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"PHARMACOLOGICAL CHARACTRIZATION AND *IN VITRO* SEEDS GERMINATION AND PLANTLETS DEVELOPMENT OF *GYMNEMA SYLVESTRE* (RETZ.) R.BR. EX. SCHULT."

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ABSTRACT

Gymnema sylvestre (Retz.) R.Br. ex. Schult. belongs to family Asclepiadaceae. It is a perennial woody climber and found in tropical and subtropical regions of India. It is commonly known as Gurmar. The present paper reveals the botanical standardization on leaf of *Gymnema sylvestre*. The pharmacognostic studies include the phytochemistry, histochemistry, microscopic, macroscopic evaluation, percentage extractives, ash and acid insoluble ash, fluorescence analysis and estimation of Protein, Carbohydrate and Flavonoid. The phytochemical and histochemical test includes starch, proteins, Saponins, alkaloids, glycosides, sugar and flavonoid. Due to increase in demand and destructive harvesting, the plant has become vulnerable. Hence micro propagation has been done by using various combinations of auxin and Cytokinins. The different auxins used are IAA and IBA and cytokinin used are BA and Kinetin. About 70% seed germination were achieved on MS medium within 30 days with a desirable shoot and number of roots. MS medium containing 0.2 mg L^{-1} Kinetin, 0.2+0.5+0.1 and 0.5+0.5+0.2mg L^{-1} BA+ Kinetin+ IBA showing good results for Shoot and root induction and 0.2+0.5+0.5mg L^{-1} BA+Kinetin+IAA showing good result for seed germination. The developed plantlets were transferred into pot having sterile soil + sand in the ratio of 3:1 in the laboratory. These were kept under observation for 15 days and finally transplanted for hardening in the garden. All transplanted seedlings showed satisfactory growth and all are surviving.

KEYWORDS: *Gymnema sylvestre*, Pharmacognosy, *In Vitro*, Plantlets, Seed germination.

INTRODUCTION

Gymnema sylvestre (Retz.) R.Br. ex. Schult. belongs to family Asclepiadaceae. It is a perennial woody climber. Mostly found in tropical and subtropical regions of India (Anonymous, 1997). Leaves are usually used in the preparation of herbal medicine. It is commonly known as Gurmar, means sugar killer. The leaves are simple, opposite, and hairy. The flowers are small, yellow with umbellate cymes type of inflorescence. *Gymnema Sylvestre* leaves contain an active principle i.e. gymnemic acids. It is also useful in the treatment of asthma, eye complaints, family planning and snake bite (Bishayee *et al*., 1994). As the natural propagation takes a long span of time (Reddy *et al*., 1998) hence, in the present study pharmacological aspects and *in vitro* propagation of seed germination is carried out by using MS medium supplemented with different concentration of hormones for the development of plantlet of *Gymnema sylvestre*. Hence, in the present study method of Pharmacognosy and *in vitro* propagation of seed germination is elaborated.

MATERIAL AND METHODS

Collection and identification of plant material

The Pods was collected from Mulashi Taluka. Efforts were made to collect the plant in flowering and fruiting condition for the correct botanical identification and authentication. It was identified with help of Flora of Presidency of Bombay (Cooke T., 1967).

Pharmacognosy

Microscopic and macroscopic evaluation

Leaf was examined macroscopically according to (Wallis, 1967 and Trease and Evans 2002) which includes shape and size, colour (inner and outer), odour and taste. For microscopic evaluation thin (25µ) hand cut sections were taken from fresh stem and leaf, permanent double stained and finally mounted in Canada balsam as per the plant micro-techniques method of Johansen (1940) and Krishnamurthy (1988).

Phytochemical study

Some material were dried under the shade so as to avoid the decomposition of chemical constituents, powdered in blender and finally stored in dry air tied containers for phytochemical screening. Ash, acid insoluble ash and percentage extractives were accomplished by following standard pharmacopoeia techniques of Anonymous (1955). Fluorescence analysis was carried out as per Chase and Pratt (1949). Qualitative

phytochemical tests were carried out by standard method of Harborne (1973) and Trease and Evans (2002). Quantitative phytochemical analysis was determined for proteins, carbohydrates, Starch, alkaloid and Flavonoid by the methods of Lowry *et al.* (1951), Nelson (1944), Harborne (1973) and Boham and Kocipai (1994) respectively. The phytochemical screening is also detected by the High Performance Thin Layer Chromatography (HPTLC). HPTLC study was carried out on instrument comprising of Linomat5 for application using Densitometer- TLC scanner 3 with "WINCATS" software (Camag, Switzerland). These studies were carried out on HPTLC precoated aluminum fluorescent plates (E. Merck). For HPTLC studies, an extract of methanol (25% GR) solvent system was used and after development, plate was scanned at 254 and 366nm (Wagner and Bladt, 1966; Reich and Schibii, 2007).

