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

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Abstract
Phyllanthus amarus has a long history of use in traditional medicine and phyllanthin is one of its potent compounds. Although various extraction methods are available but newer methods with increased efficiency are needed in the modern era. The objective of this study was to predict the optimum conditions for microwave-assisted extraction (MAE) of phyllanthin from *Phyllanthus amarus* using response surface methodology (RSM). A central composite design was applied to determine the effect of extraction time, irradiation power and methanol concentration on yield of phyllanthin. Optimum extraction conditions were predicted as 65% methanol concentration, 60% irradiation power and 3 minute extraction time. The phyllanthin content of 0.063% w/w was obtained which was higher than that of soxhlet and maceration extraction. The results were analysed statistically using one way analysis of variance (ANOVA).

Keywords: Microwave-assisted extraction; Response surface methodology; Optimization; HPTLC; Phyllanthin; *Phyllanthus amarus*

1. Introduction

Phyllanthus amarus is commonly known as Bhumi amla and it mainly contains phyllanthin and hypophyllanthin as active ingredients [1]. *Phyllanthus amarus* has about 750-800 species found in tropical and subtropical countries of the world including in India [2]. It is most commonly used in the Indian Ayurvedic system of medicine in problem of stomach, gentiurinary system, liver, kidney and spleen [3, 4]. *Phyllanthus amarus* is a plant of the family Euphorbiaceae. The plant has been found in Philippine, Cuba, Nigeria and among others.[5] It is commonly called 'stone breaker', 'carry me seed', 'windbreaker', 'gulf leaf flower', or 'gala of wind'.[6, 7] Phytochemical studies have shown the presence of many valuable compounds such as lignans, flavonoids, hydrolysable tannins, polyphenols, triterpenes, sterols & alkaloids [8, 9, 10].

In many countries around the world plants in the genus Phyllanthus are used in folk remedies; therefore this genus is of great importance in traditional medicine [11]. In a number of countries, the aerial part of *Phyllanthus amarus* is highly valued in traditional medicine for its healing properties. This plant is traditionally used around the world in the treatment of liver ailments and kidney stones. *Phyllanthus amarus* has also shown to work as an antifungal, antibacterial and antiviral agent [12]. *P.amarus* is always sold as fresh and dry plant material in the herb markets. In traditional medicine an herbal decoction is taken to treat bladder and kidney disorders, cramps and uterus complaints (with other herbs) [13, 15]. This plant decoction works also as an appetizer. Pharmacological screening revealed that phyllanthin is a hepatoprotective [16, 17]. antioxidant, [18] antihyperuricemic, antimicrobial,[19] antigenotoxic, [20] anti-inflammatory, [21] and vasorelaxant compound [22].

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.

Solid-liquid extraction with methanol is the conventional method used to recover phyllanthin from *Phyllanthus amarus;* however, this procedure needs several purification steps, is time consuming, thermally unsafe and requires a large volume of solvent. Hence with the increasing demand for environmental friendly methods, Microwave assisted extraction has been developed and optimized for the fast extraction of phyllanthin [23]. Microwave assisted extraction has received increasing attention as a potential alternative to traditional solid-liquid extraction methods, mainly due to considerable saving in processing time and solvent consumption. Microwave-assisted extraction (MAE) or simply microwave extraction is a relatively new extraction technique that combines microwave and traditional solvent extraction. In the microwave heating process, energy transfer occurs by two mechanisms: dipole rotation and ionic conduction through reversals of dipoles and displacement of charged ions present in the solute and the solvent [24]. MAE is a process of using microwave energy to heat solvent in contact with a sample in order to partition analytes from the sample matrix in to the solvent [25]. Recently, many reports have been made on the application of MAE on the extraction of natural products, such as sennosides from senna leaflets [26]. antioxidants from potato peels [27]. pectin from apple pomace [28]. flavonoid from *Radix astragali* [29]. However, no information has yet been found on the application of MAE for the extraction of phyllanthin from *Phyllanthus amarus.*

Response surface methodology is an affective technique for optimizing complex processes. It is widely used in optimizing the process variables and it is better acknowledged than traditional one. RSM is a collection of mathematical and

statistical techniques useful for the modeling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize this response [30]. An experiment is a series of tests, called runs, in which changes are made in the input variables in order to identify the reasons for changes in the output response. The most common applications of RSM are in industrial, biological and clinical, social, food, and physical and engineering sciences. Since RSM has an extensive application in the real-world [31].

