EVALUATION OF THE IN VITRO ANTIOXIDANT AND IRON CHELATING ACTIVITY OF *GYMNEMA SYLVESTRE*

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Summary

The study evaluated the *in vitro* antioxidant activity of 70% methanolic extract of *Gymnema sylvestre* leaves, which is was well known for its antidiabetic and sweet suppressing properties. The extract was used to determine the antioxidant potential using different tests including total antioxidant activity; efficiencies for scavenging of hydroxyl, superoxide, nitric oxide, singlet oxygen radicals and hypochlorous acid. Iron chelating, inhibition of lipid peroxidation and DNA protective properties were measured along with total phenolic and flavonoid content determination. The extract was found to be an antioxidant with a TEAC value 0.386 ± 0.19. The extract had shown its scavenging activity for different radical and 184.96 ± 2.44 µg/ml, 15.56 ± 0.8 µg/ml, 65.35 ± 2.56 µg/ml, 1.89 ± 0.084 mg/ml and 143.09 ± 55.49 µg/ml were determined as IC_{50} values for hydroxyl, superoxide, nitric oxide, hypochlorous acid radicals and lipid peroxidation inhibition, respectively. It was also observed that the plant extract (100 mg) has 23.7 ± 0.006 mg/ml gallic acid equivalent phenolic and 81.73 ± 0.008 mg/ml quercetin equivalent flavonoid content. The present results provide evidence that 70% methanol extract of *Gymnema sylvestre* leaves acts as an antioxidant and free radical.

Key Words: Antioxidant activity, DNA protection, free radical scavenging, *Gymnema sylvestre*, iron chelating, phenolic & flavonoid content

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Introduction

A number of reactive molecules such as superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2) and nitric oxide ($NO^{\cdot}$) are regarded as reactive oxygen species (ROS) **(1)** that are generated through various biological redox reactions and can directly react with biological macromolecules such as proteins, lipids and DNA of healthy human cells and cause cell membrane disintegration, DNA mutation and protein damage, which can further create cancer, atherosclerosis, cardiovascular disease, liver injury, ageing and inflammatory disease **(2)**. Almost all human cells are well protected against free radical damage by enzymes such as superoxide dismutase (SOD) and catalase or compounds such as ascorbic acid, tocopherol and glutathione **(3)**. But, sometimes, excessive generation of ROS beyond the capacity of antioxidant defense system leads to a variety of pathological processes such as inflammation, diabetes, genotoxicity and cancer **(4)**. Antioxidants can interrupt the oxidation process by reacting with free radicals, chelating catalytic metals and also by acting as oxygen scavengers. However antioxidant supplements are vital for human body due to their ability to combat oxidative damage. In the past few years, some synthetic antioxidants were developed but they are suspected of having some adverse effects. Therefore, the search for natural antioxidants has received much attention to identify and develop more potent antioxidants of natural origin to replace synthetic ones. Different kinds of plant material have already been reported as natural antioxidant **(5)**.

Gymnema sylvestre R. Br. (Asclepiadaceae) is a slow growing perennial, woody, climbing plant native to central and peninsular India and also be found in Australia and in tropical Africa **(6)**. In India, it is known as "gur-mar" or "sugar destroyer" because the leaves of this plant have sweet taste suppressing activity **(7)** and used in folk, *Ayurvedic* and homeopathic systems of medicine **(8)**. The plant is used for the treatment of diabetes mellitus; eye complaints **(9)** and snake bite **(10)**. Numerous studies have confirmed the hypoglycemic effect of *G. sylvestre* **(11, 12)**. It is regarded as one of the plants with potent antimicrobial activity **(13)**. The plant is also used as food additives for controlling obesity **(14)** and cholesterol level **(15)**. The main constituents isolated from *G. sylvestre* leaves are gymnemic acids **(6, 16)** and conduritol A **(17)**. Gymnemic acids are saponins with a triterpenoid structure and involved in inhibition of intestinal glucose absorption **(18)** and lowering of plasma glucose **(16)**. Another phyto-constisuents of *G. sylvestre*, gumerin, which is a peptide in nature, is also shown to involve in selective suppression of sweetness in rats **(19)**.

The present study is performed to investigate the *in vitro* antioxidant, free radical scavenging and iron chelating activity of 70% methanol extract of *G. sylvestre* leaves.

Methods

Chemicals: 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was obtained from Roche diagnostics, Mannheim, Germany. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Fluka, Buchs, Switzerland. Potassium persulfate ($K_2S_2O_8$), 2-deoxy-2-ribose, mannitol, lipoic acid, quercetin and ferrozine were obtained from Sisco Research Laboratories Pvt. Ltd, Mumbai, India. Folin-ciocalteu reagent and *N*,*N*-dimethyl-4-nitrosoaniline were obtained from Merck, Mumbai, India. DPPH, gallic acid and curcumin were obtained from MP Biomedicals, France. Catalase was obtained from HiMedia Laboratories Pvt. Ltd, Mumbai, India. Evans blue was purchased from BDH, England. Diethylene-triamine-pentaacetic acid (DTPA) was obtained from Spectrochem Pvt. Ltd, Mumbai, India. Thiobarbituric acid (TBA) was obtained from Loba Chemie, Mumbai, India. Desferal was bought from Novartis Pharma Stein AG, Switzerland.

Plant material: The plants were collected from Bankura district of West Bengal, India. The plants were identified by the Central Research Institute (Ayurveda), Kolkata, India, where a specimen (CRHS 122/08) was deposited.

Extraction: The powder (100 g) of the dried leaves of *G. sylvestre* was stirred using a magnetic stirrer with 500 ml mixture of methanol:water (7:3) for 15 hours; then the mixture was centrifuged at 2850 x *g* and the supernatant decanted. The process was repeated again with the precipitated pellet. The supernatants were collected, concentrated in a rotary evaporator and lyophilized. The dried extract was stored at -20°C until use.

