ANTIOXIDANT, ANTIMICROBIAL, AND PRESERVATION ACTIVITIES EVALUATION OF TROPICAL FRUIT PEEL EXTRACTS

Mascellia Tifanny Agung, Filiana Santoso, Hery Sutanto

Department of Food Technology, Faculty of Life Sciences and Technology
Swiss German University, EduTown BSD City, Tangerang 15339, Indonesia

Abstract

The ethanolic peel extract of five different fruits i.e. mangosteen, custard apple, passion fruit, pomelo and rambutan were analyzed for their antioxidant activity, total phenolic content, flavonoids content, proanthocyanidins content, cytotoxicity, and antimicrobial activity. The antioxidant activity analysis was done using DPPH radical scavenging method. Rambutan was found to have the highest antioxidant activity (IC_{50}=40.26ppm). Moreover, the antioxidant stability was observed over a six-week storage period at 4^{o}C. Pomelo was found to have the most stable antioxidant compounds. The highest total phenolic content was found in rambutan peel extract (101.17mg/g GAE). Whereas, mangosteen peel extract had the highest flavonoids and proanthocyanidins contents (110.56mg/g QE; 483.26mg/g CE). The cytotoxicity of the fruit peel extracts was also examined, where custard apple and passion fruit were found to exhibit the highest cytotoxicity potential (LC_{50}=124.97ppm). The antimicrobial activity of all fruit peel extracts was assessed using broth macrodilution method against *Salmonella sp.* Custard apple, passion fruit and pomelo peel extracts were then applied to mayonnaise product as preservatives as they had the strongest antimicrobial activity. The TPC results showed no preserving activity of the fruit peel extracts that could be found. The stability of the antimicrobial activity of the fruit peel extracts was examined at room temperature over four days of storage period. It was found that the antimicrobial agent of pomelo was the most stable among those three extracts.

Keywords: antimicrobial, antioxidant, extract, fruit peels

Introduction

Salmonellosis has been known as a foodborne disease ever since the 1800s. The New South Wales OzFoodNet (2012) reported 248 foodborne illness cases due to *Salmonella.* Among these cases, raw egg products, including mayonnaise, were noted as the carriers of the bacteria. *Salmonella* outbreaks due to mayonnaise were also reported in Germany in 2010 (Wissmann et al., 2012). Particularly for

cases caused by *Salmonella thyphi,* it was reported that in the year of 2000 approximately 21.6 million cases happened in a year where 90% of the cases happened in Asia (Crump et al., 2004).

Indonesia is one of the biggest fruit-producing countries in the world. In 2011, Indonesia was in the third rank of tropical fresh fruit producer (FAO, 2011). People commonly only consume the fruit meat and not the fruit peel, thus, the latter is treated as a waste. However, researchers have proven that antioxidants are not present only in fruit meat but also in its peel. Besides antioxidant activity, researches have also shown positive results towards the antimicrobial activity of fruit peel extract. Food marketing and distribution demand food to have a long shelf life. Thus, it is important to observe plant extracts with antimicrobial activity as they can be used as natural additives to protect food from pathogens. Therefore, in this research, the possibility to use different fruit peel extracts as preservative was studied, as it may lead to utilizing a waste and consequentially add value to the fruits and naturally reduce the amount of waste materials.

The present research has three objectives: to evaluate the fruit peel extracts regarding their antioxidant, antimicrobial activity against *Salmonella*, and cytotoxicity; to evaluate the stability of the antioxidant activity of the fruit peel extracts over a certain storage period; and to determine the microbial inhibitory effects of fruit peel extract addition in mayonnaise.

Materials and Methods

Materials

Pomelo madu (*Citrus grandis*) from a plantation in Kudus, Central Java; mangosteen (*Garcinia mangostana* L.*)* and custard apple (*Annona squamosa)* from Taman Bunga Mekarsari, Cileungsi, Bogor; rambutan aceh-rapiah (*Nephelium lappaecum)* from a plantation in Depok, West Java; and passion fruit (*Passiflora edulis var. Flavicarpa)* from a plantation in Ambarawa, Central Java. Ethanol 96% food-grade (Multijaya Kimia, Indonesia), Ethanol 70% technical-grade (Multijaya Kimia, Indonesia), Methanol (Bratachem, Indonesia), Folin-Ciocalteau Phenol Reagent (Merck, Germany), Sodium Carbonate (Bratachem, Indonesia), Aluminum chloride (BDH, England), Potassium acetate (BDH, England, Acetic acid (Bratachem, Indonesia), Hydrochloric acid (Bratachem, Indonesia), Ascorbic acid (BDH, England), Quercetin (Aktin Chemicals, China), Gallic acid (Aktin Chemicals, China), DPPH radical (Aktin Chemicals, China), Nutrient broth (Merck, Germany), Plate count agar (Merck, Germany), *Salmonella-Shigella* agar (Merck, Germany), Buffered peptone water (Merck, Germany), Whatman Filter Paper no. 41 (Sartorius, Germany), eggs (Giant, Indonesia), sugar (Gulaku, Indonesia), mustard (Dijon, France), *Salmonella sp.* (Bio Farma, Bandung).

