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Phytochemical Screening, TLC analysis, Antibacterial and Antioxidant Activity of methanol extract of Leaf in *Ledebouria revoluta (L.f.)* Jessop

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Abstract

The in-vitro antimicrobial and antioxidant activities of methanol extract of leaf in *Ledebouria revoluta (L.f.)* Jessop (Asparagaceae) were investigated. TLC analysis of methanol extract of Leaf resulted in the identification with a concentration 0.000001 g reported the presence of six major components with R_f values of 0.97, 0.62, 0.57, 0.47, 0.35 and 0.15. The larger R_f values of a compound, the larger the distance it travel on the TLC plate. The leaf reduce the DPPH (diphenylpicrylhydrazyl) radical (IC_{50} = 35.13µg/ml), ABTS radical (IC_{50} = 32.88 µg/ml) and total antioxidant (IC_{50} = 38.83µg/ml). The methanol leaf extract showed antibacterial activity against *Bacillus subtilis, Staphylococcus aureus and Streptococcus pneumoniae*. Phytochemical analysis of revealed the presence of Alkaloids, Flavonoids, Carbohydrates, Terpenoids, Saponin and Oils and Resins. A very few works have been done in this species. This study confirms that the leaf of *Ledebouria revoluta* possesses antioxidant and antimicrobial properties in vitro.

Key words: *Ledebouria revoluta*; Antioxidant activity; Antimicrobial activity; Methanol extract (leaf); TLC and Phytochemical Screening.

1. INTRODUCTION

It has long been recognized that naturally occurring substances in higher plants have antioxidant activity. Recently, there is a growing interest in oxygen-containing free radicals in biological systems and their implied roles as causative agents in the aetiology of a variety of chronic disorders. Accordingly, attention is focused on the protective biochemical functions of naturally occurring antioxidants in the cells of the organisms containing them (Larson, 1998; Hallidisc, 1997). The antioxidant plays an important role to protect the human body damage by reactive oxygen species (Lollinger, 1981).

The *Ledebouria revoluta* Jessop [Syn:Scillaindica (wight) baker or *Scilla hyacinthina* (Roth) J.F.Macbr and Drimiopsisbotryoids baker[syn: Drimiopsiskirkii baker] are Leafaceous species of the family Asparagaceae (The Plant List 2013). Traditionally used medicinal plants have recently attracted the attention of pharmaceutical and scientific communities (Muleya *et al.*, 2014). To the best of our knowledge, information concerning the in vitro antioxidant features of *Ledebouria revoluta* has not been found in the literature. To aim of this study was to evaluate the in vitro antioxidant and antimicrobial properties of methanol extracts of leaf in *Ledebouria revoluta (L.f.)* Jessop (Asparagaceae).

2. MATERIALS AND METHODS

2.1. Collection f plant materials

The plant parts of *Ledebouria revoluta* were collected in Periyar University Campus, Salem. The plant identified and authentication from Botanical Survey of India, Coimbatore.

2.2. Preparation of Methanolic Extracts

The fresh plant samples (Leaf) collected were washed individually under tap water and dried in room temperature for seven days. The plants were collected and chapped small pieces of shadow dry. The dried plant materials were grind into powder. About 50 gm of dry powdered plant material from each plant was extracted by soxhlate apparatus using methanol solvent. The plant extracts was then concentrated using a rotary evaporator and the concentrated residual extracts were stored at 4^0 c in a dry airtight container until further use.

2.3. Phytochemical Screening

Preliminary phytochemical analysis was carried out for methanol extracts of *Ledebouria revoluta* as per standard methods described by Brain and Turner 1975 and Evans 1996.

2.3. 1. Detection of alkaloids

Extracts were dissolved individually in dilute hydrochloric acid and filtered. The filtrate was used to test the presence of alkaloids.

Mayer's test: Filtrates were treated with Mayer's reagent. Formation of a yellow cream precipitate indicates the presence of alkaloids.

