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Research Article

Invitro Antimicrobial Activity, DNA Sequencing and Phytochemical Screening of A Medicinal Plant- *Ruellia patula Jacq*

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ABSTRACT

RuelliapatulaJacq (syn: *Dipteracanthus patulus*) belongs to the family Acanthaceae, has numerous medicinal properties but is not exploited much in modern medicine. Invitro antimicrobial activity of the *Ruelliapatulajacq* leaves extracted with Ethanol, Methanol and Acetone was checked for *Bacillus subtilis*, *Escherichia coli* and *Aspergillus niger* by disc diffusion method. Ethanol was found to be the better solvent that its extract showed more activity against *Aspergillus niger*, *Escherichia coli and Bacillus subtilis* respectively. Preliminary phytochemical screening of ethanolic and methanolic extracts showed positive results for alkaloids, steroids, phenols, flavonoids, tannins and terpenoids. Genomic DNA was extracted from *RuelliapatulaJacq* leaves using the standard Cetyl Trimethyl Ammonium Bromide extraction method. The DNA extracted responded well during PCR amplification and RAPD analysis with three gene specific primers and five random Medicinal aromatic plant primers respectively. Chromatographic fingerprinting of the ethanolic extract of the plant leaves was analyzed for compounds present in the sample using analytical type HPLC using a C-13 Column and a UV- detector of the *RuelliapatulaJacq* for the detection of the chemical constituents in the plant. Analytical type chromatogram revealed the presence of 8 compounds in the extract under scanning at 205 nm. The preparative type HPLC chromatogram revealed the presence of 2 major compounds in the extract. The collected fractions were subjected to FT-IR spectroscopy and UV-Visible spectrum analysis. FT-IR Spectrum studies on *RuelliapatulaJacq* ethanolic extract showed the possibility of harbouring secondary metabolites with higher pharmaceutical value.

Key words: *RuelliapatulaJacq* , PCR, RAPD, Phytochemical analysis, HPLC, FTIR spectroscopy.

INTRODUCTION

Medicinal plants contain large varieties of chemical substances which possess important therapeutic properties that can be utilized in the treatment of human diseases [(1)]. Thus, there is need to search for new and more potent antimicrobial compounds of natural origin to combat the activities of these pathogens which is the basis for this study. Though there are methods in the identification of phytochemicals from plant family Acanthaceae, it is limited. Pharmacological studies of *RuelliapatulaJacq* indicated its cardiotonic [(2)], wound healing (3), antiulcer and antioxidant [(4)] activities. Spectroscopic analysis of *Ruellia sp.* [(5)] results three new flavonoid glycosides, demethoxycentaureidin 7-*O*-b -D-galacturonopyranoside, pectolinarigenin 7-*O*-a -L-rhamnopyranosyl-(1_ 4_)-b -D-glucopyranoside and 7-*O*-a -L-rhamnopyranosyl-(1_ 4_)-b -D-glucuronopyranoside, a new megastigmane glucoside, byzantionoside B 6_-*O*-sulfate, and a new (*Z*)-hex-3-en-1-ol *O*b -D-xylopyranosyl-(1_ 2_)-b -D-glucopyranoside, were isolated from leaves of *Ruellia patula jacq* together with 12 known compounds. The hepatoprotective activity of methanolic Extract of DP seems to be related to its antioxidant activity possibly through free radicals scavenging mechanism by activating antioxidant enzymes [(6)]. Natural products are frequently isolated following the evaluation of a relatively crude extract in a biological assay in order to fully characterize the active entity. The biologically active entity is often present only as minor component in the extract and the resolving power of HPLC is ideally suited to the rapid processing of such multi component samples on both an analytical and preparative scale. In this study we are trying to identify the phytochemical compounds present in the plant leaves by basic analytical and preparative HPLC, spectroscopic analysis, PCR and RAPD.

