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Antimicrobial Activity of Some *Trigonella* Species

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Abstract

The genus *Trigonella* includes many medicinal and aromatic plant species used in traditional as well as veterinary medicines for different diseases, alone or in combination with other remedies. The crude methanol extracts of 15 *Trigonella* species were assayed for antimicrobial activity against four medicinally important multidrug resistant clinical isolates, five plant pathogenic bacteria and five fungi.

Three species showed a broad spectrum of antibacterial activity inhibiting all the test bacteria. Acidified ethanolic extract of the most promising species, *T. suavissima* was purified by column chromatography. Characterization of a partially purified ethyl acetate fraction by LC/ESI/MS showed the presence of 7'4' dihydroxy flavone and (2E)-3-{5-[4-(Ethoxycarbonyl)phenyl]furan-2-yl}prop-2-enoate) Three species showed strong antifungal activity against *Aspergillus niger* and *Fusarium solani*. The saponin extract of *T. spicata* showed a selective activity against *A. niger* and the sapogenin extract against *F. solani*. A polyhydroxylated alkaloid was isolated from the sapogenin extract of *T. spicata* using chromatographic techniques with structural characterization done by LC/ESI/MS, ^{13}C and ^{1}H NMR. The study reveals that *Trigonella* species are potential sources of natural compounds that may act as antimicrobial agents. It represents the most extensive survey of antimicrobial activity in *Trigonella* done to date..

Keywords: Antimicrobial activity; *Trigonella suavissima* Lindl; T. spicata Sm; Saponins; Sapogenin

Introduction

Plants are rich source of a variety of bioactive secondary metabolites reported to have in vitro antimicrobial properties. The revival of interest in plant derived antimicrobial compounds is mainly due to the current wide spread belief that "green medicine" is more safe than costly synthetic drugs, many of which have adverse side effects. Active agents from plants are not only non-toxic but also easily biodegradable. Many of the earliest drugs and pesticides were plant extracts and several plants have been exploited for their antimicrobial activity [1].

The genus Trigonella includes medicinal and aromatic plant species at a risk of genetic erosion [2]. Fenugreek (*T. foenum-graecum*) has been referred to as a medicinal herb both in Indian "Ayurvedic" and traditional Chinese medicines. Medicinal uses vary from wound healing to promotion of lactation in weaning mothers and a sex stimulant. Fenugreek seeds in human diet can reduce blood sugar, blood cholesterol and sickness due to air pollution [3].

Some sources of fenugreek seeds have been reported to possess antimicrobial activity against both gram-positive and gram-negative bacteria [4] while extracts from fenugreek seeds examined by De et al. [5] were unable to prevent the growth of either bacteria or yeast. Fatty oil from fenugreek seeds (*T. foenum-graecum*) has been reported to be useful for developing plant derived antifungal drugs [6]. Though much attention has been paid to *T. foenum-graecum*, nevertheless there are a number of other species of this genus, known to occur as weeds but need more studies [7]. Wild and weedy legume species of Trigonella are used in traditional as well as veterinary medicines for different diseases, alone or in combination with other remedies. Normally, these plants are picked up by the poorer class and used as vegetable with some species used since centuries in traditional "ayurvedic" or ethnomedical practices as alternative source of medicine [7, 8]. Many are included in Indian herbals along with *T. foenum-graecum* [9]. Since Trigonella species have received little assessment for their medicinal potential, the present paper describes the results of screening organic extracts of Trigonella against a wide range of

microorganisms including human pathogens, phytopathogens and filamentous fungi. LC-MS analysis was carried out to identify the major biologically active phytoconstituents from the most promising species showing antibacterial and antifungal activity.

Material and Methods

Plant material

Seeds of 15 *Trigonella* species were obtained from The Genetic Resource Centre, South Australian Research and Development Institute's (SARDI) Australian Medicago Research Centre (AMGRC), Urrbrae, South Australia. The accessions along with their SA, EC and herbarium voucher numbers are listed in Table 1. The entire plants were uprooted from the pots at flowering, washed thoroughly with water and shade dried. The dried plants were powdered in a blender and stored at 4ºC till further use.

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Determination of antimicrobial activity

The organisms used are listed in Table 1. Antibacterial activity was determined by disc diffusion method while antifungal activity was determined by the agar well diffusion method as described by [2]. Bacteria with a clear zone of inhibition of eight mm or more were considered to be sensitive. The antibacterial activity was expressed as the mean ± SD of three measurements. Antibiotic gentamycin (Sigma, 50 µg per disc) was used as positive control for all test bacteria except Staphylococcus aureus. In antifungal activity assays, fungi with a clear zone of inhibition of 14 mm or more were considered to be sensitive. The antifungal activity was expressed as the mean ± SD of two measurements. Antibiotic nystatin (Sigma, 100 µg / ml) was used as a positive control. In some assays, the zone diameter was monitored further up to 35th day.

Preparation of plant extracts

Five grams of powdered plant material was soaked in 50 ml of methanol for 96 h. The mixture was stirred every 18 h using a sterile glass rod. The extract was filtered through Whatman filter paper No. 1 and the filtrate was evaporated to dryness in vaccuo and weighed. The dried extract was dissolved in dimethyl sulphoxide (DMSO) to get a final concentration of 500 mg/ml. Chloroform and petroleum ether extracts were also prepared using a similar procedure.

Saponin extracts

Saponins were extracted by the method of Rajpal [10]. The dried extract was dissolved in DMSO to a final concentration of 100 mg/ ml.

Sapogenin extracts

Twenty grams of dried plant material was added to 500 ml of 2.5 M ethanolic sulfuric acid in a round bottom flask. The mixture was refluxed for 4 h at 80 ºC, cooled and filtered through Whatman filter No.1. The filtrate was diluted with 100 ml of distilled water and then extracted with chloroform (3 x 10 ml). The pooled chloroform extract was evaporated on a water bath below 60 ºC. The dried sapogenin extract was dissolved in DMSO to a final concentration of 200 mg/ml. Saponin and sapogenin extracts were evaluated for antifungal activity.

Sapogenin extract was used to prepare polar and non-polar extracts. The dried extract (3 g) was refluxed with hexane for 5 h. After filtration, the residual extract was again refluxed with hexane and filtered. The process was repeated total three times. Hexane was evaporated under reduced pressure to obtain hexane soluble extract. The dried extract was dissolved in DMSO to get a final concentration of 50 mg/ml. Using a similar procedure, hexane insoluble fraction of the sapogenin extract was further extracted sequentially with toluene, ethyl acetate (EtOAc) and ethanol. For *T. suavissima* the yields of extracts were 0.3 g of hexane extract, 0.5 g of toluene extract, 1.0 g of ethyl acetate extract and 0.5 g of ethanol extract. While for *T. spicata* the yields of extracts were 0.5 g of hexane extract, 1.2 g of toluene extract, 0.8 g of ethyl acetate extract and 0.5 g of ethanol extract. All the extracts were tested for antimicrobial activity..

Fractionation of extract

One gram of ethyl acetate extract that showed the highest total antibacterial activity was eluted through a of silica gel column (mesh size 60-120; 2 cm dia x 35 cm length) with an eluent comprising a gradient of chloroform (CHCl3): methanol with increasing polarity (100% CHCl3 , 1:99, 2:98, 5:95, 10:90, 20:80, 40:60, 80:20 methanol:CHCl3 and 100% methanol). Thirty seven fractions of 100 ml each were collected. These were concentrated to one ml on a water bath and evaluated for antimicrobial activity. The fractions were also monitored through silica gel thin layer chromatography (TLC; Merck TLC Silica Gel Plate 60 F254), using the solvent system CHCl3: methanol: water (70:26:4). The plates were visualized using iodine vapor or by spraying 5% methanolic sulphuric acid and heating at 110 ºC. Fractions with similar spots and showing antibacterial activity were combined and purified further by chromatography over small silica gel column using a gradient of EtOAc and methanol (100 % EtOAc, 1:99, 2:98, 5:95, 10:90, 20:80, 40:60, 80:20 methanol: EtOAc and 100 % methanol). Twenty five fractions of 50 ml each collected were concentrated to one ml and evaluated for antibacterial activity. The fractions were also monitored by TLC, using the solvent system CHCl3: ethyl acetate: methanol (1:1:1).

