

Antibacterial Activity of *Rhazya stricta* Non-alkaloid Extract against Methicillin-Resistant *Staphylococcus aureus*

Raziuddin Khan^{1*}, Mohammed N Baeshen^{2,3}, Kulvinder S Saini^{1,4}, Roop S Bora^{1,4}, Ahmed M Al-Hejin¹, Salim M. El-Hamidy¹ and Nabih A Baeshen¹

¹Department of Biological Sciences, Faculty of Sciences, King Abdulaziz University, Jeddah, Saudi Arabia

²Department of Biological Sciences, Faculty of Sciences, University of Jeddah, Saudi Arabia

³Center of Nanotechnology, King Abdulaziz University, Jeddah, Saudi Arabia

⁴School of Biotechnology, Eternal University, Baru Sahib, 173101, Himachal Pradesh, India

Abstract

The effect of a crude, organic, non-alkaloid extract derived from *R. stricta* leaves were evaluated against methicillin-resistant *Staphylococcus aureus* (MRSA) pathogens. Antibacterial activities were determined by zones of inhibition using a new 1% agarose well-diffusion method. Structural and morphological alterations of MRSA cells were investigated using transmission electron microscopy. The extract showed antibacterial activities against MRSA pathogens. The extract inhibited growth, with zones of inhibition ranging between 6 and 19 mm. Transmission electron microscopy revealed that the extract distorts the cellular architecture of MRSA pathogens. Crude organic non-alkaloid extract derived from *R. stricta* leaves might serve as a novel antibiotic agent to prevent or treat life-threatening MRSA infections.

Keywords: *Rhazya stricta*; Non-alkaloids; MRSA; TEM

Introduction

One of the most problematic and economically relevant nosocomial pathogens in the world is Methicillin-resistant *Staphylococcus aureus* (MRSA) [1]. The problem has been intensified in last two decades as multidrug-resistant MRSA has emerged with resistance to a wide range of antibiotics and antimicrobial agents [2]. Several classes of drug-resistant *S. aureus* have been identified. Penicillin G-resistant *S. aureus*, which produces penicillinase, was identified in the mid-1940s, and multidrug-resistant (MDR) *S. aureus*, which is resistant to penicillin G, chloramphenicol, tetracycline, and erythromycin, was identified in the late 1950s. Since 1961, MRSA continues to be a life-threatening MDR bacterium, changing its resistance patterns by acquiring resistance to each new antibiotic used against it. Generally, MRSA is a causative agent of many infectious diseases of the skin, soft tissue, respiratory tract, bones and joints, surgical wounds, urinary tract, and bloodstream. Such infections are challenging to treat because they are resistant to several commonly used antibiotics [3].

According to the World Health Organization, medicinal herbs could be important resources for obtaining new antimicrobial drugs [4]. Medicinal plants are certainly better equipped at metabolic bioengineering of its specific active pharmaceutical ingredients than traditional laboratory approaches. Several studies have shown that herbal extracts commonly used in Folkloric Medicine have high potential to be developed as bactericidal or bacteriostatic agents. Medicinal plant extracts have been used as natural therapeutic alternatives to antibiotics in order to reduce or overcome the continuous rise of antibiotic resistance.

The genus *Rhazya* belongs to the Apocynaceae family [5] and comprises two species: *R. stricta* Decne (Decaisne) and *R. orientalis* (Harmal in Arabic). It is a medicinal plant commonly used to cure various ailments in humans and animals [6,7] in many Asian countries, including Saudi Arabia. *R. stricta* and its metabolites are used to treat an array of diseases including cancer, skin diseases, hypertension, rheumatism, sore throat, syphilis, helminthiasis, inflammatory conditions, and fever [8,9]. Several studies have shown that different parts of *R. stricta* contain plentiful phytochemical constituents like alkaloids, flavonoids, triterpenes, and volatile bases [9], which have curative properties toward a wide range of ailments [10]. Studies from

our lab have shown that the aqueous extract of *R. stricta* has potential antimicrobial activities against *Neisseria meningitidis* [11]. Recently, Baeshen et al., [10] reviewed several studies on therapeutic potential of *R. stricta*'s compounds including non-alkaloids such as oleanolic acid. Oleanolic acid is a pentacyclic triterpenoid phyto-compound which showed potential antimicrobial activity against several Gram-positive pathogens [12] predominantly, methicillin-resistant *Staphylococcus aureus* (MRSA) [13]. Oleanolic acids had been shown to affect peptidoglycan metabolism and prevent peptidoglycan cross-linkage and thus altered bacterial cell morphology, which led to increased autolysis of pathogenic cells [12].