MATERIALS AND METHODS

Invitro Antimicrobial Activity and preliminary phytochemical analysis: The collected leaves were cleaned and shade dried. The dried plants were pulverized by an electrical blender and passed through the 20 mesh sieve. The powdered dried leaves (20g) were extracted with ethanol, acetone and methanol by using Soxhlet apparatus. The extraction was carried out for 24 hours at room temperature. The extracts were filtered and concentrated at room temperature. After the completion of solvent evaporation, each of these solvent extract were

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Table 1: Modified Primers for the amplification of Specific plant gene

Gene	F/R	Primer sequence (5'-3')	bases	Tm (°C)	GC%	Ta (°C)	Amplicon (bp)
Modified matK	F	CAT CTG GAA ATC TTG GTT C	19	53	42	50	777
	R	R AT AAT GAG AAA GAT TTC TGC	21	53.4	33		
Modified 18S rRNA	F	AAC GGC TAC CAC ATC CAA	18	53.8	50	50	546
	R	CCG AAG GCC AAC ACA ATA	18	53.8	50		
Modified rbcL	F	TTG GCA GCA TTC CGA GTA	18	53.8	50	50	483
	R	TCC ACC GCG AAG ACA TT	17	52.4	53		

Table 2: Invitro antimicrobial activity of leaf extracts of *RuelliapatulaJacq*

	Zone of inhibition in mm		
	Escherichia coli	*Bacillus subtilis*	*Aspergillus niger*
Ethanolic extract	27	20	33
Methanolic extract	20	12	20
Acetone extract	7	7	7

weighed and preserved at 5°C in airtight bottles until further use. Disc diffusion method was used to determine the zone of inhibition against *Aspergillus niger* (MTCC 1344), *Bacillus subtilis* (MTCC 441) and *Escherichia coli* (MTCC 433) by the *Ruelliapatula* leaf extracts with Amoxillin as control. The test for alkaloids, steroids, glycosides, phenols, flavonoids, tannins and terpenoids in *Ruelliapatula* were performed by the standard methods of Harborne, 1973.

Genomic DNA extraction, quantification, PCR-RAPD analysis: Sample extracted with ethanol was used for further analysis. Genomic DNA was extracted from the plant leaves using the standard CTAB method [8]. The quantity and purity of the DNA present in the sample were checked by spectrophotometry. The nucleic acid show absorption maxima around 260 nm if the DNA sample is pure without significant contamination from proteins and organic solvents whereas proteins shows peak absorbance at 280 nm. The absorbance is recorded at both wavelength and purity is indicated by the ratio OD_{260}/OD_{280}. If the value lies between 1.8 and 2.0, the DNA preparation is pure and free from proteins. If the sample is contaminated with protein the value is below 1.8 and if it is RNA contaminated the value lies above 2.0 [9]. The extracted DNA was subjected to PCR analysis with 20ng of DNA , randomly selected *MAP* primers (5'-3') *MAP* 02- GTCCTACTCG , *MAP* 08 - CTATCGCCGC , *MAP* 13-GTGCAATGAC, *MAP* 17 – TTGTCTCAGG, *MAP* 18 -CATCCCGAAC and gene specific primers *matK* (F- 5'CCC RTY CAT CTG GAA ATC TTG GTT C3', R-5'GCT RTR ATA ATG AGA AAG ATT TCT GC3') , *18S rRNA* (F-AAC GGC TAC CAC ATC CAA GG, R-CCG AAG GCC AAC ACA ATA GG), *rbcL* (F-TTG GCA GCA TTC CGA GTA AC, R-AAG TCC ACC GCG AAG ACA TT) and also with modified *matK, 18S rRNA, rbcL* primers. Amplified products were sequenced using AB-prism capillary DNA sequencer. The DNA sequence obtained from sequencing analysis was counter checked by employing NCBI-BLAST search.

