HPTLC Profile and Screening of Antimicrobial Activity of Pongamia Pinnata Pierre

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Authors’ contributions

This work was carried out in collaboration among all authors. Author RS performed study methodology and wrote the original draft of the manuscript. Author AR conceptualized the study and performed the formal analysis of the study. Author AK reviewed and edited the manuscript. All authors read and approved the final manuscript.

ABSTRACT

Aim: Plant secondary metabolites are the alternatives for the control of the antibiotic resistant pathogens which are highly infectious to human and plants. With respect to these aspects present work was carried out with phytochemical studies and antibacterial screening of Pongamia pinnata Pierre.

Materials: The Preliminary phytochemical screening and HPTLC studies were performed with methanolic extracts of seeds and callus. The extracts of the seeds and callus was developed in the mobile phase of toluene: ethyl acetate (90:10) using standard procedures and scanned under UV at 254 nm, 366 nm and visible light at 540 nm. Further, the extracts of seeds and callus were made in diethyl ether and methanol used for in vitro antibacterial activity. It was performed against multi drug resistance organisms such as Klebsiella pneumoniae, Escherichia coli, Bacillus subtilis and Staphylococcus aureus. The organic extracts at a concentrations of 0.02 mg/ml - 0.1 mg/ml were taken to study the inhibition properties.

Results: The Pongamia pinnata Pierre HPTLC fingerprint analysis from the extracts showed a wide range of secondary metabolites. The methanolic extracts of seed and callus showed significant inhibition zones.

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Conclusions: The study reveals the new antimicrobial agent in the form of Pongamia pinnata callus extracted with methanol will be definitely an alternative to synthetic drugs or antibiotic agents.

Keywords: Pongamia pinnata; antibacterial activity; callus; phytochemical screening HPTLC.

1. INTRODUCTION

Leguminosae (legumes) are the third largest plant family, with approximately 19,400 species [1] and they are found throughout the world in all biomes [2]. The family is economically very important being the major source of food and forage and its great diversity (the third largest family in flowering plants) has also attracted much interest in ecological as well as systematic studies [3].

Pongamia pinnata is one of the most well known species from Papilionoideae, sub family of Leguminosae. It is a medium sized glabrous tree popularly known as Karanja in Hindi, Indian Beech in English. It is originated in tropical and temperate Asian countries [4]. It is widely distributed throughout tropical Asia and the Seychelles Islands, South Eastern Asia, Australia, India and locally distributed throughout the State of Maharashtra (India) along the banks of rivers; very common near the sea-coast in tidal and beach-forests in Konkan and also along Deccan rivers [5]. The Sangwan et al. [6] reported about ecological importance of Pongamia pinnata that, is a preferred species for controlling soil erosion and binding sand dunes because of its dense network of lateral roots. Root, bark, leaves, flower and seeds of this plant also have medicinal properties and traditionally used as medicinal plants. Pongamia pinnata is reported to have anti hyperglycaemic [7-10] properties. Along with these medicinal values seeds of pongamia pinnata could be a good source of biodiesel as seeds possess 40% oil, which can be converted to biodiesel by transesterification method [11].

Keeping in view the great medicinal importance of the plant. The current work was undertaken to provide HPTLC fingerprinting profile for authentication of material used for herbal preparation and also investigates its antibacterial multidrug resistance species of bacteria.

2. MATERIALS AND METHODS

2.1 Collection of Plant Material

The seeds of Pongamia pinnata were collected in the month of May from the Parle village located at Satara district in Maharashtra. The seeds were separated from fruit. After washing with tap water, they were sun dried and stored in plastic bags.

2.2 Studies of HPTLC Profile of Pongamia pinnata Seed and Callus Extract

The seeds of Pongamia pinnata were dried in an oven for 30 minutes. After, grinding them to form a fine powder, it was stored in air tight containers. A silica pouch was kept in the containers to keep the material dry so as to prevent microbial contaminations. The air tight containers were kept in a refrigerator till further use.

