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Advances in Bioresearch

ORIGINAL ARTICLE

Establishment of Quality Parameters in the Leaf Extract of *Morus. nigra L.*

Aparna Saraf* and Nikki Huria
The Institute of Science, Dr. HomiBhabha State University, 15, Madame Cama Road, Fort, Mumbai-400032
*Corresponding author: E-mail address: draparnasaraf@yahoo.co.in

ABSTRACT
Standardized criteria for herbal assessment are urgently needed because of advancements in analytical technology and increased popularity of herbal remedies. The present study aims to formulate a chemical fingerprint of leaf extract of Morus nigra L. using High Performance Thin Layer Chromatography (HPTLC) and Fourier Transform Infrared Spectroscopy (FTIR). Preliminary phytochemical analysis confirms the existence of bioactive phytoconstituents. To determine whether dried leaf powder complied with international standards, Proximate Analysis and Scanning Electron Microscopy (SEM) was performed. Optimization studies further develop and improve chemical fingerprinting norms. Morus nigra L. leaf extracts are known to possess promising phytochemical compounds necessary for the advancement of multiple plant derived bioactive drugs. The leaf consists of minerals, vitamins, dietary fiber, amino acids, phytosterols, flavonoids, benzofurans and stilbenes. Qualitative phytochemical screening and HPTLC fingerprinting was carried out using CamagLinomat V applicator, CamagReprostar 3, a photo-documentation chamber, CamagTlc Scanner 3 and Hamilton syringe and Win Cats-4 software. FTIR analysis in mid infrared region (4000-400 cm^{-1}) was carried out using Alpha II Compact FTIR spectrometer by Bruker. Preliminary qualitative phytochemical screening revealed the presence of alkaloids, glycosides, tannins, triterpenes, phenolic compounds, phytosterols and flavonoids in the methanolic extract of the plant's leaves. The pharmacognostic profile of Morus nigra L. was determined by an HPTLC fingerprinting analysis that expressed the total number of peaks, peak heights, peak area, peak percent area, and Rf values. The FTIR spectroscopic analysis indicated the presence of various functional groups like alcohols, phenols, esters, amines, ethers, carboxylic acid, phosphate ion, oximes, ketones, ammonium ion, etc. by showing distinctive peak values that ranged from 3500-1000 cm^{-1} Both HPTLC fingerprinting and FTIR analysis methods are reliable, accurate, cost-effective methods correctly suitable for qualitative analysis, quantification and identification of bioactive and pharmaceutic phytocompounds which are abundant in therapeutic value and have a promising future ahead in the treatment of several disorders. This study can be utilized as a tool for the verification, authenticity, and standardization of plant material.
***KEYWORDS**: Morus nigra L. leaves, HPTLC fingerprint, FTIR, Methanolic extract, physicochemical analysis, Functional groups.*

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INTRODUCTION

Mulberry leaves are specially cultivated for feeding silkworms and have been used in traditional medicines for thousands of years.[1][2]. Mulberry is considered as a Kalpavruksha due to its multiple uses such as foliage for domesticated animals [3], edible berries,[4] source of pharmaceutically important compounds [5], role in green synthesis of nanoparticles [6], environmental protection [7] and its availability all over the world [8]. The leaves of *MorusnigraL* are known to express therapeutic potential and reduce blood pressure and cholesterol level, are traditionally used for the treatment of arthritis, diabetes, rheumatism, dysentery, hypertension, and anemia.[9]and can also act as a laxative, anthelmintic, expectorant and emetic agent. This pharmaceutical potential may be due to a high content of secondary metabolites in black mulberry.[10].

For a medicinal plant to be considered as herbal medicine, standardization of quality parameters is of utmost importance. Many pharmacopoeias and WHO guidelines specify the process for conducting such analyses and the acceptable outcomes.

An essential quality control method used globally, is, the proximate examination of raw herbal materials. In order to get better results when chemically fingerprinting herbal products, preliminary assessment of secondary metabolites in different extracts, solvent optimization, and quantitative determination of polar, non-polar extract are very important.

FTIR is one of the most widely used techniques to identify the bio active compounds, determine their functional group and the chemical structure.[11]. FTIR has been demonstrated to be a precise, quick and easy method for phytochemical analysis. [12]

HPTLC (High performance thin layer chromatography) is a sophisticated, reliable, quick and effective method of phytochemical screening and crude drug analysis. HPTLC fingerprint of botanically verified crude herb material serves as a basic benchmark against which an unidentified material can be evaluated. HPTLC analysis can be used to identify and isolate the various bioactive compounds found in the natural plant extracts, and their pharmaceutical properties can be discovered which can be used to treat many diseases. [13]**.** Not much literature is available on Standardization protocols, HPTLC and FTIR fingerprint of *Morus nigra L.* leaves. Present study is based on the Proximate, SEM and phytochemical analysis, FTIR and HPTLC analysis of methanolic extract of *Morus. nigra L.* leaves.

