Pure Appl. Biol., 11(2): 411-420, June, 2022
http://dx.doi.org/10.19045/bspab.2022.110041

Research Article

An *in-vitro* antibacterial activity of *Aloe vera* and gentamicin against *Escherichia coli* and *Klebsiella pneumoniae* isolates from mastitis milk samples, from Tandojam, Sindh Pakistan

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Citation

Ubaidullah Jokhio, Rehana Shahnawaz Buriro, Shamsuddin Bughio, Saeed Ahmed Soomro, Muhammad Bilawal Arain, Ali Gul Soomro, Faiza Habib, Zainab Lanjar, Wali Khan and Faisal Khan. An *in-vitro* antibacterial activity of *Aloe vera* and gentamicin against *Escherichia coli* and *Klebsiella pneumoniae* isolates from mastitis milk samples, from Tandojam, Sindh Pakistan. Pure and Applied Biology. Vol. 11, Issue 2, pp411-420.
http://dx.doi.org/10.19045/bspab.2022.110041

Received: 05/02/2021 Revised: 23/04/2021 Accepted: 28/07/2021 Online First: 26/08/2021

Abstract

The current study was conducted to examine the in vitro antibacterial activity of pure *Aloe vera* extract and compared with commonly used antibiotic, Gentamicin against *Escherichia coli and Klebsiella pneumonia* isolated from mastitis milk. For this purpose, 50 milk samples were collected from dairy farms in the vicinity of Tandojam and processed the samples for culture, identification, and Minimum inhibitory concentration of pure *Aloe vera* extract and gentamicin in the laboratory of department of Veterinary Pharmacology, Sindh Agriculture University Tandojam, to record the MIC against isolated organisms at different concentrations i-e 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.081 and 0.039µl. The MIC of the isolates was determined by the turbidity and translucency of the cultured medium Results of present study revealed that out of 50 milk samples, 35 (70%) and 15 (30%) were found positive for *E. coli* and *K. pneumonia*, respectively. It was found that *E. coli* stopped growth at 10µl of pure *Aloe vera* and 1.25µl of Gentamicin. Whereas *K. pneumoniae* failed to grow at 2.5µl of pure *Aloe vera*, but it was found highly susceptible for gentamicin and stopped growth at lowest conc: i-e 0.03µl. The comparative results revealed that the concentrations of *Aloe vera* used in this study were not as effective as that of commercial product, but *Aloe vera* stopped the growth of isolated organisms at certain level as that of gentamicin. Therefore, due to emergence of resistance towards antibiotics, *Aloe vera* can be used as a substitute of antibiotics.

Keywords: *Aloe vera*; Antibacterial activity; *Escherichia coli*; Gentamicin; Mastitis milk

Introduction

Dairy animals affected from different types of diseases among them bovine mastitis is a main problem of dairy sector generating monetary losses, decrease milk production, need costly medicine and if remedy is unsuccessful then the animals are culled. For consuming and processing, it has been found that milk from mastitis animals has lot of organism which lowered the quality of milk products and affected the health of consumer [1].

Mastitis in buffalo is defined as response of parenchyma cells of mammary gland towards the traumatic injury and infection, which is characterized by five signs, i.e, swelling, redness, loss of function, pain and hotness [2]. In buffalo, vast numbers of microorganisms have been reported to cause mastitis and among those major etiological agents are bacteria which include either gram positive or gram-negative. Mastitis has been classified as contagious and environmental based on causative organisms. Environmental mastitis is caused by gram negative bacteria which include *Klebsiella pneumoniae*, *Klebsiella aerogenosa*, *Escherichia coli*, Citrobacter spp., Pseudomonas spp and Proteus spp. [3].

Bovine mastitis is mostly treated with antimicrobial agents through intra-mammary infusion, as well as some systemic drugs are also used. Commonly different antibiotics are used in dairy animals to prevent or control bacterial infections among those Gentamicin is mostly used to treat mastitis This antibacterial belongs to aminoglycoside class and has bactericidal as well as bacteriostatic activity and clinically effective in treatment of gram-negative infections [4].

