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Research Article

EVALUATION OF SELECTED MEDICINAL PLANTS FOR THEIR POTENTIAL ANTIMICROBIAL ACTIVITIES AGAINST ESKAPE PATHOGENS AND THE STUDY OF P-GLYCOPROTEIN RELATED ANTIBIOSIS; AN INDIRECT APPROACH TO ASSESS EFFLUX MECHANISM

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ABSTRACT

ESKAPE pathogens have significantly contributed the high mortality rate of hospital borne infections. Multidrug resistance and biofilm formation are the prime hurdles for controlling infections of ESKAPE pathogens. In vivo toxicity of the allopathic chemotherapeutic agents limits the complete eradication of ESKAPE pathogens. P-glycoprotein is one of the efflux proteins present in the plasma membrane and partially regulates multidrug resistance of ESKAPE pathogens. Certain plant extracts in processed form keeps potential to block the activities of P-glycoprotein. Present study is aimed towards exploring natural plant derived substances for the control of ESKAPE pathogens and thus keeps multidisciplinary approach.

INTRODUCTION

Antibiotics, one of the greatest discoveries of 20th century was soon followed by the development of resistance to antibiotics leading to drug resistant strains of bacteria. Evolution of multidrug resistance in microorganisms is rising at an alarming rate and the rate of discovery of new antibiotics is slowing down. This has resulted in an urgent need to look upon the unintentional consequences of antibiotic use. ESKAPE pathogens are the common causes of life threatening nosocomial infections amongst critically ill and immunocompromised individuals [(17)]. The term ESKAPE pathogens encompasses drug resistant bacteria namely *Enterococcus faecium* (Gram positive), *Staphylococcus aureus* (Gram positive), *Klebsiella pneumoniae* (Gram negative), *Acinetobacter baumannii* (Gram negative), *Pseudomonas aeruginosa* (Gram negative) and *Enterobacter cloacae* (Gram negative)[(17)]. A two-year review commissioned on antimicrobial resistance by the UK government concluded in 2016 that approximately 700,000 deaths each year globally can be attributed to antimicrobial resistance [(8)]. World Health Organization has classified ESKAPE pathogens based on its type of resistance by classifying them into three priority tiers as 'Critical, High and Medium' in the year 2017 [(18)]. ESKAPE pathogens maintain lower saturation values of antibiotics within the cytoplasm and these are attributed towards rapid efflux of antibiotics within the cytoplasm [(5)]. Carbapenem resistance in ESBL producing *Klebsiella pneumoniae* is due to the combined effect of β lactamases with porin impermeability and/or efflux pump activity [(4)].β Lactamase mediated resistance in *Enterobacter* spp. is increasingly associated with plasmid encoded 'Extended Spectrum β Lactamases' (ESBLs) and carbapenemases, specifically the CTX-M family of ESBLs, the KPC family of serine carbapenemases, and the VIM, IMP, and NDM-1 metallo β lactamases [(3)]. Reduction in porin expression in *Acinetobacter baumannii* and *Pseudomonas aeruginosa* is one of the reasons for development of drug resistance. Modification of drug targets, inactivation of therapeutic agents,

