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RESEARCH ARTICLE

Influence of Microwave and Ultrasound pretreatment on Solvent Extraction of Bio-components from Walnut (*Julgans regia L.*) Shells

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Abstract

Walnut (Julgans regia L.) shells, an agro-forestry waste, are a rich source of phytochemicals with anti-oxidative and medicinal properties. Whilst recent research efforts focus on waste valorization, the present investigation demonstrates a process to extract and characterize bio-components from physically pretreated walnut shells. Pretreatment was undertaken using microwave irradiation and ultra-sonication with two different solvents (methanol and acetone). The extract obtained was optimized using Response Surface Methodology (RSM). Further, the bio-components in the extract were identified using GCMS and FTIR analyses. Through the experimental runs, it was observed that pretreatment enhanced the total extract yield from the process relative to conventional Soxhlet extraction. As a method of pretreatment, microwave irradiation was found to suit the extraction better than ultra-sonication. In addition, acetone performed better as a solvent than methanol in the bio-components recovery. Relative to conventional Soxhlet extraction, microwave pretreatment allowed for enhanced separation by 4.06 folds for methanol and 5.25 for acetone. The yield was found to be the highest (46 mg/g) while using acetone, when pretreated with microwaves at 180 W for 4 min.

Keywords

Walnut, Microwave Irradiation, Bioactive compounds, Soxhlet Extraction

1 Introduction

Walnut (*Julgans regia L.*) is a popular cash crop with great economic importance attached to its cultivation. They are nutrient-dense foods containing several bioactive compounds including; polyunsaturated fatty acids, antioxidants, plant proteins, dietary fibers, sterols, folates, tannins and polyphenols [1-3]. Earlier investigations through human supplementation have demonstrated the potential health benefits associated with the consumption of walnuts [4, 5]. These benefits include reduced risk of coronary heart diseases, treatment of tuberculosis, cancer and diabetes [4, 6, 7] However, the target compounds found in walnut are usually a part of a vast complex and its direct study and approximation is highly improbable. The qualitative and quantitative studies on these bioactive compounds from plant materials mostly rely on the selection of proper extraction method [8]. Over the past decade, several novel analytical techniques, such as hydro-distillation, enfleurage, maceration and pressing have been used for the extraction of essential oils from herbs and medicinal plants. However, strict operating conditions, long extraction period and low extraction efficiency have made such methods unviable for practical applications [9]. Solvent extraction remains one of the most favored methods for isolating bioactive components from plant materials [10].

Conventional Soxhlet extraction offers salient advantages such as repeated contact of the sample with fresh portions of the solvent and an improved penetration of solvent within the extraction cavity [11]. On the other hand, extraction processes consume large volume of solvent, time and cost in addition to their inherent heat and mass transfer limitations [12]. A recent modification to conventional extraction has focused on selectively increasing the accessibility of the pores that contain the targeted active compounds [13-16]. This can be achieved either by sample pretreatment before solvent extraction or by combining auxiliary energy to the existing extraction units [17-20]. Both processes reduce analysis time, energy consumption and solvent usage, while producing higher yields relative to conventional extraction [21]. Literature survey indicated that, very few studies have been performed on either pretreatment or process optimization on the extraction yield of bio-components from walnut shells.

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Hence, the objective of the present investigation is to: (i) subject walnut shells to physical pretreatment – Microwave (MW) irradiation and Ultrasonication (ii) compare the yield obtained from pre-treated samples with conventional Soxhlet extraction; (iii) perform structural and physical characterisation of the product obtained and (iv) optimize the process parameters chosen for maximum extraction of total bioactive components from walnut shells using Response Surface Methodology (RSM).

2 Methods and Materials

2.1 Sample preparation and pretreatment

Walnut shells were supplied by Kashmir Walnut Industries, Bohri, Jammu, India. The shells were washed thoroughly with deionized water and dried overnight in a hot air oven at 105°C. Further, the shells were subjected to size reduction in a ball mill. The ground walnut powder was passed through the standard sieves to collect fractions of 200, 350 and 500 μm and stored at 4°C in airtight bags until further use. Samples of 5 g were placed in a glass beaker and mixed with 50 ml of a solvent (methanol or acetone). Consequently, this suspension was physically activated using MW irradiation (Samsung CCE 108MDF, Malaysia) or ultra-sonication (Sonic Vibra Cell VC 130, United States). All solvents and chemicals used in this study were of analytical grade, and purchased from Nice Chemicals, Cochin, India.

In MW pretreatment, the specimen was irradiated at different output power (100, 180 and 300 W) and exposure time (2, 4 and 6 min). The activated suspension was then filtered through a Whatman No. 1 filter paper lined in a Buchner funnel. The supernatant was collected in a volumetric flask (250 mL) and made up to the mark using adequate solvent. The residue shell powder remaining in the filter paper was packed into a cellulose thimble. Similarly, for ultrasound pretreatment, the process was repeated at different amplitudes (P30, P50 and P70) with constant pulse duration of 2 s.

2.2 Process Description

Extraction was performed using conventional Soxhlet apparatus. Samples of 5 g (after MW or ultrasound pretreatment) were placed in the 250 ml extraction chamber. Samples were extracted for an average of 8 h reflux with acetone or methanol. Subsequently, the cellulose cartridge was removed from the chamber and cooled to room temperature in a desiccator. The crude extract obtained was concentrated at 40°C under reduced pressure (72 mbar) in a Rotavapor (BUCHI Labortechnik AG R-215, Switzerland). The concentrated samples were collected in 15 ml airtight screw-cap glass bottles and stored at -20°C for further studies. All the extraction runs were carried out in triplicate. Mean values have been reported here with all deviations observed to be within 5%.

