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# Antifungal and Antibacterial Efficacy of Solvent Extracts from *Ramalina Conduplicans* and *Roccella* Montagnei against Clinically Relevant Pathogens

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## ABSTRACT

**Introduction:** *Ramalina conduplicans* and *Roccella montagnei* represent ecologically resilient symbiotic systems that synthesize diverse secondary metabolites with potent antimicrobial potential.

**Aim/Objective:** The present study aimed to evaluate the antifungal and antibacterial activities of solvent extracts from *Roccella montagnei* and *Ramalina conduplicans*, traditionally known for their therapeutic properties, against clinically relevant pathogens .

**Methods:** *Lichen thalli* collected from the Western Ghats of Tamil Nadu were sequentially extracted using diethyl ether, acetone and methanol via Soxhlet hot percolation. Antifungal activity was assessed against *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxysporum* and *Fusarium solani* using disk diffusion and broth macrodilution methods. Antibacterial activity was determined by agar well diffusion and broth microdilution assays. Total phenolic and flavonoid contents were quantified spectrophotometrically as gallic acid and quercetin equivalents, respectively. Statistical significance was evaluated using two-way ANOVA ($p < 0.05$).

**Results:** *Ramalina conduplicans* and *Roccella montagnei* exhibited notable antifungal and antibacterial efficacy in a solvent dependent manner. The acetone extract of *Ramalina conduplicans* produced the largest inhibition zones (10–12 mm) and the lowest MIC values (2–8 µg/mL), particularly against *Aspergillus flavus* and *Fusarium solani*. The methanolic extract of *Roccella montagnei* showed strong antifungal activity (MIC 2–4 µg/mL) with high phenolic (37.28 mg GAE/g) and flavonoid (48.24 mg QE/g) contents. Acetone extracts of both lichens displayed broad-spectrum antibacterial activity, most pronounced against *Escherichia coli* (21–24 mm ZOI; MIC 4–8 µg/mL).

**Conclusion:** This study highlights *R. montagnei* and *R. conduplicans* as promising sources of phenolic rich metabolites with potent antifungal and antibacterial properties. The acetone and methanol extracts demonstrated broad spectrum inhibitory effects, underscoring their potential for developing novel natural antimicrobial agents.

**Key Words:** Lichens, *Roccella montagnei, Ramalina conduplicans*, antifungal activity, MIC, secondary metabolites, phenolic compounds

## INTRODUCTION

Lichens are intricate, self-sustaining consortia formed through the intimate symbiosis of a fungal mycobiont and its algal or cyanobacterial photobiont, enabling autonomous survival in diverse habitats.[1] They yield an astonishing array of applications across cultures for traditional medicinal remedies, livestock forage, natural dyes and perfumes, culinary spices, and other practical uses that underscore their enduring ecological and cultural ingenuity.[2] Lichens and their extracts have been key parts of traditional medicines around the world for a long time, and they still draw interest as helpful alternative treatments today. Their unique secondary metabolites, confer broad bioactivities including antimicrobial, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and cytotoxic effects.[3] The relentless overuse of antimicrobials has driven the emergence and dissemination of multidrug-resistant pathogens, posing a threat to global public health and thereby underscoring the urgent need for novel antimicrobial agents. Lichens with their arsenal of secondary metabolites exhibiting potent antibacterial and antimycotic activities, emerge as frontrunners in the fight against antimicrobial resistance.[4]

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*Ramalina conduplicans Vain* . is an edible fruticose lichen prevalent in central and southeastern Asia, used as a traditional delicacy in Chinese and Nepalese dishes and as a spice in Indian cuisines.[5] *R. conduplicans* demonstrates profound metabolic adaptability by producing an array of structurally unique secondary metabolites including sekikaic acid and homosekikaic acid, such as the depside compounds sekikaic acid and homosekikaic acid. These depsides constitute major fractions of its methanol extract and confer superior antioxidant capabilities through efficient free radical scavenging and elevated total phenolic contents that surpass other lichens.[6] *Roccella montagnei* Bél., a member of the Roccellaceae family is a common epiphyte to the coastal trees along the Coromandel Coast in Tamil Nadu and the Western Ghats. They have an impressive lineup of secondary metabolites such as orcinol, montagnetol, β-carotene, β-sitosterol, erythritol, roccellic acid, lecanoric acid and methyl orsellinate. Studies have revealed the strong antimicrobial, insecticidal and anti-inflammatory effects, making it a compelling candidate for combating antimicrobial resistance.[7]

The current investigation is aimed to evaluate the antifungal and antibacterial properties of diethyl ether, acetone and methanol extracts from *Roccella montagnei* and *Ramalina conduplicans* collected from Western Ghats of Tamil Nadu against fungal and bacterial pathogens.

