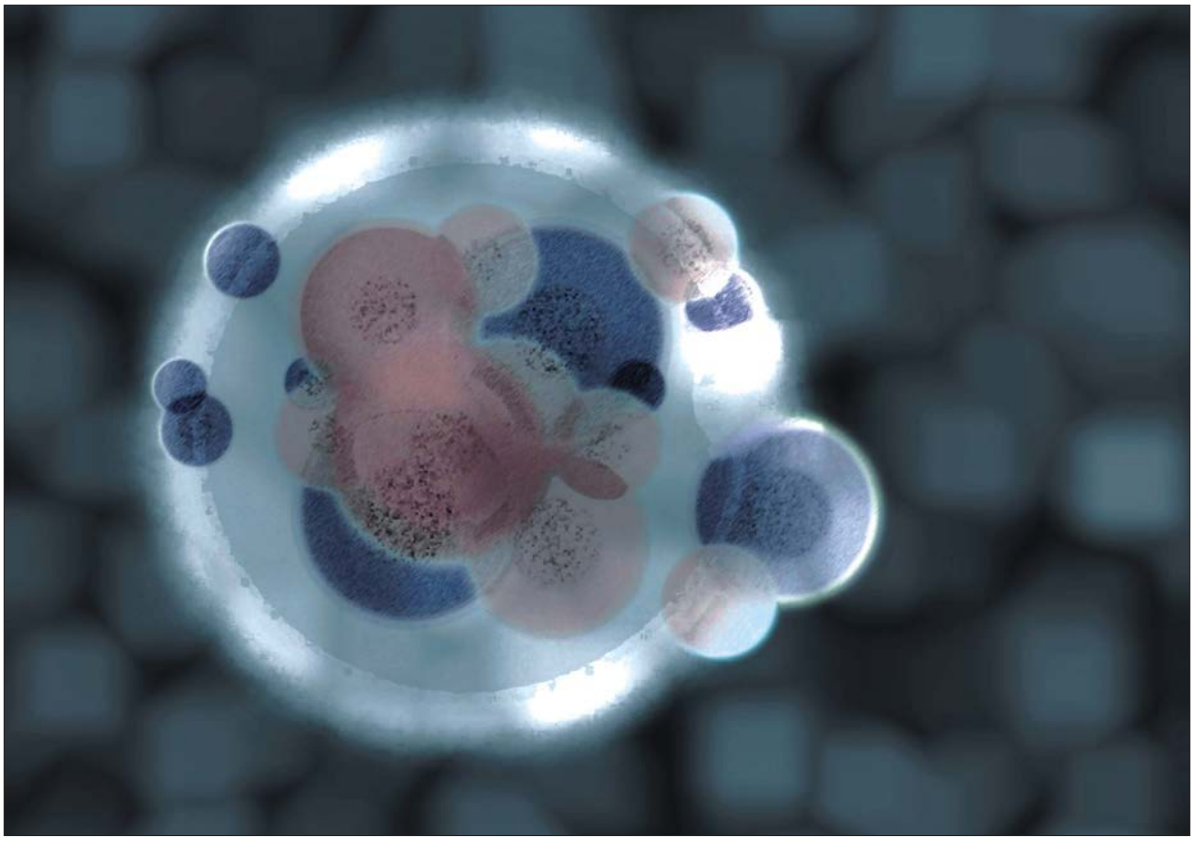

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Microwave Assisted Extraction of *Tinospora cordifolia* and Optimization through Central Composite Design

Saurabh Satija, Preeti Bansal, Harish Dureja and Munish Garg
Department of Pharmaceutical Sciences, Maharshi Dayanand University, Rohtak, Haryana, 124001, India

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Corresponding Author:
Munish Garg
Department of Pharmaceutical Sciences,
Maharshi Dayanand University,
Rohtak, 124001, Haryana, India
Tel: +919812588857

ABSTRACT
Present study was conducted to develop a new optimized Microwave-Assisted Extraction (MAE) method for *Tinospora cordifolia* in order to improve the efficiency and yield of chief bioactive compounds. Stems of the *Tinospora cordifolia* were subjected for extraction using MAE technique using three variable factors (extraction time, irradiation power and solvent concentration) optimized through central composite design. Berberine which was used, as marker was estimated in prepared extract by High Performance Thin Layer Chromatography (HPTLC) and compared with extracts prepared by conventional techniques like maceration and soxhlation. The results revealed that MAE of *Tinospora cordifolia* at 60% irradiation power, 80% ethanol concentration and at 3 min extraction time produced highest extract yield (91.3% better yield than maceration and 25.7% than soxhlation) as well as berberine content (492.8% better than maceration and 59.6% than soxhlation) as compared to extracts prepared with conventional techniques. Efficiency of the MAE method was considerably better than the conventional procedures, especially in terms of shortening extraction time (3 min as compared to 3 h for soxhlation and 7 days fort maceration), reduction of solvent used and energy consumption. The optimized microwave extraction method can provide a valuable extraction alternative of *Tinospora cordifolia* stem at industrial scale.