Animals: The Animal Ethical Clearance Committee of the Institute (Registration number: 95/1999/ CPCSEA) approved the use of adult male Swiss Albino mice [*Mus musculus*], weighing 20-25 gm for experimentation. Each polypropylene cage contained 4 mice at a time; supplied with *ad libitum* laboratory diet and water. The mice were kept at 25 ± 2°C and 60 ± 5% humidity and normal photo cycle (12 h dark/12 h light).

Total antioxidant activity: The assay was performed on the ability of test sample to scavenge $ABTS^{\cdot+}$ radical cation in comparison to trolox standard **(20)**. The $ABTS^{\cdot+}$ radical cation was pregenerated by mixing ABTS solution with potassium persulfate for overnight. Then 10 µl sample solution was mixed with 1 ml $ABTS^{\cdot+}$ solution and the absorbance was measured at 734 nm after 6 min. All experiments were repeated for six times. The percentage inhibition of absorbance was calculated and plotted as a function of concentration of standard and sample to determine the trolox equivalent antioxidant concentration (TEAC).

DPPH radical scavenging assay: The complementary study for the antioxidant capacity of the plant extract was confirmed by the DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging assay according to Mahakunakorn et al. **(21)**, with slight modification. Different concentrations (0-100 µg/ml) of the extract and the standard trolox were mixed with equal volume of ethanol. Then 50 µl of DPPH solution (1 mM) was pipetted into the previous mixture and stirred thoroughly. The resulting solution was kept standing for a few minutes before the O.D. was measured at 517 nm. The measurement was repeated with six sets. The percentage of scavenging was calculated from the values of the control and the test samples.

Hydroxyl radical scavenging: Hydroxyl radical scavenging assay was performed by a standard method **(20)**. Hydroxyl radical was generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system which is known as Fenton reaction. The assay is based on the quantification of the 2-deoxyribose degradation product, by its condensation with TBA. All tests were performed for six times. Mannitol, a classical $^{\cdot}OH$ scavenger, was used as a positive control. Percent inhibition was evaluated by comparing the results of the test and blank solution.

Superoxide radical scavenging: Measurement of superoxide anion scavenging activity was done based on the reduction of NBT according to a previously described method **(20)**. The non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide radicals. These radicals reduce nitro blue tetrazolium (NBT) into a purple colored formazan which was measured spectrophotometrically at 562 nm. All tests were performed for six times. Quercetin was used as positive control.

Nitric oxide radical scavenging: At physiological pH, nitric oxide generated from sodium nitroprusside (SNP) aqueous solution interacts with oxygen to produce nitrite ions measured by Griess Illosvoy reaction **(20)**. The chromophore generated was spectrophotometrically measured at 540 nm against blank sample. All tests were performed for six times. Curcumin was used as a standard.

Peroxynitrite anion scavenging: Peroxynitrite ($ONOO^-$) synthesis was carried out by the method as described by Beckman et al. **(22)**. Acidic solution (0.6 M HCl) of 5 ml H_2O_2 (0.7 M) was mixed with 5 ml of 0.6 M KNO_2 on an ice bath for 1 s and 5 ml of ice-cold 1.2 M NaOH was added to the reaction mixture. Excess H_2O_2 was removed by the treatment of granular MnO_2 prewashed with 1.2 M NaOH and the reaction mixture was left overnight at -20°C. Peroxynitrite solution was collected from the top of the frozen mixture and the concentration was measured spectrophotometrically at 302 nm ($\varepsilon = 1670\ M^{-1}\ cm^{-1}$).

Evans blue bleaching assay was used to measure the peroxynitrite scavenging activity. The assay was performed according to a standard method **(20)**. The percentage of scavenging of $ONOO^-$ was calculated by comparing the results of the test and blank sample. All tests were performed for six times. Gallic acid was used as reference compound.

Singlet oxygen scavenging: The production of singlet oxygen ($^1O_2$) was determined by monitoring *N*,*N*-dimethyl-4-nitrosoaniline (RNO) bleaching, using a earlier reported method **(20)**. Singlet oxygen was generated by a reaction between NaOCl and H_2O_2 and the bleaching of RNO was read at 440 nm. The scavenging activity of sample was compared with lipoic acid used as a reference compound. All tests were performed for six times.

Hypochlorous acid scavenging: Hypochlorous acid (HOCl) was prepared just before the experiment by adjusting the pH of a 10% (v/v) solution of NaOCl to pH 6.2 with 0.6 M H_2SO_4 and the concentration of HOCl was determined by taking the absorbance at 235 nm using the molar extinction coefficient of 100 $M^{-1}\ cm^{-1}$. The assay was done according to a previously described method **(20)**. The scavenging activity was evaluated by measuring the decrease in the absorbance of catalase at 404 nm. All tests were performed for six times. Ascorbic acid, a potent HOCl scavenger, was used as reference compound.

Fe^{2+} ion chelating: The ferrous ion chelating activity was evaluated by a standard method **(20)**. The reaction was carried out in hepes buffer (20 mM, pH 7.2). Briefly, the plant extract was added to ferrous sulfate solution (12.5 µM) and the reaction was initiated by the addition of ferrozine (75 µM). The mixture was shaken vigorously and left standing for 20 min at room temperature. Then the absorbance was taken at 562 nm. All tests were performed for six times. EDTA was used as a positive control.