MATERIAL AND METHODS

Plant material

The plant material was prepared from fresh and healthy leaves of *Morus. nigra L* obtained and authenticated by Central Seri cultural Germplasm Resources Centre, Central Silk Board, Hosur, Tamil Nadu. They were rinsed thoroughly with deionized water to remove sand and debris, and then air-dried in shade at ambient temperature and ground to a fine powder using a mechanical grinder and stored in air tight glass bottles for further analysis.

Phytochemical analysis

Methanol, Ethanol, water and Petroleum ether extracts of dried leaf powder of *Morus nigraL.* were tested for the presence of phytochemical compounds, namely flavonoids, phenols, tannins, anthocyanins, glycosides, coumarins, alkaloids, anthraquinones, saponins, terpenoids, and steroids. These tests were performed according to the method described by Software, Trease and Evans and Harborne. The phytochemicals were identified on the basis of a visible change in color or precipitate formation on treatment with specific reagents.

Proximate analysis

Foreign organic matter, total ash content, acid insoluble ash, water soluble ash, sulphated ash, loss on drying and determination of crude fibers were performed according to the standard protocols featured in Ayurvedic Pharmacopoeia of India.

Optimization studies

Six solvents of different polarities used for extraction. The solvent showing maximum solubility was further utilized to determine the optimum volume of solvent, optimum time for best extraction and number of extractions needed for best extractive values.

Scanning electron microscopy (SEM)

Dried leaf powder was placed on the stubs with the help of double-sided tape and put into a gold sputtering system to form a thin gold film on the surface of the sample, images were captured using, The FEI Quanta 200 scanning electron microscope.

HPTLC fingerprinting

Sonicated extract of 500 mg leaf powder in 10 ml of methanol was used for the fingerprinting analysis. 5µl of methanolic extract was applied on the TLC plate by a robotic TLC sampler IV using a micro-syringe, nitrogen was injected at a speed of 150nl/s. Samples were applied at a temperature of around 25^{o}C. The mobile phase used was Chloroform: Toluene: Ethanol (4:4:1v/v/v), Plate was developed up to 8 cm and then scanning done at 254 nm, 366 nm and 540 nm using CAMAG visualizer, followed by derivatization using Anisaldehyde sulphuric acid and drying for a few minutes on the plate heater at 100•C. TLC scanner was used to scan the plate. Win Cats software was used to note the Rf values and establish the chemical fingerprint.

FTIR fingerprinting

10 mg of the dried *Morus. nigra L.* leaf powder was grounded with KBr salt to remove scattering effect from large crystals. This mixture was used to produce a translucent pellet through which a light beam was transmitted. The transmittance spectra within the range of 4000 to 400cm^{-1} (mid-IR spectrum) wave number was recorded.

RESULT AND DISCUSSION

Phytochemical analysis

Phytochemical screening of leaf extract of *Morus. nigra L.* indicated the presence of various classes of secondary metabolites, namely, flavonoids, tannins, glycosides, alkaloids, quinones, phenols, saponins, terpenoids, coumarins, sterols and steroids. This proves that the leaves of *Morusnigra L*. are a rich source of bioactive compounds. Some classes of compounds like anthocyanins, phlobatanins and anthraquinones were shown to be lacking.

Table 1: Preliminary phytochemical evaluation of *Morus nigraL.*

Phytochemicals	*Morusnigra L.* Extraction solvents			
	Methanol	Ethanol	Petroleum Ether	Water
Flavonoids	+	+	+	+
Sterols	+	+	+	+
Alkaloids	+	-	+	+
Tannins	+	+	+	+
Saponins	+	+	+	+
Phenols	+	-	+	+
Glycosides	+	+	+	-
Anthraquinone	-	-	-	-
Anthocyanins	-	-	-	-
Terpenoids	+	-	+	+
Coumarins	+	+	+	+
Phlobatannins	-	-	-	-
Carbohydrate	+	+	+	+

Key: + = present; − = absent

Proximate analysis

Improper and prolonged storage of Herbal medicines may alter their bioactive properties. Proximate analysis of crude herbal products is a quality control parameter approved by World Health Organization (1998). The results of proximate analysis are displayed in Table 2.

Table 2: Proximate analysis of leaf extract of*MorusnigraL*.