The callus which was obtained with In vitro cultures of different explants of Pongamia pinnata like seeds and stem generate proliferation in callus when Murashige and Skoog [12] medium was supplemented with 2,4-D at 3mgL⁻¹. It was collected aseptically in the sterile petriplates. The plates were sealed with parafilm and stored in a refrigerator till further use.

2.3 Preparation of Extracts

The extracts were prepared by cold infusion method of Handa [13]. One gram of the sample (seed powder/callus) was macerated with 10ml of methanol. It was kept for 48 hours. After that, the solution was filtered and evaporated to dryness. Prior to spotting, the dry residue was dissolved in 1ml of solvent. Similarly for callus 1gm of the callus was macerated with 10 ml of methanol. It was kept for 48 hours.

2.4 Assay

The HPTLC analysis was carried out following the method of Wagner and Bradt [14]. For the present study, CAMAG HPTLC system equipped with Linomat V applicator, TLC scanner III, REPROMER III with 12 bit CCD camera for photo-documentation was used. The software used was WinCATS-4. HPTLC profile screening done using 1:10 ratio of solute to solvent used for the preparation of sample. The reconstituted samples were spotted in the form of the bands of 5mm width with a CAMAG microliter syringe on
pre-coated silica gel TLC plates. Each sample was loaded in two tracks with 10µl and 20µl quantity respectively. The seed oil of Pongamia pinnata was used as standard for comparative studies (Track 1 and 4: seed extract, Track 2 and 5: callus extract, Track 3 and 6: seed oil). The sample loaded plate was kept in TLC twin trough developing chamber with mobile phase (for terpenoids). The optimized chamber saturation time for mobile phase was 30 minutes at a temperature of 25+2°C. On the basis of preliminary TLC studies, the solvents systems were selected for the better separation of different constituents and to get good resolution of phytochemicals.

The solvent system or mobile phase selected for Pongamia pinnata was toluene: ethyl acetate (90:10). The developed plates were dried by hot air to evaporate solvents from the plate. The plates were photodocumented in visible light, UV light of 254nm and UV light of 366nm. The developed plates were sprayed with Anisaldehyde-Sulphuric acid reagent and dried at 100°C in hot air oven for 10 minutes. Finally, the plate was kept in Photo-documentation chamber (CAMAG REPROSTAR 3) and the images were captured under white light, UV light of 254nm and 366nm. Densitometric scanning was performed on CAMAG TLC scanner III which was operated by WinCATS software.

2.5 Studies of antimicrobial activities of Pongamia pinnata Seed and Callus Extracts

2.5.1 Extract preparation

The seed powder and callus of Pongamia pinnata was used to screen their antibacterial potential. Seeds were separated from the testa and were sun dried. The kernels of seeds were ground into fine powder. The callus obtained with in vitro callus studies was used. The extracts were prepared by cold infusion method, suggested by Handa [13]. Five gram of the seed powder was soaked in 50 ml of organic solvents. Organic solvents selected for the present study were methanol and diethyl ether at a concentration of 40%. It was kept for 48 hours. Similarly, five gram of callus was homogenized in minimum quantity of methanol/diethyl ether. The volume was made to 50 ml using respective solvents. After 48 hours the extracts were filtered using Whatman filter paper. The extracts were evaporated to dryness. Prior to use the residue were dissolved in known quantity of solvents (stock solutions) so as to maintain the concentration.

2.6 Bacterial Strains

The Gram-positive bacteria Bacillus subtilis, Staphylococcus aureus and Gram-negative bacteria Escherichia coli, Klebsiella pneumonia were selected because of their highly pathogenic nature. The required bacterial cultures were obtained from Department of Pharmacology, Bombay college of Pharmacy, Kalina, Santacruz, (Mumbai). These clinical isolates as pure cultures were maintained by regular sub culturing on nutrient agar slants. The bacteria were sub cultured 48 hours prior to use so as to get vigorously growing microorganisms.

