

Evidence of potent antibacterial effect of fermented papaya leaf against opportunistic skin pathogenic microbes

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Abstract

The papaya leaf juice has been long practised as a traditional remedy to cure ailments due to its medicinal properties. The objective of this research is to study the effectiveness of fermented papaya leaf to inhibit the growth of pathogenic bacteria and yeast: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Propionibacterium acnes* and *Candida albicans*. The efficacy of fermented papaya leaf against selected pathogenic microbes was evaluated using agar well diffusion assay, broth microdilution assay and time-kill test. Evidence from data collected confirmed that fermented papaya leaf supernatant showed more pronounced antibacterial and antifungal effect than papaya leaf alone. Generally, fermented papaya leaf supernatant demonstrated potent antimicrobial effect against all bacterial pathogens tested particularly *P. aeruginosa* followed by *P. acne* and *S. aureus*. However, it was found that fermented papaya leaf was less effective against *Candida albicans*. It needs 4- to 7-folds higher concentration to inhibit 50% *C. albicans* growth than the bacteria. The antibacterial compounds produced in the supernatant appeared to have some bactericidal effect against *P. aeruginosa*, *P. acne* and *S. aureus* with the minimum inhibitory concentration (MIC_{>99}) of 16%, 50% and 60%, respectively. Particularly, the fermented papaya leaf supernatant at 60% concentration showed 100% inhibition rate within 30 mins against *P. aeruginosa*. However, it needs a longer time to show the same inhibition effect against *S. aureus* and *P. acne*, which was about 2-6 h. The potent killing effect of fermented papaya leaf showed a potential use in skincare application to control pathogenic microbe infection

1. Introduction

Papaya (*Carica papaya* Linn) is cultivated mainly in all tropical and sub-tropical countries around the world. Various parts of papaya such as the leaves, seeds, flesh, latex and skin were known to have many benefits to human being and have been long used as a traditional remedy to cure ailments. Many studies have been conducted to look at the medical properties of papaya leaf not only on diseases related to human but also to animals. The papaya leaf was found to have antidiabetic (Juárez-Rojop *et al.*, 2014), antimicrobial (Baskaran *et al.*, 2012; Jafari *et al.*, 2016), anti-inflammatory (Gupta *et al.*, 2017) and anti-tumour (Otsuki *et al.*, 2010) and antioxidant activities (Okoko and Ere, 2012). Both aqueous and solvent papaya leaf extract was reported to contain numerous bioactive compounds such as alkaloids, steroids, quinones, tannins (Juárez-Rojop *et al.*, 2014), prunasin, sambunigrin (Seigler *et al.*, 2002) and flavonoids (Vuong *et al.*, 2013). Nevertheless,

papaya leaf juice is very bitter and hard to be consumed even though many people knew its various pharmacological properties.

Skin infection caused by either bacteria or yeast is a worldwide problem to human and animal. All age group can be infected, either normal or immunocompromised patients. Some of the common bacteria and yeast isolated were from *Staphylococcus*, *Pseudomonas*, *Propionibacterium* and *Candida* genera (Cogen *et al.*, 2008). At present patients are treated with antibiotics. However, some of the bacterial and yeast are getting resistant to the antibiotics (Cogen *et al.*, 2008). In addition, patients with drug therapy are generally exposed to the risk of drug-drug interaction which may reduce the efficacy of the drug or affect the health of the patients (Gupta *et al.*, 2013). This is a serious problem as antibiotics are used clinically either for oral or topical treatment for both bacterial and yeast infection. Therefore, the use of natural antimicrobial compounds

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which are generally safe would be beneficial.

Fermentation is a degradation of nutritional compounds in raw commodities through the action of a multiple hydrolytic enzymes secreted by the microorganisms. Depending on the strains, numerous primary and secondary metabolites with various functional properties and that contribute to the flavour were produced by selective microorganisms used during the fermentation process. Thus, many food industries use single or mixtures of pure cultures under controlled fermentation condition for the production of a fermented product with the desired characteristics which are safe for the consumer. Some of the pure cultures used for the preparation of known fermented foods include non-toxicogenic *Aspergillus oryzae* strains for preparation of soy sauce (Hoang et al., 2016), *Rhizopus* for tempeh (Hartanti et al., 2015), *Lactobacillus* for yoghurt (Ertem and Cakmakci, 2018) and *Saccharomyces* for wine (Marsit and Dequin, 2015). In addition to this, many studies are now applying various new raw materials as a substrate to produce a novel fermented product with better health properties. Therefore, the objective of this research is to study the effectiveness of fermented papaya leaf to inhibit the growth of opportunistic bacterial and yeast pathogens: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Propionibacterium acnes* and *Candida albicans*.