Equipments

Autoclave Hi Clave HV-50 (Hirayama, Germany), UV-Vis Spectrohotometer (Thermo Electron Corporation-Genesys 10uv, USA), Digital balance PA 214 (Ohaus, China), Digital balance (Sartorius, Germany), Blender (Phillips, Indonesia), Moisture content analyzer MA 35 (Sartorius, Germany), Vortex (Top Heidolph, Germany), Bio-Safety Cabinet MSC12 (Jouan, Germany), Incubator (Memmert, Germany), Rotary evaporator (Ika, Germany), Oven (Wiseven, Germany)

Methods

Fruit Peel Extraction

The fruit peels were dried at 45^{o}C until the moisture content reached 10%. The peels were grounded prior to the ethanol extraction. The solvent was separated using vacuum rotary evaporator. The mangosteen and rambutan were extracted according to the method used by Samuagam et al. (2013). The custard apple peel was extracted according to Seema et al. (2008). The pomelo peel was extracted according to Zarina and Tan (2013). The passion fruit peel was extracted according to Oliveira et al. (2009). All extraction procedures were done with slight modifications.

DPPH Radical Scavenging Activity

A 0.02mM DPPH radical solution was prepared with ethanol as the solvent. Each extract sample was prepared by making three different concentrations for the analysis. Sample solution amounted to 200µl was mixed with 400µl DPPH solution in the cuvette. The mixed solutions were placed in the dark room for 30 minutes. The absorbance was read at 515nm with UV-vis Spectrophotometer with the solvent as the blank (Faustina, 2013).

Broth Macrodilution Method

Standard solution was made by diluting the culture broth with nutrient broth. The diluted solution was adjusted to 0.5 McFarland turbidity standard in which the absorbance of the solution at 625nm should have lied between 0.08-0.13. The samples were made into solutions with concentration range of 10ppm - 2000ppm. One ml of each sample concentration was mixed with 1 ml diluted culture broth. The positive control was made by replacing the sample with nutrient broth whereas the negative control was made by putting 2 ml of nutrient broth in the test tube. The solutions were incubated at 37^{o}C in the incubator for 24h. The absorbance of each solution was read at 625nm (Faustina, 2013).

Folin-Ciocalteu Method

1:10 Folin reagent was prepared by diluting the mother solution of Folin-Ciocalteu Phenol reagent. Standard gallic acid solutions were made. As much as 0.3 ml of each standard solution was mixed with 1.5mL of Folin reagent and 1.2 ml of 7.5% sodium acetate solution. The mixed solutions were kept in the dark for 1 hour before the absorbance at 765nm was read with UV-vis spectrophotometer. For the analysis, the standard solutions were replaced by the sample solutions (Faustina, 2013).

Aluminum-chloride Method

Standard quercetin solutions were made as follows: 10% aluminum chloride solution and 100 ml 1 M potassium acetate solution (pH= 7.5) was prepared. Each quercetin solution (0.5 ml) was mixed with 1.5 ml methanol, 0.1 ml aluminum chloride solution, 0.1 ml potassium acetate solution, and 2.8 ml distilled water. The solutions were kept in the dark for 30 minutes before the absorbance at 415nm was read with UV-vis spectrophotometer. For the analysis, the standard solutions were replaced by the sample solutions (Chang et al., 2002).

Bate-Smith Method

Two ml of each sample was mixed with 1 ml distilled water and 3 ml hydrochloric acid in two different test tubes. The first group of the test tubes was placed in boiling water for 30 minutes whereas the other group was left in the dark. After 30 minutes, 0.5 ml of ethanol was added to each tube. The absorbance of the samples was read at 550nm. The absorbance values of the first group were subtracted to the second group and the concentration of proanthocyanidins was expressed according to catechin standard (Caceres-Mella et al., 2013).

Brine Shrimp Lethality Test

Brine shrimp eggs were incubated in filtered sea water which was covered with aluminum foil and aerated for 48h at room temperature under illumination. After incubation, the larvae were attracted to the other side of the container with a light source. The larvae were collected with Pasteur pipette and put into other vials which contain more sea water and also the solution of each extract with various concentrations. The percent death of each data was collected and the LC_{50} value was determined (Juniarti et al., 2009).

Mayonnaise production

Egg yolks were mixed with mustard before the oil was added drop by drop with constant curling. After half of the oil had been added to the egg yolk and the texture of the mixture had become thick already,

the spices and acid ingredients were then added. The rest of the oil was added drop by drop still with constant curling.

