

Isolation and Characterization of flavonoids Luteolin and Quercetin from the leaves of *Piper betle* var *Magahi*

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ABSTRACT

Background and Objectives: Plants are a rich source of diverse chemical compounds with potential therapeutic properties. Phytopharmaceuticals offer a vast array of bioactive constituents that can be used to develop new drugs or as alternative treatments to conventional medicines. Plant-derived compounds often exhibit diverse pharmacological activities, such as anti-inflammatory, antimicrobial, antioxidant, anticancer, analgesic, and immunomodulatory properties. S

Methods: Isolated ethanolic extract from *Piper betle* var *Magahi* was dissolved in 5.0 ml of 2.0 N hydrochloric acid, 0.04% (w/v) ascorbic acid as an antioxidant was added to 50 mL of semi solid sample then heated at 90°C for 2 hours. Allowed to cool and sonicated for 5 minutes to remove the dissolved oxygen. The extract was cooled, filtered using Buchner filter. By using column chromatography, HPTLC, NMR, IR spectroscopy and mass spectroscopy process of isolation was completed.

Result: Isolated sample were compared with standard one and also compared with previous reported data of isolated Luteolin and Quercetin.

Conclusion: The result of present study established the presence of biologically active compounds Luteolin and Quercetin in the leaves of *Piper betle* variety *Magahi*. The data suggest that the significant amount of biologically active compound present in the leaves of *Piper betle* variety *Magahi*.

Keywords: Flavenoids, Luteolin, Quercetin, Mass spectroscopy, Chromatography.

1. INTRODUCTION

Plants are a rich source of diverse chemical compounds with potential therapeutic properties. Phytopharmaceuticals offer a vast array of bioactive constituents that can be used to develop new drugs or as alternative treatments to conventional medicines. Integrating traditional knowledge with modern scientific research can lead to the development of effective and safe treatments. Phytopharmaceuticals have been used to treat a wide range of ailments, including common health issues like cough, cold, and indigestion, as well as chronic conditions like diabetes, cardiovascular diseases, inflammatory disorders and gastrointestinal disorder. Plant-derived compounds often exhibit diverse pharmacological activities, such as anti-inflammatory, antimicrobial, antioxidant, anticancer, analgesic, and immunomodulatory properties.

This broad spectrum of actions makes them valuable for managing complex diseases and promoting overall health. Phytopharmaceuticals serve as a valuable source of lead compounds in drug discovery. Researchers often isolate active constituents from plants to create new drugs or modify existing ones. The isolation and characterization of phytopharmaceuticals often involve a combination of different analytical techniques, including NMR (Nuclear Magnetic Resonance), IR (Infrared Spectroscopy), MS (Mass Spectrometry), and HPTLC (High-Performance Thin-Layer Chromatography). The first step is the extraction which can be achieved by various extraction methods, like maceration, percolation, or soxhlet extraction. Present research involves the method of soxhlet extraction.

After extraction, the crude extract is often fractionated using various techniques like, column chromatography, or preparative thin layer chromatography to separate different compounds present in the extract. NMR spectroscopy is a key technique used for structural elucidation and characterization of isolated compounds. The characteristic

absorption bands in the IR spectrum can help S to confirm the presence of specific functional groups, providing additional structural information. Mass spectrometry is employed to determine the molecular weight and fragmentation pattern of the isolated compounds. By combining the information obtained from these different analytical techniques, researcher gain a comprehensive understanding of the phytopharmaceuticals present in the plant extract.

2. MATERIALS AND METHODS

2.1 Collection and Authentication of Plants Material

Magahi Pan Leaf was collected from the Magahi Pan Research Institute Islampur Nalanda, in the month of July 2021. The plant was authenticated by Dr. Shivnath Das a senior scientist in the Magahi Pan Research Institute, Islampur Nalanda, with voucher specimen No. T-01512.

2.2 Preparation of Ethanolic Extract

For the obtainment of the ethanolic extract, first of all leaves were washed properly with tap water and then dried in a hot air oven at 40°C for two days. The dried leaves were grind into powder using a scientific mixing grinder. These powered leaves were used for further soxhlet extraction using ethanol as a solvents. 100 grams of powered leaves were subjected to continuous soxhlet extraction with ethanol as a solvent. It takes almost 10 hours to completely exhaust the crude leaves powder. The requirements for solvent in this process are less. The obtained ethanolic extract was filtered using whatman No -1 filter paper and then dried properly.

2.3 Acid Hydrolysis of Isolated Ethanolic extract

Isolated ethanolic extract (semi solid 50mL) was dissolved in 5.0 ml of 2.0N hydrochloric acid and 0.04% (w/v) ascorbic acid as an antioxidant was added to 50mL of semi solid sample then heated at 90°C for 2 hours and allowed to cool and sonicated for 5 minutes to remove the dissolved oxygen. The extract was cooled filtered using Buchner filter. The extract was kept in airtight amber bottles and stored in freezer.

2.4 Thin layer chromatography (HPTLC) procedure

The complete CAMAG TLC equipment consists of a fully automatic sample Linomat V sample applicator and a developing chamber. A CAMAG TLC scanner is allowing densitometric evaluation of chromatograms and CATS 4 software for interpretation of data. About 10 mg of the extracts of ethanolic extract of *Piper betle* var *Magahi* was taken and dissolved in respective solvents and volume was made up to 10 ml in standard flask. (1000µg/ml). The fraction were run on silica gel 60 F 254 pre coated aluminum plate of 0.2mm thickness.

