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Study on Extraction and Purification of Gymnemic Acid from *Gymnema sylvestre* R.Br.

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Abstract

Gymnema sylvestre R. Br is one the important medicinal plant of India widely used in indigenous medicine in the treatment of diabetes mellitus. The active principle of the drug is complex mixture of Gymnemic acids found in the leaves. Presently there is a huge demand for the plant in national and international market. The recent studies have shown that the extract of *Gymnema sylvestre* is useful in controlling blood sugar to treat type II diabetes. It increases the insulin producing b-cells of pancreas and significantly reduces the metabolic effects of sugar by preventing the intestine from absorbing the sugar molecules during the process of digestion. The objective of the present investigation was to isolate and characterize the Gymnemic acid from *Gymnema sylvestre* leaves with different solvent systems like petroleum ether, benzene, and methanol. The defatted leaves were extracted under continuous hot extraction in Soxhlet apparatus with 90% methanol gave the maximum yield of gymnemic acid (42%). Gymnemic acid was purified by preparative chromatographic methods i.e., TLC and HPLC, SDS-PAGE and NMR.

Keywords

Gymnema sylvestre, gymnemic acid, extraction, HPLC, SDS-PAGE, NMR

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INTRODUCTION

The conservative estimate shows that there are 250,000 to 500,000 species of plants on Earth [1] and a relatively small percentage (1 to 10%) of these are used as food but substantial number of plants are used for medicinal purposes [2]. Medicinal plants or their parts are primary source of products for the pharmaceutical Industry. It is estimated that even today, two third of the World population relies on plant-derived drugs. The herbal medicine represents probably the first and certainly the oldest system of human health care.

Gymnema sylvestre R.Br. is a valuable medicinal plant belonging to the family *Asclepiadaceae*, native to central and western India and can also be found

growing in tropical Africa and Australia [3]. The Hindi name for *G. sylvestre* is 'Gur-mar', meaning 'sugar-destroying', which is suggestive of the anti-sweet agent. Many monographs on pharmacognostic have emerged as an aid in the pharmacognostic investigations [1; 4; 5]. The process of standardization can be achieved by stepwise pharmacognostic studies. These studies help in identification and authentication of the plant material [6].

Recent clinical trials conducted in India have shown that an extract of *Gymnema sylvestre* is useful for controlling Blood Sugar**.** Today, *Gymnema sylvestre* has become increasingly popular in the United States as a supportive treatment for diabetes. Use of gymnema was well-known to the Indian people since ancient days ("Meshashring") as a source of anti diabetic drugs. In recent years, it became one of the better known names in the world of herbal medicine. It is rich source of many bioactive compounds such as gymnemic acid, stigmasterol, quercitol gymnemin,, lupeol**,** gurmarin, gymnemagenin, etc. which are mainly effective in lowering of blood sugar. Gymnemic acid, the active ingredient of this plant, is extracted from leaves and used widely as anti-diabetic [7], anti-sweetner [8; 9] and anti-hypercholesterolemia [10]. It also has stomachic, diuretic and cough suppressant property [11]. The plant has been reported to possess antimicrobial [12; 13] and ethno-veterinary medicinal properties [14]. In addition, it possesses antimicrobial, hepato-protective, and anti-saccharine activities [15; 16; 4; 17; 18; 19, 20]. Hence, because of these properties, *Gymnema sylvestre* is most important for plant prospecting.

The leaves of gurmar are used medicinally, for its unique property to directly mask the tongue's ability to taste sweet foods; at the same time suppresses glucose absorption from the intestine. This is the reason, it is known as "destroyer of sugar". The fresh leaves when chewed have the remarkable property of paralyzing the sense of taste of sweet substance for some time [21, 22]. The atomic arrangement of gymnemic acid molecules is similar to that of glucose molecules. These molecules fill the receptor locations on the taste buds thereby preventing its activation by sugar molecules present in the food. This prevents craving for sugar. Similarly, Gymnemic acid molecules fill the receptor location in the absorptive external layers of the intestine thereby preventing the sugar molecules absorption, which results in low blood sugar level [23].

