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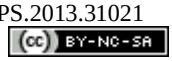
ISSN 2231-3354 

Evaluation of antibacterial potential of medicinal plant *Spilanthes acmella* Murr. and its *in vitro* raised callus against resistant organisms especially those harbouring *bla* genes

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ABSTRACT

Alcoholic extracts of stem of an endangered medicinal plant *Spilanthes acmella* and its *in vitro* raised callus were evaluated for antibacterial potential against various gram positive and gram negative bacteria including resistant isolates harbouring *bla* genes by agar well diffusion method. The alcoholic extract of parent plant as well as its callus showed good antibacterial activity against gram positive and gram negative bacteria and also efficiently controlled the growth of most of the resistant bacteria harbouring *bla* genes. Minimum inhibitory concentrations (MIC) of the extracts was determined by broth microdilution method. MIC against gram positive bacteria ranged from 12.0 to 49.0 µg/ml, while MIC against gram negative bacteria ranged from 1.53 to 12.0 µg/ml and MIC against resistant bacteria harbouring *bla* genes ranged from 6.1 to 98.0 µg/ml. The present study shows that extracts of *Spilanthes acmella* contain good antibacterial activity which can be used to obtain novel antibacterial compounds for the treatment of infectious diseases that otherwise pose problem of drug resistance to currently used antimicrobials. This is the first report of antibacterial activity of *S. acmella* through *in vitro* callus and also it's potential against gram negative bacteria harbouring *bla* genes.

INTRODUCTION

Infectious diseases are the leading cause of morbidity and mortality throughout the world. Bacteria have evolved numerous defense mechanisms against antimicrobial agents. One such mechanism is acquisition of enzyme β-lactamase which is produced due to presence of *bla* genes in the chromosome or plasmid (extrachromosomal genetic material) of these organisms. β-lactamase hydrolyze the β-lactam ring of the broad specrum β-lactam antibiotics such as penicillins, cephalosporins and carbapenems, rendering them inactive. The clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug-resistant pathogens. Incidents of epidemics due to such drug resistant micro-organisms are now a common global problem posing enormous public health concerns.

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Even though pharmaceutical industries have produced a number of new antimicrobial drugs in the last years, resistance to these drugs by micro-organisms is on rise. This is due to indiscriminate use of commercial antimicrobial drugs commonly used for the treatment of infectious diseases. Also majority of synthetic antibiotics are highly toxic at their optimum dosage level (Bush *et al.*, 1995; Philippon *et al.*, 2002; Bandow *et al.*, 2003, Parekh and Chanda, 2008). This shows that there is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action. Among many proposed strategies, a good understanding of plants offers the potential of developing potent broad spectrum antibiotics. There are several reports in the literature regarding the antimicrobial activity of crude extracts prepared from plants (El-Seedi *et al.*, 2002; Rojas *et al.*, 2003; Duraipandiyan *et al.*, 2006; Parekh and Chanda, 2007). *Spilanthes acmella* Murr. an endangered plant belongs to the family Asteraceae. It is commonly knownas'Akarkara'or 'toothache plant'.

Both the names denote the analgesic alkylamides the plant contains which is used to numb the toothache. It is a flowering herb native to Brazil, and is grown as an ornamental plant in various parts of the world. It is found throughout India, up to 5000 feet in the Himalayas and other mountains. Its growth peaks in November to March and requires frequent watering. The entire plant is medicinally active and non-toxic to humans. The Indian traditional healers use the flower heads of this plant in dental and gum care. It is one of the major ingredients in popular herbal tooth powders and paste.

It increases the flow of saliva (sialogogue) and is useful in fever especially during summers. Its root decoction has been used as a laxative and diuretic drug. The flower heads and roots have been used in the past for treatment of scabies, psoriasis, scurvy, toothache, infections of gums and throat, paralysis of tongue and as a remedy for stammering in children. Its extracts also possess properties which cause muscle relaxation and repair functional wrinkles, hence it is used in anti-age creams. The leaves and flower heads contain analgesic, antifungal, anthelminthic, antimalarial, antibacterial, and immunostimulating activity. This plant has also been used to cure dysentery and rheumatism. (Ratnasooriya *et al.*, 2004; Chakraborty *et al.*, 2004; Rani and Murty, 2006; Chandra *et al.*, 2007; Barman *et al.*, 2009; Prachayasittikul *et al.*, 2013).

