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PHYTOCHEMICAL SCREENING AND ANTIBACTERIAL ACTIVITY OF *GYMNEMA SYLVESTRE* R.BR. EX SCHULT.

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ABSTRACT: The present investigation deals with preliminary phytochemical analysis and *in vitro* antibacterial potentials of different solvent extracts of *Gymnema sylvestre* were demonstrated. The phytochemical tests of the extracts have revealed the presence of alkaloids, flavonoids, phenols, tannins, terpenoids carbohydrate, tannin, and saponin. Five gram-positive (*Bacillus cereus*, *Bacillus subtilis*, *Streptococcus cremoris*, *Streptococcus fecalis*, *Staphylococcus aureus*) and five gram-negative (*Klebsiella pneumoniae*, *Proteus vulgaris*, *Salmonella paratyphi*, *Shigella boydi*, *Shigella dysentriae*) bacterial strains were tested. The ethanol extracts have been showed encouraging results. The maximum inhibition were recorded 42.3mm in *Bacillus cereus* at 250µl concentration followed by *E. coli* (38.4mm), *Streptococcus fecalis* (37.2mm), *Klebsiella pneumonia* (35.6mm), *S. aureus* (33.3mm), *P. aeruginosa* (30.5mm), *S. cremoris* (28.1mm) *Proteus vulgaris* (26.9mm) *B. subtilis* (23.5mm) and least inhibition was observed in *S. typhi* (21.7mm). Moderate activity was observed in chloroform extract. Minimum activity was observed in hexane at different concentration tested. Compared to synthetic antibiotic Ampicillin (50mg), solvent extracts showed significant antibacterial activity. The present findings support to the traditional knowledge of the medicinal plants to the local users and plants used as therapeutic agents for treat several diseases caused by the pathogenic bacterial populations. This study confirms significant antibacterial activity of *G. sylvestre*.

INTRODUCTION: Plants have played a significant role for mankind mainly as food and medicine. Medicinal plants have been used for many centuries for human diseases because they contain bioactive components of therapeutic value because of their antimicrobial properties and they contains secondary metabolites such as alkaloids, phenolic compounds, etc [1]. Countries like India have been using crude plants as medicine since Vedic period.

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A major part of the total population in developing countries still uses traditional folk medicine obtained from plant resources [2]. According to World Health Organization, medicinal plants are the best source to obtain a variety of newer herbal drugs. About 80% of individuals from developed countries use traditional medicine, which has compounds derived from medicinal plants. Therefore, such plants should be investigated to better understand their properties, safety and efficacy. About three quarter of the world's population relies on plants and their extracts for their healthcare. India represented by rich culture, traditions and natural biodiversity, offers a unique opportunity for drug discovery researchers.

In the last few decades, there has been an exponential increase in the field of herbal medicine

for the treatment for chronic diseases [3], but there is still a vital need to screen novel substances that are bioactive towards pathogens with high resistance [4]. The last three decades have seen the development of several synthetic drugs, but the resistance towards these drugs is also developing at a faster stroke, as the bacteria can acquire and transmit genes responsible for antibiotic resistance. The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. Many plants have been used because of their antimicrobial traits, which are chiefly due to synthesized during secondary metabolism of the plant.

Plants for potential antibacterial activity because of the side effects and the resistance that pathogenic microorganisms build against antibiotics, recently much attention has been paid to extracts and biologically active compounds isolated from plant species used in herbal medicine [5]. On other hand, traditional medicinal plants with their various biological constituents have been used effectively by the communities since long time to treat diabetes. Several natural products such as alkaloids, flavonoids, terpenoids, saponins, polysaccharides and glycosides are isolated from medicinal plants and are being reported to possess anti-diabetic activities [6].

Herbal medicines used to treat infectious diseases were screened for their antibacterial activity against both gram positive and gram negative bacteria [7, 8]. However, a majority of traditionally used Indian medicinal plants have not yet been systematically screened against various microbial pathogens. The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has lead to the screening of several medicinal plants for their potential antimicrobial activity.

