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SCREENING THE LEAF EXTRACT OF *PERGULARIA DAEMIA* (FORSSK.) CHIOV., FOR ITS ANTIOXIDANT PROPERTY AND POTENTIAL ANTIMICROBIAL ACTIVITIES ON MEDICINALLY IMPORTANT PATHOGENS

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ABSTRACT

Pergularia daemia (Forssk.) chivo., is a shrub of Asclepiadaceae family found in the plains throughout India and in tropical countries worldwide. The present study aims at comparative analysis of qualitative and quantitative secondary metabolite components and to determine the biological activities of the leaf by extracting the methanolic soluble components of *Pergularia daemia* in order to establish a theoretical relationship between its constituents, biological activity and use. *Pergularia daemia* showed its anti-bacterial activity on *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa and Escherichia coli* and among fungi, *Candida albicans* and *Candida glabrata*. Ampicillin and nystatin are used as standards. The extract is having more activity on *Escherichia coli* and *Candida glabrata*. The MIC and MBC values for the extract are determined. MIC and MBC values for *Bacillus subtilis*, *Bacillus cereus*, and *Escherichia coli* are found to be lowest ie.,15.62/31.25 and 7.81/15.62 and *Candida glabrata* it was found to be 31.25/125. In case of staphylococcus and pseudomonas species the concentration is 62.5/>500 and 62.5/125. These activities are found to be due to phenols and flavonoids present in the extract.

Key words: *Pergularia daemia*, Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC).

INTRODUCTION

The present study aims at comparative analysis of qualitative and quantitative secondary metabolite components and to determine the biological activities of the leaf by extracting the methanolic soluble components of widely distributed and used weed *Pergularia daemia* of Asclepiadaceae member in order to establish a theoretical relationship between its constituents, biological activity and use.

Pergularia daemia (Forssk) Chiov. is a shrub of Asclepiadaceae family found in the plains throughout India and in tropical countries worldwide. Asclepiadaceae members are known for their rich source of glucosides (Deepak D, 1995). The plant is used in different folk medicine and in Indian ayurvedic system it has been documented for Antifertility, wound healing, antidiabetic, hepatoprotective, cardiovascular effects and antibacterial activities. The plant is used in vatha, convulsion, asthma, poisoning. The root of *Pergularia daemia* is used in mental disorders, anaemia, leprosy and piles. The plant is also reported to possess stomachic, laxative and diuretic properties. It is also used in cough, biliousness and sore eyes. Leaf paste is mixed with castor oil and applied on joints in inflammation, liver complaints, spleen enlargement; the juice of the leaves is given in asthma and rheumatic swelling in combination with lime or ginger. It is also used in the preparation of medicinal oil given in rheumatism, amenorrhea, and dysmenorrhea.

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The plant was a screened for its antimicrobial activities against some of the pathogenic agents known to cause many of the gastrointestinal tract related ailments and extra intestinal infections. These organisms are also reported to cause central nervous system, blood stream, skin and opportunistic infections in animals as well as in humans. The extract is also subjected for MIC and MBC determination. This extract is further screened for its antioxidant properties.

Collection of plant sample

The plant samples were collected from Govada village of Prakasm district where the plant materials are commonly used for snake bites. The plant is identified as *Pergularia daemia* by Dr. Ramana murthy. Voucher specimen is deposited. The collected plant leaves were stored in sterile polythene bags and transferred to the laboratory and stored at 4^0C until use. The surface of the leaves is then sterilized by 1% HgCl2, rinsed with sterile distilled water, dried and powdered for further use.

Chemicals

Tannicacid, methanol, folin ciocalteau reagent, sodium carbonate, distilled water. aluminium chloride, potassium acetate, quercetin, Ascorbic acid, phosphate buffer, potassium ferri-cyanide, 10% tri chloro acetic acid, ferric chloride. All the chemicals are purchased from Sigma.

Microorganisms

All the organisms were collected from MTCC, Chandigarh, in a lyophilised form and revived for the purpose of our experiment.

