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Dye yielding potential and antibacterial properties of lichens from Kerala, India

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Lichens are a group of lower plants that adapt to extreme environmental conditions. The utility of lichens is due to the wide range of secondary metabolites produced by them. The present study is to analyse the dye yielding potential and anti-bacterial properties of lichen species *Heterodermia boryi*, *Hypotrachyna cirrhata*, and *Cladonia fruticulosa* collected from different parts of Kerala. Dyes were extracted using methods such as Ammonia fermentation method (AFM), Di-methyl sulphoxide extraction method (DEM) and the boiling water method (BWM). The dye extracts were experimented on mulberry silk and banana fibre. The fibre stability was tested by exposure to sunlight and action with detergent and shampoo. Most of the dyed colours appeared to be stable. The colours were detected using COSMIN software. The colours that appeared were mainly shades of brown, khaki, tan and gold. AFM and DEM are better extraction methods than BWM. To investigate the antibacterial activity, methanol, ethanol, acetone, chloroform and distilled water extracts of lichens were tested against Gram-positive and negative bacteria along with positive control. Chloroform and acetone extracts showed exceptional inhibition activity followed by methanol and ethanol and the least activity by distilled water extracts. Extracts of *H. cirrhata* and *C. fruticulosa* showed better antibacterial activity than *H. boryi*. These results indicate the presence of different secondary metabolites.

Keywords: Antibacterial activity, Banana fibre, Fabric dyeing, Kerala, Lichen, Silk.

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Introduction

Lichens are symbiotic organisms composed of photobiont (algae or cyanobacteria) and phycobiont (mostly ascomycete) together form an independent physiological unit[1]. They show slow growth and can produce a wide variety of secondary metabolites, which are believed to serve as antiherbivore, antimicrobial and antigrowth agents, they have the inherent ability to produce dye colours. The secondary metabolites known as "lichen acids" are the main source of lichen dyes[2]. Lichen dyes are UV protective[3]. The earliest report on the use of lichens as a source of dyeing came from Romans who used orchil, purple colour pigment from *Roccella spp*., for dyeing. During the eighteenth-century dyeing stuffs made from lichens were economically important in the Canary Islands. Lichens were also used for the preparation of litmus paper to estimate pH values. Research was conducted on *Usnea ghattensis* and its cultured mycobiont to extract dye for silk fibre, using ammonia fermentation method (AFM), boiling water method (BWM) and cow urine method (CUM). Purple dye from AFM was obtained from natural thalli and cultured mycobiont, the other two methods yielded light shade colours[4]. Working on eleven species of lichen collected from different sites of the Gharwal region of the Indian Himalayas, confirmed that AFM and DEM are better extraction methods than BWM. As the former methods produced darker shades in silk, tussar silk, absorbent cotton[5]. Recently, in an investigation on dye yielding potential of nineteen lichen species belonging to the Himalayan region of Pakistan, wool and silk were dyed using the methods AFM, DEM and BWM and produced different colours[2]. In India, studies related to the use of lichens as a source of the colouring agent is meagre[5].

Lichens are used in several traditional systems of medicines such as Ayurveda and Unani. Lichens are said to cure dyspepsia, bleeding piles, bronchitis, stomach disorders, scabies and many disorders of blood and heart. Recently much attention has been paid to the biological activities of secondary metabolites produced by the lichen. The lichen extracts and the purified metabolites are known to