Key words: Berberine, HPTLC, irradiation power, quantitative analysis

INTRODUCTION

Extraction is an important step in the itinerary of phytochemical processing for the discovery of bioactive constituents from plant materials. Selection of a suitable extraction technique is also important for the standardization of herbal products, as it is utilized in the removal of desirable soluble constituents, leaving out those not required with the aid of the solvents. Further, selection of suitable extraction process and optimization of various parameters are critical for upscaling purposes i.e., from bench scale to pilot plant level. Various extraction techniques most commonly used include conventional techniques, such as; maceration, percolation, infusion, decoction, hot continuous extraction and soxhlet extraction etc. Recently, alternative methods like ultrasound assisted solvent extraction (USE), microwave assisted solvent extraction (MAE), Accelerated Solvent Extraction (ASE) and Supercritical Fluid Extractions (SFE) have gained increasing interest during the last three decades (Co *et al*., 2012) but associated with certain limitations like during USE, ultrasound waves affect the extraction yield and during ASE high temperature leads to degradation of thermolabile constituents. Economics of the SFE processes has restricted the application to some very specialized fields, such as; essential oil extraction, coffee decaffeination etc. (Wang and Weller, 2006). Microwave Assisted Extraction (MAE) is a relatively new extraction technique have shown promising results with certain advantages over other techniques like drastic reduction in organic solvent consumption and extraction time (Delazar *et al*., 2012).

In MAE, the heating by microwaves is because of interaction of the radiation with the dielectric field associated with polar molecules and ions. The heated solvent accelerates, desorption of target compound from the matrix into the solvent (Kaufmann *et al*., 2001). The concerted forces applied by the electric and magnetic components of the microwave radiation are rapidly changing in direction ($2.4\text{-}10^9$ sec^{-1}) at a frequency of 2450 MHz, causing the heating of the polar molecules as these molecules try to orient themselves in the direction of the field. Because solids, semisolids and liquids cannot respond instantaneously to the changing directions of the microwave field, the friction among the molecules manifests itself as heat (Galema, 1997). The microwave region of electromagnetic spectrum lies between infrared and radio frequency waves with frequencies from 0.3-300 GHz. The frequencies of 2450 MHz and 915 Hz are generally, used frequencies in industries and domestic microwave oven (Sticher, 2008). The microwaves heating leads to expansion or rupture of cell walls and is followed by liberation of chemicals into the solvent (Eskilsson and Bjorklund, 2000). It is found impossible to perform a good MAE for completely dry as well as for very wet samples when a non polar solvent, such as; hexane is used as extraction solvent (Molins *et al.,* 1997). Thus optimization step is highly necessary to get good results through this technique.

Tinospora cordifolia (Common name-Guduchi), is a medicinal plant of the family Menispermaceae is a deciduous climbing shrub indigenous to tropical Indian subcontinent. *Tinospora cordifolia*, being a rasayana drug from Ayurveda is widely used in the Ayurvedic system of medicine for its varieties of therapeutic indications (Rao *et al*., 2008). The stem is the official medicine as listed by the Ayurvedic Pharmacopoeia of India. A variety of constituents are present in TC plant belonging to different classes such as alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds and polysaccharides. Water soluble isoquinoline alkaloids viz., jatrorrhizine, palmatine, berberine, tembetarine, magnoflorine, choline, tinosporine, isocolumbine and hydrastine are present (Chandrasekaran *et al*., 2009; Patel and Mishra, 2011). *Tinospora cordifolia* has been reported to have various therapeutic activities like antiulcer (Bairy *et al*., 2002), immunomodulatory (Manjrekar *et al*., 2000), antioxidant and hypoglycemic properties (Stanely *et al*., 2000). The drug is reported to possess 20% of the analgesic effect of sodium salicylate and reported to improve the immune system and body resistance against infections (Singh *et al*., 2003).

Central Composite Design (CCD) is the most popular design to provide optimum conditions that improve a process (Haaland, 1989). The central composite design reduces the cost of expensive analysis methods and decreases the associated numeric noise. It is relatively simple to determine the exact optimum condition for a single response using response surface methodology (Bezerra *et al*., 2008).

