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Research Article

Assessment of Bactericidal Activity of Some Lichen Extracts by Disc Diffusion Assay

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Abstract

In the presented study, thirty-two natural extracts obtained from lichen species; *P. reticulatum*, *P. tinctorum*, *Herpothallon* sp., *H. leucomelos*, *R. celastri*, *Leptogium* sp. and *P. crinitum,* were evaluated for their bactericidal activity against three gram positive and three gram negative human pathogens. The investigation was performed by agar-disc diffusion assay and bactericidal activity of each lichen extract was measured using zone of inhibition. It was observed that methanol lichen extracts had the maximum efficacy whereas hexane extracts had the least bactericidal potential. Overall, *P. reticulatum* found to be the most anti-bacterial effective species and *C. subradiata* was the least potent species. According to all the result, it was concluded that these lichen species can be studied further as great source of bactericidal agents due to their high potency as antibiotics in *in vitro* models.

Keywords: Lichens; Bactericidal; Disc-diffusion; Natural extracts

Introduction

The search for novel bioactive secondary metabolites is of primary concern since infectious diseases are continuously emerging and re-emerging [1]. Pharmaceutical industries are forced to develop new pharmacological molecules. Similar to higher plants, lichens are considered as potential source of novel biologically active compounds [2]. Historically, a large portion of the world's medicine has been derived from plants and fungi. Important antibiotics such as penicillin are derived from fungi. Lichens are another type of organism that may hold the potential for medical exploration [3]. Interest in the antibiotic potential of lichen compounds was extremely high during the post-World War II era through the end of the 1950's. Once again there is an interest in the potential uses of antibiotics derived from lichens, as they may be a valuable source of antibiotics for the pharmaceutical industries in the future [3]. Antibiotic properties of the lichens are of special interest to the scientists [4,5]. According to one estimate, 50% of all lichens have antibiotic properties [5,6]. Burkholder was pioneer initiating research on lichens as antibacterial agents. He tested 42 lichens for antibiotic property and 27 were reported to inhibit growth of bacteria [5,7-15]. Based on wide screening of antimicrobial activity of lichen extracts, it seems that bacterial inhibitions can vary within the lichen extract, solvent used for extraction and bacteria tested [15]. The emergence of drug-resistant bacterial strains has currently led to severe clinical complications. Hence, new antimicrobial agents are urgently needed and as the search is being extended to new sources, lichens are clearly an interesting source of compounds in pharmaceutical research [8]. Lichens are known to synthesize variety of secondary metabolites with wide range of biological activities [10]. Yamamoto [9] had reviewed the folklore and pharmacological studies on biological activities of lichen.

Materials and Methods

Lichen materials

Lichen species *Heterodermia leucomelos*(L.) Poelt. (Voucher No. 34755), *Cladonia subradiata* (Vainio) Sandst. (Voucher No. 34756), *Parmotrema tinctorum* (Delise ex Nyl.) Hale (Voucher No. 34757), *Leptogium* sp. (Ach.) *Gray*. (Voucher No. 34758), *Parmotrema crinitum* Choisy (Voucher No. 34759), *Herpothallon* sp. Tobler (Voucher No. 34760), *Parmotrema reticulatum* (Taylor) M. Choisy (Voucher No. 34761) and *Ramalina celastri* (Sprengel) Krog & Swinscow (Voucher No. 34762) were collected from Ooty, India and a part of each identified species are preserved in NBRI- Lucknow, India.

Microorganisms: Bacterial strains *Salmonella typhimurium* (NCIM 2501), *Pseudomonas aeruginosa* (NCIM 2200), *Bacillus subtilis* (NCIM 2063), *Escherichia coli* (NCIM 2065), *Proteus vulgaris* (NCIM 2027) and *Staphylococcus aureus* (NCIM 2654), were used in this study. All the microorganisms had been obtained from National Collection of Industrial Microorganisms (NCIM) at National Chemical Laboratory, Pune, India and were maintained on recommended Muller-Hinton medium.

Extraction

Lichen materials were washed, air-dried and grinded for the extraction process. The powdered lichen thallus were placed in a thimble and extracted by soxhlet apparatus using four organic solvent; hexane, ethyl acetate, methanol and ethanol.

Determination of microbial concentration

In order to determine the suitable concentration of microbial culture for antimicrobial activity of lichen extract, microorganisms were inoculated in Muller-Hinton broth and incubated at 34°C for 24 hrs. After completion of incubation period microorganisms were serially diluted in 0.86% sodium chloride saline in the concentration of 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}. From each concentration 100 µl and 10 µl of microbial suspension were inoculated in separate petri plates containing Muller Hinton agar media and colony-forming units (CFU/mL) were calculated.

Bactericidal activity using agar-disc diffusion method

Preliminary antimicrobial activity was done by disc diffusion method [11,12]. In this method two sets of microbial suspension; 6 Log_{10} CFU/mL (10 µl) and 9 Log_{10} CFU/mL (100 µl) with 10^{-2} concentration (as the most suitable one) were used to examine efficacy of lichen extracts on different concentration of bacteria. Muller Hinton agar plates were inoculated by microbial suspension and spread completely by glass spreader, then kept for 30 min for adsorption of

microorganism on the surface of the media. Whatman filter paper No. 1 were used to prepare 0.6 cm diameter discs and soaked in different lichen extract (200 µg/ml) and implanted on surface of media [14]. All the petri plates were incubated at 34°C for 24 hrs. After completion of incubation period, zone of inhibition were measured. Tetracycline (30 µg/ml), Streptomycin (10 µg/ml), Ofloxacin (5 µg/ml) and Cefixime (5 µg/ml) were used as positive controls which are most potent standard antibiotics available against tested bacteria to compare lichen extracts with highly effective antibiotics [13].

