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# Effect of extraction conditions of *Carica papaya* leaves aqueous extracts and its resulting infusion with "kelulut" honey to its antioxidant activity

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*Carica papaya* is a tropical plant belonging to the *Caricaceae* family. Rich in phytochemical content, its leaves especially are renowned for having many health benefits, namely anticancer properties and as supplement in treating dengue disease. This study thus aimed to optimise aqueous extraction and to determine the effect of honey infusion on the leaves extract which was analysed using TPC, TFC, FRAP and DPPH assays respectively. The optimal extraction conditions for aqueous extraction were determined to be at 70°C for 20 minutes where its TPC was 9.97±0.47 mg GAE/mL ($p<0.05$), TFC was totaled at 2.63±0.52 mg QUE/mL ($p<0.05$) while its FRAP assay was amounted at 16.84±1.10 mg TE/mL. Radical scavenging values using DPPH assay was recorded to be at 87.53% with its $IC_{50}$ at 492.54±2.45 mg TE/mL ($p<0.05$). Study on the infusion of "kelulut" honey from *Trigona* species with the leaves extract provides evidence that not only does it improve the taste of the bitter papaya extract; the positive synergy also increases the overall antioxidant activity. Antioxidant values are found to increase in accordance with increasing honey dosage (max 4 tbsp.), where its TPC was valued at 21.66±0.54 mg GAE/mL ($p<0.05$), FRAP test at 24.02±0.87 mg TE/mL ($p<0.05$), radical scavenging activity at 98.2% and lower $IC_{50}$ value at 408.02± 5.0 mg TE/mL. Future study can therefore be done to improve and perhaps release the product commercially.

**Keywords:** Total Phenolic Count (TPC), Total Flavonoid Count (TFC), Ferric Reducing Antioxidant Power (FRAP), (2,2-diphenyl-1-picryl-hydrazyl-hydrate) DPPH, Carica papaya leaves

## I. INTRODUCTION

*Carica papaya* leaves are regarded highly for its many practical and healthy purposes. Rich in phytochemical and vitamins, further study on the leaves extract shows that it possess excellent antioxidant, anticancer, anti-inflammation, antibacterial and antibiotic property (Aravind *et al.*, 2013). Interestingly, in the recent years, consumption of *Carica papaya* leaves extract is increasingly popular especially among health conscious consumers due to its success in curbing dengue disease (Hettige, 2008; Yunita *et al.,* 2012; Subenthiran *et al.*, 2013). Although there are no successful studies yet to document the exact properties of the leaves that majorly contributed to the health recovery of dengue patients, research by Datan *et al.* (2016); Liu *et*

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*al.* (2017) found out that virus infection causes spike of reactive oxygen species (ROS) production, consequently causing further oxidative stress in body cell- which, if left untreated may be fatal to patients. Therefore, it is crucial to consume food rich in antioxidants such as *Carica papaya* leaves to help balance, combat and prevent oxidative stress based diseases (Koruk *et al.*, 2004; Dhivya *et al.*, 2016), since it provides the necessary nutrients to help repair and rejuvenate cell damage against virus infection (Assinger, 2014; Soundravally *et al.*, 2008; Valero *et al.*, 2013 Tsao, 2010). Thus, in order to maximise the antioxidant yield, this study first aims to focus on optimising the preparation of *Carica papaya* leaves with time and temperature as the manipulating factors. Subsequently, preparation of *Carica papaya* leaves extract either by clinical trials or home remedy are usually added with sugar (Ahmad *et al.*, 2011) or honey to abate its bitter taste. This study therefore will replicate and study the preparation of infusing honey ("kelulut" honey) with aqueous *Carica papaya* leaves extract. The study of infusing "kelulut" honey is interesting since should there be a positive synergy between the two, it might result in increasing antioxidant properties. This is because "kelulut" itself has high content of propolis, making it a potent antioxidant, antibacterial, antifungal and antivirulant-inflammatory agent as well (Surendra *et al.*, 2012; Ibrahim *et al.*, 2016).

