3

EXPERIMENTAL PROCEDURE

Green leafy vegetables constitute a major part of any balanced diet and are good sources of phytonutrients. Green plants absorb all the major nutrients from the soil which they require for diverse anabolic processes. Leafy vegetables are vital dietary sources of minerals, trace elements and phytochemicals with health protective and immune strengthening properties (Ali *et al.,* 2008).Widespread empirical use of wild plants demands accurate and reliable information on their phytochemicals and antioxidant activity, as well as on the potential benefits and prospective products, such as nutraceuticals. The complex organic compounds thus manufactured in plant leaves contain antioxidant molecules for protection against oxidative stress (Aquil *et al.,* 2005).

Phytochemical extracts from fruits, vegetables and whole grains have strong antioxidant activity and the major part of total antioxidant is from the combination of phytochemicals. Antioxidants act by free radical mediated process and therefore they protect the human body from numerous diseases affecting different tissues and organs. They help to prevent the deleterious effects caused by free radicals by acting as scavengers. Free radicals and reactive oxygen, nitrogen species (ROS and RNS) are formed as a result of normal cellular oxidative metabolic reactions (Boussaada *et al.,* 2008).The problem of environmental pollution on account of essential industrial growth is due to the problem of disposal of industrial waste whether solid, liquid or gaseous. Polluted water, in addition to other effects, directly affects soil not only in industrial areas but also in plants. However, in excessive amounts, these can be detrimental to soils or plant growth. Effluent can pose environmental, public health or agricultural resource risks if not managed properly. Treatment of effluent is difficult and represents a severe environmental and technological problem due to the presence of a series of chemicals with low biodegradability. So the treatment of effluents is a matter of great concern in the country. The ubiquitous nature of bacteria makes important tools in effluents bio-treatment (Maier *et al*., 2004). The treated effluent irrigation is encouraged when it is safe and practicable to do so and where it provides the best environment.

Hence, attention has been devoted to the commonly available green leafy vegetables, which are exploited to different conditions of fresh water, untreated crude effluent and biotreated effluent.

The study on **"Biofertilizer mediated remediation of silk dyeing industrial effluent and its effect on selected green leafy vegetables"** was studied in four phases.

Phase 1

- **Growth studies of selected GLVs grown in fresh water**
  - Five green leafy vegetables such as mustard (*Brassica juncea*), fenugreek (*Trigonella foenum*), sirukeerai (*Amaranthus polygonoides*), araikeerai (*Amaranthus tristis*) and agati (*Sesbania grandiflora*) were selected and grown in fresh water under normal conditions which serve as the control.
  - The plants were uprooted on the 45th day without any damage and analysed for various parameters like biometric, phytochemicals, proximate principles, minerals, antioxidant and pigments.
  - Physico-chemical analysis of untreated control soil was carried out.
  - Histological study was performed on the stem, the leaf and the root sections of GLVs grown in fresh water.

Phase 2

- **Characterization of silk dyeing effluent and studies of its effect on the growth of the selected GLVs**
  - The physico-chemical analysis of the collected silk dyeing industrial effluent was carried out.
  - A bioassay test was carried out using fish to check the toxicity of the collected effluent.
  - *Invitro* preliminary studies were performed for the randomly selected three biofertilizers *(Rhizobium sp., Pseudomonas fluorescens* and *Azospirillum sp.)* and decolorization of the silk dyeing industrial effluent was calculated.
  - Based on the results, the decolorizing biofertilizers were treated with different concentrations of effluent supplemented with co-substrate (Glucose).

- The best decolorizing biofertilizers (*Pseudomonas fluorescens* and *Azospirillum sp.*) were inoculated in silk dyeing industrial effluent separately and incubated for 7 days. Then they were subjected to phytohormones analysis such as IAA and GA_3.
- The selected green leafy vegetables (GLVs) were exposed to different concentrations (25%, 50%, 75% and 100%) of the effluent and the highest concentration of effluent in which the GLVs survived were analysed for the various parameters as in phase 1.
- Physico-chemical analysis of silk dyeing industrial effluent contaminated soil was carried out.
- Histological study was performed on the stem, the leaf and the root sections of GLVs grown in the highest concentration of effluent at which the GLVs survived.

