

**COMPARATIVE PHARMACOGNOSTIC, PHYTOCHEMICAL
SCREENING OF *MORINDA TINCTORIA* (ROXB.) LEAF EXTRACT
COLLECTED AT DIFFERENT SEASONS**

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ABSTRACT

Morinda tinctoria (Roxb.) (Fam: Rubiaceae) is a medicinally important plant being used in different indigenous systems of medicine such as Ayurveda, Siddha, Unani.^[1] Extract of its leaves, stems and fruits are used in the treatment of gastropathy, dyspepsia, diarrhoea, stomach ulcer, wounds, gout, inflammation, hernia, and fever. Its comparative pharmacognostic and phytochemical study based on seasonal variation has not been explored fully so far. In this study a detailed pharmacognostic and phytochemical investigation of the crude extracts of the leaf (collected at winter and summer) were executed using standard methods. In quantitative analysis the active constituents like flavanoids, steroids, tannins, phenols, alkaloids showed significant variation in winter and summer season. The glycosides are absent in

summer season. As these active metabolites are associated with antiviral, antibacterial, anticonvulsant, psycho-pharmacological, antidiabetic and anti-inflammatory properties, they are widely used in treatment of various diseases.^{[2][3][4]} Thus, the potential of the plant as a source of the active metabolites is self-explanatory.

KEYWORDS: *Morinda tinctoria*, Pharmacognosy, Phytochemicals, Metabolites, Indigenous medicine.

INTRODUCTION

The kingdom Plantae contains about 300–315 thousands of plant species, and many of them are used as medicines for years. The genus *Morinda* are one of the ethnic plants of tropical countries and are used in folk medicine since ages. *Morinda tinctoria*, commonly known as Nunaa, is grown in several parts of Southeast Asia, especially in the agricultural lands and unrefined lands.^[5] Many species of the genus *Morinda* (*Rubiaceae*) are found to be rich in secondary metabolites having medicinal value.^[6] The drug is reported to be used as styptic, digestive, carminative, febrifuge, tonic, dysentery, inflammations, boils, antioxidants, and anticancer. Hence, it is used in gastropathy, dyspepsia, diarrhoea, stomach ulcer, wounds, gout, inflammation, hernia, and fever.^[7] Some bioassay research conducted on *Morinda tinctoria* showed it to display a wide spectrum of antibacterial activity.^[8] Few phytochemical studies on *Morinda tinctoria* reported the presence of glycoside (morindone), tinctomorone, damnacanthal and nor-damnacanthal, ursolic acid, anthraquinones, soranjidol, ibericin.^[9] Another species *Morinda citrifolia* L. (Great *Morinda*, Indian Mulberry) a drug widely used in indigenous systems of medicine for antibacterial, antiviral, antifungal, antitumour, antihelminthic, anti-inflammatory, analgesic and immune enhancing properties. Its use in treatment of hypertension, painful menstruation, arthritis, gastric ulcers, diabetes gout, gingivitis, and depression was reported.^[10] Hence, *Morinda tinctoria* can be used as a substitute for *Morinda citrifolia* in medicine if it contains active metabolites or phytochemicals like the latter. The use of substitutes for genuine drugs is an important practice in the drug market. Generally, substitutes are the drugs with the same active constituents as found in the original one or a little less percentage of the active constituents or of less therapeutic value. Substitutes may serve the purpose of the original drug if the latter is not available. Therefore, detailed phytochemical screening of *Morinda tinctoria* was carried out in the present investigation. A number of variants in the atmosphere like dyes, ammonia, nitrite etc, a variety of living organisms (bacteria, fungi, virus, nematodes etc.,) and abiotic factors (temperature, humidity, pH etc.,) interact with the plants and greatly affect the metabolites /constituents production rate. Involvement of plants in defense mechanism shows immense qualitative and quantitative variability in their secondary metabolites. The domains of variability includes the chemical classes of the secondary metabolites such as alkaloids, terpenoids, flavonoids, steroids, functional, conjugational and multimeric varieties exist within the same class of compounds. The metabolic processes leading to accumulation of the active constituents in the plant are basically controlled by the physiological age of the plant, the surrounding environmental conditions, seasons as well as the genetic factors. Therefore, it

is of great importance, to follow up the seasonal variation, growth parameters and chemical composition of the plant throughout the growing season.

