

Anticancer Effects of Medical Malaysian Leech Saliva Extract (LSE)

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Abstract

Leech saliva contains biologically active compounds that are mainly proteins & peptides. Small cell lung cancer (SCLC) is a form of cell lung carcinoma. In this study a modified and smooth extraction method of saliva was used without leech scarification. Trying to find out the biological activity of medical Malaysian Leech Saliva Extract as cytotoxic *in vitro*, the SW 1271 cell line was grown and maintained in Leibovitz's L-15 medium supplemented with 10% foetal bovine serum at 37°C in normal atmospheric air. Serial dilutions of LSE were added to the cell line SW 1271 media for testing the cytotoxic activities. Result revealed that the LSE has a cytotoxic activity against small cell lung cancer (SW 1271 cell line) with IC₅₀ of 119.844 µg/ml compared with IC₅₀ values of two reference standard drugs Irinotecan (5.81 µg/ml) and Carboplatin (18.754 µg/ml). In a combination regimen, LSE reduced the IC₅₀ of Carboplatin & Irinotecan by 65% & 11.5% respectively. Carboplatin reduced the IC₅₀ of LSE by 4.6%, while Irinotecan reduced it by 57%. This results provides a promising novel agent for treatment of small cell lung cancer (SCLC) at least *in vitro*, more researches are needed.

Keywords: Cancer; Carboplatin; Cytotoxic; Irinotecan; Leech

Introduction

The concept of the medical application of leeches can be traced back to the beginning of civilization. Traditionally, in many countries including Malaysia leech application had been used for many human body disorders starting from the conventional usage of leech for bloodletting. Moreover, many reports mentioned the usage of leech in skin diseases, nervous system abnormalities like brain congestion, urinary and reproductive system problems (nephritis, vaginitis). In addition, ocular inflammation, dental problems and hemorrhoids were also treated by leech therapy [1-3].

In the 20th century, leeches have been a model for many extensive studies to evaluate the usage of leech products, especially leech saliva, for therapeutical purposes [4,5]. Consequently, a large number of peptides and proteins with tremendous clinical applications have been identified and characterized in leech extract, such as antithrombin agents [6,7], antiplatelet compounds [8-10], etc. Nowadays, leeches have been introduced as promising tools in microsurgery and reconstructive operations after many reports about better salvage of grafted tissues and amputated digits after leech application [11,12].

With regard to cancer and metastasis therapy, many researchers delineated the effective usage of leech saliva and leech salivary gland extract as an anti-metastatic agent. It has been outlined that salivary gland extract from *Haementeria ghilianii* and *Haementeria officinalis* inhibited the metastatic colonization of lung tumor cells which were injected intravenously into the experimental animals [13]. Other research described a booming synthetic *hirudin* preparation as an efficacious metastasis inhibitor of a wide range of malignant tumor cells, such as pulmonary carcinoma, osteocarcinoma, breast carcinoma, leukemia, etc. [14]. Recently, intensive researches led to the isolation of a protein named *ghilanten* from the leech *H. ghilianii* salivary gland extract with factor Xa inhibitory and anti-metastatic activities [15].

Putting in mind that leech saliva was not established for tumor treatment as a cytotoxic agent, we aimed from this research to examine the antiproliferative activity of leech saliva from the medicinal

Malaysian leech against human small cell lung cancer cell line SW1271 *in vitro* and to evaluate the effectivity of its combination with known antitumor drugs.

Materials and Methods

Chemicals, reagents and instrumentation

Leibovitz's L-15 medium, bovine serum albumin and arginine hydrochloride were supplied by Sigma Aldrich. Phosphate buffered saline (PBS, 1X sterile solution) was procured from Amresco. The Cell Culture Company PAA was the manufacturer of L-glutamine (L-Glu, liquid, 200mM), penicillin/streptomycin (pen/strep, 100X), fetal bovine serum FBS mycoplex and accutase[®]. CellTiter-Glo[®] luminescent cell viability assay was obtained from Promega. Trypan blue dye and sodium chloride were the products of Merck. Amresco was the supplier of Bradford reagent kit. Carboplatin (cis-Diamine [1,1-cyclobutanedicarboxylato]platinum II) was from Calbiochem. Irinotecan hydrochloride (USP reference standard) was the product of Rockville, MD.

