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Extraction of Phytochemicals from *Eucalyptus Spp.* & *Withania Somnifera* and Their Biological Testing

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Abstract Disease incidence and prevalence is increasing in developing countries leading to high mortality and morbidity rates. Since most of the developing countries rely on traditional plant based medicine, there is huge demand for identifying the bioactive compounds from plant origin with medicinal properties owing to their safety, availability and reduced side effects. This project focuses on extraction of two different phytochemicals from *Eucalyptus Spp* and *Withania Somnifera*. The present study narrows down on extraction of sideroxylonals from *Eucalyptus Spp* and withaferin A from *Withania Somnifera*. Formulated phloroglucinol compounds (FPC) of *Eucalyptus Spp* includes Sideroxylonal which possess strong antimicrobial, antioxidant and anticancer properties. Withaferin A belonging to withanoloides of *Withania Somnifera* has potential antimicrobial, antioxidant, anti-inflammatory and anticancer properties. Both the bioactive compounds are extracted from residual foliage from *Eucalyptus Spp* and *Withania Somnifera*. The extraction is optimized by using different solvents or combination of solvents in various proportions to increase yield. The extracted compound is studied for its antimicrobial and antioxidant activity and characterized using FTIR analysis, TLC and HPLC analysis. Antioxidant assay performed by DPPH method and phosphomolybdenum method showed similar IC50 values for both extracts. Antimicrobial assay performed using agar disc diffusion method showed potential antimicrobial activity in both the extracts.

Keywords: *phytochemicals, Formulated phloroglucinol compounds, sideroxylonal, withaferin A, FTIR, HPLC, TLC*

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1. Introduction

The mortality and morbidity rate in developing countries are estimated to grow to 8.9 million by 2030. The main reasons which contribute to the rise of diseases in developing world include population ageing, rapid unplanned urbanization, and globalization of unhealthy lifestyles. Since developing countries rely on traditional plant based medicine for their primary health care, there is an increasing interest dedicated towards identifying safe and cheap bioactive compounds from plant origin that might help in prevention and treatment of different types of diseases(including therapy and prophylaxis) [1]

1.1. Therapeutic Properties of *Eucalyptus Spp*

Eucalyptus is a large genus of evergreen aromatic trees, particularly cultivated in sub-tropical and warm regions. The extracts isolated from different Eucalyptus species revealed anti-inflammatory, antioxidant, antibacterial and anticancer activity. Recently phenolic compounds from Eucalyptus has gained interest and is focused on a newly identified group called the formylated phloroglucinol compounds (FPCs) [1]. The biological activities of these phenolic compounds include antitumor, antimalarial, cancer chemopreventive, HIV-RTase inhibition and anti- fouling. This FPC group is further divided in to three broad groups based on their chemical structure known as euglobals, macrocarpals and sideroxylonals. The sideroxylonals are strongly antimicrobial, inhibitors of human plasminogen activation and most potent natural antifouling agents. The sideroxylonals can be used as potent chemotherapeutic agent to treat certain forms of cancer because of its reported antiproliferative effect, which exhibits low cytotoxicity against normal cell lines indicating their selectivity [2].

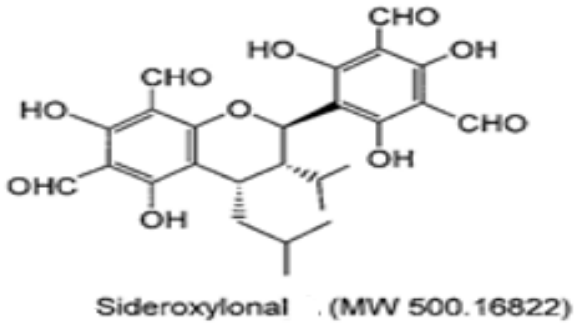

Figure 1. Chemical structure of Sideroxylonal[3]

