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Cytotoxicity study and antioxidant activity of crude extracts and SPE fractions from *Carica papaya* leaves

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A R T I C L E I N F O

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A B S T R A C T

Millions of people in various traditional systems resorted to the use of medicinal plants for their ailments and recently, substances with antioxidant properties have been given unprecedented attention as possible therapeutic and preventive agents. The present study focused on cytotoxicity and antioxidant assessment of crude and fractions of *C. papaya* leaves as an important medicinal plant. Cytotoxicity was measured using the MTS colorimetric assay while antioxidant activity of the crude water extract and solvent fractions obtained from *C.papaya* leaves was evaluated in terms of capacity to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals. The crude water extract showed no cytotoxic effects within the tested concentrations compared with methanol and ethanol extracts. *C.papaya* fraction 1, 2 and 3 showed no cytotoxicity effect up to 2000 μg/ml, whereas fraction 4 and 5 showed no cytotoxicity up to 1000 μg/ml. The crude extract and the fractions showed considerable antioxidant activity with the most potent radical scavenger in the order of fraction 3, fraction 2, fraction 1, crude water, fraction 4 and fraction 5, respectively. The results obtained from this study provide a clear rationale for the medicinal uses of *C.papaya*.

1. Introduction

Carica papaya Linn (*C.papaya*) belongs to genus family of Caricaceae. It is soft-stemmed perennial plant which can grow up to 20 m without branch. It is well known as most cultivated plants in tropical and subtropical countries as Malaysia, Philippine, Thailand and Myanmar. Usually, the fruit of *C.papaya* is taken as a daily nourishment and the leaves is boiled by an old practitioners as an immune booster especially during dengue and malarial fever. Almost each part of *C.papaya* plant including fruits, leaves, roots, and seeds has contributed to its medicinal values. The green leaves possess great content of mineral elements (Ayoola and Adeyeye, 2010). It has been showed that the polar extracts of C.papaya exert various biological properties such as an anti HIV-1 (Rashed et al., 2013), as an analgesic (Akbar and Rizki, 2016), as a wound healing potential (Nayak et al., 2012), anticancer and immunomodulatory effect (Otsuki et al., 2010). Moreover, the aqueous extract of C.papaya leaves taken by dengue infected patients increased the platelet count (Subenthiran et al., 2013), white blood cells and neutrophils. Although C.papaya leaves known to be non-toxic and safe for oral consumption (Afzan et al., 2012), there is still no information on their cytotoxicity.

Antioxidants are substances that can prevent or delay the damage caused by free radicals and diminishing oxidative stress (Dai and Mumper, 2010). Normally, antioxidant system occurring in human body can scavenge these radicals, however exposure to radicals or environmental toxins induces the production of excessive reactive oxygen species and reactive nitrogen species which result in some chronic and degenerative diseases (Waris and Ahsan, 2006). The intake of exogenous antioxidants such as fruits and vegetables would improve the damage caused by oxidative stress. Polyphenols are secondary metabolites of plants and antioxidant activities increase proportionally with the polyphenol contents. Among the diverse roles of polyphenols is protecting cell constituents against destructive oxidative damage, thus limiting the risk of various degenerative diseases by acting as potent free radical scavengers (Pandey and Rizvi, 2009). Chen et al. (2011) validated in their study that C6/36 cells may survive DENV infection

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through an antioxidant defence mechanism.

The scientific evidence of *C.papaya* demonstrated against cancer cells also have been documented in various in vitro studies. *C.papaya* juice and pure lycopene caused cell death in HepG2 cell (liver cancer cell line) with an IC_{50} of 20 µg/ml and 22.8 µg/ml, respectively (Rahmat et al., 2002). C.papaya leaf juice exhibited a stronger cytotoxic effect on SCC25 cancer cells and produced a significant cancer-selective effect compared with the leaf decoction (Nguyen et al., 2016b). C.papaya seed extract also exhibited anticancer activity on PC-3 prostate cancer cells (Alotaibi et al., 2017) and in acute promyelotic leukemia HL-60 cells (Nakamura et al., 2007). Many cellular replication and repair are similar between cancer and normal cells, however typically all cancer drugs exhibit some degree of cytotoxic effects on normal cells (Alotaibi et al., 2017). It would be interesting to determine response occurs in cells derived from mosquitoes that are also essential for virus replication in nature.