S. No.	Parameters	% Content
1	Foreign organic matter	1.92 ± 0.02
2	Total ash content	18.58 ± 0.04
3	Acid insoluble ash	4.76 ± 0.05
4	Water soluble ash	12.86 ± 0.01
5	Sulphated ash	12.48 ± 0.02
6	Loss on drying	7.30 ± 0.01
7	Percentage crude fibres	10.82 ± 0.02

Values; mean ± SD

Optimization studies

Table 3 summarizes the result of optimization studies. Methanol was found to be the most optimum solvent amongst ethanol, n-hexane, water, chloroform and petroleum ether as its extractive value was the highest. Best extraction was obtained at 120 min with 25ml of methanol, extracted four times.

Table 3. Optimized conditions for extraction of leaf extract of *Morus. nigra L.*

Sample	Type of solvent	Amount of solvent ml	Time for extraction min	Number of extractions
Morus nigra L.	Methanol	25	120	4

Scanning electron microscopy

SEM analysis of leaves of *Morus. nigra L.* revealed the following morphological characteristics in its powder: Idioblasts: The adaxial leaves of *Morus. nigra L*. exhibited different types of large epidermal, secretory and spherical idioblasts (alkaloid storage bodies); Polygonal secretory idioblast with flat surface (Plate 10) and Circular secretory idioblast with protruding surface (Plate 3)

Trichomes: Three types of trichomes were identified in the leaves of *Morus. nigra L*, namely, glandular, smooth single celled and multicellular trichomes. (Plate 1,5&6)
Calcium oxalate drupes, rosettes and prismatic crystals were found to be abundant in the leaves of *Morus. nigra L* (Plate 5&9).
Starch granules, Striated cuticle, salt glands and leaf veinlets were also visible through SEM analysis (Plate 7 & 8).
Stomata were present only on the abaxial surface of the leaf (Plate 4)

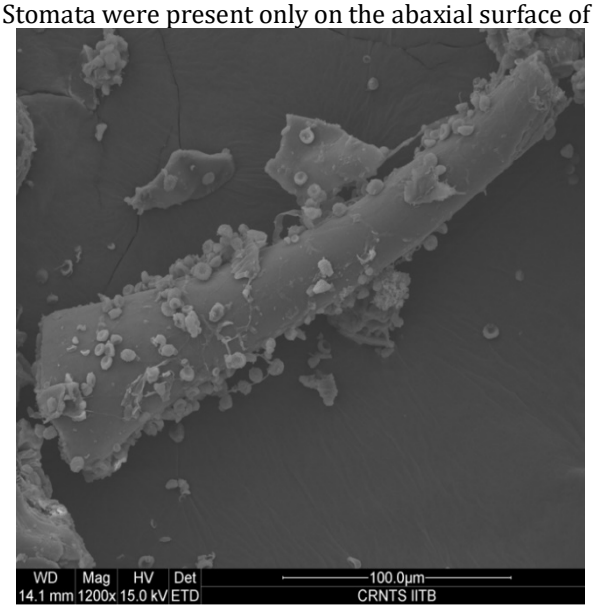

Plate: 1. Smooth Single Celled Trichome.

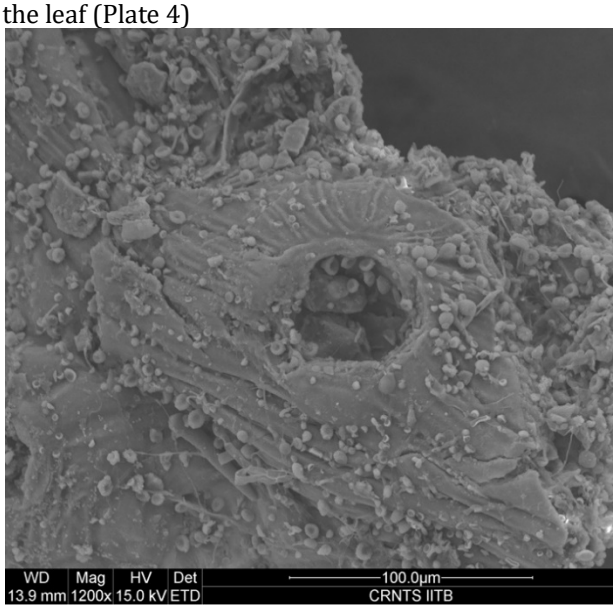

Plate: 2. Origin of Glandular trichome surrounded by striated cuticle and salt glands.

