Antioxidative and Neuroprotective Activities of Wild and Tissue Cultured *Helicteres angustifolia* L.

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Antioxidative and Neuroprotective Activities of Wild and Tissue Cultured *Helicteres angustifolia* L

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Abstract

Helicteres angustifolia L. (*H. angustifolia*), a genus belongs to the plant family of Sterculiaceae, is distributed in Southeast Asia and Southern China. The roots of the plant are commonly used to treat diabetic and cancer for a long history in Laos. Many reports reveal that *H. angustifolia* contains abundant secondary metabolites with significant anti-diabetic and anti-cancer activities. The major challenges in development and utilization of it are scarcity, unstable yield and food safety of its wild resource. Plant tissue culture technology is submitted against above-mentioned issues. The present study explored the tissue culture of wild *H. angustifolia*, and compared the antioxidative and neuroprotective potential between wild and tissue cultured *H. angustifolia*.

Firstly, ethanolic extract (EE) from roots of *H. angustifolia* was obtained with heat reflux extraction method. Then five organic solvents fractions were separated from EE, which were petroleum ether extract (PE), chloroform extract (CE), ethyl acetate extract (EAE), n-butyl alcohol extract (NE) and water extract (WE), respectively. Their extraction ratios were 14.13%, 19.33%, 6.13%, 11.27% and 47.40%, respectively. Among these five fractions, EAE contained the highest total phenolic content (TPC, 356.65 ± 6.03 mg GAE/g extract) and highest total flavonoid content (TFC, 962.27 ± 10.84 mg RE/g extract), presented strongest antioxidant activities. NE contained 308.93 ± 13.85 mg/g extract of total carbohydrate content (TCC), 251.72 ± 4.18 mg GAE/g extract of TPC, 689.22 ± 11.55 mg RE/g extract of TFC and 48.31 ± 1J.76 mg /g extract of total proteins content (TPSC). NE exhibited strongest protection effect against oxidative stress-induced damage in IMR32 cell with the cell viability increased by 14.98 ± 1.08% after treatment for 24 h.

Next, the plant tissue culture of this plant was studied, including adventitious shoot cultivation and callus suspension cultivation. The maximum biomass accumulation of adventitious shoots was observed on medium with Kinetin (KIN) 0.1 mg/L, 3% (w/v) sucrose, 0.7% (w/v) agar after cultivation for 30 days. The number of adventitious shoot reached 2.00 ± 0.30 shoots per explant, the fresh weight reached 0.054 ± 0.005 g per

explant with dry weight of adventitious shoots reached to 0.010 ± 0.002 g per explant. On the other hand, callus suspension cultures of *H. angustifolia* was established successively in medium (MS + 3.00 mg/L NAA + 0.07 mg/L BA 0.40 mg/L vitamin C + 3% (w/v) sucrose, pH 5.80). After cultivation for 2 months, dry weight of cultures reached to 16.62 ± 0.92 g/L (fresh weight: 148.70 ± 8.91 g/L) which was equal to 3-fold of the inoculum concentration (fresh weight: 50.20 ± 6.40 g/L).

Finally, comparative study on bioactive potential between the tissue cultured and wild *H. angustifolia* was conducted. The tissue cultured *H. angustifolia* showed obvious antioxidant abilities and protection effect against oxidative stress-induced damage in IMR32 cell line. Tissue cultured plants contained abundant phytochemicals and bioactive functional groups which were similar with wild plants, such as OH, C–H, C=C and C–O.

Results revealed that the roots of *H. angustifolia* are great natural antioxidant resources for improving human health, which might also have a neuroprotection potential. Moreover, the tissue cultured *H. angustifolia* presented a prospective pharmaceutical potential; it's feasible to produce multiple bioactive substances contained in wild plants by using plant *in vitro* cultivation. This study provides a new pharmaceutical resource substitute for wild *H. angustifolia*.

Keywords: *Helicteres angustifolia* L.; Plant organ culture; Callus suspension culture; Antioxidant; Neuroprotection potential.

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Acronyms and Abbrevations

$ABTS^{\cdot+}$	2,2‘-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
BA	6-Benzylaminopurine
DMEM	Dulbecc’s modified eagle’s medium
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
FBS	Fetal bovine serum
FW	Fresh weight
H. angustifolia	*Helicteres angustifolia* L.
H_2O_2	Hydrogen peroxide
HO•	Hydroxyl radical
KIN	Kinetin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAA	a-Naphthalene acetic acid
NDDs	Neurodegenerative diseases
PBS	Phosphate buffered saline
TCC	Total carbohydrate content
TFC	Total flavonoid content
TPC	Total phenolic content
TPSC	Total protein content
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
Vc	Vitamin C
WHO	World health organization

Chapter 1 Introduction

1.1 Previous studies on *H. angustifolia* (Sterculiaceae)

The family Sterculiaceae is one of the most important families among flowering plants. *Helicteres*, a genus belongs to the plants of family Sterculiaceae, is shrub or small tree. It includes about 60 species (Kubitzki and Bayer, 2003). Many species in this family have been used in traditional medicine system such as folk medicine in China, Brazil and Laos for centuries. They displayed multiple medicinal usages as shown in Table 1-1. Previous studies mainly investigated the phytochemicals of these species. Results show that, these species contain a wide range of phytochemicals such as alkaloids, phenyl propanoids, flavonoids, terpenoids, hydrocarbons, phenolic acids, lactones, lignans, amine and amides (Al-Muqarrabun and Ahmat, 2015).

H. angustifolia is one of these species in this genus as shown in Figure 1-1. It attracts increasing attentions due to many usages in traditional medicine system. The plants of *H. angustifolia* are wildly distributed in Southeast Asia and Southern China, which is used to treat flu, diabetes mellitus and cancer in China and Laos. Previous studies mainly focus on bioactivities of its aqueous extract or ethanolic extract and identification of phytochemicals. Up to now, the aqueous and ethanolic extracts from *H. angustifolia* showed obvious antioxidant abilities, antidiabetic, anticancer, anti-inflammation, antiviral, etc. And methyl helicterate, two pregnane derivatives, a quinolone alkaloid, three lupane type triterpenoids, mansonone and naphthoquinone were successfully isolated from this plant (Li et al., 2015; Hu et al., 2016).

Table 1-2 Medicinal uses of some *Sterculiaceae*, *Helicteres* species.

Species name	Medicinal usages	Reference
Helicteres angustifolia L.	Used to against analgesic, inflammatory, bacterial, flu, tumor, cancer, diabetes, viruses	Al-Muqarrabun and Ahmat, 2015 Li et al., 2016; Huang et al., 2013 Hu et al., 2016
Helicteres gardneriana	Used to against protozoal disease caused by promastigote forms of *L. (V.) braziliensis*.	Truiti et al. 2005
Helicteres isora L.	Used as expectorant, demulcent, astringent, antigalactagogue, anthelmintic. Used in treatment of snake bites, chronic nephritis, gastric ulcers, flu, diabetes and gastrospasm, etc.	Chakrabarti, 2002; Truiti et al., 2005
Helicteres sacarolha Juss.	Used as a depurative. Used in treatment of syphilitic inflammations	Truiti et al. 2005
Helicteres ovata Lam.	Used as emollient, depurative. Used in treatment of syphilitic inflammations	Truiti et al. 2005

Figure 1-1 *H. angustifolia* (from Laos)

1.2 Antioxidants and neuroprotection

Generally, antioxidant capability is considered as the foundation of many bioactive functions and has been proven to be involved in the prevention of multiple chronic diseases, such as diabetes, cancer, inflammation, cardiovascular disease and neurodegeneration diseases (Akbar et al., 2016; Zou et al., 2016).

1.2.1 Neurodegenerative diseases (NDDs)

Neurodegenerative diseases (NDDs) usually include Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS), attack more than 30 million individuals worldwide (Maiese, 2014). The disease is a time-dependent course. Initially, the neurons in the human brain were impacted for a series of causes, and finally, resulting in degeneration and/or death of nerve cells and reduction of cognition and memory function. With the increasing lifespan and age of individuals, the incidence of disease is continuing to rise. It was estimated that it will be of greatest health concern in this century and the second leading cause of death by 2050, more hazardous than cancer (Buendia et al., 2016; Luo et al., 2016). The NDDs demand

intensive care, usually at a high economic cost for society. The statistical results show that the cost per year of neurodegenerative diseases could be as much as 71.62 billion Euros, in 28 selected European countries in 2004, including direct and indirect costs (Andlin-Sobocki et al. 2005; Nieoullon, 2011). The health or society issues, no matter from which aspect to consider, great attention should be paid to study on the therapy strategies of NDDs.

1.2.2 Cause of neurodegenerative diseases

In recent years, it was frequently reported that NDDs were commonly accompanied by the following pathological events: abnormal protein aggregation, proteasomal or autophagic dysfunction, inflammation, neuronal apoptosis, oxidative stress, mitochondrial dysfunction, and abnormal interactions between neurons and glia. Among them, mitochondrial dysfunction, reactive gliosis and oxidative damage to lipids, proteins and DNA, have been commonly mentioned in various types of NDDs cases (Buendia et al., 2016). Especially, increasing evidences indicated that mitochondrial dysfunction is a major culprit in NDDs. In fact, mitochondrial dysfunction itself is not a disease. After it was caused by many chemical and environmental factors such as inherited mutation, oxidative stress, toxins and aging, it can break cellular calcium and protein homeostasis and promotes accumulation of detrimental proteins in the brain. Eventually, these consequences will result in cell death and neurodegeneration, contributing to the pathogenesis of many neurodegenerative diseases.

1.2.3 Application of antioxidants in therapy of neurodegenerative diseases

Up to now, no more therapeutic approaches can be used to cure neurodegeneration, except certain medicines that temporarily alleviate symptoms, facilitating the improvement of a patients' quality of life. These medicines include synthetic agents and natural antioxidants which represent obvious neuroprotection against neurodegeneration (Akbar et al., 2016). For synthetic agents, few drugs are approved to treat NDDs, such as AchEIs, NMDA receptor antagonist for the cognitive manifestations and dopamine agonists. But they were limited in the treatment process

of NDDs, because of the various adverse effects and complicated pathology mechanism. In contrast, natural antioxidant agents were considered as an alternative for neuroprotection agents in treatments of NDDs, attracting increasing attentions (Nieoullon, 2011). *H. angustifolia* shows a prospective potential in pharmaceutical field due to the reported antioxidant ability from the ethanol or aqueous extracts of *H. angustifolia.* Few reports are published about bioactive-guided isolation of the ethanol or aqueous extracts from *H. angustifolia* and neuroprotective potential of *H. angustifolia.* More works should focus on the antioxidant, neuroprotection and the sustainable development and utilization of the plants.

1.3 Challenges faced in development and utilization of *H. angustifolia*

Medicine herbs and their preparations have been widely used in developing and developed countries for thousands of years. Nowadays, they are still in great market demand worldwide to solve various health care problems due to their efficacy, safety and lesser side effects. However, the development of medicine herbs are facing a lot of constraints, such as biodiversity conservation, excavation pressure of wild plants resources, lack of research on the development of high-yielding varieties and domestication, poor agriculture and propagation methods, a host of environmental factors (e.g. soil, altitude, seasonal variation in temperature, atmospheric humidity, length of daylight, rainfall pattern, shade, dew, and frost conditions), infections, insects, planting density, competition with other plant species, seeding time, and genetic factors, etc (Thillaivanan and Samraj, 2014). In order to solve these problems, the plant tissue culture method has been used to offer substitute resources of wild plant herbs for production of useful substances for a long history.

1.4 Application of tissue culture method in the development of wild medicine herbs

Multitudinous functions of wild plants are attributed to natural secondary metabolites, frequently accompanied by less side effects and lower rates of secondary

failure than synthetic ones. Recently, more attentions were paid to the production of natural secondary metabolites, of which the raw materials were mainly obtained by excavation and plant *in vitro* culture. Traditional obtaining manner is likely to cause resources stress, resulting in species endangering and even extinction. Furthermore, the wild plant usually showed a slow biomass accumulation in nature, and its target secondary metabolites may be only found at low level. With the increasing demanding for secondary metabolites, it is of great significance to utilize the plant and explore a sustainable and high-efficient artificial cultivation approach for useful compounds. The plant cell and tissue culture method includes organ culture, callus culture, cell culture, protoplast culture etc. They can offer the alternative for the substitutable resources of wild plants, and also offers the establishment possibility of high-yield production system of useful compounds. In culture process, environment and nutritional factors, as well as growth regulators, can be easily controlled to produce useful compounds successfully.

1.4.1 Plant organ culture

The organ differentiation of plant is closed with production of secondary metabolites. Generally, the plant organ culture can maintain the differentiated characteristic structure of the organ and then affect the metabolic process of secondary metabolites Previous studies exhibited that the *in vitro* cultured plant organ (e.g. shoots, roots, etc.) possessed promising biosynthesis capability for useful compounds (Santos-Gomes et al., 2002; Sivanandhan et al., 2012). And many factors can change the useful compounds contents, such as addition of elicitors, types and concentrations of PGRs, etc. The establishment and optimization of organ culture system provide a possibility for high-yield production system of useful compounds.

1.4.2 Callus suspension culture

The plant callus suspension culture, an *in vitro* culture manner based on the cell totipotency, is considered as a feasible approach for a large-scale production with both sustainability and high-efficiency. It was employed as “green factories” to produce assorted nutraceutical and medicinal compounds (Matkowski, 2008). This method can

not only produce valuable ingredients against resources stress, independent of environmental conditions or yield limitations, but also enhance the yields of secondary metabolites that are either difficult to synthesize chemically or are produced in limited quantities in wild plants by optimization of culture conditions, addition of elicitors, precursors and biotransformation etc. (Ali et al., 2013; Loganathan and Bai, 2014; Albert et al., 2014; Ali et al., 2013). Therefore, it is interesting to consider the plant cell and tissue cultivation system as the feasible alternative sources for the future development and utilization commercially of *H. angustifolia* and to study on the metabolism.

1.5 Objectives and structure of thesis

H. angustifolia is used to treat various diseases for a long history in developing countries. It shows a promising potential in pharmaceutical field. The major challenges in development and utilization of it are the scarcity, the unstable yields and food safety of wild resources. The plant tissue culture technology is expected to solve the above-mentioned issues. The present study was conducted to investigate the phytochemicals, antioxidant and neuroprotection effects of the isolated fractions from ethanolic extract of wild *H. angustifolia* L. And then in order to solve the resource pressure, we attempted to establish the tissue cultivation of wild *H. angustifolia* to produce new raw materials capable for bioactive compounds. Eventually, comparation on the bioactive potential between tissue cultured and wild plants was conducted. The study aims to offer a substitutable alternative for wild medicine herbs and explore the pharmaceutical value of tissue cultured and wild *H. angustifolia* in antioxidant and neuroprotection aspects.

This study was conducted following to the technology route shown in Figure 1-2. The antioxidant and neuroprotection potential of isolated fractions from ethanolic extracts of roots of *H. angustifolia* were first investigated, then the cultivation method of organ (the plant aerial parts: adventitious shoots) and callus suspension culture of *H. angustifolia* were established. Finally, this study compared the antioxidant and neuroprotection potential of cell and tissue cultured and wild *H. angustifolia*.

