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Nasopharyngeal Carcinoma Cell Proliferation and Apoptosis Induced by the Standardised Ethanolic Extracts of *Mucuna bracteata*

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Abstract

Mucuna bracteata is a wild leguminous cover crop in oil palm and rubber plantations around Malaysia. It is also popular in Ayurveda medicine. There are scientific reports on the antiproliferative activities of plant species belong to *Mucuna* genus. However, there is no scientific report on *M. bracteata*. Hence, we investigated the antiproliferative activities of the ethanol extracts from young and matured leaves, young and matured stems, seeds and roots on nasopharyngeal cancer cells (TWO1) and non-cancerous cells (3T3) through MTT assay. The extracts were standardised through determination of its total phenolic content and total flavonoid content. The young leaves extract exhibited IC_{50} of 148µg/mL and 91µg/mL on TWO1 and 3T3 cells respectively. While, the matured leaves extract found to have IC_{50} at 165µg/mL and 225µg/mL on TWO1 and 3T3 cells respectively. The IC_{50} values for other plant parts were more than 250µg/mL on TWO1 and 3T3 cells. Both young leaves and matured leaves extracts induced slightly higher degree of apoptosis than necrosis on TWO1 cells. Being a potent extract which was also selective towards TWO1 cells, the matured leaves extract was further found to induce apoptosis through the phosphatidylserine translocation and caspase-3 activation.

Introduction

The *Mucuna* genus belongs to the Leguminosae family. It is consisting of about 100 different species that can be found all over the world. These plants have been widely used as food and medicinal products. For example, *M. pruriens* was found to have antineoplastic and other pharmacological properties. Plants from the same genus will usually share some similar pharmacological profile [1,2].

Mucuna bracteata (M. bracteata) is abundantly used as cover crop in oil palm plantation. It has also traditionally being prescribed as Ayurvedic medicine for constipation, oedema, fever, delirium and dysmenorrhoea. To our knowledge, there is no scientific publication on *M. bracteata* was carried out to determine if this species is able to suppress growth of tumour cells in any cancer cells [1,2].

Nasopharyngeal cancer is one of the common cancers found in Asia. The treatment options available for nasopharyngeal carcinoma are limited and commonly results in complications. There is much need for the discovery of plant extract compounds as a possible new treatment options for nasopharyngeal carcinoma patients [3,4]. Since the high prevalence of nasopharyngeal cancer in this region, this study investigated the antiproliferative activity of the standardised *M. bracteata* ethanol extracts of young and matured leaves, young and matured stems, seeds and roots on the nasopharyngeal carcinoma (TWO1) and the normal murine fibroblast (3T3) cells. The extracts were standardised by determining the total phenolic content (TPC) and total flavonoid content (TFC). The mode of cell deaths induced by the extracts on TWO1 cells were also investigated in this study.

Experimental Procedures

Materials and Reagents

The plant materials (young leaves, matured leaves, young stems, matured stems, seeds and roots) were collected from Jendarata Estate, Perak, Malaysia and were authenticated by Professor Dr. Zulkifli Hj. Shamsuddin from Universiti Putra Malaysia (UPM), Malaysia. The TWO1 cells were obtained from Professor CK Sam's University Malaya collection, courtesy of Professor WL Chu of International Medical University. The 3T3 cells were purchased from the American Type Cell Culture (ATCC) (Rockville, MD, USA). The following reagents and chemicals were obtained from respective suppliers: 5-fluorouracil (Calbiochem, China); dimethylsulfoxide, 95% ethanol, sodium carbonate anhydrous, gallic acid monohydrate, quercetin dihydrate, 2M Folin-Ciocalteu Reagent (Sigma, USA); potassium chloride (Darmstadt, Germany); aluminium chloride, sodium acetate anhydrous (Systerm, Malaysia); Dulbecco's Modified Eagle Medium-F12 (DMEM-F12), phosphate-buffered saline (PBS) (Mediatech Inc., USA); fetal bovine serum (FBS), penicillin-streptomycin, sodium pyruvate (PAA Laboratories, Austria); accutase™ (Innovative Cell Technologies, USA); MTT reagent and Dual Apoptosis Kit (Biotium, USA); Cell Death Detection ELISA^{PLUS} Kit (Roche, Germanay).