Sample Treatment

The three extracts with the strongest antimicrobial activity were added separately to freshly made mayonnaise with two different concentrations. Each sample was stored at 25^{o}C. The microbial number was counted every 24h using TPC. According to Indonesian Standard (SNI), the mayonnaise is considered unsafe to be consumed if the TPC is more than 1x10^{4} CFU/ml.

Total Plate Count

Samples with dilutions of 10^{-2} to 10^{-4} were prepared. One ml of each diluted sample was put in the petri dish using micropipette. The TPC agar was poured into the petri dish. The petri dishes were incubated at 37^{o}C for 1-2 days (Faustina, 2013).

Results and Discussion

Antioxidant activity analysis

The IC_{50} values of the fruit peel extracts ranged from 40.26 ppm to 5724.60 ppm (Table 1). Rambutan had the lowest IC_{50} value which means that it had the highest antioxidant activity among the fruit peel extracts.

Table 1. Antioxidant activity of the fruit peel extracts

Fruit	IC_{50}, ppm
Mangosteen	46.14 ± 1.15^{a}
Custard apple	512.06 ± 22.47^{b}
Passion fruit	2123.42 ± 58.33^{c}
Pomelo	5724.60 ± 152.28^{d}
Rambutan	40.26 ± 2.52^{a}
L-ascorbic acid	20.71 ± 0.55^{a}

Compared with the IC_{50} of L-ascorbic acid as the antioxidant standard, only the IC_{50} values of the mangosteen and rambutan were found not to be significantly different. Thus, they had the strongest antioxidant activity compared to the other fruit peel extracts and it can be concluded that the antioxidant activity of the mangosteen and rambutan peel extracts were close to that of vitamin C. The passion fruit, custard apple and pomelo peel extracts were significantly different from one another. Hence,

mangosteen and rambutan peel extracts had the highest antioxidant activity, followed by custard apple, passion fruit, and pomelo peel extracts.

Table 2. Phenolic, flavonoid, and proanthocyanidins content of the fruit peel extracts

Fruits	Phenolic content, mg/g dry base(GAE)	Flavonoid content, mg/g dry base(QE)	Proanthocyanidin content, mg/g dry base(CE)
Mangosteen	65.72 ± 0.83[a]	110.56 ± 5.03[a]	483.26 ± 3.86[a]
Custard apple	7.14 ± 0.19[b]	8.31 ± 0.91[c]	145.17 ± 9.78[b]
Passion fruit	2.19 ± 0.04[c]	5.43 ± 0.78[c,d]	47.13 ± 4.67[c]
Pomelo	14.85 ± 0.65[d]	5.94 ± 0.67[c]	37.98 ± 2.56[c]
Rambutan	101.17 ± 3.86[e]	61.90 ± 1.53[b]	441.54 ± 23.83[a]

The phenolic content of the fruit peel extracts (Table 2) had a weak negative correlation with the IC_{50} value (R^2= -0.52923). Phenolics are responsible for antioxidant activity, thus, samples with higher phenolics normally have higher antioxidant activity. However, it does not always happen that way. Pomelo peel extract had a higher phenolic content than the custard apple peel extract but lower antioxidant activity. This might indicate that the compounds which were responsible for the antioxidant activity in the fruit peels were not primarily due to the phenolic compounds, but other non-phenolic substances which also exhibit antioxidant activity such as vitamin C and tocopherol. The present results were similar to the results obtained by an experiment conducted by Sousa and Correia (2012).

The flavonoid content (Table 2) of the fruit peel extracts was moderately correlated with their phenolic content (R^2=0.77936). This was caused by the fact that flavonoids is a type of phenolics. However, it can still be obseved that not necessarily the fruit peel extract with higher phenolic content had also higher flavonoid content. For instance, the phenolic compounds in the rambutan peel might be comprised of non-flavonoids phenolic compounds, which might explain why it had lower flavonoids than mangosteen although it had higher phenolic content.

The flavonoids content of the fruit peel extracts had a weak negative correlation with the IC_{50} value (R^2=-0.58417). Flavonoids are responsible for antioxidant activity. However, since the correlation was low, it might as well indicate that the flavonoids were not the components which primarily contribute to

the antioxidant activity in the fruit peel extracts. It can be observed in rambutan which had less flavonoids yet higher antioxidant activity than mangosteen.

The proanthocyanidins is also a type of phenolic compounds which might explain its high correlation with the phenolic content (R^2=0.90744). The amount of detected proanthocyanidins (Table 2) was relatively higher than flavonoids and phenolics content. This might be caused by the sensitivity of the method used. The presence of non-tannin compounds whose chemical structure was related to proanthocyanidins such as flavonoids may have interfered the spectrometer reading and thus resulted in a bias result (Vermerris and Nicholson, 2008). This might as well cause the high correlation between proanthocyanidins and flavonoids (R^2=0.93950).

