

Original Article

POTENTIAL ANTIOXIDANT AND CYTOTOXIC PROPERTIES OF SECONDARY METABOLITE EXTRACTS FROM *CARICA PAPAYA* FRUITS AND SEEDS

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Received: 11 Apr 2014 Revised and Accepted: 16 May 2014

ABSTRACT

Objective: This study was done to examine antioxidant and cytotoxic potential in the extracts of fruits and seeds from the traditionally used medicinal plant, *Carica papaya*.

Methods: Antioxidant and cytotoxic potential of crude methanol, hexane fraction and ethyl acetate fraction from both fruits and seeds of *Carica papaya* were compared and assessed by DPPH free-radical scavenging assay and brine shrimp lethality assay. Total phenolic and flavonoid contents were determined by Folin-Ciocalteu and aluminium chloride colorimetric methods respectively. Bioactive fractions were then characterized and analyzed by silica thin layer chromatography.

Results: Results showed that both ethyl acetate fractions from the fruits and seeds of *Carica papaya* are high in their antioxidant activities (IC₅₀ values of 30.61 µg/ml and 25.97 µg/ml respectively) as well as cytotoxic (LC₅₀ of 163.96 µg/ml and 142.27 µg/ml respectively). High antioxidant activities of ethyl acetate fractions obtained from fruits and seeds are strongly correlated to the total phenolic contents and moderately correlated to the total flavonoid contents.

Conclusion: This study suggested that both ethyl acetate fractions from fruits and seeds of *Carica papaya* may have the potential to be further developed into therapeutic option for treating cancer, of which the ethyl acetate fraction from seeds raises a slightly higher prospect.

Keywords: Carica Papaya, Antioxidant, Cytotoxicity, Total phenolic and favonoid content, Ethyl acetate fraction.

INTRODUCTION

Traditional medicine plants remained as the most relevant pool of candidates that may have anticancer properties [1]. Unfortunately, available anticancer drugs today encountered the problem of cytotoxicity to the normal cells. The threat of detrimental side effects of conventional chemotherapy drugs had driven researchers worldwide to look for new compounds with cytotoxic activity from traditional medicine plants.

Different plant extracts or the bioactive constituents from secondary metabolites have been reported to be responsible for many medicinal activities [2]. Papaya (*Carica papaya*) belongs to Caricaceae family, and is tropical evergreen fruit tree originated from the southern part of Mexico and Central America. It has been used as medicinal plant since ancient time [3]. Papaya fruit is rich in carotenoid and soluble dietary fiber, known to be helpful in preventing atherosclerosis and reducing constipation problem [4]. Vitamin A and C that were found in ripe papaya fruits can act as protective scavengers against damaged cells from free radical, immune booster and anti-inflammatory actions. Moreover, papain content in papaya fruits is a remedy in dyspepsia and kindred ailments [5]. Studies also illustrated that extracts of fruits and seeds showed to have bacteriostatic properties in vitro [6, 7]. Past research on the present of bioactive compounds from papaya is rather divergent. Some studies indicated that fruit extracts are more potent [8, 9] while others reported that seed extracts are more biologically active [10, 11]. Oloyede and co-workers demonstrated that papaya fruit extracts showed a promising result in antioxidant activity due to the present of quercetin and β-sitosterol in ethyl acetate fraction [12], while other studies illustrated bioactive compounds present in aqueous extracts or methanol extracts [13, 14]. On the contrary, Dwikatet *et al* (2010) reported aqueous extracts of the papaya seeds have the anti-apoptotic effect due to its antioxidant activity [15] and Zhou *et al* (2011) showed that ethyl acetate fractions from papaya seeds had strong DPPH and hydroxyl free radical scavenging activities [11]. All these findings are rather contradictory, despite vast investigation on various parts of *Carica papaya* including fruit and seed extracts over the past 10 years [11,

13, 16, 17]. Hence, this study aims to investigate antioxidant and cytotoxicity of secondary metabolites extracts from both papaya seeds and fruits.

MATERIALS AND METHODS

Preparation of plant material

Papaya fruits were bought from market in Kota Kinabalu, Sabah. They were washed with distilled water and the peels were removed. They were cut into two longitudinal halves and the wet seeds are separated out. They were then gently but thoroughly rinsed with tap water twice.

