Antibacterial and Phytochemical Studies with Cytotoxicity assay of *Crataeva nurvala* Stem bark extract against Multi-drug Resistant Human Pathogens Isolated from UTI

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ABSTRACT

Emerging antibiotic resistance is a worldwide problem that has led to the need for development of novel antimicrobials and use of natural products which is safe and effective. The objective of this study is to investigate antibacterial activity of different solvent (Methanol, Ethanol, Acetone, Petroleum ether and Water) extract of *Crataeva nurvala* stem bark extract against multi-drug resistant UTI pathogens isolated from urine samples collected from patients suffering from UTI. Acetone extract has greater antibacterial activity against all tested uropathogens. Qualitative phytochemical test showed presence of Alkaloid, tannin, Saponin, Flavonoids, Terpenoids, Cardiac glycoside and oil. HPTLC-bioautography confirms the antibacterial potential of the plant extract. Phytochemical compounds were identified by HPLC-MS and its result showed presence of Cuscohygrine, Diuron, Myricetin, Dihydromyricetin, Rutin, Nefopam. Results of Cytotoxicity test performed by MTT assay and Viability assay showed less toxicity and more than 80% viability.

Key words: **UTI, Antibacterial activity, Phytochemical, *Crataeva nurvala*, HPTLC-bioautography, Cytotoxicity assay**

I. INTRODUCTION

Urinary Tract Infections (UTIs) are the serious health problem affecting millions of people every year. It is the second most common infection. UTIs are more common in persons aged 20-50 years (Griffiths, 2003). Women are more likely to get UTI than men because of their urinary tract's design. Nearly half of all women will have a UTI at some point in their lives (Foxman, 2003). Several potent antibiotics are available for treatment of UTI, but increasing drug resistance among bacteria has made therapy of UTI difficult. Bacteria have the genetic ability to transmit and acquire resistance to drugs (Soulsby, 2005). One major drawback to the use of antibiotic is the potential for development of antibiotic resistance among uropathogens (Head, 2008). The increasing prevalence of antibiotic resistant bacteria, escalating costs of antibiotic therapy and unsatisfactory therapeutic alternatives in recurrent UTIs have developed an interest in novel, non antibiotic based methods for preventing and controlling UTIs (Vaughan, 2007).

Plant's secondary metabolites have already demonstrated their potential as antimicrobials when used alone as synergists of other antimicrobial agents. Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity (Rojas *et. al*., 2003). Therefore researchers are increasingly turning their attention to folk medicine (Benkeblia, 2004). Even the World Health Organization (WHO) supports the use of medicinal plants, provided it is proven to be efficacious and safe (WHO 1995). The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity (Colombo, 1996). A large number of plants are being used as medicinal agent all over the world (Choudhury *et. al.*, 2012).

The present study reletes to phytochemical screening, antibacterial activity, HPTLC-bioautography and cytotoxicity assay of *Crataeva nurvala* stem bark extracts against multi-drug resistant (MDR) uropathogens isolated from urine sample of patients suffering from UTI.

II. MATERIALS AND METHODS

2.1. Collection of urine samples and Selection of MDR bacterial strain from urine:

Urine samples from the patients suffering from UTI were collected from various laboratories: Bhanumati laboratory and Parsi Hospital, Navsari and Advanced Diagnostic Laboratory, Surat. The isolated bacterial UTI pathogens were identified on the basis of gram staining, morphological and biochemical characteristics (Holt *et. al.*, 1994).

From the identified bacterial isolates, MDR bacterial strain was selected by performing antibiotic susceptibility test (Baris *et. al.*, 2005) using Pathoteq 'Bio-Disc-12' (Pathoteq Biological Laboratories, India), which includes 12 antibiotics (Ampicillin/Sulbactam, Co-trimoxazole, Ceftizoxime, Chloramphenicol, Cephalexin, Tetracycline, Ciprofloxacin, Nitrofurantoin, Sparfloxacin, Gatifloxacin, Norfloxacin and Ofloxacin) used for treatment of UTI. The zones of growth inhibition were then measured. The diameter of the zone is related to the susceptibility of the test microorganism and to the diffusion rate of the drug through the agar medium. The results were interpreted using the criteria of the Clinical and Laboratory Standards Institute (CLSI, 2007).

2.2. Plant Extraction:

Crataeva nurvala was collected from road side near Veraval, Navsari, India. Taxonomic identification of the plant was confirmed by Dr. B. K. Dhaduk, Horticulture Department, Agriculture University, Navsari. The plant material used for the study was washed under running tap water thoroughly, air dried and homogenized to fine powder and 10 g of powdered plant material was extracted using 150 ml five different solvents; methanol, ethanol, acetone, petroleum ether and water using Soxhlet extraction apparatus (Superfit Continental Pvt. Ltd.) for 8 h. The extracts were concentrated and extractive % yield was calculated. The dried extracts were re-dissolved in minimum volume of DMSO and then preserved in refrigerator for further studies.

