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Full Length Research Paper

Study on cow urine and *Pongamia pinnata* Linn seed in farmyard: A natural, cost effective, ecofriendly remedy to bacterial leaf blight (BLB) of paddy

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Bacterial leaf blight (BLB) of paddy is caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo), which leads to yield loss from 6 to 60%. Though chemicals and bactericides control BLB, they also cause several health and environmental hazards. Therefore, an alternative, cost effective, eco-friendly pest and pathogen control is the need of the hour. In the present investigation, cow urine extract, hexane, chloroform, ethyl acetate, alcohol, methanol and aqueous fractions of *Pongamia pinnata* Linn seed were tested against *X. oryzae* pv. *oryzae* for its antibacterial activity. Streptomycin sulphate (30 µg) and dimethyl sulfoxide (DMSO, 15 µL) are used as positive and negative control. All the extracts and fractions were effective and showed 10 to 13 mm zone of inhibition. Phytochemical analysis also showed the presence of terpenoids, quinine, coumarin, tannin and phenol, with flavonoid available in higher quantity (1.56 mgkg^{-1}).

Key words: Cow urine, Crude extract, Organic fraction, *Xanthomonas*, Zone of inhibition.

INTRODUCTION

Pongamia pinnata L. is a medium-sized evergreen or briefly deciduous, drought resistant, glabrous shrub or tree 15 to 25 m high. The *P. pinnata* seeds contain *pongam* oil which is bitter, red brown, thick, non-drying, non edible, 27 to 36% by weight, and used for tanning leather, soap, as a liniment to treat scabies, herpes, rheumatism and as an illuminating oil (Brijesh et al., 2006). *Pongamia* cake prepared from seed is used as 'green manure' as it is rich in protein and nitrogen; it contains 4.0% N, 1.0% P, and 1.0% K, and enhances the soil fertility, nutrient availability and yield of crops. *P. pinnata* seed have antifeedent and also antibacterial activity (Meshram, 2010).

The bacterial leaf blight (BLB) which is caused by *Xanthomonas oryzae* pv *oryzae* is one of the drastic disease affecting paddy field all over the world, causing 6 to 60% yield loss. Synthetic organic bactericides such as nickel dimethyl dithiocarbamate, dithianone, phenazine and phenazine N-oxide (Gnanamanickam et al., 1999) are used regularly in careful crop management (Dobermann, 2004; Sehgal et al., 2001 and Lyndon et al., 2010). The use of resistant cultivars and seed treatments (David et al., 2003) like dipping rice seedlings in antibiotic solution during transplantation (Fátima et al., 2010; Vijay Krishna Kumar, 2010) are also used frequently. Spraying techlofthalam readily inhibits *X. oryzae* pv. *oryzae* in rice

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plants (Gnanamanickam et al., 1999 and Nelson et al., 2001). However, the incessant and indiscriminate application of chemicals causes several health and environmental hazards. Moreover, killing of non target flora, fauna, pest resurgence, acquired resistance in plant pathogen and residual toxicity in animals and humans are the additional undesirable factors of chemicals (Madhiazhagan et al., 2002). Mitigation of all these problems necessitated the search for an alternate means of cost- effective, eco-friendly pest and pathogen control.

In such a scenario, herbs (botanicals) and bio-pesticides have emerged as viable alternatives endowed with potential bactericidal, fungicidal and viridical properties (Hrishikesh et al., 2012; Uradangarin et al., 1999). Similarly, dung and urine of cattle restrict pests, insects and plant pathogens (Raja and Kurucheve, 1997). In rural areas, bitter tasted and obnoxiously odoured plant leaves are soaked in cow urine in an earthen pot and sprayed to control plant pathogen, pests and insects. In order to scientifically validate this claim, the present investigation was carried out. The seeds of *P. pinnata* were extracted in cow urine and assayed for its antibacterial activity against *X. oryzae* pv. *oryzae.*

MATERIALS AND METHODS

Isolation and identification of plant pathogen

Bacterial leaf blight (BLB) infected paddy leaves were collected from the Plant Pathology Department of Agriculture College, Ramji Nagar, Trichy, in zip locks cover and transported to the laboratory within 2 h. Leaves were successively surface-sterilized with distilled water and 0.1% mercuric chloride. Plant pathogen was isolated from infected area by standard techniques using nutrient agar and selective medium. Characteristic colonies were subjected to standard biochemical screening.

