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Evaluation of Mutagenic Profile of Shrimp Paste Extracts by Using Ames Test

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Abstract: Shrimp paste (belacan) has been used as a condiment in many Malaysian cooking. This study aimed to determine the mutagenicity of aqueous extract of shrimp paste from three different places in Pulau Pinang, Malaysia (Balik Pulau, Juru and Pulau Aman) by using Ames test. The test was performed using *Salmonella typhimurium* strain, TA98 and TA100, in presence and absence of S9 metabolic activation system. Five concentrations of the samples tested were 50, 25, 12.5, 6.25 and 3.125 mg/ml. Macronutrients and mineral content of shrimp paste tested were complied with the Malaysian food act 1983 and Recommended Nutrient Intake (RNI) 2005. Ames test result of shrimp paste from all three places showed number of revertant colonies did not exceed the value of double-fold of negative control either in presence and absence of S9. No significant difference ($p > 0.05$) was found for each concentration tested on the number of revertant colonies between TA 98 and TA 100.

Key words: Shrimp paste, mineral content, mutagenicity

INTRODUCTION

Intake of fatty foods, alcohol and some methods of preparing such as smoked, salting and pickled food and cooking meat at high temperatures are a direct correlation to the risk of cancer (Rohi *et al.*, 2005; WCRF, 2007). To date, many food components were reported to be potentially genotoxic (Manson and Benford, 1999). Genotoxicity refers to an adverse effect on the genetic material (DNA) of living cells either through micronuclei, mutations, or chromosomal aberration (Ghazali *et al.*, 2005).

Belacan is the term used by Malaysian which refers to shrimp paste, widely used as condiment in many local dish. Previous study shows that shrimp paste is listed as one of the eight foods that are associated with cancer as well as salted fish, anchovies, dried fish, pickles and pickled vegetables (Arshad *et al.*, 2005). Factor that influences the potential of shrimp paste to cause cancer is the use of salt, specifically the production of nitrite or nitrate. Salt leads to chromosomal damage through indirect genotoxic effect (Sharif *et al.*, 2008).

Salt is often used as a preservative for dried foods such as salted fish, dried shrimp and shrimp paste. Preservation process using salt and drying can also lead to the formation of N-nitroso compounds. Nitrite in salts will form a reaction with amides and amines in meats or proteins which produces N-nitroso compounds. N-nitroso compounds are carcinogens and can provide mutagenic effects in humans (Cohen and Roe, 1997; Phukan *et al.*, 2006). Previous study by

Tsugane (2005) shows that consuming vegetable preserved with salt has a positive correlation with gastric cancer mortality. Salt intake is also associated with *H. pylori* infection and this encourages the development of gastric cancer. Higher intake of salt can also cause stomach cancer through the gastric mucosa lining damage (Wang *et al.*, 2009).

Salted foods have strong association with cancer that related to digestive tract, commonly gastric cancer (Lin *et al.*, 2014, 2015), colorectal cancer (Vargas and Thompson, 2012), nasopharyngeal carcinoma (Lau *et al.*, 2013) and esophageal squamous cell carcinoma (Lin *et al.*, 2014, 2015). Several epidemiology studies conducted on cancer patients on their consumption of salted and fermented food (Armstrong *et al.*, 1983; Armstrong *et al.*, 1998; Tsugane *et al.*, 2004; Kurosawa *et al.*, 2006; Wang *et al.*, 2009; Takachi *et al.*, 2010; Murata *et al.*, 2010; Peleteiro *et al.*, 2011; Lau *et al.*, 2013; Lin *et al.*, 2014, 2015). These study involves Asian countries including Malaysia, observed that the consumption of salt in cancer patients were higher than control group which the dietary pattern and cooking style is almost similar (Ruddle and Ishige, 2009).

Mutations occurs as gene mutations where only a single base is modified or one relatively few bases are inserted or deleted (Ames *et al.*, 1973). Hence, we decided to use bacteria strain *Salmonella typhimurium* TA 98 and TA 100 to determine the mutagenicity activities on shrimp paste extracts in Balik Pulau, Juru and Pulau Aman reflecting both frame shift and base pair mutation. This

study aimed to investigate the mutagenicity profile of shrimp paste from Juru, Balik Pulau and Pulau Aman with and without the presence of metabolic activator S9. The percentage of macronutrients was also determined via proximate analysis as well as mineral content (iron, calcium, sodium) in the samples.

