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Research Article

OPTIMIZATION OF MICROWAVE ASSISTED EXTRACTION OF WITHANOLIDES FROM ROOTS OF ASHWAGANDHA AND ITS COMPARISON WITH CONVENTIONAL EXTRACTION METHOD

DIVYA JYOTHI[1], SALMA KHANAM[2], ROKEYA SULTANA[3]

[1]Department of Pharmacognosy, Nitte Gulabi Shetty Memorial Institute of Pharmaceutical Sciences, Paneer, Deralakatte, Mangalore-574160, Karnataka, India [2, 3]Department of Pharmacognosy, Al Ameen College of pharmacy Bangalore-560027, Karnataka, India

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ABSTRACT

A simple and rapid microwave –assisted extraction (MAE) procedure was developed and optimized for fast extraction of withanolides from *Withania somnifera*. Several variables that can potentially affect the extraction efficiency, namely temperature, irradiation time, power of irradiation and powder size were optimized by means of orthogonal array design procedure. Quantification of withaferinA was done by validated HPTLC at 223nm. Under optimum conditions, MAE showed significantly higher recoveries and drastic reduction in extraction time than those obtained by conventional extraction method (soxhlet). In addition, a drastic reduction of the extraction time (4 min versus 14h) and solvent consumption (20ml versus 50ml) was achieved with MAE when compared with that provided by the soxhlet extraction as a reference method. The effect of microwave on cell destruction of plant material was observed by scanning electron microscopy (SEM).

Keywords: Microwave assisted extraction, WithaferinA, Taguchi approach, High performance thin layer chromatography, Scanning electron microscopy.

INTRODUCTION

Ashwagandha has been an important herb in the Ayurvedic and indigenous medical systems and plant has been used as an antioxidant, adaptogen, antitumor, aphrodisiac, immunomodulatory, liver tonic, anti-inflammatory agents and astringent[1-3].The major constituents of the plant include withanolides namely withnone, withaferinA, withanolides I, II, III, A, D, E, F, G, H, I, J, K, L, M, WS-I, P, S, sitoindosides, withanolide C and withsomidioenone and alkaloids[4].

The soxhlet is one of the conventional method for extraction of withanolides from *Withania somnifera*. Soxhlet extraction and other conventional methods operate through cell permeation followed by solubilising the active constituents by the extracting solvent. These conventional methods are time and solvent consuming, thermally unsafe. With the increasing demand for more environmental friendly methods, Microwave assisted extraction has been developed and optimized for fast extraction of withanolides[5].

Microwave assisted extraction(MAE) 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 consist of heating the solvent (extractant) in contact with sample with microwave energy. The process involves disruption of hydrogen bonds, as a result of microwave induced dipole rotation of molecules and migration of ions which enhance the penetration of solvent into matrix, allowing the dissolution of components to be extracted [6].

Present study describes the development and optimisation of rapid, reliable and sensitive method of extraction of withanolides from *Withania somnifera* roots using MAE and its comparison with conventional method of extraction for amount of withaferin A.

EXPERIMENTAL

Plant materials

Dried roots of *Withania somnifera* were obtained from local market and authenticated at RRI Bangalore (RCBI9101). Drug was dried at 60˚C, powdered, defatted and sieved through mesh size #22, 44, 60, immediately before the experiment.

Reagents

Analytical grade Methanol was used for the extraction. Chloroform and methanol used in HPTLC analysis were all of HPLC grade. Precoated silica gel $60F_{254}$ plates for HPTLC analysis were from E. Merck .WithaferinA standard of 95% (w/w) purity was obtained from Natural Remedies, Bangalore.

Apparatus

The extraction system comprised of a microwave extractor (CATA R) manufactured by Catalyst Systems (Pune, India) equipped with a magnetron of 2450MHz with a nominal maximum power of 700W, a reflux unit, 10 power levels, time controller, exhaust system, beam reflector and a stirring device. A Camag (Switzerland) HPTLC system was used for quantification of WithaferinA.