METHODOLOGY

The plant material and extraction

The plant *Morinda tinctoria* leaf was authenticated by Dr.M.V.Krishnaraj, Assistant Professor, Department of botany, Baselius college, Kottayam, Kerala. The leaf of *Morinda tinctoria* were carefully collected in the different season viz Winter (December-2013) and Summer (April-2014) from Wadakanchery, Thrissur (Dt.) and Pattambi, Palakkad (Dt.) Kerala, India. It was cleaned thoroughly with distilled water and was dried under shade. The shade dried leaf pulverized in a mechanical grinder to obtain coarse powder, extracted with ethanol and used for the study. The research involves pharmacognostic evaluation, qualitative and quantitative phytochemical analysis of the leaf of *Morinda tinctoria* (Rubiaceae) collected at different season (MTS1 and MTS2) following standard methods.

Macroscopic evaluation

Organoleptic parameters like size, shape, colour, odour, taste and other external characteristics of *Morinda tinctoria* leaves collected at winter(MTS1) and summer (MTS2) season were studied. These organoleptic characters provides the simplest and quickest means to establish the identity and purity and there by ensure the quality of a particular sample.

Microscopic evaluation

Section of the leaf

The leaf samples were cut and fixed in FAA (formalin-5ml+ acetic acid-5ml+70% ethyl alcohol-90ml). After 24 hrs of fixing, the specimens were dehydrated with graded series of tertiary butyl alcohol. Infiltration of the specimen was carried out by gradual addition of paraffin wax (melting point-58-60°C) until tertiary butyl alcohol solution attained super saturation. The specimens were cast into paraffin blocks. The paraffin embedded specimens were sectioned with the help of rotary microtome. The thickness of the sections was 10-12 µm. The sections were dewaxed and stained with 10% safranin. The sections were mounted with 10% glycerin on clean glass slides, and observed under microscope.^[11]

Powder microscopy

The powder of the dried leaves was studied for the identification of various cellular components. Powdered leaves were cleared with chloral hydrate solution (50g of chloral

hydrate dissolved in 20ml distilled water) and mounted in glycerin medium after staining. Different cell components were studied and measured. Microscopic description of tissues was supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nikon lab photo 2 microscopic units. For normal observations bright field was used.

Flourescent Microscopy

The powdered drug was treated with different reagents like 1N NaOH in water, 1N NaOH in methanol, 50% KOH, 1N HCl, 50% H₂SO₄, 50% HNO₃, Con. HNO₃, Con. H₂SO₄ and Iodine water and was examined under UV light in UV chamber and visible light to observe fluorescence. One of the important parameter of pharmacognostical evaluation is Fluorescence test. It is the phenomenon exhibited both in visible and UV- light by various chemical constituents present in the plant powders. Qualitative assessment of crude drug can be done by using this method.^[12]

Physicochemical evaluation^[13]

Determination of ash values

Total ash: 2-3g of accurately weighed drug was incinerated in a tarred platinum or silica dish at a temperature not exceeding 450°C until free from carbon. It was cooled and weighed. The percentage of ash was calculated with reference to the air dried drug.

Acid insoluble ash

The total ash obtained was boiled for 5 minutes with 25 ml of dilute HCl. The insoluble matter was collected in a Gooch crucible or on an ash less filter paper. It was washed with hot water and ignited to constant weight. The percentage of acid insoluble ash was calculated with reference to the air-dried drug.

