

# The Antibacterial Activity of Isolated Flavonoid Fractions from Ethanol Ethanolic Peel of *Citrus Sinensis* (Valencia Orange) with *Citrus Limon* (Lemon) against *Staphylococcus Aureus* and *Pseudomonas Aeruginosa*

Nik Nur Shamiha N. D.<sup>1,2\*</sup>, Ghayatery Nagatamby<sup>1</sup>, Fadli Asmani<sup>1</sup>, Eddy Yusof<sup>2</sup>

<sup>1</sup>School of Pharmacy, Management and Science University,

40100 Shah Alam, Selangor Darul Ehsan, Malaysia

<sup>2</sup>ICHLAS, Management & Science University,

40100 Shah Alam, Selangor, Malaysia

**Keywords:** Citrus sinensis; Citrus limon; Combination antibacterial activity; Flavonoid; Skin and soft tissue infections (SSTIs)

**Abstract:** Background: Skin and soft tissue infections (SSTIs) are most common infections encountered by all physicians. Eventhough pharmacological industries have produced a number of new antibiotics, resistance to these drugs by microorganisms has increased. Objective: The aim of this study is to evaluate the antibacterial activity of isolated flavonoid fractions from ethanolic peel extract of *C.sinensis* (Orange), *C.limon* (Lemon) and its combination against *S.aureus* and *P.aeruginosa*. Methods: The ethanolic peel extract of both plant were screened for phytochemical identification of flavonoid by lead acetate test and shinoda test. The extract of both plants were evaluated for preliminary antibacterial activity using disk diffusion method. Thin-layer chromatography and column chromatography was performed to isolate flavonoids. Isolated flavonoids were subjected to determination of minimum inhibitory concentration and antibacterial assay by disk diffusion method. Results: Isolated flavonoids of *C.sinensis* (17mm, 8mm), *C.limon* (20mm, 9mm) and its combination (24mm, 14mm) produced antibacterial activity that is comparable to the Ciprofloxacin disc (30mm, 9mm) against *S.aureus* and *P.aeruginosa* respectively. Thus, these results suggested that *C.limon* produced a better antibacterial activity against both bacteria compared to *C.sinensis*. However, the combination of both plants isolated flavonoid fractions produced much better antibacterial activity against *S.aureus* and *P.aeruginosa* in comparison with individual flavonoid fractions of both plants. Conclusion: Therefore, Citrus fruits peels that is being as primary waste in juicing industries can be further developed as marketable natural source of antibiotic as a treatment of SSTIs.

## 1 INTRODUCTION

Skin and soft tissue infections (SSTIs) are reflect inflammatory microbial invasion of the epidermis, dermis and subcutaneous tissues (Matthew S.Dryden, 2010). Some skin colonizing microorganisms, in particular *Staphylococcus aureus* (*S.aureus*) and  $\beta$ -haemolytic group A streptococci but also Gram-negative bacteria such as *Pseudomonas aeruginosa* (*P.aeruginosa*), viruses and fungi, have the potential to cause infection, particularly when the skin barrier is breached (Lacey et al., 2015). SSTIs are treated by using antibiotics. The problem arises as these bacteria are developing

resistance strains against antimicrobial agents as a result, there is an urgent need to find an alternative way to cure the bacterial infections. Plant would be a better alternative because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism and it has lesser side effects compared with synthetic antibiotics (Kailash D. Sonawane et al., 2011).

Citrus fruits have been of interest for extraction of antimicrobial metabolites by large numbers of researchers but the peels are less studied (Akhilesh et al., 2012). Moreover, the peels part of citrus fruits is abundance with secondary metabolites which serves as plant defense mechanisms against predation by microorganisms, insects, and herbivores (Xinmiao Lv et al., 2015). Among the

secondary metabolites, flavonoids are known for its antimicrobial properties. Since, they are known to be synthesized by plants in response to microbial infection, it should not be surprising that they have been found in vitro to be effective antimicrobial substances against a wide array of microorganisms (Xinmiao Lv et al., 2015).