Soxhlet extraction

Soxhlet extraction was used as conventional method of extraction and it was compared with MAE for amount of constituents. Soxhlet extraction was performed using classical soxhlet apparatus with accurately weighed 10g drug powder (screened through mesh 22) for 14hr. Extraction was performed with 500ml methanol as the extracting solvent. Finally extracts were evaporated under vacuum.

Microwave assisted extraction

Accurately weighed 5g of powder of mesh size 22, 44, 60 were placed individually in extraction vessel along with 100ml of methanol solvent and placed inside the microwave cavity and MAE was carried out for different time of irradiation, temperature and at different power levels. After extraction, extracts were filtered and evaporated in a rotary evaporator under vacuum.

Optimisation method- Taguchi design

The Taguchi-based optimization technique was adapted for the process optimization of MAE of *Withania somnifera*. Taguchi-based optimization technique is a unique and powerful optimization discipline that allows optimization with minimum number of experiments[7].Thus by this method, it is possible to reduce the time and cost for experimental investigations and improve the performance characteristics. In the present study, three levels are defined for each of the factors as summarized in Table1. A L9 orthogonal array scheme was adapted which needs 9 experiments to complete the optimization process[8]. The extraction results performed under orthogonal design conditions are shown in Table 2. The sequence in which the experiments were carried out was randomized to avoid any kind of personal or subjective bias. All the results at each step of the design are expressed as the mean of three experiments. The optimum level for each factor was determined

from the graphical representation of the analysis of mean values from each level for a particular factor.

HPTLC analysis

The samples were spotted 15µl in the form of bands of width 6 mm, positioned 10mm from the bottom of the plate, with a Camag microlitre syringe on precoated silica gel aluminium plate $60F_{254}$ (20 cm×10 cm). The mobile phase consisted of chloroform: methanol (9:1, 10 ml). Linear ascending development was carried out in a twin trough glass chamber pre-saturated with mobile phase for 10 min at room temperature (25±2˚C) at relative humidity of 55±5%. The height of the solvent (mobile phase) front was 80 mm. Quantification was done in absorbance/reflectance mode of a Camag TLC scanner III at 223nm. Standard solution (0.1mg/ml in methanol) volumes of 5-9 µl was used for the preparation of a 5-point calibration curve corresponding to an amount of 500– 900ng.

Scanning electron microscopy (SEM)

In order to understand the extraction mechanism, root powder of *Withania somnifera* untreated samples, samples obtained after conventional and microwave assisted extraction methods were subjected to SEM. Untreated sample and samples obtained after conventional and microwave assisted extraction methods were plunged in liquid nitrogen and then cut with a cold knife. The sectioned particles were fixed on a specimen holder with aluminium tape and then sputtered with gold in a JEOL JEC-1200 sputter-coater. All the specimens were examined with a JEOL JSM-5600 LV scanning electron microscopy under high vacuum condition and at an accelerating voltage of 20 kV (10µm, lower and higher magnification).

RESULTS AND DISCUSSION

Optimization of extraction conditions

The optimization of extraction parameters was investigated with an orthogonal array design. The factors were microwave power (A), irradiation temperature (B), irradiation time (C) and particle size (D). For each variable, the influence on the yield of withaferinA was considered from three levels. To analyze the influence of each variable on the extraction results, Fig.1 was constructed based on the mean values obtained for each level from a particular factor.

Effect of microwave power and irradiation temperature

Fig.1shows when microwave power level was increased from 20% to 100%, there was 0.15% decrease in amount of withaferin A. It was also observed that the highest yield of withaferinA was obtained with the value of 0.493%, when the sample was extracted with methanol at 50˚C and yield was decreased at 60˚C. Graphical representation of the analysis of means (Fig.1) indicates that 20% microwave power and irradiation temperature of 50˚C were ideal to obtain maximum withaferinA content. MAE offers a rapid delivery of energy to a total volume of the solid matrix, efficiently and homogenously. Because natural moisture present within the plant 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[9]. This increased pressure will cause microscopic fractures in the cell, due to reduction in mechanical strength of cellulose present in the cell wall. With high microwave power and high irradiation temperature there might have been intense internal superheating of the plant matrix resulting in degradation of the active constituents which because of polar nature is more prone to damage due to intense microwave heating. Hence low microwave power of 20% with low irradiation temperature of 50˚C will be ideal for extraction.

