Potency of *Citrus reticulata* Peel Extract as Active Compound of Non-Alcohol Based Gel Hand Sanitizer

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Abstract

Hand sanitizer is nowadays known as a part of one's personal hygiene kit because of its practical use and effectivity against skin microbes, such as *Staphylococcus aureus*. The common commercial hand sanitizers are made from alcohol which may have negative side effect like skin irritation. Utilization of active compounds from a plant that has antibacterial compounds might be applied to the substitution of alcohol in the formulation of hand sanitizer. In this study, phytochemical compounds from *Citrus reticulata* and *Citrus aurantifolia* peel extracts were tested qualitatively and their antibacterial acitivity on *Staphylococcus aureus* were tested using disk diffusion method. The results showed that *Citrus reticulata* peel extract at the concentration of 70% was the most effective concentration in inhibiting *Staphylococcus aureus*. This certain concentration of *Citrus reticulata* peel extract was then chosen in the gel hand sanitizer formulation. Application of gel hand sanitizer with *Citrus reticulata* peel extract as its active compound on the hands of respondents were also observed to inhibit bacterial growth. The use of peel extracts from Citrus spp, especially from *Citrus* reticulata might be potential in the formulation of non-alcohol based gel hand sanitizer.

Keywords: *Citrus reticulata, e*xtracts, antimicrobial, *Staphylococcus aureus,* hand sanitizer.

Introduction

Nowadays, people are familiar with instant lifestyle, including the application of personal hygiene that aimed to prevent contagious and opportunistic pathogen to induce infection. One example of personal hygiene practiced by people is shown by the use of hand sanitizer as an alternative way to clean hands, despite of using water and soap. As a commercial product, hand sanitizers are largely available in the market and considered to be effectively worked due to its alcohol content. Unfortunately, the application of alcohol based hand sanitizer may cause skin irritation, therefore an alternative strategy to search for non-alcohol based hand sanitizer should be done.

Indonesia was granted with abundant natural resources, such as plant-based resources that are potential to be used as antimicrobial compounds. These antimicrobial compounds in medicinal plants is referred to secondary metabolites. (Schiff *et al*., 2012). Secondary metabolite compounds in plants generally consist of flavonoids, alkaloids, saponins, steroids, tannins, and terpenoids (Ergina, 2014). There are several species of Genus *Citrus* plants, which are were known as oranges, limes and lemons, including *Citrus reticulata* and *Citrus aurantifolia* that are able to inhibit the growth of bacteria that cause skin infections. (Ayoola *et al*. 2008; Lauma, *et al* 2015). Both of these Citrus species are common and easy to find in the daily life. Research to study about active compounds content of these Citrus species that has any antimicrobial activity, especially against opportunistic *Staphylococcus* spp *(*Rahmi *et al,* 2015), is reasonable to be conducted in order to provide antimicrobial compound from cheap and abundant sources. This research was aimed to study about antibacterial potencies of *Citrus reticulata* and *Citrus aurantifolia* peel extracts and their possibilities to be used in the formulation of non-alcohol based gel hand sanitizer.

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Material and Methods

Material

Materials from the study consist of each 1 kg of mandarin orange (*Citrus reticulate*) peel and lime orange (*Citrus aurantifolia*) peel. It is important to note that peels of both

Citrus species were collected from waste product of culinary activities. Peels were then dried up at a temperature of 60-80^{0}C. Dried simplisia was crushed with a blender to get fine powder.

Methods

Extraction of Active Compound from C. reticulata and C. aurantifolia

Blended simplisias in the form of fine powder were placed into a glass jar. Simplisias were macerated for 7 days using 96% ethanol solvent in a ratio of 1: 5 (w / v), in which the weight of each simplisias was 200 g each and the volume of ethanol was 1000 ml. The extracts were filtered with a cloth filter and separated onto filtrate and residue. Filtrate was put into a jar and evaporated with an evaporator at a temperature of 50^{0}C until a thick extract from each fruit peel was formed. Yield was calculated using Equation 1 (Eq. 1).

$$\%\text{Yield} = \frac{\text{The weight of obtained extract (g)}}{\text{The initial weigt of simplisia}} \times 100\% \quad \text{(Eq.1)}$$

Phytochemical Compound Screening

Each crude extract of fruit peel was tested by a qualitative test of phytochemical compounds such as Alkaloids, Flavonoids, Saponins, Steroids, Tannins and Terpenoids (Harborne, 1987).

