IJPSR (2021), Volume 12, Issue 10 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 15 October 2020; received in revised form, 14 February 2021; accepted, 24 May 2021; published 01 October 2021

ANTIMICROBIAL ACTIVITY OF *TERMINALIA BELLERICA* (GAERTN.) ROXB. AGAINST MULTIDRUG-RESISTANT *STAPHYLOCOCCUS SAPROPHYTICUS*

Tamalika Chakraborty [1], Sumana Chatterjee [*2], Lopamudra Datta [2] and Abhijit Sengupta [2]

Department of Life Science [1], Department of Pharmacy [2], Guru Nanak Institute of Pharmaceutical Science and Technology, 147/F, Nilgunj Road, Panihati, Kolkata - 700110, West Bengal, India.

Keywords:

Antimicrobial, Screening, Multi-Drug Resistance, Phytochemicals, Purification

Correspondence to Author:
Dr. Sumana Chatterjee

Professor,
Department of Pharmacy,
Guru Nanak Institute of Pharmaceutical Science and Technology, 147/F, Nilgunj Road, Panihati, Kolkata - 700110, West Bengal, India.

E-mail: sumana.chatterjee@gnipst.ac.in

ABSTRACT: The present study investigates the isolation and identification of Multi-Drug Resistant bacteria from Hospital Effluent and the determination of Antimicrobial activity of *Terminalia bellerica* (Roxb.) against the isolated Multi Drug-Resistant Bacteria. Hospital effluent was collected and screened for the presence of Drug resistance phenotype against five different antibiotics, and the isolated bacterium was identified by 16s rRNA sequencing. *Terminalia bellerica* Roxb., Baheda has established antimicrobial activity against Gram-negative and Gram-positive bacteria hence the above plant was selected for the present investigation. Ethanolic extract of *Terminalia bellerica* outer coat, fruit, and the seed was tested for antimicrobial activity against the isolated Multi Drug-Resistant Bacteria, and the MIC_{90} value was determined. Ethanolic extract of *T. bellerica* outer coat having the highest zone of inhibition was further fractionated, and four individual fractions, F1 (water), F2 (50% ethanol and water), F3 (ethanol), and F4 (acetone) were tested for antimicrobial activity against isolated Multi Drug-Resistant Bacteria. *Staphylococcus saprophyticus*, which was isolated from Hospital effluent and identified by 16 s-rRNA sequencing, was found to be resistant to five antibiotics with the highest resistance against Cefixime MIC_{90} 24 ± 0.00 (µg/ml). Crude extract of *Terminalia bellerica* outer coat obtained by maceration gave the highest zone of inhibition of 3.4 ± 0.17(cm). Fraction F4 gave highest zone of inhibition 3.2 ± 0.17 (cm) with MIC_{90}12 ± 0.00 (µg/ml). The extractive value and yield of fraction F4 are 1500mg and 0.030%. Phytochemical test of F4 was found to contain tannin and alkaloid.

INTRODUCTION: Antibiotics are boon to society as these drugs have potentially reduced the incidence of infectious diseases and death due to them. Antibiotics can be effective when prescribed correctly and used rationally, but unfortunately, the present scenario is different [1]. There is the emergence of Antibiotic-resistant bacteria, which is a present threat to the environment and society.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.12(10).5353-63

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.12(10).5353-63

Antibiotic resistance stems from the abuse and irrational use of antibiotics; moreover, antibiotic resistance among bacteria can spread over several Genera making sensitive bacteria resistant [2].

Some bacteria are resistant to a single antibiotic, whereas others are resistant to multiple different antibiotics and are often referred to as Multi Drug-Resistant Bacteria (MDR). There are several groups of bacteria that have been proved to be a potential MDR, such as MRSA (Methicillin-Resistant *Staphylococcus aueus*), *Escherichia coli*, *Haemophilus* sp and many other β- Lactamase producer. *Pseudomonas aeruginosa* is known to have multidrug efflux pump, which can forcefully

expel nonspecific antibiotics out of the cell, whereas *Escherichia coli* has accumulated many antibiotic-resistant gene in a cassette located in an R(resistant) plasmid [1, 3-4]. The present scenario demands a new antimicrobial agent to combat multidrug-resistant bacteria. There are many newly synthesized antimicrobial agents of chemical origin but with a short life expectancy; hence, herbal products from Indian Medicinal Plants can be an alternative remedy for the present problem. A new lead molecule of herbal origin needs to be identified which will be a potential antimicrobial agent or a good resistance modifier [5, 6.]

Terminalia bellerica belongs to the family "Combretaceae" and is commonly known as belleric Myrobalan is a fast-growing deciduous tree with a rounded crown. It can even grow up to 50 meters [7]. The bark is bluish or ash-grey, whereas the inner bark is yellow. Fruits are sub-globular to broadly ellipsoid, light yellow, and have brown tomentosa. Leaves are found to belong, oval, long, and usually clusters towards the end of the branch. Flowers are white and yellow with an offensive odour. It is also called as Baheda in India and has been long used in Indian Herbal Medicine. It is anti-helminthic, digestive, laxative and astringent useful in ailments like cough, diarrhoea, dyspepsia, asthma, bronchitis *etc*. [8, 9] Many Phyto-constituents have been isolated from the fruit such as Anthraquinones, Chebulagic acid, Gallic acid *etc*.; hence, this research is an attempt to study the antimicrobial activity of *T. bellerica* fruit against Multi Drug Resistant *Staphylococcus saprophyticus* which is responsible for community-acquired urinary tract infection.

METHODS:

Source of the Pathogen: Effluent from a Dental hospital (Guru Nanak Institute of Dental Science and Research, Sodepur) was collected for the isolation of MDR. Hospital effluent was collected because many antibiotics were applied to the patient during surgery and other treatments, which were ultimately discharged into the wastewater, and hence, the probability of getting a drug-resistant pathogen was more.

Isolation of Microbial Pathogen: The sample was subjected to serial dilution in 0.9% Saline and plated in Nutrient Agar using Pour Plate Technique for isolation of individual colony. The colonies obtained after incubation at 37 °C for 24 h were further screened for antibiotic resistance [1].

Screening of Colonies for Antibiotic Resistance: Two well-distinguished colonies having opaque white and golden yellow colour respectively were selected for further investigation and hence, pure cultures were prepared in slant as well as broth. The focus was given to the Golden yellow colony as *Staphylococcus* species. Produces Golden yellow colony and, moreover, there is an increased emergence of MRSA (Methicillin-Resistant *Staphylococcus aureus*) [6, 10].

