doi.org/10.1002/cbdv.202301229 Research Article CHEMISTRY & BIODIVERSITY

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In Vitro and *in Silico* Studies of Lichen Compounds Atranorin and Salazinic Acid as Potential Antioxidant, Antibacterial and Anticancer Agents

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Lichens are symbiotic organisms made up of alga/cyanobacterium and fungus. We investigated antioxidant, antibacterial and anticancer properties of two lichen compounds, atranorin and salazinic acid, and five lichen species: *Heterodermia boryi, Heterodermia diademata, Heterodermia hypocaesia, Parmotrema reticulatum*, and *Stereocaulon foliolosum*. Free radical scavenging, Ferric reducing potential, Nitric oxide scavenging, and Trolox equivalent capacity were used to measure antioxidant activity. Strong radical scavenging action was demonstrated by atranorin and salazinic acid, with IC_{50} values of 39.31 μM and 12.14 μM, respectively. The Minimum Inhibitory Concentration (MIC) assay based on resazurin, was used to measure antibacterial activity. *Parmotrema reticulatum* demonstrated significant antibacterial activity against *Raoultella planticola* with MIC of 7.8 μg/mL. Cytotoxicity assay on breast cancer cell line was used to assess anticancer activity. To further understand the binding locations on the target proteins Er (Estrogen Receptor alpha), EGFR (Epidermal Growth Factor Receptor), mTOR (Mammalian Target of Rapamycin), and PgR (Progesterone Receptor), molecular docking experiments were conducted. Docking study showed that the binding energies of atranorin and salazinic acid with mTOR were −5.31 kcal/mol and −3.43 kcal/mol, respectively. The results suggest that atranorin has the potential to be a multitargeted molecule with natural antioxidant, antibacterial, and anticancer properties.

Introduction

A lichen is a composite ecosystem made up of filaments of fungal species and algae or cyanobacteria that coexist in a mutually beneficial relationship. They are slow-growing organisms with small biomass known to cover 8% of the land on Earth.[1] They are dominant life forms available globally. Similar to many other organisms, they also synthesize structurally unique secondary metabolites.[2] For a long, lichens have been used for various purposes by humans and animals. Regardless of the advances in medical sciences, tribal people continue to use these organisms for different purposes depending on their nutritive, medicinal, decorative, brewing, distilling, dying, cosmetic, and perfumery properties.[3,4,5]

The biological role of lichen secondary metabolites is receiving considerable research attention because they have several bioactivities, such as antibacterial, antifungal, antioxidant, antitumor, anti-proliferative, antiviral, anti-inflammatory, cardio-protective, hepato-protective, probiotic growth-promoting potential, α- and β-glucosidase and prolyl endopeptidase inhibitory activities.[6,7,8]

Free radical generation in the human system causes oxidative stress, harming various macro-molecules, cells, and tissues. Oxidative stress also causes gene mutations, thus leading to diseases, such as arteriosclerosis, diabetes, cancer, cardiovascular disease, Alzheimer's disease, and arthritis. Antioxidants scavenge free radicals, stabilize them, and maintain homeostasis and balance in the body. Antioxidants, thus facilitate the prevention and treatment of these life-threatening diseases.[9,8]

Worldwide, resistant bacterial infections are a significant public health issue; this necessitates searching for novel treatment agents. Lichens stand out in this situation as they create structurally different compounds with desirable biological properties, such as antibacterial activity.[10] The spread of multiple drug-resistant bacteria has increased and has evolved with antibiotic-resistant factors due to the rigorous and uncontrolled use of antibiotics.[11]

Breast cancer is the most frequent cancer among women worldwide. In 2020, female breast cancer surpassed lung cancer as the top cause of global cancer incidence with 2.3 million new cases, 11.7% of all cancer cases. It is the fifth biggest cause of cancer deaths worldwide, with 685,000 deaths. Breast cancer accounts for 1 in 4 cancer cases and 1 in 6 deaths caused by cancer in women.[12] Multiple factors induce carcinogenesis, such as improper transcriptional activity and other genetic factors, an imbalance between cell multiplication and death, defense mechanism suppression, and increased angiogenesis that leads to metastases.

An unstable redox equilibrium is a cell's innate defense system that generates and scavenges Reactive Oxygen

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Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbdv.202301229

Species (ROS), causes DNA damage, prevents cell death, and increases cell survival, leading to the development and propagation of cancer. Antioxidants inhibit cancer by targeting intracellular ROS generation. Many plants exhibit high ROS-scavenging action and cytotoxicity toward cancer cells, making them potential therapeutic and preventative agents. High-throughput screening programs for cytotoxic natural compounds may benefit from finding a correlation between plant extract free radical scavenging and cytotoxicity.[13]

Molecular docking is used extensively in drug discovery to study molecular behavior on target protein binding. ERα (Estrogen receptor), EGFR (Epidermal growth factor receptor), mTOR (mammalian target of rapamycin), and PgR (Progesterone receptor) are the target receptors to study the anti-cancer potential as they play a considerable role in the development and spread of breast cancer.[12,14] Taking into consideration the role of given specific target receptors in the initial development and progression of breast cancer, we proposed docking studies to investigate the anti-cancer effects of Lichen species from High altitude Himalayan Lichens and their major secondary metabolites Atranorin and Salazinic acid.

Results and Discussion

The secondary metabolites of lichens are a distinctive collection of naturally occurring organic chemicals that have biological significance because of the specifics of the lichen symbiosis.[15] The lichen compounds Atranorin and Salazinic acid are lichen-derived depside and depsidone, which are acetyl-poly malonyl-derived polyketides, and their basic structure has been preserved exceptionally well. Ester linkages connect the two or three aromatic rings that make up a depside, and in the depsidone's ether bond is present, each ring being synthesized by a polyketide synthase (PKS). Orcinol compound biosynthesis required a minimum of one PKS for a depside and a PKS plus a Cytochrome P450 for a depsidone.[16]