2. Materials and methods

2.1 Preparation of non-fermented and fermented papaya leaf

Non-fermented and fermented papaya leaf was prepared as described by Koh et al. (2017). After fermentation, papaya leaf was subjected to filtration and centrifugation at 10,000 rpm, 4°C for 10 mins. The supernatant was collected and filtered sterilized using sterile 0.22 µm cellulose acetate syringe filter and kept at -80°C until used.

2.2 Preparation of bacterial and yeast cell suspension

Staphylococcus aureus (ATCC 49775™), *Propionibacterium acnes* (ATCC 6919™) and *Candida albicans* (ATCC 26310™) were purchased from American Type Culture Collection (ATCC), whereas, *Pseudomonas aeruginosa* was isolated from mastitis cow. The glycerol stock of *S. aureus* and *P. aeruginosa* was inoculated in Mueller Hinton (MH) broth and *C. albicans* was inoculated in potato dextrose (PD) broth, followed by incubation at 30°C with the agitation rate of 200 rpm for 18 hrs. *P. acnes* was grown in a mixture of tryptic soy (TS) and Lab-Lemco broth in an anaerobic condition at 35°C for four days. The cells were harvested

by centrifugation and washed with sterile 0.85% sodium chloride (NaCl). Finally, the density of the cell suspension was adjusted with sterile 0.85% NaCl to 10⁸ CFU/mL.

2.3 Agar well diffusion assay

A bacterial and yeast cell suspension was spread uniformly on the solid agar medium using cotton swab and left dried at room temperature. The wells were cut using sterile Pasteur pipet and soft agar was loaded to seal the bottom edge of the well. An amount of 50 µL of the fermented papaya leaf supernatant (FPLS) was loaded and kept in chilled condition for two hours to allow diffusion of the sample into the agar. Then another 50 µL FPLS was added into well. The agar plate was then incubated at 37°C for 24 hrs for *S. aureus*, *P. aeruginosa* and *C. albicans*, while *P. acnes* was incubated under anaerobic conditions for a duration of 4 days. A clear zone diameter around the well which indicated the microbial inhibition was measured at two perpendicular directions. Penicillin-Streptomycin and acetic acid were used as a positive control. All experiments were done with three replicates

2.4 Determination of minimum inhibitory concentration at level of MIC₅₀ and MIC_{>99}

A mixture of different concentration of FPLS (0-100%) and growth medium broth was loaded into sterile microtiter plate (total volume 90 µl/well). Later, 10 µL of cell suspension was added (final CFU 10⁶/mL) and the plate was sealed with paraffin film to avoid spillage. The culture was incubated as mentioned above and shaking at 50 rpm. After incubation, a serial dilution was performed in sterile 0.85% NaCl and plated on solid agar medium followed by incubation for 24 hrs to determine the number of viable cells after the treatment. The medium with no viable cell after 24 hrs were further incubated and observed up to 48 hrs. The concentration of FPLS inhibited 50% of microorganism growth (MIC₅₀) was calculated using GraphPad Prism 5 software. The MIC_{>99} was determined based on the lowest concentration that totally killed the microorganisms. All experiments were done with three replicates.

2.5 Time-kill study

A tube containing a mixture of sterile growth medium broth and FPLS (final concentration 60%) was inoculated with the bacterial cell suspension. The final concentration of cell suspension was approximately 10⁶ CFUs/mL. The broth without FPLS was used as a control. The mixture was incubated at 37°C for 10 hrs and at certain time interval, a total of 100 µL sample was removed for CFU analysis. A serial dilution was

conducted before the cell was grown on agar medium for 24-48 hrs after which the visible colonies were counted. All experiments were done with three replicates.

2.6 Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) and t-test to compare the means for each treatment. $p < 0.05$ was accepted as a significant difference. The GraphPad Prism 5 (GraphPad Software Inc. California, USA) was used in the analyses.

