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Optimization of Microwave Assisted Extraction of Andrographolide from *Andrographis paniculata* and its Comparison with Refluxation Extraction Method

Manvitha Mohan^{*1}, Salma Khanam², B.G. Shivananda²

1. Department of Pharmacognosy, East West College of Pharmacy, Bangalore 91, India.
[E-mail: manvitha11@gmail.com]
2. Department of Pharmacognosy, Al-Ameen college of Pharmacy, Bangalore-27, India.

A new method using Microwave assisted extraction technique has been developed for extraction of Andrographolide from *Andrographis paniculata*. *Andrographis paniculata* is a well-known plant of Ayurveda which is also known as king of bitters because of different bitter principles present in different parts of the plant and exhibits a wide spectrum of biological activities. The MAE conditions such as irradiation time, temperature and coarseness of powder were optimized by means of orthogonal array design. The results suggested that the selected parameters were statistically significant. A comparative study was carried out to know the yield of andrographolide from the extracts prepared by microwave assisted extraction and refluxation using methanol and water as solvents. The amount of Andrographolide was estimated using HPTLC method. The results indicate that the extracts prepared by Microwave assisted extraction contained more amount of andrographolide when compared with refluxation extraction method.

Keyword: *Andrographis paniculata*, Andrographolide, Heat reflux extraction, HPTLC, MAE.

1. Introduction

Andrographis paniculata commonly known as “Kalmegh” belonging to the family Acanthaceae, is traditionally used as a medicinal herb to treat different diseases in India, China and Southeast Asia. The plant is also known as king of bitters because the plant consists of different bitter principles present in different parts of the plant. *Andrographis paniculata* is an annual shrub which grows abundantly in India and the plant extract is known to contain diterpenes, flavonoids and stigmaterols [1]. The therapeutic activity of this plant is attributed to andrographolide and its related diterpenes [2]. The extracts and constituents exhibit a wide spectrum of biological activities including antimicrobial [3],

antimalarial[4], antihypertensives and antidiabetics[5,6], antifilarial [7] activities etc. Extraction is a process whereby the desired constituents of a plant are removed using a solvent [8]. Microwave Assisted Extraction has been used as an alternative to conventional methods in the extraction of active constituents from plant materials and foods. It is based upon the selective and rapid localized heating of moisture in the sample by microwaves. MAE is a process of using microwave energy to heat solvents in contact with a sample in order to partition analytes from the sample matrix into the solvent. The ability to rapidly heat the sample solvent mixture is inherent to MAE and the main advantage of this technique. MAE method is

especially useful for the extraction of the samples with plant origin. Microwaves are non-ionizing electromagnetic waves of frequency between 300MHz to 300GHz and positioned between the x-ray and infrared rays in the electromagnetic spectrum^[9]. Several multivariate approaches had been used as optimization designs including L₉ Orthogonal array design^[10], L₁₆ array design^[11], Box-Behnken experimental design^[12], two-level factorial design^[13]. The scanning electron microscope (SEM) is a type of electron microscope that images the sample surface by scanning it with a high-energy beam of electrons in a raster scan pattern. The electrons interact with the atoms that make up the sample producing signals that contain information about the sample's surface topography, composition and other properties such as electrical conductivity^[14]. (HPTLC) was used for estimation of chemical constituents. High performance thin layer chromatography (HPTLC) method was reported to be the most suitable method for the estimation of active constituents of extracts, plant species (raw material) or polyherbal formulations^[15].

2. Materials and Methods

2.1 Plant Material:

Aerial parts of *Andrographis paniculata* was obtained from local market and authenticated by RRI Bangalore Ref.No.RRI/BNG/SMP/Drug Authentication/2008-09/229. Microwave oven (CATA-R) manufactured by Catalyst Systems (Pune, India) equipped with a magnetron of 2450 MHz with a nominal maximum power of 700W, a reflux unit, 10 power levels, time controller, exhaust system, beam reflector and a stirring device was used. Andrographolide standard of 98 % (w/w) purity was obtained from Sigma Aldrich, Bangalore.

2.3 Extraction Procedure:

Drug was dried at 60 °C, powdered and sieved through mesh size #44, 22 and 12 to obtain 3 samples A1, A2 and A3 respectively. All the samples were defatted using petroleum ether for 8hrs.

2.3.1 Refluxation was performed in Round bottomed flask using water and methanol separately as solvents for 12hrs by taking 10g of defatted powdered material of 3 samples. Percentage yield obtained by refluxation method was calculated and tabulated in **Table 1**.

2.4 MAE method:

10g of the defatted powdered material was mixed with appropriate amount of the extracting solvent 100ml methanol and placed inside the microwave cavity. Power of irradiation was set and extraction was carried out. Temperature sensor was inserted into the container to measure and control the internal temperature, the exact temperature was detected by the sensor. When the temperature was just 2-3 °C under the desired temperature time of irradiation was noted. During the extraction, solvent will be evaporated and condensed by cold water running through the condenser. Extraction was stopped after completion of exposure time at desired temperature. The extracts obtained was filtered and evaporated in a rotary evaporator. Percentage yield is shown in **Table 2**.

2.5 Optimization method of MAE:

The MAE method was optimized using L₉ orthogonal array design procedure. All the samples were subjected to MAE. Four main factors i.e. microwave power, irradiation time, particle size and extraction temperature were the optimized variables with the constant sample amount (10g) and volume of extracting solvent (100ml). The design technique needs 9 experiments to complete the optimization process. MAE was carried out for different time of irradiation at different temperature with the microwave oven operating at different power levels as shown in **Table 2**. The extracts obtained were filtered and evaporated in a rotary evaporator. All the results at each step of the design are expressed as the mean of three experiments. Samples from aerial parts subjected to MAE trial 1-9 under optimization with water and methanol and denoted as trial 1-9. The summaries of optimized conditions are tabulated in **Table 3**.

2.6 HPTLC Analysis

HPTLC method was used for the estimation of Andrographolide in all the extracts.

2.6.1 Development of HPTLC method for estimation of Andrographolide

Instruments: CAMAG Linomat 5 sample applicator, CAMAG TLC Scanner 3, CAMAG Reprostar 3, CAMAG TLC plate heater, Twin trough chamber, winCATS software –version 1.3.3.

- **Stationary Phase:** Merck TLC plates silica gel 60F254
- **Mobile Phase:** Chloroform: Methanol in the ratio of 7:1.
- **Standard Preparation:** 1mg of the standard andrographolide was prepared in 1ml methanol.
- **Application:** 1 μ l - 5 μ l of the standard solution was applied as bands on the plate.
- **Development and Scanning:** The spotted plates were individually developed upto 90mm in a previously saturated Twin Trough chamber. Chamber saturation was done for 10min. The developed plates were scanned at 200nm to 500nm and λ max was determined, then the chromatogram was scanned at λ max.
- **Linearity:** Linearity of an analytical method is its ability to elicit test results that are directly or by a well-defined mathematical transformation proportional to the concentration of analytes in sample within a given range. Detection of linearity involves determination of concentration required to prepare a linear graph. 1mg of Andrographolide standard was dissolved in 1ml of methanol. From this standard stock solution, 1 μ l - 5 μ l were spotted. The chromatogram was developed and scanned at 231 nm.

2.6.2 Estimation of Andrographolide in Extracts by HPTLC Method

The validated HPTLC method was applied to quantify Andrographolide in extracts prepared from conventional and MAE methods.

- **Sample Preparation:** 10mg/ml solution of methanolic extracts was prepared by dissolving it in methanol and filtered through Whatmann No. 1 filter paper.
- **Standard Preparation:** 1mg/ml solution of the standard andrographolide was prepared.
- **Application:** 1 μ l, 3 μ l and 5 μ l of the standard solution and 5 μ l of all sample solutions were applied as bands on the plate.
- **Development and Scanning:** The spotted plates were individually developed up to 90mm in a previously saturated Twin Trough chamber. Chamber saturation was done for 10 minutes. The developed plate was scanned at 231nm.
- **Documentation:** The images of the plates were recorded using CAMAG Reprostar 3. Plate was placed in CAMAG Reprostar, suitable light was selected and release button was clicked. The electronic image of the chromatogram was documented in the system.

The result of quantification of Andrographolide in extracts are shown in **Table 1 and 2**.

2.7 Comparison of MAE and refluxation extraction method

The refluxation extraction method and MAE methods were compared for amount of Andrographolide. The graph representing comparison of MAE and Conventional method is depicted in **Fig 1**.