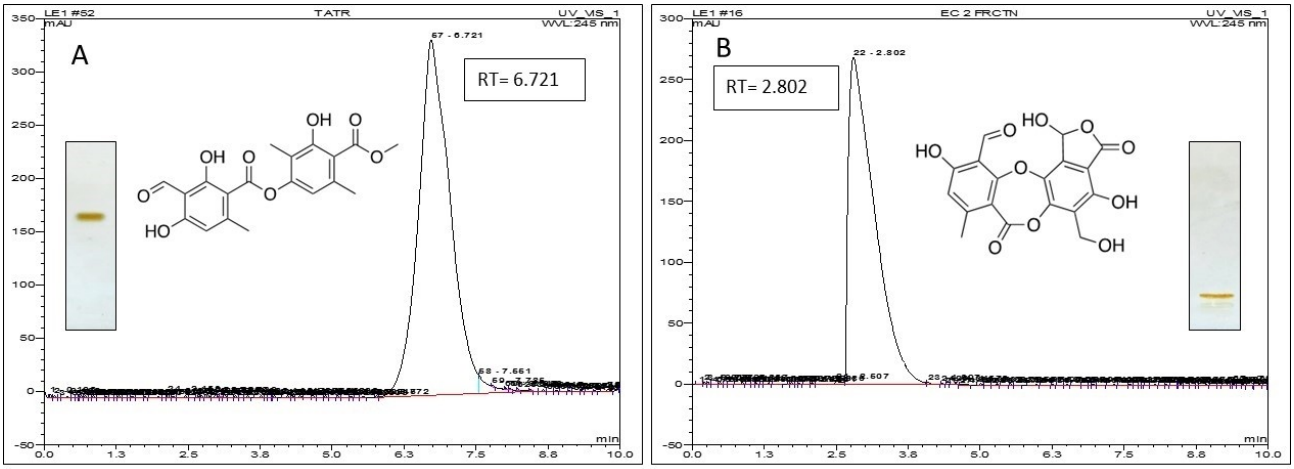
Physiochemical content

Total phenolic, polysaccharide and protein content in lichen extracts were estimated. The extracts of *Heterodermia boryi* (HB), *Heterodermia diademata* (HD), *Heterodermia hypocaesia* (HH), *Parmotrema reticulatum* (PR) and *Stereocaulon foliolosum* (SF) contain 10–110 μg/mg of phenolic compounds. The five lichen extracts had a range of 19–420 μg/mg of polysaccharides; and 10–230 μg/mg of protein content.

Isolation, purification, and characterization of lichen compounds

The Thin Layer Chromatography (TLC) and High-Performance Liquid Chromatography (HPLC) analysis of these five species showed the presence of atranorin and salazinic acid as major compounds. The secondary compounds atranorin (β-orcinol depside) and salazinic acid (β-orcinol depsidone) were isolated and purified. The retention time for atranorin obtained from the HPLC profile was 6.721 min, and for salazinic acid was 2.802 min. The HPLC chromatograms of the isolated compounds and their chemical structures are presented in Figure 1.

The High Resolution-Mass Spectroscopy (**HR-MS**) analysis revealed that the molecular weight and molecular formula of atranorin was 374 and $C_{19}H_{18}O_8$ (HR-MS calculated for $[M+Na]+C_{19}H_{18}O_8Na+$, m/z 397.0899) (*Supporting Information Figure S1*) and the salazinic acid was 388 and $C_{18}H_{12}O_{10}$ (HR-MS calculated for $[M+Na]+C_{18}H_{12}O_{10}Na+$, m/z 411.0328) (*Supporting Information Figure S2*), which matched with the available standard data.



No.	Ret. Time (min)	Height (mAU)	Area (mAU*min)	Rel. Area %
57	6.72	333.389	211.863	96.71

No.	Ret. Time (min)	Height (mAU)	Area (mAU*min)	Rel. Area %
22	2.8	268.457	140.516	98.7

Figure 1. U-HPLC graph of **a**. Atranorin, **b**. Salazinic Acid

Nuclear Magnetic Resonance (**NMR**) data obtained for atranorin (*Supporting Information Figure S3 & S4*) and salazinic acid (*Supporting Information Figure S5 & S6*) were validated using previous reports[17,18] and are given in the *Supporting Information*.

Thermo-Gravimetric Analysis (TGA)

Thermo-Gravimetric Analysis (TGA) was performed in order to evaluate the thermal stability of these isolated lichen compounds. Result were obtained by plotting graph between percent weight loss and temperature (Figure 2).

The compounds are stable and maintaining the mass at temperature 180 °C for atranorin and 155 °C for salazinic acid. The thermal decomposition is believed to occur in two steps and above 200 °C. In case of atranorin almost 72 percent of the decomposition occurs in the first step (181–300 °C), and 14 percent in the second step (300–800 °C).

Decomposition of salazinic acid occurs rather slowly, 53 percent in the first step (155–555 °C), and 9.5 percent in the second step (555–800 °C). Towards the end of this thermal treatment the mass/weight remains constant while temperature increases, thus the ash residue is formed and no further thermal decomposition is possible. Residual ash remains of atranorin and salazinic acid were 14% and 37% of the original weight respectively. An inverse relationship between mass and temperature is observed as time progresses. In a previous study of atranorin isolated from the lichen *Cladina kalbi*, thermogravimetric analysis revealed two phases of thermal decomposition (193.4–280 °C & 280–900 °C).[19] A similar thermal degradation scheme was reported by Zayed and Manojlović in which they characterized and tested lichen compounds, Evernic acid and Norstitic acid. Evernic acid was decomposed to nearly 72.68% up till 373.61 °C in two stages, whereas, norstictic acid decomposed in three stages.[20] In the first stage of thermal degradation, elemental carbon is formed, and in the second, it is liberated.[19,20]

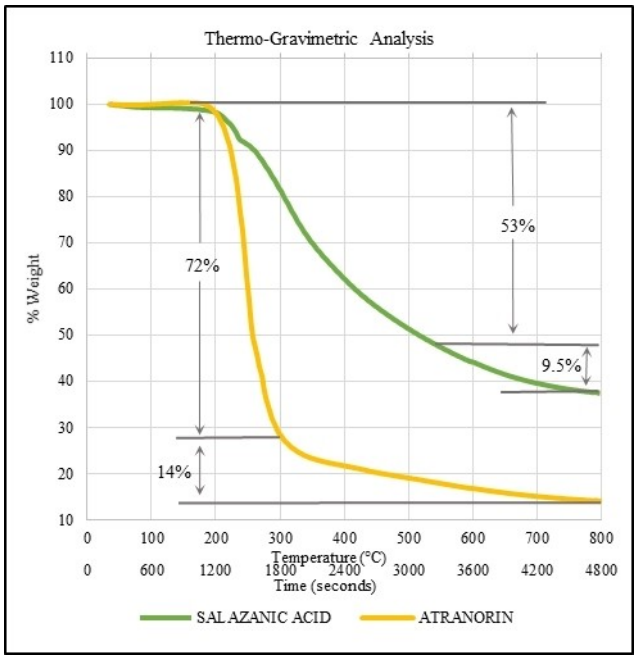


Figure 2. TGA graph of atranorin and salazinic acid. The graph is plotted of Percentage weight loss against Temperature (°C) and Time (Seconds).

Antioxidative potential

In the current study, lichen species and their compounds atranorin and salazinic acid showed moderate to strong antioxidative potential and have been listed in Table 1.

Lichen species *S. foliolosum, H. hypocaesia*, and *H. boryi* showed 1,1-diphenyl-2-picryl hydrazil (DPPH) radical scavenging activity at IC_{50} values of 44.46, 53.37, and 54.72 μg/mL, respectively. In prior antioxidant studies, the compounds atranorin and salazinic acid exhibited IC_{50} values of 131.48 and 91.57 μg/mL, respectively, when tested against DPPH radicals.[21,22] In contrast, the IC_{50} value of isolated atranorin and salazinic acid in the present study is 39.31 μM and 12.14 μM, indicating a stronger radical scavenging ability. Results demonstrated of lichen species extracts exhibited Ferric Reducing Antioxidant Potential (FRAP) with IC_{50} 24.61 to 275.9 μg/mL. The isolated compounds salazinic acid and atranorin (IC_{50} 11.91 μM and 33.79 μM respectively) exhibited strong antioxidant activity in terms of ferric reducing potential which is much higher than activity of standard Trolox (IC_{50} 71.39 μg/mL).