It has been observed that gram negative organisms such as *Klebsiella pneumoniae*, *Klebsiella aerogenosa* and *Escherichia coli* are susceptible towards the Gentamicin [5]. It has been reported that this antibiotic has broad activity against gram negative bacteria i.e *E. coli, Klebsiella pneumoniae*, Proteus spp and *Enterobacter aerogene* [6]. It has been examined that gram-negative pathogens were more sensitive towards the gentamicin [7].

Aloe vera generally known as Aloe, has more than 300 species throughout the world. It is endless succulent xerophyte plant which has developed water storage cells in the leaves to live in dry area and belongs to the Liliaceae family. The internal part of leaf is a clear, soft, moist and slippery tissue that consist of large thin-walled parenchyma cells in which water is held in the form of a viscous mucilage in the form of gel [8]. Due to its curative and therapeutic properties, it has been used since ages and so far, more than 75 active ingredients have been identified from inner gel. It has several therapeutic properties and used as an anti-allergic, healing activity, anti-carcinogenic, immuno- modulatory effect as well as having antifungal, antibacterial, antiviral and ant-inflammatory activity [9]. It has been evaluated that *Aloe vera* extract possesses medicinal activity including antioxidant, triggering of hematopoiesis, anti-neoplastic effect, promotion of radiation damage repair, anti-diabetic and antimicrobial, due to presence of polysaccharides, found in parenchymal tissue of inner leaf [10].

So far, very little work has been done on the in vitro antibacterial activity of *Aloe vera* against *Escherichia coli* and *Klebsiella pneumoniae* and its comparison with Gentamicin through MIC. Therefore, present study has been designed to check the antibacterial activity of *Aloe vera* and compared with Gentamicin against *E. coli* and *Klebsiella pneumoniae*.

Materials and Methods

Sampling and bacteriological isolation and identification

A total of 50 clinical mastitis milk samples of buffaloes were collected under aseptic condition, in bijous bottle from the dairy

farms of the vicinity of Tandojam and were brought to post graduate research laboratory of the department of Veterinary Pharmacology, Sindh Agriculture University Tandojam.

Preparation of media for culture

The different media were used to culture the bacteria to isolate the bacterial species such as *E. coli* and *Klebsiela pneumoniae*, isolated from mastitis milk. Those included Nutrient, Blood and MacConkey's agar.

Nutrient agar, MacConkey agar, Muller Hinton Broth are prepared according to manufacturing company.

Culture characteristics of bacteria

Samples were cultured onto nutrient agar, for primary culture. Purification of the isolates was done by subculturing onto blood agar and MacConkey's agar then incubated at 37 ºC for 24 hrs. After 24 hours, colonies from nutrient and blood agar were picked up by sterilized wire loop for subculture and the process of sub-culturing was continued till pure colonies growth was obtained. Identification of organisms was performed by cultural, morphological and biochemical characteristics. Grams' staining was done to check the staining reaction of the isolates. After that, pure colonies were transferred to sterile nutrient agar slant, which were then incubated for 24 hours and refrigerated at 4 ^{0}C as stock culture.

Isolation and identification of pathogens

Gram staining characteristic of bacteria

Smears was prepared form the pure isolated colonies, stained with grams stain and the characteristics of the organisms were recorded as either gram-negative or gram-positive.

Biochemical test for the identification of isolated organisms

Bacterial organism was further confirmed by following biochemical tests.

Catalase test

The catalase test was used to differentiate catalase positive bacteria from catalase negative bacteria because enzyme catalase is produced by bacteria that respire using oxygen and protects them from the toxic byproducts of oxygen metabolism. Such as *E. coli* and *Pseudomonas*

Oxidase test

The test was conducted to examine the ability of bacterial organisms to produce the oxidase, an enzyme. A positive reaction was indicated by an intensive blue coloration of the paper within 10 seconds. The oxidase reagent was prepared by adding Tetraphenylene diamine dihydrochloride (dye) 0.1gram and ascorbic acid 0.01gram in 10 ml distilled water.

