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TO ISOLATE ANTIBACTERIAL AND ANTI - INFLAMMATORY ACTIVITIES IN THE FLOWERING PART OF IXORA COCCINEA

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ABSTRACT

The study investigates the anti-inflammatory and antibacterial properties of ixora coccinea a medicinal plant traditionally used for treating various ailments, especially inflammatory conditions and infections. Bioassay-guided fractionation using column chromatography led to the isolation of Kaempferol 3-O-β- D-glucopyranoside, from the methanol extract, which were further characterized using FTIR and NMR spectroscopy.

Keywords: Anti-inflammatory, Antibacterial, Ixora coccinea, FTIR, NMR.

1.INTRODUCTION

Ixora coccinea Linn is a perennial herb that typically grows to a height of 1.5 meters. Ixora coccinea Linn. Belongs to the kingdom Plantae, phylum Angiosperms, class Dicotyledons, and the order Lamiales. It is classified under the Rubiaceae family, which includes many aromatic plants commonly used in culinary and medicinal applications.

2.MATERIALS AND METHODS

2.1 Collection and Processing of Plant material

The flowers of Ixora coccinea Linn. were gathered from area around palayamkotai,

Tamil Nadu, and authenticated by Dr. S. Mutheeswaran at Xavier Research Institution.

2.2 Extraction

The process began with the preparation of consecutive solvent extracts. Coarsely powdered dried flowers (500 g) of Ixora coccinea Linn. were extracted using pet ether at 60–80°C in a Soxhlet apparatus, followed by extraction with solvents of increasing polarity at 60–70°C, including chloroform, ethyl acetate, methanol, and water.

2.2.1Isolation and characterization

Ch/romatography is high efficient and powerful technique for resolution of the mixtures. TLC is an effective chromatographic method for separation. It requires simple instrumentation and having low cost.

2.2.2 Column Chromatography

The bioassay-guided isolation technique was employed to determine the activity in the various fractions. After several trials, a mixture of Dichloromethane and Ethanol (9:1) was selected as the optimal solvent phase. 2.3

InvitroinflammatoryActivity Chemicals:

Reagents used in the experiments were procured from SD fine chem ltd, India and all the other chemicals and reagents procured were of analytical grade.

In-Vitro Anti-inflammatory activity using Inhibition of protein denaturation Assay:

Principle:

The primary goal of the egg albumin denaturation assay is to evaluate whether certain agents or compounds can prevent or reduce the denaturation of egg albumin under specific conditions. Denaturation refers to the alteration of a protein's structure, leading to a loss of its biological function. This assay operates on the principle that substances with anti-inflammatory properties might stabilize protein structures and prevent denaturation, which is commonly associated with inflammation and tissue damage. Therefore, agents or compounds that significantly reduce egg albumin denaturation in this assay may possess potential anti-inflammatory properties (Clark, 1943).

Chemicals:

Test drug: Compounds coded
Standard drug: Diclofenac

Chemicals: Egg albumin, Phosphate-buffered saline (pH 6.4), Distilled water

Procedure:

Preparation of egg albumin

Fresh hen's eggs were utilized to prepare a 1% egg albumin solution. The preparation involved carefully cracking a fresh egg, transferring 1 mL of the transparent egg white into 100 mL of distilled water, and stirring the mixture thoroughly. The resulting clear solution was then stored in a refrigerator for subsequent use.

Assay :

The sample drugs were weighed and dissolved in distilled water to achieve a concentration of 1 mg/mL. This solution was then serially diluted to prepare concentrations of 3.125, 6.25, 12.5, 25, 50, 100, 250, and 500 µg/mL. Corresponding solutions of the standard drug, Diclofenac, were prepared at similar concentrations. The anti-inflammatory activity was assessed in vitro using a standard procedure adapted from Leelaprakash and Dass (2011), with minor modifications. Aliquots of

0.5 mL from different concentrations of the test and standard drugs were transferred

into test tubes, each containing 2.5 mL of phosphate-buffered saline (PBS). To this, 2 mL of egg albumin was added, and the mixture was incubated at 27°C. After 15 minutes, the tubes were transferred to a water bath at 70°C for 10 minutes. Once cooled, the absorbance was measured using a UV-Vis spectrophotometer at 660 nm, with distilled water as the blank. The reaction mixtures without the drugs served as control solutions. The percentage inhibition was calculated using the following formula:

Percentage inhibition = Abs Test - Abs control/ Abs control × 100

Where Abs control=absorbance of the control; Abs Test=absorbance of the test.