Results

The suitable concentration of microbial cultures to grow fully on Muller-Hinton agar was found to be 10^{-2} in 0.86% sodium chloride saline. Therefore, 10 and 100 µl of 10^{-2} microbial suspension concentration was taken and after 24 hrs of inoculation, colony-forming units (CFU/mL) were calculated. For all the inoculated organisms, 100 µl had 9 $\log_{10}$ CFU/mL and 10 µl had 6 $\log_{10}$ CFU/mL of microbial density. For susceptibility test by agar disc diffusion method, both volumes were used in two different sets to examine the efficacy of lichen extract on higher number of bacteria. But for all further studies, 10 µl of 10^{-2} concentration (10^6 CFU/mL) was used as it was found more suitable for antimicrobial experiments (Plates 1-7).

Preliminary study on antibiotic activity of lichen extracts showed overall good efficacy of tested extracts on 6 $\log_{10}$ CFU/mL of bacterial suspensions and moderate activity on 9 $\log_{10}$ CFU/mL. Methanol followed by ethyl acetate extracts were the most bactericidal in nature. The order of each sample for their antibiotic activity according to average of their zone of inhibition was as follow: *P. tinctorum* (maximum ZOI: 32 mm) followed by *P. reticulatum* (maximum ZOI: 28 mm), *R. celastri* (maximum ZOI: 22 mm), *Herpothallon sp.* (maximum ZOI: 26 mm), *H. leucomelos* (maximum ZOI: 21 mm), *P. crinitum* (maximum ZOI: 18 mm), *Leptogium* sp. (maximum ZOI: 17 mm) and *C. subradiata* (maximum ZOI: 16 mm) (Table 1; Figure 1).

As far as susceptibility of bacteria is concerned, *B. subtilis* was the most sensitive bacteria by tested extracts and *P. aeruginosa* was the least (Figure 1). Tetracycline was most potent positive standard antibiotic but still in 9 $\log_{10}$ CFU/mL of *S. typhimurium* did not have any effect whereas all methanolic tested lichen extracts in same concentration of this bacteria were effective (Table 1).

Conclusion

Extensive literature survey indicates that lichens are good source of antibiotic secondary metabolites and can be utilized to cure various infectious diseases. Hence, here bactericidal activities of thirty-two extracts from eight lichen species were investigated. According to the results, lichen extracts had demonstrated moderate to highly potent antibacterial activity with maximum inhibition in methanol extracts, therefore, it was concluded that these methanolic lichen species might be used further for *in vivo* studies due to their broad-spectrum bactericidal activity.

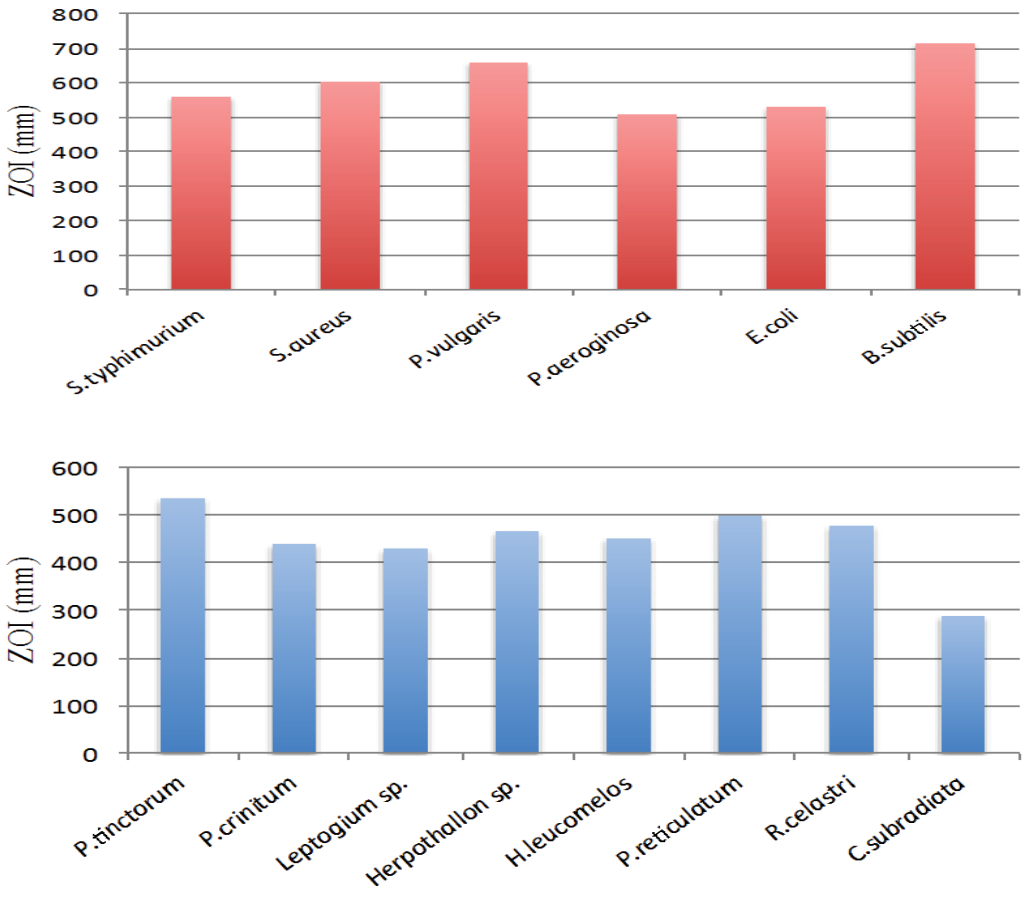

Figure 1: Preliminary bactericidal test of lichen extracts, Up: Susceptibility of tested pathogens treated with lichen extracts, down: Total inhibition zone of lichen extracts.