Phase 3

❖ **Growth studies of selected plants grown in biotreated effluent**

- Physico-chemical analysis of biotreated silk dyeing effluent by best percentage decolorizing biofertilizers (*Azospirillum sp.* and *Pseudomonas fluorescens*) was performed.
- A bioassay test was carried out using fish to check the removal of toxicity in biotreated effluent.
- The best decolorizing biofertilizer (*Pseudomonas fluorescens*) was chosen and mixed with crude effluent which was treated to the selected GLVs and analysed for parameters as mentioned in phase 1.
- Physico-chemical analysis of biotreated effluent soil was carried out.
- Histological study was performed on the stem, the leaf and the root sections of the selected GLVs grown in biotreated effluent.

Phase 4

❖ **Identification of functional groups and compounds in selected GLV plants of different treatments using spectroscopic and chromatographic techniques**

- UV visible analysis was carried out on the methanolic extract of the selected GLVs.

- FT-IR analysis of the selected GLVs grown in fresh water, crude effluent and biotreated effluent and selected dyes in silk dyeing effluent was performed.
- HPLC analysis was carried out on the selected GLVs grown in fresh water, crude effluent and biotreated effluent and untreated silk dyeing effluent and biotreated effluent.

The detailed experimental design of the four phases of the study are as follows:

3.1. Collection of silk dyeing effluent

The silk dyeing effluent was collected from the effluent disposal site of small scale silk dyeing industry in airtight plastic containers, located at Seelanaickenpatti in Salem district and the technical details such as the type of dyes used and their composition from the silk dyeing industry were also obtained.

3.2. Collection of Biofertilizers

The biofertilizers such as *Rhizobium sp., Pseudomonas fluorescens* and *Azospirillum sp.* were collected from the Tamil Nadu Agricultural University, Coimbatore.

3.3. Physico-chemical analysis of the silk dyeing effluent

The physico-chemical characterization was analyzed in samples of crude silk dyeing effluent, treated effluent with biofertilizers (*Azospirillum sp.* and *Pseudomonas fluorescens*) of higher percentage decolorization rate.

3.3.1. Physical characterization

The appearance of the small silk dyeing effluent was analysed for the presence or absence of turbidity. The color of the silk dyeing effluent was determined by visual comparison. The odour in the silk dyeing effluent was determined by qualitative Human receptor. The silk dyeing effluent was checked for the presence or absence of foam.

The reference for methodology and the appendix number for the evaluation of turbidity, total dissolved solids, BOD, COD and electrical conductivity are given in Table 1.

3.3.2. Chemical characterization

The pH of the silk dyeing effluent was noted by potentiometric method. The temperature in the silk dyeing effluent was noted by mercury thermometer.

The reference for methodology and the appendix number of the estimation of pH alkalinity, total alkalinity, total hardness, calcium, magnesium, sodium, potassium, iron,

manganese, free ammonia, nitrite, nitrate, chloride, fluoride, sulphate, phosphate, chromium, lead, nickel, zinc and cadmium are given in Table 1.

3.3.3. Bioassay Test

Effluent water discharges to receiving water bodies characterized by alkaline pH values could exacerbate NH_3–N toxicity and threaten the viability of various fish species (Follett, 2001). The heavy metals when exposed to organisms constitute a particular risk that they have the tendency to accumulate in vital organs (Akoto *et al.,* 2008). Hence a bioassay test was conducted by collecting fish from an aquarium and stored in well aerated tanks. The fish were fed with protein foods for a week. Two days prior to the start of the experiment the protein food was stopped (Natarajan and Gowri, 1991). One group of individuals was acclimated in fresh water and they acted as the control, the second group in crude silk dyeing effluent and the third group in biotreated effluent. About 96 hrs was chosen for the bioassay and the mortality rate was noted (Finney, 1971).

3.4. Decolorization of silk dyeing effluent by *Rhizobium sp., Pseudomonas fluorescens* and *Azospirillum sp.*

3.4.1. Preliminary studies

a) Screening for decolorization and determination of biodegradation activity of silk dyeing effluent

0.02g of the different biofertilizers like *Rhizobium sp., Pseudomonas fluorescens* and *Azospirillum sp.,* were inoculated in three different conical flask containing general yeast mannitol (YM broth), King's B medium and Azospirillum medium respectively. The medium compositions are given in Appendix 1.

Dyeing effluent was taken as the sole source of carbon and energy. The crude effluent was diluted and 10% of the effluent was inoculated with a loop of culture from different cultivated media in three different conical flasks and incubated at 28 ^{o}C in shaking incubator at 150 rpm for 20 days. The percentage of decolorization was determined spectrophotometrically at 600 nm (Thiripurasundari *et al.,* 2013).

