

Biofilm Inhibition of Citrus Fruits against Nosocomial Pathogens: A Systematic Review

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ABSTRACT

Aim/Background: A mechanism common to nosocomial pathogens to survive on varying environments is the ability to form biofilms. Biofilms adhere to biotic or abiotic surfaces and house mono or poly cell communities and allow them to survive hostile environments. This systematic review intends to discuss biofilm inhibitory effects of various citrus fruits against nosocomial pathogens and its phytochemical contents. **Materials and Methods:** Studies focusing on biofilm inhibition in nosocomial pathogens, those that were able to inhibit the biofilm, citrus fruit available in the Philippines, studies that utilized solvent for obtaining the citrus extract, and those that were written in English were sought. All available findings obtained from the filtered studies under the said criteria were further analyzed, and then sorted out. **Results:** Of the seven studies, with one study utilizing two extracts; two extracts were able to completely inhibit biofilm growth at 100%; while one was able to inhibit at 90%; another was able to inhibit at 81%; followed by 68% inhibition; then two extracts were able to inhibit biofilm at 53.85%; lastly was at 50%. **Conclusion:** Rutin was shown to be able to downregulate *luxS* gene and *wabG* gene which are responsible for quorum-sensing and capsular polysaccharide respectively. Naringin exhibited reduce exopolysaccharide (EPS) and biofilm biomass. Lastly of the flavonoids, naringenin downregulates *tfB*, *gtfC*, *comD*, *comE*, and *luxS* expression which are essential in the formation of biofilm. Limonin, a type of limonoid, was suggested to target and disrupt cell-to-cell signaling of a biofilm. Limonene, a monoterpene, acts by reducing the attachment of a biofilm to surfaces.

Keywords: Biofilm, Biofilm inhibition, Nosocomial pathogens, Citrus, Phytochemicals.

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INTRODUCTION

Nosocomial pathogens are opportunistic organisms that exist within hospitals which could potentially induce diseases in patients or healthcare professionals during their stay within its vicinity, leading to hospital-acquired infection. On countries like the Philippines, nosocomial infection is common with an incidence rate ranging from 5.7% to 19.1%.^[1] With a low ratio of 13.5 hospital beds per 10,000 population in the NCR, the need to

reduce the sources of transmission is imperative.^[2] A common pathogen responsible to nosocomial infection is *Pseudomonas aeruginosa*; ST235 were isolated across 13 hospitals ($n=48$) in the Philippines— in which it is responsible for nosocomial infections worldwide.^[3]

One of the mechanisms that bacteria undergo is the formation of biofilm; wherein an aggregation of microbe communities consisting of extracellular polymeric molecules enables its cells to firmly adhere to living and nonliving interfaces and develop robust bonds, which allow them to survive hostile environments such as antibiotics and host defenses.^[4] The formation of biofilm allows the pathogen, or a community of pathogen, to survive and makes it highly resistant to antimicrobial agents.

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Citrus spp. has several bioactive compounds including those that are able to inhibit biofilm growth. Citrus extracts are a rich source of polyphenols, specifically flavonoids, which have been found to have a strong inhibitory activity against biofilm forming nosocomial pathogens. Flavonoids and limonoids are able to inhibit the formation of biofilms. Other than flavonoids, phenolic acids in citrus extracts, primarily chlorogenic and ferulic acids contribute to the destruction of a biofilm matrix.^[5]

This comprehensive review intends to discuss the inhibitory effect of various citrus fruits against biofilm forming nosocomial pathogens by assessing existing data from different database. With this, it will provide data and further knowledge on the possible uses of citrus fruits extract in inhibiting biofilm forming nosocomial pathogens, thereby adding to the information available to preventing the formation and growth of biofilm in the hospital setting and limiting the spread of diseases.