Traditionally it was recommended for stomach problems, constipation, liver disease but the recent studies have shown that the extract of *Gymnema sylvestre* is useful in controlling blood sugar to treat type-II diabetes (NIDDM). When Gymnema leaf extract is administered to a diabetic patient it stimulates the pancreas to increase release of insulin [22]. These compounds have also been found to increase fecal excretion of Cholesterol [24; 25], but further studies to prove clinical significance in treating hypercholesterolemia (high serum cholesterol) are required. However, the present study is the first ever attempt for the isolation, purification and characterization of gymnemic acid from four ecotypes of *Gymnema sylvestre* with the purpose to obtain its maximum yield using various techniques.

MATERIALS AND METHODS

Seventeen ecotypes of *Gymnema sylvestre* R.Br. (Asclepiadaceae) were collected from various parts of the country and maintained in our garden. The plant material was properly identified and confirmed with help of various floras [26; 27]. All the chemicals and reagents were used analytical grade purchased from Sigma Chemical Co. and Merck.

Processing of Plant Material

About 3 kg cleaned leaves from each ecotype dried under shade, powdered and passed through 40 meshes and stored in closed vessel for further use. The dried powder material was subjected to soxhelt extraction with petroleum ether, chloroform, methanol for continuous hot extraction.

Extraction of Gymnemic Acid by Hoopers's Method [28]

Step1: Extraction with petroleum ether

100 grams of dry leaf powder was packed into a clean soxhlet extraction unit. One liter of petroleum ether (60-80^{o}C) was added and extracted for 3-6 hours till all the components are soluble in petroleum. Petroleum extract is collected and distilled in a distillation unit. Then a net weight of 25gm of petroleum ether extracts was obtained. Petroleum ether extraction was used for defatting dried leaf power.

Step2: Extraction with 90% methanol

The plant dry powder material was then extracted with 90% methanol. 90% methanol was added and the extraction was carried out for 24-36 hours till the total methanol soluble extract was obtained. The methanol soluble extract was distilled and finally 150gm of the thick paste were obtained.

Step3: Isolation of pure gymnemic acid from methanol extract

150gm dry leaf powder paste of methanol soluble extract was dissolved in 1% of KOH solution on

continuously stirring for 45min to 1 hour. The solution was then filtered through filter paper to separate the undissolved particles. Diluted HCl acid was added slowly under constant stirring, during which the gymnemic acids were precipitated. Precipitated solution was filtered under suction and precipitate was dried. The pure gymnemic acid was obtained.

Biochemical Tests to Confirm the Gymnemic acid

Gymnemic acid gave positive test for phenolics, steroids and glycoside.

Phenolic test: A pinch of gymnemic acid was taken into a clean test tube and dissolved 2ml of methanol. Then a few drops of 1% alcoholic ferric chloride were added.

Steroid test: A pinch of Gymnemic acid was added to a solution of 2ml $CHCl_3$ and 1ml of acetic anhydride. A few drops of Conc. H_2SO_4 were added from the sides of the tubes.

Glycoside test: A pinch of Gymnemci acid was taken in a dried test tube and dissolved in 2ml of methanol. 1ml of alpha naptholalcoholic solution was added from the sides of the test tube.

Thin Layer Chromatography (TLC)

The identification and separation of the components present in different extracts of *Gymnema sylvestre* was carried out by Thin Layer Chromatography. The TLC of Gymnemic acid was performed using different solvent systems i.e., Chloroform: Aceton, Chloroform: Methanol, Toulene: Ethyl acetate: Diethylamine, Ethyl acetate: Petroleum ether. The chromatograms were dried to remove the solvent, cooled and sprayed with the detecting reagents. The plates were dried at 105°C for 5 minutes to enable the full colour of the spots to develop.

Electrophoresis

Known weight of *Gymnema sylvestre* leaf samples were homogenized with 70% ethanol followed by centrifugation at 2000 x g for 10 min. Supernatant was collected and incubated at –20 °C for 12 h. Further the content was centrifuged at 10,000 x g for 15 minutes and the supernatant was discarded. The pellet was dissolved in 100 µl of sample buffer containing 5 ml of Tris-HCl buffer (pH 6.8), 0.5g of SDS, 5g of sucrose, 0.25 ml of Mercaptoethanol and 1ml of 0.5% Bromophenol Blue (W/V). The content was made upto 10ml with distilled water. About 30 µl of samples were loaded onto the 15% SDS-polyacrylamide gels prepared by the method of Laemmli (1970) and run at 70 mA for 2 h [29]. Protein bands were visualized by silver staining procedure.

