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Research on Biological Activity of some Extracts from Vietnamese *Carica papaya* Leaves

Do Thi Hoa Vien *[*,1]
Phung Thi Thuy [1]
[1] *School of Biotechnology and Food Technology Hanoi University of Science and Technology, 1 - Dai Co Viet, Hanoi, Vietnam*.
[*] *e-mail :* vien.dothihoa@hust.edu.vn

Carica papaya leaves have long been used in some folk remedies to treat cancer. This folk remedy originated from Queensland - Australia from 1978 (Harald W. Tietze, 1997). In Vietnam, there are some successful cases of using *Carica papaya* leaves for cancer treatment even though this mediation do not always work out. In this study, the soluble substances in water (aqueous extract), the carotenoid and the polar substances (polar extract) were extracted from *Carica papaya* leaves grown in Vietnam; the bioassays such as cytotoxicity on some tumor cell lines as well as antioxidant activity were also tested with obtained extracts. The results showed that all obtained extracts had antioxidant activity stronger than the control substance - vitamin C at the same tested concentration. At concentration of 100 µg/ml, the inhibitive activity on lung cancer cell line LU-1 of obtained extracts was highest, from 43.47% (aqueous extract) to 62.88% (polar extract). On carcinoma cell line KB, inhibitive ability of extracts was lower: 2.8% (polar extract) and 20.6% (aqueous extract). On the breast cancer cells MCF7, inhibitive ability of above extracts was quite high, from 33.95% to 56.19%. On the leukemia cell line HL 60, inhibitive ability of these extracts was also high, from 39.56 to 60.64%. In addition, the result pointed out that on stem cells isolated from mouse embryos (ESC), the obtained extracts was not toxic to healthy cells of this line. This suggested that extracts from *Carica papaya* leaves has the potential to become one of the natural substance products that can support cancer treatment in certain cases.

Keywords: Aqueous substances, Polar substances, Extract, Anticancer, Antioxidant, Cytotoxic assay.

INTRODUCTION

Carica papaya L (papayaceae family) was originated from Central America, and then widely planted in tropical and subtropical countries. Chemical composition of papaya tree is quite diverse and rich. The main components in papaya plants are protease enzymes, carotenoids, alkaloids, mono-terpenoids, flavonoids, glucosinolates, minerals and vitamins, etc. The content of these components was different in the

different parts of tree. For example, while the main component of *Carica papaya* (CP) latex is protease enzyme, ripe CP fruit contains many of carotenoids (A.U. Ogan, 1971; Antonella Canini, *et al.*, 2007).

CP leaves is a byproduct of the process of harvesting CP fruits. In our country, some people used aqueous extract of CP leaves to support cancer treatment. Papaya leaves were also used to wrap beef to grill (to soften the meat) or as fertilizers after harvesting ripe fruit. In Australia, aboriginal inhabitants of Gold Cost – Queensland has been effectively use papaya leaves (paw paw leaves) as folk remedy to the treatment of lung cancer from 1962 (Hulda Clark, 2010). In 2010, the research of Dr. Nam Dang (Florida University, US) and colleagues (Tokyo University, Japan) showed that the fraction with M.W. less than 1000 of aqueous extract from CP leaves inhibited tumor cell growth on 10 tested tumor cell lines and mediated Th1-type cytokines in human immune system. Especially aqueous extract from CP leaves is harmless to normal cells, thereby avoiding side effects which are common with many cancer treatments today (N. Otsuki, Nam H. Dang *et al.*, 2010).

To examine the potential role of CP as anti-cancer therapy, we estimated in this report the anti-tumor activity of the aqueous extract, the carotenoid and the polar substances (polar extract) of CP leaves against various cancer cell lines, as well as its antioxidant activity.

