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Potential antioxidant and antimicrobial activity of *Gymnema sylvestre* related to diabetes

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Abstract
Diabetes Mellitus is characterised by an elevated blood sugar level and is often associated with damage to multiple organ systems mediated by free radicals. *Gymnema sylvestre* is a traditional medicinal plant useful in diabetics because of its ability to suppress sweet taste and anti-obesity effects. This study was conducted to assess its antioxidant and antimicrobial properties. Methanolic extract was prepared from dried *Gymnema* leaves. Three different methods – DPPH free radical quenching activity, reducing power assay and hydroxyl free radical quenching activity, were used to study its antioxidant activity. All three methods showed significant antioxidant activity of the extract. *Gymnema* extract also showed significant antimicrobial activity against the bacteria P aeruginosa, S aureus and the fungus F oxysporum. *Gymnema sylvestre* has strong antioxidant and antimicrobial activity as tested by different methods. This raises the possibility that it can provide long term benefits in diabetics by reducing long term complications.

Keywords: Diabetes mellitus, Gymnema, antioxidant, antimicrobial

Introduction
Diabetes Mellitus is a term referring to a group of diseases that have in common elevated levels of glucose in the blood. Most important morbidity associated with diabetes is damage to various organ systems. Cardiovascular, neurological and renal complications are especially devastating in long standing diabetics. Most of the complications of long standing diabetes have been linked to oxidative stress which damages various tissues. [1, 2] Prolonged increased blood sugar levels result in increased production of reactive oxygen species. A drug with antioxidant effects is likely to be valuable in reducing organ damage and long term complications [3].

Diabetes mellitus also has an increased chance of infections due to a combination of an immuno-compromised state and micro-vascular complications. Diabetic polyneuropathy is also a common and important contributing factor to the morbidity of diabetic patients, especially with non-healing foot ulcers. Prevention and early treatment of such complications is desirable as it becomes irreversible once advanced and can end up in foot amputations. The most important characteristic of diabetic foot infections is that it is poly-microbial in nature. The wound synergistically harbours anaerobic along with aerobic microbes. In a study in patients with diabetic foot ulcers, the most frequent bacterial isolates were *Staphylococcus aureus* (38.4%), *Pseudomonas aeruginosa* (17.5%) and *Proteus* (14%) [4]. It is important to consider a drug which has both anti-diabetic and anti-microbial properties.

Gymnema sylvestre (GS), taxonomic serial number 506007, is a plant belonging to the family *Asclepiadaceae*, order *Gentianales*, that is used in Indian traditional medicine for patients with diabetes. It is a woody climber (figure 1) distributed throughout India at an altitude between 300 and 700 metres. It is also distributed in Asia, tropical Africa, Malaysia and Srilanka [5]. Well known for suppressing sweet taste, it has also been shown to be an excellent anti-obesity drug, reducing weight gain and fat accumulation. It is considered a promising drug for the treatment of diabetes due to its multiple enzyme inhibiting properties [6]. Anti-oxidant properties of this plant and its extracts have not been well studied. Apart from other therapeutic properties, *Gymnema sylvestre* also possesses potential anti-microbial property and the same has been practiced in folk medicine for various infections. *Gymnema sylvestre* is therefore considered as a good candidate to evaluate both its anti-oxidant and anti-microbial potential and these could be beneficial in patients with diabetes.

Fig 1: *Gymnema sylvestre*

Figure shows the gymnema plant
This study was therefore undertaken to systematically assess the extracts of *Gymnema sylvestre* for anti-oxidant and antimicrobial properties.

Materials and Methods

Preparation of plant extract

Gymnema sylvestre leaves were collected from Tirupati hills in Chittoor district, Andhra Pradesh, India. Fresh leaves were collected, washed and dried in shade. Dried leaves were ground into fine powder which was then used for extraction.
To 5 g of GS leaf powder, 250 ml of methanol was added and kept in agitator for 24 hours. It was then filtered and re-extracted under the same conditions to ensure complete extraction. Combined solvent was then evaporated by rotor evaporator to get the residue. This methanol extract was weighed, aliquoted and refrigerated.