Preparation of plant extract

1gm of fresh leaves is taken and grinded with 20ml of 20% methanol and filtered with filter paper. The filtrate was taken and made the volume upto 50ml with distilled water.

Phytochemical analysis

Phytochemical screening helps to reveal the chemical nature of the constituents of the plant extract and the one that predominates over the others. It may also be used to search for bioactive agents that could be used in the partial synthesis of some useful drugs (Yakubu *et al*., 2005). The plant extract was screened for the presence of phenols and flavonoids. These constituents are estimated quantitatively.

Estimation of phenols by folins method

Phenols in the extract are estimated by following the method described (Singleton *et al*., 1965) with slight modification. Folin ciocalteau (1:10) is prepared by diluting with distilled water. Sodium carbonate (75gms/lit) is prepared. Tannic acid 0.1mg/ml is taken as standard. 20% methanol is used. Different aliquots of tannic acid were taken in a series of 50, 100,150, and 200, 250 in a series of test-tubes. To that 5ml of Folin - ciocalteau was added and mixed well. Later 4ml of sodium carbonate was added and all the test-tubes were vortexed for 50sec. All the tubes were allowed to stand for 30min in water bath at 40°c. The colour development was measured by using the optical density values at 680nm. A graph (graph 1)was plotted by taking the optical density values on y- axis and concentration of tannic acid on x-axis. The results are given in table no 1. The concentration is calculated using the following formula.

Calculation:

Test-1=OD of test-1 X conc. of tannic acid/OD of standard

Estimation of Flavonoids by using Quercetin standard curve

Total flavonoids in the extract are estimated by following the method described Zhishen *et al*., 1999. 200mg of quercetin was dissolved in 200ml of methanol and is taken as standard. Different aliquots (15, 30, 60, 90, 120, 150,180, 210, 500, 1000, and 1500 µL) of quercetin were taken as standards in a series of test-tubes. To the each dilution, methanol was added and made the volume up to 1500µL and mixed well. Later 0.1ml of 2% aluminium chloride and 0.1ml of 1M potassium acetate were added and mixed well. All the test-tubes were allowed to stand for 30min at room temperature. Finally measured the colour developed at 420nm by using colorimeter. A graph (graph 2) was plotted by taking concentration of quercetin on x-axis and optical density values on y-axis. The results are given in the table no2. The concentration is calculated using the following formula.

Calculation: Conc. Of flavonoids = OD of test X conc of standard/OD of standard.

Estimation of Anti-Oxidants by using Ascorbic Acid standard curve

Phosphate buffer (pH-6.6) is used. Potassium ferric cyanide (1%) and Tri chloro acetic acid (0.1%) are freshly prepared. Different concentrations of ascorbic acid were taken as standards (200, 400, 600,800, 1000, 1200) into a series of test-tubes. To this 2.5ml of phosphate buffer (pH-6.6) and 2.5ml of 1% potassium ferric cyanide were added and mixed well. Then all test-tubes were incubated in water bath for 20min at 50°c. Later 2.5ml of 10% trichloro acetic acid was added. Then all the test-tubes were centrifuged at 10,000rpm for 2min and observed the formation of protein precipitation. 2.5ml of supernatant was taken and to that equal amount of distilled water was

added. Later 0.1% of 0.5ml freshly prepared ferric chloride was added and mixed well and observed for the colour change. Finally the optical density was measured at 680nm (Murray PR *et al*., 1995). A graph is plotted (graph 3) by taking the optical density values on y-axis and concentrations of ascorbic acid on x-axis. The concentration of antioxidants is calculated by using the following formula. Results are given in table 3.
Caliculation: Test-1= OD of test-1 X conc. of standard/OD of standard

Antimicrobial activity assay

Standard agar disc diffusion method (Murray PR *et al*., 1995) was used for performing antibacterial assay. 20ml of nutrient agar medium was transferred into Petriplates. Dried extract is dissolved in DMSO and different concentrations are prepared. Bacterial strains like *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa and Escherichia coli* and among fungi, *Aspergillus niger*, *Aspergillus foetidus*, *Candida albicans* and *Candida glabrata* are tested for their sensitivity towards the extract, in vitro. Ampicillin and nystatin are used as standard drugs for antibacterial and antifungal activity. DMSO solvent is used as control. The results are given in the table no.4 and 5.