MATERIALS AND METHODS

Food samples: Five kg of shrimp paste were purchased from three different places which are Balik Pulau, Juru and Pulau Aman, Pulau Pinang as suggested by the Fisheries Development Authority of Malaysia. These three places are known as the main producer and distributor of shrimp paste in Malaysia. Samples were then mixed, cut into small pieces and blended into powdery form before extracted.

Extraction of shrimp paste samples: All the shrimp paste samples were collected from three different locations in Pulau Pinang (Balik Pulau, Juru and Pulau Aman). The shrimp paste samples were blended and soaked in distilled water overnight at 4°C. The mixture was filtered and the extract was freeze dried using freeze dryer (Heto lyolab 3000, Denmark). Then, the samples were kept in a refrigerator at a temperature of 4°C prior to the test (modified from Sakanaka *et al.*, 2005).

Treatment: Five test concentrations were used in this experiment which begins at 3.125, 6.25, 12.5, 25 and 50 mg/ml. These test concentrations were prepared from main stock with concentration of 500 mg/ml by using serial dilution in distilled water. The test concentration was chosen according to previous study by Abdullah (2012) and guideline provided by OECD 471 for testing of chemicals via bacterial reverse mutation test.

Bacterial strain: Bacterial strains used were mutant *Salmonella typhimurium* strain TA98 and TA100 with histidine-dependent bacteria as previously described by Maron and Ames (1983). The mutant genotype criteria were routinely checked for their histidine-dependent, biotin-dependent, *uvrB* mutation, *rfa* mutation and presence of plasmid pkm101. Strain TA98 represents frameshift mutation while TA100 represents base-pair substitution mutation.

Proximate food analysis: Food proximate analysis was conducted to evaluate the percentage content of macronutrients such as moisture content, ash, crude protein, fat and carbohydrates (Cunniff, 1995). Moisture content was determined by drying 5 g samples in a vacuum oven at 105°C for 24 h to a constant weight. Ash content was determined by ignition at 550°C in an electric furnace (Carbolyte, United Kingdom). The crude protein content was estimated by micro-kjeldhal techniques using Tecator System (Tecator, United

Kingdom). Fat content was determined using Soxtec system (Soxtec, United Kingdom) and total carbohydrate was calculated using difference of the value of each nutrient.

Determination of mineral contents: Mineral analysis was conducted which involved three types of minerals; iron (Fe), calcium (Ca) and sodium (Na). This analysis was carried out using Atomic Absorption Spectrophotometer (AAS) instrument using samples that have undergone a process of wet dry ashing.

Ames test: Ames test is a screening tool specifically to determine the mutagenicity as previously described by Maron and Ames (1983). A mixture of 0.5 ml of phosphate buffer or S9 mix, 0.1 ml of sample and 0.1 ml of strain bacteria were added in the culture tube. The mixtures were incubated in 37°C waterbath shaker for 20 min at 100 rpm. Then, 2 ml of melted top agar supplemented with histidine/biotin were added to the culture tube containing the treatment mixture. The mixture were gently mixed and poured onto Glucose Minimal (GM) agar. The plates were incubated at 37°C for 48 h and colonies revertant were counted, following incubation, the data were collected in mean (Standard Error Mean, SEM) (n = 9).

Statistical analysis: The test was performed three times which each time the plates for each concentration are in triplicates (n = 9). One way ANOVA and Kruskal Wallis test were used to measure significant differences between the means of five different concentrations for each place. Subsequently, two-way ANOVA test were applied to analyze significant difference among three difference places. The level of confidence was set at 95% level and level of significant applied was $p < 0.05$.

