Research Article

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Antioxidant activities, cytotoxicity activity against A549 cell lines and HPTLC fingerprinting of *Pseudodrynaria coronans* (Wall. Ex Mett.) Ching rhizome

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ABSTRACT

Phytochemicals obtained from the plants include the secondary metabolite synthesized by the plants for its own utilization. Exploration of plants phytoconstituents for the prevention and treatment of various disease leads to the discovery of new drug entity. Utilization of proper authentication of the plant phytoconstituents. In this study, methanolic extract of rhizome of *Pseudodrynaria coronans* (Wall. Ex Mett.) Ching was studied for its antioxidant and cytotoxicity studies against A549 cell lines. HPTLC fingerprint analysis of the methanolic extract was performed using the developed solvent of methanol: chloroform: formic acid (10:0.5:0.5 v/v/v). DPPH free radicals scavenging activity and reducing power assay showed that the extract contains a remarkable amount of antioxidant activity possessing IC50 values of 286.7 and 181.36 µg/mL respectively. IC50 value of 88.64 µg/mL for MTT assay also revealed that the plants also contain significant amount of cytotoxicity action on A549 cell lines, which needs to be explored further. The development of the HPTLC fingerprint indicated the presence of different classes of phytochemicals, which are steroids, flavonoids, tannins, saponins, triterpenoids, amino acids, and carbohydrates; the absence of alkaloids, glycosides, reducing sugar, fats and fixed oils, and provided a quality control tool for the authentication of plant material.

Keywords: Phytochemicals, Antioxidant, Cytotoxicity, HPTLC, A549.

1. Introduction

Although it is evident that plants are an effective source of food and shelter, their potential as a source of medicine is often overlooked. Plants have been used by human civilization for almost the same amount of time as a source of food, shelter, and medicine[1]. For treating cancer and other harmful diseases, about 20% plant based were studied in pharmaceutical fields. Different types of bioactive phytochemicals are obtained from plant sources. Various phytochemicals present in the fruits and vegetables are used to protect our body from free radicals[2]. Fruits, vegetables and grains have good protective functions against different chronic diseases. This protective role of phytochemicals helps in treating different diseases. These phytochemicals are defined as bioactive non-nutrient component present in vegetables, fruits, grains, and other plants. Tannins, flavonoids, steroids, triterpenoids, alkaloids, and saponins are examples of phytochemicals that have been identified[3]. Medicinal plants are significant because of their ability to heal and cure human diseases and phytochemicals in plants attribute to this healing ability. Phytochemicals are bioactive naturally occurring chemicals that protect plants from disease and external damage and provides color, aroma, and flavor to the plants. Alkaloids, flavonoids, tannins, glycosides, saponins, phenolics, and terpenoids are examples of plant phytochemicals. Phenolic compounds are phytoconstituents with one or more aromatic rings and attached with at least one hydroxyl group showing antioxidant properties[4].

In recent years, food industry shown interest in the use of plant extracts, primarily due to their antioxidant property along with health-promoting effects for humans. These extract properties are linked to have vitamins, minerals, and various phenolic components in plants[5]. Diabetes, cancer, cardiovascular, inflammatory, and neurodegenerative diseases are all caused by oxidative stress. The condition is caused by an excess of free oxygen and nitrogen species or their inefficiency scavenging in the cell. Unstable free oxygen and nitrogen species are atoms that are prevalent in the external environment (exogenous) and are also synthesized in the body (endogenous) during normal aerobic biotranformation[6].