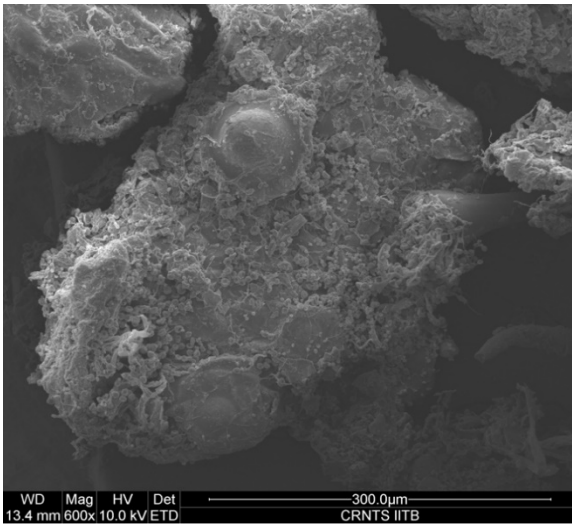

Plate: 3. Circular secretory idioblast with protruding surface

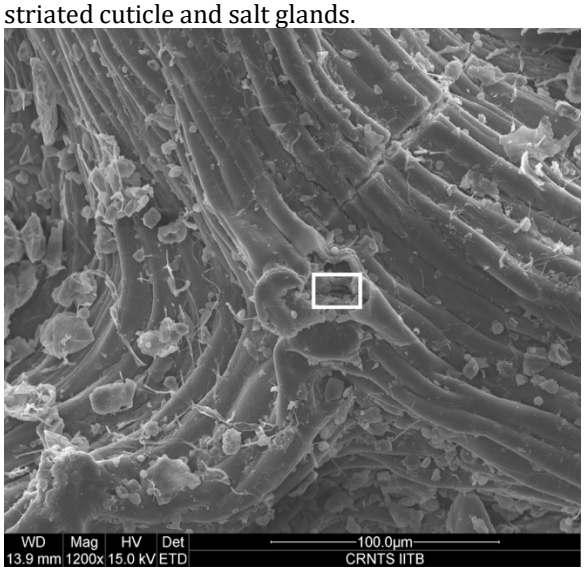

Plate: 4. Stomata surrounded by microfibres

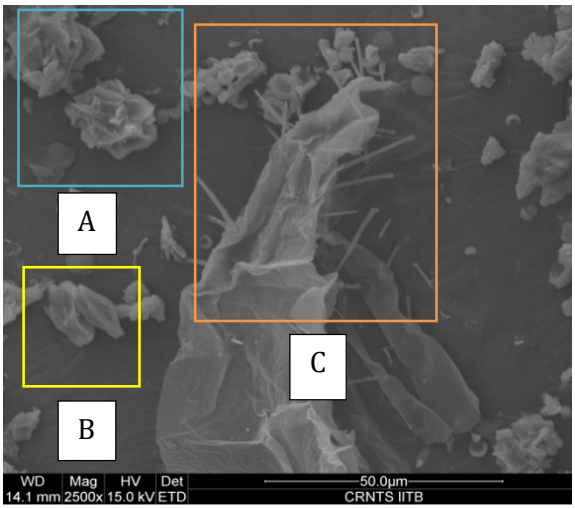

Plate: 5. **A**. Calcium oxalate rosette crystals
B. Prismatic crystals of calcium oxalate
C. Smooth unicellular trichomes

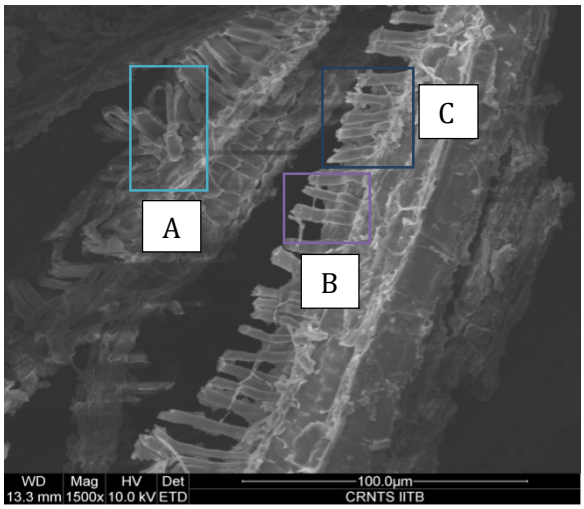

Plate: 6. **A**. Glandular trichome
B. Multicellular trichomes
C. Single celled trichomes

