ISOLATION AND STRUCTURAL ELUCIDATION OF FLAVONOLIGNAN DERIVATIVE FROM *Melochia corchorifolia*: EVALUATION OF THEIR BIOLOGICAL, ANTICANCER ACTIVITY AND MOLECULAR DOCKING STUDIES

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ABSTRACT

Ethanol extract of leaves *Melochia corchorifolia* subjected to silica gel column chromatography and the compounds obtained are analyzed for antioxidant activity. Among the compounds collected and tested, compound four showed a maximum antioxidant potential of 90.74±0.65% by DPPH activity. Flavonolignan, a flavonoid derivative compound, was isolated and structurally characterized using various spectral analyses. The antimicrobial activity of the compound showed maximum activity of 16mm and 20mm in 75µg/ml and 100µg/ml concentrations against the human pathogenic microorganisms. Molecular docking studies of the compound showed better binding affinity and H-bond interaction with the human breast cancer estrogen receptor (3EQM) with binding energy of about -9.91 kcal/mol. We observed that the isolated compound showed a potent anticancer effect against MCF-7 cells from the preliminary results.

Keywords: *Melochia corchorifolia*, Spectroscopic Techniques, Antimicrobial, MCF-7 Cell, Flavonolignan Derivative, Antioxidant Activity.

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INTRODUCTION

The emergence of new pathogens along with antibiotic resistance and the rising failure of chemotherapy is the biggest problem increasing mortality rate in tropical countries. The unavailability of vaccines for most of these diseases and the adverse side effects of chemotherapy enables us to find natural ways which can be used against the pathogens.¹ The secondary metabolites are flavonoids, terpenoids, phenolic, tannins, and alkaloids compounds from plant origin are said to protect against the microbes as most of the compounds have antimicrobial and anti-inflammatory properties. They also effectively work as an antioxidant which prevents or slow down the damages of the free radicals or the unstable molecules that the body produces as a reaction and other pressures. These secondary metabolites are mainly synthesized from various parts of the plants such as leaf, root, stem, flower, fruit, twig, and seeds and are used to develop new novel drugs.²

Melochia corchorifolia (L.) is a perennial aromatic plant, mostly sighted in the wastelands. The leaves and roots of these plants, are utilized for treating several complications such as headache, ulcers, helminthiasis, abdominal swellings, food poisoning, and dysentery.³ Pharmacologically, the leaf extract has antioxidant, anticancer, antibacterial, antiurolithiatic, Anthelmintic, and antidiuretic activities.⁴ The Phytoconstituents analysis of *M. corchorifolia* leaves has exposed the presence triterpenes, flavonol, glycosides, flavonoids, β-D-glucoside, and alkaloids.⁵ The components such as cyclopeptide alkaloids franganine, adouetine-y, frangufoline, and novel cyclopeptide were identified and even chronicled earlier from this plant.⁶ Human breast cancer is the second most common cancer found among women due to their lifestyle changes such as dietary changes, late childbearing in developing countries leads to the risk-factor of cancer development.⁷ The MCF-7 cell line has proven to be a useful model of hormone-responsive breast cancer. These cells contain receptors for both estrogen and progesterone and respond to

the addition of estrogen *in vitro* by an increase in the rate of cell proliferation and the induction of several specific proteins. The purpose of molecular docking is to obtain preliminary information about the affinity of any compound before the start of *in-vitro* experiments.^{8, 9} In this study, ligand affinity was assessed by three parameters such as H-bonding energy, binding energy, and inhibition constant.¹⁰ The present study aimed to isolate a compound from antioxidant potential fraction obtain through column chromatography and screen for antimicrobial activity from the above aspects. Further, docking analysis was performed to study the binding energy of purified compounds against the human breast cancer estrogen receptor to control cell proliferation. Besides, the study was experimented with *in vitro* method by MTT assay on MCF-7 cells to analyze the anticancer activity.