MATERIALS AND METHODS

Eligibility Criteria

The systematic review of Biofilm Inhibition of Citrus Fruits Against Nosocomial Pathogens includes experimental studies that met the following criteria: (1) Citrus fruits that were able to inhibit biofilm formation; (2) Biofilm forming nosocomial pathogens; (3) Studies that utilized a solvent for obtaining the citrus extract; (4) Studies that utilized citrus fruits that are available in the Philippines; (5) Studies published on credible search engines such as PubMed, Researchgate, Biomed Central, Google Scholar, and ScienceDirect; (6) Studies that are published in English; (7) Studies that are published in 2017 to present

Published studies that are excluded in this systematic review has one of the following criteria: (1) Studies utilized citrus fruits that are not available in the Philippines; (2) Studies that are published from predatory/non-credible search engines; (3) Studies produced in a foreign language aside from English; (4) Studies conducted before the year 2017; (5) Secondary resources such as review and critique paper.

Information Sources

The writing of this review was done following the preferred reporting items for systematic reviews and PRISMA guidelines. The authors made use of credible sites to retrieve studies regarding biofilm inhibition of citrus fruits against nosocomial pathogens. Related Literature was retrieved on credible search engines such as PubMed, Researchgate, Biomed Central, Google

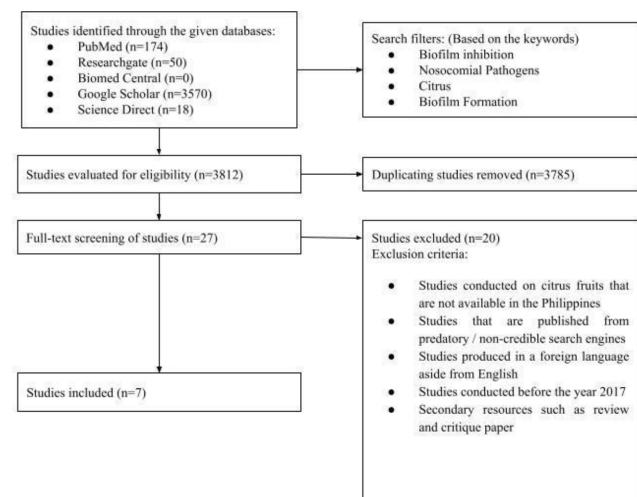
Scholar, and ScienceDirect. Additionally, documents published by the World Health Organization were used as valid sources. The authors thoroughly sorted and only considered research articles published from the year 2017 to 2022.

Search Strategy

Combinations of search terms including “Biofilm Inhibition”, “Nosocomial Pathogens”, “Phytochemicals”, “Citrus”, and “Biofilm Formation” were utilized in order to obtain studies focusing on the inhibition of various citrus fruit extract that are capable of inhibiting biofilm formation of nosocomial pathogens. Boolean operators “AND” and “OR” were included in the search terms if needed be. The authors reviewed the eligibility and discrepancies of the selected studies identified for inclusion and carefully screened the title and abstract of the research before full-text assessment of each potential eligible research article. For the processing and listing of the references, Zotero reference manager was utilized.

Selection Process

The authors reviewed the discrepancies and eligibility of each study by first assessing the title and the abstract of the research articles before evaluating the entirety of each article. All available findings obtained from the filtered studies under the said criteria were further analyzed to determine inhibition activity of *citrus* fruits against biofilm-forming nosocomial pathogens. The papers are then further sorted out, exempting papers that are non-experimental and other review papers or secondary references. Each reference was then assessed for eligibility following the said criteria. Duplicates and those that do not meet the criteria were removed. Paper



Prisma Diagram.

information such as (1) name of the author, (2) year of publishing, and (3) data relevant to the review were listed in Microsoft Excel for further analysis.

RESULTS

During the initial search, 3,812 articles were generated from the following search engines— PubMed, Researchgate, Biomed Central, Google Scholar, and ScienceDirect. A total of 27 studies were selected for full text screening, while 20 studies were excluded for the primary reason of not meeting the required criteria. The characteristics of the remaining studies are further explained in the following subsection. Shown on Table 1 are the summary of the findings of the studies utilized for the writing of this review.