Selection of medicinal plant

P. pinnata seeds were collected from an herbal shop in Trichy and authenticated by the herbal research division of Srimad Andavan Arts and Science College, Tiruchirappalli, Tamil Nadu, South India. Shade dried seeds were crushed by electrical blender (Butterfly Mixer–Grinder-Indian Made) and used throughout the study.

Organic solvent extraction

100 g of coarse powder of *P. pinnata* seeds were successively extracted in room temperature with hexane, chloroform, ethyl acetate, alcohol, methanol, alcohol and water based on increasing polarity. Duration of incubation was 3 days at each solvent. The extracts were collected, evaporated on a water bath without shaking at atmospheric pressure and the solvents were completely removed *in vacuo* and stored at 4°C for further use.

Cow urine extraction

Briefly, 3 kg of *P. pinnata* seeds were surface-sterilized with sterile distilled water and 0.1% mercuric chloride. The crushed seeds were placed in an earthen pot filled with 10 L of fresh cow-urine collected from a single cow fed with the same type of feed throughout the study. The pot was incubated for 20 days in a pit dug in the soil. At every 24 h intervals, 500 ml of crude extract was collected for 20 days and condensed into a dry powder using hot air oven at 40°C without shaking. In rural areas of south India, traditionally this method has been followed to prepare the extract and incubation of pot inside the soil providing ambient temperature for fermentation which enhances the extraction.

Antibacterial assay

Antibacterial activity of different forms of cow urine, organic fractions and cow urine extract of *P. pinnata* seeds were assayed using the well diffusion method (Perez et al.,1990). Petri plate containing 20 ml of nutrient agar medium was seeded with 18 h old culture of bacterial strain isolate. To analyze whether the cow urine itself has any effect on pathogen, condensed cow urine (10 ml of fresh cow urine was condensed into 1 ml using water bath), fresh cow urine, chloroform extract of both fresh and incubated cow urine (20 days in earthen pot) was tested on the pathogen. Different concentrations like 5, 10 and 15 µL were added in the well and incubated for 24 h at 37°C. The extracts and organic fractions were dissolved in dimethyl sulfoxide (DMSO) and sterilized by using Sartorious syringe filter of pore size 0.22 µM (stock solution (0.04 g/1 ml)). Various concentrations of the extracts (400, 800, 1200 and 1600 µg) were added into 6 mm diameter well. Streptomycin sulphate (30 µg) and DMSO (15 µl) were used as positive and negative control. Incubation was made at 37°C for 24 h. Antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well using Hi-Media standard scale.

Phytochemical analysis

All the extracts were subjected to preliminary phytochemical screening as per the standard method (Harborne, 1984).

Separation of active compounds

Cow urine extracts which showed maximum bactericidal activity was subjected to standard separation procedure. Briefly, 30 g of crude cow urine extract was packed in the column made up of silica gel (Acme's mesh: 60-120µ Hi-media). Hexane, chloroform, ethyl acetate, methanol, alcohol and water were used in different proportions in the order of increasing polarity. All the fractions obtained were purified by thin layer chromatography (TLC) using readymade silica gel plates (Merck- TLC Silica gel 60 F_{254}) and assayed for antibacterial activity by the standard method. The fraction obtained in ethyl acetate: methanol (20:80) showed maximum antibacterial activity. Hence this fraction was subjected to HPLC and FT-IR spectral analysis to elucidate the structure of the active principle.

RESULTS

The pathogen isolated from BLB infected paddy leaves was identified as *X. oryzae* pv. *oryzae* based on standard morphological and biochemical screening (John et al.,1994). Successive cold extractions of coarse powder of *P. pinnata* seeds revealed that the extractive values in hexane and chloroform fractions were little more (4%) when compared to water (3%). Higher hexane and chloroform extractive values indicated the presence of non polar chemical constituents, while the aqueous

Table 1. Extraction value of *Pongamia pinnata* seed in different organic solvents.