A number of attempts have already been made regarding optimized MAE techniques for the extraction of withanolides from *Withania somnifera* (Jyothi *et al*., 2010), phenolic compounds from *Pistachia vera* (Rajaei *et al*., 2010), dihydromyricetin from *Ampelopsis grossedentata* (Li *et al*., 2007), artemisinin from *Artemisia annua* (Hao *et al*., 2002), piperine from *Piper nigrum* (Raman *and* Gaikar, 2002), essential oil from eucalyptus (Saoud *et al*., 2006), saponins from *Centella asiatica* (Desai *et al*., 2011) and peurarin from *Radix puerariae* (Guo *et al*., 2001). However, this technique was still not employed on *Tinospora cordifolia*. Therefore it was hypothesized that MAE might be a useful method for extraction of *Tinospora cordifolia* thus, an attempt was made in this regard. Since open vessel microwave oven has produced good results in some previously conducted studies (Shukla *et al*., 2010) the same was used in the present study.

MATERIALS AND METHODS

Plant materials: The stems of *Tinospora cordifolia* were collected from herbal garden of the Maharshi Dayanand University, Rohtak and identified by Dr. Surender Yadav, Department of botany of the university. Voucher specimen of the plant having No. MDU/Phcog/111 was kept in the department for future reference.

Chemicals: HPLC grade Methanol (Spectrochem Pvt. Ltd.), Ethanol (CDH, New Delhi) and Glacial acetic acid (CDH, New Delhi) were used in the present study. Standard berberine was obtained from Sigma Chemicals (Fig. 1).

Instrumentation: Microwave extraction experiments were performed in an open vessel microwave oven (LG MC2149BB) equipped with five power levels (800, 640, 480, 320 and 160 W) to give maximum flexibility and control for extraction. A CAMAG High Performance Thin Layer Chromatography (HPTLC) system equipped with Camag Linomat 5 applicator system, TLC scanner 3 and integrated software WINCATS version 1.4.1 was used for the analysis.

Maceration: Extraction was carried out by placing 20 g of coarsely powered sample drug in a closed vessel at room temperature. Added 200 mL of ethanol and allowed for extraction for 7 days with occasional stirring at regular

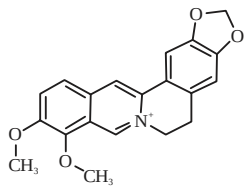

Fig. 1: Chemical structure of berberine

intervals. The liquid was filtered and the percentage yield of extract was calculated, after completion of the extraction process.

Soxhlet extraction: The 20 g powder of dried stems of *Tinospora cordifolia* was placed in thimble holder. About 300 mL of ethanol was filled in the flask. The thimble was clogged with cotton in order to avoid transfer of sample particles to the distillation flask. The drug was extracted with ethanol in soxhlet apparatus for 3 h. The ethanolic extract was filtered and concentrated on rotaevaporator to give the ethanolic extract. Percentage yield of extract was calculated.

Selection of relevant variables and experimental ranges: The variable parameters selected were extracting solvent, time and irradiation power. These parameters were optimized using Central Composite Design (CCD). In MAE, selection of solvent is a key factor affecting the recovery of analyte. Mixed solvents of lower alcohol and water have been generally, used for the extraction of alkaloids. Among the various alcohols, ethanol was selected because it is permitted in pharmaceutical and food industries. Therefore, the effect of ethanol concentration in aqueous ethanol extractant on berberine yield was evaluated. Ethanol concentration was varied in range from 60-100% (v/v). Microwave irradiation power ranged from 320-640 W. The chosen power limits were function of solvent and function of regulation limitations in the microwave apparatus. From the literature survey the extraction time chosen from 2-4 min. The upper and lower limits of the variables were selected on the basis of the previously conducted experiments.

Optimization studies: A Central Composite Design (CCD) with three variables was used to determine the response pattern and then to establish a model. Three variables-ethanol concentration (X_1), irradiation power (X_2) and extraction time (X_3) were studied at three levels (-1, 0, 1). The factors and levels are shown in Table 1, while the dependent variable was the yield of berberine. All variables were taken at a central coded value considered as zero. 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 2 were performed.