Antibiotics Susceptibility Profiling of the Isolated Colonies: The above-selected colonies were screened for antibiotic resistance against five following antibiotics, namely Azithromycin, Clarithromycin, Amoxicillin, Cefixime, and Tetracycline. Considering the MIC_{90} according to Clinical and Laboratory Standards Institute (CLSI) guidelines 2016, following concentrations of 4 µg/ml, 8 µg/ml, 12 µg/ml, 16 µg/ml, 24 µg/ml and 30 µg/ml were prepared. All the tests were performed in Muller Hinton (HI-Media) Agar Plates [11, 12-13].

Biochemical Characterization of Selected Colony Following Bergey's Manual: The selected colony which was found to be resistant was subjected to morphological identification using Gram's staining method followed by its bio-chemical characterization through various Bio-chemical tests such as Catalase, Oxidase, Glucose Fermentation, and Lactose fermentation, Mannitol Fermentation, Methyl red, Voges Proskauer and Indole test. *Staphylococcus* sp. was finally confirmed by growing in Blood agar and determining of nature of its hemolysis. The pathogenicity of *Staphylococcus* species was determined by performing Coagulase Test. All the tests were performed according to Standard Bergey's Manual of Bacteriology to characterize the microorganism accurately [14, 15].

Identification of *Staphylococcus* sp by 16s rRNA Sequencing: DNA was isolated from the isolated culture. The DNA quality obtained was evaluated on Agarose Gel of 1.0% and found to have one single band. Fragment of the 16S rDNA gene was further amplified by 27F using 1492R primers.

Further analysis resulted in a single discrete band of 1500bp when resolved in Agarose Gel. The PCR (Polymerase Chain Reaction) amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forwarding primer and reverse primers using BDT v3.1 Cycle sequencing kit with ABI 3730xl Genetic Analyzer. A consensus sequence of the 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the database of NCBI GenBank database. A maximum identity score was obtained and the first ten sequences are further selected and subjected to alignment using Clustal W, a multiple alignment software. A distance matrix was generated, and the phylogenetic tree was constructed using MEGA 7.

Preparation of Plant Extract: Fresh dry fruit of *Terminalia bellerica* was collected from the areas of North 24 Parganas. The fruit was authenticated from the Central National Herbarium-Botanical Survey of India, Kolkata. The fruit was washed 3-4 times and further dried under shade. The fruit was further crushed and separated into Exocarp (Outer Coat), Endocarp (Fruit) and seed. Each part of the fruit is converted to a fine powder and subjected to extraction.

Extraction Procedure: All the parts of Baheda fruit were extracted by Maceration, whereas the Outer Coat was extracted both by Maceration and Ultrasonic Assisted Extraction (UAE) [16].

Maceration: 10g of finely grounded powder was taken and dissolved in 100ml of 95% Ethanol. The samples were subjected to shaking at 120 rpm at 25 °C for 7 days until the extraction was complete. The extract was filtered and evaporated in Rotary Vacuum Evaporator at 45 °C until dry. 1 g of ethanol extract was mixed in 5ml of ethanol to give a concentration of 0.2mg/ml

Ultrasonic Assisted Extraction: 5g of Baheda Outer coat was taken and suspended in 95% Ethanol in the ratio of (1: 20 w/v). The content was mixed thoroughly, and the Ultrasonic bath was set at 25 °C for 15 min. Ultra-sonication was done for 30 min. After the completion of extraction, the extract was centrifuged at 3000 rpm for 15 min. The supernatant was collected, filtered and evaporated in Rotary Vacuum Evaporator at 45 °C until dry. 1 g of ethanol extract was mixed in 5ml of ethanol to give a concentration of 0.2mg/ml [17].

Determination of Antimicrobial Activity of *Terminalia bellerica* against MDR *Staphylococcus saprophyticus*: Crude extract of Outer coat, fruit, and seed extracted by Maceration and Ultrasonic Assisted extraction was charged against isolated *Staphylococcus saprophyticus* inoculum volume (0.1 ml) containing 10^8 cells for determination of antimicrobial activity. The test was performed using the standard Disc Diffusion method using sterile paper discs (5mm diameter). The Test Disc with crude extract, Ethanol as Control, and Cefixime as Standard were placed in a lawn of *S. saprophyticus* swabbed in Muller Hinton Agar Plates. Bacterial cultures were further incubated at 37 °C for 24 h. Zone of Inhibitions was measured and all the tests were performed in triplicates and the average zone of inhibition was noted [18].

Phytochemical Study of *Terminalia bellerica*: The extract with the highest zone of inhibition was selected and phytochemical constituents were identified by various phytochemical tests. The yield and extractive values were calculated according to Indian Pharmacopoeia 2014 [19, 20-21, 32].

Fractionation of *Terminalia bellerica* Outer Coat: 5g of Ethanolic extract of Baheda Outer Coat was absorbed with 10g of silica, the adsorbed material was washed with 50 ml of Acetone. The Acetone soluble portion was collected, and the insoluble portion was washed with 50 ml 100% Ethanol. Ethanol soluble portion was collected, and insoluble portion was washed with 50% Ethanol/ water followed by 100% demineralized water. The following fractions were dried and the extractive value, yield of the fractions was further determined [22].

Determination of Antimicrobial Activity of *Terminalia bellerica* Fractions against MDR *Staphylococcus saprophyticus*: The test was performed using the standard Disc Diffusion method using sterile paper discs (5mm diameter). Test Discs with crude extract of all the four fractions, Ethanol as Control and Cefixime as Standard, were placed in a lawn of *S. saprophyticus* swabbed in Muller Hinton Agar Plates and the

cultures were further incubated at 37 °C for 24 h. Zone of Inhibition was measured and all the tests were performed in triplicates and the average zone of inhibition was noted [23].

Determination of MIC_{90} (Minimum Inhibitory Concentration) of *Terminalia bellerica* Fractions against MDR *Staphylococcus saprophyticus*: Each fraction was dissolved in 10% DMSO (Dimethyl Sulfoxide) for the determination of Minimum Inhibitory Concentration of the Extract against the isolated MDR. Concentrations of 4 µg/ml, 8 µg/ml, 12 µg/ml, 16 µg/ml, 24 µg/ml, 30 µg/ml were prepared for all the fractions, and the minimum inhibitory concentration was determined against the MDR *Saprophyticus* [24, 25-26].

Determination of Phytochemicals Associated with *Terminalia bellerica* Fractions: Phyto-chemical tests for all the fractions were performed namely Ferric chloride test and Gelatin test for Tannins, Shinoda test, Ferric chloride test and Zn/HCL reduction test for Flavonoids, Mayer's test, and Wagner's test for alkaloid, Solubility test and filter paper test for Fats, Ninhydrin test and Biuret test for proteins and Salkowski test for steroid [19, 20-27, 28].

