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Antimicrobial activity of medicinal plant *Balanites aegyptiaca* Del. and its *in vitro* raised calli against resistant organisms especially those harbouring *bla* genes

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Alcoholic extracts of fruit of an endangered medicinal plant *Balanites aegyptiaca* and its *in vitro* raised calli were analyzed for antimicrobial potential against various Gram positive and Gram negative bacteria including those harbouring *bla* genes by agar well diffusion method. The alcoholic extract of parent plant as well as its callus showed good antibacterial activity against both Gram positive and Gram negative bacteria. Minimum inhibitory concentrations (MIC) of the extracts were determined by broth microdilution method. MIC against Gram positive bacteria ranged from 3.05 to 24.0 µg/ml, while MIC against Gram negative bacteria ranged from 1.53 to 49.0 µg/ml and MIC against resistant bacteria harbouring *bla* genes ranged from 12.0 to 49.0 µg/ml. The present study shows that extracts of *B. aegyptiaca* contain good antibacterial activity which can be used in the treatment of various infectious diseases. As its calli also gave good results, *in vitro* cultivation of the explants may be used to obtain novel antimicrobial compounds especially at places where it does not grow naturally. This is the first report of antibacterial activity of *B. aegyptiaca* against Gram negative bacteria harbouring *bla* genes.

Key words: *Balanites aegyptiaca*, *in vitro* raised callus, antimicrobial activity.

INTRODUCTION

Infectious diseases account for high proportion of health problems and are the leading cause of death worldwide (Parekh and Chanda, 2007). Even though pharmaceutical industries have produced a number of new antimicrobial drugs in the last years, resistance to these drugs by micro-organisms has increased. This is due to indiscriminate use of commercial antimicrobial drugs commonly used for the treatment of infectious diseases (Davies, 1994).

Beta-lactam antibiotics are typically used to treat a broad spectrum of Gram positive and Gram negative bacteria. Bacteria develop resistance to commonly used β-lactam antibiotics such as penicillins, cephalosporins and carbapenems, because of acquisition of enzyme β-lactamases whose synthesis is governed by presence of β-lactamase genes (*bla* genes). These *bla* genes are

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located either on the chromosome or plasmid (extrachromosomal genetic material) of bacteria and are responsible for production of β-lactamase enzyme that hydrolize the β-lactam ring present in the antibiotic, rendering it inactive (Bush et al., 1995; Philippon et al., 2002).

In general, bacteria have the ability to acquire and transmit resistance to drugs used as therapeutic agents (Nascimento et al., 2000). Incidents of epidemics due to such drug resistant micro-organisms are now a common global problem posing enormous public health concerns. The global emergence of multidrug resistant bacterial strains is increasingly limiting the effectiveness of current drugs and significantly causing treatment failure of infections (Davies, 1994; Hancock, 2005).

This situation has forced the researchers to search for new antimicrobial substance from various sources including medicinal plants (Bauer et al., 1966; Colombo and Bosisio, 1996; Scazzocchio et al., 2001; Erdogrul, 2002; Bandow et al., 2003; Parekh and Chanda, 2008).

The use of medicinal plants as traditional or folk medicine for the treatment of common infections is well known in rural areas of many developing countries (Sandhu and Heinrich, 2005; Gupta et al., 2005; Rojas et al., 2006).

Medicinal plants contain large varieties of chemical substances which possess important therapeutic properties that can be utilized in the treatment of human diseases (Akinpelu et al., 2008). There are several reports in the literature regarding the antimicrobial activity of crude extracts prepared from plants which may be less toxic to humans and possibly with a novel mechanism of action (Dimayuga and Garcia, 1991; El-Seedi et al., 2002; Rojas et al., 2003; Duraipandiyan et al., 2006; Parekh and Chanda, 2007).