2.3 Characterisation

Surface organic structures of the samples were identified through Fourier Transform Infra-Red (FTIR) spectroscopy. Recording of the infrared spectra was performed after 1 h of incubation using AgBr windows on a Thermo/Nicolet Magna-IR 750 spectrometer (DTGS detector, Ni-Chrome source and KBr beam splitter) with a total of 100 scans and a resolution of 4 cm^{-1}. Furthermore, the extract was analyzed by Gas Chromatography/Mass Spectrometry (GCMS) analyses (Perkin-Elmer Clarus 680 GC and Clarus 600 MS, United States). An Elite-5MS column (30 m, 0.25 mm ID, 250 μm df) with He as carrier gas at a flowrate of 1 mL min^{-1} was used. The injection was set at 250°C with a split ratio of 10:1 and aliquot volume of 1 μL. The column temperature was programmed at an initial temperature of 60°C (holding time of 2 min) to a final temperature of 300°C (holding time of 6 min) at a heating rate of 10°C.min^{-1}.

2.4 Experimental design

Response surface methodology (RSM) was used to achieve the level of design at which an optimum response was reached. The significant advantage of this method is: i) to understand how the process variables affect the selected response, ii) to determine existence of any possible interrelationships and iii) to characterize the combined effect that all variables may have [22]. Process optimization was performed using a three factor three stage RSM employing Central Composite Design (CCD). RSM was executed for the two different polar solvents used in Soxhlet extraction (acetone and methanol) in addition to the pretreatment methodologies (MW and ultra-sonication) individually. The three factors considered were; X_1 - size of the particle (200 to 500 μm), X_2 - pretreatment intensity (MW power – 100 W to 300 W, sonicator amplitude - P30 to P70) and X_3 - pretreatment time (2 to 6 min) (Table 1).

Table 1 Level of variables considered for the extraction of bioactive components from Walnut shells using Central Composite Design (CCD).

No	Variables	Independent Variables	**Variable levels**		
			-1	**0**	**1**
1	X_1	Particle Size (μm)	200	350	500
2	X_2	Pretreatment intensity(W/P)	100/30	180/50	300/70
3	X_3	Pretreatment time (min)	2	4	6

In all four instances, to minimize the effects of uncontrolled factors in observed responses, a randomized sequence of 20 experimental runs were performed (Table 2). Overall, the experimental design included a series of 80 experiments.

Table 2 Coded and uncoded Central Composite Design (CCD) of independent variables and their corresponding experimental and predicted values.

Run	Coded/Actual Factors				Microwave Yield Response (mg.g^{-1})				Ultrasound Yield Response (mg.g^{-}1)			
	X_1	X_2		X_3	Acetone		Methanol		Acetone		Methanol	
	(µm)	(W)	(P)	(min)	Predicted	Actual	Predicted	Actual	Predicted	Actual	Predicted	Actual
1	+1 (500)	-1 (100)	-1 (30)	+1 (6)	1.5	1.5	1.9	1.9	1.5	1.5	2.8	2.8
2	0 (350)	0 (180)	0 (50)	0 (4)	4.5	4.6	2.9	2.9	3.5	3.5	3.8	3.8
3	0 (350)	0 (180)	0 (50)	0 (4)	4.5	4.6	2.9	2.9	3.5	3.5	3.8	3.8
4	-1 (200)	-1 (100)	-1 (30)	-1 (2)	1.1	1.1	3.1	3.2	1.3	1.3	2.3	2.2
5	-1 (200)	+1 (300)	+1 (70)	-1 (2)	1.9	1.9	1.8	1.9	2.0	2.0	0.8	0.8
6	0 (350)	0 (180)	0 (50)	0 (4)	4.5	4.6	2.9	2.9	3.5	3.5	3.8	3.8
7	+1 (500)	+1 (300)	+1 (70)	-1 (2)	1.5	1.6	0.8	0.8	1.1	1.1	0.1	0.2
8	-1 (200)	+1 (300)	+1 (70)	+1 (6)	2.5	2.5	2.3	2.3	2.8	2.8	1.5	1.5
9	-1 (200)	-1 (100)	-1 (30)	+1 (6)	1.8	1.8	3.7	3.8	2.0	2.0	3.0	3.0
10	0 (350)	0 (180)	0 (50)	-1 (2)	3.6	3.4	2.1	2.0	2.5	2.4	2.8	2.8
11	0 (350)	0 (180)	0 (50)	+1 (6)	4.3	4.2	2.7	2.7	3.2	3.2	3.5	3.5
12	+1 (500)	0 (180)	0 (50)	0 (4)	3.7	3.5	1.8	1.8	2.4	2.3	3.4	3.2
13	-1 (200)	0 (180)	0 (50)	0 (4)	4.0	4.1	3.2	3.0	3.2	3.2	3.8	4.0
14	0 (350)	+1 (300)	+1 (70)	0 (4)	3.3	3.1	2.4	2.4	3.2	3.2	1.6	1.5
15	+1 (500)	+1 (300)	+1 (70)	+1 (6)	2.1	2.2	1.3	1.3	1.7	1.7	0.9	1.0
16	0 (350)	0 (180)	0 (50)	0 (4)	4.5	4.6	2.9	2.9	3.5	3.5	3.8	3.8
17	0 (350)	0 (180)	0 (50)	0 (4)	4.5	4.6	2.9	2.9	3.5	3.5	3.8	3.8
18	0 (350)	-1 (100)	-1 (30)	0 (4)	2.5	2.5	3.3	3.2	2.7	3.5	3.3	3.4
19	+1 (500)	-1 (100)	-1 (30)	-1 (2)	0.7	0.8	1.3	1.3	0.9	0.9	2.0	2.0
20	0 (350)	0 (180)	-1 (30)	0 (4)	4.5	4.6	2.9	2.9	3.5	3.5	3.8	3.8

