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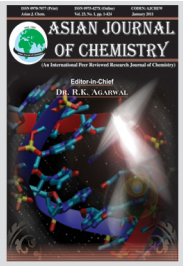

Microwave-Assisted Extraction of Gymnemic Acid: A Potent Antidiabetic Agent from *Gymnema sylvestre* R.B. and its Comparison with Conventional Extraction Technique

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Microwave assisted extraction and liquid-liquid extraction have been investigated to determine the content of gymnemic acid from leaves of *Gymnema sylvestre* R.Br. The extraction of gymnemic acid is commercially very important because it is a potent antidiabetic agent and used in various pharmaceutical products. The effect of single factors such as microwave power, microwave irradiation time, extraction solvent volume, sample size, *etc*. are evaluated and standardized. Microwave assisted extraction and liquid-liquid extraction has been comparatively evaluated for their efficiency to extract the content of gymnemic acid from leaves of *Gymnema sylvestre*. The extracts obtained by various extraction techniques were analyzed for the content of gymnemic acid by validated RP-HPLC method. Deacylgymnemic acid was used a reference standard and the amount of gymnemic acid was calculated by using molecular weight correction factor. Taking into account the extraction yield, extraction time, solvent and cost of extraction, better results were obtained by microwave assisted extraction (50 % methanol 5 min at 20 % power with minimum sample size). By applying microwave assisted extraction the use of organic solvents was minimized. Toxicity and comparative bioavailabilty of the microwave assisted extraction extract were also evaluated on animal model.

Key Words: Gymnemic acid, *Gymnema sylvestre* R.Br, Microwave-assisted extraction, Antidiabetic agent.

INTRODUCTION

Gymnema sylvestre is a plant used in Ayurvedic and folk medicine in India and parts of Asia as a natural treatment for diabetes[1,2]. *Gymnema sylvestre* was noted for its ability to rid the body of excess sugars and was thus called Gurmar meaning destroyer of sugar [3,4]. The major bioactive constituents of *Gymnema sylvestre* are a group of oleanane type triterpenoid saponins known as gymnemic acids. The latter contain several acylated (tigloyl, methylbutyroyl *etc*.,) derivatives of deacylgymnemic acid (DAGA), which is 3-O-glucuronide of gymnemagenin (3,16,21,22,23,28-hexahydroxy-olean-12-ene). The individual gymnemic acids (saponins) include gymnemic acids **I-VII**, gymnemosides **A-F**, gymnemasaponins and are reported to lower and balance blood sugar levels (Fig. 1)[5-7]. It blocks the sugar absorption in small intestine and stimulates the β-cells of pancreas. Gymnemic acid from *Gymnema sylvestre* even rejuvenates the pancreas[8,9]. In clinical studies of animals with diabetes, *Gymnema sylvestre* also appeared to reduce body weight, blood cholesterol and triglyceride levels therefore recently it is also used in antiobesity treatment[10, 11].

On the basis of gymnemic acid extracts content of plant raw materials can be finalized for use in herbal formulation where by the quality and efficacy of the formulations can be enhanced. Presently gymnemic acid is extracted by conventional extraction techniques like liquid-liquid extraction, but these techniques are much time consuming and require large volume of extraction solvents. Moreover, many natural products are thermally unstable and could be degraded during the extraction[12]. Traditionally for extraction of gymnemic acid, liquid liquid extraction had been mainly used. These techniques are time consuming and require large volume of organic solvents, whose subsequent disposal creates several environmental hazards[13,14].

In contrast microwave assisted extraction is known as one of the best green technologies. The main advantages of microwave assisted extraction with respect to other techniques are both the considerable reduction in extraction time and solvent consumption, if compared to conventional extraction also it have advantage of high extraction efficiency and better reproducibility[15]. Microwave assisted extraction is based on the selective and rapid localized heating of moisture in the sample by microwave. Due to localized heating, pressure builds

up within the cells of the sample, leading to a fast transfer of compounds from the cells into the extraction solvent[16]. Microwave energy offers numerous potential processing advantages over the conventional heating methods to provide a rapid and volumetric heating to an absorbing medium[17]. For most materials, in particular biological tissues, the maximum penetration of electromagnetic energy occurs in the microwave range[18]. In literature many reports have been published on the application of microwave assisted extraction to extract bioactive constituents and secondary metabolites from plants such as artemisinin from *Artmisia annua*[19-22].