Water soluble ash

The total ash obtained was boiled for 5 minutes with 25ml of water. The insoluble matter was collected in a Gooch crucible or on an ash less filter paper. It was washed with hot water and ignited for 15 minutes at a temperature not exceeding 450°C. The weight of insoluble matter was subtracted from the weight of ash and the difference in weight represents the water soluble ash. The percentage of acid insoluble ash was calculated with reference to the air-dried drug.

Determination of extractive values

In the present study alcohol soluble and water soluble extractive values of *Morinda tinctoria* were determined.

Alcohol soluble extractive value

The air dried and coarsely powdered leaf (5gm) was macerated with 100ml of 95% alcohol in a Stoppard conical flask for 24 hours. It was shaken frequently for six hours and allowed to stand for 18 hours. This extract was filtered rapidly into a 50 ml glass beaker. Then sufficient filtrate was collected, 25 ml of the filtrate was transferred to an evaporating dish of known weight. Then it was dried in an oven at 100°C, cooled and weighed. The percentage w/w of alcohol soluble extractive value was calculated with reference to air-dried drug.

Water soluble extractive value

The air dried and coarsely powdered leaf (5gm) was macerated with 100 mL chloroform-water in a Stoppard conical flask for 24 hours. It was shaken frequently for six hours and allowed to stand for 18 hours. When sufficient filtrate was collected, 25 mL of the filtrate was transferred to an evaporating dish of known weight. Then it was dried in an oven at 100°C cooled and weighed. The percentage w/w of water soluble extractive value was calculated with reference to air-dried drug.

Loss on drying

The air dried and powdered leaves of *Morinda tinctoria* was weighed and dried in a hot air oven at 100°C until a constant weight was obtained. The loss in weight was recorded as loss on drying.

Determination of crude fiber

2g of accurately weighed drug was extracted with petroleum ether or ether to remove fat contents (initial boiling temperature between 37 -38°C). After the extraction with ether 2g of dried material was boiled with 200mL of H₂SO₄ for 30mts with bumping chips. Whole mixture was boiled for 30mts under reflux in 500mL flask. After boiling, it was filtered through muslin cloth and washed with boiling water until washings are no longer acidic. Again it was boiled with 200mL of NaOH solution for 30mts and filtered through muslin cloth and washed with 25mL of 1.25% H₂SO₄, 50mL portion of water and 25mL of alcohol. The residue was removed and transferred to an ashing dish (pre-weighed dish W₁). It was dried for 2 hours at 130±2°C and then cooled in a desiccator and weighed (W₂). It was ignited for

30mts at $600\pm 15^{\circ}\text{C}$ and then cooled in a desiccators and reweighed (W_3). The percentage crude fiber content in the ground sample was calculated.

Phytochemical evaluation

Preliminary phytochemical screening

The Ethanolic extracts of *Morinda tinctoria* leaf collected at winter and summer season (EMTS1 and EMTS2) was subjected to preliminary qualitative phytochemical screening as per the standard procedures to determine the presence of various phytoconstituents such as Steroids, tannins, alkaloids, flavanoids, phenols, saponins, glycosides, carbohydrates in the extracts.^[14]

Quantitative estimation of Steroids, Tannin, Alkaloids, flavonoids, Phenols

The quantitative estimation of steroids, tannin, alkaloids, flavonoids, phenols, in ethanolic extracts of *Morinda tinctoria* winter season (EMTS-1) and summer season (EMTS-2) was performed by prescribed methods.^[15]

Quantitative estimation of steroids by HPTLC Method

Sample preparation

50mg of sample (EMTS1 and EMTS2) was taken in a separate beaker and dissolved in 1ml methanol each, filter through a syringe filter, injected to HPTLC.

Standard preparation

100 mg of standard compound was dissolved in 100 ml methanol. Stigmasterol was used as the standard.

Mobile phase

Toluene: Chloroform: Methnol in a ratio of 8:3:1.