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exhibit a wide array of bioactivities such as antibacterial, antifungal, antioxidant, anti-tumour, antiherbivore, phytotoxic wound healing, insecticidal activity and other uses[6]. Lichens have been acknowledged as a potent antimicrobial agents since ancient times. Prabhu and Sudha (2015) experimented with anti-bacterial activity of lichens such as *Hetrodermia boryi, Parmotrema stuppeum, Usnea nilgirica, Pyxine sp.,* and *Parmotrema melanothrix* against *E. coli, Pseudomonas sp., Klebsiella sp.*, coagulase-negative *Streptococcus aureus, Staphylococcus aureus, Acinetobacter sp*. using disc diffusion assay[7]. Recently, the antimicrobial potential of three fruticose lichens viz., *Ramalina hossei, R. conduplicans*, and *R. pacifica.* in methanol extracts against *B. cereus*, *B. subtilis, E. Coli*, and *P. aeruginosa* was done by agar well diffusion[8]. Macrolichens appear to be a promising source of bioactive compounds[9]. The present study aims to investigate the dye yielding potential and the antibacterial properties of two foliose forms of lichens *viz*. *Heterodermia boryi* (Fée) K.P Singh & S. R. Singh (Family Physiaceae) and *Hypotrachyna cirrhata* (Fr.) Divakar, A. Crespo, Sipman, Elix & Lumbsch (Family Parmeliaceae) and one species of fruticose form, *Cladonia fruticulosa* Kremp. (Family Cladoniaceae).

Materials and Methods

Collection and identification of lichen samples

The species *Heterodermia boryi* (Voucher specimen no. MCH 1804) and *Hypotrachyna cirrhata* (Voucher specimen no. MCH 1175) were collected from Meesapulimala, Munnar, Idukki (Dt.), Kerala (Lat 10.0974° N, Long 77.2034° E, Alt 2400 m). *Cladonia fruticulosa* (Voucher specimen no. MCH 2555) was collected from Thalapuzha, Mananthavady, Wayanad (Dt.), Kerala (Lat 11.8488°N, Long 75.9521°E, Alt 800m). The collected specimens were air-dried and herbarium specimens were prepared as per the standard method. The collected specimens were identified by examining the external morphology of the thallus using a dissection microscope; chemical colour tests and Thin layer Chromatography (TLC)[10]. The voucher specimens were deposited at Lichen Herbarium of Maharaja's College, Ernakulam, India.

Preparation of plant material

The identified samples were cleaned properly, air-dried under shade, and were ground into a fine powder using an electric blender. Finely powdered samples were then stored in airtight containers at ambient temperature until required.

Fibres used

Mulberry silk fibres and banana fibres were used for the study. Silkworm cocoons were procured from Hassan district of Karnataka, India. The banana fibres were procured from a vendor in Tamil Nadu, India.

Fibre preparation

Silk threads were spooled out by adding cocoons to boiling water in a vessel. The silk fibres separated out, the unwounded filaments were collectively wound up to form resultant thread. The banana fibres were combed to make them tangle free before using.

Extraction of dyes from lichens

Ammonia fermentation method (AFM), Dimethyl sulphoxide method (DEM), and Boiling water method (BWM) were used to dye the fibres. No mordants were used in the study. These three methods were employed for the extraction of dyes with a slight modification of the original methodology[2].

Ammonia fermentation method (AFM)

Four grams of powdered lichen samples were added to the diluted ammonium hydroxide solution (4 mL ammonium hydroxide in 40 mL distilled water,1:10 ratio). The contents were mixed thoroughly and were left for one month in the glass bottle, maintained at room temperature. After one month the extracts were centrifuged at 5000 rpm for 15 minutes and filtered into a 100 mL Erlenmeyer flask using a Whatman grade 1 filter paper. Mulberry silk and banana fibres of 1.5 g each, pre-soaked in distilled water were added to the extract. After one month, fibres were removed from the flasks and dried. The colours of the dried threads were noted.

Di-methyl sulphoxideextraction method (DEM)

Four grams of powdered lichen samples were added to 50 mL di-methyl sulphoxide in a glass bottle. The content was mixed thoroughly and kept for one month at room temperature. After one month, the extracts were centrifuged at 5000 rpm for 15 minutes and filtered into a 100 mL Erlenmeyer flask using a Whatman grade 1 filter paper. Mulberry silk and banana fibres were weighed (1.5 g), pre-soaked in distilled water and added to the extract. After one month, fibres were removed from the flasks, dried and colours were noted.

