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Microwave Assisted Extraction of *Phyllanthus amarus*

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Research Article

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ABSTRACT

Extraction is the separation of medicinally active portions of plant tissues using selective solvents through standard procedures. Extraction is defined as the process in which the animal or plant tissues are treated with specific solvents whereby the medicinally active constituents are dissolved out, cell tissue and most of inactive or inert components remain or undissolved. In pharmacy, the solvent used for extraction purpose is known as menstruum and residue left after extracting the desired constituents is known as marc [1]. Microwave assisted extraction is better method for extraction in terms of high yield and takes less time for extraction.

INTRODUCTION

Extraction

There are two types of extraction techniques, conventional techniques and the recent techniques. Conventional techniques are older and require more time for extraction while the recent techniques are newer extraction techniques which require less time for extraction with less solvent consumption. The types and principle of the extraction of both techniques described below:

Conventional techniques

1. Maceration: In this process extraction of drug is carried out by placing the solid drug in contact with whole of the menstruum in a closed vessel for 2 to 7 days with occasional stirring. Closed vessel is used to prevent evaporation of the menstruum [2].

2. Soxhlation: Soxhlet extraction is only required where the desired compound has a limited solubility in the solvent, and the impurity is insoluble in that solvent. If the desired compound has a high solubility in a solvent then a simple filtration can be used to separate the compound from the insoluble substance [1]. In a conventional soxhlet system plant material is placed in thimble-holder, and filled with condensed fresh solvent from distillation flask. When the liquid reaches the overflow level, a siphon aspirates the solution of the thimble-holder and unloads it back into the distillation flask, carrying extracted solutes in to the bulk liquid. In the solvent flask, solute is separated from the solvent using distillation. Solute is left in the flask and fresh solvent passes back in to the plant solid bed. The operation is repeated until complete extraction is achieved. This method cannot be used for thermolabile compounds as prolonged heating may lead to degradation of compounds [3].

3. Microwave assisted extraction: Microwave-assisted extraction (MAE) or simply microwave extraction is a relatively new extraction technique that combines microwave and traditional solvent extraction. Application of microwaves for heating the solvents and plant tissues in extraction process, which increases the kinetic of extraction, is called microwave-assisted extraction. Microwaves are non-ionizing electromagnetic waves of frequency between 300 MHz to 300 GHz and positioned between the X-ray and infrared rays in the electromagnetic spectrum. Owing to their electromagnetic nature, microwaves possess electric and magnetic fields which are perpendicular to each other. The electric field causes heating via two simultaneous mechanisms, namely, dipolar rotation and ionic conduction. Ionic conduction refers to the electrophoretic migration of ions under the influence

of the changing electric field. The resistance offered by the solution to the migration of ions generates friction, which eventually heats up the solution. Dipolar rotation is due to the alignment on the electric field of the molecules possessing a dipole moment in both the solvent and the solid sample. This oscillation produces collisions with surrounding molecules and thus the liberation of thermal energy into the medium [4].

Pressurized and focused MAE

The application of microwave energy to the samples may be performed using two technologies: either closed vessels under controlled pressure and temperature, or open vessels at atmospheric pressure. The two technologies are commonly named pressurized MAE (PMAE), with a multi-mode cavity, or focused MAE (FMAE) using the waveguide as a single-mode cavity, respectively [5]. In open vessels the temperature is limited by the boiling point of the solvent at atmospheric pressure, in closed vessels the solvent can be heated above its boiling point at atmospheric pressure by simply applying suitable pressure, thus enhancing both extraction speed and efficiency. However, after extraction with closed vessels, one needs to wait for the temperature to decrease before opening the vessel, thereby increasing the overall extraction time (by approximately 20 min.). Open systems use focused microwaves, resulting in homogeneous and very efficient heating of the sample. In closed systems using diffuse microwaves, the electric field in the cavity is non-homogeneous, and therefore the vessels are placed on a turntable. Recently, the respective advantages of high-pressure vessels and focused microwave heating have led to the development of systems that combine both approaches. These so-called "focused high-pressure, high temperature microwave system" comprise an integrated closed vessel and a focused microwave-heated system operating at very high pressure and temperature **(Figure 1).**

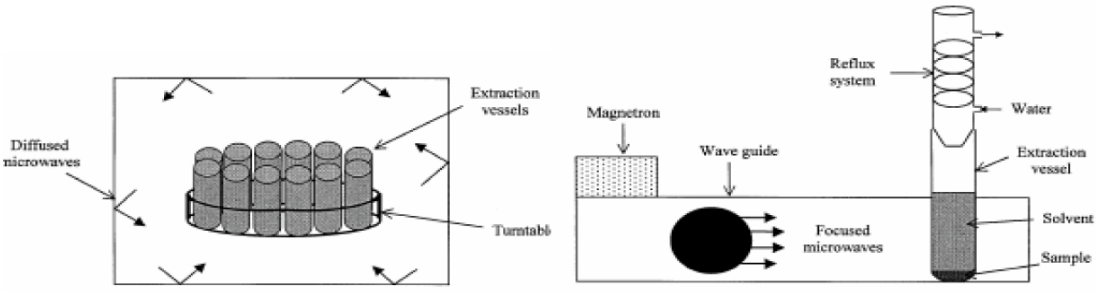

Figure 1. Scheme of the two microwave systems, using diffused or focused microwaves.