The proanthocyanidins content also had a moderate negative correlation (R^2=-0.73088) with the IC_{50} value of the fruit peel extracts. This might lead to a conclusion that as the antioxidant activity increased, the proanthocyanidins content increased as well. However, since the correlation value was not high, it could be concluded that the compounds that responsible for the antioxidant activity in the fruit peel extracts were not constituted primarily by proanthocyanidins in the fruit peel extracts.

The stability of the antioxidant activity of the fruit peel extracts was also observed (Figure 1-3). The observation was also conducted using DPPH radical scavenging method for six weeks with extracts storage condition at 4°C as it is the most common storage temperature.

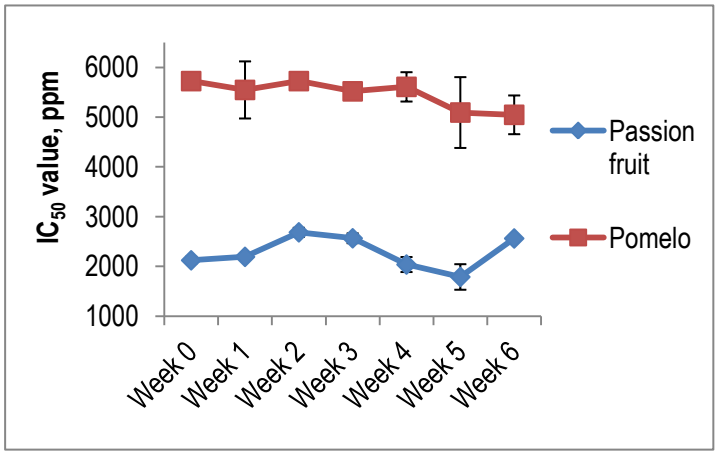

Figure 1. Stability of the antioxidant activity of passion fruit and pomelo peel extracts

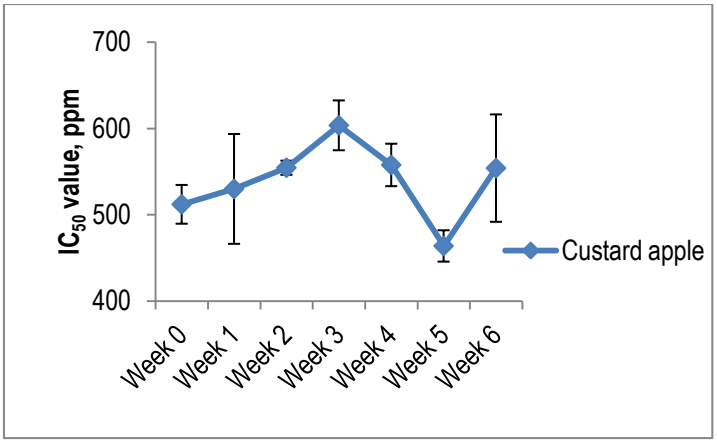

Figure 2. Stability of the antioxidant activity of custard apple peel extracts

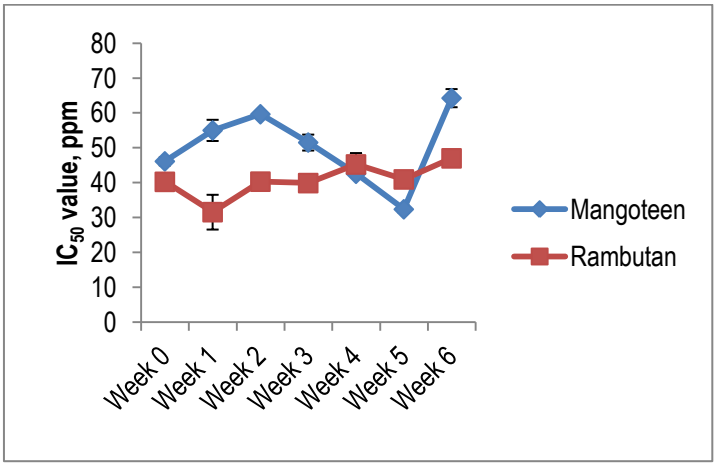

Figure 3. Stability of the antioxidant activity of mangosteen and rambutan peel extracts

In general, similar trends occurred between mangosteen and custard apple peel extracts. The IC_{50} values increased until the second week and then decreased until the fifth week of observation. Both values increased once again on the final week of observation. According to the statistical analysis using ANOVA, the antioxidant activity of the custard apple peel extract was more stable than mangosteen as there was generally no significant difference between the values in each week. The IC_{50} values of rambutan and passion fruit peel extracts both decreased on the first week and increased on the second week. The IC_{50} value of the passion fruit peel extract kept decreasing until the fifth week. However, the IC_{50} value of the rambutan peel extract increased on the fourth week before it decreased again on the fifth week. Both values increased once again on the last day of observation. Different from the other fruit peel extracts, the IC_{50} value of the pomelo peel extract seemed to be stable even after six week of storage period.

