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International Journal of Pharmaceutical and Phytopharmacological Research

(ICV-5.09)

ISSN (Online) 2249 – 6084

ISSN (Print) 2250 – 1029

Int.J.Pharm.Phytopharmacol.Res. 2012, 2(3): 143-147

(Research Article)

Pharmacognostic and Phytochemical Studies on *Gymnema sylvestre* R. Br. Hairy Variant

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Received on: 02/11/2012 ***Accepted on:*** 15/11/2012

ABSTRACT

Gymnema sylvestre (Gs) is a well proven anti diabetic plant and used in Indian system of medicine for long period. A new variant of Gs was identified having hairy leaf. This Gs hairy variant species was subjected for pharmacognostical studies and screened for phytochemical analysis and correlated with the previous work done normal non hairy species. The leaves were analyzed for macroscopic and microscopic characteristics by taking microtome sectioning and stained for viewing in slides. The dried powdered leaf was subjected to fluorescent study using chemical reagents and viewed under visible and UV light to study the coloring pattern of the powdered particles interacting with the reagents. The powder was subjected for physicochemical studies using Indian Ayurvedic Pharamacopoeia procedure. The different extractions were done by using 100%, 50% ethanol and water. These extracts were subjected for preliminary qualitative analysis, quantitative estimation of terpenoides, alkaloids and marker compound Glycemnic acid. Further, TLC studies using different mobile phase and GCMS studies were carried out on 50% ethanolic extract. The stomatal frequency, index, vein islet number and palisade ratio values of Gs variant were varies in comparing with Gs normal. Test for flavanoides and, fixed oil and fats were given negative inference in three extracts. Almost, other tests for compounds gave positive result in three extracts. Quantitative estimation results higher concentration of terpenoides rather than alkaloids and presence of lower concentration of glycemnic acid comparing with previous work in extract of normal species. Chromatographic studies gave the presence of 33 different compounds in 50% ethanolic extract can be used in creating monograph for this species. All the results of pharmacognostical, physico, phytochemical analysis gave some variation while correlating with the results of same above studies on normal Gs species.

Key Words: *Gymnema sylvestre*, Pharmacognosy, Qualitative and quantitative analysis, Gymnemic acid

INTRODUCTION

Gymnema sylvestre (Gs) is an Indian medicinal plant used for Diabetes mellitus in Siddha, Ayurveda and Folk medicine. Modern scientific studies also proved its efficacy against Diabetes (Persaud1999)[1]. While collecting *G. sylvestre*, we found a new variant differ in morphological character from normal. This variant was multiplied by stem cutting and maintained at Tamil University Herbal Garden, Tanjore. We noticed that the new variant has hairy leaf, but leaf of normal Gs doesn't have hair in both upper and lower surface. More over, flowering was not noted in the hairy new variant for the past two year during its growth. Sangeetha and Jagadeesan, 2012 screened for phytochemical characterization of the leaf of new variant and normal Gs and reported the variations found between these two species[2]. For validating the above variations, this work was carried out to investigate the leaf and its different extracts of Gs hairy variant under macroscopic and microscopic features, physicochemical parameters, phytochemical analysis and chromatographic analysis.

MATERIALS AND METHODS

Plant Collection

The leaves of *Gymnema sylvestre* R. Br variant were collected from Tamil University Herbal Garden, Thanjavur, Tamil Nadu, India. The botanical identity was also authenticated by Professor Dr. M. Jagadeesan, Department of Environmental and Herbal Science, Tamil University, Thanjavur.

Morphological Studies

The collected leaves were taken free hand as well as microtome sections and were double stained. All slides were stained initially by alcoholic safranin (0.5%) and dehydrated by employing graded series of ethyl alcohol (30%, 50%,

70%, 90% and absolute alcohol). After washing, slides were stained with 0.25% fast green in clove oil and xylol-alcohol (50-50) and passed through xylol and mounted in glycerin. Stained hand sections and macerated materials were examined under compound microscope. Vein-islet number, stomatal index and palisade ratio was found on using samples treated in 5% KOH solution. For determining stomatal index, epidermal pealing from both surfaces of a fresh leaf was taken and counting was recorded from different areas of each piece (i.e. number of stomata as well as epidermal cells per 1 sq. mm area). Stomatal index value is then calculated by using the formula [S/(E+S)]100 where E and S stand for number of epidermal cells and number of stomata of unit area respectively. Palisade ratio was determined by using fresh leaves.

Preparation of Powder for Analytical Studies

The leaves of *Gymnema sylvestre* R.Br variant were dried under shade and mechanically powdered after keeping them in an oven at 35ºC for 24 h. These powdered materials were used for further physicochemical, fluorescent and phyto-chemical analysis.