EXPERIMENT

Materials

- *Carica papaya* (CP) was collected from Dong Hoi, Dong Anh, Hanoi, Vietnam. The studied samples were extracted from two types of CP leaves: fresh leaves and dried leaves.
- Cancer cell trains:
  Human epidermic carcinoma cell: KB
  Human lung carcinoma cell: LU-1
  Human breast carcinoma cell: MCF-7
  Human acute promyelocytic leukemia cell: HL-60
  Embryotic stem cell (ESC) from mouse stem
- Tested organisms: *E. coli*, *Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus*.
- Chemicals: Hexane, dichloromethane, ethanol, methanol, DPPH: diphenylpicrylhydrazyl (Sigma), ellipticine (Sigma).

Methods

Extraction of aqueous extracts from papaya leaves

The aqueous extracts from the CP leaves were extracted in the stainless steel pot (this is a normal method) in order to examine the antioxidant and anti-cancer activities of obtained extracts. From there, we can evaluate the scientific basis of the folk remedy.

Extracted by the folk method

Washed and drained CP fresh leaves after harvesting. Weighed an amount of leaf and stem, put them in stainless steel pot, and added distillated water into the pot with the ratio: raw material/solvent = 1/5 (w/v). Boil in 5 minutes, cooled, then filtered to obtain the extract. Concentrated extract by using rotavapor. Lyophilized dried the extract to obtain powder of extract.

Extracted by the folk method (but extract sample was crushed):

Washed and drained CP fresh leaves after harvesting. Weighed an amounts of leaf and stem, broke to small, grounded the raw material by using blender, then put them in stainless steel pot, and added distillated water into the pot with the ratio: raw material/solvent = 1/5 (w/v). Boiled in 5 minutes, cooled, then filtered to obtain the extract. Concentrated extract by using rotavapor. Lyophilized dried the extract to obtain powder of extract.

Extraction of carotenoids from CP leaves

Weighed 40 grams powder of dried CP leaves and put them in flask 250 ml that containing 120 ml of n - hexane. Extracted at 30°C and 24 hours by using shaking thermostat machine with shaking speed of 50-100 rpm/min. Then, filtered to obtain extract, concentrated extract by using rotavapor, and vacuum dried at 45°C to constant mass to obtain carotenoid extract.

Extraction of polar substances

Weighed 40 grams powder of CP leaves and put them into a flask 250 ml, added 120 ml dichloromethane 100%. Extracted 3 times/24 hours for each time on shaker at room temperature, filtered extraction by vacuum filter, then evaporated the solvent to obtain the unpolar substances extract and recovered the solvent. Put extractive residue in the fume hood to evaporated dichloromethane, and continued to extract extractive residue by methanol 100%. Extracted 3 times/24 hours for each time on shaker at room temperature, the ratio of extractive residue/solvent was 1/3 (g/ml). Filtered extraction by vacuum filter, then evaporated the solvent to obtain extract and recovered the solvent. Continued to dry the extract at temperature 45°C to constant mass to obtain the polar substances extract.

Identification of antioxidant activity by the method of scavenging free radical DPPH (David Grassi *et al*, 2010; Srikanth G *et al.*, 2010).

Took 5.4 ml of CP leaf extract (experimental sample) and put in falcon tube 15 ml, added 0.6 ml of DPPH 0.8 mM, vortex of sample and put it in the dark for 30 minutes. Then measured the absorbance of sample at wavelength 517 nm. Carried out simultaneously with both negative control sample (extract was replaced by distilled water) and positive control sample (extract was replaced by vitamin C). The antioxydant activity of experimental sample was calculated as following formula:

$$PI = \left(1 - \frac{A_{Exp}}{A_{control}}\right) x100\% \qquad (1)$$

In the formula (1), PI is antioxydant activity (%), A_{Exp} is absorbance (OD value) of the experimental sample, and $A_{control}$ is absorbance of the negative control sample.

