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Preliminary phytochemical analysis and antibacterial activity of leaf and leaf derived callus extracts of *Passiflora caerulea*: An important medicinal plant

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Abstract

In the current study the Preliminary phytochemical analysis and antibacterial activity of leaf and leaf derived callus extracts of *Passiflora caerulea* were carried out in different solvent extracts.MS medium showed a better response than B5 medium and White's medium for callus induction. The plant and callus extracts showed the presence of carbohydrates, tannins and glycosides and leaf extracts showed the presence of carbohydrates, whereas stem extracts showed the presence of carbohydrates and phlobatannins. . The methanol, chloroform, leaf extract showed an inhibition zone of 8.00 mm against *B. cereus* and *E. coli*. Methanol and hexane Callus extract showed an inhibition zone of 7.00mm against *E. coli* and *P. aeruginosa*. The presence of antibacterial property of the solvent extracts against selected microorganisms might be due to the presence of some compounds. Further phytochemical studies are required to explore compounds responsible for the activity.

Keywords: *Passiflora caerulea*; Phytochemistry; TLC; Callus

1. Introduction

Plants were the basis for nearly all medicinal therapy until synthetic drugs were developed since prehistoric times, (Smith, 1996). Some people value these plants due to the ancient belief which says plants are created to supply man with food, medical treatment, and other effects. It is thought that about 80% of the 5.2 billion people of the world live in the less developed countries and the World Health Organization estimates that about 80% of these people rely almost exclusively on traditional medicine for their primary healthcare needs. Medicinal plants are the "backbone" of traditional medicine, which means more than 3.3 billion people in the less developed countries utilize medicinal plants on a regular basis (Davidson-Hunt, 2000).

The Indian subcontinent is one of the oldest civilizations which include many traditional health care systems. One of the ancient classics, "Charak Samhita" is the oldest text available on the complete treatment of diseases which specifies the use of hundreds of herbs in the complete treatment of diseases. The 500 B.C old Ayurveda is one of the ancient health care systems which are a potential source of indigenous drugs. A large number of such herbs are mentioned in "Indian Materia Medica" for the treatment of various diseases (Anon, 1994; Ayensu, 1996; Kumar *et al*., 2000). In India alone, less than 10 % of the medicinal plants traded in the country are cultivated, about 90% are collected from the wild, very often in a destructive and unsustainable manner (Natesh, 2000).

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Bioactive compounds extracted from plant can be used as stimulants, hallucinogens, insecticides, poisons, therapeutic agents, food additives, pesticides, dyes, flavors, cosmetic and pharmaceuticals. The biological activities of the secondary metabolites can be tested using different kinds of bioassays. Bioassay is a biological testing procedure for estimating the concentration of active substance in the extract. A large number of plant secondary metabolites have been shown to possess antioxidative and anticancer properties. As an example, a new anticancer drug, taxol, is derived from the bark of *Taxus brevifolia.* Taxol is a complex diterpene alkaloid known due to its unique mode of action on the microtubular cell system (Jordan and Wilson, 2004). There are many bioassay systems to evaluate the plant chemicals such as *in vitro* antimicrobial, anti-proliferative, antioxidant and radical scavenging test .

Passiflora caerulea belongs to the familyPassifloraceae,A perennial tendril climber grows over large trees and bushes (fig. 03). Leaves palmately 3-7 lobed and alternate. Leaves are opposed by tendrils. Flowers bluish purple colored, axillary and very attractive. It is used in the treatment of inflammation, pain, insomnia, depression and anxiety disorders. Plant pacifies vitiated pitta, Useful part is Whole plant.

2. Material and methods

2.1. Collection of plant materials

The fresh leaves of *P. Caerulea* were collected from Bangalore botanical garden and brought to the laboratory and processed for further studies. The collected plant materials were washed under running tap water thoroughly in order to remove the dust particles and all the materials were shade dried.

2.2. Callus induction

Murashige and Skoog's medium (1962), Gamborg's B-5 medium (1968) and White's medium (1943) were used to test the response of explants. The response on MS medium was found to be better so it was used as the basal medium for all experiments. The nutrient medium containing inorganic and organic compounds and 3 percent sugar was stabilized with 0.8 percent Difco-bacteriological grade agar - agar. The pH of the medium was adjusted between 5.6 to5.8 either with 0.1 N. NaOH or 0.1 N HCL prior to homogenization of the medium. Different concentrations of auxins and cytokinins were used individually and also in various combinations.