2.7 Media

Media used for antibacterial studies was Mueller-Hinton’s [15] agar and the technique used was agar well diffusion [16]. The stock solution of methanol and diethyl ether extract made from seed powder and callus of Pongamia pinnata was used. The extracts were further diluted so as to get concentrations in the range of 0.02 mg/ml-0.1 mg/ml. Each culture suspension was prepared in saline by scraping vigorously growing bacteria from nutrient agar slants of pure culture. Optical density of 0.05 at 540 nm was maintained for all the cultures in order to get equal cell density. One ml of culture suspension was added in sterile molten agar butts of MH media, having approximate temperature of 45°C. The butts were mixed thoroughly and the media was poured into sterile petriplates. The plates were left undisturbed for media to set. Wells were punched with the help of sterile cork borer of 6mm diameter. Wells were filled with 20µl of different concentrations of each extracts. Plates were refrigerated for 10 minutes without shaking. After 10 minutes plates were transferred to an incubator, maintained at 37°C for 48 hours. After 48 hours zone of inhibition was observed and results were recorded by measuring the diameter in millimeter. Reported inhibition zones are the average values calculated from at three replicate. Observations Positive control, negative control and medium control were also maintained.

3. RESULTS AND DISCUSSIONS

3.1 HPTLC of P. Pinnata

3.1.1 Before derivatization

When the plate was observed under 254nm, several distinct bands were observed in Track 1
and 4 (methanolic seed extract) and Track 3 and 6 (seed oil) with two distinct blue fluorescent bands Fig.1. The banding pattern was similar in all the four tracks. Track 2 and 5 (methanolic callus extract) did not show any prominent bands. Similarly, when the plate was observed under 366nm, Track 1, 4 and 3, 6 showed many distinct bands with blue fluorescence Fig.2. However, under 366nm, weakly fluorescent bands were observed even in Track 2 and 5. Of the bands seen, three bands between Rf 0.36-0.71 (Table 1; Table 2; Table 3) were common in the tracks of all the samples. The intensity of the bands was more for Track 1 and 4 (seed extract) and 3 and 6 (seed oil), indicating higher concentration of the compounds.

3.1.2 After derivatization

When the plate was derivatized with Anisaldehyde-Sulphuric acid reagent, distinct green fluorescent banding pattern was seen in Track 1 and 4 (seed extract) and 3 and 6 (seed oil). As with the observation before derivatization, the banding pattern was similar for both the tracks. No distinct bands were seen in Track 2 and 5 (callus extract). Under 366nm, the banding pattern for the six tracks showed common bands between the Rf 0.36-0.71 (Fig.3). The bands in Track 2 and 5 (callus extract) were less intense than Track 1 and 4 (seed extract) and 3 and 6 (seed oil). When the plates were observed under visible light, very few bands were seen in all the tracks (Fig.4). However, one common band was observed in each track (at Rf 0.36). HPTLC densitograph of methanolic seed extract, methanolic callus extract and seed oil of Pongamia pinnata are depicted in Figs 5, 6 and 7 respectively.

The comparative HPTLC analysis of Pongamia pinnata showed that the seed oil and methanolic seed extract of P. pinnata showed similar banding pattern, but the callus methanolic extract differed from these samples. Though three bands were common between all three extracts, i.e. P. pinnata seed oil, methanolic callus and methanolic seed extract. This could be an indication of novel compounds being produced in the callus cultures in plant which is not produced under ex vitro conditions.

The phytochemical studies were reported by Chopade et al. [17] stated that there are six compounds (two sterols, three sterol derivatives a disaccharide) together with fatty acids (three saturated and five unsaturated) have been isolated from seeds. They have also stated that, Karangin, pongamol, pongagalabrone and pongapin, pinnatin and kanjone have been isolated from seeds. The findings can be correlated with HPTLC studies of methanolic extracts of callus and seed. The number of peak denoted might be due to the presence of such a secondary metabolites.

