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Antimicrobial activity of Roccella montagnei against pathogenic microorganisms

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ABSTRACT

An attempt was made to study the antimicrobial activity of saxicolous lichen Roccella montagnei under invitro conditions. The antimicrobial activities of Methanol, Ethanol, Ethyl Acetate and Acetone extracts of Roccella montagnei were assayed against nine pathogenic microorganisms using the standard welldiffusion method. The Acetonic extract was found most effective antibacterial whereas the Ethanolic extract was found most effective antifungal against most of the organisms. The maximum inhibition zone was recorded in E. coli with inhibition zone 34 mm. fungal pathogens showed their inhibition zones in varying levels as 32 mm in Candida albicans and Fusarium oxysporium and 30 mm in Aspergillus niger. The present study reveals that extracts obtained from R. montagnei have potential compounds that can lead to control of human pathogenic microorganisms in the future.

Keywords— *Lichens, Well diffusion, Inhibition zone, Antimicrobial, Roccella montagnei*

1. INTRODUCTION

The search for novel natural bioactive compounds leading to new drug discovery is increasing as reliable standard drugs become less effective against new strains of multi drug resistant pathogens (Muller, 2001). Lichens are considered as a potential resource since these compounds function as a chemical defence against biotic and abiotic stresses and they are antibacterial (Lawrey,1986)., anticancer (Williams et al.,1998), anti HIV (Huneck and Yoshimura,1996), analgesic and antipyretic (Muller, 2001). It has been documented that more than 1050 secondary metabolites were found so far (Huneck and Yoshimura, 1996) and among them, 550 are unique in lichens. Lichens produce a large number of primary and secondary metabolites. Most lichen substances are phenolic compounds, dibenzofuranes and usnic acids, depsidones, depsones, lactones, quinines and pulvinic acid derivatives (Boustie and Grube, 2005). Lichen extracts have been used for various remedies in folk medicines and screening of compounds has shown potentiality as antimicrobial, anticancer, antioxidant, antitumour and analgesic. India is among the richest biodiversity centres contributing about 15% of 13,500 species of the world (Negi, 2000). Total of 2450 species of lichens was present in India and was abundant in temperate and alpine regions of Peninsular India (Nayaka et al, 2010).

The lichen species *R. montagnei* (Roccellaceae) is fruticose growth form, found common as epiphytes along the Coromandel Coast, Tamil Nadu, India and is abundant in Pichavaram Mangrove forests (Awasthi, 1988). They are greenish or grey in colour, erect or pendulous, attached by basal holdfast, branched, irregularly widened, tapering with pedicellate apothecia. They contain erythrin and traces of lecanoric and roccellic acid. It contains about 14% protein and also contains significant amounts of calcium (S. Ramakrishnan and S. Sankara, 1964). *R. montagnei* has been examined for free and combined amino acids. *Roccella montagnei* contains leucine, valine, tyrosine, alanine, glycine, threonine and arginine in Free State and acid and alkali hydrolysates of residue after extraction of free amino acids contain in addition to lysine and phenylalanine (S. Ramakrishnan and S.Sankara, 1964). *R. montagnei* possesses a wide array of secondary compounds such as Roccellic acid, Orcinol, Lecanoric acid, Montagnetol, Methylorsellinate, Mesoerythritol, Beta carotene and Beta sitoserol (Neelakantan and Seshadri, 1952). *R. montagnei* is the only lichen that may be converting Erythrin to strongly antifungal Methylorsellinate whenever required (V. Karunaratne et al, 2005). The present study reports the extraction of secondary compounds from *R. montagnei* using solvents Methanol, Ethanol, Ethyl Acetate and Acetone, subjected to test the antimicrobial activity using bacterial and fungal pathogens under laboratory conditions.

