Antiproliferative effect of Gymnema sylvestre in In Vitro assays

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Abstract

Gymnema is popularly known as gurmar *or* Madhunashini (destroyer of sugar), as chewing the leaves causes a loss of sweet taste.

Objectives: The aim of this study was to assess the Antiproliferative effects of *Gymnema sylvestre leaves* extract in In Vitro test systems such as dimethylthiazol diphenyltetrazolium bromide (MTT) Assay, neutral red uptake (NRU) Assay, DNA fragmentation Assay .

Materials and Methods: HEPG2 cells were exposed to 0.1, 1, 10, 100, 1000 µg/ml concentration of *Gymnema extract* in a 96-cluster-well-culture plate for 24 h. The cell viability after exposure to *Gymnema sylvestre* was determined by MTT and NRU assays in separate tissue culture plates.

Results: *Gymnema sylvestre at* concentrations of 0.1, 1.0,10,1000 and 1000 ug/ml showed dose dependent inhibition of HEPG2 cells lines and caused cytotoxicity .

Conclusion: In general, the MTT and NRU assays performed similarly, exhibiting moderate to good correlation for the evaluation of the cytotoxicity of *Gymnema sylvestre*

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Gymnema Sylvestre is a woody, climbing plant of tropical forests of central and southern India and in parts of Africa. *Gymnema* has played an important role in Ayurvedic medicine for centuries. *Gymnema* is popularly known as gurmar *or* Madhunashini (destroyer of sugar), as chewing the leaves causes a loss of sweet taste. (Gloria *et al* 2003). Gymnemic acid, extracted from leaves and roots of *G.sylvestre* is mainly used in India and parts of Asia as a natural treatment for diabetes as it helps to lower and balance blood sugar levels. The major chemical constituents of *Gymnema* are gymnemic acid and gurmarin. Gymnemic acids have antidiabetic, anti sweetener and anti-inflammatory activities. The leaves of this plant showed the pharmacological activity i.e. Antidiabetic, antimicrobial, antibiotic, anti-inflammatory,

Few studies showed that G. Sylvester leaves to cause hypoglycemia in laboratory animals and shown use in herbal medicine to treat diabetes mellitus in adults [Kanetkar *etal*, 2004, Persaud *et al*, 1999]. Preliminary phytochemical screening and in vitro anti-oxidant of *Gymnema sylvestre* R.Br. leaf extract were reported (Rachh *et al*, 2009). *Gymnema sylvestre* was also reported to have antidote property against snake venom and was tabulated under the list. Its activity was thought to be due to presence of gymnemgenin. (Walter *etal*, 2000). It has been reported that the gurmarin peptide block the ability to taste sweet or bitter flavors and thus reduces sweet cravings. (Preuss *etal*, 2004). The wound healing activity of carbopol gels prepared from hydro alcoholic extracts of *Gymnema sylvestre* were checked by excision wound model and burn wound models in albino mice. (Kiranmai *et al*, 2011). *In vitro*, the inhibitory effects of DPPH radicals and LDL oxidation were found with aqueous extract of *G. sylvestre*. (Ohmori *et al*, 2005). In vivo studies of aqueous extract of GSE containing Gymnemic acids have shown muscle relaxant properties (Luo *et al*, 1999). *Gymnema* preparations have shown to possess anti-allergic activity (Sawabe *et al*, 1992). Aqueous extract of GSE have been shown to be significantly effective in controlling *Culex* larvae. (Tandon *et al*, 2010).

The plant was investigated for immunomodulatory activity by assessing neutrophil locomotion, chemotaxis test, phagocytosis of killed Candida albicans and nitroblue tetrazolium tests (Jitender *et al*, 2009). The radio protective effect of Gymnemic acid was evaluated on Swiss albino mice induced by radiation. (Bhatia *et al*, 2008). The aqueous extract of *Gymnema sylvestre* leaves was investigated of anti inflammatory activity in rats at a dose of 200,300 and 500mg/kg in carageenan induced paw edema and cotton pellet method. (Jitender *et al*, 2008). One study

reported the anticancer activity of Gymenema sylvestre on MCF 7 (epithelial cells of human breast cancer) and A 549 (epithelial cells of human lung cancer) under in vitro conditions by MTT assay method (Srikanth *et al*, 2010).