In-vitro Studies

Preparation of Seeds

The mature pods of *Gymnema sylvestre* were harvested for the germination. The outer covering of the pods were removed by hand and Seeds are collected.

Sterilization of Seeds and development of seedlings.

The fresh mature seeds were treated with cold water for 24 hr. Wash by using the Savlon and Tween 20 than two washes given with distilled water for 2 minutes. Then the seeds were taken under aseptic condition in laminar air flow and washed with sterilized distilled water for 2 minutes. Then treated with 0.1% $HgCl_2$ for 1 minute and again washed with sterile distilled water for 5-6 times. The seeds were inoculated on different concentration of Murashige and Skoog medium, plain MS, and MS medium supplemented with Kinetin, BA+ Kinetin + IBA; BA+Kinetin+IAA and IBA. The pH of all the concentrations was adjusted to 5.7 with 1N NaOH or 1N HCl and autoclaved at 15 lb/Inch pressures and 121^{o}C temperature for 20 minutes. For seed germination firstly cultures were kept for 4-5 days in dark at 25^{o}C and 90% humidity inside the Environmental test chamber. Then the cultures were transferred to culture room and maintained at 25^{o}±2^{o}C and 16/8 hours (light/dark) photoperiod provided through white fluorescent tubes with light intensity of 3000 Lux (Kumar *et al.*, 2002).The effect toward seeds germination was weekly recorded and daily observed.

RESULTS AND DISCUSSION

Macroscopic evaluation

The studied part is leaf which is green in color with 2-6cm in length and 1-5 cm in width. The leaves are simple, opposite, petiolate with acute apex; margin is entire, venation reticulate and pubescent on both sides. Stem observed is hairy and brown. The taste of the leaf is bitter and odour is characteristics.

Microscopic evaluation

Transverse section of leaf: The transverse section of leaf shows dorsiventrally structure. The epidermis layer is interrupted with uniseriate multicellular trichomes on both surfaces. Epidermis is covered with the cuticle. The cuticle is moderately thick. Under the epidermal region single layer of palisade cells are arranged closely. Mesophyll cells thick with 3-5 large cells, large vacuoles and small intercellular space. Vascular bundle is amphicribal.

Phytochemical study

G. sylvestre Leaf contains the total ash 90.9% and acid insoluble ash 4.3 (Table 1). The value of percentage extractive is higher by using solvent distilled water and lower by using Petroleum ether (Table 2). Fluorescence analysis was carried out to check the purity and potency of the drugs. The powdered drug was then observed in ultraviolet light, it was treated with reagents like Nitrocellulose, 1N NaOH, 1N NaOH in methanol, 1N NaOH in methanol dry it for 30 min + Nitrocellulose and observed under UV light to emits the color as shown in (Table 3). Qualitative analysis of the leaf drug indicated the presence of proteins, sugars, saponins, fats, tannin, flavonoid and alkaloids in the plant (Table 4). The quantity of proteins, carbohydrate, starch, alkaloid and flavonoid are mentioned in (Table 5).

Seeds Morphology for In-Vitro

The explants used for *In-Vitro* studies of *G. sylvestre* were seeds which are brownish in color. The seed has an average weight of 0.58-1.126mg. The length of the seed varies from 1.1-1.5 cm and the breadth from 0.8-1cm.

Observation during Seeds germination and Seedlings development

During the germination of seeds firstly the color of seeds was changed in around six- seven days after inoculation in the culture medium. Secondly swelling of seeds is observed due to absorption of the water and nutrient which create pressure on testa and it breaks. After that full development was observed by globular and protocorm formation. For seed germination

and seedling development in *G.sylvestre.* the best result is observed in medium i.e. Full MS, MS with Kinetin, MS with BA+KIN+IAA and MS with BA+KIN+IBA.

