3

MATERIALS AND METHODS

A study on the efficiency of agro industrial wastes paddy and coffee husk as organic manure and its influence on the growth of some selected plants was carried out to find the solution for the profitable utilization and to reduce environmental hazards. The ultimate goal of this study is to reduce the intensive use of pesticides, chemical fertilizers and bring about sustainable technologies with the production of enriched agro industrial wastes with composting. The details of enrichment of agro industrial wastes paddy and coffee husks composting and its physico - chemical evaluation, incorporation into various treatments, pot culture experiments, biometric characters, biochemical parameters, yield, leghaemoglobin content in the nodules of the selected test plants, phytochemical studies, antioxidant, antibacterial activity, pre and post harvest soil analysis along with statistical analysis are presented in this chapter.

PHASE - I

3.1 COLLECTION OF AGRO INDUSTRIAL WASTES

The agro industrial wastes paddy husk was collected from near by villages in Visakhapatnam District of Andhra Pradesh. The coffee husk was collected from Araku valley, a hill station in Alluri Sitha Ramaraju District, Andhra Pradesh. The collected raw samples were sun dried completely and preserved for the further study.

Collection of Microorganisms

The microorganisms *Pleurotus eous*, *Pleurotus florida* and *Trichoderma asperelloides* for the biocomposting process were obtained from Tamil Nadu Agricultural University, Coimbatore.

Collection of Earth worms

The earth worms *Eisenia fetida* were collected from regional Agricultural research station, Acharya N.G Ranga Agricultural University, Anakapalli, Andhra Pradesh.

3.1.1 Procedure for Composting

Preparation of the Compost pit

The raw samples of paddy and coffee husks were partially decomposed by using pit composting method in six pits (Plate - 3A & B) each with the measurement of 4 square feet width and 2.5 feet height, named as Compost 1 - Raw paddy husk + 20g *Pleurotus eous* + *Eisenia fetida* (5t/ha), Compost 2 - Raw paddy husk + 20g *Pleurotus florida* + *Eisenia fetida* (5t/ha), Compost 3 - Raw paddy husk + 6.5g *Pleurotus eous*, 6.5g *Pleurotus florida*, 7g *Trichoderma asperelloides* + *Eisenia fetida* (5t/ha), Compost 4 - Raw coffee husk + 20g *Pleurotus eous* + *Eisenia fetida* (5t/ha), Compost 5 - Raw coffee husk + 20g *Pleurotus florida* + *Eisenia fetida* (5t/ha), Compost 6 - Raw coffee husk + 6.5g *Pleurotus eous*, 6.5g *Pleurotus florida*, 7g *Trichoderma asperelloides* + *Eisenia fetida* (5t/ha).

Preparation of Paddy husk compost

To achieve good quality of compost, paddy husk was facilitated to decomposition process with the incorporation of various microorganisms.

Compost 1

5 kg of paddy husk and cow dung was loaded in the 1st compost pit (C_1) 20 g of *Pleurotus eous* spawn was equally spread and above this layer 5 kg of paddy husk and 5 kg of cow dung was spread, the process was repeated till it reached the height of 1 m. Water was sprinkled to maintain temperature and moisture content (65 - 70 %) and once in a week the piles were turned. After 30 days vermicompost process was adopted.

Compost 2

The pit (C_2) was filled with 5 kg of paddy husk, 5 kg of cow dung and equally sandwiched with 20 g of *Pleurotus florida* spawn. It was allowed for decomposition process for 30 days and vermicompost process was adopted further. The moisture content was maintained at 65 - 70 %. Temperature was maintained by sprinkling of water. To accelerate decomposition turning was carried out every week.

Compost 3

In this pit (C_3) paddy husk (5 kg) and cow dung (5 kg) was filled. It was sandwiched with 20g *Pleurotus eous*, *Pleurotus florida* and *Trichoderma asperelloides.* The process was continuously repeated till it reached the height of 1m. Decomposition

process was accelerated by turning the heap every week and the temperature and moisture content (65 - 70 %) was maintained by sprinkling water. Predigested compost was then subjected to vermicomposting process.

Preparation of Coffee husk (Silver skin) compost

The above same procedure was used for the composting process of coffee husk. Instead of paddy husk (C_1, C_2 and C_3) the following composting pits were used for coffee husk and named as compost 4 (C_4), compost 5 (C_5) and compost 6 (C_6).