Fingerprinting of plants extracts helps in quick identification of the plant species, although differentiation of each phytochemical is necessary. Therefore, even regulatory bodies frequently advise fingerprinting as the foundation for accurately identifying herbal products, for instance. Planar chromatography is typically capable of completing this task; the most popular method is high performance thin layer chromatography HPTLC[7]. HPTLC profiling is a straightforward, quick, and inexpensive method for determining the authenticity of natural extracts based on a set of distinctive chromatographic signals that can be compared to identify the sample[8]. High Performance Thin Layer Chromatography (HPTLC) has been identified as an efficient and suitable analytical tool due to its high flexibility, versatility, accuracy, and reproducibility, as well as the ability to obtain a faster fingerprint of multiple compounds in a single chromatographic run. Indeed, the majority of HPTLC literature reports focus on comparative discrimination based on fingerprint of secondary metabolites, which is unique to each plant species[9]. Thus, "fingerprint analysis" utilizing high-performance thin-layer chromatography (HPTLC) could be a useful parameter for quality control of plant extracts and a different approach, especially when analyzing crude plant extracts[10]antioxidant, and antigout activity of Asparagus racemosus, Withania somnifera, Vitex negundo, Plumbago zeylanica, Butea monosperma and Tephrosia purpurea extracts were investigated. The chemical fingerprinting were carried out by high performance thin layer chromatography (HPTLC).

P. coronans is found primarily in Asia and southern China. In China, it has been used as a folk medicinal plant to treat traumatic injury, toothache, rheumatoid arthritis and tinnitus. It has been reported to have antioxidant activity and polyphenol content[11]. *P. coranans* is known as "Awmvel" among the Mizo. This Polypodiaceae-family epiphytic basket fern has a white rhizome that resembles a paw encircling the host plant[12]. Whole plant extract traditionally used for back pain, scabies and Herpes simplex virus infections[13]. This study was performed to explore *P. coronans* plant for its cytotoxicity activity against lung cancer cell lines along with its potential antioxidant activity. HPTLC fingerprint of the plant extract serves as a quality control standard for the identification of the plant.

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2. Materials and Methods

2.1. Collection and authentication of the plant materials

Rhizomes of the *P. coranans* was collected from in and around from Aizawl town, Mizoram, India. The plant parts were authenticated from Botanical Survey of India (BSI), Eastern Regional Centre, Shillong and voucher specimen (no. BSI/ERC/TECH/2018-19/688) was submitted to specimen library at RI-PANS for future reference. All the solvents and chemicals were of analytical grades and purchased from sigma aldrich.

2.2. Preparation of extract

Powdered rhizomes of the plant were sequentially extracted with petroleum ether, chloroform and methanol using soxhlet extractor for about 72 hours or till the extractive becomes colorless. Solvents were evaporated using rotary vacuum evaporator under negative pressure at slightly above the solvent boiling point until the semisolid mass of the extracts were obtained. All the extracts were dried and weighed. Methanol extract was selected for the activity because it contains all the secondary metabolites in it.

Percentage yield of extracts was calculated using the following formula:

$$\frac{\text{Total amount of extract obtained}}{\text{Amount of powdered plant taken}} \text{ X100}$$

2.3. Phytochemical screening

Phytochemical screening of methanol extracts were performed for various phytochemicals like alkaloids, glycosides, carbohydrates, tannins, saponins, steroids, flavonoids, reducing sugar, triterpenoids, amino acids, fats and fixed oils. [14-16].

Figure 1. P. coronans with rhizome

2.4. Antioxidant activity

In this study, the antioxidant activity of the methanolic extracts of *P. coronans* (PCM) were performed using 2,3-Diphenyl-1-picrylhydrrazyl (DPPH) free radical scavenging assay and reducing power assay. Butylated hydroxyanisole(BHA) and ascorbic acid were used as reference standard for antioxidant.

2.5. DPPH-Free radical scavenging assay

DPPH free radical scavenging assay method is widely used for the estimation of antioxidant activities of plant phytoconstituents. Antioxidants activity of methanol extract of *P. coronans* and standard antioxidant were determined using method illustrated by Kim *et al.*(2017) with slight modifications. 2.7 mL of DPPH solution (0.15mM) in methanol was mixed with 0.3 mL of the different aliquots of concentrations (20, 40, 60, 80, 100 µg/mL) of extracts and 3 mL of standard Butylated hydroxyanisole (BHA) solution were prepared with different aliquots of concentrations similar to extracts. Incubation of both standard and extract were performed at 20°C for 30 minutes under dark condition. Absorbance was calculated at 517 nm for extracts, standard and control using Ultraviolet-visible (UV) spectrophotometer (Thermo Fisher Scientific). All measurements were carried three time. Free radical scavenging capacity of DPPH was calculated using the following equation:[17]

$$\%\text{DPPH radical scavenging} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \text{ X 100}$$

Where $Abs_{control}$ stands for absorbance of control and Abs_{sample} signifies absorbance for extract or standard[17].

