Evaluation of the Biochemical Profile and Biological Activity of Budu (A Local Fermented Fish Product) Extracts on HepG2 Hepatoblastoma Cells


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Abstract: Budu is a fish product which is used as one of the condiments in daily Malaysian dish. As part of a safety evaluation of ingredients for use in everyday dishes, there is a need to determine its nutrient composition and toxicological profile. This study documented its biochemical profile in terms of macronutrients, food additives and heavy metals. The biological activity of budu was investigated in terms of its cytotoxic effects and antioxidant capacity. The macronutrients of budu were determined by proximate analysis. Both budu samples from Bachok and Tumpat, Kelantan showed high moisture contents. Detected food additives were colourants (Sunset Yellow FCF) and preservatives (benzoic acid). Heavy metals (mercury and lead) were not detected in both samples. All parameters in the biochemical profile conformed to the regulations of the Malaysian Food Act 1983. When investigated with the MTT (Microculture Tetrazolium Test) Assay, aqueous extracts of both samples did not exhibit significant cytotoxic effects on HepG2 cells at low (60 μg/ml), moderate (500 μg/ml) or high (2000 μg/ml) concentrations. Weak antioxidant capacities were detected in the aqueous extract of both budu samples by the FRAP (Ferric Reducing Antioxidant Power) Assay. However, this capacity was not significant when the aqueous extract was tested in the presence of HepG2 cells. In conclusion, budu was considered not cytotoxic to HepG2 cells and had weak antioxidant capacities.

Key words: Budu, proximate analysis, MTT assay, FRAP assay, HepG2 cells.

INTRODUCTION

Nutrient composition and toxicological profile are important parameters in food science research. Composition of nutrient determines the nutritional value of the food while analysis of chemical toxicants and contaminants in foods will give ideas on safety and any toxic effects that could be exerted from the food products (Helferich and Winter, 2000).

In Malaysia, budu is a fish sauce that is a product of liquefaction of anchovies in salt. It is used as condiment in many local dishes. Documentation of macronutrient in budu is important because it is regularly consumed by population. From the Malaysian Foods Composition Database, budu contains high concentration of carbohydrate and minerals (Tee et al., 1997). However, epidemiology study in China showed a correlation between fish sauce and the incidence of gastric cancer (Deng, 2000; Cai et al., 2000; Ye et al., 1998). This evidence was supported by several reports on mutagenicity characteristic of fish sauce (Deng et al., 1991; Ye et al., 1998; Zhang et al., 1991).

Hence, this study was conducted to evaluate the composition of macronutrients and level of food additives and heavy metals in budu samples. The biological activity of budu was investigated in terms of its cytotoxic effects and antioxidant capacity.

MATERIALS AND METHODS

Food Sample:
Budu was purchased from Kelantan (Tumpat and Bachok) which is its main production and distribution centre for Malaysia. Budu was sampled for food analysis and extracted for its biological activity.

Extraction Of Budu:
Liquid form of budu was homogenized and suspended with distilled water at ratio 1:2 for 48 hour at 4°C. The mixture was then filtered and freeze-dried. The dry extract was kept at 4°C in an air-tight jar prior to the bioassays.

Cells and Reagents:
HepG2 cells were obtained from ATCC (Rockville, MD, USA). Cells were grown as monolayer 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 μg of streptomycin/ml) and 10% foetal bovine serum. The cell culture medium and their
supplements were purchased from Life Technologies, Gibco BRL Products (Rockville, MD). The 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.

**Proximate Analysis:**
Moisture, total ash, crude protein, fat and total carbohydrate were determined using standard method (AOAC, 1995). Moisture was determined by drying 100 g samples in a vacuum oven at 100 °C for 24 h to a constant weight. Crude protein was determined using Tecator Digestion System (Foss, USA). Ash was determined by ignition at 550 °C in an electric furnace (Carbolyte, United Kingdom). Fat was determined using Soxtec system HT6 1043 (Foss, USA) and total carbohydrate was calculated using difference of the value of each nutrients.

**Determination Of Heavy Metals, Dye and Food Additives Level:**
All the analysis was conducted at the National Health Laboratory in Sungai Buloh, Selangor. Determination of mercury was conducted by flow injection analysis system atomic absorption spectrophotometer (FIAS-AAS). Lead was determined using graphite furnace atomic absorption spectrophotometer (GF-AAS). Food colouring was determined using paper chromatography. Benzoic acid and sorbic acid in foods were determined using liquid chromatography (internal standard).

**Cytotoxic Activity:**
The viability of the HepG2 cells was determined using the MTT cytotoxicity assay as described previously (Mosmann, 1983). The cell monolayer in exponential growth was harvested and 5 x 10⁴ cells in 100 µl were placed into each well of the 96-well plates. The plates were incubated for 24 h at 37 °C in 5% CO₂. The medium was discarded and 200 µl of the test extracts in different concentrations were loaded into the 96-well plates. After 72 h incubation, 20 µl of the MTT solution was added to each well and reincubated for 4 h at 37 °C. Then, 150 µl of medium was discarded and 150 µl of DMSO was added to dissolve the formazan crystals. The plate was shaken for 15 min to dissolve the crystals formed and the absorbance was measured at 570 nm with a microplate reader. Assays for each concentration were repeated three times.

