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Antimicrobial Potential and *In Vitro* Cytotoxicity Study of *Tabernaemontana Divaricata* (L.) Stem Bark Extract Against HEK 293 Cell Line

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ABSTRACT: *Resistance of antibiotics against pathogenic microbes is a growing clinical concern, and the spread of resistant strains worldwide has been discombobulating public health. The ability of most infectious microbes evolving antimicrobial resistance necessitates discovery of novel antimicrobial agents from natural sources. The present study was focused on identification of multi drug resistant (MDR) microorganisms of clinical isolates and that were subjected to methanolic extracts of Tabernaemontana divaricata stem bark crude extract for antimicrobial efficacy by agar well diffusion method. Phytochemical analysis and the minimum inhibitory concentration (MIC) testing were also determined. In vitro cytotoxic effects of the stem bark extract was observed through MTT reduction assay on normal cell line. In our study we have found antimicrobial activity against all test isolates by agar well diffusion methods. The less MIC value of methanolic extract was profound against MRSA as compared to other isolates by microtitre plate-based assay. GC-MS analysis revealed that the presence of two most probable active antimicrobial compounds viz. cyclotetrasiloxaneoctamethyl and cyclopentasiloxanedecamethyl respectively. To our knowledge, this is the first report of the presence of the said compounds with antimicrobial activity in T.divaricata. We have also evaluated possible cytotoxic effect on Normal cell lines (IC50= 56.52µg/mL). Our study suggests the possibility of exploring antimicrobial property in the active compound of Tabernaemontana divaricata as a prospective alternative therapy that could help in combating growing antibiotic resistance trend worldwide. So, in traditional medicine our investigation provides a good insight for drug development process and clinical practices.*

KEY WORDS: antimicrobial, multi drug resistant (MDR), phytochemicals, GC-MS analysis, cytotoxicity

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I. Introduction

World Health Organisation (WHO) has mentioned that in the treatment of many therapeutic purposes drug derived from medicinal plants are mainly used [1]. Medicines derived from plant sources are widely used and form a vital part in many developing countries across the globe for primary health care [2].

Compounds that are produced naturally can be more biodegradable compared to synthetic compounds. Thus, in recent year's application and positive image of natural antioxidants, antibacterial, cytotoxic, antiviral, fungicidal agents have gained popularity, and are spreading among consumers [3].

Now a day, increase in antimicrobial resistance by pathogens necessitates search for newer antimicrobial agents. Medicinal plants may provide a natural source of antimicrobial drug compounds that can be applied in controlling infections [4]. An increase in the use of commercially available antimicrobial agents in the treatment of diseases leads to the increasing multidrug resistance in microbial pathogens [5].

So, in recent years, naturally occurring antibacterial compounds have gained more importance. Natural sources like medicinal plants have the ability to retard the growth of clinically important microorganisms due to the presence of specific constituents of secondary metabolites and, are considered to be of pharmaceutical importance. As reported by World health organization (WHO), traditionally used medicinal plants are the best reservoirs of phytochemicals to develop newer pharmaceuticals [6].

Tabernaemontana divaricata of the family apocynaceae is one of the garden plants known for its antimicrobial and other medicinal values in the traditional Indian system of medicine. The beneficial properties of *T. divaricata* are anti-infection, antioxidant, anti-tumour action, analgesia activity and activity of cholinergic in peripheral as well as central nervous systems. The augmentation of cholinergic function may be of curative benefit for many neurodegenerative diseases, particularly Alzheimer's and myasthenia gravis disease [7].

Other therapeutic activities are alexipharmic, anticancer, hepatoprotective, digestive and antimicrobial properties [8]. Traditionally the bark of this plant is used as a treatment of toothache, and it is an antipyretic. A number of phytochemicals have been reported in this particular plant species consisting of alkaloids, flavonoids, triterpenoids, glycosides, lipids and saponins [9, 10 & 11].

II. Materials And Methods

Plant material

The plant material i.e. *T. divaricata* (L.) was collected from Botanical garden of Gauhati University, Guwahati, Assam.

Plant Identification

The plant material was identified by Botany Department; Gauhati University, Guwahati, Assam, with **Reference No. Herb/Bot/GU/2015/122; Acc.No. 18079**

Preparation of crude extract

The stem bark of this plant were collected and washed with distilled water to remove dust particles. Air dried barks are then powdered with the help of mixture grinder. In a soxhlet apparatus, 100 grams of dry powered bark were extracted for 6 hours at 62ºC with methanol (Merck). Under reduced pressure the extracts was evaporated and dried by using a rotary evaporator (BUCHI TYPE, IKA). Finally dried extracts were weighted and stored in a sterile screw capped bottle at –20ºC [12].

