AMERICAN JOURNAL OF PHARMTECH RESEARCH

Journal home page: http://www.ajptr.com/

Qualitative and Quantitative estimation of Deacylgymnemic acid by HPLC methods in Aqueous extract followed by Hydro alcoholic and Alcoholic extract.

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ABSTRACT

Aqueous Extract Followed By Hydroalcoholic And Alcoholic Extract Gymnema Sylvestre Roxb.[1,2] Family Asclepiadaceae And Stevia Rebaudiana Bertoni[3]amily Compositae. Literature survey reveals that till date, no combination of Stevia rebaudiana and Gymnema sylvestre are available in any dosage form. In the present work, the main objective is to formulate, evaluate and validate anti-diabetic liquid oral preparation of Gymnema using Stevia as sweetener. Stevia, non-caloric natural sweetener has added advantage than artificial sweetener due to number of beneficial effects Successive solvent extraction of Gymnema sylvestre and Stevia rebaudiana were carried out with the solvents like petroleum ether (60-80^{0}C), ethanol, hydroalcohol and water. Phytochemical screening of aqueous extract of formulation was performed and their purity was checked by TLC of aqueous extract of Gymnema sylvestre, formulation and deacylgymnemic acid were done. While preparing liquid oral formulation, aqueous extract of Gymnema sylvestre and Stevia rebaudiana were selected, because of their maximum yield and presence of phytochemicals, which were present in alcoholic and hydroalcoholic extract. Stevia rebaudiana is a natural sweetner. In the presented formulation, stevia was used as a sweetner to make the formulation sweet. Validation of liquid oral preparation was done. Physical, chemical evaluations were carried out. The amount of deacylgymnemic acid present was found to be 22.327 mg/ml by HPLC.

Keywords: Gymnema sylvestre, hydroalcoholic extract, Stevia rebaudiana, deacylgymnemic acid

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Received 25 April 2013, Accepted 08 May 2013

Please cite this article in press as: Patil PM. *et al.*, Qualitative and Quantitative estimation of Deacylgymnemic acid by HPLC methods in Aqueous extract followed by Hydro alcoholic and Alcoholic extract..American Journal of PharmTech Research 2013.

INTRODUCTION

Diabetes is the major disorder of carbohydrate metabolism and characterized by high blood sugar level. The name is from Greek with the meaning 'syphon' (Diabetes) 'sweet' (mellitus).

In 1939, Himsworth divided diabetes into two groups as insulin sensitive in which there was a severe deficiency of insulin production and insulin insensitive in which insulin though present in variable quantities sometimes even higher than normal does not have its biochemical effects in lowering blood sugar.

Diabetes is broadly classified into two categories on the basis of age of patient at onset, as juvenile or growth diabetes and adult or maturity diabetes. In the juvenile diabetes; the disease is more severe with more sudden onset below the age of thirty with symptoms of polyuria, polydipsia, wasting and weakness. They respond well to insulin and are more likely to develop hypoglycemia by insulin over dosage while upon inadequate administration they show ketosis. This group is called as insulin dependent diabetes mellitus (IDDM) or Type I Diabetes.

On the other hand insulin insensitive is usually elderly, obese and often has vascular diseases. The onset is insidious mostly after fourty years of age and symptoms are relatively mild. In this group there is only functional deficiency of insulin. This group is designated as non-insulin dependent diabetes mellitus (NIDDM) or Type II Diabetes.

MATERIALS AND METHOD

The plants were collected from the local region (Hingna Taluka) and authenticated from the Department of Botany, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur. The Specimen sheet of Gymnema sylvestre (Voucher No.7642/MA) and Stevia rebaudiana (Voucher No. 7606/MA) were submitted to the same.

Extraction of active constituents from Gymnema sylvestre and Stevia rebaudiana

The crude drugs (Gymnema sylvestre and Stevia rebaudiana) were air dried in a shade, under the normal environmental conditions and then subjected to size reduction to get coarse powder. Such powdered materials were charged into Soxhlet apparatus and extraction was carried out with petroleum ether (60-80^{O}C) for defatting. The materials were defatted with petroleum ether (60-80^{O}C) to remove primary metabolites, fats and oil fractions.