Preparation of Extracts

All the plant materials (young and matured leaves, young and matured stems, seeds and roots) were washed and were dried in a hot air oven at a temperature of not more than 45°C for a period of 7 days. Each plant part was cut into smaller pieces and ground into a powder-like consistency using a hammer mill before extracted with 95% ethanol in a Soxhlet extractor. Extracts were then concentrated in rotary evaporator and were lyophilised in freeze dryer until a constant weight was obtained. Ethanol (95%) was then used to dilute the extracts. The extracts were then filtered ascetically through the 0.22µm filters and kept sterile before further experiment processes.

Standardisation of Extracts

Total phenolic content (TPC) of extracts was determined using the Folin-Ciocalteu assay reported by Singleton and Rossi [5]. The absorbance of the solution was measured at 765nm. TPC was then expressed as gallic acid equivalents (GAE) in milligram per gram of dried extract of *M. bracteata*. The calibration equation for gallic acid was

$$y = 0.7075x - 0.0595\ (R^2 = 0.9922)$$.

Total flavonoid content (TFC) of extracts was determined using the aluminium chloride colorimetric method as reported by Woisky and Salatino [6]. The absorbance of solution was measured at 415nm. TFC was expressed as quercetin equivalent (QE) in milligram per gram of dried extract of *M. bracteata*. The calibration equation for gallic acid was $y = 0.033x - 0.021\ (R^2 = 0.9922)$.

Cell Culture

TWO1 and 3T3 cells were cultured in DMEM-F12 and RPMI-1640 complete medium respectively, with 10% FBS, 1% penicillin-streptomycin and 1% sodium pyruvate in a humidified 5% CO_2 incubator. Accutase™ was used to detach the cells from the culture flasks. The cells were monitored daily under an inverted light microscope. The cells became confluent in three to four days and were either sub-cultured or harvested for further studies.

Antiproliferative Assay

Inhibition of cell proliferation by ethanol extracts of *M. bracteata* was measured by the MTT assay. Briefly, TWO1 and 3T3 cells were plated at the concentration of 10,000 cells per well, and were incubated for 24 hours before the cells were treated with standardised ethanol extracts (50 to 250µg/mL). Ethanol (less than 1%) was used as negative controls, while wells containing cells with 5-fluorouracil were incubated as positive controls. Each experiment was performed with a minimum of triplicate. After another 48 hours of incubation, MTT reagent was added as followed manufacturing instruction. The absorbance was measured using a microplate reader at wavelength of 570nm and reference wavelength 630nm. The

$$\text{Percentage of cell viability} = \frac{\text{Absorbance of the treated cells - Absorbance of the blank}}{\text{Absorbance of the negative control - Absorbance of the blank}} \times 100\%$$

The percentage of cell viability of different plant parts were then compacted in a dose-response curve to enable the determination of IC_{50}, or the concentration of extract inhibiting cell growth by 50%. Only the extract which exhibited IC_{50} in the testing concentration range, was further tested on 3T3 cells to determine its selectivity towards cancerous and non-cancerous cells.

Determination of the Mode of Cell Death Induced by Extracts

Cell death induced by extracts can also be attributed to the induction of apoptosis or necrosis. In this study, the extract which exhibited IC_{50} in the testing concentrations, was further tested for its mode of cell death. In this study, two apoptosis assays were applied, which were the Cell Death Detection ELISA^{PLUS} kit and the Dual Apoptosis Assay.

The Cell Death Detection ELISA^{PLUS} kit was used to quantify the degree of apoptosis and necrosis activity in the cells after treated with extracts for 48 hours as according to the manufacturer's instructions. Details of the experimental procedures were published in previous study [7]. The degree of apoptosis and necrosis was quantified by using microplate reader at wavelength of 405nm and reference wavelength of 490nm. The enrichment factor was calculated using the following formula:

$$\text{Enrichment Factor} = \frac{\text{Absorbance of the sample - Absorbance of blank}}{\text{Absorbance of the negative control - Absorbance of blank}}$$

In order to further confirm the apoptosis pathways, the extracts with the highest apoptotic induction activity and was further tested on the Dual Apoptosis Assay. This assay detects two important of apoptosis markers in a single assay study. The sulforhodamine 101-annexin V marker which stains the apoptotic cell membrane bright red to detect the early phosphatidylserine (PS) translocation and the NucView™ 488 caspase-3 substrate marker which stains the apoptotic nucleus with caspase-3 activity. Experimental details were as published in previous study [7]. The cells were lastly observed for morphological changes under fluorescent microscope using fluorescein isothiocyanate (FITC) filter (450nm to 490nm) and Texas-Red filter (510nm to 560nm).