Lichen species solvent extract	*B. subtilis*		*E. coli*		*P. aeroginosa*		*P. vulgaris*		*S. aureus*		*S. typhi*	
Volume (µl)	100	10	100	10	100	10	100	10	100	10	100	10
P.tinctorum												
Hexane	9	7	10	10	9	8	7	-	9	8	7	8
Ethyl acetate	23	28	-	9	7	10	15	28	7	10	7	15
Methanol	12	32	10	16	9	18	10	12	12	12	7	14
Ethanol	10	10	-	12	8	7	8	7	16	16	10	15
P. crinitum												
Hexane	9	10	7	10	7	7	7	10	-	-	8	8
Ethyl acetate	11	12	7	8	-	12	11	16	-	18	7	7
Methanol	12	14	7	16	7	15	12	15	8	9	9	14
Ethanol	9	11	7	10	8	11	7	14	7	8	7	-
Leptogium sp.												
Hexane	-	10	7	8	10	9	7	7	-	-	7	7
Ethyl acetate	10	-	8	8	7	7	8	13	10	11	8	11
Methanol	12	13	10	15	10	12	10	17	9	14	8	10
Ethanol	10	10	7	8	7	8	8	16	10	9	8	12
Herpothallon sp.												
Hexane	7	7	10	10	7	7	7	7	7	7	8	7
Ethyl acetate	10	26	8	10	7	10	7	7	8	7	7	12
Methanol	9	20	10	16	10	14	12	12	8	14	7	14
Ethanol	9	11	8	10	7	7	9	7	9	9	8	15
H.leucomelos												
Hexane	10	10	10	7	8	7	7	11	7	13	7	8
Ethyl acetate	8	8	8	10	7	7	8	14	7	11	8	8
Methanol	15	16	9	12	9	13	12	16	8	7	7	15
Ethanol	-	-	8	10	10	7	8	14	10	21	7	8
P.reticulatum												
Hexane	12	7	9	11	9	7	8	8	10	12	11	8
Ethyl acetate	20	22	8	9	7	7	10	20	8	8	7	12
Methanol	8	20	12	28	10	16	14	15	8	12	9	7
Ethanol	-	-	8	11	7	7	11	12	9	10	7	7
R. celastri												
Hexane	17	20	7	-	8	18	7	12	9	9	7	7
Ethyl acetate	9	25	7	7	10	12	7	20	7	20	7	7
Methanol	13	15	12	12	10	13	9	22	7	11	7	7
Ethanol	8	7	-	7	7	9	7	7	7	7	7	7
Cladonia subradiata												
Hexane	7	8	-	-	-	-	-	12	8	16	7	10
Ethyl acetate	-	8	-	-	-	-	-	8	9	16	-	12
Methanol	14	15	-	15	-	-	-	17	11	16	12	16
Ethanol	-	8	-	-	-	-	-	9	-	16	7	10
Standard antibiotics												
Tetracycline	30	32	27	28	19	25	22	23	32	30	-	42
Streptomycin	-	-	26	26	29	42	30	30	27	28	-	-
Ofloxacin	35	36	38	42	42	50	42	50	42	46	-	45
Cefixime	-	-	24	25	-	18	-	-	-	16	-	-

Table 1: Antibacterial activity (zone of inhibition, mm) of various solvent fractions of lichen species and standard antibiotics with two different concentrations (10, 100 µl).

