Phytochemical Analysis of Extract from Spontaneous Fermentation of Papaya Leaf

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Abstract— **In this study, phytochemical analysis was carried out on spontaneously fermented papaya (*Carica papaya*) leaf extract in terms of total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity. Moreover, the aim was to relate the presence of the metabolic compounds with the therapeutic benefits of the naturally fermented *C. papaya* leaf. Extraction method used solvent extraction by using methanol. Estimation of TPC and TFC used Folin-Ciocalteu's reagent and aluminium chloride respectively, whereas antioxidant activity used DPPH and Trolox as radical and antioxidant models respectively. The results showed dissimilarity of TPC, TFC, and antioxidant activity of different samples taken at different sampling points throughout the 90 days fermentation period. The highest TPC and TFC were recorded at day 30 (79.84±0.29 mg GAE/g) and day 20 (51 ± 2.17 mg QE/g) respectively, whereas the highest antioxidant activity was recorded at day 30 (42.41 mM TE/g).**

Keywords—Carica papaya, spontaneously fermented, total phenolic content, total flavonoid content, antioxidant activity.

I. INTRODUCTION

The papaya tree belongs to *Caricaceae* which is a small family that has four genera (Canini, Alesiani, Arcangelo, & Tagliatesta, 2007). This *Carica papaya Linn* is said to be one of the four species in India that is under genus *Carica Linn* (Krishna et al., 2008). It is also known as paw-paw and is the best species and most commonly cultivated. Historically, in 16th century, papaya was introduced in India (Yogiraj et al., 2014). Subsequently, it was introduced in Hawaii, Philippines, Sri Lanka, South Africa, Australia, and in all other tropical and subtropical regions as a plantation crop. However, the origin of this plant is in Costa Rica and southern Mexico. Meanwhile, in Malaysia, it is known as non-seasonal and evergreen plant.

Even though *C. papaya* leaves can be considered as waste, with optimum extraction conditions, their extracts can give variety of health advantages. Basically, for mineral composition, among other parts of the plant, *C. papaya* leaves contain high mineral elements source. Minerals in *C. papaya* leaves such as Mg, Ca, Na, K, Fe and Mn are beneficial to human. Those minerals estimation can be done by using Inductively coupled plasma-Atomic Emission Spectroscopy (ICP AES) (Prod et al., 2014).

For *C.papaya* leaf, preliminary qualitative tests can be done to determine the metabolites present in greater proportion. The qualitative metabolite analysis normally uses the *C. papaya* leaves extracts. Nevertheless, the presence or absence of those metabolic compounds depend on solvent polarity used for the extraction (Roade et al., 2014). *C. papaya* leaves contain carpaine, alkaloids, phenolic acids, pseudocarpaine, cystatin, tocopherol, flavonoids, ascorbic acids, and cyanogenic glucosides which are metabolic compounds or secondary metabolites that able to treat any diseases due to its pharmacological properties (Elgadir, Salama, & Adam, 2014). However, Canini et al., 2007 reported that compared to coumarin and flavonoids, the main compounds in papaya leaves are phenolic acids and also trace amount of chlorogenic acid. *C. papaya* leaves extracts contain phenolic compound which is active compound of this plant. The example of phenolic compound that exist in *C. papaya* leaves are kaempferol, quercetin, protocatechuic acid, p-coumaric acid, 5, 7-dimethoxycoumarin, caffeic acid, and chlorogenic acid (Aruljothi, Uma, Sivagurunathan, & Bhuvaneswari, 2014). Since those important active compounds have high antioxidant ability and free radical scavenging capacity, it can act as chemo preventive and also protect human body against oxidative damage by free radicals (Am, Asmah, & Fauziah, 2014). Moreover, those active compounds can be used to treat other variety type of diseases.