Statistical Analysis

All data were reported as means ± standard deviation (S.D.) from a minimum of triplicate. Statistical difference between treated and negative control was analysed using one-way analysis of variance (ANOVA) followed by *post hoc* Dunnet's *t*-test by using the SPSS (version 15.0) for Windows. If *p* value less than 0.05 ($p<0.05$), the difference was considered as statistical significant.

Results

Standardisation of Extracts

The total phenolic content (TPC) and total flavonoid content (TFC) of the ethanol extracts of young leaves, matured leaves, young stems, matured stems, seeds and roots were shown in table 1. Seeds extract was found to have the highest TPC and TFC among the six parts tested.

Effect of M.bracteata on TWO1 and 3T3 Cells Proliferation

The cells proliferation induced by the extract of *M. bracteata* was determined by the MTT assay. Among all the standardised extracts, only the young leaves and matured leaves extracts were potent enough to exhibit IC_{50} at 148 µg/mL and 165µg/mL respectively on TWO1 cells. Other extracts did not shown IC_{50} at concentration less than 250µg/mL (Figure 1).

In order to determine the selectivity of the extracts, both young and matured leaves were again tested on 3T3 cells, a non-cancerous cell lines. As illustrated in Figure 2, the matured leaves extract had IC_{50} more than 250µg/mL on 3T3 cells but not the extract of young leaves. Thus, the extract of matured leaves, but not the extract of young leaves, was selective to TWO1 cells.

Table 1. Total Phenolic Content and Total Flavonoid Content of Extracts

Plant Parts	Total Phenolic Content (mg gallic acid equivalent/g of dried extract)	Total Flavonoid Content (mg quercetin equivalent/g of dried extract)
Young Leaves	113.18 ± 3.85	2.10 ± 0.24
Matured Leaves	98.48 ± 4.57	0.14 ± 0.56
Young Stems	146.55 ± 4.00	10.54 ± 0.50
Matured Stems	98.48 ± 4.57	-
Seeds	156.63 ± 10.00	11.57 ± 0.11
Roots	75.49 ± 7.68	6.39 ± 0.78

The values are mean± SD. The symbol "-" indicates the matured stems extract did not contain any flavonoid.