HPLC Analysis of Gymnemic sylvestre

The leaf extracts were filtered through Sartorius RC-membrane syringe filter (0.20 µm) and 20 µl was injected. Chromatography was carried out using Shimadzu HPLC (Model SPD-10A UV-VIS Detector) and supelcosil LC-18 C 18 column (25 cm x 4.6 mm, 5 µm) with mobile phase consisting of acetonitrile, water and acetic acid (50 : 50 : 0.1). Flow rate was 1.0 ml/min; back pressures 250 psi and compounds read at 210 nm in an UV detector. The total run time was 40 min, but it is preferable if the time is extended to 60 min [30].

Extraction and Purification of the Active Principle for ^{1}H-NMR Study

The leaves of *G. sylvestre* was air-dried at 70°C and stored at –20 °C before use. The fine powder of the biomass weighing about 100 g was immersed in one liter of water at about 40 °C for 1 h with continuous stirring and filtered with thin tissue paper lined with gauge. The water extraction was repeated three times. The combined filtrate was acidified to pH 2.0 by adding 4N H_2SO_4. The resulting precipitate was collected by centrifugation at 10,000 rpm for 10 minutes. The precipitate was then subjected to extraction with ethanol repeatedly to remove hydrophobic materials including Gymnemic acids. The remaining ethanol insoluble fraction was suspended in 300 ml distilled water and neutralized with 2N NaOH. The deep brown supernatant obtained by centrifugation was used for NMR studies [31].

Nuclear Magnetic Resonance (^{1}H-NMR) studies

The use of Nuclear Magnetic Resonance (NMR) measurement was carried out by 400 MHz Fourier transforms NMS system (Jeol GSX 400 NB 400 Hz FT-NMR system, RSIC, IIT, Madras). The sample was prepared by dissolving about 0.2 mg of the semi purified material in 0.4 ml of D_2O and adjusting pH to 7.5 with NaOD. Temperature of the sample was kept at 23°C by blowing temperature controlled air into the cavity.

RESULTS AND DISCUSSION

The detailed and systematic pharmacognostical evaluation would give valuable information for the future studies. The work carried out on this plant was mainly on the methods of extraction of Gymnemic acid in order to obtain its higher yields, separation, identification and purification of the Gymnemic acid by TLC. The extractions were carried out with different solvent systems like petroleum ether, benzene and methanol and were extracted under continuous hot extraction in Soxhlet apparatus. Out of all the three solvents tested, the extraction with 90% methanol gave the maximum yield of Gymnemic

acid. The yields of Gymnemic acid from five ecotypes were calculated and presented in Table 1.

Table 1. Acquisition of 4 ecotypes of *Gymnema sylvestre* from different parts of South India and the percentage of Gymnemic acid

S. No.	Name of the ecotype	Place of collection	Percentage of Gymnemic acid
1	Silent Valley	Kerala	40.8
2	Kollihills	Tamil Nadu	38.6
3	Venkatachalam	Nellore	27.9
4	Rapur	Nellore	25.6

The results obtained on conducting the phenolic test a dark blue color was developed which is the positive test indicating the presence of -OH group in the molecule. A pink/red color ring was formed when few drops of Conc. H_2SO_4 were added from the sides of the tube containing a pinch of Gymnemic acid in a solution of 2 ml $CHCl_3$. This is the positive test for steroids presence in the Gymnemic acid. The glycosidic nature of Gymnemic acid was a disputed question when it was first isolated. Hooper [28] isolated it and proved it to be a glycoside. To confirm the glycosidic nature in the present study, a small pinch of Gymnemic acid was taken in a dried test tube and dissolved in 2ml of methanol. 1ml of alpha naptholalcoholic solution was added from the sides of the test tube. A bluish red ring was developed at the junction of the two layers indicating the presence of glycoside.

Thin layer Chromatography studies were carried with different solvent systems i.e., Chloroform: Acetone, Chloroform: Methanol, Toluene: Ethyl acetate: Diethylamine and Ethyl acetate: Petroleum ether. All the samples of Gymnemic acid with different Rf values (Table. 2). The solvent system Chloroform: Methanol (6: 5) gave better results when compared with the other solvent systems. TLC studies revealed that the profiles are similar when compared with the standard Gymnemic acid having Rf 0.71.