Centrifugation was carried out using Jouan CR22 refrigerated centrifuge (Jouan, France). Memmert incubator type BE-400 (Mettler, Germany) was used for incubation. The inverted microscope was manufactured by Olympys model CK30. Luminescence was measured using TECAN microplate luminometer (TECAN, USA). Infinite M200, NanoQuant TECAN multi detection microplate reader was the product of TECAN (USA). Lyophilization was performed using Christ freeze-drier model Alpha 1-4LD (Germany).

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Received March 08, 2012; Accepted March 27, 2012; Published March 29, 2012

Citation: Merzouk A, Ghawi AM, Abdulkader A, Abdullahi AD, Alaama M (2012) Anticancer Effects of Medical Malaysian Leech Saliva Extract (LSE). Pharm Anal Acta S15. doi:10.4172/2153-2435.S15-001

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Leech sampling and saliva collection

Leeches, *Hirudinaria manillensis* (Lesson 1842), were collected from Cheneh Lake, located in Trengganu, Malaysia. They were maintained in well-aerated plastic containers filled with un-chlorinated tap water. Water was regularly changed every two days. The collected leeches were kept under 12 h light and dark cycle at the room temperature ($25 \pm 1^\circ\text{C}$). Samples of the collected leeches were sent to Leeches Biopharm (UK) Ltd, for taxonomy and identification.

Leech saliva extract (LSE) was collected from the starved leeches as we already described using 0.011 M arginine in normal saline as a phagostimulatory solution [16].

Only the colorless salivary fluids vomited by the fed leeches were pooled and centrifuged at $+4^\circ\text{C}$ and at 2500 rpm for 10 min, and the supernatant was filtered using 0.45 μm Sartorius® filter paper. The resultant LSE was aliquoted in 1 ml glass tubes and lyophilized for 24 h. The lyophilized LSE was dissolved in a proper volume of distilled water to be concentrated ten folds, and the resultant solution was termed as ten-time concentrated leech saliva extract ($10 \times$ LSE) which was used during the experimental procedures after filtration using a sterile 0.2 μm Sartorius® filter paper.

Total protein estimation

Total protein assay was performed according to the standard protocols of Bradford assay [17] and Amresco Bradford reagent kit using BSA as a standard protein and the phagostimulatory solution as a blank.

Cancer cell line and cell culture

Human small cell lung cancer SW1271 cell line was obtained from the American Type Cell Collection ATCC. Cells were cultivated at an initial inoculum cell concentration of 10^4 cells/cm² in 15 ml complete growth media (CGM) which consists of Leibovitz's L-15 medium supplemented with 10% FBS (v/v), 0.3 g/L of L-Glu, and 1% (v/v) pen/strep in Corning® 75 cm² canted neck cell culture flask. The cultivated cells were incubated at 37°C in CO₂-free humidified atmosphere. Media was changed as needed. When the monolayer of anchorage-dependent cell line is near 90% confluent, they were subcultured using accutase® as dissociating agent [18]. Cell counting was performed using trypan blue dye exclusion method [19].

The antiproliferative activity of LSE

Cells were seeded at a density of 10^4 cells/well in a sterile Corning Costar® 96-well flat bottom cell culture microplate containing 200 μl of CGM using 8-channel eppendorf® micropipettor. After all, the microplates were incubated at 37°C in free-CO₂ humidified environment for 24 hours [18]. After 24-hour incubation period; the medium was discarded and replaced by new 180 μl of CGM. After that, volumes of 20 μl , a series of double dilution of the sterile $10 \times$ LSE were pipetted.

Other plates were prepared following the same protocols replacing $10 \times$ LSE by Carboplatin and Irinotecan as positive controls and the ten-time concentrated phagostimulatory solution $10 \times$ PHS as a vehicle control with serial twofold dilution of each. Another negative control plate was prepared containing untreated cells (10^4 cells/well) cultivated in 200 μl of CGM.

In addition, two plates were prepared using 20 μl of series of double dilution of mixtures consisting of 10 μl of $10 \times$ LSE mixed with 10 μl

of 100 μM Carboplatin or 10 μl of 100 μM Irinotecan. Finally, all plates were incubated at 37°C in free-CO₂ humidified atmosphere for 5 days.