The fraction with the lowest IC50 was identified as most potent, and HPTLC analysis was conducted using an Ethylacetate-Formic acid-Acetic acid-Water (10:1.1:1.1:2.6) solvent system.

2.3 Invitro anti-bacterial Activites:

The microbes tested were Salmonella typhi and E. coli and they were collected from the microbiology department of local degree college. This culture was let to incubate for 24 hr in oven at 35ºC from this mother culture, sub cultures were taken and used for further experiments.

Antibacterial activity

The anti-bacterial activity of all isolates obtained from various solvents was tested using the disc diffusion method, following the standard procedure proposed by Theivasanthia and Alagar (2009). Petri plates were sterilized in an oven, and freshly prepared agar medium was poured into the plates, allowing it to cool and solidify. The nutrient broth medium, containing the bacterial culture, was poured onto the solidified agar medium to ensure an even thin film of bacteria was spread across the plate. The plates were then incubated. Diffusion discs of approximately 0.5 cm in radius were dipped into each isolate from different extracts and gently placed on the agar plates at equal distances from each other. The plates were appropriately marked for future identification. The plates were incubated in an oven at 40°C for 24 hours, after which they were removed and examined for the zones of inhibition. The diameter of the clear zone, where bacterial growth was inhibited, was measured from the edge of the diffusion disc to the edge of the inhibition zone. The results were recorded for each isolate.

3.1 Characterization of the isolated compound from Ixora extract (ICIC)

3.1.1 Fourier transform infrared (FTIR)

The distinctive functional groups in the ethanol extract were identified using Fourier transform infrared (FTIR) spectroscopy. FTIR allows the determination of a molecule's structure based on its absorption spectra. The isolated compound was diluted and mixed with dry potassium bromide (KBr). The mixture was thoroughly combined in a mortar before being compressed at 6 bars for 2 minutes to form a thin KBr disc. The disc was then placed in a sample cup of a diffuse reflectance accessory for analysis. The IR spectra were obtained using the Bruker Vertex 70 infrared spectrometer, Germany. The scanning range for the sample was from 4000 to 400 cm^{-1}. The UV-VIS and FTIR peak values were recorded.

3.1.2 1H and 13C NMR

1H and 13C NMR spectrum were re-corded on Bruker 300 MHz instrument using $CDCl_3$ solvent. Chemical shifts are re-ported in parts per million (ppm) using Tetramethylsilane (TMS) as internal standard and using DMSO as solvent. The Topspin software was used for this analysis.

4. RESULTS

4.1 Physicochemical parameters

4.2 Preliminary phytochemical screening

The Preliminary phytochemical screening of various extracts was performed and the presence of carbohydrates, alkaloids, glycosides, tannins, terpenoids, phenols and flavonoids was reported.

Sl. No.	Test	Pet. Ether	Benzene	Ethyl acetate	Methanol	Water
1.	Carbohydrates	-	-	-	+	+
2.	Alkaloids	-	+	-	+	+
3.	Glycosides	-	-	-	+	+
4.	Tannins	-	-	-	+	+
5.	Steroids	+	-	-	-	-
6.	Triterpenoids	-	-	-	+	-
7.	Volatile oils	-	-	-	-	-
8.	Fats and fixed Oils	-	-	-	-	-
9.	Flavonoids	-	-	-	+	+
10.	Polyphenols	-	-	-	+	+
11.	Saponins	-	-	-	+	+
12.	Amino acids	-	-	-	+	+
13.	Gums and Mucilage's	-	-	-	-	+

Anti-Inflammatory activity

The in vitro anti-inflammatory activity of the isolated fractions from the methanol extract of Ixora coccinea Linn. is presented in Table 4.1. The percentage inhibition of inflammation at various concentrations (6.25 µg/mL, 12.5

µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL, 250 µg/mL, and 500 µg/mL) for each fraction, along with the methanol extract as a reference. The data demonstrates a dose-dependent increase in antiinflammatory activity across all fractions, with Fraction 1 showing the highest inhibition at the lower concentrations, particularly at 6.25 µg/mL (8.56% inhibition). However, as the concentration increased, Fraction 1 exhibited significant improvement in inhibition, reaching 99.44% at 500 µg/mL. Fractions 2 and 3 displayed comparatively lower inhibition across the concentrations, with Fraction 2 showing 7.12% inhibition at 6.25 µg/mL and 73.42% inhibition at 500 µg/mL. Fraction 3 demonstrated a slightly lower inhibition rate at 6.25 µg/mL (5.55%) but exhibited a significant increase to 70.12% inhibition at 500 µg/mL. Fraction 4 and 5 showed more consistent antiinflammatory effects, with Fraction 4 reaching 83.32% inhibition at 500 µg/mL, while Fraction 5 demonstrated the highest inhibition among these fractions, reaching 89.26% at 500 µg/mL.