EXPERIMENTAL

Extraction of Plant Leaves

The leaves of *Melochia corchorifolia* were washed, shade dried and finely powdered using a mechanical blender and extracted exhaustively by Soxhlet apparatus (8h) using ethanol at 60°C stored in an air-tight container for further use.¹¹

Silica Gel Column Chromatography

The dried leaves *Melochia corchorifolia* (500g) was ethanol refluxed using 1000 ml and the extract was fractioned using silica gel column (100-200 mesh size) chromatography. The separation of the extract was carried out by successive elution with gradually increasing the polarity of the solvents from n-hexane and ethyl acetate. Pre-coated TLC plates were used for analyzing the purity and separation of the fractions.¹²

Spectral Analysis

A UV-Vis spectrophotometer (Shimadzu, Japan) was observed in the range of 200 to 800 nm at 25°C in ethanol. IR spectrum was recorded in Shimadzu-FTIR spectrometer using the KBr pellet. The spectrum of ¹H NMR (500 MHz) ¹³C NMR (125MHz) was reported on a 5 mm TUBES 500 MHz Bruker Avance II spectrometer using CDCl₃ as a solvent and TMS as an internal standard (chemical shift in ppm). The regular recording of the DEPT135 spectrum (θ= 135 pulse program) was performed. Using the standard Bruker pulse system, the 2D ¹H-¹H COSY and HSQC spectra recorded. HPLC was done in a Shimadzu LC solution equipped with the quaternary pump and UV-Vis spectrophotometer (Shimadzu, Japan). The column used for the study was C18 (250 4.6 × 5µm). The mobile phase was toluene: ethyl acetate: methanol (7:3:1) with the scanning wavelength at 260 nm. Mass analysis was carried out by using Q-T of Mass Spectrometer (Micro mass) instrument with high-resolution double focus. Total resolution (6000), Daltons Source options- Electron impact (EI), calibrated Total mass (1500), chemical ionization (CI).¹³

Antioxidant-DPPH Assay

The DPPH assay is based on a stable free radical reduction method and maximum absorption at 517 nm. The quantity of 4.3mg 1, 1-Diphenyl-2-picrylhydrazyl was dissolved 3.3mL ethanol and shielded from light by aluminum foil covering the test tubes. About 50 µL concentrations (10-100 µg/mL) of ethanol extract leaf of *Melochia corchorifolia* with ascorbic acid were added and the volume solution was made uniformly to 150µL using ethanol followed by addition 150 µL of DPPH.¹⁴ The absorbance was recorded at 517 nm using ethanol as blank. The value of the samples and standard and calculated the percentage of inhibition.

High Performance Liquid Chromatography Analysis

HPLC (Shimadzu LC) column was used for the study is C18250 4.6 × 5 µm. The mobile phase solvent was a 7:2:1 combination of toluene, ethyl acetate, and ethanol.¹⁵

UV-Visible Spectra Analysis

UV-Visible spectrum analysis performed in the range of 200 to 800 nm, control ethanol was used.¹⁶

In-vitro Activity-Antimicrobial Studies

The agar well diffusion methodology was performed for human pathogenic bacteria organisms. The antimicrobial activity plates were concocted with 20 ml of sterile Muller Hinton agar for bacteria. The

solidified media was topped with the test culture and was left to dry for 10 min. The sterile well borer was used to make wells in the seeded plates as soon as the solidification of media was done. Wells are filled with 25, 50, 75, and 100 µg/ml of the compound flavonolignan derivative compound 4, negative control as Streptomycin and Clotrimazole well served as a positive control. The plates are incubated at about 37°C for 24 h and activity was calculated in the range of diameter and zone of inhibition formed around the well. In antifungal activity, the same method as for bacteria was adopted, but potato dextrose agar (PDA) was used for both *C. albicans* and *A. niger*. The medium was incubated at 25°C for 2 days for *C. albicans* and 3 days for *A. niger* test.¹⁷

MTT Assay

The assay was executed using (3 (4, 5dimethyl thiazol-2yl) 2, 5 diphenyl tetrazolium bromide (MTT) (Sigma Aldrich, Bangalore). The MTT is partitioned by mitochondrial succinate dehydrogenase and followed by reductase in viable cells. That accumulates formazan for a measurable purple substance. Then following 48 h incubation, MTT assays were added in wells for 3h and medium extracted from wells and 100 µl of SDS were applied in DMSO to dissolve the formazan crystals and absorbance were read at 540nm.¹⁸

