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Extraction and characterization of red pigment from *Talaromyces australis* and its application in dyeing cotton yarn

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ABSTRACT
The use of natural dyes in dyeing industry is increasing day by day due to the toxic environment pollution and allergic nature of the synthetic dyes. The natural pigments are alternate to synthetic dye which is non toxic to environment and human. Hence the present study was aimed to produce the organic dye from soil fungus. In this study red pigment producing fungus, was isolated from the soil, collected from Attuvampatti, Kodaikanal. The red pigments were extracted and tested for their colour fastness on cotton fabric in both pre-mordant and unmordant condition which was undergone washing, perspiration and rubbing. The extracted red pigments were non toxic to vero cells and it was proved by MTT assay. Further, 2,4-Di-tert-butylphenol is a major compound present in the coloured fractions and was identified by GC-MS analysis.
Key word: *Fungus, natural dye, GC-MS analysis, fraction.*

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INTRODUCTION

A dye is a coloured substance that has an affinity to the substrate to which it is being applied. There are two types of pigments, organic pigments produced from natural sources and inorganic pigments derived from coal tars and other petro chemicals. The synthetic dyes like tartazine, colchineal red and sunset yellow contains chemicals like mercury, lead, chromium, copper, sodium chloride, toluene and benzene which are harmful to environment and human beings on their own or in combination with other colourant. The drawback of synthetic colour has increased the global demand for natural pigments [1].

Natural dyes are non toxic, non polluting and less health hazardous [2]. They can exhibit better bio-degradability hence higher compatibility with the environment. Because of increasing environmental pollution by synthetic dyes, dyeing industries were under increasing pressure to minimize this damage. So, the industries are continuously looking for cheaper, more environment friendly routes to existing dye [3]. The main sources of natural pigments are plants and microorganisms. Several technologies have been made to synthesize bacterial and fungal pigments to be used in the textile and leather industry [4].

Microbial dyes are better preferred than plant and animal dyes, as the microbes are fast growing and they have standardized commercially. Microbes can also produce a large amount of pigments such as anthraquinones, carotenoids, flavonoids, quinines and rubramines. Of these microbes, fungi are reported as potent pigment producing microorganisms [5]. Fungi are either of the group of unicellular, multicellular or syncytial spore producing organisms. They perform important services in pigment production, water dynamics, nutrient cycling and disease suppression. The application of fungal pigments in

dyeing of cotton, silk and wool has been reported in several studies [6]. Filamentateous fungi are known to produce an extra ordinary range of pigments such as carotenoids, melanin, flavius, phenazines, quinines, monascins, violacein and indigo. There are more than 200 fungal species reported for carotene production [7]. In the reason times, these pigments are increasingly used in textile industry.

In the present work attempts have been taken to assess the dyeing capability of fungal pigments on cotton fabrics and their cytotoxicity effect on vero cells. Besides, the main compound present in red pigment also identified by GC-MS.

MATERIALS AND METHODS

Sampling and fungal isolation

Soil samples were collected randomly from Attuvampatti, Kodaikanal, Tamil Nadu, India during the winter season in 2015. About 50 g of soil was collected in sterile container from 10 cm depth with a help of sterile shovel. The collected samples were transported immediately into laboratory and stored at 4˚C until further use. Potato Dextrose agar media was purchased from Himedia Laboratories, Mumbai, India and was used for preparation of culture plates. Pigment producing soil fungus was isolated by the standard serial dilution plate technique. The inoculated plates were incubated at 25±2°C for 4 to 5 days. After incubation, the fungi colonies were individually picked with sterile inoculation loop and inoculated on a sterile PDA plate. The inoculated plates were incubated for 10 to 14 days at 25±2°C. The pigment producing fungus was identified based on the colour change of media compare to the control plate (Fig. 1). The pigment producing fungus was identified based on the microscopic characters by lactophenol cotton blue wet mound [8].

