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# Assessing the Prospective Antibacterial Qualities of Cow Urine against the Pathogen Accountable for Ear Infections in Contrast to Commercially Accessible Antibiotic Eardrops

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**ABSTRACT**

Ear infections, a significant health concern particularly among young children in low-income countries, often pose challenges due to the adverse effects associated with currently approved therapies. This study aimed to explore the potential antibacterial properties of cow urine as an alternative treatment for ear infections. Disc diffusion method was used with the main purpose to discover effectiveness of antibacterial activity from cow urine to fight against the bacteria that result in ear infections (*Staphylococcus aureus* and *Pseudomonas aeruginosa)*. Both bacteria were cultured and examined using Mueller Hinton agar (MHA) to check for their response against different concentration of cow urine (15, 45, 75 and 100 %). The plates were incubated overnight at 37°C while the diameter of inhibition zone was measured in the following day in millimeters using ruler. The collected data were analysed using one-way analysis of variance (ANOVA) test. Cow urine showed a pronounced antibacterial effect against *Staphylococcus aureus*, with significant inhibition zones across all concentrations: 23.5 mm for 15 %, 14 mm for 45 %, 16 mm for 75 % and 14.5 mm for 100 %. In contrast, *Pseudomonas aeruginosa* displayed minimal inhibition at most concentrations, with only the 15 % cow urine showing a 9.5 mm inhibition zone. These findings were statistically significant ($p < 0.05$), demonstrating the potency of cow urine at various concentrations. The variation in inhibition is likely due to the differing structures of the cell walls in Gram-positive and Gram-negative bacteria. Based on the findings, cow urine exhibits potential antimicrobial effect against *Staphylococcus aureus* and *Pseudomonas aeruginosa* through disc diffusion method. Thus, it can be said that cow urine is good to combat bacteria causing ear infections.

## 1. Introduction

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Otitis media, which can manifest as either acute or chronic, is a condition predominantly affecting young children [1]. It is a leading cause of medical consultations, antibiotic use, and surgical interventions, even in high-income nations. Although it rarely results in mortality, it can lead to hearing loss and significantly impact an individual's long-term quality of life [2].

The condition primarily affects the middle ear cavity and the ossicles—malleus, incus, and stapes—which are connected to the tympanic membrane. The middle ear cavity is also linked to the nasopharynx via the Eustachian tube [3]. The aetiology of otitis media is often associated with inflammation of the Eustachian tube, which obstructs the drainage of fluids from the middle ear cavity. A hallmark clinical feature of otitis media is purulent effusion, resulting from the accumulation of these trapped fluids [4]. A study conducted in Iraq reported that *Pseudomonas aeruginosa* accounted for approximately 26.3% of cases, while *Staphylococcus aureus* was responsible for 23% of cases in patients diagnosed with chronic suppurative otitis media (CSOM) and acute otitis externa (AOE). These two pathogens are commonly associated with these ear infections. Pseudomonas aeruginosa has been identified as the primary causative agent of CSOM, particularly in immunocompromised individuals presenting with symptoms such as purulent discharge and eardrum perforation [5].

CSOM is a chronic infection in the middle ear cleft that is aggravated by partial or complete ossicles and tympanic membrane. This long-term infection will frequently results in the consequence of deafness and permanent discharge from the ears [6]. *Pseudomonas aeruginosa* are often cultivated using accessible blood agar plates or those plates that present with eosin-methylthionine blue [7]. This particular species has the potential to be detected using numerous methods including gram staining, biochemical analyses and their capability to endure at the temperature that ranging from 4 to 42$^{o}$C [8].

*Staphylococcus aureus* is the second organism that will be examined in this study after *Pseudomonas aeruginosa*. When it penetrates an individual, it usually results in acute otitis externa (AOE), an otologic condition which can be observed by infection in both soft tissue and skin that positioned at the external acoustic meatus [9]. Up to 40% of the cases are known to be caused by *S. aureus,* making it the second pathogen that commonly led to AOE [10]. An individual will typically be observed with the specific modest symptoms as well as few severe side effects upon the diagnosis with AOE that infected by *S. aureus*, including osteomyelitis and necrotizing infection death in a small ratio [11]. With the main purpose to distinguish this bacterium from other *Staphylococcus* species, coagulase test, catalase test and gram staining techniques are all can be applied for its identification [12]. Via gram staining approach, they will be noticed as colonies in cluster shape which grow in purple or blue appearance [13]. In the group of *Staphylococcus* species, it reacts positively in catalase test nonetheless, the positive result in coagulase test is the major alternative that set *S. aureus* apart from other species [14].

