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Anti-tumor Promoting Activity of Some Malaysian Traditional Vegetable (Ulam) Extracts by Immunoblotting Analysis of Raji Cells

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Abstract – The extracts of *Carica papaya* (flower), *Barringtonia macrostachya* (leaves), *Coleus tuberosus* (tuber), *Mangifera indica* (fruit skin) and *Eugenia polyantha* (leaves) showed strong *in vitro* anti-tumor promoting activity when assayed using Raji cells (Mooi *et al.*, 1999). The antitumor promoting activity of the crude extracts was further analyzed by immunoblotting analysis of Raji cells carrying Epstein-Barr virus genome. The expression of early antigens diffuse (EA-D) and early antigens restricted (EA-R) was determined by performing western blotting of treated Raji cells with human sera of nasopharyngeal carcinoma patients. All the plant extracts were shown to be able to suppress both EA-D and EA-R.

Key words – Anti-tumor promoting activity, Cancer chemoprevention, Traditional vegetables, Epstein-Barr virus activation, Raji cells

Introduction

Raji cells are human B lymphoblastoid cell line carrying genome of Epstein-Barr virus (EBV) early antigen (EA) without producing the EBV (Roubal *et al.*, 1984). The diterpene ester-type promoter of mouse skin tumors, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) efficiently induces persisting genomes of oncogenic herpes viruses resulting in more than 50% exhibition of viral antigen and particle-positive cells (Yamamoto *et al.*, 1979; Hausen *et al.*, 1979). A short-term *in vitro* assay for antitumor promoting activity by inducing early antigen in Raji cells was reported by Ito *et al.* (1981). In this assay system the inhibition of the induced early antigen of the EBV by phorbol 12-myristate 13-acetate was determined by immunofluorescence technique using human antisera of nasopharyngeal carcinoma patients. The test is simple, reproducible and applicable for mass-screening of anti-tumor promoters (Murakami *et al.*, 1995, 1997; Mooi *et al.*, 1999). The EBV-EA has been classified into diffuse EA-D that was detected both in the nucleus and the cytoplasm, and the restricted EA-R that was only detected in the cytoplasm. Both EBV EA-D and EA-R are polypeptides of about 50 to 52 kDa and 85 kDa, respectively (Kondo *et al.*, 1998). The tumor promoters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and teleocidin B-4 can induce the synthesis of EBV EA-D and R in Raji cells.

In this study, immunoblotting for the detection of EA-D and R was employed as a confirmation test for anti-tumor promoting activity of the plant extracts that showed positive activity when screened using *in-vitro* assay system with Raji cells (Mooi *et al.*, 1999).

Materials and Methods

Chemicals – Phorbol 12-myristate 13-acetate (PMA) (Sigma) was used as an inducer of the EBV activation in the EBV-activation assay. Sodium-*n*-butyrate was added to enhance the EBV activation. Early antigen (EA)-positive sera from nasopharyngeal carcinoma (NPC) patients were obtained from the University Hospital, University of Malaya, and FITC-labeled IgG was purchased from Sigma. Other

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chemicals used were the enzyme-coupled secondary antibody (affinity purified Goat anti Human IgG Horseradish Peroxidase Conjugate (KPL, USA) and 2% of 4-chloro-1-naphthol (4CN) (Bethesda Research Lab., USA) as substrate for Horseradish Peroxidase (HP) which was used as a color development solution.

Plant materials – Dr. Kamaruddin Mat Salleh of Department of Botany, Universiti Kebangsaan Malaysia, established the taxonomic identification of plant samples. The plant samples obtained from Serdang, Banting were cut into small pieces and macerated in 80% ethanol at room temperature for a week. The crude extract was then filtered and evaporated *in vacuo* at 45°C using rotary evaporator. The extract was dissolved in 100% ethanol or dimethyl sulfoxide (DMSO) and subjected to the bioassay. The inhibitory effect of each plant extract was tested at the concentrations of 400 μg/ml, 200 μg/ml, 100 μg/ml, 50 μg/ml, 25 μg/ml and 12.5 μg/ml.

Inhibitory assay of Epstein-Barr virus activation – The assay was carried out as described by Mooi *et al.* (1999). Briefly, Raji cells at a concentration of 5×10^5 cells/ml were incubated in 1ml of RPMI 1640 medium (supplemented with 10% fetal calf serum) containing sodium-*n*-butyrate (3 mM), phorbol 12-myristate 13-acetate (0.05 μM) and test substance (5 μl) at 37°C under 5% CO_2 for 48 hours. EA expressed in Raji cells was detected by an indirect immuno-flourescence method with EA-positive sera from NPC patients and FITC-labeled IgG. The average EA induction was compared to a control with only PMA and sodium-*n*-butyrate, where the induction rate of the control was less than 40%.