Molecular identification of pigment producing fungus

The pigment producing fungus was also identified based on the Internal Transcribed Spacer (ITS) region sequence as described by White et al. [9]. DNA was extracted from the fungal mycelium as described by Doyle and Doyle [10]. Purity and quantity of the isolated DNA was estimated by UV spectrophotometer (LABMAN LMSP-UV 1200). The forward and reverse primers used were ITS1-5'-TCCGTAGGTGAACCTGCGG-3' and ITS4-5'-TCCTCCG-CTTATTGATATGC-3'. The ITS amplification reaction mixture (30µl) consisted of 2X master mix (Amplicon, Denmark) with 10 ng of template DNA and 10 pmol of each primer. PCR amplification was carried out in Agilent SureCycler 8800® gradient PCR machine. The following cyclic conditions were executed: initial denaturation for 5 min at 95^{o}C followed by 35 cycles of denaturation for 45 sec at 94 ˚C; annealing for 1 min at 59 ˚C; extension for 1 min at 72 ˚C followed by a final extension at 72 ˚C for 5 min.

The PCR amplified ITS product was purified and sequenced using DNA sequencing services (Eurofins Scientific, Bangalore) employing the same primer used for PCR amplification.

The obtained sequence was subjected for noise editing using Bioedit software v 7.0.9 [11] and was blast with GenBank Nucleotide Database (http://www.ncbi.nlm.nih.gov/BLAST/) using the algorithm Blast N [12] to identify matches with existing reference sequences. The ITS –rDNA sequence of the isolate was deposited in GenBank and accession number was obtained.

Screening of Dye

The cotton fabrics used in these studies was purchased from Sakthi Knit, Tirupur, Tamil Nadu, India. The cotton fabric having 105 GSM, 40 count, 17 diameter and 34 inches. The cotton fabric was sliced into pieces of 30 cm × 30 cm dimension. The red coloured culture filtrate from fungus was used to dye cotton fabrics. The pH of culture filtrate was measured and adjusted to pH to 5, 7 and 9 with vinegar and ammonium solution.

Pre-mordant of fabric

The cotton fabrics were pre mordant with 5% of alum (ammonium potassium sulphate) and 10% ferrous sulphate on weight of fabric (o.w.f). Initially, the cotton fabrics were boiled in alum with sodium carbonate (0.03 g) at 70 °C for 1 hour and left in the bath at room temperature for overnight. For iron mordanting, the cotton fabrics were boiled with ferrous sulfate solution at 70 °C for 10 min and left to cool in the bath. After pre-mordanting, the cotton fabrics were squeezed and air dried at room temperature [13].

The pre-mordant and unmordant fabrics were immersed in the fungal filtrate and was heated in a water bath at 70 to 80 °C for 1 hour. Then, the dyed cotton fabrics were washed with cold water to remove the unfixed pigment and dried at room temperature overnight.

Colour Fastness

All the dyed cotton fabrics were assessed for colour fastness to washing, perspiration and rubbing according to standard methods, ISO 105: C 06-1997 (washing), ISO 105: E04-1996 (perspiration) and ISO 105: X12-2002 (Rubbing) described by The Regional Laboratory, Textile Committee, Chennai, India.

Purification of fungal pigment

The fungus culture filtrate was mixed with ethyl acetate 1:3 ration and agitated 24 hours at 100 rpm. After, the solvent was separated from culture filtrate by separation funnel. The pigment extract was concentrated using rotary vacuum evaporator.

About 25 cm of glass column (34 × 2.5 cm dimensions) was filled with Silica gel (60-120 mesh size, Merck, India). The crude pigment extract was loaded on to the top of the column. The following solvents were used as elution solvent such as, 100% hexane, hexane : chloroform (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9), 100% chloroform, chloroform : ethyl acetate (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9), 100% ethyl acetate, ethyl acetate : methanol (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9) and 100% methanol. The coloured fractions were collected individually and further used for pigment identification.

GC-MS Analysis of coloured fraction

The eluted coloured fractions were subjected to analyse in gas chromatography connected with mass spectrometry (GC-MS) using Agilent Technologies GC systems (Model GC-7890A/MS-5975C, Agilent Technologies, Santa Clara, CA, USA). Relative quantity of the chemical compounds present in each of the elution mixtures were expressed as percentage based on peak area produced in the chromatogram**.**

Cytotoxicity of crude fungal pigment

The normal cell line (Vero) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). One hundred microlitres of cell suspension (10,000 cells) was seeded per well of 96-well plates and incubated at 37 ˚C, 5% CO_2, 95% air and 100% relative humidity to allow for cell attachment. After 24 h the cells were treated with serial concentrations of the test samples. The plates were incubated for an additional 48 hours at 37 ˚C, 5% CO_2, 95% air and 100% relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations.