RESULTS:

Characterization of Isolated Microorganism from Hospital Effluent:

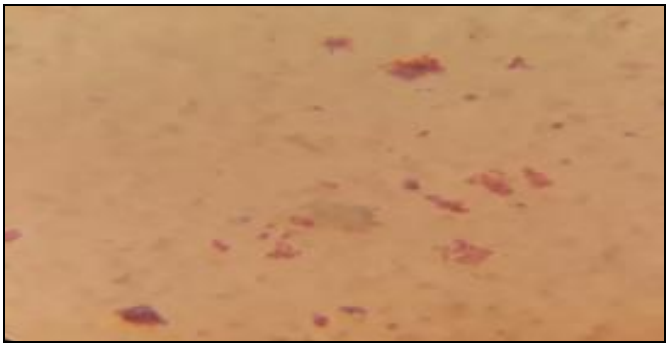


FIG. 1: GRAM POSITIVE COCCI IN BUNCHES AS OBSERVED IN 100X OBJECTIVE

TABLE 1: BIOCHEMICAL TESTS DONE ACCORDING TO STANDARD BERGEY'S MANUAL

S. no.	Biochemical Test	Results
1	Catalase	Positive
2	Oxidase	Positive
3	Glucose Fermentation	Negative
4	Lactose Fermentation	Positive
5	Mannitol Fermentation	Positive
6	Methyl Red	Positive
7	Voges Proskauer	Positive
8	Indole	Positive
9	Coagulase	Negative

Determination of Phenotypic Antibiotic Resistance against *Staphylococcus saprophyticus:*

TABLE 2: REFERENCE RANGE OF ANTIBIOTICS SUSCEPTIBILITY OF *STAPHYLOCOCCUS SAPROPHYTICUS* ACCORDING TO CLSI GUIDELINES 2016

Antibiotics	Sensitive (µg/ml)	Intermediate Resistant (µg/ml)	Resistant (µg/ml)
Amoxicillin	≤ 0.25	-	≥ 0.5
Cefixime	≤ 8	16	≥ 32
Azithromycin	≤ 2	4	≥ 8
Clarithromycin	≤ 2	4	≥ 8
Tetracyclin	≤ 4	8	≥

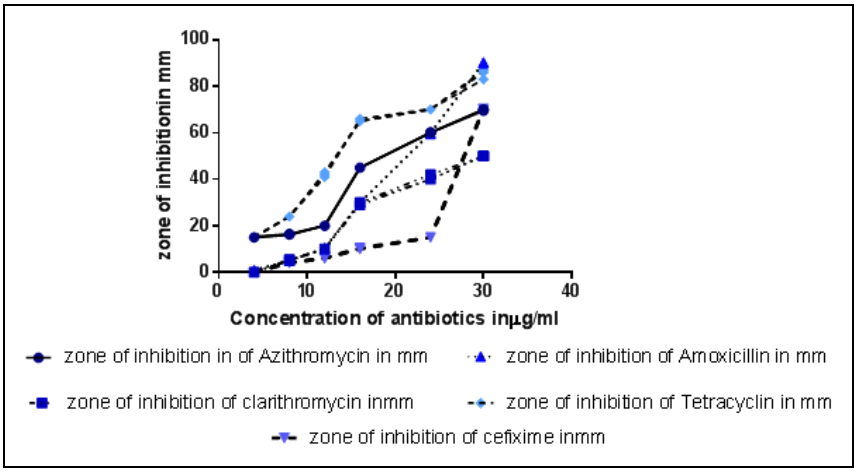


FIG. 2: COMPARATIVE STUDY OF ZONE OF INHIBITION IN (mm) OF 5 ANTIBIOTICS AGAINST ISOLATED *STAPHYLOCOCCUS SAPROPHYTICUS*

TABLE 3: AVERAGE ZONE OF INHIBITION (mm) OBTAINED FROM CHARGING 5 ANTIBIOTICS AGAINST *STAPHYLOCOCCUS SAPROPHYTICUS*. AVERAGE ZONE OF INHIBITION OBTAINED BY PERFORMING THE EXPERIMENTS IN TRIPLICATE

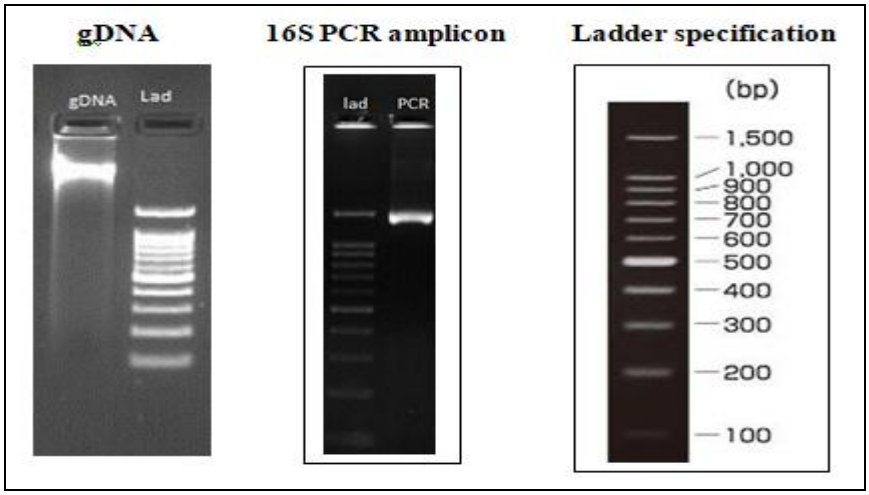
Concentration of Antibiotics (µg/ml)	Azithromycin Zone of Inhibition (mm)	Clarithromycine Zone of Inhibition (mm)	Amoxicillin Zone of Inhibition (mm)	Cefixime Zone of Inhibition (mm)	Tetracycline Zone of Inhibition (mm)
4	15	0	1	0	15
8	16	5	5	4	24
12	20	10	10	6	42
16	45	30	30	10	65
24	60	40	60	15	70
30	70	50	90	70	86

TABLE 4: OVERALL RESISTANCE PATTERN OF ISOLATED MDR *STAPHYLOCOCCUS SAPROPHYTICUS* ACCORDING TO CLSI 2016

Antibiotics	Experimental MIC (µg/ml)	Standard MIC (µg/ml)	Result
Azithromycin	16	8	Resistant
Clarithromycin	16	8	Resistant
Amoxicillin	16	2	Resistant
Cefixime	24	4	Resistant
Tetracycline	12	4	Intermediate Resistant

Identification of the Isolated Multidrug-Resistant Bacteria: The above specimen showed high similarity with Staphylococcus *saprophyticus* based on nucleotide homology and phylogenetic analysis.

gDNA and 16S Amplicon QC Data:



Sanger Sequence Chromatogram File Data: >Forward Seq data GGCCAAGATGAATGCTAGTG TTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACG ACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTT TAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGAAAACTCTAGAGATA GAGCTTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTG AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAAGTTGG GCACTCTAGGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCAT GCCCCTTATGATTTGGGCTACACACGTGCTACAATGGACAATACAAAGGGCAGCTAAACCGC GAGGTCATGCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAACTCGACTACAT GAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATACGTTCCCGGGTCTTGT ACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGCCGGTGGAGTAACCATTTAT GGAGCTAGCCGTCGAAGGTGGGACAAATGATTGGGGTGAAGTCGTAACAAGGTAACCGTAA GC.