2.3. Preliminary phytochemical analysis:

All the plant extracts were subjected to preliminary phytochemical analysis to study the presence of phytoconstituents *viz*., alkaloids, tannins, saponin, anthocyanide, phenolic flavonoids, flavonoids, carbohydrate, protein, terpenoids, cardiac glycosides, oil by standard methods described by Trease and Evans (1989).

2.4. Determination of antibacterial activity and MIC:

All the solvent extracts of *C. nurvala* were subjected to antibacterial screening test by well diffusion assay (Magaldi *et. al*., 2004). The plates containing Muller Hinton Agar medium were inoculated with the selected MDR UTI pathogens and 8 mm wells were prepared. The wells were filled with 100 µl of respective solvent extract and control well with DMSO. Plates were then incubated at 37 ^{0}C for 24 h. Antibacterial activity was evaluated by measuring the zone of inhibition against the selected UTI pathogens. The experiment was performed in triplicate and the mean of zone diameter was calculated. MIC was also determined by well diffusion assay.

2.5. HPTLC bioautography to find antibacterial compound:

HPTLC of *Crataeva nurvala* acetone extract was performed on the CAMAG HPTLC System. 10 µl of sample was applied on the HPTLC silica merk 60F 254 graded plate sized 8 cm x 10 cm as 8 mm band width using CAMAG Linomat 5 injector (CAMAG, Switzerland) which was programmed by WinCATS Planner Chromatography Manager. The plate was developed in Toluene: Ethyl acetate: Methanol (7.5: 1.5: 0.7) solvent system (Gallo *et. al*., 2009). The plates were dried and examined under the UV chamber at 254 nm and 366 nm. Rf value was found using following formula:

Rf value = Distance travelled by solute / Distance travelled by solvent

For bioautography, the developed TLC plates were placed in 9 cm x 9 cm sterile petry dishes and melted and cooled Muller Hinton Agar (Hi-Media) was overlaid over it. After solidification of medium, bacterial suspension was spreaded over it and plates were incubated at 37 ^{0}C for 24 h. For visualization of bacterial growth, the bioautogram was flooded with 2% nitroblue tetrazolim chloride. Antibacterial activity was indicated by the appearance of clear zone against a purple/black background on the TLC plate and the Rf value was compared with Rf value of separated phytoconstituents (Silva *et. al*., 2005).

2.6. HPLC-MS (High Performance Liquid Chromatography - Mass Spectrometry) analysis:

In this study, HPLC-MS was carried out at IIT-Bombay, Powai, to know the phytoconstituents present in *Crataeva nurvala* acetone extract, showed maximum antibacterial action. Chromatographic separation was performed using 5 µl injection of sample onto an Agilent G6550A iFunnel Q-TOFs technology, which combines highly efficient electrospray ion (ESI) generation and focusing of Agilent Jet Stream (AJS) technology with a hexabore capillary sampling array and dual stage ion funnel for increased ion sampling

and transmission. The mobile phase consisted of (A) ultra pure water and (B) 95:5 acetonitrile/water at a flow rate of 0.5 ml/mimute. The mass spectrometry analysis was performed on Quadrapole Time-Of-Flight (Q-TOF). The data was analyzed using Masshunter Qualitative analysis software package (Agilent Technology). The compounds were identified by comparison against a database of the Metlin metabolomics database.

2.7. Cytotoxicity assay:

The solvent extract of plant showing maximum antibacterial activity was selected for *in vitro* cytotoxicity assay to determine toxicity of the plant extract. Cytotoxicity assay (MTT and Viability Assay) of acetone extract of *Crataeva nurvala* was performed on HeLa Cervical Cell line at Department of Toxicology GeneXplore Diagnostics and Research Centre Pvt. Ltd., Ahmedabad. % Inhibition and % viability was found by using following formula:

% Inhibition = 100 - Abs (sample)/Abs (control) x100

% viability = (live cell count/total cell count) x 100

III. RESULTS AND DISCUSSION

3.1 Selection of MDR bacterial strain from urine:

In this study, out of 550 mid-stream urine samples, 543 uropathogens were recovered. Out of 543 isolates, 515 isolates were bacteria and 28 isolates were found to be *Candida albicans*. As the aim of the study was to check the antibacterial activity of medicinal plants, only bacterial isolates were used for further studies. The urinary isolates were identified based on morphological and biochemical characteristics. According to the current study, gram negative bacteria were responsible for 100% of UTIs.

This study reported *Escherichia coli* was the most predominant uropathogen with 85.40%, followed by *Pseudomonas aeruginosa* 5.04%, *Klebsiella pneumonia* 4.27%, *Proteus vulgaris* and *Enterobacter aerogens* 1.74%, *Acinetobacter baumannii* 1.55% and *Alkaligenes fecalis* 0.10% (Figure-3.1). Studies carried out by the University of Florida, USA, with 81 patients having UTI. It was found that 89% of infection was due to *Escherichia coli*, 3.7% due to *Klebsiella*, 1.2% due to *Proteus*, 1.2% due to *Citrobacter*, 1.2% due to *Staphylococcus* and *Enterococcus* 3.7%.

