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Wound Stress Induced Secondary Metabolites in *Passiflora foetida:* Exploration of Antimicrobial Compounds

Anita S. Patil, Bipin D. Lade*
Department of Biotechnology, Lab no 106, Microbial Biotechnology and Secondary Metabolite Lab, Sant Gadge Baba Amravati University, Amravati 444602 (M.S), India

Original Research Article

***Corresponding author**
Bipin D. Lade

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Abstract: The wound stress is applied to leaves of *P. foetida* and ethyl actetate extracts is optimized using mobile phase: ethyl acetate: chloroform: glacial acetic acid: benzene (25:15:2:10) and TLC bioautographic depict two antimicrobial compounds at Rf 0.46 and 0.73. The extract shows antimicrobic activity 12 mm against *S. aureus at* 5 mg/ml and 14 mm against *E. coli* at 10 mg/ml. Their HR-LCMS chromatogram elucidates the structure of two possible antimicrobial compounds as nitrofurazone (RT: 0.644 min) or 4-tridecynoic acid (RT 7.616 min) and dihydrodeoxystreptomycin (RT:9.22min). The HPLC-DAD detected dihydrodeoxystreptomycin at 254 nm and ibuprofen at 220 nm. The GCMS confirmed 4-chloro 7-nitrobenzofurazan (RT: 18.39), methyl jasmonate (RT: 18.84), 10.03 PPM and 12-hydroxy dodecanoic acid (RT: 23.79), 82.65 PPM in 96 extract. These compound's viz 4-tridecynoic acid and dihydrodeoxystreptomycin, ibuprofen, 4-chloro 7-nitrobenzofurazan, methyl jasmonate and 12-hydroxy dodecanoic acid could be possible raised as antimicrobial drugs in pharmaceutical industries.

Keywords: Wound stress, antimicrobial secondary metabolites, *Passiflora foetida*, HP-TLC, TLC-Bioautography, HR-LCMS and GCMS.

INTRODUCTION

Healing plant's contents enormous chemical compounds that ultimately polish to potent drug in medicinal industry attracting researcher from different nations to identify novel drug. The chromatography technologies have been challenged to optimize standardization, separation, extraction and purification of pure compound to formulate product for delivering improved health care in a worldwide [1].

The *Passiflora* a native of Brazil and covers a tropical area of South America to Australia, Asia and Africa. In India, *Passiflora* is termed as Krishna kamal and rakhi flower [2]. Its extracts is used for treating patients with adjustment disorder and anxious mood, asthma, burns (skin), cancer, chronic pain, cough, drug addiction, epstein-barr virus and fungal infections. Beside gastrointestinal discomfort (nervous stomach), high blood pressure, menopausal symptoms, nerve pains are some of the other traditional uses [3].

The majority of the active components in *Passiflora* are Harman's alkaloids, *o*-diphenol, *C*-glycosyl flavones based on apigenin and luteolin, In particular, the catechin derivatives are known strong antimicrobial properties [4]. The polyacetylenic compounds in extract of *P. foetida* show antibacterial potential [5-7]. The other phytoconstituents found in *P. foetida* are alkaloids, phenols, glycoside flavonoids, and cyanogen, passifloricins, polyketides, and alpha-pyrones [8].

Plants sometimes could not prevent injuries caused by abiotic stress and herbivore's animals. In defense, plants innate and acquired system gets into an act by preserved secondary metabolites, simultaneously plugging elevated wound induced compounds activated by complex phenylpropionate pathway (PPP) [9]. Starting with expression of phenyl ammonia lyase (PAL) gene a series of cross talk between diverse pathways produces coumarin, phenolic volatiles, coumaroryl, caffeoly CoA ester [10], cauramic acid derives flavones, phenols [11] lignin and flavonoid [12, 13]. The initial idea of classes of secondary metabolites in plant species influenced the choice of sample preparation procedures, chromatographic and detection methods [14].

Previous photochemical study on *P. foetida* has focused on secondary metabolites extraction techniques [6], focused on isolating antimicrobic and antifungal compounds from *passiflora* species. In view of the [6] work we have developed a modified secondary metabolite extraction protocol in search of antimicrobial compounds, specifically in *Passiflora foetida*. The extraction of wound stress induced secondary metabolite from leaf of *P. foetida* was implemented and traditional TLC; HP-TLC technology

is applied for optimization of mobile phase [15]. In continuation to that, current research work deals with the antimicrobial activity of extracted secondary metabolites using TLC bioautography and agar well diffusion method. The antimicrobic compounds were fetched for UV-VIS spectroscopic analysis and FTIR analysis for determination of a functional group that has essence of bactericidal property. The GCMS, HPLC and high resolution liquid chromatography and mass spectrometry (HR-LCMS) are used for obtaining retention time and elucidation of structure of compound from extracts.

EXPERIMENTAL

The present research work was conducted in Department of Biotechnology, Sant Gadge Baba Amravati University, and Amravati 444602 Maharashtra, India.

General reagents, materials and solutions

The chemicals such as methanol, ethyl acetate, chloroform, benzene, glacial acetic acids, acetonitrile, triethylamine, phosphorous acid and syringe filter were purchased from Fisher scientific. The TLC silica gels with 60-120 mesh, normal chamber (V =1000 cm^3), HPTLC silica gel 60 F 254, spraying reagents MTT (3-(4,5-dimethyl-2-yl)- 2,5-diphenyltetraAzoleium bromide) and 2, 3, 5-triphenyltetrazolium chloride (TTC). The GCMS/MS column- TG-5MS (30 m X 0.25 mm X 0.25 µm thickness). The HPLC-PDA column (HYPERSIL GOLD C18 100 mm X 4. 6 mm X 5µ). The antimicrobial study use *Escherichia coli* (ATCC no. 11105) and *Staphylococcus aureus* (ATCC no. 6538).

Instruments for study

The ultra-violet transilluminator and UV spectrophotometer was purchased from Cleaver Korea and Shimadzu from Japan respectively. The fourier transform infrared spectroscopy (FTIR) was done in a department of chemistry, Nersimha college Amravati. The TLC and HPLC were performed in the lab of Department of Biotechnology, SGBA University. The HP-TLC facility was avail from Qualichem lab Nagpur. The GCMS/MS triple quadruple and HPLC-PDA were done in Anacon lab, Nagpur and HR-LCMS facility was availed from SAIF, IIT Bombay, Maharashtra India.

Collection, Preparation and Extraction of wound induced extract

The *P. foetida* runners from campus of Sant Gadge Baba Amravati University collected, the wounded and un-wounded leaves of *P. foetida* were harvested, processed and extraction of secondary metabolites was done as per the protocol given in figure 1.