Auto Docking Studies

Auto dock 4.2 tools were used for docking to have a better understanding of the analysis. The human breast cancer estrogen receptor protein was subjected to forecast the mechanism of the purified compound. The PDB files were edited using chimera tools, non-protein compounds were removed, and receptor protein saved. Then PDB files edited by adding hydrogen and water molecules removed for accuracy. The docking analysis was performed with Auto dock scripts of ADT tools.¹⁹

RESULTS AND DISCUSSION

Antioxidant Activity

The compounds obtained from column chromatography were evaluated for DPPH assay, which showed that all the compounds possess free radical scavenging ability. The percentage of inhibition compounds lied between 80-90%. Among all the compounds tested, compound 4 showed a remarkable antioxidant activity of 90.74±0.65% in Fig.-1 to scavenge DPPH radicals and hence the compound 4 was structurally elucidated. The DPPH ability to free radical scavenging of all compounds (C1-C5) is tabulated shown in Table-1.

Table-1: DPPH Assay of the Isolated Compounds

Compounds	DPPH Free Radical Scavenging* (%)
Compound 1	82.77±1.39
Compound 2	79.30±1.19
Compound 3	82.20±1.04
Compound 4	90.74±0.65
Compound 5	80.49±1.38

* Each DPPH free radical scavenging compound value represents mean ± SD

FT-IR

The flavonolignan derivative compound 4 spectral analyses showed in Fig.-2 Functional group peaks are tabulated in Table-2. The intense peak for hydroxyl group stretching vibration at 3377 cm⁻¹ indicates the hydroxyl functional group. Then aromatic and aliphatic C-H stretching frequency vibration showed at the range of 2961 and 2840 cm⁻¹. The carbonyl and vinyl alkyl ether stretching vibration frequency was observed at the range 1650 and 1610cm⁻¹. The C-H bending vibration showed at 1439 cm⁻¹ and Hydroxyl (OH) bending vibration at 1325cm⁻¹. The previous studies demonstrated that the alcoholic extracts would exhibit the FTIR spectra at 2900-3500 cm⁻¹. This interprets the presence of OH and C-H vibrations with the flavonoids and phenolic compounds.²⁰

Spectroscopic Characterizations Flavonolignan Derivative

¹H NMR spectrum flavonolignan derivative compound showed Fig.-3 aromatic methine protons at δ 7.62 (s, 1H), 7.44 (s, 1H), 7.19 (s, 1H), 7.14 (t, *J* = 10 Hz, 1H), 6.97 (d, *J* = 10 Hz, 1H), 6.81 (d, *J* = 10 Hz,

1H), 6.76 (d, $J = 10$ Hz, 1H), 6.55 (d, $J = 10$ Hz, 1H) ppm. The flavonoid ring methine protons were observed at 5.77-5.67 ppm as a multiplate. The aromatic attached hydroxyl protons were observed at 6.17 ppm. Five membered furan ring methylene proton were observed at the range is 4.92 (dd, $J_{1,2} = 10$ Hz, 25 Hz), 4.28 (d, $J = 5$ Hz), 4.25 (t, $J = 10$ Hz), 4.06 (q, $J = 10$ Hz) ppm multiplate respectively. Five membered furan ring methine proton were observed at the range is 3.66-3.50 multiplate.

Fig.-1: DPPH Scavenging Activity against the isolated Compounds

Table-2: FT-IR Spectral Analysis Data of Purified Compound

Peak Wavenumber cm^{-1}	Vibration Type	Functional Groups
3377	-O-H (stret)	Alcohol
2961	-C-H (Ar stret)	Aliphatic
2840	-C-H (Sp^3 stret)	Methylene
1650	-C=O (stret)	Carbonyl
1610	-C=C (stret)	Alkyl Ether
1439	-C-H (bend)	Aromatic
1325	-O-H (bend)	Alcohol