HPLC Analytical and Preparative Analysis: Plant sample crude extracted with ethanol is used for the analysis. The HPLC analytical system is UFLC + SHIMADZU LC-8A make. The column used was Luna 10u, ^{C} 18 Silica 100 and Size->250X10.00mm 10 micron. Wavelength was 205 nm and injection volume 20 µl with flow rate 1.0 ml/ min. 100 % Methanol was used as mobile phase. The pressure was maintained at the minimum of 42kg f/ cm^2 and maximum of 300 kgf/cm^2. Ethanolic extract was used for the preparative HPLC analysis with the same conditions as in analytical HPLC. Two fractions were collected from the system in separate tubes and the collected fractions were subjected to FT-IR and UV spectrum analysis.

UV and FT-IR Spectroscopic analysis: The crude extract and extracts obtained from HPLC preparative annalysis were examined under visible and UV light for proximate analysis. The sample is diluted to 1:10 with different solvent. The extracts were scanned in the wave length ranging from 190 – 1100 nm using Perkin Elmer Spectrophotometer and characteristics peaks were

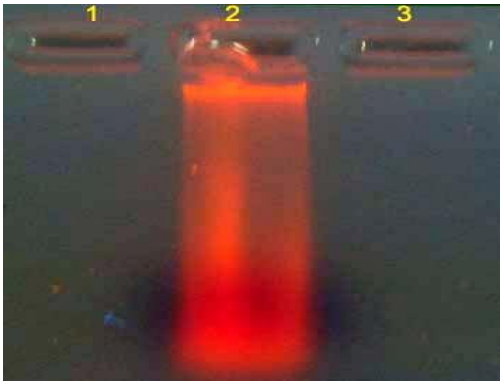


Fig.1: Genomic DNA extracted from RuelliapatulaJacq leaves

Genomic DNA of Ruellia patula: Lane 2 - Agarose gel electrophoresis of undigested genomic DNA of the plant where the 3 µl of genomic DNA was loaded from sample. Lane 1-3 Empty wells.

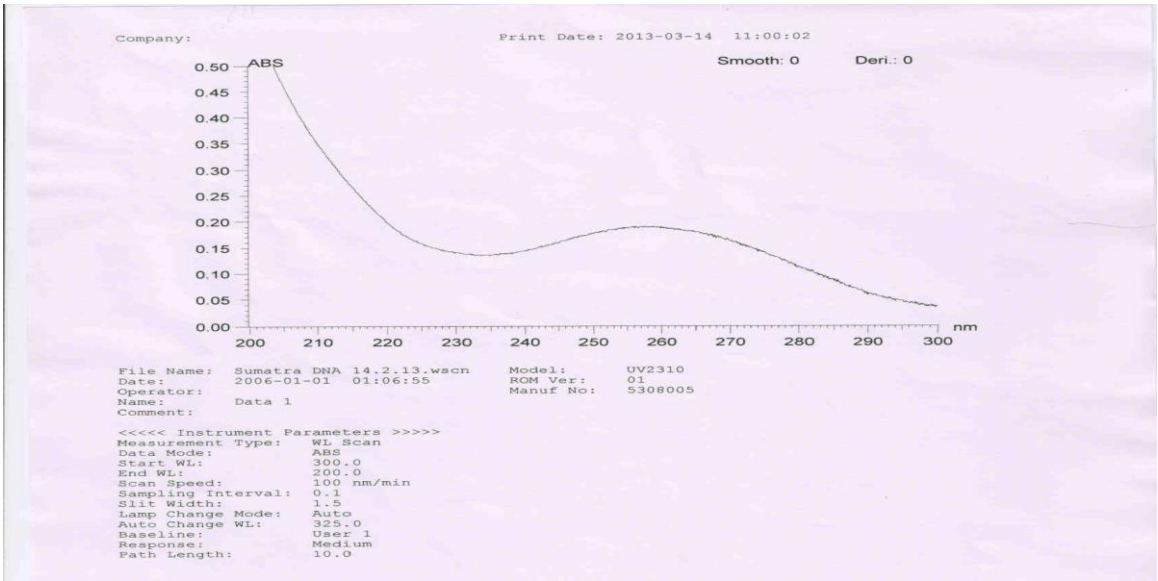


Fig.2: Quantification of DNA isolated from Ruellia patula Jacq. Absorption maxima at 260 nm showing pure DNA . OD 260/OD 280 ratio was about 1.65 indicating the level of purity of DNA