## II. MATERIALS AND METHODS

The purchase of Folin–Ciocalteu's phenol reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox) and potassium acetate ($CH_3CO_2K$) were done through Sigma-Aldrich (USA). Fluka provided gallic acid and anhydrous sodium carbonate ($Na_2CO_3$). Subsequently, 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), ferric chloride ($FeCl_3$), hydrochloric acid (HCl) and quercetin hydrate with ≥95% purity was supplied by Thermo Fisher Scientific while sodium acetate trihydrate ($CH_3COONa.3H_2O$) was supplied by Merck. Methanol AR grade was supplied by Qrec. Deionised water was self-produced using a Purelab Maxima water purifier. Consequently, 96-well microplate on the other hand was purchased from Thermo Scientific™ Nunc™ Microwell™. Ultrasonic bath used in this study was Bransonic 5510E-DTH. Analysis was done using Multiskan™ Go 1510 microplate spectrophotometer (ThermoFisher Scientific).

### A. Sample Collection

Sampling was done in the area of Analytical Chemistry Laboratory in Universiti Malaysia Sabah. To maintain data accuracy and reliability, all samples of young, healthy third leaf from the uppermost shoot of the *Carica papaya Linn.* tree were freshly harvested from one chosen source to maintain data accuracy and reliability.

### B. Sample Preparation

Optimal sample preparation of plant material was done by taking two manipulating variables into account; temperature and time factor.

#### *1. Effect of Temperature*

Based on a slightly modified method employed by (Vuong *et al.*, 2013), fresh plant material weighing 1 g was washed, cut into small pieces and pounded using pestle and mortar (n=3, in triplicate for each sample and conditions). Ten milliliters (10 mL) of water was added to aid in the maceration process. The extract was then transferred to a beaker and topped off to 100 mL for aqueous extraction. Extraction process was first done in room temperature for five minutes. After the exact time has passed, the aqueous extract collected was strained using cloth filter for extracted leaves removal. Consequently, 10 mL methanol was added to 4 mL of the aqueous extract and sonicated for 15 minutes using ultrasonic bath. Aqueous extract was then immediately used for selected assay analyses. The experiments were repeated at different temperature of 40$^{o}$C, 50$^{o}$C, 60$^{o}$C, 70$^{o}$C, 80$^{o}$C, 90$^{o}$C and 100$^{o}$C which were regulated using water bath. The optimal temperature obtained (70$^{o}$C) was then used to determine the optimal time for sample preparation.

#### *2. Effect of Time*

Using similar procedure, the experiment was repeated at 10, 15, 20, 25 and 30 minutes instead. With the data of optimal temperature (70$^{o}$C) and time (20 minutes) factor, the best sample preparation method at 1 g sample extracted with 100 mL) was therefore obtained.

### C. Assay Analysis Methodology

The methodology of applied test and assay are shown below:

#### *1. Total Phenolics Count (TPC) Test*

The total polyphenol content was determined with slight modification (Ainsworth and Gillespie, 2007). Gallic acid was used as the standard for a calibration curve (0, 20, 40, 60, 80, 90 and 100 µg/mL) and the results were expressed as mg of gallic acid equivalents per mL of sample (mg GAE/mL).

#### *2. Total Flavonoid Count (TFC) Test*

Total flavonoid count of the samples was done with slight modification (Chia-Chi Chang *et al.*, 2002). Quercetin was used as the standard for a calibration curve (0, 20, 40, 60, 80 and 100 µg/mL) and the results were expressed as mg of quercetin equivalents per gram of sample (mg QUE/mL).

*3. Ferric Reducing Ability of Plasma (FRAP) Assay*

Preparation of FRAP reagent (Russo *et al.*, 2013) was carried out with slight modification with Trolox as a standard (0, 20, 40, 60, 80 and 100 μg/mL). The results were expressed as mg of trolox equivalents per gram of sample (mg TE/mL).