Immunoblotting analysis for the detection of EBV-early antigen on PVDF membrane – Plant extract-treated Raji cells, including control were harvested after 48 hours. The cells were washed twice with PBS at 4°C. The cells were spun down at 3,000×*g* for 5 minutes at 4°C and were resuspended in 5 volume of ice-cold suspension buffer. As soon as possible, an equal volume of 2X SDS gel-loading buffer was added, followed by boiling the sample for 10 minutes. The lysates were centrifuged at 10,000×*g* for 10 minutes. A volume of 15 μl each of the samples and 3 μl of prestained SDS-PAGE standards of mid range molecular weight markers (Bio-Rad, USA) were loaded into the predetermined order wells on a 12% SDS-polyacrylamide slab gel (Laemmali, 1970). The SDS-polyacrylamide gel containing separated proteins were electrophorectically transferred to a PVDF membrane. The membrane was then stained with NPC serum (polyclonal antibody) obtained from NPC patients in the ratio of 1:20, followed by the enzyme-coupled secondary antibody (affinity purified Goat anti Human IgG Horseradish Peroxidase Conjugate (KPL, USA) in 1:200 with a final concentration of 0.5 μg/ml. The immunoreactive bands in the PVDF membranes were developed with color development solution consisting of 2% of 4-chloro-1-naphthol (4CN) (Bethesda Research Lab., USA) as substrate for Horseradish Peroxidase (HP), 50 ml of Tris HCl buffer, pH 7.4 and 30 μl of 30% of H_2O_2.

Results and Discussion

The immunoblotting analysis of EBV-early protein diffuse (D) and restricted (R) from Raji cells induced by PMA and sodium-*n*-butyrate were studied with different concentrations of extracts of flower of *Carica papaya*, leaves of *Barringtonia macrostachya* and *Eugenia polyantha*, tuber of *Coleus tuberosus* and fruit skin of *Mangifera indica* that showed strong antitumor promoting activity when assayed with short term *in-vitro* assay system with Raji cells (Mooi *et al.*, 1999). All the plant extracts were tested at the concentrations of 12.5, 25, 50, 100, 200 and 400 μg/ml. The untreated and treated Raji cells only with PMA and sodium-*n*-butyrate (3 mM) were used as controls. Two EA protein bands, 50-52 kDa of high intensity band (EA-D) and 85 kDa less intense band (EA-R), were found together with two non-specific bands at about 66.2 kDa and 40 kDa, which were observed after immunoblotting analysis (Fig. 1). The same observation was reported by Kondo *et al.* (1998).

The extracts of *Coleus tuberosus, Mangifera indica* and *Barringtonia macrostachya* completely suppressed the EA-R at the concentrations tested ranging from 12.5 to 400 μg/ml. For the crude extract of *Eugenia polyantha*, the complete suppression of EA-R was observed only at the concentrations ranging from 50 to 200 μg/ml and partial suppression was observed at 25 and 12.5 μg/ml. However for the extract of *Carica papaya*, the EA-R was found to cause only partial suppression at all concentrations.

Higher concentration of extracts were required for the suppression the EA-D. A complete suppression of EA-D was observed when 200 and 400 μg/ml of