Table 2. Different Rf values of Gymnemic acid

S.No.	Name of the ecotype	Rf value
1	Silent Valley	0.84
2	Kollihills	0.81
3	Venkatachalam	0.75
4	Rapur	0.73

HPLC analysis of active principles of* Gymnema sylvestre *R.Br.

In this study the samples of active principles extracted from dried leaves were used for HPLC analysis (Shimizu *et al.,* 1997). HPLC chromatogram of *G.sylvestre* extract samples of are presented (Figure 1)

1) *Gymnema sylvestre* R.Br. (Silent Valley) acid precipitate (GSA, concentrations of Gymnemic acids: 258 mg/g gymnemic acid II or 161.6 mg/g as gymnemagenin) (Figure 1a).
2) *Gymnema sylvestre* (Kollihills) leaf extract: (Figure 1b).
3) *Gymnema sylvestre* (Venkatachalam) leaf extract (Figure 1c).
4) *Gymnema sylvestre* (Rapur) leaf extracts (Figure 1d).

In this study, the overall active components from *G. sylvestre* leaf sample eluted through HPLC were separated into four fractions based on the retention time of standard samples. Each fraction and cumulative value of all four fractions of leaves was taken as percent. Water soluble compounds in leaf extract sample were found to be higher than other samples.

SDS-PAGE

SDS PAGE was carried out to study the polypeptides or proteinacious based compound present in the *G. sylvestre*. The samples were taken from the callus grown in different combination of growth hormones

supplemented media and elicitor treated (Figure 2) as follows: Lane-1 Standard; Lane-2 Silent Valley sample; Lane-3 Kollihills sample; Lane-4 Venkatachalam sample and Lane-5 Rapur sample.

The Kollihills sample showed more prominent bands approximately at 4, 18, 22, 37 and 56 kDa (Lane-2). Venkatachalam samples showed bands approximately at 5, 8, 12, 14, 16, 18, 45, and 62 kDa (Lane-3). Rapur sample showed bands approximately at 1, 2, 4, 8, 14, 16 and 18 kDa (Lane-4). Silent Valley sample showed bands at 17, 20, 22, 29 and 50 kDa (Lane-5). Lane-1 is standard sample.