There are previous studies on antimicrobial activity of essential oil from peels of orange and lemon against several microorganisms (Shalu Hasija et al., 2015). However, there are lack of studies on antibacterial activity of flavonoid isolated from peels of orange and lemon. Moreover, there are no studies on combined antibacterial activity of flavonoid isolated from orange peel and lemon peel. Hence, this study is mainly focused on investigating the antibacterial activity of combined flavonoid fractions from ethanolic peel extract of C.sinensis with C.limon against a gram-positive organism (S.aureus) and a gram-negative organism (P.aeruginosa) that is linked with SSTIs.

## 2 METHODOLOGY

### 2.1 Collection and Preparation of Plant Materials

The peels of lemon and oranges part was separated from the fruits. The peels were air-dried at room temperature for almost a week until constant weight. Then, it was grinded into coarse powder by using a mechanical blender (Sapna B.Shetty et al., 2015).

Both peels' powder was macerated for a week with 96% ethanol. Filtered and evaporated the solvent by using rotary evaporator to obtain a concentrate extract of the peels. The extract were stored in cold room until further use (Augustine Ahmadu & Ufuoma Omonigho, 2013).

### 2.2 Preliminary Screening for Flavonoid

Both of the plants extract were subjected for two tests: Lead acetate and Shinoda test, for identification of flavonoids (Sangha R. Bijeka et al., 2015).

### 2.3 Preliminary Antibacterial Assay

Both of the plants extract was tested on its antibacterial activity against S.aureus and P.aeruginosa by disk diffusion method with three

different concentration (1mg/mL, 10mg/mL and 100mg/mL).

### 2.4 Thin-layer Chromatography (TLC)

TLC was conducted on both plants extract by using five different solvent mixture (Oyvind M. Andersen & Kenneth R. Markham, 2006) as described in Table 1.

Table 1: Different solvent mixture for TLC.

No	Solvent mixture
1	Chloroform-Acetic acid 100:4
2	Chloroform-Methanol-Acetic acid 90:5:5
3	Chloroform-Methanol-Water 40:10:1
4	Chloroform-Methanol-Water 65:45:12
5	Ethyl acetate-Methanol-Water 50:3:10.

TLC was conducted to determine the number of flavonoid components in both plants extract and also to identify solvent mixture that provide best fractionation or separation between those components (Abe Rita Temidayo, 2013).

### 2.5 Column-chromatography (Fractionation/Isolation of flavonoid)

Best solvent mixture identified from TLC was used for isolating flavonoid fractions from both plants extract (Abe Rita Temidayo, 2013). Chloroform-Acetic acid 100:4 solvent system was used for C.Limon while Chloroform-Methanol-Water 40:10:1 was selected for C. Sinensis. Silica gel was used as adsorbent. Fractions were collected in several test tubes and tested on TLC to ensure all the flavonoid components are collected. Moreover, the collected fractions were also tested on lead acetate test again to ensure it is flavonoid fractions.

### 2.6 Determination of Minimum Inhibitory Concentration (MIC)

Stock solution of flavonoid fraction was prepared for both plants and its combination: 100mg/ml. Then, from this stock solution transferred 1ml into test tube that contains 1ml ethanol (50mg/ml), these procedure were repeated until several dilution from 25 mg/ml - 0.2mg/ml. Each concentration was tested on selected bacteria by disk diffusion method. The first lowest concentration that produced zone of inhibition represent the MIC.

## 2.7 Antibacterial Assay

The in- vitro antibacterial activities was carried out using the disk diffusion method. Mueller Hinton Agar (MHA) plate was inoculated with selected bacteria; *S.aureus* and *P.aeruginosa*. The MHA was prepared and stored at 4°C. Three different concentration of flavonoid fraction were placed on the plate (lemon, orange and its combination). Positive control was Ciprofloxacin and negative control was Ethanol. Plates were incubated at 37°C for 24 hours in the incubator. Zone of inhibition was measured and repeated for three times.

