

ISOLATION AND CHARACTERIZATION OF GYMNEMIC ACID FROM INDIGENOUS *GYMNEMA SYLVESTRE*

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ABSTRACT

The present study was conducted for the isolation and characterization of gymnemic acid from indigenous *Gymnema sylvestre* leaves. Four different methods of extraction were employed to obtain the maximum yield. The method where the defatted leaves were extracted under continuous hot extraction in Soxhlet apparatus with 95% ethanol gave the maximum yield of gymnemic acid (6.15% m.f.b.). The yield was minimum in aqueous extraction method (1.66% m.f.b.) Gymnemic acid was purified by preparative chromatographic method in two solvent systems. Its circular Thin Layer Chromatography (TLC) also resulted in to a single concentric ring. Gymnemic acid was hydrolyzed by two methods to confirm its glycosidic nature. The glycon portion thus obtained gave a positive test with Fehling's solution showing the presence of reducing sugars and indicating that gymnemic acid was a glycoside. The aglycone portion on thin layer chromatographic examination in two systems showed the presence of four genins. Therefore the gymnemic acid was a glycoside and was triterpenoid saponins in character. The resinous extract obtained on defatting the leaves with the petroleum ether in first method of extraction separated out in to four spots on TLC. The presence of stigmasterol, β -amyrin, β -amyrin acetate and lupeol was indicated when the reference samples of these compounds were run on the same chromatoplate.

Key Words: *Gymnema sylvestre*; extraction; gymnemic acid; Thin Layer Chromatography.

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INTRODUCTION

Medicinal plants which form the backbone of traditional medicines have in the last few decades been the subject for very intense pharmacological studies. These medicinal plants are the potential source of new compounds of therapeutics value and as sources of lead compounds in the drug development. In the developing countries, it is estimated that about 80% of the population rely on traditional medicine for their primary health care. There arises a need therefore to screen medicinal plants for bioactive compounds as a basis for further pharmacological studies [3].

Gymnema sylvestre is a slow growing, perennial, medicinal woody climber native to Central and Western India, Tropical Africa and Australia. The plant is also found in certain hilly areas of Pakistan. It is regarded as one of the plants with potent anti-diabetic properties and being used in folk, ayurvedic and homeopathic systems of medicine. It is also used in the treatment of asthma, cough, eye complaints, inflammations, family planning and snakebite. In addition, it possesses antimicrobial, diuretic, stomachic, anti-hypercholesteremic, hepatoprotective and anti-saccharine activities [7, 11].

The "destroyer of sugar" is a traditionally used term for *Gymnema sylvestre* because chewing the leaves will abolish the taste of sweetness. The plant belongs to the family *Asclepiadaceae* or milk weed family and occasionally cultivated as medicinal plant. Leaves are opposite, usually elliptic or ovate (1.25-2.0 inch

× 0.5-1.25 inch). Flowers are small, yellow, in umbellate cymes and follicles are terete, lanceolate, up to 3 inches in length [5].

Gymnema sylvestre leaves contain triterpene saponins belonging to oleanane and dammarene classes. Oleanane saponins are gymnemic acids and gymnemasaponins, while dammarene saponins are gymnemasides. Beside this, other plant constituents are flavones, anthraquinones, hantri-acontane, pentatri-acontane, α and β -chlorophyll, phytin, resins, *d*-quercitol, tartaric acid, formic acid, butyric acid, lupeol, β -amyrin related glycosides and stigmasterol. The plant extract also test positive for alkaloids. Leaves of the species yield acidic glycosides, anthraquinones and their derivatives. Among these bioactive compounds of *Gymnema sylvestre*, gymnemic acids have anti-diabetic, anti-saccharine and anti-inflammatory activities [2, 9, 10].

Lot of work has been done on this plant around the world particularly in India regarding to its anti-diabetic activity. However the present study is the first ever attempt for the isolation, purification and characterization of gymnemic acid from the indigenous plant with the purpose to obtain its maximum yield by applying various techniques.