>Reverse Seq Data GGGGGGGTCTCCAGGCGGAATGCTTAATGCGTTAGCTGCAGCACTAAGGG
GCGGAAACCCCCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCC
TGTTTGATCCCCACGCTTTCGCACATCAGCGTCAGTTACAGACCAGAAAGTCGCCTTCGCCAC
TGGTGTTCCTCCATATCTCTGCGCATTTCACCGCTACACATGGAATTCCACTTTCCTCTTCTGC
ACTCAAGTTTCCCAGTTTCCAATGACCCTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTT
AAGAAACCGCCTACGCGCGCTTTACGCCCAATAATTCCGGATAACGCTTGCCACCTACGTATT
ACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGATTAGGTACCGTCAAGATGTGCACA
GTTACTTACACATTTGTTCTTCCCTAATAACAGAGTTTTACGAGCCGAAACCCTTCATCACTCA
CGCGGCGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAG
GAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGTATCGT
CGCCTTGGTAAGCCGTTACCTTACCAACTAGCTAATACGGCGCGGGTCCATCTATAAGTGATA
GCAAAACCATCTTTCACTTTAGAACCATGCGGTTCTAAATGTTATCCGGTATTAGCTCCGGTTT
CCCGAAGTTATCCCAGTCTTATAGGTAGGTTACCCACGTGTTACTCACCCGTCCGCCGCTAAC
TTCAAAGGAGCAAGCTCCTTATCTGTTCGCTCGACTTGCATGTATTAGGCACGCCGCCAGCGT
TCATCCTGAGCCAGGAATCAAACTCTACGGTTACCTTGTTACGAGTTACTGGGTCAGGATTAA
AGAAAACGCTGAAAAGT.

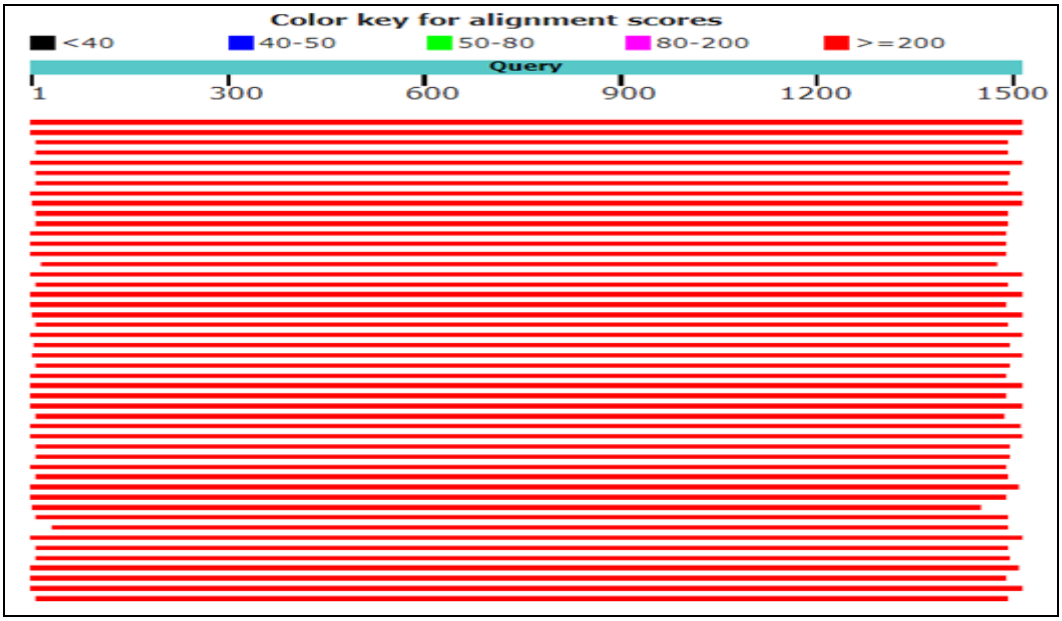
>Reverse complement ACTTTTCAGCGTTTTCTTTAATCCTGACCCAGTAACTCGTAACAAGGTA
ACCGTAGAGTTTGATTCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTC
GAGCGAACAGATAAGGAGCTTGCTCCTTTGAAGTTAGCGGCGGACGGGTGAGTAACACGTGG
GTAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAACATTTA
GAACCGCATGGTTCTAAAGTGAAAGATGGTTTTGCTATCACTTATAGATGGACCCGCGCCGTA
TTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATACGTAGCCGACCTGAGAGGGTG
ATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCT
TCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGGTTTCGGCTCGT
AAAACTCTGTTATTAGGGAAGAACAAATGTGTAAGTAACTGTGCACATCTTGACGGTACCTA
ATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTA
TCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCAC
GGCTCAACCGTGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAAAGTGGAA
TTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTT
CTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGG
TAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGC
TAACGCATTAAGCATTCCGCCTGGAGACCCCCCC.