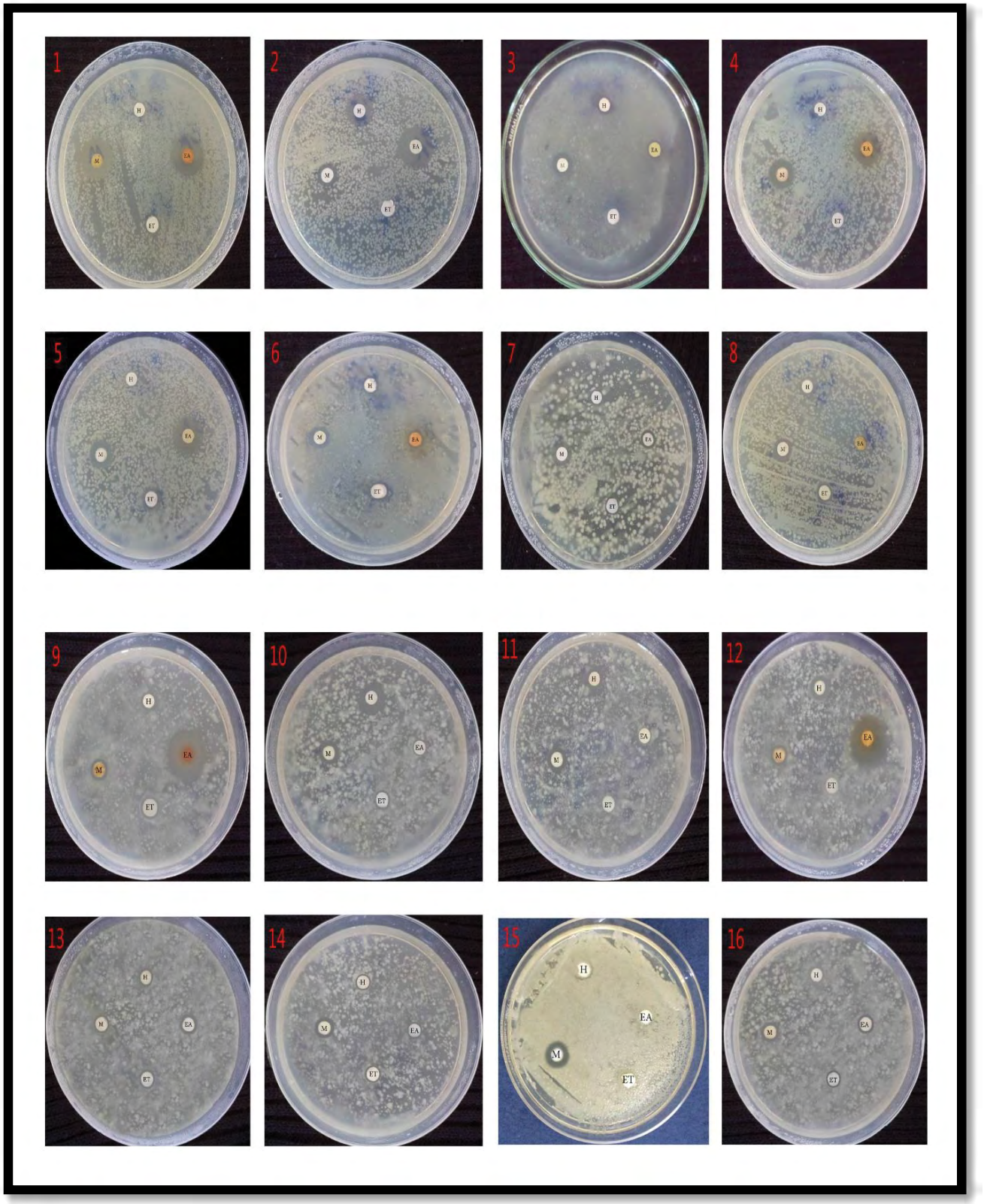

Plate 1: Antibacterial activity of lichen extracts against *B. subtilis* using agar-disc diffusion method. Up (1-8): 10 µl of bacterial suspension, Down (9-16): 100 µl of bacterial suspension. 1,9: *P. tinctorum*, 2,10: *R. celastri*, 3,11: *H.leucomelos*, 4,12: *P.reticulatum*, 5,13: *Herpothallon* sp., 6,14: *P.crinitum*, 7,15: *C.subradiata*, 8,16: *Leptogium* sp.