Balanites aegyptiaca Del. (Zygophyllaceae or Simaroubaceae), also known as 'desert date' in English, 'Hingoli' in Hindi and 'Angavriksha' in Sanskrit (Iwu, 1993; Gaur et al., 2008), is a medicinal plant that grows in Sahel-Savanna regions and drier parts of middle-belt zones of Nigeria, Ghana, and Ivory Coast, and is cultivated as a fruit tree in semisavanna parts of the continent. This plant is a small evergreen thorny tree found in drier parts of India (Gaur et al., 2008). It grows to 6 to 10 m in height, is highly resistant to stresses such as sandstorms and heat waves, and grows with minimal available moisture. The trees produce date-like fruits between March and October.

In ethnomedicinal studies, it has been found that various parts of the *Balanites* tree have been used as folk medicine in many regions of Africa and Asia (Hall and Walker, 1991; Mohamed et al., 2000, 2002). Literature has revealed antifeedant, antidiabetic, molluscicide, anthelminthic, and contraceptive activities in various *Balanites* extracts (Liu and Nakanishi, 1982; Kamel et al., 1991; Ibrahim, 1992; Rao et al., 1997). The bark, unripe fruits, and leaves of this plant are reported to have anthelminthic, antifertility, purgative and antidysenteric properties (Kirtikar and Basu, 1996; Chopra et al., 1956a, b). Aqueous suspensions of dried fruits of this plant are being used as abortifacient by local healers (Gaur et al., 2008). The root has been indicated for the treatment of malaria, herpes zoster, and venereal diseases (Irvine, 1961; Ayensu, 1978).

But unfortunately, these plants are disappearing at an alarming rate due to indiscriminate deforestation and uncontrolled collection of plant materials (Vanila et al., 2008). Through *in vitro* cultivation it would be possible to preserve and conserve these endangered plants and obtain phytotherapeutic compounds especially at places where the plant does not grow naturally due to adverse atmospheric conditions (Shahid et al., 2009b).

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

MATERIALS AND METHODS

Collection of plant

The fruit pulp of a 15 years old parent plant of *B. aegyptiaca* was obtained from Tissue Culture Laboratory, Department of Botany, Gujarat University, Ahmedabad (Gujarat). For *in vitro* cultivation of the same plant, seed materials initially procured from Ahmedabad were grown in Botanical Garden, Department of Botany, Aligarh Muslim University, Aligarh and plant materials were taken from a 5 year old plant.

In vitro culture of explants for callus induction

Sterilization of the collected explants

The young explants (seed) were isolated from the plant and washed under running tap water for 30 min and then washed thoroughly in sterile double-distilled water (DDW). Then these explants were 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 (Shahid et al., 2007, 2009b).

Then the treated explants were washed in sterilized DDW 3 to 4 times under an aseptic condition, to remove the chemical inhibitors. The explants were then treated with 70% (v/v) ethanol for 30 to 40 s, followed by rapid washing with sterile DDW and then surface sterilized by emersion in a freshly prepared aqueous solution of 0.1% (w/v) $HgCl_2$ (Qualigens Fine Chemicals, India) (Bhojwani and Razdan, 1996), for 4 min under laminar flow. Finally, the explants were washed 5 to 6 times with sterile DDW with intervals of 5 min to remove all traces of sterilants (mercury ions).

Inoculation of sterilized explants on MS medium

Murashige and Skoog's (MS) medium (Murashige and Skoog,

1962) containing 2.5 µM of 2,4-D [2,4-Dichlorophenoxy acetic acid] was taken for inoculation of explants. 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 and incubated at 25±2°C with relative humidity of 55±5% and were exposed to photocycle of 2,500 Lux intensity for 16 h (Shahid et al., 2009b). Five weeks old callus was used for evaluation of the antimicrobial effect.