2.6. Reducing Power assay

Reducing power of methanol extracts of *P. coronans* was estimated using method described by Oyaizu (1986) and Yen and Chen (1995) using ascorbic acid as reference standard. Each 1 mL of extract and standard with different aliquots of concentrations (20, 40, 60, 80 and 100 µg/mL) were mixed with 2.5 mL of potassium ferricyanide (1%) and 2.5 mL of phosphate buffer (0.2 M, pH 6.6). Mixture was incubated at 50°C for 30 minutes and 2.5 mL of trichloroacetic acid (10%) was added to the mixture to cease reaction. The mixture was centrifuged for at

3000 rpm for 10 minutes. 2.5 mL of distilled water was taken and mixed with 2.5 mL of supernatant and 0.5 mL of 0.1% ferric chloride solution. Absorbance of the resulting solution was observed at 700 nm. All determinants were measured for three times[18,19].

2.7. Cytotoxicity assay

MTT Assay

MTT dissolves in water (10 mg/mL), ethanol (20 mg/mL), culture media (5 mg/mL), and buffered salt solutions. However, it is recommended to use a PBS solution containing 5 mg/mL of MTT. After adding MTT, sonicate or vortex the content, filter it, and sterilize the solution. Cell viability study was determined by the capability of tetrazolium salt MTT [3-(4,5- dimethylthiazol2-yl)-2,5- diphenyl tetrazolium bromide] (Sigma, USA) using mitochondrial succinate dehydrogenase enzyme. 1 mg/mL concentration of stock solution of extract was prepared in dimethyl sulphoxide (DMSO). Different aliquots of concentrations (5, 10, 20, 40, 80, and 100 µg/mL) of extract were prepared using the stock solution. The 96-well microtiter plate (Tarsons India Pvt. Ltd., India) was seeded with A549 cells (NCCS, Pune) and allowed to multiply for 24 hours. Different concentrations of extract were applied in the 96-well microtiter containing cells and incubated (37°C) in a CO_2 environment for 48 hours. Doxorubicin (DOX) was used as a positive control and was applied at different concentrations of 1, 2, 4, and 8 µg/mL. After 48 hours, the supernatant was removed and 20 µL of MTT (5mg/mL in DMEM) was added and incubated for 4 hours again. Each well received 100 µL of DMSO. The plate was put on a plate shaker and gently agitated for 15 minutes to dissolve formazan crystals produced by growing cells. The absorbance was determined by utilizing a Spectramax M2 Microplate Reader (Molecular Diagnostic, Inc.) at 570 nm. Relative viability was determined using wells with non-treated cells as a 100% control, and the standard deviation of the percentage cytotoxicity was calculated. The 50% cytotoxic concentration of the extracts and a positive control were measured and compared[20].

2.8. HPTLC fingerprint analysis

Methanol extract of *P. coronans* was selected for HPTLC fingerprint profile. Extract was redissolved in chromatographic grade methanol. A number solvent systems were tried and solvent system chloroform: methanol: formic acid(10:0.5:0.5 v/v/v) showing satisfactory resolution under TLC visualizer was selected for HPTLC fingerprinting. Bands(10mm) were applied with different volume of extracts (10, 12, 15, 20 and 25 µL) on a pre-coated silica gel 60 F254 glass plate (20x10 cm) using Hamilton syringe (100 µL) with Linomat 5 applicator connected to CAMAG HPTLC system equipped with WINCATS software. The plate was run in Twin trough glass chamber (20x10 cm) previously saturated with solvent system for 30 minutes till 80% migration of solvent front. The plates were air dried and recorded with digital documentation system under UV light at 254 nm (slit dimension: 5x0.45 mm, scanning speed: 20 mm/s). The chromatogram was scanned for densitometric evaluation of HPTLC chromatograms. The fingerprint data and Rf values were recorded by WINCATS software[21].

