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INHIBITION OF E.CANCEROGENUS MAB-1 β -LACTAMASE BY SYNERGISTIC FORMULATION: AN IN VITRO HERBAL SHOTGUN STUDY

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ABSTRACT

β-lactamases are increasing worldwide speedily by creating problem to currently available UTI therapies and hence there is an urgent need of research to find the effective therapy. In the present study we propose to develop drug formulation for the reversal of drug resistance in MDR *E.cancerogenus MAB-1* by using bioenhancer property of isolated different phytochemicals (lead compounds). β-lactamase enzyme of *E.cancerogenus MAB-1* was produced and purified up to 92.5 fold, by using Ion-Exchange chromatography(DEAE-Cellulose -52) techniques with 80.23 U/mg of specific activity. Iso-electric focusing and Sodium Dodecyl Sulfate –Polyacrylamide gel electrophoresis (SDS-PAGE) study revealed enzyme with PI value 8.5 with Molecular weight ~6.5 KDa. Threonine (9.27 ppm) and Serine (3.94 ppm) were detected in highest quantity when analyzed by GC-MS technique. Organic and Water extracts of selected plants were investigated for GC-MS, IR, NMR analysis for the isolation of pure lead compounds i.e. Theobromine, Myricetin, Gallic acid, Ellagic acid, Kaempferol and Genistein. *In vitro Enterobacter cancerogenus MAB-1* β-lactamase inhibition by Dug-phytochemical combinations was studied by Checker board and Time-Kill curve at their ½ to 1/32 MIC values. The Theobromine+ Cefaclor synergy inhibited β-lactamases by 91%, Myricetin +Cefaclore (90%), Genistein+Cefaclore (92%), Genistein + Cefotaxime(90%), Genistein+Penicillin (91%) , Ellagic acid+Cefaclor (87%), Kaempferol+Ceftriaxone(87%), Kaempferol+Cefaclor (90%) and Clavulanic Acid (96%) Competitively, Gallic acid+Cefotaxime (92%) Non-Competitively. Thus the resulting data would be helpful to pick up the most effective and suitable combination therapy for further research in the area of drug development against UTI caused by *E.cancerogenus MAB-1* or any member of *Enterobacteriaceae* family.

KEYWORDS: β-lactamases, UTI infection, Phytochemicals, Synergy, Enzyme Inhibition, Drug resistance.

INTRODUCTION

Community or Nosocomial Urinary Tract Infections (UTI) are increasing worldwide speedily by creating problem to currently available β-lactam antibiotic therapies. The clinical efficacy of many existing antibiotics are being threatened by the emergence of multidrug-resistant pathogens [1]. The saga of *Enterobacter* as a nosocomial pathogen is closely linked to the logarithmic increase in the use of extended-spectrum Cephalosporin in the 1980's. The clinically isolated novel multidrug resistant strain *Enterobacter cancerogenus MAB-1* (GenBank: JX827386) belongs to the family *Enterobacteriaceae* which possess TEM,SHV, BLA type β-lactamases is our study of interest [2]. Bacterial resistance to β-lactam antibiotics is often associated with the production of β-lactamase (Penicillin amidohydrolase; EC 3.5.2.6), which hydrolyses the β-lactam ring (4 atom cyclic amide rings, 6 APA- 6-Aminopenicillanic acid) at C_4 position. This reaction gives rise to biologically inactive products and represents the most widespread mechanism of resistance. Thus it is an urgent need of research to find the effective therapeutic options to treat / manage the UTI.

Synergy is the basis of using combination antimicrobial therapy. Now a days there are considerable studies pertaining to the role of antimicrobial synergy in the treatment of serious infections caused by both Gram-positive and Gram negative organisms. In search of more effective stratergies, elaborating new antimicrobial agents and developing combination therapies to improve the efficacy and to reduce the toxicity of various drugs the synergistic combination will be helpful in treating infections caused by ESBL producing bacteria. It has been documented that some cellular

targets of bacterial resistance to antimicrobial drugs are consisting of active antibiotic efflux from the cell interior and prevention of entry of compound in to the cell, altering permeability of the cell membrane to reduce drug uptake, inactivating antimicrobial agents such as hydrolyzing their β-lactam ring and changing bacteria's cellular targets by mutation of target sites [3]. One specific hypothesis to combat multidrug resistant bacteria is to increase the uptake of antibacterial agents into the bacterial cells by interfering with cellular permeability barriers of cell membrane and cell wall structures of microorganisms. Here we hypothesized that phytochemicals can improve the entrance of antibiotics through bacterial membrane to target their sites and bypass bacterial resistance. Secondary metabolites from medicinal herbs and dietary plants possess a range of bioactivities like - antibacterial, antifungal, antiviral, antimutagenic and anti-inflammatory activities. Moreover, extensive clinical evidence has shown that chemoprevention by phenolic phytochemicals is an inexpensive, readily applicable approach in the chemotherapy and management [4]. It is speculated that due to herb-antibiotic synergy the inhibition of efflux pump and bacterial resistance mechanism could be altered [5]. Though exact mechanism of these non antibiotic drugs are not well understood but these compounds can modulate ion channel activity and make microbial adaptability difficult [6,7]. The rich chemical diversity in plants as a potential source of antibiotic resistance modifying compounds are yet to be adequately explored. Definitely herbal medicines will not replace antimicrobials but helps to revert bacterial resistance. Thus the synergistic effect from the association of antibiotics with phytochemicals against multi-drug resistant bacteria may emerge as new approach for the treatment of infectious diseases [8]. Totarol, Epicatechin, Berberin, Reserpine *etc* are some resistance modifiers derived from plants [9]. Modulators are compounds that potentiate antibiotic activity against resistant strains.

Thus the present study we propose to develop drug formulation for the drug resistance reversal in MDR *E.cancerogenus* MAB-1 by using biopotentiators (bioenhancers) properties of isolated six different phytochemicals or lead compounds ((Theobromine, Myricetin, Ellagic acid, Gallic acid. Kaempferol and Genistein) with seven different β-lactam antibioitcs (Cefotaxime, Ceftazidime, Ceftriaxone, Cefaclor, Amoxicillin, Ampicillin and Penicillin). All antibiotics under investigation have earlier been reported as β-lactam drugs and may have different mechanisms of action (have different targets).

MATERIALS AND METHODS

Production of β-lactamases from *Enterobacter cancerogenus* MAB-1

For optimum production of β-lactamases, *Enterobacter cancerogenus* MAB-1 strain was cultivated aerobically in 2-lit batch culture of optimized Muller-Hinton broth media with 0.1% Nitrocefin. The initial pH of media was adjusted to 7.5. A 2.5 % (v/v) of inoculum was added in total broth culture and incubated at 37^0C temperature for 24 h on rotary shaker (120rpm/min).At the end of fermentation; bacterial cells were harvested by centrifugation at 8,000 rpm for 20 min. The resulting cell pellets were resuspended in 0.1M phosphate buffer (pH 7) and disrupted by lysis buffer containing 05 mg/ml lysozyme and incubated at 37^0C for 30 min on shaker [10]. The cell debris was removed by centrifugation at 10,000rpm for 20 min at 4^0C and cell –free supernatant was designed as crude β-lactamase and used for further purification process. All the preperations were carried out at 4^0C temperature.

β-lactamase crypticity

β-lactamase crypticity was defined as the ratio of the activity of the disrupted cell to that of the intact cells.The cells were disrupted by treatment with 1% toluene for at least 30 min.

$$\beta\text{-lactamase crypticity} = \frac{\text{Activity of disrupted cells}}{\text{Activity of intact cells}}$$

Enzyme purification

Purification of the enzyme was carried out according to the standard method with some modifications [11]. The crude β-lactamase was fractionated with addition of ammonium sulfate to obtain saturations (30, 40, 50, 60 and 70% w/v). The precipitate was collected by centrifugation at 10,000 rpm/20min at 4^0C and dissolved in 0.05M potassium phosphate buffer (pH 7.5), followed by dialysis (10 KDa molecular weight cut off) against same buffer of low molarity (0.01m M) for overnight at 4^0C temperature. The dialyze was passed through a DEAE (Diethylaminoethyl)-cellulose 52 column pre-equilibrated with 0.05 M phosphate buffer (pH 7.5).The enzyme elution was performed with a linear gradient of 0.1 to 0.5 M NaCl. The active enzyme fractions were pooled, concentrated with ammonium sulfate

treatment and dialyzed. The dialyzed was again passed through DEAE-cellulose 52 column with same buffer. Fractions in milliliter were collected per minute and estimated for protein concentration as well as β-lactamase activity.

Protein estimation
Protein concentration was determined by the standard method using Bovine Serum albumin (BSA) as the standard [12].

Assay of β-lactamase
β-lactamase activity was determined Spectrophotometrically [13] with some modifications. The rate of breakdown of substrate (Nitrocefin) in the presence of β-lactamase was determined by measuring the rate of change of ultraviolet light associated with β-lactam ring. The standard mixture contains total volume of 3 ml reaction mixture containing 1.9ml of 51.6 µg/ml of Substrate (Nitrocefin) in 0.05 M Phosphate buffer (pH 7), 1 ml 0.1.M Phosphate buffer (pH 7). The reaction was started by addition of 0.1ml enzyme solution in a total volume of 2.9 ml. Enzyme concentration at which the rate of reaction complete in 5 min was found to be the most convenient and the change in absorbance at 386 nm wavelength was measured over first 2 min. One unit of activity of the enzyme was defined as micromoles of substrate destroyed per minute per milliliter of enzyme at 37^0C at pH 7 at 386 nm.
Enzyme Activity = X/1.03 × 0.3× v µmole destroyed per min per milliliter of enzyme
where: X= is the ΔOD/minute observed, 0.3 is the number of micromoles of Nitocefin present and v is the factor for adjusting enzyme volume to 1ml.

Iso-electric focusing
Isoelectric point (PI) value of β-lactamase from *E.cancerogenus* MAB-1 was determined by Iso-electric focusing by using 2-D PAGE with ready IPG strips (Ampholine pH gradient: 3-10) using the Bio-Rad PROTEAN IEF cell. The focusing was carried out for three days i.e. Day 1 : Rehydration of IPG strips were done with *Enterobacter cancerogenus* MAB-1 β-lactamase for 12 h. Day 2: Enzyme focusing and Day 3: Destaining for 120 min. β-lactamase focusing bands were detected by overlaying the gel with solution of Commassie Brilliant blue. The gel was observed for development of blue bands. The coordinate of active bands were recorded.