1.2. Therapeutic Properties of *Withania Somnifera*

Withania somnifera is commonly called Ashwagandha, Indian ginseng, or winter cherry, it is a well-known herb, used for centuries in Ayurvedic medicines for increasing longevity, vitality, and as a health-promoting tonic to boost body's resistance against diseases. It has broad therapeutic applications, including anti-inflammatory activities, action on the immune system, circulatory system, diabetes, central nervous system, etc. A metabolic profiling of crude extracts of leaves and roots of *W. somnifera* reported a total of 62 different major and minor primary and secondary metabolites from leaves and 48 from roots. Out of these, 29 metabolites were common to both leaves and roots which include fatty acids, organic acids, amino acids, sugars, flavones, and sterol derivatives and quantitative differences were also noticed between the leaf and root tissues [4]. It improves learning ability and memory capacity. It helps counteract chronic fatigue, weakness, dehydration, bone weakness, loose teeth, thirst, impotency, premature aging emaciation, debility, convalescence [5]

Withania somnifera is an indigenous plant and its main phytoconstituent is WA which has been used for years ago in traditional medicines for its therapeutic use against many disorders. Withaferin A (4β,27-dihydroxy-1-oxo-5β,6β-epoxywitha-2-24-dienolide, Figure 3) was the first member of this group of compounds to be isolated from *W. somnifera*. Withaferin A (WA) has been identified as a major biologically active constituent with many pharmacologically useful properties against many cancer types in vitro/in vivo, including breast, colon, prostate and ovarian cancers [6]

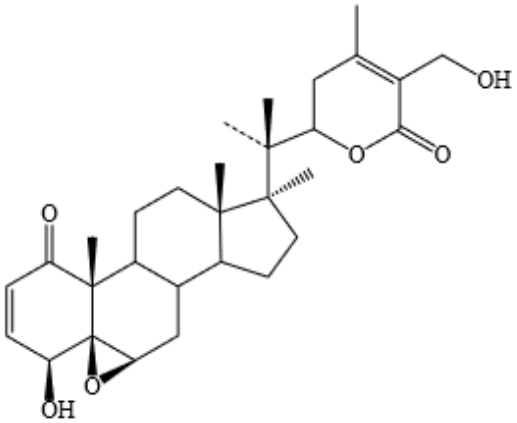

Figure 2. Structure of withaferin A [6]

2. Materials and Methods

2.1. Materials

Ascorbic acid, DMSO (Dimethyl sulfoxide), H_2SO_4, Ammonium Molybdate, Sodium Phosphate, Methanol, Acetone, Chloroform, DPPH (1, 1-diphenyl-2-picryl hydrazyl), Gentamicin, Ciprofloxacin, trifluroacetic acid, acetic acid and HPLC grade acetonitrile all were procured from HiMedia.

2.2. Sample Preparation

Residual foliage was collected from individual tree within a circle of 100 m diameter. 20gm of leaves were collected and dried at 75 degree Celsius for 4 hrs in hot air oven. The dried foilage were powdered to get a particle size of 1mm.[7].

2.3. Extraction and Isolation

To extract sideroxylonal and withaferin A from eucalyptus foliage and ashwagandha leaves different solvents of varying polarity were chosen to test the best extraction solvent to maximize the extractive value and the percentage of extract.

Acetone and combination of chloroform and methanol in different proportions were used for optimization of extraction of sideroxylonal [8] Ethyl alcohol and combination of chloroform and hexane in different proportions were used for optimization of extraction of withaferin A [8].

Conventional extraction was carried out wherein the dried sample was weighed (5gm) and homogenized with 100ml of solvent. It was later incubated in hot water bath at 85 degree C for 2hrs. The crude preparation was left overnight in magnetic stirrer at 60 degree Celsius for 24hrs. The crude extracts were filtered using Whatman filter paper to remove plant particles, transferred into pre weighed beaker and concentrated by evaporating the solvent at 60. Dried sample was scraped, weighed and extraction yield is calculated.

2.4. Antioxidant Assay

2.4.1. Phosphomolybdenum Assay

The method was developed by Prieto et al (1999) antioxidant activity of samples was evaluated by the green phosphomolybdenum complex formation. In this method phosphomolybdic acid is reduced to phosphomolybdenum blue complex by sodium sulfide. The obtained phosphomolybdenum blue complex is oxidized by the addition of nitrite and this causes a reduction in intensity of the blue color.

In the present study 5.5 mg of test sample was taken to which 500 µl of DMSO was added and similarly 5.5 mg of standard ascorbic acid was taken to which 500 µl of DMSO was added. 100 µl of the above two sample solution was taken to which 1ml of reagent mixture was added. Tubes were capped and incubated in hot water bath at 90 degree C for 90 min. It was later cooled to room temperature and reading observed @ 630 nm.

