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Optimization of curcumin extraction by microwave assisted *in vitro* plant cell bursting by orthogonal array designed extraction process and HPTLC analysis

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ABSTRACT - The present work proposes the use of orthogonal design in the development of microwave assisted extraction of curcumin from *Curcuma longa* L. rhizomes. Extraction was brought about by plant cell bursting under microwave effect at 2450 MHz, followed by leaching out of the active constituents from the irradiated sample under constant stirring in a closed container with acetone. Curcumin was quantified using a Camag HPTLC system. The optimized conditions as obtained from the graphical representation of analysis of means showed that at 20% microwave power, 4 min irradiation time and with particles screened from sieve20, extraction efficiency of 98.05% was obtained from the first 3 hrs of extraction as compared to the curcumin yield obtained from unirradiated sample with constant stirring in acetone for 24 hours. Analysis of different chromatographic parameters showed that curcumin was not degraded under microwave effect.

KEYWORDS - Orthogonal design, Microwave 2450 MHz, Curcumin, HPTLC, Optimization, Analysis of means

INTRODUCTION

India has rich history of using plants for medicinal purposes. Turmeric (*Curcuma longa* L.) is a medicinal plant extensively used in Ayurveda, Unani and Siddha medicine for various diseases (1). Turmeric belongs to the Zingiberaceae family along with the other noteworthy members like Ginger, Cardamom and Galangal. Turmeric became a very important spice to mankind when it was observed that the addition of turmeric powder in food preparation preserved its freshness and nutritive value. The yellow pigmented fraction of *C. longa* contains curcuminoids, which are chemically related to its principal ingredient, curcumin. The three main curcuminoids isolated from turmeric are curcumin, demethoxy curcumin and bis-(demethoxy) curcumin (1). Curcumin has been shown to possess wide range of pharmacological activities including anti-inflammatory, anticancer, antioxidant, wound healing, hypoglycemic, anticoagulant, antifertility and antimicrobial activities (1,2,). The other salient feature of turmeric/curcumin is that despite being consumed daily for centuries in Asian countries, it has not been shown to cause any toxicity. Classical techniques for the solvent extraction of active constituents from plant matrices are based on the choice of solvent coupled with the use of heat and / or agitation. Existing classical techniques used include: Soxhlet, hydro distillation and maceration. The conventional techniques reported for extraction of curcumin from *C. longa* are all time consuming and require the usage of a large amount of organic solvents (3,4,5). So in order to solve the problem, a novel extraction technique based on the use of microwave was designed and optimized using engineering design. The application of microwave in the extraction of secondary metabolites from plants has gained considerable importance in recent times as evident from its use in the extraction of biologically active compounds from different matrices, such as extraction of camptothecin from *Nothapodytes foetida (*6), extraction of tanshinones from *Salvia miltiorrhiza (*7), extraction of solanesol from tobacco leaves (8), and so on. In this study the main objective was to design a extraction process by exposing the plant matrix to microwave to bring about cell rupture that will facilitate leaching out of active constituents when later extracted with continuous stirring. Four main factors namely microwave power, irradiation time, particle size and stirring time were studied using an orthogonal array L_9 design. The process was optimized

for maximum yield of curcumin based on the graphical representation of the analysis of means.

MATERIALS AND METHODS

Plant materials

Dried authenticated rhizomes of *C. longa* L. was provided as gift sample by herbal exporter M/S Ram Traders, Mumbai (India) and was used as received without any pretreatment. A voucher specimen (CL-MAE-0106) has been deposited in the Pharmacognosy Research Laboratory of Department of Pharmaceutics, BHU. Rhizomes were powdered to homogenous 40, 20, 10 mesh powder (selected by sieve), immediately before the experiment.

Reagents

Acetone and methanol used in the experimental were all of analytical grade (Merck, India). Chloroform and methanol used in HPTLC analysis were all of HPLC grade. Precoated silica gel $60F_{254}$ plates for HPTLC analysis were (E.Merck, Germany) used without pretreatment. Curcumin standard of (99% w/w) purity was obtained as a gift sample from Centre for Cellular and Molecular Biology, Hyderabad (India).