Molecular mass determination and Purity checking
The relative molecular mass and purity of the β-lactamase was determined by Sodium Dodecyl-Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) on 10% polyacrylamide gel using standard protein markers: Myosin (205KDa), Phosphorylase b (97.4KDa), Bovin serum albumin (66KDa), Ovalbumin (43KDa),Carbonic unhydrase (29KDa), Soyabean Trypsin inhibitor (20.1KDa), Lysozyme (14.3KDa), Apoprotin (6.5KDa) and Insulin (3.5KDa). After electrophoresis the gel was stained with Commassie Brilliant blue stain for 1 h, followed by destaining for 30 min in distilled water and photographed.

Bioactivity guided fractionation for isolation of lead compounds
Fresh plants of *Acacia catechu (Khair), Hemidesmus indicus (Anantmul) , Santalum album (Chandan) , Acorus calamus (Vekhand) , Vetivera zinanoides (Ushirwala) , Justicia adhatoda (Adulsa) , Withania somnifera (Ashwagandha), Tinospora cordifolia (Gulwel) , Hibiscus rosa-sinensis (Jashwandpushp), Asparagus racemousus (Shatawari)* were collected (January - December 2012) randomly ,extracted by solvent extraction method and employed for GC-MS analysis for confirmation of probable phytochemicals [14]. Preliminary TLC was done of each extract for determining the column solvent system. Selected each extract was mixed with 20 g of Silica gel (100-200 mesh) to become a slurry. The slurry was loaded onto the wet packed column and continuously eluted with the mobile phase or solvents starting with n-Hexane, Petroleum ether, benzene, chloroform, ethyl acetate and methanol by increasing or decreasing polarity individually. Each sample yielded 20 fractions of 25 ml aliquots of eluent which was collected by observing the distance travelled by the sample down to the column. In addition, bands of the each fraction formed on TLC were also monitored. The fractions showing TLC mobility and band formation were pooled and the solvent evaporated under a steady air current at room temperature. Fractions which did not give single sharp band on the TLC plates were re-fractionated using same silica gel column in different solvent system.

Assaying fractions for antibacterial testing
All fractions of all extracts were assayed against test strain *Enterobacter cancerogenus* MAB-1 for potent antimicrobial activity [15]. Fraction no F3,F5,F7,F4 of *Hemidesmus indicus* extract,F9,F3,F1 of *Acacia catechu*

extract,F11,F5,F8,F10 of *Santalum album* extract,F4 of *Vetivera zinanoides* extract,F15,F17 of *Acorus calamus* extract,F5 of both *Justicia adhatoda* extract and *Withania somnifera* extract were tested for potential antimicrobial activity against the test strain and hence these fractions from each plant extract was combined according to their Rf value in to six main fractions and named F1,F2,F3,F4, F5 and F6 respectively. These six fractions employed for IR, NMR anlaysis for the identification of lead bioactive compounds as per standard procedure.

Determination of Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) of isolated six phytochemicals and Standard β-lactam antibiotics for *Enterobacter cancerogenus MAB-1* was determined by Tube Dilution method [16]. Varying concentrations of phytochemicals and Antibiotics ranging from 240-0.01 µg/ml were prepared in 3 ml nutrient broth, 0.5Mac Farland turbidity adjusted *Enterobacter cancerogenus* MAB-1culture was added into each test tube. Test tubes were incubated at 37^0C for 24 h and examined for turbidity. After incubation, tube containing least concentration of phytochemicals showing no visible sign of growth was considered as the minimum inhibitory concentration (MIC).

Synergistic interaction of isolated Phytochemicals with Standard Antibiotics (The Checkerboard analysis)

To get MIC of the combination i.e. all seven standard β-lactam Antibiotics and Theobromine, Myricetin, Ellagic acid, Galiic acid, Kaempferol and Genistein against *Enterobacter cancerogenus* MAB-1, the cells were inoculated in MHB and incubated under following different combinations *viz*: Cefaclor + Gallic acid, Theobromine, Ellagic acid, Kaempferol, Myricetin, Genistein of 240-0.001ug/ ml concentration; Cefotaxime + Gallic acid, Theobromine, Ellagic acid, Kaempferol, Myricetin, Genistein of 240-0.001ug/ ml concentration; Ceftazidime + Gallic acid, Theobromine, Ellagic acid, Kaempferol, Myricetin, Genistein of 240-0.001ug ml^{-1} concentration; Ceftriaxone + Gallic acid, Theobromine, Ellagic acid, Kaempferol, Myricetin; Genistein of 240-0.001ug/ ml concentration; Ampicillin+ Gallic acid, Theobromine, Ellagic acid, Kaempferol, Myricetin, Genistein of 240-0.001ug/ ml concentration; Amoxicillin+ Gallic acid, Theobromine, Ellagic acid, Kaempferol, Myricetin, Genistein of 240-0.001ug/ ml concentration and Penicillin+ Gallic acid, Theobromine, Ellagic acid, Kaempferol, Myricetin, Genistein of 240-0.001ug/ ml concentration. To eliminate other random factors all assays were carried out in triplicates to determine accurate MIC of antibiotic and Phytochemicals in combination and individually.

Synergistic interactions between Third Generation Cephalosporin antibiotics viz. Cephotaxime, Ceftazidime, Ceftriaxone, Cefaclor, Amoxicillin, Ampicillin and Penicillin with Theobromine, Myricetin, Ellagic acid, Gallic acid, Kaempferol and Genistein, were studied using Broth Dilution Checkerboard method. In this method, 0.5 Mac Farland turbidity adjusted inoculum of *E.cancerogenus* MAB-1 strain was inoculated in to 3 ml of sterile nutrient broth. Phytochemicals and antibiotic combinations at concentration of 1/32 × MIC to 2 × MIC (combined above cited MIC) were added into tubes of culture. The fractional inhibitory concentration was derived from the lowest concentration of antibiotics and phytochemical combination showing no visible growth of the test organism after an incubation of 24 h. FIC was calculated by using the formula,

FIC index = (MIC of antibiotic in combination/MIC of antibiotic alone) + (MIC of phytochemical in combination /MIC of phytochemical alone).

The results were interpretated as synergistic - if the FIC indices were < 0.5; Additive - if FIC indices were between 0.5- 4 and Antagonistic - if the FIC indices were > 4 [17].

Time –Kill curve

Time-Kill synergy assay [18] was carried out to find out the enhanced antibacterial effect of Phytochemicals and Antibiotic combination against Test strain. Tubes containing 10 ml of MHB broth with above mentioned antibiotics and Phytochemicals (at concentration of 1/2,1/4,1/8,1/16 and 1/32 times of their MIC) alone and in combinations and one tube without antibiotics were inoculated with 100 µl of actively growing *E.cancerogenus* MAB-1 culture adjusted to yield a final inoculums of 10^5 cells / ml**.** All above cited combinations were incubated at 37^0C. The negative control was maintained with Phytochemicals and antibiotics individually. Growth rates of bacterial species were assessed at every ½ hr interval by measuring absorbance (OD) at 600 nm (Shimadzu, Japan).

Post Antibiotic Effect (PAE)

The post antibiotic effect was tested in Phytochemical +Antibiotic synergistic combinations only. 20 ml of 1:20 diluted overnight grown and 0.5 Mac Ferland turbidity adjusted culture of *E.cancerogenus* MAB-1 in MH broth was inoculated for 2 h at 37^0C with antibiotics and Phytochemicals (at a concentration of 1/2,1/4,1/8,1/16 and 1/32 times of their MIC) alone and in combinations. In order to remove the antibiotics and phytochemicals, the exposed bacteria was washed twice with the phosphate buffered saline (pH 7) by centrifugation for 7,000 rpm. Control was handled similarly.The pellets were resuspended in 20 ml of MHB followed by incubation at 37^0C and samples (1 ml) were obtained at 1/2 h of exposure to antibiotics, Phytochemicals and their combinations for corrections that were made to ensure that all the cultures would start with the same bacterial count. Samples were removed at time 0 (immediately after washing and correction) and then hourly up to 12 h and the OD values were determined (reflecting the bacterial growth).The OD values were converted in to CFU (bacterial growth) by using a standard curve which was constructed relating the bacterial count to the OD. The Post Antibiotic Effect (PAE) is defined according to -

$$PAE = T- C$$

Where T is the time required for the viable counts of the exposed bacteria to increase by 1 $\log_{10}$ of above the counts observed immediately after washing and C is the corresponding time for the sample unexposed to antibiotic and phytochemical alone and in combination (control) [19].

Inhibition study of *E.cancerogenus* MAB-1 β-lactamases

Inhibitory activity of each Antibiotic, Phytochemical and Antibiotic+ Phytochemical combination was determined with slight modifications [20]. The assay mixture contains various concentrations of inhibitors i. e. Antibiotic, Phytochemicals alone, Antibiotic + Phytochemical in combination and Standard inhibitor Clavulanic acid in concentration range of 0.01µm-35 µm. The assay mixture was incubated with 0.5 ml of purified β-lactamases separately for different time period from 0.5-120 min. In case of Phytochemical + Antibiotic combination the synergistic combination of Time –Kill study formulation was used for inhibition. About 0.1 ml sample was removed periodically and 1ml nitrocefin was added to the reaction mixture to record the absorbance.The blank was prepared without enzyme solution. The test was prepared in triplicate. β-lactamase activity was expressed as percent inhibition of β-lactamase as-

$$\% \text{ inhibition} = 100 \times (C-r) / c$$

Where; C=Activity in control incubated without inhibitor;
r= Remaining activity in samples incubated with inhibitor and

$$IC_{50} = I(Vi)/(Vo-Vi)$$

Where; I=Concentration of inhibitor which gives rate of Vi
Vo=Rate of control lacking inhibitor
Vi=rate of sample with inhibitor

Lineweaver-Burk plot of V_{max} (is maximal velocity) and K_m (is concentration at 50% V_{max}) was calculated.The IC_{50} value i.e. mM concentration of inhibitor is necessary for 50% inhibition were determined from the above mentioned formula.

RESULTS AND DISCUSSION

β-lactamase crypticity is the ratio of activity of disrupted cells/ intact cells of isolate *E. cancerogenus* MAB-1. Nitrocefin exhibited highest crypticity i.e.52 whereas other substrates Cefaclor (32), Ceftriaxone (42), Amoxicillin (36), Amikacin (47), Chloramphenicol (30) and Ciprofloxacin (40) revealed moderate crypticity. Cefalexin (50) alone exhibited crypticity similar to Nitrocefin. Remaining all substrates revealed very less crypticity. Thus according to crypticity data it can be concluded that Nitrocefin is the ideal one for the maximum β-lactamase production in disrupted as well as intact cells of *Enterobacter cancerogenus* MAB-1 rather than any other substrates tested (Table 1). The purified β-lactamase from *Enterobacter cancerogenus* MAB-1 revealed 92.5 purification fold which is similar with other microbial β-lactamases [Table 2 & Fig.1]. The enzyme showed optimum activity at pH 7 and 30^0C temperature as well as broad stability over a wide range of pH (7-7.5) and temperature (20^0C-30^0C) [Fig. 1 &2 Supplementary Data].The enzyme is ~6.5KDa having PI value 8.5[Fig.2 &3].