The IC50 values, which indicate the concentration required for 50% inhibition, further reveal that Fraction 1 has the lowest IC50 value of 46.36 µg/mL, indicating superior anti-inflammatory activity compared to the other fractions. Fraction 4 had an IC50 of

72.25 µg/mL, and Fraction 5 exhibited an IC50 of 116.01 µg/mL. Fraction 2 and Fraction 3 demonstrated higher IC50 values, with 104.37 µg/mL and 128.14 µg/mL, respectively, reflecting their comparatively lower efficacy.

Table 4.1 Invitro anti-inflammatory activity of isolated fractions of the methanol extract of Ixora coccinea flower

Sl no	Conc (µg/ml)	%inhibition					
		Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Methanol Extract
1	6.25	8.56±0.45	7.12±0.45	5.55±0.45	7.25±0.47	2.34±0.67	5.64±0.95
2	12.5	19.44±1.34	15.42±1.75	13.39±1.31	17.749±1.52	6.35±1.84	10.46±1.48
3	25	31.07±2.28	24.36±2.15	22.18±2.11	28.15±2.36	14.51±2.24	21.73±2.75
4	50	50.96±3.58	39.66±4.03	36.63±3.65	42.43±3.43	30.25±4.29	33.11±3.25
5	100	67.98±3.55	50.03±3.25	46.04±4.34	58.91±3.24	45.44±4.75	42.59±5.71
6	250	95.33±4.15	61.25±4.06	57.28±5.29	72.34±5.12	68.51±5.11	58.54±5.29

7	**500**	99.44±5.26	73.42±5.43	70.12±6.61	83.32±6.08	89.26±5.54	72.37±5.77
IC50		46.36	104.37	128.14	72.25	116.01	135.58

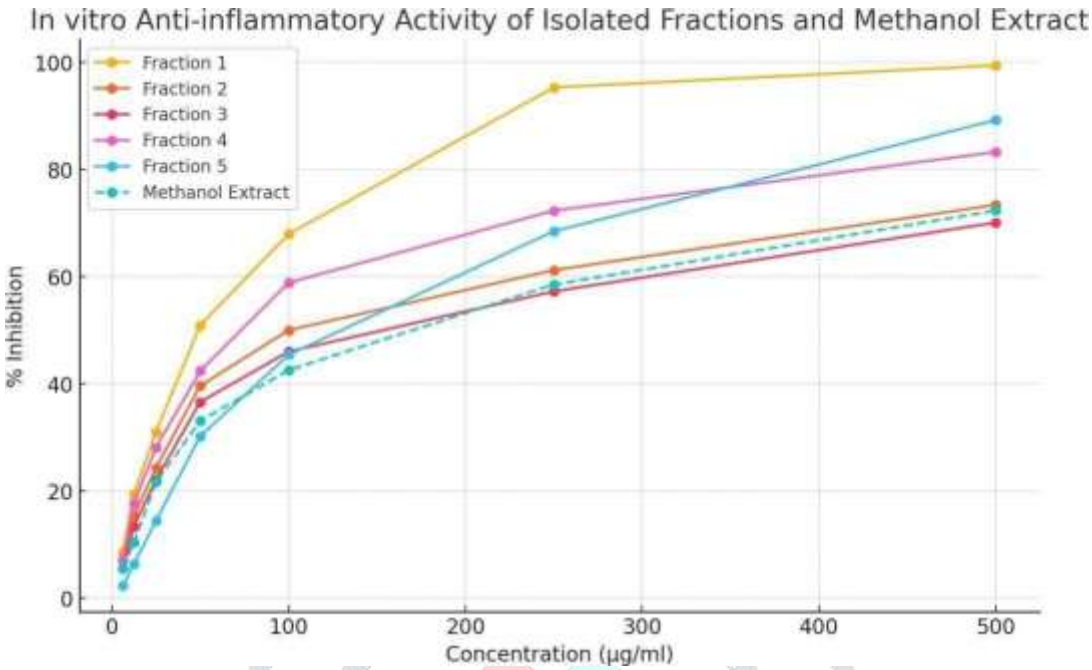

Figure 4.2 Anti-inflammatory activity of isolated fractions of Methanol extract of Ixora coccinea flowering part