Test Microorganisms

Clinical strains of MDR *E. coli*, *K. pneumoniae*, MRSA (Methicillin Resistant *Staphylococcus aureus*) and *Candida albicans* were used as test microorganisms, collected from various clinical specimens received at bacteriology laboratory, Gauhati Medical College [Ethical Committee No. GU/ACA/Ethics/2012/3993 dated 10/01/2012]. All isolates were isolated, identified by standard methodology and stored as glycerol stock in -80˚ C deep freezer.

Media

Muller Hinton agar, Mackonky agar and Blood agar base, Sabouraud dextrose agar was purchased from Himedia Laboratories Pvt. Ltd., Mumbai. RMPI culture media, FBS, Antibiotic-antimycotic solution was purchased from Life Technologies India Pvt Ltd. and MTT (expansion) from Himedia Laboratories Pvt. Ltd., Mumbai.

Phytochemical analysis

Qualitative phytochemicals analysis were performed by standard methodology [13,14] to detect the different phytochemicals by using different phytochemical test viz. Mayer's test, Carbohydrate test, Salkowski test, Cardiac Glycosides, Foam test , Shinoda's test & Ferric chloride test.

Antibiotic sensitivity patterns and determination of ESBL producers

All bacterial strains were subjected to antibiotic sensitivity tests on Muller-Hinton agar by the disc diffusion method developed by Kirby-Bauer by using different groups of 21 antibiotics. The bacterial culture was swabbed on MH agar, wherein antibiotic discs were placed 20 mm apart. Upon incubation at 37 °C for ~16 hours, the different diameter of zone of inhibition was measured in millimeters (mm). For fungal isolate i.e. *C. albicans*, 5 different antibiotics were used in MH agar with chloramphenicol.

Cefotaxime, ceftaxidime and aztreonam resistant or intermediately resistant isolates were selected for phenotypic detection of ESBL production by the Double Disk Synergy Test (DDST) using cefotaxime and cefotaxime + clavulanic acid (30/10 µg) and ceftazidime and ceftazidime + clavulanic acid (30/10µg) as per the CLSI guidelines, 2011.

Determination of Antimicrobial Activity

Methanolic bark extracts of *Tabernaemontana divaricata* were tested for antimicrobial activity by agar well diffusion method [15] against four different clinical isolates of MDR *E. coli*, *K.pneuminiae.*, MRSA, and *C. albicans*. I*n vitro* antimicrobial screened was performed by using Muller Hinton agar media (MHA) and Sabouraud dextrose agar (SDA) purchased from Himedia. Muller Hinton & Sabouraud dextrose agar plates were prepared by pouring 20 ml of molten media into sterile petriplates. Then to solidify the plates for 10 mins and 0.5% inoculums suspension was swabbed uniformly on to the individual plates using sterile cotton swabs. For antimicrobial activity, from the total dried 200 microgram of stock crude extracts was diluted to 50 µg/mL in DMSO. Wells of 6 mm diameter were made on Muller Hinton & Sabouraud dextrose agar plates**.** Now by

using a micropipette 20 µl of plant extract was poured on to the each well of four different agar plates & one SDA plate for five different strains. 20 µl of DMSO was used as a control. The plates were then incubated at 37ºC for 24 hrs. At the end of the incubation period different level of zone of inhibition of bacteria and fungus were measured by zone scale & in the control there was no zone of inhibition.

Minimum Inhibitory Concentration (MIC)

By microtitre plate-based assay MIC value was evaluated using resazurin as an indicator of cell growth [16]. After overnight incubation of the microbial isolates in Luria Bertani (LB) Broth, the bacterial and fungal isolates were centrifuged at 4000 RPM for 5 minutes. Then discarded the supernatant and pellet was re-suspended in 20ml of normal saline and re-centrifuged at 4000 rpm for 5 minutes. The pellet was then dissolved in 20 ml sterile normal saline for use in the MIC assay . Resazurin dye was prepared as per manufacturer's instructions (HiMedia Pvt. Ltd., Mumbai). In a 96 well microtitre plate, the first row of the plate was filled with 100µL of the test extract and all the wells were filled with 100µL of LB broth. Two fold serial dilutions were achieved by transferring 100µL from the first well to the subsequent wells such that each well has 100µL of sample in decreasing concentration. 10µL of resazurin dye was added to each well as an indicator. Finally 10µL of the bacterial and fungal suspension was added to all the wells and the plate was loosely wrapped with cling film to make sure that bacteria and fungus did not become dehydrated. Each plate had two controls: 1. a well with all solutions with the omission of the test extract and 2. a well with all solutions except bacterial and fungal suspension, replaced by LB Broth. The plate was incubated overnight at 37°C and then observed for any visible colour change. Change of colour from purple to pink or colourless was taken to be positive.

