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Research Article

Formulation and Evaluation of Eugenol/Glycyrrhizic Acid -Loaded Nano-Lipid Carrier Gel for Treating Multidrug-Resistant Oral Infections: Histological Changes in a Mouse Model

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Abstract:

Methicillin-resistant Staphylococcus aureus (MRSA), Pseudomonas aeruginosa, and Candida albicans are pathogens that are drug-resistant and biofilm-forming. Therefore, they are responsible for severe infections, high morbidity, and mortality all over the world. Their ability to form biofilms and develop resistance against conventional antibiotics calls for a big need for new antimicrobial strategies. In this study, a nano-lipid carrier (NLC) gel containing eugenol and glycyrrhizic acid extract (EGAE) was developed as a topical treatment system and it was evaluated for its efficacy against multidrug-resistant (MDR) clinical isolates. The EGAE-NLC gel showed a very good physico-chemical stability by maintaining its visual appearance, uniform consistency, and drug content (98.9%) over a period of 12 weeks standard storage conditions. The gel formulation demonstrated a thermodynamically stable microemulsion with a globule size ranging between 29.1 and 60.5 nm. The zeta potential ranged from -35 to -43 mV and a pH range of 6.5–6.7. All these make the gel suitable for topical administration. The antimicrobial evaluation showed that the EGAE-NLC gel exerts a significant inhibitory activity against the studied pathogens, where a marked reduction in the minimum inhibitory concentrations (MICs) compared to individual components was attained. It is believed that the gel disrupted the biofilms and increased microbial cell membrane permeability. Eventually, leading to leakage of intracellular materials, cell lysis, and death. The anti-biofilm activity was seen as the highest against S. aureus, in which severe disruption of cell-to-cell connections was observed. In conclusion, this study suggests that EGAE-NLC gel represents a promising alternative antimicrobial therapy for fighting against infections caused by MDR pathogens. The mechanism of direct bactericidal action and biofilm disruption can provide a strong basis for further development into a clinically useful formulation.

1. Introduction

The discovery and introduction of antibiotics have resulted inadramatic reduction in mortality from bacterial infections and enabled many medical advances. Today, these advances are threatened by the emergence of resistant bacteria, estimated to cause >1.2 million deaths per year worldwide [40]. Therefore, antimicrobial resistance is acknowledged as one of the major global public health threats by the World Health Organization (WHO) and the U.S. Centers for Disease Control and Prevention (CDC). The burden of untreatable infections is projected to increase in the coming decades at immense economic costs [34], due to the dissemination of multidrug-resistant (MDR) bacteria and the lack of an innovative research and development (R&D) pipeline for antibiotics. The increase in infections caused by multi¬drug-resistant (MDR) bacteria has culminated in a global public health crisis, increasing morbidity and mortality rates and making the diagnosis and

treatment of these infections challenging [44]. Numerous studies on *Syzygium aromaticum* Myrta¬ceae extracts have been shown to have inhibitory effects on both gram-positive and gram-negative bacteria. Clove essential oil inhibited Escherichia coli and Klebsiella, two bacteria that produceβ-lactamase [22], and extended-spectrum beta-lactamase, metallo-beta-lactamase, and Amp C beta-lactamase-producing gram-negative uro¬pathogens [19]. Moreover, clove oil-based nano emulsions exhibit antibacterial properties against Staphylococcus aureus [41]. The major active ingredient of clove essential oil is eugenol, a phenolic compound, which is responsible for its antimicrobial activity by acting on cell membrane phospholipids and denaturing proteins [23]. Eugenol or 2-methoxy-4-[2-propenyl] phenol, a phenolic aromatic compound mainly derived from Cinnamomum and Clove essential oil, is one of these natural compounds belonging to a novel class of microbiocidal phenylpropanoids and has been used for a long time as an analgesic in dentistry [Hartnolletal.,2018, 56]. Eugenol has also indicated anesthetic, neuroprotective, antidiabetic, insecticidal, analgesic, anti-inflammatory, and antifungal properties that make this compound a versatile natural ingredient that helps prevent and cure various disorders [26, 42]. Glycyrrhizic acid is a triterpene compound primarily obtained from perennial plants of glycyrrhiza species, especially from the root part. Several positions in the chemical structure of GL appear to be suitable for generating a variety of active pharmacological derivatives [32]. The majority of antimicrobial effects from licorice is due to isoflavonoid components particularly hispaglabridin and ß,4'-O-methylglabridin, glabridin, glabriol and 3-hydroxyglabrol [24, 38]. In recent years, different methods of synthesis of glycyrrhizic acid and glycyrrhetinic acid have been widely used to synthesize many derivatives for many pharmacological applications, especially anti-viral and anti-bacterial uses [5].

Nanoemulsion gels are a type of formulation that combines the advantages of both nanoemulsions and gels. Nanoemulsions are thermodynamically stable, transparent or translucent dispersions of oil and water stabilized by an emulsifying agent. They have a droplet size in the nanometer range (typically less than 200 nm), which makes them highly stable and able to penetrate biological barriers. Nanoemulsions have been extensively studied for drug delivery applications due to their ability to improve the solubility, stability, and bioavailability of drugs. However, they are often difficult to apply topically due to their liquid nature [1, 35, 53]. Gels, on the other hand, are semisolid formulations that are easy to apply topically and provide sustained release of active ingredients. They are often used in topical drug delivery applications due to their ability to adhere to the skin and mucous membranes, which allows for prolonged contact time and increased absorption. However, conventional gels often have limited penetration due to their high viscosity. Nanoemulsion gels combine the advantages of both nanoemulsions and gels, resulting in a formulation that is easy to apply, highly stable, and able to penetrate biological barriers. The droplet size of the nanoemulsion in the gel allows for improved penetration of the active ingredient into the affected area, while the gel matrix provides sustained release and increased contact time [54]. In this study, we developed a nano-lipid carrier (NLC) gel containing eugenol and glycyrrhizic acid extract (EGAE) and evaluated its antimicrobial and healing effects against multidrug-resistant (MDR) pathogens. The gel was characterized for its physicochemical properties, tested in vitro for antimicrobial activity, and assessed in vivo using infected animal models, with histopathological analysis to confirm tissue healing.

2. Materials and Methods

2.1 Materials

EGAE-NLCs (Previously prepared), MDR Bacterial isolate (*pseudomonas aeruginosa*, Methicillin-resistant *Staphylococcus aureus* (MRSA)) and fungal isolate (*Candida albicans*) from oral cavity (Previously isolate), Tween 80 (Merk-Germany), carbopol-934 (CB) (Himedia-India), Hydrochloric Acid (HCl) (Merk-Germany), hydroxypropyl methylcellulose (HPMC K4M) (Merk-Germany), Methylparaben (Merk-Germany), Distilled water (D.W).