Biofilm Inhibition of Citrus Extracts

Figure 1 shows the results gathered from 7 studies with their respective Citrus extract concentration and their corresponding percent inhibition with one study utilizing two different Citrus extracts. A concentration of 200 µg/mL (1/4 MIC) of rutin isolated from *Citrus sinensis* peels at a maximum reduction of biofilm by 50% of *Pseudomonas aeruginosa*.^[6] On another study that utilized

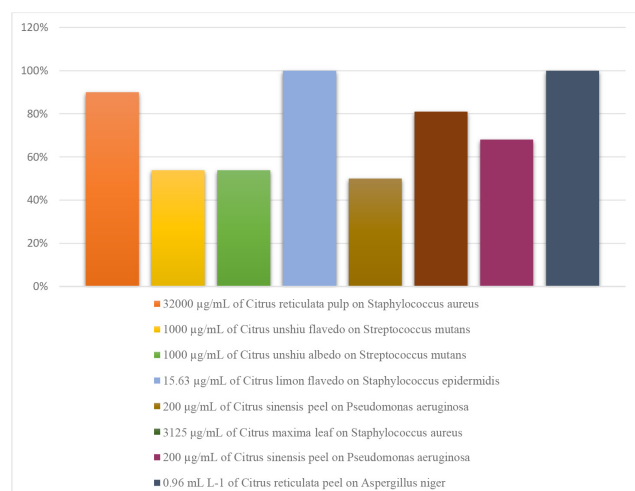


Figure 1: Biofilm Inhibition of Citrus Extracts.

Citrus sinensis peel extracts, it was able to inhibit various strains of ESBL-producing *P. aeruginosa* PS11 by 68% at 200 µg/mL.^[3] On a study that utilized *Citrus reticulata* pulp extracts, it was able to inhibit *Staphylococcus aureus* biofilm by 90% at a concentration of 32000 µg/mL.^[7] Similarly, *Citrus reticulata* peel extracts were able to inhibit *Aspergillus niger* biofilm by 100% at a concentration of 0.96 mL L⁻¹.^[8] Another citrus fruit that was shown is *Citrus unshiu* that both the flavedo and albedo showed biofilm inhibition by 53.85% at a concentration of 1000 µg/mL each on *Streptococcus mutans* biofilm.^[9] *Citrus limon* was able to completely eradicate *Staphylococcus epidermidis* biofilm with its flavedo essential oil by 100% at a concentration of 15.63 µg/mL.^[10] *Citrus maxima* leaf extracts were shown to be able to inhibit biofilm formation of *Staphylococcus aureus* at a concentration of 3125 µg/mL by 81%.^[11]

Citrus Extracts

Shown in Figure 2 are the results gathered that exhibiting the various part of citrus plant used as extracts against biofilm forming nosocomial pathogens. Of the different parts of a citrus fruit, the most part that was utilized and was shown on three studies was the peel by *C. sinensis*; on two studies was *C. reticulata*; two studies used the flavedo by *C. unshiu* and *C. limon*; the leaves were utilized once by *C. maxima*; the albedo by *C. unshiu*; and pulp by *C. reticulata*.

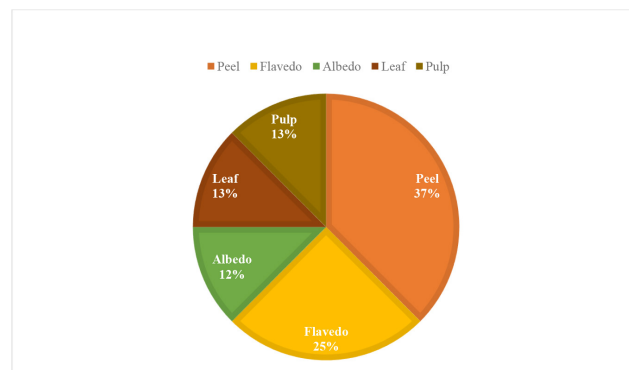


Figure 2: Citrus Parts.

