Research Article

Phytochemical and Antimicrobial Examination of the Root Extracts of *Morus Indica*

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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* roots on conventional extraction and various chromatographic methods**,** led to the isolation of six compounds- β-sitosterol, α-amyrin acetate, salvigenin, morusin, cyclomorusin and cirisimaritin. 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 roots 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: Antibacterial activity, Antifungal activity, *Morus indica*, Phytochemical.

INTRODUCTION

The family Moraceae (Mulberry family) with approximately 53 genera and 1400 species was mostly of pantropical distribution,[1,2] widespread in tropical and subtropical regions and less common in temperate climates. The plants of this family elaborate many interesting secondary metabolites, including prenylated flavonoids, arylbenzofurans and stilbene derivatives.[3-5] The genus *Morus*, commonly called Mulberry is a fast growing deciduous, woody, perennial plant. The genus is widely distributed in Asia, Europe, North America, South America, and Africa extending from temperate and sub-tropical regions of the northern hemisphere, as well as in the tropics of the southern hemisphere, growing in a wide variety of climatic, topographical and soil conditions.[6-8] It is an economically important plant being used for sericulture, as it is the sole food plant for the domesticated silkworm, *Bombyxmori* and is cultivated extensively in the eastern, central and southern Asia for silk production.[9]

Some of the species of mulberry were mainly known for their edible fruits (*Morus alba*, *Morus indica*, *Morusnigra*, and *Morus laevigata*), timber (*Morus laevigata* and *Morus serrata*) preparation of marmalades, juices, liquors, natural dyes (*Morus nigra, Morus rubra*, *Morus alba*) and in cosmetics industries.[10,11] Recent studies have been reported on the chemical composition and nutritional potentials of some mulberry species worldwide.[12-16] The genus is well-known for its richness in isoprenylated phenolic secondary metabolites including sterols, terpenes, flavonoids, anthocyanins and carotenoids[17-22] and is reported to exhibit antibacterial, antifungal, anti-viral, anti nematodal, anti-cancer, anti-oxidant, anti-inflammatory, anti proliferative, Cytotoxic, hypoglycemic, analgesic, cardio protective and Immuno regulating activities.[23-29] In view of the medicinal and economic interest of the family Moraceae, especially the genus *Morus,* the present investigation has been taken up on the Phytochemical analysis and antimicrobial studies on the root extracts of *Morusindica* ,since no work has been reported till date.

MATERIALS AND METHODS

Plant Material

The roots 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

About 1kg of M. indica roots were collected and air dried. The dried roots were coarsely powdered and extracted with chloroform (4lit.x3). The chloroform extract was concentrated to a small residue (15gm.) under vaccum. The crude extract gave positive color reactions with Ferric Chloride solution (green color) and Shinoda's test (orange color) 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 six 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% H_2SO_4 in methanol. Six compounds β-sitosterol, α-amyrin acetate, salvigenin, morusin, cyclomorusin and cirisimaritin were isolated, purified by crystallization and characterized by spectroscopic methods.

Characterization of the compounds

β-sitosterol

It was obtained from petroleum-ether benzene as needles, m.p. 136-137^0, $[\alpha]_D^{30}$ -37^0 (Chloroform) and analyzed for the formula $C_{29}H_{50}O$. It showed play of colors (pink-blue-green), in L.B.test. Its IR showed peaks at 3440 (-OH), 1380 and 1385 cm^{-1} (gem dimethyl). It formed a monoacetate, m.p.124-126^o, $[\alpha]_D^{30}$ -38.2^O (chloroform). From the above data the compound was identified as β-sitosterol(Figure 1) and the identity was confirmed by comparison with an authentic sample of β -sitosterol (mmp, and Co-TLC).