After 48 hours of incubation, 15 µl of MTT (5 mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37 ˚C for 4 hours. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 µl of DMSO and then measured the absorbance at 570 nm using micro plate reader.

The percentage of cell viability was calculated with respect to control as follows

% Cell viability = [A] Test / [A]control × 100

RESULTS

The microscopic identification revealed the red pigment producing fungus exhibited typical morphological characteristics of *Talaromyces australis* such as soft ascocarps with a cleistothecial wall of interwoven hyphae and typically yellow ascomata, with ovate to globose asci containing spiny ascospores.

Molecular identification of pigment producing fungus.

The amplified PCR product was subjected to Agarose Gel Electrophoresis using a DNA Ladder and was visualised by Gel Documentation instrument. The amplified PCR product was resolved in gel at molecular weight of ~ 600 bp. The amplified PCR product of red pigment fungus was purified and sequenced. Further, the sequence was subjected to NCBI blast. The result showed that red pigment producing fungus rDNA –ITS sequences 100% similarity with previously submitted NCBI nucleotide sequence of *Talaromyces australis*. The red pigment producing fungus rDNA – ITS sequence was deposited in NCBI and accession number KY611854 was obtained.

Screening of Dye

The effect of washing, perspiration and rubbing on red fungal pigment dyed cotton fabrics was shown in Table 1.

Colour Fastness to Washing

After washing, the red fungal pigment dyed all cotton fabric samples showed colour changes of fastness which were rated from slight change to much change. The cotton fabric which was pre-mordant with iron and dyed with red fungal pigment at pH 9 showed slightly colour

change compare to other dyed cotton fabrics. All the three different pH (9, 7 and 5) red fungal pigment dyed cotton fabrics of unmordant, pre-mordant with iron and pre mordant with alum showed negligible to slightly staining on white fabrics were observed.

Colour Fastness to Perspiration

In acid perspiration, negligible to slightly colour change was observed in cotton fabric which was pre-mordent with iron and dyed with red pigment at pH-9 whereas, the cotton fabric which was pre-mordant with alum and dyed with red pigment at pH-7 showed considerable colour change, respectively. In acid perspiration study noticed that the variation in staining of red pigment dyed cotton fabrics on test white fabrics ranged from negligible to slightly. The alum pre-mordant cotton fabric dyed at pH -7 showed negligible colour staining whereas, the iron pre-mordant cotton fabric dyed at pH -7 observed slight colour staining, respectively.

In alkali perspiration test, the iron pre-mordant cotton fabric dyed with red fungal pigment at pH-9 exhibited slightly colour change whereas the following dyed cotton fabrics; unmordant at pH- 5 and 9, iron pre- mordant at pH-7 and alum pre-mordant at pH-9 revealed noticeable colour change, respectively. Negligible to slight colour staining on test white fabrics were observed in unmordant cotton fabrics dyed at pH-5 & 7 and alum pre-mordant cotton fabric dyed at pH -7.

Colour Fastness to Rubbing

After dry rubbing, the colour fastness of red fungal pigment dyed cotton fabrics at pH- 5 (without pre-mordant) and 9 (with iron pre-mordant) recorded slight colour change compare to other dyed cotton fabrics. The cotton fabrics dyed (pH -7) without pre-mordant, with iron pre-mordant (pH-5) and with alum pre-mordant (pH -9) exhibited negligible to slight staining on test white fabrics.

The red pigment dyed cotton fabrics at pH- 5 and 9 (with iron pre-mordant) rated noticeable to slight colour change compare to other dyed cotton fabrics in wet rubbing test. The cotton fabrics dyed (pH -7 and 9) without pre-mordant, with iron pre mordant (pH -5 and 9) and with alum pre mordant (pH-9) demonstrated slight staining on test white fabrics.