2. MATERIALS AND METHODS

Fig. 1: Roccella montagnei sample was crushed to powdered form

2.1 Lichen material

R. montagnei thalli were collected in the month of November on Ceriops in Gujrat, Jamnagar District, and Marine National Park of Cheneda Islands and were identified based on standard literature (Awasthi, 1988; Swinscow and Krog, 1988). The collected material was washed thoroughly with distilled water followed by tween 80 and made air dried. The dried material was weighed and made into powdered form.

2.1.1 Extraction of Lichen material: The powdered lichen (10gms) was wrapped in 8 x 6 cm cylindrical pouch made of Whatman filter paper grade 1 and kept inside the extractor arm of Soxhlet apparatus (Balaji, 2005). A series of solvents as Methanol, Ethanol, Ethyl acetate and Acetone was used for extraction based on their polarity and each extraction was carried out at the specific boiling temperature for a period of 48 hrs for the complete extraction of secondary compounds. The final filtrate of each of the extraction obtained was concentrated using Rotatory Evaporator or Rotavapour.

2.3 Culture media

Nutrient Agar (NA) and Potato Dextrose Agar (PDA) medium were used to culture pathogens and for bacterial and fungal susceptibility test (Balaji, 2005).

2.4 Microorganisms source

Total of Six bacterial cultures (Pseudomonas *aeruginosa, Agrobacterium tumefaciens, Escherichia coli, Streptococcus mutans, Staphylococcus aureus and Klebsiella pneumoniae*) and three fungal cultures of *Aspergillus niger, Candida albicans and Fusarium oxysporium* was used in this testing and screening process. All the cultures were obtained from the Pharmacological Laboratory, National Botanical Research Institute (NBRI), Lucknow. The cultures were maintained at 4 degrees Celsius and subcultured in solid and semisolid nutrient agar slants.

2.5 Determination of Antimicrobial Activity

Antimicrobial activity was tested using well-diffusion method (Bauer et al., 1966). The Nutrient Agar medium was transferred into one-fourth volume of petriplates for antibacterial activity. Potato Dextrose Agar medium was transferred into one-fourth volume of petriplates for antifungal activity. Inoculation of cultures (100 mg/ml) to this medium was carried out uniformly using glass spreader. Five wells were made in each petriplate. Different concentration of crude extracts of Methanol, Ethanol, Ethyl acetate and Acetone (i.e. 2.5%, 5%, 10%, 15% and 20%) were prepared as individual stock solutions by mixing Dimethyl Sulfoxide (DMSO) and Distilled Water. These stock solutions of different concentrations were filled in their respective wells along with DMSO as negative control and Streptomycin (in antibacterial testing) and Ketoconazole (in antifungal testing) as a positive control. The plates were labelled and incubated for 24 hrs at 37 degree Celsius in BOD.

3. RESULTS AND DISCUSSION

The inhibitory zones were recorded and measured with the help of Hi-Antibiotic Zone Scale. The results of antimicrobial activity of extracts are given in Table.1. Among the four different extracts, Acetonic extract exhibited growth inhibition on the bacterial organisms and Ethanolic extract exhibited growth inhibition on the fungal organisms whereas Methanolic and Acetonic extracts exhibited growth inhibition on seven organisms and no inhibition against *Agrobacterium tumefaciens* and *Klebsiella pneumonia.* There was a least inhibitory activity for Ethyl acetate extract against *Staphylococcus aureus, Escherichia coli* and *Fusarium oxysporium* only.

The Ethanolic extract inhibited the growth of all the organisms tested and especially exhibited 28-34 mm zones of inhibition against *Staphylococcus aureus, Streptococcus mutans, Escherichia coli, Candida albicans and Fusarium oxysporium*. The various concentrations (5-20%) of Ethanolic extracts exhibited more effective zone of inhibition compared to the antibiotic standard Streptomycin (25-28 mm) against *Staphylococcus aureus* and *Escherichia coli (*28-32 mm) also compared to the antifungal standard Ketoconazole (14-15 mm) against *Aspergillus niger, Candida albicans and Fusarium oxysporium* (25-28 mm).

