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Bioassay Guided Phytometabolites Extraction for Screening of Potent Antimicrobials in *Passiflora foetida* L.

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ABSTRACT

Passiflora is a genus belonging to the family Passifloraceae having varied species with highly therapeutic values. Tribes with traditional medicinal knowledge suggested *Passiflora foetida* L. as a source of high value pharmaceutical plant. Present study deals with the Isolation, Purification, characterization and bioassays of antimicrobial secondary metabolites. In spite of traditional soxhlet extraction, Cold percolation proved suitable extraction scheme. Bioassay guided TLC characterization, and purification led the effective collection of bioactive natural products. Disc diffusion method shows a potent inhibitory activity of compounds against multi drug resistant pathogenic microorganisms like *S. aureus(OARS).* For structural characterization bioactive products were analyzed using UV. The results confirmed the presence of polyacetylenes as active constituents in the plant.

INTRODUCTION

The genus *Passiflora* belongs to Passifloraceae family and includes the passion fruit, is the largest and the most widespread genus of tropical flora. About 400 species of this genus are grouped into 21 subgenera (Cronquist, 1981). More than 350 species have been found in tropical regions and rain forests of South America (Killip, 1938) and 60 of them are edible species. Passion fruit is an important fruit crop in many exotic and subtropical countries due to its edible fruits, ornamental use and medicinal properties. Some species (*P. edulis*, *P. quadrangularis* and *P. ligularis*). are chiefly cultivated for the production of fruit juice. *P. incarnata* is reputed for its sedative properties, and several other species are known for their ethnobotanical uses.

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P. amethystina Mikan, found in the rain forest of the Brazilian south-eastern coast (Fouqué, 1972), is a wild passion fruit species. It has purple-blue flowers, which are very aromatic. The genus also contains some species of ornamental use and medicinal properties as sedatives, antispasmodics and antibacterial (Nhut *et al.,* 2007). Several species have edible fruits and attractive flowers, about 40 species have been cultivated, but fewer than 6 are fruit crops in the neotropics and only one, *P. edulis* (and its varieties, such as the yellow favicarpa), is economically important. A few species, such as *P. foetida* and *P. lonchocarpa*, are extremely foul smelling (Benson *et al.,* 1976). Eleven species, including *P. foetida* and *P. tripartita* (= *P.* mollissima) are recorded as weeds in distinctive parts of the world (Swarbrick 1981). Both *P. foetida* and *P. tripartita* are closely related taxonomically, whereas, *P. edulis* belongs to a different subgenus (Waage *et al.,* 1981) and is the only economic crop at risk from oligophagous insects attacking

P. foetida. In recent years, a significant revival of interest in natural products as a potential source for new medicines has been observed throughout the world. Several modern drugs ~40% in use have been developed from natural products. Nowadays, multiple drug resistance in human pathogenic microorganisms develops due to indiscriminate use of commercial chemical antimicrobial drugs commonly used in the treatment of human diseases. Over the last three centuries, intense efforts have been made to discover clinically useful antimicrobial drugs (Ahmed *et al.,* 1998; Sarker *et al.,* 2006; Werner *et al.,* 1999). The increasing interest on traditional ethno medicine may lead to discovery of novel therapeutic agents. The World Health Organization (2000) estimates that 80% of the population of developing countries still relies on traditional medicines, mostly plant drugs, for their primary health care needs. Herbs are supposed to be safe, but many unsafe and fatal side effects have recently been reported (Ikegami *et al.,* 2003; Izzo, 2004).

Traditionally, fresh or dried whole plants as well as their preparations are accepted for medicinal use in America, Germany, France, and other European countries for the treatment of nervous anxiety (Blumenthal, 1997; Speroni and Minghetti, 1988). Pharmacological studies show that passion flower has antispasmodic, sedative, anxiolytic, and hypotensive activity (Akhondzadeh *et al.,* 2001; Abascal and Yarnell, 2004; Dhawan *et al.,* 2001a; 2001b; Dhawan *et al.,* 2003; Weiss, 1988; Wolfman *et al.,* 1994).

