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PHYTOCHEMICAL CONTENT IN Polyalthia bullata King AND THE EFFECT OF AUXINS, ELICITORS AND PRECURSORS ON TOTAL ALKALOID CONTENT IN CALLUS

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By

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Polyalthia bullata or locally known as Tongkat Ali Hitam is one of the species belongs to genus of *Polyalthia*. The plant has been reported to possess an ability to treat many diseases and enhance human health and life quality, which might be contributed by the presence of bioactive compounds. However, due to limited reports on phytochemical compounds present in *P. bullata*, the phytochemical profiling can help in clarifying the types of phytocompounds, therefore, choosing the right extraction solvent is important in order to get optimum yield. One of the factors that might affect the extraction yield is the polarity of extraction solvent. Apart from that, overcollection of *P. bullata* from wild habitat has become serious problem that may lead to species extinction. The establishment of *P. bullata* callus culture and addition of elicitors and precursors can help in enhancing the production of phytochemical compounds, therefore reducing the extinction of *P. bullata* from native habitat. Hence, the aims of this study were to determine the total alkaloid content (TAC), total phenolic content (TPC), total flavonoid content (TFC), and total terpenoid content (TTC) as well as antioxidant activity of hexanic, ethanolic, methanolic, and distilled water extracts of *P. bullata* root, leaf and stem, to profile biochemical compounds using gas chromatography- mass spectrometry (GC-MS), to induce callus from *P. bullata* using different explants, basal media, and plant growth regulators (PGRs), and to determine the effectiveness of auxins, precursors, and elicitors in enhancing alkaloid production in callus. For callus induction, the sterilized leaf and midrib explants were used and cultured on Murashige and Skoog (MS) and Woody Plant Medium (WPM) basal media supplemented with B5 vitamin, and different concentrations and types of auxins (2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthaleneacetic acid (NAA), picloram and dicamba). The MS and WPM basal media supplemented with different types and concentrations (10, 20, 30, 40 50 µM) of auxins (2,4-D, NAA, picloram, dicamba, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA)) were used to determine the best multiplication medium and alkaloid content after six weeks of culture. The elicitors (methyl jasmonate (MeJA), salicylic acid (SA), and chitosan) and precursors (L-phenylalanine, L-tyrosine, and L-tryptophan) at concentration of 50, 100 and 150 µM was respectively added into the best alkaloid production medium to enhance the alkaloid production. The results from the studies revealed that the methanolic extract of *P. bullata* leaf exhibited the highest TPC, TFC, TTC and total antioxidant activity at 1042.52 ± 1.97 mg GAE/g DW, 80.88 ± 0.24 mg QE/g DW, 0.19 ± 0.00 mg LE/g DW and 85.19 ± 1.16%, respectively. Meanwhile, the methanolic extract of *P. bullata* stem showed the highest TAC at 7.71 ± 0.00 mg AE/g DW. The fatty acids, phenolics, and carboxylic acid were found in methanolic stem extract; carbohydrates, alkaloids, and fatty acids were found in methanolic root extract; and terpenoids, phenolics, and alcohol were found in methanolic leaf extract of *P. bullata* using GC-MS. Among the media tested for *in vitro* callus induction, WPM basal medium supplemented with 16.56 µM picloram exhibited the highest callus induction percentage with 53.33 ± 22.06%. As for the callus multiplication, the callus cultured on MS + 30 µM dicamba was found to significantly produce the highest fresh weight (1180.00 ± 159.43 mg FW) and dry weight (58.00 ± 6.66 mg DW) of callus after three weeks of culture. The addition of auxins into culture medium managed to enhance the alkaloid production in callus with the highest alkaloid content was observed in callus cultured on MS medium supplemented with 30 µM 2,4-D (31.07 ± 0.05 µg/g DW). Among auxins, elicitors, and precursors tested, the MS + 30 µM 2,4-D and MS + 30 µM 2,4-D + 50 µM chitosan were found to be the best media for alkaloid production with the amount of 31.07 ± 0.05 and 31.30 ± 0.23 µg/mg DW after six weeks of culture, respectively. As a conclusion, methanol was found to be the best extraction solvent to extract phytochemical compounds from *P. bullata*. The incorporation of auxins like 2,4-D into the culture medium is the best strategy to enhance alkaloid production in *P. bullata* callus. Therefore, the data obtained from this study can be used to further investigate biological activities of phytochemical compounds present in *P. bullata*, and therefore can reduce overcollection of this plant from the forest.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Ijazah Master Sains

KANDUNGAN FITOKIMIA DALAM *Polyalthia bullata* King DAN KESAN AUKSIN, PENGELISIT DAN PREKURSOR TERHADAP JUMLAH KANDUNGAN ALKALOID DALAM KALUS

Oleh

MUNIRAH ADIBAH BINTI KAMARUL ZAMAN

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Polyalthia bullata atau dikenali sebagai Tongkat Ali Hitam adalah salah satu spesies tergolong dalam genus Polyalthia. Tumbuhan ini dilaporkan mempunyai keupayaan untuk merawat pelbagai penyakit dan meningkatkan kesihatan dan kualiti hidup manusia, di mana ia mungkin disumbangkan oleh kehadiran sebatian bioaktif. Walau bagaimanapun, disebabkan oleh laporan yang terhad mengenai sebatian fitokimia yang terdapat dalam *P. bullata*, pemprofilan fitokimia dapat membantu dalam menjelaskan jenis-jenis fitosebatian, oleh itu, pemilihan pelarut pengekstrakan yang tepat adalah penting untuk mendapatkan hasil yang optimum. Salah satu faktor yang boleh menjejaskan hasil pengekstrakan ialah polariti pelarut pengekstrakan. Selain itu, pengambilan berlebihan *P. bullata* dari habitat liar telah menjadi masalah serius di mana boleh mengakibatkan kepupusan spesies. Penubuhan kultur kalus *P. bullata* dan penambahan pengelisit dan prekursor boleh membantu dalam meningkatkan pengeluaran sebatian fitokimia, seterusnya mengurangkan kepupusan *P. bullata* dari habitat asal. Oleh itu, matlamat kajian ini adalah untuk menentukan jumlah kandungan alkaloid (TAC), jumlah kandungan fenolik (TPC), jumlah kandungan flavonoid (TFC), dan jumlah kandungan terpenoid (TTC) serta aktiviti antioksidan ekstrak heksana, etanol, metanol, dan air suling dalam akar, daun, dan batang *P. bullata,* untuk memprofil sebatian biokimia menggunakan kromatografi gas-spektroskopi jisim (GC-MS), untuk menghasilkan kalus dari *P. bullata* menggunakan eksplan, media asas, dan pengawal atur pertumbuhan (PGRs) yang berbeza, dan untuk menentukan keberkesanan auksin, precursor, dan pengelisit dalam meningkatkan penghasilan alkaloid dalam kalus. Bagi induksi kalus, eksplan daun dan tulang daun yang telah disterilkan digunakan dan dikultur di atas media asas Murashige dan Skoog (MS) dan Woody Plant Medium (WPM) yang ditambah dengan vitamin B5, dan kepekatan dan jenis auksin yang berbeza (asid 2,4-diklorofenoksiasetik (2,4- D), asid α-naftalenaasetik (NAA), pikloram dan dikamba). Media asas MS dan WPM ditambah dengan jenis dan kepekatan (10, 20, 30, 40 50 μM) auksin yang berbeza (2,4-D, NAA, pikloram, dikamba, asid indol-3-asetik (IAA), asid indol-3-butirik (IBA)) telah digunakan untuk menentukan media

penggandaan kalus dan kandungan alkaloid yang terbaik selepas enam minggu kultur. Pengelisit (metil jasmonat (MeJA), asid salisilik (SA), dan kitosan) dan prekursor (L-fenilalanina, L- tirosina, dan L-triptofan) masing-masing pada kepekatan 50, 100, 150 µM ditambah ke dalam media penghasilan alkaloid yang terbaik untuk meningkatkan pengeluaran alkaloid. Keputusan dari kajian ini menunjukkan bahawa ekstrak metanol daun *P. bullata* menunjukkan jumlah TPC, TFC, TTC dan jumlah aktiviti antioksidan tertinggi masing-masing pada 1042.52 ± 1.97 mg GAE / g DW, 80.88 ± 0.24 mg QE / g DW, 0.19 ± 0.00 mg LE / g DW dan 85.19 ± 1.16%. Sementara itu, ekstrak metanol batang *P. bullata* menunjukkan TAC tertinggi pada 7.71 ± 0.00 mg AE / g DW. Asid lemak, fenolik dan asid karboksilit telah dijumpai dalam ekstrak metanol batang; karbohidrat, alkaloid dan asid lemak telah dijumpai dalam ekstrak metanol akar; dan terpenoid, fenolik dan alkohol dijumpai dalam ekstrak metanol daun *P. bullata* menggunakan GC-MS. Antara media yang diuji untuk induksi kalus *in vitro*, media asas WPM yang ditambah dengan 16.56 µM pikloram menunjukkan peratusan induksi kalus tertinggi dengan 53.33 ± 22.06%. Manakala bagi penggandaan kalus, kalus yang dikultur di atas media MS + 30 µM dikamba didapati dengan ketara menghasilkan kalus dengan berat segar (1180.00 ± 159.43 mg FW) dan berat kering (58.00 ± 6.66 mg DW) tertinggi pada minggu ketiga. Penambahan auksin dalam medium kultur berjaya meningkatkan penghasilan alkaloid dalam kalus dengan kandungan alkaloid tertinggi dihasilkan dalam media MS ditambah dengan 30 µM 2,4-D (31.07 ± 0.05 µg /g DW). Antara auksin, pengelisit, dan prekursor yang diuji, MS + 30 µM 2,4-D dan MS + 30 µM 2,4-D + 50 µM kitosan merupakan media yang terbaik unuk penghasilan alkaloid masing-masing dengan jumlah 31.07 ± 0.05 and 31.30 ± 0.23 µg/mg DW selepas enam minggu kultur. Kesimpulannya, metanol telah didapati menjadi pelarut pengekstrakan yang terbaik untuk mengekstrak sebatian fitokimia dalam *P. bullata.* Penggabungan auksin seperti 2,4-D ke dalam medium kultur adalah strategi terbaik untuk menningkatkan penghasilan alkaloid dalam kalus *P. bullata*. Oleh itu, data yang diperolehi daripada kajian ini boleh digunakan untuk mengetahui dengan lebih lanjut aktiviti biologi sebatian biokimia yang terdapat dalam *P. bullata* dan dengan itu dapat mengurangkan pengambilan tumbuhan ini dari hutan.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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2,4-D	2,4-dichlorophenoxyacetic acid
AE	Atropine equivalent
$Bi(NO_3)_3.5H_2O$	Bismuth nitrate pentahydrate
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DR	Dragendorff's reagent
DW	Dry weight
FW	Fresh weight
GAE	Gallic acid equivalent
GC-MS	Gas chromatography – mass spectrometry
HCl	Hydrochloric acid
HNO_3	Nitric acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
LE	Linalool equivalent
MeJA	Methyl jasmonate
MS	Murashige and Skoog
Na_2S	Disodium sulfide
NAA	α-Naphthaleneacetic acid
NaOH	Sodium hydroxide
PGRs	Plant growth regulators
QE	Quercetin equivalent
rpm	Revolutions per minute
SA	Salicylic acid
SE	Standard error
TAC	Total alkaloid content
TFC	Total flavonoid content
TPC	Total phenolic content
TTC	Total terpenoid content
UV-VIS	Ultraviolet - Visible
WPM	Woody plant medium

CHAPTER 1

INTRODUCTION

1.1 Introduction

Plant bioactive compounds can be defined as compounds that have biological activities and give positive and negative effects towards humans and animals (Šaponjac *et al.,* 2016). These metabolites are produced when plants responding to biotic and abiotic stresses, and therefore, they are involved in the plant adaptation process (Ncube and Van, 2015; Ahmed *et al.,* 2017). Plant bioactive compounds can be classified into three major categories which are terpenes, alkaloids, and phenolics (Azmir *et al.,* 2013). Many studies have reported that the plant-derived antioxidant compounds are mainly from phenolic group like flavonoids, polyphenols and tannins (Cartea *et al.,* 2010; Nagavani *et al.,* 2010). They have capacity to combat the free radicals with minimal side effects on human (Patel *et al.,* 2012). According to Gangwar *et al.* (2014), the consumption of natural antioxidant can reduce the risk of cancer and many chronic diseases. Commonly, the solvent extraction is a method used for separation and isolation of plant bioactive compounds (Barchan *et al.,* 2014). The use of suitable solvent extraction is a key factor to extract maximum yield of the phytochemical and antioxidant compounds because the compounds have different characteristics and polarities that make them soluble in the solvent used (Fatiha *et al.,* 2012; Pham *et al.,* 2015).

Polyalthia bullata or locally known as Tongkat Ali Hitam is a medicinal plant belongs to genus *Polyalthia* and Annonaceae family. The plant is a shrub that can grow up to two- to three-meter height mainly in lowland of primary or secondary forest located in Peninsular Malaysia and Sabah. The genus *Polyalthia* has been reported to contain numerous types of bioactive compounds that are mostly derived from alkaloids, flavonoids, acetogenin and triterpenoids groups (Paarakh and Khosa, 2009). The *P. bullata* flower, root, and leaf extracts are reported to have capabilities in treating high blood pressure, diabetes and liver diseases, while its root is involved in boosting men sexual desires (Virmala, 2013). These factors, therefore, lead to the exploitation of *P. bullata* plant from the wild habitat, which may become a reason of species extinction in the near future. Apart from that, the accumulation of the plant secondary metabolites is naturally time consuming, and most of the bioactive compounds in *P. bullata* are remain unknown.

Among the bioactive compounds, alkaloids play a crucial role in pharmaceutical industries as important drugs for controlling numerous diseases (Dias *et al.,* 2012; Perviz *et al.,* 2016). These compounds have been used as a source of remedies to treat diverse range of human diseases (Amirkia and Heinrich, 2014). However, the alkaloid content in the intact plant is low, and the accumulation of this compound is influenced by environmental factors (Bienaimé *et al.,* 2015; Gupta *et al.,* 2015). Thus, the *in vitro* technique can be utilized to mass produce bioactive compounds without harvesting the entire plant (Efferth, 2019).

Plant tissue culture technique has been widely used due to its ability to produce disease free plant and pharmaceutically important secondary metabolites such as phenolics, alkaloids, and terpenoids (Adhikari and Pant, 2013; Upadhyay and Koche, 2015; Shahzad *et al.,* 2017). Callus culture is one of the techniques that can be used to facilitate the mass production of these compounds. The presence of auxins is significant in callus formation and mass production of bioactive compounds (Ahmad *et al.,* 2013; Ikeuchi *et al.,* 2013). The application of exogenous auxins may affect the biosynthesis of bioactive compounds including alkaloids in callus culture (Raj *et al.,* 2015).

The yield of bioactive compounds especially alkaloid in callus culture can be improved by adding the elicitors and precursors into the callus culture media. Elicitors are the chemical compounds that can promote stress responses in plants and lead to the mass production of secondary metabolites (Naik and Al-Khayri, 2016). As for precursor feeding, the addition of precursors that is involved in the metabolic biosynthetic pathway has stimulates the production of bioactive compounds (Zuldin *et al.,* 2013).

1.2 Hypothesis

The hyphoteses of this study were:

1) Polar extraction solvent is the best solvent to extract high amount of bioactive compounds from *P. bullata* leaf, stem, and root

2) The different concentrations of auxins applied in callus induction media can induce the formation of *P. bullata* callus grown under dark condition

3) Application of different types of auxins, elicitors, and precursors can enhance the production of total alkaloids in callus

1.3 Objectives

The objectives of this study are:

1) To determine the efficiency of different extraction solvents in extracting phytochemical content and their antioxidative capacity in leaf, stem, and root of *P. bullata*

2) To profile phytochemical compounds in leaf, stem, and root of *P. bullata* using GC-MS

3) To induce callus from *P. bullata* using different explants, culture media, and auxins

4) To determine the effectiveness of auxins, elicitors, and precursors in enhancing alkaloid production in callus of *P. bullata*

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