Identification of an antibacterial withanolide (dinoxin b) from leaf of *Datura inoxia* mill.

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A b s t r a c t

Natural medicines are gaining popularity due to the alarming incidence of bacterial resistance even in regions with improved healthcare systems. Hence, there is a distinct and constant need for isolating medicinally active compounds from different natural sources. Taking this into consideration, experiment was done in order to isolate and identify an antibacterial compound from *Datura inoxia* through bioassay and Mass spectroscopy technique. The ethanol, hexane and aqueous leaf extracts of *Datura inoxia* were screened for their antibacterial activity against *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli*. All extracts showed inhibitory effect against *Staphylococcus aureus* and *Escherichia coli,* while no inhibitory effect was found by aqueous and hexane extracts against *Pseudomonas aeruginosa*. Out of the three extracts, ethanolic extract was found more susceptible against *Staphylococcus aureus*. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the ethanolic extract was also determined. Crude ethanolic extract was further fractioned through column chromatography. All the fractions showed antibacterial activity against the tested strains. The most active fraction was further analysed by LC-ESI-MS and identified six major compounds as withanolides and their genins. Our findings confer the utility of *Datura inoxia* in developing a new class of antibiotics that might be useful for treating as well as controlling infectious diseases.

Keywords: *Datura inoxia*, antibacterial compound, column chromatography, LC-ESI-MS, Withanolide.

Introduction

Herbal medicine has been widely used as an integral part of primary health care in many countries [1]. According to the World Health Organization (WHO), as much as 80% of the population of developing countries depends on medicinal plants as their only affordable source of medication [2]. Although antibiotics provide the main basis for the treatment of many bacterial infections, but due to the alarming incidence of bacterial resistance to commercially available antibiotics, there is an urgent need for development of new antibiotic therapies [3]. However, despite the need for development of new antibiotic therapies, the newly approved drugs are continuously declining [4,5]. Hence, investigations on plants as a source of human disease management [6,7] and efforts to promote novel technologies to intensify research in combating drug resistant microbes are increasing rapidly [8,9]. During the past 40 years, many biologically active novel compounds have been isolated from different plants and marine organisms, some of which could be utilized for potential drug development [10,11].

Datura inoxia is one of the medicinal plant known to exhibit potential antibacterial activity and has been widely used as phytomedicine to cure various diseases [12-16]. It is a vespertine flowering plants belonging to the family Solanaceae (Nightshade family).The species was first described by English botanist Philip Miller in 1768 and commonly called as thorn-apple, moonflower, sacred *Datura* etc. All *Datura* plants were commonly known to contain tropane alkaloids such as scopolamine, hyoscyamine, and atropine, primarily in their seeds and flowers [17]. Although there are many scientists who have studied antibacterial properties of different species of *Datura* against different microorganisms [18,19,20,21], but very few studies has been found on characterization of its antibacterial compound [22]. Steroidal (5[1], 7[1] dimethyl 6[1]– hydroxyl 3[1], phenyl 3 α - amine ß – ynesitosterol) and β-carboline (1, 7 dihydroxy-1- methyl 6, 8 dimethoxy ß-carboline) alkaloids are the two antibacterial compounds isolated from *Datura metel* leaf [23,24]. However, no such attempt has ever been made to identify and isolate compounds responsible for unleashing the antibacterial activity of *Datura inoxia*. Hence, in this study an attempt was made to isolate and characterize an antibacterial compound from *Datura*

inoxia. The study was made possible by collaboration with three institutions i.e., Amity Institute of Biotechnology, Amity University Uttar Pradesh, Lucknow; Department of Microbiology, Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow and Sophisticated Analytical Instrument Facility (SAIF), CSIR-Central Drug Research Institute, Lucknow.

Material and Method

Sample collection and preparation of plant extracts

Datura inoxia were collected in April, 2014 (hot dry climate in northern India), from nearby area of Amity University Lucknow Campus. The plant leaves were washed thoroughly with tap water followed by distilled water to remove the dust particles and allowed to air dry at room temperature on laboratory bench. The dried plant leaves were pulverized with the help of liquid nitrogen and stored at -20°C till further use. For preparing extract, 10 gm of the pulverized sample was mixed with 100 ml hexane (Merck, India), ethanol and distilled water. The mixture was macerated in mortar and pestle and kept for 48 hrs at room temperature to ensure maximum metabolite extraction. The extract obtained was filtered and concentrated. The final concentration was maintained as 100mg/ml by redissolving the crude extracts in 10% dimethylsulfoxide [25] for bioassay analysis.

Test organisms and bacterial inoculum preparation

The bacteria used in this study included *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922. These strains were obtained from HiMedia Laboratory Pvt. Ltd. and maintained by sub culturing on blood agar base No. 2 (HiMedia Laboratory Pvt. Ltd.) and macconkey's agar (HiMedia Laboratory Pvt. Ltd.) plates prior to use. To prepare bacterial inoculum, pure isolates of bacteria were diluted in test tubes containing 0.9% normal saline solution followed by 15min incubation in an ambient air incubator (Thermo Scientific, Thermo electron LED GmbH) so as to meet the 0.5 McFarland turbidity standard (TULIP DIAGNOSTICS (P) LTD.) which is equivalent to 1.5×10^8CFU/ml before applying onto the plates. For MIC testing, standardized inoculum should have a desired concentration of 5×10^5CFU/ml which is prepared by delivering 1ml of the adjusted suspension (1.5×10^8CFU/ml) in 25ml of water [26].

Antibacterial activity assay of crude extracts

The agar gel diffusion [27] was carried out on Muller Hinton Agar No. 2 (HiMedia Laboratory Pvt. Ltd.) plates to assess antibacterial activity assay.The media were prepared as per the supplier's instructions and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min (High-pressure steam sterilizer ES-315, TOMY KOGYO CO., LTD). The plates were then inoculated with the diluted bacterial suspensions using sterile swab sticks dipped in it. Wells of 6 mm size were dug with the help of a sterile cork borer on these plates. In each wells, 100 µl of the hexane, ethanol and aqueous extracts were loaded taking care not to allow spillage of the solutions onto the surface of the agar. The culture plates were allowed to stand on

the laboratory bench for 1 h to allow proper diffusion of these extracts before being incubated at 37°c for 24 h. After incubation period, the antibacterial activity was calculated in terms of zone of inhibition (diameter in mm). Determinations were done in triplicates and mean of all values was taken. Standard antibiotic (Gentamicin) was used as positive control, while DMSO (10%) was used as negative control to compare the antibacterial activity of the extracts.