Toluene extract (1.2 g), showing antifungal activity, was eluted through silica gel column with a gradient of $CHCl_3$: methanol as described above. Thirty four fractions of 50 ml each were concentrated to 1 ml and evaluated for antifungal activity. The fractions were monitored by TLC using hexane: ethyl acetate

Table 1: List of *Trigonella* species and organisms used to evaluate antimicrobial activity

Species	SA Number [a]	EC Number[b]	AHMA Number[c]	Organism	Designation[d]	Phenotype[e]
Trigonella anguina Delile.	40210	583495	28553	*Agrobacterium tumefaciens*	MTCC-413	-
T. balansae Boiss. et Reut.	32204	583507	28552	*Acidovorax* spp.	MCMB-835	-
T. caelesyriaca Boiss.	5043	583557	28557	*Pectobacter caratovorum*	MTCC-1428	-
T. calliceras Fisch. ex M. Bieb.	32202	583570	28563	*Pseudomonas syringae*	MTCC-1604	-
T. filipes Boiss.	13459	583580	28565	*Xanthomonas campestris*	MTCC-2286	-
T. foenum-graecum L.	35684	583588	28559	*Bacillus subtilis*	MTCC-1558	K^r, N^r
T. schlumbergeri Boiss.	5044	583609	28549	*Escherichia coli*§	MCMB-813	-
T. maritima Delile ex Poir.	9841	583600	28556	*Salmonella typhi*§	MCMB-814	-
T. mesopotamica Hub.-Mor.	14518	583604	28550	*Staphylococcus aureus* (VRSA) §	MCMB-818	G^r,Va^r Ro^r, K^r, T^r, A^r
T. spinosa L.	34549	583620	28554	*Fusarium solani*	NFCCI 0747	-
T. stellata Forssk.	34597	583621	28561	*Aspergillus niger*	NFCCI 1666	-
T. suavissima Lindl.	27514	583623	28551	*Colletotrichum gloeosporioides*	NFCCI 1296	-
T. coerulescens (M.Bieb.) Halácsy.	14939	583573	28562	*Sclerotium rolfsii*	NFCCI 1002	-
T. caerulea (L.) Ser.	32197	583567	28564	*Saccharomyces cerevisiae*	NFCCI 1248	-
T. spicata Sm.	14495	583614	28560			

[a] Accession identity number - South Australian Research and Development Institute's (SARDI) The Australian Medicago Resource Centre (AMGRC), Urrbrae, South Australia

[b] Accession identity number - National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India

[c] Herbarium identity number- Agharkar Herbarium of Maharashtra Association (AHMA), Pune, India

[d] A, ampicillin; G, gentamicin; K, kanamycin; N, neomycin; Ro, roxithromycin; T, tetracycline; Va, vancomycin. Superscript letter 'r' following antibiotic indicates resistance of the bacteria to the particular antibiotic.

[e] MTCC-Microbial Type Culture Collection (Institute of Microbial Technology, Chandigarh, India), MCM- MACS Collection of Microorganisms (Agharkar Research Institute, Pune, India), NFCCI-National Fungal Culture Collection of India

§ Clinical isolates, VRSA, vancomycin-resistant *Staphylococcus aureus*.

solvent system (9:1). Fractions with similar spots and showing antifungal activity were pooled and further resolved by preparative TLC. The dried extract was dissolved in acetonitrile and extracted with 5 ml HPLC grade petroleum ether to separate waxy impurities. The concentrated acetonitrile fraction was streaked across TLC plates in narrow band 2 cm from the edge. The chromatograms were developed with the solvent dichloromethane: petroleum ether: ethyl acetate (7:2:1). The bands were visualized using ultraviolet (254 nm) lamp. Eluates of each band were evaporated to dryness and tested for activity against *Fusarium*. The eluate from the band showing activity, when re-chromatographed with several other solvents showed no further separation and hence was considered as pure and characterized further.

UPLC-QTOF-MS

Metabolite analysis was carried out using a UPLC-PDA-qTOF-MS instrument (Waters Premier qTOF, Milford, MA, USA). Instrument control, data acquisition, and data processing were done using Masslynx software, version 4.0. The instrument was operated in "full-scan" MS with a spectrum integration time of 1 sec in the range of m/z 50-500 ("interscan" time 0.1 sec). Acquisition was performed in continuum mode. The electrospray source conditions were as follows: capillary voltage, 3.0 kV; sample cone voltage, 35 V; extraction voltage, 4V; source temperature, 120°C; desolvation temperature, 400ºC; cone gas, 50 L/h; and desolvation gas, 900 L/h. The injection volume of samples was 10 µl. Masses of the eluted compound were detected by the Q-TOF equipped with electrospray ionization (ESI) source. The MS was calibrated using sodium formate, and leucine enkephalin was used as the lock mass. The molecular formula suggested by the MassLynx software based on the accurate mass and isotopic pattern recognitions, was used for screening putative molecules from the Dictionary of Natural Products (Chapman & Hall/CRC Chemical Database) and the SciFinder Scholar databases (SciFinder ScholarTM 2007).

Spectroscopy

The 1D ^{13}C NMR and DEPT NMR spectra were recorded on JEOL ECS -400 MHz spectrometer (JEOL Inc, Peabody, MA) in $CDCl_3$. The ^{13}C NMR spectrum was recorded with 4 s delay time and 6K scans were accumulated. To obtain methylene sub-spectra DEPT -135 spectra was recorded using standard pulse sequence with J modulation time of 3.7 ms (J_{CH} =135Hz) with 2 s delay time. ^{1}H NMR, performed at 200 MHz, was recorded with a Varian Inova 400 FT-NMR spectrometer (Varian, Palo Alto, CA) with $CDCl_3/CD_3OD$ (2:1, v/v) as the solvent and tetramethylsilane as the internal standard.

Results

Antibacterial activity

In comparison of activities, methanol extracts were more active than chloroform and petroleum ether extracts. The antibacterial activity of methanol extracts measured as zone of inhibition against test organisms is shown in table 2. Broad spectrum antibacterial activity inhibiting all the test bacteria was shown by only three species namely *T. coerulescens, T. suavissima,* and *T. stellata.* Based on the diameter of zone of inhibition, *T. suavissima* was selected for further studies.

Preliminary phytochemical tests demonstrated that saponins, steroids and alkaloids were a common feature of all the extracts (data not shown). Antibacterial activity was observed only in the sapogenin extract of *T. suavissima*. In the polar and non-polar extracts prepared from the dried sapogenin extract antibacterial activity was seen in hexane, toluene, ethyl acetate as well as ethanol extract. However maximum activity, in terms of diameter of zone of inhibition was seen in the ethyl acetate extract with the least activity observed in the hexane extract.

In chromatographic fractionation of ethyl acetate extract, fractions 7, 8, 9, 16, 17, 19, 21 and 22 showed antibacterial activity. On the basis of similar TLC profiles fractions 21 and 22 were combined to get 0.4 g of dried extract which was further fractionated by silica gel column chromatography. Fractions 2 to 10 obtained in second–step chromatography showed antibacterial activity (Fig 1S, Support Information). Based on the diameter of zone of inhibition, fraction 6 was subjected to LC-MS analysis to identify the antibacterial compound(s) present. The chromatogram of fraction 6 showed a mixture of at least 7 compounds. Compounds corresponding to peak 1 and 2 accounted for 74% of the total mass (Fig 1a, 52% by peak 1 and 22% by peak 2).