The present study describes the development and optimization of rapid, reliable and sensitive methods of extraction of phyllanthin from leaves of *Phyllanthus amarus* using MAE and its comparison with conventional methods of extraction for amount of phyllanthin.

2. Materials and methods

2.1 Plant materials

The leaves of *Phyllanthus amarus* were collected from the herbal garden of Maharshi Dayanand University, Rohtak in the month of July-August. The leaves were dried at room temperature in shade, powdered and sieved through mesh size #24 immediately before the experiment. Voucher specimen no. MDU/Pharmacognosy/100/2014 was kept in the departmental research laboratory for future reference.

2.2 Reagents

Analytical grade methanol (Spectrochem Pvt. Ltd.) was used for extraction. Hexane, ethylacetate & methanol used in HPTLC analysis were all of HPLC grade. Pre-activated silica gel $60F_{254}$ plates for HPTLC analysis were from E. Merck. Standard phyllanthin was obtained from Sigma Chemicals.

2.3 Apparatus

Microwave extraction experiments were performed in a U-Wave-1000 Microwave-Ultraviolet- Ultrasonic synthesis 3-in-1 extraction reactor of SINEO Microwave Chemistry Technology Co., Ltd. equipped with 0-1000W power levels to give maximum flexibility and control for extraction. For conventional extraction soxhlet apparatus was used. A Camag HPTLC (High performance thin layer chromatography) system equipped with Camag Linomat 5 applicator system, TLC scanner 3 and integrated software WINCATS version 1.4.1 was used for the analysis.

2.4 Conventional extraction techniques

Soxhlet extraction and maceration were used as conventional methods of extraction and they were compared with MAE.

2.5 Maceration

Extraction of drug was carried out by placing 10g 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.

2.6 Soxhlet extraction

The 10g powder of dried leaves of *Phyllanthus amarus* was placed in thimble holder. 150ml 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.

2.7 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 gm 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 600W. 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).

2.8 Experimental design

Central composite design was used in order to optimize the dependent variable (Phyllanthin yield) as a response while the independent variables chosen were extraction time (2 to 4 mins.), methanol concentration (50 to 80% v/v) & microwave irradiation power (400 to 1000W).The coded levels of the independent variables are given in Table 1. According to this design the total no. of treatment combinations were calculated as 2^k+2k+4 where k is the no. of factors to be studied. Therefore, the eighteen experiments were obtained for the optimization of extraction procedure for the enhancement of phyllanthin yield.

The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). This analysis included the Fisher's F-test (overall model significance), its associated probability P(F), correlation coefficient R, and determination coefficient R^2 which measures the goodness of fit of regression model. It also includes the Student's t-value for the estimated coefficients and associated probabilities, P (t). The relative effect of each process parameter on individual response was compared from the β values corresponding to that parameter. The quadratic models were represented as response surface graphs, which gives infinite number of combinations of the two factors selected keeping the other constant. The optimization of the process was aimed at finding the optimum values of independent variables (power, time & methanol concentration), which would give maximum phyllanthin yield. The optimum values of the selected variables were obtained by solving the regression equation. For solving the equation, the Design-Expert 8.0.7.1. was used.

2.9 Quantitative analysis method

High performance thin layer chromatography was used for quantitative analysis of phyllanthin. 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). Subsequent to the development, TLC plates were dried with the help of an air dryer. 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 HPTLC chromatogram were analysed based on R_f value. The equivalent R_f values of test samples and marker compound were confirmed through absorption spectra [32].

Table 1: Factors and levels for CCD test

Level	Methanol conc.(%v/v) (X1)	Irradiation power (%) (X2)	Extraction time (min.) (X3)
-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

3. Results and discussion

The experimental data of the process variables for yield of phyllanthin under different extraction conditions are shown in Table 2. After the response surface regression procedure, the results of the analysis of variance, regression coefficient, along with the corresponding P-value, and the adequacy for the models of phyllanthin yield from the *Phyllanthus amarus* showed that the model data could adequately predict the experimental phyllanthin yield. The analysis of variance showed that this regression model was highly significant with an F-value of 4.22, implying a good fit between the predicted model and the experimental data. The value of 1.92 for lack of fit implied that it is not significant relative to the pure error, but non-significant lack of fit is good. The yield of phyllanthin changed significantly with all the quadratic term and linear coefficients. The importance of the independent variables on the yield could be ranked in the following order: methanol concentration (A) > irradiation power (B) > extraction time (C) according to the F-value of analysis of variance (Table 3). The results were analysed by polynomial quadratic regression method which describes the effect of variables in the model derivatized. The regression coefficients were obtained after fitting the experimental data in the selected model given in Table 3. The individual effect of each variable and also the effect of the interaction terms in coded level of variables were determined by polynomial quadratic equation.