The present work was carried out to evaluate the capability of C.papaya extracts and its fractions to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals and potential cytotoxic effects in C6/36 cells, an Aedes albopictus cell line, which is useful for the replication of flaviviruses and also commonly used for arboviral studies.

2. Materials and methods

2.1. Plant materials and extraction

Fresh leaves of *Carica papaya* were collected locally, washed thoroughly with water, dried and homogenized to a fine powder. A voucher specimen (SK3143/17) was identified by Dr. Shamsul Khamis (Universiti Putra Malaysia, Malaysia) and deposited at Herbarium of Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia, Malaysia. The fresh leaves of *C.papaya* was expressed using a juicer. The resulting juice was then subjected to lyophilisation to produce powder forms of the extracts and stored in air tight bottle at 4 °C. Extraction using methanol and ethanol was performed in a Soxhlet extractor. The solvents were then removed under pressure to obtain sticky residues.

2.2. HPLC analysis

The quantification of compounds in *C.papaya* extract was performed by a high performance liquid chromatography system (Waters 2690) coupled with a photodiode array detector (Waters 996) and a C18 synergy column (Synergy 4u fusion-RP 80A, 150 × 4.60 mm, 4 µm). Identification and separation of compounds from *C.papaya* was performed as per described by Wittenauer et al. (2015). Briefly, the mixture of 2% acetic acid in water (A) and 0.5% acetic acid in water and acetonitrile (50:50, B) was used as a mobile phase. The separation was performed in gradient elution with proportions of solvent B 35 min, 0–5%; 45 min, 5–20%; 30 min, 20–100%; 3 min, 100%, 10 min, 100-0% at the flow rate of 0.8 ml/min and injection volume of between 10 and 80 µl. The standard solutions were prepared by dissolving 1 mg of standards in 1 ml of methanol. The stock standard solution was serially diluted to the desired concentrations and filter sterilized with 0.22 µm syringe filter before injection.

2.3. Solid phase extraction (SPE)

SPE was performed by using Strata® C18-E cartridges (500 mg/6 ml). The cartridge was first pre-conditioned with 1 ml of absolute methanol to trigger the sorbent and then equilibrated using deionized distilled water. The crude extract was dissolved in water and filtered through 0.22 µm syringe filter. One ml of the crude extract was loaded into the SPE column and left in the sorbent matrix for a while. The SPE was performed in gradient elution of methanol-water mixture. Separation and collection of fraction 1 was composed by eluting 6 ml of 20% methanol followed by 40%, 60%, 80% and 100% methanol for fraction 2, 3, 4 and 5, respectively.

2.4. Cell culture conditions

C6/36 cell is a continuous mosquito cell line derived from larvae of *Aedes albopictus*. C6/36 cells were maintained in L15 medium supplemented with 10% heat-inactivated foetal bovine serum (FCS), 1% penicillin-streptomycin and 10% tryptose phosphate buffer. The cells were grown in a room temperature at 27 °C. Cells were passaged every four days and cultures were plated in 96 wells plate before experiments were performed.

2.5. Determination of maximum non cytotoxicity dose in C6/36 cells

The MTS cell proliferation assay was performed to determine the maximum tolerating dose which do not cause any cytotoxic effect to the cells following exposure to the tested extracts and fractions. C6/36 cells were seeded into 96 wells plate at 2×10^4 cells per well in L15 complete medium. Various concentrations of each extract were added to wells containing cell monolayer and incubated for 96 h. After 96 h incubation, the numbers of viable cells were measured using The CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA). The optical density of each well was measured at 490 nm using 96-well plate reader. The 50% cytotoxic concentration (CC_{50}) values for the extracts were calculated by plotting the percentage of cell viability against the concentration to quantify the activity.