But unfortunately, these medicinal plants are disappearing at an alarming rate because of destruction of their natural habitats due to urbanization, indiscriminate deforestation and uncontrolled collection of plant materials for purposes other than medicinal use, thus making these plants endangered and threatened for extinction (Vanila *et al.*, 2008). Through *in vitro* cultivation it would be possible to preserve and conserve these important endangered plant species and also *in vitro* cultivation of explants may be used to obtain phytotherapeutic compounds, even at places where the parent plant does not grow due to adverse atmospheric conditions (Shahid *et al.*, 2009).

The present study was done to evaluate the antibacterial activity of medicinal plant *S. acmella* as well as its *in vitro* raised callus against an exhaustive range of bacteria, including both standard as well as clinical strains, with special reference to those possessing resistant *bla* genes responsible for β-lactamase production.

MATERIALS AND METHODS

Collection of plant materials

Stem segments were collected from 4 months old parent plant of *Spilanthes acmella* (Figure 1A) grown in the Medicinal Plant Nursery of Tamnaar, Raipur, Chhattisgarh, and nodal segments of this plant initially procured from Raipur were raised by *in vitro* callus induction, in the Department of Botany, Aligarh Muslim University, Aligarh and plant materials were taken from a 4 weeks old callus (Figure 1B).

Fig. 1: shows barious of plant *Spilanthes acmella* included in our study A) stem of parent plant and B) *in virto* raised callus.

IN VITRO CULTURE OF EXPLANTS FOR CALLUS INDUCTION

The nodal segments (explants) were washed under running tap water for 30 min and then washed thoroughly in sterile double-distilled water (DDW). These explants were then kept in 1% (w/v) Bavistin (Carbendazim Powder, BASF India Limited), a broad spectrum fungicide for 25 min, followed by thorough washing with 5% (v/v) Teepol (Qualigens Fine Chemicals, India), a liquid detergent for 15 min by continuous shaking method. The treated explants were washed in sterile DDW 3-4 times to remove the chemical inhibitors. The explants were then treated with 70% (v/v) ethanol for 30-40 sec, followed by rapid washing with sterile DDW and then surface sterilized by emersion in a freshly prepared solution of 0.1% (w/v) $HgCl_2$ (Qualigens Fine Chemicals, India), for 4 min under laminar flow. Finally, the explants were washed 5-6 times with sterile DDW for 5 min to remove all traces of sterilants (Shahid *et al.*, 2007). These sterile nodal segments were then inoculated in Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962) containing 5µM of TDZ (Thidiazuron) and 1µM of BA (6-Benzyladenine). The medium was prepared in culture tubes (25×150 mm, Borosil) and pH was adjusted at 5.8±0.2. The medium was solidified by adding 1% agar (HiMedia Lab. Ltd., India) and sterilized by autoclaving at 15 lb pressure per square inch, 121°C temperature for 15 min. The sterilized explants were then inoculated aseptically into the medium and incubated at 25 ± 2°C with relative humidity of 55 ± 5% and exposed to photocycle of 2,500 Lux intensity for 16 hrs. 4 weeks old callus was used for evaluation of the antimicrobial effect.

Preparation of plant extracts

The alcoholic extracts of the plant were tested for antibacterial activity. The extracts were prepared according to the method of Singh and Singh (2000) with some modifications as described below. To prepare alcoholic extracts, fresh stem segments (15 g) from parent plant were surface sterilized in 70% ethyl alcohol for 1 min and then washed 3 times with sterilized double distilled water (DDW). The callus was aseptically removed from the culture tube and all the plant materials, including callus,

were grounded separately with a sterilized pestle and mortar in 150 ml of 95% ethanol. The homogenized tissues were then centrifuged at 5000 rpm for 15 min. The supernatants were filtered and taken as alcoholic extracts which were immediately used for experimentation.