MATERIALS AND METHODS

1. Procurement and identification of plant material:

About 4 kg of dry leaves of *Gymnema sylvestre* were procured from the local market. The material consisted of leaves in broken and crumpled form, twigs and follicles. A small amount of dust present as contamination was removed by shifting through a sieve of mesh number 30. The material was initially identified by chewing few leaves for a minute or two. The mouth was rinsed clean with water, few grains of sugar were placed in the mouth and disappearance of sugars' sweetness was felt. For further identification, the leaves along with powdered material were studied for their macroscopic & microscopic characteristics and compared with literature. Finally 1gm powdered sample was shaken vigorously with water and examined for more than 30 minutes for froth test.

2. Processing of plant material:

The leaves were freed from the follicles, coiled twigs and other extraneous matter. About 3kg cleaned leaves were dried over night in hot air oven at 50°C and ground to a powder which was passed through a sieve of mesh number 40. The woody fibers of the stem were discarded. The powdered material was again dried at 50°C and stored in air-tight, paper-lined tin on cooling. The moisture contents of *Gymnema sylvestre* leaves were determined by drying and toluene determination methods. The ash contents were also determined.

3. Extraction of saponins:

The extraction was carried out using ethanol as well as water. Four different methods were employed for the extraction of gymnemic acid from the leaves of indigenous plant.

Method 1: In this method, the leaves already defatted with petroleum ether were subjected to continuous extraction for 24 hours with 95% ethanol in Soxhlet apparatus. The solvent was removed under reduced pressure to yield a water soluble brown gum. About 200gm of this extract was dissolved in a small volume of ethanol and used for column chromatographic separation by eluting with water saturated chloroform ethanol mixture (2:1 v/v) through a column of Merck's alumina. On the evaporation of the solvent, the eluent gave a greenish amorphous powder. The powder was weighed and labeled as sample A.

Method 2: A portion of extract from method 1 of the plant material was separated into its acidic and neutral fractions. Briefly, the extract was dissolved in ethanol and made acidic to pH 3 with 1N sulphuric acid. This mixture was extracted with chloroform ethanol ((3:2 v/v) and lower organic layer was poured in to another separating funnel containing 60ml water. This was then washed twice with 60ml 1N sodium hydroxide solution and twice with 60ml of half saturated sodium sulphate solution. From neutral chloroform ethanol fraction solvent was evaporated to give a greenish amorphous powder. This was dried over sodium sulphate to yield sample B.

Method 3: In this method, the gymnemic acid was extracted from *Gymnema sylvestre* leaves without prior defatting. A 500gm of powdered dry plant material was packed in a Soxhlet thimble and extracted continuously with 80% ethanol until the material was completely exhausted. The final product was a dark green amorphous powder i.e. sample C, after the evaporation of solvent.

Method 4: The aqueous extraction was carried out as described by Kurihara [8]. Briefly, 500gm of dried powdered leaves were extracted twice with 4L of water at 60°C for 5 hours. Two extracts after filtration were combined and acidified to pH 2 with 2N sulphuric acid. The precipitated principle was filtered, dried and then extracted with ethanol and acetone. The insoluble matter was eliminated by filtration and solvents were evaporated in a big china dish. The dry dark green powder left behind was scrapped out and weighed as sample D.

4. Tests for identification of gymnemic acid:

The identification of gymnemic acid extracted through all four methods was carried out by its property of abolishing the sense of sweetness, froth test, test for acidity, Liebermann-Burchard color test, melting point, optical rotation and solubility.

5. Gymnemic acid as glycoside:

Gymnemic acid was hydrolyzed in order to see if it was a glycoside by two different methods i.e. Rangawami-Reichastein system and Kiliani system. An equal amount (0.8gm) of acid was taken for both acid hydrolysis. In first method, gymnemic acid was refluxed for 30 minutes with 50% methanol in 0.05 N sulphuric acid while in Kiliani system it was refluxed for 30 minutes with a mixture of acetic acid, water and concentrated hydrochloric acid (35:55:10 v/v). The glycon portion thus obtained in both systems was tested with Fehling's solution for the presence of sugars. The yield of genin after hydrolysis of gymnemic acid by both methods was calculated.