A second order polynomial equation was obtained using the independent variables as mentioned in Eq. (1)

$$Y = \quad A_0 + A_1.X_1 + A_2.X_2 + A_3.X_3 + A_{12}.X_1.X_2 + A_{13}.X_1.X_3 + A_{23}.X_2.X_3 + A_{11}X_1^2 + A_{22}X_2^2 + A_{33}X_3^2 \tag{1}$$

where, Y is the predicted response, A_o the intercept coefficient, A_i the linear terms, A_{ii} the squared terms, A_{ij} the interaction terms and X_i represents the coded independent variables for the model (Table 1). Analysis of variance (ANOVA) was performed to identify interaction between the variables and the responses. The statistical significance was checked by Fischer-Test value and the quality of fit was expressed by the Coefficient of Determination (R^2).

3 Results and Discussion

3.1 Choice of process variables and conditions

The experimental range for all process variables performed in the current investigation was based on the results of preliminary single-factor experiments and observations as mentioned below:

a) Although a wide range of organic solvents (acetone, chloroform, ethanol, hexane and methanol) were examined, based on our initial investigations, only acetone and methanol were chosen. These solvents were chosen as they gave optimum peaks for hentriacontane and antioxidants (acetic acid and benzene dicarboxylic acids) in GC analysis. Further, earlier investigations on walnuts also advocate the use of acetone and methanol as solvents [23, 24].

b) The addition of solvents in extraction is performed with the intention to increase the yield of extract. Pretreatment of the sample with MW or ultra-sonication, results in dissipation of electrical or mechanical energy into heat, reducing the extraction time, solvent volume and increases the total yield [25]. Our initial experiments also indicated that to ensure non destructibility of the samples, MW power must be maintained below 300 W and sonication intensity lower than P70. Any further increase, either in MW output power (>300 W) or ultrasound amplitude (>P70), resulted in denaturation of the target compounds due to development of high internal stresses and diffusion rate [26, 27].

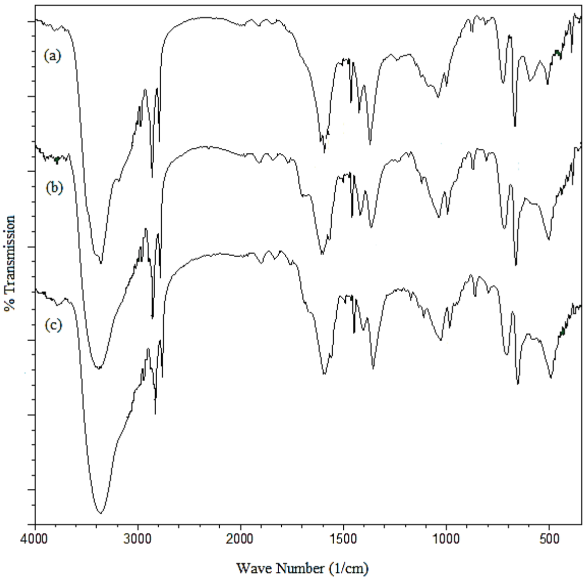

Fig. 1 FT-IR spectra for MW pretreated extraction using methanol (a) 100 W (b) 180 W and (c) 300 W

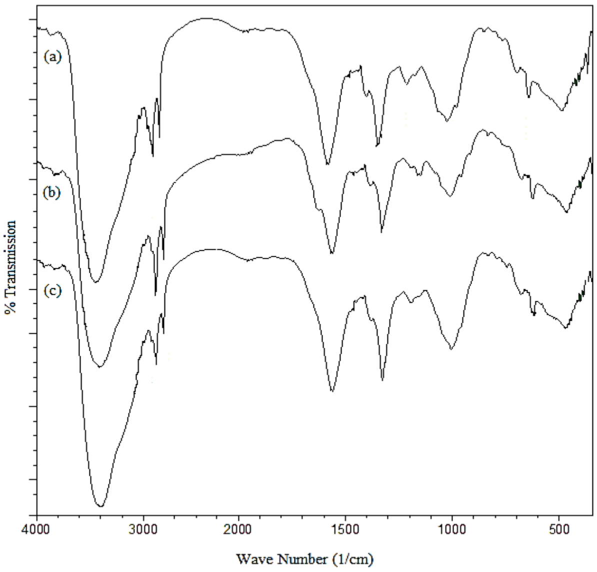

Fig. 2 FT-IR spectra for MW pretreated extraction using acetone (a) P30 (b) P50 and (c) P70

c) Initial studies carried out allowed for the particle size to be varied as 200, 350 and 500 µm. Extraction yield was found to increase with decreasing particle size as it allowed greater surface area for molecular transport between the phases. Decreasing the size below 200 µm led to reduced extraction yield which may be due to increased diffusive resistance in the solution. Similar observations have also been made by Chen and his coworkers [28]. Furthermore, lower particle sizes (>200 µm) produced slurries which were not feasible to be packed into the laboratory scale Soxhlet apparatus used for experimentation.
d) As noticeable changes were recorded only between 2 and 6 min for both MW irradiation and ultra-sonication, the pretreatment time was varied within this range. Based on initial single factor experiments for pretreatment time, it was observed that the extract yield increased only up to 6 min after which it dropped drastically. It may be inferred that thermal accumulation within the extraction solution allowed for enhanced separation of the bio-compounds only until 6 min. Moreover, excessive pretreatment time resulted in degradation of the target compounds [29].