Alkaloid Assay

Zero point three (0.3) g of orange peel extract was added with 5 ml of 2N HCl and heated for 2-3 minutes. After chilling, 0.3 g of NaCl and 5 ml of 2N HCl were added. Next, serial tube labelled as A1, A2, and A3 was prepared. Solution was divided onto tubes labelled as A1, A2 and A3. The A1 tube was used as a blank, the A2 tube was added with 3 drops of Mayer reagent, and A3 tube was added with 3 drops of Wagner reagent. An indication of the presence of alkaloids was characterized by the presence of feculent solutions.

Flavonoid Assay

Zero point three (0.3) g of orange peel extract was added with 3 ml of n-hexane and stirred until dissolved. Next, 20 ml of 96% ethanol was added and solution was divided into 3 tubes (B1, B2, and B3). Tube B1 contained a blank, 0.5 ml of concentrated HCl was added onto tube B2 and heated in a waterbath. Color changes indicated the presence of leukoanthocyanin compounds. Zero point five (0.5) ml of HCl and small pieces of magnesium tape were added into tube B3. A change in color to orange in B3 tube indicated the presence of flavonols, while a red color indicates the presence of flavonone compounds.

Saponin Assay

Zero point three (0.3) g of orange peel extract was added with 5 ml of distilled water and shaken vigorously for 30 seconds. If a stable froth formed for more than 30 seconds, it indicates the presence of saponin in the extract.

Steroid and Terpenoid Assay

Zero point three (0.3) g of extract was added with 15 ml of ethanol, homogenized and divided into three tubes : C1, C2, and C3 tubes. C1 solution was used as a blank. C2 solution was added with 3 drops of anhydrous acetic acid and 1 drop of concentrated H_2SO_4 then shaken slowly. A positive steroid result was marked by a discoloration to blue or green. A positive triterpenoid result is marked by a red or purple discoloration. Positive results of saturated steroids are characterized by yellow discoloration. C3 solution was added with 1-2 ml concentrated H_2SO_4 through the tube wall. Unsaturated steroids are characterized by the formation of red rings.

Tannin Assay

Zero point three (0.3) g of each extract was added with 10 ml of hot distilled water then homogenized, 3-4 drops of 10% NaCl was added and homogenized. Solution was divided into 3 tubes : D1, D2, and D3. The D1 tube was used as a blank. Solution in D2 tube was added with 1-2 drops of 1% gelatin solution and 5 ml of NaCl 10%. The presence of white sediment indicates the presence of tannins. If no white precipitate was formed and the addition of $FeCl_3$ solution change the color from green-blue to black, it indicates the

presence of polyphenol compounds. Solution in D3 tube was added with 3 drops of $FeCl_3$ solution and color changes were observed. The change in color from green blue to black indicates the presence of tannin compounds.

Antibacterial Activity Assay

Antibacterial activity of *C. reticulata and C. aurantifolia* peel extracts was performed using disk-diffusion method. Twenty microliter (20 µl) of crude peel extracts or sample were absorbed using sterile Whatmann No.1 filter paper (6 mm diameter) and dried up on sterile empty petri dishes. Twenty milliliter (20 ml) of Mueller Hinton Agar (MHA) was poured onto petri dishes and let them until being condensed. The bacterial culture with density of 10^8 cells / ml was inoculated onto MHA in petri dishes using cotton swab, then allowed to dry for 5 minutes. Pieces of Whatmann paper that contain orange peel extract were then placed on the MHA. Petri dishes would be then incubated at 37°C for 24 hours. Antibacterial activities of extracts were observed by the presence of clear zones (inhibition zones) surrounded Whatmann filter paper on the medium. Inhibition zone diameters were measured in millimeters using a ruler. It should be noted that antibacterial activity assay were performed in two step : first step aimed to test antibacterial activity of oranges extracts (single extract and combined extract) and the second step purposed to test antibacterial activity of formulated hand sanitizer that contained those oranges extracts.