The homogenate medium was dispensed into culture tubes and conical flasks and plugged with non-absorbent cotton. Approximately 30 to 40 ml of medium was poured into 100ml Erlenmayer flasks and the Cornning test tubes measuring 25mm X 200 mm and 75 ml to 100 ml in 250 ml Erlenmayer flasks. After plugging with cotton they were autoclaved at 15-lbs/inch^2 pressures for 15 to 20 minutes at 121 $^\circ$C. The inoculation of the explants and subcultures were carried out in a laminar airflow chamber. The cultures were incubated at a temperature of 25$\pm$ 2^{o}C and relative humidity of 60-70 percent under 16 hours daily illumination of approximately 1000-2000 lux provided by cool white fluorescent tubes.

2.3. Sample extraction and Phytochemical analysis

The shade dried plant material and callus was powdered. The powdered material was placed in the extraction tube of the soxhlet apparatus for further extraction. Solvents like hexane (non polar), ethyl acetate (mid polar) and methanol (polar) were used for extraction. The obtained extracts were scraped and transferred in to a tube labeled and stored for further studies. Quantitative phytochemical analysis was done by the following the method of Harborne (1984).

2.4. Antibacterial activity

Antimicrobial activity was done by disk diffusion method (Jorgrnsen and Turnidge, 2007). The leaf and callus solvent extracts from *P. caerulea* were screened for antibacterial activity against test bacterial strains like *Salmonella typhi* (MTCC-531), *Pseudomonas aeruginos*a (MTCC- 1034), *Escherichia coli* (MTCC-118), *Bacillus cereus* (MTCC-430), *Streptococcus epidermis* (MTCC-435) obtained from MTCC Chandigarh, India. The zones of inhibition for the entire tested organism were measured in mm.

2.5. Disk Diffusion Assay

A suspension of testing microorganisms was spread on nutrient agar medium. The filter paper discs (5mm in diameter) was placed on the agar plates which was inoculated with the tested microorganisms and then impregnating with 20µL of plant extract (concentration 200 mg/mL). The plates were subsequently incubated at 37$_o$ C for 24 Hrs. After incubation the growth inhibition zone were quantified by measuring the diameter of the zone of inhibition in mm (Kumar *et al*., 2009).

2.6. Determination of MIC of plant extract by Microdilution Method

The 96-well plates were prepared by dispensing 50 µL of nutrient broth for bacteria, into each well. A 50 µl from the stock solution of tested extracts (concentration of 200 mg/mL) was added into the first row of the plate. Then, two fold, serial dilutions were performed by using a micropipette. The obtained concentration range was from 100 to 0.1953 mg/ml, and then added 10 µL of inocula to each well except a positive control (inoculum were adjusted to contain approximately 1.5 x 10^8CFU/mL. Plant extract with media was used as a positive control and inoculum with media was used as a negative control. The test plates were incubated at 37 ˚C for 18 h. After 18 h 50 µL of a 0.01% solution of 2, 3, 5- triphenyl tetrazolium chloride (TTC) was added to the wells and the plate was incubated for another hour. Since the colorless tetrazolium salt is reduced to red colored product by biological active bacteria, the inhibition of growth can be detected when the solution in the well remains clear after incubation with TTC. MIC was defined as the lowest sample concentration showing no color change (clear) and exhibited complete inhibition of growth (Abu-Shanab *et al*., 2004 and Abou Elkhair *et al*., 2010 Radojevic *et al*., 2012).

2.7. Phytochemical analysis of *in vitro* callus and *in vivo* plant material through TLC

The methanolic extract showed good antimicrobial activity so the same solvent extract was sleeted for TLC. The methanolic extracts were filtered through Whatmann No. 1 filter paper and filtrate was evaporated to dryness.

2.8. Sample Preparation

10 mg of dry methanol extract was dissolved in 1ml of methanol. From the stock 2.5 µL of sample was used for TLC analysis. The readymade TLC plates of dimension 24x24 cm were used and the solvent system used was Chloroform and Methanol (1:9). The TLC chamber was subjected to saturation for about 20-30 min with mobile phase. The TLC plates loaded with a extracts were put in to the TLC chamber previously saturated. The eluted chromatogram was observed under UV light (254 nm). The Rf value is the constant and characteristic of the substance which indicates its movement relative to the solvent front in a given chromatographic system.

2.9. Statistical Analysis

All data will be subjected to statistical analysis wherever necessary.

3. Results

3.1. Callus induction from leaf

Leaf explants ofPassiflora caerulea were testedfor callus induction on MS, B5 media and Whites medium containing different concentrations of 2,4-D (0.5mgL^{-1}, 1mgL^{-1} and 2mgL^{-1}) in order to test the best suitable medium for morphogenetic potentiality. Leaf segments failed to show any response on basal media, however, remained green for some times and eventually necrosed. On all the three media containing growth regulators curling of explants was noticed as an initial step in both the species. Further it was noticed that MS medium elicited a better response than B5 medium and White's medium. MS medium supplemented with different concentration of growth regulators and other additives was used for further experiments.