Crude secondary metabolites extraction and liquid-liquid extraction

100g of powdered papaya fruit and seed were macerated in 1 litre of pure methanol for one day. After filtration, it was concentrated under vacuum in rotary evaporator at 40°C and stored at 4°C. The crude methanol was suspended in 20ml distilled water and liquid-liquid extracted sequentially with hexane and ethyl acetate in the ratio of 1:1. It was extracted until the colorless of each organic layer were observed.

Total phenolic content (TPC) test

Sample was prepared at (5mg/ml) in methanol. Then, 200µl of sample was mixed with 1.5ml of Folin-Ciocalteu reagent solution (10-folded with distilled water). They were mixed and left at room temperature for 5 minutes. After that, 1.5ml of sodium bicarbonate (60g/L) was added to the mixture. The mixture was left at room temperature for 90 minutes. The sample was prepared in triplicate. The absorbance was measured at 725nm

Total flavonoid content (TFC) test

Sample was prepared at (5mg/ml) in methanol. Then, 0.5ml of this solution was pipetted in a test tube consists of 1.5ml of methanol, 0.1ml of 10% aluminium chloride, 0.1ml of 1M potassium acetate aqueous solution and 2.8ml of distilled water. The mixture was

incubated at room temperature for 30 minutes. The sample was prepared in triplicate. The absorbance was measured at 415nm.

DPPH free-radical scavenging assay

At first, 0.2M of DPPH solution was prepared by dissolving 7.8mg of DPPH powder in 100ml of methanol. Then, 1mg/ml of extract of each test sample was prepared by dissolving in suitable solvent to obtain different concentration (12.5 – 200µg/ml). After that, 2ml of sample solution was mixed with 0.5ml of DPPH solution. The mixture was shaken vigorously and kept in dark for 30 minutes at room temperature. The sample was prepared in triplicate. The absorbance was measured at 517nm. The negative control containing water instead of the sample while the positive control containing (12.5 – 200µg/ml) ascorbic acid. Blank solution of crude secondary metabolite contains 2ml of sample and 0.5ml of methanol without DPPH.

$$\% \text{ radical scavenging} = \frac{(Ac - Acb) - (As - Asb)}{(Ac - Acb)} \times 100$$

Ac: the absorbance of the water, DPPH in methanol

Acb: the absorbance of the water, methanol without DPPH

As: the absorbance of the sample, DPPH in methanol

Asb: the absorbance of the sample, methanol without DPPH/ methanol without DPPH

Artemia salina (brine shrimp) lethality assay

For optimum condition of brine shrimp egg hatching, artificial seawater (3.8% NaCl in distilled water) was adjusted to pH 8.8. It was then aerated and incubated for 48 hours in room temperature. After 48 hours, 10 shrimps were transferred to a new plate and incubated with total 5ml of mixture of 4.5ml new aerated seawater and 0.5ml sample extracts. The secondary metabolite extracts were prepared by dissolving in distilled water and 1% DMSO respectively. Three different concentrations (10, 100 and 1000µg/ml) of extracts were prepared. 1% mercury chloride acts as positive control while 1% DMSO and distilled water act as negative control. It was made in triplicate. In cases where negative death occurred, the data was corrected using Abbott's formula (% deaths = [(dead larvae in test - dead larvae in control)/survivors in control] x 100. The LC₅₀ values were determined from the 24 hour counts. The extract or isolated compounds were considered bioactive when LC₅₀ value was lower than 30µg/ml.

Thin layer chromatography

TLC solvent containing hexane and ethyl acetate (1:1) by volume was added into the jar. Filter paper was added into the TLC jar to aid equilibration. After closed for 5 to 15 minutes for equilibration, ethyl acetate fraction from seeds which has the maximum DPPH scavenging activity was dissolved in the extraction solvent before spotted on the silica coated aluminium TLC plate. A line that is higher than the solvent in the TLC jar was marked by using pencil on the TLC plate. Then, the sample was spotted onto the TLC plate using a capillary tube with diameter less than 1 mm. The TLC plate was placed in the beaker at one end of the slide immersed in the solvent prepared. It was eluted until the solvent migrates up to a few centimetres before reach the end of the plate. Finally, the spot on the plate was visualized under short UV 254nm.

Statistical analysis

All the experiments were conducted in three replicates. The results were expressed in mean ± SD and data obtained was analyzed statistically by one way ANOVA using SPSS software (SPSS 21, IBM). P values less than 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSIONS

Crude secondary metabolites extraction and collection

The yield of the crude secondary metabolites extracted using methanol solvent from fruits is higher than that from the seeds of *Carica papaya*, which was about five-fold higher, with the methanol yield of fruits 25.6% compared to the methanol yield of seeds 5.2% Liquid-liquid extraction from the fruits indicated that the hexane fraction (24.61%) was about eight-fold higher than that in ethyl acetate fraction (3.52%) (Table 1).