The present work standardizes the extraction of gymnemic acid from *Gymnema sylvestre* by using microwave assisted extraction technique. The effect of single factors such as microwave power, microwave irradiation time, extraction solvent volume, sample size *etc*. are evaluated and standardized. Microwave assisted extraction and liquid-liquid extraction have been comparatively evaluated for their efficiency to extract the content of gymnemic acid from leaves of *Gymnema sylvestre*. Toxicity and bioavailabilty of the extracts is evaluated using an animal model.

EXPERIMENTAL

The leaves of *Gymnema sylvestre* were collected in October, 2009 from Mumbai, India. The freshly cut leaves were sorted out, dried in a drying room with active ventilation at room temperature (23-25 ºC) until constant weight. The leaves were ground with a grinder to obtain a homogeneous drug powder. The powder was stored in closed airtight container and preserved in the refrigerator[23-25]. Plant Material was authenticated by Botanical Survey of India, Pune.

All solvents were from Merck fine chemicals (Mumbai, India) and were HPLC grade. The buffer was of analytical grade. Standard of deacylgymnemic acid was obtained from Natural Remedies, India.

Conventional extraction technique: Liquid-liquid extraction (LLE) was performed in a clean stopper volumetric flask with different methanol as a extraction solvent; the exhaustive extraction was performed on accurately weighted 0.5 g drug powder, add 10 mL of 2 M NaOH and let it dissolve. Heat it on a water bath for 1 h. After cooling, add conc. HCl so that pH of the solution is under 8.0 ± 0.05. Add methanol to make up the solution to 50 mL. Filter through 0.45 m membrane filter.

Microwave assisted extraction: Microwave assisted extraction was performed on a modified domestic microwave oven (Model GMC25E09MRGX, Godrej, India) equipped with a magnetron of 2450 MHz with nominal maximum power of 1400 W operated at 3 power levels and the time controller, 0.50 g of plant powder was placed into the round bottom flask fitted with a water condenser in addition with 10 mL of 2 M NaOH subjected to different time of irradiation and irradiation power. Before extraction the crude powder was soaked for 2 min. After the extraction time had elapsed the vessels were allowed to cool to room temperature and conc. hydrochloric acid was added so that pH of the solution is less than 8.0 ± 0.05. After this the volume of solution was made up to 50 mL with methanol, this solution was filtered through 0.45 µ membrane filter.

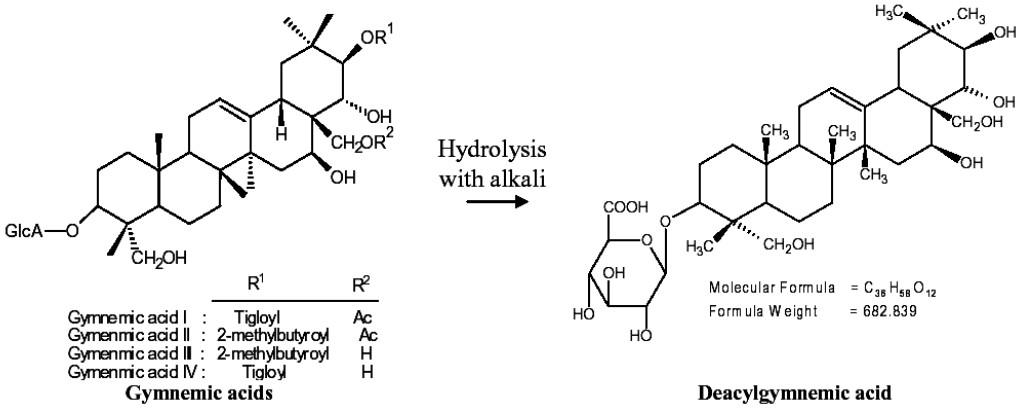
HPLC- analysis: Gymnemic acid was quantified by high performance liquid chromatography with ultraviolet detector (RP-HPLC-UV/PDA). A Jasco HPLC system (Japan) consisting of PU-980 pump and MD-910 detector (210 nm) was used. Sample solutions were injected using an auto sampler AS 2057. Experimental data were acquired and processed by borwin PDA and borwin chromatography software.

