CHAPTER – III
MATERIALS AND METHODS

Materials

Mature fresh leaves of *Jatropha curcas* were collected from the *Jatropha* field, dried in an oven at 60ºc ± 2ºc for four days, powered (40 meshes) and used for bioassay and pot experiment studies.

The *Jatropha curcas* seeds were collected from Tamilnadu Agricultural University, Coimbatore, Tamilnadu, India. These seeds were used for *Jatropha* intercropping.

Seeds of the following crop plants were collected from Tamilnadu Agricultural University, Coimbatore, Tamilnadu, India

1. *Capsicum annum* L. (Green chilli)
2. *Phaseolus aureus* L. (Green gram)
3. *Sesamum indicum* L. (Sesame)
4. *Phaseolus mungo* L. (Black gram)

The following phenolic acids were purchased from sigma chemicals for bioassay studies on selected crop plants.
1. Coumarin
2. Catechin
3. Kaempferol

Methods

Extraction procedure

The dried leaves were ground to a fine powder in a wiley Mill (40 mesh). Using this powder, aqueous extracts were prepared by the methods of Heisey (1990).

Preparation of aqueous extract

Ten gram of leaf powder was dissolved separately in 100ml of distilled water in a beaker and kept for 24 h at room temperature (28°C ± 2°C) with occasional swirling. The solution was filtered through Whatman No. 1 filter paper and the volume was made up to 100ml with distilled water. The aqueous extract was diluted with water to get 5, 10, 15, and 20% concentrations. The dilutions thus obtained corresponded to 0.05, 0.1, 0.15, 0.2% water extractable materials.

Experiments on crop plants

The seeds of crops were surface sterilized with 0.1% mercuric chloride for 1 min. to remove the fungal spores on the seeds. Then the seeds were washed with distilled water several times to remove the mercuric chloride.
Bioassay studies

Bioassay studies were carried out following the methods of Heisey (1990). Ten seeds of each crop were placed on Whatman No.1 filter paper in petriplates (9cm × 2cm). Petriplates were moistened with 2ml/plate of leaf aqueous extract of *Jatropha curcas* with different concentrations (5%, 10%, 15% and 20%). The control plant were treated with distilled water and incubated in dark at 28°C ± 2°C. The experimental design was a randomized complete block with five replicates for each treatment and control. Germination percentage, plumule length and radical length were measured after 7 days.

\[
\text{Germination (％) } = \frac{\text{Number of seeds germinated}}{\text{Number of seeds placed for germination}} \times 100
\]

Preparation of phenolic acid for bioassay studies

Bioassay studies were carried out following the methods of Heisey (1990). Ten seeds of each crop were placed on Whatman No.1 filter paper in petriplates (9cm × 2cm). Petriplates were moistened with 2ml/plate of each phenolic acid coumarin, catechin, and kaempferol with different concentrations of 0.05mM, 1.0mM, 2.0mM, 0.3mM, 4.0mM and 5.0mM. Distilled water used as control and incubated in dark at 28°C ± 2°C. The experimental design was a randomized complete block with five replicates for each treatment and control.
Germination percentage, radicle length and plumule length were measured after 15 days.

**Pot culture studies**

The seeds of *Capsicum annum* (Green chilli), *Phaseolus aureus* (Green gram), *Sesamum indicum* (Sesame) and *Phaseolus mungo* (Black gram) were sown in earthen pots (24cm ± 24cm). The soil consisted of a mixture of silt, humus and river sand in the ratio of 1: 1:2. The seedlings were thinned to a three plant/pot. The treatment was started after the 3\textsuperscript{rd} leaf emerged (15\textsuperscript{th} day after sowing). Before sowing, the seeds were surface sterilized with 0.1% mercuric chloride. All the experimental plants were grown under outdoor conditions in sunlight in the garden of S.T. Hindu College, Nagercoil, Tamilnadu. The day temperature was 28\degree C ± 2\degree C and the night temperature was 26\degree C ± 2\degree C. Natural photoperiod varied between 10 and 12 h. The seedlings were irrigated with different concentration of aqueous extracts of the test plants (500ml/pot) on alternate days. Control plants were irrigated with water. The experimental design employed was factorial CRD with three replications for each treatment and control. Shoot length, root length, yield, biomass weight and root nodules were determined at the end of the life span of each crop plan (Manikandan, M., 2000).
Field experiments