2.6. DPPH radical scavenging assay

The ability of *C.papaya* extracts to scavenge 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical was measured by the addition of 0.1 mM DPPH solution in various concentrations of the extract samples. Absorbance reading was measured at 517 nm after 30 min of incubation in the dark. A mixture of methanol and extract served as the blank. The percent inhibition was calculated by measuring the absorbance of the extract or ascorbic acid treated samples against the blank. The 50% effective concentration (EC_{50}) values for the extracts were calculated by plotting the percentage of inhibition against the concentration to quantify the activity.

3. Results and discussion

3.1. Phytochemicals analysis in C.papaya water extract

The crude water extract of *C.papaya* leaves showed the presence of catechin, quercetin and cinnamic acid (Fig. 1). Though the HPLC chromatogram of the water extract was found to contain several well resolved peaks with distinct retention time, only three prominent peaks corresponding to the standard were identified. The results showed that the retention time of the catechin, quercetin and cinnamic acid standards matched with that of the retention time in the water extract components with retention time of 11.55 min, 37.55 min and 38.03 min, respectively. Phytochemicals analyses and screening of some past studies reported that *C.papaya* contain anthraquinone, amino acids, terpenoid, alkaloids, tannins, saponins, flavonoids, glycosides, phenol, vitamins, minerals, proteins and enzymes (Sherwani et al., 2013) and the leaves reported to have alkaloids carpain, pseudocarpain and dehydrocarpaine I and II, choline, carposide, vitamin C and E (Krishna et al., 2008).

Julianti et al. (2014) reported that flavonoids and alkaloids were the active constituents of C.papaya leaves by using HPLC. Previous studies reported by Muhamad et al. (2017),Asghar et al. (2016), and Tay and Chong (2016), mentioned that C.papaya contained abundance of polar compounds rather than non-polar compounds including flavonoid and phenolic compounds which suggested to contribute to the antioxidant activity. Similarly, previous bioinformatics work done by Senthilvel

