

COMPARISON OF CONVENTIONAL AND NON CONVENTIONAL METHODS OF EXTRACTION OF HEARTWOOD OF *PTEROCARPUS MARSUPIUM* ROXB.

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Abstract: The renewed interest in plant-derived drugs has led to an increased need for efficient extraction methods. The present investigation was an attempt to evaluate and compare the conventional methods of extraction with non conventional methods of extraction, such as ultrasonic-assisted extraction (UAE) and microwave-assisted extraction (MAE) methods. *Pterocarpus marsupium* Roxb. has been reported to contain bioactive phytochemicals, e.g., pterostilbene (3',5'-dimethoxy-4-stilbenol). The results showed that among the conventional extraction methods, percolation gave the highest yield. The non conventional methods were optimized. The extraction yield was the highest in case of MAE. The phytochemical screening of the extracts indicated similar groups of compounds in all the extracts. The thin layer chromatography showed the presence of pterostilbene in the extracts obtained by using percolation, MAE and UAE. In these extracts the quantification of pterostilbene was conducted by high performance liquid chromatography and the method was validated. The MAE method extracted significantly higher amount of pterostilbene.

Keywords: *Pterocarpus marsupium* Roxb., ultrasonic-assisted extraction, microwave-assisted extraction, high performance liquid chromatography

An increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extraction and development of several drugs/chemotherapeutics from the plants as well as from traditionally used herbal remedies (1). The increasing interest in plant secondary metabolites is accompanied by a need to expand and modify the arsenal of plant-extraction protocols. There are several conventional methods of extraction, such as infusion, decoction, digestion, maceration and percolation (2). The conventional extraction processes are time consuming, e.g., maceration done for 2–7 days; these involve bulk amount of solvents (3). The demand for new extraction techniques has encouraged the development of alternative extraction techniques such as ultrasonic-assisted extraction (UAE) and microwave-assisted extraction (MAE). These techniques have enabled automation, shortened extraction time and reduced organic solvent consumption (4).

The UAE method involves the usage of ultrasound which refers to mechanical vibrations, which

are essentially the same as sound waves but of a higher frequency. Ultrasound causes rapid extraction due to:

- 1) increase in the permeability of the cell wall,
- 2) spontaneous formation of bubbles in the liquid below its boiling point, i.e., cavitation effect, due to dynamic stressing and
- 3) increase in the mechanical stressing, i.e., internal friction of the cells (3).

The UAE depends upon many factors like: (a) intensity, (b) time, (c) solvent, (d) temperature, (e) pulsation, (f) matrix (5–8).

The MAE method involves the use of microwaves, which are electromagnetic waves whose frequencies range from approximately 300 megahertz to 1000 gigahertz (9). The MAE is based upon the selective and rapid localized heating of moisture in the sample by microwaves. Due to the localized heating, pressure builds up within the cells of the sample, leading to a fast transfer of the compounds from the cells into the extracting solvent (10). The MAE depends upon many factors like: (a)

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solvent, (b) time, (c) power, (d) temperature, (e) matrix (11–15).

The UAE and MAE are influenced by many factors as described above and there exists interaction among these factors, thus attention has to be focused on optimization of procedures (16).

The *Pterocarpus marsupium* (Family: Fabaceae) is widely distributed on the earth. Kino is the juice obtained by incision in the trunk and is composed of a peculiar kino-tannic acid (17). As astringent it is used in diarrhea, dysentery, etc. Bruised leaves are applied on skin diseases, sores and boils. Wood is useful in treating diabetes (18, 19). The heartwood of *Pterocarpus marsupium* is golden yellowish-brown in color with darker streaks. It is very hard and brittle. In water it gives yellow colored solution with blue fluorescence (20). Several methods have been used for the extraction of the heartwood, like infusion (17), decoction (20), maceration (21), percolation (22) and hot water extraction (23). The ethyl acetate extract of powdered dried heartwood of *Pterocarpus marsupium* revealed the presence of following constituents: pterostilbene, (2S)-7-hydroxyflavanone, isoliquiritigenin, liquiritigenin, 7,4'-dihydroxyflavone, marsupin, pterosupin, *p*-hydroxybenzaldehyde, (2R)-3-(*p*-hydroxyphenyl)-lactic acid, pm-33, retusin-8-O- α -L-arabinopyranoside, naringenin, lupeol, pterocarpol (24–26). Various glucoside have also been isolated (23, 27–29). Two sterols, sitosterol and stigmasterol were also isolated (30). *Pterocarpus marsupium* has been reported as an antidiabetic, an anti-hypertriglyceridemic, a cardiogenic, an anticataract, a COX-2 inhibitor and a hepatoprotective agent (17, 26, 31–34).