Table 4.3 presents the in vitro anti-inflammatory activity of the isolated compounds from Fraction 1 of the methanol extract. The table shows the percentage inhibition of inflammation at various concentrations for Isolate 1a, Isolate 1b, Isolate 1c, and a standard anti-inflammatory compound. Among the isolates, Isolate 1a demonstrate an increase in inhibition with concentration, starting at 8.37% inhibition at 6.25 µg/mL and reaching 97.89% at 500 µg/mL. Isolate 1c exhibited moderate inhibition at lower concentrations but showed a higher inhibition (81.24%) at 500 µg/mL. The best antiinflammatory compound in this group, however, was Isolate 1c, with an IC50 of

99.74 µg/mL, significantly lower than Isolate 1a (IC50 = 136.74 µg/mL) and Isolate 1b (IC50 = 168.39 µg/mL). The standard drug, with an IC50 of 34.82 µg/mL, displayed superior anti-inflammatory activity at all concentrations.

These data suggest that Isolate 1c from Fraction 1 of the methanol extract of Ixora coccinea Linn. demonstrates promising anti-inflammatory activity, with an IC50 value close to that of the standard drug. This highlights Isolate 1c as the most potent bioactive compound among the isolates, potentially contributing to the observed anti-inflammatory effects of the plant. The overall data from Table 4.1 and 4.3 emphasize the potential of Ixora

coccinea Linn. as a source of anti-inflammatory agents, with Isolate 1c being a key candidate for further pharmacological exploration.

Table 4.3 Invitro anti-inflammatory activity of isolated compounds of the Fraction 1 of methanol extract of Ixora conninea flower

Si no	Conc (µg/ml)	%inhibition			
		Isolate 1a	Isolate 1b	Isolate 1c	Standard
1	6.25	8.37±0.45	2.95±0.54	5.43±0.45	10.07±0.64
2	12.5	16.47±1.33	6.03±1.73	11.19±1.58	24.24±1.63
3	25	28.29±3.69	11.56±3.64	21.78±2.36	47.52±2.75
4	50	51.52±4.28	23.15±3.55	37.56±3.25	60.34±4.14
5	100	69.71±3.45	41.79±4.49	50.38±4.37	71.36±4.05
6	250	87.46±5.78	58.48±4.64	67.51±4.74	88.65±4.62
7	500	97.89±5.58	71.24±5.95	81.24±5.55	99.84±4.98
IC50		136.74	168.39	99.74	34.82

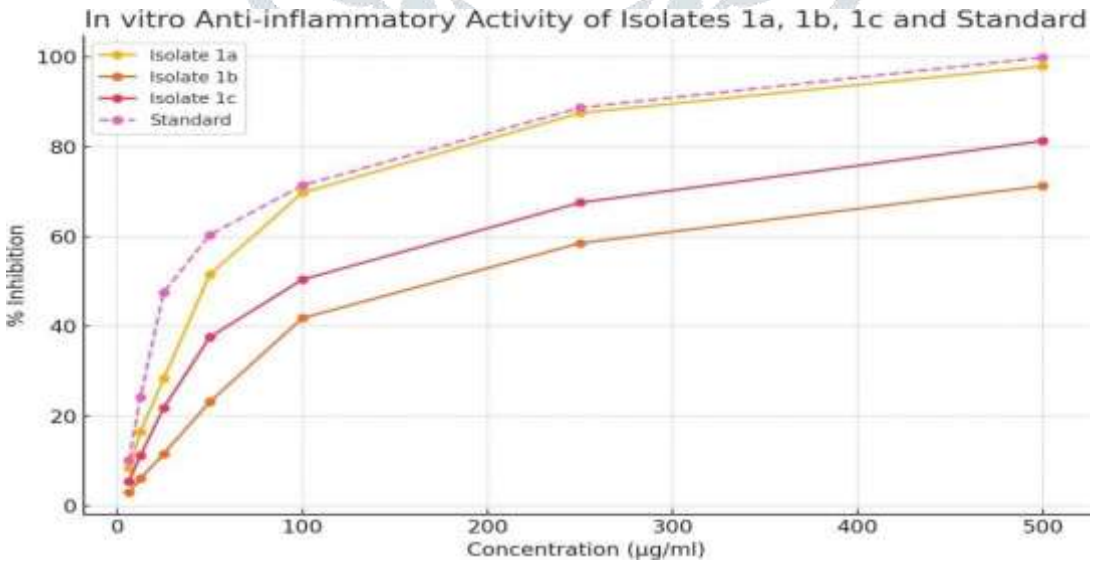

Figure 4.4 Anti-inflammatory activity of isolated compounds of Methanol extract of ixora