DPPH free radical quenching activity

The organic chemical compound 2,2-diphenyl-1-picrylhydrazyl (DPPH) is composed of stable free radical molecules. DPPH possesses a stable free radical which is reduced by accepting hydrogen or electrons from other compounds. The DPPH radical has a deep violet color in solution with an absorption band centered at 520 nm. When neutralized, it becomes colorless and pale and thus the change in optical absorption allows measurement of radical scavenging activity. The DPPH radical quenching activities of methanolic extract of *Gymnema sylvestre* leaf was assessed using the method developed by Chang *et al*. [7]. Different concentrations of extracts were made up to 180 µl with the solvent methanol. This was added to 60 µl of DPPH (0.1 mM) solution and the system was kept for 10 min in dark. After incubation, the absorbance of the samples at 517 nm was measured using methanol as a blank. The same way, standard samples were also run with control being the DPPH in methanol and standard as known concentrations of antioxidant ascorbic acid. The DPPH radical inhibition percentage was calculated as stated in the below formula

$$\% \text{ Inhibition} = (ODc - ODt / ODc) \times 100$$

Wherein, ODc = control optical density and ODt = test optical density.

Reducing power assay:

Reducing power assay measures the anti-oxidant activity of compounds by measuring the conversion of iron in ferric chloride from ferric to ferrous form. The total antioxidant capacity of the extract was detected by performing the reducing power assay as described by Makari *et al*. [8] Various concentrations of extract were separately added to 2.5 ml of 0.2M phosphate-buffered saline (PBS - pH 7.4) followed by 2.5 mL of 1% potassium ferricyanide and then kept at 50 °C for 20 minutes. To the above mixture, 2.5 ml of 10% trichloroacetic acid (TCA) was added and then centrifuged at 3000 rpm for 10 min. Finally, 2.5 mL of supernatant was collected to a new centrifuge tube and to it 2.5 mL of double distilled water was added followed by 0.5 mL of aqueous ferric chloride solution (0.01%). The UV - visible spectrophotometer was used to estimate the optical density of the samples at 700 nm against phosphate buffer blank and ascorbic acid as standard. The reducing power activity is directly proportional to the increase in absorption of sample with increasing concentrations.

Hydroxyl free radical quenching activity

Hydrogen peroxide inactivates few thiol group containing enzymes by oxidizing thiol groups. Hydrogen peroxide quickly diffuses through cell membranes and can be converted into hydroxyl radicals through catalysis. These hydroxyl radicals can cause oxidative stress and damage to the cell membrane.
The hydroxyl free radical quenching ability of the GS extract was assessed based on the previously reported method [9] with slight modifications. In this experiment, freshly prepared 600 µl of hydrogen peroxide (100 mM) was separately added to various concentrations of (100-200 µg) solutions and then made up to 1 mL volume with Phosphate buffer saline. The UV-visible spectrophotometer was used to measure the absorbance of the above mixture against appropriate reagent blank (extract and PBS) at 230 nm and rutin was used as a standard. The hydroxyl free radical inhibition percentage was calculated based on the below formula

$$\% \text{ Inhibition} = (ODc - ODt / ODc) \times 100$$

wherein, ODc = control optical density and ODt = test optical density

Antibacterial activity

The anti-bacterial activity was assessed by micro dilution method. Nutrient broth was prepared by dissolving standard concentrations of sodium chloride, peptone, beef/yeast extract, pyruvic acid, fructose/dextrose, alanine and phenyl alanine in distilled water and the broth was autoclaved and kept. The media was aliquoted 5ml in each pre-autoclaved test tube, plugged and sterilised under ultraviolet light.
Under aseptic conditions, mother broth was prepared freshly by inoculating 100 µl of freshly sub cultured *Pseudomonas aeruginosa* (MTCC 3541) and *Staphylococcus aureus*(MTCC 737) bacterial strains in 5ml of this Nutrient broth and incubating for 4 hours at 37 °C.
Methanolic GS extract in four different concentrations (2, 3, 4 and 5mg/ml) were taken in test tubes. Methanol was taken in a different tube as solvent control. Broth alone was used in a tube as a blank. Silver nitrate and Gold chloride were used as positive controls. Mother broth (100 µl) was added to all the test tubes except the blank. The tubes were incubated at 37 °C for 16-20 hours. From each test tube, 100 µl of the sample was added to individual wells in a 96-well-plate and read at 600 nm under UV spectrophotometer.