3.2 Characterization

The FTIR spectrum of the MW pretreated extract using methanol is shown in Fig. 1. The Absorption spectra revealed distinct peaks at 2918.30, 2956.87, 1402.00 and 746.45 cm^{-1} indicating the presence of long-chain alkanes (possibly hentriacontane) and suggested the presence of C-C stretches within a ringed structure. The peaks between 1000- 650, at 1641.12 and 2848.86 cm^{-1} showed the presence of alkene bends, long chain stretch alkenes and long-chain aldehydes respectively. Moreover, the characteristic peaks at 1219.01 and 1080.14 cm^{-1} predicted the presence of -C-O- bonds of alcohols, esters or carboxylic acids. The peak at 698.23 cm^{-1} corresponds to the presence of alkyl halides in the extract. Further, the presence of the above mentioned functional groups are validated by GCMS analysis.

Both samples revealed the presence of similar functional groups except the absence of alkyl halides in acetone extract (Fig. 1 and Fig. 2). In spite of similar polarities (5.1), the alcohol with a hydroxyl ion was more easily replaced by halide compounds from the shell. Peaks observed at 1153.43, 1070.49 and 1028.06 cm^{-1} (indicating the presence of esters, carboxylic acids and alcohols) were substantiated by the GC analysis. The peaks in the three regions 3026.31 cm^{-1} (3100-3000 region), 1600.92 cm^{-1} (1600-1585 region) and 1492.90 cm^{-1}, 1450.47 cm^{-1} and 1402.25 cm^{-1} (1500-1400 region) elucidated the possible presence of ringed aromatic carbon structures. The presence of sp^3 carbon atoms which could be hentriacontane and 3-methyl-heneicosane can be identified at 2954.95, 2918.3 and 2848.86 cm^{-1}. A similar trend was observed for walnut shells pretreated with ultra-sonication for acetone and methanol (Figure not shown).

The observed chromatogram showed similarities in residence time for all samples pretreated with MW and ultra-sonication. It was noticed from Fig. 3 and Fig. 4 that the residence time for most components was found to be in the range of 21.5 and 31.5 min. Although, the intensity of peaks increased with an increase in MW output power, they dropped significantly above 300 W, this may be due to the degradation of the biomolecules caused by intense irradiation and the internal heat generated by microwaves [30]. Whereas, the peak at 24.40 min indicated the presence of hentriacontane ($CH_3(CH_2)_{29}CH_3$) with no noticeable reduction in peak intensity for treatment above 300 W. Hentriacontane can be separated almost exclusively from walnut extract at 300 W or above. This is a significant inference, as hentriacontane is a commercial anti-inflammatory drug used in, among others, treatment of tuberculosis [31].

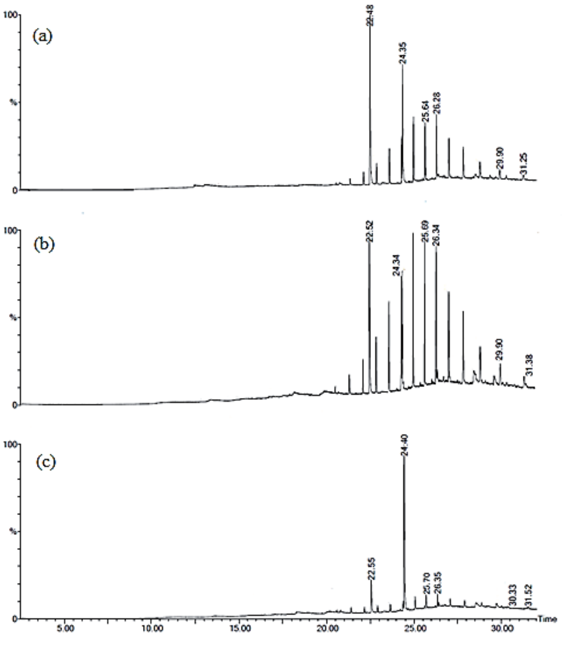

Fig. 3 GC plots for MW pretreated methanol extraction at (a) 100 W (b) 200 W and (c) 300 W

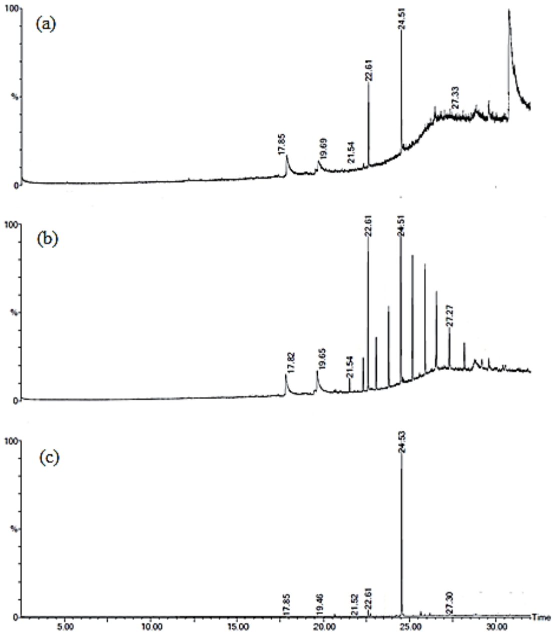

Fig. 4 GC plot for MW pretreated acetone extraction at (a) 100 W (b) 200 W and (c) 300 W.