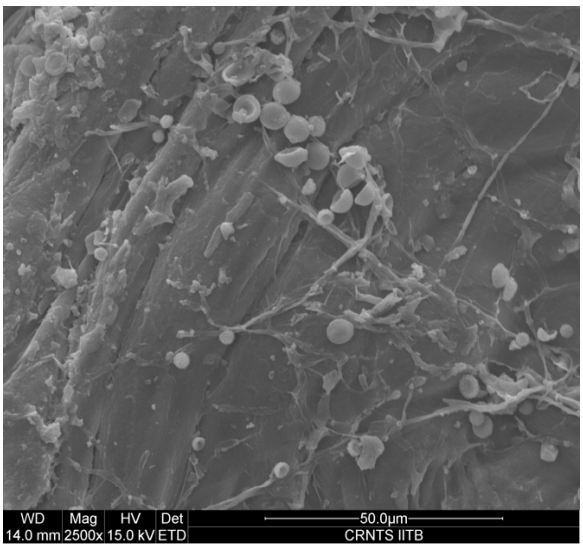

Plate: 7. Starch granules, salt glands and leaf veinlets

Plate: 8. Striated cuticle

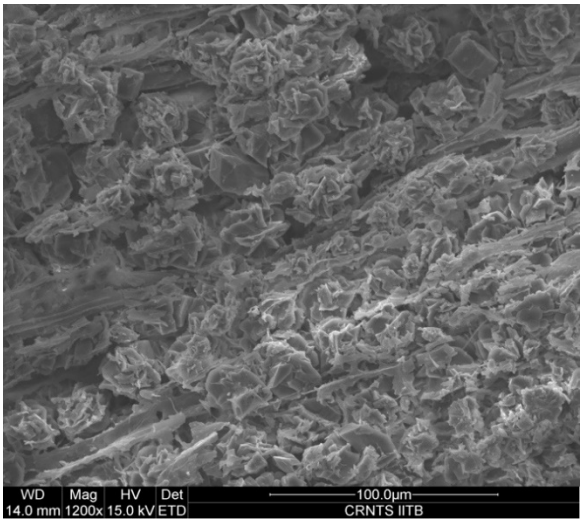

Plate: 9. Calcium oxalate drupes

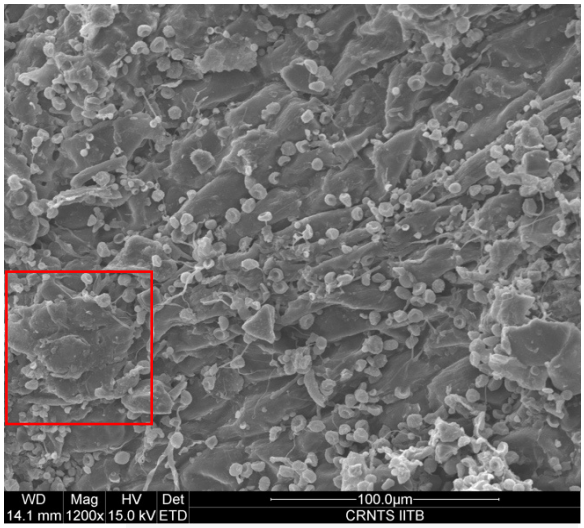

Plate: 10. Polygonal secretory idioblast with flat surface

HPTLC fingerprinting analysis

HPTLC fingerprint of methanolic leaf extract of *Morus. nigra L.* (100mg/ml in methanol) scanned at 540 nm after derivatization with AnisaldehydeSulphuric acid showed the presence of 7 components with Rf values 0.071, 0.439, 0.500, 0.553, 0.629, 0.758, 0.858[14]. (Figure: 1-6, Table: 4). Out of these 5 components with Rf 0.439, 0.500, 0.553, 0.629 and 0.858 were found to be predominant having percentage areas 13.69, 14.27, 19.06, 26.40, 13.10 respectively. Highest percentage area found to be 26.40 at Rf 0.629.

HPTLC fingerprint of leaves of *Morusnigra L.* was best observed in visible light after derivatization with ASR. The best solvent system was found to be Chloroform: Toluene: Ethanol (4:4:1v/v/v). The best loading volume was found to be 5 µl.

The HPTLC fingerprint of the methanolic leaf extract of *Morus. nigra L.* exhibited the existence of several bioactive phytoconstituents in different concentrations. The total number of peaks obtained reveals the presence of different phytoconstituents present in the sample. The Rf values tabulated for the different phytoconstituents present in the methanolic leaf extract of *Morus. nigra L.* can be utilized for identification of the unknown compounds by matching them with different reference standards, and from the peak area values, the concentration of the bioactive compounds can be deduced.