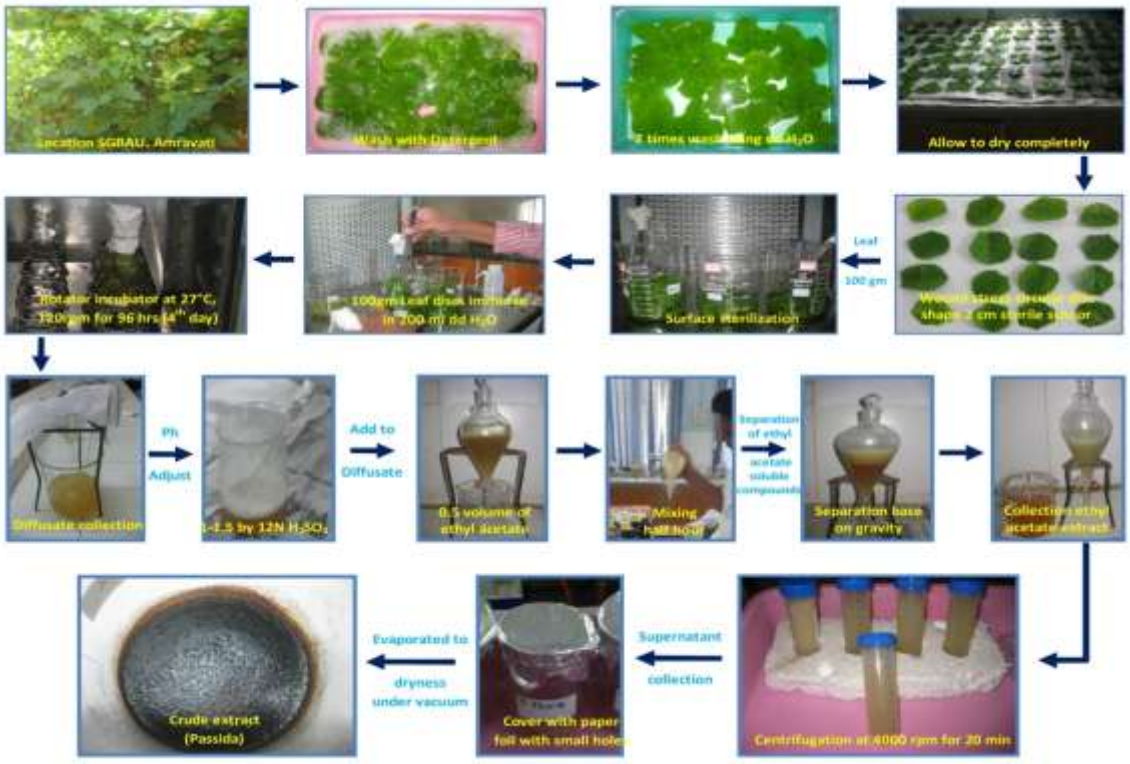


Fig-1: Outline the complete extraction procedure from leaves of *P. foetida*

UV spectroscopic analysis

The pinch of extract or pure compound was dissolved in distilled water and set in UV spectroscopic instrument. The scan range was set for 190 nm - 800 nm and took around 3 - 8 min to complete and results were retrieved and saved.

TLC and **HPTLC analysis**

The extract is spotted in TLC plate and various mobile phases tried to develop fingerprints for study of antimicrobial activity. In HPTLC, 100 µl extracts was dissolved in methanol, applied on instrument using a syringe. The parameters such as inert gas spray, dosage speed: 150 nl/s, predose volume: 0.2 ul was set. The scanning speed was adjusted as 20 mm/sec with wavelength of 254 nm and 366 nm.

FTIR analysis

The stress induced extract and pure compounds are applied on the spot of FTIR instrument, and the needle-like arm was fixed above it. The FTIR scan start in wavelength of 667-4000 cm^{-1} range and the spectrum results were fetched into PDF files.

TLC-Bioautography method

The 100 ml of *E. coli and S. aureus* suspensions was mixed with nine ml of agar and poured on profiled TLC plate that ran with chloroform: ethyl acetate: glacial acetic acid: benzene (25:15:2:10). The plate was incubated overnight at 37^{o} C. Next day, TLC plate was sprayed with 3-(4,5-dimethyl-2-thiazolyl)- 2, 5-diphenyl tetrazolium bromide (MTT) and pure compound plate sprayed with 2, 3, 5-triphenyltetrazolium chloride (TTC).

Agar well diffusion method

The 20 ml of nutrient agar was inoculated with 0.4 ml of 24 hr *E. coli and S. aureus cultures*, which was mixed and poured into sterile petriplates. After solidification of the medium, the cork borer was used to punch wells of eight mm diameters in each plate. The different concentration of *P. foetida* extracts as 0.1 mg/ml, 0.5 mg/ml, 1 mg/ml, 5 mg/ml and 10 mg/ml were added to the wells. Further the plates were incubated at 37^{o}C for 24 hr. Next day the zone of inhibition (ZOI) was measured along with standard control amikacine sulphate.

HR-LCMS analysis

The 3µl of extract was injected and solvent composition A: B (100 % water : 100 % acetonitrile) with 0.1% FA in water : 90 % ACN+10 % H_2O+0.1 % FA in ratio 95 % : 5% was used. The details for HR-LCMS acquisition method is performed for 30 min (see table 3).

Table-3: Acquisition method for HR-LCMS analysis of ethyl acetate extract of *P. foetida.*

Sr.n o.	Time	Function	Parameters
1	2min	Change solvent composition	Solvent composition A:95% and B 5%
2	2min	Change flow	Flow: 0.3ml/min
3	2min	Change Maximum pressure limit	Max pressure limit 1200 bar
4	20min	Change solvent composition	Solvent composition A: 5% and B 95%
5	20min	Change flow	Flow: 0.3ml/min
6	20min	Change Maximum pressure limit	Max pressure limit 1200 bar
7	25min	Change solvent composition	Solvent composition A: 5% and B 95%
8	25min	Change flow	Flow: 0.3ml/min
9	25min	Change Maximum pressure limit	Max pressure limit 1200 bar
10	26min	Change solvent composition	Solvent composition A:95% and B 5%
11	26min	Change flow	Flow: 0.3ml/min
12	26min	Change Maximum pressure limit	Max pressure limit 1200 bar
13	30min	Change solvent composition	Solvent composition A:95% and B 5%
14	30min	Change flow	Flow: 0.3ml/min
15	30min	Change Maximum pressure limit	Max pressure limit 1200 bar

HPLC analysis

The 96 hr extract of 0.02 gm dissolved in two ml of methanol, sonicate for 15 min and filter in HPLC vial and applied on HPLC instrument via an injector portal. The mobile phase in combination of A-acetonitrile (40%) and B-1% triethylamine + 0.5% phosphoric acid (60%) was used. The flow rate for solvent was adjusted to 1.5 ml / min and detector scan was set at absorbance of 253 nm, 269 nm, 254 nm. The retention times of compounds were measured for individual compounds, and HPLC chromatograph were recorded.

GCMS analysis

The 0.02 to 0.03 gm of 96 hr extract mixed with 2 ml of methanol and sonicated for 15 min. further, sample was filtered with syringe filter in a vial and applied to GCMS instrument. The GCMS instrument parameters such as source temperature was adjusted to 220 ˚C, with scan ranges of 35-500 (m/z) and MS run time of 48.86 min, for adapted method (table 4).

Table-4: Shows instrument method adopted for GCMS-MS analysis of *P. foetida* extract

Sr. No.	Function	Rate (^{0}C/Min)	Temperature (^{o}C)	Hold Time
1.	Initial	-	40	2.00
2.	Ramp-1	7.00	200	4.00
3.	Ramp-2	10.00	300	10.00

RESULTS AND DISCUSSION

The wounded and unwounded leave's ethyl acetate 96 hr extract of *P. foetida* are processed for UV, FTIR, GCMS, HPLC, HR-LCMS analysis and their bactericidal property are evaluated against *E. coli* and *S. aureous pathogens.*

Wounded and Un-wounded secondary metabolites

The 100 gm of wounded leaf yields 1 gm of dry extract from *P. foetida* for 96 hr sample. Figure 2 shows the isolated extracts from un wounded and wounded leaf discs at 24 hr, 48 hr, 72 hr, 96 hr and 120 hr.