About ten different plants viz. *Acacia catechu (Khair), Hemidesmus indicus (Anantmul) , Santalum album (Chandan) , Acorus calamus (Vekhand) , Vetivera zinanoides (Ushirwala) , Justicia adhatoda (Adulsa) , Withania somnifera (Ashwagandha), Tinospora cordifolia (Gulwel) , Hibiscus rosa-sinensis (Jashwandpushp), Asparagus racemosus (Shatawari)* were collected for isolation of bioactive compounds to treat *Enterobacter cancerogenus* MAB-1 caused UTI infection. The plants were from different families i.e. *Apocynaceae, Santalaceae, Acoraceae, Poaceae, Acanthaceae, Solanaceae, Menispermaceae, Malvaceae* and *Liliaceae*. Most of the plants of these families are well known for their antimicrobial activity against common as well as drug resistant pathogens but yet no report is present about these plants regarding UTI infection. The bark of *Acacia catechu* and *Santalum album*, roost of *Hemidesmus indicus* and *Asparagus racemousus*, whereas Rhizome of *Acorus calamus*. Grass of *Vetivera zinanoides* and flowers of *Hibiscus rosa-sinensis* and shrub of *Justicia adhatoda, Withania somnifera, Tinospora cordifolia* were used.

Our main objective was to find out active lead compound to kill MDR *E.cancerogenus* MAB-1 so all extracts were employed for GC-MS analysis for probable determination of the phytochemicals in each plant extract. GC-MS analysis revealed large number of phytochemicals but Gallic acid, Ellagic acid, Threobromine, Genistein, Myricetin were the prominent peak [Table 1.,supplimentary data]. For identification of phytochemicals all fractions were subjected for antimicrobial tests and only 6 fractions revealed promising AST [Fig.4] against *E.cancerogenus* MAB-1. F1: Fraction of *Hemidesmus indicus* (15±0.22 mm) ,F2: Fraction of *Acacia catechu* (17±0.04 mm), F3&F4: Fraction of *Santalum album*(21±0.86 mm ; 13±0.94mm) ,F5: Fraction of *Acorus calamus* (11±0.00 mm),F6: Fraction of *Justicia adhatoda* (10±0.07mm). After IR and NMR analysis [Table 3] the fractions were identified as F1: Ellagic acid, F2: Myricetin, F3: Theobromine, F4: Gallic acid, F5: Kaempferol and F6: Genistein etc. Results of antimicrobial assay revealed that acetone and ethanol extract of plant exhibited broad spectrum activity against tested isolates as compared to aqueous extract. Susceptibility of pathogens varied to solvent and aqueous extract. This indicates the involvement of more than one active principles of biological significance [21]. The traditional healers use primarily water as the solvent, but we observed that plant extracts prepared in ethanol and acetone as solvents provided more consistent antimicrobial activity, as also reported earlier [22,23]. These observations can be rationalized in terms of the polarity of the compounds being extracted by each solvent and, in addition to their intrinsic bioactivity, by their ability to dissolve or diffuse in the different media used in the assay. In the present study negligible inhibitory activity with aqueous extract was observed in most of the plants which may be due to loss of some active compounds during extraction process of the sample or there may be lack of solubility of active constituents in aqueous solution [24]. Alternatively, active compounds may be present in insufficient quantities in the crude extracts to show activity with the dose levels employed [25].

Thus the six lead phytochemicals having promising antimicrobial activity against MDR *E.cancerogenus* MAB-1 as a resistance modulators were isolated and identified. The isolated lead compounds and β-lactam antibiotics were subjected for determination of Minimum Inhibitroy Concentration (Table.4) as per standard procedure. The results revealed MIC values for Theobromine 240 µg/ml, Myricetin >10 µg/ml, Ellagic acid 1 µg/ml, Gallic acid 240 µg/ml and Kaempferol 1 µg/ml whereas in case of antibiotics Ceftriaxone 0.1 µg/ml, Ceftazidime 0.1 µg/ml, Cefotaxime 0.032 µg/ml, Cefaclor 480 µg/ml, Amoxicillin 128 µg/ml, Ampicillin 0.1 µg/ml and Penicillin 240 µg/ml MIC against the test strain *E.cancerogenus* MAB-1. MIC determination of test compounds against test strain is the most essential criteria for calculating FIC index as well as formulation therapy.

The combination effects of β-lactam drugs and phytochemicals are depicted (Table.5). The results showed that combinations of Genistein + Penicillin, Theobromine + Ceftazidime, Theobromine + Cefotaxime, Myricetin + Cefaclor, Ellagic acid + Cefaclor, Ellagic acid+ Ceftazidime, Gallic acid + Cefotaxime, Kaempferol + Ceftriaxone, Kaempferol + Cefaclor , Theobromine + Cefaclor revealed synergistic mode of interactions for *E.cancerogenus* MAB-1under investigation. Whereas other combinations were additive in nature but only one combination i.e. Gallic acid + Ceftriaxone revealed Antagonastic effect against the test strain. (Table.5). It is noteworthy, that only one antibiotic-phytochemical combination showed antagonism. The FIC indices of the combinations Genistein+ Penicillin, Theobromine + Ceftazidime, Theobromine + Cefotaxime, Myricetin + Cefaclor, Ellagic acid + Cefaclor, Ellagic acid+ Ceftazidime, Gallic acid + Cefotaxime, Kaempferol +Ceftriaxone, Kaempferol + Cefaclor , Theobromine + Cefaclor were ranged from 0.10 – 0.5. The growth inhibitory properties of all the test compounds and additive combinations assayed over 20 h periods at sub-inhibitory concentrations. Moreover, the synergistic combinations were found to inhibit the test strain growth at similar concentrations.

Time-kill assays are illustrated for the most potent synergistic combinations (Genistein+Penicillin, Theobromine+Ceftazidime,Theobromine+Cefotaxime,Myricetin+Cefaclor,Ellagic acid + Cefaclor, Ellagic acid+ Ceftazidime, Gallic acid+Cefotaxime, Kaempferol +Ceftriaxone, Kaempferol + Cefaclor , Theobromine + Cefaclor) on *E.cancerogenus* MAB-1(Fig.5: a-f). The net reduction in colony count was seen consistently (throughout 24 h) at (1/2th + 1/32th) x MIC for the identified synergistic drug-phytochemical combinations. Any single active agent alone at 1/2th x MIC did not show significant inhibition of the tested isolates. The rate of killing was higher at 6 h with maximum reduction in the colony count at 24 h. The ½ MIC of Theobromine+1/4 MIC of Cefaclor significantly decrease bacterial count between 2.5 h with maximum reduction in the growth baseline by 6 – 7 $\log_{10}$ CFU/ml. However, when ¼ MIC Gallic Acid + ½ MIC Cefotaxime combined a reduction of 7 $\log_{10}$ decrease in viable count occurred at 11 h. ½ MIC EA+1/32MIC Ceftazidime decreased viable count by 7 log 10 at 6 h; ¼ MIC Myr+ ¼ MIC Cefaclor decreased viable count by 7 log 10 at 9 h , ½MIC GEN+ ½MIC Penicillin decreased viable count by 7 log 10 at 5 h and combination of ¼ MIC KEM+1/2 MIC Cefaclor decreased viable count by 7 log 10 at 6.5 h(Fig.5: a-f). A strong synergy observed between the Genistein + Penicillin, Theobromine + Ceftazidime, Theobromine + Cefotaxime, Myricetin + Cefaclor, Ellagic acid + Cefaclor, Ellagic acid + Ceftazidime, Gallic acid + Cefotaxime, Kaempferol + Ceftriaxone, Kaempferol + Cefaclor , Theobromine + Cefaclor is a significant finding demonstrating the therapeutic potentials of these phytochemicals. The concentrations relative to MIC may have a major role to play in the outcomes of the combinational experiments.

Combined antibiotic therapy has been shown to delay the emergence of bacterial resistance and may also produce desirable synergistic effects in the treatment of bacterial infection. Drug synergism between antibiotics and bioactive phytochemicals is a novel concept and could be beneficial (Synergistic or Additive interaction) or deleterious or Toxic outcome. The antimicrobial and resistance modifying potentials of naturally occurring compounds have been reported in studies [26]. This would suggest that the synergy with antibiotics observed could be attributable to some compounds, like polyphenols which exert their antibacterial action through membrane perturbations. This perturbation of the cell membrane coupled with the action of β-lactams on the transpeptidation of the cell membrane could lead to an enhanced antimicrobial effect of the combination [27]. These mechanisms of action can be obtained by the combination of antibiotics with extract at a sub-inhibitory concentration applied directly to the culture medium [28].This strategy is called “Herbal shotgun” or “synergistic” “multieffect targeting” and refers to the utilization of plants and drugs in an approach using more or multi phytochemical combinations, which affect not only a single target but various targets. The action involves different therapeutic components collaborate in a synergistic combinations between natural or synthetic products with antibiotics. Sometimes the use of single antibiotic does not produce the desired or the effective inhibitory effects and to overcome this, combination of drugs often exercise their synergistic effect which surpasses their individual performance.

Bacterial efflux pumps are responsible for a significant level of resistance to antibiotics in pathogenic bacteria. Some plant derived components have been observed to enhance the activity of antimicrobial compounds by inhibiting MDR efflux system in bacteria [29]. 5’-methoxy hydanocarpin is an example of an inhibitor of the nor A efflux pump of *S.aureus* isolated from *Berneries fremontii* [30]. It is likely that the Theobromine,Myricetin, Ellagic acid,Genistein,Kaempferol and Gallic acid could contain potential efflux pump inhibitors. Such compounds are likely to be broad spectrum efflux inhibitors as synergistic effect was observed on MDR *E.cancerogenus* MAB-1 as well as in combination with cell wall inhibiting and protein synthesis inhibiting antibiotics.

Antibiotics could interfere with bacterial cell wall synthesis, increase bacterial membrane permeability and /or inhibit bacterial protein synthesis at the 30S subunit of ribosome; therefore the different modes of action of the phytochemical with the antibiotics may be an important factor in the enhanced bactericidal efficacy observed when used in combination.

