HAZARD ANALYSIS, RISK ASSESSMENT OF WESTERN TOILETS AND ANTIBACTERIAL ACTIVITY OF PERGULARIA DAEMIA LEAF EXTRACT AGAINST BACTERIAL ISOLATES FROM TOILETS

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ABSTRACT: Microorganisms are present on all surfaces, to which they were carried by many direct and indirect methods of transmission. Regular cleaning of any surface, even using good disinfectants, will not prohibit organisms from reoccupying these surfaces again and in a very brief period of time. This study aimed at risk assessment, detecting the presence of bacteria on the surfaces of public western toilets and testing of antimicrobial activity of Pergularia daemia leaves extract. Samples collected from doors of 5 public toilets were tested for the presence of bacteria. The results showed that almost all the toilets were contaminated with potentially pathogenic bacteria. The organisms such as Staphylococcus sp, Pseudomonas sp, Bacillus sp, E.coli and Klebsiella sp were isolated and tested for biofilm formation using Congo Red Agar Method and Tube Method. The leaf extract of Pergularia daemia shows good activity against Staphylococcus sp, Pseudomonas sp, Bacillus sp, E.coli and Klebsiella sp. The disinfectant Harpic shows high activity against Staphylococcus sp, Pseudomonas sp, Bacillus sp, E.coli and Klebsiella sp and lyzol shows anti-bacterial activity only on Bacillus sp. The Phenol shows no antibacterial activity against the isolates from Toilet.

KeyWords: Pergulardia daemia, Pseudomonas sp, Klebsiella sp, Staphylococcus sp, E.coli, Bacillus sp, Congo Red agar, Tube method, Biofilm.

1.INTRODUCTION:
Western toilets usage is increasing in both public and private sectors. Chance of getting disease or infection from western toilets is high due to the skin contact to the surface. Many factors have been shown to influence the bacterial transfers between surfaces, including the source and destination surfaces feature, bacterial species involved, moisture levels, pressure and friction between the contact surfaces and inoculums size on surfaces (Chen et al., 2002; Rusin et al., 2002). Studies have also shown that household surfaces can easily be contaminated with bacteria and that viruses can be easily transferred to hands and hands to mouth (Rusin et al., 2002).

Previous studies have shown that door handles may be contaminated with different types of microorganisms including Enterococcus fecalis, Coagulase negative staphylococci, Streptococcus spp., Klebsiella spp., Bacillus spp., Escherichia coli, Proteus mirabilis, Proteus vulgaris, Pseudomonas aeruginosa and Staphylococcus aureus (Nworie et al., 2012; Onwubiko and Chinyeaka, 2015). Various bacteria have been isolated from public surfaces, there by providing information on the relative hygiene of commonly encountered public surface, identifying the environments with contaminants and risk of exposures (Reynolds et al., 2005).

The United State (US) center for disease control (CDC) and prevention indicate that most of the microorganisms which were wide spread problems in contemporary world and is responsible for about one third of death world-wide through infections, with adverse effects which can reduce economic productivity (WHO, 2002). Many enteric pathogens are spread by the fecal-oral route and it has been suggested that the fallout of droplets containing fecal material, is the important factor in transmission of many classic disease and urinary, vaginal, anal infections. In recent years, there has been a remarkable interest in understanding the implications of enterobacteria because of its resistance mechanisms and their ability to be transmitted among other groups. Some of these mechanisms are the production of β- lactamases A class (TEM, SHV, CTX) and D class (OXA), enzyme expression, mutation and high rate transference plasmids which provides to bacteria the possibility to survive the action of several antibiotics. The control of infections in public toilets especially during mass gathering is a matter of great concern and a major challenge.

Risk assessment of public toilets establishes the risk factors and risk areas where the chance of infection is high in toilets. Identifying the risk factors helps in reduce the chance of getting infection. Risk
Hazard assessment indicates the lack of facilities, high risk areas which contains more level of contaminants and used to analyse hazards from the risk factors.

Hazard are things which are harmful to us. Hazards are of three types, chemical, physical and biological hazards. Physical hazards indicate foreign objects and manmade objects. Chemical hazards indicate chemical products like disinfectant, detergent and others. Biological hazards indicate microbial contaminants bacteria, fungi, protozoa and viruses etc. The major hazard acquire in toilets is biofilm. Bacteria generally exist in two types of population: planktonic, freely existing in bulk solution, and sessile, as a unit attached to a surface or within the confines of a biofilm. Biofilms were observed as early as 1674, when Antonie van Leuwenhoek used his primitive but effective microscope to describe aggregates of "animalcules" that he scraped from human tooth surfaces. Since then, many advances in technology and laboratory working practices have allowed more accurate descriptions of biofilms to be made, although even today there is still ambiguity. A biofilm consists of cells immobilised at a substratum and frequently embedded in an organic polymer matrix of microbial origin. Biofilms are a biologically active matrix of cells and extra-cellular substances in association with a solid surface. Microcolonies are discrete matrix enclosed communities of bacterial cells that may include cells of one or many species. Depending on the species involved, the micro-colony may be composed of 10–25% cells and 75–90% of extracellular polymeric substances (EPS) matrix. Bacterial cells within the matrix are characterised by their lack of Brownian motion, and careful structural analysis of many micro-colonies often reveals a mushroom-like shape.