The antioxidant activity of all fruit peel extracts during the six week of observation was expected to have a gradual decrease as what happened in the experiments done by Nekvapil et al. (2012) in assessing the stability of antioxidant activity in tea extracts and Polydera et al. (2004) on orange juice. However, the antioxidant activity of the fruit peel extracts was generally fluctuated.

The increase of antioxidant activity under storage condition happened before in the research by Kevers et al. (2007) as he assessed the antioxidant stability of several fruits and vegetables. There are two possible reasons for this. Firstly, antioxidant agents may undergo reversible reaction with radicals. For example, Metodiewa et al. (1999) stated that the flavonoids oxidation reaction with radicals was reversible. And secondly was the fact that some antioxidants may regenerate other antioxidant such as catechin and vitamin C which may regenerate other antioxidants like vitamin E or tocopherol (Mukai et al., 2005).

Cytotoxicity Analysis

The LC_{50} value of the fruit peel extracts varied from 124.97ppm to 737.12ppm (Table 3). Custard apple and passion fruit had the same LC_{50} value which was 124.97ppm. Lower LC_{50} value indicated that both extracts had stronger toxicity potential compared to the other extracts. Conversely, rambutan had the least toxicity potential.

Table 3. LC_{50} value of the fruit peel extracts

Fruits	LC_{50}, ppm
Mangosteen	159.47
Custard apple	124.97
Passion fruit	124.97
Pomelo	625.67
Rambutan	737.12

The toxicity potential of the fruit peel extracts was due to the compounds in each extract. According to Simanjuntak (2013), saponin, tannin, flavonoids, and xanthons in certain concentration may cause the death of the *Artemia salina.* In his experiment, it was also found that the LC_{50} of mangosteen peel extract was much lower than that in this experiment which was generally lower than 1 ppm. However, it was probably caused by the high temperature drying process applied by Simanjuntak. As for the other fruit peel extracts, to the author's knowledge, there are no cytotoxicity analysis with BSLT method applied. Nevertheless, several researches have indicated toxicity potential of rambutan peel (Khonkarn

et al., 2010; Ampasavate et al., 2010). The research by Ampasavate et al. (2010) also showed cytotoxic potential in passion fruit peel with LC_{50} higher than 100 ppm which was agreed by the result of this experiment. The research regarding cytotoxicity of custard apple peel and pomelo peel is still uncommon. However, cytotoxicity potential has been detected in the leaf extract of custard apple (Saha, 2011).

Antimicrobial activity analysis

Figure 4 shows that the MICs of the extracts varied among one another. The MIC of pomelo peel extracts was the lowest which was lower than 25ppm. The MICs of mangosteen and passion fruit peel extract lied between 25 ppm and 50 ppm. Whereas, the MICs of rambutan and custard apple peel extracts were the highest lied between 50 ppm and 100 ppm. At this point, only approximate MICs could be determined. Narrower range of concentrations is needed in order to obtain more precise MIC.