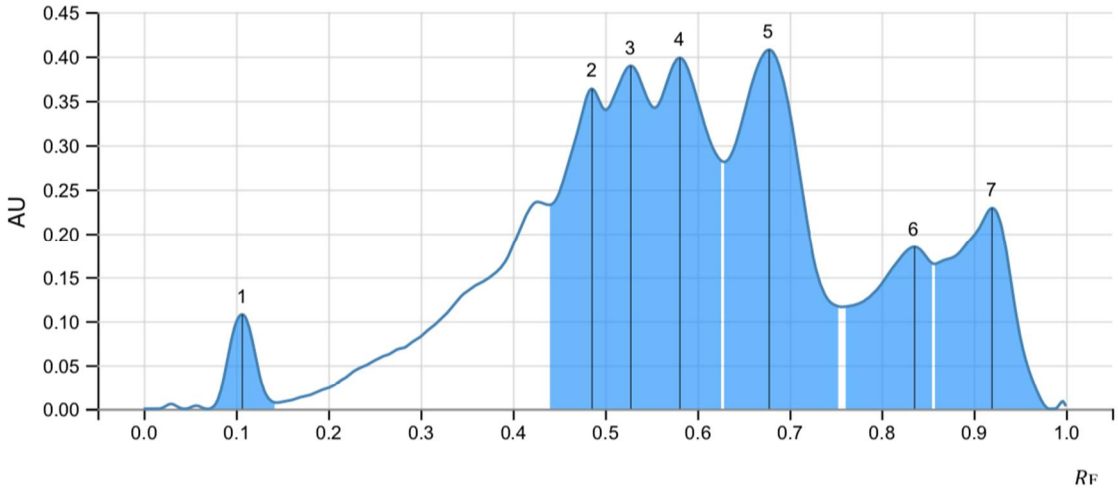

Figure 1: Densitogram of *Morus nigra L.* leaf extract

Table 4: RF values for fingerprint of *Morusnigra L.* leaf extract

Peak #	Start		Max			End		Area		Manual peak	Substance Name
	R_F	H	R_F	H	%	R_F	H	A	%		
1	0.071	0.0000	0.106	0.1074	5.17	0.144	0.0070	0.00359	2.63	No	
2	0.439	0.2325	0.485	0.3634	17.48	0.500	0.3398	0.01866	13.69	No	
3	0.500	0.3398	0.527	0.3893	18.73	0.553	0.3420	0.01945	14.27	No	
4	0.553	0.3420	0.581	0.3983	19.16	0.627	0.2814	0.02598	19.06	No	
5	0.629	0.2810	0.677	0.4074	19.60	0.756	0.1160	0.03599	26.40	No	
6	0.758	0.1160	0.835	0.1842	8.86	0.856	0.1649	0.01480	10.85	No	
7	0.858	0.1648	0.919	0.2287	11.00	0.982	0.0000	0.01785	13.10	No	

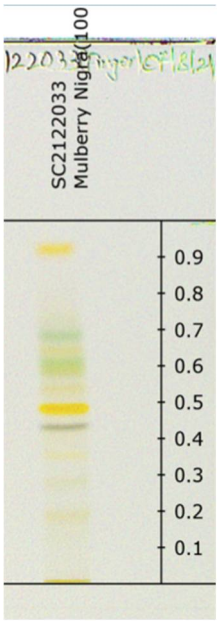

Figure 2: HPTLC finger print profile of *Morus nigra L* in visible light before derivatization

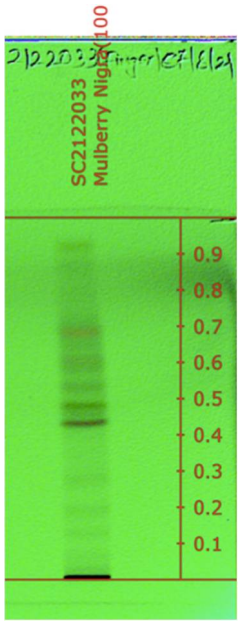

Figure 3: HPTLC finger print profile of *Morus nigra L* in UV light before derivatization

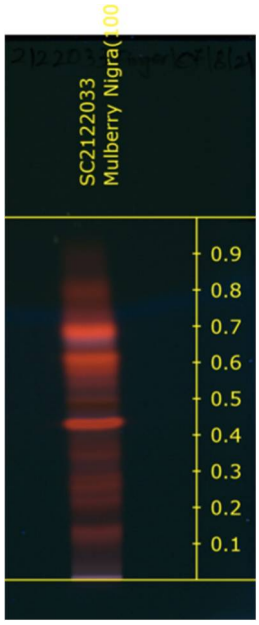

Figure 4: HPTLC finger print profile of *Morus nigra L* at 366 nm before derivatization