The process of fermentation has been traditionally used worldwide because of its advantages such as to help the certain microbes maintains their nutrition and also increase the value of plant and at the same time avoid any postharvest losses. One of the advantages of fermentation process towards *C. papaya* leaves is that their metabolite components can be increased which gain their vitamin, amino acids compound, and antioxidant. As the main compound in the *C. papaya* leaves, phenolic compounds have been considered as xenobiotics that are contingent on complexity of their chemical structure which has unfortunate resulted bioavailability 5-10% approximately and reduce their therapeutic effects. But, the other findings are still unavailable to carry out fermentation process that can develop *C. papaya* leaves bioavailability. Ayoola et al., 2010 stated that research has found the comparison between nutrients content and phytochemical for different used of papaya leaf from the same plant which undergo fermentation process.

To our knowledge, spontaneous fermentation of *C. papaya* leaf, hence its phytochemical analysis has never been carried out. With the perspective of innovating a standardised, highly functional dietary supplement, this study aims to evaluate the phenolic profiles and antioxidant activity of spontaneously fermented *C. papaya* leaf.

II. METHODOLOGY

A. Materials

C. papaya leaves were bought from the supplier in Shah Alam, Malaysia. The leaves were washed and then shredded using blender to become smaller pieces to be used as the feedstock in 50 L fermenter at 10% (w/v). With the quantity of 10% (w/v), unrefined sugar was used as substrate. In addition, distilled water was added to make up 50 L volume. The mode of fermentation was anaerobic and the fermentation was done for 90 days at room temperature condition. The broth samples were collected at day 0, 2, 5, 10, 20, 30, 40, 60, and 90 of fermentation for the analysis.

B. Chemical and Instruments

The main chemical used for this research is methanol (CH_4). For analysis, chemicals used are Folin-Ciocalteu's reagent, gallic acid, sodium carbonate (Na_2CO_3), quercetin, sodium nitrite ($NaNO_2$), Aluminium chloride ($AlCl_3$), Sodium hydroxide (NaOH), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox).

The extract preparation process used centrifuge and rotary evaporator (Heidolph). Meanwhile, the UV-Vis Spectrophotometer (SECOMAM) was used to read the absorbance value.

C. Preparation of extract for total phenolic, flavonoid and DPPH scavenging effect analysis.

Fifteen milliliters (15 ml) of *C. papaya* leaves suspension were collected at day 0, 2, 5, 10, 20, 30, 40, 60, and 90 of fermentation. Next, solid debris was removed by centrifuge (10,000 g) for 20 minutes and 5°C. Then, the supernatant was collected and dried by rotary evaporator at 30°C for 45 minutes to remove water content (Filannino, Cavoski, 2016) and re-suspended 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.

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 milliliter 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 was measured at 765 nm using UV-vis spectrophotometer. Gallic acid standard calibration curve was constructed by plotting absorbance against known concentrations.

For the estimation of TPC of 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.

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 milliliters 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 milliliters 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 against concentration (Zhishen et.al, 1999).

For sample analysis, 20 mg of dried sample was dissolved in 10 ml of MeOH. Then, one milliliters 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 milliliters 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.

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. 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, 10 mg of Trolox was added into 20 ml of absolute 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:

$$Scavenging\ effect\ (\%) = \left(\frac{A_c - A_s}{A_c}\right) \times 100$$

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 absolute 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.

III. RESULTS AND DISCUSSION

A. The Determination of total phenolic content

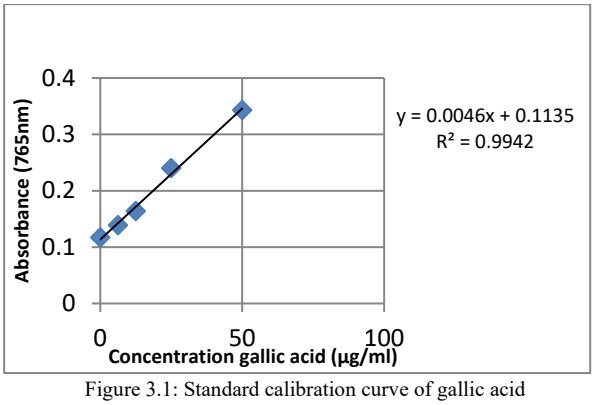

Figure 3.1: Standard calibration curve of gallic acid

Figure 3.1 shown gallic acid calibration curve that was used to analyze and measure amount of phenolic content of samples. This graph has gallic acid linearity range from 0 to 50 µg/ml. Standard curve equation is y = 0.0046x + 0.1135 with R^2 = 0.9942.