3. Results and Discussions:

3.1. Yield of extract

Percent extractive yield and appearance of the plant extract were shown in the table 1. Extract was appeared to be sticky brown in color has a characteristic odor.

3.2 Phytochemical screening

Preliminary phytochemicals screening of the extracts shown the presence of various phytochemicals. Phytochemical screening of the extract revealed the presence of steroids, flavonoid, reducing sugar, tannins, saponins, triterpenoids, amino acids and carbohydrates (Table. 1).

3.3. DPPH free radical scavenging activity

DPPH free radical scavenging capacity of the extracts were determined against the standard butylated hydroxyanisole (BHA) with different concentration. The IC_{50} value of the standard and extracts were found to be 65.98 µg/mL and 286.7 µg/mL respectively (Figure. 2). DPPH free radical test depends on neutralization of DPPH, which is a stable radical. DPPH scavenging shows that the plant have mild antioxidant activity[22].

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Table 1. Percentage yield and appearance of extract

Sl. No	Type of extract	Appearance	Color	Extractive yield (%w/w)
1	Methanolic	Sticky	Brown	15.79

Table 2. Phytochemical Screening of the extracts

Sl. No.	Phytochemicals	Present/Absent
1	Alkaloids	Absent
2	Steroids	Present
3	Flavonoid	Present
4	Reducing Sugar	Present
5	Tannins	Present
6	Saponins	Present
7	Triterpenoids	Present
8	Amino acids	Present
9	Fats and fixed oils	Absent
10	Carbohydrates	Present
11	Glycosides	Absent

3.4 Reducing power activity

Reducing power assay of the extracts were determined and compared against standard ascorbic acid. The IC_{50} values of the standard and extract was found to be 109.55 and 181.36 µg/mL. The comparison graph of reducing power assay of standard and extracts were shown in Figure. 3. The results showed that extract contains some amount of reducing power which may attribute to the antioxidant activity of the extracts. The reducing assay methods also provides an estimation of antioxidant capacity of plants extracts. Reducing power increases with increase in the absorbance for both standard and extract[23] (Figure. 3).

3.5. In vitro cytotoxicity activity

Cytotoxicity studies of the extracts were studied using MTT assay for A549 lung cancer cell lines against standard Doxorubicin (Dox). The percentage cytotoxicity of the extract and standard were compared and shown in Figure. 4.

The plant extract showed significance amount of cytotoxicity. The percentage cytotoxicity and IC_{50} value of the standard and extract were found to be 3.73 and 88.64 Concentration (µg/mL) respectively. The plant phytochemicals attribute to the possible cytotoxicity in the plant. The presence of various phytochemicals like flavonoids and tannins also indicates the possible cytotoxic nature of the plant[24].

3.6. HPTLC fingerprinting analysis

HPTLC fingerprint analysis of the extract were performed by optimizing a suitable mobile phase. The mobile phase selected was chloroform: methanol: formic acid in the ratio of 10: 0.5: 0.5 v/v/v for the analysis.

HPTLC fingerprint analysis showed 12 peaks in the peak diagram (Figure 5.). R_f values and the peak area for each peak were shown in the peak table (Table 3.). Three-dimensional (3D) representation of the chromatogram was shown in the figure (Figure 6.).

