Int J Pharm Bio Sci 2016 Jan; 7(1): (B) 127 - 136

**Research Article** **Microbiology**

**International Journal of Pharma and Bio Sciences**

**ISSN 0975-6299**

# ASSESSMENT OF PARACHOLOROMETACRESOL (PCMC), A FUNGICIDE AGAINST THREE COMMON FUNGAL SPECIES IN LEATHER MANUFACTURING

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## ABSTRACT

Raw hide is the most susceptible substrate for the attack of microorganisms. The microbial attack is high in the vegetable tanned leather than the chrome tanned leather. The present study is designed to isolate, standardize, study and to perform application studies on the antifungal efficacy of PCMC on the isolated fungal species from vegetable tanned leather. The three isolated fungal species such as *Aspergillus niger*, *Aspergillus flavus* and *Trichophyton rubrum* were presently used for the study. Antifungal susceptibility and physical characteristics of leather was tested and the minimum inhibitory concentration (MIC) of PCMC against *Aspergillus niger* and *Aspergillus flavus* was found to be 80 µl, whereas against *Trichophyton rubrum* was found to be 40 µl. Application study conducted showed that 0.1% of PCMC is sufficient to control the growth of isolated fungal species on leather. The physical properties of leather showed moderate deterioration on fungal attack. All these studies were performed indicate that PCMC was found to be effective in protecting leather against attack by all these three isolated species.

**KEYWORDS**: ***Aspergillus niger, Aspergillus Flavus, Trichophyton rubrum,* fungicide, leather manufacturing.**

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# INTRODUCTION

Fungi, a distinguished group of eukaryotic microorganisms differentiated from plants by the absence of chlorophyll, and cell walls. Many fungi along with bacteria play a key role as decomposers and bring rotting and decay of dead organic matter. All these together bring in various detrimental consequences during leather production. Leather, is an organic material that consists of various nutrients for growth of fungi that was obtained through absorption. Fungal growth is influenced by many factors in the environment such as humid conditions, high storage temperature, low pH value which causes the growth of the mold[1]. Krishnamurthi, (1956)[2] isolated five species of Aspergillus namely *Aspergillus amstalodhnmi, Aspergillus sydoni, Aspergillus versicolor, Aspergillus niger and Aspergillus flavus* from finished leather. According to the report of Tancaus (1969)[3], ring worm was a parasitic disease caused by a fungus of Trichophyton genus. The lesions from ringworm persist in the leather, made from infected skins and hides; appear as smooth shiny spots, making it impossible to obtain a uniform finish. The damage occurs irregularly in lots of skins and hides and greatly reduces the value of the leather. Several damages such as pigmentations in pelts under fungal attack, dyeing, finishing defects and fat acid spew on leather may generate bad smell[4-7]. Under optimum mildew growing conditions, the mould had attacked the carboxyl groups of the fatty acids and caused loss of fatty grease with a consequent fall in tensile strength and firmness. pH level measuring between 1.4 and 1.6 in leathers was found to inhibit the growth of fungus. In such situation, during pH increase addition of fungicide was found to be beneficial[8]. Fungicides which are effective against deteriorating fungal organisms are also necessary for protection and long term storing of leather[9]. Currently the fungicides which are popularly used to protect leather against fungi include thiocyanomethylthiobenzothiazole (TCMTB), sodium mercapbenzothiazole (MBT), ortho-phenylphenol (OPP), Para-chloro-meta-cresol (PCMC), Octylisothiazolin (OIT). In the present study three different fungal species were isolated from leather industries. The three fungal species used for the study includes *Aspergillus niger, Aspergillus flavus* and *Trichophyton rubrum*. The aim and objective of the present study is to study the antifungal effect of PCMC on these three isolated fungal species and to perform the application study of PCMC on the infected leather.

# MATERIALS AND METHODS

***(i) Leather-for sourcing microorganisms***

The microorganisms (fungal species) were isolated from the Mildew attacked EI (East India) Cow calf leather obtained from the leather industry.

***(ii) Haemocytometer***

Haemocytometer was used for counting the spore suspension to know the number of spores.

**(iii) EI leather-for application study**

Vegetable tanned (EI) cow calf leather tanned using Wattle and Myrabolan were used as a substrate for the application study of the various fungicides (to study the extent, degree and pattern of the inhibition of the identified fungal species).