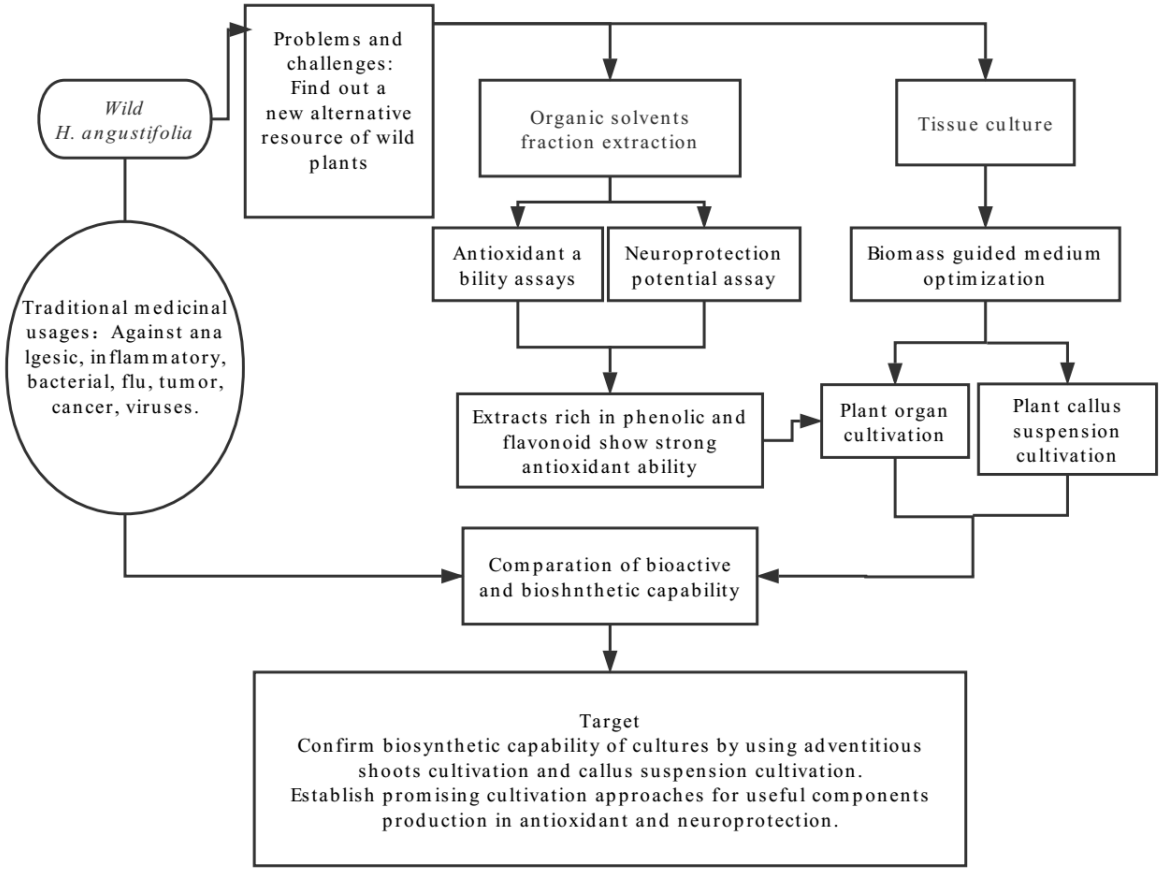


Figure 1-2 Technology route

Chapter 2 Characterization of phytochemicals, antioxidant and neuroprotection potential of roots of wild *H. angustifolia*

2.1 Introduction

Reactive oxygen species (ROS) play a key role in physiological processes, excessive ROS might cause oxidative stress to damage cellular DNA, oxidize fatty acids in lipids and amino acids in proteins, which result in tissue destruction and are associated to chronic diseases, including diabetes, neurodegeneration, and cancer. Antioxidant compounds play an important role against these diseases, with promising commercial potential in pharmaceutical and food industries. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commonly toxic and harmful to human body (Costa et al., 2012). Whereas, natural antioxidants derived from plants have low cytotoxicity, which mainly include phenolic compounds (flavonoids, phenolic acids and alcohols, stilbenes, tocopherols, tocotrienols), minerals, organosulfur compounds, vitamins and derivatives and carotenoids (Ali et al, 2008; Carocho and Ferreira, 2013). The quest for natural antioxidants for dietary, cosmetic and pharmaceutical uses has become a major industrial and scientific research challenge over the last two decades. Our studies focus on antioxidant and neuroprotection potential of *H. angustifolia*. This chapter was performed (i) to investigate the phytochemicals characteristic of five organic solvents fractions of ethanolic extract from *H. angustifolia* roots, (ii) to evaluate their antioxidant potencies, and (iii) to investigate their neuroprotection for the first time.

2.2 Materials and methods

2.2.1 Chemicals and reagents

Rutin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), ferric chloride, and picric acid were purchased from Sigma-Aldrich (Sigma). The conc. sulphuric acid, $NaNO_2$, $AlCl_3$, NaOH, petroleum ether, chloroform, ethyl acetate, and n-butyl alcohol were purchased from Wako.

2.2.2 Plant materials

The *H. angustifolia* were collected from the rural area near to the city of Vientianei, Laos in June, 2013 and then was planted at 25 ℃. It was identified by Dr. Ende Liu from Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China, of which a voucher specimen (No. 090155) was deposited at the herbarium of the same institute. This study collected tender leaves grown in spring as explants.

2.2.3 Samples preparation

The roots of *H. angustifolia* were ground into fine powders. 400 g of root powders were soaked into 4 L of 99.5% (v/v) ethanol. After 5 days the mixtures were extracted by hot reflux method for 2 hours, which was repeated 3 times. The supernatants were concentrated into 15g of ethanolic extract via vacuum evaporation and frozen-dried, stored at - 20 ℃ for tests. The dry sample (15g) was re-dissolved in hot distilled water with ultrasonic assistance, then sequentially extracted with petroleum ether, chloroform, ethyl acetate and n-butyl alcohol (300 mL) for three times. The fractions were evaporated to dryness. The extracts, petroleum ether extract (PE), chloroform fraction extract (CE), ethyl acetate extract (EAE) and n-butyl alcohol extract (NAE), were obtained and stored at - 20 ℃ for tests. The extraction yield was expressed as:

$$\text{Extraction yield (\%)} = \frac{\text{Weight of the extract (g)}}{\text{Weight of the ethanolic extract (g)}} \times 100 \qquad (2\text{-}1)$$

2.2.4 Preliminary phytochemical assay

The extracts were subjected to standard methods of phytochemical analyses to detect the presence of phyto-constituents, viz. tannins, alkaloids, steroids and triterpenoids.

(1) Test for tannins

A small quantity of the extracts was boiled with 5 mL of 45% ethanol solution for 5 min. The mixture was cooled and filtered. 1 mL of filtrate was added with two drops of ferric chloride (0.1%). A dark green color indicates the presence of tannins (Singh and Bag, 2012).

(2) Test for alkaloid

The extract was warmed with 2% H_2SO_4 for two minutes. The mixture was filtered and added with a few drops of picric acid (1%). A yellow precipitation indicates the presence of alkaloid (Singh and Bag, 2012).

(3) Test for steroids and triterpenoids

The extract was dissolved with chloroform and filtered. The filtrate was added with a few drops of conc. sulphuric acid, shaken and allowed to stand. A red color in the lower layer indicates the presence of steroids. A reddish brown color in interface indicates the presence of triterpenoids after addition of conc. sulphuric acid without shaking (Singh and Bag, 2013).

(4) Test for polysaccharide

The extract was subjected to test for polysaccharide with determination method of total polysaccharide content as described by DuBois et al. (1956).

2.2.5 Quantification of several bioactive components

(1) Total phenolic content

The total phenolic content (TPC) was determined with the Folin - Ciocalteau assay. Briefly, 1 mL of extracts or a standard solution of gallic acid (20, 40, 60, 80 and 100 mg/L) was added to a volumetric flask, containing 9 mL of distilled deionized water. 1 mL of the Folin-Ciocalteu's phenol reagent was added to the mixture and shaken. After 5 min, 10 mL of 70 g/L Na_2CO_3 solution was added to the mixture. The solution was diluted to 25 mL with distilled deionized water and mixed. After incubation for 90 min at room temperature, the absorbance was determined at 725 nm with a UV-vis spectrophotometer. The TPCs of samples were expressed as milligrams of gallic acid equivalents (GAE) per 100 grams extract (mg GAE/g dry extract).

(2) Total flavonoid content

The total flavonoids content was measured with an aluminum chloride colorimetric assay. 1 mL of extracts or a standard solution of Rutin (20, 40, 60, 80 and 100 mg/L) was added to a 10 mL volumetric flask, containing 4 mL of distilled water. Subsequently, 0.3 mL of 50 g/L $NaNO_2$ was added to the flask. After 5 min, 0.3 mL of 100 g/L $AlCl_3$ was added. At the sixth minute, 2 mL of 1 mol/L NaOH was added and

the total volume was made up to 10 mL with distilled water. The solution was mixed well and the absorbance was measured against a prepared reagent blank at 510 nm with a UV-vis spectrophotometer. The total flavonoid contents were expressed as milligrams of rutin equivalents (RE) per gram extract (mg RE/g dry extract).

(3) Total carbohydrate content

The total carbohydrate contents were determined according to the method of McCseady (1970) and Dubois et al. (1956). 1mg of extract was mixed with 2 mL of distilled water into test tubes in triplicates; then was added with 1 mL phenol (5%) into each test tube and mixed, and 5 mL of concentrated sulphuric acid was added into the mixture. The tuber was shaken well and allowed to stand for 20 min, and the absorbance was measured at 490 nm. 1 mL of distilled water was used as blank. The glucose solution was prepared as different concentrations (0-0.9 mg/mL). A standard curve of glucose was plotted, based on which total carbohydrate concentration was calculated.

(4) Total protein content

The total protein contents were determined according to Bradford (1976). 0.1 g Coomassie Brilliant Blue G250 (CBB) was dissolved in 50 mL ethanol (95%) and 100 mL phosphoric acid (85%, w/v) was added. Then, the resultant solution was diluted to 1000 mL. The final concentrations in the reagent were 0.01% (w/v) for CBB, 4.7% (w/v) for ethanol, and 8.5% (w/v) for phosphoric acid, respectively. 10 mg sample was dissolved into 5 mL phosphate buffer solution (pH 7.6), vortex homogenization, then stand for at least 5 min. 1 mL supernatant was diluted into a test tube and added 5 mL CBB solution, and using distilled water as the blank. After 2 min the absorbance was measured at 595 nm using an UV-Visible spectrophotometer. The BSA (Bovine serum albumin) solution was prepared as different concentrations (0.25-4.00 mg/mL) using 0.15 M NaCl solution. 100 μL standard protein solution and 5 mL CBB solution were pipetted into a test tube. The sample was shaken and stand for at least 5 min. Distilled water was used as the blank. The absorbance was measured at 595 nm after 2 min. A standard curve was plotted, and total proteins concentration was calculated out.

2.2.6 Measurement of antioxidant activity

(1) DPPH radical scavenging ability

The DPPH radical scavenging ability of extracts was determined as described by Blois (1958) with some modifications. Briefly, different concentrations (0.0780, 0.1560, 0.3125, 0.6250, 1.2500 and 2.5000 mg/mL) of samples were prepared to determine the DPPH antioxidant activity. 0.1 mL of sample was mixed with 3.9 mL of 0.025 mg/mL DPPH solution in ethanol. The mixture was shaken, and then was kept at room temperature for 30 min in the dark. The absorbance was measured at 517 nm in a UV-vis spectrophotometer. The absorbance of the blank was obtained by replacing the sample with 80% methanol. Ascorbic acid was used as a control. The DPPH radical scavenging activity of the sample was calculated as follows:

$$\text{DPPH radical scavenging ratio (\%)}=(1-\frac{As}{Ao})\times 100 \qquad (2-2)$$

where A_o is the absorbance of the blank and A_s is the absorbance of the sample at 517 nm.

(2) $ABTS^{\cdot+}$ radical scavenging assay

$ABTS^{\cdot+}$ radical scavenging ability was determined as described by Debnath et al. (2011) with slight modifications. $ABTS^{\cdot+}$ was dissolved in ethanol solution with concentration reaching 7 mM, and then mixed with a potassium persulphate solution to get a final concentration of 2.45 mM. $ABTS^{\cdot+}$ stock solution was produced by leaving the mixture at 25 ℃ for 12-16 h in dark till complete reaction indicated by stable absorbance. For each test, the $ABTS^{\cdot+}$ stock solution was diluted with 0.01 M phosphate buffer saline (pH 7.4) to keep its absorbance at 734 nm within 0.70 ± 0.02. Before determination, 0.15 mL of each sample was mixed with 2.85 mL of $ABTS^{\cdot+}$ working solution, and the absorbance was measured at 734 nm after incubation for 10 min at 25 ℃ in the dark. Trolox was used as the positive control. The $ABTS^{\cdot+}$ radical scavenging activity was calculated according to Eq.

$$ABTS^{\cdot+}\text{ scavenging ratios (\%)} = 100 \times (A_{Control}-A_{Sample}) / A_{Control} \qquad (2-3)$$

2.2.7 Cell culture, cytotoxicity assay and cell viability

IMR 32 cell was purchased from RIKEN Bioresource Center (Tsukuba, Japan), were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 ℃ and 5% CO_2 incubator. The medium was changed every 2-3 days.

The cytotoxic effects of samples on IMR 32 cells were determined by MTT assay (Zhang et al., 2011). Briefly, Human IMR32 Neuroblastoma cell were seeded into 96-well plate at a density of 5×10^3 cells/well to incubate for 24 h. The various concentrations of extracts were added into 96-well plate. After incubation for another 24 h, 10 μL MTT (5 mg/mL) was added into the mixture to react for 4 h, and then 100 μL of DMSO was added into the wells before culture medium removed. After shaking for 10 min, the optical density (OD) was determined at 570 nm with a microplate reader (Bio-Rad, Japan).

IMR32 cell were seeded into 96-well plate at a density of 5×10^3 cells/well. After incubation for 24 h, DMEM culture medium was replaced by fresh medium (without FBS) with final concentrations of H_2O_2 from 0 to 500 μM for 2 h. Followed by cultivating them for 24 h in the fresh medium. Finally, cell viability was determined by MTT. The concentration of H_2O_2 (400 μM), at which the cell viability can achieve to 50% for 2 h pretreatment, was used to do the following experiments. IMR32 cells were seeded into 96-well plate at a density of 5×10^3 cells/well. Culture mediums were replaced with fresh serum-free mediums containing 400 μM H_2O_2 for 2 h. The various concentrations of extracts were added into 96-well plate. After incubation for another 24 h, cell viability was measured by MTT.

2.2.8 FT-IR spectroscopy analysis

The dry extracts were ground with potassium bromide (KBr) to fine powder, placed under high pressure until formation of a euphotic film. Then the films were placed on the sample chamber of FT-IR spectrophotometer (FT/IR-300, JASCO) and the spectra were recorded in the range of 4000-400 cm^{-1}, to detect various functional groups responsible for biological activities.

2.2.9 Statistical analysis

All the experimental results were presented as mean ± S.E. of three parallel measurements. Results were processed by Microsoft Excel (2010) and OriginPro 8.

2.3 Results and discussion

2.3.1 Preliminary phytochemical investigation

17.05 g of ethanolic extract was obtained from 400 g of dry roots powders. Then the yield, color, consistency and extraction yield of five fractions extracts from ethanolic extracts were investigated and shown in Table 2-1. The maximum yield was observed about 47.4% in water fraction extraction, whereas ethyl acetate extraction yielded only 6.13%. Then, preliminary phytochemical screening indicated the presence of tannin, triterpenoids and steroids in extracts as shown in Table 2-2. The presence of tannins was observed in EAE, NE and WE. PE and CE revealed the presence of both triterpenoids and steroids. EAE contained triterpenoids without steroids. Otherwise, alkaloid were found to be absent in all the extracts.