Determination of minimum inhibitory and bactericidal concentrations

The minimum inhibitory concentration (MIC) of the extract was tested by two-fold serial dilution method (Bajpai et al 2009). The dried extract was dissolved in dimethyl-sulphoxide (DMSO), incorporated into nutrient broth for bacterial pathogens to obtain a concentration of 1000µg/ml and is serially diluted to achieve 500,250,125,62.5,31.25,15.62 and 7.81µg/ml. the final concentration of DMSO in the culture medium was maintained at 0.5% v/v. A 10µl of standardized suspension of each tested organism (which approximately produce 10^7 CFU/ml) was added to each tube. Controls are maintained by incubating only the bacterial suspension at 37^0 C for 24 hrs. The lowest concentration of the compound, which did not show any growth of the organism after macroscopic observation, was determined as MIC and the concentration was expressed in µg/ml. 50 µl of each culture broth showing no growth was transferred on to agar plates and incubated for 24 hrs at 37^0 C. The minimum bactericidal concentration (MBC) was thus determined and is the lowest concentration of the sample that shows no growth of bacterial colonies on the agar surface after incubation.

RESULTS AND DISCUSSION

Phytochemical screening

The phytochemical screening of the plant extract shows the presence of important phytoconstituents ie., phenols and flavonoids. From the standard graph the plant extract was found to contain total phenol content (>232 µg/mL of tannic acid equivalents) and total flavonoids (>708.75µg/mL quercetin equivalents).

Antioxidant property

The extract was found to contain 980µg and 1.940 µg of antioxidants. It is found to have similar activity as that of ascorbic acid.

Antimicrobial activity

The plant extract is found to have antimicrobial and antifungal activities in a concentration dependent manner. *Pergularia daemia* showed its anti-bacterial activity on *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa and Escherichia coli* and among fungi, *Candida albicans* and *Candida glabrata*. Ampicillin and nystatin had shown good antimicrobial activity. It was found that *E.coli* is more sensitive towards the extract than to the standard. Among bacteria, the plant extract is having more activity on *Escherichia coli*, *Bacillus subtilis*, *Bacillus cereus*, than on pseudomonas and staphylococcus species. The extract had shown its antifungal activity only on *Candida albicans* and *Candida glabrata*. Among them *candida glabrata* is found to be more susceptible than albicans. At a concentration of 10 and 20 the µg/ml the extract is more effective on *C.glabrata*. After series of pilot experiment it is found to have no activity on Aspergillus species and saccharomyces. Distilled solvent had shown no activity on the microbes.

Table 1. Estimation of phenol content of standard

S.No	Tannic acid(in µl)	FC reagent (in ml)	Na2CO3(in ml)	OD at 680nm
1	0	5	4	0.00
2	200	5	4	0.59
3	400	5	4	1.16
4	600	5	4	1.62
5	800	5	4	1.92
6	1000	5	4	
T1	200 µL	5	4	0.68
T2	400 µL	5	4	1.28

Table 2. Estimation of Flavonoids

S. No	Vol of Quercetin (µL)	Methanol (µL)	CH3COOH(µL)	OD at 420nm
1	0	1500	100	0.00
2	15	1485	100	0.05
3	30	1470	100	0.08
4	60	1440	100	0.12
5	90	1410	100	0.18
6	120	1380	100	0.22
7	150	1350	100	0.30
8	180	1320	100	0.32
9	210	1290	100	0.37
10	240	1260	100	0.42
11	270	1230	100	0.47
12	300	1200	100	0.51
13	500 T	1500	100	1.26

Table 3.Determinatioon of antioxidant property by using ascorbic acid standard curve.