Minimum inhibitory concentration (MIC)

MIC is defined as the lowest concentration which results in the reduction of inoculums viability [28]. The MIC of the crude extract (ethanolic) of *Datura inoxia* was determined by broth macro dilution method [26,29]. The growth media, nutrient broth (HiMedia Laboratory Pvt. Ltd.) were prepared as per the supplier's instructions and sterilized by autoclaving. The sterilized media was allowed to cool to 50°C and 2ml was added to each labeled test tubes as per concentrations taken. Serial dilution of the plant extract (100mg/ml) was done by transferring 2ml from each test tube to obtain two fold dilutions (100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml, 3.125mg/ml 1.56mg/ml). The mixture of the media and the test crude extract were thoroughly mixed and 100µl of the test organism (*Staphylococcus aureus*) having desired inoculum concentration of 5×10^5 were added to all the test tubes. The tubes were then incubated at 37°c for 24 h and MIC were expressed as the highest dilution which inhibited growth, determined by lack of opacity in the tubes. Two blank nutrient broth tubes, with bacterial inoculation were used as the growth controls of which one is kept overnight at 4°C in a refrigerator for determination of complete inhibition, as very faint turbidity may be given by the inoculums itself. Also a nutrient broth tube without bacterial inoculation was used as the sterility control. 10% DMSO was used as negative control while broth containing standard drug (gentamicin) was used as positive control.

Minimum bactericidal concentration (MBC)

The MBC is the lowest concentration of an antibacterial agent required to kill a particular bacteria [30]. It was determined by sub culturing from each tubes of MIC showing no apparent growth. Before being subcultured, the tubes were gently mixed by flushing them with a sterile pipette. Each aliquot was then spread over the fresh Muller-Hinton Agar plates by lawning technique. The MBC lawned plates were incubated at 37°C for 24 h. After the incubation period, the lowest concentrations of the extract that did not produce any bacterial growth on the solid medium were regarded as MBC values for this extract [31]. This observation was matched with the MIC test tube that did not show evidence of growth after 48 h of incubation.

Fractionation of extract through column chromatography

Crude ethanolic extract of *Datura inoxia* was further fractioned through column chromatography using a single solvent throughout the process. Column was filled with silica gel (60-120 mesh) which acts as a stationary phase and packed by passing eluant (ethanol)

acting as a mobile phase. After sample was added (ratio stationary phase to crude extract 5:1), each fraction was collected by passing eluant from top of the column until silica gel appears colorless. Antibacterial activity assay was performed for each fraction through the same procedure as for crude extract.

Ultra performance liquid chromatography electrospray ionization mass spectrometry

The LC-MS analysis of active fraction of ethanolic extract was performed on Waters TQD triple quadrupole mass spectrometer (USA). It was equipped with waters, H-Class Acquity UPLC system and electrospray ionization source. To achieve separation of analytes used waters BEH C-18 50 x 2.1 mm, 1.7um and 1 µl of sample was injected through autosampler into UPLC flow. For the separation of individual compounds, the mobile phase used (A) acetonitrile and (B) 5 mM ammonium acetate in 95% water. An isocratic elution was performed at the flow rate of 0.250 ml min^{-1} as 40:60 for 10 min. Nitrogen was used as the nebulizing and drying gas at flow rates of 50 and 650 L h^{-1} respectively. The ESI source potentials were capillary voltage 3.5 kV; cone potential at 30 V for every experiments. Source and desolvation temperature was at 120 and 350 ^{0}C respectively. The mass analyzer was scanned between 150 to 1200 Th in 0.6 sec. Data acquisition and processing was carried out using MassLynx V4.1 SCN 714 software. The spectra were accumulated from the top of EIC (Extracted ion chromatogram) peak.

Results and Discussion

In the present study, antibacterial activity of hexane, ethanol and aqueous extracts of *Datura inoxia* leaf were observed using agar well diffusion method by measuring the diameter of the zone of inhibition (Table 1). Out of three extracts of the plant, ethanolic extract showed significant (25mm) antibacterial activity against *Staphylococcus aureus*, while moderate activity against *Pseudomonas aeruginosa* (17mm) and *Escherichia coli*(16mm). Hexane extract of *Datura inoxia* showed mild antibacterial activity against *Escherichia coli* (10mm) and *Staphylococcus aureus* (15mm) but found ineffective against *Pseudomonas aeruginosa.* Aqueous extract showed mild antibacterial activity against *Staphylococcus aureus* (14mm), *Escherichia coli* (12mm) respectively and no activity against *Pseudomonas aeruginosa*. All the isolates were susceptible to standard antibiotic tested with appreciable zone of inhibition measured as- for *Pseudomonas aeruginosa*-28mm and *Staphylococcus aureus*- 30mm and *E.coli*-28mm, but the negative control dimethyl sulfoxide (DMSO) was found ineffective against all the tested strains. The extracts were found to be less potent than the standard antibiotic. The differences between the activities of the extracts and the standard drug may be due to the mixture of compounds present in the extract compared to the pure compound contained in the standard antibiotics [32].

Table 1: Zone of inhibition of crude ethanolic, aqueous and hexane extracts of *Daturainoxia* against *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus.*

Test organisms	Zone of Inhibition (mm)				DMSO (10%)
	Standard antibiotic	Extracts (100mg/ml)			
	Gentamicin (85mg)	Hexane	Ethanol	Aqueous	
Pseudomonas aeruginosa	28	-	17	-	-
Staphylococcus aureus	30	15	25	14	-
Escherichia coli	28	10	16	12	-

Avg. ZOI :< 15mm (mild); <25mm (moderate); >25mm (strong)
Values are average mean of the triplicate samples

Table 2: MIC of the *Datura inoxia* leaf ethanolic extract against *Staphylococcus aureus*.

Tested organism	Concentration (mg/ml)						
	100	50	25	12.5	6.25	3.125	1.56
Staphylococcus aureus	+	+	+	+	_	_	_

(+): Clear broth, indicating no growth, (-): Turbidity in the broth, indicating growth

Table 3: MBC of the *Datura inoxia* leaf ethanolic extract against *Staphylococcus aureus*.

Test organisms	Concentration (mg/ml)			
	100	50	25	12.5
Staphylococcus aureus	+	+	+	+

(+): No colonies on petriplates observed.