2.8 Scanning Electron Microscopy

In order to study cell damage during the MAE experiments, the plant samples of *Andrographis paniculata* was examined by scanning electron microscopy before and after extraction methods. Untreated samples obtained after conventional and MAE 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 20kV(10 μ m and 100 μ m, \times 75, \times 100, \times 250, \times 400 and \times 1000 magnification) **Fig 2A-F** shows the micrographs of the untreated samples, Reflux extraction sample and MAE sample respectively.

3. Results and Discussion

Comparison of the results indicated that Refluxation with methanol yields highest

extractive value. However the sieve size did not show any significant influence on the extractive value. The results of percentage yields are shown in **Table 1**. The results indicate that extraction of kalmegh is effective with methanol refluxation rather than water refluxation. The highest yield with methanol refluxation was 19.21 % with mesh size # 44.

Table 1: Data showing yield of extracts, amount of Andrographolide by heat-reflux extraction

Sample identity	Water Reflux		Methanol Reflux	
	% Yield	% Andrographolide	% Yield	% Andrographolide
A1	19.060	1.6335	19.210	1.654
A2	19.055	1.279	19.063	1.254
A3	19.004	0.796	18.396	0.523

Note: A1: Aerial powder passed through mesh size # 44, A2: Aerial powder passed through mesh size # 22 and A3: Aerial powder passed through mesh size # 12

3.1 Quantification of Andrographolide in Extracts Obtained by Refluxation

Percentage of Andrographolide obtained with Methanol Refluxation was found to vary between 0.523 to 1.654 % with maximum amount of Andrographolide with mesh size #44. Percentage of Andrographolide obtained with Water Refluxation was found to vary between 0.796 to 1.633 % with maximum amount of Andrographolide with mesh size #44. Comparison

of the results indicates that with both water and methanol, yielded more amount of Andrographolide. The orthogonal array design was adapted for the process optimization of MAE of *Andrographis paniculata*. Four factors i.e. microwave power, temperature, irradiation time and particle size was taken up for the study. **Table 2** lists the factors and levels used in the tests and the yield of extracts obtained under orthogonal conditions.

Table 2: Data showing yield of extracts, amount of Andrographolide by Orthogonal test with Water and methanol as solvents

Tr. No.	Power (W)	Temp. ($^{\circ}$ C)		Time (Min)	Sieve No.	% yield		% Andrographolide	
		Water	Meth.			Water	Meth.	Water	Meth.
1	700	100	60	1	12	19.01	17.01	0.7667	0.773
2	700	80	50	2	22	29.82	19.62	1.1024	0.968
3	700	60	40	4	44	21.39	20.10	1.19	2.198
4	420	100	60	2	44	25.38	18.08	1.573	1.537
5	420	80	50	4	12	44.70	21.60	3.9	1.474
6	420	60	40	1	22	19.12	18.32	0.443	1.348
7	140	100	60	4	22	17.90	14.61	0.7707	0.213
8	140	80	50	1	44	18.29	22.70	0.2677	0.240
9	140	60	40	2	12	15.61	14.32	0.4639	0.075

3.2 Quantification of Andrographolide in extracts obtained by MAE:

Percentage of Andrographolide obtained from Methanol extracts by MAE was found to be 0.075 % to 2.198 %. Percentage of Andrographolide obtained from Water extracts by MAE was found to be 0.267 % to 3.900 %. MAE trial 5 gave the maximum amount 8.725 % of extractive as well as amount of Andrographolide 3.9 % with water as solvent. In case of methanol as solvent Trial 3 gave maximum amount 9.52 % of extractive as well as amount of Andrographolide 2.198 %. Methanol was found to be a better solvent compared to water as it yielded higher extractive as well as more amount of Andrographolide. Comparison of extraction methods indicates that MAE was better than refluxation for extraction of Andrographolide.

3.3 Effect of MAE parameters on percentage yield of extracts of aerial part of the plant:

3.3.1 Effect of Irradiation power on yield:

Yield obtained with water was maximum at microwave power 420 W (60%) followed by 700W (100%) and 140W (20%) and yield obtained with Methanol was maximum at 420W (60%)microwave power followed by 700W (100%) and 140W (20%).These results indicate that microwave power is factor which has variable effect on the yield of the extracts. Hence trials have to be carried out to choose appropriate power level for extraction.