Preparation of plant extracts

The alcoholic extracts of the plant were tested for antimicrobial activity. The extracts were derived according to the method of Singh and Singh (2000) with some modifications (Shahid et al., 2007, 2009a, b). To prepare alcoholic extracts, fresh fruit (15 g) from parent plant were surface sterilized in 70% ethyl alcohol for 1 min and then washed 3 times with sterilized DDW. The calli of the explants were aseptically removed from the culture tubes and all the plant materials, including calli, were grounded with a sterilized pestle and mortar in 150 ml of 95% ethanol and centrifuged at 5000 rpm for 15 min, and the supernatant was filter sterilized and taken as the alcoholic extract. The extracts were immediately used for experimentation.

Microorganisms tested

Gram positive and Gram negative bacteria isolated from various clinical specimens in the Department of Microbiology, Jawaharlal Nehru Medical College and Hospital, Aligarh Muslim University, Aligarh, India, were included in the study. The clinical bacterial species tested were *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Enterococcus faecalis*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Proteus mirabilis*, *Salmonella typhi*, *Salmonella paratyphi A*, *Salmonella typhimurium*, *Shigella dysenteriae* type 1, *Citrobacter freundii* and *Vibrio cholerae*. Among these clinical strains, those which were highly resistant against β-lactam antibiotics were genetically analyzed for the presence of β-lactamase gene (*bla* gene). The resistant clinical isolates along with their respective *bla* genes included in our study were *E. coli* (*bla* $_{ampC}$), *Klebsiella* species (*bla* $_{CTX-M}$), *Klebsiella* spp. (*bla* $_{SHV}$), and *E. coli* (*bla* $_{TEM}$). 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.

Antimicrobial susceptibility testing

Antibacterial susceptibility tests were performed as per Clinical and Laboratory Standards Institute, formerly National Committee for Clinical Laboratory Standards (2000). Muellar-Hinton agar (M 173; HiMedia, India) was used for determining antibacterial susceptibility testing and for fastidious organisms such as *Streptococci*, 5% sheep blood agar was used. Antimicrobial activity was determined using agar well diffusion method as described by Vanden-Berghe and Vlietinck (1991) and Akinpelu (2001), with some modifications (Shahid et al., 2007). Bacterial suspension containing 10^6 cfu/ml of bacteria was used for inoculating the susceptibility plates. Muellar-Hinton agar plates were lawn cultured with the bacterial suspension with the help of sterile swabs. Wells of 5 mm diameter were made in each plate using a sterile borer. 20 µl of alcoholic plant extract was poured in the wells using a sterile micropipette. 20 µl of 95% ethanol was used as negative control, whereas, antibacterial agent gentamicin (500 µg/20 µl) was used as positive control. The plates were kept upright for 5 to 10 min until the solution diffused into the medium and then they were incubated aerobically at 37°C for 24 h. The zone of inhibition (in mm) was measured and recorded. All the experiments were performed in triplicate.

Determination of minimum inhibitory concentrations (MIC)

MIC was determined by broth micro-dilution method performed according to Clinical and Laboratory Standards Institute (CLSI), formerly NCCLS (2000), with minor modifications (Shahid et al., 2007). Doubling dilutions of the extract was prepared using RPMI-1640 (HiMedia, India) broth supplemented with 0.3 g/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). 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 100 µl of undiluted extracts in the first well, followed by doubling dilutions of extracts from second well onwards. The 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 25×10^3 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 h with their upper surface covered by sterile sealers. The lowest concentration of the extract that did not show any visible growth was considered as MIC of the extract for the tested 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 analyzed statistically by one way analysis of variance (ANOVA) followed by Tukey's multiple analysis test using 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

Antimicrobial activity of alcoholic extracts of parent plant as well as its *in vitro* raised calli against the tested bacterial species is shown in Tables 1 to 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 extracts derived from both parent plant as well as callus gave good antibacterial activity against Gram positive bacteria. Alcoholic fruit extract gave significant ($P<0.05$) antibacterial activity against most of the tested Gram positive bacteria except *S. agalactiae* (Table 1). It showed significant activity against *S. aureus* (P=0.003), *S. epidermidis* (P=0.024), *S. pyogenes* (P=0.012), *E. faecalis* (P=0.038) and *B. subtilis* (P=0.017), with its MIC ranging from 3.05 to 24.0 µg/ml (Figure 1).