The practice of complementary and alternative medicine is now on the increase in developing countries in response to World Health Organization directives culminating in several pre-clinical and clinical studies that have provided the scientific basis for the efficacy of many plants used in folk medicine to treat infections [9].

Gymnema sylvestre R.Br. (Asclepiadaceae) a large woody, much branded, climber with pubescent young parts, found throughout India in a dry forest up to 600m, Deccan peninsula leaves opposite, usually elliptic or ovate. Leaves contain lupeol, β-amyrin, stigmasterol, pentriacontane, hentricontane, α and β chlorophyll, resin, tartaric acid, gymnemic acid (anti sweet compounds) the mixture of triterpene saponins, anthraquinone derivatives, alkaloids, betain, choline and trimethylamine **Fig. 1** [10].

FIG. 1: NATURAL HABIT OF *GYMNEMA SYLVERSTRE*.

In the Ayurvedic system of medicine, *G. sylvestre* is referred to as "mesasrngi," and both the dried leaf (mesasrngi leaf) and dried root (mesasrngi root) are used therapeutically. The leaves of the plant in particular are used as antiviral, diuretic, antiallergic, hypoglycemic, hypolipidemic, for the treatment of obesity and dental caries [11]. It is also used as Antibiotic, in stomach pains, as a blood purifier and in rheumatism.

The active principle is gymnemic acid also possesses antimicrobial and sweet suppressing activities[12] and *G. elegans* is used as a substitute for *G. sylvertre* in traditional medicine for the treatment of diabetes and snake bite [13]. The saponin gymnemic acid, constituent of the leaves, was shown to suppress sweet taste sensation and to inhibit glucose absorption in the small intestine [14].

The individual gymnemic acids (saponins) **Fig. 2** include gymnemic acids I-VII, gymnemosides A-F, gymnemasaponins[15, 16].

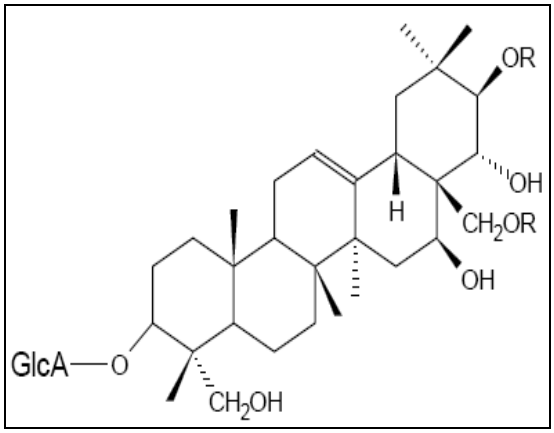

FIG.2: CHEMICAL STRUCTURE OF GYMNEMIC ACID.

Chemical constituents of the plant include the gymnemic acids (gymnemosides), saponins, stigmasterol, quercitol, and the amino acid derivatives betaine, choline, and trimethylamine. The present study has been made to investigate the phytochemical analysis and *in vitro* antimicrobial activity of different extracts of *G. sylvestre*. Here an attempt has been made to study the *in vitro* antibacterial activity of important medicinal plants used in India.

MATERIALS AND METHODS:

Collection of Plant Materials:

Plant parts were collected from different areas of Dharmapuri district, Tamilnadu, India. Collection was based on information given by local in habitats during ethnobotanical surveys in 2012 to 2014, and is supported by different references. Specimen was labeled, numbered, annotated with the date of collection, the locality and their medicinal uses. The voucher specimens were identified and deposited in the herbarium of PG and Research Department of Botany, Government Arts College, Dharmapuri for the future reference. After authentification leaves were collected in bulk, washed, shade dried and extracted with different solvents such as hexane, chloroform, ethyl acetate and ethanol for 48 hrs in a Soxhlet assembly.