Table 4: Zone of inhibition of purified ethanolic extract of *Datura inoxia*

Test organisms	ZOI of Purified fractions						
	1	2	3	4	5	6	7
Pseudomonas aeruginosa	15	12	16	19	13	13	16
Staphylococcus aureus	21	24	27	28	17	11	13
Escherichia coli	12	14	13	11	12	18	10

Avg. ZOI :< 15mm (mild); <25mm (moderate); >25mm (strong)
Values are average mean of the triplicate samples

Table 5: Mass spectrometry data of identified withanolides and their aglycone

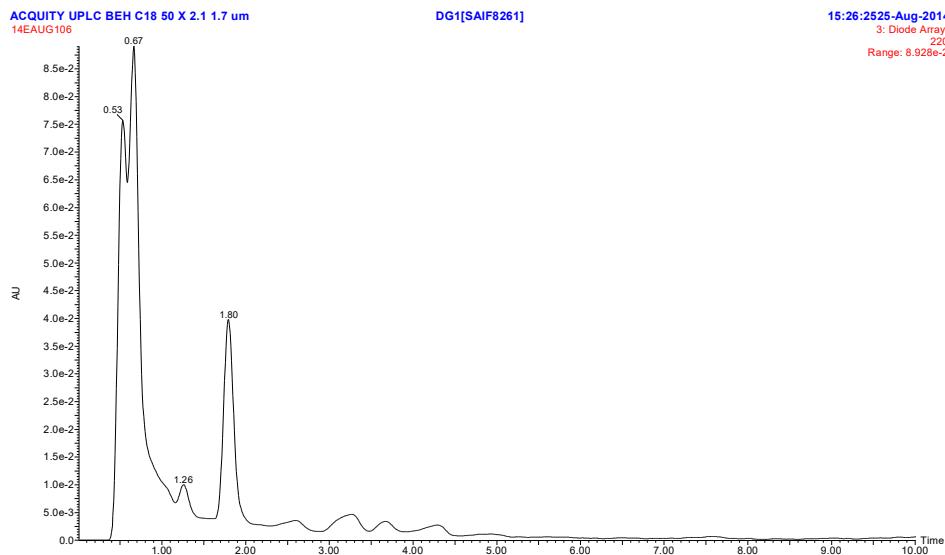| S.No.	RT	M.W.	ESI (+)			Fragments		
			$[M+H]^+$	$[M+NH_4]^+$	$[M+Na]^+$	Aglycone unit ion (*m/z*)	Others characteristic ions (*m/z*)	Glycone unit loss (Da)
1	1.25	632	633	-	-	471	453, 435	162
2	1.79	616	-	634	639	455	437	162
3	2.61	454	455	-	-	455	435	-
4	3.20	616	617	634	639	455	437	162
5	3.66	452	453	470	-	453	435, 417	-
6	7.44	452	453	470	475	452	435, 417	-

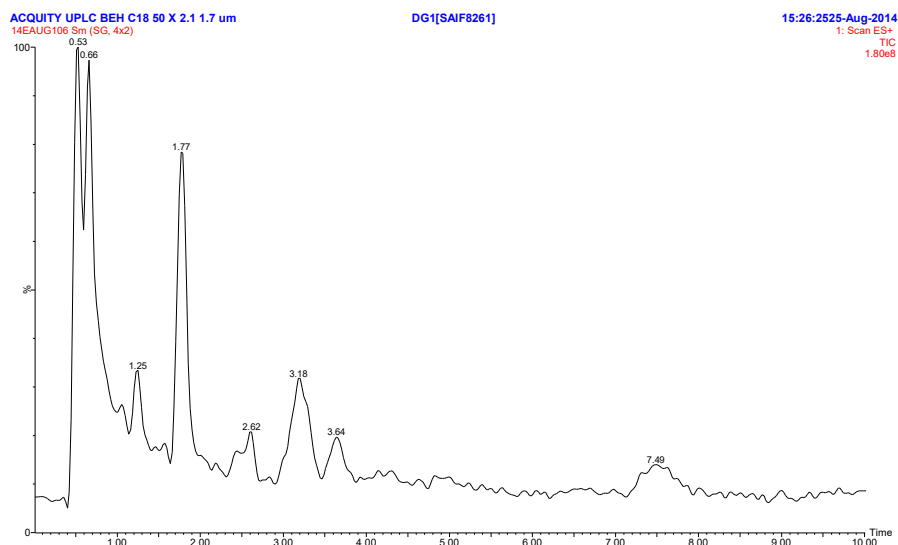**Figure 1: HPLC chromatogram of active purified ethanolic fraction**

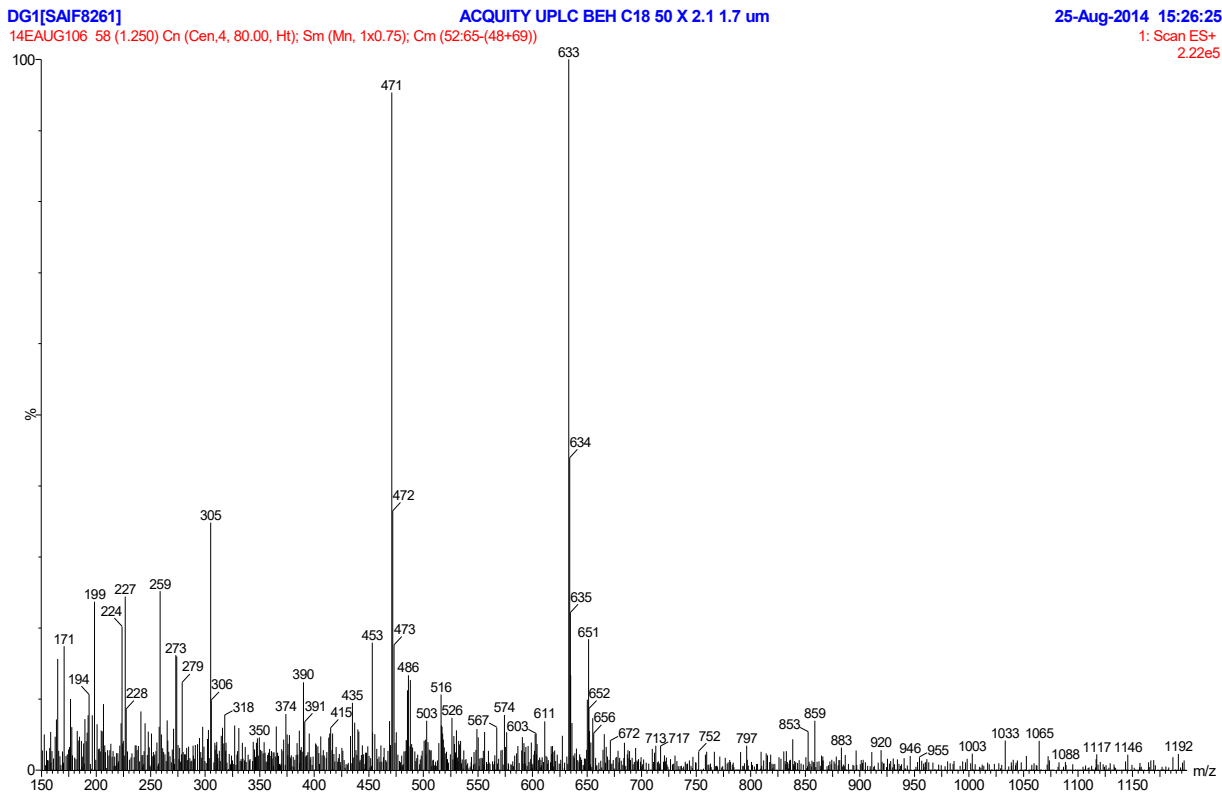**Figure 2: Mass chromatogram of active purified ethanolic fraction**