In this paper, the extraction yield of heartwood of *Pterocarpus marsupium* has been compared using various conventional methods and two non conventional methods, i.e., UAE and MAE. The UAE and MAE methods were optimized. The preliminary phytochemical screening, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC), taking pterostilbene as reference standard marker compound, were performed and the results were compared (35).

EXPERIMENTAL

The *Pterocarpus marsupium* (Family: Fabaceae) heartwood was purchased from Yucca Enterprises, Mumbai (India) and was identified at the Department of Raw Materials Herbarium and Museum, National Institute of Science Communication and Information Resources (NIS-

CAIR), New Delhi (India). It was assigned reference no. NISCAIR/RHMD/Consult/-2010-11/1469/67 and was deposited in the Departmental Herbarium. The heartwood was shade dried and was powdered in an electric grinder. The powder was passed through no. 36 mesh and was used for extraction.

Pure standard of pterostilbene was purchased from Chromadex Inc. (LGC Promochem India Pvt. Ltd., Bangalore, India). Ethyl acetate (Qualigens Fine Chemicals, Navi Mumbai, India), *n*-hexane (Loba Chemie Pvt. Ltd., Mumbai, India), trifluoroacetic acid, acetonitrile and methanol (Merck, Mumbai, India) were of HPLC grade. The ultrapure water (Organo Biotech Lab. Pvt. Ltd., Mumbai, India) was used to make the solutions. For ethanolic extractions absolute ethanol (Hayman Ltd., Essex, England) was used. Equipment used included: microwave oven (Model MG- 555 F, LG, Greater Noida, India), ultrasonic bath (Model UCB- 5200 D, Macro Scientific Works Pvt. Ltd., Delhi, India), Agilent HPLC system (Agilent Technologies India Pvt. Ltd., Haryana, India) comprising of Agilent HPLC pump (JP 94174012), Agilent auto sampler (DE 62973678), column oven, Agilent UV-VIS detector (VWDDE 71366452), Zorbax C-18 column (5 μ m, 150 \times 4.60 mm) and Agilent chemstation software used for data analysis and data processing. The TLC silica gel 60 F₂₅₄ Aluminum sheets (Merck, Mumbai, India) was used for TLC.

Sample extraction

(A) *Conventional methods*: 200 g of the powdered heartwood of *Pterocarpus marsupium* was extracted (aqueous and ethanolic) by infusion, decoction, maceration and percolation. The various methods were statistically compared using Student's *t*-test and analysis of variance (ANOVA), followed by Dunnett's *t*-test.

(B) *Microwave-assisted extraction method (MAE)*: 50 g of the powdered heartwood of *Pterocarpus marsupium* was extracted (aqueous and ethanolic) using microwave oven, subjected to microwave irradiation (1350 W at 100% power). The constrained optimization of aqueous MAE was adopted by obtaining the experimental data using factorial design experiments, the effects of the two parameters, i.e., microwave power (Factor-A) and irradiation time (Factor-B) were studied. The low (–) level and the high (+) level of the factors A and B were predefined as 20–100% and 5–25 min, respectively. A simple factorial design experiment (two levels – two factors) was utilized to assess the relative importance of these two factors. The effect of any factor was the change in the response pro-

duced by altering the level of that factor, averaged over the levels of all the other factors. The effect of any factor was calculated using the formula:

$$\text{Effect of factor A} = \frac{1}{4} [ab + a - b - (1)]$$

$$\text{Effect of factor B} = \frac{1}{4} [ab + b - a - (1)]$$

The magnitude of the factors interaction term was calculated in the same way as that of the main factors, i.e., the mean of the results of all the experiments with a + in the interaction column minus the mean of all those with a - in that column. The formula used was:

$$\text{Interaction Term} = \frac{1}{4} [(1) + ab - a - b]$$

The factor B was optimized by performing various experiments taking factor A constant at 100%. The yield was calculated and the results were statistically estimated using ANOVA.