Antibacterial activity

Table 4.5 presents the antibacterial activity of the isolated compounds from Fraction 1 of the methanol extract of ixora coccinea Linn. The antibacterial activity was measured by the zone of inhibition against two bacterial species: Escherichia coli and Staphylococcus aureus. The data shows the zone of inhibition for Isolates 1a, 1b, and 1c, as well as the standard antibiotic used for comparison. For Escherichia coli, Isolate 1c exhibited the largest zone of inhibition (8.38 ± 0.72 mm), followed by Isolate 1b (5.28 ± 0.47 mm) and Isolate 1a (4.55 ± 0.39 mm). The standard antibiotic displayed the highest zone of inhibition (9.06 ± 0.66 mm), indicating strong antibacterial activity against this bacterium.

In the case of Staphylococcus aureus, Isolate 1c again showed the most significant antibacterial activity, with a zone of inhibition of 9.85 ± 0.81 mm. Isolate 1b also demonstrated good activity with a zone of inhibition of 5.83 ± 0.33 mm, while Isolate 1a showed the least activity with a zone of inhibition of 3.59 ± 0.52 mm. The standard antibiotic again produced a comparable zone of inhibition (9.54 ± 0.49 mm), highlighting its strong effectiveness against Staphylococcus aureus. Isolate 1c demonstrated the highest antibacterial activity against both Escherichia coli and Staphylococcus aureus, with inhibition zones that were relatively close to or higher than the standard antibiotic. This suggests that Isolate 1c is a promising antibacterial agent, particularly against Staphylococcus aureus, and warrants further investigation into its potential as a therapeutic compound.

Table 4.5 Antibacterial activity of the isolated compounds from the fraction 1 of methanol extract of Ixora coccinea flowering part

Sino	Bacterium sps	Zone of inhibition			
		Isolate 1a	Isolate 1b	Isoalate1c	Standard
1	Escherichia coli	4.55±0.39	5.28±0.47	8.38±0.72	9.06±0.66
2	Staphylococcus aureus	3.59±0.52	5.83±0.33	9.85±0.81	9.54±0.49