Figure 3 (a)   LC-MS spectra of component 1 identified from ethanolic extract

Figure 3 (b)   LC-MS spectra of component 2 identified from ethanolic extract

Figure 3 (c) LC-MS spectra of component 3 identified from ethanolic extract

Figure 3 (d) LC-MS spectra of component 4 identified from ethanolic extract
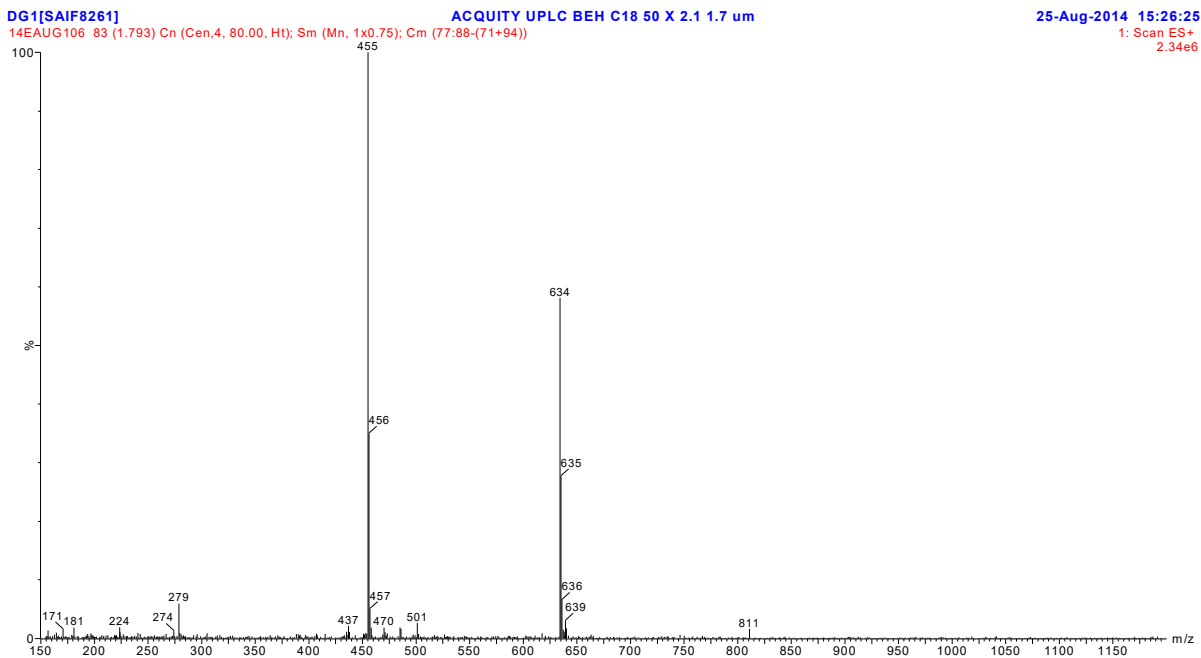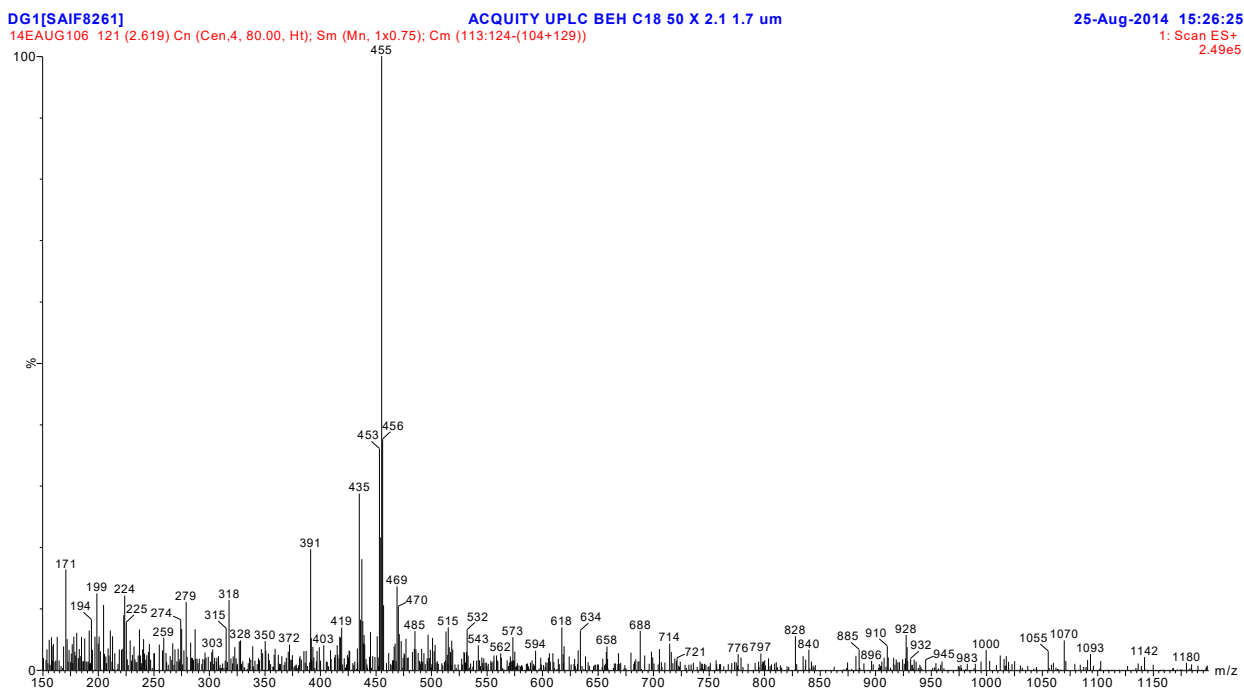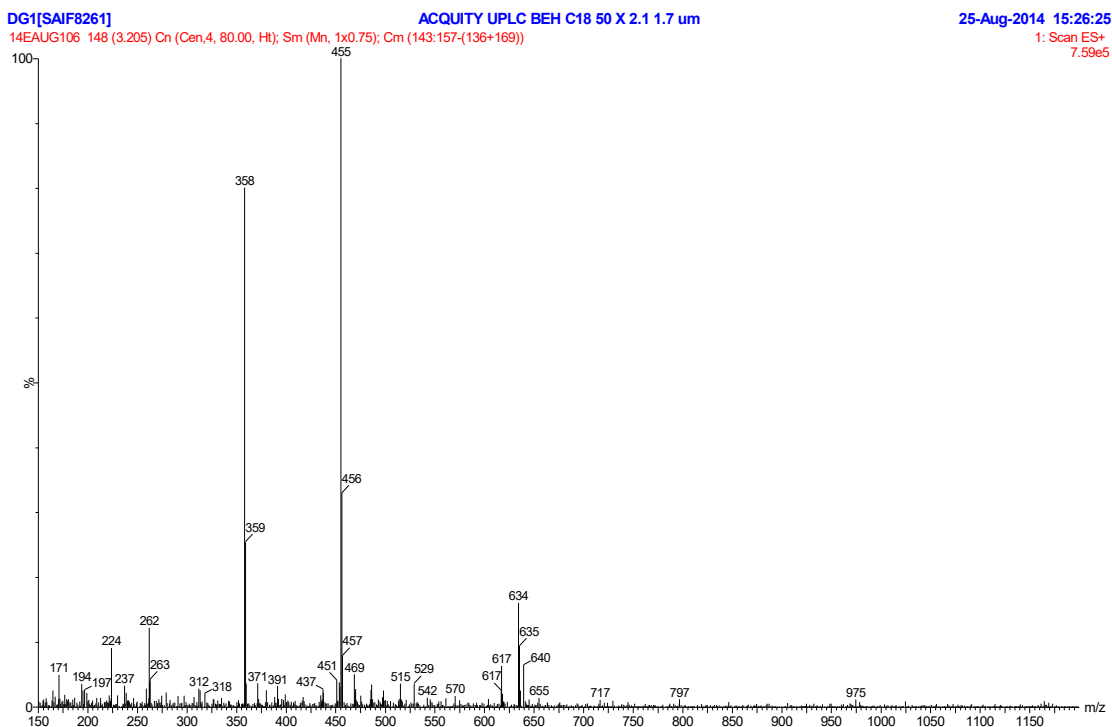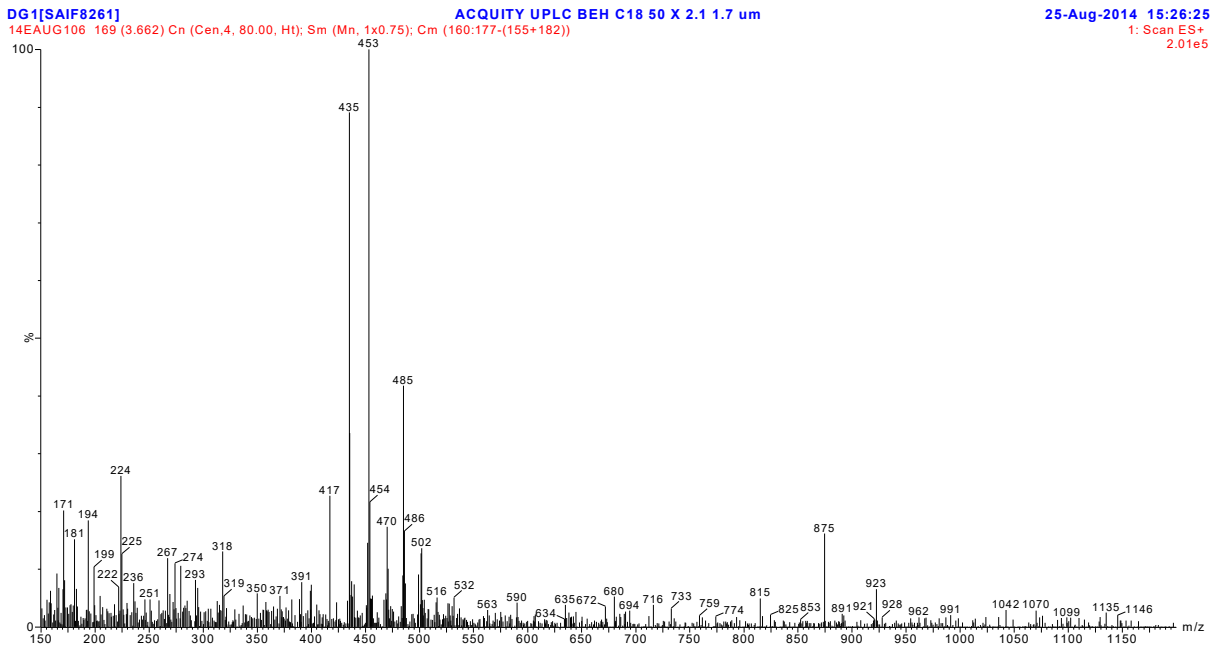
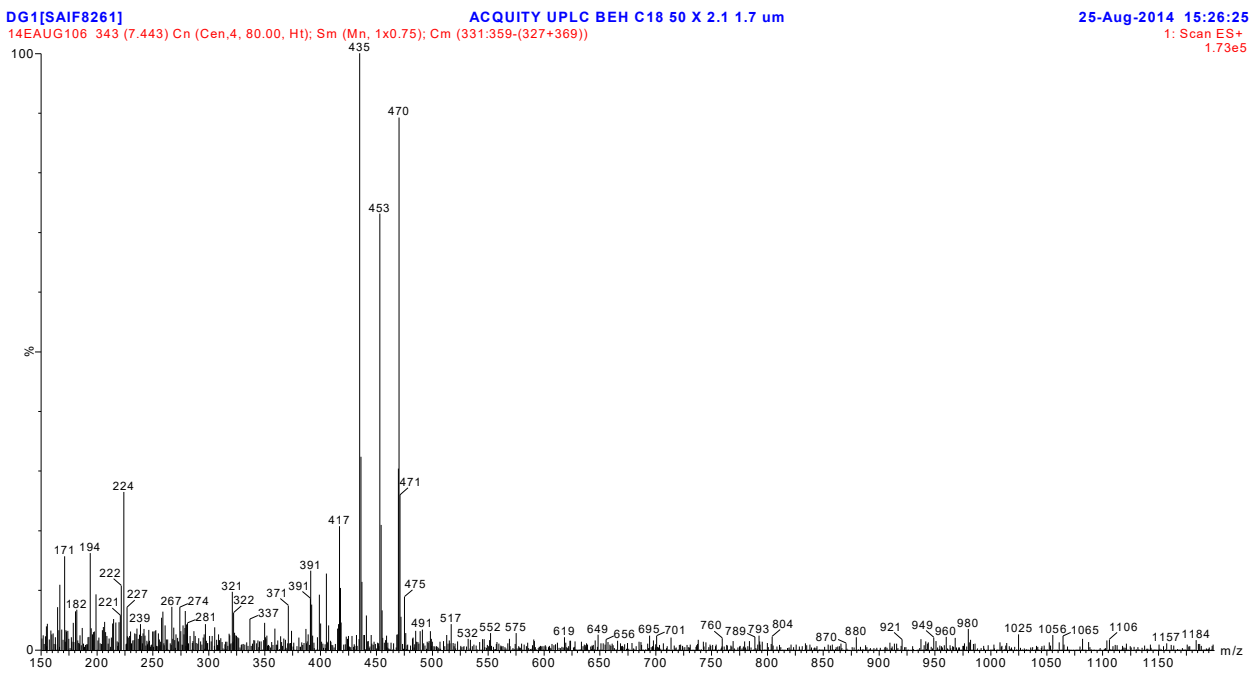**Figure 3 (e)   LC-MS spectra of component 5 identified from ethanolic extract**