For bacteria, the advantages of biofilm formation are numerous. These advantages includes: protection from antibiotics, disinfectants and dynamic environments. Intercellular communications within a biofilm rapidly stimulate the up and down regulation of gene expression enabling temporal adaptation such as phenotypic variation and the ability to survive in nutrient deficient conditions. About 99% of the world's population of bacteria are found in the form of a biofilm at various stages of growth and the films are as diverse as the bacteria are numerous. Biofilm mutate the bacteria to be resistant to disinfectant and antibiotics. These types of biofilm are hard to remove and they cause several UTI infection and disease.

**Pergularia daemia**

Medicinal plants represent a rich source of antimicrobial agents. Plants are used medicinally in different countries and are a source of many potent and powerful drugs. A wide range of medicinal plant parts is used for extract as raw drugs and they possess varied medicinal properties. The different parts used include leaf, stem, flower, fruit and root exudates and modified The Pergularia daemia (Forsk.) Chiov. belongs to the family Asclepiadaceae. Traditionally, the plant Pergularia daemia is used as anthelmintic, laxative, antipyretic and expectorant, and is also used to treat infantile diarrhoea and malarial intermittent fever; latex of this plant is for toothache. Stem bark of this plant is remedy for cold and fever. Aerial parts of this plant are reported to have various pharmacological activities like hepatoprotective, antifertility, anti-diabetic, analgesic and anti-inflammatory. A number of phytochemical studies have demonstrated the presence of several classes of chemical compounds. It is not our intention in this review to cover all the many compounds reported for P. daemia, but to summarize the major components that have been implicated in the pharmacological activities of the crude drug. Most commonly found phytochemicals from the leaves of P. daemia are flavonoids, alkaloids, terpenoids, tannins, steroids and carbohydrates (Karthishwaran et al., 2010). Although, a large number of compounds have been isolated from various parts of P. daemia, Phytochemical studies have shown the presence of cardenolides, alkaloids, triterpenes (lupeol), saponins, steroidial compounds (Aanjaneely et al., 1998). The seeds of P. daemia contain uzarigenin, coroglaucigenin, calactin, calotropin, other cardenolides and a bitter resin, Pergularin and have a cardiotoxic action (Patel and Rowson, 1964; Rowson, 1965). It has been suggested that the plant seed action on the uterus is similar to that of pituitrin and is not inhibited by progesterone (Dutta and Gosh, 1947; Paris and Moyse, 1971). The promising antibacterial activity was observed in ethyl acetate and ethanol extracts of Pergularia daemia which showed significant antibacterial activity against S. aureus, P. aeruginosa, A. hydrophila, E. coli and S. typhi. (Senthilkumar et al., 2005).

2.AIM

To evaluate the Pergularia daemia leaf extract against bacterial isolates from public western toilets in comparison with the effectiveness of commercially available disinfectants.

**OBJECTIVES:**

- To assess the risk in public western toilets.
- To establish a minimum standard requirements chart.
To analyze the microbiological hazard associated with western toilets.
To Collect the plant leaves
To screen the antibacterial activity of plant extract in comparison with commercial disinfectant.

3. MATERIALS AND METHODOLOGY

Risk assessment
Risk assessment taken by auditing the western toilets for identifying the chances of contamination and infection in Dr. N.G.P Arts and Science College.

Minimum standard required for toilets
- Buckets, Cup
- Cleaning Brush
- Hanger
- Handwash
- Disinfectant
- Toilet usage board
- Tab and Sink
- Clean water
- Exhaust fan
- Broad space
- Foot wears

Sample collection:
The bacteriological survey was conducted randomly in five toilets within the institution campus. The samples were taken from five parts of western toilets as shown in the Picture 1.1. The samples were collected at noon to maximize chances of isolation (Amala et al., 2015). The swabs were moistened with 5ml of normal saline and excesses were removed by pressing the swab stick against the inner wall of the tube (Chesebrough et al., 2000). The properly labelled samples were promptly transported to Microbiology laboratory of the Department of Microbiology, Dr. N.G.P Arts and Science College for analysis.

