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Preliminary Studies of Phenolic and Flavanoid Content in Leaf Galls and Leaves of *Pongamia pinnata* (L.) Pierre

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Abstract: Plants have been forming galls to ward off the attacks of bacteria, fungi, mistletoe, mites, nematodes, viruses, and insects. Although most galls probably do not harm the host too much, they do involve some form of parasitism and generally do not benefit the host. In the present paper galls found on the leaves of *Pongamia pinnata* were examined. Investigations were carried out for the presence of flavanoids and phenolics and compared with the ungalled leaf tissues.

Key Words: Leaf galls, HPTLC, Flavanoids, Phenolics

Introduction

Galls are abnormal plant growths caused by various organisms' insects, mites, nematodes, fungi, bacteria, and viruses for the feeding or egg-laying activity. They are plant tissue which is controlled by the insect. Galls act as both the habitat and food source for the maker of the gall. The interior of a gall can contain edible nutritious starch and other tissues. Some galls act as "physiologic sinks", concentrating resources in the gall from the surrounding plant parts. Galls may also provide the insect with physical protection from predators.

It is common to see galls on the leaves of *Pongamia pinnata* (L) Pierre, the tree which has multiple uses. Commonly known as Karanj, its seeds yield oil which is useful in making soaps, leaves are used as poultice and being a leguminous plant it also improves fertility of the soil. Insect galls are usually induced by chemicals injected by the larvae or the adults of the insects into the plants, and possibly mechanical damage. After the galls are formed, the larvae develop inside until fully grown, when they leave. The meristems, where plant cell division occurs, are the usual sites of galls, though insect galls can be found on other parts of the plant, such as the leaves, stalks, branches, buds, roots, and even flowers and fruits. Gall-inducing insects are usually species-specific and sometimes tissue-specific on the plants they gall. Since insects derive their nutrition from gall tissue, the gall becomes a sink for different nutrients and energy that will be vital for the insect's growth (Raman, A 2003). A majority of gall-inducing insects stimulate the host-plant tissue to develop into galls by their feeding action, whereas species of Hymenoptera trigger gall development via oviposition. Even the vascular tissues can be modified by gall induction, so that they supply nutrients and water subserving the needs of the inducing insect (Mani M S 1974). Some of the gall-inducing gall midges and cynipids show an unusual ability to induce differentiated tissues to revert to a meristematic state and resume cell-division activity

In this paper the leaf galls on leaves of *Pongamia pinnata were* examined for their morphology and microscopic characteristics. Investigations were carried out to find out the changes in the flavanoids and phenolics in the galls, galled leaf and ungalled leaf tissues. Biochemical analyses were done using standard analytical techniques.

Materials and Methods

The leaf galls and leaves of *Pongamia pinnata,* were collected from Sanjay Gandhi national park and Tungareshwar during June and July after the onset of monsoon. The galls were preserved in FAA [formalin-alcohocol-glacial acetic acid]. Section of galls were taken to study the anatomical

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characteristics. The plant materials were sorted as galls, galled leaf and ungalled leaf. The samples were taken for standard extraction procedure for analysis of phenolic and flavonoids compounds.

***Pongamia pinnata* (L) Pierre**

The colour of the gall varies from green to yellowish-green to brownish-green. The surface of the gall is glabrous. Galls are epiphyllous, pouch-gall usually stalked, hard unilocular and indehiscent. Ostiole is minute and hypophyllous and obstructed by the downwardly directed erineal hairs. Gall cavity is large with numerous irregularly projecting smaller or larger fleshy emergences, simple, multicellular and downwardly erineal hairs. The gall is 5-10mm long and 1-2mm thick at base and 5 mm thick apically.

Epidermis is single layered followed by thin walled parenchymatous cells. 17-20 vascular bundles are present between the parenchymatous layer and sclerenchymatous layers. A large number of short or long multicellular erineal hair linning the inner cavity. Coloured cells are also present which may be either tannin or oil.

Preliminary Tests for Phenolic acids and flavanoid

Fresh leaf extract was added to fresh aqueous mixture of 1% $FeCl_3$ and 1% potassium feriicyanide which gave purple colouration indicating presence of phenolic acids.