Instrumentation

Both multi-mode and focused microwave devices comprise four major components:

a) Microwave generator: magnetron, which generates microwave energy.

b) Wave guide: Which is used to propagate the microwave?

c) The applicator: Where the sample is placed.

d) Circulator: This allows the microwave to move only in the forward direction.

However, the applicator in case of multi-mode system can be a closed cavity inside which microwaves are randomly dispersed. Uniform distribution of microwave energy inside the cavity can be achieved by using beam reflectors or turntable that makes heating of the sample independent of the position. In focused microwave systems, the extraction vessel is however kept directly in a microwave waveguide and that acts as the applicator. The bottom few inches of the vessel are directly exposed to the microwaves, whereas the upper region of the vessel remains cool as glass is transparent to microwaves and hence does not get heated up in the process. This results in an effective condensing mechanism inherent in the design [6].

Types of experimental designs

The (statistical) design of experiments is an efficient procedure for planning experiments so that the data obtained can be analyzed to yield valid and objective conclusions. Design of experiments begins with determining the objectives of an experiments and selecting the process factors for the study. An experimental design is the laying out a detailed experimental plan in advance of doing the experiment. Well-chosen experimental design maximizes the amount of "information" that can be obtained for a given amount of experimental effort. Some most commonly used types of experimental designs are as follows.

1. Full factorial designs

   Two level full factorial designs

   Three level full factorial designs

2. Fractional factorial designs

3. Plackett Burman designs

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4. Response surface methods

   Central composite designs

   Box behnken designs

5. Taguchi designs

Central composite design in microwave-assisted extraction

By using the central composite design method we can apply the variable parameters in MAE in a scientifically approved manner. It is a powerful tool provides the optimum conditions that improve a process [7]. A second-order (quadratic) model can be constructed efficiently with central composite designs (CCD). CCD is first order (2^n) design augmented by additional centre and axial points to allow estimation of the tuning parameters of a second-order model. It decreases the associated numeric noise. It is relatively simple to determine the exact optimum condition for a single response using response surface methodology [8].

Phyllanthus amarus

Phyllanthus amarus is commonly known as Bhumi amla and it mainly contains phyllanthin and hypophyllanthin as active ingredients [9]. *Phyllanthus amarus* has about 750-800 species found in tropical and subtropical countries of the world [10] including in India. It is most commonly used in the Indian Ayurvedic system of medicine in problem of stomach, gentiurinary system, liver, kidney and spleen [11]. *Phyllanthus amarus* is a plant of the family Euphorbiaceae. Phyllanthus is one of the genus that falls under this family [12]. It is commonly called 'stone breaker', 'carry meseed', 'windbreaker', 'gulf leaf flower', or 'gala of wind' **(Figure 2)** [13]. Phytochemical studies have shown the presence of many valuable compoundssuch as lignans, flavonoids, hydrolysable tannins, polyphenols, triterpenes, sterols & alkaloids [14].

Figure 2. *Phyllanthus amarus* leaves.

Origin and distribution

Plants in the genus Phyllanthuscan be found around all tropical regions of the world: from Africa to Asia, South America and the West Indies. Phyllanthuscontains about 550 to 750 species in 10-11 subgenera. *P. amarus*can is found in all the tropical regions of the world: through the roads, valleys, on the riverbanks and near lakes. This plant is a common weed of disturbed ground in southern Florida, the Bahamas, the West Indies and tropical America and is naturalized in the Old World tropics. *Phyllanthus amarus* is usually misidentified with the closely related *Phyllanthus niruri* L. in appearance, phytochemical structure and history of use. *Phyllanthus niruri* reaches a length of 60 cm, the fruits are larger, and the seeds are dark brown and warty [15].

Chemical constituents

Phyllanthus amarus contains numerous lignan derivatives such as phyllanthin (0.5%), hypophyllanthin (0.2%), nyrphyllin, nirurin, phyllnirurin, hydroxyl niranthin, lintetralin, seco-4 hydroxyl lintetralin and seco-isolaricresinoltrimethyl ether. Two Indolizidine alkaloid, epi-bubbialine (0.010%) and iso-bubbialine (0.012%) have been reported from the aerial parts of the herb. Two new alkaloidal compounds entnorsecurinine, 4-methyl norsecurinine have also been reported [16]. The herb also contains hydrolysable tannins like phyllanthusiin-D, amariin, amarulone, amarinic acid, furosin, corilagin and elaeocarpusin [17,18]. Tetracyclic triterpenoids such as phyllanthanol, phyllanthanone and phyllanthiol have also been reported from the herb. Foo isolated and identified a number of compounds from the aerial parts of *Phyllanthus amarus* like, tannin, amarin, corilagin, geranin (1.02702%); benzenoid, gallic acid (0.08648%), 1-6-d-galloyl-glucopyranose (00.00189%); flavonoid, (+) gallocatechin (0.00675%); flavonol, isoquercitrin (0.01216%) and rutin (0.13783) **(Table 1).**

Table 1. Phytochemicals in *Phyllanthus amarus.*

Secondary Metabolites	Phytochemicals
Alkaloids	Securinine, Norsecurinine, Epibubbialine and Isobubbialine, Phyllanthine, 4-Methoxy-NOR-Securinine, Dihydrosecurinine, Tetrahydrosecurinine, Securinol, Allo-Securine, 4-Methoxy Dihydrosecurinine & Phenazine [18]
Flavonoids	Catechin, Gallocatechin, Quercetin, Quercitoside, Rutin, Astragalin, Kaempferol, Quercetin-3-O-Glucoside & Quercitrin