Table 3: Preliminary phytochemical screening of leaf extracts of *RuelliapatulaJacq*

Compound	Ethanolic extract	Methanolic extract	Acetone extract
Alkaloids	+	+	+
Steroid	+	+	+
Glycosides	-	-	-
Phenols	+	+	+
Flavanoids	+	+	-
Tannin	+	+	+
Terpenoids	+	+	-
Proteins	-	-	-
Amino acid	-	-	-

(+) indicates presence; (-) indicates absence

detected. FT-IR analysis was performed using Perkin Elmer Spectrophotometer system, which was used to detect the characteristic peaks and functional groups present in the sample. The peak values of the UV and FT-IR were recorded.

RESULTS AND DISCUSSION

DNA sequence from the leaves of *Ruelliapatulajacq*

rbcL partial gene sequence (483bp)

ttggcagc attccgagta actcctcaac ctggagttcc acctgaagaa gcaggggctg cggtagctgc tgaatcttct actggtacat ggacaactgt gtggaccgat gggcttacca gccttgatcg ttacaaagga cgatgctacc acatcgagcc cgttccagga gaagaaactc aatttattgc gtatgtagct tatcccttag acctttttga agaaggttcg gttactaaca tgtttacctc gattgtgggt aatgtatttg ggttcaaagc cctggctgct ctacgtctag aggatctgcg aatccctcct gcttatacta aaacttttca aggaccacct catggtatcc aagttgaaag agataaattg aacaagtatg gacgtcccct attaggatgt actattaaac caaaattggg gttatccgct aaaaactatg gtagagcagt ttatgaatgt ctacgtggtg gactt

18SrRNA partial gene sequence (546bp)

aacggc taccacatcc aaggaaggca gcaggcgcgc aaattaccca atcctgacac ggggaggtag tgacaataaa taacaatacc gggctcttcg agtctggtaa ttggaatgag tacaatctaa atcccttaac gaggatccat tggagggcaa gtctggtgcc agcagccgcg gtaattccag ctccaatagc gtatatttaa gttgttgcag ttaaaaagct cgtagttgga ccttgggttg ggtcgaccgg tccgcctcgc ggtgtgcacc tgtcggctcg tcccttctgc cggcgatgcg ctcctggcct taactggccg ggtcgtgcct ccggcgctgt tactttgaag aaattagagt gctcaaagca agcctacgct ctggatacat tagcatggga taacatcata ggatttcggt cctattgtgt tggccttcgg

matK partial gene sequence (766bp)

atcttggtt caaacccttc gatactgggt gaaagatgcc tcttcttttc atttattaag gctctttctt tatgagtatt ttaattggaa taggaatagt cttattactc caaaaaatgg gatttctact **ttttcaaaaa ggaatccaag attattcctg ttcctatata atttttatgt atgtgaatac gaatctatct ttctttttct ccgtaacaaa tcttcttatt tacgattaac atcttctgga gtccttttg agcgaatcta tttctatgca aaaatagaac atttttaga agtctttgat aaggattttc cgtccaccct atggttcttc aaggacccctt tcattcatta tgttagatat caaggaaaat ccattctggc ttcaaagaat acgccctttt tgatgaataa atggaaatac tatcttatcc atttatggca atgtcatttt tatgtttggt ctcaaccagg aaagatccat ataaaccaat tatccgagca ttcattttac tttttgggct atttttcaaa tgtgcggcta aatccttcag tggtacggag tcaaatgctg gaaaattcat ttctaattga aaatgttatg aaaaagcttg atacaataat tccaattatt ccactaatta gatcattggc taaagcgaaa ttttgtaatg tattagggca tcccattagt aagccggtct gggccgattc atccgatttg gatattattg accgattttt gcggagatgc agaaatcttt ctcattat**