$Y = 16.03 + 0.79X_1 - 0.047X_2 - 2.25X_3 + 1.50X_1X_2 + 0.00X_1X_3 + 1.00X_2X_3 - 1.55X_1^2 - 1.19X_2^2 - 1.55X_3^2$ (where X_1 is methanol conc., X_2 is irradiation power, X_3 is extraction time)

The goodness of fit for the model was expressed by the coefficient of determination R^2 and was found to be 0.989, indicating that 98.9% of the variability in the response could be explained by the model. This suggests that the predicted values exhibit a good correlation with the experimental data and that the model is suitable and practicable.

Table 2: 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

Table 3: ANOVA (Analysis of variance) model statistics

Source	Sumof 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			

3.1 Verification of models

In order to optimize the process conditions for yield of phyllanthin extraction, equal importance was given to all the process variables and response (% yield). The optimum operating conditions for yield of phyllanthin extraction were of irradiation power 600W, time 3 min & methanol concentration 65% v/v.

3.2 Interaction between factors influencing yield of phyllanthin

Three dimensional response surface (Fig.2) and contour (2D) plots (Fig.3) for responses were generated to study the effect of independent variables and their interactions on phyllanthin yield according to the results of regression equations. A pareto chart of standardized effect (Fig.4) was carried out in order to show significant of all variables (linear, quadratic and interactions between variables). 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.

3.3 HPTLC analysis

The HPTLC analysis of extract of *Phyllanthus amarus* was performed for the estimation of phyllanthin content in the test samples. The plates were analyzed densitometrically and area under curve was considered for quantification of phyllanthin in the samples. Quantitative analysis was carried out by calibration curve method. For that purpose, standard plot of phyllanthin was plotted at different concentrations (Fig.5). The R^2 value = 0.989. From the regression equation the content of phyllanthin was calculated. HPTLC chromatogram of the standard marker compound phyllanthin at 366 nm (Fig.6). Figure 7, 8 and 9 has shown the HPTLC chromatograms of the *P.amarus* extracts obtained by MAE, soxhlation and maceration at 366 nm. The peak of extract of *Phyllanthus amarus* matches with standard phyllanthin was confirmed using spectra comparison. Figure 10 shows the spectral comparison of phyllanthin with extract of *Phyllanthus amarus*. The absorption pattern of standard phyllanthin and one of the components of extract of *Phyllanthus amarus* coincide, which confirms the presence of phyllanthin in the extract. The λmax value of the phyllanthin was found to be 366 nm which also matched with standard literature. The phyllanthin content in microwave assisted extraction was found as 0.063 % w/w.

3.4 Comparison of MAE with conventional methods

During the experiments, the amount of sample and HPTLC analysis were kept as the same as that in MAE. It can be seen in Table 4 that the extractive values and phyllanthin content 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. MAE is a good alternative to soxhlet and maceration method for sample preparation. Therefore, MAE was found to be the most efficient extraction methods as compared with the other conventional methods. It also can 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.

Table 4: 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.