Mayer's reagent: Mercuric chloride (1.358g) is dissolved in 60ml of water and potassium iodide (5g) is dissolved in 10ml of water. The two solutions are mixed and made up to 100ml with water.

2.3. 2. Detection of Flavonoids

H2SO4 test: Extracts were treated with few drops of H_2SO_4. Formation of orange colour indicates the presence of flavonoids.

2.3. 3. Detection of Phenols

Ferric chloride test: Extracts were treated with few drops of 5% ferric chloride solution. Formation of bluish black colour indicates the presence of phenol.

2.3. 4. Detection of Terpenoids

Salkowski's test: 0.2g of the extract of the whole sample was mixed with 2ml of chloroform and concentrated H2SO4 (3ml) was carefully added to form a layer. A reddish brown colouration of the inner face was indicates the presence of terpenoids.

2.3. 5. Detection of Saponins

Froth test: About 0.2g of the extract was shaken with 5ml of distilled water. Formation of frothing (appearance of creamy stable persistent of small bubbles) shows the presence of saponins.

2.3. 6. Detection of Tannins

Ferric chloride test: A small quantity of extract was mixed with water and heated on water bath. The mixture was filtered and 0.1% ferric chloride was added to the filtrate. A dark green colour formation indicates the presence of tannins.

2.3. 7. Detection of Carbohydrates

Fehling's test: 0.2gm filtrate is boiled on water bath with 0.2ml each of Fehling solutions A and B. A red precipitate indicates the presence of sugar.

Fehling's solution A: Copper sulphate (34.66g) is dissolved in distilled water and made up to 500ml using distilled water.

Fehling's solution B: Potassium sodium tartarate (173g) and sodium hydroxide (50g) is dissolved in water and made up to 500ml.

2.3. 8. Detection of Amino acid

Ninhydrin Test: take few drops of extracts and added with 0.25% ninhydrin reagent. Blue colour indicates the presence of amino acid.

2.3. 9. Detection of Quanins

Sulphuric acid test: take few drops of extracts and added with sulphuric acid with form a red colour. Red colour indicates the presence of quanins.

2.3. 10. Detection of Oils and Resins

Spot test: Test solution was applied on filter paper. It develops a transparent appearance on the filter paper. It indicates the presence of oils and resins.

2.4. Quantitative Phytochemical analysis

2.4.1 Estimation of Alkaloids

Alkaloid determination by using Harborne (1973) method. One gram of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and it's covered and allowed to stand for 4 h. It was filtered and the extract was concentrated on a water bath to one quarter of the original volume. Concentrated NH4OH was added by drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute NH4OH and then filtered. The residue is the alkaloid, which was dried and weighed.

2.4.2 Quantitative Estimation of Flavonoids

Total flavonoid content was determined by Aluminium chloride method using catechin as a standard. 1ml of test sample and 4 ml of water were added to a volumetric flask (10 ml volume). After 5 min 0.3 ml of 5 % Sodium nitrite, 0.3 ml of 10% Aluminium chloride was added. After 6 min incubation at room temperature, 2 ml of 1 M Sodium hydroxide was added to the reaction mixture. Immediately the final volume was made up to 10 ml with distilled water. The absorbance of the reaction mixture was measured at 510 nm against a blank spectrophotometrically. Results were expressed as catechin equivalents (mg catechin/g dried extract).

