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Research Article

Standardisation and Estimation of Hispidulin – A Potent Antidiabetic Constituent Present in Leaves of Millingtonia Hortensis and Scoparia Dulcis by Hptlc and Hplc Methods as Per Ich Appliance & Bioassay Guided in Vitro Study of Antidiabetic Activity

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ABSTRACT

The present work was undertaken with the objective to quantify the hispidulin and estimate from the successive leaf extracts of *Millingtonia hortensis* and *Scoparia dulcis* with the help of standard marker hispidulin by using HPTLC and HPLC methods for the first time and to carry out the evaluation of their antidiabetic activity. Camag HPTLC system equipped with TLC Linomat V applicator, Camag TLC scanner and winCATS software and Shimadzu HPLC prominence UFLC were used. HPTLC of standard marker and petroleum ether, chloroform, ethyl acetate and methanol leaf extracts of *Millingtonia hortensis* and *Scoparia dulcis* were developed in suitable mobile phase of toluene: ethyl acetate: methanol (5:3:1%v/v/v) using standard procedures and scanned under UV at 335nm. HPLC of standard marker and successive leaf extracts of *Millingtonia hortensis* and *Scoparia dulcis* were carried out by using acetonitrile: phospohoric acid (pH-4.05) of ratio 40:60 and chromatograms were recorded at 335nm. Standardisation of hispidulin were carried out by HPTLC, HPLC methods and linearity was found to be 0.9982 and 0.9989 respectively. Quantification of hispidulin in successive leaf extracts of two plants were carried out.

Keywords: *Millingtonoa hortensis, Scoparia dulcis*, Hispidulin, HPTLC, HPLC, *In vitro* antidiabetic activity.

INTRODUCTION

Herbal medicines include herbs, herbal materials, herbal preparations and finished herbal products that contain active ingredients which are present in different parts of plants, other plant materials or combinations and are used especially for the prevention and treatment of disease. Herbal drug standardisation is a much needed public and private interactive that will help in elevating traditional medicine to the levels it probably deserves internationally.

According to WHO guideline markers (marker substances) are reference substances that are chemically defined constituents of a herbal material. They may or may not contribute to the therapeutic activity. However, even when they contribute to the therapeutic activity, evidence that they are solely responsible for the clinical efficacy may not be available.

Chromatographic fingerprinting techniques are playing an ever more important role in the standardisation of herbal products. They are being used for confirming the presence of different herbs in poly herbal formulations as well as the quantification of marker compounds.

HPTLC is very simple technique used for the separation of plant constituents. It is a qualitative tool for separation of simple mixtures where speed, low cost and simplicity are required and it is also a tool for quantitative analysis with high simple throughput. HPLC is a very sensitive analytical technique most widely used for quantitative and qualitative analysis of pharmaceuticals.

Diabetes mellitus, one of the major public health problems, is a metabolic disorder of multiple etiologies distinguished by a failure of glucose homeostasis with disturbance of carbohydrate, fat and protein metabolism as a result of defects in insulin secretion and/or insulin action. Alpha-amylase is a prominent enzyme found in the pancreatic juice and saliva which breaks down large insoluble starch molecules into absorbable molecules. On the other hand, mammalian -glucosidase in the mucosal brush border of the small intestine catalyzes the end step of digestion of starch and disaccharides that are abundant in human diet. Inhibitors of α-amylase and β-glucosidase delay the breaking down of carbohydrates in the small intestine and diminish the postprandial blood glucose excursion.

Millingtonia hortensis (Family- Bignoniaceae) is also called as Cork tree, Akas nim, Nim chameli. It is a tall deciduous tree and contains Hispidulin, scarotene,

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dinatin, rutinoside etc. Flower buds are used in the treatment of asthma, sinusitis and cholagogue. Leaves and roots of cork tree are used for their antiasthmatic, antidiabetic and antimicrobial activities. *Scoparia dulcis* (Family- Scrophulariaceae) is a rich source of flavones, terpenes and steroids, phenols, tannins, saponins, amino acids, coumarins and carbohydrates. The main chemicals in it include hispidulin, scopadulcic acids A and B etc. The leaves of *Scoparia dulcis* is used for diabetes in India. Plant is also reported to possess cytotoxic, anti-cancerous, antimicrobial, anti-malarial, anti-ulcer, antacid, anti-cholesterol and antioxidant actions**.** Hispidulin (4, 5, 7-trihydroxy 6-methoxyflavone) is a monomethoxyflavone. Its pharmacological activities include antidiabetic, anticonvulsant, antioxidant, anticonvulsant, antimycobacterial, antiasthma, antimicrobial, antiproliferative and insect larvicidal activities. Hence, the present work was undertaken with the objective to quantify the hispidulin from the plant extracts with the help of standard marker by using HPTLC and HPLC and to carry out the evaluation of their antidiabetic activity

MATERIALS AND METHODS

Chemicals and solvents used

AR/HPLC grade methanol, water, acetonitrile, petroleum ether, ethyl acetate, toluene, formic acid, potassium dihydrogen phosphate, sodium hydroxide, starch solution, dinitrosalicylic acid, sodium potassium tartrate tetrahydrate and alpha amylase were supplied by S.D. Fine chemicals Ltd., Mumbai, Reachem laboratory chemicals Pvt. Ltd., Chennai and Merck Pvt. Ltd., Mumbai. Hispidulin is purchased from ChemSci innovation Pvt. Ltd, Pune, Maharashtra.

Plant material

The leaves of *Millingtonia hortensis* were collected from Sri Ramakrishna Institute of Paramedical Sciences college of pharmacy campus and the whole plants of *Scoparia dulcis* were collected from Marthandam, Nagercoil, Tamil nadu. The collected leaves and whole plant of *Millingtonia hortensis* and *Scoparia dulcis* were identified and authenticated by Dr.Subramanian, Scientist E, Institute of Forest Genetics and Tree Breeding, Coimbatore, Tamil Nadu.

Extraction

Preparation of powder for extraction

The leaves of the plants were collected, separated from extraneous materials, cleaned, washed and well dried at room temperature to avoid the degradation of phytoconstituents. The dried leaves were ground well for getting a semi coarse powder.

Process of extraction

- The crude drug powders were extracted by successive extraction methods using four organic solvents viz., Petroleum ether, chloroform, ethyl acetate and methanol
- The solvents were selected based on the solubility of the selected marker hispidulin
- The extraction process was carried out by continuous hot percolation method using soxhlet apparatus
- The extraction process was carried out for 3 days for each solvent so as to effect complete extraction of the plant material
- After extraction the extracts were collected and dried at room temperature to get well dried extract

Extraction

Method : Successive extraction
Principle : Continuous hot percolation
Apparatus : Soxhlet extractor
Temperature maintained : 30-45^0 C
Quantity of leaves powder used : 100g
Volume of solvent used : 1000ml
Duration of each extraction process: 3 days

The percentage yields of the extracts are mentioned in Table: 1a, b. The extracts were stored in the refrigerator at 4^0 C until further analysis. The collected extracts were subjected to preliminary phytochemical screening (Table: 2, 3), TLC, HPTLC, HPLC analyses and *in vitro* antidiabetic studies.

Thin Layer Chromatographic analysis of leaf extracts of *Millingtonia hortensis* and *Scoparia dulcis*.