On the basis of elemental composition and accurate masses of some fragment ions the chemical structure of m/z=254.0653 (peak 1, Fig 2S, Support Information) corresponds to 7' 4' dihydroxy flavone. The identity of 7'4' dihydroxy flavone was confirmed using a standard (1 ppm, Indofine Chemical Company, Inc. Hillsborough, NJ, USA. Fig 3S, Support Information). The chemical structure of m/z=285.0775 (peak 2, Fig.1) was (2E)-3-{5-[4-(Ethoxycarbonyl)phenyl]furan-2-yl}prop-2-enoate) according to database. However, a comparison with a standard would be essential to confirm this report indicating the occurrence of (2E)-3-{5-[4-(Ethoxycarbonyl)phenyl]furan-2-yl}prop-2-enoate) in *T. suavissima.* Remaining minor compounds could not be identified.

Antifungal activity

Polar and non polar extracts of all the species were not active against Colletotrichum gloeosporioides, Saccharomyces cerevisiae and Sclerotium rolfsii while antifungal activity was observed in the methanol extracts of some Trigonella species only against Aspergillus niger and Fusarium solani (Table 3).

T. coerulescens, T. caerulea and *T. spicata* showed antifungal activity against both *A. niger* and *F. solani.* Based on the diameters of the zone of inhibition, maximum activity was observed in *T. spicata.* The zone diameter was monitored till the 14th day to see the potency of the extract and development of resistance. While the diameter of zone of inhibition reduced significantly with incubation for *A. niger*, it remained constant for *F. solani* (data not shown).

Table 2: Antibacterial activity of crude methanol extracts of the screened *Trigonella* species

Species	*Agrobacterium tumefaciens*	*Acidovorax* spp	*Pectobacter caratovorum*	*Pseudomonas syringae*	*Xanthomonas campestris*	*Escherichia coli*	*Salmonella typhi*	*Staphylococcus aureus*	*Bacillus subtilis*
Trigonella anguina Delile.	nil	nil	8.00 ± 0.00	Nil	10.00 ± 0.00	Nil	Nil	Nil	8.00 ± 0.00
T. balansae Boiss. &Reut.	nil	nil	Nil	Nil	8.00 ± 0.00	Nil	Nil	Nil	8.00 ± 0.00
T. coelesyriaca Boiss.	10.66 ± 0.5	nil	10.00 ± 0.00	Nil	11.33 ± 1.54	10.00 ± 0.00	Nil	Nil	8.00 ± 0.00
T. calliceras Fisch ex M Bieb.	10 ± 0.0	nil	10.00 ± 0.00	11.33 ±1.5	11.33 ± 1.54	10.66 ± 1.15	Nil	13.33 ± 1.54	10.00 ± 0.00
T. filipes Bioss.	10 ± 0.0	nil	10.00 ± 0.00	8.66 ± 1.50	Nil	Nil	Nil	Nil	8.00 ± 0.00
T. foenum-graecum L.	10 ± 0.0	nil	Nil	Nil	Nil	Nil	Nil	Nil	10.00 ± 0.00
T. schlumbergeri Boiss.	10 ± 0.0	nil	8.00 ± 0.00	Nil	10.00 ± 0.00	10.00 ± 0.00	Nil	12.00 ± 0.0	10.00 ± 0.00
T. maritima Delile ex Poir.	12.33 ± 2.5	nil	9.33 ± 1.54	10.00 ± 0.00	Nil	Nil	Nil	11.33 ± 1.54	10.00 ± 0.00
T. mesopotamica Hub.-Mor.	10.00 ± 0.0	nil	9.33 ± 1.54	9.33 ± 1.54	10.33 ± 0.57	10.66 ± 1.15	Nil	10.66 ± 1.15	8.00 ± 0.00
T. spinosa L.	10.22 ± 0.57	nil	Nil	Nil	9.33 ± 1.54	Nil	Nil	10.66 ± 1.15	10.00 ± 0.00
***T. stellata* Forssk.**	**12.0 ± 0.0**	**10.00 ± 0.0**	**10.00 ± 0.00**	**8.66 ± 1.15**	**11.00 ±1.15**	**10.00 ± 0.00**	**10.00 ± 0.00**	**12.66 ± 1.15**	**10.00 ± 0.00**
***T. suavissima* Lindl.**	**14.0 ± 1.7**	**10 ± 0.0**	**10.00 ± 0.00**	**11.33 ± 1.54**	**10.00 ± 0.00**	**12.00 ± 0.00**	**12.00 ± 0.00**	**13.66 ± 1.15**	**10.00 ± 0.00**
***T. coerulescens* (M.Bleb) Hal.**	**10.66 ± 1.1**	**10.66 ± 1.1**	**10.66 ± 1.15**	**10.66 ± 1.15**	**10.00 ± 0.00**	**10.66 ± 1.15**	**10.00 ± 0.00**	**11.33 ± 1.15**	**12.00± 0.00**
T. caerulea (L) Ser.	8.66 ± 1.15	nil	8.00 ± 0.00	12.00 ± 0.00	10.33 ± 0.58	10.00 ± 0.00	Nil	10.00 ± 0.0	10.66 ± 1.15
T. spicata Sm.	9.33 ± 1.15	nil	10.00 ± 0.00	10.66 ± 1.15	Nil	11.33 ± 1.15	9.33 ± 1.15	10.66 ± 1.15	12.00 ± 0.00
Gentamycin sulfate(50µg)	35.00 ± 5.0	26.66 ± 2.8	25.00 ± 0.00	32.33 ± 2.51	25.00 ± 3.00	28.33 ± 2.88	26.66 ± 2.88	Nil	26.66 ± 1.73

*Zone diameter expressed as mean of three replicates ± standard deviation including 6 mm diameter of disc

