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Evaluation of Antioxidants, Antidiabetic, Antiinflammatory Active Compounds from *Leptogium rivurale* Through *In-Vitro* and *In-Silico* Studies

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Abstract: *Leptogium rivurale* is a flooded jelly skin lichen as the surface becomes jelly on wet. It is a cyanolichen with a cyanobacterium *Nostoc*. In this present study, *Leptoguium rivurale* were collected from Kodaikanal and extracted using methanol and distilled water. Qualitative analysis of phytochemicals from the extracts showed the presence of carbohydrates, glycosides, phenols, terpenoids, saponins, and proteins. The methanol extract was found to inhibit the α-amylase enzyme activity better than aqueous extract. The methanol extract was found to have better DPPH radical scavenging activity and anti-inflammatory activity than aqueous extract. Then the extract was subjected to GC-MS analysis. The molecules obtained through GC-MS analysis were subjected to *in-silico* molecular docking simulation using AutoDock software. Cyclohexanol and oxirane were the potential drug candidates identified.

Keywords: *Leptogium rivurale*; antioxidants; antidiabetic; anti-inflammatory.

1. Introduction

Inflammation is a healing process wherein chronic inflammation causes rheumatoid arthritis, cancers, obesity, etc. [1]. There are remedies to combat inflammation once such are microbes-derived metabolites, vitally used in the medical field. They address problems from environmental clean-up to treating diseases. Microbes show enhanced activities when associated with other microbes of different community communities, including mycorrhiza, Azolla, and lichens [2].

Lichens are composite organisms that comprise algae or cyanobacteria living among the filaments (hyphae) of the fungi exhibiting symbiotic relationships [3]. The dominating partner fungus gives lichens most of its features. The alga can be either a green alga or a blue-green alga, otherwise known as cyanobacteria and most of the lichens have both types of algae[4]. Apart from having the ability to survive in a harsh environment, Lichens possess the capability to absorb everything in their atmosphere, especially pollutants, thereby acting as one

of the indicators of environmental condition. The total numbers of lichens species are 18,500 are reported in the world [5].

Lichens grow on rocks, plastics, tree bark, leaves, etc. Among lichens, *Leptogium* is a genus belonging to the family Collemataceae. It appears to be blue-grey to brown blackish with the jelly-like surface in wet conditions [6-10]. This is associated with a cyanobacterium belonging to the genus *Nostoc*. The secondary metabolites produced by lichens possess significant biological activities like antimicrobial, antioxidant, antiviral, antiproliferative, and anti-inflammatory activities [11-13]. This study aims to extract and identify bioactive compounds from Leptogium spp and evaluate the bioactivity of these compounds, especially anti-inflammatory activity by *in vitro* and *in silico* analysis.

2. Materials and Methods

2.1. Sample collection and extraction.

The sample was collected from Kodaikanal, India, in sterile polythene bags, thoroughly washed with tap water, and shade dried. 50 g of dried sample was taken in two conical flasks. 50 ml of methanol was added to one flask and another with 50 ml of distilled water. The soaked samples were placed in a shaker for three days. After 3 days, it was centrifuged at 10000 rpm for 10 min. The supernatant from the methanolic extract was taken and evaporated in a Petri plate in a fume hood, whereas water extract was subjected to lyophilization. Thus, prepared extracts were stored at 4 ^{0}C till further use.

2.2. Phytochemical screening and quantification.

Phytochemical examinations were carbohydrates, alkaloid, Glycosides, saponins, protein, etc. were performed following the [14]. Further, the lichens extract were subjected to quantify carbohydrates by Anthrone method, Protein content by Lowry's method [15], and phenol content by Folin-Ciocalteu assay [16].

2.3. Antioxidant activity by DPPH method.

The free radical scavenging activity of the ethanol extract was measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) [14].

2.4. Estimation of antidiabetic activity by α-amylase inhibition assay.

α-amylase inhibitory activity of methanol and aqueous extract was carried out according to the standard method [17].

2.5. Estimation of anti-inflammatory activity by egg albumin denaturation assay.

Egg albumin denaturation activity of methanol and aqueous extracts was carried out according to standard method with minor modifications [18]; the reaction was prepared by adding 0.5ml of egg albumin and 1.5ml of phosphate buffer saline (PBS, 0.02mol/L, pH 6.8). Various concentrations of sample (10, 20, 40, 60, 80, 100 µg/ml) of both extracts were added (as separate reaction) and incubated at 37 °C for 15 min. Then the reaction mixture was heated at 70 °C for 10 min. The absorbance at 660 nm was recorded by a spectrophotometer after the reaction mixture was cooled down. The inhibition of percentage was calculated [18].