Calculation: Capacity of total antioxidant = Absorbance (test) / absorbance (antioxidant) x 100 = µg / equivalence of antioxidant = antioxidant capacity

The graph was plotted based on y = mx + c. The final optimum IC 50 value was calculated.

2.4.2. DPPH Assay

Activity of the leaf extracts were determined on the basis of their scavenging activity of the stable 1, 1-diphenyl-2-picryl hydrazyl (DPPH) free radical. DPPH has an odd electron which makes it a stable free radical

usually used for detection of the radical scavenging activity.

6 test tubes were taken to make aliquots of 6 concentration (800, 400, 200, 100, 50 and 25µg/ml) with the samples. Extracts and ascorbic acid standard were weighed accurately and dissolved in methanol to make required concentration by dilution technique. DPPH was weighed and dissolved in methanol to make 0.004% (w/v) solution. To dissolve homogenously magnetic stirrer was used. After making the desired concentrations, 3ml of 0.004% DPPH solution was added to each test tube by pipette. The room temperature was recorded and kept the test tubes for 30 min in dark to complete the reactions. DPPH was also applied on the blank test tubes at the same time where only methanol was taken as blank. After 30 minutes, the absorbance of each test tube was taken on a UV spectrophotometer. IC50 value was measured from % Inhibition vs Concentration graph. [9]

% Inhibition was calculated from the following equation:

% Radical scavenging activity = (Absorbance of blank – absorbance of sample)/(absorbance of blank)*100.

2.5. Determination of Antibacterial Activity by Agar Disc Diffusion Assay

Activities of the Eucalyptus and ashwagandha extracts were evaluated by means of agar-disc diffusion assay with some modifications. Fifteen milliliters of the molten agar (45°C) were poured into sterile Petri dishes. Cell suspensions were prepared and 100 µL was evenly spreader onto the surface of the agar plates of agar. Once the plates had been aseptically dried, different extracts (10 mg/mL) were dissolved in dimethylsulfoxide/water (1/9) and 80 µL were placed into the 6mm sterile discs with a sterile Pasteur pipette and the plates were incubated at 37°C for 24 h. Gentamicin (25 µL/wells at concentration of 4 µg/mL) and ciprofloxacin (5 µg/mL) were used as positive control for bacteria. The diameter of the circular zone of inhibition was measured and antimicrobial activity was evaluated around the well. Tests were performed in triplicate and values are the averages of three replicate.[10]

2.6. Thin Layer Chromatography

2.6.1. TLC analysis of Eucalyptus Extract

A silica gel TLC plate was prepared and allowed to air-dry and was then activated in an oven for 3-4 h. Samples (ca. 1g) were dissolved in chloroform-methanol (90:10) and loaded onto the TLC plate and allowed to air-dry for 5 min. The chromatography chamber was saturated (chloroform- methanol-acetic acid (90: 10: 0.5)) for 30 min and the plate was run as above (height 15 cm; run time: 40 min). The plate was allowed to air-dry and observed under UV at 254 nm. [11]

2.6.2. TLC Analysis of *Withania Somnifera* Extract

100mg of aqueous extracts of all Ashwagandha samples were dissolved in 10ml of methanol and filtered through Whatman No.1 filter paper. 5µl-9µl of the standard solution and 10µl of all sample solutions were applied as bands on the plate. The spotted plates were individually developed up to 90mm in a previously saturated chamber. Chamber saturation was done for 10 minutes with Chloroform: Methanol(9:10). The developed plate was scanned at 254nm. The nm at which the peak obtained maximum height and area was considered as λ max [12].

2.7. FTIR Analysis

The powdered extract was subjected for FTIR analysis. The powder was filled in a 2 mm internal diameter micro-cup and loaded onto FT-IR spectrophotometer at 26°C±1°C. The samples were scanned using infrared rays in the range of 4000-750 cm-1[13]

2.8. HPLC Analysis

2.8.1. HPLC Analysis of Sideroxylonal from *Eucalyptus Spp*

The chromatographic separations were carried out on an column(250 ×4.0 mm i.d,;3 µm) connected to Waters Alliance HPLC system consisting of a 2690 separation module auto sampler fitted with a 250µL syringe and a 100µL sample loop and a photodiode array detector. Optimal separation was achieved through isocratic elution with acetonitrile: water containing 0.1% trifluroacetic acid (93:7). The flow rate was 0.75mL/min with a column temperature of 40 degree Celsius and a run time of 15min. the typical operating pressure was 90bar with a pressure ripple of 1bar. Waters millennium software was used for both data collection and integration [14]