Fig.-2: FT-IR Spectrum of Flavonolignan Derivative

Aliphatic methylene proton was observed at the range at δ 2.00 (s), 1.95 (s), 1.32 (s), 1.16-0.77 (m) ppm respectively. ^{13}C NMR of the flavonolignan derivative compound showed carbonyl carbon peak at 199.07

respectively it shows flavanone group Fig.-4. The aromatic carbon signal observed at the range is δ 139.2, 130.9, 129.5, 128.8, 123.9, 123.3, 120.1, 115.8, 115.3, 114.0 ppm and aliphatic carbon peak were observed at the range is 71.7, 70.4, 65.5, 60.4, 33.8, 31.9, 31.6, 30.5, 29.6, 29.6, 29.4, 29.3, 29.1, 28.9, 27.7, 22.6, 20.9, 20.6, 19.1, 19.1, 14.1, 14.0 ppm. ^1H NMR (500MHz, CDCl_3 , ppm): δ 7.62 (s, 1H), 7.44 (s, 1H), 7.19 (s, 1H), 7.14 (t, $J = 10$ Hz, 1H), 6.97 (d, $J = 10$ Hz, 1H), 6.81 (d, $J = 10$ Hz, 1H), 6.76 (d, $J = 10$ Hz, 1H), 6.55 (d, $J = 10$ Hz, 1H), 5.77-5.67 (m, 1H), 4.92 (dd, $J_{1,2} = 10$ Hz, 25 Hz, 2H), 4.28 (d, $J = 5$ Hz, 1H), 4.25 (t, $J = 10$ Hz, 2H), 4.06 (q, $J = 10$ Hz, 17H), 3.66-3.50 (m, 3H), 2.00 (s, 9H), 1.95 (s, 17H), 1.32 (s, 3H), 1.16-0.77 (m, 62H) ^{13}C NMR Data (125MHz, CDCl_3 , ppm): δ 199.07 176.46, 171.34, 139.23, 130.91, 129.50, 128.83, 123.91, 123.39, 120.18, 115.81, 115.32, 114.04, 71.73, 70.47, 65.59, 60.45, 33.80, 31.90, 31.60, 30.54, 29.67, 29.60, 29.49, 29.33, 29.13, 28.93, 27.70, 22.66, 20.99, 20.67, 19.15, 19.12, 14.14, 14.07 ppm DEPT 135 NMR (125MHz, CDCl_3 , ppm): δ 139.24, 131.05, 128.85, 114.05, 60.46, 60.44, 33.80, 31.91, 31.60, 29.68, 29.61, 29.47, 29.34, 29.14, 28.94, 22.67, 21.03, 20.69, 19.12, 14.15, 14.08 ppm. Investigation of the correlations in the COSY spectrum further assisted to elucidate the structure of the compound 4 one bond coupling with carbon and hydrogen was observed in HSQC. HSQC was used to obtain the complete assignment of proton and carbon resonances. From the HSQC correlations, the aromatic and furan ring moiety couplings are confirmed and shown in the Table-3, the long-range correlation of aromatic methine and furan ring moiety methine protons in the compound 4 as showed in DEPT 135 spectrum of flavonolignan compound 4 Fig.- 5. ^1H - ^1H COSY NMR Spectrum of flavonolignan compound 4 is shown in Fig. -6 and HSQC Spectrum of flavonolignan compound 4 is shown in Fig.-7. NMR chemical shifts together with 2D NMR correlations confirm that the predicted molecular structure is flavonolignan derivative compound 4 which is in consistent with proposed structure of the flavonolignan derivative. The Mass spectral analysis flavonolignan compound 4 showed Fig.-8 the intense base peak at 301.2852 is because of the presence of taxifolin, a flavonoid moiety and $(\text{M}+1)^+$ molecular ion peak at 713.8269, the molecular mass of compound 4 and corresponds to the calculated theoretical molecular mass of the isolated compound m/z ; 712.2520. The HR-MS, flavonolignan derivative compound 4 deduced is molecular formula $\text{C}_{40}\text{H}_{40}\text{O}_{12}$ and name (2R,3R)-2-(5'-((1S,3aR,4S,6aR)-4-(4-(hex-5-en-1-yl)-3-methoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2',3',5,6-tetrahydroxy-[1,1'-biphenyl]-3-yl)-3,5,7-trihydroxychroman-4-one, Fig.-9. ²¹