RESULTS

Proximate analysis: Proximate analysis was conducted to determine the percentage content of shrimp paste extracts at three different locations in Penang, Malaysia namely are Balik Pulau, Juru and Pulau Aman. The components that were analyzed include percentage of carbohydrate, crude protein, fat, moisture content and ash. Table 1 shows the percentage of proximate analysis on shrimp paste extracts. Moisture content for shrimp paste from Pulau Aman was found to be higher than shrimp paste from Balik Pulau and Juru (40.27 ± 0.15 , 37.71 ± 0.18 and $31.93 \pm 0.06\%$). The highest level of total ash was reported in shrimp paste from Juru with $21.69 \pm 0.70\%$ compared to other two places. The highest value for crude protein was presented by the shrimp paste from Balik Pulau with $34.99 \pm 0.15\%$. Minimal percentage of carbohydrate and fat was found in all samples, indicated that all the food samples were low in fat and carbohydrates.

Table 1: Mean percentage of proximate analysis for shrimp paste sample

Shrimp paste sample	Water (% per 100 g)	Ash (% per 100 g)	Crude fat (% per 100 g)	Crude protein (% per 100 g)	Carbohydrate (% per 100 g)
Balik Pulau	37.71±0.18	19.23±0.55	0.36±0.10	34.99±0.15	7.71±0.56
Juru	31.93±0.06	21.69±0.70	0.70±0.04	33.80±0.27	11.87±0.75
Pulau Aman	40.27±0.15	16.44±0.50	0.47±0.06	32.60±0.05	10.22±0.51

Table 2: Mean percentage of mineral content from shrimp paste extracts in three different locations in Pulau Pinang

Shrimp paste sample	Iron (mg/100 g)	Calcium (mg/100 g)	Sodium (mg/100 g)
Balik Pulau	5.4±0.17	156±17.21	1345±251
Juru	5.0±0.11	139±17.56	1005±360
Pulau Aman	5.8±1.47	140±13.32	951±162

Mineral content: Percentage of mineral content from shrimp paste extracts in three different locations in Pulau Pinang was presented in Table 2. The highest value of iron content was shown in shrimp paste from Pulau Aman with 5.8±1.47 mg/100 g sample. On the other hand, the highest value of calcium and sodium content was found in shrimp paste from Balik Pulau with 156±17.21 and 1345±251 mg/100 g sample, respectively.

Ames test: Figure 1(a,b) represent mutagenic activities of shrimp paste from Pulau Pinang (strain TA98). Test with strain TA98 showed no mutagenic activities 2 observed as the revertant colonies did not exceed the double-fold of negative control for both in the absence and presence of S9. The highest revertant colonies produced was by shrimp paste from Balik Pulau at the concentration of 25 mg/ml (15.33±3.06) compared to Pulau Aman dan Juru. Two-way ANOVA showed there were significant difference between revertant colonies from Balik Pulau and Pulau Aman at all concentration (p<0.05). Pre-treatment with S9 into bacterial culture showed that the highest revertant colonies were observed from Juru at concentration of 12.5 mg/ml (18.33±1.88). No significant difference among three places was found.

Figure 2(a,b) represent mutagenic activities of shrimp paste from Pulau Pinang tested on strain TA100. The result was found to be below the double-fold of negative control. These result indicated that there were no mutagenic activities found for samples from Balik Pulau, Juru and Pulau Aman tested with and without presence of S9. At concentration of 50 mg/ml, shrimp paste from Balik Pulau showed high revertant colonies with 81.33±16.42 and 54.33±4.48, without and with the presence of S9, respectively. Significant difference of revertant colonies between Balik Pulau and Pulau Aman at concentration of 3.125, 6.25 and 12.5 mg/ml (without S9) (p<0.05) was observed, while no significant difference reported for the culture with S9.

DISCUSSION

This study is being conducted using samples from Pulau Pinang due to its large contribution in the market of commercial shrimp paste. Thus, this study will able to

