EVALUATION OF *Justicia gendarussa* CRUDE LEAF EXTRACT FOR ENHANCEMENT OF FLAVONOIDS PRODUCTION VIA ADVENTITIOUS ROOT CULTURE AND GENETIC MODIFICATIONS

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Special dedication to

My loving parents

Ayob Mat

Fatimah Sulieman

My beloved sisters

Nurhidayah Ayob

Siti Nuradha Ayob

My sweet nieces

Wan Nur Hannah

Wan Nur Hanis

My future soulmate

MJ

For all your love, prayers, support and sacrifice.

Thank you so much

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ABSTRACT

Justicia gendarussa extract possesses various bioactivities associated with the availability of flavonoids. Low availability of flavonoids could limit or even hinder the bioactivities effects. Therefore, attempts to enhance the flavonoids production via tissue culture approaches are being studied. This study aimed to optimize flavonoids contents in *J. gendarussa* using different tissue culture systems (*in vitro* plant regeneration and adventitious root culture) and genetic transformation methods. The cytotoxicity of plant extracts against various cancer cell lines was also evaluated. Detection and quantification of naringenin and kaempferol were performed using GC-FID. Cytotoxicity tests of crude extract against cancer cell lines (MCF-7, MDA-MB-231, MDA-MB-468, HT-29, HeLa and BxPC-3) were determined by MTT assay. The optimization of elicitors used including yeast extracts (YE), casein hydrolysate (CH) and proline (P) at various concentrations (0, 0.2, 0.4, 0.6, 0.8 or 1.0 mg/L) were examined using nodal explants from *in vitro* plants. Adventitious roots were inoculated into MS liquid medium supplemented with IBA (2.0-4.0 mg/L). For genetic transformation studies, plasmids pCAMBIA 1305.2, which harbour the *PKS* gene and plant selectable marker, *HPT* for hygromycin resistance was used to transform nodal explants of *J. gendarussa* under the optimized transformation protocol using biolistic and *Agrobacterium tumefaciens*-mediated transformation. Results showed that mature leaves extract, JG1 had the highest naringenin (444.35 ± 81.43 mg/kg) and kaempferol (1591.80 ± 94.91 mg/kg), while the cytotoxicity against BxPC-3 cell was the strongest (IC_{50}~16 µg/mL). The highest naringenin and kaempferol contents were obtained in leaf crude extracts when treated with 0.6 g/L of CH (1180.30 ± 50.23 mg/kg) and 0.6 g/L of P (385.01 ± 13.10 mg/kg), respectively. Adventitious root culture produced high naringenin (97.54 ± 5.47 mg/kg) and kaempferol (853.82 ± 56.52 mg/kg) when treated with 2.0 mg/L IBA. The optimal parameters for biolistic method were established at 1100 psi helium pressure and 12 cm target distance with 95% of transformation efficiency. Meanwhile, the optimal transformation condition of *A. tumefaciens* method was bacterial concentration at OD_{600}nm ~ 0.8, 20 minutes of inoculation time, 500 µM AS and 1 cm explant size with 90% transformation efficiency. Even though *A. tumefaciens* method produced lower percentage of transient GUS expression than biolistic method, a few transformed explants were successfully produced. The integration of the *PKS* gene with band size of 1200 bp into the genome of transgenic plants were verified by PCR, sequencing and subsequently confirmed by Southern blot analysis. The content of kaempferol were found to be higher in stem extracts of transgenic plants (450.40 ± 7.82 mg/kg) than non-transgenic plants (197.13 ± 2.29 mg/kg). In conclusion, addition of elicitors, establishment of adventitious root culture and *A. tumefaciens*-mediated transformation could enhance flavonoid contents in *J. gendarussa*.