Apparatus

The extraction system comprised a commercially available multimode microwave oven (LG electrical equipment Co., Ltd., India) equipped with a magnetron of 2450 MHz with a nominal maximum power of 800 W.

Microwave assisted extraction (MAE)

For MAE accurately weighed 2 g of the homogenous 40, 20, 10 mesh drug powder was used. The samples were spread as a thin layer over a petri dish. The sample was then placed inside the microwave cavity and MAE was carried out for different time of irradiation with the microwave oven operating at different power levels. In all MAE a dummy water load was kept beside the sample inside the microwave cavity to absorb unwanted radiations that were not being focused on the sample. This helped to reduce the microwave load inside the microwave cavity. To avoid charring of sample and facilitate uniform exposure of the sample to microwave the sample was treated in an intermittent way, i.e. irradiation – mixing – irradiation. The irradiation time was kept for 1 min and 30 sec was taken to mix the sample between two irradiations. After microwave exposure the powdered drug was extracted with acetone in a closed container with continuous stirring for different time interval according to the experimental design. After extraction the samples were centrifuged for 15 min at 4000 rpm. The supernatant was filtered, concentrated under vacuum, dissolved in methanol for quantification of curcumin by HPTLC

Stirring extraction

To determine the effect of microwave on the powder drug a continuous stirring extraction was carried out for 24 hrs with 40 ml of acetone using unirradiated sample of 40, 20, 10 mesh drug powder. Since 40 mesh drug powder showed the highest curcumin yield of 1.56% w/w (equivalent to 31.20 mg) so it was considered as the highest achievable target and the extraction efficiency of MAE was calculated by comparison with this target value. In the present work extraction efficiency (%) for MAE was defined as follows

$$\text{Extraction efficiency (\%)} = \frac{\text{Curcumin (mg) obtained from MAE under orthogonal conditions} \times 100}{31.20}$$

HPTLC analysis

The samples were spotted in the form of bands of width 8 mm, positioned 10 mm from the bottom of the plate, with a Camag microlitre syringe on precoated silica gel aluminum plate $60F_{254}$ (20 cm × 10 cm) with 200 µm thickness, using a Camag Linomat V (Switzerland) automated sample applicator with nitrogen flow. A constant application rate of 150 nls^{-1} was employed and space between two bands was 10 mm. The slit dimension was kept at 6 mm × 0.3 mm and 20 mms^{-1} scanning speed was employed. The mobile phase consisted of chloroform: methanol (98:2 v/v, 20 ml). Linear ascending development was carried out in a twin trough glass chamber pre-saturated with mobile phase for 30 min at room temperature (25 ± 2°C) at relative humidity of 55 ± 5 %. The length of the chromatogram run was 80 mm. The plate was removed from the chamber, dried in air and scanned in absorbance/reflectance mode of a Camag TLC scanner III at 366 nm. Peak area data were recorded using Camag win CATS software.

Thin layer chromatography

To determine the formation of any decomposition products during MAE, a two-dimensional 'diagonal chromatography' approach was used (9). The sample (crude extract dissolved in methanol) was applied at one corner of the plate, and developed in a normal linear ascending mode. The plate was removed from the chamber; air-dried and rotated 90°. It was then developed again in the same solvent system. Curcumin was isolated using flash column chromatography (FC) with chloroform as the eluent. The different solvent system and the parameters studied to ascertain its

chromatographic similarity with that of standard curcumin are presented in Table 4. The $I_{P\ (Chr)}$ value was calculated according to the rules of coordinate geometry as described by Snyder and Nyiredy (10). It characterizes the goodness of the chromatographic identification. The higher the value of IP $_{(Chr)}$, the better is the probability that two compounds are identical. If the IP $_{(Chr)}$ *value* is less than 0.1, the identification is inadequate; for routine laboratory work it has to be between 0.1 and 0.5. If the value of $I_{P(Chr)}$ is higher than 0.5 then the substances are chromatographically identical with a high degree of probability. A chamber saturation time of 30 min at room temperature (25 ± 2°C) at relative humidity of 55 ± 5 % was allowed. Linear ascending development was followed in all cases. To avoid variation due to influence of moisture the plates were activated before use. The samples were well dried after spotting. An equal volume of ethanol was applied over the dried initial zone, followed by redrying to allow the alcohol to azeotrope off any remaining moisture (9).

