Antibacterial Activity and Mechanism of Action of an Ayurvedic formulation Khadirarishta

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Research paper

Antibacterial activity and mechanism of action of an ayurvedic formulation Khadirarishta

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ABSTRACT

Aim: To evaluate the antibacterial effect of Khadirarishta (An Ayurvedic traditional formulation) against some of the bacterial strains as well as to investigate the probable mechanism of action against sensitive bacterial strains by using standard procedures.

Methods: The antibacterial activity of Khadirarishta against some of the bacterial strains was evaluated by using bacterial susceptibility assay and the broth Microdilution method by using microplate reader and p-iodonitrotetrazolium chloride (INT) reagent assay. The mechanism of action of Khadirarishta was observed by its effects on the bacterial membrane followed by quantification of effluxes components. Resistance analysis and Combination effect of Khadirarishta was also performed to explore its antibacterial dimensions. HPLC analysis of Khadirarishta was carried out for identifying the different marker compounds.

Results: Khadirarishta revealed a significant ($p < 0.05$) antibacterial spectrum against *E. coli*, *S. aureus*, and *S. enterica* bacterial strains. The mechanism of action of Khadirarishta seems to be linked with the disruption of bacterial membrane permeability which leads to the smooth entry of active compounds, efflux of nucleotide material and K^+ ions. The four major marker compounds *viz.*, gallic acid, catechin, ellagic acid, and eugenol were identified by HPLC analysis.

Conclusion: This study shows that Khadirarishta has antibacterial potency and has a vigorous effect on the bacterial cell wall and membrane permeability. This could be due to the presence of identified marker compounds in this herb-decoction based Ayurvedic traditional formulation.

1. Introduction

Since olden times, Ayurveda has recommended a number of herbs and natural products which have become an important resource to maintain a healthy life. Natural products are today as important as raw materials in alternative medicine, where chemical structures have been isolated with proven bioactivity. Ayurveda comprises of diverse types of medicines from natural sources, among them the fermented forms namely *arishtas* (fermented decoctions) has a great pharmacological importance. The medicinal utility of these fermented forms has been greatly mentioned in detail in Ayurveda. Fermentation in Ayurveda is set as a method for drug preparation (Shrivastava, 1998; Sabu and Haridas, 2015). The poly-herbal materials used in the preparation of these drugs by fermentation may increase the desired properties by biotransformation with specific activity and mimic the processes of 'digestive and metabolic transformation' (Chandra et al., 2015). Fermentation not only splits the plant cells to release the microbial enzymes, but also produces an active transportation network with dissolved components from the herbal material (Mishra et al., 2010).

Arishta is a poly-herbal Ayurvedic liquid medicine. It is prepared by a natural fermentation process using herbal decoction and contains about 5–10 % of self generated alcohol, which is often referred to as medicated wine. Arishthas are widely used in the treatment of several disorders. Khadirarishta is one such Arishta, made from the decoctions

Abbreviations: INT, p-iodonitrotetrazolium chloride; HPLC, High Performance Liquid Chromatography; MCC, Microbial Culture Collection; MTCC, Microbial Type Culture Collection and Gene bank; NCL, National Chemical Laboratory; CFU, Colony Forming Units; PBS, Phosphate Buffer Saline; MHA, Mueller Hinton Agar; ZOI, Zone of Inhibition; MIC, Minimum Inhibitory Concentration; CLSI, Clinical and Laboratory Standards Institute; MHB, Mueller Hinton Broth; rpm, revolutions per minute

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of various herbs. The gum and bark of *Acasia catechu* are the main contributory sources of Khadirarishta (Table 1). The alcohol and the water present in the product acts as a medium to deliver soluble active herbal components in to the body. Ancient Ayurvedic literature "Sharangdhara Samhita" has described in detail the Khadirarishta composition, preparation method, therapeutic application and mode of action. Khadirarishta is beneficial in various skin problems such as psoriasis, vitiligo and eczema. Besides, it is effective in gastric disorders and acts as an anthelmintic agent (Shinde and Bhangale, 2017; Parulkar, 2016).

A previous study on the therapeutic application of Khadirarishta has also mentioned its traditional use for heart disorders, anaemia, abdominal pain, jaundice and leprosy (Sajjad et al., 2015). In addition, more medicinal uses and applications of Khadirarishta in different disease were mentioned in ancient Ayurvedic literatures as summarized by (Shinde and Bhangale (Shinde and Bhangale, 2017). Along with its therapeutic applications, some of the quality control parameters of Khadirarishta have also been previously studied (Nahar et al., 2015). However, based on the authors knowledge and literature mining, it was found that the antibacterial property of Khadirarishta has not evaluated, despite its huge medicinal importance. The aim of the current study was to evaluate the *in vitro* antibacterial activity and to investigate the antibacterial mechanism of action of Khadirarishta.

2. Material and methods

2.1. Collection of Khadirarishta

Ayurvedic medicine Khadirarishta (Batch no. KDR033, Divya Pharmacy, Haridwar) was used in this study since it is used as an Indian folk medicine which contains compounds with antimicrobial properties. Khadirarishta was purchased from Patanjali Mega Store, PY-I, Haridwar and its antibacterial studies were carried out in the laboratory.

2.2. Test Bacteria for susceptibility assay

Khadirarishta was evaluated for its antibacterial potency against Gram-negative **bacteria** *viz; Escherichia coli* (MCC2414), *Pseudomonas aeruginosa* (MCC2080), *Salmonella enterica* (NCL5284), *Shigella boydii* (MTCC11947) and Gram-positive *viz; Bacillus cereus* (MCC 2086), *Listeria monocytogen* (NCL5260), *Rhodococcus* sp. (MCC2645) and *Staphylococcus aureus* (MCC2408), *Lactobacillus spp*. (MCC2889) bacterial strains. These bacterial cultures were maintained in nutrient agar slants at 37.0 °C. Each of the bacteria was reactivated prior to susceptibility testing by transferring them into a separate test tube containing nutrient broth and incubated overnight at 37.0 °C. These bacterial strains were procured from different culture collection centres *viz;* Microbial Culture Collection (MCC) NCCS, Pune, India, National Chemical Laboratory (NCL), Pune, India and MTCC (Microbial Type Culture Collection), IMTECH, Chandigarh, India.