The activity was calculated based on the below formula

% Activity = (ODc − ODt / ODc) × 100

wherein, ODc = control optical density and ODt = test optical density

Antifungal activity

The methanolic extract of GS was assessed for anti-fungal activity using qualitative method. 30 gm of Sabourad's dextrose agar (SDA) was dissolved in 1000 ml of distilled water and then autoclaved. 2.5 ml of this mixture was poured on a six well plate and allowed to solidify.

Under aseptic conditions, mother broth was prepared by scraping the mycelium of the fungus, *Fusarium oxysporum* (MTCC 3322) and suspending in 10 ml of SDA broth.

100µl each of the methanolic extract in different concentrations ranging from 3 to 5 mg/ml were spread on to different wells of solidified SDA followed by 100µl of mother broth. Methanol solvent control was also inoculated. Silver nitrate and Gold chloride were used as positive controls. The plates were then incubated at 37˚C for three days and read.

Statistical analysis

All the measurements were done in three independent repetitions of the experiments and were expressed as the mean and standard deviation.

Results

The results obtained using the methanolic extract of *gymnema* in the various experiments was as follows.

DPPH radical scavenging activity

Methanolic extract of the GS leaves showed good DPPH scavenging activity. At higher concentrations its radical quenching ability was identical to the standard ascorbic acid (figure 2). The IC50 (concentration with 50% inhibition) for methanolic extract was 0.5 µg/µl. The extract showed a maximum of 87.3% inhibition at the concentration of 2 µg and the inhibition plateaued thereon. The activity found for methanolic extract at its highest concentrations of 6 µg was 82.5%.

