ORIGINAL ARTICLE

A comparative study of phytochemical content in air dried and lyophilized *Carica papaya* leaves

Nusrat Hossain[1], Palanirajan Vijayaraj Kumar[1], Yeong Siew Wei[2]

[1]Department of Pharmaceutical Technology, Faculty of Pharmacy, UCSI University, Kuala Lumpur Campus (South Wing), No. 1, Jalan Menara Gading, UCSI Heights (Taman Connaught), Cheras 56000, Kuala Lumpur, Malaysia, [2]Department of Clinical Pharmacy, Faculty of Pharmacy, UCSI University, Kuala Lumpur Campus (South Wing), No. 1, Jalan Menara Gading, UCSI Heights (Taman Connaught), Cheras 56000, Kuala Lumpur, Malaysia

Abstract

Background: Recently, large numbers of *Carica papaya* leaves supplementary products are hitting the market for increasing the platelet counts among the dengue patients. The thermoliable active compounds of *C. papaya* leaves present in these products can be degraded during the manufacturing process. **Objective:** In this study, air drying and lyophilization techniques were used to prepare the dry *C. papaya* leaves powder to improve the stability of thermoliable compounds. **Materials and Methods:** Thermoliable compounds like flavonoids and saponin were estimated using ultraviolet spectrophotometer. Furthermore, high performance liquid chromatography method was developed for estimating the phenols and vitamin C content and validated using system precision, linearity, inter- and intra-day precision. A comparative analysis was performed among the air dried and lyophilized *C. papaya* leaves samples. **Results:** The amount of phenols, vitamin C, saponin, flavonoids in the air dried *C. papaya* leaves present at the concentration of 45.47 ± 0.05, 3.14 ± 0.03, 22.63 ± 0.04, and 13.47 ± 0.12 mg/g. Similarly, 58.5 ± 0.16, 8.02 ± 0.04, 32 ± 0.5 and 20 ± 0.30 mg/g of phenols, vitamin C, saponin and flavonoids were identified in lyophilized *C. papaya* leaves. **Conclusion:** The presence of high amount of ascorbic acid, phenols, saponins, and flavonoids in lyophilized *C. papaya* leaves indicates that the thermoliable compounds are degrading in air-dried samples. Therefore, lyophilized *C. papaya* leaves can be used to prepare better supplementary products for dengue treatment.

Key words: *Carica papaya* leaves, dengue, high performance liquid chromatography analysis, lyophilization, supplementary products

INTRODUCTION

Dengue is a severe arthropod-borne viral disease.[1] Dengue virus can be transmitted by mosquitoes named *Aedes aegypti*.[2] *Carica papaya* leaves juice has shown positive effects among the dengue suffering patients.[3,4] Various active phytochemicals including flavonoids, phenols, carbohydrates, saponins, terpenoids, alkaloids, and ascorbic acid have been identified in *C. papaya* leaves by phytochemical screening process.[5]

A variety of analytical methods were used to analyze the active phytochemicals of *C. papaya* leaves. Afzan *et al.* conducted phytochemical analysis of *C. papaya* leaves using ultra performance liquid chromatography time-of-flight mass analyzer with an electrospray ionization source for fingerprinting.[6] High-performance thin layer chromatography (TLC/HPTLC) phytochemical screening had been used by Anjum *et al.* on different extracts of *C. papaya* leaves. In another study, fingerprinting was done by determining the r_f values of the prepared extracts.[7] Vuong *et al.* and Maisarah *et al.* conducted ultraviolet (UV)

Address for correspondence:
Palanirajan Vijayaraj Kumar, Department of Pharmaceutical Technology, Faculty of Pharmacy, UCSI University, Kuala Lumpur Campus (South Wing), No. 1, Jalan Menara Gading, UCSI Heights (Taman Connaught), Cheras 56000, Kuala Lumpur, Malaysia.
E-mail: palanirajan@ucsiuniversity.edu.my

Received: 10-05-2016
Revised: 04-09-2016
Accepted: 21-09-2016

spectrophotometer for analyzing the composition of saponins and flavonoids in *C. papaya* leaves.[8,9] Fadare *et al.* analyzed *C. papaya* leaves and flowers using attenuated total reflectance-Fourier transform infrared spectroscopy and high performance liquid chromatography (HPLC) spectroscopic methods.[10]

Papaya leaves extract formulations are available on the global market by companies, namely as Rochway, iowa select herbal, herbal papaya, and sidomuncul herbal. According to the product information's of the companies, they are preparing *C. papaya* leaves products by micronization, fermentation and extraction techniques.[11] Vandana *et al.* mentioned that the micronization technique involves controlling the uniform particle size of formulation and avoiding hydrophilic particles to form agglomeration.[12] During this process, due to mechanical stress and heat applied on the natural products can degrade the active phytochemicals. Furthermore, micronization with supercritical fluids like CO_2 can be difficult, due to oxidative stresses.[13] The most noticeable drawback of supercritical fluids is the fact that several molecules (specially, flavonoids) are not soluble in CO_2.[14] Similarly, the products made by fermentation will face the problems associated with purification of end products, balancing the yields, and biomass estimation.[15] On contrast, Vuong *et al.* mentioned that *C. papaya* leaves samples prepared using freeze-drying method showed a higher percentage of total phenols and saponins.[8] Maisarah *et al.* found that lyophilized *C. papaya* leaves had high phenolic and flavonoid content compare to fruit (both ripe and unripe) and seeds.[9] Marques *et al.* identified freeze dried technique can protect the vitamin C content in freeze-dried fruits such as guava, mango, papaya, and pineapple comparing conventional drying methods.[16]

In this study, lyophilization technique was chosen to prepare the *C. papaya* leaves powder. Moreover, HPLC method was developed to determine the thermoliable compounds present in the *C. papaya* leaves. However, UV spectrophotometer and HPLC method were used to estimate the phytochemicals such as phenols, vitamin C, saponin, and flavonoids present in air dried and lyophilized *C. papaya* leaves.

MATERIALS AND METHODS

Plant Material

The fresh leaves of *C. papaya* were collected from a private plantation belong to Exotic Star Sdn Bhd, Selangor, Malaysia. The botanical origin of this sample was taxonomically confirmed by Dr. Shamsul Khamis, University Putra Malaysia. A voucher specimen (SK 2742/15) had been deposited at the Forest Research Institute Malaysia.

Reagents and Materials

Acetonitrile (Sigma-Aldrich, Sweden), aescin (Sigma-Aldrich, Sweden), aluminum chloride (R&M Marketing, UK), aluminum foil (Diamond brand, USA), ascorbic acid (Fisher Scientific, UK), ethanol (Sigma-Aldrich, Sweden), hydrochloric acid (R&M Marketing, UK), methanol (Sigma-Aldrich, Sweden), phosphoric acid (Merck, Germany), potassium dihydrogen phosphate (Fisher Scientific, UK), rutin (R&M Marketing, UK), sephadex g-15 (Sigma-Aldrich, Sweden), sodium hydroxide (Merck, Germany), sodium chloride (NaCl), sulfuric acid (R&M Marketing, UK), tannic acid (R&M Marketing, UK), and vanillin (R&M Marketing, UK). For HPLC analysis, HPLC grade reagents were used.

Preparation of Lyophilized *C. papaya* Leaves

Fresh *C. papaya* leaves were washed thoroughly 2-3 times with running tap water.[17] After washing, the leaves were cleaned properly using sterile water and grounded into several pieces using sharp knife. Grounded *C. papaya* leaves were added in a juicer (ELBA-9811A) for preparing the juice. 50 g of *C. papaya* leaves was used to prepare the juice. The prepared juice was lyophilized using alpha 1-4 LO plus lyophilizer (Christ, UK). First, the samples were covered in aluminum foil and freezed overnight at −80°C. Afterward, the primary drying was done for 24 h at −33°C and 0.28 mbar. In the primary drying process, there was a possibility of having 0.5% moisture in the lyophilized sample. Therefore, secondary drying was done for 4 h at −10°C.[18]

Preparation of Air Dried *C. papaya* Leaves Powder

Total 500 g of *C. papaya* leaves was dried under shade for 1 week and crushed to powder manually and sieved (mesh size 10) properly for removing fibers.

Comparative Studies of Air Dried and Lyophilized *C. papaya* Leaves

Sample preparation

Total 200 mg of air dried and lyophilized *C. papaya* leaves samples were weighted separately using an electronic balance. The powdered samples were soaked in 10 ml of ethanolic water (75% ethanol and 25% water) for 72 h, after which it was filtered using sepadex g-15 column and Whatman no. 1 filter paper for removing the fibers and minor particles.[19]

Qualitative analysis

Qualitative analysis of air-dried and lyophilized *C. papaya* leaves samples were carried out according to standard methods to identify the presence of phenols, alkaloids, flavonoids, and saponins.[20] However, vitamin C was identified using titration method.[21]

Quantitative analysis

Quantitative analysis of air-dried and lyophilized *C. papaya* leaves was done by both UV spectrophotometer (L600000B, Perkin Elmer, USA) and HPLC analysis (Perkin Elmer, USA).