## 3 RESULTS AND DISCUSSION

Both plants peel extracts showed positive result for flavonoid screening by producing yellow precipitate (Lead acetate test) and orange (*Shinoda* test). The result of preliminary antibacterial assay was, both plants peel extracts showed antibacterial effect against *S.aureus* and *P.aeruginosa* except *C.limon* extract does not show any antibacterial effect against *P.aeruginosa* at lowest concentration (1mg/mL). Upon TLC for both plants extract on different solvent mixture (Table 1), solvent mixture number 3 was selected for orange while solvent mixture 1 was selected for lemon to isolate its flavonoid components. Solvent mixture was chosen based on two factors which are good retention factor (0.25 – 0.35) and spot on TLC (considerable distance between one spot to another). Table 2 and Table 3 shows the result of isolating flavonoid fractions from orange and lemon peel extract respectively.

Table 2: Flavonoid fractions of *C.sinensis* peel extract (orange)

Fractions ( <i>C.sinensis</i> )	Flavonoid test (Lead acetate test)
1-5 (Presence spot)	Yellow precipitate (Component A)
11-15 (Presence spot)	Yellow precipitate (Component B)

Table 3: Flavonoid fractions of *C.limon* peel extract (Lemon).

Fractions ( <i>C.limon</i> )	Flavonoid test (Lead acetate test)
1-5 (Presence spot)	Yellow precipitate (Component A + B)
12-14 (Presence spot)	Yellow precipitate (Component C)
17-19 (Presence spot)	Yellow precipitate (Component D)
21-23 (Presence spot)	Yellow precipitate (Component E)

Table 4 shows the MIC of flavonoid fractions from the peel extract of orange, lemon and its combination respectively against the selected bacteria. The result is suggestive that when the flavonoid fractions are combined (lemon with orange), a lesser concentration of flavonoid is needed to inhibit the growth of both *S.aureus* and *P.aeruginosa* than individual flavonoid fraction of orange and lemon. Furthermore, it is noted that a higher concentration of flavonoid is needed to inhibit growth of *P.aeruginosa* compared to *S.aureus*.

Table 4: MIC of flavonoid from orange, lemon and its combination.

Microor- ganism	MIC (mg/mL)		
	<i>C.sinensis</i>	<i>C.limon</i>	Combi- nation
<i>S.aureus</i>	12.5	0.78	0.2
<i>P.aeruginosa</i>	50	50	25

Figure 1 shows graph represent comparison of flavonoid fractions antibacterial activity against *S.aureus* and *P.aeruginosa*. All three flavonoid fractions produced antibacterial effect against *S.aureus* and *P.aeruginosa*. It is noted that antibacterial effect of flavonoid against *S.aureus* is more compared to *P.aeruginosa*. It is associated with impermeability of gram-negative bacteria (*P.aeruginosa*) towards antimicrobial agents, in this case, it is flavonoids. The wall structure of Gram-negative bacteria, and specifically the presence of an outer envelope, is often responsible for the impermeability of these micro-organisms to antimicrobial agents (S.P. Denyer & J.Y. Maillard, 2002). When the antibacterial effect of *C.sinensis* and *C.limon* is compared, *C.limon* produced a better antibacterial effect against both bacteria. This can be due to presence of more flavonoid components in *C.limon* (Component A-E) than *C.sinensis* (Component A & B). Moreover, the combination of both plants flavonoid produced a further increase in antibacterial effect against *S.aureus* and *P.aeruginosa*. Combined flavonoid fractions produced an antibacterial effect against *S.aureus* (24mm) which was nearly equal to positive control, Ciprofloxacin disc (30mm). Both flavonoid fraction of *C.sinensis* (8mm) and *C.limon* (9mm) produced effective antibacterial effect against *P.aeruginosa* comparable to positive control (9mm). Also it was found that combination of *C.sinensis* and *C.limon* flavonoid fractions produced an antibacterial effect

against *P.aeruginosa* (14mm) better than the positive control (9mm).

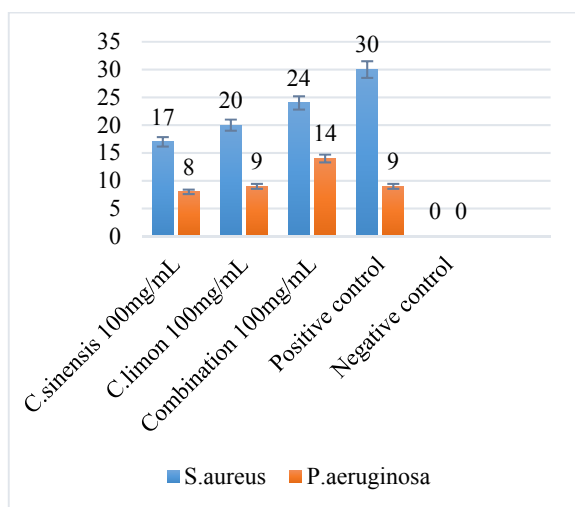


Figure 1: Graph that compare the antibacterial activity of flavonoid fractions from orange, lemon and its combination against *S.aureus* and *P.aeruginosa*.