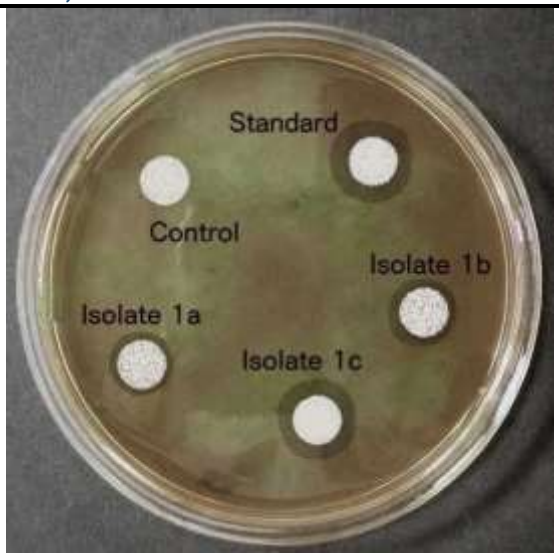

Figure 4.6 Zone of inhibition of isolated compounds on Staphylococcus aureus

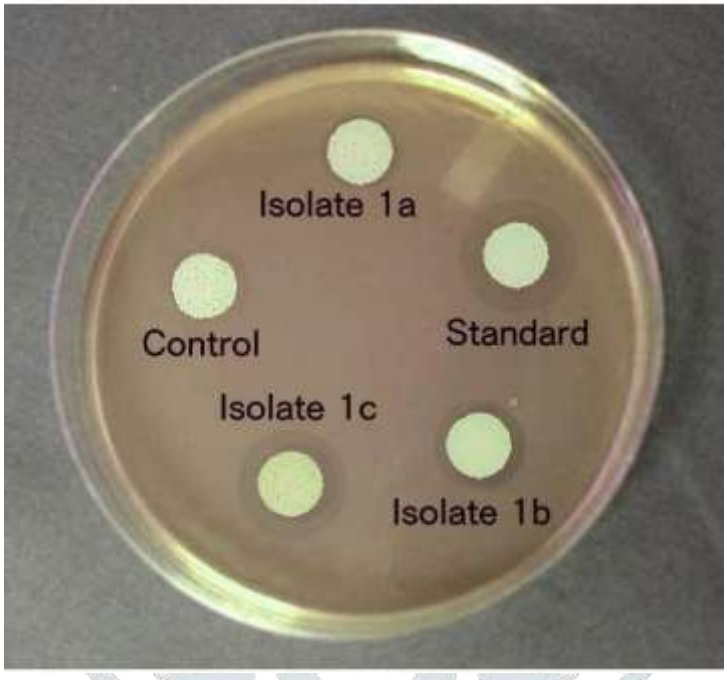

Figure 4.7 Zone of inhibition of isolated compounds on E. coli

4.2 FTIR Analysis

Fourier-transform infrared (FTIR) spectroscopy was conducted to identify the functional groups in Isolate 1c, which was obtained from the methanol extract of Ixora coccinea Linn. flowers. The analysis provided valuable insights into the chemical structure of the isolated compound, facilitating its characterization. Figure 4.2 summarizes the key absorption peaks and their corresponding functional group assignments. The prominent peaks observed at 1448.09 cm^{-1} and 1511.68 cm^{-1} were attributed to phenyl OH groups, indicating the presence of aromatic hydroxyl functionalities. The peak at 1605.48 cm^{-1} was associated with C=C stretching vibrations, characteristic of aromatic or unsaturated carbon-carbon bonds. The absorption at 1649.91 cm^{-1} suggested the presence of carbonyl (C=O) groups. Additionally, the peak at 1368 cm^{-1} indicated CH_3 stretching vibrations.

Bands observed between 730–820 cm^{-1} were attributed to C-H plane bending, while the broad absorption between 2856–3327 cm^{-1} was linked to aromatic C-H stretching.

These FTIR spectral features confirm the presence of aromatic hydroxyl, carbonyl, and alkyl groups in Isolate 1c, supporting its identification as a bioactive phytochemical constituent of Ixora coccinea Linn. flowers.

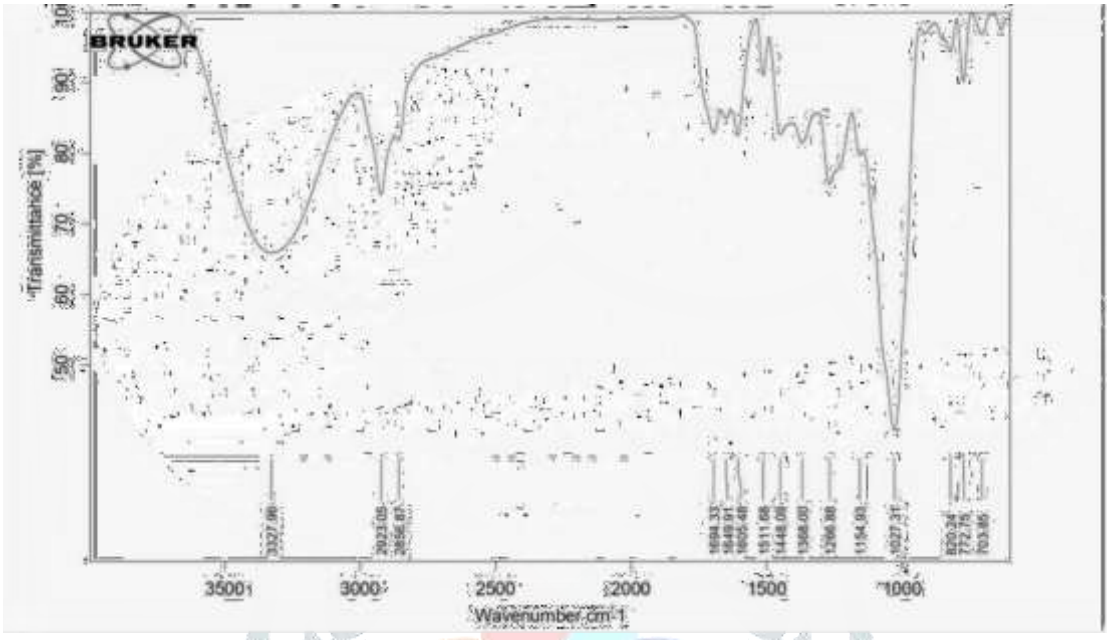

Fig 4.2 FTIR chromatogram of Isolate 1c

4.3 NMR Analysis

4.3.1 Carbon and Hydrogen correlation based on NMR

The structural elucidation of Isolate 1c was performed using a combination of ^13C and ^1H

nuclear magnetic resonance (NMR) spectroscopy, enabling the assignment of specific carbon and proton environments within the molecule. The chemical shifts (δ ppm), multiplicities, and coupling constants (J Hz) associated with each atom allowed for a detailed interpretation of the compound's molecular architecture.