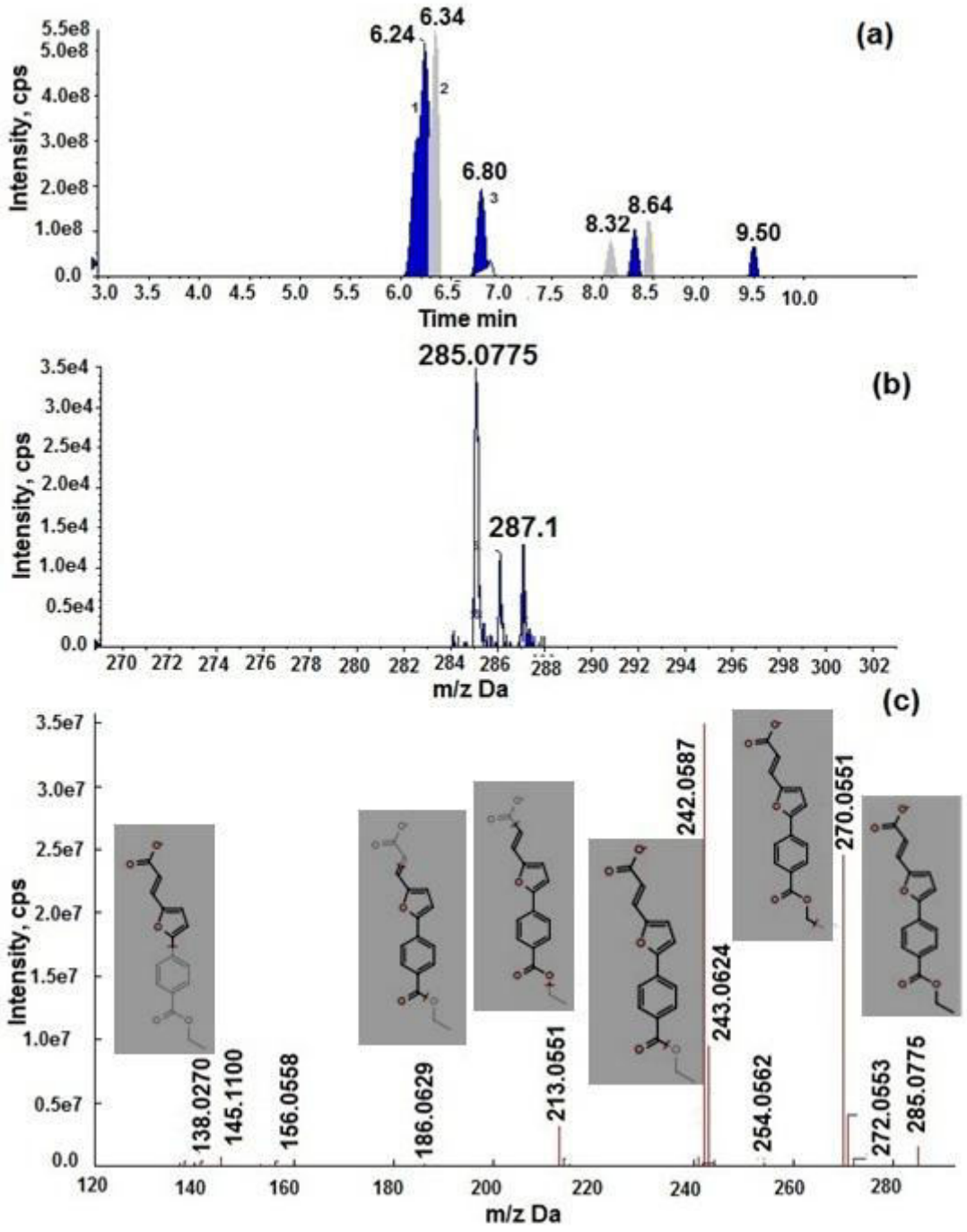

Figure. 1: (a) Total chromatogram of fraction 6 obtained at low cone voltage (b) Accurate mass measurements of ions acquired by ESI-QqTOF-MS/MS for m/z= 285.0775 and fragmentation pathway of (2E)-3-{5-[4-(ethoxycarbonyl) phenyl]furan-2-yl}prop-2-enoate).

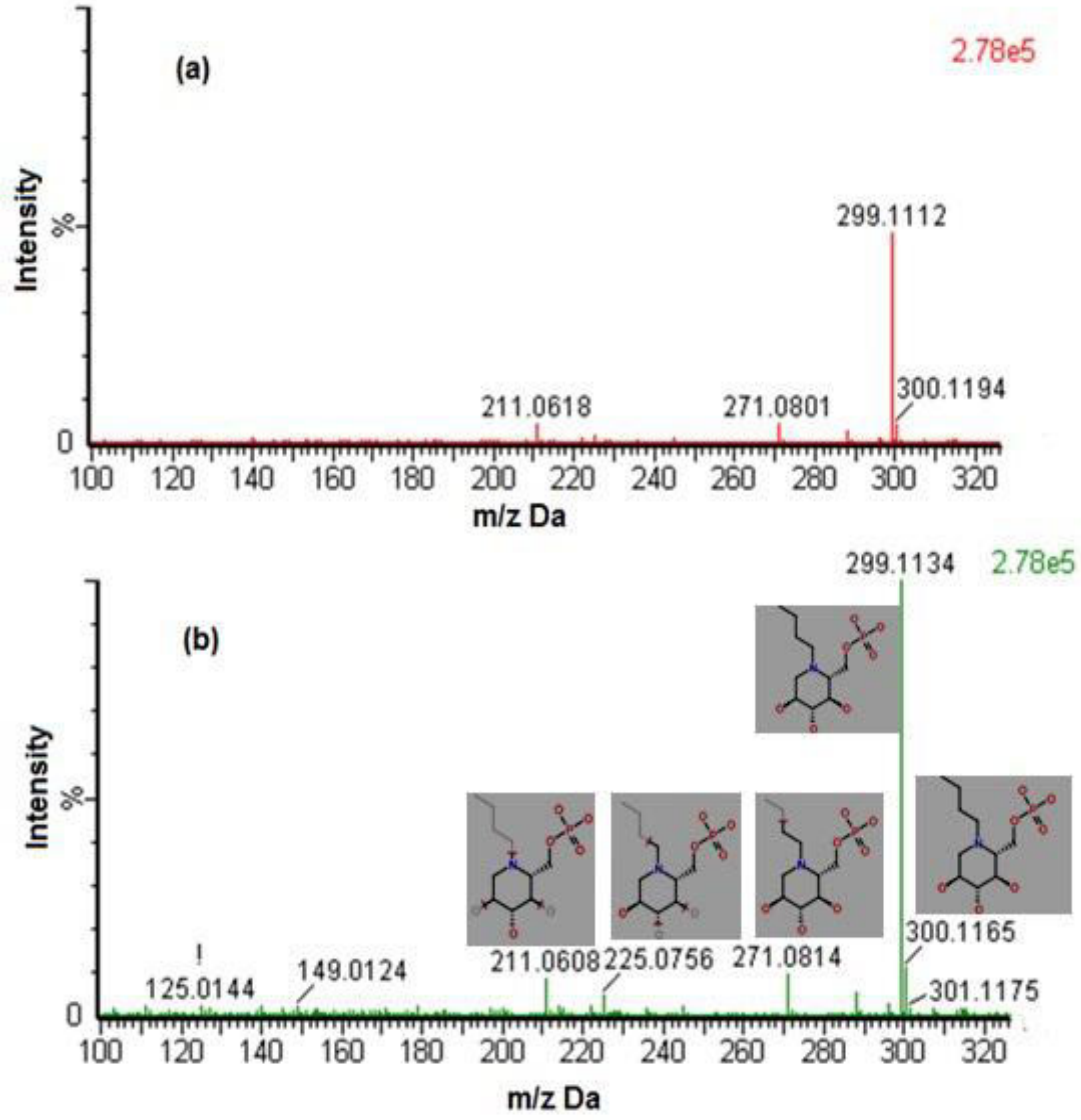

Figure 2: (a) Product ion mass spectrum obtained by ESI-QqTOF-MS/MS and (b) Mass error of the fragment ions in the product ion mass spectrum obtained by ESI-QqTOF-MS/MS and Proposed fragmentation pathway of [(2S, 3R, 4S, 5R, 6R)-1-butyl- 3, 4, 5 trihydroxypiperidine -2yl] methyl dihydrogen phosphate

Table 3: Antifungal activity of crude methanol extracts of screened *Trigonella* species

Sr. No	Species	Diameter of zone of inhibition (mm) [a]	
.		*Aspergillus niger*	*Fusarium solani*
1	*Trigonella anguina* Delile.	Nil	Nil
2	*T. balansae* Boiss. et Reut.	Nil	Nil
3	*T. caelesyriaca* Boiss.	Nil	Nil
4	*T. callicerus* Fisch. ex M. Bieb.	19.33 ± 1.15	Nil
5	*T. filipes* Boiss.	Nil	Nil
6	*T. foenum-graecum* L.	Nil	Nil
7	*T. schlumbergeri* Boiss.	15.33 ± 1.15	Nil
8	*T. maritima* Delile ex Poir.	15.33 ± 1.15	Nil
9	*T. mesopotamica* Hub.-Mor.	Nil	Nil
10	*T. spinosa* L.	Nil	Nil
11	*T. stellata* Forssk.	Nil	Nil
12	*T. suavissima* Lindl.	Nil	Nil
13	*T. coerulescens* (M. Bieb.) Halácsy.	31.66 ± 2.88	26.33 ± 1.15
14	*T. caerulea* (L.) Ser.	20.00 ± 0.00	33.33 ± 2.88
15	*T. spicata* Sm.	48.33 ± 5.77	65.00 ± 5.00
	Nystatin (100µg) Control	35.00 ± 0.00	30.00 ± 0.00

