

Gene Mutation Effect of Aqueous and Methanol Extracts of Salted Fish from Pulau Pinang, Malaysia towards V79 Lung Fibroblast Cells

(Kesan Mutasi Gen Ekstrak Akues dan Metanol Ikan Masin dari Pulau Pinang, Malaysia terhadap Sel Fibroblas Paru-paru V79)

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ABSTRACT

Salted fish is a locally processed raw food which is used in everyday cooking among Malaysians. Previous studies suggested that salted fish intake was a risk of nasopharyngeal cancer. Hence, this study was carried out to evaluate gene mutation effects through the induction of mutagenic effect of aqueous and methanol extracts of salted fish from Balik Pulau, Pulau Pinang, Malaysia. Balik Pulau was chosen for sampling purpose due to its popularity as a commercial centre for local raw fisheries in Malaysia. Evaluation of mutagenic effect was carried out by hprt Gene Mutation Assay towards V79 lung fibroblast cells. It was found that the aqueous and methanol extracts of salted fish were not cytotoxic towards V79 lung fibroblast cells. It was also found that the extracts of salted fish from Balik Pulau were not mutagenic towards hprt gene of V79 lung fibroblast cells as the mutation frequency of the extracts did not exceed 3 times of the value for negative control mutation frequency. In conclusion, both aqueous and methanol extracts of salted fish from Balik Pulau did not have gene mutation effect towards hprt gene in vitro. However, other toxicological profile could be assessed to determine the mechanism of toxicity of salted fish.

Keywords: Cytotoxic; gene mutation; hprt; Pulau Pinang; salted fish

ABSTRAK

Ikan masin merupakan salah satu makanan tempatan terproses yang kerap digunakan dalam masakan harian penduduk Malaysia. Kajian terdahulu menunjukkan perkaitan antara pengambilan ikan masin dengan risiko menghidap penyakit kanser nasofarinks. Oleh itu, kajian ini telah dijalankan untuk menilai kesan mutasi gen melalui induksi aktiviti mutagenisiti pada ekstrak akues dan metanol ikan masin dari Balik Pulau, Pulau Pinang, Malaysia. Balik Pulau telah dipilih sebagai kawasan persampelan kerana popularitinya sebagai pusat pengeluaran ikan mentah tempatan di Malaysia. Penilaian kesan mutagenik telah dijalankan dengan menggunakan Asai Mutasi Gen hprt menggunakan sel selanjur V79 iaitu sel fibroblas paru-paru. Kajian ini mendapati bahawa ekstrak akues dan metanol ikan masin tidak sitotoksik terhadap sel fibroblas paru-paru V79. Hasil kajian ini juga mendapati bahawa ekstrak ikan masin dari Balik Pulau tidak mutagenik ke arah gen hprt sel fibroblas paru-paru V79 dengan frekuensi mutasi ekstrak tidak melebihi 3 kali nilai frekuensi mutasi berbanding kawalan negatif. Kesimpulannya, kedua-dua ekstrak akues dan metanol ikan masin dari Balik Pulau tidak menyebabkan mutasi pada gen hprt secara in vitro.

Kata kunci: hprt; ikan masin; mutasi gen; Pulau Pinang; sitotoksiti

INTRODUCTION

Cancer is the main cause of death which accounts for 7.9 million or 13% of all deaths worldwide in 2007 (Jemal et al. 2009). Carcinogens have the ability to cause irreversible DNA changes which are expressed as mutation, chromosomal aberration or activation of oncogene (Perera & Weinstein 2000).

Diet plays a major role in cancer aetiology and prevention. Salt intake and pickled food are suggested to be the factors that can contribute to cancer (Ghazali et al. 2005; Sharif et al. 2008). Armstrong et al. (1998) showed that there was an association between salted fish

and nasopharyngeal cancer in Malaysia. Yuan et al. (2000) also suggested that salted fish was also a risk factor for nasopharyngeal cancer in China. The frequency of salted fish intake in Malaysia was mostly high in some parts of the country (Armstrong & Chan 1983).