In-vitro cytotoxicity assay and dose-responsive curves

The antiproliferative effect of LSE was performed using CellTiter-Glo® luminescent cell viability assay based on measuring the luminescence signal resulted from the reaction between the Ultra-Glo™ recombinant luciferase and the ATP molecules produced by the metabolically viable cells in the presence of Mg⁺² and molecular oxygen. CellTiter-Glo assay was fulfilled according to the standard protocols provided by the manufacturer [20]. The percentage inhibition was calculated from the following equation:

$$\%inhibition = \frac{Control\ signal - Sample\ signal}{Control\ signal} \times 100$$

The concentration of the test sample which inhibits 50% of cell growth (IC₅₀) was averaged from three replicates and estimated from plotting the percentage of cell growth inhibition against test sample concentration [21]. Plots were carried out using Four Parametric Logistic Equation using Sigma Plot 11.0 software.

Results

LSE collection and total protein estimation

A total volume of 45 ml (pH 6.39) of LSE was collected and lyophilized. Prior to the experiment, the lyophilized LSE was dissolved in 4.5 ml distilled water. The total protein estimation exhibited that the collected LSE contained a total protein of 62.549 ± 3.840 $\mu\text{g}/\text{ml}$. Consequently, it was assumed that the initial concentration used during the cytotoxic activity was 625.49 $\mu\text{g}/\text{ml}$ because LSE was concentrated ten folds ($10 \times$ LSE) after freeze-drying.

The cytotoxic activity of LSE

Results exhibited that LSE had a remarkable anti-proliferation activity against human small cell lung cancer (SW1271 cell line). The concentration of the total protein of leech saliva extract that inhibits the growth of 50% of the treated cells after 5 days of incubation (IC₅₀) was 119.844 $\mu\text{g}/\text{ml}$ (Figure 1). On the other hand, the cytotoxic effect of leech

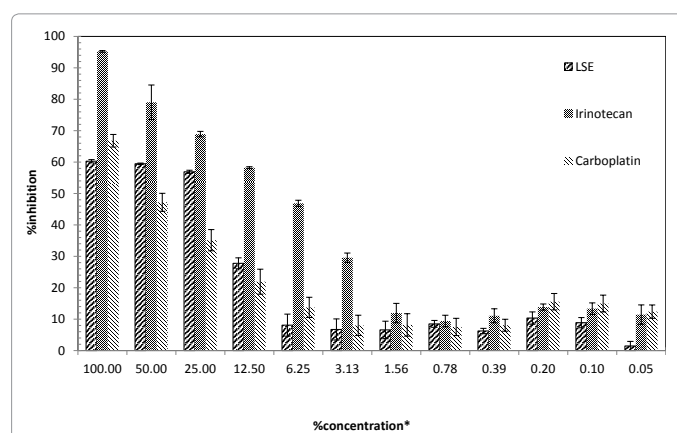


Figure 1: Dose responsive curve of the cytotoxic activity of serial double dilution of leech saliva extract, Irinotecan and Carboplatin on small cell lung cancer cell line SW127. *%concentration = (concentration / initial concentration) \times 100. Cell viability after 5 days of incubation was determined using CellTiter-Glo® luminescent cell viability assay. Inhibition of proliferation is expressed as the mean of three separate replicates \pm SEM (n=3). IC₅₀ was estimated by Four Parametric Logistic Equation using Sigma Plot 11.0 software.

saliva extract was compared with two other reference standard drugs, Irinotecan and Carboplatin, which are currently in use as anticancer agents. It was found that the IC₅₀ of these agents were 5.813 µg/ml and 18.754 µg/ml, respectively (Figure 1). Furthermore, findings of the experiment showed that the ten folds concentrated phagostimulatory solution (10 × PHS) had no effects on cell proliferation.