The yield of berberine 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 extraction time). 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 (coded±1), superimposed by centre points (coded 0) and "star points" (coded±α). The group of "star points" axial experiments located at a distance α from the centre, allow rotatability. 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

Table 1: Factors and levels for CCD test

Level	Ethanol conc. (%v/v) X_1	Irradiation power (%) X_2	Extraction time (min.) X_3
-α (-1.6818)	46.4	60	3
-1	60.0	40	2
0	80.0	60	3
+1	100.0	80	4
+α (+1.6818)	113.6	60	3

Table 2: Fully coded central composite design matrix of three variables and experimental results from response variables

Run order	Ethanol conc. (%v/v)	Irradiation power (%)	Extraction time (min)	Yield (%w/w)
1	-1 (60)*	-1 (40)	-1 (2)	0.5
2	+1 (100)	-1 (40)	-1 (2)	1.35
3	-1 (60)	+1 (80)	-1 (2)	0.10
4	+1 (100)	+1 (80)	-1(2)	0.30
5	-1 (60)	-1(40)	+1(4)	2.30
6	+1 (100)	-1 (40)	+1(4)	1.55
7	−1 (60)	+1 (80)	+1(4)	0.60
8	+1 (100)	+1(80)	+1(4)	1.95
9	-1.68 (46.4)	0 (60)	0 (3)	2.85
10	+1.68 (113.6)	0 (60)	0 (3)	3.6
11	0 (80)	-1.68 (13.2)	0 (3)	2.4
12	0 (80)	+1.68 (107.4)	0 (150)	0.45
13	0 (80)	0 (60)	-1.68 (1.32)	2.0
14	0 (80)	0 (60)	+1.68 (4.68)	3.0
15	0 (80)	0 (60)	0 (3)	4.3
16	0 (80)	0 (60)	0 (3)	4.25
17	0 (80)	0 (60)	0 (3)	4.40
18	0 (80)	0 (60)	0 (3)	4.1

*Values in brackets indicate the real values

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: $-\alpha$ (= -1.68), -1, 0, +1, $+\alpha$ (=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: For MAE, the dried stems of *Tinospora cordifolia* was crushed and screened through 24 mesh sieve. Twenty gram of the powdered drug was transferred to a 500 mL conical flask. Two hundred milliliter of 80% (v/v) ethanol-water was added. The mixture was shaken well and kept for some time so that the drug absorbs the solvent. In this way the bumping of solvent was avoided and 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 temperature was set at 3 min and irradiation power set at 480 W. After the extraction completed, the conical flask was taken out from the oven. Sufficient quantity of solvent was added to make a solution and then filtered. Concentration of extract was then carried out on water bath and calculated the percentage yield of extract (%w/w).

Quantitative analysis method: High performance thin layer chromatography was used for quantitative estimation of berberine in the obtained extract. The development of the TLC layer was performed, using a CAMAG twin trough glass tank, which had been pre-saturated with mobile phase of methanol: acetic acid: water (8: 1: 1 v/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 366 nm. The source of radiation utilized was a deuterium lamp. The peaks obtained in HPTLC chromatogram were analysed based on R_f values and confirmed through overlay absorption spectra.

RESULTS AND DISCUSSION

Optimisation studies: Three variables i.e., "ethanol concentration", "microwave irradiation power" and "extraction time" were involved in central composite design in order to evaluate, optimize and conduct relevant microwave-assisted extraction of alkaloid from stem of *Tinospora cordifolia*. These three controlled variables were studied in a multivariate study with 18 experiments as shown in the Table 2.

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 3).

The Model F-value of 4.77 implies that the model is significant. There is only a 2.49% 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. The estimated model, therefore, can be used as a response surface for percentage yield of drug. Figure 2 and 3 shows the response surfaces estimated from central composite design.

The "Lack of Fit F-value" of 111.83 implies the Lack of Fit is significant. There is only a 0.14% chance that a "Lack of Fit F-value" this large could occur due to noise. Experimental data allowed us to fit the yield of extracted as a function of extraction time, ethanol concentration and applied power. The optimum regression equation of berberine is as follows:

$$Y\ (\%\ \text{yield}) = 4.33+0.21X_1-0.44X_2+0.43X_3+0.18X_1X_2-0.056X_1X_3+0.019X_2X_3-0.63X_1^2-1.26X_2^2-0.88X_3^2+0.34X_1X_2X_3$$

where, X_1 is ethanol conc., X_2 is irradiation power, X_3 is extraction time.