A passion flower (*Passiflora foetida*), an exotic and fast-growing perennial vine, is found in the Western USA and in Asian countries like India. Ethnobotanical reviews of *P. foetida* report the decoction of leaves and fruits to treat asthma and biliousness (Ambasta, 1986); and leaf paste is applied on the head for giddiness and headache (Chopra *et al.,* 1956). In Brazil, the herb is used in the form of lotions or poultices to treat erysipelas and skin diseases with inflammation (Chopra *et al.,* 1944). The major phytoconstituents of this plant are alkaloids, phenols, glycoside flavonoids, and cyanogenic compounds, passifloricins, polyketides, and alpha-pyrones (Dhawan *et al.,* 2004; Echeverri *et al.,* 2001). One chemical component of a passion flowers Passicol, a polyacetylenic compound has antimicrobial activity (Birner and Nicolls, 1973; Nicolls, 1970; Nicolls *et al.,* 1973), which is still not reported in *P. foetida* L. The majority of the active components in this plant are C-glycosyl flavones based on apigenin and luteolin; Harman alkaloids are found in trace amounts along with sucrose and trace amounts of volatile oil (Bradley, 1992; Leung and Froster, 1996; Newall *et al.,* 1996).

MATERIALS AND METHODS

Leaves and fruits of *P. foetida* L. was collected from the Melghat forest area, Amravati, India. The plant was authenticated using standard flora and cross-checked with herbarium records at the NBRI, Lucknow, India as *Passiflora foetida* L. with an accession number- 98181. Silica gel-GF 254 was purchased from Himedia Laboratories, India and all other reagents and solvents were purchased from Sigma-Aldrich, St. Louis, MO, USA. The UV spectrophotometer-1800 (Shimadzu, Japan), and UV Transilluminator (Cleaver, Korea) was used for study and analysis.

PRELIMINARY PHYTOCHEMICAL SCREENING

The extracts of *Passiflora foetida* were analyzed for the presence of Alkaloids, Saponins, Tannins, Cardiac Glycoside, Anthraquinones, Steroid, coumarin, carbohydrates and flavonoids, according to standard methods (Odebiyi and sofowora, 1978; Sofowora, 1982; Williamson et. al., 1996; Banso and Ngbede, 2006; Ngbede et. al., 2008). For the study of phytochemical analysis, the ethanol extract of the plant leaves was prepared according to standard methods (Sofowora, 1982). The plant leaves were air dried and powdered. Transferred the powdered material into solvent extractor and extracted it with 95% ethanol for 72 h. The extract was obtained as a brown gummy solid. The extract was stored and used for phytochemical screening.

Screening for alkaloids

Three grams of extract was stirred with ethanol containing 3% tartaric acid. The filtrate was shared into 3 beakers and tested for alkaloids as follows-

In to the first beaker, Hagar's reagent was added into the second beaker, Mayer's reagent was added and into the last beaker Marquins reagent was added. Precipitations in any of 3 tests indicate the presence of an alkaloid (Odebiyi and sofowora, 1978; Banso and Ngbede, 2006)

Screening for saponins

About 0.5 g of the plant extract was shaken with water in a test tube, frothing, which persists on warming was taken as preliminary evidence for the presence of saponins.

Few drops of olive oil were added to 0.5 g of extract and vigorously shaken; formation of soluble emulsion in the extract indicates the presence of saponins (Odebiyi and sofowora, 1978).

Screening for tannins

Into 10ml of freshly prepared 10% potassium hydroxide (KOH) in a beaker, 0.5 g of extract was added and shaken to dissolve. A dirty precipitate observed indicated the presence of tannin (Odebiyi and sofowora, 1978; Willimson *et al.,* 1996).

Screening for steroid

Total 100mg of *Passiflora foetida* extract was dissolved in 2 ml of chloroform. Sulphuric acid was carefully added to form a lower layer. A reddish-brown color at the interface is indicative of the presence of steroidal ring (Sofowora, 1982)

Screening for flavonoids

About 2 g of the powdered leaves was completely detanned with acetone. The residue was extracted in warm water after evaporating from the acetone in water bath. The mixture was filtered while still hot. The filtrate was cooled and used.

Sodium hydroxide test

Five ml of 20% sodium hydroxide was added to equal volume of the detanned water extract. A yellow solution indicates the presence of flavonoid.