***(iv) Fungicide***

Known concentration of PCMC was taken and dissolved in sterile distilled water. Various concentrations (10 µl, 20 µl, 40 µl, 80 µl, 100 µl, 200 µl) were prepared by serial dilution method.

***(v) Media used***

***a. Sabouraud Dextrose Agar***

The medium used in the study is Saboraud Dextrose Agar, which is of the following composition.

- Peptone - 10 g
- Dextrose - 40 g
- Agar - 15 g
- Distilled water - 1000 ml

The above mentioned ingredients were weighed and dissolved in distilled water and the pH was adjusted to 5.6. Then the agar was dissolved by boiling and then sterilized at $121^0$c for 10 minutes.

**b. Aspergillus flavus / parasiticus agar**

- Yeast extract - 20 g
- Peptone - 10 g
- Ferric ammonium citrate - 0.5 g
- Agar - 15 g
- Dichloran - 0.02 mg
- Distilled water - 100 ml

The above mentioned ingredients were weighed and dissolved in distilled water and sterilized at $121^0$c for 15 minutes.

***c. Urease medium:***

- Urea agar base (Christensen) - 2.9 g
- Distilled water - 10 ml

***This was dissolved in the water and sterilized by filtration.***

- Agar - 1.5 g
- Distilled water - 90 ml

The agar was dissolved in the water and sterilized by autoclaving at 15 lb/in$^2$ for 15 minutes. Then the urea agar base were added to the cooling agar (approximately at 500 and mixed well in aseptic condition. This was dispensed in the sterile tubes as slant.

***d. Corn meal agar***

- Corn meal - 2 g
- Peptone - 2 g
- Dextrose - 2 g
- Agar - 1.5 g
- Distilled water - 100 ml

The corn meal was heated with 50 ml of distilled water and maintained at $60^0$c for 30 minutes. The other mentioned ingredients were mixed and dissolved in 50 ml of distilled water separately and mixed with the cornmeal extract and made upto 100 ml. Then the medium was autoclaved at $121^0$c for 15 minutes.

***(vi) Reagent used***

***Lactophenol cotton blue stain***

- Lactic acid - 20 ml
- Phenol crystals - 20 g
- Glycerol - 40 ml
- Distilled water - 20 ml
- Cotton blue - 0.05 g

Phenol was dissolved in lactic acid, glycerol and water by gentle heating. Then the cotton blue was added and mixed well.

***(vii) Materials for application study***

Cow calf leathers were collected for studies which do not have any preservative, biocide or any other fungicides.

***(viii) Isolation of fungal species from mildew attacked leather (Meral Birbir et al., 1994)[10]***

***(ix) Slide culture technique-for morphology study (Sundarajan T, 1995)[11]***

***(x) Antifungal susceptibility test***

**a.** Inoculum preparation (Eva Petrikkou et al., 2001) [12]
**b.** Inoculum standardization (Aneja 2000)[13]
**c.** Assessment of antifungal susceptibility (Imhof et al., 2003)[14]

***(xi) Application of fungicides on leather :***

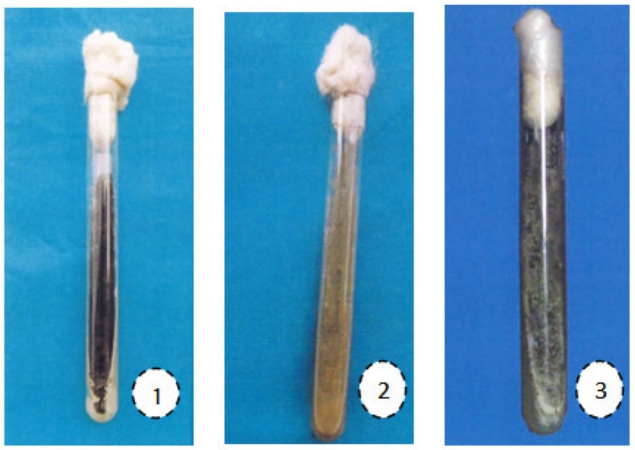
Four EI cow calf leathers were taken. No preservative or fungicide or biocide had been administered during the manufacturing of the EI leathers. Each leather is marked as 1, 2, 3, 4 and cut along the back cone. The four right halves are the control leathers, where no fungicide is administered. Four left halves were treated with varying amounts of the fungicides with 100% water in drum for 60 minutes. The leathers (both controlled and experimented) were dried and inoculated with fungal spores. They were incubated at room temperature for 30 days.