The antimicrobial potential of Ethanolic extracts of *R.montagnei* is much more than the methanolic and acetonic extracts of *R.montagnei*. The thallus of *R.montagnei* is known to contain a class of compounds Depsides and Terpenes (Rundel,1978) while Acetone and Methanol extracts contain Terpenoids, Depsides, Polyols, Aromatic, Aliphatic and Cycloaliphatic compounds (Bombuwala,2000).

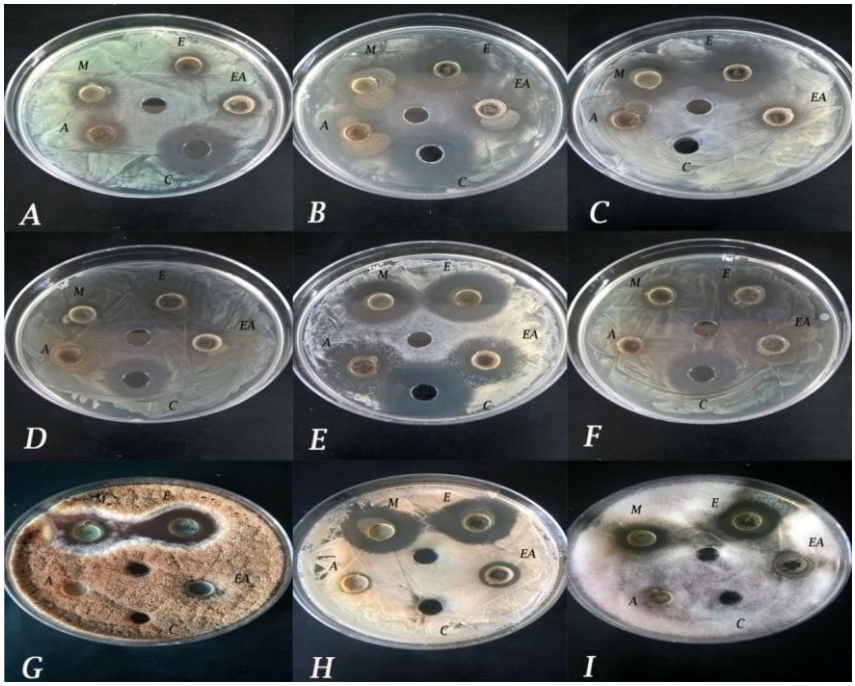


Fig. 2: Inhibition zones after Antimicrobial test activity where A. *Pseudomonas aeruginosa* B. *Staphylococcus aureus* C. *Streptococcus mutans* D. *Agrobacterium tumefaciens* E. *Escherichia coli* F. *Klebsiella pneumonia* G. *Aspergillus niger* H. *Candida albicans* and I. *Fusarium oxysporium*

In figure 2, M denotes Methanolic Extract, E denotes Ethanolic extract and A denotes Acetonic extract. C is the positive control used as Streptomycin for antibacterial and Ketoconazole for antifungal testing. In the middle, DMSO or DimethylSulphoxide has been used as a negative control in antibacterial as well as antifungal testing. The results of the antimicrobial activities of extracts are given in table 1.