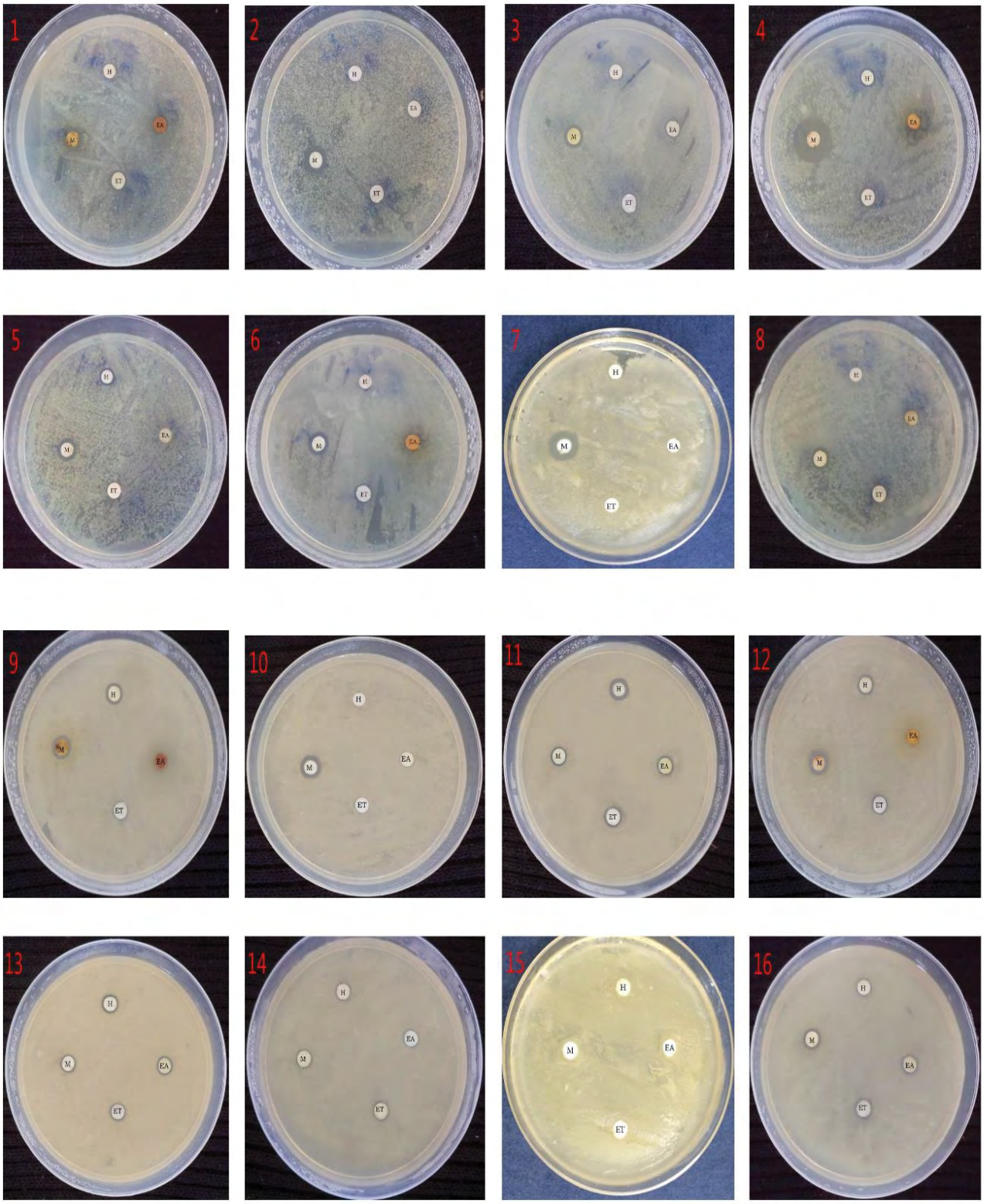

Plate 2: Antibacterial activity of lichen extracts against *E. coli* using agar-disc diffusion method. Up (1-8): 10 µl of bacterial suspension, Down (9-16): 100 µl of bacterial suspension. 1,9: *P. tinctorum*, 2,10: *R. celastri*, 3,11: *H.leucomelos*, 4,12: *P.reticulatum*, 5,13: *Herpothallon* sp., 6,14: *P.crinitum*, 7,15: *C.subradiata*, 8,16: *Leptogium* sp.

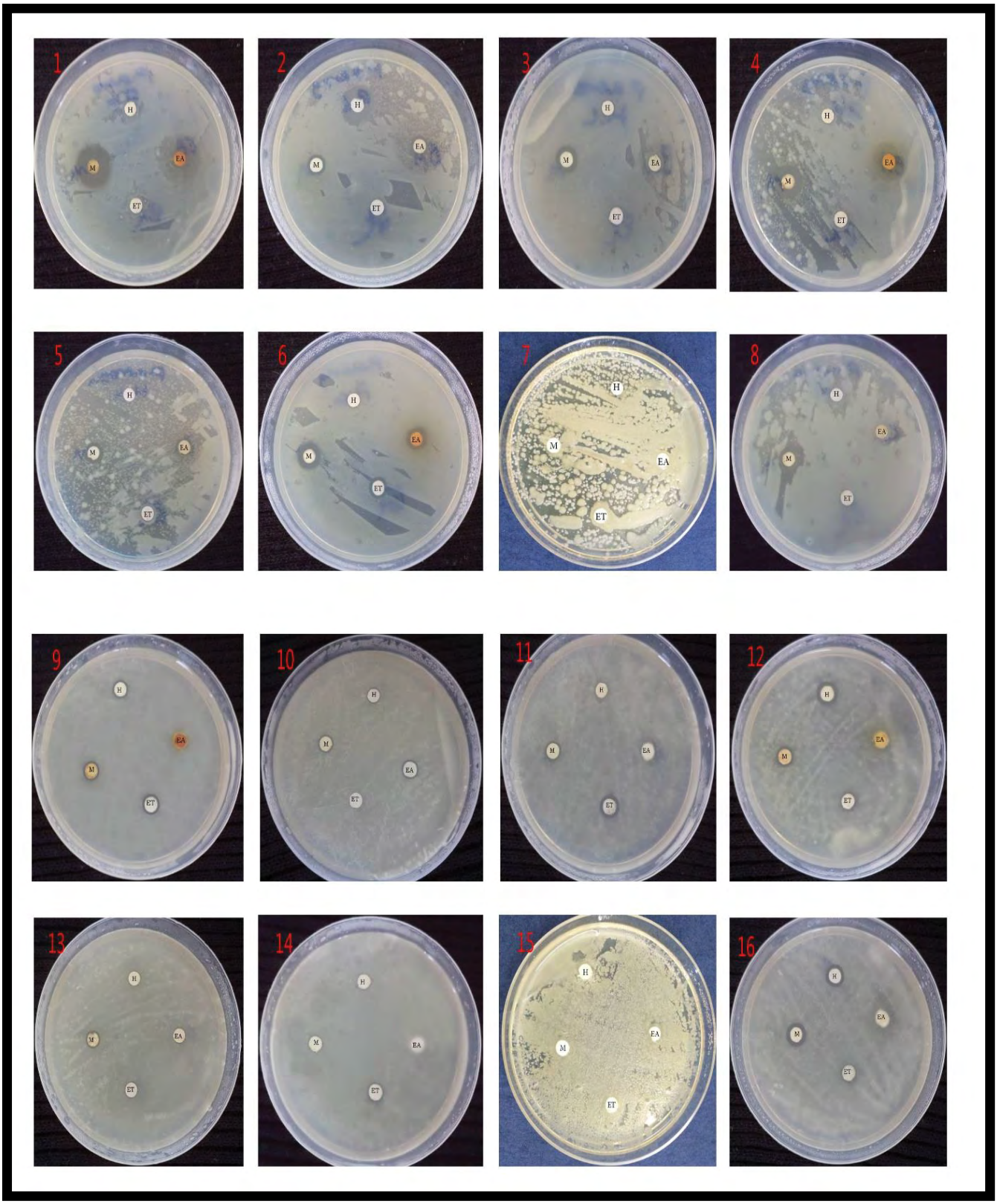

Plate 3: Antibacterial activity of lichen extracts against *P. aeroginosa* using agar-disc diffusion method. Up (1-8): 10 µl of bacterial suspension, Down (9-16): 100 µl of bacterial suspension.1,9: *P. tinctorum*, 2,10: *R. celastri*, 3,11: *H.leucomelos*, 4,12: *P.reticulatum*, 5,13: *Herpothallon* sp., 6,14: *P.crinitum*, 7,15: *C.subradiata*, 8,16: *Leptogium* sp.