The basic thin layer chromatography was done in order to compare R_f value of the marker with the plant extract. The collected successive extracts of *Millitonia hortensis*, *Scoparia dulcis* and marker were applied as a spot 2cm from the edge of the plate using capillary tubes. The plates were then kept aside for the evaporation of the solvent. Then plates were placed in a closed container previously saturated with the vapors of developing solvent toluene: ethyl acetate: formic acid (5:3:1%v/v/v) with care being taken to avoid direct contact between the sample and the developer. After developing the plates up to two-third of the length of the plate, it was removed from the chamber and dried. After development, all the plates were examined in ultra-violet light in order to identify various spots.

Development of validated HPTLC method for the estimation of hispidulin in successive leaf extracts of *Millingtonia hortensis* and *Scoparia dulcis* as per ICH guidelines.

Preparation of standard stock solution of marker

A quantity of 5mg of hispidulin was transferred into 10ml standard flask, dissolved in a small quantity of DMSO and the volume was made up with methanol to 10ml get a concentration of 500µg/ml.

Fixed experimental conditions:

Stationary phase : Pre-coated Silica gel $60F_{254}$ on aluminium sheets
Mobile phase : Toluene: Ethyl acetate: Formic acid (5:3:1%v/v/v)
Chamber saturation time : 20 minutes
Migration distance : 80 mm
Band width : 6 mm
Slit dimension : 5 x 0.35 mm

Source of radiation	:	D_2
Detection wavelength	:	335 nm
R_f value	:	0.53

Validation of the method

Validation of the developed method was carried out in accordance with ICH guidelines in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), interday and intraday precision, repeatability of sample application and measurement, stability studies and selectivity.

Linearity and range

The linearity of an analytical procedure is its ability (within a range) to obtain test results that are directly proportional to the concentration of the analyte in the sample.

The marker was prepared in various concentrations and analysed by HPTLC method in order to establish that the linear regression data shows a good linear relationship over the concentration under study. From the standard stock solution of marker 50 to 250 ng/band of hispidulin (Figure: 4-8) were applied using CAMAG semi applicator. The R_f value was found to be 0.53 ± 0.03. The slope, intercept and correlation co-efficient values were found from the calibration graph (Figure: 9). Calibration data are presented in Table 7.

Limit of detection and limit of quantification

The limit of detection and limit of quantification of the standard were determined by the application on the plate of decreasing amounts of the drug in triplicate. The lowest concentration at which the peak is detected is referred to as the "Limit of Detection" and the lowest concentration at which the peak is quantified is referred to as the "Limit of Quantification". Table 8 shows the LOD and LOQ values of the marker. The detection limit (DL) may be expressed as:

$DL = 3.3\ \sigma / S$

Where, σ = the standard deviation of the response and S = the slope of the calibration curve.

The quantitation limit (QL) may be expressed as:

$QL = 10\ \sigma / S$

Where, σ = the standard deviation of the response and S = the slope of the calibration curve

Precision

The precision of the analytical procedure indicates the closeness of the agreement between a series of multiple sampling measurements of the same homogeneous sample under the prescribed conditions.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

Precision of the method adopted in the present work was demonstrated by

a) Intraday precision
b) Interday precision
c) Repeatability
i. Repeatability of sample application
ii. Repeatability of sample measurements

(a) Intraday precision

Intraday precision was studied by performing an analysis of the standard drug at two different concentrations in the linearity range of marker three times on the same day and the %RSD was calculated (Table:9).

(b) Interday Precision

Interday precision was studied by carrying out the analysis of the standard drug at different concentrations in the linearity range of the drug for three days over a period of one week and %RSD was calculated (Table:10)

(c) Repeatability

(i). Repeatability of sample application

Repeatability of sample application was assessed by spotting 100 ng concentration of standard marker solutions six times on pre-coated TLC plate. Plate was then developed, scanned and %RSD was calculated (Table: 11)

(ii). Repeatability of sample measurement

Repeatability of sample measurement of peak area was assessed by spotting100 ng concentration of standard marker solution on pre-coated TLC plate. After development of the plate, the separated spots were scanned six times without changing position of the plate and %RSD was calculated (Table: 12)

Stability studies

The analyte are may be prone to decompose when developed chromatographic plate is exposed to the atmosphere. Therefore, after development, the stability of the plates must be confirmed.

The stability of the plate was studied at different time intervals and the peak areas were compared with the peak area of the freshly scanned plate. The developed plate was found to be stable for 2 hours under room temperature (Table: 13).

HPTLC analysis of successive leaf extracts of *Millingtonia hortensis* and *Scoparia dulcis*

Preparation of stock solution of the petroleum ether extract of *Millingtonia hortensis* and *Scoparia dulcis*:

The petroleum ether extract (10mg) of each plant was weighed and made up to 10ml with methanol as the solvent to get a concentration of 1000µg/ml solution and the solution was filtered through whatmann filter paper. A quantity of 10µg/band of the solution was applied on the TLC plate.

Preparation of stock solution of the chloroform extract of *Millingtonia hortensis* and *Scoparia dulcis*:

The chloroform extract (10mg) of each plant was weighed and made up to 10ml with methanol as the solvent to get a concentration of 1000µg/ml solution and the solution was filtered through whatmann filter paper. A quantity of 10µg/band of the solution was applied on the TLC plate.

Preparation of stock solution of the ethyl acetate extract of *Millingtonia hortensis* and *Scoparia dulcis*:

The ethyl acetate extract (10mg) of each plant was weighed and made up to 10ml with methanol as the solvent to get a concentration of 1000µg/ml solution and the solution was filtered through whatmann filter

paper. A quantity of 10µg/band of the solution was applied on the TLC plate.

Preparation of stock solution of the methanol extract of *Millingtonia hortensis* and *Scoparia dulcis*:

The methanol extract (10mg) of each plant was weighed and made up to 10ml with methanol as the solvent to get a concentration of 1000µg/ml solution and the solution was filtered through whatmann filter paper. A quantity of 10µg/band of the solution was applied on the TLC plate.

Recording of the chromatogram

The peak areas of the chromatogram of the two plant extracts (Figure: 10-17) were compared with standard chromatogram and the amounts of hispidulin present in the extracts were calculated (Table: 14, 15) from the calibration graph (Figure: 9)

Development of validated RP-HPLC method for estimation of hispidulin in successive leaf extracts of *Millingtonia hortensis* and *Scoparia dulcis*

Preparation of standard stock solution of marker

An accurately weighed quantity of 5 mg of hispidulin was transferred into 10 ml standard flask and dissolved in small quantity of DMSO and the final volume was made up with methanol to 10 ml to get a concentration of 500 µg/ml.

Fixed chromatographic conditions

Stationary phase: Shimack RP-C_{18} (50 mm*4.6 id, 5 µm)

Mobile phase : Acetonitrile: phosphoric acid (pH-4.05)

Mobile phase ratio	:	40:60%v/v
Flow rate	:	1ml/min
Injection volume	:	20 µl
Elution mode	:	Gradient mode
Detection wavelength	:	335 nm
Operating temperature	:	25^0 C
Operating pressure	:	77 kgf

Validation of RP-HPLC method

Validation of the developed method was carried out in accordance with the ICH guidelines in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), interday and intraday precision, repeatability of sample injection and measurement and stability studies.

Linearity and range

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration of the analyte in the sample.