GC-MS Analysis

For the analysis, Shimadzu GC 2010 plus with triple MS (TP-8030) fitted with EB-5MS column was used. The temperature program for the column was set at 100º C hold for 1 min, 15º C/min to 160º C and 5º C/min to 300º C hold for 7 min. The GC injector was held isothermally at 280ºC with a split less period of 3 min. As the carrier gas Helium was used, at a flow rate of 1 mL/ min by using electronic pressure control. The interface temperature for GC–MS was maintained at 280 ºC. In electron impact (EI) ionization mode, the MS was operated with 70 eV electron energy and the scan to determine appropriate masses for selected ion monitoring ranged from 50 to 500amu (atom to mass unit). GC–MS library search was used to confirm the metabolites (Fig.1).

Identification of Phytocomponents

For identification of the compounds National Institute Standard and Technology (NIST) 11 library database was used.

Cell line

The human HEK-293 normal cell line was provided by National centre for cell science, Pune. Both cells were cultured in RPMI medium, supplemented with 10% Fetal Bovine Serum and 1% antibiotic-antimycotic solution, in a T25 culture flask. The flask is incubated at 37ºC in 5% CO2 for 24 hours. The cell cytotoxicity assay was performed when cell growth reached an optimum number of 1×10^5 cells/ml.

In-vitro cytotoxicity studies

The evaluation of cytotoxicity was performed using MTT assay [17] . The cells were seeded in a 96well culture plate with various concentrations (3.12–100 µg/ml) of the methanol extract. The cultured plates were incubated for 24h at 37°C and 5% CO_2. Following incubation, 20 µl MTT solutions in phosphate buffered saline (PBS) were added to each well at a final concentration of 0.5 mg/ml followed by further incubation for 3 h at 37°C. The medium was then removed, and 100 ml DMSO were added to each well for solubilizing the formazan. The absorbance was measured at 570 nm using an ELISA reader (in Multiskan Go, Nanodrop (Thermo scientific). Three independent experiments were carried out for each concentration. The concentration of the methanol extract which resulted in a 50% reduction of cell viability, the half maximal inhibitory concentration (IC50 value), was calculated using the following formula: % inhibition = (control abs - sample abs)/(control abs) × 100 (Fig.2).

Statistical analysis

To enter and capture data Microsoft Excel® was used & from this data various graphs and tables were extracted. The zone size for each microorganism was analyzed using t-test. P value < 0.05 was considered as the significance level. For statistical analysis R 2.6.2 (2008-02-08) software was used.

III. Result

Analysis of Phytochemicals

Phytochemical analysis was carried out by using standard qualitative methods. Phytochemical analysis of the methanol extract of *T.divericata* bark [**Table No 1]** tested positive for the presence of alkaloids, terpenoids, saponins, flavonoids, and tannins except for carbohydrates and Cardiac Glycosides.

Table No 1 Preliminary phytochemical analysis of plant extracts in methanol solvent system.
+ve:- Presence / –ve:- Absence

Phytochemicals	**Bark**
Alkaloids	+ve
Carbohydrate	-ve
Terpenoids	+ve
Cardiac Glycosides	-ve
Saponins	+ve
Flavonoids	+ve
Tannins	+ve

Antibiotic susceptibility test

By using disc diffusion of Kirby-Bauer's method different antibiotics (HiMedia Laboratories Pvt. Ltd., India) presented in [**Table No 2]** were used for antimicrobial susceptibility testing according to CLSI guidelines (CLSI, 2011) on Muller-Hinton agar. For the determination of ESBL producers in bacteria double-disc synergy method was followed. Aztreonam, cefotaxime and ceftaxidime resistant or intermediately resistant isolates were selected for phenotypic detection of ESBL production by the Double Disk Synergy Test. An increase in the zone diameter of the antibiotic disc supplemented with clavulanic acid by ≥5mm was recorded as ESBL producer isolate.