Hydrolysable tannins (elagitannins)	Amariin, Amariinic acid, Amarulone, Corilagin, Elaeocarpusin, Furosin, Geraniin, Geraniinic acid B, Glucopyranose and Glucopyranoside derivatives, Phyllanthusiin D and Repandusinic acid
Lignans	Phyllanthin, Hypophyllanthin, Niranthin, Phyltetralin, Nirtetralin, Isonirtetralin, Hinokinin, Lintetralin, Isolintetralin, 5-Demethylenedioxy-Niranthin & 4,5-Demethoxy-Niranthin
Polyphenols	Ellagic acid, Phenazine and Phenazine derivatives [15]

Phyllanthin is a major bioactive component of *Phyllanthus amarus*, with several known biological activities. Phyllanthin in a solid state was found to undergo significant thermal decomposition above 200°C. The compound demonstrated good stability in aqueous solution over a pH range of 1.07-10.02 for at least 4h. The solubility of phyllanthin appeared to be pH-independent of pH range from 1.07 to 10.26.

Chemical properties of phyllanthin

Chemical name: 4-[(2S,3S)-3-[(3,4-dimethoxyphenyl)methyl]-4-methoxy-2(methoxymethyl)butyl]-1,2-dimethoxybenzene.

Molecular formula: $C_{24}H_{34}O_6$ **(Figure 3)**

Molecular weight: 418.529 g/mol

Density: 1.069

Log P: 3.2

Category: Anti-cancer.

Description: It is a white colour, odourless, crystalline powder.

Solubility: Soluble in organic solvents like chloroform, methanol, ethanol and poorly soluble in water.

Melting point: 96°C.

Storage: Store in a well closed, light resistant container.

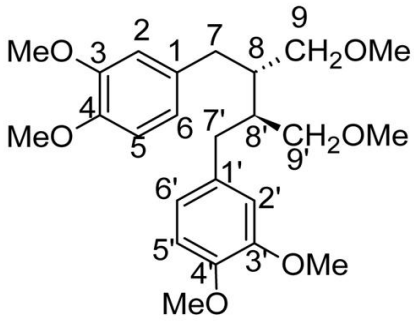

Figure 3. Chemical structure of Phyllanthin.

OBJECTIVE

Phyllanthin is an important chemical constituent found in all parts of *Phyllanthus amarus*. It has wide spectrum of pharmacological activities including antiviral, antibacterial, antiplasmodial, anti-inflammatory, antimalarial, antimicrobial, anticancer, antidiabetic, hypolipidemic, antioxidant, hepatoprotective, nephroprotective and diuretic properties. It is useful in the treatment of kidney problems, urinary bladder disturbances, diabetes, pain, gonorrhea, chronic dysentery & skin ulcers. Microwave-assisted extraction (MAE) technique has been used for the extraction of large number of herbal drugs and has been found to be effective and efficient technique as compared to the conventional methods. Thus looking at the potential of the microwave-assisted extraction technique, it was thought to develop optimized MAE protocol for an important bioactive compound phyllanthin for efficient and effective extraction procedure using central composite design.

The aim of our present study was to carry out:

a. Microwave-assisted extraction of bioactive compound phyllanthin from *Phyllanthus amarus* and optimization using central composite design.

b. Quantitative estimation of phyllanthin using high performance thin layer chromatography (HPTLC).

c. Comparison with conventional extraction techniques like maceration and soxhlation.

MATERIALS AND METHODS

Collection of the plant material

The leaves of *Phyllanthus amarus* were collected from the herbal garden of Maharshi Dayanand University, Rohtak in the

month of July-August and a voucher specimen no. MDU/Pharmacognosy/100/2014 was kept in the departmental research laboratory for future reference.

Drying and extraction by conventional methods

The leaves were shade dried at room temperature. Extraction was carried out by maceration and soxhlet extraction techniques.

Maceration

Extraction of drug was carried out by placing 10 g of coarsely powdered drug *(Phyllanthus amarus)* in a closed vessel at room temperature. Added 150 ml of methanol and allowed the extraction of drug for 7 days with occasional stirring. The liquid was filtered and the percentage yield of extract was calculated after completion of the extraction process.

Soxhlation

The 10 g powder of dried leaves of *Phyllanthus amarus* was placed in thimble holder. 150 ml of methanol was filled in distillation flask. The thimble was clogged with cotton in order to avoid transfer of sample particles to the distillation flask. The drug was extracted with methanol in soxhlet apparatus for 3 hours. The methanolic extract was filtered. After filtration the solution was evaporated on a water bath to give the methanolic extract. Percentage yield of extract was calculated as follows:

The percentage yield of extract = (weight of extract/weight of drug taken) × 100

Optimization of parameters by using experimental design

The variable parameters selected were solvent concentration, time and irradiation power. These parameters were optimized by using central composite design (CCD).