***(xii) Physical testing of leather samples***

Samples of standard dimension of various physical tests were obtained as per international union of leather technology chemist's society (IULTCS) methods. The leather specimen was conditioned at 200c and 65 ± 2% relative humidity over a period of 48 hours. The physical properties such as tensile strength, tongue tear strength and stitch tear strength were then investigated with the help of instruments called Instran.

## RESULTS AND DISCUSSION

Leather, a biological product which is very suitable for the growth of microorganism (Orlita, 2004)[16] due to the presence of protein and lipids in the form of glycerides. Presence of higher fat content in the leather decreases their resistance to mould growth. In comparison with chrome tanned leather, the vegetable tanned sole leather had the heaviest growth of mildew[15].




**Figures (1-3)**
***showing macroscopic appearance of isolated fungi***

From the different sources of vegetable tanned (EI) leathers obtained from the leather industries, three different fungal species were isolated. The isolated organisms were inoculated in SDA (Sabouraud dextrose agar) agar medium, and designated as J1, J2, and J3. Figure 1, 2 and 3 shows the macroscopic pictures of isolated organism which was later identified and confirmed. The isolated organisms (J1, J2, and J3) were studied for cultural and morphological characteristics to ascertain the species. The fungi *Aspergillus niger* was designated as J1. The Table 1 presents the culture and morphological characters of *Aspergillus niger*. Figure 4 shows the microscopic observation of Aspergillus niger under 40X.

**Table 1**
***Identification of fungal species Aspergillus niger***

| Species isolated | Characteristics | | Species Inferred |
|---|---|---|---|
| | **Macroscopic** | **Microscopic** | |
| J1 | • Media was covered with white fluffy mycelia when immature | • Conidial head were large black to brownish black | ***Aspergillus niger*** |
| | • On maturation colonies exhibit “salt and pepper” effect | • Conidial head appear globose | |
| | • Covered with black spores | • Conidiophores was brownish near the vesicle | |
| | • Reverse of the colony was buff colour | • Vesicle had a concave under the surface | |
| | | • Brownish sterigmata was produced in two series | |
| | | • Primary sterigmata were long | |
| | | • Secondary sterigmata were short | |
| | | • Conidia were globose and echinulate | |

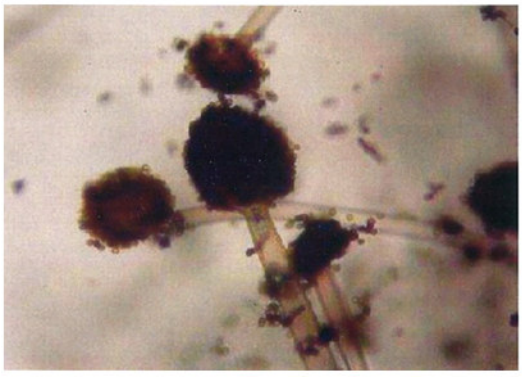


**Figure 4**
***Microscopic observation of Aspergillus niger (Microscopic observation under 40X)***

The presence of fungi varies depending upon the materials used for processing, the environment from where the leather is obtained[16]. The leather susceptibility depends on the sex of the animals (The Wealth of India, 1970), breed used[17], hair[18], due[19], age of the animal used[20] castration and season[21], all these factors brings difference in the number of fungal species.