Boiling water method (BWM)

Powdered 4 g of lichen sample was added to 50 mL distilled water in a flask and heated till boiling. The mixture was maintained at simmer for half an hour with periodic stirring. Then the content was filtered through Whatman grade 1 filter paper. 1.5 g of mulberry silk and banana fibres were weighed and then pre-soaked in distilled water was added to the filtrate and was progressively heated up to a maximum of 90 °C for one hour. The dye bath was cooled after dyeing, the threads were rinsed in cold water, dried and colours were noted.

Stability test

The dyed threads were then subjected to test the stability and fastness of the dye. The stability of the dyes was tested by exposing the dyed threads to sunlight for 8 hours per day. This was maintained for 5 days. On the second and fourth day, the threads were divided and half of them were washed with a mild shampoo (5 mL of protein shampoo in 500 mL water) and the other half with premium consumer Grade 1 detergent (5 g/half teaspoon of detergent in 500 mL water) separately for five minutes to test the fastness of the dye. COSMIN software was used to detect the HEX and RBG colour code.

Antibacterial activity

Preparation of plant extracts

One gram of the powdered lichen samples was weighed and soaked each in 10 mL of methanol, ethanol, acetone, chloroform, and distilled water in capped glass bottles, the bottles were washed and autoclaved before use. The bottles were shaken periodically for 72 hours at room temperature. The crude extracts were then filtered. The extracts collected were stored in airtight bottles at 4 °C. These were then used for anti-microbial screening.

Antibacterial activity of lichen extracts

Antibacterial activity of lichen extracts was tested against six strains of bacteria *viz*. *Escherichia coli* (MTCC 1610), *Vibrio harveyi* (BCCM 4044), *V. Parahaemolyticus* (MTCC 451), *Bacillus cereus* (MTCC 1272), *Staphylococcus aureus* (MTCC 3061) *and Edwardsiella tarda* (MTCC 2400), collected from the School of Marine Sciences, CUSAT, Ernakulam, Kerala. The bacterial strains were inoculated in nutrient broth. After 24 hours, the bacterial culture in the nutrient broth was swabbed on the solidified agar medium on the culture plates using a cotton bud. Wells were prepared on the medium using a cork borer of the size 0.4 cm. 20 µL of lichen extracts and solvents (negative control) were filled into the wells using a micropipette. Amikacin 30 µg anti-biotic discs were used as positive control. The culture plates were kept in the incubator. After 24 hours, the culture plates were observed for the inhibition zone. The measurements of the inhibition zone were recorded using a scale. Percentage of inhibition was calculated using the formula:

Magnitude of inhibition zone on the bacteria by lichen extract X 100

Magnitude of inhibition zone on the bacteria by the antibiotic disc

Results and Discussion

Dye yielding potential of lichens

The lichens proved to be potential dyeing agents. Colours obtained from AFM were bole, old gold, lemon chiffon, dark khaki and dark tan while through DEM golden brown, zinnwaldite brown, olive green, flax and dark khaki were obtained. BWM produced colours like citron, raw umber, dark goldenrod, dark tan and ecru (Table 1). HEX and RBG colour codes were also noted for each colour using COSMIN software (Table 2). AFM and DEM showed better results than BWM[5]. The threads used for dyeing were pure white silk fibres, while banana fibre had a natural buff colour hence after dyeing it appeared different colour from silk. A study stated that tussar silk is known to have a buff colour. So after dyeing the tussar silk appeared different from white silk and cotton fibres[1].

Hypotrachyna cirrhata produced colours like old gold in silk and dark khaki in banana fibres through AFM (Fig. 1a) while extraction through DEM produced colours like zinnwaldite brown on silk, and banana fibres remained with natural flax colour (Fig. 2a). However, the same lichen through BWM produced dark golden rod colour in silk and a dark tan in banana fibres (Fig. 3a). *Everniastrum cirrhatum* (=*H. cirrhata*) is known to have secondary metabolites like salazinic acid, atranorin and protolichesteric acid[11]. Salazinic acid in lichens was responsible for orange and brown dyes and both salazinic acid and atranorin produced yellow colour. The presence of brown shade instead of yellow colour may be due to the increased fermentation time[2].