The variables that affect the extraction of phyllanthin from the leaves of *Phyllanthus amarus* were studied namely, "extraction time", "microwave irradiation power", "extracting solvent concentration". In a MAE procedure, extraction solvent is a key factor affecting the recovery of analytes. Mixed solvents of lower alcohol and water have been generally used for the extraction of lignans. Among the various alcohols, methanol was selected because it is widely acceptable during experimental studies and also permitted in pharmaceutical industries. Methanol concentration was varied in range from 50% to 80% (v/v). Microwave irradiation power ranged from 400 W to 1000 W. The chosen power limits were function of solvent and function of regulation limitations in the microwave apparatus. Based upon literature survey and several trials made in the laboratory, the extraction time was chosen from 2 to 4 minutes because during this period the maximum response was obtained. The upper and lower limits of the variables were selected on the basis of the previous experiments.

Optimization of MAE process of Phyllanthus amarus by CCD-RSM

A central composite design (CCD) with three variables was used to determine the response pattern and then to establish a model. Three variables used in this study were methanol concentration (X_1), irradiation power (X_2) and time (X_3) with five levels (-α, -1, 0, 1, +α) of each variable, the experimental factors and levels of coding were shown in **Table 2**, while the dependent variable was the yield of phyllanthin. In general, CCD is constructed in such a way that 2^k+2k+4 experiments were required where k represents the number of factors to be studied. Therefore, the eighteen experiments listed in Table 3 were performed. The experiments were randomly assigned to avoid unobserved error.

Table 2. Factors and levels for CCD test.

Level	Methanol conc. (% v/v) (X_1)	Irradiation power (%) (X_2)	Extraction time (min.) (X_3)
-α (-1.6818)	39.77	26.36	1.3
-1	50	40	2
0	65	60	3
1	80	80	4
+α (+1.6818)	90.23	93.64	4.7

The yield of phyllanthin was estimated by quadratic response surface model. A box Wilson procedure, commonly called central composite design (CCD), was used to evaluate the relevance of the three controlled factors (namely extraction time, irradiation power and solvent concentration). The multivariate study allows the identification of interactions between variables and provides a complete exploitation of the experimental domain to be studied with a reduced number of experiments.

The CCD comprise a two-level full factorial design, in which "cube points" (coded ±1), superimposed by centre points (coded 0) and "axial points" (coded ±α). The group of "axial points" axial experiments located at a distance α from the centre; allow rotatability **(Figure 4)**. They also establish new extremes for the low and high settings for all factors, allow estimation of experimental error and provide estimation of the curvature for the model. The precise value of α depends on the number of factors involved and on certain properties desired for the design. A CCD can be represented by a cube where each factors corresponds to an axis. The three key variables studied were pointed at five separate coded levels: -α (=-1.68), -1, 0, +1, +α (=1.68) and their values were selected on the basis of previous experiments. The statistical analysis of experimental results was performed by the software Design-Expert 8.0.7.1.

Microwave-assisted extraction

Microwave-assisted extraction was performed with a U-Wave-1000 Microwave-Ultraviolet-Ultrasonic synthesis 3-in-1 extraction reactor of SINEO Microwave Chemistry Technology Co., Ltd. The dried leaves of *Phyllanthus amarus* were crushed and screened through 24 mesh sieve. 5 g of powdered drug was transferred to a 100 ml 4-necked round bottom flask. 50 ml of 65% (v/v) methanol-water was added. The mixture was shaken well and kept for some time so that active constituents leached out in the solvent. In this extraction reactor, stirrer is also used for shaking the mixture. Place the flask with sample on the cushion block in the extraction reactor. Extraction was better when the flask kept in the microwave oven and treated for microwave process. The best suited combination obtained after central composite design were applied. Extraction time was set at 3 min. and irradiation power set at 600 W. After the extraction completed, flask was taken out from the oven and then filtered. Concentration of extract was then carried out on water bath and calculated the percentage yield (% w/w) **(Figure 5).**

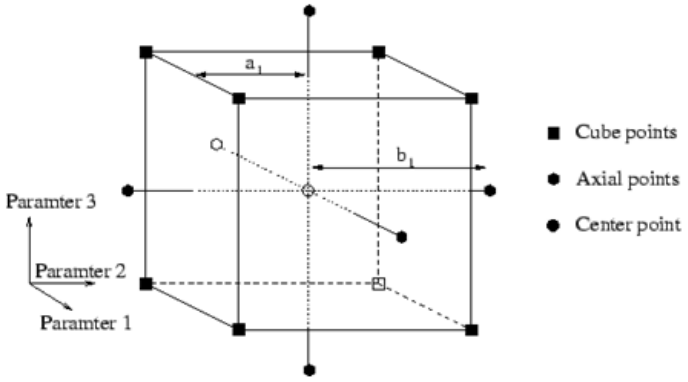

Figure 4. Distribution of experimental points in a three variable central composite design.

Figure 5. Sineo microwave oven.

Estimation of phyllanthin by high performance thin layer chromatography

Apparatus

A Camag HPTLC (High performance thin layer chromatography) system equipped with Camaglinomat 5 application system, TLC scanner 3 and integrated software WINCATS version 1.4.1 was used for analysis.

Mobile phase

A mixture of hexane & ethylacetate was used as mobile phase. The mobile phase was filtered using 0.45 µm filter. The mobile phase was degassed by sonication for 20 min. at 25°C and immediately used for HPTLC. The mobile phase (Hexane: Ethylacetate) was used in a ratio of 2:1 v/v.