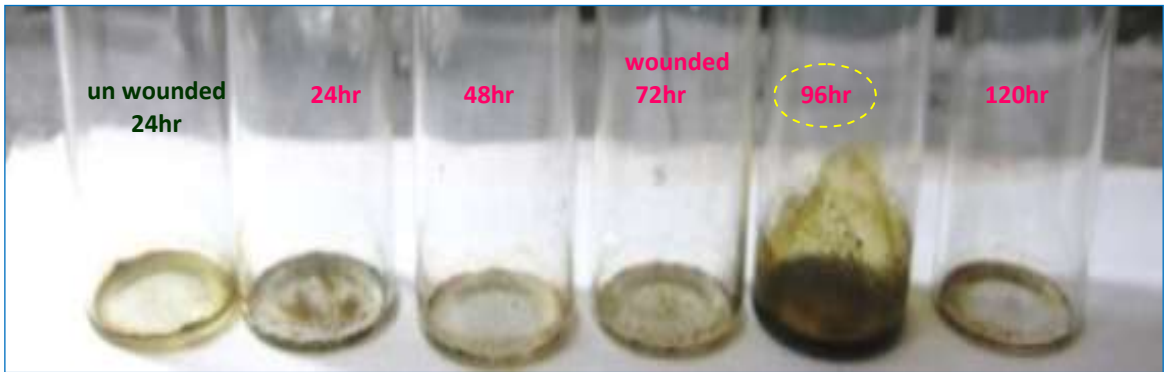


Fig-2: Shows the UN wounded and wounded extracts of *Passiflora foetida*

TLC profiling

The TLC fingerprinting of crude extracts was tested using forty five mobile phases and one silica as stationary phase, details are given in [15]. The solvent system chloroform: ethyl acetate: glacial acetic acid: benzene [C: EA: GAA: BZ] (25:15:2:10) mobile phase was selected that generates eight bands of secondary metabolites.

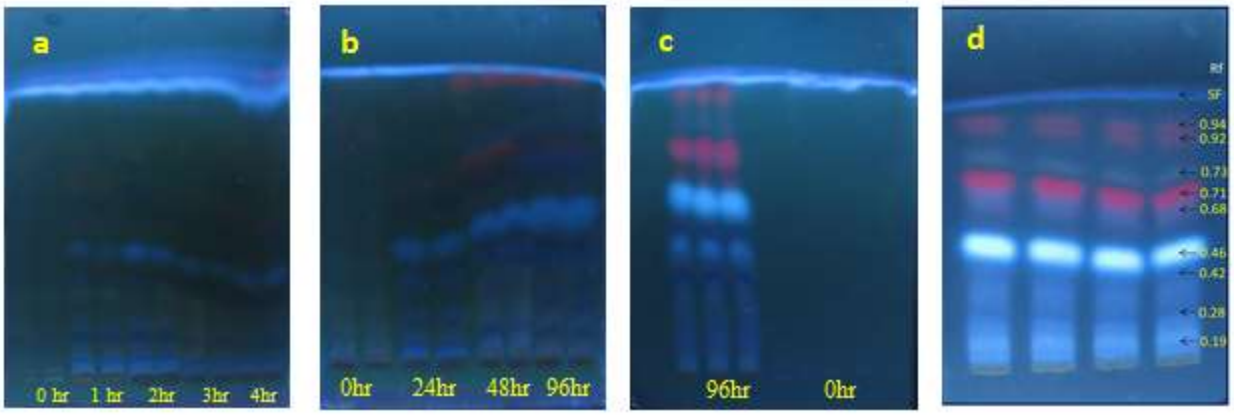


Fig-3: Displays TLC profiling of wounded leaf extract in optimized system chloroform: ethyl acetate: glacial acetic acid: benzene (25:15:2:10) at 365 nm, a-b: 0 hr, 1 hr, 2 hr, 3 hr, 4 hr, 24 hr, 48 hr, 96 hr, c: wounded and unwounded at 96 hr, d: wounded

HP-TLC fingerprinting

The extract and pure compound are HP-TLC profiled by mobile phase that used for TLC i.e C: EA: GAA: BZ (25:15:2:10) gives alike bands (see figure 4) with TLC as shown in figure 3d.

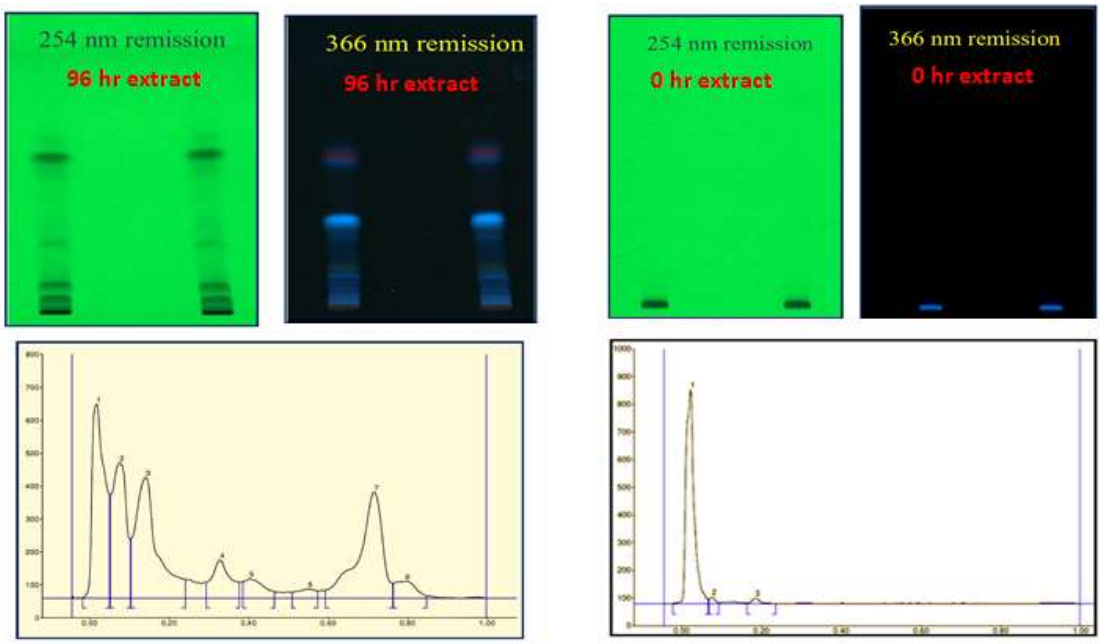


Fig-4: It shows HP-TLC generated bands of secondary metabolites from extract and pure compound by optimized solvent system, plate under a: llumination type, 255 nm and 365 nm

TLC of pure compound

The pure compound isolated and run on TLC along with the unwounded and 96 hr sample in benzene: chloroform (10:1) and chloroform: ethyl acetate: glacial acetic acid: benzene (25: 15:2:10) see figure 5.

Ultraviolet–visible spectroscopic qualities

The UV spectroscopic qualities of extract and pure compounds were determined in range of 800 nm - 200 nm (see figure 6)

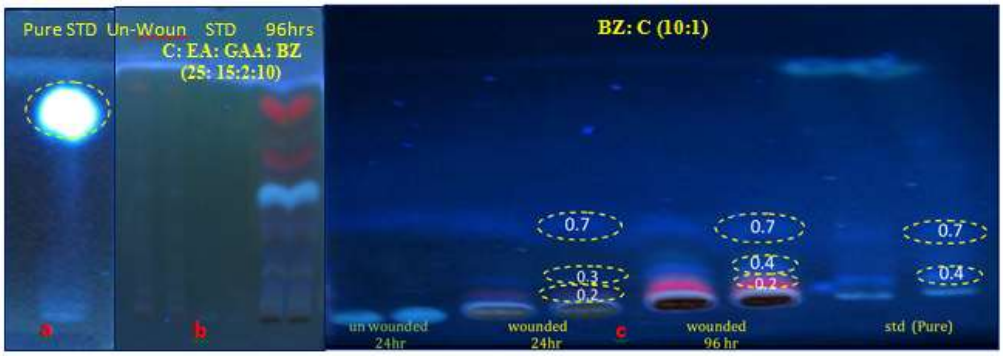


Fig-5: Show TLC run of stress induced pure compound (Rf 0.46) in a: an optimized solvent system a: benzene: chloroform (10:1), b: furthermore with unwounded and 96 hr in C: EA: GAA: BZ (25:15:2:10), c: extract of unwounded, wounded 24 hr and 96 hr along with pure compound in benzene: chloroform (10:1), under UV transilluminator at 365 nm.