The saponin extract of *T. spicata* showed activity only against A. niger. The diameter of zone of inhibition reduced with increasing incubation time. However, sporulation was greatly delayed in the presence of the extract. While the control plate showed sporulation on the 5th day, the test plate showed uneven sporulation by the 28th day. In contrast, the sapogenin extract showed antifungal activity only against *F. solani*. There was no evidence for the development of resistance as the zone diameter remained constant till the 35th day (Figure 4S, Support Information). The sapogenin extract was therefore selected for the purification of antifungal compound with selective activity against *F. solani.*

In the polar and non-polar extracts prepared from dried sapogenin fraction, activity against Fusarium was seen only in the toluene soluble fraction. Fractions 11 and 12 collected by silica gel chromatography showed activity against *F. solani*. On the basis of similar TLC chromatograms these two fractions were pooled and 0.4 g of dried extract was obtained. In TLC analysis of the pooled fractions, three major bands were observed under ultra-violet light. Compound eluted from the band at Rf of 0.18-0.20 showed antifungal activity while compounds from other bands had little or no activity. The isolated compound showed a positive Dragendroff test. In LC-ESI-MS analysis the chemical structure of m/z 299.1134 on the basis of elemental composition and accurate masses of some fragment ions was [(2S, 3R, 4S, 5R, 6R)-1-butyl- 3, 4, 5 trihydroxypiperidine -2yl] methyl dihydrogen phosphate according to database (Figure 2).

In the NMR analysis [13C NMR & DEPT NMR (400 MHz, CDCl3): δ 14.18 ppm (CH3), 29.80ppm (CH2), 43.42ppm(CH2), 60.87ppm(CH2),73.28ppm(CH), Figure 5S, Support Information] one terminal methyl carbon CH3 resonating at δC 14.18 ppm is attached to one methylene carbon CH2 resonating at δC 29.30ppm which gives upfield carbon signal. The other two methylene carbon CH2 resonating at δC 43.42ppm & δC 60.87ppm are connected to tertiary amine. Five methines carbons CH resonating at δC 73.28ppm are assigned to the ring system. DEPT 13C NMR also gives supportive confirmation for three methylene carbon group, one methyl carbon. The 1H NMR spectrum [1H NMR (200MHz, CDCl3): δ 1.26 (t, 3H, J = 7.33 Hz), 1.32 (t, 4H, J = 7.33 Hz), 1.58 (m, 3H), 2.84 (ABq, 2H, J = 15.6 Hz), 3.49 (bs, 1H), 4.16 (dd, 2H, J = 7.3, 14.2 Hz), 4.15 (s, 1H), 4.29 (dd, 1H, J = 6.9, 14.2 Hz) ppm, Fig 6S, Support Information] further supported the structure. For example, a triplet at δ 1.26 corresponds to the terminal -CH3, triplet at δ 1.32 showed the presence of -CH2 and the multiplet at 1.58 ppm can be assigned as those protons connected to the tertiary amine. ABq at 2.84 ppm can be attributed to the protons on the C6 carbon linked to phosphate group. Singlets at 3.49 and 4.15 can be interpreted as the hydrogen attached to a carbon next to an amine. The double doublets at 4.16 and 4.29 ppm can be seen as those

protons connected to hydroxyl groups. Given the perfect match of LC-MS and 13C and 1H NMR studies, one can speculate the structure as shown in figure. 2. However, further confirmation by x-ray crystallography and other spectroscopic techniques would be highly required before assigning the structure unambiguously.

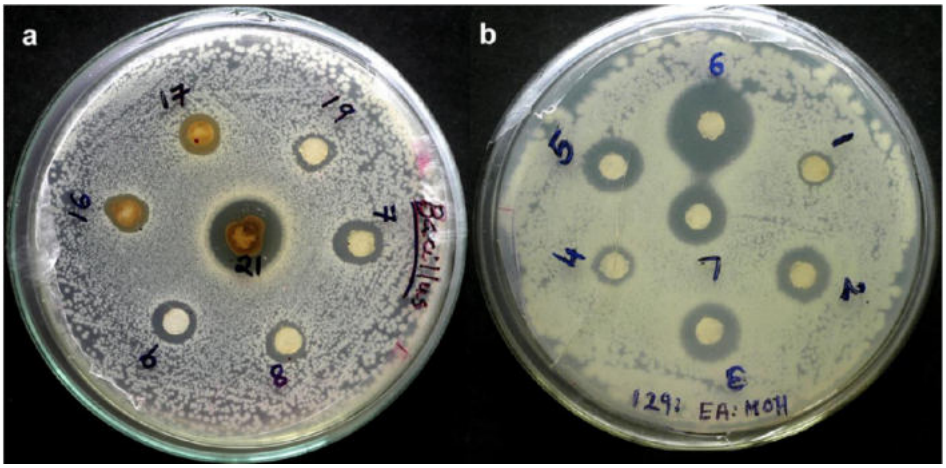

Figure. 1S: Antibacterial activities of fractions collected from- (a) first and (b) second silica gel column chromatography against *B. subtilis*

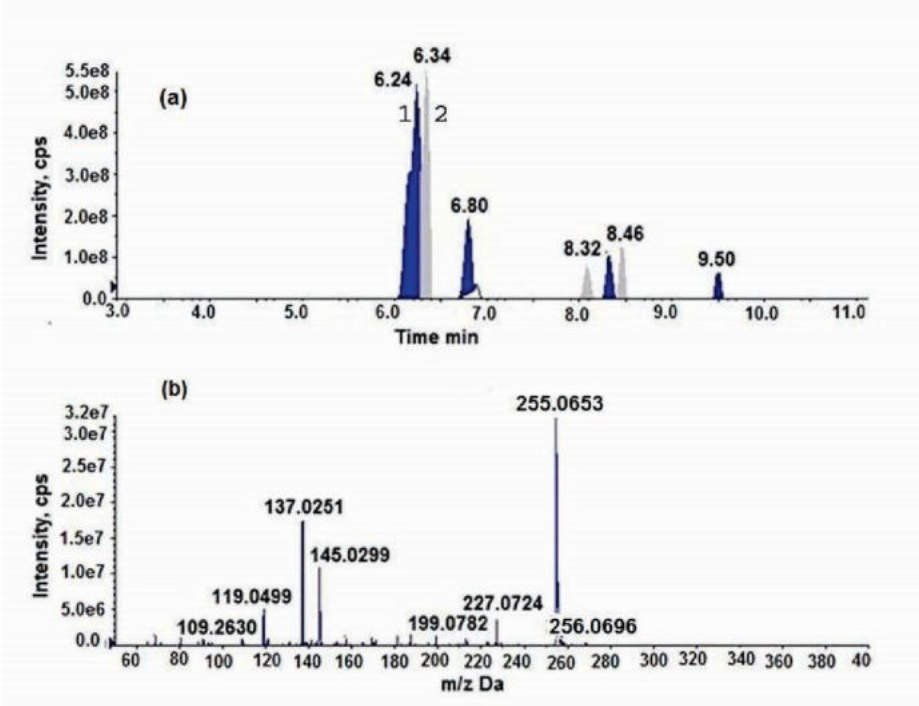

Figure. 2S: (a) Total chromatogram of fraction 6 (b) Accurate mass measurements of ions acquired by ESI-QqTOF-MS/MS for *m/z*=255.0653