>S_AUREUS consensus seq CCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGT
CGAGCGAACAGATAAGGAGCTTGCTCCTTTGAAGTTAGCGGCGGACGGGTGAGTAACACGTG
GGTAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAACATTT
AGAACCGCATGGTTCTAAAGTGAAAGATGGTTTTGCTATCACTTATAGATGGACCCGCGCCGT
ATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATACGTAGCCGACCTGAGAGGGT
GATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGAGGCAGCAGTAGGGAATCT
TCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGGTTTCGGCTCGT
AAAACTCTGTTATTAGGGAAGAACAAATGTGTAAGTAACTGTGCACATCTTGACGGTACCTA
ATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTA
TCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCAC
GGCTCAACCGTGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAAAGTGGAA
TTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTT
CTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGG
TAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGC
TAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGAC
GGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCA
AATCTTGACATCCTTTGAAAACTCTAGAGATAGAGCTTTCCCCTTCGGGGGACAAAGTGACAG

GTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTAAGCTTAGTTGCCATCATTAAGTTGGGCACTCTAGGTTGACTGCCGGTGACAAACCG
GAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTA
CAATGGACAATACAAAGGGCAGCTAAACCGCGAGGTCATGCAAATCCCATAAAGTTGTTCTC
AGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAGATCAG
CATGCTACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTGT
AACACCCGAAGCCGGTGGAGTAACCATTTATGGAGCTAGCCGTCGAAGGTGGGACAAATGAT
TGGGGTGAAGTCGTAACAAGGTAACCGTA

DATA: (Alignment view using a combination of NCBI GenBank):

Distribution of 100 Blast Hits on the Query Sequence:



Sequences Producing Significant Alignments:

TABLE 5: IDENTIFICATION OF *STAPHYLOCOCCUS SAPROPHYTICUS*

Description	Max Score	Total Score	Query Cover	E value	Per. Ident.	Accession
Staphylococcus saprophyticus subsp. saprophyticus ATCC15305 16S ribosomal RNA, complete sequence	2756	2756	100%	0	99.67%	NR_074999.2
*Staphylococcus edaphicus*strain CCM 8730 16S ribosomal RNA, partial sequence	2750	2750	100%	0	99.60%	NR_156818.1
*Staphylococcus xylosus*strain KL 162 16S ribosomal RNA, partial sequence	2723	2723	98%	0	99.93%	NR_036907.1
*Staphylococcus xylosus*strain JCM 2418 16S ribosomal RNA, partial sequence	2719	2719	97%	0	99.93%	NR_113350.1
*Staphylococcus succinus*subsp. succinus strain AMG-D1 16S ribosomal RNA, partial sequence	2717	2717	100%	0	99.20%	NR_028667.1
Staphylococcus saprophyticus strain NBRC 102446 16S ribosomal RNA, partial sequence	2700	2700	98%	0	99.59%	NR_114090.1
Staphylococcus saprophyticus subsp. saprophyticus ATCC 15305 16S ribosomal RNA, partial sequence	2700	2700	98%	0	99.66%	NR_115607.1
*Staphylococcus equorum*subsp. linens strain RP29 16S ribosomal RNA, partial sequence	2700	2700	100%	0	99.00%	NR_041926.1
*Staphylococcus succinus*subsp. casei strain SB72 16S ribosomal RNA, partial sequence	2700	2700	99%	0	99.07%	NR_037053.1
Staphylococcus saprophyticus strain JCM 2427 16S ribosomal RNA, partial sequence	2695	2695	97%	0	99.59%	NR_113349.1

Phylogenetic Tree:

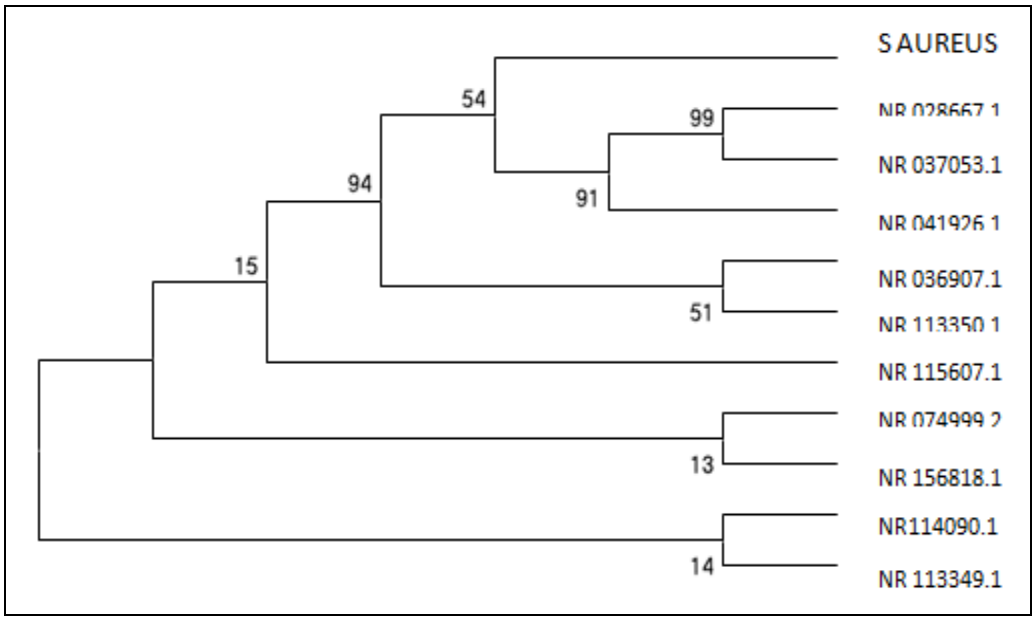


FIG. 3: MOLECULAR PHYLOGENETIC ANALYSIS WAS BASED ON MAXIMUM LIKELIHOOD METHOD AND THE EVOLUTIONARY HISTORY WAS DETERMINED BY MAXIMUM LIKELIHOOD METHOD FOLLOWING KIMURA 2 PARAMETER MODEL [29]

1000 replicates were considered for drawing inference of bootstrap consensus tree and the following data is taken to determine the evolutionary history of the taxa analyzed [30]. Branches associated with the partitions reproduce in less than 50% replicates of bootstrap are collapsed. Next to the initial tree(s) comprise of the percentage of replicate trees which are associated with the taxa clustered together in the bootstrap test. The pairwise distance was analyzed and estimated using the Maximum Composite Likelihood (MCL) approach followed by a selection of topology with superior log likelihood value. The above analysis includes 11 nucleotide sequences. The following codon positions were included as 1^{st} + 2^{nd} + 3^{rd} + Non-coding, followed by elimination of positions containing gaps and missing data. The final data set contained 1434 positions, and the evolutionary analysis was performed in MEGA 7 [31].