High performance thin layer chromatography conditions

Stationary phase: Silica gel aluminum plate 60 F 254 (plate size 5×10.0 cm), Injection volume: $10\mu\text{l}$ (0.5mg), each in the case of extracts (EMTS1 and EMTS2), $4\mu\text{l}$ (0.004mg) in the case of standard Band width: 8 mm, Chamber type: Twin trough glass chamber 20×10 cm, Visualization: after derivatisation (using Anisaldehyde sulfuric acid-50ml at 105°c for 10Minutes) under 366 nm and visible light, Scanner: Camag TLC scanner. (Instrument Details: HPTLC – CAMAG make with Parts Linomat V, Scanner V and Visualizer).

The concentration was calculated by using the following equation

$$\frac{\text{Sample area}}{\text{Std area}} \times \frac{\text{standard concentration}}{\text{Sample concentration}} \times \frac{\text{purity of standard}}{100} \times 100$$

RESULTS AND DISCUSSION

Macroscopic evaluation

The leaves are green, simple, opposite, oblong to lanceolate, rough surface due to presence of trichomes on both surfaces. length 15-21cm (winter), 15-25cm (summer), width 4.8cm (winter) 4cm (summer) Odour not characteristic and taste slightly bitter. All the above characters are same in both sample (MTS1 and MTS2) except length and breadth, its shows slight difference.

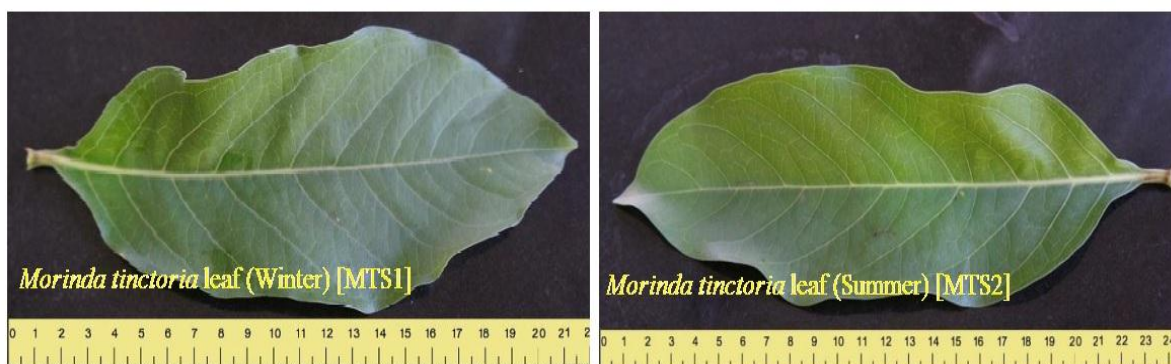


Fig. 1: *Morinda tinctoria* leaf (Winter) [MTS1] and *Morinda tinctoria* leaf (Summer) [MTS2].

Microscopic evaluation

Transverse section of *Morinda tinctoria* leaf passing through midrib shows upper and lower epidermis with trichomes. 3-5 layers of Collenchymatous tissues are seen under both upper and lower epidermis. Chollenchymatous cells are followed by the parenchymatous cells. Vascular bundle is located at the center portion. Mesophyll cells contain palisade and spongy parenchyma. The study shows no much variation in microscopic characters of *Morinda tinctoria* leaf in winter [MTS1] and summer season [MTS2].