Identification of antibacterial activity by the agar diffusion assay (R.C. Jagessar *et al.*, 2008)

Added tested microorganism (1 ml broth containing 105 CFU/ml) in 100 ml of LB medium that contained 1.2% of sterile agar and kept it at 50°C. Shook gently until bacteria distributed evenly, poured out to plates, waited for the freezing medium, then used agar perforative tube to perforate with the hole size of 0.5 cm. Added into the holes 100 µL of extract sample at different concentrations (10 mg/ml, 20 mg/ml, 30 mg/ml, 40 mg/ml, 50 mg/ml, 100 mg/ml, 150 mg/ml and 200 mg/ml). Positive control sample was amoxicillin antibiotic. Cultured at 37°C in incubator within 24-48 hours, then checked the antimicrobial round.

Tested the anti-cancer activity by the cytotoxic assay (R. H. Shoemaker *et al.*, 2002; A. Monks *et al.*, 1991)

The principle of the method

Stained tumor cells with sulforhodamine B (SRB), then measured the optical absorbance of the SRB coloured complex attached to cells. Protein content of cells was proportional to the measured OD values. Tumor cells were inhibited or destroyed, so the protein content was decreased. Based on the determination of total cellular protein to assess inhibitive ability on tumor cells. Medium of tumor cell consisted 2 mM L-glutamine, 1.5 g/L sodium bicarbonate; 4.5 g/L glucose; 10 mM HEPES (chemical buffer system) and 1.0 mM pyruvate sodium; plus 10% of fetal bovine serum - FBS (GIBCO).

Proceeding

Dissolved 20 µl of extract in 10% DMSO, then added them into the plate 96 wells to attain final concentrations: 100 µg/ml; 50 µg/ml; 25 µg/ml; 12.5 µg/ml and 6.25 µg/ml. Trypsin of the cells to leave them and adjusted to reach the density of 3-7 x 10^4 CFU/ml by counting in the erythrocyte counting chamber. Added the suitable cells to the wells (contained 180 µl of medium) such as in the table 3.1. The tumor cells were cultured in CO_2 incubator within 3-5 days. Then they were fixed to the bottom of wells with TCA for 30 minutes, and were stained with SRB at 37°C in 1 hour. Removed excessive SRB and washed 3 times by 5% acetic acid and then dried in air at room temperature. Control sample of zero day was tested on another plate (96-wells) that was added reagent but was added tumor cells. After 1 hour, the tumor cells in the control plate were fixed with trichloracetic acid - TCA. Finally, used 10 mM of Tris buffer solution to dissolve the SRB that was sticked and stained the protein molecules, shaked plate gently for 10 minutes on the shaker. Using ELISA Plate Reader (Bio-Rad) to read results at a wavelength of 515 nm. The viability of cells in the presence of reagent was determined through the following formula:

$$\text{The rate of living cells (\%)} = \left(\frac{OD_1 - OD_0}{OD_2 - OD_0}\right) x100\% \quad (2)$$

In the formula (2), OD_1 is optical density of the tested tumor cell line, OD_2 is optical density of the negative control sample, whereas OD_0 is optical density of the cell sample at the start of experiment (at zero day).

Ellipticine (Sigma) was used as positive control. DMSO10% was used as negative control. IC_{50} value (inhibitive concentration of 50% of the development of tumor cells) was determined by the standard curve equation that was established based on the growth of tumor cells and the concentration of tested sample. IC_{50} value calculated by the formula:

$$\frac{1}{Y} = a + b \ln X \quad (3)$$

In the formula (3), Y is concentration of tested extract sample, and X is the percentage of living tumor cells.

If the tested sample has $IC_{50} \leq 20$ µg/ml (with crude extract and with extractive fraction) or has $IC_{50} \leq 5$ µg/ml (with purified compound) it will be considered to have cellular cytotoxic activity and have inhibitive capacity of the growth of tumor cells or have killed capacity of tumor cells.

Cultured method of tumor cell lines *in vitro* was carried out by conventional standards of ATCC cells bank (American Type Culture Collection). The tumor cell lines were cultured in the medium to suit each type of cell; added 1.0 mM sodium pyruvate, 10% fetal bovine serum - FBS (GIBCO), 1% PSF (penicillin streptomycine fungizone). Cells were subcultured after 3-5 days, with ratio 1: 3 depending on cell density. The culture was carried out in the CO_2 incubator at 37°C and 5% CO_2.