In earlier studies from our laboratory, a significant amount of work has been done on examining the alkaloid extracts of *R. stricta*, therefore, the aim of this study was to explore the antibacterial activities of organic, non-alkaloid extract derived from *R. stricta* leaves against MRSA using a 1% agarose well-diffusion method as well as transmission electron microscopy (TEM).

Material and Methods

Chemicals for antimicrobial assays

Chloroform (Sigma-Aldrich,USA), Ethanol(Sigma-Aldrich,USA), Glacial acetic acid(Sigma-Aldrich,USA), Dimethyl sulphoxide (Sigma-Aldrich,USA), Phosphotungstic acid (PTA; Sigma-Aldrich,USA). Bacterial culture media were purchased from Himedia (India).

Bacterial strains

All *S. aureus* strains used in this study were obtained from the

*Corresponding author: Raziuddin Khan, Department of Biological Sciences, Faculty of Sciences, King Abdulaziz University, Jeddah, Saudi Arabia, Tel: +966547848091; E-mail: razisiraj@gmail.com

Received March 08, 2016; Accepted April 02, 2016; Published April 10, 2016

Citation: Khan R, Baeshen MN, Saini KS, Bora RS, Al-Hejin AM, et al. (2016) Antibacterial Activity of *Rhazya stricta* Non-alkaloid Extract against Methicillin-Resistant *Staphylococcus aureus*. Biol Syst Open Access 5: 157. doi:10.4172/2329-6577.1000157

Copyright: © 2016 Khan R, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Strains S.No.	Pathogens	Barcode	Source	Gram reaction	Meropenem	Pipracillin / Tazobactam	Amikacin	Ciprofloxacin	Colistin	Cefepime	Gentamicin	Trimethoprim/sulfa	Ceftazidime	Tigecline	Clindamycin	Erythromycin	Oxacillin	Vancomycin	Ampicillin	Cefuroxime	Imipenem	Amoxicillin/Clavulanic Acid	Nitrofurantoin	Cefazolin	Ilevofloxacin	Teicoplanin
6	<i>S.aureus</i>	-	ATCC #43300	GPC	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
27	<i>S. aureus</i> (MRSA)	600018	Superf icial wound	GPC	X	X	X	X	X	X	X	S	X	X	S	S	R	R	X	X	X	X	X	X	X	X
31	<i>S. aureus</i>	877736	Superf icial wound	GPC	X	X	X	X	X	X	X	S	X	X	R	R	S	X	X	X	X	X	X	S	X	X
39	<i>S. aureus</i> (MRSA)	452652	Superf icial wound	GPC	X	X	X	X	X	X	X	S	X	X	R	R	R	S	X	X	X	X	X	R	X	X

ATCC: American Type Culture Collection; R: resistant; S: susceptible; I: intermediate

Table1: Antibiogram of clinical *Staphylococcus aureus*.

clinical microbiology laboratory of King Abdulaziz University Hospital, Jeddah, KSA. The *S. aureus* isolates were obtained from 3 different patients and one reference strain (ATCC #43300). Strains 27, 31, and 39 were obtained from superficial wound samples of different patients, and strain 03 was obtained from an ATCC source. Strains 27 and 39 were found to be MRSA while strain 31 belongs to non-MRSA (Table 1).

Plant material

Leaves of *R. stricta* were collected from an area near Bahara village along the Jeddah-Makkah road, KSA. The species was identified and authenticated to the genus and species level by Professor Nabih A. Baeshen (Department Biology, Faculty of Science, King Abdulaziz University, Jeddah, K.S.A.). *R. stricta* is also grown in the green house of Department of Biological Sciences, University of Texas at Austin, TX, USA in the laboratory of Prof. Robert Jansen, one of our collaborator.

Preparation of organic, non-alkaloid extract from *R. stricta* leaves

The leaves were washed and dried under shade and ground to a fine powder. Five hundred grams of powder was measured and kept in 800 ml absolute ethanol (95%) for 2 days. The mixture was then filtered through Whatmann filter paper and evaporated by a rotary evaporator. Twenty-five grams of solid extract was then taken and added to 200 ml 1M HCl and an equal volume of chloroform. The chloroform layer containing non-alkaloids were separated and stored at 4°C.