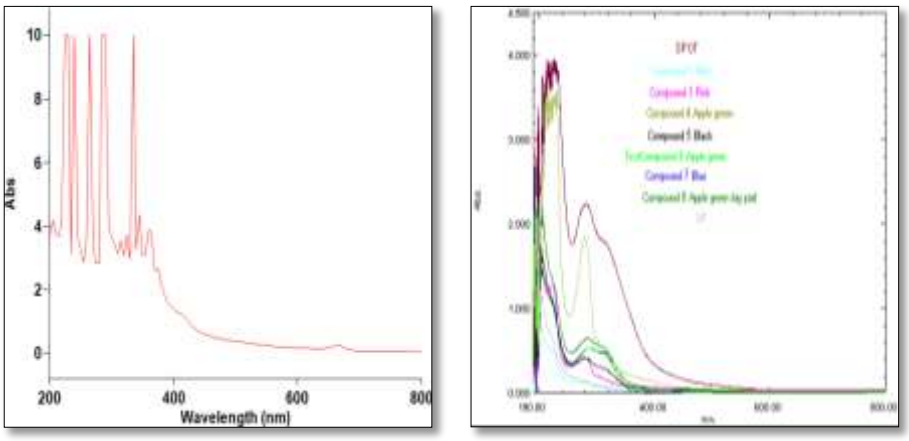


Fig-6: Shows a characteristics uv-visible spectrum for ethyl acetate extracts (a) and pure compounds (b) of *P. foetida.* Note. Compound 2nd is not shown in results, due to loss of it during scraping process

Functional group detection in extract

The characteristic FTIR absorptions in scan range of 500 nm - 4500 nm for 5th, 6th, 7th and 8th was given preferences due to their more probability of being antimicrobic potential and respective function group are drawn in table 5.

Table-5: Shows functional group detected on a basis of FTIR for pure secondary metabolites with its absorption range, absorption frequency strength and characteristic bond

Sr. No.	Stress – induced compound name	Absorptions	Characteristic bond	Characteristic functional group	Frequency Absorption strength
1	5 Blue	3402.43	N–H stretch	1°, 2° amines	medium
		2991.58	C–H stretch	alkanes	medium
		1511.90	N–O asymmetric stretch	nitro compounds	strong
		1429.29	C–C stretch (in–ring)	aromatics	medium
		1166.53	C–H wag (–CH_2X)	alkyl halides	medium
		1028.06	C–N stretch	aliphatic amines	medium
		929.88	O–H bend	carboxylic acids	medium
		863.37	C–H	aromatics	strong
2	6 Apple green	3439.08	O–H stretch, H–bonded	alcohols, phenols	Strong, broad
		3419.79	O–H stretch, H–bonded	alcohols, phenols	Strong, broad
		3394.72	N–H stretch	1°, 2° amines	medium
		1513.91	N–O asymmetric stretch	nitro compounds	strong
		1415.75	C–C stretch (in–ring)	aromatics	medium
3	7 Blue	3500.20	O–H stretch, H–bonded	alcohols, phenols	Strong, broad
		3441.01	O–H stretch	alcohols, phenols	Strong, broad
		3417.06	O–H stretch, H–bonded	alcohols, phenols	Strong, broad
		1417.06	C–C stretch (in–ring)	aromatics	medium
4	8 Apple green	3404.36	O–H stretch, H–bonded	alcohols, phenols	Strong, broad
		2905.53	C–H stretch	alkanes	medium
		1517.77	N–O asymmetric stretch	nitro compounds	Strong
		1417.68	C–C stretch (in–ring)	aromatics	medium
		1280.16	C–H wag (–CH_2X)	alkyl halides	medium
		1170.79	C–H wag (–CH_2X)	alkyl halides	medium
		1122.57	C–N stretch	aliphatic amines	medium
		1037.70	C–N stretch	aliphatic amines	medium
		929.99	O–H bend	carboxylic acids	medium
		852.54	C–H	aromatics	Strong
		771.53	C–Cl stretch	alkyl halides	medium
		667.33	–C≡C–H: C–H bend	alkynes	Strong, broad
		563.57	C–Br stretch	alkyl halides	medium

MTT analysis

The MTT sprayed on TLC profiled using C : EA: GAA : BZ (25: 15:2:10) shows clear results as dark violet backgrounds with colorless spot observed against *E. coli* at Rf 0.7 and 0.4 confirming bacteriocidal property(see figure 7a). A solution of 2,

3, 5-triphenyl tetrazolium chloride (TTC) was sprayed on BZ : C (10:1) run pure compound shows dark pink colored background with colorless spots confirms the presence of potent antimicrobic property against *S. aureus*.

Agar well diffusion analysis

It is clearly seen that the *P. foetida* extract have a bactericidal activity against *E. coli* at 10 mg/ml concentration with 14 mm zone of inhibition. In contrast, 5 mg/ml extracts concentration show 12 mm zone of inhibition against *S. aurous.* Figure 7ba, 7bb shows antibacterial activity of ethyl acetate extracts of *P. foetida* against *E. coli* and *S. aurous.*

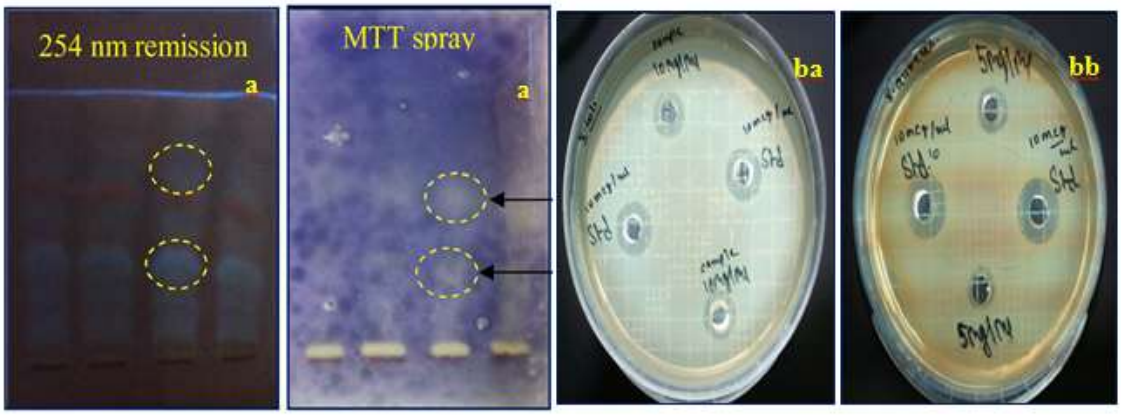


Fig-7 A: Shows TLC bioautography of leaf extract of *P. foetida against E. coli* using tetrazolium salt 3-(4,5-dimethyl-2-yl)- 2,5-diphenyltetra azoleium bromide (MTT) spray. b. Determination of minimum inhibitory concentration (MIC) of *P. foetida* leaf extracts (Passida) against ba: *E. coli* and bb: *S. aurous*

HR-LCMS determination of wound stress induced antimicrobial compounds

The pure compound 5th (Rf 0.46) and 8th (Rf 0.73) possessed bactericidal property confirm by FTIR and bioautography method sent for HR-LCMS analysis.