Standard solution of drug

To make standard solution, 10 mg of standard phyllanthin was weighed and transferred to 10 ml volumetric flask. Volume was made up to mark with mobile phase to obtain a solution of 1000 µg/ml. Appropriate aliquots of stock solution of 1000 µg/ml were taken in a 10 ml volumetric flask and diluted up to the mark with mobile phase to obtain final concentrations 2, 3, 4, 5, 6, 7, 8 µg/ml of model drug.

Sample preparation

For the preparation of sample, 100 mg of extract of *Phyllanthus amarus* was dissolved in HPLC grade methanol to make the final volume of 10 ml (10 mg/ml).

Chromatographic conditions

Chromatography was performed on pre-activated (at 110°C) silica gel 60 F_{254} HPTLC plates (10×10 cm; 0.25 mm layer thickness). Samples and standard compounds were applied to the layer as 8 mm wide bands, positioned 10mm from the bottom

of the plate, using an automated TLC applicator Camaglinomat 5 with nitrogen flow providing delivery from the syringe at a speed of 10 s/µl. These critical parameters were maintained for all analysis performed.

Detection and analysis of compounds

The development of the TLC layer was performed using a Camag twin trough glass tank which had been pre-saturated with mobile phase of hexane: ethylacetate (2:1 v/v). The optimized chamber saturation time for mobile phase was 20 min. at room temperature (25°C). The solvent front was allowed to run to a height of 8cm. The composition of mobile phase was made under laboratory conditions of 25°C and 50% relative humidity. Subsequent to the development, remove the plates from the chamber, dry, spray with the spray reagent, heat for 3 min. at 120°C and examine under visible light. Densitometric scanning was performed on a Camag TLC scanner 3 in the absorbance mode at 254 nm. The source of radiation utilized was a deuterium lamp. The peaks obtained in the HPTLC chromatogram were analyzed based on R_f value. The equivalent R_f values of test samples and marker compound were confirmed through absorption spectra.

Quantitative analysis

Quantitative analysis was carried out by calibration curve method. Different concentrations of standard were prepared and run as mentioned above and developed same as above. The plates were analysed densitometrically and area under curve was considered for quantification of phyllanthin in the samples.

RESULTS

Collection of the plant material

Phyllanthus amarus leaves were collected from the herbal garden of Maharshi Dayanand University, Rohtak in the month of July-August and a voucher specimen no. MDU/Pharmacognosy/100/2014 was kept in the departmental research laboratory for future reference.

Extraction by conventional methods

The percentage yield of *P. amarus* leaves obtained from maceration and soxhlation processes were found to be 14% and 6% w/w respectively.

Optimization of MAE using central composite design

Three variables that affect extraction of phyllanthin from leaves of *P. amarus* were studied: namely, "methanol concentration", "microwave irradiation power" and "extraction time". These key variables were involved in a central composite design in order to evaluate, optimize and conduct microwave-assisted extraction of phyllanthin from leaves of *P. amarus*. Microwave irradiation power ranged from 400 W to 1000 W. The chosen power limits were function of solvent use and function of regulation limitations in the microwave apparatus. The extraction time range chosen from 2 to 4 minutes were relatively short yet competitive with conventional extraction. These three controlled variables were studied in a multivariate study with 18 experiments as shown in the **Table 3.**

Table 3. Fully coded central composite design matrix of three variables and experimental results from response variables.

Run Order	Methanol conc. (% v/v)	Irradiation power (%)	Extraction time (min.)	Extract yield (% w/w)
1	-1(50)	-1(40)	-1(2)	16
2	+1(80)	-1(40)	-1(2)	16
3	-1(50)	+1(80)	-1(2)	12
4	+1(80)	+1(80)	-1(2)	14
5	-1(50)	-1(40)	+1(4)	10
6	+1(80)	-1(40)	+1(4)	6
7	-1(50)	+1(80)	+1(4)	6
8	+1(80	+1(80)	+1(4)	12
9	-1.682(39.77)	0(60)	0(3)	10
10	+1.682(90.23)	0(60)	0(3)	14
11	0(65)	-1.682(26.36*)	0(3)	12
12	0(65)	+1.682(93.64**)	0(3)	14
13	0(65)	0(60)	-1.682(1.3)	14
14	0(65)	0(60)	+1.682(4.7)	10
15	0(65)	0(60)	0(3)	18
16	0(65)	0(60)	0(3)	16
17	0(65)	0(60)	0(3)	16
18	0(65)	0(60)	0(3)	14

*40 was the irradiation power in the microwave oven
**100 was the irradiation power in the microwave oven

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Values in brackets indicate the real values.

An analysis of variance (ANOVA) was carried out in order to test the model signification and suitability. Thus, various statistical data such as standard error, sum of squares, F-ratio or p-value are given in ANOVA **(Table 4).**

Table 4. ANOVA (Analysis of variance) model statistics.