3.1.2 Vermicompost experimental tray preparation

Plastic trays of 40 x 20 x 15cm were used for the experiment with proper draining system. After 30 days, the preprocessed coffee husk and paddy husk were filled in separate plastic trays (C_1, C_2, C_3, C_4, C_5 & C_6). The trays are preferred to have controlled environment, ease of harvesting, reducing pest and predator risk and less risk of waterlogging. Vermibeds were prepared with the incorporation of dry cow dung in the ratio of 1:2. The bedding was kept moist 50 - 60 % throughout the experiment by regular sprinkling of water. About 20 healthy, exotic earthworms (*Eisenia fetida*) were released on the upper layer of the respective vermibeds to stabilize the active organic materials, convert them into a valuable soil amendments and source of plant nutrients. After the inoculation water was sprinkled to keep beds moist (60 - 70 %). Experimental beds were kept in shaddy area and undisturbed for 60 days (Plate - 8B). Beds were turned once in after 30 days for the maintenance of aeration and proper decomposition. At the end of the composting process, it is found less bedding and more castings in the trays, adding of water was stopped before 1 week of harvesting. The earthworms were separated by sieving and clear vermicompost which is dark in color was collected from the tray on 90^{th} day.

3.2 MICROBIAL POPULATION DURING COMPOSTING PROCESS

Compost sample (1g) was mixed with 9 ml of distilled water in conical flask and were shaken for 30 min in vortex mixer. It is used as stock solution to prepare the dilutions. The serial dilution method was adopted which was described by Kannan, (1996) and viable colony count of bacteria, fungi and actinomycetes were carried out by colony counter during 0-20, 20-40, 40-60 and 60-80 days of composting.

Methods used for the total population colony forming units of bacteria, fungi and actinomycetes

Name of the microbial group	Medium used	Dilution factor (CFU/ml)	Growth temperature (0^C)
Bacteria	Nutrient agar	10^{-6}	37
Fungi	Potato dextrose agar	10^{-4}	28
Actinomycetes	Casein agar	10^{-5}	37

3.2.1 Evaluation of Compost maturity

Raw and composted agro-industrial waste was analyzed based on the standard methods for its physical and chemical parameters. The methods were used to determine the degree of maturity as well as the instrumental characterizations of organic wastes and vermicompost.

3.2.2 Morphological study of the compost by using Field Emission Scanning Electron Microscope (FESEM)

The surface morphology or micro structural studies of the samples were done in a Field Emission Scanning Electron Microscope (TESCAN - MIRA3 XMU.).

Gold Sputtering

The final dried vermicompost of each compost (C_1, C_2, C_3, C_4, C_5 & C_6) along with the raw samples of agro industrial wastes (Paddy and Coffee husks) were grounded separately into fine powder. A spec of the sample was placed on a double side carbon tape and placed on an Aluminium stub. Gold sputtering was done for 90 seconds to provide an uniform coating of gold plasma on the film surface. Images of surface texture of samples were recorded at different magnifications.

3.2.3 Fourier Transform Infrared (FTIR) spectroscopy analysis

FT-IR Spectroscopy (FTIR - SHIMADZU (Miracle 10) is used for the samples to analyze the decomposition process.

Procedure (Williams and Fleming, 1973**)**

About 1 mg sample with 100 mg potassium bromide (KBr) are mixed and pressed into a pellet at a pressure of 1 M Pa (megapascal). The pellets were about 1mm in thickness and 10mm in diameter. The different bands obtained in the graphs were analyzed to quantify the biodegradation process.