Natural components, e.g. tannins, triterpenoids, steroids and saponins are commonly found to possess multiple bioactive functions such as antioxidant, neuroprotection, antibacterial and anti-inflammation, etc (Tan et al., 2012; Luo et al., 2011; Garcia-Segura and Balthazart, 2009). In this context, our works on *H. angustifolia* are quite valuable due to the presence of above-mentioned phytochemicals in the plants. The present phytochemicals screening is beneficial for future research activities on isolation and identification of these bioactive compounds.

Table 2-1 Yield, color and consistency of five fractions from ethanolic extract of *H. angustifolia* roots.

Extracts	Extraction yield (%)	Color and consistency
PE	14.13	Reddish gum
CE	19.33	Reddish solid
EAE	6.13	Reddish solid
NE	11.27	Yellowish solid
WE	47.40	Yellowish solid

Table 2-2 Preliminary phytochemical screening

Phytochemical group	PE	CE	EAE	NE	WE
Tannins	-	-	+	+	+
Alkaloids	-	-	-	-	-
Triterpenoids	+	+	+	-	-
Steroids	+	+	-	-	-

+: Present and -: Not present

PE: petroleum ether fraction extract; CE: chloroform fraction extracts. EAE: ethyl acetate fraction extract; NE: n-butyl alcohol fraction extracts; WE: water fraction extracts.

2.3.2 Contents of several bioactive compounds

The extracts were subjected to quantitative tests of the total flavonoid, phenolic, carbohydrate and proteins contents. Results are tabulated in Table 2-3. The maximum flavonoid and phenolic contents were detected in EAE, reaching 962.27 ± 10.84 mg RE/g extract and 356.65 ± 6.03 mg GAE/g extract, respectively. While PE contained the minimum flavonoid and phenolic contents only 3.78 ± 0.96 mg RE/g extract and 23.14 ± 0.78 mg GAE/g extract, respectively. The total flavonoid contents showed a positive relationship with the total phenolic contents of extracts. On the other hand, the maximum carbohydrate content was detected in WE reaching 439.08 ± 2.92 mg/g extract, and CE had the minimum carbohydrate content, only reaching 7.46 ± 0.08 mg/g extract.

Table 2-3 Quantitative determination of several phytochemicals

Extracts	The total flavonoid contents (mg RE/g extract)	The total phenolic contents (mg GAE/g extract)	The total carbohydrate contents (mg/g extract)	The total proteins contents (mg/g extract)
PE	3.78 ± 0.96	23.14 ± 0.78	34.54 ± 3.15	0
CE	248.74 ± 2.55	65.12 ± 4.71	7.46 ± 0.08	0
EAE	962.27 ± 10.84	356.65 ± 6.03	22.85 ± 0.38	66.83 ± 0.65
NE	689.22 ± 11.55	251.72 ± 4.18	309.23 ± 13.69	48.31 ± 1.76
WE	347.61 ± 3.33	109.20 ± 2.52	439.08 ± 2.92	25.17 ± 0.65

PE: petroleum ether fraction extract; CE: chloroform fraction extracts. EAE: ethyl acetate fraction extract; NE: n-butyl alcohol fraction extracts; WE: water fraction extracts.

Phenolic compounds act as antioxidants with mechanisms involving both free radical scavenging and metal chelation. They are widely existed in all plant organs, and are considered more potential than Vitamin C, E and carotenoids in anti-oxidation (Ali et al., 2013). Studies showed they might have beneficial effects against oxidative stress, neurodegeneration, inflammation, cancer, diabetes, etc. (Kim et al., 2016). Flavonoids in numerous plants, as natural antioxidants, were also reported to possess beneficial effects to human body. It has been one of the most promising agents to treat oxidant stress, inflammations, pathogenic bacteria, cancer, etc (Koirala et al., 2016). Carbohydrates mainly include monosaccharide, oligosaccharide, and polysaccharide. Many studies indicated that natural carbohydrate polymers possess antioxidant, hypoglycemic, anti-inflammation, etc (Yan et al., 2016). The natural bioactive proteins are reported to have antioxidant, immunomodulatory potential and antibacterial activity (Al-Akeel et al., 2014; Pereira et al., 2015; Zhao et al., 2015).

2.3.3 Antioxidant activity assays

Synthetic antioxidants are commonly toxic and harmful to body (Costa et al., 2012). Natural antioxidants are mainly derived from plants such as phenolic compounds (flavonoids, phenolic acids and alcohols, stilbenes, tocopherols, tocotrienols), minerals, organosulfur compounds, vitamins and derivatives and carotenoids (Ali et al., 2008; Carocho and Ferreira, 2013). In this study, the radical scavenging activity and EC_{50} values of different extracts from *H. angustifolia* roots are shown in Figure 2-1, Figure 2-2 and Table 2-4, respectively.

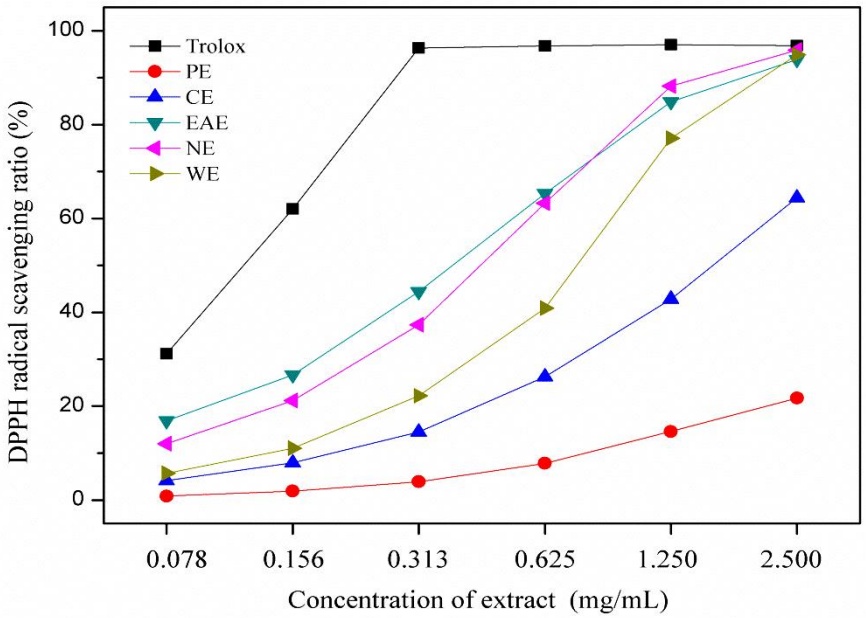


Figure 2-1 DPPH radical scavenging assay of the five extracts. Values are mean of three replicate (Standard Error < 0.5).

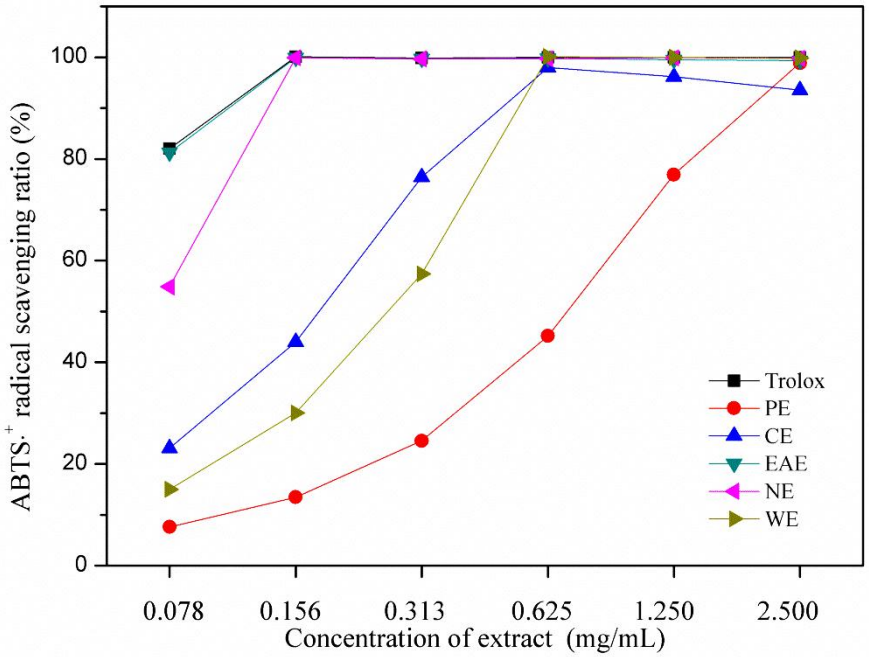


Figure 2-2 $ABTS^{\cdot+}$ radical scavenging assay of the five extracts. Values are mean of three replicate (Standard Error < 0.5).

Table 2-4 EC_{50} values for different extracts in antioxidant abilities assays

Radical scavenging	Trolox (mg/mL)	PE (mg/mL)	CE (mg/mL)	EAE (mg/mL)	NE (mg/mL)	WE (mg/mL)
DPPH	0.14 ± 0.03	> 2.5	1.55 ± 0.02	0.35 ± 0.01	0.40 ± 0.01	0.58 ± 0.02
$ABTS^{\cdot+}$	< 0.078	0.54 ± 0.03	0.20 ± 0.01	< 0.078	≈ 0.078	0.26 ± 0.03

Trolox was used as the positive control. A lower EC_{50} value indicates a higher antioxidant activity.

Trolox was selected as a positive control. A lower EC_{50} value indicates a higher antioxidant activity. All extracts showed increasing antioxidant activities in a dose-dependent manner. Among them, EAE exhibited excellent DPPH and $ABTS^{\cdot+}$ radical scavenging abilities with EC_{50} values of 0.350 ± 0.010 mg/mL and < 0.078 mg/mL, respectively, showing similar $ABTS^{\cdot+}$ radical scavenging abilities compared with trolox. PE displayed very weak DPPH and $ABTS^{\cdot+}$ radical scavenging abilities with EC_{50} values greater than 2.500 mg/mL and 0.540 ± 0.030 mg/mL, respectively. The observed antioxidant abilities might be attributable to the presence of various chemical components including tannins, phenolic, flavonoids, carbohydrate, etc. Moreover, the antioxidant ability of the extract showed positive correlations with the concentrations of phenolic contents and flavonoids contents.

Plant extracts and their components as a natural source of antioxidants present a promising potential in pharmaceutical field. In human body, free radicals at high concentration level can cause oxidative stress to damage the cellular macromolecular, eventually, resulting in cell death and various diseases such as neurodegenerative diseases. Many researches have proven that antioxidant capability of natural antioxidant agents involves the prevention of neurodegeneration diseases, cancer, inflammation, cardiovascular disease and diabetes (Zou et al., 2016; Akbar et al., 2016). Hence, natural antioxidants attract increasing attentions due to multiple bioactive functions and their lower adverse effects than synthetic antioxidants.

2.3.4 Neuroprotection potential of extracts on H_2O_2-injuried IMR32 cells

IMR32 cell line, a human neuronal cell line, which is considered as a good model to study the stress-induced cell damage and neurodegeneration in humans, is used to screen the extracts from medicinal plants capable for neuroprotection potential (Shivapriya et al., 2015). Our study aims to investigate the preliminary phytochemical components, and then evaluate the *in vitro* antioxidant and neuroprotective activity of *H. angustifolia* roots extracts using IMR32 human neuroblastoma cell line.

(1) Cytotoxicity

MTT assay was performed to test the cytotoxic effects of the extracts on IMR32 cell growth. As shown in Figure 2-3, a wide range of drug doses (0.00–0.10 mg/mL) were selected, CE exhibited relatively obvious reduction in cell proliferation after drug treatment for 24 h. While the reduction in cell proliferation was not observed upon drug treatment of other extracts.

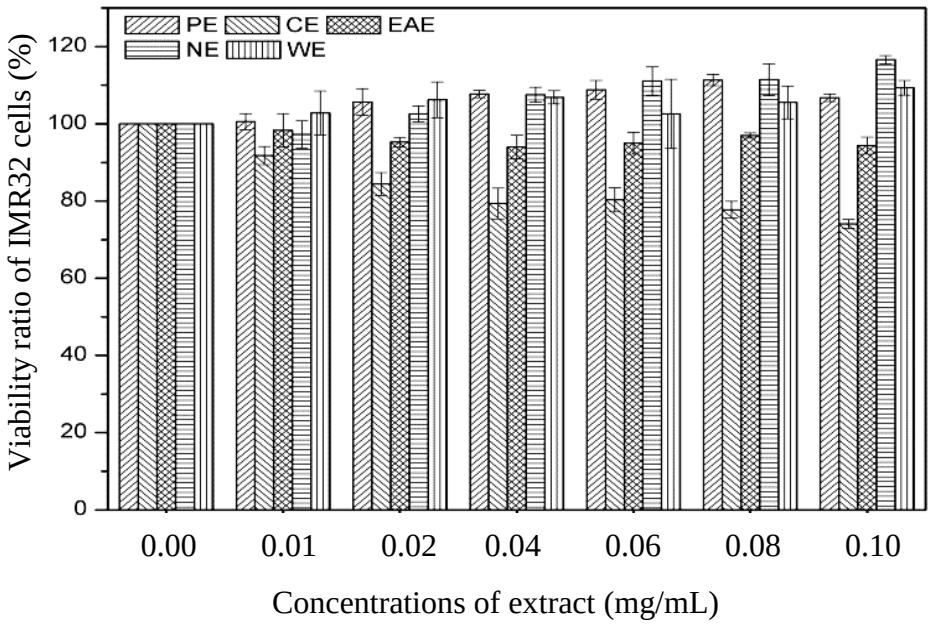


Figure 2-3 Cytotoxicity of extracts on IMR32 cell viability

PE: petroleum ether fraction extract; CE: chloroform fraction extracts. EAE: ethyl acetate fraction extract; NE: n-butyl alcohol fraction extracts; WE: water fraction extracts.

(2) H_2O_2-injuried IMR32 cells model

MTT results revealed that cell viability reduced in a dose-dependent manner. The cell viability ratio of IMR 32 cells reduced to 52.60 ± 4.46 % with H_2O_2 (400 μM) treatment for 2 h. This concentration of H_2O_2 (400 μM) was selected to detect neuroprotection effect of extracts in the following experiments.

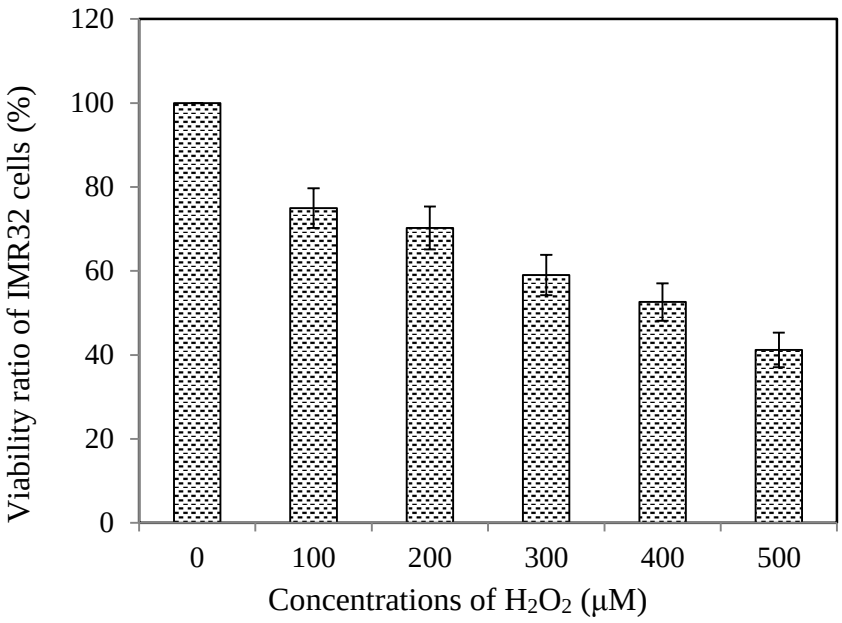


Figure 2-4 H_2O_2-injuried IMR32 cells model

The concentration of H_2O_2, at which (400 μM) the cell viability can achieve 50% after 2 h pretreatment, was used to detect the neuroprotection effect of extract on H_2O_2-injuried IMR32 cells.

