

In Vitro Antibacterial Activity and Phytochemical Analysis of Leaves of *Gymnema sylvestre* Retz. R. Br.

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Abstract: To investigate the antibacterial activity and phytochemical screening of the hexane, chloroform and methanol extracts of leaves of *Gymnema sylvestre*. The antibacterial activity was evaluated by agar well diffusion method against four Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus vulgaris*) and five Gram-positive bacteria (*Bacillus subtilis*, *Enterococcus faecalis*, *Micrococcus luteus*, *Staphylococcus aureus*, *Streptococcus pneumoniae*). Phytochemical screening was performed according to the Harborne method. Methanol extract showed good antibacterial activity with the high inhibition zones, while chloroform extract exhibited mild to moderate activity and hexane extract was found to be less active. Phytochemical screening revealed the presence of various secondary metabolites like steroids, alkaloids, phenols, flavonoids, coumarins, saponins, tannins and triterpenoids. The results of the present study suggest that leaves of *Gymnema sylvestre* can be used to treating infectious diseases caused by *Escherichia coli* and *Staphylococcus aureus*.

Keywords: *Gymnema sylvestre*, Antibacterial activity, Phytochemistry, Minimum inhibition concentration.

Introduction

The resistance of microbes and the appearance of strains with reduced susceptibility to antibiotics are continuously increasing due to the indiscriminate use of commercial antibacterial treatment of infectious diseases(1). In addition, high cost and adverse side effects (such as hypersensitivity, allergic reactions, immunosuppression, etc.) are commonly associated with popular synthetic antibiotics and are high major burning global issues in treating infectious diseases (2). Therefore, there is a need to search new infection fighting strategies to control bacterial infections.

In concern to drawbacks of the conventional medicine, the use of natural products as an alternate to the conventional treatment of various diseases still remain as one of the best reservoir of new structural types(3). They are directly used as therapeutic agents as well as starting material for the synthesis of synthetic drugs or as models for pharmacologically active compounds. In recent years this interest to evaluate plants possessing antibacterial activity for various diseases is growing (4). About 80% of individuals from developed countries use traditional medicine which has compound derived from medicinal plants (5). Therefore, such plants should investigate for a search of new potent antibacterial compounds (6).

Gymnema sylvestre Retz. R.Br. (Asclepiadaceae) is a medicinally important branched woody climber, distributed throughout India. The plant is popularly known as 'Gudmar' (gud-jaggary, mar-kills) for its distinctive property of temporarily destroying the taste of sweetness. Leaves are tasteless with a faint pleasant aromatic odour and are said to be used as a remedy for Diabetes. The plant reported to treat various ailments,

such as stomachic, stimulant, laxative and diuretic, cough, biliousness and sore eyes (7, 8). Pharmacological studies have shown that *G. sylvestre* possesses properties like anti atherosclerotic (9), antihyperglycemic effect (10), larvicidal effect (11), hepatoprotective activity (12), antimicrobial effects (13-16). Earlier workers have reported that the presence of pentriacontane, hentriacontane, phytin, chlorophyll a, chlorophyll b, tartaric acid, formic acid, butyric acid, anthraquinone derivatives, inositol, d-quercitol, gymnemic acids (anti-sweet compound) in this plant (17). This study aims to investigate the antibacterial activity and phytochemical screening of leaves of *Gymnema sylvestre*.

Materials and Methods

The present study was done in the Department of Botany, Andhra University, Visakhapatnam, Andhra Pradesh, India. Leaves of *G. sylvestre* were collected from University premises.

Chemicals, Media and Antibiotic

The organic solvents i.e., hexane, chloroform, methanol and Dimethyl sulphoxide (DMSO), were obtained from Rankem company, India. Nutrient broth and Nutrient agar were obtained from Hi-media, Mumbai, India. The antibacterial agent Ciprofloxacin was obtained from Axiom Laboratories Ltd., India.

Test organisms

Nine human pathogenic bacteria were selected to assess the antibacterial activity and include Gram-negative (*Escherichia coli* MTCC B1560, *Klebsiella pneumoniae* MTCC B4030, *Pseudomonas aeruginosa* MTCC B2297, *Proteus vulgaris* MTCC B7299) and Gram-positive (*Bacillus subtilis* MTCC B2274, *Enterococcus faecalis* MTCC B3159, *Micrococcus luteus* MTCC B1538, *Staphylococcus aureus* MTCC B3160, *Streptococcus pneumoniae* MTCC B2672) bacteria. All the bacterial strains were pure isolates obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India and were maintained in nutrient agar.

Extraction procedure

Leaves of *G. sylvestre* were washed thoroughly under running tap water, dried on paper towel, then shade dried and finally crushed to fine powder in mixture grinder. The dried powder of the leaves was allowed to Soxhlet for sequential extraction with hexane, chloroform and methanol. The liquid extract was collected, filtered and evaporated to dryness using Rotary evaporator (Heidolph, Heizbad, Laborota 4001, Germany 2002). A semisolid or dried crude extracts of leaves so obtained were re-suspended in an inert solvent, DMSO (18). The extracts were dissolved in DMSO to reach final concentration (100mg/ml), and were kept in refrigerator till used.