Different concentration of marker were prepared by making the final volume with methanol and injected into HPLC system with auto-sampler. Linear regression data showed a good linear relationship over a concentration range of 0.1 to 0.5 µg/ml for hispidulin. The peak areas were noted and a linear graph was plotted between concentration (x) versus peak area (y). The UV spectrum in figure 18 and standard chromatogram of hispidulin are shown in figure 19-23. Calibration graphs are given in figure 24. Calibration data are presented table: 16.

Limit of detection (LOD) and Limit of quantification (LOQ)

The limit of detection and limit of quantification of the standards were determined by the application on the plate of decreasing amounts of the drug in triplicate. The lowest concentration at which the peak is detected is referred to as the "Limit of Detection" and the lowest concentration at which the peak is quantified is referred to as the "Limit of Quantification". The detection limit (DL) may be expressed as:

$DL = 3.3\ \sigma / S$

Where, σ = the standard deviation of the response and S = the slope of the calibration curve.

The quantitation limit (QL) may be expressed as:

$$QL = 10\ \sigma / S$$

Where, σ = the standard deviation of the response and S = the slope of the calibration curve.

Precision

The precision of the analytical procedure indicates the closeness of the agreement between a series of multiple sampling measurements of the same homogeneous sample under the prescribed conditions.

Precison of the method adopted in the present work was demonstrated by,

a) Intraday precision
b) Interday precision
c) Repeatability
i. Repeatability of sample injection
ii. Repeatability of sample measurement

a) Intraday precision

Intraday precision was studied by performing an analysis of the standard drugs at two different concentrations in the linearity range of marker three times on the same day and the %RSD was calculated (Table:18)

b) Interday Precision

Interday precision was studied by carrying out the analysis of the standard drug at different concentration in the linearity range of the drug for three days over a period of one week and %RSD was calculated (Table :19).

c) Repeatability of sample injection

Repeatability of sample application was assessed by injecting same concentration of standard marker solution six times and %RSD was calculated (Table: 20)

Stability

The drug solutions were subjected to stability studies under refrigeration and at room conditions. Stability were assessed by looking for any changes in retention time, resolution, peak shape, etc. when compared to chromatogram of freshly prepared solution. The drugs were found to be stable at room temperature for two days and under refrigeration condition up to 30 days.

System suitability studies

System suitability parameters like number of theoretical plates (N), tailing factor, resolution (Rs) etc. were studied. The results were shown in (Table: 21).

Robustness

In order to demonstrate the robustness of the method, the following optimized conditions were slightly varied.

- ±1 in ratio of acetonitrile: water
- ± 0.1 in units of flow rate

The slight changes obtained with the variation in conditions were found almost same with the standard solution under optimized conditions. So the developed method was found to be robust.

Analysis of successive leaf extracts of *Millingtonia Hortensis* and *Scoparia dulcis* by RP-HPLC

Preparation of stock solution of the petroleum ether extracts of *Millingtonia hortensis* and *Scoparia dulcis*:

The petroleum ether extract (10mg) of each plant was weighed and made up to 10ml with methanol as the solvent to get a concentration of 1000µg/ml solution and the solution was filtered through whatmann filter paper. A quantity of 10µg was injected.

Preparation of stock solution of the chloroform extract of *Millingtonia hortensis* and *Scoparia dulcis*:

The chloroform extract (10mg) of each plant was weighed and made up to 10ml with methanol as the solvent to get a concentration of 1000µg/ml solution and the solution was filtered through whatmann filter paper. A quantity of 10µg was injected.

Preparation of stock solution of the ethyl acetate extracts of *Millingtonia hortensis* and *Scoparia dulcis*:

The petroleum ether extract (10mg) of each plant was weighed and made up to 10ml with methanol as the solvent to get a concentration of 1000µg/ml solution and the solution was filtered through whatmann filter paper. A quantity of 10µg was injected.

Preparation of stock solution of the methanol extract of *Millingtonia hortensis* and *Scoparia dulcis*:

The methanol extract (10mg) of each plant was weighed and made up to 10ml with methanol as the solvent to get a concentration of 1000µg/ml solution and the solution was filtered through whatmann filter paper. A quantity of 10µg was injected.

Study of biological activity of *Millingtonia hortensis* and *Scoparia dulcis*

Based on ethno pharmacological data, the plants were reported to possess antioxidant and anti-inflammatory and antidiabetic activities. Therefore, *in vitro* antidiabetic activity was carried out using α-amylase inhibitory assay.

***In vitro* antidiabetic study**

The study was carried out with porcine pancreatic alpha amylase with starch as substrate. Acarbose was used as the standard drug and the two plant extracts were prepared in methanol.

Principle

Alpha amylase digests the starch in reaction mixture to yield maltose and maltose will reduce the 3, 5 dintrosalicylic acid coloring agent to 3-amino-5-nitrosalicylic acid. This reaction will produce a colour change from orange to red. The intensity of red colour produced will be directly proportional to the amount of maltose formed. When an enzyme inhibitor is present in the reaction mixture, digestion of starch, production of maltose and intensity of red colour produced will be less.

Reagents

- 20 mM phosphate buffer of pH 6.9 in methanol (prepared with sodium phosphate monobasic and sodium chloride)
- 1.0% starch solution (prepared in phosphate buffer by boiling)
- Colour reagent was prepared by slowly adding sodium potassium tartarate solution (prepared in the ratio 12g of solid dissolved in 8ml of 2M sodium hydroxide) to 20ml of 96nM 3,5-dintrosalicylic acid (prepared in distilled water) and then diluting the mixture with distilled water
- Enzyme solution was (0.5mg/ml) prepared in phosphate buffer

Procedure

From 1mg/ml stock solution different concentration (2-40 µg/ml) of two plant extracts were prepared in methanol. About 500µl of test / standard was added to 500µl of alpha amylase (0.5mg/ml) and was incubated for 10 minutes at room temperature. 500µl of 1.0% starch solution was added and incubated for another 10 minutes. After that, 1ml of the coloring reagent was added to the reaction mixture and heated in a boiling water bath for 5 minutes. After cooling, the volume was made upto 10ml distilled water and absorbance was measured. Blank was prepared for each set of concentration of test sample by replacing the enzyme solution with buffer. The absorbance was then measured at 540nm. The alpha amylase inhibition was expressed as percentage of inhibition and the IC_{50} values were determined by linear regression plots with varying concentration of plant extract against percentage inhibition (Table: 24-26). All determinations were done in triplicate and acarbose was used as standard. The percentage inhibition was calculated for each individual plant extracts and also combined two plant extracts by using the formula:

Percentage inhibition = 100-[(absorbance of control – absorbance of test)/absorbance of control] x 100

RESULTS AND DISCUSSION

The leaves of *Millingtonia hortensis* and *Scoparia dulcis* contain significant amounts of flavonoids like hispidulin which have pharmacological actions like antidiabetic, antiinflammatory, antimutagenic, antioxidant activities etc. Hence, these plants were selected for the present study.