Plate - 7

Graphical representation of raw paddy and coffee husk composting process

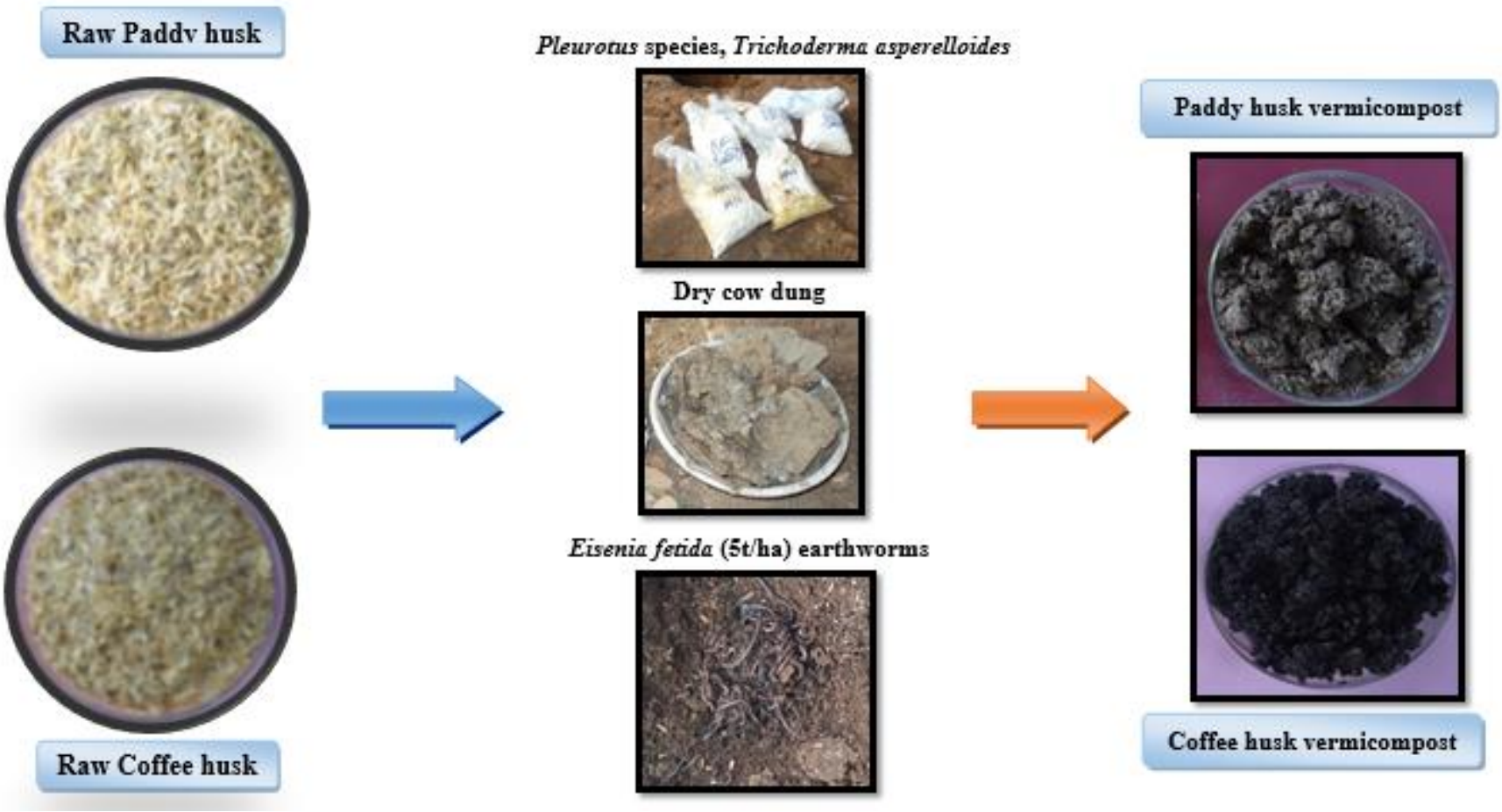
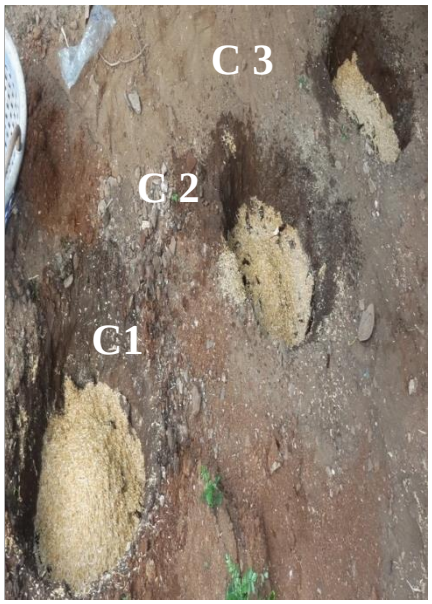