Table 1 — List colours obtained through three methods and stability test

Lichen sample	Method of dye extraction	Colours obtained on fibres		Stability of colours to sunlight and washing
		Silk (A)	Banana fibre (B)	
Heterodermia boryii	Ammonium fermenation method	Bole	Bole	Slightly faded up in sunlight
Hypotrachyna cirrhata		Old gold	Dark khaki	Stable colour
Cladonia fruticulosa		Lemon chiffon	Dark tan	Stable colour
Heterodermia boryii	Dimethyl sulphoxide extraction method	Golden brown	Flax	Stable colour
Hypotrachyna cirrhata		Zinnwaldite brown	Flax	Stable colour
Cladonia fruticulosa		Olive green	Dark khaki	Stable colour
Heterodermia boryii	Boiling water method	Citron	Raw umber	Stable colour
Hypotrachyna cirrhata		Dark golden rod	Dark tan	Stable colour
Cladonia fruticulosa		Ecru	Ecru	Stable colour

Table 2 — List of colours with HEX code and RGB values

Colours	HEX	RGB
Bole	#7E5C50	126,92,80
Citron	#DDCC9A	221,204,154
Dark khaki	#BAA56D	186,165,107
Dark golden rod	#B89758	184,151,88
Dark tan	#907F59	144,127,89
Ecru	#COAF7D	192,175,125
Flax	#EBC87B	221,204,154
Golden brown	#99692E	153,105,46
Lemon chiffon	#FFFACD	255,250,205
Old gold	#CDBF9C	205,191,156
Olive green	#B4A269	180,162,105
Raw umber	#836C40	131,108,64
Zinnwaldite brown	#4F351C	79,53,28

Cladonia fruticulosa produced light colours in all three methods; it produced lemon chiffon in silk and dark tan in banana fibres through AFM (Fig. 1b), while colours like olive green and dark khaki were developed on silk and banana fibres respectively using DEM (Fig. 2b). Based on BWM, the lichen produced ecru colour on both silk and banana fibres (Fig. 3b). Metabolites like Fumarprotocetraric acid, protocetraric acid, usnic acid or atranorin present in the genus *Cladonia* produce mostly shades of pink[2]. However, *Cladonia fruticulosa* contain Beta-orcinoldepsidones compound - Psoromic acid in their thallus[12]. The presence of psoromic acid might be the reason that *Cladonia fruticulosa* produced varied shades on silk and banana fibres. Colours obtained from *Heterodermia boryi* through AFM was bole on both silk and banana fibres (Fig. 1c), DEM produced golden brown colour on silk fibres and banana fibres remained with the natural flax colour (Fig. 2c) and with BWM the lichen produced citron and raw umber colours on silk and banana fibres respectively (Fig. 3c). Genus *Heterodermia* is known to have secondary metabolites zeorin and atranorin which may have resulted in the varied colour production[5,13]. The dye colours may vary upon the soil properties, the season of harvesting of lichen thallus and fermentation time[1]. It is also observed that environmental, climatic and geographical factors can change the secondary metabolite production in lichen-forming fungi[14].

After the exposure to sunlight for 40 hours followed by detergent and shampoo washing for the determination of stability and fastness of dyed threads, it was noticed that threads treated with

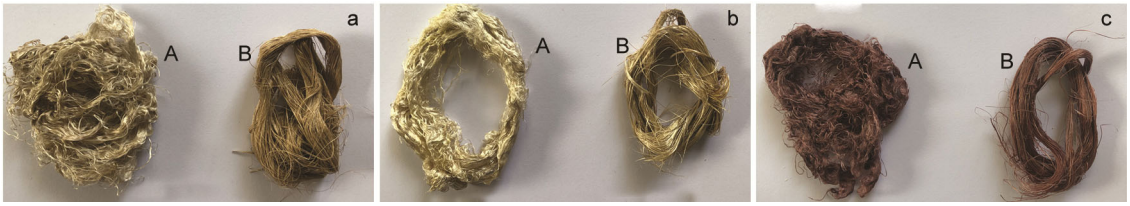

Fig. 1 — Ammonium fermenation method: A) silk and, B) banana fibre.

