ISOLATION AND CHARACTERIZATION OF A PIGMENT FROM FUNGUS, *Colletotrichum falcatum*

A PROJECT REPORT

Submitted by

VINOTH KISHORE, V
(Reg. No.1120203017)

in part fulfillment for the requirement of award of the degree

of

M.TECH (BIOTECHNOLOGY)

FACULTY OF TECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY, COIMBATORE 641 049
(An Autonomous Institution Affiliated to Anna University, Chennai)

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BONA FIDE CERTIFICATE

Certified that this project report entitled **ISOLATION AND CHARACTERIZATION OF A PIGMENT FROM FUNGUS, *Colletotrichum falcatum*** is the bona fide work of **Mr. VINOTH KISHORE, V (Reg. No. 1120203017)** who carried out the research under my supervision. Certified further that, to the best of my knowledge, the work reported herein does not form part of any other project report or dissertation, of the basis of which, a degree or award was conferred on an earlier occasion on this or any other candidate.

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ABSTRACT

Pigments are present in most of the living organisms providing attractive colors, and play an important role in their development. Use of synthetic pigments is not eco-friendly; the demand for natural pigments, especially for fabrics, foods, feeds, cosmetics and printing inks is increasing. Recently, microbial sources of pigment have found application in the medical field, food and textile industries. The aim of the present study was to isolate and characterize the pigments from the fungal source, *Colletotrichum falcatum.* The fungus was isolated from 'red rot' infected sugarcane, and grown on PDA agar. The effects of carbon and nitrogen sources as well as pH were examined on the pigment production. Four types of carbon sources - fructose, sucrose, maltose and soluble starch were tried. Of these, the soluble starch supported maximal dry fungal mass weight (3.0 g/100 ml) and with the pigment production of about 0.467 g/100 ml. Soy peptone as a nitrogen source yielded maximal dry cell weight (4.6 g/100 ml) and pigment production of about 0.365 g/100 ml among different nitrogen sources - beef extract, $NaNO_3$, KNO_3, $(NH_4)_2SO_4$, tried. Increased dry cell weight (4.3 g/100 ml) and pigment production 0.38 g/100 ml was found at optimum pH 5.0. The presence of flavonoid was confirmed using $FeCl_3$ and $AlCl_3$ tests. Total polyphenolic compound was estimated to be 41.08 mg/g of *Colletotrichum falcatum* mass using the total polyphenol assay expressed gallic acid equivalent. Dyeing along with premordanting, simultaneous mordanting and post mordanting was carried out and examined for shades.

Key words: *Colletotrichum falcatum,* Dyeing, Flavonoid, Pigment, Soluble starch, Soy peptone, sugarcane red-rot.

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(VINOTH KISHORE, V)

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1
INTRODUCTION

1.1 PIGMENTS

Pigments are chemical compounds that reflect light in the wavelength range of the visible region. The color produced is due to a molecule-specific structure (chromophore), this structure captures the energy and the excitation of an electron from an external orbital to a higher orbital is produced, the non-absorbed energy is reflected and/or refracted to be captured by the eye, and generated neural impulses are transmitted to the brain where they could be interpreted as a color.

The colorants become the most sensitive part of any commodity not only for its appeal but also, it enhances consumer acceptability. Although, Aristotle and other ancient scientists attempted to understand the nature of light and color vision, it was not until Sir Newton, that light was identified as the source of color sensation and defined as a class of spectra that reciprocates to the same color sensation. Such classes vary widely among different species, and to a lesser extent among individuals within the same species.

The production of the synthetic colorants is economically efficient and technically advanced but many artificial synthetic colorants, usually used in foodstuff, dyestuff, cosmetics and pharmaceutical manufacturing processes, cause various hazardous including diseases such as cancer and other behavioral problems in children. To counteract the ill-effects of synthetic colorants, there is a widespread interest in process development for the production of colorants from natural sources due to the serious environment and safety problems caused by many artificial colorants (Kim *et al*., 1995).

Biological pigments, also simply known as pigments or biochromes, are substances produced by living organisms that have a color resulting from selective color absorption. Natural colors are generally extracted from fruits, vegetables, roots of plants and microorganisms and often called 'BIOCOLORS' because of their origin. A number of natural carotenoids, prodigiosin and violacein pigments, produced by microbes have found application in medical arena due to their activities as immunosuppressive, anticancer, antibacterial and antifungal agents. According to a latest review, fungal pigments could be used as a reliable source of natural colorants. Fungal pigments are generally biosynthesized as secondary products during metabolism.

TABLE 1.1.1 LIST OF MICROBIAL PIGMENTS

Sl. No	Microorganism	Molecule	Color
1	*Penicillium oxalicum*	Anthroquinone	Red
2	*Xanthophyllomyces dendrorhous*	Astaxanthin	Pink-red
3	*Xanthomonas oryzae*	Xanthomonadin	Yellow
4	*Monascus roseus*	Canthaxanthin	Orange-pink
5	*Pacilomyces farinosus*	Anthroquinone	Red
6	*Serratia rubidaea*	Prodigiosin	Red
7	*Serratia marcescens*	Prodigiosin	Red
8	*Blakeslea trispora*	Lycopene	Red
9	*Dunaliella salina*	ß-carotene	Orange

1.1.1 RED ROT DISEASE

The importance of sugar in human diet needs no introduction; it has become part and parcel of daily life. Sugar is produced mainly from sugarcane (*Saccharum officinarum*) and sugar-beet and over 75 per cent of the world sugar comes from sugarcane.

Sugarcane is a tropical, perennial grass that forms lateral shoots at the base to produce multiple stems, typically three to four meters high and about five cm in diameter. The stems grow into cane stalk, which when mature constitutes approximately 75% of the entire plant mass. A mature stalk is typically composed of 11–16% fiber, 12–16% soluble sugars, 2–3% non-sugars, and 63–73% water. The sugarcane crop is sensitive to the climate, soil type, irrigation, fertilizers, insects, diseases control, varieties, and the harvest age. The average yield of cane stalk is 60–70 tonnes per hectare per year. However, this figure can vary between 30 and 180 tonnes per hectare depending on the knowledge and crop management approaches used in sugarcane cultivation. Sugarcane is a cash crop, but it is also used as a livestock fodder.

Fig. 1.1.1 Red-rot infected sugarcane.

Numerous pathogens infect sugarcane, such as sugarcane grassy shoot disease caused by *Phytoplasma*, whiptail disease or sugarcane smut, *pokkah boeng* caused by *Fusarium moniliforme*, *Xanthomonas Axonopodis* bacteria causes Gumming Disease, and red rot disease caused by *Colletotrichum falcatum*. Viral diseases affecting sugarcane include sugarcane mosaic virus, maize streak virus and sugarcane yellow leaf virus.