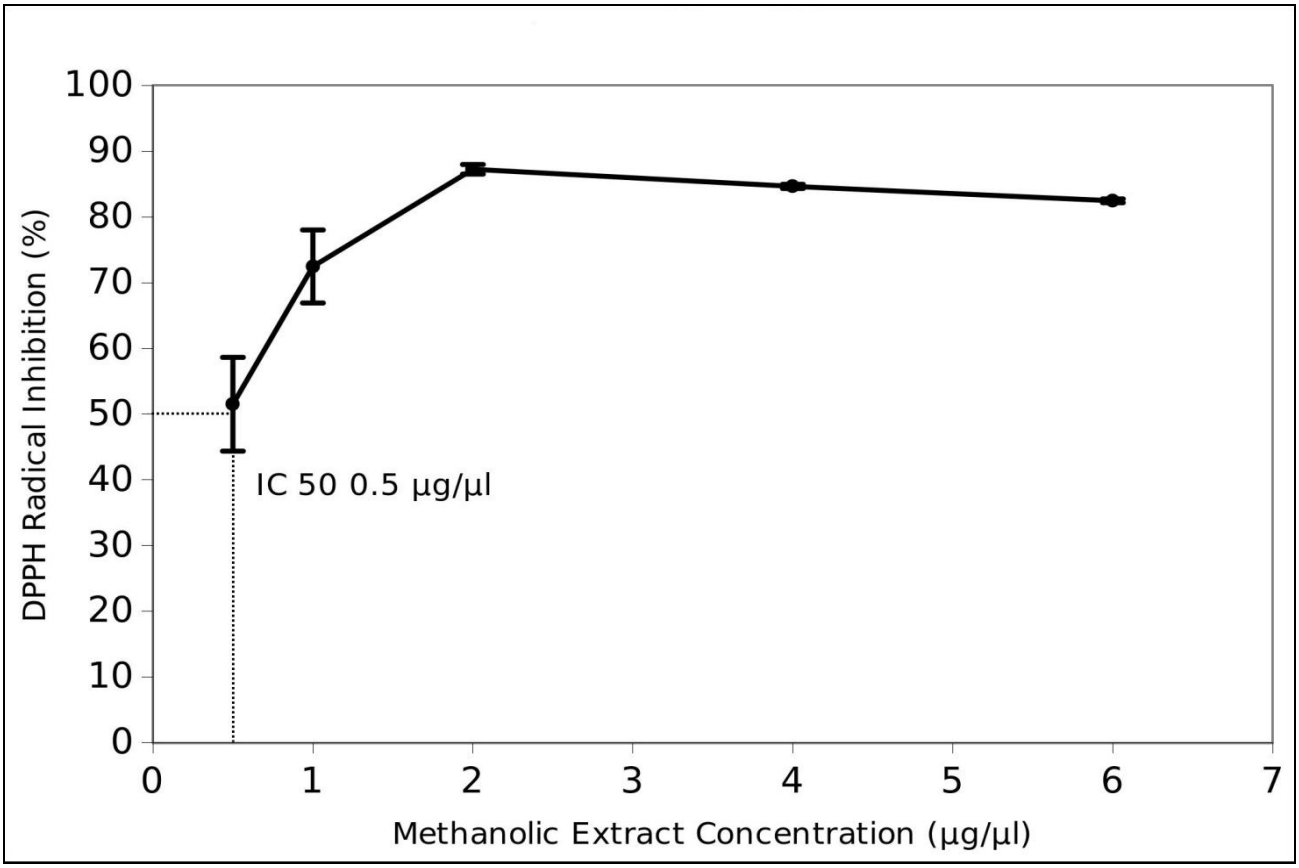

Fig 2: DPPH radical scavenging activity

This graph shows the percentage inhibition of DPPH radicals at different concentrations of methanolic extract of Gymnema sylvestre.

Reducing power assay

Antioxidant activity was seen by the reducing power assay also. The methanolic extract of *Gymnema* when treated with appropriate reagents, in increasing concentrations resulted in significant reduction of iron in ferric chloride from ferric to ferrous form. This was confirmed by increasing absorbance showing increasing anti-oxidant activity (figure 3).

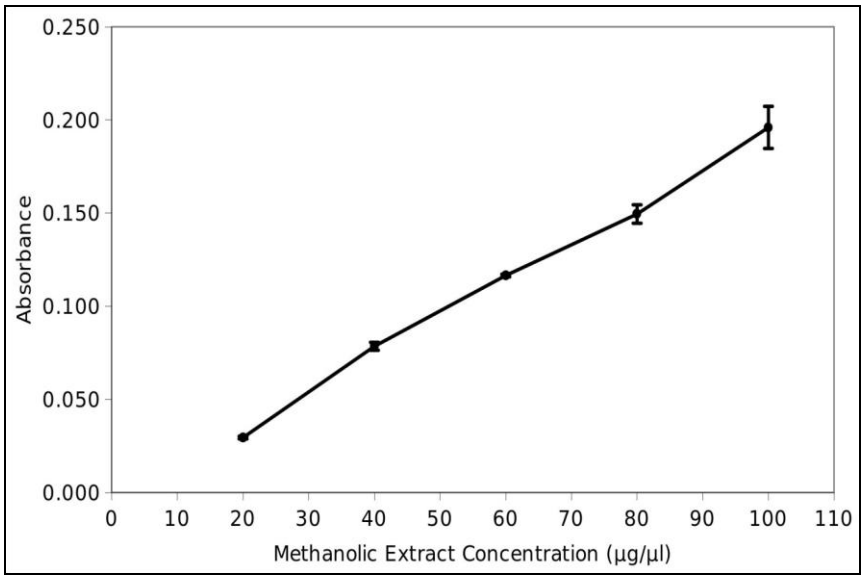

Fig 3: Reducing power assay

Bar chart shows increasing absorbance indicating increase in the reducing activity of methanolic extract of Gymnema sylvestre at increasing concentrations

Hydroxyl free radical scavenging activity

Figure 4 shows the hydroxyl free radical scavenging activity of the extract. The methanolic extract exhibited increasing hydroxyl free radical inhibition with increasing concentrations. The IC50 was 155 µg and at 200 µg concentration, there was 59.8% inhibition. There was a consistent increase in the scavenging activity from 100 to 200 µg concentration.