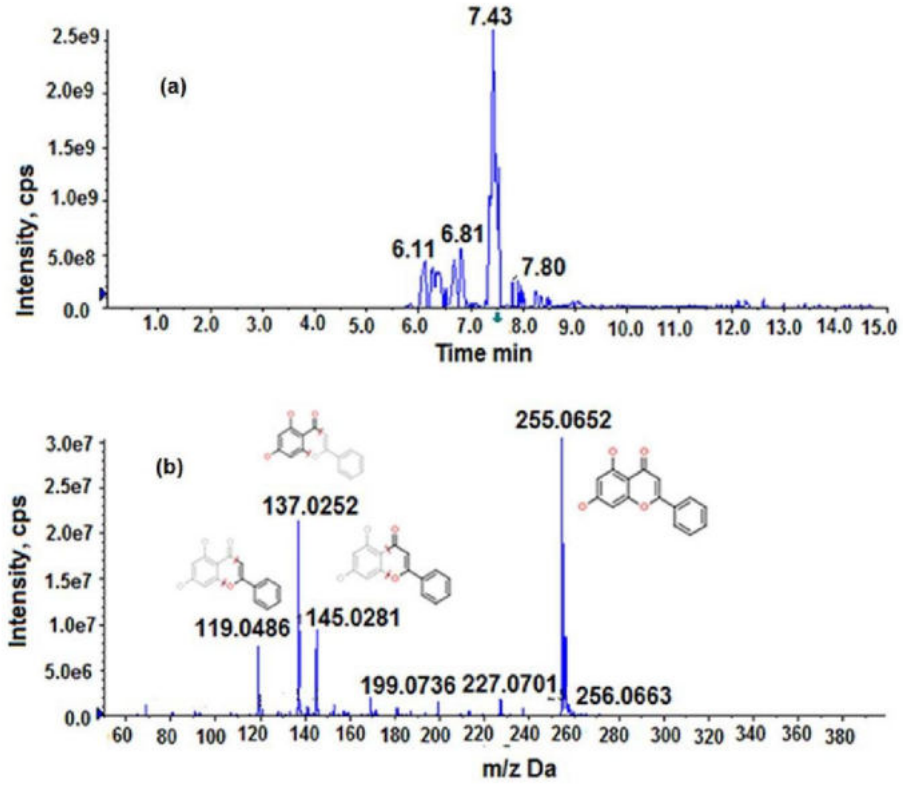

Figure. 3S: (a) Chromatogram of a standard solution of 7'4' dihydroxy flavone. (b) Fragmentation pathway of 7'4' dihydroxy flavones

To carry out a comprehensive study of species from genus *Trigonella*, 15 species were screened for the antimicrobial activity. Ten species showed antibacterial activity against *Staphylococcus aureus* (VRSA) resistant to gentamycin, kanamycin, roxithromycin, tetracycline, vancomycin and ampicillin to varying extent. Other clinical isolates like *Salmonella typhi* and *Escherichia coli* were inhibited by four and nine species, respectively. Three species showed anti-fungal activity against both *A. niger* and *F. solani. T. coerulescens* was the only species showing both antibacterial and antifungal activity. These results indicate that *Trigonella* species can be potential sources for the isolation of natural antimicrobial compound(s) from plants. Although antimicrobial activity from *T. foenum-graecum* has been reported earlier, to our knowledge, the antimicrobial activity in the other *Trigonella* species has been reported for the first time.

Antibacterial flavonoids are one of the largest classes of naturally-occurring polyphenolic compounds which are increasingly becoming the subject of biomedical research. However, most of these like chrysin, apigenin, vitexin, saponarin, apigenin, lucenin 2-O-glycoside and luteolin 7-O-glycoside acted selectively only towards certain gram-negative bacilli like Proteus vulgaris, Proteus mirabilis, Psuedomonas aeruginosa, E. coli, Klebsiella pneumoniae and Enterobacter cloacae and showed little or no activity against the gram-positive cocci like Staphylococcus aureus and Enterococcus faecalis [11]. Although antibacterial activity of 7'4' dihydroxy flavone was known previously [12] its prominent activity against gram positive bacteria like VRSA has been reported for the first time. It is possible that the synergistic action of 7'4' dihydroxy flavone and (2E)-3-{5-[4-(Ethoxycarbonyl)phenyl]furan-2-yl}prop-2-enoate) may have contributed to observed activity. A number of phenyl acrylate derivatives are known to exert excellent antibacterial activity due to the presence of phenyl and acryl group [13, 14].

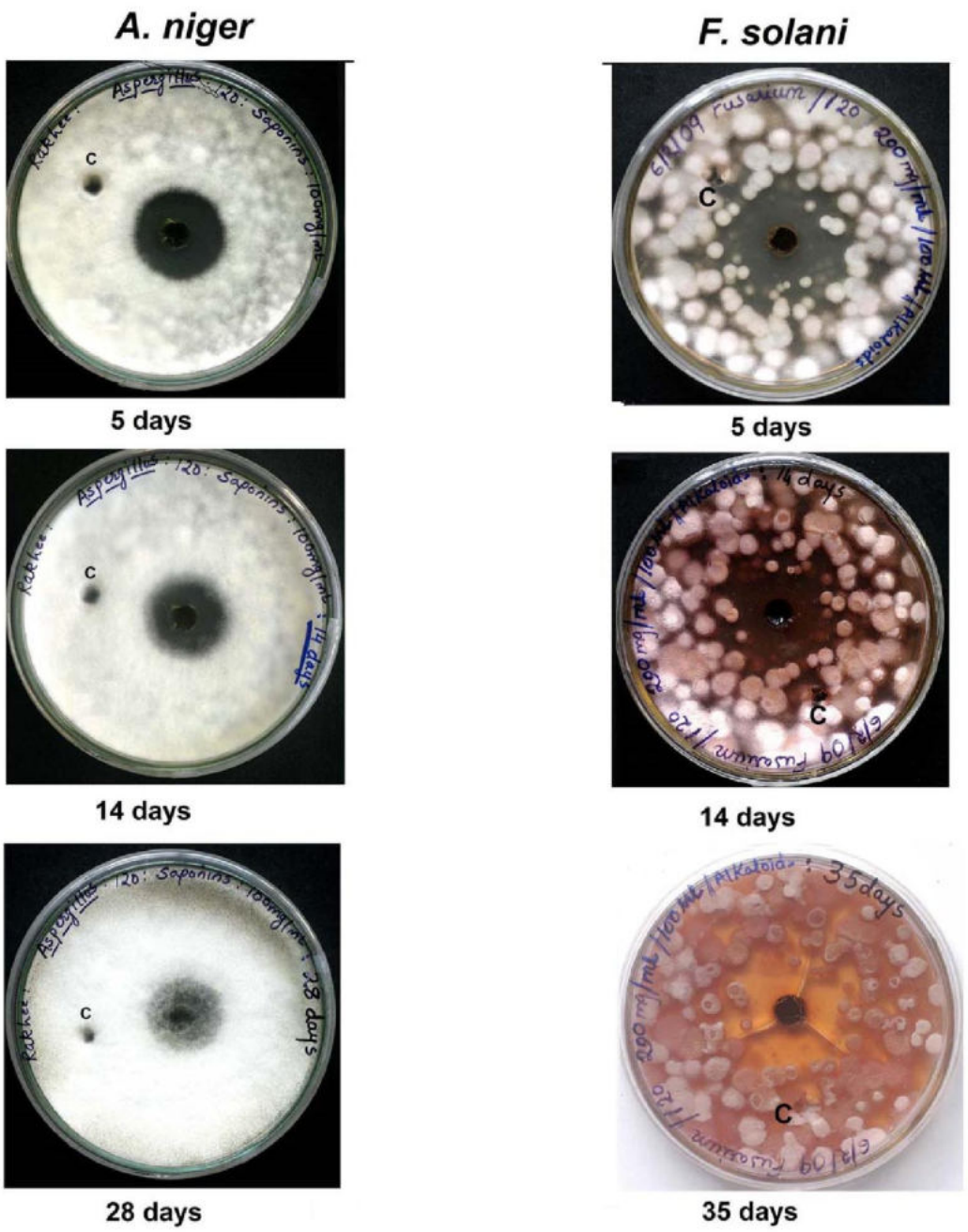

Figure 4S: Inhibition of *A. niger* by the saponin extract of *T. spicata.* C denotes DMSO control