Table 1: Summary of Findings.

Citrus Fruit	Fruit Extract	Target Biofilm	Result
<i>Citrus sinensis</i>	Peel	<i>Pseudomonas aeruginosa</i>	68% biofilm inhibition at 200 µg/mL concentration of the extract. ^[3]
<i>Citrus sinensis</i>	Peel	<i>Pseudomonas aeruginosa</i>	Maximum reduction of biofilm by 50% at a concentration of 200 µg/mL. ^[6]
<i>Citrus reticulata</i>	Pulp	<i>Pseudomonas aeruginosa</i>	Inhibition of biofilm was at 90% at a concentration of 32000 µg/mL. ^[7]
<i>Citrus limon</i>	Flavedo	<i>Staphylococcus epidermidis</i>	Total biofilm inhibition by 100% at a concentration of 15.63 µg/mL. ^[8]
<i>Citrus reticulata</i>	Peel	<i>Aspergillus niger</i>	Biofilm inhibition was at 100% at a concentration of 0.96 mL L ⁻¹ . ^[9]
<i>Citrus unshiu</i>	Flavedo Albedo	<i>Streptococcus mutans</i>	Biofilm inhibition was at 50% concentration of 1000 µg/mL. ^[10]
<i>Citrus maxima</i>	Leaf	<i>Staphylococcus aureus</i>	3125 µg/mL exhibited the highest activity at an 81% inhibition in biofilm. ^[11]

DISCUSSION

Studies published about citrus fruit extracts as a biofilm inhibiting treatment were assessed to evaluate the efficacy of such to inhibit different biofilm forming nosocomial pathogens. Presented data confirm the ability of various citrus fruits to be able to inhibit the growth and formation of biofilm forming nosocomial pathogens— of the seven studies that exhibited biofilm inhibition, two utilized *Citrus sinensis*, two used *Citrus reticulata*, then the remaining are *Citrus unshiu*, *Citrus limon*, and *Citrus maxima* plant extracts.

Of the collated studies, *C. sinensis* was able to inhibit the formation of biofilm. The study was able to show that at a concentration of 200 µg/mL (1/4 MIC) of rutin isolated from *C. sinensis* peels at a maximum reduction of biofilm by 50% of *Pseudomonas aeruginosa*.^[6] Upon quantifying the reduction of exopolysaccharides (EPS) on *P. aeruginosa*, the maximum reduction was at 40.16% when compared to the untreated control. As the EPS is essential for a biofilm to survive under varying conditions, a significant reduction to the EPS will lay the pathogen bare and expose the cells to antimicrobial treatment to act upon it. On a study that assessed the inhibitory ability of rutin on *Klebsiella pneumoniae* biofilm, it was shown that rutin possess the highest ability to scavenge free radicals among other flavonoid in that same study.^[12] The same study then assessed the mechanism of rutin in biofilm inhibition and it was shown that it downregulates *luxS* gene and *wabG* gene that functions for quorum-sensing and capsular polysaccharide respectively. As rutin disrupts the expression of *luxS*, it inhibits the cellular communication and downgrades the formation of biofilm leading to inhibition of growth. On another study that utilized *C. sinensis* peel extracts, it was able to inhibit various strains of ESBL-producing *P. aeruginosa* PS11 by 68% at 200 µg/mL.^[3] It was conferred that flavonoids found on the plant were able to inhibit cell-to-cell contact within the biofilm, which is essential for its survival, leading to inhibition of the formation of biofilm. In a study investigating the phytochemical profile of *C. sinensis*, it was shown that the peels were at least 98.7% flavonoid.^[13] *C. sinensis* peels being rich in flavonoid may be a factor to its biofilm inhibitory capabilities.