ABSTRAK

Ekstrak *Justicia gendarussa* mempunyai pelbagai bioaktiviti berkaitan dengan ketersediaan flavonoid. Ketersediaan kurang flavonoid boleh menghadkan atau menghalang kesan bioaktiviti. Oleh itu, percubaan untuk meningkatkan kandungan flavonoid melalui teknik kultur tisu sedang dikaji. Kajian ini bertujuan untuk mengotimumkan kandungan flavonoid di dalam *J. gendarussa* menggunakan sistem kultur tisu yang berbeza (pertumbuhan pokok *in vitro* dan kultur akar adventitus) dan kaedah transformasi genetik. Kesitotoksikan ekstrak pokok terhadap pelbagai titisan sel kanser juga dinilai. Pengesanan dan pengkuantitian naringenin dan kempferol telah dijalankan menggunakan GC-FID. Ujian kesitotoksikan ekstrak daun mentah *J. gendarussa* terhadap titisan sel kanser (MCF-7, MDA-MB-231, MDA-MB-468, HT-29, HeLa dan BxPC-3) telah ditentukan oleh asai MTT. Pengoptimuman elisitor iaitu ekstrak yis (YE), kasein hidrolisat (CH) dan prolin (P) pada pelbagai kepekatan (0, 0.2, 0.4, 0.6, 0.8 or 1.0 mg/L) telah diperiksa menggunakan eksplan nodal dari pokok *in vitro*. Akar adventitus telah diinokulasi di dalam cecair media MS yang ditambah dengan IBA (2.0-4.0 mg/L). Bagi kajian transformasi genetik, plasmid pCAMBIA 1305.2 yang mempunyai gen *PKS* and gen penanda pemilihan pokok, *HPT* untuk rintangan higromisin telah digunakan untuk transformasi eksplan nodal *J. gendarussa* di bawah kaedah transformasi menggunakan biolistik dan transformasi berperantarakan *Agrobacterium tumefaciens*. Hasil kajian daun matang ekstrak, JG1 menunjukkan naringenin tertinggi (444.35 ± 81.43 mg/kg) dan kempferol (1591.80 ± 94.91 mg/kg), manakala kesitotoksikan menentang sel BxPC-3 yang terkuat (IC_{50}~16 µg/mL). Kandungan tertinggi naringenin dan kempferol diperolehi di dalam ekstrak daun apabila dirawat dengan CH 0.6 g/L (1180.30 ± 50.23 mg/kg) dan P pada 0.6 g/L (385.01 ± 13.10 mg/kg). Kultur akar adventitus menghasilkan tinggi naringenin (97.54 ± 5.47 mg/kg) dan kempferol (853.82 ± 56.52 mg/kg) apabila dirawat dengan 2 mg/L IBA. Parameter optimum bagi kaedah biolistik adalah tekanan helium 1100 psi dan 12 cm jarak sasaran dengan keberkesanan tranformasi sebanyak 95%. Manakala, parameter optimum bagi kaedah *A. tumefaciens* adalah apabila dirawat dengan kepekatan bakteria pada OD_{600}nm~0.8, 20 minit masa inokulasi, ditambah dengan 500 µM kepekatan AS dan saiz eksplan iaitu 1 cm dengan keberkesanan transformasi sebanyak 90%. Walaupun kaedah *A. tumefaciens* menghasilkan peratusan gen GUS transien lebih rendah berbanding kaedah biolistik, beberapa eksplan tertransformasi telah berjaya dihasilkan. Kehadiran dan integrasi gen *PKS* dapat dikenalpasti dengan pengesanan saiz jalur 1200 bp di dalam genom pokok transgenik berdasarkan PCR, penjujukan dan seterusnya disahkan oleh analisis pemblotan Southern. Kandungan kempferol didapati lebih tinggi di dalam ekstrak batang pokok transgenik (450.40 ± 7.82 mg/kg) berbanding pokok tanpa tertransformasi (197.13 ± 2.29 mg/kg). Kesimpulannya, penambahan elisitor, penghasilan kultur akar adventitus dan transformasi berperantarakan *A. tumefaciens* boleh meningkatkan kandungan flavonoid di dalam *J. gendarussa*.

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LIST OF SYMBOLS

rpm	-	Rotation per minute
v	-	Volume
v/v	-	Volume per volume
w/v	-	Weight per volume
µg/mL		Microgram per milliliter

