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Lichenomics: Exploring bioactive compounds with anti-tumor potential in colon cancer cell lines

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ABSTRACT

Lichens, a diverse group of Ascomycetes within the major fungal phylum Lecanorales, include various forms such as Crustose, Foliose, Squamulose, and Fruticose, with the latter being the most common photobionts. This study aims to provide novel in-sights into the extraction and acquisition of bioactive compounds from lichens, emphasizing their potential for future advancements in analytical techniques. This research highlights the pharmaceutical potential of secondary metabolites from lichens, with a focus on GC-MS analysis, which identified 80 compounds belonging to various functional groups. Many lichens have demonstrated bioactive properties, including novel drug candidates such as Falcarinol, Oleic Acid methyl ester, Benzoic acid, 2,4-dihydroxy-3,6-dimethyl, Glycerol 1-palmitate, Guanosine, 2(3 H)-Furanone, 3-butyldihydro, Eudesma-5,11(13)-dien-8,12-olide, Phthalic acid butyl undecyl ester, Phthalic acid ethyl pentadecyl ester, Tridecanoic acid 12-methyl methyl ester, Undecanoic acid 10-methyl methyl ester, Aromandendrene, Dodecahydroacenaphthylene, 3-Bromo-7-methyl-1-adamantanecarboxylic acid, Estra-1,3,5(10)-trien-17β-ol, 3-Methoxy-5-propylphenol, and Phenol. Consequently, *P. aurata* and *P. reticulatum* exhibited the highest antimicrobial activity, effectively inhibiting bacterial strains such as *E. coli, K. pneumoniae*, and *S. aureus*, particularly in terms of zones of inhibition. Additionally, the lichen extracts showed notable anti-tumor effects against HT-29 colon cancer cells, demonstrating a clear dose-dependent response for (10 mg/mL). Novel scientific methodologies involving biological entities have significantly contributed to a deeper understanding of lichenology. The research hypotheses are: (a) the collection of higher fungi, selective isolation of lichens, *in vitro* culture, and conservation; (b) extraction of lichen secondary metabolites and GC-MS analysis using innovative bioactive techniques.

1. Introduction

Lichenologists and mycologists have eagerly embraced the challenge of classifying lichens into four main growth forms: Foliose, Fruticose, Crustose, and Leprose. These forms are found within the subclass of Ascomycetes, which belongs to the largest fungal phylum, Lecanorales. The symbiotic relationship between lichens was first described in 1867 (Ahmadjian, 1967; Singh, 2023), and their name is derived from the species name of the fungal partner. Approximately 130 photobiont species are associated with the 29,000 reported lichenized and lichenicolous fungal species (Coppins, 1993; Muggia, & Grube, 2018). Globally, many unique mycobiont species share the same photobiont (Singh and Sinha, 2010). About 3005 lichen species have been documented on the Indian subcontinent, including those found in Kerala, Karnataka, Gujarat, Maharashtra, Goa, and Tamil Nadu (Rajaprabu and Ponmurugan, 2022). These species have been surveyed across various ecosystems worldwide (about 9.8 %), including tropical deciduous, subtropical temperate forests, evergreen forests, grasslands, and deserts (Matthews

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and Owen, 2008; Nayaka and Upreti, 2021; Shukla et al., 2010).

Further classification of lichen species has been carried out, such as *C. aurata P. aurata* and *S. fuliginosa* (Family Lobariaceae). These species are typically found on corticolous and saxicolous substrates, often in moist and humid environments like rainforests in the central part of the Western Ghats, including Kodaikanal and Ooty (Aragón et al., 2010; Gauslaa, 2014; Kaasalainen et al., 2023; Rajaprabu and Ponmurugan, 2022; Palmqvist and Sundberg, 2000). Another species, *L. leucomelos* (Family Pannariaceae), and the foliose lichen *P.reticulatum* (Family Parmeliaceae), as well as *H. leucomela* (Family Physciaceae), have been found in various parts of the world, including North America, Europe, and Asia (Del-Prado et al., 2016; Lücking et al., 2017; Moncada et al., 2014). The list of taxa has been examined in recent reports by international lichenologists, who are paying increasing attention to taxonomy (Lange and Galloway, 2015; Lücking et al., 2017; Ranft et al., 2018).

Lichens have recently been found to produce a diverse spectrum of metabolites, some of which are specific to lichen symbiosis, such as depsides, depsidones, dibenzofurans, and pulvinic acid (Brisdelli et al., 2013; Ghate et al., 2013; Liu et al., 2012; Cordeiro et al., 2020). However, caution is advised when employing lichens due to potential toxicity and sustainability concerns. Species like *P. aurata, S. fuliginosa, L. leucomelos, P. reticulatum*, and *H. leucomelos* play important ecological roles and exhibit antiviral, antibiotic, antioxidant, antitumor, allergenic, anticancer, and plant growth inhibitory activities (Asplund and Wardle, 2017; Manojlovic et al., 2010; Ranković et al., 2011; Elix & Stocker-Wörgötter, 2008). These species, including *P. reticulatum, P. aurata*, and *Parmotrema tinctorum*, have been investigated for various types of chemical extraction using ethanol, ethyl acetate, acetone, and methanol (Jain et al., 2016; Rajaprabu and Ponmurugan, 2023). Biological studies have examined their inhibitory effects, cytotoxic activities on different cancer cells, and carrageenan-induced inflammation (Behera et al., 2009; Pol et al., 2017; Shrestha and St. Clair, 2013;Kosanić et al., 2023; Tomović et al., 2024). Lichens are economically important sources of bioactive chemicals with a wide range of biological activities, including anticancer, anti-inflammatory, antibacterial, cytotoxic, and herbicidal properties (Poulsen-Silva et al., 2023; De Jesus, 2016; Gazo et al., 2019; Santiago et al., 2010; Timbreza et al., 2017; Molnár & Farkas, 2010).

Finally, secondary metabolic activity significantly impacts antioxidant activities, total polyphenols, polysaccharides, food flavor, and lichen-derived metabolic triterpenes. These demonstrate distinct sectorial localizations, such as Succinic acid, Pulvinic acid, Lecanoric acid, Chrysophanol, Conorlobaridone, and 5,7-Dihydroxy-6-methylphthalide (Kanwar and De, 2010; Luo et al., 2009; Marfatia et al., 2019; Pandhi et al., 2014; Rambhia et al., 2018). In this chapter, research on *H. leucomelos* species has detected triterpenoids, 6a-hydroxy-hop-21pH-22, Atranorin, zeorin, Glyceryl trilinolate, 3,6-dimethyl-2-hydroxy-4-methoxybenzoic acid, Cabraleadiol monoacetate, 4–0-methylcryptochlorophaeic acid, lichexanthone, and 3,6-dimethyl-2-hydroxy-4-methoxybenzoic acid (Poulsen-Silva et al., 2023; Tomović et al., 2024; Kathirgamanathar et al., 2006; Spribille et al., 2016). Several lichen species have been identified as promising sources for developing anticancer drugs, particularly for colon, breast, and lung cancer treatments. Ongoing research aims to optimize extraction methods and assess the bioavailability and pharmacokinetics of these compounds *in vivo*.

This study aims to investigate the bioactive compounds present in lichen species, particularly *P. aurata, S. fuliginosa, L. leucomelos, P. reticulatum,* and *H. leucomelos*, and their potential ecological and pharmacological significance. By identifying and characterizing key metabolites extracted using acetone solvent, this study explores their biological activities, including anticancer properties. Compounds such as falcarinol, oleic acid methyl ester, and benzoic acid have been identified as secondary metabolites with potential therapeutic agents.In conclusion, the study of lichen-derived bioactive compounds represents a promising avenue for cancer research and drug development. As lichens continue to be explored for their pharmaceutical potential, understanding their secondary metabolites and refining extraction techniques will enhance their applicability in modern medicine.

2. Materials and methods

2.1. Samples

Lichens were collected from various environmental conditions in Coimbatore, including *P. aurata* (Ach.) Vain., *S. fuliginosa* (With.) Ach., *L. leucomelos* (L.), *P. reticulatum* (Taylor) (Foliose lichen), and *H. leucomelos* (Physciaceae). These samples were taken from soil, rock, tree bark, and moss plants during the rainy season from August to December-2023. The lichen specimens were carefully plucked from tree bark to a measurable height and sliced with a chisel. Lichen thalli attached to rocks were cut from the base using a chisel and hammer. The terricolous lichens were extracted from the soil and transported to the Lichen Herbarium at the Biomedical Research Laboratory, Department of Botany, Bharathiar University in Coimbatore, Tamil Nadu, India.

2.2. Phenotypic characterization of Lichen

The lichens were identified at the Lichen Herbarium, Biomedical Research Laboratory, Department of Botany, Bharathiar University, Coimbatore, Tamil Nadu, India. Individual lichen species were authenticated by comparing the specimens to those in the herbarium at the National Botanical Research Institute in Lucknow. Identification processes included morphological characterization, microscopic examination, chemical tests, and thin-layer chromatography analysis of secondary metabolites. Compound light microscopy was used to identify the thalli morphologically and anatomically. The lichens were identified following the key identification manual by (Coppins, 1993; Singh and Sinha, 2010; Wolseley, 2008).

2.3. Phytochemical extraction of secondary metabolites in lichens

Lichen samples were thoroughly washed using distilled water to removed surface impurities and extraneous materials. Then lichen samples were air-dried with shadow at room temperature for incubation for five days with make grounding powder by a grinder to a fine powder, followed by the same sample were maintain in container for the further experiments. An acetone solvent extraction method was used to elute and recover secondary metabolites from the lichen thalli crude extract. A known amount of lichen (5 g) was packed in a fat-free thimble and placed in the extraction tube at 26 °C. The receiving beaker was filled with 100–150 mL of acetone, chloroform, and distilled water, using cold extraction for 24 hours at a room temperature of 26 ± 2 ºC. The ether extract was transferred to a clean glass dish and concentrated for 30 minutes in a 103°C water bath. A desiccator was used to cool the concentrate. The same method was used to collect the lichen's acetone and methanolic extracts. Based on the qualitative results, a quantitative assay was conducted for primary and secondary metabolites extracted from the lichen samples.