Screening for anthraquinones

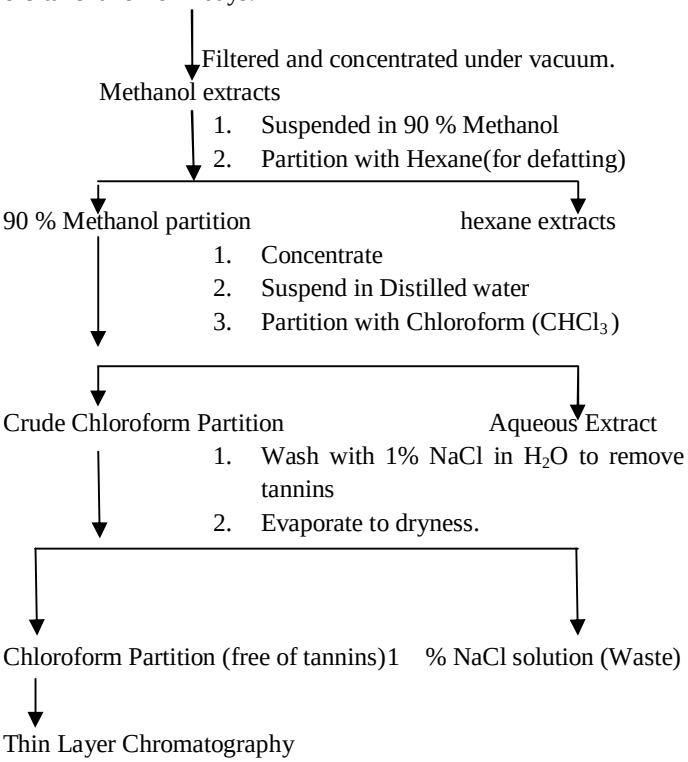
Bontrager's test- About 0.5 g of the extract was taken in order to dry test tube and 5 ml chloroform was added and shaken for 5 min. The extract was filtered, and the filtrate shaken with an equal volume of 100% ammonia solution. A pink violet or red colour in the ammoniacal layer (lower layer) indicates the presence of free anthraquinones.

Screening for cardiac glycoside

(Keller Killiani test)- Total 100 mg of extract was dissolved in 1 ml of glacial acetic acid containing one drop of ferric chloride solution. This was then underlayerd with 1 ml of concentrated sulphuric acid. A brown ring obtained at the interface indicates the presence of de-oxysugar characteristics of cardenolides.

PREPARATION OF TANNIN FREE BIOACTIVE EXTRACT

The schematic representation of procedure was given below as proposed by Wall *et al.*, (1996).



TLC Characterization

The presence of active metabolites in *P. foetida* L. extract was evaluated by TLC plates. The preparative normal phase TLC was performed on 15x10 cm glass plates coated with 1 mm layer of silica gel, dried in room temp. for 30 minutes and activated in the oven at 110^{o} C for next 30 minutes. The solvent system chloroform: ethyl acetate: formic acid: methanol (5:3:2:0.4) 4 were used (Harborne, 1973). 10 µl of extract was spotted on the silica plates. The experimental plates were developed in a chromatographic chamber, saturated by 100 ml of the solvent system. Plate development required about 15 to 20 minutes; it was then visualized at 365 nm under UV transilluminator (Cleaver). Rf values of bands were recorded.

TLC Bioautography

In order to find out the bioactive fraction from TLC plate the TLC Bio-autography by agar overlay method was performed (Hostettmann et al, 1990). In N.A medium, a suspension of test pathogen Oxacilin resistant *S. aureus (OARS)* i.e. 10mg/10mm zone was added aseptically in the molten agar, further spread on the TLC plate. The plate kept in moist chambers and incubated at 37^{0} C for overnight. Next the solution of tetrazolium salt MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide, 2mg/ml) was sprayed on the plate.

Spectroscopic Analysis

The bioactive fractions were scrapped from the TLC plate and Centrifuged in methanol, and the Purified fractions were UV analyzed in the range of 200-400nm while the baseline was corrected keeping methanol as blank.