Effect of irradiation time

Maximum amount of withaferinA was obtained with irradiation time of 2 min and yield of withaferinA was dropped when irradiation time was increased to 4min (Fig.1) This indicates that a irradiation time of 2min is sufficient to ring about the extraction of withaferinA and reduction in amount of withaferinA may be due to decomposition of constituent at high temperature for long period of time.

Effect of particle size

Powder mesh size powder # 44 gave higher amount of Withaferin A followed by # 60 and #22 (Fig.1). This indicates that powder of medium mesh size 44 is suitable for extraction. With the use of fine particles, microwaves will be facilitated with deep penetration ability resulting in thermal degradation of active constituents.

According to largest donating rule, namely, as far as each investigated factor, the largest value which affects the extraction yield of withaferinA should be the selected value. In Table 3, K_1- K_3 was the average yield of withaferinA under every level of an investigating variable, respectively the largest value was the optimized value. Therefore the optimized experimental conditions were as following: The microwave power- 20%, irradiation temperature -50˚C, irradiation time- 2min and powder of mesh size-44. The preferred conditions were namely the second group in Table 2. Under the optimum conditions, the total extraction yield of withaferinA was 0.69%. This indicated that the extraction yield of withaferinA could be enhanced using a combination of those factors at different levels in the extraction process.

Table 1: Shows factors and levels for the orthogonal design (A-D are the respective code for each factors)

Levels	Microwave power (%) A	Irradiation temperature(˚C) B	Irradiation time(min) C	Sieve number D
1	20	60	1	22
2	60	50	2	44
3	100	40	4	60

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

Tests	A	B	C	D	Withaferin A (mg)	Percentage Withaferin A
1	1	1	1	1	19.83	0.39
2	1	2	2	2	34.51	0.69
3	1	3	3	3	21.92	0.43
4	2	1	2	3	15.69	0.31
5	2	2	3	1	15.69	0.31
6	2	3	1	2	15.86	0.44
7	3	1	3	2	22.04	0.44
8	3	2	1	3	22.45	0.48
9	3	3	2	1	24.28	0.37

Table 3: Shows average yield of withaferinA at different levels for each factors

Levels	A	B	C	D
K1	0.503	0.380	0.436	0.293
K2	0.366	0.493	0.456	0.520
K3	0.353	0.350	0.393	0.400

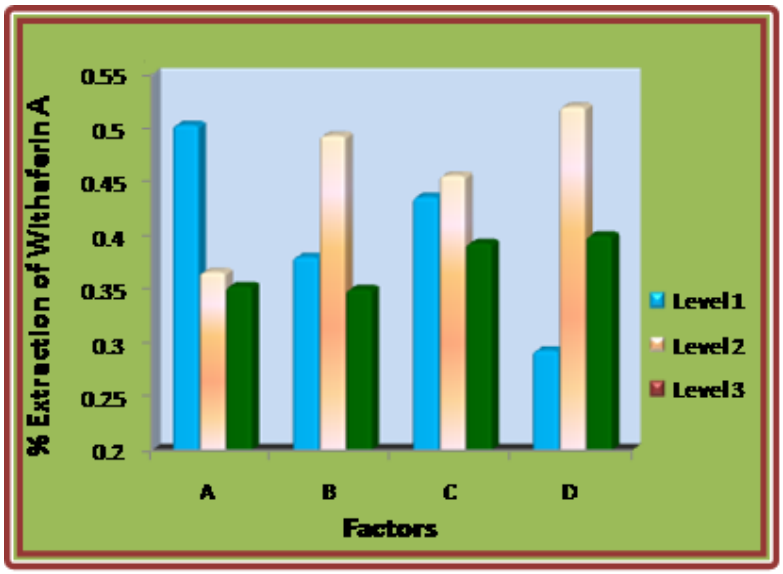

Fig. 1: Graph showing Percentage extraction of withaferin A, obtained under orthogonal condition of MAE.