The ^13C-NMR spectrum exhibited characteristic resonances for oxygenated aliphatic carbons in the δ 62.4–78.0 ppm range (atoms 23, 24, 25, 26, and 27), all appearing as singlets. These signals are indicative of carbons bonded to electronegative groups, such as hydroxyl moieties, which are commonly found in sugar units or hydroxylated side chains. The corresponding ^1H signals in the range of δ 3.10 to 3.82 ppm exhibited multiplicities including triplets (t), doublet of doublets (dd), and doublet of triplets (dt), reflecting vicinal and geminal couplings within an aliphatic spin system. This pattern strongly suggests the presence of a sugar-like moiety or a hydroxylated carbon chain attached to the main structure. Aromatic and olefinic carbons were observed between δ 96.3 and 134.4 ppm, corresponding to atoms 1, 2, 5, 6, 8, 10, 11, 12, 16, and 21. The ^1H signals in this region ranged from δ 5.25 to 7.50 ppm, with complex splitting patterns such as

doublets (d) and doublet of doublet of doublets (ddd). The coupling constants in the aromatic region indicated ortho, meta, and para interactions, confirming the presence of a substituted aromatic ring system, a hallmark of flavonoid or phenolic compounds. The presence of multiple coupling constants further suggests substitution patterns that may contribute to the bioactivity of the molecule. Deshielded carbons observed between δ 156.9 and 163.3 ppm correspond to aromatic carbons bonded to electronegative atoms, such as phenolic hydroxyl or carbonyl groups. The carbon resonance at δ 178.7 ppm is characteristic of a conjugated carbonyl group, likely a ketone or ester, which plays a critical role in defining the molecule's electronic properties and biological interactions. The combined ^13C and ^1H NMR data indicate that Isolate 1c is a complex polyphenolic compound, possibly a flavonoid glycoside, based on the presence of hydroxylated aliphatic carbons (likely sugar residues), substituted aromatic rings, and conjugated carbonyl functionalities. The sugar moiety inferred from the aliphatic region likely enhances the molecule's solubility and bioavailability, while the aromatic and carbonyl groups contribute to its antioxidant and cytotoxic properties. This structural framework aligns well with the observed anticancer activity in vitro, further supporting Isolate 1c as a bioactive principle responsible for the pharmacological effects of Ixora coccinea Linn. flowers.

This comprehensive NMR analysis, when combined with FTIR and bioactivity studies, strengthens our understanding of the molecular basis for the therapeutic potential of Ixora coccinea Linn. and provides a solid foundation for future chemical and pharmacological investigations.