Successive extractions were carried out by continuous hot percolation method for extraction of powdered leaves of *Millingtonia hortensis* and *Scoparia dulcis.* Petroleum ether, chloroform, ethyl acetate and methanol have been chosen as appropriate solvents for drug extraction. The percentage yield of the successive extracts of *Milligtonia hortensis* and *Scoparia dulcis* petroleum ether, chloroform, ethyl acetate and methanol extracts were found out (Table: 1, 2)

Table 1: The amount and percentage yield of extracts obtained

S.No	Successive leaf extracts of *Millingtonia hortensis (*100gm)	Amount of extract obtained (g)	% of extract obtained (% w/w)
1	Petroleum ether extract	5.2	5.2
2	Chloroform extract	5.5	5.5
3	Ethyl acetate extract	6.7	6.7
4	Methanol extract	7.8	7.8

Table 2: The amount and percentage yield of extracts obtained

Si.No	Successive leaf extracts of *Scoparia dulcis* (100gm)	Amount of extract obtained (g)	% of extract obtained (% w/w)
1	Petroleum ether extract	3.8	3.8
2	Chloroform extract	6.5	6.5
3	Ethyl acetate extract	7.3	7.3
4	Methanol extract	9.6	9.6

For the successive extracts, preliminary phytochemical tests were done which confirmed the presence of flavonoid, alkaloids, tannins and carbohydrates, etc. The preliminary TLC studies showed that *Millingtonia hortensis* and *Scoparia dulcis* methanol, chloroform, ethyl acetate extract spots were near to the standard hisipidulin spot as shown in figure 1, 2. Hence, the further analyses were carried out based on these comparison.

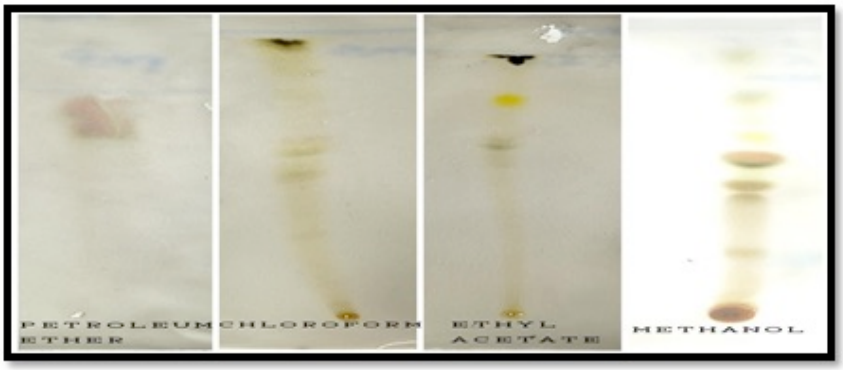

Figure 1: TLC of successive extracts of *Millingtonia hortensis*.

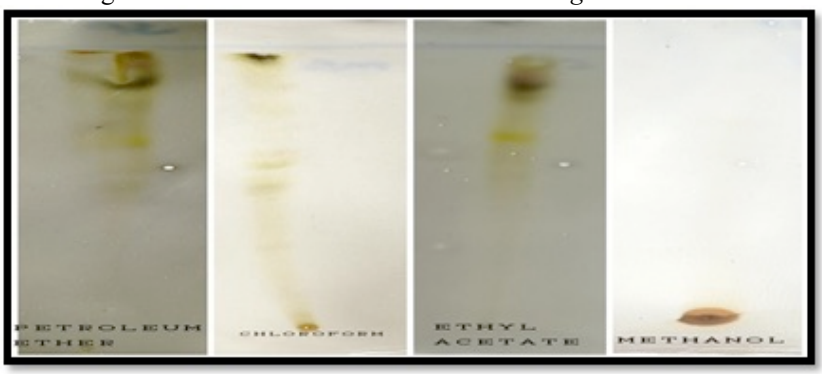

Figure 2: TLC of successive extracts of *Scoparia dulcis*.

HPTLC method

A mobile phase system consisting of toluene: ethyl acetate: formic acid (5:3:1%v/v/v) was selected for the determination of hispidulin by HPTLC. The system gave symmetric peaks with Rf value 0.53 (Figure: 4) for hispidulin at the selected wavelength of 335 nm (Figure: 3). Calibration graph were plotted (Figure: 5). The linearity was found to be in the concentration range of 50-250 ng/spot for hispidulin (r = 0.9922). From the calibration graph slope and intercept values were found to be 29.769 and 1623.880 respectively. The validation parameters were carried out for marker and they are tabulated (Table: 3). HPTLC fingerprintings were obtained for successive leaf extracts of *Millingtonia hortensi*s and *Scoparia dulcis* (Figure: 6-13)

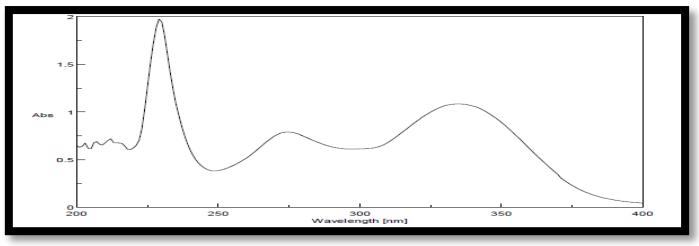

Figure 3: Spectrum of hispidulin on pre-coated TLC plate.

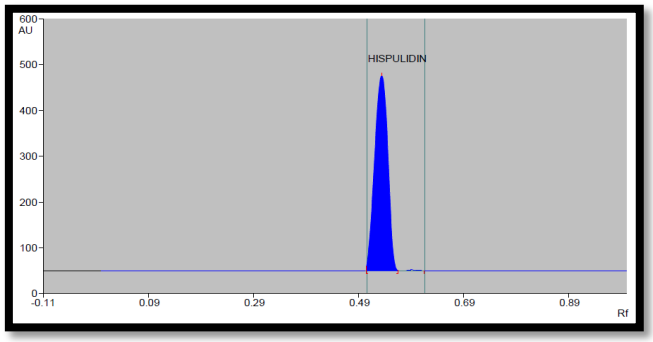

Figure 4: standard chromatogram of hispidulin (250 ng/band)

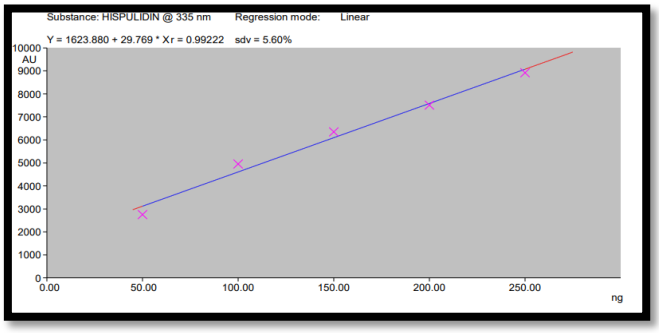

Figure 5: Calibration graph of hispidulin (50 – 250ng/band)

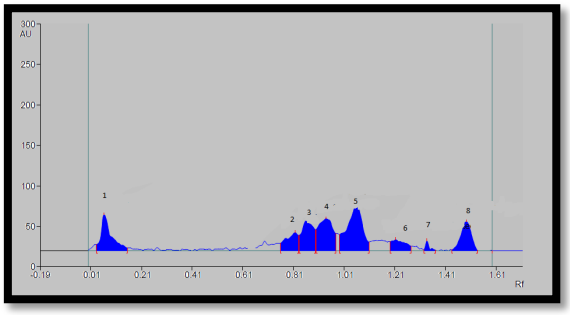

Figure 6: HPTLC fingerprint of *Millingtonia hortensis* petroleum ether extract at 335nm.