(3) Effects on cell viability in H_2O_2-injuried IMR32 cells

In our present work, MTT results presented that n-butyl alcohol fraction of ethanolic extracts from *H. angustifolia* roots which possessed obvious inhibition effects on H_2O_2-induced reduction of IMR32 cell viability with IMR32 cells viability increased by 14.98±1.08 % after treatment with NE for 24 h. Otherwise, the petroleum ether fraction and water fraction also exhibited inhibition effects on H_2O_2-induced reduction of IMR32 cell viability to some extent, with IMR32 cells viability increased by 10.42±3.54 % and 8.34±0.37 % after treatment with PE and WE for 24 h, respectively. MTT data indicated great pharmaceutical potential of *H. angustifolia* roots in neuroprotection field.

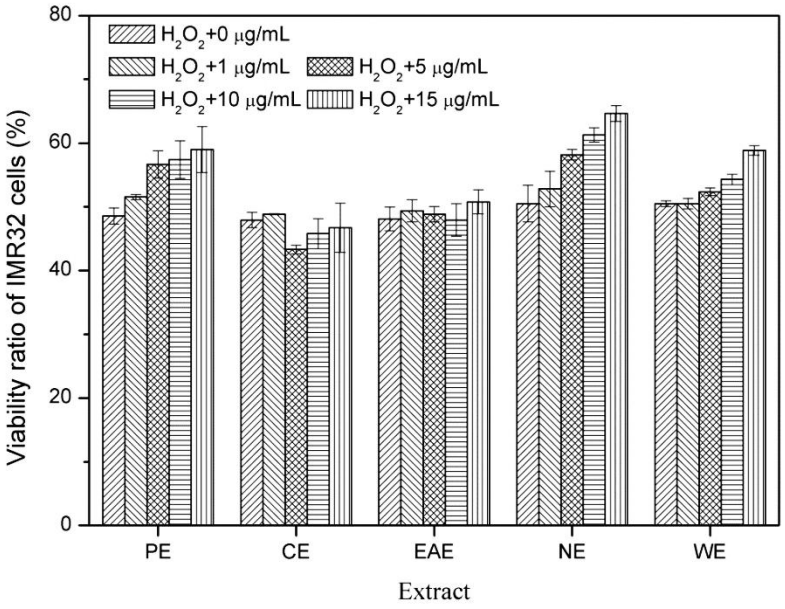


Figure 2-5 Effect of extract on cell viability in H_2O_2-injuried IMR32 cells
PE: petroleum ether fraction extract; CE: chloroform fraction extracts. EAE: ethyl acetate fraction extract; NE: n-butyl alcohol fraction extracts; WE: water fraction extracts.

2.3.5 Functional groups analysis

Functional groups can affect the physical and chemical characteristics of bioactive molecules such as acid-base properties, solubility, crystal structure, stereochemistry, and influence the absorption, toxicity and bioactivities (Poojary et al., 2015). FT-IR spectral analysis data indicated that the extracts contain multiple functional groups such as OH, C–H, CH_2, CH_3, C–O, C=C which are presented in Table 2-5 (a, b). All extracts revealed the presence of broad peak for hydrogen bonded –OH stretching. The OH group suggested that there might be some phenolic compounds in the extracts. Strong and wide absorption band of about 3400 cm^{-1} for OH group, the peak of about 2926 cm^{-1} for C–H group, the peaks of about 1711, 1605 cm^{-1} for C=C group, the peaks of about 1458, 1384 cm^{-1} for C–H group and the peak of about 1073, 1018 cm^{-1} for C–O group indicated that the probable presence of saponin (Borah et al., 2016; Motz et al.,

2015; Kareru et al., 2008). The FT-IR spectral analysis data reflected that the extracts might be composed of phenolic, saponin and others.

Table 2-5 (a) Major bands observed in the FT-IR spectra of various extracts.

Range (cm^{-1})	Groups and class of compound	Assignment and remarks	PE (cm^{-1})	CE (cm^{-1})	EAE (cm^{-1})	NE (cm^{-1})	WE (cm^{-1})
3450–3250	-OH In alcohols and phenols	O-H Stretch	3422.06	3426.89	3404.71	3377.71	3344.93
3000–2850	C-H In alkanes	C-H Stretch	2924.52, 2852.20	2922.59, 2852.20	2925.48, 2851.24	2933.2	2931.27
2936–2913	CH_3-, CH_2- In aliphatic compounds	CH_3-, CH_2- Anti-symmetric stretch	2924.52	2922.59	2925.48	2933.2	2931.27
1724–1700	C=O In carboxylic acids, ketones	C=O Stretch			1716.34	1716.34	
1650–1580	N-H, 1◦ Amines	N-H Bend		1602.5	1607.38	1625.7	1625.7
1600–1520	NH^{3+} In NH_4OH	NH_3 Deformation					
1618–1498	Benzene ring in aromatic compounds	C=C Aromatic ring stretch		1602.5, 1515.77	1607.38, 1519.63	1519.63	

PE: petroleum ether fraction extract; CE: chloroform fraction extracts. EAE: ethyl acetate fraction extract; NE: n-butyl alcohol fraction extracts; WE: water fraction extracts.

Table 2-6 (b) Major bands observed in the FT-IR spectra of various extracts.

Range (cm^{-1})	Groups and class of compound	Assignment and remarks	PE (cm^{-1})	CE (cm^{-1})	EAE (cm^{-1})	NE (cm^{-1})	WE (cm^{-1})
1550–1475	N-O Nitro compounds	N-O Asymmetric stretch		1515.77	1519.63	1519.63	
1360–1290	N-O Nitro compounds	N-O Symmetric stretch					
1300–1150	Alkyl halides	C-H wag (CH_2X)	1246.75, 1172.51	1248.68	1281.47, 1188.90	1286.29	
1095–1074	NH_2 in NH_4OH; C-OH In secondary alcohols	NH_2 Groups in-plane rocking vibrations; C-O stretch			1074.16		
1026-1075	C-O bond stretching		1026.91	1027.87	1074.16	1073.19	1053.91
1072–1034	C-O-C In aliphatic ethers	C-O-C Anti-symmetric stretch					1053.91
900–700	=CH In aromatic hydrocarbons	=C-H Out-of-plane bending	711.6	871.67, 828.28, 754.99, 713.53	812.85	868.77, 812.85	
700–610	Alkynes	-C≡C-H: C-H Bend			633.5	633.5	

PE: petroleum ether fraction extract; CE: chloroform fraction extracts. EAE: ethyl acetate fraction extract; NE: n-butyl alcohol fraction extracts; WE: water fraction extracts.

2.4 Summary

H. angustifolia contained abundant phytochemicals like tannins, triterpenoids, steroids, phenolics, flavonoids, carbohydrates, proteins and other chemicals. The five fractions from ethanolic extracts of *H. angustifolia* roots exhibited different levels of antioxidant activity. A descending order of DPPH radical scavenging ability was found among these five extracts: EAE > NE > WE > CE > PE. And a descending order of $ABTS^{\cdot+}$ radical scavenging ability was detected as EAE > NE > CE > WE > PE. Among the five fractions, ethyl acetate fraction (EAE) contained the maximum phenolic and flavonoid contents with the concentrations reaching 356.65 ± 6.03 mg GAE/g extract and 962.27 ± 10.84 mg RE/g extract, respectively. Meanwhile, EAE presented the strongest DPPH radical scavenging activity and $ABTS^{\cdot+}$ radical scavenging activity. The fraction rich in phenolics and flavonoids possessed strongest antioxidant activity. Otherwise, among these five fractions, petroleum ether soluble fraction (PE), n-butyl alcohol soluble fraction (NE), water soluble fraction (WE) exhibited different levels of inhibition effect on H_2O_2-induced reduction of IMR32 cells viability. NE exhibited the strongest inhibition effect on H_2O_2-induced reduction of IMR32 cells viability which increased by 14.98 ± 1.08 % after NE treatment for 24 h. In summary, our study revealed that wild *H. angustifolia* can be used as a potential source of natural antioxidant and neuroprotection agents for improving human health, due to richness of bioactive phytochemicals.

Chapter 3 Tissue culture of *H. angustifolia*

3.1 Introduction

Nowadays, medicinal herbs play a very important role worldwide. They are widely used in various industry sectors, such as phytochemicals, pharmaceuticals, nutraceutical, herbal remedies, food supplements and perfumes and cosmetics, among other uses (Tripathy, 2015). Especially in pharmaceutical industry, increasing attentions were paid to the development and application of wild medicinal plants due to their considerable health and economic values in clinical treatment of various diseases. According to WHO estimates, the demand for medicinal plants will increase to $ 5 trillion by the year 2050 (Tripathy et al., 2015). Huge demand and rapid climate change are decreasing the wild populations of medicinal plants (Wan et al, 2014). Meanwhile, the tremendous demand also has raised concerns for the quality control and safety of medicinal herbs. Currently, environmental contaminants have become one of the major factors resulting in poor quality and safety of medicinal herbs, such as pesticides, heavy metals, microbial load, polycyclic aromatic hydrocarbons (PAHs). Environmental contaminants accumulations in plants during cultivation have a direct effect on quality and safety of medicinal herbs with high risk for health. There is an urgent demand for providing the sufficient and safe raw materials capable of accumulation of bioactive secondary metabolites in order to sustainably develop and utilize wild plants herbs.

The plant *in vitro* tissue and callus culture methodology was submitted basing on the core theory of cell totipotency (Dias et al., 2016), as an alternative scheme for supplying sufficient and safe raw materials and production of bioactive secondary metabolites. Tissue and callus culture have many advantages such as species diversity protection, independence from climatic and geographical conditions, independence from environmental contaminants, more control over biosynthetic routes for target components, simpler extraction and purification, shorter production cycles and more effective production of biosynthetic secondary metabolites (Matkowski, 2008). *H. angustifolia* has been confirmed to possess many pharmacological functions such as

anti-oxidant, anti-bacterial, anti-inflammatory, anti-cancer and anti-viral activities, etc. (Wang, 2012; Li, 2015). These functions are considered to come from biosynthetic secondary metabolites, which are found frequently accompanied by less side effects and lower rates of secondary failure than synthetic ones in chemical methods. At present, few reports were about plant *in vitro* culture of *H. angustifolia.* Our previous studies mainly contributed to establishment of shoot and callus induction medium. More attentions should be paid to explore the tissue and cell suspension cultures of *H. angustifolia* for its sustainable and high-efficient industrial utilization. The callus and tissue cultivation could be a viable alternative for germplasm conservation of rare and endangered plants and sustainable utilization of wild medicinal herbs.

The objective of this chapter was to (i) investigate the effects of types, concentrations and proportions of PGRs on shoot induction and callus induction, (ii) establish the adventitious shoot and callus suspension culture approaches of *H. angustifolia,* and (iii) provide a basic cultivation system for the large-scale cultivation of the substitutes for wild medicinal herbs.

3.2 Materials and methods

The living plants of *H. angustifolia* were collected in the rural area near by Vientianei city in Laos in 2013. The species of plant was identified by Dr. Ende Liu employed in Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China, of which a voucher specimen (No. 090155) was deposited at the herbarium of the institute. The living plants were cultivated in phytotron with 3000 lux light at 25 ± 1 ℃ for the following experiments.

3.2.1 Surface sterilization of explants

Healthy and disease free young explants were washed thoroughly by detergents for 5 minutes without damaging the tissues. The explants were surface sterilized with 70% (v/v) ethanol for 30 s. Then the explants were treated with 100 mL of 2% (m/v) sodium hypochlorite solution combined with 5-6 drops of Tween 80, and shaken continuously. After 8-13 min, the disinfectant agent was removed. The explants were washed with sterile water for 5 times at 5 min internal.

3.2.2 Induction of adventitious shoots

The sterilized nodal segments were cultured on MS medium supplemented with different concentrations of cytokinins and auxins (BAP, KIN and NAA), 3% (w/v) sucrose, 0.7% (w/v) agar, and incubated at 25 ± 1 ℃ under a 16/8 h light/dark photoperiod using cool-white fluorescent light (3000 lx). The pH value of media was adjusted to 5.8 ± 0.03 by 1 M NaOH and 1 M HCl before autoclaving. After 1 month, the number, length, fresh weight and dry weight of adventitious shoots were calculated.

3.2.3 Callus induction

Surface sterilized leave explants were cultured on MS medium supplemented with different concentrations of PGRs (0 - 5.0 mg/L NAA), 0.4 mg/L vitamin C, 3% (w/v) sucrose and 0.6 % agar, and maintained at 28 ℃ in dark. The pH value of media was adjusted to 5.80 ± 0.03 by 1 M NaOH and 1 M HCl before autoclaving. The induction ratios, callus morphology and intensity of callus were recorded after 30 days. After optimization of NAA concentration, effects of the addition of 0 - 1.0 mg/L BA on callus induction were investigated.

3.2.4 Growth curve and callus suspension culture

4-month-old callus were inoculated into a liquid MS medium supplemented with 3.0 mg/L NAA, 0.07 mg/L BA, 0.4 mg/L vitamin C, 3% (w/v) sucrose. The pH value of media was 5.80 ± 0.03. The cultures were placed on a rotary shaker set at 80 rpm, maintained at 28 ℃ in dark with an inoculum density of 6 g/L. The kinetic curve of biomass formation by *H. angustifolia* callus cultures was investigated in 60 days.

Next, 4-month-old callus into an optimized liquid MS medium supplemented with 3.0 mg/L NAA, 0.07 mg/L BA, 0.4 mg/L vitamin C, 3% (w/v) sucrose. The pH value of media was 5.80 ± 0.03. The cultures were placed on a rotary shaker set at 80 rpm, maintained at 28 ℃ in dark with an inoculum density of 50 g/L for 60 days. Callus suspension cultures were established from the 4-month-old callus. Fresh weight and dry weight of cultures were detected.

3.2.5 Determination of suspension cultures and shoots biomass

The suspension cultures were separated from the media by using 0.45 μm filters (Sigma). The callus and shoots were dried at 60 °C till constant weight and their dry weights were recorded.

3.2.6 Statistical analysis

All the experimental results were presented as mean ± S.E. of three parallel measurements. Results were processed by Microsoft Excel (2010).