LSE in combination with Irinotecan and Carboplatin

Furthermore, findings exhibited that there is a kind synergism between the cytotoxicity activity of LSE and that of the experimental reference standards, Irinotecan and Carboplatin. The concentrations of LSE that can suppress 50% of cell growth when combined with another anticancer agent (IC_{50comb}) were calculated from the plots. It was found that LSE has IC_{50comb} of 51.463 µg/ml when combined with Irinotecan which is approximately 57.1% less than the IC₅₀ of LSE used alone (Figure 2 and Table 1). However, Irinotecan experienced about 11.5% decline in the IC₅₀ value to be 5.146 µg/ml. On the other hand, roughly 4.6% reduction in IC₅₀ of LSE was observed when combined with Carboplatin to have an IC_{50comb} of 114.261 µg/ml. On the contrary, Carboplatin showed a dramatic decline in IC₅₀ value by 65%, meaning that IC_{50comb} of carboplatin when combined with LSE was 6.449 µg/ml (Figure 3 and Table 1).

Discussion

The cytotoxic activity of LSE

Our findings revealed that leech saliva extract of the medicinal Malaysian leech *H. manillensis* possessed a dose-dependent cytotoxic effect against small cell lung cancer cell line SW1271. The total protein concentration of LSE that could inhibit 50% of cell growth was 119.844 µg/ml. The maximum percentage inhibition (60%) was observed at a total protein concentration of 625.490 µg/ml corresponding to the ten-time concentrated LSE, which clarifies the reason of concentrating the extract by ten folds before performing the experiment, where as protein concentrations less than 40 µg/ml did not exhibit a remarkable anti-proliferation activity, corresponding to the fifth dilution.

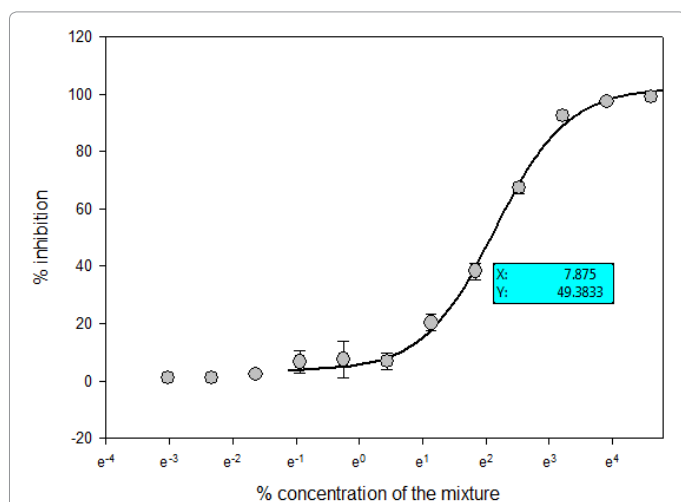


Figure 2: Dose responsive curve of the cytotoxic activity of serial double dilutions of a mixture of LSE and Irinotecan on small cell lung cancer cell line SW1271. It was found that 7.88% of the mixture inhibited 50% of cell growth. Cell viability after 5 days of incubation was determined using CellTiter-Glo® luminescent cell viability assay. Inhibition of proliferation is expressed as the mean of three separate replicates ± SEM (n=3). IC₅₀ was estimated by Four Parametric Logistic Equation using Sigma Plot 11.0 software.

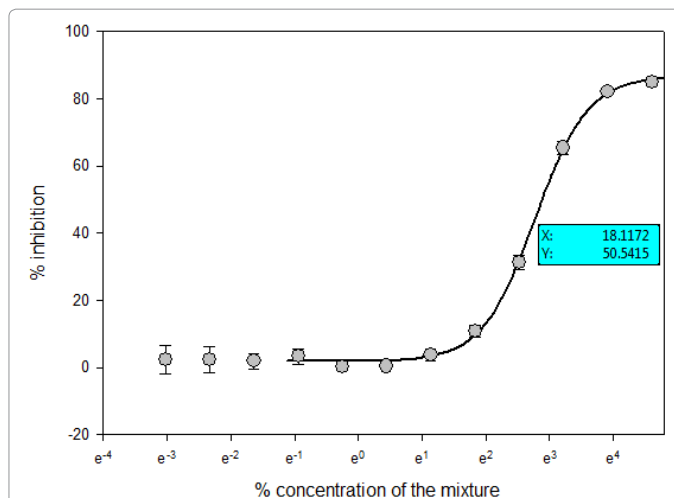


Figure 3: Dose responsive curve of the cytotoxic activity of serial double dilutions of a mixture of LSE and Carboplatin on small cell lung cancer cell line SW1271. It was found that 18.12% of the mixture inhibited 50% of cell growth. Cell viability after 5 days of incubation was determined using CellTiter-Glo® luminescent cell viability assay. Inhibition of proliferation is expressed as the mean of three separate replicates ± SEM (n=3). IC₅₀ was estimated by Four Parametric Logistic Equation using Sigma Plot 11.0 software.