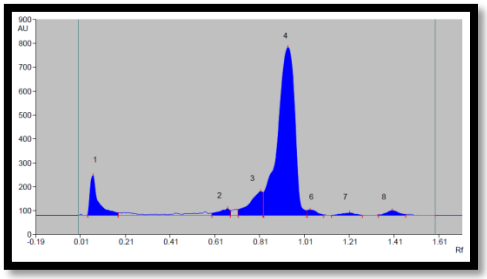

Figure 7: HPTLC fingerprint of *Millingtonia hortensis* chloroform extract at 335nm.

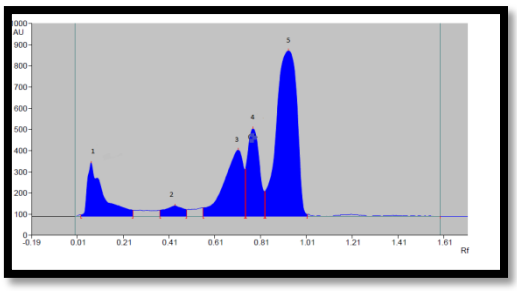

Figure 8: HPTLC fingerprint of *Millingtonia hortensis* ethyl acetate extract at335nm.

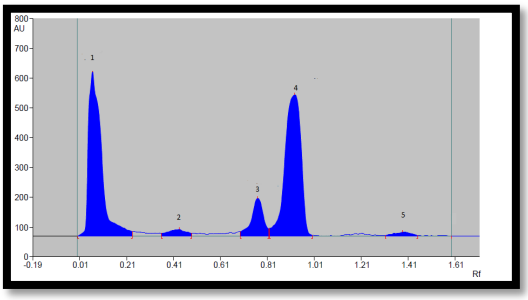

Figure 9: HPTLC fingerprint of *Millingtonia hortensis* methanol extract at 335nm.

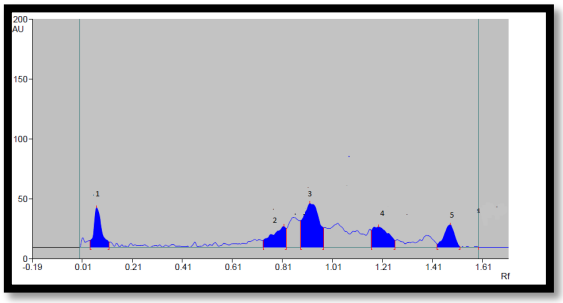

Figure 10: HPTLC fingerprint of *Scoparia dulcis* petroleum ether extract at 335nm.

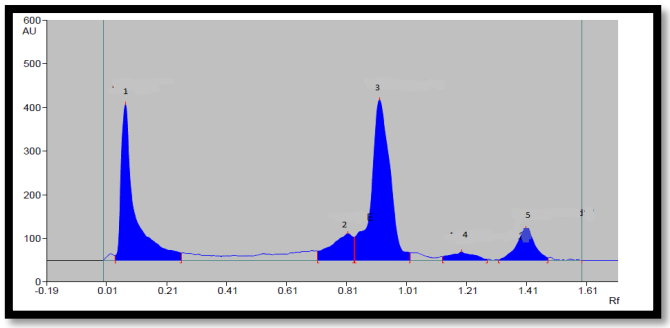

Figure 11: HPTLC fingerprint of *Scoparia dulcis* chloroform extract at 335nm.

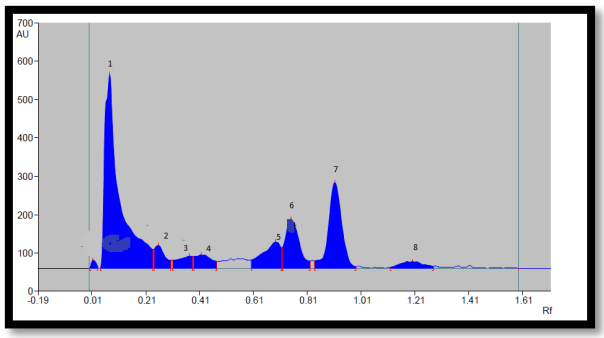

Figure 12: HPTLC fingerprint of *Scoparia dulcis* ethyl acetate extract at 335nm.

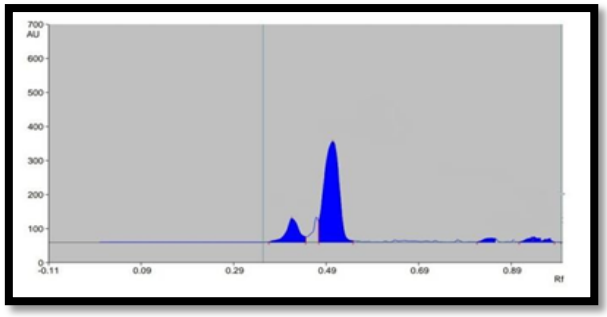

Figure 13: HPTLC fingerprint of *Scoparia dulcis* methanol extract at 335nm.

Table 3: Validation parameters for hispidulin by HPTLC method

Parameters	HPTLC
Linearity	50-250 ng/band
Correlation coefficient	0.9922
LOD	0.0414 ng/band
LOQ	0.1255 ng/band
Precision,	
❖ **Intraday**	0.6057
❖ **Interday**	0.524
❖ **Repeatability**	0.527
Plate stability	5459

RP-HPLC method

In RP-HPLC methods, a mobile phase system containing acetonitrile: phosphoric acid (pH-4.05) (40:60%v/v) was employed for the determination of hispidulin because this system gave symmetric peak shape (Figure: 14) and minimum of tailing with a retention time of 4.8. Linearity of hispidulin was found to be over the range of 0.1 – 0.5 µg/ml and correlation coefficient values for the hispidulin were found to be 0.9989 (Figure: 15). From the calibration graph slope and intercept values were found to be 185360 and 2078 respectively showing good correlation between concentration and peak area response. The validation parameters were carried out for marker hispidulin and are tabulated (Table: 4). HPLC fingerprinting of ethyl acetate extracts of *Millingtonia hortensis* and ethyl acetate, chloroform, methanol extract of *Scoparia dulcis* were performed (Figure: 16-19).