Antimicrobial activity

The antimicrobial activity of extracts was determined by using agar well diffusion technique (19). Nutrient agar plates were each seeded with 0.1 ml of an overnight culture of each bacterial (equivalent to $10^7 - 10^8$ CFU/mL) strain.

The 24 hours broth culture of each bacterium used to seed sterile molten nutrient agar at 45 °C, allowed to solidify at room temperature and well made by sterile standard cork borer and 50 µl (100mg/ml) solution of extract added to each well. Then bacterial plates incubated at 37 °C for 24 hours after which diameter of zones of incubation were measured (mm) by using HiAntibiotic ZoneScale-C (Himedia). Each assay was performed in at least triplicate and mean values (\pm standard deviation) are reported. Standard antibiotic strip of Ciprofloxacin (5 µg/disc) for each bacteria along with DMSO were used as positive and negative controls, respectively.

Minimum inhibitory concentration (MIC) was determined by the broth dilution method (20). A quantity of 0.6g of each extract was dissolved in 300 ml sterile nutrient broth, which yields an initial concentration of 2000 µg/ml. Subsequently, two-fold serial dilution was made from the stock to obtain following concentrations 1000, 500, 250, 125, 62.5, 31.5 and 15.6 µg/ml. Different concentrations of leaf extract in hexane, chloroform and methanol were tested separately for each bacterium and inhibition zone of microbial growth in the plates containing test solutions was judged by comparison with blank control plates. MIC is defined as the lowest

concentration of test samples that result in a complete inhibition of visible growth. Experiments were carried out in triplicate.

Phytochemical screening

The phytochemical analysis for steroids, flavonoids, carbohydrates, proteins, lipids, iridoids, coumarins, phenols, tannins, alkaloids, saponins and triterpenoids was carried out to all test extracts of leaves of *G. sylvestre* using standard procedures given by Harborne (21).

Results and Discussion

The inhibition zones and MIC values of leaves of *G. sylvestre* are presented in Table 1. Methanol extract exhibited good antibacterial activity with the high inhibition zones, while chloroform extract showed mild to moderate activity and hexane extract found to less effective. Methanol extract showed fairly high degree of antibacterial activity against *S. aureus* followed by *B. subtilis*, *E. faecalis* and *S. pneumoniae*, while chloroform extract against *P. aeruginosa*, *S. aureus* followed by *B. subtilis* and hexane extract against *S. aureus* followed by *B. subtilis*. Hexane extract did not shown inhibition values against *E. coli*, *K. pneumoniae*, *P. vulgaris* and *M. luteus*, whereas chloroform extract against *P. vulgaris*. On comparison standard antibiotic ciprofloxacin, methanol extract exhibited higher inhibition zones against *P. aeruginosa*, *P. vulgaris*, *B. subtilis*, *E. faecalis*, *M. luteus*, *S. aureus*, *S. pneumoniae* and similar value against *E. coli*, while chloroform extract showed higher and similar value against *P. aeruginosa* and *S. aureus*, respectively. DMSO, a negative control did not show any inhibition zones and indicated that it is not interfering zone formation. The low MIC values of methanol extract were 15.6 µg/ml against *B. subtilis*, *S. aureus*; 31.2 µg/ml against *E. faecalis*, *M. luteus*, *S. pneumoniae* whereas chloroform extract showed low MIC against *S. aureus*, it was 62.5 µg/ml.

Qualitative analysis of solvent extracts of leaves of *G. sylvestre* revealed the presence of different class of phytochemicals in different proportions (Table 2). Methanol extract displayed positive results for the presence of steroids, flavonoids, coumarins, phenols, tannins, alkaloids, saponins and triterpenoids while chloroform extract showed positive results for steroids, flavonoids, lipids, coumarins, phenols, alkaloids, triterpenoids and hexane extract showed positive results for steroids, lipids and triterpenoids. In addition, three extracts showed negative results for iridoids, carbohydrates and proteins.

Table 1. Antibacterial activity and MIC of leaves of *G. sylvestre*.

Bacteria	Hexane extract		Chloroform extract		Methanol extract		Cip	D
	ZOI	MIC	ZOI	MIC	ZOI	MIC		
<i>E. coli</i>	-	-	14±0.54	500	20±1.71	125	20	-
<i>K. pneumoniae</i>	-	-	16±2.28	500	19±2.44	250	20	-
<i>P. aeruginosa</i>	14±0.47	>1000	22±1.63	125	22±0.54	62.5	20	-
<i>P. vulgaris</i>	-	-	-	-	20±0.81	500	18	-
<i>B. subtilis</i>	18±1.84	500	21±0.87	250	24±1.64	15.6	22	-
<i>E. faecalis</i>	14±2.85	>1000	18±2.35	250	24±2.36	31.2	22	-
<i>M. luteus</i>	-	-	16±2.44	1000	23±1.50	31.2	19	-
<i>S. aureus</i>	21±1.47	250	23±3.26	62.5	25±1.63	15.6	23	-
<i>S. pneumoniae</i>	14±1.50	1000	19±2.16	125	24±0.73	31.2	23	-

ZOI: Zone of inhibition

MIC: Minimum inhibition concentration

Cip: Ciprofloxacin

D: DMSO

-: negative

Values are represented as mean ±SD

Table 2. Phytochemical analysis of leaves of *G. sylvestre*.