Plate - 8A

Preparation of paddy husk and coffee husk biocompost

Compost pits of paddy husk and coffee husk



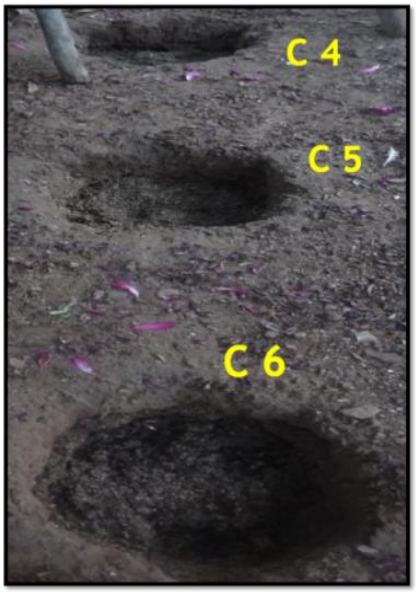
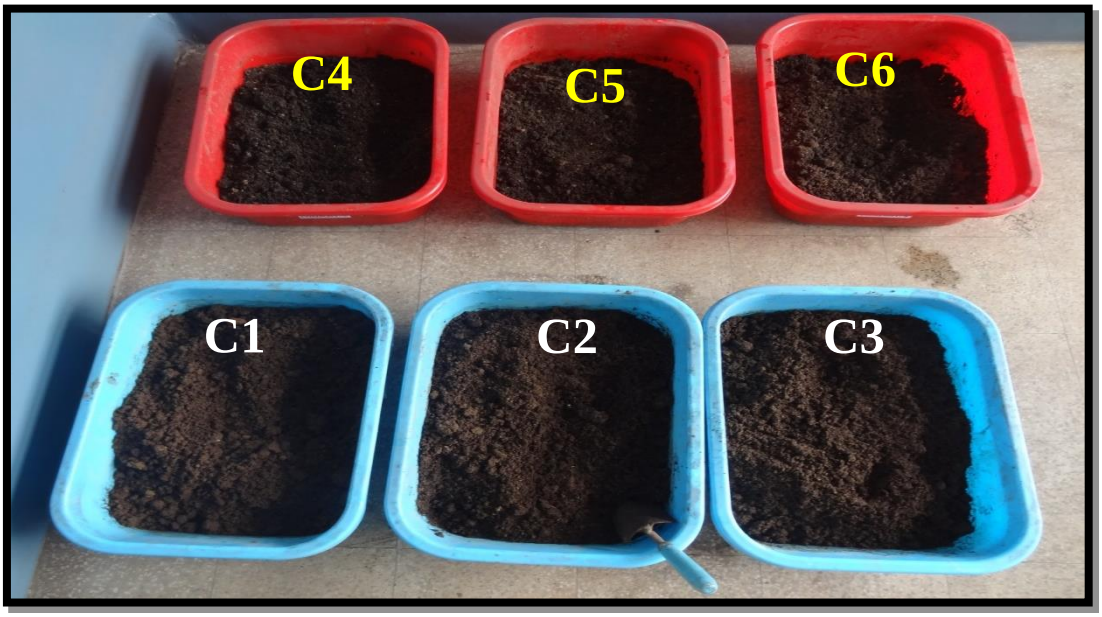


Plate - 8B

Vermicomposting process



C_1 - Raw Paddy husk +20g *Pleurotus eous* + *Eisenia fetida* (5t/ha)
C_2 - Raw Paddy husk + 20g *Pleurotus florida* + *Eisenia fetida* (5t/ha)
C_3 - Raw Paddy husk + 6.5 g *Pleurotus eous*+ 6.5 g *Pleurotus florida* +7g *Trichoderma asperelloides*+*Eisenia fetida* (5t/ha)
C_4 - Raw Coffee husk +20g *Pleurotus eous* + *Eisenia fetida* (5t/ha)
C_5 - Raw Coffee husk + 20g *Pleurotus florida* + *Eisenia fetida* (5t/ha)
C_6 - Raw Coffee husk + 6.5 g *Pleurotus eous*+ 6.5 g *Pleurotus florida* +7g *Trichoderma asperelloides*+*Eisenia fetida* (5t/ha)

3.2.4 Physico - chemical Analysis of raw and prepared Biocomposts

The physico - chemical characterization of raw and biocomposted samples were analyzed using standard procedures.