Bacteria Tested

The clinical bacterial strains included in our study were *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Salmonella typhi*, *Shigella dysenteriae* type 1, and *Vibrio cholerae*, which were isolated from various clinical specimens in the Department of Microbiology, Jawaharlal Nehru Medical College and Hospital, Aligarh Muslim University, Aligarh, India.

In addition, resistant clinical isolates harbouring *bla* genes (identified by genetic characterization done earlier) were also included in our study. These strains were *Escherichia coli* (*bla* $_{ampC}$), *Klebsiella* spp. (*bla* $_{CTX-M}$), *Klebsiella* spp. (*bla* $_{SHV}$), *Escherichia coli* (*bla* $_{TEM}$) and *Escherichia coli* (*bla* $_{ampC+SHV}$). The control bacterial species tested were *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853) obtained from National Institute for Communicable Diseases (NICD), New Delhi, India. All the bacterial strains were grown on Blood agar or MacConkey agar plates at 37°C and maintained on nutrient and blood agar slants.

Antibacterial susceptibility testing

Antibacterial activity was determined using agar well diffusion method (Akinpelu, 2001) as per the guidelines of Clinical and Laboratory Standards Institute (formerly known as National Committee for Clinical Laboratory Standards, 2000). Muellar-Hinton Agar (M 173; HiMedia, India) and 5% sheep blood agar (for fastidious organisms such as Streptococci) were used for testing the antibacterial activity. The plates were lawn cultured with inoculum of bacterial suspension containing 10^6 cfu/ml with the help of sterile swabs and wells of 5mm diameter were made in each plate using a sterile borer. Plant extracts (20µl) were poured in the wells using micropipette. 20µl of 95% ethanol was used as negative control, whereas, antibacterial agent gentamicin (500µg/20µl) was used as positive control. The plates were kept upright for 5-10 min until the solution diffused into the medium and then incubated aerobically at 37°C for 24 hours. Later, the zone of inhibition was measured and recorded. All the experiments were performed in triplicate.

Determination of minimum inhibitory concentrations (MIC)

MIC of the alcoholic extracts was determined by broth microdilution method, performed according to Clinical and Laboratory Standards Institute (formerly known as National Committee for Clinical Laboratory Standards, 2000), with minor modifications as described below. Doubling dilutions of the extracts were prepared using RPMI-1640 broth (HiMedia, India) supplemented with 0.3g/L L-glutamine (HiMedia, India), 0.165 mol/L of 3-[N-morpholino] propanesulfonic acid (MOPS) buffer (HiMedia, India) and 0.01% of Dimethyl sulphoxide (DMSO) (Qualigens Fine Chemicals, India). The extracts were dissolved in DMSO, and further diluted 1:50 in RPMI-1640 medium, and each resulting solution was used for a doubling dilution series. Microtitre plates were prepared containing 100µl of undiluted extracts in the first well, followed by doubling dilutions of extracts from second well onwards. Standardized inoculum of each bacterial species was added to the respective dilution wells including the first well.

The final concentrations of the extracts ranged from 25×10^3µg/ml to 48×10^{-3}µg/ml. For each test there was a sterility control well containing alcoholic extract in RPMI-1640 broth plus DMSO and a growth control well containing bacterial suspension without alcoholic extract. The microtitre plates were incubated at 35 ± 2°C for 24 hours with their upper surface covered by sterile sealers. The lowest concentration of the extract that did not show any visible growth was considered MIC of the extract for that bacterial species. All the MIC experimentations were performed in duplicate.

Statistical analysis

All the experiments of antimicrobial susceptibility testing were performed in triplicate. The results were expressed as the mean ± standard error (SE). Data were statistically analyzed by using one way analysis of variance (ANOVA) followed by Tukey's multiple analysis test (SPSS Software, Chicago, III, version 10). P values were calculated by one-sample T-test and $P < 0.05$ was taken as statistically significant.

RESULTS AND DISCUSSION

Antibacterial activities of alcoholic extracts of *S. acmella* as well as its *in vitro* raised callus against the tested bacterial species are shown in Table 1, 2 and 3. Negative control (ethanol) showed the zone of inhibition in the range of 0.00 to 8.67±0.33 mm. Positive control (gentamicin) showed the zone of inhibition in the range of 9.33±0.33 to 13.00±0.58 mm.