Sowing in nursery beds

Raised beds (10 cm high) were prepared by digging and mixing soil with sand and FYM (farmyard manure) in 1:1:1 ratio. Each bed was prepared having 1 m x 5 m dimensions. Shallow furrows of 2 cm depth was made by finger or using a stick. Soaked seeds were placed in furrows at an interval of 5 cm. and it was covered with a thin layer of soil. Care was taken to avoid deep sowing. Light irrigations were given after the seed germination and bare rooted seedlings were transplanted after 3-4 months in the field during the rainy season.

Jatropha curcas field practices

The disease free and bold seedlings of Jatropha curcas were transplanted in rows at spacing of 3x2 meter. It was recommended for under irrigated condition. The land was deep ploughed, because the soil was heavy in nature. The 30 cm x 30 cm pits were prepared in the field at required spacing and then filled with a mixture of soil, FYM (2-3 kg) and fertilizer (20 g urea, 120 g single super phosphate and 16 g murate of potash). Eight to ten week old seedlings were to be planted in 30 cm x 30 cm pits dug in the field at required spacing’s and it was filled with a mixture of soil, FYM (2-3 kg.) and fertilizer (20 gm Urea, 120 g Single Super Phosphate and 16 g Murate of Potash).

Intercropping in Jatropha curcas field

To study the allelopathic effect of Jatropha curcas on intercropping plants, the selected intercropping plants such as green chilli, green gram, sesame
and black gram were planted in between the *Jatropha* rows with proper spacing after one year in the *Jatropha* field. The crops were managed with proper agriculture practices.

**Qualitative and quantitative determination of allelochemicals in *Jatropha curcas***

The plant materials viz., leaf, stem, seed and root of the *Jatropha curcas* were collected from *Jatropha* experimental field, S.T. Hindu College, Nagercoil, Tamilnadu.

*Jatropha curcas* samples of leaf, stem, seed and root powder were then extracted using methanol and water as solvents with the soxhlet apparatus. The extracts were stored in sterile bottles at 4 degree in a refrigerator.

**Screening of allelochemicals**

The methanolic and aqueous extract and leaf, stem, seed and root powder of *Jatropha curcas* were used as samples for qualitative and quantitative phytochemical screening for phenols, flavonoids, tannin and alkaloids following the standard procedures described by Trease and Evans (1989) and Faraz *et al.*, (2003).

**Test for phenols**

One millilitre of each sample of *Jatropha curcas* (leaf, stem, seed and
root) was added to 1 mL of 10% FeCl$_2$ and mixed together. The presence of blue precipitate confirmed the presence of phenols (Trease and Evans, 1989).

**Test for flavonoids**

0.5g of each sample was dissolved in 2ml dilute NaOH solution. A few drops of concentrated H$_2$SO$_4$ were then added. The presence of flavonoids was indicated by the disappearance of color (Trease and Evans, 1989).

**Test for Tannins**

0.5g of each sample was stirred with 10ml of boiling distilled water. This was filtered and a few milliliters of 6% ferric chloride added to the filtrate. Appearance of deep green coloration indicated the presence of tannins. The second portion of the filtrate was treated with a few milliliters of iodine solution. Appearance of a faint bluish coloration confirmed the presence of tannins (Trease and Evans, 1989).