Studies that were carried out by Wakayabashi et al. (1985) and Fong et al. (1979) found that salted fish has the potential to induce mutagenic effect via Ames Test. This study was carried out to assess the potential of aqueous and methanol extracts of salted fish from Balik Pulau, Pulau Pinang to induce gene mutation by *hprt* Gene Mutation Assay.

MATERIALS AND METHODS

FOOD SAMPLES

Two kg of salted fishes (*Nibea soldado* or 'ikan gelama') were purchased by convenient sampling from Balik Pulau, Pulau Pinang as suggested by the Fisheries Development Authority of Malaysia (LKIM). Balik Pulau was chosen as it was well known centre of local raw fisheries in Malaysia. Samples were then mix, cut into small pieces and blended into powdery form before extracted.

EXTRACTION OF FOOD SAMPLES

For methanol extraction, the ground powder of salted fish (200 g) was soaked successively in 500 mL of methanol (Fisher Scientific, United Kingdom) for two days. The mixture was then filtered and evaporated using rotary evaporator (Buchi Rotavapor R-114, Switzerland) at 50°C under reduced pressure to evaporate the solvent from the extracts. The resulting pellet was then freeze-dried (Heto LyoLab 3000, Denmark) and the powder extract were kept at 4°C in an air-tight jar prior to the bioassays.

For aqueous extraction, the ground powder (200 g) of the salted fish was soaked in 500 mL of distilled water for 2 days and stored at 4°C in the dark to prevent microbial activity. The mixture was then filtered, freeze-dried and the dry extract was kept at 4°C in an air-tight jar prior to the bioassays (Sakanaka et al. 2005).

REAGENT AND CELLS

V79 lung fibroblast cells were obtained from ATCC (Rockville, MD, USA) and cultured in DMEM media (ATCC Catalogue Details No CCL 93, 2009). Cells were grown as monolayers in a T-25 cm² culture flask. The medium was supplemented with 2.0 g/L sodium bicarbonate, antibiotics (100 U of penicillin/mL, 100 mg of streptomycin/mL) and 10% foetal bovine serum. The cell culture medium and their supplements were purchased from Thermo-Fisher Biochemical Co. Ltd (Beijing, China). Cell cultures were maintained in a humidified atmosphere of 5% CO₂ at 37°C and were harvested when they reached 80% confluency, i.e. in their exponential growth phase. For gene mutation activity, aqueous and methanol extracts of salted fish were dissolved in 5% dimethyl sulphoxide (DMSO) (Sigma-Aldrich, Germany) and media DMEM (Thermo-Fisher Biochemical Co. Ltd, Beijing, China) to a final concentration of 10 mg/mL and 50 mg/mL. These solutions were then filtered using sterile 0.22 mm syringe filter. Hydrogen peroxide at 100 mM was used as the positive control for MTT assay and 1 mg/mL of ethyl methanesulfonate (EMS) (Sigma-Aldrich Company, USA) was used for *hprt* Gene Mutation assay.

MTT CYTOTOXICITY ASSAY

The viability of V79 lung fibroblast cells was used to determine the cytotoxic effect of the extracts of salted

fishes as described previously (Mosmann 1983). The cell monolayers in exponential growth were harvested and 5×10^4 cells/mL in 200 mL were placed into each well of the 96-well plates (Nunclon™, VWR International Inc., MD). The plates were incubated for 24 h at 37°C in 5% CO₂. The medium was discarded and 200 mL of the test extracts for each extracted sample in different concentrations were loaded into the 96-well plates. After 72 h incubation, 20 mL of the MTT solution was added into each well and reincubated for 4 h at 37°C before the medium was discarded and 200 mL of DMSO was added to dissolve the formazan crystals. The plate was agitated for 30 min to dissolve the crystal formed and the absorbance was measured at 570 nm with a microplate reader. Assays with each concentration were repeated three times.