2.4. GC-MS analysis

Secondary metabolites were extracted and analyzed using gas chromatography-mass spectrometry (GC–MS). The foliose lichen samples, including *P. aurata* (Ach.) Vain., *S. fuliginosa* (With.) Ach., *L. leucomelos* (L.) (Physciaceae), and *P. reticulatum* (Taylor) Joisy (Parmeliaceae), were identified through morphological traits and thallus reactivity. While common spot tests can detect the presence of lichen metabolites, proper analytical research is necessary to quantify metabolite levels. The Crop Protection Division Entomology Lab at the ICAR-National Research Centre for Bananas analyzed the sample using a TRACE 1300 GC Ultra Gas Chromatograph (Thermo Fisher Scientific, Austin, TX, USA) with a Thermo mass spectrometer detector (ISQ Single

Quadrupole Mass Spectrometer). The GC-MS system was equipped with a TR-5 MS column (30 m × 0.32 mm i.d., 0.25 μm film thickness). Analyses were conducted using helium as the carrier gas at a flow rate of 1.0 mL/min and a split ratio of 1:10, under the following temperature program: 60 °C for 1 minute, then increasing by 4.0 °C per minute to 240 °C, with a 1-minute hold. The injector and detector temperatures were maintained at 210 °C. Mass spectra were obtained using electron ionization (EI) at 70 eV, with a spectral range of *m/z* 40–450.

2.5. Screening for antibacterial activities

These lichen compounds were tested for antibacterial activity against *Staphylococcus aureus* and *Klebsiella pneumoniae* pathogens obtained from the MTCC, using acetone-solvent-eluted samples. Experiments were conducted on three bacteria: *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Escherichia coli*. Additional antibacterial testing was performed by the Department of Biotechnology at Nehru Arts and Science College (Autonomous). The extracted lichen samples were tested against bacterial pathogens, including antibiotic-sensitive *S. aureus*, *K. pneumoniae*, and *E. coli*, using the paper disc diffusion method on LB agar, followed by an 18–24-hour incubation at 37 °C. Standardized test microorganisms were first swabbed onto solid Mueller-Hinton Agar (Hi-Media) plates (4 mm deep). Lichen crude extracts (5 μL, 10 μL, 15 μL, and 20 μL) were applied to sterile paper discs and air-dried for 10–15 seconds until the solvent evaporated, resulting in a final concentration of 200 μg per disc. The discs (Whatman) were then carefully placed on the culture agar swabbed with the test microorganisms. Positive and negative controls were placed alongside the test lichen extracts, and each extract was tested in triplicate. The culture plates were incubated at 37 °C for 18–24 hours, after which the zones of inhibition were measured using a 90-mm Vernier caliper, as described.

2.6. In Vitro anticancer and cytotoxicity assay

The colon adenocarcinoma (HT-29) cell line was obtained from India's National Centre for Cell Science (NCCS) and maintained in the laboratory for methyl thiazolyl tetrazolium (MTT) cytotoxicity assays. The HT-29 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10 % fetal bovine serum (FBS), 10 mM 2-hydroxyethyl disulfide, Penicillin G (100 U/mL), Streptomycin (50 mg/mL), and Amphotericin B (2.5 mg/mL), with the pH adjusted to 7.0–7.4. The cell lines were kept in a CO_2 incubator at 37 °C in a humidified atmosphere containing 5 % CO_2. A 200 μL suspension of cancer cells (1×10^4 cells/mL) was added to each well of a 96-well microplate and pre-incubated for 24 hours. In the 96-well plate, HT-29 cells were treated for 48 hours with tannic acid, catechin, purpurin, and reserpine at concentrations ranging from 0 to 300 mg/ mL. After treatment, 10 μL of cell proliferation reagent was added to each well and incubated for 2 hours at 37 °C. After 72 hours of treatment, 50 μL of MTT saline solution (1 mg/mL) was added to each well, and the cells were incubated for an additional three hours under the same conditions to assess viability. Following the removal of the growth medium, 150 μL of DMSO was added to dissolve the cells. Cell proliferation and viability were measured at 460 nm using a microplate ELISA reader (Thermo Electron Corporation, USA). The viability of the treated cells was compared to that of the untreated control cells using the following formula: Cell viability % = [100 ×Sample (Abs)/Control (Abs)]

2.7. Statistical analysis

Values are presented as mean ± SD with ANOVA results (n = 3). Different letters represent significant differences between different concentrations ($P < 0.05$; Tukey's test).

3. Results

3.1. Lichen

Our results revealed four basic growth forms of lichens: Crustose, Foliose, Squamulose, and Fruticose, through the subspecies *P. aurata*, *S. fuliginosa*, *L. leucomelos*, *P. reticulatum*, and *H. leucomelos* (Fig. 1). Based on phenotypic variation, *P. aurata* exhibits a foliose (leafy) growth form with lobes that are often irregularly shaped and overlapping. Like other lichens, *P. aurata* is a symbiotic organism consisting of a fungus and algae or cyanobacteria living together in a mutually beneficial relationship. The fungus provides structure and protection, while the algae or cyanobacteria perform photosynthesis to produce energy for both organisms. *P. aurata* is known for its yellow to orange coloration and foliose growth form. *P. reticulatum* is found growing on rocks, bark, or soil in a variety of habitats, including forests, woodlands, and urban areas. It is relatively tolerant of environmental disturbances and can thrive in moderately polluted environments. Like many lichen species, *P. reticulatum* plays important ecological roles, such as nutrient cycling, soil stabilization, and habitat provision for other organisms. It is also sensitive to changes in air quality and habitat conditions, making it a useful bioindicator species for assessing environmental health.

H. leucomelos, belonging to the family Physciaceae, is known for its foliose growth form and typically grows on rocks, bark, or soil in various habitats. This species has a grayish thallus with white sporidia or isidia. *S. fuliginosa* typically grows on the bark of trees, especially in humid and shaded environments such as old-growth forests. It has a foliose growth form, with lobes that are broad and somewhat strap-like, often overlapping. The upper surface of the lobes is typically dark green to brownish-black, while the under surface is pale with black dots or pustules (reproductive structures). Like other lichens, S. fuliginosa is a symbiotic organism, with the fungus providing structure and protection and the algae or cyanobacteria performing photosynthesis. S. fuliginosa is sensitive to air pollution and habitat disturbance (Fig. 1).

3.2. Secondary metabolite elution via GC-MS analysis

A total of 80 secondary metabolites were identified from various lichen species, including *P. aurata* (Fig. 2A), *S. fuliginosa* (Fig. 2B), *L. leucomelos* (Fig. 2C), *P. reticulatum* (Fig. 2D), and *H. diadamata* (Fig. 2E) through GC-MS analysis (Table 1). These lichen species were distinguished a diverse array of chemical compounds, including alcohols, hydrocarbons, phenols, ketones, aldehydes, carboxylic acids, and esters. *P. aurata* showed the highest reliability, containing a significant number of secondary metabolites. Followed by, *P. reticulatum* exhibited alcohols, esters, hydrocarbons, sugar compounds, carboxylic acids, and various functional groups, demonstrating moderate reliability across sixteen compounds. In addition, *S. fuliginosa* displayed fifteen chemicals, including phenols, alcohols, hydrocarbons, esters, carboxylic acids, nitrogen and sulfur-containing compounds, with low reliability. Meanwhile, *L. leucomelos* contained fourteen chemicals, including hydrocarbons, alcohols, esters, ketones, carboxylic acids, and a variety of functional groups and sugar compounds, with low reliability. Finally, *H. diadamata* contained alcohols, hydrocarbons, esters, and carboxylic acids, with low reliability across nine compounds (Fig. 3 & 4).

Most of the lichens possess bioactive properties that are significant for their antimicrobial, anti-inflammatory, and other biological properties which are unique to this symbiosis (over 90 %). The highest number of secondary metabolites was found in the esters group, with fourteen compounds, followed by hydrocarbons in twelve and alcohols groups in eleven. Carboxylic acids and diverse functional groups were represented by a smaller number of compounds. Ketones had the lowest reliability, while sugar compounds, aldehydes, and nitrogen-containing compounds were the least represented, with only one or two compounds (Fig. 3). The traditional understanding of lichen symbiosis is evolving my concentric viewpoint to a more expansive one, viewing lichens as a