Preparation of extracts:

The clean and air-dried leaves of *G. sylvestre* were ground well using mechanical pulverizer. Fifty grams of powdered material was soaked in 250mL of 95% ethanol for 24hours and filtered using standard filter paper. The material was again mixed with 250mL of fresh ethanol and filtered after 24 hours. This process was repeated 3 times. The extracts transferred into clean vials and allowed to evaporate until completely dry. Once dry, the extract was dissolved in 20mL of ethanol. The final concentration of the extract was calculated for 1g/1mL. Air-dried and powdered leaves were Soxhlet-extracted with four solvents. Different concentrations (50-250mg/ml) of all extracts were tested for antimicrobial activity.

Preliminary Phytochemical Screening:

Preliminary phytochemical screening of the *Gymnema* leaf extract was carried out for the detection of the various plant constituents [17]. Shaded dried and powdered of aerial part of plant samples were successively extracted with hexane, chloroform, ethyl acetate and ethanol. The extracts were filtered and concentrated using vaccum distillation. The different extracts were subjected to qualitative tests for the identification of various phytochemical constituents as per standard procedure[17].

Thin layer Chromatography:

One gram of *G. sylvestris* leaves powder was dissolved in 100 ml of 50% (v/v) ethanol, and then 20 ml of KOH was added and heated on a boiling water bath under relux for an hour and then cooled. To this 18 ml of 12N HCl was added and heated on water bath. After cooling the pH was adjusted to 7.5-8.5 with 10% KOH. This solution was dissolved with 50% (v/v) ethanol and filtered.

The samples was applied on TLC plates and the solvents were used to separate compounds (Chloroform:Methanol:Acetic acid) in the ratio (5:3:2). To spray vanillin sulphuric acid reagent for detecting the gymnemic acid. A small portion of the dry extract was used for phytochemcial screening test. Dragendorffs reagents were used to test for alkaloids, ferric chloride for tannins, while Benedict's solution was used to test for saponins Harborne[18] (1998).

Antimicrobial activity:

Ten species of bacteria, five gram-positive (*Bacillus cereus*, *Bacillus subtilis*, *Streptococcus cremoris*, *Streptococcus fecalis*, *Staphylococcus aureus*) and five gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella typhi*)

obtained and identified specimens from the Government medical college Hospital, Dharmapuri. All the cultures were maintained on nutrient agar medium at ambient temperature. They were cultured in nutrient broth for 24 hours and the fresh inoculums were taken for the test. The antibacterial screening of the extract was carried out by determining the zone of inhibition using disc diffusion method [19].

Bioassays:

The density of the bacterial suspension was standardized by standard McFarland method. The bioassay used was the standard disc diffusion assay. Test discs were prepared by dipping and saturating sterilized filter paper discs in plant extracts. The extract solution was made at a concentration of 100 mg/ml and finally sterilized by filtration using 0.45µm Millipore filters. Same sized filter paper discs (6 mm diameter) absorbed the different volume of extract (50-250µL). For negative control solvent paper discs were used, prepared by dipping the disc into the 95% ethanol, while ampicillin paper discs were used as positive control. For ampicillin paper discs 50µL (50µg) solution was poured on the discs.

The ampicillin stock solution was prepared at the concentration of 1mg/mL. The controls were prepared using the same solvents employed to dissolve the extracts. The inoculated plates with the test and standard discs on them were incubated at 37°C for 24 h.

Culture media and inoculums:

Muller Hinton (MH) media (Hi-media Pvt. Ltd; Bombay, India) was used for growth of bacteria. The inoculum for bacteria was prepared by transferring a large number of bacteria from fresh culture plates to tube containing 10mL of liquid media (DIFCO, Bacto: dehydrated nutrient broth) and incubating over night at 37°C. The tubes were shaken occasionally to aerate and promote growth.