Table No 2 Antibiotic susceptibility pattern of selected bacterial and fungal strains for screening of antimicrobial property of plant extracts

ORG	AMP	MEC	PIT	CPM	CPZ	CFM	IMP	DOR	ERT	MRP	GEN	KA	TE	GEM	NA	COT	FO	NIT	AT	CAZ	CTX	POLYB	CIP	TIC
E.coli	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	I	R	R	R	-	R	-
K.P	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	-	R	-
P.A	-	-	R	R	R	-	I	-	-	I	R	-	-	-	-	-	-	-	R	R	R	R	R	R

ORG	OX	MET	PIT	CPM	CTX	CAZ	CPD	CDR	IMP	MRP	GEN	VA	RP	CIP	CX	CD	E	C	TE	NIT
S.A	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	I	S

Organisms	AmphotericinB 20 µg/disc	Fluconazole 10 µg/disc	Ketoconazole 30 µg/disc	Itraconazole 10 µg/disc	Voriconazole 20 µg/disc
C. albicans	R	R	R	R	R

Key#: ORG- Organism; *E.coli- Escherichia coli ,K.P:Klebsiella pneumoniae, P.A: Pseudomonas aeruginosa, S.A: Staphylococcus aureus,* AMP- Ampicilin 10µg/disc, MEC- Mecillinam 10 µg/disc PIT- Piperacillin Tazobactum 100/10 µg/disc, CPM-Cefepime 30 µg/disc, CPZ –Cefoperazone 75 µg/disc, CFM- Cefixim 5 µg/disc, IPM- Imipenem 10 µg/disc, DOR- Doripenem 10µg/disc, ERT- Ertapenem 10 µg/disc , MRP- Meropenam 10 µg/disc, GEN- Gentamicin 10 µg/disc, KA- Kanamycin10 µg/disc, TE- Tetracyclin 30 µg/disc, GEM- Gemifloxacin 5 µg/disc, NA- Nalidaxic Acid 30 µg/disc, COT- Cotrimoxazole 25 µg/disc, NIT- Nitroflurantoin 300 µg/disc, AZT- Aztreonam 30 µg/disc, CAZ- Ceftazidime 30 µg/disc, CTX- Cefotaxime µg/disc, POLYB- PolymyxinB 300units , TIC- Ticarcillin 75 µg/disc OX- Oxacillin 1 µg/disc, CPD- Cefpodoxime 10 µg/disc, CDR- Cefdinir 5 µg/disc, VA- Vancomycin, CIP- Ciprofloxacin 5 µg/disc, CX- Cefoxitin 30 µg/disc, CD- Clidamycin 2 µg/disc, E- Erythormycin 15 µg/disc, RP- Trimethoprim-Sulfamethoxale 1.25/23.75 µg/disc.

Determination of Antimicrobial Activity: antimicrobial screening against each different organisms and their antimicrobial zone of inhibition was recorded in [**Table No 3]** which was found to be more profound against MRSA with zone of inhibition 14.6 ±0.3 mm compared to *E. coli, K. pneumoniae*, & *C. albicans* with zone size 13.3 ±0.3 mm, 11.3 ±0.5mm & 12.6± 0.3 mm respectively and to found to be significant consider the p value at <0.05.

Table No 3 Inhibition zone of different bacterial and fungal strains. Assay was performed in triplicate and results are the mean of three values and ±SE of mean.

Plant extract	Organisms	Zone Of Inhibition (mm)	DMSO (Control)	P value
Tabernaemontana divaricata	*E.coli*	13.3 ±0.3	0	0.0006291
	K. pneumoniae	11.3 ±0.5	0	0.0008716
	MRSA	14.6 ±0.3	0	0.0005197
	C .albicans	12.6± 0.3	0	0.0006973

Foot note: **Organisms:** *E.coli- Escherichia coli; K. pneumoniae-Klebsiella pneumoniae;* MRSA- Methicillin resistant *staphylococcus aureus; C .albicans- Candida albicans;* mm- millimetre, DMSO- Dimethyl sulfoxide

Minimum Inhibitory Concentration (MIC)

The lowest concentration of the extract at which the colour change was observed was recorded as the MIC value .The values for minimum inhibitory concentrations (MIC) for *T. divericata* against the test organisms are represented in [**Table No 4].** The MIC of extracts against all tested isolates fall in the range of 50–1.562 µg/ml as determined by microtitre plate based assay using resazurin as an indicator.

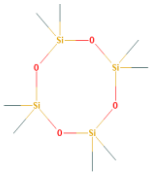
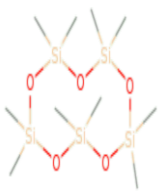
Table No 4 Values of minimum inhibitory concentration (MIC) by resazurin based dye method.