## METHODS

The present research was conducted at Department of Microbiology, Sarvepalli Radhakrishnan University, Bhopal, India, from April 2024 to November 2024. Lichen samples were gathered during the summer from Kodayar in the Western Ghats, Kanyakumari District, Tamil Nadu, India. Specimens were authenticated at the Plant Biodiversity and Conservation Biology Division, National Botanical Research Institute (NBRI-CSIR), Lucknow, India. The species included *Roccella montagnei* Bél. em. D.D. Awasthi (10028) and *Ramalinaconduplicans* Vainio (10031). Fungal isolates comprised *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxysporum*, *Fusarium solani* and the bacterial isolates included *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli*.

### Preparation of Lichen Extracts

The dichotomously or irregularly branched, greenish gray to yellowish gray lichen thalli collected from Western Ghats were thoroughly cleaned to remove the debris, air dried at room temperature and pulverized into a coarse powder. 100 g of powder was sequentially extracted with solvents of increasing polarity such as diethyl ether, acetone, and methanol, which are non-polar , intermediate and polar respectively. Extractions employed the hot percolation technique for 72 hours, with temperatures maintained below each solvent's boiling point using a Soxhlet apparatus. The extracts were concentrated by rotary evaporation and preserved in sealed glass vials within a desiccator containing anhydrous calcium chloride. For the subsequent bioactivity assays, extracts were redissolved in their original extraction solvents.

### Evaluation of Antifungal activity

#### *Fungal Strains and Inoculum Preparation*

Fungal strains used in the study included *Aspergillus niger* MTCC 1344, *Aspergillus flavus* MTCC 277, *Fusarium oxysporum* MTCC 284, *Fusarium solani* MTCC 350. Strains were maintained on Sabouraud dextrose agar (SDA) slants at 4°C. Inocula were prepared by suspending conidia in sterile saline with 0.05% Tween 80, adjusted to $5 \times 10^4$ CFU/mL and confirmed by plating serial dilutions on SDA.

#### *Disk Diffusion Assay*

Antifungal efficacy was screened using the Kirby-Bauer disk diffusion method, adhering to guidelines of CLSI M44-A2.[8] Sterile 6 mm paper disks were impregnated with 50 µL of each extract, applied in aliquots with evaporation intervals to avoid solvent residue. Potato dextrose agar (PDA) plates were lawn cultured with the fungal suspensions at $10^6$ spores/mL. Impregnated disks were placed on the plates, with Amphotericin B (5 mg/mL) serving as the positive control. Plates were incubated at 25°C for 72 hours and zones of inhibition were measured in millimeters.

#### *Broth Macrodilution Assay and MIC Determination*

MICs were determined by performing Broth Macrodilution as per CLSI M38- Ed3 guidelines.[9] Extracts were serially diluted in dimethyl sulfoxide (DMSO) and incorporated into Sabouraud dextrose broth with a final volume of 2 mL per tube. 20 µL of fungal inoculum was added to each tube. Positive controls included Amphotericin B and negative controls contained DMSO alone. The tubes were incubated at 30°C and monitored visually every 48 hours for up to 14 days. MIC was visually determined as the lowest concentration preventing visible growth, relative to controls. The mean MIC value from three replicates was noted.

### Evaluation of Antibacterial activity

#### *Agar well diffusion assay*

Bacterial strains used in the study included *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli*. Antibacterial efficacy was determined by performing agar well diffusion method. Mueller-Hinton agar plates were lawn cultured with 100 µL of 0.5 McFarland bacterial suspension. 6 mm wells were created in the agar using a sterile well-cutter and each well was filled with 10 µL fungal suspension. The plates were incubated at 37°C for

24 hours and the zones of inhibition around each well were measured post incubation in mm. The assay was performed in triplicate and ZOI values were reported as mean ± standard deviation.