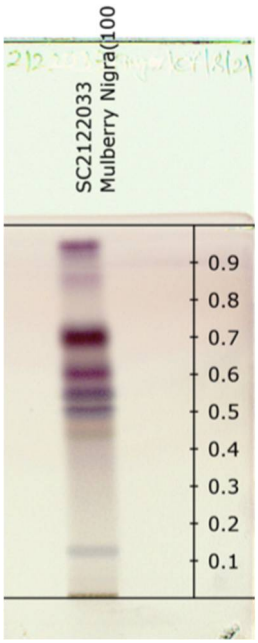

Figure 5: HPTLC finger print profile of *Morus nigra L* in visible light after derivatization

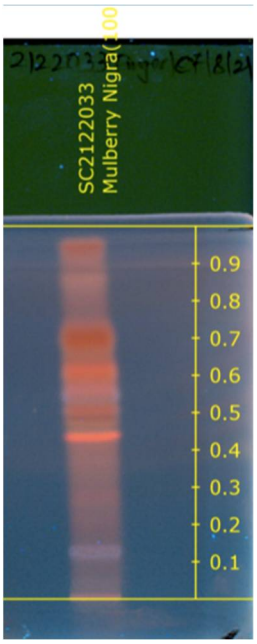

Figure 6: HPTLC finger print profile of *Morus nigra L* at 366 nm after derivatization

FTIR fingerprinting analysis

FTIR analysis of *Morus. nigra L.* leaf powder revealed peaks at 3285, 2917, 2849, 1620, 1542, 1415, 1314, 1246, 1046cm^{-1}. (figure: 7 Table:5)

The broad peak present at 3285 cm^{-1} lies within the range of 3550-3200 cm^{-1} this indicates: O-H stretching[15], N-H stretching vibration of peptide groups (Amide A)[16], C=O stretching vibrations overtone[17], and shows the presence of alcohols, phenols, carboxylic acid, aliphatic primary amine, hydrogen bonded amines, amino group, ammonium ion[18] and quinone oximes.

The sharp peak at 2917 cm^{-1} exhibits C-H stretching[19] vibration of methylene group which corresponds to alkanes, lipids and hydroxyl compounds[20]. The C-H asymmetric methylene group is characteristic of aliphatic compounds. [21]. Carboxylic group also shows absorbance in this region. This peak also corresponds to O-H Stretching and Symmetrical bending[22].

The sharp peak obtained at 2849 cm^{-1} denotes symmetrically CH_3 stretching vibration, C-H stretching vibration, CH_2 stretching vibration and corresponds to aliphatic ethers, aldehydes[23]. The bands between 2800–3000 cm^{-1} are characterized due to asymmetric and symmetric stretching vibrations of CH_2 and CH_3 groups (CH_2 and CH_3 symmetric stretching).[24].

No peaks were indicated in the region within the range of 2220-2260 cm^{-1} which denotes the absence of cyanide group therefore no toxic substances are found in *Morusnigra L.* leaves. [25]

Sharp peak at 1620 cm^{-1}lies in the range of 1600–1690 cm^{-1} corresponds to C=O stretching amide I band.[26] and C=C stretching vibration, A heavy element, group with a heavy element directly attached to C=C[27].

The peak obtained around 1547 cm^{-1}, is a characteristic of amide II band with C-N stretching and N-H bending. (C-N stretching and N-H bending vibration of amide II band).[28].

The peak obtained at 1415 cm^{-1} lies between the range of 1400 to 1420 wave number region and indicates C-H deformation vibration, stretching vibrations of C-O amide and C-C stretching from phenyl group and corresponds to ketones, acetates, amides etc.[29]

The peak at 1314 cm^{-1} shows C-H deformation vibration corresponding to amines[30], alkynes, cellulose and Amide III band components of proteins collagen[31]

Two medium peaks found between the range of 1250-1020 cm^{-1} (phosphodiester region), 1246 cm^{-1} and 1046 cm^{-1} indicate C-N stretching (amine)[32], stretching vibrations of carbonyl C-O (alkyl aryl ether) or OH bending[33], corresponding to amine group and phosphate ion[34].

The peak at 1046 cm^{-1}corresponds to C-C, C-OH, C-H ring and side group vibrations[35].