Lipid peroxidation inhibition: The antioxidant capacity of the plant extract was alternatively measured by lipid peroxidation inhibition, following an earlier method **(23)**. Brain homogenate was prepared by centrifuging Swiss Albino mice brain with 50 mM phosphate buffer and 120 mM KCl. An aliquot of the supernatant homogenate was mixed with plant extract of various concentrations (2.5-25 µg/ml), followed by addition of 0.1 mM FeSO4 and 0.1 mM ascorbic acid, and incubated for 1 hr at 37 °C to generate the TBARS (Thiobarbituric acid reactive substance). After stopping the reaction with TCA, TBA was added and the absorbance of the supernatant was taken at 532 nm. All tests were repeated six times. Trolox was used as the standard.

DNA Protection: The protection of the pUC-18 plasmid DNA damaged by Fenton reaction generated $OH^\cdot$ radicals was studied by quantifying the decrease of supercoiled DNA after oxidative attack, following an earlier reported method **(24)**, with minor modifications. In Hepes buffer (pH 7.2, 100 mM), $FeSO_4$ solution (750 µM), plant extracts of varying concentrations, DNA (10 µg/ml) and water were added to make an initial reaction mixture. Finally, H_2O_2 solution (7.5 mM) was added to start the reaction. After 10 min, the reaction was stopped by adding Desferal as stopping reagent followed by loading buffer. 25 µl of each reaction mixture was loaded in 1% agarose gel. After migration, the gel was stained with ethidium bromide and visualized in a UV transilluminator. The DNA bands were quantified through densitometry and the following formulae were used to calculate the percentage of protection.

$$\% SC = [1.4 X SC/(OC+(1.4 X SC))] X 100$$

where, SC = supercoiled; OC = open circular; 1.4 = correction factor

% protection = 100 X [(control SC – chelator SC)/(control SC – no chelator SC) – 1]

The ability of the plant extract to protect the DNA supercoil can be expressed by the concentration of sample required for 50% protection, designated as the $[P]_{50}$ value.

Assay of total phenolic content: The total phenolic content present in the 70% methanol extract of *G. sylvestre* leaves was determined using Folin-Ciocalteu (FC) reagent by a formerly reported method **(20)**. The phenolic content was evaluated from gallic acid standard curve.

Assay of total flavonoid content: The amount of total flavonoid content was determined with aluminium chloride ($AlCl_3$) according to a known method **(20)**. The flavonoid content was calculated from quercetin standard curve.

Statistical analysis: All data were reported as the mean ± SD of six measurements. The statistical analysis was performed by KyPlot version 2.0 beta 15 (32 bit). The IC_{50} values were calculated by the formula, Y = 100*A1/(X + A1) where A1 = IC_{50}, Y = response (Y = 100% when X = 0), X = inhibitory concentration. The IC_{50} values were compared by paired t test. $p < 0.05$ was considered significant.

Results and Discussion

Although organisms are bestowed with antioxidants and repair systems that have evolved to protect them against oxidative damage and free radicals which for the sake of stability damage proteins, lipids and DNA of healthy human cells, these systems are insufficient to prevent the damage totally. Natural antioxidants are important as possible protective agents to help the human body to reduce oxidative damage and hence disease propagation. Formerly, we have shown that *Cajanus cajan* leaf can be used as antioxidant supplement **(23)**. The present study focused on the evaluation of antioxidant and free radical scavenging activity of 70% methanol extract of *G. sylvestre.*

Antioxidant capacity: The reaction between ABTS and potassium persulfate results in the formation of a blue colored chromophore, $ABTS^{\cdot+}$. The total antioxidant activity of extract was calculated based on the decolorization of the $ABTS^{\cdot+}$, which was measured spectrophotometrically at 734 nm. After addition of the plant extract and trolox, this pre-formed radical cation was converted to ABTS on a concentration dependant manner. The percentage inhibition of the absorbance was shown in Figure 1(a) and (b) respectively. The results are compared with trolox and the TEAC value was found to be 0.386 ± 0.19 (Table 1). The obtained TEAC value demonstrates the extract as a potent antioxidant.

The effect of the plant extract in the scavenging assay of DPPH radical furthermore assured the fact that the extract smoothly acts as an antioxidant, since the study on TEAC and DPPH scavenging can be observed as complementary to each other **(25)**. As is also evident in the Figure 2 and the IC_{50} value of the sample (150.55 ± 3.57 µg/ml) in comparison to the ascorbic acid (5.29 ± 0.28 µg/ml) standard (Table 1) to scavenge the radical, it can be put forward as a fact that the extract truly works as an antioxidant.

Scavenging of hydroxyl radicals - Inhibition of Fenton reaction: The hydroxyl radical is one of the reactive free radicals formed in biological systems. It causes enormous damage on biomolecules of the living cells **(26)**. In course of the Fenton reaction, hydroxyl radicals are formed by incubating Ferric-EDTA with ascorbic acid and H_2O_2 at pH 7.4 that cause 2-deoxy-2-ribose damage and generate malondialdehyde (MDA) like product.

This compound forms a pink chromogen upon heating with TBA at low pH. As the *G. sylvestre* extract or standard mannitol is added to the reaction mixture the hydroxyl radicals are scavenged and thereby sugar damage can be blocked. The results are shown in Figure 3. The IC_{50} values of extract and standard were found as 184.96 ± 2.44 µg/ml and 571.45 ± 20.12 µg/ml, respectively (Table 1) which indicates that the plant extract is a better hydroxyl radical scavenger than standard mannitol.