Standard (10mg) was taken and dissolved in methanol. This was transferred into a standard flask and volume was made up to 100 ml to prepare 100µg/ml solution. Silica gel 60 F254 coated on HPTLC aluminum sheet were used as an adsorbent (Stationary phase). The extract were applied point wise from 1000µg/ml sample solution. 10µl of the sample was applied on HPTLC aluminium sheet as a different track in the form of 6 mm wide bands by using a Camag semi-automatic Linomat 5 spotter at a distance of 12 mm. Nitrogen gas was also supplied for simultaneous drying of bands and then using drier for completely drying of bands. Mobile phase used for extract were developed on v/v basis to saturate the chamber. 10 ml mobile phase was placed in each flat bottomed CAMAG twin chamber 30 minute before the development of the HPTLC plate.

2.5 Fourier Transform Infrared (FT-IR) spectrum analysis

FAR-MID-NIR-FTIR spectrophotometer (Model Frontier) was used for the sample analysis. The sample (1-2 mg) was crushed in KBr (3-4mg) pellets using FTIR (Fourier transform Infrared spectroscopy)

2.6 NMR spectral analysis

Bruker advance III HD NMR 500 MHz instrument was used for the sample analysis. First samples were dissolved in glass vials using DMSO D6 as a solvents. After closing the lid, glass vials were shaken properly to ensure proper dissolution of samples in given solvent then dissolved samples were transferred to NMR tube. NMR tubes were wipe out properly with tissue paper so no water molecule or anything remain present on the NMR tube.

2.7 Mass spectroscopy Analysis

For mass spectroscopy Agilent 6550 UHD accurate mass QTOF-MS was used. The detailed analysis conditions was given below.

Mass spectroscopy parameters

Table 1

MS Ion source	Dual AJS ESI
Polarity	Negative and Positive
MS Scan range	at m/z 60 to 1600
Ion source Parameters Standard Electrospray (ESI)	
Nebulizer Gas Temperature	350°C
Sheath Gas Temperature	300°C
Drying gas	8.1/min
Nebulizer Gas	40 psig
Sheath Gas Flow	101/min
Capillary Voltage	2500V
Nozzle Voltage	500V

3. RESULT AND DISCUSSION

3.1 HPTLC chromatogram

The TLC developing was set as twin through chamber were examined in various solvents and best resolution was obtained with the cyclohexane: dichloromethane: ethyl acetate: formic acid in the ratio of 20:5:8:1 v/v. Retention factor (Rf) was calculated using the formula

$R_f = \text{Distance moved by the solute} / \text{Distance moved by the solvent}$. The Rf value reported to quercetin and luteolin was 0.39 and 0.32