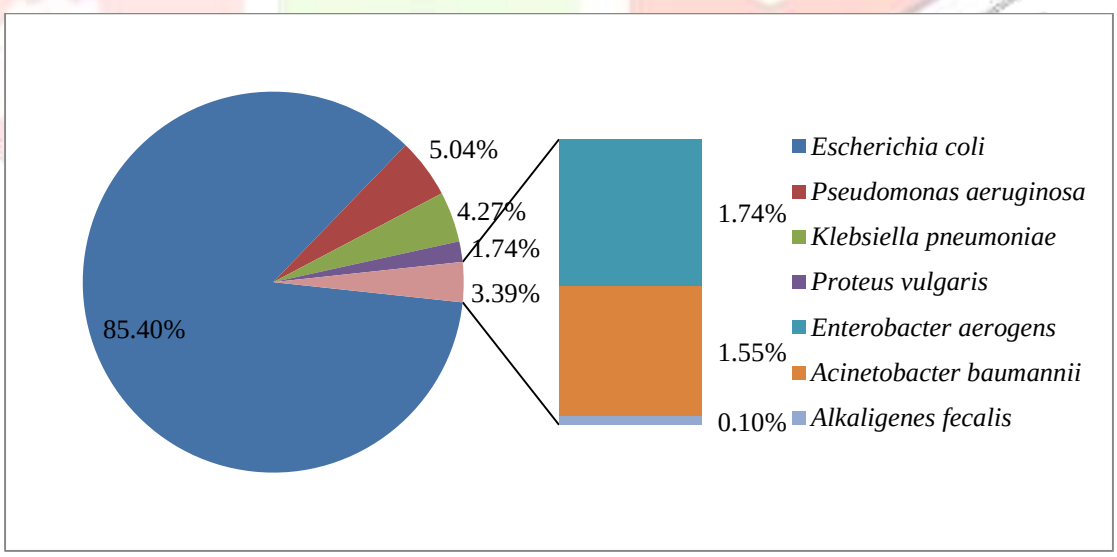


Figure 3.1: Percentage occurrence of bacterial pathogens in UTI

All the identified isolates were studied for antibiotic resistance profile to select MDR UTI pathogens. The results are shown in Table-3.1. *K. pneumoniae* showed maximum resistance, was sensitive to only 2 antibiotics, Nitrofurantoine and Gatifloxacin. *E. coli* showed sensitivity against three antibiotics i.e. Nitrofurantoin, Gatifloxacin and Chloramphenicol. *P. vulgaris* was also sensitive to three antibiotics; Nitrofurantoin, Sparfloxacine and Gatifloxacin while *A. baumannii* was sensitive to Co-trimoxazole, Chloramphenicol, Sparfloxacin and Gatifloxacin. Minimum resistance was observed with *E. aerogens* which showed sensitivity to all tested antibiotics except Co-trimoxazole and Norfloxacin followed by *A. fecalis* which was resistant to only four antibiotics i.e. Ampicillin/Sulbactam, Co-trimoxazole, Ceftizoxime and Tetracycline. Comparing the effect of all antibiotics on the tested seven

bacterial isolates, Gatifloxacin was the only drug which was effective on all the selected UTI pathogens followed by Nitrofurantoin which was effective on all bacterial isolates except *A. baumannii* and Chloramphenicol which showed effectivity on all except *P. vulgaris* and *K. pneumoniae.*

Table 3.1: Antibiotic resistance profile of urinary isolates

Sr. No.	Name of antibiotic	Diameter of zone of inhibition (mm)						
		A.b.	***A.f.***	***E.a.***	***P.v.***	***K.p.***	***E.c.***	***P.a***
1.	**Ampicillin/ Sulbactam**	00	00	27	00	00	00	00
2.	**Co-trimoxazole**	30	00	10	00	00	00	00
3.	**Ceftizoxime**	00	00	00	00	00	00	00
4.	**Chloramphenicol**	11	14	20	00	00	11	21
5.	**Cephalexin**	00	20	16	00	00	00	00
6.	**Tetracycline**	00	00	16	00	00	00	17
7.	**Ciprofloxacin**	00	19	10	00	00	00	00
8.	**Nitrofurantoin**	00	10	15	19	10	17	18
9.	**Sparfloxacin**	15	21	10	10	00	00	12
10.	**Gatifloxacin**	11	22	15	11	11	11	14
11.	**Norfloxacin**	00	17	00	00	00	00	00
12.	**Ofloxacin**	00	18	12	00	00	00	11

A.b.-Acinetobacter baumannii, A.f.-Alkaligenes fecalis, E.a.-Enterobacter aerogens, P.v.-Proteus vulgaris, K.p.-Klebsiella pneumoniae, E.c.-Escherichia coli, P.a.-Pseudomonas aeruginosa.