3.4.2. Decolorization of silk dyeing effluent by *Rhizobium sp., Pseudomonas fluorescens* and *Azospirillum sp.,* supplemented with co-substrate

Four different concentrations of crude silk dyeing effluent (25%, 50%, 75% and 100%) supplemented with glucose (0.002g) as co-substrate were selected in three sets of conical flask. To each flask, a specific microbe about 2×10^5 colony forming units (CFU)

was inoculated with a loop of culture from three different cultivated medium of *Rhizobium sp., Pseudomonas fluorescens* and *Azospirillum sp.* and incubated at 28^0C in shaking incubator at 150 rpm for 10 days. At 24 hours interval the samples were analyzed for degradation.

5.0 ml was centrifuged at 5000 rpm for 15 minutes and the optical density of the supernatant was measured spectrophotometry at 620 nm (Tripathi and Srivastava, 2011).

The percentage decolorization was calculated for each flask by the formula $(A_0-A_t) / A_0 \times 100$.

Where A_0 = Absorbance of raw effluent at time zero, A_t = Absorbance of treated effluent at the end of 20 days post microbial inoculation. From which the microbe with the highest percentage decolorization was selected for further studies.

3.5. Quantification of Phytohormones (IAA, GA_3)

A loop of culture from the cultivated medium of plant growth promoting *Azospirillum sp.* and *Pseudomonas fluorescens* was inoculated separately to each flask of 100% crude silk dyeing industrial effluent with freshly prepared, sterilized solution of L-tryptophan. It was incubated at 37^oC in dark for seven days. After incubation, the cultures were centrifuged at 6,000 rpm for 5 minutes to remove the bacterial cells. The supernatant was brought to pH 2.8 with 1N HCl (Megala and Elango, 2013). The *invitro* production of phytohormones such as indole acetic acid (IAA) and gibberellic acid (GA_3) was determined by the procedures as given in appendices 27 and 28 respectively.

3.6. Soil preparation for the study

The red soil and the sand were mixed at the ratio of 3:1. Each pot was filled with 7 kg of soil. In Phase 1 and Phase 3, five GLVs were grown with four replicates. In phase 2, three pots for each of the four different concentrations (25%, 50%, 75% and 100%) were used. The biofertilizer, *Pseudomonas fluorescens* was mixed at the rate of 5 tonnes ha^{-1} with crude effluent and used in Phase 3. The bacterial concentration of the biofertilizer was 10^8 Colony forming units (CFU) ml^{-1}.

3.7. Selection of GLVs for the study

Five green leafy vegetables namely *Brassica juncea* (mustard), *Trigonella foenum* (fenugreek), *Amaranthus polygonoides* (sirukeerai), *Amaranthus tristis* (araikeerai) and

Sesbania grandiflora (agati) have been selected to study their growth in normal, effluent and biotreated effluent conditions as pot study in Phase 1, 2 and 3.

Brassica juncea

Scientific classification

Kingdom : Plantae

Order : Brassicales

Family : Brassicaceae

Genus : *Brassica*

Species : *Brassica juncea*

Brassica juncea

Trigonella foenum

Scientific classification

(unranked) : Eudicots

(unranked) : Rosids

Order : Fabales

Family : Fabaceae

Genus : *Trigonella*

Species : *Trigonella foenum*

Trigonella foenum

Amaranthus tristis

Scientific classification

Kingdom : Plantae

(unranked) : Angiosperms

(unranked) : Eudicots

(unranked) : Core eudicots

Order : Caryophyllales

Family : Amaranthaceae

Genus : *Amaranthus*

Species : *Amaranthus tristis*

Amaranthus tristis

Amaranthus polygonoides

Scientific classification

Kingdom : Plantae

Order : Caryophyllales

Family : Amaranthaceae

Genus : *Amaranthus*

Species : *Amaranthus polygonoides*

Amaranthus polygonoides

Sesbania grandiflora

Scientific classification

Kingdom : Plantae

(unranked) : Angiosperms

(unranked) : Eudicots

(unranked) : Rosids

Order : Fabales

Family : Fabaceae

Genus : *Sesbania*

Species : *Sesbania grandiflora*

Sesbania grandiflora

3.8. Collection of Seeds

Seeds of mustard (*Brassica juncea*), fenugreek (*Trigonella foenum*), sirukeerai (*Amaranthus polygonoides*), araikeerai (*Amaranthus tristis)* and agati (*Sesbania grandiflora*) were collected from Superseeds Nursery, Coimbatore.