For solving the equations, the Design-Expert 8.0.7.1. was used to obtain the optimal conditions. The maximum yield computed by the software is calculated by the maximum value

Table 3: ANOVA (Analysis of variance) model statistics

Source	Sum of squares	df	Mean square	Mean square	p-value Prob>F
A-A	0.62	1	0.62	0.89	0.3768
B-B	2.66	1	2.66	3.82	0.0916
C-C	2.49	1	2.49	3.57	0.1006
A^2	4.99	1	4.99	7.17	0.0317
B^2	20.23	1	20.23	29.03	0.0010
C^2	9.90	1	9.90	14.20	0.0070
ABC	0.95	1	0.95	1.36	0.2823
Residual	4.88	7	0.70	significant	significant
Lack of Fit	4.85	4	1.21	111.83	0.0014
Pure Error	0.033	3	0.011	-	-
Cor Total	38.10	17	-	-	-
Model	33.22	10	3.32	4.77	0.0249 (significant)

Standard deviation: 0.83, R^2: 0.8791, Adjusted R^2 0.6890**,** ANOVA for response surface reduced cubic model where, A: Ethanol concentration, B: Irradiation power, C: Extraction time

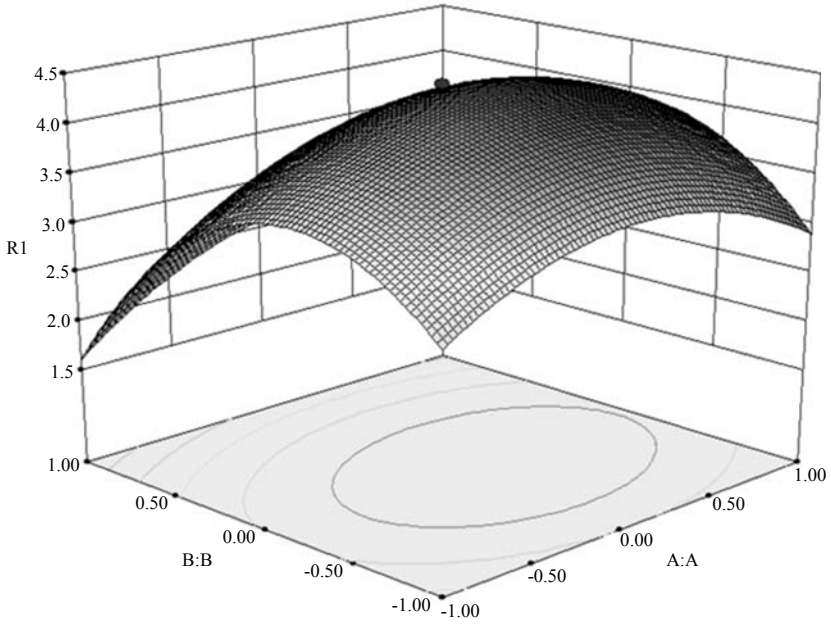

Fig. 2: 3D view of response surfaces estimated from central composite design

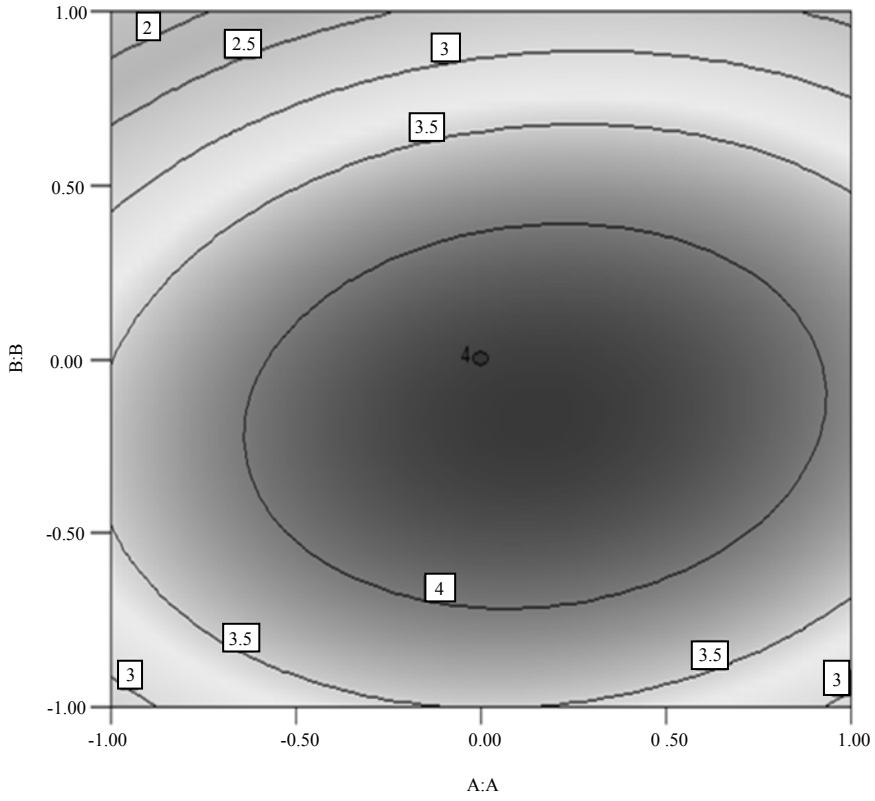

Fig. 3: 2D-contour view of response surfaces estimated from central composite design