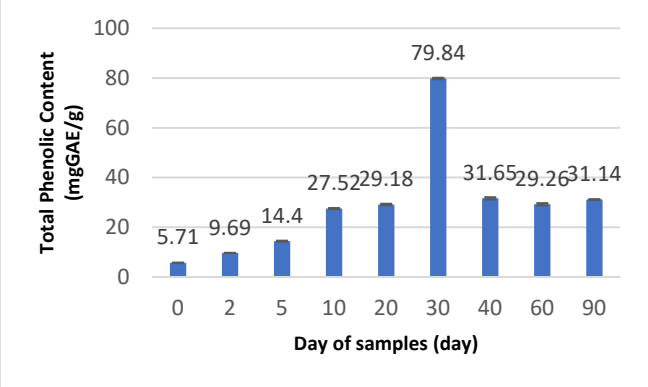

Figure 3.2: Total Phenolic content in fermented *C. papaya* leaves broth

Based on Figure 3.2 above, the trend of the TPC of the fermented samples is increasing. Although the increased result in TPC is not steady, there is still a pattern of increasing TPC day by day of fermentation. The highest TPC in fermented sample was at day 30 which is 79.84±0.29 mg GAE/g. Although it was higher than day 40 (31.65±0.44 mg GAE/g), but the consistency of amount TPC in fermented samples was still increasing across the time. The last sample (day 90) shown higher TPC (31.14±0.00 mg GAE/g) than sample of day 60 (29.26±0.45 mg GAE/g).

In comparing with fermented and unfermented papaya leaves, the highest TPC of fermented papaya leaves was at day 30 which is 79.84±0.29 mg GAE/g. However, Vuong et al., 2013 stated that the highest TPC of unfermented papaya leaves was 28.61±0.03 mg GAE/g sample. From this comparison, it is proven that fermented *C. papaya* leaves broth samples contained highest TPC value that will give advantage to consumers' health. This is because higher TPC implied better therapeutic effect to consumer. Hence, novel idea of spontaneously fermented *C. papaya* leaves can be supported.

B. The Determination of total flavonoid content

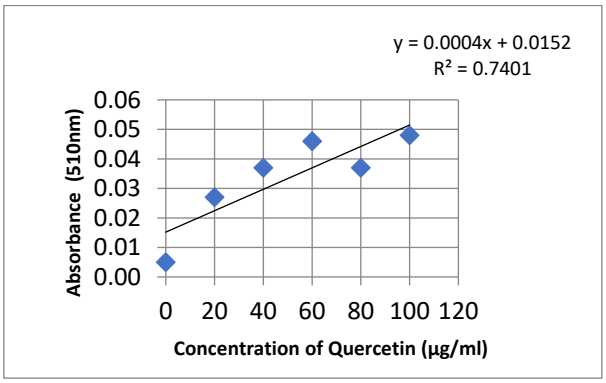

Figure 3.3: Standard calibration curve of quercetin

Figure 3.3 shown quercetin calibration curve that was used to analyze and measure amount of flavonoid content of samples. This graph has quercetin linearity range from 0 to 100 µg/ml. Standard curve equation is y = 0.0004x + 0.0152 with R^2 = 0.7401.