6. 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 resinous extract, gymnemic acid, genins isolated after acid hydrolysis and sugar portion was performed using different solvent systems. 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 color of the spots to develop.

7. Purification of gymnemic acid:

Preparative Thin Layer Chromatography was used for the final purification of gymnemic acid. The bands which corresponded with the colored spot were quickly scrapped off the plates along with the adsorbent. The gymnemic acid was recovered from the adsorbent by extraction in a Soxhlet apparatus using ethanol as the solvent. On completion of the extraction, the solvent was removed by distillation under reduced pressure. The purified acid thus obtained was allowed to dry, weighed and its yield was calculated.

RESULTS AND DISCUSSION

The disappearance of the sugar's sweetness gave the identification that the material was *Gymnema sylvestre*. This effect lasted for about 2 hours and during this period the sugar gave only a gritty feeling when present in the mouth. The macroscopic and microscopic characteristics studied were also comparable to those given in the literature [5]. A froth which persisted for more than 30 minutes was produced which also confirmed that the plant material was *Gymnema sylvestre*. 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 acid by chromatography; hydrolysis of gymnemic acid and thin layer chromatography of the genins thus separated. The four different methods were employed for the extraction of gymnemic acid from the leaves of *Gymnema sylvestre*. The yields of gymnemic acid, the antisaccharine principle were calculated on moisture free basis (m.f.b.) (Table 1).

Table 1: Yields of antisaccharine principle; gymnemic acid from *Gymnema sylvestre* leaves by different extraction methods

Extraction method	Sample	Weight of powdered leaves (gm)	Gymnemic acid isolated (gm)	Yield in % age on moisture free basis (m.f.b.)
Method-I: With ethanol 95% after defatting & chromatography	A	200	10.7	6.15
Method-II: By separating in to acidic, alkaline & neutral fractions	B	200	7.3	4.2
Method-III: With ethanol 80% with out defatting	C	500	15.9	3.65
Method-IV: Aqueous extraction	D	500	7.2	1.66

In first method, where the leaves were initially defatted with petroleum ether and then extracted with 95% ethanol under Soxhlet extraction for 24 hours, the yield of gymnemic acid (Sample A) was 10.7gm (6.15% m.f.b.). This was obtained after column chromatographic separation by eluting with chloroform ethanol mixture (2:1 v/v) through a column of alumina. Khastgir et. al. [6] had reported this method but did not mention the yield of gymnemic acid. In second method, isolation of gymnemic acid from defatted plant material was achieved by separating the ethanolic extract in to alkaline, acidic and neutral fractions. Drying and evaporation of solvents from the above fractions showed the presence of gymnemic acid (Sample B) in the neutral fraction. The yield was less i.e. 7.3gm (4.2% m.f.b.) as compared to the first method. It is likely that some quantity of gymnemic acid was partially destroyed or hydrolyzed by the alkaline and acidic reagents used in this method.

In third method, the powdered leaves were subjected to continuous hot extraction in the Soxhlet apparatus using 80% ethanol. The yield of gymnemic acid (Sample C) obtained was 15.9gm (3.65% m.f.b.). This was greater than the yield (13gm, 2.6% m.f.b.) obtained by Chopra et al. [1] by the same method of extraction using 95% ethanol. This was most probably due to greater solubility of saponins in 80% ethanol than in 95% and also due to the continuous hot extraction in the Soxhlet apparatus instead of repeated extraction. The yield by this method was however far less than the yield obtained by the first two methods. It is possible that some gymnemic acid was lost with the resins in the ethanol extracts during the repeated defatting process while purifying.