Physicochemical Analysis

The procedures recommended in Indian Ayurvedic Pharmacopoeia were followed for the determination of total ash, water-soluble ash, acid-insoluble ash and sulfated ash, loss on drying at 110ºC and extractive values[3]. *Total ash value:* 5g of plant powder was ignited in an electric furnace at 600ºC in silica crucible until the sample reaches a constant weight. *Water-soluble ash value:* Total ash obtained was heated up to 600ºC with addition of 25ml of water for 10 minutes and filtered in an ashless filter paper (whatman no.41) and the residue was ignited in the furnace to get a constant weight. *Acid-insoluble ash value:* Total ash obtained was heated with addition of the 25ml of dil. HCl for 10 min and filtered in an ash less filter paper (whatman no. 41) and the residue was ignited in the furnace to get a constant weight. *Sulphated ash value:* 1g of plant powder was ignited in an electric furnace until the drug gets charred. The crucible was cooled and the residue was moistened with 1ml of H_2SO_4, heated gently until the white fumes were no longer evolved and ignited at 800ºC ± 25ºC until all black particles disappear. After cooling, few drops of H_2SO_4 was added and again heated. The ignition was carried as before, allowed to cool and then weighed. This was repeated until the sample reaches a constant weight. *Alcohol solubility percentage* (Kokate, 1994): 5g of powdered material along with 100ml of alcohol was shaken well occasionally for the first 6 h and kept undisturbed for 18 h. The liquefied extract thus obtained was concentrated in a vacuum pump and the percentage was calculated with the weight of the drug powder taken[4]. *Water solubility percentage* (Kokate, 1994): The procedure adopted for the solubility percentage of the plant powder in alcohol is used with chloroform water instead of alcohol to get the water solubility percentage[4]. *Fluorescent powder analysis:* Florescent analysis was carried out by using the method of Chase and Pratt, 1949[5]. Behavior of different chemical reagents was carried out as mentioned by Kay (1938) and Johansen (1940)[6,7]. *Successive extraction:* 250g of dried leaves were extracted successively with various solvents in the increasing order of polarity viz., Petroleum ether (60-80'C), Benzene, Chloroform, Ethanol and Water. Each extract was concentrated to a small volume and allowed to dry. After drying, and the respective extracts were weighed and percentage of extractive values were determined.

Qualitative and Quantitative Analysis

Qualitative analysis for carbohydrates, alkaloids, phytosterols, tannins and phenols, fixed oil and fat, saponin, gums and mucilage, and flavanoids were done in aqueous, 100% and 50% ethanolic extracts of Gs variant leaves following Kokate (1994)[4]. *Estimation of total Terpenoids* (Ferguson, 1956): 100g of plant powder was taken separately and soaked in alcohol for 24 h. Then filtered, the filtrate was extracted with petroleum ether; the ether extract was treated as total terpenoids[8]. *Estimation of total Alkaloids*` (Agrawal, 2007): 100 g of plant powder was moistened with aqueous alcohol and treated with dil. HCl. To this, sufficient chloroform was added and shacked for 15 min and filtered. Residual matter was collected. The same process was repeated for another two times and the residuals were collected. All the residual extracts were dissolved in water and basified by adding Sodium bicarbonate. Then, this was extracted with chloroform and the free alkaloids were separated[9].

Gravimetric Estimation of Gymnemic acid

The air-dried leaf of *Gymnema sylvestre* R.Br variant was coarsely cut into pieces to aid the extraction and soaked in distilled water and kept for 48 hours. The obtained extract was decanted and filtered. So, subsequently the extracts were concentrated over a boiling water bath on china-dishes by free evaporation. 25g of this water extract was dissolved in 10ml of 1N NaOH solution. The solution was acidified with required quantity of 1N HCl so as to get the precipitate. It was filtered and washed with water completely free from acid. The precipitate was dissolved in methanol and filtered. The filtrate was evaporated to remove the methanol and the residue was weighed.