Formulation of Gel Hand Sanitizer

Carbopol was developed in distilled water for 24 hours. Zero point zero six gram (0.06 g) of propyl paraben was dissolved in beaker glass using 15 ml of propylene glycol. These solution would be then mixed with carbopol, and homogenized using a mixer for 1 minute. Each orange peel extract was put into the mixture and stirred until homogeneous. Eight drops of triethanolamine were added to the gel preparation until pH value became 6. Formulation of gel hand sanitizer gel was shown in Table 1.

Table 1. Formulation of gel hand sanitizer

Composition	Formula
Peel Extract	5 g
Carbopol	0.7 g
Triethanolamin	8 drops
Propil paraben	0.06 g
Propilen glikol	15 g
Aquades *ad*	100 ml

Gel hand sanitizer products were evaluated based on organoleptic (color, aroma, and texture), then tested for its pH value, dispersion, and stickiness based on SNI 06-2588-1992 standard.

Result

Extraction of Active Compound from C. reticulata and C. aurantifolia

Yield of crude oranges peel extract is shown in Table 2. Yield of *C. aurantifolia* extract that was obtained in this research was higher than the yield of *C. reticulata* extract.

Tabel 2. Yield value of *C. reticulata* dan *C. aurantifolia.*

Species	Weigth of Simplisia (g)	Weight of Extract (g)	Yield (%)
C. reticulata	1709	291.3	17.04
C. aurantifolia	869.441	153.080	17.60

Phytochemical Screening

Alkaloid, Flavonoid, Saponin, and Tanin compounds were detected in peel extract of *C. reticulata* and *C. aurantifolia*. It was observed that *C. reticulata* has triterpenoid compounds but *C. aurantifolia* has not. On the other hand, *C. aurantifolia* contains steroid, but not *C.reticulata*. The result of phytochemical assay iss shown in Table 3.

***Antibacterial Activity Assay of Single C. reticulata* and *C. aurantifolia* Extracts**

The result of antibacterial activity of *C. reticulata* and *C. aurantifolia* againts *S. aureus* are shown in Table 4 and Table 5. The results showed that both *C. reticulata* and *C. aurantifolia* had the best inhibition at extract concentration of 70%. The lowest inhibitory value of *C. reticulata* extract is at concentration of 100%, meanwhile the lowest inhibitory value of *C. aurantifolia* extract is at concentration of 10%. It is also observed that

Table 3. Phytochemical compound of *C. reticulata* and *C. Aurantifolia* extracts

Phytochemical Compounds	Results	
	CR	CA
Alkaloid	+	+
Flavonoid		
Leukoantosianin	+	+
Flavonol	+	+
Saponin	+	+
Steroid	-	+
Triterpenoid	+	-
Tanin	+	+

CR : *Citrus reticulata*, CA : *Citrus aurantifolia*

the increasing concentration of extract (above 70%) resulted a decrease in the inhibitory activity.

Table 4. Antibacterial activity of *C. reticulata* againts *S. Aureus*

Extract	Inhibition zone (mm)
CR 10 %	8.33
CR 20 %	8.33
CR 30 %	8.33
CR 40 %	8.33
CR 50 %	8.33
CR 60 %	10.33
CR 70 %	11.33
CR 80 %	11
CR 90 %	9
CR 100 %	8

*) CR (*C. reticulata* extract)

Table 5. Antibacterial activity of *C. aurantifolia* againts *S. aureus*

Extract	Inhibition zone (mm)
CA 10%	7
CA 20 %	7.66
CA 30 %	8.33
CA 40 %	8
CA 50 %	8.33
CA 60 %	8.66
CA 70 %	9.66
CA 80 %	9
CA 90 %	8.66
CA 100 %	8.33

*) CA (*C. aurantifolia* extract)

Antibacterial Activity Assay of C. reticulata and C. aurantifolia Extracts Combination

Combination of these two extracts showed that CR: CA = 25%:75% proportion has the best inhibition to *S. aureus* (9 mm). However, the diameter of inhibition results from the combination of the two extracts were lower compared to single extract result, especially *C. reticulata* extract (Table 6). Based on these single and combined extract test results, the extract of *C. reticulata* with concentration 70% was chosen in the formulation gel hand sanitizer gel.