Being a saponins gymnemic acid is more readily soluble in water, thus an aqueous method of extraction was also carried out. The powdered leaves were extracted twice with water at 60°C for 5 hours. The yield of gymnemic acid (Sample D) was the lowest as compared to other methods (7.2gm, 1.66% m.f.b.). This may be due to the incomplete extraction of the saponins with water from the powdered leaves. The aqueous method of extraction at 60°C for 5 hours was also used by Kurihara, [8] for the extraction of gymnemic acid. The moisture contents of the leaves were found to be 13% by Toluene method. It was observed that the ash contents of the leaves were fairly high (11.27%). The possible reason for these high contents may be due to the presence of alkalies, phosphorus pentaoxide, ferric oxide, manganese and other inorganic matters.

All the samples of gymnemic acid gave a single pinkish spot by Thin Layer Chromatography (TLC) in the two solvent systems ethanol water concentrated ammonia (18:2:1), butanol ethanol water (10:1:1) and using ceric sulphate solution in concentrated sulphuric acid as the detecting reagent. The R_f values were same for all the samples in the same solvent system. Gymnemic acid has been reported to be a mixture of A₁, A₂, A₃ and A₄ which were separated by TLC using butyl formate methyl ethyl ketone formic acid water (5:3:1:1). Variations have been reported on the solubility of gymnemic acid in different organic solvents. In this study all the samples were found to be soluble in chloroform and partially in ether, benzene and ethyl acetate. The appearance of the characteristic honey comb froth which persisted for more than 30 minutes after shaking the aqueous solution of gymnemic acid for 3 minute was a positive indication for its saponins

character. The results obtained on conducting the Liebermann Burchard test with several non-aqueous solutions of different samples of gymnemic acid showed that the color produced varied from light green to bluish green. The melting points of all the samples varied between 195⁰C to 198⁰C, whereas the angle of rotation was 163.50⁰ (Table 2). The conversion of the blue color of the litmus to red was a positive test for the acidic nature of gymnemic acid. The addition of a small pinch of sodium bicarbonate in aqueous solution of all four samples evolved carbon dioxide which confirmed that the gymnemic acid was an acid.

Table 2: The identification tests of gymnemic acid extracted from *Gymnema sylvestre* by different extraction methods

Gymnemic acid sample	Solvent with color developed in Liebermann Burchard test		Melting point	Optical rotation
A	Ethanol (95%) Ethyl acetate	Light green Bluish green	195 ⁰ C- 198 ⁰ C	163.50 ⁰
B	Ethanol (95%) Ethyl acetate	Light green Bluish green	195 ⁰ C- 198 ⁰ C	163.49 ⁰
C	Ethanol (95%) Ethyl acetate	Light green Light green	194 ⁰ C- 196.4 ⁰ C	163.50 ⁰
D	Ethanol (95%) Ethyl acetate	Light green Bluish green	196 ⁰ C- 198 ⁰ C	163.50 ⁰

The glycosidic nature of gymnemic acid was a disputed question when it was first isolated. Hooper, [4] isolated it and proved it to be a glycoside. To confirm the glycosidic nature in the present study, its hydrolysis was carried out by two different methods. In first method where the gymnemic acid was refluxed for 30 minutes with 50% methanol in 0.05N sulphuric acid, the yield of genin was 67.52%. With Kiliani system in which gymnemic acid was refluxed for 30 minutes with a mixture of acetic acid water concentrated hydrochloric acid (35:55:10 v/v), the genin yield was 73.37%. The higher yield in the later case was possibly due to the better hydrolysis of gymnemic acid. The glycon portion thus obtained in both the systems gave a positive test with Fehling's solution showing the presence of reducing sugars and indicating that gymnemic acid was a glycoside. The TLC results of the genins separated by two different hydrolysis methods showed them to be identical because in both cases after using chloroform methanol (9:1) and chloroform ethanol (19:1) as solvent systems four spots separated out. Their colors varied from grey to black. Stoecklin et al. [13] has reported four genins G, J, K and L which he obtained after the enzymatic hydrolysis of gymnemic acid. A number of saponins have been identified in the gymnemic acid fraction out of which gymnemagenin has been shown to inhibit glucose absorption [12].