Optimization method – Analysis of Means / Taguchi approach

The optimum level for each factor was determined from the graphical representation of the analysis of mean values obtained from each level for a particular factor (11). Quality of performance of different factors mentioned in the L_9 design was measured by calculating the variation (Mean square deviation) around the target (12). The factor which resulted in reduced variation was considered to have shown the best performance. Mean square deviation (MSD) was calculated by adding the square of deviations of all individual numbers from a fixed target value. In this case 1.56% w/w (equivalent to 31.20 mg) curcumin was taken as the target value as this was considered to be the maximum yield obtained from 24 hrs of continuous stirring extraction using acetone. All performances were measured by measuring the deviation from the maximum target value.

RESULTS AND DISCUSSION

Extraction conditions

During MAE of curcumin from *C. longa*, there are many parameters influencing the extraction efficiency. Four main factors i.e. microwave power, irradiation time, particle size and stirring time was taken up for this study. For each variable, the influence on the yield of curcumin and extraction efficiency (%) was considered from three levels. Table 1 lists the factors and levels used in the tests. In general, a full evaluation of the effect of four factors from three levels on the yield of curcumin and extraction efficiency (%) needs 81 (3^4) experiments. In order to reduce the number of experiments, a L_9 (3^4) orthogonal design was used (Table 2) (13). Orthogonal array can be defined as a matrix with the columns representing the number of parameters to be studied with their different levels in different combinations of experiments and the number of rows equal to the number of experiments. According to this design only nine experiments were needed. The sequence in which the experiments were carried out was randomized to avoid any kind of personal or subjective bias. All the tests at each step of the design were carried out in triplicate. The percentage extraction of curcumin, w/w and extraction efficiency (%) of curcumin obtained under orthogonal conditions are shown in Table 2. To analyze the influence of each variable on the extraction results, Fig.1 and 2 was constructed based on the mean values obtained for each level from a particular factor.

Effect of microwave power and irradiation time

Fig.1 shows that when microwave power level was decreased from 60% to 20%, there was a 6.3% increase in curcumin yield. It was also observed that when irradiation time was increased from 1 min to 4 min there was a increase in 4.2% curcumin yield. Graphical representation of the analysis of means (Fig.1) indicates that 20% microwave power and 4 min irradiation time were ideal to obtain the maximum yield. 20% microwave power and 4 min irradiation time showed an extraction efficiency of 97.94% and 96.27% respectively (Fig.2). MAE offers a rapid delivery of energy to a total volume of the solid plant matrix with subsequent heating of the solid matrix, efficiently and homogenously. Because natural moisture present within the plant matrix absorbs microwave energy, cell disruption is promoted by internal superheating, which facilitates desorption of active constituents from the matrix, thus improving the final yield. The cell disruption phenomenon can be accounted for the sudden build up of pressure due to rapid heating of the moisture present in the plant cells (14). This increased pressure will cause microscopic fractures in the cell, due to reduction in mechanical strength of cellulose present in the cell wall. With higher microwave power and longer time of irradiation there might have been intense internal superheating of the plant matrix resulting in degradation of the active constituent which because of its polar nature is more prone to damage due to intense microwave heating. Hence a

Table 1: Factors and levels for the orthogonal design

Levels	Microwave power (%)	Irradiation Time (min)	Sieve number	Stirring time (hrs)
	A	B	C	D
I	60	1	10	3
II	40	2	20	6
III	20	4	40	9

A, B, C, D represents the respective code for each factor

Table 2: The results of orthogonal test L_9 (3^4)