Table 6. Antibacterial Activity extract combination againts *S. aureus*

Extract	Average of inhibition zone (mm)
CR:CA= 100:0	7.3
CR:CA = 75:25	7.6
CR:CA = 50:50	8.6
CR:CA = 25:75	9
CR:CA = 0:100	7.6

*) CR: *C. reticulata* extract, CA: *C. aurantifolia* extract
*) Inhibition zone measurements carried out three times using Whatmann No. 1 paper disk (diameter : 6 mm)

Visualization of Hand Sanitizer Gel

Gel hand sanitizer that contained *C. reticulata* peel extract was observed to have a yellow color, if compared to the base gel (Figure 1).

Figure 1. Color comparison of base gel and gel hand sanitizer gel contained *C. reticulata* peel extract

Antibacterial Activity Assay of Formulated Hand Sanitizer

Formulated gel hand sanitizer that contained *C. reticulata* extract was used in the second step of antibacterial activity to confirm the steadiness of *C. reticulata* antibacterial potency. Result showed that formulated gel hand sanitizer that contain *C. reticulata* extract was still able to inhibit *S. aureus* due to the active compound of *C. reticulata* extract (Table 7).

Table 7. Antibacterial activity of hand sanitizer gel againts *S. aureus*

Extract	Average of inhibition zone (mm)
Hand sanitizer gel of *Citrus reticulata* extract concentration 70%	10.6 mm
Commercial hand sanitizer	7.6 mm

*) Inhibition zone measurements carried out three times using Whatmann No. 1 paper disk (diameter: 6 mm)

Quality Test of Hand Sanitizer Gel

As a final product of this research, gel hand sanitizer was organoleptically tested. Organoleptic test parameters were consisted of color and aroma. Formulated gel hand sanitizer produced in this research has yellow color with a distinctive smell of mandarin orange. Table 8 showed the organoleptic test result in comparison with commercial hand sanitizer.

Table 8. Organoleptic Test of Gel Hand Sanitizer

Parameter	Formula		Commercial Hand Sanitizer
	Hand Sanitizer CR 70%	Base Gel	
Colour	Yellow	Clear	Clear
Aroma	Mandarin orange	odorless	Alcohol

CR = *C. reticulata*

Another quality test was also performed on the formulated gel hand sanitizer product. These quality test was referred to SNI 06-2588-1992 standard. Some parameters that were measured consist of pH value, dispersion, and adhesion of hand sanitizer preparations. Result in Table 9 showed this formulated gel hand sanitizer that contain *C. reticulata* peel extract fulfilled SNI 06-2588-1992 standard.

Discussion

Raw Material Availability and Extract Yield

Because of its abundant availability in daily life, mandarin oranges (*Citrus reticulata*) and lime (*Citrus aurantifolia*) could be potential source of bioactive compounds, specifically those which are capable of inhibiting microbial growth. This research used peels of *Citrus reticulata* and *Citrus aurantifolia* fruit that is considered as waste product from culinary activities. By showing antibacterial effect from *Citrus* spp peels waste means giving an additional useful value to unwanted materials.

In attempt to obtain raw extract from parts of plants, some factors may affect the value of final extract yield include surface area of the material, type of solvent that is used, temperature, and duration of the extraction process. Simplisia of *Citrus reticulata* and *Citrus aurantifolia* were mashed onto powder and macerated in 96% ethanol solvent. These small size of peel powder was aimed to increase contact between the surface of simplisia and the solvent. According to Hasnaeni and Wisdawati (2019), the active compound present in plant extract is proportional to the yield of the extract. Based on the result in Table 2, the yield value between these two species of Citrus is similar, and indicated the similar amount of their active compounds that were dissolved by solvent.

Phytochemical Compounds from Citrus Peel Extracts

Based on the results of the phytochemical assay in Table 3, it was found that alkaloid test on both Citrus extracts gave positive results. Alkaloid that is detected in these Citrus extract could play role as antibacterial. According to Darsana *et al*. (2012), alkaloids are secondary metabolites that can damage bacterial cell walls.