Figure 3 (f)   LC-MS spectra of component 6 identified from ethanolic extract

Figure 3 (a-f): LC-MS spectra of different components identified from ethanolic extract
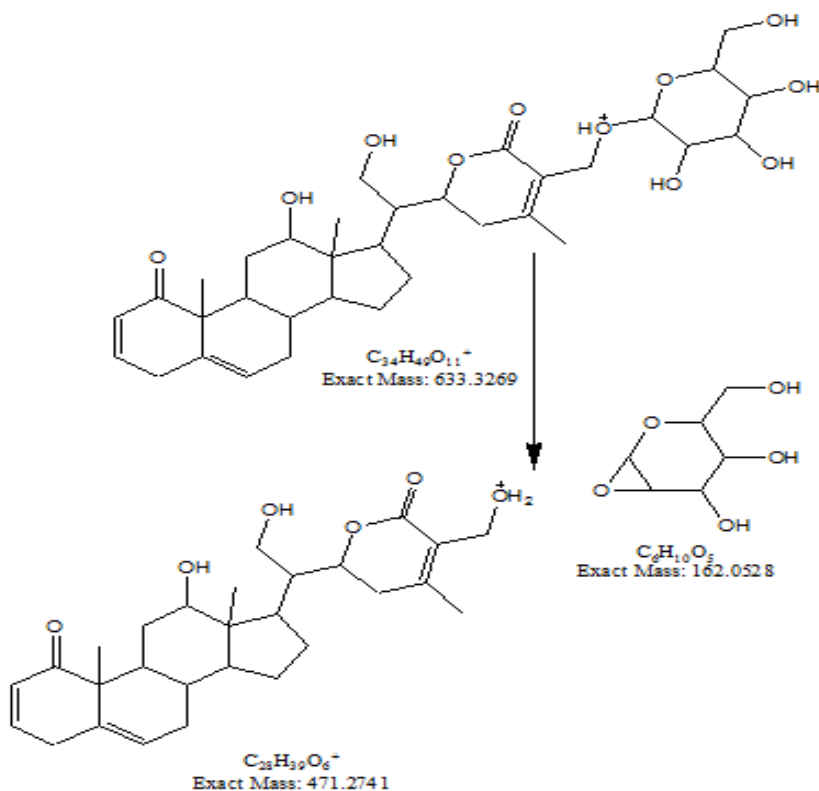

Figure 4: Cleavage of O-glycoside bonding identified withanolide (Dinoxin B)

Table 2 and 3 shows the MIC and MBC values observed against *Staphylococcus aureus* (12.5mg/ml), exerted by the ethanolic leaf extract of *Datura inoxia*. A similar type of study was found with leaf extract of *Andrographis paniculata* [33]. MBC assay result confirms the data of agar well diffusion assay (Table 1) and the MIC determination assay (Table 2). These results further confirmed that *Datura inoxia* leaf ethanolic extract is the most potent extract among all the tested extracts in present experiment.

Ethanolic extract of the plant was selected for further fractionation and purification due to its strong antibacterial activity in present experiment. From column chromatography seven fractions were collected. Different purified fractions showed mild to significant antibacterial activity against the tested strains. Inhibitory effects of different fractions are shown as zone of inhibition against Pseudomonas aeruginosa, Staphylococcus aureus and Escherichia coli (Table 4). It has been observed that fraction 4 was most effective

(broad spectrum) against Staphylococcus aureus & Pseudomonas aeruginosa. It was also observed that the purified fractions showed greater antibacterial activity than the crude extracts. This may be due to compounds become more active in purified state and may be acting synergistically to produce good antibacterial effects [34].

It has been observed from the study that gram-positive bacteria were more susceptible in both crude as well as in purified extract (Table 1 and 4). The greater susceptibility of gram-positive bacteria has been previously reported for South American [35] African [36] and Australian [37] plant extracts. Susceptibility differences between Gram-positive and Gram-negative bacteria may be due to cell wall structural differences between the two and/or in their genetic contents [38] Gram-positive bacteria have an outer peptidoglycan layer which is not an effective permeability barrier. The cell walls of Gram-negative organisms are more complex in lay out than the Gram-positive ones acting as a diffusion barrier and making them

less susceptible to the antimicrobial agents than Gram-positive bacteria [39-41]. Other studies have also shown Gram-negative bacteria to be less susceptible to plant extracts than Gram-positive bacteria [42,43].