Table 1. Antibacterial activity of alcoholic extracts of *Balanites aegyptiaca* and its *in vitro* raised calli against pathogenic Gram-positive bacteria.

Bacteria tested	Zone of inhibition (mm) ± SE			
	Alcoholic fruit extract (2 mg/20 µl)	Alcoholic callus extract (2 mg/20 µl)	Ethanol† (negative control)	Gentamicin (positive control) (500 µg/20 µl)
Staphylococcus aureus	15.33±0.67^{a}	12.67±0.33^{b}	7.33±0.33^{d}	11.67±0.33^{c}
Staphylococcus epidermidis	13.00±0.58^{c}	12.33±0.33^{c}	8.33±0.33^{b}	11.33±0.33^{d}
Streptococcus pyogenes	14.67±0.33^{b}	0.00±0.00^{c}	8.67±0.33^{a}	13.00±0.58^{a}
Streptococcus agalactiae	0.00±0.00^{e}	0.00±0.00^{c}	7.67±0.33^{c}	10.67±0.33^{e}
Enterococcus faecalis	12.33±0.33^{d}	0.00±0.00^{c}	7.33±0.33^{d}	9.67±0.33^{f}
Bacillus subtilis	15.33±0.67^{a}	12.67±0.33^{b}	8.33±0.33^{b}	12.33±0.33^{b}
S. aureus ATCC 25923	14.67±0.33^{b}	13.00±0.58^{a}	8.67±0.33^{a}	13.00±0.58^{a}

† = 20 µl of 95% ethanol was poured in well as negative control. Figure within parentheses denotes the concentration used in the test. 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 significant. The mean represented by same letter is not significantly different within the column.

Table 2. Antibacterial activity of alcoholic extracts of *Balanites aegyptiaca* and its *in vitro* raised calli against pathogenic Gram-negative bacteria.

Bacteria tested	Zone of inhibition (mm) ± SE			
	Alcoholic fruit extract (2 mg/20 µl)	Alcoholic callus extract (2 mg/20 µl)	Ethanol† (negative control)	Gentamicin (positive control) (500 µg/20 µl)
Escherichia coli	12.33±0.33^{cd}	11.67±0.33^{cd}	7.67±0.33^{c}	11.33±0.33^{c}
Klebsiella pneumoniae	11.67±0.33^{d}	11.33±0.33^{d}	7.33±0.33^{d}	10.67±0.33^{d}
Proteus vulgaris	11.33±0.33^{e}	0.00±0.00^{e}	8.33±0.33^{b}	10.67±0.33^{d}
Proteus mirabilis	0.00±0.00^{f}	0.00±0.00^{e}	7.67±0.33^{c}	11.33±0.33^{c}
Pseudomonas aeruginosa	11.33±0.33^{e}	0.00±0.00^{e}	7.33±0.33^{d}	10.67±0.33^{d}
Salmonella typhi	13.00±0.58^{bc}	0.00±0.00^{e}	7.67±0.33^{c}	10.33±0.33^{e}
Salmonella paratyphi A	15.33±0.67^{a}	13.00±0.58^{b}	8.67±0.33^{a}	12.33±0.33^{b}
Salmonella typhimurium	11.00±0.58^{e}	0.00±0.00^{e}	7.33±0.33^{d}	10.67±0.33^{d}
Shigella dysenteriae type 1	0.00±0.00^{f}	0.00±0.00^{e}	8.33±0.33^{b}	9.67±0.33^{f}
Vibrio cholerae	11.33±0.33^{e}	0.00±0.00^{e}	7.67±0.33^{c}	10.33±0.33^{e}
E. coli ATCC 25922	13.67±0.33^{b}	13.33±0.33^{a}	8.67±0.33^{a}	12.67±0.33^{a}
P. aeruginosa ATCC 27853	12.67±0.33^{c}	12.33±0.33^{c}	8.33±0.33^{b}	11.33±0.33^{c}

† = 20 µl of 95% ethanol was poured in well as negative control. Figure within parentheses denotes the concentration used in the test. 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 significant. The mean represented by same letter is not significantly different within the column.