Extraction of gel from *Aloe vera* leaves

The *Aloe vera* plant leaves were collected from the local plant nursery, of Tandojam. The plant leaves were cleaned with 70% alcohol. The leaves were incised, and gel was separated with the help of sterile knife. Further, the gel was blended to make it homogenous and filtered with muslin cloth and sterilized by using autoclave at 121ºC for 15 minutes at 15lbs pressure, then sterilized stock solution (100% concentration) was used as an antibacterial activity.

Determination of MIC (Minimum Inhibitory Concentration)

Aloe vera and Gentamicin were used during this study, to evaluate the susceptibility of the organisms. The susceptibility test was done by micro broth dilution method on Muller-Hinton (MH) medium. 1:1000 dilution was prepared for MIC. For this, 6µl of bacteria cultured Tryptic Soy Broth (TSB) were added into 6 ml of Muller Hinton (MH) medium, 96 well plates were used to determine minimum inhibitory concentration (MIC) of *Aloe Vera* and Gentamicin against *Escherichia coli* and *Klebsiella pneumoniae*. In 1stwell, 100µl of the (MH) medium were added and repeated in all wells, 80µl concentration of bacterial culture was added in 1st and 2nd well and 20µl of *Aloe vera* were added in 2nd well then 100µl concentration

of *Aloe vera* were taken from 2nd well and added in 3rd well, this procedure was followed up to 12th well respectively. For Gentamicin, concentration of 15mg/15ml (stock solution) was used against the isolates and same procedure as mentioned above was followed for Gentamicin. The MIC plates were incubated at 37°C overnights. After that, the break points were recorded by observing the transparency and turbidity of the cultured medium in order to determine the minimum inhibitory activity of *Aloe vera* and Gentamicin. [11]. The obtained data was compared with control and the concentrations where the isolated organisms stopped to grow with *Aloe vera* and Gentamicin.

Data analysis

The break points, where the bacteria stopped to grow were recorded as MIC for antibiotic as well as pure *Aloe vera* extract by turbidity or non-turbidity of culture.

Results

The percentage prevalence of bacterial organisms isolated from mastitic milk

A total 50 milk samples were examined, and all were recorded positive. Out of those 35 and 15 were found positive for *Escherichia coli* and *Klebsiella pneumoniae* respectively (Table 1). All organisms were identified on their morphological, cultural characteristics and staining reactions.

Susceptibility of isolated bacteria at different concentrations of pure *Aloe vera* extract

The concentrations of 40µl, 20µl, 10µl, 5µl, 2.5µl, 1.25µl, 0.62µl, 0.31µl, 0.15µl, 0.07µl, 0.03µl, and 0.01/µl of pure *Aloe vera* extract were used to check the susceptibility of isolated organisms. *Escherichia coli* presented break points at 40µl, 20µl and 10µl concentrations while at lower concentration it showed resistance, which was observed by turbidity of the medium. Whereas *Klebsiella pneumoniae* displayed break points at 40µl, 20µl, 10µl, 5µl and 2.5µl concentrations while at lower concentrations the organism showed resistance by growth at that concentration (Table 2).

Susceptibility of isolated bacteria at different concentrations of Gentamicin

The concentrations of 40µl, 20µl, 10µl, 5µl, 2.5µl, 1.25µl, 0.62µl, 0.31µl, 0.15µl, 0.07µl, 0.03µl and 0.01/µl of Gentamicin were used to determine the susceptibility of *Escherichia coli* and *Klebsiella pneumoniae*. *Escherichia coli* was recorded, susceptible at 40µl, 20µl, 10µl, 5µl, 2.5µl and 1.25µg/µl and this susceptibility was observed by clear/translucence appearance of the medium. While at lower concentrations, the isolated organism showed resistance, Whereas *Klebsiella pneumoniae* showed susceptibility even at lowest concentration of the gentamicin i-e 0.03/µl (Table 3) which was observed by turbidity of the medium in 96 well plates.