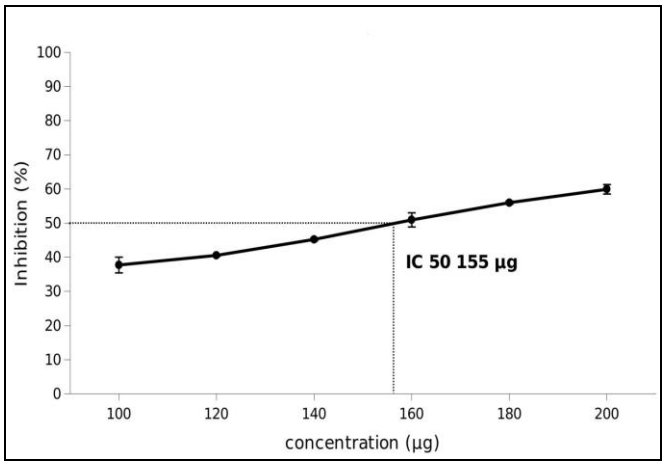

Fig 4: Hydroxyl free radical scavenging activity

Bar chart showing hydroxyl free radical scavenging activity at different concentrations of the methanolic extract of Gymnema sylvestre.

Anti-bacterial activity

Against the gram negative bacterial strain *Pseudomonas aeruginosa*, the methanolic extract showed significant anti-bacterial activity which increased with increase in concentration. At the concentration of 5 mg/ml, the anti-bacterial activity was comparable to that of the known antiseptic agent silver nitrate, that was used as control (figure 5).

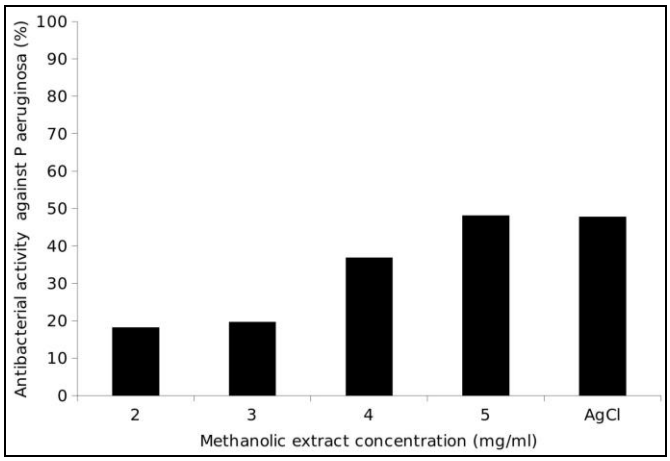

Fig 5: Antibacterial activity against P aeruginosa

Bar chart shows the antimicrobial activity of the methanolic extract of gymnema at various concentrations.

AgCL – Silver Chloride

Against the gram positive bacterial strain *Staphylococcus aureus*, the GS extract showed significant activity with the IC50 at 3 mg/ml (figure 6). At 5 mg/ml, the methanolic extract showed 60.6% inhibition compared to 87.6% with gold chloride which was the control.

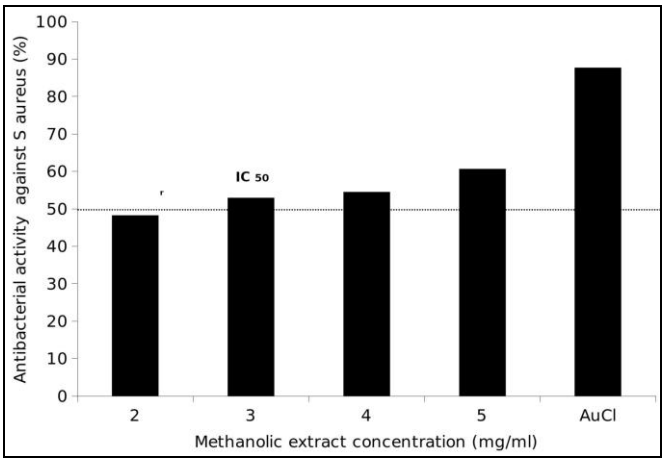

Fig 6: Antibacterial activity against S aureus

Bar chart shows the antimicrobial activity of the methanolic extract of gymnema at various concentrations against S aureus.