3. Results and discussion

3.1 Agar well diffusion assay

Agar well diffusion assay was used as a preliminary assay to examine the potential of non-fermented and fermented papaya leaf for growth inhibition of *S. aureus*, *P. aeruginosa*, *P. acne* and *C. albicans*. It was noted that none of these microbes were affected by the non-fermented papaya leaf (Table 1). However, fermented papaya leaf showed inhibition activity toward these selected pathogenic microorganisms, particularly against *S. aureus* and *P. aeruginosa* but no inhibition was observed against *P. acne* and *C. albicans* using this assay. There was no significant different ($p > 0.05$) in inhibition zone diameter between *S. aureus* (1.50 cm inhibition zone) and *P. aeruginosa* (1.40 cm inhibition zone) on agar plate treated with fermented papaya leaf supernatant. Indeed, the inhibition activity against *P. aeruginosa* was not significantly different when compared to Penicillin-Streptomycin (1%) and 2% acetic acid (control). However, the inhibition zone of *S. aureus* was significantly smaller ($p < 0.05$) than Penicillin-Streptomycin (1%) which about 28%. Previous studies had reported that the antimicrobial activity of papaya leaf was varied depending on the extraction method (Khan et al., 2012) and the bacterial strains (Cho and Maung, 2017). The antibacterial activity was high when the extraction was carried out using ethanol but has no effect with water against gram-positive and negative strains (Awah et al., 2017). In this study, the negative antimicrobial effect of the non-fermented papaya leaf could be due to the application of the water extraction

method that resulted in the release of water-soluble compounds with weak or no antimicrobial activity.

Fermentation is always associated with the production of primary and secondary metabolites by the organisms and the production of new compounds through the degradation of complex substances in the substrate. This includes organic acids (Pejin et al., 2017), hydrolytic enzymes (Rashad et al., 2017), phenolic compounds (Sheih et al., 2014), peptides (Souza et al., 2015) and others bioactive compounds (Parvez et al., 2019). The high antibacterial activity of fermented papaya leaf could be due to the presence of organic acids such as acetic acid, citric acid, kojic acid, quinic acid, etc. which may work synergistically to inhibit *S. aureus* and *P. aeruginosa*. Those organic acids were found increase drastically in FPLS by microorganism action after the papaya leaf gone thru fermentation process (Koh et al., 2017)

3.2 Determination of MIC_{50} and $MIC_{>99}$

The efficacy of fermented papaya leaf supernatant to inhibit bacterial and yeast growth was further determined using microbroth dilution assay. Figure 1 shows the growth inhibition pattern of *P. aeruginosa*, *P. acne*, *S. aureus* and *C. albicans* after treated with different concentration of fermented papaya leaf supernatant. It was found that gram-negative bacteria (*P. aeruginosa*) was the most susceptible to fermented papaya leaf supernatant (FPLS) followed by *P. acne* and *S. aureus* (gram-positive) and the least was *C. albicans* (Figure 1). There was a small reduction in *P. aeruginosa* viable cell count when treated with 4% FPLS but reduced drastically to more than 95% after treated with 6% FPLS. This result was in parallel with the earlier finding by Velićanski et al. (2014). The author reported the antibacterial activity of fermented lemon balm tea using a mixture of bacteria and yeasts was higher against gram-negative than gram-positive microbe. Table 2 shows the MIC_{50} and $MIC_{>99}$ of FPLS against tested selected pathogenic microorganisms. The result demonstrated that the low concentration (<10%) of FPLS was required to inhibit at least 50% growth of *P. aeruginosa*, *P. acne* and *S. aureus*. However, it needs about 4 – 7 fold higher

Table 1. Antimicrobial activity of fermented papaya leaf supernatant against opportunistic bacterial and yeast pathogen using agar well diffusion assay.

Antimicrobial agent	Inhibition zone diameter (cm)			
	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>P. acne</i>	<i>C. albicans</i>
Penicillin- Streptomycin (1%)	2.10±0.12*	1.95±0.42*	1.70±0.36	NI
Acetic acid (2%)	1.80±0.22	1.60±0.21*	1.10±0.16	NI
Papaya Leaf:				
Non-fermented	NI	NI	NI	NI
Fermented	1.50±0.15 †	1.40±0.10* †	NI	NI

Data are means of triplicate±standard deviation. (*) indicate significant different ($p < 0.05$) within treatments and (†) between pathogenic microorganisms. NI: no inhibition

concentration to give the same effects on *C. albicans*. Nevertheless, FPLS cannot kill *C. albicans* totally with no MIC_{>99} value observed. On the other hand, the antibacterial compounds produced in the FPLS appeared to have some bactericidal effect against *P. aeruginosa*, *P. acne* and *S. aureus* with the minimum inhibitory concentration (MIC_{>99}) of 16%, 50% and 60%,