On a study that utilized *C. reticulata* pulp extracts, it was able to inhibit *Staphylococcus aureus* biofilm by 90% at a concentration of 32000 µg/mL.^[7] Among the other treatment utilized in the study, *C. reticulata* was able to inhibit the highest. Similarly, *C. reticulata* peel extracts were able to inhibit *Aspergillus niger* biofilm by 100% at a concentration of 0.96 mL L⁻¹.^[8] The limonene

constituent of *C. reticulata* played a major role in inhibiting the biofilm growth. Upon observation, it was seen that the extracts were able to considerably alter morphological characteristics and cell collapse of the biofilm when treated with the essential oil extract. Another finding was that the albedo has the most polyphenols among the extracts which may contribute to its effectivity in inhibiting biofilm. On a study that focused on the biofilm inhibitory activity of limonene, it was shown that it is efficient in inhibiting biofilm. In the presence of limonene, *Candida albicans* biofilm was reduced by 87%.^[14] Scanning electron microscope of biofilm treated by limonene shows that the structure and morphology was greatly damaged. Molecular mechanics/Poisson–Boltzmann surface area method showed that limonene has shown a high binding energy against Als3. Als3 comes from ALS gene family which plays a crucial role in the formation and growth of biofilm on surfaces.^[14]

Another citrus fruit that was shown to inhibit biofilm growth is *C. unshiu*. Among the extracts that were utilized, flavedo, albedo, fruit, leaf; it was shown that both the flavedo and albedo showed significantly greater biofilm inhibition by 53.85% at a concentration of 1000 µg/mL each.^[9] In the images of the *Streptococcus mutans* biofilm, it was shown that with the presence of the flavedo and albedo extract was able to obscure the biofilm and damage the cell surface of *S. mutans*. Most citrus peels contain the highest number of phenolic compounds than other parts of the fruits.^[15] *C. unshiu* flavedo and albedo contain high amounts of hesperidin, narirutin, rutin, naringin, naringenin, and hesperetin. The activities of the said phytochemicals are beneficial to one's health.^[16] Naringenin, a type of flavonoid, was shown to downregulate several gene relating to biofilm structure of *S. mutans* such as *tfb*, *gjfC*, *comD*, *comE*, and *luxS* expression.^[17] Another type of flavonoid found in *C. unshiu* flavedo and albedo were naringin. It was observed that naringin was able to reduce and disturb *Pseudomonas* spp. EPS leading to inhibition of biofilm.^[18] It was also shown that when observed under scanning electron microscope, there was visible decrease in the biofilm aggregation and disturbed biofilm morphology. The mechanism of naringin to disturb the EPS of biofilms is an essential finding as the EPS are the structure that protects biofilm from external stress and antimicrobial agents. Reduction in the EPS interferes the biofilm matrix and can synergize the effects of antimicrobial agents as it can easily enter and attack the cellular components inside the biofilm.

C. limon, locally known as lemon or *dayap*, was able to completely eradicate *Staphylococcus epidermidis* biofilm

with its flavedo essential oil by 100% at a concentration of 15.63 µg/mL.^[6] It was hypothesized that the D-limonene constituent of *C. limon* was able to pose antibiofilm activity on *S. epidermidis*. Although there may be more compounds present on the essential oil as it has a complex chemical composition thus the conclusion is not limited to D-limonene. Limonene, the major component of the extract from the essential oil of *C. limon*, has been attributed as the key to the inhibition of biofilm formation.^[6] A surface-coating experiment revealed that limonene works by reducing bacterial attachment to surfaces, blocking subsequent biofilm development pathways.^[19] Although limonene extracts from *C. limon* were found to have only a moderate effect (MBEC = 15.63–62.50 µg/mL) against the growth of the biofilm of *S. epidermidis*. Analysis showed that the gram-positive peptidoglycan cell wall of the *S. epidermidis* allows hydrophobic molecules to penetrate its membrane. The limonene together with the hydrophobicity of the essential oil allows for biofilm inhibition. It is carried out by the disintegration of the cell wall and disruption of the respiratory chain that triggers the leakage of important cell contents.^[20]

