Research Paper

In Vitro and In Vivo Evaluation of Hepatoprotective Activity of Gymnema Sylvestre

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ABSTRACT: Leaves of Gymnema sylvestre were extracted by hot and cold maceration. Qualitative phytochemical analysis of the plant extract showed the presence of majority of compounds like alkaloids, carbohydrates, flavonoids, terpenoids, glycosides, proteins, amino acids, phenolic compounds and tannins. Among the two extracts, the hydro-alcoholic extract prepared by the hot maceration has shown high phenol content 6.51 ± 0.231 mg/g, high total flavonoid content 89.51 ± 0.100 mg/g. By DPPH method, total antioxidant capacity, reducing power ability of the hydro-alcoholic extract prepared by hot maceration process was evaluated. The freshly prepared rat hepatocytes were treated with different concentration of hydro-alcoholic extract prepared by the hot maceration process. The antihapatotoxicity produced by the extract at the concentration of 200, 400,600 µg/ml was found effective against the D-galactosamine-induced hepatotoxicity, whereas at the concentration of 800 µg/ml was found to be cytotoxic. A significant increase in the levels of ASAT, ALAT,ALP, total bilirubin, direct bilirubin (P< 0.001) were observed. The cells were treated with the hydro-alcoholic extract different extracts of Gymnema sylvestre showed a significant restoration of the altered biochemical parameters towards the normal (P<0.001) when compared to D-galactosamine treated groups and were found to be dose dependent. A similar result was obtained when D-galactosamine intoxicated hepatocytes were treated with silymarin.

KEYWORDS: Hepatoprotective activity, Silymarin, D-Galactosamine, Gymnema sylvestre

Introduction

Drugs continue to be taken off the market due to late discovery of hepatotoxicity. Due to unique metabolism and close relationship with the gastrointestinal tract, the liver is susceptible to injury from drugs and other substances. 75% of blood coming to the liver arrives directly from the gastrointestinal organ and spleen via portal veins which brings drugs and xenobiotics in concentrated form. Several mechanisms were responsible for either inducing hepatic injury or worsening the damage process. Many chemicals damage mitochondria an intra cellular organelle that produces energy. Its dyes functional release excessive amount of oxidant which in turn injures hepatic cells. Activation of some enzymes in the cytochrome p-450 systems such as CYP2E1 also lead to oxidative stress. Injury to hepatocytes and bile duct cells leads to accumulation of bile acid inside liver. This aggravates further liver damage. Non-parenchymal cells such as kuffer cells, fat storing stellate cells and leucocytes also have role in mechanism (Chaterjee T, Pal S, Roy R, Pathak M 1991) Gymnema sylvestre belongs to the family Asclepiadaceae. The leaves have pleasant and aromatic odour. The leaves contain pentriacontane, hendriacotane, phytin, d-quercitol, gymnemic acids (antisweet compound). According to the Ethanomedical information of Gymnema sylvestre they is being used as antidiabetic, stomachic, stimulant, laxative and diuretic. It was also found to be useful in hepatosplenomegaly, dyspepsia, constipation, jaundice helminthiasis, cardiopathy and amenorrhea. Leaves were found to contain phenol in high concentration which is responsible for the antidiabetic activity. In this study the hepatoprotective activity of this plant was assessed using in vitro and in vivo methods.

Materials and Methods

The leaves of Gymnema sylvestre were collected in Kannur, Kerala during the month of May 2008. The plants was authenticated by Dr. S. Rajan, Botanical Plant Survey and Collection Unit, Government Arts College, Ootacamund, Tamil Nadu, India. The collected leaves were dried under shade, chopped and coarsely powdered. The hydro alcoholic extracts were prepared by cold maceration and hot maceration.

Qualitative Phytochemical Analysis

(Evans WC 2004, Kokate CK, Purohit, Gokhale SB 2002)
A systematic and complete study of crude drugs should include a complete investigation of both primary and secondary metabolites derived from plant metabolism. The different qualitative chemical tests were performed for establishing profiles of given extract for their nature of chemical composition. The ethanol extracts obtained as above were subjected to qualitative chemical test for identification of various phyto constituents (Jahangir rumana et al., 2008)

Quantitative Phytochemical Analysis

(Raman N 2006)