Table 1: Inhibitory zones of extracts of lichen

S no.	Bacterial and Fungal Pathogens	Diameter Of Inhibition Zones (mm)				
			Solvent systems			
		Control (+)	Methanol	Ethanol	Acetone	Ethyl Acetate
1.	*Pseudomonas aeruginosa*	20 ± 1.4	16 ± 1.4	15 ± 0.7	18 ± 1.4	16 ± 0.7
2.	*Staphylococcus aureus*	25 ± 1.4	25 ± 0.7	28 ± 2.1	30 ± 1.4	25 ± 0.7
3.	*Streptococcus mutans*	12 ± 0.7	20 ± 0.0	28 ± 1.4	16 ± 0.7	15 ± 0.7
4.	*Agrobacterium tumefaciens*	16 ± 1.4	14 ± 0.7	15 ± 1.4	12 ± 2.1	14 ± 0.7
5.	*Escherichia coli*	28 ± 1.4	28 ± 0.7	30 ± 1.4	34 ± 1.4	26 ± 1.4
6.	*Klebsiella pneumonia*	18 ± 0.0	16 ± 0.0	18 ± 0.7	12 ± 0.7	14 ± 1.4
7.	*Candida albicans*	14 ± 0.7	28 ± 1.4	32 ± 2.1	16 ± 1.4	18 ± 0.7
8.	*Aspergillus niger*	14 ± 0.0	28 ± 1.4	30 ± 1.4	0.0 ± 0.7	0.0 ± 0.7
9.	*Fusarium oxysporium*	14 ± 0.7	25 ± 0.7	32 ± 1.4	14 ± 0.7	24 ± 1.4

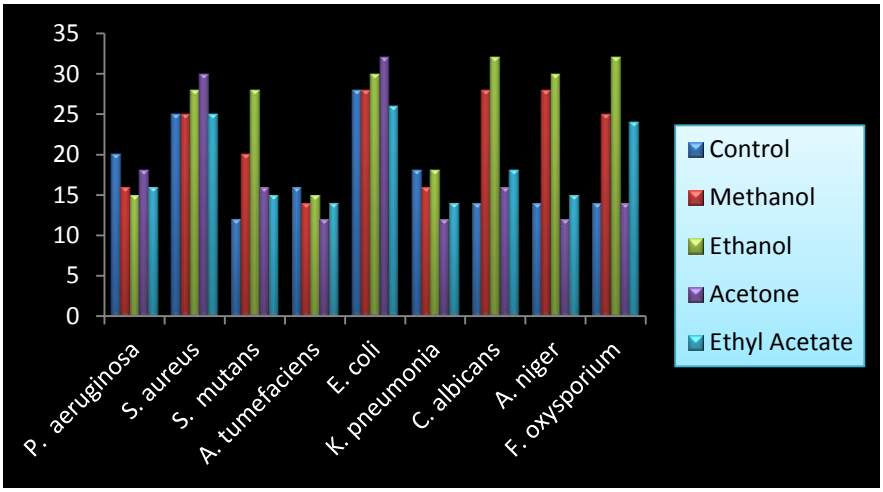
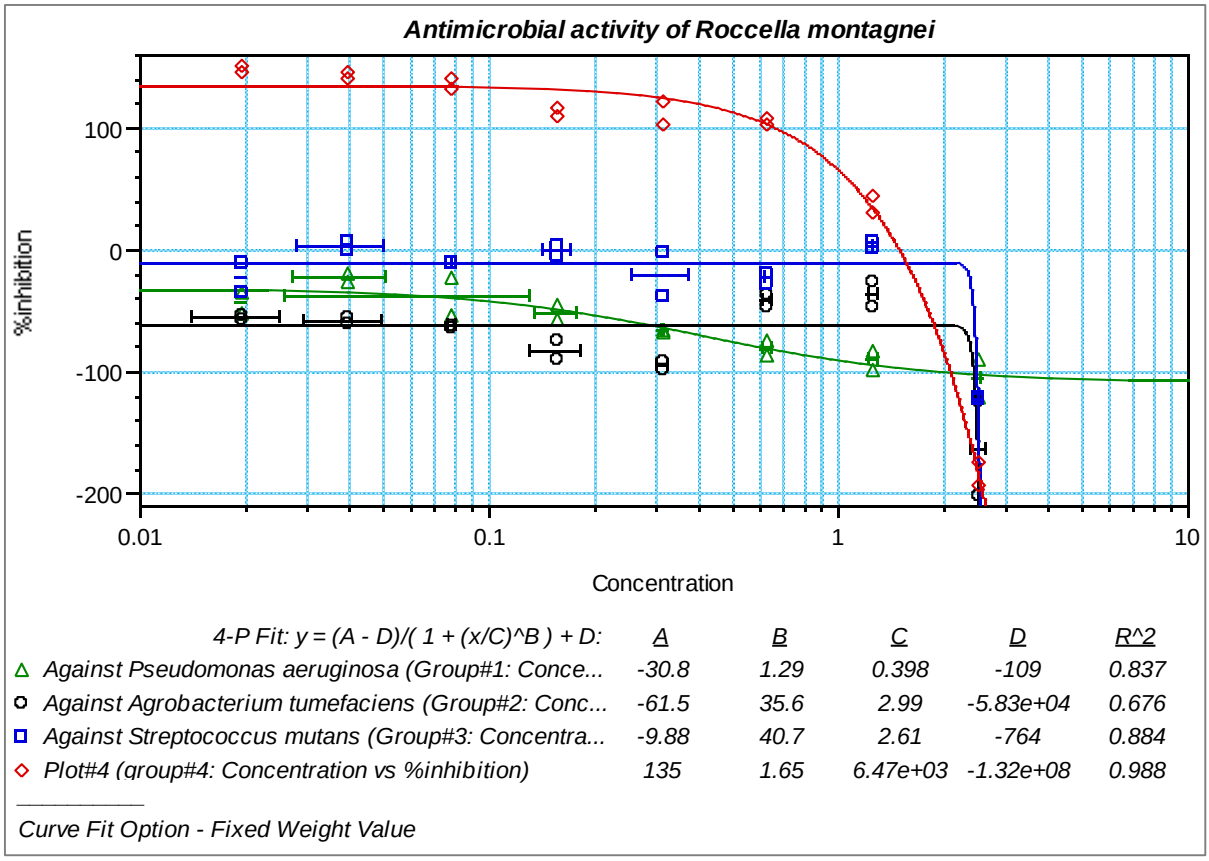