3.2 Extractive yield:

The quantity and composition of metabolite of an extract depends on type of extraction, time of extraction, temperature and nature, concentration and polarity of the solvent (Ncube *et. al.*, 2008). In the present study, powdered plant materials of stem bark of *C. nurvala* was extracted individually with different solvent (methanol, ethanol, acetone, petroleum ether and water) by Soxhlet extraction method showing extractive yield varied among different plant material and different solvent as the extractive values depends on the phytoconstituents present in plant and their solubility in a particular solvent.

The extractive % yield of different solvent extract is shown in Figure-3.2. Solvent wise rank can be given from high to low % extractive yield in following order:

C. nurvala: Water > Acetone > Methanol > Ethanol > Petroleum ether

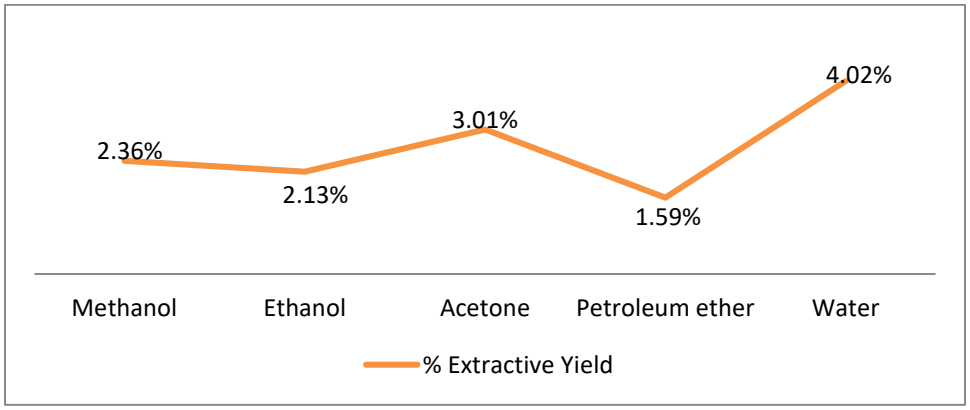


Figure 3.2.: % Extractive yield of *Crataeva nurvala* with different solvent

3.3. Preliminary phytochemical analysis:

Phytochemical screening of different solvent extract of the *C. nurvala* showed variations in presence of phytoconstituents such as alkaloid, tannin, flavonoids, carbohydrates, terpenoids, cardiac glycosides and saponin and the results are summarized in Table-3.2. Terpenoids and cardiac glycoside were present in all the solvent extracts while anthocyanide, phenolic flavonoids and protein were absent in all the solvent extracts. Petroleum ether extract showed minimum phytoconstituents.

Table 3.2: Preliminary phytochemical analysis of *C. nurvala* solvent extracts

"+" = Present and "-" = Absent.

Sr. No.	Phytochemicals	Methanol	Ethanol	Acetone	Petroleum ether	Water
1.	Alkaloid	+	+	+	-	+
2.	Tannin	+	+	+	-	+
3.	Saponin	+	-	+	-	+
4.	Anthocyanide	-	-	-	-	-
5.	Phenolic flavonoids	-	-	-	-	-
6.	Flavonoids	+	+	+	-	-
7.	Carbohydrate	+	-	-	-	+
8.	Protein	-	-	-	-	-
9.	Terpenoids	+	+	+	+	+
10.	Cardiac glycoside	+	+	+	+	+
11.	Oil	+	+	+	-	-

3.4 Determination of antibacterial activity and MIC:

Antibacterial activity of *C. nurvala* is represented in Table-3.3. All the selected MDR urinary isolates was inhibited by all solvent extracts of *C. nurvala* and zone of inhibition was not observed with control. Among five extracts of *C. nurvala*, acetone extract showed greater antibacterial activity against all tested MDR uropathogens, except *K. pneumoniae* (12 mm). Maximum inhibition zone was obtained against E. *coli* (27 mm) followed by *A. fecalis, P. vulgaris* and *P. aeruginosa* (26 mm), 21 mm against *A. baumannii* and 20 mm against *E. aerogens*. Petroleum ether extract showed minimum antibacterial activity as it possesses minimum phytochemical compounds. Ethanol extract was not effective on *A. fecalis* and *P. aeruginosa* was not inhibited by methanol extract while *A. baumannii* was not inhibited by water extract.

Table 3.3: Antibacterial activity of *Crataeva nurvala*

Sr. No.	Solvent	Diameter of zone of inhibition in mm						
		A.b.	***A.f.***	***E.a.***	***P.v.***	***K.p.***	***E.c.***	***P.a***
1.	Methanol	15	12	10	11	17	12	00
2.	Ethanol	12	00	12	10	16	12	14
3.	Acetone	21	26	20	26	12	27	26
4.	Petroleum ether	14	00	09	00	13	00	00
5.	Water	00	11	14	11	18	13	12
6.	Control	00	00	00	00	00	00	00

A.b.-Acinetobacter baumannii, A.f.-Alkaligenes fecalis, E.a.-Enterobacter aerogens, P.v.-Proteus vulgaris, K.p.-Klebsiella pneumoniae, E.c.-Escherichia coli, P.a.-Pseudomonas aeruginosa.