S/N	Solvent	Volume of solvent added (ml)	Powder taken (g)	Incubation (days)	Volume of solvent collection (ml)	Wet weight (g)	Final dry weight (g)
1	Hexane	300	100	3	280	95.2	4.5
2	Chloroform	285	95.2	3	270	85	3.9
3	Ethyl acetate	275	85	3	260	79	3.5
4	Methanol	270	79	3	250	70	3.2
5	Alcohol	265	70	3	240	65	3.0
6	Water	260	65	3	250	60	2.5

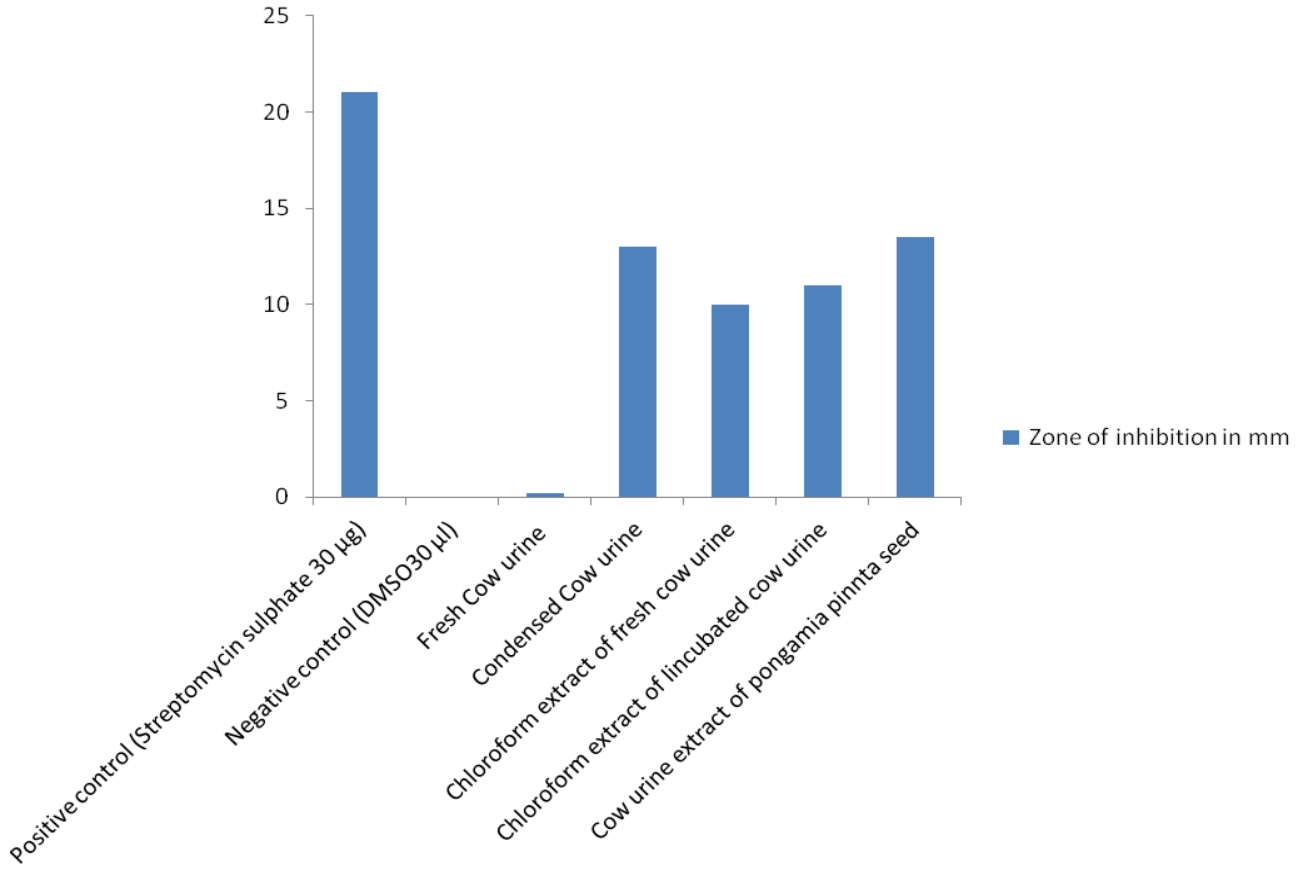


Figure 1. Antibacterial activity of Cow urine under different condition and cow urine extract of *P. pinnata* seed on *Xanthomonas oryzae* pv. *oryzae*

extractive values revealed the presence of high polar constituents (Table 1).