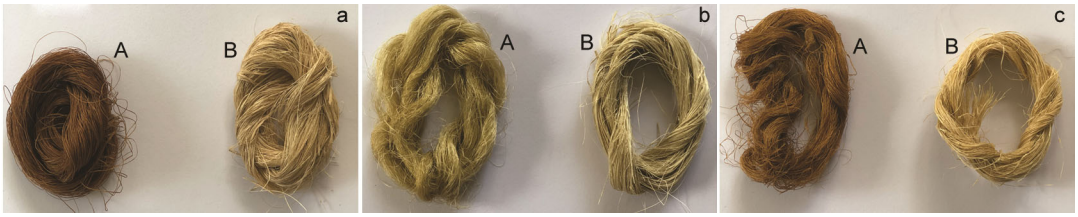

Fig. 2 — Dimethyl sulphoxide extraction method: A) silk and, B) banana fibre.

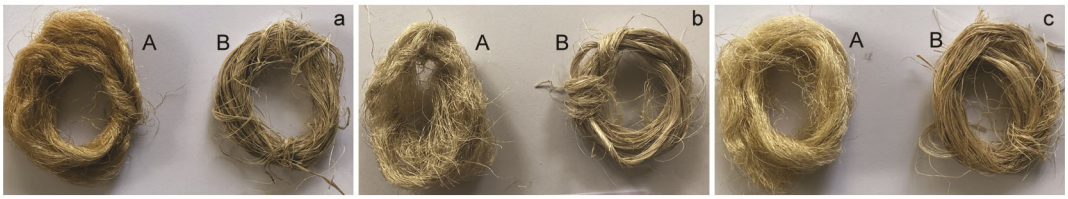

Fig. 3 — Boiling water method: A) silk and, B) banana fibres.

DEM have shown much stability than AFM. Since BWM produced lighter colours, it did not show any comparative results. A degree of colour change was observed in threads dyed-through AFM of *Heterodermia boryi* after exposure to sunlight. The change in colour mainly occurs due to the oxidation of phytochrome[1,2]. Lichen dyes impart musky odour to the fibres. The dyed products are reputed to be insect-proof as the secondary metabolites make the fibres distasteful for the insects[15].

Antibacterial activity of lichen

In the present study, all solvent extracts of lichens showed variable anti-bacterial activity (Fig. 4). Chloroform and acetone extracts of the lichen showed exceptional activity followed by methanol and ethanol. Water extracts of lichen showed poor antibacterial activity. The methanol and chloroform extracts of *Hypotrachyna cirrhata* showed the highest activity against *E. tarda* (3.8 and 2.7 cm respectively) (Fig. 4b & 4g) which made the percentage of inhibition higher than the reference antibiotic (2.3 cm) (Fig. 5b). All the extracts of *Hypotrachyna cirrhata* marked an average activity against most of the bacteria. All the extracts of *Cladonia fruiticulosa* exhibited an above-average antibacterial activity against most of the bacteria. Acetone extracts of *C. fruticulosa* displayed the highest inhibition against *V. harveyi* (2.8 cm) (Fig. 4f). The activity of *Heterodermia boryi* was marked highest in acetone extract against *V. paraheamolyticus* (1.7 cm) (Fig. 4e). Antibiotic discs (positive control) formed inhibition zones with high magnitude (Fig. 5). Overall high and consistent antibacterial activity was recorded for *H. cirrhata* and *C. fruticulosa* than *H. boryi* (Table 3).

In many studies, it has been proved that chloroform extracts show the highest inhibition than other solvents. Acetone and methanol are also considered as potential solvents[7,16]. Distilled water extracts of the lichen samples showed the least activity against all the tested bacteria. The probable reason for the less activity of water extracts is that majority of active substances in lichen thalli are either insoluble or poorly soluble inwater[16].