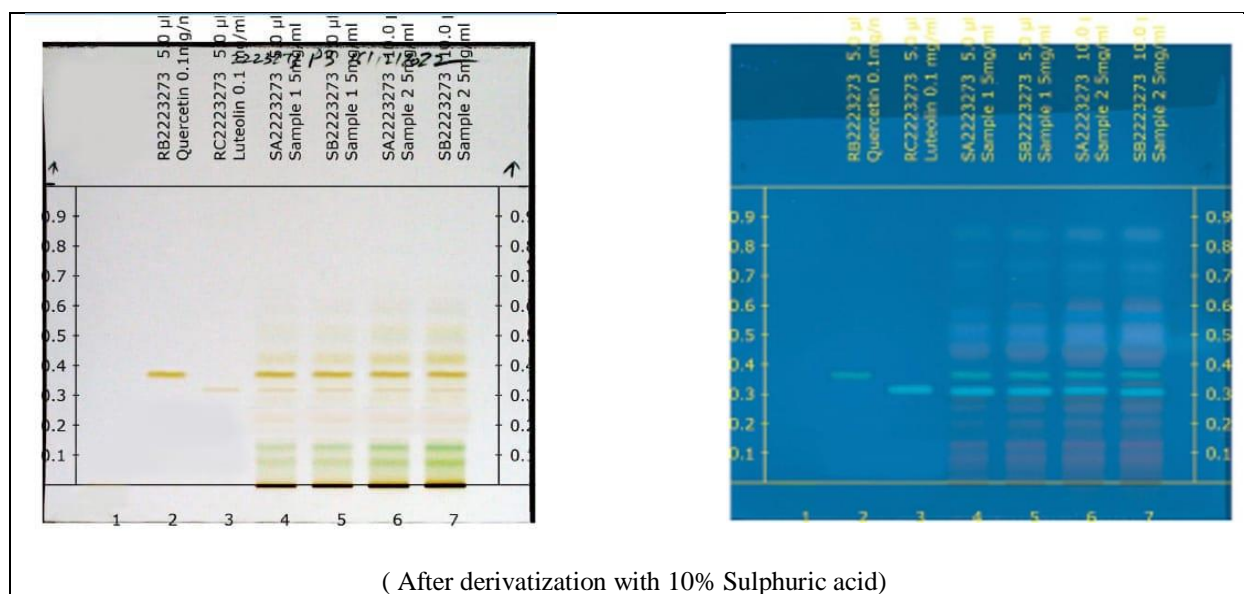


Fig. 1. Image of HPTLC plates

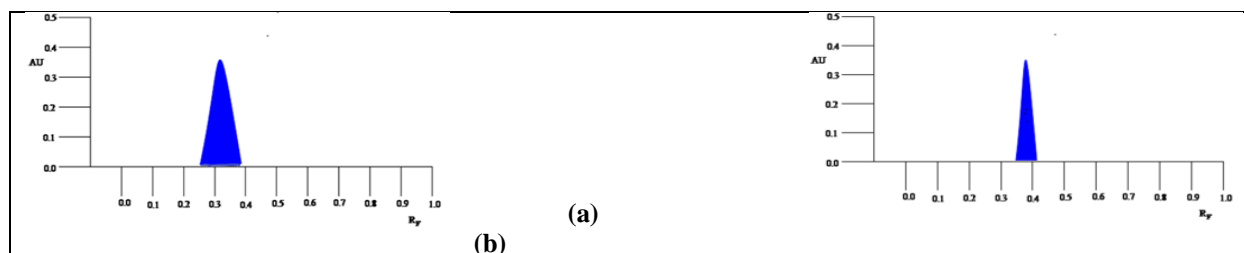


Fig. 2. Chromatogram of standard luteolin(a) and Quercetin (b)

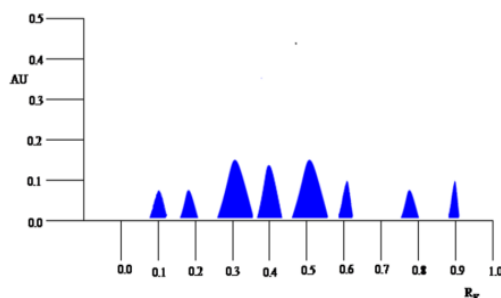


Fig. 3. Chromatogram of ethanolic extract of *Piper betle* variety Magahi

3.2 Fourier Transform Infrared (FT-IR) spectrum analysis of sample 01 and Sample 02

3.2.1 IR spectra of sample 01

FT-IR spectrum of sample 01 is well represented through figure 5 and their corresponding peak positions were explained as follows. The absorption peak at around 3490cm^{-1} was observed for OH stretching in sample 01 which indicates the presence of an OH functional group in flavonoids. The absorption peak at 1616cm^{-1} was observed for $\text{C}=\text{O}$ stretching of a carbonyl group in sample 01. The absorption peak position at around 3114cm^{-1} represents OH stretching of a carboxylic acid. The peak positions at 1470cm^{-1} , 1640cm^{-1} and 2950cm^{-1} represent the C-H stretching of an aromatic group, $\text{C}=\text{C}$ stretching of an aromatic and C-H stretching of an aliphatic group respectively. Various absorption bands due to stretching vibration of carbon-carbon and carbon-oxygen can be observed at 1000cm^{-1} and 1600cm^{-1} . They are particularly useful in identifying the presence of an aromatic ring and oxygen-containing functional groups. The extreme right in figure 5 indicates an absorption peak below 1000cm^{-1} that is often represented as “fingerprint region” and contains a complex pattern of absorption bands and provides information about the specific molecular structure and arrangement of atoms in the molecule. In table 2, observed peak positions and reported peak positions of interatomic bonds present in sample 01 were compared.

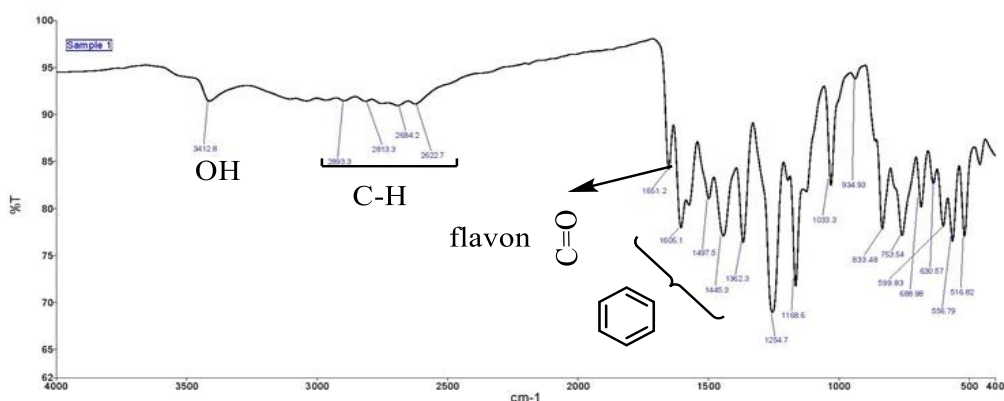


Fig. 4. FT-IR spectra of isolated sample 01

Table 2.