3.9. Seed sowing and maintenance of plants

About 20 seeds were sown in each pot and were allowed to germinate. Neem cake was mixed with water and poured around the pots as pest control.

Fresh water, silk dyeing effluent of different concentrations (25%, 50%, 75% and 100%) and crude silk dyeing effluent treated with *Pseudomonas fluorescens* have been used in Phase 1, Phase 2 and Phase 3 respectively. After germination, 100% moisture condition was maintained throughout the study.

3.10. Harvest methodology

The plants were harvested on the 45th day without any damage. The adhering soil particles were removed by washing gently with water and the water droplets were removed by blotting with the filter paper. Then these plants were subjected to various analysis like biometric parameters, phytochemical constituents, proximate principles, minerals contents, antioxidants, pigments, histological studies and phytohormone quantification.

3.11. Biometric observations

Biometric observations of the GLV plants were recorded on the 45th day of growth. Measuring plants' vegetative growth is by capturing enough data on the overall health of the plants.

3.11.1. First cotyledon

The number of days from the date of the seed sowing till the first cotyledon emerged was recorded.

3.11.2. Germination percentage

The seeds that germinated from 20 sown seeds were counted and the percentage germination was calculated.

3.11.3. Seedling length

The height of the uprooted plant from the tip of the root to top of main plant stem at 45th day of growth was measured and was expressed as cm per plant.

3.11.4. Number of leaves

Every visible leaf on the plant was counted, including the tips of the new leaf just beginning to emerge. The plant was kept over some graph paper to avoid counting errors. The number of leaves of each plant was counted on the 45th day after sowing and was expressed as number of leaves per plant.

3.11.5. Surface area of leaves

The leaves of different plants were hand-drawn onto the grid paper. The 2 cm grid paper showed both the basic leaf parts (blade and petiole). Since the petiole did not contribute much to photosynthesis, it was removed from the other drawings. The squares

covered by each leaf were counted and the surface area was estimated. For each calculated surface area, whole squares located within the leaf area drawing were identified and multiplied by the appropriate area of each grid size (i.e. the area for each square using 2 cm grid paper = 4 cm^2). Squares which included part of the leaf surface were added up and then divided by 2 since only part of the surface was included within the square (Gerber and Mccool, 2014).

3.11.6. Root length

The Root length was measured from the crown region of the plant to the tip of the root and expressed as cm per plant.

3.11.7. Shoot length

The Shoot length was measured from the point of first cotledonary node to the tip of the longest leaves and expressed as shoot length in cm per plant.

3.11.8. Root - Shoot ratio

The harvested plants were dried in an oven overnight at low heat. The plants were left to cool in a dry environment by keeping it in a ziploc bag (which will keep the moisture out) whereas in a humid environment the tissue will take up water). Once the plants got cooled, they were weighed. The root and the shoot were separated and weighed. The Root - Shoot ratio was calculated

Root - Shoot ratio = Dry weight of roots / Dry weight of shoot

3.11.9. Root mass

The plant from the soil was removed. The adhering soil particals were removed by washing gently with water and the water droplets were removed by blotting with the filter paper and the roots were separated, weighed and expressed as grams per plant.

3.11.10. Dry matter production

The plants were dried in an oven at low heat overnight. The plants were cooled in a dry environment, than in a humid environment. Once the plants got cooled, weighed and calculated by the formula.

Total Dry Matter (Total DM) = (Dry weight of the plant / Initial weight of the plant) x 100.

3.11.11. Number of Seed bunch

The number of the seed bunch was counted and recorded as seeds per plant.

3.11.12. Distance between nodes

The distance between the nodes of the plant were measured and expressed in cms.

3.12. Phytochemical analysis

3.12.1. Preparation of plant extracts

The leaf, the stem and the seed of the selected plants were air dried and powdered separately using a blender. The phytochemicals were analyzed as described in Appendix 29.

3.13. Proximate analysis

The proximate priniciples namely moisture content, ash content, crude fibre, total carbohydrate, total protein and cellulose were analyzed in the samples on the 45th day of the GLV plants grown in fresh water, crude effluent (75%) and biotreated effluent.

