Total Phenolic Content, Flavonoid Content, Antioxidant activity and Phenolic Acid Profile of Naturally Fermented *Carica Papaya* Leaves

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ABSTRACT

In this study, the total phenolic content (TPC), total flavonoid content (TFC), antioxidant activity and phenolic acids of naturally fermented Carica papaya (C. papaya) leaves were evaluated. The total phenolic compounds were extracted by liquid-liquid extraction (LLE) using methanol while free phenolic acids were extracted by acidic hydrolysis followed by LLE using ethyl acetate. The results showed variation of TPC, TFC, antioxidant activity and phenolic acids levels of different samples taken at different sampling points throughout the 100 fermentation period. The highest results, measured in terms of the sample dry mass (dm) relative to standard compounds were as follows: 48.42 ± 0.63 mg GAE/g for TPC at days 75, 14.47 ± 7.94 mg QE/g for TFC at days 33, 2.39 ± 0.55 mg CE/g for Proanthocyanidin at days 100 and finally, 93.82 ± 0.14 % for DPPH radical scavenging at days 60. The phenolic acids decreased with fermentation period.

I. INTRODUCTION

Carica papaya (C. papaya) has wide range of therapeutic properties such as anti-inflammatory (Patil *et. al,* 2014) , antioxidant and immunomodulatory (Canini *et.al,* 2007). Across different region of the world, different parts of C. papaya is recognized in folk medicines as cure for myriad diseases and sicknesses such as asthma, stomachache, rheumatoid, skin burn as well as antimalarial, antifertility and antiseptic (Patil *et. al,* 2014). In the tropical and sub-tropical region, C. papaya leaf extract is infamous to the anti-dengue efficacy as indicated by numerous scientific findings on the increase of platelet counts and other hematological parameters as a result of its intake (Subenthiran *et.al,* 2013, Ismail *et.al*, 2014). Studies also purported its role in preventing chronic diseases such as cardiovascular disease, cancer, diabetes and bacterial and parasitic infections (Canini *et.al,* 2007). These medicinal properties are associated with its prominent polyphenolic compounds such as alkaloids, saponins, flavanoids, tannins, proanthocyanidins, and phenolic acids Sudhalar *et.al*, 2014). Of these, flavonoids and phenolic acids stand out as the most important phytochemicals that are attributed to antioxidant and anticancer roles (Ghasemzadeh *et.al*, 2011). While most studies on C. papaya leaf only address the effect of its intake on bioassays and animal and clinical subjects, a study highlighted the presence of several phenolic acids such as protocatechuic acid, p-coumaric acid, caffeic acid and chlorogenic acid as well as flavonoids such as kaempferol and quercetin (Canini *et.al,* 2007). Another study revealed some twelve major phytochemical compounds comprising of alkaloid, organic acids, malic acid derivatives and flavonol glycosides in C. papaya leaf extract, with alkaloid carpaine and two flavonols (clitorin and manghaslin glycosides) were reported as its dominant constituents (Afzan *et.al*, 2012). The study also highlighted the glycosylated flavonols and esterified phenolic acids in natural C. papaya leaf extract rather than a glycone as reported in earlier study (Canini *et.al,* 2007).

Fermentation has been exploited by several to enhance bioactivity of several medicinal plants or their bioactive compounds. Studies highlighted the ennhanced bioactivity of fermented plants. Lactic acid fermentation of *Myrus communis* berries using *L. plantarum* and yeast extract was reportedly enhanced the antioxidant activity of the fruit with respect to DPPH scavenging activity, inhibition of linoleic acid peroxidation and increase of phenolic acids and flavonols content (Curiel *et.al*, 2015). Comparable outcomes were observed during fermentation of *Echinacea spp* using the same method. Increased level of antioxidant and anti-inflammatory were reported from fermentation of cactus pear (Opuntia ficus-indica L.) by lactic acid fermentation using several *Lactobacillus* strains, while increased level of its flavonols bioactive compounds were implicated to esterase enzyme activities which converted the flavonol glycosides into a glycones (Arunrattiyakorn *et.al*, 2011). Fermentation of mangosteen's α-mangostin by *Colletotrichum gloeosporioides* (EYL131) and *Neosartorya spathulata* (EYR042) fungi resulted several novel metabolites with the expectation of their higher bioactivity (Arunrattiyakorn *et.al*, 2011).

