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IN-VITRO, EVALUATION OF ANTIBACTERIAL ACTIVITIES OF SOME MEDICINAL PLANT EXTRACTS AGAINST METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) ISOLATED AND GENETICALLY IDENTIFIED FROM ACUTE TONSILLITIS

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ABSTRACT : Methicillin-resistant *Staphylococcus aureus* (MRSA) is a common infection associated tonsillitis. The aim of this research is to genetically identify MRSA in tonsillitis patients and evaluate herbal medicinal plant extracts against Methicillin-resistant *Staphylococcus aureus* (MRSA) as multidrug-resistant bacteria, 34 (60.71%). *Staphylococcus aureus* was isolated from tonsillitis, phenotipcally identified on chrome agar media, and genotyped using the Staph 16S rRNA gene specific for the *Staphylococcus aureus* and *mecA* gene for determining *Staphylococcus aureus* resistance to methicillin (MRSA). The result of amplification of the *staph 16S rRNA* and *mecA* genes confirmed that all 34 (60.71%) were *Staphylococcus aureus* and resistant to methicillin (MRSA). Seven plant extracts, *Nigella sativa* seeds, *Punica granatum* peel, *Peganum harmala*, *Eugenia caryophyllus*, *Cinnamomum zeylanicum*, *Brassica juncea* and *Zingiber officinal* were tested for antibacterial efficacy against *Staphylococcus aureus* (MRSA) isolates, as well as testing the cytotoxicity of crude extracts. A well diffusion method was used to complete the primary screening on *Staphylococcus aureus* (MRSA), both extracts [aqueous and alcoholic] had a broad-spectrum impact, while alcoholic extracts had the greatest effect. However, both extracts had varied effects against all bacterial strain at the minimal inhibitory concentrations. The two extracts had a bactericidal effect, and the growth of all bacteria isolates in this investigation was monitored for seven days with no growth. The cytotoxicity of the extracts on red blood cells (RBCs) was tested, and the results revealed that there was no cytotoxicity in the absence of alcohol. An extract of *Cinnamomum zeylanicum* enhanced red blood cell volume, swelling, and agglutination. *Zingiber officinale* and *Punica granatum* peel alcoholic extract produced the opposite effect. The cells expanded and erupted at concentrations of 200 and 100 of the *Peganum harmala* alcoholic extract. As a result, the seven plants can be safely employed as a natural bactericidal agent source.

Key words : *Staphylococcus aureus* (MRSA), tonsillitis, alcoholic extract, cytotoxicity, medicinal plant.

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INTRODUCTION

Tonsillitis is an inflammation of the tonsils that usually develops quickly, the tonsils are the two lymph nodes on each side of the back of the throat, they play a role in defensive mechanisms and hence help to prevent infections, tonsillitis is a disorder that occurs when a bacterial or viral infection is followed by inflammation (Windfuhr *et al*, 2016). Most cases of tonsillitis are caused by bacterial infection. Bacterial tonsillitis can be caused by a variety of bacteria, mainly *The Streptococcus* group is thought to be the cause of chronic tonsillitis (Anderson and Paterek, 2019). Several researchers have suggested that antibiotic therapy failure could be due to an underestimation of resistant microorganisms, which could be explained by low antibiotic concentrations in the tonsillar tissue, possibly combined with the presence of resident bacteria producing protective enzymes, or specific antibiotic resistance patterns of the pathogenic bacteria involved.

Bacterial biofilms may play a role in a variety of

recurrent/chronic upper respiratory tract diseases, such as chronic tonsillitis. With chronic infections, biofilm has been detected in the tonsillar tissue , the prevalence of beta-lactamase-producing bacteria in the tonsils microbiota, such as *Staphylococcus aureus*, has been linked to Multidrug-resistant-bacteria (MDRB) in recent years, MDRB are Microorganisms that are resistant to one or more antimicrobial treatments (Torretta *et al*, 2013). Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the MDRBs of clinical interest, MRSA has been found in the tonsil's exterior and interior tissues. (Okwu *et al*, 2019).