Table 1
Herbal components per 100 mL of Khadirarishta.

No.	Components	Quantity
1	*Acacia catechu*	19.52 g
2.	*Cedrus deodara*	19.53 g
3.	*Berberis aristata*	7.81 g
4.	*Terminalia chebula*	7.81 g
5.	*Terminalia belerica*	7.81 g
6.	*Emblica officinalis*	7.81 g
7.	*Woodfordia fructicosa*	7.81 g
8	*Psoralea corylifolia*	4.68 g
9	*Piper longum*	1.56
10	*Piper cubeba*	0.39 g
11	*Mesua ferrea*	0.39 g
12	*Myristica fragrans*	0.39 g
13	*Syzgium aromaticum*	0.39 g
14	*Elettaria caradamomum*	0.39 g
15	*Cinnamomum zeylanicum*	0.39 g
16	*Cinnamomum tamala*	0.39 g
17	Honey	78.12 g
18	Sugar	39.06 g
19	Water	Q.S.

*Q.S.: Quantum satis.

2.3. Antibacterial assay

2.3.1. Bacterial susceptibility assay

The initial antibacterial screening of Khadirarishta was checked by agar well diffusion method. Culture plates were prepared by pouring 20 mL sterilized Muller Hinton Agar (MHA) into pre-sterilized Petri dishes. 0.1 mL (inoculums size were adjusted by using 0.5 McFarland standard with approximately 1.0×10^8 $CFUmL^{-1}$) of inoculums suspension of each of nine bacterial strains and were spread uniformly over the agar medium using a sterile glass rod in plates. Wells in the agar plates were made by using sterile cork borer (6 mm). A concentration (100 $mgmL^{-1}$) of Khadirarishta (1X PBS buffer pH 7.4 solvent use) was separately added to wells in the plates. The plates were incubated at 37.0 °C for 24 h. The mean diameters of zone of inhibition (mm) was measured and recorded. Standard antibiotic Tetracycline 30 mcg/disc and Gentamicin 10mcg (only for *P. aeruginosa*) (Hi-Media, Mumbai) were used as positive control while blank MHA media plates and 1X PBS buffer alone were used as control respectively (Gupta et al., 2016). Experiments were carried out in triplicate and the average diameter for the zone of inhibition (ZOI) was recorded. The percent inhibition was calculated using the formula of Vincent (1947).

Inhibition (%) = (C-T)/C ×100

Where C is the growth in control in mm and T is growth in treatment in mm

2.3.2. Broth microdilution method

The antibacterial potency of Khadirarishta was also evaluated by measuring the minimum inhibitory concentration (MIC) values as per the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2003). 96-well micro plates were used to determine the MIC of Khadirarishta against sensitive bacterial strains. A stock solution of the test was prepared by dissolving 100 mg and 33 mg of Khadirarishta into 1 mL of 1XPBS. Both the stock solutions were diluted to obtain serial log by tenfold dilutions; 125 μL of these dilutions were added to each well containing Muller-Hinton broth medium (MHB–Himedia), resulting in test concentrations ranging from 100 to 0.003 mg mL^{-1} and 100 μL of the bacterial suspension of each sensitive strain *E. coli*, *S. aureus* and *S. enterica* prepared in MHB (final concentration, 10^7 $CFUmL^{-1}$) was added to each well, with a final volume of 250 μl per well. All the plates were covered with a sterile plate sealer and incubated at 37.0 °C and 180 rpm agitation for 18 h. After the incubation period, MIC values were quantified by taking absorbance at 600 nm by using Envision microplate reader (Perkin Elmer, USA). MIC-80, minimum concentration of extract that caused ≥80 % inhibition in bacterial growth, was considered as an active MIC value of extract. The percentage of bacterial growth inhibition by extract was calculated using the following equation:

% bacterial growth inhibition = [(Ac-At)/Ac] 100

Where Ac: Absorbance of the control; At: Absorbance of test extract

The MIC qualitative of the Khadirarishta was also performed by using p-iodonitrotetrazolium chloride (INT) assay. After taking absorbance, above micro plates were followed to add (50 μL) of 0.4 mg mL^{-1}p-iodonitrotetrazolium chloride (INT) and kept at room temperature for 30 min. viable bacteria produced a pink color by the reduction of yellow dye. Here, MIC value was determined with the lowest test sample concentration that prohibited the color change in the medium which results as the inhibition of bacterial growth and also determined by observing the consistency of turbidity in the wells.

2.4. Mechanism of action

2.4.1. Time killing curve of Khadirarishta

All the three sensitive bacterial strains, *viz E. coli*, *S. aureus* and *S. enterica* were selected for the time-killing studies. The time-killing curve was performed as per the Su et al. (2015) protocol with some modifications. An inoculate of 7.0 log $CFUmL^{-1}$ (1.0×10^7 $CFUmL^{-1}$) of overnight grown bacterial culture was used in these experiments. The concentration of Khadirarishta in the Mueller-Hinton broth medium was set at a concentration equal to double of the MIC level for each bacterial strain. Aliquots (200 μL) of the cultures were taken at 0, 2, 4, 6, 8, 10, 12 and 24 h and serially diluted in the Mueller-Hinton broth, and then plated on nutrient agar media plates. Following 24 h of incubation, the numbers of colonies were counted to determine the total viable bacterial number. A bacterial culture without test sample was assayed as the control.