The antimicrobial resistance modifying potentials of naturally occurring flavonoids and polyphenolic compounds have been reported in other studies such as [26]. It has also been shown that some plant derived components can improve the *in vitro* activity of some peptidoglycan inhibiting antibiotics by directly attacking the same site (i.e.peptidoglycan) in the cell wall. Here we recommended the evaluation of the exact Drug-Phytochemical ratio at which the interaction was maximum between the phytochemical and antimicrobial drug. A wider study with increase in the number of drugs,

increase in number of clinical isolates especially MDR is necessary to establish the mode of action against the *E.cancerogenus MAB-1*.

Post-Antibiotic Effect (PAE) (Table.6) is a well-established pharmacodynamic parameter that reflects an arrested bacterial growth, following the removal of the active antibacterial agent from the growth medium [31]. The duration of the PAE is mainly influenced by the bacterial species, and the nature of the antibacterial drug and its concentration. Environmental factors such as temperature, pH, O_2, growth medium, the kind of body fluid *etc* also affect PAE. Addition of Genistein + Penicillin, Theobromine + Ceftazidime, Theobromine + Cefotaxime, Myricetin + Cefaclor, Ellagic acid + Cefaclor, Ellagic acid+ Ceftazidime, Gallic acid + Cefotaxime, Kaempferol + Ceftriaxone, Kaempferol + Cefaclor , Theobromine + Cefaclor seems to prolong the post antibiotic effect of all above combined β-lactam antibiotics. Interestingly Theobromine, Myricetin, Ellagic acid, Amoxicillin, Kaempferol, Genistein or Cefaclor, Ceftazidime, Cefotaxime, Ceftriaxone, Penicillin, Ampicillin, Gallic acid when used alone does not depress the growth of the microorganism after they were removed.

The kinetic behavior of the compounds that inhibit the β-lactamase activity was studied [Table.7]. The type of inhibition was determined by Lineweaver—Burk and Dixon plots. The reciprocals of the velocity of the reaction was plotted against the substrate concentrations of the inhibitors in the Lineweaver-Burk plot and Dixon plot respectively for the inhibitor constant(K*i*).Percentage inhibition was calculated from the standard formula. Phytchemical + β-lactam Antibiotic combination inhibited β-lacatamase *in vitro* in a dose dependent manner with minimum inhibitory activity [Table.7].

The combinations of Theobromine+Cefaclore, Theobromine + Ceftazidime, Theobromine + Cefotaxime, Myricetin + Amoxicillin, Myricetin + Cefaclore, Genistein + Cefaclor, Genistein + Ceftazidime, Genistein + Cefotaxime, Genistein + Penicillin, Gallic acid + Ceftriaxone, Gallic acid + Cefotaxime, Ellagic acid + Cefaclor, Ellagic acid + Ceftazidime,Kaempferol + Cefaclor, Kaempferol+Amoxicillin, Kaempferol+Ceftriaxone, Kaempferol+Penicillin displayed significant concentration dependent β-lactamase inhibition with respect to control from 79-90% [Table.7]. These 10 combinations posses the most potent inhibitory activity with IC_{50} values ranging from 0.95 - 3.1, than the positive control Clavulanic acid (0.115). The Kaempferol +Ampicillin, Kaempferol+ Ceftazidime, Kaempferol + Cefotaxime, Ellagic acid + Ceftriaxone, Gallic Acid + Amoxicillin, Gallic Acid + Penicillin, Gallic Acid + Ampicillin, Myricetin + Ampicillin, Myricetin + Ceftazidime, Theobromine + Ceftriaxone, Theobromine + Ampicillin combinations revealed moderate β-lactamase inhibitory activity in the range of (60-75%). Kinetic studies of Phytochemical and Antibiotic alone exhibited non-competitive and competitive inhibition respectively. Percentage inhibition activity of the Phytochemicals and Antibiotics alone revealed 8-89%, while standard showed 96% of inhibition at their highest concentration with minimum substrate concentration. The activity of these compounds greatly dependednt upon the nature of the substuents in the Phytochemical + Antibiotic . Upon close inspection of the inhibitory activity exerted by these compounds, a biological profile on the Structure – Activity relationship could be deduced as follows- The position of the hydroxyl (-OH) is probably responsible for such inhibition. The Phytochemical+ Antibiotic combinations that are non-competitive have OH in positions C3', C4', C5 and C7, while those are competitive have a hydroxyl in positions C4 ', C5 and C7. The hydroxyl at C3 'seems to be able to modify the interaction of combination with the β-lactamase and thus the type of inhibition. The Genistein, Cefaclor and Cefotaxime had low effect on the enzyme activity. This could be due to the absence of hydroxyl groups on the C5 and C7. Ellagic Acid and Gallic acid had inhibitory effect on β-lactamase, suggesting that the structure C6-C3-C6 is important for this inhibition.

Thus the combinations i.e. Theobromine + Cefaclore, Theobromine + Ceftazidime, Theobromine + Cefotaxime, Myricetin + Amoxicillin, Myricetin + Cefaclore, Genistein + Cefaclor, Genistein + Ceftazidime, Genistein + Cefotaxime, Genistein + Penicillin, Gallic acid + Ceftriaxone, Gallic acid + Cefotaxime, Ellagic acid + Cefaclor, Ellagic acid + Ceftazidime, Kaempferol + Cefaclor, Kaempferol+Amoxicillin, Kaempferol+Ceftriaxone and Kaempferol + Penicillin with their proper formulation could be a lead compounds for anti-UTI drug discovery.

CONCLUSION

The present study clearly highlights the low toxic potential of phytochemicals as antibacterial compounds and suggest the possibility of use of the above mentioned synergistic drug-herb combinations for combating infections caused by this pathogen. The drug-herb network presented in this study shows the level of interactions between various classes of antibiotics and phytochemicals and provides a baseline to identify the potential mechanism of action of these potential phytochemicals. Moreover, phytochemicals are reported to have the capability of increasing the susceptibility of the pathogen to various drugs and also reduce the toxicity of the drugs when used in combination. The synergistic effect may be due to certain complex formation which becomes more effective in the inhibition of a particular species of microorganisms either by inhibiting the cell wall synthesis or by causing its lysis or death. Finally, the experimental findings encourage further studies such as *in vivo* animal experiments to validate these interesting observations before clinical test. This study probably suggests the possibility of concurrent use of these antimicrobial drugs and phytochemicals in combination for treating Urinary Tract infections caused by *E.cancerogenus MAB-1*.

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Tables:

Table 1. β-lactamase Crypticity

Sr.No.	Drugs (Con.100 um)	Ratio of activity of disrupted cells/Intact cells of isolate *E. cancerogenus* MAB-1
1	Imipenem	18
2	Cefoxitin	21
3	Nitrocefin	57
4	Cefaclore	32
5	Ceftazidime	27
6	Cefotaxime	24
7	Ceftriaxone	42
8	Ampicillin	25
9	Amoxycillin	36
10	Penicillin	29
11	Amikacin	47
12	Aztreonam	22
13	Cefpirome	18
14	Cefalexin	50
15	Norfloxacin	30
16	Chloramphenicol	40
17	Kanamycin	20
18	Tetracycline	15
19	Piperacilline	17
20	Ciprofloxacine	12

Table 2. Purification of β- lactamases from E. cancerogenus MAB-1

Purification step	β-lactamases activity (U/min/ml)	Total protein (mg/ml)	Specific activity (U/mg)	Yield (%)	Purifiaction fold
Crude extract	2,260	62	36.45	100	1
Dialysis (Ammonium Sulphate)	3,170	35	58.02	88	1.8
DEAE-Cellulose-52	85	11	80.23	16.4	92.5

Table 3. Antibiogram Pattern of Fractions on Test Strain Enterobacter cancerogenus MAB-1

Test Strain	Fractions (Zone Diameter in mm)					
	F1	F2	F3	F4	F5	F6
Enterobacter cancerogenus MAB-1	15±0.22 mm	17±0.04 mm	21±0.86 mm	13±0.94 mm	11±0.00 mm	10±0.07 mm

Data given are mean of 3 replicates ±S.D. P<0.05; F1: Fraction of *Hemidesmus indicus* ,F2: Fraction of *Acacia catechu* ,F3&F4: Fraction of *Santalum album* ,F5: Fraction of *Acorus calamus* ,F6: Fraction of *Justicia adhatoda*.

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Table 4. Determination of Minimum Inhibitory Concentration (MIC) of isolated lead compounds

Test compounds	**MIC (µg/ml)**
Theobromine,	240
Myricetin,	>10
Ellagic acid,	1
Gallic acid.	240
Kaempferol	1
Genistein	32
Ceftriaxone	0.1
Ceftazidime	0.1
Cefotaxime	0.032
Cefaclor	480
Amoxicillin	128
Ampicillin	0.1
Penicillin	240

Table 5. In vitro Checkerboard FIC Index (Interpretation) of all Antibiotics + Phytochemicals combinations against E.cancerogenus MAB-1

Sr.No.	**Antibiotics + Phytochemicals combination**	**Checker board FIC Index (Interpretation)**
1	**Theobromine+Cefaclor**	**0.5 (S)**
2	**Theobromine+Ceftazidime**	**0.25(S)**
3	**Theobromine+Cefotaxime**	**0.5 (S)**
4	Theobromine+Ceftriaxone	1 (A)
5	Theobromine+Ampicillin	2.1 (A)
6	Theobromine+Amoxicillin	0.7 (A)
7	Theobromine+Penicillin	3 (A)
1	Myricetin+Penicillin	3(A)
2	Myricetin+Ampicillin	2.5(A)
3	Myricetin+Ceftazidime	1(A)
4	Myricetin+Ceftriaxone	2.1(A)
5	Myricetin+Amoxicillin	0.99(A)
6	Myricetin+Cefotaxime	0.75(A)
7	**Myricetin+Cefaclor**	**0.12(S)**
1	Ellagic acid+Amoxicillin	3.3(A)
2	Ellagic acid+Penicillin	2.4(A)
3	Ellagic acid+Ceftriaxone	1.8(A)
4	Ellagic acid+Cefotaxime	0.92(A)
5	Ellagic acid+Ampicillin	0.67(A)
6	**Ellagic acid+Cefaclor**	**0.37(S)**
7	**Ellagic acid+ Ceftazidime**	**0.10(S)**
1	Gallic acid+Ceftriaxone	4.5(ANT)
2	Gallic acid+ Ampicillin	3.8(A)
3	Gallic acid+Penicillin	3.1(A)
4	Gallic acid+Amoxicillin	2.5(A)
5	Gallic acid+Cefaclor	1.7(A)
6	Gallic acid+Ceftazidime	0.73(A)
7	**Gallic acid+Cefotaxime**	**0.5(S)**
1	Kaempferol +Cefotaxime	3.4(A)
2	Kaempferol+ Ceftazidime	2.2(A)