Because of their potential to relieve the signs and symptoms of tonsillitis, herbal remedies are the most commonly used medications, natural herbal products, such as medicinal plants have made a significant contribution to human health, well-being and medicine development, they're useful natural blueprints for the production of novel medications (particularly in Western countries) or refined phytomedicines for illness therapy (mostly in underdeveloped countries and Europe), medicinal plants can be quite beneficial in the treatment of a variety of ailments, in many underdeveloped nations, 80% of people treat infectious infections with homemade phytomedicines, medicinal plant use has remained strong despite the advent of modern treatment in some cultures because to their efficacy, popularity and low cost, they also represent potential sources of new medicinal chemicals, as all portions of the plant are used in traditional treatment and can thus act as lead compounds (Mahady, 2005; Elkammoshi *et al*, 2016; Okwu *et al*, 2019).

The aim of this research is to prove that herbal medicinal plants extracts are effective against Methicillin-resistant *Staphylococcus aureus* (MRSA) a multidrug-resistant-bacteria that causes tonsillitis and has fewer side effects.

MATERIALS AND METHODS

Bacterial isolation

34 (60.71%) *Staphylococcus aureus* was isolated from tonsillitis and previously diagnosed phenotipcally on chrome agar media by researchers Al-Tameemi *et al* (2020).

Molecular identification

DNA extraction : According to the manufacturer's instructions, using the genomic DNA mini kit (Geneaid, Taiwan).

Staph 16srDNA, *mecA* gene Primer

Staph 16S rRNA gene used for determining *Staphylococcus aureus* with primers *Staph756F* (5-AACTCTGGTTATTAGGGAAGAACA-3) and *Staph750R* (5- CCACCTTCCTCCGGTTTGTCACC-3) (Zhang *et al*, 2004). For determining methicillin resistance *Staphylococcus aureus* (MRSA) species used the *mecA* gene with primers *MecA1* (5'-GTAGAAATGACTGAACGTCCGATAA-3') and *MecA2* (5'-CCAATTCCACAATTGTTTCGGTCTAA-3') (Eed *et al*, 2016). The set of thermocycling conditions for *Staph 16S rRNA* gene at 95°C for 5 min, followed by a 35 cycle for 95°C for 30s; 55°C for 30s; 72°C for 1min and extension 72°C for 5 min, for *mecA* genes for 3 min at 95°C, followed by a 30 cycle for 1min at 94°C; 30s at 53°C; 1 min at 72°C and extension 6 min at 72°C.

Sampling and extraction of plants

Twenty-five grams (25g) of powdered plant materials (flowers and stem bark) were weighed into clean bottles, extracted separately in tightly closed bottles using (125ml) distilled water and ethanol, and kept at room temperature for 48 hours, according to the methods of (Umeh *et al*, 2005 and Gberikon *et al*, 2015). Suspensions were filtered and placed in beakers using Whatman No. 1 filter paper. The filtrates were collected, and the filtrates were poured into dry Petri plates for desiccation at room temperature.

Antimicrobial activity test

The antibacterial properties of the examined extracts were assessed using the well diffusion method against Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, and Bacillus sp., according to the principles of Bauer *et al* (1966), Miles and Amyes (1996). Each of the test microorganisms was cultured on Muller Hinton Agar [MHA, Merck] plates with 0.1 ml of bacterial suspension containing 3.0 × 108 CFU/ml [McFarland standard 1], then spread evenly using an L-shape spreader. In the cultured MHA agar, the cork borer was used to create 6 mm diameter wells, which were then filled with 0.1 ml of each extract at a 200 mg/ml concentration. Controls included sterilized distilled water and 70% ethanol. After a 24-hour incubation period at 37°C, the antibacterial activity was assessed by measuring the mean of the inhibitory zone diameters around each well. The test was repeated three times (Al-Manhel and Niamah, 2015).