Purification of red fungal pigment

The ethyl acetate solvent extract of red fungal pigments was purified by silica gel column chromatography and yielded 16 different coloured fractions. The fraction containing colour pigment was analysed by GC-MS analysis, based on their polarity order. The 16 different coloured fractions contained 25 different compounds. The retention time, molecular formula, molecular weight, and the fragmentation of these compounds were also presented in Table 2. Based on the abundance, the highly photo-sensitized 2,4-Di-*tert*-butylphenol (Fig. 2) compound is majorly present (more than 70%) at all the elution mixtures. The peak at *m/z* 206 is corresponds to the molecular ion $[M]^+$ of 2,4-Di-*tert*-butylphenol. The peak at *m/z* 191 is corresponds to the species $[M\text{-}OH]^+$ formed by the removal of one hydroxide from the molecular ion of 2,4-Di-*tert*-butylphenol.

Cytotoxicity of crude fungal pigment

The cytotoxicity effect of crude red pigment against normal vero cell line was carried out by MTT assay. Result of per cent viability vero cell line treated with red pigment shown in Table 3. The cell viability of vero cells was slightly decreased with increasing concentration of red pigment. The cell viability was 97.96, 96.88, 95.17, 94.47 and 93.89 percentage with red pigment concentrations 12.5, 25, 50, 100 and 200 mg, respectively. The result showed there was no significant cytotoxic effect by red pigments on the normal vero cell line.

DISCUSSION

In this study, red pigment producing fungus was isolated from soil. Soil is the rich source of microorganisms. Soil is consists of multilayered mineral and organic constituents which present in solid, liquid and gaseous state. Soil also comprises plants, animals carcasses and manmade materials. Soil fungi are break down the organic materials. Ten to thirty percent of soil fungi are habitat in soil rhizosphere [14-15].

Similarly many authors previously isolated pigment producing fungi from soil. Velmurugan et al. [16] isolated five fungi namely, *Monascus purpureus, Isaria farinosa, Emericella nidulans,Fusarium verticillioides and Penicillium purpurogenum* from soils collected from Western Ghats, Nilgris. The isolated fungi produced pigments used for dye to cotton fabric and leather samples. Sharma et al. [17] isolated various fungi from different habitats such

as soil, leaf surface, air and vermicompost from the premises of college (Institute of Home Economics, University of Delhi). The isolated pigment producing fungi were identified as *Trichoderma virens*, *Alternaria alternate* and *Curvularia lunata*. The fungi grown pigmented culture media was used to test their dying capacity on wool and silk fabrics. Poorniammal et al. [18] isolated pigment producing fungus, *Thermomyces* sp. from soil. The pigments were extracted and tested their dyeing capacity on cotton, silk and wool fabrics. Gupta et al. [19] isolated *Trichoderma* spp. from soil, collected from premises of Institute of Home Economics, University of Delhi, and screened their dying ability on silk and wool fabrics. Aishwarya and Devi [20] isolated two fungi species, *Trichoderma* sp. and *Aspergillus* sp. from soil samples randomly collected from Chennai. The pigments were extracted and tested their dying property to silk, cotton and silk cotton fabrics. Sastrawidana et al. [21] isolated red pigment producing fungus, *Penicillium purpurogenosum* from goat milk contaminated soil at Sepang village, Buleleng Bali, Indonesia and the pigments were extracted and tested their ability to dye on silk and cotton fabrics. Sarkar et al. [22] collected 113 samples from soil, air and water from different regions at Jessore, Bangladesh and isolated wide range of bacteria and fungi. Among them, only two fungal species, *Aspergillus* sp. and *Penicillius* sp. produced green and red pigments with greater solubility. The pigments were extracted and tested their dying capacity to cotton and silk fabrics.

Molecular identification of pigment producing fungi

The conventional fungal identification mainly based on morphological characters such as spore-producing structures formed as a result of asexual (mitosis) or sexual (meiosis) reproduction [23]. The understanding the evolution of morphological characters very important to identification of fungal species. However, this approach helped to classification of fungi at the ordinal or familial level [24], may not always perform well for species level [25]. Also, the morphological characters which misleading due to the hybridization [26-27], cryptic speciation [28-32] and convergent evolution [33]. Thus, the identification of fungi based on morphology alone can be challenging, especially when nonexperts are dealing with cultures of fungi.