2.6. Thin-layer chromatography.

Commercial TLC plates made with silica gel on aluminum foil were used [19-22]. Where 2:1 chloroform and methanol were used as mobile phase. Following separation, the plate dried; the spots were detected under UV light. Rf value was calculated.

2.7. GC-MS analysis.

The GC-MS analysis of the extract was carried out using a Triple quadrupole mass spectrometer with Fused silica 30mm of the length of the capillary column, diameter and film thickness is 0.25mm, where the condition was set as described previously [23].

2.8. Molecular docking.

Molecular docking is the *in-silico* technique used to determine the binding affinity between the optimum ligand and the target receptor, which is a protein the majority of times. The lichen *Leptogium rivurale* showed high ani-inflammatory activity through *in-vitro* studies. Hence, the docking is done with receptor target as an enzyme involved in the inflammation process in the human body, and the selected ligands are the molecules obtained in GC-MS analysis. The compound identified from the GC-MS analysis used in this study was nitric oxide synthase enzyme (iNOS) complexed with imidazole (PDB ID 3EJ8). AutoDock 4.6 tools were utilized, and docking simulation was done as previously reported [24].

3. Results and Discussion

Microbial products are highly potent active compounds since secondary metabolites have more potential than primary metabolites. Lichen metabolites are different than normal microbes because lichens are associated with fungi and algae. These metabolites are exhibiting excellent bioactive compounds in terms of antioxidants, anticancer, antimicrobial, antidiabetic and anti-inflammatory activities, etc. [25-27]. In this study, *Leptogium rivurale* metabolites were extracted by aqueous and methanol extractions. This study reveals that lichens have a specific phytochemical activity exhibited in a screening of phytochemicals (Table 1). Aqueous and methanol extracts showed carbohydrates, glycosides, phenol, terpenoids, steroids, tannin, alkaloids, and protein. Among them, steroids, tannins, and alkaloids were absent in both extracts. Rashmi *et al*. [28] suggested methanol is highly polar to dissolve most of the secondary metabolites of lichens. Few previous studies support this study that reported the presence of alkaloids, saponins, tannins, and terpenoids in the methanolic extracts of macro lichen *Ramalina conduplicans* [29].

The proximate composition of *Leptogium rivurale* was evaluated, and results are shown in Figure 1. Total carbohydrates, crude protein, and total phenol content from methanol and aqueous extracts. Carbohydrates are a function of cell energy and cell structural components. It is a major component of lichen species, including *P. tinctorum*, *Parmotrema pseudotinctorum,* etc. [30]. Carbohydrate and protein content was found to be a little high in aqueous extracts than methanol extract (Figure 1). Certain lichen species recorded high crude protein content [31]. In this present study, the highest level of total phenolic contents observed in the aqueous extract was 20.4%, whereas the methanol extract had 13.5% (Figure 1). A similar study was carried out by Aoussar *et al*. [32] with acetone extract of *P. furfuracea,* which

showed much antioxidant activity. Parizadeh and Garampalli [33] reported 2.96 mg/g of phenolic content in *Leptogium* sp.

Table 1. Qualitative analysis of phytochemicals from *Leptogium rivurale*.

S. No	Phytochemicals	Aqueous Extracts	Methanol Extracts
1	Carbohydrates	++	+++
2	Glycosides	++	+++
3	Phenol	+	++
4	Terpenoids	+	++
5	Saponins	++	+++
6	Steroids	-	-
7	Tannin	-	-
8	Alkaloids	-	-
9	Proteins	++	+++

+++ Faster reaction, + Slower reaction, - No reaction

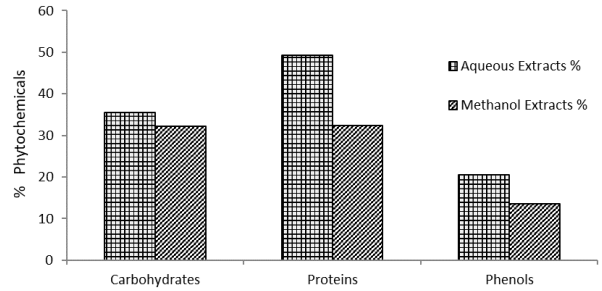

Figure 1. Quantitative estimation of Carbohydrates, Proteins, and Phenols from *Leptogium rivurale.*

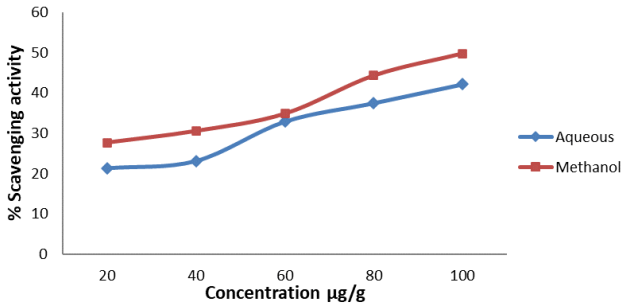

Figure 2. Estimation of antioxidant activity.