### Broth Microdilution Assay and MIC Determination

MIC of the extracts against was determined using the broth microdilution method in MHB, following CLSI M07 Ed11 guidelines.[10] Serial twofold dilutions of the methanol extracts were prepared in 96-well microtiter plates. Bacterial inocula were adjusted to a 0.5 McFarland standard and diluted 1:10 in MHB prior to inoculation. Each well received 100 µL of the extract and 100 µL of bacterial suspension, followed by incubation at 37 °C for 24 h. MIC was visually determined as the lowest concentration showing no visible growth compared to untreated controls. All assays were performed in triplicate.

### Quantification of Total Phenolic Content

Total phenolic content was measured spectrophotometrically using modified Folin-Ciocalteu assay.[11] 1 mL of the extract was combined with 9 mL distilled water, followed by 1 mL Folin-Ciocalteu reagent. 10 mL of 7% $Na_2CO_3$ was added after agitation and the volume adjusted to 25 mL with water. Mixtures were incubated in darkness at 23°C for 90 minutes and absorbance recorded at 750 nm. Total phenolic content was calculated by extrapolation of the calibration curve generated using Gallic acid standards (20–100 µg/mL) and results were expressed as mg gallic acid equivalents (GAE)/g dry weight. All measurements were performed in triplicates.

### Quantification of Total Flavonoid Content

Flavonoid content was determined via the aluminum chloride colorimetric method.[12] 0.5 mL aliquot of extract was mixed with 1.5 mL methanol, 0.1 mL 10% $AlCl_3$, 0.1 mL 1 M potassium acetate and 2.8 mL distilled water. This was followed by incubation at room temperature for 30 minutes and absorbance was measured at 510 nm using a UV-visible spectrophotometer. The flavonoid content was quantified using a quercetin calibration curve (20-100 µg/ml in methanol) and results reported as mg quercetin equivalents (QE)/g dry weight, with all measurements in triplicates.

### Statistical Analysis

Data from antifungal and antibacterial assays are presented as mean ± standard error from triplicates. Differences among extract types and fungal species were analysed using two-way analysis of variance (ANOVA) with $p < 0.05$.

## RESULTS

### Antifungal activity of *R. conduplicans* and *R. montagnei* extracts

The acetone, methanol and diethyl ether extracts of *R. conduplicans* demonstrated variable antifungal activity against *A. niger, A. flavus, F. oxysporum* and *F. solani*. The acetone extract demonstrated the strongest antifungal activity among the extracts, with zone of inhibition values ranging from 10 ± 0.22 mm to 12 ± 0.98 mm, followed by methanol and diethyl ether extracts (Table 1 and Figure 1). Acetone extract showed the largest zone of inhibition against *F. solani* with a diameter of 12 ± 0.98 mm, followed by 12±0.36 against *A. flavus*. 11 ± 0.50 mm against *A. niger* and 10 ± 0.22 mm against *F. oxysporum*. The antifungal activity of acetone extract against *A. flavus* (12±0.36) was comparable to the standard Amphotericin B (12 ± 0.14 mm). The methanol extract exhibited intermediate activity, with ZOI values between 9 ± 0.14 mm and 11 ± 0.96 mm. Highest activity was recorded against *A. flavus* (11 ± 0.96 mm), though lower, yet comparable to the standard (12 ± 0.14 mm). ZOI values against *F. oxysporum*, *A. niger* and *F. solani* were found to be 10 ± 0.7 mm, 10 ± 1.2 mm and 9 ± 0.14 mm respectively. Diethyl ether extract showed the least antifungal activity, with ZOI ranging from 8 ± 0.56 mm to 10 ± 0.4 mm. The highest inhibition was observed against *A. flavus* (10 ± 0.4 mm) and the lowest against *F. oxysporum* (8 ± 0.56 mm). Zone of inhibition with a mean diameter of 9 ± 0.88 mm was observed for *A. niger* and *F. solani* exhibited ZOI value of 8 ± 1.4 mm. The methanolic extract of *R. montagnei* showed a marked antifungal activity against *A. flavus* and *A. niger* as indicated in the Figure 2. It demonstrated a mean zone of inhibition 12±0.36 mm against *A. flavus* and 11±0.50 against *A. niger*. The standard zone of inhibitions by Amphotericin B (5mg/mL) was found to be 11±0.97 and 15±0.08 respectively for *A. flavus* and *A. niger*. Statistical evaluation confirmed significant extract-pathogen interactions. Methanol extract of *R. montagnei* contained the total phenolic content of 37.28± 2.12 gallic acid equivalents mg/g and total flavonoids of 48.24±1.92 quercetin equivalents mg/g.