2.4.2. K^+ leakage

The K^+ leakage from the sensitive bacterial strains viz; *E. coli*, *S. aureus* and *S. enterica* cells was measured. Overnight broth cultures of *E. coli*, *S. aureus* and *S. enterica* were taken *and the cells* were centrifuged at 8000 rpm for 10 min. The centrifuged pellet was resuspended at a concentration of 1.0×10^8 $cellsmL^{-1}$ in 1XPBS buffer (pH 7.2). The cells were washed twice with the same buffer. One millilitre of this suspension was mixed with Khadirarishta at a concentration of 2X MIC (2X MIC required for lyses of cell) and then incubated at 37.0 °C for different time intervals from 0–4 h. Quantity of leaked K^+ ions was measured by using a spectrophotometer method (Rajawat et al., 2014). This technique begins with the addition of sodium cobaltinitrite to the control and test samples. The precipitate subsequently formed was centrifuged and incubated at room temperature for 45 min. Afterwards, it was washed with 95 % ethanol followed by the addition of concentrated HCl. This solution was kept at room temperature for 15 min. The resultant green colour in the form of K^+ efflux was quantified at 623 nm by UV–vis spectrophotometer (Shimadzu UV 1800). Bacterial strains incubated with 1XPBS buffer were only used as controls.

2.4.3. Nucleotide efflux

E. coli, *S. aureus* and *S. enterica* 6 h old logarithmic phase growth culture were washed and resuspended in 1XPBS buffer (pH 7.2). These bacterial strains were incubated with Khadirarishta at its MIC in 1:1 ratio for different time intervals from 0–12 h. Afterwards, the mixture was filtered and absorbance of filtrate was estimated at 260 nm by using UV–vis spectrophotometer. Strains incubated with 1XPBS buffer were only used as a control (da Silva et al., 2014).

2.4.4. Resistance analysis of Khadirarishta

The tested bacterial strains *E. coli*, *S. aureus* and *S. enterica* were subcultured in a sub-MIC concentration of the Khadirarishta for five successive sub culturing series to examine their ability to develop any kind of resistance. Then, the MIC of the Khadirarishta was determined at subculture series six and the results were recorded (Su et al., 2015).

2.4.5. Combination effect of Khadirarishta with antibiotics

All the three sensitive bacterial strains, *E. coli*, *S. aureus* and *S. enterica* were selected for the combination studies by using the disc diffusion method (Su et al., 2015). The disc impregnated with Khadirarishta (100 $mgmL^{-1}$) was placed on the Muller-Hinton agar plate at a standard distance within the antibacterial discs. The plate was incubated at 37.0 °C for 18 h. After the incubation, the zone of inhibition pattern was observed. The inhibition pattern was observed for any type of synergism in terms of confluent or bridging in zones of inhibition between the discs of the test sample and the antibiotics. The antibiotics used in this assay were Ofloxacin (5mcg/disc), Tetracycline (30mcg/disc), Cephalosporin (30mcg/disc), Faropenem (30mcg/disc).

2.5. HPLC analysis

2.5.1. Column

HPLC column Shodex C-18-4E column [4.6 MM ID x250 mm L] was used to analyze the Khadirarishta

2.5.2. Mobile phase

The elution was carried out by using gradient elution of mobile phase A (1.0 % Ortho phosphoric Acid, adjust pH 2.5 by Diethyl amine) and mobile phase B (Acetonitrile).

2.5.3. Chromatographic conditions

All analysis was carried out at 35 °C temperature under isocratic condition. The flow rate and injection volume was 1.0 $mlmin^{-1}$ and 10 μL respectively. Before injecting the sample, each sample was filtered through 0.45 μm filter. The resultant chromatograph was recorded at 270 and 365 nm wavelength.

2.5.4. Test sample preparation

5.0 gm of sample was weighted and dissolved in 10 mL methanol. This solution was centrifuged at 8000 rpm for 5 min and filtered (Sol A). This solution was used for the analysis of catechin, ellagic acid and eugenol. For the analysis of gallic acid 1.0 mL of sol A was diluted to 25 mL methanol and subjected to HPLC.

2.6. Statistical analysis

All the experiments were performed in triplicates. The mean and standard deviation of the results were calculated by GraphPad Prism 7 software. In the graphs, the standard deviation was represented as error bars. Data were subjected to significant differences between the means tested using Tukey method at $p < 0.05$ by using GraphPad Prism 7 software.

3. Results

3.1. Antibacterial Activity of the Khadirarishta by the Well diffusion method

Initially nine bacterial strains, Gram + ve *viz; S. aureus* (MCC2408); *Lactobacillus* sp. (MCC2889); *Bacillus cereus* (MCC2086), *Listeria monocytogen* (NCL5260), *Rhodococcus* sp. (MCC2645), *Lactobacillus* spp. (MCC2889) while in Gram-ve *viz; E. coli* (MCC2414); *Salmonella enterica* (NCL5284); *P. aeruginosa* (MCC2080) and *Shigella boydii* (MTCC11947) were selected for the antibacterial analysis. As shown in Table 1, the well diffusion analysis results (100 μL/well; 100 $mgmL^{-1}$ dissolved in 1XPBS) revealed that Khadirarishta showed antibacterial activity against the test strains of *E. coli*, *S. aureus*, *S. enterica* and *Rhodococcus* sp. with average inhibition zones of 21.90 mm, 22.0 mm, 19.30 mm

and 12.0 mm while % inhibition was 86 %, 92 %, 75 % and 35 % respectively. Among these bacterial strains *S. aureus* was found more sensitive in comparison to other test strains. However, *Rhodococcus* sp. Did not show prominent sensitivity towards Khadirarishta and thus was not selected along with the other sensitive test bacterial strains for further examinations. The negative control, 1XPBS, did not show any inhibition zone against all the test strains. The positive control, tetracycline, showed a mean inhibition zone of 25.50 mm, 23.90, 25.70 mm and 34.50 mm against the strains of *E. coli*, *S. aureus*, *S. enterica* and *Rhodococcus* sp., respectively (Table 2).