The synthesis of plant secondary metabolites if often linked to specialized cells and/or at specific developmental stages [18-19]. Therefore, undifferentiated tissue, such as callus, may not be capable of producing the secondary metabolites which are produced by specific cells in a plant. Due to this, a compound produced in ex vitro grown plants
may be absent in *in vitro* callus cultures. The terpenoids in the oil and seed extract of the plant is known to have a wide range of activities [20-23] with numerous compounds being isolated from the seed oil of the plant. However, the terpenoid compounds produced in the callus cultures have not been studied in detail. Further work on characterization of these compounds, unique to the callus cultures of each plant, must be carried out to understand possible applications of these compounds.

![Fig. 3. HPTLC of *Pongamia pinnata* after derivatization a. Under UV 366nm](image)

*Fig. 3. HPTLC of *Pongamia pinnata* after derivatization a. Under UV 366nm. Track 1 and 4: seed extract, Track 2 and 5: callus extract and Track 3 and 6: seed oil*

![Fig. 4. HPTLC of *Pongamia pinnata* after derivatization a. in visible light.](image)

**Table 1. HPTLC peak values of methanolic extract of *Pongamia pinnata* seed**

| Peak | Start Rf | Start Height | Max Rf | Max Height | Max % | End Rf | End Height | Area | Area % |
|------|----------|--------------|--------|------------|-------|--------|------------|------|--------|
| 1    | -0.04    | 8.8          | 0      | 662        | 14.78 | 0.05   | 113.9      | 15561.9 | 8.98   |
| 2    | 0.08     | 88.1         | 0.09   | 101.4      | 2.26  | 0.11   | 70.4       | 2241.2 | 1.29   |
| 3    | 0.13     | 109.2        | 0.16   | 389.2      | 8.69  | 0.2    | 92.2       | 12794.5 | 7.38   |
| 4    | 0.2      | 92.2         | 0.26   | 571.1      | 12.75 | 0.29   | 212.9      | 22895.4 | 13.21  |
| 5    | 0.29     | 212.9        | 0.3    | 248.8      | 5.55  | 0.32   | 181.6      | 4867.1  | 2.81   |
| 6    | 0.32     | 181.6        | 0.36   | 730.4      | 16.31 | 0.45   | 91.7       | 40357.9 | 23.28  |
| 7    | 0.45     | 91.7         | 0.47   | 109.3      | 2.44  | 0.52   | 62         | 4805.6  | 2.77   |
| 8    | 0.52     | 62           | 0.56   | 215.9      | 4.82  | 0.57   | 212.4      | 5827.8  | 3.36   |
| 9    | 0.57     | 212.4        | 0.61   | 597.3      | 13.33 | 0.66   | 188.3      | 26270.5 | 15.15  |
| 10   | 0.66     | 188.3        | 0.71   | 461.4      | 10.3  | 0.75   | 154.1      | 21690.6 | 12.51  |
| 11   | 0.75     | 154.1        | 0.79   | 246.2      | 5.5   | 0.83   | 117.1      | 11266   | 6.5    |
| 12   | 0.83     | 117.1        | 0.85   | 146.5      | 3.27  | 0.9    | 6.7        | 4775.9  | 2.75   |
Fig. 5. HPTLC densitograph of methanolic extract of *Pongamia pinnata* seed showing different peaks of phytoconstituents.

Fig. 6. HPTLC densitograph of methanolic extract of *Pongamia pinnata* callus showing different peaks of phytoconstituents.
Fig. 7. HPTLC densitograph of *Pongamia pinnata* seed oil showing different peaks of phytoconstituents