3.13.1. Determination of Percentage moisture content

The uprooted plant was weighed on the 45th day. The plant sample was air dried and the dry weight was noted. The percentage moisture content was calculated as follows:

Wet weight = Total weight - Dry weight

Percentage moisture content = (Wet weight/Total weight) x 100

3.13.2. Estimation of Ash content

Five grams of the plant sample was weighed accurately into a platinum or porcelain crucible, which had been previously heated to about 600˚C and cooled. The crucible was placed over a low flame till all the material was completely charred followed by heating in a muffle furnace for about 3½ hrs at 600˚C. It was then cooled in a dessicator and weighed. To ensure completion of ashing, the crucible was again heated in the muffle furnace for 1 hr, cooled and weighed. This was repeated till the two consecutive weights were the same and the ash was almost grayish white in colour (A.O.A.C, 1990).

Ash content (g/ 100g sample) = (Weight of the ash/Weight of the sample taken) X 100

The reference for methodology and the appendix number for the estimations of crude fibre, total carbohydrate, total protein and cellulose are given in Table 1.

3.14. Mineral analysis

The minerals such as iron, phosphorus, calcium and magnesium were analyzed in samples of the plants grown in fresh water, crude effluent (75%) and biotreated effluent.

The reference for methodology and the appendix number for the estimations of iron, phosphorus, calcium and magnesium are given in Table 1.

3.15. Antioxidant status and Lipid peroxidation

The enzymic antioxidants namely superoxide dismutase and peroxidase and the non-enzymic antioxidants namely total phenol, ascorbic acid and tocopherol and lipid peroxidation (hydrogen peroxide scavenging activity and DPPH activity) were analyzed in the plants grown in fresh water, crude effluent (75%) and biotreated effluent.

3.15.1. Enzymic and Non-enzymic antioxidants

Natural antioxidants can protect the human body from free radicals and retard the progress of many diseases. Many mineral and vitamins have antioxidant properties.

3.15.2. Lipid peroxidation

The toxicity of reactive oxygen species is to initiate cascade of reactions that in turn leads to the production of the hydroxy radical which can cause lipid peroxidation that can be prevented by antioxidants.

The reference for methodology and the appendix number for the estimations of superoxide dismutase, peroxidase, total phenol, ascorbic acid, tocopherol, hydrogen peroxide scavenging activity and DPPH activity are given in Table 1.

3.16. Pigment analysis

The total chlorophyll (Chl_a and Chl_b) and carotenoids were analyzed in the post harvested plants grown in fresh water, crude effluent (75%) and effluent biotreated water.

The reference for methodology and the appendix number for the estimations of total chlorophyll and carotenoids are given in Table 1.

3.17. Soil analysis

The soil which was treated with freshwater for the growth of the GLVs served as the control soil. The crude effluent soil treated with 100% of the crude silk dyeing effluent

without dilution and the effluent biotreated soil is the soil treated with the biotreated effluent. The analysis of control soil, crude effluent soil and effluent biotreated soil samples in the initial stages of treatment was carried out.

The reference for methodology and the appendix number for the measurement of soil pH and electrical conductivity are given in Table 1.

3.17.1. Measurement of soil texture

The soil texture refers to the relative proportion of sand, loam, silt and clay present in the soil. Based on these proportions, the soil used in this study was classified and identified into various textural classes. Clayey soil has a larger percent of clay. They were considered more fertile than the sandy soil but were difficult to work. Clay has small particles which are less than 0.002 mm in diameter and are referred to as soil colloids. Sandy soil is easy to work but is less fertile. They have low water retention capacity. Sand has large particles which are coarse and individual particles are easily visible (0.02 - 2 mm in diameter). Loamy soil is in between sandy and clayey soils. They are best for airable cropping. Silt soil has medium-sized particles which are 0.002 - 0.02 mm in diameter.

The reference for methodology and the appendix number for the estimations of calcium, total nitrogen, available phosphorus, available potassium, zinc, copper, iron and manganese in soil are given in Table 1.

3.18. Histological studies

The leaf, the stem and the root of the tissues of *Brassica juncea*, *Trigonella foenum*, *Amaranthus polygonoides*, *Amaranthus tristis* and *Sesbania grandiflora* in all the three phases on the 45th day were stained with Giemsa and Saffranin as described in Appendices 53 and 54. The images were examined under the microscope and snapped using the Motic digital camera associated with Motic Image plus 2.0 software.