***Shenoda test**:* Fresh leaf extract was added to zinc and concentrated H_2SO_4 which gave pink colouration indicating presence of flavonoids.

Thin Layer Chromatography for Phenolic Compounds

10gm of fresh leaf samples were taken into 25ml of methanol for extraction and were kept on shaker for 24 hours. Then the extract was filtered. Acid hydrolysis was carried out with 2M HCl for 30 mins. The resultant solution was cooled and filtered before extraction. To this ether was added and the ether extract was washed, dried and evaporated to dryness. The residue was dissolved in ether and then chromatographed by 2D TLC on silica gel plates. The solvent systems used was Acetic acid: Chloroform (1:9) and Ethyl acetate: benzene (9:11). The spots were visualized using Folin-Ciocalteu reagent. The phenols were also observed under UV light before spraying the visualizing agent. (Harborne 1998). The extracts were also chromatographed using Toluene: Ethyl acetate: Formic acid (5:4:0.2). The visualizing agent was alcoholic $FeCl_3$.

Thin Layer Chromatography for Flavonoids

10gm leaf sample were immersed into 2M HCl and heated in test tube for 30 mins. The cool extract were then filtered and extracted with Ethyl acetate. Ethyl acetate extract was conc to dryness taken up in 1-2 drops of Ethanol for TLC. There were 5 solvent system used such as Forestal-Acetic acid: concHCl: Water (30:3:10), 50%HOAC (50%Acetic acid), BAW-n-Butanol: Acetic acid: Water (4:1:5), PhOH-Phenol saturated with water and Water (Harborne 1998).

The extracts were also chromatographed using Toluene: Ethyl acetate: Formic acid (5:4:0.2). The visualizing agent was Aluminum chloride.

High Performance Thin Layer Chromatography

Preparation of extract: 1gm of plant samples was extracted in 10ml of methanol for 5 mins on water bath at 60°C and then filtered. This rapid method extracts both lipophillic and hydrophilic flavanoids. The chromatogram was processed in CAMAG Linomat 5, 20 x 10 HPTLC silica gel 60 F254 plates were used The plates were pre run with methanol till 90mm in twin trough chambers saturated with methanol. The mobile phase was Ethyl acetate-formic acid-glacial acetic acid-water [10:1.1:1.1:2.6]. The mobile phase or solvent front was allowed to run till 80mm. Photodocumentation was done using densitometeric scanning at 254nm, 366nm.

Derevatization was done using alcoholic $FeCl_3$ and inspected in visible light and ultra violet light. Phenolics form blue or dark blue (visible) azo dyes. The Peak table, Peak display and Peak densitogram were noted**.** Derevatization was done using Folin-Ciocalteu and inspected in visible light and ultra violet light. Flavonoids form blue or blue -violet (visible) azo dyes. The Peak table, Peak display and Peak densitogram were noted**.**

Results and Dicussion

The plant materials were sorted as galls, galled leaf and ungalled leaf. The samples were extracted in methanol and acid hydrolysed for analysis of phenolic and flavonoids compounds. Extracts of galls-P1, galled leaf tissue-P2, ungalled leaf tissue-P3 were tested for phenolics and flavanoids. Preliminary test with $FeCl_3$ gave purple colouration indicating the presence of phenolic substances in all the 3 extracts.

TLC for phenolic acids the samples were chromatographed by 2D TLC on silica gel plates. The solvent systems used was Acetic acid: Chloroform (1:9) and Ethyl acetate: benzene (9:11). The spots were visualized using Folin-Ciocalteu reagent.

The phenols were also observed under UV light as bright blue fluorescence before spraying the visualizing agent. When sprayed with 5% ethanolic ferric chloride solution it showed bluish grey spots when plates were placed in a chamber saturated with ammonia vapours.