Bacterial culture and Agarose well diffusion method

Two to three isolated colonies of each *S. aureus* strain were taken from chocolate agar, and culture suspensions were prepared in Mueller Hinton Broth. The 0.5 McFarland inoculum (1.5×10^8 CFU/ml) was adjusted between 0.08 and 0.13 using a spectrophotometer at 625 nm optical density [14]. The inoculum was spread on Mueller Hinton agar plates using sterile cotton swabs. Wells were made on the agar plates using 10 mm diameter sterile tips. The base of each well was sealed with molten agarose to prevent the loss of extract due to leakage. Each well was then filled with 50 µl extract. Extract concentrations of 4.9, 6.1, 7.3, and 8.5 mg/ml were prepared with 0.04% methanol and tested for antibacterial activity against the *S. aureus* strains with 0.04% methanol used as a control. All plates were kept at room temperature for 2 h after inoculation with the bacterial strains and the extract. After that, the plates were incubated at 37°C overnight. Zones of inhibition were then measured in mm by a ruler.

Transmission electron microscopy

All mentioned *Staphylococcus aureus* strains were investigated by TEM using a negative-staining procedure with PTA [15]. Briefly, one

drop of MRSA were applied to carbon-coated, 300-mesh copper grids (Electron Microscopy Sciences, Hatfield, UK), incubated for 2 min, and fixed with a 2% PTA for 2 min. The specimens were examined by TEM using a JEM-1011 microscope (JEOL, Tokyo, Japan) at 80 KV accelerated voltage. All the data were presented as the mean ± standard deviation (mean ± SD) where appropriate.

Results

Determination of antibacterial activity using Agarose well diffusion method

The antibacterial activity of the extract depended on the concentration of the test material as well as on the quality of the solid base media. In this study, we used agarose instead of agar. As the *Rhazya* extract concentration increased, there was an increase in bactericidal activity of the extract, resulting in the enlarged diameter of the inhibition zone (Table 2; Figures 1A-D).

Evaluation of bacterial cellular alterations by TEM

The morphological alterations in the bacteria after exposure to the extract were analyzed by TEM. The MRSA cells exhibited changes compared with the untreated cells (Figures 2A-C). The coccoid shape of the cells was not maintained, and deformations and indentations

Strain	Concentration (mg/ml)	Zone of Inhibition (Diameter; mm) mean ± standard deviation
<i>S. aureus</i> ATCC #43300	4.9	12.50 ± 2.12
	6.1	13.00 ± 1.41
	7.3	14.50 ± 2.12
	8.5	15.50 ± 3.54
MRSA (27)	4.9	12.00 ± 0.00
	6.1	15.00 ± 0.00
	7.3	14.50 ± 0.71
	8.5	15.50 ± 0.71
<i>S. aureus</i> (31)	4.9	12.00 ± 1.41
	6.1	13.50 ± 0.71
	7.3	14.00 ± 1.41
	8.5	15.00 ± 0.00
MRSA (39)	4.9	12.00 ± 1.41
	6.1	13.00 ± 0.00
	7.3	15.50 ± 3.54
	8.5	18.00 ± 0.00

Table 2: Antibacterial activity of non-alkaloid extract from *Rhazya stricta* leaves.

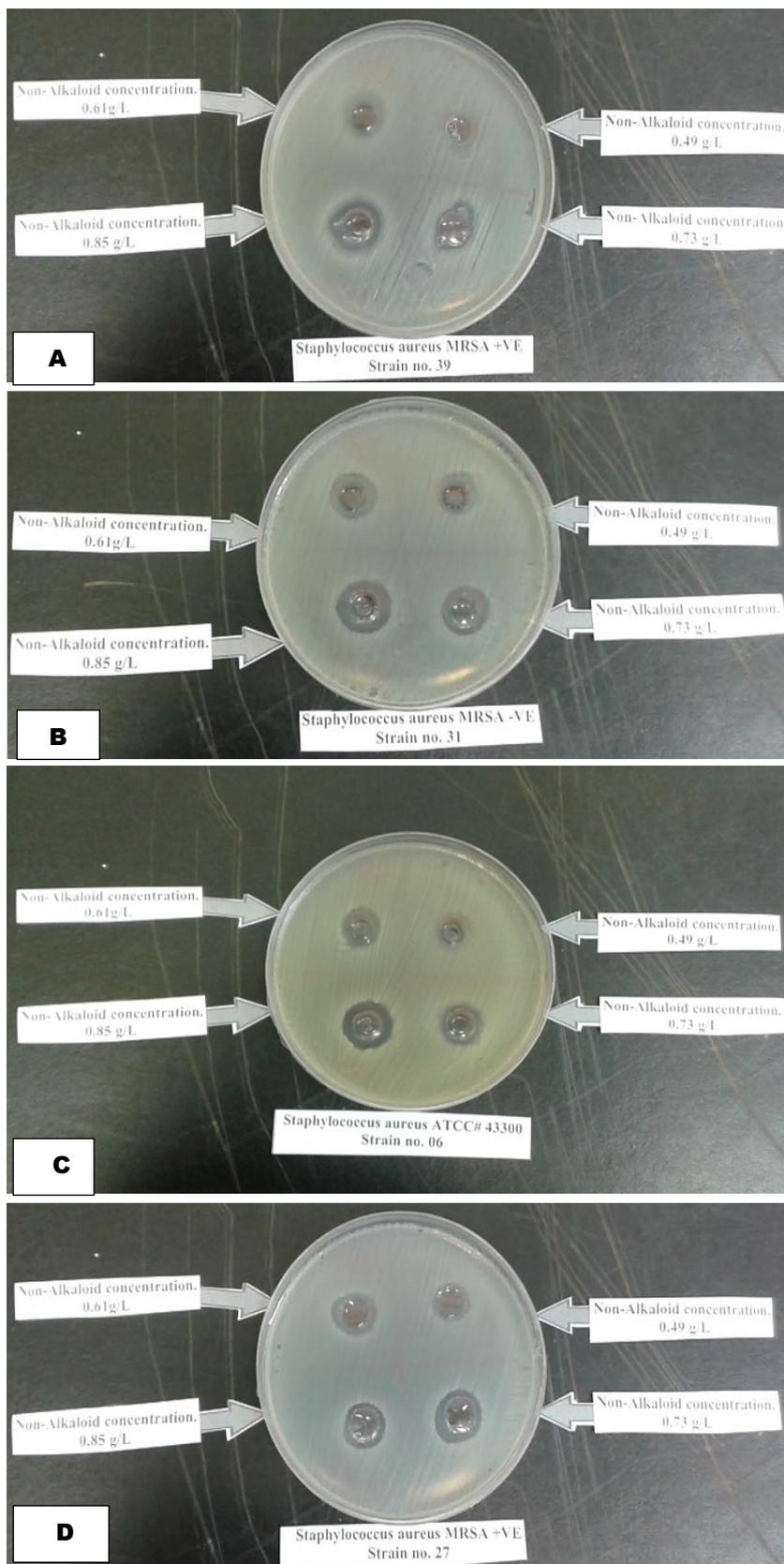


Figure 1: Inhibition of bacterial growth using a non-alkaloid extract derived from *R. stricta* leaves. The non-alkaloid extract derived from *R. stricta* leaves was a potent inhibitor of different isolates of MRSA obtained from a wound sample (A, B, C) and a standard ATCC # 43300 strain (D) at concentrations of 4.9, 6.1, 7.3, and 8.5 mg/ml.