In the present study, the isolated red and yellow pigment producing fungi were identified as *Talaromyces australis* and *Pencillium bilaiae* by rDNA – ITS sequencing. Similarly, Chadni et al. [34] isolated pigment producing fungi from spoiled mango and tested against cotton fabrics. They have used rDNA-ITS sequencing techniques to identify the pigment producing fungus, *Talaromyces verruculosus*. Hernández et al. [35] isolated two pigments producing fungi from rotten wood samples and identified as *Talaromyces australis* (red) and *Penicillium murcianum* (yellow) by rDNA–ITS sequencing.

Fastness properties of Dyed cotton fabrics

The present study, the cotton fabric pre mordant with iron and dyed with red pigment at pH 9 showed negligible to noticeable colour changes and negligible to slight staining was observed in wash, perspiration and rubbing fastness studies. This result corroborate with previous of finding of Sharma and Grover [36] that the cotton fabric pre mordant with iron (5%) and dyed with walnut bark recorded negligible to noticeable colour changes and staining in wash, perspiration and rubbing fastness studies. Velmurugan et al. [16] extracted pigments from five fungi, *Monascus purpureus, Isaria farinosa, Emericella nidulans, Fusarium verticillioides, and Penicillium purpurogenum* and dyed on cotton yarn. High level of shade, colour depth and wash fastness obtained by pre mordant with alum and ferrous sulphate when compared with post-mordanting or without a mordant.

This result of red pigment dyed with cotton fabric controversy to previous finings of Devi and Karuppan [37] that cotton fabric without pre-mordant dyed with brown pigment extracted from *Alternaria alternata* showed excellent to good colour fastness in perspiration and rubbing. Also, it showed least staining in washing, perspiration and rubbing.

Purification of fungal pigment by silica gel column chromatography

In the present study, purified coloured fractions were analysed by GC-MS and identified 2, 4-Di-*tert*-butylphenol is the major compound present in the red coloured pigments. The phenolic compounds 2, 4-Di-tert-butylphenol showed antiplasmodial activity [38]. In the present study, 2, 4-Di-*tert*-butylphenol is a phenolic compound isolated from fungus it produced the colour which is used for dye in cotton cloth and found it is quickly binding with cloth and confirmed by washing. Our knowledge this is the first report, the 2, 4-Di-*tert*-butylphenol isolated from fungi.

Cytotoxicity of crude fungal pigment

Textile manufacturing industry used wide range of chemicals, which are harmful to the environment, workers who working in textile processing and most importantly to consumers. The level of toxicity is varied depends on the textile processing stage [39]. The most common diseases reported among the textile workers are allergic reactions and irritation to the skin and respiratory tract [40]. Some research finding reported textile dyes have potential for mutagenicity [41-42] and genotoxicity [43].

In the present investigation was aimed to study the adverse effect of fungal pigments on vero cells by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. This is an advance significant quantitative assay over traditional techniques which have been used commonly for proliferation and cytotoxicity assays. In this technique, the yellow compound tetrazolium ring is reduced by mitochondrial dehydrogenases to the water insoluble blue formazan compound, depending on the living cells [44].

Similarly, many authors previously studied the cytotoxicity effect of dyes. Klemola et al. [45] investigated the cytotoxicity of reactive dyes and dyed fabrics using human keratinocyte HaCaT cells *in vitro*. Kadir et al. [39] explored cytotoxicity and neurotoxicity effect of natural dyes in the form of liquid and dyed silk fabrics.