After extraction with petroleum ether, the materials were dried at room temperature to remove the traces of petroleum ether. Again, the defatted materials were charged into Soxhlet apparatus and extraction was carried out with alcohol followed by maceration with hydro alcohol and water. Each time before extracting with next solvent, the materials were dried at room

temperature. After extraction and maceration, the liquid extracts were distilled on water bath to remove the traces of solvent. The semisolid extracts were kept in a separate container for further study.

Formulation of liquid oral preparation[9]

Number of formulation we had tried in their different proportional and consistency, Depends upon the results of evaluation we have finalized the following formula for liquid oral preparation on the basis of its stability and the consistency.

Table 1 Formulation of liquid oral preparation

Antidiabetic liquid oral preparation	
Each 100 ml contains	
Name	Quantity
Aqueous extract of Gymnema sylvestre	8.0 g
Aqueous extract of Stevia rebaudiana	2.0 g
Methyl paraben	0.1 g
Propyl paraben	0.01 g
Disodium EDTA	0.5 g
Chocolate flavour	1 ml
Distilled water	q.s.

EVALUATION AND VALIDATION

Physical evaluation

Organoleptic evaluation -

Colour Dark brown

Odour Chocolate

Taste Sweet

pH 6.8

Density 1.242 g/ml

Clarity clear

Viscosity 1.118 poise

Chemical evaluation.

Thin layer chromatography[10, 11]

Active alcoholic, hydroalcoholic and aqueous extracts were subjected to thin layer chromatography to find out the number of compounds present in them. The details of the procedure were given in table 2 and 3.

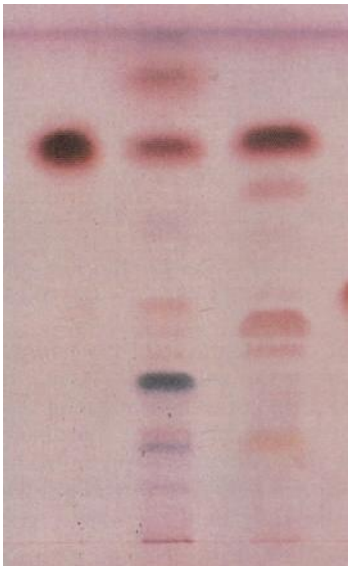
Table 2. Thin layer chromatography of *Gymnema sylvestre* extract and *Stevia rebaudiana*

Plants	**Extracts**	**Solvent system**	**UV Fluorescence (No of spots)**	**Iodine Vapour(Rf)**	**50% H_2SO_4 And No of spot**	**Plants**	**Extracts**	**Solvent system**
Gymnema sylvestre	Aqueous	n-Butanol:Acetic acid : Water (6:3:1)	2	0.71 0.80	5	Light yellow Light pink	5	0.24 0.44 0.45 0.71
	Hydroalcoholic	Chloroform : Methanol : Acetic acid : Water (6:1:1:1)	1	0.70	3	Dark blue Light blue Light brown	3	0.70 0.56 0.55
			1	0.70	3	Dark blue Light blue Light brown	3	0.70 0.56 0.55
	Alcoholic	Methanol : Acetone (4:6)	1	0.77	2	Dark blue Light yellow	2	0.77 0.60
Stevia rebaudiana	Aqueous	n-Butanol:Acetic acid : Water (6:3:1)	1	0.66	2	Light yellow Dark blue	2	0.50 0.66
	Hydroalcoholic	Chloroform : Methanol : Acetic acid : Water (5:1:1:1)	2	0.55 0.60	4	Light yellow Dark yellow Light brown Dark brown	4	0.45 0.50 0.55 0.60
	Alcoholic	Toulene: Ethylacetate : Methanol (5:4:1)	2	0.56 0.66	4	Light yellow Dark yellow Light brown Dark brown	4	0.45 0.50 0.56 0.66

Table 3: Results of thin layer chromatography of aqueous extract of Gymnema sylvestre, formulation, standard (Deacylgymnemic acid)