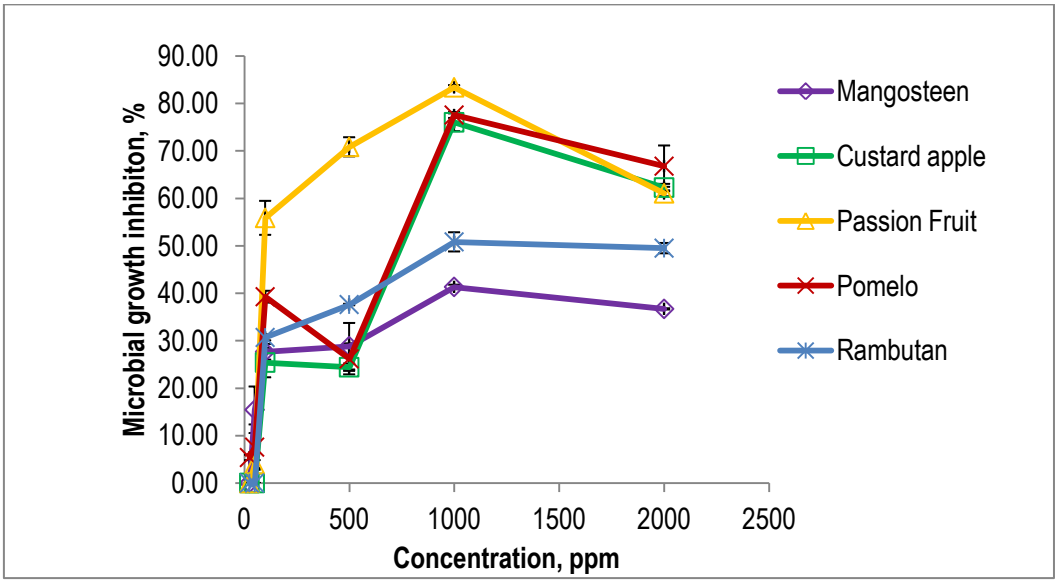

Figure 4. Microbial inhibition of the fruit peel extracts

Normally, the trend of the graph is most likely increasing as there are more antimicrobial compounds in higher concentration of the fruit peel extracts. However, in some fruits like mangosteen and pomelo, the inhibition percentage at 500 ppm was lower than the inhibition at 100 ppm. This might be caused by the presence of the substances in the extracts other than the antimicrobial agent such as metal ions for example, which at certain concentration might promote the growth of the microorganisms. It can be observed also that the inhibition percentage of all extracts at 2000 ppm were lower than that of 1000 ppm. This might be due to over concentrated of the extract solutions themselves leading to the

agglomerates formation of the molecules of the antimicrobial agents, and caused the surface area for the inhibition reaction became smaller.

According to the statistical analysis using ANOVA, it was found that there was no significant difference between pomelo and passion fruit. There was also no significant difference between mangosteen, custard apple and rambutan. Yet, a significant difference can be found between the two groups. Thus, it can be concluded that passion fruit and pomelo had the strongest antimicrobial activity compared to mangosteen, custard apple, and rambutan. The concentration's influence to the bacterial growth inhibition was found to be highly significant ($p<0.05$). In all extracts, 1000 ppm was the most effective concentration to inhibit the bacterial growth. However, in pomelo and rambutan peel extracts, the result was not significant between concentrations of 1000 ppm and 2000 ppm.

According to Palakawong et al. (2010), mangosteen peel extract had antimicrobial activity against *Salmonella sp.* with MIC of 3.13mg/ml which was higher than the result obtained in this research. In another study (Thitilertdecha et al., 2008), it was found that no inhibition performed by rambutan peel extract to the growth of *Salmonella sp.* In this research, the result was the contrary. This might indicate that the difference of plantation environment influences the antimicrobial agent compounds in the plants.

Total plate count

After three days of observation, the mayonnaise control reached the limit given by the SNI which is presented by the red line (Figure 5-6). The mayonnaise with addition of 5% custard apple and passion fruit peel extracts reached also the microbial number limit, with the microbial number higher than the control (Figure 5). The microbial number of the mayonnaise with addition of 10% of custard apple and passion fruit peel extracts even passed the limit since the second day of observation (Figure 6). The total plate count result was more than 300 colonies and stated as "too many to be counted" or TMTC since the second day. On the fourth day, all samples exceeded the microbial limit given by the SNI.