Furthermore, the Trolox Equivalent Antioxidant Capacity (TEAC) of crude extract of *P. reticulatum* (IC_{50} 23.36 μg/mL) exhibited stronger TEAC than Trolox (IC_{50} 29.78 μg/mL). Atranorin and salazinic acid exhibited IC_{50} values of 40.36 μM and 59.29 μM, respectively. *H. hypocaesia* and *P. reticulatum* extracts were the most potent, as they eliminated the nitrite radical at a lower concentration than the other extracts when tested using Scavenging of Nitric Oxide (SNO) assay. Atranorin and salazinic acid showed IC_{50} values of 44.2 μM and 35.19 μM, respectively (Table 1). The results shows that *H. boryi*, *H. hypocaesia*, *P. reticulatum* and *S. foliolosum* might be used as potential radical scavengers. Atranorin isolated from *Cladonia* spp. exhibited strong antiradical activities, against DPPH, TEAC, nitric oxide (NO) radicals and reduced ferric ions.[23] The results indicated synergistic properties of polyphenols and polysaccharides together towards antioxidant activity which also have been reported in our previous studies.[24]

Antibacterial activity

The antibacterial activity of the lichen extract and compounds tested against five bacterial strains, namely *Bacillus subtilis* (MTCC 121), *Escherichia coli* (MTCC 739), *Pseudomonas aeruginosa* (MTCC 2453), *Raoultella planticola* (MTCC 530), and *Staphylococcus aureus* (MTCC 2940).

In this study, noteworthy activity was observed against *R. planticola*, a gram-negative bacterium that was formerly classified under *Klebsiella* genera. It is reported as an emerging

Table 1. Antioxidant and Antibacterial potential of lichen extracts and compounds.

Lichen/Compound	Antioxidant Potential* IC_{50}				Antibacterial Potential** (MIC)				
	DPPH	FRAP	TEAC	SNO	*B. subitilis*	*E. coli*	*P. aeruginosa*	*R. planticola*	*S. aureus*
Heterodermia boryi (HB)	54.72 ± 5.52	51.567 ± 0.060	42.451 ± 1.225	146.08 ± 2.93	31.25	31.25	31.25	62.5	62.5
Heterodermia diademata (HD)	112.05 ± 3.76	275.90 ± 0.794	62.01 ± 1.636	107.63 ± 7.43	31.25	31.25	31.25	62.5	62.5
Heterodermia hypocaesia (HH)	53.37 ± 4.50	24.61 ± 2.815	34.81 ± 0.442	19.01 ± 4.59	62.5	31.25	31.25	62.5	125
Parmotrema reticulatum (PR)	65.40 ± 5.91	28.362 ± 3.478	23.36 ± 0.927	19.31 ± 0.185	31.25	31.25	31.25	7.81	62.5
Stereocaulon foliolosum (SF)	44.46 ± 2.86	180.10 ± 0.819	124.44 ± 6.569	24.45 ± 0.427	62.5	31.25	31.25	62.5	62.5
Atranorin (in μM)	39.31 ± 5.35	33.791 ± 2.541	40.36 ± 1.508	44.2 ± 1.113	125	62.5	62.5	62.5	125
Salazinic Acid (in μM)	12.14 ± 1.89	11.916 ± 3.88	59.29 ± 2.562	35.19 ± 0.716	125	62.5	125	62.5	125
Trolox (Vitamin-E)	2.794 ± 0.301	71.39 ± 1.566	29.783 ± 1.896	52.34 ± 3.379	–	–	–	–	–
Streptomycin (in μM) (Antibiotic)	–	–	–	–	7.81	3.91	7.81	1.95	62.5
Erythromycin (in μM) (Antibiotic)	–	–	–	–	31.25	31.25	3.91	62.5	62.5

*Data presented are the mean of three consecutive readings of samples in the assays performed. The mean value (± Standard deviation) of each concentration of different lichen metabolite was used for IC_{50} value calculation. IC_{50} value of lichen extracts and standard (μg/mL) and lichen metabolites (μM) showing 50% scavenging in antioxidant activity.
** Data presented are the mean of three consecutive readings of samples in the assays performed. MIC value of lichen extracts and standard (μg/mL) and lichen metabolites (μM) showing antibacterial activity.

pathogen worldwide and has caused severe human infections in recent years. In Africa, a case of nosocomial infection at a surgical site caused by *R. planticola* with extended antimicrobial resistance was reported recently.[25] We believe, our study is the first to report the antibacterial activity of lichen compounds against *R. planticola*. All lichen extracts showed an equal MIC value of 31.25 μg/mL against *E. coli* and *P. aeruginosa*. The activity shown by the isolated compounds is lower than streptomycin but is higher than antibacterial activity reported by others. Micheletti with others were isolated atranorin and salazinic acid from different lichen species and were effective against *S. aureus* and *E. coli*; the MIC value for both lichen compounds ranged from 125–250 μg/mL.[10] The MIC values of the extracts and compounds are given in Table 1.

In a previous study, lichen compounds were examined for their potential antimicrobial effects against five bacterial strains, including *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922), and *Staphylococcus aureus* (ATCC 25923). The sensitivity of these bacterial strains towards atranorin was determined, with MIC of 0.0312, 1, and 0.25 mg/mL, respectively.[21] Additionally, the sensitivity of the same bacterial strains towards salazinic acid was assessed, resulting in MIC values of 0.0312, 1, and 0.125 mg/mL, respectively.[22] The present study aligns with prior research findings and demonstrates enhanced antibacterial efficacy. The possible antimicrobial mechanisms of lichen compounds include inhibition of protein and nucleic acid synthesis, cell wall inhibition, and metabolism inhibition; and could be a reason for different bacterial sensitivity.[1] In the view of search for new antibiotics lichen compounds prove to be a strong candidate.

Cytotoxic activity and Molecular Docking

The cytotoxic activity of the extract of five lichen species was tested against MCF-7. Lichen extracts in varying concentrations (in μg/mL) were plotted against cell viability (in percentage) of MCF-7 cells, representing decreasing cell viability in a concentration-dependent manner. *H. diademata* and *S. foliolosum* showed the highest inhibition against MCF-7 cell lines with IC_{50} values ranging from 0.1 to 0.2 mg/mL (Figure 3).

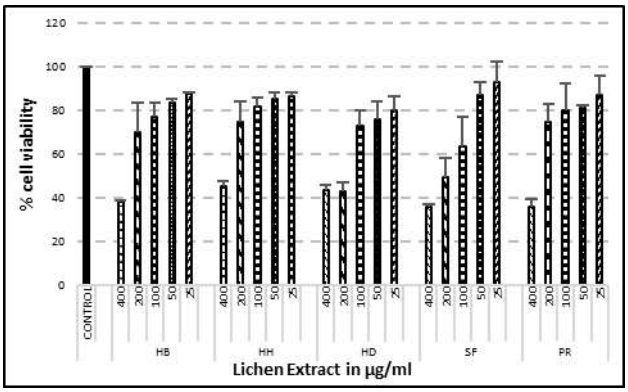


Figure 3. Cell viability of Human breast cancer cell line (MCF-7) against the extract of five lichen species. Data presented are the mean of three consecutive readings of samples in the assays performed. The mean value of each concentration of different lichen species was used for IC_{50} value calculation by linear regression between metabolite concentrations versus biological activities.