Sl No	Inter atomic bond	Peak position observed in cm^{-1}	Reported by JOLANTA PATORA and BARBARA KLIMEK (2002)
1	O-H stretching vibration	3412.8cm^{-1}	$3490\text{--}3400\text{cm}^{-1}$
2	C-H vibrations	$2893.3\text{--}2627.7\text{cm}^{-1}$	$2923\text{--}2617\text{cm}^{-1}$
3	$\text{C}=\text{O}$ in flavon	1651.2cm^{-1}	1655cm^{-1}
4	Aromatic ring	$1605.1\text{--}1445.3\text{cm}^{-1}$	$1610\text{--}1490\text{cm}^{-1}$
5	$\text{C}=\text{C}$ (stretching aromatic)	1640cm^{-1}	1642cm^{-1}
6	C-H bending of aromatic hydrocarbon	934.93cm^{-1} , 833.48cm^{-1} , 753.54cm^{-1} , 688.98cm^{-1} , 630.57cm^{-1} , 556.79cm^{-1} , 516.82cm^{-1}	

3.2.2 FT-IR spectra of sample 02

The FT-IR spectrum of isolated sample 02 is shown in figure 6 and their corresponding peak position were listed in table 3. The broad absorption peak at around 3290 cm^{-1} was assigned to the OH stretching vibration of phenols. The multiple peaks in this region indicates more OH groups present in the molecule which confirm the given molecule is flavonoids. The carbonyl group (C=O) such as ketonic functional group can produce absorption bands at around 1668 cm^{-1} which is close to the reported value. The absorption peak position at 1612 cm^{-1} , 1516 cm^{-1} and 1429 cm^{-1} are assigned to the C-C, C=O, and C=C aromatic stretching vibrations respectively. The OH bending vibration of phenols were observed at 1359 cm^{-1} . The absorption peak at 1315 cm^{-1} and the peak at the lower frequency between 950 cm^{-1} and 600 cm^{-1} were assigned to the C-H bending vibration of aromatic hydrocarbons. C-O stretching vibrations of aryl ether and phenols were observed at 1240 cm^{-1} and 1210 cm^{-1} respectively. The C-CO- stretching and bending vibrations of ketone were observed 1163 cm^{-1} which confirm that the isolated compounds is quercetin. C-H Stretching: aliphatic and aromatic C-H stretching vibrations will result in absorption bands in the $2800\text{--}3100\text{ cm}^{-1}$ range. C-C and C-O Stretching: In the $1000\text{--}1600\text{ cm}^{-1}$ range, you may observe various absorption bands due to stretching vibrations of carbon-carbon (C-C) and carbon-oxygen (C-O) bonds. These can be particularly useful for identifying the presence of aromatic rings and oxygen-containing functional groups.

Fingerprint Region: The region below 1000 cm^{-1} is often referred to as the "fingerprint region" and contains a complex pattern of absorption bands that can provide information about the specific molecular structure and the arrangement of atoms in the molecule.

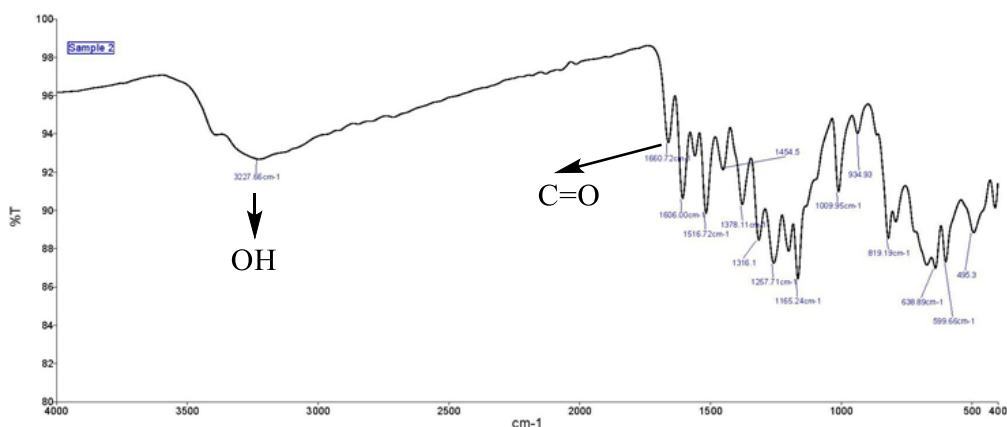


Fig. 5. FT-IR spectra of isolated sample 02

Table 3

Sl No	Inter atomic bond	Peak position observed cm^{-1}	Reported by MACIEJ HENECZKOWSKI et al (2001)
1	O-H stretching vibration of Phenol	3227.66 cm^{-1}	3300 cm^{-1}
2	C=O aryl ketonic stretch	1660.72 cm^{-1}	1662 cm^{-1}
3	C=C aromatic stretch	1554.5 cm^{-1}	1560 cm^{-1}
4	O-H bending of phenols	1378.11 cm^{-1}	1400 cm^{-1}
5	C-H bond in aromatic hydrocarbon	1316.1 cm^{-1}	1320 cm^{-1}
6	C-H bending of aromatic hydrocarbon	934.93 cm^{-1} , 819.19 cm^{-1} , 638.89 cm^{-1} , 599.66 cm^{-1} , 495.30 cm^{-1}	

3.3 NMR spectrum of sample 01 and Sample 02

3.3.1 ^1H and NMR spectrum of sample 01

The following observation can be made in sample 01. Multiple signals which include multiplets and multiplets cluster reported in the range of (6.0 ppm to 8.5 ppm) indicating the presence of neighboring proton in the aromatic

ring. Peaks in the range of 9ppm to 12ppm indicate the hydrogen in hydroxyl group (-OH). The series of peaks in the range 100ppm to 160ppm corresponds to the various carbon atoms in the aromatic ring. The peaks in the range of 160- 190ppm indicates the carbonyl carbon present in the sample 01.