The potential of fermentation as evidence in the existing studies may overcome the poor absorption of native glycosylated polyphenols which are abundant in plant matrices including *C.papaya* leaf which currently relies on colonic microbiome for hydrolysis at small intestine (Cardona *et al.,*2013, Manach *et al.,* 2004). To our knowledge, natural fermentation of *C. papaya* leaf and its resulting phytochemical components analysis have never been conducted. With the perspective of innovating a standardised, highly functional dietary supplement with potential commercial application, this study aims to evaluate the phenolic profiles and antioxidant activity of naturally fermented *C. papaya* leaf.

II. MATERIALS AND METHODS

Sample preparation and extraction

A. Preparation of extract for total phenolic, flavonoid and proanthocyanidin content and DPPH scavenging effect analysis.

Fifteen milimetre of broth samples were collected at day 0, 2, 4, 8, 15, 30, 33, 45, 60, 75, 90 and 100 of fermentation. Solid debris was removed by centrifuge (10,000g) for 20 minutes and 4 °C. Then, the supernatant was collected and dried by rotary evaporator at 30 °C for 45 minutes to remove water content (Filannino, Cavoski et.al, 2016) and resuspended with 80% of methanol at 1:1 (v/v) to yield methanolic (MeOH) extract (ME). The ME was purged with nitrogen for 30 minutes followed by re-centrifugation (4,600g) for 20 minutes to remove the residue. The supernatant was collected and finally purged with nitrogen for 45 minutes.

B. Preparation extract for free phenolic acid analysis.

Fifteen milimetre of broth samples was mixed with 80% MeOH at 1:1 (v/v). The mixture was shaken for one hour and followed by centrifugation (4,225g) for 10 minutes to remove solid residue (Rizzello, 2013). The supernatant was collected and dried by rotary evaporator at 30 °C for 30 minutes. The residue was re-dissolved in 30 ml of MilliQ water and acidified to pH 1.5. by several drops of 5M HCl. The mixture was subjected to liquid-liquid extraction (LLE) using ethyl acetate (EtOAc) at 1:1 (v/v) and shaken every 10 minutes for 30 minutes resulting organic layer (top) and aqueous layer (bottom). The aqueous layer was re-extracted as before. The EtOAc extracts were later combined and dried using rotary evaporator at 30 °C for one minutes to remove EtOAc. Finally, the sample was purged by nitrogen to remove EtOAc residue and stored in the desiccator. The dried sample was redissolved in 5 ml MeOH prior to analysis.

Analysis compound in different method

A. Determination of total phenolic content

Total Phenolic content (TPC): For the construction of gallic acid equivalent (GAE) standard curve, 10 mg of gallic acid was dissolved in 100 ml of 50% MeOH (100 µg/ml) of stock solution and further diluted into 0, 6.25, 12.5, 25 and 50 ug/ml of working solutions. One ml of each working solution was added into 10 ml of distilled water. Then 1.5 ml of Follin & Ciocalteu's reagent was added into each working solution and incubated at room temperature for five minutes followed by addition of four mililitre of 20% w/v Na_2CO_3. Then, distilled water was added to bring to 25 ml volume and left to stand for 30 minutes at room temperature (Madaan et.al, 2011). The absorbance value of standard assays were measured at 765 nm using UV-vis spectrophotometer. Gallic acid standard calibration curve was constructed by by plotting absorbance vs known concentrations.

For the estimation of TPC od sample, 20 mg of dried sample was added into 10 ml of 50% MeOH. Then, 1 ml of mixture was added into 10 ml of distilled water and 1.5 ml of Follin & Ciocalteu's reagent followed by incubation for five minutes at room temperature. Next, four ml of 20% w/v Na_2CO_3 was added into the mixture followed by addition of distilled water to bring 25 ml volume. The assay was left for 30 minutes at room temperature prior to absorbance reading at 765 nm. All readings were done in triplicate.

Total phenolic content (TPC) of samples was calculated in terms of gallic acid equivalent (GAE) per sample dry mass (dm) i.e. TPC= C x V/M where TPC is total phenolic content, C is concentration of gallic acid (µg/ml), V is volume of extract solution (ml) and M is mass of sample (g) (Abdelhady et.al, 2011).