Type	Solvent system	UV-fluorescence		Iodine vapor	50% H_2SO_4		
		No. of spots	R_f	No. of spots	Colour	No. of spots	R_f
Aqueous extract of *Gymnema sylvestre*	n-Butanol : Acetic acid : Water (6 : 3 : 1)	1	0.80	5	Light yellow Light pink Light brown Light pink Dark brown	5	0.24 0.44 0.45 0.71 0.80
Formulation	n-Butanol : Acetic acid : Water (6 : 3 : 1)	2	0.44 0.79	6	Light blue Light pink Dark blue Light pink Dark brown Light brown	6	0.10 0.16 0.19 0.23 0.44 0.79
Standard (Deacyl gymnemic acid)	n-Butanol : Acetic acid : Water (6 : 3 : 1)	1	0.79	1	Dark brown	1	0.79

Thin layer chromatography of aqueous extract of Gymnema sylvestre, formulation, standard (deacylgymnemic acid)



A B C

Figure. 1. Thin layer chromatography of aqueous extract of Gymnema sylvestre, formulation, standard (Deacylgymnemic acid)

A – Standard (Deacylgymnemic acid)

B – Formulation (Marketed preparation)

C – Aqueous extract of Gymnema sylvestre

Chemical Evaluation

Gravimetry analysis[13]

Weighed 3.00 gm of the extract into a beaker. Dissolved in 50 ml distilled water, filter and to the filtrate add 10% hydrochloric acid till pH 1.5. Allowed to stand for 30 minutes at room temperature. Filtered on Whatman No. 1 filter paper. Washed with 20 ml distilled water and discard the filtrate. Collected the precipitate and dissolved in 20 ml 80.0% v/v ethanol or methanol. Combined the filtrate and washed, evaporated in pre-weighed beaker and dry in oven under vacuum at 70^0C to a constant weight. Weighed and calculated the percentage of total deacylgymnemic acid.

Table 4. Results of qualitative estimation of deacylgymnemic acid by gravimetric analysis

Extracts	Percentage of deacylgymnemic acid
Aqueous extract of Gymnema sylvestre	76%w/w
Hydroalcoholic extract of Gymnema sylvestre	65% w/w
Alcoholic extract of Gymnema sylvestre	40%w/w

UV-Visible and HPLC Method for Deacylgymnemic acid

All the chemicals and solvents used were of AR grade. Distilled water was used during this work and Whatman filter paper no. 41 was used for filtration. The pure drug Deacylgymnemic acid used for present investigation was purchased from Natural Remedies, Bangalore. UV spectrophotometric method for determination of Deacylgymnemic acid. Selection of solvent : solvent was selected by determining the solubility of Deacylgymnemic acid.

In various solvents namely sodium hydroxide, hydrochloric acid, distilled water, methanol. Finally methanol was chosen as the solvent for Deacylgymnemic acid, depending on the desorption at its analytical wavelength. Study of spectra selection of scanning range and wavelength

Preparation of Deacylgymnemic acid: An accurately weighed quantity of about 2.0 mg Deacylgymnemic acid was taken in 10.0 ml volumetric flask and was dissolved in methanol and volume was made upto the mark with methanol to get the concentration of 200 u/ml. A 0.5 ml of this solution was diluted to 10.0 ml with methanol to get the concentration of 10 ug/ml.

This solution was taken in 1 cm cell and scanned in the range 400 nm to 200 nm and spectrum was recorded as shown in Figure. 2.

The λmax of Deacylgymnemic acid was found to be at 217.0 nm. Hence the estimation of Deacylgymnemic acid was done at its λmax i.e. 217.0 nm. Study of Beer-Lamberts law: An accurately measured aliquot portion of 200 ug/ml to 0.5 ml were taken in series of 10.0 ml

volumetric flask and diluted upto the mark with methanol to get concentration in the range of 10-50 ug/ml The absorbance of each solution was measured at 217.0 nm against the blank.