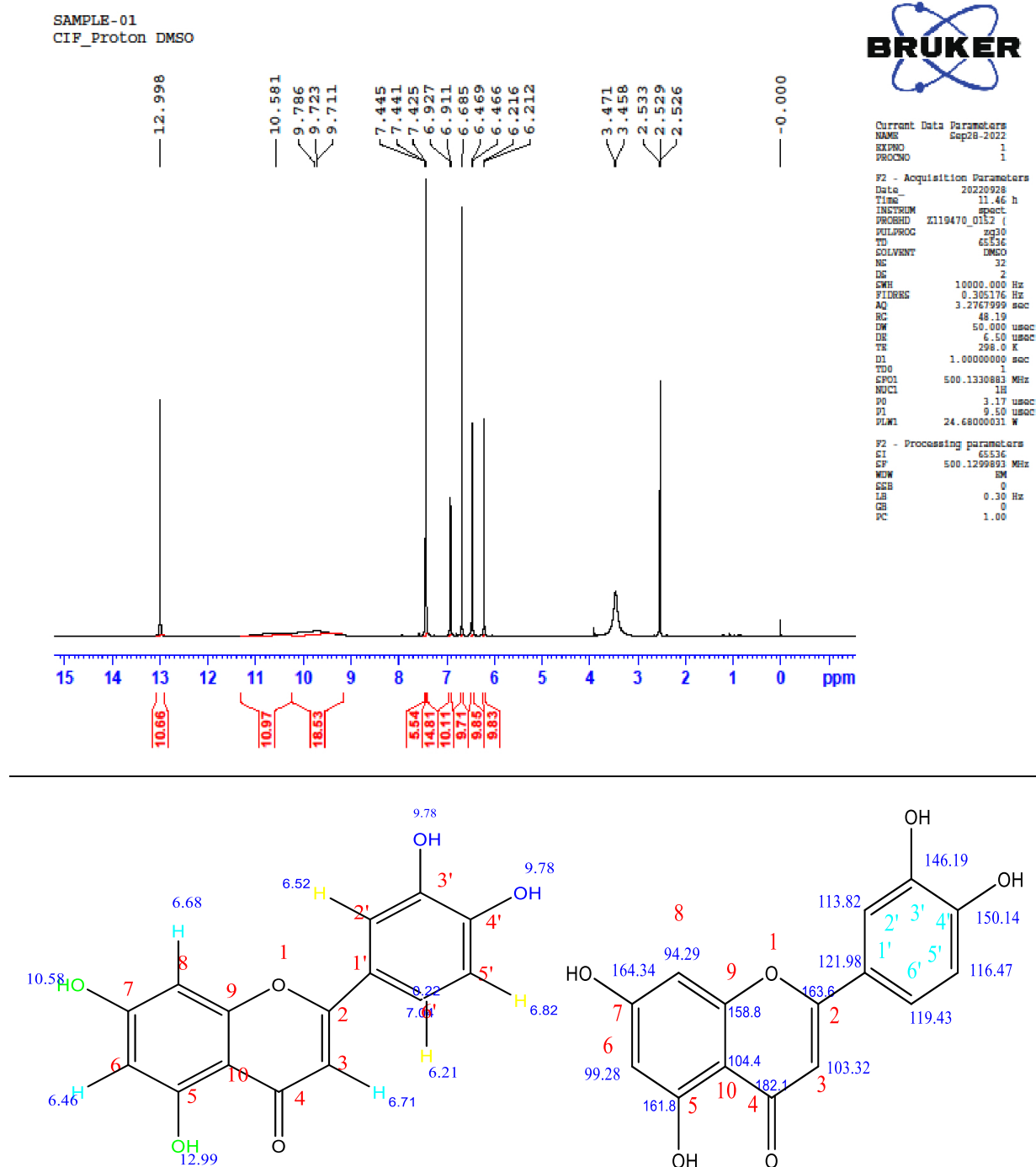
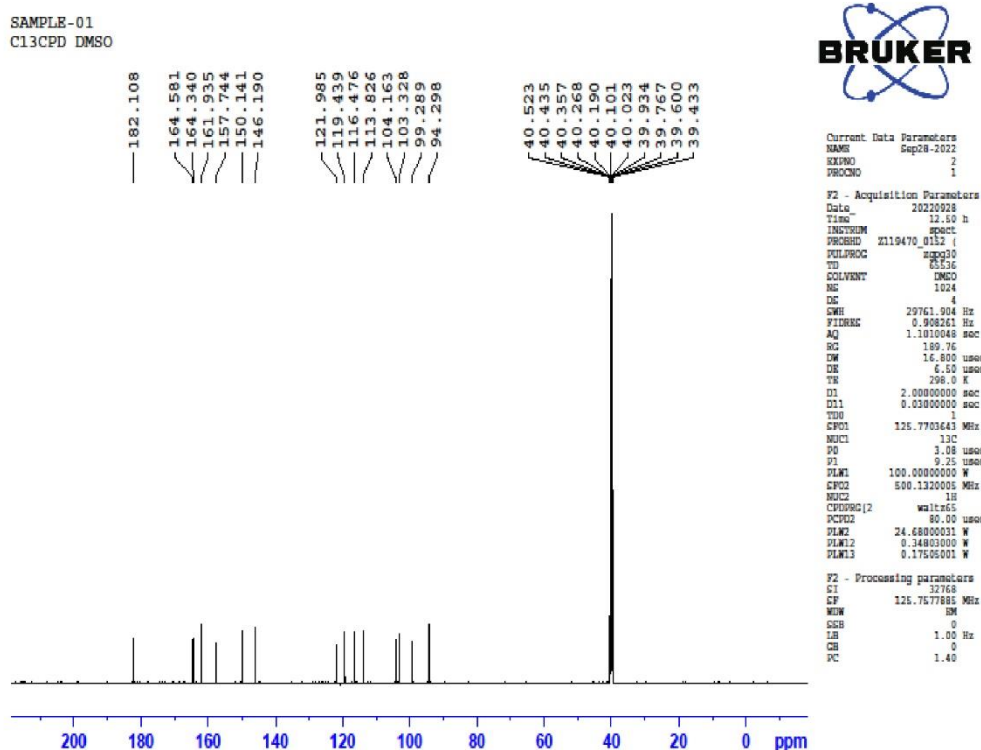


