CHEMICAL AND BIOLOGICAL EXAMINATION OF LEAVES OF *MORUS INDICA*Pethakamsetty Lakshmi^{1*}, Seru Ganapaty² and K. Mary Bharathi³¹Department of Microbiology, Andhra University, Visakhapatnam, A.P. India²College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, A.P. India³Department of Botany, Andhra University, Visakhapatnam, A.P. India

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ABSTRACT

Mulberry belongs to the genus *Morus* of the family Moraceae. It is an economically important plant being used for sericulture. Studies have been reported on the chemical composition and nutritional potentials of some mulberry species worldwide. In the present study the chemical examination of *Morus indica* leaves on conventional extraction and various chromatographic methods, led to the isolation of five compounds- β -sitosterol-3-O- β -D-glucoside, β -sitosterol, salvigenin, cirisimaritin and quercetin. All the compounds were characterized by 2D NMR, MS spectral data and comparison with the published data for the known compounds. All the compounds were reported for the first time from the leaves of this species. The work was further extended to test the crude extracts for antibacterial and antifungal activities. The results from the present study have shown that the species have considerable activity against selected bacterial and fungal strains which can be attributed to the presence of steroidal and phenolic compounds in the crude extracts of *Morus indica*.

Keywords: *Morus indica*, Phytochemical, Antibacterial activity, Antifungal activity

INTRODUCTION

Morus indica (Mulberry) of the family Moraceae, is a fast growing deciduous, woody, perennial tree. Plants are generally dioecious with catkin inflorescence bearing unisexual flowers in scaly clusters. The leaves are simple, alternate, stipulate, petiolate, entire, or lobed and the number of lobes varies from one to five^{1,2}. The genus *Morus* is native to south-western Asia and is widely distributed in Asia, Europe, North America, South America, and Africa occurring naturally in sparse forests on hillsides at a wide range of elevation³. The genus is valued for its foliage, which constitutes the chief feed for silkworms *Bombyxmori* and is cultivated extensively in the eastern, central and southern Asia for silk production. The leaves are nutritious, palatable and nontoxic and are proved to improve milk yield when fed to dairy animals^{4,5}. *Morus* is an economically important genus evaluated for their edible fruits used in the preparation of marmalades, juices, liquors, natural dyes and their usage in sericulture, timber, medicine and cosmetic industries⁶⁻⁸.

The genus is well-known for its richness in secondary metabolites including prenylated flavonoids, sterols, terpenes, anthocyanins, polyphenolic and Isoprenoid-substituted phenolic compounds, phenyl bromides, arylbenzofurans and stilbene derivatives⁹⁻¹⁸ which exhibited diverse biological activities like antibacterial, antifungal, antiviral, antinematodal, anti-cancer, anti-platelet, antioxidant, anti-inflammatory, antiproliferative, cytotoxic, hypoglycemic, analgesic, cardio protective and immuno regulating activities¹⁹⁻²⁴. As a part of ongoing search for novel natural products of plant origin with potential biological activity, the current study was undertaken to investigate the chemical analysis and antimicrobial studies on the leaves of *Morus indica*, since no work has been reported from this species.

MATERIALS AND METHODS

Plant Material

The leaves of *Morus indica* were collected from Araku Valley, Visakhapatnam District, A.P., India where it is

cultivated for sericulture. The plant specimen was authenticated by Prof. T. Pulliah, Taxonomist, Department of Botany, Sri Krishna devaraya University, Anantapur, India. A voucher specimen has been deposited at the Herbarium, Department of Botany, Andhra University, Visakhapatnam, India. All chemicals and solvents used were of analytical grade and obtained from Ranbaxy Fine Chemicals and Merck Ltd., Mumbai.