2.Materials and Methods:

2.1Chemicals

70% ethanol, Minimum Essential media with Earl's modification, Foetal bovine serum, Trypsin EDTA, Dimethylthiazolyldiphenyltetrazolium bromide, Neutral red uptake dye, Dimethyl sulphoxide, 1% Glacial Acetic acid, Cell lysis buffer, RNAse H, Proteinase K, Phenol, chloroform and Isoamyl alcohol mixture, Sodium acetate, Isopropanol, , TAE buffer, Agarose, Ethidium bromide, DNA Loading dye, DNA Ladder

2.2 Preparation of *Gymnema sylvestre* **extract** Plant material (*Gymnema sylvestre*) was collected locally and identified and the specimen was authentified at Department of Botany, Safia college, Bhopal(MP), India. . The leaves was washed, air dried, powdered and extracted separately, with 50 % methanol using separating funnel. Extract thus obtained were vacuum evaporation.

2.3 Preparation of Stock Solution For *G.sylvestre*extract stock solution**,** take 50 g of dried leaf powder of *gymnema sylvestre* in a separate container. Now, 250 ml of 50%methanol was added to a separating funnel and kept for 24 h. The container was shaked periodically. Then it was filtered and the filtrate was taken out and fresh solvent was again added to the residue powder. The procedure was repeated three times and the filtrates collected were pooled. Filtrate was concentrated in water bath at 40º C. Concentrated extract was dried at 40º C in incubator. Dried extract was powdered and packed in an air tight container.

Dose preparation

The *G. sylvestre* extract was then prepared by 50mg of extract in 1ml aqueous solution in treatment 1 by dissolving 400 µl of above prepared extract and 600 µl of distilled water. 900 µl of distilled water was added to each eppendroff. 100 µl of Gymnema extractadded to treatment 2 from treatment 1. Now remaining eppendroffs were serially diluted. The eppendroffs were labeled in terms of treatment numbers.

Cell culture procedure

The target cells used in this experiment were HEPG2 Cell were maintained at 37°C under 5% CO_2 and 100% humidity in DMEM and supplement with 10% fetal calf serum and antibiotics (200 µg/ml penicillin G, 200 µg/ml streptomycin, 2 µg/ml fungizone). The medium was changed every other day. When cells reached confluency, they were detached using 0.2% (w/v) trypsin and transferred to new culture flasks.

After sufficient growth for experimentation, the cells were trypsinized and plated in 96-cluster-well-culture plates at a concentration of 1×10^4 cells/well. Each well contained 100 µl of cell suspension and the plates were incubated for 24 h at 37°C under 5% CO_2 to obtain a monolayer culture. After 24 h of incubation, the old media was removed from each well. Then, 100 µl of elute from the *G.sylvestre* solution at concentrations of 0.1, 1.0,10,1000 and 1000 ug/ml the positive control or negative control was inserted into 96-cluster-well-culture plates (8 wells/test material). Two 96-cluster-well-culture plates were separately prepared to evaluate cell viability using the MTT and NRU assays. The experiments were repeated in triplicate. Following a 24-h incubation period at 37°C under 5% CO_2, cell viability of both plates was assessed.

Dimethylthiazol diphenyltetrazolium bromide assay

The test materials were removed from each well of the first plate. Then, 50 µl of MTT reagent (5 mg/mL) was added and incubated for 2 h at 37°C in the CO_2 incubator. MTT solution was then discarded, and 100 µl of isopropanol was added. The plates were swayed on a shaker to solubilize the formations of purple crystal formazan. The absorbance was measured using a

microplate reader at a wavelength of 570 nm. The results were used to construct a graph of the cell viability percentage against extract concentrations.