Standard Ascorbic acid	Concentration of Ascorbic acid (µl)	OD at 680 nm
Blank	0.00	0.00
S1	200	0.20
S2	400	0.58
S3	600	0.79
S4	800	0.96
S5	1000	1.04
S6	1200	1.23
T1	200µL	0.98
T2	400 µL	0.97

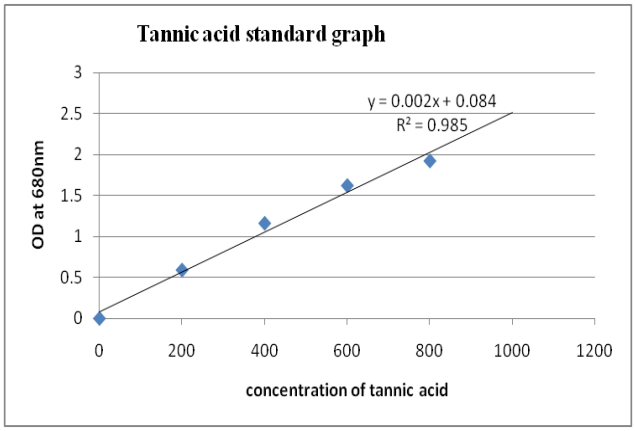
Table 4. Antibacterial activity of leaf extract of *Pergularia daemia*

S.no	Plant extract	Concentration of extract µg/ml	Zone diameter in mm					
			B. subtilis	*B. cereus*	*P. aeruginosa*	*S.aureus*	*S. epidermidis*	*E. coli*
1	methanol	10	10	9	7	8	7	12
2		20	14	13	8	10	10	15
3		30	15	13	10	13	12	17
4		40	18	16	11	15	13	19

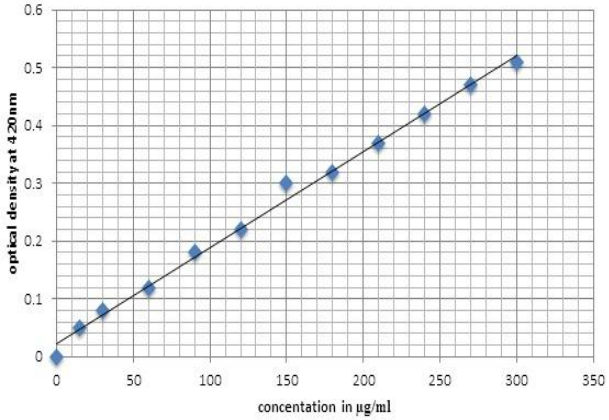
Table 5. Antifungal activity of leaf extract of *Pergularia daemia*

S.no	Plant extract	Concentration of extract µl	Zone diameter in mm				
			Aspergillus niger	*Aspergillus foetidus*	*Candida glabrata*	*Candida albicans*	*Saccharomyces cervaseae*
1	methanol	10	-	-	15	12	-
2		20	-	-	17	13	-
3		30	-	-	14	15	-
4		40	-	-	13	15	-