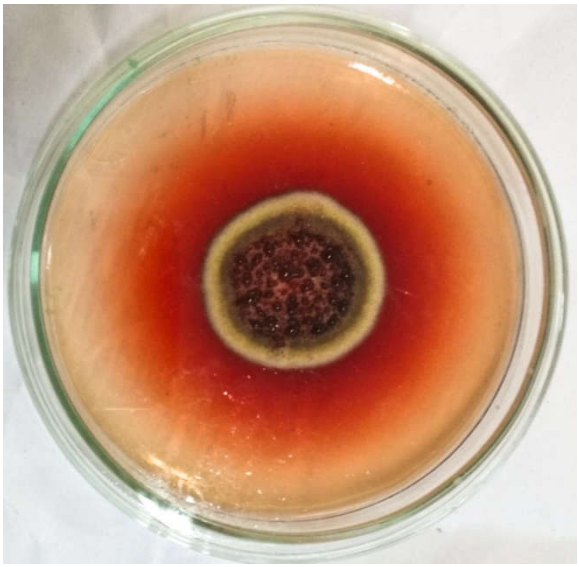

Fig. 1. Pure culture of red pigment producing fungus, *Talaromyces australis*

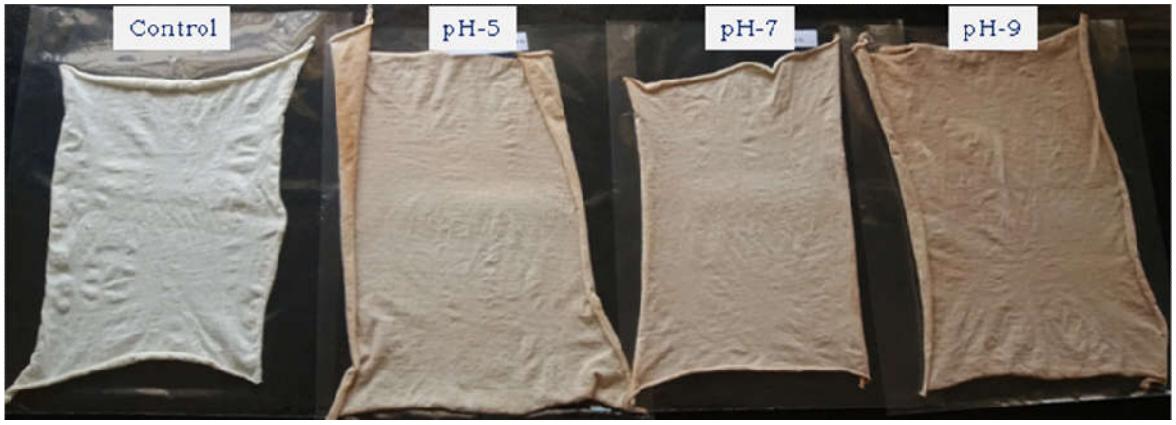

Fig 2. Unmordant cotton fabrics dyed with red fungal pigment at different pH

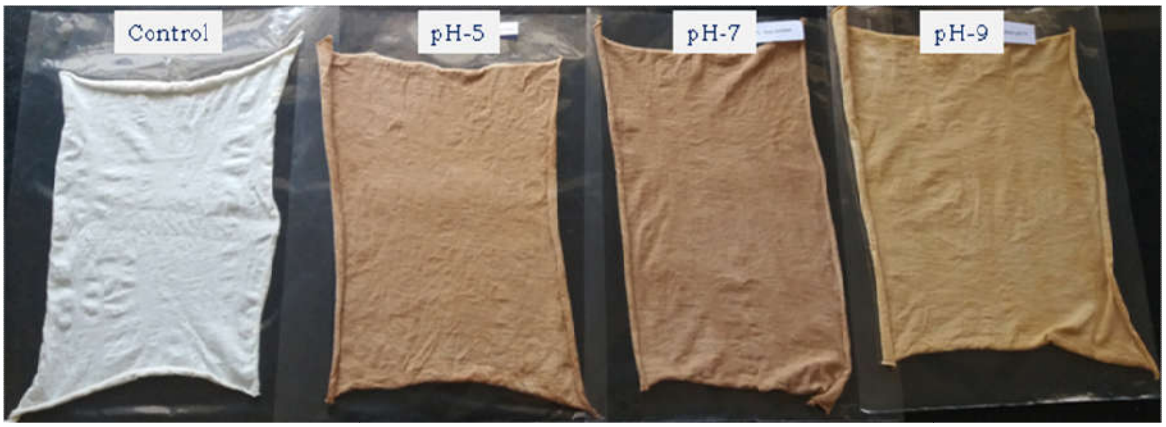

Fig 3. Iron pre-mordant cotton fabrics dyed with red fungal pigment at different pH

Fig 4. Alum pre-mordant cotton fabrics dyed with red fungal pigment at different pH

Table 1. Fastness properties of dyed cotton fabrics.