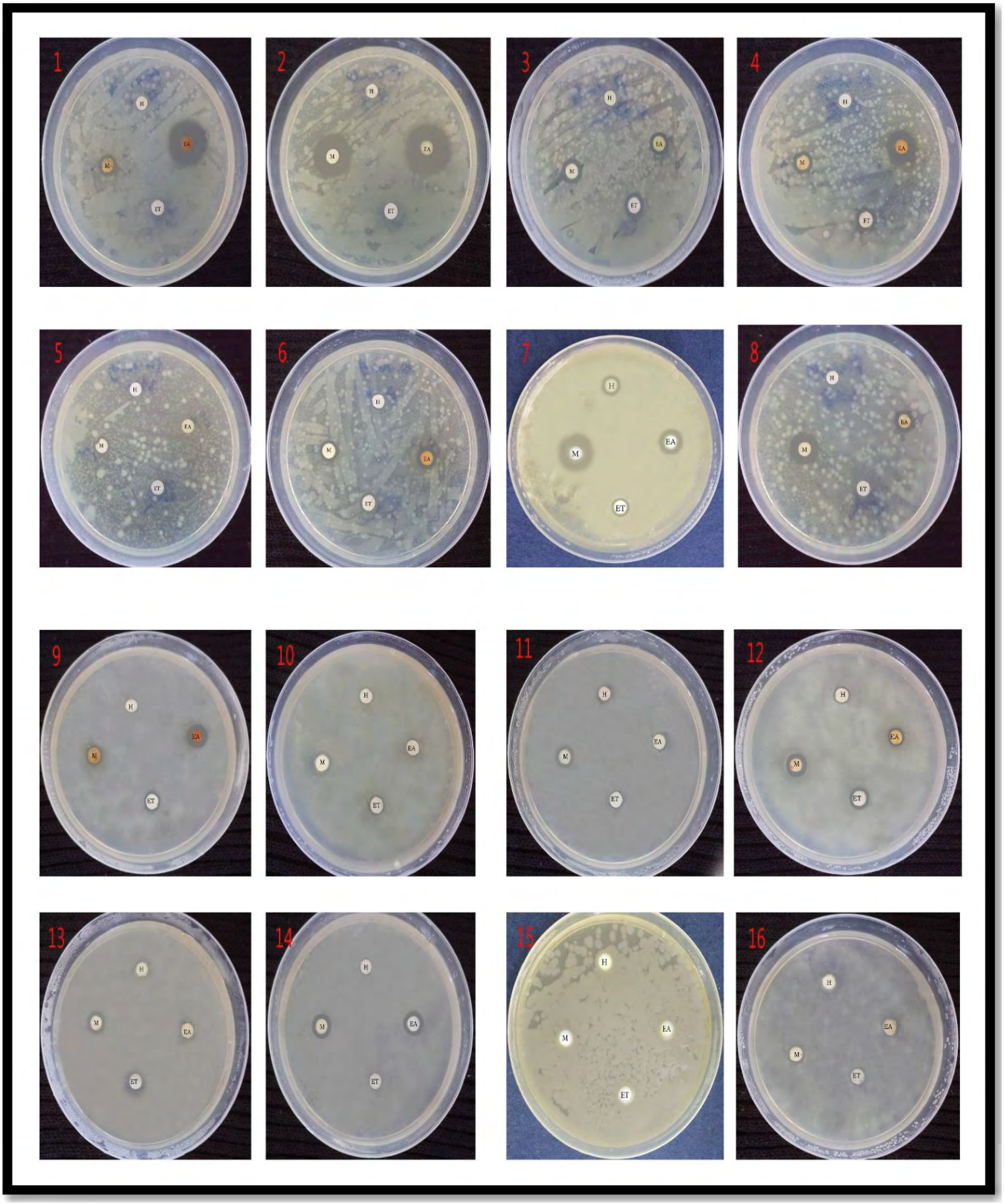

Plate 4: Antibacterial activity of lichen extracts against *P. vulgaris* using agar-disc diffusion method. Up (1-8): 10 µl of bacterial suspension, Down (9-16): 100 µl of bacterial suspension.1,9: *P. tinctorum*, 2,10: *R. celastri*, 3,11: *H.leucomelos*, 4,12: *P.reticulatum*, 5,13: *Herpothallon* sp., 6,14: *P.crinitum*, 7,15: *C.subradiata*, 8,16: *Leptogium* sp.