Previous researches proposed that antibiotics can be administrated topically or taken orally to treat AOE. The antibiotics that regularly used to treat otitis media are macrolides, amoxicillin and cephalosporin. Amoxicillin is the first-line medication applied in majority of patients unless if they have allergic reaction after consume it [4]. The combination usage of it with clavulanic acid is recommended for people with recurrent otitis media diagnoses and those who have taken amoxicillin in the past several months. At the same time, the patient receives cephalosporin as another alternative when they exert allergic reaction to penicillin. The following option is macrolides which is used when the patient is intolerant to cephalosporin and penicillin [4].

Even though amoxicillin is usually observed as a successful first-line treatment, it may possess certain side effect, including nausea, diarrhoea and vomiting [15]. Arumugham [16] stated that after the ingestion of cephalosporin, individuals may experience nephrotoxicity, diarrhoea or hypersensitive reaction. While the application of commercially available antibiotic eardrops by a minority are often seen with side effects that regarding on their gastrointestinal issues. Additionally, predicated on research published in 2019, approximately 81.9% of the bacterial strains are the main contribution to the AOM that exhibit amoxicillin resistance [17] while 84.5% of the organisms show resistance to cephalosporin [18].

Hence, we propose that cow urine may possess antibacterial activities that could serve as an alternative remedy for otitis media without resorting to the currently prescribed pharmaceutical. In this study, cow urine will be tested using the disc diffusion method to validate the statement and determine the presence and efficacy of the antibacterial properties in cow urine.

## 2. Methodology

Cow urine was kept under 4°C refrigerator all the time before use and covered entirely by a thick plastic bag to avoid sunlight exposure to avoid potential compound degradation in the urine. All studies were done in triplicate to ensure the integrity of the data for statistical analysis. Figure 1 showed the flowchart of the experiments.