2.2 Method

2.2.1 Preparation of Gel Formulation

EGAE-NLCs (previously prepared) were dissolved in a mixture of 0.1 N (hydrochloric acid) and 1% w/v Tween 80. Methylparaben was added as a preservative to the resulting drug solution, which was then added to the polymer solution while being continuously stirred with a magnetic stirrer until a uniform solution was obtained and the volume was finished with distilled water (D.W.). The polymer solution was made using carbopol-934 (CB) in a concentration of 0.7% w/v in combination with hydroxypropyl methylcellulose (HPMC K4M) (1%, 1.5% w/v) using the hot method, which involved

heating 70 ml of water until it boiled, then gradually adding the desired amount of HPMC part-wise while stirring on a hot plate. Once HPMC was fully added, the solution was allowed to cool to obtain a clear colorless viscous dispersion. After that, CB was weighed and added to the HPMC dispersion in a different beaker while being continuously stirred on a magnetic stirrer that was heated to about 70°C. The drug solution was then added to polymer solution with steady stirring and the volume finished with D.W. (Nief *et. al*., 2019). Formulation was prepared in triplicate.

2.3 Evaluation Parameter of the Prepared Gel

2.3.1 Appearance and pH determination

The formulations were examined for overall appearance, including color, and the presence of any suspended particles. The preparation's clarity was assessed using both black and white backgrounds (Meshram and Thorat, 2015). On the other hand, the pH values of the prepared emulsions were tested at 25°C using digital pH meter by dipping the electrode into the liquid bulk [31].

2.4 In Vitro Release Assessment

A dissolution test using type II apparatus (paddle type) was conducted for the in vitro release study. Each formulation had 1 ml placed in a dialysis membrane (0. 08 μm pore size), which had been soaked in phosphate buffer at pH 6. 8 overnight [23]. This membrane was then secured to the paddle shaft and submerged in 150 ml of salivary fluid, serving as the dissolution medium, which was rotated at 50 rpm and kept at a temperature of 37 ± 1°C (Peh and Wong, 1999). A volume of 1 ml was extracted at defined time intervals of 0.5, 1, 2, 3, 4,5, 6 and 7 hours and replaced by 1 ml of fresh PBS to maintain the sink condition. EGAE-NLC suspension was subjected to in-vitro release test for comparison. The experiment was conducted in triplicate. The samples were analyzed for drug concentration using HPLC technique at 215 nm for eugenol and GA was detected at a wavelength of 254 nm.

2.5 Laboratory Animals and Experimental Designs

A total 40 male albino mice were used currently with 8-10 weeks old and 21±6 g average weight was purchased from Iraqi Center for Cancer Research and Medical Genetics / Ministry of Higher Education and Scientific Research. The mice were housed in polypropylene cages in the animal house / College of Veterinary Medicine under controlled conditions at temperature (25-28) C°. Mice were acclimatized condition for 7 days before commencement of the experiment. A total of 30 mice divided into three group according to the type of infection (Group -A- *C. albicans*, Group-B- *P. aerogenes* and group-C- MRSA) that were starved for 3 days with free access to salted water were studied (Morris *et. al*., 2011). Infections were induced by oral tube feeding with the respective multidrug-resistant (MDR) isolates, and mice were monitored for 48 hours for any abnormal changes. Following infection, the mice were treated with the EGAE-NLC oral gel via oral tube feeding for seven consecutive days and monitored for clinical signs and abnormalities throughout the treatment period (Figure 1).

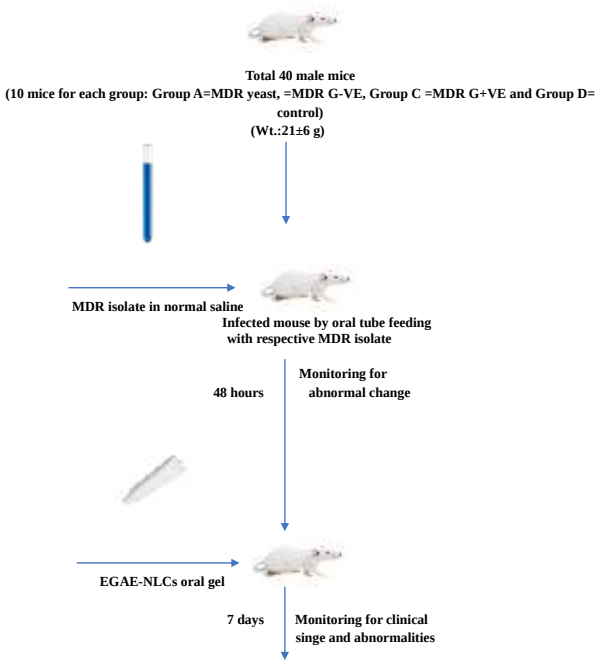

Figure 1. *Experimental Protocol for MDR Infection and EGAE-NLC Gel Treatment in Mice*

2.6 Collection of Organ Samples

The muscle tissues were excised then placed in a petri dish. The dish contained physiological saline solution for the removal of any adipose tissue and connective tissue that are adherent to the muscles with the aid of anatomical microscope. The cleaned tissues were then weighed using a sensitive balance and afterward were transferred to a 10% formalin solution for fixation. The following was histological sectioning and examination [13].

2.7 Histopathological Assessment

The fixed tissue samples prepared as above were processed by embedding them in paraffin wax. The

samples then sectioned into thin slices using a microtome. The sections were mounted on a glass microscope slide and were stained with hematoxylin and eosin (H&E) for the visualization of cellular and tissue structures. Histopathological examination was carried out under a light microscope to assess tissue architecture, inflammatory response, and signs of infection or healing [13].

3. Results and Discussion

3.1 Visual Appearance and pH Determination

All prepared *in situ* gelling systems were evaluated for their visual appearance and clarity at room temperature. That was done to ensure uniformity of the gels and absence of any undesirable particles. The formulations exhibited a clear and homogeneous texture, thus indicating a success of dispersion of the components. Added to that, the pH of the prepared oral gels was measured and found to fall between 6.2 and 6.9, which is within the normal physiological pH range of the oral cavity (6.0–7.5) [31]. This pH compatibility suggests that the gels can be suitable for oral administration without causing irritation to the mucosal tissues, as presented in Table 1.