Thin Layer Chromatographic Analysis

TLC studies on alcohol, water and 50% alcohol extracts of the powdered drugs of all three samples were carried out. TLC plates were prepared by using Silica Gel-G as adsorbent. 100g Silica Gel-G was mixed with sufficient quantity of distilled water to make slurry. The slurry was immediately poured into a spreader and plates were prepared by spreading the slurry on glass plates of required size. The thickness of the layer was fixed 1.5mm. Plates were allowed to air dry for one hour and layer was fixed by drying at 110'C for two hours. Using a micropipette, about 10µml of 1% w/v solution of extracts were loaded gradually over the plate. The loaded plated was eluted by suitable mobile phase like TBA (t-BuOH-AcOH-H2O – 3:1:1 ratio), BAW (n-BuOH–AcOH–H2O – 4:1:5 ratio- Upper Phase), Forestal (AcOH – Con. HCl – H2O – 30:3:10 ratio), 60% AcOH and Water. Before elution, the tank was allowed 30 min for saturation with mobile phase. The extracts showed separation into bands. The chromatograms were observed under visible light and were photographed. The Rf value of the band was calculated by the ratio between the distance traveled by the substance [cm] and the distance traveled by the mobile phase [cm].

Gas Chromatographic-Mass Spectroscopic Analysis

The 50% ethanolic extract was examined in GC-MS for its chemical composition by GC-MS engine model, GC–Clarus 500; Perkin Elmer and Computer Mass Library (Wiley 138L) of 80,000 compounds with a GC column Elite – 1 (100% Methyl Poly Siloxane). The other conditions were as follows: Injector: GC-Clarus – 500; Perkin Elmer; Carrier gas flow Helium 1ml/min; Split ratio – 1:25; Sample injected 1µl; Oven temperature – 110°C – 2 min hold; Up to 270°C at the ratio of 5°C /min – 4 min hold; Injector temperature 250°C; Total GC- time 38 min; MS inlet line temperature 200°C; Source temperature 200°C; Electron energy 70eV; Mass Scan 25- 400; MS time 39 min.

RESULTS AND DISCUSSION

Macroscopic Characteristics

Gymnema sylvestre has two varieties, one is normal leaf and another one is leaf with hair on both surfaces. In our observation, the hairy variant type could be distinguished by shape, size and hairyness of leaf. Flowering in hairy variant was not observed. Leaf of normal variety is lanceolate to ovate, apex acute and base truncate, less pubescent on both sides. The leaf of hairy variant is ovate to cordate in shape, apex acute and base cordate. Both upper and lower leaf surface of hairy leaf variant have pubescent hairs.

Microscopic Characteristics

The microscopic analysis result (Table 1) that the stomatal frequencies in both upper and lower leaf surfaces were two fold higher in hairy variant than normal variant. But the stomatal index was higher in normal variety than thin hairy variety. The vein-islet number (44.4) was higher in hairy variant than normal variant. But the vein termination number was greater in normal variant (43.5) than in hairy variant. The hairy variant has high palisade ratio (16.7) than normal leaf.

Physicochemical Analysis

The values of physicochemical parameters were shown in the table 2. The water insoluble ash value was higher (58.44) compared to water soluble ash (26.24). The total ash value (8.24) and loss on drying (13.76) was higher compared to acid insoluble ash (0.41) and sulphated ash value (11.78). The solubility percentage of the plant powder was higher in 50% alcohol (22.2%) compared in 100% alcohol (10.4%) and water (13.2%). The above all values of Gs variant in the present investigation vary with those observed for the normal *G. sylvestre* species and hairy variant in the previous work done[2]. Analytical values also differ from those of normal and hairy species[2]. The differences in physicochemical values between variants of same species are due to genetic, edaphic and climatic factors and can be used as diagnostic tool. However, the difference in the values for Gs variant might be due to time and season of collection, physiological phase of the plant and of course in human error in analysis. To eliminate human error, both variants should have been analysed simultaneously.

Behavior of powder of the leaves of the Gs variant on treatment with different chemical reagents and their fluorescent behavior were shown in the table 3. Behavior of plant powders to different chemical reagent revealed diagnostical colouration. Gs variant leaf powder treated with H_2So_4, HNO_3, HCl, NH_4OH, AcOH, I_2, $Fecl_3$, Picric acid and NaOH gave different colours in both UV and visible light. These present investigations of Gs variant vary with those observed for the same variant in previous work[2]. The result of successive extraction of leaf powder by hot continuous extraction technique using petroleum ether, benzene, chloroform, alcohol and water (Table 4) revealed high percentage of yield extraction got using the solvent alcohol having dark green, pungent character.