Distance Matrix:

TABLE 6: ESTIMATES OF EVOLUTIONARY DIVERGENCE BETWEEN SEQUENCES

S_AUREUS		0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.002	0.003	0.001
NR_074999.2	0.003		0.000	0.001	0.001	0.002	0.000	0.000	0.003	0.003	0.000
NR_156818.1	0.003	0.000		0.001	0.001	0.002	0.000	0.000	0.003	0.003	0.000
NR_036907.1	0.001	0.002	0.002		0.000	0.002	0.001	0.001	0.002	0.002	0.001
NR_113350.1	0.001	0.002	0.002	0.000		0.002	0.001	0.001	0.002	0.002	0.001
NR_028667.1	0.008	0.009	0.009	0.007	0.007		0.002	0.002	0.002	0.001	0.002
NR_114090.1	0.003	0.000	0.000	0.002	0.002	0.009		0.000	0.003	0.003	0.000
NR_115607.1	0.003	0.000	0.000	0.002	0.002	0.009	0.000		0.003	0.003	0.000
NR_041926.1	0.009	0.010	0.010	0.010	0.010	0.010	0.010	0.010		0.003	0.003
NR_037053.1	0.009	0.010	0.010	0.008	0.008	0.001	0.010	0.010	0.011		0.003
NR_113349.1	0.003	0.000	0.000	0.002	0.002	0.009	0.000	0.000	0.010	0.010	

"The number of base substitutions per site from between sequences is shown. Standard error estimate(s) are shown above the diagonal. Analyses were conducted using the Kimura 2-parameter model [29]. The analysis involved 11 nucleotide sequences. Codon positions included were 1^{st} + 2^{nd} + 3^{rd} + Noncoding. All positions containing gaps and missing data were eliminated.

There were a total of 1434 positions in the final dataset. Evolutionary analyses were conducted in MEGA7" [31].

Antimicrobial Activity of *Terminalia bellerica* Roxb. against Multi-Drug Resistant *Staphylococcus saprophyticus*:

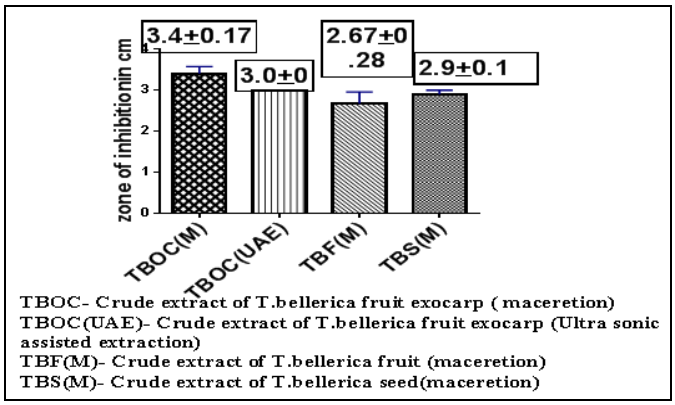


FIG. 4: ANTIMICROBIAL ACTIVITY OF *TERMINALIA BELLERICA* CRUDE EXTRACT AGAINST MDR *STAPHYLOCOCCUS SAPROPHYTICUS*

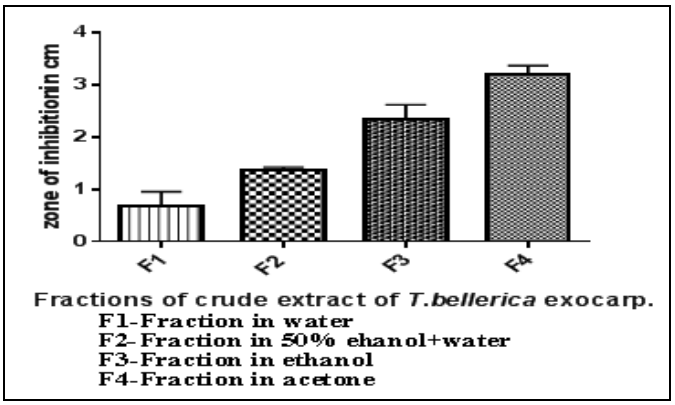


FIG. 5: ANTIMICROBIAL ACTIVITY OF 4 DIFFERENT FRACTIONS OF *TERMINALIA BELLERICA* EXOCARP AGAINST MDR *STAPHYLOCOCCUS SAPROPHYTICUS*

TABLE 5: PHYTOCHEMICAL CONSTITUENTS OF 4 FRACTIONS OF *TERMINALIA BELLERICA* EXOCARP

Fractions	Tannin	Flavonoid	Alkaloid	Fat	Protein	Steroid
F1	+	-	-	+	-	+
F2	+	+	+	-	-	-
F3	+	+	-	-	-	-
F4	+	-	+	-	-	+

TABLE 6: EXTRACTIVE VALUE AND YIELD OF *TERMINALIA BELLERICA* FRACTIONS

Fractions	Extractive value (mg)	Yield
F1	325	0.065%
F2	300	0.060%
F3	350	0.070%

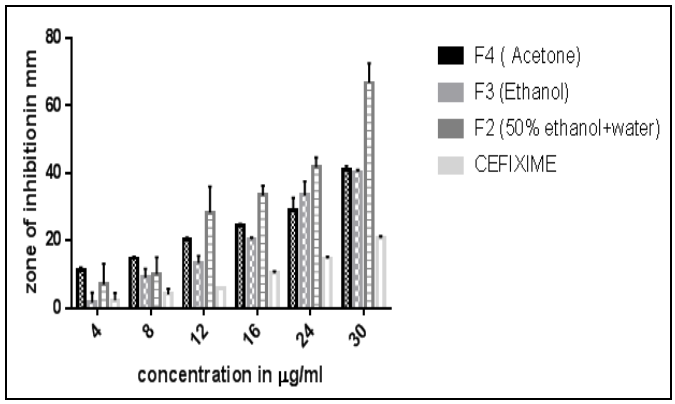


FIG. 6: COMPARATIVE ZONE OF INHIBITION STUDY OF ALL 4 FRACTIONS OF *TERMINALIA BELLERICA* EXOCARP TAKING CEFIXIME AS STANDARD

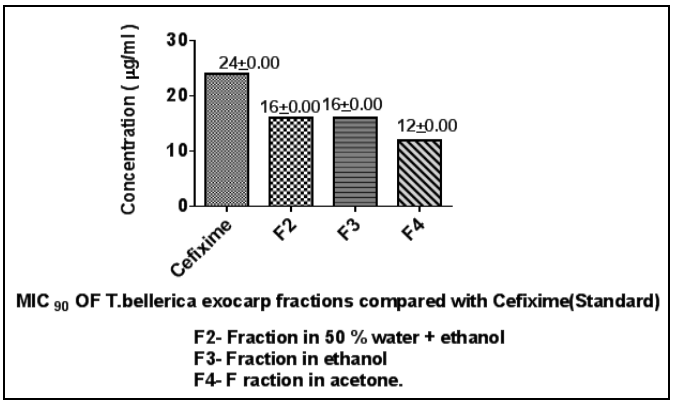


FIG. 7: COMPARISON OF MIC OF *T. BELLERICA* FRACTION (EXOCARP) WITH RESPECT TO STANDARD CEFIXIME

DISCUSSION: Microscopic examination of the isolated bacteria from effluent was found to be Gram-positive Coccus, looked like a bunch of grapes under 100 x objectives, which was characteristics of *Staphylococcus* species **Fig. 1**. The following microorganism was further characterized by the Biochemical tests according to the Bergey's Manual. Microorganism was found to be positive for the following tests such as Catalase, Oxidase, Lactose Fermentation, Mannitol Fermentation, Methyl Red, Voges Proskauer and Indole test **Table 1**. The above results proved that the microorganism was *Staphylococcus* sp. It was further confirmed by growing the bacteria in blood agar showing no hemolysis (Gamma reaction). The bacteria were found to be pathogenic as it was Coagulase-negative as well; the organism was further confirmed and identified by 16 s rRNA