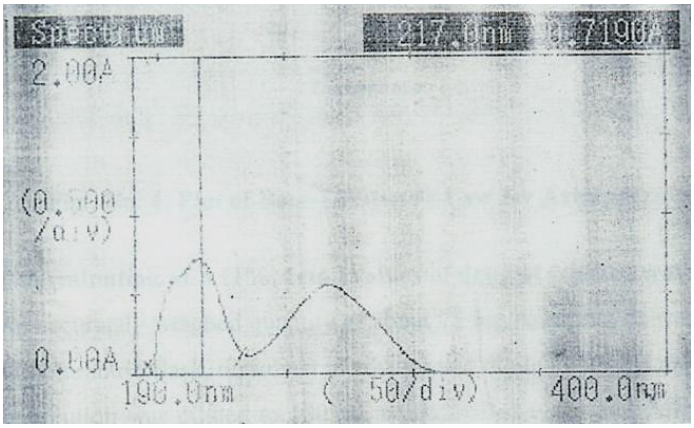


Figure. 2. UV-Visible spectrum of Deacylgymnemic acid

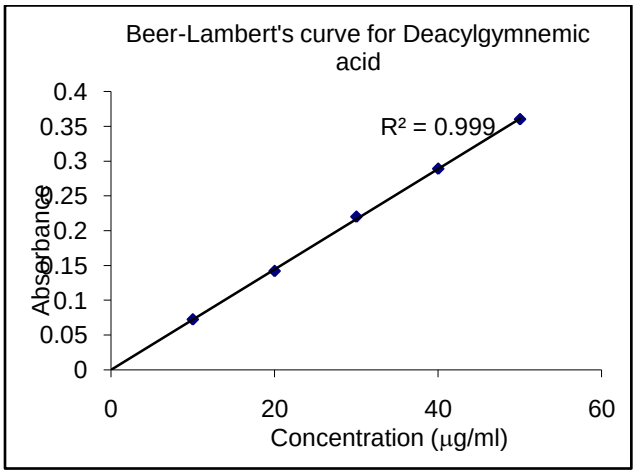


Figure. 3. Plot of Beer-Lamberts Law for Deacylgymnemic acid.

Determination of E(1%, 1cm) values of drug at selected wavelength An accurately weighed quantity of about 2.0 mg Deacylgymnemic acid was taken in 10.0 ml volumetric flask and was dissolved in methanol and volume was made upto the mark with methanol to get to the concentration of 200 ug/ml. A 0.5 ml of this solution was diluted to 10.0 ml with methanol to get the concentration of 10 ug/ml of Deacylgymnemic acid. Absorbance of final dilution was measured at 217.0 nm and E (1%, 1 cm) values were calculated by using formula

Table 5 Observations and results of E (1%, 1 cm)

S.No.	Concentration g/100 ml	E (1%, 1 cm)
1.	0.000997	724.97
2.	0.000991	725.52
3.	0.000983	726.24
4.	0.001001	720.87
5.	0.000979	725.02
Mean		724.52

$$A\ 1\%, 1\ cm) = \frac{\text{Absorbance}}{\text{Concentration in g/100 ml}}$$

B. HPLC method development for Deacylgymnemic acid

1. Preparation of standard solution

An accurately weighed quantity of about 2.0 mg Deacylgymnemic acid was taken in 10 ml volumetric flask and was dissolved in methanol (5.0 ml) and volume make upto the mark with the Mobile phase. A 1.25 ml of this solution was taken in 10 ml volumetric flask and diluted upto the mark with mobile phase.

2. Selection of mobile phase:

Using the following chromatographic parameters, various mobile phases were tried to choose the suitable one.

Column - C18

Detection wavelength -217 nm

Flow rate - 1.2 ml/min

Temperature - Ambient

Injection volume - 20 ul

Mobile phases -

1. 0.1% acetic acid in methanol: Acetonitrile (80:20)
2. 0.1% acetic acid in methanol: Acetonitrile (70:30)
3. 0.6% acetic acid in methanol: Acetonitrile (80:20)
4. 0.1% acetic acid in methanol: Acetonitrile (75:25)
5. 0.1% acetic acid in methanol: Acetonitrile (90:10)

The prepared mobile phases were filtered through 0.45u membrane filter and vacuum degassed.