The major peaks at 22.48 and 24.35 min, indicative of benzene-1,2-dicarboxylic acid ($C_6H_4(CO_2H)_2$) and decanedioic acid ($HO_2C(CH_2)_8CO_2H$) remained unchanged till 180 W. Thereafter, the intensity dropped from 100% to 30% for benzene-1,2-dicarboxylic acid and from 70% to 10% for decanedioic acid (Fig. 4). Other peaks remained nearly constant as the MW power output increased from 100 W to 180 W and thereafter, it reduced below 20%. In addition, hentriacontane (36%), benzene dicarboxylic acid (35%) and decanedioic acid (20%) were identified to be the most detectable components in the extract.

The observance of characteristic red color of the extract from methanol may be due to the presence of bromine, in 1-bromotriacontane ($C_{30}H_{61}Br$) (2%) and 1,54-dibromo-tetrapentacontane ($C_{54}H_{108}Br_2$) (2%) [32].

Most of the components extracted from walnut shells using acetone had a residence time between 17.5 and 27.5 min (Fig 4). The peaks observed were found to correspond with major antioxidant components of the extract. The intensity of the peaks at 22.61 min (benzenedicarboxylic acid) and 24.51 min (hentriacontane) were seen to increase with MW output power from 60% and 85% (at 100 W) to 90% and 100% (at 180 W) respectively. After 180 W, the peak at 22.61 min suddenly dropped to below 10% while, the peak at 24.51 remained at 100% peak intensity. This increase was not observed for the peaks at 17.87 min (ascorbic acid - $C_6H_8O_6$), 19.67 min (stearic acid - $C_{18}H_{36}O_2$), 21.54 min (octadecane - $CH_3(CH_2)_{16}CH_3$) and 27.27 min (heneicosane - $CH_3(CH_2)_{19}CH_3$). This may be due to the negligible effect of MW irradiation on the extraction yields of these components. Again, hentriacontane was the largest component with an average area of 59%. Other major components included anti-oxidants like ascorbic acid (10%) and benzene dicarboxylic acid (14%). Waxy long chain hydrocarbons like stearic acid (9%) and heneicosane (5%) were also present in considerable amounts. Ultrasonication as a pretreatment method, allowed for the identification of similar components trends. The peaks are not as prominent as the extract using MW pretreatment, especially in the case of hentriacontane that only reached 67 % purity when extracted by this method at P70 compared to 87 % for MW at 300 W (Figure not shown).

3.3 Optimization of total extraction yield

A three factor-three level CCD was used to develop a correlation between the process variables and yield of extracts by multiple regression analysis. The raw experimental data were fed into the Design Expert 9.0.4.1 software (Stat-Ease, Inc., 2021 East Hennepin Ave., Suite 480, MN, USA) for further analysis. Without performing any transformation on the response, examination of the Fit Summary revealed that the quadratic model is statistically significant for all cases within the scope of this study and therefore it was used throughout the investigation. Design of Experiment helps reduce the number of experimentations without impairing the exactitude of results. The set of experiments, generated by Design of Experiments fixing the various levels of process parameters and pretreatment processes, were performed (Table 2). The following correlations were generated for the extraction process under study. MW pretreated Soxhlet extraction with methanol (Eq. (2)) and acetone (Eq. (3)) as the solvent:

$$\begin{aligned} Y = \quad & \mathbf{1.9967} + 0.0043X_1 - 0.0094X_2 + 1.0356X_3 \\ & -0.000014X_1X_2 + 0.000041X_1X_3 - 0.000188X_2X_3 \\ & -0.000017X_1^2 + 0.00000013X_2^2 - 0.1091X_3^2 \end{aligned} \tag{2}$$

$$
\begin{aligned}
Y = \quad & \mathbf{-}9.0113 + 0.01980X_1 + 0.0692X_2 + 1.3445X_3 \\
& -1.5342X_1X_2 - 7.5989X_1X_3 - 0.000125X_2X_3 \\
& -0.000030X_1^2 - 0.000162X_2^2 - 0.1443X_3^2
\end{aligned} \tag{3}
$$

Ultrasound pretreated Soxhlet extraction with methanol (Eq. (4)) and acetone (Eq. (5)) as the solvent:

$$
\begin{aligned}
Y = \quad & 1.9967 + 0.0043X_1 - 0.0094X_2 + 1.0356X_3 + \\
& 0.000014X_1X_2 + 0.0000416X_1X_3 - 0.000187X_2X_3 \\
& -0.000017X_1^2 - 0.0000013X_2^2 - 0.1091X_3^2
\end{aligned} \tag{4}
$$

$$
\begin{aligned}
Y = \quad & -9.0113 + 0.0198X_1 + 0.0691X_2 + 1.3445X_3 \\
& -1.5342X_1X_2 - 7.5989X_1X_3 - 0.000125X_2X_3 \\
& -0.00003X_1^2 - 0.000162X_2^2 - 0.1443X_3^2
\end{aligned} \tag{5}
$$