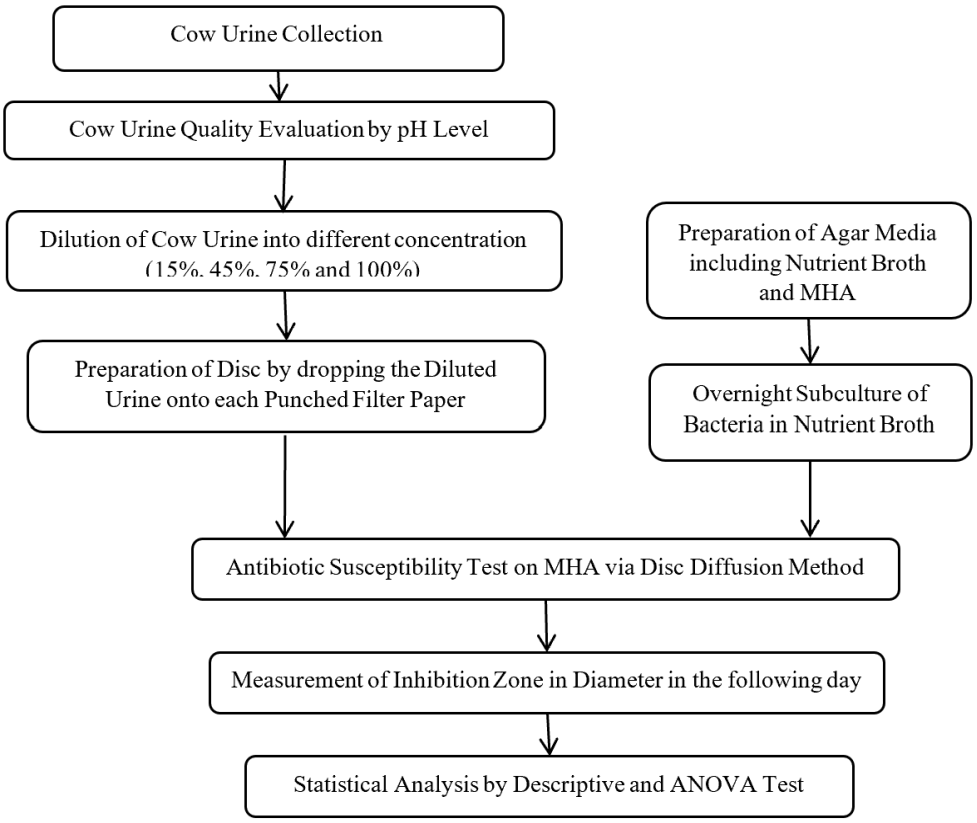


**Fig. 1.** Project experimental design

### *2.1 Collection, Preparation and Storage Condition for Cow Urine Samples*

The cow urine utilized in this study was freshly obtained from Farm Fresh at University Putra Malaysia, Serdang after obtaining the approval and permission of the farm manager, who provided oversight and ensured their staff adhered to a proper handling protocol throughout the sample collection. While this study did not apply any formal ethical approval since there is no invasive sampling procedure conducted to the cow, but all procedures complied with institutional and local guidelines to ensure ethical and responsible practices.

In the meantime, the fresh sample of cow urine was assembled by professional staff there into the pre-autoclaved Scott bottle from a female Holstein cow at the early morning when the cow stays awake and gives its first micturition in a new day [19]. The cow urine-filled bottle would be sealed appropriately by parafilm to ensure its integrity and being kept it in a cold condition throughout the entire delivery process from the farm to laboratory. Immediately, in order to maintain its freshness and antibacterial activity, the urine was stored under 4$^{o}$C fridge for its durability for future use [20].

Before starting the experiment, the biosafety cabinet was cleaned with alcohol wipe and UV light to eliminate any possible contamination. The cow urine underwent a simple filtration process using Whatman filter paper in order to ensure it is free of any particulates [19]. The pH level of cow urine was measured to ensure its quality and usability, as it is expected to fall within the optimal range of pH 6.0–7.0 [21]. Table 1 presents the pH measurements of both cow urine and distilled water, recorded five times. Distilled water was used as a baseline to verify the proper functioning of the pH meter, as its pH should be approximately 7.0 [22]. If the pH of distilled water deviated significantly from this value, recalibration of the pH meter was performed to ensure accurate measurements.

**Table 1**
Measurement of pH level for distilled water and cow urine for 5 times for the accuracy before starting the experiment

| | Distilled water | Cow Urine |
|---|---|---|
| pH level 1 | 7.3 | 6.6 |
| pH level 2 | 7.7 | 6.8 |
| pH level 3 | 7.1 | 6.7 |
| pH level 4 | 7.3 | 6.9 |
| pH level 5 | 7.4 | 6.7 |
| Average | 7.4 | 6.7 |

Subsequent to pH testing, the cow urine samples were equally divided into two glass beakers for the sake of undergoing two distinct processing method, which were heated until 100 °C and exposed to direct sunlight for reducing the number of microbiota and triggering the photo-activated compounds in the cow urine.

### *2.2 Cow Urine Dilution*

After ensuring the good quality of cow urine by measuring their pH level, the cow urine was diluted into few different concentrations using sterile distilled water. The formula of forming cow urine in various concentration was demonstrated in the Table 2 below.

**Table 2**
The formulation of different concentration of cow urine

| Concentration of Cow Urine Diluted (%) | Volume of Cow Urine (mL) | Volume of Sterile Distilled Water (mL) |
|---|---|---|
| 15 | 0.75 | 4.25 |
| 45 | 2.25 | 2.75 |
| 75 | 3.75 | 1.25 |
| 100 | 5 | 0 |

### *2.3 Bacterial Strains Involved and Storage*

Both bacterial strains, Staphylococcus aureus and Pseudomonas aeruginosa were obtained from the Applied Microbiology, Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, University Putra Malaysia (UPM) with each identification of ATCC 25923 and ATCC 27853 respectively. The plates were sealed properly using parafilm and kept in a sterilized zipped plastic bag to be stored in a 4$^{o}$C fridge.

### *2.4 Procedures to Prepare and Subculture the Test Organism*

An inoculation loop was heated to decontaminate it before use to collect a colony from the plate. The loop was allowed to cool down for a few seconds to avoid killing the bacteria then only the collection of colonies was performed.

It was carried out gently to prevent scraping on the surface on the agar media which may assemble those undesired materials during subculturing. The colony was then transferred into the nutrient broth carefully, swirling mildly for the bacteria to spread evenly in the broth. Then the falcon tube was recap instantly after inoculation to avoid possible contamination and sealed appropriately using parafilm. The tube was left overnight for incubation at 37$^{o}$C which is a suitable temperature for most of the microbes to propagate.