Tests	A	B	C	D	Curcumin(mg)	% curcumin (w/w)	Extraction Efficiency (%)
1	1	1	1	1	29.46	1.47	94.42
2	1	2	2	2	28.67	1.43	91.89
3	1	3	3	3	28.30	1.41	90.70
4	2	1	2	3	29.12	1.45	93.33
5	2	2	3	1	29.52	147	94.61
6	2	3	1	2	29.02	1.45	93.01
7	3	1	3	2	28.28	1.41	90.64
8	3	2	1	3	30.60	1.53	98.07
9	3	3	2	1	32.80	1.64	105.12

Table 3: MSD values obtained between different factors

Factors	MSD value
Microwave power	0.115
Irradiation time	0.109
Sieve no.	0.109
Stirring time	0.111

Table 4: Comparison of chromatographic similarity between standard curcumin and isolated curcumin crystals

Mobile phase	R_f		hR_f		Flow constant (κ) mm^2/sec
	Std.	Sample	Std.	Sample	
Chloroform: Methanol (9.8:0.2)	0.45±0.01	0.44±0.01	45	44	5.16±0.04
Dichloromethane: Methanol (9.9:0.1)	0.44±0.01	0.44±0.01	44	44	6.45±0.03
Dichloromethane: Methanol (9.5:0.5)	0.81±0.02	0.81±0.02	81	81	6.45±0.04

All values represent Mean ± SD; n = 3

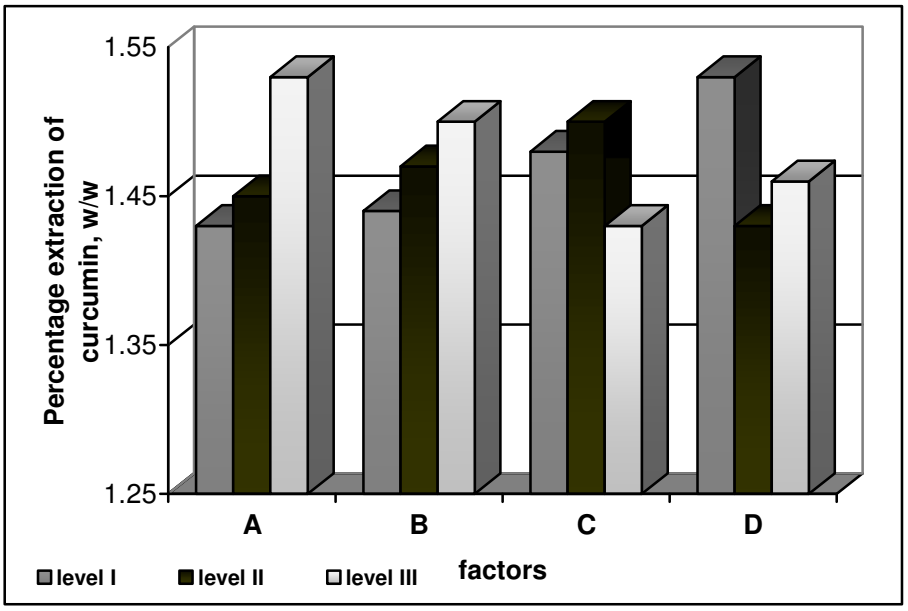

Fig. 1 Percentage extraction of Curcumin, w/w obtained under orthogonal design conditions by MAE.

Percentage extraction of curcumin, w/w = mass of curcumin in crude extract × 100/mass of raw material. ***A****= Microwave power [level I= 60%, level II= 40%, level III= 20%].* ***B****= Irradiation time [level I= 1 min, level II= 2 min, level III= 4 min].* ***C****= Sieve number [level I= 10, level II= 20, level III= 40].* ***D****= Stirring time [level I= 3 hrs, level II= 6 hrs, level III= 9 hrs].*