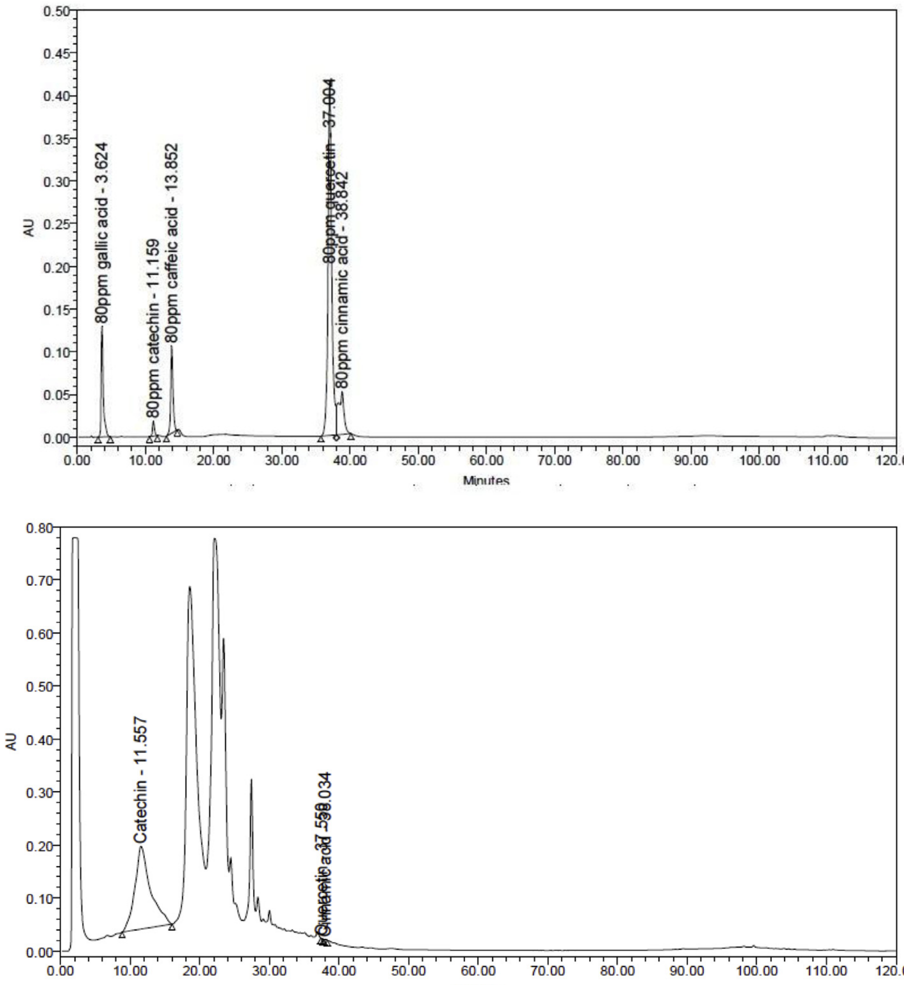

Fig. 1. Chromatogram of the standards and compounds in crude water extract of *C.papaya* leaves.

et al. (2013) has proved that the presence of quercetin in the leaves of C.papaya possessed the highest binding energy against NS2B-NS3 protease which has become a main target for the inhibition of viral assembly.

Several other studies have performed identification of the flavonoid constituents in C.papaya leaves. As stated by Nugroho et al. (2017), seven flavonoids from butanol fraction of C.papaya methanol extract have been identified and isolated. The compounds were quercetin 3-(2^G-rhamnosyrutinoside), quercetin 3-rutinoside, myricetin 3-rhamnoside, kaempherol 3-rutinoside, quercetin and kaempherol which possessed potent antioxidant properties by peroxynitrite scavenging assays. Afzan et al. (2012) also identified four flavonoids in C.papaya leaves extract known as quercetin 3-(2^G-rhamnosylrutinoside), kaempferol 3-(2^G-rhamnosylrutinoside), quercetin 3-rutinoside and kaempferol3-rutinoside.

Tan et al. (2012) found apigenin, kaempferol, quercetin, myricetin, isorhamnetin, catechin, hesperitin and naringenin while Nguyen et al. (2016a) reported kaempferol β-D-glucopyranoside, luteolin β-D-glucopyranoside, myricetin 3-rhamnoside, quercetin and rutin in C.papaya leaves. Phenolic acids are derivatives of benzoic and cinnamic acids, continuously getting interest due to their antioxidant activity and potential health benefits. The quantitative analysis by Canini et al. (2007) has shown the presence of phenolics acids as the main compound in *C.papaya* leaves using gas chromatography-mass spectrometry (GC-MS). The identified compounds comprised of procatechric acid, *p*-coumaric acid, 5,7- dimethoxycoumarin, caffeic acid, kaempherol, quercertin, and chlorogenic acid.

3.2. Maximum non toxicity dose of C.papaya crude extracts and fractions

The toxicological effects of *C.papaya* extracts were determined through MTS cell proliferation assay. Increasing concentrations of extracts were added to the cell monolayers in triplicate and absorbance reading was measured after 96 h. The maximum concentration at which no visible toxicity observed needs to be determined to ensure that concentrations used is not toxic to the cells and affect the cell proliferation. Cells that were incubated with extracts within the non-cytotoxic dose showed a normal cell morphology compared with cells incubated over the range of non-cytotoxic dose (Fig. 2). Toxicity to the cells can be visualized with a signs of rounding, clumping and detachment from the surface as depicted in Fig. 2(b), while normal healthy cells showed no observable decrease in cell number and morphology as depicted in Fig. 2(a).