B. Determination of total flavonoid content

Total Flavonoid content (TFC): For the construction of quercetin equivalent (QE) standard calibration curve, 20 mg of quercetin was added into 20 ml of MeOH to obtain 1000 ug/ml of stock solution.. The stock solution was further diluted to 0, 20, 40, 60, 80 and 100 ug/ml working solutions. Then, 1 ml of each working solution was added into four mililitre of distilled water and 0.3 ml of 5% w/v $NaNO_2$. The mixture was left to stand for five minutes prior to addition of 0.3 ml 10% w/v $AlCl_3$ at sixth minute. Finally, two mililitre of 1M NaOH and 4.8 ml of distilled water were added.. The absorbance was measured at 510 nm using UV-vis spectrophotometer following the color change from colorless to orange yellowish. The standard calibration curve was constructed by plotting absorbance vs concentration (Zhishen et.al, 1999).

For sample analysis, 20 mg of dried sample was dissolved in 10 ml of MeOH. Then, one mililitre of the mixture were mixed with 4 ml of distilled water and 0.3 ml of 5% w/v $NaNO_2$ in test tube. Next, the mixture was incubated for five minutes followed by addition of 0.3 ml of 10% w/v $AlCl_3$ at sixth minute. Finally, two mililitre of 1M sodium hydroxide and 4.8 ml of distilled water were added. The absorbance was measured at 510 nm using UV-vis spectrophotometer. All readings were done in triplicate.

Total flavonoid content (TFC) of sample was calculated in terms of quercetin equivalent (QE) per sample dry mass (dm) i.e. TFC = C x V/M where TFC is total flavonoid content, C is concentration of quercetin (µg/ml), V is volume of extract solution (ml) and M is mass of sample (g)

C. Determine of proanthocyanidin content

Total Proanthocyanidin content: For the construction of catechin equivalent (CE) standard calibration curve, ,five miligram of catechin was added into 20 ml of absolute MeOH to obtain stock solution of 250 µg/ml and further diluted into 0 to 25 ug/ml of working solutions. Then, two mililitre of each woking solution was added to five mililitre of 4% (w/v) of vanillin and five mililitre of 6M HCl. Finally, the mixture was incubated for 15 min at room temperature to allow reaction before absorbance reading 500 nm using UV-vis spectrophotometer (Sun et.al, 1998).

For sample analysis, 20 mg of dried sample was re-disolved in 10 ml of absolute methanol. Then, one mililitre of the suspension was added into 2.5 ml of 4% w/v vanillin in a test tube. Next, 2.5 ml of 6M HCl was added into the mixture and incubated for 15 minutes at room temperature before absorbance reading. All readings were done in triplicate.

Proanthocyanidin content was expressed in terms of catechin equivalent (CE) per sample dry mass (dm) i.e. Proanthocyanin content = (C x FV x d x F)/W where C is concentration of catechin (µg/ml), FV is volume of extract (ml), d is dilution factor and W is weight of extract samples (g).

D. Determination of DPPH scavenging activity

DPPH scavenging activity: The radical scavenging activity was expressed in terms of (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) equivalent (TE) per sample dry mass (dm) (mM TE/g). For the construction of standard calibration curve, 44 mg of Trolox was added into 100 ml of 80% methanol and further diluted into 0 to 800 uM of working solutions. Meanwhile, the free radical model was prepared by dissolving 24 mg of DPPH in 100 ml of MeOH and stored at -20 °C until needed. The DPPH working solution was prepared by mixing 10 ml of stock solution with 45 ml of MeOH which gave

1.1 ± 0.02 absorbance unit at 515 nm with UV-vis spectrophotometer (Shimamura et.al (2014).

For DPPH scavenging activity analysis, 0.15 ml of trolox sample was mixed with 2.85 ml of DPPH solution. The reaction of trolox and DPPH was allowed for 24 h under darkness absorbance reading at 515 nm. The free radical scavenging effect (%) was calculated as follows:

$$\text{Scavenging effect}(\%) = \left[1 - \left\{\frac{A_c - A_s}{A_c}\right\}\right]$$

where Ac is control absorbance (MeOH) and As is sample absorbance (after reacting with DPPH) (Vuong et.al, 2013).

For free radical scavenging activity of the sample, 20 mg of dried sample was dissolved in 10 ml of methanol. Then, 0.15 ml of sample was added into 2.85 ml of DPPH solution. Finally, the absorbance reading at 515 nm was taken after 24 h incubation under darkness. All readings were done in triplicate.