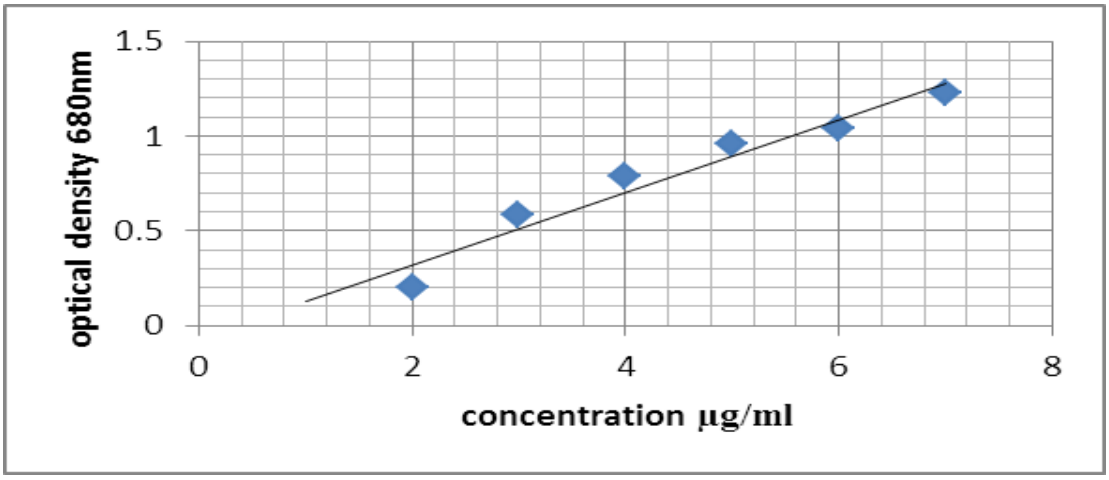
Graph 1. Tannic acid standard graph for the estimation of phenols from *Pergularia daemia*



Graph 2. Standard graph of quercetin for the estimation of flavonoids from *Pergularia daemia*



Graph 3. Standard graph for estimating antioxidant ability of Ascorbic acid



MIC and MBC

The MIC and MBC values for the extract are determined. MIC and MBC values for *Bacillus subtilis* and *Escherichia coli* are found to be lowest ie.,15.62/31.25 and 7.81/15.62. for *Bacillus cereus*, *Candida albicans and Candida glabrata* it was found to be 31.25/62.5 and 31.25/125.in case of staphylococcus species the concentration is 62.5/>500 and 62.5/125. For pseudomonas it is 62.5/> 500

DISCUSSION

There exists a strong correlation between total phenols, flavonoids and their biological activities. Due to the presence of phenols as major constituents the extract had shown good antimicrobial activity and presence of flavonoids in a minor concentration showed a good antioxidant property. Hence a strong correlation exist between the total phenols, flavonoids, antioxidant and antimicrobial activities.

The methanolic extract is effective on most of the pathogens which are said to cause food poisoning. Entero toxic E.coli (ETEC) is the most common cause of traveller's diarrhoea and can cause fatal consequences for children less than 5 years. ETEC is also important in the farming industry, as post-weaning piglets are highly susceptible to infection. *Pseudomonas aeruginosa* is an increasingly prevalent opportunistic pathogen and is the most common Gram –negative bacilli found in nosocomial and life threatening infections of immunocompromised patients (Donlan RM *et al*., 2002). Pseudomonas is also known for its natural resistance to a large number of antibiotics like penicillins, 1st-3rd generation cephalosporins and also against antipseudomonals- like carboxy penicillins, carbapenems, gentamycin and ciprofloxacin (Mariana CC *et al*., 2007). *Staphylococcus aureus* which can infect meat, dairy and other carbohydrate rich foods, produces powerful exotoxins which cause necrosis, hemolytic, scalded skin

syndrome and toxic shock syndrome. *Bacillus cereus* found mostly in rice products toxins that are emetic and diarrheal type.

Candida species are involved in causing skin infections, superficial candidiasis in AIDS, pregnancy, diabetes, young or old age, birth control pills, and trauma during burns, maceration of the skin. In AIDS especially the treatment using corticosteroids or antibiotics may lead to immunodeficiency. Systemic candidiasis may involve kidneys, eyes, heart and meanings.

The leaf extract of *Pergularia daemia* is found to be more effective on these species that cause serious diseases and it is found to have no activity on Aspergillus species and saccharomyces.

Phytochemicals are classified as antimicrobial agents on the basis of their susceptibility tests that produce MIC in the range of 100 to 1000 µg/ml (Simoes M *et al*., 2009; Gibbons S, 2005) and for the plant extracts activity is considered to be significant if MIC values are below 100 µg/ml for crude extracts and moderate when 100<MIC<625µg/ml (Kuete V *et al*., 2011; Fabry W *et al*., 1998). Therefore the activity of the plant extract is considered almost significant.

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