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overexpression of efflux pumps and cell envelope adaptive response that promotes survival in the human host and the nosocomial environment are the antibiotic resistant mechanisms observed in multidrug resistant *Enterococcus* species [11]. Drug resistance of ESKAPE pathogens is mainly relied on rapid removal of antibiotics from the cytoplasm [14]. Rapid efflux of the antibiotics is the function of P-glycoprotein efflux proteins present in the plasma membrane and its expression is regulated by multiple genes [9].For improving intracytoplasmic concentration of antibiotics, the activity of P-glycoproteinsare required to get regulated. Certain plant extracts keeps potential to block the activity of P-glycoproteins [14]. In this context, present study is aimed towards exploring selected natural plant derived substances for the control of ESKAPE pathogens and thus keeps multidisciplinary approach. *Aloe vera* is a perennial plant belonging to family Liliaceae (Indian Aloe or Korphad) [13]. It is distributed in tropical and subtropical regions. Itis reported for its antiviral, anti-inflammatory, immunity, antidiabetic, wound healing, antibacterial, antifungal, antioxidant, laxative and antitumor effects [4,13]. *Cymbopogon citratus*, a member of Poaceae family (Lemon grass or Gavti chaha) is a perennial tropical grass. It is cultivated in temperate climates, leaves are long, green and has a pleasant taste and aroma [7]. *Cymbopogon citratus* is reported for treatment against hypertension, epilepsy, gastrointestinal and central nervous system disorders [7].*Cynodon dactylon*, a member of Poaceae family is commonly known as 'Bermuda grass' or 'Doorva' [2]. It is a perennial grass mainly found in tropical regions with a warm climate. *Cynodon dactylon* is documented for its central nervous, anti-inflammatory, antipyretic, anticancer, immunological, protective, antimicrobial, antiparasitic, dermatological, diuretic, cardiovascular, antidiabetic, gastrointestinal, antioxidant, analgesic and antiallergic properties [1]**.** *Piper longum* is documented for antioxidant potential and anti-inflammatory properties [6, 14]. *Piper longum* belongs to family Piperaceae. It is commonly known as 'Indian Long Pepper' or 'Pimpli mool'. It is a flowering vine which is mainly cultivated for its fruit [14].

MATERIALS AND METHODS

Collection of plant resources

Cynodon dactylon (L.) Pers., *Cymbopogon citratus* (DC.) Stapf. and *Aloe vera* (L.) Burm. f., were collected from the Village Narhe, situated in the Taluka Haveli in the Pune district. These plants were collected from Pune territory. *Piper longum* Linn. was collected from a registered Ayurved medicine supplier from Pune district. All the plant derivatives were identified using standard identification keys and authenticated from Agharkar Research Institute, Pune.

Preparation of extracts of selected medicinal plants

All the selected plant resources namely *Aloe vera* (L.) Burm. f., *Cymbopogon citratus* (DC.) Stapf. , *Cynodon dactylon* (L.) Pers. and *Piper longum* Linn. were carefully washed using tap water followed by washing with sterile distilled water. The samples were shed dried. Leaves of *Aloe vera* (L.) Burm. f., *Cymbopogon citratus* (DC.) Stapf, *Cynodon dactylon* (L.) Pers. and root of *Piper longum* Linn. were subjected to chloroform, hexane, ethanol and aqueous extraction process using Soxhlet apparatus. Extracts were homogenized using magnetic stirrer and it was followed by concentration of extracts using rotary vacuum evaporator to dryness. The extracts were stored at 4°C.

Collection of microbial cultures

Staphylococcus aureus is obtained from Golwilkar Metropolis Health Service (India) Pvt. Ltd. Pune. *Enterococcus faecium* is obtained from Sahyadri Speciality Labs, Paud Road, Kothrud, Pune - 411038. *Klebsiella pneumoniae* NCIM 2706, *Acinetobacter baumannii* NCIM 5654, *Pseudomonas aeruginosa* NCIM 5210 and *Enterobacter cloacae* NCIM 2164 were obtained from NCIM Resource Center, Pune vide Proforma Invoice No. 2018 / NCIM - 2271.

Evaluation of drug resistance of the cultures obtained

1 ml suspension of the cultures obtained were prepared and compared with 0.5 McFarland standard as per NCCLS guidelines, subjected to 'Antibiotic Susceptibility Test' using 'Kirby-Bauer Disc Diffusion method' using 'Muller Hinton agar' (pH adjusted to 7.4 ± 0.1 at 25°C) incubated at 37°C for 24 hours.