Comparison of antibacterial activity of *Aloe vera* and Gentamicin against isolated bacteria

The concentrations of 40µl, 20µl, 10µl, 5µl, 2.5µl, 1.25µl, 0.62µl, 0.31µl, 0.15µl, 0.07µl, 0.03µl, 0.01µl of *Aloe vera* extract as well as Gentamicin were used to compare the susceptibility of isolated bacterial organisms. The *E. coli* exhibited susceptibility at 40µl, 20µl and 10µl of pure *Aloe vera* extract while it showed susceptibility to Gentamicin at 40µl, 20µl, 10µl, 5µl, 2.5µl, 1.25µl. whereas, The *Klebsiella pneumoniae* was found susceptible at 40µl, 20µl, 10µl, 5µl and 2.5µl concentrations and 40µl, 20µl, 10µl, 5µl, 2.5µl, 1.25µl, 0.62µl, 0.3µl, 0.15µl, 0.07µl, 0.03µl of *Aloe vera* extract and Gentamicin respectively (Table 4).

Hence, it is proved from this study that pure *Aloe vera* can be used as an alternate of antibiotics as it stopped the growth of isolated bacterial organisms at certain levels.

Pure Appl. Biol., 11(2): 411-420, June, 2022
http://dx.doi.org/10.19045/bspab.2022.110041

Table 1. The percentage prevalence of bacterial organisms isolated from mastitic milk samples

Bacterial species	Total No. of mastitic milk samples	No. of positive milk samples	Percentage
Escherichia coli	50	35	70%
Klebsiella pneumonia		15	30%

Table 2. Susceptibility of isolated *Escherichia coli and Klebsiella pneumoniae* at different concentrations of Pure *Aloe vera* extract

Bacterial species	Control	40 µl	20 µl	10 µl	5 µl	2.5µl	1.25µl	0.62µl	0.31 µl	0.15 µl	0.07 µl	0.03 µl
Escherichia coli	+	-	-	-	+	+	+	+	+	+	+	+
Klebsiella pneumoniae	+	-	-	-	-	-	+	+	+	+	+	+

- = susceptibility
+ = Resistance

Table 3. Susceptibility of isolated *Escherichia coli* and *Klebsiella pneumoniae* against various concentrations of Gentamicin

Bacterial species	Control	40 µl	20 µl	10 µl	5 µl	2.5 µl	1.25 µl	0.625 µl	0.3125 µl	0.15625 µl	0.078125 µl	0.0390625 µl
Escherichia coli	+	-	-	-	-	-	-	+	+	+	+	+
Klebsiella pneumonia	+	-	-	-	-	-	-	-	-	-	-	-

- = susceptibility
+ = Resistance

Table 4. Comparative Susceptibility of *Escherichia coli and Klebsiella pneumonia* at various concentrations of Gentamicin and pure *Aloe vera* extract

CONCENTRATION		*Escherichia coli*		*Klebsiella pneumonia*	
	Control	*Aloe vera*	Gentamicin	*Aloe vera*	Gentamicin
40µl	+	-	-	-	-
20µl	+	-	-	-	-
10µl	+	-	-	-	-
5µl	+	+	-	-	-
2.5µl	+	+	-	-	-
1.25µl	+	+	-	+	-
0.62µl	+	+	+	+	-
0.31µl	+	+	+	+	-
0.15µl	+	+	+	+	-
0.07µl	+	+	+	+	-
0.03µl	+	+	+	+	-
0.01µl	+	+	+	+	+

- = Susceptibility
+ = Resistance

Discussion

The percentage prevalence of bacterial organisms isolated from mastitis milk

During this study, fifty clinical mastitic milk samples of buffaloes were collected under aseptic condition. Out of 50 samples, 35 and 15 were found positive for *Escherichia coli* and *Klebsiella pneumoniae* respectively, which are most prevalent bacteria found in mastitis milk of buffalo. It is reported in this study that *Escherichia coli* and *Klebsiella pneumoniae* were having prevalent percentage as 70% and 30%.