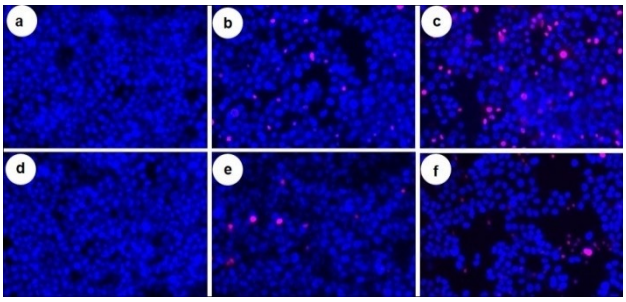


Figure 4. Morphological observation of MCF-7 cells, (a & d) Control, (b) 50 μM Atranorin, (c) 100 μM Atranorin, (e) 100 μM Salazinic acid, (f) 200 μM Salazinic acid. Images are representative of three independent experiments

The anticancer potential of the isolated compounds was determined, significant cytotoxicity showed by atranorin with IC_{50} value 82.79 μM. Morphological observation of MCF-7 was done using NucBlue® Live Reagent, which stains the nuclei of all cells (Hoechst 33342). Propidium iodide stains the nuclei of dead cells with compromised plasma membrane (100 μM Atranorin, 200 μM Salazinic acid) as shown in Figure 4, red cells represented dead cells.

Several cellular downstream signaling pathways, triggered by the activation of ER, PgR, EGFR, and HER-2 receptors, are responsible for the onset and progression of breast cancer.[26]

PgR is an essential steroid hormone receptor in breast carcinogenesis. PgR-positive breast cancer has a greater endocrine response in the clinic. Exposure to progesterone raises breast cancer risk and causes hormone receptor-negative tumors. Increased postmenopausal progesterone levels increase breast cancer risk by 16 percent. In luminal cells, progesterone binds to PgR, causing an increase in RANKL (Receptor activator of nuclear factor kappa-B ligand) protein primarily through mRNA stabilization. Then, RANKL upregulates its receptor RANK to induce proliferation or differentiation.[27] During our research, we found that Atranorin had the most favorable docking confirmation with a binding energy of −5.32 kcal/mol towards PgR. This suggests that Atranorin could be a potent inhibitor of PgR, which could lead to a reduction in cancer cell proliferation or differentiation.

Mammalian target of rapamycin (mTOR) is a key regulator of cell growth and metabolism. The mTOR complexes are functionally distinct. mTORC1 promotes mRNA translation and protein synthesis by phosphorylation of ribosomal protein S6 kinase (S6 K1) and eIF4E binding protein-1 (4E-BP1) and inhibits autophagy. Atranorin and salazinic acid both the lichen compounds exhibited best docking confirmation with the binding energy of −5.31 kcal/mol and −3.43 kcal/mol respectively towards mTOR, and their interactions with amino acids on active sites as shown in Table 2. mTOR inhibitors could improve patient outcomes with hormone receptor-positive or human epidermal growth factor receptor-2-positive breast cancer.[28]

The epidermal growth factor receptor (EGFR) levels are elevated in half of triple-negative breast cancer (TNBC) and inflammatory breast cancer (IBC) patients. Estrogen promotes the PI3 K/AKT/mTOR signalling pathway by binding to extranuclear ERα in ER+breast cancer, causing resistance to therapies and EMT (Epithelial-to-Mesenchymal Transition), tumor invasion and metastasis. Targeting ERα and EGFR are important antitumor targets as it alters PI3 K/AKT protein kinase Cα pathway components causing cell death and it activates major signalling pathways mediated by PI3 kinase, Ras-Raf-MAPK, JNK, and PLC.[29,30] Atranorin showed favorable docking confirmation with a binding energy of −3.99 kcal/mol towards ERα.

In previous studies, atranorin induces apoptosis associated with the activation of caspase-3, PARP cleavage and depletion of Bcl-xL protein in 4T1 cells.[31] It was suggested that it might suppress RhoGTPase activity and influence AP-1, Wnt, and STAT signalling to inhibit lung cancer cell motility and carcinogenesis.[32] A recent study has shown that salazinic acid modulates the Nrf2, NF-κB, and STAT3 pathways in colorectal cancer.[33]

This is the first study in which Molecular docking experiments were carried out to determine the binding orientation of atranorin and salazinic acid to ERα, EGFR, mTOR, and PgR to estimate the stable binding conformation and binding affinity of the lichen compounds with proteins. Among the two lichen metabolites screened, atranorin exhibited docking energy values above −5 Kcal/mol for receptor ERα, mTOR, and PgR. Atranorin exhibited the best docking confirmation with the binding energy of −5.32 kcal/mol towards PgR, followed by −5.31 kcal/mol towards mTOR. Salazinic acid showed binding energy of −3.43 and −0.40 kcal/mol towards mTOR and PgR, respectively, and their Interactions with amino acids on active sites as shown in Table 2.

Regarding the cytotoxic activity of atranorin and salazinic acid against MCF-7, atranorin exhibited higher cytotoxic potential than salazinic acid. Based on the binding affinities shown by the docking studies, atranorin is a potent inhibitor of mTOR and PgR, making it an effective anti-breast cancer drug. Furthermore, the compounds can be studied in-vitro and in-

doi.org/10.1002/cbdv.202301229

Table 2. 3D structure of the docked Atranorin and Salazinic acid against EGα, EGFR, mTOR and PgR; and their specific interaction with EGα, EGFR, mTOR and PgR.