*4. 2,2-diphenyl-1-picrylhydrazyl (DPPH) Assay*

With slight modification, the radical scavenging activity of the sample extract was implemented (Chan et al., 2012). Trolox was used as the standard for a calibration curve (3.125, 6.25, 12.5, 25, 50 and 100 μg/mL) and the results were expressed as mg of trolox equivalents per g of sample (mg TE/mL).

**D. Infusion of "kelulut" honey with water**

Honey weighing 7.5 g (corresponds to 1 tablespoon) was infused with 100 mL of water maintained at optimal temperature obtained from earlier study.

The experiment was then repeated at increasing dosages starting with 2, 3 and 4 tablespoon (tbsp.). For ease of data presentation, 1-4 tbsp. of honey corresponds namely to samples A-D respectively. Consequently, the honey was allowed to dissolve by stirring for 2 minutes. 4 mL of the sample was then added to 10 mL of methanol and allowed to sonicate for 5 minutes using ultrasonic bath. The dissolved honey infused with water sample were then immediately tested with TPC, TFC, FRAP and DPPH assay.

**E. Sample infusion with "kelulut" honey**

Young *Carica papaya* leaf extract was infused with 1, 2, 3 and 4 tbsp. of honey with its preparation maintained at optimal temperature. The honey was allowed to dissolve by stirring for 2 minutes. 4 mL of the sample was then added to 10 mL of methanol and was further sonicated for 5 minutes using ultrasonic bath. The resulting results of TPC, TFC and antioxidant based in FRAP and DPPH assays was recorded and analysed.

**F. Statistical Analysis**

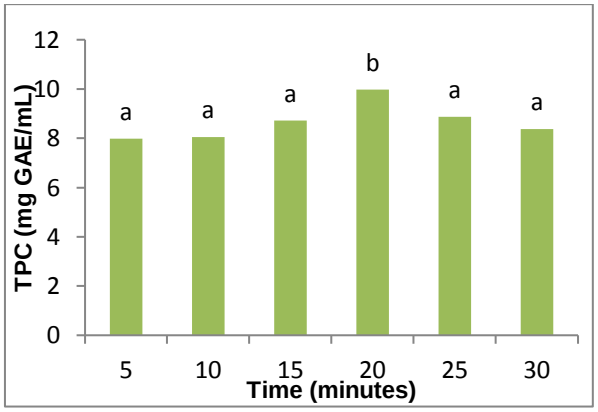
One way ANOVA followed with post hoc Tukey test analysis was implemented using SPSS statistical software version 22. Pearson correlation test analysis was also used to determine the relationship between total phenolic compounds and antioxidant of the samples used in this study. Differences between the mean levels of *Carica papaya* leaves and its infusion with "kelulut" honey were considered to be statistically significant at $p < 0.05$.

## III. RESULTS AND DISCUSSIONS

### A. Result of temperature and time manipulation on antioxidant activities of young Carica papaya leaves

It is widely accepted that the biosynthesis of

phenolic is interrupted by enzyme's destruction and/or cell structure degradation. One of the most important factors that influence them is thermal treatment (Visioli *et al.*, 2011). Based on the results achieved via TPC, TFC, FRAP and DPPH assay as shown in Figure 1, it was found out and also supported by other study on *Carica papaya* leaves as well that the optimum temperature and length of extraction time to achieve the highest antioxidant extraction rate is at 70°C and 20 minutes (Vuong *et al.*, 2013).

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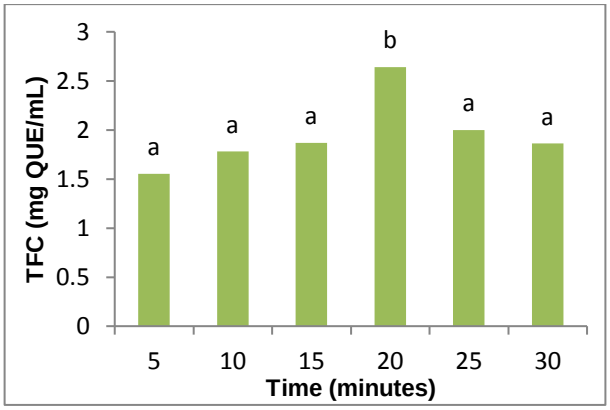