sequencing; hence, it was concluded from the above evidence that the isolated bacterium was none other than a pathogenic *Staphylococcus saprophyticus* **Table 7**. Antibiotics were charged against the isolated *Staphylococcus saprophyticus,* and the bacteria were found to be resistant to 5 different antibiotics such as Azithromycin, Clarithromycin, Amoxicillin, Cefixime and Tetracycline **Fig. 2**, **Table 4**. The Minimum Inhibitory Concentration (MIC) of the antibiotics were compared with the standard MIC according to CLSI Guidelines 2016 **Table 2**. The bacteria were proved to be Multi-Drug Resistant with the highest resistance against Cefixime $MIC_{90}24\pm0.00$ µg/ml **Table 3, Table 4, Fig. 3**.

Terminalia bellerica fruit was selected for the screening of antibacterial activity against Multi-Drug Resistant *Staphylococcus saprophyticus* and was found to have potent antibacterial activity against isolated MDR *S.saprophyticus*. Fruit of *T. bellerica* showed good antimicrobial activity against the MDR and the highest activity was shown by the exocarp extracted by maceration with zone inhibition of 3.4 ± 0.17cm **Fig. 4**. The Exocarp extract with the highest antibacterial property was further fractionated to separate the phytochemical constituents responsible for the activity. The acetone fraction of the exocarp gave the highest antimicrobial activity against the isolated MDR with a zone of inhibition of 3.2 ± 0.17 cm **Fig. 5**. All, the fractions were tested for phytochemical constituent **Table 5**. Phytochemical constituents present in F4 fraction were Tannin Alkaloid and Steroid. Minimum Inhibitory Concentration of all the fractions against MDR *S saprophyticus* was determined and compared with standard Cefixime (with the highest MIC). Comparison of MIC of 4 purified fractions of *T. bellerica* exocarp with respect to Cefixime showed acetone fraction has the highest antimicrobial activity with the lowest MIC-12 ± 0 µg/ml and Cefixime with MIC 24 ± 0µg/ml **Fig. 5, Fig. 6**. The extractive value and Yield of all the fractions were determined in **Table 6**. The P-value for the MIC of *T. bellerica* fractions obtained by ONE WAY ANOVA was $P<0.0001$, which was statistically significant.

CONCLUSION: *Staphylococcus saprophyticus* is associated with uncomplicated Urinary Tract Infection (UTI) in humans. It is one of the leading causes of cystitis in young women. The organisms are also associated with some severe complications like pyelonephritis; septicemia, nephrolithiasis, and endocarditis. Antibiotics are the only choice for treating those diseases. Unfortunately, if Multi-Drug Resistant pathogenic *Staphylococcus saprophyticus* thrives in the environment, then it can cause a severe form of the above diseases with no fruitful treatment and the ultimate consequence will be increased mortality in the population; hence, an alternative remedy is required to combat Multi Drug Resistance. The above study revealed a potential antimicrobial activity of acetone fraction of *T. bellerica* outercoat against isolated multi-drug resistant *Staphylococcus saprophyticus*. The acetone fraction comprises Alkaloids and tannins as primary phytochemicals. This work can be further proceeded by identification and characterization of the active phytoconstituents of *T. bellerica* exocarp, which can have an antibacterial property or can be good resistance modifier for Multi Drug-Resistant Bacteria. Mechanism of resistance of the above bacteria also need be determined for the formulation of potential therapeutic agent against MDR *Staphylococcus saprophyticus.*

ACKNOWLEDGEMENT: The authors are grateful to the Institution Guru Nanak Institute of Pharmaceutical Science and Technology for all the support and the lab facilities provided to complete the research work.

CONFLICTS OF INTEREST: There is no potential conflict of interest.

REFERENCES:

1. Abu GO and Wondikom AC: Isolation, characterization and antibiotic resistance profile studies of bacteria from an excavated pond in Port Harcourt Metropolis, Nigeria. J of Applied Sciences and Envl Manag 2018; 22(8): 1177.
2. Oladeinde A, Cook K, Lakin SM, Woyda R, Abdo Z, Looft T, Herrington K, Zock G, Lawrence JP, Thomas JC, Beaudry MS and Glenn T: Horizontal gene transfer and acquired antibiotic resistance in *Salmonella enterica* serovar heidelberg following *in-vitro* incubation in Broiler Ceca. App and Environ Microbiol 2019; 85(22): 1-46.
3. Ghotaslou R, Yekani M and Memar MY: The role of efflux pumps in *Bacteroides fragilis* resistance to antibiotics. Microbiological Research 2018; 210: 1-5.
4. Seukep AJ, Kuete V, Nahar L, Sarker SD and Guo M: Plant-derived secondary metabolites as the main source of efflux pump inhibitors and methods for identification. Journal of Pharmaceutical Analysis 2020; 10(4): 277-90.
5. Shriram V, Khare T, Bhagwat R, Shukla R and Kumar V: Inhibiting bacterial drug efflux pumps *via* phyto-