Evaluation of antimicrobial activities of the plant extracts against ESKAPE pathogens by comparing with selected standard antibiotics

1 ml suspension each of the ESKAPE pathogens were prepared and compared with 0.5 McFarland standard as per NCCLS guidelines and inoculated on 'Muller Hinton agar' (pH adjusted to 7.4 ± 0.1 at 25°C) using 'Spread Plate Method'. 1 mg/ml concentration of aqueous, ethanol, chloroform and hexane extracts of all the four selected plant resources were evaluated for their antimicrobial activities against ESKAPE pathogens on 'Muller Hinton agar' (pH adjusted to 7.4 ± 0.1 at 25°C) incubated at 37°C for 24 hours using 'Agar well method'. The standards selected were Amoxycillin, Tetracycline and combination of Amoxycillin plus Clavulanic acid. 20 µg/ml concentration of the selected standard antibiotics were also assessed for their antimicrobial activities against ESKAPE pathogens using 'Agar well method' on 'Muller Hinton agar' (pH adjusted to 7.4 ± 0.1 at 25°C) incubated at 37°C for 24 hours.

Evaluation of antimicrobial activities of the plant extracts in combination with selected antibiotics (In direct approach to assess P-glycoprotein binding capacity)

1 ml suspension each of the ESKAPE pathogens were prepared and compared with 0.5 McFarland standard as per NCCLS guidelines and inoculated on 'Muller Hinton agar' (pH adjusted to 7.4 ± 0.1 at 25°C) using 'Spread Plate Method'. 25 µg/ml concentration of aqueous, ethanol, chloroform and hexane extracts of all the four selected plant resources in combination with 25 µg/ml concentration of standard antibiotic Amoxycillin and Tetracycline were assessed for their antimicrobial activities against ESKAPE pathogens using 'Agar well method' on 'Muller Hinton agar' (pH adjusted to 7.4 ± 0.1 at 25°C) incubated at 37°C for 24 hours.

