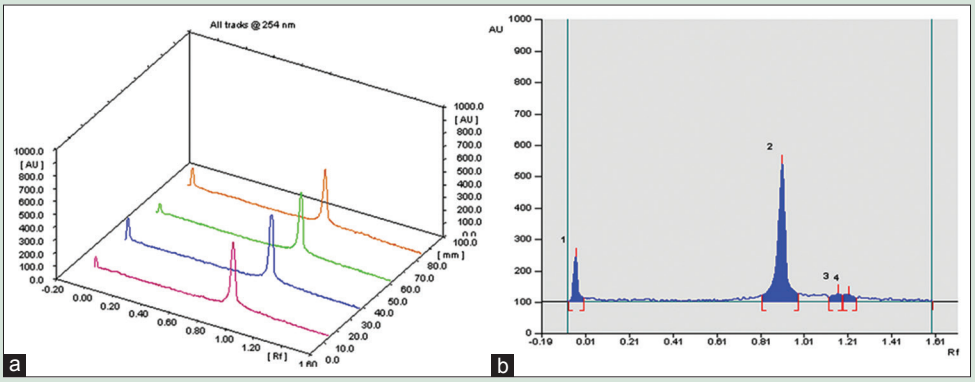

Figure 10: High-performance thin-layer liquid chromatography chromatogram showing (a) linearity tracks of Oleuropein standard recorded at 254 nm; (b) high-performance thin-layer liquid chromatography chromatogram for Olive leaf extract showing maximum extraction efficiency at optimized conditions which was recorded at 254 nm

Table 12: Summary of validation parameters

Validation parameters	Method property value
Linearity for Oleuropein (ng/spot)	100-600
Correlation coefficient for Gallic acid (r)	0.9994
Intraday precision (RSD % *n*=6) on different days for Oleuropein	1.08
Interday precision (RSD % *n*=6)	1.13
Limit of quantitation of gallic acid (ng/µl)	0.0667
Limit of detection of gallic acid (ng/µl)	0.045
Specificity	Specific

RSD: Relative standard deviation

formation of microstreams. These changes in leaf were very intense, and it causes rupturing of glandular trichomes.

High-performance thin-layer liquid chromatography studies

The validation parameters for HPTLC were tabulated in Table 12. Linearity tracks of Oleuropein standard and HPTLC chromatogram for Olive leaves extract were recorded at 254 nm as shown in Figure 10. Oleuropein content was estimated qualitatively and quantitatively. The results show that the extraction efficiency of Oleuropein was found to be in the range of 35%–38% w/w after blanching. From this analytical study, it was observed that blanching accelerates the content of Oleuropein from Olive leaves.

CONCLUSION

Hot blanching technique assisted with UAE causes microstructural changes in the leaf which will augment the extraction efficiency of Oleuropein from Olive leaf. Blanching of leaf causes rupturing of cell wall; thereby, it promotes the interaction of solvent with bioactive compounds of leaf. From this study, optimized conditions were developed to extract Oleuropein from Olive leaf and comparative assessment was done between conventional and novel techniques to maximize the extraction efficiency and minimize the processing loss of secondary metabolites.

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Conflicts of interest

There are no conflicts of interest.

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