2.8.2. HPLC Analysis of Withaferin A from *Withania Somnifera*

HPLC analysis was carried out on a LC-20AT system (Shimadzu, Japan) using a reverse-phase C18 column (4.6 mm x 250 mm; Separation Methods Technologies). Elution was carried out at a flow rate of 1 mL min-1 with water: glacial acetic acid (99.9:0.1, v/v) as solvent A and acetonitrile : glacial acetic acid (99.9:0.1, v/v) as solvent. The following gradient elution was used: 0-5 min with 80% A, 5-30 min with 80-0% A, 30-35 min with 0% A, 35-37 min with 0-80% A and 37-40 min with 80% A. 20 µL of the sample solutions were injected. PDA detector was used for detection at 230nm [15].

3. Results and Discussion

3.1. Extraction Yield

Table 1. Extraction yield of sample foilage with different solvents

Sample number	Solvent/ Combination	Extraction value(gms)
1.*Eucalyptus spp* leaves powder	Acetone	14
2. *Eucalyptus spp* leaves powder	Chloroform: Methanol (80:20)	11.6
3. *Eucalyptus spp* leaves powder	Chloroform: Methanol (50:50)	7
4. *Eucalyptus spp* leaves powder	Chloroform: Methanol (20:80)	6
5. *Withania Somnifera* leaves powder	Ethyl Alcohol (70%)	9.7
6.*Withania Somnifera* leaves powder	Chloroform : Hexane (50:50)	6.5

3.2. Antioxidant Assay

3.2.1. Phospho-molybdenum Assay

3.2.1.1. Phospho-molybdenum Assay of *Eucalyptus Spp*

Table 2. IC50 value of phosphomolybdenum assay

Antioxidant Assay						% Inhibition			
Conc (µg/ml)	Test tube t1	Test tube t2	Blank B	1-B	2-B	1	2	Average	Std Deviation
1.10000	0.168	0.172	0.166	0.002	0.006	98.14	96.32	97.23	1.29
2.8000	0.148	0.152	0.147	0.001	0.005	77.57	83.90	80.66	4.48
3.6000	0.13	0.143	0.129	0.001	0.014	65.00	54.97	59.54	7.14
4.4000	0.122	0.12	0.119	0.003	0.001	62.00	56.90	59.00	4.24
5.2000	0.072	0.078	0.07	0.002	0.008	54.08	52.00	53.23	1.74
Control	0.068	0.068	0.029	0.07	0.07	0.00	0.00	0.00	0.00
IC50						816.9761	815.8878	**816.43**	0.77

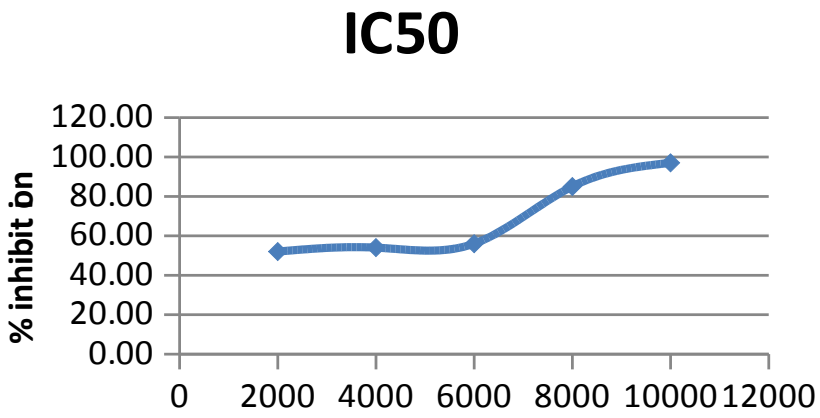

Figure 3. %Inhibition vs Concentration graph for phosphomolybdenum assay (IC50 value – 99.643µg/m)