The fungus causing red rot of sugarcane is commonly known by its imperfect state, *Colletotrichum falcatum* Went. The disease was first described to the scientific world in 1893 (Went) from Java (now Indonesia). Later Von Arx and Muller from Germany transferred this fungus *Physalospora tucumanensis* to the genus *Glomerella* and renamed it as *Glomerella tucumanensis*.

Red rot is not easily identified from the external appearance of the cane unless it has caused severe damage by rotting the internal tissues. When sufficient stalk and sugar have formed, typical stalk rot phase occurs and red color is due to the production of phenolic compound by the fungus. Depending on the age of the stalk, time of infection and susceptibility of the cane genotype, it produces different types of symptoms. The typical stalk symptoms i.e., the presence of white spots in otherwise rotten (red spot) internodal tissues. The red spot is due to the production of acervuli and white spot is due to conidiospores. Red rot infected cane exhibits an increase in amino acids and phenolic compounds. The resistance of genotype is correlated with the intensity of red pigment production.

Red-rot causes poor stands of both plant and stubble crops as a result of the deterioration of the seed cuttings and the stubble rhizome and the inversion of sucrose in mill cane, resulting in low recovery of sugar at the factory. The effect of diseased crop is felt not only in reduced tonnages of cane but also in the lower sucrose content of the juice as well as resulting from the delayed maturity of the cane.

1.1.2 EXTRACTION

Extraction is the process of separating biologically active compounds using selective techniques through standard procedure. The compounds present in the red substance were found to be 3-deoxyanthocyanidin flavonoids, luteolinidin, apigeninidin (Viswanathan *et al*., 1996). Phenolic compounds constitute one of the main classes of secondary metabolites. They are responsible for the major organoleptic characteristics of colors and taste properties. They also contribute to the nutritional qualities of food. The complex mixture obtained from the red rot fungal extracts are polyphenols and flavonoid derivatives.

1.1.3 POLYPHENOLS/FLAVONOID

Flavonoids are polyphenolic compounds that are ubiquitous in nature and are categorized, according to chemical structure into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones. Over 4,000 flavonoids have been identified, many of which occur in fruits, vegetables and beverages (tea, coffee, beer, wine and fruit drinks). The flavonoids have arousen considerable interest, recently, because of their potential, beneficial effects on human health; they have been reported to have antiviral, anti-allergic, anti-platelet, anti-inflammatory, anti-tumor and antioxidant activities. Polyphenols, rather than exerting direct antioxidant effects, the mechanisms by which they express these beneficial properties appear to involve their interaction with cellular signaling pathways and related machinery that mediate cell function under both normal and pathological conditions.

1.1.4 ANTIOXIDANT ACTIVITY

Antioxidant compounds are necessary to protect the cells from the damage caused by free radicals. Free radicals are generated due to the imbalance between the antioxidant and reactive oxygen species. Hence, dietary intake of antioxidant is important. Plants are the major source of antioxidant compounds. Several reports indicated various novel antioxidant compounds isolated from endophytic fungi and they exhibited potent radical scavenging activity. Phenolic compounds are the principal antioxidant constituents of natural products and composed of phenolic acids and flavonoids, which are potent radical terminators acting by donating hydrogen radicals. It is found that apigeninidin has diverse pharmacological activities and has demonstrated antioxidant and anti-carcinogenic properties.

Antioxidants are compounds that protect cells against the damaging effects of reactive oxygen species, such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite. An imbalance between antioxidants and reactive oxygen species results in the oxidative stress, leading to cellular damage. Oxidative stress has been linked to cancer, aging, atherosclerosis, ischemic injury, inflammation and neurodegenerative diseases (Parkinson's and Alzheimer's).

1.1.5 FOOD COLORANTS

Color is the main feature of food, which determines its appeal to the consumers. Biocolorants are those coloring agents, which are obtained from the biological sources. Biocolorants are mainly derived from pigments like anthocyanidin, carotenoids etc. However, there are biocolorants, that are not pigments in any state like structural color and light emitting luciferin (Chattopadhyay *et al*., 2008). Color is added to food for one or more of the following reasons: to replace color in the food which is lost during processing, to enhance color of the food already present, to minimize batch-to-batch variations, to color otherwise uncolored food, and to supplement food with nutrients (Mortensen *et al.*, 2006).

Colors derived from minerals (lead chromate, copper sulphate) cause serious health problems and environment hazards (Francis *et al.,* 1989). Thus, in the last few decades, synthetic additives have been severely criticized, and consumers show reluctance towards these products; consequently they prefer to use the natural colorants. The demand for natural colours is thus increasing day-by-day because of health-promoting properties of biocolorant food; Natural colors have been the consumer priority. Low-fat content is the objective for many new or improved food formulations, replacing fats with thickeners or other food additives and increased consumer preferences for organic food.

1.1.6 DYEING

Dyeing is the process of adding color to textile products like fibers, yarns and fabrics. Dyeing is normally done in a special solution containing dyes and particular chemical material. After dyeing, dye molecules have uncut chemical bond with fiber molecules. The temperature and time controlling are two key factors in dyeing.

Fig 1.1.6 Pigment.

Production of synthetic dyes is dependent on petrochemical sources, and some of the synthetic dyes contain toxic or carcinogenic amines which are not ecofriendly (Samanta *et al.,* 2001). Moreover, global use of textiles is estimated at around 30 million tonnes, which is expected to grow at the rate of 3% per annum.

Microbial dyes have some advantages over plant-animal based dyes as microbes are fast-growing and have the potential of being standardized, commercially. Synthetic dyes, if at all degraded, are full of by-products that are directly or indirectly proven to be health hazards; such hazardous compounds have so far not been detected in the microbial dye degraded byproducts. It is possible that natural dyes completely degrade under natural conditions. Though all natural dyes are not 100% safe, they are less toxic than their synthetic counterparts. Many of the natural dyes like turmeric, annatto and saffron are permitted as food additives. Many natural dyes have pharmacological effects and possible health benefits.

1.1.7 APPLICATIONS

The major active ingredients in plants are flavonoids. As is typical for phenolic compounds, they can act as potent antioxidants and metal chelators. They have also been recognized to possess anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral and anticarcinogenic activities.

Anthocyanins are interesting pigments regarding their chromate features. Anthocyanins have recently attracted even more interest due to their possible health attributes, such as reduced risk of coronary disease, reduce risk of stroke, anticarcinogenic activity and improved cognitive behavior.

The use of bio-colorants may show benefits over synthetic colors**.** Natural dyes are less toxic, less polluting, less hazardous, non-carcinogenic and non-poisonous (Siva *et al.,* 2007) and prevent chronic diseases such as prostate cancer. In addition to this, they are harmonizing colors, gentle, soft and subtle, and create a restful effect. Most of them are water-soluble (anthocyanins), which facilitate their incorporation into aqueous food systems. These qualities make natural food colorants attractive (Liu *et al.,* 2004). Above all, they are environment-friendly and can be recycled after use. Thus, they attribute to food both for aesthetic value and quality judgement and also they tend to yield potential positive health effects, as they possess potent antioxidant and improve visual acuity properties.

1.2 OBJECTIVES

To replace the synthetic colors used in various industries which are harmful to environment with natural colorant derived from natural source, the project is initiated with the following objectives:

- To isolate and screen the pigment producing microbes from plant sources
- To characterize the screened pigments
- To identify the compounds present in the screened pigment fraction and
- To apply the extracted natural dye on cotton fabric using natural mordant.

REVIEW OF LITERATURE

Pigments produce the colors that we observe at each step of our lives, because pigments are present in each one of the organisms in the world; among them plants are the principal producers. They are in leaves, flowers, vegetables, fruits; they are also present in skin, eyes, and other animal tissues as well as in bacteria and fungi. Natural and synthetic pigments are used in medicines, foods, clothes, furniture, cosmetics and in other products. However, natural pigments have important functions other than the imparted beauty.

Pigments can be classified by their origin as natural, synthetic or inorganic. Natural pigments are produced by the living organisms such as plants, animals, fungi and microorganisms. Synthetic pigments are chemically synthesized from laboratories. Natural and synthetic pigments are organic compounds. Inorganic pigments can be found in nature or produced by synthesis (Delgado-Vargas *et al*., 2000).

1.3 RED ROT FUNGUS

Ranking among one of the worst sugarcane diseases in some countries is red rot, caused by the fungus, *Colletotrichum falcatum.* It is widely distributed throughout the tropics, and is one of the most serious diseases in India, Louisiana and Australia. It has been attributed with losses of from 25 to 50 per cent of the crop. Within the past decade; it has caused the complete failure of one of the leading commercial sugarcane varieties and has shown indications of increasing severity on other varieties after a few years of large scale commercial cultivation. It is found that the presence of 3-deoxyanthocyanidin flavonoids, luteolinidin and apigeninidin in the red substance (Viswanathan *et al., 1996*). These compounds may also serve as phytoalexins in sugarcane.

1.3.1 FLAVONOIDS

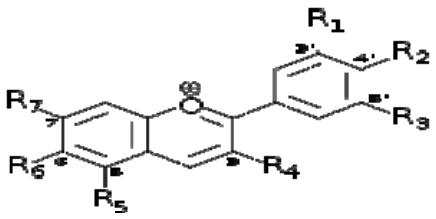
Anthocyanins are water-soluble vacuolar pigments that may appear red, purple or blue depending on the pH. They belong to a parent class of molecules called flavonoids synthesized via the phenylpropanoid pathway. They are odorless and nearly flavorless contributing to taste as a moderately astringent sensation. Today, interest in anthocyanin pigments has intensified because of their possible health benefits as dietary antioxidants. Over 300 structurally distinct anthocyanins have been identified in nature. Anthocyanins are one class of flavonoid compounds, widely distributed as plant polyphenols. Flavonols, flavan-3-ols, flavones, flavanones, and flavanonols are additional classes of flavonoids that differ in their oxidation state from the anthocyanins.

There are six major anthocyanidins acting as central chromophores of anthocyanins: pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin which differ only in the extent of ß-ring hydroxylation.

Recent studies using purified anthocyanins or anthocyanin-rich extracts *invitro* experimental systems have confirmed the potency of these pigments. Demonstrable benefits include protection against liver injuries, significant reduction of blood pressure, improvement of eyesight, strong anti-inflammatory and antimicrobial activities, inhibition of mutations caused by mutagens from cooked food, and suppression of proliferation of human cancer cells (Izabela Konczakl *et al*,. 2004).

1.3.2 DISTRIBUTIONS OF FLAVONOIDS

Anthocyanins are water-soluble glycosides of polyhydroxyl and polymethoxyl derivatives of 2-phenylbenzopyrylium or flavylium salts.



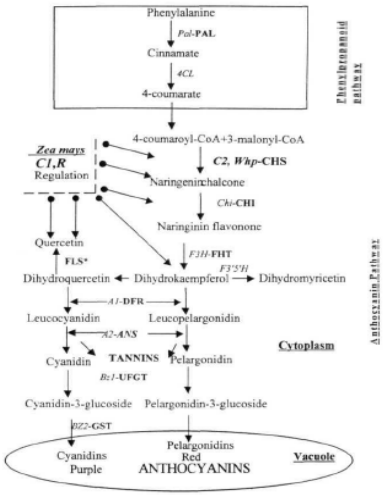
1.3.2 Anthocyanidin structure.

The six anthocyanidins commonly found in plants are classified according to the number and position of hydroxyl groups on the flavan nucleus, and are named - cyanidin (cy), delphinidin (dp), malvidin (mv), peonidin (pn), pelargonidin (pg) and petunidin (pt). The differences between individual anthocyanins come from the number and the position of hydroxyl groups, the degree of methylation of these hydroxyl groups, the nature, number and location of sugars attached to the molecule, and aliphatic or aromatic acids attached to the sugars in the molecule (Mazza *et al*., 2004). Glycosylation confers increased structural stability and water solubility to the parent anthocyanidin.

Acylation of the sugar residues with cinnamic (*p*-coumaric, caffeic, ferulic) or aliphatic (acetic, malonic, succinic) acids further improves anthocyanin stability. Generally, di-, tri-, or polyacylated anthocyanins have increased stability over simple and monoacylated anthocyanins (Cacace *et al*., 2004).

1.3.3 FLAVONOID BIOSYNTHESIS

The biosynthesis pathway leading to the production of flavonoids and anthocyanins has been described and the responsible enzymes have been identified, characterized and reviewed (Heller and Forkmann, 1988). Flavonoids are produced in the cytosol of cell. It is assumed that the enzymes of flavonoids biosynthesis form a super-molecular complex through protein-protein interaction and held in the endoplasmic reticulum membrane. These biosynthetic enzymes are categorized in various enzyme families, such as 2-oxoglutarate-dependent dioxygenases (OGD), cytochromes P450 (P450) and glycosyl transferases (GT) (Tanaka *et al*., 2010). The enzymes associate as loose aggregate and interaction among the constituent enzymes allow the direct transfer of substrates from one enzyme to enzyme and the channeling of intermediates. As a result, the final products are collected into the various places like hydrophilic derivatives are accumulated in the vacuole and lipophilic compounds in epidermal cells or exuded from roots (Dinelli *et al*., 2006). The key precursors for the synthesis of flavonoids are phenylalanine and malonyl-CoA produced from shikimate pathway and the TCA cycle, respectively. Phenylalanine is converted into cinnamic acid by phenylalanine- ammonia lyase (PAL), a generally tetrameric, ubiquitous enzyme in the plant kingdom. Cinnamic acid undergoes hydroxylation reaction to form p-coumaric acid mediated by cinnamic acid 4-hydroxylase (CA4H) which requires molecular oxygen and NADPH.



1.3.3 Schematic representation of the flavonoid biosynthesis pathway.

The *p*-coumaric acid may be converted to 4- coumaroyl-CoA by *p*-coumarate CoA ligase (4CL) (Davies *et al*., 2009). In the phenylpropanoid pathway, *p*-coumaroyl CoA is located at the junction of the metabolic routes resulting to flavonoids or to phenylpropanoid compounds. The *p*-coumaroyl CoA is a substrate of chalcone synthase which catalyzes the production of the flavonoid skeleton by condensation of p-coumaroyl CoA with three malonyl CoA, under release of three carbon dioxide molecules. A linear phenyl propanoid tetraketide 4, 2′, 4′, 6′- tetrahydroxy chalcone is formed via intramolecular cyclization and aromatization. The formation of flavanones from chalcones then occurs through an isomarization catalyzed by the enzyme chalcone isomerase (CHI). Various biosynthesis enzymes further down this pathway are accountable for catalyzing the conversion of flavanones into the different flavonoid molecules by hydroxylation, oxidation, reduction, glycosylation, methylation and acylation. All flavonoids are synthesized from a common precursor, (2S)-naringenin (flavanones), through modifications by various tailoring enzymes e.g. flavone synthase 1 (FLS 1) synthesizes apigenin from (2S)-naringenin and flavanone 3 α-hydroxylase (F3H) and flavonol synthase (FLS) sequentially convert (2S)-naringenin into dihydrokaempferol and kaempferol. FLS 1, F3H, and FLS belong to the 2- oxoglutarate dependent oxygenases that are nonheme iron dioxygenases utilizing 2-oxoglutarate as a cofactor.

1.3.4 EXTRACTION

Extraction is the separation of biologically active portions of plants or microorganisms using selective solvents through standard procedures. The products obtained from microbes contains complex mixtures of metabolites, in liquid or semisolid state (after removing the solvent) in dry powder. During extraction, solvents diffuse into the solid plant material and solubilize compounds with similar polarity (Handwa *et al*., 2008). The products obtained contains complex mixture of many metabolites such as alkaloids, glycosides, terpenoids, flavonoids and lignans.

Anthocyanin, like flavonoids in general, have aromatic rings containing polar substituent groups (hydroxyl, carboxyl, and methoxyl) and glycosyl residues that altogether produce a polar molecule. Consequently, they are more soluble in water than in nonpolar solvents, but depending on the media conditions anthocyanins could be soluble in ether at a pH when the molecule was unionized. These characteristics aid in the extraction and separation of anthocyanin compounds (Harborne *et al*., 1988).

1.3.5 ANTIMICROBIAL PROPERTY

Antimicrobial compounds are low molecular weight natural substances synthesized by microbes that inhibit the growth of other microbes even at low concentrations (Tang *et al*., 2008). These are used to treat various infectious diseases caused by bacteria, fungi, virus, protozoa, etc. Many pathogen become multi drug resistances, they developed resistant mechanism against existing antibiotics. Hence, the treatment against these resistant species becomes ineffective. Endophytic microorganisms are capable of synthesizing novel antimicrobial compounds that belong to several structural classes like alkaloids, flavonoids, terpenoids, peptides, steroids, phenols and quinines (Zhang *et al.,* 2010).

Extraction of biologically active compounds from the culture broth was done using suitable solvent after fermentation. The bioactive compounds from the *Colletotrichum falcatum* were extracted using ethyl acetate. The extract was screened for antibacterial activity against pathogens. Ethanol extract was more active against 80% of the organisms tested. It was followed by methanolic extract (70%), benzene (50%) and chloroform extract (40%) in inhibiting the growth of the organisms tested. Petroleum ether and hexane extracts did not show any antibacterial activity (Kandhasamy *et al*., 2008).

Antimicrobial activity of anthocyanin compounds extracted from fruits was evaluated by Schaefer *et al.,* (2008). It showed the highest antioxidant activity and high level of phenolics. Anthocyanins reduced fungal growth by 50% in the concentrations that typically characterize unripe blackberries and by 95% in the concentrations that typify ripe blackberries.

1.3.6 MORDANTS

A mordant is a substance used to set dyes on fabrics or tissue sections by forming a coordination complex with the dye which then attaches to the fabric or tissue. It is used for dyeing fabrics, or for intensifying stains in cell or tissue preparations. In the past, it was thought that a mordant helped the dye bind onto the fiber so that it would hold fast during washing. A mordant is often a polyvalent metal ion. The resulting coordination complex of dye and ion is colloidal and can be either acidic or alkaline.

Mordants and dyes may be applied in three ways:

i. Pre-mordanting, where the mordant is applied first, followed by dyeing,
ii. Post-mordanting, where the dyeing is done first and then mordanting is carryout, and
iii. Simultaneous mordanting, where mordant and dye are mixed together and applied.

It is often remarked that the addition of a mordant to an appropriate dye solution results in a very sudden, dramatic change in color. This is due to the incorporation of the metal atom into the delocalized electron system of the dye. Metals have relatively low energy levels, so their incorporation into a delocalized system results in lowering of the overall energy. The absorbance of the hue and thus its color is related to this phenomenon.

The stability of the mordants for printing fibres with natural dyes varies from dye to dye. In the study of marigold flower dye, mustard color was produced when chrome was used as a mordant and olive green color was obtained on mordanting with copper sulphate (Radhika *et al*., 2007).

CHAPTER 2

MATERIALS AND METHODS

2.1 COLLECTION OF SAMPLE MATERIALS

Plant samples were collected from Saravanampatti, Coimbatore, and the samples were stored in sterile containers and maintained at 4°C.