The Minimum Inhibitory Concentration [MIC]

The MIC was determined using the well diffusion method, as reported previously, with five concentrations of both alcoholic and aqueous extracts [200, 100, 50, 25 mg/ml] (Paarhusip and Sitanggang, 2011).

Cytotoxicity test

According to Nair *et al* (1989) for maintaining osmotic pressure in RBCs, aqueous and alcoholic extracts

were examined for cytotoxicity on human red blood cells (RBCs). A Ringer solution the extracts were introduced to 1 ml of RBCs with 19 ml of Ringer solution in consecutive test tubes at concentrations of 25, 50, 100, and 200 mg/ml and the mixtures were evaluated every hour for 12 hours (Al Hawani *et al*, 2020).

RESULTS

Genomic DNA extraction

Electrophoresis technique showed DNA bands for isolates in the Fig. 1.

Staph I6srDNA gene test results

Under the UV transilluminator visualized the PCR products gave (100%) positive results and a single band at the position 756bp and compared with the DNA ladder is shown in Fig. 2.

mecA gene test results

All 35 isolates (100%) showed *Staphylococcus aureus* resistance to methicilline (MRSA), which confirmed the amplification of the *mecA* genes and gave bands at positions 310bp, as shown in Fig. 3.

The minimum inhibition zone [MIC] assay

In both aqueous and alcoholic extracts, the minimal inhibitory concentration of *Nigella sativa* seeds, *Punica granatum* Peel, *Peganum harmala*, *Eugenia caryophyllus* and *Cinnamomum zeylanicum* was 25 mg/ml. The alcoholic extract of the *Brassica juncea* had a minimum inhibitory concentration of 50 mg/ml, while the aqueous extract had a concentration of 100 mg/ml. The alcoholic *Zingiber officinale* extract's the MIC was 25 mg/ml. The aqueous extract, on the other hand, did not have a minimal inhibitory concentration since it did not prevent bacterial growth as mentioned in Tables 1 and 2. Some results sample shown in Fig. 4.

The cytotoxicity trails

The cytotoxicity test on extracts of the seven aqueous and alcoholic plants revealed that all extracts of all plants had no effect on red blood cells at all concentrations.

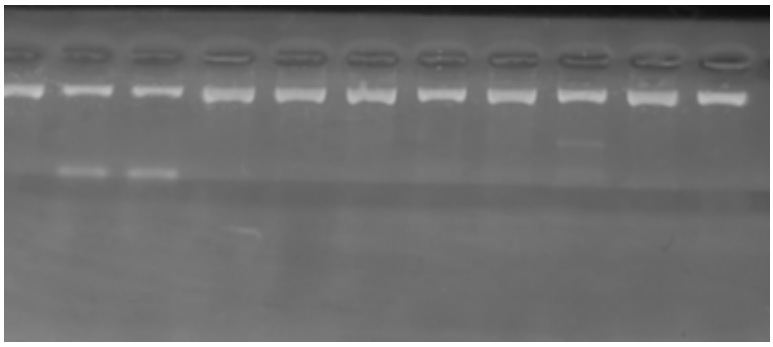

Fig. 1 : Agarose (0.8%) gel electrophoresis for DNA bands.