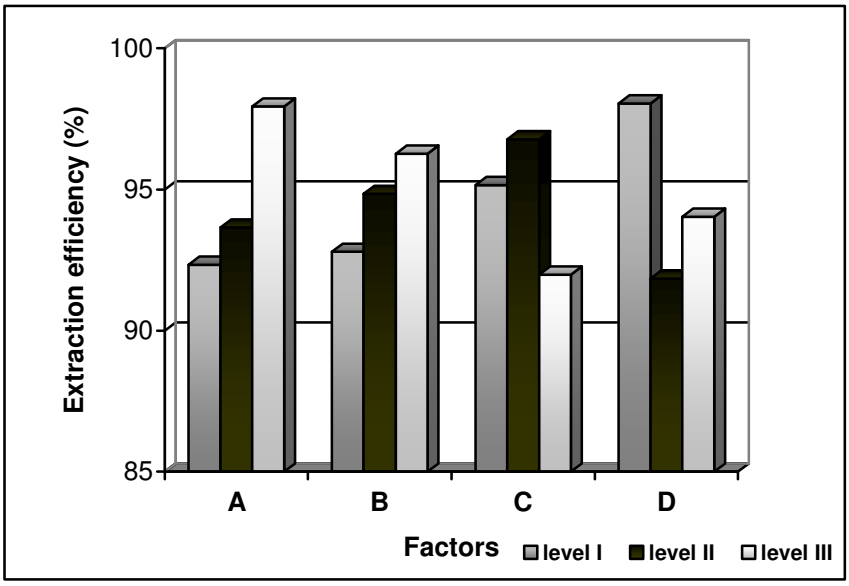

Fig. 2 Extraction efficiency (%) obtained under orthogonal design conditions.

Extraction efficiency (%) calculated by comparison with the yield of curcumin (mg) obtained from conventional stirring experiment (formula mentioned in text). ***A****= Microwave power [level I= 60%, level II= 40%, level III= 20%].* ***B****= Irradiation time [level I= 1 min, level II= 2 min, level III= 4 min].* ***C****= Sieve number [level I= 10, level II= 20, level III= 40].* ***D****= Stirring time [level I= 3 hrs, level II= 6 hrs, level III= 9 hrs].*

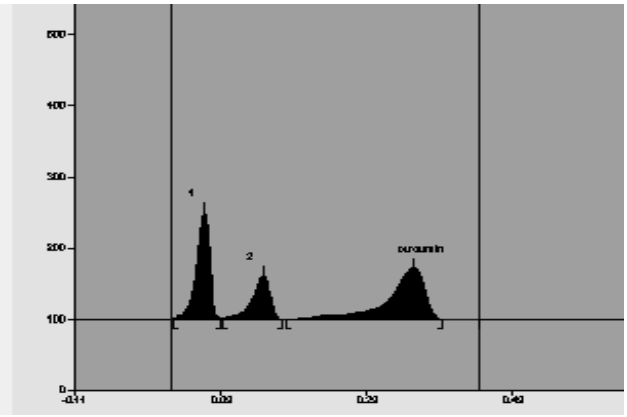

Fig. 3 Chromatogram of standard curcumin (sigma)

Peaks in the order from left to right: bis - (demethoxy) curcumin, demethoxy curcumin and curcumin

1: *bis - (demethoxy) curcumin*

2: *demethoxy curcumin*

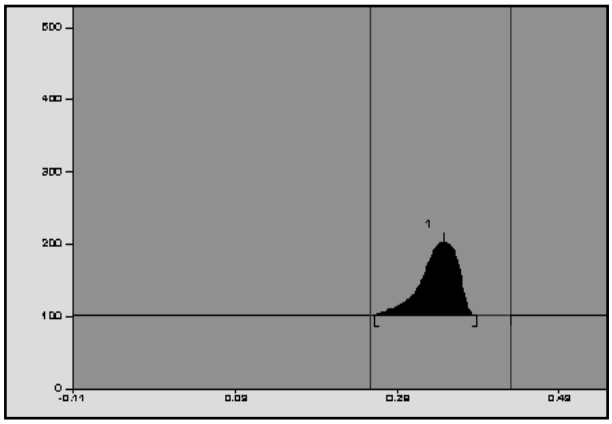

Fig. 4 Chromatogram of pure curcumin obtained from MAE.