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 effects was carried out in order to show significant effects of all variables

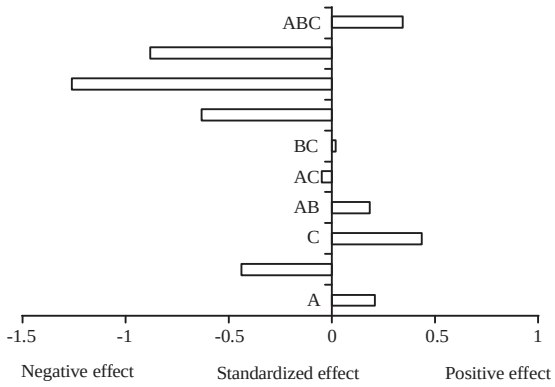

Fig. 4: Standardized Pareto chart

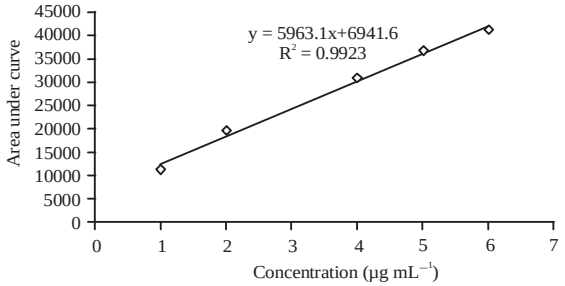

Fig. 5: Standard plot of berberine by HPTLC

(linear, quadratic and interactions between variables) as shown in Fig. 4. The length of the bars is proportional to the absolute magnitude of the estimated effect coefficients. It can be seen from the chart that extraction time has the most important influence on yields followed by ethanol concentration and irradiation time.

HPTLC analysis: The HPTLC analysis of extract of *Tinospora cordifolia* was performed for the estimation of berberine content in the test samples. The plates were analyzed densitometrically and area was considered for quantification of berberine in the samples. Quantitative analysis was carried out by calibration curve method. For that purpose, standard plot of berberine was plotted at different concentrations (Fig. 5). The R^2 value of 0.992 was obtained from the regression equation. HPTLC chromatogram of the standard marker compound berberine at 366 nm (Fig. 6). Figure 7-9 show HPTLC chromatograms of the *T. cordifolia* extracts obtained by MAE, soxhlation and maceration respectively at 366 nm. The peak of extract of *Tinospora cordifolia* matched with standard berberine was confirmed using spectra comparison. Figure 10 shows the spectral comparison of berberine with extract of *Tinospora cordifolia*. The absorption pattern of standard berberine and one of the components of extract of *Tinospora cordifolia* coincide, which confirms the presence of berberine in the extract. The λmax value of the berberine was found to be 348 nm,

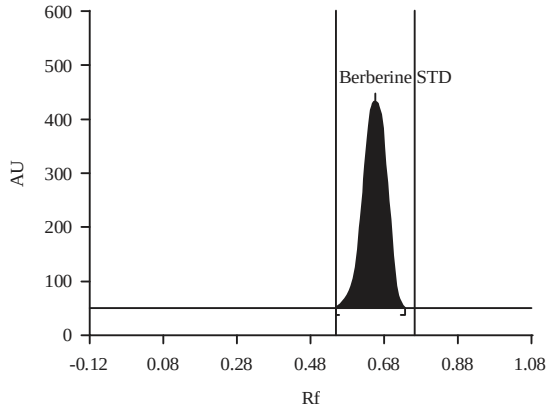

Fig. 6: HPTLC chromatogram of the standard marker compound berberine at 366 nm

Table 4: Extraction yield of berberine of different extraction methods

Extraction	Extractive value (%w/w)	Yield of berberine (%w/w)	Extraction time
Maceration	2.3	0.28	7 days
Soxhlation	3.5	1.04	3 h
MAE	4.40	1.66	3 min

which also matched with standard literature. The berberine content in microwave assisted extraction was found as 1.66% w/w.