3.19. Identification of functional groups, organic compounds and pigments in the selected GLVs.

3.19.1. UV visible analysis of selected GLVs

The *Brassica juncea*, *Trigonella foenum*, *Amaranthus polygonoides*, *Amaranthus tristis* and *Sesbania grandiflora* plants were air dried and 20gms of finely powdered material of each were taken in a thimble and extracted using 200ml of

HPLC grade methanol in Soxhlet apparatus. The methanolic extracts of selected GLVs were subjected to Bio-nano UV visible spectrophotometer at different wavelength to find its maximum absorbance peak.

3.19.2. FT-IR analysis of selected GLVs plants and selected dyes

FT-IR (Fourier Transform Infrared) analysis provides spectral information that is essentially a molecular fingerprint for organic, polymeric and in some case inorganic materials. This technique is extremely useful for identifying base polymer compositions and organic contaminants. The FT-IR spectrum of the unknown material can be compared for "best matches" with libraries of spectra that have been cataloged for known materials. The FT-IR analysis was carried out for the selected plants of the GLVs grown in fresh water, crude effluent and biotreated effluent to identify the organic compounds.

1. **In Phase 1,** the selected plants Mustard (*Brassica juncea*), Fenugreek (*Trigonella foenum*), Sirukeerai (*Amaranthus polygonoides*), Araikeerai (*Amaranthus tristis*) and Agati (*Sesbania grandiflora*) treated with the fresh water were removed on the 45th day, air dried, powdered and subjected to FT-IR.

2. **In Phase 2,**

   a) The selected plants were grown in crude silk dyeing effluent till 45 days. The effluent exposed plants were air dried, powdered and subjected to FT-IR.

   b) From the technical information obtained from the small scale industry, the dyes (Direct 2y2, Direct yellow 5gll, Procell pineapple) used were subjected to FT-IR.

3. **In Phase 3,** the selected plants were treated with the biotreated silk dyeing effluent. The effluent biotreated plants were air dried, powdered which was subjected to FT-IR.

3.19.3. HPLC analysis of selected GLV plants.

3.19.3.1. Sample preparation for HPLC analysis

1. **In Phase 1,** the selected plants *Brassica juncea*, *Trigonella foenum*, *Amaranthus polygonoides*, *Amaranthus tristis* and *Sesbania grandiflora* treated with the fresh water, on the 45th day were removed, air dried and ground into powder. The HPLC grade methanol was purchased from Fischer Scientific & Co.

20 grams of each dried powder sample was weighed and then packed in Whatmann filter paper placed in thimble of soxhlet apparatus. The HPLC grade methanol of 200 ml each was taken in round bottom flask and the extract was prepared, then stored in the dark at 4°C which was subjected to HPLC analysis at 450 nm.

2. **In Phase 2,** the selected plants were treated with the crude silk dyeing effluent. At the end of the 45th day, the effluent exposed plants were removed, air dried and powdered. The methanolic extracts of the plants were obtained from soxhlet apparatus as in phase 1 and subjected to HPLC analysis at 450 nm.

   i) The collected crude silk industrial effluent and degraded effluent by *Pseudomonas fluorescens* were filtered and subjected to HPLC analysis at 510 nm.

3. **In Phase 3,** the selected plants were treated with the biotreated silk dyeing effluent. The plants grown in biotreated effluent were uprooted on the 45th day and air dried and powdered. The methanolic extracts of selected plants were obtained from soxhlet apparatus as in phase 1 and subjected to HPLC analysis at 450 nm.

All the peaks were analysed between the phases and were compared with the standards of pigments (chlorophyll C2, chlorophyll C3, chlorophyll B, carotene), alkaloid (caffeine) and monosaccharides (glucose, fructose, mannose and galactose) which was subjected to HPLC analysis at 450 nm.

3.19.3.2. Chromatographic conditions

The chromatographic system was equipped with column C18 with 3µl particle size (50×4.6 mm I.D) and detector UV- VIS model SPD 20A at specific nanometer at a flow rate of 1ml/min. The solvent HPLC methanol was used with the stream of liquid N_2 until it reached nearly 0.5 ml and then some mobile phase was added to reach 1ml. Then 20µl of the methanolic extract of the sample were injected into HPLC column. The presence of each compound was determined by comparison of peak area of the samples with that of the standard.

3.19.3.3. Mobile phase and solutions

1. The mobile phase prepared with a mixture of methanol: water (70:30) was used for the HPLC analysis of methanolic extracts of the untreated, crude effluent and biotreated plants.