The resinous extract obtained on defatting the leaves with the petroleum ether in Method-I separated out in to four spots on TLC using benzene ethanol (19:0.2 v/v) as the solvent system and antimony trichloride in chloroform as detecting reagent. The presence of stigmasterol, β -amyrin, β -amyrin acetate and lupeol was indicated when the reference samples of these compounds were run on the same chromatoplate. Kanetkar et al. [5] also reported these compounds as the other plant constituents in addition to gymnemic acid.

CONCLUSION

On the basis of the results of the present study, it was concluded that the first method in which defatted leaves with petroleum ether were extracted with 95% ethanol under Soxhlet extraction for 24 hours, the yield of antisaccharine saponin, gymnemic acid was maximum (10.7gm, 6.15% m.f.b.). The gymnemic acid thus obtained can be further identified, purified and characterized using TLC, preparative TLC and circular TLC techniques. The glycosidic nature of gymnemic acid was confirmed on acid hydrolysis by two different methods. The stigmasterol, β -amyrin, β -amyrin acetate and lupeol were also identified as the other leave constituents of *Gymnema sylvestre*.

REFERENCES

1. Chopra, R.N.; Bose, J.P.; Chatterjee, N.R. (1928). *Gymnema sylvestre* in diabetes mellitus. *J. Med. Res.* 16(1):115-124.
2. Dateo, G.P.; Long, L. (1973). Gymnemic acid, the anti saccharine principle of *Gymnema sylvestre*. Studies on isolation and heterogeneity of gymnemic acid A₁. *J. Agri. Food Chem.* 21:899-903.
3. Gurav, S.; Gulkari, V.; Duragkar, N.; Patil, A. (2007). Systemic review: Pharmacognosy, phytochemistry, pharmacology and clinical applications of *Gymnema sylvestre* R Br. *Pharmacog. Rev.* 1(2):338-343.
4. Hooper, D. (1887). Isolation and antiviral activity of gymnemic acid. *Pharm. J. Trans.* 17:867-868.
5. Kanetkar, P.; Singhal, R.; Kamat, M. (2007). *Gymnema sylvestre*: A Memoir. *J. Clin. Biochem. Nutr.* 41(2): 77-81.
6. Khastgir, H.N.; Gupta, S.S.; Gupta, P.S. (1958). Effects of gymnemic acid on sweet taste perception in primates. *J. Indian Chem. Soc.* 35: 650-652.
7. Komalavalli, N.; Rao, M.V. (2000). *In vitro* micrpropagation of *Gymnema sylvestre*: multipurpose medicinal plant. *Cell tissue organ culture.* 61:97-105.
8. Kurihara, Y. (1969). Antisweet activity of gymnemic acid A and its derivatives. *Life Sci.* 8(9): 537-543.
9. Liu, H.M.; Kiuchi, F.; Tsuda, Y. (1992). Isolation and structure elucidation of Gymnemic acids, antisweet principles of *Gymnema sylvestre*. *Chem. Pharm. Bull.* 40:1366-1375.
10. Masayuki, Y.; Toshiyuki, M.; Hisashi, M. (1997). Structures of new triterpene glycosides, gymnemasides C, D, E and F from the leaves of *Gymnema sylvestre* R Br.: Influence of Gymnema glycosides on glucose uptake in rat small intestinal fragments. *Chem. Pharm. Bull.* 45:2034-2038.
11. Nadkarni, A.K.; Nadkarni, K.M. (1976). *Indian Materia Medica*, Vol.1. Popular Prakashan: Bombay, pp.569.
12. Puratchamani, V.; Jha, S. (2004). Standardization of *Gymnema sylvestre* R. Br. With reference to gymnemagenin by high performance thin layer chromatography. *Phytochem. Anal.* 15:164-166.
13. Stoecklin, W.; Weiss, E.; Reichstein, T. (1967). Gymnemasäure, das antisaccharine Prinzip von *Gymnema sylvestre* R. Br. Isolierungen Identifizierungen. *Helv. Chim. Acta.* 50 (2): 474 – 490.