S. No	Sample	Wash Fastness		Perspiration Fastness				Rubbing Fastness			
		CC	S	Acid		Alkali		Dry		Wet	
				CC	S	CC	S	CC	S	CC	S
1	Without Mordant @ pH9	3	4/5	3/4	4/5	3	2/3	3/4	4	3	4
2	Without Mordant @ pH7	3/4	4/5	3	4/5	2/3	4/5	3/4	4/5	3	4
3	Without Mordant @ pH5	3	4/5	3/4	4/5	3	4/5	4	4	3	3/4
4	Iron Mordant pH9	4	4/5	4/5	4/5	4	4	4	4	3/4	4
5	Iron Mordant pH7	2/3	4/5	3	4	3	3/4	3	4	2/3	3/4
6	Iron Mordant pH5	3	4/5	3	4/5	2/3	4	3/4	4/5	3/4	4
7	Alum Mordant pH9	2/3	4/5	3	4/5	3	4	3/4	4/5	3	4
8	Alum Mordant pH7	2/3	4/5	2	5	2/3	4/5	3	4	2/3	3/4
9	Alum Mordant pH5	1/2	4/5	3	4/5	2/3	2/3	2/3	3/4	2	3

CC – Change in colour; S – Staining on white fabric
1-Much change, 2-Considerable change, 3-Noticeable change, 4-Slight change and 5-Negligible change

Table 2. Fungal colour compounds of ethyl acetate extract at different elution mixtures.

