ANTIPROLIFERATIVE STUDY OF *B. JAVANICA* EXTRACTS AGAINST HEAD AND NECK CANCER CELLS

(Kajian antiproliferatif ekstrak-ekstrak B. javanica terhadap sel-sel kanser kepala dan leher)

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Abstract

Brucea javanica or locally known as Meladapahit, are being used in Malaysia as traditional medicine mainly for the treatment of diabetes mellitus and hypertension. In order to study the potential use of this plant for cancer treatment, we have prepared crude extracts of the leaves and fruits, and assessed them for antiproliferative activities against head and neck cancer cell line which is HTB-43. The dried and ground leaves and fruits of the plant were successively extracted using hexane, chloroform, methanol and water, respectively. Inhibition of growth of the cultured cancer cells line was measured using a standard Microculture Tetrazolium Technique (MTT) assay. The crude extracts were also subjected to toxicity test using brine shrimp lethality assay. Most of the tested crude extracts exhibited significant antiproliferative activities against the HTB-43 cell with IC_{50} ranging from 8.46 µg/ml to 47.25 µg/ml. The chloroform extract from the leaves gave the highest antiproliferative activity (IC_{50}, 8.46 µg/ml). Hexane extract from the fruits, aqueous and hexane extracts from *B. javanica* leaves showed low antiproliferative activities to the HTB-43 cell line with an IC_{50} values >100 µg/ml. The chloroform extracts from fruits and leaves and methanol extract from fruits induced toxicity against brine shrimps with LC_{50} values of 118.7 µg/ml, 512.44 µg/ml and 75.27 µg/ml respectively. It indicated that bioactive components presence in the crude extracts for its pharmacologic effects against head and neck cancer cells. Methanolic extract of *Brucea javanica* fruit was selected as the most effective extract to inhibit the growth of head and neck cancer cells (HTB-43) by the two different assays used.

Abstrak

Brucea javanica atau lebih dikenali tempatan sebagai Meladapahit, digunakan di Malaysia sebagai ubat tradisional terutamanya untuk rawatan penyakit diabetis mellitus dan hipertensi. Bagi mengkaji potensi kegunaan pokok ini untuk rawatan kanser, kami telah menyediakan ekstrak-ekstrak mentah daun dan buah, seterusnya membuat penilaian untuk aktiviti-aktiviti antiproliferatif terhadap sel kanser kepala dan leher iaitu HTB-43. Daun-daun dan biji- biji buah pokok tersebut yang telah dikeringkan dan dikisar masing-masing dengan jayanya telah diekstrak menggunakan heksana, kloroform, metanol dan air mengikut turutan. Pembendungan perkembangan sel-sel yang dibiak telah diukur dengan menggunakan ujian piawai Teknik Mikrokultur Tetrazolium. Ekstrak-ekstrak mentah tersebut juga telah diuji ketoksikannya dengan menggunakan ujian kematian udang air garam. Hampir semua ekstrak mentah yang telah diuji menunjukkan aktiviti-aktiviti antiproliferatif terhadap sel kanser HTB-43 dengan IC_{50} antara 8.46 µg/ml hingga 47.25 µg/ml. Ekstrak kloroform daripada daun telah memberikan aktiviti antiproliferatif yang paling tinggi (IC_{50}, 8.46 µg/ml). Ekstrak heksana daripada buah, ekstrak air dan heksana daripada daun-daun *B. javanica* memberikan aktiviti-aktiviti antiproliferatif yang rendah terhadap sel kanser HTB-43 dengan nilai IC_{50} >100 µg/ml. Ekstrak-ekstrak krloroform daripada buah dan daun serta ekstrak metanol daripada buah menyebabkan ketoksikan terhadap udang air garam dengan nilai-nilai LC_{50} masing-masing 118.7 µg/ml, 512.44 µg/ml dan 75.27 µg/ml. Ini menunjukkan bahawa terdapatnya komponen-komponen bioaktif di dalam ekstrak-ekstrak mentah tersebut untuk memberikan kesan-kesan farmakologi terhadap sel-sel kanser kepala dan leher. Ekstrak metanol buah *Brucea javanica* telah dipilih sebagai ekstrak yang paling efektif untuk menghalang perkembangan sel-sel kanser kepala dan leher (HTB-43) oleh kedua-dua ujian yang telah dijalankan.