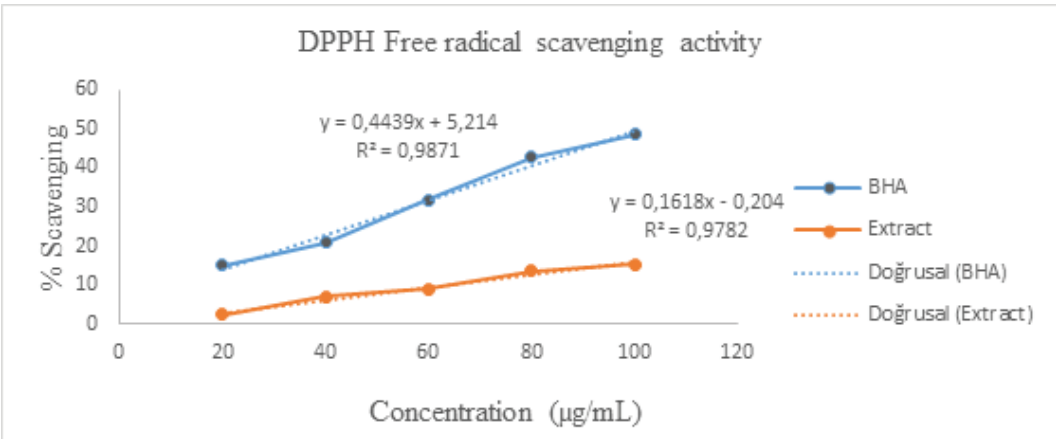

Figure 2. DPPH free radical scavenging activity of extract against Standard Butylated hydroxyanisole (BHA)

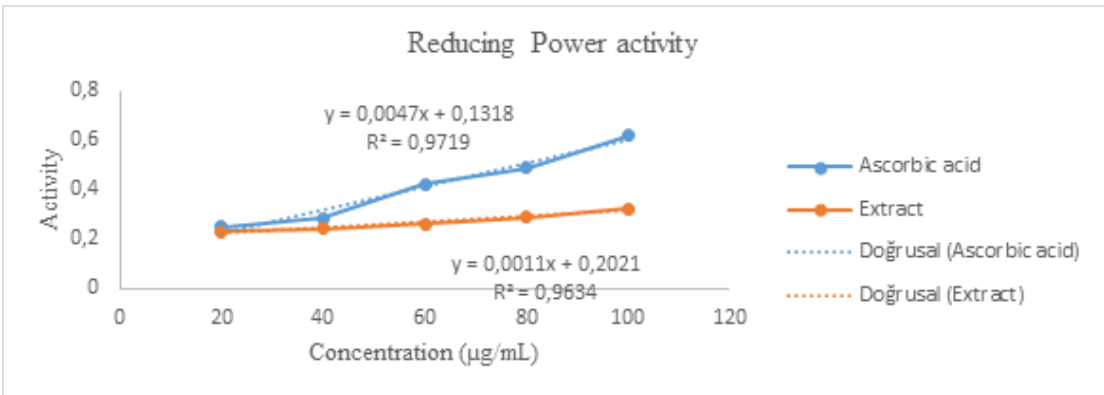

Figure 3. Reducing power activity of extract against standard Ascorbic acid

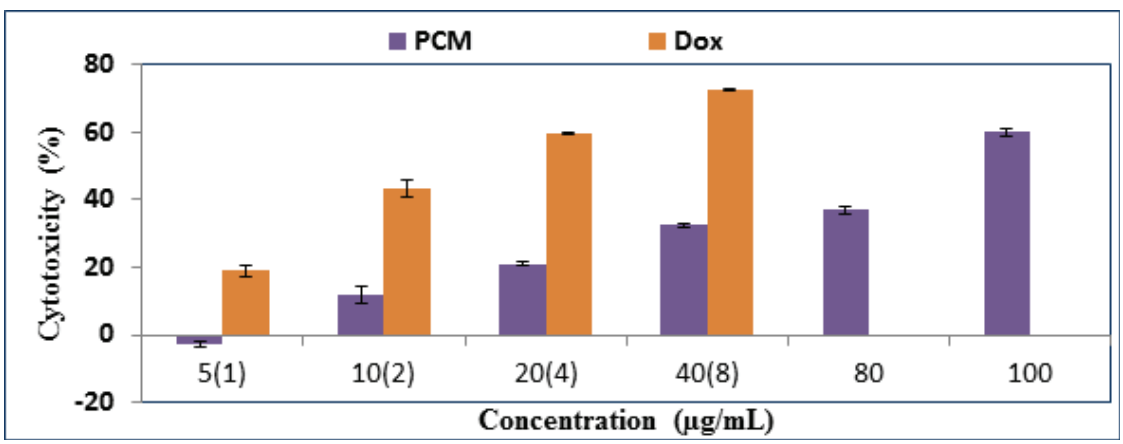

Figure 4. Percentage cytotoxicity activity of methanolic extract of *P. coronans (*PCM) against A549 cell lines. *Parenthesis () indicates concentration of doxorubicin(Dox).*