Determination Of Minimum Inhibitory Concentration

The antibacterial potency of the bioactive fraction in variable concentrations was determined in terms of minimum inhibitory concentration (MIC) using the filter paper disc diffusion method (Pelczar *et al.*, 1998; Patil, 2010). The lawn of the log phase of *Staphylococcus aureus, Salmonella typhi, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* were prepared using a glass spreader. The variable concentrations of bioactive fraction were prepared in 1 % DMSO (dimethyl sulphoxide) so that a single disc contained 0.5, 1, 2, 3, 4 and 5 µg/ml. The filter paper discs (3 mm) were presoaked in their respective dilutions for 30 min and then kept in the test bacterial lawn. The plates were left on a bench for 1 hr before incubation at 37°C for 24 h to allow prediffusion of the extract (Esimone *et al.*, 1998), control was maintained by soaking paper disc in 1% DMSO. The zone of inhibition was measured in mm using the zone measuring scale and compared with the control set (i.e. disc with solvent). The MIC value was taken as the lowest concentration of the extract showing complete lack of growth after the incubation period (EUCAST, 2000) or maximum inhibition of test microorganisms each experiment was performed in triplicates.

Statistical Analysis

Data acquired was statistically analyzed using Microsoft Excel 2007.

RESULTS AND DISCUSSION

Phytochemical Analysis

The preliminary phytochemical analysis of *P. foetida* resulted in presence of all the principal classes of compounds discriminated in Table.1.

Table. 1: Preliminary phytochemical screening of ethanol extract of *Passiflora foetida* L.

Phytochemical constituents	Results
Saponins	+
Tannins	+
Cardiac Glycosides	+
Alkaloid	+
Anthraquinones	+
Steroid	+
Flavonoid	+

Key + = present

Extraction Of Material

The initial weight of leaf powder before extraction was 50 gm, after extraction the pure crude extracts was of 1. 11gm. The extraction scheme resulted in efficient method as it removed the tannins, Saponins and other interfering compounds.

Spectroscopic Analysis

The UV analysis of bioactive fractions showed multiple absorption maxima in the range of 230-370nm, confirming their polyacetylenic nature (Birner and Nicolls, 1973; Sorensen, 1968; Bohlman *et al.,* 1961). The finger like appearance of spectrum (PA1) confirmed the presence of polyacetylenes (Fig.1).

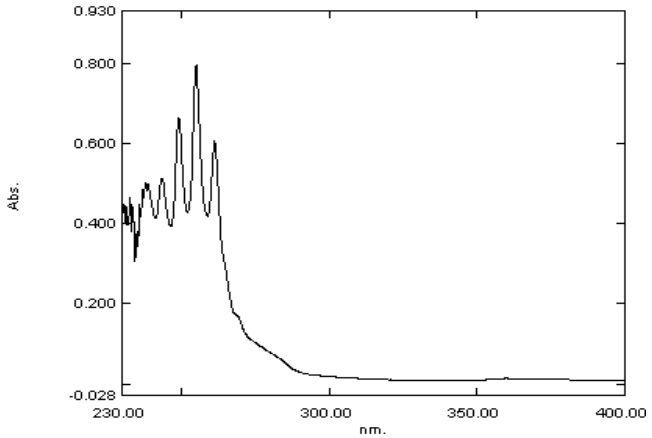


Fig. 1: UV spectrum of bioactive fraction PA1.

Confirmative Test for Unsaturation

To confirm the polyacetylenic nature of fraction, a bromine water test was performed, in which a bioactive spot from TLC plate was scrapped off and eluted with 2 ml distilled water; plain distilled water with an equal amount of scrapped silica was kept as the control.. To 1 ml of the test sample along with the control, 1 ml of bromine water was added drop wise, and the color change was observed; the disappearance of bromine water color indicated unsaturation confirming the presence of polyacetylenes in fractions (Jamode *et al.*, 1998).

TLC Analysis

The TLC analysis of extract resulted in showing two prominent green coloured band named as **PA1** and **PA2,** i.e. Polyacetylene 1 and Polyacetylene 2 with Rf value 0.32 and 0.70 respectively (Fig. 2).

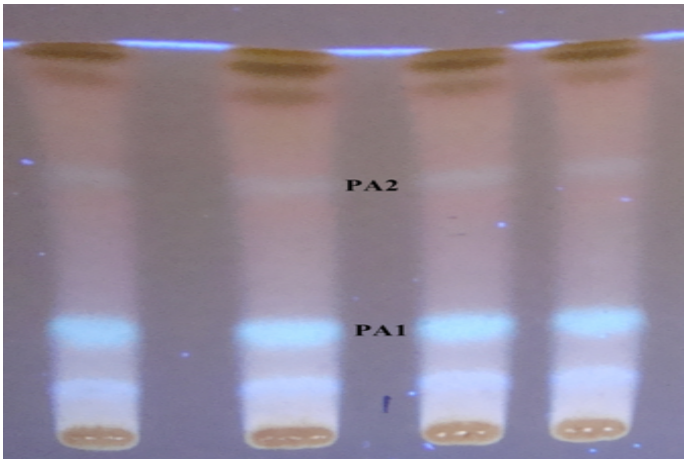


Fig. 2: TLC characterizations of bioactive fractions.