The acetone extract of *R. conduplicans* exhibited the lowest MIC values, ranging from 2 µg/mL. The most susceptible species were *A. flavus* and *F. solani* (2 µg/mL) followed by *A. niger* and *F. oxysporum* (4-8 µg/mL). Methanol extracts showed moderate inhibition with MICs from 4 to 16 µg/mL, while diethyl ether extracts exhibited the weakest activity, with MICs ≥ 32 µg/mL for all tested fungi. *R. montagnei* methanolic extract demonstrated strong inhibitory effects, with MICs of 2 µg/mL against *A. flavus* and 4 µg/mL against *A. niger*. The acetone and diethyl ether extracts of *R. montagnei* showed comparatively higher MIC values (4–64 µg/mL).

### Antibacterial activity of *R. conduplicans* and *R. montagnei* extracts

The diethyl ether, acetone and methanol extracts of *R. conduplicans* exhibited potent antibacterial activity against *S. aureus*, *K. pneumoniae*, *P. aeruginosa* and *E. coli*. Acetone extracts demonstrated the strongest activity (ZOI: 9-21 mm),

particularly against *E. coli* (21 ± 0.4 mm), surpassing methanol (9–17 mm) and diethyl ether (7–13 mm) extracts (Table 2). *S. aureus* showed the least sensitivity across extracts (ZOI: 7–9 mm). All extracts of *R. montagnei* displayed significant antibacterial activity, with acetone extracts showing the broadest spectrum (ZOI: 11–24 mm). Highest inhibition was observed against *E. coli* (24 ± 1 mm). Methanol extracts were moderately effective (9–17 mm), while diethyl ether showed the least potency (9–15 mm) (Table 2 and Figure 3). One-way ANOVA for each extract confirmed significant differences across bacteria.

The MIC values demonstrated marked variation among both species and bacterial strains. For *R. conduplicans*, the lowest MIC was recorded against *E. coli* (8 µg/mL), whereas *K. pneumoniae* and *P. aeruginosa* exhibited higher MICs of 32 µg/mL and 64 µg/mL, respectively. *R. montagnei* methanol extract showed potent inhibition against *E. coli* (4 µg/mL), while *K. pneumoniae*, *P. aeruginosa* and *S. aureus* were less susceptible (32-128 µg/mL).

## DISCUSSION

The present investigation elucidates the antifungal and antibacterial potential of acetone, methanol and diethyl ether extracts derived from *R. montagnei* and *R. conduplicans*, underscoring the significance of lichens as reservoirs of bioactive metabolites with pharmacological relevance. The solvent extracts from *R.montagnei* and *R.conduplicans* demonstrated robust antifungal activity against opportunistic fungal pathogens. Among the tested solvents, acetone and methanol extracts exhibited pronounced antifungal efficacy, while diethyl ether extracts demonstrated comparatively lower activity. Solvent polarity profoundly influenced the extract potency, with intermediate polarity acetone and polar methanol outperforming non-polar diethyl ether, consistent with the lipophilic nature of fungal cell walls favouring moderately polar bioactives. The superior activity of polar and intermediate polarity solvents may be attributed to their enhanced ability to solubilise phenolic, depside and depsidone constituents which are widely recognized for disrupting microbial membranes and interfering with ergosterol biosynthesis.

The acetone extract of *R. conduplicans* revealed inhibition zones comparable to the standard antifungal, suggesting the presence of potent fungitoxic compounds. Similarly, *R. montagnei* methanolic extracts showed significant inhibition against *A. flavus* and *A. niger*, correlating with high phenolic and flavonoid concentrations. Earlier studies employing different methodology have reported stronger inhibition against *Fusarium* spp. and *A. niger*.[13,14] The MIC trends paralleled the diffusion assay results, confirming that acetone and methanol extracts possessed the most potent fungistatic effects, particularly against *A. flavus* and *F. solani*. The antifungal response variability among fungal species reflects the intrinsic differences in cell wall composition, ergosterol content and the metabolic tolerance to oxidative stress. The relatively higher susceptibility of *A. flavus* and *F. solani* suggests that phenolic enriched extracts may target ergosterol or β-glucan synthesis pathways. Phytochemical quantification substantiates that higher total phenolic and flavonoid contents parallel enhanced antimicrobial activity, emphasizing their functional role as natural defense molecules.