Percentage extraction of withaferinA= mass of withaferinA in crude extract ×100/ mass of raw material.

A=Microwave power [level1=20%, level2=60%, level3=100%], B=Irradiation temperature [level1=60˚C, level2=50˚C, level3=40˚C], C=Irradiation time [level1=1min, level2=2min, level3=4min], D=Sieve number [level1=22, level2=44, level3=60].

Comparison of MAE and other conventional techniques

The selection of extraction method would mainly depend on the advantages and disadvantages of the processes such as extraction yield, complexity, production costs, environmental friendliness and safety. In general refluxation and soxhlation are the most commonly used extraction methods. The drawbacks of refluxation and soxhlation are the large amount of solvent and long extraction time needed.

MAE is a relatively new method, which has received increasing attention as an alternative method. MAE operates through cell bursting due to localized internal superheating followed by leaching out of the active constituents. Cell bursting phenomenon probably facilitates entry of the extracting solvent to solubilize out the target compound, thus lead to faster and efficient extraction. Compared with conventional extraction methods, refluxation and soxhlation, MAE method showed advantages with strong penetration force, high selectivity, high extraction efficiency and better products with lower cost.

In this study, MAE was compared with soxhlet extraction method for extraction of withaferinA from *Withania somnifera*. The conditions of soxhlet extraction method and optimised MAE and their results are shown in Table 4.

Table 4: Shows comparison of MAE with Soxhlation.

Number	Extraction methods	Extraction time	Solvent consumption (ml/g)	Yield of withaferinA (%)
1	Soxhlation	14h	50	0.16
2	MAE	2min	20	0.69

Table 4 showed that in terms of amount of withaferinA, the best results were obtained by MAE which gave significantly higher values when compared to other conventional methods. Significant benefits in terms of extraction time, solvent consumption indicated that MAE provides a very good and reliable extraction method.

Scanning electron microscopy

In order to study cell damage during the MAE experiments, the Ashwagandha root samples were examined by a scanning electron microscopy. Fig.2A–F present the micrographs of the untreated sample, soxhlet extraction sample and MAE sample, respectively. The changes observed for soxhlet extraction were not considerably different from those of untreated samples, and only few slight ruptures were seen on the surface of the sample. However, the surface of the sample was greatly destroyed after MAE. This observation suggests that microwave treatment affects the structure of the cell due to the sudden temperature rise and the internal pressure increase[10]. During the rupture process, a rapid exudation of the chemical substance within the cell into the surrounding solvents takes place. This mechanism of MAE based on exposing the analytes to the solvent through cell rupture is different from that of soxhlet extraction that depends on a series of permeation and solubilization processes to bring the analytes out of them.