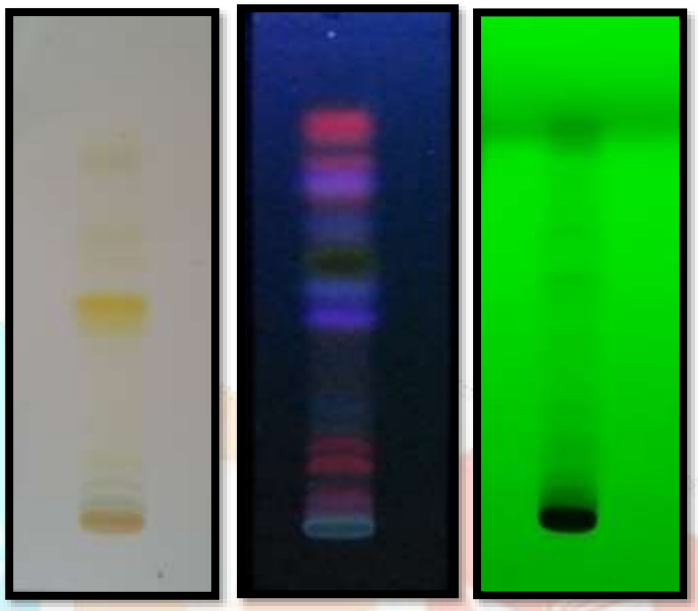
The zone of the inhibition noted with acetone extract against *E. coli* was approximately 1.5 times higher than inhibition zone obtained with Nitrofurantoine (17 mm) and 2.5 times more active than zones obtained with Chloramphenicol and Gatifloxacin. Similarly, the zone of inhibition obtained against *K. pneumoniae* (12 mm) which was nearer to the inhibition zone obtained with Nitrofurantoin (10 mm) and Gatifloxacin (11 mm). Chloroform extract of stem bark of *Varun* (*Crataeva nurvala*) is found to be effective against gram negative (*E. coli*) mediated urinary tract infection (Bal *et. al*., 2016). MIC was also determined by well diffusion assay and the lowest MIC was observed with acetone extract of *C. nurvala* against *E. coli* (0.31 mg/ml) and maximum MIC was obtained against *K. pneumoniae* (2.50 mg/ml).

3.5. HPTLC bioautography to find antibacterial compound:

The acetone extracts of *Crataeva nurvala* was subjected to HPTLC analysis by specific solvent system, toluene: ethyl acetate: formic acid (5: 4: 1) and Rf values of separated phytoconstituents were calculated at visible light, 254 nm and 366 nm. The HPTLC images shown in Figure-3.3 indicate that the phytoconstituents were clearly separated. In the acetone extract of *C. nurvala*, nine bands were observed at visible light having Rf values of 0.051, 0.063, 0.265, 0.620, 0.683, 0.721, 0.765, 0.821 and 0.835, fourteen bands

were observed at 366 nm showing Rf values of 0.043, 0.059, 0.074, 0.135, 0.172, 0.419, 0.469, 0.525, 0.584, 0.592, 0.668, 0.695, 0.734 and 0.839 while only seven bands were detected at 254 nm with Rf values of 0.232, 0.320, 0.385, 0.708, 0.753, 0.805 and 0.820.

HPTLC bioautography of *C. nurvala* stem bark acetone extract has been done to find antibacterial compounds against *E. coli* and *K. pneumoniae.* No growth of *E. coli* was observed on TLC plate while the growth of *K. pneumoniae* was not inhibited (Figure-3.4**)** indicating that the acetone extract of *C. nurvala* has antibacterial potential against *E. coli* due to combined effect of all phytoconstituents present in it.



Visible light 366 nm 254 nm

Figure 3.3: HPTLC chromatogram of acetone extract of *Crataeva nurvala*

It is interesting to note that in the HPTLC bioautography studies, no antibacterial activity has been recorded for acetone extract of *C. nurvala* against *K. pneumoniae,* while the same extract has shown zone of inhibition against the respective organisms during antibacterial activity assessment using agar well diffusion method. This might be due to the synergistic activity is more than one substance or compounds separated on TLC plate or may be due to application of less amount of plant extract, as 10 µl extract was applied on TLC plate while 100 µl was in well diffusion method . During the separation of these extract components on TLC plate, the compounds responsible for the activity at synergistic level might have separated from each other and lost their antimicrobial activities as an individual substance or compound.