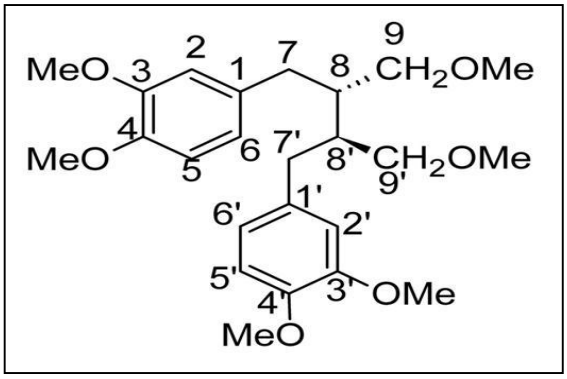

Fig 1: Chemical structure of Phyllanthin

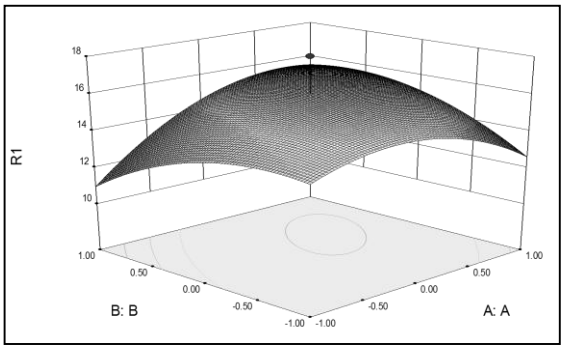

Fig 2: 3D view of response surfaces from central composite design

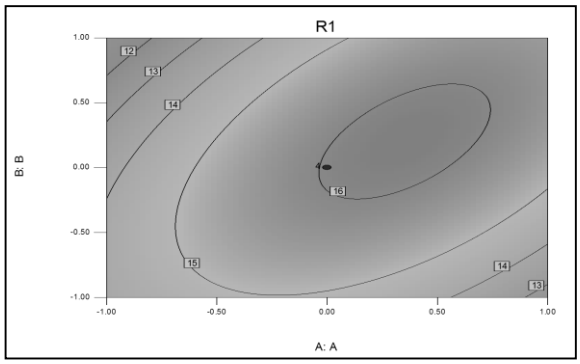

Fig 3: 2D- contour view of response surfaces from central composite design

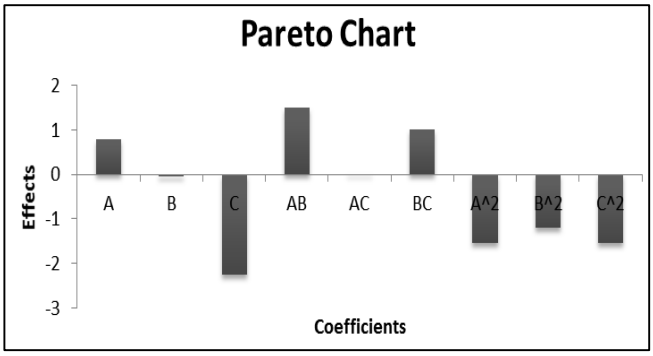

Fig 4: Standardized Pareto chart

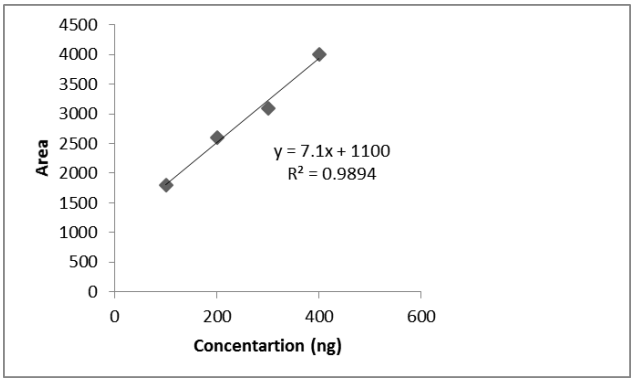

Fig 5: Standard plot of phyllanthin by HPTLC

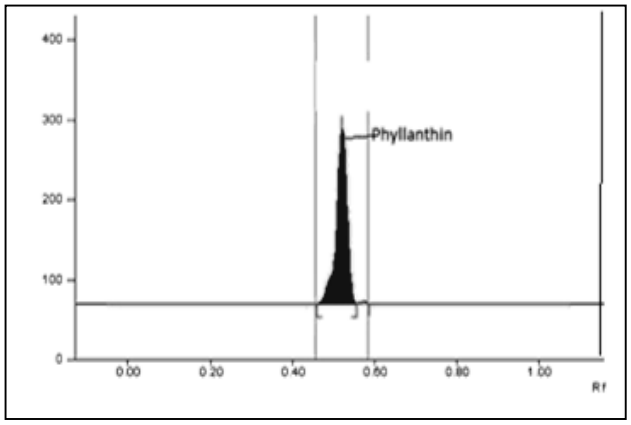

Fig 6: HPTLC chromatogram of the standard marker compound phyllanthin at 366 nm

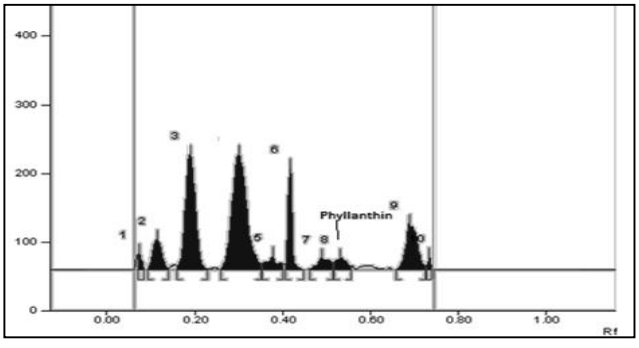

Fig 7: HPTLC chromatogram of the *P.amarus* extract obtained by MAE at 366 nm

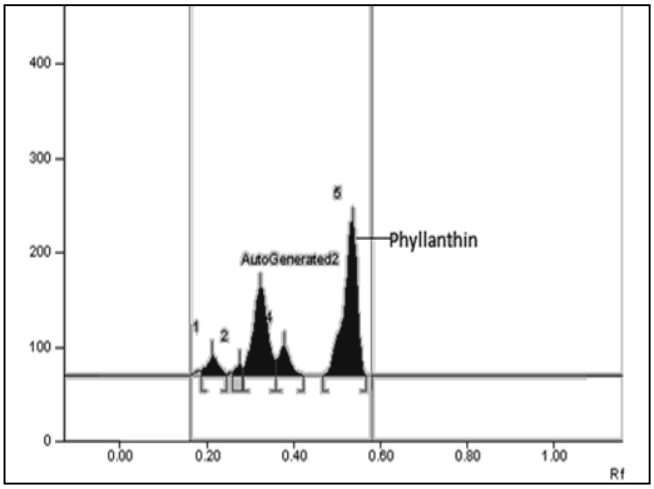

Fig 8: HPTLC chromatogram of the *P.amarus* extract obtained by soxhlation at 366 nm

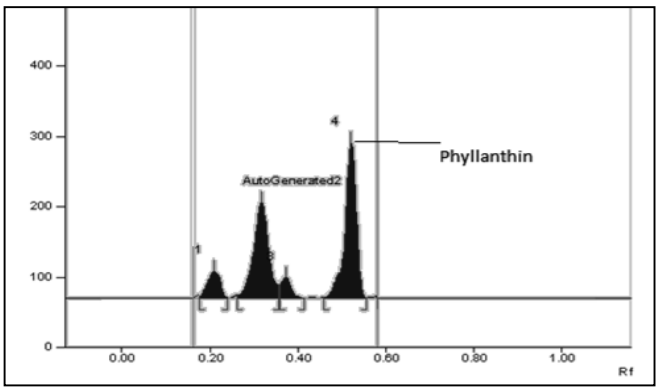

Fig 9: HPTLC chromatogram of the *P.amarus* extract obtained by maceration at 366 nm