Neutral red uptake assay

The test material was removed from each well of the second plate. Then, 150 µl of neutral red dye (100 µg/ml) dissolved in the serum of free medium (pH 6.4) was added to the culture medium and incubated for 3 h at 37°C. Cells were washed with phosphate-buffered saline, and 150 µl of elution medium (EtOH/AcCOOH, 50%/1%) was added followed by gentle shaking for 60 min for complete dissolution. Absorbance was recorded at 540 nm using a microtiter plate reader.Cell viability was expressed as a percentage of the control values.

3. RESULTS

Fig:2.3

Confluent

HepG2 cells

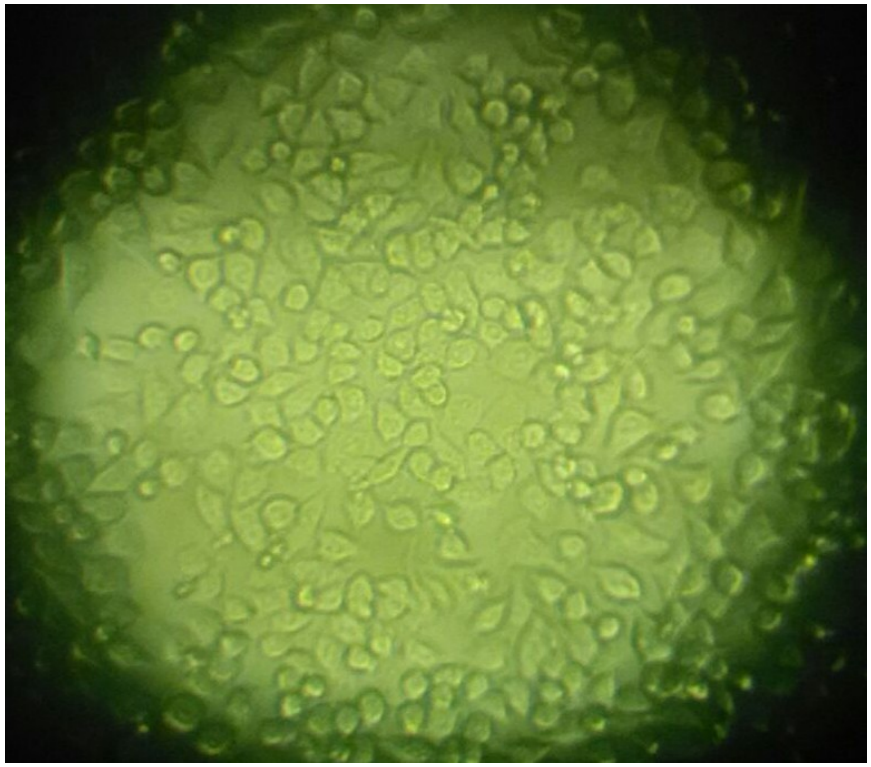

Fig:2.4HepG2 Cell seen under microscope

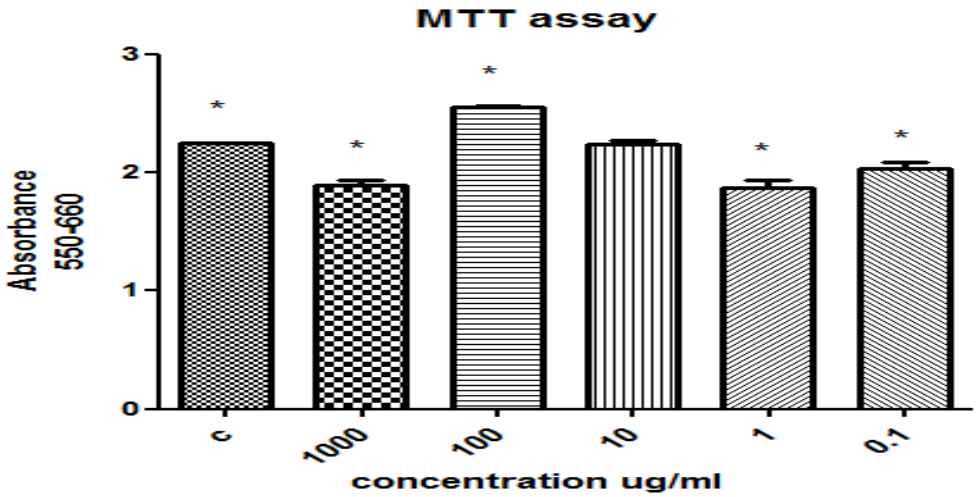

Fig: 2.5 MTT dose response curve of methanolic extract of *G. sylvestre*

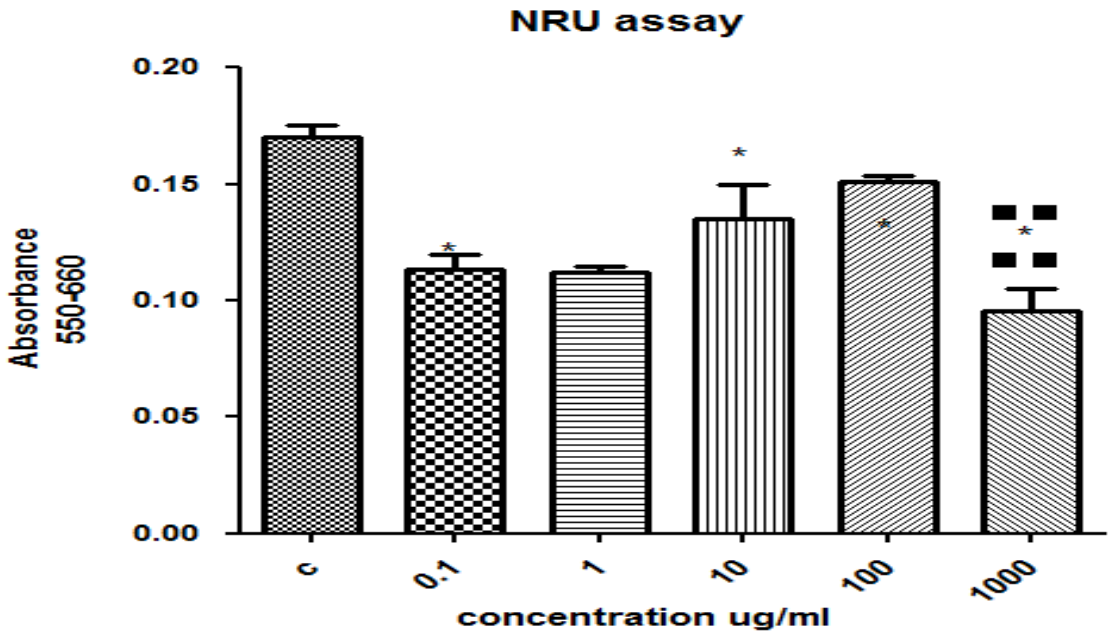

Fig: 2.6 NRU dose response curve of methanolic extract of *G. Sylvestre*

The methanolic extract of gymnema sylvestre were tested for cytotoxicity effect against HEpG2 cell line by MTT and NRU assay. The methanolic extract were tested at 0.1, 1, 10, 100, 1000 µg/ml concentration. The *G. Sylvestre* has good cytotoxic activity which was increasing with concentration.

DNA Fragmentation Assay- The result of DNA fragmentation analysis are as under (Nanodrop readings 260/280)

sample	C	T1	T2	T3	T4	T5
260/280	1.44	1.30	1.53	1.54	1.35	1.50

These reading show that DNA in all the concentration is in pure form

DNA Fragmentation Assay

DNA Fragmentation Assay In this, same initial steps were followed as in MTT and NRU process till passaging and plating of cells except that the cells were plated in the 6 well plate instead of 96 well plate. After incubation for 24h approx. the contents in the plate were pipette out without disturbing or scratching the confluent cells in the plate.