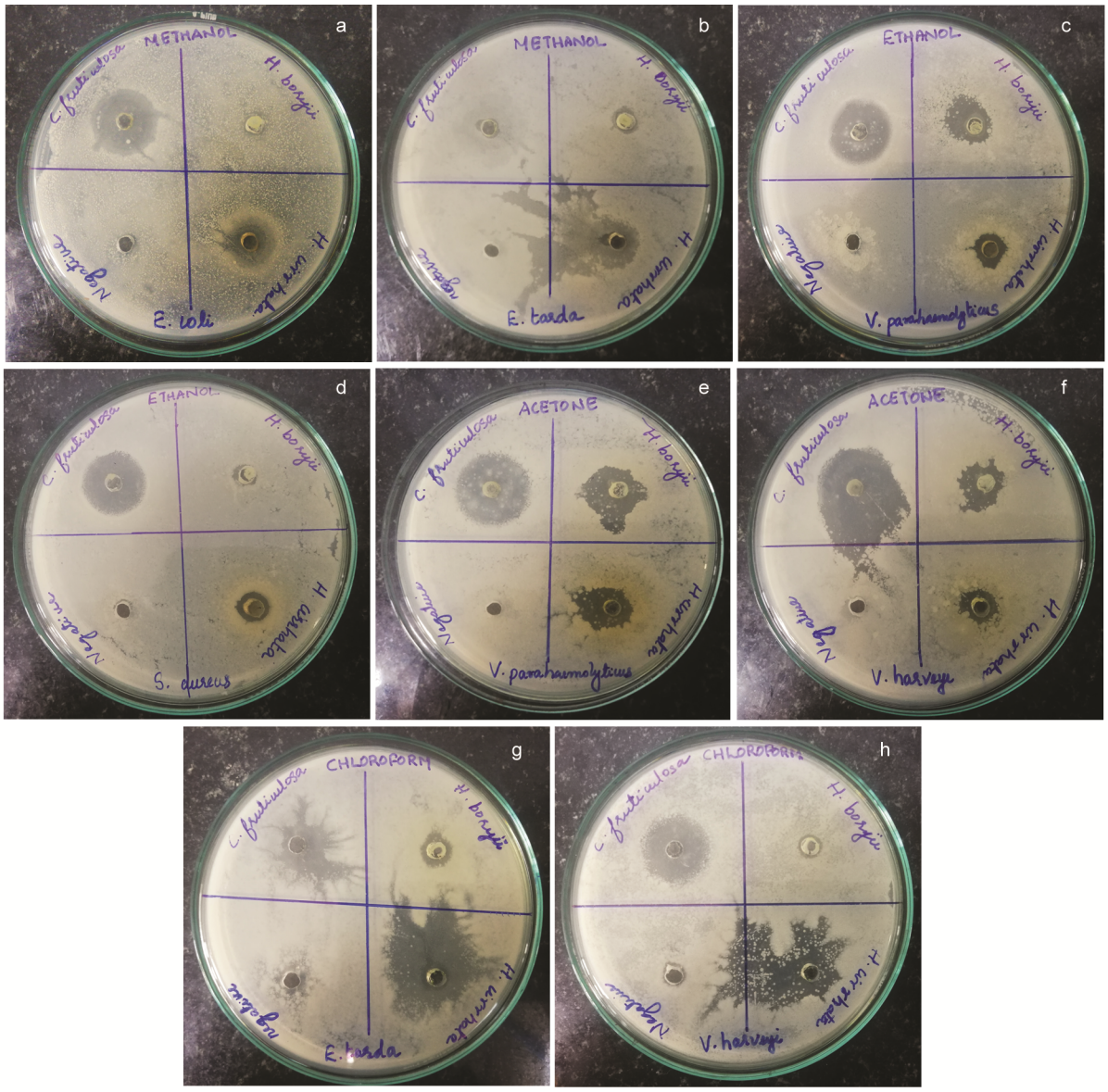

Fig. 4 — a-b) Antibacterial activity of methanol extracts *E. coli* and *E. tarda*, c-d) Antibacterial activity of ethanol extracts agains *V. parahaemolyticus* and *S. aureus*, e-f) Antibacterial activity of acetone extracts against *V. paraheamolyticus* and *V. harveyi* g-h) Antibacterial activity of chloroform extracts against *E. tarda* and *V. Harveyi*.