Inhibition of *F. solani* by the sapogenin fracti of *T. spicata.* C denotes DMSO control

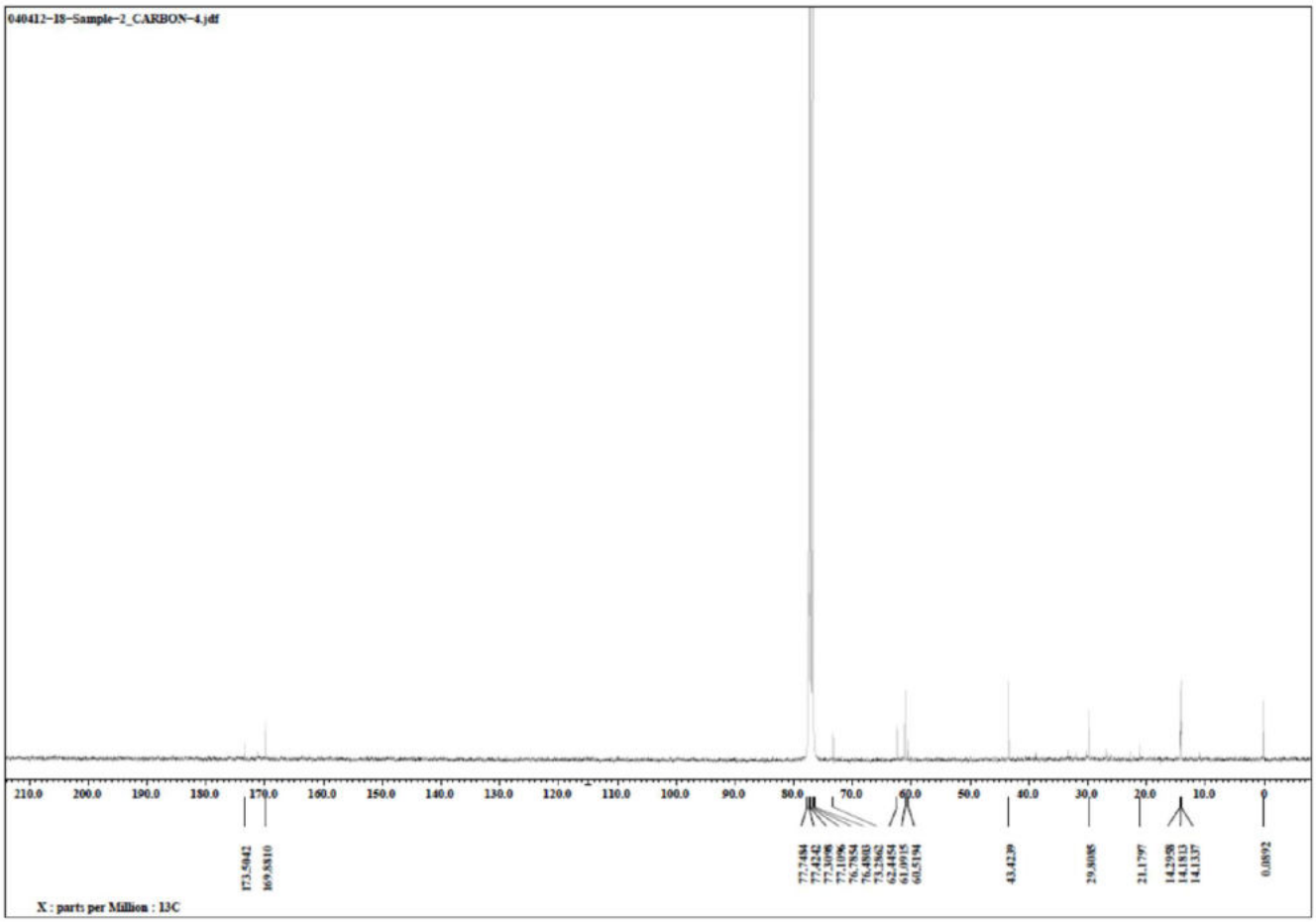

Figure. 5S-a: DEPT ^{13}C NMR analysis.

Antimicrobial, especially antifungal activities of many saponins have been reported [15]. The primary mode of action of saponins towards fungi involves the formation of complexes with membrane sterols, which results in pore formation and loss of membrane stability [16]. In general there are two main mechanisms of resistance of fungi to saponins. The first one involves intrinsic resistance by the virtue of membrane composition [17]. This probably may explain the high degree of resistance shown by F. solani to the saponin extract of T. spicata. The second major mechanism of resistance of fungi to saponins involves production of saponin detoxifying enzymes. Many phytopathogenic fungi like Stagonospora avenae, Gaeumannomyces graminis, Septoria lycopersici and Botrytis cinerea are known to produce saponin inhibitory enzymes [18]. The reduction in diameter of zone of inhibition with increasing incubation observed in A. niger may be due to the production of saponin detoxifying enzymes by the fungus over the time. The saponin fraction or the by-products of saponin detoxification may be the bioactive molecule(s) that lead to metabolic changes, which delays sporulation in A. niger. This opens the possibility of extracting sporulation inhibiting substance(s) from T. spicata.

Among alkaloids, polyhydroxylated piperidines are low molecular weight compounds where the nitrogen-containing six-member (piperidine) ring, is substituted with one or more hydroxyl groups. Many are naturally occurring structural analogues of 'true' sugars, called azasugars (also known as iminosugars) in which the ring oxygen atom is replaced by a nitrogen atom. Iminosugars constitute leads for the development of new therapeutic agents in many bacterial, fungal and viral diseases [19]. Similar to other azasugars, in the polyhydroxylated alkaloid isolated from *T. spicata,* the presence of multiple hydroxyl functionality (responsible for secondary interactions) as well as the shape of the iminosugar may be responsible for its inhibitory activity against a number of enzymes including glycosidases [20], glycosyltransferases [21], metalloproteinases [22] or nucleoside processing enzymes [23]. To our knowledge, this is the first report on the isolation of this polyhydroxylated alkaloid with selective antifungal activity against *F. solani* from a natural source like *T. spicata*.

