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FULL PAPER

Effect of extraction solvents on antioxidant and wound healing properties of *Carica papaya* leaves extract

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Abstract

Carica papaya is a well-known plant that has been reported to exert various pharmacological activities including antioxidant and wound healing properties. However, to date, the lack of scientific evidence has been explored on the efficiency of the solvents towards *C. papaya* extract as a potential wound healer. The selection of proper extraction solvent plays a pivotal role in extracting the bioactive compounds from the plant. Therefore, the present study was aimed to examine the effect of three types of extraction solvents (methanol, ethanol and aqueous) on the antioxidant activity and wound healing potential of *C. papaya* leaves. In this study, the effect of different solvents of *C. papaya* leaves extracts were determined through 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, cytotoxicity assay and scratch migration assay on Human Skin Fibroblast cells (HSF1184). The result showed that the methanolic extract of *C. papaya* possessed a significant antioxidant activity as compared to ethanol and aqueous extract. The result also demonstrated that methanolic extract significantly stimulates the highest migration rate of HSF1184 cell at a concentration of 3.9 µg/mL, 7.8 µg/mL and 15.6 µg/mL ($p \leq 0.05$) after 48 hrs with no cytotoxicity observed at the concentration lower than 1000 mg/mL as compared to other solvents. HPLC analysis of methanol extract identified the presence of two flavonoids (catechin, quercetin) and two phenolic acids (caffeic acid, cinnamic acid). The findings suggest that the methanolic extract of *C. papaya* is effective in fighting free radicals and it has good wound healing activities. It also has the potential to be further explored for its medicinal values owing to the abundance of bioactive compounds from the extract.

1. Introduction

A proper extraction process of herbal plants for isolation of its bioactive compounds is essential in pharmaceutical, food and cosmetic application. Richard (1998) stated that two important points should be considered during the extraction process; 1) the purpose in conducting the extraction, and 2) the nature of the targeted compound that needs to be isolated. Many contributing factors play a part in a successful extraction procedure, such as part of the plant used, types of solvent, and extraction technique. The selection of a suitable solvent is critical as it greatly affects the active compounds that need to be isolated. "Like dissolves like" is the basic principle in which the solvent polarity influences the solubility of active compounds. If the compound of interest belongs to the polar compound; hence, a polar solvent is the most appropriate choice to use and vice versa. Studies have reported that extracting bioactive compounds using a different type of solvent significantly affect phytochemical content and biological activities (Perumal *et al*., 2013; Thouri *et al*., 2017). Moreover, a good solvent should possess a less toxic effect, can preserve the active compound and enhance the extraction yield and its bioactivities.

Carica papaya Linn belongs to the genus *Carica* and the family of Caricaceae. It is the most cultivated plants in tropical and subtropical countries such as Malaysia, Indonesia, Australia, Brazil, India and China. The young leaves of *C. papaya* exert the most prominent antioxidant activities and contained the highest total phenolic and

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flavonoid content as compared to other parts of the plant (Maisarah *et al*., 2013; Gogna *et al.,* 2015; Hadadi *et al*., 2018). Asghar *et al*. (2016) reported that the *C. papaya* leaves have the highest scavenging activity and inhibition of peroxidation as compared to its bark, root and pulp. Additionally, the young leaves of *C. papaya* contained high levels of secondary metabolites including caffeic acid, cinnamic acid, chlorogenic acid, quinic acid, coumaric acid, vanillic acid, protocatechuic acids, naringenin, hesperidin, rutin, and kaempferol as compared to old leaves (Gogna *et al.,* 2015).

Various literature studies have reported on the effect of *C. papaya* for its wound healing activities (Prashant *et al.*, 2011; Ancheta and Acero, 2015; Nafiu and Rahman, 2015; Ajani and Ogunbiyi, 2015; Nafiu *et al.,* 2016). The wound healing properties have been attributed to its antioxidant, anti-inflammatory and antimicrobial activities (Gosh and Gaba, 2013). Wound healing is a complex and dynamic process involving overlapping interactions among cellular structures, tissue layers, and different types of cells. The wound can be interpreted as a disruption of the functional continuity of cells and tissues due to physical, chemical, microbial infection or immunology process. In general, the wound healing process comprises three distinct phases; inflammation, proliferation and remodeling (Kurahashi and Fujii, 2015). Recently, the current application for wound healing treatment involves autographs, allografts, cultures epithelial autographs and wound dressing (Dreifke *et al.*, 2015). However, distinct types of treatment differ in the category of the wound, such as acute or chronic wounds. Adaptation of patients to the treatment also varies with age, sex, lifestyle, health status, and severity of the wounds (Ghosh and Gaba, 2013).