The alcoholic stem extract of *S. acmella* showed significant activity ($P<0.05$) against both gram positive and gram negative bacteria (Table 1 and 2). It showed significant activity against *Staphylococcus aureus* (P=0.010), *Staphylococcus epidermidis* (P=0.015), *Streptococcus pyogenes* (P=0.007), *Escherichia coli* (P=0.024), *Klebsiella pneumoniae* (P=0.027) and *Salmonella typhi* (P=0.017). It is interesting to note that the extract efficiently controlled the growth of pathogenic organisms responsible for causing various diseases like wound infection, stitch abscess, sore throat, urinary tract infection, diarrhea, pneumonia, enteric fever and nosocomial infections. The alcoholic extracts from parent plant also controlled the growth of most of the tested resistant bacteria harbouring *bla* genes (Table 3). It showed significant activity against *Escherichia coli* (*bla* $_{ampC}$) (P=0.038), *Klebsiella* spp. (*bla* $_{SHV}$) (P=0.027) and *Escherichia coli* (*bla* $_{ampC+SHV}$) (P=0.044).

Table. 1: Antibacterial activity of alcoholic extracts of *Spilanthes acmella* and its *in vitro* raised callus against pathogenic gram-positive bacteria.

Bacteria tested	Zone of inhibition (mm) ± SE			
	Alcoholic stem extract$^{\Delta}$	Alcoholic callus extract$^{\Delta}$	Ethanol† (negative control)	Gentamicin$^{£}$ (positive control)
Staphylococcus aureus	13.00 ± 0.58^{d}	13.33 ± 0.33^{d}	7.33±0.33^{c}	11.67±0.33^{b}
Staphylococcus epidermidis	13.33 ± 0.33^{c}	14.67 ± 0.33^{b}	8.33±0.33^{b}	11.33±0.33^{c}
Streptococcus pyogenes	14.33 ± 0.33^{a}	15.33 ± 0.67^{a}	8.67±0.33^{a}	13.00±0.58^{a}
Enterococcus faecalis	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	7.33±0.33^{c}	9.67±0.33^{d}
S. aureus ATCC 25923	13.67 ± 0.33^{b}	14.00 ± 0.58^{c}	8.67±0.33^{a}	13.00±0.58^{a}

† = concentration of negative controls used in test i.e. 20 µl each of 95% ethanol and DDW. Δ = concentration of extracts used in the test i.e. 2 mg / 20 µl. £ = concentration of gentamicin used in test i.e. 500 µg / 20 µl. Diameter of zone of inhibition is a mean of triplicates ± SE (mm). Differences were assessed statistically using one way ANOVA followed by Tukey's test. P<0.05 was considered as significant. The mean represented by same letter is not significantly different within the column.

Table. 1: Antibacterial activity of alcoholic extracts of *Spilanthes acmella* and its *in vitro* raised callus against pathogenic gram-negative bacteria.

Bacteria tested	Zone of inhibition (mm) ± SE			
	Alcoholic stem extract$^{\Delta}$	Alcoholic callus extract$^{\Delta}$	Ethanol† (negative control)	Gentamicin$^{£}$ (positive control)
Escherichia coli	13.33±0.33^{a}	14.67±0.33^{a}	7.67±0.33^{c}	11.33±0.33^{b}
Klebsiella pneumoniae	12.67±0.33^{c}	13.67±0.33^{b}	7.33±0.33^{d}	10.67±0.33^{c}
Proteus mirabilis	0.00±0.00^{e}	0.00±0.00^{f}	7.67±0.33^{c}	11.33±0.33^{b}
Pseudomonas aeruginosa	0.00±0.00^{e}	11.67±0.33^{e}	7.33±0.33^{d}	10.67±0.33^{c}
Salmonella typhi	13.00±0.58^{b}	13.33±0.33^{c}	7.67±0.33^{c}	10.33±0.33^{d}
Shigella dysenteriae type 1	0.00±0.00^{e}	0.00±0.00^{f}	8.33±0.33^{b}	9.67±0.33^{e}
Vibrio cholerae	0.00±0.00^{e}	0.00±0.00^{f}	7.67±0.33^{c}	10.33±0.33^{d}
E. coli ATCC 25922	13.33±0.33^{a}	13.67±0.33^{b}	8.67±0.33^{a}	12.67±0.33^{a}
P. aeruginosa ATCC 27853	12.33±0.33^{d}	12.67±0.33^{d}	8.33±0.33^{b}	11.33±0.33^{b}