Transformation of bacteria and test discs:

The bacteria of petridish with nutrient agar a swab dipped in standard inoculum was used. After dipping, the swab was used to spread the bacteria on the media in a confluent lawn. Prepared dried discs were then transferred on bacterial lawn using flame-sterilized forceps. The petridish with these test discs were then incubated upside down for 24 hours at 37°C.The sensitivity testing of the extracts were determined using disc diffusion method [19]. The MIC of the extracts was also determined using a two-fold dilutions method. The bacterial isolates were first grown in nutrient broth for 18h before use.

Observation of results:

Results were recorded as presence or absence of zone of inhibition. The inhibitory zone around test paper discs indicated absence of bacterial growth and it was reported as positive (growth inhibition observed) and absence of zone as negative. The test was repeated three times to insure reliability of the results. Then, the plates were examined for any zone of growth inhibition. Inhibition zones were recorded as the diameter of growth free zones including the diameter of the disc in mm at the end of incubation period.

$$\text{Percentage of disc was calculated by the formula: inhibition} = \frac{I}{90} \times 100$$

Where I = Diameter of the inhibition zone.

Statistical analysis:

Phytochemical estimation and quantification were performed in five replicates under standard procedures to ensure consistency of all conclusions. Data of all experiments were statistically analysed and expressed as Mean ± Standard Deviation.

RESULTS:

In this study, the results of preliminary phytochemical screening of leaves extracts of *G. sylvestre* are presented in **Table 1**. Investigations on the phytochemical screening of *G. slvyestre* leaf ethanol extract revealed the presence of alkaloids, Tannins, flavonoids, saponins, phenols, anthraquinones, quinones, carbohydrate and glycosides. Whereas the metabolites like terpenoids, Steroids, coumarins and anthraquinones were found to be absent in the ethanolic extracts. In ethyl acetate extracts of leaves showed the presence of flavonoids, quinines, anthraquinones, phenol, carbohydrates and glycosides. Very few numbers of phytochemicals were found in hexane extract like terpenoids, coumarin, tannin and phenol.

TABLE 1: PRELIMINARY PHYTOCHEMICAL ANALYSIS OF *GYMNEMA SYLVERSTRE* LEAVES.

S. No.	Tests	Hexane	Chloroform	Ethyl acetate	Ethanol
1.	Alkaloids	-	+	+	+
2.	Terpenoids	+	+	-	-
3.	Steroids	-	+	-	-
4.	Coumarin	+	-	-	-
5.	Tannin	+	-	-	+
6.	Saponin	-	-	-	+
7.	Flavonoids	-	-	+	+
8.	Quinones	-	+	+	-
9.	Anthraquinones	-	-	+	+
10.	Phenol	+	+	+	+
11.	Carbohydrate	-	-	+	+
12.	Glycosides	-	-	+	+

+ : Present - : Absent

In the present studies thin layer chromatographic analysis of the metabolites, indicated the separation at different Rf values. The alkaloids separated showed two fractions (Rf=0.80, 0.82) and saponins three (Rf=0.81, 0.83, 0.84) under visible light. TLC chromatogram for cardiac glycosides showed the separation of two fractions (Rf =0.88, 0.79) and two bands of flavonoids (Rf =0.78 and 0.82). The sample concentration 10µl is found to be showing more bands in case of saponins and cardiac glycosides whereas 5µl of sample concentration was sufficient to generate the bands in case of alkaloids and flavonoids.

TABLE 2: ANTIBACTERIAL ACTIVITY OF ETHANOL EXTRACTS FROM *GYMNEMA SYLVERSTRE* AGAINST HUMAN PATHOGENIC BACTERIA.