Present findings are in line with **[3]**, who reported that different microorganisms are cause of buffalo mastitis and among them *Escherichia coli* was most prevalent followed by *Klebsiella pneumonia* with the 40% and 25% respectively. Somewhere resent findings are agreement with **[12]** who reported different type of bacteria including both g+ve and g-ve, and among gram negative pathogens, *Escherichia coli* and *Klebsiella pneumoniae* were most prevalent having 55 and 19% prevalence, respectively. Similar type of study reported that environmental mastitis in buffalo is mostly caused by gram negative bacteria including *Klebsiella pneumoniae, Pseudomonas aerogensa Escherichia coli, Serratia marcescens*, Citrobacter spp. Pseudomonas spp. and other gram-negative bacteria include Proteus, and among all organisms *E. coli* was highly prevalent with 49% **[13]**. Present findings are in line with **[14]** who reported that, among g-ve bacteria, major prevalent bovine mastitis pathogens were *Escherichia coli* and *Klebsiella pneumonia* having 32 and 14% prevalence. Therefore, in the light of previous studies, it has been confirmed that *Escherichia coli* and *Klebsiella pneumoniae* are highly prevalent in bovine mastitis milk samples in this study.

Susceptibility of isolated organisms to different concentrations of *Aloe vera* extract

In present study, various concentrations of *Aloe vera* extract i-e from 0.039µl to 40µl were used to check the susceptibility of isolated organism. *Escherichia coli* and *Klebsiella pneumoniae* showed break points at 10µl and 2.5µl, respectively.

Present results are comparable with **[15]** who reported that *Aloe vera* possessed the antibacterial activity against gram negative bacterial organisms i,e, *Escherichia coli*. **[16]** also observed that growth of *Escherichia coli* and *Klebsiella pneumoniae* was inhibited in the medium which is containing *Aloe vera*. Another study was conducted to examine the antibacterial activity of *Aloe vera* extract against *E. coli* as well as *Klebsiella pneumoniae*, which showed that it has moderate to high activity against both organisms mentioned earlier **[17]**. Present results are in accordance with **[18]**, who suggested that *Aloe vera* extract has also exhibited antimicrobial effect against gram-negative bacteria such as *Pseudomonas aeruginosa, E. coli* and *Klebsiella pneumonia* at 10 and 5µl respectively but disc diffusion method was used. Similarly, kind of study, was conducted by **[2],** who reported that *Aloe vera* possesses the antibacterial activity against gram negative organisms including, *E. coli and Klebsiella pneumoniae*.**[8]** also reported that *Aloe vera* possessed strong antibacterial effect. Present results are in accordance with **[15]** who reported that *Aloe vera* has potent antibacterial activity against different gram-negative organisms including *Escherichia coli* and *Klebsiella pneumonia* and (MIC) values ranged from 31.25 to 4000 and 62.5 to 8000µg/ml. Similar type of study was conducted to evaluate the antibacterial activity of *Aloe vera* and it was observed from result that gram-negative bacteria are found susceptible, to *Aloe vera* such as *Escherichia coli* and *Klebsiella pneumoniae*

[9]. Present results are in line with previous study by **[19]** who reported that *Aloe vera* possessed inhibitory activity against *Salmonella typhi, Klebsiella pneumoniae, Escherichia coli, Bacillus subtilius* and *Pseudomonas epidermidis*. Furthermore, it has potent antibacterial effect against gram negative organisms, and this activity is principally due to presence of biological elements such as Aloin and anthraquinone, which block the bacterial protein synthesis. Current findings are comparable with previous studies **[9, 20]** who used Aloe liquid and found its efficacy as an antibacterial agent against wide range of gram positive and gram-negative bacteria. The antimicrobial agents of *Aloe vera* gel was reported to either effectively kill or greatly reduce growth of *Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pyogenes, Pseudomonas aeruginosa, Escherichia coli, Propionibacterium acne, Helicobacter pylori* and *Salmonella typhi*.

Current results are in accordance with **[17, 18]** who found that *Aloe vera* possessed antibacterial activity against *Salmonella typhi, Klebsiella pneumoniae, Escherichia coli, Bacillus subtilius*, and *Pseudomonas epidermidis*. It has direct antimicrobial activity due to presence of various components in *Aloe vera* such as saponins, anthraquinones and pyrocatechol **[21]**. The antibacterial activity of *Aloe vera* is due to presence of Anthraquinone which works as protein synthesis inhibitor like tetracycline and which acts by retarding the synthesis of bacterial protein through blocking active site of ribosomes **[22, 23]**.