3.2 Drug Content Uniformity

To assess the uniformity of drug distribution within the prepared gel formulations, samples equivalent to 10 mg of the active compounds were collected from each formula. The drug concentrations were quantified using a validated high-performance liquid chromatography (HPLC) method, with eugenol detected at 215 nm and glycyrrhizic acid (GA) at 254 nm. The results were expressed as a percentage of the theoretical drug content (w/w). All three replicates demonstrated high consistency, as shown by low standard deviation (SD) values. This high degree of uniformity indicates that the preparation method used here was good ensuring reproducible drug loading throughout the prepared batches. The results are summarized in Table 1.

3.3 In vitro drug release studies

The *in vitro* release profiles of the prepared gel formulations were evaluated over a period of three hours. The cumulative percentage of active constituent (eugenol and GA) released ranged from 22% to 100%, as shown in Figure 2. The results revealed a noticeable retardation in drug release, which can be attributed to the entrapment of the drug within the lipid-based oil globules of the nano-lipid carrier system.

Table 1. *pH, Globule Size, PDI, Zeta Potential and Drug contents percentage*

Formula code	pH	Particle Size	PDI	Zeta potential(mV)	Drug contents %
EGAE-NLC gel - 1	6.5	29.14 nm	0.003	- 36	99.5%
EGAE-NLC gel - 2	6.9	45 nm	0.0012	- 43	98.1%
EGAE-NLC gel - 3	6.7	60.5 nm	0.002	- 35	99%±
P-value	0.569 NS	0.0001 **	0.437 NS	0.0372 *	0.807 NS
* (P<0.05), ** (P<0.01) , NS: Non-Significant.					

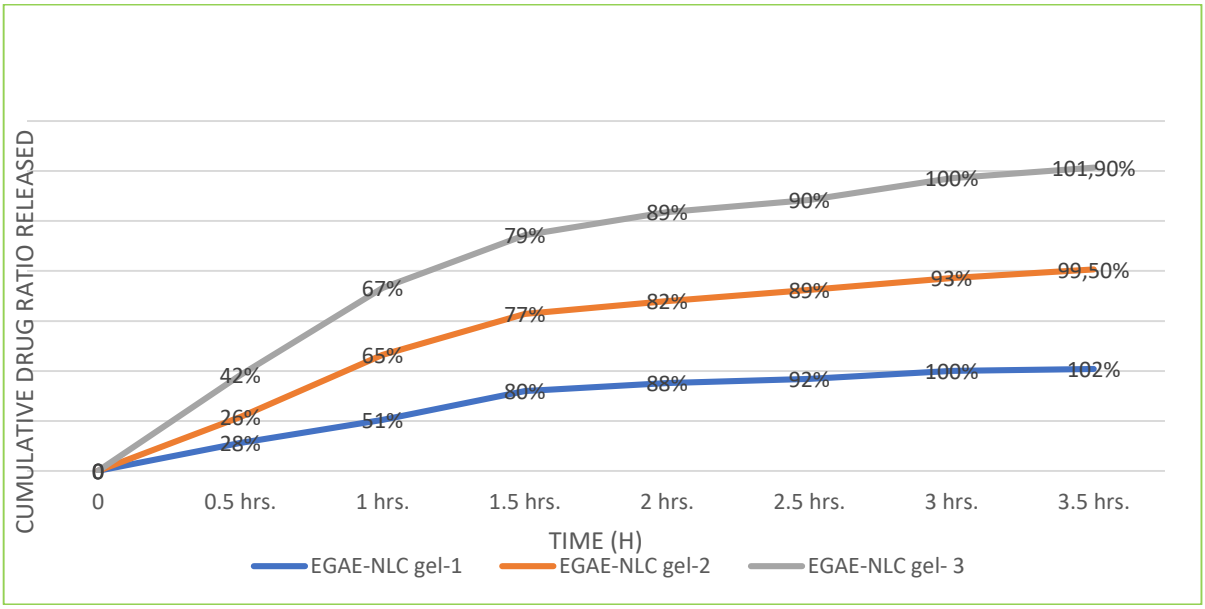

Figure 2. *In vitro release profiles of EGAE-NLC Oral Gel.*

Since the active compounds exhibit higher solubility in the lipid phase, their diffusion into the aqueous release medium was controlled and sustained over time. This controlled release behavior suggests that the developed formulation could provide prolonged therapeutic action, potentially reducing the frequency of dosing [27].

3.4 The Stability Study Of Formula

The stability of the selected gel formulation was monitored over six months under standard storage conditions (25°C and 60% RH). Throughout the study period, the formulation maintained its clear apperance, with no signs of phase seperation, precipitation, or color change. Drug content analysis showed that 98.9% of the active ingredients remained intact by the end of the study, indicating excellent stability. These results suggest that the gel can retain its physical and chemical properties over time, making it a relieable option for long-term use.

3.5 Histopathological Changes

Histological readings showed muscular necrosis and mononuclear cells infiltration in infected mice with MDR *Candida albicans* (group A) Figure-(2)., in compare to control show normal epidermis layer and salivary gland section in mice mouth Figure-(1), In figure (3) Showed area of cartilage with newly chondrocyte surrounded by inflammatory cells and necrotic muscle with inflammatory cells. While Figure (4) showed hypercellularity and vacuolation of epidermal layer, odema with mononuclear cells infiltration, odema in subepidermal layer, hypercellularity and hyperplasia of salivary glands and necrotic muscular layer.

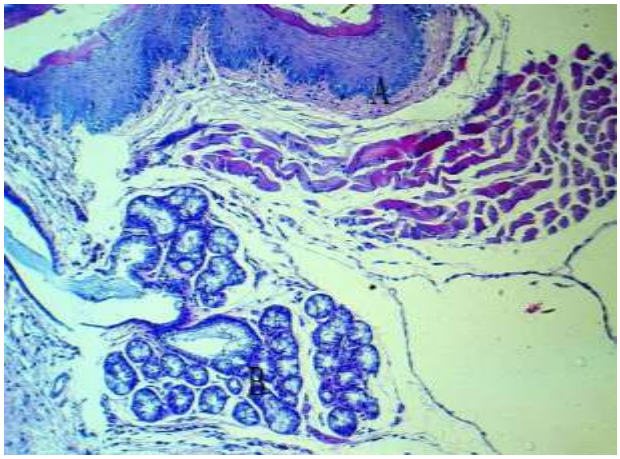

Figure 3. *section in normal mice mouth: A) epidermis layer. B) salivary gland. (control) (X 200 H and E stain.)*