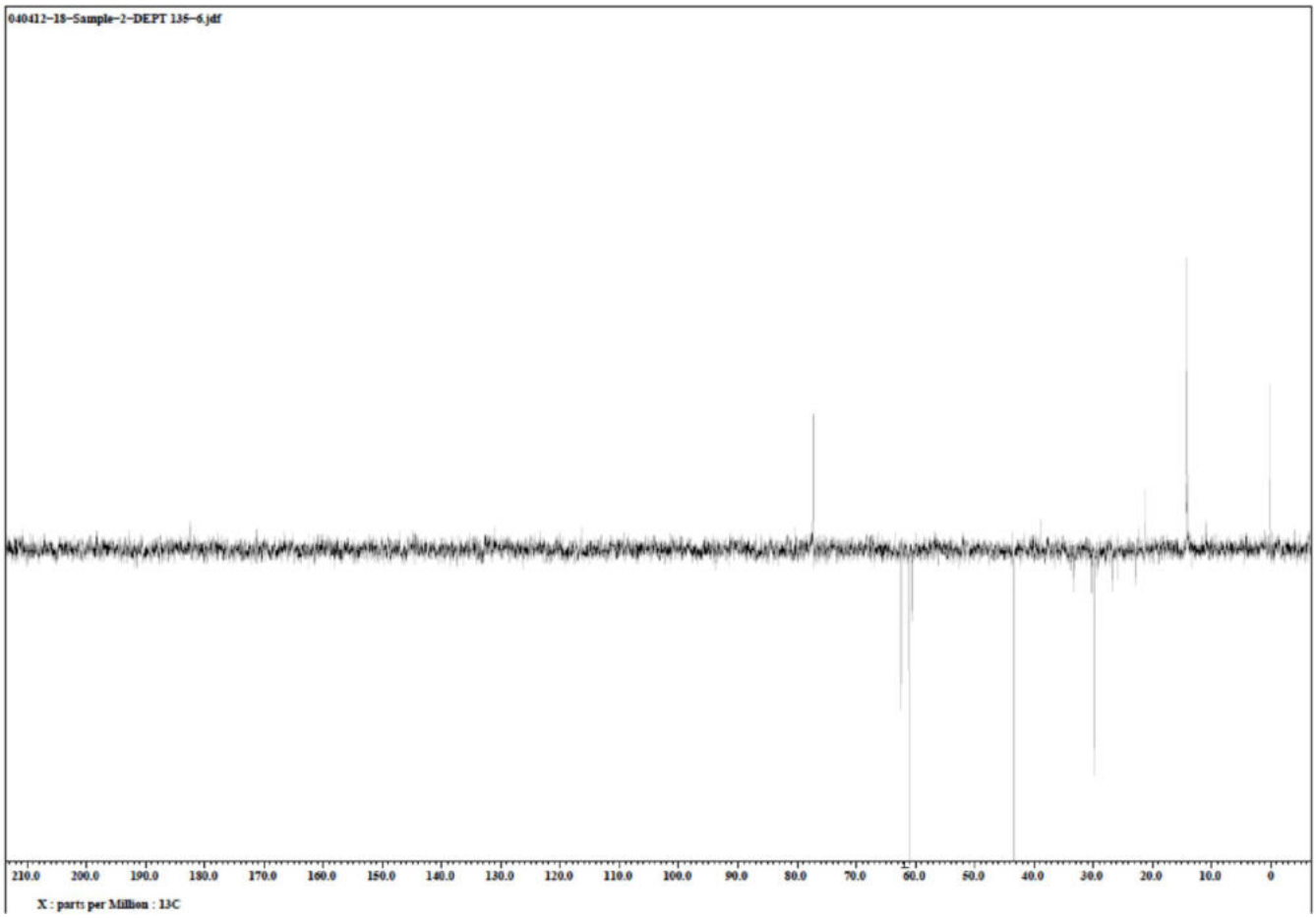

Figure. 5S-b: ^{13}C NMR analysis of the purified compound

In summary, laboratory screening of *Trigonella* species for antimicrobial activity could identify species with antibacterial and antifungal activity indicating that a more extensive chemical prospecting of *Trigonella* genus is likely to identify compounds with potential use in medicine and agriculture. Of particular interest is *T. coerulescens* which has shown both antibacterial and antifungal activity. A combination of 7'4' dihydroxy flavone and (2E)-3-{5-[4-(ethoxycarbonyl) phenyl] furan-2-yl}prop-2-enoate characterized from *T. suavissima* can be used to prepare potent pharmaceutical formulations along with other antibiotics to treat infections caused by multidrug resistant bacteria. Complete structural characterization and mode of action of the antifungal compound identified from *T. spicata* needs to be elucidated before considering it as a useful lead product for disease control.

Authors' Contribution

RD: Conceived the study, performed the experiments, compiled the results, and wrote the manuscript. DO: Performed LC-MS analysis. PK: Provided drug resistant clinical isolates and monitored the results for antibacterial activity. SKS: Provided fungal isolates and monitored the results for antifungal activity. KB: Analysed LC-MS results. DN: Monitored experiments and results related to fractionation and column chromatography. ST: Monitored the overall work, and gave critical inputs in the manuscript. SR: conceived the study, participated in its design and co-ordination and monitored the experiments and the results.

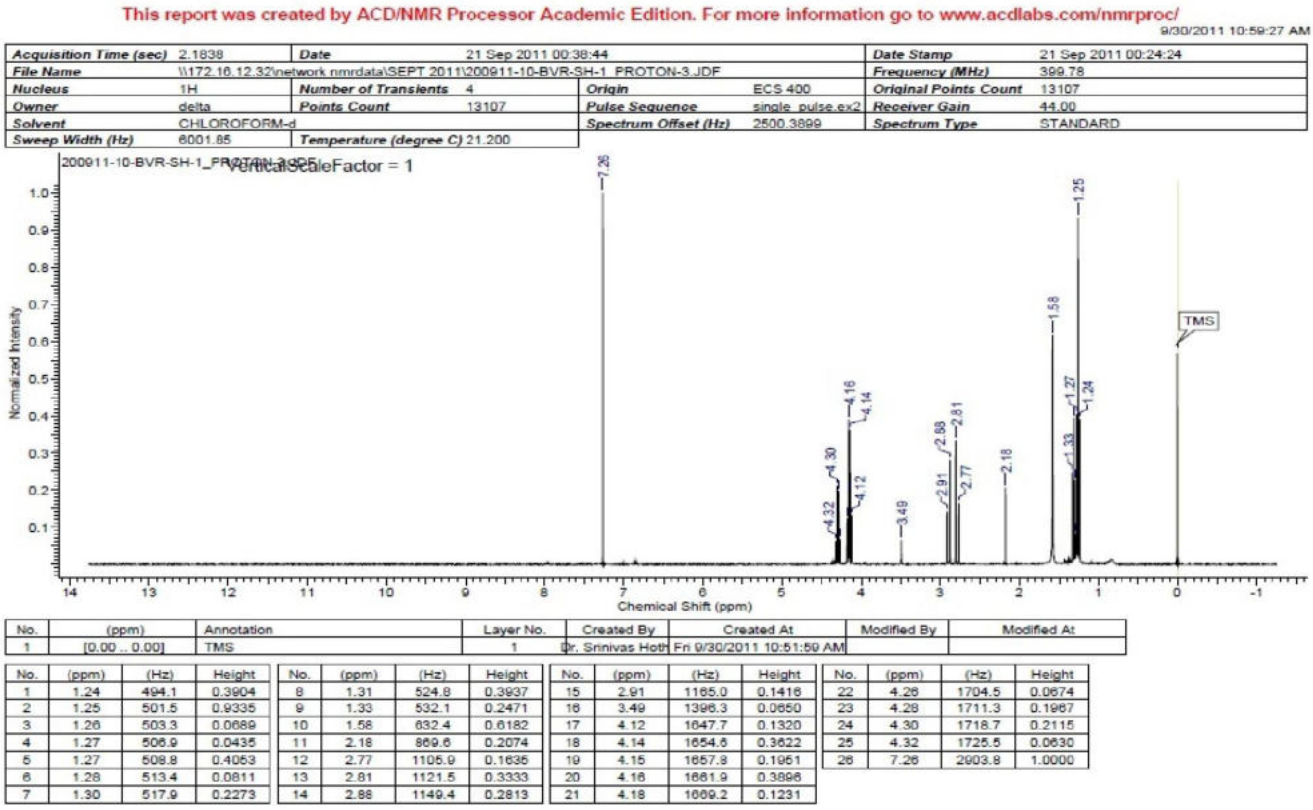

Figure. 6S: ^{1}H NMR spectrum of the purified compound

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