Estimation of total Phenolic Content

Phenols are the largest group of plant secondary metabolites. They range from simple phenol to larger molecules such as tannins, anthroquinones, flavonoids and coumarins. Phenolic compounds are commonly found in both edible and inedible plants and they have been reported to have multiple biological effects, including antioxidant activity. Total phenols was determined in powder crude drugs, extracts and beverages by the folin - ciocaltem method. The test is based on the oxidation of phenolic groups with phosphomolybdic and phosphotungstic acids. After oxidation a green - blue colored complex is formed whose absorbance was measured at 750 nm. The total phenol content of a tested material is being related to the antioxidant activity shown by it (Dash Deepak Kumar,2006)

Estimation of total Flavonol Content

Flavonoids are water-soluble polyphenolic compounds which are common and wide spread in the plant kingdom as their glycosides. The world “flavonoids” is derived from Latin: flavin meaning yellow and many flavonoids are indeed yellow in colour. It consists of a single benzene ring formed to benzo-gamma pyrone and binds to proteins such as enzymes and structural proteins. They include catechins, leucanthocyanidins, flavanones, flavones, anthrocyanidins, flavonol, chalcones aurones and isoflavones. Total flavonol was determined by aluminum chloride forms acid stable complexes with C-4 keto group and either the C-3 or C-5 hydroxyl groups with phosphomolybdic and phosphotungstic acids. After oxidation a green - blue colored complex is formed whose absorbance was measured at 750 nm. The total phenol content of a tested material is being related to the antioxidant activity shown by it (Dash Deepak Kumar,2006)

In vitro Anti-oxidant Activity

In vitro methods are based on the inhibition of free radical action. Samples are added to a free radical generating systems, inhibition of free radical action is measured and this inhibition is related to antioxidant activity of the samples. All the methods developed for in vitro antioxidant have strengths and limitations and a single measurement of the antioxidant capacity usually is not sufficient. A number of different methods may be necessary to adequately assess in vitro antioxidant activity of a specified compound or antioxidant capacity of a biological fluid. So, in this research paper we have performed the in vitro anti-oxidant activity by grayish method ( Sanvarinda et al., 2008), Nitric oxide radical inhibition activity (Nanjan M.J et.al 2007), Reducing power assay ( Arora S et.al 2007, Zhao M et.al 2007) and total antioxidant capacity ( Singab A.N. B et.al 2006).

In vitro Antihapatotoxic Activity on Isolated Rat Hepatocytes

The availability of methods for isolation of large quantities of intact cells had made isolated hepatocytes culture a favorite experiment system for pharmacological, toxicological and biological research. The HEPES buffer and collagenase solution (0.075 %) were warmed in a water bath (38°C – 39 ºC to achieve 37 ºC in the liver). The pump flow rate was adjusted to 30 ml/minutes. The rat (180-200 gms) was anesthetized by intraperitonial administration of Phenobarbital sodium 35 mg/K.g B.W. The abdomen was opened and loosely tied ligature was placed around the portal vein and the cannula was inserted up to the liver and then the ligature was tightened and heparin (1000 IU) was injected into the femoral vein. Sub hepatic vessels were rapidly incised to avoid excess pressure and 500 ml of calcium free HEPES buffer was perfused at a low rate of 30 mL/min for 20 minutes. The liver swells during this time slowly changing colour from dark red to grayish white. 200 ml of collagenase solution (0.075 %) were perfused at a flow rate of 15 mL/min for 20 minutes during which the lobes swell. The lobes were removed and washed HEPES buffer, after disrupting the glisson capsule. The cells were dispersed in 100 ml of Ham’s F12 medium. The cell suspension was centrifuged at 1000 rpm to remove collagenase damaged cells and non-parenchyma cells. The hepatocytes were collected in Ham’s F12 medium enriched with 0.2 % bovine albumin, 10 µg/ml bovine insulin and 0.2 dexamethasone. Cell counting was performed by tryphan blue dye exclusion techniques because this method gives an exact number of dead cells and viable cells.