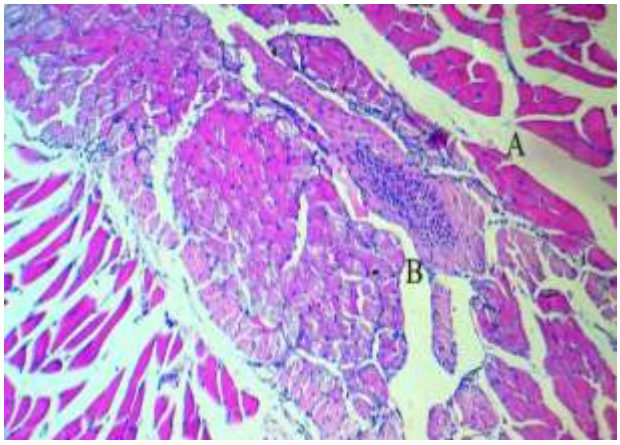

Figure 4-A. *section in mice mouth infected with Candida albicans: A) muscular necrosis. B) mononuclear cells infiltration. (group A). (X 200 H and E stain).*

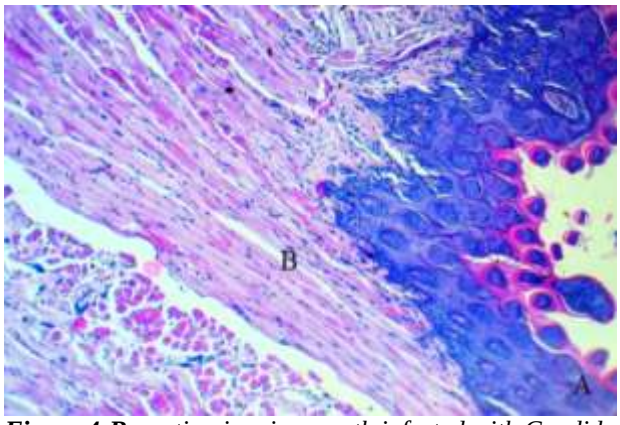

Figure 4-B. *section in mice mouth infected with Candida albicans: A) area of cartilage with newly chondrocyte surrounded by inflammatory cells. B) necrotic muscle with inflammatory cells. X 200 H and E stain.*

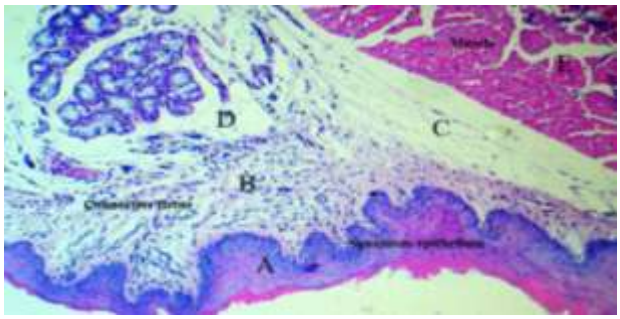

Figure 4-C. *section in mice mouth infected with Candida albicans: A) hypercellularity and vacuolation of epidermal layer. B) odema with mononuclear cells infiltration. C) odema in subepidermal layer D) hypercellularity and hyperplasia of salivary glands. E) necrotic muscular layer. X 200 H and E stain.*

Recently, *Candida albicans* has become an important opportunistic pathogen, responsible for several infections, especially among elderly individuals with weakened immune systems. One of the major challenges in treating *Candida infections* is its ability to form and thrive in biofilms, which show resistance to standard antifungal treatments, making management more

difficult. Although clove oil has been found to have potential antifungal properties, its specific effects on Candida albicans biofilms have not been thoroughly investigated. Thus, a thorough review of the available literature on clove oil's anti-biofilm activity and its mechanisms against *Candida albicans* biofilms is necessary. This review will help shed light on the possible therapeutic uses of clove oil in addressing biofilm-related *Candida infections* [51].

In group B Histological readings showed section in mice mouth muscles infected with MDR *Pseudomonas aeruginosa* appear Multiple area of necrosis, Mononuclear cells infiltration, fibrine network and hemorrhase, figure (5A), and Enlargement of muscles with necrosis, Mononuclear cells infiltration and Oedema in section in mice mouth muscles figure (5B), While figure (5C) showed dilated and congested blood vessels in submucosal layer, necrotic muscle with inflammatory, odema, fibrinous and focci of inflammatory cells ,figure (5D) showed necrotic area, sever macrophages infiltration, dilated, congested blood vessels, odema and fibrinous network with inflammatory cells infiltration , figure (5E) showed hyperplasia of epidermis layer (papillae) like, hyperplasia of salivary gland and surrounded by odema, odema and necrotic muscular , and increase thickening of epidermis, hyperplasia of salivary glands with debri, mononuclear cells infiltration mostly macrophages and necrotic muscular figure (5F)

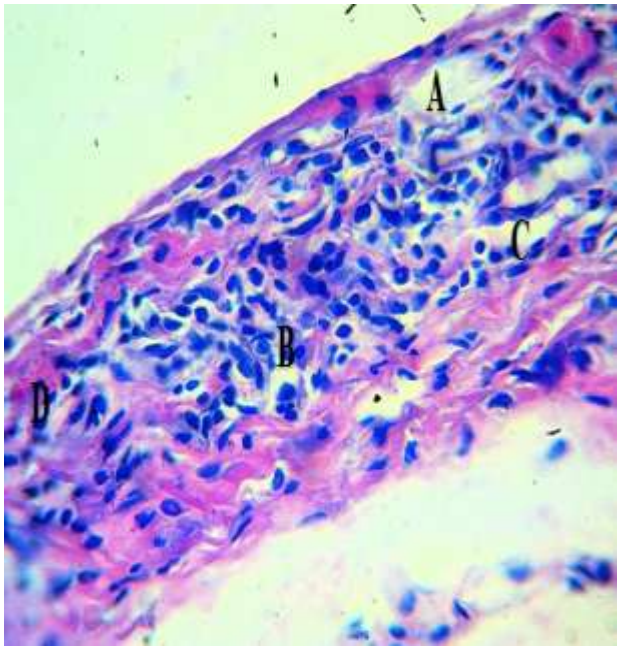

Figure 5-A. *section in mice mouth muscles infected with Pseudomonas aeruginosa: A) Multiple area of necrosis. B) Mononuclear cells infiltration. C) fibrine network. D) hemorrhase. X 400 H and E stain.*