2.4.3 Quantitative Estimation of Saponins

Test extract were dissolved in 80% methanol, 2ml of Vanilin in ethanol was added, mixed well and the 2ml of 72% sulphuric acid solution was added, mixed well and heated on a water bath at 600 c for 10min, absorbance was measured at 544nm against reagent blank. Diosgenin is used as a standard material and compared the assay with Diosgenin equivalents. (Journal of Pharmacognosy and Phytochemistry 2016; 5(2): 25-29)

2.4.4 Estimation of Carbohydrate

100 mg of sample was hydrolysed in a boiling tube with 5 ml of 2.5 N HCl in a boiling water bath for a period of 3 hours. It was cooled at room temperature and solid sodium carbonate was added until effervescence ceases. The contents were centrifuged and the supernatant was made to 100 ml by using distilled water. From this 0.2 ml of sample was pipetted out and made up the volume to one ml with distilled water. Then one ml of phenol reagent was added and followed by 5.0 ml of sulphuric acid. The tubes were kept at 25-30 C for 20 min. The absorbance was read at 490 nm (Krishnaveni et al., 1984).

2.5. TLC analysis

Thin layer chromatography, or TLC, is a method for analyzing mixtures by separating the compounds in the mixture. TLC can be used to help determine the number of components in a mixture, the identity of compounds, and the purity of a compound. By observing the appearance of a product or the disappearance of a reactant, it can also be used to monitor the progress of a reaction. TLC is a sensitive technique - microgram (0.000001 g) quantities can be analyzed by TLC - and it takes little time for an analysis (about 5-10 minutes). TLC consists of three steps - spotting, development, and visualization. Photographs of each step are shown on the course website. First the sample to be analyzed is dissolved in a volatile (easily evaporated) solvent to produce a very dilute (about 1%) solution. Spotting consists of using a micro pipette to transfer a small amount of this dilute solution to one end of a TLC plate, in this case a thin layer of powdered silica gel that has been coated onto a plastic sheet. The spotting solvent quickly evaporates and leaves behind a small spot of the material.

Development consists of placing the bottom of the TLC plate into a shallow pool of a development solvent, which then travels up the plate by capillary action. As the solvent travels up the plate, it moves over the original spot. A competition is set up between the silica gel plate and the development solvent for the spotted material. The very polar silica gel tries to hold the spot in its original place and the solvent tries to move the spot along with it as it travels up the plate. The outcome depends upon a balance among three polarities - that of the plate, the development solvent and the spot material. If the development solvent is polar enough, the spot will move some distance from its original location. Different components in the original spot, having different polarities, will move different distances from the original spot location and show up as separate spots. When the solvent has traveled almost to the top of the plate, the plate is removed, the solvent front marked with a pencil, and the solvent allowed to evaporate. Visualization of colored compounds is simple the spots can be directly observed after development. Because most compounds are colorless however, a visualization method is needed. The silica gel on the TLC plate is impregnated with a fluorescent material that glows under ultraviolet (UV) light. A spot will interfere with the fluorescence and appear as a dark spot on a glowing background. While under the UV light, the spots can be outlined with a pencil to mark their locations. A second method of visualization is accomplished by placing the plate into iodine vapors for a few minutes. Most organic compounds will form a dark-colored complex with iodine. It is good practice to use at least two visualization techniques in case a compound does not show up with one particular method.

$$Rf = \frac{\text{Distance traveled by the substance}}{\text{Distance traveled by the solvent.}}$$

2.6. Assay for antibacterial activity

Agar disc diffusion method

The screening of alcoholic extracts of different plant species for antibacterial activity was determined by agar disc diffusion method (Perez et al., 1990; Parekh et al., 2005). Culture Preparation: 1st Day : All testing culture (GSTP1, GT132, HVT234) & pathogen (***B.subtilus S. aureus S.pneumonia)*** was inoculated in 5ml of Nutrient broth. Then the tube was incubated in shaking incubator at 37°C in 200rpm speed for 24 hrs. After incubation testing culture (GSTP1, GT132, HVT234) broth was centrifuged at 10,000rpm for 20 mins and collected culture supernatant used for further process. 2nd Day: MHA medium was prepared & sterilized. Then the medium was poured in to the

sterile petriplates. After medium solidification the pathogen was swapped on MHA plate for culture lawn. Disc Diffusion Method: disc was formed into the agar medium. 100µl of culture supernatant was loaded into the disc as disc as disc plate also, then the plate was incubated at 37ºC for 24hrs. After incubation clear zone of inhibition was observed on around the disc.