3.2. Minimum inhibitory concentration (MIC)

Since the well diffusion results showed that the Khadirarishta (100 μL/well; 100 mgmL^{-1} dissolved in 1XPBS) revealed a significant antimicrobial activity against the three sensitive bacterial strains *viz; E. coli*, *S. aureus* and *S. enterica*. These three bacterial strains were thus selected as test strains for determination of minimum inhibitory concentration (MIC) of Khadirarishta. Fig. 1 shows that all sensitive bacterial strains growth inhibited ≥ 80 % significantly ($p < 0.05$) at the average concentration range of 1.0–10.0 mg mL^{-1} of Khadirarishta. Active MIC values of Khadirarishta against bacterial strains *E. coli*, *S. aureus* and *S. enterica* were found 3.3, 1.0 and 10.0 mg mL^{-1} respectively (Table 3). The Gram positive strain (*S. aureus*) with MIC of 1.00 mg mL^{-1} was more susceptible to the test extract than the Gram negative strains (*E. coli* and *S. enterica*) with MIC ranges 3.30–10.0 mg mL^{-1}.

Khadirarishta antibacterial spectrum: S. aureus > *E. coli* > S. enterica

3.3. Time-killing assay

The bactericidal ability of the Khadirarishta with significant antimicrobial activity was determined by time-killing curves. All the three sensitive bacterial strains *E. coli*, *S. aureus* and *S. enterica* were used for this assay. The time killing curve of negative control (only bacterial strain) revealed that the colony increased over time while time killing curve in the presence of Khadirarishta revealed a significant ($p < 0.05$) growth inhibition effect on *E. coli*, *S. aureus* and *S. enterica* bacterial strains at 8, 6 and 12 h respectively. Time killing curve of Khadirarishta showed that the *E. coli*, *S. aureus* and *S. enterica* colony counts went from 7.0 log CFUmL^{-1} (1×10^7 CFUmL^{-1}) down to 3.40 log CFUmL^{-1} (5.0×10^3 CFUmL^{-1}), 2.65 log CFUmL^{-1} (4.40×10^2 CFUmL^{-1}), 4.95 log CFUmL^{-1} (8.9×10^4 CFUmL^{-1}) at 8, 6, and 12 h respectively (Fig. 2). All of the time the killing effect was maintained for 24 h, thus show-

Table 2
Antibacterial sensitivity of Khadirarishta against tested bacterial strains.

No.	Bacterial strains	Accession No.	Khadirarishta (100 mgml^{-1})	Tetracycline (30mcg)	Gentamicin (10 mcg)	% Inhibition
1.	*E. coli*		21.9 ± 0.5	25.5 ± 1.0	–	86 %
2.	*P. aeruginosa*	MCC2080	NI	–	24.8 ± 0.7	NI
3.	*S. aureus*	MCC2408	22.0 ± 1.0	23.9 ± 0.6		92 %
4.	*S. enterica*	NCL5284	19.3 ± 0.7	25.7 ± 0.5		75 %
5.	*Rhodococcus spp.*	MCC2645	12.0 ± 0.5	34.5 ± 0.5		35 %
6.	*Listeria spp.*	NCL5260	NI	37.5 ± 0.4		NI
7.	*Lactobacillus spp.*	MCC2086	NI	33.0 ± 0.7		NI
8.	*B. cereus*	MCC2086	NI	32.0 ± 0.5		NI
9.	*S. boydii*	MTCC11947	NI	27.0 ± 0.5		NI

Sample size n = 3; *Mean of three values ± Standard Deviation. *NI: No Inhibition.

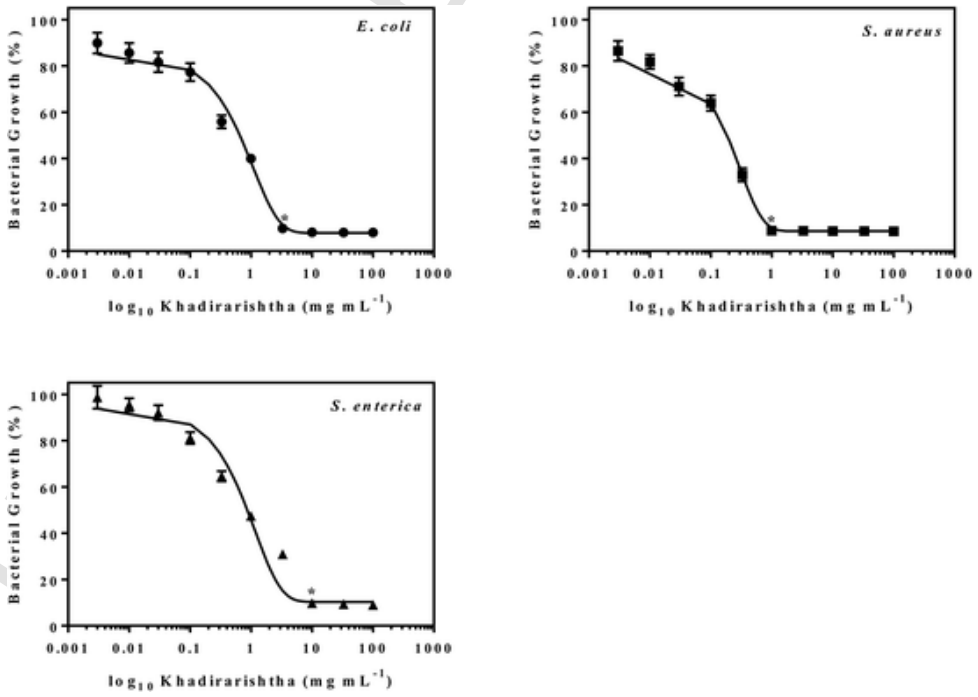

Fig. 1. MIC determination of Khadirarishta by bacterial growth (%) of sensitive bacterial strains viz; *E. coli*, *S. aureus* and *S. enterica* against $\log_{10}$ values of Khadirarishta (mg mL^{-1}) against A inoculate of 1×10^7 CFUmL^{-1} of each bacterial strain was used. Means of bacterial growth (%) marked by * is significantly different (Tukey test, $p < 0.05$).

Table 3
MIC of Khadirarishta against sensitive bacterial strains.