3.3.2 Effect of temperature: Yield obtained with water was maximum at temperature of 80 °C, followed by 100 °C and 60 °C, the yield obtained with methanol was maximum at temperature of 50 °C followed by 40 °C and 60 °C. From the results obtained it can be concluded that yield of extract varies with extraction temperature with different solvents and hence preliminary trials have to be carried out in order to select appropriate temperature.

3.3.3 Effect of microwave irradiation time:

Yield obtained with water was maximum at 4

minute followed by 2minute and 1 minute. Yield obtained with methanol was maximum at 1 minute followed by 4minute and 2 minute. Results shows that in order to get maximum yield of the extracts appropriate irradiation times is necessary and it must be selected by carrying out extraction trials with different irradiation time.

3.3.4 Effect of particle size: Mesh size # 12 showed maximum % yield with water followed by #22 and # 44, Mesh size # 44 showed maximum % yield with methanol followed by # 12 and # 22. These results indicates that size of powder has significant effect on yield of extracts and yield of extracts were found to vary with size of powder .Hence in order to get maximum yield it is necessary to choose the required mesh size .

The optimized experimental conditions for MAE in order to get high yield of extracts using with water as solvent were found to be as follows: Power of irradiation 420W (60%), extraction temperature was 80 °C with 4 minutes irradiation time using powder of mesh size #12.The optimized experimental conditions for MAE in order to get high yield of extracts using with methanol as solvent were found to be as follows: Power of irradiation 420W (60%), extraction temperature was 50 °C with 1 minute irradiation time using powder of mesh size #44.The summary for optimized condition for better extraction of Andrographolide are given in **Table 3**.

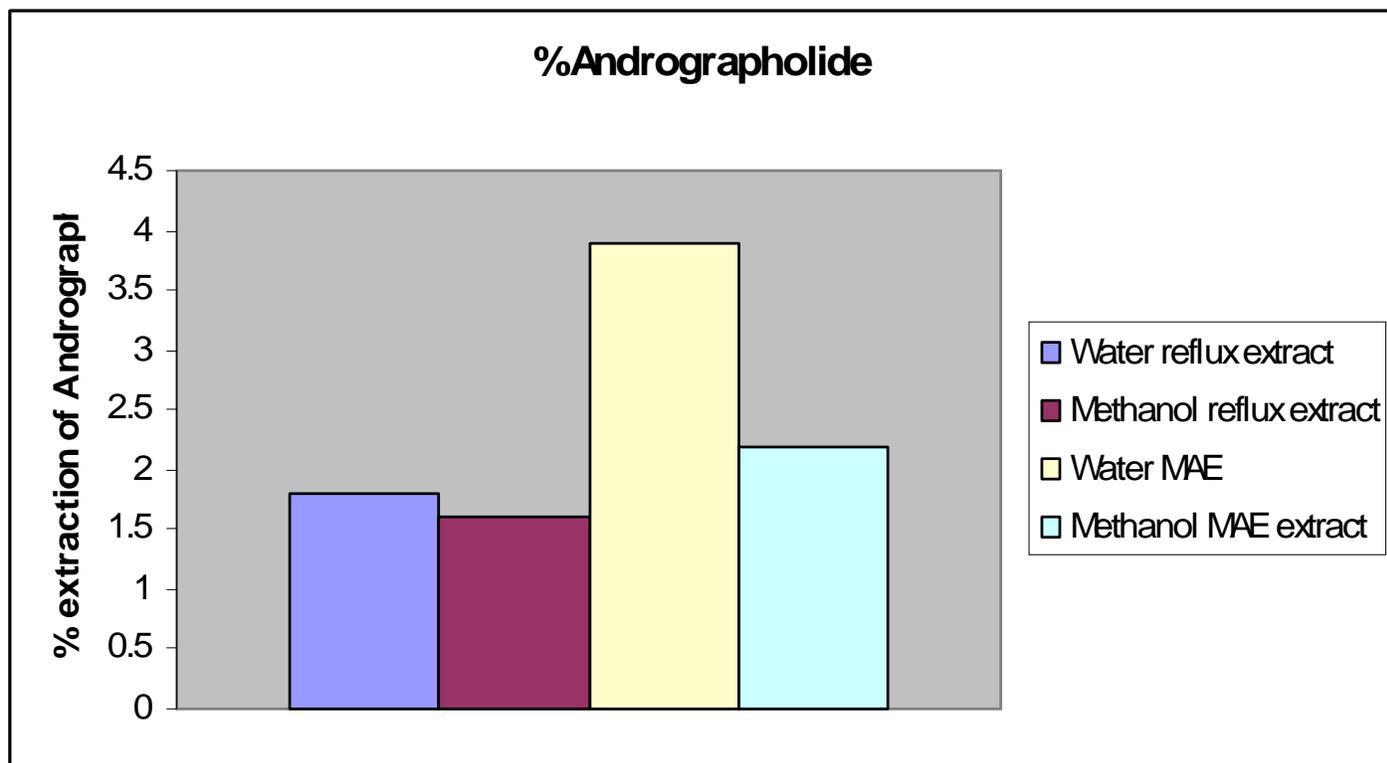
Table 3: Optimized conditions for better extraction of Andrographolide