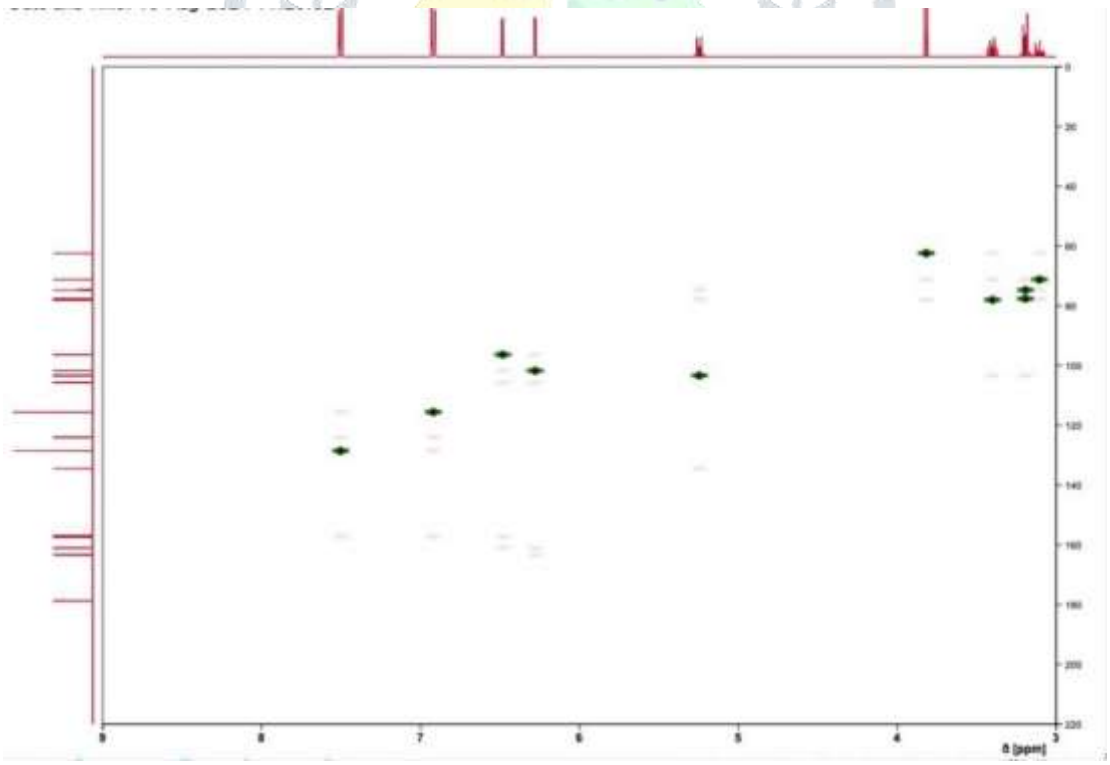

Figure 4.4 HMBC Spectra of Isolate 1c

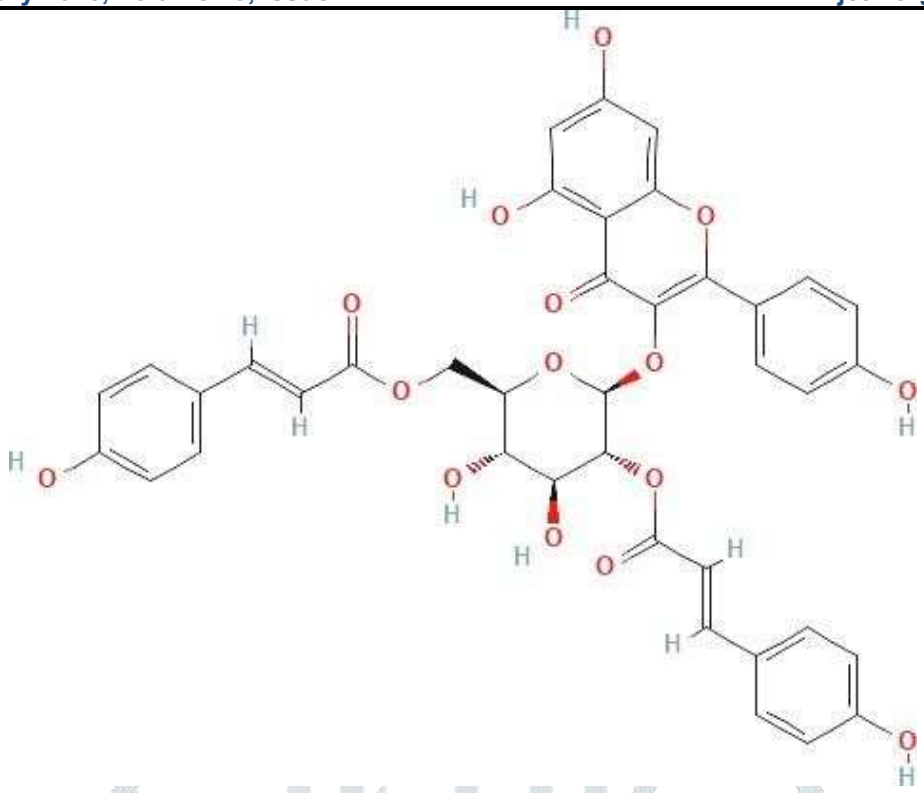

Figure 4.5 Probable structure of Isolate 1c

The data, in combination with FTIR, ^1H-NMR, and ^13C-NMR analyses, strongly suggest that Isolate 1c contains an aromatic and oxygen-substituted system with probable hydroxyl, methoxy, or ketonic functionalities, indicating that it may be a flavonoid or phenolic derivative. Based on the analysis, it was elucidated that the molecule is Kaempferol 3-O-β-D-glucopyranoside, as confirmed by literature (Leaves,

2003). **The study provides a solid foundation for future investigations into the development of plant-based treatments that could help address the increasing challenges of inflammation and antibiotic resistant.**

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