**Antioxidant Capacity:**
The FRAP (Ferric Reducing Antioxidant Power) assay was employed to determine the antioxidant activities in cells using 96 well plate with slight modification from Benzie and Strain (1996). The cells were plated as in MTT assay in 96 wells plate. The volume of the sample used for treatment was 50 µl/well in a series of concentration. After 2 hours incubation, the plate was shaken for 30 seconds. Then 175 µl of freshly prepared FRAP reagent and warmed at 37 °C was added to each well. The absorbance at 590 nm was monitored by ELISA plate reader. Aqueous solutions of known Fe²⁺ concentration in the range of 100-1000 µM (FeSO₄·7H₂O) were used as a standard. FRAP Assay was also done on sample (without cell) where 50 µl/well of these solutions were added with the reagent immediately after placed in 96 wells plate and the absorbance recorded.

**Results:**
Proximate analysis was conducted to evaluate the macronutrients of food analysis. Figure 1 shows the moisture content of budu from Tumpat (58.13 ± 1.06%) and Bachok (62.93 ± 0.50%) was differed. The highest level of total ash was found in budu from Bachok (21.81 ± 0.14%) compared to budu from Tumpat (18.95 ± 3.97%). The highest value for crude protein, fat and carbohydrate was presented by budu from Tumpat with 10.23 ± 0.29% crude protein, 0.63 ± 0.01% fat and 10.3% carbohydrate whereas budu from Bachok contained the lowest percentage of crude protein, fat and carbohydrate with 7.80 ± 0.18% crude protein, 0.56 ± 0.01% fat and 6.90% carbohydrate respectively.

Food additives analysis showed that sorbic acid was absence of in both samples but benzoic acid was found in budu from Tumpat (68 mg/kg) and budu from Bachok (66 mg/kg). Permitted colour was also found in both samples which was Sunset Yellow FCF. Heavy metals such as mercury (Hg) and lead (Pb) were present in each sample but lower than 0.07 mg/kg for mercury and lower than 0.1 mg/kg for lead (Table 1).

The cytotoxic effect of budu on the viability of HepG2 cells was determined using the MTT assay. Following 72 hours of treatment, aqueous extracts of both samples did not exhibit significant cytotoxic effects on HepG2 cells at low (60 µg/ml), moderate (500 µg/ml) and high (2000 µg/ml) concentrations (Fig. 2, 3 and 4).

Antioxidant activity was determined using FRAP assay. FRAP value of L- ascorbic acid (LAA) was determined as the positive control. The results showed that FRAP value of LAA without cells were significantly increased (100 µM to 1000 µM) in concentration dependent manner as compared with negative control (Fig. 5). FRAP value of LAA with cells also significantly increased (500 µM to 1000 µM) in concentration dependent manner as compared with the negative control (Fig. 5). FRAP value of aqueous extract of budu from Tumpat was significantly increased from 1.875 µg/ml to 2000 µg/ml as compared with the negative control except at
concentration 3.75 µg/ml, 15 µg/ml and 60 µg/ml (Fig. 6). Whereas, FRAP value of aqueous extract of budu from Bachok was significantly increased from 1.875 µg/ml to 2000 µg/ml as compared with the negative control except at concentration 15 µg/ml and 60 µg/ml (Fig. 6). For aqueous extract with cells, FRAP values of both samples were not significantly increased as compared to negative control (Fig.7).

**Fig. 1:** Percentage of main nutrient components in budu from Tumpat and budu from Bachok (mean ± SD), n = .

**Table 1:** Detection of dye, food additives and heavy metals on budu from Tumpat and budu from Bachok.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Budu from Tumpat</th>
<th>Budu from Bachok</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dye</td>
<td>Detectable</td>
<td>Detectable</td>
</tr>
<tr>
<td>Sunset Yellow FCF</td>
<td>Detectable</td>
<td>Detectable</td>
</tr>
<tr>
<td>Food additives</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>68 mg/kg</td>
<td>66 mg/kg</td>
</tr>
<tr>
<td>Sorbic acid</td>
<td>Not detectable</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Heavy metals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mercury (Hg)</td>
<td>&lt;0.07 mg/kg</td>
<td>&lt;0.07 mg/kg</td>
</tr>
<tr>
<td>Lead (Pb)</td>
<td>&lt;0.1 mg/kg</td>
<td>&lt;0.1 mg/kg</td>
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**Fig. 2:** Percentage cell viability (%) following incubation with budu extracts at lowest concentration (<60 µg/ml). Each point represents the mean ± SEM, n = 4. (a p<0.05 for Budu from Tumpat; b p<0.05 for Budu from Bachok).
Fig. 3: Percentage cell viability (%) following incubation with budu extracts at moderate concentration (<500 μg/ml). Each point represents the mean ± SEM, n = 4.