The bactericidal effect of fresh cow urine, condensed cow urine, incubated cow urine and their chloroform extracts are given in Figure 1. Among various samples, condensed cow urine exhibited maximum zone of inhibition (13 mm) followed by chloroform extract. Incubated cow urine is also effective. On the other-hand, chloroform extract of fresh and incubated cow urine (20 days) produced only 10 and 11 mm respectively. Among the various fractions, methanol fraction exhibited 12.33 mm at 1600 µg, while all other organic fractions showed more or less similar inhibition to bacterial growth (10 mm) (Table 2). The antibacterial activity of cow urine extracts of *P. pinnata* seed and organic solvent fractions were assayed against the phytopathogen *X. oryzae* pv. *oryzae.* Results indicate that the antibacterial activity of *P. pinnata* seed incubated in cow urine was analyzed at different concentration and different days of incubation (1, 3, 10, 11 and 20 days). Extracts were not effective even at the highest concentration. Only after 6 days was appreciable activity found. Maximum zone of inhibition

Table 2. Antibacterial activity of organic fraction of *Pongamia pinnata* seed against *Xanthomonas oryzae* pv *oryzae*.

Name of the fraction	Concentration of extract in µg/zone of inhibition in mm					
	400 µg	800 µg	1200 µg	1600 µg	Positive control	Negative control
Hexane	10	10	10	10	21.00	-
Chloroform	-	10	10	10.5	21.00	-
Ethyl acetate	10	10.66	10	10	21.00	-
Methanol	11.66	11.66	10.66	12.33	21.00	-
Alcohol	10	10	10	10	21.00	-
Aqueous	10	10	10	10	21.00	-

Table 3. Antibacterial activity of cow urine extract of *Pongamia pinnata* seed against *Xanthomonas oryzae* pv *oryzae*.

Days of incubation	Concentration of extract in µg/zone of inhibition in mm					
	400 µg	800 µg	1200 µg	1600 µg	Positive control	Negative control
1	-	-	-	-	21.00	-
2	-	-	-	-	21.00	-
3	-	-	-	-	21.00	-
4	10	10	10	10	21.00	-
5	-	10	10	10	21.00	-
6	10	10	10.5	13.5	21.00	-
7	10	11	10.5	11	21.00	-
8	-	-	-	-	21.00	-
9	10	-	11	11.66	21.00	-
10	-	-	-	-	21.00	-
11	-	-	-	-	21.00	-
12	-	-	10	10	21.00	-
13	-	-	-	10	21.00	-
14	10	10	10.33	10	21.00	-
15	-	-	10	10	21.00	-
16	-	10	10	10	21.00	-
17	-	-	10	10	21.00	-
18	10	10	10	10.66	21.00	-
19	10	10	10	10	21.00	-
20	-	-	-	-	21.00	-

was observed in 1600 µg of the extract (13.5 mm). The positive control streptomycin sulphate showed 21.00 mm at 30 µg concentration (Table 3). When the antibacterial activity of all the extract and fractions were compared, cow urine extracts was showed to be highly effective in controlling the pathogen (13.5 mm) followed by incubated condensed cow urine (13 mm), methanol extract (12.33 mm) and the aqueous extract (10mm) (Figure 2).