The chromatographic separation was carried out by using chromolith performance RP-18 100 × 4.6 mm column, (Merck, Germany). The mobile phase consisted of 0.1 % trifluroacetic acid: acetonitirle in the ratio of (80:20) v/v, total run time was 10 min and flow rate was set at 1 mL/min.

All the mixtures obtained by liquid-liquid extraction and microwave assisted extraction was centrifuged (REMI, R-8C DX)) at 4000 rpm for 5 min, before injection each sample solution was filtered through 0.45 µ membrane filter paper. The injection volume was 50 µL; each determination was carried out in triplicate.

RESULTS AND DISCUSSION

Extraction methods: Extraction procedures with liquid-liquid extraction and microwave assisted extraction were investigated in order to exhaustively extract gymnemic acid from *Gymnema sylvestre*. Because of unavailability of gymnemic acid reference standard, deacyl gymnemic acid reference standard was used to determine the yield of gymnemic acid. The extraction was carried out by using alkali hydrolysis of the plant material (Fig. 1). Three extractions were carried out with each method. At first we tested the liquid-



	R^1	R^2
Gymnemic acid I :	Tigloyl	Ac
Gymenmic acid II :	2-methylbutyroyl	Ac
Gymenmic acid III :	2-methylbutyroyl	H
Gymenmic acid IV :	Tigloyl	H

Gymnemic acids **Deacylgymnemic acid**

Fig. 1. Gymnemic acid and deacylgymnemic acid

TABLE-1
LIQUID-LIQUID EXTRACTION (LLE) AND MICROWAVE ASSISTED EXTRACTION (MAE)

Extraction technique	Extraction solvent	Sample size extraction time	Gymnemic acid (%)Yield + SD (Average ± S.D) [a]
LLE	50 mL methanol	0.5 g crude drug powder for 1 h	2.06 ± 0.21

Microwave assisted Extraction, effect of extraction variables [b]

Irradiation time (min)	% Extraction yield of gymnemic acid (average ± SD) [a]							
	Microwave assisted extraction irradiation power (%)							
	10	20	30	50	60	70	90	100
5	2.19 ± 0.10	2.61 ± 0.098	1.89 ± 0.098	2.10 ± 0.15	1.53 ± 0.13	1.28 ± 0.12	1.14 ± 0.13	1.1 5 ± 0.10
10	2.24 ± 0.12	2.24 ± 0.12	2.10 ± 0.13	1.87 ± 0.14	1.45 ± 0.19	1.22 ± 0.16	0.98 ± 0.11	0.95 ± 0.12
15	2.19 ± 0.18	2.24 ± 0.099	1.80 ± 0.13	1.51 ± 0.14	1.23 ± 0.15	1.39 ± 0.18	-	-
20	2.16 ± 0.17	2.21 ± 0.11	2.05 ± 0.12	1.44 ± 0.18	1.29 ± 0.14	0.82 ± 0.20	-	-
30	2.21 ± 0.14	2.20 ± 0.12	2.15 ± 0.11	1.28 ± 0.17	1.035 ± 0.19	0.85 ± 0.19	-	-

[a] Triplicates extraction.
[b] Extraction solvent, 50 % aqueous methanol, 0.5 g crud powder.