In this study the ethanolic extract of leaves of *Ruellia patula jacq* showed higher antimicrobial activity against *A.niger* rather than *E.coli* and *B.Subtilis* with a zone of inhibition of 33 mm. Preliminary phytochemical screening of ethanolic and methanolic extracts showed positive results for alkaloids, steroids, phenols,

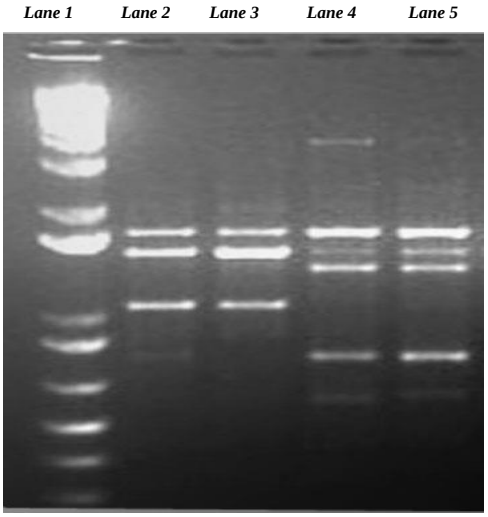


Fig.3: RAPD with MAP 2, MAP 13 Primers
Lane :1 - Molecular weight marker (100 bp)
Lane : 2 -3 Rp –DNA + MAP 2 Primer (3 bands)
Lane : 4 - 5 Rp –DNA + MAP 13 Primer (5 bands)

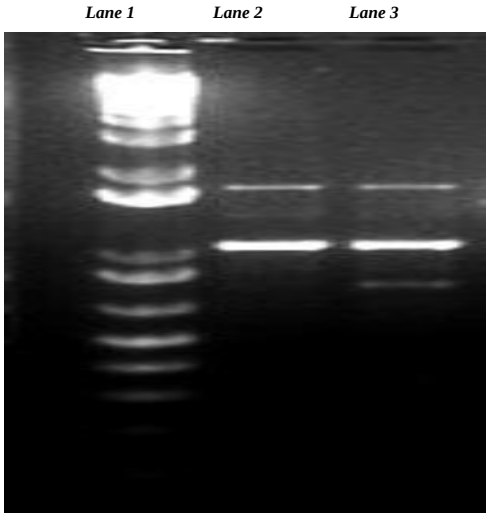
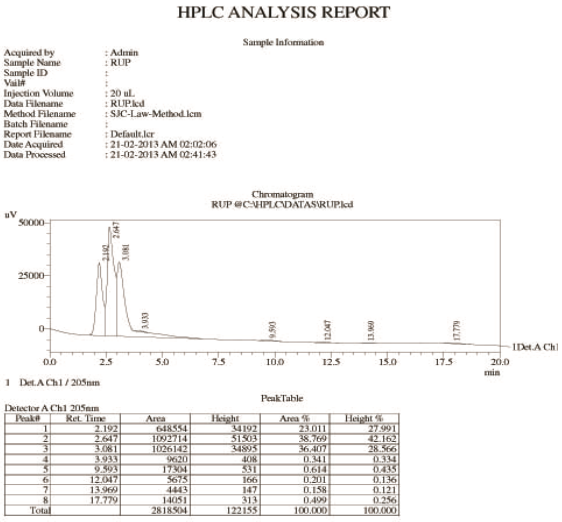


Fig.4: RAPD with MAP 18 Primer Image
Lane 1 - Molecular weight marker (100 bp)
Lane 2,3 Rp –DNA + MAP 18 Primer (3 bands)



HPLC ANALYSIS REPORT

Sample Information

Acquired by	: Admin
Sample Name	: RUP
Sample ID	:
Vial#	:
Injection Volume	: 20 uL
Data Filename	: RUP.lcd
Method Filename	: SJC-Law-Method.lcm
Batch Filename	:
Report Filename	: Default.lcr
Date Acquired	: 21-02-2013 AM 02:02:06
Data Processed	: 21-02-2013 AM 02:41:43