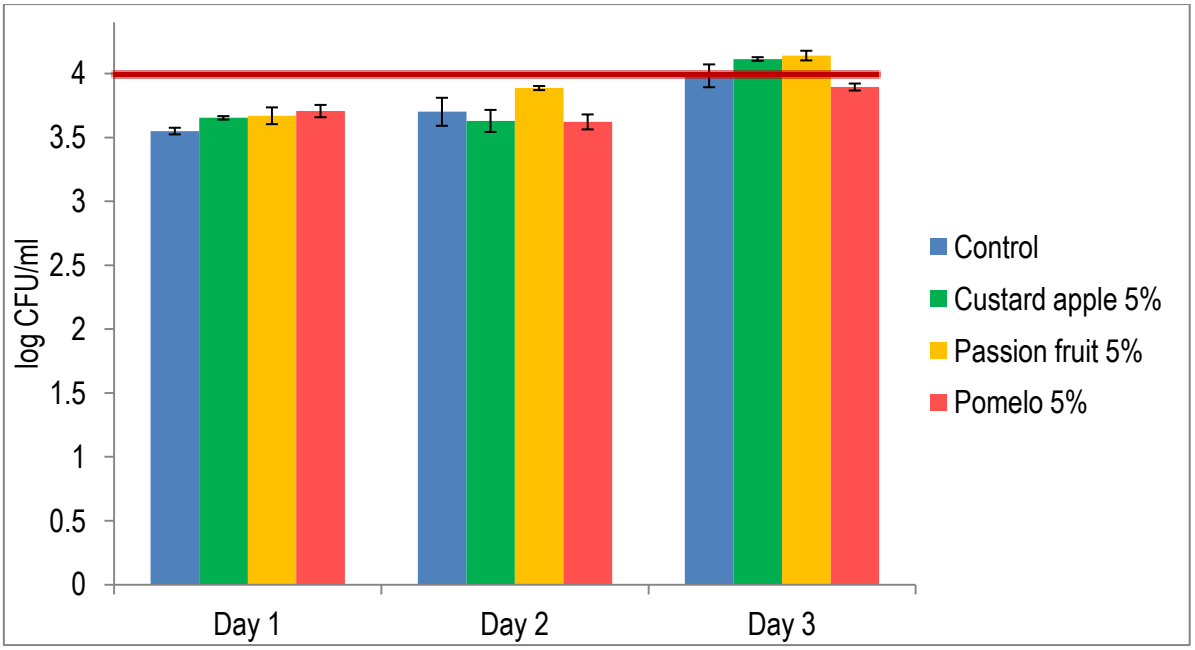

Figure 5. Total plate count result of mayonnaise with 5% (w/w) extracts addition

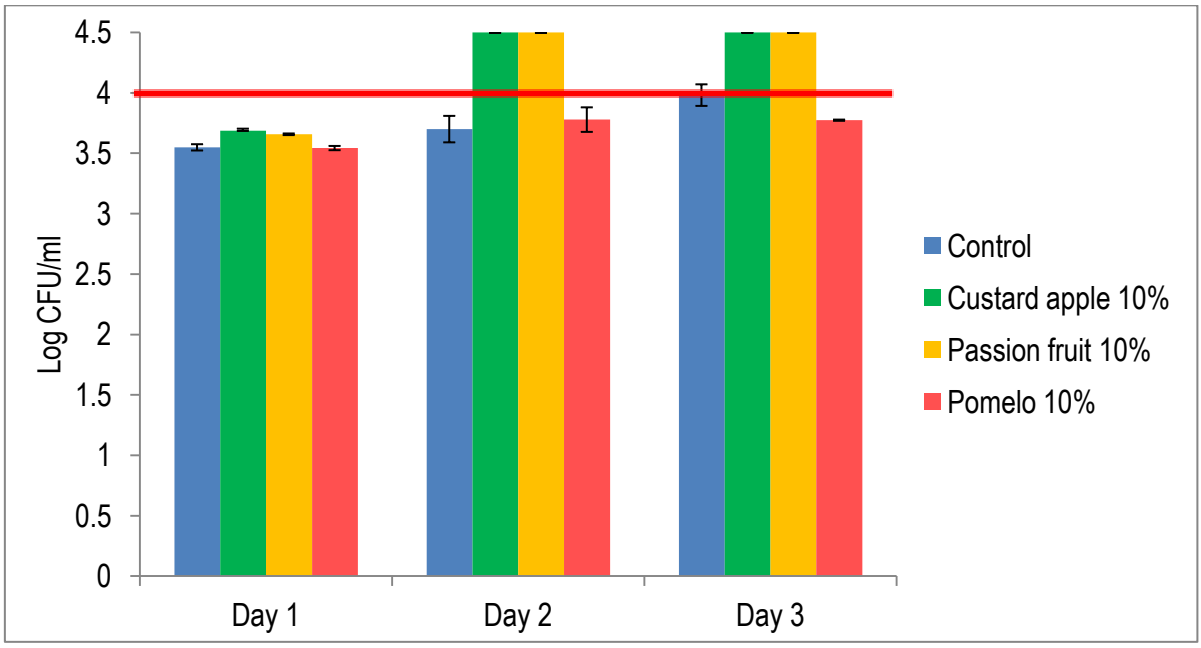

Figure 6. Total plate count result of mayonnaise with 10% (w/w) extracts addition

Compared to the other fruit peel extracts, pomelo fruit peel showed a better result in terms of inhibiting the microbial number of the mayonnaise. After three days of observation, both mayonnaise products with additional pomelo peel extract had not reached the microbial limit given by the SNI. However, the statistical analysis using ANOVA shows that there was no significance difference between the microbial number of the control mayonnaise and the mayonnaise with the pomelo peel extract addition. Thus, it cannot be said that the pomelo peel extract had the ability to preserve the mayonnaise.

The custard apple, pomelo, and passion fruit peel extract were subjected to another antimicrobial activity evaluation. The extracts were stored at room temperature and the antimicrobial activity was checked every 24h to see if there was any indication that the antimicrobial agent in the extracts were destroyed, or at least, any decrease in the antimicrobial activity can be observed. The observation was done for four days since the mayonnaise products were unsafe on the fourth day of observation.