Further analysis of most active fraction 4 (Table 4) of ethanolic extract was carried out by LC-MS (Figure 1, 2 and 3). The fraction was observed as mixture of six major phytoconstituents [Table 5; Figure 3(a-f)]. From the literature review [44] and characteristic mass spectrums, these compounds were identified as withanolide and their aglycone. The phytoconstitute1 eluted Rt 1.25 min was showed [M+H- 162] ion m/z 471, indicated the loss of haxose sugar unit and detected as aglycone unit. It can be predicted from structure Dinoxin B (previously reported data of withanolide) by cleavage of O-gycosidic bond (Figure 4). Similarly, the compounds 2 and 4 having same fragmentation characteristic as 1 indicated same structure connectivity and expected as isomer of each others. Apart from this, compounds 3, 5 and 6 were identified as genin units of withanolides.

Withanolides are a group of at least 300 naturally occurring chemical compounds acting as secondary metabolites. They occur mainly in genera of the Nightshade family. They constitutes a large family of plant steroids and steroid glycosides typified by a fused tetracyclic cholestane core and a side-chain unsaturated δ-lactone [45]. Within the genus Datura, withanolides have been isolated from several species, reports on this class have appeared for D. fastuosa [46], D. ferox [47,48], D. inoxia [49,50], D. metel [51,52], D. quercifolia [51,52] and D. stramonium [51,52]. In all cases, the withanolides were obtained from leaves but in one case the flowers [44]. Kagale et al. in 2004 [53] found withanolide as the most abundant compound from extracts of D. metel leaves which inhibited the growth of plant pathogens. Whereas, withanolides from D. metel flowers were found cytotoxic against three human cancer cell lines [52].

Conclusion

The present research work evidently showed that the ethanolic extract of Datura inoxia was effective against all the tested strains, showing its broad spectrum antibacterial activity. This extract was found to contain Dinoxin B, withanolide as an active compound. This compound can be considered as a candidate drug (antibacterial) in appropriate concentrations, recommended for therapeutic purpose. It may be considered that active compounds obtained from natural sources have complex structure, thus have lesser cases of bacterial resistance. Through this research paper, we are the first to report, withanolide (Dinoxin B) as an antibacterial compound from Datura inoxia. There is a need of further research on structure-activity relationship (SAR) of compound to make it more valuable for pharmaceutical industries.

Authors' contributions

This work was carried out in collaboration between all authors. Each author has participated sufficiently in the work to take public responsibility for appropriate portions of the content. All authors read and approved the final manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

References

[1]. Akinyemi KO, Oladapo O, Okwara CE, Ibe C, Fasure KA. Screening of crude extracts of six medicinal plants used in South-West Nigeria unorthodox medicine for antimethicilin resistant-*Staphylococcus aureus* activity. BMC Compl Alt Med. 2005; 5: 5-8.

[2]. Mahady GB. Global harmonization of herbal health claims. J Nutr. 2001; 131: 1120S-3S.

[3]. Austan DJ, Kristinsson KG, Anderson RM. The relationship between the volume of antimicrobial consumption in human communities and the frequency of resistance. Tro J Phar R. 1999; 3(2): 225-228.

[4]. Bachi BB. Resistance mechanisms of Gram positive bacteria. J Med Microb. 2002; 292: 27-35.

[5]. Nagi AAH, Mashan NI, Shamsudin MN, Mohamad H, Vairappan CS. Antibacterial activity of marine source extracts against multidrug resistance organisms. Am J Pharm Toxicol. 2010; 5: 95-102.

[6]. Paul C, Arnold JV, Dirk VB, Louis M. Anti-infective potential of natural products: How to develop a stronger in vitro 'proof-of-concept'. J Ethnopharmacol. 2006; 106: 290-302.

[7]. Abutbul S, Golan-Goldhirsh A, Barazani O, Ofir R, Zilberg D. Screening of desert plants for use against bacterial pathogens in fish. Isr J Aquacult Bamidgeh. 2005; 57: 71-80.

[8]. Finch R, Hunter PA. Antibiotic resistance-action to promote new technologies. J Antimicrob Chemother. 2006, 58(suppl 1): 3-22.

[9]. Ariyo AO. Antimicrobial activity of the indigenous liverwort, Riccia nigerica Jones, from Southwestern Nigeria. Evansia. 2011; 28(2): 43-48.

[10]. Gerald B. Biologically active compounds from marine organisms. Phytother Res. 2001; 15: 89-94.

[11]. Houghton PJ. Old yet new-pharmaceuticals from plants. J Chem Educ. 2001; 78: 175.

[12]. Duke JA, Ayensu ES. Medicinal plants of China. Houghton Mifflin China. 1985: 90 – 91.

[13]. Ali M, Shuab M. Characterization of the chemical constituents of *Datura metel* Linn. Ind J Pharm Sci. 1996; 5(6): 243 - 245.

[14]. Dabur R, Ali M, Sigh H, Gupta J, Sharma G. A novel antifungal pyrole derivative from *Datura metel*. Pharmazie CODEN Pharlet. 2004; 59: 568 - 570.

[15]. Chopra RN, Nayar SL, Chopra LC. Glossary in Indian medicinal plants. Council of Scientific and Industrial Research: New Delhi; 1968. p. 121-124.

[16]. Chopra RN, Nayar SL, Chopra LC. Glossary of Indian medicinal plants. Council of Scientific and Industrial Research: New Dehli; 1986. p. 238 - 240.

[17]. Preissel U, Preissel HG. *Brugmansia and Datura:* Angel's Trumpets and Thorn Apples. Buffalo, NY: Firefly Books; 2002. p. 106-129.

[18]. Mathur P, Singh A, Srivastava V, Singh D, Mishra Y. Antimicrobial activity of indigenous wildly growing plants: Potential source of green antibiotics. Afr J Microbiol Res. 2013; 7(29): 3807-3815.

[19]. Jamdhade M, Survase S, Kare M, Bhuktar A. Antibacterial activity of genus *Datura L.* in Marathwada. Maharashtra J phytol. 2010; 2(12): 42-45.

[20]. Bharathi B, Sharmila devi R, Daniel G. Studies on antibacterial activity and phytochemical analysis of *Datura metel* L against bacterial pathogens associated with HIV. J Advanced Biotech. 2010; 10(3): 21-25.

[21]. Sakthi S, Saranraj P, Geetha M. Antibacterial evaluation and phytochemical screening of *Datura metel* leaf extracts against bacterial pathogens. Int J Pharm Biol. 2011; 2(4): 1130-1136.