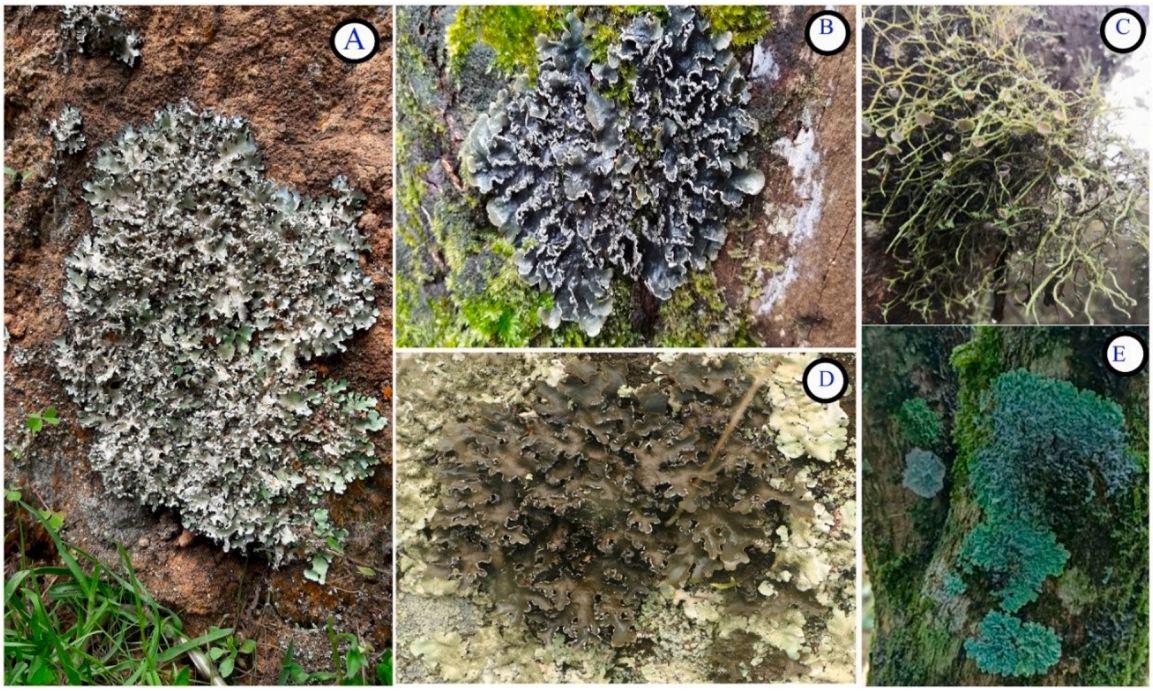


Fig. 1. Pictures of some species of lichen (a), *P. reticulatum,* (b), *P. gaurata* (Ach.). (c). *L. leucomelos* (L.) (d). *S. fuliginosa* (With.) Ach., (e) *H. diadamata.*

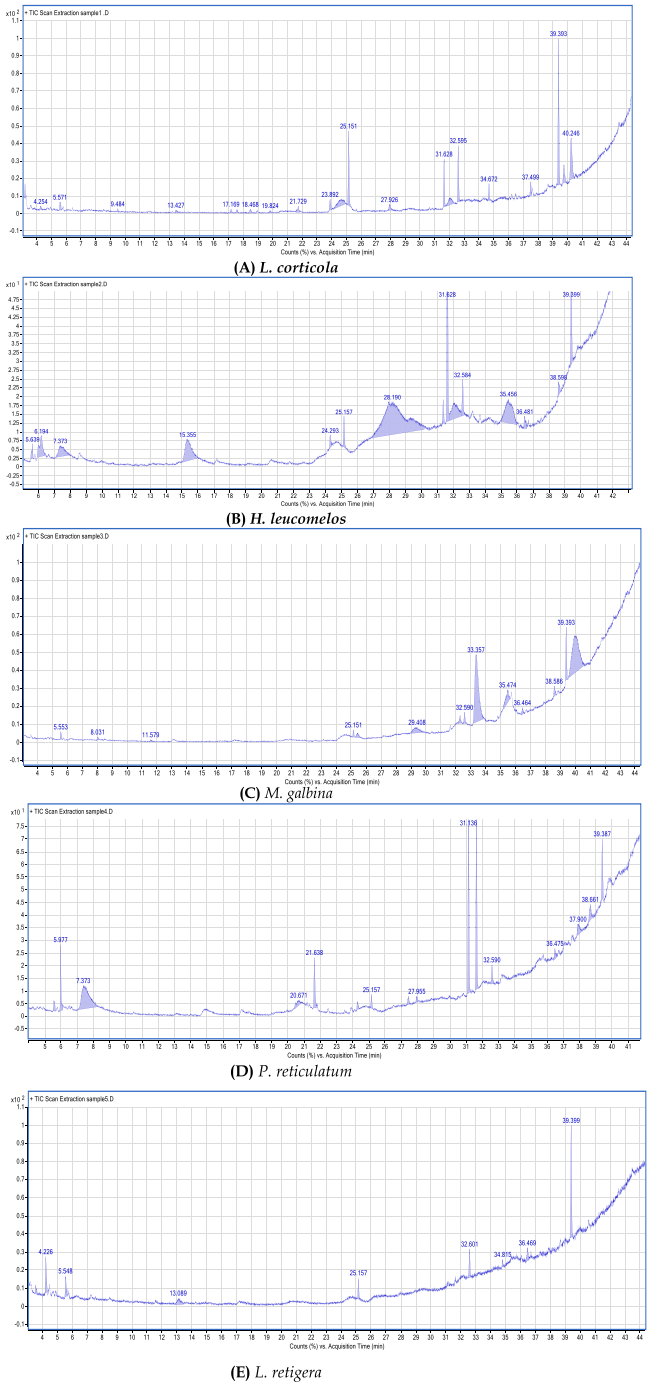
microbial community comprising numerous related taxa. Industrial activities involve the production and utilization of various chemical compounds such as 1,3,5-Pentanetriol, 3-methyl, Cyclohexanecarboxylic acid, Phthalic acid, ethyl pentadecyl ester, Estra-1,3,5 (10)-trien-17β-ol, 3-Methoxy-5-propylphenol, Spiro [2.3] hexan-4-one, 5,5-diethyl-6-methyl-, Phenol, Cyclohexanecarboxylic acid, and 1H-3a,7- Methanoazulene, octahydro-1,4,9,9-tetramethyl-, and Octadecane, 6-methyl-.

Interestingly, some lichen metabolites are also synthesized in animals and humans, identified as 2,5-Cyclohexadiene-1,4-dioneBicyclo [3.1.1] hept-2-ene, 3,6,6-trimethyl-, 2,5-Cyclohexadiene-1,4-dione, 4-Isopropenyl-4,7-dimethyl-1-oxaspiro [2.5]octane 8-Heptadecene, Dodecanoic acid, Cyclopropanetetradecanoic acid, 2-octyl-, methyl ester, and 2-(acetyloxy)-1-[(acetyloxy)methyl]ethyl ester, among others. The medicinal benefits of lichens are largely attributed to these active compounds, which are specific to their symbiosis (Fig. 3). Lichens have become a prominent area of research for their medicinal applications, including anti-cancer compounds like Estra-1,3,5(10)-trien-17β-ol, Falcarinol, Eudesma-5,11(13)-dien-8,12-olide, and 3-Bromo-7-methyl-1-adamantanecarboxylic acid. Antimicrobial activity was determined for compounds such as 2,5-Octadecadiynoic acid, methyl ester, Phthalic acid, butyl undecyl ester, 1-Octyn-3-ol, 4-ethyl, 6-epi-shyobunol, Falcarinol, Aromandendrene, Decane, Guaia-1(10),11-diene, and 1-Pentanol, 5-(methylenecyclopropyl) these are chemicals showing in cytotoxicity against cancer cells and anti-proliferative effects.

In this study, transplanted lichens were exposed to insecticides such as cyclopropanecarboxylic acid, ethyl ester, cis, cyclopropanetetradecanoic acid, 2,2-dimethyl-3-(2-methyl-2-propenyl), and 2-octyl-, methyl ester. Oleic acid, 6-epi-shyobunol, Eudesma-5,11(13)-dien-8,12-olide, and 8-Heptadecene have shown potential for future herbicide and fungicide research. The study's findings contribute to a better understanding of insect pheromones, including 1,5-Heptadiene, 2,5-dimethyl-3-methylene, 1-Octanol, 2,7-dimethyl, 2(1 H)-Pyridinone, 3-methyl, Aromandendrene, and Estra-1,3,5(10)-trien-17β-ol. Certain steroids have been identified as pheromones in various insects, with compounds also exhibiting antiherbivore and antiparasitic characteristics (Fig. 3 & 4). To analyze the biological role of these lichen compounds, we can examine their potential pharmacological activities based on existing research. For example, 2-Methoxybenzyl alcohol, 4-Ethylcyclohexanol, and Phellandrene are monoterpene compounds studied. Guanosine, a nucleoside, plays a role in neurotransmission and energy metabolism, and has been studied for its potential neuroprotective and anti-inflammatory effects. Eudesma-5,11(13)-dien-8,12-olide, a sesquiterpene lactone, exhibits antioxidant properties with potential anti-inflammatory and cytotoxic activities. Estra-1,3,5(10)-trien-17β-ol, a steroid, has diverse biological activities, including anti-inflammatory, immunosuppressive, and hormonal effects. Benzoic acid and its derivatives are known for their antioxidant, antimicrobial, and anti-inflammatory properties. Their pharmacological activities are well-documented (Fig. 3 & 4).