Antibacterial activity results further highlight the broad-spectrum potential of lichen extracts, with acetone extracts of both species excelling against *E. coli*, positioning these lichens as multifaceted AMR countermeasures. *R. montagnei* exhibited stronger antibacterial activity than *R. conduplicans*, particularly against Gram-negative bacterial strains. The observed MIC pattern was consistent with the zone of inhibition data from the agar well diffusion assay, suggesting a concentration dependent bactericidal effect of the methanolic extracts. Previous studies have reported higher ZOI values for the antibacterial activity of *R. conduplicans* by well diffusion assay, but *E. coli* strains are found to be more susceptible to the extract in our study. The variations in antibacterial activity could be possibly attributed to the antimicrobial resistance profiles of the test strains.[5,13]

Results of the current study validate *R. montagnei* and *R. conduplicans* as promising bioprospecting candidates for natural antifungal development. Given the alarming rise of invasive fungal infections and antifungal resistance globally, along with the threat of MDR bacteria, these natural metabolites may represent as sources of novel antimicrobial agents. However, *in vitro* limitations of the study include pharmacokinetic studies and metabolic profiling. Future efforts should isolate active depsides for structure-activity profiling, synergy with antimicrobials and *in vivo* validations.

## CONCLUSION

This study unveils the significant antifungal and antibacterial activities of *R. montagnei* and *R. conduplicans*, with acetone and methanol extracts exhibiting the most potent effects. The findings of our study not only validate the pharmacological relevance of these lichens, but also highlight their potential as sources of novel antimicrobial agents with broad-spectrum efficacy. Future work should focus on metabolite profiling, structure elucidation of active components and evaluation of cytotoxicity and mechanistic pathways *in vivo*. The integration of molecular insights could accelerate the discovery of next-generation antimicrobial therapeutics derived from natural lichen resources.

## DECLARATIONS

**Acknowledgement:** Authors acknowledge the immense help received from the scholars whose articles are cited and

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**Authors' Contribution:** Georgy Antony and Anusha Bhaskar conceptualized the study. Georgy Antony performed the sampling, sample collection, and bacteriological analysis. Georgy Antony, Anusha Bhaskar, Sudheesh M, and Manikkumar R contributed to the writing, review, and editing of the manuscript. All authors have read and approved the final manuscript.

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**Table 1: Zones of Inhibition (mm) for *Ramalina conduplicans* extracts against fungal pathogens**

| Sl. No | Fungal strains | Standard* | Acetone extract | Methanol extract | Diethyl ether extract |
|---|---|---|---|---|---|
| 1 | *Aspergillus flavus* | 12±0.14 | 12±0.36 | 11±0.96 | 10±0.4 |
| 2 | *Aspergillus niger* | 16±0.28 | 11±0.50 | 10±1.2 | 9±0.88 |
| 3 | *Fusarium oxysporum* | 11±1.1 | 10±0.22 | 10±0.7 | 8±0.56 |
| 4 | *Fusarium solani* | 14±1.2 | 12±0.98 | 9±0.14 | 8±1.4 |

**Table 2: Zones of Inhibition (mm) for *R. conduplicans* and *R. montagnei* extracts against bacterial strains**

| Bacterial strains | *R. conduplicans* Diethyl Ether (mm) | *R. conduplicans* Acetone (mm) | *R. conduplicans* Methanol (mm) | *R. montagnei* Diethyl Ether (mm) | *R. montagnei* Acetone (mm) | *R. montagnei* Methanol (mm) |
|---|---|---|---|---|---|---|
| *S. aureus* | 7 ± 1 | 9 ± 1 | 9 ± 0.5 | 9 ± 1.1 | 12 ± 0.6 | 9 ± 1.5 |
| *K. pneumoniae* | 8 ± 0.7 | 14 ± 0.6 | 11 ± 0.5 | 7 ± 1.2 | 11 ± 1.1 | 11 ± 1.1 |
| *P. aeruginosa* | 7 ± 0.3 | 12 ± 0.7 | 10 ± 0.9 | 8 ± 1.2 | 13 ± 0.6 | 12 ± 1 |
| *E. coli* | 13 ± 0.6 | 21 ± 0.4 | 17 ± 1.3 | 15 ± 0.5 | 24 ± 1 | 17 ± 2.1 |