The following day, both tubes were observed. *S. aureus* and *P. aeruginosa* showed turbidity as displayed in Figure 2 and 3 indicating the growth of the bacteria. The culture was only applied for maximum 7 days to ensure the viability of the bacteria.

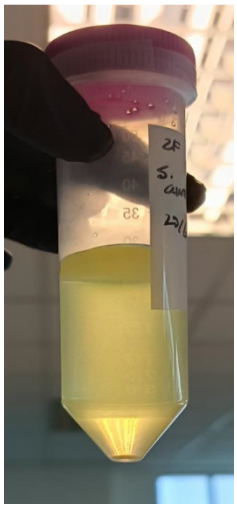

**Fig. 2.** Subculture of *S. aureus* in nutrient broth

**Fig. 3.** Subculture of *P. aeruginosa* in nutrient broth

### *2.5 Agar Media Selection and Preparation*

Nutrient broth is often used as a medium to isolate microbes in microbiology lab as it is developed from a series of vital nutrients which provide a preferable environment for microbes to grow and propagate [23]. The ingredients involved in the production of nutrient broth include peptone as the major source of nutrients for bacterial growth [24] while yeast extract as the second source that supplies various nutrients including nitrogen compounds, vitamin B and carbohydrates [25]. Besides, it also consists of sodium chloride to regulate the osmotic balance thus form a habitat that suitable for bacteria growth as in natural condition [26]. The addition of distilled water was utilized to create a homogeneous liquid medium, ensuring uniform distribution and consistency during the experiments [27]. Moreover, Song *et al*. [28] suggested the advantages of nutrient broth in growing *S. aureus,* while the growth of *P. aeruginosa* culture in nutrient broth was also reported by Al-Saffar and Jarallah [29].

Nutrient broth was utilized to subculture the bacteria while MHA is beneficial for the antibiotic susceptibility test. The media was prepared according to the manufacturer's instructions. Mueller Hinton agar (MHA) is the most recommended media for antibiotic susceptibility test due to its non-selective characteristic and permit the evenly diffusion of antibiotic from disc into agar thus allow the inhibition zone to be observed accurately [30].

### *2.6 Storage Condition of Prepared Agar Media*

Based on the manufacturer protocol, the prepared nutrient broth able to be stored for a maximum of half year duration under 4$^{o}$C with the condition of prevent the possibility of any contamination and light exposure. On the other hand, the MHA agar can be preserved for approximately 3 months under low temperature with properly sealed using parafilm.

### *2.7 Preparation of Positive and Negative Control*

In this study, doxycycline (Pharmaniaga™) as demonstrated in Figure 4 was utilized as positive control while sterile distilled water for irrigation (RinsCap®W) acted as the negative control due to availability. Meanwhile, doxycycline was categorised as semi-synthetic tetracycline antibiotic [31], which able to against both Gram-positive and Gram-negative bacteria [32] and is potent to break off the synthesis of bacterial protein by interacting with the bacterial ribosome and creates allosteric blockage at the acceptor site [33]. Consequently, the zone of inhibition formed by this antibiotic is accepted and trustworthy. For the sake of preparing this antibiotic solution, 100mg of it in a capsule form would be mixed thoroughly with 1mL of sterile distilled water by using micropipette. However, the sterile distilled water for negative control was prepared into a decontaminated test tube.


**Fig. 4.** Doxycycline used in this research

### *2.8 Anti-microbial Sensitivity Test with Disc Diffusion Method*

The procedure was carried out primarily by preparing multiple 6 mm diameter filter paper disc with the help of a sterilized 1-hole puncher which then covered using an aluminium foil as a support under high temperature autoclave process. Within the biosafety cabinet, a sterile cotton swab was immersed into the sub-cultured test organism then carefully swabbed and spread evenly over the MHA plate. One drop of each solution including the positive control, negative control and each different concentration of cow urine was collected for the filter paper disc to be soaked within them. Soon after the dispersal of the solution into the filter paper, each filter paper was placed onto the MHA plate that has been spread with test organism accordingly to their labelled components. The plates were sealed appropriately with parafilm then kept for overnight incubation at 37$^{o}$C. The inhibition zone was observed in the following day using a ruler while the results were recorded as in the below section.

### *2.9 Statistical Analysis*

All date were analysed with GraphPad Prism version 8.0.2 software using ANOVA and $p < 0.05$ was considered statistically significant.