E. Determination of phenolic acid contents by High Performance Liquid Chromatography (HPLC)

Free phenolic acids were analysed in high performance liquid chromatography (HPLC) (Perkin Elmer) according to Curie et al. with some modifications (Curiel *et al.*,2015). The individual phenolic acid was separated by C18 column. The dry matter of each sampling point was re-dissolved in 5 ml methanol using ultrasonic mixer, filtered through 0.2 µm filter and injected into HPLC at 20 µl. Isocratic elution was carried out at 30 °C with a flow rate of 0.8 ml/min using mobile phase comprising of solvent A (water/acetic acid, 98:2, v/v) and solvent B (water/acetonitrile/acetic acid, 78:20:2, v/v) at constant A:B; 10:90 v/v. The compounds were detected at 280 nm. Samples were spiked with mixture of all standard compounds. About 5 mg of each standard compound was dissolved in 5 ml methanol, then serially diluted into several concentrations (1.0, 0.5, 0.25, 0.1, 0.55 mg/ml) to build linear calibration curves by plotting peak area vs concentration.

III. RESULTS AND DISCUSSION

Total Phenolic Content

Figure 3.1 shows the linearity standard calibration curve of gallic acid in range 0 µg/ml to 150 µg/ml (R^2 = 0.9945).

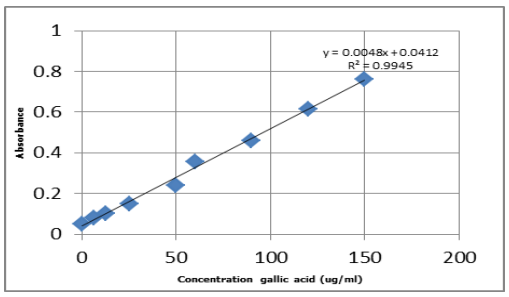

Figure 3.1 Standard calibration curve of gallic acid.

A. Total phenolic content

The total phenolic content in those samples fermented Carica papaya leaves were in range 12.13 ± 0.39 mgGAE/g to 47.49 ± 0.18 mgGAE/g. Phenolic content for samples can be calculate by using method T = C x V/M (Abdelhady et.al, 2011) which equivalent to amount gallic acid that contain in the samples. T is stand for total phenolic content, C is concentration of gallic acid in calibration curve (µg/ml), V is volume of extract solution (ml) and M is weight of extract samples (g).

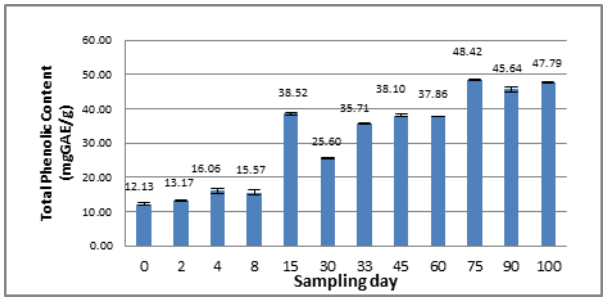

Figure 3.2 Total phenolic content (TPC) of fermented C. papaya leaf in mg GAE/g dm (mean±SD).

The **Figure 3.2** shows total phenolic content (TPC) content of the fermented C. papaya leaf samples throughout fermentation period. The highest TPC was at 100 day i.e. 47.79 ± 0.18 mg GAE/g dm. The TPC was relatively increased with fermentation day despite a discontinuation on day 15 i.e. 38.52 ± 0.28 mgGAE/g.

By comparison, fermented C. papaya leaf showed higher TPC (48.42 ± 0.31 mg GAE/g at day 75) than aqueous papaya leafs extract (28.61 ± 0.03 mg GAE/g) by Quan et.al, 2013. This can be related to enzymatic action by microorganisms such as esterase that freed the phenolic compound from their glycosidic and ester bonds. Such effect was more pronounced as the various microoganisms accumulated in the fermentation ecosytem as fermentation progressed. Higher TPC per amount of product implied greater therapeutic effect to the consumer thus supported the novel idea of naturally fermented C. papaya leaves.

Total Flavonoid content

Figure 4.2 shows the linearity standard calibration curve of quercetin indicated by R^2 = 0.9767.

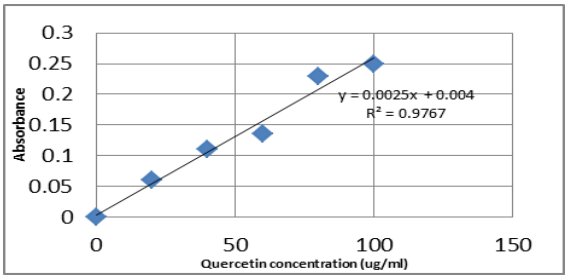

Figure 3.3 Standard calibration curve for quercetin.

B. Total flavonoid content

The lowest TFC was 0.27 ± 1.15 (mgQE/g) at day 0 while the highest was recorded at 14.47 ± 3.97 mgQE/g at day 33.