Amongst the tested Gram negative bacteria, alcoholic fruit extract showed significant (P<0.05) activity against most of the tested species with best activity against *S. typhi, S. paratyphi* A and *E. coli* (Table 2). It showed significant activity against *E. coli* (P=0.015), *K. pneumoniae* (P=0.012), *P. vulgaris* (P=0.044), *P. aeruginosa* (P=0.012), *S. typhi* (P=0.010), *S. paratyphi* A (P=0.003), *S. typhimurium* (P=0.044) and *V. cholerae* (P=0.038). MIC of the alcoholic fruit extract against Gram negative bacteria ranged from 1.53 to 49.0 µg/ml (Figure 2).

It is interesting to note that these bacteria which are found to be susceptible to the extracts of *B. aegyptiaca* are important human pathogens responsible for wound infection, sore throat, enteric fever, urinary tract infection and diarrhea.

Various studies have been under taken previously by different researchers to analyze the antimicrobial potential of *B. aegyptiaca* (Doughari et al., 2007; Maregesi et al., 2008; Parekh and Chanda, 2008). They showed significant antibacterial activity of this plant against Gram positive bacteria like *S. aureus*, *S. epidermidis* and *B. cereus*, and Gram negative bacteria like *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *S. typhi* and *S. typhimurium*, which supports our present research findings. Since we tested an exhaustive range of both Gram positive and Gram negative bacteria as compared to previously done studies, we were able to detect a wide spectrum of

Table 3. Antibacterial activity of alcoholic extracts of *Balanites aegyptiaca* and its *in vitro* raised calli against genetically characterized resistant isolates harbouring *bla* genes.

Bacteria tested*	Zone of inhibition (mm) ± SE			
	Alcoholic fruit extract (2 mg/20 µl)	Alcoholic callus extract (2 mg/20 µl)	Ethanol† (negative control)	Gentamicin (positive control) (500 µg/20 µl)
Escherichia coli (*bla* $_{ampC}$)	12.33±0.33^{b}	11.33±0.33^{b}	0.00±0.00^{a}	10.67±0.33^{b}
Klebsiella spp. (*bla* $_{CTX-M}$)	12.67±0.33^{a}	12.33±0.33^{a}	0.00±0.00^{a}	11.33±0.33^{a}
Klebsiella spp. (*bla* $_{SHV}$)	0.00±0.00^{d}	0.00±0.00^{c}	0.00±0.00^{a}	10.33±0.33^{c}
Escherichia coli (*bla* $_{TEM}$)	11.67±0.33^{c}	0.00±0.00^{c}	0.00±0.00^{a}	9.33±0.33^{d}

*Figure within the parenthesis denotes the *bla* genes harboured by the respective clinical strains of gram-negative bacteria. † = 20 µl of 95% ethanol was poured in well as negative control. Figure within parentheses denotes the concentration used in the test. 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 significant. The mean represented by same letter is not significantly different within the column.