Another citrus fruit, *C. maxima*, was shown to be able to inhibit biofilm formation. It was shown that at a concentration of 3125 µg/mL, *C. maxima* leaf extracts were able to inhibit *Staphylococcus aureus* biofilm by 81%.^[11] *C. maxima* leaf extracts inhibits the biofilm at an increasing concentration and was able to completely inhibit at 3125 µg/mL. In the same study, the diameter of zones of inhibition produced by varying concentrations was also measured to evaluate the relationship between the volume of concentration and the number of clinical isolates. It was revealed that as the concentration of the methanol leaf extract of *C. maxima* increases, the diameter of zones of inhibition also increases. A study conducted with the same principle also showed this kind of activity, indicating that the relationship between the diameter of the inhibition zone and the concentration of extracts is proportional.^[21] The biofilm inhibitory activity of methanol leaf extract of *C. maxima* may be due to its rich source of phytochemicals such as alkaloids, flavonoids, terpenoids, tannin, phenol and glycosides which are known to have antibiofilm properties. Flavonoids are the main phytochemical component of *C. maxima* not just in its leaves but also in peel and pulp. It is commonly present in the form of glycosides (phloridzin, myricitrin, hesperidin) and aglycones (phloretin, hesperitin, myricetin). Available studies have demonstrated that the aglycone form of flavonoids exhibits a higher antioxidant effect than the glycone forms, however, their inhibitory effects against

biofilm formation are still not available.^[22] Another reason for the effective inhibition of biofilm growth by the methanol leaf extracts, may be also that the bacterial specie used in the study was Gram-positive bacteria, which means that it only has an outer peptidoglycan layer which is not an effective permeability barrier, thus allowing the extracts to penetrate its cell membrane easily.^[21]

Among the seed, pulp and peels; the peels of *C. sinensis* have shown to have the highest amount of bioactive compound and is mostly represented by flavonoids by 98.7%.^[13] Next to the peels are the pulp which contain rich amounts of flavonoid, particularly hesperidin. Of the biofilm inhibitory ability of hesperidin, it was particularly able to inhibit biofilm formation.^[23] Hesperidin was able to disrupt aerogenesis, which is a key factor in the formation of biofilm. Another mechanism of hesperidin was that it was able to downregulate the gene that regulates and activates biofilm formation, *sarA*. Another finding was that hesperidin downregulated *icaA* and *icaD* expression, which are key genes in production of the EPS on biofilms. Pertaining to the phytochemical analysis of *C. sinensis*, the seeds were found to contain the highest amount limonoids— and was mostly limonin.^[13] On the mechanism of limonin, a study has suggested that it acts by disrupting cell-to-cell signaling, thus inhibiting biofilm formation.^[24]

Strengths in this review rely on the objective inhibitory activity of citrus fruits against biofilm forming nosocomial pathogens. This systematic review provides an update and summary on the potency of citrus fruit extracts as a biofilm inhibiting treatment against nosocomial pathogens. The presence of various phytochemicals on citrus, especially on the peel, have shown to be correlated to biofilm inhibition. The studies included demonstrated evidence that could further be modified and utilized in the clinical setting, by formulating and manufacturing byproducts that can prevent the growth of the said microorganisms, which are not only acquired in the hospital, but also in the environment and community.