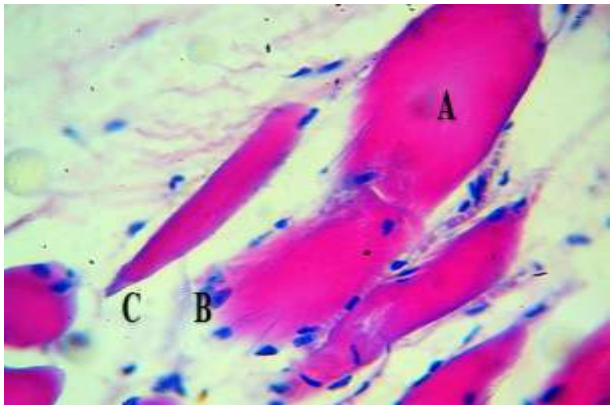

Figure 5-B. *section in mice mouth muscles infected with Pseudomonas aeruginosa: A) Enlargement of muscles with necrosis. B) Mononuclear cells infiltration. C) Oedema, X 400 H and E stain.*

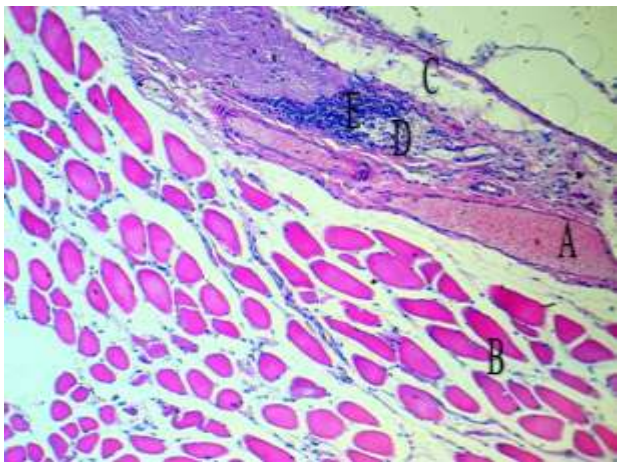

Figure 5-C. *section in mice mouth infected with pseudomonas aeruginosa: A) dilated and congested blood vessels in submucosal layer. B) necrotic muscle with inflammatory C) odema D) fibrinous E) focci of inflammatory cells. X 200 H and E stain.*

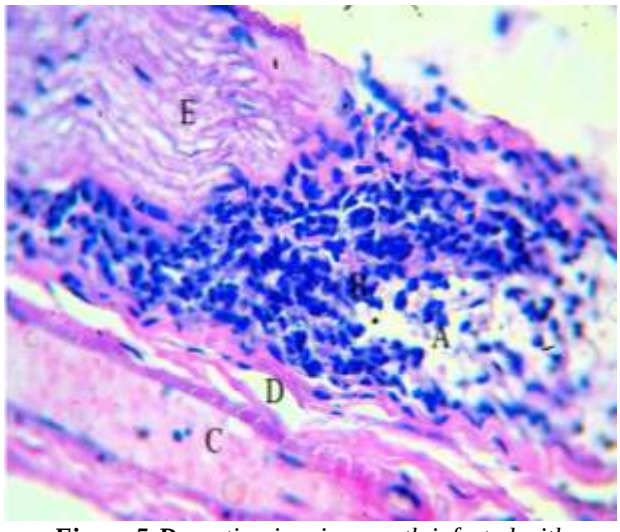

Figure 5-D. *section in mice mouth infected with pseudomonas aeruginosa: A) necrotic area. B) sever macrophages infiltration C) dilated and congested blood vessels D) odema E) fibrinous network with inflammatory cells infiltration. X 400 H and E stain.*

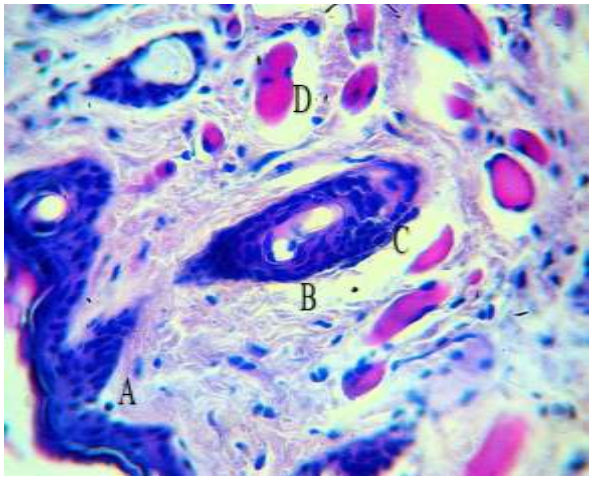

Figure 5-E. *section in mice mouth infected with pseudomonas aeruginosa: A) hyperplasia of epidermis layer (papillae) like. B) hyperplasia of salivary gland and surrounded by odema C) odema D) necrotic muscular. X 200 H and E stain.*

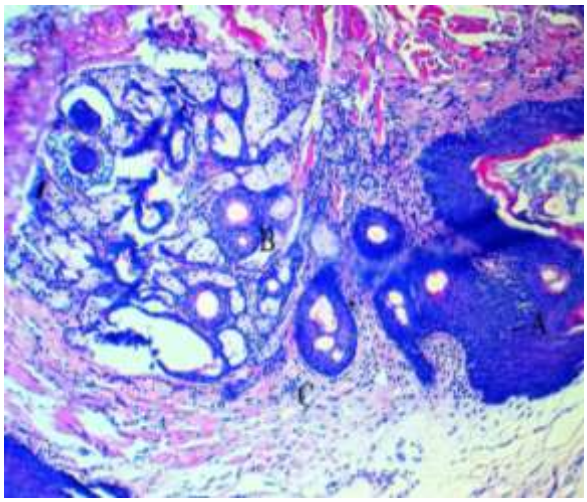

Figure 5-F. *section in mouth tissue of mice infected with pseudomonas aeruginosa: A) increase thickening of epidermis. B) hyperplasia of salivary glands with debri. C) mononuclear cells infiltration mostly macrophages D) necrotic muscular. X 200 H and E stain.*

Pseudomonas aeruginosa is an opportunistic pathogen from the γ-proteobacteria family and is known to cause pneumonia associated with ventilator use and hospital-acquired infections. As antibiotic-resistant bacteria continue to rise, it is essential to discover alternatives to traditional antibiotics. Plant-derived substances (PDSs) provide potential benefits not only as antibacterial agents but also in combating antibiotic resistance [5].

