



An assessment of various disinfectants using the Kirby-Bauer Method with disc diffusion to determine their effectiveness against locally isolated pathogens

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Abstract

This study aimed to assess the effectiveness of various disinfectants against locally isolated pathogens using the Kirby-Bauer Method with disc diffusion. Indigenous strains of *Salmonella typhimurium*, *E. coli*, *Campylobacter*, *Citrobacter freundii*, and *Staphylococcus aureus* were obtained from the bacterial depository bank and sub-cultured on their respective selective media. A 0.5 McFarland Turbidity Standard was prepared, and eight commercially available disinfectants were tested for efficacy. The results of this study will inform the selection of appropriate disinfectants for use in preventing the spread of disease in various settings, including home sanitation, healthcare, and industrial manufacturing processes. The disinfectants used in this study produced different results against the targeted pathogens. Hydrogen peroxide and Formalin produced larger zones of inhibition, while Povidone Iodine and Hydrochloric acid produced intermediate zones. Ethanol, Methanol, and Dettol produced smaller zones of inhibition. Benzalkonium Chloride was effective only against *S. aureus*, while all other indigenous isolates resisted it.

Keywords: Disinfectants, *Sal. typhimurium*, *E.coli*, *Campylobacter*, *Citrobacter freundii*, *Staphylococcus auerus*, Antibiotic sensitivity test

INTRODUCTION:

Disinfectants are chemicals that kill microorganisms on surfaces and prevent their growth in specific areas. These biocides eliminate or suppress bacterial contamination in various settings, including home sanitation, healthcare, and industrial manufacturing processes. Disinfectants can be bacteriostatic or bactericidal but are primarily known for their bactericidal effect^{1,2}. Disinfectants are crucial for biosafety and biosecurity and are used to prevent the spread of disease in livestock and poultry houses³. Many natural and synthetic disinfectants are available, but chlorinated compounds are considered exceptionally safe and effective in livestock and poultry production^{4,5}. The characteristics of each disinfectant, such as its concentration, application time, pH of the surface, and environmental temperature, determine its strength against targeted pathogens⁶.

Disinfectants have different chemical properties that can affect their effectiveness when applied in particular cases⁷. Therefore, choosing the proper disinfectant is crucial for the disinfection process. Factors to consider when selecting a disinfectant include user satisfaction, compatibility with the equipment, and compliance with Control of Substances Hazardous to Health

(COSHH) regulations⁷. If the disinfectant concentration is unsuitable or the contact time is insufficient, the disinfection process may be ineffective and provide favorable conditions for pathogens to grow⁸. The efficacy of disinfectants can vary depending on the interaction with different types of microbes. Thus, it is essential to pay attention to this interaction to ensure the success of the disinfection process⁹.

Disinfectants are classified into different categories: alcohols, aldehydes, quaternary ammonium, halogens, chlorhexidine, and oxidizing agents^{10,11}. Alcohols, phenols, and quaternary ammonium compounds are the most commonly used disinfectants¹². Their active ingredients and mechanism of action vary, and they perform their functions in two stages: primary and secondary. The efficacy of disinfectants depends on their shelf life, which can be affected by temperature, sunlight exposure, and the presence or absence of organic matter.

Microorganisms, especially bacteria, are becoming increasingly resistant to antimicrobial agents due to plasmids in their bodies. These organisms are highly diverse genetically, which helps them survive even in unfavorable environments. However, excessive use, under or over-dosage, and

inappropriate selection of antimicrobial agents can propel them towards antimicrobial resistance. In light of this, a study has been conducted to evaluate the antimicrobial potential of selected disinfectants against antibiotic-resistant bacteria to prevent further spread of these organisms in the environment. Disinfection is a potent way to control the spread of infection, but this control is at risk due to increasing microbial resistance. Therefore, it is essential to evaluate the efficacy of antimicrobial agents to prevent the spread of such infections.

MATERIALS AND METHODS:

Source of Bacterial Cultures:

Indigenous strains of bacterial cultures, including *Sal. typhimurium*, *E. coli*, *Campylobacter*, *Citrobacter freundii* and *Staphylococcus aureus*, were obtained from the bacterial depository bank of the research and development division of Ottoman Pharma, located 10 km away from Raiwind Road Lahore. Each bacteria was sub-cultured and reactivated on their respective selective media to produce fresh growth for 18 to 20 hours, followed by Gram staining and microscopy. Details of the bacterial cultures used are described in Table 1.