2.7. Antioxidant Assay

2.7.1. DPPH (1, 1-diphenyl-2- picrylhydrazyl) radical scavenging assay (Jain & Agrawal, 2008)

The antioxidant activity (free radical scavenging activity) of the *L. revoluta* Leaf extract on the stable radical 1, 1-diphenyl-2- picrylhydrazyl (DPPH) was determined according to above mentioned methods. The following concentrations of extract were prepared in methanol; 30 µl, 40µl & 50µl and mixed with 50µl of 0.659mM DPPH dissolved in methanol solution was added make up to one with double distilled water. The tubes were incubated at 25 ºC for 20minutes. The absorbance value was recorded at 510 nm using shimadzu UV 1800 spectrophotometer. Above same procedure followed for control without sample.

2.7.2. ABTS radical scavenging Activity

Different concentrations (30 µl, 40µl & 50µl) of sample were taken and 0.3 ml of ABTS radical caption and 1.7 ml of phosphate buffer pH 7.4 was added. [ABTS solution: ABTS 2 mM (0.0548 gm in 50 ml) was prepared in distilled water. Then the Potassium per sulphate 70 mM (0.0189g in 1ml) was prepared in distilled water. Then it is added 200 µl of potassium persulphate and 50 ml of ABTS were mixed and used after 2 hrs]. The tubes were incubated at 25 ºC for 20minutes. The absorbance value was recorded at 734 nm using shimadzu UV 1800 spectrophotometer. Above same procedure followed for control without sample.

2.7.3. Total antioxidant capacity assay (Rajamanikandan et al 2011)

Different concentrations (30 µl, 40µl & 50µl) of extracts were taken and 1ml of reagent solution was added. [Reagent solution: 0.6M sulphuric acid, 28mM sodium phosphate and 4mM Ammonium molybdate]. The tubes were capped and incubated in thermal block at 95ºC for 90 minutes. After the time interval the tubes were cool down at room temperature**.** The absorbance was recorded at 695 nm using shimadzu UV 1800 spectrophotometer.

2.7.4. Calculation of 50% Inhibitory Concentration (IC50)

The concentration (µg/ml) of the fractions that was required to scavenge 50% of the radicals was calculated by using the percentage scavenging activities at five different concentrations of the fractions.

Percentage inhibition (I %) was calculated using the formula,

$$I\ \% = \frac{(Ac-As)}{Ac} \times 100$$

Where Ac is the absorbance of the control and as is the absorbance of the sample.

3. RESULTS AND DISCUSSION

3.1 Phytochemical Screening

3.1.1 Qualitative analysis

In the present study, methanol extract of *Ledebouria revoluta* leaf were screened for its phytochemical constituents (Table: 1) The methanol extracts were screened for the presence of Alkaloids, Flavonoids, Terpenoids, Saponins, Carbohydrates and oils and resins. The saponin has the property of precipitating and coagulating red blood cells. Some of the characteristic of saponins include information of foams in aqueous solutions, hemolytic activity, cholesterol binding properties and bitterness (Sodipo, O.A., et al., 2000). Examined that qualitative phytochemical tests for the identification of alkaloids, flavonoids, steroids, and terpenoids were carried out for all the extracts by the method described.(Sazada *et al*., (2009).

3.1.2. Quantitative analysis

The total Alkaloid content of the methanol extract of leaf parts of *Ledebouria revoluta* was varying widely 2.268 mg/ml. The total Flavonoids content of the methanol extract of leaf parts of *Ledebouria revoluta* was varying widely 1.571 mg/ml. The total saponin content of the methanol extract of leaf parts of *Ledebouria revoluta* was varying widely 3.321 mg/ml. And the total carbohydrate content of the methanol extract of leaf parts of *Ledebouria revoluta* was varying widely 3.382 mg/ml and it is shown in table:2. The maximum (5.06) saponins content was recorded in methanolic extract of *T. offcinale* while the *E. hirta* possessed the minimum (0.55) saponins content (Amin et al., 2013).