2.2 ISOLATION OF FUNGUS

Red rot infected sugarcane stalks were inoculated on Potato Dextrose Agar (PDA). The composition of PDA include potato infusion from 200 g/l, Dextrose 20 g/l, agar 15 g/l and adjucted to final pH 5.6±0.2.

2.3 CULTIVATION OF PURE CULTURE

The fungus *Colletotrichum falcatum* was isolated and cultivated individually in 500 ml Sabouraud Dextrose Broth (yeast – 1 g, malt extract – 1 g, glucose – 6 g, peptone – 2 g, K2HPO4 - 0.5 g, MgSO4 - 0.5 g, $FeSO4.7H_2O$ - 0.01 g). Final pH 6.0. The fermentation was carried out in 500 ml flasks containing basal medium at room temperature on a rotary shaker at 150 rev/min for 12 days.

2.4 EXTRACTION

After the incubation for 12 days, the cultured broth was centrifuged at 10,000 rpm for 10 min, then the supernatant saved and stored at 4°C. Equal amount of ethyl acetate and the supernatant was transferred into a separating funnel, shaken well and left undisturbed for 20 min. The upper organic phase was saved.

2.5 LYOPHILIZATION

The organic phase separated was freeze-dried in a lyophlizer. The lyophilized sample was dissolved in distilled water and used for further studies whenever required.

2.6 MORPHOLOGICAL IDENTIFICATION

The isolates were identified using the Lactophenol Cotton Blue method (LPCB). Lactophenol Cotton Blue wet mount preparation is the most widely used method of staining and observing fungi. It can also be used to examine at filaments and higher life forms under a microscope.

i. A drop of wastewater sample was placed on a microscope slide
ii. One or utmost two drops of the LPCB stain was added.
iii. The cover slip was folded between forefinger and thumb, one edge of the drop of sample was touched with the cover slip edge, and lowered gently, avoiding air bubbles.
iv. Morphological character was examined under a light microscope and recorded.

2.7 IDENTIFICATION OF FLAVONOIDS –CHROMATOGRAPHIC ANALYSIS

Glass plates (20 × 10 cm) were placed on an even surface. Silica gel dissolved in water was applied as a thin slurry onto the glass plate using an applicator. The plates were dried for 24 h to remove moisture and other adsorbed substances from the surface so as to activate the plate. Forty µl of organic solvent extract of fungal samples was spotted on each plate using a micropipette. Spot was placed 2 cm above the base of the plate and the spotted area should not dip in the mobile phase. The chromatography tank was saturated for 24 h with mobile phase - ethyl acetate: formic acid: acetic acid: distilled water (25: 2.75: 2.75: 6.5). The plates were carefully placed to develop the chromatogram and air-dried before spraying liquid ammonia (detecting agent). Moderate amount of the reagent was sprayed onto the plate so that it always appeared dull and flat. The plates were then viewed under while light, and short UV to detect the phytoconstituents present.

2.8 ANTIMICROBIAL ASSAY

For the assay of antimicrobial susceptibility of crude extract, standard Kirby-Bauer (1956) methodology was followed. Antibacterial activities of red pigment were estimated by solid media bioassay test. Assay plates were prepared using Mueller Hinton Agar (MHA); (25 ml) poured in 90 mm x 15 mm Petri plates. The agar surface of the Petri plates was seeded with 0.1 ml of saline suspension of a 24 h grown test bacteria. A paper disc impregnated with the sample compound was placed at the centre of the seeded agar plates and incubated at 37°C. Size of inhibition zones around the paper discs were measured after 24 h of incubation.

2.9 DETERMINATION OF PHENOL BY FOLIN-CIOCALTEAU'S METHOD

Total phenolic content in the crude ethyl acetate extract was determined following to the method of Folin Ciocalteau's (Singleton & Rossi, 1965) method. (1998).

i. 0.1 to 0.5 ml of working solution (gallic acid) was taken in a series of test tubes
ii. 0.1 ml of sample was taken in another test tube
iii. The volume was made up to 4 ml with distilled water
iv. 0.5 ml of Folin's reagent was added to each test tube and the contents incubated at room temperature for 3 min
v. 2 ml of 20% sodium carbonate solution was added to each test tube and warmed in boiling water bath for one minute
vi. Absorbance was read at 750 nm
vii. Phenolic content present in the sample was expressed as gallic acid equivalent.

2.10 DETERMINATION OF TOTAL ANTI-OXIDANT ACTIVITY BY PRIETO METHOD

This assay was based on the principle of reduction of Molybdenum (VI) to Molybdenum (V) by the extract and the subsequent formation of green phosphate /Molybdenum complex at acidic pH.

The reagents used were as follows:

- Reagent solution (0.6 M Sulphuric acid, 28 mM Sodium phosphate and 4 mM Ammonium molybdate)
- Ascorbic acid
- Stock solution (1 mg/ml)

- Working solution (1 ml of stock solution was taken and made up to 10 ml with distilled water 100µg/ml).

Procedure

The working solutions (100-500 µg/ml) of samples were prepared by diluting the extracts in water. 0.1 ml of the extract was mixed with 1ml of reagent solution. The tubes were incubated at 95˚C for 90 min. The tubes were then cooled to room temperature and the absorbance measured at 695 nm using water blank. Ascorbic acid was used as standard. The total antioxidant capacity expressed as ascorbic acid equivalent.

Total antioxidant activity is calculated using the following formula (Kumar *et al*., 2008)

Ascorbic acid equivalent (µM/g) = (T/S)*C*(V/P)*(RS/E)*(1*MW)
T – OD of test solution,
S – OD of standard,
C – Concentration of test (µg),
V – Volume of solvent used for extraction (ml),
P – Amount of powder (g),
RS – Volume of reagent solution (ml),
E – Volume of extract (ml) and
MW – Molecular weight of ascorbic acid (176.13 g/ mol).

- The test microbial suspensions were prepared by dispersing a loopful of culture in 2 ml sterile saline and turbidity adjusted to McFarland standard solution
- The lawn cultures of test pathogens were made on Muller-Hinton agar (MHA) using sterile cotton swab with the prepared bacterial suspensions
- Four wells were cut out using a sterile cork borer in the MHA plates
- 25µl of crude extract and negative control (ethanol) was placed into different wells. The MHA plates were incubated for overnight at 37°C and examined.

2.13 FABRIC DYEING METHOD

The colored filtrate from the broths were then used for dyeing fabrics. The cotton fabrics were desized at 120°C for 30 min to prepare for dyeing. The samples were pre-mordanted with 5% of natural mordants like orange, grape and pomegranate, separately. Samples were subjected to simultaneous mordant and post mordanting. Finally, unmordanted and mordanted fabric, each weighing 1g, was dyed in 50 ml of colored filtrate using MLR 1:30, dyeing time 45 min and temperature 90°C. After dyeing, washing of the samples was carried out followed by rinsing in cold water.