The extracts were also chromatographed using Toluene: Ethyl acetate: Formic acid (5:4:0.2). The visualizing agent was alcoholic $FeCl_3$. The dark blue spots indicate the presence of phenolic acids. (Fig. 1)

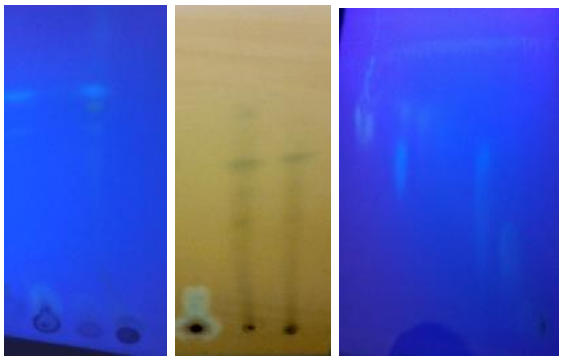


Fig.1: TLC PHENOLICS in TEF, 2D TLC of PHENOLICS

Shenoda test for the 3 samples gave pink colouration indicating the presence of flavanoids.

TLC for flavanoids using There were 5 solvent system used such as Forestal - Acetic acid: concHCl: Water (30:3:10), 50%HOAC (50% Acetic acid), BAW-n-Butanol:Acetic acid: Water (4:1:5), PhOH-Phenol saturated with water and Water. Flavanoids were detected as bright blue fluorescence.

The extracts were also chromatographed using Toluene: Ethyl acetate: Formic acid (5:4:0.2). Flavanoids were detected as bright blue fluorescence and after spraying with the visualizing agent Aluminum chloride flavanoids formed bluish gray spots. (Fig 2)

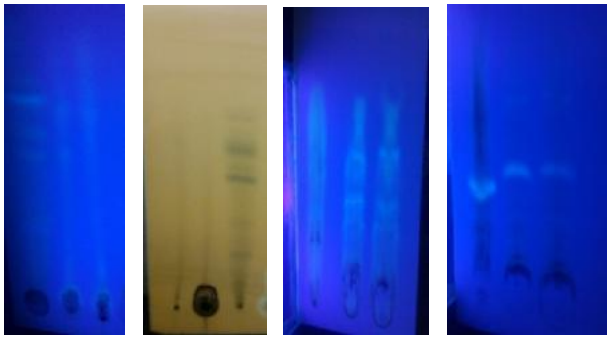


Fig.2: TLC Flavanoids in TEF, BAW, Phenol and Water

HPTLC silica gel 60 F254 plates were used The plates were pre run with methanol till 90mm in twin trough chambers saturated with methanol. The mobile phase was Ethyl acetate-formic acid-glacial acetic acid- water [10:1.1:1.1:2.6]. The mobile phase or solvent front was allowed to run till 80mm. Photo-documentation was done using densitometeric scanning at 366nm.

Phenolics form blue or green-blue fluorescent bands (figs. 3&5) having Rf values for P1 are 0.73, 0.78, 0.87, P2 are 0.72, 0.86 and P3 are 0.66, 0.72, 0.86. No significant changes in the phenolic content observed in the galls, galled leaf and ungalled leaf tissues.

Flavanoids form light blue to dark blue fluorescent bands (figs. 6&7) having Rf values for P1 are 0.10, 0.14, 0.22, 0.31, 0.34, 0.45, 0.55, P2 are 0.10, 0.14, 0.27, 0.30, 0.74, P3 are 0.10, 0.14, 0.22, 0.27, 0.30, 0.74. There is a significant increase in the number of flavanoid content observed in the galls as compared to galled leaf and ungalled leaf tissues. This could be to provide nutrition and protection to the growing insect in the galls.

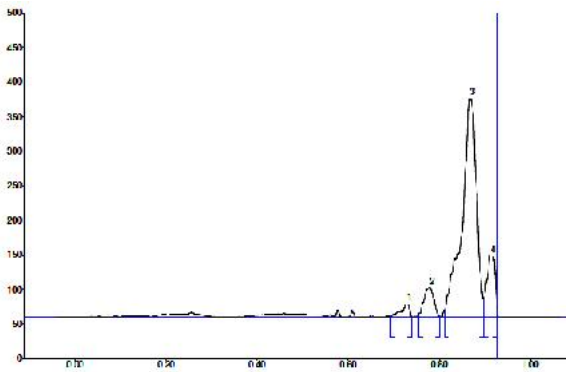
Derevatiztion using alcoholic $FeCl_3$ and inspected in visible light and ultra violet light. 254nm, Phenolics form dark blue bands. The Peak table, Peak display and Peak densitogram were noted. (Figs. 4 & 5) having Rf values for P1 are 0.73, 0.88, P2 are 0.88

and P3 are 0.87. There were a few additions in the phenolic content observed in the galls as compared to galled leaf and ungalled leaf tissues. This could have been triggered due to the mechanical injury to the plant tissue leading to gall formation.