Chromatogram
RUP @C:\HPLC\DATAS\RUP.lcd

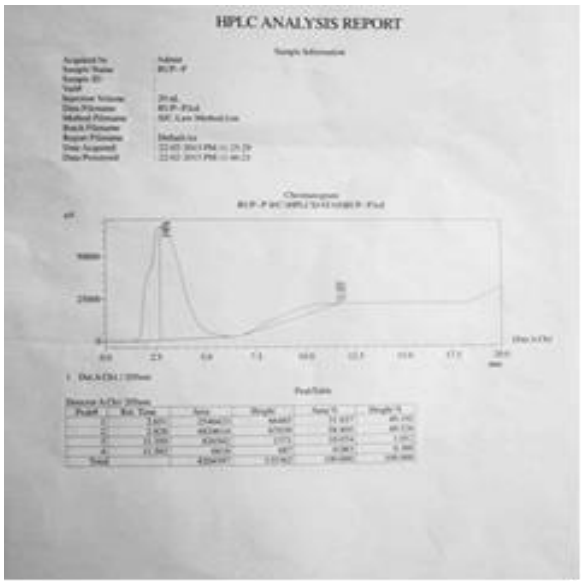
1 Det.A Ch1 / 205nm

PeakTable

Detector A Ch1 205nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.192	648554	34192	23.011	27.991
2	2.647	1092714	51503	38.769	42.162
3	3.081	1026142	34895	36.407	28.566
4	3.933	9620	408	0.341	0.334
5	9.593	17304	531	0.614	0.435
6	12.047	5675	166	0.201	0.136
7	13.969	4443	147	0.158	0.121
8	17.779	14051	313	0.499	0.256
Total		2818504	122155	100.000	100.000

Fig.5: HPLC Analytical Analysis



HPLC ANALYSIS REPORT

Fig.6: HPLC Preparative Analysis

flavonoids, tannins and terpenoids. The quality of DNA extracted was analysed by UV spectrophotometer at 260 nm and found to be pure without much contamination. The quantity of the DNA was estimated to be 13 µg /ml. The amplified DNA samples were subjected to PCR-RAPD analysis with different primers MAP (2, 8, 13, 17and 18). In RAPD analysis only three primers (2, 13 &18) responded well and two (8, 17) were unable to amplify any partial genes of the target plant DNA. This shows that the primers sequences MAP 2, 13 & 18 complementary sequences were available in more than two locations in the target. MAP 2 primers yielded 3 bands and MAP-13 primers gave 5 bands. MAP-18 primer combination got amplified in 3 regions. Because of the absence of complementary sequence in the target genome against the MAP 8 & 17 they failed to amplify and resulted with no products. Specific gene locus based primers were designed for the three plant DNA finger printing genes such as 18s *rRNA, matK, rbcL* genes. Primers were designed with few modifications of bases at 5' and 3'ends. Three partial genes were analyzed and the amplicons were checked on the agarose gel (1.5 % w/v). PCR products were resolved distinctly at various size 546 bp, 777 bp, 483 bp. Amplified products were sequenced and counter checked by NCBI-BLAST search. The HPLC analytical chromatogram revealed the presence of 8 compounds in the extract under 205 nm. Among the constituents the compounds at 2.647, 3.081 were found to be the major compounds around 38.76% and 36.40% among the total constituents at this detection nanometer. The UV profile of ethanolic extract of *RuelliapatulaJacq* chosen wavelength of 200 to 350nm and the profile showed peaks at 205 and 331 respectively and another chosen wavelength 400-700 nm showed the peak at 421 and 658. The HPLC preparative chromatogram revealed the presence of 2 major compounds in the extract under