Table 9. Value of pH, Dispersion and Stickiness of Gel Hand Sanitizer samples

Parameter	Formula		*Commercial Hand sanitizer*	*SNI*
	HSE	Base Gel		
pH	6	5.9	7	4.5-8
Dispersion (cm)	5.3	5.1	5.2	5-7
Adhesion (second)	268.26	188.99	177.55	150-300

*) HSE (Hand Sanitizer from Extract)

Flavonoid test of *Citrus reticulata* and *Citrus aurantifolia* peels extract informed that leukoantocyanin and flavonol compounds are owned by these Citrus species. Choi *et al*. (2007) states that naringin, hesperidin, tangeretin, nobiletin, are common flavonoid compounds contained in *C. aurantifolia*. Jasim (2012) showed that *Citrus reticulata* have flavonoid, flavon, flavonon, phenol, and polyphenol compounds which have antibacterial, antioxidant and anti-inflammatory properties. Flavonoids themselves are able to work as an antibacterial by damaging cells and denaturing cell proteins so that bacterial metabolism is inhibited (Pelczar and Chan, 1988).

Saponin assay results on *C. reticulata* and *C. aurantifolia* extracts showed positive results. Saponin compounds contained in an extract can damage bacterial cell walls resulting in leakage of cytoplasm that leads to bacterial cell death (Harborne, 1987).

Based on the phytochemical test of steroids and terpenoids, both peels of *C. reticulata* and *C. aurantifolia* contained unsaturated steroids. Peels of *C. reticulata* showed contain of triterpenoid compound, but it doesn't contain in peel of *C. aurantifolia*. On the other hand, *C. reticulata* does not have common steroid in its peel extract compare to *C. aurantifolia* peel extract. Harborne (1987) revealed that triterpenoid compounds belong to 4 groups including triterpenes, saponins, glycosides and steroids. The terpene compounds contained in *C. reticulata* are fat soluble compounds. The content of common steroid compounds found in *C. aurantifolia* works as an antibacterial by damaging bacterial cell membrane so cells cannot grow well (Ahmed, 2007).

The tannin test results showed that both *C. reticulata* and *C. aurantifolia* contained tannin and polyphenol compounds. The antibacterial properties of tannins work by entering into bacterial cells, disrupting the protoplasm and result in cell death (Nuria et al., 2009).

Taken together, all of those phytochemical compounds owned by both *C. reticulata* and *C. aurantifolia* peel extracts are capable to provide antibacterial action.

Antibacterial Potency of Citrus Peel Extracts

As mentioned in the material and method section that antibacterial assays were grouped into two steps : step one is aimed to test antibacterial activity of *C. reticulata* and *C. aurantifolia* peel extract (single or combined treatment) and step two is aimed to study about antibacterial activity of formulated gel hand sanitizer that already contained chosen Citrus extract. Our research showed that both *C. reticulata* and *C. aurantifolia* peel extract could individually inhibit the growth of *S. aureus* (Table 4 and 5), specifically on the concentration value of 70%. According to Brooks *et al*. (2013), factors that effects antibacterial activity consist of the content of antibacterial compounds, the concentration, and the spread/diffusion capability of the extract. Secondary metabolites that is contained in the extract is able to inhibit bacterial growth properly at certain concentrations. Faozi (2013) revealed that high concentration of extract was followed by large diameter of formed inhibition zone, but if the concentration exceeds the maximum point limit, the formed inhibition zone may decrease due to saturation. Based on this statement, it can be possible that the extract of *C. reticulata* and *C. aurantifolia* reached optimum concentrations in inhibiting bacterial growth at concentration of 70%. Furthermore, saturation point of the extract was observed at concentration point above 70% (80% to 100%) and resulted in the decrease of *S. aureus* inhibition.

Results in Table 6 informed the diameter of the inhibitory zone against *S. aureus* from both Citrus extracts combination were smaller compared to single extract treatment. (*C. reticulata* or *C. aurantifolia* alone, Table 4 and Table 5). This data suggested that *C. reticulata* extract and *C. aurantifolia* extract did not have a synergistic effect to inhibit the growth of *S. aureus* bacteria.

