

Antioxidant and Antidiabetic potentials of *Mussaenda tomentosa* Wight Ex Hook. F.

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ABSTRACT

Ethnobotanical significances of *Mussaenda tomentosa* has been documented from the tribes, during the exploration conducted from July to December 2014 on the Gingee hills belong to Pakkamalai Reserve Forests at Villupuram District, Tamil Nadu, India. The present investigation focus on the comparative analysis of phytochemical potential with special reference to antioxidant and antidiabetic activities. The active compounds were extracted using methanol. The preliminary qualitative phytochemical screening of *Mussaenda tomentosa* has revealed for the presence of Phenols, Flavanoids, Glycosides, Terpenoids, Tannins, Reducing sugars, Proteins and absence of Alkaloids in methanol extract. The quantitative determination of phenol and flavanoids was carried out and found that the total phenolic content as 79.30 mg GAE/gram and flavonoids 36.49 mg QE/gram respectively. The IC₅₀ value of DPPH assay in methanol extract was recorded as 80.33 µg/mL. The Phosphomolybdenum assay and reducing power assay indicated that the plant extract has a potential antioxidant compared with the standard drug ascorbic acid. The IC₅₀ value for the antidiabetic assay in methanol extract were recorded as 364.03 µg/ml and non enzymatic glycosylation of hemoglobin shows 62% - 94% in the concentration of 250 - 1500 µg/mL. The inhibitory activity of Glucose uptake by yeast cells in methanol extract of *Mussaenda tomentosa* was found and compared with the standard drug acarbose. The details are presented in the present study.

Keywords: Phytochemical analysis, DPPH assay, Phosphomolybdenum assay, TLC, Pakkamalai reserve forest, hillocks.

INTRODUCTION

India is the largest producer of medicinal plants and is called as "Botanical garden of the world"¹. Plant-derived substances have recently become of great interest owing to their versatile applications². The utility of traditional medicine is widespread in India³. The plant kingdom represents an enormous reservoir of biologically active phytochemicals with various chemical structures and protective properties. These phytochemicals are often secondary metabolites present in the plants which are including alkaloids, steroids, flavonoids, terpenoids, tannins, and other groups. Many of these groups have scavenging activity for free radicals therefore; they can reduce the tissue injury⁴⁻⁶. Scientists estimated that, there may be as many as 10,000 different phytochemicals with the potential effects against diseases such as cancer, stroke, or metabolic syndrome. In addition, all the higher plants produce one or several secondary metabolites, which are not essential for their metabolism⁷. Phyto-constituents are the natural bioactive compounds found in plants. These phyto-constituents work with nutrients and fibers to form an integrated part of defense system against various diseases and stress conditions⁸. Besides, phenolic compounds and flavonoids are also widely distributed in plants which have been reported to exert multiple

biological effects, including antioxidant, free radical scavenging abilities, anti-inflammatory, anticarcinogenic etc⁹. In recent years, the use of natural antioxidants has been promoted because of the concerns on the safety against synthetic drugs¹⁰. The antioxidants preserve and stimulate the function of immune cells against homeostatic disturbances¹¹. It has been established that oxidative stress is among the major causative factors in induction of many chronic and degenerative diseases including atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, immune suppression, neurodegenerative diseases and others¹². A healthy cell has a mortal enemy which is called a "free radical." Free radicals are known to cause defects in normal RNA as well as in life perpetuating DNA, the genetic material of the cells¹³. Free radicals, namely Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS), are known to cause damage to lipids, proteins, enzymes and nucleic acids leading to cell or tissue injuries implicated in the process of ageing¹⁴. Free radicals are highly unstable and reactive due to the presence of an odd number of electrons in the outermost orbit of their atoms; their aggressive action derives from their attempts to attain "balance" by binding with electrons of neighboring atoms, giving rise to chain reactions¹⁵. Among the numerous naturally occurring antioxidants;