Optimized parameters	Water	Methanol
Microwave power	420W (60%)	420W (60%)
Irradiation temperature	80°C	50°C
Irradiation time	4 minute	1 minute
Particle size	mesh size #12	mesh size #44

In order to compare the amount of andrographolide a cumulative graph representing the amount of andrographolide obtained by

refluxation and optimized MAE with water and methanol is shown in **Fig 1**; results indicate that yield of water extracts obtained from MAE were higher than Refluxation.

Fig 1: Results Indicate that Yield of water extracts obtained from MAE were higher than Refluxation.



3.4 Scanning Electron Microscopy

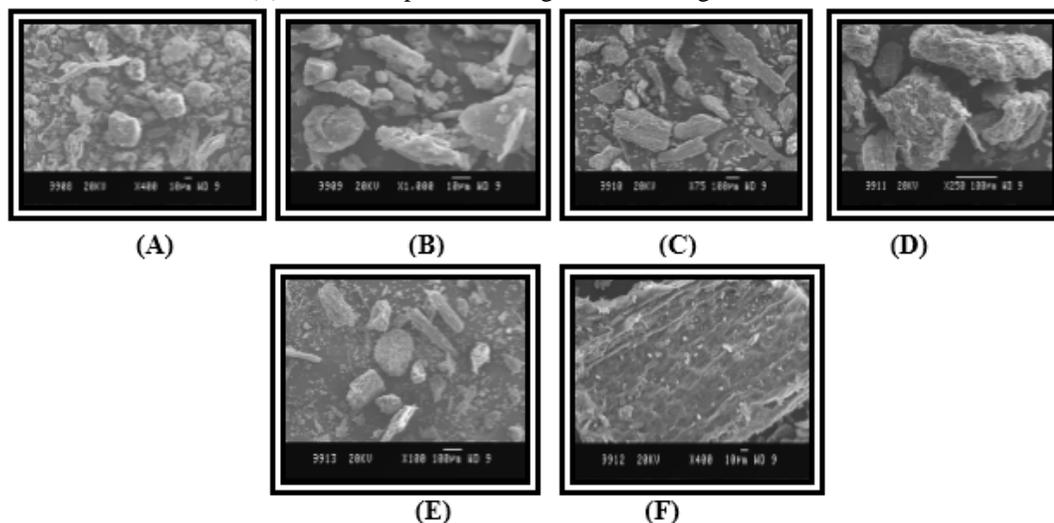
In order to study cell damage during the MAE experiments, the kalmegh samples were examined by SEM. **Figure 2A - F** shows the micrographs of the untreated samples, Heat Reflux sample and MAE sample respectively. The untreated sample **Fig 2A and 2B** exhibited smooth particles without any damage. The samples analyzed after refluxation **Fig 2C and 2D** were swollen with ruptured surfaces. However the surface of the sample after MAE (**Fig 2E and 2F**) was greatly destroyed. This

observation suggested that microwave treatment affects the structure of the cell.

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Fig 2: Scanning electron micrographs of Kalmegh; (A) and (B): untreated sample, (C) and (D) : Heat reflux sample, (E) and (F): MAE samples under high and low magnifications



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