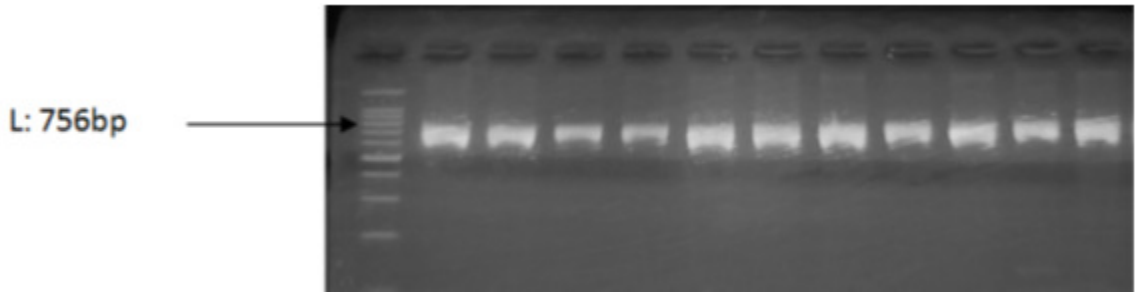

Fig. 2 : Agarose (1%) gel electrophoresis for *Staph 16SrDNA* PCR products at position756bp, L: (Ladder:100-1000bp).

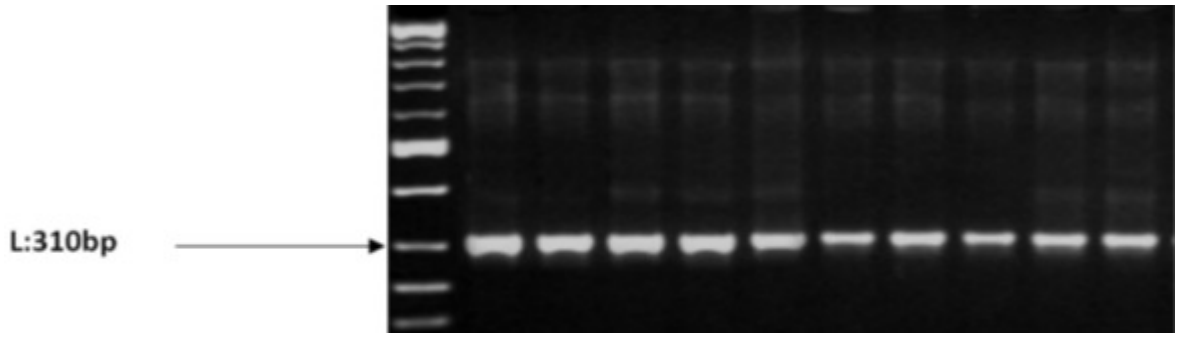

Fig. 3 : Agarose (1%) gel electrophoresis for *mecA* gene products at position 310 bp, L: (Ladder:100-1000bp).