Determination of Hepatoprotective Activity on Freshly Isolated Rat Hepatocytes by Estimating the Biochemical Parameters

Hepatocytes isolated were incubated for 30 minutes at 37 0 C for stabilization. The cells were then diluted in Hame’s F12 medium to obtain a cell count of 2 X 10 5 cells/ml. after 2 hours or preincubation, the medium was replaced with fresh medium. Hepatocytes injury was induced with ( 50 µl of 30 nM D- Galactosamine for 24 hours at 37 0 C. After incubation, the toxicant and drug treated cell suspension were pooled into eppendorf’s tubes and
Biochemical parameters were determined by using Ecoline diagnostic kits. Assays of aspartate amino transferase (ASAT), alanine amino transferase were carried out by measuring the NADH consumption determined by means of photometer at 340 nm and found to be directly proportional to aspartate amino transferase level in the serum. Assay of alkaline phosphate were identified by finding out the increase in 4-nitrophenolate which was determined photometrically at 405 nm which is directly proportional to the alkaline phosphate activity. Total protein content were identified with the absorbance of the colored complexes (violet color) sample and the standard against biuret reagent at 546 nm. Albumin was identified by measuring the absorbance of the blue green complex formed by diazotized sulfanilic acid and measured photometrically at 625 nm. The direct bilirubin(mg/dl) is measured, as the red azodye at 555 nm (Fernandas Ferreira M et al 2004, Dhanaraj SA et al 2006, Suresh B et al 2003, Sathish Kumar MN et al 2006).

In vivo Hepatoprotective Studies

Selection and Maintenance of Animals:
Healthy adult albino rats of Wistar strain weighting 150-200 gm were obtained from JSS college of Pharmacy, Animal house, Ooty, India. The animal house was well ventilated and the animals had 12±1 hour day and night schedule with temperature between 20 ±2 0 C. The animals were housed in large spacious hygienic cages during the course of the experimental period. The animals were fed with the rat feed supplied by M/s Hindustan Lever Ltd, Bangalore.

Preparation of the Extract Dose
Hydro alcoholic extract prepared by the hot maceration process from the leaves of Gymnema sylvestre were prepared at the concentration of 200 and 400 mg/ml.

Preparation of the Standard:
Single dose of 25 mg/kg b.w of silymarin for 13 days.

Preparation and Induction of Hepatotoxicity
D-Galactosamine hydrochloride (400 mg/kg b.w) was administered intra peritoneally on the 14th day to induce liver damage. The animals were divided into 5 groups with 6 animals in each (Equal number of both male and female).

Group – I: Served as control which received double distilled water (1 ml/Kg b.w) and 0.3 % sodium carboxyl methyl cellulose (CMC).

Isolation of Blood
The rat were anesthetized using thiopental and blood was collected from the abdominal artery and after collection, the blood was kept at 370 C in the incubator for 30 minutes later, it was cold centrifuged at 2000 rpm for 15 minutes to get clear supernatant serum, which was used for the biochemical estimation.

Preparation of Liver and Kidney Homogenates
The liver and kidney were removed, weighed and homogenized immediately with Elvenjan homogenizer fitted with plunger, in chilled 10 % KCl solution (10 ml/g of tissue). After centrifugation at 2000 rpm for 10 minutes, clear supernatant was used for the determination of SOD, CAT, TBARS and protein estimation. SOD and CAT levels were determined immediately after centrifugation and Malondialdehyde (MDA) as TBA-RS and protein content were estimated in frozen samples.

In vivo Antioxidant Evaluation
Catalase is present in all the major organs specially being concentrated in liver, kidney and erythrocytes. Catalase catalyses the rapid decomposition of hydrogen peroxide to water. It was estimated by measuring the change in the absorption at 240 nm was measured for 2-3 min and dy/dx for 1 minute was calculated and the results are expressed as CAT units/mg of tissue. SOD was estimated by measuring the color intensity of the chromo gram in the butanol by spectrophotometrically at 560 nm and concentration of SOD was expressed as U/mg of protein. Thiobarbituric acid reactive substances (TBA-RS) was
estimated by measuring the intensity of the pink chromogram formed due to MDA which is an end product of lipid peroxidation which reacts with thiobarbituric acid. It was calculated using a molar extinction coefficient of 1.56 X 10^5 M^-1 and expressed as TBARS/ mg of protein. The assay of protein estimation by bradford method is based on the ability of proteins to bind with the coomassie brilliant blue G 250 and to form a complex whose extinction coefficient is much greater than that of the free dye. Estimation of biochemical parameters such as ASAT, ALAT, ALP, total protein, albumin, LDH, total bilirubin, direct bilirubin and triglycerides were assayed using Ecoline diagnostic kit. (Cervinkova Z et al 2003 and Nishida Norihisa 2002)

Statistical Analysis
The result of in vitro and in vivo antihapatotoxic activity were analyzed statistically using one way analysis of variance (ANOVA) followed dunuatt’s t- test.