**Test for Alkaloids**

0.5g of each sample was stirred with 5ml of 2N HCL in a steam bath. This was filtered and 1ml of the filtrate was tested with a few drops of Dragendorff's reagent and a second 1ml portion was treated similarly with Wagners reagent. The formation of a precipitate was an indication of the presence of alkaloids (Faraz et al., 2003).
After preliminary analysis to determine the presence of these phytochemicals, the samples were further subjected to quantitative analysis to determine the percentage of each of these secondary metabolites in each plant part of each species. The following procedures were adopted.

- **Quantitative analysis of phenol using the follin – ciocaltean colorimetric method of Harborne (1993)**

\[
\text{Phenols (\%)} = \frac{100 \times C \times VF \times D}{W \times 1000 \times VA \times 1}
\]

Whereas

- \(W\) = Weight of sample analyzed
- \(C\) = Concentration of standard in mg/ml
- \(VF\) = Total filtrate volume
- \(VA\) = Volume of filtrate analyzed
- \(D\) = Dilution factor where applicable

- **Quantitative analysis of Flavonoid by the hydrolysis gravimetric methods of Harborne (1993)**

Percentage flavonoid was calculated by

\[
\text{Flavonoids (\%)} = \frac{(W_2 - W_1)}{\text{Weight of Sample}}
\]

Whereas \((W_2 - W_1)\) = Weight of residues
Quantitative analysis of tannin by the Van Burden and Robinson (1981)

Tannins were computed thus:

Whereas \( X = \text{Absorbance} \)

\[
\text{Tannins (mg/100mL)} = \frac{X - \text{blank}}{\text{Standard} - \text{blank}}
\]

Quantitative analysis of alkaloids by the gravimetric methods of Harborne (1993)

Percentage alkaloids were computed as follows:

\[
\text{Alkaloids (\%)} = \frac{(W_2 - W_1)}{\text{Weight of Sample}}
\]

Whereas \((W_2 - W_1) = \text{Weight of residues}\)

Identification of phenolic acid by HPTLC method

Extraction and preparation of test solution for HPTLC analysis

Dried leaf sample (5gm) of *Jatropha curcas* was extracted with methanol in soxhlet apparatus for 3hrs and then allowed to cool, filtered the content and concentrated using vacuum flash evaporator. The concentrated solution was dissolved with 1ml of methanol solution and centrifuged at 3000rpm for 5min. This solution was used as test solution for HPTLC analysis.
Preparation of standard phenolic acid solution for HPTLC analysis

One gram of standard phenolic acids such as kaempferol, coumarin, catechin and quercetin, were dissolved in 1ml of methanol solution and centrifuged at 3000rpm for 5min. These standard solutions were used as standard for HPTLC analysis.

Activation of pre-coated plates

The TLC plates were pre washed with methanol and activated by keeping an oven at 110-120º C for 30 minutes.

Plates were exposed to high humidity or kept on hand for long time to be activated.

Aluminum sheets were kept in between two glass plates and placed in oven at 110-120º C for 15 minutes.

Samples

2µl of the above test solution and 2µl of standard solutions were loaded as 5mm band length in the 6 x 10 Silica gel 60F₃₄ TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument.

Pre- conditioning (Chamber saturation)

Unsaturated chamber causes high R F values. Therefore, the chamber was saturated by lining with filter paper for 30 minutes prior to the development and uniform distribution of solvent vapours, less solvent for the sample to travel lower R F values.
Spot development

The samples loaded plate was kept in TLC twin trough developing chamber (after saturated with solvent vapour Toluene-Acetone-Formic acid (4.5: 4.5: 1) with mobile phase (Toluene-Acetone-Formic acid (4.5: 4.5: 1)) and the plate was developed in the mobile phase up to 90mm.

Retention factor (R F)

Retention factor (R F) is defined as the amount of separation due to the solvent migration through the sorbent layer as shown in the formula. It depends on time of development and velocity coefficient or solvent front velocity.

\[
R_f = \frac{\text{Migration distance of substance}}{\text{Migration distance of solvent front from origin}}
\]

Photo-documentation

The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in Photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at White light, UV 254nm and UV366nm.