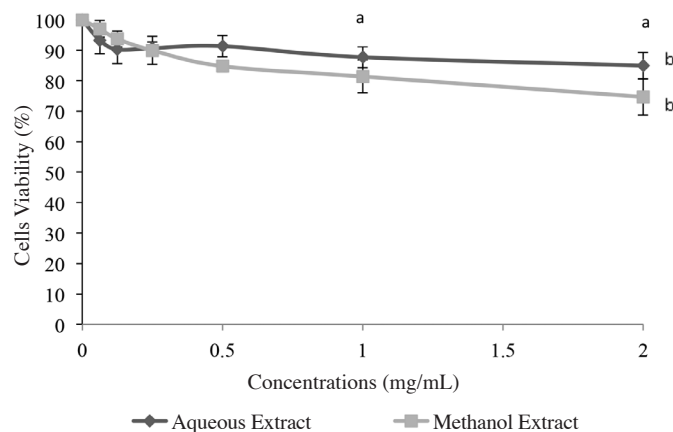
The MTT (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyl tetrazolium bromide) (Sigma-Aldrich Company, USA) was used to dissolved in phosphate buffered saline (PBS) solution at concentration of 5 mg/mL and filtered through a 0.22 mm filter to sterilize and remove the insoluble residues.

Hprt GENE MUTATION ASSAY

The *hprt* gene mutation test was performed as previously described (OECD 1998). For the induction of *hprt* mutations, 5×10^5 V79 cells were seeded and 3 h later exposed to the test substance for 4 h. After treatment, the cultures were split into parallel subcultures to ensure the growth of independent mutants. Survival (relative cell growth) was determined via MTT assay as mentioned above (Mosmann 1983) and then read 3 days later. The treated cultures were transferred (5×10^4 cells/mL) as needed during the expression period of 7 days. Thereafter, 5×10^4 cells/mL were replated into 10 petri-dishes (100 mM) with selective medium (6-thioguanine; 5.6 mg/mL). At the time of replating into selective medium, the plating efficiency was also determined in non-selective medium (three replica well plates (6-well plates); 100 cells each). After 8 days, the 6-thioguanine-resistant colonies were fixed with methanol (Fisher Scientific, United Kingdom), Giemsa-stained (LabStain LabChem Sdn. Bhd, Malaysia). The viability of mutant colonies that grow in the presence of 6-thioguanine are stained in blue after treated with Giemsa. Each colony that has more than 50 cells are counted and mutant frequencies (MF) were calculated by correcting for non-selected cell survival (plating efficiency). Three independent experiments were performed for each compound and concentration tested, and mean MF (numbers of mutants/ 5×10^5 cells) were calculated. The test compound was classified as a mutagen when it was able to enhance in a concentration-dependended manner colonies and exceed three times or more value of relative frequency mutation than the negative control plate (Bradley et al. 1981; Kreja & Seidel 2002).

RESULTS AND DISCUSSION

Based on the graph of the cell viability (Figure 1), both extracts were not considered to be cytotoxic towards V79



(a = significant compared with negative control at $p < 0.05$; b = significant compared with type of extract at $p < 0.05$)

FIGURE 1. Percentage of cell viability (%) following incubation with the aqueous and methanol extracts of salted fish at different concentrations. Each point represents mean \pm SEM of three different independent experiments, $n=3$

cells with absence of IC_{50} . The results were similar to the studies that has been carried out by Ahmad Rohi et al. (Ghazali et al. 2005) and Sharif et al. (2008).

We choose 5 mg/mL of aqueous and methanol extracts of salted fish as maximum concentration for *hprt* Gene Mutation Assay (OECD 1998). Prior to gene mutation assay, the relative cell growth for cell survival after treatment was carried out (Figure 2). The results stated that both extracts showed relative cell growth reduction from 5 to 2.5 mg/mL of extracts with the absence of IC_{50} compared with the negative control. The results for relative cell growth are crucial as they may affect the plating efficiency test. Relative plating efficiency is to test for cell viability that is able to form a single colony with a minimum of 50 cells per colony. In this research, it is performed to determine the cell death due to mutation after treated with salted fish extracts thus may affect the plating efficiency. The results are compared relatively to the colonies of negative treated

group. The plating efficiency for treated group should be higher than 80% of the plating efficiency of negatively treated group. Plating efficiency was associated with the presence of cytotoxic agents which with its presence, the cytotoxic agents can prevent colony formation during plating efficiency test (Freshney 1994).