(C) *Ultrasonic-assisted extraction method (UAE)*: 50 g of the powdered heartwood of *Pterocarpus marsupium* was extracted (aqueous and ethanolic) using ultrasonic bath. The aqueous and ethanolic methods were statistically compared using Student's *t*-test. The effects of the two parameters, i.e., temperature (Factor-A) and time (Factor-B) were studied. The low (-) level and the high (+) level of the factors A and B were predefined as 25–60°C and 5–60 min, respectively. The effect of factors and the magnitude of the interaction term were calculated in the same way as that for MAE. The simplex search method is an optimization procedure which adopts an empirical approach. The results of the previous experiments were used to define the experimental conditions of subsequent experiments. The optimum was approached by moving away from the low values of the response. A simplex of two variables was a triangle. All the variables must be put on the same unitary basis, and this was achieved by normalization. Normalization was carried out by using the following equation:

$$N = \left[\frac{(X - L)}{(H - L)} \right] \times 100\%$$

where: N was the normalized value, X was the original uncorrected value of that variable, L and H were the lowest and highest values of that factor which were likely to be of interest.

The simplex was constructed by selecting three combinations of these two variables, temperature and time. The three experiments were carried out

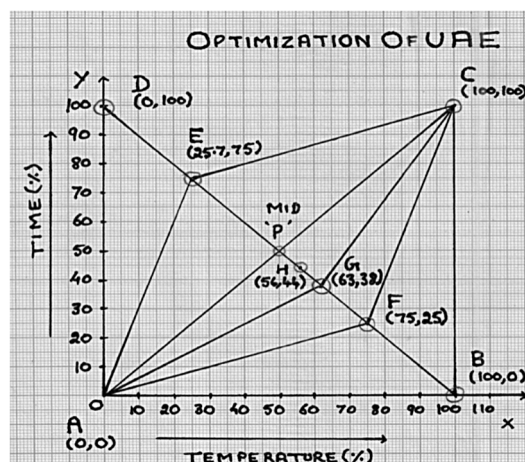


Figure 1. Simplex optimization of UAE

and the response (Ra, Rb and Rc) was measured in each case. The worst response was Rb and the values of the independent variables for the next experiment D were chosen by moving away from point B (reflection). This was achieved by reflecting the triangle ABC about AC axis. Hence BP = DP. The experiment at point D was performed. The response at D was lower than response at A and at C whereas it was greater than response at B. The procedure was to locate the next experiment (E) along the DP axis at P + 0.5 P (contraction). The response at point E was greater than that at D and B but it was less than that at A and C. Thus considering the triangle AEC, the values of the variables for the next experiment were chosen by reflecting the Δ AEC along the AC axis and the point F was chosen such that EP = PF. The response at F was lower than that at A and C and was greater than that at E, the next point G was located along the EF axis at EP + 0.5 P (contraction). The yield at point G was maximum. Now taking Δ AGC into consideration, the lowest yield was at point C, reflecting the point C moves the parameters out of the boundaries. Thus, moving to next lower point A, reflecting the point A also moves the parameters out of the boundaries. So, point G was the only point left. In order to compliment, another point H was chosen, the yield at H was nearly equal to Rg. The yields at last two points were virtually the same indicating that the maximum was nearby. Thus the process was optimized (Fig. 1).

Preliminary phytochemical screening

The phytochemical screening involved testing of extracts prepared by using percolation, optimized MAE and UAE for their contents of different class-

es of compounds. The well documented tests were used to detect various phytochemicals (36, 37).

Thin layer chromatography

The extracts obtained by aqueous percolation, optimized MAE and optimized UAE were taken and ethyl acetate soluble fractions were prepared. The silica gel 60 F₂₅₄ aluminum sheets were used and taking pterostilbene as reference, a mobile phase comprising of *n*-hexane and ethyl acetate (7.5:2.5, v/v) was prepared. The plate was developed and visualized using UV chamber and iodine chamber. The comparison was done using retention factors.

HPLC analysis

Aliquots of sample extracts were filtered through nylon filter (45 µm) prior to HPLC analysis. The mobile phase consisted of (A) 0.1% trifluoroacetic acid in Milli-Q water and (B) acetonitrile. The elution profile was as follows: 0 min, 95% A, 5% B; 5 min, 95% A, 5% B; 20 min, 5% A, 95% B; 25 min, 5% A, 95% B; 30 min, 95% A, 5% B. A constant flow rate of 0.5 mL/min was maintained. Method validation (specificity, linearity, system suitability tests: calibration range, plate number, tailing factor, relative standard deviation or precision, limit of detection, limit of quantification) was performed with pure standard. The sample injection volume was 2.5 µL. The eluates were monitored at 250 nm. Quantification of pterostilbene was done by using the following formula:

$$\text{Pterostilbene (\%)} = \frac{\text{Sample Area}}{\text{Avg. Std. Area}} \times \frac{\text{Std. Conc.}}{\text{Sample Conc.}} \times \frac{\text{Std Potency}}{100} \times 100$$

where: Avg. Std. Area = average standard area, Std. Conc. = standard concentration, Sample Conc. = sample concentration and Std. Potency = standard potency.