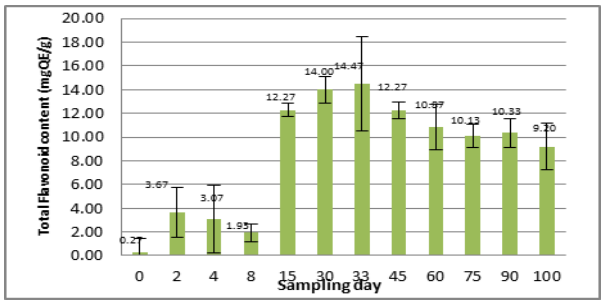

Figure 3.4 Total flavonoid content (TFC) of fermented C. papaya leaf in mg GAE/g dm (mean±SD).

Similar to TPC results, TFC of fermented C. papaya leaf was relatively higher than conventional C. papaya leaf extract. By comparison, the lowest TFC during fementation was at day 0 when the fermentation was at very initial stage. In contrast, significantly higher TFC were observed at later stage of fermentation. Other finding by Agung Nugroho et.al (2017) recorded 7.23 mg QE/g sample which was apparently lower TFC level than any fermented C. papaya leaf samples taken on day 15 onwards.

Similar to TPC results, the fermenting microorganisms synthesised enzymes that might hydrolysed the bound flavonoid compounds into aglycones (free flavonoids) thus enhancing their calorimetric detection. Thus, given the fact that flavonoids is well-known antioxidant compound that are beneficial to cancer prevention and other degenerative diseases (Agung Nugroho et.al, 2017), better therapeutic effect and higher functionality of the fermented C. papaya leaf is likely due to its richer TFC as compared to conventional extract (Srivastava & Singh 2016).

Total Proanthocyanidin

Figure 3.5 shows the linearity standard calibration curve of catechin indicated by (R^2 = 0.9961)

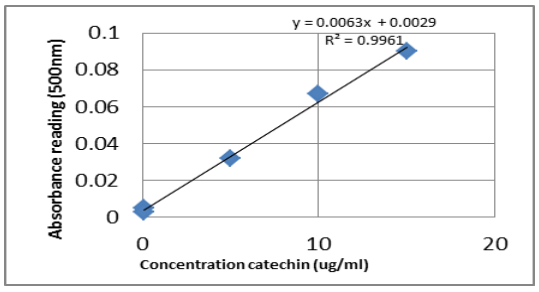

Figure 3.5 Standard calibration curve for Catechin.

C. Total proanthocyanidin content

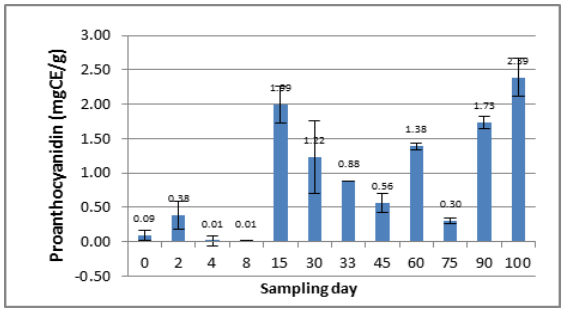

Figure 3.6 Total proanthocyanidin of fermented C. papaya leaf in mg QE/g dm (mean±SD).

The result in **Figure 3.6** shows the lowest proanthocyanidin content of 0.01 ± 0.08 mgCE/g and 0.01 ± 0.00 mgCE/g at day 4 and day 8 respectively whereas the highest value was recorded at 2.39±0.27 at day 100. By comparison with aqueous extract conducted by (Quan et.al, 2013), the proanthocyanidin content of of fermented *C. papaya* extract at any sampling point was lower than findings by Quan et.al, 2013 which was at 4.86 ± 0.16 mg CE/g.

DPPH Scavenging activity

Figure 3.7a shows the linear standard calibration curve of Trolox (R^2 = 0.9678).

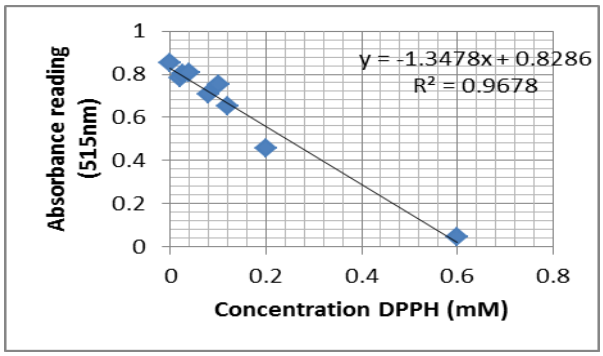

Figure 3.7a Standard calibration curve for Trolox concentration.