2. The mobile phase with a binary mixture of acetonitrile: water (60:40) was used for crude effluent and biotreated silk dyeing industrial effluent.

3. The mobile phase for standards such as pigments, alkaloid caffeine and monosaccharides were prepared with 20% methanol and 0.2% phosphoric acid, methanol: water (80:20) and acetonitrile: water (90:10) respectively.

3.20. Statistical analysis

One way Anova using DMRT (Duncan's Multiple Range Test) method was employed to predict the results of various analysis of all the untreated, effluent exposed and effluent biotreated plants.

Table 1

Analysis of physico-chemical parameters of silk dyeing effluent, phytohormones, proximate principles, minerals, antioxidants, pigments, soil analysis and histological studies

Parameters	Sample	Reference	Appendix Number
PHYSICO-CHEMICAL ANALYSIS OF SILK DYEING EFFLUENT			
Turbidity NT units	Untreated and biotreated effluent	APHA, 1995	2
Total Dissolved solids			3
BOD_5 at 20◦C		APHA, 2005	4
COD		APHA, 2005	5
Electrical conductivity		APHA, 1995	6
pH Alkalinity as $CaCO_3$		Natarajan *et al*., 1988	7
Total Alkalinity as $CaCO_3$		APHA, 1995	8
Total Hardness as $CaCO_3$		APHA, 2005	9
Calcium			10
Magnesium			11
Sodium		Natarajan *et al.,*1988	12
Potassium			
Iron		Shanmugam *et al.,* 1994	13
Manganese		APHA, 1995	14
Free ammonia		APHA, 1995	15
Nitrite		APHA, 2005	16
Nitrate		APHA, 2005	17

Parameters	Sample	Reference	Appendix Number
Chloride		Vogel, 1978	18
Fluoride		APHA, 2005	19
Sulphate		APHA, 2005	20
Phosphate		APHA, 1995	21
Chromium	Predigested untreated and treated effluent	Photometric kit method	22
Lead			23
Nickel			24
Zinc			25
Cadmium			26
PHYTOHORMONES QUANTIFICATION			
Indole acetic acid (IAA)	*Pseudomonas fluorescens* and *Azospirillum sp.,* inoculum in Silk dyeing effluent	Gordon and Paleg, 1957	27
Giberrellic acid (GA_3)		Borrow *et al.,*1955	28
ANALYSIS OF PROXIMATE PRINCIPLES			
Crude fiber	Plant dry sample	Maynard, 1970	30
Total Carbohydrate	Fresh plant sample	Hedge and Hofreiter, 1962	31
Total Protein		Lowry *et al*., 1951	32
Cellulose		Elena, 2012	33
MINERAL ANALYSIS			
Iron	Plant dry sample	Wong, 1928	34
Phosphorus		Fiske and Subbarow, 1925	35
Calcium		A.O.A.C, 1990	36
Magnesium			
ANTIOXIDANT STATUS			
Superoxide dismutase(SOD)	Fresh Plant sample	Misra and Fridovich,1972	37
Peroxidase activity		Reddy *et al*., 1995	38
Total phenol		Malick and Singh, 1980	39
Ascorbic acid		Roe and Kuether, 1953	40
Tocopherol		Rosenberg, 1992	41
H_2O_2 Scavenging assay		Ruch *et al.,* 1989	42
DPPH activity		Mensor *et al*., 2001	43

Parameters	Sample	Reference	Appendix Number
PIGMENT ANALYSIS			
Chlorophyll	Fresh leaves	Witham *et al.,* 1971	44
Carotenoid	Fresh leaves	Zakaria *et al*., 1979	45
SOIL ANALYSIS			
pH	Control soil, Effluent contaminated soil, and biotreated soil	Jackson, 1962	46
EC		Jackson, 1962	47
Calcium		Cheng and Bray, 1951	48
Available Nitrogen		Johan Kjeldahl,1883	49
Available Phosphorus		Olsen *et al.*, 1954	50
Available Potassium		Toth and Prince, 1949	51
Iron		Jackson, 1962	52
Manganese			
Copper			
Zinc			
HISTOLOGICAL STUDIES			
Saffranin O staining	Leaf, stem and root of GLVs grown in control soil, untreated effluent soil and biotreated soil	Jong Kwiton, 1970	53
Giemsa staining		Giemsa, 1904	54