## 4 CONCLUSIONS

This research has found that flavonoid fractions of all three (*C.sinensis*, *C.limon* and its combination) significantly ( $p < 0.05$ ) produced inhibitory effect against *S.aureus* and *P.aeruginosa*. Thus, *C.sinensis*, *C.limon* and its combination holds promise as a potential to be developed into solely herbal based antimicrobial agent to cure SSTIs.

## ACKNOWLEDGEMENTS

The author is very thankful and grateful towards the research committee and lecturers of Management and Science University, Malaysia for providing all the needed materials, equipment as well continuous guidance and support throughout completing this research project.

## REFERENCES

Akhilesh Khushwaha, Raghvendra Pratap Singh, Vikas Gupta, and Madhulika Singh. (2012). Antimicrobial properties of peels of Citrus fruits. International Journal of Universal Pharmacy and Life Sciences 2(2).  
 Augustine A Ahmadu and Ufuoma Omonigho (2013). Flavonoids from the leaves of *Physalis angulata* Linn.

African Journal of Pharmaceutical Research & Development, 5, 40-43.  
 Kailash D. Sonawane, Maruti J. Dhanavade, Chidamber B. Jalkute, and Jai S. Ghosh (2011). Study Antimicrobial Activity of Lemon (Citrus lemon L.) Peel Extract. British Journal of Pharmacology and Toxicology 2(3): 119-122, 2011.  
 Lacey, K. A., Geoghegan, J. A., & McLoughlin, R. M. (2016). The Role of *Staphylococcus aureus* Virulence Factors in Skin Infection and Their Potential as Vaccine Antigens. Pathogens (Basel, Switzerland), 5(1).  
 Matthew S.Dryden. (2010). Complicated skin and soft tissue infection. J Antimicrob Chemother 2010; 65 Suppl 3: iii35-44.  
 Oyvind M. Andersen & Kenneth R. Markham. (2006). Flavonoids: Chemistry, Biochemistry and applications. S.P. Denyer and J.-Y. Maillard. (2002). Cellular impermeability and uptake of biocides and antibiotics in Gram-negative bacteria. Journal of Applied Microbiology Symposium Supplement 2002, 92, 35S-45S.  
 Sangha R Bijekar, M.C Gayatri, L Rajanna. (2015). Antimicrobial Activity of Isolated Flavonoid Fractions from Drypetes Roxburghii (Wall.) Huresawa and Its Phytochemical Fingerprinting. International Journal of Innovative Research in Science, Engineering and Technology, 4, 6214-6224.  
 Sapna B. Shetty, Prabu Mahin-Syed-Ismail, Shaji Varghese, Bibin Thomas-George, Pathinettam KandathilThajuraj, Deepak Baby, Shaista Haleem, Sreeja Sreedhar, Darshan Devang-Divakar. (2016). Antimicrobial effects of Citrus *sinensis* peel extracts against dental caries bacteria: An in vitro study. Journal of clinical and experimental denstistry.  
 Shalu Hasija, Geeta Ibrahim, and Ashok Wadia. (2015). Antimicrobial Activity of Citrus Sinensis (Orange), Citrus Limetta (Sweet Lime) and Citrus Limon (Lemon) Peel Oil on Selected Food Borne Pathogens. International Journal of Life Sciences Research, 3, 35-39.  
 Xinmiao Lv, Siyu Zhao, Zhangchi Ning, Honglian Zeng, Yisong Shu, Ou Tao, Cheng Xiao, Cheng Lu, and Yuanyan Liu (2015). Citrus fruits as a treasure trove of active natural metabolites that potentially provide benefits for human health. Chemistry Central Journal, 9, 68.