Preliminary phytochemical analysis of *P. pinnata* seeds were performed for both cow urine and organic solvent fractions (Table 4). Secondary metabolites like terpenoids, flavonoid, quinine, coumarin, tannin and phenol were found in all the 20 days extracts. In organic solvent fractions, ethyl acetate extract showed positive result for terpenoids, methanol extract showed the positive result for terpenoids and phenol, while alcohol extract showed the positive result for flavonoid, terpenoids and phenol. Even though aqueous extract showed positive for most of the secondary metabolites, it produced the lowest zone of inhibition. All organic fractions were effective in controlling the *X. oryzae* pv *oryzae.* HPLC result of the crude cow urine extract of *P. pinnata* seeds revealed the presence of flavonoids (1.56 mg kg^{-1}), quinine (0.06 mg kg^{-1}), terpenoids (0.16 mg kg^{-1}), tannins (0.32 mg kg^{-1}), coumarin (0.05 mg kg^{-1}) and phenol (0.49 mg kg^{-1}).The crude cow urine extract of *P. pinnata* seed showed higher quantity of flavonoids. This extract which gives maximum antibacterial activity was due to flavonoid (I) (Figure 3) as shown by the FTIR results. In the FTIR spectrum of cow urine extract of *P. pinnata* seed, a broad absorption at 3404 cm^{-1} due to NH and O-H stretching frequency while at 2358 and 2089 cm^{-1} the C=N stretching vibrations were found. The frequencies at 1389 cm^{-1} were assigned to C-N bending,

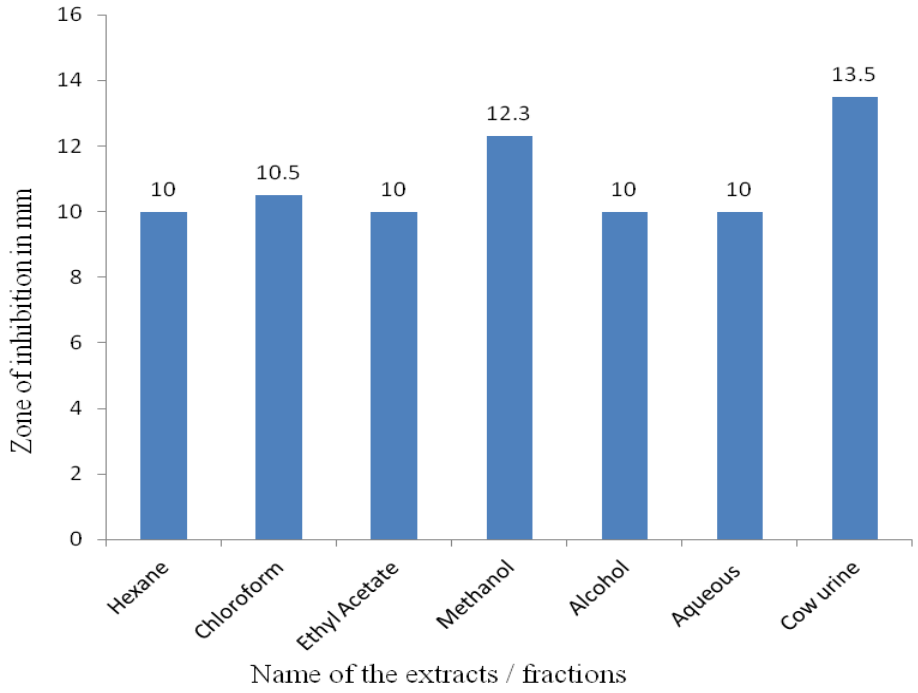


Figure 2. Comparative analysis of antibacterial activity of fresh cow urine extract and organic solvents fractions of *Pongamia pinnata* seed on *Xanthomonas oryzae pv. oryzae* at 1600 mg concentration

Table 4. Preliminary phytochemical analysis of cow urine extracts of *Pongamia pinnata* seed.

S/N	Test	Cow urine extract of *Pongamia pinnata* seed																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1.	Terpenoids	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2.	Flavonoid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3.	Sugar	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4.	Alkaloid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5.	Quinine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6.	Coumarin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7.	Tannin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8.	Saponin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9.	Glycosides	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	Anthraquinones	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	Phenol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