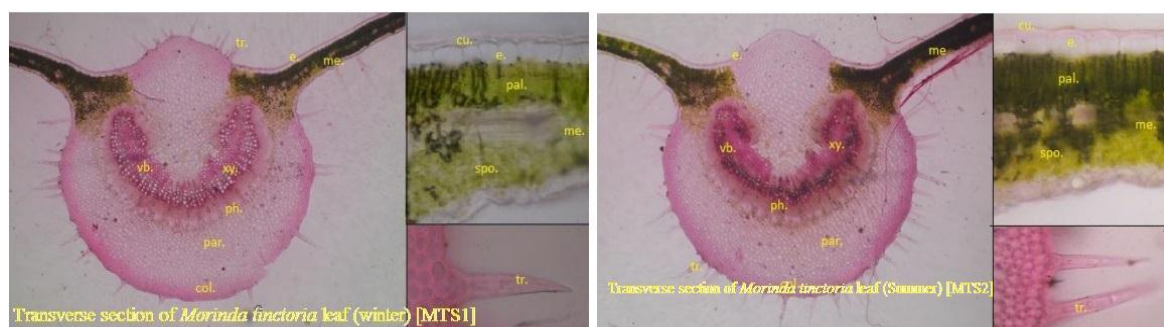


Fig. 2: Transverse section of *Morinda tinctoria* leaf [tr.: Trichome; e.: Epidermis; cu.: Cuticle; vb.: Vascular bundle; ph.: Phloem; xy.: Xylem; par.: Parenchyma cells; col.: Collenchyma cells; pal.: Palisade cells; spo.: Spongy parenchyma cells].

Powder Microscopy: The powder microscopy of *Morinda tinctoria* leaf shows fragment of mesophyll cells; parenchyma cells; crystals of calcium oxalate; trichomes and fragments of spiral vessels. The study shows no significant variation between (winter) [MTS1] and (Summer) [MTS2] sample.

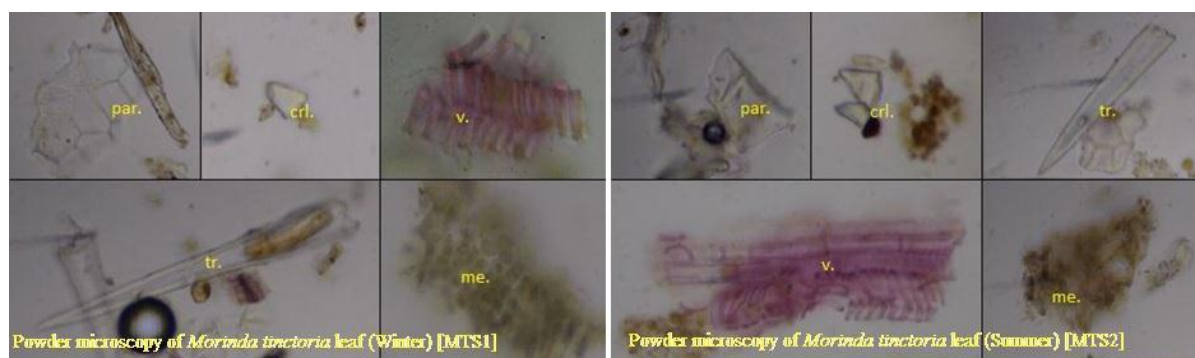


Fig. 3: Powder microscopy of *Morinda tinctoria* leaf [t.: Trichome; v.: Vessel; me.: Mesophyll cells; par.: Parenchyma cells; crl.: Crystals of calcium oxalate.].

Fluorescent Analysis: The fluorescence analysis of the leaf extracts were observed under ordinary visible light and also under UV light and recorded in Table 1. The fluorescence analysis of leaf powder of two samples (MTS1 and MTS2) of *Morinda tinctoria* showed both samples produce the same characteristic coloration, depending upon the presence of various chemical constituents of plant material. Some metabolites show fluorescence in the visible range in daylight. Ultraviolet light produces fluorescence in many natural products (e.g. alkaloids) which do not visibly fluoresce in daylight. These findings indicate both samples contain active chemical constituents showing fluorescence in UV and visible light.^[12]

Table. 1: Fluorescent Analysis of *Morinda tinctoria* leaf in (Winter) [MTS1] and (Summer) [MTS2].