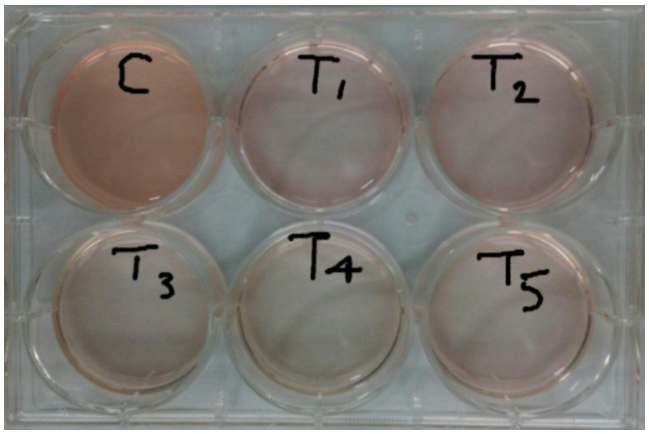

Fig:1.8 Six well plate for DNA fragmentation

Then lysing buffer was added once about 100 µl for extracting the cells through scratching. Same step was followed again to take out the remaining cells from the plate and were collected in eppendroffs which were labeled in terms of treatment no. To the eppendroff, 2µl RNAse was added was kept for incubation for 1 hr. After incubation, again 2µl Proteinase K was added and the eppendroff was kept for 1hr incubation. After this, buffered phenol/chloroform/isomyl propanol was added followed by centrifugation at 10,000 rpm or g for about 10 mins. After centrifugation, the supernatant was isolated by the help of pipette. To the supertanant, 50 µl sodium acetate and 200 µl isopropanol was added and was again centrifuged.

This time the pellet was isolated and 70% ethanol was added and again centrifuged. Discard the supernatant and collect the pellet and keep it for air drying for 1 day in laminar air flow. After this nuclease free water was added to the dried pellet and was taken for quantifying DNA using Nanodrop that is type of nano-spectrophotometer.

SpectrophotometerNanodrop Calculations for preparations of loading samples

sample	Concentration ng/ul	Sample vol for 200 ng DNA	Amount of loading dye
C	523.69	0.3819	0.03819
T1	26.59	7.52	0.752
T2	301.70	0.6629	0.06629
T3	176.40	1.133	0.1133
T4	159.87	1.251	0.1251
T5	508.45	0.393	0.0393

Now the readings were entered on an excel sheet and the amount of DNA sample and amount of Dye to be mixed were calculated after which the mixture was added to fresh eppendroffs and samples were refrigerated for a while. The preparations were then made for Gel Electrophoresis for which 0.3 gm of agarose powder was dissolved in 1X TAE buffer. The solution was then kept in the water bath and heated until a clear solution was visible after which the solution was poured into the casting tray with combs to make wells into the gel(15ml volume) and left for 15-20 mins. After the agarose gel was solidified the casting tray was separated from the combs carefully without damaging the wells made on the gel. The tray was then kept in the electrophoresis unit and the wells were loaded with the DNA sample and Dye mixture. The unit was then filled with 1X TAE buffer up to a volume that the gel was completely immersed. The tray was kept on the negative terminal of the unit. The power supply was then turned on and after1 h the gel was taken out and the readings were taken on the Gel Documentation System.

Discussion

The methnanolic extract of *gymnema sylvestre* were tested for cytotoxicity assay on Hep G2 (epithelial cell of human liver cancer) cells invitro by MTT and NRU assay. Methanolic extract was tested at 0.1µg/ml, 1 µg/ml,10 µg/ml, 100 µg/ml and 1000 µg/ml. All extract show concentration dependent activity. The effect of methanolic extract of gymnema on Hep G2 cell lines was very good at concentration 1000 µgm/ml. The anticancer activity determined in this study may also be due to gymnemic acid and this aspect should be established.

CONCLUSION

Two *in vitro* cytotoxicity assays, MTT and NRU, were compared to determine their correlation in Thai herbal plant evaluation. Good to moderate agreement was noted for the evaluation of *G.sylvestre* cytotoxicity. Thus, both MTT and NRU assays can be used for cytotoxic screening of this herbal species