Source	Sum of squares	Df	Mean square	F-value	p-value Prob>F
A-A	8.43	1	8.43	2.01	0.1944
B-B	0.030	1	0.030	7.061E-003	0.9351
C-C	69.13	1	69.13	16.46	0.0036
AB	18.00	1	18.00	4.29	0.0722
AC	2.842E-014	1	2.842E-014	6.767E-015	1.0000
BC	8.00	1	8.00	1.90	0.2049
A2	30.25	1	30.25	7.20	0.0278
B2	18.00	1	18.00	4.29	0.0722
C2	30.25	1	30.25	7.20	0.0278
Residual	33.60	8	4.20		
Lack of Fit	25.60	5	5.12	1.92	0.3136 (not significant)
Pure Error	8.00	3	2.67		
Cor Total	193.11	17			
Model	159.51	9	17.72	4.22	0.0274 (significant)
Standard Deviation		2.05			
R-Squared		0.8260			
Adjusted R-Squared		0.6303			
ANOVA for Response Surface Reduced Cubic Model where: A: Methanol concentration; B: Irradiation power; C: Extraction time					

The Model F-value of 4.22 implies the model is significant. There is only a 2.74% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case C, A_2, C_2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The estimated model, therefore, can be used as a response surface for percentage yield of drug **(Figures 6 and 7).**

The "Lack of Fit F-value" of 1.92 implies the Lack of Fit is not significant relative to the pure error. There is a 31.36% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good. We want the model to fit. The optimum regression equation of phyllanthin is as follows:

$$Y = 16.03 + 0.79X_1 - 0.047X_2 - 2.25X_3 + 1.50X_1X_2 + 0.00X_1X_3 + 1.00X_2X_3 - 1.55X_1^2 - 1.19X_2^2 - 1.55X_3^2$$

(Where X_1 is methanol conc., X_2 is irradiation power, X_3 is extraction time).

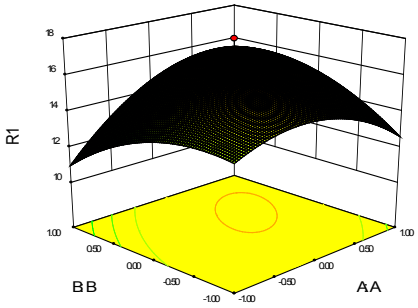

Figure 6. 3D view of response surfaces from central composite design.

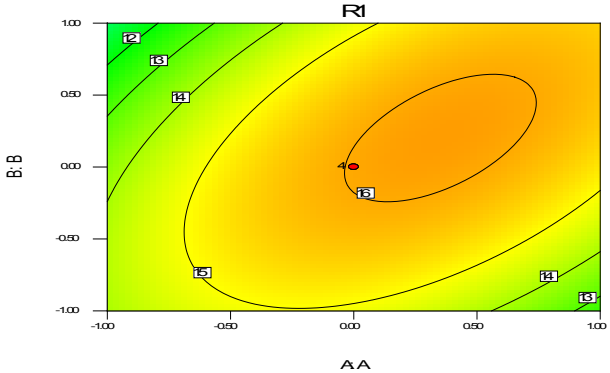

Figure 7. 2D-contour view of response surfaces from central composite design.

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For solving the equation, the Design-Expert 8.0.7.1. was used. The maximum yield computed by the software is calculated by the maximum value of the surface response for a set of variables lying between the minimum and maximum value of the CCD plan.

A pareto chart of standardized effect **(Figure 8)** was carried out in order to show significant of all variables (linear, quadratic and interactions between variables).

A = Methanol concentration

B = Irradiation power

C = Extraction time

The length of bars is proportional to the absolute magnitude of the estimated effects coefficients. It can be seen from the chart that extraction time has the most important influence on yields followed by methanol concentration and irradiation power.

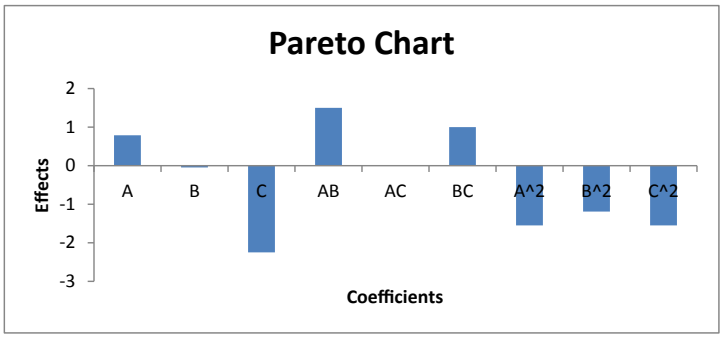

Figure 8. Standardized Pareto Chart.

HPTLC studies

High Performance Thin Layer Chromatography (HPTLC) is primarily used as an inexpensive method for separation, for qualitative identification, or for the semi quantitative visual analysis of samples. The HPTLC analysis of extract of *Phyllanthus amarus* was performed for the estimation of phyllanthin content in the test samples. The R_f value of one of the component of extract matches with standard phyllanthin **(Figures 9-14)** which shows that phyllanthin is present in these extracts. Further confirmation was done on the basis of overlapped UV spectra of the corresponding peaks.

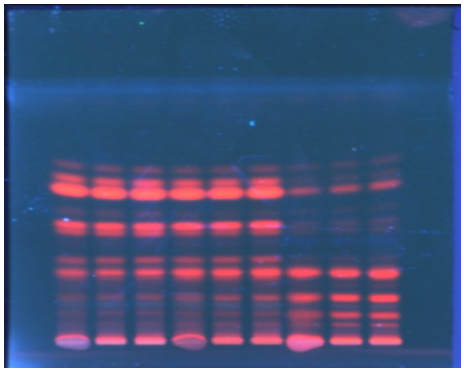

Figure 9. Compounds eluted from *P. amarus* extract shown at 366 nm.