HPLC method validation

(A) *Determination of specificity*: The specificity was assessed by comparing analytical results obtained from reference containing the analyte only with results obtained from samples containing excipients, related substances or degradation products, and including or excluding the analyte.

(B) *Determination of linearity*: For the establishment of linearity, five solutions of different concentrations (25, 45, 55, 110, and 220 ppm) of pterostilbene were injected; chromatograms were obtained and the calibration plot was drawn between concentration and area. The linearity was evaluated by the visual inspection of the plot and also by calculating correlation coefficient.

(C) *Determination of calibration range*: The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analytes in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. The specified range was derived from linearity studies and depends on the intended application of the procedure.

(D) *Determination of number of theoretical plates*: The system was injected with 6 replicate injections of pterostilbene in the working concentration of 45 ppm. The number of theoretical plates is the measure of the sharpness of the peaks and therefore the efficiency of the column and was calculated using the half width method.

(E) *Determination of tailing factor*: Tailing factor is the distance from the front slope of the peak to the back slope divided by twice the distance from the centre line of the peak to the front slope, with all measurements made at 5% of the maximum peak height.

(F) *Determination of relative standard deviation or precision*: The retention time precision and the peak area precision were calculated to determine the repeatability of the system.

(G) *Determination of limits of detection and quantification*: The limit of detection (LOD) and limit of quantification (LOQ) were determined by analyzing pure standards. The lowest concentration of analyte that gave a measurable response with a signal-to-noise ratio lying in between 2 to 3 was determined.

RESULTS AND DISCUSSION

Conventional extraction methods

The powdered heartwood of *Pterocarpus marsupium* was extracted with aqueous and ethanolic solvents using four different conventional methods, i.e., infusion, decoction, maceration and percolation. The extraction yield using aqueous solvent was significantly greater than extraction yield using ethanolic solvent, ($p < 0.05$) (Fig. 2). Now taking aqueous extractions into consideration, the analysis of variance (ANOVA) was applied. Since decoction is the official extraction method (20), thus it was taken as control and the Dunnett's *t*-test was applied. The aqueous percolation brought significant change ($p < 0.05$) in the extraction yield when compared to the aqueous decoction as well as other conventional extraction methods (Table 1).

Table 1. Extraction yield using conventional and non conventional extraction methods.

No.	Weight of dried extract (g)						Conventional methods	Expt. in non conventional methods
	Aqueous			Ethanollic				
	Conventional	MAE	UAE	Conventional	MAE	UAE		
1	21.50	6.00*	6.19*	7.10	2.20	1.78	Infusion	(1)
2	21.00	6.18*	6.23*	8.68	2.50	1.76		
3	24.91	6.23*	5.82*	13.86	2.03	1.77	Decoction	a
4	21.53	6.21*	5.76*	13.10	1.99	1.77		
5	24.00	7.04*	6.00*	10.92	3.05	1.98	Maceration	b
6	24.54	7.30*	6.12*	10.30	3.05	1.90		
7	28.50*	7.50*	5.90*	11.90	2.18	2.00	Percolation	ab
8	26.45*	7.44*	5.92*	12.62	2.16	2.04		

*Values are significant ($p < 0.05$).

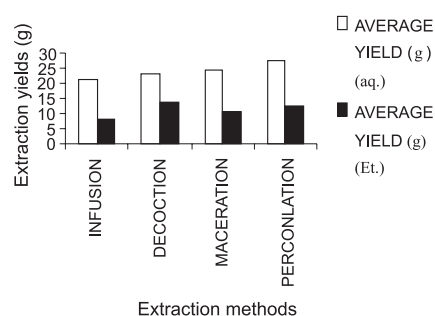


Figure 2. Comparison of extraction yields among conventional extraction methods using aqueous and ethanolic solvents

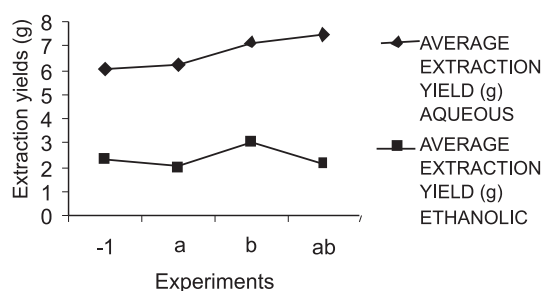


Figure 3. Comparison of extraction yields between aqueous and ethanolic microwave-assisted extraction method.