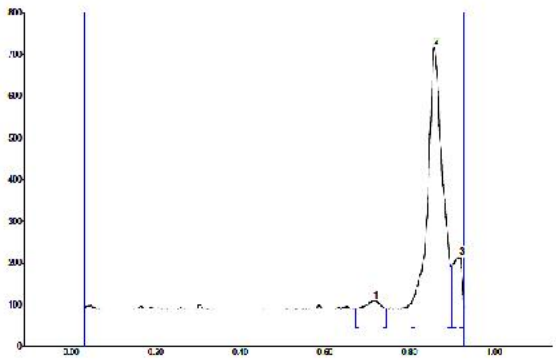
Derevatiztion using Folin-Ciocalteu and inspected in visible light and ultra violet light. 254nm. Flavonoids form dark blue or gray blue bands. The Peak table, Peak display and Peak densitogram were noted**.** (Figs. 6 & 8) having Rf values for P1 are 0.11, 0.15, 0.21, 0.23, 0.31, 0.35, 0.45, 0.55, P2 are 0.10, 0.14, 0.23, 0.30, 0.42, P3 are 0.11, 0.14, 0.24, 0.30, 0.42. There is a significant increase in the number of flavanoid content observed in the galls as compared to galled leaf and ungalled leaf tissues. This could be to provide nutrition and protection to the growing insect in the galls.

Conclusion

There are more than one type of galls observed on the *Pongamia pinnata* tree, but no two types of galls on the same tree. The gall inducer must be host specific and organ specific. As per the findings of the present study the phenolics and the flavanoids are modified or new types are initiated after the deposition of the eggs by the female insect. Though the galls were present in many leaves of the same tree the density of the galls were not so much to ham the tree, There seems to be some sort of harmonious association between the gall insect and the host tree. The galls contain additional flavanoids which acts as a chemical shield to the growing insect and at the same time not harmful to the host tree. Polyphenols are in the galls as well as the leaves probably a defensive mechanism which prevents the invasion of other fungi or bacteria. Further studies in this concept is in progress.



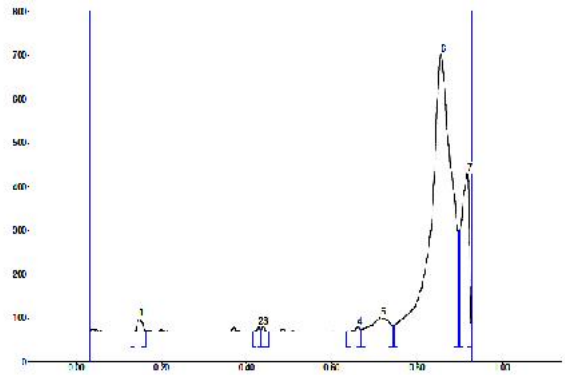
P1 366nm



P2 366nm

Peak P1	Rf Value	Max Height	Area	Substance
1	0.73	19.7	303.2	Phenolic
2	0.78	43.8	798.3	Phenolic
3	0.87	317.7	8535.0	Phenolic
4	0.92	89.7	1376.9	unknown

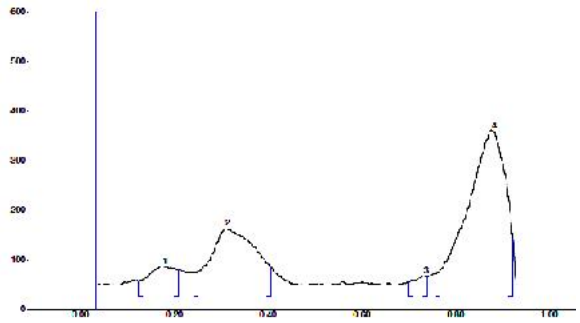
Peak P2	Rf Value	Max Height	Area	Substance
1	0.72	19.8	479.7	Phenolic
2	0.86	627.5	17617.3	Phenolic
3	0.92	124.5	2134.0	unknown