Physical parameters

The pH and Electrical conductivity of raw and composted wastes were analyzed by using pH meter (Microprocessor Infra Digi pH Meter, Model: IR 501A) and EC meter (Digital Infra Digi Conductivity Meter, Model: IR 503).

Table - 1

Chemical parameters of the compost

Parameters	Method used	References	Appendix No
Lignin (%)	Acid detergent lignin method	Goering & Van soest, (1975)	**Appendix-I**
Cellulose (%)	Neutral detergent fiber method	Updegroff, (1969)	**Appendix-II**
Organic Carbon (%)	Chromic acid wet digestion method	Wakley and Black, (1934)	**Appendix-III**
Total N (%)	Microkjeldhal Method	Humphries, (1956)	**Appendix-IV**
Total P (%)	Bray 1 Method	Jackson, (1973)	**Appendix-V**
Total K (%)	Flame photometer Method	Jackson, (1973)	**Appendix-VI**
Ca & Mg (%)	Versanate method	Jackson (1973)	**Appendix-VII**

PHASE -II

3.3 Cultivation of the selected plants

The plants selected for the experiment were moth bean (*Vigna aconitifolia* (Jacq.) Marechal) Var. RMO-40, black gram (*Vigna mungo* L. Hepper) Var. CO - 6, fenugreek (*Trigonella foenum-graecum* L.) Var. Lam Selection - 1 and Kalmegh (*Andrographis*

paniculata (Burm.f.) Nees) Var. CIM - Megha. Pot culture experiments were carried out using the test crops to study the efficiency of paddy and coffee husk vermicompost.

3.3.1 Collection of seeds

Seeds of moth bean were obtained from Jaipur, Rajasthan Agriculture Institute, black gram from Tamil Nadu Agriculture University, Coimbatore, fenugreek from N.G Ranga Agicultural University, Guntur and Kalmegh from CIMAP, Hyderabad, Telangana.

3.3.2 Details of the Treatments used

Bio composted paddy husk and coffee husk were incorporated into different treatments and used for pot culture experiments, are as follows.

Treatment Code	Treatments Composition	Biocompost
C	Control (without treatment)	Control
T_1	Raw paddy husk + 20g *Pleurotus eous* + *Eisenia fetida* (5t/ha)	Biocompost - 1
T_2	Raw paddy husk + 20g *Pleurotus florida* + *Eisenia fetida* (5t/ha)	Biocompost - 2
T_3	Raw paddy husk + 6.5g *Pleurotus eous* + 6.5g *Pleurotus florida* + 7g *Trichoderma asperelloides* + *Eisenia fetida* (5t/ha)	Biocompost - 3
T_4	Raw coffee husk + 20g *Pleurotus eous* + *Eisenia fetida* (5t/ha)	Biocompost - 4
T_5	Raw coffee husk + 20g *Pleurotus florida* + *Eisenia fetida* (5t/ha)	Biocompost - 5
T_6	Raw coffee husk + 6.5g *Pleurotus eous* + 6.5g *Pleurotus florida* + 7g *Trichoderma asperelloides* + *Eisenia fetida* (5t/ha)	Biocompost - 6

3.3.3 Pot culture Experiment with application of biocomposts

All the experimental pots were filled with 7 kg of red sandy loam soil incorporated with biocompost and mixed thoroughly while control was maintained with only soil. Nearly 20 viable seeds of *Vigna aconitifolia* (Jacq.) Marechal Var. RMO - 40, *Vigna mungo* (L.) Hepper Var. CO-6, *Trigonella foenum-graecum* L. Var. Lam Selection - 1 and *Andrographis paniculata* (Burm.f.) Nees Var. CIM - Megha were sown in each pot

for pot culture studies. Three replications were used for each treatment. Experiments were carried out at St. Joseph's College for Women (A) campus, Visakhapatnam, A.P. at the latitude 17.719185 and longitude 83.286938. All the cultural practices and protection measures were carried out according to the instructions of Krishi vigyan Kendra of N.G.Ranga Agricultural University.