Derivatization

The developed plate was sprayed with Folin Cio-calteu spraying reagent and dried at 100°C in hot air oven. The plate was photo-documented at day light
and UV366nm using Photo-documentation (CAMAG REPROSTAR 3) chamber (Sherma and Fried, 2003; Peter, 2005).

**Preparation of spraying reagent Folin Cio-calteu**

10 g sodium tungstate and 2.5 g sodium molybdate were dissolved in 70 ml water and added 5 ml of 85% phosphoric acid and 10 ml concentrated hydrochloric acid were added and refluxed for 10 hr. To this 15 g lithium sulfate, 5 ml water and 1 drop of bromine were added and then again refluxed for 15 min. Finally allowed to cool in room temperature and the volume were made to 100 ml with water. After the preparation, the Folin & Ciocalteu’s phenol reagent was stored tightly capped at room temperature (Peterson, 1979).

**Scanning**

Before derivatization, the plate was fixed in scanner stage (CAMAG TLC SCANNER 3) and scanning was done at UV 254nm and UV366nm. The peaks were tabulated, peak display and peak densitogram were observed and recorded (Sherma and Fried, 2003; Peter, 2005)

**Detection**

The blue colored zones at day light mode present in the standards (kaempferol, coumarin, catechin and quercetin) and sample track (*Jatropha curcas* leaf) observed in the chromatogram after derivatization, which confirmed the presence of phenolics in the sample and standards.
Identification of alkaloids by HPTLC method

Extraction and preparation of test solution for HPTLC analysis

*Jatropha curcas* dried leaf sample (5gm) was extracted with methanol in soxhlet apparatus for 3hrs and then allowed to cool. The content was filtered and concentrated using vacuum flash evaporator. The concentrated solution was dissolved with 1ml of methanol solution and centrifuged at 3000rpm for 5min. This solution was used as test solution for HPTLC analysis.

Preparation of standard phenolic acid solution for HPTLC analysis

One gram of standard alkaloids such as colchicine, emetine and scopolamine, was dissolved in 1ml of methanol solution and centrifuged at 3000rpm for 5min. These standard solutions were used as standard for HPTLC analysis.

Activation of pre-coated plates:

- The TLC plates were prewashed with methanol and activated by keeping an oven at 110-120º C for 30 minutes.
- Plates were exposed to high humidity or kept on hand for long time to be activated.
- Aluminum sheets were kept in between two glass plates and placed in oven at 110-120º C for 15 minutes.
Samples

3µl of the above test solution and 2µl of standard solutions were loaded as 5mm band length in the 6 x 10 Silica gel 60F$_{254}$ TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument.

Pre-conditioning (Chamber saturation)

Unsaturated chamber causes high R F values. Therefore, the chamber was saturated by lining with filter paper for 30 minutes prior to development and uniform distribution of solvent vapours, less solvent for the sample to travel lower R F values.

Spot development

The samples loaded plate was kept in TLC twin trough developing chamber (after saturated with solvent vapour Ethyl acetate-Methanol-Water (100: 13.5: 10) with mobile phase (Ethyl acetate-Methanol-Water (100: 13.5: 10) and the plate was developed in the mobile phase up to 90mm.

Retention factor (R F)

Retention factor (R F) is defined as the amount of separation due to the solvent migration through the sorbent layer as shown in the formula. It depends on time of development and velocity coefficient or solvent front velocity.

\[
R_f = \frac{\text{Migration distance of substance}}{\text{Migration distance of solvent front from origin}}
\]
Photo-documentation

The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in Photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at White light, UV 254nm and UV366nm.

Derivatization

The developed plate was sprayed with Dragendorff’s reagent followed by 10% sodium nitrite reagent and dried at 100°C in hot air oven. The plate was photo-documented at day light and UV366nm using Photo-documentation (CAMAG REPROSTAR 3) chamber (Sherma and Fried, 2004; Peter, 2005).