## 3. Results

### *3.1 The Effectiveness of Diluted Cow Urine against Staphylococcus Aureus using Disc Diffusion Method*

Based on the result demonstrated in Table 3 below, with the purpose to evaluate the antibacterial activity of the heated and sunbathed cow urine against *Staphylococcus aureus*, it was found that the average diameter of inhibition zone measured for the positive control, doxycycline is 36 mm and 6 mm for the negative control, sterile water. A duplicate experiment was conducted following the same procedures, as shown in Figures 5 and 6, to confirm the antibacterial activity of cow urine against Staphylococcus aureus. The largest inhibition zone, measuring 16 mm in diameter, was observed with 75% cow urine, while the smallest inhibition zone of 14 mm was seen with 45% cow urine. In comparison, the negative control exhibited a 6 mm inhibition zone. These results indicate that cow urine has a significant antibacterial effect, as it inhibited bacterial growth on the agar plates. The calculated *p*-value of < 0.05 further supports the significance of the results, suggesting that the observed differences are not due to chance and that cow urine effectively inhibits Staphylococcus aureus growth.

**Table 3**
Evaluation results of diluted cow urine's antibacterial activity against *Staphylococcus aureus* using disc diffusion method

| Test Components | Diameter of Inhibition Zone in millimetre (mm) | | | | | |
|---|---|---|---|---|---|---|
| | Doxycycline (Positive Control) | 15% Cow Urine | 45% Cow Urine | 75% Cow Urine | 100% Cow Urine | Sterile Water (Negative Control) |
| Mean | 36.00 | 23.50 | 14.00 | 16.00 | 14.50 | 6.00 |
| Standard Deviation | 1.00 | 0.50 | 1.00 | 6.00 | 0.50 | 0.00 |

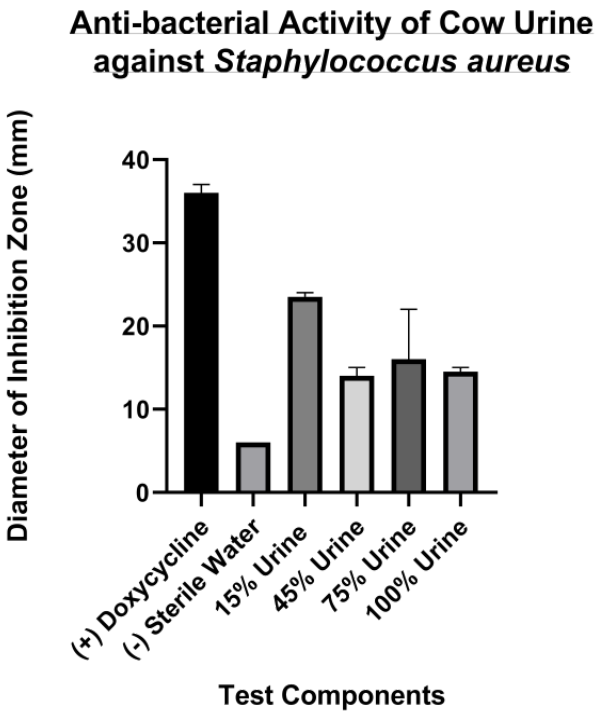


**Fig. 5**. Antibacterial activity of cow urine against *S. aureus* triplicate. 15% cow urine concentration, 45% cow urine concentration, 75% cow urine concentration and 100% of cow urine concentration