Pure curcumin isolated from the crude extract by using flash column chromatography with chloroform as the eluent.

1: *curcumin*

low microwave power with intermittent heating upto 4 min will serve the purpose best.

Effect of particle size

Maximum yield was produced by particles screened through sieve 20 (Fig.1). Curcumin yield dropped by 4.7% with the increase in particle fineness as obtained from sieve 40. An extraction efficiency of 96.78% was reported for particles screened through sieve 20. With the use of finer particles, microwave will be facilitated with deeper penetration ability resulting in thermal degradation of active constituent.

Effect of stirring time

Stirring was used to facilitate the leaching of the active constituent from the ruptured cells into the solvent, without the formation of any saturation layer around the drug particle. Graphical representation of the mean values (Fig.1) shows that major part of the extraction took place in the first 3 hrs, with 98.05% extraction efficiency being achieved.

In vivo cell rupture

In vivo plant cell rupture was carried out under the optimized conditions as obtained under orthogonal design conditions. In vivo plant cell rupture was brought about by immersing the powder drug (sieve 20) in 40 ml chloroform contained in a long neck conical flask. The suspension was then irradiated for 4 min at 20% microwave power. Since throughout theexperiment heat has not been used to accelerate the extraction, so in case of *in vivo* cell rupture chloroform was used which shows no heating effect under microwave due to its lower dielectric constant (15). The cell rupture was facilitated, while being immersed in the solvent, whereas in case of *in vitro* cell rupture, cell rupture was brought about in the dry powder drug itself. The in *vivo* cell rupture method showed a curcumin yield of 53.4 mg which is 71.15% more than the target value.

TLC analysis

In case of diagonal chromatography all spots occurred along a 45° line indicating no decomposition products. Spots in other areas would have indicated the presence of decomposition products formed during MAE and along the course of chromatography. The $I_{P\ (Chr)}$ was calculated to be 5.06 which indicate that isolated curcumin and standard curcumin are chromatographically similar with high degree of probability. The chromatogram of standard curcumin showed the presence of three characteristics peaks (Fig. 3) whereas the curcumin isolated from the extract showed only one characteristic peak of curcumin (Fig. 4). The UV spectra of standard curcumin showed three characteristics peak for bis-demethoxy curcumin, demethoxy curcumin and curcumin with peak absorbance at 416, 419, 421 nm respectively. Isolated pure curcumin showed only one characteristics peak for curcumin which was found to overlap exactly on the standard curcumin peak. These observations reveal that curcumin was not degraded

under microwave effect.

MAE method repeatability

The repeatability of the proposed method for extraction of curcumin was determined under the optimum extraction conditions (Fig. 1) and results expressed by relative standard deviation (RSD) value. Five samples with same weight (2 g) were processed under the optimum conditions of 20% microwave power, 4 min irradiation, sieve 20 screened particles and 3 hrs stirring. The mean yield of curcumin from the five replicates was found to be 39.22 mg (equivalent to 1.96% w/w curcumin) which is 26% more than the curcumin yield obtained from conventional stirring extraction for 24 hrs performed with unirradiated sample. The calculated RSD value was 4.59% which shows that the proposed method has an acceptable precision.

CONCLUSION

In measuring the performance of different factors towards producing a output equivalent to that of the target value, MSD data (Table 3) showed that irradiation time and particle size to have produced the best performance in terms of curcumin yield (% w/w) because of its minimum deviation from the target value. Stirring time and microwave power followed next. It has been successfully reported in this work that prior exposure of powdered crude drug of appropriate particle size to microwave definitely accelerates the extraction process due to *in vitro* cell rupture. Since *in vivo* cell rupture might prove out to be more effective tool. Further investigations to this regard are in progress by authors, which requires careful selection of extracting solvent, as solvents heat up differently under microwave. Also at the same time, with no compromise being made regarding the solubility aspect of the constituent in that particular solvent.

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