3.2. Antimicrobial activity

The antimicrobial activity if the investigated of this plant against gram positive bacteria used by agar disc diffusion methods were shown in (Table: 3). The plant showed the varying degrees of antibacterial potential. The bacterial tested *B.subtilis*, S. aureus, *S. pneumonia* (Figure: 1). The antibacterial activity of methanol extracts were in the range of less than 5mm and not higher than 14mm. The highest zone inhibition was observed at 50µl/mm with all the three bacteria were shown in figure: 2. The lowest zone of inhibition was observed at 20 µl/mm with all the three bacteria. The zone of inhibition given by tetracycline as a control at different concentration. The eucalyptol showed antibacterial activity against some pathogenic bacteria in the respiratory tract and because of its antimicrobial properties. It is also used in dental care and soaps. (Salari *et al.,* 2006)**.** The antibacterial potential of phthalate derivatives from plant, bis (2-ethylhexyl) extracted from *Streptomyces bangladheshiensis* has been reported to show antibacterial activity against gram +ve bacteria (Camila *et al.,* 2013).

Table: 1 Qualitative phytochemical analysis

Phytochemical	Observation	Extract
		L.R.B
Alkaloids Mayer's Test	Yellow cream precipitate	+
Flavonoids H_2SO_4	Orange colour precipitate	+
Phenols Ferric Chloride	Deep blue to Black colour formation	-
Terpenoids Salkowski's Test	Reddish Brown colour Precipitate	+
Saponin Forth Test	Bubbles are formed	+
Tannins Ferric Chloride Test	Dark Green Colour Precipitate	-
Carbohydrates Felling's Test	Green colour formed	+
Amino acid Ninhydrin Test	Blur colour formed	-
Quanins Sulphuric acid Test	Red colour fromed	-
Oils And Resins	Filter paper method	+

Table 2: Quantitative phytochemical Analysis of Methanol extracts of *Ledebouria revoluta* (Leaf) Sample Concentration (µg/ml)

S. No	Constituents	*Ledebouria revoluta* Leaf (µg/ml)
1.	Alkaloids	2.268
2.	Flavonoids	1.571
3.	Saponin	3.321
4.	Carbohydrate	3.382

Table: 3 Antibacterial activity of *Ledebouria revoluta* (Leaf) Methanol Extract Againstpathogenic Bacteria

S.No	Sample Marking	Concentration	G+ve		
			B.subtilis	*S. aureus*	*S. epidermidis*
1	*Ledebouria revoluta* (Leaf) Methanol Extract	Negative Control DMSO	NA	NA	NA
2		Positive Control Tetracycline	7mm	14mm	13mm
3		20µl	4mm	4mm	4mm
4		30µl	5mm	6mm	5mm
5		40µl	7mm	7mm	6mm
6		50µl	8mm	8mm	7mm

Table: 4 Free radical scavenging activities of *Ledebouria revoluta* (Leaf) determined by DPPH assay (%).

S.No	Sample marking and Compound	Radical scavenging activity at different concentrations (µg/mL)	% Inhibition of DPPH Antioxidant Activity	
			DPPH (µg/ml)	IC50
1	*Ledebouria revoluta* Methanol Extract	30	55.20	35.13
2		40	62.05	
3		50	71.15	