The presence of different peaks in the chromatogram signifies the presence of various types of phytochemicals in the extracts[25] HPTLC fingerprint analysis is an important plant analysis tool used for the rapid separation and identification of the plant phytoconstituents. The chromatogram obtained in this analy-

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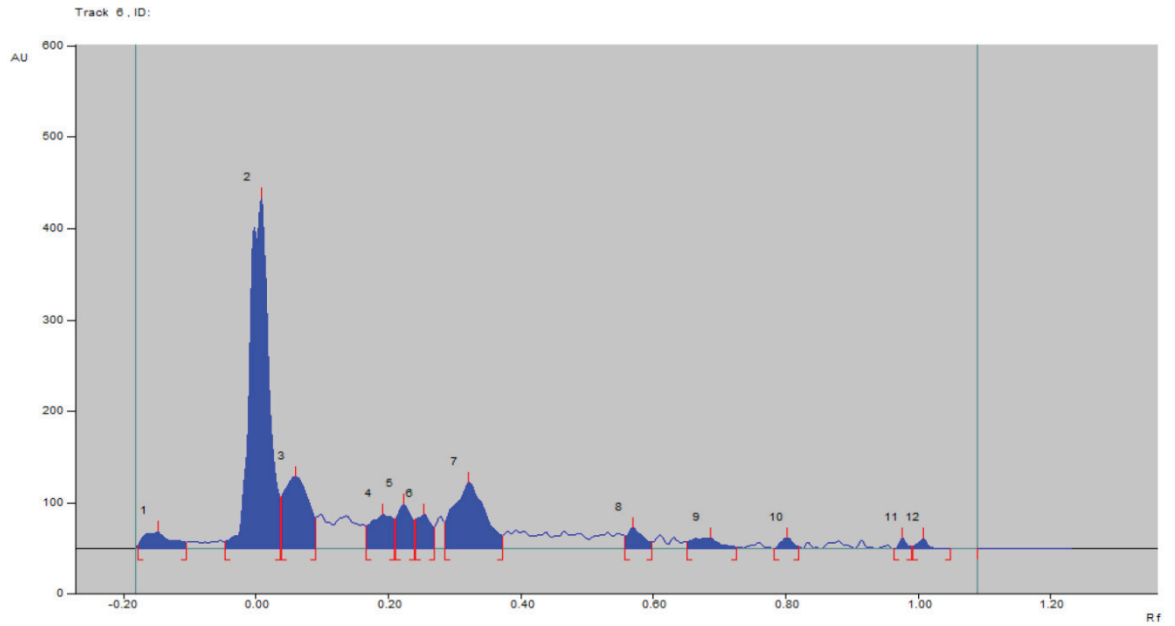

Figure 5. Peak diagram for methanolic extract of *P. coronans*

Table 3. Peak table for methanolic extract of P. coronans

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.18	3.6	-0.15	18.0	2.43	-0.11	6.8	468.1	3.34	unknown *
2	-0.05	7.8	0.01	383.6	51.79	0.04	56.1	6666.4	47.53	unknown *
3	0.04	56.5	0.06	78.2	10.56	0.09	33.7	1878.6	13.39	unknown *
4	0.17	24.6	0.19	37.1	5.01	0.21	32.3	776.5	5.54	unknown *
5	0.21	32.6	0.22	48.1	6.49	0.24	31.1	672.9	4.80	unknown *
6	0.24	31.8	0.25	36.7	4.96	0.27	23.6	505.2	3.60	unknown *
7	0.29	28.4	0.32	71.5	9.65	0.37	14.1	2113.5	15.07	unknown *
8	0.56	14.0	0.57	22.7	3.07	0.60	7.1	335.1	2.39	unknown *
9	0.65	7.5	0.69	11.5	1.56	0.73	1.4	311.2	2.22	unknown *
10	0.78	0.1	0.80	11.5	1.55	0.82	2.0	124.4	0.89	unknown *
11	0.96	0.0	0.98	11.0	1.48	0.99	1.9	71.7	0.51	unknown *
12	0.99	2.0	1.01	10.6	1.43	1.05	0.0	101.5	0.72	unknown *