Fig. ^7H NMR spectra of isolated sample 01

Table 4: ^1H and ^{13}C NMR chemical shifts (ppm) for sample 01

^1H	Chemical shift (ppm)		^{13}C	chemical shift(ppm)
3H	6.71C2	163.9		
			C3	102.8
5H	12.99C4	181.6		
6H	6.46C5	161.4		
7H	10.58		C6	98.80
8H	6.68C7	164.80		
C8	93.80			
C9	157.30			
	C10	103.30		
2'H	6.52C1'	121.50		
3'H	9.78C2'	113.30		
4'H	9.78C3'	145.70		
5'H	6.82C4'	149.70		
6'H	6.21C5'	116.00		
C6'	119.00			

3.3.2 ^{13}C NMR spectrum of sample 01

**Fig. 8:** ^{13}C NMR spectra of sample 01

3.3.3 ^1H NMR spectrum of sample 02

Some significant observation can be made in sample 02. Multiple signals in the aromatic range of 6.0 to 8.58 ppm can be noted in the given spectrum. These signals are indicating the presence of protons in the aromatic range, multiple peaks in the range of 9 ppm to 12 ppm indicate the protons in the hydroxyl group. The ^{13}C NMR spectrum showed carbonyl group at 176.20 ppm that represents the carbon environment.

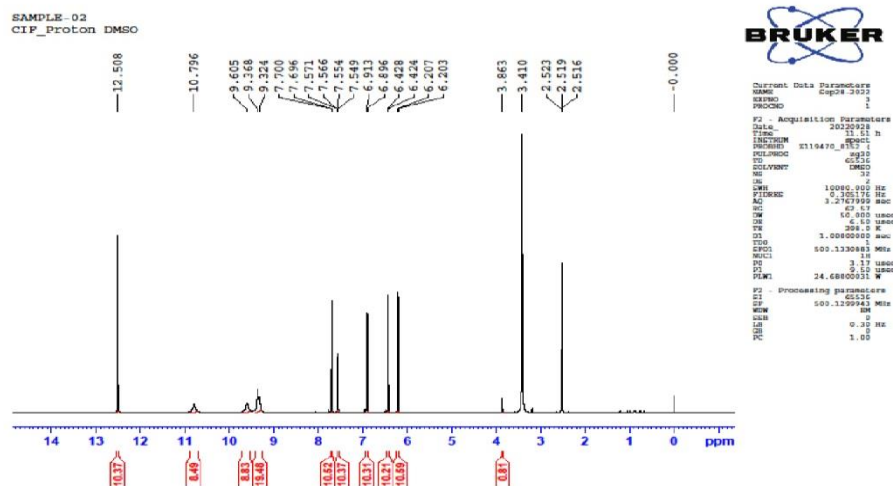
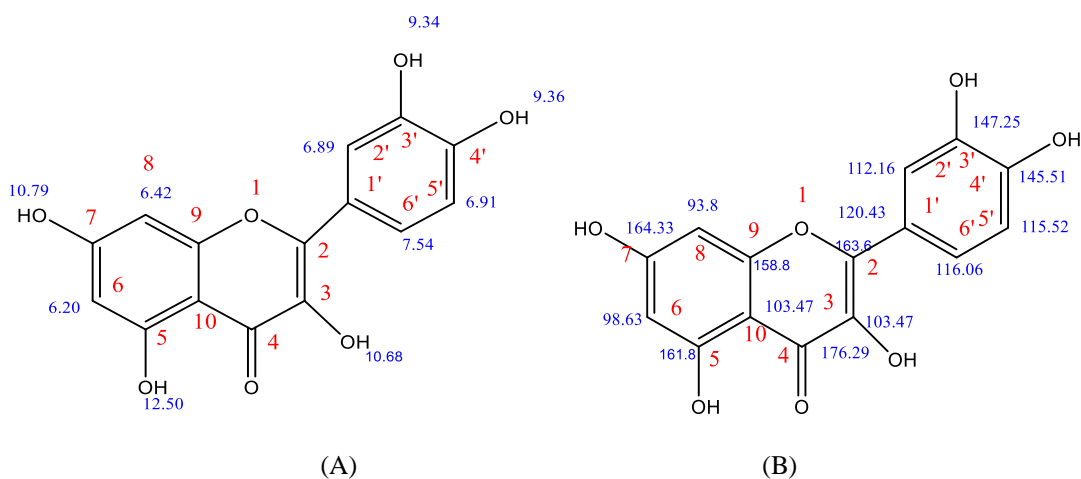
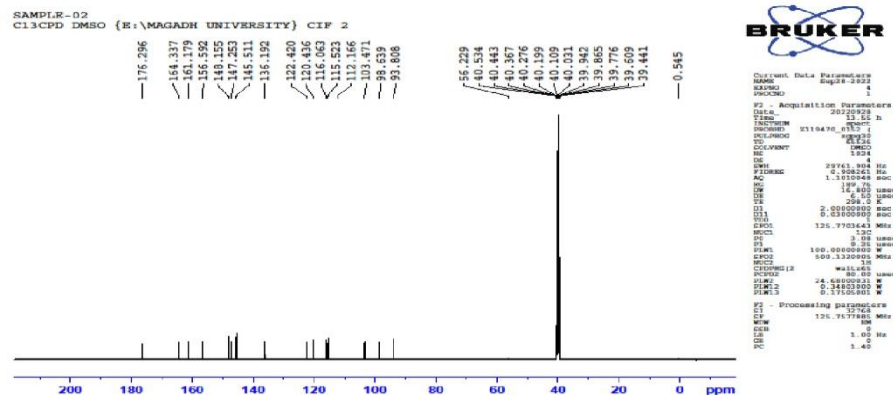
Fig. 9: ^1H NMR spectra of sample 02Fig. 10: ^1H and ^{13}C NMR spectra of sample 02 and chemical shift in (ppm)3.3.4 ^{13}C NMR spectrum of sample 02:Fig. 11: ^{13}C NMR spectra of sample 02.