† = concentration of negative controls used in test i.e. 20 µl each of 95% ethanol and DDW. Δ = concentration of extracts used in the test i.e. 2 mg / 20 µl. £ = concentration of gentamicin used in test i.e. 500 µg / 20 µl. Diameter of zone of inhibition is a mean of triplicates ± SE (mm). Differences were assessed statistically using one way ANOVA followed by Tukey's test. P<0.05 was considered as significant. The mean represented by same letter is not significantly different within the column.

Table. 3: Antibacterial activity of alcoholic extracts of *Spilanthes acmella* and its *in vitro* raised callus against genetically characterized resistant isolates harbouring *bla* genes.

Bacteria tested$^{\beta}$	Zone of inhibition (mm) ± SE			
	Alcoholic stem extract$^{\Delta}$	Alcoholic callus extract$^{\Delta}$	Ethanol† (negative control)	Gentamicin$^{£}$ (positive control)
Escherichia coli (*bla* $_{ampC}$)	11.67±0.33^{a}	12.33±0.33^{b}	0.00±0.00^{a}	10.67±0.33^{b}
Klebsiella spp. (*bla* $_{CTX-M}$)	0.00±0.00^{d}	12.67±0.33^{a}	0.00±0.00^{a}	11.33±0.33^{a}
Klebsiella spp. (*bla* $_{SHV}$)	11.33±0.33^{b}	11.67±0.33^{c}	0.00±0.00^{a}	10.33±0.33^{c}
Escherichia coli (*bla* $_{TEM}$)	0.00±0.00^{d}	0.00±0.00^{e}	0.00±0.00^{a}	9.33±0.33^{e}
Escherichia coli (*bla* $_{ampC+SHV}$)	10.33±0.33^{c}	10.67±0.33^{d}	0.00±0.00^{a}	9.67±0.33^{d}

β = Figure within the parenthesis denotes the *bla* genes harboured by the respective clinical strains of gram-negative bacteria. † = concentration of negative controls used in test i.e. 20 µl each of 95% ethanol and DDW. Δ = concentration of extracts used in the test i.e. 2 mg / 20 µl. £ = concentration of gentamicin used in test i.e. 500 µg / 20 µl. Diameter of zone of inhibition is a mean of triplicates ± SE (mm). Differences were assessed statistically using one way ANOVA followed by Tukey's test. P<0.05 was considered as significant. The mean represented by same letter is not significantly different within the column.