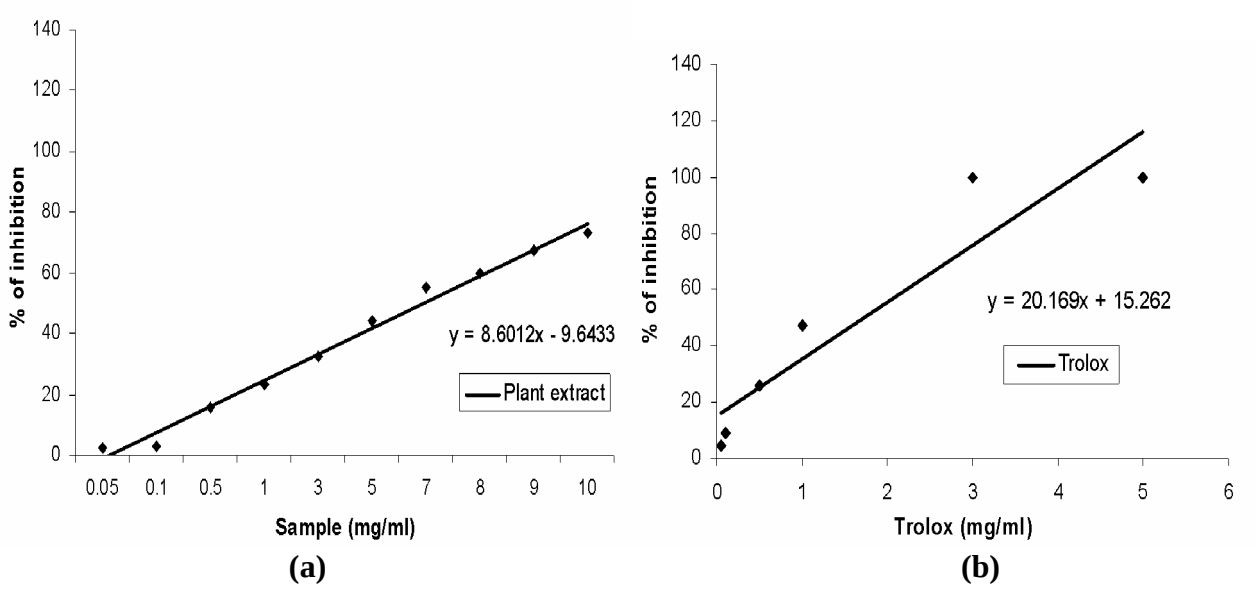


Figure 1. Total antioxidant activity: Total antioxidant activity of plant extract and trolox. Effect of (a) *Gymnema sylvestre* extract and (b) reference compound trolox on ABTS radical cation decolorization assay. The percentage of inhibition was plotted against concentration of sample. All data are expressed as mean ± S.D. (n=6).

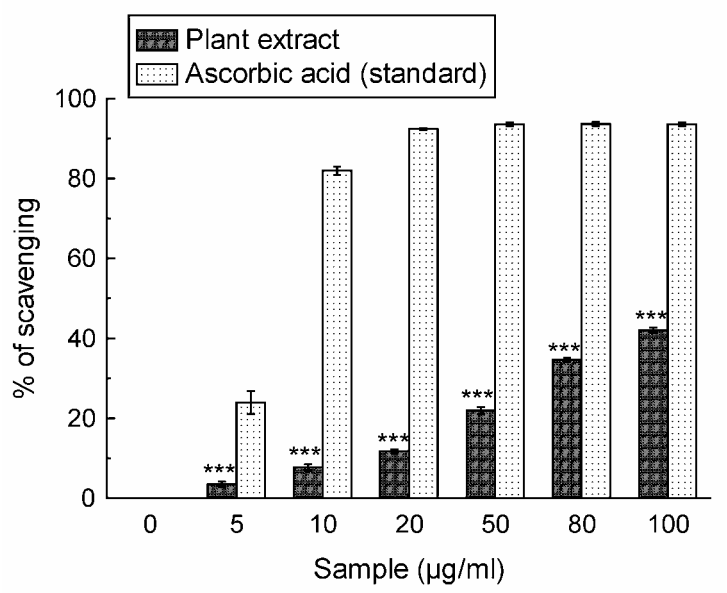


Figure 2. DPPH Scavenging Activity: Effect of the plant extract and standard ascorbic acid on DPPH radical scavenging study. The data is expressed as % scavenging of DPPH radicals. The results are mean ± S.D. of six parallel measurements. ***$p < 0.001$ vs 0 µg/ml.

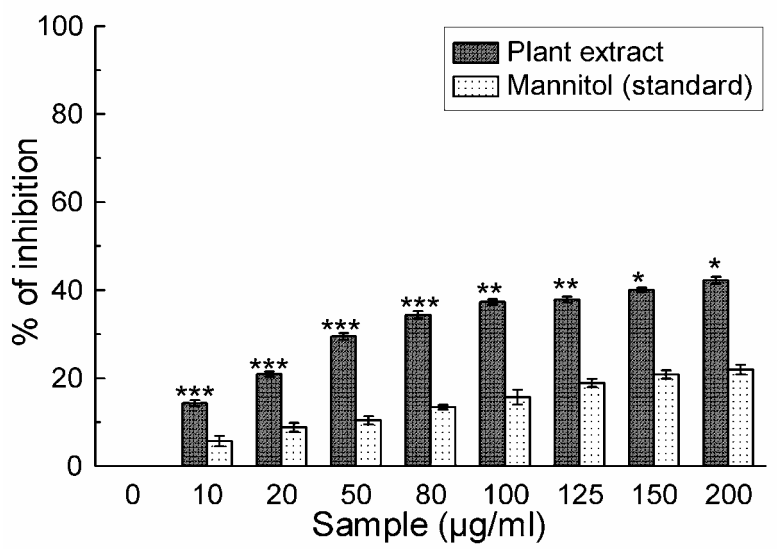


Figure 3. Hydroxyl radical scavenging: Hydroxyl radical scavenging activity of the *Gymnema sylvestre* extract and the reference compound mannitol. The data represent the percentage of inhibition of deoxyribose degradation. The results are mean ± S.D. of six parallel measurements. *p < 0.05, **p < 0.01 and ***p < 0.001 vs 0 µg/ml.

Superoxide anion scavenging capacity: Superoxide anion is also implicated as harmful reactive oxygen species. In biological system cellular components get damaged in contact with superoxide anion. PMS-NADH coupling reaction yields dissolved oxygen which in turn changed into superoxide radicals. These superoxide radicals can be measured by its ability to reduce NBT. The ability of the plant extract and the reference compound quercetin to quench superoxide radicals from reaction mixture is reflected in the decrease of the absorbance at 560 nm. In accordance to Figure 4, the IC_{50} values of the plant extract and quercetin on superoxide scavenging activity were found to be 15.56 ± 0.8 µg/ml and 42.06 ± 1.35 µg/ml, respectively (Table 1). The results suggest that the plant extract is more potent scavenger of superoxide radical than standard quercetin.

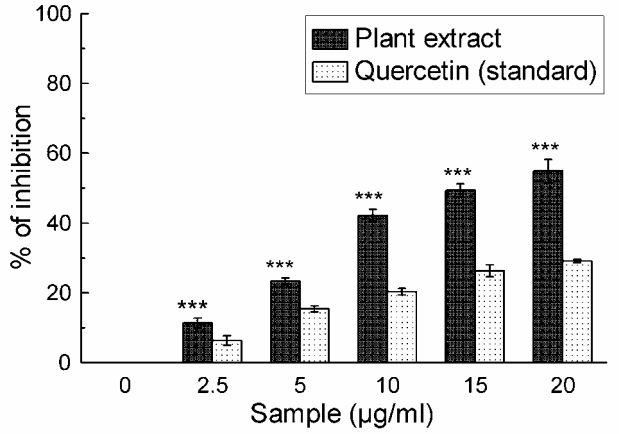


Figure 4. Superoxide radical scavenging: Scavenging effect of *Gymnema sylvestre* plant extract and standard quercetin on superoxide radical. The data represents the percentage of superoxide radical inhibition. All data are expressed as mean ± S.D. (n=6). ***p < 0.001 vs 0 µg/ml.

Effect on nitric oxide scavenging: Nitric oxide radical is well known as it has an important role in various types of inflammatory process. The production of nitric oxide radical at a sustained levels result in direct tissue toxicity and contribute to the vascular collapse associated with septic shock, whereas chronic expression of nitric oxide radical is associated with various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis **(27)**. The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. *G. sylvestre* extract also moderately inhibits nitrite formation by directly competes with oxygen to react with nitric oxide. Curcumin was used as a reference compound. The scavenging activity of extract and curcumin was shown in Figure 5 and 65.35 ± 2.56 µg/ml and 90.82 ± 4.75 µg/ml were determined as IC_{50}, respectively (Table 1). The present study proved that the nitric oxide scavenging activity of the studied extract is better than the standard curcumin.

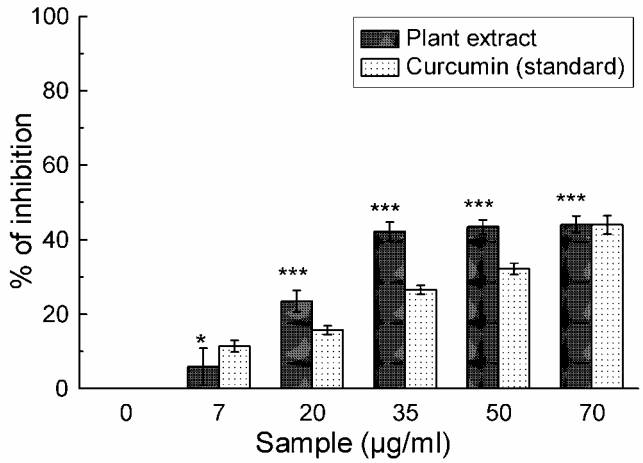


Figure 5. Nitric oxide radical scavenging: The nitric oxide radical scavenging activity of *Gymnema sylvestre* extract and standard curcumin. The data represents the % of nitric oxide inhibition. Each value represents mean ± S.D. (n=6). *p < 0.05 and ***p < 0.001 vs 0 µg/ml.

Moreover, the toxic effect of NO increases greatly upon reaction with superoxide radical and resulting formation highly reactive peroxynitrite anion ($ONOO^-$), especially its protonated form peroxynitrous acid (ONOOH) **(28)**. However, the studied extract did not show any substantial result to raise the interest of study.

Singlet oxygen scavenging activity: Singlet oxygen, a high energy form of oxygen, was generated in the skin upon UV-radiation. It induces the hyperoxidation, oxygen cytotxicity and decreases the antioxidant activity **(29)**. The IC_{50} value (Table 1) of test sample was found to be 460.53 ± 11.98 µg/ml whereas that of lipoic acid was found to be 46.15 ± 1.16 µg/ml. The IC_{50} value of the extract was higher than the reference compound. The present study indicates that the *G. sylvestre* extract has singlet oxygen scavenging activity but very poor compared to standard lipoic acid, as reflected in Figure 6.

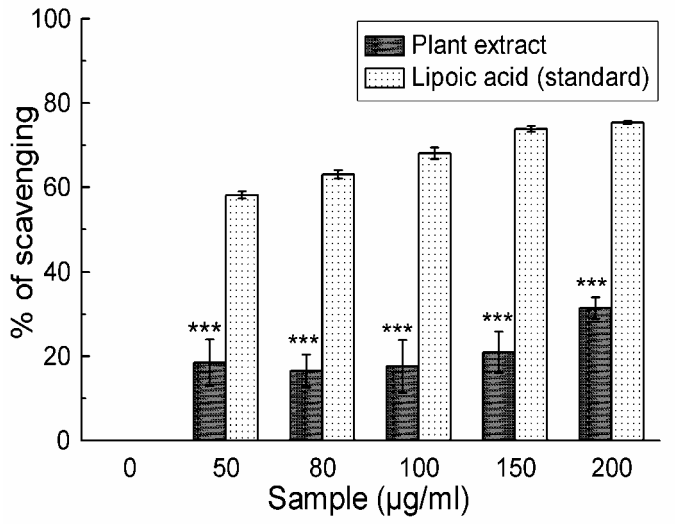


Figure 6. Singlet oxygen scavenging: Effect of *Gymnema sylvestre* plant extract and standard lipoic acid on the scavenging of singlet oxygen. The results are mean ± S.D. of six parallel measurements. ***p < 0.001 vs 0 µg/ml.

Activity in scavenging HOCl: Hypochlorous acid is another harmful ROS. At the sites of inflammation, the oxidation of Cl^- ions by the neutrophil enzyme myeloperoxidase results in the production of this ROS **(1)**. HOCl has the ability to inactivate the antioxidant enzyme, catalase through break down of heme prosthetic group. The inhibition of catalase inactivation in the presence of the extract signifies its HOCl scavenging activity. The obtained results (Figure 7) indicate that the standard ascorbic acid (IC_{50} = 0.24 ± 0.006 mg/ml) is the better scavenger than the plant extract (IC_{50} = 1.89 ± 0.084 mg/ml) (Table 1). So, it is anticipated that *G. sylvestre* is not efficient scavenger of HOCl.

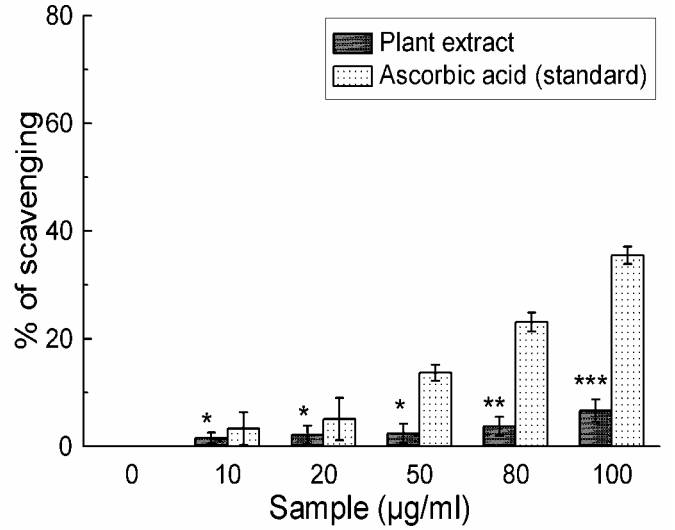
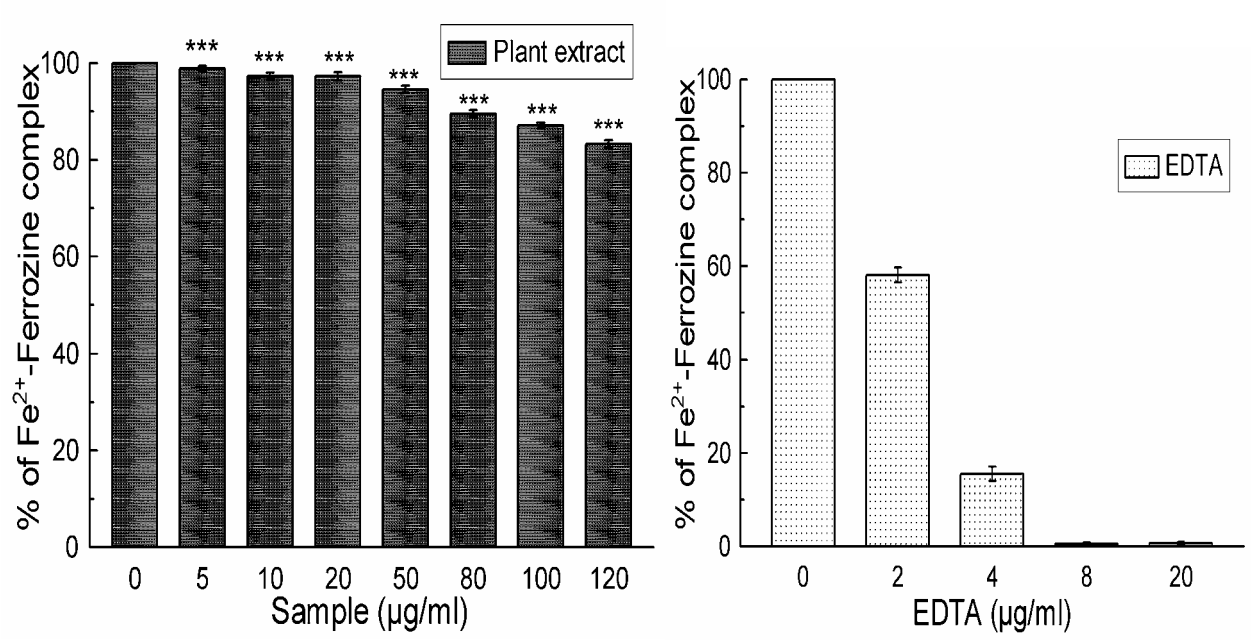


Figure 7. HOCl scavenging: Hypochlorous acid scavenging activity of *Gymnema sylvestre* plant extract and standard ascorbic acid. All data are expressed as mean ± S.D. (n=6). **p < 0.01 and ***p < 0.001 vs 0 µg/ml.