Results and Discussion
Leaves of Gymnema sylvestre were separated from the other parts and shade dried and coarsely powdered. Leaves of Gymnema sylvestre were extracted by hot and cold maceration. All the extracts were stored in refrigerator till it was used. The percentage yield of the extracts obtained by various extraction process were tabulated in the Table 1. Qualitative phytochemical analysis of the plant extract showed the presence of majority of compounds like alkaloids, carbohydrates, flavonoids, terpenoids, glycosides, proteins, amino acids, phenolic compounds and tannins. The results were tabulated in the Table 2. Among the two extracts, the hydro alcoholic extract prepared by the hot maceration has shown high phenol content 6.51 ± 0.231 mg/g, high total flavanol content 89.51 ± 0.100 mg/g. and their result were tabulated in Table 3. By DPPH method, total antioxidant capacity, reducing power ability, the hydro alcoholic extract prepared by hot maceration process showed higher antioxidant activity with the IC50 value of 132.46 ± 2.145 µg/ml and the results are tabulated in Table 4. The freshly prepared rat hepatocytes were treated with different concentration of hydro alcoholic extract prepared by the hot maceration process. The antihapatotoxicity produced by the extract at the concentration of 200, 400,600 µg/ml was effective against the D- Galactosamine-induced hepatotoxicity, whereas at the concentration of 800 µg/ml was found to be cytoxic. The effect of different extracts of Gymnema sylvestre on freshly isolated rat hepatocytes intoxicated with D- galactosamine are recorded in Table 5. A significant increase in the levels of ASAT, ALAT,ALP, total bilirubin, direct bilirubin (P= 0.001) and a significant reduction in the levels of TGL, total proteins and albumin (P=0.001) was observed in hepatocytes. The cells treated with different extracts of Gymnema sylvestre showed a significant restoration of the altered biochemical parameters towards the normal (P<0.001) when compared to D-galactosamine intoxicated treated groups and were dose dependent. A similar result was obtained when D-galactosamine intoxicated hepatocytes were treated with silymarin.

Summary and Conclusion
Plant drugs are known to play a vital role in the management of liver diseases. There are numerous plants and poly herbal formulations claimed to have hepatoprotective activities. Nearly 150 phyto constituents from 101 plants have been claimed to possess liver protecting activities. The preventive action in the liver damage induced by D-galactosamine has been widely used as an indicator of the liver protective activities of drugs or medicinal plants extracts, by in vitro and in vivo techniques. D-galactosamine produces an experimental liver damage, which histologically resembles that of viral hepatitis. During hepatic injury, superoxide radical generates at the site of damage and modulate SOD and CAT, resulting in the loss of activity and accumulation of superoxide radical, which damages liver. Decreased CAT activity is linked up to exhaustion of the enzyme as a result of oxidative stress caused by D-galactosamine. The SOD and CAT activities were brought to near normal after pretreatment with the D-galactosamine treated rats evidently shows the antioxidant property of the extract against oxygen free radical. The hydro alcoholic extract of Gymnema sylvestre was evaluated for anti-hepatotoxic activity against D-galactosamine-induced toxicity in isolated rat hepatocytes and in animal models. Freshly isolated hepatocytes have a distinct advantage that the cells express differentiated functions of liver and more importantly the activities of hepatic cytochrome P 450 involved in the activation of drugs and chemicals including D-galactosamine that is being used as a toxicant in this study. Various concentrations of the plant extracts of Gymnema sylvestre ranging from 200 µg /ml to 800 µl were tested on freshly isolated rat hepatocytes which are intoxicated with 15D- galactosamine (30 mm) for its hepatoprotective activities. The hydro-alcoholic extract prepared by hot maceration process showed good hepatoprotective activity when compared to the hydro-alcoholic extract prepared by cold maceration process.
Table 1. Percentage yield of the extracts from leaves of Gymnema sylvestre extracts.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Extraction Process</th>
<th>Powder Weight</th>
<th>Solvent used</th>
<th>Weight of the extract</th>
<th>Percentage of yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Hot maceration</td>
<td>200 g</td>
<td>Hydro Alcoholic</td>
<td>24.55 g</td>
<td>12.27 %</td>
</tr>
<tr>
<td>2.</td>
<td>Cold Maceration</td>
<td>100 g</td>
<td>Hydro Alcoholic</td>
<td>9.42 g</td>
<td>9.42 %</td>
</tr>
</tbody>
</table>

Table 2. Qualitative Phyto Chemical Analysis of Gymnema sylvestre extracts.