Effect of *C.papaya* extracts on cell proliferation of C6/36 cells are shown in Fig. 3. *C.papaya* leaves was extracted with water, methanol and ethanol and the best extraction solvent which showed no cytotoxicity to cells was further examined. *C.papaya* water extract was shown to have no cytotoxicity to C6/36 cells within the range of concentrations tested, whereas methanol extract was more tolerable by the cells compared with ethanol extract with CC_{50} of 6952 μg/ml and 1995 μg/ml, respectively. CC_{50} is the concentration of the tested sample able to cause the death of 50% of the cells and can be predictive to the degree of cytotoxic effect. A high CC_{50} value indicated that the extract was less toxic to the cells. The assay showed that water extract possessed the highest maximum non toxicity dose in C6/36 cells followed

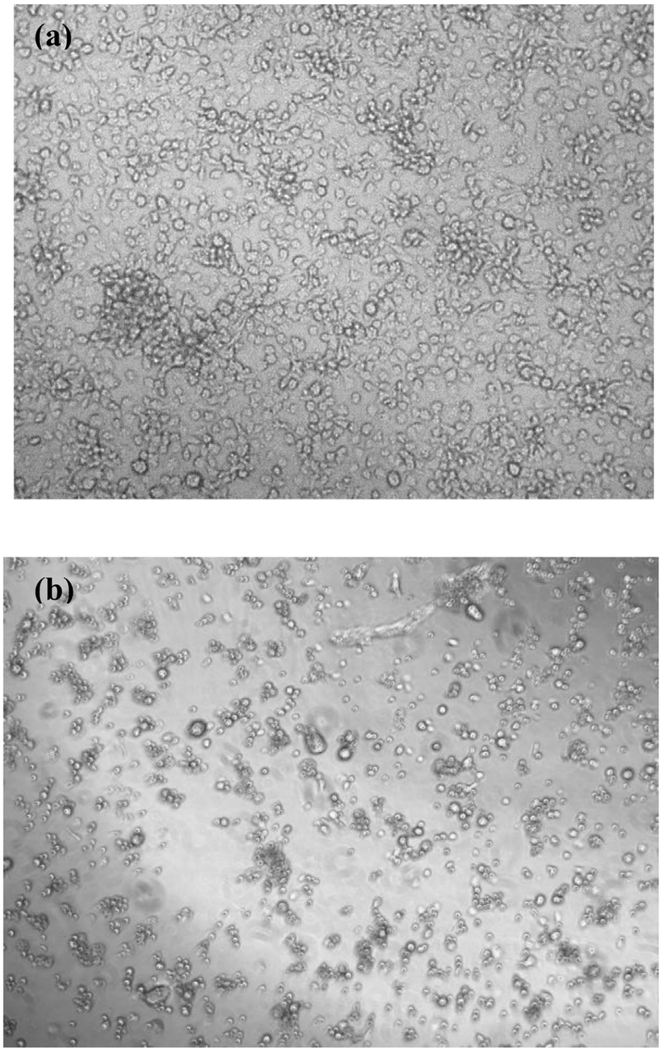

Fig. 2. Morphology of C6/36 cells incubated with *C.papaya* extract, (a) healthy cells and (b) cytotoxic effect in cells incubated beyond the maximum non cytotoxicity dose range of concentrations.