**Table 2**
***Identification of fungal species Aspergillus flavus***

| Species isolated | Characteristics | | Species Inferred |
|---|---|---|---|
| | Macroscopic | Microscopic | |
| J2 | • Colonies were yellowish green in colour | • Conidial head were radiate and loosely columnar<br>• Conidiophore was hyaline and thick walled<br>• Vesicle were elliptical<br>• Sterigmata were covered entire surface of the vesicle<br>• Monoseriate sterigmata were seen<br>• Primary and secondary sterigmata were nearly equal in size<br>• Conidia were elliptical and echinulate<br>• Conidia appeared yellow green | ***Aspergillus flavus*** |

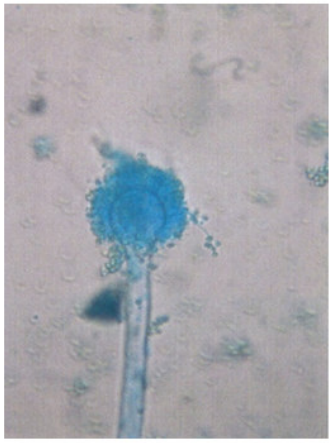


**Figure 5**
***The microscopic observation of Aspergillus flavus***
***(Microscopic observation under 40X)***

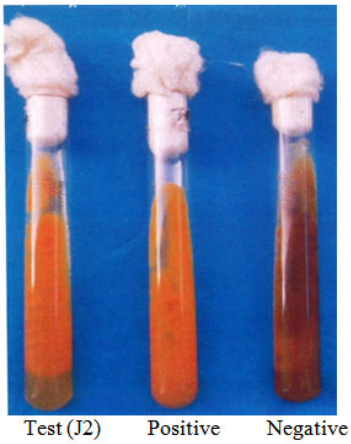



**Figure 6**
***Confirmatory test for Aspergillus flavus in AFPA medium***

The species designated as J2 was inferred to be *Aspergillus flavus* and also the organism (J2) was inoculated in AFPA medium (*Aspergillus flavus/Parasiticus* Agar) and the observation of growth by yellow orange pigmentation on the reverse of the media and absence of the pigmentation in negative control

confirmed that the species was *Aspergillus flavus*. Table 2 presents the culture and morphological characteristics of *Aspergillus flavus*. Figure 5 shows the microscopic observation of *Aspergillus flavus* under 40X. Figure 6 shows the confirmation of *Aspergillus flavus*. *Trichophyton rubrum* species was designated as J3 was inoculated in urease agar medium and the observation of red colour pigmentation which was weakly positive confirmed the presence of *Trichophyton rubrum*. Table 3 shows the cultural and morphological characteristics of Trichophyton rubrum. Figure 7 shows the conformation of *Trichophyton rubrum.*

**Table 3**
***Identification of fungal species Trichophyton rubrum***

| Species isolated | Characteristics | | Species Inferred |
|---|---|---|---|
| | **Macroscopic** | **Microscopic** | |
| | • Surface is granular or fluffy, white to buff. | • Clavate tear drop microconidia were seen along the sides of the hyphae. | |
| | • Reverse is deep red or purplish; occasionally it is brown, yellow orange, or even colourless. | • Long, septate hyphae were seen. | |
| | • The pigment production is best seen on cornmeal dextrose agar. | • Pencil shaped macroconidia were seen. | |
| **J3** | • Reverse of the colony was buff colour | • Conidia were elliptical and ectinulate | ***Trichophyton rubrum*** |

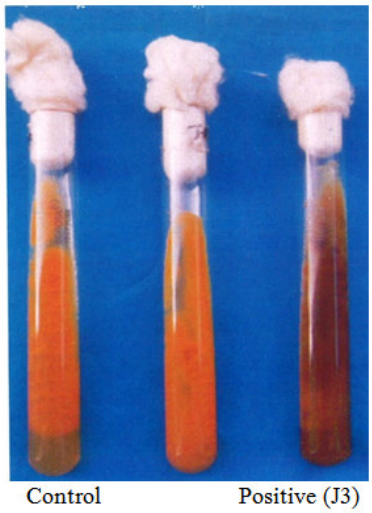



**Figure 7**
***Confirmatory test for Trichophyton rubrum in Urease agar medium***

Table 4 shows the results of standardization of isolated fungal species. The standardized inoculum was adjusted to 4.91 x $10^6$ spores/$cm^3$ for Aspergillus niger, 5.25 x $10^6$ spores/$cm^3$ for Aspergillus flavus and 2.8 x $10^6$ spores/$cm^3$ for Trichophyton rubrum. 5 µl of standardized inoculum was taken for antifungal tests of selected fungicides.