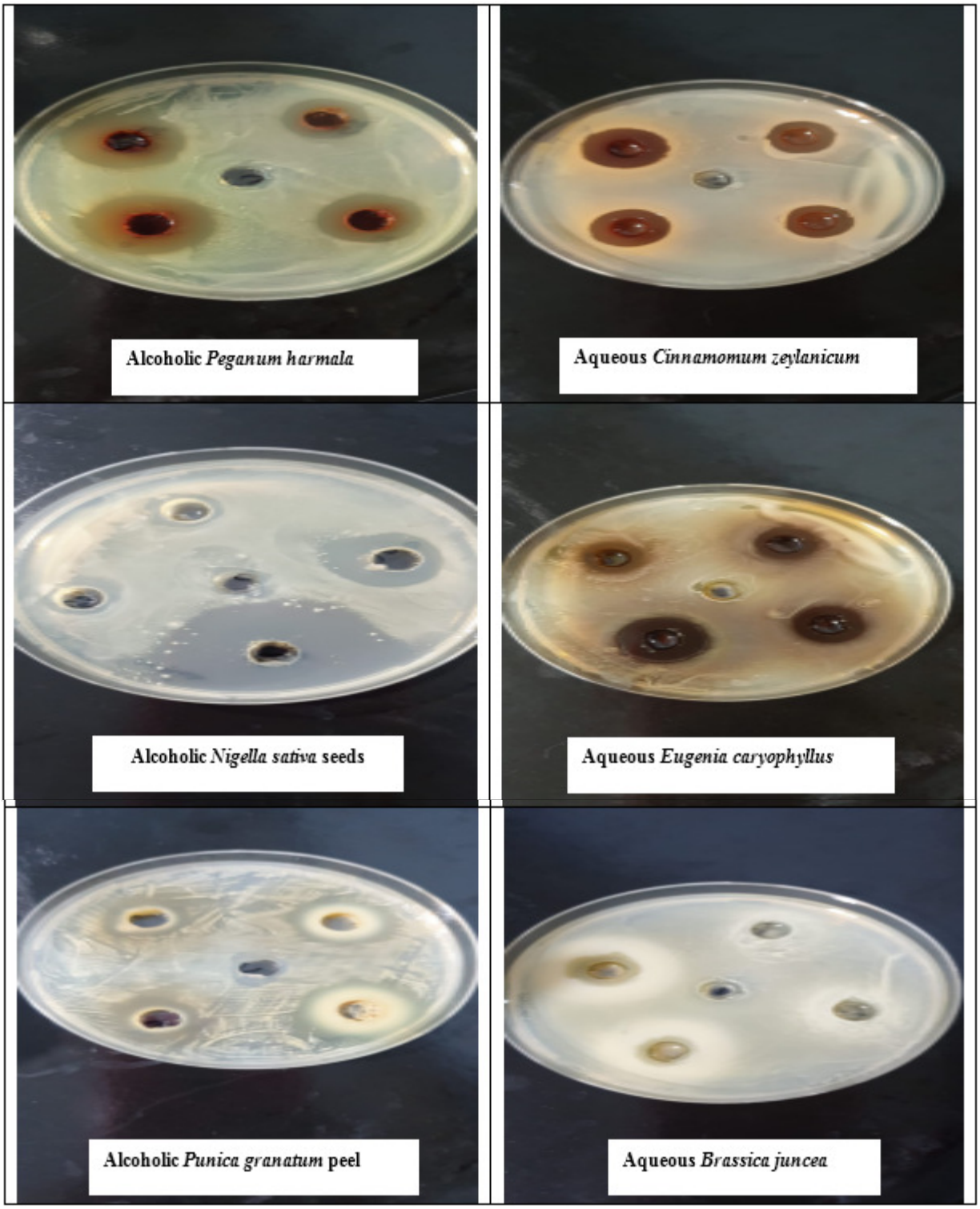

Fig. 4 : Results of alcoholic and aqueous extracts.

There was no agglutination, swelling, or explosion of red blood cells, indicating that they are safe and non-toxic as aqueous extracts for treating colds and relieving tonsil congestion. The alcoholic extracts of the *Nigella sativa* seeds, *Eugenia caryophyllus* and *Brassica juncea* had no effect on red blood cells at any concentration, and no cell lysis was observed. It was observed that the alcoholic extract of the *Cinnamomum zeylanicum* increased the volume of red blood cells and their swelling and agglutination at a concentration of 200 mg/ml. As for *Zingiber officinale*, a decrease in the number of red blood cells and agglutination occurred at the concentrations of 200 and 100 mg/ml. Agglutination and disintegration of red blood cells were found in the *Punica*