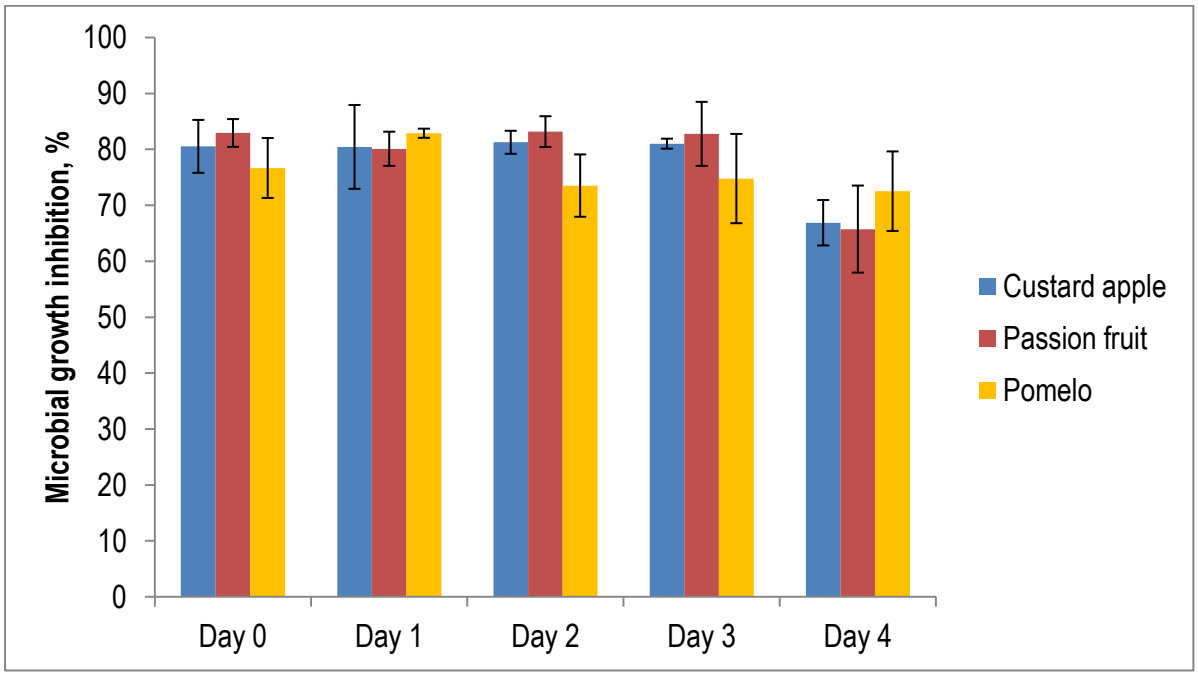

Figure 7. Microbial growth inhibition of the custard apple, passion fruit, and pomelo peel extract over a time period

Figure 7 shows the inhibition percentage of the three fruit peel extracts applied to the product during four days observations. It can be seen that generally, the custard apple and passion fruit peel extract showed a decreasing antimicrobial activity as the inhibition percentage decreased as well, particularly on the fourth day of observation. The pomelo peel extract had a more stable inhibition percentage over the time period. However, the antimicrobial activity of the extracts was generally stable.

Each of the fruit peel extract data was analyzed statistically using ANOVA single factor. It was found that custard apple peel extract was significantly different among others. Therefore, Tukey's HSD analysis was done to analyse further. The results showed that the inhibition percentage at the first day and the fourth day of observation were significantly different. It indicated that there was a decrease in the antimicrobial activity of the custard apple peel extract during storage at room temperature. The

inhibition of the microbial growth on the third day of observation was not significantly different compared to the first day of observation. There was a significant difference, however, between the inhibition of the microbial growth on the third and fourth day of observation.

Similar trend occurred also to the passion fruit peel extract. The microbial growth inhibitions of the passion fruit peel extract on first and second day of observation were not significantly different. The microbial growth inhibition on the fourth day of observation was significantly different from the first day which implies that there was a decrease in the performance of the antimicrobial agent of the passion fruit peel after four days of observation.

The observation result of the pomelo peel extracts after four days of storage at room temperature did not have significant differences. Thus, it can be concluded that the compounds that responsible for the antimicrobial property in pomelo peel extract were stable at room temperature even after four days. Hence, for the other two fruit peel extracts, the instability of the antimicrobial agents might be the reason of their inability to perform preserving action. Whereas, the performance of the antimicrobial agent in the pomelo peel extract might be retarded by the mayonnaise components such as the high fat content or the substances in the mustard.

Conclusion

The extracts of mangosteen, custard apple, passion fruit, pomelo and rambutan peel were found to exhibit antioxidant activity. Rambutan peel extract had the strongest antioxidant activity with the IC_{50} value of 40.26ppm. Compared to the L-ascorbic acid which has IC_{50} value of 21.46ppm, the antioxidant of rambutan peel extract was approximately half of the L-ascorbic acid. The antioxidant activity of the extracts was generally fluctuated from week to week.

Custard apple and pomelo peel extracts were found to have the highest cytotoxicity potential with LC_{50} value of 124.97ppm. The same extracts and passion fruit had the strongest antimicrobial activity. Application of the extract in mayonnaise showed that the mayonnaise spoiled more quickly than the control. Hence, no microbial inhibitory effect can be observed from the extracts when added to mayonnaise, thus, the extracts cannot be used as preservatives for mayonnaise.

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