TABLE-2
COMPARISON OF EXTRACTION YIELDS OF GYMNEMIC ACID BY MICROWAVE ASSISTED EXTRACTION AND LIQUID-LIQUID EXTRACTION

Extraction technique	Solvent volume (mL)	Sample size	Extraction time (min)	Yield of gymnemic acid ± SD
Liquid-liquid extraction	50 mL MeoH	0.5 g crude drug powder	60	2.06 ± 0.21[c]
Microwave assisted extraction	50 mL 50 % Aq. MeOH	0.5 g crude drug powder	5	2.61 ± 0.098[c]

[c] Compared for the yield, used for statistical analysis

liquid extraction method by using methanol; the extraction was performed for 1 h on a boiling water bath. A result reported in Table-1 showed that extraction yield with liquid liquid extraction is not satisfactory and requires more extraction time.

Microwave assisted extraction (MAE): Finally, we have studied the applicability of microwave assisted extraction to the extraction of gymnemic acid. In order to obtain grater extraction yield, effects of microwave on the compound yield and different irradiation times were compared. Effect of solvent volume, sample loading and preleaching time also studied to achieve better extraction of gymnemic acid. The optimal extraction conditions for gymnemic acid are 0.5 g sample in 50 mL of 50 % aqueous methanol, irradiation of 20 % microwave power (300 W) for 5 min. The repeated extraction was observed reproducible (Table-1). The short extraction time required by microwave assisted extraction allowed better extraction of gymnemic acid as compared to conventional extraction method.

It is worth nothing that microwave assisted extraction is the best technique to extract gymnemic acid, being the differences respect to the amount extracted and time required with, liquid-liquid extraction (1 h) was significant (Table-2).

Effect of microwave power and irradiation time: Fig. 2 shows the yield power plots for the extraction of gymnemic acid. It was observed that the yield of gymnemic acid increases with increase in microwave power between 10 % (140 W) to 40 % (600 W) with respect to time. The significant improvement in extraction yield was achieved at 20 % power (300 W) with very lees irradiation time 05 min. However, there was no increase in yield after 10 min and the yield decrease significantly as we increase the microwave power; probably this was due to thermal degradation of active constituent (Fig. 2).

Microwave irradiation time of 5 to 30 min at various power levels was optimized. Based on above observation, extraction at 20 % microwave power (300 W) with 5 min irradiation time considered optimum for the extraction of gymnemic acid.

Effect of extraction solvent volume: The solvent volume always must be sufficient to ensure that entire sample is immersed during process. In traditional extraction techniques the performance increases with increase in solvent volume, but in microwave assisted extraction higher solvent volumes are not required. To investigate influence of solvent with sample size on extraction yield solvent volume was varied from 20 to 150 mL. It can see in Fig. 3 that there was increase in yield with amount of solvent, but after 50 mL it was felt down, the large volume of solvent will cause more absorption of microwave energy and thus sufficient microwave energy may not be available for facilitating the cell breakage for effective leaching out of target analyte[26,27].

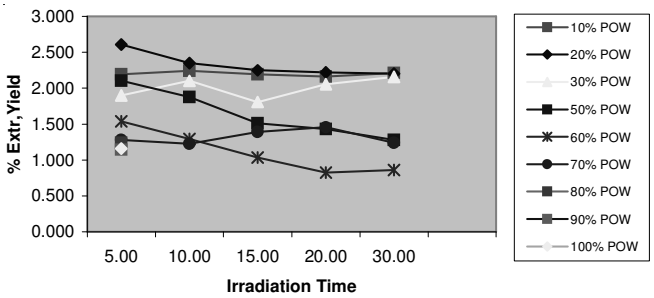


Fig. 2. Effect of microwave irradiation power and time on extraction yield

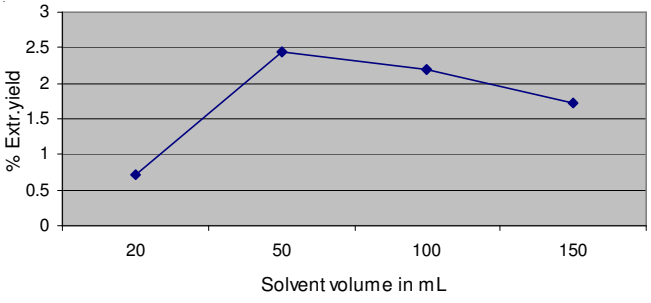


Fig. 3. Effect of solvent volume on extraction yield

In order to study cell damage during microwave assisted extraction experiments, the *Gymnema sylvestre* leaf powder sample were used for microscopic observation. There is significant difference in cells of liquid-liquid extraction and microwave assisted extraction processed samples were observed Fig. 4.