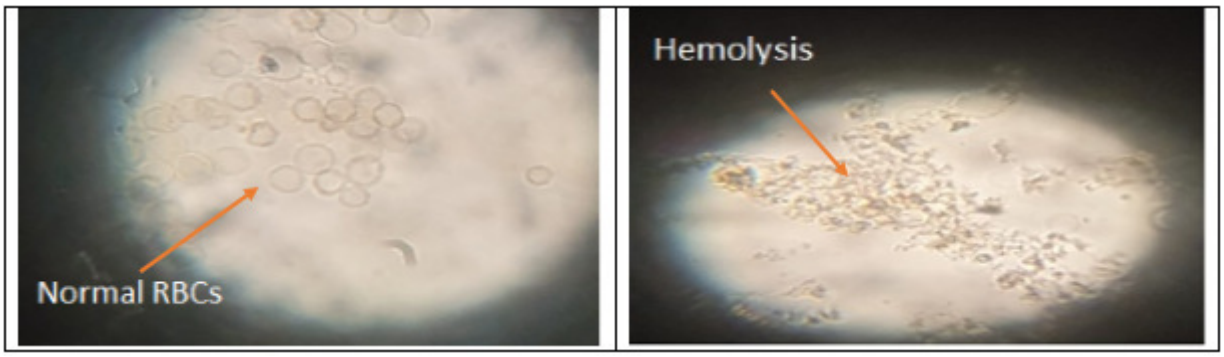

Fig. 5 : Cytotoxicity test show the RBC shape under microscope.

Table 1 : The diameter of the inhibition zone and MIC measured in mm for aqueous extraction for *Staphylococcus aureus.*

Concentration strain	25 mg/ml	50 mg/ml	100 mg/ml	200 mg/ml
Nigella sativa seeds	9.5	10.5	14.5	17.5
Punica granatum peel	11	13.5	15	19
Peganum harmala	10	15	18.5	19
Eugenia caryophyllus	11	12	14	17
Cinnamomum zeylanicum	13.5	14.5	15.5	19.5
Brassica juncea	-	-	10	12
Zingiber officinale	-	-	-	-

Table 2 : The diameter of the inhibition zone and MIC measured in mm for alcoholic extraction for *Staphylococcus aureus.*

Concentration strain	25 mg/ml	50 mg/ml	100 mg/ml	200 mg/ml
Nigella sativa seeds	11	13	20	32.5
Punica granatum peel	12	15	20	22.5
Peganum harmala	16	18	20.5	22
Eugenia caryophyllus	12	15	18.5	22.5
Cinnamomum zeylanicum	11.5	13	15	16
Brassica juncea	-	10.5	11.5	13
Zingiber officinale	9.5	10	11	12

granatum peel alcoholic extract at concentrations of 200 and 100 mg/ml. At concentrations of 200 and 100 of the *Peganum harmala* alcoholic extract, the cells swelled and erupted, and agglutination occurred, indicating that aqueous extracts of *Cinnamomum zeylanicum*, *Zingiber officinal*, *Punica granatum* peel and *Peganum harmala* are safer than alcoholic extracts.

The extracts' cytotoxic effect on red blood cells (RBCs) was assayed, the result showed no cytotoxicity in the absence of alcohol. It was observed that the alcoholic extract at the concentration of 200 mg/ml, an extract of *Cinnamomum zeylanicum* enhanced red blood cell volume, swelling, and agglutination. At 100 mg/ml, *Zingiber officinale* and *Punica granatum* peel alcoholic extract produced the opposite effect. The cells expanded and erupted, and agglutination occurred at concentrations of 200 and 100 of the *Peganum harmala* alcoholic extract as shown in Fig. 5.

DISCUSSION

Plants have been used as potential medication sources to prevent and cure human ailments by biomedical research. Antimicrobial resistance has been identified by the World Health Organization as a worldwide health security problem that necessitates action from all levels of government and society. It is self-evident that the rise in MDR microorganisms helps to alleviate socioeconomic issues and improve public health around the world. Many currently available and affordable antimicrobials, particularly in developing countries, are jeopardized by these MDR bacteria. It is for these reasons that researchers are looking for novel antimicrobials from medicinal plants to address the growing of antibiotic-resistant bacteria (Kebede *et al*, 2021).

Methicillin-resistant *Staphylococcus aureus* (MRSA)was the most common and abundant bacterial isolate, which could indicate virulence factors for tonsillitis (Al-Tameemi, 2020). The amplification of the staph 16SrDNA and mecA genes in all of the extracted DNA bacterial isolates reveals that they are all *Staphylococcus aureus* that are methicillin resistant. In addition to confirming identification, the genotypic identification prevents bacterial loss (Abed *et al*, 2021).