The scan and library matching of mass peaks showed the high abundance of nitrofurazone and 4-tridecynoic acid (for structure see figure 8a, b) along with the existence of other compounds in compound 5th.

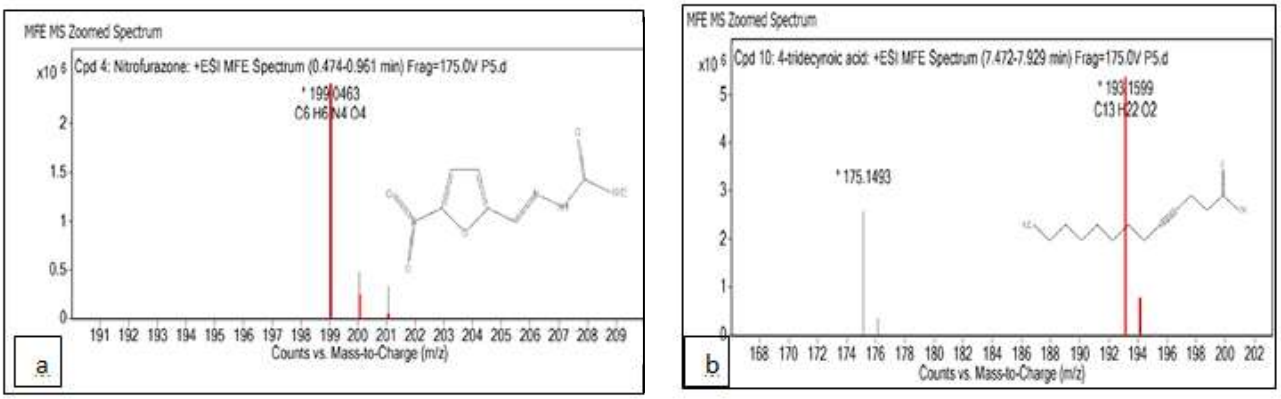


Fig-8: Show the structure of nitrofurazone and 4-tridecynoic acid

The purified compound 8th is processed through scanning of HR-LCMS, and its chromatogram is shown in figure 9. The library matching of mass peaks showed the high abundance of dihydrodeoxystreptomycin the possible antibacterial compound along with the existence of other compounds in minor quantity. HR-LCMS analysis of pure compound 5th and 8th along with retention time, formula, database hits and activities are given in table 7 and 7

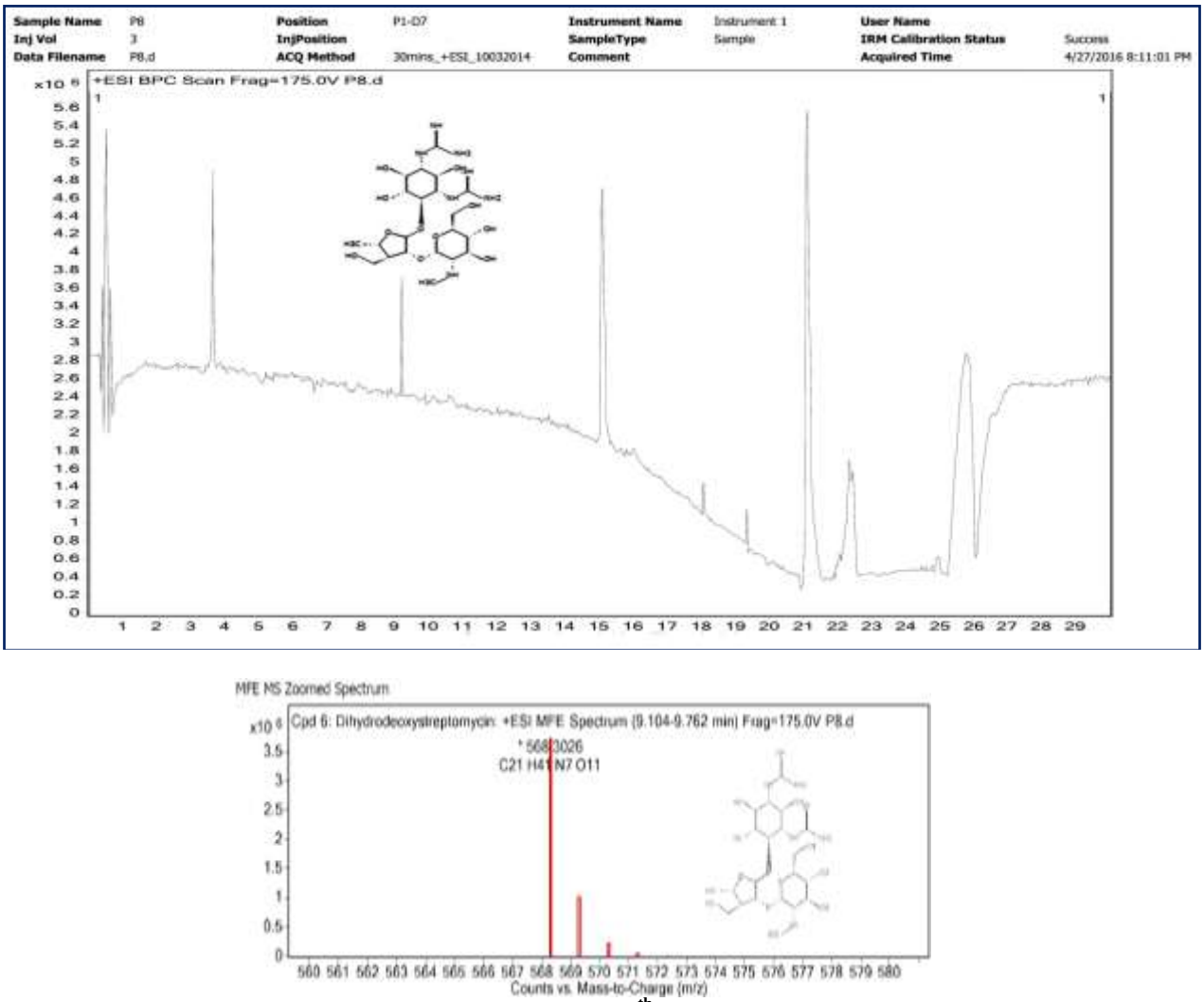


Fig-9: HR-LCMS scanning chromatogram of compound 8th showing main antimicrobial bioactive compound, dihydrodeoxystreptomycin (RT: 9.22 min)

HPLC profiling

The HPLC chromatogram of extract for 96 hr, and 0 hr are shown in figure 10. The 96 hr extract shows an HPLC chromatogram at 0.87 minute (6.28PPM) similar to standard of ibuprofen. Figure 10b shows HPLC chromatogram of ibuprofen in standard 100 ppm, 96 hr and 0 hr extract and Figure 10c shows HPLC chromatogram of dihydrodeoxystreptomycin in standard, 96 hr and 0 hr extracts. The 96 hr sample show peak of dihydrodeoxystreptomycin at 7.39 minutes (12.98PPM).

GC-MS/MS analysis

The ethyl acetate extracts of *P. foetida* is applied for GCMS analysis, its chromatogram is represented in figure 11. The 96 hr extract run for GCMS analysis, and is matched with a database library to identified compounds with specific RT is given in table 8.