Hexane fraction from seeds (3.85%) was only about four-fold higher than the seed ethyl acetate fraction (1.15%) (Table 1). This suggested that hexane can extract more secondary metabolites compared to ethyl acetate. Krishna *et al* (2008) also reported that the secondary metabolites present in papaya fruits is more than that in seeds [18].

Meanwhile, similar study by Kibria *et al* (2012) reported that the yield of hexane fraction (1.5g) was 3 fold higher than the yield of the ethyl acetate fraction (0.5g) that partitioned off from the crude methanol extract of *Terminalia chebula* bark [19].

Table 1: Secondary metabolites yield percentage of hexane and ethyl acetate fractions of *Carica papaya*

Extraction solvents	Sample	Symbol	Initial crude methanol extract weight (g)	Fraction extract weight (g)	Secondary metabolites yield (%)
Hexane	Fruit	FHF	25.6	6.3	24.61
	Seed	SHF	5.2	0.2	3.85
Ethyl acetate	Fruit	FEAF	25.6	0.9	3.52
	Seed	SEAF	5.2	0.06	1.15

Total phenolic content: Total phenolic content of crude methanol, hexane and ethyl acetate fractions from both fruits and seeds of *Carica papaya* was determined by using the Folin-Ciocalteu reagent and were expressed as gallic acid equivalents per 100 gram of plant extracts. The total phenolic contents of the different sample extracts were calculated using the standard curve of gallic acid ($y = 10.155x - 0.0665$; $R^2 = 0.9799$) (Figure 1). It was found that the fruit ethyl acetate fraction (FEAF) contains the highest total amount of phenols and the hexane fractions from fruits and seeds exhibited the lowest amount of total phenolic content. In a comparison of all extracts, the total phenolic content was demonstrated in a trend of FEAF > SEAF > FCM > SCM > FHF > SHF (Table 2). Similar result reported by Oloyede *et al* (2012) showing that the highest amount of total phenolic content was observed in fruit ethyl acetate fraction [12]. Phenols are known to possess a wide range of therapeutic uses such as antioxidant and free radical-scavenging activities [20]. This result suggested that ethyl acetate fractions are rich in polar phenolics compared to other extracts which could be related to its antioxidant potential.

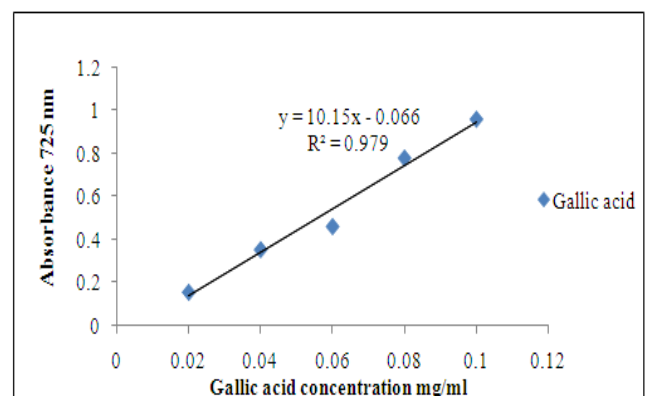


Fig. 1: Standard curve of total phenolic content estimation with gallic acid.

Table 2: Total phenolic and flavonoid content of crude methanol, hexane fraction and ethyl acetate fraction of *Carica papaya*

Sample	Extracts	Symbol	Total phenolic content (mg GAE/100 g)	Total flavonoid content (mg QE/100 g)
Fruit	Crude methanol	FCM	1255.64 ± 103.06	818.78 ± 2.58
	Hexane fraction	FHF	815.97 ± 20.52	617.34 ± 4.42
	Ethyl acetate fraction	FEAF	1979.67 ± 126.93	1716.01 ± 11.63
Seed	Crude methanol	SCM	980.45 ± 30.26	449.79 ± 1.53
	Hexane fraction	SHF	613.42 ± 25.49	412.35 ± 5.57
	Ethyl acetate fraction	SEAF	1972.42 ± 125.98	685.08 ± 2.54