P. aeruginosa is a Gram-negative opportunistic bacterium that frequently contributes to nosocomial infections, with an increasing rate of multidrug resistance to antibiotics commonly prescribed [46]. The rise in bacterial resistance has become a worldwide challenge, diminishing the effectiveness of existing medications [37]. Developing drugs that are based on plant components may offer a solution to this significant issue of drug resistance [9]. The current study aimed to assess the combined antibacterial effectiveness of plants and antibiotics, along with the possible lowering of the minimum inhibitory concentration (MIC) of antibiotics used to tackle Pseudomonas-related hospital infections. The effects of plant extracts (clove, eucalyptus, ginger) when combined with ceftazidime were evaluated and resulted in a notable decrease in the MIC of ceftazidime. This study also focused on the antibacterial properties of the selected antibiotics.A recent study by Ghafil and Zgair, (2022) [21] reported that the effect of serial bacterial secretion in the growth media after inoculation with *P. aeruginosa t*hat was isolated from infected wounds (cut wound) stimulated the pro-inflammatory immune response and had a slight effect on the histological feature of an animal model, while in the present study, the *P. aeruginous* was isolated from oral cavity. Ali and Heydarlou, (2023) [4] support the concept of the relationship between high levels of pro-inflammatory cytokines in the inflammatory area and the presence of leukocyte infiltration

In group C that infected with MRSA Histological readings figure (6A) showed dilated, enlargement, congested with monocytes and rounded and enlargement of muscles with necrosis, figure (6B) showed sever odema in submucosal layer and Mononuclear cells infiltration in submucosal layer with thrombosed artery, While figure (6C) showed necrotic salivary gland, focci of mononuclear cells and odema, and figure (6D) showed dilated and congested blood vessels, necrotic muscular layer with dark eosinophilic cytoplasm and mononuclear cells infiltration.

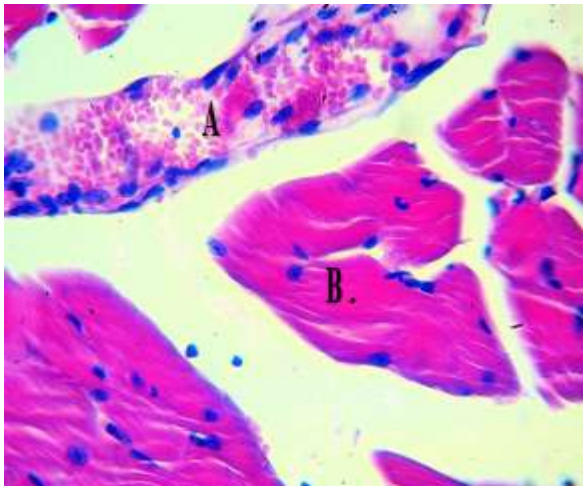

Figure 6-A. *section in mice mouth muscles infected with MRSA: A) dilated and enlargement and congested with monocytes. B) rounded and enlargement of muscles with necrosis. X 400 H and E stain.*

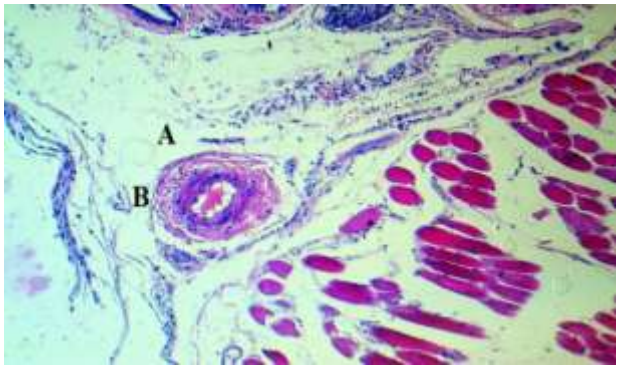

Figure 6-B. *section in mice mouth muscles infected with MRSA: A) sever odema in submucosal layer. B) Mononuclear cells infiltration in submucosal layer with thrombosed artery. X 200 H and E stain.*

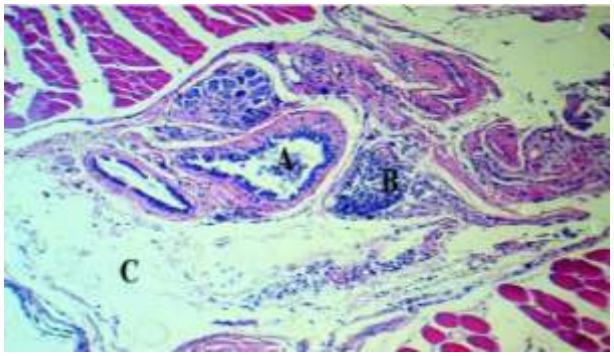

Figure 6-C. *section in mice mouth muscles infected with MRSA: A) necrotic salivary gland. B) focci of mononuclear cells C) odema. X 200 H and E stain.*

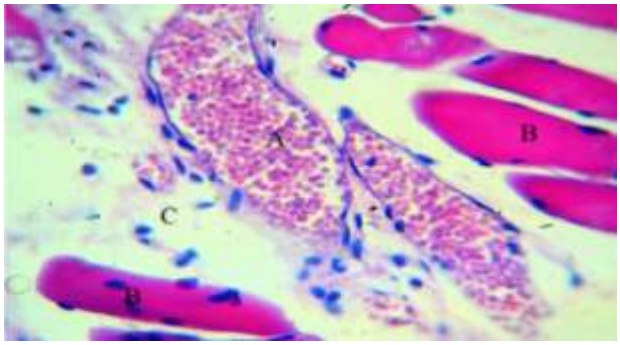

Figure 6-D. *section in mice mouth infected with MRSA: A) dilated and congested blood vessels. B) necrotic muscular layer with dark eosinophilic cytoplasm C) mononuclear cells infiltration. X 400 H and E stain.*

MRSA is the most frequently found drug-resistant Gram-positive bacteria responsible for infections related to healthcare settings. Therefore, MRSA is a critical target for measures aimed at controlling and preventing infections [50, 57]. S. aureus ranks among the three main pathogens linked to infections acquired in hospitals. These bacteria can lead to various infections, ranging from skin lesions to endocarditis. Currently, VISA and VRSA are not widely prevalent pathogens, but their potential rise is concerning (Goud et al. 2011). The community-acquired strains of VRSA raise even more alarm (Limbago et al. 2014). Essential oils, which are hydrophobic hydrocarbons, interact with the hydrophobic elements of bacterial cell walls, showcasing their antibacterial properties (Sikkema et al. 1992). Nanoemulsions, containing oil droplets at the nanoscale, possess antibacterial capabilities that far exceed those of standard bulk oil (Ghosh et al. 2013).

After 5 days getting infection with three group of MDR bacterial and fungal isolate the mice treated orally with gel loaded with EGAE- NLCs two times a day for a week. Then, the vital activities of the mice were observed and tissue samples were taken from the oral tissue of the treated mice. Group A after treatment, Figure (7A-B) showed normal muscle with slight lymphocytic and normal epidermal layer, and necrotic glands.