3.4 Evaluation of Biometric parameters of the selected plants

Biometric parameters of test plants root length (cm), shoot length (cm), number of leaves, number of nodules, plant fresh weight (g), plant dry weight (g) for moth bean (*Vigna aconitifolia* (Jacq.) Marechal) Var.RMO-40, black gram (*Vigna mungo* (L.) Hepper) Var. CO- 6, fenugreek (*Trigonella foenum-graecum* L.) Var. Lam Selection -1 on 20, 40 and 60 DAS. Kalmegh (*Andrographis paniculata* (Burm.f.) Nees) Var. CIM-Megha on 30, 60 and 90 DAS were recorded.

3.4.1 Evaluation of the yield parameters of the test crops

The yield parameters of the test plants including number of pods per plant, number of seeds per pod, length of the pod (cm), weight of the pod (g), weight of the seeds per pod (g), fresh weight of the pod (g), dry weight of the pod (g) were recorded on 90^{th} day (moth bean, black gram and fenugreek) and 120^{th} day (kalmegh) plants. The plants were uprooted during the study as it is given above on the mentioned days after sowing (DAS).

3.4.2 Authentication of test plants

All the selected plants were prepared as herbarium and were identified by Botanical Survey of India, Deccan regional center, Hyderabad. *Trigonella foenum-graecum* L. with reference number BSI / DRC / 2022-23 / Identification / 653 (**Annexure-I).** *Vigna aconitifolia* (Jacq.) Marechal, *Vigna mungo* (L.) Hepper and *Andrographis paniculata* (Burm.f.) Nees with reference number BSI / DRC / 2022-23 / Identification / 580 (**Annexure-II).**

PHASE -III

3.5 Biochemical analysis

The leaves and seeds of selected test crops were subjected to biochemical evaluation.

3.5.1 & 2 Estimation of Protein and Carbohydrate (mg/g)

Estimation of protein and carbohydrate in leaves of moth bean, black gram and fenugreek (20, 40 and 60 DAS) kalmegh (30, 60 and 90 DAS) was recorded. In seeds it was estimated on 90 DAS (moth bean, black gram and fenugreek) and on120 DAS (kalmegh). The method used for the protein estimation is Lowry *et al*., (1951) and carbohydrate based on the method of Hedge and Hofreiter, (1962) as described in **Appendix-VIII** & **IX.**

3.5.3 Estimation of Chlorophyll (mg/g)

Estimation of chlorophyll a, chlorophyll b and total chlorophyll was recorded in the leaves of moth bean, black gram, fenugreek (20, 40 and 60 DAS) and kalmegh (30, 60 and 90 DAS). The analysis was done according to the method of Arnon, (1949) as described in **Appendix-X.**

3.5.4 Estimation of Leghaemoglobin content (mg/g)

Leghaemoglobin content was estimated in nodules of moth bean, black gram and fenugreek (20, 40 and 60 DAS) by using the method of Appleby and Bergersen, (1980) as given in **Appendix-XI.**

3.5.5 Preliminary Phytochemical assessment

The active secondary metabolites present in methanol leaf extracts *Andrographis paniculata* (Burm.f.) Nees and the seeds of *Trigonella foenum-graecum* L. were determined using the standard technique (Shaikh and Patil, 2020). The preliminary phytochemical test for alkaloids, flavonoids, sterols, anthraquinone, aminoacids, saponins, cardiac glycosides, phenols, steroids, terpenoids, tannins and glycosides were performed in accordance with the techniques given in **Appendix- XII.**

3.5.6 Preparation of the Plant Extract

Medicinally active plants of kalmegh (*Andrographis paniculata* (Burm.f.) Nees) and fenugreek (*Trigonella foenum-graecum* L.) leaves and seeds were selected for phytochemical screening. The best treatment (T_6 - Raw coffee husk + 6.5g *Pleurotus eous* + 6.5g *Pleurotus florida* + 7g *Trichoderma asperelloides* + *Eisenia fetida* (5t/ha) of kalmegh and fenugreek were used in this experiment. Fresh leaves and matured seeds were collected, cleaned and dried in the shade for 24 hours in a hot air oven at 60°C. The dried leaves and seeds were then powdered using an electric mixer to obtain a fine powder. The phytochemical elements of these powders were examined. The plant was extracted overnight with methanol extracts and filtered through Whatman No.1 filter paper. Using this extracts, qualitative tests were performed.