Phytochemicals	Hexane extract	Chloroform extract	Methanol extract
Steroids	+	+	+
Flavonoids	-	+	+
Carbohydrates	-	-	-
Proteins	-	-	-
Lipids	+	+	-
Iridoids	-	-	-
Coumarins	-	+	+
Phenols	-	+	+
Tannins	-	-	+
Alkaloids	-	+	+
Saponins	-	-	+
Triterpenoids	+	+	+

+: positive

-: negative

Plants are important sources of potentially useful substances for the development of new therapeutic agents. Various phytochemical compounds which are naturally present in plants as secondary metabolites have been implicated in plants as the conferment of antibacterial activities (22, 23). The inhibition of bacterial growth *in vitro* by the extracts of *G. sylvestre* leaves could be due to the presence steroids, alkaloids, phenols, flavonoids, coumarins, saponins, tannins and triterpenoids in the extracts. These active compounds may act alone or in combination to inhibit bacterial growth and conferred the strong antibacterial activity.

The results from the present study screening revealed that the strongest antibacterial activity was exhibited by the methanol extract and the followed by the chloroform and least by hexane. Methanol proved as the most effective solvent for extracting broad spectrum of antimicrobial compounds from plants (24). It is worth mentioning to note that a correlation was observed between the extract solubility and antibacterial activity of different fractions. This suggests that in sequential extraction, maximum antibacterial compounds were soluble in polar solvent as methanol extract displayed the highest antibacterial activity followed by chloroform and hexane extracts. These results further confirm that significant antibacterial compounds are polar in nature as evidenced by the higher degree of antibacterial activity of methanol extracts of *G. sylvestre* leaves.

The present study shows that relationship of inhibition zone and MIC values of crude extracts of *G. sylvestre* leaves varies against tested bacteria. The relationship between inhibition zone and MIC value may or may not be related in crude extracts. Because crude extracts have mixture of phytoconstituents, which may influence the diffusion power of active constituents. But the direct relationship of inhibition zone size and MIC value is expected with pure compounds not with crude extracts. Several workers have made similar observations by using essential oils or complex mixture from higher plants (25-28).

In general, the plant antibiotic substances appear to be more inhibitory to Gram-positive than Gram-negative organisms. Unlike Gram-positive bacteria, the lipopolysaccharide layer along with proteins and phospholipids are the major components in the outer surface of Gram-negative bacteria (29). The outer lipopolysaccharide layer hinders access of most compounds to the peptidoglycan layer of the cell wall. This explains the resistance of Gram-negative strains to the lytic action of most extracts. In contrary, the present study results showed that the crude extracts are more susceptible to Gram-positive bacteria than Gram-negative. These results suggest the possible exploitation of *G. sylvestre* leaves in the management of the infectious diseases caused by *E. coli* and *S. aureus*.

Earlier, it was reported that ethanol extract of *G. sylvestre* leaves demonstrated antimicrobial activity against *B. pumilis*, *B. subtilis*, *P. aeruginosa* and *S. aureus* and inactivity against *P. vulgaris* and *E. coli* (13). Deb Roy et al (15) reported that various extracts of *G. sylvestre* showed zone of inhibition against *B. subtilis*, *S. aureus* but not against *E. coli* whereas in the present study we observed chloroform and methanol extracts exhibited inhibition zones against *E. coli*. The results of the present study suggest that the chloroform and

methanol extracts of *G. sylvestre* leaves may be used to treat urinary tract infections, diarrhea, pyogenic infections and septicemia. Hydro alcohol extracts effective at low concentration against *S. aureus*, *S. mitis*, *S. mutansi* and *C. albicans* (16) whereas in the present study stated that *S. aureus* was the most sensitive to the hexane, chloroform and methanol extracts of *G. sylvestre* leaves and suggest that extracts of *G. sylvestre* may be used to treat diseases like sepsis in wounds and burns, septicemia, pharyngitis, sinusitis, and tonsillitis. Therefore, *G. sylvestre* may be a future drug candidate to prove its efficacy as a preventive and therapeutic agent against *E. coli* and *S. aureus*. However, further studies needed to isolation and identification of compounds responsibility for antibacterial activity.

Conclusion

In conclusion, the results provide a scientific base for the traditional use of *G. sylvestre* as an antibacterial agent. It is suggested that, *G. sylvestre* may possess promising therapeutic action in the treatment of infectious diseases caused by the species like *E.coli* and *S. aureus*. Further work will emphasize the isolation and characterization of active principles responsible for antibacterial activity.

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