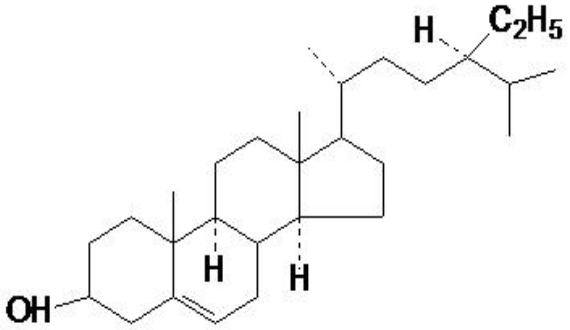

Figure 1: β-sitosterol

α-amyrin acetate

It was crystallized from chloroform-methanol as needles, m.p. 216^{O}C $[\alpha]_D^{30}$ +78.2^O (chloroform) and analyzed for the formula $C_{32}H_{52}O_2$. It gave positive Lieberman Burchard reaction for triterpenoids and yellow colour with tetranitromethane. IR showed absorption bands at 0.86, 0.95 and 1.10 (24 H, 8 x Me), multiplets at 3.1(12-H) and 4.36(3α-H) each integrating for one proton and a three proton singlet at 1.94 for an acetoxyl function suggesting the compound is a monoacetate, The compound was deacetylated, when a crystalline substance, m.p. 185-186^O, $C_{30}H_{50}O$, λ_{max}^{KBr} +81^O (chloroform). From the above data, the compound MIR-02 was identified as α- amyrin acetate and the identity was further confirmed by comparison with an authentic sample (Co-TLC and mmp).

Salvigenin

It was obtained as lemon yellow rectangular crystals (250mg) from benzene-chloroform, m.p 196-197^{0}C, analyzed for $C_{18}H_{16}O_6$ (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, 162-163^{0}C and with sodium acetate and acetic anhydride, it gave an acetate m.p, 170-172^{0}C. The UV spectrum of the compound showed peaks at λ_{max}^{KBr} 275, 329nm. With $AlCl_3$ / 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 ^{1}H NMR spectrum showed three methoxyls at δ3.90, 3.92 and 3.98 and two doublets (J=9 H_3 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 C_8 and $C_{3\ protons}$ of Ring A. From the foregoing data, the compound MIL -03 was identified as 5-OH-6, 7, 4'-trimethoxy flavone (Salvigenin-Figure 2) and the identity were confirmed by comparison with an authentic sample. The 1H NMR spectral data of the compound was recorded in Table 1.

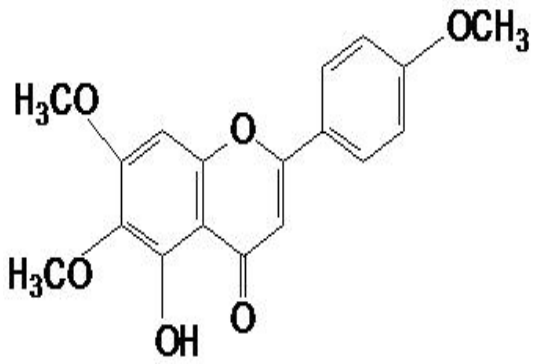

Figure 2: Salvigenin

Morusin

It was obtained as pale yellow powder from benzene-petroleum ether,m.p. 212-214^{O}C, It gave olive green colour with Ferric chloride solution and positive Gibb's test, The compound showed UV absorption at $\lambda_{max}^{Et.OH}$nm (log E) .205 (4.49), 220 (sh, 4.41), 270 (4.58), 300 (sh, 3.90), 320 (sh, 3.89), 350 (3.80). IR spectrum showed absorptions at υ_{KBr}^{max} cu^{-1} :3250, 1660. The ^{1}HNMR (cocl$_3$, 90) showed signals at 1.42(9H,S, CH_3X 2(C-4) CH_3(C-H), 1.56 (3H,S,(C_{11}-CH_3), 3.02(24,*d*,*J*,8H_2, C-9),5.03(1H,*t*,*J*= 7.5 H_2, C-6'H), 9.76, 9.85,13.17 (each 1H,S,3 XOH) disappeared on addition of D_{20}). The Mass spectrum showed molecular

or m/c 420 M^+,$C_{25}H_{24}O_6$), 405 (M^+-CH_3), It formed a dimethyl ether m.p. 144-146^O and diacetate,$C_{29}H_{28}O_6$, m.p.137-138^{O}C. From the above data the compound was identified as morusin (Figure 3). It was further identified by comparing with the authentic sample, Co TLC and m, m.p.

