Phytochemical analysis and in vitro propagation of Gymnema sylvestre R.Br. a valuable medicinal plant

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A B S T R A C T

The present paper reports the phytochemical and micropropagation studies of a threatened plant, Gymnema sylvestre. Gymnema sylvestre which belongs to the family Asclepiadaceae is a perennial slow-growing medicinal woody climber commonly called as “Gudmar”. There is a growing demand for leaves of G. sylvestre in the pharmaceutical trade due to its use as a remedy for diabetes and also as a tonic of the nerves and as a laxative. Propagation of this plant is often hard and expensive. In the present study the qualitative analysis confirmed the presence of various phytochemicals like alkaloids, flavonoids, phenols, tannins, terpenoids and glycosides. Quantitative estimation of flavonoids and phenols was also carried out. In vitro propagation is an alternative method of propagation of the threatened and endangered plant which can aid its conservation. The nodal explants were cultured on MS medium containing different concentration and combinations of growth regulators like 6-benzylaminopurine (BAP) and indoleacetic acid (IAA). Multiple shoot buds were regenerated successfully from the nodal explants which were efficiently rooted on ½ strength MS medium supplemented with IBA. The regenerated plantlets were successfully transferred to the glasshouse, acclimatized and transferred to the field.

Introduction

Gymnema sylvestre R.Br. which belongs to the family Asclepiadaceae, is a vulnerable species. It is a perennial slow-growing woody climber of tropical and subtropical regions (Anonymous, 1997). It is a potent antidiabetic plant and used in folk, homeopathic and ayurvedic systems of medicine (Mitra et al., 1995). It is also used in treating of asthma, eye complaints, inflammations, family planning and snake bite (Anonymous, 1997; Selvanayagam et al., 1995). In addition, it possesses antimicrobial, antihypercholesterolemic (Bishayee and Chatterjee, 1994), hepatoprotective (Rana and Avadhoot, 1992) and sweet suppressing (Kurihara, 1992) activities. It also acts as feeding deterrents to caterpillar, Prodenia eridania (Granich et al., 1974), prevent dental caries caused by Streptococcus mutans (Hiji Yasutake, 1990) and in skin cosmetics (Maeda et al., 1996). There is a growing demand for leaves of G. sylvestre in the pharmaceutical trade due to its use as a remedy for diabetes and also as a tonic of the nerves and as a laxative (Shanmugasundaram et al., 1983), as an anti-sweetner (Kurihara, 1992) and as an antihypercholesterolemic (Bishayee and Chatterjee, 1994). It also has stomachic, diuretic and cough suppressant property (Kapoor, 1990). Increasing awareness of the side effects of Western drugs have made general public turn towards the herbal medicine, thus the demands for medicinal plants have drastically increased. Due to over exploitation, this plant species has become threatened and is listed in International Union for Conservation of Nature (IUCN) red data book (Shailasree et al., 2012).

Gymnema sylvestre is a slow growing, perennial woody climber (Shrivastava and Singh, 2011). Seeds lose viability in a short period of storage (Reddy et al., 1998). Conventional propagation methods are hampered due to its poor seed viability, low rate of germination and poor rooting ability of vegetative cuttings (Komavali and Rao, 2000). The propagation of plant through seed results in less survivability under natural conditions (Anonymous, 1950). Therefore, to fulfill the increasing demand of this potent medicinal plant and population, in vitro culture and micropropagation could be an alternative method to aid in its conservation. Therefore, the propagation of this plant species by alternative methods is needed.

The present study of Gymnema sylvestre has been taken up to carry out qualitative phytochemical analysis for alkaloids, flavonoids, tannins, saponins, phenols, steroids, terpenoids and glycosides present in leaves and quantify the flavonoids and phenols and in vitro propagation through direct organogenesis using nodal segments as explants.
Materials and Methods

Material: Gymnema sylvestre plants were collected from Herbal garden, N. G. Ranga Agricultural University at Hyderabad and planted in the Botanical Garden at Department of Botany, Osmania University, Hyderabad. Gymnema sylvestre is a slow growing, perennial woody climber of tropical and subtropical regions with a twining woody stem and opposite petiole leaves, entire, smooth shiny, varying in shape and size according to their age. Flowers are small, in axillary sessile racemes. The root is long, rigid and cylindrical. These plants were subjected to phytochemical analysis (qualitative and quantitative) for the presence of important secondary metabolite compounds. Further, a good protocol for micropropagation was developed to aid in its multiplication and conservation.