Table 1: Indigenous Bacterial Cultures and NCBI Data of Accession Number.

Name of Bacterial Culture	NCBI Data (Accession Numbers)
<i>Salmonella typhimurium</i>	PP511204
<i>E. coli</i>	PP327376
<i>Campylobacter</i>	PP465710
<i>Citrobacter freundii</i>	PP218315
<i>Staphylococcus aureus</i>	OR232960

Table 2: Details of disinfectants commercially used and their recommended concentrations by the manufacturer

Sr. No.	Disinfectant Group	Concentration used	Mechanism of Action
1.	Ethyl Alcohol	70%	Disturb membrane permeability
2.	Methyl Alcohol	70%	Disturb membrane permeability
3.	Chloroxylenol	5%	Disruption of cell membrane
4.	Benzalkonium Chloride	25%	Deformation of negatively charged bacterial membrane
5.	Hydrogen Peroxide	3%	Ribosomes, Action on enzymes with -SH groups, Thiol groups
6.	Hydrochloric Acid	12%	Disruption of cell wall formation
7.	Povidone Iodine	10%	Disrupt the metabolic pathways
8.	Formalin	0.5%	Disrupt the cell wall

Standard disinfectant concentrations were prepared with distilled water according to manufacturer recommendations.

Kirby-Bauer Disc Diffusion Method:

The Kirby Bauer disc diffusion method was used to test the antimicrobial susceptibility of antibiotics and disinfectants. This involved preparing Muller Hinton Agar plates from Condalab, Canada. Fresh bacterial cultures of different indigenous pathogens and a 0.5 McFarland standard were also prepared. Filter paper discs were sterilized using a Hot Air Oven from Binder B 20, Germany. This method helped to determine the most effective disinfectant that showed its

Preparation of 0.5 McFarland Turbidity Standard

The McFarland (MF) turbidity standard is a commonly used reference for determining the approximate amount of bacteria in a suspension. This standard is often used to test the susceptibility of bacteria to antimicrobial agents. A specific amount of barium chloride is added to sulfuric acid to prepare the standard to obtain a slightly turbid barium sulfate precipitate. To get a 0.5 MF standard, 0.05 ml of 1% Barium Chloride is added to 9.95 ml of 1% Sulfuric Acid to make a final volume of 10 ml. The suspension's optical density (OD) is then measured at a wavelength of 625 nm using a visible spectrophotometer (721-Vis USA).

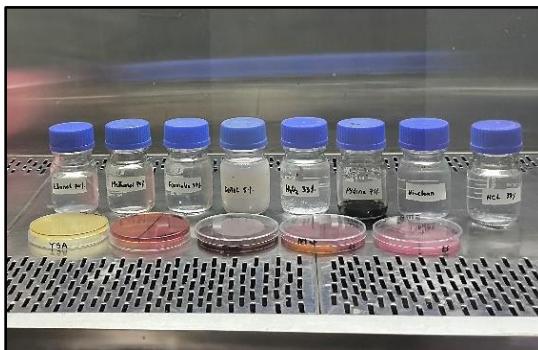


Figure 1: Preparation of Recommended concentrations of the Disinfectants

Preparation of Disinfectants:

The study obtained eight commercially available disinfectants. These disinfectants contain different components, such as Ethyl Alcohol, Methyl Alcohol, Chloroxylenol, Benzalkonium Chloride, Hydrogen Peroxide, Hydrochloric Acid, Povidone Iodine, and Formalin. The manufacturer's recommended concentration and mechanism of action are described in Table 2 and can be seen in Fig. 2.

efficacy against all the mutated pathogens or at least against the most prevalent pathogens, which may mutate over time.

Preparation of Muller Hinton Agar Plates:

Muller Hinton Agar (Condalab Canada) plates were prepared by preparing a Mixture of 11.4g of powder and 300 ml of distilled water, which was then mixed well and brought to boil by a magnetic stirrer hot plate (Model 78-1 China). This was followed by autoclaving (Model 50-ATC-60) for 15 minutes at 121°C and 15 Pascal pressure and then cooled at room temperature. 20 ml of biochemical media was poured into each of the 15 petri plates.