Table-6: Shows the qualitative HR-LCMS analysis of pure compounds 8th along with retentation time, formula, database hits and activities

HRLCMS analysis of pure compound 8th							
Sr no	**Compound name**	**RT**	**Mass**	**Formula**	**DBH**	**DB Diff (ppm)**	**Activity**
1	Norcotinine	0.434	162.0785	C9 H10 N2 O	1	5.23	
2	11-amino-undecanoic acid	0.553	201.1756	C11 H23 N O2	1	-13.45	
3	Ethyl Oxalacetate	3.338	188.0714	C8 H12 O5	4	-15.4	
4	Methyl jasmonate	5.136	224.1446	C13 H20 O3	2	-14.87	phytoalexin (antimicrobial)
5	Desmethylpirenzepine	6.184	337.1483	C18 H19 N5 O2	3	16.42	Pirenzepine: antigungal
6	Dihydrodeoxystreptomycin	9.22	567.2955	C21 H41 N7 O11	3	-16.05	Anti-Infective Anti-Bacterial
7	Lactone of Prostaglandin F2 α Main urinary metabolite	15.122	296.1672	C16 H24 O5	6	-16.24	Anti aggregator in human blood antifertility
8	DL-PDMP (ceramide analog) DL- threo-1 phenyl-2 decanoylamino-3 morpholino-1-praponal (www.scbt.com)	21.135	390.2832	C23 H38 N2 O3	15	12.87	
Note : DBH : Data base hits							

Table -7: Shows the qualitative HR-LCMS analysis of pure compound 5th along with retention time, formula, database hits and activities

HRLCMS analysis of pure compound 5th						
Sr No	Compound name	R T	Mass	Formula	DBH	Activity
1	1,2-Diacetylhydrazine	0.424	116.0587	C4 H8 N2 O2	2	Insecticidial
2	Niacinamide	0.432	122.0461	C6 H6 N2 O	1	Nicotinamide's antimycobacterial,
3	Norcotinine	0.432	162.0767	C9 H10 N2 O	1	
4	Nitrofurazone	0.644	198.0384	C6 H6 N4 O4	6	Anti-infective, antibiotic, antiseptics, disinfectants, antimicrobial, antibacterial drug.
5	4-(2-hydroxypropoxy)-3,5-dimethyl-Pheno	5.411	196.1111	C11 H16 O3	10	
6	Benzenemethanol, 2-(2-aminopropoxy)-3-methyl	5.814	196.1113	C11 H16 O3	2	
7	Zolpidem Metabolite II	6.189	337.1452	C19 H19 N3 O3	8	Sleep disorder, drug-facilitated sexual assault
8	DIHYDROJASMONIC ACID, METHYL ESTER	7.317	226.1581	C13 H22 O3	12	Flavouring ingredient, aroma compound
9	2-Methyl-5-isopropylhexa-2Z,5-diena	7.614	152.1212	C10 H16 O	15	No literature
10	4-tridecynoic acid	7.616	210.1632	C13 H22 O2	15	Antimicrobial activity against
11	3-oxo-tridecanoic acid	7.625	228.1739	C13 H24 O3	15	-
12	Methyl jasmonate	7.689	224.142	C13 H20 O3	15	Wound response metabolite, Biotic and abiotic stresses, herbivory and wounding induce metabolites.
13	TETRAHYDROTRIMETHYL HIS PIDIN	7.697	292.1325	C16 H20 O5	8	Antibiotics, Novel hispidin-based compound : enoyl-reductase-inhibiting, antimicrobial activity
14	3-Hydroxymorphinan	9.798	243.1641	C16 H21 N O	3	Neurotrophic to dopaminergic neurons and is also neuroprotective against LPS-induced neurotoxicity.
15	Lactone of PGF-MUM	15.123	296.1639	C16 H24 O5	2	
16	1-Linoleoylpho-sphatidylcholine	16.931	520.3287	C26 H51 N O7P	5	In lipid bilayer
17	GPGro(16:0/0:0)[U]	16.933	484.2838	C22 H45 O9 P	2	Glycerophosphoglycerol
18	3beta,6alpha,7alpha-Trihydroxy-5beta-cholan-24-oic Acid	21.16	408.2892	C24 H40 O5	15	

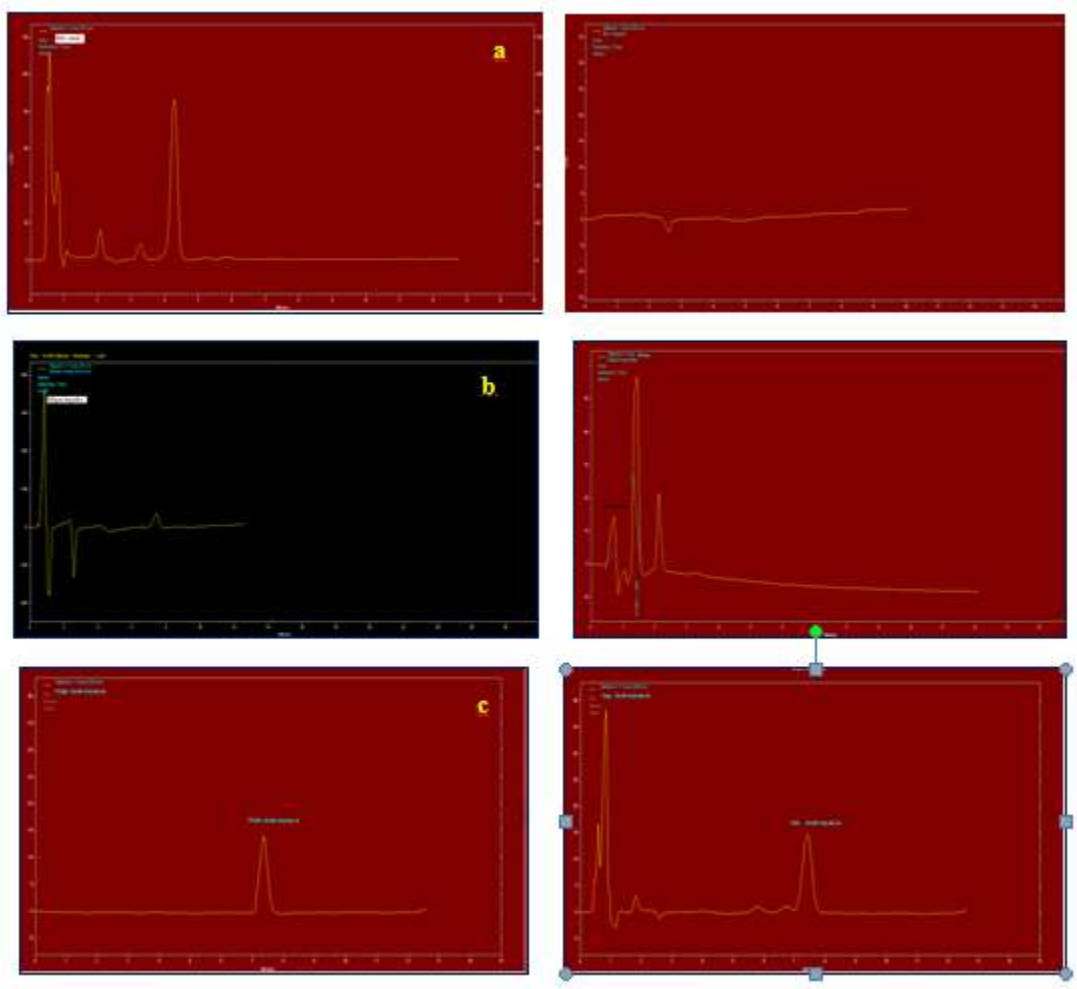


Fig-10a: Shows the HPLC chromatogram of *P. foetida* ethyl acetate extracts from 96 and 0 hr at 269 nm. b. ibuprofen in standard 100 ppm and 96 hr, c. standard, 96 hr and 0 hrs sample for dihydrostrptomycin.