Fig.-3: ^1H NMR Spectrum Flavonolignan Derivative

Fig.-4: ^{13}C NMR Spectrum Flavonolignan Derivative

Fig.-5: DEPT 135 Spectrum Flavonolignan Derivative

Table-3: ^1H NMR, ^1H - ^1H COSY, HSQC NMR (1J) Data of Purified Flavonolignan Derivative

Proton Number (^1H NMR)	^1H - ^1H Cosy	HSQC NMR (1J)
H1-7.62 (s, 1H)	H1-H2	128.70
H2-7.44 (s, 1H)	H2-H1	130.94
H3-7.19 (s, 1H)		123.84
H4-7.14 (t, $J = 10$ Hz, 1H)	H4-H6,H7	129.41
H5-6.97 (d, $J = 10$ Hz, 1H)	H5-H8	123.33
H6-6.81 (d, $J = 10$ Hz, 1H)	H6-H4	120.26
H7-6.76 (d, $J = 10$ Hz, 1H)	H7-H4	115.31
H8-6.55 (d, $J = 10$ Hz, 1H)	H8-H3	115.79
H9-5.77-5.67 (m, 1H)	H9-H10	139.14
H10-4.92 (dd, $J_{1,2} = 10$ Hz, 25 Hz, 2H)	H10-H9	114.12, 114.03

H11-4.28 (d, $J = 5$ Hz, 1H)	H11-H19	61.57
H12-4.25 (t, $J = 10$ Hz, 2H)	H12-H17	65.53
H13-4.06 (q, $J = 10$ Hz, 17H)	H13-H19	60.39
H14-3.66-3.50 (m, 3H)	H14-H19	70.36, 47.91, 41.23, 44.16
H15-2.00 (s, 9H)	H15-H16	20.63
H16-1.95 (s, 17H)	H16-H15	20.92

Fig.-6: ^1H - ^1H COSY Spectrum Flavonolignan Derivative

Fig.-7: HSQC Spectrum Flavonolignan Derivative

Fig.-8: Mass Spectrum of isolated Compound 4

(2R, 3R)-2-(5'-((1S,3aR,4S,6aR)-4-(4-(hex-5-en-1-yl)-3-methoxyphenyl) hexahydrofuro[3,4-c] furan-1-yl)-2',3',5,6-tetrahydroxy-[1,1'-biphenyl]-3-yl)-3,5,7-trihydroxychroman-4-one

Fig.-9: Proposed Structure of the Compound 4

HPLC Analysis

The purified compound showed a sharp peak at 9.945 min (retention time). The peak area is 42857 coverage of 100% flavonolignan derivative compound 4, Fig.-10.

Fig.-10: HPLC Analysis of the isolated Flavonolignan Derivative

UV-Vis Spectrum of Purified Compound

The spectrum of the purified flavonolignan derivative compound 4 in ethanol solution Fig.-11 exhibited maximum absorption at UV area 277 nm, indicating flavonolignan derivative compound 4 containing flavanone and polyphenol group.

In-vitro Antimicrobial Activity

The flavonolignan derivative compound 4 was observed to be active against pathogenic microorganisms with a zone of inhibition represented in Table- 4. Among the four concentrations used, both 25µg/ml and 50 µg/ml of the sample showed an antimicrobial activity against the organisms. The higher concentrations of 75 µg/ml and 100 µg/ml maximum inhibition activity against *E. coli* (16 mm and 20 mm respectively) and *B.subtilis* (16 mm and 19 mm). The isolated compound 4 showed the least activity against *A.niger*, while *C.albicans* was the most sensitive species (16 mm in 100 µg/ml concentration). The MIC value of flavonolignan derivative, the lowest MIC values (>50 µg/mL), was recorded against *B.subtilis* *P. aeruginosa* and *E.coli*. Then the moderate response of MIC was registered against *S.aureus* and *C.albicans* (>75 µg/mL). As suggested by previous reports, *M. corchoifolia* has been presented with numerous biological activities like Hepatoprotective antimicrobial.²²