Determination of cell death due to gene mutation after treatment with the extracts was carried out by plating efficiency test. Based on the relative plating efficiency graph (Figure 3), both aqueous and methanol extracts of salted fish showed more than 80% of plating efficiency compared with the negative control used. Hence, V79 cells were able to form colonies efficiently during *hprt* Gene Mutation Assay and there was no significant cell death occurred after treatment with control group and test extracts.

The gene mutation effect of aqueous and methanol extracts of salted fish via the ability of V79 cells to form mutant colonies towards 6-thioguanine (6-TG) were

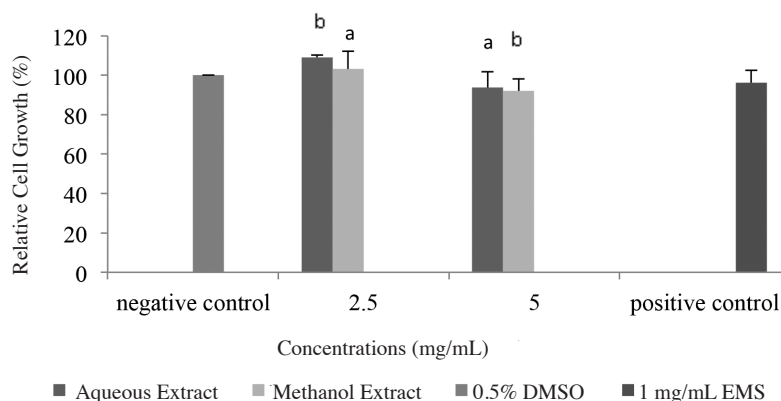


FIGURE 2. Percentage of cell survival via relative cell growth (%) following incubation with the aqueous and methanol extracts of salted fish at 2.5 mg/mL and 5 mg/mL. (mean \pm SEM, $n=3$) (a = significant compared with negative control at $p < 0.05$; b = significant compared with type of extract at $p < 0.05$)

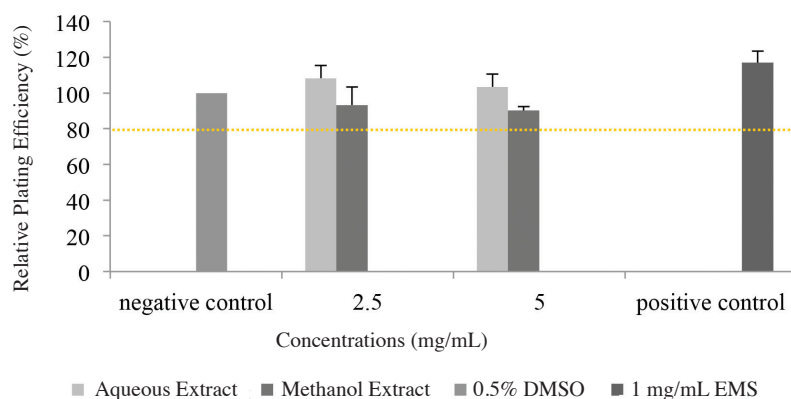


FIGURE 3. Percentage of relative plating efficiency (%) following incubation with the aqueous and methanol extracts of salted fish at 2.5 mg/mL and 5 mg/mL (mean \pm SEM, $n=3$)

observed. Figure 4 shows that there was an increase in the number of mutant colonies towards 6-TG for both extracts used (Figure 4). There was also a significant increase in the number of mutant colonies for 2.5 mg/mL of aqueous extract and 5 mg/mL of both aqueous and methanol extracts compared with the negative control. The increase in the number of mutant colonies toward 6-TG with the increased of the extract concentration might be due to the presence of nitrosamine compounds or other carcinogens that can contribute to the occurrence of nasopharyngeal cancer (Armstrong & Chan 1983). There was also evidence that the foods which has been processed would contain nitrite that can cause direct mutagenic effect (Sharif et al. 2008; Wakabayashi et al. 1985).