3.3. Antimicrobial assay of lichen species

The lichen extracts exhibited varying inhibitory effects against the tested bacterial strains, including *E. coli, K. pneumoniae,* and *S. aureus*. The results, presented as zones of inhibition (cm), were observed for extracts from *P. aurata, S. fuliginosa, L. leucomelos, P. reticulatum*, and *H. leucomelos* against three different bacterial strains (Fig. 5). Lichen extracts were tested at different concentrations (5, 10, 15, and 20 mg/mL) along with a control. For *E. coli*, the zone of inhibition ranged from 0.2 cm to 0.9 cm, with the maximum inhibition observed at a concentration of 20 mg/mL (Fig. 5A). For pathogenic *K. pneumoniae*, the inhibition ranged from 0.2 cm to 1.3 cm, with the strongest effect also observed at 20 mg/mL (Fig. 5B). S. aureus showed inhibition ranging from 0.3 cm to 1.3 cm, with the highest inhibition again at 20 mg/mL (Fig. 5C). The picture shows the antibacterial activity at different concentrations (5, 10, 15, 20 μg/mL and control) of crude extracts amended with LB medium in LB agar diffusion assays (200 and 100 μg/mL of crude extracts) showed zones of inhibition (ZOIs) exhibited by the lichen extracts of for different bacterial Strains such as (A) *E. coli,* (B) *K. pneumoniae*, (C) *S. aureus* dorsal view and bottom view of (D) *E. coli,* (E) *K. pneumoniae*, (F) *S. aureus*. Overall, extracts from *P. aurata, S. fuliginosa, L. leucomelos, P. reticulatum,* and *H. leucomelos* exhibited promising antibacterial activity against the tested bacterial strains, with stronger effects observed at higher concentrations. Similar to *P. aurata,*



(A) *L. corticola*

(B) *H. leucomelos*

(C) *M. galbina*

(D) *P. reticulatum*

(E) *L. retigera*

Fig. 2. GC-MS fingerprint of methanolic extracts of species of lichen (a), *P. reticulatum,* (b), *P. gaurata* (Ach.). (c). *L. leucomelos* (L.) (d). *S. fuliginosa* (With.) Ach., (e) *H. diadamata.*

the extract from *P. reticulatum* demonstrated inhibitory effects against all tested bacterial strains. The zone of inhibition for *E. coli* ranged from 0.5 cm to 1.0 cm, with the maximum inhibition at 20 mg/mL (Fig. 5E, F). *K. pneumoniae* inhibition ranged from 0.2 cm to 1.3 cm, with the highest inhibition at both 20 mg/mL and 15 mg/mL concentrations (Fig. 5B). S. aureus inhibition ranged from 0.3 cm to 1.2 cm, with the strongest effect observed at 20 mg/mL

3.4. Anti-Tumor effect on colon cancer line

The data suggests that lichen extract possesses significant anti-tumor potential against HT-29 colon cancer cells, with higher concentrations exhibiting greater efficacy in inhibiting cell growth. The results illustrate the anti-tumor activity of *P. aurata, S. fuliginosa, L. leucomelos, P. reticulatum*, and *H. leucomelos* lichen extracts on the HT-29 colon cancer cell line at varying concentrations (Fig. 6 & 7). As the concentration of the extract increased, there was a notable decrease in cell viability, indicating a dose-dependent response. At the highest concentration tested (10 mg/mL), cell viability was substantially reduced to 17.07 %. Conversely, the lowest concentration (0.156 mg/mL) showed a relatively minor decrease in cell viability to 85.36 %. The curve exhibited a clear dose-response relationship, indicating that as the concentration of lichen extract increased, cell viability decreased. Below a concentration of approximately 2.5 mg/mL, the reduction in cell viability was relatively gradual. However, as the concentration increased further, the curve steepened, indicating a more substantial reduction in cell viability. At the highest concentration tested (10 mg/mL), the curve reached its lowest point, indicating maximum inhibition of cell viability (17.07 %). This concentration demonstrated the highest efficacy of *P. aurata* and *H. leucomelos* lichen extracts in suppressing the growth of HT-29 colon cancer cells. Overall, these results demonstrate the dose-dependent anti-tumor activity of the treatment on HT-29 colon cancer cells.

To perform the LC50 analysis for cell viability and determine the concentration at which 50 % of the cells are viable, a dose-response curve or interpolation between data points can be used. Since the provided data points do not directly give the LC50 value, linear interpolation was used to estimate the LC50 value, using points (1.25, 48.78) and (0.625, 53.65). The estimated LC50 value for this experiment is approximately 0.939 mg/mL, suggesting that at a concentration of 0.939 mg/mL, approximately 50 % of the HT-29 colon cancer cells would be viable (Fig. 6 & 7). The S-shaped curve, commonly observed in dose-response relationships in pharmacology, visually confirms the findings from the tabulated data, demonstrating the concentration-dependent anti-tumor effect of lichen extracts on HT-29 colon cancer cells. Higher concentrations of lichen extract led to significant inhibition of cell viability, indicating its potential as a therapeutic agent against colon cancer (Fig. 6 & 7)

4. Discussion

Identifying lichens can be more challenging than characterizing them, especially when separating them from other vascular plants under the microscope. Generally, lichens are classified based on fungal characteristics, such as those found in species like *P. reticulatum*, *P. aurata*, *L. leucomelos*, *S. fuliginosa*, and *H. diadamata*. Recent genomic research, which began a year ago, has focused on the larger and more complex genomes of primary mycobionts, although data is still lacking (Balaji and Hariharan, 2004; Upreti et al., 2015). A lichen species was documented and submitted as a voucher specimen at the CSIR-National Botanical Research Institute, Uttar Pradesh, India, with samples collected from various locations across India (Rajaprabu and Ponmurugan, 2022; Rajaprabu et al., 2021; Upadhyay et al., 2020).

More recently, chemical ecology techniques have been applied for spatial mapping of small molecules at the intersection of biology and chemistry using GC-MS (Kim et al., 2021). This innovative technique, which began developing over twenty years ago, was first used for studying secondary metabolism in medicine (Kim et al., 2021). Lichens have also been synthesized as new options for extracting ethanol, ethyl acetate, acetone, and methanol from *P. reticulatum* lichen, which could alleviate carrageenan-induced inflammation (Jain et al., 2016; Luo et al., 2009). These extracts have been studied for their inhibitory effects and cytotoxic activities against different cancer cells (Ghate et al., 2013; Pol et al., 2017; Rani et al., 2018).

Interestingly, the biology of lichens, such as *P. aurata*, has been reviewed, revealing derivatives in metabolic triterpenes and different sectorial localizations according to compounds like succinic acid, 5,7-

Table 1
Secondary metabolites of pharmaceutical interest found in lichens from Tamil-Nadu.

Chemical class	Secondary Metabolites	RT	MW	*P. aurata*	*S. fuliginosa*	*L. leucomelos*	*P. reticulatum*	*H. diadamata*	Biological Activity	References
Alcohols	1,3,5-Pentanetriol, 3-methyl-	3.19	134	1.32					Mild pleasant odor, soaps, dyes, chemicals	Cordeiro et al., 2020
	1-Octanol, 2,7-dimethyl-	5.56	158				1.61	8.47	Metabolite, a protic solvent	Huneck & Yoshimura, 1996
	1-Octyn−3-ol, 4-ethyl-	3.56	154					3.63	Antioxidant, anti-cancer, and neuroprotective	Elix & Stocker-Wörgötter, 2008
	1-Pentanol, 5-(methylenecyclopropyl)-	19.82	140	0.31					Toxicity, pharmacological, and Environmental	Ranković & Kosanić, 2015
	2-Methoxybenzyl alcohol	25.15	138	12.27					Antioxidant, antimicrobial, and anti-Inflammatory	
	4-Ethylcyclohexanol	20.67	128				2.28		Antimicrobial, anti-inflammatory, and analgesic	
	5-cis-Methyl−1R,3-cis-cyclohexanediol	8.03	130			0.38			Toxic ingestion, inhalation and skin absorption	Molnár & Farkas, 2010
	6-epi-shyobunol	27.42	222				1.03		Plant metabolite, Fungal and Antimicrobial	
	1,2,3-Butanetriol	7.37	106		3.53				Eyes and mucous toxic by ingestion	Muggia et al., 2018
	Falcarinol	31.37	244		1.1				Microbial metabolite	Ranković et al., 2020
	Glycerin	7.37	92				20.91		Human metabolite, Algal, *E.coli,* mouse metabolite	
	1,3,5-Pentanetriol, 3-methyl-	3.19	134	1.32					Mild pleasant odor, soaps, dyes, chemicals	
Aldehydes	8-Hexadecenal, 14-methyl-, (Z)-	34.67	252	2.57					Plant metabolite	Kosanić & Ranković, 2015
Carboxylic acids	Cyclohexanecarboxylic acid	11.57	128			0.5	0.6		Coatings, caulks, sealants, adhesives	
	Hexanoic acid, 2-methyl-	17.16	130	0.54					Burn skin, eyes, and mucous membranes in toxic	
	Oleic Acid	36.47	282				1.48	1.67	Herbicide, insecticide, and fungicide.	Molnár & Farkas, 2010
	Propanoic acid	39.39	74	24.83	5.59	6.41		39.62	Antimicrobial, Neurotransmitter, And Inflammatory Regulation	
	Propanoic acid, di (tert-butyl) silyl ester	28.19	216		38.99				Pharmaceuticals, agrochemicals, and materials science	
Diverse functional	Benzoic acid, 2,4-dihydroxy−3,6-dimethyl-, methyl ester	33.35	196			37.17			Anti tumor, Active ingredient in spanish fly, Aphrodisiac	
	Glycerol 1-palmitate	39.38	330				6.31		Food, pharmaceutical, and cosmetic metabolism)	
	Guanosine	29.4	283			4.56			Fundamental metabolite	Ranković, 2019
Esters	2(3 H)-Furanone, 3-butyldihydro-	13.08	142					8.82	Antimicrobial, anti-virulence, anti-biofilm, anti-inflammatory, and anticancer activities	Elix & Stocker-Wörgötter, 2008
	2,5-Octadecadiynoic acid, methyl ester	39.75	290	4.31					Human & Mouse metabolite, anticonvulsant, pheromone,	
	Cyclopentaneundecanoic acid, methyl ester	32.04	268	5.67	1.43	4.57			Pharmacological, anti-inflammatory, antimicrobial, anticancer	
	Cyclopropanecarboxylic acid, 2,2-dimethyl−3-(2-methyl−2-propenyl)-, ethyl ester, cis-	24.28	196				0.98		Anti-Inflammatory, Antimicrobial, Anticancer and Neuroprotective	
	Cyclopropanetetradecanoic acid, 2-octyl-, methyl ester	35.45	394		9.77	5.07			Pharmacological activities, anti-inflammatory and antimicrobial	
	Dodecanoic acid, 2-(acetyloxy)−1-[(acetyloxy) methyl]ethyl ester	40.08	358			37.11			Pharmaceutical Formulations, Antimicrobial Activity,	Kosanić et al., 2014
	Dodecanoic acid, 2,3-bis(acetyloxy)propyl ester	38.59	358		0.92	1.57	0.7	2.66	Antimicrobial, antiviral, and Antifungal (lipid metabolism)	
	Eudesma−5,11(13)-dien−8,12-olide	40.24	232	12.93					Anthelminthic drug, Antineoplastic antiparasitic, antiviral drug metabolite	
	Methyl tetradecanoate	32.6	242					10.68	Plant metabolite, food component, & algal metabolite.	