Fig.-11: UV-Vis Spectrum purified Flavonolignan Derivative

Table-4: Antibacterial and Antifungal Activity

Pathogenic microorganism	30 STD	25	50	75	100	MIC
<i>E. coli</i>	25.32±0.05	8.05±0.17	10.23±0.03	16.03±0.03	20.33±0.14	>50
<i>P. aeruginosa</i>	28.16±0.03	0.00±0.00	10.12±0.05	12.98±0.25	15.90±0.05	>50
<i>B. subtilis</i>	20.25±0.05	8.23±0.14	14.16±0.03	16.03±0.10	19.10±0.15	>50
<i>S. aureus</i>	20.13±0.14	0.00±0.00	08.56±0.06	10.06±0.03	12.16±0.03	>75
<i>C. albicans</i>	19.45±0.03	7.37±0.09	08.24±0.09	11.00±0.07	16.42±0.14	>75
<i>A. niger</i>	15.41±0.08	0.00±0.00	0.00±0.00	08.06±0.03	09.10±0.12	>125

* Streptomycin; # Clotrimazole

ZOI: Zone of inhibition

In-vitro Cytotoxicity Activity

The cytotoxicity effect of the flavonolignan derivative compound 4 against suspension of adherent cell line (Breast cancer cell line MCF-7) is given in Table-5. Compound 4 showed an excellent anticancer reactivity against MCF-7 cell line. There was dose-dependent manner activity observed. MCF-7 cell lines showed a low level of cell viability (12.41%) registered in 400 µg/ml at 48h, whereas 48 h MCF-7 cell line showed a low level of cell viability registered (94.27%) in 3.125 µg/ml. The IC₅₀ value of the isolated compound 4 was observed at 68.59 µg/ml against MCF-7. Another study has found that the flavonoids and phenolic compounds isolated from the extract of *M. corchorifolia* showed anticancer and cytotoxicity activities against HCT-116 and MCF-7 cells.²³

Table- 5: Cytotoxicity Assay - MCF-7 Cell Line

Concentrations (µg/ml)	Cell Viability (%)
0	100.00
3.125	94.27
6.25	83.85
12.5	77.57
25	59.82
50	53.62
100	38.58
200	19.17
400	12.41
IC ₅₀	68.59

Docking Studies

The ligand flavonolignan derivative compound 4 of *M.corchorifolia* is docked with Human Breast Cancer Estrogen Receptor protein (3EQM) and binding energy was determined. The isolated ligand compound 4 showed H-bond energy of -10.81, the binding affinity of -9.91 kcal/mol and the inhibition constant 988.69 μm Table-6. The potential binding site of amino acid residues of protein receptors is PHE147, LYS150, ALA151, SER153, PRO155, VAL158, ARG159 these amino acid residues act as strong binding region with the purified compound 4 Fig.-12. ²⁴

Table-6: Binding Strength of Flavonolignan Ligand with the Protein Receptor (3EQM)

Protein Pdb ID	Ligand	Hydrogen Bond	Binding Energy (kcal/ mol)	Inhibition Constant (μm)
3EQM	Flavonolignan	-10.81	-9.91	988.69

Fig.-12: Molecular Interaction of 3EQM with Flavonolignan Derivative Compound

CONCLUSION

The flavonolignan derivative compound 4 was isolated and separated from ethanol extract of *Melochia corchorifolia* leaf the compound possess both flavanone and lignan parts molecular formula $\text{C}_{40}\text{H}_{40}\text{O}_{12}$, molecular name (2R,3R)-2-(5'-((1S,3aR,4S,6aR)-4-(4-(hex-5-en-1-yl)-3-methoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2',3',5,6-tetrahydroxy-[1,1'-biphenyl]-3-yl)-3,5,7-trihydroxychroman-4-one, are further characterized and confirmed through spectral analysis and by the Pubchem database structure similarity search possess 95% similarities with pseudotsuganol compound. In silico results, flavonolignan derivative compound 4 was docked with estrogen receptor protein, which showed stable interaction with the lowest binding energy (-9.91 ΔG , kcal/mol). The biological activities such as antimicrobial activity and anticancer activity of the flavonolignan derivative compound 4 showed promising effects. Moreover, there is a need for elaborated study on the biological aspects.

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