(a) **(b)**

Figure 3.4: HPTLC Bioautography of *Crataeva nurvala* acetone extract against (a) *E. coli* and (b) *K. pneumoniae*

3.6. HPLC-MS analysis:

The *C. nurvala* acetone extract chromatogram (Figure-3.5) revealed numerous overlapping peaks. Total 144 unique mass signals were noted for *C. nurvala* acetone extract out of which, 92 compounds were identified by comparison against the Metlin metabolomics database. The result showed greater phytochemical diversity. Several phytochemicals were identified out of which, Cuscohygrine, SNAP, Nefopam, Diuron, Myricetin, Dihydromyricetin, Rutin, Dihydrodeoxystreptomycin were the major compounds (Table-3.4).

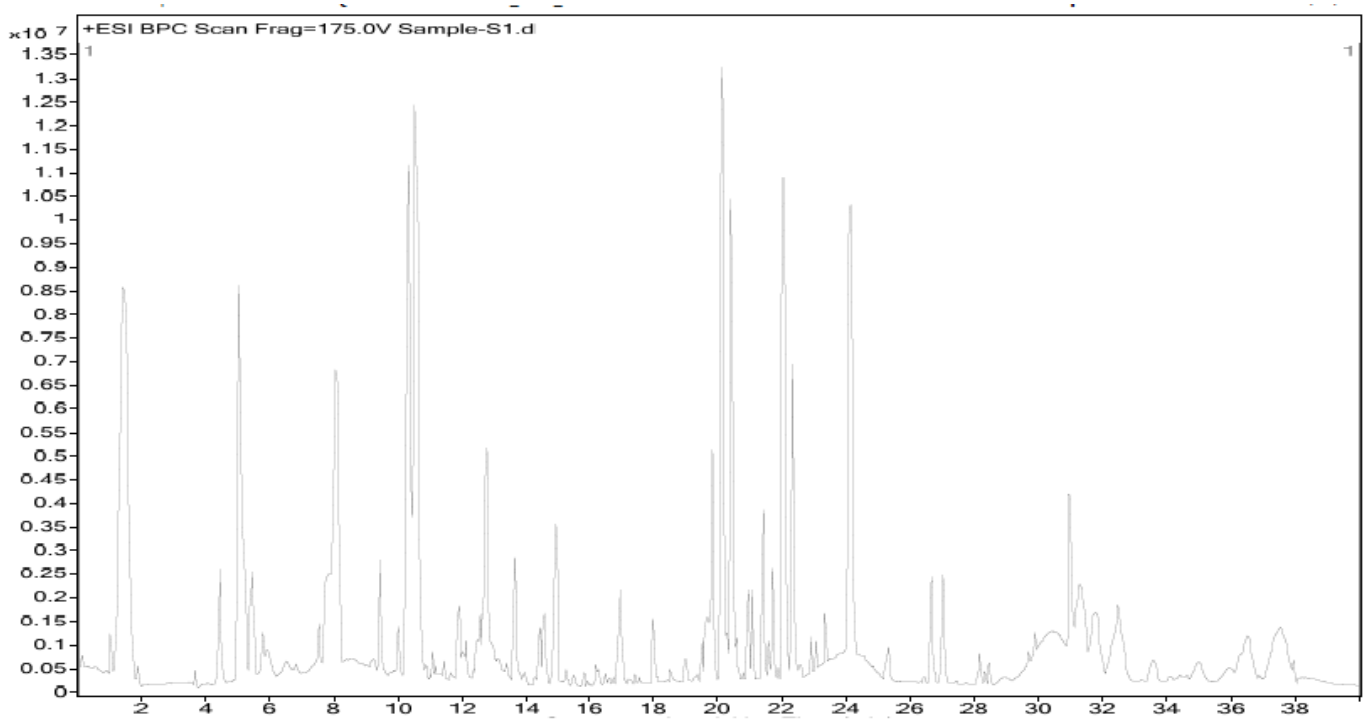


Figure 3.5: HPLC-MS spectra of *C. nurvala* acetone extract

Table 3.4: Major compounds of *C. nurvala* acetone extract identified by HPLC-MS analysis

Sr. No.	Retention Time	Name	Formula
1.	0.168	Cuscohygrine	$C_{13}H_{24}N_2O$
2.	5.166	SNAP (S-Nitroso-N-Acetylpenicillamine)	$C_7H_{12}N_2O_4S$
3.	5.323	Nefopam	$C_{17}H_{19}NO$
4.	8.073	Diuron	$C_9H_{10}Cl_2N_2O$
5.	14.582	Dihydromyricetin	$C_{15}H_{12}O_8$
6.	14.582	Rutin	$C_{27}H_{30}O_{16}$
7.	15.265	Myricetin	$C_{15}H_{10}O_8$
8.	21.892	Dihydrodeoxystreptomycin	$C_{21}H_{41}N_7O_{11}$

Cuscohygrine is a type of alkaloid; Myricetin, Dihydromyricetin and Rutin are types of Flavonoids. In a study using radioactive precursors, Mori and colleagues showed that DNA synthesis was strongly inhibited by Myricetin, Epigallocatechin and robinrtin in *P. vulgaris*, whilst RNA synthesis was most affected in *S. aureus*. Protein and lipid synthesis were also affected but to a lesser extent. The authors suggested that B ring of flavonoids may play a role in intercalation or hydrogen bonding with the stacking of nucleic acid bases and that this may explain the inhibitory action on DNA and RNA synthesis (Mori *et. al*., 1987). The antibacterial activity of *C. nurvala* plant extract might be due to presence of these phytoconstituents and might be due to one of the mechanisms mentioned above.