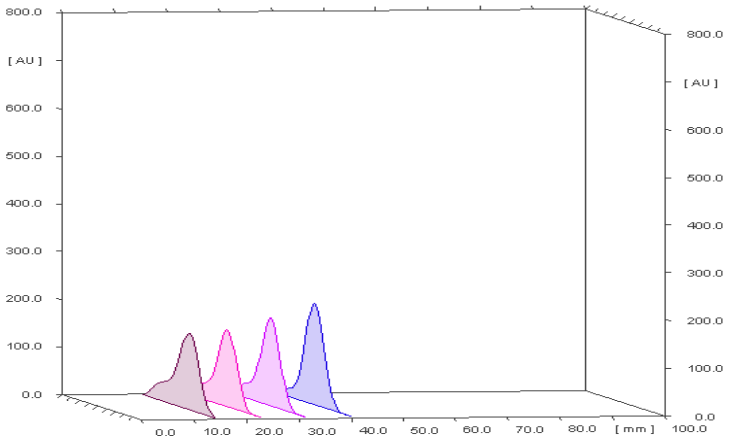

Figure 10. Three dimensional presentations of standard marker compound phyllanthin in *Phyllanthus amarus* extract at 366 nm.

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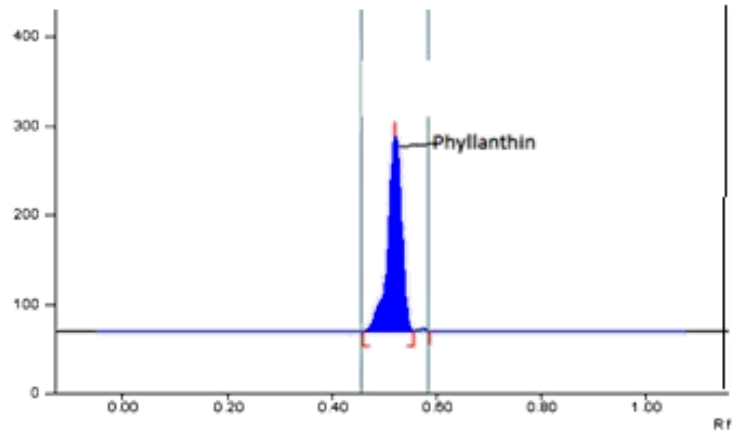

Figure 11. HPTLC chromatogram of the standard marker compound phyllanthin at 366 nm.

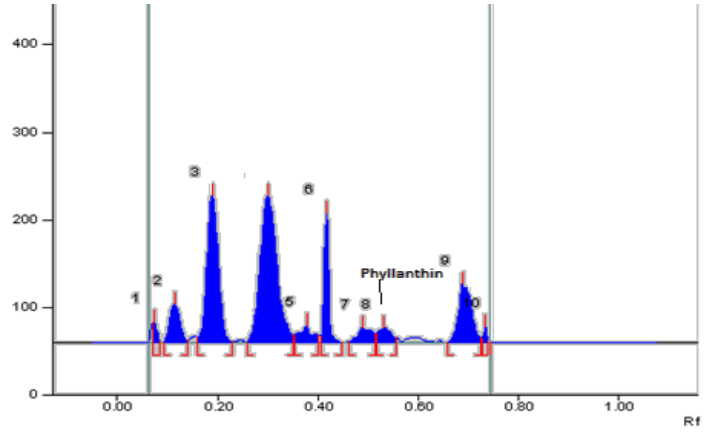

Figure 12. HPTLCchromatogram of the *P. amarus* extract obtained by MAE at 366 nm.

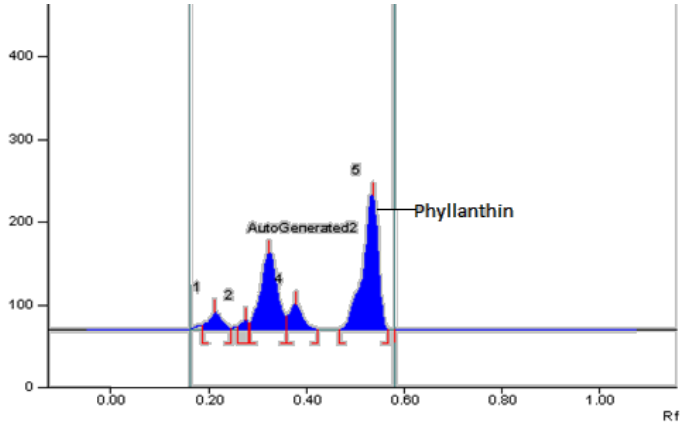

Figure 13. HPTLC chromatogramof the *P. amarus* extract obtained by soxhlation at 366 nm.

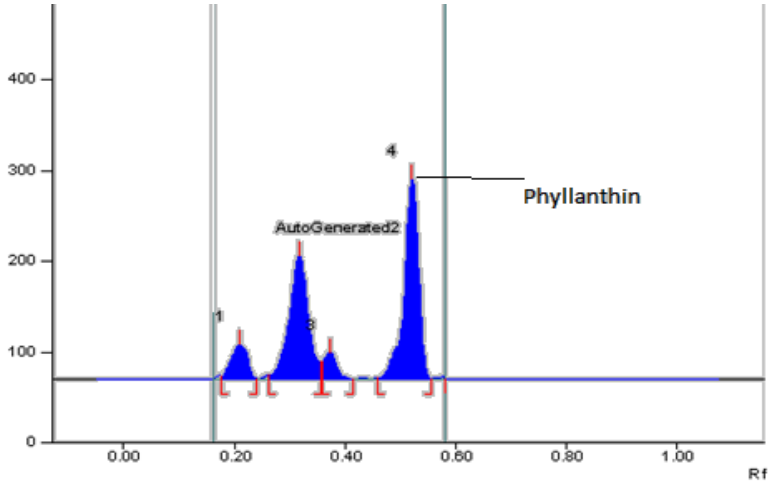

Figure 14. HPTLC chromatogram of the *P. amarus* extract obtained by maceration at 366 nm.