Table 2. HPTLC peak values of methanolic extract of *Pongamia pinnata* callus

| Peak | Start Rf | Start Height | Max Rf | Max Height | Max % | End Rf | End Height | Area | Area % |
|------|----------|--------------|--------|------------|-------|--------|------------|------|--------|
| 1    | -0.04    | 3.4          | 0      | 701.3      | 66.38 | 0.07   | 48.1       | 14009.6 | 50.17  |
| 2    | 0.14     | 20.2         | 0.16   | 31.9       | 3.02  | 0.19   | 23.2       | 936.5 | 3.35   |
| 3    | 0.21     | 27.8         | 0.25   | 49.9       | 4.73  | 0.29   | 14.2       | 1972.7 | 7.06   |
| 4    | 0.33     | 14.4         | 0.36   | 30.6       | 2.89  | 0.38   | 4.1        | 754.8  | 2.7    |
| 5    | 0.53     | 6.1          | 0.56   | 19.3       | 1.83  | 0.57   | 17.1       | 406.9  | 1.46   |
| 6    | 0.67     | 29.6         | 0.71   | 60         | 5.68  | 0.74   | 48.2       | 2498.7 | 8.95   |
| 7    | 0.79     | 60           | 0.85   | 163.5      | 15.48 | 0.9    | 6.5        | 7347.5 | 26.31  |

Table 3. HPTLC peak values of *Pongamia pinnata* seed oil

| Peak | Start Rf | Start Height | Max Rf | Max Height | Max % | End Rf | End Height | Area | Area % |
|------|----------|--------------|--------|------------|-------|--------|------------|------|--------|
| 1    | -0.02    | 4.1          | 0      | 421.9      | 10.33 | 0.01   | 159.7      | 3847.3 | 2.87   |
| 2    | 0.01     | 159.7        | 0.03   | 306        | 7.49  | 0.04   | 120.8      | 4308.7 | 3.21   |
| 3    | 0.04     | 120.8        | 0.05   | 130.1      | 3.18  | 0.06   | 74.2       | 2177.1 | 1.62   |
| 4    | 0.06     | 74.2         | 0.08   | 122        | 2.99  | 0.11   | 56.2       | 2873.1 | 2.14   |
| 5    | 0.11     | 55.8         | 0.16   | 336.6      | 8.24  | 0.19   | 62.7       | 11115.1 | 8.28   |
| 6    | 0.19     | 62.7         | 0.22   | 284.8      | 6.97  | 0.23   | 267.3      | 6325.5 | 4.71   |
| 7    | 0.23     | 267.3        | 0.26   | 516.5      | 12.65 | 0.29   | 173.2      | 15383  | 11.46  |
| 8    | 0.29     | 173.2        | 0.3    | 201.4      | 4.93  | 0.31   | 180.4      | 3384.2 | 2.52   |
| 9    | 0.31     | 180.4        | 0.36   | 689.7      | 16.89 | 0.45   | 71.2       | 36556.4 | 27.23  |
| 10   | 0.45     | 71.2         | 0.46   | 85.5       | 2.09  | 0.51   | 48.9       | 3091.6 | 2.3    |
| 11   | 0.52     | 50.1         | 0.6    | 405.3      | 9.92  | 0.64   | 153.6      | 19399.9 | 14.45  |
| 12   | 0.64     | 153.6        | 0.68   | 348.9      | 8.54  | 0.73   | 84.2       | 14591.6 | 10.87  |
| 13   | 0.73     | 84.2         | 0.75   | 93.7       | 2.29  | 0.78   | 76.3       | 3215.4  | 2.4    |
| 14   | 0.78     | 77           | 0.84   | 141.8      | 3.47  | 0.89   | 9.2        | 7974   | 5.94   |
3.2 Antimicrobial Activity of *Pongamia pinnata* Pierre

The methanol extract from seed powder of *Pongamia pinnata* found to inhibit the growth of all the pathogens at 0.06mg/ml concentration. At 0.1mg/ml concentration *E. coli*, *K. pneumoniae* and *B. subtilis* were found to be inhibited maximum with 18mm,(Fig.8) 18mm and 20mm(Fig.9) zone of inhibition respectively. It was observed that, *S. aureus* was more tolerant as at 0.1mg/ml concentration of methanolic extract as the zone of inhibition was only 13mm. The extract of *P. pinnata* seed powder in diethyl ether does not show any antimicrobial activity for the selected pathogens at any of the concentrations tested (Table 4). The result obtained for *P. pinnata* can be correlated with the report by Kumar et al. [24] which states that ethanolic extract of *Pongamia pinnata* seeds exhibited moderate antimicrobial activity against acne inducing bacteria such as *Propionibacterium acnes* and *Staphylococcus epidermis*.