Extraction and Isolation

The leaves of *M. indica* (1.8 kg) were dried and coarsely powdered. The powdered leafy material was extracted with chloroform (4lit) and concentrated under reduced pressure and kept overnight in refrigerator. A pale brown solid settled down (325 mg) which was separated. It gave a prominent spot on TLC, M.P. 289-291⁰ and gave a positive test for L.B reaction (play of colours) and based on the spectral data it was identified as β -sitosterol-3-O- β -D-glucoside. The chloroform extract was concentrated to a small residue (16gm.) under vacuum. The crude extract gave positive colour reactions with Ferric Chloride solution (green colour) and Shinoda's test (orange colour) indicating the presence of phenolic constituents especially flavonoids. It also gave Lieberman-Burchard reaction for triterpenes and sterols. The crude extract of *M. indica*, when examined on TLC (80:20 chloroform: benzene), showed four prominent spots after spraying 5% alcoholic sulphuric acid. Hence, the extract (14gm.) was column chromatographed over silica gel (>100gm), eluting with solvents Petroleum ether, Benzene, Chloroform and Methanol mixtures in the order of their polarity. Fractions of 250 ml each were collected and monitored on the TLC (Acme-Silica gel was used for thin layer chromatography) and the spots on chromatogram were detected under UV light (254 and 365 nm) and by spraying with 5% H₂SO₄ in methanol. The chromatogram reported four compounds as β -sitosterol, Salvigenin, Cirisimaritin and Quercetin. All the compounds were isolated, purified and identified by chemical tests and spectral analysis.

Characterization of the compounds

β -sitosterol-3-O- β -D-glucoside

The compound which was settled (325mg) from chloroform extract was separated and crystallized from methanol-chloroform mixture m.p. 289-291⁰C, $[\alpha]_D^{30}$ - 27.5⁰ (Pyridine). It gave a play of colours in Liebermann- Burchard reaction and a positive Molisch test for carbohydrates, suggesting that it could be a steroid glycoside. Further hydrolysis with 6% methanolic sulphuric acid for four hours furnished a crystalline aglycone, M.P - 135-136⁰, $[\alpha]_D^{30}$ - 36.5⁰ (chloroform) and was identified as β - sitosterol by direct comparison and through its acetate, M.P- 126-128⁰. The filtrate from the aglycone showed single spot in descending paper chromatography technique (R_F 0.18) butanol: acetic acid: water 6:4:3) identified as glucose by co-chromatography with an authentic sample. Enzymatic hydrolysis with β -glucosidase also yielded glucose as sugar moiety. Therefore, the compound was identified as β -sitosterol-3-O- β -D-glucoside (Figure 1) and confirmed by comparison with the authentic sample.

β -sitosterol

It was crystallized as needles from pet-ether (200mg), m.p 139-140⁰, $[\alpha]_D^{30}$ -36⁰, (chloroform) and analyzed for the formula C₂₉H₅₀O. It showed colour reaction in L.B test, characteristic of sterols (play of colours) and gave a single spot on TLC. Its IR showed peaks at 3440 (-OH), 1380 and 1385cm⁻¹ (gem dimethyl). It formed a monoacetate, m.p 125-27⁰, $[\alpha]_D^{30}$ -38.2⁰ (chloroform). ¹H NMR spectrum showed the peaks at δ 0.80 - 1.25 (methyl), δ 4.45 (1 H broad C 3H) and δ 5.30 (1H, m (-5H)). From the above properties the compound was identified as β -sitosterol (Figure 2) and the identity was confirmed by comparison with an authentic sample (M.P and Co-TLC).