INTRODUCTION

Head and neck cancer is a squamous cell carcinoma cancer type and most common form of skin cancer. It can be categorized by the area of origin of the head or neck involving upper aerodigestive tract (UADT). There are six overall sites of head and neck region: nasal cavity, pharynx, oral cavity, oropharynx, larynx and hypopharynx (Shane and Woo, 2012). This type of cancer also consists of heterogenous groups of tumours with a multitude of histologies (Lee *et al.,* 2011). The number of head and neck cancer cases is increasing every day and quite markedly, due predominately to the ageing of the populations and the population growth. It is about 500, 000 new cases of the disease reported every year (Lee *et al.,* 2011). Patients in the age group of 60-69 years were the largest percentage of patients with the cancer (Shashinder *et al*., 2008). Besides, men showed predominance compared with women in the disease (Shashinder *et al*., 2008). According Scottish Intercollegiate Guidelines Network (SIGN) (2006), there is evidence now days the cancer incidence is increasing amongst young people of both sexes. Smoking and alcohol consumption become well known risk factors for the head and neck cancer (Janne *et al.,* 2014). People who are very interested to leave their cigarette on the lip are vulnerable to have lip cancer irrespective of cumulative tobacco consumption. On the other hand, alcohol consumption increases the risk of developing cancers of oral cavity, pharynx and larynx (SIGN, 2006). There is a strong relationship between the quantity of alcohol consumption and the level of risk. Fanucchi *et al.* (2006) stated that early diagnosis and treatment are important to increase the survival rate of the cancer patients. Besides, any delay may lead to more severe disease, difficult to treat, leading to higher morbidity and mortality (Kowalski and Carvalho, 2001).

In Malaysia, there are several treatments for head and neck cancer conducted by clinical specialist such as surgery, chemotherapy, radiotherapy and palliative care. Those treatments depend on the stage of the cancer had by a patient. According Kahairi *et al.* (2014), most of the patients with head and neck cancer were being treated by radiotherapy and reconstructive surgery. The delicate nature of the tissues of the UADT is difficult to replace or reconstruct once damage by the disease or the treatment (Shane and Woo, 2012). Therefore, numerous studies of medicinal plants have been carried out in order to discover new biologically active compounds to reduce the risk of side effects.

Brucea javanica or locally known as Meladapahit, are being used in Malaysia as traditional medicine mainly for the treatment of diabetes mellitus and hypertension. It is a member of Simaroubaceae family. It is a shrub tree with 1 to 3 meters and younger parts softly pubescent. It also has compound-paripinnate leaves and the flowers are minute, purple, in numerous small cymes or clusters collected into axillary panicles. The fruit become black when ripened. Recent studies found this plant has potential for the treatment of inflammatory diseases and induced cytotoxicity and apoptosis in many cancer cell lines. But, it has been done only with single fraction (usually methanol or ethanol) instead of trials by using different solvents for comparison purposes. The objectives of the study are to evaluate the cytotoxicity of the plant extracts against the selected head and neck cancer cell line which is HTB-43 (pharynx cancer cells) and to determine the most potential extracts to inhibit the growth of the cancer cells in vitro. Sequential extraction method has been used to fractionate the plant compounds according to their solvents.

MATERIALS AND METHODS

Preparation of *B. javanica* extracts

B. javanica fruits and leaves were collected from a local farmer at Gemencheh, Negeri Sembilan, Malaysia. The collected specimens were thoroughly washed under running tab water and then oven-dried at 60^{o}C for three days. The dried fruits (200 g) and leaves (150 g) were finely ground by using a basic microfine grinder machine. Each plant powder was sequentially extracted with different organic solvents according to Pathmanathan *et al.* (2010). The extraction started with non-polar solvents which are hexane, chloroform, methanol and ended with polar solvent which is water. 100 g of dried powder of each plant material was soaked into 500 ml of hexane in an Erlenmeyer flask resulting 1:5 ratio used. They were intermittently shaken for 24 hours and then vacuum filtered with Whatman No.54 filter paper. The residue was further extracted for the second time by using fresh hexane solvent. All the

filtrates were pooled together to be concentrated under reduced pressure and low temperature. The resulting residue was used for further extraction with chloroform and followed by methanol and ultra-purified water similar to the procedure that carried out for the hexane extraction. Finally, the solvents were removed from the extracts by storing in vacuum oven at 60^0C. It is important to keep the extracts in condition without air circulation as a precaution to prevent the growth of microorganisms. The yield of each extraction was measured separately after completely dried and the crude extracts were kept back in the vacuum oven for further study.

General Cell Culture Methods

Chemicals and reagents used in the cell culture experiments are Gibco products that purchased from Bio-Diagnostics, Malaysia. The human pharynx cancer cell line used in the study, HTB-43 was purchased from American Type Culture Collection (ATCC). For general cell culture, the HTB-43 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS), Glutamax (100X) and Penicillin and Streptomycin (100X) in a humidified atmosphere of 5% CO_2 at 37^0C.

Antiproliferative activity assay

Inhibition of growth of the cultured cancer cells line was measured using a standard MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay. The assay detects the reduction of yellow MTT dye by metabolically active cells, in part by action of mitochondrial dehydrogenase of viable cells to purple formazan crystals (Wiratchanee *et al.*, 2010).

The cells were seeded in 96-well flat bottom plates at a density of 10^5 cells/well in 100 µl culture medium and allowed to attach during 24 hours incubation time. The cells were then treated with different concentration of plant extracts (100 µl each well) and incubated for 72 hours. There were eight concentrations prepared from each extract (500, 250, 125, 62.5, 31.25, 15.625, 7.8125 and 3.9 µg/ml). The extracts that derived from non-polar solvents were dissolved in dimethylsulfoxide (DMSO) and the aqueous extract was dissolved in distilled water before the treatment. Untreated cells were used as a negative control in the study.

Next, 20 µl of 5 mg/ml of MTT reagent was added into each well, and the plates were incubated for 4 hours at 37^0C. After the incubation, the remaining MTT solution removed and 100 µl DMSO was added into each well of the plates to dissolve the purple formazon and lysed the cell to release the mitochondrial residues of formazon. Absorbance measurements were made at 570 nm and IC_{50} values (concentration that inhibit cell proliferation by 50%) were obtained by using EnSpire Multimode Plate Reader. All the experiments were performed in triplicate and repeated three times in order to perform the statistical analysis.

Brine Shrimp Lethality Assay

Brine shrimp (*Artemia salina* Leach) dried eggs were hatched in a shallow two compartment rectangular plastic box filled with artificial sea water (36 g/L) which was prepared from commercial sea salt (Sigma Chemical Co., UK) and sterilized distilled water. A divider with several holes was placed in between the covered and the open compartment. The eggs were placed into the dark section, while the open compartment was illuminated. After 48 hours of incubation at room temperature (30^0C), nauplii (larvae) were collected from the lighted side whereas their shells and other unhatched eggs were left in the light tight side.

The brine shrimp lethality test was conducted by using the 96-well microplates procedure described by Solis *et al.* (1993). An aliquot (100 µl) of the 2 mg/ml sample solution was dispensed in triplicate into the first and second well of the microplate row. Two fold serial dilutions with 100 µl sea salt solution were made in triplicate across the plates starting from well number 2 to 8 to give final concentration of 7.8 µg/ml. 2% of DMSO diluted with sea salt water was used as a solvent and also as a negative control. 7 – 10 mature nauplii in suspension were then added into each well and the covered plates were incubated in room temperature for 24 hours. The numbers of survivors were

counted and LC_{50} values (lethality concentration by 50%) were analyzed after the incubation. The LC_{50} values were determined using the probit analysis by IBM SPSS 20.

RESULTS

Antiproliferative Activity of *Brucea javanica* extracts

Most of the *Brucea javanica* crude extracts exhibited antiproliferative activities against the HTB-43 cells as showed in **Table 1**. From the results, it can be suggested that chloroform extract (leaves) of the plant expressed the highest inhibition towards the HTB-43 cell line with IC_{50} value of 8.46±2.3 µg/ml. Other potential extracts are chloroform extract of the fruit and methanol extract of the fruit and leaves with IC_{50} values of 15.86±4.54 µg/ml, 8.52±1.61 µg/ml and 26.48±4.42 µg/ml respectively **(Table 1 and Figure 1)**. Hexane extract from the fruits, hexane and aqueous extracts from *B. javanica* leaves showed low antiproliferative activities to the HTB-43 cell line with an IC_{50} values >100 µg/ml. Statistical differences among the replicates in each potential extract were determined by one way ANOVA using GraphPad Prism 6.0. The results found that there were no significant differences ($p>0.05$) among them.