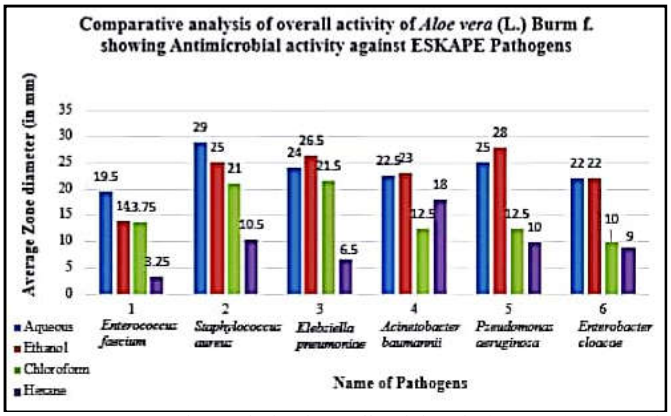
OBSERVATION AND RESULTS

Evaluation of drug resistance of the cultures obtained

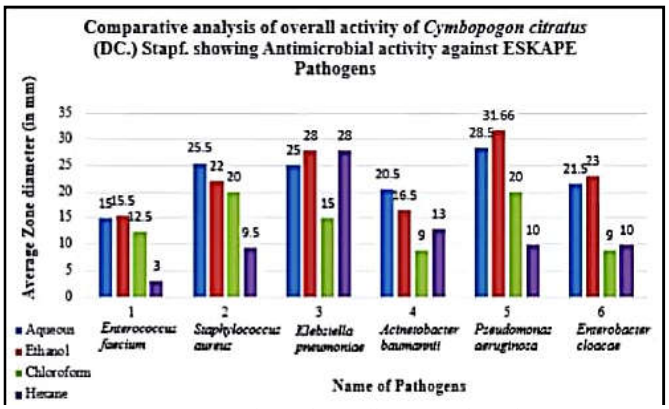
Enterococcus faecium was observed to be resistant to Amikacin (AN), Calithromycin (CLR), Cefotaxime (CF), Sparfloxacin (SF), Cefuroxime (CR), Cefoperazone (CFP), Ampiclox (ACX), Cefadroxil (CD), Roxythromycin (RX), Gentamycin (G) and Azithromycin (AZ); intermediate to Ciprofloxacin (CIP). *Staphylococcus aureus* was resistant to Amikacin (AN), Sparfloxacin (SF), Cefuroxime (CR) and Ampiclox (ACX); intermediate to Ciprofloxacin (CIP), Cefotaxime (CF) and Roxythromycin (RX); sensitive to Calithromycin (CLR), Cefoperazone (CFP), Cefadroxil (CD), Gentamycin (G) and Azithromycin (AZ). *Klebsiella pneumoniae* was resistant to Gentamycin (G), Netilmycin (NET), Lomefloxacin (LM) and Ampicillin + Sulbactam (SLB); sensitive to Amikacin (AN),Ciprofloxacin (CIP), Cefotaxime (CF), Sparfloxacin (SF), Cefoperazone (CFP), Cefadroxil (CD), Ceftriaxone (CTX) and Ceptazidime (CPZ). *Acinetobacter baumannii* was observed to be resistant to Cefotaxime (CF), Cefoperazone (CFP), Cefadroxil (CD), Netilmycin (NET) and Ceptazidime (CPZ); intermediate to Ceftriaxone (CTX) and Ampicillin + Sulbactam (SLB); sensitive to Amikacin (AN),Ciprofloxacin (CIP), Sparfloxacin (SF), Gentamycin (G) and Lomefloxacin (LM). *Pseudomonas aeruginosa* was resistant to Cefotaxime (CF), Sparfloxacin (SF), Cefoperazone (CFP), Cefadroxil (CD), Netilmycin (NET), Ampicillin + Sulbactam (SLB) and Ceptazidime (CPZ); intermediate to Ceftriaxone (CTX) and Lomefloxacin (LM); sensitive to Amikacin (AN),Ciprofloxacin (CIP) and Gentamycin (G). *Enterobacter cloacae* was observed to be resistant to Cefadroxil (CD), Netilmycin (NET), Lomefloxacin (LM), Ampicillin + Sulbactam (SLB) and Ceptazidime (CPZ); intermediate to Sparfloxacin (SF) and Cefoperazone (CFP); sensitive to Amikacin (AN),Ciprofloxacin (CIP), Cefotaxime (CF), Gentamycin (G) and Ceftriaxone (CTX).

Evaluation of antimicrobial activities of the plant extracts against ESKAPE pathogens by comparing with selected standard antibiotics

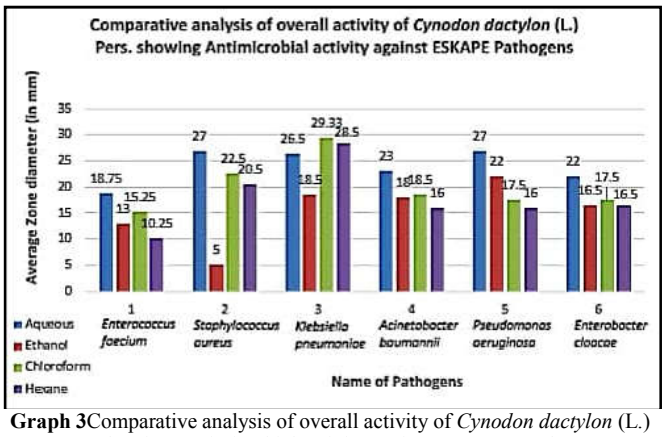
Aqueous, Ethanol, Chloroform and Hexane extracts of selected medicinal plants in the concentration of 1 mg/ml were separately tested for their antimicrobial activities. Broad spectrum antibiotics Amoxycillin, combination of Amoxycillin and Clavulanic acid and Tetracycline in the concentration of 20 µg/ml was selected as standard antibiotics against which antimicrobial activities of plant extracts were assessed. The antibiotics selected were totally drug resistant against *Enterococcus faecium* whereas aqueous extract of *Piper longum* Linn. exhibited maximum antimicrobial activity at a zone diameter of 21.33 mm against *Enterococcus faecium.* Amoxycillin, Tetracycline and blended dose of Amoxycillin and Clavulanic acid showed zone diameter of 15 mm, 11 mm, 22 mm respectively against *Staphylococcus aureus* whereas maximum antimicrobial activity was observed of aqueous extract of *Aloe vera* (L.) Burm f. at a zone diameter of 29 mm. Amoxycillin and blended dose of Amoxycillin and Clavulanic acid were totally resistant to *Klebsiella pneumoniae* and Tetracycline showed zone diameter of 19 mm. Maximum antimicrobial activity of hexane extract of *Cynodon dactylon* (L.) Pers. was observed against *Klebsiella pneumoniae* at a zone diameter of 29.33 mm. Amoxycillin and blended dose of Amoxycillin and Clavulanic acid were totally resistant to *Acinetobacter baumannii*and Tetracycline showed zone diameter of 19 mm. Maximum antimicrobial activity of aqueous extract of *Piper longum* Linn. was observed against *Acinetobacter baumannii*at a zone diameter of 29.33 mm. *Pseudomonas aeruginosa* was found to be totally resistant to the selected antibiotics whereas ethanol extract of *Cymbopogon citratus* (DC.) Stapf. showed maximum antimicrobial activity at a zone of 31.66 mm. Amoxycillin and blended dose of Amoxycillin and Clavulanic acid were totally resistant to *Enterobacter cloacae*and Tetracycline showed zone diameter of 17 mm. Ethanol extract of *Cymbopogon citratus* (DC.) Stapf. showed maximum antimicrobial activity at a zone of 23 mm against *Enterobacter cloacae*.