Nearly 65 antioxidants compounds were extracted from Lichens substances [26], where the antioxidant activity act through electron-donating substituents and their mutual positioning on the aromatic ring [34]. The scavenging DPPH radicals of the studied *L. rivurale* extracts are shown in Figure 2. Both the aqueous and methanol extracts of *L. rivurale* showed a good scavenging activity EC_{50} = 42.2 µg and EC_{50} = 49.8µg, respectively (Figure 2). Kosanić *et al*. [34] estimated *Lasallia pustulata* to have better antioxidant activity.

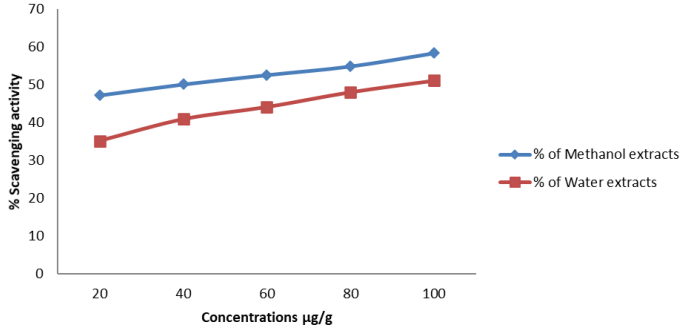

Figure 3. α-amylase inhibition activity of *L. rivurale.*

L. rivurale was showing antidiabetic activity through inhibition of α-amylase activity (Figure 3). α-amylase is an important enzyme involved in carbohydrate digestion. It hydrolyses the starch and is converted into glycogen and maltose, which exhibits increased blood sugar. Diverse lichens species have anti-diabetic potential activity [35].

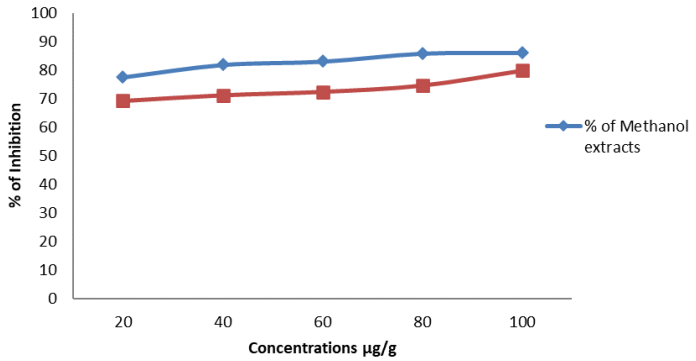

Figure 4. Inflammation inhibition activity of *L. rivurale.*

Methanol extract of *L. rivurale* showed maximum inflammation inhibition activity at 86.01%, while it was minimum in the aqueous extract (79.8 %) (Figure 4).

Thin-layer chromatography (TLC) is a good choice to identify the components [36]. Methanol extracts showed three bands with Rf values 0.95, 0.75, and 0.62, and aqueous extract showed Rf values were 0.69, 0.59, and 0.45 (Table 2). Different bioactive compounds were determined by GC-MS (Figure 5 and Table 3).

Table 2. Thin Layer Chromatography.

S.no	Extracts	Rf value	Rf value	Rf value
1	Methanol extract	0.95	0.75	0.62
2	Aqueous extract	0.69	0.59	0.45

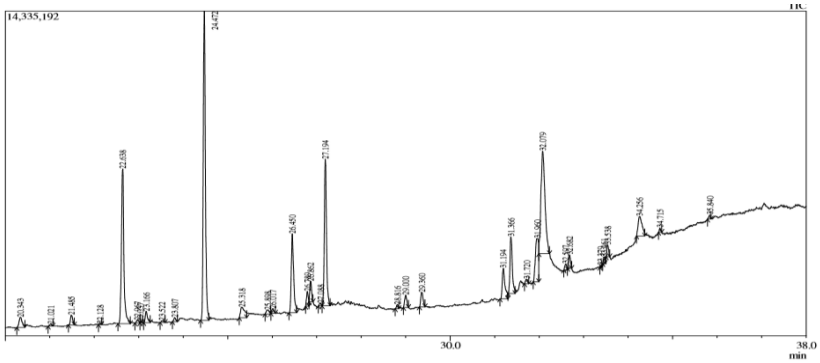

Figure 5. GC-MS analysis of extract.