3.3 Results and discussion

3.3.1 Effects of cytokinins on adventitious shoot induction

For clonal propagation of plant species, it is a better approach to induce adventitious shoots from plant explants than from callus. Generally, adventitious shoots derived directly from explants can form uniform diploid individuals while callus produces abnormal plants (Jalali et al., 2012). In our study, after about 1 week the adventitious shoots germinated from internode explants. Results are as shown in Table 3-1. BA and KIN increased germination ratio and mean number of shoot, and promoted the accumulation of biomass to some extent. After treatment of the different cytokinin types, the maximum biomass accumulation during cultivation of internodes was observed at 0.50 mg/L of BA and 0.10 mg/L of KIN, the biomass accumulations reached 6.96 ± 0.44 mg/explant and 9.8 ± 0.75 mg/explant, respectively. The effect of cytokinin on biomass accumulation weakened with increasing of concentrations of cytokinin (BA and KIN).

Table 3-1 Effects of BA and KIN on shoot induction of *H. angustifolia*.

BA (mg/L)	KIN (mg/L)	Germination ratio (%)	Number (shoot/explant) ± S.E.	FW (mg/explant) ± S.E.	DW (mg/explant) ± S.E.
0	0	78	1.00 ± 0.00	23.43 ± 1.13	3.17 ± 0.55
0.1		100	1.89 ± 0.19	35.82 ± 1.68	4.92 ± 0.25
0.5		100	2.11 ± 0.19	49.29 ± 2.12	6.96 ± 0.44
1.0		100	2.00 ± 0.00	42.38 ± 0.64	5.84 ± 0.47
2.0		78	1.33 ± 0.88	27.87 ± 2.20	3.87 ± 0.46
	0.1	100	2.00 ± 0.30	53.51 ± 1.64	9.8 ± 0.75
	0.5	100	1.00 ± 0.00	28.75 ± 0.05	5.6 ± 0.33
	1.0	100	1.78 ± 0.39	28.12 ± 0.78	5.05 ± 0.05
	2.0	100	1.11 ± 0.19	19.14 ± 1.69	3.43 ± 0.44

S.E.: standard error.

3.3.2 Effects of relative ratio of auxin to cytokinin on adventitious shoot induction

Usually, the relative ratio of auxin to cytokinin is an important factor for organ differentiation. Low relative ratio of auxin to cytokinin is beneficial for differentiation of adventitious shoots while high relative ratio of auxin to cytokinin is beneficial for differentiation of adventitious roots. In our study, the germination ratios of adventitious shoots were inhibited to 89% at a low relative ratio of auxin to cytokinin (1:20). Results did not reveal facilitation effect on differentiation and biomass accumulation of adventitious shoots at low relative ratio of auxin to cytokinin (1:10 and 1:20). On the other hand, high relative ratio of auxin to cytokinin promoted adventitious shoots form and their biomass accumulation (1:1 and 1:5).

Table 3-2 Effect of relative ratio of auxin to cytokinin on shoot induction

Relative ratio of auxin to cytokinin	NAA (mg/L)	BA (mg/L)	Germination ratio (%)	Number (shoot/explant) ± S.E.	FW (mg/explant) ± S.E.	DW (mg/explant) ± S.E.
1: 1	0.1	0.1	100	1.78 ± 0.44	41.68 ± 1.56	6.61 ± 0.44
1: 5	0.1	0.5	100	1.89 ± 0.60	23.00 ± 1.58	4.78 ± 0.66
1: 10	0.1	1.0	100	1.33 ± 0.71	19.11 ± 0.62	3.03 ± 0.58
1: 20	0.1	2.0	89	1.22 ± 0.67	14.11 ± 1.72	3.00 ± 0.55

S.E.: standard error.

3.3.3 Effects of auxins on callus induction

The effect of different auxin types on callus induction was investigated. The callus generated in MS with NAA revealed longer survival time than the callus which generated in MS with IBA or IAA (Data not shown). In this study we mainly investigated the effect of NAA on callus induction. For callus induction, among the selected nodal, intermodal and leaf as explants, leaf was the best explants for callus induction (Data not shown). Therefore, further studies were carried out with leaf. As shown in Table 3-3, the presence of NAA in the culture media induced callus formation. During 0.50 - 5.00 mg/L of NAA the callus induction ratio was improved with increasing concentrations of NAA. Low concentrations of NAA induced poor and compact callus while high concentrations of NAA induced the profuse and wet callus. The friable callus was observed in MS media with 3.00 mg/L of NAA. Friable callus was reported to be more suitable for suspension culture.

Table 3-3 Effects of auxin on callus induction

NAA(mg/L)	Induction ratio (%)	Intensity of callus formation	Callus morphology
0	0	-	-
0.1	0	-	-
0.5	20	+	Compact
2.0	60	+	Compact
3.0	80	++	Friable
4.0	100	+++	Wet
5.0	100	++	Wet

+++: Profuse callus, ++: Moderate callus, +: Poor callus, -: No response.
Culture conditions: MS medium suppled with plant growth regulator (NAA) and cultured at 28 ℃ in dark.

3.3.4 Effects of auxin combined with cytokinin on callus induction

The effects of NAA (3.00 mg/L) combined with BA (0-1.00 mg/L) on callus induction were also investigated. Results indicated that profuse callus was observed in MS suppled with 3.0 mg/L NAA and 0.07 mg/L of BA. Callus induction was inhibited with increasing concentrations of BA.

3.3.5 Growth kinetic curve of callus suspension culture

The growth of cells goes through four phases including lag, log, stationary and decline phases. Lag phase is characterized by slow increase in dry biomass of cultures. And a relatively quick growth is observed in log phase, followed by a stationary phase without obvious variation in dry biomass of cultures. The final decline phase was characterized by decrease in dry biomass (Ali et al., 2013). Callus was induced in MS media with 3.00 mg/L of NAA. They were inoculated in suspension culture system at an inoculum density of 0.425 ± 0.009 g/L (DW). The curve of biomass formation of the callus suspension culture of *H. angustifolia* was displayed in Figure 3-1. The callus initiated a continuous growth phase of 21 days at day 7 (DW: 0.595 ± 0.040 g/L). Followed by a stationary phase of 28 days, the maximum dry weight reached to 1.054 ± 0.100 g/L. Biomass accumulation of callus culture showed two-fold increase in dry weight. In this study, the stationary phase of suspension culture was initiated in the 28 day. The growth cycle was similar to *Habenaria edgeworthii* and *Artemisia absinthium* L (Giri et al., 2012; Ali and Abbasi, 2014). But biomass accumulation was significantly lower than these two kinds of plant cell cultures, which might be attributed to culture conditions including light, pH, temperature, inoculum density, et al. These conditions were reported to influence the fluctuation of biomass accumulation of callus (Nagella and Murthy, 2010).

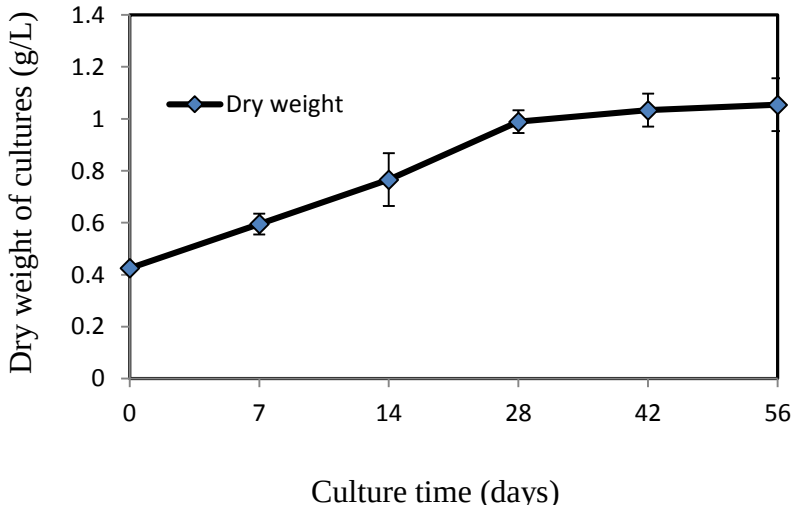
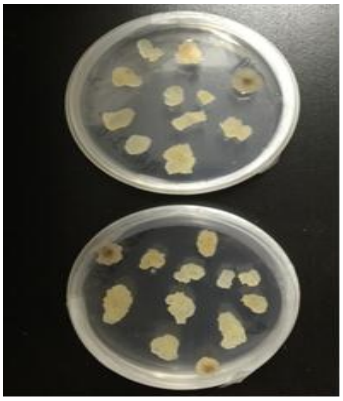


Figure 3-1 The growth curve of suspension culture

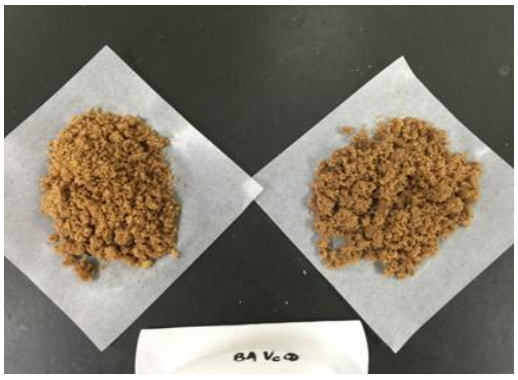
3.3.6 Establishment of callus suspension culture

Optimization results of callus culture medium exhibited that profuse callus was obtained in MS suppled with 3.0 mg/L NAA and 0.07 mg/L of BA. Thus, this study attempted to establish the callus suspension culture of *H. angustifolia* in liquid medium with 3.0 mg/L NAA and 0.07 mg/L of BA. As shown in Figure 3-2 (a), 4-months-old callus generated in MS culture media supplemented with 3.00 mg/L NAA, 3% (w/v) sucrose, 0.6% agar, in the dark, which was sub-cultured at an interval of two weeks to extend culture. Then 2.50 g (fresh weight) callus were inoculated into 50 mL liquid culture media (pH = 5.8 ± 0.03) supplemented with 3.0 mg/L NAA, 0.07 mg/L BA, 0.4 mg/L vitamin C, 3% (w/v) sucrose with an inoculum density of 50 g/L . The cultures were placed on a rotary shaker set at 80 rpm, maintained at 28 ℃ in dark for cultivation of 60 days. Callus growth initiated and entered a stationary phase with obvious accumulation of biomass, as shown in Figure 3-2 (b). Finally, Figure 3-2 (c) showed that callus were collected. After 2 months of cultivation time, the fresh weight of suspension cultured callus reached to 148.7 ± 8.91 g/L (DW: 16.62 ± 0.92 g/L) in optimized liquid culture medium with supplement of BA 0.07 mg/L, as shown in Table 3-4. A three-fold increase in biomass accumulation of callus was detected.



(a) Callus cultured on solid medium

(b) Callus inoculated in suspension culture medium



(c) Plant callus suspension cultures

Figure 3-2 The callus suspension culture in MS media suppled with 3.0 mg/L NAA and 0.07 mg/L BA

Table 3-4 Callus suspension cultures

Type of PGR	Inoculum size (FW: g/L)	Culture time (days)	FW (g/L) ± S.E.	DW (g/L) ± S.E.
NAA (3.0 mg/L) + BA (0.07 mg/L)	50.2 ± 6.4	60	148.7 ± 8.91	16.62 ± 0.92

S.E.: standard error.

3.4 Summary

This study investigated the effects of PGRs on adventitious shoots induction and callus induction, and established the induction culture medium of aerial growth part and suspension culture protocol by using axillary bud and callus of *H. angustifolia* The maximum biomass accumulation during cultivation of internodes was observed at 0.10 mg/L of KIN, and the biomass accumulations reached to 9.8 ± 0.75 mg/explant. The callus suitable for suspension culture was induced in MS with 3.00 mg/L NAA and 0.07 mg/L BA. After approximately 2 months of cultivation time, the callus suspension culture system reached stationary phase. In this phase, the fresh weight of suspension cultured callus reached to 148.7 ± 8.91 g/L (DW: 16.62 ± 0.92 g/L) with 3-fold growth than the initiate inoculum (50.20 ± 6.40 g/L). Therefore, the *in vitro* cultured aerial growth part and callus were obtained successfully. This cultivation method offered an alternative substitute for wild medicinal plants resources. Further research is necessary to improve the biomass accumulation capability of aerial growth part and suspension cultured callus *H. angustifolia* by optimizing the culture conditions and addition of elicitors.

Chapter 4 Comparation in antioxidant and neuroprotective potential of tissue cultured and wild *H. angustifolia*

4.1 Introduction

It is worth mentioning that plant *in vitro* callus suspension culture is considered as a feasible approach for large-scale production with both sustainability and high-efficiency. It was employed as "green factories" to produce assorted nutraceutical and medicinal compounds (Davies and Deroles, 2014; Matkowski, 2008; Thomas De Vijlder, 2015; Thanh et al., 2014). Both plant *in vitro* tissue and callus cultures can enhance the yields of secondary metabolites that are either difficult to synthesize chemically or are produced in limited quantities in wild plants by the optimization of culture conditions, addition of elicitors, precursors and biotransformation etc. (Loganathan and Bai, 2014; Davies and Deroles, 2014; Ali et al., 2013; Dias et al, 2016). At present, they have been used to produce a serious of high-value and therapeutic metabolites which can be used as flavourings, colourants, essential oils, sweeteners, antioxidants, nutraceuticals and pharmaceuticals, etc (Murthy et al., 2014; Murthy et al., 2015). Furthermore, many invaluable phytochemicals can also be synthesized and are about 4 times as much as microorganisms (Gueven and Knorr, 2011). Thus, plant *in vitro* tissue and callus cultures show a novel potential for commercial production of bioactive secondary metabolites of medicinal herbs.

Plant tissue and callus suspension culture emerges as a viable biotechnological tool for the production of bioactive compounds. The objective of this chapter was to investigate the potential biosynthetic capacity of tissue cultures and callus suspension cultures of *H. angustifolia* to accumulate and produce anti-oxidative and neuroprotection substances. In the present work, we evaluated (i) the effect of PGRs on the total phenolic and total flavonoids contents, (ii) free radical scavenging activity, (iii) neuroprotection effect on H_2O_2-induced IMR32 cells damage model, (iv) investigate functional groups existed in extracts and (v) to compare with wild plants.

4.2 **Materials and methods**

4.2.1 Extracts preparation

The wild roots, adventitious shoots and callus of *H. angustifolia* were ground into fine powders, and soaked into 500 mL of 80% (v/v) ethanol for 24 h. The mixtures were boiled for 2 h, repeated 3 times. The supernatants were concentrated via vacuum evaporation and frozen-dried, stored at – 20 ℃ for tests. The 80% ethanolic extracts of wild plants roots (RE), *in vitro* cultured shoots (SE) and suspension callus (SCE) were obtained, and subjected to the following tests.

4.2.2 Determination of TPC and TFC in cultures

10 mg cultures powders were extracted using 5 mL of 80 % ethanol solution in a tube. The tube was placed on a rotary shaker set at 120 rpm at room temperature for 24 h. The supernatant were performed to total phenolic and flavonoids contents measurements as described in Chapter 2 (2.2.5).

4.2.3 Phytochemical analysis

Extracts were performed to preliminary phytochemical analysis to detect the presence of phytochemicals, i.e. tannins, alkaloids, Steroids and triterpenoids, and also subjected to quantitative determination of several important bioactive compounds such as phenolic, flavonoid, carbohydrate and proteins. The standard methods can be referred to Chapter 2 (2.3, 2.4).

4.2.4 Quantification of several bioactive components in extracts from wild and tissue cultured *H. angustifolia*

The determination methods have been described in Chapter 2 (2.2.5).

4.2.5 Antioxidant activity

Extracts were subjected to DPPH and OH• scavenging activity assays. DPPH scavenging activity assay was conducted as described in Chapter 2 (2.5.1).