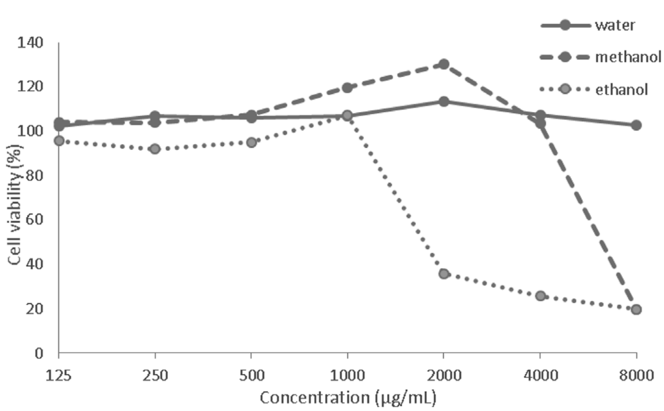

Fig. 3. Cell viability of C6/36 cells incubated with *C.papaya* extracts as measured by MTS assay. Cells were incubated with increasing concentrations of *C.papaya* extracts and cell viability was measured after 96 h. Each value represents the mean of triplicate assays ± S.E.

by methanol and ethanol extract, respectively, hence only water extract was further fractionated by using gradient concentrations of solvent on SPE C18 column.

Water extract of *C.papaya* was fractionated with different

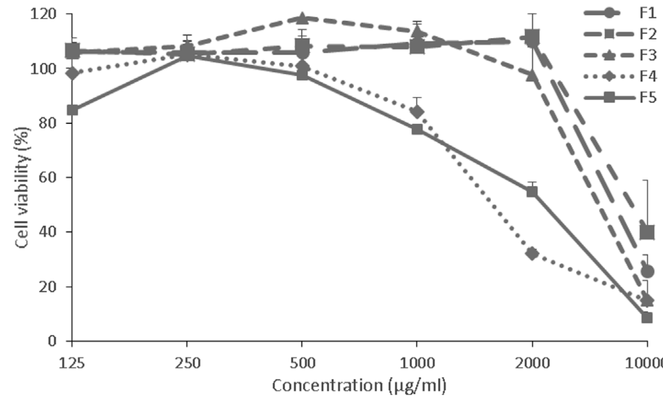

Fig. 4. Cell viability of C6/36 cells incubated with *C.papaya* fractions as measured by MTS assay. Cells were incubated with increasing concentrations of *C.papaya* fractions and cell viability was measured after 96 h. Each value represents the mean of triplicate assays ± S.E.

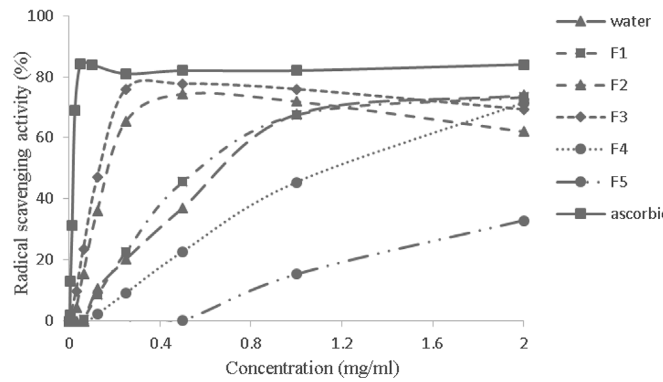

Fig. 5. DPPH radical scavenging activity of *C.papaya* fractions and ascorbic acid as a reference standard. Data are presented as means ± S.E of triplicate assays.

Table 1
EC_{50} values of DPPH radical scavenging activity from *C.papaya* extract and fractions.

Extract	EC_{50} (mg/ml)
Ascorbic acid	0.025
Water	0.718
F1	0.681
F2	0.268
F3	0.216
F4	1.306
F5	2.769

percentages of methanol, so that five separate fractions were obtained at the end of the process. The five different fractions were designated as F1, F2, F3, F4 and F5. The cytotoxicity effects of *C.papaya* fractions on proliferation of C6/36 cells was determined by using MTS assay. In general, the result showed that the fractions did not inhibited cell proliferation of C6/36 cells up to 1000 µg/ml (Fig. 4). Fraction 1, 2 and 3 were found to be less cytotoxic in C6/36 cells compared to fraction 4 and 5. The CC_{50} of the fractions in order of increasing cytotoxicity was 8994 µg/ml, 7481 µg/ml, 6640 µg/ml, 4890 µg/ml and 1672 µg/ml for fraction 2, 1, 3, 5 and 4, respectively. To our knowledge, this is the first report on cytotoxicity of crude and fractions from *C.papaya* in C6/36 cells.