LIST OF ABBREVIATIONS

JG	-	*Justicia gendarussa*
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PKS	-	Polyketide synthase
CHS	-	Chalcone synthase
GUS	-	β-glucuronidase
DNA	-	Deoxyribonucleic acid
RNA	-	Ribonucleic acid
PCR	-	Polymerase Chain Reaction
PBS	-	Phosphate buffer saline
FBS	-	Fetal bovine serum
DMSO	-	Dimethyl sulfoxide
LB	-	Luria Bertani
IBA	-	Indole-3-butyric acid
vir	-	virulence
GC-FID	-	Gas Chromatography-Flame Ionization Detector
MS	-	Murashige and Skoog
X-gluc	-	5-bromo-4-chloro-3-indolyl *β*-D-glucuronic acid
HCl	-	Hydrochloric acid
Na_2CO_3	-	Sodium carbonate
$NANO_2$	-	Sodium nitrile
$AlCl_3$	-	Aluminium chloride
NaOH	-	Sodium hydroxide
CTAB	-	Cetyltrimethyl ammonium bromide
PVP	-	Polyvinyl pyrrolidone

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CHAPTER 1

INTRODUCTION

1.1 Background of the problem

The updated strategy of World Health Organisation (WHO) from 2014 to 2023 devotes more attention than its predecessor to prioritizing health services and systems, including traditional and complementary medicine products, practices and practitioners (WHO, 2013). Medicinal plants have been used as traditional treatments for numerous human diseases for thousand years. In rural areas of the developing countries, they continue to be used as the primary source of medicine since western pharmaceuticals are often expensive or inaccessible (Ekor, 2014). The natural products derived from medicinal plants have proven to be an abundant source of biologically active compounds, many of which have been the basis for the development of new leading chemicals for pharmaceuticals (Enzo, 2011).

Numerous researchers have shown high interest in plant secondary metabolites particularly flavonoids which have possessed diverse bioactivities and contributed valuable prospects to the pharmaceutical industries. Flavonoids exhibit various biological effects including lowering plasma levels of low-density lipoproteins, inhibiting platelet aggregation, promoting scavenging free radicals and reducing cell proliferation (Woodman and Chan, 2004). Therefore, in this study, flavonoids such as naringenin (flavanone) and kaempferol (flavonol) were given greatattention due to their potential beneficial effects on the human health. Naringenin was reported as strong antioxidant and showed cytotoxicity against human breast cancer cell line, MCF-7 (Cavia-Saiz *et al*., 2010; Park *et al*., 2010).

Previous studies reported kaempferol as strong antioxidant, able to prevent arteriosclerosis, inhibits cell proliferation and induces apoptosis in pancreatic cancer cells (Tu *et al*., 2007; Zhang *et al*., 2008).

Justicia gendarussa, which is also known by its common name Gendarussa has been investigated as potential medicinal plants in this study. These plants have distributed in many countries such as India, Indonesia, Malaysia and Sri Lanka. The root and leaf extracts of *J. gendarussa* have been used traditionally to treat many ailments such as chronic rheumatism, inflammations, bronchitis, headache, arthritis, vaginal discharges, dyspepsia, eye disease and fever (Janarthanam and Sumanthi, 2010). Leaf and stem extracts of *J. gendarussa* were reported to possess anti-inflammatory, antioxidant, antibacterial, antifungal, antiangiogenis, antiplatelet, antiarthritic, anthelmintic and hepatoprotective activities (Navarro *et al*., 2001; Paval *et al*., 2009; Krishna *et al*., 2010; Saha *et al*., 2012). Phytochemical studies on leaf extracts of *J. gendarussa* revealed the presence of flavonoids, alkaloids, triterpenoid saponins, amino acids, aromatic amines and sterols (Chakravarty *et al*., 1982; Ratnasooriya *et al*., 2007; Bambang Prajogo *et al*., 2009; Mustafa *et al*., 2010; Uddin *et al*., 2011; Kiren *et al*., 2014).

According to statistics in Malaysia, the incidence of breast and cervical cancers are common among female patients while colon and pancreas cancers are prevalence among men patients (Bachok *et al*., 2012; Farooqui *et al*., 2013). Unfortunately, cancer is a public health problem in all over the world affecting all categories of persons (Iweala *et al*., 2015). Despite the advancement in cancer therapies such as surgery, radiotherapy, hyperthermia, hormone therapy and chemotherapy, these therapies are ineffective in destroying cancer cells and may cause damage to the healthy cells. Examples of the adverse side effects of cancer treatments include mouth sore, tiredness, hair loss, nausea and vomiting (Jones *et al*., 2004). For many years herbal medicines have been used and are still used in developing countries as the primary source of medical treatment. Thus, research has developed into investigating the potential properties and uses of terrestrial plants extracts for the preparation of potential nanomaterial based drugs for diseases including cancer (Zakaria *et al*., 2011b). Many plant species are already being used