3.2.1.2. Phospho-molybdenum Assay of *Withania Somnifera*

Table 3. IC50 values of phophomolybdenum assay of *Withania Somnifera*

Antioxidant Assay						% Inhibition			
Conc (µg/ml)	Test tube t1	Test tube t2	Blank B	1-B	2-B	1	2	Average	Std Deviation
1.10000	0.198	0.196	0.197	0.001	0.002	97.92	96.32	97.12	1.13
2.8000	0.164	0.168	0.161	0.007	0.007	85.42	83.90	84.66	1.07
3.6000	0.143	0.144	0.122	0.022	0.021	56.00	56.97	56.54	0.78
4.4000	0.122	0.122	0.101	0.021	0.021	55.25	54.90	55.05	0.18
5.2000	0.98	0.097	0.075	0.023	0.023	52.08	52.00	52.04	0.06
Control	0.068	0.068	0.029			0.00	0.00	0.00	0.00
IC50						1000	999.32	**999.66**	0.48

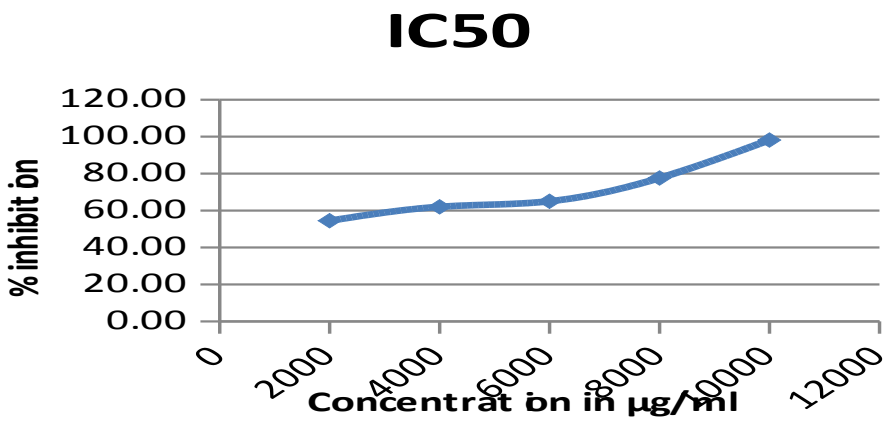

Figure 4. % Inhibition vs concentration graph of phoshomolybdenum assay of *Withania Somnifera* (IC50 value – 81.643µg/ml)

3.2.2. DPPH Assay

3.2.2.1 .DPPH Assay of Eucalyptus Spp

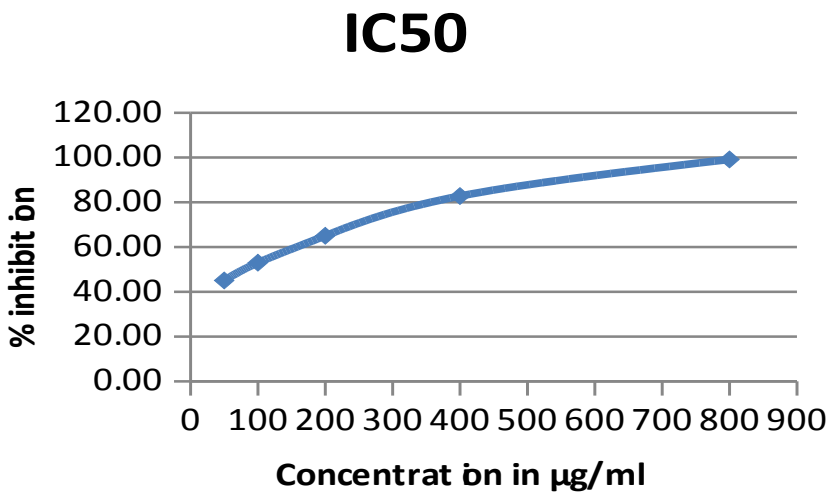

Figure 5. %Inhibition vs Concentration graph for DPPH assay (IC50 value – 92.443µg/ml)