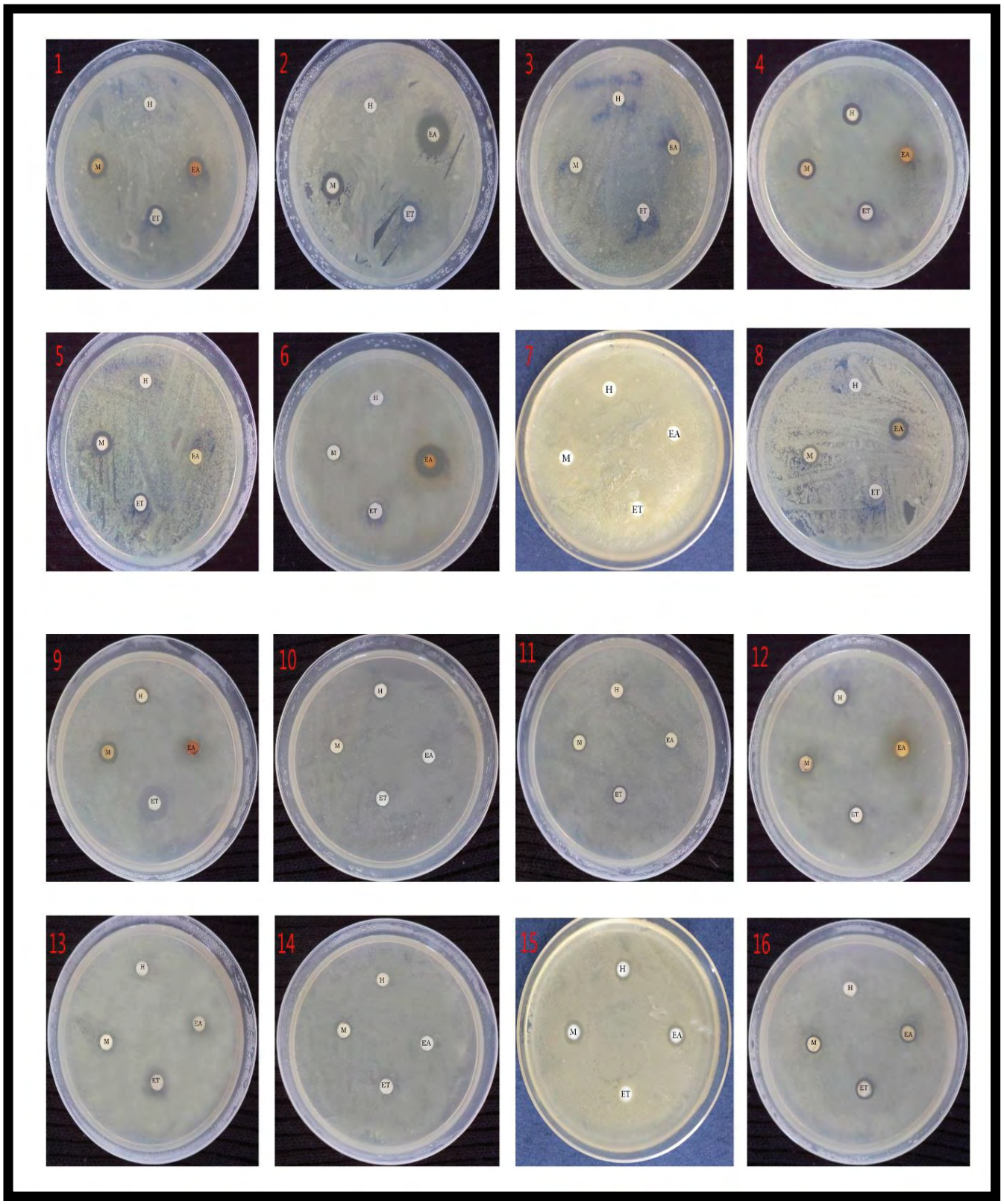

Plate 5: Antibacterial activity of lichen extracts against *S. aureus* using agar-disc diffusion method.Up (1-8): 10 µl of bacterial suspension, Down (9-16): 100 µl of bacterial suspension.1,9: *P. tinctorum*, 2,10: *R. celastri*, 3,11: *H.leucomelos*, 4,12: *P.reticulatum*, 5,13: *Herpothallon* sp., 6,14: *P.crinitum*, 7,15: *C.subradiata*, 8,16: *Leptogium* sp.