Fig. 4: Percentage cell viability (%) following incubation with budu extracts at highest concentration (<2000 μg/ml). Each point represents the mean ± SEM, n = 4.

Fig. 5: FRAP value of L-ascorbic acid (LAA) with and without HepG2 cells at the concentrations of 100 μM to 1000 μM. Each value represents the mean ± SEM, n = 3. (*p<0.05 compared with negative control).
Fig. 6: FRAP value of budu extracts without HepG2 cells. Each value represents the mean ± SEM, n = 3. (*p<0.05 compared with negative control).

Fig. 7: FRAP value of budu extracts with HepG2 cells. Each value represents the mean ± SEM, n = 3.

Discussion:
In this present study, determination of biochemical profile, percentages of macronutrient and food additive analysis were done. The level of moisture and protein in budu samples were higher than value in Malaysian Foods Composition Database (57.6% moisture, 5.1% protein). Protein level also complied with Malaysian Food Act 1983 which stated that budu should contain not less than 5% protein. Ash represents inorganic components and minerals in food and there was a high level of minerals in budu products. The value of carbohydrate was also high because of the addition of sugar in budu processing or cleavage of budu component to oligosaccharides. However, the percentage of fat in both local fermented fish product was lower than the value in Malaysian Foods Composition Database (1.4%). The fat content was originated from the addition of salt into fermented fish.

Benzoic acid and sorbic acid are weak organic acids. Both are the most common preservative agents (Brul and Coote, 1999) used in fermented food at pH value below 7 (Park et al., 2001; Dissaraphrong et al., 2006). These preservative agents inhibit the outgrowth of bacterial and fungal cells at the low pH (Brul and Coote, 1999). The level of benzoic acid for both budu samples was lower than maximum permitted proportion from Malaysian Food Act 1983 while sorbic acid was not detected in all samples. Following heavy metals analysis, the level of mercury and lead were very low. Synthetic colouring is added into foods to replace natural colour lost during processing, to reduce batch-to-batch variation and to produce products with consumer appeal where no natural colour exists. The presence of Sunset Yellow FCF was found in both budu samples. Sunset Yellow is water soluble synthetic azo dyes and a permitted food colour (Tripathi et al., 2007). Some synthetic azo dyes can be noxious to the human health and when in contact with some drugs, it can cause allergic and asthmatic reactions to some people (Vidotti et al., 2006).
Based on the MTT graph, budu extracts did not show a reduction in the percentage of cell viability except for budu from Tumpat at the concentration of 15 µg/ml and 60 µg/ml. However, this reduction was less than 20%. Based on the classification of cytotoxicity by Abbas et al. (2002), budu extracts showed no cytotoxic effect.

The FRAP assay was quick and simple to perform, and the reaction was reproducible and linearly related to the molar concentration of the antioxidant(s) present. This assay can detect antioxidant when a ferric tripyridyltriazine (Fe(III)-TPTZ) complex is reduced to the ferrous tripyridyltriazine (Fe(II)-TPTZ) (Benzie and Strain, 1996; Firuzi et al., 2005). The positive control for this assay was L-ascorbic acid (LAA). In this study, FRAP value of LAA without cells increased linearly with LAA concentration. At the highest concentration of LAA (1000 µM) without cells, the FRAP value was 2009.78 ± 14.83 µM FeSO₄7H₂O. The previous report found that 1 mole of LAA can reduce 2 mole of Fe(III) ion to Fe(II) ion (Benzie and Strain, 1996; Huang et al., 2005).

Antioxidant capacity value of LAA with cells was significantly increased at concentration 500 µM to 1000 µM. However, the value was lower than the FRAP value of LAA without cells. This could be due to the reaction of LAA in cell activity such as generation of reactive oxygen species (ROS) (Seifried et al., 2007) or LAA involved in cell metabolic activity (Mello and Kubota, 2007). LAA was also not stable in the culture medium and can reduce the GSH levels in cells (Gehin et al., 2006).

When the aqueous extract of both fermented fish product was tested with FRAP assay without cells, the FRAP value was significantly increased. However, at the lowest concentration of budu extracts, the increasing of FRAP value were not stable because of the possible interference due to the UV-Vis absorption at 593 nm by other compounds than Fe (TPTZ)₂ (II) (Ou et al., 2002). FRAP values of both budu extracts was significant as compared to the negative control but this value was lower than LAA at the same concentration. This showed that budu extracts had weak antioxidant capacities.

For aqueous extract with cells, FRAP values of both samples were not significantly increased as compared to the negative control. However, this value was increased 4-fold higher than the value of budu extract without cells. This was because of the endogenous antioxidant present in HepG2 cells. This result was supported by Morel et al. (2000) which stated that HepG2 cells had endogenous antioxidant system such as glutathione.

Conclusion:
In conclusion, the biochemical profile showed that fermented fish product contain high nutrient composition and the aqueous extracts of budu were not cytotoxic to HepG2 cells and had weak antioxidant capacities as compared with L-ascorbic acid.

ACKNOWLEDGEMENT

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REFERENCES