This shows that extracts derived from this plant could be used in future as novel antibacterial agents to treat infections caused by such resistant organisms, which otherwise pose problems of drug resistance to the currently used antimicrobial agents. Few studies have been done in the past to analyze the antibacterial potential of *Spilanthes acmella* . A study done by Prachayasittikul et al. (2009) showed that extracts from *Spilanthes acmella* gave significant antibacterial activity against *Streptococcus pyogenes* and *Staphylococcus epidermidis*, which supports our research findings. Another study done by Voravuthikunchai et al. (2005), tested the extracts of *Spilanthes acmella* against *Salmonella typhi*, but no activity was observed against this bacterium. This is in contrast to our study. The difference in results could be due to the different method of extraction used in their study as compared to that used in our study as well as variation in active metabolites present in plant extracts derived from different places. The alcoholic extract of *in vitro* cultivated callus of *S. acmella* also gave excellent antibacterial activity against both gram positive and gram negative bacteria (Table 1 and 2). It showed activity against *Staphylococcus aureus* (P=0.009), *Staphylococcus epidermidis* (P=0.012), *Streptococcus pyogenes* (P=0.003), *Escherichia coli* (P=0.012), *Klebsiella pneumoniae* (P=0.024), *Pseudomonas aeruginosa* (P=0.038) and *Salmonella typhi* (P=0.015). In addition, it also showed significant activity (P<0.05) against wide range of tested resistant organisms harbouring *bla* genes (Table 3). It showed significant activity against *Escherichia coli* (*bla* $_{ampC}$) (P=0.015), *Klebsiella* spp. (*bla* $_{CTX-M}$) (P=0.027), *Klebsiella* spp. (*bla* $_{SHV}$) (P=0.024) and *Escherichia coli* (*bla* $_{ampC+SHV}$) (P=0.038). The antibacterial activity of alcoholic extract of *in vitro* raised callus was found to be better in comparison to the activity of parent plant extract. This enhanced activity of *in vitro* raised callus could be due to nutritional enhancement and hormonal manipulations done in the culture medium. To the best of our knowledge this is the first study analyzing the antibacterial potential of *in vitro* raised callus and that too against resistant isolates harbouring *bla* genes, therefore, our findings could not be compared. MIC was determined for alcoholic extracts of *in vitro* raised callus. The MIC values against tested gram positive bacteria ranged from 12.0 to 49.0 µg/ml (Figure 2), while MIC values against tested gram negative bacteria ranged from 1.53 to 12.0 µg/ml (Figure 3) and MIC values against resistant bacteria harbouring *bla* genes ranged from 6.1 to 98.0 µg/ml (Figure 4).

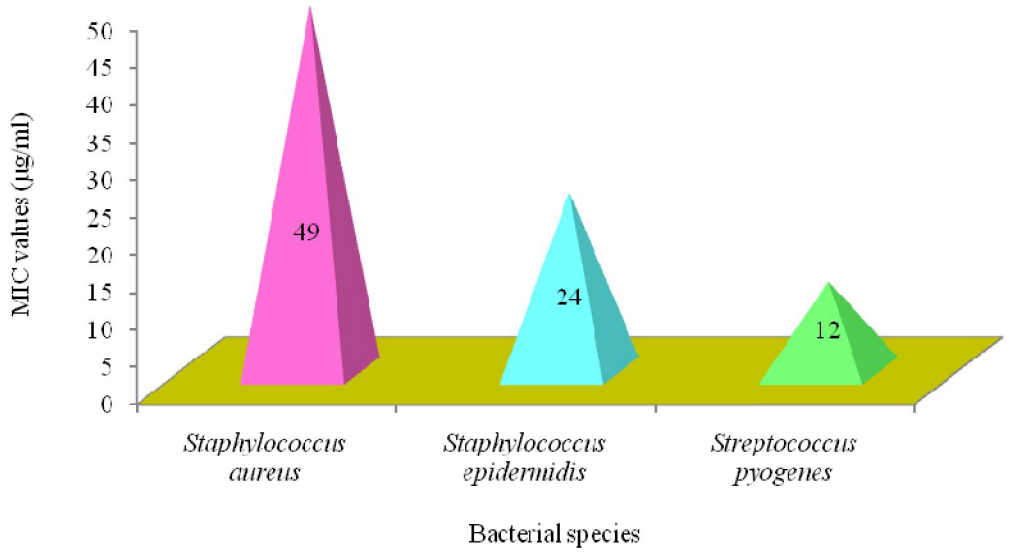

Fig. 2: MIC determination of alcoholic ectract of in vitro raise callus of *Spilanthes acmella* against tested gram positive bacteria.