Susceptibility of isolated organisms against different concentration of Gentamicin

Gentamicin is an aminoglycoside with wide spectrum antibacterial activity and has been used for diverse decades for therapy of severe infections caused by gram negative bacteria such as *E. coli, Enterobacter* spp. as well as g+ve including *Staphylococcus* spp. and *Streptococcus* spp. **[24]**. Its mode action revealed that it inhibits the bacterial protein synthesis through binding to 30s ribosomes, and effective in severe infections caused via gram negative organisms **[25]**. It has been stated that gram negative organisms such as *E. coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were found susceptible to Gentamicin **[26]**. It has extended spectrum towards the *Klebsiella pneumoniae, Klebsiella aerogens, Proteus spp* and *E. coli* **[18]**. It has been determined that gram negative bacteria do not resistant towards it **[7]**.

In present study various concentrations of Gentamicin i-e from 0.039µg/µl to 40 µg/µl were used to check the susceptibility of isolated organism. MIC at which growth of *Escherichia coli* and *Klebsiella pneumoniae* inhibited was 1.25µg/µl and 0.0390625 µg/µl respectively. In current study it was found that *Escherichia coli* was less susceptible as compared to the *Klebsiella pneumoniae* which is inhibited even at lowest concentration i-e, 0.039µg/µl and hence considered as MIC for *Klebsiella pneumoniae*. In current result, MIC of *Escherichia coli* was recorded as 1.25µg/µl. The present results showed agreement with the previous finding in which it has been reported that the *Escherichia coli* showed susceptibility against Gentamicin and its MIC ranges between 0.25–4.0 µg/ml **[11, 27, 28]**. In another study the MIC of *Escherichia coli* was determined, and it was found that *Escherichia coli* was susceptible at 0.5 mg/L. Present result are comparable with **[18]** who reported that MIC at which growth of *Escherichia coli* was inhibited ranged between 0.25 to 0.5µg/ml, Whereas, MIC for *Klebsiella pneumoniae* was determined as 0.039µg/µl. Present result are in line with **[28]** who reported that *Klebsiella pneumoniae* was susceptible against Gentamicin and MIC at which growth

inhibited ranged between 0.25–4mg/l. Present findings are comparable with [5] who reported that gram negative organisms such as *E. coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, were found susceptible. Current results are comparable with previous study of [18] who suggested that the extended spectrum effect of antibacterial towards the *Klebsiella pneumoniae*, *Klebsiella aerogens*, Proteus spp and *E. coli*. Present findings can be supported with [8, 29]. Who determined that gram negative bacteria do resistant towards gentamicin and *E. coli* Present results are comparable with [28, 30] who found that gram negative bacteria i.e., *E. coli* and *Klebsiella pneumoniae* are susceptible. Another study conducted by [31] reported that different bacterial species are susceptible toward Gentamicin such as *E. coli*, Salmonella spp. Klebsiella spp. Pseudomonas spp. Clostridium spp. and *Neisseria gonorrhea.*

The results of present study are an indication that *Aloe vera* can be used as substitute of antibiotics due to its antibacterial effect and it helps to prevent bacterial resistance in the environment.

Conclusion

Isolated *E. coli* was found MIC susceptible at 10µl of *Aloe vera*. Isolated *K. pneumoniae* was recorded MIC susceptible at 2.5µl of *Aloe vera*. Both isolated organisms showed MIC against Gentamicin at 1.25µg/µl and 0.039062µg/µl.

Authors' contributions

Conceived and designed the experiments: U Jokhio, RS Buriro, S Bughio & SA Somroo, Performed the experiments: U Jokhio, AG Soomro, Z Lanjar & F Khan, Analyzed the data: U Jokhio, F Habib & W Khan, contributed materials/ analysis/ tools: RS Buriro, S Bughio, Wrote the paper: MB Arain.

Acknowledgement

The authors are thankful to the team of Molecular Parasitology Laboratory, Sindh Agriculture University, Tando Jam for providing Laboratory facilities, which are essential for successful completion of the present research work.

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