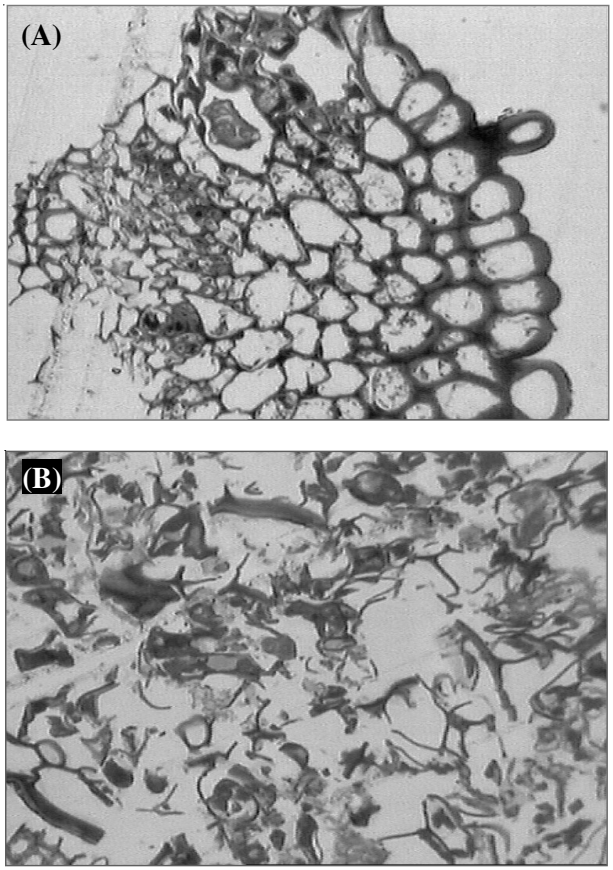


Fig. 4. Semi thin sections of plant powder (A) liquid-liquid extraction, (B) microwave assisted extraction residue after extraction

Effect of sample loading: The effect of sample loading on extraction of gymnemic acid was studied with extraction solvent at 20 % power level. Fig. 5 shows that the extraction of gymnemic acid is decreased as sample loading increases. The consequence of the shorter penetration depth is that the material in the interior of the irradiated sample will not be subjected to the same level of microwave irradiation and hence is affected to lesser degree by the incident microwaves. Because of internal waves created in microwave oven a small variation in the sample thickness could make a large difference in heating rates of sample[28,29].

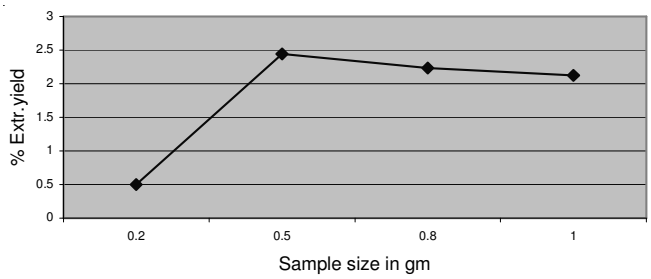


Fig. 5. Effect of sample loading on extraction yield

Quantitative analysis by HPLC: Analyses of extracts of *Gymnema sylvestre* were carried out by isocratic HPLC method[30]. The method was optimized to get better resolution and peak shape for deacylgymnemic acid. A typical HPLC-UV chromatograms of MAE processed sample and gymnemic acid standard, respectively are shown in Fig. 6.