Comparison of MAE with conventional methods: The extraction yield and berberine content were compared in all three experimental techniques. The results are shown in Table 4, which reveal that the extractive values and berberine 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 10 days and soxhlation for 3 h. 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.

In analytical chemistry applications, it has been observed that microwave extraction obtains similar or greater yields than other available novel extraction techniques i.e., ultrasonic extraction in the case of phytoconstituents such as flavonoids (Song *et al*., 2007). Microwave-assisted extraction has been reported to be an economical substitute for routinely used homogenization and vortexing extraction techniques (Akhtar, 2004). Also it has been observed that the MAE method followed by chromatographic separation technique like HPLC-UV-ELSD determination is a simple, rapid and reliable method for the quality assessment of the extracts (Song *et al*., 2007). The conventional extraction of various

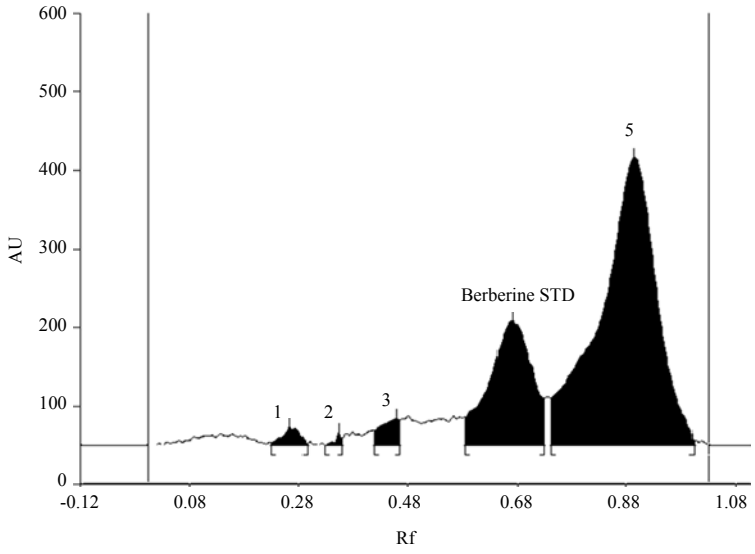

Fig. 7: HPTLC chromatogram of the *T. cordifolia* extract obtained by MAE at 366 nm

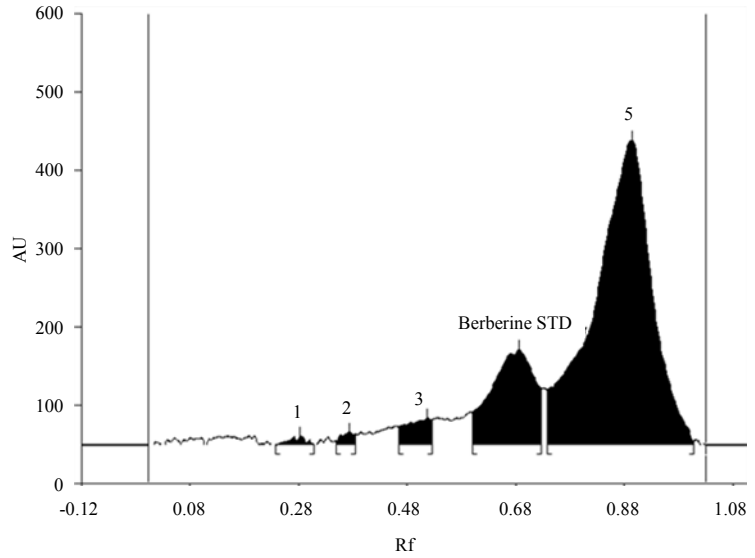

Fig. 8: HPTLC chromatogram of the *T. cordifolia* extract obtained by soxhlation at 366 nm

phytoconstituents often involves the addition of an acid. Using microwave extraction, in most of the cases, reduce the need for an acid (Gao *et al*., 2006; Kothari and Seshadri, 2010; Xiao *et al*., 2008). However, in cases where acids are used, such as for the extraction of anthocyanins (Sun *et al*., 2008; Yang and Zhai, 2010), less concentrated acids can be used with microwave as compared with conventional methods of extraction, without affecting the final yield. However compared to other modern extraction techniques like Super Critical Fluid Extraction (SFE), an additional filtration or centrifugation is necessary to remove the solid residue during MAE. Moreover, the efficiency of microwaves can be very poor when either of the target compounds or the solvents are non-polar, or when they exists in volatile state (Wang and Weller, 2006). In general, microwave-assisted extractions processes are either better or comparable with conventional solvent extraction methods. Optimization of process parameters and the use of modern technologies can significantly improve extraction efficiencies.