The OH• scavenging activity was measured by using the Fenton method (Smirnoff and Cumbes, 1989) with some slight modifications. The reaction mixture (2.5 mL) contained 0.5 mL of 1.5 mM $FeSO_4$, 0.35 mL of 6 mM H_2O_2, 0.15 mL of 20 mM sodium salicylate and 1 mL of sample solution. Ascorbic acid (Sigma, Saint Louis, MO, USA)

was used as the positive control. The reaction was initialed by adding H_2O_2 into the system. After incubation at 37 ℃ for 1 h, the absorbance of the hydroxylated salicylate complex was measured at 562 nm. The OH• scavenging activity was calculated as follows:

$$\text{OH• scavenging activity (\%)} = (1-\frac{A_1-A_2}{A_0})\times 100 \qquad (4\text{-}1)$$

where A_0 is the absorbance of the solvent control, A_1 is the absorbance of the sample or ascorbic acid, and A_2 is the absorbance of the reagent blank without sodium salicylate, respectively.

4.2.6 Cytotoxicity and cell viability

The cytotoxicity of 80% ethanolic extracts on IMR32 cells were determined by MTT assay (Zhang et al., 2011) as described in Chapter 2 (2.8.2).

IMR32 cells were seeded into 96-well plate at a density of 5×10^3 cells/well. After 12 h, cells was treatment using serum free medium with 400 μM H_2O_2 for 2 h. Culture medium was replaced with fresh medium combined with various concentrations of extracts for 24 h. The cell viability ratio was measured by MTT.

4.2.7 FT-IR spectral analysis

As a widely used method for structure elucidation, FT-IR has been gradually applied into the classification and identification of various natural products from herbal and microorganism, etc. Especially for the mid-infrared spectra scanned in the region of 4000-400 cm^{-1}, FT-IR spectra can be used to identify the presence of relevant functional groups of natural products (Fan et al., 2013). Here, FT-IR spectroscopy analysis was conducted to investigate the functional groups in the 80% ethanolic extracts from *in vitro* cultivated cultures, finally to explore and clarify the differences of bioactive functional groups and fingerprint between wild plants and *in vitro* cultures. The extracts were ground with potassium bromide (KBr) to fine powder, placed under high pressure until formation of a euphotic film. The sample was scanned in the range of 4000-400 cm^{-1} in FT/IR 300 (JASCO).

4.2.8 ^{1}H NMR spectra analysis

All extracts extracted by 80% ethanol solution were analyzed on a Bruker Avance 600 MHz spectrometer. 7 mg of samples were added to 0.6 mL deuterium oxide (D_2O) in a 5 mm NMR tube. Data were recorded between 0.00-10.00 ppm of the chemical shift region.

4.3 Results and discussion

4.3.1 Effects of PGR on important bioactive components (phenolic and flavonoid) production in *in vitro* cultures

In Chapter 2, extracts of *H. angustifolia* rich in total phenolic and flavonoid components showed strong antioxidant abilities. Many reports show biosynthetic phenolic and flavonoid are important plant secondary metabolites with multiple bioactive functions such as anti-oxidation, anticancer, anti-inflammation, anti-microbial and neuroprotection abilities, etc. They were widely used in pharmaceutical, food and cosmetics industries, due to multiple bioactivities and low cytotoxicity than chemosynthetic compounds (Sarvestani et al., 2013; Martinsa et al., 2016; Roleira et al, 2015; Cheng et al., 2015). Their commercial production has become a valuable research field attracting increasing attentions. In plant *in vitro* cultivation process, types and concentrations of PGR which can affect plant cell and organ differentiation, furthermore, influence the biosynthetic process of target products including phenolic and flavonoid components. Eventually, PGR become a key factor resulting in the different levels of the useful products accumulation. In this context, our study investigated the effects of PGR on TPC and TFC in *in vitro* cultures and optimized the culture medium to produce shoots and callus.

(1) Effects of PGR on TPC and TFC in adventitious shoots

The effects of the PGR (BA and KIN, in dose range of 0.0 - 2.0 mg/L) on TPC and TFC in adventitious shoots were investigated and tabulated in Table 4-1. TPC and TFC in shoots showed a positive relationship with biomass accumulation. Both BA and KIN exhibited improvement effects on phenolic and flavonoid accumulation in shoots. KIN showed a stronger facilitation on phenolic and flavonoid accumulation than BA. The

maximum TPC (126.41 ± 1.36 mg GAE/g sample) and TFC (530.91 ± 6.67 mg RE/g sample) were detected in culture media with the supplementation of 0.1 mg/L KIN and accompanying with maximum biomass accumulation. With the increase in PGR concentrations, the promotion effect on phenolic and flavonoid accumulation decreased obviously. Our study indicated that, in cultivation of the axillary bud-mediated adventitious shoots, the types and concentrations of PGRs influenced their production of secondary metabolites such as total phenolic and flavonoids as described by Paula et al. (2002).

Table 4-1 Effects of PGR on TPC and TFC in adventitious shoots

BA (mg/L)	KIN (mg/L)	DW (mg/explant) ± S.E.	TPC (mg GAE/g sample) ± S.E.	TFC (mg RE/g sample) ± S.E.
0	0	3.17 ± 0.55	95.67 ± 3.53	334.83 ± 5.36
0.1		4.92 ± 0.25	78.18 ± 1.13	273.45 ± 0.83
0.5		6.96 ± 0.44	104.90 ± 3.40	389.27 ± 5.00
1.0		5.84 ± 0.47	93.12 ± 0.23	331.78 ± 4.17
2.0		3.87 ± 0.46	81.80 ± 1.59	313.45 ± 0.83
	0.1	9.8 ± 0.75	126.41 ± 1.36	530.91 ± 6.67
	0.5	5.6 ± 0.33	106.71 ± 2.04	436.76 ± 0.83
	1.0	5.05 ± 0.05	103.09 ± 2.04	432.59 ± 1.67
	2.0	3.43 ± 0.44	88.14 ± 2.04	352.61 ± 1.67

S.E.: standard error.

(2) Effects of PGR on TPC and TFC in callus

As shown in Table 4-2, in range of 0-1.0 mg/mL, the increasing BA promoted the accumulation of phenolic and flavonoid components in callus. The results indicated that cytokinin played an important role in secondary metabolite. In future works, more efforts should be paid to the optimization of cytokinin types and concentrations in order to get more useful compounds.

Table 4-2 Effects of PGR on TPC and TFC in callus

NAA (mg/L)	BA (mg/L)	TPCs (mg GAE/g sample) ± S.E.	TFCs (mg RE/g sample) ± S.E.
3.0	0.01	62.32 ± 0.91	128.26 ± 3.85
3.0	0.05	69.57 ± 0.45	138.81 ± 3.33
3.0	1.00	155.78 ± 0.94	354.61 ± 5.89

S.E.: standard error.

4.3.2 Biosynthetic capability study of *in vitro* cultures

(1) Preliminary phytochemical screening of *in vitro* cultures

All extracts were subjected to preliminary phytochemical assay. The results are shown in Table 4-3. Alkaloids and steroids were found to be absent in both 80% ethanolic extracts of cultures and wild plants. The presence of tannins was observed in all extracts. The presence of triterpenoids was detected in extracts from the callus suspension cultures (SCE) and the roots of wild plants (RE), while it was not formed in extracts of adventitious shoots (SE).

Table 4-3 Preliminary phytochemical assay of *in vitro* cultures

Phytochemicals	SE	SCE	RE
Tannins	+	+	+
Alkaloids	-	-	-
triterpenoids	-	+	+
Steroids	-	-	-

+: Present and -: Not present

SE: Adventitious shoots extracts. SCE: Callus suspension cultures extracts. RE: Roots extracts of wild plants.

(2) Contents of several important bioactive components in *in vitro* cultures

Compared with the roots of wild plants, the cultures exhibited different biosynthesis potential in phenolic, flavonoid, carbohydrate and proteins production. As shown in Table 4-4, the extracts of *in vitro* cultured adventitious shoots (SE) contained 126.41 ± 1.36 mg GAE/g extract of TPC, 530.91 ± 6.67 mg RE/g extract of TFC, 75.77 ± 1.34 mg/g of TCC and 50.38 ± 1.57 mg/g of TPSC. The extracts of *in vitro* cultured callus (SCE) contained 72.74 ± 1.13 GAE/g extract of TPC, 230.13 ± 0.83 mg RE/g extract of TFC, 151.38 ± 1.56 mg/g of TCC and 56.09 ± 1.94 mg/g of TPSC. The

extracts of wild plants (RE) contained 122.11 ± 1.59 GAE/g extract of TPC, 518.41 ± 2.50 mg RE/g extract of TFC, 459.69 ± 4.35 mg/g of TCC and 44.70 ± 1.85 mg/g of TPSC. The cultures also showed a promising biosynthetic capability.

Table 4-4 Preliminary phytochemicals assay in *in vitro* cultures

Sample	TPCs (mg GAE/g extract) ± S.E.	TFCs (mg RE/g extract) ± S.E.	TCCs (mg/g extract) ± S.E.	TPSCs (mg/g extract) ± S.E.
SE	126.41 ± 1.36	530.91 ± 6.67	75.77 ± 1.34	50.38 ± 1.57
SCE	72.74 ± 1.13	230.13 ± 0.83	151.38 ± 1.56	56.09 ± 1.94
RE	122.11 ± 1.59	518.41 ± 2.50	459.69 ± 4.35	44.70 ± 1.85

S.E.: standard error. SE: Adventitious shoots extracts. SCE: Callus suspension cultures extracts. RE: Roots extracts of wild plants.

4.3.3 Antioxidant activity

The DPPH radical scavenging ability of cultured wild *H. angustifolia* was detected. Ascorbic acid as the control with the EC_{50} value of 0.10 ± 0.00 mg/mL, all extracts possessed DPPH radical scavenging activities in a dose-dependent manner (Figure 4-1). A descending order of DPPH radical scavenging activity was determined as RE > SE > SCE. Their EC_{50} values were 0.50 ± 0.00 mg/mL, 0.54 ± 0.01 and 0.93 ± 0.01 mg/mL, respectively. The cultured shoots and callus exhibited strong antioxidant ability. The cell and tissue culture systems of this plant were proven with promising biosynthetic capability of antioxidant compounds for the first time.

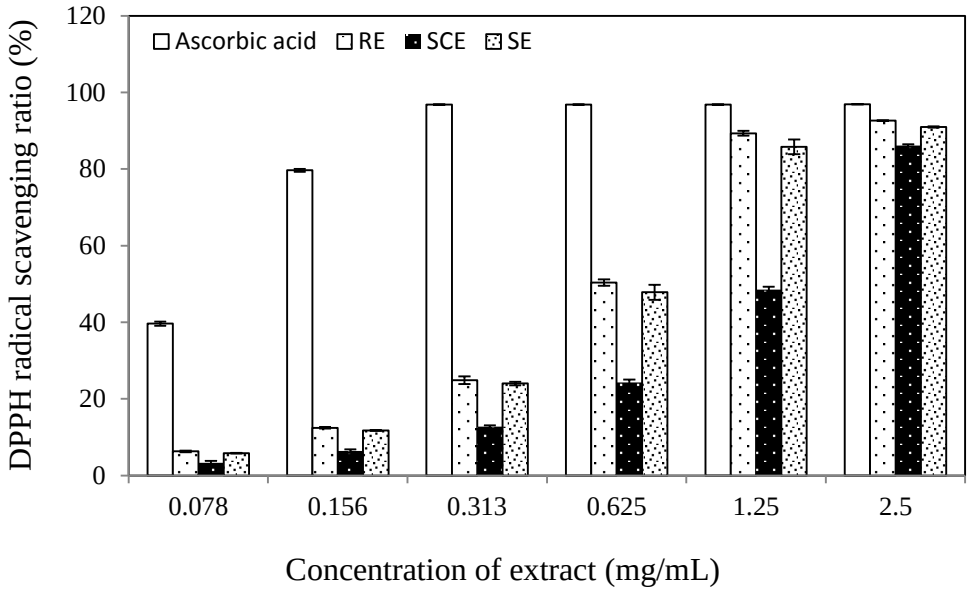


Figure 4-1 DPPH radical scavenging ability

The OH radical, one of the reactive oxide species, can react with a series of biological molecules such as proteins, lipids and DNA, ultimately resulting in cell damage and even death (Nandhakumar and Indumathi, 2013). Thus, OH scavenging activity, very important for screening natural protective agents of living systems, confirms bioactive value of natural protective agents to some extent. The present study evaluated OH radical removing capability of cultures and compared with the ones of wild plants shown in Figure 4-2. The results revealed that extracts exhibited obvious scavenging activities in a dose-dependent manner at a concentration of 0.078-2.500 mg/mL. SE (EC_{50} = 0.33 ±0.05 mg/mL) showed scavenging ability similar to RE (EC_{50} = 0.21 ±0.03 mg/mL). SCE showed an obvious scavenging ability with EC_{50} value of 0.97 ±0.08 mg/mL. Moreover, the variation of antioxidant activity in different assays is attributed to differences in the mechanism, which also has been observed by other researchers (Giri et al., 2012)

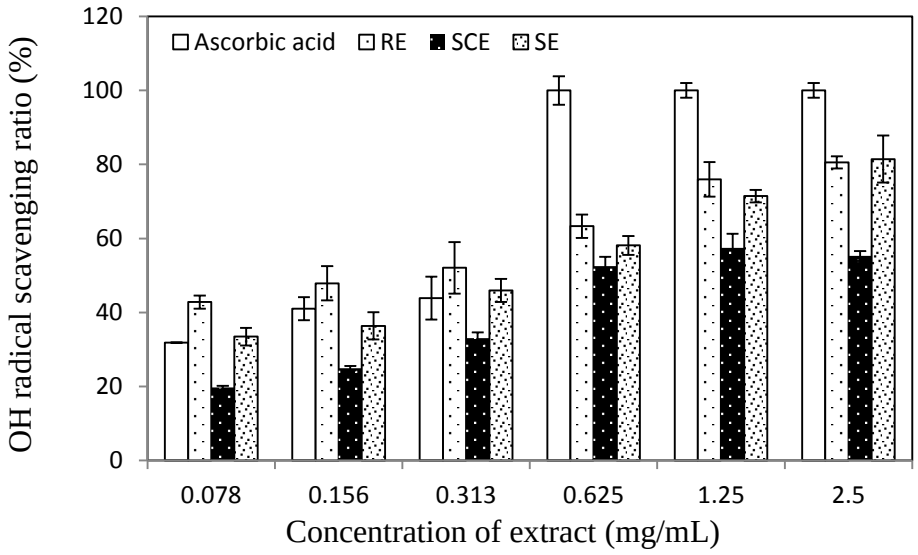


Figure 4-2 Hydroxyl radical scavenging ability

In summary, SE showed the slight greater antioxidant ability than SCE, although they possessed similar extraction ratios. The results might be related to degree of plant cell differentiation. Organs possessed higher differentiation level than plant callus. They might generate more amounts of secondary metabolites or more complex in classification. Thus, it is necessary to study the classification and improvement of secondary metabolites in different culture methods. Otherwise, in many researches the obvious improvement of antioxidant activities has been observed by enhancing accumulation of secondary metabolites caused by addition of elicitors and optimization of culture conditions (Gueven and Knorr, 2011; Nagella and Murthy, 2010). It is thus clear that *in vitro* cultures show a promising biosynthetic potential for natural antioxidants production of *H. angustifolia*. More efforts should focus on enhancing of useful compoents.