References

- Agrawal, R.C., Jain, R., Wasim Raja, Ovais, M. (2009): Anticarcinogenic Effects of *Solanum lycopersicum* Fruit Extract on Swiss Albino and C57 BL Mice. *Asian Pacific Journal of Cancer Prevention.*10:379-381.
- Berenblum, J., Shubik, P. (1947): A new quantitative approach to the study of stages of chemical carcinogenesis in the mouse skin. *Br. J. Cancer.* 1:373-391.
- Bhatia AL, Raka K, Gulshan V, Sharma KV, Sharad V, Megha J (2008). Radio protective role of Gymnemic acid on mice: Study on Hepatic Biochemical Alterations. Asian J. Exp.Sci; 22(3):427-32.
- Gloria Y Yeh, David M. Eisenberg, Ted J. Kaptchuk, Russell S. Phillips (2003). Systematic Review of Herbs and Dietary Supplements for Glycemic Control in Diabetes. Diabetes Care; 26 (4): 1277-1294.
- Jitender KM, Manvi FV, Alagawadi KR and Noolvi M (2008): Evaluation of anti-inflammatory activity of *Gymnema sylvestre* leaves extract in rats. Int. J. Green Pharmacy: 114-115.
- Jitender KM, Manvi FV, Nanjwade BK, Alagawadi KR and Sanjiv S (2009): Immuno - modulatory activity of *Gymnema sylvestre* leaves extract on in vitro human neutrophils. J. Pharmacy Res; 2(8):1284-6.
- Kanetkar, P.V. Laddha, K.S. Kamat. M.Y (2004). Gymnemic acids: A molecular perspective of its action on carbohydrate metabolism. Poster presented at the 16th ICFOST meet organized by CFTRI and DFRL, Mysore, India.
- Kiranmai M, Kazim SM and Ibrahim M (2011). Combined wound healing activity of *Gymnema sylvestre* and *Tagetes erecta Linn*. Int. J. Pharmaceu. Appl,; 2(2):135-140.

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- Luo H (1999). Possible participation of NO and EDHF in the relaxation of rat intestinal circular muscle induced by Gymnema water extracts containing gymnemic acids. Journal of the Yonago Medical Association; 50(1): 22-31.
- Morse MA, Stoner GD. Cancer chemoprevention: principles and prospects, *Carcinogenesis*. 1993; 14(9) :1737-1746
- Murakami A, Ohigashi H, Koshimizu K. Anti-tumor promotion with food phytochemicals: a strategy for cancer chemoprevention, *Bioscience, Biotechonolgy,and Biochemistry*.1996; 60(1) :1-8,
- Ohmori, R Iwamoto, T Tago, M Takeo, T (2005). Unno, T Itakura H. *Lipids***,** 40 (8), 849-53.
- Pitot HC, Dragan YP, Facts and theories concerning the mechanisms of carcinogenesis, *The FASEB Journal*.1991; 5(9) :2280-2286
- Preuss, H.G. Bagchi, (2004). D Bagchi, M.. Rao, C.V. Dey, D.K Satyanarayana. S. *Diabetes Obes Metab,* **,** 6(3), 171-80.
- Rachh PR, Patel SR, Hirpara HV, Rupareliya MT, Rachh MR, Bhargava AS, Patel NM and Modi DC (2009): In-vitro evaluation of antioxidant activity of *Gymnema sylvestre* R.Br.leaf extract. Rom. J.Biol.Plant Biol,; 54(2):141-8.
- Sawabe Y, Nakagomi K, Iwagami S, Suzuki S, Nakazawa H (2009). Inhibitory effects of pectic substances on activated hyaluronidase and histamine release from mast cells. Biochim Biophys Acta 1992; 1137(3): 274-278.
- Sporn MB, Approaches to prevention of epithelial cancer during the preneoplastic period, *Cancer Research*.1976;36(7): 2699-2702,
- Srikanth AV, Sayeeda M, Lakshmi NM, Ravikumar P and Madhava RB (2010): Anti cancer activity of *Gymnema sylvestre* R.Br. Int. J. Pharmaceu. Sci. Nanotech; 3(1):897-899.
- Tandon Pankaj, Sirohi Anita (2010). Assessment of larvicidal properties of aqueous extracts of four plants against Culex quinquefasciatus larvae. JJBS; 3(1):1-6.
- Walter BM, Maria CDN, Bettina MRP and Nuno AP (2000). Plant natural products active against snake-bite-the molecular approach. Phytochemistry; 55:627- 642.