3.2. Preliminary phytochemical analysis

Preliminary phytochemical analysis revealed that the presence of some of the secondary metabolites in different parts of the plants.LThe plant and callus extracts showed the presence of carbohydrates, tannins and glycosides. *P. caerulea* leaf extracts showed the presence of carbohydrates. In *P. caerulea* stem extracts showed the presence of carbohydrates and phlobatannins (Table 1). The TLC profile characteristics of the leaf, stem and callus sample were presented in the tabale 2 and fig. 1.

Table 1 Phytochemical profile of different parts of *P. caerulea*

Sl. No.	Phytochemicals	PCL	PCC	PCS
1	Carbohydrates	+	+	+
2	Amino acids	-	-	-
3	Alkaloids	-	-	-

4	Tannins	-	+	-
5	Phlobatannins	-	-	+
6	Saponins	-	-	-
7	Flavonoids	-	-	-
8	Terpenoids	-	-	-
9	Glycosides	-	-	-
10	Steroids	-	-	-

PCL- P.caerulea leaf, PCC- P.caerulea callus; PCS- P.caerulea stem, +: Present, -: Absent

Table 2 TLC characteristics of the sample

Sample code	TLC Band	Retention Factor	TLC Profile characteristics		
			Visible light	Shortwave UV 254 nm	Longwave UV 366 nm
			Figure-1	Figure-2	Figure-3
PCL	1	0.61	Black	Black	Black
	2	0.68	Green	Green	Red
	3	0.71	Yellow	Yellow	Brown
PCC	1	0.8	No band	No band	Pink
PCS	1	0.73	Green	Green	Pink
	2	0.76	Yellow	Green	Light pink

PCL- *P.caerulea* leaf PCC- callus PCS- *P.caerulea* stem

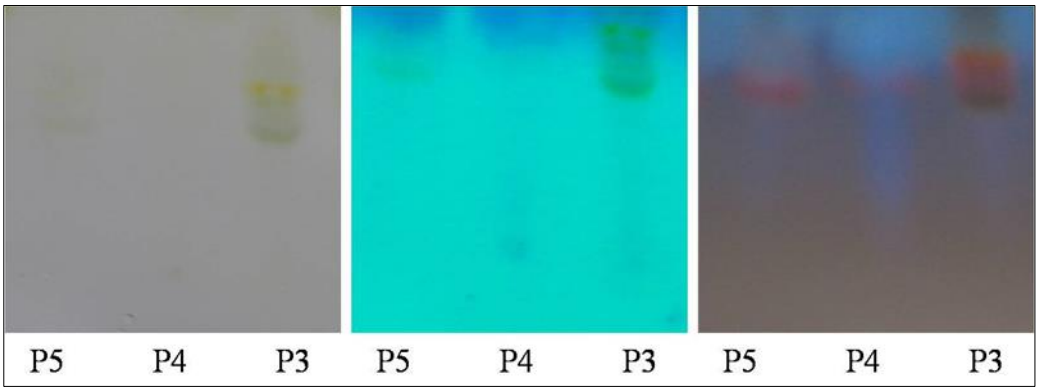

Figure 1 TLC patterns under different wavelengths; P3- leaf; P4- callus P5-stem

3.3. Antimicrobial activity

Among the different solvents extracts tested, methanolic leaf extract showed good antimicrobial activity followed by hexane and chloroform. The methanol leaf extract of *P. caerulea* showed an inhibition zone of 8.00±0.00 mm against *B. cereus* and the chloroform leaf extract showed an inhibition zone of 8.00±0.00 mm against *E. coli.* The methanol callus extract showed an inhibition zone 8.00±0.0 against *E. coli.* Callus extract showed an inhibition zone of 7.00±0.00 mm against *E. coli*. The hexane callus extract showed an inhibition zone of 7±0.00 mm against *P. aeruginosa and* methanol callus extract showed an inhibition zone of 7.0±0.00 mm against *P. aeruginosa.* For all the studied extracts negative antifungal activity was observed (Table 3).

The minimum inhibitory concentration (MIC) was also determined for the extracts showing better results in the antimicrobial activity.MIC determination was done against *Bacillus cereus* by micro broth dilution technique. Leaf extract of *P. caerulea* showed MIC of 1024 µg/mL against *B. cereus* compared to 1 ug $^{-mL}$ observed for ciprofloxacin

Table 3 Inhibitory activity of methanolic leaf extracts of *P. caerulea*

Test Samples	**Con. (mg/disk)**	**Zone of inhibition (mm)**				
		B. cereus	***S. typhi***	***P. aruginosa***	***S. epidermis***	***E. coli***
		18.00± 0.00	**-**	**-**	**-**	**-**
P3	1	-	-	7.00	-	7.00
P4	1	-	-	-	-	-
Ciproflaxacin	0.01	27.00± 0.50	28.00 ± 0.25	20.00 ± 0.50	28.00 ± 0.50	20.00 ± 0.25
Crystal Violet	0.1	-	-	-	-	-