CONCLUSION

Nosocomial pathogens that exist within the hospital pose a huge threat for people seeking for the betterment of their health. Formation of biofilm by these nosocomial pathogens encourage these pathogens to thrive and survive in varying conditions. Citrus fruits are shown to be able to inhibit biofilm by disrupting different biochemical functions and targeting the biofilm matrix

due to the presence of bioactive compounds. The findings in this review confirms that extracts and from various citrus fruits were able to inhibit biofilm by 50%-100%—with *C. limon* flavedo and *C. reticulata* peel being able to inhibit biofilm by 100%. Several phytochemicals and compounds are found to be contributing to the inhibitory properties of the extracts such as flavonoids (rutin, naringin, and naringenin), limonoids (limonin), and limonene; that are able to disrupt several biofilm mechanisms and leads to biofilm inhibition. Rutin was shown to be able to downregulate *luxS* gene and *wabG* gene which are responsible for quorum-sensing and capsular polysaccharide respectively. Naringin exhibited reduce EPS and biofilm biomass. Lastly of the flavonoids, naringenin downregulates *tffB*, *gtfC*, *comD*, *comE*, and *luxS* expression which are essential in the formation of biofilm. Limonin, a type of limonoid, was suggested to target and disrupt cell-to-cell signaling of a biofilm. Limonene, a monoterpene, acts by reducing the attachment of a biofilm to surfaces.

RECOMMENDATIONS

The authors would like future researchers to explore the utilization of peel extracts of citrus fruits on the inhibition of biofilm. That aside from biofilm inhibitory concentration, minimum biofilm inhibitory and eradication concentration must also be given focused so that the best method to monitor and assess biofilm inhibition is fully analyzed and evaluated to develop a more sophisticated conclusion. Lastly, focusing on the found chemicals and compounds in this study and their synergistic effect on biofilm should be explored.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

EPS: Exopolysaccharide; **NCR:** National capital region; ***C. sinensis:*** *Citrus sinensis*; ***P. aeruginosa:*** *Pseudomonas aeruginosa*; ***C. reticulata:*** *Citrus reticulata*; ***C. limon:*** *Citrus limon*; ***S. epidermidis:*** *Staphylococcus epidermidis*; ***A. niger:*** *Aspergillus niger*; ***C. unshiu:*** *Citrus unshiu*; ***S. mutans:*** *Streptococcus mutans*; ***C. maxima:*** *Citrus maxima*; ***S. aureus:*** *Staphylococcus aureus*; **MIC:** Minimum inhibitory concentration; **ESBL:** Extended-Spectrum beta-lactamases.

SUMMARY

Citrus spp. has shown to inhibit biofilm growth and formation by nosocomial origin. As nosocomial pathogens pose a huge threat to immunocompromised patients and people subject to invasive medical procedures, it is detrimental to a patient's health. Such nosocomial pathogens have developed an ability to adhere to abiotic surfaces and survive hostile environments such as antibiotics and host defenses. Journals collated from various databases were assessed to accomplish the criteria set by the authors. Studies were focused on *Citrus* spp. that were able to inhibit growth and formation of biofilm forming nosocomial pathogens. The results were as follow: two extracts were able to completely inhibit biofilm growth at 100%; while one was able to inhibit at 90%; another was able to inhibit at 81%; followed by 68% inhibition; then two extracts were able to inhibit biofilm at 53.85%; lastly was at 50%. Several phytochemicals and compounds are found to be contributing to the inhibitory properties of the extracts such as flavonoids (rutin, naringin, and naringenin), limonoids (limonin), and limonene; that are able to disrupt several biofilm mechanisms and leads to biofilm inhibition.

Authors' Contributions

The authors were able to divide the work in writing this paper. Each author has individually contributed to the accomplishment and completion of the paper. Authors A, F, G, H worked on the writing of the introduction. The methodology was accomplished by Author A, B and C. Author D constructed the Prisma Diagram. Authors A, D, E, F, G, H participated in forming the results. The writing of the discussion was done by all authors. Author A constructed the abstract, conclusion, and recommendations, and was responsible for the citation and proper referencing. Lastly, Author I was

responsible for supervising and guidance throughout the journey of writing this paper.

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