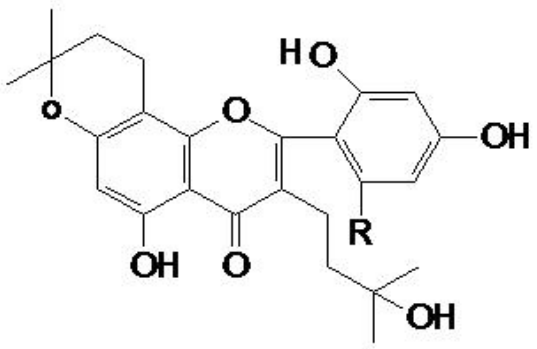

Figure 3: morusin

Table 1: 1H NMR spectral data of compound MIR -03 (salvigenin) Solvent: $CDCl_3$, TMS; Internal standard (90 MHz spectra)

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	*d* J = 9Hz	C-3', 5'H^s
7.73-7.82	2H	*d* J = 9Hz	C-2', 6'-H^s

Cyclomorusin

It was obtained as yellow crystals from methanol, m.p 242-244^{O}C, $[\alpha]_D^{30}$ +20^O (C= 0.01 in methanol). The compound showed UV absorptions at 223 (4.4), 225 (4.29), 283(4.42), 383(4.18). It showed IR bands in 3500, 1660, 1620 and 1590 cm^{-} The ^{1}HNMR in C6(CD_3)$_2$ Co,90 MH_2) showed signals at 1.59 (64,*S, c*-14-(CH_3)$_2$), 1.72, 1.98 (each 3H,*S*,C-H,CH_3 x2), 5.49(1H *d*,*J*=10H_2, C-104), 5.78 (1H,*d, J*= 10 H_2, C-13H), 6.17 (1H, *S*,C-64), 6.24 (1H,*d*,*J*=10H_2,C-9H), 6.45(1H,*d*,*J*=2H_2, C-3'H), 6.67 (1H,*dd*, *J*=2 and 9H_2,C-5'H), 6.95(1H, *d, J*=10H_2,C-12H), 7.82 (1H, *d*,*J* =9H_2,C-6'H). This data suggested that the compound was cyclomorusin(Figure 4). It was further confirmed by comparison with the authentic sample, Co.TLC and m.m.p.

Cirisimaritin

It was obtained from methanol as lemon yellow crystals (200mg), m.p 248-250^0, $C_{17}H_{14}O_6$ (m/e 314 $H^{+)}$. A positive ferric reaction and orange red color with Mg+HCl suggested flavone nature of the compound. It formed a diacetate m.p, 202-203^{o}C and tetra methyl ether, m.p 162-163^o. U.V showed absorption bands at λ^{MeOH}_{max} (nm): 325, 270. A 35nm bathochromic shift in Band I of $AlCl_3$ / HCl spectrum indicated the presence of a free 5-OH group and 6-OCH_3 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 ^{1}HNMR spectrum showed a clear A_2B_2 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 and the identity was confirmed by comparison with an authentic sample. The ^{1}H NMR spectral data of compound cirisimaritin (Figure 5) was recorded in the Table 2.