RESULTS AND DISCUSSION

Antioxidant activity

The tested result of antioxidant activity of extractive samples was showed on Figure 1.

The obtained result demonstrated that all extractive samples had antioxidant activity. While aqueous extract from fresh papaya leaves using folk method has the highest antioxidant activity (it was up to 60% at concentration of 1000 µg/ml), the one that was extracted after being crushed in a blender has lowest antioxidant activity. This is because natural enzymes in crushed papaya leaves contacted directly with the substate, and therefore, fasten the decomposition process of antioxidant

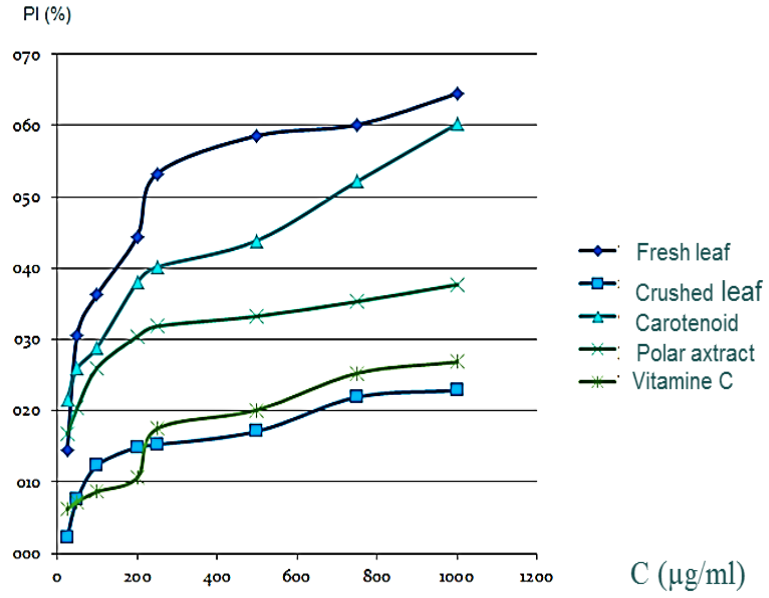

Fig.1: Antioxidant activity of extractive samples

compounds in fresh papaya leaves. Meanwhile, in folk extractive process, fresh papaya leaves wasn't crushed and was boiled so natural enzymes that can decompose the antioxidant compounds were inactivated at the beginning. The result also showed that most extractive samples has higher antioxidant activity than vitamin C at respective tested concentration. This characteristic may enable papaya leave extract to support cancer prevention and treatment, particularly the cancer types caused by free radicals.

Antibacterial activity

In this study, the extractive samples were tested antibacterial activity at concentrations up to 200 mg/ml. The obtained result showed that the extractive samples didn't have antibacterial activity with four bacterial species as *E. coli*, *Staphylococcus aureus*, *Pseudomonas*, *Streptococcus* at the tested concentrations.

Anticancer activity

Tested with the lung tumor cell line LU – 1

The data in Table 1 indicated that all four extracts from papaya leaves and stems were inhibiting capable of lung tumor cell line LU - 1 at the studied concentrations. Among them, polar compound extract was highest inhibiting capable of tumor cell LU-1 (the inhibition reaching 62.88% at concentration of 100 µg/ml), the IC_{50} value of this extract was 78.02%. These tumor-inhibiting results were not high enough to