Sl. No.	Fluorescent Analysis of MTS1			Fluorescent Analysis of MTS2			
	Treatment	Visible light	Short wave	Long wave	Visible light	Short wave	Long wave
1	Powder as such	Blackish Green	Pale green	Dark brown	Blackish Green	Pale green	Dark brown
2	Powder +1 N NaOH in water	Blackish Green	Green	Black	Blackish Green	Green	Black
3	Powder + 1 N NaOH in methanol	Blackish Green	Dark green	Dark brown	Blackish Green	Dark green	Dark brown
4	Powder +50%KOH	Blackish Green	Green	Blackish brown	Blackish Green	Green	Blackish brown
5	Powder + 1N HCl	Grayish black	Green	Black	Greyish black	Green	Black
6	Powder +50% H ₂ SO ₄	Grayish black	Green	Black	Greyish black	Green	Black
7	Powder +50% HNO ₃	Reddish brown	Green	Blackish brown	Reddish brown	Green	Blackish brown
8	Powder +Con: HNO ₃	Reddish brown	Green	Blackish brown	Reddish brown	Green	Blackish brown
9	Powder + Con: H ₂ SO ₄	Black	Green	Black	Black	Green	Black
10	Powder +Iodine water	Black	Green	Black	Black	Green	Black

Physicochemical evaluation

Table 2 shows the details of physicochemical evaluation *Morinda tinctoria* leaf in Winter [MTS1] and Summer seasons [MTS2]. The results illustrates that Acid soluble ash, PH, Water soluble Extract, Water soluble Ash, Crude Fiber contents are more in MTS1 sample than MTS2. The other parameters like Alcohol soluble extract Lose on drying, Total ash are more in MTS2 than MTS1.

Table. 2: Physicochemical evaluation –*Morinda tinctoria* leaf Winter[MTS1] and Summer[MTS2].

Sl No	Parameters	MTS1	MTS2
1	Acid soluble ash	0.88 %	0.76%
2	Alcohol soluble extract	12.67 %	13.27%
3	Lose on drying	9.94 %	10.24%
4	PH	5.54	5.37
5	Total ash	8.03 %	9.30%
6	Water soluble Extract	26.99 %	26.53%
7	Water soluble Ash	2.72 %	1.98%
8	Crude Fibre	18.40 %	17.37%

Phytochemical analysis: Table: 3 shows the presence or absence of phytoconstituents in ethanolic extracts of *Morinda tinctoria* Leaf in winter season (EMTS1) and summer season (EMTS2). The study shows the presence of steroids, tannin, alkaloids, flavonoids and phenols in both extracts. The saponins and carbohydrates are absent in both extracts. The glycosides present only in winter season (EMTS1).

Table. 3: Shows the presence /absence of phytoconstituents in ethanol extract of *Morinda tinctoria* in winter [EMTS1] and summer [EMTS2] after preliminary phytochemical screening.

Sl. No	Phytoconstituents	Method used	EMTS1	EMTS2
1	Steroids	Salkoswki's test	+	+
2	Tannins	Ferric chloride test	+	+
3	Alkaloids	Dragendroff's reagent test	+	+
4	Flavanoids	Shinoda test	+	+
5	Phenols	Folinciocalteu reagent test	+	+
6	Saponins	Foam test or froth test Heamolysis test	-	-
7	Glycosides	Picric acid test	+	-
8	Carbohydrates	Benedicts's test	-	-

Quantitative Estimations: Quantitative analysis of EMTS1 and EMTS2 shows seasonal variation in relative quantity of phytoconstituents. The result shows that in winter the phytoconstituents like steroids, tannins, alkaloids and phenols are more than summer season. There are significant variation in alkaloids, and phenols. Concentration of flavonoids is slightly more in summer season when compared with winter season. There is no much variation in the concentration of steroids in both seasons.

Table. 4: The concentration of steroids, tannin, alkaloids, flavanoids. phenols, present in EMTS1 and EMTS2 extract of leaf of *Morinda tinctoria*.