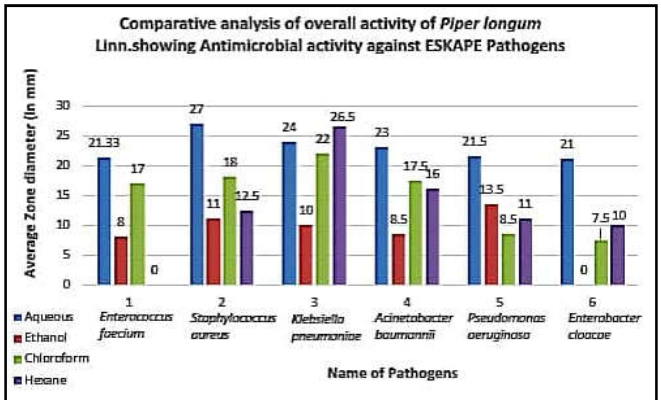
Graph 1: Comparative analysis of overall activity of *Aloe vera* (L.) Burm f. showing Antimicrobial activity against ESKAPE pathogens



Graph 2: *Comparative analysis of overall activity of Cymbopogon citratus (DC.) Stapf. showing Antimicrobial activity against ESKAPE pathogens*



Graph 3 Comparative analysis of overall activity of *Cynodon dactylon* (L.) Pers. showing Antimicrobial activity against ESKAPE pathogens



Graph 4 Comparative analysis of overall activity of *Piper longum* Linn. showing Antimicrobial activity against ESKAPE pathogens

Evaluation of antimicrobial activities of the plant extracts in combination with selected antibiotics (In direct approach to assess P-glycoprotein binding capacity)

Plants may alleviate the degree of antibiosis by down regulating the activity of P-glycoprotein. For the confirmation of potential effect of selected plant derivatives, plant extracts combined with Amoxycillin and Tetracyclineand their antimicrobial activities were assessed. Aqueous extract of *Piper longum* Linn. showed maximum antimicrobial activity in combination with antibiotics Amoxycillin and Tetracycline at a zone diameter of 21.33 and 14 respectively against *Enterococcus faecium*. Aqueous extract of *Aloe vera* (L.) Burm f. showed maximum antimicrobial activity in combination with antibiotics Amoxycillin and Tetracycline at a zone diameter of 29 and 17.66 respectively against *Staphylococcus aureus*. Hexane extract of *Cynodon dactylon* (L.) Pers. showed maximum antimicrobial activity in combination with antibiotics Amoxycillin and Tetracycline at a zone diameter of 29.33 and 27.00 respectively against *Klebsiella pneumoniae.* Aqueous extract of *Piper longum* Linn. showed maximum antimicrobial activity in combination with antibiotics Amoxycillin and Tetracycline at a zone diameter of 23.00 and 21.00 respectively against *Acinetobacter baumannii*. Ethanol extract of *Cymbopogon citratus* (DC.) Stapf. showed maximum antimicrobial activity in combination with antibiotics Amoxycillin and Tetracycline at a zone diameter of 31.67 and 28.66 respectively against *Pseudomonas aeruginosa*. Ethanol extract of *Cymbopogon citratus* (DC.) Stapf. showed maximum antimicrobial activity in combination with antibiotics Amoxycillin and Tetracycline at a zone diameter of 23 and 17.33 respectively against *Enterobacter cloacae*.