Qualitative analysis:
Preparation of plant extract:
The plant extract was prepared by grinding 0.5 gm of the plant part (leaf, shoot or root etc...) in 100 ml distilled water. This extract was filtered through a fine mesh into a test tube. This crude extract was used for the qualitative tests given below (Karthikeyan et al., 2009, Lozoya et al., 1989) and the tests were carried out in triplicate.

Test for identification of Alkaloids: About 0.5 gm of methanol extract was taken in a test tube and was diluted and homogenized with 10 ml distilled water, dissolved in 20 ml dilute HCl solution and clarified by filtration. The filtrate was tested with Drangendorff’s and Mayer’s reagent. The treated solution was observed for precipitation of white or creamy colour.

Test for identification of Flavonoids: About 0.5 gm of extract was introduced into 10 ml of ethyl acetate in a test tube and heated in boiling water for 1 min. The mixture was then filtered. About 4 ml of the filtrate was shaken with 1 ml 1% aluminium chloride solution and incubated for 10 min. Formation of yellow colour in the presence of 1 ml dilute ammonia solution indicated the presence of flavonoids.

Test for identification of Phenols: About 0.5 gm of extract was taken in a test tube, mixed with 100 ml distilled water and heated gently. Ferric chloride solution of 2 ml volume was added and observed for the formation of green or blue colour.

Test for identification of Saponins: About 0.5 gm of methanol extract was taken in a test tube and 5 ml distilled water was added. Persistent froth was observed upon vigorous shaking of solution. 3 drops of olive oil was added to the frothing and shaken vigorously after which it was observed for the formation of an emulsion.

Test for identification of Steroids: About 0.5 gm of methanol extract was taken in a test tube and 2 ml of acetic anhydride was added to it and 2 ml of sulphuric acid was added along the sides of the test tube and observed for the colour change to violet or blue green.

Test for identification of Tannins: Five grams of the ground powder was extracted with 10 ml ammonical chloroform and 5 ml chloroform. The mixture was filtered and the filtrate was shaken with 10 drops of 0.5 M sulphuric acid. Creamish white precipitate was observed for the presence of tannins.

Test for identification of Terpenoids: 5 ml of the methanol extract was mixed with 2 ml of chloroform and 2 ml concentrated sulphuric acid. The layer interface was observed for reddish brown coloration which indicates the presence of Terpenoids.

Quantitative analysis:
Quantitative analysis was carried out to estimate total phenols and total flavonoids.

Determination of total phenols: Folin Ciocalteau reagent method (Mc Donald et al., 2001) with some modifications was adopted for total phenolic content determination. The root extract (1.0 ml) was mixed with Ciocalteau reagent and allowed to stand for 15 min and 5 ml of saturated Na2CO3, was added. The mixture was allowed to stand for 30 min at room temperature and the total phenols were determined spectrophotometrically at 760 nm. Gallic acid was used as a standard. Total phenol values are expressed in terms of gallic acid equivalent (mg/g of extracted compound).

Determination of total flavonoids: Aluminium chloride - colorimetric method (Chang et al., 2002) with some modifications was used to determine flavonoid content. 1.0 ml root extract was mixed with 1.0 ml methanol, 0.5 ml aluminium chloride (1.2 %) and 0.5 ml potassium acetate (0.1176%). The mixture was allowed to stand for 30 min at room temperature. Later the absorbance was measured at 415 nm. Quercetin was used as standard. Flavonoid content is expressed in terms of quercetin equivalent (mg/g of extracted compound).