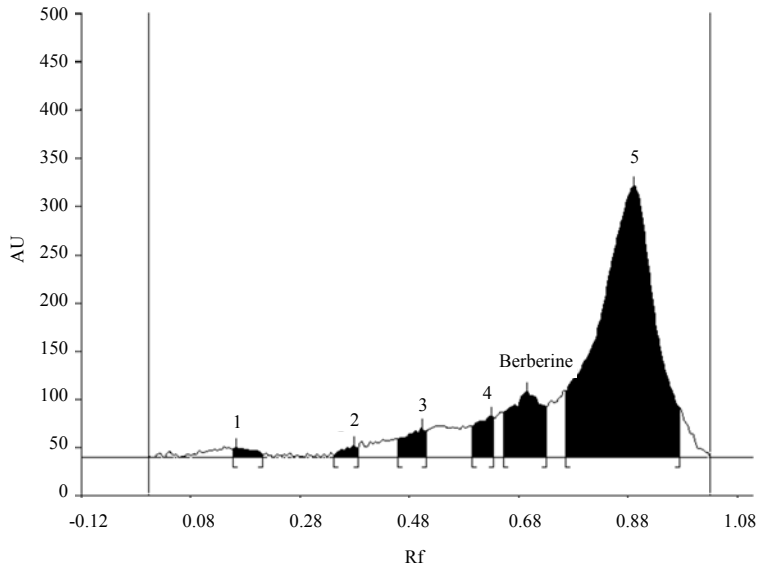

Fig. 9: HPTLC chromatogram of the *T. cordifolia* extract obtained by maceration at 366 nm

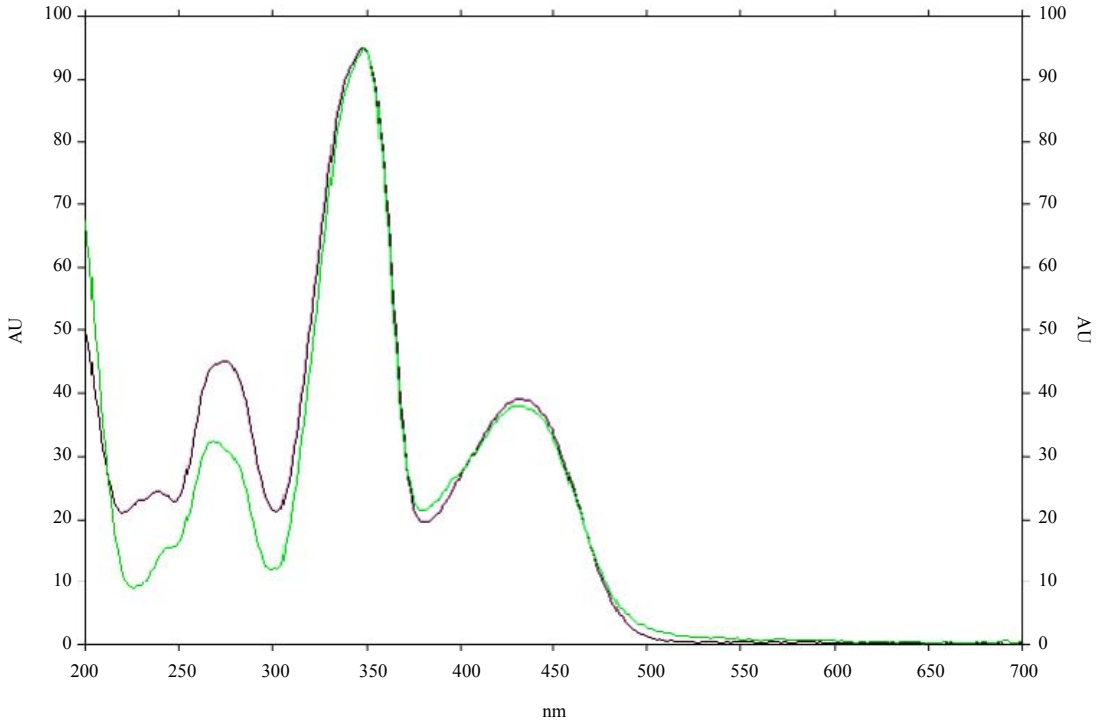

Fig. 10: Overlapped UV spectra of standard berberine and sample

CONCLUSION

The developed microwave assisted extraction method during the present study is more efficient than the previous methods thus can be used as promising tool for extraction of *Tinospora cordifolia* because of high yield and fast extraction ability with less consumption of solvent as well as time.

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