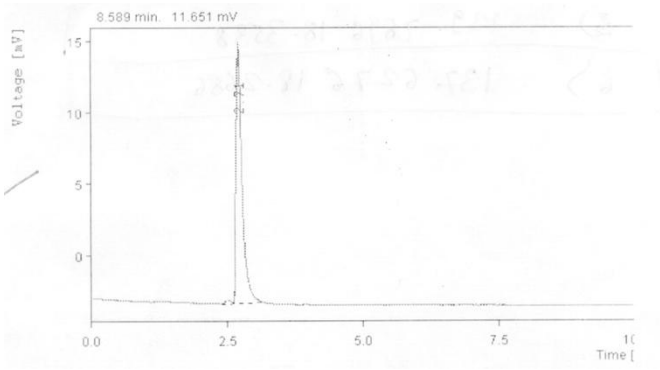


Figure. 4. Chromatogram of Deacylgymnemic acid and mobile phases were allowed to equilibrate with stationary phase. Standard drug solutions were injected and chromatograms have been obtained as follows.

3. Study of system suitability parameters

Study of system suitability parameters was carried out using the following chromatographic condition, which was maintained same throughout the method development.

Column - C18

Detector - UV-VIS (LC 6500)

Detection wavelength - 217 nm

Flow rate - 1.2 ml/min

Temperature - Ambient

Injection volume - 20 ul

Mobile phase - 0.1% acetic acid in methanol: acetonitrile (80:20)

The chromatographic conditions were set as per the given parameters and mobile phase was allowed to equilibrate with stationary phase. Five replicate injections of standard drug solutions were injected separately. The chromatograms were recorded and the response i.e., peak area of major peaks were measured and results are depicted in table no. 6

Table 6 Results system suitability parameters

Parameter		Results
Area under curve		134.0885
		138.3451
		139.2450
		139.7896
		140.4161
Mean		138.3769
S.D. (Standard deviation)		2.515
R.S.D. (Relative standard deviation)		0.0182
Capacity factor		1.71
Tailing factor		2.125
Theoretical	Per column	3366
plates	Per meter	33665

VALIDATION OF PROPOSED METHOD

Accuracy

Standard solution: An accurately weighed quantity of about 2.0 mg Deacylgymnemic acid was taken in 10 ml volumetric flask and was dissolved in methanol (5.0 ml) and volume make upto the mark with the Mobile phase. A 1.25 ml of this solution was taken in 10 ml volumetric flask and diluted upto the mark with mobile phase.

Sample solution: Near about 2.0 mg of pure Deacylgymnemic acid were taken in 10.0 ml volumetric flask dissolved in methanol and volume adjusted to mark. A 1.25 ml of this solution was taken in 10 ml volumetric flask and diluted upto the mark with mobile phase.

The solution was filtered using Grade 1 filter paper. Standard chromatograms were obtained and % recovery was calculated. Results of recovery studies. (a)Accuracy, (b) Precision

Table 7 Results of recovery studies

Sr. No.	Weight of pure drug taken (mg)	Weight of pure drug recovered (mg)	% recovery
1	2.0	2.02	101.00
2	2.1	2.1	100.00
3	1.9	1.96	103.15
4	1.9	1.81	95.25
Mean			99.85
S.D.			3.336
R.S.D.			0.0334
S.E. (Standard error)			1.668

ii) Precision

Precision of an analytical method is expressed as the S.D. or R.S.D. of series of measurements. It was ascertained by replicate estimation of drugs by proposed method. The results are shown in Table 7

Linearity and range

Accurately weighed quantities of Deacylgymnemic acid to 1.6, 1.8, 2.0, 2.2 and 2.4 mg (i.e., representing 80, 90, 100, 110 and 120% of standard solution claim) were taken in five different volumetric flasks and were dissolved in mobile phase with vigorous shaking. The solutions were then filtered and aliquots of filtrate were diluted to get the final concentration and areas under curve were recorded.