In the present study, six bacterial strains were used as test bacteria, of these two were Gram-positive (*B. cereus* and *S. aureus*) and four were Gram-negative bacteria (*E. coli, E. tarda, V. Harveyi* and *V. parahaemolyticus*). Many reports from elsewhere have stated that lichens were more active against Gram-positive bacteria[17-20]. In the present investigation, the lichens were active against both Gram-positive and negative bacteria.

Hypotrachyna cirrhata contain active compounds like atranorin, protolichesteric acid and salazinic acid. Also, many species of *Cladonia* like *C. foliacea, C. furcata, and C. crispatula* have active compounds like usnic acid, atranorin, fumarprotocetratic acid and depsides. These compounds are active against Gram-positive and Gram-negative bacteria, usually, extracts of the lichen are taken using solvents like acetone, methanol, ethanol, chloroform, petroleum ether and diethyl ether[21]. *Heterodermia sp*. is known to have the compound, Zeorin, which is active against *Bacillus sp.* and *Staphylococcus sp*.[16]. The presence of these secondary metabolites in lichens is responsible for the antimicrobial activity of the lichens. The results indicate the differences in antimicrobial activity

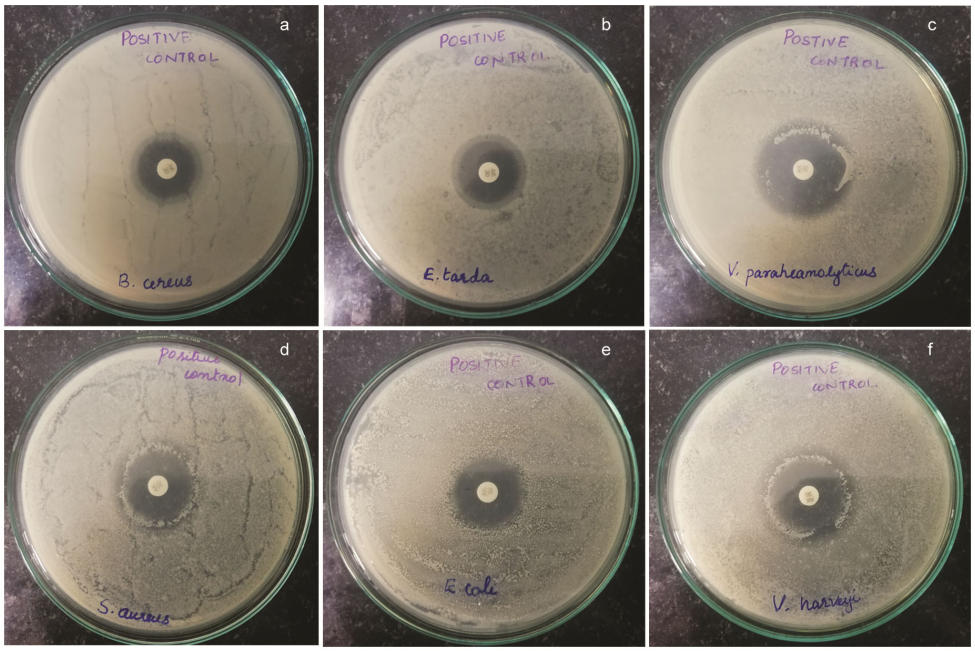

Fig. 5 — Antibiotic positive control on different strains of bacteria, a) *B. aureus*, b) *E. tarda*, c) *V. parahaemolyticus*, d) *S. aureus*, e) *E. coli*, f) *V. harveyi*.