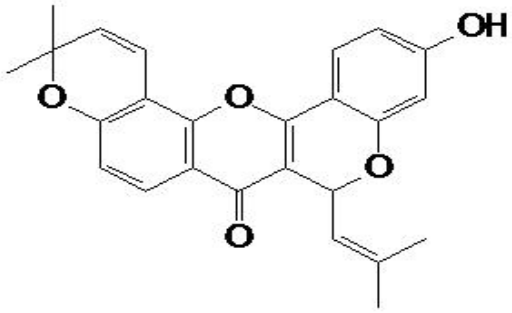

Figure 4: Cyclomorusin

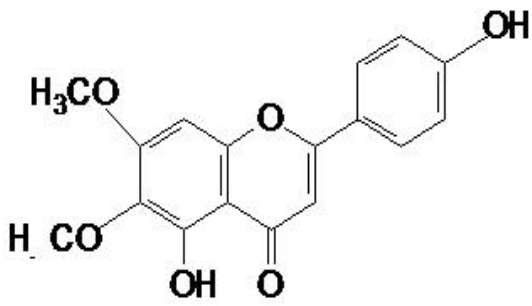

Figure 5: Cirisimaritin

Antimicrobial activity of the root extracts of *Morusindica*

Some of the *Morus* species were found to contain antibacterial and antifungal activities. Species of M*orus, M.bombycis, M.alba, M.nigra, M.mongolica*exhibited proven antimicrobial activity.[30,31] Hence, the author has attempted to study the antimicrobial activity of the chloroform extract of *M.indica*roots 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 method.[32] The chloroform extract of the roots of *M. indica* were used in two dose levels of 100mg/ml and 300mg/ml. Benzyl penicillin(100µg/ml) was used as standard.

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.

Table 2: ^{1}H NMR spectral data of compound cirisimaritin

Solvent: $CDCl_3$, TMS; Internal standard (90 MHz spectra)

Chemical shift (δ)	Proton integration	Multiplicity	Assignment
3.75 3.92	6H		6-OCH3 7-OCH3
6.82	1H	*s*	C-3H
6.87	1H	*s*	C-8H
7.00	2H	*d J*= 8.5 Hz	C- 3' 5' H^{s}
8.01	2H	*d J* = 8.5 Hz	C-2' 6' H^{s}

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 the 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^{o}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.

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

Extracts	Zones of inhibition							
	Gram+ ve				Gram-ve			
	B.S	*B.P*	*S.P*	*M.L*	*E.C*	*K.P*	*P.V*	*E.F*
Chloroform extract(100mg/ml)	10	19	-	10	16	16	08	-
Chloroform extract(300mg/ml)	12	21	-	12	19	18	10	-
Benzylpenicillin(100µg/ml)	36	32	35	36	36	40	34	

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- Klebsiellapneumoniae, P.V- Proteus vulgaris, E.F- Enterococcus faecalis*)

Antifungal activity

Test Samples

Antifungal activity was carried out by the cup- plate agar diffusion method.[32] 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, Saccharomyces ceriviseae, Pencillium excelsa, Pencillium chrysogenum were used as test fungal strains.

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^{o}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^{o}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.

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

Extracts	Zones of inhibition (mm)			
	A.F	*S.C*	*P.E*	*P.C*
Chloroform extract(100mg/ml)	-	16	12	11
Chloroform extract(300mg/ml)	-	18	15	13
Nystatin(100µg/ml)	19	19	17	15

Cup diameter: 6mm, Zone of inhibition in mm; **(***A.F – Aspergillus fumigans, S.C* **-** *Saccharomyces ceriviseae, P.E. – Pencillium excelsa, P.C – Pencillium chrysogenum)*

RESULTS AND DISCUSSION

The chemical examination of the roots of *M.indica* on conventional extraction and a series of chromatographic methods gave β-sitosterol, α-amyrin acetate, salvigenin, morusin, cyclomorusin and cirisimaritin. Occurrence of flavones like salvigenin and cirisimaritin were recorded for the first time in this species. The chloroform extract of *Morusindica* roots howed 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 ceriviseae*,mild activity towards *Pencillium excelsa, Pencillium chrysogenum* and showed no activity against *Aspergillus fumigans*.

CONCLUSION

The compound morusin was reported in almost all the *Morus* species, probably indicating some chemotaxonomic significance. 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. Further 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 *Morusindica.*

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