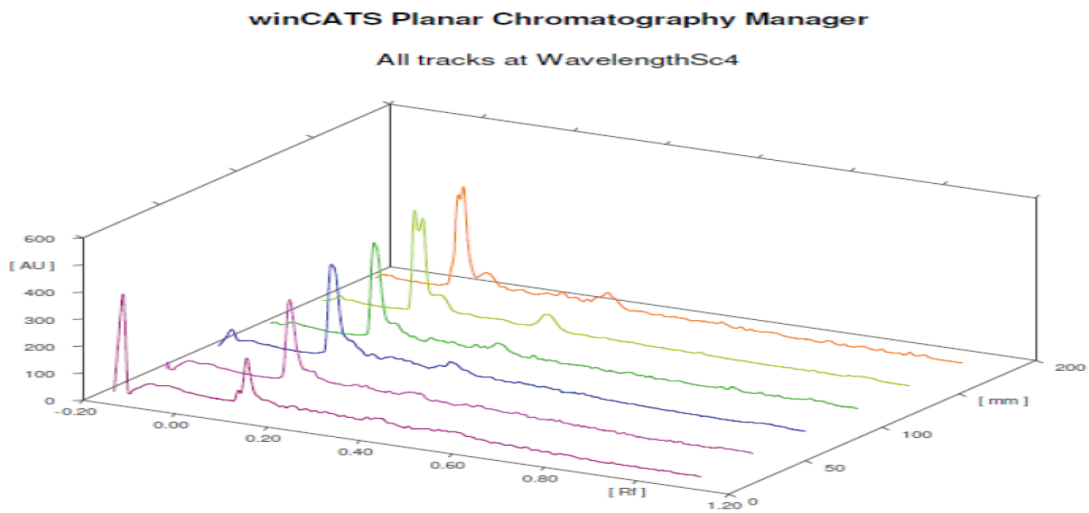

Figure 6. Three-dimensional (3D) representation of HPTLC chromatogram for methanolic extract of *P. coronans.*

sis is unique for the developed mobile phase system and this can be used as a quality control parameter for the selected plant[26]. HPTLC fingerprint analysis of the plants is helpful in standardization of plant extract. This analysis also useful for the elimination of possible adulteration of the plant material due to its flexibility and cost effectiveness[27].

4. Conclusion

The selected plant extract exhibited significant antioxidant activity against the standard drugs. IC_{50} values for DPPH free radical scavenging activity was found to be 65.98 and 286.7 µg/mL respectively for butylated hydroxyanisole(standard) and plant extract respectively, which suggest that the plant carries antioxidant activity. IC_{50} values of 109.55 and 181.36 µg/mL for ascorbic acid(standard) and plant extract indicate the presence of some antioxidant activities. The cytotoxicity study against A549 cell lines revealed that the plant contains cytotoxicity properties. The IC_{50} values of 3.73 and 88.64 µg/mL for the standard doxorubicin and plant extract justified the presence of cytotoxicity properties of the plant, thus it can be further studies for its cytotoxic properties. HPTLC fingerprint analysis of the plant extract showed the presence of various phytochemicals and it also developed a quality control parameter for plant authentication, which can be further utilized in future for the reference.

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Conflict of Interest

There is no conflict of interest among the authors.

Statement of Contribution of Researchers

Concept, Experimentation and Manuscript writing-MUM; Manuscript writing and suggestions-TL; Manuscript writing-AT; Manuscript writing-BJS; Manuscript writing: IH; Manuscript writing and revision-MM.

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