Determination of Saponin Content

Vuong *et al.* was used to quantify the saponin content of lyophilized *C. papaya* leaves.[8] The same method was used to identify total saponin content in air dried and lyophilized *C. papaya* leaves with modification. 0.5 ml of sample solutions from each serial dilution was added to 0.5 ml of total 8% vanillin and 5 ml of 72% sulfuric acid. Afterward, the mixture was incubated at 60°C for 15 min in the oven. After incubation, a sample solution of both air-dried and lyophilized *C. papaya* leaves containing saponin reacts with reagents and changes the color from yellow to green. However, before taking absorbance, the mixture was cooled on ice bath to return at room temperature. The absorbance of the prepared serial dilutions was measured at 590 nm a using a UV spectrophotometer. Aescin was used as a standard for determining the total saponin content, and the results were expressed as mg of aescin equivalents per g of sample.

Determination of Flavonoid Content

The method of Maisarah *et al.* was used to estimate the flavonoid content of *C. papaya* leaves extracts.[9] The same method was used to identify total flavonoid content in air dried and lyophilized *C. papaya* leaves with modification. Total 1 ml of sample solutions from each serial dilution was added with 5 ml of 2% aluminum trichloride. The prepared mixture degrades quickly; therefore all absorbances were taken immediately after preparation. The absorbance of the prepared serial dilutions was measured at 425 nm a UV spectrophotometer. Rutin was used as a standard for determining the total flavonoid content, and the results were expressed as mg of rutin equivalents per g of sample.

Determination of Phenols and Vitamin C

HPLC experiments were performed on a Perkin Elmer HPLC system equipped with Phenomenex Luna C_{18}, 5 µm (4.6 mm × 250 mm) column, two LC-20AD pumps, SCL-10AVP system controller, SIL-20A autoinjector, SPD-20A UV-visible detector and chemstation software was used. The chromatographic conditions were developed using 0.1M phosphate buffer, methanol and acetonitrile (90:2:8) as mobile phase.[10] However, 1 ml/min flow rate, wavelength of 221 nm and 20 µl sample injection was used for further analysis. The method was validated and developed to identify the total phenol and vitamin C contents of lyophilized *C. papaya* leaves extract. In the developed method, tannic acid for phenols and ascorbic acid for vitamin C were used as standards. The retention times of both standards and samples were noted. Correlation coefficient curve was prepared for standards and samples using plot area versus concentration. The developed HPLC method was validated according to the guidelines of the International Conference on Harmonization for validation of linearity, system precision, inters day and intraday precision.[22] However, the method was validated for lyophilized *C. papaya* leaves sample.

Statistical Analysis of Data

The results were expressed as mean ± standard deviation and statistical analysis was performed using SPSS. One-way analysis of variance was used in inter-day precision of the samples. A comparative study was done using paired sample *t*-test. The difference was considered to be significant when $P < 0.05$.

RESULTS AND DISCUSSION

Collected *C. papaya* leaves were authenticated as scientific name *C. papaya* L. belongs to family Caricaceae. In the preparation of *C. papaya* leaves juice, 50 g of sample leaves gives 10.7 ± 0.681 ml of fresh juice. The prepared fresh juice was air dried and lyophilized. Lyophilization technique has many advantages compared to another drying, especially for heat sensitive products. The lyophilized products can be stored at ambient temperature over a 2 years shelf life and it can enhance product stability in a dry state. Therefore, in this study, lyophilized technique was used for preparing the sample.[23]

In qualitative method, the presence of alkaloids, phenols, saponins, flavonoids and vitamin C was identified in both air dried and lyophilized *C. papaya* leaves shown in the Table 1. Furthermore, Adachukwu *et al.* reported the presence of saponin, tannin, flavonoid, and alkaloids in the *C. papaya* leaves.[20]

In the quantitative analysis, 22.63 ± 0.04 mg/g of saponin and 13.47 ± 0.12 mg/g of flavonoids was quantified in the air dried *C. papaya* leaves using UV spectrophotometer. Similarly, 32 ± 0.5 mg/g of saponin and 20.73 ± 0.30 mg/g of flavonoids was quantified in the lyophilized *C. papaya* leaves [Figures 1 and 2].