No.	Pathogens	MIC ($mgml^{-1}$)
1.	*E. coli*	3.3
2.	*S. aureus*	1.0
3.	*S. enterica*	10.0

ing that the Khadirarishta not only possessed antibacterial activities, but also had significant ($p < 0.05$) bactericidal abilities.

3.4. Intracellular K^+ leakage

All the three sensitive bacterial strains (24 h old grown) were initially resuspended in sodium phosphate buffer and negligible potassium leakage was observed initially at 1 h while the maximum potassium leakage of 3.3–4.1 ppm was observed after 3–4 h incubation time (Fig. 3.). In all three sensitive bacterial strains, addition of 2X MIC Khadirarishta resulted in vigorous potassium leakages, which reached to the maximum level at 4 h after the incorporation of active compounds ($p < 0.05$). *E. coli* was observed to possess greater sensitivity towards Khadirarishta in terms of K^+ leakage (Fig. 3).

3.5. Nucleotide efflux

All the three sensitive bacterial strains (6 h old grown) were initially re-suspended in sodium phosphate buffer and negligible nucleotide efflux was observed throughout the study (Fig.3). In this Nucleotide efflux study, nucleotide efflux of *E. coli*, *S. aureus* and *S. enterica* begins at 3, 6 and 6 h respectively (Fig.4). Addition of Khadirarishta (1XMIC) to a medium containing sensitive bacterial strains caused 3.7, 6.6 and 4.1 significant ($p < 0.05$) fold changes in the nucleotide efflux of *E. coli*, *S. aureus* and *S. enterica* respectively. This increment in nucleotide efflux was observed at 12 h compared to control cells (Fig. 4)

3.6. Resistance analysis

The drug induced resistance was determined by six series of sub culturing of the bacterial strains with the sub-MIC concentration of the Khadirarishta followed by MIC determination. The result showed that the MIC for the test strains, including *E. coli*, *S. aureus* and *S. enterica* were unchanged, thus demonstrating that the tested bacterial strains would not be expected to induce resistance to the active substances from Khadirarishta.

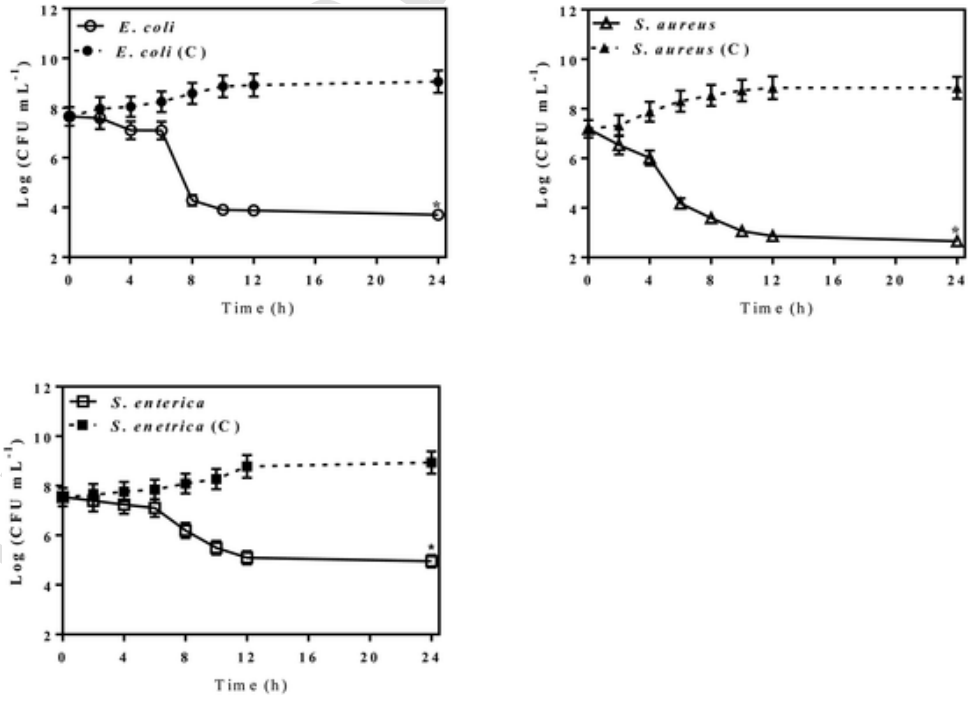

Fig. 2. Bactericidal effect of 2X MIC of Khadirarishta on the *E. coli*, *S. aureus* and *S. enterica*. A inoculate of 1×10^7 $CFUmL^{-1}$ of each bacterial strain was used. Error bars represent the standard deviations. Means of bactericidal effect marked by * is significantly different (Tukey test, $p < 0.05$).

