Effects of methanolic plant extracts on cell proliferation and HIF activity under hypoxic condition in vitro

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Abstract. Low oxygen tension is termed as hypoxia. Hypoxia will lead to transcription of hypoxia-inducible factor (HIF) and regulation of downstream gene expression. Underexpression or overexpression of HIF was found to be responsible for various diseases. Proper regulation of this transcriptional factor will aid in treatment of the related diseases. Nowadays, many different approaches are used to modulate HIF, including the usage of naturally-derived plant extracts. Plant extracts are widely accepted compared to other treatments as they are less harmful to the patient and are widely available. In this study, the cytotoxicity of eight different plant extracts under two different gaseous conditions, hypoxic and normoxic, were examined. We also examined the HIF activity shown by the cells under treatment of various concentrations of plant extracts. All eight plants were dried, blended, extracted using methanol, and evaporated to form crude plant extracts. MTT assay was performed by treating the cells with different concentrations of plant extracts and cell viability was determined. Meanwhile, HIF activity of the cells was evaluated by using single luciferase reporter assay. Relative cytotoxicity shown by the cells was different for each plant extract under the various concentration. *Pereskia bleo*, *Orthosiphon aristatus*, and *Clinacanthus nutans* showed high cell viability, 80% of cell viability, within the range of concentration tested. In contrast, *Gynura procumbens*, *Hydrocotyle sibthorpioides*, *Pereskia grandifolia*, *Strobilanthes crispus*, and *Melastoma malabathricum* showed low cell viability. Most of the cells showed activation of HIF activity when treated with different concentrations of the plant extracts. When cells were treated with high concentrations of plant extracts, inhibition of HIF activity were seen and was correlated with low cell viability after treatment. The most notable part of the study was that more than 100% HIF activation was observed for *Clinacanthus nutans*. However, the cell viability remained high. This might indicate that *Clinacanthus nutans* is a promising candidate to activate HIF at a transcriptional level with minimal cytotoxicity.

Keywords: HIF activation, hypoxia, plant extracts

INTRODUCTION

Oxygen is a basic survival need for living organisms. Oxygen deprivation in cells or tissues is termed as hypoxia (Harris, 2002). Cellular responses during hypoxia are executed by a transcription factor known as hypoxia-inducible

factor (HIF). HIF is a dimeric transcriptional factor that consists of an oxygen dependent HIF- α , and constitutively expressed HIF- β isomers (Stroka *et al.*, 2001). The aftermath of HIF expression can be deleterious or protective

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towards our body. HIF was claimed to be responsible to various diseases such as human cancers and chronic kidney disease (Mabjeesh and Amir, 2007; Gunaratnam, 2009). Upregulation of HIF was found to activate the transcription of downstream genes that intensify disease progression. However, appropriate suppression of HIF can therapeutically target these HIF-related diseases. On the other hand, HIF activation is reputed to be useful in treating ischemia and aid in wound healing for diabetic patients as upregulation of HIF stimulates angiogenesis (Ziello *et al.*, 2007; Mace *et al.*, 2007). Angiogenesis is said to be able to deliver more oxygen supply to specific regions by promoting formation of new blood vessels from pre-existing vasculates (Birbrair *et al.*, 2015).

Identification of new chemical entities (NCEs) is essential for the development of new drugs. In recent years, plant extracts have become new targets for drug discovery and a source of NCEs, which are isolated from plant extracts (Katiyar *et al.*, 2012). Drug discovery based on natural products mainly target these predominant therapeutic areas: anticancer activity, immunosuppression, antimicrobial activity, and cardiovascular health (Pan *et al.*, 2013). Alternative complimentary medicine, especially naturally-derived plant extracts, turn out to be a preferable choice compared to current treatment modalities due to better tolerance and acceptance in patients (Paul *et al.*, 2013). In addition, plant extracts are naturally available, thus making them more accessible.

There are many diseases related to HIF. Plant extracts might be the key to developing more drugs to combat HIF-related diseases. The main objective of this study is to examine the relationship between cell cytotoxicity of the medical plant extracts and the corresponding HIF activity level. Therefore, we investigated the concentration-dependent effect of the relevant plant derived extracts towards HIF activity using an established HIF luminescent cell-based assay system (Shafee *et al.*, 2015). The plant extracts were used to treat Saos-2 cells that were stably transfected with hypoxia-driven firefly luciferase plasmids containing four copies of the erythropoietin (EPO) hypoxia response elements (HRE; Liew *et al.*, 2015).

MATERIALS AND METHODS

Cell culture and culture condition. Cultured Saos-2 human osteosarcoma cells and C3, the genetically-modified Saos-2 cells (MY Patent filing no. PI2012003492, PCT no. WO2014021705 A1, U.S. filing no. 14/415,064, India filing no. 205/DELNP/2015, Europe filing no. 13713255.1 and China filing no. 2015021200444780) which stably expressed firefly luciferase under the control of four copies of the HRE of the EPO gene upon hypoxia induction were used in the study. The low oxygen environment at 0.5% O₂ was created by culturing the cells at 37°C in the hypoxic incubator (Galaxy 48R, New Brunswick, USA). The incubator was set to 94.5% N₂, 5% CO₂ and 0.5% O₂.

Preparation of plant extracts, cell culture treatment condition and cell proliferation assay. Eight types of medicinal plants were evaluated in this study. They are *Gynura procumbens*, *Hydrocotyle sibthorpioides*, *Pereskia bleo*, *Pereskia grandifolia*, *Orthosiphon aristatus*, *Clinacanthus nutans*, *Strobilanthes crispus* and *Melastoma malabathricum*. The plants were identified by the botany taxonomist from Institute of Bioscience (IBS), Universiti Putra Malaysia (UPM). Leaves of the tested plants were washed with distilled water, dried and subjected to methanolic extraction as described in Liew *et al.*, 2012. Before treatment, the plant extracts were dissolved in serum-free media with a final DMSO concentration of less than 0.5% (v/v). Then, the mixtures were centrifuged at 1 000 xg and filtered through 0.22 mM filters (Sartorius, USA). For plant extracts treatment, cells were seeded at 1.5×10^4 cells/well in a 96-well plate. After the cells were treated with selected concentrations of plant extracts, the cells were incubated in hypoxic conditions. After 24 h of incubation, a viability MT_T assay (Vijayakumar & Ganesan, 2012) was performed to measure the cell viability.

Single luciferase reporter assay. In order to ensure the HIF regulatory effect of the selected medicinal plants, C3 cells, the genetically-modified Saos-2 cells as mentioned above was utilized to measure the HIF activity. C3 cells were seeded at 1.5×10^4 cells/well in DMEM

supplemented with 10% FBS in 96-well plate for 24 h. After overnight incubation, cells were treated with selected plant extracts in a dose-dependent manner and followed exposure to hypoxia (0.5% O₂) for another 24 h. Bright-Glo™ single luciferase reporter assay was

conducted as described according to the manufacturer's instruction to evaluate firefly luciferase expression which was an indication of the level of HIF activity of C3. HIF activity was calculated by using the formula below:

$$\text{HIF activity} = \frac{\text{HIF activity of the desired concentration} - \text{HIF activity of control}}{\text{HIF activity of control}} \times 100\%$$

Positive value of HIF activity indicates HIF inhibition whereas negative value indicates HIF activation.

Statistical analysis. The Student t-test was used to analyse the experimental data in this study. Results were expressed as mean $\pm$ SEM by using Graph Pad Prism 5.

RESULTS AND DISCUSSION

Cell viability under hypoxia. In this study, we intended to study the concentration-dependent effect of the above mentioned methanolic plant extracts towards cell viability and HIF activity of Saos-2 cells under hypoxia. In order to determine cell viability and HIF activity, C3, the stable cell-based HIF assay system was employed. Cisplatin (Duyndam *et al.*, 2007; Figure 1A) and bortezomib (Befani *et al.*, 2012; Figure 1B) were included as positive controls. During treatment under hypoxic condition, cell viability decreases upon increasing concentrations of cisplatin, but bortezomib does not show any cytotoxic effect.

For *G. procumbens* and *H. sibthorpioides*, they displayed different cytotoxicity levels when treated with different concentrations under hypoxia. However, both plant extracts demonstrated a high induction in cell viability when cells were treated with the first two concentrations used, 500 and 750 $\mu\text{g/ml}$ for *G. procumbens*, and 125 and 250 $\mu\text{g/ml}$ for *H. sibthorpioides* (Figures 1C and 1D). A decrease in cell viability for both methanolic plant extracts was observed for treatments beyond these two concentrations. Similar killing curve patterns were obtained for cells treated under normoxic

conditions (Supplementary Figure 1). The increase in cell viability was supported by few researchers who showed that both plant extracts assisted in the wound healing process (Somboonwong *et al.*, 2012, Zahra *et al.*, 2011). Our findings in cell proliferation under either hypoxia or normoxia may contribute to an understanding of the mechanisms underlying the wound healing process.

P. bleo and *P. grandifolia* are both from the cactus genus. Within the *Cactaceae* family, these are the only ones with green leaves (Sharif *et al.*, 2013). Even though both of the plants are from the same genus, the extracts exhibited different cytotoxic effects on the cells. *P. bleo* showed no obvious cytotoxicity, but a gradual increase in cell viability even though C3 was treated with a high concentration under hypoxia (up to 1500 $\mu\text{g/ml}$; Figure 1E). Similar conditions were also observed in the plant extract treated cells that were incubated under normal oxygen levels (Supplementary Figure 1). The findings, done under normoxia, contrasted a study conducted by Tan *et al.* (2004) that displayed how *P. bleo* killed almost 90% of the cells at a very low concentration, 3 $\mu\text{g/ml}$. Under hypoxic conditions, cells treated with concentrations of *P. grandifolia* lower than 50 $\mu\text{g/ml}$ retained almost 100% cell viability (Figure 1F). Then, a decrease in cell viability was observed when cells were treated with 75 $\mu\text{g/ml}$ of plant extract and remained at the same level when higher concentrations were used. This was dissimilar to what was observed for the *P. grandifolia*-treated cells when incubated in normoxic condition. High mortality, and less than 50% cell viability were detected when concentrations higher than 75 $\mu\text{g/ml}$ of methanolic plant extract were used (Supplementary Figure 1). Results obtained from

the cells treated with *P. grandifolia* under normoxia were in agreement with a study by Nurestri *et al.* (2009), in which cytotoxicity was observed towards several cancer cell lines by having IC₅₀ values ranging from 34 to 88 µg/ml.

For *O. aristatus* and *C. nutans*, both plants exhibited minimal cytotoxicity towards C3 under hypoxic conditions (Figures 1G and 1H). Overall, C3 treated with various concentrations under hypoxia showed cell viability of more than 70%. A similar concentration-dependant curve was observed for *C. nutans* treated cells incubated under normal oxygen concentrations, but the opposite was observed for *O. aristatus*. In normoxic condition, there was a drop in cell viability for cells treated with 400 µg/ml of *O.*

aristatus plant extract (Supplementary Figure 1). Vijayan *et al.* (2013) reported that *O. aristatus* extract had limited cell inhibitory activity, showing high IC₅₀ values for several treated cancer cell lines under normoxia. Several studies reported severe cytotoxicity for methanolic *C. nutans* plant extracts towards different cancer cell lines by providing IC₅₀ values that ranging from 47 µg/ml to 96 µg/ml when the cell was treated under normal oxygen concentrations (Yong *et al.*, 2013; Arullappan *et al.*, 2014). Results from the prior research were in contrast to our current study. One of the possible explanations for the contrast is that different types of cancer cell lines were used.

Figure 1. MTT assay for concentration-dependant of positive control drug and each plant extract towards cell viability under hypoxia. (A) Cisplatin (B) Bortezomib (C) *Gynura procumbens* (D) *Hydrocotyle sibthorpioides*.

E **F** **G** **H** **I** **J**

Figure 1. (continued). MTT assay for concentration-dependant of positive control drug and each plant extract towards cell viability under hypoxia. (E) *Pereskia bleo* (F) *Pereskia grandifolia* (G) *Orthorsiphon aristatus* (H) *Clinacanthus nutans* (I) *Strobilanthes crispus* (J) *Melastoma malabathricum*.

In hypoxia, *S. cripus* showed no prominent cytotoxicity under low plant extract concentrations of less than 100 µg/ml (Figure 1I). However, at concentrations higher than 200 µg/ml, the cells treated with plant extract exhibited evident decrease in cell viability. A similar curve pattern was noted for cells treated in normoxia at concentrations of 300 µg/ml (Supplementary Figure 1). In addition, cell viability was shown to be less than 15% when the cells were treated with 500 µg/ml of plant extract. In year 2005, Yaacob *et al.* demonstrated that *S. cripus* was able to induce almost 100% cell death when cells were treated with 100 µg/ml of plant extract under normal oxygen concentrations. In the current study, cells cultured both hypoxic and normoxic conditions showed minimal cell viability after treatment with *S. cripus* plant extract. *M. malabathricum* exhibited a gradual decrease in cell viability when the cells were treated with increasing concentrations of plant extracts. The same phenomena was observed for both hypoxic and normoxic incubated cells (Figure 1J). However, plant extract concentrations used to treat the cells were low, with a maximum concentration of 40 µg/ml. Previous studies from Devehat *et al.* (2002) and Zakaria *et al.* (2011) reviewed this plant extract, stating that under normal oxygen concentration, the IC₅₀ of the extract ranging from 13 to >88 µg/ml for a panel of cancer cell lines. This suggested the cytotoxicity of this plant extract varied according to the cancer cells.

HIF activity. HIF is the master regulator for cells under low oxygen concentrations. HIF activity is the indication of cellular response for cells treated with various plant extracts under hypoxic conditions. To investigate HIF activity, a hypoxia-driven reporter assay (Kaluz *et al.*, 2008) was performed in C3 samples treated with different concentrations of plant extracts. The signal intensity emitted from the assay is directly proportional to HIF activity. Positive values indicate inhibition of HIF activity while negative values showed HIF activation. The positive controls, cisplatin and bortezomib were shown to inhibit HIF activity tremendously (Figures 2A and 2B). Both cisplatin and bortezomib are well

known HIF inhibitors (Duyndam *et al.*, 2007; Onnis *et al.*, 2009).

For *G. procumbens* and *H. sibthorpioides*, HIF activation was seen when the cells were treated with plant concentrations of 1000 and 750 µg/ml respectively (Figures 2C and 2D). Whereas, for *P. bleo*, HIF activation was observed for all treatment concentrations (Figure 2E). *P. grandifolia* only showed HIF inhibition at 100 µg/ml (Figure 2F). The concentrations that displayed the highest HIF activation for the above mentioned four plants were 750, 500, 1500, and 50 µg/ml respectively. For all four plants, cell viability exceeded 100% when the cells exhibited highest HIF activation levels. *O. aristatus* and *C. nutans* both displayed HIF activation for all concentrations of plant extracts (Figures 2G and 2H). HIF inhibition was observed when C3 cells were treated with concentrations of *S. cripus* exceeding 300 µg/ml, and with *M. malabathricum* exceeding 1500 µg/ml (Figures 2I and 2J). HIF inhibition seen in *G. procumbens*, *H. sibthorpioides*, *P. grandifolia* and *S. cripus* were due to low cell viability after treatment. Throughout the study, *C. nutans* was the only plant extract that displayed more than 100% of HIF activation alongside minimal cell cytotoxicity.

Adaptation and cellular responses towards hypoxic environments are complex. These adaptations play an important role in normal cellular physiology. HIF is the master regulatory upon all the hypoxia cellular responses (Semenza, 2011). This regulatory is thought to be responsible for the occurrence of many diseases. The use of plant extract is considered an optional alternative for drug discovery research targeted at curing these diseases. Therefore, the screening of different plant extracts dosages in this study helps in understanding the effect of the extracts towards cells viability and HIF activity under hypoxia. To conclude, evidence of more than 100% HIF activation and minimal cell cytotoxicity towards cells when treated with *C. nutans* makes it as a primary candidate for HIF activation-related drug screening. Potent HIF activators exhibit low toxicity and can be identified by showing activation of HIF activity at a transcriptional level (Nagle & Zhou, 2006).

Figure 2. HIF activity shown by C3 cells when treated with plant extracts of various concentration and incubated under hypoxia. (A) Cisplatin (B) Bortezomib (C) *Gynura procumbens* (D) *Hydrocotyle sibthorpioides* (E) *Pereskia bleo* (F) *Pereskia grandifolia*.

Figure 2. (continued). HIF activity shown by C3 cells when treated with plant extracts of various concentration and incubated under hypoxia. (G) *Orthorsiphon aristatus* (H) *Clinicantus nutans* (I) *Strobilanthes crispus* (J) *Melastoma malabathricum*.

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