Microbial Strain	Methanol Extract (µg/mL)
MRSA	4.1 ±1.04
Escherichia coli	5.2 ±1.08
Klebsiella pneumoniae	12.5 ±0.0
Candida albicans	12.5 ±0.0

GC-MS analysis

By comparison of mass spectra on NIST 11 library database, two compounds were identified and characterized, shown in [**Table No 5]**. GC-MS analysis revealed that the presence of two most probable active antimicrobial compounds viz. cyclotetrasiloxaneoctamethyl and cyclopentasiloxanedecamethyl. To our knowledge, this is the first report of the presence of the said compounds with antimicrobial activity in *T.divaricata.*

Table No 5 Compounds of *Tabernaemontana divericata* are summarized below

Peak	R.TIME	I.TIME	F.TIME	AREA	AREA%	HEIGHT	HEIGHT %	A/H	NAME	STRUCTURE
1	6.439	6.375	6.505	113986	75.28	42075	70.95	2.71	CYCLOPENTASILOXANE -OCTAMETHYL	 $C_8H_{24}O_4Si_4$
2	9.114	9.080	9.155	37426	24.72	17226	29.05	2.17	CYCLOPENTASILOXANE -DECAMETHYL	 $C_{10}H_{30}O_5Si_5$

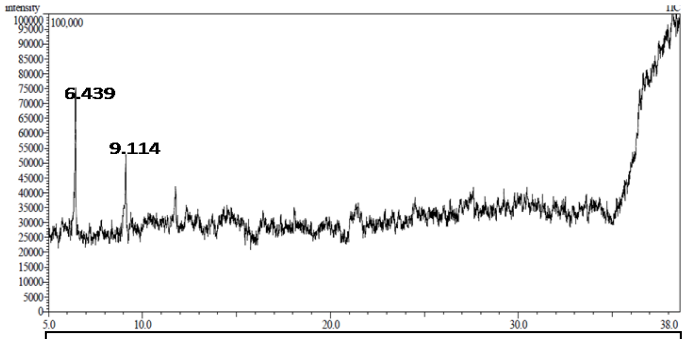


Fig.1 GC Profile of Tabernaemontana divericata

In-vitro cytotoxicity studies

In-vitro cytotoxic effects of the plant crude extract were carried out through MTT reduction assay at different plant drug concentration ranging from (3.12–100 µg/ml) in a drug response curve against normal cell line which showed IC_{50} value 56.52µg/ml presented in [**Table No 6]**. The IC_{50} value of the plant extract was found to be non toxic i.e IC_{50} < 20 µg/mL in the preliminary assay as per the criteria of cytotoxicity for the crude extract, as established by the U.S. National Cancer Institute [18].

Table No 6 Determination of cytotoxicity by MTT Assay

PLANT	Plant Concentration (µg /ml)	% inhibition	IC_{50}
T.divaricata	100	64.71	
	50	45.36	
	25	23.03	
	12.5	15.88	
	6.25	12.19	
	3.125	62.30	56.52 µg/mL

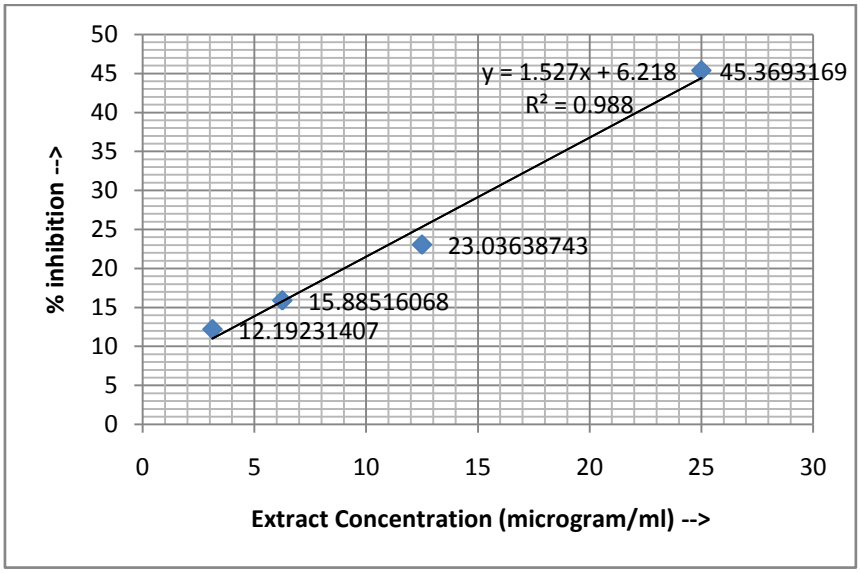


Fig. 2 Percentage growth inhibition of methanolic extract of *Tabernaemontana divericata* against HEK-293 cell line