3.2.2.2. DPPH Assay of Withania Somnifera

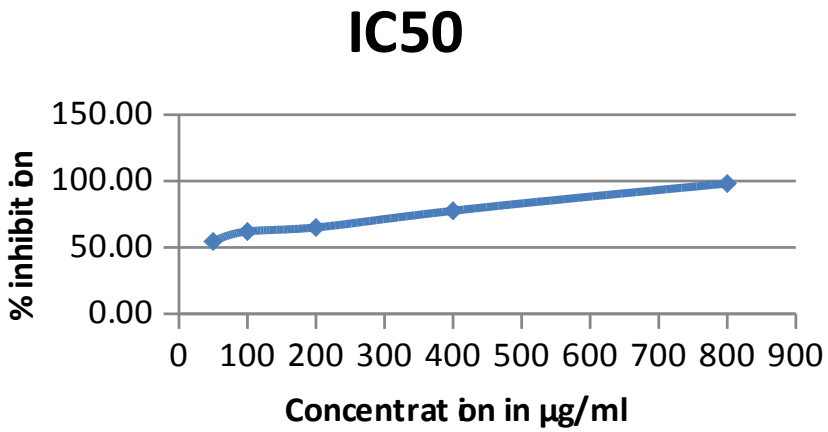

Figure 6. % Inhibition vs concentration graph of DPPH assay of *Withania Somnifera* (IC50 value – 81.523µg/ml)

3.3. Antimicrobial Results

Table 4. Presence or absence of Zone of inhibition at different concentration

Concentration in mg/ml	Zone of Inhibition	
	Eucalyptus Spp	***Withania Somnifera***
2	-	-
4	+	+
6	+	+
8	+	+

(-) in Table showed the growth of bacteria on culture and the lack of antibacterial activity of aqueous extract.
(+) in Table showed zone of inhibition and no bacterial growth and antibacterial activity of aqueous extracts.

Table 5. Average diameter (mm) of microbial free zone area of *Eucalyptus Spp* and *Withania Somnifera* leaves extract on E. coli (Agar Disc diffusion method)

Concentration in mg/ml	Diameter(mm) of Zone of inhibition	
	Eucalyptus Extracts	*Withania Somnifera* extracts
2	-	-
4	1.1±0.5	8.2±0.5
6	2.3±0.5	4.63±0.5
4) 8	-	12.1±0.5

(-) in Table showed no inhibitory effects was shown.

3.4. TLC Results

- The Rf value of extracts of *Eucalyptus Spp* - 0.86.
- The Rf value of leaf extracts of *Withania Somnifera* - 0.66

Figure 7. TLC plate

The R_f values observed were found close to standard R_f value for Sideroxylonal and withaferin A confirming its presence in the samples.

3.5. FTIR Results

3.5.1. FTIR Analysis of *Eucalyptus Spp*

The peak observed at 2925 cm^{-1} was associated with the variable -C-H aldehydic region and stretching vibrations of C-H bond The peaks around 1688.57cm^{-1} correspond to strong C=O amide region. The intense peak at 1032.58cm^{-1} cm^{-1} corresponded to the C-F alkyl halide region. The last peak at 633.42cm^{-1} refers to the strong C-Cl alkyl halide moiety. An IR Spectra of Standard sideroxylonal was used as the reference to compare the spectra which were obtained for the samples.

3.5.2. FTIR Analysis of *Withania Somnifera*

The peak observed at 3378.73cm^{-1} refers to strong alcohol OH stretch. The peaks at 2920.96 cm^{-1} and 2853.59cm^{-1} indicate a weak -C-H stretch of alkanes and alkyls. The peak observed at 1739.35cm^{-1} indicates strong C=O ester containing aldehyde. The peak at 633.42cm^{-1} refers to the strong C-Cl alkyl halide moiety. The intense peak at 1396.29 cm^{-1} and 1054.59cm^{-1} indicate strong CF alkyl halide moiety.