Cytotoxic agents	IC50 (µg/ml)		
Agent 1	LSE	Irinotecan	Carboplatin
Agent 2			
LSE	119.844	5.146	6.449
Irinotecan	51.463	5.813	-
Carboplatin	114.261	-	18.754

Table 1: The effect of leech saliva extract on the cytotoxic activity of Irinotecan and Carboplatin in SW1271 cell line SW1217 was treated by a serial double dilution of LSE combined with Irinotecan/Carboplatin. Cell viability was determined by CellTiter-Glo® luminescent cell viability assay after 5days of incubation. It was found that LSE enhanced the cytotoxic activity of Carboplatin. Whereas, Irinotecan enhanced the antiproliferative activity of LSE.

As far as we know, we are the first who demonstrated the cytotoxic activity of medicinal leech salivary gland secretion, at least against small cell lung cancer. The previously published data evidenced the antimetastatic activity of leech extract from *Haementeria ghilianii* and *Haementeria officinalis* which had been referred to the anticoagulant factors existed in these extracts [13,22,23]. Other reports outlined the usage of the antithrombin peptide, *hirudin*, from *H. medicinalis* as a metastatic inhibitor against a wide range of cancers [13,14]. On the other hand, natural products and their synthetic derivatives currently play a leading role in cancer therapy. The sources of natural anticancer include plants, animals, microorganisms and marine creatures [24]. From annelids, several antitumor components were identified from the body tissue and coelomic fluids of the earthworm *Eisenia fetida*. It was reported that the coelomic fluids of earthworm caused HeLa cell growth inhibition in a dose dependent manner with a concentration of 1 mg/ml was able to inhibit 84% of cell growth [25].

Other studies delineated that the antimicrobial and antitumor activities are related to each other. The first anti-tumoural antibacterial compound was discovered in 1940 from *Actinomyces antibioticus* and then named actinomycin D [24,26]. Additionally, some proteins extracted from the annelid earthworm exhibited both cytotoxic and antimicrobial effects besides to their hemolysis activity [27,28]. Thus,

we can suggest that the antiproliferative activity of LSE maybe due to the existence of antimicrobial components which we previously evidenced [16].

Nowadays, the controversial role of antioxidants in cancer prevention has been arisen from many reports which linked lower cancer incidence with higher consuming of antioxidants-containing diets, such as fruits and vegetables [29]. Many reports outlined that natural antioxidants may display this prophylactic activity via many suggested mechanisms, like the prevention of free radicals-induced DNA damage, cellular components destruction, etc. [30]. Considering all, the current research revealed that the medicinal Malaysian leech saliva extract possessed both antioxidant (not published data) and cytotoxic activities which make it as a promising tool in cancer therapy and prophylaxis.

Furthermore, Irinotecan which we used in our research as a reference standard drug showed a more potent cytotoxic activity than LSE against SW1271 cell line. Irinotecan growth inhibitory activity was dose-dependent with IC_{50} of 5.813 $\mu\text{g/ml}$. It was found that a concentration of 62.315 $\mu\text{g/ml}$ resulted in about 95% cell growth inhibition and a concentration of about 2 $\mu\text{g/ml}$ was able to induce a considerable percentage inhibition of approximately 30%. Dose responsive curve of Irinotecan became less important after the seventh dilution. Irinotecan is the drug of choice for lung cancer patients. SN-38 is the active metabolite of Irinotecan *in vitro*. It was reported that IC_{50} values of SN-38 ranged from 1-300 nM for many lung cancer cell lines (PC-9, PC-9/ZD, PC-14, SBC-3, a549, H69 and PC-7) [31]. Whereas, we demonstrated that the IC_{50} of Irinotecan against small cell lung cancer was about 5.813 $\mu\text{g/ml}$, corresponding to about 1000 nM.