Ability to chelate Fe^{2+}: Iron can accelerate lipid peroxidation by decomposing lipid hydro-peroxides into peroxyl and alkoxyl radicals that can also perpetuate the chain reaction of lipid peroxidation. The metal chelating capacity is significant since it reduces the concentration of the catalyzing transition metal in lipid peroxidation. The results [Figure 8(a) and Figure 8(b)] demonstrated that the formation of ferrozine-Fe^{2+} complex is inhibited in the presence of test and reference compound. The IC_{50} value (Table 1) of the plant extract and EDTA was found 659.55 ± 33.33 µg/ml and 1.27 ± 0.05 µg/ml, respectively. According to the results, the plant extract is not as efficient as standard EDTA; but the decrease in the concentration dependent color formation in presence of extract indicate that it has iron chelating property.



(a) **(b)**

Figure 8. Fe^{2+} chelating: Effect of (a) *Gymnema sylvestre* plant extract and (b) standard EDTA on ferrozine-Fe^{2+} complex formation. The data expressed as % inhibition of chromogen formation. The results are mean ± S.D. of six parallel measurements. ***$p < 0.001$ vs 0 µg/ml.

Effect in the inhibition of lipid peroxidation: Lipid peroxidation is initiated through iron catalysed generation of ferry-perferryl complex or hydroxyl radicals that accelerates peroxidation by decomposing lipid hydro-peroxides into peroxyl and alkoxyl radicals. The hydroxyl radical is highly reactive and can damage biological molecules, when it reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids that eventually yield carbonyl products like malondialdehyde (MDA), which generate a pink chromogen with TBA. As can be seen in Figure 9 and from the IC_{50} values of the plant extract and standard trolox being 143.09 ± 55.49 µg/ml and 13.52 ± 0.33 µg/ml, respectively, the gradual decrease of the MDA measured as inhibition of lipid peroxidation with increasing concentration of the sample establishes the role of the sample as an antioxidant.

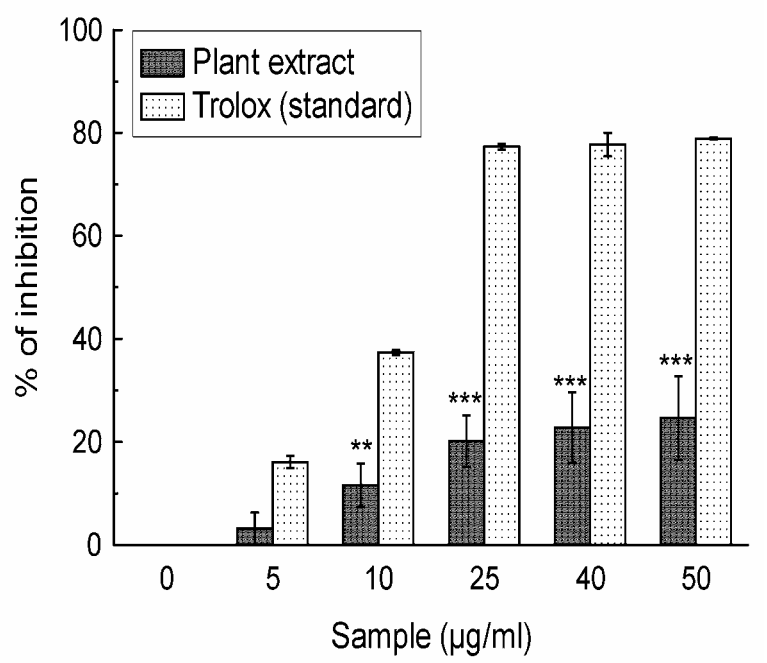


Figure 9. Lipid peroxidation inhibition: The inhibitory effect of *Gymnema sylvestre* plant extract and standard trolox on lipid peroxidation phenomenon. The data expressed as % inhibition of peroxidation of lipids from mice brain homogenate. The results are mean ± S.D. of six parallel measurements. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs 0 µg/ml.

DNA protective ability: At the cellular level, subjecting cells to oxidative stress can result in severe metabolic dysfunction, including DNA damage with a characteristic pattern of modification of all bases, production of base-free sites, deletions, strand breaks, DNA-protein cross-links, and chromosomal rearrangement. An important reaction involved in DNA damage involves generation of hyrodxyl radical through Fenton chemistry. Hydroxyl radical is known to react with all components of the DNA molecule: the purine and primidine bases as well as the deoxyribose backbone. When DNA was exposed to Fenton reaction, H_2O_2 will be generated to hydroxyl radicals, and then the supercoiled (SC) form of DNA would cleave to give rise open-circular (OC) form.

DNA damage protective activity of *G. sylvestre* extract showed a considerable result in retaining the SC from OC, gradually with increasing concentration. As can be observed in the Figure 10(a) where lane 1 is the control with only plasmid (pUC-18) and hence contains only SC-DNA, lane 2 is the Fenton reaction without extract and having only the OC form, and lane 3-9 contains plant extract of varying concentrations (30 - 300 µg/ml) in the Fenton reaction. Result suggested that the gradually increasing concentration of the plant extract is retaining the SC form, thus protecting DNA effectively. Figure 10(b) also corroborates the fact with giving about 80% protection at the highest concentration, as is also evident from the $[P]_{50}$ value of the plant extract being 128.79 ± 2.23 µg/ml (Table 1)