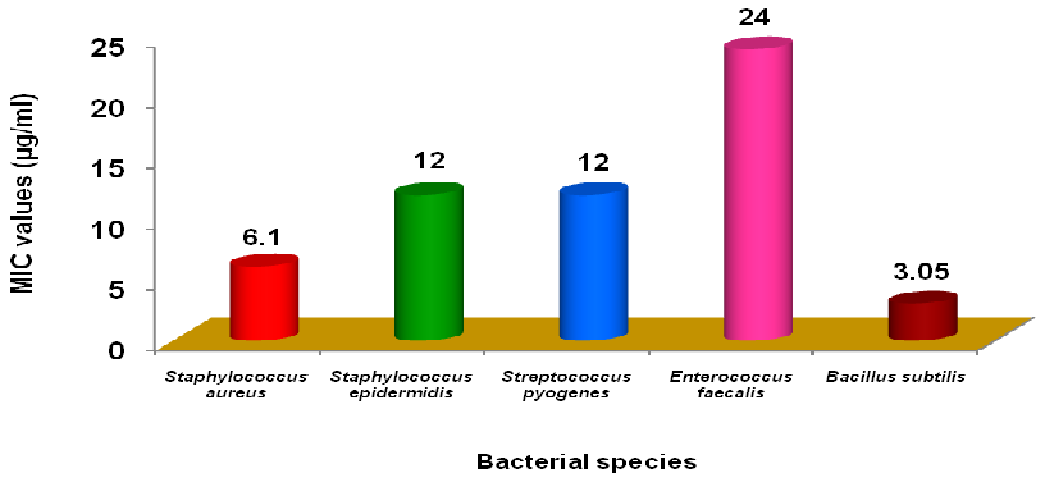

Figure 1. MIC determination of alcoholic fruit extract of *Balanites aegyptiaca* against tested Gram positive bacteria.

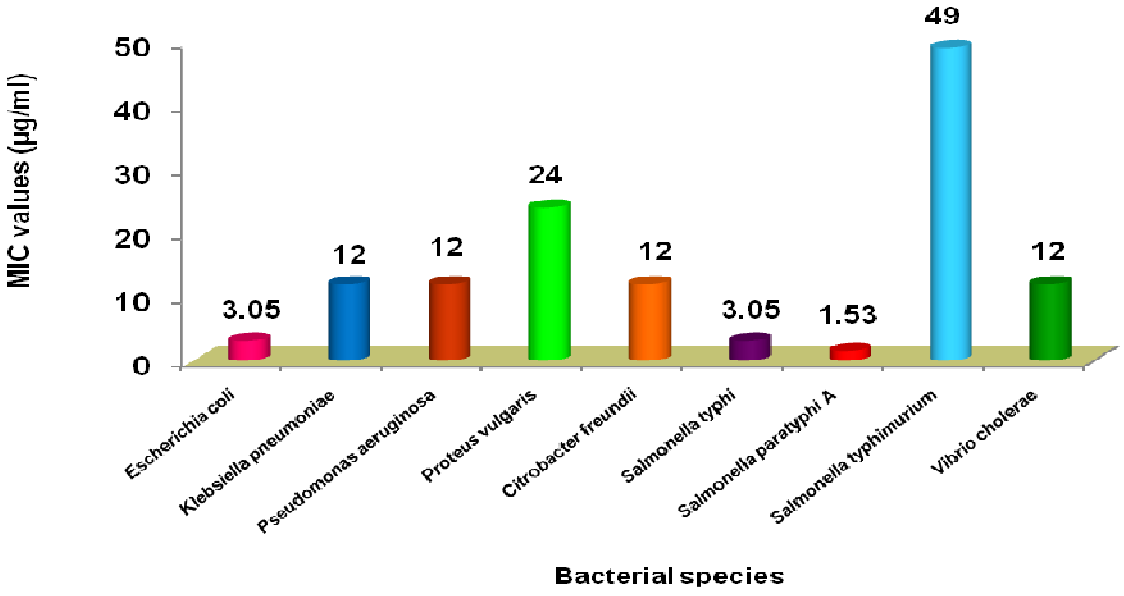

Figure 2. MIC determination of alcoholic fruit extract of *Balanites aegyptiaca* against tested Gram negative bacteria.