3.7 Cytotoxicity assay:

From the results obtained with MTT Assay of acetone extract of *Crataeva nurvala* (Table-3.5), LC_{50} value was obtained at maximum exposed dose i.e. 500.0 µg / ml. The minimum concentration required to inhibit *E. coli* was 0.31 mg/ml at which 40 % inhibition was obtained indicating less cytotoxic effect of *C. nurvala* acetone extract. Moderate antibacterial and cytotoxic activities was also obtained with chloroform crude extract of stem bark of *Crataeva nurvala* indicate that the extract can be used as a remedy against urinary tract infections caused by pathogenic bacteria (Shumaia *et. al*., 2012).

Results of % viability of *Crataeva nurvala* acetone extract on *HeLa* cell line was carried out by using Trypan blue dye Exclusion technique and the results are described in Figure-3.6. The results showed dose dependent response. Less inhibition at minimum exposed concentration (5%) i.e. 94%, cell viability was obtained, while with highest exposed dose (20%), 82% viability was obtained which is higher than the % viability obtained with positive control (73%).

Table 3.5: MTT assay of *Crataeva nurvala* acetone extract in different concentration

% Dose concentration	Concentration in µg/ml	Average OD	% Inhibition
Control	0.0	1.82	0.0
1	5.0	1.75	3.888
2	10.0	1.736	4.629
4	20.0	1.74	4.444
6	30.0	1.616	11.29
10	50.0	1.526	16.29
15	75.0	1.536	15.74
25	125.0	1.47	19.44
50	250.0	1.253	31.48
75	375.0	1.073	41.48
100	500.0	0.906	50.74

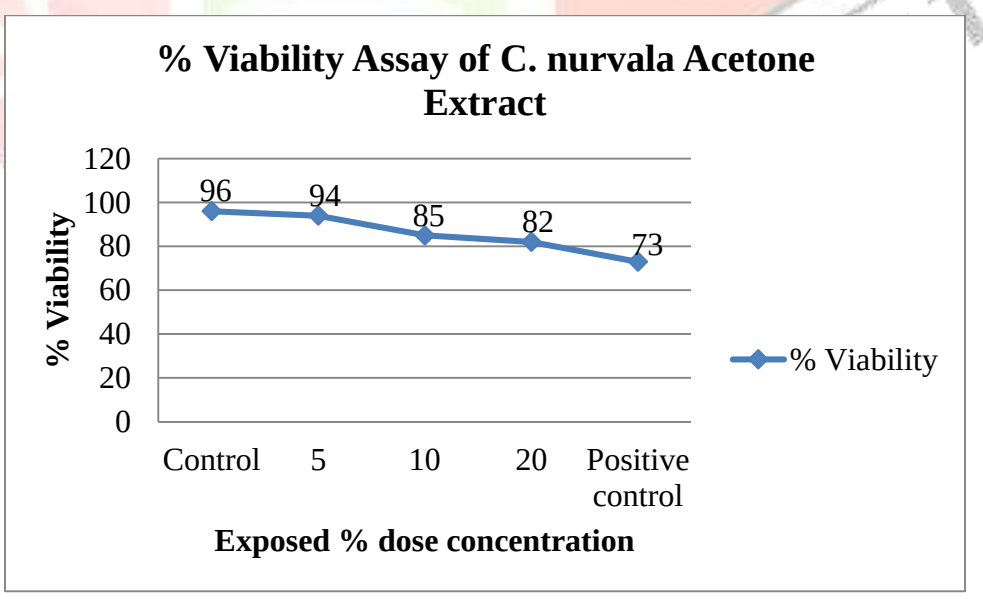


Figure 3.6: Viability assay of *Crataeva nurvala* acetone extract exposed in different concentration

Results of MTT and Viability Assay of acetone extract of *C. nurvala* showed less cytotoxic effect which indicates that this extract can be used as a remedy against urinary tract infections caused by multi drug resistant pathogenic bacteria.

IV. CONCLUSION

Crataeva nurvala acetone extract has remarkable antibacterial activity as compare to synthetic antibiotics. It may be considered as a potential source of new chemotherapeutic drugs because of their diverse phytochemicals and little or no toxic effect and may have potential to use as an alternative therapy to treat UTI because of having good antibacterial activity against highly resistant UTI pathogens.