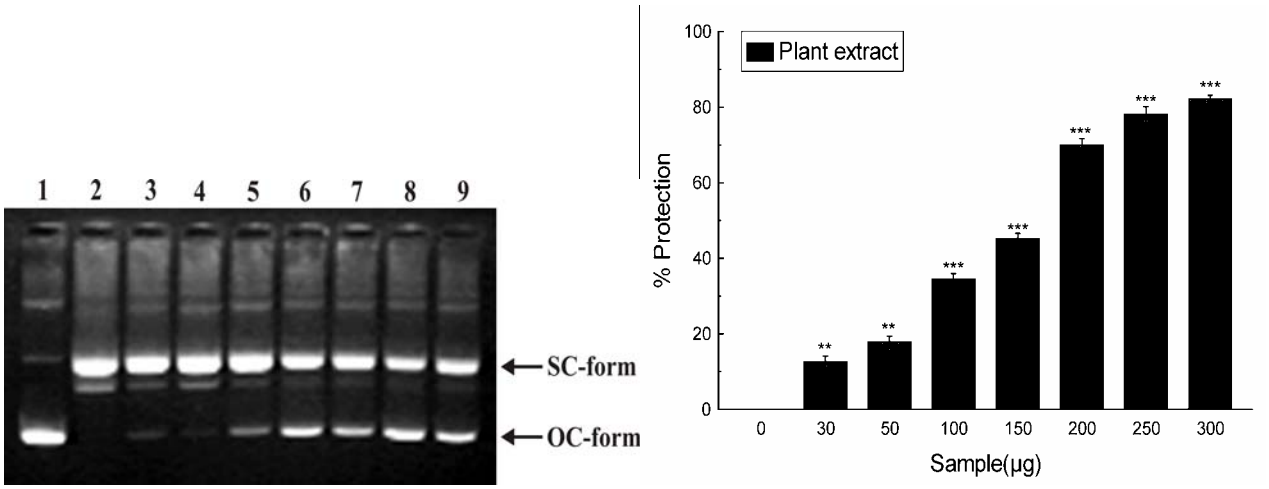


Figure 10. DNA protective ability: Ability of *Gymnema sylvestre* plant extract to protect pUC-18 plasmid DNA from undergoing cleavage due to Fenton reaction. The 1% agarose gel figure (a) shows the gradual retention of supercoiled DNA from the open-circular form. Lane 1 contained the plasmid DNA along with $FeSO_4$, but not H_2O_2, so only the SC-form is found. Lane 2 consisted of the same along with 4 µl 7.5 mM H_2O_2, thus resulting complete transformation to the OC-form. Lanes 3-9 contained the plant extract of varying concentrations (30, 50, 100, 150, 200, 250, 300 µg/ml), which clearly shows the protective effect of the plant extract through retention of the SC-form from the OC-form gradually with increasing concentration. This is also corroborated by the adjoining curve (b) which shows that there is nearly 80% protection activity of the sample at the highest concentration. The results are mean ± S.D. of three parallel measurements. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs 0 µg/ml.

Determination of total phenolic and flavonoid content: Phenolic contents are very important plant constituents because they can act as reducing agents, hydrogen donors and metal chelator **(30)**. They have also scavenging ability due to their hydroxyl groups. Flavonoids show their antioxidant action through scavenging or chelating process **(31)**. Both of these compounds have good antioxidant potential and their effects on human nutrition and health are significant. The phytochemical analysis showed that 100 mg of *G. sylvestre* extract has 23.7 ± 0.006 mg/ml gallic acid equivalent phenolic content and 81.73 ± 0.008 mg/ml quercetin equivalent flavonoid content (Table 1). The results indicate that *Gymnema sylvestre* plant extract contains significant amount of flavonoids and phenolic content.

Table 1 - Relative activities of *Gymnema sylvestre* and reference compounds:

Activity	***Gymnema sylvestre***
Trolox equivalent antioxidant capacity (TEAC)	0.386 ± 0.19 (6)
Phenolic content (mg/ml gallic acid equivalent)	23.7 ± 0.006 (3)
Flavonoid content (mg/ml quercetin equivalent)	81.73 ± 0.008 (3)
Concentration required for 50% DNA protection; $[P]_{50}$ (µg/ml)	128.79 ± 2.23 (3)

IC_{50} values for the sample and reference compounds in scavenging different ROS and for Iron chelating activity and Lipid peroxidation inhibition (#)

Activity	***Gymnema sylvestre***	**Reference compound**	**Value for reference compound**
DPPH radical scavenging	150.55 ± 3.57 (6)	Ascorbic acid	5.29 ± 0.28 (6) ***
Hydroxyl radical ($OH^{\cdot}$) scavenging	184.96 ± 2.44 (6)	Mannitol	571.45 ± 20.12 (6) ***
Superoxide anion ($O_2^{\cdot-}$) scavenging	15.56 ± 0.8 (6)	Quercetin	42.06 ± 1.35 (6) ***
Nitric oxide radical (NO) scavenging	65.35 ± 2.56 (6)	Curcumin	90.82 ± 4.75 (6) *
Singlet oxygen ($^1O_2$) scavenging	460.53 ± 11.98 (6)	Lipoic acid	46.15 ± 1.16 (6) ***
Hypochlorous acid (HOCl) scavenging	1.89 ± 0.084 (6)	Ascorbic acid	0.24 ± 0.006 (6) ***
Iron Chelating Activity	659.55 ± 33.33 (6)	EDTA	1.27 ± 0.05 (6) ***
Lipid Peroxidation Inhibition	143.09 ± 55.49 (6)	Trolox	13.52 ± 0.33 (6) **

Unit of IC_{50} values of all activities is µg/ml, except HOCl scavenging where unit is mg/ml.
Data expressed as mean ± S.D. Data in parenthesis indicate number of independent assays.
EDTA, Ethylene diamine tetra acetic acid. *$p< 0.05$; **$p< 0.01$; ***$p< 0.001$ vs. *Gymnema sylvestre.*

Conclusions

In conclusion, the present study demonstrates that the 70% methanolic extract of *G. sylvestre* leaves, which contains high amount of flavonoid and phenolic content, exhibits high antioxidant activity and free radical scavenging activity. These *in vitro* assays demonstrate that this plant extract is an important source of natural antioxidant, which might be beneficial to prevent the various oxidative stresses. Therefore, further investigation should be carried out to identify the antioxidant compound present in the plant extract to evaluate the *in vivo* antioxidant activity for clinical use.

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