Preparation of Bacterial Lawn:

To determine the turbidity of a freshly prepared culture of *Sal. typhimurium*, one to two bacterial colonies were mixed with 3ml of sterile average saline tube and compared with a 0.5 MF turbidity standard. This process was repeated until the 0.5 MF standard and the test organism's suspension turbidity were matched entirely. The turbidity was subsequently checked at 625nm wavelength of light with a Visible Spectrophotometer (721-Vis, USA). Turbid suspensions of *E. coli*, *Campylobacter*, *Citrobacter freundii*, and *Staph. aureus* were prepared similarly.

A sterile cotton swab was used to collect the inoculum of *Sal. typhimurium*, which was then swabbed onto a Muller Hinton Agar Plate to prepare the bacterial lawn. Pre-sterilized filter paper discs were soaked for 20-30 seconds to their respective disinfectant concentrations and applied on the bacterial lawn surface at an equal distance to other discs within 10 minutes. The same procedure was repeated for *E. coli*, *Campylobacter*, *Citrobacter freundii*, and *Staph. aureus*. All the plates were incubated at 37°C for 24 hours.



Figure 2: Application of disinfectant discs

RESULTS:

Optical Density of 0.5 MF

The optical density of the prepared 0.5 MF turbidity standard at 625nm was 0.09.

Disc Diffusion Test:

Using a digital vernier caliper, the study measured the Zones of Inhibition (ZOI) produced by different disinfectants against different pathogens. The results are as follows:

- Hydrogen Peroxide produced a ZOI of 31.82mm against *Sal. typhimurium*, 33.6mm against *E. coli*, 27.64mm against *Campylobacter*, 24.01mm against *Citrobacter freundii*, and 30.5mm against *Staph. aureus*.
- Ethanol produced a ZOI of 10.53mm against *Sal. typhimurium*, 7.2mm against *E. coli*, 8.4mm against *Campylobacter*, 7.3mm against *Citrobacter freundii*, and 7.5mm against *Staph. aureus*.
- Methanol produced a ZOI of 8.5mm against *Sal. typhimurium*, 9.2mm against *E. coli*, 8.6mm against *Campylobacter*, 7.9mm against *Citrobacter freundii*, and 8.3mm against *Staph. aureus*.
- Formalin produced a ZOI of 29.32mm against *Sal. typhimurium*, 20.06mm against *E. coli*, 19.1mm against *Campylobacter*, 18.72mm against *Citrobacter freundii*, and 19.49mm against *Staph. aureus*.
- Chloroxylenol produced a ZOI of 7.2mm against *Sal. typhimurium*, 8.3mm against *E. coli*, 7.2mm against *Campylobacter*, 7.6mm against *Citrobacter freundii*, and 8.3mm against *Staph. aureus*.
- Povidone Iodine produced a ZOI of 11.64mm against *Sal. typhimurium*, 11.88mm against *E. coli*, 10.42mm against *Campylobacter*, 10.38mm against *Citrobacter freundii*, and 13.49mm against *Staph. aureus*.
- Hydrochloric Acid produced a ZOI of 12.97mm against *Sal. typhimurium*, 13.98mm against *E. coli*, 13.40mm against *Campylobacter*, 11.72mm against *Citrobacter freundii*, and 16.23mm against *Staph. aureus*.

- Benzalkonium Chloride produced a ZOI of 8.50mm against *Sal. typhimurium*, 7.60mm against *E. coli*, 8.30mm against *Campylobacter*, 7.20mm against *Citrobacter freundii*, and 11.73mm against *Staph. aureus*. All measurements were plotted in the corresponding figures.

Cumulative ZOI against all pathogen of Hydrogen Peroxide, Ethanol, Methanol, Formalin, Chloroxylenol, Povidone Iodine, Hydrochloric Acid and Benzalkonium Chloride were 29.51 ± 3.77 , 8.19 ± 1.39 , 8.50 ± 0.47 , 21.34 ± 4.49 , 7.72 ± 0.55 , 11.56 ± 1.28 , 16.66 ± 1.66 and 8.67 ± 1.79 respectively as shown in (Fig. 7).