Salvigenin

It was obtained as lemon yellow rectangular crystals (250mg) from benzene-chloroform, m.p 196-197⁰, analyzed for C₁₈H₁₆O₆ (m/e 328 M⁺). It gave olive green ferric- chloride reaction, orange colour in Shinoda's test, and yellow colour in Wilson's boric-citric acid test, suggesting that it was a 5-OH flavone. On PC, the spot appeared purple under UV and intense purple under UV/NH₃. On methylation with dimethyl sulphate and potassium carbonate, it gave a tetra methyl ether m.p, 162-163⁰ and with sodium acetate and acetic anhydride, it gave an acetate m.p, 170-172⁰. The UV spectrum of the compound showed peaks at λ_{max}^{MeOH} 275, 329nm. With AlCl₃/ HCl a 22nm bathochromic shift in Band I is observed, which indicated the presence of a free 5-hydroxyl group and a shift of 40nm in Band I of NaOMe spectrum suggested 4-substitution. With NaOAc there was no bathochromic shift in Band II of methanol spectrum which implies that position C-7 was substituted. IR spectrum exhibited bands at 3450 (OH) and at 1660 cm⁻¹ (C=O). The ¹H NMR spectrum showed three methoxys at δ 3.90, 3.92 and 3.98 and two doublets (J=9 Hz each) centered at δ 7.73 and 6.93, integrating for 4 protons assigned to 2',6; & 3',5' protons of the ring B. The two other singlets at δ 6.50 and 6.53 were attributed to C₈ and C₃ protons of Ring A. From the foregoing data, the compound was identified as 5-OH-6, 7, 4'-trimethoxy flavone (Salvigenin-Figure 3) and the identity were confirmed by comparison with an authentic sample. The ¹H NMR spectral data of compound was recorded in Table 1.

Cirisimaritin

It was obtained from methanol as lemon yellow crystals (200mg), M.P 248-250⁰, C₁₇H₁₄O₆ (m/e 314 H⁺). A positive ferric reaction and orange red colour with Mg+HCl suggested flavone nature of the compound. It formed a diacetate m.p, 202-203⁰ and tetra methyl ether, M.P 162-163⁰. U.V showed absorption bands at λ_{max}^{MeOH} (nm): 325, 270. A 35nm bathochromic shift in Band I of AlCl₃/HCl spectrum indicated the presence of a free 5-OH group and 6-OCH₃ and a shift of 53nm in Band I of NaOMe spectrum showed the presence of a free 4'- hydroxyl. Absence of Band II shift with NaOAc suggested that C-7 is substituted. IR exhibited bands at 3450 (OH), 2880 (CH stretch), 1640 (C=O), 1500, 835 and 765cm⁻¹. The ¹H NMR spectrum showed a clear A₂B₂ pattern with doublets centered at δ 7.00 and 8.01 (J = 8.5 Hz), 2 methoxylations at δ 3.75 and 3.92 and flavone proton singlet at δ 6.82 (C-3H). The above data coincided well with that of Cirisimaritin (Figure 4) and the identity was confirmed by comparison with an authentic sample. The ¹H NMR spectral data of compound was recorded in the Table 2.

Quercetin

It was obtained from CH₃OH as yellow crystalline solid, M.P 312-314⁰ and analyzed for the formula C₁₅H₁₀O₇. In paper-chromatography, it was yellow under UV & intense yellow under UV/NH₃ with FeCl₃ it gave green colour and with magnesium and HCl (Shinoda's test) magenta colour, characteristic for flavonoids. An orange red precipitate with neutral lead acetate confirmed the presence of 3-hydroxyl group. Presence of 5-hydroxyl group was inferred through Wilson's boric & citric acid reaction. It formed a penta acetate, m.p 194-196⁰ & a penta methyl ether, M.P 150-151⁰. UV showed absorption at λ_{max} 257, 267sh, 301sh, 370nm. A nm Band I bathochromic shift with AlCl₃/HCl suggested the presence of 5-hydroxyl group. Presence of B- ring ortho-dihydroxyl bathochromic shift with NaOAc/ H₃BO₃ reagent. A bathochromic shift of 18nm in Band II with NaOAc suggested the presence of 7-hydroxyl group. ¹H NMR exhibited peaks at δ 6.15 (d, 6H), 6.4 (d, 8H), δ 6.90 (d, 5'H), δ 7.60 (d, 6 H) and δ 7.75 (d, 2'H). The elemental analysis of the compound and its acetate and UV spectral data indicated that the compound is quercetin (Figure 5) and the identity was confirmed by comparison with an authentic sample (M.P and Co-PC).