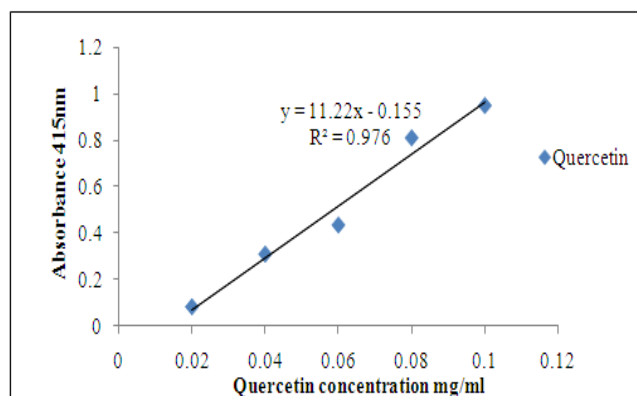


Fig. 2: Standard curve of total flavonoid content estimation with quercetin

Total flavonoid content

Aluminium chloride colorimetric method was used to determine total flavonoid content of crude methanol, hexane and ethyl acetate fractions from both fruits and seeds of *Carica papaya*. The total flavonoid contents of the different sample extracts were calculated using the standard curve of quercetin ($y = 11.225x - 0.1557$; $R^2 = 0.9761$) (Figure 2) and was expressed as quercetin equivalents per 100 gram of the plant extracts. The highest amount of total flavonoid content was observed in the ethyl acetate fraction from fruit (FEAF) and the hexane fraction from seeds (SHF) demonstrated to be of the lowest content of total flavonoid.

In a comparison of all extracts, the total flavonoid content was demonstrated in a trend of FEAF > FCM > SEAF > FHF > SCM > SHF (Table 2). It was consistent with the study by Afidah *et al* (2010) reported that ethyl acetate fraction contains the most amount of flavonoid as compared to methanol and hexane extracts [21]. Flavonoids acquired antioxidative mechanisms such as scavenging of free radicals and chelating of metal ions [22]. According to our finding, the high contents of flavonoids in *Carica papaya* can explain its high radical scavenging activity.

Antioxidant of DPPH free-radical scavenging assay

Antioxidant property of the sample extracts from both fruits and seeds of *Carica papaya* was evaluated by its ability to reduce the DPPH through observing the stoichiometric discoloration in spectrophotometric method and was denoted as IC_{50} value ($\mu\text{g/ml}$). A lower IC_{50} value indicates higher antioxidant activity. In the present study, SEAF ($25.97 \pm 0.30 \mu\text{g/ml}$) showed to have the lowest IC_{50} value and SHF ($143.11 \pm 3.22 \mu\text{g/ml}$) exhibited to have the highest IC_{50} value. In comparison to all sample extracts, the IC_{50} value was demonstrated in a trend of SEAF < FEAF < FCM < SCM < FHF < SHF (Figure 3 & Table 3).

According to our study, ethyl acetate fraction is seen to have the highest antioxidant activity while the hexane fraction is seen to have the lowest antioxidant activity. Similar results of high antioxidant activity was reported by Zhou *et al* (2011), who showed that ethyl acetate fraction of papaya seeds has a low IC_{50} value ($64.61 \mu\text{g/ml}$). This study demonstrated that the high antioxidant activity of the ethyl acetate fraction of seeds and fruits is very likely attributable to the high amount of total phenolic and total flavonoid content. The presence of *p*-hydroxybenzoic acid and vanillic acid might be among the phenolic compounds that have been isolated [11].

Table 3: IC_{50} value ($\mu\text{g/ml}$) of different extracts of *Carica papaya* compared with ascorbic acid

Sample	Extracts	Symbol	IC_{50} value ($\mu\text{g/ml}$)
Fruit	Crude methanol	FCM	65.62 ± 1.33
	Hexane fraction	FHF	78.51 ± 0.83
	Ethyl acetate fraction	FEAF	30.61 ± 0.27
Seed	Crude methanol	SCM	78.42 ± 0.88
	Hexane fraction	SHF	143.11 ± 3.22
	Ethyl acetate fraction	SEAF	25.97 ± 0.30
Standard	Ascorbic acid	AA	25.09 ± 2.13

Correlation between total phenolic content (TCP) or flavonoid and antioxidant activity

Relationship between the total phenolic or flavonoid content and antioxidant activity of all sample extracts was evaluated using Pearson Correlation test. A best fit straight regression line was plotted with coefficient of determination, $R^2 = 0.801$ and $R^2 = 0.358$ for total phenolic content and total flavonoid content, respectively (Figure 4 & 5).