Figure 4: Zone of inhibition of different disinfectants against different pathogens

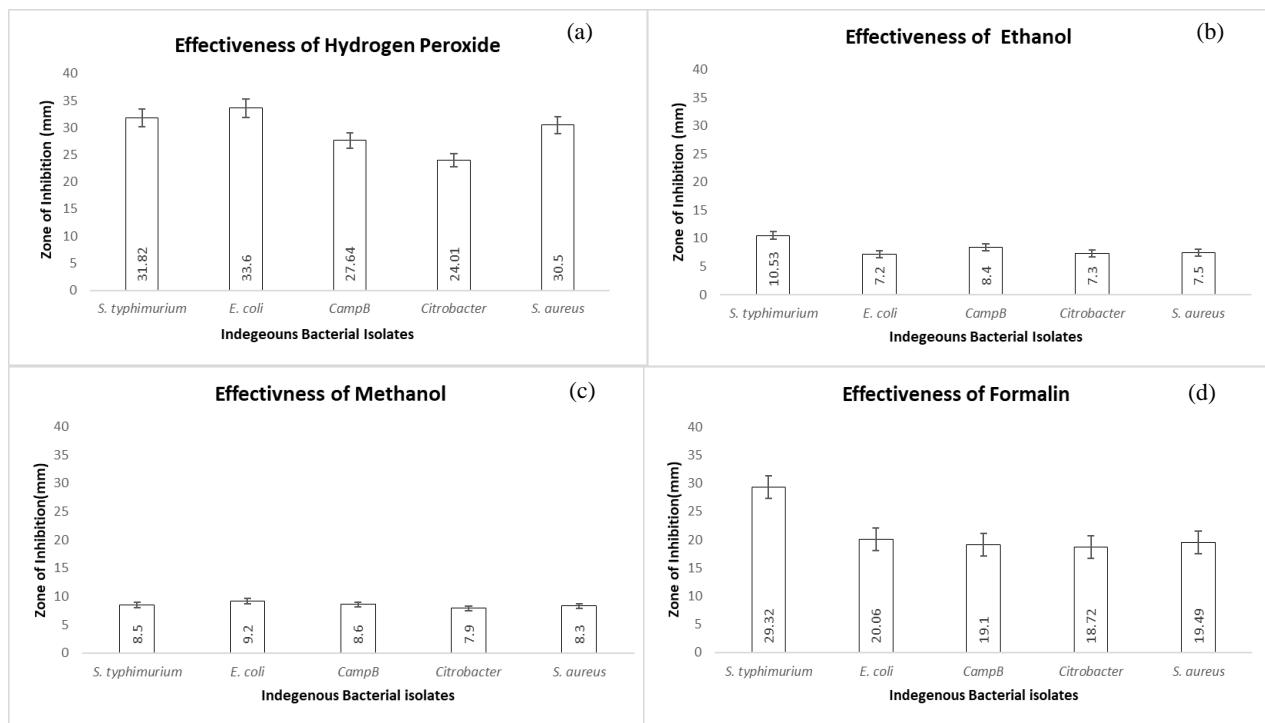


Figure 5: (a) Effectiveness and zone of Inhibition (ZOI) of Hydrogen Peroxide, (b) Ethanol, (c) Methanol (d) Formalin against the five indigenous isolates. As shown in this figure in term of efficacy Hydrogen Peroxide comes first followed by Formalin, Ethanol and Methanol respectively.