Table 1. Inhibitive activity for lung tumor cell line LU – 1

Tested sample	Aqueous-extract of fresh leaves		Aqueous-extract of crushed leaves		Carotenoid		Polar extract		Elipticine		
Concentration (µg/ml)	OD of cells	Inhibition (%)	OD of cells	Inhibition (%)	OD of cells	Inhibition (%)	OD of cells	Inhibition (%)	Concentration (µg/ml)	OD of cells	Inhibition (%)
100	0.370	43.47	0.331	50.45	0.288	58.23	0.262	62.88	20	0.079	95.53
20	0.507	18.96	0.488	22.45	0.521	16.46	0.495	21.11	4	0.124	87.48
4	0.520	16.64	0.545	12.25	0.542	12.70	0.541	12.88	0.8	0.495	21.20
0.8	0.544	1..34	0.560	9.57	0.561	9.30	0.556	10.20	0.16	0.563	8.94
IC_{50} (µg/ml)	> 100		98.34		94.67		78.02		1.59		
		DMSO	0.613		0 day	0.054					

Table 2. Inhibitive activity for carcinoma cell line KB

Tested sample	Aqueous-extract of fresh leaves		Aqueous-extract of crushed leaves		Carotenoid		Polar extract		Elipticine		
Concentration (µg/ml)	OD of cells	Inhibition (%)	OD of cells	Inhibition (%)	OD of cells	Inhibition (%)	OD of cells	Inhibition (%)	Concentration (µg/ml)	OD of cells	Inhibition (%)
100	0.977	20.60	1.116	8.20	1.035	15.41	0.963	2.80	8	0.080	100.16
20	1.498	-20.64	1.395	-16.53	1.258	-4.42	1.156	4.71	1.6	0.654	49.23
4	1.398	-16.81	1.419	-18.63	1.511	-26.86	1.231	-2.00	0.32	1.109	8.82
0.8	1.375	-14.79	1.390	-16.11	1.497	-25.62	1.339	-11.54	0.064	1.129	7.08
IC_{50} (µg/ml)	> 100		> 100		> 100		> 100		1.58		
		DMSO	1.203		0 day	0.0818					

Table 3. Inhibitive activity for breast tumor cell line MCF7

Tested sample	Aqueous-extract of fresh leaves		Aqueous-extract of crushed leaves		Carotenoid		Polar extract		Elipticine		
Concentration (µg/ml)	OD of cells	Inhibition (%)	OD of cells	Inhibition (%)	OD of cells	Inhibition (%)	OD of cells	Inhibition (%)	Concentration (µg/ml)	OD of cells	Inhibition (%)
100	0.490	41.51	0.539	33.95	0.445	48.58	0.396	56.19	8	0.134	97.03
20	0.659	15.18	0.706	7.96	0.686	11.02	0.658	15.37	1.6	0.381	58.50
4	0.693	9.93	0.732	3.86	0.792	-5.41	0.786	-4.52	0.32	0.584	26.94
0.8	0.714	6.66	0.753	0.59	0.781	-3.81	0.766	-1.40	0.064	0.868	-17.30
IC_{50} (µg/ml)	> 100		> 100		> 100		> 100		1.04		
		DMSO	0.7568		0 day	0.1146					

Table 4. Inhibitive activity for leukemia cell line HL60

Tested sample	Aqueous-extract of fresh leaves		Aqueous-extract of crushed leaves		Carotenoid		Polar extract		Elipticine		
Concentration (µg/ml)	OD of cells	Inhibition (%)	OD of cells	Inhibition (%)	OD of cells	Inhibition (%)	OD of cells	Inhibition (%)	Concentration (µg/ml)	OD of cells	Inhibition (%)
100	0.590	45.69	0.639	39.56	0.472	60.64	0.491	58.24	8	0.141	102.25
20	0.787	21.01	0.791	20.51	0.734	27.59	0.822	16.60	1.6	0.426	66.32
4	0.881	9.06	0.857	12.15	0.889	8.14	0.878	9.54	0.32	0.665	36.25
0.8	0.963	-1.24	0.985	-3.99	0.938	1.92	0.933	2.61	0.064	0.909	5.56
IC_{50} (µg/ml)	> 100		> 100		57.96		86.31		0.68		
		DMSO	0.9535		0 day	0.1589					

achieve the standard of inhibitors and destroy tumor cells as expectance (≤ 20 mg/ml); however this initial results can confirm that papaya leaves extract were preventing capable of some cancers.