PHASE-IV

3.6 Analysis of Antioxidant activity of test plants

Antioxidant activity was performed using the assays on the best treatment (T_6) and control grown test plants seeds (moth bean, black gram and fenugreek) and leaves (kalmegh). The ability of test plants aqueous and methanol seed and leaf extracts to scavenge free radical was assessed by hydrogen peroxide scavenging activity, nitric oxide radical scavenging activity, reducing power assay and DPPH radical scavenging activity.

The seeds (moth bean, black gram and fenugreek) and leaves (kalmegh) were collected, shade dried, powdered and were progressively extracted using methanol and water with soxhlet extractor. The solvent extracts were concentrated by rotary evaporator and then air dried. The extracts were freeze - dried and stored in desiccators for the further use.

3.6.1 Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity of methanol and aqueous seeds and leaf extracts of test plants was carried out according to the method of Ruch *et al*., (1989) as given in **Appendix-XIV**

3.6.2 Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity of methanol and aqueous seed and leaf extracts of test plants were assessed based on the method of Green *et al*., (1982) as described in **Appendix-XV**.

3.6.3 Reducing power assay

Reducing power assay was determined in methanol and aqueous seed and leaf extracts of test plants based on the method of Oyaizu, (1986) as elaborated in **Appendix-XVI**.

3.6.4 DPPH radical scavenging activity

DPPH (2, 2 - diphenyl-1-picrylhydrazyl radical scavenging activity) radical scavenging assay was carried out in methanol and aqueous extracts (seed and leaf) of test plants. The extracts were estimated by using the method of Mensor *et al*., (2001) given in (**Appendix-XIII**)

3.7 Antibacterial activity

The antibacterial activity of selected test plants methanol and aqueous extracts were determined using the standard method of (Bauer *et al.,* 1996), as given in **Appendix- XVII.** Mueller – Hinton agar was used to assess the antibacterial activity of moth bean, black gram and fenugreek (seeds) and kalmegh (leaf). Antibacterial activity of the test crops was evaluated using the bacterial strains such as gram negative bacterial strains *Escherichia coli*, *Vibrio cholerae* and gram positive bacterial strains *Staphylococcus aureus.* These strains were obtained from KGH, Microbiology Department, Visakhapatnam, Andhra Pradesh.

Antibacterial activity was evaluated for the best treatments (T_6 - Raw coffee husk + 6.5g *Pleurotus eous*,6.5g *Pleurotus florida*,7g *Trichoderma asperelloides* + *Eisenia fetida* (5t/ha) and control of all the four test plants (moth bean, black gram, fenugreek and kalmegh) by using Agar well diffusion method with triplicates. The safety measures followed during antibacterial testing in the lab to prevent contamination were sterilization of all equipment, glassware and media before and after use. Lab coat, sterile surgical gloves, safety shield was used.

3.8 Experimental pre and post-harvest soil analysis

Soil analysis	Reference	Method
Available Nitrogen	Subbiah and Asija, (1956)	Alkaline permanganate (**Appendix-XVIII)**
Available Phosphorus	Jackson, (1973)	Colorimetry **(Appendix- XIX)**
Available Potassium	Stanford and English, (1949)	Flame photometry **(Appendix-XX)**

Statistical nalysis of the Data

The research findings are provided in the form of tables and graphs using Microsoft Office Excel (Version 2007) soft ware. The obtained data of the microbial population during biocomposting, vegetative and biochemical parameters for moth bean (*Vigna aconitifolia* (Jacq.) Marechal) Var. RMO - 40, black gram (*Vigna mungo* (L.) Hepper) Var. CO - 6, fenugreek (*Trigonella foenum-graecum* L.) Var. Lam Selection - 1 on 20, 40, 60 DAS and 30, 60 and 90 DAS for kalmegh (*Andrographis paniculata* (Burm.f.) Nees) Var. CIM - Megha, yield parameters on 90 DAS (moth bean, black gram and fenugreek) and 120 DAS (kalmegh) antioxidant and antibacterial activity of the seed extracts of the best treatment and control plants were subjected to SPSS (Version Sigma stat 3.1) statistical analysis of variance (One -way and Two - way ANOVA). Conclusions were drawn based on the results. The inference was drawn according to the results obtained and these values of tables and figures are provided in chapter 4 (Results and