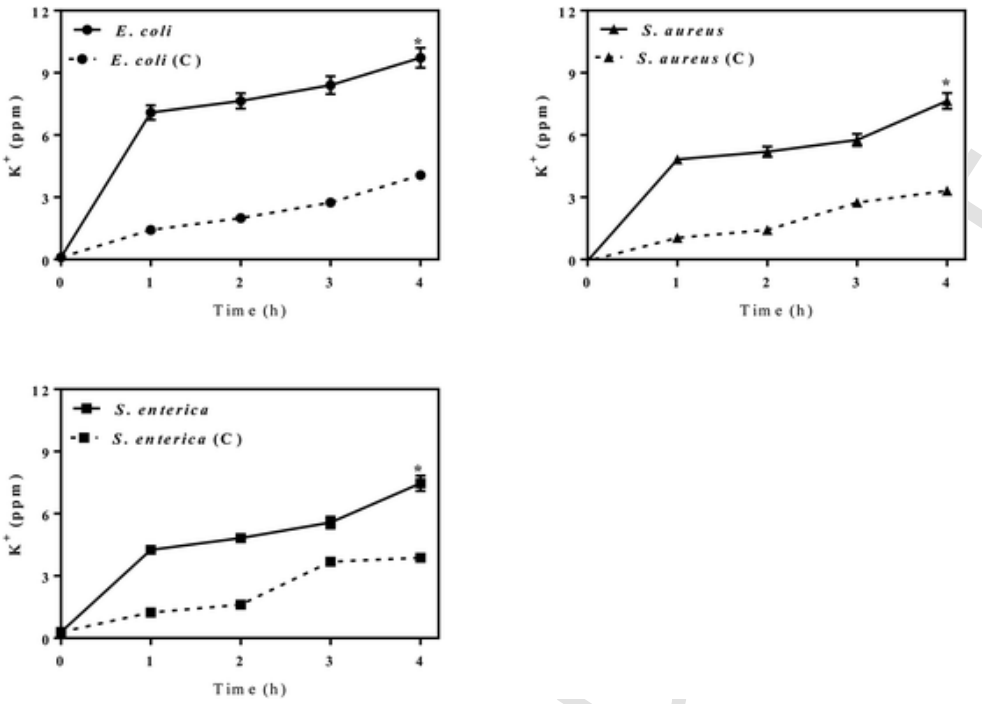

Fig. 3. Effect of 2X MIC of Khadirarishta on the amount of intracellular K^+ leakage from *E. coli*, *S. aureus* and *S. enterica.* C: Control. Error bars represent the standard deviations. Means of K^+ leakage marked by * is significantly different (Tukey test, $p < 0.05$).

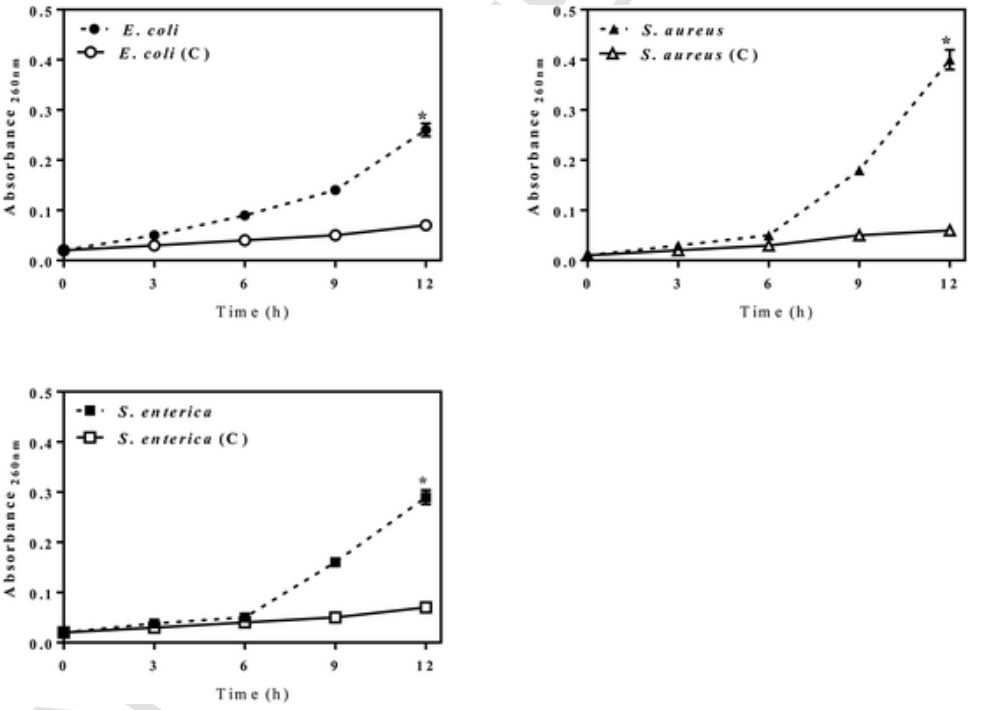

Fig. 4. Effect of MIC of Khadirarishta on the amount of total Nucleotide efflux from *E. coli*, *S. aureus* and *S. enterica*. Error bars represent the standard deviations. Means of nucleotide efflux marked by * is significantly different (Tukey test, $p < 0.05$).

3.7. Combination effect of the Khadirarishta with antibiotics

Combination effects of the Khadirarishta with antibiotics were determined by the disc diffusion method. The strains of *E. coli*, *S. aureus* and *S. enterica* were selected as the test strains; a total of 4 antibiotics, included Ofloxacin, Tetracycline, Cephalosporin and Faropenem were used for the combination effect analysis. The results revealed that all of the test antibiotics neither showed any obvious combination nor synergistic effect with Khadirarishta against the test strains (Table 4).

3.8. HPLC analysis

HPLC analysis of Khadirarishta revealed four different marker compound *viz.,* gallic acid, catechin, eugenol and ellagic acid by using the standard method. All four compounds were confirmed based on their

Table 4
Combination effect of Khadirarishta s with Antibiotics.

No.	Pathogens	ZOI of Khadirarishta (100 mg ml^{-1}) /Standard antibiotics					Combi.Effect
		Khadirarishta	Ofloxacin	Tetracycline	Cephalosporin	Faropenem	
1.	*E. coli*	22.0 ± 0.5	27 ± 0.8	25 ± 0.5	21 ± 0.5	34 ± 0.3	N
2.	*S. aureus*	20.9 ± 0.3	25 ± 0.6	20 ± 0.7	18 ± 0.4	30 ± 0.8	N
3.	*S. enterica*	20.3 ± 0.5	25 ± 0.6	24 ± 0.3	20 ± 0.5	29 ± 0.5	N

*'N'-No Combination Effect; Sample size n = 3; *Mean of three values ± Standard Deviation; Combi.: Combination.

respective retention time, and UV spectrum at photodiode array (PDA) detector (Fig. 5A-D). The quantity of these marker compounds is represented in Table 5.