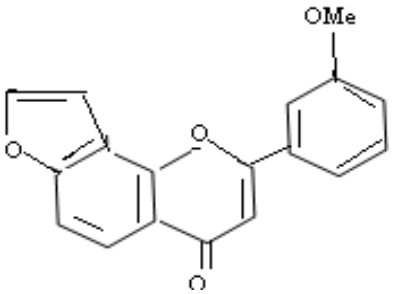
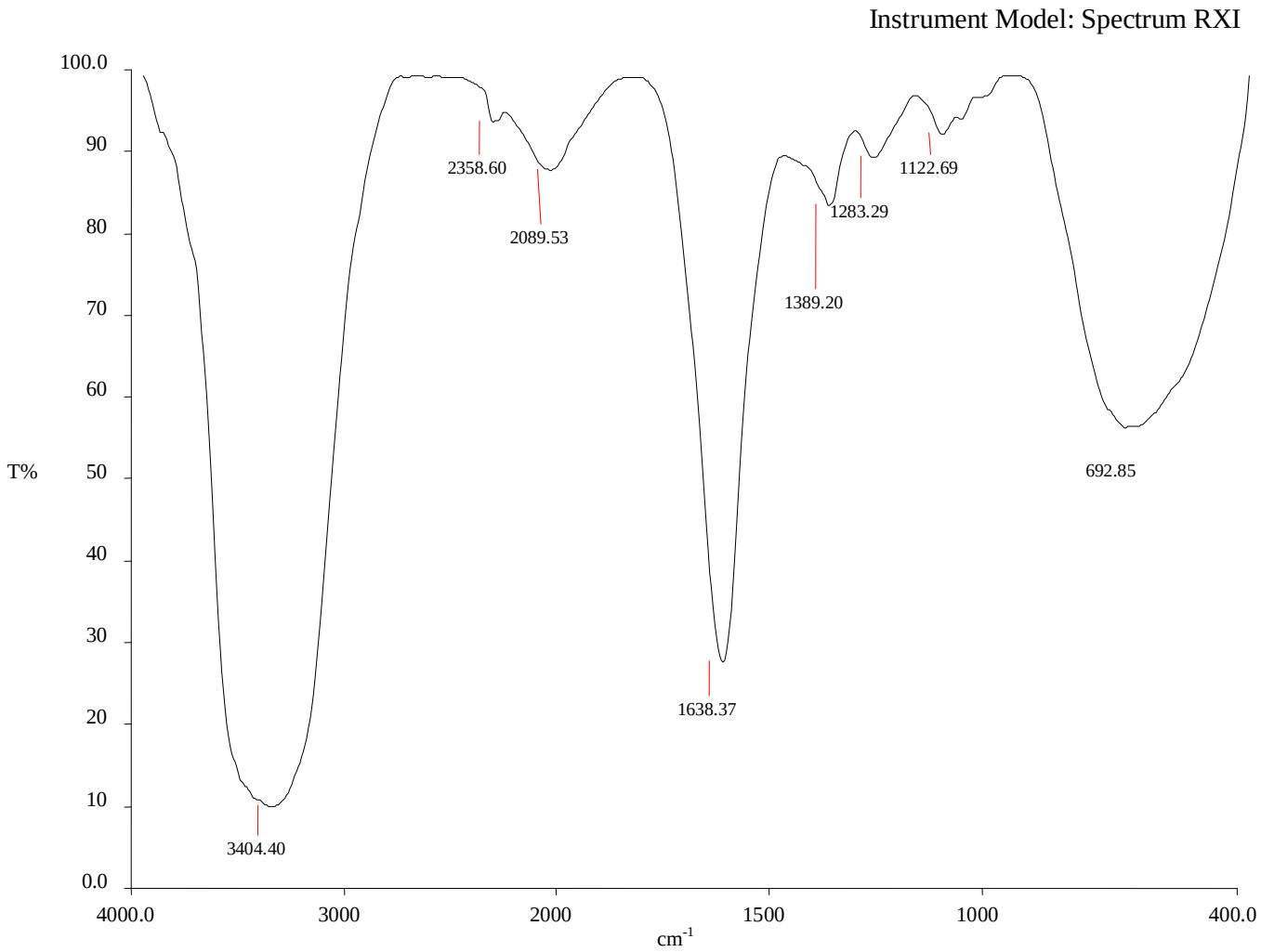


Figure 3. Structure of flavonoid (I).

while at 1638 cm^{-1}, stretching vibrations were observed for the C-N group. The presence of a small stretching vibration at 1122 cm^{-1} may be due to C-O and at 1289 cm^{-1} maybe due to C=C of aromatic rings (Figure 4).