Biological Studies

Some of the *Morus* species were found to contain antibacterial and antifungal activities. Species of *Morus*, *M. bombycis*, *M.alba*, *M.nigra*, *M.mongolica*²⁵ exhibited proven antimicrobial activity. Hence, the author has attempted to study the antimicrobial activity of the chloroform extract of *M. indica* leaves for their possible activity. The test organisms used in the study were obtained from Institute of Microbial technology (IMTECH) Chandigarh, India.

Antibacterial Activity

Test Samples

Antibacterial activity was carried out by the cup- plate agar diffusion method²⁶. The chloroform extract of the leaves of *M. indica* were used in two dose levels of 100mg/ml and 300mg/ml. Benzyl penicillin (100 μ g/ml) was used as standard.

Table 1: ¹H NMR spectral data of compound Salvigenin

Chemical shift(δ)	Proton Integration	Multiplicity	Assignment
3.90 3.92 3.98	9H	s	6-OCH ₃ 7- OCH ₃ 4'- OCH ₃
6.50	1H	s	C-3H
6.53	1H	S	C-8H
6.93-7.02	2H	<i>d J</i> = 9Hz	C-3', 5'H ^s
7.73-7.82	2H	<i>d J</i> = 9Hz	C-2', 6'-H ^s

Solvent: CDCl₃, TMS; Internal standard (90 MHz spectra)

Table 2: ¹H NMR spectral data of compound Cirisimaritin

Chemical shift (δ)	Proton integration	Multiplicity	Assignment
3.75 3.92	6H		6-OCH ₃ 7-OCH ₃
6.82	1H	s	C-3H
6.87	1H	s	C-8H
7.00	2H	<i>d J</i> = 8.5 Hz	C- 3' 5' H ^s
8.01	2H	<i>d J</i> = 8.5 Hz	C-2' 6' H ^s

Solvent: CDCl₃, TMS; Internal standard (90 MHz spectra)

Table 3: Antibacterial activity of chloroform extract of leaves of *Morus indica*

Extracts	Zones of inhibition							
	Gram+ ve					Gram-ve		
	<i>B.S</i>	<i>B.P</i>	<i>S.P</i>	<i>M.L</i>	<i>E.C</i>	<i>K.P</i>	<i>P.V</i>	<i>E.F</i>
Chloroform extract(100mg/ml)	10	21	-	12	17	16	08	-
Chloroform extract(300mg/ml)	12	23	-	15	19	18	10	-
Benzylpenicillin (100µg/ml)	34	30	33	34	34	39	32	-

Cup diameter: 6mm, Zone of inhibition in mm.

(*B.S*-*Bacillus subtilis*, *B.P*- *Bacillus pumilis*, *S.P*-*Streptococcus pyogenes*, *M.L*. *Micrococcus luteus*, *E.C*- *Escherichia coli*, *K.P*- *Klebsiella pneumoniae*, *P.V*- *Proteus vulgaris*, *E.N*- *Enterococcus faecalis*)

Table 4: Antifungal activity of chloroform extract of leaves of *Morus indica*

Extracts	Zones of inhibition (mm)			
	<i>A.F</i>	<i>S.C</i>	<i>P.E</i>	<i>P.C</i>
Chloroform extract(100mg/ml)	-	17	13	11
Chloroform extract(300mg/ml)	-	19	15	13
Nystatin(100µg/ml)	21	23	22	23

Cup diameter: 6mm, Zone of inhibition in mm.

(*A.F*-*Aspergillus fumigans*, *S.C* - *Saccharomyces cerevisiae*, *P.E*-*Pencillium excelsa*, *P.C*-*Pencillium chrysogenum*)

Figure 1: β-sitosterol-3-O-β-D-glucoside

Figure 2: β- sitosterol

Figure 3: Salvigenin

Figure 4: Cirisimaritin

Figure 5: Quercetin

Test Organisms

For testing the antibacterial activity, the following Gram (+) ve (*Bacillus subtilis*, *Bacillus pumilis*, *Streptococcus pyogenes*, *Micrococcus luteus*) and Gram (-) ve (*Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Enterococcus faecalis*) bacterial strains were selected.