Tested with the carcinoma cell line KB

The obtained result (data in Table 2) showed that all four tested extract samples were inhibiting capable of carcinoma cells KB, but these activity were weak at tested concentration of extracts (both IC_{50} > 100 µg/ml). Similar to extracts of fresh leaves and crushed leaves, carotenoid extract had the inhibitive activity with carcinoma cells KB at concentrations of 100 µg/ml; but polar extract had the inhibitive activity at concentration of 100 µg/ml and 20 µg/ml. The lower concentration did not show activity.

Tested with the breast tumor cell line MCF7

The data (Table 3) indicated that all four tested samples were inhibiting capable of breast tumor cells MCF7. Extracts derived from fresh leaves and from crushed leaves had inhibitive activity at the tested concentrations. Polar extract and carotenoid extract had inhibitive activity at concentration of 100 µg/ml (equivalent to 4 mg of fresh leaves/ml or 1.2 mg of dried leaves/ml) and at concentration of 20 µg/ml. These extracts did not has inhibitive activity at lower tested concentrations (4 and 0.8 µg/ml). IC_{50} values of the obtained extracts were greater than 20 µg/ml. However, these results showed higher

Table 5. Inhibitive activity for original cell isolated from mouse embryo (ESC)

Tested sample	Aqueous-extract of fresh leaves		Aqueous-extract of crushed leaves		Carotenoid		Polar extract		Elipticine		
Concentration (µg/ml)	OD of cells	Inhibition (%)	OD of cells	Inhibition (%)	OD of cells	Inhibition (%)	OD of cells	Inhibition (%)	Concentration (µg/ml)	OD of cells	Inhibition (%)
100	0.299	- 4.00	0.333	- 15.65	0.303	- 5.39	0.318	- 10.43	0.064	0.289	- 0.52
		DMSO	0.288								

inhibitive activity compared with the research at the University of Tokyo which showed that aqueous extract of *Carica papaya* leaves (with a concentration equivalent to 10 mg dried leaves/ml) had inhibitive capable of 50% breast tumor cells MCF7 (Noriko Otsuki, Nam H. Dang *et al.*, 2010).

Tested with the leukemia cell line HL 60

The obtained result that is shown in Table 4 demonstrated that all four tested samples have inhibitive activity with leukemia cells HL60. Polar extract and carotenoid extract had inhibitive activity at all tested concentrations. Extracts from fresh leaves and from crushed leaves had inhibitive activity at concentrations of 100 µg/ml, 20 µg/ml and 4 µg/ml; these extracts did not has inhibitive activity at concentration of 0.8 µg/ml . IC_{50} values of obtained extracts were greater than 20 µg/ml.

Tested with the embryo stem cells (ESC) isolated from mouse embryo

The extractive samples in this study did not show lethal toxicity for stem cells at the highest tested concentration (100 µg/ml). This result is shown in Table 5. The result showed that using papaya leaf extracts in cancer prevention will decrease unwanted side effects caused by anti-tumor drugs that were currently used.

CONCLUSION

Initially, the obtained results indicated that the obtained extracts from papaya leaves in this study have quite good antioxidant activity. The inhibitive activity for tested tumor cell lines of these extracts was relatively high. The inhibitive activity of carotenoid extract for HL60 leukemia cell line was strongest with IC_{50} reached 57.96 µg/ml (Table 4); then it was the inhibitive activity of polar substance's extract for LU-1 lung tumor cell line with IC_{50} reached 78.02 µg/ml (table 1). The inhibitive activity of all extracts for KB carcinoma cell line was lowest comparing to the other tested tumors: the polar extract inhibited only 2.8% of KB carcinoma cells, and the aqueous- extract inhibited 20.6% of KB carcinoma cells. Tested result for stem cells (ESC) isolated from mouse embryos demonstrated that the obtained extracts were not toxic to ESC stem cell line. This may suggested that papaya leaf extracts have potential to become one of the preparations that can be used to prevent or support the treatment of some cancers.

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