Elution mixtures	Name of the compounds	Retention Time	Mol. Weight	Mol. Formula
CH 100	2,4-Di-*tert*-butylphenol; 3,5-Di-*tert*-butylphenol; 2,6-Di-*tert*-butylphenol	12.359	206	$C_{14}H_{22}O$
CH90:HEX10	1,3-Dioxalane	5.369	74	$C_3H_6O_2$
CH90:ET10	2,4-Di-*tert*-butylphenol; 3,5-Di-*tert*-butylphenol; 2,6-Di-*tert*-butylphenol; 2,5-Di-*tert*-butylphenol	12.368	206	$C_{14}H_{22}O$
CH80:ET20	1-Tetradecane; Cyclotetradecane	10.812	196	$C_{14}H_{28}$
	2,4-Di-*tert*-butylphenol	12.405	206	$C_{14}H_{22}O$
	Cetene	13.493	224	$C_{16}H_{32}$
CH70:ET30	4-Nitrobenzaldehyde	9.830	151	$C_7H_5NO_3$
	3-Nitrobenzaldehyde; 5-Nitrobenzaldehyde	10.208		
	2,4-Di-*tert*-butylphenol	12.442	206	$C_{14}H_{22}O$
	12-Oxatetracyclo[5,2,1,1 (2,6).1(4,10)]dodecane-11-one	13.388	178	$C_{11}H_{14}O_2$
CH60:ET40	4-Nitrobenzaldehyde	10.245	151	$C_7H_5NO_3$
	Pentadecane	10.931	198	$C_{14}H_{30}$
	2,4-Di-*tert*-butylphenol	12.423	206	$C_{14}H_{22}O$
CH50:ET50	1-Tetradecane	10.803	196	$C_{14}H_{28}$
	2,4-Di-*tert*-butylphenol	12.405	206	$C_{14}H_{22}O$
	Cetene	13.500	224	$C_{16}H_{32}$
	1-Octadecane	16.308	252	$C_{18}H_{36}$
CH40:ET60	1-Dodecane	7.601	168	$C_{12}H_{24}$
	1-Tetradecane	10.821	196	$C_{14}H_{28}$
	Tetradecane	10.913	198	$C_{14}H_{30}$
	2,4-Di-*tert*-butylphenol	12.368	206	$C_{14}H_{22}O$
	Cetene	13.479	224	$C_{16}H_{32}$
	Hexadecane	13.535	226	$C_{16}H_{34}$
	E-15-Heptadecanal	16.282	252	$C_{17}H_{32}O$
CH30:ET70	1-Decane	5.265	140	$C_{10}H_{20}$
	1-Dodecane	7.619	168	$C_{12}H_{24}$
	1-Tetradecane	10.803	196	$C_{14}H_{28}$
	2,4-Di-*tert*-butylphenol	12.387	206	$C_{14}H_{22}O$
	Cetene	13.493	224	$C_{16}H_{32}$
	E-15-Heptadecanal	16.394	252	$C_{17}H_{32}O$
ET30:MET70	2,4-Di-*tert*-butylphenol	12.359	206	$C_{14}H_{22}O$
ET40:MET60	2,4-Di-*tert*-butylphenol	12.387	206	$C_{14}H_{22}O$
ET50:MET50	2,4-Di-*tert*-butylphenol	12.389	206	$C_{14}H_{22}O$
	3,4,5-Trimethoxy benzoic acid	14.157	212	$C_{10}H_{12}O_5$
ET60:MET40	2,4-Di-*tert*-butylphenol	12.369	206	$C_{14}H_{22}O$
	3,4,5-Trimethoxy benzoic acid	14.122	212	$C_{10}H_{12}O_5$
ET80:MET20	2,4-Di-*tert*-butylphenol	12.368	206	$C_{14}H_{22}O$
	1-(2,4,6,6-Trimethyl-1-cyclohexen-1-yl)-2-butene-1-one	14.926	192	$C_{13}H_{22}O$
ET90:MET10	2,4-Di-*tert*-butylphenol	12.378	206	$C_{14}H_{22}O$
HEX60:CH40	2,6-bis(1,1-dimethylethyl)-2,5-Cyclohexadiene-1,4-dione	11.830	220	$C_{14}H_{20}O_2$
	2,4-Di-*tert*-butylphenol	12.341, 12.387	206	$C_{14}H_{22}O$
	4-Ethoxy ethylester benzoic acid	12.633	194	$C_{11}H_{14}O_3$
	2-(1-Cyclohexenyl)cyclohexanone	13.395	178	$C_{12}H_{18}O$
	2.6-Bis(1,1-dimethylethyl)-4-cyanomethyl phenol	14.332	245	$C_{16}H_{23}NO$

Chloroform; Hex-Hexane; ET-Ethyl acetate; MET-Methanol.

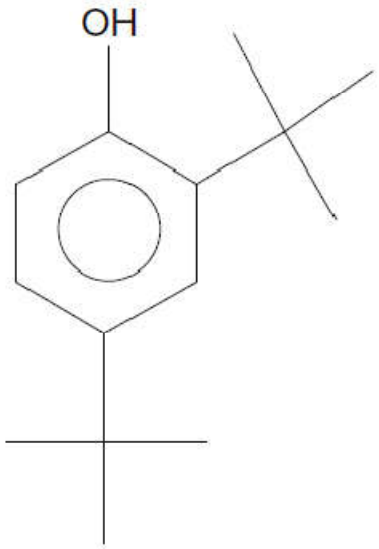

Fig. 5. Structure of 2,4-Di-*tert*-butylphenol

Table 3. Cytotoxicity of fungus red pigments Against Vero Cell Line at Different Concentrations by MTT Assay

Crude red pigment concentration	Cell viability*
12.5 mg	97.96 ± 1.04
25 mg	96.88 ± 0.47
50 mg	95.17 ± 0.37
100 mg	94.47± 0.64
200 mg	93.89 ± 0.48
Control (without red pigment)	100 ± 00

Average of three replications

CONCLUSION

2, 4-Di-tert-butylphenol is a major compound present in the fungal extract. The cotton fabric pre-mortant with iron and dyed at pH -9 exhibited better colour fastness compare to other dyed fabrics. The fungal pigment is an alternative to synthetic dye. The dye did not showed any Cytotoxicity effects against tested cell lines.

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