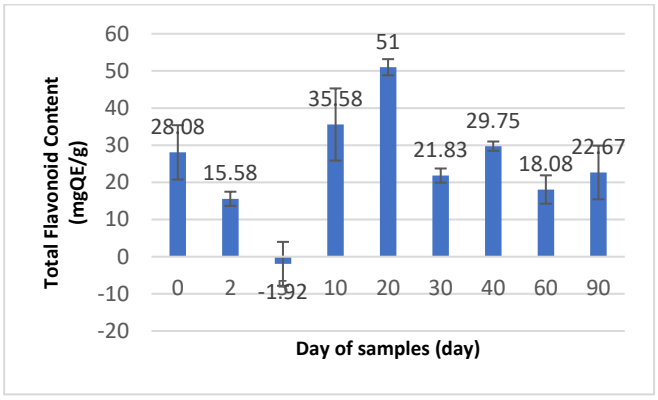

Figure 3.4: Total Flavonoid content in fermented *C. papaya* leaves broth

Based on the Figure 3.4 above, the amount of TFC is not consistent as it is increasing and decreasing across the time until fermentation day 90. However, TFC also seems to increase as day increase. This graph shown that day 20 has the highest TFC which is 51±2.17 mg QE/g. From previous research conducted by Nugroho, Heryani, Choi, & Park, 2017, the highest TFC amount in unfermented papaya leaves samples was 7.23 mg QE/g sample which lower than fermented samples. Moreover, TFC amount of sample at day 90 also higher which is 22.67±7.22 mg QE/g compared to unfermented sample.

In comparison, it is proven that TFC of fermented *C. papaya* leaves samples was higher than conventional *C. papaya* leaves extract. During fermentation, the lowest TFC was at day 5 which is still considered as initial stage of fermentation. At later fermentation stage, the TFC were significantly higher than TFC at day 5. On day 10 onwards, TFC of fermented samples were higher than 7.23 mg QE/g which is value of the highest TFC number of unfermented samples. Therefore, fermented *C. papaya* leaves has better therapeutic effect as it can prevent cancer (Nugroho, Heryani, Choi, & Park, 2017).

C. The Determination of DPPH Scavenging or Antioxidant activity

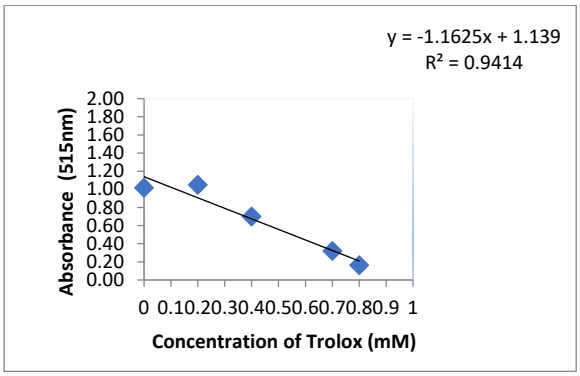

Figure 3.5: Standard calibration curve of Trolox concentration

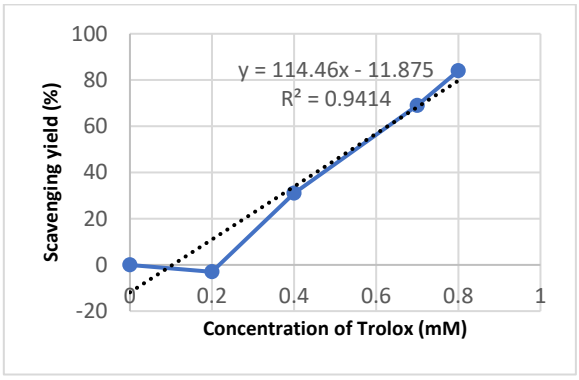

Figure 3.6: Scavenging activity for DPPH in Trolox concentration

Figure 3.5 shown Trolox calibration curve that was used to analyze and measure amount of antioxidant activity of samples. The equation that equivalent to Trolox standard is y = -1.1625x + 1.139 with R^2 = 0.9414. From the graph, it shown that the Trolox concentration ranges from 0 to 0.8 mM.