Table 4. Effect of *Pongamia pinnata* seed extract on Gram positive and Gram negative bacteria

| Extracts       | Conc. (mg/ml) | Zone of inhibition (mm) | Gram negative | Gram positive |
|----------------|---------------|-------------------------|---------------|---------------|
|                |               |                         | *E. coli*     | *K. pneumoniae* | *B. subtilis* | *S. aureus* |
| Methanol       | 0.02          | Nil                     | Nil           | Nil           |
|                | 0.04          | 9                       | Nil           | Nil           |
|                | 0.06          | 12                      | 10            | 9             |
|                | 0.08          | 13                      | 12            | 10            | 9            |
|                | 0.1           | 18                      | 18            | 20            | 13           |
| Diethyl Ether  | 0.02          | Nil                     | Nil           | Nil           |
|                | 0.04          | Nil                     | Nil           | Nil           |

Fig. 8. Effect of methanolic seed extract of *P. pinnata* on *E. Coli*
Fig. 9. Effect of methanolic seed extract of *P. pinnata* on *B. Subtilis*

Fig. 10. Effect of methanolic callus extract of *P. pinnata* on *B. subtilis*

Table 5. Effect of *Pongamia pinnata* callus extract on Gram positive and Gram negative bacteria

| Extracts      | Conc. (mg/ml) | Zone of inhibition (mm) | Gram negative | Gram positive |
|---------------|---------------|-------------------------|---------------|---------------|
|               |               |                         | *E. coli*     | *K. pneumoniae* | *B. subtilis* | *S. aureus* |   |
| Methanol      | 0.02          | 9                       | Nil           | 10            | Nil           |            |   |
|               | 0.04          | 10                      | Nil           | 11            | Nil           |            |   |
|               | 0.06          | 10                      | 11            | 11            | Nil           |            |   |
|               | 0.08          | 14                      | 13            | 12            | 13            |            |   |
|               | 0.1           | 15                      | 15            | 20            | 26            |            |   |
| Diethyl Ether | 0.02          | Nil                     | Nil           | Nil           | Nil           |            |   |
|               | 0.04          | Nil                     | Nil           | Nil           | Nil           |            |   |
|               | 0.06          | Nil                     | Nil           | Nil           | Nil           |            |   |
|               | 0.08          | Nil                     | Nil           | Nil           | Nil           |            |   |
|               | 0.1           | 10                      | Nil           | 10            | 12            |            |   |

Nil: No inhibition

Taraquzzman et al. [25] observed that there was no significant activity with methanol extract of *Pterospermum semisagittatum* against gram positive and gram negative bacterial cultures. Contrary to this there are several reports suggesting the methanol extracts to have antibacterial properties. Kachhawa et al. [26] reported that *Pterocarpus marsupium*, a member of family Leguminosae, showed a potent antibacterial activity with methanolic extract...
against *E. coli*. Kamraj et al. [27] reported significant antimicrobial activity of *Acacia karroo* with methanolic extract. *Pongamia pinnata*, methanolic extracts of seed as well as callus had shown a potent antibacterial activity against gram negative and gram positive bacteria. Hence, seeds and callus of both the plants can be a good source of antimicrobial agent.

4. CONCLUSIONS

The seeds of *Pongamia pinnata* is a wide source of secondary metabolites as HPTLC profile had shown different bands representing different biomolecules. Also callus can be an alternative source for production of secondary metabolites. The studies in terms of qualitative and quantitative analysis need to be done. The methanolic extracts of seed and callus showed significant inhibition zones. The study reveals the new antimicrobial agent in the form of *Pongamia pinnata* callus extracted with methanol will be definitely an alternative to synthetic drugs or antibiotic agents.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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