Table 5: ^1H and ^{13}C NMR chemical shift (ppm) for sample 02

^1H	Chemical shift (ppm) ^{13}C	chemical shift(ppm)	
3H	10.68	C2	163.6
		C3	103.47
5H	12.50	C4	176.29
6H	6.20	C5	161.8
7H	10.79	C6	98.63
8H	6.42	C7	164.33
C8	93.80		
C9	158.80		
C10	103.47		
2'H	6.89	C1'	120.43
3'H	9.34	C2'	112.1
4'H	9.36	C3'	147.51
5'H	6.91	C4'	145.51
6'H	7.54	C5'	115.51
		C6'	116.06

NMR studies were carried out to confirm the position of proton in sample 02. The isolated compounds displayed a better and sharp ^1H NMR and ^{13}C NMR spectra. The ^1H NMR spectrum of the isolated compounds showed aromatic hydrogen group from 6.18 -7.66ppm. The phenolic OH group shows deflection from 9.36 -12.48ppm respectively.

The ^{13}C NMR spectrum showed carbonyl group at 176.2ppm aromatic carbon group from 93.8 -164.3 ppm . The cross ponding ^1H NMR and ^{13}C NMR peak position for isolated compounds were shown in figure 2

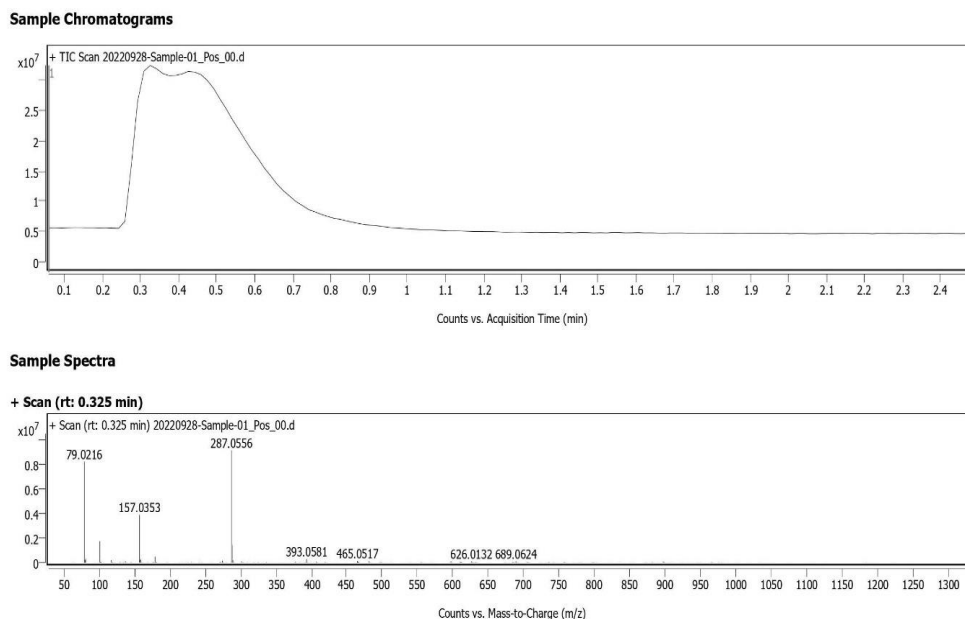
3.4 Mass Spectroscopy of sample 01 and sample 02

3.4.1 Mass spectroscopy of sample 01

Mass spectroscopy:

Mass spectroscopy is a power full analytical technique used to identify and quantify compounds based on their mass to charge ratio. When applied to quercetin a flavonoid with numerous health benefits MS can provide detailed information about its structure and composition.

Isolated plant compounds was analyzed by the mass spectroscopy the chromatogram of the sample 01 was shown in Figure 11

**Fig. 11.** Total ion chromatogram of isolated sample 01

Flavonoid commonly found in many plants and has several biological activity. The fragmentation pattern of sample 01 when analyzed by mass spectroscopy can provide insight into its structure and name of its substituents.