3	Kaempferol+Ampicillin	1.9(A)
4	Kaempferol+Penicillin	1(A)
5	Kaempferol+Amoxicillin	1(A)
6	**Kaempferol+Ceftriaxone**	**0.5(S)**
7	**Kaempferol+Cefaclor**	**0.25(S)**
1	Genistein+ Cefaclor	3.1(A)
2	Genistein+Ceftazidime	2.5(A)
3	Genistein+Cefotaxime	2(A)
4	Genistein+Ceftriaxone	1.6(A)
5	Genistein+Ampicillin	0.97(A)
6	Genistein+Amoxicillin	0.73(A)
7	**Genistein+Penicillin**	**0.13(S)**

S= Synergy; A= Additive; ANT= Antagonastic

Table 6. Post antibiotic effect of Antibiotic and Phytochemicals alone and in combination against E.cancerogenus MAB-1

Sr.No.	Treatment	Post antibiotic effect (PAE) (in h)
1	Theobromine	0.5
2	Myricetin	0
3	Ellagic acid	0
4	Gallic acid	0.5
5	Kaempferol	1
6	Genistein	0
7	Cefaclor	0
8	Ceftazidime	0
9	Cefotaxime	0.5
10	Ceftriaxone	0
11	Penicillin	0
12	Ampicillin	0.5
13	Amoxicillin	0
14	Theobromine+Cefaclor	7
15	Theobromine+Ceftazidime	4.5
16	Theobromine+Cefotaxime	5
17	Myricetin+Cefaclor	5
18	Ellagic acid+Cefaclor	3.5
19	Ellagic acid+ Ceftazidime	4
20	Gallic acid+Cefotaxime	6
21	Kaempferol+Ceftriaxone	2.5
22	Kaempferol+Cefaclor	5.6
23	Genistein+Penicillin	3

Table 7. In vitro β-lactamase inhibitory activity of Phytochemicals, Antibiotics and Antibiotic + Phytochemical combination

Sr. No.	Compounds	Km	Ki(mM)	IC_{50}	% Inhibition	Inhibition type
1	Theobromine	0.07	0.76	0.61	89%	Non-Competitive
2	Myricetin	0.078	0.62	0.43	58%	Non-Competitive

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3	Ellagic acid	0.09	0.48	0.50	74%	Non-Competitive
4	Gallic acid	0.07	1.15	0.69	63%	Non-Competitive
5	Kaempferol	0.18	2.32	0.54	77%	Non-Competitive
6	Genistein	0.5	0.60	0.51	41%	Non-Competitive
7	Cefaclore	0.45	1.4	0.73	27%	Competitive
8	Ceftazidime	0.41	2.30	1.5	13%	Competitive
9	Cefotaxime	0.55	2.1	1.65	8%	Competitive
10	Ceftriaxone	0.55	1.7	1	21%	Competitive
11	Ampicillin	0.48	0.85	2.9	19%	Competitive
12	Amoxicillin	0.32	0.01	0.39	19%	Competitive
13	Penicillin	0.39	0.42	3.7	39%	Competitive
14	**Theobromine + Cefaclore**	**0.12**	**0.33**	**0.15**	**91%**	**Non-Competitive**
15	**Theobromine + Ceftazidime**	**0.09**	**0.25**	**0.22**	**86%**	**Non-Competitive**
16	**Theobromine + Cefotaxime**	**0.15**	**0.45**	**0.90**	**87%**	**Non-Competitive**
17	Theobromine+Ceftriaxone	0.36	0.05	0.18	75%	Non-Competitive
18	Theobromine+Ampicillin	0.2	0.43	0.21	62%	Non-Competitive
19	Theobromine+Amoxicillin	0.23	0.65	0.33	50%	Competitive
20	Theobromine +Penicillin	0.05	2.1	0.10	43%	Non-Competitive
21	Myricetin +Penicillin	0.003	1.6	1.5	54%	Competitive
22	Myricetin+Ampicillin	0.23	0.9	0.17	67%	Competitive
23	Myricetin+Ceftazidime	0.1	1.1	0.17	73%	Non-Competitive
24	Myricetin+Ceftriaxone	0.06	0.36	1.3	78%	Competitive
25	Myricetin+Amoxicillin	0.034	0.07	21	83%	Non-Competitive
26	Myricetin+Cefotaxime	0.4	0.73	4.8	79%	Non-Competitive
27	**Myricetin+Cefaclore**	**0.23**	**0.24**	**3.7**	**90%**	**Competitive**
28	**Genistein+Cefaclore**	**0.12**	**0.53**	**1.01**	**92%**	**Competitive**

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29	Genistein+ Ceftazidime	0.34	0.17	1.97	89%	Competitive
30	**Genistein+Cefotaxime**	**0.67**	**0.5**	**0.39**	**90%**	**Competitive**
31	Genistein+Ceftriaxone	0.78	0.16	0.83	86%	Non-Competitive
32	Genistein+Ampicillin	0.09	1.3	0.45	86%	Non-Competitive
33	Genistein+Amoxicillin	0.07	0.21	2.3	70%	Competitive
34	**Genistein+Penicillin**	**0.03**	**0.36**	**1.65**	**91%**	**Competitive**
35	Gallic acid+Ceftriaxone	0.012	0.83	0.43	80%	Competitive
36	Gallic Acid+Ampicillin	0.34	0.39	0.15	77%	Non-Competitive
37	Gallic Acid+Penicillin	0.65	0.06	3.7	71%	Non-Competitive
38	Gallic Acid+Amoxicillin	0.78	0.48	3.4	65%	Competitive
39	Gallic Acid+Cefaclore	0.98	0.09	4.7	53%	Competitive
40	Gallic Acid+Ceftazidime	0.23	0.37	1.5	40%	Non-Competitive
41	**Gallic acid+Cefotaxime**	**0.45**	**1.32**	**0.77**	**92%**	**Non-Competitive**
42	Ellagic acid+Amoxicillin	0.76	1.22	2.3	41%	Competitive
43	Ellagic acid+Penicillin	0.81	0.76	0.9	57%	Competitive
44	Ellagic acid+Ceftriaxone	0.54	0.81	1.3	62%	Non-Competitive
45	Ellagic acid+Cefotaxime	0.76	0.13	3.9	81%	Non-Competitive
46	Ellagic acid+Ampicillin	0.53	0.4	0.04	87%	Competitive
47	**Ellagic acid+Cefaclor**	**0.04**	**0.50**	**0.27**	**87%**	**Competitive**
48	**Ellagic acid + Ceftazidime**	**0.7**	**0.18**	**1.6**	**88%**	**Non-Competitive**
49	Kaempferol +Cefotaxime	0.38	0.63	0.13	63%	Competitive
50	Kaempferol+ Ceftazidime	0.75	0.27	0.09	70%	Non-Competitive
51	Kaempferol+Ampicillin	0.62	0.24	0.47	77%	Competitive

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52	Kaempferol+Penicillin	0.07	2.32	0.15	80%	Non-Competitive
53	Kaempferol+Amoxicillin	0.35	0.76	1.9	81%	Competitive
54	**Kaempferol+Ceftriaxone**	**0.1**	**0.35**	**0.24**	**87%**	Competitive
55	**Kaempferol+Cefaclor**	**0.24**	**0.45**	**0.50**	**90%**	Competitive
56	**Clavulanic Acid**	**0.33**	**0.067**	**0.115**	**96%**	**Competitive**

Figures:

Fig.1. Elution profile (DEAE-Cellulose-52) of β-lactamase from E.cancerogenus MAB-1

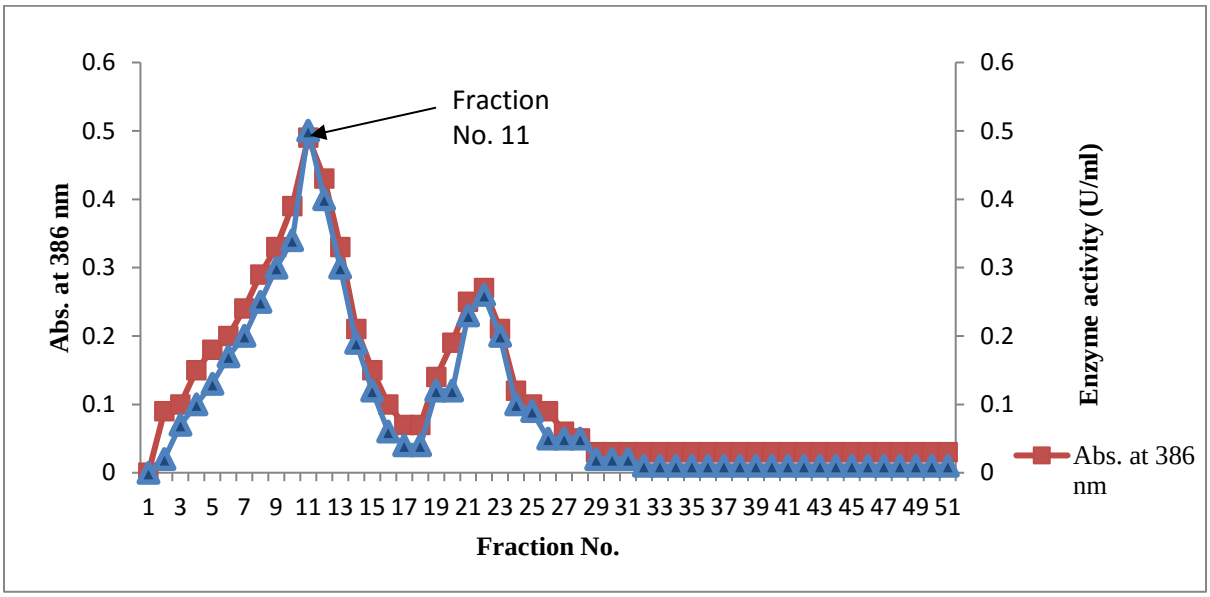

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Fig 2.Determination of Isoelectric point (PI) of β-lactamase from E.cancerogenus MAB-1 by Isoelectric Focusing