Nitrosamine which may contain in salted fish, need activation of the metabolic enzyme in the liver for the compound to act as mutagenic agent and react towards DNA (Cooper & Porter 2000). Nevertheless, there are also nitrosamine compounds which can cause mutation without metabolic activation such as unsaturated

nitrosamines (Lijinsky 1992). Hence, the nitrosamine which may contain in salted fish from Balik Pulau may act towards DNA through alkylating activity such as addition of ethyl group on purine or pyrimide bases.

The frequency of *hprt* gene mutation is then evaluated by plotting the graph of relative frequency mutation (Figure 5) and it shows that both aqueous and methanol extracts at concentration 2.5 and 5 mg/mL are not classified as mutagen because they cannot enhance a concentration-depended manner of spontaneous *hprt* frequency (negative control) by a factor of three or more (Bradley et al. 1981; Kreja & Seidel 2002).

CONCLUSION

Aqueous and methanol extracts of salted fish from Balik Pulau, Pulau Pinang were not classified as the compounds that can induce mutation on *hprt* gene of V79 fibroblast cells. Other toxicological profile could be assessed to determine the mechanism of toxicity induced by salted fish.

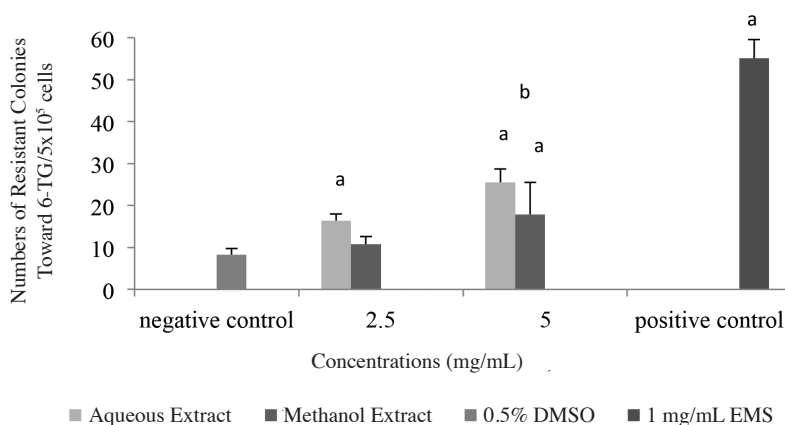
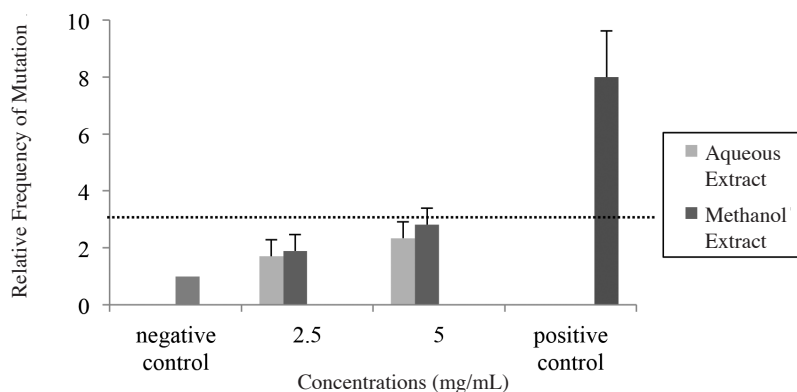


FIGURE 4. Number of resistant V79 colonies toward 6-TG after being treated with the aqueous and methanol extracts of salted fish. (mean \pm SEM, $n=3$) (a = significant compared with negative control at $p<0.05$; b = significant compared with type of extract at $p<0.05$)



Three times the value of relative frequency mutation of negative control

(a = significant compared to negative control at $p < 0.05$)

FIGURE 5. Relative frequency of mutation of V79 colonies toward 6-TG after being treated with the aqueous and methanol extracts of salted fish. (mean+SEM, $n=3$)

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