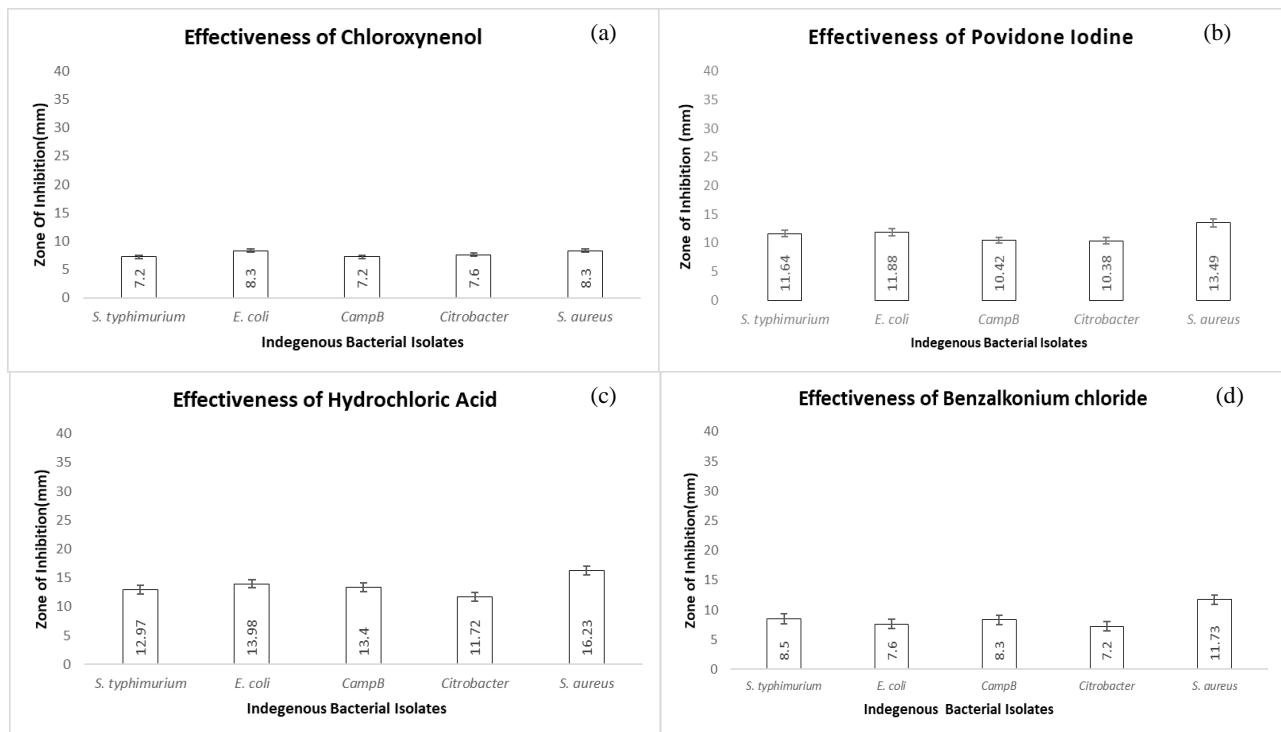


Figure 6: (a) Effectiveness and zone of inhibition (ZOI) of Chloroxylenol, (b) Povidone Iodine, (c) Hydrochloric acid (d) Benzalkonium chloride. AS shown in this figure in term of efficacy Hydrochloric acid comes first followed by povidone Iodine,

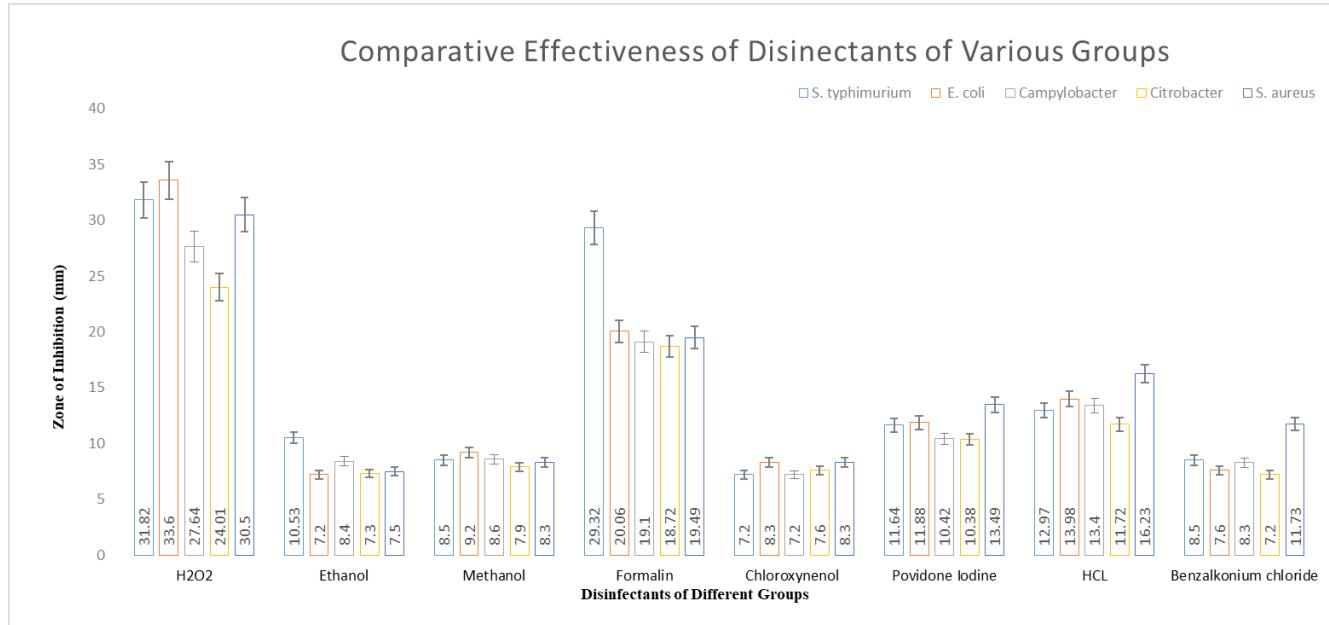


Figure 7: Comparative Effectiveness of Disinfectants of Various Groups

DISCUSSION:

Different disinfectants are available on the market, but they undergo thorough testing to ensure their efficacy before being introduced. If contact surfaces are contaminated with resistant microorganisms, the results can differ from the manufacturer's claims. Hence, evaluating disinfectants' effectiveness at the required concentration is crucial¹⁴. The development of mutation and plasmid existence can lead to extensive resistance to antiseptics and disinfectants¹⁵. Additionally, disinfectant resistance is linked to antibiotic resistance due to cross-sensitivity¹⁶.

Bactericidal agents usually target multiple types of pathogens rather than just one. The reaction between biocides and microorganisms can vary depending on the surface membrane characteristics of the microorganism. Once disinfectants penetrate the cell wall, they can destroy the target organism by causing coagulation, oxidation and denaturation of proteins and enzymes¹⁷. The biological process of forming biofilms is one of the primary reasons for developing resistance to

disinfectants. Many factors contribute to this process, such as the formation of Exopolymeric substances (EPS) that surround the pathogen. These substances bind with biocides and make the biofilm impervious to them. EPS also reduce the efficacy of disinfectants by secreting enzymes that inactivate them. A biofilm of various microorganisms is much more resistant to disinfectants than a monomicrobial biofilm¹⁸.

In this study, formalin demonstrated the highest efficacy against all indigenous isolates. This result was consistent with Abed, 2016 and Amiri, 2011 studies^{19,20} respectively, where formalin also showed proficiency as a bactericidal agent. On the other hand, Chloroxylenol showed less efficacy, which was in agreement with¹⁹. Therefore, due to its greater efficacy, formalin is highly recommended for disinfection. Ethanol showed less activity compared to formalin and chloroxylenol, and abed also demonstrated similar results regarding the efficacy of ethanol¹⁹. Methanol's efficacy was identical, but all of the indigenous isolates had developed resistance against it. Hydrogen peroxide and hydrochloric acid showed the highest efficacy.

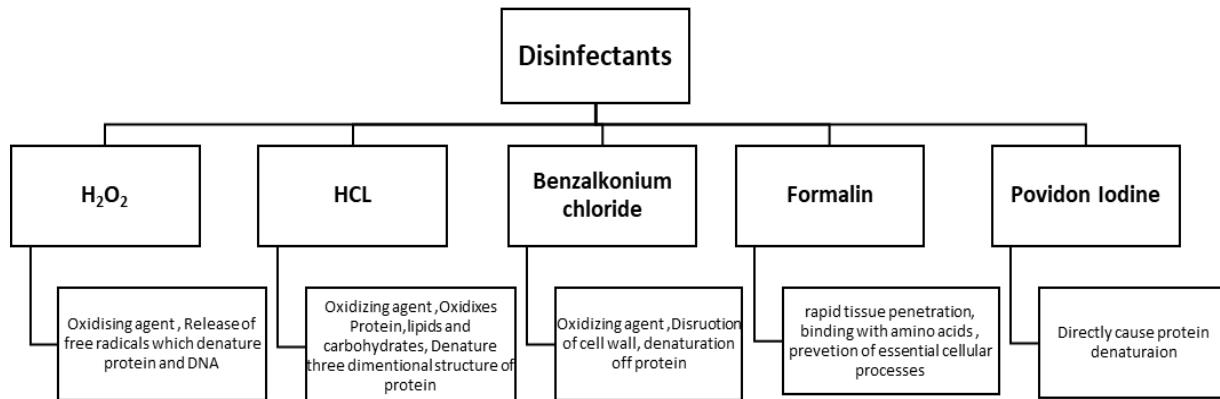


Figure 8: Greater zones of inhibition were obtained due to following characteristics of the disinfectants