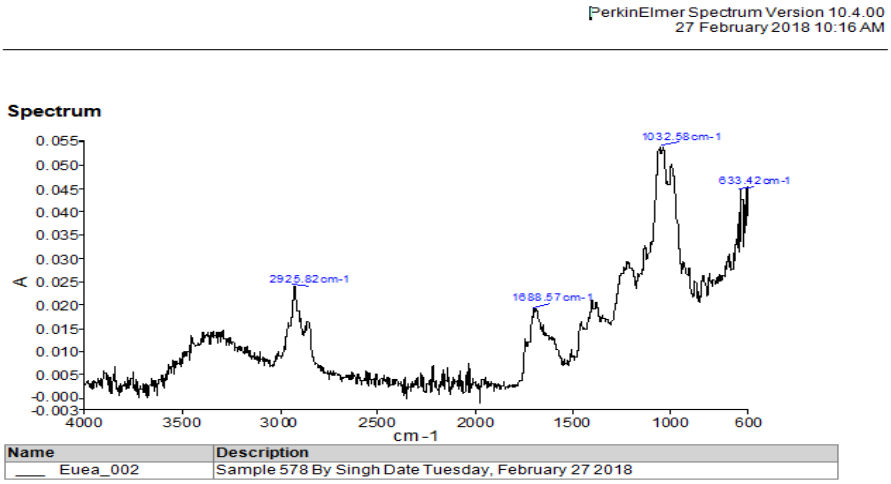

Figure 8. FTIR Spectra of extracts of *Eucalyptus Spp*

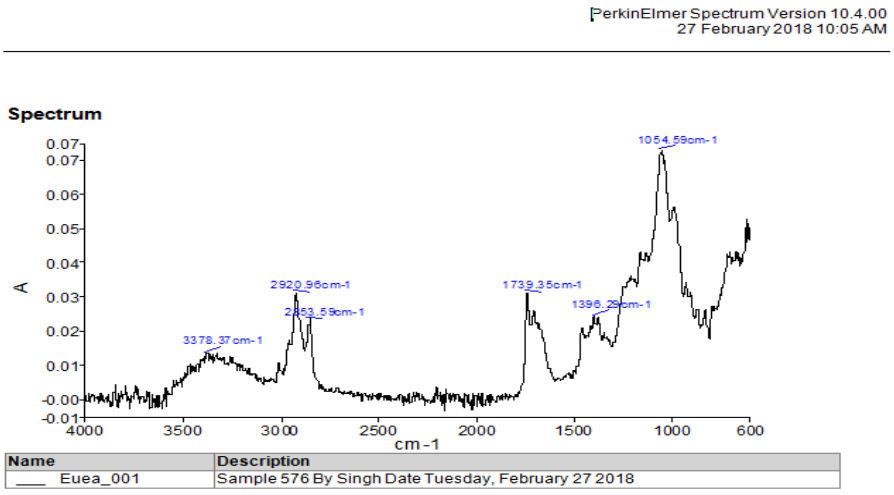

Figure 9. FTIR spectra of *Withania Somnifera*

3.6. HPLC Results

3.6.1. HPLC results of Sideroxylonal from *Eucalyptus Spp*

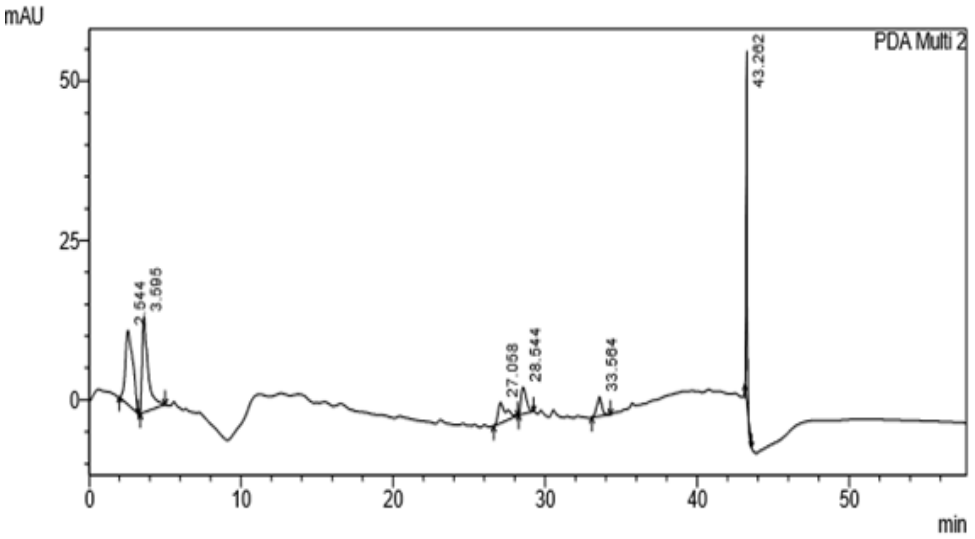

Figure 10. HPLC separation of samples

Table 6. Peak table of extracts of *Eucalyptus Spp*

Peak	Ret Time	Area	Height	Area%	Height%
1	2.742	5849715	154979	46.758	32.818
2	3.636	3743213	156610	29.920	33.164
3	5.662	166116	9975	1.328	2.112
4	10.652	946266	23041	7.564	4.879
5	12.216	534488	11609	4.272	2.458
6	13.625	456336	9866	3.648	2.089
7	33.559	300027	14703	2.398	3.113
8	43.264	514581	91448	4.113	19.365
		12510743	472230	100	100