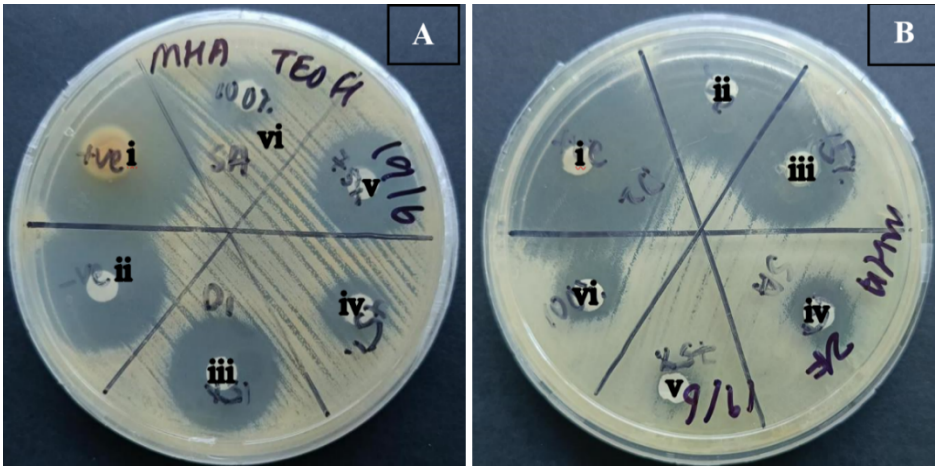


**Fig. 6**. Disc diffusion method using MHA agar on *S. aureus*, where (A) The first testing plate and (B) The second testing plate with inhibition zones. (i) Positive control, (ii) Negative control, (iii) 15% cow urine concentration, (iv) 45% cow urine concentration, (v) 75% cow urine concentration and (vi) 100% of cow urine concentration

### *3.2 The Effectiveness of Dilute Cow Urine against Pseudomonas aeruginosa using Disc Diffusion Method*

Table 4 revealed that the positive control, doxycycline gives an average inhibition area of 35 mm in diameter while the inhibition zone of negative control, sterile water only able to be measured with 6 mm in diameter. Similar to the procedures carried out against *S. aureus* in the previous section, duplicate experiment was also performed with the exactly same procedures for both as shown Figures 7 and 8 provide evidence of the antimicrobial activity of cow urine against Pseudomonas aeruginosa. Among the tested concentrations, only 15% cow urine demonstrated activity, resulting in an inhibition zone measuring 9.5 mm in diameter. Yet, the remaining concentration of the cow

urine (45%, 75%, 100%) were measured with 6 mm in diameter for the inhibition zone, which is identical with the result obtained from negative control, thus indicating that no antibacterial effects towards the bacteria being observed. Meanwhile, the *p*-value that counted from this result was less than 0.05 which is suggesting that the result is appreciable. Additionally, the experiment was suspected to be influenced by human error, as only the lowest concentration of cow urine showed antibacterial activity, while the other concentrations might also exhibit minimal antibacterial activity that went undetected.

**Table 4**
Evaluation results of diluted cow urine's antibacterial activity against *Pseudomonas aeruginosa* using disc diffusion method

| Test Components | Diameter of inhibition zone in millimetre (mm) | | | | | |
|---|---|---|---|---|---|---|
| | Doxycycline (Positive Control) | 15% Cow Urine | 45% Cow Urine | 75% Cow Urine | 100% Cow Urine | Sterile Water (Negative Control) |
| Mean | 35.00 | 9.50 | 6.00 | 6.00 | 6.00 | 6.00 |
| Standard Deviation | 2.00 | 2.50 | 0.00 | 0.00 | 0.00 | 0.00 |

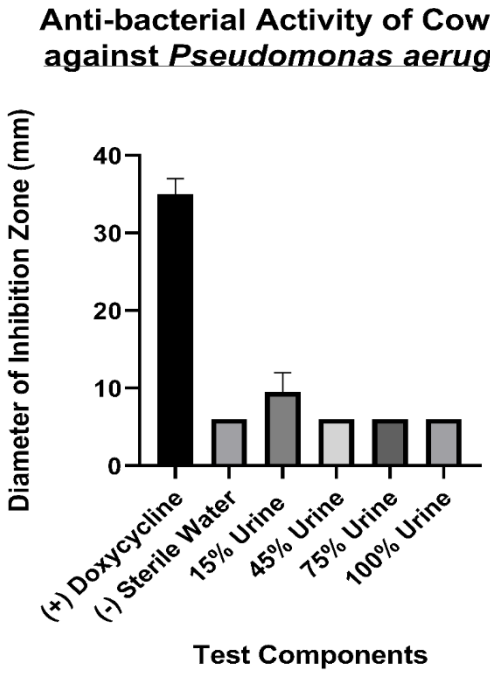


**Fig. 7.** Antibacterial activity of cow urine against *P. aeruginosa* triplicate. 15% cow urine concentration, 45% cow urine concentration, 75% cow urine concentration and 100% of cow urine concentration