Non-conventional extraction methods

(A) MAE: The extraction yield (Table 1) using aqueous solvent was significantly greater ($p < 0.05$), than the extraction yield using ethanolic solvent (Fig. 3). The effect of factor A (power) was found to be 0.125 and that of factor B (time) 1.165. Thus, factor B was more significant out of the two. The interaction term comes out to be 0.085 which was very low. Thus, there was negligible interaction between the two factors. The MAE of the powdered heartwood of *Pterocarpus marsupium* was carried out, taking factor A, i.e., power at 100% and varying the factor B, i.e., time. The total weights of the dried extract were recorded (Table 2). The MAE in the time range of 8 to 40 min produced significant results ($p < 0.05$) when compared with the MAE done for 2 min. The results showed that the extraction efficacy of MAE (aq) was maximal when the *Pterocarpus marsupium* was extracted at 100% power and for 30 min (Fig. 4).

(B) UAE: The extraction yield (Table 1) using aqueous solvent was significantly greater ($p < 0.05$), than the extraction yield using ethanolic solvent (Fig. 5). The effect of factor A (temperature) was found to be -0.285 and that of factor B (time) -0.015 . Thus, the effects were shown by both factors; however, the difference between the effects was small. The interaction term comes out to be 0.135 which was very low. Thus, there was negligible interaction between the two factors. The experiments were performed by taking various combinations of these factors and the reflection, contraction or expansion were done (Fig. 1). This ultimately led to the point G (temperature 47°C and time 26 min), which were the optimized conditions for UAE (aqueous) (Table 3).

The total extract contents obtained by MAE (aqueous, 100% power, 30 min) was 33% and 38% higher than those obtained by using percolation (aqueous) and UAE (aqueous, 47°C , 26 min), respectively (Table 4).

Table 2. Optimization of MAE (aqueous) at 100% power.

Experiment No.	Time (min)	Extraction yield (g)	Average yield (g)
1	2	6.17	6.19
2	2	6.20	
3	5	6.21	6.22
4	5	6.23	
5	8	6.76	6.79*
6	8	6.82	
7	11	7.00	7.04*
8	11	7.08	
9	15	7.35	7.40*
10	15	7.45	
11	20	7.45	7.43*
12	20	7.41	
13	25	7.44	7.47*
14	25	7.50	
15	30	9.20	9.15*
16	30	9.10	
17	35	8.03	8.02*
18	35	8.01	
19	40	7.54	7.58*
20	40	7.62	

*Values are significant ($p < 0.05$).

Table 3. Optimization of aqueous UAE by simplex search.

Points	Temperature		Time		Yield (g)
	Original value (°C)	Normalized value (%)	Original value (min)	Normalized value (%)	
A	25	0	5	0	6.21
B	60	100	5	0	5.79
C	60	100	60	100	5.91
D	25	0	60	100	5.81
E	34	25.7	46	75	5.86
F	51	75	19	25	5.90
G	47	63	26	38	6.63
H	45	56	29	44	6.62

Table 4. Comparison of extraction yield by percolation (aqueous) and MAE (aqueous, 100% power and 30 min) and UAE (aqueous, 47°C, 26 min).

Average extraction yield (%)		
Percolation (aqueous)	MAE (aqueous, 100% power, 30 min)	UAE (aqueous, 47°C, 26 min)
13.73	18.3	13.26

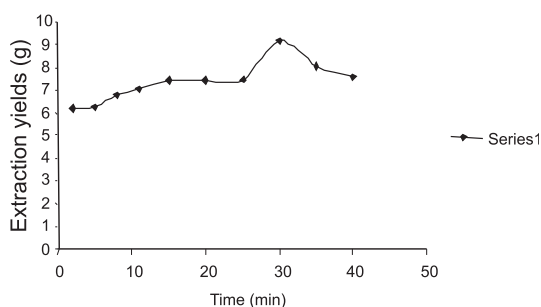


Figure 4. Optimization curve of MAE (aq.)