Procedure

The Nutrient medium prepared was inoculated at 1% level with 18 hours culture of the above mentioned test organisms and transferred in to sterile 15 cm diameter Petri dishes. The medium in the plates were allowed to settle at room temperature for about 10 minutes and allowed to solidify in a refrigerator for 30 min. 4 cups of 6mm diameter were made in each plate at equal distance. Stock solutions of the test residual extract were prepared in concentrations of 100mg/ml and 300mg/ml. 100µg/ml of each concentration were placed in the cups by means of sterile pipettes. In each plate one cup was used for control (solvent) and one for standard. Antibiotic Benzyl penicillin (100µg/ml) was used as standard. The plates thus prepared were left for 2 hrs at room temperature for diffusion. After incubation for 18 hrs at 37°C the plates were examined for zones of inhibition. The experiments were run in duplicate and the average diameter of the zones of inhibition was recorded and noted in Table 3.

Antifungal Activity

Test Samples

Antifungal activity was carried out by the cup- plate agar diffusion method²⁶. The chloroform extract of the roots of *M. indica* were used in two dose levels of 100mg/ml and 300mg/ml. Nystatin (100µg/ml) was used as standard.

Test organisms

Aspergillus fumigans, *Pencillium excelsa*, *Pencillium chrysogenum*, *Saccharomyces cerviseae* were the fungal test organisms.

Procedure

Peeled potatoes are cut in to small chips and boiled with 200 ml of water for the 30 minutes. The chips are crushed during boiling and the pulp is removed after cooling by filtration through muslin. Dextrose and agar were added slowly by stirring and the volume is made up to 1000 ml. It was distributed in 40ml quantities into 100ml conical flasks and were sterilized in an autoclave at 121°C (151bs/sq.in) for 20 minutes. Then the medium was inoculated with 0.5 ml of an aqueous suspension of the organism which was prepared from 48 hrs culture. Then the inoculated agar medium was poured in to sterile 15 cm diameter Petri dishes and allowed to settle at room temperature for about 15 minutes. 4 cups of 6mm diameter were made in each plate at equal distance. Stock solutions of the test residual extract were prepared in concentrations of 100mg/ml and 300mg/ml. 100µg/ml of each concentration were placed in the cups by means of sterile pipettes. In each plate one cup was used for control (solvent) and standard. Antibiotic Nystatin (100µg/ml) was used as standard. The plates thus prepared were left for 2 hrs at room temperature for diffusion. After incubation for 48 hrs at 37°C the plates were examined for zones of inhibition. The experiments were run in duplicate and the average diameter of the zones of inhibition was recorded and noted in Table 4.

RESULTS AND DISCUSSION

The chemical examination of the leaves of *M.indica* gave five compounds β -sitosterol-3-O- β -D-glucoside, β -sitosterol, salvigenin, cirisimaritin and quercetin. All the compounds were first reported from the leaves of *M.indica*. The occurrence of prenylated flavonoids in *Morus* genus was recorded earlier but in this species the author could not come across any such flavonoids. All the above compounds were isolated for the first time from the leaves of this species. The isolation of the above bioactive chemical constituents renders the medicinal significance of the genus, which leads to the discovery of novel lead molecules which are presently been the subject of human curiosity and need.

The chloroform extract of *Morus indica* roots showed good amount of activity against *Bacillus pumilis*, *Escherichia coli* and *Klebsiella pneumoniae*, but showed mild response towards *Bacillus subtilis*, *Micrococcus luteus* and *Proteus vulgaris* and no activity against *Streptococcus pyogenes* and *Enterococcus faecalis*. The root extracts also displayed promising antifungal activity against *Saccharomyces cerviseae*, mild activity towards *Pencillium excelsa*, *Pencillium chrysogenum* and showed no activity towards *Aspergillus fumigans*. The results from the antimicrobial studies have shown that the species have considerable antibacterial and antifungal activity against selected test organisms, which can be attributed to the presence of steroidal and phenolic compounds in the crude extracts of *Morus indica*.

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