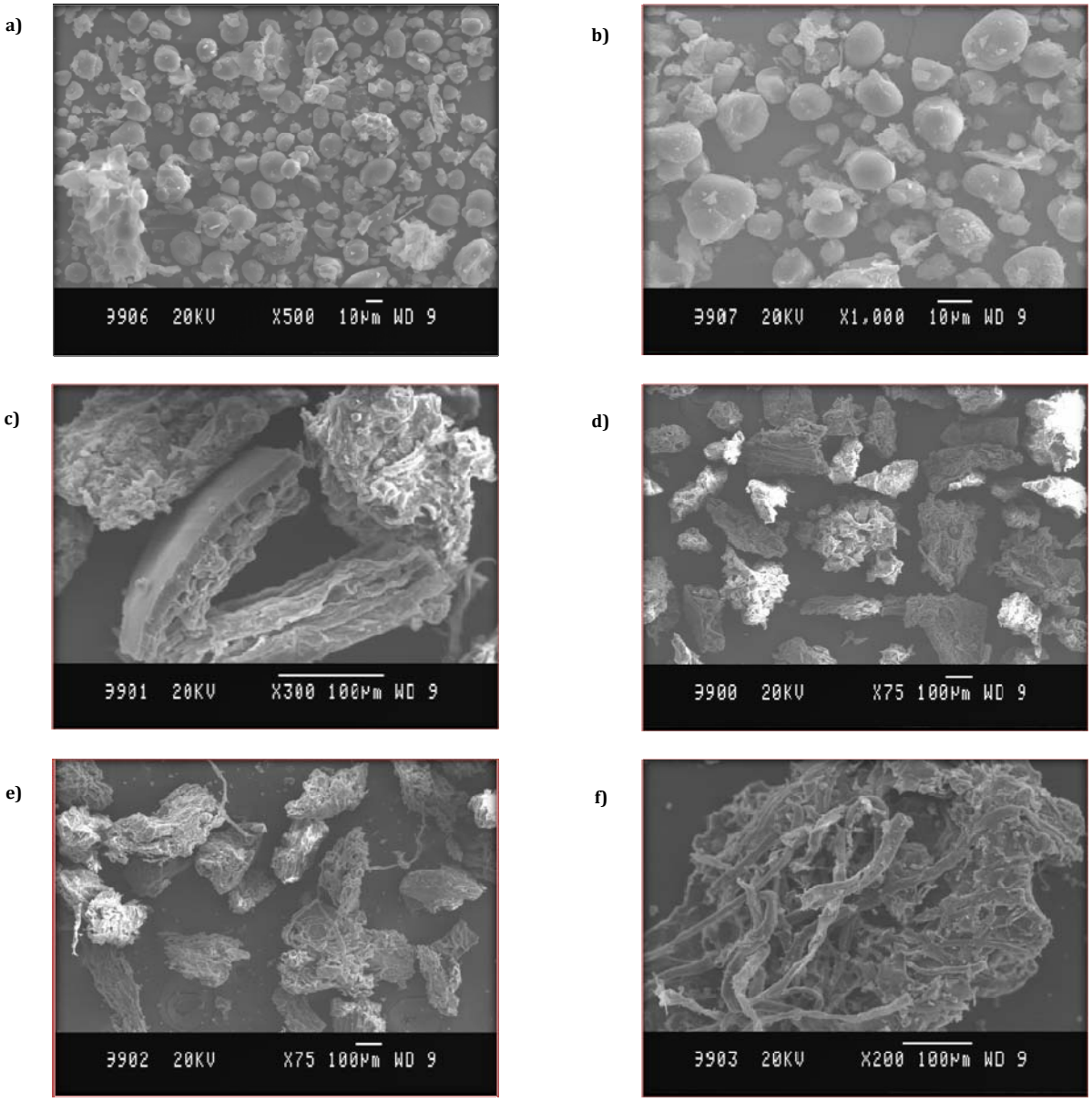

Fig. 2: Shows scanning electron micrographs of Ashwagandha root samples: (A) and (B) untreated Ashwagandha root; (C) and (D) after soxhlet extraction; and (E) and (F) after MAE under high and lower magnification.

CONCLUSION

MAE method for isolation of withaferinA was optimized using orthogonal array design which has allowed to find optimum values for variables that can potentially affect the extraction with a limited number of experiments. It can be concluded that the amount of withaferinA extracted is highly dependent on microwave power, irradiation temperature, and irradiation time and powder size. In comparison with conventional extraction method employed in this study the main advantages of the proposed MAE procedure are the low consumption of organic solvent and particularly, the rapid extraction which was performed in only 2min, achieving higher amount of withaferinA .SEM study confirm that the extraction mechanism of each method is different and MAE operates through cell bursting where as soxhlet method operates through cell permeation. With all these merits, MAE should be considered for wider application in the extraction and purification of phytochemicals from plants.

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