The developments of seedlings were divided into three stages, shoot elongation, shoots and leave expanding and roots elongation. The three stages were observed that it developed within 30-38 days after inoculation of seeds (Table 6). The pods of the collected plant material were shown in Figure 1. The microscopic study of *G. sylvestre* was shown in Figure 2. The appearance of seeds germination and seedlings development of *G. sylvestre* were shown in Figure 3 and 4. The transplantation of fully expanded plantlets in pot with sterile Soil: Sand ratio was shown in Figure 5 and 6. The seeds from the *G.sylvestre* were successfully germinated. The HPTLC study is carried out for gymnemic acids estimation by using growing *in-vitro* plants. For HPTLC 1g powder was cold macerated in 50 ml of mixture of 95 % ethanol: water (1:1 v/v) for 48 h. The obtained solution was filtered and concentrated under vacuum, re-dissolved in methanol (25 ml) in a water bath at 55ºC. The final volume was made up to 50 ml and used as an application for quantification of gymnemic acid. For each application 10µl extracts were used and loaded on instrument comprising of Linomat5 for application using densinometer-TLC scanner3 with "WINCATS" software (Camag, Switzerland). These studies were carried out on precoated aluminum fluorescent plates (E. Merck). The plates were scanned at 254 and at 366nm (Wagner and Bladt, 1996; Reich and Schibii, 2007) (Fig.7).

Table No. 1: Ash analysis

Wt. of crucible (A)	Wt. of crucible after ignition(B)	% of ash (A-B) X 100	Wt. of acid insoluble ash + crucible (C)	% Acid insoluble ash (B-C) X100
34.319 gm	33.410 gm	90.9%	33.367 gm	4.3%

Wt. of powder taken =1gm

Initial Wt. of crucible = 34.319 gm…….. (A)

Wt. of crucible after ignition = 33.410 gm…..…… (B)

Wt. of acid insoluble ash + crucible =33.367 gm …...(C)

Table No. 2: Percentage extractive

Sr. No.	SolventUsed 50ml + 0.5gm.	Wt. of Porcelain dish (A)	Wt. Of sample + Porcelain dish (B)	B-A	% extractive
1	Abs. Alcohol	57.413	57.602	0.189	47.3%
2	Chloroform	59.362	59.436	0.074	18.5%
3	Diethyl ether	63.451	63.525	0.074	18.5%
4	Acetone	57.380	57.506	0.126	32 %
5	Distilled Water	59.101	59.372	0.271	67.8%
6	Petroleum Ether	55.714	55.736	0.022	5.5%

Table No.3: Fluorescence Analysis

SN	Test	Visible Light (short wavelength)
1	Powder as such	Grayish green
2	Powder as such in UV light	Greenish black with tinge of grey
3	Powder + Nitrocellulose	Greenish black
4	Powder + 1N NaOH in Methanol +Nitrocellulose	Grayish black
5	Powder + 1N NaOH in Methanol dry it for 30 min. + Nitrocellulose	Greenish black

Table No. 4: Phytochemical tests

Alcohol extract

Sr.No.	Extract ml.	Reagent	Test for	Result
1	1	Wagner's	Alkaloid	+ve
2	1	Mayer's	Alkaloid	-ve
3	1	Dragendorff's	Alkaloid	+ ve
4	1	Hager's	Alkaloid	+ ve
6	1	Millon's	Protein	+ ve
8	1	Mg turning +HCL	Flavonoid	+ ve

Water extract

Sr.No.	Extract ml.	Reagent	Test for	Result
1	1	I_2KI	Starch	+ ve
2	1	Acidic $FeCl_3$	Tannin	- ve
3	1	Conc H_2SO_4	Saponins	+ve
4	1	Millon's	Protein	+ ve
5	1	Benedicts reagent	Sugar	+ve

Table No. 5: Quantitative estimation

Quantitative estimation (mg/gm)	Protein	Reducing sugar	Non reducing sugar	Starch	Alkaloid	Flavonoid
Leaf	2.36	0.46	0.72	0.04	0.03	0.07

**Results are the mean of three determinants of the weights of dry drug powder in mg/g.*

Table 6: Seedling development by using solidified full MS medium supplemented with different concentration of Hormone

Different concentration of MS medium	Concentration	Shoot initiation	Shoot and Leaves initiation	Plantlets in 30 days high (cm)	Roots in 30 days	% of seed germination
Full MS	-	+++	+++	10.3	+++	70%
MS with Kinetin	0.2	+++	+++	9.7	+++	63%
	0.5	+	+	7.3	+	35%
	0.7	-	-	-	-	0%
	0.8	-	-	-	-	0%
	1	+	+	7.4	+	24%
MS with BA+ Kinetin+ IBA	0.2+0.5+0.1	++	++	8.1	++	55%
	0.5+0.5+0.2	+++	+++	9.3	+++	61%
	0.5+0.5+0.5	-	-	-	-	0%
	0.5+0.2+0.2	-	-		-	0%
MS with BA+ Kinetin+ IAA	0.2+0.5+0.5	++	++	10.2	++	56%
	1.2+1+0.5	+	+	10.6	+	23%
	0.5+0.5+0.5	-	-	-	-	0%
	0.5+0.2+0.2	-	-	-	-	0%
MS with IBA	0.2	+	+	0.9	+	22%
	0.5	+	+	0.8	+	23%
	0.8	-	-	-	-	0%
	1.0	-	-	-	-	0%