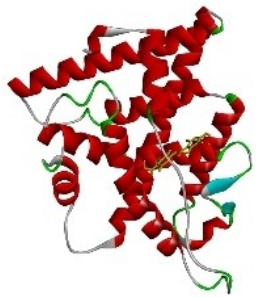
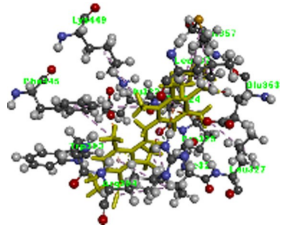
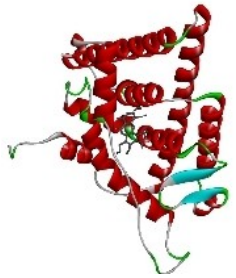
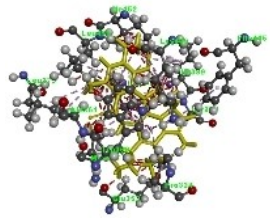
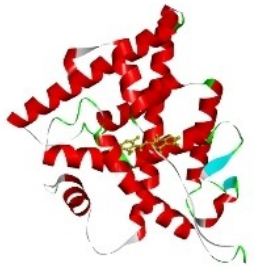
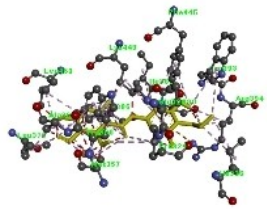
Ligand (Lichen compound) with Binding Energy (kcal/mol) and Estimated Inhibition Constant (Ki) [Temp. = 298.15 K]	Nature of interactions	Interactions with Amino acids on active sites	3D structure of the docked compound with receptors	Specific interaction of lichen compound with receptors
		Target receptor: ERα		
Atranorin −3.99 kcal/mol 1.20 mM	Electrostatic	:ATRANORIN0 – A:ARG394:NH1; :ATRANORIN0 – A:GLU323:OE1; :ATRANORIN0 – A:GLU353:OE1		
	Hydrogen Bond	:ATRANORIN0:H3 – A:PRO324:O		
	Hydrophobic	:ATRANORIN0:C6 – A:PRO324; :ATRANORIN0:C6 – A:ILE326; :ATRANORIN0:C18 – A:LYS449; :ATRANORIN0:C19 – A:PRO324		
Salazinic Acid +137.18 kcal/mol Nil	Electrostatic	:SALAZINIC ACID0 – A:LYS449:NZ		
	Hydrophobic	:SALAZINIC ACID0 – A:TRP360 :SALAZINIC ACID0:C27– A:ALA361; :SALAZINIC ACID0 – A:LYS449; :SALAZINIC ACID0:C8 – A:ILE386; :SALAZINIC ACID0:C27 – A:LEU379		
		Target receptor: EGFR		
Atranorin +65 kcal/mol Nil	Electrostatic	:ATRANORIN0 – A:ARG394:NH1; :ATRANORIN0 – A:GLU323:OE1		
	Hydrogen Bond	:ATRANORIN0:O7 – A:GLY390:O		
	Hydrophobic	:ATRANORIN0:H42 – A:ALA361; :ATRANORIN0:O6 – A:PRO324; :ATRANORIN0:O6 – A:MET357		
	Hydrogen Bond; Electrostatic	:ATRANORIN0 – A:LYS449:NZ		

Table 2. continued

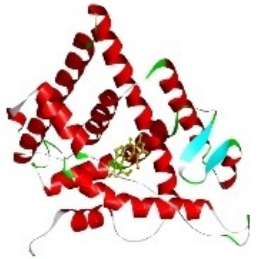
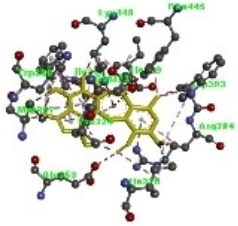
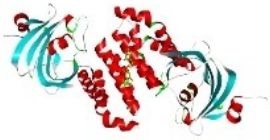
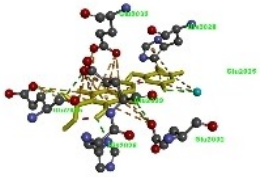
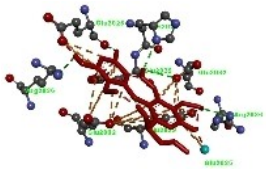
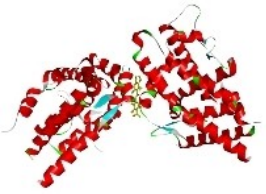
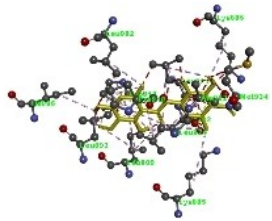
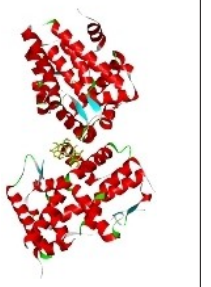
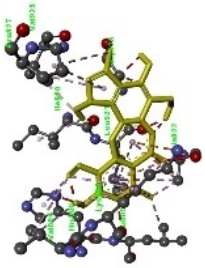
Ligand (Lichen compound) with Binding Energy (kcal/mol) and Estimated Inhibition Constant (Ki) [Temp. = 298.15 K]	Nature of interactions	Interactions with Amino acids on active sites	3D structure of the docked compound with receptors	Specific interaction of lichen compound with receptors
Salazinic Acid +135 kcal/mol Nil	Electrostatic	:SALAZINIC ACID0 – A:ARG394:NH1; :SALAZINIC ACID0 – A:GLU323:OE1		
	Hydrogen Bond	:SALAZINIC ACID0:O10 – A:GLU353:OE1		
	Hydrophobic	:SALAZINIC ACID0 – A:MET357; :SALAZINIC ACID0:O7 – A:PRO324; :SALAZINIC ACID0:O8 – A:ILE389; :SALAZINIC ACID0:O8 – A:LYS449; :SALAZINIC ACID0:H39 – A:ILE386		
	Hydrogen Bond; Electrostatic	:SALAZINIC ACID0 – A:LYS449:NZ		
		Target receptor: mTOR		
Atranorin −5.31 kcal/mol 127.41 uM	Electrostatic	:ATRANORIN0:C16 – B:HIS2028; :ATRANORIN0:C21 – B:HIS2028; :ATRANORIN0:C23 – B:HIS2028; :ATRANORIN0 – B:GLU2032:OE1		
	Hydrogen Bond	:ATRANORIN0:H39 – E:HIS2028:ND1; :ATRANORIN0:C18 – E:GLU2032:OE2; :ATRANORIN0:C25 – B:GLU2025:OE1:B; :ATRANORIN0:O7 – E:GLU2025:OE1; :ATRANORIN0:O7 – E:GLU2029:OE2		
Salazinic Acid −3.43 kcal/mol 3.07 mM	Electrostatic	B:GLU2032:OE1 – :UNK0; B:GLU2032:OE2 – :UNK0; E:GLU2029:OE2 – :UNK0		
	Hydrogen Bond	B:ARG2036:NH2 – :UNK0:H40; E:HIS2028:ND1 – :UNK0:O8; E:ARG2036:NH2 – :UNK0:H39; :UNK0:O8 – E:GLU2032:OE2 :UNK0:C27 – E:GLU2025:O :UNK0:H39 – B:GLU2029:OE2 :UNK0:H33 – :UNK0:O1		
		Target receptor: PgR		
Atranorin −5.32 kcal/mol 125.79 uM	Hydrophobic	:ATRANORIN0:C19 – A:ALA922; :ATRANORIN0:H41 – A:ALA922; :ATRANORIN0:O6 – A:LEU921; :ATRANORIN0:O6 – B:LEU889; :ATRANORIN0:O6 – B:LEU921; :ATRANORIN0:C19 – B:LYS885; :ATRANORIN0:C25 – A:LEU892; :ATRANORIN0:C25 – B:PRO918; :ATRANORIN0:C25 – B:LEU921		

Table 2. continued

Ligand (Lichen compound) with Binding Energy (kcal/mol) and Estimated Inhibition Constant (Ki) [Temp. = 298.15 K]	Nature of interactions	Interactions with Amino acids on active sites	3D structure of the docked compound with receptors	Specific interaction of lichen compound with receptors
Salazinic Acid −0.40 kcal/mol 509.49 mM	Electrostatic Hydrophobic	:SALAZINIC ACID0 – A:LYS885:NZ :SALAZINIC ACID0 – A:LEU921:CA; :SALAZINIC ACID0 – A:VAL925; :SALAZINIC ACID0 – A:PRO927; :SALAZINIC ACID0:O8 – B:ALA922; :SALAZINIC ACID0:C27 – B:ALA922; :SALAZINIC ACID0:O7 – A:PRO927; :SALAZINIC ACID0:O8 – A:LYS885; :SALAZINIC ACID0:O8 – A:LEU921		

vivo using breast cancer model organisms to treat and prevent the disease. The results indicated that lichen metabolite atranorin could be good natural phytochemicals with cytotoxicity against the MCF-7 cancer cell line.