Sample analysis:
Each collected sample was inoculated onto the nutrient agar plate. (Bashir et al., 2016) All the plates were incubated at 37 ºC for 24 hours. After the overnight incubation, the plates were taken from the incubator and presumptively observed for cultural characteristics. Identification of isolates:
Isolated colonies were then sub-cultured onto fresh nutrient agar, Eosin-methylene blue and MSA agar plates for proper preliminary identification (Chesebrough et al., 2000). Single isolated colonies from these plates were subjected to Gram’s staining and standard biochemical tests (catalase, coagulase, IMViC- indole, methyl red, voges-proskauer and citrate utilization tests).

Gram’s staining:
1. Smear of isolates was prepared and heat fixed in a sterile slide. Add crystal violet stain over the fixed culture. Let stand for 10 to 60 seconds; for thinly prepared slides, it is usually acceptable to pour the stain on and off immediately. Pour off the stain and gently rinse the excess stain with a stream of water from a faucet or a plastic water bottle. Note that the objective of this step is to wash off the stain, not the fixed culture.
2. Add the iodine solution on the smear, enough to cover the fixed culture. Let stand for 10 to 60 seconds. Pour off the iodine solution and rinse the slide with running water. Shake off the excess water from the surface.
3. Add a few drops of decolorizer so the solution trickles down the slide. Rinse it off with water after 5 seconds. The exact time to stop is when the solvent is no longer colored as it flows over the slide. Further delay will cause excess decolorization in the gram-positive cells, and the purpose of staining will be defeated.
4. Counterstain with basic fuchsin solution for 40 to 60 seconds. Wash off the solution with water. Blot with bibulous paper to remove the excess water. Alternatively, the slide may shaken to remove most of the water and air-dried.

Biochemical characterization:
Indole:
Bacterium to be tested was inoculated in peptone water, containing amino acid tryptophan and incubated overnight at 37ºC. After incubation few drops of Kovacs reagent were added. Kovacs reagent
consists of para-dimethyl aminobenzaldehyde, isoamyl alcohol and conc. HCl. Formation of a red or pink colour indicated the positive reaction.

**Methyl red:**

The bacterium to be tested was inoculated into glucose phosphate broth containing glucose and phosphate buffer, and incubated at 37°C for 48 hours. Over the 48 hrs the mixed- acid producing organisms produce sufficient acid to overcome the phosphate buffer and remain acidic. The pH of the medium was tested by addition of 5 drops of MR reagent. Development of red colour was taken as positive. MR negative organisms produced yellow colour.

**Voges proskauer:**

Bacterium to be tested was inoculated into glucose phosphate broth and incubated for 48 hours. 0.6 ml of alpha-naphthol was added to the test broth and shaken. 0.2 ml of 40% KOH was added to the broth with shaking. The tube was allowed to stand for 15 minutes. Appearance of red colour was taken as a positive test. The tubes showing negative result were held for one hour, since maximum colour development occurs within one hour after addition of reagents.

**Citrate utilization test:**

Bacterial colonies were picked up by a nichrome loop and inoculated into slope of Simmon's citrate agar and incubated overnight at 37°C. If the organism has the ability to utilize citrate, the medium changes its color from green to blue (Pommerville 2010).

**Identification of biofilm formation:**

<table>
<thead>
<tr>
<th>Method</th>
<th>Aim</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube method (TM)</td>
<td>Qualitative detection by observing biofilm lined on bottom and walls of tube</td>
</tr>
<tr>
<td>Congo red agar (CRA)</td>
<td>Qualitative detection by observing colony color change</td>
</tr>
</tbody>
</table>

The isolated samples were proceeded for identification of biofilm formation using Tube method and congo red agar method

**Processing of plant material**

The fresh leaves were collected, washed thoroughly and dried in shade for 14 days. The leaves were cut into smaller pieces and powdered in an electrical blender. The leaf powder was stored at room temperature until further use.

**Plant extraction**

The leaf powder of Pergularia daemia was extracted by using continuous hot extraction method. The leaf powder of Pergularia daemia was charged into thimble of Soxhlet apparatus and extracted by using ethyl acetate as a solvent by maintaining a temperature [60-780°C] extraction was continue till a colour less solvent appears from siphon tube. Then the extract was dried and concentrated to yield solid material. The ethyl acetate extract of Pergularia daemia were subjected to antibacterial assay. The antibacterial activity of leaf extract was compared with commercially available disinfectants such as harpic, lyzol and phenol. The extract were immersed in DMSO solvent in appropriate concentration and then used in antibacterial screening.

**Agar well diffusion method:**

Agar well diffusion method was employed for checking the antimicrobial activity of the disinfectant samples and leaf extract. 24 hour cultures of Staphylococcus, Pseudomonas, Bacillus, Citrobacter, Klebsiella and E.coli were used for the sensitivity test. The Mueller Hinton Agar was prepared and autoclaved at 121°C for 15 minutes. The plates were swabbed with respective organisms and marked according to the organism. With sterile well cutter the 5 wells of 5mm were cut and 60μl of each disinfectants and plant extract were added onto to each well. After that the plate were incubated at 37°C for 24 hours. A zone of inhibition is indicative of microbial activity against the organism. Presence of zone of inhibition indicates that the plant extract and disinfectant is effective, the zone was measured and recorded in millimetres using transparent meter rule.