ascorbic acid, carotenoids and phenolic compounds are more effective¹⁶. Diabetes mellitus is a clinical syndrome characterized by inappropriate hyperglycemia caused by a relative or absolute deficiency of insulin or by a resistance to the action of insulin at the cellular level. It is the most common endocrine disorder^{17,18}. About 170 million of population diabetic throughout the world and it has no known permanent cure so far¹⁹. Diabetes is arising from complex interactions between multiple genetic and environmental factors. The characteristic high blood sugar levels result from either lack of the hormone insulin (type 1 diabetes T1D) or because body tissues do not respond to the hormone (type 2 diabetes T2D) both are common and serious metabolic disorder throughout the world. The insulin deficiency results in increased concentration of glucose in the blood. Diabetes acquiring around 2.8 % of the world's population and is anticipated to cross 5.4 % by the year 2025. It is a growing health concern worldwide and now emerging as an epidemic world over. India has a rich source of indigenous medicinal plants which are traditionally being used in various health care purposes. It has the largest number of diabetic patients in the world and has been infamously known as the 'diabetic capital of the world'²⁰. The prevalence of diabetes mellitus is on increase and needs to be addressed appropriately. Phytochemical potential, antihyperglycemic activity and antioxidant activity of *Mecycylon umbellatum* Burn.F., *Cleistanthus collinus* Roxb., *Polygonum glabrum* Wild., *Melia azedarch* Linn., *Indigofera trifoliata* Linn., *Cassia absus* Linn., *Cassia auriculata* Linn. and *Cassia fistula* Linn. in our earlier studies showed the methanol extracts of the plant parts like leaves and seeds had moderate antidiabetic and antioxidant activity²¹⁻²⁵. The present study focuses on the tribal knowledge of *Mussaenda tomentosa* Wight ex hook.f. belong to Rubiaceae habited on the Gingee hills belong to Pakkamali Reserve forests, Villupuram District, Tamil Nadu, India. With reference to the above scientific information the present study was planned to assess the phytochemical potential, antioxidant and antidiabetic efficacy of *Mussaenda tomentosa*.

MATERIALS AND METHODS

Plant collection

Fresh leaves of *Mussaenda tomentosa* were collected from Pakkamalai hills and Gingee hillocks of Villupuram district of Tamil Nadu, India. *Mussaenda tomentosa* belong to the family Rubiaceae.

Preparation of Plant extracts

The leaves of *Mussaenda tomentosa* were carefully washed in tap water, rinsed with distilled water and air-dried in room temperature for few days till the leaves were completely dried. Then the dried leaves were crushed and ground in to fine powder. The powdered leaf samples were subjected to direct extraction with chloroform ethyl acetate and methanol in the ratio of 1:10 (w/v) by repeated extraction. The extracts were filtered through the Whatmann No. 1 filter paper and the solvent was condensed by steam bath to obtain concentrated sample²⁶. These extracts were diluted with respective solvents and

used to perform various *in vitro* antioxidants, antidiabetic assays and other phytochemical parameters.

The cleaning and preparation of glassware were done following Mahadevan and Sridhar method. General laboratory techniques recommended by Purvis *et al*²⁸ was followed for the preparation of media inoculation and maintenance of cultures.

Antioxidant Assays

Dot blot assay

The samples were applied on a 2.5 mm silica gel 60 F254 TLC plate. After the plate had been developed, it was dried at room temperature and then sprayed with 0.2 mm DPPH in methanol. The DPPH solution was freshly prepared and stored in darkness. After the plate was dried at room temperature, an active spot indicating antioxidant was a colorless spot on the purple background.

DPPH assay

DPPH assay: (2,2-diphenyl-1-picrylhydrazyl)

The Radical Scavenging Activity of different plant extracts was determined by using DPPH assay according to Chang *et al*²⁹. with a little modification. The decrease of the absorption at 517nm of the DPPH solution after the addition of the antioxidant was measured in a cuvette containing 2.96ml of ethanolic DPPH (0.1 mm) solution and 20 to 200 µg/ml of plant extract. The setup was kept in dark in room temperature and the absorption was monitored after 20 minutes. Ascorbic acid was used as standard. The ability of the plant extract to scavenge DPPH radical was calculated by the following equation:

$$\text{Percentage of DPPH Radical Scavenging Activity (\% RSA)} = \frac{(\text{Abs. control} - \text{Abs. sample})}{\text{Abs. control}} \times 100$$

Phosphomolybdenum assay

The antioxidant activity of samples was evaluated by the green phosphomolybdenum complex formation according to the method of (Prieto *et al*)³⁰. An aliquot of 100µl of sample solution was combined with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in a 4 ml vial. The vials were capped and incubated in a water bath at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 695 nm against a blank. The results reported (Ascorbic acid equivalent antioxidant activity) are mean values expressed as g of ascorbic acid equivalents/100g extract.