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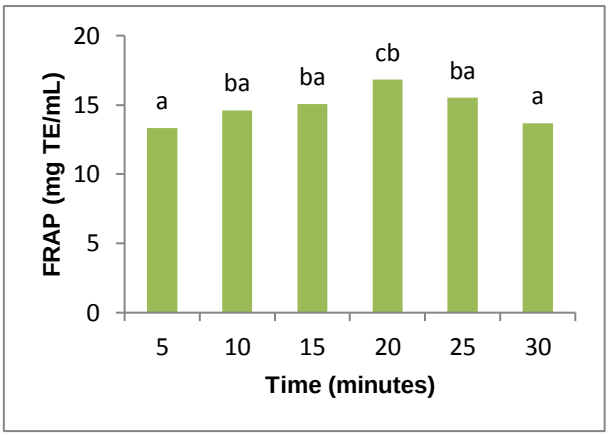


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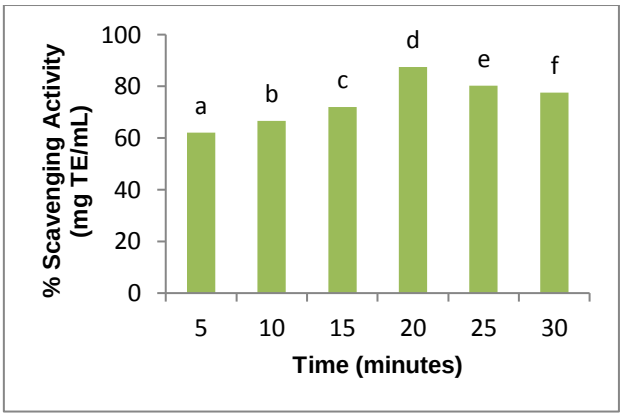


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Figure 1. Effect of extraction time on (A) TPC; (B) TFC; (C) FRAP and (D) DPPH scavenging activity(%) of aqueous extract of *Carica papaya* leaves. The tabulated values not sharing a letter are significantly different at $p<0.05$.

Heat implementation at 70°C is ideal since it supplies ample activation energy needed to break down the plant cell structure, allowing the release and subsequent activation of the phytoconstituents of the plants, while at the same time keeping the enzyme from denaturing (Visioli *et al.*, 2011; Okoduwa *et al.*, 2016). On the other hand, 20 minutes was suggested as the best extraction time since it provides balance by allowing extraction at optimum temperature while at the same time reducing the competing process of thermal induced decomposition of

bioactive compounds (Gertenbach, 2001; Sultana *et al.*, 2009; Vuong *et al.*, 2013).

It is worth to note however, that despite the comparatively short extraction time, the total phenolics and antioxidant yield obtained is satisfactory when compared with other study (Maisarah *et al.*, 2013; Vuong *et al.*, 2013). This study also incorporates two combination types of extraction, where after the sample was extracted in the water bath, it was then followed up by ultrasound-assisted extraction (ultrasound bath). Ultrasound-assisted extraction is chosen in this study since it enables reduced extraction time with lower energy consumption while concurrently being environmental friendly (Mason, 2005). Nevertheless, extraction using ultrasound assisted extraction should proceed in controlled temperature. Constant monitoring should be exercised since elevated temperature due to prolonged extraction time might lower the yield of extract.

The aqueous extract for all samples for TFC values in contrast is lower compared to the total phenolic yield. This might be due to the fact that most flavonoids are non-polar, making it less readily to be extracted by water, which is a strong polar solvent. Pearson correlation shows that there is a strong, positive correlation (r=1.0) between all antioxidant tests, concluding that phenolics plays a significant role in the total antioxidant value of the *Carica papaya* plant.