Test organisms	Ampicillin		Zone of inhibition in diameter (mm)							
	50 µg		100 µL		150 µL		200 µL		250 µL	
	I	%I	I	%I	I	%I	I	%I	*I*	%I
B. cereus	28.3	31.44	25.7	28.55	30.4	33.77	35.2	39.11	38.3	42.55
B. subtilis	20.4	22.66	21.0	23.33	23.2	25.77	27.4	30.44	30.1	33.44
S. cremoris	23.3	25.88	22.3	24.77	25.4	28.22	29.8	33.11	32.0	35.55
S. fecalis	22.6	25.11	23.5	26.11	25.4	31.55	31.4	34.88	34.6	38.44
S. aureus	21.3	23.66	23.7	26.33	27.5	30.55	31.5	35.00	33.5	37.22
E. coli	18.6	20.66	18.5	20.55	21.5	23.88	24.5	27.22	27.5	30.55
K. pneumoniae	19.4	21.77	18.7	20.77	19.5	21.66	20.2	22.44	21.2	23.55
P. vulgaris	17.5	19.44	16.2	18.00	18.2	20.22	21.2	23.55	24.2	26.88
P. aeruginosa	18.5	20.55	16.2	18.00	19.3	21.44	22.4	24.88	25.3	28.11
S. typhi	12.2	13.55	11.2	12.44	13.4	14.88	16.3	18.11	19.6	21.77

I : Inhibition in mm %I: Percentage of inhibition

The ethanol extract of *G. sylverstre* was used for bacterial susceptibility test by disc diffusion method. After 18 h of incubation, the zone of inhibition was measured **(Table 2)**. The numbers of bacteria used in screening have been restricted to ten: five gram-negative and five gram-positive.

The extract was found to exhibit the maximum antibacterial activity against *Bacillus cereus* (42.55%) followed by *Streptococcus fecalis* (38.44%), *Staphylococcus aureus* (37.22%), *Streptococcus cremoris* (35.55%), *Bacillus subtilis* (33.44%), *Escherichia coli* (30.55%), *Pseudomonas aeruginosa* (28.11%), *Proteus vulgaris* (26.88%), *Klebsiella pneumonia* (23.55%) and *Salmonella typhi* (21.77%). The extract was tested against 10 human pathogenic bacteria, of which all the 7 were found to be sensitive. Of the tested 7 sensitive bacteria, 4 were gram positive and 3 was gram negative. The extract was found to be effective against gram-positive than gram-negative human pathogenic bacteria. In the present investigation, ethanol extract have been showed encouraging percentage of inhibition against both types of bacteria gram-positive (*Bacillus cereus*) 42.55% (250 mg/mL) and gram-negative (*Escherichia coli*) 30.55% (250 mg/mL). The observed activity may be due to the presence of potent phytoconstituents in the extracts.

DISCUSSION: The preliminary phytochemical screening results were revealed the presences of

alkaloids, Tannins, flavonoids, saponins, phenols, anthraquinones, quinones, carbohydrate and glycosides in leaves extracts of *G. sylvestre*. Sukesh *et al*.[20] reported the presence of steroids/terpenoids and coumarins by using thin layer chromatography of hexane and chloroform extract of *G. sylvestre*. The presence of different active fractions in the TLC studies. Medicinal plant can be poisonous if wrong plant parts or wrong concentrations are used. A glycoprotein isolated from *G. sylverstre* exhibits good antibacterial activity against methicillin resistant *Staphylococci* and multi resistant *Enterococc i* [21].

Several flavonoids and phenolic acids were isolated from the aerial parts which exhibit interesting antiviral and antimicrobial properties both *in vitro* and *in vivo*. The various phytochemical compounds detected are known to have beneficial importance in medicinal sciences. These phytochemicals may be responsible for different therapeutic properties of *Gymnema* like antidiabetic, anti-oxidant, anti-pyretic and antimicrobial role. For instance saponin is used as mild detergents and in intracellular histochemical staining. It is also used to allow antibody access in intracellular proteins.

This is comparable with values reported for several medicinal plants such as *Gynandropsis gynandra* and *Buchholzia coriaceae*; *Erythrina senegalensis*; *Vitex negundo*; *Terminalia glaucescens*.