To date, most of the extract preparations of *C. papaya* for wound healing potential used varieties of solvents from polar to mid polar. Nevertheless, it is difficult to find a relationship of *in vitro* study based on the effect of different solvent against antioxidant and wound healing activities. Therefore, the present study was aimed to examine the effect of different extraction solvents on antioxidant and wound healing activities of *C. papaya* leaves extracts.

2. Materials and methods

2.1 Materials

2, 2-diphenyl-1-picrylhydrazyl. (DPPH), and thiazolyl blue tetrazolium bromide (MTT), catechin, and ascorbic acid were purchased from Sigma-Aldrich®, USA. HPLC- grade methanol, ethanol and Folin-Ciocalteu reagent were purchased from Merck®. Penicillin streptomycin (PS), Dulbecco's Modified Eagle Medium (DMEM), trypsin EDTA, phosphate-buffered saline (PBS), fetal bovine serum (FBS) were purchased from GIBCO®, USA. The green leave of *C. papaya* cultivar 'Eksotika' was collected from the Malaysian Agriculture Research and Development Institute (MARDI), Serdang, Selangor, Malaysia. The sample was identified and authenticated by botanist Dr. Shamsul Khamis and a voucher specimen (SK 3143/17) was deposited to Herbarium Institute of Bioscience, Universiti Putra Malaysia, Serdang, Selangor, Malaysia.

2.2 Preparation of the extracts

The method of extraction was conducted according to Vuong *et al*. (2013) with slight modification. Initially, the leaves were washed with tap water and dried in the oven for two (2) days at 50°C. Afterward, the dried leaves were ground using blender (Model: CB15V, Waring Commercial®, USA) to obtain a fine powder. 7.5 g of *C. papaya* leaves powder was subjected to a reflux extraction system for 20 mins (70 °C) using three different solvents; methanol, ethanol, and aqueous (100 mL). Extracts were filtered and concentrated using a rotary vacuum evaporator at 60°C. The dried extracts were stored at -20°C until further use.

2.3 DPPH radical scavenging activity

The scavenging activity of extracts against DPPH radical was performed based on Maisarah *et al*. (2013) with modifications. 100 µL of DPPH solution (0.1 mM) was added into each well plate containing different concentrations of extracts (0.5, 0.25, 0.125, 0.063, 0.031, 0.016, 0.008 mg/mL). The mixture was kept in the dark at room temperature for 30 mins. Ascorbic acid and catechin were used as the reference standard. The absorbance was measured at wavelength 517 nm whereas the activity of radical scavenging was calculated using the equation:

(%) Inhibition of DPPH scavenging activity = $[(A_0 - A_1)/A_0] \times 100$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample extract or standard.

2.4 Cell culture maintenance

Human Skin Fibroblast 1184 cell line (ECACC, United Kingdom) was maintained in DMEM supplemented with 10% FBS and 1% PS. The cell was incubated under 5% CO_2 at 37°C. Cell confluence at 80-90% was used for seeding and treatment.

2.5 Cytotoxicity assay

Cytotoxicity assay was conducted according to

Ismail *et al.* (2017) with modifications. Cells at a concentration of 1.0 x 10^5 cell/mL were seeded into 96-well plates and incubated overnight. Treatment was initiated by replacing the media with 200 µl of various concentrations of extracts ranging from 3.9 µg/mL - 1000 µg/mL. After 24 hrs of treatment, the MTT solution was added and re-incubated for another 4 hrs. Formazan crystal was dissolved in 100 µl of DMSO and measured at 570 nm using an ELISA reader (Model Erba: Mannheim).

2.6 Cell migration rate assay

Cell migration assay was carried out according to Ahmad *et al.* (2017) with modifications. Before treatment, post-confluent cells were scratched using 200 µL pipette tip and washed with PBS to remove cell debris. Extracts of 3 mL (3.9 µg/mL, 7.8 µg/mL and 15.6 µg/mL) were added and incubated for 48 hrs. Cells without treatment were regarded as negative control while ascorbic acid (5 µg/mL) was regarded as a positive control. Images were captured and analyzed using ImageJ analysis software using the following equation.

$$(\%)\text{Migration rate} = [(D_0 - D_{24,48}) / D_0] \times 100$$

Where D_0 is the distance between scratch at 0 hr, D_{24} is the distance of scratch after 24 hrs of treatment and D_{48} was the distance of scratch after 48 hrs of treatment.

2.7 Phytochemical quantification by HPLC

The quantification of phytochemicals present in the active solvent extract was analyzed using a Waters HPLC system (Model 2690; Milford, MA, USA). HPLC protocol was based on the previous study (Wittenauer *et al*., 2015) with some modifications. Chromatographic separation of compounds was achieved on a reversed-phase Luna®C18 column (100 x 4.6 mm, 5 µm; Phenomenex, Torrance, CA) operated at 25°C at a flow rate of 0.8 mL/min. The mobile phase consisted of 2% (v/v) acetic acid in water [A] and 0.5% acetic acid in water and acetonitrile (50:50,v/v) [B] using the following gradient program: 0-5% B (35 mins), 5-20% B (45 mins), 20-100% B (30 mins), 100% B isocratic (3 mins), 100-0% B (10 mins). The detection wavelength was set at 280 nm and UV/Vis spectra were recorded in the range of 200 to 600 nm. The total run time used was 123 mins and the injection volume of each sample was 30 µL The stock solutions of samples were prepared by dissolving 1 mg of standards/sample in 1 mL of methanol. The identification of compounds was confirmed by comparing the retention time of the standard with the sample. The calibration curves of every standard were constructed for quantification, and the compounds were further calculated based on the linearity of calibration curves standard; Y = mx +c in which x is the concentration of the compound while Y is a peak area (Table 1). The results were expressed as mg/g of extract.

2.8 Statistical analysis

All data were collected and analyzed by using one-way ANOVA using SPSS software (version 17.0). The significances of the results were determined by Tukey's test and the $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$ were set as the limit of significant difference. The results were expressed as mean ± standard deviation (STDEV) or standard error mean (SEM).

3. Results and discussion

3.1 DPPH radical scavenging activity of Carica papaya leaves

In this study, the leaves extracts of *C. papaya* were examined to identify the most active extract reflecting on antioxidant potential and wound healing activity. Determination of the antioxidant capacity of *C. papaya* leaves extracts on different solvents was carried out using DPPH scavenging assay. DPPH which is a stable free radical and can reduce its color from dark purple to pale yellow by accepting an electron from an antioxidant agent (Boligon *et al.,* 2014). EC_{50} was defined as an effective concentration to inhibit 50% of free radical DPPH. The lowest value of EC_{50} postulates the highest antioxidant capacities. Under normal physiology of the wound healing process, the exposure of reactive oxygen species (ROS) in low level helps to combat the invading microbial. Unfortunately, the imbalance may lead to oxidative stress and even can delay the healing process (Rasik and Shukla, 2000). At this point, antioxidant capacity plays a major role in avoiding cellular damages and stimulating the migration of cells towards the wound area. Figure 1 presents the dose-dependent trends of antioxidant scavenging activity of *C. papaya* leaves

Table 1. Quantitative analysis of compounds from *C. papaya* leaves extract (mg/g)

Compounds	R*t* (min)	Calibration equation	Quantity (mg/g)	Class of compounds	R^2
(1) Catechin	11.096	y = 7595.4x + 0.6667	4.98±0.78	Flavonoid	1
(2) Caffeic acid	14.476	y = 59887x - 604000	10.82±0.52	Phenolic acid	0.9962
(3) Quercetin	37.711	y = 141986x - 691660	4.92±0.41	Flavonoid	0.9768
(4) Cinnamic acid	38.014	y = 89417x - 1000000	11.28±1.21	Phenolic acid	0.9872

R*t*: Retention time

extracts. At the highest tested concentration (0.5 mg/mL), methanol extract scavenged 93.57% of the free radicals, 85.54% for ethanol and 82.04% for aqueous extract. The calculated EC_{50} value conformed the similar trends where methanol extract demonstrated the lowest EC_{50} value (0.193 mg/mL) followed by ethanol (0.249 mg/mL) and aqueous (0.284 mg/mL). Meanwhile, the EC_{50} value of standards; catechin and ascorbic acid were much lower than the crude extracts with an EC_{50} value of 0.005 mg/mL and 0.014 mg/mL, respectively. It has been shown by Asghar *et al.* (2016) in their study that ethanol and methanol were found to be the best solvent as compared to water, n-butanol, dichloromethane, ethyl acetate, and n-hexane to extract compounds which are responsible to the antioxidant activity. This could be explained by the efficiency of the solvent used during extraction.

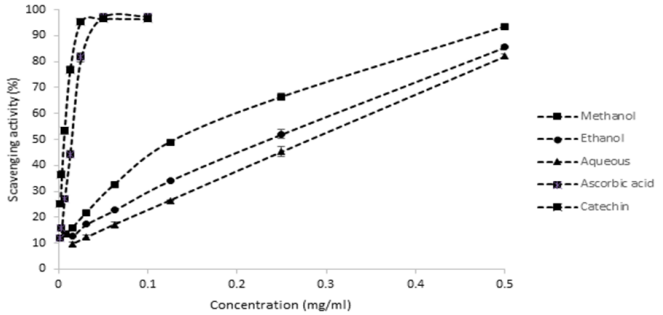

Figure 1. Percentage of scavenging activities of *C. papaya* extracts on DPPH radicals. Results were expressed as the mean of three independent experiments ± standard deviation. Catechin and ascorbic acid were used as standards

3.2 Effect of Carica papaya leaves extracts on cells viability and cells proliferation

MTT assay involves an enzymatic reduction of yellow soluble MTT into purple insoluble formazan by the mitochondria enzyme of viable cells. in this study, the cytotoxicity and proliferative study of the *C. papaya* extracts on HSF1184 were evaluated using MTT assay for 24 hrs. As shown in Figure 2, the viability of the cells of crude extract of *C. papaya* regardless of any solvents was not cytotoxic to HSF1184 cells. Interestingly, all extracts promote cell proliferation at different degrees. Aqueous extract dose-dependently promotes cells proliferation with the highest percentage of 150% at 500 µg/mL. Conversely, with methanol, this extract promoted cells proliferation at low concentrations (3.9 µg/mL to 62.5 µg/mL) with a maximum proliferation of 125%. Meanwhile, the ethanol extract promotes cells proliferation at the highest of 114% at 125 µg/mL. The result obtained was in agreement with the result reported by Afzan *et al.* (2012) that emphasized the pre-clinical safety of *C. papaya* leaves extract as a medicinal agent. The extract produces an insignificant effect between the treatment and the control. Moreover, it appears that the extract does not cause mortality and no morphological alteration on rats' organs has been detected upon treatment.

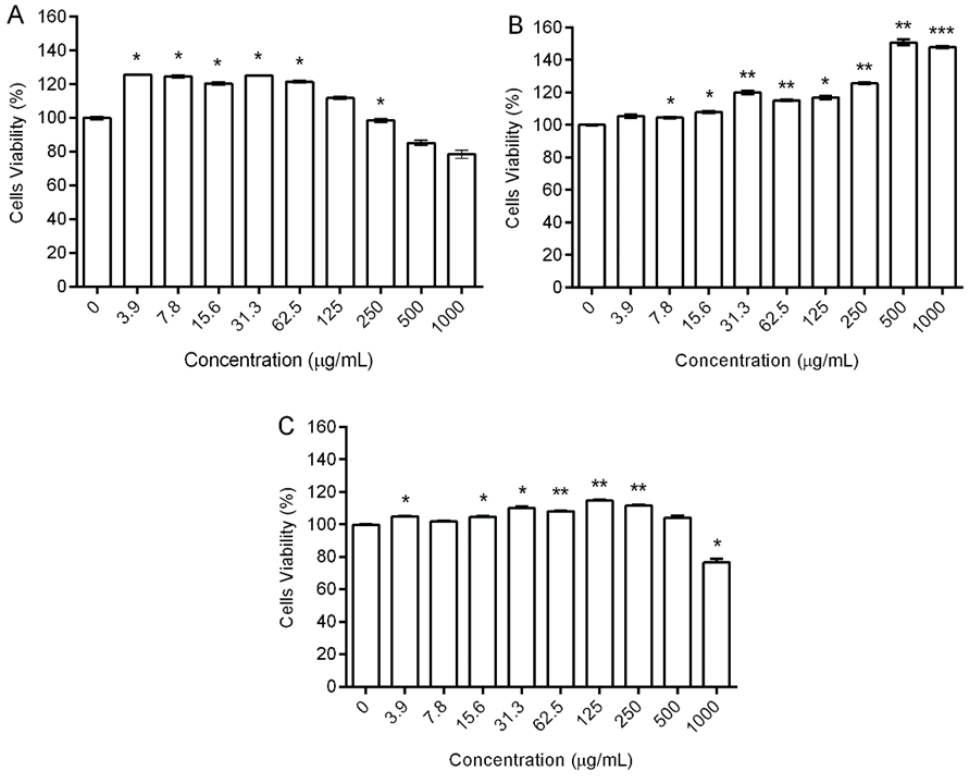

Figure 2. Effect of various concentrations of crude extract of C. papaya leaves extracts from different solvents (A); methanol, (B); aqueous, (C); ethanol on the cell viability of HSF1184 cell line after 24 hrs treatment. Results were expressed as the mean of three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$ and ***$p \leq 0.001$ compared to negative control.

3.3 Effect of Carica papaya leaves extract on wound scratch assay

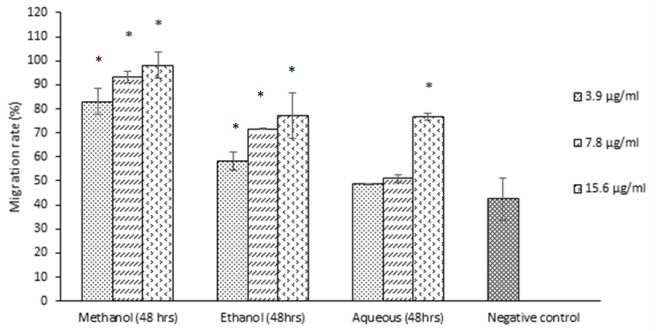

Figure 3. Effect of various concentrations of *C. papaya* (3.9, 7.8, 15.6 µg/mL) extracts of different solvents on migration rate in HSF1184 cell. The migration rate was analyzed by using Image-J software. Results were expressed as mean±SEM of three independent experiments. '*' indicates significant differences ($p \leq 0.05$) as compared to the negative control.

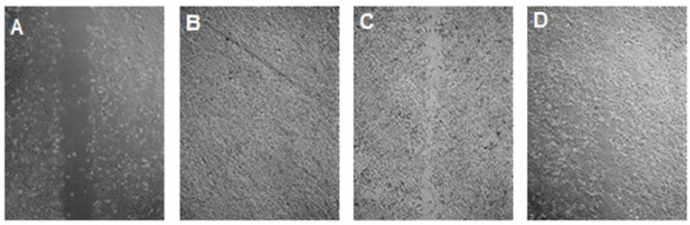

Figure 4. Digital image (x5 magnification) showing the effects of 15.6 µg/mL of *C. papaya* extracts obtained from different solvents (A); Control, (B); Methanol, (C); Aqueous and (D); Ethanol on the migration of HSF1184 cell line in wound scratch assay. The result shows that the scratch area was completely closed when cells treated with methanol extract at 15.6 µg/mL.

The cells migration assay was conducted to discover the effect of *C. papaya* extracts on the migration of HSF1184. During the study, a single scratch line was made on the cell to mimic the wound area. The migration of the fibroblast cells to fill the scratched area indicates the features of wound healing. The effect of *C. papaya* extracts of different solvents on the cell migration rate in HSF1184 cells was shown in Figure 3 and Figure 4. The non-cytotoxic concentrations of 3.9 µg/mL, 7.28 µg/mL and 15.6 µg/mL were used based on the previous result of MTT assay. The highest cells migration rate was observed when the cells were treated with methanol extract of *C. papaya* leaves at all concentrations used ($p \leq 0.05$) followed by ethanol and aqueous extract. A rapid migration rate was found at the highest concentration for all extracts (15.6 µg/mL) as compared to the scratch area treated with negative control (without treatment) as illustrated in Figure 4. The result was consistent with antioxidant activity indicating that the methanolic extract possesses the highest activities compared to other extracts. These findings were supported by other studies that revealed the relationship between antioxidants and wound healing processes (Agar *et al*., 2015; Yuslianti *et al*., 2015). It is believed that the highest antioxidant capacity may reduce the amount of free radical at the wound area.

3.4 HPLC analysis of active solvent of Carica papaya extract

It has been previously confirmed that the methanolic extract of *C. papaya* possessed the most potent antioxidant activities and promoted the fastest migration rate against HSF1184 cells as compared to other extracts. Therefore, further analysis was carried out for the quantitative detection of compounds present in the methanolic extract using the HPLC. The phytochemical analysis identified the presence of two flavonoids compounds (catechin, quercetin) and two phenolic acid compounds (caffeic acid, cinnamic acid) in the methanol extract of *C. papaya* (Figure 5). Cinnamic acid (11.28 mg/g) was found to be the highest compound in the extract followed by caffeic acid (10.82 mg/g), catechin (4.98 mg/g) and quercetin (4.92 mg/g). The HPLC result was in line with the result reported by other studies whereby they also have successfully identified many compounds from *C. papaya* including catechin, caffeic acid, quercetin and cinnamic acid (Canini *et al*., 2007; Gogna *et al*., 2015; Nguyen *et al*., 2016; Nugroho *et al*., 2017).