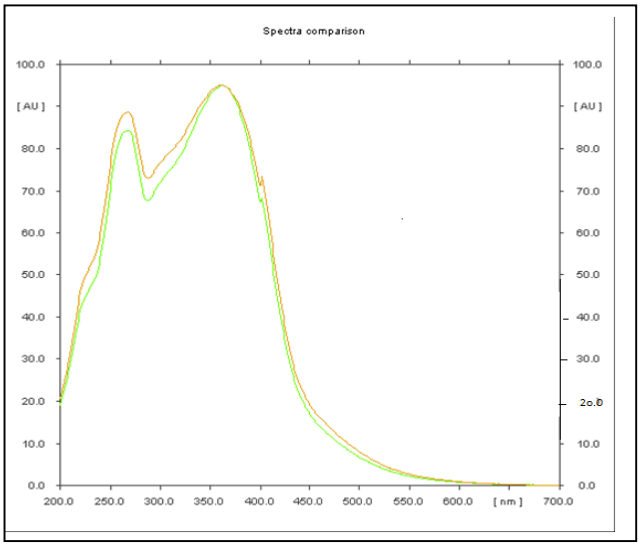

Fig 10: Overlapped UV spectra of standard phyllanthin and sample at 366 nm

4. Conclusion

The response surface methodology was successfully employed to optimize the phyllanthin extraction from *Phyllanthus amarus*. The second-order polynomial model gave a satisfactory description of the experimental data. An optimized condition for extraction of phyllanthin was determined.

Extraction time and methanol concentration were the most important factors affecting extraction. The optimal predicted phyllanthin content of 0.063%w/w was obtained when the extraction conditions were irradiation power 60%, methanol concentration 65% and extraction time 3 minutes. Comparing with the conventional methods MAE resulted in good yield with high efficiency which indicated it an effective alternative to sample preparation of *Phyllanthus amarus* and promising development for industrial extraction processes.

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