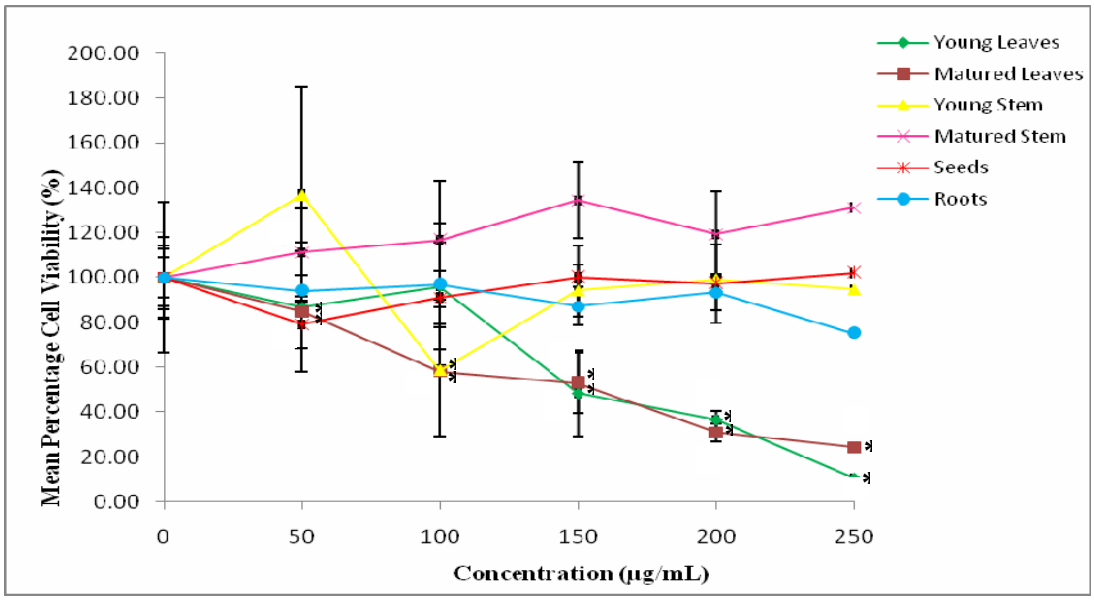

Figure 1. The cell viability of TWO1 cells when tested with *M. bracteata* ethanol extracts at different concentrations. The symbol '*'indicates a statistical significantly different from negative control with *p* value less than 0.05 through Dunnett's t-test.

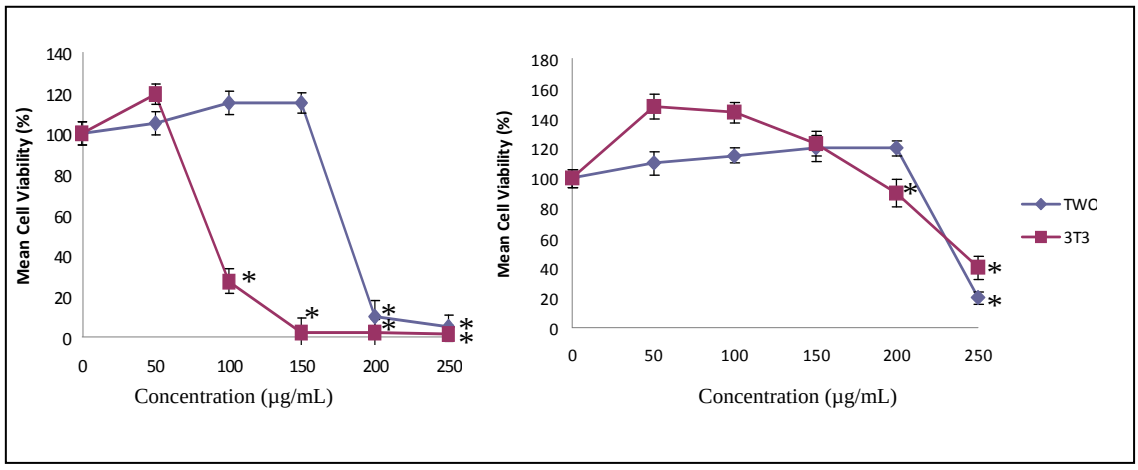

Figure 2. The cell viability of HT-29 and 3T3 cells after treated with ethanol extracts of young (left) and matured leaves (right). The symbol '*'indicates a statistical significantly different from negative control with *p* value less than 0.05 through Dunnett's t-test.

Apoptosis Induction by the Ethanol Extracts

From the proliferation study, we found that the young and matured leaves extracts had potent antiproliferative activities on TWO1 cells. The TWO1 cells were treated with standardised young and matured leaves extracts at a concentration closest to their IC_{50} values (150µg/mL) for 48 h. The degree of apoptosis was found to be higher in matured leaves extracts, as shown in Figure 3. Both extracts were found to have higher degree of apoptosis than necrosis.

The mode of cell death induced by matured leaves extract was further confirmed as apoptosis (Figure 4). Most of the treated TWO1 cells were found as red ring at 6 hours which indicated the PS translocation, while most cells presented with a red ring and a green core at 12 h, indicated a complete apoptosis induction with both PS translocation and caspase-3 activation respectively.

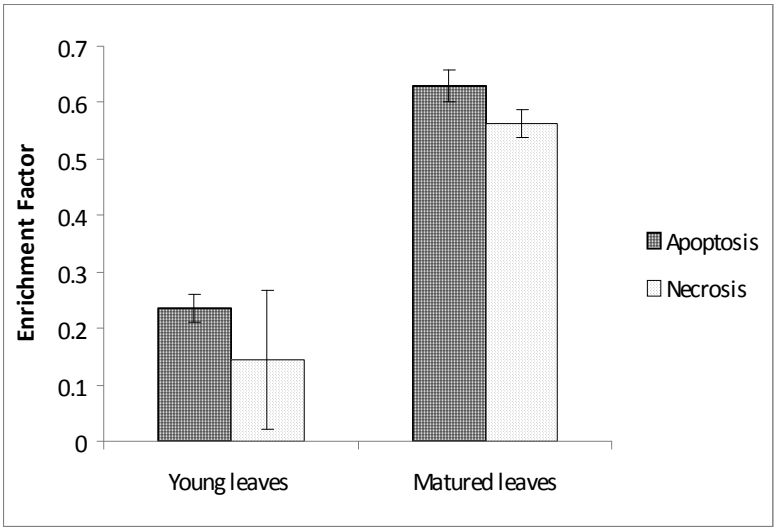

Figure 3 The degrre of apoptosis and necrosis induced by standardised young and matured leaves extracts on TWO1 cells.