Fig. 3: Yield of concentration of different lichen extracts against 9 pathogens

Assayed microtitre plates incubated at 35 + 2 degree Celsius for 24 hours. After incubation, Optical density or OD was taken at 492 nm from Spectramax plus 384 spectro for growth inhibition and quantitative data, in the form of IC and MIC (mg/ml). All the results in the form of Standard deviation error calculated by Softmax Pro-5 software. Lichens studied for antibacterial against observation on the basis of antibacterial susceptibility assay of lichen belongs to family Roccellaceae with acids Roccellic acid,

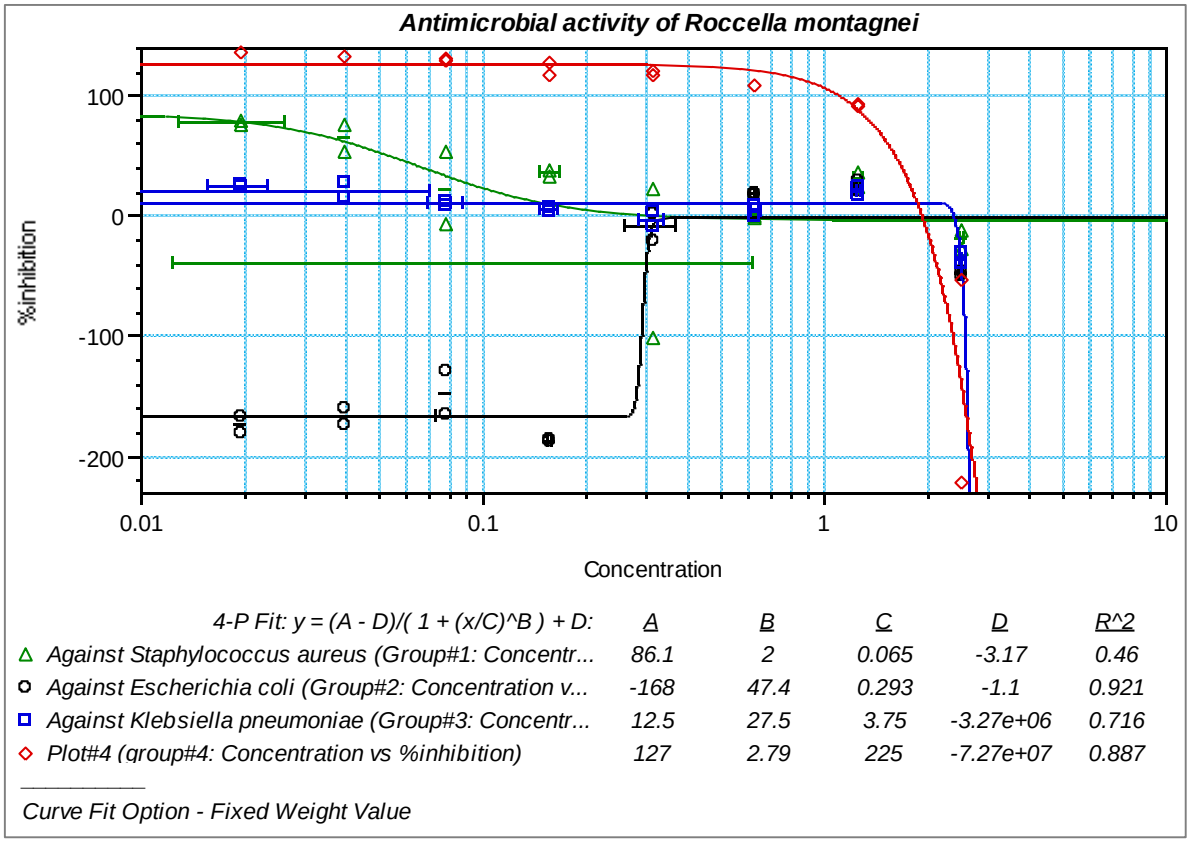
Lecanoric acid, Lepraric acid and Pulvinic acid as Lichen acids. Tested against Bacteria as well as fungi. Lichen was found to have activity only against *Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus mutans, Escherichia coli, Candida albicans, Fusarium oxysporium and Aspergillus niger* with MIC values percentage growth inhibition at various concentrations and graph for growth inhibitory activity as in figures 4, 5 and 6. Absorbance data depicts the colour of the drug can be a factor hindering spectrophotometer means of quantitative analysis.



4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:	A	B	C	D	R^2
△ Against Pseudomonas aeruginosa (Group#1: Conce...	-30.8	1.29	0.398	-109	0.837
○ Against Agrobacterium tumefaciens (Group#2: Conc...	-61.5	35.6	2.99	-5.83e+04	0.676
□ Against Streptococcus mutans (Group#3: Concentra...	-9.88	40.7	2.61	-764	0.884
◇ Plot#4 (group#4: Concentration vs %inhibition)	135	1.65	6.47e+03	-1.32e+08	0.988

Curve Fit Option - Fixed Weight Value

Fig. 4: MIC against bacterial pathogens



4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:	A	B	C	D	R^2
△ Against Staphylococcus aureus (Group#1: Concentr...	86.1	2	0.065	-3.17	0.46
○ Against Escherichia coli (Group#2: Concentration v...	-168	47.4	0.293	-1.1	0.921
□ Against Klebsiella pneumoniae (Group#3: Concentr...	12.5	27.5	3.75	-3.27e+06	0.716
◇ Plot#4 (group#4: Concentration vs %inhibition)	127	2.79	225	-7.27e+07	0.887