Table 1: IC_{50} values of the *Brucea javanica* extracts against human pharynx cancer cell line (HTB-43) by MTT assay.

IC50 value (µg/ml)		
Fraction	Plant Parts	
	Fruits	Leaves
Hexane	>500	103.95±10.38
Chloroform	15.86±4.54	8.46±2.3
Methanol	8.52±1.61	26.48±4.42
Aqueous	47.25±8.24	195.68±6.99

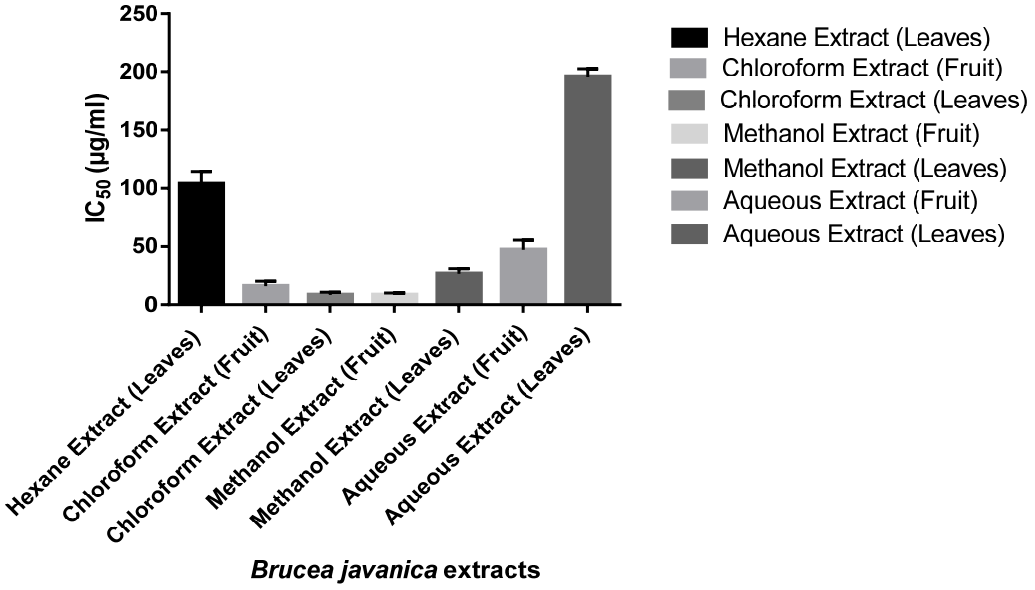

Figure 1: There were four potential extracts (chloroform and methanol extracts from plant parts, fruit and leaves) with antiproliferative activities against the cancer cell line. Data represents mean ± SD values in three replicates.

LC_{50} Values of Brine Shrimp Lethality Assay

The brine shrimp lethality test results are presented in **Figure 2**. Out of eight extracts screened for activity against brine shrimp larvae, three of the crude extracts demonstrated activity below 1000 µg/ml. There are chloroform extracts from fruits and leaves and methanol extract from fruits with LC_{50} values of 118.7±11.32 µg/ml, 512.44±7.9 µg/ml and 75.27±4.33 µg/ml respectively. Other extracts were considered to be non-toxic towards the *Artemia salina* larvae (LC_{50} values >1000 µg/ml).