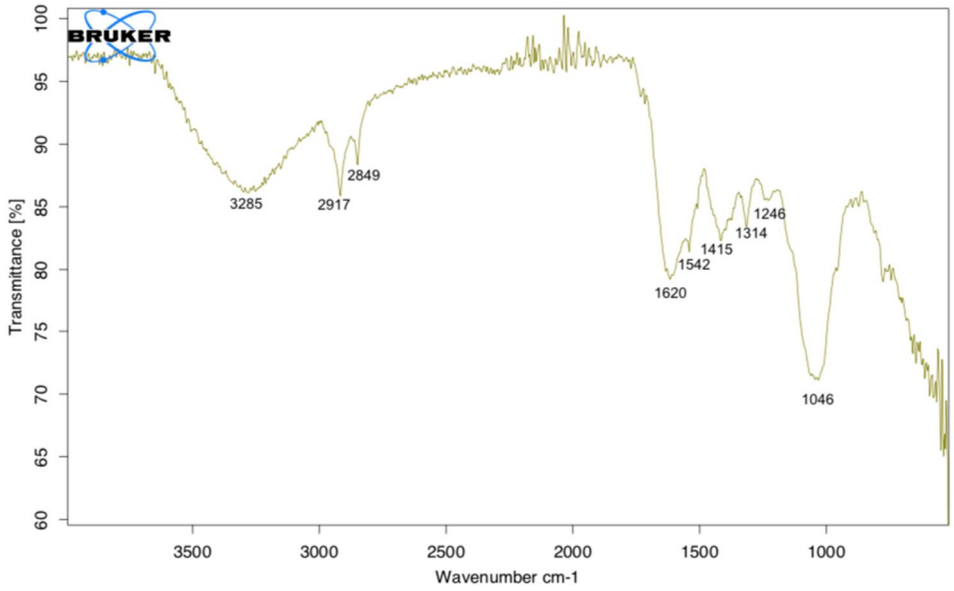

Figure 7: FTIR spectra of *Morus nigra L.* leaves

Table 5: Wavenumber (cm^{-1}) of dominant peaks obtained from absorption spectra

Wave number of dominant peaks cm^{-1}	Functional groups and bond stretching
3285	N-H stretching (amines) O-H stretching
2917	O-H (carboxylic acid)
2849	C-H, CH_2, CH_3 stretching vibration (aliphatic ethers, aldehydes) O-H (carboxylic acid)
1620	C-O (alkenes) stretching amide I band
1542	C-N stretching and N-H bending vibration of amide II band
1415	C-O (amide), C-C stretching (phenyl)
1314	cellulose and Amide III band components of proteins collagen N-acetylglucosamine (Chitin)
1246	C-N stretching (amine) C-O (carbonyl), O-H bending
1046	C-C, C-OH, C-H rings, C-O

CONCLUSION

The study, identification, isolation, quantification and chemical fingerprinting of the bioactive phytoconstituents found in the natural and herbal products is the need of the hour for the development of drug discovery programs.

This study was able to show the existence of numerous bioactive phytochemicals in the methanolic leaf extract of *Morus. nigra L.* which may be the reason for its therapeutic properties and justify its use as herbal medicine in curing several diseases.[36] .

The proximate analysis is used to establish the precision and genuineness of a crude plant extract during the formulation of drug. The use of optimized conditions for best possible extraction ensures maximum therapeutic potential from the crude drug and no wastage of solvent and other resources.[37]

SEM is an effective technique for examining the surface characteristics of herbal products. This method has been used extensively to examine the surface morphology of a wide range of plant materials, and the resulting three-dimensional images play a crucial role in the validation and certification of medicinal plants.[38]

Comparing the Rf values of the compounds with the reference standards will enable identification of bioactive organic compounds and biomarkers based on the derived HPTLC chemical fingerprint.[39]

This investigation produced an FTIR chemical fingerprint for the therapeutically valuable plant, *Morus. nigra L.*The FTIR analysis indicated the presence of alkaloids due to N-H stretching, and polyphenols and flavonoids because O-H stretching, terpenes due to C-H group, esters, ethers, alkenes, amines, amides, alcohols, phenols, aromatics and carboxylic acids.[40] The presence of such characteristic functional groups justifies the medicinal properties shown by *Morus. nigra L.*[41] These biologically active compounds can be further analyzed by isolation, quantification, characterization and structural elucidation techniques and utilized for pharmaceutical research and development[42].

Thus, the present study confirms that *Morus. nigra L* plant shows pharmacogenetic properties which can be used as resources of different bioactive compounds [43].*M. nigra L.* can be used as a promising nutraceutical resource to prevent and cure chronic diseases. Since almost all the researches are conducted in vitro and use animal models, further studies at the clinical level are required to distinguish the potency, effectiveness and reliability of *M. nigra L.* in the human body.[44]

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SEM analysis: SAIF, IIT Bombay

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CONFLICTS OF INTEREST: The authors declare no conflicts of interest.

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