3.3. Antioxidant capacity of water extract and fractions of C.papaya leaves

Plants are the potential source of antioxidants with capability to scavenge free radicals. The broad range effects of these antioxidant has drawn the attention of many experimental works as they are able to neutralize the free radicals or oxidants responsible for the onset of damage. There are many reports which support findings of antioxidant from various plants (Bhatt et al., 2013; Djeridane et al., 2006; Li et al., 2008). Antioxidant reacts with DPPH by changing the color from purple to yellow and the degree of discoloration indicates the scavenging potentials of the antioxidant. The antioxidant activity of the ascorbic acid as a standard was evaluated to compare the potential of the natural antioxidant with the fractions. The crude water extract was also tested to evaluate whether the fractionation process yielded fractions that were more or less active than the crude extract. Purification of the water extract yielded fractions with higher antioxidant activity than the crude water extract for fraction 1, 2 and 3 only whereas fraction 4 and 5 showed lower antioxidant activity than the crude water extract (Fig. 5).

All the tested extract and fractions showed scavenging activity in a concentration dependent manner. The strongest antioxidant potency in *C.papaya* was in order of F3 > F2 > F1 > crude water > F4 and the weaker was F5 when compared with ascorbic acid. These results suggest that F3 which was fractionated using 60% methanol might contain the strongest free radical scavenger compounds because different solvent has different extraction capacity. In general, methanol has been generally found to be more efficient in extraction of lower molecular weight polyphenols (Dai and Mumper, 2010). DPPH scavenging activity of water extract and fractions, expressed in term of EC50 was in the range of 0.216–2.769 mg/ml (Table 1). In this assay, F3 showed lowest EC_{50} value compared to other fractions. The lowest EC_{50} value indicates the presence of the highest antioxidants in that sample. Oloyede (2005) reported that quercetin and β-sitosterol may be responsible for the antioxidant potential in methanolic extract in mice treated with orally dose of 100 mg/kg of unripe fruits of *C.papaya*.

The third fractions (F3) which exhibited the highest antioxidant activity in this assay may contained the highest polyphenolic compounds compared to the other fractions. Papaya has been shown to contain many active components that can increase the total antioxidant power in blood and reduce the lipid peroxidation level such as α-tocopherol, ascorbic acid, beta carotene, flavonoids, vitamin B1, papain and niacin (Ross, 2003). The juice of ripe *C.papaya* displayed in vitro activities against oxidative stress and efficient scavenger of highly reactive hydroxyl radicals formed during ^{60}Co irradiation (Webman et al., 1989). Compounds with flavonoid structure such as catechin generally display a higher level of antioxidant activity than non-flavonoid compounds and the activity of flavonoids to act as antioxidants depends upon their molecular structure (Zhao et al., 2010). Quercetin on the other hands are able to bind transition metal ions which allow it to inhibit lipid peroxidation, which interfere by reacting with the radicals formed (Baghel et al., 2012).

4. Conclusion

In summary, this study demonstrated that the *C.papaya* crude water extract was less cytotoxic in C6/36 cells compared with its fractions, however the fractions showed better antioxidant activity than the crude water extract when fractionated with lower percentage of methanol-water ratio. This allows for new perspectives on its use in situations involving oxidative stress and cell proliferation. The present study also confirms the free radical scavenging activity of *C.papaya* which can be accounted for the traditional uses of this plant and it may be prudent to further explore the leaves extract for its usefulness in the oxidative stress induced disorders. The fractions should be further separated by using different solvent, purified to characterize and identify the bioactive compounds and re-evaluated for their properties.

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