205 nm. The compounds at Retention time 2.651, 2.828 were the major compounds which are around 31.037 % and 58.805 % among the total constituents at this detection nanometer .Two fractions were collected fraction and subjected to FT-IR and UV spectrum analysis. In the FT-IR spectrums the wave number 3548 cm^{-1} corresponds to *O-H stretch of alkenes*. The peak at 3380 cm^{-1} corresponds *to O-H stretch alcohols (or) phenols*. The peak at 2815.16 cm^{-1}corresponds *to –CHO aldehyde or [N-CH3]*. The peak at 2729 cm^{-1} corresponds to *–CHO (aldehyde).* The assignment 2101 cm^{-1} corresponds *– C Ξ CH* and the peaks at 1629 cm^{-1} corresponds *C=C -- C=O (or) –O-NO2.* The bands observed at 1350.12 cm^{-1} and 670 cm^{-1} corresponding *C-F (alkyl) (C-C1) (C-Br) --> alkyl halides, esters and alkenes*. The plant secondary metabolites such as Terpenoids, Flavanoids, Alkaloids and Steroids having functional group of alcohols, phenols, aldehyde, ketone , alkyl halides, alkenes, carboxylic acids, Aliphatic amines. FT-IR spectrum studies on *Ruellia patula* Jacq crude ethanolic extract revealed that the possibility of harbouring specific secondary metabolites with higher pharmaceutical value. But this provisional data has to be confirmed with the additional characterization of the fraction using Nuclear Magnetic Resonance, Liquid Chromatography-Mass Spectroscopy and X-Ray Diffraction analysis.

IMPORTANT ABBREVIATIONS

DP- *Dipteracanthus patulus jacq*
CTAB- Cetyl Trimethyl Ammonium Bromide
RAPD-Random Amplification of Polymorphic DNA
HPLC-High Performance Liquid Chromatography
FTIR – Fourier Transform Infrared Spectrometry
MAP-Medicinal Aromatic Plants
Rt-Retention time
NCBI-BLAST- National Center for Biotechnology Information -Basic Local Alignment Search Tool.