The ethanol extract of *G. sylverstre* leaves have showed the encouraging percentage of inhibition against both types of bacteria gram-positive *Bacillus cereus* and gram-negative *Escherichia coli*. Similar kind of results were observed by Paz *et al*.[22] and have been reported antibacterial activity in some native medicinal plants and most of the plants showed activity against *Pseudomonas aeruginosa* and *E. coli*. Generally gram-negative organisms, particularly *Pseudomonas aeruginosa* are more resistant than gram-positive organism.

The ethanolic extract of *G. sylvestre* leaves showed good antimicrobial activity against *Bacillus pumilis, B. subtilis, Pseudomonas aeruginosa* and *Staphylococcus aureus* and no activities were found against *Proteus vulgaris* and *Escherichia coli* [23]. Ethanolic, Chloroform and Ethyl acetate extracts of the aerial parts of *G. sylvestre* also reported to have antibacterial effects against *P. vulgaris, E. coli, P. aeroginosa, Klebsella pneumoniae* and *S. aureus*. The aqueous and methanolic extract of *G. sylvestre* leaves also showed moderate activity against the three pathogenic *Salmonella* species (*Salmonella typhi, S. typhimurium* and *S. paratyphi*). Out of the two extracts used, aqueous extract showed higher activity against the *Salmonella* species [24].

The observed activity may be due to the presence of potent phytoconstituents in the extracts. Similar results are reported in *Gymnema* by Kalidas and Mohan [25]. The main active compound of *G. sylvestre* is gymnemic acid, saponins and oleanane type of triterpenoid. The efficiency of plant extracts of *Funaria hygrometrica* (Bryophyte). *Cardiospermum halicacabum* and *Cynodon dactylon* against gram-positive bacteria has also been reported. In classifying the antibacterial activity as gram-positive or gram-negative, it would generally be expected that a much greater number would be active against gram-positive than gram-negative bacteria. The growth of *E. coli* was controlled by extract of *G. sylverstre*, which indicated that they could inhibit the activity of bacteria, which can cause diarrhoea and dysentery.

From this study we can conclude that the plant *G. sylverstre* possessed the highest antimicrobial activity and described in the Chinese Materia Medica [26]. Despite many published reports dealing with bioactivity of compounds isolated from *G. sylverstre*, little was known about its antimicrobial activity prior to our investigation. The results provide justification for the use of the plant in folk medicine to treat various infection disorders. From the above results it can be concluded that plant extract have great potential as antibacterial compounds against microorganisms and that they can be used in the treatment of infectious diseases caused by resistant microorganisms [27].

Bacillus cereus showed maximum antibacterial activity and so this plant can be used to discover bioactive natural products that may serve as leads for the development of new pharmaceuticals that address hither to unmet therapeutic needs. Such screening of various natural organic compounds and identifying active agents is the need of the

hour, because successful prediction of lead molecule and drug like properties at the onset of drug discovery will pay off later in drug development. These findings support the traditional knowledge of local users and it is a preliminary scientific validation for the use of these plants for antibacterial activity.

To promote proper conservation and sustainable use of such plant resources, awareness of local communities should be enhanced incorporating the traditional knowledge with scientific findings. Since *Gymnema sylvestre* is used in various medicinal preparations; Hence, the present study may be useful to supplement information in respect to its identification, authentication and standardization. The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. Many plants have been used because of their antimicrobial traits, which are chiefly due to synthesized during secondary metabolism of the plant.

CONCLUSION: The results clearly indicated that research works carried out on the medicinal plant, possessing traditional claims of effectiveness in bacterial infections, provided fruitful results Therefore, ethanol extract of *Gymnema sylvestre* possessing antibacterial activity could be applicable in therapeutic procedure. The main focus of our work is on the antibacterial potential of medicinal plants which we plan to study further with the ultimate objective of providing scientifically validated herbal remedies against bacterial infections. However, the present study of *in vitro* antibacterial activity of *Gymnema sylvestre* forms primary platform for further phytochemical and pharmacological studies.

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