4. Discussion

The minimal side effects and toxicity level in Khadirarishta or any plant based medicines have played a crucial role to combat various infections or diseases. In India, Khadirarishta is mainly used to treat many skin disorders, cyst, tumours or any chronic diseases. In this study, the efficacy of Khadirarishta is evaluated by using in-vitro approaches against some of Gram-positive and Gram-negative bacterial strains. The antibacterial mechanism of action has also been included to determine that the active ingredients of Khadirarishta may possibly cause damage to bacterial cells. Currently there is no information in the literature regarding the antibacterial activity of Khadirarishta. However, the ancient Ayurvedic text "Sharangdhara Samhita" clearly states Khadirarishta is beneficial in curing any skin disorder, urticaria, wounds, splenomegaly, and intestinal worms (Parulkar, 2016). This is the first research on Khadirarishta to show its notable antibacterial activity against a no. of bacterial strains.

The antibacterial sensitivity test of Khadirarishta showed its clear antibacterial activity against *E. coli*, *S. aureus* and *S. enterica* among the tested nine bacterial strains. The MIC results of Khadirarishta by broth micro-dilution assay (qualitative and quantitative) of *E. coli*, *S. aureus* and *S. enterica* (MIC 1–10 mg mL^{-1}) are in accordance with previously reported antibacterial activities of plant extracts/ natural compounds against these or other bacterial strains with the MIC values ranges from 1 to 20 mg mL^{-1} (Negi and Dave, 2010; Bhattarai and Bhuju, 2011; Mostafa et al., 2011; Saxena et al., 2014). This result indicates that Khadirarishta has a broad spectrum antibacterial activity because of its effectiveness against both Gram-positive *viz; S. aureus* and Gram-negative *viz; E. coli* and *S. enterica* bacterial strains. Hence, these three sensitive bacterial strains were chosen to elucidate the antibacterial mechanism of action of Khadirarishta.

Included in this study, is how Khadirarishta and its active marker compounds may affect the bacterial cells which can lead to the disruption of bacterial ion transportation and nucleotide system. To confirm its antibacterial mechanism of action; time killing assay, combination study and resistance analysis of Khadirarishta were also performed. In earlier studies, a similar type of antibacterial mechanism of action of *Piper umbellatum* (da Silva et al., 2014) and *Polygonum cuspidatum* (Su et al., 2015) herbal extracts were studied. In the present study, the HPLC analysis of Khadirarishta showed the presence of diverse plant phenolic compounds *viz.,* gallic acid, catechin and ellagic acid as well as a monoterpenoid eugenol. The phenolic or polyphenols marker compounds are generally considered to disrupt the cytoplasmic membrane of bacterial cells and their antibacterial potency mainly attributed to the presence of hydroxyl functional groups (Gyawali and Ibrahim, 2014) while the eugenol which is a monoterpene, alters the bacterial cell morphology by changing the membrane permeability of the affected bacterial cell (Nazzaro et al., 2013). Most of the natural compounds from active herbal extracts cause disruption of bacterial membranes of sensitive bacterial strains (Lou et al., 2011).

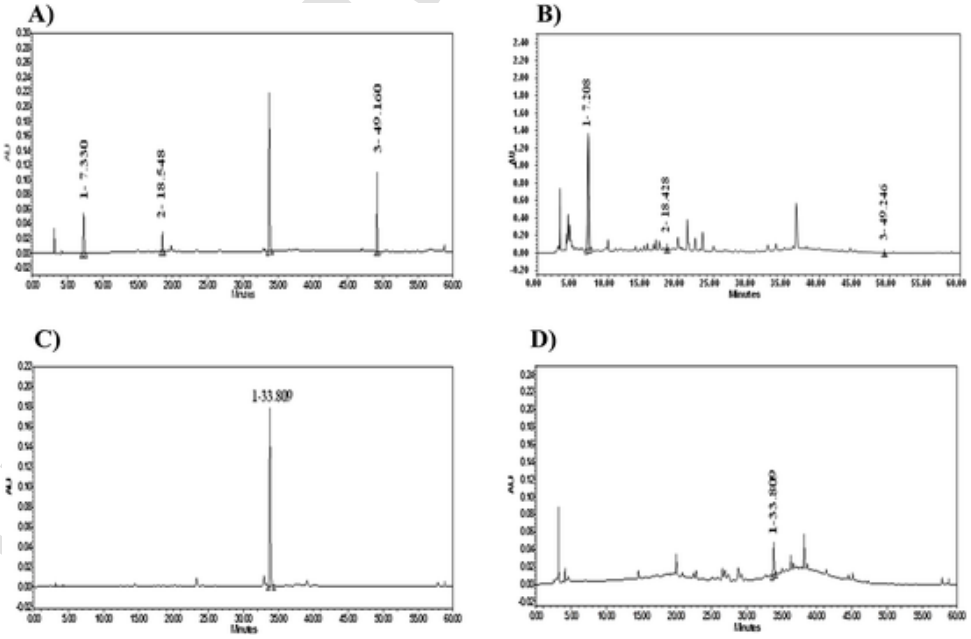

Fig. 5. (A) HPLC chromatogram of standard marker compounds Peak details 1. Gallic acid; (B) HPLC chromatogram of marker compound in Kumaryasava Peak details 1. Gallic acid; (C) HPLC chromatogram of standard marker compounds Peak details 1. Ellagic acid; (D) HPLC chromatogram of marker compound in Kumaryasava Peak details 1. Ellagic acid; (E) HPLC chromatogram of standard marker compounds Peak details 1. Ellagic acid; (D) HPLC chromatogram of marker compound in Kumaryasava Peak details 1. Ellagic acid.

Table 5
Quantification of marker compounds in Khadirarishta.

No.	Name of marker compound	Quantity ($mgml^{-1}$)
1.	Gallic acid	1.41
2.	Catechin	0.080
3.	Ellagic acid	0.040
4.	Eugenol	0.006

The cytoplasmic membrane of the bacterial cell system plays an important role in controlling the entry and exit of compounds of the cell. Any damage to the cytoplasm membrane will cause a leakage of small molecules from the intracellular system which will eventually make it one of the favourite sites for antibacterial agents (Yi et al., 2010). Once the cytoplasmic membrane of bacteria is affected, the intracellular components and small molecules are leaked, and can also be quantified. Following the concept of bacterial membrane disruption, it is important to quantify the K^+ ions and total nucleotides leakage from the sensitive bacterial cell system (Hao et al., 2009). Both of these quantification assays were choosen to outline the probable mechanism of action of Khadirarishta. Moreover, K^+ plays an essential role in regulation of cytoplasmic pH, pressure, electric potential and bacterial physiology (Gries et al., 2013).