Table 1: Qualitative analysis of air dried and lyophilized *Carica papaya* leaves

Phytochemicals	Description	Results
Alkaloids	Yellow precipitated	Positive
Saponin	Foam formed	Positive
Tanins	Color changed to green	Positive
Flavonoids	Yellow precipitated	Positive
Vitamin C	Color change	Positive

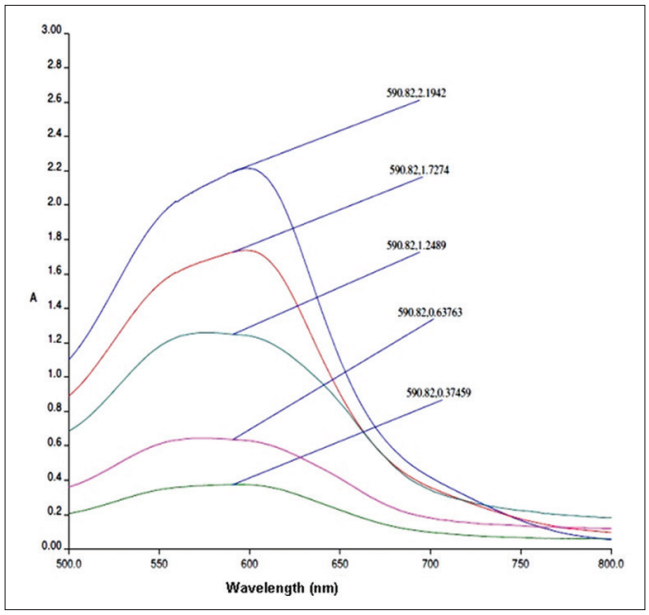

Figure 1: Ultraviolet spectrum of standard aescin at different concentration (590 nm of wavelength)

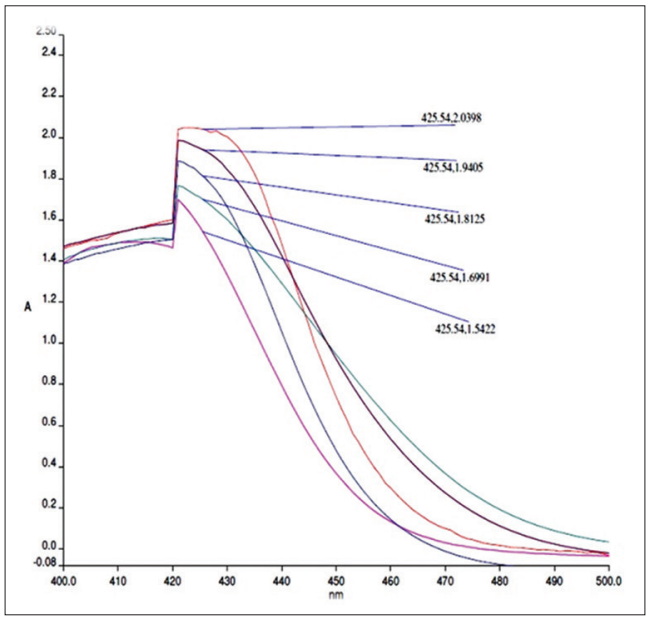

Figure 2: Ultraviolet spectrum of standard rutin at different concentration (425 nm of wavelength)

The HPLC method was developed to estimate the amount of total phenols and vitamin C in the air dried and lyophilized *C. papaya* leaves. The retention times of the standards ascorbic acid and tannic acid under the optimized conditions were found to be of 3.2 and 4.3 min, respectively shown in Figures 3 and 4. The chromatogram of lyophilized *C. papaya* leaves extract was shown in Figure 5. The chromatogram clearly shows the separation of vitamin C and phenol having the retention time of 3.2 and 4.3 min. The calibration curves of tannic acid and ascorbic acid showed good linearity [Tables 2 and 3]. In this analysis, 45.47 ± 0.05 of phenols and 3.14 ± 0.03 mg/g of vitamin C was quantified in the air dried