Statistical analysis

One Sample t Test was performed (Degrees of freedom, N = 3). Statistical analysis was performed of the plant extracts that showed maximum antimicrobial activity. It revealed that the plant extracts showed significant impact against ESKAPE pathogens [P value < 0.05^{*} (5 % level of significance), P value < 0.01^{**} (1 % level of significance) and P value < 0.001^{***} (0.1 % level of significance)] as observed in Table 1.

Table 1 One sample t Test of plant extracts against ESKAPE pathogens

Sr. No.	Test			P value
	Name of plant	Nature of extract	Name of microorganism	
1	*Piper longum* Linn.	Aqueous	*E. faecium*	0.007^{**}
2	*Aloe vera* (L.) Burm f.	Aqueous	*S. aureus*	0.002^{**}
3	*Aloe vera* (L.) Burm f.	Aqueous	*S. aureus*	0.007^{**}
4	*Aloe vera* (L.) Burm f.	Aqueous	*S. aureus*	0.001^{**}
5	*Cynodon dactylon* (L.) Pers.	Hexane	*K. pneumoniae*	0.004^{**}
6	*Cynodon dactylon* (L.) Pers.	Hexane	*K. pneumoniae*	0.028^{*}
7	*Piper longum* Linn.	Aqueous	*A. baumannii*	0.000^{***}
8	*Piper longum* Linn.	Aqueous	*A. baumannii*	0.001^{**}
9	*Cymbopogon citratus* (DC.) Stapf.	Ethanol	*P. aeruginosa*	0.004^{**}
10	*Cymbopogon citratus* (DC.) Stapf.	Ethanol	*E. cloacae*	0.004^{**}
11	*Cymbopogon citratus* (DC.) Stapf.	Ethanol	*E. cloacae*	0.05^{*}

Statistical analysis was performed of the plant extracts in combination with antibiotic Amoxycillin that showed maximum antimicrobial activity. It revealed that the plant extracts showed significant impact against ESKAPE pathogens [P value < 0.05^{*} (5 % level of significance), P value < 0.01^{**} (1 % level of significance) and P value < 0.001^{***} (0.1 % level of significance)] as observed in Table 2.

Table 2 One sample t Test of plant extracts in combination with antibiotic Amoxycillin against ESKAPE pathogens

Sr. No.	Test			P value
	Name of plant	Nature of extract	Name of microorganism	
1	*Piper longum* Linn.	Aqueous	*E. faecium*	0.000^{***}
2	*Aloe vera* (L.) Burm f.	Aqueous	*S. aureus*	0.001^{**}
3	*Cynodon dactylon* (L.) Pers.	Hexane	*K. pneumoniae*	0.000^{***}
6	*Piper longum* Linn.	Aqueous	*A. baumannii*	0.000^{***}
7	*Cymbopogon citratus* (DC.) Stapf.	Ethanol	*P. aeruginosa*	0.000^{***}
8	*Cymbopogon citratus* (DC.) Stapf.	Ethanol	*E. cloacae*	0.001^{**}

Statistical analysis was performed of the plant extracts in combination with antibiotic Tetracycline that showed maximum antimicrobial activity. It revealed that the plant extracts showed significant impact against ESKAPE pathogens [P value < 0.05^{*} (5 % level of significance), P value < 0.01^{**} (1 % level of significance) and P value < 0.001^{***} (0.1 % level of significance)] as observed in Table 3. Only aqueous extract of *Piper longum* Linn. in combination with Tetracycline did not show any significant impact against *A. baumannii* (P value >0.05).