Sl. No.	Name	EMTS1(g/100gm)	EMTS2(g/100gm)
1	Steroids	1.06	0.99
2	Tannin	5.20	4.49
3	Alkaloids	17.26	14.70
4	Flavanoids	3.31	3.40
5	Phenols	7.80	4.59

The medicinal properties like action on cvs, antiulcer may be due to the presence of Steroids.^[16] Tannins were found in slightly high quantity in the leaf collected at winter 5.20%. Tannins are polyphenolic compounds, which can be either hydrolysable and condensed tannins. The content of tannins in *Morinda tinctoria* help in its use anti-

inflammatory, cardioprotective, antitumour, antioxidants antidiabetic etc.^[17] Amount of alkaloids in the leaf collected at winter season was found to be 17.26%. The medicinal properties of *Morinda tinctoria* like analgesic, antibacterial, and antifungal and antispasmodic actions and antiulcer also due to the presence of alkaloids.^[18]

The content of Flavanoids was found 3.40% in summer season. The Antioxidant, anti-inflammatory activities of *Morinda tinctoria* may be due to the presence flavanoids.^[19] The content of phenols was found to be 7.80% in winter. Phenols, aromatic compounds with a hydroxyl group are widespread in plant materials. They occur in all parts of the plants. The use of *Morinda tinctoria* in the treatment of, antibacterial, antifungal, anti-inflammatory due to the presence and cooling properties of phenols.^[20]

Quantitative estimation of steroids by HPTLC Method^[21]

Quantitative analysis of steroids on EMTS1 extract and EMTS2 extract shows that EMTS1 extract have 1.06 g/100gm of sample and EMTS2 extract have 0.99g/100gm of the sample respectively in terms of sterol as standered.

Table. 5: The Rf value and area of EMTS1 EMTS2 and standard Sterol.

Sl. No.	Name	Rf Value	Area
1	EMTS1	0.49±0.02	3673.0
2	EMTS2	0.48±0.02	4321.7
3	STEROL	0.47±0.02	2771.8

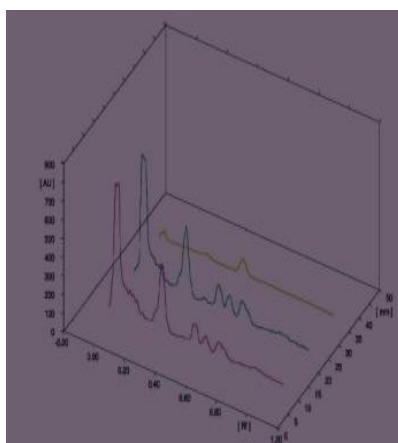


Fig. 4: Densitometric chromatogram of stigmasterol and ethanolic extracts of *Morinda tinctoria* (EMTS1andEMTS2) Position 12.5mm, 25.0 and 37.5mm shows the chromatogram of EMTS1, EMTS2 and STEROL respectively.

Fig-5,6 and 7-below shows the HPTLC chromatogram of EMTS1 EMTS2 and STEROL. In the 9th position peak of EMTS1 and EMTS2 at an Rf value of 0.49 and 0.48 respectively is matched with the 9th peak in the chromatogram of STEROL (Rf value-0.47).