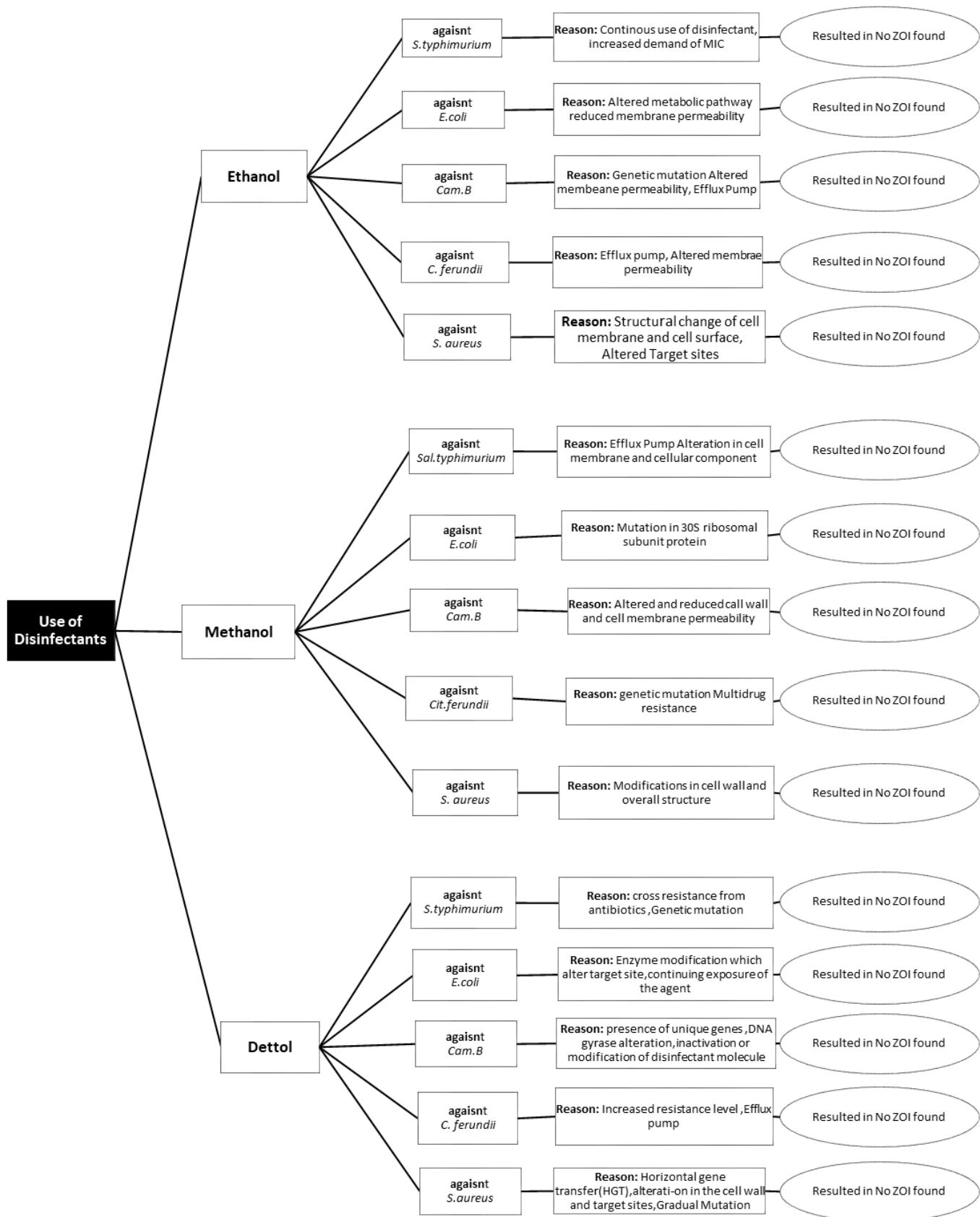


Figure 9: The Disinfectants which made no zone against the indigenous Isolates

CONCLUSION:

The study aimed to evaluate the effectiveness of various disinfectants against locally isolated pathogens using the Kirby-Bauer Method with disc diffusion. The results showed that different disinfectants produced varying degrees of inhibition against the targeted pathogens. Hydrogen peroxide and Formalin produced larger zones of inhibition, while Povidone Iodine and Hydrochloric acid produced intermediate zones. Ethanol, Methanol, and Dettol produced smaller zones of inhibition. Benzalkonium Chloride was effective only against *S. aureus*, while all other indigenous isolates resisted it. The study provides valuable information for selecting appropriate disinfectants for use in various settings, including home sanitation, healthcare, and industrial manufacturing processes, to prevent the spread of disease.

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