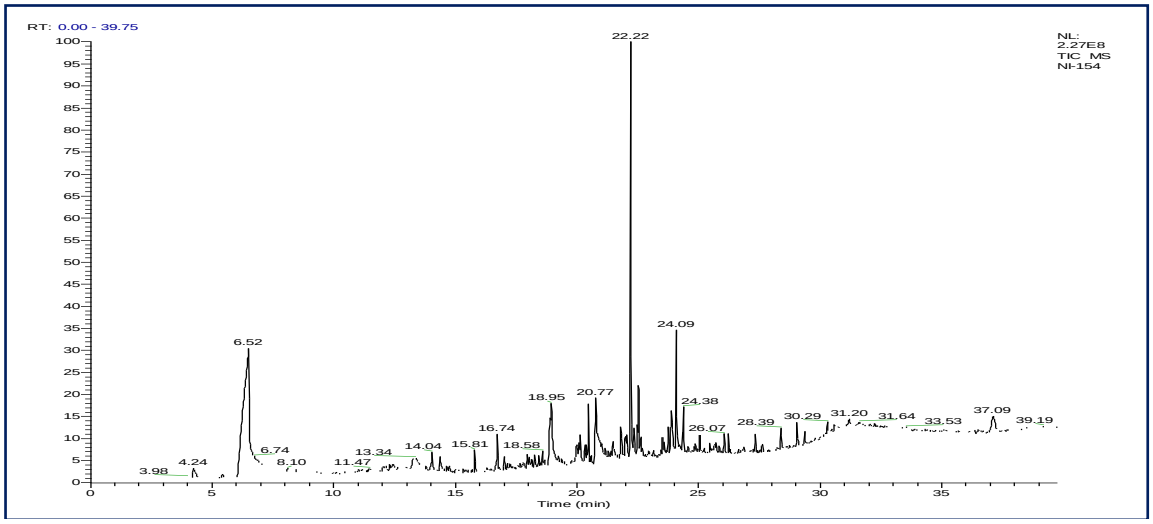
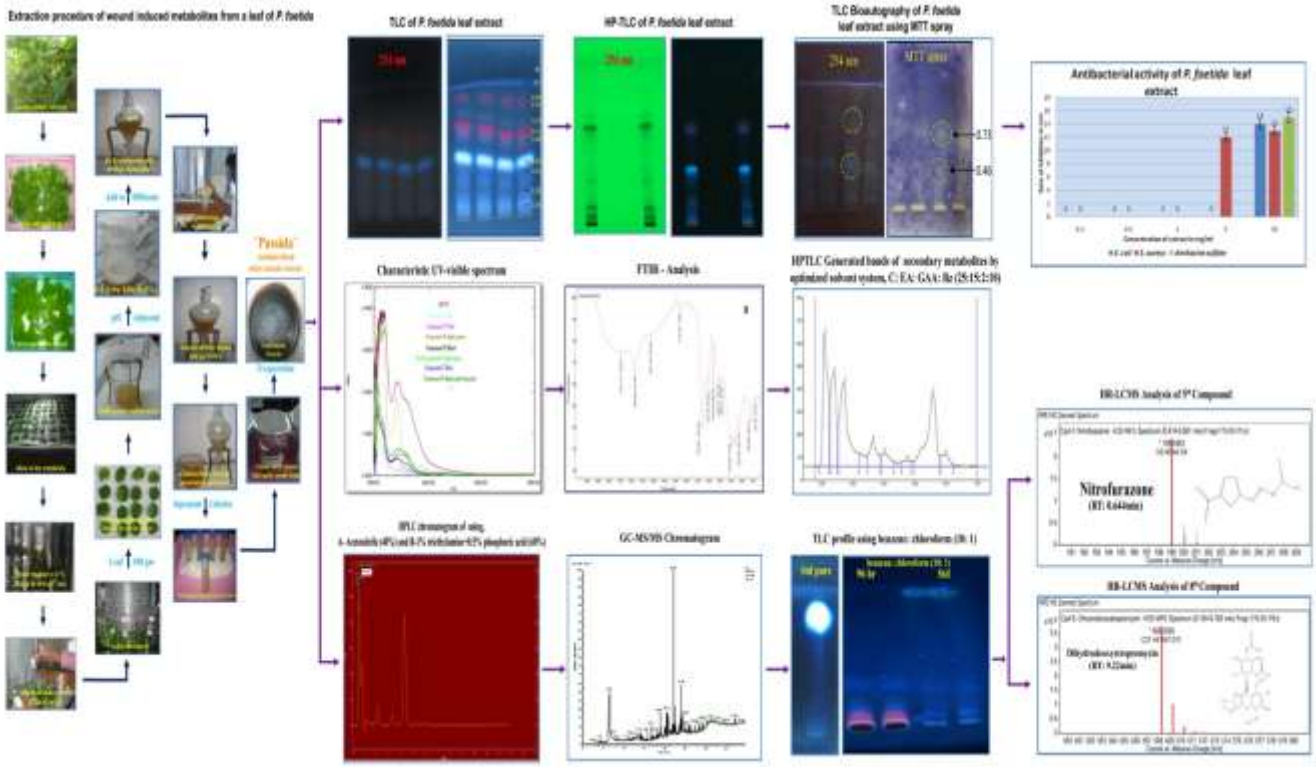


Fig-11: GC-MS/MS Chromatogram of ethyl acetate extracts of *P. foetida* (96-hours)

Table-8: Shows the list of GCMS library search for compound with respective RT along with their possible role identified in 96 hr *P. foetida* extract

Sr. No.	RT (min)	Identified compound	Activity (role)
1.	6.52	Lactic acid	Disinfectant
2.	14.36	Benzene propanoic acid	Antioxidant to prolong shelf life foods, flavoring, spices, fragrance, fixative agent, sweetener emulsifier, perfumes, bath gels.
3.	14.67	Acetyl eugenol	Acaricidal , antileishmanial.
4.	15.81	Fumaric acid	Antibacterial.
5.	16.74	3,6 Dimethoxyaurone	Phytoalexins, antioxidant, antibacterial, antiplasmodial, antiviral, antimalarial, antiflammatory. Aurones; insect antifeedant, antiparasitic, antileishmanial, antifungal, inhibitory activities, cancer chemotherapy agent.
6.	17.03	Dimethylpyridine	Antituberculosis
7.	18.95	Methyl Jasmonate	mediates defense responses against biotic and abiotic stresses, seed germination, root growth, flowering, fruit ripening, and senescence, antimicrobial, nicotine, proteinase inhibitors, antioxidant capacity, storage life leading to decreased postharvest losses, enhance the fruit postharvest life.
8.	20.47	Eicosane	antioxidant
9.	20.59	Pyridine	antiviral, anticancer, antimicrobial, antidiabetic, antitubercular, antithrombin , anticoagulant.
10.	20.76	Coumaric acid	antioxidant and anti-inflammatory, antiplatelet.
11.	22.2	Dodecanoic acid	antibacterial and anti-inflammatory
12.	22.55	Sulfurous acid, butyl nonyl ester	-
13.	24.09	Stearic acid	antidepressant and antimicrobial
14.	29.03	hydroxymethyl 2-hydroxy-2-methylpropionate	-