Micropropagation studies: Gymnema sylvestre plants were subjected to tissue culture and a good protocol for micropropagation was developed to aid in its multiplication and conservation. The micropropagation studies comprised the culture of nodal explants on defined culture media under standard growth conditions. The nodal explants were collected from mature and healthy field grown plants. They were washed under running tap water for 20 min followed by soaking in 0.1% (v/v) liquid detergent Tween-20 for 5 min and then subsequently washed with tap water. The explants were then soaked in 70% ethanol for 5 min followed by washing with water. Finally the explants were surface sterilized with 0.1% solution of mercuric chloride for 3 to 5 min. They were allowed by thorough rinsing in sterile distilled water. A total of thirty explants were inoculated in culture tubes containing MS medium (Murashige and Skoog, 1962) augmented with 2 % sucrose and 0.8 % agar and different combinations and concentrations of various plant growth regulators. The experiment was carried out in triplicates. Prior to that, the medium was adjusted to pH 5.8, autoclaved at 121°C for 15 lbs / cm2 for 15 min and allowed to cool before inoculation. The culture media comprised of the following: MS + BAP (0.5, 1.0, 1.5 and 2.0 mg/l) and MS + BAP (0.5, 1.0, 1.5 and 2.0 mg/l) + IAA (0.5 mg/l). All the inoculated cultures were incubated in sterile growth room under controlled conditions of 22 ± 1°C temperature, 75 % humidity and 2000 lux illumination of 16 hr / 8 hr L/D cycle. The 2 cm long regenerated shoots were transferred to root inducing media comprising half MS medium supplemented with IBA (0.5, 1.0 and 1.5 mg/l). The regenerated plantlets were later transplanted to pots containing a mixture of soil and vermicompost in the ratio of 2:1. The plantlets were gradually acclimatized on the laboratory bench by covering with a plastic bag with holes (to maintain high humidity), which were opened up gradually over a period of one week. The plants in the pots were moved to the glasshouse to a shaded area and gradually acclimatized.
Table – 1: Qualitative analysis of the plant extracts of Gymnema sylvestre to screen for the presence of phytochemicals.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Test for Phytochemicals</th>
<th>Plant Parts</th>
<th>leaf</th>
<th>Stem</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>Ethanol</td>
<td>Aqueous</td>
</tr>
<tr>
<td>1.</td>
<td>Alkaloids</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>2.</td>
<td>Flavonoids</td>
<td>+ve</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>3.</td>
<td>Tannins</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>4.</td>
<td>Saponins</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>5.</td>
<td>Phenols</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>7.</td>
<td>Terpenoids</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>8.</td>
<td>Glycosides</td>
<td>+ve</td>
<td>+ve</td>
<td>-ve</td>
</tr>
</tbody>
</table>

+ve= present, -ve= absent

Table - 2: Quantitative analysis of the methanol extracts of Gymnema sylvestre for estimation of Flavonoids and Phenols.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Plant extract</th>
<th>Phytochemicals</th>
<th>Leaf</th>
<th>Average Estimated value (mg/gm) (Mean ± S.E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Flavonoids</td>
<td>Leaf</td>
<td>17.98 ± 0.55</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Phenols</td>
<td>Leaf</td>
<td>40.22 ± 0.74</td>
<td></td>
</tr>
</tbody>
</table>

* Phenols are expressed as Gallic acid equivalent (GAE) and Flavonoids are expressed as Quercetin equivalents (QE) in mg/100 gm

Table – 3: Efficiency of shoot regeneration and production of multiple shoots from nodal explants of Gymnema sylvestre, on different culture media

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>No. of explants with shoot induction</th>
<th>Percentage of shoot induction* (Mean±S.E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS + BAP (0.5 mg/l)</td>
<td>56</td>
<td>3.14±1.00</td>
</tr>
<tr>
<td>MS + BAP (1.0 mg/l)</td>
<td>60</td>
<td>3.58±1.79</td>
</tr>
<tr>
<td>MS + BAP (1.5 mg/l)</td>
<td>68</td>
<td>4.23±2.01</td>
</tr>
<tr>
<td>MS + BAP (2.0 mg/l)</td>
<td>77</td>
<td>4.80±1.81</td>
</tr>
<tr>
<td>MS + BAP (0.5 mg/l) + IAA (0.5 mg/l)</td>
<td>51</td>
<td>1.55±0.22</td>
</tr>
<tr>
<td>MS + BAP (1.0 mg/l) + IAA (0.5 mg/l)</td>
<td>52</td>
<td>1.76±0.45</td>
</tr>
<tr>
<td>MS + BAP (1.5 mg/l) + IAA (0.5 mg/l)</td>
<td>54</td>
<td>2.00±0.89</td>
</tr>
<tr>
<td>MS + BAP (2.0 mg/l) + IAA (0.5 mg/l)</td>
<td>58</td>
<td>2.15±0.92</td>
</tr>
</tbody>
</table>

*The value was calculated as the percentage of nodal explants that have produced shoots out of a total number of inoculated explants (90).

Table – 4: Percentage of root induction from multiple shoots regenerated from nodal explants of Gymnema sylvestre.