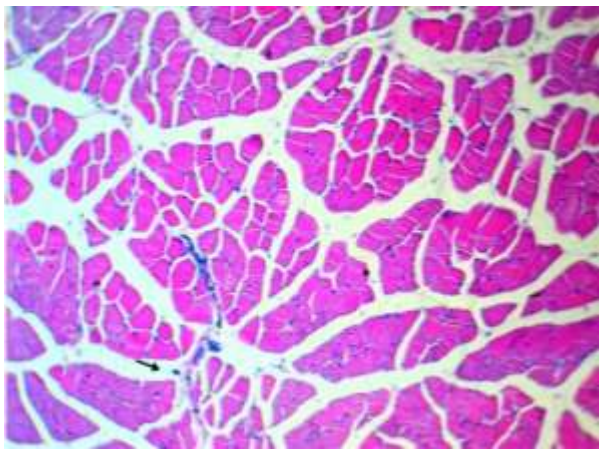

Figure 7-A. *section in mice mouth infected with Candida albicans and treated: normal muscle with slight lymphocytic infiltration, X 200 H and E stain.*

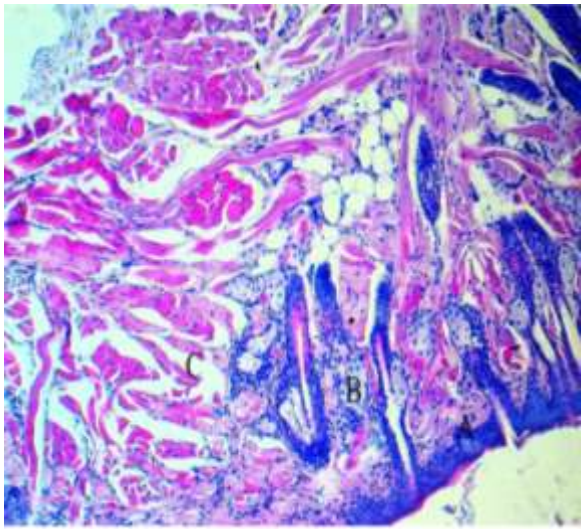

Figure 7-B. *section in mice mouth infected with Candida albicans and treated : A) normal epidermal layer. B) necrotic glands C) normal muscle. X 200 H and E stain.*

Group B after treatment, Figure (8A-B) showed normal muscle normal, epidermal layer, normal glands and necrotic muscular layer.

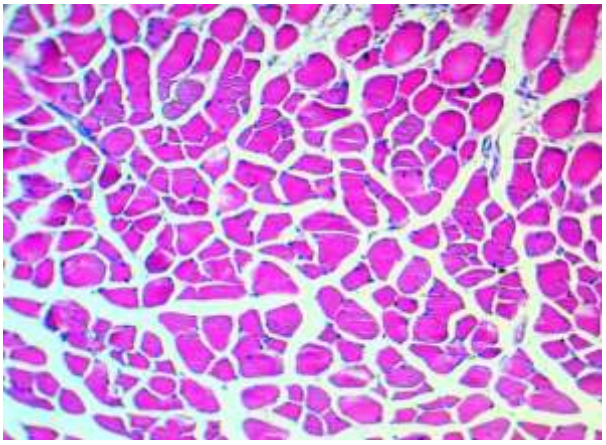

Figure 7-A. *section in mice mouth infected with Pseudomonas aeruginosa and treated: normal muscle with, X 200 H and E stain.*

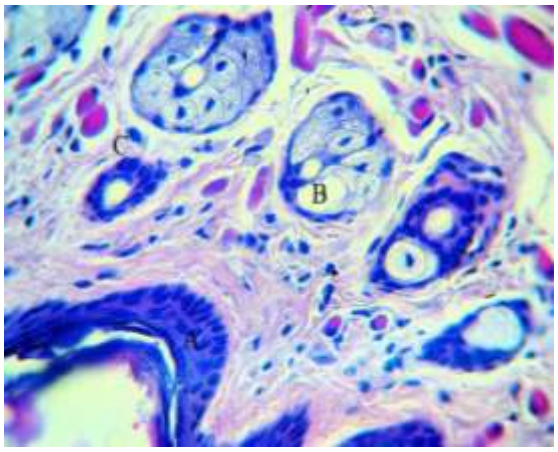

Figure 7-B. *section in mice mouth infected with pseudomonas aeruginosa and treated : A) normal epidermal layer. B) normal glands C) necrotic muscular layer. X 400 H and E stain.*

Group C after treatment, Figure (8A-B) showed normal muscle layer.

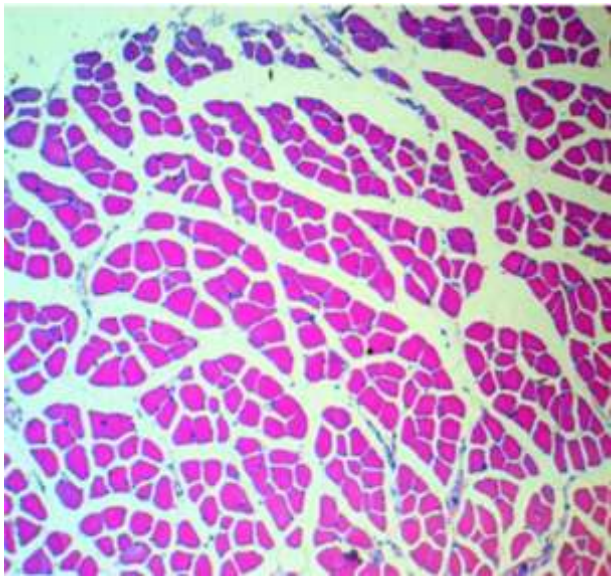

Figure 8-A. *section in mice mouth infected with MRSA and treated: normal muscle layer, X 200 H and E stain.*

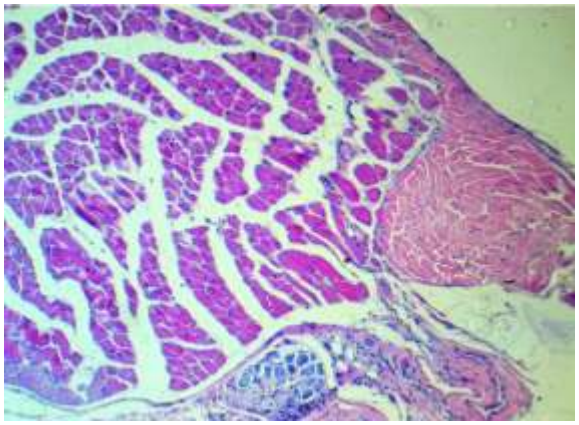

Figure 8-B. *section in mice mouth muscles infected with MRSA and treated: normal mucosal layer. X 200 H and E stain.*