2.11 FLAVONOID CONFIRMATION TEST

Aluminium chloride test (Harborne, 1969)

Two drops of 1% $AlCl_3$ was added to 1 ml of the extract. Dark brown color indicates the presence of flavonoid.

Ferric chloride test (Harborne, 1969)

To a few ml of extract, add a pinch of $FeCl_3$ added. Indication of brown color confirms the presence of flavonoid.

2.12 ANTI-MICROBIAL ACTIVITY

The ethanolic extract of the red rot fungus *Colletotrichum falcatum* was tested against the following pathogenic bacteria:

- *Escherichia coli*
- *Pseudomonas aeruginosa*
- *Staphylococcus aureus*
- *Bacillus subtilis* and
- *Klebseilla pneumonia*

The test organisms above were inoculated in nutrient broth and incubated at 37˚C for 8 h.

Antimicrobial assay

- 0.5M McFarland solution was prepared and its turbidity measured and adjusted at 680nm

CHAPTER 3

RESULTS AND DISCUSSION

3.1 ISOLATION AND IDENTIFICATION OF ORGANISM

The fungus has been isolated from red rot sugarcane. It has been identified as *Colletotrichum falcatum* and its morphological characters were studied under a light microscope.

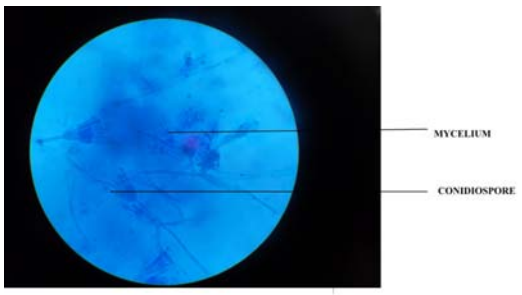


Fig 3.1.1 Microscopic view of *Colletotrichum falcatum*.

3.2 DETERMINATION OF ANTIMICROBIAL ACTIVITY

Disc diffusion method was performed on crude fungal extracts. Test microorganisms used were Gram negative bacteria such as *E. coli, P. aeruginosa, K. pneumoniae* aa well as Gram positive bacteria such as *B. subtilis* and *S. aureus.* These are all common human pathogens that cause urinary tract infection (UTI), diarrohea, upper respiratory tract infection, wound infection and meningitis. The fungal extracts were, hence, screened for any novel antimicrobial compounds which inhibit the growth of human pathogens.

Positive control: Streptomycin 10µg/disc

Negative control: Methanol

Table 3.2.1 Disc marking and extract name

Sl.No	Disc marking	Samples
1	+ve	Streptomycin (10µg/disc)
2	-ve	Methanol (20µl)
3	F1	*Colletotrichum falcatum* extract (20µl)

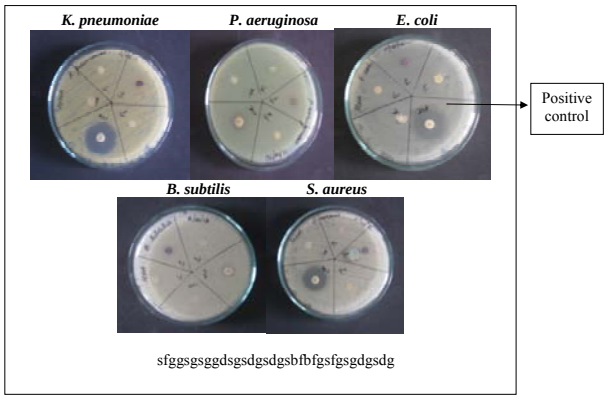
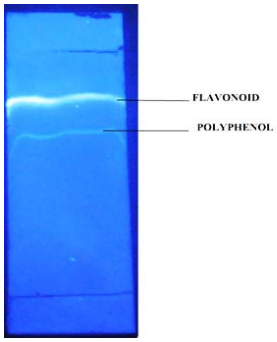


Fig. 3.2.1 Antimicrobial activity assay of *Colletotrichum falcatum*.

The fungal extracts showed no positive results. This observation indicates that the fungus did not possess any antibacterial activity. The zone of inhibition was observed only in positive control. i.e., streptomycin.

3.3 IDENTIFICATION OF COMPOUNDS

The crude *Colletotrichum falcatum* extract was separated by Preparative Thin Layer Chromatography (PTLC). The separated spot was detected by UV transilluminator.



3.3.1 Identification of flavonoid and phenolic acids of *Colletotrichum falcatum* extract by TLC under UV light.

Separation of the soluble extracts by silica gel in TLC demonstrated the presence of compounds. The UV detection of the fungal extract has proved the presence of phenolic acids and flavonoids. The spot observed in the TLC of fungal extract was successfully recovered and subjected for LC-MS analysis.

3.4 LIQUID CHROMATOGRAPH-MASS SPECTROMETRY (LC-MS)

Phenolic acids and flavonoids seem to be universally distributed in the plant kingdom. Due to the abundance of different classes of flavonoids and phenolic acids and their diverse properties, a variety of separation and identification methods have been developed using Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) and Gas Liquid Chromatography (GLC).

The TLC fraction of aqueous fungal extract was sent to IISC BENGALURU to perform LC-MS with ESI for first peak.

The MS profile is as follows:

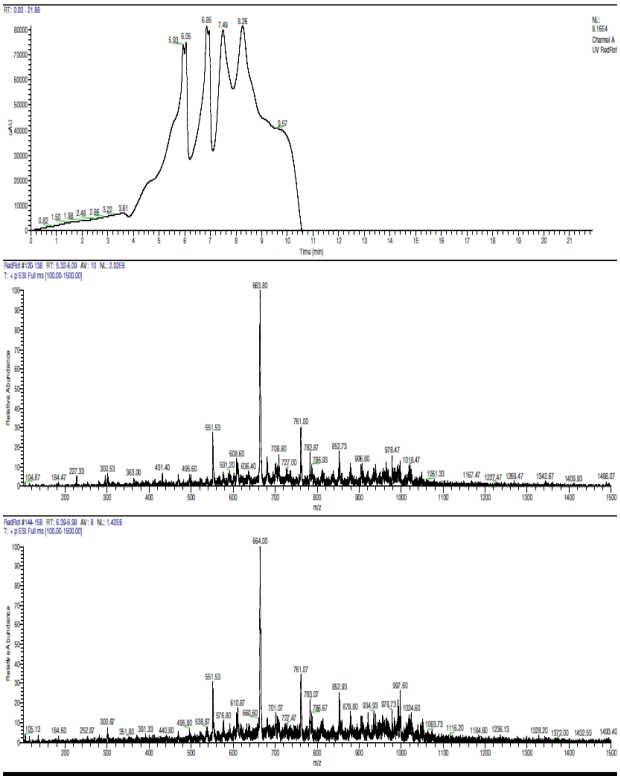


Fig 3.4.1 Mass spectrograph of pigment on LC-MS.