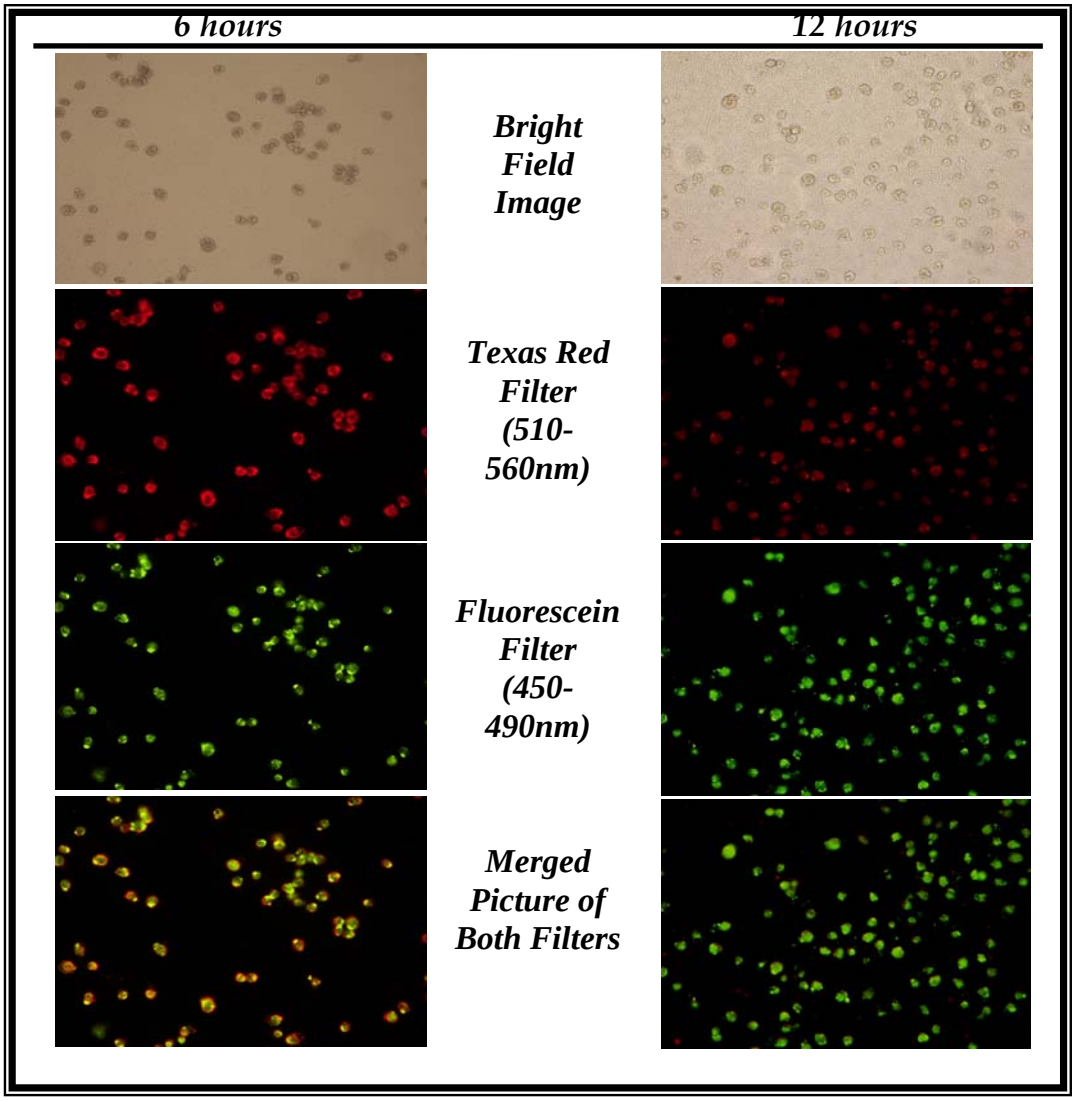

Figure 4 Apoptosis induction by ethanol extract of matured leaves extract through phosphatidylserine translocation and caspase-3 activation on TWO1 cells. The images were taken using Nikon Eclipse 80i fluorescent microscope at 200× magnification.

Discussion

The introduction of *M. bracteata* as a cover crop in oil palm plantations has resulted in an abundance of the *Mucuna* plant in Malaysia. Since *M. pruriens*, the plant from the similar genus of *M. bracteata* has shown to have a potential of antiproliferative property [8], we investigated the antiproliferative activities of standardised ethanol extracts of *M. bracteata* on a nasopharyngeal carcinoma TWO1 cells. Moreover, so far the antiproliferative activity of the extracts from different plant parts of *M. bracteata* had not yet been published and no study was found to report on its mode of cancer cell death.

In this study the young leaves and matured leaves extracts were found to have potent antiproliferative activities on TWO1 cells. However, both extracts were not the highest amount of TPC and TFC among all the tested plant parts. These results suggested that phenolic and flavonoid compounds that present in *M. bracteata*, may not be the solely active compounds that responsible for their antiproliferative activities. Other reason could be a combination of phenolic and non-phenolic compounds are responsible for their antiproliferative activity.

The young leaves extracts was found to be non-selective in inhibiting the proliferation of cancerous (TWO1) and non-cancerous (3T3) cells. The IC_{50} exhibited by the young leaves extract was lower on 3T3 than TWO1 cells. In other words, this extract did not spare the normal cells while inhibiting cancer cells proliferation. However, the matured leaves extracts had a higher IC_{50} on 3T3 than TWO1 cells. Its antiproliferative effect was more selective towards the cancerous cells than the normal healthy cells. Between the young and matured leaves extracts, we found that the young leaves extracts had higher TPC and TFC than the matured leaves extracts (Table 1). It may be due to the present of certain phenolic and flavonoid compounds in young leaves that may not present in the matured leaves extracts, was responsible for the non-selective antiproliferative effect. Another reason could

be the amount of TPC and TFC in an extract may have an important role in determining their selectivity index. High level of TPC and TFC may be toxic to the normal healthy cells.

Apoptosis is an important mechanism that controls the homeostasis of cell populations in the body, as well as having roles in the pathogenesis and development of various disease processes. In apoptosis, membrane damage is much slower and these dead cells are engulfed by phagocytes, causing little or no inflammation. In cancer cells, there is an accumulation of mutated cells due to excessive proliferation or faulty apoptosis mechanisms, or both. The induction of apoptotic pathways is an important step towards control of cancer cells proliferation [9]. In this study, we found that both young and matured leaves extracts had higher degree of apoptosis than necrosis activity. Thus, it strongly suggests the apoptotic activity induced by the ethanol extracts of *M. bracteata*.

Being able to induce the highest degree of apoptosis and had a good selectivity on TWO1 cells, the matured leaves extract was further tested for the PS translocation and caspase-3 activation, two of the important apoptosis pathways. Almost all the TWO1 cells had undergone PS translocation as early as after 6 hours of treatment (Figure 4). After 12 h of treatment with the extract, almost all the TWO1 cells had now undergone PS translocation and caspase-3 activation. This results supports that the apoptosis activities by the matured leaves extract of *M. bracteata*.

Conclusion

M. bracteata extracts showed antiproliferative activities on TWO1 cells. The ethanol extract of matured leaves was found to be selective towards the cancerous cells, specifically TWO1 cells. Its apoptosis induction was confirmed through the PS translocation and caspase-3 activation. Further study is necessary to determine the active compounds that responsible for its antiproliferative and apoptosis induction.

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