V. SUGGESTIONS FOR FURTHER RESEASRCH

Further work can be focused on extraction, purification and identification of bioactive phytocompounds and their mode of action on these bacterial pathogens as well as *in vivo* stability, toxicity and efficacy of the bioactive phytoconstituents in the management of UTI caused by such multi drug-resistant bacteria.

REFERENCES

Bal, N., Chaubey, S., Tiwari, R.C., Kour, G.D., and Dhyani, S. (2016). Varun (*Crataeva Nurvala* Buch-Ham): A Critical Review w.s.r. to Urinary Tract Disorder. *International Journal of Ayurveda and Pharma Research*, 4(4), 49-52.

Baris, O., Gulluce, M., Sahin, F., Ozer, H., Kilic, H., and Ozkan, H. (2006). Biological activities of the essential oil and methanol extract of Achillea Biebersteinii Afan. (Asteraceae). *Turk J Biol*, 30, 65-73.

Benkeblia, N. (2004). Antimicrobial activity of essential oil extract of various onions (*Allium sativum*). *Lebensm-Wiss-u-Technol*, 37, 263-268.

Choudhury, K. D. (2012). Anti Bacterial Activity of Some Plants Belonging to the Family Rubiaceae: A Review. *World Journal of Pharmacy and Pharmaceutical Sci-ences*, 1(3), 1179-1194.

Clinical, L.S.I. (2007). Performance Standards for Antimicrobial susceptibility testing, Nineteenth Informational Supplement. *CLSI Document*, 27.

Colombo, M. L., and Bosisio, E. (1996). Pharmacological activities of *Chelidoniummajus L* (Papaveraceae). *Pharmacol Res*, 33, 127-134.

Foxman, B. (2003). Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. *Dis Mon*, 49(2), 53-70.

Gallo, M.B.C. and Sarachine, M.J. (2009). Biologibal activities at Lupeol. International *Journal of Biomedical and Pharmaceutical Sciences*, 3(1), 44-46.

Griffiths, P. (2003). The Role of Canberry Juice in the Treatment of Urinary Tract Infection. *Br J Community Nurses*, 8, 557-561.

Head, K.A. (2008). Natural approaches to prevention and treatment of the lower urinary tract. *Alternative Medicine Review*, 13(3), 227.

Holt, J.G., Kreig, N.R., Sneath, P.H.A., Staley, J.T. and Williams, S.T. (1994). In: *Bergy's Manual of Determinative Bacteriology*, ninth ed. Williams and Wilkins Pub., MD, USA.

Magaldi, S., Mata-Essayag, S., Hartung de Capriles, C., Perez, C., Colella, M., and Olaizola, C. (2004). Well diffusion for antifungal susceptibility testing. *International journal of infectious diseases*, 8, 39-45.

Mori, A., Nishino. C., Enoki, N., and Tawata, S. (1987). Antibacterial activity and mode of action of plant flavonoids against *Proteus vulgaris* and *Staphylococcus aureus*. *Phytochemistry*, 26, 2231-2234.

Ncube, N.S., Afolayan, A.J., and Okoh, A.I. (2008). Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. *African Journal of Biotechnology*, 7 (12), 1797-1806.

Rojas, R., Bustamante, B., and Bauer (2003). Antibacterial activity of selected Peruvian medicinal plants. *J Ethnopharmacol*, 88, 100-204.

Shumaia, P., Md. Abdul, K., Md. Ajijur, R., Mir, I.B.W., and Md. Ekramul, H. (2012). Antibacterial Activities and Brine Shrimp Lethiality Bioassay of the Chloroform extract of stem bark of *Crataeva nurvala buch ham. Int J Pharm Sci & Res*, 3(3), 830-34.

Shumaia, P., Md. Abdul, K., Md. Ajijur, R., Mir, I.B.W., and Md. Ekramul, H. (2012). Antibacterial Activity and Brime Shrimp Lethiality Bioassay of the Chloroform Extract of stem bark of *Crataeva nurvala* buch ham. *Int J Pharm Sci & Res*, 3(3), 830-834.

Silva, M.T., Simas, S.M., Batista, T.G., Cardarelli, P., and Tomassini, T.C. (2005). Studies on antimicrobial activity, *in vitro*, of *Physalis angulata L*. (Solanaceae) fraction and physalin B bringing out the importance of assay determination. *Memorias do Instituto Oswaldo* Cruz, 100, 779-782.

Soulsby, J. (2005). Resistance to antimicrobials in humans and animals. *Braz J Med*, 331, 1219- 1220.

Trease, G.E. and Evans W.C. (1989). *A Textbook of Pharmacognosy*. 13th edition; 83, 685. Brailliere Tiridall Ltd., London, ISBN: 0702013617.

Vaughan, V. (2007). *C. difficile* endemic in health service. *Health Serv J*, 117(6038), 6.

WHO (1995). Research Guidelines for Evaluating the Safety and Efficacy of Herbal Medicines. Manila.