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>No. of shoots with root induction</th>
<th>Percentage of root induction* (Mean±S.E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS + IBA (0.5 mg/l)</td>
<td>50</td>
<td>8.82±0.043</td>
</tr>
<tr>
<td>MS + IBA (1.0 mg/l)</td>
<td>60</td>
<td>10.00±0.124</td>
</tr>
<tr>
<td>MS + IBA (1.5 mg/l)</td>
<td>80</td>
<td>12.93±0.116</td>
</tr>
</tbody>
</table>

*The value was calculated as the percentage of shoots with root induction out of a total number of inoculated shoots.
**Figure 1 A-F:** A. *Gymnema sylvestre* plant. B. Shoot regeneration from nodal explants, 12 days after inoculation. C. Multiple shoots, 25 days after inoculation. D. Rooting from regenerated shoot, 15 days after inoculation of shoot. E. Acclimatization of regenerated plantlet. F. Regenerated plant transferred to the field.
Results and discussion

The present study contributes valuable information of bioactive compounds in *G. sylvestre*. Qualitative analysis of plant extract was carried out for Alkaloids, Flavonoids, Saponins, Phenols, Tannins, Steroids, Terpenoids and Glycosides. All of the phytochemicals like Alkaloids Flavonoids, Phenols, Tannins, Terpenoids and Glycosides were present in *Gymnema sylvestre* except Saponins and Steroids (Table-1) which is similar to the reports of Vaghasiya et al. (2011) and Han et al. (2007). The plant extracts were quantitatively analyzed for Flavonoids and Phenol (Table-2). Whereas, our study reports the absence of Saponins, Kalidas et al. (2010) and Ajayeeoba (2000) indicated that Saponins were present in *G. sylvestre* in the aqueous extract. Several medicinal properties have been attributed to Saponins (Gopinath, 2012) and Kalidas et al. (2010) but surprisingly, Saponins were not found in the present study. Flavonoids and Phenol are however reported in the present study which agrees with the findings of Vaghasiya et al. (2011) who has attributed antidiabetic, anti-aging anti-inflammation and bactericidal effects.

An efficient micropropagation protocol was developed with a high percentage of shoot regeneration and multiple shoots (fig. 1-A to C). The highest response of 4.80±1.81 for production of multiple shoots was recorded with MS + BAP (2.0 mg/l) followed by 4.23±2.01 in MS + BAP (1.5 mg/l) (Table-3). The explants proliferated for 5-8 days and shoot regeneration was observed by 10-15 days (fig. 1-D). Shoots of about 2 cm with 2-3 nodes were produced by 25 days. These were cultured on root induction media containing different concentrations of IBA (0.5, 1.0, 1.5 mg/l) to induce roots. The higher concentration of IBA (1.5 mg/l) produced better rooting efficiency of 12.93±0.116 (Fig. 1-E; Table-4). The regenerated plants were transferred to the greenhouse for acclimatization (fig. 1-F). Out of a total of 720 explants (pooled from triplicates) inoculated, 476 explants could regenerate shoots and 190 shoots were inoculated on rooting media for root induction out of which 152 shoots could develop roots to enable 121 plants to be transplanted out of which 96 plants survived in pots. In the present study, different concentrations of BAP and BAP with IAA were used to induce regeneration. Komalavalli N & Rao M.V (2000) however, reported the regeneration of *Gymnema sylvestre* through the use of BAP and KN individually and combined with NAA. The present results agree well with the above report with supplementation of BAP individually or in combination but a higher frequency of regeneration was obtained with BAP (2.0 mg/l) presently. Manonmani and Francisca, (2012) reported the plant regeneration of *Gymnema sylvestre* from nodal explants on MS medium supplemented with BAP (1.0 mg/l) and NAA (2.0 mg/l) whereas, in our present report use of BAP individually produced the highest shoot regeneration frequency without any additional supplementation of NAA. In the present study it was observed that MS + IBA combination produced efficient rooting compared to other reports where they achieved rooting on MS medium supplemented with NAA. This efficient high frequency plant regeneration protocol developed presently can be taken up for large scale micropropagation for its multiplication and conservation. Further, the information of phytochemical analysis would be useful to the pharmaceutical industry.

Conclusion

It is concluded that *Gymnema sylvestre* is a plant with a variety of medicinal uses. The qualitative and quantitative analysis of *G. sylvestre* shows the presence of bioactive compounds such as Alkaloids Flavonoids, Phenols, Tannins, Terpenoids and Glycosides. This is valuable information for preparation of drugs in pharmaceutical industry and stress the need for more intensive research since they play a great role in healthcare. The present study describes the successful development of rapid micropropagation protocol of *Gymnema sylvestre*. This protocol provides a successful technique for multiplication and conservation of the valuable medicinal plant which is used in treating various disorders. The protocol developed presently can be taken up in large scale to produce the planting material for development of medicinal plant cultivation programmes and it can also help the pharmaceutical industry.

References


Komalavalli N & Rao MV., 2000. In vitro micropropagation of *Gymnema sylvestre* – A