Curve Fit Option - Fixed Weight Value

Fig. 5: MIC against bacterial pathogens

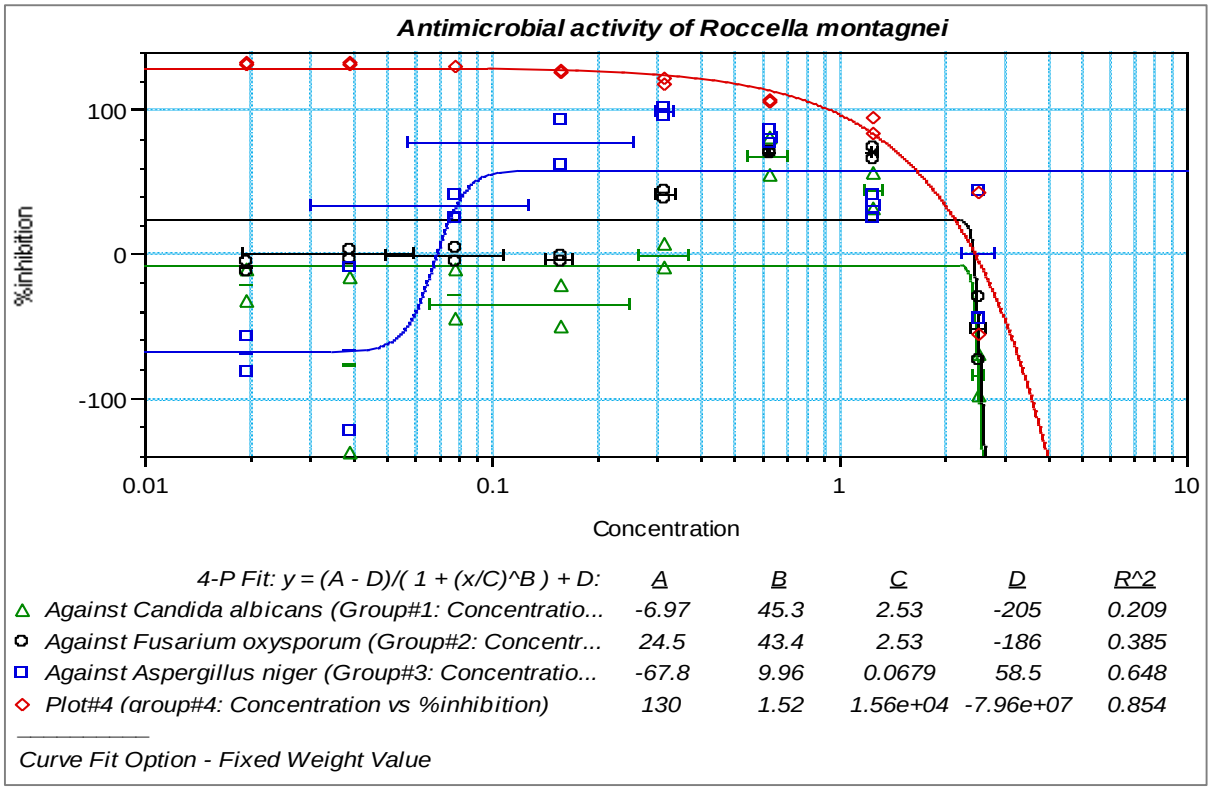


Fig. 6: MIC against fungal pathogens

4. CONCLUSIONS

The ethyl acetate extract (Standard Lichenological Procedure) showed minimum inhibitory effect or activity against the pathogens tested. However, The Methanolic and acetonic extracts showed significant antimicrobial activity while the Ethanolic extract showed maximum inhibitory zones against the fungal pathogens tested. Therefore, this study proves the antimicrobial potential of acetonic and ethanolic extracts of *R. montagnei* and in the discovery of the novel potential biomolecules from lichens, application of different solvents in combination with extraction procedures. Further processing and investigation into fractionation and purification of ethanolic extract may result in the isolation of viable alternate source to the presently available antibiotics. Lichens hold great potential that needs to be fully explored and utilized for the benefit of human health and our society. This will definitely provide a new base and ray of light for the future perspectives and highlight the need for further studies of this promising source to harvest more beneficial in the field of bioprospection. This work is intended to contribute to the current research and development trends in the bioprospection of lichens and their bioactive compounds in the applications of commercial interest as well.

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