This study, seven extracts of medicinal plants commonly used in daily life, especially for the treatment of colds and chronic tonsillitis, were tested on *Staphylococcus aureus* isolates. It was isolated from the pulp of the tonsils of patients with chronic inflammation and it was found by conducting antibiotic sensitivity using the well diffusion method. According to the findings, bacteria were sensitive to both alcoholic and aqueous of *Nigella sativa* seeds extracts in all concentrations, with the alcoholic extract having a stronger effect on bacteria than aqueous extract (Chaieb *et al*, 2011). In the case of the *Punica granatum* peel, both extracts had an effect on bacteria (Devanesan *et al*, 2018) and the alcoholic extract of *Peganum harmala* had a stronger

effect on bacteria than water, where the effect was for both extracts at all concentrations (Jasim, 2019). Likewise, for the *Eugenia caryophyllus* plant, the effect of the two extracts was in all concentrations, and the effect of the alcoholic extract was greater than the concentration of the aqueous extract (Abid, 2009). The *Cinnamomum zeylanicum* plant had an effect in all concentrations, although the aqueous extract had a better effect than the alcoholic extract this time (Salih *et al*, 2014). The alcoholic extract had a better effect on the *Brassica juncea* extract than the aqueous extract, but it had no effect at a concentration of 25 mg/ml, at concentrations of 50 and 25 mg/ml, the aqueous extract had no impact (Engels *et al*, 2012). The alcoholic extract of *Zingiber officinal* had an effect on the bacteria at all concentrations, whereas the aqueous extract had no effect on the bacteria at any concentration (Sivasothy, *et al*, 2011). Al-Manhel and Niamah (2015) tested the effect of aqueous and alcoholic plant extracts on the suppression of several types of microbes that cause food spoiling, and discovered that the alcoholic extract has substantially greater action on the analyzed fungi and bacteria than the water extract.

Nigella sativa seeds, *Punica granatum* Peel, *Peganum harmala*, *Eugenia caryophyllus* and *Cinnamomum zeylanicum* had a minimum inhibitory concentration of 25 mg/ml in both aqueous and alcoholic extracts, the minimum inhibitory concentration of the alcoholic *Brassica juncea* extract was 50 mg/ml, while the aqueous extract had a concentration of 100 mg/ml. The MIC was 25 mg/ml for *Zingiber officinale* extract. However, there was no minimum inhibitory concentration in the aqueous extract. This means it didn't stop bacteria from growing.

The extracts' cytotoxic effect on red blood cells (RBCs) was assayed, the result showed no cytotoxicity in the absence of alcohol. It was observed that the alcoholic extract at the concentration of 200 mg/ml, an extract of *Cinnamomum zeylanicum* enhanced red blood cell volume, swelling and agglutination. At 100 mg/ml, *Zingiber officinale* and *Punica granatum* peel alcoholic extract produced the opposite effect. The cells expanded and erupted and agglutination occurred at concentrations of 200 and 100 of the *Peganum harmala* alcoholic extract (Madhkoor *et al*, 2021) reported that the leaking of the minor cation K+ from cells produced hemolysis in ethanol-treated RBCs due to a disruption in osmotic pressure, implying that membrane pores may be implicated in the ethanol-treated RBCs' defective cytoskeletal network. This finding backs up our findings.

CONCLUSION

The aqueous and alcoholic crude extracts of seven medicinal plants exhibit a strong antibacterial action on Methicillin-resistant *Staphylococcus aureus* (MRSA), there was a greater effect of the alcoholic extracts on the isolates than the aqueous extracts, and the diameters of inhibition were greater around the wells containing the concentrations of the different extracts. with varying minimum inhibitor concentrations and no cytotoxicity with the aqueous extracts, while it was observed that red blood cells were degraded in high concentrations of four plant extracts, it is preferable to use them in low concentrations, and it is preferable to use aqueous extracts of these plants because they are safer. Plants that are used locally can be used safely as disinfectants.

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