3.6.2. HPLC Result of withaferin A from *Withania Somnifera*

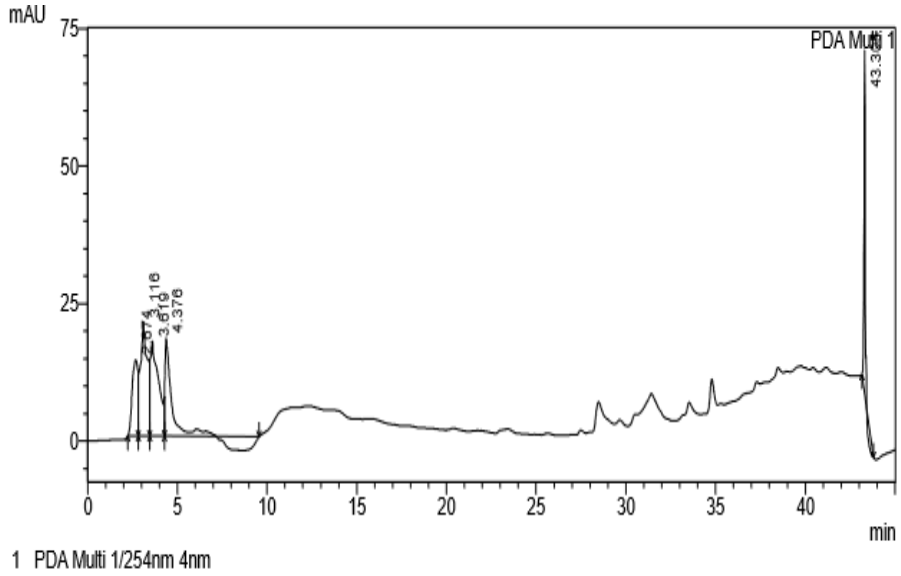

Figure 11. HPLC separation of extracts of *Withania Somnifera*

Table 7. Peak table of extracts of *Withania Somnifera*

Peak	Ret Time	Area	Height	Area%	Height%
1	2.674	308131	13841	16.269	10.477
2	3.116	573052	20625	30.257	15.613
3	3.619	534460	17224	28.219	13.039
4	4.376	230399	17767	12.165	13.450
5	43.305	247901	62644	13.089	47.422
Total		1893943	132101	100	100

4. Conclusion and Future Prospects

Sideroxylonal and withaferin A was extracted from residual foliage. The optimization of extraction was achieved by using various solvents and combination of solvents in different proportions. The method described for extraction is simple, rapid and low-cost procedure amenable to preparative scale. The isolated compounds were characterized and confirmed by HPLC, FTIR and TLC analysis by comparing the results obtained with the standards. Antioxidant assay performed using DPPH method and phosphomolybdenum method showed effective antioxidant activity in the extracts and both the methods resulted in similar IC50 values. Agar disc diffusion method carried out to study antimicrobial testing showed good zone of inhibition at concentration of 4 and 6mg/ml of extracted sample suggesting that the extracts have potential antimicrobial effect.

The optimization process of extraction can be further extended by using various solvents and controlling other parameters like time, temperature and pH to increase the yield. Antimicrobial capacity can be extensively studied by studying the effects of these phytochemicals in various strains of bacteria and fungi. The effective dose of sideroxylonal and withaferin A for its antibacterial activity can also be assessed in vitro by extended studies. The extracted compound can be further characterized using Scanning electron microscopy, NMR, XRD and GC-MS to confirm the presence of sideroxylonal and withaferin A in the extracts. The much claimed tumor inhibitor and cytotoxic effects of sideroxylonal and withaferin A can be further studied by testing the crude extracts on cancer cell lines to assess its anticancer activity. The extracts should be vigorously investigated for its health promoting, potential anti-cancer benefits.

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