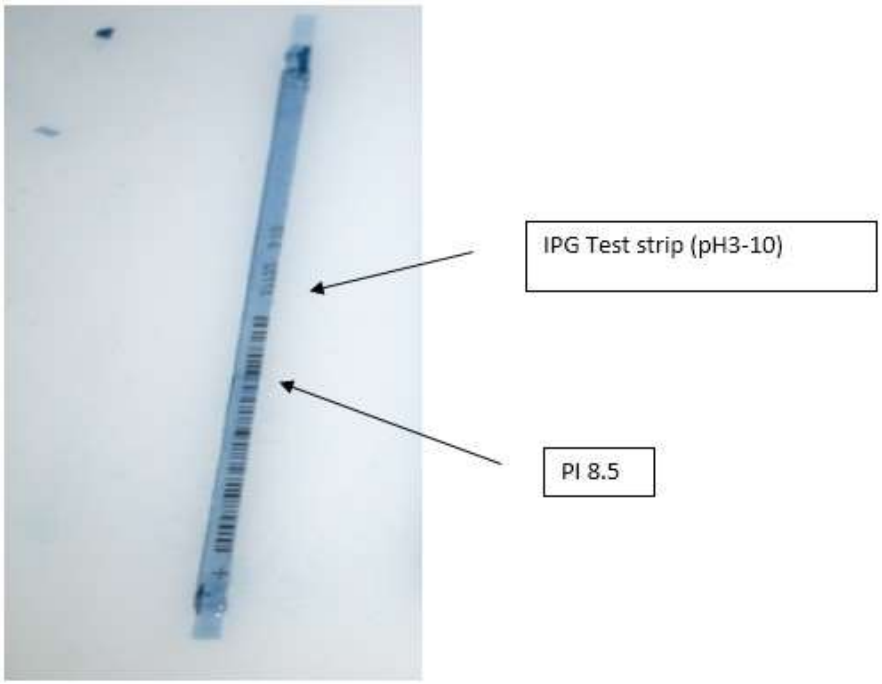

Fig.3. SDS- PAGE of purified β-lactamase from E.cancerogenus MAB-1 on 10% polyacrylamide gel.

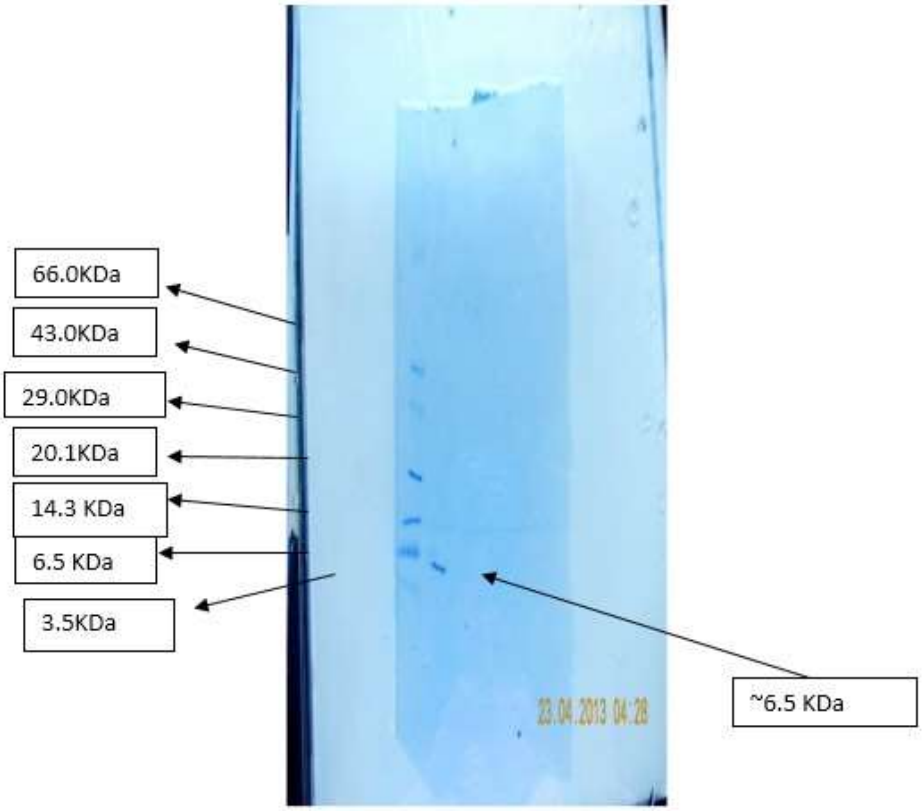

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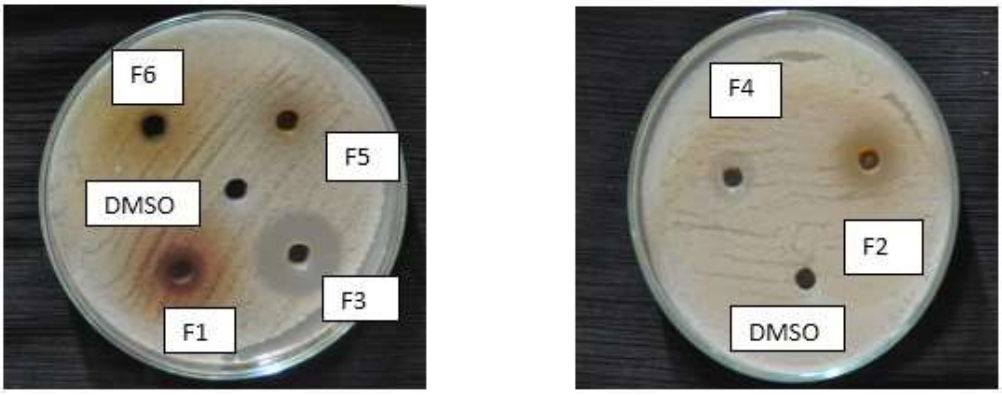
Fig.4. Antibiogram Pattern of Fractions on Test Strain Enterobacter cancerogenus MAB-1



F1: Fraction of Hemidesmus indicus ,F2: Fraction of Acacia catechu ,F3&F4: Fraction of Santalum album ,F5: Fraction of Acorus calamus ,F6: Fraction of Justicia adhatoda.

Fig. 5. Time-kill curve of E.cancerogenus MAB-1

Fig. 5 (a) Time-kill curve of E.cancerogenus MAB-1 with Theobromine + Ceftriaxone, Ceftazidime, Cefotaxime, Cefaclor, Amoxicillin, Ampicillin, Penicillin

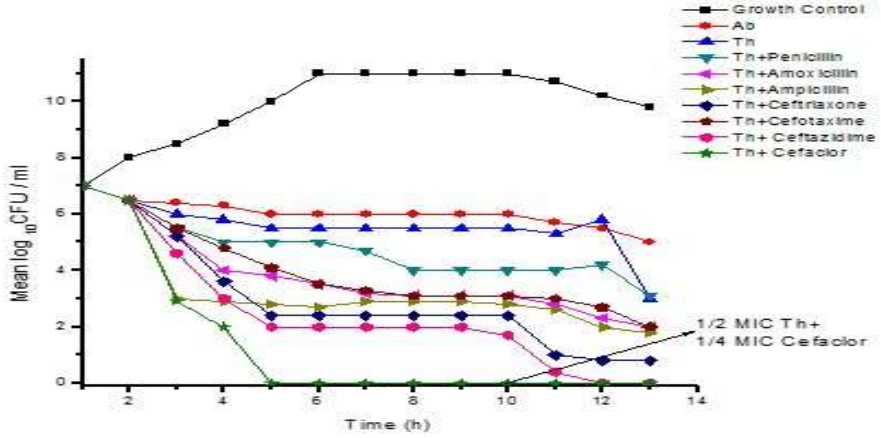


Fig. 5 (b) Time-kill curve of E.cancerogenus MAB-1 with Myricetin + Ceftriaxone, Ceftazidime, Cefotaxime, Cefaclor, Amoxicillin, Ampicillin, Penicillin

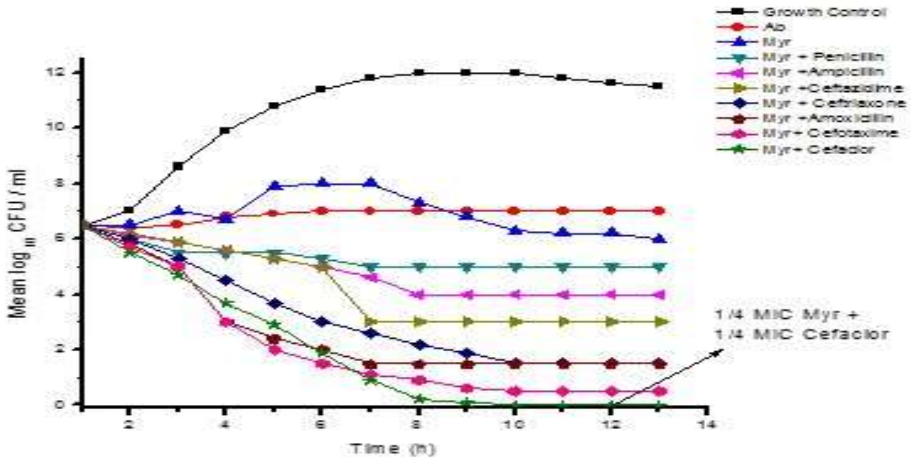


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Fig. 5(c) Time-kill curve of E.cancerogenus MAB-1 with Ellagic acid + Ceftriaxone, Ceftazidime, Cefotaxime, Cefaclor, Amoxicillin, Ampicillin, Penicillin

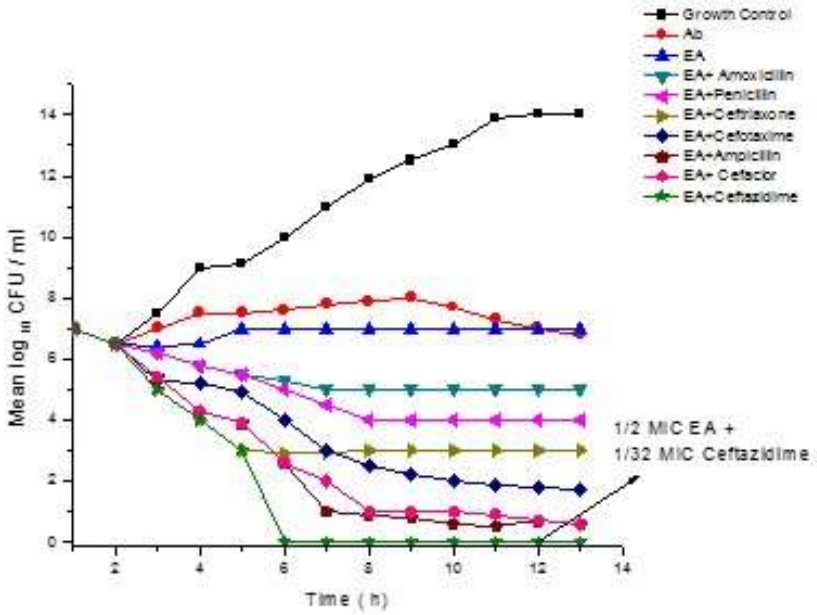

Fig. 5 (d) Time-kill curve of E.cancerogenus MAB-1 with Gallic acid + Ceftriaxone, Ceftazidime, Cefotaxime, Cefaclor, Amoxicillin, Ampicillin, Penicillin