In *P. pinnata* aqueous extract, the stretching vibration at 3429 cm^{-1} can be assigned to N-H vibration and intra-molecularly H-bonded O-H group. The intense peak at 2360 cm^{-1} indicates the presence of C=N group and at 2119 and 1386 cm^{-1} may also be assigned to this group. The well defined wedge pattern at 1115 and1076 may be assigned to C-O stretch that resonates. Presence of



PSCE.sp 3601 4000.00 400.00 10.08 100.00 4.00 %T 5 0.50

REF 4000 99.35 2000 91.26 600
3404.40 10.08 2358.60 93.71 2089.53 87.90 1638.37 27.69 1389.20 83.51
1283.29 89.40 1122.69 92.23 692.85 56.48

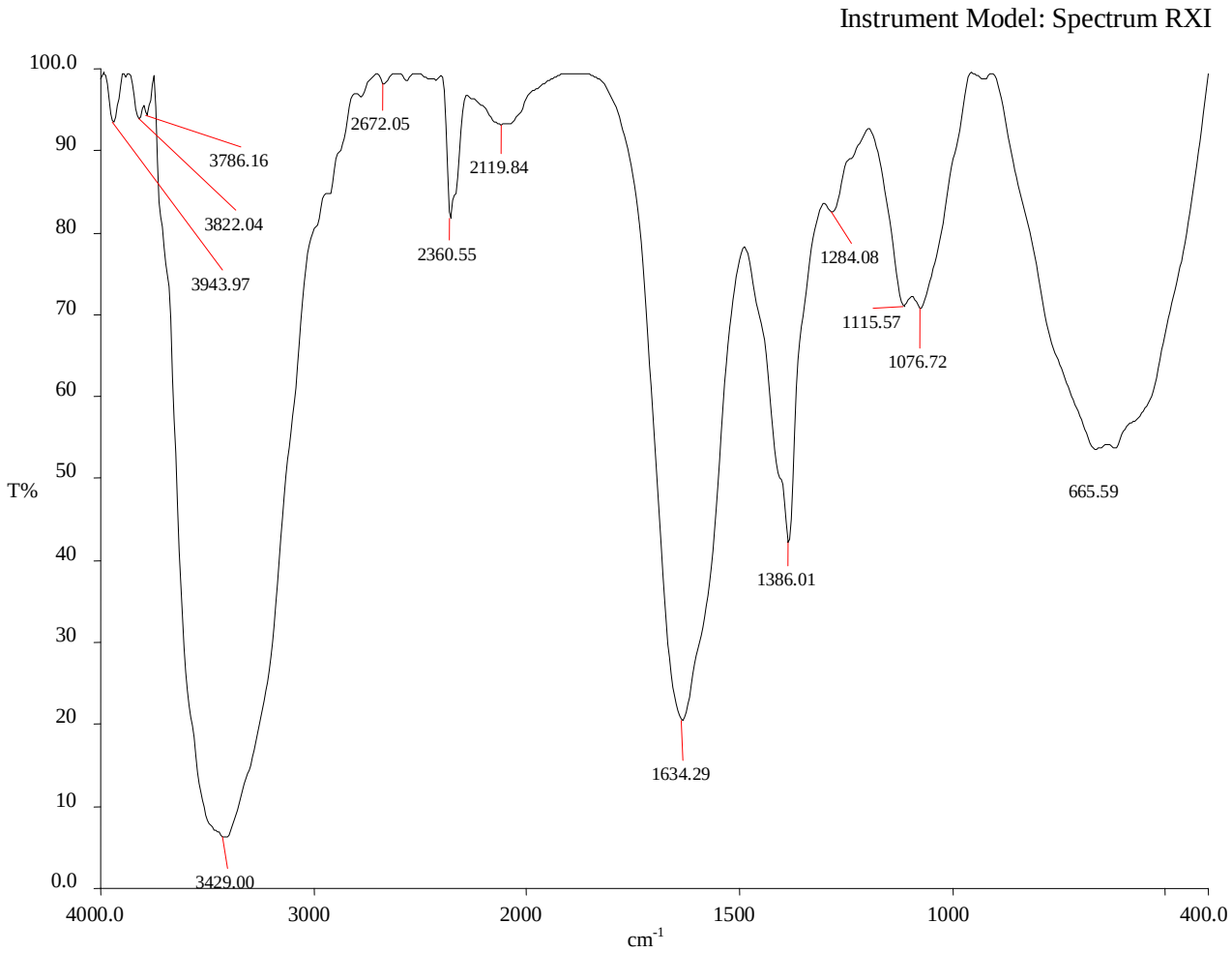
Figure 4. FT-IR spectrum of alcohol: water (70:30) fraction of *Pongamia pinnata* seed incubated in cow urine.