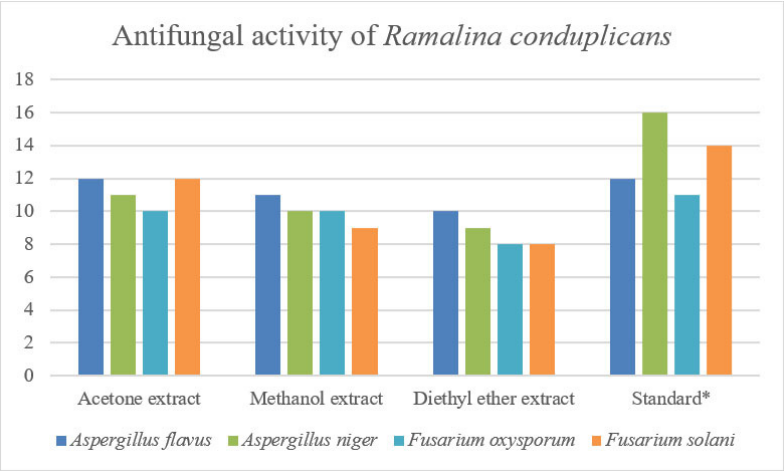


**Figure 1:** Antifungal activity of *Ramalina conduplicans* against *A.flavus, A.niger, F.oxysporum* and *F. solani*

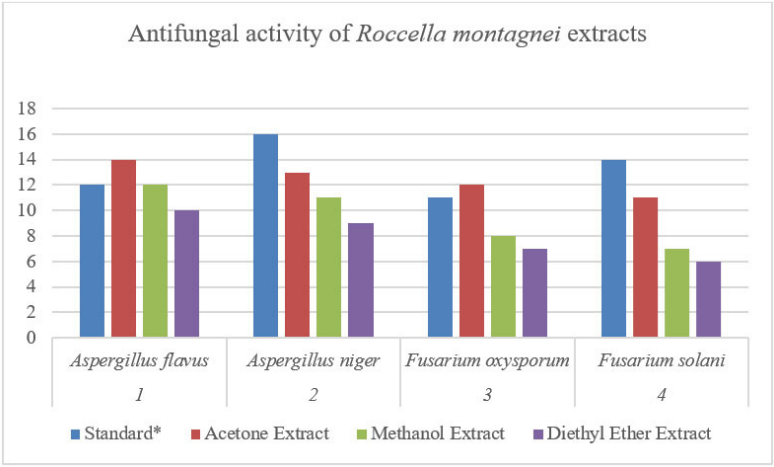


**Figure 2:** Antifungal activity of Methanol, Acetone and Diethyl ether extracts of *Roccella montagnei*

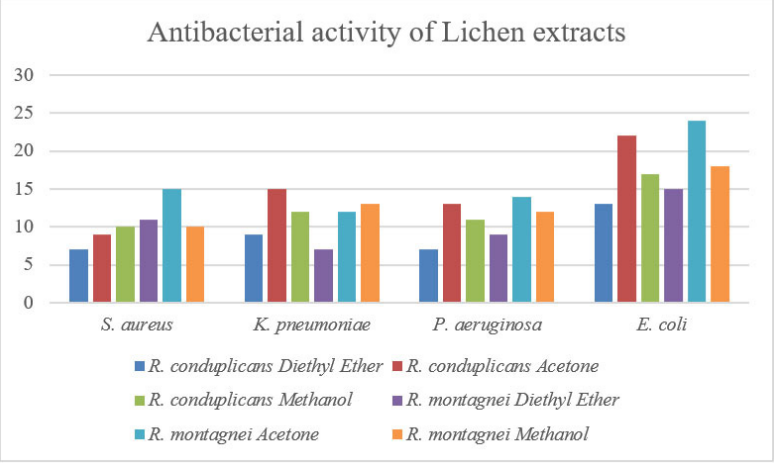


**Figure 3:** Antibacterial activity of *R. conduplicans* and *R. montagnei* extracts