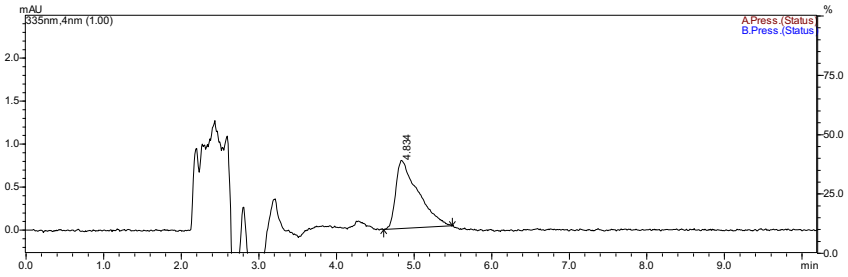

Figure 15: Hispidulin 0.1 µg/ml

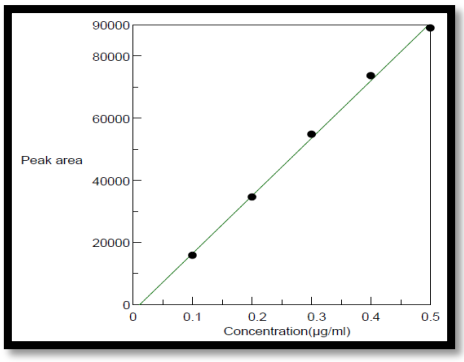

Figure 16: Calibration Graph Of Hispidulin

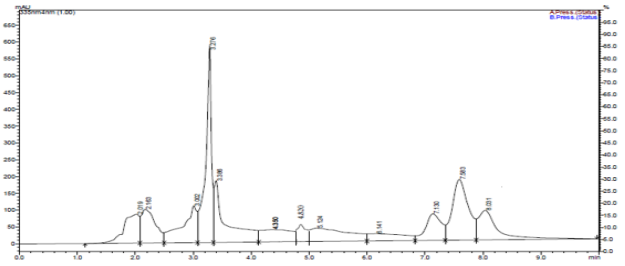

Figure 17: Chromatogram of ethyl acetate extract of *Millingtonia hortensis*

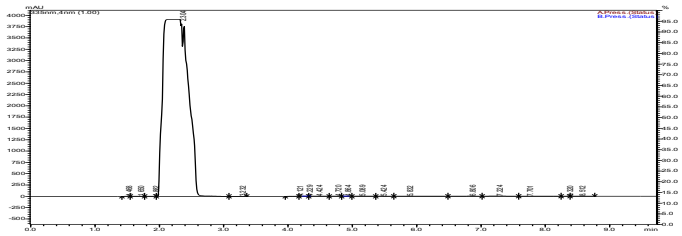

Figure 18: Chromatogram of chloroform extract of *Scoparia dulcis*

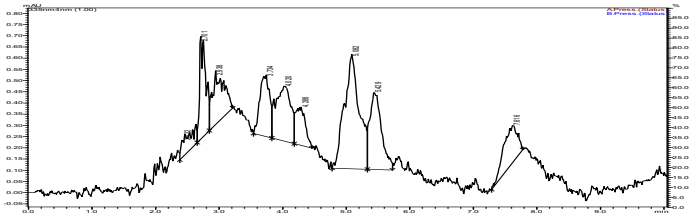

Figure 19: Chromatogram of ethyl acetate extract of *Scoparia dulcis*

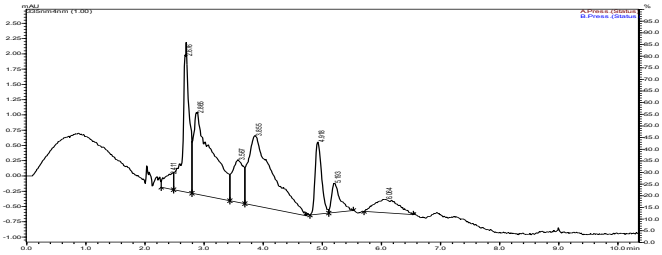

Figure 20: Chromatogram of methanol extract of *Scoparia dulcis*

Table 4: Validation parameters for hispidulin by RP-HPLC method

Parameters	HPLC
Linearity	0.1-0.5 µg/ml
Correlation coefficient	0.9989
LOD	6.5 µg/ml
LOQ	19.7 µg/ml
Precision	
❖ **Intraday**	0.045
❖ **Interday**	0.136
❖ **Repeatability**	0.049
Stability in solution	30 days
Resolution	5.001
No. of theoretical plates	1241.96
Tailing factor	1.45

Biological activity of the extract

The biological activity of the extracts were confirmed by carrying out *in vitro* antidiabetic activity. The antidiabetic activity was confirmed by alpha amylase inhibitory activity which is shown in figure: 20-26. The plants extracts were compared with a standard acarbose for inhibition of amylase and good activity was observed (Table: 5-7). It was seen that with increasing

concentration of the plant extract the activity considerably increased. So, the activity was found to be proportionally increasing with increase in concentration of all the successive extracts of *Millingtonia hortensis* and *Scopria dulcis.* Methanolic and ethyl acetate extracts of *Millingtonia hortensis* have showed higher inhibition activity of 89.98% and 74.20% respectively. Methanolic, chloroform and ethyl acetate extracts of *Scoparia dulcis* have showed inhibition activity of 69.16%, 75.23%, 61.59% respectivity. Combined methanolic and petroleum ether extracts of *Millingtonia hortensis* and *Scoparia dulcis* have showed synergistic activity. Combined chloroform and ethyl acetate extracts of *Millingtonia hortensis* and *Scoparia dulcis* was found to possess antagonistic activity.

Table 5: % Inhibition of successive extracts of *Millingtonia hortensis* extracts

Plant extracts	% Inhibition concentration (µg/ml)							IC_{50} (µg/ml)
	2	4	6	8	10	20	40	
Petro ether extract	0.35±0.1	0.72±0.01	5.53±0.22	8.27±0.52	8.92±0.48	22.22±0.96	24.14±0.76	-
Chloroform extract	33.44±0.18	50.46±0.26	56.31±0.13	56.15±0.42	63.21±0.96	65.01±0.13	68.45±0.18	4.98±0.41
Ethylacetate extract	20.00±1.16	29.74±0.62	29.75±0.01	66.36±0.19	66.73±0.67	73.03±0.77	74.20±0.19	5.98±0.33
Methanol extract	2.64±0.29	24.43±0.55	40.38±0.23	57.97±0.63	69.86±0.78	71.00±0.81	89.98±0.91	6.67±0.14
Acarbose (control)	34.09±0.06	46.11±0.3	53.09±0.47	65.90±0.58	78.45±0.45	87.56±0.76	99.87±0.18	5.87±0.19

Table 6: % Inhibition of successive extracts of *Scoparia dulcis* extracts

Plant extracts	% Inhibition concentration (µg/ml)							IC_{50} (µg/ml)
	2	4	6	8	10	20	40	
Petro ether extract	3.95±0.45	8.31±0.01	31.11±0.06	38.16±0.45	51.24±0.54	57.78±0.02	64.84±0.37	9.47±0.75
Chloroform extract	10.73±0.78	21.90±0.24	33.44±0.78	37.78±0.2	48.08±0.34	52.01±0.24	75.23±0.87	11.5±0.45
Ethylacetate extract	21.72±0.01	26.83±0.32	31.44±0.45	32.45±0.45	40.20±0.41	43.78±0.64	61.59±0.54	30.25±0.15
Methanol extract	28.03±0.54	28.29±0.45	50.54±0.21	53.79±0.64	57.16±0.49	57.34±0.78	69.16±0.36	5.9±0.24
Acarbose (control)	34.09±0.04	46.11±0.43	53.09±0.47	65.90±0.58	78.45±0.45	87.56±0.76	99.87±0.18	5.87±0.16

Table 7: % Inhibition of combined extracts of *Millingtonia hortensis* and *Scoparia dulcis*