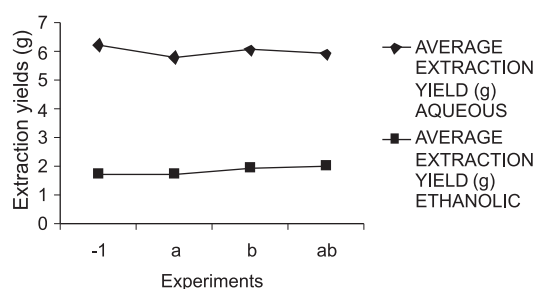


Figure 5. Comparison of extraction yield between aqueous and ethanolic UAE

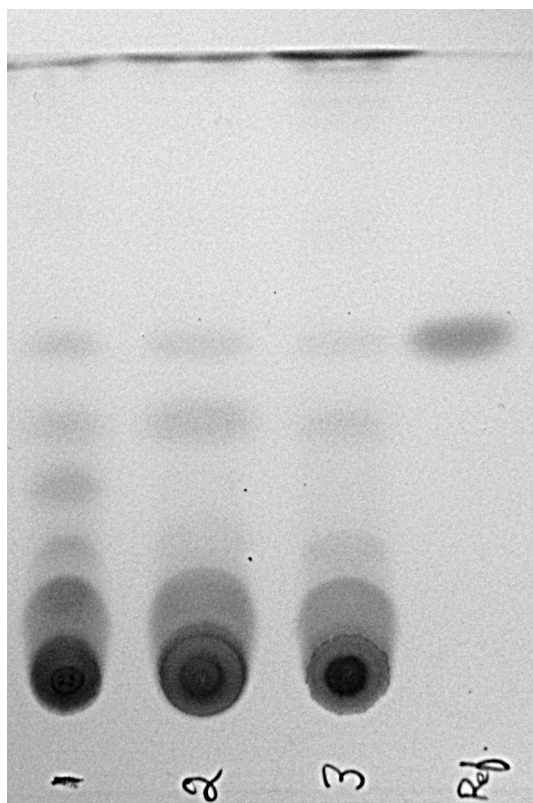


Figure 6. TLC of ethyl acetate soluble fractions of the three extracts and pterostilbene standard. Extract 1: percolation extract; Extract 2: MAE extract; Extract 3: UAE extract and Ref.: pterostilbene

Preliminary phytochemical screening

All the three aqueous extracts showed the absence of alkaloids, glycosides, sterols and oils and fats and the presence of carbohydrates, phenolic compounds and tannins, saponins, flavonoids, acidic compounds and proteins and free amino acids. The

chemical composition of the extract obtained by the conventional method of extraction matched with the literature survey and the chemical composition of the extracts obtained by the non conventional methods matched with that of the conventional methods.

Thin layer chromatography

The chemical composition of the ethyl acetate soluble fraction of the extracts prepared by using non conventional methods was similar to that which was prepared by using conventional method. All the three samples show the Rf value of the spot similar to that of the reference standard pterostilbene (0.538). This confirmed the presence of pterostilbene in extracts prepared by using non conventional methods as well as that prepared by using conventional method (Fig. 6).

HPLC method validation

(A) *Specificity*: The pterostilbene was used as a marker compound. The chromatogram of the standard pterostilbene was obtained (Fig. 7). To test the specificity, a blank run was conducted which showed no peak at 20.32 min retention time.

(B) *Linearity*: The linearity of the method was determined. The chromatograms of the five solutions with different concentrations of pterostilbene were plotted. The peak area was calculated and plotted against concentration. A linear relationship was observed and correlation coefficient was calculated to be 0.996 (Fig. 8).

(C) *Calibration range*: The calibration ranged between 25–220 µg. In between this range, the system had a suitable level of precision, accuracy and linearity.

(D) *Theoretical plates*: The number of theoretical plates came out to be fairly high, 141,877, ensuring that the column was reasonably efficient.