AuCl – Gold Chloride

Anti-fungal activity:
Excellent antifungal activity was seen against *Fusarium oxysporum* with the methanolic extract of GS. At the highest concentration of 4 mg/ml, no colonies were seen after three days of incubation (figure 7).

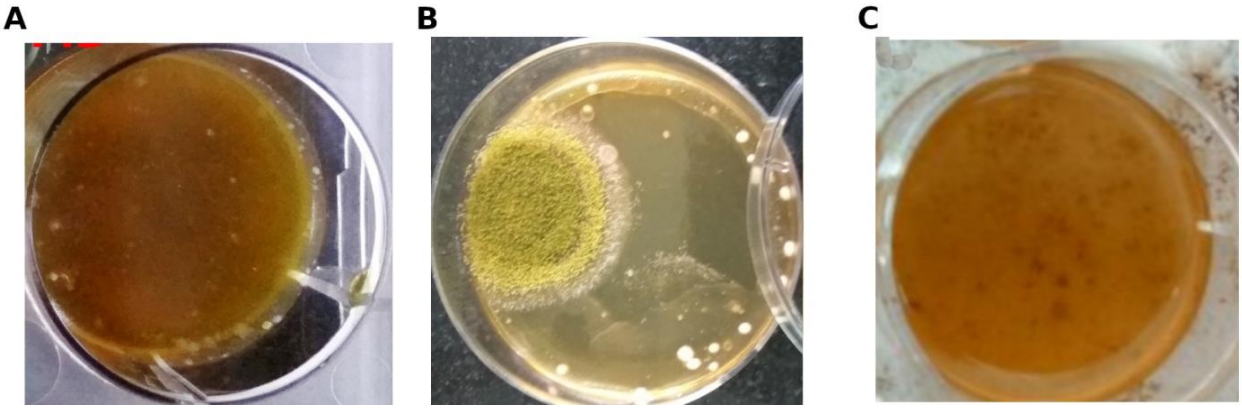

Fig 7: Antifungal activity

Figures shows complete absence of fungal colony in highest concentration of gymnema extract with methanol only as negative control and Gold chloride as positive control.
A – Methanolic extract of GS –at 4 mg/ml. B – Negative control (methanol), C- Positive control (Gold chloride).