As illustrated in Table 4, there were strong negative correlation ($r = -0.902$) between the phenolic content and IC_{50} , while it is only a moderately negative correlation ($r = -0.626$) between the flavonoid content and IC_{50} . The negative correlations revealed that the antioxidant activity of *Carica papaya* were in accordance with their amount of phenolic and flavonoid contents. The higher total phenolic

and flavonoid contents resulted in a higher antioxidant activity (with lower IC_{50}). The phenolic and flavonoid compounds were the main micro constituents contributing to the antioxidant activity of papaya [23]. One way ANOVA significance analysis indicated that the total phenolic content of the crude methanol, hexane and ethyl acetate fractions from both fruit and seed extracts are strongly correlated with their antioxidant activities, with correlation coefficient $r = -0.902$ ($p < 0.01$).

This is in agreement with other studies indicating that there is a strong correlation of polyphenol compounds with antioxidant activity [10, 11, 13, 17, 23] and that phenolic phytoconstituents are strongly attributed to the antioxidant activity of *Carica papaya*. It is interesting to note that, the correlation between the total flavonoid contents of all extracts and their antioxidant activities was found to be moderately negative linear with correlation coefficient $r = -0.626$

($p < 0.01$), suggesting that the antioxidant activity of extracts are to a lesser extent correlated to flavonoid contents, as compare to phenolic contents.

Cytotoxicity of *Artemia salina* (brine shrimp) lethality assay

Cytotoxic activity of all the sample extracts from both fruits and seeds of *Carica papaya* was evaluated by its ability to cause lethality in brine shrimp through observing the number of dead brine shrimp under light microscope and was denoted as LC_{50} value ($\mu\text{g/ml}$). A lower LC_{50} implies higher cytotoxic activity.

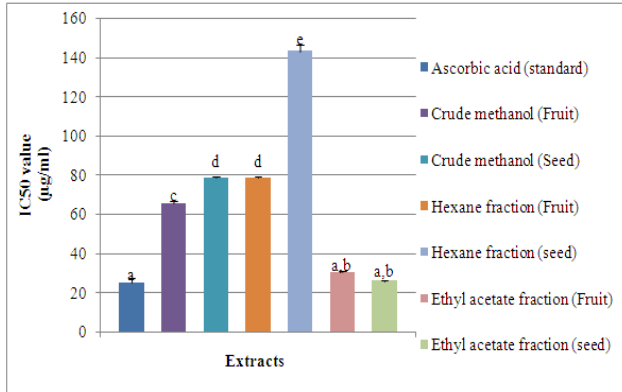


Fig. 3: IC₅₀ value ($\mu\text{g/ml}$) of various extracts of *Carica papaya* compared with ascorbic acid

Table 4: r-values of Pearson Correlation Test

	IC ₅₀ value (antioxidant activity)
Total phenolic content	-0.902**
Total flavonoid content	-0.626**

** . Correlation is significant at the 0.01 level (2-tailed).

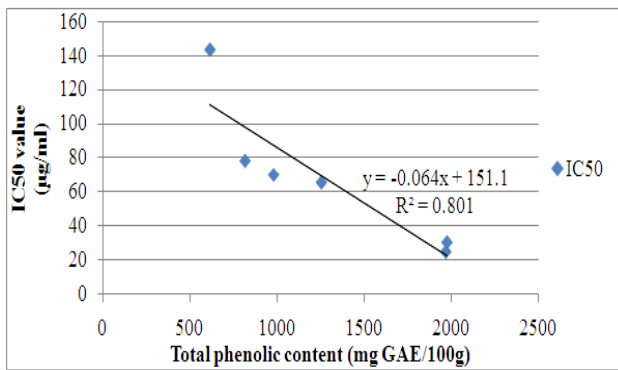


Fig. 4: Linear correlations between total phenolic content (mg GAE/100g) and IC₅₀ value ($\mu\text{g/ml}$) of various extracts of *Carica papaya*

Table 5: LC_{50} value ($\mu\text{g/ml}$) of different extracts of *Carica papaya* compared with 1 % mercury chloride

Sample	Extracts	Symbol	LC_{50} ($\mu\text{g/ml}$)
Fruit	Crude methanol	FCM	415.76 ± 51.48
	Hexane fraction	FHF	441.11 ± 3.80
	Ethyl acetate fraction	FEAF	163.96 ± 76.77
Seed	Crude methanol	SCM	374.86 ± 42.38
	Hexane fraction	SHF	357.26 ± 47.56
	Ethyl acetate fraction	SEAF	142.27 ± 40.35
Standard	1% Mercury chloride	MC	2.95 ± 0.63