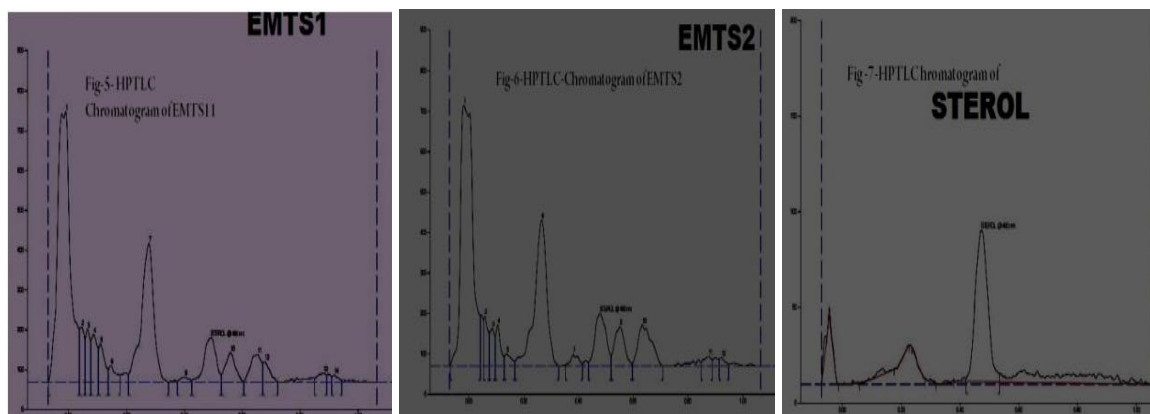


Fig. 5,6 and 7-HPTLC chromatogram of EMTS1 EMTS2 and STEROL.

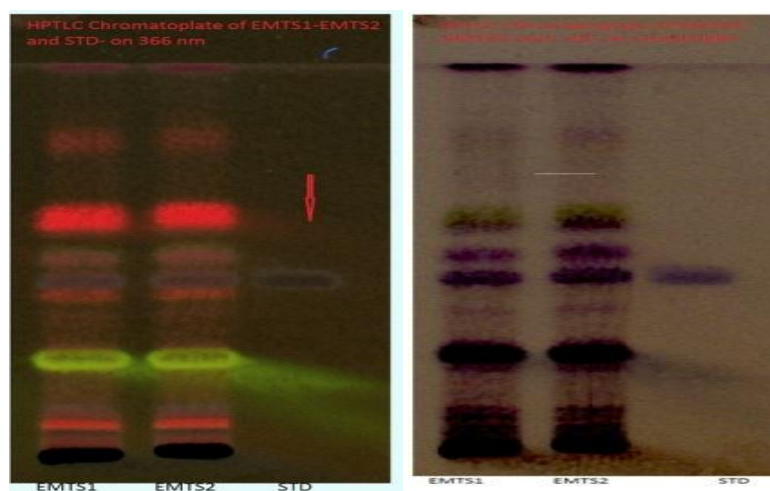


Fig. 8 and 9 Shows the HPTLC Chromatoplate of EMTS1 and EMTS2 and STEROL after derivatization (using Anisaldehyde sulfuric acid-50ml at 105°C for 10 Minutes) under 366nm and visible light respectively.

CONCLUSION

In this study the *Morinda tinctoria* leaf (MTS1 and MTS2) and its alcoholic extracts (EMTS1 and EMTS2) subjected pharmacognostic and phytochemical evaluation to assess the effect of winter and summer seasons in pharmacognostic characteristics and concentration of important phytochemical constituents. The macroscopical and microscopical analysis of the leaf of *Morinda tinctoria* collected at winter and summer revealed that length of leaf collected in summer season longer than winter. Breadth is wider in winter compared to summer. All the other parameters are relatively same. Physicochemical characteristics showed

marked difference in sample collected in both seasons. The extractive values are useful to evaluate the chemical constituents present in crude drug and also help for estimation of specific constituents soluble in a particular solvent. The Steroids, tannins, alkaloids, phenols and flavonoids present in both season. The study reveals that alkaloids and phenols present in high concentration in winter season compared to summer season. The pharmacological activities of natural drugs mainly depends phytoconstituents present in it The quality and purity of phytocostituents determines the intensity and effectiveness the plant drug. Moreover *Morinda tinctoria* has been found to be rich in active phytochemicals,. Also the in winter season the constituents are more compared to summer. More studies like isolation and charecterisation of the compounds and the studies on the materials which collected in other season is also needed. to confirm these findings.

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