Fig.1: Pod of *Gymnema sylvestre*

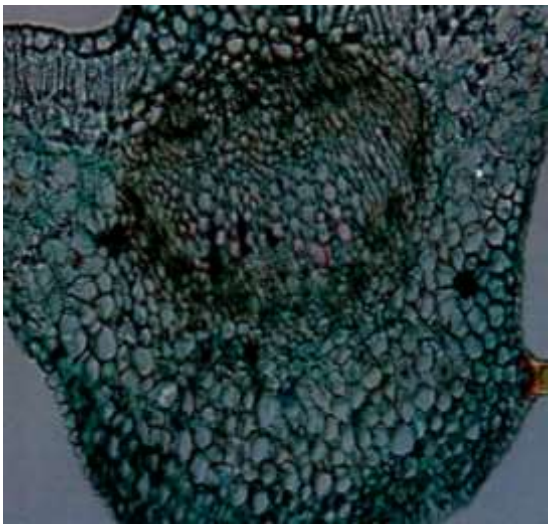

Fig.2: T.S of leaf *Gymnema sylvestre*

Fig.3. Plantlet grow in Full MS medium

Fig.4. MS medium with BA+ Kin+ IAA, 0.2 Kin and BA+ Kin+ IBA

Fig.5.Transplanted with Soil: Sand (3:1)

Fig.6. Transplanted in the pot.

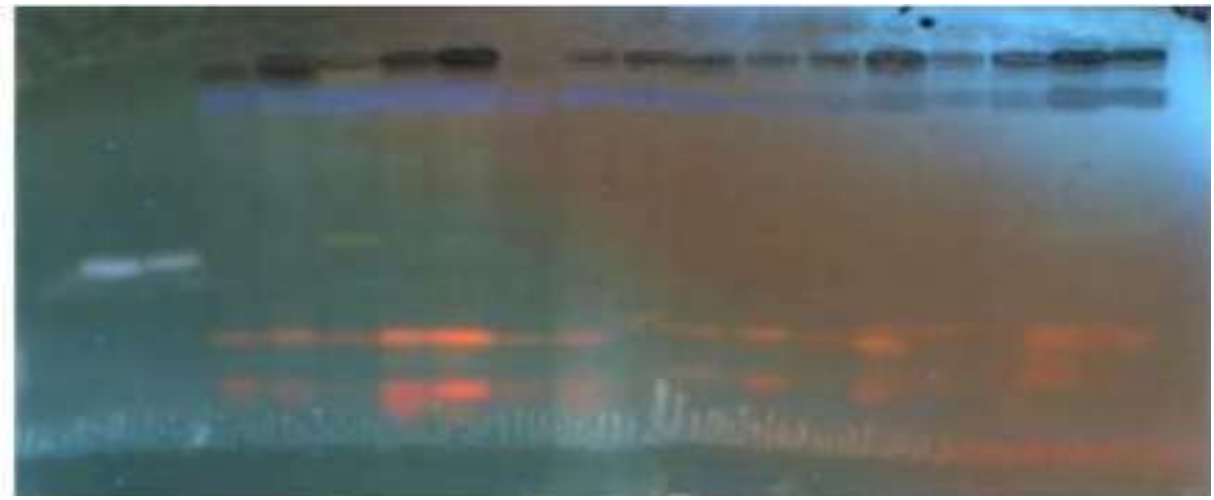

Fig.7: HPTLC plate showing Gymnemic acid.

CONCLUSIONS

From the study we concluded that when the seeds are dormant for several months we can use the *in vitro* seeds germination method for propagation of *G.sylvestre.* The best medium for seed germination of *G.sylvestre* was Full MS medium, MS with Kinetin, MS with BA+KIN+IAA and MS with BA+KIN+IBA. This study will be helpful for the conservation of plants and to meet the more demand of *G.sylvestre* for the preparation of traditional and other pharmaceuticals remedies.

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