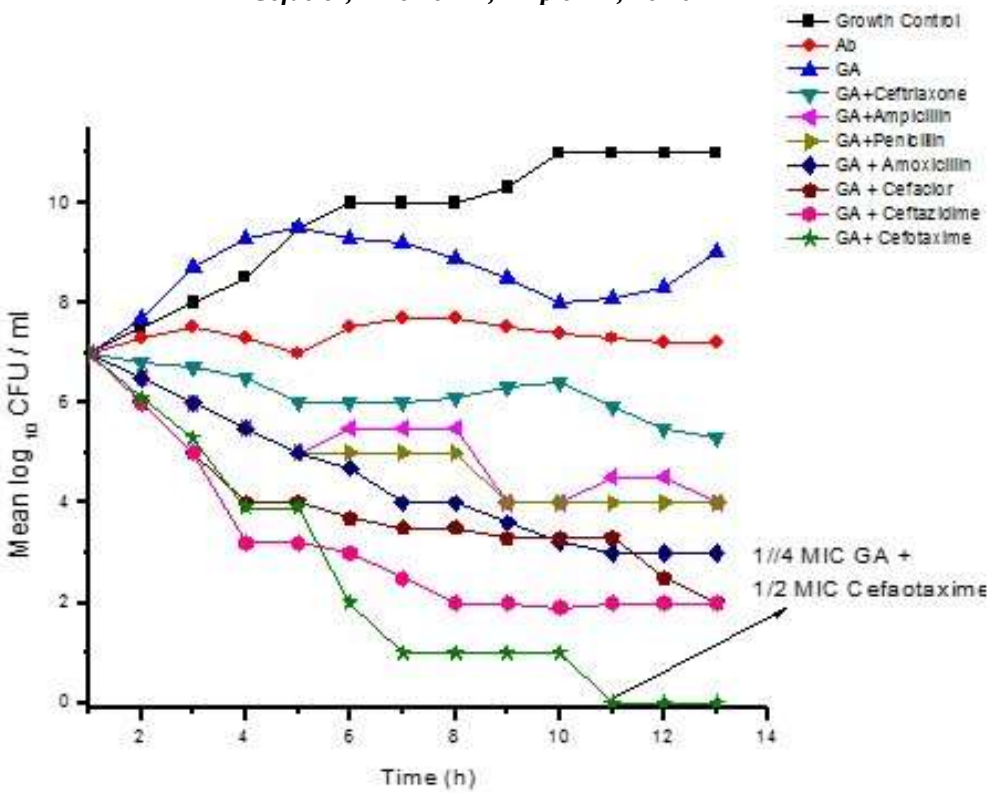

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Fig. 5 (e) Time-kill curve of E.cancerogenus MAB-1 with Kaempferol + Ceftriaxone, Ceftazidime, Cefotaxime, Cefaclor, Amoxicillin, Ampicillin, Penicillin

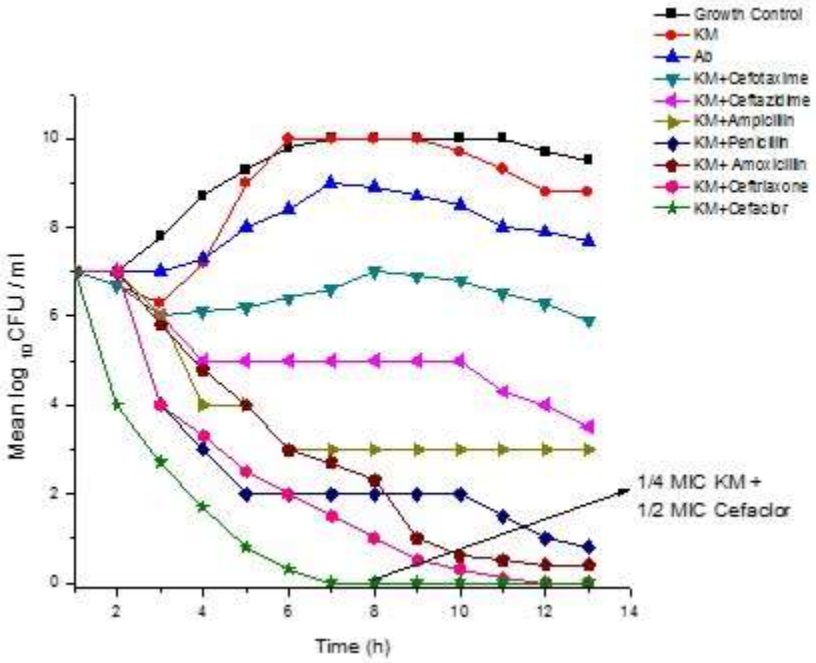

Fig. 5 (f) Time-kill curve of E.cancerogenus MAB-1 with Genistein + Ceftriaxone, Ceftazidime, Cefotaxime, Cefaclor, Amoxicillin, Ampicillin, Penicillin

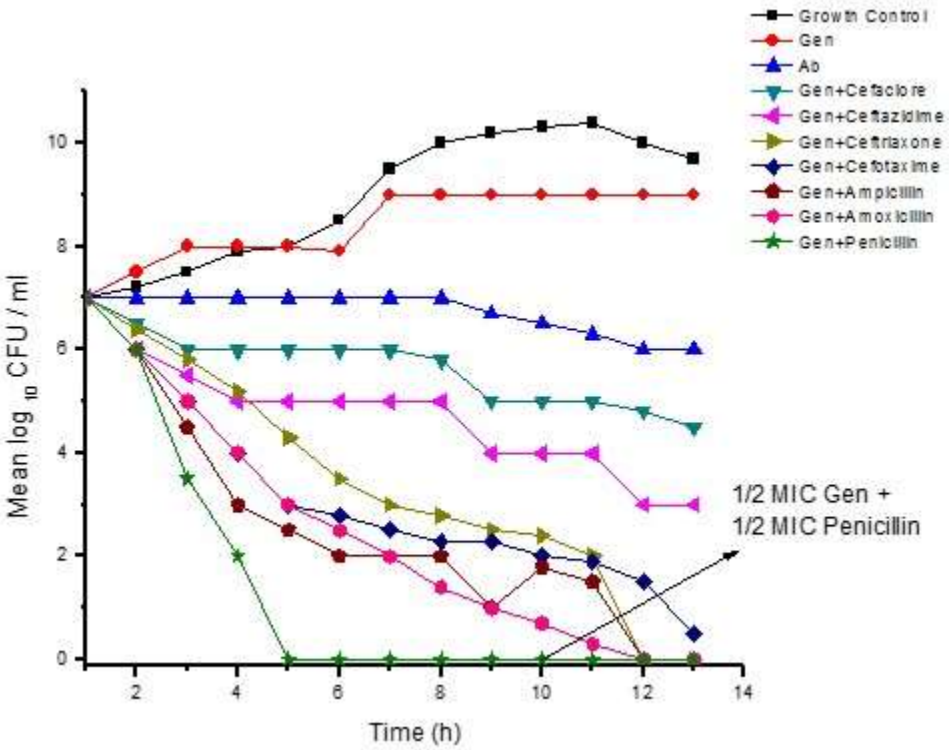

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Fig.1. Effect of pH on purified β-lactamase activity