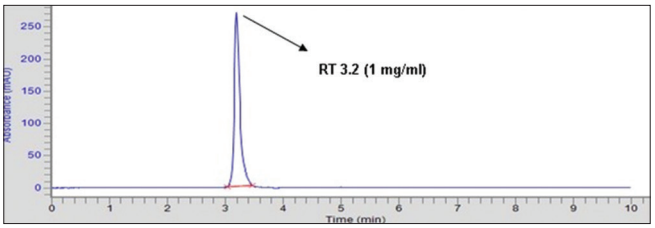

Figure 3: High performance liquid chromatography chromatogram of ascorbic acid

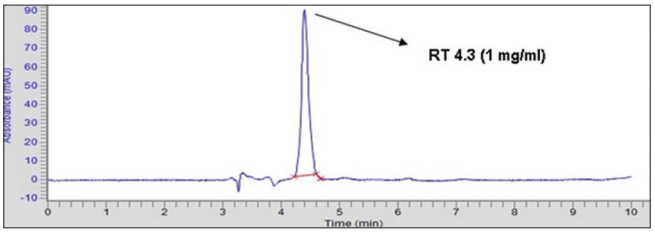

Figure 4: High performance liquid chromatography chromatogram of tannic acid

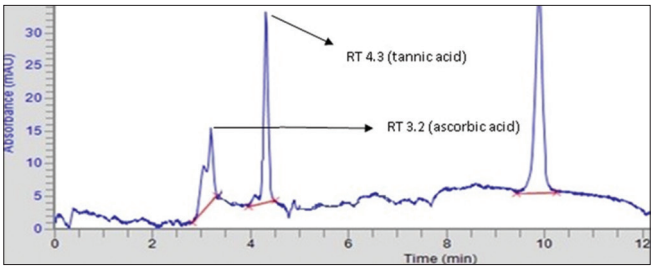

Figure 5: High performance liquid chromatography chromatogram of lyophilized *Carica papaya* leaves

Table 2: Linearity of tannic acid

Parameter	Value
Calibration range (mg/mL)	0.4-1
Regression equation slope*	951991
Y intercept*	248624
Correlation coefficient (*r*)	0.99

*Linear regression equation: y = 951991x -248624

Table 3: Linearity of ascorbic acid

Parameter	Value
Calibration range (mg/mL)	0.25-3
Regression equation slope*	2409904.49x
Y intercept*	10129.05
Correlation coefficient (r)	0.99

*Linear regression equation: y = 2409904.49x-10129.05

C. papaya leaves. Similarly, 58.5 ± 0.16 of phenols and 8.02 ± 0.04 mg/g of vitamin C was quantified in the lyophilized leaves. In this study, the HPLC method was validated for lyophilized *C. papaya* leaves using system precision, intra- and inter-day precision shown in Tables 4-11. According to the

Table 4: System precision of tannic acid

Parameter	Tannic acid 1 mg/ml	Accuracy
Peak area	750645.33	100.87
	750495.63	100.85
	741329.63	99.62
Mean	744140.20	100.45

Table 5: System precision of ascorbic acid

Parameter	Ascorbic acid 1 mg/ml	Accuracy
Peak area	2303272.20	101.11
	2217199.32	97.33
	2313152.39	101.55
Mean	2277874.64	99.99

interday precision data, no significant difference was found in the developed HPLC method.

In the comparative analysis, the air dried *C. papaya* leaves showed degradation of active phytochemicals while comparing with lyophilized *C. papaya* leaves showed in Table 12. The p value was below 0.05, which indicates that there was a significant difference among air dried and lyophilized *C. papaya* leaves. Similarly, Zainol *et al.* used freeze dried, vacuum dried and air dried *Centella asiatica* (Spadeleaf) to analyze the degradation of flavonoids.[24] The result revealed that the percentage of flavonoids were degraded to 35%, 66%, and 76%, respectively which indicates, freeze dried sample shows the least degradation of flavonoids in spade leaves. Orphanides *et al.* investigated the total phenolic content of spearmint leaves using five different drying treatments which are convection oven drying, freeze-drying, microwave drying,

Table 6: System precision of ***Carica papaya*** leaves (tannic acid)

Parameter	*Carica papaya* leaves sample 7.5 mg/ml	Standard area of *Carica papaya* leaves 7.5 mg/ml	Accuracy %
Peak area	177133.89	176461.84	100.38
	179321.90	176461.84	101.62
	173889.12	176461.84	98.54
Mean	100.18		

Table 7: System precision of ***Carica papaya*** leaves (ascorbic acid)