Phytochemical Analysis

The preliminary qualitative analysis (Table 5) results most compounds were found in both 100% alcoholic and aqueous extract of Gs variant leaf powder except flavanoids and, fixed oil and fats. The powder extract has 0.0046% of brown, oily total alkaloid, 12.1947% of dark greenish, oily total terpenoid and 2.5092% of Gymnemic acid content (Table 6). So, Gs variant contains higher content of total terpenoid than total alkaloid. Sangeetha 2012 reported Gymnemic acid content in hairy variant was 3.75%[2]. In this work, we found decreased content of Gymnemic acid (2.5%). This variation is due to the change in the time and season of collection. Collection time and season influence the bioactive compounds in both qualitatively and quantitatively (Trease and Evans) [10]. TLC run for 100% ethanolic, 50% ethanolic and aqueous extract of Gs variant using mobile phase such as BAW, Forestal, TBA, 60% AcOH, Water gave Rf values were shown in the table 7. These values can be used as one of the method for standardization of drugs having this plant as one of the ingredients. Sangeetha 2012 reported aqueous extract of hairy variant species produced a major spot at 0.49 in BAW, 0.74 in Forestal, 0.95 in AcOH and 0.79 in Water[2]. Here, we found the major spots produced by the same extract using above mobile phase were varies (Table 7). So, the TLC study showed different band with different Rf values on correlating these two species and proved these species are phytochemically different. The GC-MS analysis on 50% ethanol extract revealed the presence of 33 organic compounds (Table 8). Among them, 1,2,3,4,5-Cyclopentanepentol found in higher concentration than other major compounds found such as 2, 2-Dimethyl -3-heptanone, Heptanediamide, N,N' – di-benzoyloxy, benzene (ethenyloxy),1,2,3,4 – Cyclohexanetetrol, n-Hexadecanoic acid, Phytol and Oleic acid.

CONCLUSION

This present work reveals that there is some variation in the concentration of active compounds in *Gymnema sylvestre* hairy species on correlation with normal species. So, further work shall be carried out in proving that Gs hairy variant has efficacy against Diabetic mellitus since the normal species possess antidiabetic activity. Once, we proved its efficacy as an antidiabetic drug we can use the hairy variant in place of normal one.

Table – 1: Microscopic values of two variables of *Gymnema sylvestre*

Parameter	Normal leaf	Hairy leaf
Stomatal frequency – Upper surface Lower surface	66.5-75.0-80.0 68.5-75.0-80.0	151.0-162.0-170.0 151.0-162.0-170.0
Stomatal index – Upper surface Lower surface	37.5-39.1-45.0 36.8-42.1-47.1	34.8-38.1-40.9 41.2-42.9-44.4
Vein – islet number	24.7-32.3-33.5	35.2-38.8-44.7
Vein termination number	39.8-40.3-43.5	31.8-38.5-40.0
Pallisade ratio	13.7-15.2-16.3	14.2-15.8-16.7

Table – 2: Analytical values of leaf of *G. sylvestre* R. Br. Hairy Variant Species

Parameters	Values (%)
Water soluble	26.24
Water insoluble	58.44
Total ash	8.24
Acid insoluble ash	0.41
Sulphated ash	11.78
Loss on drying	13.76
Solubility in 100% alcohol	10.4
Solubility in 50% alcohol	22.2
Solubility in water	13.2

Table – 3: Fluorescent Analysis of Dried leaf powder of Gs variant

Treatment with reagent	Ultraviolet (256nm)	Visible light
Powder + H_2So_4	Dark green	Dark red
Powder + HNO_3	Light green	Orange
Powder + HCl	Fluorescent yellow	Dark green
Powder + NH_4OH	Fluorescent yellow	Green
Powder + CH_3COOH	Dark green	Dark red
Powder + I	Fluorescent yellow	Green
Powder + $FeCl_3$	Fluorescent yellow	Yellowish green
Powder + $C_6H_3N_3O_7$	Green	Green
Powder + NaOH	Fluorescent yellow	Light green

Table – 4: Characters of different extracts of the leaf of Gs Variant

Parameters	Ether	Benzene	Chloroform	Ethanol	Water
Colour	Dark brown	Dark brown	Dark green	Dark green	Dark brown
Odour	Pleasant	Pungent	Pungent	Pungent	Pungent
Extractive values	1.6006%	1.4389%	1.3004%	20.6687%	7.7323%

Table – 5: Preliminary qualitative phytochemical analysis of Gs variant

Test compound	Reagents used	100% Ethanol	50% Ethanol	Aqueous
Reducing sugars	Fehling's	+	-	+
Carbohydrate	Molisch's	+	+	+
Alkaloids	Wagner's	+	+	+
	Meyer's	-	-	-
	Hager's	+	+	+
	Draggendroff's	+	+	+
Phytosterols	Libermann-Burchard test	+	+	+
Tannins and Phenols	10% Lead acetate	+	+	+
	5% Ferric chloride	+	+	+
	1% Gelatin solution	+	+	+
Fats and Fixed oils	Spot test	-	-	-
Saponins	Foam test	+	-	+
Gums and Mucilages	Absolute Alcohol	+	+	+
Flavanoids	Shinoda test	-	-	-
	Alkaline reagent test	-	-	-