It was found that SEAF ($142.27 \pm 40.35\mu\text{g/ml}$) showed to have the lowest LC_{50} value and FHF (411 ± 3.80) demonstrated to have the highest LC_{50} value. In comparison to all extracts, LC_{50} value was demonstrated in a trend of SEAF < FEAF < SHF < SCM < FCM < FHF (Figure 6 & Table 5). Crude extracts resulting in the LC_{50} values less than $250\mu\text{g/ml}$ was considered significantly active and had the potential for further investigation [24, 25, 26]. The finding of this study indicated that the LC_{50} values of both SEAF and FEAF are less than $250\mu\text{g/ml}$, hence it is promising to have potent bioactive compounds present.

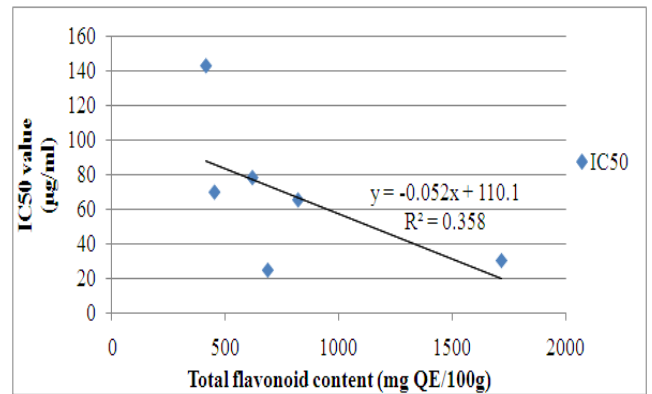


Fig. 5: Linear correlations between total flavonoid content (mg QE/100g) and IC₅₀ value ($\mu\text{g/ml}$) of various extracts of *Carica papaya*

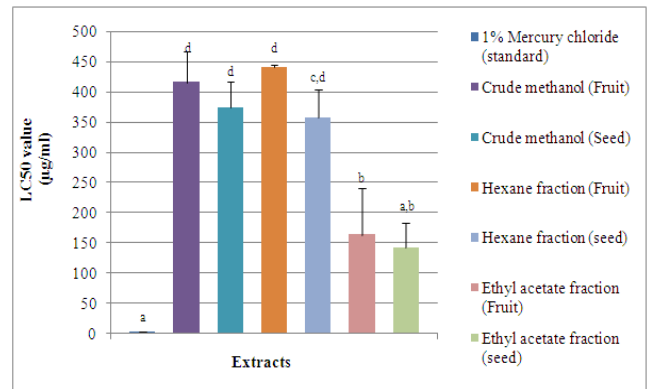


Fig. 6: LC_{50} value ($\mu\text{g/ml}$) of various extracts of *Carica papaya* compared with 1 % mercury chloride

Thin layer chromatography analysis

Bioactive compounds in the ethyl acetate fraction of crude methanol extract from the seeds of *Carica papaya* was identified using thin layer chromatography. TLC plate was developed in different ratio of solvent (hexane: ethyl acetate) and visualized at short UV-254nm. The best separation was observed in the ratio of 20: 80 (hexane: ethyl acetate) solvent system. TLC separation demonstrated that two non-polar compounds were eluted to the highest top of TLC plate, three moderately polar compounds were moderately eluted to the middle part of the TLC plate and three polar compounds were eluted the least and remained at the bottom of TLC plate.

The polar compounds eluted may most likely be the phenolic compounds; *p*-hydroxybenzoic acid and vanillic acid which were isolated from ethyl acetate fraction of seeds from *Carica papaya*[11]. According to Costa *et al* (2012), phenolic acids and flavonoid compounds were eluted in the solvent system of hexane: ethyl acetate = 1: 1[27].

CONCLUSION

The study proves that the ethyl acetate fractions of both fruits and seeds from *Carica papaya* were comparably high in antioxidant activity. Ethyl acetate fractions from both seeds and fruits of *Carica papaya* were significantly active and possess pharmacologically active principles for anti-cancer. Nonetheless, ethyl acetate fraction from seeds exhibited slightly higher potential. On the contrary, the hexane fractions of either fruits or seeds have the least potential.

CONFLICT OF INTERESTS

Declared None

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