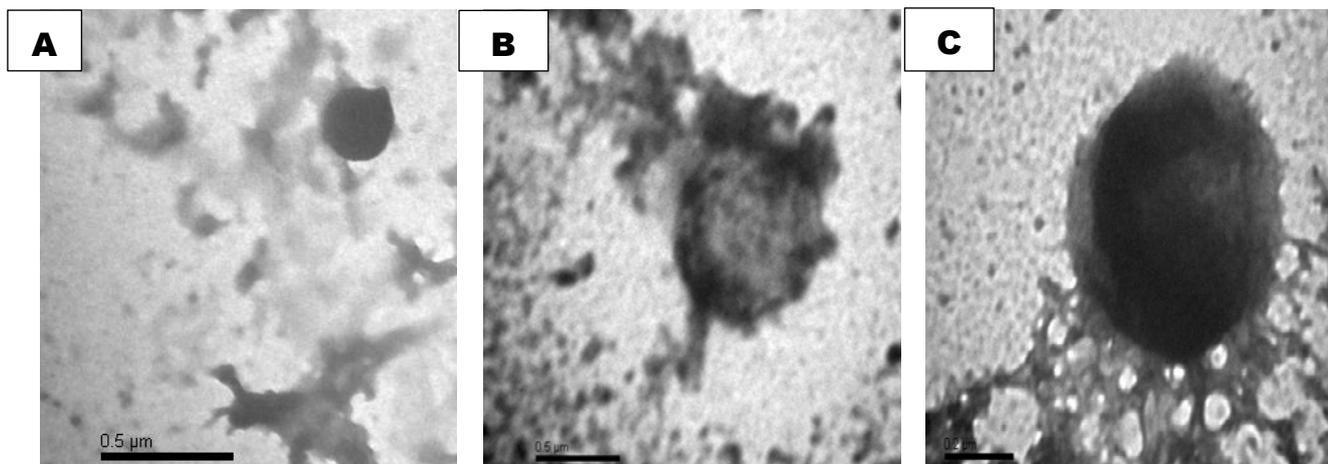


Figure 2: The non-alkaloid extract caused changes in MRSA cells observed using electron microscopy. The morphology of treated MRSA cells was altered (B, Bar = 0.5 μm ; C, Bar = 0.2 μm) compared with that of control cells (A, Bar = 0.5 μm). The coccoid shape of the cells was not maintained, and cells with a rough surface (B) and shrunken cytoplasm (C) were clearly visible.

on the cell surface were clearly observed. Cells with a rough surface were clearly visible (Figure 2B). Shrinkage of the cytoplasm was also observed (Figure 2C).

Discussion

In the past century or so, natural products have proven to be an important source of new chemical entities, which are now driving pharmaceutical inventions. Plant extracts have been the main source of crude therapeutic formulations for pharmaceutical companies. Several studies have established the antimicrobial properties of plant products [2,4,15-18]. Our study revealed the activity of non-alkaloid extract of *R. stricta* against MRSA using growth-inhibition assays and morphological assessments. Microbiological examinations revealed zones of inhibition with diameters depending on the extract concentration. Electron microscopy revealed morphological as well as intracellular alterations after cells were treated with the extract. Our results corroborate previous work done by us and other laboratories [4,16,19] and are in general agreement with the current knowledge on the molecular basis of antimicrobial activity. It is possible, however, non-alkaloid extract contained different compounds and different proportions of compounds. To exploit the full therapeutic potential of *R. stricta* non-alkaloid extracts and the purified compounds that could be derived from *R. stricta* extracts, additional *in vitro* and *in vivo* pharmacological and toxicological studies are required.

The mechanism of the antibacterial activity of the non-alkaloid extract from *R. stricta* remains largely unknown, but it is likely to be related to the various phytochemicals, including flavonoids, terpenoids, and tannins, that were reported previously by several researchers [20]. Additionally it has been reported that non-alkaloids from other plants also show antimicrobial activities [13,21]. It is possible that nonalkaloid extracts might contain different chemical compounds which provide stable antimicrobial activities as seen here against MRSA. An earlier study by Horiuchi et al., [13], revealed that the oleanolic acid, a pentacyclic triterpenoid isolated from the leaves of *Salvia officinalis* showed potential antimicrobial activity against vancomycin-resistant enterococci (VRE) [13]. Oleanolic acids had been shown to affect peptidoglycan metabolism and prevent peptidoglycan cross-linkage and thus altered bacterial cell morphology, which led to increased autolysis of pathogenic cells [12]. Oleanolic acid is also known

to be present in the leaves of *R. stricta* which might be responsible for the antimicrobial activity of non-alkaloid fraction.

Although the crude extract showed dose-dependent antibacterial activity, the use of crude extracts from *R. stricta* to control MRSA pathogens requires further research for economic and therapeutic development. Our findings support the overall value of potential medicinal extracts from *R. stricta* leaves. Further pharmacological and toxicological studies should be performed to determine the purified fractions and bioactive compounds responsible for the antibacterial activities, which could serve as a useful resource for new antibiotics.

Conclusion

The non-alkaloid extract from the leaves of the medicinal plant *R. stricta* proved to have antimicrobial activities against MRSA clinical isolates based on TEM and 1% agarose well-diffusion method. Those findings indicate that *R. stricta* leaves might be a source of new antibacterial compounds for the treatment of MRSA infections.