4.3.4 Neuroprotection potential of *H. angustifolia* detected by using H_2O_2-injuried IMR32 cells model

MTT assay was performed to test the cytotoxic effects of the extracts on IMR32 cell growth in a wide range of drug doses (0 - 100 μg/mL). All extracts did not exhibit obvious reduction effects on cell proliferation after drug treatment for 24 h.

Next, neuroprotection effects of extracts were evaluated in H_2O_2-injuried IMR32 cell model, and results were shown in Figure 4-3. All extracts presented protection effects against H_2O_2-induced reduction of cell viability in a dose-dependent manner. After RE, SCE and SE treatments for 24 h, cell viability ratio of IMR 32 cells were 16.40 ± 3.29 %, 13.61 ± 0.32 %, 11.75 ± 2.79 %, respectively. The results indicated that the tissue cultured *H. angustifolia* possessed neuroprotection potential. SE and SCE showed slight weaker effects than wild *H. angustifolia* on cell viability in H_2O_2-injuried IMR32 cells. The results might be related to the amounts and classification of secondary metabolites in cultures. Fortunately, many reports have confirmed that the yields of adventitious shoots can be improved significantly by callus-mediated plantlet regeneration, the yields of callus can be improved significantly by optimization of culture conditions, the control in metabolize route, etc. Hence, we can obtain more useful components by enhancing yields. Meanwhile, it is necessary to study the classification and improvement of secondary metabolites in different culture methods.

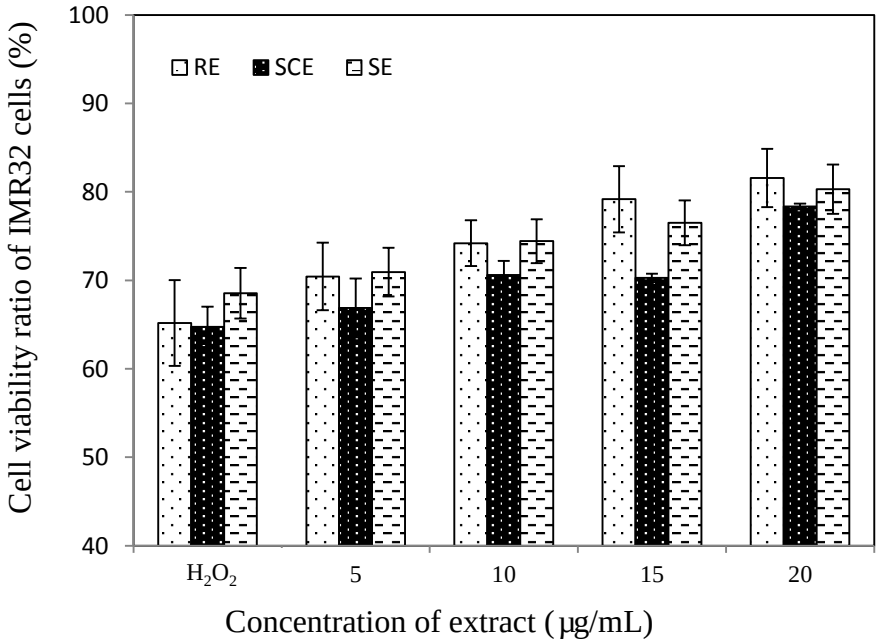
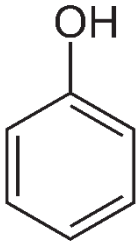


Figure 4-3 Effect on cell viability of H_2O_2-injuried IMR32 cells

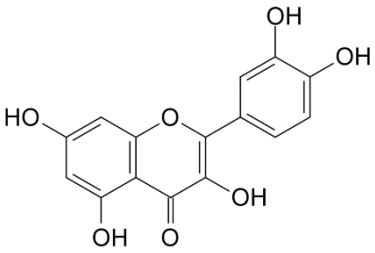
4.3.5 Bioactive functional groups

The bioactive functions are attributable to phytochemicals including phenol, flavonoids, terpenoids and tannins et al. Their chemical structures are shown in Figure 4-4. These phytochemicals have various functional groups which affect their chemical and physical properties and have close inner link with their bioactive functions (Poojary et al., 2015). This study investigated the functional groups in extracts from cultured shoots, callus and wild plant roots to better understand medicinal properties and activities potential. As shown in Figure 4-5. FT-IR spectral data ranging in 4000-400 $cm^{-1}$of extracts were recorded. Results showed all extracts exhibited broad intense peaks at around 3400 cm^{-1} attributed to the OH group. These OH stretching ranging from 3500-3100 cm^{-1} were characteristic of polyphenolic extracts (Siu et al., 2016; Grasel et al., 2016). The peaks ranging from 2962 cm^{-1} to 2848 cm^{-1} (around 2920 cm^{-1}) were assigned to the weak C–H stretching vibration (Raveendra et al., 2016; Siu et al., 2016; Pawlaczyk et al., 2013). The peaks ranging from 1477 cm^{-1} to 1625 cm^{-1} were attributed to the C=C aromatic stretch (Sert et al., 2015). These peaks appeared in region 1135-1031 cm^{-1} which were attributed to the polysaccharides, glycosides carbohydrate bending vibrations of C–O. These observed functional groups suggested the possible presence of monodesmosidic saponins as described by Kareru et al (2008). Otherwise, peaks distributed in the range of 940-810 cm^{-1} and at 778.14 cm^{-1}, which can be attributed to the presence of polysaccharides (Zhu et al., 2014; Pawlaczyk et al., 2013). Phenolics, polysaccharides and saponins, these bioactive compounds were observed in FTIR spectra data of three extracts. Cultures and wild plant roots had presence of five common functional groups which were amide/amine, alkane, alcohol, alkyl halide and sulfonates except aldehyde, aromatic (nitro) and phosphines (Table 4-5). Among them, the cultures showed more similar IR spectra. But they exhibited different levels of antioxidant activity. This result might be attributed to the fluctuation in bioactive compounds which responsible for antioxidant activity (phenolics and flavonoids), as shown in Table 4-4. SE contained equal TPC and TFC to RE, SCE contained lower TPC and TFC. In summary, both cultivated shoots and callus showed a promising

biosynthetic capability, and had a very high medicinal value and industrial usability. But the differences between wild and tissue cultured cultures need to be investigated.

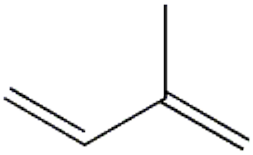


Phenol

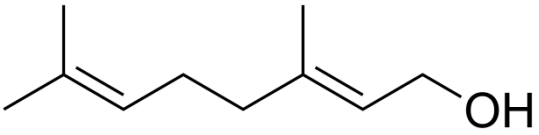


Flavonoids

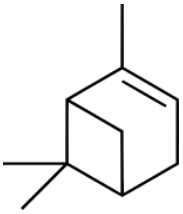
Types of terpenoids



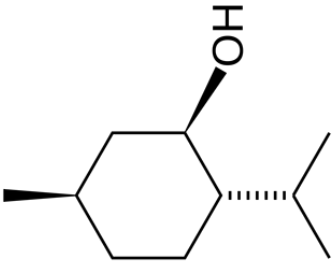
lsoprene



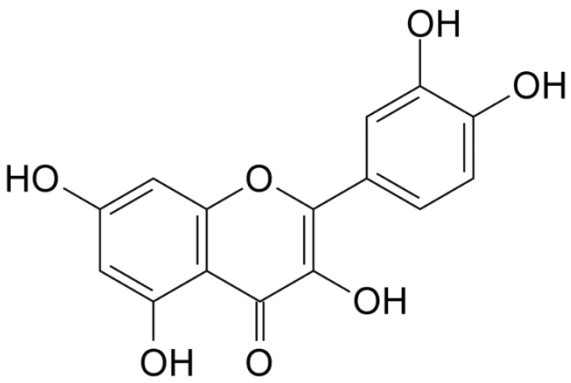
Geraniol (in geranium)



Pinene
(in pine resin)



Menthol (in mint)



Tannins

Figure 4- 4 Chemical structures of the phytochemicals (Vignesh et al., 2015)

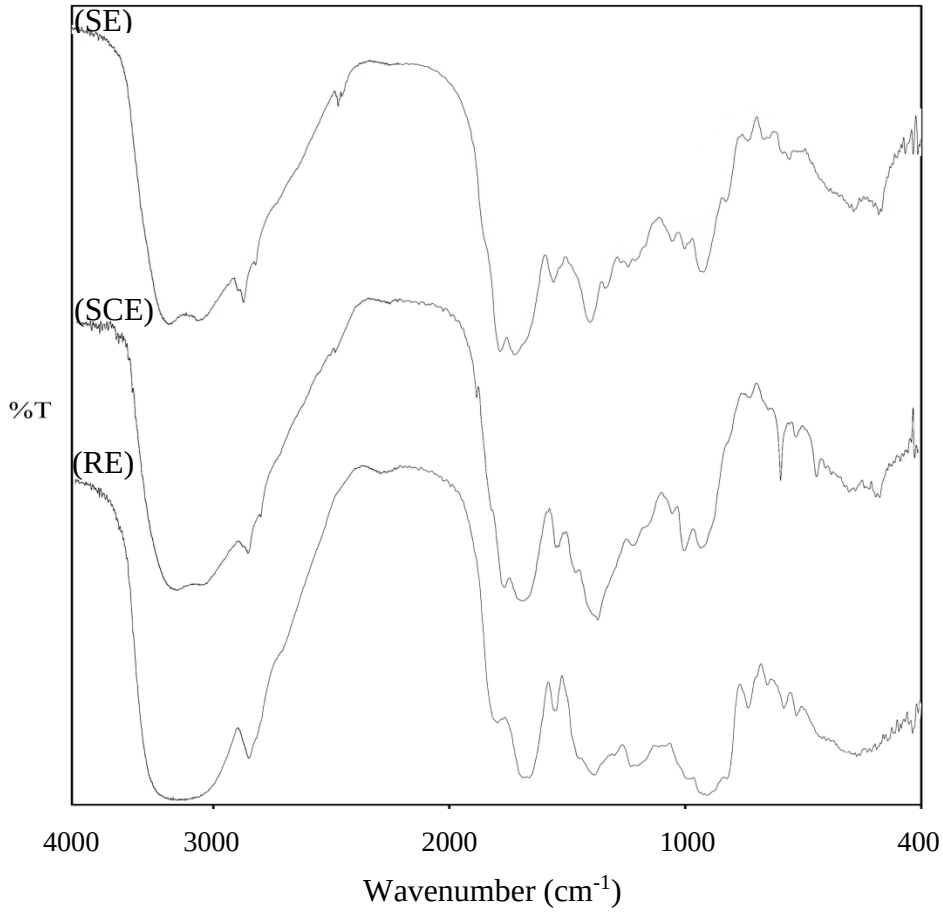


Figure 4- 5 FT-IR spectral analysis data of the extracts recorded in the range of 4000-400 cm^{-1}

SE: Adventitious shoots extracts. SCE: Callus suspension cultures extracts. RE: Extracts of wild plant roots.

Table 4-5 The functional groups in three extracts (SE, SCE, RE)

Wavelength (cm^{-1})	Functional groups	SE	SCE	RE
3500-3180	Amide/Amine	+	+	+
2950-2800	Alkane	+	+	+
1690-1630	Alkene	+	+	-
~2850 & ~2750	Aldehyde	-	-	-
1260-1000	Alcohol	+	+	+
1690-1640	Nitro or Imine	+	+	-
785-540	Alkyl halide	+	+	+
1550-1490 & 1355-1315	Aromatic (Nitro)	-	-	-
2320-2270	Phosphines	-	-	-
1000-750	Sulfonates	+	+	+

+: Present and -: Not present; SE: Adventitious shoots extracts. SCE: Callus suspension cultures extracts. RE: Roots extracts of wild plants.
Note: Wavelength and functional groups refer to the paper written by Vignesh et al. (2015).

4.3.6 NMR analysis

^{1}H NMR spectroscopy analysis method, which is commonly used to analyze the metabolic profiles of the phytoconstituents of medicinal plants, can provide a holistic view compared to the following-mentioned analysis methods such as liquid or gas chromatography, infrared spectrometry and mass spectrometry. It is a powerful and non-destructive tool for metabolic research to confirm the presence of the plants primary and secondary metabolites, to identify new metabolites, to study the structure of organic constituents from the plants and their quantitative determination (Gogna et al., 2015; Sharma et al., 2014). Our research allowed a comparison on ^{1}H NMR spectroscopy data between cultures and wild plant roots. As is shown in Figure 4-6, the ^{1}H NMR spectra were recorded in region δ_H 0-10.00 ppm. The spectra are often divided into four regions including (1) the aliphatic methyl and methylene region from δ_H 0.00

ppm to 1.60 ppm, (2) the region from δ_H 1.60 ppm to 3.20 ppm (protons of the methyl and methylene groups α to aromatic rings, protons on carbons in α position to carbonyl, carboxylic acid, ester or amino acid), (3) the region from δH 3.20 ppm to 4.30 ppm (protons on carbon of hydroxyl, ester and ether, and protons on methyl, methylene and methylene carbons directly bonded to oxygen and nitrogen), (4) the aromatic region from δH 6.00 ppm to 9.50 ppm (aromatic protons including quinones, phenols, oxygen containing hetero-aromatics) (Rodr ǵuez et al., 2016; Zhao et al., 2016). In this study, the extracts of cultures and wild plants roots exhibited some dominant peaks in regions δH 1.00-4.50 ppm and δH 6.00-8.00 ppm. According to ^{1}H NMR spectra, an obvious peak ranging from δH 4.50 ppm to 4.80 ppm were attributed to the presence of D_2O (Rodr ǵuez et al., 2016). The peaks ranging in region from 1.00 to 3.00 ppm were mainly consisting of amino acids and acyl peaks of lipids. The peaks ranging in region from 3.00 to 4.50 ppm were mainly attributed to carbohydrates. The peaks ranging in region from 6.0 ppm to 8.00 ppm contained mainly aromatic resonances which might be attributed to polyphenols and flavonoids (Gogna et al., 2015; Saifudin et al., 2016). Both cultures and wild plant roots presents characteristic peaks in these three regions. These results indicated that the cultures had capability to synthesize phytochemicals having the same types with wild plants roots. They all synthesized lipids, carbohydrates, polyphenols and flavonoids. Furthermore, the amplified peaks ranging in feature spectrum regions (δH 2.00 - 2.80 ppm, 3.00 - 4.00 ppm and 6.00 - 8.00 ppm) which were exhibited in Figure 4-7, 4-8, 4-9. Some peaks which not existed in wild plant roots were observed within SE and SCE in amplified regions, as shown in red regions in Figure 4-7 and 4-8. The results indicated that cultures contained different structures with phytochemicals from wild plant roots, might have different classifications of polyphenols, flavonoids and carbohydrates. Bioactive ability of cultures might be associated to classifications of polyphenols, flavonoids and carbohydrates. More efforts are expected to study classifications of secondary metabolites in cultures including polyphenols, flavonoids and carbohydrates, etc. And then it becomes possible to improve contents of effective constituents of secondary metabolites.