Reducing power assay

The reducing power of the extracts was evaluated according to Oyaizu *et al*³¹. Different amounts of methanol extracts were perched in methanol solvent and diverse with 2.5 ml of 0.2 M phosphate buffer (pH 6.6), and 2.5 ml of 1% K₃Fe (CN)₆. This mixture was incubated at 50°C for 20 min, 2.5 ml of 10% TCA was added to the blend and centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was assorted with methanol (2.5 ml) and FeCl₃ (0.5ml, 0.1%), and the absorbance was measured at 700 nm. Increase in absorbance of the reaction mixture indicated increased reducing power.

Antidiabetic assays

Starch-iodine assay α-Amylase activity was measured by the decreasing of blue colour after action of the enzyme on

soluble starch according the method of Jones and Varner³². The iodine stock solution was prepared by mixing 6 g of potassium iodide and 0.6 g of iodine in 100 ml of distilled water. 1 ml of stock solution was added to 99 ml of 0.05N hydrochloric acid. This is used to stop the α -amylase activity and giving the colour reaction. The reaction mixture was incubated at 37°C for 10 min in tubes containing 10 mg soluble potato starch, 50 mM sodium acetate buffer, pH 4.5, appropriate amount of enzyme solution and distilled water to give a final volume of 1 ml. The reaction was stopped and the colour was developed by addition of 1.0 ml of diluted iodine reagent. The absorbance was recorded Spectrophotometrically at 620 nm.

Non-enzymatic Glycosylation of Haemoglobin

To measure non-enzymatic glycosylation test 1 ml each of Glucose (2%) haemoglobin (0.06%) and Gentamycin (0.02%) in phosphate buffer 0.01M at pH 7.4. were taken and mixed in a test tube. The methanol extract was weighed and dissolved in DMSO to obtain stock solutions of 1-5 μ g/ml. Then 1 ml of each concentration was added to above mixture. The Mixture was incubated in dark at room temperature for 72 hrs. The degree of glycosylation of haemoglobin was measured calorimetrically at 520 nm³⁴. Metformin was used as a standard drug for assay and % inhibition was calculated using the formula

$$\% \text{ inhibition of glycosylation} = \frac{\text{Abs. of sample} - \text{Abs. of control}}{\text{Abs. of Sample}} \times 100$$

Where Abs control is the absorbance of the control reaction (containing all reagents except the test sample) and Abs sample is the absorbance of the test sample.

Glucose uptake by Yeast cells

Yeast cells were prepared according to the method of Cirillo³⁵. Commercial baker's yeast was washed by repeated centrifugation (3,000 \times g; 5 min) in distilled water until the supernatant fluids were clear and a 10% (v/v) suspension was prepared in distilled water. Various concentrations of extracts (1–5 mg) were added to 1ml of glucose solution (5, 10 and 25 mM) and incubated together for 10 min at 37 °C. Reaction was started by adding 100 μ l of yeast suspension, vortex and further incubated at 37 °C for 60 min. After 60 min, the tubes were centrifuged (2,500 \times g, 5 min) and glucose was estimated in the supernatant. Acarbose was taken as standard drug³⁶. The percentage increase in glucose uptake by yeast cells was calculated using the following formula;

$$\text{Increase in glucose uptake (\%)} = \frac{\text{sample} - \text{control}}{\text{sample}} \times 100$$

where, Abs control is the absorbance of the control reaction (containing all reagents except the test sample), and Abs sample is the absorbance of the test sample.

Qualitative phytochemical screening

Detection of Alkaloids

Solvent free extract (50mg) was stirred with 2 ml of diluted hydrochloric acid (1ml HCL+1ml H₂O) and filtered. The filtrate was tested carefully with various alkaloid reagents. *Mayer's Test*

To small quantity of the extracts Mayer's reagent was added. Presence of creamy white precipitate indicates the presence of alkaloids³⁷.

*Detection of Phenolic compound (Ferric chloride test by Mace 1963)*³⁸

The extract (50 mg) was dissolved in 5mL of distilled water. To this few drops of neutral 5% ferric chloride solution were added. A dark green color indicated the presence of Phenol.

Detection of Glycosides

About 50 mg of extract was hydrolysed with 5mL of concentrated hydrochloric acid for 2h on a water bath filtered and the hydrolysate 2 mL and 3mL of chloroform were taken and shaken. Chloroform layer was separated and 10% ammonia solution was added to it. Pink colour indicated the presence of glycosides³⁷.

Detection of Terpenoids (Salkowski test)

About 0.5 g of the extract was added in 2 ml of chloroform. Concentrated H₂SO₄ (3ml) was carefully added to form a layer. A reddish brown coloration of the interface indicates the presence of terpenoids.

Detection of Flavonoids

The sample extract 0.5g was dissolved in 5mL of Distilled water and filtered. Dilute ammonia (5mL) was added to 1mL of the extract filtrate. Concentrated sulphuric acid (1mL) was added. Yellow colorations that disappear on standing indicate the presence of flavonoids.

Detection of Tannins

To 0.5 g of the extract was boiled in 10 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black coloration

*Detection of Reducing Sugars*³⁹

The extract (100mg) was dissolved in 5mL of water and filtered. The filtrate was subjected to the Fehling's test for identification of reducing sugars. For this test 1 mL of filtrate was boiled on water bath with 1mL each of Fehling's solution I and II and a red precipitate indicated the presence of sugar.

*Detection of Saponins (Foam test by Kokate)*⁴⁰

About 50mg of extract was diluted with 5mL distilled water. The suspension was shaken in a graduated cylinder for 15 min. A 2cm layer of foam indicated the presence of saponins.

*Millon's test*⁴¹

About 100mg of extract was dissolved in 10mL distilled water and filtered through Whatmann No.1 filter paper and the filtrate was subjected to tests of proteins. To 2 mL of filtrate few drops of millon's reagent were added. A white precipitate indicated the presence of proteins. To prepare a Millon's reagent Mercury (0.1g) was dissolved in 0.9mL of fuming nitric acid. When the reaction was completed equal volume (0.9mL) of distilled water was added.

Quantitative Phytochemical analysis

*Determination of Flavonoids - Aluminium chloride test*⁴².

To 1 ml of varying concentrations of extract 3 ml of methanol 0.2ml of 1 M potassium acetate 0.2ml of 10% aluminium chloride and 5.6ml of distilled water were

Figure.1: Dot plot assay of *M. tomentosa*

added and left at room temperature for 30 minutes. Absorbance of the mixture was read at 415 nm using UV–VIS spectrophotometer. Calibration curve was prepared using Quercetin as standard.

Determination of Phenolic compound - FolinCiocalteu's method⁴³.

The total phenol content of the extract was measured at 765 nm by Folin–Ciocalteu reagent (McDonald *et al.* 2001). The dilute methanolic extract (0.5 ml of 1:10 g ml⁻¹) and or gallic acid (standard phenolic compound) was mixed with 5ml of Folin–Ciocalteu reagent (1:10 diluted with distilled water) and added 4ml of aqueous sodium carbonate (1 M). The mixture was allowed to stand for 15 min and the total phenols were determined by spectrophotometer at 765 nm. The standard curve was prepared using 0.50 100 150 200 250 mg/ml⁻¹ solutions of gallic acid in methanol: water (50:50 v/v). Total phenol values are expressed in terms of gallic acid equivalent (mg per gm of dry mass) which is a common reference compound.

Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) technique for separation of active compounds extracted from *Euphorbia hirta* and *Strychnos nux-vomica* plants was achieved after the method of Hao *et al.*⁴⁴. Silica gel TLC plate obtained from Emerck laboratories about 1.5cm wide and 5cm long was used. A small spot of solution containing the sample is applied to a plate and dried. A small amount of an appropriate solvent (eluent) poured in to a TLC chamber to a depth of less than 1 centimeter. The container is closed with a cover glass or lid and is left for 10 minutes for saturation. The TLC plate is then placed in the chamber and allowed to run the chromatogram. The solvent moves up the plate by capillary action meet the sample mixture and carries it up the plate (elutes the sample). The dried plate is placed in a chamber containing a few crystals of iodine. The iodine vapor in the chamber oxidizes the substances in the various spots making them visible to the

Table 1: DPPH Radical scavenging assay of *Mussaenda*

tomentosa Methanol Extract.

S.No	Conc. (µg/ml)	Percentage of Inhibition	
		Plant extract	Standard
1	20	16.47±0.69	27.09±0.54
2	40	26.58±0.46	42.08±0.74
3	60	38.85±0.55	51.04±0.87
4	80	48.62±0.57	63.97±0.46
5	100	64.89±0.50	80.94±0.91

IC₅₀ value of methanol extract = 80.33 µg/ml

Table 2: Phosphomolybdenum assay of *Mussaenda tomentosa*

S.No	Concentration (µg/ml)	Absorbance
1	50	0.015±0.002
2	100	0.025±0.001
3	150	0.057±0.004
4	200	0.083±0.004
5	250	0.116±0.002
6	300	0.144±0.004

Table 3: Ferric oxide Reducing Power assay

S.No	Concentration (µg/ml)	Absorbance
1	20	0.030±0.005
2	40	0.066±0.003
3	60	0.122±0.007
4	80	0.147±0.001
5	100	0.172±0.001
6	120	0.217±0.005

Table 4: Alphaamylase inhibition assay

S.No	Concentration (µg/ml)	% of Inhibition
1	100	19.40±1.02
2	200	38.46±0.82
3	300	49.29±0.80
4	400	54.94±0.76
5	500	65.09±1.04
6	600	69.52±0.35
7	700	75.08±0.77

The IC₅₀ value = 364.03µg/ml

Table 5: Nonenzymatic glycosylation of haemoglobin

S.No	Concentration (µg/ml)	% of Inhibition
1	250	62.83±0.79
2	500	78.05±1.46
3	750	86.47±2.05
4	1000	91.10±0.50
5	1250	93.19±0.46
6	1500	94.41±0.32

IC₅₀ value = 198.94 µg/ml

eye. Once the spots are visible they may be outlined with a pencil before the iodine coloration fades.

RESULTS AND DISCUSSION

Collection of plant

Leaves of *Mussaenda tomentosa* were collected from Pakkamalai reserve forest and hillocks Gingee of Villupuram district of Tamilnadu, India.

Evaluation of In vitro antioxidant Potential

Dot blot assay

Table 6: Glucose uptake by yeast cells – *Mussaenda tomentosa*

S. No	Conc. (µl)	Percentage of Glucose uptake by yeast cells		
		5mM	10mM	25mM
1	250	01.51±0.89	14.91±0.54	03.64±0.87
2	500	09.72±0.16	17.30±0.78	07.85±1.43
3	750	14.37±1.93	21.44±0.93	10.62±1.54
4	1000	18.32±0.78	23.10±1.43	11.52±0.96
5	1250	22.69±2.01	26.87±0.65	13.55±0.43
6	1500	28.96±1.32	29.29±1.92	15.95±0.81

Table 7: Qualitative and Quantitative phytochemical analysis of *Mussaenda tomentosa*

S.No	Compound	Test	Result
1	Phenols	Ferric chloride (79.30 GAE/mL)	+++
2	Glycosides	Evans test	+
3	Flavonoids	Alkaline reagent (36.45 QUE/mL)	++
4	Tannins	Neutral ferric chloride	+++
5	Reducing sugars	Fehling solution test	+
6	Alkaloids	Wagners test	-
7	Saponins	Foam test	++
8	Proteins	Kokate test	+
9	Terpenoids	Salkowski test	+

+++ (Presence in major amount)

+ (Present in less amount)

- (presence not detected)

Table 8: Thin layer chromatography

No of the spot	UV 254nm (Rf value)
1	0.22
2	0.32
3	0.47
4	0.60
5	0.90
6	0.97

The result of dot- plot assay showed (Fig 1) that the Methanolic extracts of *Mussaenda tomentosa* changed the purple background of DPPH to yellow, it indicated that the leaves of *M. tomentosa* has antioxidant active compounds. **DPPH Radical Scavenging Activity**

The DPPH radical scavenging activity in the methanolic extract of *Mussaenda tomentosa* showed (Table 1) effective activity (64.89%) at 100µg/mL concentration. The degree of discoloration indicates that the *Mussaenda tomentosa* showed free radical scavenging potentials due to the hydrogen donating ability. The Phosphomolybdenum assay and Reducing Power assay of methanolic extract of *Mussaenda tomentosa* showed (Table 2&3) high absorbance of 0.144 at 300µg/mL and 0.0217 at 120 µg/mL concentration respectively. The antidiabetic activity of methanolic extract of *Mussaenda tomentosa* showed in Alpha amylase inhibition activity, Non enzymatic glycosylation of Haemoglobin and

Glucose uptake by yeast cells (Table 4,5,6) maximum activity 75.08% at 700 µg/ml, 94.41% at 1500 µg/ml and 28.96% at 1500 µg/ml respectively. The IC₅₀ value of methanolic extracts of *Mussaenda tomentosa* for DPPH radical scavenging activity, Alpha amylase inhibition assay and Non enzymatic glycosylation of hemoglobin was 80.33, 364.03 and 198.94 µg /mL respectively, The qualitative and quantitative phytochemical analysis of methanolic extracts of *Mussaenda tomentosa* showed (Table. 7) in the presence of Phenol (79.30 GAE/mL), flavonoid (36.45 QUE/mL) and terpenoid. The R_f value of the eluted spots for the *Mussaenda tomentosa* were 0.22, 0.32, 0.47, 0.60, 0.90 and 0.97 as exposed under UV at 254 nm in the solvent ratio of 1:1 of Ethyl Acetate : Hexane showed in Table 8 and Fig.2.

DISCUSSION

The methanol extract of leaves of *Mussaenda tomentosa* have shown significant antioxidant and antidiabetic properties. Based on the above mentioned phytochemical results, it is evident that the presence of Phenols, Flavonoids, Glycosides, Saponins, Tannins, Terpenoids and Reducing sugars. Further studies are needed to explore *in vivo* response for better understanding.

CONCLUSION

Antioxidant and antidiabetic studies of *Mussaenda tomentosa* indicate its remarkable role in free radical scavenging activity and the possibilities of possessing anticancerous therapeutic property and also antidiabetic curing property. The methanol extract of leaves of *Mussaenda tomentosa* was an effective extract. The results stimulate that further mechanistic studies are required to ascertain its anticancer property and antidiabetic property. The present study has provided a platform for further research to probe scientifically bring out a potent drug for cancer and Diabetes mellitus. The DPPH radical scavenging ability of *Mussaenda tomentosa* methanolic leaf extract was significant when Ascorbic acid was used as standard; it reveals the proton donating capacity of the extract. Phytochemical screening of the crude extracts of *Mussaenda tomentosa* revealed that the presence of secondary compounds such as Phenols, Flavonoids, Glycosides, Saponins, Tannins, Terpenoids and Reducing sugars. Presence of these phytochemicals in methanol extract of *Mussaenda tomentosa* attributed to their excellent antioxidant activity. The results of the present study indicate that methanol crude extract of *Mussaenda tomentosa* leaves are high in phenolic contents and these extract exhibit strong antioxidant activities. The scavenging activities observed against DPPH, phosphomolybdenum, Fe³⁺ reducing power assay and alpha amylase, non-enzymatic glycosylation, glucose uptake by yeast cells, lead us to propose the above plant leaves as promising natural sources of antioxidants and antidiabetic suitable for application in nutritional/pharmaceutical fields. Further studies are needed to explore *in vivo* response for better understanding.

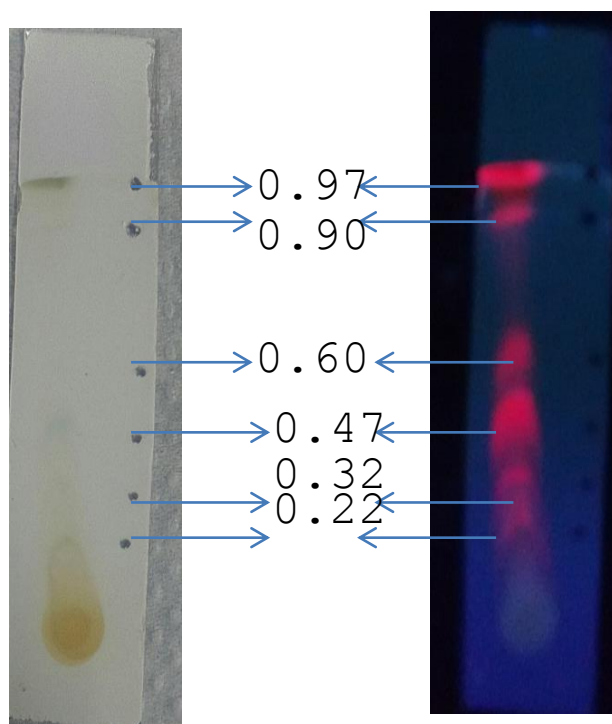


Figure 2. Thin layer chromatography of *Mussaenda tomentosa*

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