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The peak of the extract of *Phyllanthus amarus* matches with standard phyllanthin was confirmed using spectra comparison. **Figure 15** shows the spectral comparison of phyllanthin with extract of *Phyllanthus amarus*. The absorption pattern of standard phyllanthin and one of the components of the extract of *Phyllanthus amarus* coincide, which confirms the presence of phyllanthin in the extract. The maximum value of the phyllanthin was found to be 366 nm which also matched with standard.

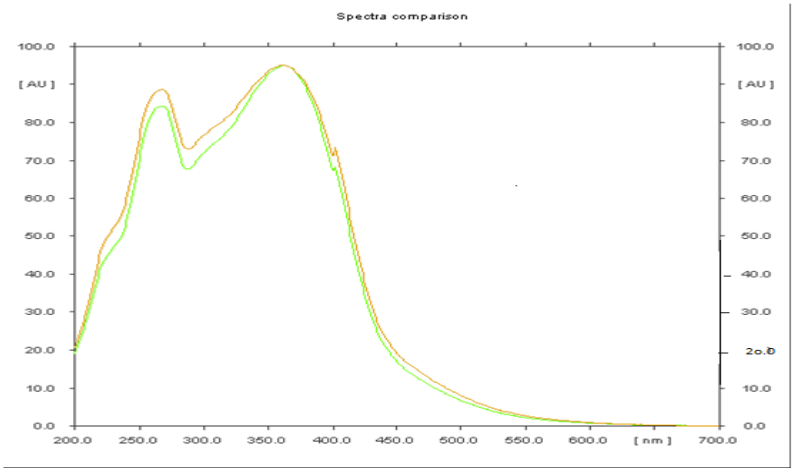

Figure 15. Overlapped UV spectra of standard phyllanthin and sample at 366 nm.

Comparison of MAE with conventional methods

In order to compare the extraction efficiency with MAE, maceration and soxhlation extraction methods were chosen as comparative methods.

Table 5. Extraction yield of phyllanthin of different extraction methods.

Extraction methods	Yield of phyllanthin (% w/w)	Yield of extract (% w/w)	Extraction time (min.)
Maceration	0.023%	14%	7 days
Soxhlation	0.026%	6%	3 hours
MAE	0.063%	18%	3 min.

During the experiments, the amount of sample and HPTLC analysis were kept as the same as that in MAE. The results are shown in Table 5. It can be seen that the extraction yield of phyllanthin was found to be much higher than other two procedures and the time taken by MAE for 3 min is much lower than that of maceration for 7 days and soxhlation for 3 hours. Therefore, MAE was found to be most efficient extraction method as compared with the other conventional methods. It can also be seen that among three extraction methods, MAE can be carried out not only in the shortest time but also in the lowest temperature; therefore the allied components of extraction by MAE might possess higher bio-activity and purity.

DISCUSSION AND CONCLUSION

In the present study, a two level central composite design (CCD) was employed for optimization of phyllanthin from *Phyllanthus amarus.* The heating time, concentration of extracting solvent and irradiation power were the optimized variables with the constant sample amount. Central composite design is the most popular design for second order model. By using the central composite design method we can apply the variable parameters in MAE in a scientifically approved manner. It is a powerful tool provides the optimum conditions that improve a process [7]. Based upon central composite design or optimized conditions, microwave-assisted extraction of *Phyllanthus amarus* was performed.

The results showed that microwave-assisted extraction of *Phyllanthus amarus* at 65% methanol concentration, 60% irradiation power and 3 minute extraction time produced higher percentage yield of extract. In order to test the model signification and suitability, an analysis of variance (ANOVA) was carried out. The model F-value of 4.22 and the “Lack of Fit F-value” of 1.92 implies that the model is significant. The estimated model, therefore, can be used as a response surface for percentage yield of drug.

Then a pareto chart of standardized effects were plotted in order to show significant effects of all variables and interactions between variables. The length of the bars in pareto chart is proportional to the estimated effects of variables on extraction yield. It can be concluded from the chart that the extraction time has a large influence on yield of phyllanthin followed by methanol concentration and irradiation time.

The content of phyllanthin was determined by high performance thin layer chromatography (HPTLC). HPTLC method is simple, rapid and accurate. The extracts of *Phyllanthus amarus* were prepared by MAE, soxhlation and maceration. Standardization of extracts was carried out by using phyllanthin as a standard marker. The chromatograms of extracts and standard phyllanthin were developed by using solvent system hexane: ethylacetate (2:1, v/v). The presence of phyllanthin in microwave-assisted extraction, soxhlation and maceration extract was found to be 0.063, 0.023 & 0.026% w/w respectively.

The results obtained during the study indicate that the microwave-assisted extraction method can be used as promising tool for extraction of phyllanthin from *Phyllanthus amarus* because of high yield and fast extraction ability with less solvent and time consumption.

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