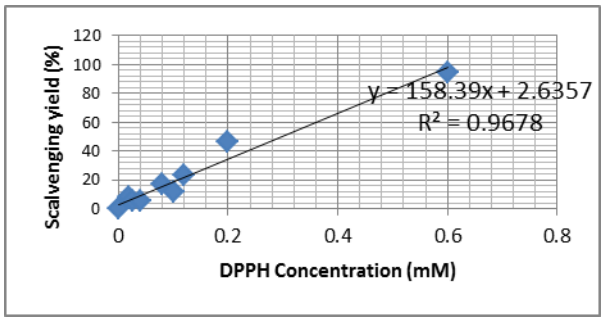

Figure 3.7b Scavenging activity for DPPH in Trolox concentration.

D. DPPH Scavenging or Antioxidant activity

Figure 3.7a and 3.7b show the total antioxidant content and DPPH scavenging activity at different sampling points. The highest total antioxidant content and DPPH scavenging activity was recorded at day 60 at 143.9±0.1 mM TE/g and 93.8±0.14% respectively. The total antioxidant content and DPPH scavenging activity were apparently increased after second week of fermentation which hinted the enrichment of antioxidant compounds resulting from the microbial activites.

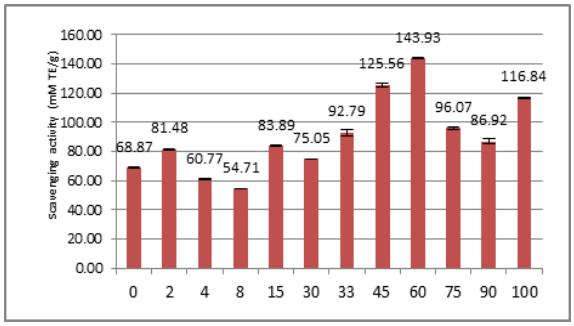

Figure 3.8a The extraction of fermented C. papaya leaf broth for antioxidant activity.

By comparison, the DPPH antioxidant activity of aqueous extract recorded by Quan et al. (2013) was 96.44 ± 4.58 µgTE/g.

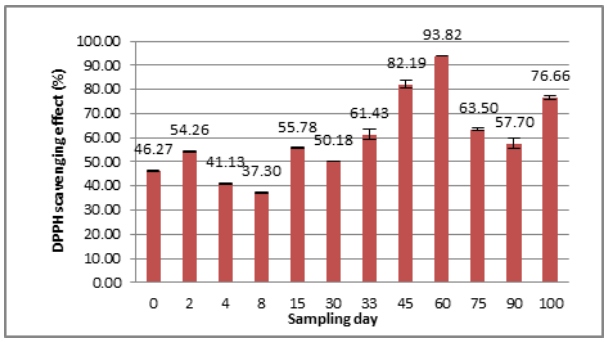

Figure 3.8b DPPH radical scavenging activity for fermented C. papaya leaf broth samples.

Free phenolic acids

Table 1 Phenolic acids at selected sampling points

	[a]Concentration (mg/g dm*)		
Phenolic compounds	Day 1	Day 60	Day 90
Chlorogenic acid	112.1±1.9	11.5±5.7	15.8
Gallic acid	26.7±0.06	2.3±0.2	0.5±0.0004
p-coumaric acid	0.8±0.001	0.6±0.1	1.0±0.0008
5,7-dimethoxycoumarin	647.1±94	346.7±74	163.7±0.3

*dm dry mass
[a]mean[n]±SD
n=2

The phenolic acids concentration at selected sampling points (Table 1) shows their decline at later fermentation stage as compared to initial stage. While the exact cause of this trend may be attributed to microbial action, Filannino et al. 2017 in their lactic acid fermentation of *cactus cladodes* pointed out that phenolic acids between fermented and unfermented materials may not be significant.

IV. CONCLUSION

Different extracts of different sampling points of naturally fermented C. papaya display varying degree of total phenolic content, total flavonoid content, total proanthocyanidin content, antioxidant acitivity and phenolic acids. Nevertheless, the presence of these phenolic compounds and their antioxidant activity highlighted the functionality and medicinal values of naturally fermented C. papaya which are comparable to other medicinal plants.

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