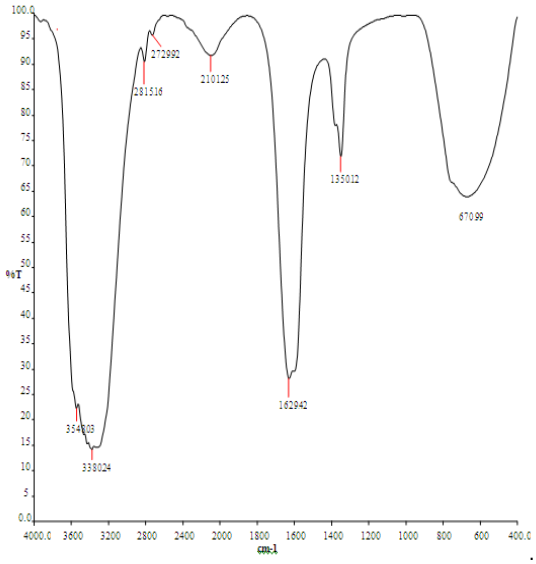


Fig. 7: FT - IR Spectrum for preparative fraction I

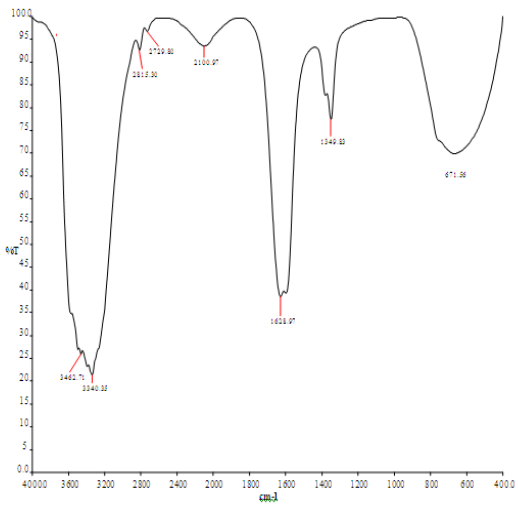


Fig.8: FT - IR Spectrum for preparative fraction II

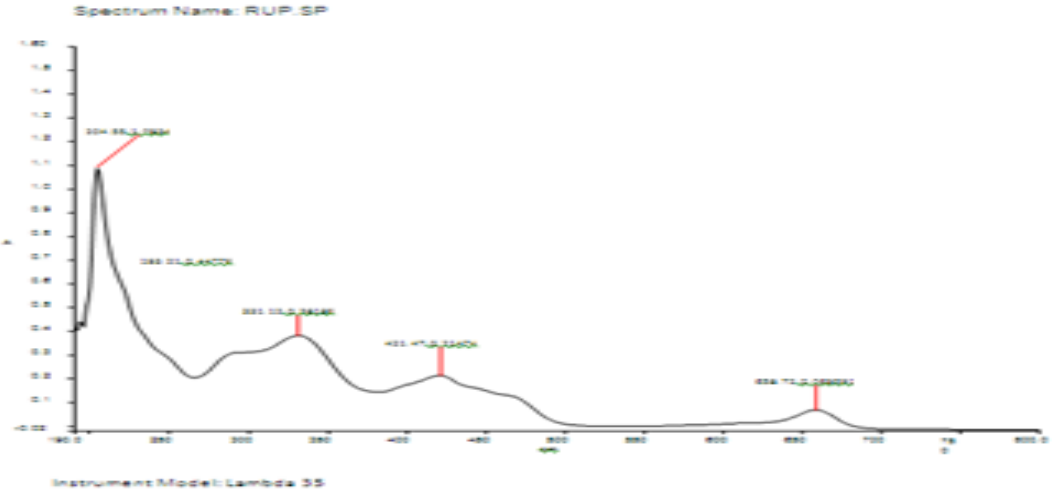


Fig 9: UV spectrum of crude ethanolic leaf extract of Ruelliapatulajacq

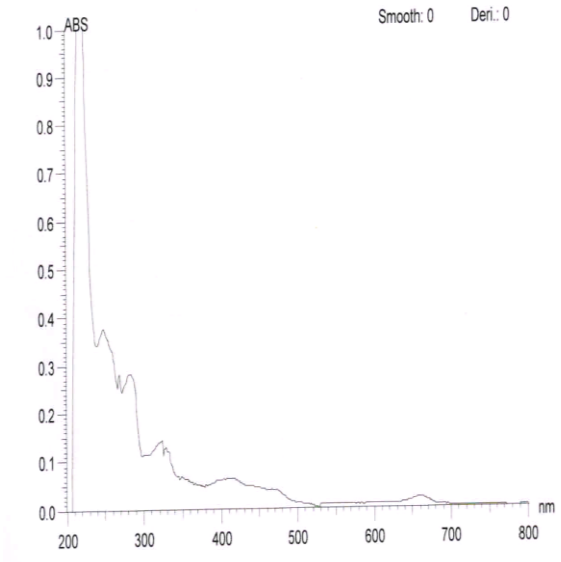


Fig.10: UVAbsorption Spectrum for fraction I

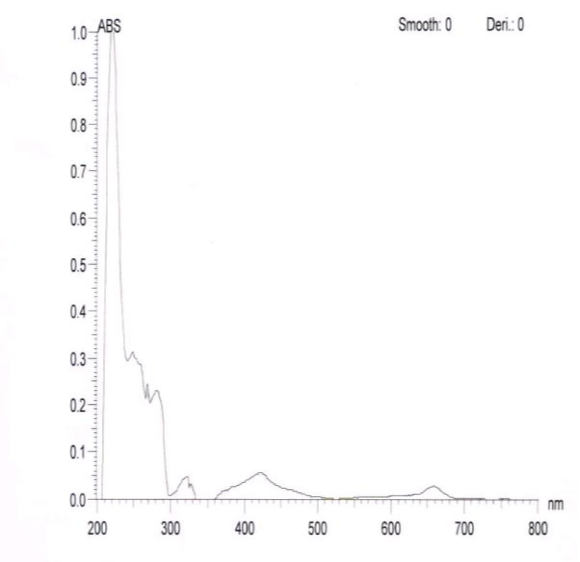


Fig.11: UVAbsorption Spectrum for fraction II

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