When sample one was analyzed using mass spectrometry particularly using technique ESI MS (Electron spray Ionization Mass spectrometry) the following key fragments are typically observed.

Parent ion [molecular ion [M-H] at m/z 285 the deprotonated molecular ion of sample one the following characteristic fragmentation pattern was observed.

- (1) m/z 217 this fragment results from the loss of a C₂H₂O (68 dalton) indicate cleavage between the A and C ring of the flavone structure.
- (2) m/z 151 this fragments cross bond to the A ring of the flavonoid structure indicating the stability of this ring during fragmentation.
- (3) m/z 133 often observed due to further fragmentation of the ion at m/z 151
- (4) m/z 175 this ion arises from the loss of a C₄H₆O₃ unit from the parent ion indicating cleavage within the C ring and loss of substituents.
- (5) A common fragmentation pattern for flavonoids when C ring undergoes a RetroDiels Alder reaction result in the formation of the fragments m/z 151 and m/z 133.
- (6) Heterocyclic ring cleavage: Fragmentation involving the cleavage of the heterocyclic ring (C- ring) results in significant ion like m/z 217 and m/z 175.

Interpretation of Fragmentation patterns: The observed fragments can be interpreted to confirm the structure of sample one is of luteolin flavonoid and can be distinguished it from other similar flavonoids. The key fragments at m/z 217, 151, 133, 175 are diagnostic for luteolin due to the specific position of the hydroxyl group and the stability of the resulting ions.

These fragments and their pathway provide a clear mass spectrometric fingerprints for luteolin aiding in its identification and structural elucidation in complex mixture.

3.4.2 Mass spectroscopy of sample 02

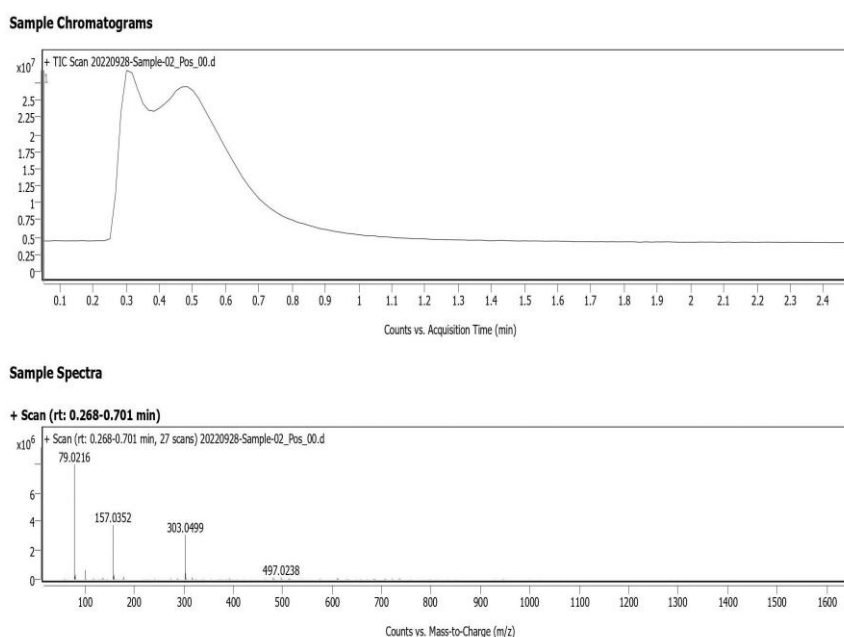


Fig. 11: Total ion chromatogram of isolated sample 02

Ionizations Electro spray ionizations (ESI) of sample two molecules are ionized by applying high voltage producing ions like [M-H] protonated molecule. The ionized molecule are then introduced into the mass analyzer which separate them based on their mass to charge ratio m/z

Characteristic fragments pattern of sample two molecule:

- (1) m/z 153, 137, 125, 107 and 95 which are indicative of the breakdown of the flavonoid structure

- (2) Interpretation of mass spectra: parent ion peak and the peak corresponding to the molecular ion $[M-H]^+$ provides the molecular weight of the 303
- (3) Fragment ions peak at lower m/z value correspond to fragments of the sample molecule. The pattern of these peaks helps in elucidating the structure.
- (4) A prominent peak at m/z 303 $[M-H]^+$ in positive ion mode peaks at m/z 285 273 and other represents various ions by interpreting the mass spectra and fragmentation patterns

These fragments and their pathway provide a clear mass spectrometric fingerprints for quercetin aiding in its identification and structural elucidation in complex mixture.

4. CONCLUSION

The result of present study established the presence of biologically active compounds Luteolin and Quercetin in the leaves of *Piper betle* variety *Magahi*. The data suggest that the significant amount of biologically active compound present in the leaves of *Piper betle* variety *Magahi*. Thus it may be concluded that the leaves of *Piper betle* variety *Magahi* may be the potential source for producing the healthy and highly nutritive products.

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Author Contribution

Singh Nadkar narayan singh participated in isolation and characterization of flavonoids Luteolin and Quercetin from the leaves of *Piper betle* var *Magahi* and writing and editing the original draft. Dr Dr Jitendra Singh² Dr Alok Mukharjee³ participated in interpretation of mass spectroscopic, Nuclear magnetic resonance and infrared spectroscopic data analysis.

Declaration of interest

The author declare that there is no conflict of interest. The author alone are responsible for the accuracy and integrity of the paper content.

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