**Table 4**
***showing standardization of isolated fungal species***

| S.NO | Fungal species isolated | Spores/ml (spores/$cm^3$) |
|---|---|---|
| 1 | *Aspergillus niger* | 4.91 x $10^6$ |
| 2 | *Aspergillus flavus* | 5.25 x $10^6$ |
| 3 | *Trichophyton rubrum* | 2.8 x $10^6$ |

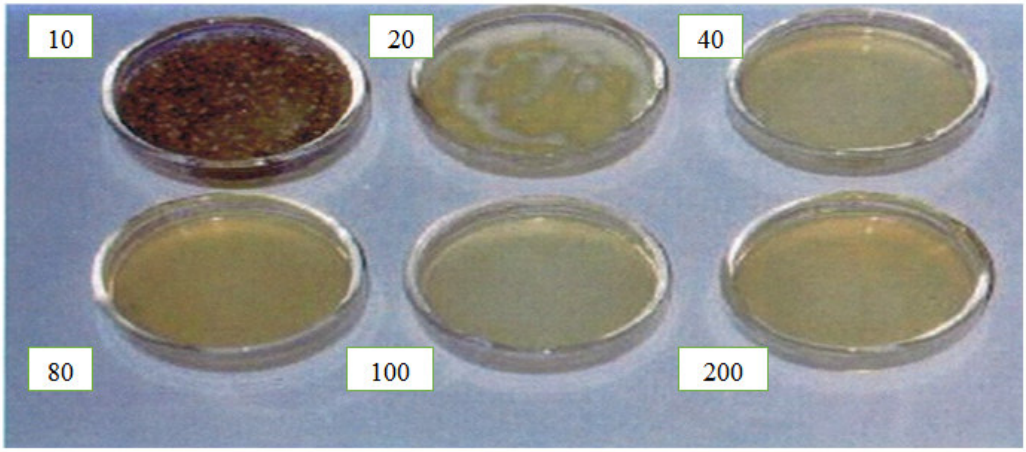



**Figure 8**
***MIC of PCMC against Aspergillus niger***

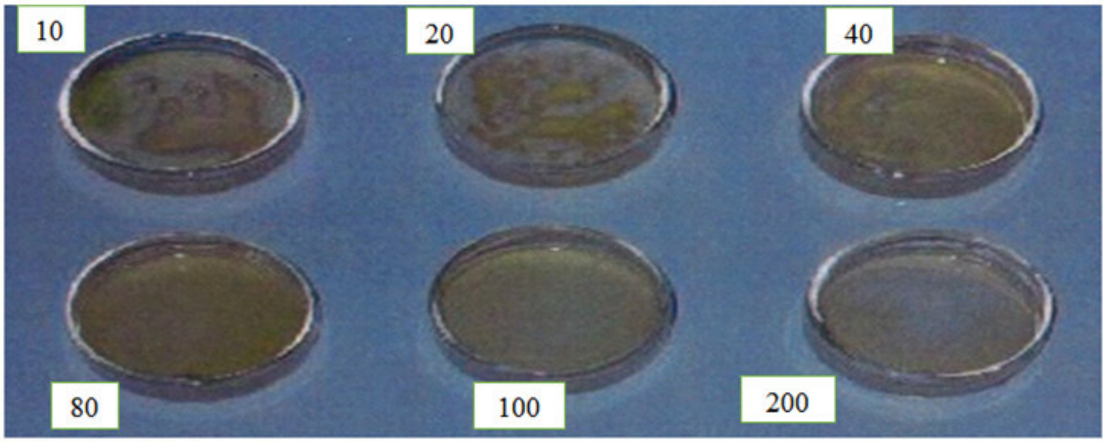



**Figure 9**
***MIC of PCMC against Aspergillus flavus***

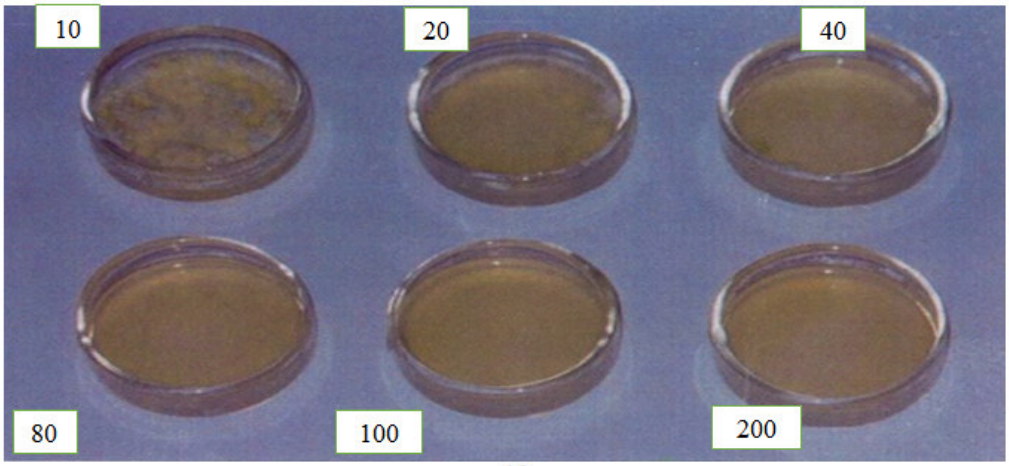



**Figure 10**
***MIC of PCMC against Aspergillus flavus***

**Table 5**
***Antifungal susceptibility of PCMC***

| Fungicide | Concentration (9 ml of distilled water + 1 ml of fungicide) in µl | *Aspergillus niger* | *Aspergillus flavus* | *Trichophyton rubrum* |
|---|---|---|---|---|
| Cumine Phenol | 10 | + | + | + |
| | 20 | + | + | + |
| | 40 | + | + | - |
| | 80 | - | - | - |
| | 100 | - | - | - |

Inhibition study was performed on commercially obtained PCMC to test antifungal susceptibility. The result of antifungal susceptibility was presented in table 5. Figures 8, 9 and 10 show the MIC of PCMC. The MIC of PCMC against *Aspergillus niger* was found to be 80 µl among the concentrations of 10, 20, 40, 80, 100 and 200 µl. The MIC of PCMC against *Aspergillus flavus* was found to be 80 µl among the concentrations of 10, 20, 40, 80, 100 and 200 µl. The MIC of PCMC against *Trichophyton rubrum* was found to be 40 µl among the concentrations of 10, 20, 40, 80, 100 and 200 µl.

**Table 6**
***Results of applications studies***

| Sample | Fungal growth | | | |
|---|---|---|---|---|
| | 0.05% | 0.1% | 0.15% | 0.2% |
| PCMC control | + | + | + | + |
| PCMC experiment | + | - | - | - |

On the basis of the base line data obtained from the anti-fungal tests, the application studies were conducted. The samples are taken and four experiments had been conducted in each case of the fungicide. Varying amounts of fungicides had been offered to the EI cow calf leathers. The concentrations offered were 0.05%, 0.1%, 0.15% and 0.2% on the weight of the EI leathers taken for the experiments. The leathers after treatment with varying amounts of the three fungicides independently and the corresponding control leathers were inoculated with the isolated fungal species independently. The leathers were inoculated and incubated for a period of 30 days. The growth of the organisms was observed. Figures 11 shows the leathers treated with selected fungicides. Table 6 presents the results of the application studies conducted. It is evident that 0.1% PCMC is sufficient to control the growth of the isolated representative fungal species on leather.