Table 3. Compounds present in the extract and their bioactivity

S.no	Compounds	Bioactivity
1	Neophytadiene	Anti-inflammatory, analgesic, and antipyretic activities [37]
2	Hexadecanoic acid, methyl ester	Antibacterial and antifungal activities [38]
3	Octadecanoic acid	Antimicrobial [39]
4	Glycidyl palmitate	Preparation of isophosphatidic acid which inhibits apoptosis [40]
5	2-methyloctacosane	Antifungal activity [41]
6	Cyclohexanol	Antimicrobial and cytotoxic activities [42]

S.no	Compounds	Bioactivity
7	Oxirane	Anti-bacterial activity [43].
8	3,7,11,15-Tetramethyl-2-hexadecen-1-oL	Antimicrobial, anticancer, anti-inflammatory, anti-diuretic activities [44].

L. rivurale extract's GCMS report showed antimicrobial activity of essential plant oil containing neophytadine [45] (Table 3). Neophytadiene is also reported to possess antibacterial activity as well as helping in the treatment of headaches, rheumatism, and some skin disease [46]. Glycidyl palmitate is a fatty acid with the molecular formula $C_{19}H_{3}6O_{3}$ is essential in the preparation of lysophosphatidic acids, which inhibit apoptosis [40,47].

3.1. Molecular docking.

To explore the possible mechanism of NO inhibition, a molecular docking approach was executed. The lower the relative binding energy, the more potent the binding affinity between 3JE8 and target molecules. The binding energy of Octadecanoic acid, 2-methyloctacosane, and Neophytadiene with the protein were –8.48, 7.62, and –6.37 kcal/mol, respectively (Table 4). The remaining 6 compounds, such as cyclopropanetetradecanoic acid, hexadecenoic acid methyl ester, glycidyl palmitate, oxacycloheptadec-8-en-2-one, cyclohexanol, and oxirane, showed the binding energy in the range of 4.52 to 5.89 kcal/mol.

Table 4. Molecular docking analysis.

Compound	Receptor	Dock score (k/cal)	Number of hydrogen bonds	Bond length(Å)	Interacting residues
Octadecanoic acid	3EJ8	-8.48	1 TRP 372	3.5 (C-H-O)	ARG 381,TYR 489,TRP 194,PHE 369,ILE 433
2-methyloctacosane		–7.62	2 PRO 350 ALA 439	2.5(N-H-O) 3.6(C-H-O)	ARG 381,TYR 489,TRP 194,PHE 369,ILE 433
Neophytadiene		–6.37	0	0	ARG 381,TYR 489,TRP 194,PHE 369,ILE 433
Cyclohexanol		-4.52	2 TRP 372 ILE240	2.1 (C-H-O) 2.3(N-H-O)	ARG 381,TYR 489,TRP 194,PHE 369,ILE 433
Oxirane		–5.28	1 VAL 304	2.9 (N-H-O) 3.4(C-H-O)	ARG 381,TYR 489,TRP 194,PHE 369,ILE 433
3,7,11,15-Tetramethyl-2-hexadecen-1-ol		–4.65	2 ASP 303 TRP 483	3.2 (N-H-O) 2.9(C-H-O)	ARG 381,TYR 489,TRP 194,PHE 369,ILE 433
Glycidyl palmitate		-5.56	TRP 372	3.3 (N-H-O)	ARG 381,TYR 489,TRP 194,PHE 369,ILE 433
Hexadecanoic acid, methyl ester		-4.93	0	0	ARG 381,TYR 489,TRP 194,PHE 369,ILE 433
Cyclopropanetetradecanoic acid		-5.76	0	0	ARG 381,TYR 489,TRP 194,PHE 369,ILE 433

4. Conclusions

In this study, various α-amylase enzyme inhibition assays ranged from 47.2 % to 58.4 % for methanol extract and 35.12 % to 51.1 % for aqueous extract. The DPPH radical scavenging activity ranged from 27.6% to 49.8% for methanol extract and from 21.3% to 42.2% for aqueous extract. The anti-inflammatory activity ranged from 40.5% to 86.2% and 49.4% to 79.2% for methanol extract and aqueous extract respectively. Cyclohexanol and oxirane were found to be potent drug candidates through molecular docking.

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Conflicts of Interest

The authors declare no conflict of interest.

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