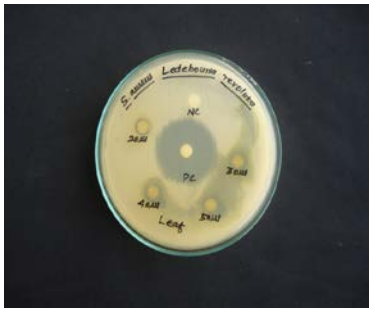


Plate 1: S. aureus

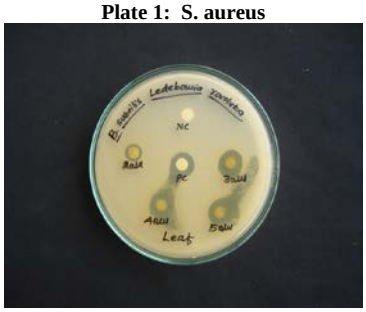


Plate 2: *B. Subtilis*

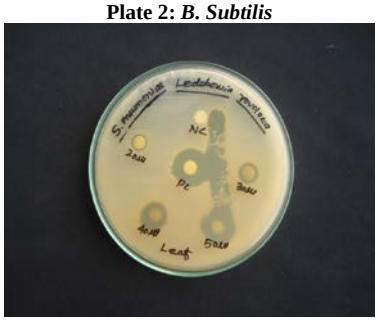


Plate 3*: S. pneumonia*

Figure: 1 Antibacterial activity of methanol extracts a *B. Subtilis,* S. aureus and *S. pneumonia*

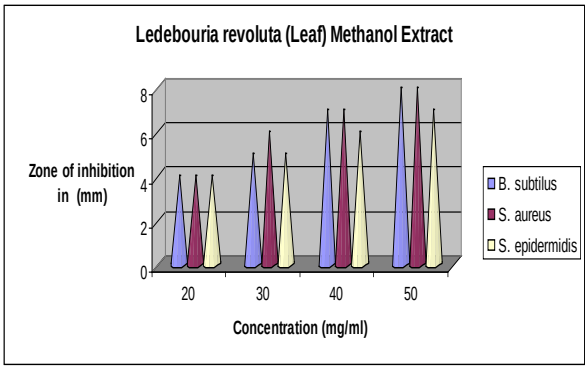


Figure: 2 Antibacterial activity

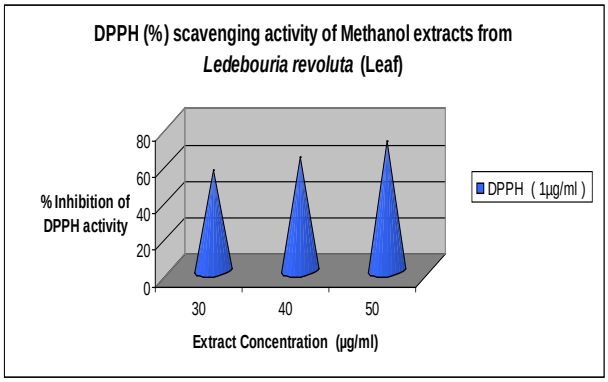


Figure: 3 DPPH Scavenging activity of Methanol extracts from *Ledebouria revoluta* (Leaf)

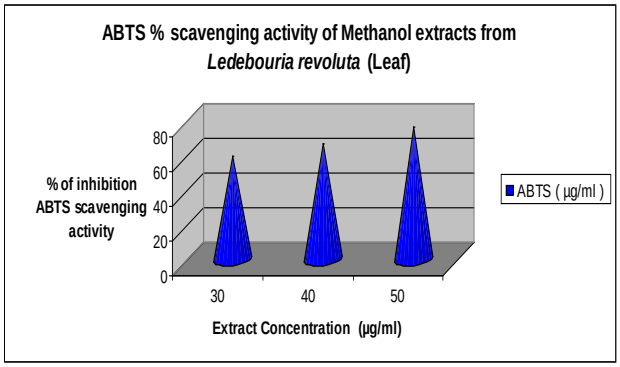


Figure: 4 ABTS scavenging activity of Methanol extract from *Ledebouria revoluta* (Leaf)

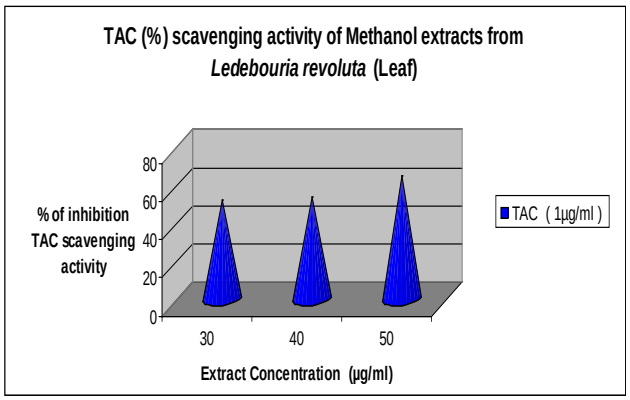


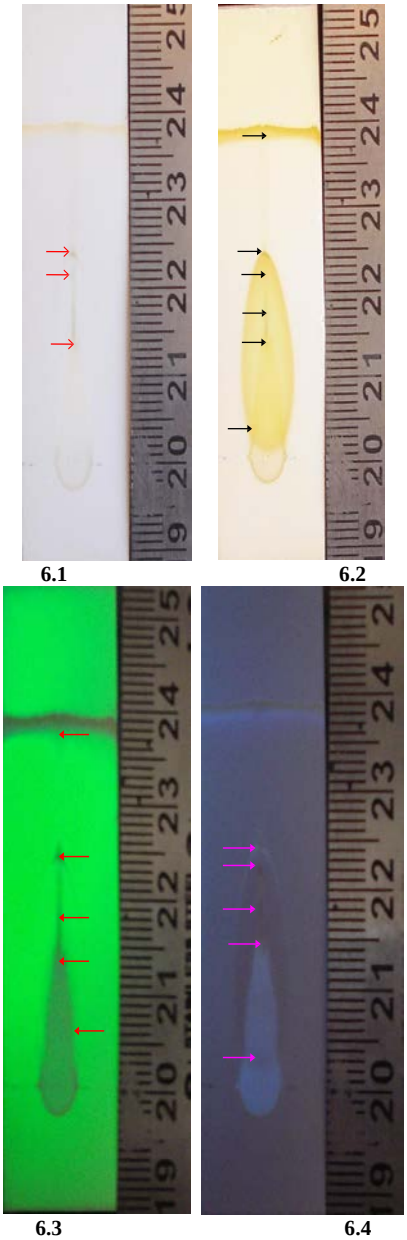
Figure: 5 TAC scavenging activity of Methanol extract from *Ledebouria revoluta* (Leaf)

Table: 5 Free radical scavenging activities of *Ledebouria revoluta* (Leaf) Determined by ABTS assay (%).

S.No	Sample marking and Compound	Radical scavenging activity at different concentrations (µg/mL)	% Inhibition of ABTS Antioxidant Activity	
			ABTS(µg/ml)	IC50
1	Ledebouria revoluta Methanol Extract	30	59.08	32.88
2		40	66.73	
3		50	76.03	

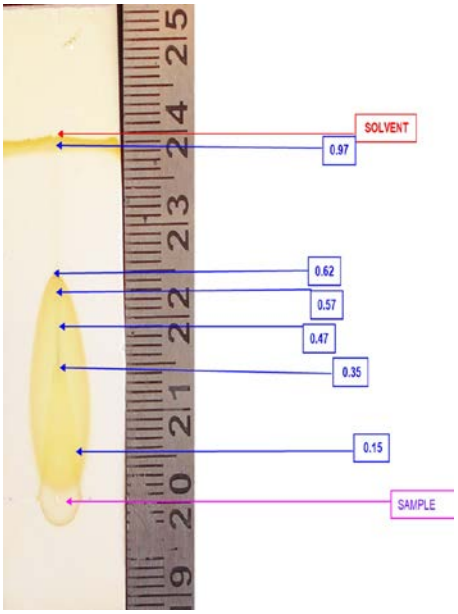
Table: 6 Free radical scavenging activities of *Ledebouria revoluta* (Leaf) determined by TAC assay (%).

S.No	Sample marking and Compound	Radical scavenging activity at different concentrations (µg/mL)	% Inhibition of TAC Antioxidant Activity	
			TAC (µg/ml)	IC50
1	*Ledebouria revoluta* Methanol Extract	30	52.31	38.83
2		40	53.61	
3		50	64.38	



6.1 6.2 6.3 6.4

Figure: 6 6.1, LRL Normal Illumination., 6.2, LRL Iodine vapor exposed., 6.3, LRL Short UV Illumination., 6.4, LRL -Long UV Illumination



Figure; 6.5, LRL Iodine vapor exposed

3.3. Antioxidant activity

The antioxidant activity of methanol extract of this plant evaluated using DPPH scavenging assay, ABTS and Total antioxidant activity.

3.3.1. DPPH radical scavenging activity

The record value (% inhibition) for the lowest concentration (30µl/ml) methanol extracts was 55.20 and respectively this value is increased to 71.15 concentration was increased 50 µl/ml it showed in the table:4., figure: 3. DPPH assay was widely used for determining the antioxidant activity in plants because it can accommodate many samples in a short period and it was sensitive enough to detect active ingredients at low concentrations (Wu, et al., 2013). Methanol showed the maximum antioxidant capacity of 93.2% revealed that the solvent (Tepe, 2008).

3.3.2. ABTS radical scavenging activity

The antioxidant activity of methanol extract of this plant evaluated ABTS assay were the record value (% inhibition) for the lowest concentration (30µl/ml) methanol extracts was 59.08 and respectively this value is increased to 76.03 concentration was increased 50 µl/ml it showed in the table:5., figure: 4. The antioxidant activities as measured by the ABTS assay were 1.85 m mol of Trolox/g DW (80%methanol) (Youg Sun et al., 2013).

3.3.3. Total antioxidant activity

The antioxidant activity of methanol extract of this plant evaluated in total antioxidant were the record value (% inhibition) for the lowest concentration (30µl/ml) methanol extracts was 55.95 and respectively this value is increased to 68.71concentration was increased 50 µl/ml it showed in the table:6., figure: 5. The total antioxidant capacity of he was determined by the linear regression equation of the calibration curve (Y=0.005x + 0.042, r^3 = 0.996) and was expressed as the number to equivalent of ascorbic acid (77.92+/- 0.65) µg/ml plant extract (Pate et., 2011).

4. TLC analysis

TLC analysis was the common method of choice for herbal analysis before instrumental chromatography methods like GC-MS and HPLC were established. The methanol extract with a concentration 0.000001 g reported the presence of six major components with R_f values of 0.97, 0.62, 0.57, 0.47, 0.35 and 0.15 as visualized under iodine chamber plate (Figure: 6.2) and Short

UV- illumination plate (Figure: 6.3), Long UV- illumination plate (Figure: 6.4) and Normal illumination plate (Figure: 6.1) and its represented in plate (Figure: 6). The larger R_f values of a compound, the larger the distance it travel on the TLC plate (Figure: 6.5). Examined that the methanol extract with a concentration of 1mg/ml reported the presence of four major compounds with Rf values of 0.48, 0.62, 0.71 and 0.8 as visualized under iodine chamber and UV light. The larger Rf values of a compound, the larger the distance it travels on the TLC plate (Hemalatha *et al*., 2015).

CONCULSION

The present study phytochemicals, antibacterial and antioxidant study have been done using *Ledebouria revoluta* plant methanol extracts. The methanol extract was purified using for TLC studies. TLC study revealed presence of compounds in the plant extract. The antibacterial studies showed the antimicrobial activity against *Bacillus subtilis, Staphylococcus aureus and Streptococcus pneumoniae*. The antioxidant activity showed the against the activity. Further investigate is nanoparticles activities in *Ledebouria revoluta* leaf.

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