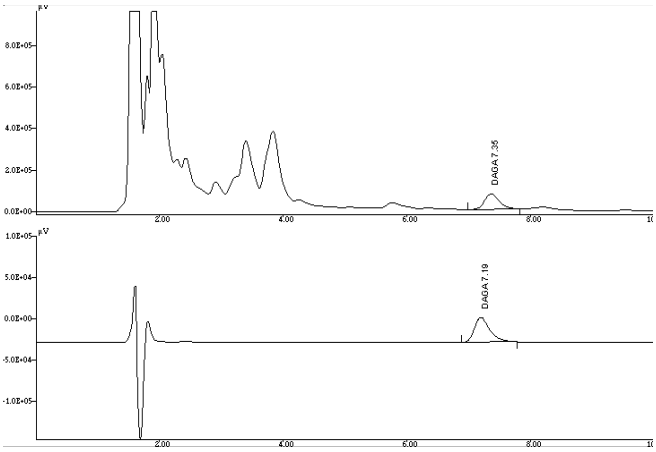


Fig. 6. HPLC chromatograms of deacylgymnemic acid (A) microwave assisted extraction processed sample (B) deacylgymnemic acid standard

The chromatographic method present good linearity in the concentration range considered. The calibration curve in the range of 10 to 200 µg/mL for deacylgymnemic acid, (seven points of calibration) was linear with a correlation coefficient (R) 0.9980. Quantification was performed by comparing the chromatographic peak areas of extracted samples with that of pure reference standard (Fig. 6). Molecular weight correction factor was used for the determination of gymnemic acid. The components of extracts were identified comparing HPLC retention time.

Method validation was performed for recovery, precision, accuracy and stability. Recovery was investigated at 80, 100 and 120 % level, 0.8, 1.0 and 1.2 mg of deacylgymnemic acid standard was added in 0.5 g of crude drug powder and processed as per the proposed method. The mean % recovery at above levels was observed 98.07, 97.11 and 98.37 %, respectively. Performing three injections on different days assessed reproducibility of the method. Finally the stability of standard and sample solution was evaluated for 24 h.

Stastical analysis: Student's t test was used to calculate the significance of differences of the gymnemic acid yield among the extraction techniques. The results of HPLC analysis were expressed as means of yield ± SD and the means of liquid-liquid extraction was compared with microwave assisted extraction using student's t-test. The p-values < 0.005 are considered significant[31].

Toxicity and bioavailabilty study: The toxicity of the extracts of *Gymnema sylvestre* was evaluated in albino wistar rats by OECD guideline 420 (fixed dose procedure)[32,33]. Neither mortality nor evident toxicity was observed for the dose of 2-g/kg body weight of animal in 14 days observation period. The comparative bioavailabilty of the extracts were evaluated by using Newzeland strain albino rabbits[34]. Two different processed (liquid-liquid extraction and microwave

assisted extraction) extracts were dosed to animals and blood samples were collected at different time interval after administration. Separated plasma samples were analyzed by validated LC-MS/MS method for the determination of gymnemic acid. No significant difference was observed between bioavailabilty of microwave assisted extraction and liquid-liquid extraction extract.

Conclusion

In the present work, effect of different extraction methods on gymnemic acid yield from *Gymnema sylvestre* R.Br. is reported. Both the method gave different yields and varied significantly in time required to attain the extraction yields. Liquid- liquid extraction showed less reproducibility and also required longer time duration (1 h). Microwave assisted extraction gave the best results in terms of less time (5 min) and better consistency in extraction yields. Moreover, microwave assisted extraction also consumed the least amount of organic extraction solvent as compared to all other conventional extraction methods evaluated.

Microwave assisted extraction thus, appears the simplest, reproducible and efficient method of extraction and it can also be applied for large commercial scale-up extraction processes. The method of microwave assisted extraction provides as an alternative more efficient technique to extract an important phytoconstituent like gymnemic acid. The bioavailabilty of gymnemic acid extracted by microwave assisted extraction confirms the potential of using more efficient extraction technique for cheaper source of gymnemic acid to manufacture formulations for the management of diabetes.

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