Natural products are an incredibly valuable source of novel bioactive substances that merit research to verify or clarify their common uses [14, 20, 15]. Eugenol has been utilized extensively in dental practice for many years owing to its properties, including antimicrobial, analgesic, antioxidant, and anti-inflammatory effects [45]. According to Bai et al. (2023), eugenol is identified as the primary constituent of clove essential oil (CEO). Both CEO and eugenol demonstrated clear antibacterial effects against *S. aureus* and *E. coli*. The way eugenol acts against S. aureus likely involves damaging the cell wall and membrane, hindering biofilm formation, inducing apoptosis via oxidative stress, and disrupting DNA synthesis. These findings suggest that CEO and eugenol could serve as alternatives to antibiotics and synthetic antimicrobial agents in the food sector. In research by Taher et al. (2015), the effects of clove extract on skin inflammation and bacteria in mice were assessed, revealing significant treatment efficacy compared to the control group. Ashjazadeh et al. (2019) [6] observed that the healing time with eugenol nanofibers was less than that of other groups. Several indicators, including collagen levels, the formation of new blood vessels, epithelial thickness, edema, leukocyte presence, and acute bleeding, were critical to their analysis.

Our findings reveal that eugenol exhibits considerable inhibitory effects on MRSA, *Pseudomonas aeruginosa*, and *Candida albicans*. The strong impact of eugenol on S. aureus biofilms arises from the disruption of cell-to-cell connections and increased cell permeability, leading to the leakage of internal contents and eventual cell lysis. The loss of membrane integrity, along with damage to the cell surface, shows that eugenol's bactericidal properties against S. aureus occur through membrane disruption and inhibition of cell growth [16, 28]. Likely owing to structural differences, the two compounds engage differently

with the cell membrane, enhancing their ability to eradicate biofilms [55, 17, 36]. Further investigation is needed to uncover the precise mechanisms that contribute to the synergistic effects of these two compounds on *S. aureus*.

Xu et al. (2016) [58] proposed that the essential oil likely first engages with the cell wall and membrane. To begin with, it disrupts these structures, leading to the loss of crucial materials within the cell, which ultimately results in the death of the bacteria. Furthermore, after damaging the cell's structure, the essential oil is able to penetrate the cytoplasmic membrane or enter the cell, subsequently inhibiting the production of DNA and proteins that are necessary for bacterial proliferation. These findings indicate that clove essential oil affects the growth inhibition of *S. aureus* at a molecular level instead of just causing physical harm. Ribeiro-Santos et al. (2016) [48] discovered that eugenol exhibits antimicrobial properties against *E. coli* and *S. aureus* strains, showing inhibition zones. In earlier research by Albano et al. (2016) [3], an MIC of 1200 μg/mL was observed for eugenol against S. aureus, which aligns with the MIC of 1000 μg/mL determined in this current study. These comparative results suggest that many derivatives of eugenol have strong antimicrobial potential, similar to eugenol itself. Haripriyan et al. (2018) [25] found that CBO reduces significant virulence factors of this crucial human pathogen while simultaneously boosting host immune functions, which could lead to advancements in topical treatments for antibiotic-resistant infections. The antioxidant capacity of cloves may be enhanced due to a greater presence of organic phenolic compounds such as eugenol and eugenyl acetate, known for their hydrogen-donating ability and effectiveness as strong free radical scavengers [12]. El-Haroun (2021) [18] concluded that delivering clove extract to Furan-treated rats could improve the histological, biochemical, and immunohistochemical issues observed in the submandibular gland based on all the reported data and findings. In light of these results, cloves display a protective effect against Furan toxicity and could potentially offer health benefits, thereby enhancing quality of life.

Nonetheless, further in-depth studies are essential to assess the potential of this plant as a protective agent against induced damage to the submandibular area in clinical research. Various extracts from plants show different levels of antimicrobial effectiveness, which may result from the presence of distinct phytochemical components such as phenolic compounds, terpenoids, tannins, and alkaloids. Each of these phytochemicals possesses its own complex mechanisms that contribute to their antimicrobial properties [49]. Although no single mechanism for the antibacterial effects of plant extracts or phytochemicals has been conclusively defined, it is likely that the variety of compounds in these extracts affects the shape and structure of cells, targets important intracellular molecules, ions, and various active sites within cells, which may account for their antimicrobial capabilities [58]. Previously, it was noted that clove methanol extract contains a high concentration of phenolic compounds [33]. The presence of these active phenolic compounds may explain the strong antibacterial properties of clove extract, which can alter membrane permeability, inhibit efflux pumps, and cause protein denaturation in microorganisms [43, 47]. Clearly, more screening of the phytochemicals found in clove is necessary for validation. Previous studies have documented synergistic effects between various antibiotics and crude plant extracts against multiple microorganisms [10, 29]. Nevertheless, research using EGAE-NLCs against multi-drug resistant isolates has not been conducted. This report is the first to investigate the use of endophytic fungi-based EGAE-NLCs as an innovative antimicrobial solution targeting MDR isolates, including *P. aeruginosa*, MRSA, and *C. albicans*, associated with oral infections. Our findings present a potent green antimicrobial agent that leverages the unique antimicrobial properties of eugenol and GA, serving as capping and stabilizing agents for EGAE-NLCs.

4. Conclusion

This research presented EGAE-NLCs derived from eugenol extracts as a potential antimicrobial solution for MDR isolates (*P. aeruginosa*, MRSA, and *C. albicans*). Our findings indicated that EGAE-NLCs hindered growth, prevented the formation of bacterial biofilms, and caused degeneration of bacterial membranes and cell walls. Various plant extracts show different levels of antimicrobial effects, which may result from the various phytoconstituents they contain, such as phenolic compounds, terpenoids, tannins, and alkaloids. Moreover, EGAE-NLCs appeared to enhance the healing process. Thus, our findings suggest that EGAE-NLCs could be a promising antimicrobial agent for treating and/or preventing MDR isolates by blocking essential virulence factors.

Author Statements:

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- **Author contributions:** M. T. Al. Musawi suggested the research idea, provided assistance, and reviewed and edited the study. for histological study. K. K. AL-Kinani suggested the research idea, provided assistance, and reviewed and edited the study for gel preparation and evolution. Y. S. Al. Jubouri prepared the samples, examined the writing and parameters, and examined the publication procedure.
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- **Data availability statement:** The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.
- **Declaration of the Authors**
  We hereby attest that every figure and table in the document belongs to us. Additionally, any figures and pictures that are not ours have been used with the required re-publication license, which is included in the manuscript.
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