Combined plant extracts	% Inhibition concentration (µg/ml)						IC_{50} (µg/ml)
	1	2	4	6	8	10	
Petroleum extracts	45.68±0.46	53.85±0.03	62.09±0.69	62.19±0.25	65.89±0.51	75.02±0.64	1.5.2±0.36
Chloroform extracts	5.45±069	10.25±0.12	34.02±0.39	44.63±0.64	50.08±0.35	50.18±0.41	8.8±0.36
Ethyl acetate extract	1.54±0.34	9.24±0.19	9.79±0.57	12.27±0.34	24.01±0.61	42.05±0.47	-
Methanol extract	43.87±0.61	69.48±0.09	72.37±0.64	83.49±0.57	97.67±0.75	100.10±0.25	1.34±0.32
Acarbose (Control)	20.45±0.37	34.09±0.16	46.11±0.24	53.09±0.16	65.90±0.45	78.45±0.42	5.87±027

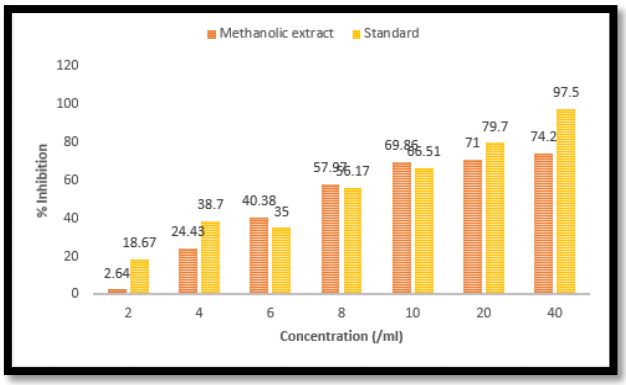

Figure 21: α-amylase activity of methanol extract of *Millingtonia hortensis*.

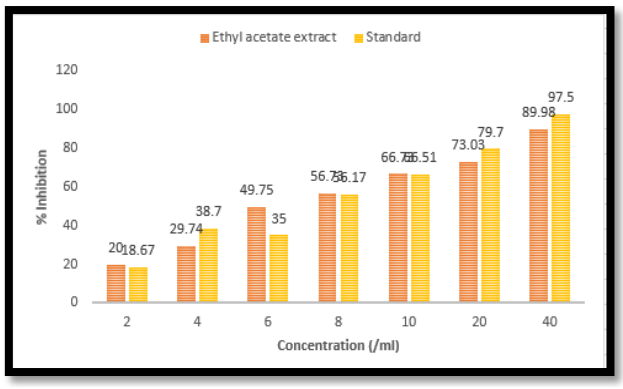

Figure 22: α-amylase activity of ethyl acetate extract of *Millingtonia hortensis*.

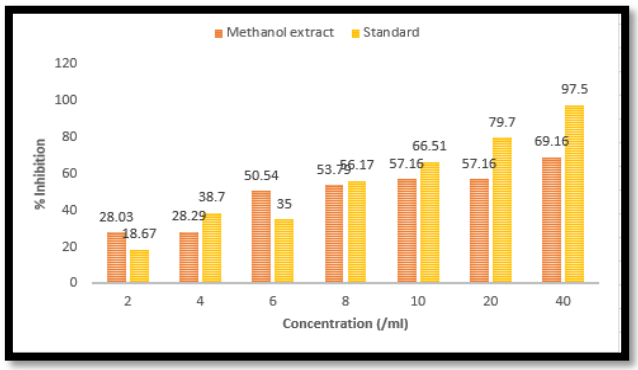

Figure 23: α-amylase activity of methanol extract of *Scoparia dulcis*.

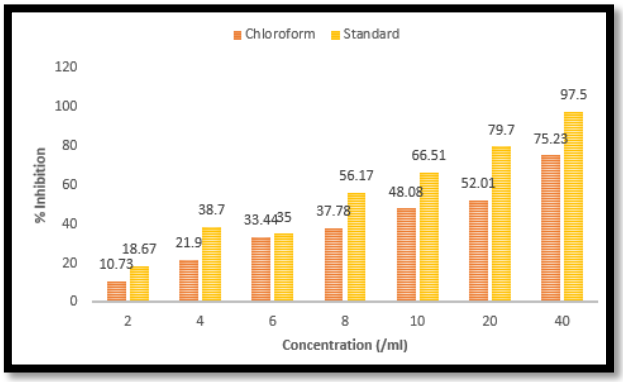

Figure 24: α-amylase activity of chloroform extract of *Scoparia dulcis*.

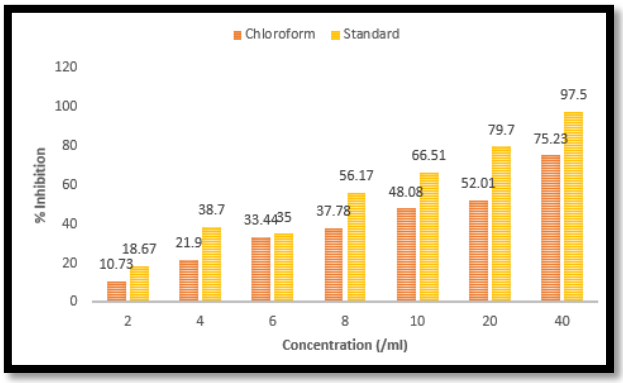

Figure 25: α-amylase activity of ethyl acetate extract of *Scoparia dulcis*.

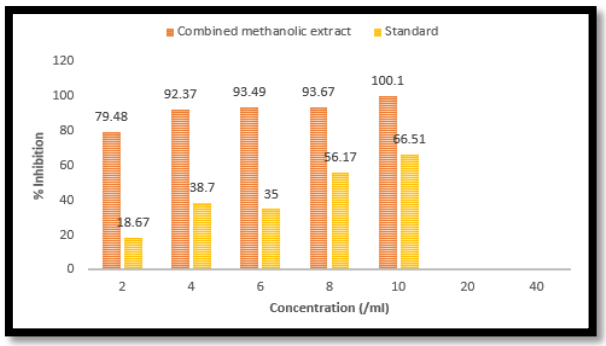

Figure 26: α-amylase activity of combined methanol extracts of *Millingtonia hortensis* and *Scoparia dulcis*.

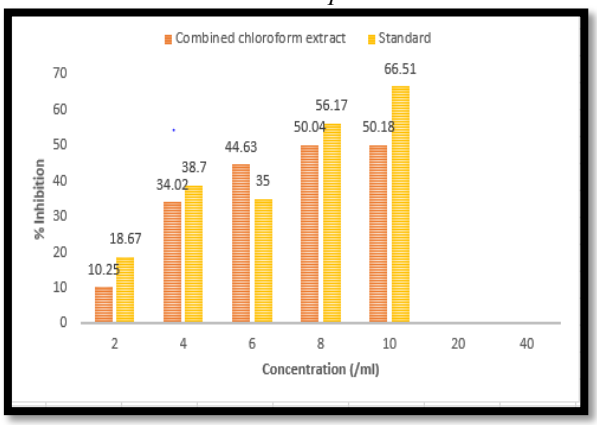

Figure 27: α-amylase activity of combined chloroform extracts of *Millingtonia hortensis* and *Scoparia dulcis*