(*continued on next page*)

Table 1 (*continued*)

Chemical class	Secondary Metabolites	RT	MW	*P. aurata*	*S. fuliginosa*	*L. leucomelos*	*P. reticulatum*	*H. diadamata*	Biological Activity	References
	Phthalic acid, butyl undecyl ester	37.49	376	2.52					Industrial Applications (Plasticizers, environmental contamination, and potential health effects)	
	Phthalic acid, ethyl pentadecyl ester	27.92	404	1.66					Bioactive substance, secondary metabolites, active enzymatic	
	Tridecanoic acid, 12-methyl-, methyl ester	32.59	242	9					Plant, food component, Human blood serum & algal metabolite.	
	Undecanoic acid, 10-methyl-, methyl ester	25.15	214		1.71	0.83	1.51	6.58	Antimicrobial Activity, Metabolism, Topical Formulations, Chemical Ecology, And Environmental	
	4-Isopropenyl−4,7-dimethyl−1-oxaspiro[2.5] octane	18.46	180	0.88					Flavouring agent, plant, Mammalia metabolite.	
Hydrocarbons	1,5-Heptadiene, 2,5-dimethyl−3-methylene-	17.56	136	0.6					Turpentine odor, Plant metabolite.	
	1H−3a,7-Methanoazulene, octahydro−1,4,9,9-tetramethyl-	21.72	206	1.65					Characteristic odor	Gong et al. 2004
	8-Heptadecene	31.13	238				22.18		Carrion beetles. animal metabolite and a pheromone.	
	Aromandendrene	21.63	204				6.71		non-steroidal anti-inflammatory drug, a fragrance, a metabolite	Ranković & Kosanić, 2015
	Bicyclo[3.1.1]hept−2-ene, 3,6,6-trimethyl-	4.22	136					14.46	*Teucrium montanum*, *Xylopia aromatica*, and plant metabolite.	
	Dodecahydroacenaphthylene	13.42	164	0.74					Insects, plants, and microorganisms cues for behavior and ecology	
	Decane	5.57	142	1.81		1.04			Pheromones, signaling molecules, or cues for behavior and ecology	
	Guaia−1(10),11-diene	23.89	204	2.2					Non-steroidal Anti-inflammatory drug	
	Heptadecane	31.62	240				20.85		Pheromones, signaling molecules, or cues for behavior and ecology organisms.	
	Octadecane, 6-methyl-	5.63	268		0.89				pheromones, signaling molecules, or cues for behavior and ecology in various organisms	
	α-Phellandrene (terpenoids)	5.97	136				5.63		Anti-Inflammatory, Analgesic, Antioxidant, and Antimicrobial Activities	
	Hexadecane	31.62	226	7.28	16.04				Plant and volatile oil metabolite	Muggia et al., 2018
Ketones	2,5-Cyclohexadiene−1,4-dione	24.65	108	6.93					Human xenobiotic and a mouse metabolite	
	Spiro[2.3]hexan−4-one, 5,5-diethyl−6-methyl-	25.4	166			1.41			Plant growth regulator, fungal, Mammalian, plant, flavouring agent, antineoplastic agent	
Low reliability	3-Bromo−7-methyl−1-adamantanecarboxylic acid	4.25	272	0.63					Biodegradability and toxicity, Pharmacological activity, drug delivery applications	Cordeiro et al., 2020
	Estra−1,3,5(10)-trien−17β-ol	36.48	256		1.09	0.95	2.74	6.07	Female Reproductive System, and Sexual Health	Molnár & Farkas, 2010
Nitrogen-containing	2(1 H)-Pyridinone, 3-methyl-	15.35	109		7.65				Antimicrobial, Anti-Inflammatory, And Neuroprotective Effects	
Phenols	3-Methoxy−5-propylphenol	9.48	166	0.43					Slight aromatic odor, Food antioxidant.	
	Phenol	6.19	94		3.93				Slimicides, disinfectant and antiseptic	
Sugar compounds	D-Fructose, pentaacetate	32.58	496		2.27	1.57	2.42		chromatography or mass spectrometry, Metabolism and functions of fructose	
	2-Propyloctahydro−1-benzothiophene	24.29	184		0.85				Antimicrobial effects, and anti-inflammatory	Kosanić et al., 2014

Structure of the compounds in secondary Metabolites from lichen

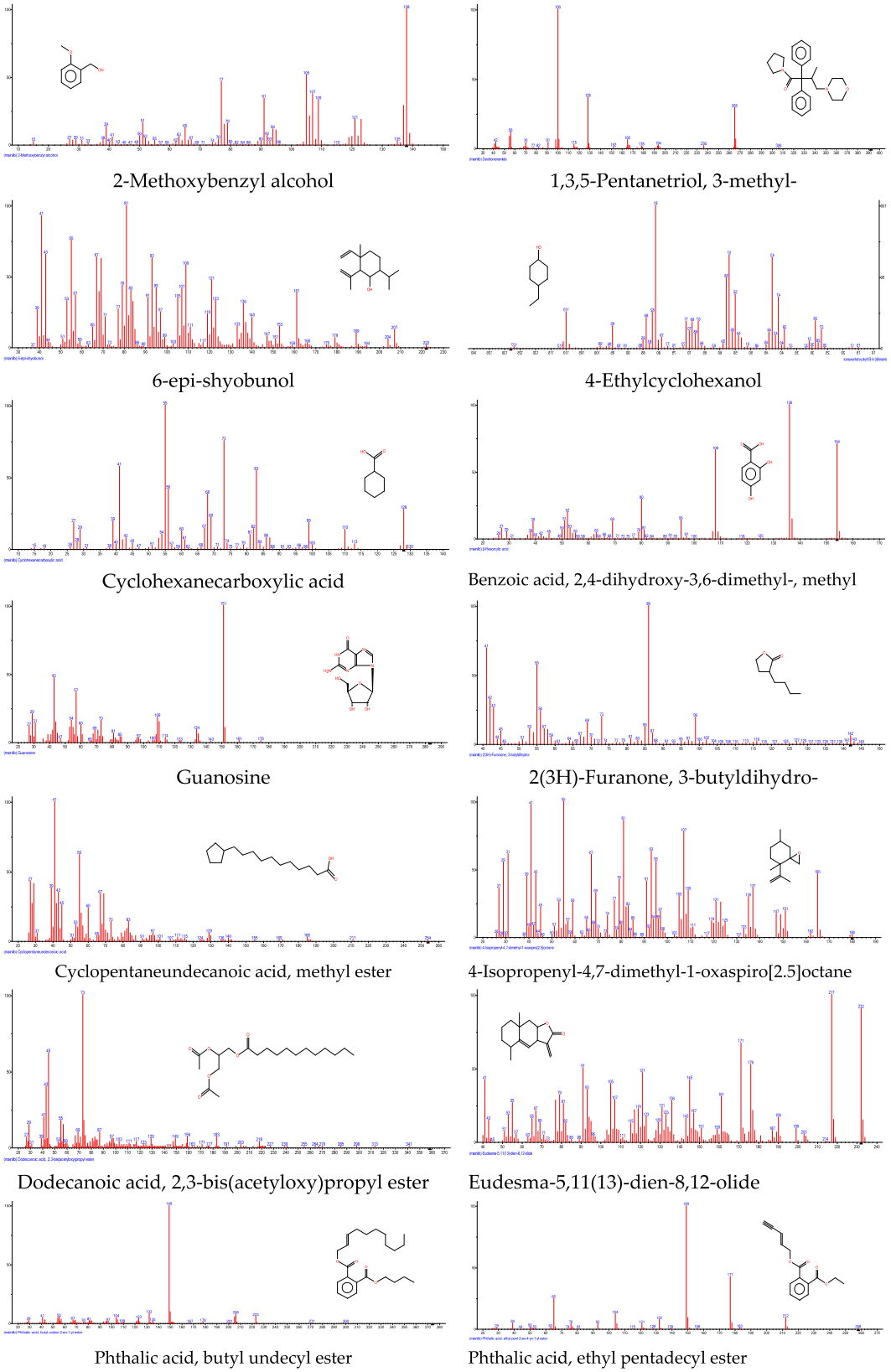
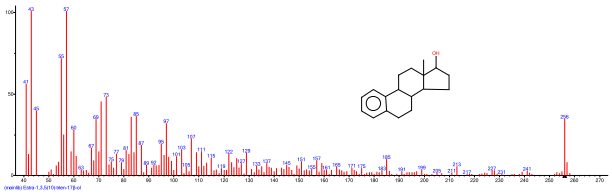
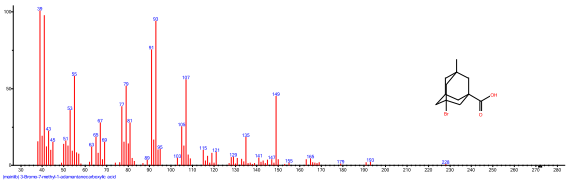


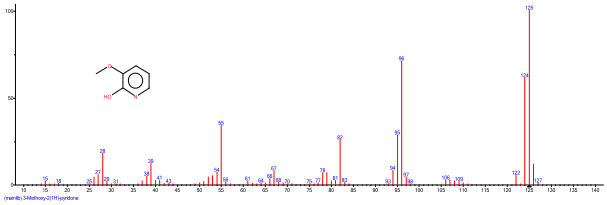
Fig. 3. List of secondary metabolites was structures present in the lichens.