In addition, we also established that Carboplatin was more potent than LSE as an anti-proliferative agent but still less cytotoxic than Irinotecan. Dose responsive curve displayed IC_{50} of 18.754 $\mu\text{g/ml}$. The maximum cell growth inhibitory activity was observed at a concentration of 37.130 $\mu\text{g/ml}$ and then activity was not significant after the fifth dilution. Carboplatin is the typical cytotoxic agent of the second generation of the platinum-based anticancer drugs [32]. Many derivative of Carboplatin was prepared and tested against a wide range of cancer cell lines. The IC_{50} of carboplatin and its synthetic derivatives ranged from 5-50 μM against many human cancer cell lines (HOS, MCF7, A2780, A2780cis, etc.) [33]. Likewise, we found that the IC_{50} of Carboplatin on SW1271 was 18.754 $\mu\text{g/ml}$, corresponding to about 50 μM .

This wide difference between the cytotoxic activity of LSE and the two reference standard drugs can be logically referred to the fact that we used in our investigation the crude LSE which contains, according to what we already published, a wide range of peptides and proteins [34].

LSE in combination with Irinotecan and Carboplatin

We found that the concurrent application, *in vitro*, of LSE and Carboplatin showed a synergistic cytotoxic effect on small cell lung cancer because of a 65% reduction in the IC_{50} value of Carboplatin, whereas, LSE did not experience a considerable decline in IC_{50} when combined with Carboplatin. On the other hand, we evidenced a kind of supra-addition between LSE and Irinotecan when cells were treated with both agents simultaneously. The IC_{50} of LSE was reduced by 57% reaching the value of 51.463 $\mu\text{g/ml}$ and the IC_{50} of Irinotecan showed 11.5% down-fall to be 5.146 $\mu\text{g/ml}$.

In general, many theories have been suggested to explain the interactions between two active drugs including the following [35] including:

- Multiple, concurrent, or complementary effects on a single enzyme.
- Alterations in drug uptake.
- Enhanced drug activity or decreased drug inactivity.

The synergism between LSE, Carboplatin and Irinotecan could be a result of one or more of the above mentioned mechanisms. Thus, more studies are needed to reveal the mechanism of action of LSE as antitumor agent. Moreover, this difference in combination effectivity might be referred to the mechanism of action of each compound and to the stage of cell cycle at which each agent functions [31]. For example, some researchers reported an antagonistic effect of the sequential administration of gefitinib and irinotecan. They argued that gefitinib administration would result in a rise in the G0-G1 phase and a decline in S phase populations. The decreased S phase population was not sensitive to irinotecan. On the contrary, treating cells with SN-38 followed by gefitinib exhibited synergistic effects. They reported that SN-38 treatment produced S phase population increase which is sensitive to irinotecan [31].

It was reported that chemotherapy of cancer by multiple drugs would produce better survival and therapeutic efficacy than the results obtained by using a single agent. Combination chemotherapy may also induce maximum cell kill and boarder spectrum of cytotoxicity [35]. Furthermore, many studies had outlined some acute toxic events (nausea, vomiting, etc.) and delayed toxicity (alopecia, myelosuppression, etc.) accompanied chemotherapy regimens by Irinotecan and Carboplatin [36]. On the contrary, literature about leech therapy did not mention serious cytotoxic conditions except for some post-leeching infections which can be handled by the conventional antibiotics [37]. Consequently, we aimed from this experiment to evaluate the capability of LSE to enhance the cytotoxic effect of the previously known anticancer agents, Irinotecan and Carboplatin, at lower doses. Lower doses would result in less adverse effects of these agents. Based on our research result, we recommend using leech or its salivary gland secretion as an adjuvant therapy, especially when using Carboplatin.

Conclusion

Aside from the well-known antimetastatic activity of leech saliva, we reported here for the first time that the salivary gland secretion obtained from the medicinal Malaysian leech, *H. manillensis*, had an antiproliferative activity against small cell lung cancer besides to its positive synergism with Carboplatin. Hence, further studies still are needed on this issue to isolate and identify the active principle, to study the mechanism of action, to evaluate its effect on other cell line types, etc.

Acknowledgment

The authors would gratefully acknowledge Roy T. Sawyer and Fred O. P. Hechtel, Leeches Biopharm (UK) Ltd, for their highly appreciated assistance in leech taxonomy.

This research was supported by the grant (EDW B 10-0393) from Research-Management Center, International Islamic University Malaysia.

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