therapeutics to combat threatening antimicrobial resistance. Frontiers in Microbiology 2018; 9: 1-18.
6. Manandhar S, Luitel S and Dahal RK: *In-vitro* antimicrobial activity of some medicinal plants against human pathogenic bacteria. J of Tropi Medicine 2019; 1-5.
7. SF, Buchanan R and Gibbons N: Bergey's Manual of Determinative Bacteriology. Taxon 1975; 24(2/3): 377.
8. Dharmaratne MPJ, Manoraj A, Thevanesam V, Ekanayake A, Kumar NS, Liyanapathirana V, Abeyratne E and Bandara BMR: *Terminalia bellirica* fruit extracts: *in-vitro* antibacterial activity against selected multidrug-resistant bacteria, radical scavenging activity and cytotoxicity study on BHK-21 cells. BMC Complementary and Alternative Medicine 2018; 18(1): 1-12.
9. Tasneem FMI and Narsegowda PN: Antimicrobial activity of different varieties of *Terminalia catappa* leaves. Intern J of Pharma Science and Research 2018; 9(10): 4430-35.
10. Chen Q and Xie S: Genotypes, enterotoxin gene profiles, and antimicrobial resistance of *Staphylococcus aureus* Associated with Foodborne Outbreaks in Hangzhou, China. Toxins 2019; 11(6): 307.
11. Kwiatkowski, P, Pruss A, Wojciuk B, Dołęgowska B, Wajs-Bonikowska A, Sienkiewicz M, Mężyńska M and Łopusiewicz Ł: The influence of essential oil compounds on antibacterial activity of mupirocin-susceptible and induced low-level mupirocin-resistant MRSA strains. Molecules 2019; 24(17): 3105.
12. Karska-Wysocki B, Bazo M and Smoragiewicz W: Antibacterial activity of *Lactobacillus acidophilus* and *Lactobacillus casei* against methicillin-resistant *Staphylococcus aureus* (MRSA). Microbiological Research 2010; 165(8): 674-86.
13. Zhang XR, Kaunda JS, Zhu HT, Wang D, Yang CR and Zhang YJ: The Genus Terminalia (Combretaceae): An Ethnopharmacological, Phytochemical and Pharmacological Review. Natural Products and Bioprospecting 2019; 9(6): 357-92.
14. Abbasi AM, Mosaffa NDN, Taheri S and Jaafari MK: Effect of antibacterial skin secretion of Rana ridibanda Frog on Methicillin resistant *S. aureus*, Yakhteh Medical Journal 2007; 9: 146-55.
15. Krieg NR, Ludwig W, Whitman W, Hedlund BP, Paster BJ, Staley JT, Ward N, Brown D and Parte A: Bergey's Manual of Systematic Bacteriology, Second Edition, Springer 2005; US.10.1007/0-387-29298-5.
16. Cappuccino-James G: Microbiology: A laboratory Manual, Pearson, Tenth Edition 2014.
17. Choudhary GP: Anti-ulcer activity of the ethanolic extract of *Terminalia bellerica* Roxb. Int J of Pharmaceutical and Chemical Sci 2012; 1(4): 1293-97.
18. Zou D, Chen T, Li H, Chen C, Zhao J and Li Y: Separation of three phenolic high-molecular-weight compounds from the crude extract of *Terminalia chebula* Retz. by ultrasound-assisted extraction and high-speed counter-current chromatography. Journal of Separation Science 2016; 39(7): 1278-85.
19. Cheesman MJ, White A, Matthews B and Cock IE: *Terminalia ferdinandiana* fruit and leaf extracts inhibit methicillin-resistant *Staphylococcus aureus* growth. Planta Medica 2019; 85(16): 1253-62.
20. Deb A, Barua S and Das B: Pharmacological activity of Baheda (*Terminalia bellerica*): A review. Journal of Pharmacognosy and Phytochemistry 2016; 5(1): 194-97.
21. Nigam M, Mishra AP, Adhikari-Devkota A, Dirar AI, Hassan MM, Adhikari A, Belwal T and Devkota HP: Fruits of *Terminalia chebula* Retz.: A review on traditional uses, bioactive chemical constituents and pharmacological activities. Phytotherapy Research 2020; 34(10): 2518-33.
22. Chakraborty T, Chettri K, Chatterjee S, Datta L and Sengupta A: Isolation and Identification of Multi-drug Resistant Bacteria from various sources and Antimicrobial Activity of Terminalia chebula Retz against isolated Multi-drug Resistant Bacteria. IJPSR 2021; 11(4): 7338-44.
23. Mahesh AR, Ranganath MK and Kumar H: Enrichment of flavonoids from the methanolic extract *of Boerhaavia diffusa* roots by partitioning technique. Research Journal of Chemical Science 2013; 3(1): 43-47.
24. Rasna G, Ram LS and Ankit G: Antioxidant, DNA protective and antibacterial activities of *Terminalia bellerica* extracts. J of Med Pla Res 2019; 13(18): 431-42.
25. Atef NM, Shanab SM, Negm SI and Abbas YA: Evaluation of antimicrobial activity of some plant extracts against antibiotic susceptible and resistant bacterial strains causing wound infection. Bulletin of the National Research Centre 2019; 43(1): 1-11.
26. Assis F, Siqueira F, Gonçalves I, Lacerda R, Nascimento R, Araújo S, Andrade J, Herrera K, Lima L and Ferreira J: Antibacterial activity of Lamiaceae plant extracts in clinical isolates of multidrug-resistant bacteria. Anais Da Academia Brasileira de Ciências 2018; 90(2): 1665-70.
27. Al-Ansari M, Al-Humaid LA, Vijayaraghavan P, Ravindran B, Chang SW, Agastian P, Rathi MA and Balamuralikrishnan B: Identification of phytochemical components from *Aerva lanata* (Linn.) medicinal plants and its *in-vitro* inhibitory activity against drug-resistant microbial pathogens and antioxidant properties. Saudi Journal of Biological Sciences 2019; 26(6): 1129-33.
28. Chandel SR, Kumar V, Guleria S, Sharma N, Sourirajan A, Khosla PK, Baumler DJ and Dev K: Sequential fractionation by organic solvents enhances the antioxidant and antibacterial activity of ethanolic extracts of fruits and leaves of *Terminalia bellerica* from North Western Himalayas, India. Pharmacognosy J 2019; 11(1): 94-101.
29. Singamaneni V, Dokuparthi SK, Banerjee N, Kumar A and Chakrabarti T: Phytochemical investigation and antimutagenic potential of ethanolic extracts of *Emblica officinalis, Terminalia chebula* and *Terminalia bellirica*. The Natural Products Journal 2020; 10(4): 488-94.
30. Kimura M: A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 1980; 16(2): 111-20.
31. Felsenstein J: Confidence limits on phylogenies: an approach using the bootstrap. Evolution 1985; 39(4): 783.
32. Kumar S, Stecher G and Tamura K: MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molecular Biology and Evolution 2016; 33(7): 1870-74.
33. Indian Pharmacopoeia 2014. The Indian Pharmacopoeia Commission, Ghaziabad, Edition 2015.

How to cite this article:
Chakraborty T, Chatterjee S, Datta L and Sengupta A: Antimicrobial activity of *Terminalia bellerica* (Gaertn.) Roxb. against multi drug resistant *Staphylococcus saprophyticus*. Int J Pharm Sci & Res 2021; 12(10): 5353-63. doi: 10.13040/IJPSR.0975-8232.12(10).5353-63.