P2: *P. caerulea* leaf methanolic extract; **P3:** *P. caerulea* methanolic leaf callus extracts; **P4:** *P. caerulea* methanolic stem extract

4. Discussion

Scientific validation of use of traditional medicinal plants has gained a peak since last few decades with the aim to search for the natural novel drug. Phytochemical analysis is the main tool for the drug discovery. It gives an idea about the most probable constituents (chemical) present in the plant with a specific biological activity. Natural products, such as plants, either in crude form or standardized extracts provide a broad opportunity for the novel drug discovery because of their unmatched chemical diversity (Cosa *et al.,* 2006).

Phytochemicals are categorized in different classes based on their chemical structure like alkaloids, phenols, steroids, terpenoids, phenols etc. Disclosure of the chemical constituents of the plant not only helps in the discovery of new therapeutic agents but also helps to search the additional resources of raw materials for pharmaceutical industry. Bioassays of plant extracts and their fractionation as per their bioactivity are the major steps in phytochemical studies. Bioassay comprises screening of biological activity of plant extracts and followed by phytochemical analysis (Iqbal, 2012).

In the present study, preliminary screening of leaf and leaf callus extracts revealed the presence of tannin, glycosides, phlobatannins, carbohydrates in methanol extract. Presence of secondary metabolites in leaf callus cultures paves an option for the production of biologically viable compounds. Our results confirmed that the determination of the therapeutic value depends on the standard extraction procedure. Ncube *et al.,* (2008) reports that the quality of the herbal drug depends on the standardization of extraction procedures. The antimicrobial activity exhibited by*P. caerulea* leaf and leaf callus extracts could be attributed to the presence of the phenolic compounds present as revealed in the phytochemical analysis. The results are in agreement with another study done on 112 traditional Chinese medicinal plants by Cai, *et al.,* (2004).

To conclude, preliminary phytochemical study lays the foundation for the new drug discovery in general and *P. caerulea* leaf and leaf callus extracts in particular. Screening of medicinal plants by *in vitro* guided bioassays followed by phytochemical analysis is the major development in the drug discovery technology. In the present study, qualitative phytochemical analysis has revealed the presence of tannin, glycosides, phlobatannins, carbohydrates, but further isolation and characterization of active fractions needs to be carried out which may lead to the development of the novel natural drug.

The multiple drug resistance menace has been recognized as serious global human and animal health problem by world health organization (Patel, 2012). The over use of commercially available antibiotics and the over the counter availability is attributed to the emergence of bacterial resistance. With the advancement of technology and discovery of various antibiotics, people were optimistic about the treatment of infectious disease , but the optimism is yet to be met. Nature has always stood by man's side whether it is food, shelter or medicine. Plants as medicine have been used ever since the existence of civilization. Plants contain diverse secondary metabolites which are effective and safe in the treatment of various human ailments (Arash Khorasani Esmaeili, 2013).

In the present study the traditionally used ethnomedicinal plants were subjected to the in vitro antin]bacterial screening against different gram positive and gram negetive bacteria Out of different extracts tested methanolic extract proved to be highly effective against the microorganisms used. In the study, *P.caerulea* methonolic leaf extract showed maximum growth inhibition against the *B.cereus*. Our results are in concurrence with thestudies earlier carried out by Eloff, 1998 and Soniya *et al.,* (2013) who reported that effectiveness of the extract largely depends on the type of solvent used in the extraction. It is widely hypothesized that inhibition of any extract is attributed to the presence of phenolic compounds as phenolics sensitize the phospholipids bilayer of the microbial cytoplasmic membrane which increases the membrane permeability and impairs the bacterial enzymes and makes them susceptible. The present study confirms the antimicrobial property of callus extracts, though less inhibition, nevertheless results showed that *in vitro* callus extracts have antimicrobial property. The study paves a way for more production of biologically viable antimicrobials using callus mediated establishment of cell lines and using callus suspension cultures. Our inference falls in agreement with the study carried out by Khanpour-Ardestani, 2015.To conclude, our study confirmed the antimicrobial efficacy of *P. caerulea* leaf, stemand leaf derived callus extracts. However, further study needs to be carried out to isolate and characterize a viable metabolite for leaf and leaf callus extracts which may lead to the development of an antimicrobial agent.

5. Conclusion

From the present study revealed that presence of different phytoconstituents and secondary metabolites, particularly Gallic acid in leaf extract. The presence of antibacterial property of the solvent extracts against selected microorganisms might be due to the presence of some compounds. Further phytochemical studies are required to explore compounds responsible for the activity.

Compliance with ethical standards

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Disclosure of conflict of interest

There is no conflict of interest.

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