Adequacy check is a prime requirement in the analysis to ensure approximation of the proposed model to real systems [33]. This was performed by the test for lack of fit and test of significance of individual and regression model coefficients. The negligible lack of fit values and low P-values indicated that the generated models could be used to predict the desired response and explain the variation by the regression equations [34]. The model accuracy and precision were verified by the coefficient of determination (R^2 >0.99). Moreover, the predicted R^2 was in good agreement with the adjusted R^2 indicating adequate approximation of the proposed model with actual [25]. In addition, the signal to noise ratio was found to be > 40 indicating goodness of fit. The regression equations (Eq. (2) - Eq. (5)) were then modified through the test for ANOVA to include only the statistically significant terms based on the P-values (< 0.001) (Table 3). The modified equations are as follows: MW pretreated Soxhlet extraction with methanol (Eq. (6)) and ethanol (Eq. (7)) as the solvents:

$$
\begin{aligned}
Y = \quad & \mathbf{1.9967} + 0.0043X_1 - 0.0094X_2 + 1.0356X_3 \\
& -0.00001X_1X_2 - 0.000017X_1^2 - 0.1091X_3^2
\end{aligned} \tag{6}
$$

$$
\begin{aligned}
Y = \quad & \mathbf{-}9.0113 + 0.0692X_2 + 1.344X_3 - 0.00003X_1^2 \\
& -0.000162X_2^2 - 0.1443X_3^2
\end{aligned} \tag{7}
$$

Ultrasound pretreated Soxhlet extraction with methanol (Eq. (8)) and ethanol (Eq. (9)):

$$
\begin{aligned}
Y = \quad & 1.9967 + 0.0043X_1 - 0.0094X_2 + 1.035X_3 \\
& -0.0000013X_2^2 - 0.1091X_3^2
\end{aligned} \tag{8}
$$

$$
\begin{aligned}
Y = \quad & -9.0113 + 0.0198X_1 + 0.0691X_2 + 1.3445X_3 \\
& -0.00012X_2X_3 - 0.00003X_1^2 - 0.00016X_2^2 \\
& -0.14431X_3^2
\end{aligned} \tag{9}
$$

From the above equations and the significance study using p-value and F-test, it was observed that most of the parameters used had a direct effect on the yield while, few did not show an interactive effect. This was confirmed using the perturbation plots (Fig. 5 and Fig. 6). When MW was used with acetone, all three parameters showed negligible interactive effects (Fig. 5). The slope for particle size (X_1) is almost horizontal in the perturbation plot hence; its contribution to optimization is insignificant, while pretreatment time (X_3) having the steepest gradient is a significant parameter in the optimization process [33]. When acetone was used in the presence of ultrasound (Fig. 6), all the parameters had a significant intensity of slope independently. The pretreatment time (X_2) and time (X_3) parameters displayed a relatively greater slope and therefore, a better significance in the model. Perturbation plots for methanol using both treatment methods showed negligible variation from the corresponding acetone plots (Figure not shown).

Table 3 Regression coefficients for the optimization studies and ANOVA for the experimental results

Source	Microwave		Ultrasonication	
	Acetone	Methanol	Acetone	Methanol
Model	2.55×10^{-10}	4.51×10^{-9}	2.80×10^{-13}	9.75×10^{-11}
X_1	1.02×10^{-3}	6.45×10^{-10}	1.11×10^{-9}	3.92×10^{-5}
X_2	5.79×10^{-6}	3.57×10^{-8}	9.46×10^{-8}	2.05×10^{-10}
X_3	1.25×10^{-5}	4.50×10^{-6}	2.47×10^{-9}	4.38×10^{-7}
X_1X_2	1.00×10^{0}	1.23×10^{-4}	4.06×10^{-5}	3.95×10^{-2}
X_1X_3	1.00×10^{0}	7.29×10^{-1}	8.82×10^{-2}	7.42×10^{-1}
X_2X_3	6.02×10^{-1}	3.11×10^{-1}	5.43×10^{-1}	7.42×10^{-1}
X_1^2	6.53×10^{-1}	7.33×10^{-5}	1.76×10^{-9}	2.36×10^{-2}
X_2^2	1.65×10^{-9}	8.24×10^{-1}	4.84×10^{-8}	1.39×10^{-9}
X_3^2	2.63×10^{-5}	2.64×10^{-5}	3.67×10^{-9}	1.91×10^{-6}
Mean	2.9692	3.1231	3.3308	2.7077
SD	0.0900	0.0866	0.0919	0.1158
Lack of Fit	0.0567	0.0524	0.0591	0.0938
R^2	0.9867	0.9956	0.9962	0.9806
Adj. R^2	0.9771	0.9925	0.9935	0.9667
Pred. R^2	0.8642	0.9586	0.9636	0.8385
C.V %	3.0302	2.7715	2.7589	4.2757

In MW extraction using acetone (Fig. 7), the particle size is observed to have negligible effect as an independent factor but, highly significant in its quadratic form. Also, MW-aided

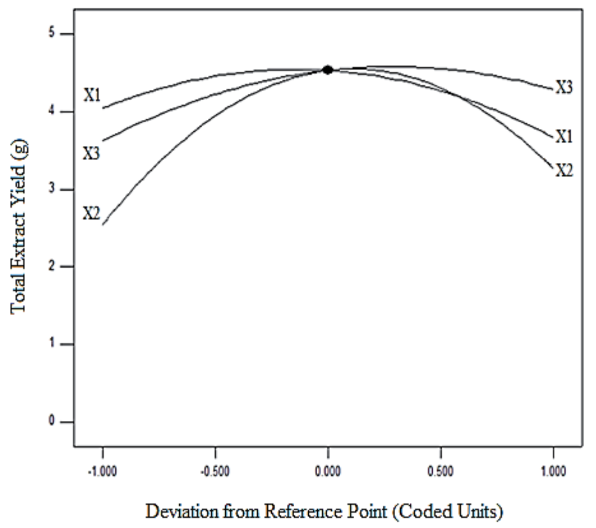

Fig. 5 Perturbation plot for MW pretreated Soxhlet extraction using acetone.