References

1. Oliveira DC, De Lencastre H (2011) Methicillin-resistance in *Staphylococcus aureus* is not affected by the overexpression in trans of the *mecA* gene repressor: a surprising observation. *PLoS One* 6: e23287.
2. Mohtar M, Johari SA, Li AR, Isa MM, Mustafa S, et al. (2009) Inhibitory and resistance-modifying potential of plant-based alkaloids against methicillin-resistant *Staphylococcus aureus* (MRSA). *Curr Microbiol* 59: 181-186.
3. Gould IM (2006) Costs of hospital-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) and its control. *Int J Antimicrob Agents* 28: 379-384.
4. Li N, Tan SN, Cui J, Guo N, Wang W, et al. (2014) PA-1, a novel synthesized pyrrolizidine alkaloid, inhibits the growth of *Escherichia coli* and *Staphylococcus aureus* by damaging the cell membrane. *J Antibiot (Tokyo)* 67: 689-696.
5. Yates SA, Chernukhin I, Alvarez-Fernandez R, Bechtold U, Baeshen M, et al. (2014) The temporal foliar transcriptome of the perennial C3 desert plant *Rhazya stricta* in its natural environment. *BMC Plant Biol* 14: 2.
6. Baeshen NA, Lari SA, Al Doghathier H, Ramadan HA (2010) Effect of *Rhazya stricta* extract on rat adiponectin gene and insulin resistance. *J Am Sci* 6: 1237-45.
7. Baeshen N, Lari S, Aldoghathier H, Elkady A (2010) Biochemical evaluation of the effect of *Rhazya stricta* aqueous leaves extract in liver and kidney functions in Rats. *Nat Sci* 8: 136-42.
8. Baeshen N, Sabir J, Qari S (2009) Cytogenetic and molecular evaluations of

- genetic effects of leaf extract of *Rhazya stricta* (Decne) on *Allium cepa* root tip meristems. Egypt J Genet Cytol 38:73-83.
9. Baeshin NA, El-Twaty NH, Al-Hebshi AM (2005) Evaluating the Genotoxicity of *Rhazya stricta* leaves extract by the *Saccharomyces cerevisiae* auxotrophic mutants test. Egypt J Nat Toxi 2: 87-100.
 10. Baeshen M, Khan R, Bora R, Baeshen N (2015) Therapeutic Potential of the Folkloric Medicinal Plant *Rhazya stricta*. Biol Syst Open Access 5: 2.
 11. Abadi F, Abdulaziz A, Hadhoud A, Baeshin N, Qari S, Alhejin A (2011) An epidemiological Survey and Evaluation of the Antimicrobial Growth Effect of *Rhazya stricta* (Decne) Leaves Extract on Different Genotypes of *Neisseria Meningitidis*. Egypt J Med Microbiol.
 12. Wolska KI, Grudniak AM, Fiecek B, Kraczkiewicz-Dowjat A, Kurek A (2010) Antibacterial activity of oleanolic and ursolic acids and their derivatives. Cent Eur J Biol 5: 543-553
 13. Horiuchi K, Shiota S, Hatano T, Yoshida T, Kuroda T, et al. (2007) Antimicrobial activity of oleanolic acid from *Salvia officinalis* and related compounds on vancomycin-resistant enterococci (VRE). Biol Pharm Bull 30: 1147-1149.
 14. Wiegand I, Hilpert K, Hancock RE (2008) Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protocol 3: 163-75.
 15. Charyulu EM, Sekaran G, Rajakumar GS, Gnanamani A (2009) Antimicrobial activity of secondary metabolite from marine isolate, *Pseudomonas* sp. against Gram positive and negative bacteria including MRSA. Indian J Exp Biol 47: 964.
 16. Zuo GY, Meng FY, Han J, Hao XY, Wang GC, et al. (2011) In Vitro Activity of Plant Extracts and Alkaloids against Clinical Isolates of Extended-Spectrum b-Lactamase (ESBL)-Producing Strains. Molecules 16: 5453-9.
 17. Prabuseenivasan S, Jayakumar M, Ignacimuthu S (2006) In vitro antibacterial activity of some plant essential oils. BMC Complement Altern Med 6: 39.
 18. Karaman I, Sahin F, Güllüce M, Ogütçü H, Sengül M, et al. (2003) Antimicrobial activity of aqueous and methanol extracts of *Juniperus oxycedrus* L. J Ethnopharmacol 85: 231-235.
 19. Rasooli I, Rezaei MB, Allameh A (2006) Ultrastructural studies on antimicrobial efficacy of thyme essential oils on *Listeria monocytogenes*. Int J Infect Dis 10: 236-241.
 20. Ullah I (2012) A review of phytochemistry, bioactivities and ethno medicinal uses of *Rhazya stricta* Decsne (Apocynaceae). Af J Microbiol Rese 6: 1629-41.
 21. Cowan MM (1999) Plant products as antimicrobial agents. Clin Microbiol Rev 12: 564-582.