CONCLUSION

Authentication, investigation and standardization of *MIllingtonia hortensis* and *Scoparia dulcis* were carried out in the current work. The study was conducted on the successive leaf extracts of *Millingtonia hortensis* and *Scoparia dulcis.* The preliminary phytochemical tests for the presence of flavonoids, alkaloids, tannins and carbohydrates were carried out for all the extracts.The current research work marches to lay down chromatographic techniques like RP-HPTLC and HPLC for the standardisation and quantification of hispidulin present in *Millingtonia hortensis* and *Scoparia dulcis* plant extracts. The fingerprint and chromatogram of HPTLC and HPLC analysis of biomarker hispidulin and herbal extracts will provide data for identification and standardisation of bioactive constituent in herbal products. The methods were developed and validated complying with ICH regulation which is of paramount importance for the herbal drug manufacturers or the importers. The α-amylase activity-based bioassay carried out for the *in vitro* antidiabetic activity has proven that the methanol extract of *Millingtonia hortensis* has high inhibition of 89.98% at 40mcg/ml concentration. Chloroform extract of *Scoparia dulcis* has highest alpha amylase inhibition of 75.23% at concentration of 40mcg/ml. The result outcomes of this project would support herbal industries and phytochemical research to use this as a primary reference documents since, till date no such studies are available in the literature for the two plants viz., *Millingtonia Hortensis* and *Scoparia dulcis.*

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REFERENCES

1. Arun R, Sravya B, Roja C. Standardisation of herbal formulation-An overview. International Journal of Phytotherapy 2012; 2(2): 74-78.
2. Bele AA, Khale A. Standardisation of herbal drugs: An overview. International Research Journal of Pharmacy 2011; 2(12): 56-60.
3. Kunle, Folsahade O, Egharevba, Henry O, Peter O. Standardisation of herbal medicines – An overview. International Journal of Biodiversity & Conservation 2012; 4(3): 101-107
4. ICH Topic Q 2 (R1) Validation of Analytical Procedures: Text and Methodology. EMEA guideline 1995.
5. Kazeem MI, Adamson JO, Ogunwande IA. Modes of Inhibition of *α*-Amylase and *α*-Glucosidase by Aqueous Extract of *Morinda lucida* Benth Leaf. BioMed Research International 2013; 1(1): 1-9.
6. Hye Jin Kim, Eun Hye Jee, Kwang Sung Ahn, Hyo Sook Choi, Young Pyo Jang.

Identification of marker compounds in herbal drug TLC with DART-MS. Archives of Pharmacal Research 2010; 33(9): 78-89.
7. Nagaraja MS, Padmaa M Paarakh. *Millingtonia hortensis* Linn. - A Review. Pharmacology online 2011 ; 2(1): 597-601.
8. Kavitha, A. and V. Mary Kensa. Pharmacognostic studies on *Millingtonia hortensis* Linn. Kongunadu Research Journal 2016; 3(2): 52-58.
9. Meera Paul, Kavitha Vasudevan, Krishnaja K. R. *Scoparia dulcis*: A review on its phytochemical and pharmacological profile. Innoriginal International Journal of Science 2017; 4(4): 17-21.
10. Kanika Patel, Dinesh Kumar Patel. Medicinal importance, pharmacological activities, and analytical aspects of hispidulin: A concise report. Journal of Traditional and Complementary Medicine 2016: 1-6.
11. https://aurigaresearch.com/herbal-testing/chromatographic-fingerprinting/
12. Alaerts G, Matthijis N, Smeyers J-Verbeke, Vander Heyden Y. Chromatographic fingerprint development for herbal extracts: a screening and optimization methodology 2007; 2(1): 1-8.
13. https://aurigaresearch.com/herbal-testing/chromatographic-fingerprinting/
14. Alaerts G, Matthijis N, Smeyers J-Verbeke, Vander Heyden Y. Chromatographic fingerprint development for herbal extracts: a screening and optimization methodology 2007; 2(1): 1-8.
15. Janaki A, Kaleena PK, Elumalai D, Hemalatha P, Babu M, Velu K, Sudha Ravi. Phytochemical screening, antioxidant and antibacterial activities of *Millingtonia hortensis* (L). International Journal of Current Pharmaceutical Research 2017; 5(9): 162-167.
16. Kanika Patel , Dinesh Kumar Patel. Medicinal importance, pharmacological activities, and analytical aspects of hispidulin: A concise report. Journal of Traditional and Complementary Medicine 2016: 1-6.
17. Jadhav S, Kadam VN, Mohite SK. development and validation of UV spectrophotometric method for estimation of rutin in extract of *Millingtonia hortensis* Linn. Journal of Current Pharma Research 2014; 4(4): 1281-1285.
18. Mageswari S, Pawan Kumar Sagar, Meera Devi Sri P, Murugeswaran R, Rampratap Meena, Shamshul Arfin and Asiya Khanum. Pharmacognostical evaluation and HPTLC fingerprinting studies of *Millingtonia hortensis* L. f. Leaf. Hippocratic Journal of Unani Medicine 2017; 12(2): 21-36.
19. Cindy Kitcher, Nana Ama Mireku-Gyimah. Pharmacognostic standardization of the leaf and stem bark of *Millingtonia hortensis* Linn. (Bignoniaceae). International Journal of Pharmaceutical Research & Allied Science. 2021; 10(1):42-49.
20. Snarma RC, Zaman. A and Kidwai AR. Chemical examination of *Millingtonia hortensis.* Phytochemistry 1968; 7: 1891-1892.
21. Ahana Sarkar, Pranabesh Ghosh, Susmita Poddar, Tanusree Sarkar, Suradipa Choudhury and Sirshendu Chatterjee. Phytochemical, botanical and Ethnopharmacological study of *Scoparia dulcis* Linn. (Scrophulariaceae): A concise review. The Pharma Innovation Journal 2020; 9(7): 30-35.
22. Naresh Kumar Kataria , Venkatanarayana, Parag Deodharb , Chandra Sekhar and Ramya Billurc. Quantitation of alpha amyrin in *Scoparia dulcis* L. whole plant powder by high performance liquid chromatography. Schlors Research Library 2013; 5(6): 234-240.
23. Qing Liu, Qi-Ming Yang, Hai-Jun Hu, Li Yang, Ying-Bo Yang, Gui-Xin Chou and Zheng-Tao Wang. Bioactive diterpenoids and flavonoids from the aerial parts of *Scoparia dulcis.* Journal of Natural Products 2014; 77: 1594−1600.
24. Osei-Safo. D, Chama MA, Addae-Mensah and Waibel. Hispidulin and other constituents of *Scoparia Dulcis* Linn. Journal of Science and Technology 2009; 29(2): 7-13.
25. Geethi Pamunuwa, Nedra Karunaratne and Viduranga Y. Waisundara. Antidiabetic properties, bioactive constituents, and other therapeutic effects of *Scoparia dulcis*. Hindawi Publishing Corporation 2016; 1(1): 1-11.
26. Champanerkar PA, Vaidya VV , Rane NJ ,Kekare MB , Pabrekar PN. Simultaneous determination of β-sitosterol and lupeol from *Scoparia dulcis* L. by HPLC. Analytical Chemistry an Indian Journal 2010; 9(1): 71-75.
27. Devang Khamar, Raviraj Devkar, Reshma KK, Shreedhara, Manjunath Setty, Smitha Hegde. Enhanced hispidulin production *in vitro* from callus culture of *Millingtonia hortensis* L.f. Iinternational Journal of Pharmacy and Biological Sciences 2013; 3(2): 633-639.
28. Alper gökbulut. Determination of hispidulin in the flowers of *Inula viscosa* (L.) *Aiton* Using HPLC and HPTLC Methods. Turkey Journal of Pharma Science 2016; 13(2): 159-166.