several stretching vibrations clustered at 665 cm^{-1} may be ascribed to p- disubstituted benzene ring and C-H bending vibrations (Figure 5). The aqueous extract was rich in quinine and coumarin, while the cow urine extract contained flavonoid, quinine, coumarin and tannin in addition to other components (Table 4) as shown in the reaction:

$$\text{I} + \text{Cow urine} \xrightarrow[\text{Incubation}]{\text{20 days}} \text{Phenol+ benzoic acid + diethylphthalate + terpenoids + quinine}$$

Phenol and benzoic acid are well known antibacterial agents. The cow urine extract contains urea, uric acid and creatinine from cow urine besides the degraded products phenol, benzoic acid etc from *P. pinnata* seeds.

The enhanced zone of inhibition observed with the cow urine extract may be due to the synergistic activity of all these active compounds.



PSWE.sp 3601 4000.00 400.00 6.32 99.78 4.00 %T 5 1.00

REF 4000 98.82 2000 96.60 600
3943.97 93.45 3822.04 93.91 3786.16 94.26 3429.00 6.32 2672.05 98.10
2360.55 81.82 2119.84 93.17 1634.29 20.58 1386.01 42.22 1284.08 82.46
1115.57 71.07 1076.72 70.77 665.59 53.58

Figure 5. FT-IR spectrum of water fraction of *Pongamia pinnata* seed.

DISCUSSION

BLB is the most serious disease of rice in South-East Asia, particularly since the widespread cultivation of dwarf high-yielding cultivars. Our results on the mode of action of cow urine and *P. pinnata* seeds extract have established strong antagonism towards the bacterial leaf blight causing microorganism *X. oryzae* pv. *oryzae* through mechanisms such as antibiosis. In our present investigation, cow urine and *P. pinnata* seed extracts showed maximum antimicrobial activity. A novel pharmaceutical composition present in cow urine distillate has been patented (Arunkumar et al., 2010). A recent work on cow urine distillate was shown to effectively control both bacteria and fungi at 15 µL concentration (Khanuja, 2002). Cow urine distillate has immuno-modulatory activity in Broiler chickens (Jojo et al., 2011).

The phytochemical investigation of *P. pinnata* also indicated the presence of abundant prenylated flavonoids such as furanoflavones, furanoflavonols, chromeno-flavones, furanochalcones and pyranochalcones (Yadav et al., 2004; Yin et al., 2005). The seeds contain a flavone derivative 'pongol'. The structures of glybanchal-cone, isopongachromene, karangin and pongal of *P. pinnata* were elucidated (Shameel et al., 1996), which have antimicrobial activity. The leaves, flowers, seeds

and stem bark of *P. pinnata* are known to have karanjin, a furanoflavanoid (Prabhu et al., 2002; Meera et al., 2003; Kokate et al., 1990).

The quantitative analysis and FTIR report discovered some important compounds of flavonoid, quinine, coumarin and tannin in addition to other components. But in our investigation, antimicrobial activity of cow urine extract may be due to the products formed from the flavonoid (I) present in the *P. pinnata* seeds during incubation. Enhanced zone of inhibition observed with the cow urine extract may be due to the synergistic activity of all these active compounds. Therefore, instead of using hazardous chemicals to control BLB, it is advisable to shift our farming from chemicals to these types of natural cost effective eco-friendly formulations.

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