Table 3 One sample t Test of plant extracts in combination with antibiotic Tetracycline against ESKAPE pathogens

Sr. No.	Test			P value
	Name of plant	Nature of extract + Antibiotic	Name of microorganism	
1	*Piper longum* Linn.	Aqueous + Tetracycline	*E. faecium*	0.002^{**}
2	*Aloe vera* (L.) Burm f.	Aqueous + Tetracycline	*S. aureus*	0.031^{*}
3	*Cynodon dactylon* (L.) Pers.	Hexane + Tetracycline	*K. pneumoniae*	0.005^{**}
6	*Piper longum* Linn.	Aqueous + Tetracycline	*A. baumannii*	0.074
7	*Cymbopogon citratus* (DC.) Stapf.	Ethanol + Tetracycline	*P. aeruginosa*	0.000^{***}
8	*Cymbopogon citratus* (DC.) Stapf.	Ethanol + Tetracycline	*E. cloacae*	0.423^{*}

DISCUSSION

Drug toxicity developed by ESKAPE pathogens leading to severe fatal complications is the cause of concern in the present era due to its spread on a global scale. Novel antibiotics are

needed for combating these rapidly evolving pathogens. Production of new antibiotics has declined progressively over the past few years, creating less possibilities to treat these drug-resistant pathogens [16]. Inappropriate use of antibiotics resulting in incomplete antibiotic therapy has led to increasing resistance of ESKAPE pathogens to antibiotics. The major factor for development of multidrug resistance in ESKAPE pathogens is the P-glycoprotein efflux mechanism [9, 10, 12]. Drug resistance of ESKAPE pathogens can be controlled if the regulation of P-glycoproteins can be inhibited. Inhibition of P-glycoprotein efflux proteins occurs through blockage of substrate binding site through competitive / non-competitive / allosteric inhibition, by interfering with hydrolysis of Adenosine Tri Phosphate (ATP) or by modifying the integrity of lipid bilayers of cell membrane[9,12]. In Ayurveda, the plants used to prevent and treat degenerative diseases are termed as rejuvenators [15]. In the present study, four different medicinal plants have been selected; *Aloe vera* (L.) Burm f., *Cymbopogon citratus* (DC.) Stapf., *Cynodon dactylon* (L.) Pers. and *Piper longum* Linn. . These have been utilized as a base of different medicines in Ayurveda. Previous studies as described were conducted to assess the potential inhibition of P-glycoprotein efflux protein. Present study differs by focusing on the synergistic action of selected plant extracts with antibiotics. The study also focuses on the effect of potential inhibition of P-glycoprotein efflux protein on the antimicrobial pattern, which is a novel area of research. In the present study, *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae* NCIM 2706, *Acinetobacter baumannii* NCIM 5654, *Pseudomonas aeruginosa* NCIM 5210 and *Enterobacter cloacae* NCIM 2164 were selected as representative ESKAPE pathogens. 'Antibiotic Susceptibility tests' of these ESKAPE pathogens were observed to be resistant and intermediate to most of the antibiotics. Aqueous, ethanol, chloroform and hexane extracts of selected medicinal plants in the concentration of 1 mg/ml were separately tested for its antimicrobial activities. The antimicrobial activities of the plant extracts were compared with the antibiotics Amoxycillin, Tetracycline and blended dose of Amoxycillin and Clavulanic acid as standard. Further, plant extracts were evaluated to study the P-glycoprotein efflux mechanism as an indirect approach by combining the plant extracts with antibiotic. Statistical analysis revealed that the plant extracts have a significant impact and can be used for combating ESKAPE pathogen infections.

CONCLUSION

Aqueous, Ethanol, Chloroform and Hexane extracts of *Aloe vera* (L.) Burm f., *Cymbopogon citratus* (DC.) Stapf., *Cynodon dactylon* (L.) Pers. and *Piper longum* Linn. were screened for their antimicrobial properties and potential P-glycoprotein inhibiting capacity. Plant extracts under study in the blended form with Amoxycillin showed better antimicrobial properties. The research indicated that the plant extracts showed "Improved intracytoplasmic concentration of antibiotics" and this is predictive towards regulation of P-glycoprotein efflux protein. Study requires further evaluation to confirm the indirect approach.

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