Discussion
In this study, we found that analysis of anti-oxidant effects of extracts of *Gymnema sylvestre* by three different methods showed consistent significant anti-oxidant activity. Damage from reactive oxygen species is implicated in organ damage seen in long standing diabetes. Therefore this raises the exciting possibility of developing drugs from *Gymnema* that may reduce or prevent the development of organ damage in diabetics.
Diabetes is associated with high levels of sugar in the blood due to inadequate production or function of insulin [10]. Free radicals are short lived chemical entities which have one or more unpaired electrons. The unpaired electrons are passed on to cell components and molecules, causing their oxidation and resulting in cell damage [11]. Free radicals are produced constantly in the body from endogenous and exogenous processes. Natural scavenging mechanisms in the body keep these in check by removing these reactive oxygen species. Increased production or inadequate removal of these can result in oxidative stress [12]. Oxidative stress forms a major factor in the development of vascular complications in diabetes mellitus [13]. Elevated levels of reactive oxygen species have been found in diabetes and are thought to result from changes in levels of catalase, superoxide dismutase and glutathione peroxidase. Oxidative stress can also further worsen diabetes by producing insulin resistance [14]. Reactive oxygen species produce tissue damage leading to the vascular and multi organ complications that are the hallmark of long standing diabetes [15].
Gymnema is a plant extract that has been used in traditional Indian medicine. Due to its property of suppressing sweet taste, it is also called gurmar. The first scientific confirmation of its use in human diabetics came more than 90 years ago when it was demonstrated that the leaves of *Gymnema sylvestre* reduced urine glucose in diabetics [16]. The drug also enters into the composition of Ayurvedic preparations like Ayaskrti, Varunadi kasaya, Varunadighrtam, Mahakalyana kartam. It is also beneficial in diabetics by reducing fat accumulation and reducing weight gain. Anti-diabetic effects are thought to be mediated by enhanced glucose uptake by altered gene expression [17]. And by Insulin receptor affinity [18, 19]. Extracts of GS also reduce blood glucose levels [20] and fat accumulation [21]. In mice. *Gymnema sylvestre* therapy also increased the activities of the enzymes affording the utilisation of glucose by insulin dependent pathways: it controlled phosphorylase levels, gluconeogenic Enzymes and sorbitol dehydrogenase. This valuable herb appears to correct the metabolic derangements in diabetic rabbit liver, kidney and muscle [22].
Since this is an endangered plant, rapidly disappearing in its natural habitat, and cultivation is difficult, alternative sources have been explored for the extraction of the active compound gymnemic acid, including cell suspension cultures [23] and hairy root cultures [24].
Antioxidants reduce the oxidative stress in cells and are therefore useful in the treatment of many human diseases, including cancer, cardiovascular diseases, diabetes and inflammatory diseases.In this study, we used three different measures of antioxidant activity. DPPH radical scavenging was almost comparable and at higher concentrations the radical quenching ability of G*ymnema* extract was identical to the standard ascorbic acid. Reducing power assay and Hydroxyl radical scavenging also showed good antioxidant activity. But DPPH radical scavenging showed best results even in very low concentrations compared to the other two. In previous studies, GS extracts significantly increased the levels of antioxidant enzymes in rats fed a high fat diet [25]. Kalyani *et al.* also studied the DPPH radical scavenging effect of GS extract *in vitro* and found a significant antioxidant effect [26].
Antibiotics are one of the most important weapons in fighting bacterial infections. Compounds extracted from plants serve as candidates to develop more effective and less toxic medicines which may encounter less resistance. It is well known that *Pseudomonas aeruginosa* is a prototypical "multidrug resistant (MDR) pathogen" that is recognized for its ubiquity, intrinsically advanced antibiotic resistance mechanisms and association with serious illnesses. *Staphylococcus aureus* is associated with skin infections and food poisoning. Anti-microbial efficacy of *Gymnema* have been shown in a few studies previously [27]. We found a dose dependent antimicrobial activity of the GS extract against both gram positive and gram negative bacterial strains.

Fusarium oxysporum is a ubiquitous fungus found in soil, air and in plants. *Fusarium* have long been recognised as a cause of localised infections in immuno-competent individuals and disseminated infections among those who are severely immuno-compromised. *Fusarium* species now represent the second most frequent mold-causing invasive fungal infections in the latter population. [28, 29] It can cause disease in humans that may be localized, focally invasive or disseminated. *Gymnema* extract was active in suppressing colony growth of this fungus.

Limitations

Although we showed that *G. Sylvestre* exhibits consistent anti-oxidant effect in different experiments, the study does not reveal the mechanisms for such an effect. However, the purpose of the study was purely descriptive and future studies may focus on the constituents responsible for the effect and the mechanism of the same. Future studies should also assess the anti-oxidant effects in in-vivo models.

Conclusions

In this study, we found that the extract of the plant *Gymnema sylvestre* showed significant antioxidant and anti-microbial activity. This suggests additional beneficial effects from the use of this plant extract for patients with diabetes. The antioxidant activity could help mitigate multi system organ damage that occurs in diabetics and the anti-microbial activity help prevention and treatment of diabetic ulcers and other infections. These promising effects need to be evaluated further in in-vivo studies.

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