Table 3 — Antibacterial activity of lichen extracts on test bacteria

Solvent	Lichen	Zone of inhibition (cm) (Percentage of inhibition)					
		B.cereus	*S. aureus*	*V. harveyi*	*V. parahaemolyticus*	*E. tarda*	*E. coli*
Methanol	*H. boryi*	0.4 (19.04)	0.4 (16)	0.6 (18.75)	0.4 (12.5)	0.3 (13.04)	0.2 (9.5)
	H. cirrhata	0.7 (33.3)	0.4 (16)	0.7 (21.8)	0.7 (21.8)	3.8 (165.2)	0.5 (23.8)
	C. fruticulosa	1.3 (61.9)	1.4 (56)	1.7 (53.1)	1.5 (46.8)	0.3 (13.04)	1.7 (80.9)
	Negative control	-	0.3 (12)	-	-	-	-
Ethanol	*H. boryi*	0.2 (9.5)	0.3 (12)	0.4 (12.5)	1.1 (34.3)	0.4 (17.3)	0.4 (19.0)
	H. cirrhata	0.8 (38.09)	0.5 (20)	0.8 (25)	0.6 (18.7)	0.2 (8.6)	0.3 (13.04)
	C. fruticulosa	1.2 (57.1)	1.4 (56)	1.2 (37.5)	1.4 (43.7)	0.3 (13.04)	1.7 (73.91)
	Negative control	0.5 (23.8)	0.2 (8)	-	-	0.1 (4.3)	0.2 (9.5)
Acetone	*H. boryi*	0.8 (38.09)	0.7 (28)	1.3 (40.6)	1.7 (53.16)	0.4 (17.39)	0.7 (33.3)
	H. cirrhata	0.8 (38.09)	1.4 (56)	0.8 (25)	1.3 (40.6)	1.9 (82.6)	1.1 (52.38)
	C. fruticulosa	1.4 (66.6)	1.6 (64)	2.8 (87.5)	1.8 (56.2)	0.3 (13.04)	1.6 (76.19)
	Negative control	-	-	-	-	0.2 (8.6)	-
Chloroform	*H. boryi*	0.8 (38.09)	0.8 (32)	0.3 (9.3)	0.7 (21.87)	0.6 (26.08)	0.3 (14.28)
	H. cirrhata	0.9 (42.85)	1.2 (48)	3 (93.75)	1.5 (46.8)	2.7 (117.3)	1.7 (80.95)
	C. fruticulosa	0.5 (23.8)	1.6 (64)	1.4 (43.7)	1.6 (50)	1.7 (73.91)	1.7 (80.95)
	Negative control	0.5 (23.8)	0.9 (36)	0.3 (9.3)	-	0.3 (13)	0.3 (14.28)
Distilled water	*H. boryi*	0.1 (4.76)	-	-	-	-	-
	H. cirrhata	0.1 (4.76)	-	-	-	0.3 (13.04)	-
	C. fruticulosa	-	-	-	-	-	-
	Negative control	-	-	-	-	-	-
Positive control (antibiotic discs)		2.1	2.5	3.2	3.2	2.3	2.1

between the extracts depending on the species of lichen and the type of extracting solvent. These results might reflect that bioactive components of lichens have different solubility in different extracting solvents[7].

Conclusion

From the study, it is evident that lichens are a promising source of dye. Most synthetic dyes use mordants that have a heavy metal components

which makes them harmful to the environment. Lichen dyes do not require mordants, making them completely natural and eco-friendly. The musky odour of lichen dyed fibres makes them insect proof thus increasing the shelf life and durability of fibres. The use of lichen dyes for commercial purposes is not viable; still, they can be used in cottage industries to some extent. The antibacterial property shown by the lichen gives promising evidence for the development of future drugs useful for humans, animals and plants. They can indeed act as protecting agents for bacterial diseases. The resistance of pathogens gradually increasing pose a threat to public health. Thus, natural products can act as a promising alternative to combat drug resistance.

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Conflict of interest

The authors declared that there is no conflict of interest.

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