P3 366nm

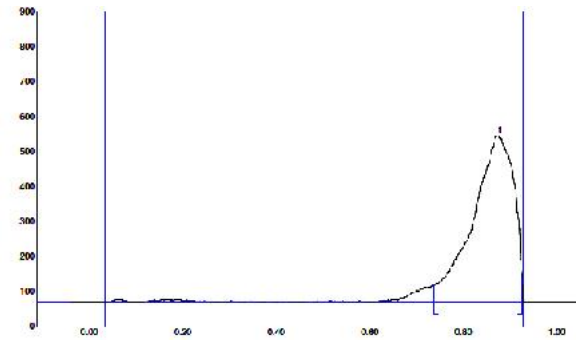
Peak P3	Rf Value	Max Height	AREA	SUBSTANCE
1	0.15	29.6	294.2	unknown
2	0.43	11.0	60.2	unknown
3	0.44	10.8	61.3	unknown
4	0.66	11.3	97.4	phenolic
5	0.72	31.9	1041.5	Phenolic
6	0.86	632.7	24637.9	Phenolic
7	0.92	360.7	5467.4	unknown

Figure 3: Peak table and densitogram of phenolics at 366nm in Pongamia *pinnata* (L) Pierre p1-gall, p2-galled leaf, p3-ungalled leaf



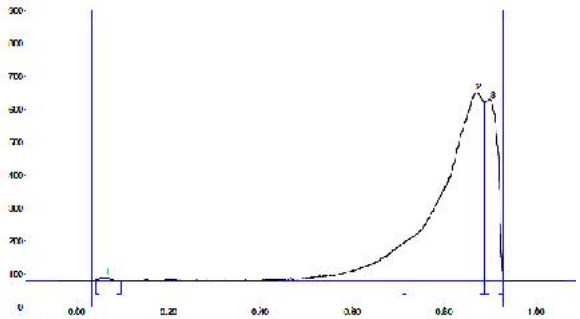
P1 254nm

Peak P1	Rf Value	Max Height	Area	Substance
1	0.17	38.1	1657.3	unknown
2	0.31	114.4	8723.0	unknown
3	0.73	18.9	370.8	phenolic
4	0.88	312.1	20334.0	phenolic



P2 254

Peak P2	Rf Value	Max Height	Area	Substance
1	0.88	476.7	34822.4	Phenolic



P3 254nm

Peak P3	Rf Value	Max Height	AREA	SUBSTANCE
1	0.06	11.6	208.7	unknown
2	0.87	572.7	40982.7	Phenolic
3	0.90	550.0	11995.0	unknown

Figure 4: Peak table and densitogram of phenolics at 254nm *Pongamia pinnata* (L) Pierre p1-gall, p2-galled leaf, p3-ungalled leaf before derivatization after derivatization

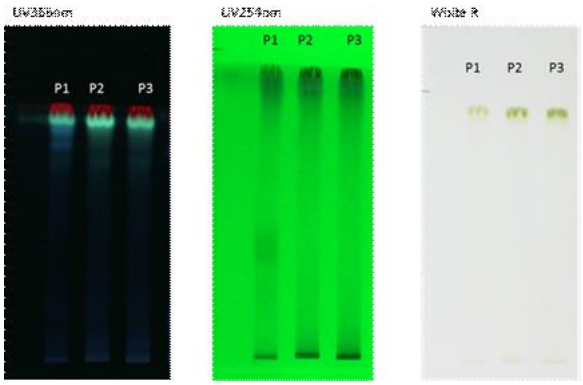


Figure 5: HPTLC Chromatogram of phenolics in *Pongamia pinnata,* p1-gall, p2-galled leaf, p3-ungalled leaf. Before derivatization after derivatization