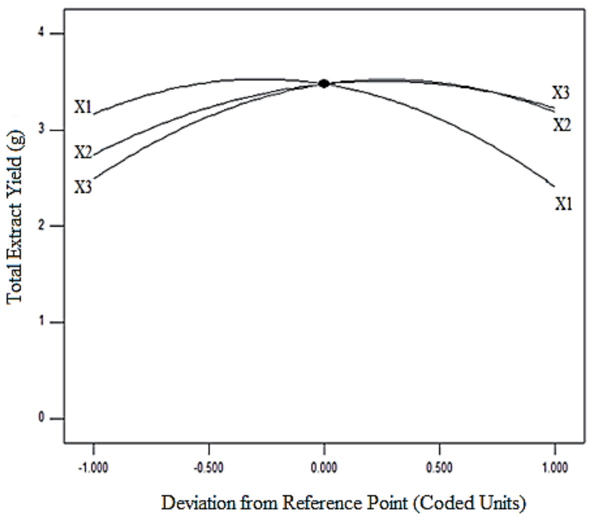

Fig. 6 Perturbation plot for ultra sound pretreated Soxhlet extraction using acetone.

extraction process generally showed a better optimized result when compared with ultra-sound. The 3D plots for extraction using acetone pretreated with ultrasound (Fig. 8) showed the yield to increase up to 4 minutes and ultra-sound amplitude of 50 and thereafter, declined. From the plot, it may be inferred that the optimum particle size is 350 μm in this case. The results observed in the 3D plots were corroborated by the observations made from the perturbation plots.

All the parameters are seen to be significant in the optimization of the yield either in the linear, interactive or quadratic term. From the above results, we can infer that acetone is the best solvent for extraction of bio-components from walnut shells using both pretreatment methodologies. MW irradiation was found to be the best pretreatment method to optimize the extraction yield for both, methanol and acetone as the solvents. For both extractions, optimum yields were observed using acetone (46 mg g^{-1}) and methanol (40 mg g^{-1}) at similar particle size (200 μm) and pretreatment time (4 min) with 180 W and 100 W respectively.