Antibacterial Activity of Formulated Gel Hand Sanitizer

Based on result that is previously mentioned (Table 4,5,and 6), peel extract of *C. reticulata* was chosen as the active ingredient for gel hand sanitizer formulation. After gel

hand sanitizer was formulated and made, antibacterial assay of this gel hand sanitizer was necessary to be conducted. Result in Table 7 shows that formulated gel hand sanitizer which contained 70 % of *C. reticulata* peel extract were still able to inhibit growth of *S. aureus*. The inhibitory zone of hand sanitizer gel with *Citrus reticulata* extract (concentration 70%) as its active compound was smaller if compared to the inhibition zone produced by extract of *Citrus reticulata* (concentration 70%) alone. This result might be caused on the reason that hand sanitizer gel was not only contain *C. reticulata* extract, but other ingredients that were used to make gel-based hand sanitizer (Table 4 and 7). Gel-based hand sanitizer composition might affect the amount of *Citrus reticulata* that was used as active ingredient in the formula. The inhibition zone of this formulated gel hand sanitizer was also larger than inhibition zone formed by commercial hand sanitizer. This finding suggested eventhough *C. reticulata* peel extract was already mixed with base gel, it is still potent to play role as antibacterial compound.

Quality of Formulated Gel Hand Sanitizer

To determine the good properties of this formulated gel hand sanitizer when applied to the skin, some tests were carried out. Organoleptic test (Table 8), probandus preference test (data not shown) and other physical quality test like dispersion/ spread test and stickiness test (Table 9) are components of the quality assays.

Garg *et al*. (2002) revealed that the ideal dispersal power of semisolid preparation on the skin should be in the range of 5-7 cm. The dispersal power of this formulated gel hand sanitizer was 5.3 cm (Table 9) which is in accordance with the this reference and SNI standard. Matrix of gel preparation is a factor that affects the diameter of the dispersal power. Weaker strength of matrix gel will result to the increase of dispersal/spread power. Matrix gel component of hand sanitizer was formed by a gelling agent in formulation.

Results of the adhesion test of gel hand sanitizer showed the longest time to be absorbed by skin compared to base gel and commercial hand sanitizers (Table 9). Commercial hand sanitizers have the fastest adhesion time due to its alcohol content, but not all of its ingredients will be absorbed by skin during adhesion test because alcohol is volatile. Body temperature also affects the evaporation of alcohol therefore the adhesion period of commercial hand sanitizer is the fastest one. The stickiness of a hand sanitizer can be affected by materials that are used in its production. The use of materials such as carbopol and triethanolamine, which are gel developer materials, could increase the stickiness. The more concentrated the gels produced, the more sticky it will be felt on skin and stay longer on the surface of the skin. Based on visualization, our formulated gel hand sanitizer had thicker texture than commercial hand sanitizer, and it causes longer adhesion period on skin.

The pH measurement of a hand sanitizer product is needed to ensure the product is safe to be applied on the skin. The pH value of our gel hand sanitizer was 6 (Table 9), while the pH of the commercial hand sanitizer is 7.

Overall, the dispersion/spread value, adhesion time, and pH value of this formulated gel hand sanitizer meets the hand sanitizer quality requirements as stated by SNI 06-2588-1992 (Table 9). These findings suggested that our formulated gel hand sanitizer which contain *C. reticulata* peel extract are effective against hand microbe (represented by *S. aureus)*, safe to be used, and promising to be further developed as commercial product as an alternative substitution to alcohol-based hand sanitizer.

Conclusion

Peel extract of *Citrus reticulata* and *Citrus aurantifolia*) were effectively inhibited the growth of *Staphylococcus aureus,* with the best inhibition potency was showed by 70% concentration of *Citrus reticulata* peel extract. The combination of both Citrus peel extract did not produce a better antibacterial activity compared to the single extract of *C. reticulata*. Peel extract from *C. reticulata* is promising to be used as active compounds in this non-alcohol based gel hand sanitizer. This formulated gel hand sanitizer is complied with the provisions issued by SNI 06-2588-1992.

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