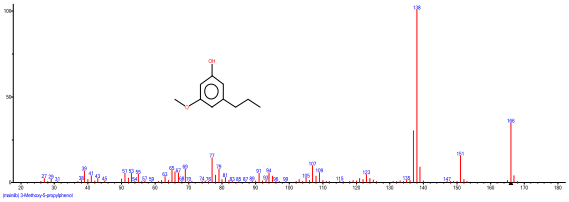
Estra-1,3,5(10)-trien-17β-ol



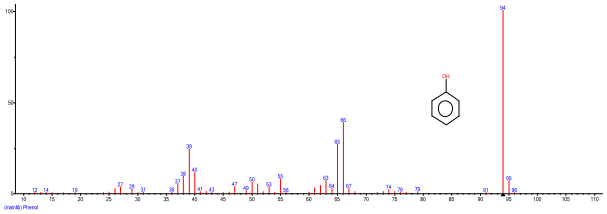
3-Bromo-7-methyl-1-adamantanecarboxylic acid



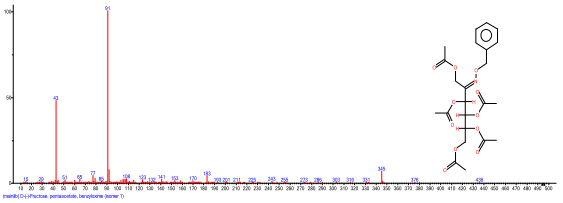
2(1H)-Pyridinone, 3-methyl-



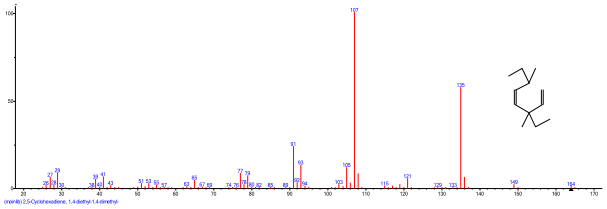
3-Methoxy-5-propylphenol



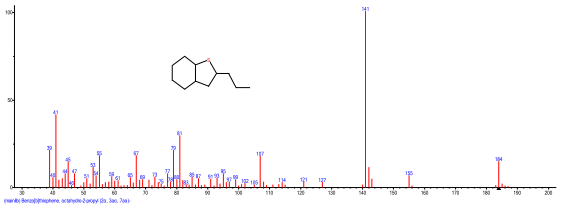
Phenol



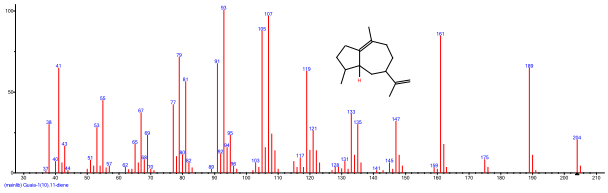
D-Fructose, pentaacetate



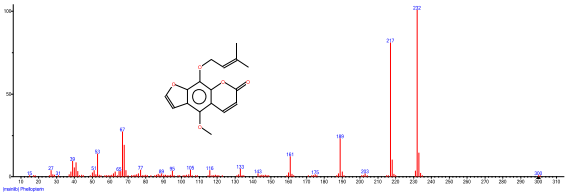
2,5-Cyclohexadiene-1,4-dione



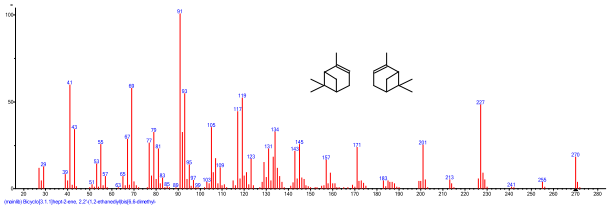
2-Propyloctahydro-1-benzothiophene



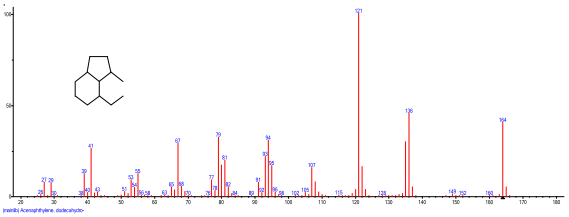
Guaia-1(10),11-diene



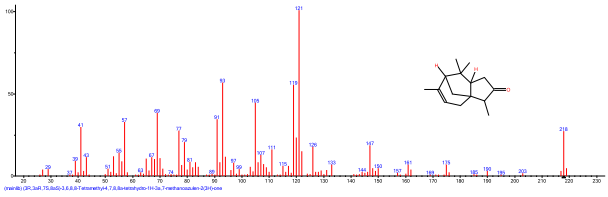
α-Phellandrene (terpenoids)



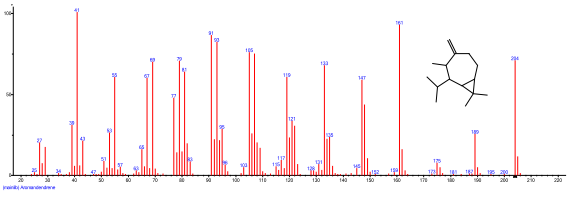
Bicyclo[3.1.1]hept-2-ene, 3,6,6-trimethyl-



Dodecahydroacenaphthylene



1H-3a,7-Methanoazulene, octahydro-1,4,9,9-tetramethyl-



Aromandendrene

Fig. 3. (*continued*).

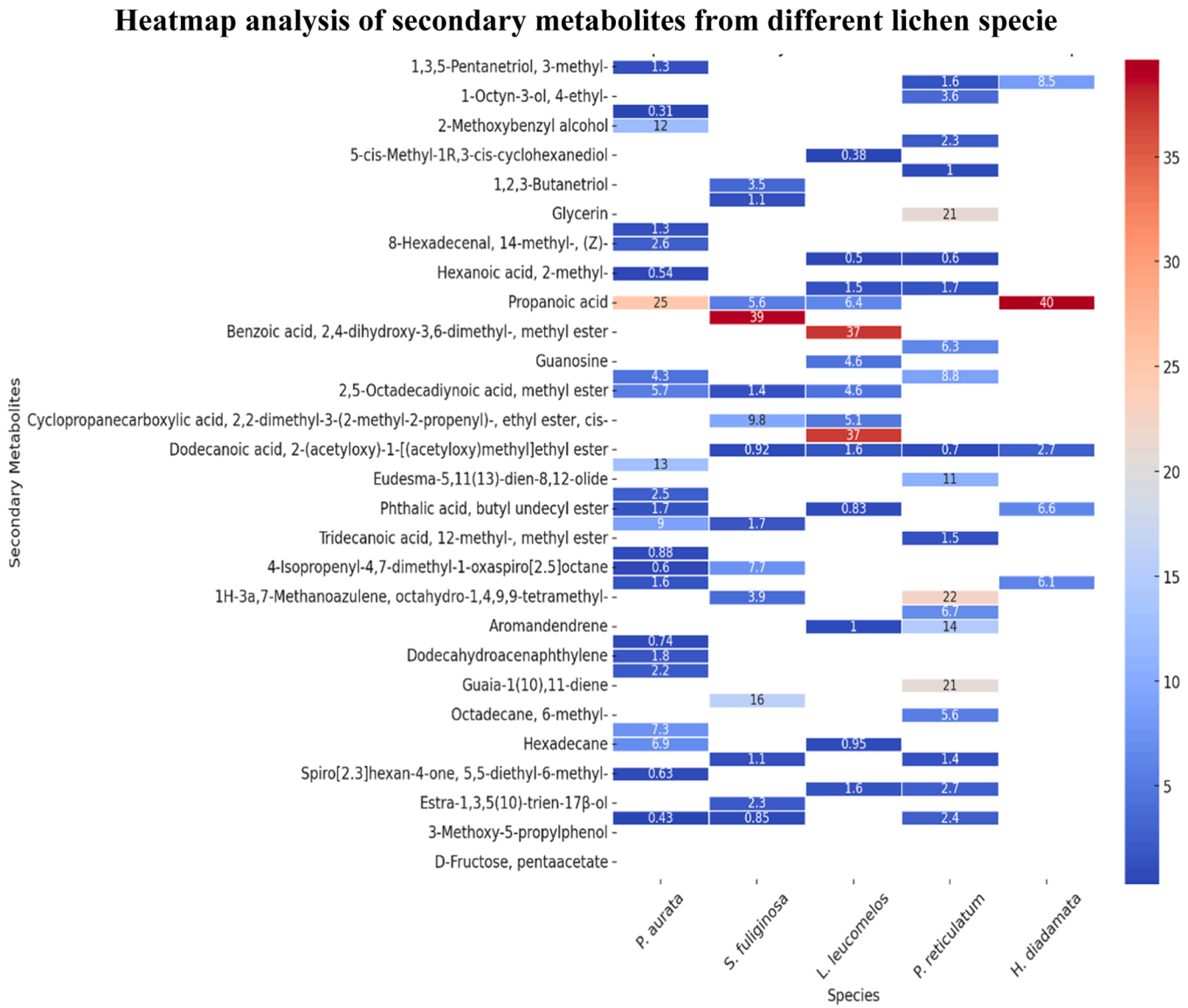


Fig. 4. Heatmap analysis of secondary metabolites from different lichen species, including *P. aurata, S. fuliginosa, L. leucomelos, P. reticulatum,* and *H. leucomelos*, extracted using ethyl acetate and acetone. The heatmap highlights the distinct metabolite profiles across the species, providing insights into the variations in compound abundance based on the extraction solvent used.

dihydroxy-6-methylphthalide, chrysophanol, conorlobaridone, and pulvinic acid (Ranković et al., 2012; Singh et al., 2017). Eventually, species like *P. tinctorum* were explored for methanol extracts and secondary metabolites, which play a significant role in antioxidant activities, total polyphenols, polysaccharides, and food flavoring (Kanwar and De, 2010, p. 100; Marfatia et al., 2019; Pandhi et al., 2014; Rambhia et al., 2018). Lecanoric acid (LeA), specifically 4-(2,4-dihydroxy-6-methylbenzoyl) oxy-2-hydroxy-6-methylbenzoic acid, is a notable compound (Luo et al., 2009).