DISCUSSION

In present study, the wide ranges of a chemical group confirmed in a ethyl acetate *P. foetida* extract are apigenin, alkanes, alkyne, alkaloids, alkyl halides, aliphatic amines, alcohols, aromatics, carboxylic acids, cyanogen compounds, glycosyl flavonoids, harman alkaloids, nitro compounds and phenols. However ethanol extract of the *P. foetida* leaves also established alkaloid, anthraquinones, cardiac glycosides, flavonoid, saponins, steroid and tannins [16]. The secondary metabolites confirmed in *P. foetida* are morphine, nicotine, 1,2-diacetylhydrazine, niacinamide, norcotinine [17]. Some compounds that is antibiotic in nature are dihydrodeoxystreptomycin, nitrofurazone, ibuprofen, undecane-2-one and hydroxydodecanedioic acid. The dimethyl caffeic acid, methylxanthine, 4-tridecynoic acid, 3-oxo-tridecanoic acid, benzenepropanoic acid, fumaric acid and 4-tridecynaic acids are antioxidant compounds [17, 18]. The work of [19] belief to have three polyketides α-a-pyrone, named passifloricins, apigenin, isovitexin, vitexin, 2-xylovitexin, 2- xylosylso vitexin in *P. foetida.*

An extraction of wound stress induced metabolites was done at an interval of 0 hr, 24 hr, 48 hr, 72 hr, 96 hr in ethyl acetate. It was observed that the diffusate extracts from 96 hr show good quality as well as quantity. Among the forty five mobile phases and one stationary phase solvent system chloroform: ethyl acetate: glacial acetic acid: benzene (25:15:2:10) was successfully optimized with specific Rf values [20]. Likewise, [21] evaluated vitexin in *P. foetida* using HP-TLC densitometric with ethyl acetate: methanol: distilled water: formic acid (50:2:3:6) as the mobile phase.

There is no result fluctuation as a band, no 5^{th}, 6^{th}, 7^{th} and 8^{th} that do resemblance with Rf of TLC. In both TLC and HPTLC a sharp band was seen at Rf 0.46 and 0.73 and 0.02 and 0.07 respectively. In TLC profiling of a pure compound (Rf 0.46) the breakdown of into two called a derivatives was noted at two different Rf values at 0.4 and 0.7. This suspected antimicrobial compounds separated, purified and identified in *P. foetida* in a present study are dihydrodeoxystreptomycin, nitrofurazone and 4-tridecynoic acid metabolites.

The UV spectroscopical wavelength for the compound 5^{th} have 280.50 nm wavelengths, and compound 8^{th} have 284.50 nm wavelength with absorbance of 0.416 and 0.648 respectively. The FTIR analysis of the pure compound 5^{th} appears blue/ black color under UV transilluminator, and FTIR confirms alkanes, alkyl halides and aromatic functional group. The compound 8^{th} appears apple green contains alkyl halide, alkanes, aliphatic amines, alkyne and aromatics functional groups that represent a role in antimicrobial activities.

In the agar well diffusion method the minimum inhibitory concentration (MIC) for *P. foetida* extract was found to be 12 mm against *S. aureus* at 5 mg/ml and 14 mm against *E. coli* at 10 mg/ml concentration. The result for MTT was confirmed with observation of dark violet background with colorless spot at Rf 0.46 and 0.73 against *E. coli.* Similarly, TTC confirmed by observation of dark pink colored background with pale spots at Rf 0.46 confirmed antimicrobial against *S. aureus* [22].

We confirmed, that the ethyl acetate extract was more effective at gram -ve than a gram +ve microorganisms revealed by zones of inhibition, similar to the observation of [24] instead used alcoholic extract. The study by [23] reveals the ethanol and acetone leaf and fruit extracts of 100 mcg/ml shows escalated antibacterial inhibition against four human pathogenic bacteria *i.e. pseudomonas putida, vibrio cholerae, shigella flexneri* and *streptococcus pyogenes* by well-in agar method. However, similarly, ethyl acetate extract of 10 mg/ml and 5 mg/ml show inhibitory activity against *E. coli* and *S. aureus* in present suggesting, ethyl acetate extract is more effective than ethanol and acetone.

The purified wound stress induced compounds consider for HR-LCMS library search and matching of mass peaks showed the high abundance of nitrofurazone (RT: 0.644 min) and 4-tridecynoic acid (RT 7.616 min) in the compound 5^{th}. Similarly, pure compound the library matching of mass peaks for pre compound 8^{th} showed the high abundance of dihydrodeoxystreptomycin (RT: 9.22 min). The qualitative HPLC optimized mobile phase A-acetonitrile (40%) and B-1% triethylamine + 0.5% phosphoric acid (60%) was used to generate a spectrum of antimicrobial compounds such as dihydrodeoxystreptomycin and ibuprofen confirmed with their retention time at respective wavelength of 254 nm and 220 nm.

We don't identify paper showing use of ethyl acetate extracts for GCMS analysis. However, [25] identify 27 bioactive compounds by employ GCMS to ethanolic seed extracts of *P. foetida*. The present work GCMS identified compounds dodecanoic acid has antioxidant and antimicrobial compound. The term passicol is coined by [5] in case of banana passion fruit for antifungal activity, similarly we denote the extract from *P. foetida* as "Passida" for antibacterial activity. This study also encourages the pure compounds (5^{th} and 8^{th}) potential to be development of drug in pharma industry.

CONCLUSIONS

The ethyl actetate leaf extract of *P. foetida* is of mixed nature, including polar, mid polar and non polar compounds. The 45 mobile phase in combination with silica tried and optimized mobile phase: ethyl

acetate: chloroform: glacial acetic: benzene in a ratio (25:15:2:10) was finalized. The characterization of secondary metabolites using TLC has generated eight bands in 96 hours under UV transilluminator at 365 nm.

The whole extracts showed antimicrobial activity of 12 mm and 14 mm against *S. aureus* at 5 mg/ml and *E. coli* at 10 mg/ml concentration. The TLC bioautography of *P. foetida* extract indicates two antimicrobic compounds at Rf 0.46 and 0.73 against *E. coli and S. aureus*. The pure compound from Rf 46 is processed and benzene: chloroform (10:1) has been optimized which on TLC bioautography show inhibition against *S. aureus.* The stressed induced antimicrobic compound initiation time is identified as 45-60 min. These confirmed the wound stress induced antimicrobial compounds in ethyl acetate extract of *P. foetida* is labeled *a*s *"Passida."* The HR-LCMS chromatogram elucidates the structure is 4-tridecynoic acid at RT 7.616 min and dihydrodeoxystreptomycin at RT: 9.22 min for pure compound 5^{th} and 8^{th} respectively. The HPLC mobile phase A-acetonitrile (40 %) and B-1 % triethylamine + 0.5 % phosphoric acid (60 %) was optimized for getting eight peaks of compounds present in *P. foetida* ethyl acetate extracts of 96 hours. The HPLC retention time for antimicrobial compounds such as dihydrodeoxystreptomycin and ibuprofen was observed at 7.39 and 0.87 min at 254 nm and 220 nm respectively. The 4-chloro 7-nitrobenzofurazan (RT: 18.39), methyl jasmonate (RT: 18.84) and 12-hydroxy dodecanoic acid (RT: 23.79) are confirmed through GCMS library search and their PPM is given as o, 10.03 and 82.65 respectively.

The wound stress antimicrobial compound confirmed in *P. foetida* using TLC, HP-TLC, TLC bioautography, HR-LCMS, HPLC and GCMS are 4-tridecynoic acid and dihydrodeoxystreptomycin, ibuprofen, 4-chloro 7-nitrobenzofurazan, methyl jasmonate and 12-hydroxy dodecanoic acid.

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Declarations

Authors' contributions

AS Patil designed, developed and improvised manuscript, BD Lade designed experimentation, wrote and edit the manuscript. Present work is a some part of Ph. D thesis of BD lade, both authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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