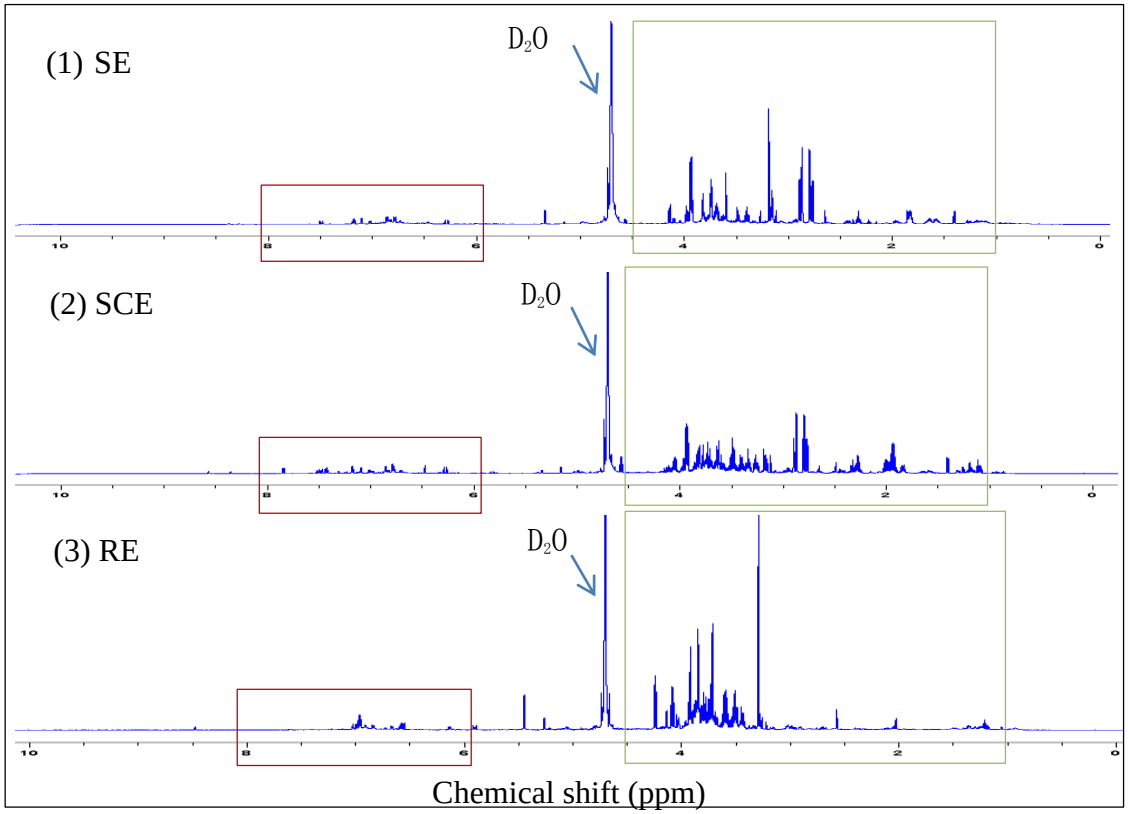


Figure 4-6 ^{1}H NMR spectrum of extracts in the region δH 0.00-10.00 ppm

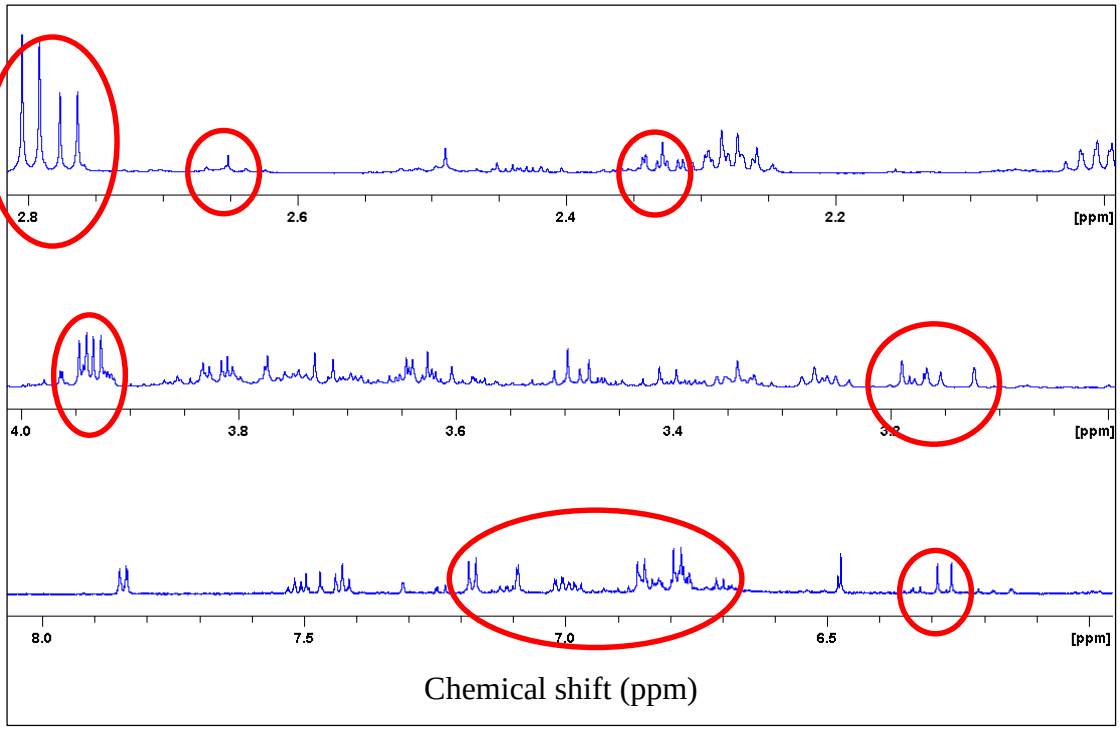


Figure 4-7 ^{1}H NMR spectrum of SE in δH 2.00-2.80, 3.00-4.00 and 6.00-8.00 ppm

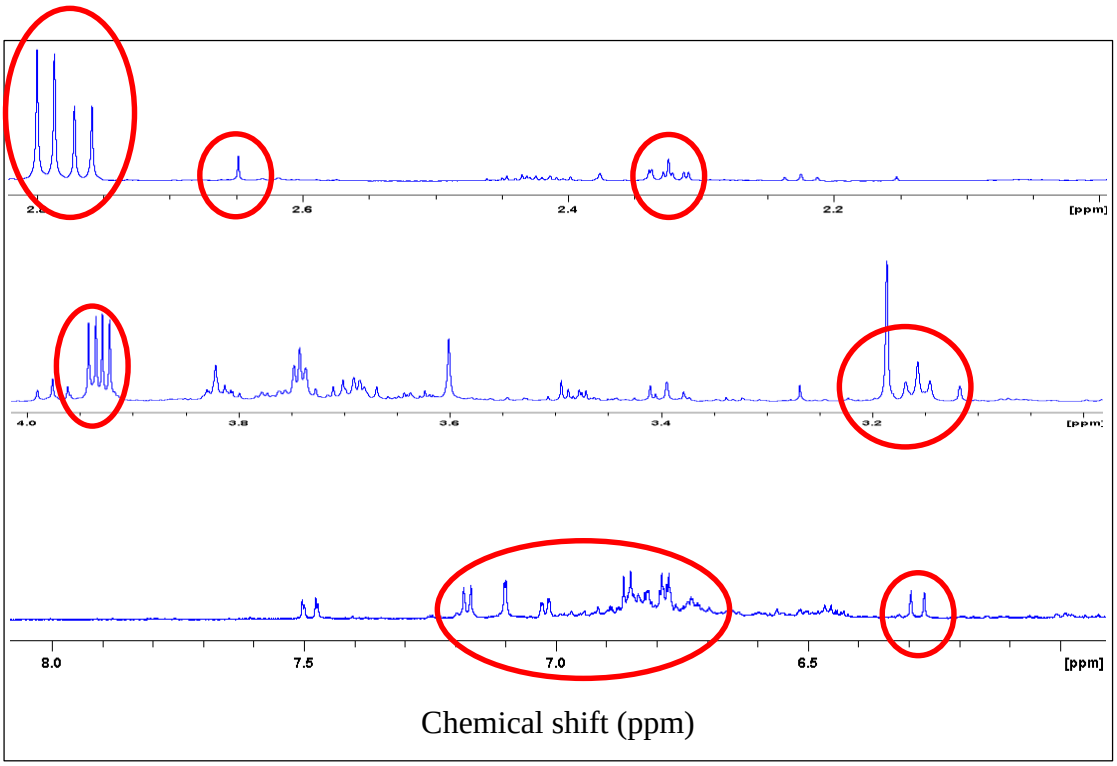


Figure 4-8 ^{1}H NMR spectrum of SCE in δH 2.00-2.80, 3.00-4.00 and 6.00-8.00 ppm

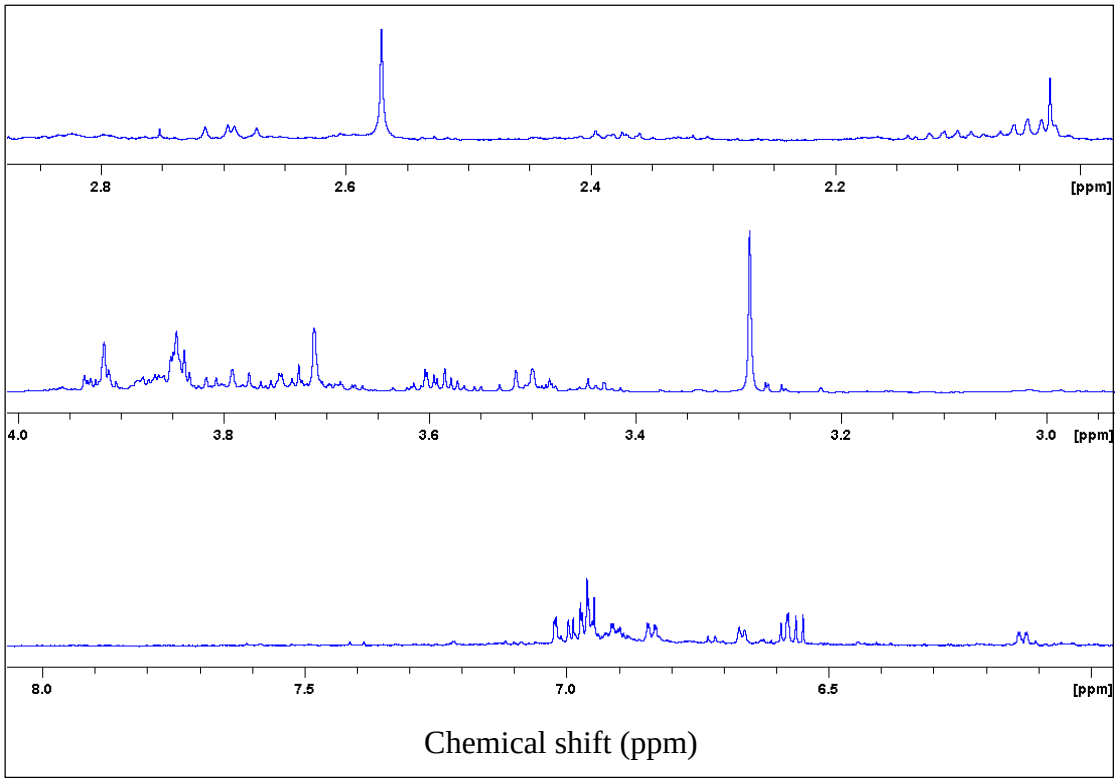


Figure 4-9 ^{1}H NMR spectrum of RE in δH 2.00-2.80, 3.00-4.00 and 6.00-8.00 ppm

4.4 Summary

Antioxidant ability, neuroprotection, FTIR analysis and NMR analysis indicated that the cultured shoots and callus possessed antioxidative and neuroprotective potential. They did not show cytotoxicity on IMR32 cell, and they synthesized phenolics, polysaccharides, proteins, saponins under the conditions of artificial cultivation. As a new substitutable resource for wild medicinal herbs, the cultured adventitious shoots and callus possessed the promising biosynthesis capability and pharmaceutical values. One the one hand, adventitious shoots showed stronger antioxidant activity and neuroprotective potential than callus. It was attributed different contents, structure and types of bioactive compounds in shoots and callus. On the other hand, callus showed lower antioxidative and neuroprotective potential, but callus culture was suitable for a large-scale production. Thus, more work should focus on optimization of culture conditions and comparation of bioactive compound between cultures and wild plants. The establishment of shoots and callus cultivation were good for production of natural antioxidants and neuroprotective agents of *H. angustifolia*. They were alternative resources for wild medicinal herbs.

Chapter 5 Conclusions and future research

The previous researches on *H. angustifolia* mainly focus on (i) identification of phytochemicals such as flavonoids, lignans, triterpenoids and steroids, and (ii) bioactive evaluation of aqueous and ethanolic extracts from *H. angustifolia* roots. Few researches were conducted to further isolate bioactive products from *H. angustifolia* roots in a bioactivity-guided isolation manner. And also almost no reports are published about sustainable utilization manner of this wild plant resource for the future industrial development. In this study the five fractions were separated from ethanolic extract of *H. angustifolia* roots by using different organic solvents, and further clarified their antioxidant, neuroprotection potentials and phytochemicals characteristic. Next, the sustainable utilization manners of *H. angustifolia* were established to aggravate the pressure of wild plant resources. Meanwhile, we explored the bioactive potential of plant resource obtained by these sustainable production manners. Finally, the study offered a sustainable manner to produce the substitute resources of wild plants without influence by climates, environmental conditions and contaminants.

5.1 Conclusion

The main results could be concluded as follows.

1. Among five fractions derived from ethanolic extract, EAE contained the maximum TPC (356.65 ± 6.03 mg GAE/g extract) and TFC (962.27 ± 10.84 mg RE/g extract) with the strongest DPPH and $ABTS^{\cdot+}$ radical scavenging activity.
2. NE exhibited the strongest inhibition effect on H_2O_2-induced reduction of IMR32 cells viability. IMR32 cells viability increased by 14.98 ±1.08 % after 15 μg/mL NE treatment for 24 h.
3. The aerial-part and callus induction culture medium of *H. angustifolia* were optimized. The maximum biomass accumulation (9.80 ± 0.75 mg/explant) was observed at 0.1 mg/L of KIN with maximum TPC (126.41 ± 1.36 mg GAE/g sample) and TFC (530.91 ± 6.67 mg RE/g sample). The most profuse callus was observed in MS supplemented with 3.0 mg/L NAA and 0.07 mg/L of BA.

4. The callus growth curve of *H. angustifolia* was obtained in MS supplemented with 3.0 mg/L NAA at an inoculum density of 12 g/L. The cells initiated a continuous growth phase of 21 days at day 7. Followed by a stationary phase of 28 days with the maximum dry weight reaching 1.054 ± 0.100 g/L. Under optimized liquid culture medium supplemented with 0.07 mg/L BA with an inoculum density of 50 g/L, the fresh weight of suspension cultured callus reached to 148.7 ±8.91 g/L (DW: 16.62 ±0.92 g/L) with 3-fold growth after 60 days of cultivation time.
5. The cultured shoots and callus possessed antioxidative and neuroprotective potential. They did not show cytotoxicity on IMR32 cell, and they synthesized phenolics, polysaccharides, proteins, saponins. Adventitious shoots and callus of *H. angustifolia* produced the natural antioxidants and neuroprotective agents. They were considered as a new substitutable resource for wild medicinal herbs, possessing the promising biosynthesis capability and pharmaceutical values.

5.2 Future research

In the followed-up experiments, more efforts and attentions should be paid to: (i) exploration of metabolic mechanism of bioactive substances, (ii) enhancement on biomass accumulation and the yields of bioactive compounds in the cultured materials by optimization of suspension culture conditions and the addition of elicitors, (iii) isolation and identification of the bioactive compounds, and (iv) comparation of bioactive compounds between the cultured and wild plants.

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