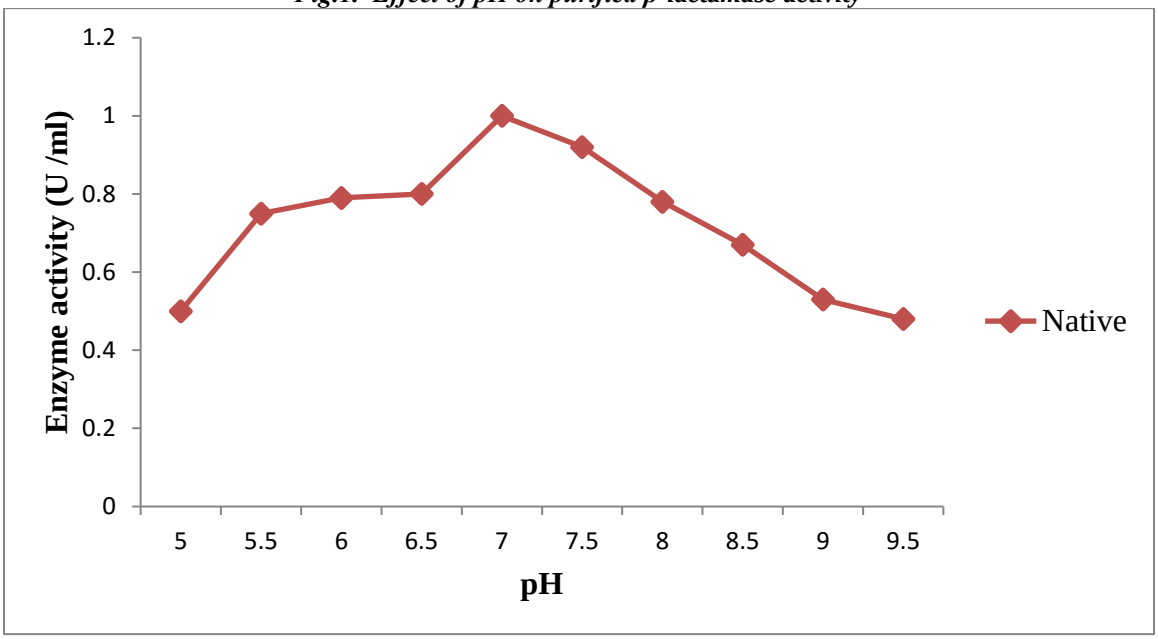

Fig.1 (a). Effect of pH on stability of purified β-lactamase

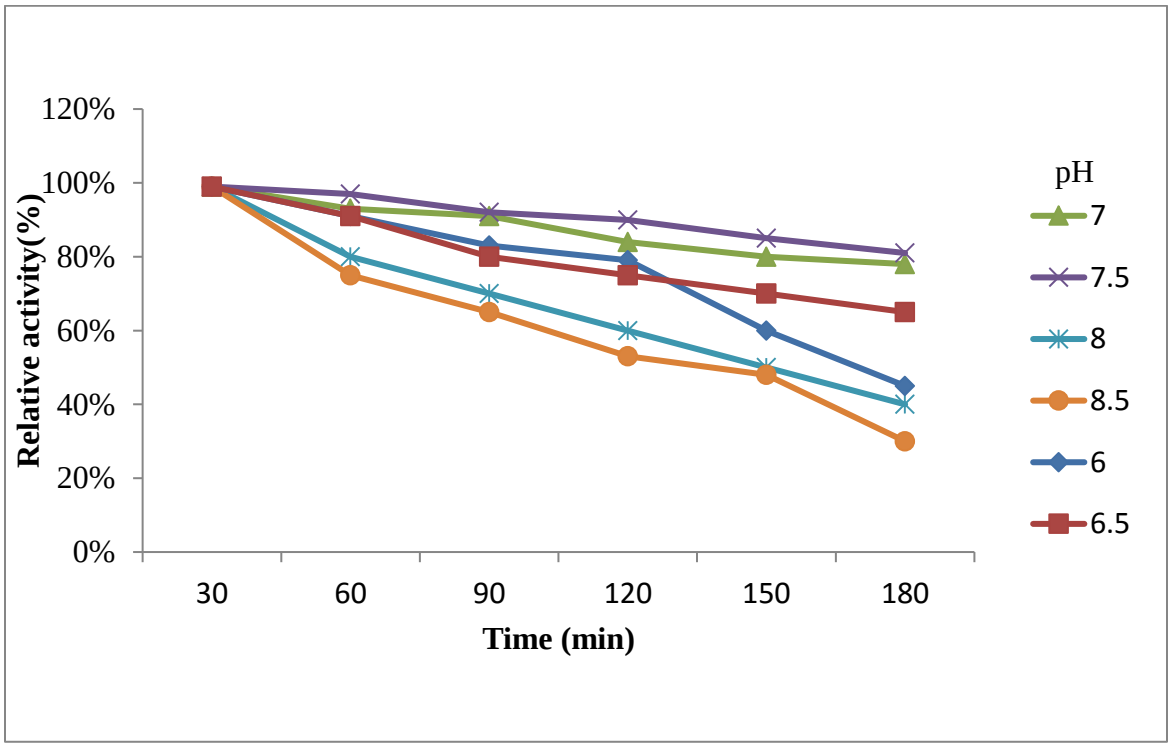

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Fig.2. Effect of Temperature on purified β-lactamase activity.

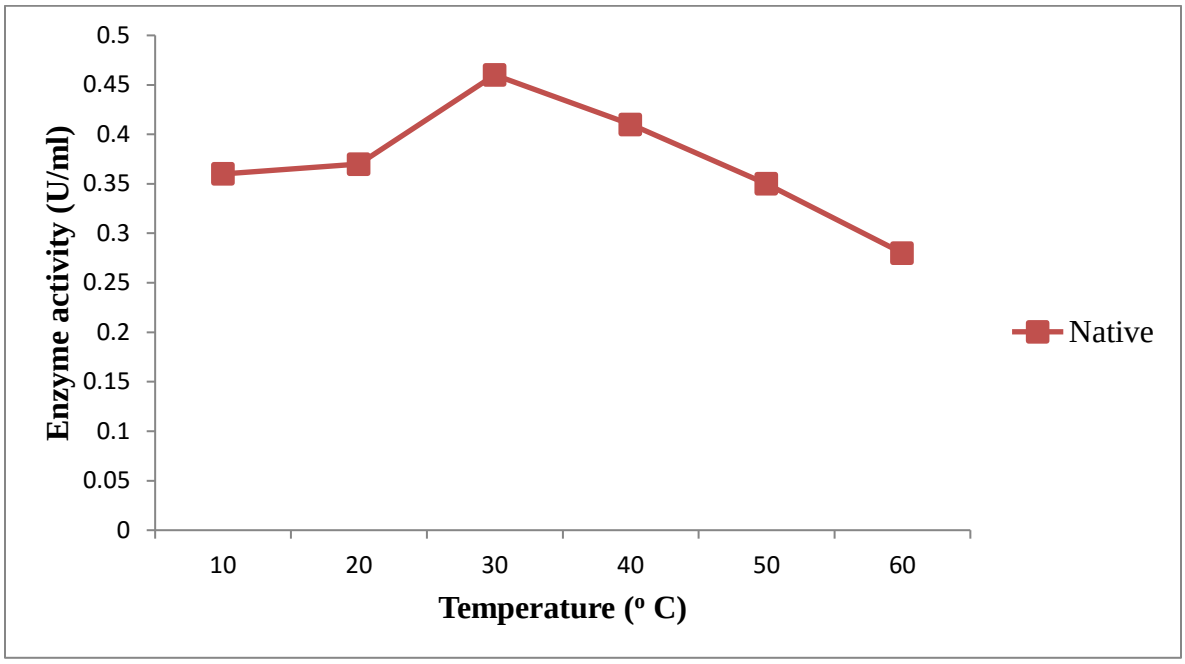

Fig.2 b) Effect of Temperature on stability of purified β-lactamase activity

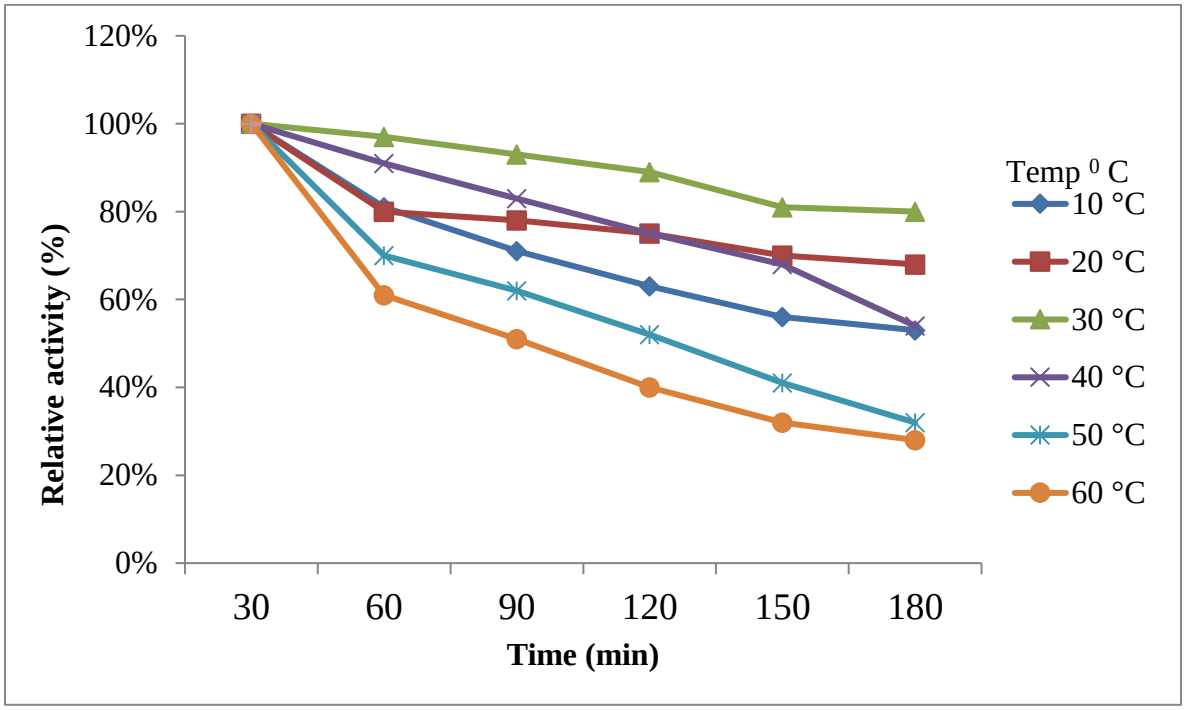

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Table 1. IR,NMR analysis for structure elucidation of lead compounds

- **Gallic acid (Mol.Wt. 170.12)**

IR (KBr):3363(-OH) cm^{-1}, 3286(Carboxylic -OH) cm^{-1}, 3063(Ar-H) cm^{-1}, 1705(>C=O) cm^{-1}. **^{1}H-NMR** (DMSO**):** δ12.0(s, 1H, acidic OH), δ9.0 (s, 2H, Ar-H), δ3.4 (s, 3H, OH).

- **Ellagic acid (Mol.Wt. 302.19)**

IR (KBr):3471(-OH) cm^{-1}, 3155(Ar-H) cm^{-1}, 1720(-OC=O) cm^{-1}. **^{1}H-NMR** (DMSO): δ3.4 (m, 4H, -OH), δ7.7(s, 2H, Ar-H).

- **Theobromine (Mol.Wt. 180)**

IR (KBr):3155(-NH) cm^{-1}, 3024(-CH, assym) cm^{-1}, 2823(-CH symm) cm^{-1}, 1689(amide), 1550(C=N) cm^{-1}. **^{1}H-NMR** (DMSO): δ11.1(s, 1H, NH), δ8.0(s, 1H, -CH), δ3.9(s, 3H, $-CH_3$), δ3.1(s, 3H, $-CH_3$).

- **Kaempferol (Mol.Wt. 286.24)**

IR (KBr):3420(bs, -OH) cm^{-1}, 3100(Ar-H) cm^{-1}, 3000(=CH), 1720(>C=O) cm^{-1}, 1135(-O-C-O) cm^{-1}. **^{1}H-NMR** (DMSO): δ12(s, 1H, OH), δ7.5(s, 2H, Ar-H), δ7.2 (m, 4H, Ar-H), δ3.5 (s, 2H, OH), δ3.0(s, 1H, OH).

- **Genistein (Mol.Wt. 270)**

IR (KBr):3500(bs, OH) cm^{-1}, 3160(Ar-H) cm^{-1}, 2950(=CH) cm^{-1}, 1675(α, β-unsaturated ketone) cm^{-1}, 1140(-O-C-O) cm^{-1}. **^{1}H-NMR** (DMSO): δ7.4(s, 2H, Ar-H), δ7.1 (q, 4H, Ar-H), δ6.5(s, 1H,=CH), δ3.8(s, 3H, OH).

- **Myricetin (Mol.Wt 318.24.)**

IR (KBr):3451(bs, OH) cm^{-1}, 3100(Ar-H) cm^{-1}, 1669(>C=O) cm^{-1}, 1138(-O-C-O) cm^{-1}. **^{1}H-NMR** (DMSO): δ12.0(s, 1H, OH), δ7.9(s, 2H, Ar-H), δ7.5(s, 2H, Ar-H), δ3.9(s, 5H, OH).