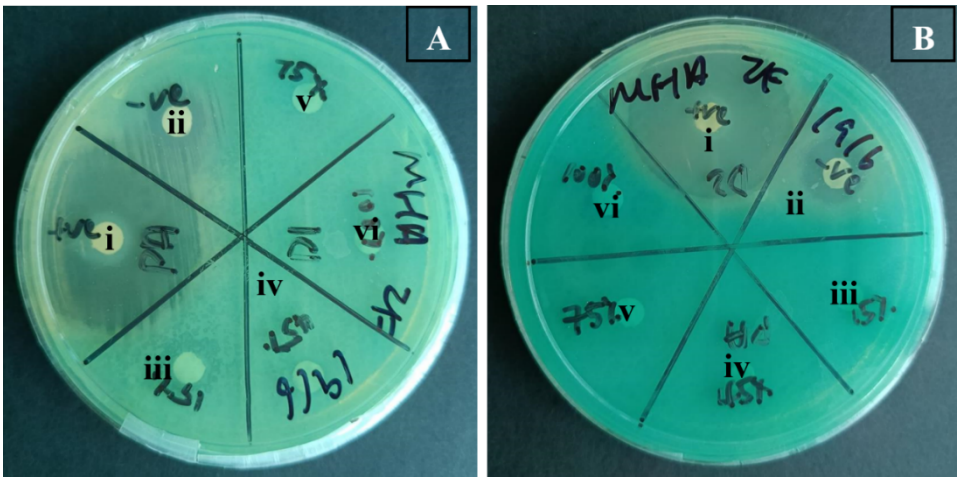


**Fig. 8**. Disc diffusion method using MHA agar on *P. aeruginosa*, where (A) The first testing plate and (B) The second testing plate with inhibition zones. (i) Positive control, (ii) Negative control, (iii) 15% cow urine concentration, (iv) 45% cow urine concentration, (v) 75% cow urine concentration and (vi) 100% of cow urine concentration

## 4. Discussion

The effectiveness of the antimicrobial activity which can be found in cow urine against ear infection will be explored in a more comprehensive way. The major discovery throughout this research is that the cow urine has been successfully observed with effective antimicrobial effect which can be exerted against *S. aureus* that led to otitis media. Consequently, this could inspire the development of cutting-edge therapeutic solutions utilizing cow urine, emphasizing reduced adverse effects and improved infection management strategies.

Using the disc diffusion method, the antibacterial effect of cow urine against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, common causes of otitis media, was evaluated. The results, shown in Figures 5–8, reveal a significant inhibitory effect, supported by statistical analysis. As reviewed above, the cow urine in each different concentration were known to have inhibition zones as response to *Staphylococcus aureus* however only the cow urine in 15% concentration demonstrated a small inhibition zone against *Pseudomonas aeruginosa* while no zone was able to be noticed in the cow urine with other concentration. It is strong evidence manifesting that *Staphylococcus aureus* is susceptible to the antibacterial activity which can be found in the cow urine while the *Pseudomonas aeruginosa* is resistant to it. In the meantime, the current experimental finding was aligned with previous relevant study which proves that a greater susceptibility to cow urine was observed among gram-positive bacteria such as *Staphylococcus aureus* compared to gram-negative bacteria such as *Pseudomonas aeruginosa* [20,34,35]. As the outer membrane in Gram-negative bacteria serves as a protective barrier, limiting the penetration of many environmental substances, whereas Gram-positive bacteria lack this outer membrane, allowing antibacterial agents to more easily pass through the peptidoglycan layer and enter the intracellular space [20].

Besides, this finding can be supported by some previous researches which had proposed that the antimicrobial effect in cow urine can participate in as an alternative to cure otitis media. One of the studies in 2019 stated that the *S. aureus* results in a quite large measurement of inhibition zone in both fresh and other type of cow urine such as sunbathed and heated [36]. According to the previous finding by Ghosh and Biswas [20], the fresh cow urine specimen have created a greater antibacterial activity compared with those cow urine which undergone sunbath or sterilisation, especially if the

urine posses a pH of 3.8 [37]. Since an acidic pH environment is possible to give a strong impact to the general development and activity of the microorganisms [38].

Meanwhile, Parkavi *et al.,* [39] mentioned that bioactive compounds and antimicrobial activity were detected in cow urine. The antioxidant and anti-inflammatory capability of cow urine also studied in another research which further proved the importance of cow urine utilization in the treatment of ear infection [40].