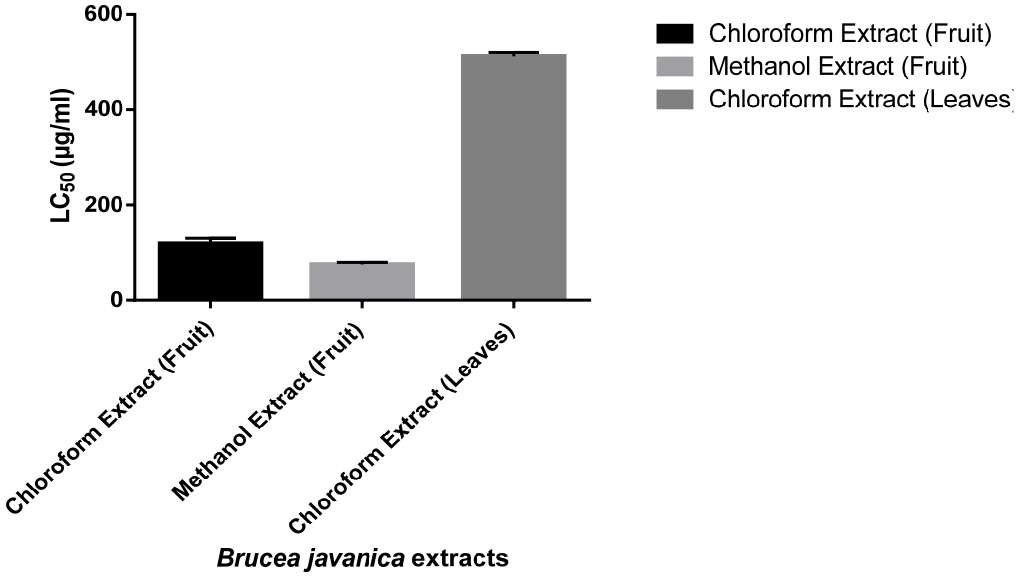

Figure 2: Brine shrimp lethality assay results of *Brucea javanica* crude extracts. Only three extracts induced toxicity against the nauplii. Data represents mean ± SD values in three replicates.

DISCUSSION

MTT assay is an adequate method to study the cell viability and proliferative activities on cell (Imbert and Cullander, 1999). The effect of extracts on the growth of cells in vitro was estimated by the reduction of the yellow MTT dye. According to American National Cancer Institute by Itharat *et al*. (2004), to meet the criteria of cytotoxic activity, any crude extracts need to have an IC_{50} <30 µg/ml. In the study, the chloroform and methanol extracts of both plant parts (fruit and leaves) were found to significantly reduced HTB-43 cells proliferation. A study by Lee *et al.*, (2008) found that methanol extract of *Brucea javanica* of the combined twigs, leaves and influorescence were showed high antiproliferative activity against MCF-7 human breast cancer cell line. Besides, *B.javanica* fruit extract by ethanol solvent induced cytotoxicity and apoptosis in pancreatic adenocarcinoma cancer cell lines (Sin *et al*., 2008). The studies were supported the results in this research, where the methanolic extract of *B.javanica* exhibit potent cytotoxic activity against tested cell line. Chloroform solvent has never been used to isolate bioactive compound in previous research. The data on **Table 1** indicated that the chloroform inhibited the cell growth with the highest IC_{50} value compared with others. It seems that the use of chloroform for extraction is strongly useful.

Brine shrimp lethality assay is one of the best and rapid test for biological and toxicological purposes in a lab (Kanwar, 2007). An extract is considered active when the LC_{50} values lower than 1000 µg/ml (Khade *et al.*, 2011). In addition, Rieser *et al*. (1996) reported that crude extract resulting in LC_{50} value less than 250 µg/ml were considered significantly active and had potential for further investigation. Based on the results, the chloroform and methanol extracts of *B.javanica* fruit have the potential to be the candidate for the investigation of cytotoxic compounds due to the LC_{50} values obtained were <250 µg/ml. Chloroform extract from the plant leaves was found to be less toxic in the study. The result was supported by Marissa *et al.* (2012) where they categorized the *B.javanica* Merril leaves extract was slightly toxic on mice.

CONCLUSION

In the study, two different methods were used for evaluation of cytotoxic activity by *B.javanica* fruit and leaves extracts using different polarity of solvents. Thus, we demonstrate that chloroform and methanolic extracts of the plant fruit exhibits potent antiproliferative property against the head and neck cancer cell line (HTB-43) by all methods used. But, methanolic extract of *Brucea javanica* fruit can be selected as the most effective extract to inhibit the growth of HTB-43 cells. However, the study will be more focus on identifying the active ingredients in chloroform extract of *B.javanica* fruit instead of compounds in methanol extract that have been elucidated by many of studies before.

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