Table 1 below tabulates the $IC_{50}$ values of *Carica papaya* leaves aqueous extract in different time length. The tabulated values are of triplicate extraction (mean ± standard deviation) and those not sharing a letter are significantly different at $p<0.05$. The lower the value of $IC_{50}$, the more potent it is as an antioxidant. Extraction time of 20 minutes yields the lowest $IC_{50}$ values.

Table 1. $IC_{50}$ Values of *Carica papaya* leaves aqueous extract in different time length.

| Time (minutes) | Aq. *Carica papaya* leaves $IC_{50}$ Values |
|---|---|
| **5** | 754.14± 11.47[a] |
| **10** | 675.79± 4.47[b] |
| **15** | 600.48± 9.06[c] |
| **20** | 492.54± 2.45[d] |
| **25** | 537.61± 4.85[e] |
| **30** | 568.91± 4.04[f] |

### B. Antioxidant activities of "kelulut" honey infused with water

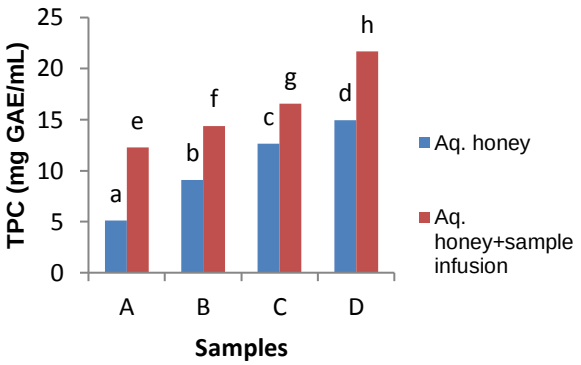
In this study, the effect of amount of tablespoon of "kelulut" honey added to 100 mL distilled water on its antioxidant values was investigated. Based on the result of this study, "kelulut" honey proved to be a potent antioxidant source since despite the extraction time being extremely short. Overall, it recorded higher value when tested on TPC, FRAP and DPPH assay compared to *Carica papaya* leaves samples.

Consequently, result of this study shows an increase in total phenolic and antioxidant values as higher dosage of honey was added into the aqueous solution. TFC values, however was lower for honey infused with water compared to the TFC value of *Carica papaya* aqueous solution. This is due to the fact that honey has a

higher viscosity compared to the aqueous solvent used, making its total extraction lower compared to the latter (Fan *et al.*, 2011). Short extraction time may also contribute to low TFC value. It is also noted though that increasing the amount of tablespoon of honey shows increasing TFC value.

### C. Antioxidant activities of young Carica papaya leaves infused with "kelulut" honey

Rather than using sugar, honey proves to be a better option as an alternative sweetener since it contains rich nutrients and high antioxidant activities. Figure 2 shows the TPC, TFC, FRAP and DPPH test of aqueous "kelulut" honey and its infusion with *Carica papaya* leaves aqueous extracts. From the figures, it was shown that there are an increasing trends of antioxidant values as the amount of tablespoon was increased. Hence, it proves the theory that infusion of *Carica papaya* leaves extract with honey increases their overall antioxidant properties; providing the much needed positive synergistic effect to the consumers. The result is in agreement with other study which focuses on the infusion of honey with other medicinal plant (Pereira *et al.*, 2015).

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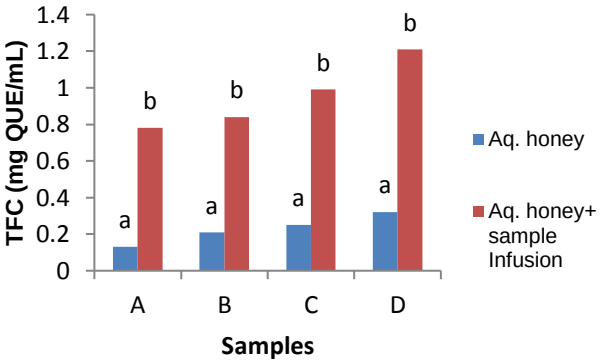