to treat or prevent development of cancer. Multiple researchers have identified species of plants that have demonstrated anticancer properties with a lot of focus on those that have been used in herbal medicine in developing countries (Greenwell and Rahman, 2015). In this regard, this is the first study to evaluate the cytotoxic activities in *Justicia gendarussa* crude leaf extracts against breast (MCF-7, MDA-MB-231 and MDA-MB-468), colon (HT-29), cervix (HeLa) and pancreas (BxPC-3) cancer cell lines have been investigated. In order to identify the plants with potential bioactive compounds against cancer cell lines, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay will be performed by using the crude leaf extracts and flavonoids (naringenin and kaempferol) which would give a plausible contribution in cytotoxicity of secondary metabolite towards cancer treatment.

It is well-known that flavonoids possessed remarkable strong anticancer and antioxidant activities (Susanti *et al*., 2007). The drawbacks of the flavonoids extraction from field-grown plants are low yields and fluctuation in flavonoids concentration due to geographical, seasonal and environmental variations. In addition, the long cultivation period resulting in high-cost commercial drug production (Murthy *et al*., 2014). Current advances in plant biotechnology offer manipulation of bioactive compounds via tissue culture approaches (Baque *et al*., 2012). Many strategies for enhancement of plant metabolites such as selection of cell clones, optimization of medium and culture environments, elicitation, nutrient and precursor feeding and biotransformation can be applied. Hence, in this study, alternative methods to enhance the production of flavonoids from *J. gendarussa* were performed through elicitation on *in vitro* plant, adventitious root cultures and genetic transformation methods.

In recent years, many studies on the production of biomass and secondary metabolites through elicitation on *in vitro* plant and adventitious root culture system have been conducted. Elicitors are compounds from various biotic or abiotic sources which may enhance the secondary metabolite in plant cells by triggering the signal in secondary metabolites production (Rao and Ravishankar, 2002). Elicitation can be used as one of the important strategies to increase secondary metabolites production and reduce production cost (Siddiqui *et al*., 2013). In addition, plant growth

regulators are also one of the most important factors in affecting cell growth, differentiation and metabolites formation (Baque *et al*., 2012). Previous studies reported that adventitious root tissues are efficient in biomass production because of fast growth rates and stable secondary metabolite productivity (Choi *et al*., 2000; Kim *et al*., 2004a; Wu *et al*., 2006). Hence, in this study, high flavonoids production and biomass can be achieved by optimizing the effects of elicitors and plant growth regulators of *in vitro* plant and adventitious root cultures conditions.

Modification or enhancement of flavonoids production in *Justicia gendarussa* plant through genetic transformation would be a powerful tool in flavonoid biosynthetic pathway. It is important to develop a genetic transformation system by optimizing the transformation efficiency parameters followed by introduction of a polyketide synthase (PKS) gene i.e chalcone synthase (CHS), which is the precursor in the flavonoid biosynthesis pathway (Jamalnasir *et al*., 2014). There are several methods available for genetic plant transformation such as *Agrobacterium tumefaciens*-mediated transformation, biolistic and electroporation (Yong *et al*., 2006). In this study, the transformation methods using biolistic and *A. tumefaciens* - mediated transformation had been optimized using *β*-glucuronidase (GUS) as a reporter gene. Enhancement of the flavonoids production in *J. gendarussa* plant is a highly valuable protocol and thereby this is an ideal platform to improve the genetic transformation system in the medicinal plant.

1.2 Problem Statements

Flavonoids such as kaempferol and naringenin have been reported to have antioxidant, anticancer, antibacterial, antiviral and anti-inflammatory properties (Kumar and Pandey, 2013). These flavonoids also act as plant protective agents against various biotic and abiotic stress and also beneficial in preventing degenerative diseases on human (Kasote *et al*., 2015). To the best of our knowledge, there is no report on the detection and quantification of naringenin and kaempferol in

young and mature leaves of *Justicia gendarussa* especially from different localities in Johor.

Until now, no study has yet been carried out to determine the cytotoxicity effects of *Justicia gendarussa* leaf extracts against human cancer cell lines. This study was intended to demonstrate the anticancer potential of local *J. gendarussa* as an alternative anticancer agent.

Nowadays, medicinal plant studies have gained considerable attention internationally especially from pharmacology industry because of the extensive research on phytochemical and biological activities. However, the quality of the bioactive compounds derived from field-grown medicinal plants may be affected by environmental factors, physiological and developmental stages of the plant. Field cultivation requires a long growth period and plant management, which is a slow, time-consuming and laborious process (Wang *et al*., 2015). Therefore, in this study, plant cell culture approach is an alternative for the enhancement of the biomass and secondary metabolites production particularly flavonoids. Studies on the effect of elicitor on *in vitro* plant cultures, plant growth regulators of adventitious root cultures and genetic transformation for the enhancement of flavonoids production were not yet explored. In addition, manipulating the medium compositions supplemented with different concentrations of elicitors and plant growth regulators for *in vitro* plant and adventitious root cultures with the introduction of polyketide synthase (PKS) gene into *J. gendarussa* via genetic transformation were attempted in order to enhance the flavonoids production.

1.3 Aim and Objectives of the Study

The aim of the study is to increase the flavonoids (i.e naringenin and kaempferol) contents of *Justicia gendarussa* using tissue culture approaches via adventitious roots cultures and genetic modifications. The study objectives include:

1. To determine flavonoids contents in young and mature leaves of *J. gendarussa* plant.
2. To evaluate the cytotoxicity of *J. gendarussa* crude leaf extract against various cancer cell lines.
3. To assess the effects of different concentrations of elicitor and plant growth regulator on flavonoids contents of *in vitro* plant and adventitious roots cultures of *J. gendarussa.*
4. To determine the effects of transformation parameters on the biolistic transformation efficiency of *J. gendarussa* nodal explants
5. To determine the effects of transformation parameters on the *A. tumefaciens*–mediated transformation efficiency of *J. gendarussa* nodal explants

1.4 Scope of the Study

This research was focused on quantification of flavonoids, namely naringenin and kaempferol in young and mature leaf extracts of *Justicia gendarussa* from different locations in Johor by GC-FID method. The mature leaves that produced high flavonoids content were subjected to cytotoxic MTT assay against various cancer cell lines, MCF-7, MDA-MB-231, MDA-MB-468, BxPC-3, HeLa and HT-29. Plants that produced high flavonoids content and strong cytotoxic activity were selected for further applications of tissue culture approach. In order to enhance the flavonoids content in *J. gendarussa* plants, *in vitro* plant culture, adventitious root culture and genetic plant transformation were applied. Firstly, *in vitro* plants were supplemented with different concentrations of elicitors, while adventitious root cultures were induced and optimized using different concentrations of plant growth regulator in shake flask system. Determination of flavonoids content on *in vitro* plant and adventitious roots cultures were done using the GC-FID method. Secondly, optimization of biolistic and *A. tumefaciens*-mediated transformation parameters for *J. gendarussa* plant by GUS histochemical assay were conducted. The presence of HPT, GUS and PKS genes in transgenic plants were verified by PCR and confirmed using Southern blot analysis. Lastly, the comparison of

flavonoids content in transgenic plants and wild-type plants were determined using the GC-FID method.

1.5 Significance of the Study

This study was conducted to determine flavonoids content in *Justicia gendarussa* plant which contributes to cytotoxic effect against cancer cell lines. High cytotoxic effect of *J. gendarussa* extracts could be served as a good candidate for the development of new anticancer agents. Besides that, the application of *in vitro* plant culture, adventitious roots culture and genetic plant transformation could be applied to enhance flavonoids content in *J. gendarussa*. The establishment of adventitious roots culture system and flavonoids biomass production could enhance the flavonoids content from adventitious roots culture by applying in various strategies such as elicitation, application in a suitable bioreactor and bioprocess technologies. In this study, a fast and reliable method of biolistic and *Agrobacterium tumefaciens*-mediated transformation of *J. gendarussa* were developed. Establishment of plant transformation system provides the first essential step in the systematic study of the flavonoid biosynthetic pathway. The introduction of desired gene i.e PKS gene into the plant genome would modify flavonoids content in putatively transformed plants. Furthermore, a suitable approach for secondary metabolite production i.e flavonoids in *J. gendarussa* plants suggested through adventitious root culture and genetic modifications.

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