'+'= Present, '-' = Absent

Table – 6: Quantitative phytochemical estimation of Gs variant

Compound	Colour and nature	Value (%)
Total alkaloid	Brown and oily in nature	0.0046
Total terpenoid	Dark green and oily in nature	12.1947
Gymnemic acid	-	2.5092

Table – 7: TLC profiles of different extracts of Gs variant

Mobile phase	100% Ethanol	50% Ethanol	Aqueous
BAW	0.62	0.50	0.49
FORESTAL	0.78	0.78	0.77
TBA	0.85	0.84	0.71
60% AcOH	0.75	0.79	0.80
WATER	0.14	0.82	0.88

Table – 8: Components identified in the 50% ethanolic extract of Gs variant using GC-MS

S. No.	RT	Name of the compound	Molecular Formula	Molecular Weight	Peak Area %
1.	1.33	Formic acid	CH_2O_2	46	1.85
2.	1.50	S)-(+)-2-Amino-3-methyl-1-butanol	$C_5H_{13}NO$	103	0.77
3.	1.73	Butanol, 2-methyl	$C_5H_{10}O$	86	1.00
4.	3.77	Glycerin	$C_3H_8O_3$	92	0.61
5.	4.01	Pyridine, 3-5-dimethyl	C_7H_9N	107	1.23
6.	4.6	2,2-Dimethyl-3-heptanone	$C_9H_{18}O$	142	2.16
7.	5.28	Hydroperoxide, 1-methylpentyl	$C_6H_{14}O_2$	118	0.22
8.	5.67	3-Penten-1-ol	$C_5H_{10}O$	86	0.06
9.	5.76	Benzaldehyde, 3-benzyloxy-2-fluoro-4-methoxy-	$C_{15}H_{13}FO_3$	260	0.48
10.	6.02	Oxirane, hexyl-	$C_8H_{16}O$	128	0.19
11.	6.2	2-Propyl-tetrahydropyran-3-ol	$C_8H_{16}O_2$	144	0.19
12.	6.49	Heptanediamide, N,N'-di-benzoyloxy	$C_{21}H_{22}N_2O_6$	398	3.77
13.	7.05	Hexanediamide, N,N'-di-benzoyloxy	$C_{20}H_{20}N_2O_6$	384	0.42
14.	7.19	Benzene, (ethenyloxy)	C_8H_8O	120	3.11
15.	7.6	1,3,5-Cycloheptatriene,7-ethyl	C_9H_{12}	120	1.35
16.	8.53	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150	1.73
17.	9.1	Bicyclo[3,3,1]non-6-ene-3,9-dione	$C_9H_{10}O_2$	150	0.31
18.	9.46	Alpha-1-rhamnopyranose	$C_6H_{12}O_5$	164	0.13
19.	9.63	1,4-Nonadiene, 2-nitro-, (Z)-	$C_9H_{15}NO_2$	169	0.07
20.	11.72	1,2,3,4-Cyclohexanetetrol	$C_6H_{12}O_4$	148	2.43
21.	12.46	2(3H)-Furanone,3-butyldihydro	$C_8H_{14}O_2$	142	0.10
22.	12.81	1,3,2,5-Dimethylene-1-rhamnitol	$C_8H_{14}O_5$	190	0.33
23.	13.44	2-Heptenoic acid	$C_7H_{12}O_2$	128	0.04
24.	14.21	2-Nitrohept-2-en-1-ol	$C_7H_{13}NO_3$	159	0.30
25.	14.50	2-Deoxy-D-galactose	$C_6H_{12}O_5$	164	0.18
26.	14.60	2-Decanynoic acid	$C_{10}H_{16}O_2$	168	0.21
27.	14.97	2-Octenoic acid (E)	$C_8H_{14}O_2$	142	0.22
28.	15.75	1,3-Propanediol, 2-ethyl-2-(hydroxymethyl)	$C_6H_{14}O_3$	134	0.10
29.	17.78	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	12.81
30.	20.14	Phytol	$C_{20}H_{40}O$	296	1.59
31.	20.72	Oleic Acid	$C_{18}H_{34}O_2$	282	13.20
32.	20.87	Trans-2-undecenoic acid	$C_{11}H_{20}O_2$	184	1.02
33.	26.88	1,2,3,4,5-Cyclophentanepentol	$C_5H_{10}O_5$	150	47.83

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