Observations for linearity and range study

(c) Linearity & Range

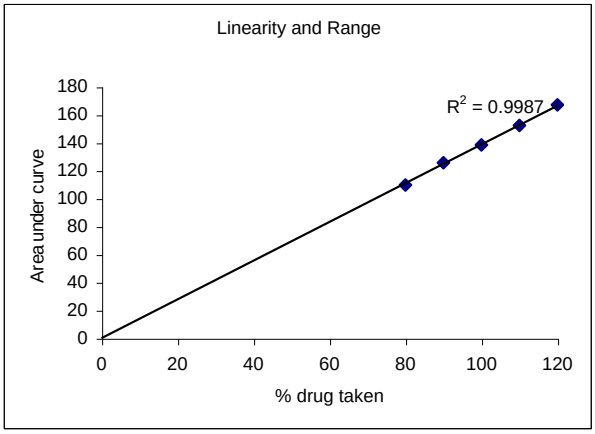


Figure. 5. Linearity and range.

Table 8 Results of linearity and range study

Sr. No.	% drug taken	Area under curve
1	80	109.7015
2	90	125.5392
3	100	138.3769
4	110	152.2146
5	120	167.0523

Ruggedness

Getting the sample analyzed from different analysts and carrying out the analysis on different days by proposed method ascertained ruggedness.

Table 9 Result of different analyst

S.No	Wt. of Sample (mg)	Area under curve	% Sample solution claim
Analyst-1	2.0	139.2759	100.65
Analyst-2	2.1	145.3957	99.93
Analyst-3	1.9	129.9827	98.88
Mean			99.82
S.D.			0.8901
R.S.D.			0.0089

Results are average of three injections.

Table 10 Result of different days

S.No.	Wt. of Sample (mg)	Area under curve	%Sample solution claim
Day-1	1.9	130.9827	99.64
Day-2	1.8	123.3769	99.07
Day-3	1.6	111.7015	100.90
Mean			99.87
S.D.			0.9419
R.S.D.			0.0094

Results are average of three injections.

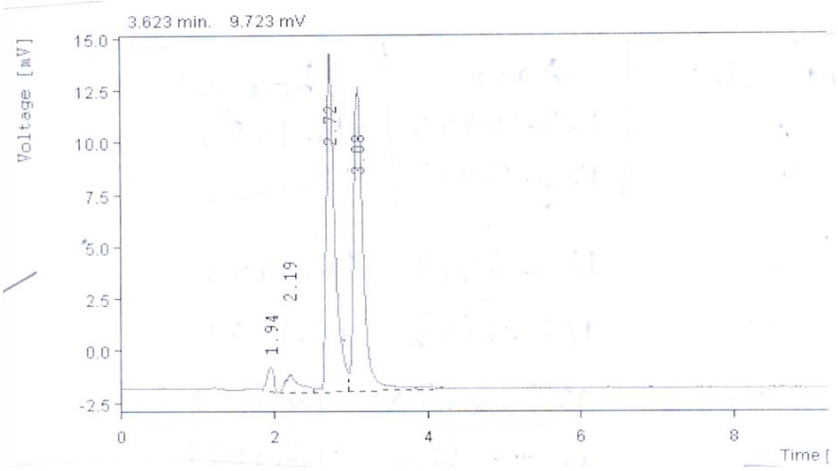


Figure. 6. Chromatogram of formulation.

Quantitative estimation of deacylgymnemic acid was found using the following formula- Liquid, mg/ml = (R/R') x (c/v) x 1000

R/R' – response ratios for test sample and std. solution respectively

c = concentration of deacylgymnemic acid standard solution, mg/ml

v – mL injection taken for analysis

Table 11 Results of the amount of deacylgymnemic acid present in formulation (peak area)

Parameter	Results
Peak area	123.3444
	123.2443
	124.6719
	125.2270
	125.2661
Mean	124.75074

The amount of deacylgymnemic acid was found to be 22.327 mg/ml

CONCULSION

The work presented here deals with formulation, evaluation and validation of liquid oral preparation of Gymnema sylvestre and Stevia rebaudiana (as sweetner), to study its antidiabetic activity. Successive solvent extraction of Gymnema sylvestre and Stevia rebaudiana were carried and formulating aqueous extracts as liquid oral preparation and validation of liquid oral preparation were carried out. Validation of liquid oral preparation was done. Physical, chemical evaluation were carried out. The amount of Deacylgymnemic acid present was found to be 22.327 mg/ml by HPLC. It may be said that the aqueous extract of Gymnema sylvestre and formulation of Gymnema sylvestre decreased the serum glucose level and improved glucose tolerance.

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