Table: 3.4.1 Validation of LC-MS report

S.No	Compound	Molecular Weight	MS^1 Base peak ion	MS^2 Parent ion
1.	3,5-dicaffeoyiquinic acid	516.45	680.33	677
2.	Rutin	610	611.33	609
3.	Leutolin	286	279.60	285
4.	Cyanidin-3-glucoside	484.83	453.73	449.10
5.	Rhamnosyl dihexosyl luteolin	756.21	757	756

According to Lin *et al.,* (2010), the MS/MS spectrum of standard luteolin revealed two distinct peaks at m/z 287 and 285 and base peaks with m/z 256, 266. A similar MS/MS spectrum has been recorded in the present study at m/z 279.0 and base peak at m/z 285 has proved the presence of **luteolin** in the sample.

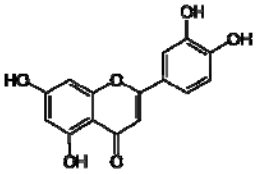


Fig 3.4.2 Structure of luteolin.

According to Hou *et al.,* (2005), the MS/MS spectrum of standard rutin revealed two distinct peaks at m/z 611. A similar MS/MS spectrum has been recorded in the present study at m/z 609 and base peak at m/z 611.33 has proved the presence of **rutin** in the sample.

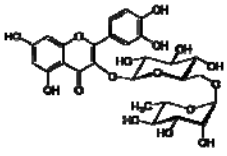


Fig 3.4.3 Structure of rutin.

According to Daise *et al.,* (2010), the MS/MS spectrum of standard cyaniding-3-glucoside revealed distinct peaks at m/z 449. A similar MS/MS spectrum has been recorded in the present study at m/z 453.73 and base peak at m/z 449. The samples were identified as cyaniding-3-glucoside.

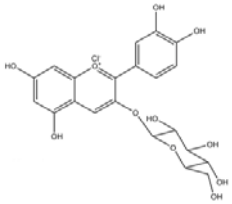


Fig 3.4.4 Structure of Cyanidin-3-glucoside.

According to Hong *et al.,* (2006), the MS/MS spectrum of standard rutin revealed distinct peaks at m/z 756. A similar MS/MS spectrum has been recorded in the present study at m/z 757 and base peak at m/z 756. The peaks were identified as rhamnosyl-dihexosyl luteolin isomers.

3.5 DETERMINATION OF TOTAL PHENOL CONTENT

Aromatic compounds present in the sample reduce Folin's reagent. Phosphomolybdo -tungstate was reduced to heteropolymolybdenum which results in dark blue colored that was read at 650 nm. For determination of phenolic content in the crude extract, gallic acid was used as a standard. Total phenolics present in the sample is expressed as gallic acid equivalent (GAE).

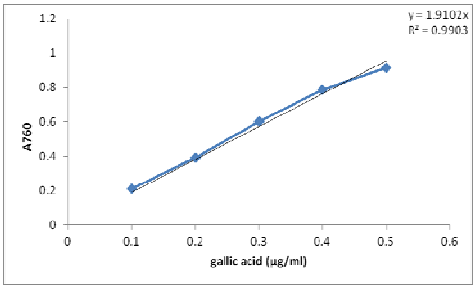


Fig 3.5.1 Standard graph of gallic acid to estimate the phenol content in the fungal extract.

Table: 3.5.1 Total phenolic content in the crude extracts

Sl. No.	Sample	Phenolic content (mg GAE/ml crude extract)
1	*Colletotrichum falcatum*	41.08±0.348

3.6 ESTIMATION OF TOTAL FLAVONOID CONTENT

Aluminium chloride forms acid-stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, it also forms acid labile complexes with the ortho-dihydroxyl groups in the α- or β-ring of flavonoids. For determination of flavonoid content in the crude extract, rutin was used as a standard. Total flavonoid present in the sample is expressed as rutin equivalent. The absorbance was read at 510 nm.

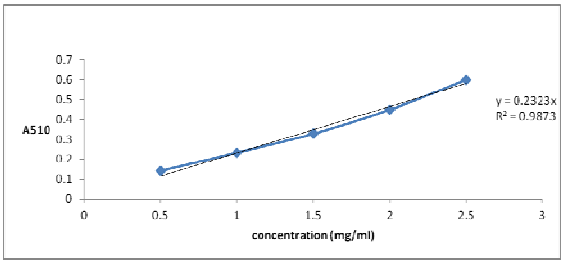


Fig 3.6.1 Standard graph of rutin to estimate total falvonoid content.

Table 3.6.1 Total flavonoid content in the crude extracts

Sl. No.	Sample	Flavonoid content (mg /g) of rutin equivalent
1	*Colletotrichum falcatum*	6.08±0.218

3.7 FLAVONOID CONFIRMATORY TEST - QUALITATIVE ANALYSIS

Aluminium chloride test

Two drops of 1% $AlCl_3$ was added to 1 ml of the extract. Dark-brown color indicates the presence of flavonoid.

Fig 3.7.1 Flavonoid confirmatory test using $AlCl_3$ method.

Ferric chloride test

To a few ml of extract, added a pinch of $FeCl_3$. Brown color confirms the presence of flavonoid.

Fig 3.7.2 Flavonoid confirmatory test using $FeCl_3$ method.

3.8 EFFECT OF CARBON AND NITROGEN SOURCES ON PIGMENT PRODUCTION

To select a suitable carbon source for the pigment production, *Colletotrichum falcatum* was cultivated in the basal medium containing various carbon sources (2%). Of the four carbon sources examined - fructose, sucrose, maltose and soluble starch were relatively favorable to the mycelial growth of *Colletotrichum falcatum* although the pigment production was low. The maximal mycelial growth (3.8 g/100 ml) was obseved in maltose medium, while the maximum pigment production (0.469 g/100ml) was obtained in soluble starch containing medium.