Based on all the authors observations it was found; that the phytochemical marker substances of Khadirarishta *viz;* gallic acid, catechin, ellagic acid, eugenol and others may act on the bacterial cell membrane or outer membrane proteins which may leads to the leakage of K^+ ions and nucleotides. Moreover, in previous studies, it was noted that the presence of poly-phenols such as ellagic acid may damage the membrane of *S. aureus* (Akiyama et al., 2001) while phenolic compounds such as gallic acid effects the different bacterial physiological indices such as membrane permeabilization and internal potassium leakage (Borges et al., 2013). This can ultimately cause the death of the Gram-negative bacteria such as *E. coli* as well as Gram-positive *S. aureus* bacterial cells (Lee and Je, 2013). Other phenolic compounds such as catechin in herbal extracts have also been reported for their broad spectrum bactericidal effect (Tsuchiya, 2015; Górniak et al., 2019). The plant phenols majorly act on bacteria by targeting their cell wall, ion channels, and disruption of outer and cytoplasmic membranes which represents the strong antimicrobial potency of any plant extracts or herbal formulations. Similarly, the presence of eugenol in an extract also causes membrane alteration which affects the ion transport system of bacterial cells (Oyedmiet et al., 2009; Nazzaro et al., 2013). The kinetic potassium leakage from *E. coli*, *S. aureus* and *S. enterica* treated with Khadirarishta showed an immediate and great release of potassium. The presence of Khadirarishta accelerated the K^+ leakage which resulted in a significant ($p < 0.05$) threefold increase of K^+ in extracellular environment. A similar increment of K^+ concentration in the outer environment was also observed in *S. aureus* during its treatment with Punicalagin (Xu et al., 2017). The nucleotide efflux studies of sensitive bacterial strains *E. coli*, *S. aureus* and *S. enterica* in the presence of Khadirarishta revealed a significant ($p < 0.05$) 3–6 fold efflux of Total nucleotides by measuring the absorbance at 260 nm. Similar findings regarding nucleotide efflux in the outer environment was also observed in *S. flexineri* (da Silva et al., 2014) and in *S. aureus* (Hao et al., 2009) during their treatment with *P. umbellatum* hydro-alcoholic extract and peptide Buforin 2 respectively.

Both these efflux assays were performed only to quantify the leaked compounds. It is not possible to determine the interaction between bacterial cells and Khadirarishta leading to the leakage of the compounds. However, a number of mechanisms for this type of interaction have been hypothesized to determine the action of antibacterial agent on a bacterial cytoplasmic membrane. Mainly these findings were related to solubilization of membrane proteins, formation of pores, and changes in the structure/function of the membrane (Chen and Cooper, 2002; da Silva et al., 2014).

During time-killing analysis, 2 x MIC's of Khadirarishta was incubated with the three sensitive bacterial strains *E. coli*, *S. aureus* and *S. enterica* for 0–24 h. The result showed a reduction of the viable cell counts ranging between $4.40 \times 10^2 CFUmL^{-1}$ and $8.90 \times 10^4 CFUmL^{-1}$. A significant ($p < 0.05$) reduction in the bacterial cell counts at 8, 6 and 12 h of incubation time period indicated that Khadirarishta had a bactericidal property which caused the 50–80 % reduction in bacterial population. The study was carried out in time dependent mode and presents an effective time-killing profile for the tested bacterial strains. The degree of antibacterial potency could be due to the presence of phytochemical marker compounds in Khadirarishta as was reported earlier in different plants or extracts based antibacterial studies (Schinor et al., 2007; Olajuyigbe and Afolayan, 2012).

Similarly, the resistance analysis of Khadirarishta also adds to its antibacterial potency since none of the sensitive bacterial strains *viz; E. coli*, *S. aureus* and *S. enterica* induce any kind of resistance against Khadirarishta. However, the combination studies of Khadirarishta with the different antibiotics didn't show any kind of synergism. Overall, all these studies showed the antibacterial potency of Khadirarishta by verifying its action on bacterial cytoplasmic membrane which leads to the leakage of important components from the sensitive bacterial cells. The result from all these parameters are in accordance with the antibacterial studies of plant extracts that were carried out previously (da Silva et al., 2014; Su et al., 2015).

5. Conclusion

The aim and objective of this study was to show the antibacterial potency and probable mechanism of action of Khadirarishta. The antibacterial potency of Khadirarishta was confirmed for the sensitive bacterial strains *viz; E. coli*, *S. aureus* and *S. enterica*. The mechanism of action of Khadirarishta against the sensitive bacterial strains appears to be allied with its effects on the bacterial cell wall and the membrane which leads to the leakage of the major bacterial components. This membrane damage could be due to the presence of active phytochemical marker compounds in Khadirarishta. Although these *in-vitro* studies do not provide a definite confirmation on Khadirarishta antibacterial potency or as suitable drug against the tested strains, they do however provide a basic antibacterial perception of Khadirarishta. It also acts as an encouragement for the elucidation of known or newer active substances to validate such traditional formulation.

CRediT authorship contribution statement

Acharya Balkrishna: Concept, Design, Definition of intellectual content. **Dr. Ashish Kumar Gupta:** Experimental studies, Data Analysis, Statistical analysis, Manuscript preparation, Manuscript editing. **Antriksh Gupta:** Data Analysis, Statistical analysis. **Priya Singh:** Literature search, Experimental studies, Data acquisition. **Kanchan Singh:** Experimental studies, Data acquisition. **Meenu Tomar:** Experimental studies, Data Analysis. **Dr. D. Rajagopal:** Manuscript editing, Manuscript review.

Declaration of Competing Interest

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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