Correlation between antioxidant potential in terms of TEAC and the cytotoxic potential of lichen species is shown in Figure 5. Scattered plot shows Pearson's correlation coefficient (R^2). A regression model accounts for more of the variance, the data points are closer to the regression line. From first investigation, there is no correlation between free radical scavenging and cytotoxicity. Differences were considered to be statistically significant $p < 0.05$. Based on the p-value, lichen species *S. foliolosum* showed significant correlation between antioxidant and cytotoxicity. Four lichen extracts (HB,HD,HH,PR) showed radical scavenging potential with $IC_{50} < 65$ μg/mL and all extracts were found to be cytotoxic at 400 μg/mL concentration with less than 45 % cell viability.

The pathophysiological correlation between cell viability and oxidative stress creates opportunities for the use of beneficial compounds referred for protective and even therapeutic purposes. Due to the correlation between oxidative stress and the development of cancer, important biopharmaceutical therapy approaches are also revealed.

Conclusions

In this study, lichen compounds atranorin and salazinic acid were isolated and purified using chromatography techniques and their characterization was done by HR-MS and NMR. These compounds have been tested for scavenging of free radical and reactive oxygen species, antibacterial activity and cytotoxic potential. We believe, this is the first report on antibacterial activity of both lichen compounds against *R. planticola* and investigating the *in-silico* cytotoxic potential of lichen compounds. Atranorin is found to be a potent inhibitor of anti-breast cancer targets mTOR and PgR. Multiple in-vitro and in-silico studies indicate that atranorin could be a promising natural antioxidant, antibacterial, and anticancer agent. Both the compounds needs in-vivo studies employing breast cancer models and a thorough research on the cytotoxic action. Furthermore, growth of lichen is slow and its bioavailability is limited. Hence, to utilize Atranorin, it is necessary to scale up its production by methods such as chemical synthesis and/or biotechnological processes.

Experimental Section

About half of the Western Himalayan region lies in the higher altitude eco-zone (temperate to subalpine), and therefore, the secondary metabolite concentration is expected to be higher because of the stressed climatology and high environmental lapse rate. Fresh lichen thalli were collected from the Western Himalayan region (Uttarakhand and Himachal Pradesh in India) during November 2021 by authors. This region covers the altitude from 1000 to 4000 m, and the present study focuses on high-altitude zones from 1800 to 4000 m. All the necessary field information and

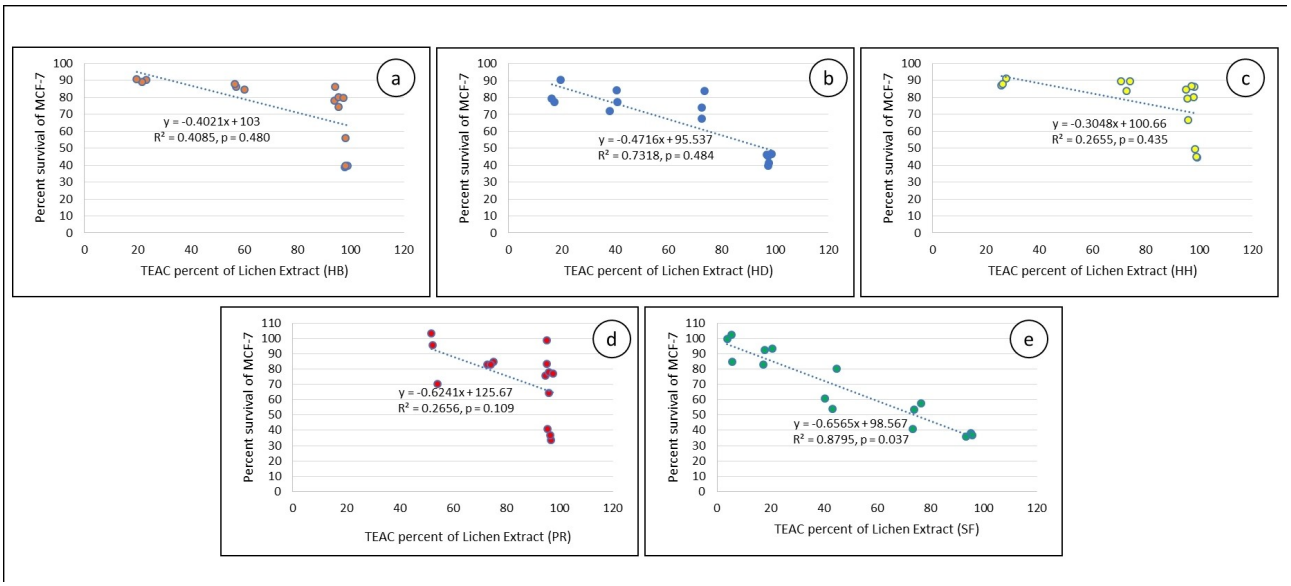
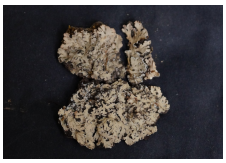


Figure 5. The cytotoxicity effect of Trolox Equivanent Antioxidant Capacity of Lichens species. (a: HB, b: HD, c: HH, d: PR, e: SF) ; significant when $p < 0.05$.

passport data of lichen species collected, along with the accession number, is documented (Table 3).

For this study, we selected five lichen species *Heterodermia boryi, Heterodermia diademata, Heterodermia hypocaesia, Parmotrema reticulatum, Stereocaulon foliolosum* (Table 3).

Table 3. Lichen species collected from various localities of high altitudinal Himalayan region.

Species Name	Habit	Accession Numbers	Collection Sites	State, Country	Altitude range (meter)
Heterodermia boryi (Fée) Kr.P. Singh & S.R. Singh 1976 (Physciaceae)		AMH-21.51	Khaliya Top	Uttarakhand, India	2000–2200
Heterodermia diademata (Taylor) D.D. Awasthi 1973 (Physciaceae)		AMH-21.17	Badrinath, Auli, Khaliya top, Bijli mahadev, kasol,	Uttarakhand and Himachal Pradesh, India	1600–3600
Heterodermia hypocaesia (Yasuda ex Räsänen) D.D. Awasthi 1973 (Physciaceae)		AMH-21.07	Auli, Khaliya top	Uttarakhand, India	2000–3700
Parmotrema reticulatum (Taylor) M. Choisy 1952 (Parmeliaceae)		AMH-21.26	Auli, Khaliya top, Bijli mahadev, kasol, Gorson buggyal	Uttarakhand and Himachal Pradesh, India	1600–3600
Stereocaulon foliolosum Nyl. 1860 (Stereocaulaceae)		AMH-21.12	Badrinath, Auli, Khaliya top, Hemkund, Bijli mahadev, Gorson buggyal	Uttarakhand and Himachal Pradesh, India	2000–4200

Lichens in this region were identified based on their morphology, anatomy and through chemical analysis. For confirming the chemotaxonomy, spot tests and a routine, standardized procedure of thin-layer chromatography was performed using solvent system – A [Toluene (180): Dioxane (45): Acetic acid (5)]. The specimens were preserved and accessioned in the Ajrekar Mycological Herbarium (AMH), Agharkar Research Institute, Pune, India.

Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 4-aminoantipyrine, horse radish peroxidase (HRP), 2,2′-azobis (2-methyl propionamide) dihydrochloride (AAPH), sodium fluorescein, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), hydrogen peroxide (H_2O_2), L-ascorbic acid, phenol,2,4,6-tris (2-pyridyl)-s-triazine (TPTZ), $FeCl_3.6H_2O$, DMSO, bovine serum albumin, sodium chloride, and magnesium chloride hexahydrate were used in the study. Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), hydrogen peroxide solution (30% w/w), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and all other reagents were obtained from Sigma-Aldrich. All other routine solvents and chemicals used were of AR grade and were obtained from the following brands: Sigma, SRL, HiMedia, and Merck.

Extraction of Lichen species

The natural thallus of all five selected lichen species were separated from its substrate and washed by keeping overnight under flow of tap water and then by distilled water. Cleaned thallus was cut into small pieces and extracted successively with different organic solvents. The resulted fractions were filtered by Whatman® filter paper no.1, dried in-vacuo and stored at 4 °C until use. Respective crude extracts were then used for various assays at 25, 50, 100, 200, and 400 μg/mL concentrations.

Physiochemical content

Lichens produce many primary and secondary compounds for their different metabolic activities. In order to know the quantity and the compound responsible for the observed antioxidant activity, total phenolics, polysaccharide and protein content present in extractive fractions of lichen species were estimated. Total polysaccharide content was determined, using the phenol-sulfuric acid method of Dubois et al.[34] Glucose was used as standard for this assay. Protein content was determined by the method of Lowry et al.[35] The protein content was estimated by a standard curve using known amount of bovine serum albumin (BSA). Total soluble phenolic content was determined with Folin-Ciocalteu reagent according to Slinkard and Singleton,[36] using pyrocatechol as a standard. Details of all the assays are reported earlier by us.[37,38,24]

Purification of secondary compounds

Lichen species were cleaned, dried at room temperature for 48 h, and powdered. This material was extracted with acetone through incubation at room temperature for 72 h and concentrated in-vacuo to yield the crude extract. Compounds were isolated using column chromatography, preparative TLC and purified by recrystallization.

Compound Characterization

The purity of isolated compounds was evaluated using High-Performance Liquid Chromatography (HPLC), and these isolated compounds were characterized using HR-MS data and NMR spectra.

HPLC: HPLC analysis was performed using the Thermo-Scientific DIONEX UltiMate 3000 instrument to check the purity of the compounds obtained after isolation. A 30 μL solution of the compound in DMSO was prepared and injected, and the flow rate was set at 1 mL/min. The solvent system used was Methanol : Water : Ortho-Phosphoric acid (80 : 19 : 1, v/v/v). Compared with available standard data, lichen compounds were identified based on their peak symmetry and retention time.

HR-MS: Impact II UHR-TOF Mass Spectrometer System and Dionex UHPLC Ultimate 3000 System were the electrospray ionization source. The mass resolution was set at 50,000 FSR, and the mass range was 100–1200 m/z.

NMR: The isolated pure compound was subjected to 1H-NMR (500 MHz, DMSO-d6) and 13 C NMR (126 MHz, DMSO) NMR in the BRUKER AVANCE III HD NMR 500 MHz Nuclear Magnetic Resonance (Liquid State 500 MHz NMR) instrument by using DMSO as a solvent for dissolving the sample and TMS as the internal standard. Chemical shifts (δ-values) are given in parts per million (ppm), and the coupling constants (J-values) are given in hertz (Hz).

TGA: Thermogravimetric Analysis (TGA) was carried out to test the thermal stability of the isolated compounds. Thermogravimetric analysis (TGA, Columbus, OH, USA) was recorded using a Mettler Toledo instrument under Nitrogen atmosphere (40 mL min^{-1}), and subjected to temperature ranging from 35 to 800 °C at a heating rate of 10 °C min^{-1}.

Antioxidative potential

The free radical scavenging activity of lichen extracts was evaluated using different antioxidant assays.

The radical-scavenging (DPPH) activity of lichen fractions was measured with 1,1-diphenyl-2-picryl hydrazil (DPPH) according to the methods of Blois et al.[39] and modified by Verma et al.[38] Five concentrations of the compound (range: 25–400 μg/mL) were added with DPPH solution (0.12 mM) in a 96-well plate to make up the final volume of 300 μL/well. The changes in absorbance were read at 517 nm after 30-min incubation in dark.

The reducing power ability of the lichen crude extract and compound was tested. Reagents were prepared as per the method described in the protocol of Khatua et al.[40] Then, 100 μL of the extract (range: 25–400 μg/mL), was added to the reaction mixture. The obtained blue color absorbance was then measured at 700 nm against a blank sample. Trolox (water soluble Vitamin-E analogue) at a concentration ranging from 25 μg/mL to 1 mg/mL was taken as the standard.

Trolox equivalent antioxidant capacity of the extracts and compound was determined using as given in the proposed modified method of Khatua et al.[40] Five concentrations of the compound (range: 25–400 μg/mL) were added to ABTS and potassium per-sulphate solution under a dark condition. Then, the change in the color of the ABTS radical was measured by determining absorbance at 734 nm. Trolox was used as a standard.

Scavenging of nitric oxide was evaluated following the protocol of Boora et al.[41] The Griess reagent was prepared by mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride in 2.5% phosphoric acid

immediately before use. Then, 0.5 mL of 10 mM sodium nitroprusside in phosphate buffered saline was mixed with 1 mL of different concentrations of the ethanol extract (100–1000 μg/mL) and incubated at 25 °C for 180 min. The extract was mixed with an equal volume of the freshly prepared Griess reagent. The absorbance was measured at 546 nm by using a Bio Tek UV-Vis microplate reader.

Antibacterial activity

The antibacterial activity of the lichen extracts was determined through the Minimum Inhibitory Concentration (MIC) assay by using the resazurin-based method as described by Teh et al. with some modifications.[42] The antibacterial activity was tested against five bacterial strains, namely *Bacillus subtilis* (MTCC 121, gram-positive soil bacterium), *Escherichia coli* (MTCC 739, gram-negative bacterium), *Pseudomonas aeruginosa* (MTCC 2453, gram-negative bacterium), *Raoultella planticola* (MTCC 530, gram-negative bacterium), and *Staphylococcus aureus* (MTCC 2940, gram-positive bacterium). The test strains were procured from Microbial Type Culture Collection (MTCC), CSIR-Institute of Microbial Technology (IMTECH), Chandigarh, India. Microdilution was performed in triplicates for each bacterial species. After an overnight incubation at 37 °C, 25 μL resazurin was added to all wells and incubated at 37 °C for another 2 h. Change in color (purple to pink or colorless) were recorded as positive. The lowest concentration prior to color change was considered as the MIC.