**5.RESULT:**

**Risk assessment:**

The risks were assessed by auditing the western toilet in Dr. N.G.P arts and Science College. The risks in toilets were shown in Figure1.2
Sample analysis:
The collected swab samples were inoculated. Different colonies were isolated. The isolates were characterised by colony morphology in nutrient agar shown in the picture 1.3. For further identification the samples were inoculated in EMB, Blood agar and MSA agar plates. The colony morphology of isolates in EMB and MSA agar were shown in the Figure 1.4

Gram staining:
The samples were primarily identified using gram staining technique to differentiate the gram positive bacteria from gram negative bacteria based on the shape and colour. Gram staining results were shown in the Figure 1.5

Biochemical characterization:
The isolates were subjected to biochemical tests. On the basis of biochemical analysis in table 1, the isolated microorganisms were identified as E.coli, Klebsiella, Bacillus, Staphylococcus, and Pseudomonas.

Biofilm identification:
The experiments in our study enabled to measure the rate of adherence and subsequent biofilm formation of tested bacteria. The test was considered positive when there was an adherent layer of stained material on the inner wall surface of the tube. The adherence was estimated as absent (0), Weak (+), Moderate (++), or strong (+++). The absence of stained material at the liquid air interface was not considered to be indicative of biofilm formation. The OD measured at 570nm by calorimeter. This shown in table 2 and Figure 1.6

Congo red agar plate:
Mucoid nature bacteria colonies was studied by cultivation all the strains on congo red plates. 18 hours old bacterial culture were taken and streaked on the CRA plates. Plates were incubated at 37°C for 24 to 48 and observed the colony morphology. The black mucoid colonies indicate the positive biofilm formation. The Pseudomonas sp, Staphylococcus sp, E.coli, Klebsiella sp, Bacillus sp. was produced biofilm showed in the Figure 1.7.

Antimicrobial activity:
Plant extract, Lyzol, Harpic and phenol were used against the bacterial isolates to compare the effectiveness of plant extract. The plant extract shows good activity against Klebsiella (13mm), Pseudomonas (12mm), E.coli (13mm), Staphylococcus (10mm) and Bacillus (13mm). The harpic possess high activity against Klebsiella (20mm), Pseudomonas (20mm), E.coli (17mm), Staphylococcus (17mm) and Bacillus (16mm). The lyzol shows only activity against Bacillus (17mm). The phenol shows no activity against all of the isolates showed.

Tube method:
Biofilm identification:

<table>
<thead>
<tr>
<th>S.No</th>
<th>MICROORGANISMS</th>
<th>OD VALUES(570nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pseudomonas</td>
<td>0.40</td>
</tr>
<tr>
<td>2</td>
<td>Staphylococcus</td>
<td>0.37</td>
</tr>
<tr>
<td>3</td>
<td>E.coli</td>
<td>0.32</td>
</tr>
<tr>
<td>4</td>
<td>Klebsiella</td>
<td>0.34</td>
</tr>
<tr>
<td>5</td>
<td>Bacillus</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Antimicrobial activity:

<table>
<thead>
<tr>
<th>Sl.no</th>
<th>Isolates</th>
<th>Lyzol mm</th>
<th>Harpic Mm</th>
<th>Phenol mm</th>
<th>P.E Mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>E.coli</td>
<td>-</td>
<td>17</td>
<td>-</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>Klebsiella</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>Staphylococcus</td>
<td>17</td>
<td>-</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Bacillus</td>
<td>17</td>
<td>16</td>
<td>-</td>
<td>13</td>
</tr>
<tr>
<td>5</td>
<td>Pseudomonas</td>
<td>20</td>
<td>-</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

6. SUMMARY AND CONCLUSION

In our study, concluded the ethyl acetate leaf extracts of Pergularia daemia has antibacterial activity against E.coli, Pseudomonas, Klebsiella, Staphylococcus and Bacillus due to the presence of the bioactive constituents. However further research will be needed to detect the bioactive compounds which are present in the plant leaf that is responsible for the pharmacological activity against the disease causing microorganism in toilets. The ethyl acetate extract of Pergularia daemia which is active against biofilm producers such as E.coli, Klebsiella, Pseudomonas, Staphylococcus and Bacillus. Also we concluded the disinfectant harpic is more effective against E.coli, Pseudomonas, Klebsiella, Staphylococcus and Bacillus comparing to the other disinfectants (ie) Phenol and Lysol. So the harpic is more effective to the toilets because of the chemical compounds.

BIBLIOGRAPHY:


