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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 cripus, and Melastoma malabathricum showed low cell viability. Most of the cells showed activation of HIF activity when treated with different concentrations of 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
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. PI201203492, 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 Gymnura procumbens, Hydrocotyle sibbodioides, Pereskia bleo, Perssokia 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 X 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 MTT 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 X 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 ± 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 μg/ml for G. procumbens, and 125 and 250 μg/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 μg/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 μg/ml. Under hypoxic conditions, cells treated with concentrations of P. grandifolia lower than 50 μg/ml retained almost 100% cell viability (Figure 1F). Then, a decrease in cell viability was observed when cells were treated with 75 μg/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 μg/ml of methanolic plant extract were used (Supplementary Figure 1). Results obtained from
Effects of methanolic plant extracts on HIF activity

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*. 
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 1). 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 (Kaluza 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. bloo*, 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) Orthosiphon aristatus (H) Clinacanthus nutans (I) Strobilanthes crispus (J) Melastoma malabathricum.

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REFERENCES


Supplemental Figure 1: MTT assay for plant extracts treated C3 under normal oxygen condition. The eight plant extracts include *Gynura procumbens*, *Hydrocotyle sibthorpioides*, *Pereskia bleo*, *Pereskia grandifolia*, *Orthosiphon aristatus*, *Clinicantus nutans*, *Strobilanthes crispus* and *Melastoma malabathricum*.
The effect of Malaysian stingless bee, *Trigona* spp. honey in promoting proliferation of the undifferentiated stem cell

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**Abstract.** Stem cells provide various potential applications in regenerative medicine through its ability of self-renewal and differentiation. Among the various stem cells, dental pulp stem cells (DPSCs) have shown encouraging results in their ability to regenerate. Honey has been used in traditional culture as a natural medicine in supporting wound healing. Yet, very few studies on honey were conducted for its potential as a proliferative agent for stem cells. The aim of this study is to evaluate the stability of two *Trigona* spp. honeys (1 and 2) added in culture media and its proliferative effect on DPSCs. Both honeys were diluted with standard culture medium through dilution process to prepare the concentrations of 0.01%, 0.04%, 0.10% and 0.25%. DPSCs were treated with the diluted honeys for 24 hours. The proliferative activity was determined through the images taken using an inverted microscope for every six hours. In addition, the MTT assay was conducted to determine the cell viability of DPSCs when treated with both honey 1 and 2 at various concentrations. The results showed a stable culture media added with honey for three days and a dose-dependent proliferative effect of both *Trigona* spp. honey samples on DPSCs. Optimum proliferative effects were observed at 24 hours for both *Trigona* spp. honey 1 and 2 on DPSCs. The optimum concentration of *Trigona* spp. honey 1 was from 0.04% to 0.10% and *Trigona* spp. honey 2 was below 0.01%. It is concluded that *Trigona* spp. honey has a promising proliferative effect on DPSCs.

**Keywords:** *Trigona* spp. honey, mesenchymal stem cells, dental pulp, DPSCs, kelulut

**INTRODUCTION**

In recent years, many studies have focused on the possible application of stem cells to mend and regenerate body structures. By definition, stem cell is a cell that has the ability to divide (self-replicate) for indefinite periods throughout the life of the organism (Chandross & Mezey, 2001). Stem cells are immature, unspecialised cells that have the potential to develop into many different cell lineages. For example, they can develop into mature cells that have characteristic shapes and
specialised function, such as heart cells, skin cells, or nerve cells. Generally, stem cell research in regenerative medicine focuses on restoring the vitality and function of diseased and traumatised tissue (Noce et al., 2014).

Mesenchymal stems cell (MSC) was obtained from the dental pulp of teeth. Dental pulp is a connective tissue that contains MSCs known as dental pulp stem cells (DPSCs) that is relatively easy to be obtained from the exfoliated primary teeth or extracted permanent teeth (Piva, Silva & Jacques, 2014) and is able to differentiate into different kinds of cells and tissues. As compared to bone marrow MSCs, DPSCs has higher proliferation rate, availability, and greater cell number (Noce et al., 2014).

In the context of regenerative therapies, DPSCs hold great promise for cell therapy approaches in dentistry with studies already showing encouraging results in their ability to regenerate bone and periodontal tissues. Specifically, the identification of DPSCs promises a great potential in the regeneration of lost dental pulp and tooth tissues because of their ability to differentiate into dentin and pulp-like tissue (Eubanks et al., 2014). Previous studies from Obeid et al. (2013) have shown that DPSCs are putative candidate cells for tooth and bone-tissue engineering. The identification of the MSCs capability in regenerating organised tooth-like structures has increased the interest in the potential use of postnatal stem cell–based therapies for dental tissue regeneration after trauma or disease.

Honey is a naturally available food with a long history of traditional use as an active medicinal compound in a large number of cultures. It contains a variety of phytochemicals with high flavonoid and phenolic content that become the main sources of high antioxidant activity of honey (Yao et al., 2003). Various findings have proven that this natural product does not only exhibit antioxidant properties, but also immunoregulatory, chemopreventive, antiatherogenic, wound healing, and antimicrobial properties (Molan, 2006; Tsiapara et al., 2009; Khalil et al., 2010; Sherlock et al., 2010).

In this respect, honey is well known for its healing potential. Understanding the healing potential in honey requires an understanding of the detail of its physico-chemical characterisation and also an analysis of biological impacts at different concentrations/dilutions at different levels (e.g. cellular and molecular) (Chaudhary et al., 2015). Research on the benefits of honey for health industry has been extensively conducted in many countries all over the world. However, there are few findings on the benefits of various honeys that exist in Malaysia, particularly Trigona spp. honey. In a comparison between the Tualang and the Trigona spp. honey, the latter has not been widely studied with only several findings on the effect of Trigona spp. propolis are reported. Thus, it is important to thoroughly study the benefits and potential contributions of the Malaysian stingless bee honey to the human health, especially as a proliferative agent.

Honey produced by stingless bee (Trigona spp.), which is also known as ‘kelulut’ in Malaysia, has been identified to have medicinal values. Trigona spp. honey is widely used by the aborigines and is considered to induce better performance than honeybee honey (Shahjahan et al., 2007). The Malaysian Agricultural Research and Development Institute (MARDI) in Malaysia conducted research on this species and found that Trigona spp. consists of over 30 species in Malaysia. Among the species of Trigona spp. are Trigona itama, Trigona apicalis, Trigona laeviceps, and Trigona thorasica (Aquaponic Cultures Malaysia [ACM], 2012).

Trigona species are distributed throughout the tropical and subtropical parts of the Afrotropical, Australasian, Indo-Malayan, and Neotropical regions (Klasikorn et al., 2005). Among the countries that have been identified to own Trigona spp. are Indonesia (37 species), Thailand (20 species), Vietnam (9 species), India (3 species), Taiwan (1 species) and Malaysia (more than 30 species) (ACM, 2012).

Healing with diluted honey rather than its raw form is also proposed considering the fact that a low concentration of hydrogen peroxide in a healing agent has bactericidal effect but is not toxic on mammalian cells (Molan, 2006). Therefore, knowledge on the optimum dilution of honey is imperative in order to determine its maximum healing efficiency. Impacts of specific honey dilutions on epithelial cell population in vitro also need to be investigated.

Herein, this study aimed to investigate the potential role of Trigona spp. honey in proliferative...
effect on dental pulp stem cells (DPSCs). Hence, this study is designed to evaluate the differential dosage effect of Trigona spp. honey. It is hypothesised that honey from this species is able to increase the proliferative activity of DPSCs in a dose- and time-dependent manners.

**MATERIALS AND METHODS**

**Honey procurement.** Trigona itama species honey from two different places were procured from Assoc. Prof. Dr. Muhammad bin Ibrahim, from Department Nutrition Science, Kulliyah of Allied Health Science, International Islamic University Malaysia (IIUM). It was available in the market and the places were not to be revealed in this study in order to avoid any conflict of interest.

**Dental pulp stem cells (DPSCs) procurement.** Human DPSCs (Passage 10, P10) were obtained from Dr. Shamsul Azlin (which was derived from his lab), The Department of Biology, University Malaya. The validations of cells were done by identifying the protein specific markers of CD105 and CD90 with fibroblast-like shape of the cells (Helena, Alencar, & Kitten, 2011; Nikolić et al., 2011). The cells were cultured and maintained in Molecular and Cellular Biology Lab, Integrated Centre for Research Animal Care and Use (ICRACU), IIUM.

**Serial dilution of honey.** The sample was kept in a tightly packed and sterilised container to avoid contamination until use and was certified by the Ministry of Health Malaysia (MOH) for marketing purposes. The absolute honey (100%) with the initial pH 3.3 and moisture content of 12% was then diluted at the concentrations of 0.01% (v/v), 0.04% (v/v), 0.10% (v/v), and 0.25% (v/v) with culture medium. The honey mixtures were prepared 24 hours before being tested to cell lines and stored at 4°C until use.

**Media stability test.** Each media was prepared with different concentrations of Trigona spp. honey 1 and 2. About 5 ml of each concentration were delivered into T25/25 cm² cell culture flasks. Then, the media added with honey was kept in 5% CO₂ incubator at 37°C for three days. Images were taken before and after three days in the incubator using EVOS XL CORE (Thermo Fisher Scientific, USA).

**Cell culture.** DPSC lines were propagated in Dulbecco’s modified Eagle’s medium (DMEM) in three different cell culture flasks, all supplemented with 10% fetal bovine serum (FBS) and 5% penicillin/ streptomycin, and maintained at 37°C in 5% CO₂ incubator.

**Proliferation test.** The proliferation test was performed based on the work of Fauzi et al. (2011) using haemocytometer with modification. In order to perform the proliferation assay for DPSC lines, at least 5,000 or 5 x 10³ cells per well are recommended. The cells were seeded in 12-well plate at three different cells density; 5 x 10³ cells, 10 x 10³ cells, and 15 x 10³ cells well. The cells were incubated overnight at 37°C with 5% CO₂ and treated for 24 hours at four different concentrations of both Trigona spp. honey samples 1 and 2 (0.01%, 0.04%, 0.10% and 0.25% respectively). Images were taken using an inverted microscope for every 6 hours interval until 24 hours of treatment at 10X magnification.

**Evaluation of cell viability in media added with honey and control.** In this study, we conducted the cell viability by using (3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide [MTT]) colorimetric assay. DPSC cell lines were seeded at a density of 5000, 10000, and 15000 cells/well in a 96-well plate. The best-seeded number of cells was analysed in this paper. The three densities were chosen based on the optimum densities obtained from the proliferation test. After an incubation period of 24 hours, the cells were washed with phosphate buffered saline (PBS). The different concentrations (0.01% (v/v), 0.04% (v/v), 0.1% (v/v), and 0.25% (v/v)) of honey were added into the cells and incubated at 37°C and 5% CO₂ for 24 hours. A 20 µl of MTT reagent (0.5 mg/ml) was dispensed into each well and incubated for 2 hours. Subsequently, MTT reagent was aspirated and 200 µl of dimethyl sulfoxide (DMSO) was added to solubilise the formazan. This technique was performed in a dark room. The absorbance was recorded using spectrophotometer under a wavelength of 450 nm. Each experiment was
replicated three times for each concentration and result obtained was compared with the control.

Data analysis. All proliferative activities of DPSCs were observed through the images taken by an inverted microscope (EVOS XL CORE, Thermo Fisher Scientific, USA) for 24 hours with the intervals at 0, 6th, 12th, and 24th hour. Images obtained were observed and compared with the control. Comparisons within the groups of data were analysed using ANOVA and the probability of p<0.05 was considered statistically significant.

RESULTS

Effect of Trigona spp. honey on culture media. Both Trigona spp. honey samples of 1 and 2 were tested for their stability with media containing DPSCs at the concentration of 0.01%, 0.04%, 0.1% and 0.25% v/v. Results showed that after 72 hours, all media did not change its appearance or colour as compared to the control medium (Figure 1).

Figure 1. Comparison of media stability based on different concentrations of Trigona spp. honey 1 and 2.

Effect of Trigona spp. honey on DPSC proliferation. All the four concentrations showed relative proliferation activities when compared to the control.
The obtained proliferative activities varied not only in concentrations and time, but also between the two different samples (Trigona spp. honey 1 and 2). The observations were made based on the result of the images captured which are more on the qualitative result and yet to be confirmed by MTT assay for quantitative result.

Figure 2 shows the overall images of $15 \times 10^3$ DPSCs in 24 hours based on the different concentrations of Trigona spp. honey 1 (result for $5 \times 10^3$ and $10 \times 10^3$ are not shown). The best-seeded number of DPSCs cultured with Trigona spp. honey 1 was $15 \times 10^3$ and showed an increase in proliferation rate from 0.04% (v/v) to 0.10% (v/v) concentration. However, the proliferation rate was slower at a higher concentration of 0.25% (v/v) (Figure 2). Meanwhile, in DPSCs treated with Trigona spp. honey 2, higher proliferation rate was only observed at the concentration of 0.01% (v/v) (Figure 3). Therefore, higher proliferation rate was achieved 24 hours after being treated with both Trigona spp. 1 and 2. Thus, it can be suggested that both Trigona spp. honey 1 and 2 are able to induce a significant proliferation activity of DPSCs in their respective range of concentrations.

![Figure 2](image-url) Overall images $15 \times 10^3$ DPSC in 24 hours based on different concentrations of Trigona spp. honey 1. Magnification X20.
Evaluation of honey on cell viability. To confirm the result obtained from the proliferation test, MTT assay was carried out to look at the optimum proliferation effects of both honeys. Only the concentrations that gave higher proliferative activity as compared to the control were chosen for cell viability test, which are Type 1 Honey 0.04% and 0.1%, and Type 2 Honey 0.01% at 15 x 10^3 seeded cell number. Figure 4 demonstrated that the optimum proliferation effects of *Trigona* spp. honey 1 are within the concentration 0.04% (v/v) and 0.10% (v/v). Meanwhile, for *Trigona* spp. honey 2, the optimum value is at concentration 0.01% (v/v) (Figure 5). In addition, both *Trigona* spp. honey 1 and 2 showed that the best-seeded number of DPSCs cultured was 15 x 10^3 cells/well.

**Figure 3.** Overall images 15 x 10^3 DPSC in 24 hours based on different concentrations of *Trigona* spp. honey 2. Magnification X20.

**Figure 4.** Effects of media added with *Trigona* spp. honey 1 on DPSCs at seeding density of 15,000 and treated at the concentrations of 0.04% and 0.10%. Data are presented as ±s.d. and represent of three experiments. Significance at p-value is less than 0.05.
Proliferative effect of Trigona spp. honey on stem cells

Figure 5. Effects of media added with Trigona spp. honey 2 on DPSCs at seeding density of 15,000 and treated at the concentration of 0.01%. Data are presented as ±s.d. and represent three experiments. Significance at p-value is less than 0.05.

DISCUSSION

Honey is a very complex product because its chemical properties and composition depend not only on the nectar-providing plant species, but also on other factors such as the bee species, geographic area, season, mode of storage, harvest conditions, and even the interactions between the chemical compounds and enzymes in the honey. It is known that the accumulation of phytochemicals in honey depends on climatic conditions (sunlight, moisture), soil characteristics, and other factors. Therefore, it is reasonable to believe that the differences between honey from country to country appear due to the different compositions of pollen or nectar, which have the greatest influence on the chemical composition of honey. For instance, essential oil composition is very dependent on the geographical location even for the same plant species thus suggesting that even the same floral origin honey composition may be quite different (Kaškonienė & Venskutonis, 2010).

Most of the available culture media include phenol red as a pH indicator, which allows constant monitoring of pH. During the cell growth, the medium changes colour as pH is changed due to the metabolites released by the cells. At low pH levels (increased acidity), phenol red turns the medium to yellow, while at higher pH levels it turns the medium to purple. The medium is bright red for pH 7.4, the optimum pH value for cell culture (Arora, 2013). From Figure 1, images showed that there were no changes in the colour of DMEM media without DPSCs after being incubated for three days. This showed that the substances from both Trigona spp. honey 1 and 2 did not alter the stability of the media.

Media supplements are the growth media recommended for certain cell lines that require additional components which are not present in the basal media and serum. These components and supplements help to sustain cell proliferation and maintain normal cell metabolism (Arora, 2013). The additional substances from both Trigona spp. honey samples did not alter or change the standard culture media of DPSCs by providing a stable colour of pH 7.4.

The addition of Trigona spp. honey might affect the osmolality of the culture media as well as its stability. For most of the cell lines, optimal osmolality for culture media should be between 260 mOsm/kg and 320 mOsm/kg (Arora, 2013). Thus, the change of the culture media osmolality will affect the DPSCs proliferative activities. This factor may be the reason why Trigona spp. honey 1 showed proliferative activity at 0.04% and 0.10% concentration, while Trigona spp. honey 2 showed only active proliferative activity at 0.01% concentration. Both of the Trigona spp. honey samples may have different osmolality due to the different chemical compositions and concentrations since the addition of supplements from honey can change the osmolality of the complete growth media that can negatively affect the growth of cells. It is always best to recheck the osmolality after supplements are added because it is essential in ensuring that the cells can keep surviving and proliferating. High osmolality could affect the water from the cells to rush out, resulting in shrinkage. Therefore, the addition of the supplements in the honey might alter the osmolality that can affect the proliferation of the cells.

MTT assay was done to determine the best seeding number for DPSCs to proliferate at optimum range. Three different ranges of cell seeding number were tested in this study; namely 5 x 10^3, 10 x 10^3, and 15 x 10^3 cells/well. The best seeding number of DPSCs was chosen by comparing the overall triplicate of the DPSCs through proliferation test and viability test using the MTT assay. Cell density or cell seeding...
number is known to control the rate of proliferation of cells in culture. The transport of small nutrients also depends on the cell density. It decreases when cell density increases and vice versa. Increases in the uptake of low molecular-weight nutrients are among the early events associated with the initiation of cell growth (Piedimonte, Borghetti & Guidotti, 1982).

From previous studies, Xia et al. (2011) stated that a wide range of endothelial cell seeding densities from $4 \times 10^3$ to $2 \times 10^5$ cells/cm$^2$ have been used for in vitro studies on cell-biomaterial interaction. Stimuli from the neighbouring cells via the interaction of cell surface receptors and secreted growth factors or cytokines are strongly dependent on the cell density. When the cell density is low, direct cell-cell contacts are limited and cell-biomaterial interaction is expected to be pre-dominantly influenced by cell-substrate contact. Compared to the low seeding density, cell-cell interaction at high seeding density is more extensive, which could contribute to cell attachment signalling activation through crosstalk between cell-substrate and cell-cell adhesion (Xia et al., 2011). Thus, this previous finding is similar to the present study in which the number of seeding cells was found to influence the cells’ proliferation activity.

Stem cells exhibit variable differentiation and proliferation characteristics of the same cell line if it is grown in different types of serum and can also present different phenotypes if cultivated in different conditions. Nevertheless, the cellular requirement for a specific micronutrient is directly correlated with the cell type, the rate of cell growth, and the stage of cell differentiation (Arigony et al., 2013).

Based on Figures 2 and 3, at concentrations between 0.04% (v/v) and 0.10% (v/v) for honey 1, the best proliferation time for DPSCs was 24 hours. Similarly, the best time for proliferation is 24 hours for Trigona spp. honey 2 at the concentration of 0.01% (v/v). Xia et al. (2011) stated that as cell density increases, cell-cell interaction also increases and it is expected to profoundly influence cellular responses to biomaterials.

The composition of honey is rather varied and depends primarily on its floral source, however, certain external factors, such as seasonal environmental factors and processing methods also play a role. The varying proliferative effect of honey may be due to the different substances and depends on the botanical origin of honey. For example, different antimicrobial chemicals were found on different bee honey; methylglyoxal was discovered to contribute to the activity of New Zealand’s Manuka honey, bee defensin-1 was detected in a Dutch honey, and melanoidins were identified in Canadian honeys (Moussa et al., 2012).

Phenolic compounds may be one of the factors that increase the proliferative activity of DPSCs as compared to the standard culture media. The compounds, such as flavonoids, caffeic acid, di-hydroxy benzoic acid, cinnamic acid, and ferulic acid are found in different types of honey at various concentrations depending on the location and botanical sources (Yao et al., 2003; Jaganathan & Mandal, 2009; Tsiapa et al., 2009). As for the stingless bee honey, specifically Trigona spp. honey, it is found that the honey contain free phenolic acids, including phenylpropanoic acid, benzoic acid, 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, vanillic acid, protocatechuic acid, and p-coumaric acid (Roowi et al., 2012). In the present study, there are possibilities that one or more of the phenolic acids or compounds mentioned above might be the component that contributed to the proliferative effect on DPSCs. Thus, further study to identify the compounds and their effects is needed.

Other factors that influence the proliferation rate of DPSCs also need to be taken into consideration. An extensive review of the antimicrobial activity of honey showed it to be derived from high sugar content, low water content, acidity, the generation of hydrogen peroxide on dilution, and phytochemical components (Moussa et al., 2012).

In this study, the MTT assay was used to observe the optimum concentration of both honey toward the cell proliferation effect of DPSCs. Based on the data that we had, at $15 \times 10^3$ seeded cell number, the optimum concentration of Trigona spp. honey 1 is between 0.04% (v/v) and 0.10% (v/v), as compared to the control. In a comparison with the control, the optimum concentration of honey 2 is 0.01% (v/v). Therefore, when the concentration is less than the optimum value, the honey might show slow
proliferation effects. However, if the concentration of honey is greater than the optimum value, it might cause a reduction in the cell proliferation activity.

CONCLUSION

This study demonstrated that *Trigona* spp. honey 1 and 2 have a promising proliferative effect on DPSCs. This honey was found to not have any effect towards the media stability within three days of incubation. It also increased the proliferation rate of DPSCs as early as 24 hours with 0.04% and 0.10% concentrations of *Trigona* spp. honey 1 and only 0.01% concentration of *Trigona* spp. honey 2 as compared to the control.

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Evaluation of acute and sub-acute oral toxicity of the aqueous extract of *Aquilaria malaccensis* leaves in Sprague Dawley rats

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Abstract. *Aquilaria malaccensis* or commonly known as ‘gaharu’ is a species of *Aquilaria* genus and belongs to the Thymelaeaceae family. It is widely distributed in Malaysia, Indonesia, and the Borneo Islands. Traditionally, its leaves were used to relieve bruises and studies have shown that they function as an antioxidant, aphrodisiac, and tranquilizer. Despite its proven beneficial medicinal properties, information regarding its toxicity is limited. Therefore, we performed a safety evaluation on the aqueous *A. malaccensis* leaves extract (AMAE) in Sprague Dawley rats. The assessment of acute toxicity based on the Organization for Economic Cooperation and Development (OECD) Guideline 420 revealed that AMAE did not influence mortality, clinical appearance, body weight gain, or necropsy findings at a dose of 2000 mg/kg body weight. In the sub-acute toxicity, all doses did not significantly modify the body weight and food and water intake. In male rats treated with 2000 mg/kg, there was a significant reduction in the relative weight of liver. Not only that, an increase in alkaline phosphatase and alanine transaminase was also observed in different groups among the female rats. A significant decrease in the creatinine level was also seen among male rats administered with different doses of AMAE. In both sexes, histopathological analysis had shown abnormalities in the liver and kidney of rats treated at the dose of 2000 mg/kg. In conclusion, the 50% lethal dose (LD₅₀) of AMAE was estimated to be greater than 2000 mg/kg. In sub-acute duration, the findings suggested that AMAE administered orally is slightly toxic at higher doses (2000 mg/kg) and could provoke functional and structural changes in the kidney and liver of rats. Thus, the extract should be used with caution.

Keywords: *Aquilaria malaccensis*, aqueous extract, acute toxicity, sub-acute toxicity

INTRODUCTION

Over the past few decades, the popularity of plant remedies has become more substantial and vital to various parts of the world. Despite the useful and medical benefits of the plants, some of the phytochemical compounds might pose lethal threats to human. Obviously, commonly
consumed plant preparations have limited data on their efficacy and potential toxicity. It must be clearly understood that all plant products should not be falsely considered as safe as they are natural. Some chemical constituents in plants may be safe to the plant but it might be deleterious to human. In addition, some chemical constituents present as naturally safe may exhibit toxic effects at a certain dose or prolonged exposure (Bode & Dong, 2014).

Toxicity assessment is an approach to evaluate the adverse effects of elements that are intended to be used or consumed by humans. These elements could be from chemical, physical, biological, animals, or the environment. The emergence of toxicity in natural products could be intrinsic, extrinsic, and from other contributing factors (Knöss, 2017). The phytochemical or metabolites in a plant could contribute to its intrinsic toxicity. The extrinsic toxicity can be derived from pesticides, environmental pollutants, preservatives, and adulterants. In addition, toxicity can also be acquired from the processing procedures such as sun drying, roasting, frying, steaming, and fumigating. Herb-drug and herb-herb interactions including prolonged consumption and high dosage could also play important roles in triggering adverse effects (Cock, 2015).

*A. malaccensis* is a native species of *Aquilaria* in Malaysia, Indonesia, and Thailand. It is classified under the family Thymelaeaceae and one of the agarwood-producing trees. It is locally known as karas, engkaras, gaharu, or depu (Lim & Noorainie, 2010). Mitra, Orbell and Muralitharan (2007) addressed the local ethnomedicinal use of *A. malaccensis* in treating general dropsy or oedema using *A. malaccensis* root infusion while its leaves are rubbed over swollen hands and legs of a dropsical patient. This plant has gained enormous interest among researchers due to its antioxidant activity (Huda, Munira, Fitrya, & Salmah, 2009; Miniyar et al., 2008; Nik Wil, Mohd Omar, Awang@Ibrahim, & Tajuddin, 2014; Ray, Leelamanit et al., 2014; Sattayasai et al., 2012; Tay et al., 2014), hepatoprotective effect (Vakati et al., 2013), anticancer activity (Ibrahim et al., 2011), antidiabetic (Zulkifle et al., 2013), and antimicrobial activity (Saad S Dahham et al., 2015; Kamonwannasit et al., 2013). Its essential oil can act as mosquito larvicidal and repellent (Zaridah, Nor Azah, & Rohani, 2006). These medicinal activities could be attributed to the presence of various phytoconstituents in the extracts.

An array of chemical classes including terpenoids, xanthones, flavonoids, benzophenones, phytosterols, phenolic acids, and fatty acids can be found in *Aquilaria* leaves. A phytochemical finding by Khalil et al. (2013) revealed the presence of flavonoids, tannins, alkaloids, triterpenoids and saponins in methanol extract of *A. malaccensis* leaves. Steroids were also identified in both methanol and water extracts of *A. malaccensis* leaves (Nik Wil et al., 2014). Acetaminophen or its synthetic form, 4’-hydroxyacetanilide also available in the leaves extract of *A. malaccensis* (Affiffudden, Alwi, & Hamid, 2015). Various phytoconstituents can be isolated from *A. malaccensis* leaves which can contribute to the health and well-being of humans.

Despite its various beneficial pharmacological activities, its potential health risk might have been overlooked. To date, several toxicity analyses of *Aquilaria* species were conducted either in vivo or in vitro. Several studies reported no evident toxicity effect was detected following single oral administration of *A. crassna* leaves extract (Saad Sabbar Dahham et al., 2016; Ghan et al., 2016; Kamonwannasit et al., 2013). Meanwhile, an ethanol extract of *A. agallocha* leaves was reported to be non-toxic on single oral administration in female Wistar rats at a dose of 2000 mg/kg (Vakati et al., 2013). Kaempferol, an isolated constituent of chloroform extract of *A. subiniegra* stem, exhibited no cytotoxicity effect on various human cell lines and the lethal concentration (LC₉₀) of the Brine Shrimp Lethality Assay (BSLA) was 762.41 µg/ml (Bahrami et al., 2014). Leaves extract of *A. sinensis*, another species of *Aquilaria*, demonstrated no acute toxicity and genotoxic effects (Li et al., 2015). Musir, Winarti, & Siti Hasnah (2016) described that the LC₉₀ of 70% ethanol extract of non-inoculated *A. bescariana* leaves was 113.73 ppm.

An acute and sub-acute toxicity study of *A. malaccensis* methanol leaves extract suggested the LD₉₀ was higher than 2000 mg/kg and the effective dose was 500 mg/kg (Zulkifle et al., 2018). Therefore, this is the very first toxicity study of aqueous extract of *A. malaccensis* carried out on Sprague Dawley rats to define its safety.
profile and ascertain its potential adverse effects as a key stepping stone for further development of the extract.

**MATERIALS AND METHODS**

*Plant collection and identification.* The leaves were collected in the morning (9 a.m. to 10 a.m.) from *A. malaccensis* tree plantation in the Forest Research Institute Malaysia (FRIM) Research Station, Maran Pahang. The leaves were collected between the month of March and April 2016. The leaves' compositions were identified and authenticated by a botanist from Universiti Kebangsaan Malaysia (UKM). The specimen voucher PIIUM 0296 was prepared by and deposited at the Natural Medicinal Product Centre, Kulliyyah of Pharmacy, IIUM.

*Preparation of the extract.* The aqueous extraction of *A. malaccensis* leaves (AMAE) was performed using hot water extraction method as established by the Phytochemistry Unit, Herbal Medicine Research Centre, Institute for Medical Research. Fresh leaves samples were dried in a solar drying oven and ground in an electrical grinder to produce the powder form. 150 g of dried leaves powder was soaked in 1500 ml of 40°C hot ultra-pure distilled water and left to fully immerse for 60 minutes followed by filtration through a Whatman No.1 filter paper. The previous steps were repeated twice. The combined filtrates were filled into a plastic centrifuge test tube and stored in a -80°C freezer, and the resulting frozen filtrates were freeze-dried in continuous operation for 144 hours. The extract powder was brown in colour and had a leafy smell with a sweet odour. The powder extract was stored at room temperature in an air-tight bottle until further use. The extract was reconstituted in distilled water to obtain the required doses for administration in rats.

*Experimental animal.* Sprague Dawley rats (2-3 months old and weight between 150-200 g) were used for both acute and sub-acute toxicity tests. 8 female rats (4 rats for sighting study and 4 rats for main study) were used for the acute toxicity study and 40 rats (20 males and 20 females) for the sub-acute toxicity study. All rats were housed singly in polypropylene cages with steel grill covers and bedded with corn cob. The rats were kept at room temperature (22±2°C), with 50±10% humidity, and a cycle of 12h light and 12h dark. Standard laboratory animal feed and water were provided ad libitum. Rats were acclimatized to the experimental conditions for a period of one week prior to dosing. All research procedures and animals were approved by the Institutional Animal Care and Use Committee (IACUC-IIUM) with approval number IIUM/ IACUC Approval/ 2016/ (9) (57).

*Acute toxicity study design.* The acute toxicity study design was in accordance with the OECD Guideline 420 with fixed dose (Organisation for Economic Co-operation and Development, 2001b). This study consisted of 2 distinct phases named the sighting study and the main study. All rats were abstained from food overnight prior to dosing. The rats’ body weight was measured followed by extract administration. Food was continually withheld for a further 3-4 hours post administration. The sighting study was intended to identify the suitable starting dose for the main study. A total of four female rats were used in the sighting study. Based on Adamson (2016) and Schlede et al. (2005), the fixed dose levels of 5, 50, 300, and 2000 mg/kg body weight were selected as starting doses to determine any evident toxicity signs. The lowest dose of AMAE (5 mg/kg body weight) was administered to the first rat. The rat was monitored closely for the first 30 minutes followed by hourly observations for four hours. Observation parameters included skin and fur changes, eyes and mucous membrane secretion, salivation, and locomotor activity. The rat was observed for 24 hours and, if there is no mortality recorded, another rat is administered for the next dose level. Similar procedures were performed throughout the dosing study. The highest dose without mortality was selected as the tested dose for the main study. As for the main study, five rats were subjected to administration of the selected dose. The five rats were made up of one rat from the sighting study dosed at the selected dose together with an additional four rats. All rats were kept and observed for the following 14 days. All life phase parameters were recorded such as daily body weight and food and water intake. All rats
were made to fast overnight and only had access to water. Rats were weighed prior to necropsy with pentobarbital injection intraperitoneally at a dose of 80 mg/kg body weight. The rats were dissected and vital organs were harvested and weighed. All organs were examined grossly and fixed in 10% neutral buffered formalin for histopathological processing.

**Sub-acute toxicity study design.** The OECD Guideline 407 with repeated dose 28-day oral was used as a study design (OECD, 2008). Each group comprised of 5 male rats and 5 female rats. The control group was given distilled water while the three different doses of AMAE (20, 200 and 2000 mg/kg) were administered daily via oral gavage for 28 days. All rats were closely monitored to observe for any evident clinical signs of toxicity during the administration period. Daily body weight and weekly food and water intake were measured and recorded. All rats were made to fast overnight and only had access to water. Rats were weighed prior to being anaesthetized with a pentobarbital injection intraperitoneally at a dose of 40 mg/kg body weight. Under the anaesthetized condition, blood was withdrawn from the rats via retro-orbital venous puncture for biochemical analyses. 4 ml of blood were collected into a plain tube (yellow cap). The rats were necropsied and dissected. The vital organs were trimmed, weighed, and harvested for gross examination. Only the kidney and liver were subjected to further histopathological finding.

**Biochemical analysis of blood serum.** Blood samples were collected into plain tubes and were centrifuged for 15 mins at 3,000 rpm to obtain the serum. The serum was analysed using a blood analyser for renal profile and liver profile. The value for total protein, albumin, urea, and creatinine were recorded to determine the biochemical status of the kidney. The liver profile was evaluated based on alkaline phosphatase, aspartate transferase, and alanine transaminase.

**Histological analysis.** The organs were cut and trimmed according to the recommendation guideline for organ sampling and trimming by the European Registry of Industrial Toxicology Animal Data (RITA) and North American Control Animal Database (NACAD) (Kittel et al., 2004; Morawietz et al., 2004; Ruehl-Fehlert et al., 2003). The liver and kidney samples were collected and fixed in 10% neutral buffered formalin. The organs were processed following the routine histological process and embedded in paraffin to produce tissue blocks. The tissue blocks were sectioned at 4 µm of thickness and stained in hematoxylin and eosin (H&E). The staining protocol was adopted from the Department of Pathology and Laboratory Medicine, Kulliyyah of Medicine with several modifications. The kidney and liver were analysed for histological abnormalities through light microscopy. Both organs were evaluated qualitatively based on the parameters or changes illustrated by Aroud (2014). The kidney was assessed based on the formation of cytoplasmic vacuolation and pyknotic nuclei while the liver was evaluated for vascular congestion and lymphocytic infiltration.

**Statistical analysis.** All data were assessed for normality using the Test of Normality. A parametric test, one-way analysis of variance (ANOVA), was used for normally distributed data followed by the Post-Hoc test. Dunnett’s test was used if equal variance is not assumed while Least Significance Difference (LSD) test was used if equal variance is assumed. The not normal distributed data were analysed using Kruskal-Wallis test followed by the Dunn-Bonferroni pairwise comparisons if there were significant trends. All data were considered statistically significant when p<0.05. The parametric data were expressed as mean ± standard deviation (SD) and the median (interquartile range) was recorded for non-parametric data. The analyses were performed using SPSS version 25.

**RESULTS**

**Acute toxicity.** All female rats survived until the necropsy day. None of the rats displayed signs of toxicity throughout the observation period. The colour of stools and urine were normal. Therefore, in accordance with OECD guideline, the LD₉₀ is greater than the highest dose used in this study, 2000 mg/kg body weight. The body weight of rats in all groups increased consistently
Throughout the 14 days and no significant variations were observed. There was no change in food and water intake of female rats administered with AMAE at the highest dose, 2000 mg/kg. The increasing pattern of food and water intake was recorded during the 2-week observation period. Macroscopic evaluation of the organs did not reveal any abnormality. Histopathological analysis of the kidney and liver showed no histological changes in the organs that might indicate abnormality as shown in Figure 1.

**Figure 1.** (A) Photomicrograph of kidney section from acute dose of AMAE 2000 mg/kg demonstrating normal and intact glomerulus and tubular epithelial cells (H&E stain, X40). (B) Photomicrograph of liver sections from acute dose of AMAE 2000 mg/kg showing portal tract which contains central vein and bile duct (H&E stain, X10). B: Bowman’s space, BC: Bowman’s capsule, EC: Tubular epithelial cell, G: Glomerulus, P: Portal tract, S: Sinusoid, V: Hepatic venule.

**Sub-acute toxicity.** All rats administered with AMAE at doses of 0 (control), 20, 200, and 2000 mg/kg body weight survived with no evident clinical changes throughout the treatment period until necropsy on day 29. All rats displayed good health status until the day of necropsy. There were no changes in body weights and body weight gains in all treated groups. Food and water intake were recorded in similar fashion among the treated groups and the control group. Statistical analysis did not show any significant difference among groups (p>0.05). The general health parameters of the rats were tabulated in Table 1.

**Absolute and relative organs weight.** Both absolute and relative organ weights in all groups were recorded as normal except the relative organ weight of liver in male rats treated with 2000 mg/kg body weight which was lower than control and other treated groups. This change was significantly different (p<0.05). The relative organ weights of adrenal glands, kidneys, and liver were presented in Table 2.

**Clinical biochemistry of blood serum.** The renal profile of the rats was assessed based on the value of total protein, albumin, globulin, urea, and creatinine. There were no significant differences between all groups for all renal parameters in male and female rats except a significantly elevated level of globulin in AMAE 2000 of male rats and significant reduction of creatinine in male rats of all AMAE administered groups. Meanwhile, the female rats in AMAE 200 exhibited an increase in urea level. The liver profile of the rats was evaluated based on the value of three liver enzymes named alkaline phosphatase, aspartate transferase, and alanine transaminase. In male rats, the level of alkaline phosphatase and alanine transaminase was not significant between all groups except for aspartate transferase, which was at a high significant level for both AMAE 200 and 200. Meanwhile, in female rats, alanine transaminase was recorded at a significantly high level for AMAE 200 and 2000. Only AMAE 200 indicated significant elevated level of aspartate transferase. The detailed renal and liver profiles were described in Table 3.
Table 1. The vital general health parameters of rats in control and administered with AMAE for 28 days.

<table>
<thead>
<tr>
<th>General parameters</th>
<th>Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Male</td>
<td></td>
</tr>
<tr>
<td>Weekly water intake (ml)</td>
<td>293.00 (14.56)</td>
</tr>
<tr>
<td>Weekly food intake (ml)</td>
<td>178.02±4.66</td>
</tr>
<tr>
<td>Daily body weight (g)</td>
<td>260.68±3.53</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>124.31±7.71</td>
</tr>
<tr>
<td>Female</td>
<td></td>
</tr>
<tr>
<td>Weekly water intake (ml)</td>
<td>210.80±13.00</td>
</tr>
<tr>
<td>Weekly food intake (ml)</td>
<td>132.71±7.25</td>
</tr>
<tr>
<td>Daily body weight (g)</td>
<td>174.48±7.53</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>81.75±10.64</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SEM

Table 2. The relative organ weight of rats in control and administered with AMAE for 28 days.

<table>
<thead>
<tr>
<th>Relative organ weight (%)</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Male</td>
<td></td>
</tr>
<tr>
<td>Right adrenal</td>
<td>0.007±0.0004</td>
</tr>
<tr>
<td>Left adrenal</td>
<td>0.008±0.0001</td>
</tr>
<tr>
<td>Right kidney</td>
<td>0.47±0.02</td>
</tr>
<tr>
<td>Left kidney</td>
<td>0.46±0.03</td>
</tr>
<tr>
<td>Liver</td>
<td>3.94±0.12</td>
</tr>
<tr>
<td>Female</td>
<td></td>
</tr>
<tr>
<td>Right adrenal</td>
<td>0.02 (0.01)</td>
</tr>
<tr>
<td>Left adrenal</td>
<td>0.02 (0.02)</td>
</tr>
<tr>
<td>Right kidney</td>
<td>0.46±0.02</td>
</tr>
<tr>
<td>Left kidney</td>
<td>0.44 (0.10)</td>
</tr>
<tr>
<td>Liver</td>
<td>4.00 (0.27)</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SEM

Histopathological analysis of kidney and liver. There were no marked abnormalities observed in gross examination of kidney and liver in all groups. However, histopathological examination revealed several cellular changes in kidneys and liver. Both kidneys and liver in the control group and treated groups (20 and 200 mg/kg) displayed normal morphology and arrangement. Kidney histological sections of the rats from the highest dose displayed cytoplasmic vacuolation and pyknotic nuclei as shown in Figure 2. Changes in both parameters including lymphocytic infiltration and vascular congestion for liver were observed in the group treated with 2000 mg/kg body weight as demonstrated in Figure 3. Table 4 summarized the histological changes recorded in the control and treated groups.
Table 3. The kidney and liver clinical biochemistry of rats in control and administered with AMAE for 28 days.

<table>
<thead>
<tr>
<th>Serum parameters</th>
<th>Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Male</td>
<td></td>
</tr>
<tr>
<td>Total protein (g/dL)</td>
<td>4.84±0.13</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>2.74±0.07</td>
</tr>
<tr>
<td>Globulin (mmol/L)</td>
<td>21.00±0.71</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>7.86±0.77</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>0.46±0.04</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>230.20±23.06</td>
</tr>
<tr>
<td>Aspartate transferase (U/L)</td>
<td>224.80±26.89</td>
</tr>
<tr>
<td>Alanine transaminase (U/L)</td>
<td>39.00±4.72</td>
</tr>
<tr>
<td>Female</td>
<td></td>
</tr>
<tr>
<td>Total protein (g/dL)</td>
<td>4.72±0.17</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>2.68±0.10</td>
</tr>
<tr>
<td>Globulin (mmol/L)</td>
<td>20.40±0.81</td>
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<tr>
<td>Urea (mmol/L)</td>
<td>7.08±0.65</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>0.46±0.04</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>214.40±18.91</td>
</tr>
<tr>
<td>Aspartate transferase (U/L)</td>
<td>214 (75) np</td>
</tr>
<tr>
<td>Alanine transaminase (U/L)</td>
<td>36 (10) np</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SEM

*Values are expressed as Median (IQR)

*p<0.05 in comparison to control

one missing value, n=4

Table 4. The histological parameters of rats in control and administered with AMAE for 28 days.

<table>
<thead>
<tr>
<th>Histological parameters</th>
<th>Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Male</td>
<td></td>
</tr>
<tr>
<td>Liver Vascular congestion</td>
<td>No</td>
</tr>
<tr>
<td>Lymphocytic infiltration</td>
<td>No</td>
</tr>
<tr>
<td>Kidney Cytoplasmic vacuolation</td>
<td>No</td>
</tr>
<tr>
<td>Pyknotic nuclei</td>
<td>No</td>
</tr>
<tr>
<td>Female</td>
<td></td>
</tr>
<tr>
<td>Liver Vascular congestion</td>
<td>No</td>
</tr>
<tr>
<td>Lymphocytic infiltration</td>
<td>No</td>
</tr>
<tr>
<td>Kidney Cytoplasmic vacuolation</td>
<td>No</td>
</tr>
<tr>
<td>Pyknotic nuclei</td>
<td>No</td>
</tr>
</tbody>
</table>
Toxicity of ‘gaharu’ leaves extract in rat

Figure 2. (A) Photomicrograph of kidney section of a control rat in subacute toxicity study of AMAE sacrificed at the end of study period showing normal morphology of glomerulus, tubular epithelial cells and Bowman’s space (H&E stain, X20). (B) Photomicrograph of kidney section of a rat AMAE 20 in subacute toxicity of AMAE sacrificed at the end of study period showing no abnormality (H&E stain, X40). (C) Photomicrograph of kidney section of a rat AMAE 200 in subacute toxicity study of AMAE sacrificed at the end of study period showing intact cellular arrangements and histology (H&E stain, X40). (D) Photomicrograph of kidney section of a rat AMAE 2000 in subacute toxicity study of AMAE sacrificed at the end of study period showing cytoplasmic vacuolation and appearance of pyknotic nuclei (H&E stain, X40). B: Bowman’s space, BC: Bowman’s capsule, G: Glomerulus, PCT: Proximal convoluted tubule, EC: Tubular epithelial cell, CV: Cytoplasmic vacuolation, PN: Pyknotic nuclei.

Figure 3. (A) Photomicrograph of liver section of a control rat in subacute toxicity study of AMAE sacrificed at the end of study period exhibiting normal hepatocytes and sinusoids (H&E stain, X20). (B) Photomicrograph of liver section of a rat AMAE 20 in subacute toxicity of AMAE sacrificed at the end of study period showing no notable abnormality (H&E stain, X15). (C) Photomicrograph of liver section of a rat AMAE 200 in subacute toxicity study of AMAE sacrificed at the end of study period showing intact cellular arrangements and histology (H&E stain, X10). (D) Photomicrograph of liver section of a rat AMAE 2000 in subacute toxicity study of AMAE sacrificed at the end of study period showing lymphocytic infiltration and vascular congestion (H&E stain, X40). H: Hepatocyte, P: Portal tract, S: Sinusoid, V: Hepatic venule, C: Vascular congestion, L: Lymphocytic infiltration.
DISCUSSION

Toxicity analysis is a fundamental and paramount step to be undertaken for any preparation intended to be used in humans. This analysis could describe any health hazards and risks associated with potential exposures resulting from its consumption or use. Safety consumption of any herbal preparation is a serious issue that needs to be highlighted. Any claimed bioactivity or medicinal benefits of plant extracts needs to be coupled with a safety analysis as there is uncertainty that the plant is free from the possibility of toxicity (Jordan, Cunningham, & Marles, 2010).

In the acute toxicity study of AMAE, only female rats were used as the experimental animal. In the light of ethical consideration to minimize the number of experimental animal and reduce variability, only one rat was used per dose in the sighting study and an additional 4 rats for the main study. Sex differences are able to manifest distinct toxicity effects in rats. A female rat has lower detoxification capacity and is more responsive to any toxic elements even at a lower dose (OECD, 2001a).

The mortality and toxicity level of acute oral dose of AMAE were evaluated. After 24 hours and 14 days of observation period, no mortality was recorded up to 2000 mg/kg. Thus, AMAE can be categorised under category-5 and LD₅₀ value was greater than 2000mg/kg in line with Globally Harmonised System of Classification and Labelling of Chemicals. This LD₅₀ finding was similar with the previous acute toxicity analysis of methanol extract of A. malaccensis leaves (Zulkifle et al., 2018). In fact, mangiferin that can be found in the Aquilaria species did not exhibit any evident toxicity (Prado et al., 2015).

Daily clinical observations were conducted on the rats for 14 days starting from the first 24 hours prior treatment. The observations were carried out based on Hippocratic signs. Qualitative findings from Hippocratic signs are helpful in preliminary investigation of toxic properties of a substance. Moreover, it can also assist the establishment of a dosing regimen for prolonged pharmacological and toxicological studies (de Azevedo Neta Mahon et al., 2014). In our acute toxicity study, there was no evident adverse clinical signs recorded at any doses tested during the observation period of 14 days. Positive findings during the 14-day observation implied no delayed toxic effect occurred (OECD, 2001b).

Increasing body weight pattern in all groups indicated the normal growth of the rats. This pattern was in line with the consistency in food and water intake percentage of consumption. This positive consistency exhibited the normal metabolism of the rats and the extract did not retard the rats’ growth (Mukinda & Eagles, 2010). Reduction in average food intake could be an indicator for sickness behaviour. This sickness behaviour can also be associated with cognitive disturbances, social withdrawal, anorexia, and lethargy (Shattuck & Muehlenbein, 2015). Meanwhile, organ weight is an important indication of the pathological and physiological status of animals. Instead of absolute organ weight, relative organ weight or organ-to-body weight ratios is highly useful when body weight confound the organ weight (Michael et al., 2007). The relative organ weight of all vital organs was normal and within the normal range. This implied that AMAE at 2000 mg/kg did not pose any pathological threat to vital organs like the liver and kidney.

Due to limited data that could be gained from the acute toxicity study, it is important to have more extensive data on repeated administration due to the fact that cumulative toxic effects could occur even at very low doses (Abotsi, Ainooson, & Gyasi, 2011). Therefore, in this present study, the sub-acute profile of AMAE was assessed in rats using a measurement of body and organ weights, biochemical and histopathological parameters. In the sub-acute toxicity study, both sexes of rat were used to accurately justify any toxic or unwanted changes across genders upon prolonged and consecutive oral treatment. Unlike the single dose treatment of acute toxicity study, prolonged oral treatment might possess different outcomes of several parameters. It is known that continuous intake of any treatment for certain periods of time affects the balance state of several hormones and induces sex specific differences. As an example, different mode of lipid and glucose metabolism among male and female associated with sex specific insulin resistance development (Stanimirovic et al., 2016).
A study demonstrated that the toxicity of triptolide, an active diterpenoid from *Tripterygium wilfordii*, was in sex-related pattern. The high distribution of CYP3A2 in male rats accelerates triptolide metabolism including rapid triptolide clearance by male rats liver microsomes up to 5-fold faster than seen in female rats (Liu *et al.*, 2010). Unless it is related to a specific reproductive system, it is vital to put sex differences into consideration as it is a biological variable that might confound the finding. In fact, this consideration can provide a better insight in the pharmacological and toxicological research to enhance the safety evaluation of any compound or extract (Gochfeld, 2016).

It was noted that daily oral administration for 28 days of AMAE up to 2000 mg/kg did not cause mortality to the rats. In fact, no prominent toxicity signs were recorded during daily observation in post oral treatment. Therefore, it was appropriately justified that AMAE has low toxicity following repeated oral administration and is relatively safe as classified by the OECD guideline. This recent finding was correlated with a previous sub-acute toxicity study of ethanol extract of similar species which reported no mortality and adverse toxicity signs (Zulkifle *et al.*, 2018).

In case of prolonged administration, it is recommended to measure weekly body weight. It is highly sensitive to detect the toxicity of materials with low toxicity level (Jigam *et al.*, 2012). The prolonged administration of AMAE did not cause any alteration in body weight and body weight gain in all treated groups. Similarly, the food intake and water consumption were documented as normal. This scenario indicated that the prolonged extract administration did not disrupt the normal growth and no significant physiological alterations occurred in the rats. The extract may not affect the digestion process of the rats. Our findings on vital health status of rats administered with *A. malaccensis* extract were consistent with the findings of Zulkifle *et al.* (2018).

Serum biochemical parameter is an essential analysis to evaluate the consequences of drugs on vital organs particularly liver and kidney. The liver is known to metabolize drugs as well as detoxify any toxic elements circulating in the blood. Two transaminase enzymes, aspartate transferase, and alanine transaminase are the important biomarkers for identifying any potential toxic effects and indicating the functional status of the liver. These enzymes are present abundantly in the hepatocytes. In the event of damaging toxic effects, hepatocytes are highly susceptible to injury and leak these enzymes into blood circulation. Any elevation of both transaminases in blood is associated with liver injury (Raina *et al.*, 2015).

In our study, an elevation of aspartate transferase was demonstrated in AMAE 200 and 2000 in male rats. Even though high aspartate transferase can be associated with liver damage, it is still arguable as there was inconsistent and not significant data for alanine transaminase. Aspartate transferase is not a convincing and highly specific marker for liver damage as it is also generated by other tissues like the muscles, heart, and brain (Adeyemi, Akindele, & Nwumeh, 2010). In addition, aspartate transferase is able to bind with an immunoglobulin that might cause serum aspartate transferase level and can be falsely linked with liver dysfunction (Yi-Chen *et al.*, 2018). On the other hand, in female rats, a significant increase of alanine transaminase was recorded in AMAE 200 and 2000. However, only AMAE 200 showed a significant increase in aspartate transferase level. The presence of possible toxic compounds trigger the liver’s adaptation or regenerative response (Schwabe & Luedde, 2018) such as hepatocyte hypertrophy, rapid production of metabolizing enzyme, and cytoplasmic organelles synthesis to assist the detoxification process following chronic exposure (Meyer, 2001). Notably, relative organ weight of the liver did not show any significant changes as compared to control except a significant reduction in liver relative weight of AMAE 2000. Hepatocytes are highly susceptible to an array of harmful elements that can cause cellular degeneration or atrophy as seen in Jiang *et al.* (2017). Apoptosis of hepatocytes is a sign of liver injury and closely associated with progression of fibrogenesis and development of cirrhosis (Schwabe & Luedde, 2018). The significant reduction in liver weight of AMAE 2000 suggested the development of liver cirrhosis which is associated with gradual shrinkage of liver size (Gill, 2018; Nath, Vishwakarma, & Bhattacharjee, 2016). In relation with the histopathology result of liver, both sexes
Toxicity of ‘gaharu’ leaves extract in rat

In AMAE 2000 demonstrated notable vascular congestion and lymphocytic infiltration that may correlate with the liver cirrhosis. Liver cirrhosis is characterized by hepatic vasculature distortion that can impair portal and arterial blood supply to the central vein. This impairment diminishes exchanging activities between the hepatic sinusoids and the adjacent liver parenchyma (Sofue, Tsurusaki, & Murakami, 2017). Despite elevations in serum liver enzymes for AMAE 200, these elevations were very slight and did not correlate with histological findings. It is important to highlight that the absence of notable damage in liver histology can manifest slight alterations in serum liver enzymes (Lee et al., 2015). With all the justifications, it can be suggested that AMAE is likely to be hepatotoxic at a dose of 2000 mg/kg.

Meanwhile, the kidney is also known as a critical target organ for noxious compounds which produce an array of nephrotoxic effects in tubular cells and glomerulus. Its function was evaluated by total protein, albumin, globulin, urea and creatinine levels and an increase in these markers is an indicator of a negative impact on renal function. The globulin level of male rats in AMAE 2000 showed a significant difference in comparison to the control group. Conversely, male rats in all groups administered with AMAE exhibited a significant reduction in creatine level as compared to control. However, it is still vague to conclude AMAE exhibited a nephroprotective effect as it is inconsistent with other renal parameters. To add, a reduction in creatinine could also be associated with muscle wastage (Aouachria et al., 2017) apart from nephroprotective properties. On the contrary, the kidney histology of highest dose of AMAE 2000 mg/kg demonstrated the presence of pyknotic nuclei which may indicate cellular necrosis or apoptosis due to inflammation. Considering the safety use of AMAE, it is reasonable to speculate that AMAE 2000 could impair the renal function. It is recommended to conduct a prolonged duration of AMAE administration to accurately evaluate its toxicity towards kidneys.

CONCLUSION

In conclusion, the present study demonstrated that the aqueous extract from A. malaccensis is non-toxic with LD$_{50}$ > 2000 mg/ kg. However, sub-acute administration of aqueous extract from A. malaccensis over a period of 28 days and at a higher dose triggered several toxicity effects in rats. At the dose of 2000 mg/kg, there were significant biochemical and histological changes that indicate hepatotoxic and nephrotoxic activities. Thus, in order to limit the adverse effects of the plant extract, it is cautioned to use this plant extract above 2000 mg/kg dose at a prolonged duration. Indeed, this study offers valuable preliminary data on the toxicity profile of A. malaccensis that is surely useful for future in vivo and clinical studies.

In future studies, it is recommended to conduct an extensive prolonged treatment such as sub-chronic and chronic duration. This will provide a better insight of this extract upon prolonged consumption as most of the herbal preparations are intended for extended duration. Upon that notion, it is highly suggested to carry out wide-ranging toxicity to determine the effects of this plant on the prenatal development, reproductive capacity, genetic, and tumor generation.

ACKNOWLEDGEMENTS

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Overview of hepatotoxicity.


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Impact of \textit{CYP3A4} and \textit{CYP3A5} single nucleotide polymorphisms on anastrozole-associated adverse events among Malaysian breast cancer patients

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\textbf{Abstract.} The catalytic activity of the cytochrome P450A (CYP3A4) enzyme is reportedly affected by the presence of single nucleotide polymorphisms (SNPs), leading to inter-individual variability in drug efficacy and adverse reactions. \textit{CYP3A4} polymorphisms can serve as potential biomarkers for predicting the efficacy of many drugs, including those used in breast cancer treatment. This study was conducted on 94 hormone receptor-positive postmenopausal breast cancer patients who received 1 mg of anastrozole per day. Anastrozole-associated adverse events (AAAEs), such as musculoskeletal adverse events (MSAEs), hot flashes, mood disturbance and vaginal dryness/dyspareunia, were assessed according to the Common Terminology Criteria for Adverse Events (CTCAE). The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was performed to determine the allelic frequency of \textit{CYP3A4*4}, \textit{CYP3A4*18A}, \textit{CYP3A4*18B}, \textit{CYP3A4*22} and \textit{CYP3A5*3}. The frequencies of \textit{CYP3A4*18A T>C} (rs28371759), \textit{CYP3A4*18B G>A} (rs2242480) and \textit{CYP3A5*3} were 0.03, 0.48 and 0.64, respectively. However, no \textit{CYP3A4*4 A>G} (rs55951658) or \textit{CYP3A4*22 C>T} (rs35599367) alleles were detected. No significant association was observed between the alleles and the development of AAAEs. We have demonstrated for the first time that \textit{CYP3A4*18B G>A} is highly prevalent among Malaysian breast cancer patients. The clinical relevance of \textit{CYP3A4*18B} is currently under investigation by our group.

\textbf{Keywords:} \textit{CYP3A4}, \textit{CYP3A5}, breast cancer, PCR-RFLP, anastrozole, adverse events

\textbf{INTRODUCTION}

Worldwide, breast cancer is the second most common cancer and by far the most frequent cancer in women, with an estimated 1.7 million cases in 2012. However, in terms of mortality, it ranks 5\textsuperscript{th}, a development believed to be a result of its fairly favourable prognosis (Ferlay \textit{et al.}, 2015). Recent advances in the early detection and treatment of breast cancer have led to a significant increase in the number of survivors, with the 5-year survival rate reaching almost 90\% (Siegel \textit{et al.}, 2014). However, a significant proportion of breast cancer survivors suffer several side effects that potentially impair their quality of life, including anastrozole-associated adverse events.
such as musculoskeletal symptoms, hot flashes, vaginal dryness/dyspareunia and mood disturbances (Cella et al., 2006; Mouridsen, 2006; Burstein, 2007; Rocha-Cadman et al., 2012; Kyvernitakis et al., 2014; Stearns et al., 2015).

Anastrozole is a selective third-generation aromatase inhibitor (AI) established as one of the drugs of choice in adjuvant therapy for postmenopausal breast cancer as well as in advanced-stage breast cancer (Ingle and Suman, 2005; Ingle, 2006). Although anastrozole has been shown to be superior and more effective than tamoxifen (Forbes et al., 2008), a significant number of patients still present with large inter-individual variability in tolerability, resulting in serious adverse effects including musculoskeletal complaints and hot flashes, which occasionally leads to patients’ withdrawal from treatments (Mouridsen, 2006; Ingle et al., 2010a). This inconsistency has been attributed to inter-individual variability in the pharmacokinetics and/or pharmacodynamics of anastrozole, partly attributable to genetic variations (Abubakar et al., 2015) and other undetermined factors.

Emerging evidence suggests that genetic variation in CYP3A4 results in functional changes that may alter the activity of the CYP3A4 enzyme, resulting in inter-patient variability in response to medication (Zanger and Schwab, 2013; Jin et al., 2015). The current database of CYP3A4 allele nomenclature (http://www.cypalleles.ki.se/cyp3a4.htm) shows that the wild type CYP3A4*1 allele constitutes 18 subtypes (CYP3A4*1A-T). There are also an additional 28 alleles designated as CYP3A4*2 to CYP3A4*26 with CYP3A4*15, CYP3A4*16 and CYP3A4*18 each having the “B” subtype. Interestingly some of these alleles have been shown to influence enzymatic activity (Werk and Cascorbi, 2014).

Two major CYP3A5 alleles (CYP3A5*3 and CYP3A5*6) are associated with functional changes in the CYP3A5 enzyme (Zanger and Schwab, 2013). CYP3A5*3 (rs776746), with a 6986A>G in intron 3, is the most frequent allele with a reported frequency of 0.12-0.35 in Africans and 0.88-0.97 in Caucasians (Werk and Cascorbi, 2014). Interestingly, its frequency among Malaysians varies based on ethnicities, with frequencies of 0.59 (Malays), 0.72 (Chinese) and 0.50 (Indians) (Hamzah et al., 2014). Recent findings among Malaysians indicate that the presence of the CYP3A5*3 allele can influence the pharmacokinetics of tacrolimus in renal-transplant patients (Hamzah et al., 2014; Mac Guad et al., 2016).

The present study investigates the allelic frequencies of CYP3A4*4, CYP3A4*18A, CYP3A4*18B, CYP3A4*22 and CYP3A5*3. This is the first study to report the presence of the CYP3A4*18B allele and its potential impact on anastrozole-associated adverse events in the Malaysian population, in particular in Malaysian breast cancer patients.

**MATERIALS AND METHODS**

**Study population.** A total of 94 unrelated breast cancer patients were recruited for this study. The patients attended the Oncology Clinic, Universiti Sains Malaysia, Kelantan, Malaysia between April 2014 and April 2015. The research protocol was approved by the Human Research Ethical Committee of the Universiti Sains Malaysia (USMKK/PPP/JEPEM [260.3,(21)]) and complied with the Declaration of Helsinki. The subjects were postmenopausal women with histologically confirmed hormone receptor-positive stage I, II or III breast cancer based on the American Joint Committee on Cancer (AJCC) staging manual (Sixth Edition). All patients received 1 mg/day of anastrozole. Patients who previously received tamoxifen and were switched to anastrozole (for at least four weeks) at the time of enrolment were also included. However, patients taking hormone replacement therapy (HRT) with an underlying psychiatric illness, chronic liver disease or renal disease were excluded. The study protocols were explained to the patients, and only those who gave informed consent were enrolled.

Patients’ demographic data, such as age, marital status, occupational status, educational level, age of menopause, years since menopause, age at time of breast cancer diagnosis, family history of breast cancer and history of contraceptive use, were ascertained. Other variables, such as weight and height, were recorded at the clinic during a routine follow-up visit. Clinical variables, such as cancer stage,
tumour grade, human epidermal growth factor (HER2) status, current anastrozole use, time since anastrozole was administered and the number of comorbidities, were also derived from each patient's case folder and verified by an oncologist for quality control.

**Anastrozole-associated adverse events.** Patient-reported anastrozole-associated adverse events (AAAEs) were assessed according to the National Cancer Institute's (NCI's) Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 (CTCAE, 2010). The musculoskeletal adverse events (MSAEs) assessed in this study, as reported elsewhere (Stearns et al., 2015). Other AAAEs assessed in this study include mood disturbance and vaginal dryness (dyspareunia).

**Blood sample collection and DNA isolation.** Peripheral blood (1 mL) was collected in EDTA tubes (BD Franklin Lakes, NJ USA) and was stored at -20°C until use. Genomic DNA was extracted from whole blood using a QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The purity of the DNA and its concentration were determined using an Infinite® 200 NanoQuant (Tecan, Switzerland).

**PCR-RFLP.** PCR to amplify CYP3A4*18A was performed in a total reaction volume of 25 μL consisting of 1 X PCR buffer, 0.2 mM of dNTP, 2.0 U of Taq polymerase, 0.2 μg of DNA template and 0.2 μM of *18A_F and *18A_R primers (Table 1). The PCR reaction was prepared in a total reaction volume of 25 μL, which consisted of 1 X PCR buffer, 0.2 mM of dNTP, 2.0 U of Taq polymerase, 0.2 μg of DNA template and 0.2 μM of *18B_F and *18B_R primers (Table 1). The final extension step was performed at 68°C for 5 min. The PCR reaction was prepared in a total reaction volume of 25 μL that consisted of 1 X PCR buffer, 0.2 mM of dNTP, 2.0 U of Taq polymerase, 0.2 μg of DNA template and 0.2 μM of *22_F and *22_R primers (Table 1). The final extension step was performed at 68°C for 5 min. The PCR reaction was prepared in a total reaction volume of 25 μL consisting of 1 X PCR buffer, 0.2 mM of dNTP, 2.0 U of Taq polymerase, 0.2 μg of DNA template and 0.2 μM of *53_F and *53_R primers (Table 1). The final extension step was performed at 68°C for 5 min. The PCR reaction was prepared in a total reaction volume of 25 μL consisting of 1 X PCR buffer, 0.2 mM of dNTP, 2.0 U of Taq polymerase, 0.2 μg of DNA template and 0.2 μM of *22_F and *22_R primers (Table 1). The final extension step was performed at 68°C for 5 min. The PCR reaction was prepared in a total reaction volume of 25 μL consisting of 1 X PCR buffer, 0.2 mM of dNTP, 2.0 U of Taq polymerase, 0.2 μg of DNA template and 0.2 μM of *53_F and *53_R primers (Table 1). The final extension step was performed at 68°C for 5 min. The PCR reaction was prepared in a total reaction volume of 25 μL consisting of 1 X PCR buffer, 0.2 mM of dNTP, 2.0 U of Taq polymerase, 0.2 μg of DNA template and 0.2 μM of *22_F and *22_R primers (Table 1). The final extension step was performed at 68°C for 5 min. The PCR reaction was prepared in a total reaction volume of 25 μL consisting of 1 X PCR buffer, 0.2 mM of dNTP, 2.0 U of Taq polymerase, 0.2 μg of DNA template and 0.2 μM of *53_F and *53_R primers (Table 1). The final extension step was performed at 68°C for 5 min. The PCR reaction was performed as reported previously (Ruzilawati et al., 2007) with slight modifications. Briefly, the PCR protocol was carried out in a total volume of 25 μL consisting of 1 X buffer, 0.2 mM of dNTP, 2.0 U of Taq polymerase, 0.2 μg of DNA template and 0.2 μM of *4_F and *4_R primers (Table 1). The buffers, dNTPs and Taq polymerase used in all the PCR reactions were supplied by New England Biolabs® Inc., MA, USA. The cycling protocol consisted of an initial denaturation step at 94°C for 30 s, followed by 35 cycles of 30 s at 94°C, 45 s at 65.3°C and 45 s at 68°C. The final extension step was performed at 68°C for 5 min. The PCR product (244 bp) (Figure 1) was digested with 2.0 U of BsmAI, followed by incubation for 60 min at 55°C. The PCR reaction to amplify CYP3A4*18A was performed in a total reaction volume of 25 μL that consisted of 1 X PCR buffer, 0.2 mM of dNTP, 2.0 U of Taq polymerase, 0.2 μg of DNA template and 0.2 μM of *18A_F and *18A_R primers (Table 1). The PCR product (388 bp) (Figure 1) was digested with 8.0 U of HpaII at 37°C for 60 min; the enzyme was then inactivated at 80°C for 20 min. The PCR method to amplify CYP3A4*18B was modified from a previously described protocol (Hu et al., 2007). The PCR reaction was prepared in a total reaction volume of 25 μL, which consisted of 1 X PCR buffer, 0.2 mM of dNTP, 2.0 U of Taq polymerase, 0.2 μg of DNA template and 0.2 μM of *18B_F and *18B_R primers (Table 1). The final extension step was performed at 68°C for 5 min. The PCR product (331 bp) (Figure 1) was digested with 4.0 U of RsaI at 37°C for 60 min. For CYP3A4*22, the PCR reaction was performed in a total reaction volume of 25 μL that consisted of 1 X PCR buffer, 0.2 mM of dNTP, 2.0 U of Taq polymerase, 0.2 μg of DNA template and 0.2 μM of *22_F and *22_R primers (Table 1). The final extension step was performed at 68°C for 5 min. The PCR product (293 bp) (Figure 1) was digested with 6.0 U of BsmAI at 37°C for 60 min; the enzyme was then inactivated at 80°C for 20 min. CYP3A5*3 was genotyped using PCR as previously described by (van Schaik et al., 2002) with slight modifications. Briefly, the PCR reaction was performed in a total reaction volume of 25 μL consisting of 1 X PCR buffer, 0.2 mM of dNTPs, 2.0 U of Taq polymerase, 0.2 μg of DNA template and 0.2 μM of *53_F and *53_R primers (Table 1). The final extension step was performed at 68°C for 5 min. The PCR product (293 bp) (Figure 1) was digested with 6.0 U of SspI at 37°C for 60 min; the enzyme was then inactivated at 65°C for 20 min.
Table 1. Primer sequences used for PCR-RFLP genotyping.

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Primer</th>
<th>Sequences (5'-3')</th>
<th>PCR product size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A4*4 A&gt;G</td>
<td>*4_F</td>
<td>CACATTTTCTACAACCATGGGACCC</td>
<td>244</td>
<td>(Ruzilawati et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>*4_R</td>
<td>TACCTGTCCCCCACAGATTCATTCT</td>
<td></td>
<td>(Ruzilawati et al., 2007)</td>
</tr>
<tr>
<td>CYP3A4*18A T&gt;C</td>
<td>*18A_F</td>
<td>AATGATTTGGCTTATTCTGGTTCTG</td>
<td>388</td>
<td>Self-designed</td>
</tr>
<tr>
<td></td>
<td>*18A_R</td>
<td>TTTCACCTCCCTCCCTCCCTCC</td>
<td></td>
<td>Self-designed</td>
</tr>
<tr>
<td>CYP3A4*18B G&gt;A</td>
<td>*18B_F</td>
<td>CCACGAGCAGGTCTTCTCTCTTC</td>
<td>331</td>
<td>Self-designed</td>
</tr>
<tr>
<td></td>
<td>*18B_R</td>
<td>ATAGAAAGCAGATGAACCGAGCC</td>
<td></td>
<td>(Hu et al., 2007)</td>
</tr>
<tr>
<td>CYP3A4*22 C&gt;T</td>
<td>*22_F</td>
<td>GCATAGAGTCTGCAGTCAGCAAT</td>
<td>793</td>
<td>Self-designed</td>
</tr>
<tr>
<td></td>
<td>*22_R</td>
<td>GATGACAGGGTTTGTGACAGGGG</td>
<td></td>
<td>Self-designed</td>
</tr>
<tr>
<td>CYP3A5*3 A&gt;G</td>
<td><em>5</em>3_F</td>
<td>CATGACTTAGTAGACAGATGA</td>
<td>293</td>
<td>(Van Schaik et al., 2002)</td>
</tr>
<tr>
<td></td>
<td><em>5</em>3_R</td>
<td>GGTCCAAAACAGGGAAGAATA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mismatch with the CYP3A5*3 sequence is underlined.

Figure 1. PCR products for CYP3A4*4 (L2), CYP3A4*18A (L3), CYP3A4*18B (L4) and CYP3A4*22 (L5) with band sizes of 244 bp, 388 bp, 331 bp and 793 bp, respectively, on a 2% agarose gel. L1: Quick-Load 100 bp DNA ladder (NEB® Inc, MA, USA).

**PCR product purification and DNA sequencing.** The PCR products were first purified before being sent for sequencing using Illustra™ ExoProster™ 1-Step Enzymatic and Sequencing Clean-Up (GE HealthCare Life Sciences, UK) according to the manufacturer’s instructions. The sequencing results were run through snpBLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) and compared against the SNP database (http://www.ensembl.org/index.html). The reference SNP (rs) IDs rs55951658, rs28371759, rs2242480, rs35599367 and rs776746 were identified for CYP3A4*4, CYP3A4*18A, CYP3A4*18B, CYP3A4*22 and CYP3A5*3 respectively. At least three representative samples from each genotype were randomly selected for sequencing.

**Statistical analyses.** Data analyses were performed using IBM SPSS Statistics (Version 22.0, Armonk, NY: IBM Corp). A simple logistic regression was used to compare the four outcomes (MSAEs, mood disturbance, hot flashes or sweating and vaginal dryness/dyspareunia), and all independent variables were assessed. Covariates with a p-value <0.25 or those with a p-value >0.25 that were considered to be clinically significant in simple logistic regression modelling were fitted into the multiple logistic regression models.
RESULTS AND DISCUSSION

A total of 94 patients between the ages of 44 and 83 years, with a mean age of 58.1 (SD, 7.3), were screened for this study (Supplementary data). The digestion of CYP3A4*4 (244 bp) with BsmAI yielded fragments of 15 bp (not shown), 88 bp and 141 bp for the wild type gene (Figure 2). The hypothetical RFLP bands for the homozygous and heterozygous variants are 15 bp, 47 bp, 88 bp and 94 bp or 15 bp, 47 bp, 88 bp, 94 bp and 141 bp, respectively. The digested PCR product of CYP3A4*18A had a length of 388 bp for the wild type and 189 bp, 199 bp and 388 bp for the heterozygous variant (Figure 3); the hypothetical RFLP bands for the homozygous variant allele are 188 bp and 200 bp. The digested PCR products of CYP3A4*18B (331 bp) were 115 bp and 216 bp for the wild type, 331 bp for the homozygous variant and 115 bp, 216 bp and 331 bp for the heterozygous variant (Figure 4). The digested wild type PCR product of CYP3A4*22 yielded 219 bp and 574 bp fragments (Figure 5). The hypothetical RFLP banding pattern should yield a band of 793 bp for the homozygous variant allele and bands of 219 bp, 574 bp and 793 bp for the heterozygous variant. The digested PCR products of CYP3A5*3 were as follows: 125 bp and 148 bp fragments for the wild type; 125 bp, 148 bp and 168 bp fragments for the heterozygous variants; and 125 bp and 168 bp fragments for the homozygous variants (Figure 6).

The allelic and genotypic frequencies of the various SNPs are shown in Table 2. In the present study, no significant association was observed between any of the alleles and the AAEEs (Table 3). However, the patients’ ages were associated with the occurrence of hot flashes (adjusted odds ratio, OR = 0.91, 95% confidence interval CI = 0.84-0.97, p = 0.021). In addition, patients receiving anastrozole for more than one year had a greater chance of developing mood disturbances (OR = 3.07, CI = 1.02-9.24, p = 0.046) and vaginal dryness/dyspareunia (OR = 18.00, CI = 3.71-87.40, p = 0.000) compared to patients receiving the drug for less than one year.

Figure 2. PCR products for CYP3A4*4 on a 3% agarose gel before (L2 and L3) and after (L4 and L5) digestion with BsmAI. L1: GeneRuler 50 bp DNA ladder (Thermo Fisher Scientific Inc, MA, USA). L2 and L3: undigested CYP3A4*4 PCR product (244 bp). L4 and L5: digested PCR products for wild type CYP3A4*4 (141 bp and 88 bp). L6: negative control. Gel picture was taken using the negative mode.

Figure 3. (a) PCR products for CYP3A4*18A on a 2% agarose gel before (L2) and after (L3) digestion with HpaII. L1: GeneRuler 50 bp DNA ladder (Thermo Fisher Scientific Inc, MA, USA). L2: undigested CYP3A4*18A PCR product (388 bp). L3: digested PCR products for heterozygous CYP3A4*18A (199 bp and 189 bp). L4: negative control. (b) Direct sequencing of the CYP3A4*18A allele showing a chromatogram (reverse sequence) of patients with wild type (I) and heterozygous variants (II). The highlighted “A” is adenine, indicating an absence of a CYP3A4*18A SNP in this subject, and the highlighted “G” is guanine, indicating the presence of heterozygous CYP3A4*18A in this subject.

Figure 4. (a) PCR products for CYP3A4*18B on a 2% agarose gel following digestion with RsaI. L1: Quick-Load 100 bp DNA ladder (NEB® inc, MA, USA). L2: digested wild type CYP3A4*18B PCR product (115 bp and 216 bp). L3: digested PCR products for heterozygous CYP3A4*18B (115 bp, 216 bp and 331 bp). L4: undigested product for homozygous CYP3A4*18B (331 bp). L5: negative control. (b) Direct sequencing results for the CYP3A4*18A allele showing a chromatogram of patients with wild type (I), heterozygous (II) and homozygous (III) variants. The highlighted “G” in (I) is guanine, indicating the presence of the wild type CYP3A4*18B, and the highlighted “G” in (II) is guanine, detected in addition to the “A” allele, indicating the presence of heterozygous CYP3A4*18B. The highlighted “A” in (III) indicates the presence of a homozygous variant.

Figure 5. PCR products for CYP3A4*22 on a 2% agarose gel before (L2) and after (L3) digestion with BseYI. L1: Quick-Load 100 bp DNA ladder (NEB® inc, MA, USA). L2: undigested CYP3A4*22 PCR product (793 bp). L3: digested wild type CYP3A4*22 PCR product (219 bp and 574 bp). L4: negative control.

Figure 6. PCR products for CYP3A5*3 on a 4% agarose gel before and after digestion with SspI. L1: Quick-Load 100 bp DNA ladder. L2: undigested PCR product (293 bp). L3: digested wild type PCR products (125 bp and 148 bp). L4: PCR product of the digested heterozygous variant (125 bp, 148 bp and 168 bp). L5: PCR products for the digested homozygous variant (125 bp and 168 bp). L6: negative control. (b) Direct sequencing results for the CYP3A5*3 allele showing chromatograms from patients with wild type (I), heterozygous (II) and homozygous (III) variants. The highlighted “A” is adenine, indicating the presence of wild type CYP3A5*3 in this subject. The highlighted “G” in (II) is guanine, detected in addition to “A” (adenine), indicating the presence of a heterozygous variant. The highlighted “G” in (III) is also guanine, indicating the presence of a homozygous variant.
In the present study, we successfully genotyped CYP3A*4, CYP3A*18A, CYP3A*18B, CYP3A*22 and CYP3A*3 in breast cancer patients (n=94) using the PCR-RFLP method. This study is the first to report the genotypes of CYP3A*18B and CYP3A*22 among Malaysians, in particular Malaysian breast cancer patients.

In this study, five out of 94 subjects had the heterozygous variant CYP3A*18A (frequency of 0.03), while the remaining 89 had the wild type allele. No individual had a homozygous variant for this allele. This was similarly reported in another study in which five out of 121 healthy Malaysian subjects were found to have the heterozygous CYP3A*18A allele (Ruzilawati et al., 2007).

The identification of the recently described intronic CYP3A*18B allele among Malaysians is a novel finding of this study. The variant “A” allele had a frequency of 0.48 with 18 (19.1%) of the subjects having the homozygous “A/A” variant (Table 2). It was reported earlier that the presence of the CYP3A*18B variant among Chinese renal transplant recipients could affect the pharmacokinetics of cyclosporine. Both trough levels and 2 h post-dose concentrations were significantly lower in patients who were homozygous for CYP3A*18B when compared to patients with the wild type allele, suggesting the presence of increased CYP3A4 enzyme activity (Qiu et al., 2008). These findings are further corroborated by additional studies reporting increased CYP3A4 activities among healthy Chinese volunteers treated with cyclosporine and tacrolimus (Hu et al., 2007; Shi et al., 2011; Tao et al., 2015).

The present study also demonstrates that the occurrence of CYP3A*5*3 at an allelic frequency of 0.64, with 47 (50%) of the subjects carrying the homozygous “G/G” variant, is very common among Malaysians. The allelic frequency of CYP3A*5*3 obtained in our study is similar to that reported for kidney transplant Malaysians, with a mean frequency of 0.60 [0.59 (Malaysian), 0.72 (Chinese) and 0.50 (Indians)] (Hamzah et al., 2014). The slight disparity in the frequencies may be attributed to a difference in the proportion of the three ethnicities. In our study, only a single Indian subject was included; a higher number of Indian subjects (n=7) were included in the report by Hamzah et al. (2014).

Interestingly, in the present study, no variants were detected for the CYP3A*4 and CYP3A*22 alleles. The absence of the CYP3A*4 variant allele in this study is in agreement with our previous study in which no CYP3A*4 variants were detected in 121 healthy Malaysians (Ruzilawati et al., 2007). Generally, this allele has a frequency of 0.015-0.033 in Asians, but it has not been reported in Caucasians or African Americans (Werk and Cascorbi, 2014).

The relatively small sample size makes it difficult to conclude that CYP3A*22 is absent in a particular Malaysian population. A larger prospective study should be undertaken to further establish the actual allelic frequency of the newly detected CYP3A*18B allele and the undetected CYP3A*22 allele in the Malaysian population.
Table 3. *CYP3A4*<sup>18A</sup>, *CYP3A4*<sup>18B</sup> and *CYP3A5*<sup>12</sup> variants and odds ratios of having anastrozole-associated adverse events (AAAEs).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Musculoskeletal adverse events</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A4&lt;sup&gt;18A&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type (TT)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterozygous (TC)</td>
<td>0.37 (0.04, 3.43)</td>
<td>0.380</td>
<td></td>
</tr>
<tr>
<td>CYP3A4&lt;sup&gt;18B&lt;/sup&gt;</td>
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</tr>
<tr>
<td>Wild type (GG)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterozygous (GA)</td>
<td>0.61 (0.22, 1.69)</td>
<td>0.342</td>
<td></td>
</tr>
<tr>
<td>Homozygous (AA)</td>
<td>0.77 (0.21, 2.80)</td>
<td>0.691</td>
<td></td>
</tr>
<tr>
<td>CYP3A5&lt;sup&gt;12&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Wild type (AA)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterozygous (AG)</td>
<td>1.05 (0.32, 3.45)</td>
<td>0.933</td>
<td></td>
</tr>
<tr>
<td>Homozygous (GG)</td>
<td>0.82 (0.26, 2.56)</td>
<td>0.728</td>
<td></td>
</tr>
</tbody>
</table>

Vasomotor symptoms

| CYP3A4<sup>18A</sup> | | | |
| Wild type (TT) | 1 | | |
| Heterozygous (TC) | 4.06 (0.64, 25.82) | 0.137 |
| CYP3A4<sup>18B</sup> | | | |
| Wild type (GG) | 1 | | |
| Heterozygous (GA) | 1.00 (0.33, 3.03) | 1.000 |
| Homozygous (AA) | 1.042 (0.25, 4.26) | 0.955 |
| CYP3A5<sup>12</sup> | | | |
| Wild type (AA) | 1 | | |
| Heterozygous (AG) | 1.04 (0.26, 4.11) | 0.955 |
| Homozygous (GG) | 1.68 (0.46, 6.06) | 0.427 |

Mood disturbances

| CYP3A4<sup>18A</sup> | | | |
| Wild type (TT) | 1 | | |
| Heterozygous (TC) | 1.23 (0.12, 11.82) | 0.856 |
| CYP3A4<sup>18B</sup> | | | |
| Wild type (GG) | 1 | | |
| Heterozygous (GA) | 0.53 (0.15, 1.87) | 0.325 |
| Homozygous (AA) | 0.67 (0.38, 3.40) | 0.644 |
| CYP3A5<sup>12</sup> | | | |
| Wild type (AA) | 1 | | |
| Heterozygous (AG) | 1.03 (0.17, 6.31) | 0.971 |
| Homozygous (GG) | 2.21 (0.43, 11.32) | 0.343 |

Vaginal dryness/dyspareunia

| CYP3A4<sup>18A</sup> | | | |
| Wild type (TT) | 1 | | |
| Heterozygous (TC) | 1.46 (0.15, 14.13) | 0.743 |
| CYP3A4<sup>18B</sup> | | | |
| Wild type (GG) | 1 | | |
| Heterozygous (GA) | 0.31 (0.80, 1.22) | 0.095 |
| Homozygous (AA) | 0.98 (0.22, 3.43) | 0.984 |
| CYP3A5<sup>12</sup> | | | |
| Wild type (AA) | 1 | | |
| Heterozygous (AG) | 0.32 (0.64, 1.66) | 0.177 |
| Homozygous (GG) | 0.61 (0.54, 2.45) | 0.490 |

Simple logistic regression; OR - odds ratio; 95%CI - 95% confidence interval.
The clinical relevance and contribution of these potential biomarkers to inter-individual variability in response to anastrozole should also be evaluated in a larger cohort.

CONCLUSION

Our study reports, for the first time that CYP3A4*18B allele has a high frequency in Malaysian postmenopausal breast cancer patients. The study also confirms a similar allelic frequency for CYP3A4*4 and CYP3A4*18A in Malaysians, as previously reported. No CYP3A4*22 SNPs were found in any of the study subjects. No significant associations were established between the CYP3A4*18B, CYP3A4*18B and CYP3A5*3 variants in terms of the development of AAAs (MSAEs, hot flashes, mood disturbance and vaginal dryness/dyspareunia). The clinical relevance of the newly detected CYP3A4*18B is currently under investigation by our group.

ACKNOWLEDGEMENTS

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REFERENCES


Expression of cytochrome P450 2C9 (CYP2C9) in *Escherichia coli* and its functional characterization

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Abstract. This study aimed to express the major human hepatic drug metabolizing cytochrome P450 (CYP), CYP2C9, together with NADPH cytochrome P450 oxidoreductase (OxR) in *Escherichia coli* and to evaluate its catalytic activities. Co-expression of CYP2C9 and OxR was achieved by means of separate, compatible plasmids with different antibiotic selection markers. The expressed proteins were evaluated by immunoblotting and reduced CO difference spectral scanning. Enzyme activities were examined using high performance liquid chromatography (HPLC) assays with probe substrates valsartan and tolbutamide. Results from immunoblotting demonstrated the presence of CYP2C9 protein in bacterial membranes and reduced CO difference spectra of the cell preparations exhibited the characteristic absorbance peak at 450 nm. Co-expressed OxR also demonstrated an activity level comparable to previously published data. Kinetic parameters, \(K_m\) and \(V_{max}\) values determined from the valsartan and tolbutamide hydroxylase assays, were also concordant with literature values. As a conclusion, the procedures described in this study provide a relatively convenient and reliable means of producing catalytically active CYP2C9 suitable for drug metabolism and interaction studies.

Keywords: Cytochrome P450, *Escherichia coli*, heterologous expression, human CYP2C9, kinetic analysis

INTRODUCTION

Cytochromes P450 (CYPs) constitute a large family of heme-containing enzymes, all of which catalyse the oxidation of a variety of endogenous and exogenous compounds, including drugs, carcinogens, and other xenobiotic chemicals. Knowledge regarding CYPs is therefore crucial to the fields of drug therapy and drug development, as well as in our understanding of the mechanisms underlying the metabolic activation of potentially toxic and carcinogenic compounds.

CYP2C9 is one of the major enzymes that constitutes approximately 20% of total human liver microsomal CYP proteins and metabolizes approximately 15-20% of therapeutically important drugs that undergo phase I metabolism, including anticoagulants (warfarin), hypoglycaemic agents (tolbutamide and glimepiride), non-steroidal anti-inflammatory drugs (flurbiprofen and diclofenac), diuretics (torsemide), antihypertensives (losartan and
valsartan), and anticonvulsants (phenytoin) (van Booven et al., 2009). As with many other CYP isoforms, CYP2C9 has been the main focus of many in vitro studies. However, conventional in vitro studies for human CYPs using human liver microsomes, liver homogenates or hepatocyte culture are facing several constraints and challenges such as limited tissue source, ethical clearance issue and concerns as well as intricate isolation and long-term storage procedures (Ong et al., 2013). As a solution to these problems, heterologous expressions of the human CYPs are made available in various host cells, including yeast, mammalian, insect, and bacterial cells. These systems have now become a routine and reliable resources for conducting drug metabolism and interaction investigations. Their use provides an effective way to further confirm results obtained from metabolism studies using microsomes or other tissue sources. Moreover, the activity of one specific human CYP can be studied in isolation. Recombinant preparations may also prove useful in cases where the enzyme in question may not be present in sufficient quantity in liver tissues. Also, due to the fact that only a single CYP enzyme is present, the need for a highly selective substrate probe is not required. These enzyme preparations generally afford a high level of reproducibility - once the expression plasmids are constructed and maintained in host cells, and the expression protocols are optimized, the cDNA clones represent a virtually inexhaustible supply of the proteins for routine use in the laboratories (Foti et al., 2010).

The present study describes the expression of recombinant CYP2C9 in E. coli together with its coenzyme NADPH cytochrome P450 oxidoreductase (OxR), as well as its functional characterization in reduced CO difference spectroscopy and high performance liquid chromatography (HPLC)-based assays using valsartan and tolbutamide as the probes. Although CYP2C9 has been expressed in different expression systems and characterized using a number of substrate probes, little is known regarding the kinetic behaviour of the bacterial expressed CYP2C9 in hydroxylation of valsartan. In this paper, the expression of CYP2C9 in E. coli and its kinetic characterization of valsartan oxidation, together with that of tolbutamide, are described.

MATERIALS AND METHODS

Materials and chemicals. Luria-Bertani (LB) broth was purchased from Conda Pronadisa (Spain). Chloramphenicol and ampicillin were purchased from Nacalai Tesque (Japan). Terrific broth (TB), sucrose, Tris, glycine, sodium chloride (NaCl), sodium dodecyl sulfate (SDS), isopropyl β-D-1-thiogalactopyranoside (IPTG), δ-aminolevulinic acid (δ-ALA), ethylenediaminetetraacetic acid (EDTA), phenylmethanesulfonyl fluoride (PMSF), glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PD), β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH), protease inhibitor, cytochrome c, magnesium chloride (MgCl2), Bradford reagent, potassium phosphate monobasic and dibasic, sodium dithionite, glycerol, bovine serum albumin (BSA), valsartan, tolbutamide, and 4-hydroxytolbutamide were purchased from Sigma (USA). 4′-Hydroxyvalsartan (mixture of diastereomers) was acquired from Santa-Cruz Biotechnology (USA). Dithiothreitol (DTT) was purchased from Promega (USA). Potassium cyanide (KCN) and methanol were purchased from Fisher Scientific (USA). Precast polyacrylamide gels, Laemmli sample buffer and pre-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) low range standards were products of Bio-Rad Laboratories (USA). Western Max horseradish peroxidase (HRP) chromogenic detection kit was purchased from Amresco (USA). The cDNAs of CYP2C9 and the OxR were gifts from Professors John Miners and Donald Birkett (Flinders University, Adelaide, Australia). The CYP2C9 cDNA was provided as an insert in pCWori+ expression vector while OxR cDNA was in pACYC vector.

Expression of recombinant CYP2C9 in E. coli. The expression of CYP2C9 and OxR was carried out following an established method (Gillam et al., 1994). The glycerol stocks containing the pCW-CYP2C9 co-transformed with pACYC-OxR into E. coli DH5α competent cells, were revived by culturing overnight in LB media supplemented with 70 μg ml⁻¹
chloramphenicol and 50 μg ml⁻¹ ampicillin at 37°C under aerobic condition. The next day, each overnight culture was used to inoculate 350 ml TB supplemented with the same concentration of chloramphenicol and ampicillin as in LB media and continued with incubation at 37°C in a shaking incubator. When the growth of the bacterial culture reached about 0.7 optical density at 600 nm wavelength, 1 mM IPTG and 0.5 mM δ-ALA were added into the culture to induce the expression of the CYPs. The CYP expressions were continued at 30°C for 24 hours at a shaking speed of 200 rpm. The growth of the 24-hour incubated culture was arrested by chilling on ice for 10 min. After that, the culture was harvested by centrifuging at 4°C for 10 min at the speed of 5,000 g. The pellet was weighed before resuspended in Tris-EDTA-sucrose (TES). Freshly prepared lysozyme was added into the suspension and mixed gently, then the suspension was diluted one-fold with chilled distilled water (dH₂O), followed by incubation on ice for 30 min with gentle shaking. After incubation, the mixture was centrifuged at 10,000 g for 10 min at 4°C to collect the spheroplast, which was then resuspended in spheroplasts resuspension buffer (SRB). PMSF (1 mM) and protease inhibitor (1 ml per 4 g wet cell) were added into the SRB suspension prior to sonication. After that, the bacterial lysate was centrifuged at 10,000 g for 20 min at 4°C. The supernatant was removed carefully and subject to ultra-centrifugation at 180,000 g for 75 min at 4°C to collect the membrane fractions. The membrane fraction containing the CYP and OxR proteins were resuspended in TES-water in a ratio of 50/50 and stored at -80°C until further use. The protein concentration for the recombinant protein was determined according to the Bradford’s method (Bradford, 1976) using bovine serum albumin at different concentrations as the standard.

**SDS-PAGE and Western blotting analysis.**

Prior to SDS-PAGE, the protein samples were treated with equal volume of sample buffer. The protein samples were then denatured at 95°C for 5 min using a heat block. After that, separation of the protein in the sample by size was conducted on Mini-PROTEAN® Tetra Cell (Bio-Rad Laboratories, USA), using 10% Mini-PROTEAN® TGX™ precast gels according to the protocols of Laemmli (Laemmli, 1970). By using micropipettor, 15 μl of samples were gently loaded into each well. Electrophoresis was carried out at 100 V for 75 min or until the blue dye front has reached the bottom of the polyacrylamide gel. The separated protein from the polyacrylamide gel was transferred onto nitrocellulose membrane (0.45 μm) through Western blotting using a semi-dry blotting system (C.B.S. Scientific Company, CA). The nitrocellulose membrane was pre-soaked in distilled water for 5 min and then briefly soaked in transfer buffer. The blotting was driven by at 5 V for 65 min under a constant current. Detection of the blotted CYP and OxR was carried out by using Western MAX HRP chromogenic kit (Amresco, USA). The nitrocellulose membrane was first rinsed in distilled water for 5 min, followed by wash buffer for another 5 min on a rotary shaker. The membrane was then put into blocking buffer and incubated for one hour at room temperature on a rotary shaker. After that, the blocking buffer was discarded and the membrane was probed with primary antibody solution for one hour at room temperature. The primary antibodies used were rabbit anti-human cytochrome P450 2C9 polyclonal antibody (Millipore, USA), and anti-cytochrome P450 reductase, rabbit monoclonal antibody (Abcam, UK), both incubated at 1:10000 dilution. The primary antibody was then aspirated and the blot was washed with an ample amount of wash buffer for 5 min with agitation. The wash buffer was then discarded and this washing step was repeated twice. The membrane was transferred into secondary antibody solution containing HRP conjugated goat anti-rabbit IgG secondary antibody (Amresco, USA) and incubated for 30 min with gentle agitation at 1:10000 dilution. The secondary antibody was then discarded and the membrane was washed for three times with wash buffer, each for 5 min. After washing, the membrane was then put into substrate solution containing 3,3’-diaminobenzidine (DAB) substrate and incubated at room temperature until brown colour bands were seen on the membrane. The residual substrates were washed away by putting the membrane in distilled water for 5 min with agitation. Finally, the membrane was air-dried and stored in the dark.
**Spectral determination of CYP content.** The CYP content of the membrane fraction, which was indicator for holoenzyme level of the expressed protein, was determined spectrophotometrically by reduced-carbon monoxide (CO) difference spectroscopy (Omura and Sato, 1964). Firstly, the cell lysate was solubilized in 50 mM phosphate buffer, pH 7.4 containing 20% glycerol and then further diluted to 1 mg ml\(^{-1}\) with phosphate buffer. This protein sample was distributed equally into two quartz cuvettes with 1 cm path length. A pinch of solid sodium dithionite was added into each cuvette containing the sample and mixed well. The baseline between 400 and 500 was recorded using a double beam Shimadzu UV-1800 UV-VIS spectrophotometer (Shimadzu Corp., Japan). After that, CO was bubbled through the sample for 1 min and the spectrum was recorded. The concentration of CYP in the cuvette was calculated using Beer’s Law equation (Equation 1):

\[
A = \varepsilon \cdot c \cdot L \quad \text{(Equation 1)}
\]

where \(A\) was the difference in light absorption at 450 nm relative to 490 nm, \(L\) was the light path of the cuvette and the extinction coefficient \(\varepsilon_{450-490}\) was 91 mM\(^{-1}\) cm\(^{-1}\).

**Determination of NADPH cytochrome P450 oxidoreductase activity.** The activity of the co-expressed OxR was determined by NADPH cytochrome c reductase assay. This assay measures the reduction of cytochrome c by the OxR in the presence of NADPH (Vermilion and Coon, 1978). A dual-beam recording spectrophotometer (Jasco V-630) was set to time scan at a wavelength of 550 nm. Two quartz cuvettes with 1 cm path length were prepared of which one served as reference cuvette and the other as sample cuvette. Each of the cuvette was filled with 1 ml of cytochrome c solution (0.125 mM in 0.3 M potassium phosphate buffer, pH7.7). After that, 200 μl of 15 mM KCN was added into each cuvette. A measured amount of sample protein (final protein concentration of 0.5 – 1.0 mg ml\(^{-1}\)) was added into each cuvette and mixed by gentle inversion. The baseline absorbance versus time at 550 nm was recorded for 2 min. After that, a volume of 100 μl NADPH solution was added into the sample cuvette and mixed gently by inversion. The increase in absorbance with time at 550 nm was recorded over 2 min. The activity of reductase was determined by Equation 2, where 0.021 is the extinction coefficient for reduced cytochrome c and 1.2 is the total volume of reaction mixture in cuvette. The specific content of reductase (nmol cytochrome c reduced min\(^{-1}\) mg protein\(^{-1}\)) was then calculated based on the concentration of protein in the sample:

\[
\text{Activity of cytochrome c reductase (nmol)} = \frac{\Delta \text{Abs at 550 nm min}^{-1}}{0.021} \times 1.2 \quad \text{(Equation 2)}
\]

**Establishment of valsartan 4-hydroxylase assay.** The HPLC-based assay was established based on the published procedure (Nakashima et al., 2005) with some modifications. In this assay, valsartan was used as the substrate probe to investigate the catalytic activity of CYP2C9. In the presence of active CYP2C9, valsartan was converted into the 4-hydroxyvaleryl metabolite of valsartan (4-OH valsartan) through 4-hydroxylation. In order to prepare the HPLC injection sample, an incubation mixture with total volume of 200 μl was prepared in a 1.5 mL microcentrifuge tube. The stock solutions (10-50 mM) for the substrate, valsartan, was prepared by dissolving the measured solid valsartan in 70% ethanol. The incubation mixture contained 0.1 mg bacterial membrane protein, valsartan and NADPH generating system (1 mM NADP, 10 mM G6P, 2 IU G6PD and 5 mM MgCl\(_2\) in 0.1 M phosphate buffer at a pH 7.4. After that, the mixture was incubated at 37°C for 30 min in a water bath with gentle shaking. The reaction was terminated by adding 1 ml of ice-cold acetoneitrile with vortex mixing. After centrifugation at 1800 g for 5 min at 25°C, the supernatant was carefully transferred into another tube and evaporated to dryness using a centrifugal evaporator with vacuum pump (Eyela, USA). The residue was dissolved in 200 μL of 10% methanol with vortex mixing. After centrifugation at 1800 g for 5 min at 25°C, an aliquot of 100 μl was injected onto HPLC to determine the amount of 4-OH valsartan formed in the catalytic reaction by CYP2C9. The analytical HPLC was carried out...
Expression of human CYP2C9 in E. coli

Western blotting of CYP2C9 and OxR proteins. Expression of the recombinant CYP and OxR was carried out in E. coli and the extraction procedures released the expressed proteins from the harvested cell in the form of semi-purified membrane fractions. Membrane fractions extracted from E. coli cells harbouring the pCWori+ plasmid without any gene inserts and expressed under the same conditions as the recombinant CYP and OxR served as control protein in this study. Separation of the protein mixtures in the membrane fractions by size was conducted using SDS-PAGE. The separated proteins were then transferred onto nitrocellulose membrane by semi-dry blotting and the target proteins were detected by matched antibodies as described under Materials and Methods. Colorimetric DAB substrates were used to detect the HRP-labelled target proteins and produced brown colour bands directly on the surface of the blots within seconds.

The results from immunoblottings showed the presence of immunoreactive band with molecular mass approximately 56 kDa in the lane loaded with CYP2C9 (Figure 1a). The detected brown-coloured band indicated molecular mass corresponding to the reported molecular mass of this CYP isoform. There were no bands detected from the control protein at the same molecular mass, indicating the absence of CYP and thus confirmed the expression of immunoreactive CYP2C9 from the recombinant stocks. The presence of an extra band with higher molecular mass in CYP2C9 (Figure 1a) might be caused by non-specific binding of polyclonal primary antibody that was used in the immunoblotting. Immunoblotting was also carried out to detect the co-expressed OxR in E. coli. As shown in Figure 1b, a single protein band was detected with a molecular mass around 75 kDa that was in accordance to the published molecular mass for OxR. Therefore, the expression of OxR in the fraction was confirmed.

Enzyme kinetic characterization of CYP2C9. Catalytic activity of the CYP2C9 was examined by incubating a series of concentrations (ranging from 10-1000 μM) of valsartan with 0.1 mg CYP2C9 in the reaction mixtures at 37°C for 30 min. Using these data, saturation plot was generated using EZ-Fit kinetic software (Perrella Scientific Inc, USA) to determine Michaelis-Menten constant (Km) and maximum velocity (Vmax) value. Saturation plot or substrate saturation curve is used to examine the change in reaction velocity as the substrate concentration is increased. The rate of reaction when the enzyme is saturated with substrate is designated as Vmax, whereas Km is expressed as half of Vmax. In addition to valsartan, the expressed CYP2C9 in this study was also examined for its kinetics using tolbutamide 4-methylhydroxylase assay. The details of this HPLC-based assay has been described in our earlier published papers (Pan et al., 2010; Pan et al., 2012).
Expression of human CYP2C9 in E. coli

Figure 1. Western blot analysis on the expression of CYP2C9 (a) and OxR (b) in E. coli. SDS-PAGE were performed on 10% denatured polyacrylamide gels and the separated proteins were transferred onto nitrocellulose membranes. Immunodetection of CYP2C9 and OxR was done by using appropriate primary antibodies. Lane 1: Prestained protein standards, low range (Bio-Rad, USA); lane 2: membrane fractions extracted from control cell (10 μg); lane 3: membrane fractions (10 μg) containing the expressed CYP2C9 (a) and OxR (b). Lanes 4 and 5 in (b) are membrane fractions containing the expressed OxR derived from different stocks.

Functional characterization of expressed CYP2C9 and OxR. The membrane fractions prepared from the harvested E. coli cultures contained semi-purified CYP2C9 and OxR. Western blotting can only detect the presence of immunoreactive CYP2C9 in each membrane protein sample but not the specific CYP content. In order to quantify the actual CYP content in each membrane sample, reduced CO difference spectral assay was performed. The principle of this assay is that the reduced form of CYP (ferrous CYP) reacts with CO to form a complex that produces a distinguished Soret band at around 450 nm, due to the signature cysteine thiolate axial ligand to heme iron in the CYP. The presence of Soret band around 450 nm in CYP2C9 protein sample (Figure 2) confirmed the expression of the active CYP. This assay was also used to characterize the recombinant CYP preparations in the context of stoichiometry of active CYP per unit protein. Using Equation 1, the spectral content of CYP2C9 was determined to be 366.0 ± 16.8 pmol mg⁻¹ protein. A relatively small absorbance peak was observed around 420 nm for CYP2C9 (Figure 2) due to low level of inactive CYP protein in the preparation. This level of inactive protein was however relatively small compared to the large absorbance peak of 450 nm which represented the active form.

Figure 2. Carbon monoxide reduced spectrum used for the quantification of CYP content in the E. coli membrane protein preparation of CYP2C9. The reduced form of CYP bound to CO and produced a characteristic spectrum with a wavelength maximum at 450 nm. The x axis represents the wavelength (nm) while axis y shows the absorbance of the CYP protein. The difference in absorbance unit was used to measure the spectral content of CYP in the tested protein samples.

On the other hand, the expression of spectrally active OxR in protein preparations was confirmed through the NADPH CYP oxidoreductase assay (Figure 3) and the level of
expression was quantified using Equation 2. In this assay, OxR accepted electrons from the biological hydride donor NADPH and transferred these to cytochrome c, therefore the activity of OxR was expressed as the molarity of cytochrome c reduced per min in one mg of protein sample. The rate of reduction of cytochrome c was measured from the absorbance difference at 550 nm wavelength per min from the spectrum recorded with spectrophotometer (Figure 3). From the results, the cytochrome c reduction activity per mg protein preparation containing CYP2C9 was found to be 1280 ± 340 nmol min⁻¹ mg protein⁻¹.

Figure 3. NADPH-cytochrome c reduction assay to measure the OxR content in the E. coli membrane protein preparation of CYP2C9. The activity of OxR was determined as the rate of reduction of cytochrome c at 550 nm wavelength per mg membrane protein. The x axis represents the time of incubation (minutes) while axis y shows the absorbance of the reduced cytochrome c solution.

Hydroxylase activities of CYP2C9 towards valsartan and tolbutamide. The chromatograms obtained from incubation mixture containing the expressed CYP2C9 in comparison to that of the control cell (membrane fractions of control cell harbouring only pCWori+ plasmid) is shown in Figure 4. From the figure, valsartan and its metabolite (OH-valsartan) were eluted at retention times of 8.1 and 2.7 min, respectively. The valsartan and OH-valsartan peaks were subsequently proven as injection of pure compounds onto the system yielded peaks with the same retention times. Having optimized the chromatographic and incubation conditions, the rate of formation of OH-valsartan from valsartan in the incubation mixture containing CYP2C9 was plotted against the substrate concentration to create a saturation plot (Figure 5). Saturation plot is used to determine the Michaelis-Menten kinetics of an enzyme, including the maximum rate of reaction (Vₘₐₓ) when the enzyme is saturated with substrate, and the Michaelis constant (Kₘ) that represents the concentration of substrate when the enzyme achieves half of its Vₘₐₓ value.

Figure 4. Representative HPLC chromatograms of incubations of valsartan (150 μM) with 0.1 mg/200 μl control cell membranes (top) and 0.1 mg/200 μl of bacterial membranes expressing CYP2C9 and OxR (bottom) at 37°C for 45 min. The x axis shows the retention time (minutes) whereas axis y represents the fluorescence absorbance of the analytes (in I.U.). Peak labelled as Val (valsartan) was eluted at retention time of 8.1 min. OH-valsartan (OH-Val) was observed as the peak with retention time of 2.7 min.
Expression of human CYP2C9 in E. coli

DISCUSSION

Impelled by the advent of recombinant DNA technology, many in vitro studies of human drug metabolism using heterologous expression systems in different host cells have been materialized. Among the many heterologous expression systems available, bacterial expression system is the most favourable in terms of high proliferation rate, high protein yield, low maintenance cost, and easy handling (Zelasko et al., 2013). E. coli is the most extensively utilized bacterial host in the production of recombinant human CYPs in attempt to generate ample amount of CYP proteins with relatively short culture periods for the study on drug metabolism and toxicology. While the advantages are clear, the drawback of this system is that the low expression of functional CYP of human origin due to their hydrophobicity, their requirements for heme incorporation and folding problems. After years of research to improve the functional expression of human CYP in E. coli, several strategies have been exploited to overcome this problem, including: (i) replacing the second codon at the N-terminal of native CYP sequence with GCT coding for Alanine; (ii) reduction of free energy for secondary structure formation by changing G:C pairs to A:T pairs in the first 10 codons; (iii) removal of hydrophobic segments at the N-terminal; (iv) alignment to insert the sequence MALLLAVFL at the N-terminal; (v) co-expression of CYP and OxR; and (vi) incorporation of δ-ALA as precursor for heme in culture media (Yun et al., 2006; Schroer et al., 2010; Zelasko et al., 2013).

Human CYPs are about 500 amino acid residues in length, including an N-terminal endoplasmic reticulum (ER) retention signal. Removal of this N-terminal ER retention signal and aligning an inserted N-terminal membrane anchor (MALLLAVFL…) have been shown to markedly increase expression of CYP in bacterial cell without altering catalytic activity (Yun et al., 2006). Our laboratory has previously generated CYP cDNAs with N-terminal modifications to overcome the low expression level of human protein expression in E. coli (Pan et al., 2011; Tiong et al., 2010). These CYPs were successfully cloned into pCWori+ expression vector and transformed.
Expression of human CYP2C9 in E. coli DH5α cells. Table 1 lists the N-terminal modifications of the CYP2C9 cDNA that was used in this study. The N-terminal modification involved replacing the second codon with GCT (substitution of ASP for ALA), deleting codons 3–20, and modifying codons 21–26 for bacterial codon bias. These modifications involved the strategies (i) and (iii) discussed above and have effectively removed the hydrophobic segment at the N-terminal and the modified construct has resulted in successful expression of CYP2C9 in this study.

Co-expression of the CYPs and OxR has been found to increase catalysis activity in bacterial cells. To achieve this purpose, expression of both proteins was carried out independently using two independent expression plasmids for the proteins. This was accomplished by using pCWori+ (with CYP2C9 cDNA inserted) and pACYC (with OxR cDNA inserted) plasmids in our system. Both pCWori+ and pACYC were two different but compatible plasmids suitable for use in bacterial expression system. pCWori+ possesses the ColE1 plasmid of origin, whereas pACYC carries the p15A origin of replication. These two replicons are commonly used control elements in bacterial cells to regulate and drive replication of plasmids and hence expression of any foreign genes inserted within. Furthermore, the two tandemly arranged tac promoter which are inducible by IPTG have facilitated the expression of CYP2C9. Besides, the presence of antibiotic resistance gene in pCWori+ vector (ampicillin resistance) and pACYC vector (chloramphenicol resistance) allow the selection and isolation of bacterial cell populations carrying both plasmids in culture media in which ampicillin and chloramphenicol have been incorporated. Figure 6 depicts the general maps of the constructed pCW-CYP and pACYC-OxR plasmids adopted in this study.

Table 1. N-terminal modifications of CYP2C9 primary sequences adopted in this study.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence/construct</th>
<th>N-terminal amino acid sequence</th>
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<tbody>
<tr>
<td>CYP2C9</td>
<td>Native</td>
<td>MDSLVLVLVLCCLLLLWLRQSS</td>
</tr>
<tr>
<td></td>
<td>Modified</td>
<td>MA……………………………RQSS</td>
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Figure 6. Map of the constructed pCW-CYP and pACYC-OxR with the insertion and the orientation of CYP gene and OxR gene. pCW-CYP contains ColE1 origin of replication (ColE1 ori), ampicillin resistance (AmpR) and two tandemly arranged tac promoter (Ptac), while pACYC-OxR harbours p15A origin of replication (p15A ori), chloramphenicol resistance (CmR) and two Ptac. The OmpA bacterial leader sequence accounts for the first 21 amino acids at the N-terminal of OxR (OmpA-OxR).
Besides the construction of functional CYPs, the incubation conditions can also affect the level of expression of recombinant CYPs. Rich media such as Terrific broth was used as the incubation media for the *E. coli* culture to facilitate high-level protein expression. Since *E. coli* is unable to biosynthesize heme intracellularly, addition of the heme precursor δ-ALA in the growth media can enhance the yield of spectrally active CYP, presumably owing to proper folding and incorporation of heme (Yun et al., 2006; Zelasko et al., 2013). A concentration of 0.5 mM ALA is sufficient to enhance the expression of CYP in *E. coli* (Guengerich et al., 1996), but the concentration required for maximal expression varies among individual CYP and thus should be optimized. Incubation temperature is essential for successful expression of human CYPs in *E. coli*. Lower temperature at incubation is assumed to slow the expression of protein sufficiently to allow time for proper folding and heme incorporation. The optimal expression temperature tends to be between 26 and 32°C (Yun et al., 2006). The incubation time to carry out protein expression was set at 30°C as this was the temperature commonly used to express the recombinant CYPs. Besides temperature, the induction time should be optimized for each CYP. Typical induction time is 24-72 hours (Yun et al., 2006), and thus the induction time in this study has been set to 24 hours. Since *E. coli* is aerobic organism, the aeration of cultures may affect the expression of protein. Routine incubation was carried out at 200 rpm on a shaking incubator and the total volume of culture media was capped at one-third of the flask volume to provide sufficient level of aeration for the bacterial growth.

Having isolated the membrane fractions from the bacterial cultures, the expression of the recombinant CYP2C9 was confirmed by Western blotting (Figure 1), indicated by detection of visible band around 56 kDa. Immunoblotting however can detect the expression of both apoprotein (CYP2C9 protein without heme prosthetic group) and holoprotein (CYP2C9 protein with heme prosthetic group), but not specifically the expression level of functional holoprotein. Therefore quantitative analysis using reduced-CO difference spectrophotometry was performed to measure the content of stoichiometrically active CYP per unit protein in the recombinant CYP2C9 preparations.

The expressed CYP2C9 protein was characterized for its absorbance through reduced-CO difference spectra. This spectral characterization has been used as a classical assay to examine CYP expression and activity since the discovery of the enzyme in early 1960s (Omura and Sato, 1964; Guengerich et al., 2009). As illustrated in Figure 2, CYP2C9 expressed in this study exhibited the characteristic absorbance peak around 450 nm and thus the expression of the holoenzyme was confirmed. A relatively small absorbance peak was observed around 420 nm and this is due to low level of inactive CYP protein in the preparation. It is customary to observe a minor peak at 420 nm in cDNA-expressed CYP preparations that represents the denatured form of the protein where there is no efficient folding in the protein tertiary structure and is likely due to the loss of cysteine thiolate linkage to the heme iron, causing instability of the ferrous/CO-bound form of the enzyme. Normally in the properly expressed CYPs, the P420 peak is rather small and does not affect the overall functionality of the CYPs (Narimatsu et al., 2004; Zheng et al., 2003). The total CYP content in membrane fractions containing CYP2C9 was found to be the high (366.0 ± 16.8), and was close to the reported values (in the range of 120 – 800 pmol mg protein⁻¹) of different CYP isoforms using the similar heterologous expression system (Blake et al., 1996; Boye et al., 2004, Pritchard et al., 2006).

Co-expression of OxR in CYP2C9 culture was also confirmed through Western blot, revealing a band around 75 kDa, corresponding to the reported molecular weight of OxR (Figure 1). Once again, immunoblotting could not conclude the expression level of functional OxR and thus spectral characterization was carried out to quantitate the amount of active OxR. The activity and level of expression of the co-expressed OxR was determined by NADPH cytochrome c reductase assay. The reduction of cytochrome c formed distinct bands in the absorption spectrum. The increase in absorbance at 550 nm was measured with time (Figure 3) and the activity of OxR was calculated using Equation 2. The membrane fractions showed cytochrome c reduction activity of 1280 ± 340 nmol min⁻¹ mg
protein\(^1\) which was in line with the reported values ranging from 300 to 1300 nmol min\(^{-1}\) mg protein\(^{-1}\) (Blake \textit{et al}., 1996, Pritchard \textit{et al}., 1998, Pritchard \textit{et al}., 2006) and thus confirmed the expression of functional OxR in the recombinant cultures.

Valsartan is an orally active specific angiotensin II receptor antagonist which causes reduction in blood pressure and is used in treatment of hypertension (Flesch \textit{et al}., 1997). It was first developed by Novartis and sold under the brand name DIOVAN which was the world’s number-one selling high blood pressure medication (Siddiqui \textit{et al}., 2011). Since valsartan is metabolised predominantly by CYP2C9 to form 4-hydroxyvaleryl metabolite, a HPLC-based assay to assess CYP2C9 activity \textit{in vitro} using valsartan as the activity marker has been established in this study. Furthermore, this CYP2C9 substrate is relatively new as compared to the other well-established substrate probes for CYP2C9 such as diclofenac and (S)-flurbiprofen.

Both the substrate and metabolite were well-separated by chromatography and distinguishable as individual peaks (Figure 4) in the assay developed in this study. Kinetic characterization of CYP2C9 was subsequently carried out by constructing the saturation plot (Figure 5) where valsartan concentration was plotted against the rate of formation of its metabolite (OH-valsartan). Metabolite formation demonstrated a hyperbolic curve in substrate concentration-velocity plot, indicating increasing reaction rate at low substrate concentrations and saturating rate at higher concentrations. This hyperbolic curve in saturation plot revealed an enzyme-catalyzed reaction involving a single substrate and that the metabolite formation was in accordance to the Michaelis-Menten kinetics, with apparent \(K_m\) value of 146.0 ± 53.6 μM and \(V_{max}\) value of 43.0 ± 4.9 pmol min\(^{-1}\) mg\(^{-1}\) as determined in triplicates. The maximum velocity of the CYP2C9 prepared in this study compares favourably with those reported \(V_{max}\) values in literature (27.2 – 216.9 pmol min\(^{-1}\) mg\(^{-1}\) protein\(^{-1}\)), implying that the CYP2C9 preparation in this study was enzymatically as active as those prepared in other laboratories (Nakashima \textit{et al}., 2005). On the other side, the CYP2C9 in this study exhibited a higher \(K_m\) as compared to the reported \(K_m\) values (41.9 – 55.8 μM) (Nakashima \textit{et al}., 2005). The deviation of \(K_m\) here was however relatively small with difference of less than one order of magnitude from the reported values, and coupled with acceptable \(V_{max}\) value, the assay can be accepted as a valid activity marker for CYP2C9. Kinetic parameters \(K_m\) and \(V_{max}\) were also determined in the present study with the second substrate probe, tolbutamide, with values of 273.0 ± 62.9 μM and 3240 ± 240 pmol min\(^{-1}\) mg protein\(^{-1}\) respectively. These values fell within the published range in literature, where \(K_m\) and \(V_{max}\) values of 80.7-348.0 μM and 115.0-9130.0 pmol min\(^{-1}\) mg protein\(^{-1}\) have been reported (Miners \textit{et al}., 1988; Doecke \textit{et al}., 1991; Veronese \textit{et al}., 1993; Hickman \textit{et al}., 1998; Shin \textit{et al}., 1999; Komatsu \textit{et al}., 2000). The difference among the \(K_m\) values and the wide range of \(V_{max}\) values reported in literature might be caused by the use of different source of CYP2C9, in which the source of enzyme in the current study was membrane fractional preparation containing recombinant CYP2C9 and in the reference studies cited, human liver microsomes and recombinant CYP2C9 expressed in different cell lines such as \textit{E. coli}, COS, and lymphoblastoid cells were the sources. Some investigators have suggested that the causes of the difference in Michaelis-Menten kinetic parameters could be due to: (a) inter-individual variation from using liver microsomes isolated from donors with different genetic/disease/drug backgrounds, (b) different heterologous expression system in the case of recombinant CYPs, owing to the unique physiological conditions in each system that affect the CYP quantity/affinity/activity profiles, and (c) inter-laboratory variations including difference in experimental conditions (Ong \textit{et al}., 2013). These variations and their causes have important implications on the validity of metabolic \textit{in vitro-in vivo} quantitative extrapolations and thus these variations should be taken into consideration when conducting such extrapolations. Considering all the facts above, both \(K_m\) and \(V_{max}\) values from this study reside close to and within the variability of reported values for CYP2C9 in the literatures, the assays (using valsartan and tolbutamide) can therefore be accepted as valid activity markers for CYP2C9.
CONCLUSION

In this study, CYP2C9 was successfully expressed in bacterial cells as indicated by the immunoblotting, reduced CO difference spectroscopy and HPLC-based assays. The kinetic data of the expressed CYP2C9 were close to the published values indicating that the protein expressed in the membranes of E. coli was catalytically active and fully functional. The established assays will serve as an investigational tool in drug metabolism and drug interaction studies involving this functionally important CYP isof orm.

ACKNOWLEDGEMENTS

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Acute gamma irradiated Stevia rebaudiana Bertoni enhanced particular types of steviol glycosides

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Abstract. Stevia rebaudiana Bertoni from Asteraceae family is commercially valuable for its steviol glycosides (SGs) contents, which is 300 times sweeter than commercial sugar. The bottleneck in Malaysia is the lack of suitable stevia varieties that are able to thrive well under her climatic conditions and still produce high SGs. Mutation induction including gamma irradiation is effective in generating genetic variations and developing new plant varieties with desired traits. This study was aimed to determine the effects of acute gamma irradiation on phenotypic changes and enhancement of SGs contents of Stevia rebaudiana Bertoni variety AKH L1 (herein after will be designated as AKH L1). In vitro shoot tip explants of AKH L1 were subjected to a gamma doses regime of 10Gy to 50Gy, following which phenotypic changes of the irradiated explants and subsequent regenerated plantlets were observed. All irradiated explants exhibited different survival rates, with the lowest at 9.33±8.33% when subjected to 50Gy, while all the control (non-irradiated explants) survived. The LD\textsubscript{50} was found to be at 23Gy. Subsequent irradiation of 900 shoot tip explants at 23Gy, produced 468 surviving shoot tips, which were all capable to develop and successfully sub-cultured until the fourth generation, M\textsubscript{4}. These M\textsubscript{4} in vitro mutant plantlets exhibited significant increase in the numbers of leaf (16.07±5.19) and average leaf size (1.12±0.26cm x 0.54±0.15cm). HPLC analysis performed in parallel further revealed the mutant plants contained higher concentrations of stevioside (387.04ppm), rebaudioside A (670.18ppm) and rebaudioside D (106.26ppm) compared to the non-irradiated plantlets, which exhibited 96.87, 194.42 and 28.25ppm, respectively.

Keywords: stevioside, rebaudioside A, rebaudioside D, HPLC, LD\textsubscript{50}, irradiation

INTRODUCTION

Stevia rebaudiana Bertoni, a perennial sweet herb belongs to the family Asteraceae, is one of the 154 members of the genus Stevia. This plant is the most important source of non-caloric natural sweeteners, and is mainly known as “Sweet Weed”, “Sweet Leaf”, “Sweet Herbs” and “Honey Leaf”. The property of the species that called attention to the plant is the intense sweet taste of the leaves and aqueous extracts (Uddin \textit{et al.}, 2006). The extract of the stevia plant contains mixture of various sweet steviol glycosides, designated as SGs, such as stevioside, rebaudioside A, B, C, D, E and F, dulcoside A, steviolbioside, steviolmonoside, and rubusoside, which accumulate in the leaves and is 300 times sweeter than sugar (Ahmad \textit{et al.}, 2011; Reis \textit{et al.}, 2011; Mathur & Shekhawat, 2012). The compounds that made up the majority

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proportions of SGs are stevioside and rebaudioside A, and they have been extensively used in the food and beverage industry. Stevioside traditionally makes up the majority of the sweetener which comprises 60-70% of the total glycosides content and has been evaluated as being 110-270 times sweeter than sugar (Dubois, 2000). It is believed to give a somewhat bitter aftertaste, or reported as a “licorice” taste or lingering effect (Carakostas et al., 2008). Rebaudioside A (30-40% of total sweet content) is of particular interest as it is 180-400 times sweeter than sugar and has more pleasant sweet taste and no bitter aftertaste (Carakostas et al., 2008). Therefore, a higher ratio of rebaudioside A to stevioside indicates a better sweetness quality, and is hence, preferred as reported by Yadav et al. (2011).

Jain (2010) proposed that mutation breeding was preferred over traditional breeding methods and genetically modified organisms (GMO) in the past few years. This is because multiple traits mutants can be isolated in mutation induction, but only single trait can be introduced into the crop in a transgenic way. Also, mutation induction can help to establish mutant lines range and determine trait specific genes in order to create molecular gene database, for molecular and functional genomics study and improve bioinformatics for future plant varieties development (Jain, 2010). Radiation treatment of plants had been reported as one of the most familiar methods for induction of plant mutations (Oladosu et al., 2016). The newly developed mutant varieties were useful in developing new plant varieties as well as for functional studies of genes (Hase et al., 2000; Tanaka et al., 2002; Shikazono et al., 2003).

In Malaysia, stevia is not recognized as a commercial crop due to lack of suitable variety when it was first introduced in the mid-1970s (Armizatul et al., 2009). Stevia is a short day plant with a critical day length of at least about 13 hours (Lester, 1999). This is in line with Armizatul et al. (2009) report that stevia plant exhibited reduced vegetative growth and lower steviosides content when the day length in Malaysia was less than the critical 13 hours. Several improvements in crop quality and bioactive metabolites production through induced mutagenesis have been reported such as rice with low amylase and protein contents for diabetes people (Chen et al., 2006), maize with better protein contents (Tomlekova, 2010), and cassava with increased amylase content (Ceballos et al., 2008). Khalil et al. (2014) reported higher stevioside contents were obtained from in vitro shoots from irradiated seeds of stevia. With these promising results, induced mutagenesis has potential to be applied on stevia to develop new mutant varieties that are suitable for growing under Malaysia’s climatic condition and increases its potential as a viable crop plant. SGs content is the key selling point for stevia, and hence, increasing or improving the yield of SGs is crucial. There is still limited report regarding research on the effect of gamma irradiation on the accumulation of SGs contents of stevia in Malaysia. Hence, the main objective of this study was to create new varieties of stevia with high particular types of sweet glycosides via acute gamma irradiation suitable for growing in Malaysia.

**MATERIALS AND METHODS**

**Plant materials preparation.** One-month-old potted *Stevia rebaudiana* Bertoni AKH L1 propagated from stem cutting were bought from nursery located at Denai Alam, Selangor (3.153028, 101.520538). The sterilization methods employed for *in vitro* plants initiation using potted stevia plants were slightly modified from Nurhidayah et al. (2014). Young, actively growing shoots were collected from one-month-old potted AKH L1 plants. Nodes measuring 2 cm were chosen as explants where each was cut and placed under running tap water for 20 minutes to remove traces of soil and dirt. Prior to treatments, the explants were shaken with 2 mg/L fungicide (Ridomil) for 5 minutes and rinsed thrice with distilled water. Under aseptic condition, the explants were surfaced sterilized with 70% (v/v) ethanol for 30 seconds, followed by 5% (v/v) commercial sodium hypochlorite for 15 minutes. Then, the explants were rinsed with sterile distilled water for four times and dried with sterile blotting paper.

**Shoot induction and multiplication.** Shoots induction was initiated using surface sterilized nodal explants cultured on full strength
Murashige and Skoog (MS) basal medium (Murashige & Skoog, 1962) containing 30 g/L sucrose and solidified with 7.5 g/L of agar (Gelrite) supplemented with 1 mg/L 6-benzylaminopurine (BAP). The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 15 minutes. The cultures were incubated at 24±2°C with a 16 hours photoperiod and allowed for regeneration and multiplication for two weeks. Two types of explants (shoot tip and nodal segment) were used to determine which explants was more suitable for micropropagation. All cultures were examined after one month of incubation for the following parameters: shooting percentage, initiation time (day), number of shoot, shoot length and number of leaf. The regenerated shoots were cut, and shoot tips with four leaflets were placed on MS medium supplemented with 1 mg/L BAP. All experiments were repeated twice.

**Acute gamma irradiation.** One-week-old AKH L1 shoot tips with four leaflets were irradiated with gamma rays at different doses of 0, 10, 20, 30, 40, and 50Gy using the Malaysia Nuclear Agency (MNA) Gamma Cell Facility, Bangi, Selangor, Malaysia at different doses of the source of gamma rays was Caesium¹³⁷. Each experiment constituted 5 replicates per dose, with 5 explants per replicate. The whole experiment was repeated twice. The irradiated explants were cultured on basal MS medium and incubated at 24±2°C with a 16 hours photoperiod. After 4 weeks of culture, data were taken based on percentage of survival, length of explants, number of new shoots formed and number of leaves. The lethal dose, LD₅₀ was calculated based on the percentage of survival, whereby further acute irradiation was carried out at the selected LD₅₀ dose. The newly regenerated shoots were subcultured up to M₄ generation for selection of steviol glycosides content.

**Plant leaf extract preparation.** The steviol glycosides content were analyzed for both M₁ generation of irradiated and non-irradiated plantlets. The plant extracts preparation methods used were slightly modified from Abou-Arab et al. (2010). The leaves were randomly picked and dried under room temperature ranging from 25±2°C on the bench-top in the laboratory for three days. All the dried irradiated and non-irradiated stevia leaves were, separately, grounded into powder form by using mortar pestle. Approximately 0.4g of powdered leaves was extracted in 10 ml of distilled H₂O in tubes placed in a beaker of water and allowed to boil for 15 minutes. The crude extracts containing steviol glycosides were cooled to room temperature for 5 minutes, and then filtered through Whatman filter paper No. 1. The filtrates were freeze-dried using the VirTis® BenchTop™ “K” Series Freeze Dryers (SP Industries, USA) for two days and stored at 4°C in airtight bottles.

**Steviol glycosides analysis.** Dried leaf extracts were subjected to High Performance Liquid Chromatography (HPLC) analysis. The HPLC system used was an Agilent 1260 Infinity (Agilent Technologies, CA, USA) equipped with an auto sampler, quaternary pump and a diode array detector. The HPLC method was performed using an Agilent Poroshell 120 C18 column (3.0 × 50 mm, particle size 2.7 µm) and a UV detector set at 210 nm. Separation was done with a ratio of ultrafiltration 80/20 H₂O:acetonitrile, set to a flow rate of 0.8 mL/min. The standards consisted of stevioside, rebaudioside A and rebaudioside D were run on the HPLC system and the retention time for each was recorded. Identification of stevioside, rebaudioside A and rebaudioside D in the plant leaf extracts were calculated by comparing the retention time of the samples with the standards. The results obtained for each sample were expressed in parts per million (ppm).

**Data analysis.** Each experiment was repeated twice and the data represented the means of three experiments. Statistical analyses of mean values, standard deviation (±) and significant difference were carried out by using IBM-SPSS software Window version 20. The data were subjected to One-Sample T Test or One Way Analysis of Variance (ANOVA) analysis and the means were compared by using Duncan’s Multiple Range Test (DMRT) at significance p<0.05.

**RESULTS AND DISCUSSION**
Establishment of in vitro stevia plantlets. *Stevia rebaudiana* Bertoni variety AKH L1 can be propagated by stem cutting, seed propagation and micropropagation (tissue culture method). It had been reported that seed propagation was not effective due to low fertility (Tadhani *et al.*, 2006) and self-incompatibility of its flowers, while propagation through stem cutting has limitation on account of the low number of new plants (Miyagawa *et al.*, 1986). Thus, by considering future needs of plant materials, micropropagation was chosen for rapid multiplication of stevia in this experiment. In vitro stevia plantlets were established from nodal explants excised from young, actively growing shoot tips of glasshouse grown, one-month-old potted AKH L1. The explants were sodium hypochlorite-ethanol sterilized prior culturing on MS medium supplemented with 1 mg/L BAP. Past findings by Thiyagarajan and Venkatachalam (2012) found that MS medium supplemented with 1 mg/L BAP was suitable for shoots multiplication of stevia. Thus this concentration of BAP was used in this study to mass propagate the AKH L1 plantlets. Shoots regenerated from nodal explants were observed after 7 days of culture, and newly regenerated plantlets were formed after one month. Shoot tips were cut and sub-cultured on fresh medium monthly to allow for regeneration (Figure 1).

Shoot tips were chosen as explants for mass propagation of stevia because it showed significantly (p<0.05) higher shooting percentage (97.78%) and shorter initiation time (3.33 days) compared to nodal explants. Figure 2 clearly showed that shoot tip explants were better than nodal explants with respect to the number of shoots formed, shoot length increment and number of leaves produced. The tallest shoots (10.50±2 cm) were induced from shoot tip explants which produced significantly greater numbers of leaf (20.67±3.06) as summarized in Table 1. These observations were similar to El-Motaleb *et al.* (2013) data whereby shoot tip explants also proved to perform better than nodal explants in terms of shooting percentage, shoot length achievement and shoot initiation time. Both Patil *et al.* (1996) and Mubarak *et al.* (2008) also reported that shoot tip explants gave the highest number of plantlets than nodal explants in micropropagation of stevia. This could probably be due to lower endogenous phytohormonal level in the nodes compared to the shoot tips. Introduction of BAP in the medium was found to elevate phytohormone concentration which enhanced shoots proliferation, and cutting off the shoot tips promoted lateral buds formation and rapid regeneration (El-Motaleb *et al.*, 2013).

Figure 1. Initiation of in vitro culture of stevia variety AKH L1. (A) Approximately 2 cm long nodal explants obtained from young, actively growing shoot tips of one-month-old stevia potted plant. (Bar=3cm). (B) Shoots multiplication was initiated on MS supplemented with 1mg/L BAP for one month. (Bar=1cm). (C) Newly regenerated plantlets were sub-cultured to new medium monthly. (Bar=1cm).
Optimization of subculture cycle is crucial in order to obtain higher plantlets biomass with regenerative potency. However, information on the effects of repeated sub-culturing cycles on the rate of shoots multiplication and regeneration of *in vitro* stevia plantlets is scanty. In this study, it was observed that repeated subculturing cycles resulted in stunted growth of the regenerated plantlets (Figure 3). Gradual yellowing in some leaves were observed after repeated subculturing for 12 cycles. This indicated that the shoot cultures reduced their regeneration capacity (Ramanand, 2006). Somaclonal variations have been long known to be induced during dedifferentiation and regeneration of plants in tissue cultures (Filipecki & Malepszy, 2006). Rapid multiplication and prolonged culturing may affect genetic stability and thus increased the frequency of somaclonal variation in *in vitro* culture (Petolino et al., 2003). The result paralleled Nakasha et al. (2016) findings whereby reduction in regenerative capacity was observed in *Chlorophytum borivilianum* after repeated subculture cycles of their callus cultures. Thus, frequent subculturing does seem to affect the stevia plantlets growth, hence is not recommended as it may compromise the quality of the *in vitro* cultures.

### Acute irradiation of in vitro stevia plantlets

In any mutational induction studies, it is critical to determine that the dose of mutagen applied is able to induce desirable alterations with minimal unintended effects. The effective dose of mutagen used will ensure the success of mutational induction for desirable outcomes. Therefore, in order to obtain the effective dose of mutagen used, fixation of LD<sub>50</sub> doses was deemed crucial before massive irradiation of similar materials was performed (Rajarajan et al., 2016). Lethal dose, LD<sub>50</sub>, was referred to a dosage at which greatest frequency of mutation occurred with half of the planting materials survived, while half died.
Acute gamma irradiation of Stevia rebaudiana Bertoni (Kangarasu et al., 2014). This LD$_{50}$ value varies according to plants, genera and cultivars (Tah, 2006). In this study, the LD$_{50}$ of in vitro shoot tips of stevia plantlets was determined to be 23Gy (Figure 4). There were significant differences (p<0.05) in the survival rates amongst the stevia shoots subjected to lower and higher doses of acute gamma irradiation. The survival rate was inversely proportional to the gamma irradiation dose. All the non-irradiated explants (control) survived while explants treated with 50Gy exhibited the lowest survival rate (9.33 ± 8.33%).

**Figure 3.** In vitro culture of stevia variety AKH L1. (A) 1$^{st}$ generation plantlet; (B) 12$^{th}$ generation clump of plantlets. (Bar=1cm).

**Figure 4.** LD$_{50}$ of stevia variety AKH L1 shoot tips after one month of culture. Data was taken from five replicates and the experiment was repeated twice. The data was the average of three experiments.

Table 2 depicts the mutagenic effects of different doses of gamma rays on in vitro growth of AKH L1 shoots and their survival rates. The data highlights that the gamma rays imposed significant effect on the regenerated shoots’ length. The control exhibited the highest length (2.53±0.36 cm) while not much difference in shoot length was seen for explants exposed to 10 Gy (2.44±0.67 cm). Nevertheless, gamma doses greater than 30Gy did significantly reduce the length of the regenerated shoots compared to the control. It was more pronounced at 40Gy and 50Gy which could be due to higher sensitiveness of stevia to higher doses (Figure 5). This observation paralleled Jamie’s (2002) data which showed that the mutation rate increased when the amount of exposure to irradiation and intensities of gamma rays used were increased. Inhibition of seeds germination, seedlings growth and other biological responses were frequent occurrences observed in plants subjected to high gamma doses (Wi et al., 2007). Preussa and Britta (2003) attributed such inhibitive effects to be caused by cell cycle arrest at G2/M phase during somatic cell division and/or various damages in the entire genome.

Likewise, in this study, the highest number of regenerated shoots with mean value of 1.84 was observed in the control and no new shoots were formed at 40 and 50Gy. Unfortunately, the number of leaves was negatively affected by the gamma doses. The non-irradiated regenerated shoots (control) showed greater number of leaves at 16.52±5.05. At 50Gy, necrosis of explants and poor development of regenerated leaves (2.81±2.47) were observed. Increasing gamma irradiation dosage resulted in reduced survival rate, length of regenerated shoots, number of new shoots formed and the number of leaves formed. The radiosensitivity test for AKH L1 was successfully performed with a LD$_{50}$ value of 23Gy.

Table 3 summarized the explants establishment percentage and the growth of control and irradiated stevia plantlets after four generations. It is recommended and a common practice for irradiated plantlets to be subjected to at least four generations/cycles of sub-culturings in order to screen off potential chimeras and to select for stable mutants. Therefore, M$_4$ generation was chosen to maximize the probability of obtaining stable mutants. Each sub-culturing cycle was counted as one generation as the excised explants regenerated into new plantlets. Irradiated stevia in vitro plantlets exhibited significantly (p<0.05) longer shoot length of 10.16±1.85 cm, higher number of leaves (16.07±5.19) and greater leaf size (1.12±0.26 cm x 0.54±0.15 cm) compared to non-irradiated in vitro plantlets. It can be said that the growth of
Acute gamma irradiation of Stevia rebaudiana Bertoni

Stevia plantlets were significantly improved when exposed to gamma irradiation of 23Gy (LD50). Similar observations were reported by Yadav (2016) where there was significant increase in leaf numbers of Canscora decurrens Dalz plants exposed to low irradiation doses than the control plants. Such “low dose-high growth” correlation was also observed in several plants such as Mollucella lavis (Minisi et al., 2013), Cucurma longa (Ilyas & Naz, 2014), Hibiscus sabdariffa (Sherif & Sarwat, 2005), and Dracaena serrulosa (Sakr, 2005). A broad range of negative activities at plant physiological levels such as water exchange, leaf gas exchange, enzymatic and hormonal imbalance, and alteration in protein synthesis were triggered at higher doses of gamma rays (Hameed et al., 2008).

Table 2. Effect of different doses of acute gamma irradiation on growth of regenerated stevia variety AKH L1 shoots after one month.

<table>
<thead>
<tr>
<th>Dose (Gy)</th>
<th>Survival rate (%±SD)</th>
<th>Length of regenerated shoot (mean±SD)</th>
<th>No. of new shoots formed (mean±SD)</th>
<th>No. of leaf (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100±0.00a</td>
<td>2.53±0.36a</td>
<td>1.84±0.63a</td>
<td>16.52±5.05a</td>
</tr>
<tr>
<td>10</td>
<td>93.33±8.33a</td>
<td>2.44±0.67a</td>
<td>1.72±0.38a</td>
<td>15.77±5.66a</td>
</tr>
<tr>
<td>20</td>
<td>58.67±12.86b</td>
<td>1.76±0.78ab</td>
<td>0.73±0.37b</td>
<td>13.35±4.86ab</td>
</tr>
<tr>
<td>30</td>
<td>37.33±16.65bc</td>
<td>1.40±0.55bc</td>
<td>0.31±0.19bc</td>
<td>8.80±3.56abc</td>
</tr>
<tr>
<td>40</td>
<td>14.67±4.62d</td>
<td>1.05±0.13bc</td>
<td>0.00</td>
<td>6.89±1.92abc</td>
</tr>
<tr>
<td>50</td>
<td>9.33±8.33d</td>
<td>0.47±0.28c</td>
<td>0.00</td>
<td>2.81±2.47c</td>
</tr>
</tbody>
</table>

Data was taken from five replicates (with five explants per replicate) and the experiment was carried out three times. Superscripts within the means of each column (a-d) indicate significant difference among means (p<0.05, using Duncan’s Multiple Range Test). SD = standard deviation.

Figure 5. Effects of acute gamma irradiation on growth of regenerated stevia variety AKH L1 shoots after one month of culture on MS medium supplemented with 1 mg/L 6-benzylaminopurine (BAP). Bar= 1 cm.
Table 3. Comparison of control and irradiated stevia variety AKH L1 plantlets at LD$_{50}$ (23Gy) after four generations.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Irradiated</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of explant survived (%)</td>
<td>95.83$^a$</td>
<td>87.18$^b$</td>
</tr>
<tr>
<td>No. of shoot / explants</td>
<td>6.20±2.39$^a$</td>
<td>2.60±0.90$^b$</td>
</tr>
<tr>
<td>Length of shoot / explant (cm)</td>
<td>8.61±3.40$^a$</td>
<td>10.16±1.85$^b$</td>
</tr>
<tr>
<td>No. of leaf / explants</td>
<td>13.33±5.42$^a$</td>
<td>16.07±5.19$^b$</td>
</tr>
<tr>
<td>Average leaf size,</td>
<td>0.81±0.22</td>
<td>1.12±0.26</td>
</tr>
<tr>
<td>Length x Width (cm)</td>
<td>0.44±0.14$^a$</td>
<td>0.54±0.15$^b$</td>
</tr>
</tbody>
</table>

The data presented was mean±SD of n=300, and the experiment was repeated twice. Superscripts within the means (a-b) indicate significant difference among means (p<0.05 using One-Sample T Test).

**Determination of steviol glycosides (SGs) using high performance liquid chromatography (HPLC) analysis.** An isocratic HPLC method was validated to determine the concentration of stevioside, rebaudioside A and rebaudioside D as described by Nishiyama et al. (1992). The retention times of the stevioside, rebaudioside A and rebaudioside D standards were recorded at 3.98 mins, 3.96 mins, and 3.56 mins, respectively (Table 4).

A standard curve was used to determine the concentration of each steviol glycoside in the irradiated and non-irradiated stevia leaf extracts. Based on Figure 6, the concentration of each steviol glycoside tested was higher (about three folds increment) in irradiated plantlets compared to the control leaf extract. From the HPLC results, a new stevia variety of AKH L1 was characterized with higher stevioside (387.04 ppm), rebaudioside A (670.18 ppm) and rebaudioside D (106.26 ppm).

Rebaudioside A and stevioside made up the majority proportion of the total steviol glycosides contents whereby the ratio of rebaudioside A to stevioside determined the sweetness quality of stevia (Yadav et al., 2011). Similarly, Khalil et al. (2014) reported that gamma irradiation at 15Gy produced slightly enhanced stevioside contents in their irradiated plants. Other reports, however, claimed that increasing gamma ray doses induced higher mutation rates while at the same time led to significant reduction in stevioside contents in the stevia plants (Predieri, 2010; Ali et al., 2015). Such conflicting findings probably could be due to different varieties of stevia used in the experiments, and the different environment and physical conditions where the experiments were conducted.

**Table 4.** Retention time of standard steviol glycosides: Stevioside, Rebaudioside A, and Rebaudioside D, all done under separation of acetonitrile/water as the elution solvent at flow rate of 0.8 mL/min, and the detection wavelength set at 210nm.

<table>
<thead>
<tr>
<th>Steviol glycosides</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stevioside</td>
<td>3.98</td>
</tr>
<tr>
<td>Rebaudioside A</td>
<td>3.96</td>
</tr>
<tr>
<td>Rebaudioside D</td>
<td>3.56</td>
</tr>
</tbody>
</table>

**Figure 6.** Stevioside contents in irradiated and non-irradiated (control) stevia leaf samples. Stev = stevioside; Reb A= rebaudioside A; and Reb D = rebaudioside D.
CONCLUSION

Acute gamma irradiation was found to be a clean and effective method to induce mutations in *Stevia rebaudiana* varieties. All irradiated *in vitro* AKH L1 plantlets exhibited different survival rates, with the lowest at 9.33±8.33% subjected to 50Gy. The high irradiation dose produced plants exhibiting poor growth and development (necrotic leaves, reduced shoot length and number of leaves). Nevertheless, determination of the LD₉₀ provided an effective dose at 23Gy whereby irradiated plantlets showed significant improvement in phenotypic characteristics and steviol glycosides contents, especially stevioside and rebaudioside A. The data obtained can be further applied to develop new stevia plants with other improved features using acute gamma irradiation.

ACKNOWLEDGEMENTS

Authors would like to thank management and staff of Malaysia Nuclear Agency for their technical assistance on irradiation. We acknowledge Universiti Putra Malaysia for funding this project under the Putra Grant No. GP-IPB/2016/9490300.

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Effect of liquid medium on shoots amplification, in vitro flowering and ex vitro rooting of *Oldenlandia umbellata* L. - A dye yielding medicinal herb

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Abstract. *Oldenlandia umbellata* L. gains importance due to its medicinal properties and the presence of anthraquinones based natural dyes in the roots. Present study describes the effect of Murashige and Skoog’s (MS) liquid medium (full strength) on in vitro regeneration, flower bud induction and ex vitro rooting in *O. umbellata*. Shoot segments with 2-3 nodes (each node with 2 axillary buds) served as explants for establishment of cultures. The liquid medium augmented with 2.0 mg L\(^{-1}\) 6-benzylaminopurine (BAP) with additives (50 mg L\(^{-1}\) of ascorbic acid and 25 mg L\(^{-1}\) each of arginine, adenine sulphate and citric acid) was effective for shoot bud induction (6.4±0.19 shoots per explant within 2-3 weeks). The shoots were further multiplied (89.3±1.07 shoots, 2-3 weeks) when the shoot clusters obtained from the culture initiation directly transferred to the full-strength MS liquid medium incorporated with 1.0 mg L\(^{-1}\) BAP and 0.5 mg L\(^{-1}\) indole-3 acetic acid (IAA) with additives. Flower buds were induced (12.0±0.15 buds per shoot) when the shoots were cultured on 1.0 mg L\(^{-1}\) BAP and kinetin (Kin, 6-furfurylaminopurine) and 0.5 mg L\(^{-1}\) of IAA at 45 µmol m\(^{-2}\) s\(^{-1}\) SFPD (Spectral Flux Photon Density) light intensity for 14/10h (light/dark) photoperiod. The adventitious roots were induced on 1/4 strength MS medium supplemented with 1.5 mg L\(^{-1}\) indole-3 butyric acid (IBA). Ex vitro rooting was achieved (16.0±0.53 roots per shoot) by pulse treatment of the shoots with 300 mg L\(^{-1}\) IBA for 2 min. The in vitro produced plantlets were acclimatized in the greenhouse and finally translocated to the in vivo conditions with 93 % success rate. This is the foremost (use of liquid MS medium) and cost-effective method for large scale multiplication of *O. umbellata*.

Keywords: Acclimation, ex vitro rooting, in vitro flowering, liquid medium, *Oldenlandia umbellata*

INTRODUCTION

*Oldenlandia umbellata* L. is a natural dye yielding plant of the family Rubiaceae. There are ten synonyms of this plant viz. *Gerontogea umbellata* (L.) Cham. & Schltdl., *Hedyotis umbellata* Lam., *H. breviclada* Sivar., Biju & P. Mathew, *H. indica* Roem. & Schult., *H. linearifolia* R.Br. ex Wall., *H. puberula* (G.Don) R.Br. ex Arn., *H. umbellata* (L.) Lam., *H. wightii* (Hook. f.) K.K.N. Nair, *H. wightii* (Hook. f.) Sivar., Biju & P. Mathew and *O. puberula* G. Don (The Plant List, 2013). It is a small, prostrate, profusely branched perennial herb; native to the Indian subcontinent but distributed in the tropical and subtropical regions such as Burma, Sri Lanka, Cambodia, Indonesia, India, Pakistan and the west tropical Africa (Siva, 2007). The plant is commonly known as Indian Madder plant, Chay root and Shaya (Yoganarasimhan and Chelladurai, 2000). Ethnopharmacological and medicinal potential of this species are explored by the tribal...
people and ethnic communities of India and China (Yoganarasimhan and Chelladurai, 2000). Traditionally, the leaves and roots of *O. umbellata* were used in the treatment of various ailments (Samy et al., 2008). The roots are used to extract chay-root red dye for calico printing and coloring of fur and silk fabrics from centuries (Siva, 2007).

Owing to over exploitation of *O. umbellata* for its roots by the pharmaceutical companies to get herbal drugs (phenolic compounds and their derivatives) and to harvest anthraquinone pigments (red dye), the population of this plant has been depleted (due to uprooting of entire plant system) in the wild (Siva et al., 2012). The natural propagation of this plant is only by seeds and the plants are harvested before seeds setting. Therefore, there is a need of alternate methods of propagation for conservation of this plant species (Krishnan and Siril, 2015). Tissue culture techniques offer powerful tool for large-scale production of important plants which could help in conservation of medicinal plants.

Some reports are available on the *in vitro* studies and biosynthesis of secondary metabolites from *O. umbellata*, but all the researchers used agar-agar as gelling agent (Siva et al., 2009, 2012; Shekhawat et al., 2012; Kumar et al., 2014; Krishnan and Siril, 2015, 2016, 2017). Agar-agar is a conventional gelling agent used in tissue culture experiments and has been reported to possess certain disadvantages in differentiation and growth of the cultures. Agar powder increases the viscosity of the medium and slows down the nutrients intake by the cultures which affect the growth of plantlets (Kuria et al., 2008). Moreover, the gelling agents in plant tissue culture media may constitute about 70% of production cost (Mohamed et al., 2010). Attempts were made with the other alternatives of agar to reduce this cost, but success was always questionable (Babbar and Jain, 2006). The use of liquid medium for *in vitro* propagation is very effective in proliferation of shoots and adventitious roots which reduce time, energy and cost of production of plantlets (Mehta et al., 2014).

*In vitro* flower induction explains the fundamental mechanism of transition phase from vegetative phase to the reproductive phase and it could further help to study the physiology of *in vitro* flowering process (Cheruvathur et al., 2015). Rooting and acclimatization can be achieved concurrently through *ex vitro* rooting method. *Ex vitro* rooting produces better adventitious roots which can again reduce the time; cost and labor input during *in vitro* rooting (Ranaweera et al., 2013).

Therefore, experiments were conducted for the first time to explore the effect of liquid medium in bud breaking, shoots multiplication, *in vitro* flowering and *ex vitro* rooting of *O. umbellata*.

### MATERIALS AND METHODS

**Plant materials and surface sterilization.** One-year old *O. umbellata* plants were collected from the natural habitats of the east coast of India (Puducherry Union Territory), the Coromandel coast (11.9416°N, 79.8083°E), maintained in greenhouse and identified by the *Institut Français de Pondichéry* (IFP), Puducherry (Accession number: HIFP 26744). The nodal segments (explants) of 3-4 cm in length (bearing 2-3 nodes, each node with 2 axillary buds) were collected at the flowering stage. Initially, the explants were treated with 0.1% (w/v) broad spectrum antifungal agent Bavistin (BASF India Ltd., India) for 5-7 min and surface sterilization was achieved with 0.1% (w/v) mercuric chloride (Hi-Media, India) solution for 4-5 min and finally rinsed (five times) with sterilized distilled water under aseptic conditions.

**Nutrient medium and culture conditions.** Full strength Murashige and Skoog’s (MS) medium (Murashige and Skoog, 1962) incorporated with 3% sucrose, additives (50 mg L\(^{-1}\) of ascorbic acid and 25 mg L\(^{-1}\) each of arginine, adenine sulphate and citric acid) (after Patel et al., 2014) devoid of agar-agar (liquid medium) was used for regeneration of cultures in this study. The pH of the medium was attuned to 5.8±0.02 with 1N HCl or NaOH before autoclaving at 108 kPa pressure and 120°C temperature for 15 min. The culture flasks were kept on a gyratory shaker at 100 rpm (Technico Pvt. Ltd, Chennai, India), incubated at 25±2°C temperature under 12/12 h (light/dark) photoperiod with light intensity of 40-50 μmol m\(^{-2}\) s\(^{-1}\) Spectral Photon Flux Density (SPFD) provided by cool white fluorescent lamps of 40 Watts each (Philips India Ltd, New Delhi). The 250 mL Borosilicate culture flasks procured from
the Technico Pvt. Ltd., Chennai, India were used in this study. Twenty-five ml liquid MS medium was poured in each culture vessel and the vessels were closed by cotton plugs before autoclaving.

**Induction and proliferation of shoots.** The sterilized nodal shoot segments (explants) were inoculated in full strength liquid MS medium (one explant per vessel) without trimming the cut ends. Liquid MS medium fortified with different concentrations of cytokinins, BAP (6-benzylaminopurine) and Kin (6-furfurylaminopurine) ranging from 0.5 to 3.0 mg L\(^{-1}\) (Sigma-Aldrich, St. Louis, USA) alone or in combinations were used for the induction of shoots from nodal explants. The cultures were transferred to the fresh medium after every 2 weeks. The *in vitro* regenerated shoots with optimized length were segmented leaving 3-4 nodes and subcultured on MS medium containing various concentrations of BAP (0.1 to 2.5 mg L\(^{-1}\)) combined with various concentrations (0.25 to 0.75 mg L\(^{-1}\)) of IAA (indole-3-acetic acid) for proliferation of shoots. The shoots were further multiplied by repeated transfer of mother explants and subculture of *in vitro* shoots. The cultures on MS medium devoid of growth regulators were treated as control.

**In vitro flower buds induction.** The *in vitro* produced shoots of 3-4 cm in length were excised and subcultured on full strength liquid MS medium augmented with various concentrations (0.5 to 2.0 mg L\(^{-1}\)) of BAP and Kin combined with IAA (0.1 to 0.75 mg L\(^{-1}\)) for *in vitro* flower bud induction. Various parameters such as percentage response and average number of flower buds were recorded after 4-5 weeks of incubation. The cultures were maintained at 25±2°C under various photoperiod regimes (10/14 to 16/8h per day light/dark) with light intensity of 40-50 μmol m\(^{-2}\) s\(^{-1}\) SPFD provided through white fluorescent tubes.

**In vitro rooting of the shoots.** Healthier shoots were separated from the multiplied shoot clumps and transferred to various strengths of nutrient medium (full, ½ and 1/4 strengths of MS liquid media) augmented with auxins [IAA, IBA (indole-3-butyric acid) and NAA (α-naphthalene acetic acid)] at various concentrations (1.0-4.0 mg L\(^{-1}\)) for induction of roots. The cultures were initially incubated under diffused light for 2-3 days thereafter shifted to the *in vitro* culture environment, and maintained at the light intensity of 40-45 μmol m\(^{-2}\) s\(^{-1}\) SPFD.

**Ex vitro rooting and hardening of plantlets.** The end base of the shoots was pulsed with various types and concentrations of auxins (IAA, IBA and NAA at 50-500 mg L\(^{-1}\)) for various time durations (1-5 min) for *ex vitro* rooting. The treated shoots were transplanted into 1.5-2.0 cm deep in paper cups (size 150 mL) containing 50 g sterilized soilrite® (Keltech Energy Limited, Bangalore, India) without any antibiotic treatment. Shoots were moistened with 15 mL 1/4 strength MS macro salts solution by the intermission of one week to evade wilting. The set up was capped by perforated transparent polythene cups and maintained in greenhouse under high relative humidity (80-90% RH) and 28±2°C temperature conditions. The caps were gradually removed over a period of two to four weeks. These plantlets were transferred to the nursery poly bags containing organic manure, sand and red soil (1:1:1; w/w) and maintained for 5 weeks. The same plants were next shifted to the earthen pots filled with garden soil, red soil and vermi-compost (2:2:1; w/w), irrigated with tap water and retained in greenhouse for another one and half months for proper hardening.

**Experimental design, data collection and statistical analysis.** The experiments were laid out in completely randomized block design (CRBD) (Compton and Mize, 1999), conducted with 20 explants for each treatment and repeated thrice (triplicates). The observations on the response, number and length of shoots and roots were recorded at 4 weeks time interval. The data were subjected to the standard error of the mean and single factor analysis of variance (one-way ANOVA). The significance of differences among mean values was carried out using Duncan’s Multiple Range Test (DMRT) at P<0.05 using SPSS software, version 16.0 (SPSS Inc., Chicago, USA). The results were expressed as Mean±Standard Error.

**RESULTS AND DISCUSSION**
Significant improvements were observed in terms of production of shoots with maximum number of nodes and roots with rapid organ developments (within 2 weeks) due to the adoption of liquid MS medium in present study.

**Axillary bud emergence from the explants.** All the explants yielded shoots on MS liquid medium with various treatments of cytokinins (Figure 1a). The MS medium supplemented with 2.0 mg L\(^{-1}\) BAP generated highest number of axillary buds (6.4±0.19 shoots per explant with 4.0±0.21 cm mean length) within 2-3 weeks from the nodal explants (Table 1, Figures 1b and 1c). Liquid MS medium combined with BAP alone significantly enhanced shoots formation from the explants as compared to the other hormonal treatments tested. The MS medium augmented with Kin at 1.5 mg L\(^{-1}\) produced 4.2±0.38 shoots. The length of the *O. umbellata* shoots was better in liquid medium as compared to semisolid medium used in previous studies (Shekhawat et al., 2012). The role of liquid medium in axillary bud induction is first time reported in this species. Liquid culture system is advantageous over to the agar gelled medium due to rapid uptake of nutrients by the cells and tissues and minimum negative effect of phenolics leach outs which ultimately enhance the growth of cultured shoots and roots (AlKhateeb and Alturki 2014). Kumar et al. (2014) induced 6 shoots (3.5 cm in length) on agar gelled MS medium containing 3.0 mg L\(^{-1}\) BAP using shoot tip explants. Krishnan and Siril (2015) observed vitiﬁed shoots with crowning of scaly leaves when nodal explants cultured on agar gelled MS medium with high concentration of 6-benzyladenine (BA).

**Proliferation of shoots in liquid MS medium.** The number of shoots was enhanced about 15 folds (from initiation stage) when the regenerated shoots and mother explants were subcultured on MS liquid medium composed of 1.0 mg L\(^{-1}\) BAP and 0.5 mg L\(^{-1}\) IAA with additives. The maximum shoot numbers (89.3±1.07) with average length of 12.5±0.30 cm were obtained using this media combination (Table 2, Figure 1d). It has been reported that cytokinin and auxin are the major plant growth regulators of core cell cycle components (Takatsuka and Umeda, 2014). Shekhawat et al. (2012) reported shedding of leaves of *O. umbellata* on semisolid media incorporated with optimized growth regulators. It was observed that liquid media facilitated the production of quality shoots in terms of shoot numbers, shoot length and thickness as compared to agar gelled medium. All the shoots appeared healthy in liquid medium and did not display any symptoms of hyperhydration or vitriﬁcation in this experiment. Pati et al. (2005) also reported that the liquid medium enhanced rate of shoots multiplication with complete elimination of vitriﬁcation in *Rosa damascena* and *R. bourboniana*. Similar results reported in propagation of apple by Mehta et al. (2014).

**In vitro ﬂowering.** Healthy and long shoots (4-5 cm) were cultured on MS medium augmented with combination of cytokinins and auxin resulted in development of terminal and axial congested umbelliform inﬂorescence. Among the growth regulators tested, the combination of full-strength MS medium containing 1.0 mg L\(^{-1}\) BAP and Kin + 0.5 mg L\(^{-1}\) of IAA at 45 µmol m\(^{-2}\) s\(^{-1}\) SFPD light intensity for 14/10 h (light/dark) photoperiod induced the highest number of flower buds in *O. umbellata* (Table 3). Three inﬂorescences per shoot were observed and 12.0±0.15 flower buds per inﬂorescence were emerged when the shoots grown on this medium (Figure 1e). The *in vitro* raised flower buds gradually opened after 2-3 days and expressed similar physical characters as of the mother plants in nature. Flowers were distylos with short pedicel, white corolla, green calyx and glabrous (Figure 1f). *In vitro* ﬂower bud induction is promoted by the plant growth regulators and photoperiods (Taylor et al., 2005). Recently, Behera et al. (2017) reported *in vitro* flowers from embryogenic calli of *O. umbellata* on MS semisolid medium. Successful *in vitro* and *ex vitro* ﬂowering is reported to be determined by various intrinsic and extrinsic factors such as florigen coupled with time duration and meristematic activity (Zeevaart, 2006). The results could be used for further analysis of ontogeny of flowers in plants.

**In vitro rooting of the proliferated shoots.** Liquid MS medium with IBA played an important role in adventitious roots induction from the
shoots (Thakur et al., 2018). The development and proliferation of roots (100%) were more rapid in 1/4\textsuperscript{th} strength of MS liquid medium (Table 4). Quarter strength MS medium with 1.5 mg L\textsuperscript{−1} IBA resulted in the emergence of 24.2±0.47 roots with the mean length of 6.1±0.22 cm within 3 weeks (Figure 1g). Comparatively, IAA detected as the weakest hormone in the root induction and NAA ranked second next to the IBA. The previous reports on the role of auxins in rooting of shoots of \textit{O. umbellata} are contradictory to the present findings. Siva \textit{et al.} (2012) reported that higher concentration of NAA in semisolid MS medium had better effect in induction of roots and production of anthraquinone pigments as compared to IBA and IAA from the roots of \textit{O. umbellata}. Enhanced induction and proliferation of adventitious roots in the liquid medium with auxins support the concept of the quick absorption of nutrients from the liquid media than the semisolid medium.

**Ex vitro rooting and hardening of the plantlets.** Comparatively IBA was found most suitable for \textit{ex vitro} root induction than IAA and NAA. The shoots pulsed with 300 mg L\textsuperscript{−1} IBA for 2 min exhibited 100\% rooting response. Maximum 16.0±0.53 roots with 4.2±0.22 cm average length were induced from the cut end of the shoots with IBA at 300 mg L\textsuperscript{−1} (Table 5, Figure 1h). Krishnan and Siril (2015) observed 14.03 roots/shoot when the shoots were treated with IBA. The roots induced via this method were superior to the \textit{in vitro} roots in terms of thickness and firmness, which helped in hardening processes in order to improve the survival percentage of \textit{O. umbellata} plantlets in the field conditions. The results are in agreed with the reports of Patel \textit{et al.} (2014) in \textit{Caralluma edulis} and Ranaweera \textit{et al.} (2013) in \textit{Camellia sinensis}. According to the cost estimation on \textit{ex vitro} rooting experiments, it is revealed that the plant production cost can be reduced up to 70\% in development of a micropropagation protocol using \textit{ex vitro} rooting technique (Ranaweera \textit{et al.}, 2013).

The rooted shoots were placed on the paper cups containing soilrite\textsuperscript{®} and uphold for 5 weeks in the greenhouse (Figure 2a). Further, the greenhouse hardened plantlets were transferred to nursery poly-bags containing organic manure, sand and red soil (1:1:1; w/w) (Figure 2b). The plantlets were shifted to earthen pots filled with garden soil, red soil and vermi-compost (2:2:1; w/w) after 4 weeks and finally planted in the natural habitat in another 4-5 weeks (Figures 2c and 2d). About 93\% plantlets survived in the field conditions after 6 months of transfer. This rate of survival is superior to the earlier reports in this plant. Krishnan and Siril (2015) achieved 81.3\% survival rate in quercetin induced shoots of \textit{O. umbellata}.
Table 1. Effect of cytokinins (BAP and Kin) on shoots induction from nodal stem segments of *Oldenlandia umbellata* after 3 weeks of cultures in MS liquid medium.

<table>
<thead>
<tr>
<th>Cytokinins (mg L(^{-1}))</th>
<th>Response (%)</th>
<th>Shoots number (Mean ± SE)</th>
<th>Shoots length (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP</td>
<td>Kin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>0.0</td>
<td>0.0 ± 0.00(^a)</td>
<td>0.0 ± 0.00(^a)</td>
</tr>
<tr>
<td>0.5</td>
<td>-</td>
<td>2.0 ± 0.23(^c)</td>
<td>1.9 ± 0.15(^b)</td>
</tr>
<tr>
<td>1.0</td>
<td>-</td>
<td>2.7 ± 0.18(^d)</td>
<td>2.5 ± 0.30(^b)</td>
</tr>
<tr>
<td>1.5</td>
<td>-</td>
<td>4.0 ± 0.39(^f)</td>
<td>3.1 ± 0.16(^d)</td>
</tr>
<tr>
<td>2.0</td>
<td>-</td>
<td>6.4 ± 0.19(^b)</td>
<td>4.0 ± 0.35(^c)</td>
</tr>
<tr>
<td>2.5</td>
<td>-</td>
<td>5.1 ± 0.47(^x)</td>
<td>3.8 ± 0.11(^c)</td>
</tr>
<tr>
<td>3.0</td>
<td>-</td>
<td>3.9 ± 0.24(^e)</td>
<td>3.0 ± 0.20(^d)</td>
</tr>
<tr>
<td>-</td>
<td>0.5</td>
<td>1.4 ± 0.11(^b)</td>
<td>1.4 ± 0.26(^b)</td>
</tr>
<tr>
<td>-</td>
<td>1.0</td>
<td>2.0 ± 0.29(^c)</td>
<td>2.0 ± 0.41(^c)</td>
</tr>
<tr>
<td>-</td>
<td>1.5</td>
<td>4.2 ± 0.38(^f)</td>
<td>3.6 ± 0.11(^d)</td>
</tr>
<tr>
<td>-</td>
<td>2.0</td>
<td>3.3 ± 0.12(^c)</td>
<td>3.1 ± 0.33(^d)</td>
</tr>
<tr>
<td>-</td>
<td>2.5</td>
<td>3.0 ± 0.20(^c)</td>
<td>2.7 ± 0.27(^c)</td>
</tr>
<tr>
<td>-</td>
<td>3.0</td>
<td>2.6 ± 0.36(^d)</td>
<td>2.0 ± 0.25(^c)</td>
</tr>
</tbody>
</table>

Note: The values represented in corresponding column followed by same letters are not significantly different by DMRT at *p* < 0.05.

Table 2. Combined effect of BAP and IAA on proliferation of multiple shoots in liquid MS medium after 2 weeks.

<table>
<thead>
<tr>
<th>Cytokinins (mg L(^{-1}))</th>
<th>Shoots number (Mean ± SE)</th>
<th>Shoots length (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP</td>
<td>Kin</td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>0.0</td>
<td>0.0 ± 0.00(^a)</td>
</tr>
<tr>
<td>0.5</td>
<td>0.25</td>
<td>43.7 ± 0.92(^b)</td>
</tr>
<tr>
<td>1.0</td>
<td>0.25</td>
<td>61.0 ± 1.00(^b)</td>
</tr>
<tr>
<td>1.5</td>
<td>0.25</td>
<td>58.1 ± 0.80(^c)</td>
</tr>
<tr>
<td>2.0</td>
<td>0.25</td>
<td>53.4 ± 0.77(^d)</td>
</tr>
<tr>
<td>2.5</td>
<td>0.25</td>
<td>45.9 ± 0.42(^e)</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5</td>
<td>60.0 ± 0.29(^g)</td>
</tr>
<tr>
<td>1.0</td>
<td>0.5</td>
<td>89.3 ± 1.07(^a)</td>
</tr>
<tr>
<td>1.5</td>
<td>0.5</td>
<td>80.0 ± 1.39(^m)</td>
</tr>
<tr>
<td>2.0</td>
<td>0.5</td>
<td>71.6 ± 1.18(^g)</td>
</tr>
<tr>
<td>2.5</td>
<td>0.5</td>
<td>63.1 ± 0.94(^i)</td>
</tr>
<tr>
<td>0.5</td>
<td>0.75</td>
<td>60.3 ± 0.65(^e)</td>
</tr>
<tr>
<td>1.0</td>
<td>0.75</td>
<td>82.8 ± 2.13(^e)</td>
</tr>
<tr>
<td>1.5</td>
<td>0.75</td>
<td>74.2 ± 1.10(^l)</td>
</tr>
<tr>
<td>2.0</td>
<td>0.75</td>
<td>69.9 ± 0.49(^j)</td>
</tr>
<tr>
<td>2.5</td>
<td>0.75</td>
<td>55.7 ± 1.00(^e)</td>
</tr>
</tbody>
</table>

Note: The values represented in corresponding column followed by same letters are not significantly different by DMRT at *p* < 0.05.
Table 3. Effect of cytokinins (BAP, Kin) and IAA in induction of flowers per inflorescence under 45 µmol m\(^{-2}\) s\(^{-1}\) SFPD for 14/10h (light/dark) photoperiod.

<table>
<thead>
<tr>
<th>Conc. of BAP + Kin (mg L(^{-1}))</th>
<th>Conc. of IAA (mg L(^{-1}))</th>
<th>Number of flower buds (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.00</td>
<td>0.0±0.00(^{a})</td>
</tr>
<tr>
<td>0.50</td>
<td>0.1</td>
<td>4.1±0.13(^{b})</td>
</tr>
<tr>
<td>0.75</td>
<td>0.1</td>
<td>4.8±0.28(^{c})</td>
</tr>
<tr>
<td>1.00</td>
<td>0.1</td>
<td>6.3±0.44(^{d})</td>
</tr>
<tr>
<td>1.50</td>
<td>0.1</td>
<td>6.0±0.37(^{e})</td>
</tr>
<tr>
<td>2.00</td>
<td>0.1</td>
<td>5.0±0.15(^{f})</td>
</tr>
<tr>
<td>0.50</td>
<td>0.5</td>
<td>6.9±0.11(^{g})</td>
</tr>
<tr>
<td>0.75</td>
<td>0.5</td>
<td>7.0±0.53(^{h})</td>
</tr>
<tr>
<td>1.00</td>
<td>0.5</td>
<td>12.0±0.15(^{i})</td>
</tr>
<tr>
<td>1.50</td>
<td>0.5</td>
<td>9.8±0.12(^{j})</td>
</tr>
<tr>
<td>2.00</td>
<td>0.5</td>
<td>5.1±0.71(^{k})</td>
</tr>
<tr>
<td>0.50</td>
<td>0.75</td>
<td>5.5±0.26(^{l})</td>
</tr>
<tr>
<td>0.75</td>
<td>0.75</td>
<td>6.7±0.59(^{m})</td>
</tr>
<tr>
<td>1.00</td>
<td>0.75</td>
<td>7.2±0.35(^{n})</td>
</tr>
<tr>
<td>1.50</td>
<td>0.75</td>
<td>6.0±0.72(^{o})</td>
</tr>
<tr>
<td>2.00</td>
<td>0.75</td>
<td>5.4±0.16(^{p})</td>
</tr>
</tbody>
</table>

Note: The values represented in corresponding column followed by same letters are not significantly different by DMRT at \(p<0.05\).

Table 4. Effect of auxins (IAA, IBA and NAA) on \textit{in vitro} root induction from the shoots on \(\frac{1}{4}\) strength MS liquid medium.

<table>
<thead>
<tr>
<th>Auxins (mg L(^{-1}))</th>
<th>Response (%)</th>
<th>Roots number (Mean ± SE)</th>
<th>Roots length (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IAA</td>
<td>IBA</td>
<td>NAA</td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0±0.00(^{a})</td>
</tr>
<tr>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>6.7±0.11(^{c})</td>
</tr>
<tr>
<td>1.5</td>
<td>-</td>
<td>-</td>
<td>7.0±0.39(^{d})</td>
</tr>
<tr>
<td>2.0</td>
<td>-</td>
<td>-</td>
<td>8.5±0.35(^{e})</td>
</tr>
<tr>
<td>2.5</td>
<td>-</td>
<td>-</td>
<td>8.0±0.72(^{f})</td>
</tr>
<tr>
<td>3.0</td>
<td>-</td>
<td>-</td>
<td>7.2±0.41(^{g})</td>
</tr>
<tr>
<td>3.5</td>
<td>-</td>
<td>-</td>
<td>6.9±0.19(^{h})</td>
</tr>
<tr>
<td>4.0</td>
<td>-</td>
<td>-</td>
<td>6.4±0.25(^{i})</td>
</tr>
<tr>
<td>-</td>
<td>1.0</td>
<td>-</td>
<td>18.6±0.18(^{j})</td>
</tr>
<tr>
<td>-</td>
<td>1.5</td>
<td>-</td>
<td>24.2±0.47(^{k})</td>
</tr>
<tr>
<td>-</td>
<td>2.0</td>
<td>-</td>
<td>19.4±0.33(^{l})</td>
</tr>
<tr>
<td>-</td>
<td>2.5</td>
<td>-</td>
<td>10.9±0.49(^{m})</td>
</tr>
<tr>
<td>-</td>
<td>3.0</td>
<td>-</td>
<td>8.3±0.82(^{n})</td>
</tr>
<tr>
<td>-</td>
<td>3.5</td>
<td>-</td>
<td>8.0±0.16(^{o})</td>
</tr>
<tr>
<td>-</td>
<td>4.0</td>
<td>-</td>
<td>7.5±0.22(^{p})</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>1.0</td>
<td>7.8±0.10(^{q})</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>1.5</td>
<td>8.5±0.32(^{r})</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>2.0</td>
<td>9.2±0.67(^{s})</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>2.5</td>
<td>10.8±0.44(^{t})</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>3.0</td>
<td>8.6±0.19(^{u})</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>3.5</td>
<td>6.0±0.61(^{v})</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>4.0</td>
<td>5.1±0.36(^{w})</td>
</tr>
</tbody>
</table>

Note: The values represented in corresponding column followed by same letters are not significantly different by DMRT at \(p<0.05\).
Table 5. Effect of auxins (IAA, IBA and NAA) on ex vitro rooting of in vitro raised shoots of Oldenlandia umbellata in Soilrite®.

<table>
<thead>
<tr>
<th>Auxins (mg L⁻¹)</th>
<th>Response (%)</th>
<th>Roots number (Mean ± SE)</th>
<th>Roots length (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IAA</td>
<td>IBA</td>
<td>NAA</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.0 ± 0.0a</td>
</tr>
<tr>
<td>50</td>
<td>-</td>
<td>-</td>
<td>4.6 ± 0.12b</td>
</tr>
<tr>
<td>100</td>
<td>-</td>
<td>-</td>
<td>5.9 ± 0.26c</td>
</tr>
<tr>
<td>150</td>
<td>-</td>
<td>-</td>
<td>6.4 ± 0.38d</td>
</tr>
<tr>
<td>200</td>
<td>-</td>
<td>-</td>
<td>6.9 ± 0.11d</td>
</tr>
<tr>
<td>300</td>
<td>-</td>
<td>-</td>
<td>8.5 ± 0.35f</td>
</tr>
<tr>
<td>400</td>
<td>-</td>
<td>-</td>
<td>7.2 ± 0.56a</td>
</tr>
<tr>
<td>500</td>
<td>-</td>
<td>-</td>
<td>6.7 ± 0.42d</td>
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<tr>
<td>-</td>
<td>50</td>
<td>-</td>
<td>7.1 ± 0.39c</td>
</tr>
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<td>-</td>
<td>100</td>
<td>-</td>
<td>8.9 ± 0.26d</td>
</tr>
<tr>
<td>-</td>
<td>150</td>
<td>-</td>
<td>9.4 ± 0.55e</td>
</tr>
<tr>
<td>-</td>
<td>200</td>
<td>-</td>
<td>12.6 ± 0.60b</td>
</tr>
<tr>
<td>-</td>
<td>300</td>
<td>-</td>
<td>16.0 ± 0.53f</td>
</tr>
<tr>
<td>-</td>
<td>400</td>
<td>-</td>
<td>14.5 ± 0.29g</td>
</tr>
<tr>
<td>-</td>
<td>500</td>
<td>-</td>
<td>11.3 ± 0.36h</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>50</td>
<td>5.5 ± 0.72c</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>100</td>
<td>7.2 ± 0.53c</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>150</td>
<td>7.7 ± 0.26d</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>200</td>
<td>8.5 ± 0.45f</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>300</td>
<td>9.6 ± 0.19g</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>400</td>
<td>8.0 ± 0.37f</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>500</td>
<td>7.3 ± 0.22g</td>
</tr>
</tbody>
</table>

Note: Data were recorded after 4 weeks in greenhouse conditions. The values represented in corresponding column followed by same letters are not significantly different by DMRT at p<0.05.

Figure 2. Hardening of plantlets of O. umbellata.
(a) Hardening of plantlets in paper cups (containing soilrite®) in greenhouse after 5 weeks, (b and c) Hardened plantlets in the nursery polybags (containing organic manure, sand and red soil (1:1:1; w/w)) and earthen pot (filled with garden soil, red soil and vermi-compost (2:2:1; w/w) after 4-5 weeks, (d) Field transferred plant of O. umbellata (6 months old). (Scale bar – 5 cm)

CONCLUSION

The cultures were established from the node explants of O. umbellata. The meristematic activity of axillary buds was activated by the liquid MS medium augmented with BAP. The shoots were further multiplied by lowering the concentration of cytokinin and addition of auxin (IAA) to the medium. Well-developed shoots were routed by in vitro and ex vitro methods. Comparatively, IBA proved better for induction of roots than other auxins. The present protocol could be implemented for the large-scale production of...
plantlets of *O. umbellata* with less input of cost as compared to the semisolid medium.

**ACKNOWLEDGEMENTS**

Authors are grateful to the Department of Science, Technology and Environment, Government of Puducherry, India for providing financial support to their laboratory under the Grant–In-Aid Scheme.

**REFERENCES**


Antifungal activities against oil palm pathogen *Ganoderma boninense* from seaweed sources

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\(^b\)Laboratory of Marine Biotechnology, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
\(^c\)Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
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**Abstract.** Basal stem rot (BSR) disease is the most devastating disease in oil palm which is caused by a fungal pathogen, *Ganoderma boninense*. However, to date, there is no reliable control for this disease. This study investigated the antifungal potential of seaweed extracts against *G. boninense* and screening of the compounds possessing this antifungal activity. Four seaweed species namely *Sargassum oligocystum*, *Caulerpa racemosa*, *Caulerpa racemosa* var. *lamouroxii* and *Halimeda macrophysa* were collected from Teluk Kemang, Port Dickson, Malaysia and their antifungal potential against *G. boninense* were evaluated. Two solvents with different polarities were used for crude extraction namely methanol and chloroform. Antifungal assay using crude methanolic and chloroform extracts from these seaweed species were carried out at various concentrations using the poisoned food technique. *Caulerpa racemosa* var. *lamouroxii* chloroform extract showed strong antifungal activity against *G. boninense* with 27.44% inhibition of the fungus followed by *C. racemosa* methanolic extract with 26.92% inhibition of the fungus at the lowest extract concentration of 0.25 mg/mL. The extracts were subjected to Gas Chromatography-Mass Spectrometry analysis and the dominant bioactive compounds detected in both extracts were phytol and 1-(+)-ascorbic acid 2,6-dihexadecanoate which were also found in plant extracts showing antimicrobial activities in previous studies. The findings suggested that local Malaysian seaweed species have high potential as a source of antifungal compounds which could be useful specifically for the application in the oil palm industry.

**Keywords:** antifungal activity, bioactive compounds, seaweeds, oil palm, basal stem rot, *Ganoderma boninense*

**INTRODUCTION**

Fungal infection is a serious case where it affects plants and interferes with the growth of plants mainly by utilizing the plants’ nutrients. It is very severe and can even cause death to plants (Tucker and Talbot 2001). *Ganoderma boninense* is a soil borne pathogen which causes the basal stem rot disease in oil palm (Hushiarian *et al.*, 2013). Neglecting the disease will affect the yield of fresh fruit bunches which will trim down the production of crude palm oil. It was estimated that in 2020, 400 thousand hectares of oil palm plantations could be affected by *G. boninense*
Fungal infection is not new in the oil palm industry of Malaysia. However, to date, no proper control measure is available, and this situation should be controlled urgently. Many approaches to prevent the fungal infection in oil palm have been done. The most common method is by the application of fungicide but these attempts require high cost and are not environmentally friendly (Idris et al., 2002). Therefore, a more effective and sustainable remedy to this disease would be worth exploring. Research suggested that the application of thiamine activated the host defense response and suppressed plant diseases (Rapala-Kozik et al., 2012; Sylvander et al., 2013). It was proven in a study by Monaim (2011), whereby thiamine treatment makes up hydrogen peroxide generation, callose disposition, and host resistance (HR) cell death in grapevine, hence, weakening the downy mildew disease. Likewise, thiamine was also able to induce defense-related enzymes in soybean plants resulting in success control of charcoal rot disease (Abdel-Monaim, 2011). Another study demonstrated the upregulation of thiamine biosynthesis as a result of the application of endophytes which also suggest the suppression of G. boninense (Kamarudin et al., 2017).

On the other hand, a study by Tay and Chong (2016) on papaya leaf extract against G. boninense demonstrated a significant inhibition activity where at highest concentration tested (45 mg/mL) results in high inhibition activity. The fungicidal agent found in the extracts contained five classes of compounds: carboxylic acid, ester, fatty acid ester, phenol and steroid (Tay and Chong 2016). The potential of antimicrobial activities has also been discovered in certain seaweed extracts. Seaweeds are rich with useful bioactive substances that play important roles in biological activities such as antimicrobial (Demirel et al., 2009), antiviral, anti-inflammatory and antifungal activities. For example, Rajasulochana and colleagues (2013) carried out a study on the potential of marine seaweed, Kappaphycus alvarezii. From the study, it was reported that K. alvarezii exhibited high antifungal activity against fungus Aspergillus fumigates and showed maximum antibacterial activity against bacteria Staphylococcus aureus (Rajasulochana et al., 2013).

In this project, four common seaweeds in Malaysia namely C. oligocystum, C. racemosa, C. racemosa var. lamouroxii and H. macrophysa were tested for their antifungal activity specifically against a disease-causing fungus in oil palm, G. boninense.

### MATERIALS AND METHODS

**Collection of seaweeds.** Brown seaweed (C. oligocystum) and green seaweeds (C. racemosa, C. racemosa var. lamouroxii and H. macrophysa) were collected from Teluk Kemang, Port Dickson, Malaysia (2° 26’ N latitude; 101° 51’ E longitude). The collected seaweeds were washed immediately with seawater and rinsed with tap water to remove any residual salt. The seaweeds were transported to the laboratory in liquid nitrogen and identified by Prof. Phang Siew Moi from Institute of Graduate Studies, Universiti Malaya. The samples were freeze-dried using a freeze-dryer for seven days, ground into fine powder using a mechanical blender and weighed (Abirami and Kowsalya, 2012).

**Methanol and chloroform extract preparation.** 250 mL of solvent methanol and chloroform were used to extract ten grams of seaweed powder via Soxhlet extraction, respectively. The liquid extract was then cooled down and concentrated using rotary evaporator at 50-60°C. The concentrated extracts were allowed to dry in a hot-air oven, weighed, and kept at 4°C until further use (Abirami, 2012). The percentage yield was calculated by using the following formula:

\[
\text{Yield (\%)} = \frac{\text{Dry weight of extract}}{\text{Dry weight of plant powder}} \times 100
\]
Preparation of fungus culture. *Ganoderma boninense* culture was provided by Dr. Nusaibah Syed Ali from Faculty of Agriculture, Universiti Putra Malaysia. The examination of the fungus structure was done by observing under light microscope at 40 x 10 magnification. The growth rate of *G. boninense* culture was measured from the colony diameter of the culture daily for 14 days by using slide caliper repeated for three times in triplicates.

**Antifungal assay.** The antifungal activity of seaweed extracts were tested using the poisoned food technique (Bussaman et al., 2012; Schmitz 1930). One percent of dimethyl-sulfoxide (DMSO) was used as co-solvent to dissolve the seaweed extracts and were reconstituted to the concentration of 0.25, 0.5 and 1.0 mg/mL. The seaweed extracts were mixed with warm potato dextrose agar (PDA) containing 100 μg/mL ampicillin and 100 μg/mL penicillin before poured in a 9 cm sterile petri dish. The agar was left to solidify, and a 6 mm agar piece of one-week old fungus mycelia was inoculated to each petri dish. The cultured plates were incubated at 27°C and the diameters of fungal colonies were measured daily for seven days. PDA plates treated with an equal quantity of DMSO were used as a negative control while triadimefon (Sigma-Aldrich, USA) was chosen as positive control due to its ability in inhibiting fungal growth (Jayaratne et al., 2001). All treatments consisted of duplicates repeated three times and the averages of the experimental results were determined. The percentage inhibition of fungal growth was obtained from the following equation:

\[
\% \text{ Inhibition} = \frac{\text{ADC} - \text{ADT}}{\text{ADC}} \times 100
\]

ADC: Average diameter of fungal culture on negative control plate
ADT: Average diameter of fungal culture on plates treated with seaweed extracts

**Identification of compounds via GC-MS analysis.** Methanol extracts were prepared by solid phase extraction (SPE) method (Abdullah et al., 2004), while the chloroform extract were subjected to syringe filtration to remove impurities. Compounds from both extracts were identified with Thermo Scientific TSQ Quantum XLS Gas Chromatography (USA) by referring to method by Upgade and Bhaskar (2013).

**Data analysis.** Each treatment was replicated three times and the results were expressed as mean ± standard deviation. The mean values were subjected to Kruskal-Wallis H tests (SPSS statistical package, version 22) was used to determine the significant differences (p < 0.05) between treatments.

**RESULTS**

**Morphological identification of seaweeds.** The collected seaweeds were authenticated by Prof. Phang Siew Moi by comparing the morphology of the seaweeds from seaweeds library. Figure 1 shows the four Malaysian seaweeds collected.

![Figure 1. Four Malaysian seaweed species collected. (a) *S. oligocystum* (b) *C. racemosa* (c) *C. racemosa var. lammaurixii* (d) *H. macrophysa*.](image)

**Microscopic identification and growth rate of *G. boninense*.** Figure 2 shows the identification of *G. boninense*. The formation of clamp connection (Figure 2a) is similar to identified *G. boninense* strain (Kandan et al., 2010). The shape of *G. boninense* is filamentous while the elevation view is raised. *Ganoderma boninense* went through lag phase starting from day one until day four followed by exponential phase on day five until day eight. At day nine and ten, the fungus undergoes stationary phase and started to die on day twelve (Figure 2b).
Antifungal compounds from seaweed sources

**Percentage yield of seaweed extracts.** Two of the different extraction solvents were used per the order of their increasing polarity as different compound gets extracted in different solvents (Figure 3). From 10 g of seaweed powder, methanol extraction relatively produced higher yield than chloroform in all seaweed species. The extraction yield of methanol extracts ranged from 7.11% to 21.20% and chloroform extracts ranged from 1.98% to 7.11%.

**In vitro inhibitory effect of seaweed extracts against G. boninense.** The results showed that most of the seaweed extracts affect the growth of *G. boninense* at all three concentrations tested and comparative effectiveness of *C. racemosa* var. lamouruxii extract is shown in (Figure 4). The highest antifungal activities for methanolic extract against *G. boninense* at concentration of 0.25 mg/mL were exhibited by *C. racemosa* var. lamouruxii and *H. macrophysa* extracts, with inhibition percentage of 27.44% and 25.74%, respectively (Figure 4b). Lower antifungal activities were detected in *S. oligocystum*-chloroform extract in which the inhibition percentage was 6.82% while the methanolic extract of *S. oligocystum* enhanced the growth of *G. boninense*.

**GC-MS analysis of seaweed extracts.** Compounds from seaweed extracts were identified by comparing the output of the analysis to the National Institute of Standard and Technology (NIST) database library software. For methanolic extract, a total of six major compounds were identified in *S. oligocystum* extract, five major compounds in *C. racemosa* extract, seven major compounds in *C. racemosa* var. lamouruxii extract, and four major compounds were identified in *H. macrophysa* extract (Table 1). For chloroform extracts, seven major compounds detected in *S. oligocystum*, *C. racemosa* var.
*lamouroxii* and *H. macrophysa* extracts while five major compounds in *C. racemosa* extract (Table 2). Several compounds identified in methanol extracts were similar to that were found in chloroform extracts. The major classes of compounds found in all of the extracts were alkaloids, terpenes, fatty acids and sterols.

![Image](image_url)

**Figure 5.** Growth of *G. boninense* on extract-incorporated PDA after 7 days. (a) Methanolic extract of *C. microphysa*, (b) chloroform extract of *C. racemosa*, (c) negative control, (d) positive control. Red bracket: 6 mm fungus inoculum, black line: diameter of fungal growth.

**DISCUSSION**

Morphological identification of *G. boninense* was carried out by using light microscopy under 40 x 10 magnification, and we observed clamp connection structure of *G. boninense*. The mycelia were white cotton-like whereas the mature sporophores on upper surface can be in light to dark brown color. These characteristics have been previously reported for *Ganoderma* species (Kandan *et al.*, 2010). The growth of *G. boninense* was observed until day fourteen by measuring the diameter of mycelia of the fungus. During the lag phase, *G. boninense* grew through spore germination and formed mycelia which then developed primordia. Commonly, a the exponential stage, *G. boninense* produces fruiting bodies known as young sporophores and grew rapidly to mature sporophores (Kandan *et al.*, 2010). The stationary phase was between day nine to day ten in which the growth was stunted and the shrunken of mycelia indicates its death phase at day twelve.

From this study, methanol extracts displayed better extraction capability by showing high extraction yield than chloroform extracts. This might be due to the polarity of methanol which makes it able to extract not only the polar compounds but also some non-polar compounds that is miscible to methanol such as phenolics, fatty acids, sterols, alkaloids, hydrocarbons and more (Abdel-Aal *et al.*, 2015). Chloroform on the other hand is a non-polar solvent which can only solublize non-polar compounds such as phenolics, diterpene alcohols, fatty acids and other non-polar compounds (Abdel-Aal *et al.*, 2015). The major classes of compounds found in both extracts were diterpene, alkaloid, vitamin, fatty acids and steroids (Table 1).

In this study, almost all the algal extracts tested showed antifungal activity against *G. boninense* proving that extractable materials from marine algae collected from Port Dickson, Malaysia contained bioactive compounds with potential remedial interest. The broad range of classes of bioactive compounds found in seaweeds are carotenoids (Yip *et al.*, 2014), phenolics (Airanthi *et al.*, 2011; Matanjun *et al.*, 2008), alkaloids (Dheeb, 2015; Pawar and Nasreen, 2016), sterols (Abdel-Aal *et al.*, 2015), essential fatty acids (Fayaz *et al.*, 2005; Rajasulochana *et al.*, 2013), vitamins (Hamid *et al.*, 2015), polyphenols, dietary fiber, polysaccharides and proteins (Ibañez *et al.*, 2012). A Kruskal-Wallis H test was conducted to know the effect of solvents and species of extracts towards growth reduction percentage of *G. boninense*. There is statistically significant difference, *r*=9.6, *p*=.002 in growth reduction percentage between the different solvent with a mean rank growth reduction percentage of 44.14 for solvent methanol and 28.86 for solvent chloroform. The effect of different seaweed species tested towards growth reduction percentage showed no significant difference, *r*=5.3, *p*=.15 with a mean rank growth reduction percentage of 40.61 for species *S. oligocystum*, 31.94 for species *C. racemosa*, 30.00 for species *C. racemosa var. lamouroxii* and 43.44 for species *H. macrophysa*. 

"Antifungal compounds from seaweed sources" by Nasreen, 2016)
All eight extracts were further analyzed for their compounds via GC-MS. Based on the results obtained (Tables 1 and 2), all eight extracts mainly consist of compounds from the classes of alkaloids, terpenes, fatty acids and sterols. Previous studies have shown that there are a few compounds that were identified in methanol and chloroform extracts such as phytol (Shobier et al., 2016) and L- (+)-ascorbic acid 2,6-dihexadecanoate (Karthikeyan et al., 2014) were correlated with strong biological activities. Phytol is a precursor of vitamin E and vitamin K and was known for their antimicrobial activity (Inoue et al., 2005; Pejin et al., 2014). Phytol is widely used in the fragrance industry, cosmetics, shampoos, toilet soaps, household cleaners, and detergents (Ghaneian et al., 2015; McGinty et al., 2010). While L- (+)-ascorbic acid 2,6-dihexadecanoate characterized from plants and marine sources was also demonstrated powerful antimicrobial activity against Staphylococcus aureus, S. faecalis, P. aeruginosa, Escherichia coli, Streptococcus pneumoniae and Proteus mirabilis (Okwu and Ighodaro, 2009). Therefore, these compounds may be associated with the observed inhibitory effect on the growth of G. boninense in this study.

To summarize, methanol produced higher yield than chloroform due to its ability to extract polar and non-polar compounds. Three different species of marine seaweeds collected, namely, C. racemosa, C. racemosa var. lamouroxii and H. macrophysa are potential species as they exhibited antifungal activities at the lowest concentration tested while S. oligoeyustum extract slightly inhibited the growth of G. boninense. Further studies to identify the antifungal compounds and their synergism should be carried out as they could be utilized as the source of antifungal compounds which could be useful to the Malaysian oil palm industry.

### Table 1. Chemical constituents of seaweed-methanol extracts.

<table>
<thead>
<tr>
<th>Type of seaweed</th>
<th>Retention time (min)</th>
<th>Component name</th>
<th>Molecular weight (m/z)</th>
<th>Area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. oligoceystum</td>
<td>8.87</td>
<td>Phenol, 2,4-bis(1,1-dimethylethyl)-</td>
<td>206</td>
<td>10.48</td>
</tr>
<tr>
<td></td>
<td>13.71</td>
<td>Hexadecanoic acid, methyl ester</td>
<td>270</td>
<td>3.01</td>
</tr>
<tr>
<td></td>
<td>14.23</td>
<td>L- (+)-Ascorbic acid 2,6-dihexadecanoate</td>
<td>652</td>
<td>21.28</td>
</tr>
<tr>
<td></td>
<td>16.66</td>
<td>cis-Vaccenic acid</td>
<td>282</td>
<td>4.46</td>
</tr>
<tr>
<td></td>
<td>22.05</td>
<td>Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester</td>
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<td>9.34</td>
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<td></td>
<td>33.70</td>
<td>Cholest-5-en-3-ol, 24-propylidene-</td>
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<td></td>
<td>13.99</td>
<td>9,12,15-Octadecatrienoic acid, (Z,Z,Z)-</td>
<td>278</td>
<td>19.73</td>
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<tr>
<td>C. racemosa</td>
<td>13.99</td>
<td>9,12,15-Octadecatrienoic acid, (Z,Z,Z)-</td>
<td>278</td>
<td>19.73</td>
</tr>
<tr>
<td></td>
<td>14.21</td>
<td>Benzenamine, 2-[2-(4-pyridiny1) ethyl]-</td>
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<td>11.59</td>
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<td></td>
<td>16.29</td>
<td>Phytol</td>
<td>296</td>
<td>2.40</td>
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<tr>
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<td>16.61</td>
<td>9,12-Octadecadienoic acid (Z,Z)-</td>
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<td>7.91</td>
</tr>
<tr>
<td></td>
<td>16.71</td>
<td>Isobutyl methylphosphonofluoridate</td>
<td>154</td>
<td>9.63</td>
</tr>
<tr>
<td>C. racemosa var. lamouroxii</td>
<td>14.19</td>
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<td>11.59</td>
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<tr>
<td></td>
<td>20.62</td>
<td>Ethyl iso-allocholate</td>
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<td>21.01</td>
<td>Hexacyclo [7.2.2.2(4,7).0(3,8).0(12,14).0(13,15)] pentadecan-1-ol-2-one</td>
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<td>33.91</td>
<td>Spirost-8-en-11-one, 3-hydroxy, (3â,5â,14â,20â,22â,25R)-</td>
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<td>35.48</td>
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<td>36.00</td>
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<td>H. macrophysa</td>
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<td>Benzenamine, 2-[2-(4-pyridinyl) ethyl]-</td>
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<td>4.02</td>
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m/z = mass per charge number of ions
Table 2. Chemical constituents of seaweed-chloroform extracts.

<table>
<thead>
<tr>
<th>Type of seaweed</th>
<th>Retention time (min)</th>
<th>Component name</th>
<th>Molecular weight (m/z)</th>
<th>Area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. oligocystum</em></td>
<td>11.72</td>
<td>Tetradecanoic acid</td>
<td>228</td>
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</tr>
<tr>
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<td>12.65</td>
<td>Phytol</td>
<td>296</td>
<td>13.02</td>
</tr>
<tr>
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<td>14.28</td>
<td>1-(-)-Ascorbic acid 2,6-dihexadecanoate</td>
<td>652</td>
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</tr>
<tr>
<td></td>
<td>22.60</td>
<td>Diisooctyl phthalate</td>
<td>390</td>
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<td>Tetratriacontane</td>
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<tr>
<td></td>
<td>28.26</td>
<td>2H-1-Benzopyran-6-ol,3,4-dihydro-2,8-dimethyl-2-(4,8,12-trimethyl tridecyl) - [2R-2R*(4R*,8R*)]-</td>
<td>402</td>
<td>3.94</td>
</tr>
<tr>
<td></td>
<td>31.68</td>
<td>á-Tocopherol, O-methyl-</td>
<td>430</td>
<td>5.61</td>
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<tr>
<td><em>C. racemosa</em></td>
<td>11.75</td>
<td>Tetradecanoic acid</td>
<td>228</td>
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<td>17.89</td>
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</tr>
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<td>á-Tocopherol, O-methyl-</td>
<td>430</td>
<td>1.72</td>
</tr>
<tr>
<td><em>C. racemosa var. lamouroxii</em></td>
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<td>1-(-)-Ascorbic acid 2,6-dihexadecanoate</td>
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<td>2.69</td>
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<td>(+)-ç-Tocopherol, O-methyl-Furo[3',4':6,7]naphtho[2,3-d]-1,3-dioxol-6(5aH)-one,5,8,8a,9-tetrahydro-5-(3,4,5-trimethoxyphenyl)-, [5R-(5a,5a,8a)]-</td>
<td>398</td>
<td>9.51</td>
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<tr>
<td><em>H. macrophysa</em></td>
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<td>Benzenamine,2-[2-(4-pyridinyl)ethyl]-</td>
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<td>6.51</td>
</tr>
<tr>
<td></td>
<td>14.27</td>
<td>1-(-)-Ascorbic acid 2,6-dihexadecanoate</td>
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<td>13.41</td>
</tr>
<tr>
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<td>16.30</td>
<td>Phytol</td>
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<td>4.55</td>
</tr>
<tr>
<td></td>
<td>20.69</td>
<td>2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-ethyl)cyclohex-1-enyl] cyclohex-1-en-1-carboxaldehyde</td>
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<td>Cholesterol</td>
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<td>4.84</td>
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m/z = mass per charge number of ions

**ACKNOWLEDGEMENTS**

The authors gratefully acknowledge Prof. Phang Siew Moi from Institute of Graduate Studies, Universiti Malaya for the authentication of seaweed species used in this research and Dr. Nusaibah Syed Ali from Fakulti Pertanian, UPM for providing the fungal materials. This research was funded by Geran Putra IPS Universiti Putra Malaysia (Project No. 9511100), Higher Institution Centre of Excellence (HICOE) Research Grant (Innovative Vaccines and Therapeutics against Fish Diseases) (Project No. 6369100), and SATREPS (JICA-JST): COSMOS-MOHE G4-B Research Grant (Microalgae for Sustainable Aquaculture Health: Microalgae Vaccine Delivery System) (Project No. 6300866). 

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Inoculation of fowlpox viruses coexpressing avian influenza H5 and chicken IL-15 cytokine gene stimulates diverse host immune responses

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Abstract. Fowlpox virus (FWPV) has been used as a recombinant vaccine vector to express antigens from several important avian pathogens. Attempts have been made to improve vaccine strains induced-host immune responses by coexpressing cytokines. This study describes the construction of recombinant FWPV (rFWPV) strain FP9 and immunological responses in specific-pathogen-free (SPF) chickens, coexpressing avian influenza virus (AIV) H5 of A/Chicken/Malaysia/5858/2004, and chicken IL-15 cytokine genes. Expression of H5 (50 kD) was confirmed by western blotting. Anti-H5 antibodies, which were measured by the haemagglutinin inhibition test, were at the highest levels at Week 3 post-inoculation in both rFWPV/H5- and rFWPV/H5/IL-15-vaccinated chickens, but decreased to undetectable levels from Week 5 onwards. CD3+/CD4+ or CD3+/CD8+ T cell populations, assessed using flow cytometry, were significantly increased in both WT FP9- and rFWPV/H5-vaccinated chickens and were also higher than in rFWPV/H5/IL-15-vaccinated chickens, at Week 2. Gene expression analysis using real time quantitative polymerase chain reaction (qPCR) demonstrated upregulation of IL-15 expression in all vaccinated groups with rFWPV/H5/IL-15 having the highest fold change, at day 2 (117±51.53). Despite showing upregulation, fold change values of the IL-18 expression were below 1.00 for all vaccinated groups at day 2, 4 and 6. This study shows successful construction of rFWPV/H5 co-expressing IL-15, with modified immunogenicity upon inoculation into SPF chickens.

Keywords: avian influenza virus, fowlpox virus, haemagglutinin, interleukin-15, interleukin-18

INTRODUCTION

Since the late 1980s, recombinant FWPVs (rFWPV)s based on attenuated FWPV strains have been developed to express antigens from several important avian pathogens, including: avian influenza virus (AIV; (Qian et al., 2012)), Newcastle disease virus (NDV; (Sun et al., 2008) and Marek’s disease virus (MDV; (Lee et al., 2003). rFWPVs expressing haemagglutinin (HA) H5 protein of AIV (rFWPV/H5), particularly derived from A/Turkey/Ireland/83 (H5N9), or A/Goose/Guangdong/96 (H5N1), have been used in South East Asia as vaccines against highly pathogenic avian influenza (HPAI) H5N1. Despite this preventive measure, HPAI H5N1 is
still a major concern due to its ongoing, sporadic re-emergence. The need to boost existing eradication efforts to limit the spread and occurrence of the outbreak has prompted development of several strategies to improve readily available avian influenza vaccines. We describe here one such strategy: to co-express host cytokines from rFWPV/H5.

In mice, recombinant vaccinia virus (rVACV) co-expressing gp160 of human immunodeficiency virus (HIV) and human interleukin 15 (hIL-15) has been shown to provide a stronger and more enduring response than rVACV expressing gp160 alone (Oh et al., 2003). Integration of hIL-15 into rVACV Wyeth strain or Modified VACV Ankara (MVA) resulted in better survival (Perera et al., 2007) and enhanced in vivo viral clearance (Zielinski et al., 2010) in vaccinated athymic nude mice upon intranasal challenge with virulent VACV strain Western Reserve, or intravenous challenge with monkeypox virus strain Zaire 79, respectively. Enhanced CD4 and CD8 T cell memory responses, along with reduction in lung mycobacterial load in lungs, was also observed in mice infected with Bacille Calmette-Guérin (BCG), supplemented with IL-7 and IL-15 recombinant proteins, but not IL-1, IL-6 or interferon (IFN)-α (Singh et al., 2010). In mice model, it has also been shown that IL-15 offers potent antiviral effects against rVACV coexpressing IL-15, with high dependency on the presence of NK cells and IFNs (Foong et al., 2009).

Almost all of the chicken cytokines that have been investigated are Th1-like. In a rare avian study, in ovo plasmid DNA vaccination against an intestinal coccidial parasite, *Eimeria acervulina*, using coccidial gene 3-1E coexpressed with chicken IL-15, was shown to induce higher serum antibody levels than immunization with 3-1E alone. Following challenge with the homologous parasite, chickens vaccinated with 3-1E plus IL-15 showed a significant decreased in oocyst shedding and had an increased body weight, compared to chickens vaccinated with 3-1E alone (Lillehoj et al., 2005). Similar results were obtained whether the construct was given subcutaneously (Min et al., 2001) or intramuscularly (Ma et al., 2013).

Studies with rFWPV coexpressing HA from AIV H5N1 and chicken IL-18 (Chen et al., 2011; Mingxiao et al., 2006) or IL-6 (Qian et al., 2012) have been described. The results showed that all chickens vaccinated with rFWPV/H5/IL-18 exhibited reduced virus shedding and replication (Chen et al., 2011), and had higher levels of cellular immunity (Mingxiao et al., 2006), compared to rFWPV/H5 alone. Study of the effect of chIL-15 coexpression by rFWPV/H5 in chickens, as reported here, is novel.

MATERIALS AND METHODS

**Ethical approval.** All animal experiments performed in this study were in accordance with the ethical standards of the local Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia (UPM) with reference number UPM/FPV/PS/3.2.1.551/AUP-R72.

**Viruses and cells.** The initial stock of parental FP9 was from M.A. Skinner laboratory (Imperial College London, UK). The development of FP9 via 438 serial passages of the wild-type fowlpox virus HP-1, followed by plaque purification, has been described (Laidlaw and Skinner, 2004). Chicken embryonic fibroblast (CEF) s used in this study were cultured in 2% newborn bovine serum (NBBS) in DMEM media (both from Gibco).

**Construction of recombinant plasmids.** Previously cloned and sequenced cDNA encoding full-length H5 of influenza strain A/Ch/Malaysia/5744/2004 (Balasubramaniam et al., 2011) was amplified by PCR with primers H5-F: 5'-ATCGGATACATGGAGAATAAGTGCGC-3' and H5-R: 5'-GACTGATATCTTTAATGCGAAAATTCTGC-3', introducing EcoRV sites as underlined. Sequence encoding the pentabasic peptide motif at the protease cleavage site of H5 was replaced with threonine (T) using mutagenic primers S(2-F): 5'-CAAAGAGAGACAAGGATTATTTGGAGCCTAGT3' and S(1-R): 5'-CAAATAATCCTTTGCTCTCTTTGAGGCTATTTC-3'.

The assembled amplicon was inserted into the Smal site of lac Z-selectable, FWPV expression/recombination vector pEFL29 (Qingzhong et al., 1994), downstream of a copy of the vaccinia virus p7.5 early/late promoter. The
Chicken IL-15 gene (supplied by the late Prof. Dr. Pete Kaiser from the then Institute for Animal Health, Compton, UK) was inserted downstream of a synthetic/hybrid promoter in vector pEFgfp12S, before being subeloned into vector pPC1.X (Abd Razak, 2011). Positive transformants were grown in LB broth (15 mL) supplemented with appropriate antibiotic(s) (750 μg) at 37°C overnight. The culture (0.5 μL) was used to provide templates for analytical PCR. The reaction mixture for a small scale PCR verification contained 10X PCR buffer (2 μL; Sigma), JumpStart Taq DNA polymerase (0.5 U; Sigma), dNTPs (0.5 μL of 10 mM) and oligonucleotide primers (0.5 μL of each 10 μM stock), in a total volume of 20 μL. PCR was conducted in 2 steps; 4 cycles of 95°C for 3 min, 95°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min followed by 26 cycles of 95°C for 30 s, 59°C for 30 s, and 7°C for 1.5 min. Final extension was performed at 72°C for 10 minutes. Verification of H5 integration into FWPV was carried out using H5-F and H5-R primers, while primers pPC1.X-F: 5'-ATGAAAAATAGTACCACTATGG-3' and IL-15R: 5'-ACAGAGTTTTGTAAAGGTTATACA GAGG-3' were used to screen for rFWPV/H5 carrying IL-15 in gene.

Recombination/transfection, selection and purification of recombinant viruses. The detailed protocol for recombination/transfection has been described (Laidlaw and Skinner, 2014), with minor modifications i.e. replacing 199 media with DMEM (Gibco), and polyfect with lipofectin (Thermo Fisher Scientific), of the same volume. Successful recovery of rFWPV/H5 carrying a LacZ gene from pEFL29 into FP9 was demonstrated by blue plaques upon X-Gal overlay (at final concentration of 0.4 mg/mL) on day 4 post-transfection. Further screening for rFWPV/H5 carrying chIL-15 was done by mycophenolic acid (MPA) selection of gpt gene and spontaneous resolving of the gpt gene by a second crossover event, as described previously (Laidlaw et al., 1998). The recombinant protein lysates were prepared by infecting CEFs, with rFWPV/H5 at a multiplicity of infection (MOI) of 3, for 48 hours. The cell pellet was subjected to 15% SDS-PAGE. The electro-transferred nitrocellulose membrane (GE Healthcare) was incubated with a goat polyclonal primary antibody against haemagglutinin H5 (Cat. No. ab62587, Abcam, USA) with the final concentration 1 μg/μL, for 1 hour. The membrane was developed using a commercial kit using the chromogenic substance, WesternBreeze (Invitrogen).

Immunofluorescence antibody test (IFAT) was performed using 80% confluent CEFs. Cells were either infected with viruses at 0.3 MOI, or left uninfected (negative control). The infection was left overnight in 2% NBBS DMEM medium, before incubation with a rabbit polyclonal primary antibody against haemagglutinin H5 (Cat. No. ab70077, Abcam, USA) with the final concentration 1 μg/μL, for 2 hours. After three washes with PBS, cells were incubated with fluorescein-labelled secondary antibodies for 1 hour. Slides were viewed under a fluorescent microscope (model Leica DMRA II).

Immunization of animals. One-day old specific pathogen-free (SPF) chickens were inoculated subcutaneously with 10³ plaque forming unit (PFU) of parental FWPV FP9 (WT FP9), rFWPV/H5 or rFWPV/H5/IL-15, diluted in PBS to a total volume of 100 μL, at the scruff of the neck, using a 27-G needle. One control group was mock-treated with 100 μL of PBS. Nine chickens were assigned for each group. Blood sampling (for serum) of each chicken was done on a weekly basis. At Weeks 2 and 5, whole blood (0.2 mL) of each chicken in each group of nine was sampled and pooled into 3 groups (0.6 mL in total), for CD4+ and CD8+ lymphocyte isolation, followed by flow cytometry analysis. As for IL-15 and IL-18 gene expression analysis, immunization of 105 PFU of aforementioned vaccine groups was done on 14-days old SPF chickens; twelve chickens for each group. At every two consecutive days’ post immunization, RNA was extracted from the spleens (four from each group) and processed for qPCR.

Serological tests. Haemagglutination inhibition (HI) tests were performed in U-bottomed 96-well microtitre plates using 4 HA units/25 μL of H5N2 virus strain A/Malaysia/Duck/8443/04 (Veterinary Research Institute Ipoh, Malaysia), and washed chicken erythrocytes (25 μL of 0.8% v/v). The antigen-antibody was incubated for 1 hour. HI titres were determined as the reciprocal
of the highest serum dilution that completely inhibited haemagglutination.

**Immunophenotyping analysis.** Fresh, non-coagulated chicken whole blood was diluted to 1 mL using cold PBS and was carefully layered on 2 mL Ficoll-Paque PLUS (GE Healthcare). Isolation of peripheral blood mononuclear cells (PBMC) was done by following the standard Ficoll-Paque PLUS protocol. Approximately 10^6 cells were incubated with mouse anti-chicken CD8a-PerCP-Cy5-conjugated (1 µg/mL), CD3-PE-conjugated (0.5 µg/mL) and CD4-FITC-conjugated (0.5 µg/mL) monoclonal antibodies (all from Southern Biotech), prior to analysis using a BD FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

**Quantitative real time polymerase chain reaction (qPCR).** The total RNA from chicken spleens was harvested using TRIzol reagent (Ambion) according to manufacturer’s recommendations. The extracted RNA was reverse-transcribed using Script cDNA synthesis kit (Jena Bioscience) in a total volume of 20 µL containing 2.5 µM primers, 1X Script RT buffer, 500 µM dNTP, 5 µM DTT stock, 40 units RNase inhibitor, 100 units Script RT and 5 µg RNA template. The reaction mix was incubated at 42°C for 10 minutes followed by 50°C for 60 minutes. Primer sequences for cytokines IL-15 and IL-18, and a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were designed from public databases (Brisbin et al., 2010; Cai et al., 2009), as shown in Table 1. The qPCR amplification was performed according to the KAPA SYBR FAST qPCR kit (KAPA Biosystem) using Bio-Rad CFX96 real-time system. The data was imported into the analysis module of the Bio-Rad CFX Manager. The expression of GAPDH gene was used as the qPCR normalization standards. All results are reported as delta-delta CT (ΔΔCT), relative to the control group.

**Statistical analysis.** Data variations between groups were analysed by one-way ANOVA or paired-samples T test using SPSS (Version 15) software. Results were expressed as the mean ± standard error of the mean (SE). P values less than 0.05 were considered statistically significant in all cases.

Table 1. Forward and reverse primer sequences used for qPCR.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’ to 3’)</th>
<th>GenBank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-15 - F</td>
<td>CGAGGCTTGTACCGCAATGT</td>
<td>AF139097</td>
</tr>
<tr>
<td>IL-15 - R</td>
<td>GCCATCCCCAGCATCTTGT</td>
<td></td>
</tr>
<tr>
<td>IL-18 - F</td>
<td>ACAAGGAATGTTCCTTGGGCTTT</td>
<td>NM_204608</td>
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<td>IL-18 - R</td>
<td>CTTACATCTTCTCTGGGCAAGTTTC</td>
<td></td>
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<tr>
<td>GAPDH - F</td>
<td>CTACACACGGACACCTCAAG</td>
<td>NM_204305</td>
</tr>
<tr>
<td>GAPDH - R</td>
<td>ACAAACATGGGGGCCATCAG</td>
<td></td>
</tr>
</tbody>
</table>

F, forward; R, reverse; IL, interleukin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

**RESULTS**

**PCR amplification of H5 gene.** The HPAIV haemagglutinin (HA) gene of the H5 virus contains multiple basic amino acids, arginine and lysine, that allow cleavage by ubiquitous proteases (furin and PC6) (Horimoto et al., 1994). To maintain compatibility with recombinant, killed H5N1 influenza vaccines (J. Wood, personal communication), and to reduce any potential biosafety issues, the pentabasic peptide motif (underlined) at the protease cleavage site of H5, S-P-Q-R-E-R-R-K-R was removed and replaced with threonine (T), leaving a monobasic arginine (R) at the site (S-P-Q-R-E-T-R). The H5-F and S(1-R) primers generated the first H5 fragment (1036 bp), while primers H5-R and S(2-R) generated the second H5 fragment (684 bp). Full length mutated H5 gene (1695 bp), was obtained through PCR overlap extension mutagenesis (Figure 1a).
Coexpression of AIV H5 and IL-15 in fowlpox viruses

Verification of H5-recombinant fowlpox viruses coexpressing chicken IL-15. Upon successful transfection, the recombinant clones were verified for presence of the inserted H5 gene by PCR of extracted FWPV genomic DNA (data not shown). Positive recombinants (rFWPV/H5) were subjected to second homologous recombination of vector pPC1.X carrying chicken cytokine gene IL-15 at a second non-essential site, the PC-1 (fpv030) homology region. The cytokine expression cassettes in pPC1.X/IL-15 were previously confirmed by restriction digests and sequencing (data not shown). Screening of positive recombinant viruses (rFWPV/H5/IL-15) was done using primers external and internal to the inserted genes (the latter resulting in PCR products exclusively for recombinant clones) (Figure 1b). H5 protein expression was analysed by western blotting (Figure 2). A faint band at ~50 kD was observed for H5 recombinant, none for uninfected cell lysate and negative control (WT FP9). This is the first report on the size of H5 protein from strain A/Ch/Malaysia/5744/2004. Further analysis using IFAT detected fluorescent signals only for CEF infected with H5 recombinant, which indicates successful H5 protein expression. No reactivity was observed for uninfected or WT FP9-infected CEF (Figure 3).
Coexpression of AIV H5 and IL-15 in fowlpox viruses

Figure 3. IFAT analysis for verification of H5 protein expression from rFPV/H5. CEFs were (A) uninfected; (B) infected with WT FP9 as negative control; (C) infected with rFPV/H5. Infected cells were incubated with a rabbit polyclonal primary antibody against haemagglutinin H5 (Cat. No. ab70077, Abcam, USA) with the final concentration 1 µg/µL. Observation was performed under visible light (ii) or UV light (ii). The images did not represent 80% of cell confluency due to repeated washing of the cells without fixation during procedure.

Haemagglutinin inhibition tests for chickens following rFPV immunizations. None of the nine control chickens inoculated with PBS or WT FP9 showed any evidence of HI antibody responses. Mean HI titres, in log2, of all groups were calculated for general comparison (Table 2). H5 antibodies reached detectable levels in chickens vaccinated with rFPV/H5/IL-15 one week earlier than those vaccinated with rFPV/H5 but, thereafter, there was no significant difference between the two groups. Responses were highest at Week 3 in both groups of recombinant vaccine-treated chickens. However, the antibodies were undetectable based on HI tests that have been carried out from Week 5 onwards.

CD3+/CD4+ and CD3+/CD8+ T cells population following rFPV immunizations. The levels of CD3+/CD4+ T cells in the control group remained relatively constant at Weeks 2 and 5. Samples from groups vaccinated with WT FP9 or rFPV/H5/IL-15 demonstrated increases in CD3+/CD4+ T cell population levels over time, of 2.06 and 3.16 point percentages, respectively. The rFPV/H5 vaccinated group showed a significantly higher CD3+/CD4+ T cell population relative to control at Week 2 (P≤0.05) but had returned to control levels by Week 5. No statistically significant difference in CD3+/CD4+ T cell levels was observed for other groups at either sampling point (Figure 4).

Animal experiments also revealed a relatively constant CD3+/CD8+ T cell population for control chickens. The same was true for the slight to somewhat higher levels observed in rFPV/H5/IL-15, WT FP9- and rFPV-vaccinated birds (significant for WT- and rFPV/H5- but not rFPV/H5/IL-15-vaccinated birds), although a fall to control levels was observed in rFPV/H5-vaccinated birds at Week 5.

Table 2. Mean of HI titre, log2, of sera from immunized chickens.

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>HI titre</th>
<th>Weeks, post immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Control</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>WT FP9</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>rFPV/H5</td>
<td>ND</td>
<td>8.11±4.60</td>
</tr>
<tr>
<td>rFPV/H5/IL-15</td>
<td>0.56±0.44</td>
<td>9.89±2.16</td>
</tr>
</tbody>
</table>

ND indicates undetected titre. Each value represents the means±SE of nine birds.

No significant difference was observed between rFPV/H5 and rFPV/H5/IL-15 at any time point (P≥0.05).

Coexpression of AIV H5 and IL-15 in fowlpox viruses

Figure 4. Immunophenotyping of CD3+/CD4 (a) and CD3+/CD8+ (b) lymphocytes from chickens after mock-treatment with PBS (control), or vaccination with WT FP9, rFWPV/H5 or rFWPV/H5/IL-15. Each value represents the mean percentages of T lymphocytes sub-population ± SE, from PBMC samples of nine chickens pooled in threes (n=3), sampled at Weeks 2 and 5. Significant differences between vaccinated and control groups (*), were determined by one-way ANOVA (P≤0.05). Significant differences within the same group at different points were determined by paired-samples T-test (P≤0.05).

Gene expression analysis of IL-15 and IL-18.
IL-15 expression in all vaccinated groups showed upregulation on day 2, notably in those vaccinated with rFWPV/H5/IL-15, which overexpress chicken IL-15 (Figure 5). WT FP9- and rFWPV/H5-vaccinated birds expressed 7- to 19-fold more IL-15 than control birds; over-expression by rFWPV/H5/IL-15 boosted IL-15 levels to 120 fold more than control. Expression of IL-15 dropped to control levels by day 4, for all tested groups.

IL-18 expression was lower in all of the FWPV-infected groups (two to five fold lower for WT FP9- and rFWPV/H5-vaccinated groups, possibly up to ten fold lower for rFWPV/H5/IL-15) and this decreased expression was extended out to 6 days.

DISCUSSION

The most important component of host immune response that confers protection in chickens against AIV is the humoral response against HA (Swayne, 2007). To achieve this, several different types of vaccines have been developed, e.g. inactivated AIV vaccines (Bublot et al., 2007; Tian et al., 2010), DNA vaccines (Lim et al., 2012), and virus-like particles (Hendin et al., 2017). In this study, a safe, lab-adapted FWPV-based vector expressing the H5 of AIV was modified to co-express a chicken IL-15 cytokine gene to test if it would enhance the host cell mediated immune response, which may be critical in clearance of AIV during infection (Foong et al., 2009). However, we did not perform protective or challenge studies for AIV, nor did we evaluate protection against FWPV.

Vaccination with rFWPV/H5/IL-15 produced HA antibody titres comparable to
vaccination with rFWPV/H5. This finding contrasts with several studies conducted in mice, including that by Perera et al. (2007) which reported induction of two-fold higher VACV-neutralizing antibody titres in hIL-15-expressing recombinant VACV. The group also showed that recombinant VACV strain Wyeth, expressing five heterologous influenza virus genes, induced stronger neutralizing antibodies against AIV H5 when adjuvanted with hIL-15 (Poon et al., 2009). The inconsistencies in HA antibody titres between our study and those by Perera et al. (2007) and Poon et al. (2009) might be due to the usage of heterologous (instead of a homologous) H5N2 virus strain A/Malaysia/Duck/8443/04 antigens against the H5 antibodies from our rFWPV recombinants. Heterologous antigens used to assay H5 antibodies induced by rFWPV were shown to produce either low (Taylor et al., 1988), highest (Bublot et al., 2010) or inconsistent (Swayne et al., 2007) HI titres. Although these studies did not use homologous antigens, which might be more suitable for their HI testing, the results provide useful comparisons of HI antibody levels elicited by rFWPV/H5 and rFWPV/H5/IL-15. Several studies have shown rFWPV expressing H5 can provide complete or nearly complete protection against lethal challenge, even when achieving pre-challenge HI titres of as low as 3 log₂ (Bublot et al., 2010; Webster et al., 1991). Post-vaccination protection of chickens against AIV may not be dependent entirely on HI antibodies but also on non-HI antibodies and possibly also on cell-mediated immunity.

rFWPV/H5/IL-15 did not increase CD4+ T cell populations, compared to rFWPV/H5, following vaccination. This finding is consistent with previous reports that IL-15 only has profound effects on the proliferation and survival of memory CD8+ T cells, not on CD4+ T cells (Marks-Konczalik et al., 2000; Zhang et al., 1998), although a significant increment of CD4+ T cell populations was observed in a DNA vaccine coexpressing H5 and chicken IL-15 genes (Lim et al., 2012). It is not known whether inherent molecular patterns of, or immunomodulatory proteins expressed by, FWPV FP9 can influence IL-15 levels in vaccinated chickens. It has been reported that IL-15 can only activate CD4+ T cell proliferation when at high concentration presence (Kanegane and Tosato, 1996). Niedbala et al. (2002) showed that 2 to 4 fold higher concentrations of IL-15 are required to achieve optimal CD4+ T cell proliferation than to promote CD8+ T cell response.

The co-stimulatory effects of IL-15 on CD8 cells have been studied widely, especially with regard to proliferation and survival of memory CD8+ T cells. IL-15 has been found to directly stimulate purified CD8+ memory cells in vitro (Zhang et al., 1998). Transgenic mice which constitutively expressed a significant level of IL-15 in the serum had higher numbers of memory CD8+ T cells (Marks-Konczalik et al., 2000; Yajima et al., 2002). In our study, chickens vaccinated with WT FP9 or rFWPVs showed low to moderate increase in levels of CD8+ T cells. The increases, at 1.6 to 2 fold, were significant for WT FP9 or rFWPV/H5 respectively but, at 1.25 fold increment, was insignificant from the rFWPV/H5/IL-15. These results suggest that FWPV enhances chicken CD8+ T cells stimulation and possibly that IL-15 has the opposite effect.

Although hIL-15 has been shown to stimulate CD8+ T cells population and promote the maintenance of CD8+ CD44hi memory T cells, the responsiveness of CD8+ T cells to IL-15 might depend on the cytokine background (Niedbala et al., 2002; Oh et al., 2003). Unfortunately, in this study, we did not measure the levels of IL-15, secreted by cells infected with an initial dose of 10⁵ PFU rFWPV/H5/IL-15, in peripheral blood prior to flow analysis. Since a strong synthetic/hybrid promoter was used for IL-15 co-expression, levels of expression might have been inconsistent with generation of the desired immune responses.

We observed elevation of the CD4+ T cell population and sustained CD8+ T cell population from WT FP9 and rFWPV/H5/IL-15 inoculated groups. However, rFWPV/H5 inoculated group showed a consistent decreasing pattern for both T cells. By way of comparison, an in vivo study examining T cell populations in the peripheral blood of rhesus macaques treated with rhesus IL-15, where the level of CD4+ and CD8+ memory, but not naive, T cells peaked at Weeks 1 to 2 and returned to baseline by Weeks 3 to 4 (Picker et al., 2006).

Acting synergistically, IL-15 and IL-18 can
perpetuate Th1 responses (Gracie et al., 1999) and enhance IL-12 stimulation of NK cell to produce IFN gamma (French et al., 2006). A DNA vaccine co-expressing H5 and chicken IL-15, induced a significant increase in IL-15, but not IL-18, levels post-vaccination (Lim et al., 2012). Our results are comparable, where enhanced expression of host IL-15 and reduced expression of host IL-18 are mediated directly by infection with WT FP9 or rFWPV/H5 co-expressing exogenous IL-15 (mediated by a strong synthetic poxvirus promoter).

The dramatic drop of IL-15 levels from day 2 to day 4 in all FWPV-infected groups might be due to clearance of these attenuated viruses by NK cells, their cytolytic activity potentially augmented by the ability of IL-15 (expressed endogenously by the host or exogenously by the recombinant FWPV) to enhance IFN expression and increase poxvirus clearance (Foong et al., 2009). However, we cannot currently explain the concomitant drop in IL-18 mRNA expression during FWPV infection but FWPV appears to express one or more IL-18 binding proteins (Laidlaw and Skinner, 2004), which might reduce steady-state levels of circulating IL-18 in a similar manner to the host-encoded regulator IL-18BP (Harms et al., 2017). It is possible therefore that the virus encodes additional mechanisms to down-regulate expression of IL-18 mRNA.

CONCLUSION

rFWPV/H5 and rFWPV/H5/IL-15 inoculated groups elicited the highest levels of anti-H5 antibodies at Week 3 post-inoculation. CD3+/CD4+ or CD3+/CD8+ T cell populations were significantly increased in both WT FP9- and rFWPV/H5-, higher than in rFWPV/H5/IL-15-vaccinated chickens, at Week 2 post-inoculation. IL-15 and IL-18 expressions were upregulated in all vaccinated groups at day 2 post-inoculation. These diverse immunogenicity findings may contribute to the limited exploration of chicken IL-15 in vaccine developments.

ACKNOWLEDGEMENTS

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Coexpression of AIV H5 and IL-15 in fowlpox viruses


Characterization of cis-elements in hormonal stress-responsive genes in *Oryza sativa*

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**Abstract.** Phytohormones play a key role in plant growth and development. The process of plant’s perception and response to abiotic and biotic stresses is controlled mainly by the phytohormones which act as an endogenous messenger in the regulation of the plant’s status. They can be activated by different signaling pathways in response to environmental stresses. Plants respond to environmental stress through interaction of transcription factors with a handful of *cis*-regulatory elements (CREs). Some examples of *cis* elements include abscisic acid-responsive element (ABRE), G-box (CACGTG) element, and W-box. In order to investigate the effects of different hormonal stresses which have a key role in response to biotic and abiotic stresses in rice, microarray data was used. Of the available data, 931 genes revealed significant differences in response to different hormonal stresses such as auxin, cytokinin, abscisic acid, ethylene, salicylic acid, and jasmonic acid. The present results showed that 388 genes were up-regulated, and 543 genes were down-regulated. Most of the genes were up-regulated in response to Indole-3-acetic acid (IAA) hormone. Genes Ontology analysis revealed that they respond to various hormones involved in *auxin*- responsive genes, auxin-activated signaling pathway and cellular responses to environmental stimuli. G-box had the highest number of *cis* elements involved in hormonal stress and was regulated by auxin signaling and various stresses. *Dehydrin* was the only gene up-regulated in response to the six hormones. This gene can be activated in response to abiotic and biotic stresses. As such, *dehydrin* gene can be used in crop breeding programs to increase tolerance to different environmental stresses in various plant species.

**Keywords:** *Cis* elements prediction, Microarray data, Phytohormones, Regulatory elements.

**INTRODUCTION**

Plants are exposed to many abiotic and biotic stresses during the course of life (Jung *et al*., 2008). Plants use phytohormones which are important regulatory elements involved in response to different conditions such as stress, defense, and adaptation (Nemhauser *et al*., 2006).

When plants are exposed to external stimuli such as pathogen and insect attack, drought, and salt stress, hormonal signal transduction pathways are induced which in turn cause changes in the hormone metabolism and its distribution within plant (Cheong *et al*., 2002). It has been reported that phytohormones such as auxin, cytokinin, gibberellin, abscisic acid, ethylene, jasmonic acid and salicylic acid can play a role as important regulators in response to different stresses. Therefore, it is important to increase our understanding about role of phytohormones in molecular and cellular mechanisms in various pathways (Là *et al*., 2016). Rice is an ideal model to

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study the regulation of gene expression in response to different stresses (Rabbani et al., 2003; Yazaki et al., 2005). Based on previous research performed, auxin and abscisic acid are the most important phytohormones in response to different hormonal stresses (Uddin and Singh, 2017). ABA has an important factor in the response of plants to abiotic stresses. Thousands of phytohormones-regulated genes are activated in response to hormonal, abiotic and biotic stresses (Depuydt and Hardtke, 2011). Numerous number of hormone-responsive genes with differential expression have been detected, offering a cross-talk between hormone and abiotic stress signaling (Garg et al., 2012).

Among up-regulated genes, genes involved in flavonoid biosynthesis are key roles in response to stresses. Auxins influence flavonoid biosynthesis and the reduction of flavonoid levels leads to IAA transport in plant. Flavonoid biosynthesis is related to the biosynthesis of aromatic compounds such as tryptophan, which is a precursor for auxin (Li and Zachgo, 2013). Analysis of gene expression profiling increase understanding to the biological pathways that one might wish to derive (Garber et al., 2001). There is a requirement for more insights of transcriptional responses of plant hormone under stresses, that extensive crosstalk and signal integration has been uncovered (Sharoni et al., 2012). High-throughput technique such as microarray has been extensively used for evaluation of plant expression profile under stress conditions (Lenka et al., 2011). Microarray data has been used to survey gene expression patterns providing new dimensions in gene expression related to growth and development (Cramer et al., 2011).

In the current study, microarray data was used to survey gene expression patterns on six hormones in rice. Interaction between cis regulatory elements and transcription factors forms transcriptional regulation network. This network can regulate gene-controlling morphogenesis, development of anatomy, and other aspects of developmental biology and hormone responses (Sato et al., 2012; Smita et al., 2011; Wilmoth et al., 2005). Gene network can detect relationships between genes and transcription factors, also co-expression genes (Wilmoth et al., 2005).

Different reporters have shown that co-expressed genes are involved in a common biological pathway and cellular processes, plant growth, and reproduction (Michalak et al., 2008; Koç et al., 2018). Integration of data with promoter structure in plants shows that promoters of co-expressed genes induced a common cis-regulatory elements (CAREs) (Reiss et al. 2006; Werner 2001). CAREs functions were predominantly associated with light response, hormonal regulation and stress response and were detected in the upstream of DGEs under drought and salinity stress conditions in rice (Shariatipour et al., 2018). Most of cis elements expressed in response to abiotic, biotic, and hormonal stresses can be suggested to be the ABA responsive elements (ABREs), also termed as G-box, with promoters of the hormonal responsive genes (Ross and Shen 2006). The hormonal regulatory elements such as Methyl jasmonate (MeJA), Ethylene (ET) and abscisic acid (ABA) were responsible for activation of ABRE, P-box, and TGACG-motif elements. These motifs are present in majority of DGEs under drought stress conditions (Shariatipour et al., 2018). This work aimed to characterize cis elements related to responsive genes in *Oryza sativa* under hormonal stress.

**MATERIALS AND METHODS**

Microarray data related to the expression patterns of hormonal stresses response of rice (*Oryza sativa* subspecies indica varietiy IR64) (GSE37557) was retrieved from NCBI GEO database (www.ncbi.nlm.nih.gov/geo/). In this study, microarray data required for control and stress conditions were collected from 16 samples in water (control) and in a 50 μM solution of IAA and BAP and 100 μM solution of ABA, ACC, SA and JA for 3 h (treatment) with 2 biological replications from gene bank (http://www.ncbi.nlm.nih.gov/gds). Analysis of data was performed using fold change which is based on log2ratio, followed by normalizing the data (the ratio expression of the genes examined under hormonal stress to control) and selected by P-value <0.05 as the statistical significance (Mehdizadeh et al., 2013). T-test was used to calculate the p-value of the expression change of each probe, and differentially expressed genes (DEGs) were detected using the Affy
package (Gautier et al., 2004). DEGs with p-values <0.05 and log fold-change values ≥2 and ≤-2 were selected for genes. The filtered data with DEGs >2 was considered as up-regulated genes and DEGs<2 were determined to be down regulated. Up-regulated and down-regulated genes in response to hormonal stress are shown in table 2. Probe sets were mapped to MSU Rice Genome Annotation Project gene set (release 6.1). Heatmap of DEGs was performed by package graphics to represent differential expression. To convert probe set to ID genes, DAVID sites (https://david.ncifcrf.gov) was used. One-way ANOVA showed significantly different genes among the studied genotypes at a five percent significance level. In addition, the corrected P-value of Benjamin-Hookberg allowed a more stringent selection of up-regulated genes showing less than five percent significance level. To obtain the possibility functions of hormone-responsive genes, their annotation was taken from the rice genome database (http://rice.plantbiology.msu.edu/). Gene ontology was performed on biological pathways of genes using Gene Ontology Enrichment Analysis Software Toolkit (Yazaki et al., 2003). STRING 10.0 (http://string-db.org/) was done for identifying co-expression genes and drawing the genes network (Szklarczyk et al., 2017). In order to predict the cis regulatory elements, genes promoter analysis for each gene was obtained from PlantCARE; database (Lescot et al., 2002) (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

RESULTS AND DISCUSSION

Differentially-expressed gene in response to hormonal stresses. According to microarray data in different hormonal stress conditions, the genes are up-regulated and down-regulated in response to IAA and ethylene, respectively (Fig. 1). The data were based on p-value > 0.05 and the threshold of 2 by which a total of 931 genes were induced. A set of 388 up-regulated and 543 down-regulated genes were detected in response to different hormonal stresses. Results of clustering of expressed genes showed that almost all genes were up-regulated in response to IAA (Table 1).

Therefore, it can be suggested that auxin may be involved with other hormones and can be a main component in regulation of reactions to environmental stresses. Similar to previous studies, several auxin-responsive genes are regulated by biotic and abiotic stresses (Ghanashyam et al., 2009). It was shown that IAA levels significantly increased in response to pathogen infection (O’Brien and Benková, 2013). Among six hormones evaluated, analysis of phytohormones transcriptome showed that auxin and ABA were the most important hormones induced under abiotic and biotic stresses (Ohme-Takagi and Shinshi, 1995).

![Figure 1. Results of clustering of expressed genes in microarray test. Microarray data was normalized based on Log 2. Up-regulated genes are represented by red color, genes showing decreased expressions are indicated by blue color, and no significant change in gene expression is shown by white colour. The colour scale representing log signal values is shown at the right of the heatmap.](image-url)
Characterization of cis-elements in *O. sativa*

Table 1. Up and down-regulated genes in response to different hormonal stress.

<table>
<thead>
<tr>
<th></th>
<th>IAA</th>
<th>ABA</th>
<th>BAP</th>
<th>SA</th>
<th>JA</th>
<th>ACC</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up-regulated</td>
<td>171</td>
<td>50</td>
<td>39</td>
<td>60</td>
<td>38</td>
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<td>388</td>
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<tr>
<td>Down-regulated</td>
<td>0</td>
<td>98</td>
<td>100</td>
<td>86</td>
<td>123</td>
<td>136</td>
<td>543</td>
</tr>
</tbody>
</table>

Detection of responsive gene-network under hormonal stresses. Figure 2 shows gene network analysis of genes involved in response to different hormonal stresses and their relationships. The responsive genes to hormonal stress include CYPs (*CYP450, CYP72A* and *CYP734A*), GSTs, the gene family of auxins (*OsIAA*), and transcription factors of auxin (ARFs). CYPs are vital for signaling molecules and environmental responses (Sato *et al.*, 2002). Cytochrome P450 genes are engaged in biosynthesis of brassinosteroids and their metabolism have been shown to be up-regulated by auxin (Fujioka and Yokota, 2003). *CYP72A1* gene is involved in brassinosteroids inactivation and regulation of BRs homeostasis (Fujioka and Yokota, 2003). Findings revealed that cytochrome P450 is the main component in the synthesis and degradation of external steroid hormones as a monoxygenase (Chapple, 1998). Recently, ARF genes have extensively been investigated using molecular and bioinformatics analysis (Spartz *et al.*, 2014). ARF19 gene acts redundantly in reining leaf expansion and lateral root growth (Wang *et al.*, 2007). *OsLAA1* gene family is induced in response to different hormonal stresses. Many auxin-responsive genes are responsive to at least two or more hormones. Similar findings indicated that *OsLAA1* genes play a key role in particular growth stages or are involved in specific stress or hormones (Hagen and Guilfoyle, 2002; Jain *et al.*, 2007; Greenham and McClung, 2015). Similar results revealed that the four *OsLAA1* genes were increased by >2.5-fold in response to auxin, suggesting that many of the *OsLAA1* genes are auxin activated (Greenham and McClung, 2015; Hagen and Guilfoyle, 2002). According to a previous study, *OsLAA1* genes were also expressed in response to six different hormonal treatments and other stresses (Hagen and Guilfoyle, 2002). Furthermore, several auxin-response gene families (*Aux/IAA* and *ARF*) showed differentially-expressed genes under various hormonal stress situations (Jain *et al.*, 2007). *IAA* genes code for short-lived proteins that are main components in cell growth (Song *et al.*, 2009). The glutathione S-transferase (GSTs) and cytochrome P450 genes exhibited, in most cases, increased expression in response to different environmental stresses (Fig. 2). *OsGSTU6* gene was expressed by different environmental stresses and *OsGSTU35* gene was induced by biotic stresses (Jain *et al.*, 2007).

Gene ontology enrichment analysis. Gene ontology at three levels of cellular components, molecular function, and biological processes is presented in Table 2. The nucleus, membrane-bound organelles and intracellular organelle were related to cellular components and that the molecular function was associated with catalytic activity, glutathione transferase activity and binding. Gene ontology of biological processes includes response to hormone, response to auxin and auxin-activated signaling pathway in rice (Table 2). Similar researches have shown that most of genes are involved in response to environmental stresses and are related to auxin signaling (Jain *et al.*, 2006).
### Table 2. Analysis of gene ontology of molecular function, biological processes and cellular components, respectively in response to different hormonal stresses in rice plant.

<table>
<thead>
<tr>
<th>GO ID</th>
<th>Ontology</th>
<th>Term</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0043226</td>
<td>Cellular_component</td>
<td>Organelle</td>
<td>0.025</td>
</tr>
<tr>
<td>GO:0043229</td>
<td>Cellular_component</td>
<td>Intracellular organelle</td>
<td>0.025</td>
</tr>
<tr>
<td>GO:0005634</td>
<td>Cellular_component</td>
<td>Nucleus</td>
<td>0.025</td>
</tr>
<tr>
<td>GO:0043227</td>
<td>Cellular_component</td>
<td>Membrane-bounded organelle</td>
<td>0.02</td>
</tr>
<tr>
<td>GO:0010181</td>
<td>Molecular_function</td>
<td>Binding</td>
<td>4.87e-05</td>
</tr>
<tr>
<td>GO:0003824</td>
<td>Molecular_function</td>
<td>Catalytic activity</td>
<td>7.50e-08</td>
</tr>
<tr>
<td>GO:0009725</td>
<td>Biological_process</td>
<td>Response to hormone</td>
<td>7.50e-08</td>
</tr>
<tr>
<td>GO:0009733</td>
<td>Biological_process</td>
<td>Response to auxin</td>
<td>1.54e-08</td>
</tr>
<tr>
<td>GO:0009734</td>
<td>Biological_process</td>
<td>Auxin-activated signaling pathway</td>
<td>1.25e-06</td>
</tr>
<tr>
<td>GO:0009755</td>
<td>Biological_process</td>
<td>Hormone-mediated signaling pathway</td>
<td>4.57e-06</td>
</tr>
<tr>
<td>GO:0032870</td>
<td>Biological_process</td>
<td>Cellular response to hormone stimulus</td>
<td>4.57e-06</td>
</tr>
<tr>
<td>GO:0071365</td>
<td>Biological_process</td>
<td>Cellular response to auxin stimulus</td>
<td>1.25e-06</td>
</tr>
</tbody>
</table>

**Up-regulated gene in response to different hormonal stress.** Analysis of microarray data showed that only *dehydrin* gene expression increased in response to the six hormones used. It has been reported that dehydrin expression may be corresponding with hormonal regulation under cold acclimation (Zhang *et al*., 2017). As a result, *dehydrin* gene may be produced in response to other stresses, such as salinity, cold and drought. A report showed that expression of dehydrins in seeds undergoing a maturation drying has to be considered as a direct response to drought stress. *OsDHN1* gene belongs to acidic dehydrin family (Kleinwächter *et al*., 2014). *Dehydrin* genes are the main component in membrane hardening and osmotic regulation. Moreover, dehydrin is the key element in drought tolerance of seeds (Hanana *et al*., 2014). Previous report has shown that *OsDHN1* gene is overexpressed in rice, hence confirmed its high tolerance to abiotic stresses (Kumar *et al*., 2014). In wheat, this gene is expressed in response to ABA (Koike *et al*., 1997). *Dehydrin* genes were up-regulated by many phytohormones, indicating the involvement in ABA dependent pathways (Allagulova *et al*., 2003).

**Cis-regulatory elements analysis.** Cis-regulatory elements detected in the 1000-bp upper region are presented in Figure 3. Cis-regulatory components in the promoter sequences provided a good understanding of plant responses under hormonal stress conditions. Gene promoter analysis revealed that TATA-box and CAAT-box were present in majority of promoter regions of genes. In the present study, many light-responsive components were revealed in the promoter sequence of all genes from rice, including ACE, Box-W1, CATT motif, G box, GC motif, Skn-1_motif, and TCT motif. The presence of circadian in many of studied genes causes plant to respond appropriately to cellular processes such as physiological processes, plant growth and reproduction. Additionally, cis-regulatory elements (G-box, Circardian, and TGACG-motif) expressed in response to hormonal stress. Other cis-elements (box E and Box-W1) are induced in response to pathogens such as fungi (Jain *et al*., 2007). Skn-1 motifs regulate specific expression pattern of endosperm and CAT-box, specifically expressed in the meristem region.

The G-box provides binding sites for specific bZIP proteins and this cis-element are regulated to environmental stresses such as UV light, ABA, red light and injuries (Garmiello *et al*., 2011; Saidi and Hajibarat, 2018). It has been reported that G-box motif related to photosynthesis, hormone (ethylene and ABA) metabolism, and stress
responses was common in grapevine and Arabidopsis (Ma et al., 2013).

Our results showed that various regulatory elements are involved in salicylic acid (W boxes) which are expressed in response to hormonal stresses. W-boxes are the main components of binding site for WRKY transcription factors and play a key role in activating transcription of auxins, salicylic acid, and light. This finding is also reported by other studies (Liu et al., 2016; Park et al., 2004).

P-box is responsive to gibberellin, TCA-element to salicylic acid, and TGACG-motif are involved in methyl jasmonate which agrees with the results obtained by other researchers as reported by Kaur et al. (2017). TGACG motif is involved in methyl jasmonate response affecting biological processes like response to abiotic and biotic stresses. MBS is activated in response to low temperature, drought and salinity stress (Jaspers et al., 2009).

**CONCLUSION**

Phytohormones are key elements in growth processes and development, as well as mediators in response to different environmental stresses. Our findings showed that the responsive genes were expressed in response to six hormones at seedling stage in rice, but the most detected genes up-regulated are in response to auxin hormone. The present study can provide a comprehensive understanding of hormonal cross-talk in plant development and stress responses. G-box was the most abundant among the cis-regulatory elements and was regulated in response to auxin and different stresses. This cis-element, a sequence-specific protein-binding site, has an interaction with transcription factors such as bZIP and dehydrin gene network. The interaction between genes and transcription factors lead to the formation of gene network. Overall, insight of relationships among these components can help researchers to engineer plants resistant to stress conditions.

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