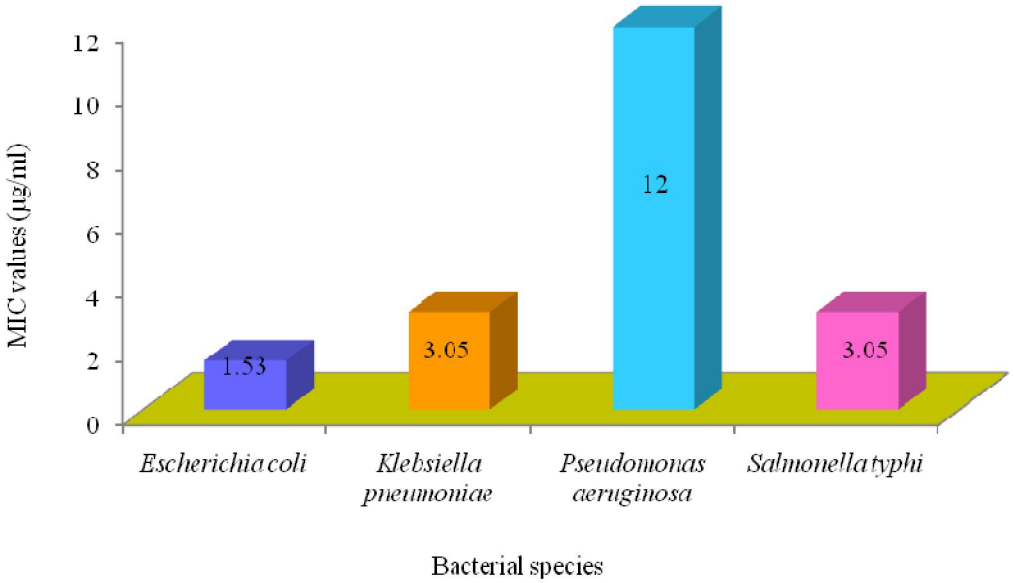

Fig. 3: MIC determination of alcoholic ectract of in vitro raised callus, of *Spilanthes acmella* against tested gram negative bacteria.

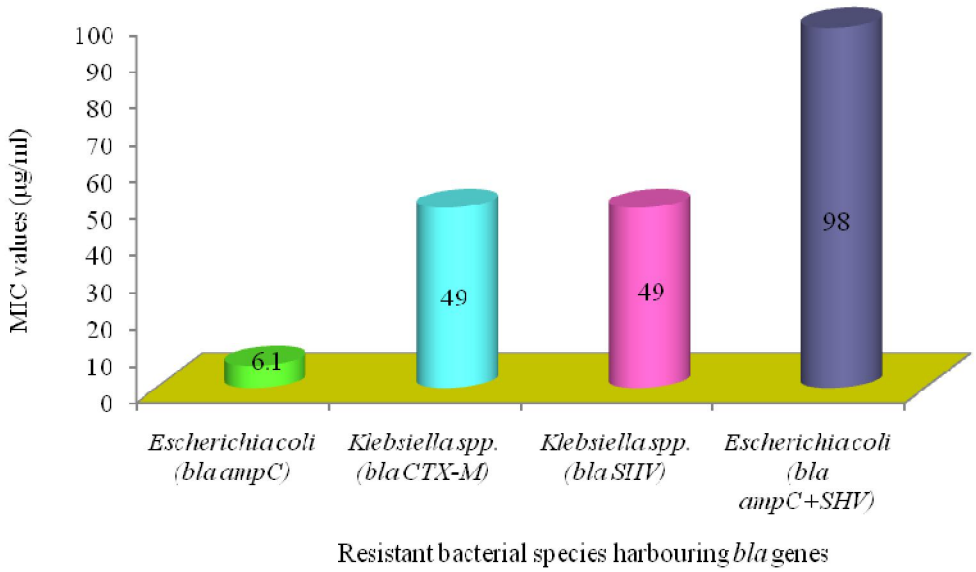

Fig. 4: MIC determination of alcoholic ectract of in vitro callus of *Spilanthes acmella* against resistant organisms harbouring bla genes.

CONCLUSION

In nutshell, extracts of *Spilathes acmella* contain remarkable antibacterial potential and thus could be used in future to derive new antimicrobial compounds to treat infections caused by resistant organisms. Since herbal medicines are easily acceptable and quite affordable to the majority of the Indian population, it could act as an alternative medicine in treating various infections as synthetic antibiotics meant to treat such resistant organisms are quite costly and produce toxic effects at optimum dosage level.

As the extracts from *in vitro* raised callus also showed significant antibacterial potential, *in vitro* cultivation of the plant may be used to obtain phytotherapeutic compounds, especially at places where the parent plant does not grow naturally because of adverse atmospheric conditions. Also by *in vitro* cultivation we could preserve this threatened plant and prevent it from extinction.

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