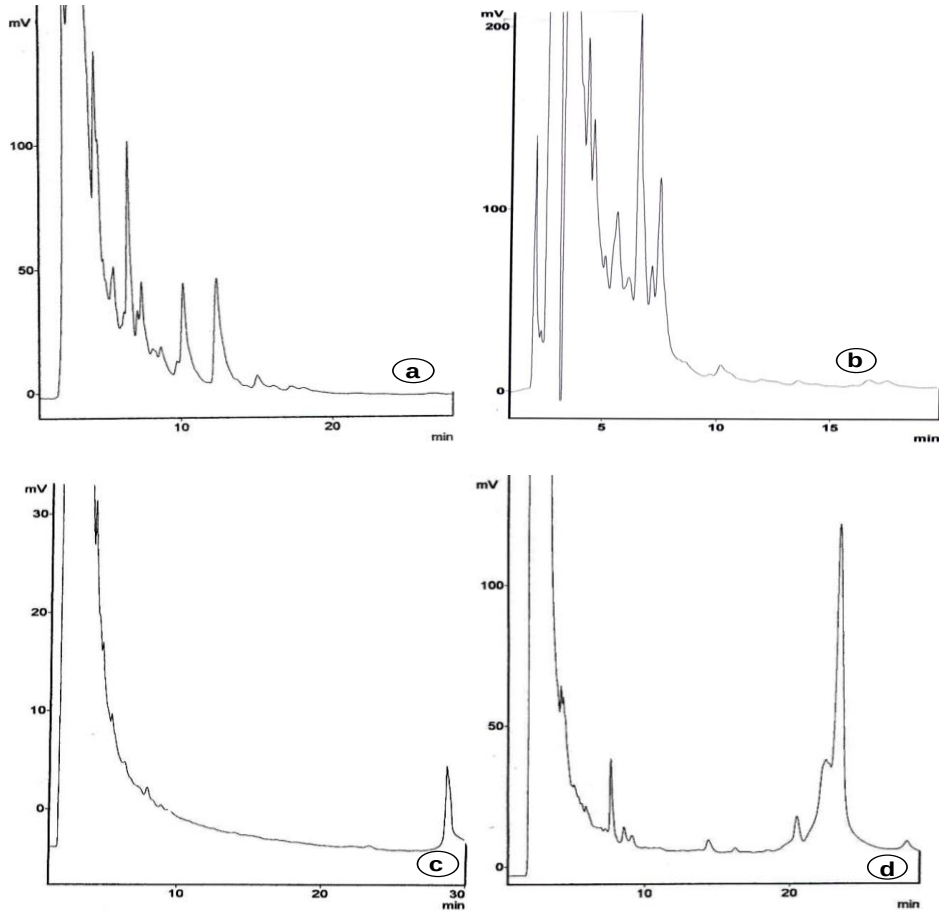
H-NMR studies

H-NMR study carried out with callus sample grown in growth hormones standardized medium was compared with leaf sample. Spectrum of the sample taken at 400 MHz also showed the resonance pattern characteristic of compounds consisting of polypeptide chain. From the results, the spectrum of callus sample was very similar to that of leaf sample (Figure 3).

CONCLUSION

On the basis of the results of the present study, it was concluded that the extraction with 90% methanol under continuous hot extraction in Soxhlet apparatus gave the maximum yield of Gymnemic acid. The Gymnemic acid thus obtained can be further identified, purified and characterized using TLC, HPLC, SDS-PAGE and NMR techniques. HPLC and NMR methods are found to be accurate, precise, and less time consuming and hence, it can be used for analysis of Gymnemic acid and for standardization of herbal drugs in general laboratory conditions. These parameters could be useful in preparation of Herbal drugs.

Figure 1. HPLC chromatogram of leaf samples



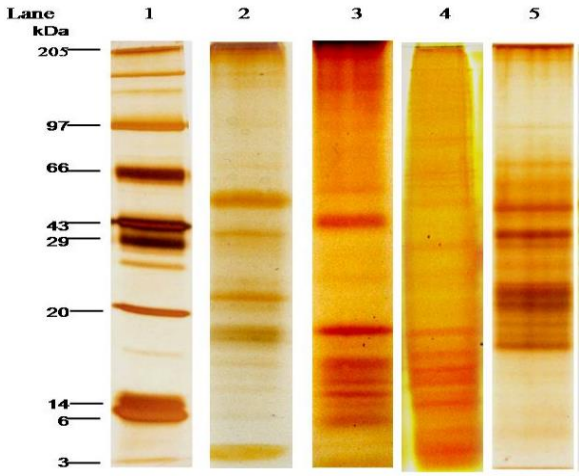


Figure 2. SDS-PAGE

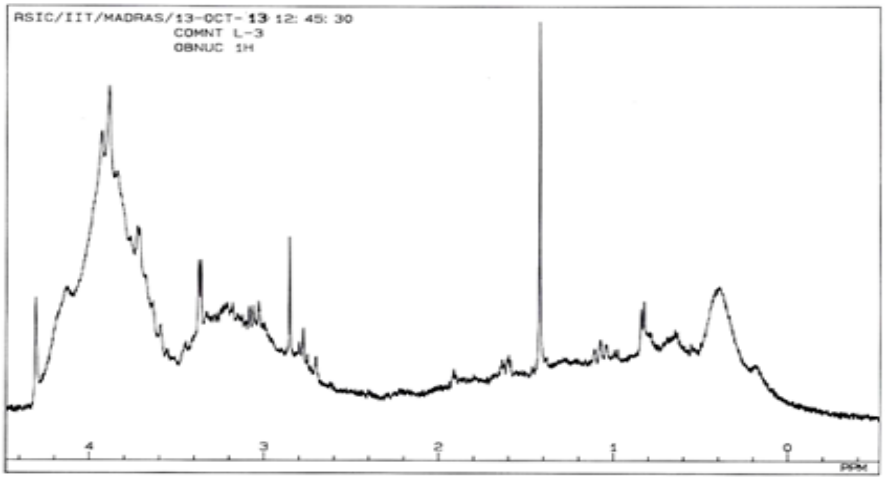


Figure 3. NMR Spectrum of Gymnema sylvestre R.Br.

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