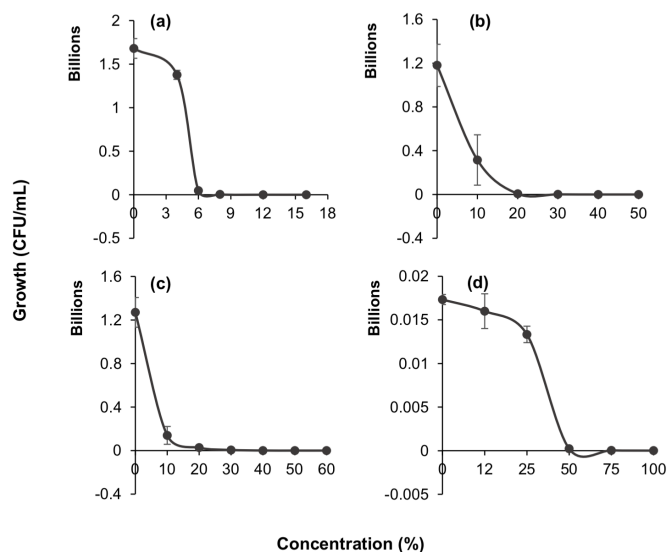


Figure 1. Effect of different concentration of fermented papaya leaf supernatant on the growth inhibition pattern of (a) *P. aeruginosa* (b) *P. acne*, (c) *S. aureus* and (d) *C. albicans*

Table 2. MIC₅₀ and MIC_{>99} of fermented papaya leaf supernatant for controlling the growth of *P. aeruginosa*, *P. acne*, *S. aureus* and *C. albicans*

Bacterial and yeast strain	MIC ₅₀ (%)	MIC _{>99} (%)
<i>P. aeruginosa</i>	4.5	16
<i>P. acne</i>	8.5	50
<i>S. aureus</i>	4.2	60
<i>C. albicans</i>	28.4	-

respectively.

3.3 Time-kill study

Time-kill pattern of fermented papaya leaf supernatant (FPLS) against the three bacteria pathogens is shown in Figure 2. The highest MIC_{>99} value of FPLS (60% concentration) was selected to compare the time needed to kill each of the bacteria completely. It was noticed that there was a 1.9 log growth reduction when *P. aeruginosa* cell was exposed to the FPLS. Indeed, the killing time was very short to eliminate *P. aeruginosa*, which was about 30 mins. In contrast, FPLS need about 2 hrs to cause 100% inhibition against *P. acne*. The different phenomenon was observed for *S. aureus* during the time-kill study. There was a gradual growth reduction of *S. aureus* during 8 hrs exposure to the FPLS. The growth was reduced from 7.3 log to 2.2 log at the initial 4 hrs and finally, no viable cell was found after 6 hrs exposure. The ability of the FPLS to kill the bacteria instantly is important. This is particularly to

reduce the possibility of the bacteria to develop resistance to the antimicrobial agents. It was reported that prolong exposure of dermatophyte *T. rubrum* to the sub-inhibitory concentration of antifungal drug has reduced the susceptibility to the antifungal drug (Ghelardi et al., 2014).

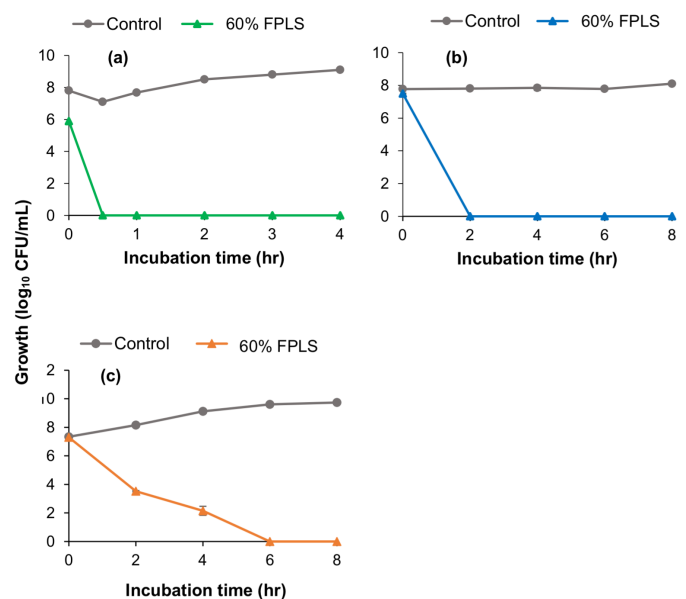


Figure 2. Time-kill pattern of fermented papaya leaf (FPLS) supernatant at 60% concentration against (a) *P. aeruginosa*, (b) *P. acne* and (c) *S. aureus*.

4. Conclusion

Fermented papaya leaf was very effective against *P. aeruginosa* growth, followed by *P. acne* and *S. aureus*. The potent killing effect of fermented papaya leaf supernatant showed a potential use as bioactive ingredient in skincare application to control pathogenic microbe infection. Future study will focus on the identification of bioactive metabolites present in fermented papaya leaf supernatant with antimicrobial properties.

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