Preparation of spraying reagent Dragendorff’s reagent

Solutions A and B were prepared. The solution (A and B) were mixed and diluted to 100ml with double distilled water

Solution A: Bismuth nitrate (0.17g) in acetic acid (2mL) and H₂O (8mL)

Solution B: KI (4g) in acetic acid 10mL) H₂O (20mL)

Preparation of spraying reagent 10% sodium nitrite

Scanning

Before derivatization, the plate was fixed in scanner stage (CAMAG TLC SCANNER 3) and scanning was done at UV 254nm. The peaks were tabulated,
peak display and peak densitogram were observed and recorded (Sherma and Fried, 2003; Peter, 2005).

**Detection**

The black colored quenching zone at UV 254nm was observed in the standard such as colchicines, emetine and scopolamine (before and after derivatization) and bright orange & brown colored zones at day light mode were present in the *Jatropha curcas* leaf sample track observed in the chromatogram after derivatization, which confirmed the presence of alkaloid in the given standard and the sample.

**Identification of flavonoids by HPTLC method**

**Extraction and preparation of test solution for HPTLC analysis**

Dried leaf sample (5gm) of *Jatropha curcas* was extracted with methanol in soxhlet apparatus for 3hrs and then allowed to cool. The content was filtered and concentrated using vacuum flash evaporator. The concentrated solution was dissolved with 1ml of methanol solution and centrifuged at 3000rpm for 5min. This solution was used as test solution for HPTLC analysis.

**Preparation of standard phenolic acid solution for HPTLC analysis**

One gram of standard flavonoid rutin was dissolved in 1ml of methanol solution and centrifuged at 3000rpm for 5min. These standard solutions were used as standard for HPTLC analysis.
**Activation of pre-coated plates:**

- The TLC plates were prewashed with methanol and activated by keeping in oven at 110-120º C for 30 minutes.
- Plates were exposed to high humidity or kept on hand for long time to be activated.
- Aluminum sheets were kept in between two glass plates and placed in oven at 110-120º C for 15 minutes.

**Samples**

1.2µl of the above test solution and 2µl of standard solutions were loaded as 5mm band length in the 6 x 10 Silica gel 60F<sub>254</sub> TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument.

**Pre-conditioning (Chamber saturation)**

Unsaturated chamber causes high R F values. Therefore, the chamber was saturated by lining with filter paper for 30 minutes prior to development and uniform distribution of solvent vapours, less solvent for the sample to travel lower R F values.

**Spot development**

The samples loaded plate was kept in TLC twin trough developing chamber (after saturated with solvent vapour Ethyl acetate-Butanone-Formic acid-Water (5: 3: 1: 1)) with mobile phase (Ethyl acetate-Butanone-Formic acid-Water (5: 3: 1: 1)) and the plate was developed in the mobile phase up to 90mm.
Retention factor (R F)

Retention factor (R F) is defined as the amount of separation due to the solvent migration through the sorbent layer as shown in the formula. It depends on time of development and velocity coefficient or solvent front velocity.

\[
R_f = \frac{\text{Migration distance of substance}}{\text{Migration distance of solvent front from origin}}
\]

Photo-documentation

The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in Photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at White light, UV 254nm and UV366nm.

Derivatization

The developed plate was sprayed with 1% ethanolic aluminium chloride spraying reagent and dried at 100°C in hot air oven. The plate was photo-documented at day light and UV366nm using Photo-documentation (CAMAG REPROSTAR 3) chamber (Sherma and Fried, 2003; Peter, 2005).

Detection

The yellow colored fluorescent zones at UV 366nm mode present in the standard (rutin) and sample (Jatropha curcas leaf) track observed in the chromatogram after derivatization confirmed the presence of flavonoids in the sample.