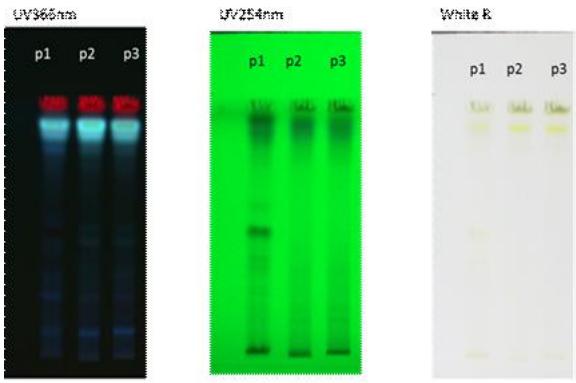
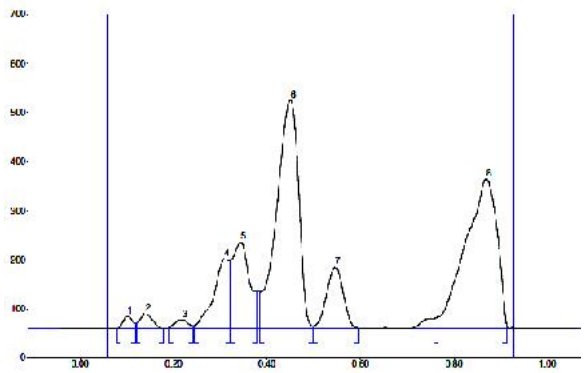
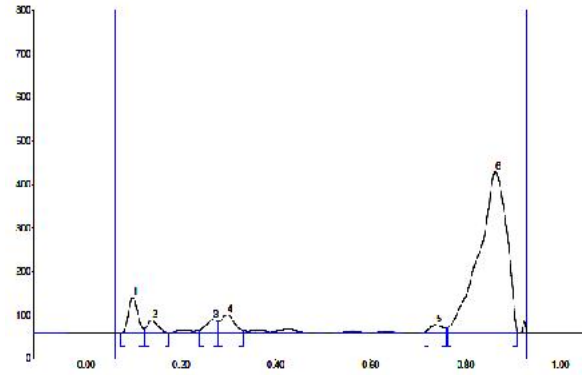


Figure 6: HPTLC Chromatogram of Flavanoides in *Pongamia pinnata,* p1-gall, p2-galled leaf, p3- ungalled leaf.

Peak P1	Rf Value	Max Height	Area	Substance
1	0.10	25.3	428.8	flavanoid
2	0.14	30.9	692.8	flavanoid
3	0.22	18.6	444.7	flavanoid
4	0.31	142.4	3961.5	flavanoid
5	0.34	177.0	5527.7	flavanoid
6	0.45	465.5	18303.6	flavanoid
7	0.55	125.1	3772.6	flavanoid
8	0.87	305.6	17412.8	unknown



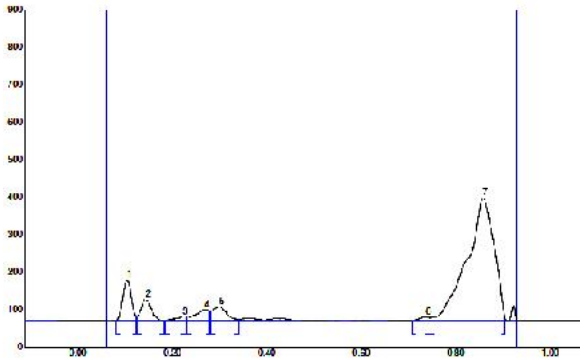
P1 366nm



P2 366nm

Peak P2	Rf Value	Max Height	Area	Substance
1	0.10	81.0	1332.4	flavanoid
2	0.14	28.9	545.8	flavanoid
3	0.27	30.2	612.4	flavanoid
4	0.30	42.4	1024.0	flavanoid
5	0.74	18.6	437.5	flavanoid
6	0.87	370.1	18056.5	unknown