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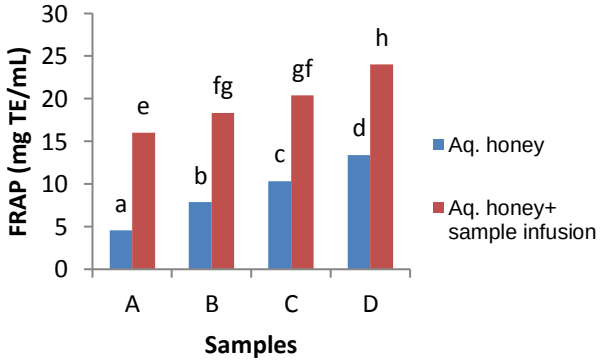


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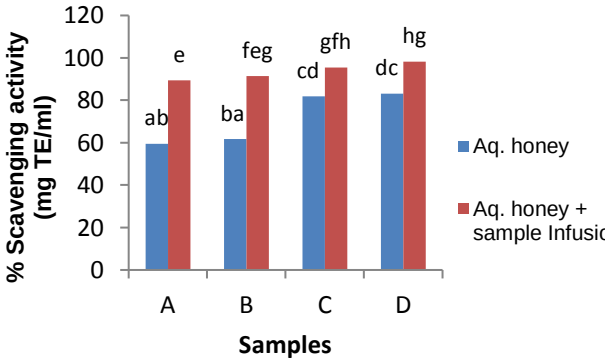


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Figure 2. Comparison of. on (A) TPC; (B) TFC; (C) FRAP and (D) DPPH scavenging activity(%) between "kelulut" honey and its infusion with aqueous extract of *Carica papaya* leaves. The tabulated values not sharing a letter are significantly different at $p<0.05$

Based on information tabulated in table 2 (tabulated values not sharing a letter are significantly different at $p<0.05$), infusion of aqueous "kelulut" honey with aqueous *Carica papaya* leaves lowers $IC_{50}$ by almost half giving evidence that adding honey into sample extract provides synergistic increase to the sample, making it more antioxidant potent.

Table 2. $IC_{50}$ Values of Aqueous honey and its infusion with Aq. *Carica papaya* leaves.

| | Aq. Honey $IC_{50}$ Values | Aq. Honey + Sample Infusion $IC_{50}$ Values |
|---|---|---|
| **1 tbsp.** | $807.84\pm 27.35^{a}$ | $437.56\pm 3.93^{e}$ |
| **2 tbsp.** | $747.08\pm 36.10^{b}$ | $431.87\pm 13.78^{e}$ |
| **3 tbsp.** | $532.10\pm 6.25^{cd}$ | $423.13\pm 1.42^{ef}$ |
| **4 tbsp.** | $504.19\pm 2.17^{dc}$ | $408.02\pm 4.98^{gf}$ |

Therefore, in this study, four tablespoons of honey was chosen as the optimum amount of dosage to be infused with *Carica papaya* leaves aqueous extract.

## IV. SUMMARY

This study shows that extraction conditions affect the antioxidant values of all samples. It was determined that the optimum temperature and length of extraction time is 70°C and 20 minutes. Infusion of aqueous "kelulut" honey with aqueous extract of *Carica papaya* leaves gives out positive synergy since there is an increase in antioxidant based on TPC, TFC, FRAP and DPPH value. Dosage of 4 tablespoon of honey is suggested for an increase of phenolics and antioxidant yield while enhancing the taste of the bitter *Carica papaya* leaves samples. Even though the extraction time for all sample in this experiment is relatively short, it still brought upon satisfactory result. Subsequently, natural medicine is worth it to consume since it brings extremely minimal to no side effect. With prevention is better than cure mind set, consuming these plants arms mankind with robust protective nutrients, making us less susceptible to diseases that might show itself predominantly in the later age. It is not a stretch to say then that with these wonderful findings, it gives a better chance of fighting against diseases and also promotes natural healing with less strain on the body; providing us with an option of not having to develop a lifelong dependency on allopathic drugs.

## V. ACKNOWLEDGMENT

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