Moreover, DPPH scavenging also been calculated in order to identify the antioxidant activation characteristic for all samples. The equation used to calculate DPPH radical scavenging yield was based on inhibition (%) = {(Ac-As)/Ac} × 100 (Vuong et al., 2013). From this equation, graph from Figure 3.6 was obtained. Therefore, calibration curve equation of DPPH radical scavenging was y = 114.46x – 11.875 with R^2 = 0.9414.

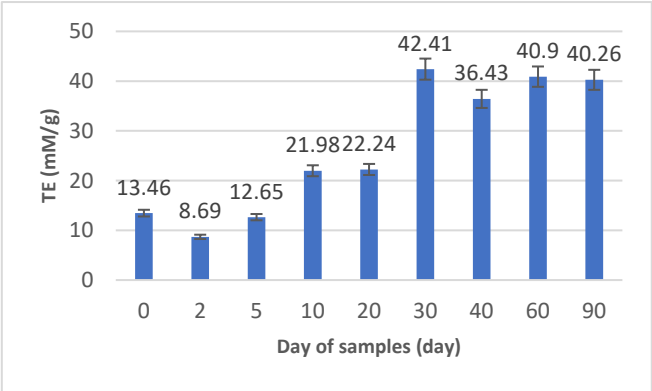

Figure 3.7: TE content in fermented *C. papaya* leaves broth

Figure above shown antioxidant activity of different sampling days. The highest antioxidant activity was observed at day 30 with 42.41 mM TE/g. From that bar graph, it has shown that the trend was increasing even not consistent. This means that there was an enrichment of antioxidant compounds which resulting from microbial activities. Vuong et al., 2013 on the other hand stated that the DPPH antioxidant activity of aqueous extract was 96.44 ± 4.58 µg TE/g.

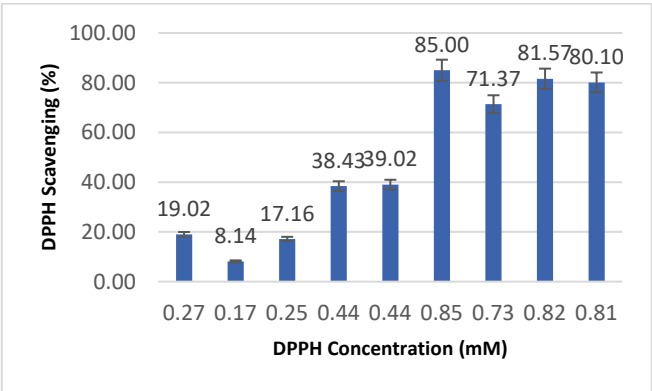

Figure 3.8: The DPPH radical scavenging activity for fermented *C. papaya* leaf extract samples

Figure 3.8 shown DPPH radical scavenging activity for fermented *C. papaya* leaf extract samples which were increased but not consistent. This DPPH radical scavenging also showed the highest antioxidant activity at day 30 (85 % scavenging). Therefore, both antioxidant activity content and DPPH radical scavenging activity were increased after fermentation day 2.

IV. Conclusion

As a conclusion, methanol and water mixture used to extract the sample is suitable since the metabolic compounds in samples can be shown. The analysis of metabolic compound which involve TPC, TFC, and the antioxidant activity have increasing trend across fermentation days even though the bar graph is not consistent. However, the values of those metabolic compound vary since there are different extract of different sampling points of spontaneously fermented *C. papaya* leaves.

Since the metabolic component of fermented samples is higher than unfermented samples, its therapeutic advantages are also higher. It can be concluded that the presence of phytochemical constituents which is phenolic compounds and their antioxidant activity emphasized the therapeutic values of spontaneously fermented *C. papaya* leaves. Therefore, efforts must be done in order to promote the fermented *C. papaya* leaves extracts for human health benefits.

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