Siva [41] stated that there is discovery of various elements like creatinine, urea, phenols, calcium, manganese and carbolic acid in cow urine are the main point that introduce the cow urine in medicinal field. Amino acids and urinary peptides are both major components that elevate the hydrophobicity on the bacterial cell surface therefore promotes their antibacterial effects against the bacteria [42]. The hydrophobicity property on the bacterial cell surface able to enhance the antibacterial effect by promoting interaction between the antibacterial agent and bacteria, destroying the membrane integrity and hinder the development of biofilm [43]. Meantime, the phagocytic activity of macrophage can be improved with the involvement of cow urine [44]. Moreover, it also had been proved that the cow urine possessed greater antimicrobial effects as compared to the currently available antibiotics such as cefpadoxime and ofloxacin against the bacterial that result in otitis media [45].

Furthermore, a previous study had verified that cow urine were able to hinder the transfer of R-factor (a plasmid that carries genes responsible for antibiotic resistance in bacteria) thus inhibit the evolution of antimicrobial resistance in the bacteria [46] which able to overcome the resistance against most of the currently available antibiotics. There are still limited evidence suggesting that cow urine has the antibacterial effects against *Pseudomonas aeruginosa*. An earlier study in year 2013 proposed that the cow urine that consists of higher level of protein will have better antibacterial effect against *Pseudomonas aeruginosa* [47]. Further studies are necessary to validate and reinforce the findings regarding the antibacterial activity of cow urine against *Pseudomonas aeruginosa*. These investigations should include more extensive experiments using various methods, such as broth microdilution for determining minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Additionally, testing against a larger number of clinical *Pseudomonas aeruginosa* isolates from diverse sources would enhance the reliability and generalizability of the results. Future research could also explore the specific bioactive components in cow urine responsible for its antibacterial properties and examine its efficacy in combination with other antimicrobial agents. This comprehensive approach will help confirm its potential as an alternative treatment against *Pseudomonas aeruginosa* infections and not limited *to Staphylococcus aureus* only.

## 5. Conclusion

In short, the study has successfully to illustrate the potential antimicrobial activity in cow urine against the bacterial that commonly causing ear infections especially *Staphylococcus aureus*. Unfortunately, the ability of cow urine to fight against *Pseudomonas aeruginosa* was not able to be detected in this approach. Meanwhile, comparative analysis was performed with the currently available antibiotic, doxycycline reveals the measurable antimicrobial effects that exerted from the cow urine. This outcome may contribute as strong supporting evidence to utilize the conventional therapies in advanced medication especially in the context of rising antibiotic resistance issue. There are few restrictions were found in this study which include the variability of the sample. The antibacterial activity in the cow urine can differ due to their diet, health and breed [48]. Different

cow maybe fed with different diets which may subsequently affect the amount of minerals, nutrients and bioactive compounds that can be detected in the cow urine therefore affecting the effectiveness of their antibacterial activities in the urine [49]. This study only emphasizes on the particular bacteria which are *Staphylococcus aureus* and *Pseudomonas aeruginosa,* thus not able to represent as evidence for cow urine to be used in the ear infection that caused by other bacteria. Additionally, the mode of action of antibacterial activity in cow urine was not mentioned in this study therefore may be challenging in the process to convert cow urine into antibiotics against otitis media. In addition, there was a lack of determination regarding the purity of the distilled water, which might lead to a false positive reaction especially when applying 15% cow urine into the antimicrobial susceptibility testing. Therefore, it is advisable to culture the distilled water on the agar media and check if there is any microbial colonies developed on the plate after overnight incubation in order to minimise the risk of contamination. To further validate the findings, advanced techniques such as flow cytometry, MIC, and MBC should be employed. MIC determines the lowest concentration of cow urine needed to inhibit bacterial growth, while MBC identifies the minimal concentration required to eradicate bacteria [50]. Flow cytometry can analyze bacterial characteristics and determine the proportion of live and dead bacteria after exposure to cow urine. Proteomics analysis should be conducted to examine antibacterial proteins in cow urine, including antimicrobial peptides, lysozyme, urease, and immunoglobulins [20]. Bioactive compounds such as phenols, aromatic acids, and vitamins should be analyzed using high-performance liquid chromatography [53]. Additionally, comparative studies should assess how cow conditions, such as diet and environment, affect the antibacterial properties of cow urine [54], while evaluating the stability of its antimicrobial activity under different conditions [55]. Mechanistic studies using molecular biology techniques will help elucidate the mode of action of cow urine [56]. Lastly, research should determine the optimal formulation and dosage for safe and effective therapeutic use.

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