<table>
<thead>
<tr>
<th>Test</th>
<th>Gymnema sylvestre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hot Maceration</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>+</td>
</tr>
<tr>
<td>Phytosterols</td>
<td>+</td>
</tr>
<tr>
<td>Protein and Amino acids</td>
<td>-</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
</tr>
<tr>
<td>Phenolic Compounds</td>
<td>-</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
</tr>
</tbody>
</table>

+ - Indicates the presence - - indicates the absence

Table 3. Quantitative phytochemical analysis of the extracts of Gymnema sylvestre.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Name of the Extract</th>
<th>Total Phenol content</th>
<th>Total favonol content</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Hydro-alcoholic extract prepared by hot maceration</td>
<td>6.51 ± 0.231</td>
<td>89.51 ± 0.100</td>
</tr>
<tr>
<td>2.</td>
<td>Hydro alcoholic extract prepared by cold maceration</td>
<td>5.55 ± 0.174</td>
<td>47.69 ± 0.113</td>
</tr>
</tbody>
</table>

Table 4. Antioxidant activity of extracts of Gymnema sylvestre leaves.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Name of the Extract</th>
<th>DPPH method IC 50 Value µg/ml</th>
<th>Reducing Power IC 50 Value µg/ml</th>
<th>Power ability equivalent to Ascorbic acid (mM)</th>
<th>TAC (mM equivalent to Ascorbic acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Hydro alcoholic Extract prepared by hot maceration process</td>
<td>211.11 ± 0.966</td>
<td>225.73 ± 0.987</td>
<td>0.81 ± 0.018</td>
<td>0.81 ± 0.018</td>
</tr>
<tr>
<td>2.</td>
<td>Hydro alcoholic extract prepared by cold maceration process</td>
<td>132.46 ± 2.145</td>
<td>137.42 ± 3.12</td>
<td>0.98 ± 0.014</td>
<td>0.98 ± 0.014</td>
</tr>
<tr>
<td>3.</td>
<td>Ascorbic acid</td>
<td>2.69 ± 0.05</td>
<td>1.62 ± 0.13</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>
Table 5. Biochemical parameters of D-Galactosamine intoxicated rats with Gymnema sylvestre.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>ASAT U/L</th>
<th>ALAT U/L</th>
<th>ALP U/L</th>
<th>Total Protein g/ml</th>
<th>Albumin mg/dl</th>
<th>Total Bilirubin mg/dl</th>
<th>Direct bilirubin</th>
<th>TGL mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>-</td>
<td>65.91± 0.70</td>
<td>33.93± 1.32</td>
<td>440± 1.08</td>
<td>7.13±0.05</td>
<td>4.90±0.04</td>
<td>0.318±0.06</td>
<td>0.166±0.06</td>
<td>77.40±0.77</td>
</tr>
<tr>
<td>D- Gal N treated</td>
<td>400 mg/kg b.w</td>
<td>140.34 ± 1.32</td>
<td>72.01±0.98</td>
<td>905.1±4.77</td>
<td>4.35±0.13</td>
<td>3.64±0.11</td>
<td>2.12±0.11</td>
<td>0.48±0.01</td>
<td>27.6 ± 1.13</td>
</tr>
<tr>
<td>Gymnema sylvestre hydro alcoholic extract prepared by hot maceration</td>
<td>200 mg/kg b.w</td>
<td>103.68 ± 0.44</td>
<td>63.57±0.87</td>
<td>469.1±2.97</td>
<td>5.73±0.01</td>
<td>3.94±0.03</td>
<td>0.63±0.03</td>
<td>0.25±0.03</td>
<td>70.48 ± 0.72</td>
</tr>
<tr>
<td>Gymnema sylvestre hydro alcoholic extract prepared by hot maceration</td>
<td>400 mg/kg b.w</td>
<td>91.07±0.32</td>
<td>58.56±0.17</td>
<td>606.70±0.54</td>
<td>5.96±0.01</td>
<td>4.12±0.01</td>
<td>0.64±0.01</td>
<td>0.22±0.03</td>
<td>72.15 ± 1.26</td>
</tr>
</tbody>
</table>

References