[22]. Tandon C, Mathur P, Sen M. Antibacterial compounds in different species of *Datura*: A review. 2014; 8(21): 2090-2098.

[23]. Okwu D, Igara E. Isolation, characterization and antibacterial activity of alkaloid from *Datura metel* Linn leaves. Afr J Pharm Pharmacol. 2009; 3(5): 277-281.

[24]. Okwu D, Igara E. Isolation, characterization and antibacterial activity screening of a new ß-carboline alkaloid from *Datura metel* Linn. Der Chemica Sinica. 2011; 2(2): 261-267.

[25]. Sule A, Ahmed QU, Samah OA, Omar MN, Hassan N, Kamal L, Yarmo M. Bioassay guided isolation of antibacterial compounds from *Andrographis paniculata*. Am J Applied Sci. 2011; 8(6): 525-534.

[26]. Sen M. Authored chapter in the Manual entitled "Vancomycin Resistant Enterococci" of CME/Workshop on update in Clinical Microbiology theme-" Emerging antimicrobial resistance: Are we doing enough?" conducted by Department of Microbiology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow in November 2006.

[27]. Perez C, Paul M, Bazerque P. Antibiotic assay by well diffusion method. Acta Biol Med Expt. 1990; 15: 113-115.

[28]. Carson CF, Hammer KA, Riley TV. Broth micro-dilution method for determination of susceptibility of *Escherichia coli* and *Staphylococcus aureus* to the essential oil of *Malaleuca alterifolia* (Tea tree oil). Microbios. 1995; 82: 181–185.

[29]. Kataky A, Handique PJ. Antimicrobial activity and phytochemical estimation of micropropagated *Andrographis paniculata* (Burm.f) NEES. Asian Journal of Science and Technology. 2010; 5: 91-94.

[30]. Mims and Playfair. Medical microbiology. Mosby Europe. 1993; 35: 31

[31]. Irkin R, Korukluoglu M. Control of *Aspergillus niger* with garlic, onion and leek extracts. Afr J Biotechnol. 2007; 6: 384–387.

[32]. Gatsing D, Nkeugoauapi CFN, Nkah BFN, Kuiate JR, Tchouanguep FM. Antibacterial activity, bioavailability and acute toxicity evaluation of the leaf extract of *Alchornea cordifolia* (Euphorbiaceae). Int. J. Pharmacol. 2010; 6: 173–182.

[33]. Hosamani PA, Lakshman HC, Sandeep KK, Hosamani RC. Antimicrobial activity of leaf extract of *Andrographis paniculata* wall. Science Research Reporter. 2011; 1(2): 92-95

[34]. Eloff JN. Which extractant should be used for the screening and isolation of antimicrobial components from plants? J. Ethnopharmacol. 1998; 60: 1–8.

[35]. Paz EA, Cerdeiras MP, Fernandez J, Ferreira F, Moyna P. Screening of Uruguayan medicinal plants for antimicrobial activity. J Ethnopharmacol. 1995; 45(1): 67-70.

[36]. Kudi AC, Umoh JU, Eduvie LO, Gefu J. Screening of some Nigerian medicinal plants for antibacterial activity. J Ethnopharmacol. 1999; 67(2): 225-228.

[37]. Palombo EA, Semple SJ. Antibacterial activity of traditional Australian medicinal plants. J. Ethnopharmacol. 2001; 77(2-3): 151-157.

[38]. Karaman I, Sahin F, Gulluce M, Ogutcu H, Sngul M, Adiguzel A. Antimicrobial activity of aqueous and methanol extracts of *Juniperus oxycedrus* L. J.

Ethnopharmacol. 2003; 85(2-3): 231–235.

[39]. Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. Microb Mol Bio Rev. 2003; 67: 593-656.

[40]. Tortora GJ, Funke BR, Case CL. Microbiology: An Introduction. 7th ed. Benjamin Cummings Publishing: San Francisco, USA; 2001. p. 88.

[41]. Gurinder JK, Daljit SA. Antibacterial and phytochemical screening of *Anethum graveolens*, *Foeniculum vulgare* and *Trachyspermum ammi*. BMC Complement Altern Med. 2009; 9: 30.

[42]. Stavri M, Piddock LVJ, Gibbons S. Bacterial efflux pump inhibitors from natural sources. J Antimicrobial Chemo. 2007; 59(6): 1247-1260.

[43]. Doughari JH, Manzara S. In vitro antibacterial activity of crude leaf extracts of Mangifera indica Linn. Afr J Microbiol Res. 2008; 2: 67-72.

[44]. Vermillion K, Holguin OF, Berhow MA, Richins RD, Redhouse T, O'Connell MA, Posakony J, Mahajan SS, Kelly SM, Simon JA. Dinoxin B, a Withanolide from *Datura inoxia* leaves with specific cytotoxic activities. J Nat Prod. 2011; 74(2): 267–271.

[45]. Abraham A, Kirson I, Lavie D, Glotter E. The Withanolides of Withania somnifera chemotypes I and II. Phytochemistry.1975; 14: 189–194.

[46]. Manickam M, Srivastava A, Ray A. Withanolides from the flowers of *Datura fastuosa*. Phytochemistry .1998; 47: 1427–1429.

[47]. Evans WC. In Solanaceae Biology and Systematics: D'Arcy WG, editor. Columbia University Press: New York; 1986. p. 179-186.

[48]. Veleiro AS, Cirigliano AM, Oberti JC, Burton G. 7-Hydroxywithanolides from *Datura ferox*. J Nat Prod. 1999; 62:1010–2.

[49]. Siddiqui BS, Afreen S, Begum S. Two New Withanolides from the aerial parts of *Datura inoxia*. Aust J Chem.1999; 52: 905–907.

[50]. Siddiqui BS, Afreen S, Begum S, Sattar F. Daturacin, a new withanolide from *Datura inoxia*. Nat Prod Res.2005; 19: 619–623.

[51]. Gupta M, Bagchi A, Ray AB. Additional Withanolides of *Datura metel*. J Nat Prod. 1991; 54: 599–602.

[52]. Pan Y, Wang X, Hu X. Cytotoxic Withanolides from the flowers of *Datura metel*. J Nat Prod. 2007; 70: 1127–1132.

[53]. Kagale S, Marimuthu T, Thayumanavan B, Nandakumar R, Samiyappan R. Induction of systemic resistance in rice by leaf extracts of *Zizyphus jujuba* and *Ipomoea carnea* against *Rhizoctonia solani*. Plant Signal Behav. 2011; 6(7): 919–923.