TLC Bioautography

The Bioautography of TLC plate revealed the presence of bioactive fractions as PA1 and PA2 both appeared as a white band against the violet background proving potent antimicrobial against multiple drug resistant *S. aureus* (Fig. 3).

Fig. 3: TLC Bioautography by agar overlay assay of bioactive fractions PA1 and PA2.

Disc Diffusion Assay

Strains of *Staphylococcus aureus, Salmonella typhi, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* were selected to study the inhibitory effect of Fractions extracted from *P. foetida* L. The antibacterial activity was analyzed by disc diffusion techniques of both fractions eluted from the PA1 and

Table. 2: Inhibitory effect of pure PA1 and PA2 fractions from *P. foetida* L. extracts.

S.No	Conc. of Passicol µg /ml	Zone of Inhibition (mm)							
		S. aureus		*S. typhi*		*K. pneumoniae*		*P. aeruginosa*	
		PA1	**PA2**	**PA1**	**PA2**	**PA1**	**PA2**	**PA1**	**PA2**
1	Control	3	3	3	3	3	3	---	---
2	0.5	12±0.16	12±0.16	13±0.16	12±0.16	**12**±0.16	11±0.24	---	---
3	1	12±0.16	12±0.16	11±0.24	12±0.16	12±0.16	12±0.16	---	---
4	2	12±0.16	12±0.16	12±0.16	12±0.16	12±0.16	12±0.16	---	---
5	3	14±0.08	13±0.16	12±0.16	**13**±0.16	12±0.16	11±0.24	---	---
6	4	14±0.08	**21**±0.08	**14**±0.08	13±0.16	12±0.16	11±0.24	6±0.24	6±0.24
7	5	**15**±0.08	11±0.24	14±0.08	13±0.16	11±0.24	**13**±0.16	6±0.24	6±0.24

[a] Values expressed as fractions concentration µg /ml.
[b] Values expressed as zone of inhibition in mm ± S.D

PA2 concentration, ranging from 0.5, 1, 2, 3, 4 and 5 µg/ml per disc. The PA2 was found to be significantly effective at *S. aureus* since the higher zone of inhibition (21 mm) was observed at 4 µg/ml concentration compared to zone (15 mm) at 5 µg/ml inPA1. PA1 inhibited slightly more (14 mm) at 4 µg/ml, compared to PA2 (13mm) at 3 µg/ml against *S. typhi.*

PA1 showed steady inhibition of *K. pneumoniae* from 0.5 to 4 µg/ml (12mm) concentration, which then lowered at higher concentrations. PA2 showed inhibition at the highest concentrations (13 mm). The same trend was observed for both types of fractions when tested against *P. aeruginosa*, where only higher concentration showed slight inhibition. The results are summarized in Table 2. The antibacterial potential of the extract of *P. foetida* L. was too largely due to polyacetylenic compounds (Nicolls, 1970; Nicolls *et al.,* 1973; Birner and Nicolls, 1973; Patil, 2010).

CONCLUSION

The proposed extraction procedure and compiled TLC data illustrate that quick and efficient extraction of bioactive fractions from *P. foetida* L. fractions characterization, followed by reliable UV analysis. The experiments can be performed on the large pool of samples to generate unique spectroscopic determination; which provides general overview on patterns of polyacetylene and their trends. The generated data could offer solid support for different applications, such as the use of these fractions in drug discovery, production and optimization of secondary metabolites, bioautography screening, and direct use in the pharmaceutical industry.

Present study concludes the importance of conventional knowledge of medicinal plants and their uses in common practices. The UV and TLC data could be proven as a prominent tool in validation and documentation of the customary treasure of herbal remedies. Moreover, the described solvent extraction procedure in this work can be used as a standard that is applicable to alike polyacetylenic compounds in related or other plant genera.

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