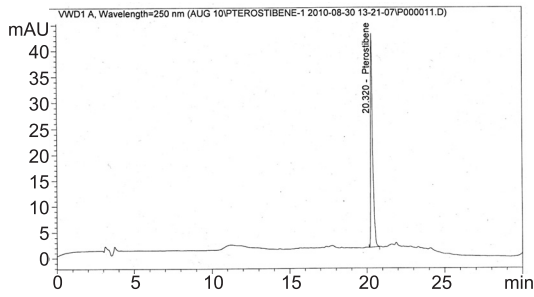


Figure 7. Standard chromatogram of pterostilbene

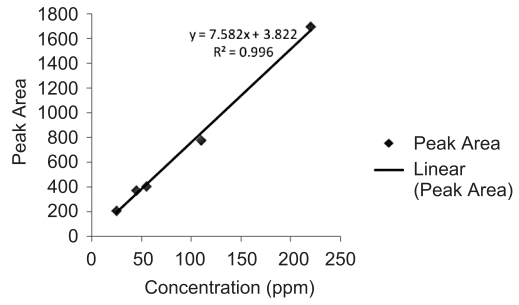


Figure 8. Linearity study

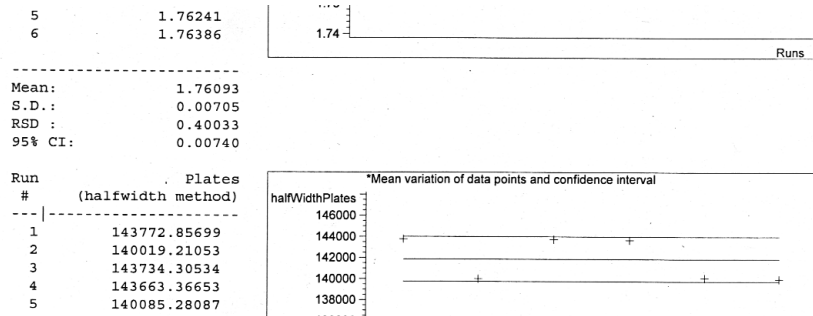
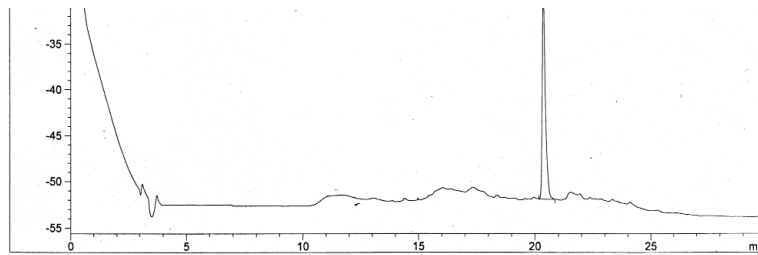


Figure 9. HPLC method validation (theoretical plates, tailing factor and % RSD)



Area Percent Report with Performance and Noise

Calib. Data Modified : 8/31/2010 9:34:29 AM
 Multiplier: 1.0000
 Dilution: 1.0000
 Use Multiplier & Dilution Factor with ISTDs

Signal 1: WVD1 A. Wavelength=250 nm

Noise determination:

Time range	Noise (6*SD)	Noise (PtoP)	Noise (ASTM)	Wander	Drift
from [min]	to [min]	[mAU]	[mAU]	[mAU]	[mAU /h]
7.000	9.000	3.336e-2	2.061e-2	2.132e-3	1.726e-2
					3.303e-1

Figure 10. HPLC method validation (LOD and LOQ)

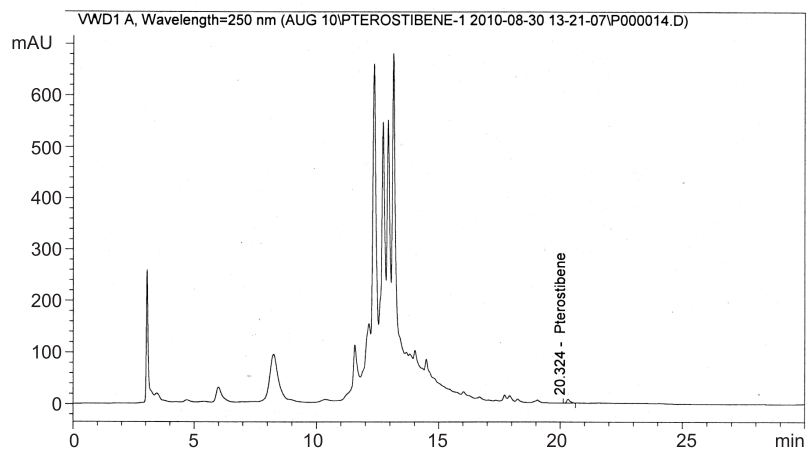


Figure 11. Chromatogram of the extract obtained by percolation (aq.)