Anticancer activity

Anticancer activity of lichen compounds was determined using the cytotoxicity (MTT) assay on breast cancer cell line (MCF-7).

Cell lines, media and treatment

The breast cancer cell line (MCF-7) was obtained from the National Centre for Cell Sciences (NCCS) cell repository, Pune, India. The cell line (MCF-7) was cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin–streptomycin in 75-cm^3 cell culture-treated flasks in 5% CO_2 and humidified atmosphere at 37 °C. The medium was replaced every 2–3 days as required. After the cells reached 80%–90% confluence, they were washed with PBS. Detached using trypsin-EDTA solution, and harvested through centrifugation at 300×g for 4 min. The cells were re-suspended in 1 mL of medium, and viable cells were counted using the hemocytometer after they were stained with trypan blue (0.4% in PBS).

Cytotoxicity analysis

The cytotoxic effect of the lichen acetone extracts and isolated compounds on MCF-7 cells, a breast cancer cell line, was determined using the MTT assay.[43] The cells (1×10^4) were seeded in 96-well plates, incubated overnight, and then treated with five concentrations of the lichen extract (25–400 μg/mL) and six concentrations of the lichen compounds (3.12–200 μM) for 24 h. After treatment, 10 μL MTT (5 mg/mL) was added to each well and kept for 4 h. Viable cells convert the MTT dye to insoluble formazan crystals, which were dissolved using 100 μL DMSO after the medium was removed. Absorbance was measured at 570 nm the using universal microplate reader Synergy TM HTX and Synergy TM 2 multi-mode readers (BioTek Instruments India (P) Limited). The percentage of cell survival was determined using the following equation 1.

Equation 1.

$$\% \text{ Cell death} = (\text{Absorbance of the sample}/\text{Absorbance of the control}) \times 100$$

Microscopic analysis

Changes in cellular morphology after treatments were examined using the Floid Cell Imaging Station by Life Technologies. Ready probes and the cell viability imaging kit (Blue/Red) were used to determine cell viability. A drop of each stain was added to 24-well microplate and incubated for 30 min. Images were taken using red and blue filters on the Floid Cell Imaging Station.

Molecular Docking Study

Molecular docking study of lichen metabolites has been carried out in order to know the binding sites on target proteins and to estimate its free energy of binding.[44] Based on the literature, ERα (Estrogen receptor), PgR (Progesterone receptor), EGFR (Epidermal growth factor receptor) and mTOR (mammalian target of rapamycin) were selected as targets for breast cancer. Three-dimensional X-Ray crystallized structure of ERα (6CHZ), EGFR (4I23), mTOR (4DRH) and PgR (1SQN) was downloaded from the protein data bank (http://www.rcsb.org). The three-dimensional structure of the testing compound was downloaded from PubChem (http://www.pubchem.ncbi.nlm.nih.gov).

The ERα, PgR, EGFR, and mTOR compound docking study was conducted using AutoDock 1.5.6, Discovery Studio Visualizer 2.1 and Cygwin64. In AutoDock, internal conformation was searching with help of Genetic Algorithm (GA) and produces an ensemble of conformations by Lamarckian Genetic Algorithm. The lowest binding energy conformer was searched out of 10 different conformers for each docking simulation and best scoring pose was judged by the Cygwin64. The chosen complex was visually analyzed using a Discovery Studio Visualize (DSV).

Statistical Analysis

All experiments were repeated in triplicate. Results were reported as Mean ± SD. The statistical significance between antioxidant activity and cytotoxicity values of the extracts was evaluated with one-way ANOVA. P-values less than 0.05 were considered statistically significant. The IC_{50} values were calculated by non-linear regression analysis. Using data analysis tools like Microsoft Excel 2013 and Sigma Plot 12, the degree of correlation between two parameters was evaluated using a regression analysis based on the coefficient of determination's (R^2) value.

Abbreviations

4E-BP1	eIF4E binding protein 1
AAPH	2,2′-azobis (2-methyl propionamide) dihydrochloride
ABTS	2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
AKT	Serine/Threonine Kinase family
BSA	Bbovine Serum Albumin
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide

DPPH	2,2-Diphenyl-1-picrylhydrazyl
EGFR	Epidermal Growth Factor Receptor
ERα	Estrogen Receptor alpha
FBS	Fetal Bovine Serum
FRAP	Ferric Reducing Antioxidant Potential
HB	*Heterodermia boryi*
HD	*Heterodermia diademata*
HH	*Heterodermia hypocaesia*
HPLC	High-Performance Liquid Chromatography
HR-MS	High Resolution-Mass Spectroscopy
HRP	4-aminoantipyrine, horse radish peroxidase
IBC	Inflammatory breast cancer
IC50	Half Inhibitory Concentration
MCF-7	Michigan Cancer Foundation-7 (Human breast cancer cells)
MIC	Minimum Inhibitory Concentration
MTCC	Microbial Type Culture Collection
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	Nuclear Magnetic Resonance
PI3K	phosphoinositide 3-kinases
PR	*Parmotrema reticulatum*
PgR	Progesterone receptor
RANKL	Receptor activator of nuclear factor kappa-B ligand
S6K1	Ribosomal protein S6 kinase
SD	Standard deviation
SF	*Stereocaulon foliolosum*
SNO	Scavenging of Nitric Oxide
TEAC	Trolox Equivalent Antioxidant Capacity
TGA	Thermo-Gravimetric Analysis
TLC	Thin Layer Chromatography
TNBC	Triple-Negative Breast Cancer
TPTZ	2,4,6-tris (2-pyridyl)-s-triazine
Trolox	(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid

Author Contributions

Subhash Gaikwad, Sachin Mapari and Ruchira Sutar conducted the experiments and drafted the manuscript. Subhash Gaikwad, Sachin Mapari, Ruchira Sutar and Dr. Roshni Khare collected and analysed the data. Dr. Roshni Khare identified and accessioned the samples. Dr. Muntjeeb Syed and Sachin Mapari contributed for molecular docking study and analysis. Dr. Roshni Khare and Dr. Bhaskar Behera reviewed and edited the manuscript. Dr. Bhaskar Behera conceived and designed the study. All authors have read and approved the final manuscript.

Acknowledgements

The authors acknowledge Agharkar Research Institute, Pune, India for providing necessary supports and facilities to carry out the present research work. We also acknowledge Central Instrumentation Facility (CIF), at Savitribai Phule Pune University, Pune for HR-MS and NMR facility. This work was supported by Department of Biotechnology, Government of India, New Delhi (for Sachin Mapari – Award letter No.: DBTHRDPMU/JRF/BET-20/I/2020/AL/1 dated 27.11.2020); University Grants Commission, Government of India, New Delhi (for Ruchira Sutar – UGC-Ref no. 835/Dec.2017); D.S. Kothari Post-Doctoral Fellowship by University Grants Commission, Government of India, New Delhi, (for Dr. Roshni Khare – BL/18-19/0479).

Conflict of Interests

The authors declare no competing interests. Authors have no financial or non-financial interests that are directly or indirectly related to the work submitted for publication.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: antibacterial · anticancer · atranorin · lichen · salazinic acid

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Manuscript received: August 14, 2023
Accepted manuscript online: October 27, 2023
Version of record online: November 15, 2023