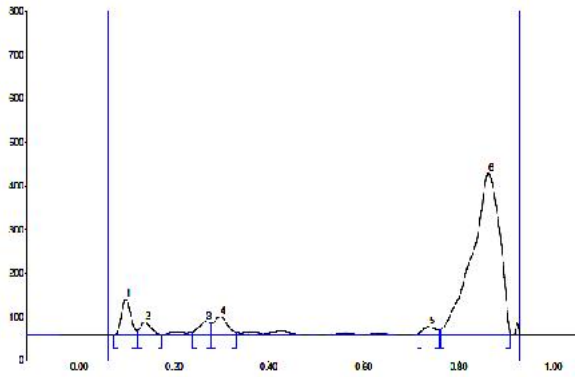
Peak P3	Rf Value	Max Height	Area	Substance
1	0.10	110.7	1695.0	flavanoid
2	0.14	57.8	1051.3	flavanoid
3	0.22	10.5	214.3	flavanoid
4	0.27	30.2	746.7	flavanoid
5	0.30	38.6	963.7	flavanoid
6	0.74	11.8	202.6	flavanoid
7	0.86	329.3	15779.1	unknown



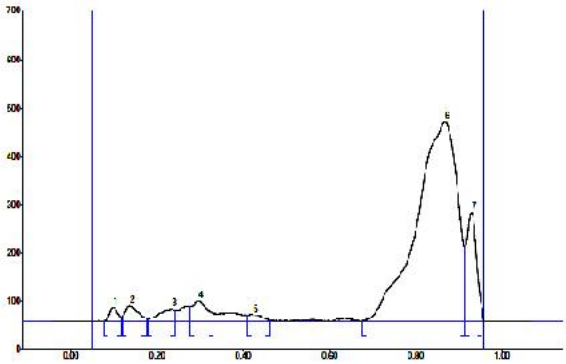
P3 366nm

Figure 7: Peak table and densitogram of Flavonoids at 366nm in Pongamia *pinnata* (L) Pierre p1-gall, p2- galled leaf, p3- ungalled leaf

Peak P1	Rf Value	Max Height	Area	Substance
1	0.09	19.7	202.5	unknown
2	0.11	22.4	273.3	flavanoid
3	0.15	28.2	762.8	flavanoid
4	0.21	58.3	763.2	flavanoid
5	0.23	33.5	476.8	flavanoid
6	0.31	84.5	2187.7	flavanoid
7	0.35	113.2	3271.7	flavanoid
8	0.45	368.2	13030.7	flavanoid
9	0.55	87.6	2458.2	flavanoid
10	0.89	476.0	44763.8	unknown

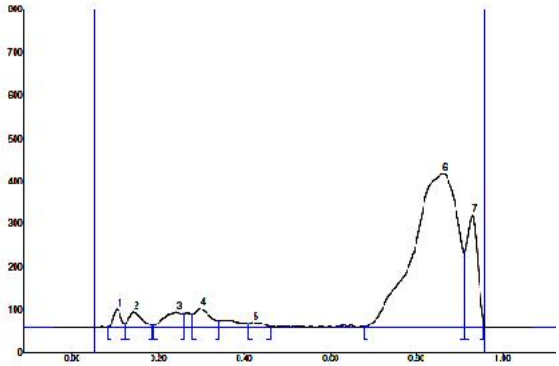


P1 254nm



P2 254nm

Peak P2	Rf Value	Max Height	Area	Substance
1	0.10	29.3	470.8	flavanoid
2	0.14	31.9	816.7	flavanoid
3	0.23	26.5	764.6	flavanoid
4	0.30	42.5	1270.9	flavanoid
5	0.42	14.6	392.9	flavanoid
6	0.87	412.0	32352.4	unknown
7	0.93	226.2	4330.4	unknown



P3 254nm

Peak P3	Rf Value	Max Height	Area	Substance
1	0.11	42.9	606.9	flavanoid
2	0.14	36.9	921.0	flavanoid
3	0.24	36.2	1327.2	flavanoid
4	0.30	44.4	1391.4	flavanoid
5	0.42	12.6	319.8	flavanoid
6	0.86	359.9	30843.8	unknown
7	0.93	264.2	5710.0	unknown

Figure 8: Peak table and densitogram of Flavanoids at 254nm in Pongamia *pinnata* (L) Pierre p1-gall, p2-galled leaf, p3-ungalled leaf

Figure 9: Galled leaf, ungalled leaf, Gall, Section of Gall of *Pongamia pinnata* (L) Pierre.

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