In this chapter, the study of *H. leucomelos* species, belonging to the Physciaceae family, has detected triterpenoids such as 6α-hydroxyhop-21βH-22, atranorin, zeorin, glyceryl trilinolate, 3,6-dimethyl-2-hydroxy-4-methoxybenzoic acid, cabraleadiol monoacetate, 4-O-methylcryptochlorophaeic acid, lichexanthone, and 3,6-dimethyl-2-hydroxy-4-methoxybenzoic acid (Kathirgamanathar et al., 2006; Spribille et al., 2016). New bioactive molecules synthesized from *P. reticulatum*, *M. fuliginosa*, and *P. aurata* lichen extracts, including methanol and acetone compounds, have shown various biological roles, such as antioxidant, antiviral, hepatoprotective, antibacterial, and anticancer activity. Chemicals like LeA and 2′-O-methyl anziaic acid, methyl 8-chloro-10-formyl-3,9-dihydroxy-1,4,7-trimethyl-6-oxobenzo [b], benzodioxepine-2-carboxylate were used in cytotoxicity assays, playing a major role in activity against normal fibroblast cells, MCF-7 cancer cells, HeLa cells, and colorectal cancer (Ghate et al., 2013; Ristić et al., 2016; Taş et al., 2019).

Secondary metabolites such as salazinic acid, evernic acid, protolichesterinic acid, divaricatinic acid, usnic acid, and isodivaricatic acid have demonstrated antibacterial effects (Furmanek et al., 2019; Podterob, 2008). Previously, these bioactive substances were tested against *H. pylori*, *P. vulgaris*, *E. cloacae*, *P.aeruginosa*, *K. pneumoniae*, *S. aureus*, and *E. coli* using disc diffusion and micro-well dilution assays (Furmanek et al., 2019; Gulluce et al., 2006; Shrestha and St. Clair, 2013; Zambare and Christopher, 2012). On the other hand, lichen extracts have shown strong antibacterial and antifungal effects, particularly against gram-positive bacteria (Grujičić et al., 2014; Gulluce et al., 2006; Kosanić et al., 2013; Mitrović et al., 2011; Zambare and Christopher, 2012). Additionally, lichen-derived toxins play a significant role in anticancer activity against various cell lines, including liver, leukemia, cervical, rectal, glioblastoma, lung, prostate, breast, melanoma, colon, pancreatic, ovarian, lymphatic cancers, and astrocytoma. Lichens have

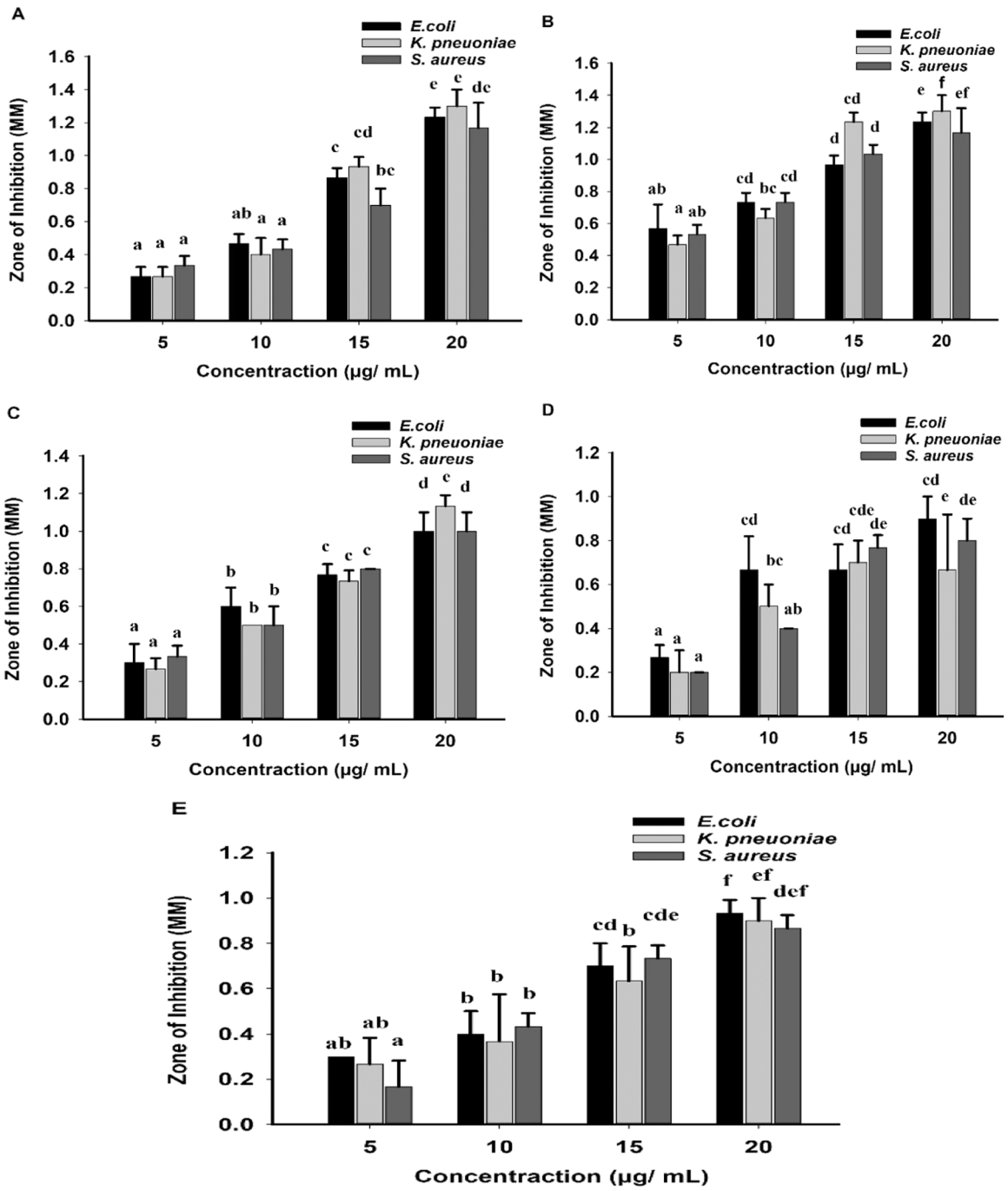


Fig. 5. The picture shows the antibacterial activity at different concentrations (5, 10, 15, 20 µg/ mL and control) of crude extracts amended with LB medium in LB agar diffusion assays (200 and 100 µg/mL of crude extracts) showed zones of inhibition (ZOIs) exhibited by the lichen extracts of for different bacterial Strains such as (A) *E. coli,* (B) *K. pneumoniae,* (C) *S. aureus* dorsal view and bottom view of (D) *E. coli,* (E) *K. pneumoniae,* (F) *S. aureus.* Different alphabets depicted in superscript indicate mean treatments that are significantly different according to Tukey's HSD posthoc test at $p < 0.05$, each value is an average of 3 replicate samples ± standard error and percentage of growth inhibition.

been studied for their effects both in vivo and in vitro (Agrawal et al., 2020; González-Burgos et al., 2019).

The anticancer properties of lichens include cytotoxic action, cell cycle regulation, anti-proliferation, anti-invasiveness, anti-migration, anti-angiogenesis, telomerase suppression, inhibition of endothelial tube formation, and other mechanisms (Reddy et al., 2019; Solárová et al., 2020). Lichen samples, including *C. islandica*, *E.prunastri*, *U.longissima*, *P.reticulata*, and *Ramalina* species, were eluted using established methanol and acetone extraction techniques. Four key compounds, including norstictic acid and doxorubicin, effectively inhibited cancer cells in the cervix, lung, melanoma, colon, and breast (HeLa, MCF-7, and DU-145) in *in vitro* studies (Bessadóttir et al., 2014; Brandão et al., 2013; Ebrahim et al., 2016; Ghate et al., 2013; Grujičić et al., 2014; Kosanić et al., 2013). The ethyl acetate extract of *U. longissima* may suppress the formation and progression of N-methyl-N′-nitro-N-nitrosoguanidine (MNNG)-induced esophagogastric cancer (Mammadov et al., 2019). Karagoz et al. (2014) (Karagoz et al., 2014) demonstrated that diffraction acid from *U. longissima* might reduce cancer cell proliferation as well as inflammation and necrosis in organs (kidney, liver, and small intestine) in an Ehrlich's ascites carcinoma model. In our ongoing search