Parameter	*Carica papaya* leaves sample 7.5 mg/ml	Standard area of *Carica papaya* leaves 7.5 mg/ml	Accuracy %
Peak area	135771.09	133729.78	101.53
	134588.90	133729.78	100.64
	133219.90	133729.78	99.62
Mean	100.60		

Table 8: Intraday precision analysis for total phenolic compounds

Capsule number	Test area	Standard area	Amount present in 500 mg	Relative amount %
1	177911.23	176461.84	29.88	100.23
2	180133.53		30.03	100.17
3	176621.80		29.78	99.93
Mean	100.11 ± 0.16			

Table 9: Inter day precision analysis for total phenolic compounds

Day 1

Number	Test area	Standard area	Amount present in 500 mg	Relative amount %
1	179321.57	176461.84	29.97	100.57
2	178221.09		29.89	100.30
3	177900.66		29.87	100.23
Mean	100.37± 0.18			

*p= 0.542

Day 2				
Number	**Test area**	**Standard area**	**Amount present in 500 mg**	**Relative amount %**
1	176456.09	176461.84	29.77	99.90
2	178329.89		29.90	100.33
3	177995.56		29.88	100.25
Mean	100.16±0.23			

*p=0.779

Day 3				
Number	**Test area**	**Standard area**	**Amount present in 500 mg**	**Relative amount %**
1	169342.98	176461.84	29.27	98.22
2	175889.62		29.73	99.76
3	177551.34		29.84	100.15
Mean	99.38±1.02			

*p=0.544

Table 10: Intraday precision analysis for total vitamin C content

Number	**Test area**	**Standard area**	**Amount present in 500 mg**	**Relative amount %**
1	135815.92	133729.78	4.04	101.57
2	135219.83		4.02	101.15
3	133945.09		3.98	100.29
Mean	101±0.65			

Table 11: Inter day precision analysis for total vitamin C content

Number	**Test area**	**Standard area**	**Amount present in 500 mg**	**Relative amount %**
1	133672.06	133729.78	3.98	100.08
2	134578.01		4.00	100.70
3	135575.90		4.03	101.40
Mean	100.73±0.66			

*p=0.311

Table 12: Comparative analysis lyophilized and air dried ***Carica papaya*** leaves.

Phenol	**n_1 (mg/ml)**	**n_2 (mg/ml)**	**n_3 (mg/ml)**	**Mean**
Drying				
Lyophilized	0.058	0.059	0.052	0.0563± 0.0038
Air dried	0.0457	0.0449	0.0452	0.0453± 0.0004
Vitamin C				
Drying				
Lyophilized	0.0080	0.0081	0.0082	0.0081± 0.0001
Air dried	0.0031	0.0034	0.0030	0.003 ± 0.0002
Saponin				
Drying				
Lyophilized	0.032	0.037	0.035	0.0347±0.003
Air dried	0.0225	0.0228	0.0221	0.0225±0.0004
Flavonoids				
Drying				
Lyophilized	0.020	0.022	0.023	0.0217±0.00153
Air dried	0.0131	0.0132	0.0127	0.0130± 0.0003

P value >0.05

air drying with and without the sun exposure.[25] The results of the study showed that freeze-dried spearmint leaves had the highest total phenolics compounds compare to other drying techniques. Marques *et al.* used freeze dried and hot air dried guava, mango, papaya, and pineapple for analyzing the total vitamin C.[26] The result showed that vitamin C losses were lower in freeze-dried samples. Considering the results, this could be attributed that phenols, flavonoids, and vitamin C compounds are degrading in air dried leaves compared with lyoplized *C. papaya* leaves. Therefore, lyophilization can be a better option for drying the *C. papaya* leaves for further uses.

CONCLUSION

The developed HPLC method established the suitability of estimating the total phenolic and vitamin C content in *C. papaya* leaves. The presence of high amount of ascorbic acid, phenols, saponins, and flavonoids in lyophilized *C. papaya* leaves indicates that the thermo-liable compounds are degrading in air-dried samples. Therefore, lyophilized *C. papaya* leaves juice extract can be used to prepare supplementary products for dengue treatment.

ACKNOWLEDGMENTS

This work was supported by post graduates research fund by Faculty of Pharmaceutical Sciences, UCSI University, and Kuala Lumpur, Malaysia.

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Source of Support: Nil. **Conflict of Interest:** None declared.