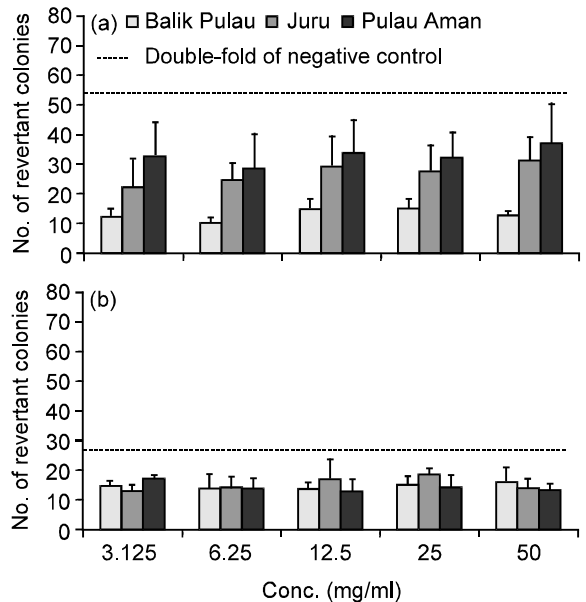


Fig. 1: Number of revertant colonies on the strain TA 98 towards Balik Pulau, Juru and Pulau Aman shrimp paste extracts. (a) Absence of S9, (b) Presence of S9

disseminate the effect of genotoxicity of shrimp paste on human health to the public, especially Malaysian. This will help to reduce the cancer risk, indirectly the risk of death caused by cancer in Malaysia.

Present study shows that the macronutrient content of shrimp paste from three locations in Pulau Pinang was complied with the guideline of the Malaysian Food Act (1983). The regulation suggested that the shrimp paste produced should not contain less than 15% of salt and 25% of protein. Meanwhile, the limit of water and ash must not exceed 40 and 35 %, respectively. Protein is the major nutrient which shows that shrimp paste is a good source of protein. Interestingly, macronutrient content in the shrimp paste from Malaysia is slightly lower than production in Thailand (Pongsetkul *et al.*, 2014) may be due to the differences in method of preparation and the amount of raw materials used (McNaught and Wilkinson, 1997).

Ash content in shrimp paste indicating the presence of inorganic materials and minerals such as sodium, calcium, potassium and iron (McNaught and Wilkinson, 1997; Pongsetkul *et al.*, 2014). These macro minerals are essential in body physiology system (Fenech, 2003). Several studies reported that macrominerals and

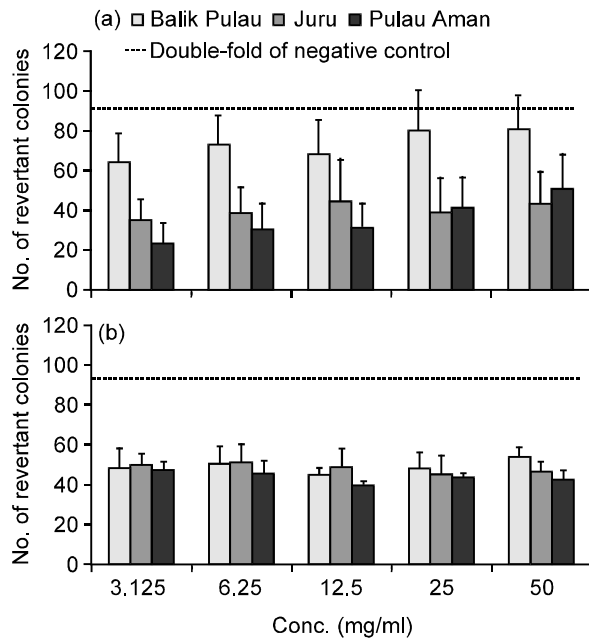


Fig. 2: Number of revertant colonies on the strain TA 100 towards Balik Pulau, Juru and Pulau Aman shrimp paste extracts. (a) Absence of S9, (b) Presence of S9

microminerals in food are associated with the cancer risk (Vargas and Thompson, 2012; Cho *et al.*, 2013). Sodium was found to be most abundant in shrimp paste in Pulau Pinang. This macromineral elements contributing 40% in the production of salt (NaCl). The experimental study have shown that mice treated with high salt concentration exert a promoter effect on cancer risk by reduce the protective mucous barrier and cause tissue damage (Tatematsu *et al.*, 1975). Human study on higher consumption of salted fish roe among Japanese population demonstrated a higher risk of colorectal and gastric cancer (Takachi *et al.*, 2010). Mutagenicity is the ability of a substance to cause mutations in genes and to induce the occurrence of cancer (Vanita *et al.*, 2011). DNA damage that cannot be repaired will form abnormal cells and divided in an uncontrolled manner to form a malignant tissue or tumour (MAKNA, 2008). A substance is considered mutagenic when it produces twice the number of colonies over the negative control (Mortelmans and Zeiger, 2000). The results of the Ames test conducted, showed that there were no mutagenic activities of the shrimp paste extract from three different locations in Penang for both bacterial strains tested. The results of the tests both in the absence and presence of S9 metabolic activation system gave negative results. Our test outcomes are parallel with the previous study conducted by MAKNA in 2007 which no mutagenic activities were detected on shrimp paste aqueous