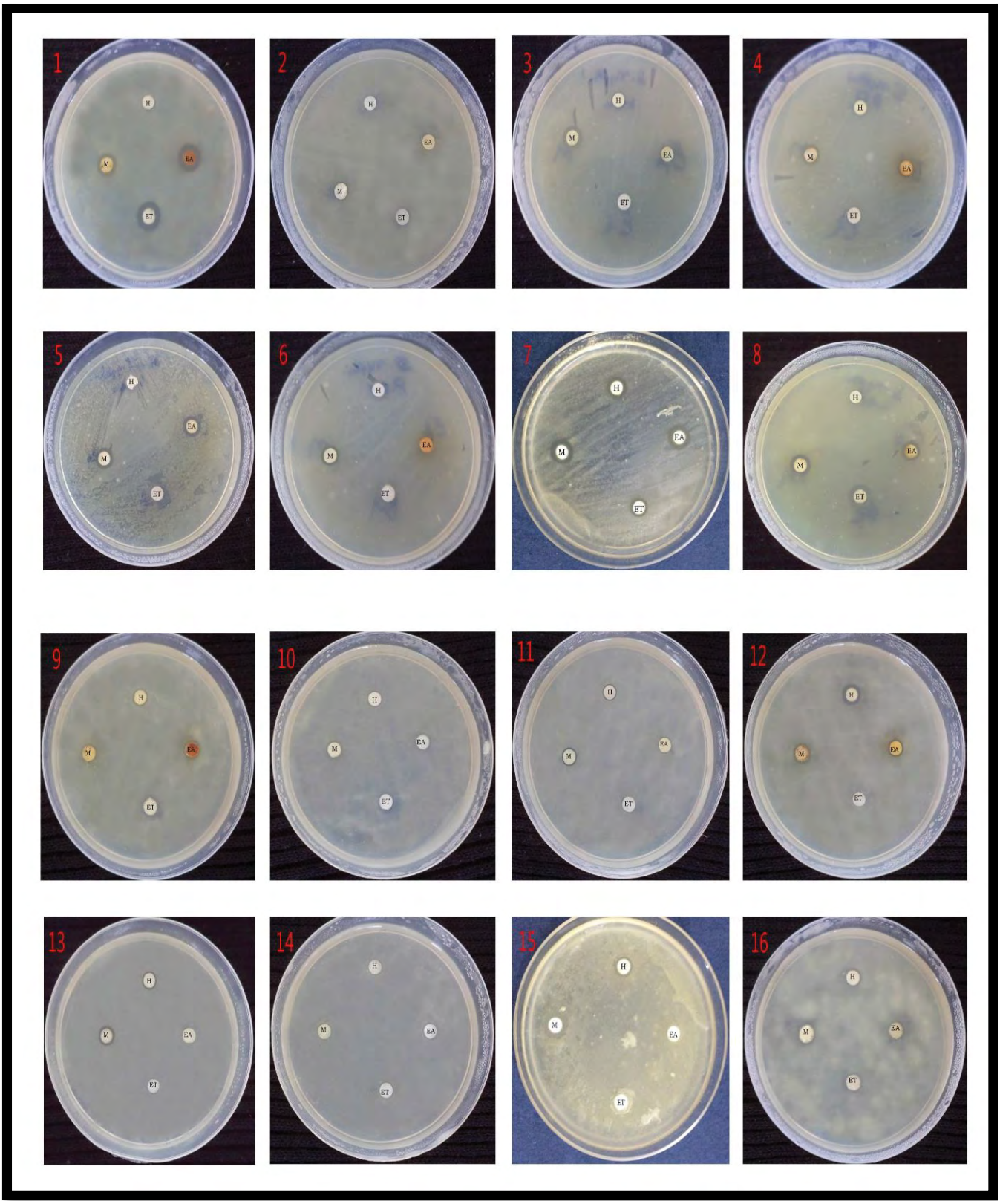

Plate 6: Antibacterial activity of lichen extracts against *S. typhimurium* using agar-disc diffusion method. Up (1-8): 10 µl of bacterial suspension, Down (9-16): 100 µl of bacterial suspension. 1,9: *P. tinctorum*, 2,10: *R. celastri*, 3,11: *H.leucomelos*, 4,12: *P.reticulatum*, 5,13: *Herpothallon* sp., 6,14: *P.crinitum*, 7,15: *C.subradiata*, 8,16: *Leptogium* sp.

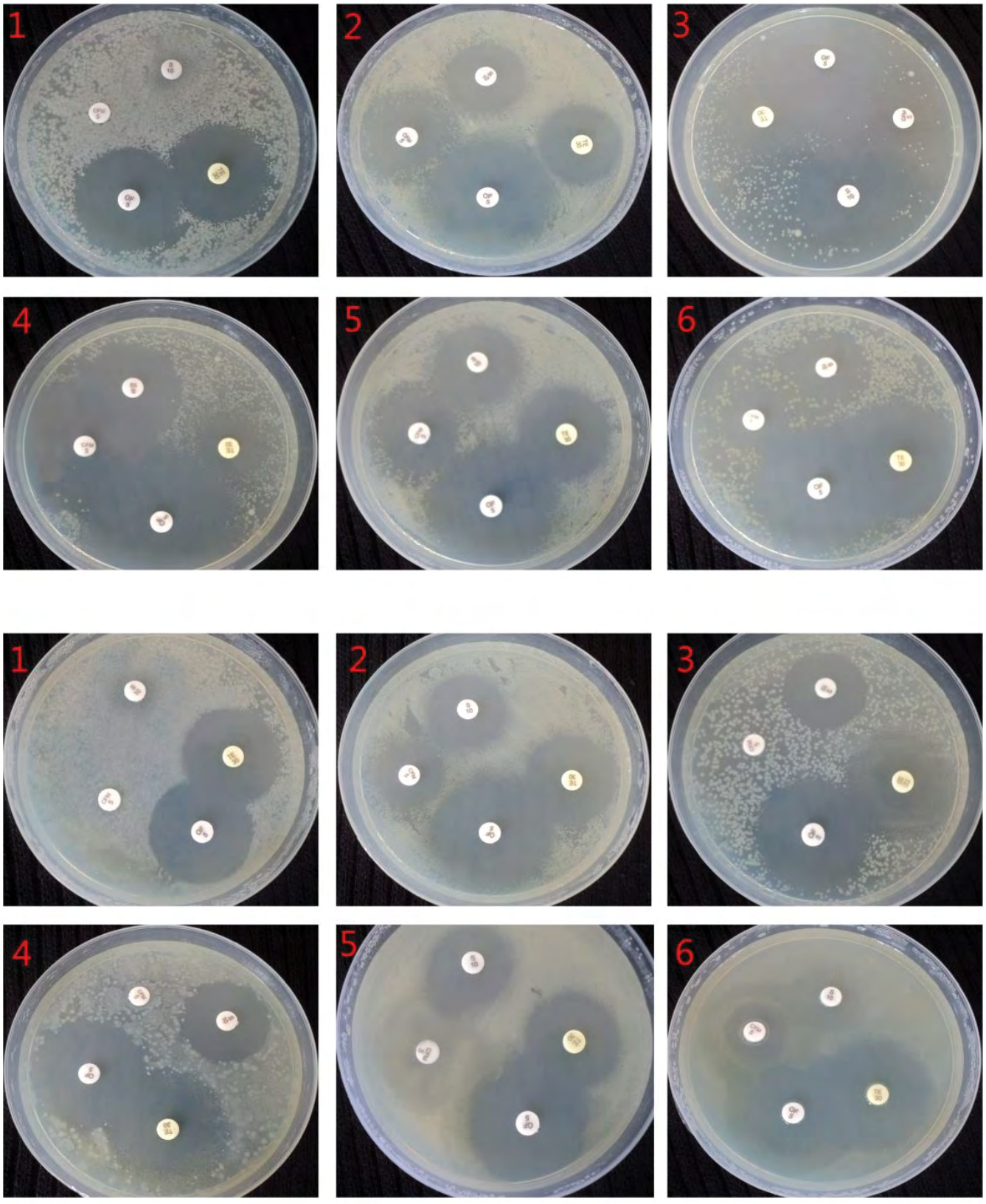

Plate 7: Standard antibiotic activity (1) *B. subtilis* (2) *E. coli* (3) *P.aeroginosa* (4) *P. vulgaris* (5) *S.aureus* (6) *S. thyphimurium.* Left 100 µl microbial suspension, Right: 10 µl microbial suspension. (T: Tetracycline, S: Streptomycin, O: Ofloxacin, C: Cefixime).

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