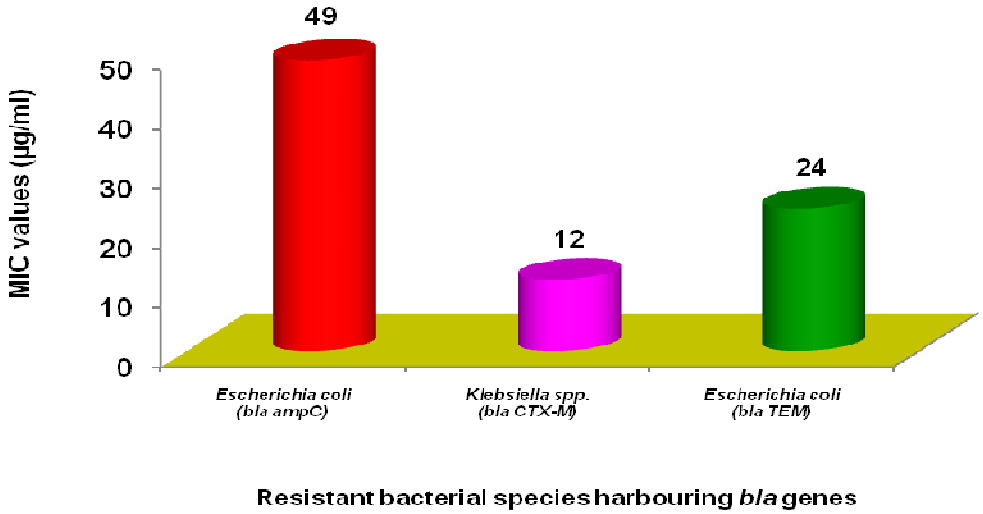

Figure 3. MIC determination of alcoholic fruit extract of *Balanites aegyptiaca* against resistant organisms harbouring *bla* genes.

antibacterial activity of this plant extract. In the present study, it was also found that *Proteus mirabilis* and *S. dysenteriae* type 1, which were not tested in earlier studies, were totally resistant to the alcoholic extracts of *B. aegyptiaca*.

It was also seen in our study that the alcoholic fruit extract had significant antibacterial activity ($P<0.05$) against wide range of tested resistant bacteria harbouring *bla* genes responsible for β-lactamase activity (Table 3). This result shows the future prospect of the extracts of this plant which can be used as antibacterial agents in infections caused by these resistant organisms, which otherwise pose problems in treatment by the currently used antimicrobial drugs. MIC against bacteria harbouring *bla* genes ranged from 12.0 to 49.0 µg/ml (Figure 3).

As seen in Tables 1, 2 and 3, the *in vitro* raised calli of this plant also gave good results. The alcoholic extract of *in vitro* cultivated calli showed significant and comparable activity to parent plant against Gram positive bacteria, significant activity against few Gram negative bacteria and slightly less but significant activity against few resistant bacteria harbouring *bla* genes. To the best of our knowledge this is the first study analyzing the antimicrobial potential of *in vitro* raised calli of this plant and also this is the first study analyzing the antibacterial potential of this plant extracts against organisms harbouring *bla* genes. Therefore, our findings could not be compared.

Conclusion

In nutshell, extracts of *B. aegyptiaca* have remarkable antibacterial potential and thus can be used to derive antimicrobial agents especially against *S. aureus*, *E. coli*, *S. typhi* and *S. paratyphi A*. Hence, it can be used in the treatment of enteric fever, diarrhea, wound infections and urinary tract infections. Nowadays, *S. typhi* is showing resistance against the commonly used drugs for the treatment of enteric fever, drugs obtained from extracts of this plant may be tried as an alternative medicine. As the extracts of this plant showed significant activity against resistant organisms harbouring *bla* genes, it may prove as a novel antimicrobial agent in the treatment of such resistant organisms. Also, the extracts from *in vitro* raised calli showed significant antibacterial potential, hence, *in vitro* cultivation of the plant may be used to obtain phytotherapeutic compounds, especially at places where it does not grow naturally, because of adverse atmospheric conditions. In future, bioactive compounds responsible for antimicrobial activity could further be enhanced by nutritional and hormonal manipulations of the cultivation medium.

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