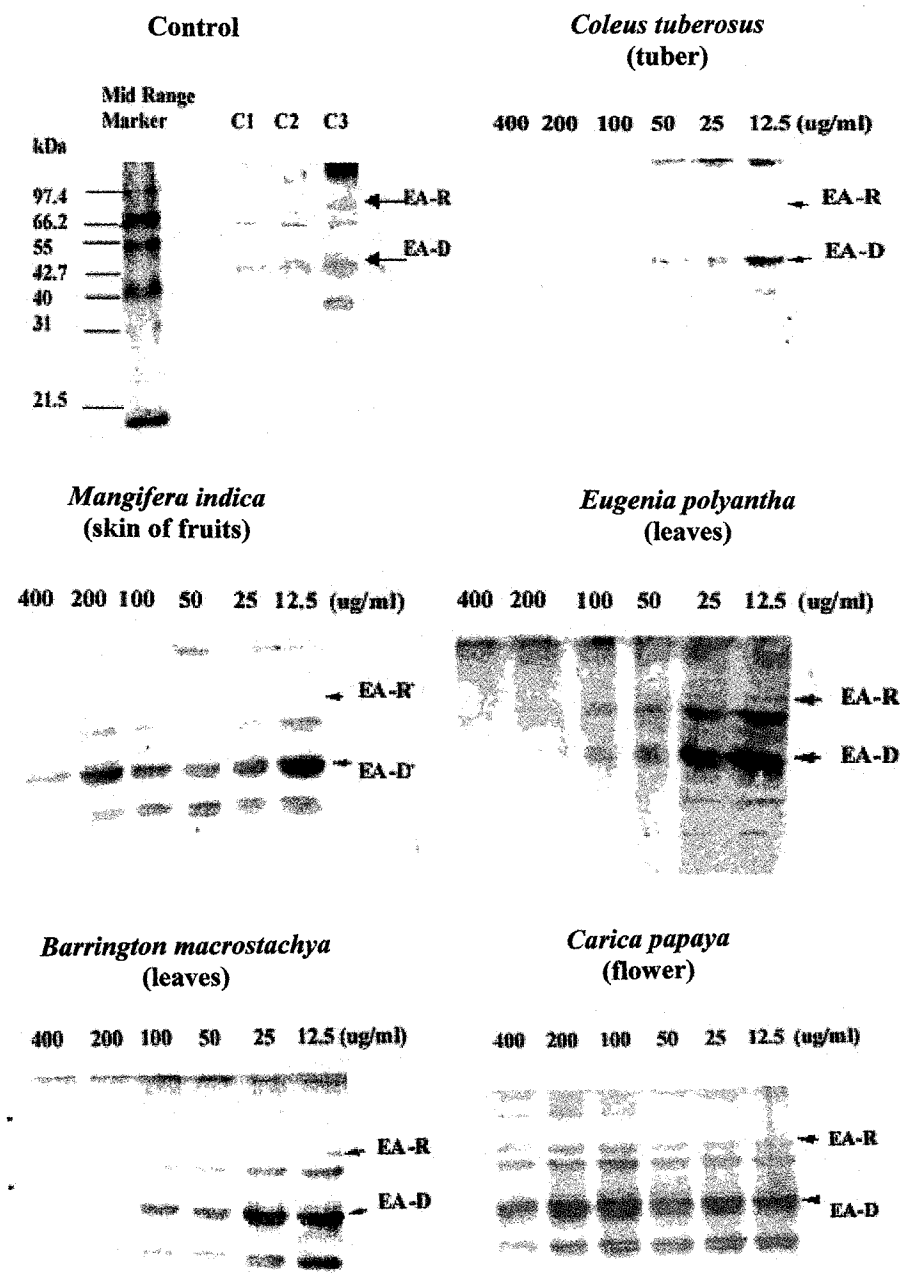

Fig. 1. Effects of edible plant extracts on the synthesis of EBV-EA in Raji cells treated with phorbol 12-myristate 13-acetate (PMA, 0.05 μM) and sodium-*n*-butyrate (3 mM) by immunoblotting analysis. Control: Lane 1 is the prestained SDS-PAGE standard, mid-range marker (Bio-Rad, USA). C1: untreated Raji cells, C2: (lane 3) sodium-*n*-butyrate-treated Raji cells, C3: PMA, sodium-*n*-butyrate and plant extracts treated Raji cells (*Coleus tuberosus, Barrington macrostachya, Carica papaya, Mangifera indica* and *Euginea polyantha*). All the plant extracts were tested at the concentrations ranging from 12.5 to 400 μg/ml .

Barringtonia macrostachya extracts were used, while partial suppression of EA-D was obtained at 50 and 100 μg/ml. The extract was not able to suppress of EA-D at 12.5 and 25 μg/ml. The extract of *Carica papaya* was not able to suppress the EA-D at all. The extract of *Mangifera indica* showed complete suppression of EA-D only at 400 μg/ml, whilst, partial suppression was observed at the concentrations ranging

from 25 to 200 μg/ml. No suppression was observed at 12.5 μg/ml. The extract of *Eugenia polyantha* was able to suppress the EA-D at the concentrations ranging from 50 to 200 μg/ml and partial suppression was observed at 12.5 and 25 μg/ml.

As compared to the immunofluorescence method, immunoblotting method is easier to perform without determining the number of EBV-EA-positive cells. Both of the EBV-early antigens can be detected separately at the specific range of molecular weight, moreover, the specific binding of the antibodies to both polypeptides can be achieved. Hence, the immunoblotting analysis can be a useful confirmation test for anti-tumor promoters detection.

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