A study conducted by Pitz *et al.* (2016) showed that the activity of wound healing of Jaboticaba fruit peel hydroalcoholic extract was promoted by its phenolic compounds. The phenolic compounds have protective effects as a result of its antioxidant activity. It also has been supported by Lodhi and Singhai (2013) that luteolin at 0.5% proved to exert wound healing by antioxidant capacity. Moreover, the phenolic compound also has been evaluated for the synthesis of collagen (Dzialo *et al*., 2016). Catechin has been abundantly found harbored in the tea plant called *Camellia sinensis*. A study reported that catechin promotes angiogenesis and up-regulate nitric oxide synthase and cyclooxygenase which responsible during the process of anti-inflammatory response (Kapoor *et al.,* 2004). As the persistence of inflammation can delay the healing process, thus the action against wound healing activities takes place. Quercetin is a powerful antioxidant. It has been proved that quercetin exerts various pharmacological effects such as wound healing, anti-inflammatory, anti-cancer, antibacterial, neurodegenerative disease and others (Anand *et al*., 2016). Isolated quercetin from *Salvia leucantha* has significantly reduced wound area and promoted epithelisation on albino rats (Rajamanickam *et al*., 2013). A report by Song *et al.* (2017) showed that the presence of caffeic acid and cinnamic acid in the extract of *Bletilla striata* promotes the action of wound healing

and antioxidant activities. Besides, caffeic acid also has been identified in the active fraction of *Prosopis cineraria* that exhibited wound healing activities attributed by antioxidant and anti-inflammatory action (Yadav *et al*., 2018).

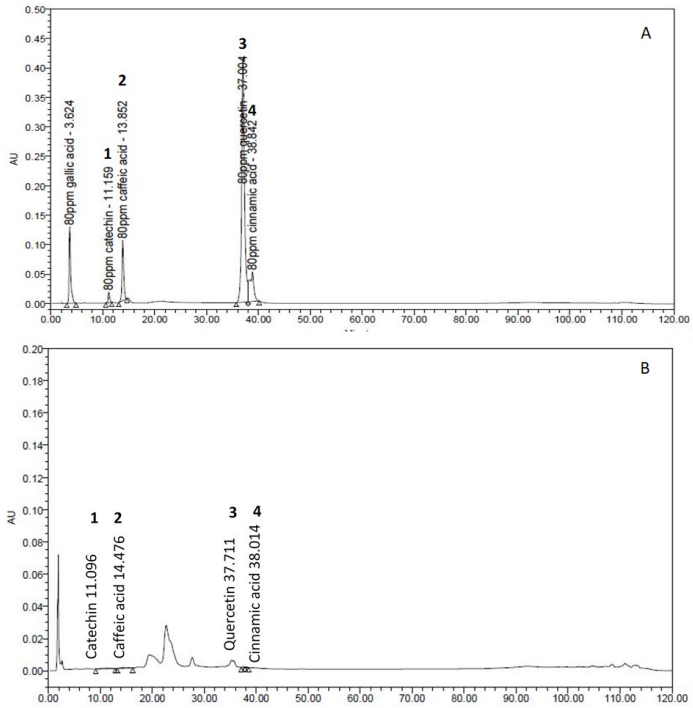

Figure 5. HPLC chromatogram detected from methanolic extract of *C. papaya* (B) compared with standards (A) at wavelength 280 nm. Peak assignment: (**1**); Catechin, (**2**); Caffeic acid, (**3**); Quercetin, (**4**); Cinnamic acid.

4. Conclusion

Our study demonstrated that the methanolic extract of *C. papaya* was effective in enhancing cells proliferation and cells migration as well as antioxidant activities on HSF1184 cells with no cytotoxicity observed upon treatment. The bioactive compounds present in the active extract have been successfully identified and confirmed by comparing the retention time of the sample with the standards using HPLC analysis. The results provide a basic consideration for further exploration on the isolation of compounds associated with the wound healing process and thereby promote a novel understanding of the mechanism involved.

Conflict of interest

The authors declare no conflict of interest.

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