extract from Pulau Pinang in which involve two locations, Balik Pulau and Permatang Pauh (MAKNA, 2008). The absence of mutagenic effects in this study still does not conclude that this product is safe to be used since other shrimp paste from other places assay using Ames test result in positive result. In agreement with our study, previous study reported that shrimp paste in Melaka demonstrated no genotoxic effect and clastogenic effect (Abdullah, 2012).

Previously, Ghazali *et al.* (2012) conducted study on shrimp paste from Kelemak and Batang Tiga, Melaka and showed mutagenic activities via Umu test at concentration of 5mg/ml and 0.625 mg/ml. According to the study, factor contributed to the mutagenicity of the sample was high content of salt that exceeding 15% limit allowed by Malaysian Food Act (1983). Furthermore, Sumino *et al.* (2003) reported that shrimp paste processed in Malaysia as higher than the limit allowed, approximately 24.1% of salt. Salt is used to preserve the shrimp, thus the shelf life of the paste produced can be prolonged. Processed foods that use salt lead to the formation of N-nitroso compound which indirectly induces chromosome breakage (Cohen and Roe, 1997), cell death and DNA damage (Ghazali *et al.*, 2005). All these end-point will lead to risk of cancer.

World Health Organization (WHO) and Food and Agriculture Organization of the United States (FAO) concluded that salt-preserve food and salt probably increase the risk of stomach cancer (WHO, 2003). In Malaysia, shrimp paste was listed as the food causing cancer out of seven other salted-processed foods (Arshad *et al.*, 2005). To date, a study of salted fish from Pulau Pinang shows no mutagenic effects toward hprt gene forward mutation assay (Ghazali *et al.*, 2013). However, a case control study shows dietary habit of consuming salted food since childhood contributed to the increase the risk of nasopharyngeal cancer among Chinese population in Selangor, Malaysia (Armstrong *et al.*, 1983; Armstrong *et al.*, 1998). This epidemiology study supported by a current study which shows that associations between salted food intakes with the occurrence of oral cancer among Malaysian who were diagnose with oral squamous cell carcinoma (Helen-Ng *et al.*, 2012).

Effects of salt on gastric cancer are not due to total of sodium chloride (NaCl), but depends on the regular consumption of highly salt-concentrated preserved food itself (Takachi *et al.*, 2010). This is because the salted food contain chemical carcinogen such as N-nitroso compound, which is the byproduct of nitrite or nitrate reaction, occurring during curing process or in the body of consumers (Dich *et al.*, 1996; Knekt *et al.*, 1999). Besides, the presence of heterocyclic amines resulted from cooking method of fish and meat, which commonly used for dried and salted fish in Asian countries (Skog *et al.*, 1998).

Some of the limitations identified in this study is from the food safety aspect of shrimp paste. Further study on genotoxicity either *in vitro* or *in vivo* need to be implemented as well as epidemiological study. These steps will initiate the changes and updates in Malaysian food guidelines or act. Moreover, the literature review on shrimp paste research in Malaysia is not broad as research done on salted-fish. However, this study is important to provide general knowledge for the public on the safety of having shrimp paste in everyday cooking. In addition, Ames test is one of the well validated methods by Organization of Economic Co-operation and Development (OECD) for food genotoxicity testing.

Conclusion: This study showed that all samples do not possess any mutagenic activity as measured via Ames test using bacteria *Salmonella typhimurium* strain TA 98 and TA100 with and without metabolic activation S9. This indicates that the aqueous extracts of shrimp paste from Balik Pulau, Juru and Pulau Aman were not mutagenic, however further studies for other genotoxicity events are warranted.

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