**Figure 11**
***Leather treated with PCMC***

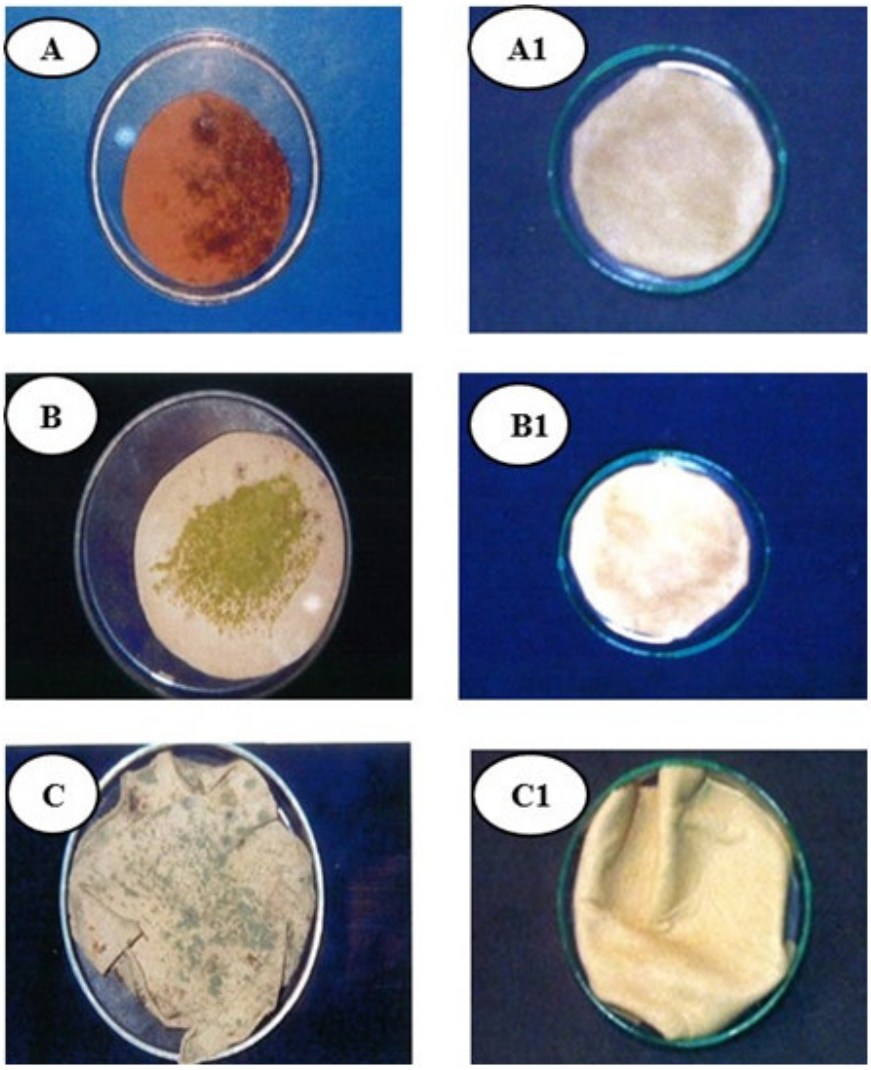



***Figure 11, the A shows leather with Aspergillus niger growth and A1 shows the leather treated with PCMC. B shows with growth of Aspergillus flavus and B1 shows the treated one. C shows the leather with the growth of Trichophyton rubrum and C1 shows the inhibitory effect of PCMC.***

**Table 7**
***Physical characteristics of leather***

| | Physical characteristics | | |
|---|---|---|---|
| **Sample** | **Tensile strength (kg/cm2)** | **Tongue tear strength (kg/cm)** | **Stitch tear strength (kg/cm)** |
| Sample treated with PCMC | 232 | 58 | 88 |
| Sample not treated with PCMC | 221 | 52 | 81 |
| % change | -4.74 | -11.1 | -7.95 |

The fungicide (independently) treated leathers were inoculated with the mixed culture containing all the three isolated species. The corresponding right halves of the leathers which were not treated with the fungicides were also inoculated with the mixed culture and incubated for 30 days. Both control and experiment were processed further to convert them into crust leathers. The leathers were then tested to understand the physical characteristics of the leathers. The physical properties of the experimental leathers and their corresponding control leathers were compared. This part of the study would indicate the pattern and degree of damage that is caused by the isolated fungal species if they are not treated with suitable biocides chosen. Table 7 represents the results. It is evident from the table that the physical characteristics of the fungal damaged leathers exhibited a moderated deterioration. Though these changes are not very serious per se the final effect that could be rendered by these effects on the finished leather in the physical requirements would be very significant.

## CONCLUSION

Finished leather which are used to prepare number of commodities are found to be highly susceptible to fungal attack. Even during storage conditions the leathers are subjected to heavy microbial infection. It has been concluded that the fungicide PCMC was found to be better in inhibiting the growth of the three isolated organisms of our interest. There was no serious deterioration but only moderate decrease in the physical properties was observed but yet it could lead to serious change in the bulk properties of the leather, which may render the leather unsuitable for the purpose. As belief fundamental works unravel the potential utility of other fungicides for leather, further research has to be conducted to standardize the application process.

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