IV. Discussion

In recent time research for a new antimicrobial agents has a great immense important, as pathogenic microbes are highly resistance against different antibiotics. So, the main focused of this study was the use of medicinal plants for possible antimicrobial agents due to its less expensive and less side effects. Thus search for effective and alternative therapeutics agents from the medicinal plants against such resistant microbes has become an important concern all over the world [19].

In most parts of the world, medicines derived from plant have been used for traditional health care and also the antimicrobial efficacy of the bioactive compounds that are mainly plant derived are well-documented [2].

Result obtained from the present study shows that the bark of *T. divaricata* has showed to have an antibacterial as well as antifungal activity against clinically important pathogenic bacterial and fungal isolates. The methanolic stem bark extract was found to be possessed maximum antimicrobial activity against MRSA with zone of inhibition 14.6mm (±0.3) and less effective against *E.coli, K.pneumoniae and C.albicans* by agar well diffusion method which suggests that they may be clinically useful.

The result of the present study also made an effort to identify the phytochemical, revealed that the presence of alkaloids, terpenoids, saponins, flavonoids and tannins. But Cardiac glycosides and carbohydrate were not present in bark of *T. divaricata*. The previous study also showed that presence of flavonoids, terpenoids, phenols, tannins, carbohydrates and protein in all parts. But in flower steroids and alkaloids were absent. In stem, Cardiac glycosides, steroids and saponins were not present [15].

In our study the less MIC value was found against MRSA compared to other clinical isolates. The zone size of each of the different organisms was found to be significant, considered $p<0.05$. A previous study has shown MIC value for both *E.coli* and *S.aureus* was 16 µg/mL [5].

In the present study, microdilution method was used because it is a quantitative reference method routinely used in clinical laboratories. In this method, susceptibility panels in 96-well microtitre plates contained various concentrations of antimicrobial agents. Then, standardized numbers of bacteria were inoculated into the wells of the microtitre plates and incubated overnight at 35 °C. The MIC value was observed as the lowest concentration where no viability was observed in the wells after incubation.

Further GC-MS analysis showed presence of (two potent) probable two active antimicrobial compounds which was first reported in bark of *T.divaricata* i.e. cyclotetrasiloxane-octamethyl and cyclopentasiloxane-decamethyl. As per published literature, the above mentioned compounds were isolated from plant parts or fruit juice of medicinal plants and showed antimicrobial activity [20,21,22,23 & 24]. In our study, we were able to detect the presence of the said compounds from the bark of *Tabernaemontana divaricata* suggesting that they might be the active constituents in our plant extract for its efficient antimicrobial activity. To our knowledge, this is the first report of the presence of the said compounds with antimicrobial activity in *T. divaricata*.

We have also carried out toxicity study for the crude extract for potential application against normal cell line. This was done using human embryonic kidney (HEK293) cell line. So, from our preliminary study we can conclude that the methanolic extracts did not exhibit any significant cytotoxic effect against human embryonic kidney (HEK293) cell line. The dose response curve showed IC50 value for normal cell line (HEK-293) 56.52µg/ml. As per the American National Cancer Institute (NCI) guidelines the limit of activity for crude extracts at 50% inhibition (IC) of proliferation of less than 30 µg/mL after the exposure time of 72 hours. On the hand a crude extract with IC less than 20 µg/mL is considered highly cytotoxic [18]. So the result of the present study showed that plant extract does not have any toxic effect against normal cell line.

This work is feasible having potential which can be exploited for the development of superior therapeutics. The obtained result provides a support for the use of this plant in traditional medicine for its antimicrobial property and can be prospected to reach potential bioactive compounds which can be further formulated for the development of the drug in the pharmaceutical industry.

V. Conclusion

In conclusion, we found that methanol extracts of the bark parts of this plant showed potential antimicrobial activity against multidrug resistant pathogenic organisms and this suggest that constituents of the plants could be useful in clinical practices. From the findings of this study, we can also recommend that the isolated compounds from bark of *T. divaricata* could be made for further drug development process.

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Conflict of Interests

Authors have no conflicts of interest.

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