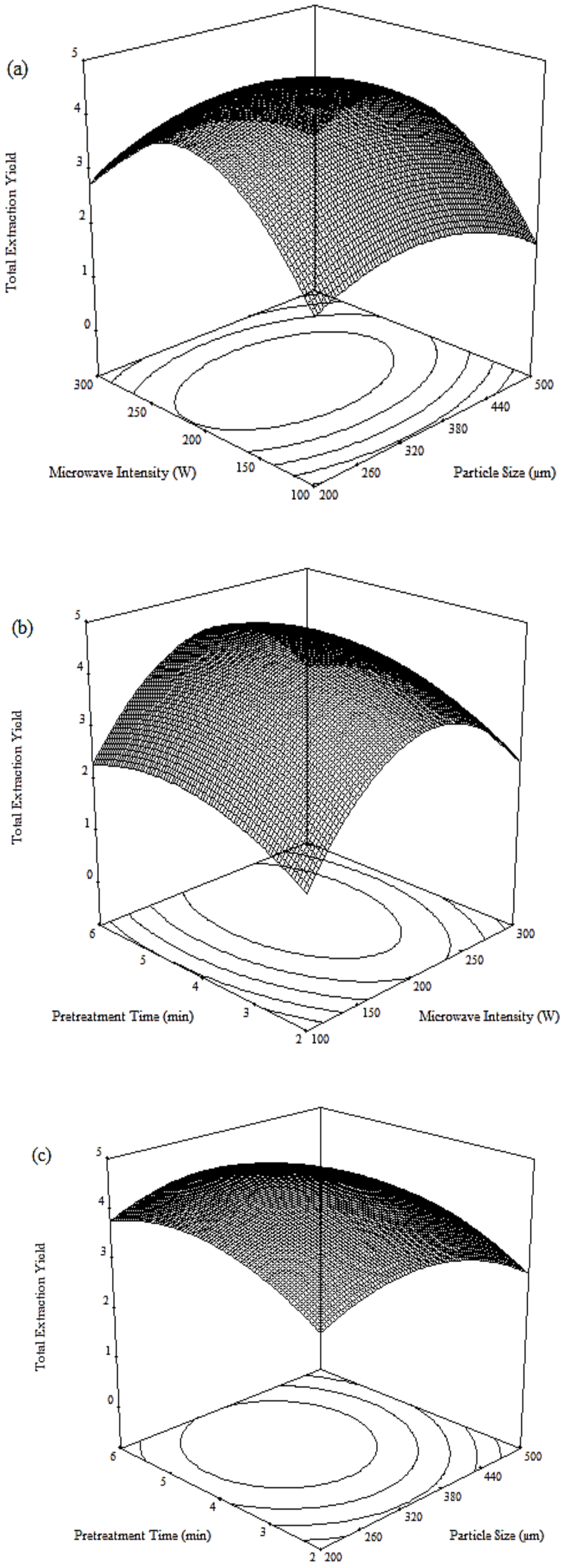

Fig. 7 3D response surface plots for MW pretreated Soxhlet extraction using acetone (a) Interactive effects of particle size and MW intensity (W) on total extraction yield (mg.g^{-1}) at pretreatment time of 4 mins; (b) Interactive effects of microwave intensity (W) and pretreatment time (min) on total extraction yield (mg.g^{-1}) at particle size 350 μm and (c) Interactive effects of particle size (μm) and pretreatment time (min) on total extraction yield (mg.g^{-1}) at MW intensity 180 W.

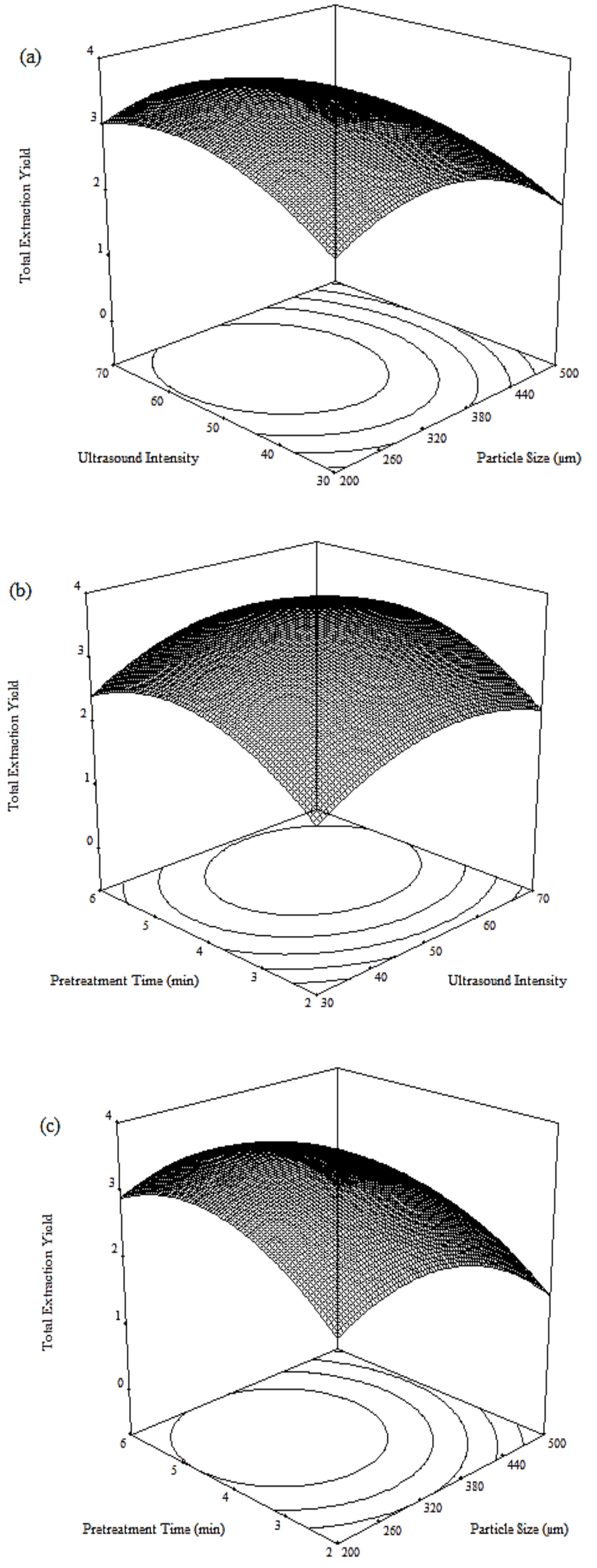

Fig. 8 3D response surface plots for ultra-sound pretreated Soxhlet extraction using acetone (a) Interactive effects of particle size and ultrasound on total extraction yield (mg.g^{-1}) at pretreatment time of 4 mins; (b) Interactive effects of ultrasound intensity and pretreatment time (min) on total extraction yield (mg.g^{-1}) at particle size 350 µm and (c) Interactive effects of particle size (µm) and pretreatment time (min) on total extraction yield (mg.g^{-1}) at ultrasound of P 50.

4 Conclusions

Walnuts contain a diverse array of compounds that enhance the nutritional value of the human diet. This also extends to auxiliary components of walnuts including shells. The detected presence of antioxidants and hentriacontane, may impart therapeutic properties to the extract. Soxhlet extraction followed by pretreatment with MW irradiation and ultra-sonication using two different solvents (methanol and acetone) was optimized using a three factor-three stage Response Surface Methodology based on Central Composite Design. Results showed that all the process variables had significant effect on the extraction yield and a quadratic mathematical model was developed. MW irradiation was found to be the best pretreatment method to optimize the yield for both methanol and acetone. Due to the pretreatment performed, net extraction yield was found to improve up to 4.06 times with methanol and 5.25 times with acetone. The optimal extract yield was determined to be 46 mg g^{-1} for a MW power of 180 W and particle size of 200 µm on using acetone for an exposure time of 4 min. Under these optimal conditions, the experimental yield agreed closely with the statistically predicted yield value.

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