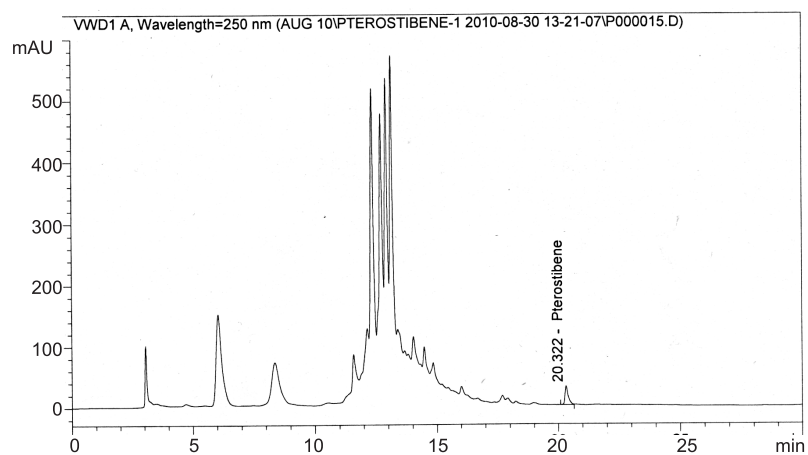


Figure 12. Chromatogram of the extract obtained by MAE (aq.)

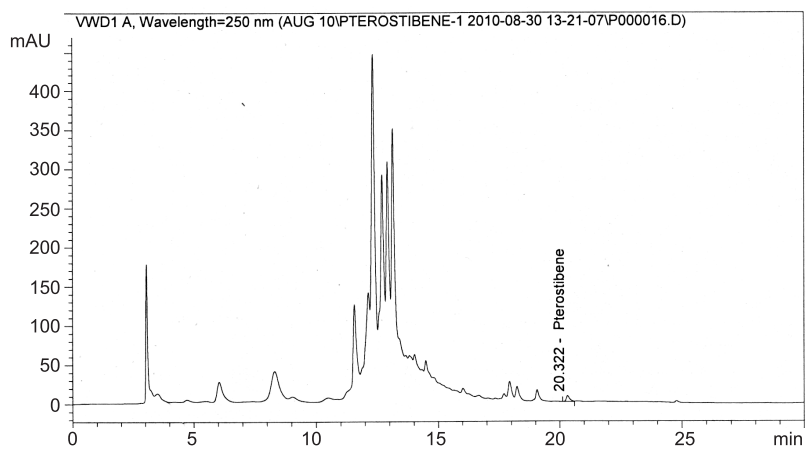


Figure 13. Chromatogram of the extract obtained by UAE (aq.)

(E) *Tailing factor*: The tailing factor came out to be 0.4% indicating that the peak was sufficiently asymmetric.

(F) *Relative standard deviation or precision*: The relative standard deviation of the retention time and peak area was 0.022 and 0.306%, respectively. This ensured the repeatability of the method (Fig. 9).

(G) *Limits of detection and quantification*: The limit of detection of the peak area and concentration was 6.39 and 0.758 ppm, respectively. The limit of quantification of the peak area and concentration was 21.3 and 2.526 ppm, respectively (Fig. 10).

Thus the analytical method was validated. The three optimized aqueous extracts, which were prepared using percolation, MAE and UAE were subjected to HPLC (Figs. 11–13). The amount of pterostilbene present in each extract was determined. The MAE extract contained maximum percentage of pterostilbene (0.667%), which was higher than that obtained using percolation (0.176 %) or UAE (0.171%). This implies that there was approximately 279 and 290% increase in the yield of pterostilbene when obtained using MAE as compared to that obtained by using percolation and UAE, respectively.

CONCLUSION

The main conclusion of this study is that microwave-assisted extraction could be a powerful technique for the extraction of phytochemical agents from the heartwood of *Pterocarpus marsupium*. The results showed that the extraction efficiency using MAE was much higher, than that for percolation or UAE. All the three extracts showed similar phytochemical constituents and TLC indicated the presence of pterostilbene (reported to be one of the marker compounds) in all three extracts, however, the proportion of pterostilbene was much higher in MAE. The results indicate that both UAE and MAE are better methods of extraction, so far as the time of extraction, and efficiency of extraction are concerned, as compared to the conventional methods. Thus, in combination with HPLC measurements, a more systematic study of extraction of heartwood of *Pterocarpus marsupium* has been given.

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