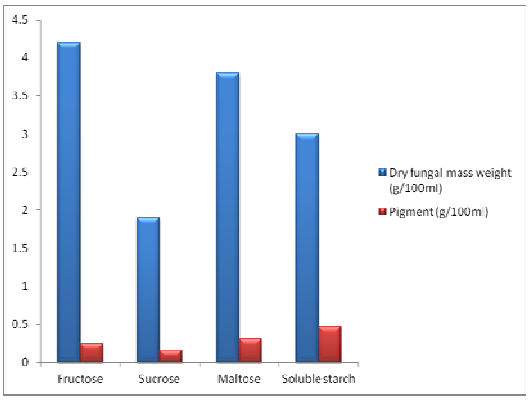


Fig 3.8.1 Effect of carbon sources on pigment production.

In this study, soy peptone, beef extract and sodium nitrate showed a positive effect on the pigment production, where as ammonium sulphate and potassium nitrate supported very minimal pigment production. Of all the nitrogen sources tested soy peptone (0.365 g/100ml) gave the maximal yield of pigment.

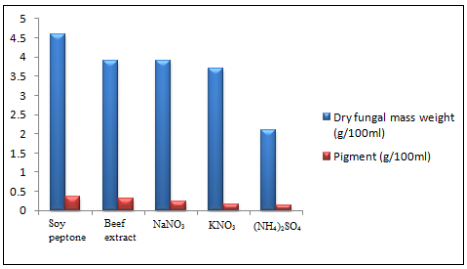


Fig 3.8.2 Effect of nitrogen sources on pigment production.

3.9 EFFECT OF pH ON PIGMENT PRODUCTION

In order to investigate the effect of pH on mycelial growth and pigment production, *Colletotrichum falcatum* was cultivated at different initial pH values (4.0-6.0) in shake flask cultures. The optimal pH for both mycelial growth and pigment production was pH 5.0.

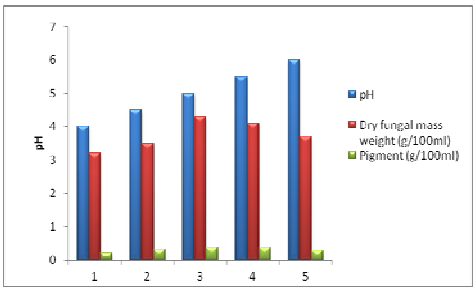


Fig 3.9.1 Effect of pH on pigment production.

3.10 DETERMINATION OF TOTAL ANTIOXIDANT ACTIVITY

The total antioxidant assay gives an estimate of the overall antioxidant potential of the plant. There is a formation of phosphomolybdenum complex, the intensity of which indicates the potential of the *Colletotrichum falcatum* as a scavenger of free radicals.

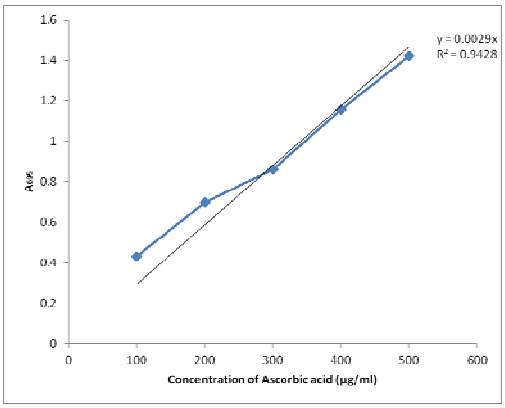


Fig 3.10.1 Standard graph for total antioxidant capacity.

Value represents mean ± SD of two replicates

AAE - Ascorbic acid equivalents (µM/g).

The results obtained showed that ethanolic extracts of *Colletotrichum falcatum* has greater antioxidant activity 552.82 µM/g in the concentration of 500 µg/ml and least activity 115.70 µM/g in the concentration range of 100 µg/ml.

3.11 DYEING

Microbes can be exploited as one of the natural sources of textile dyes. These multiply very fast and are capable of growing on large scale on a variety of raw materials requiring limited space. *Colletotrichum falcatum* was used for the study produced pigmentation in submerged cultivation.

Samples were subjected to pre-moradanting, simultaneous mordanting and post-modanting with natural mordants extracted from grape, orange and pomegranate. In this, post mordanting had better shades of color than pre-modanting and simultaneous mordanting. The suitability of mardants for cotton fabrics with natural dye varies from dye to dye. Different mordant produce different colors. Radhika *et al.,* (2007) reported that Mustard color was produced when chrome was used as a mordant and olive green was obtained on copper sulphate. While, Peach shade was produced when orange mordant was used, and light grey shade was obtained on pomegranate mordant.

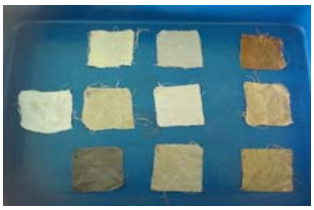


Fig 3.11.1 Various shades obtained from after dyeing.

CONCLUSION

- Pigments are present in all living matter and impart attractive colors and play basic roles in the development of organisms. The toxicity problems caused by those pigments of synthetic origin to the environment have created a mounting interest towards natural pigments. Among them, pigments from microbial sources are potentially alternative to synthetic pigments. The fungal isolates were separated from the 'red rot' infected sugarcane and the fungal culture was purified and identified following Lactophenol Cotton Blue method. Under light microscope its morphological characters were subsequently studied. The fungal culture was identified as *Colletotrichum falcatum.* Enhanced pigment production was achieved under optimal culture condition using submerged fermentation. In this study, the pigment production recorded maximum (0.467 g/100 ml) by supplementing soluble starch as a carbon source and soy peptone (0.365 g/100 ml) as a nitrogen source. The pigment production also mainly depends on the pH of the medium. Maximal pigment production was noticed at pH 5.0.
- *Colletotricum falcatum* is a significant source of antioxidants. In sugarcane, red color is due to the production of phenolic compounds by the fungus, *Colletotricum falcatum* the total phenolic content in the cultured broth was estimated at 41.08 mg/g gallic acid equivalent. The presence of flavonoid in the cultured broth was confirmed by tesing with $AlCl_3$ and $FeCl_3$ methods. The total flavonoid content was 6.08 mg/g rutin equivalent. The fungal extracts were tested against human bacterial pathogens and all extracts showed negative result. They did not exhibit antibacterial activity.
- The natural dye of *Colletotricum falcatum* produced silky beige shade without any mordant. Peach shade was produced when orange peel mordant was used, and light grey shade obtained with pomegranate as mordant. It can be conclude from the study that the fungus can be a potential source of natural dye which can be applied on textile. The fermentation conditions for maximization of pigmentation can be standardized and therefore, these natural dyes can be commercially produced on mass scale in an inexpensive and environmentally friendly approach.

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