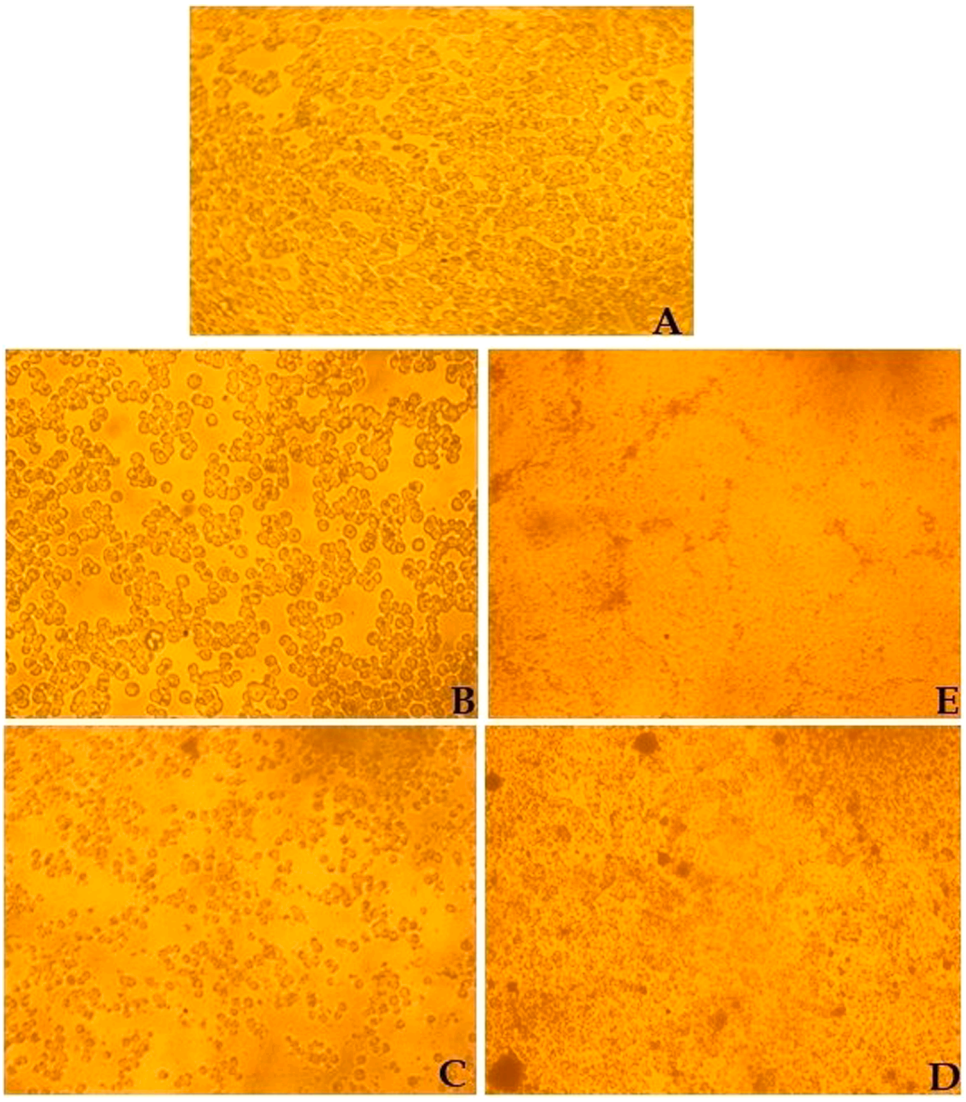


Fig. 6. Anti-tumor effect on Colon (HT-29) Cell line represents, treated with Lichen extract (10, 5, 2.5, 1.25, 0.625, 0.312 & 0.156 mg/mL) concentrations (A) *S. fuliginosa*, (B) *L. leucomelos*, (C) *H. leucomelos*, (D) *P. aurata*, (E) *P. reticulatum*.

for physiologically active compounds in tropical lichens, we examined *P. consocians* Vainio and *H. leucomelos* (L. Poelt), both foliose lichens from the Physciaceae family.

In this study, we present the isolation and structural elucidation of several metabolites, including the novel triterpenoid 6α-hydroxyhop-21βH-22(29)-en. Structurally, these compounds contain functional groups such as alcohols, including 1,3,5-pentanetriol, 3-methyl (Cordeiro et al., 2020), 1-octanol, 2,7-dimethyl (Huneck & Yoshimura, 1996), and glycerin. Falcarinol and phenol, polyacetylene compounds found in certain lichens, have been reported to induce apoptosis and inhibit the proliferation of cancer cells, particularly in colorectal cancer (Ranković et al., 2011 & 2012; Shukla et al. 2010). Additionally, compounds such as dodecanoic acid, 2-(acetyloxy)-1-[(acetyloxy)methyl] ethyl ester (Kosanić et al., 2014), oleic acid (Molnár & Farkas, 2010), Estra-1,3,5(10)-trien-17β-ol (Molnár & Farkas, 2010), steroidal estrogen, aromandendrene (sesquiterpenoid) (Ranković & Kosanić, 2015), 6-epi-shyobunol (terpenoid), α-phellandrene (monoterpene), and 2-propyloctahydro-1-benzothiophene (Ranković et al., 2012) exhibit cytotoxicity against cancer cells by inducing oxidative stress and apoptosis. Furthermore, 8-hexadecenal, 14-methyl-, (*Z*)- (Kosanić & Ranković, 2015) enhances reactivity and solubility, impacting biological interactions that play a role in cellular signaling and cytotoxic activity. Compounds such as propanoic acid (Kosanić et al., 2014), cyclohexanecarboxylic acid, and 4-ethylcyclohexanol contain hydroxylated cyclic derivatives, which are crucial in metabolic pathways and may contribute to anti-proliferative effects. These properties suggest potential pharmacological relevance in drug design.

The symbiotic relationship between algae and fungi has given rise to a new life form known as lichen. Our research may lead to the development of genetic transformation systems for certain medically important lichen species. Our study investigated the lichen species *P. aurata, S. fuliginosa, L. leucomelos, P. reticulatum,* and *H. leucomelos*, revealing significant antibacterial and anti-tumor properties. The secondary metabolites of these lichens, analyzed via GC-MS, demonstrated a rich diversity of chemical compounds, including alcohols, hydrocarbons, esters, and carboxylic acids. *P. aurata* and *P. reticulatum* showed the highest antimicrobial activity, effectively inhibiting bacterial strains *E. coli, K. pneumoniae*, and *S. aureus*, particularly at higher concentrations. Additionally, the lichen extracts exhibited notable anti-tumor effects against HT-29 colon cancer cells, with a clear dose-dependent response. The highest concentration tested (10 mg/mL) led to a significant reduction in cell viability, with an estimated LC50 of approximately 0.939 mg/mL. These results underscore the potential of lichen extracts as sources of bioactive compounds with therapeutic

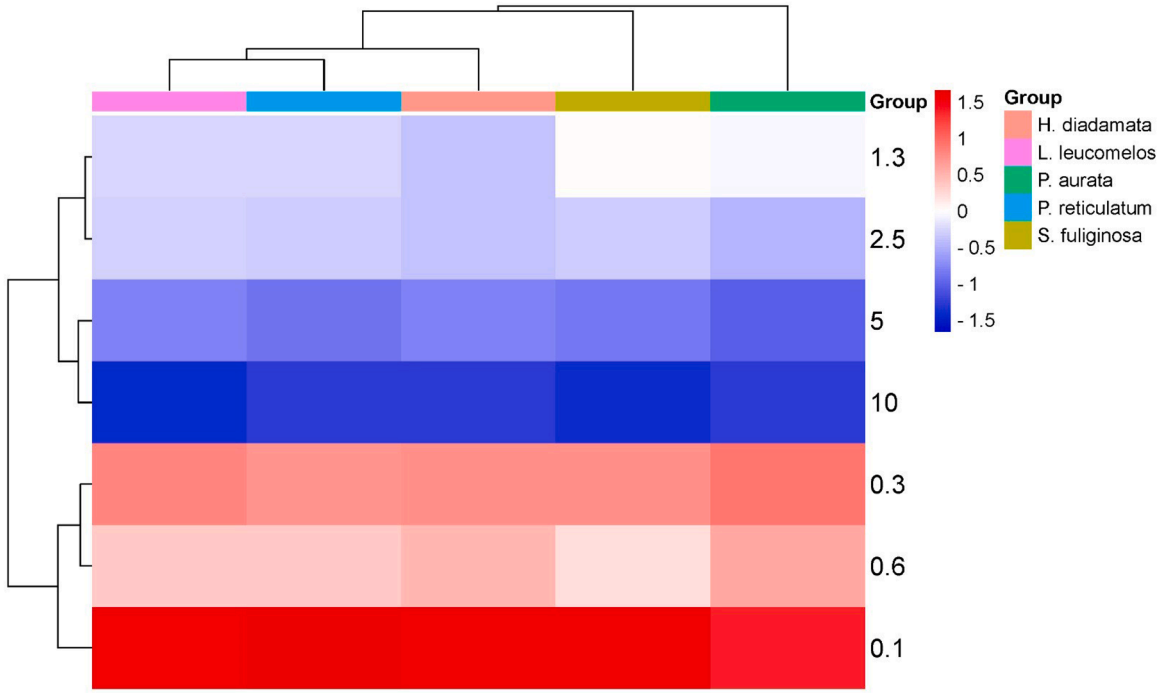


Fig. 7. Anti-tumor effect on-colon cancer (HT-29) Cell Line.

applications in both antimicrobial and anti-cancer treatments. Our study highlights the potential of lichen secondary metabolites for various biological applications. The compounds analyzed showed significant antimicrobial, antioxidant, and anti-inflammatory properties, supporting their possible use in medicine and agriculture. Further research is needed to explore their mechanisms of action and practical applications.

CRediT authorship contribution statement

Krishnaswamy Ezhilan Vivekanandan: Writing – review & editing, Validation. **Nagaraj Rajaprabu:** Software, Resources, Investigation. **Kanagaraj Kalaiarasi:** Formal analysis, Data curation, Conceptualization. **Viswakethu Velavan:** Writing – original draft, Resources, Project administration, Investigation. **Velusamy Sharmila:** Writing – review & editing, Software. **Periyasamy Thirunavukkarasu:** Writing – review & editing, Software, Resources. **Vasan Suvetha:** Formal analysis, Data curation, Conceptualization. **Raj Joel:** Resources, Methodology, Conceptualization. **Asokan Aiswarya:** Writing – original draft, Software, Data curation, Conceptualization. **Bhagwandas Ranjini:** Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.microb.2025.100287.

Data availability

No data was used for the research described in the article.

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