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In vitro production of secondary metabolites from *Pseuderanthemum palatiferum*

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Declaration

I hereby declare that I have done this thesis entitled "*In vitro* production of secondary metabolites from *Pseuderanthemum palatiferum*" independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA.

In Prague, 15. 5. 2020

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Kryštof Knejp

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Abstract

Pseuderanthemum palatiferum is an Acanthaceae plant used in Vietnamese and Thai traditional medicine and has been proven to have (at least some) anti-bacterial, anti-fungal, anti-inflammatory, anti-oxidative, anti-diabetic, anti-thrombotic, analgesic, bradycardic, hypotensive, cancer-inhibiting, gastric ulcer preventing and acetylcholinesterase activity inhibiting physiological effects. Which secondary metabolites are responsible for the mentioned physiological capabilities has not yet been revealed. *In* vitro cultivation of *Pseuderanthemum palatiferum* was optimized and plantlets and callus were successfully micropropagated. The plantlets were then oven-dried and subjected to GC-MS analysis, based on which 25 unique chemical compounds were detected and identified. Dopamine and noradrenaline, which were among the identified compounds, were not previously reported to be present in *Pseuderanthemum palatiferum.* 'Precursor feeding' is the practice of supplementing culture medium with a precursor of a specific compound or group of compounds in order to increase their production in the plant tissues. Precursor feeding of *Pseuderanthemum palatiferum* plantlets and callus cultures was conducted using different concentrations of L-tyrosine in culture media – 25, 50, 100 and 200 mg.l^{-1}. After 6 weeks of cultivation on medium supplemented with tyrosine, the plantlets and calli were oven-dried and subjected to a GC-MS analysis.

Key words: · catecholamines · medicinal plant · plant tissue culture · *Pseuderanthemum latifolium* · SouthEast Asia

Abstrakt

Pseuderanthemum palatiferum je rostlina z čeledi Acanthaceae, která se používá v rámci vietnamské a thajské tradiční medicíny. Bylo vědecky dokázáno, že tato rostlina a výtažky z ní mají řadu fyziologických účinků. Konkrétně má (alespoň nějaké) analgetické, anti-trombotické a protizánětlivé, působí proti otokům, může snižovat tlak a srdeční frekvenci, inhibitovat růst rakoviny, a dále může pomáhat proti nepříznivým důsledkům diabetu, proti následkům Alzheimerovy choroby a může pomáhat předcházet tvorbě žaludečních vředů. Které sekundární metabolity jsou zodpovědné za tyto léčivé účinky, nebylo doposud vyzkoumáno. Rostliny a kalus *Pseuderanthemum palatiferum* byly úspěšně pěstovány a namnoženy pomocí *in vitro* mikropropagace, a jejich kultivace byla optimalizována. Vypěstované rostliny a vzorky kalusu byly vysušeny a podrobeny GC-MS analýze, na základě níž bylo z tohoto rostlinného materiálu identifikováno 25 různých chemických látek. Přítomnost dopaminu a noradrenalinu v *Pseuderanthemum palatiferum* nebyla do té doby známa. „Precursor feeding” je metoda sloužící ke zvýšení produkce konkrétní látky nebo skupiny látek v rostlině, pomocí přidání odpovídajícího prekurzoru do kultivačního média. Byl proveden experiment, ve kterém byly *Pseuderanthemum palatiferum* rostliny a vzorky kalusu pěstovány na médiích s různými koncentracemi L-tyrosínu – 25, 50, 100 a 200 $mg.l^{-1}$. Po šesti týdnech kultivace byl tento rostlinný materiál vysušen a podroben GC-MS analýze.

Klíčová slova: · jihovýchodní Asie · katecholaminy · kultura rostlinných explantátů · léčivá rostlina · *Pseuderanthemum latifolium* ·

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List of the abbreviations used in the thesis

MS	Murashige & Skoog medium (1962)
GCMS	gas chromatography - mass spectrometry
BAP	6-benzylaminopurine
NAA	α-naphthaleneacetic acid
TYR	L-tyrosine

1. Introduction

Pseuderanthemum palatiferum is an Acanthaceae herbaceous plant native to Southeast Asia (Wood et al. 2002) and is used in Vietnamese traditional medicine and Thai traditional medicine (Padee et al. 2010). It has in the past twenty years become of interest to researchers, due to its assumed remedial effects. It has since been proven to have some of the proposed physiological capabilities, such as anti-bacterial, anti-diabetic, anti-inflammatory, hypotensive and cancer growth inhibiting effects (Dieu et al. 2006; Khonsung et al. 2011; Sittisart & Chitsomboon 2014).

Plant secondary metabolites are organic compounds naturally synthesized by plant cells. Their presence is not unconditionally required for the plant's survival (unlike primary metabolites), though they provide the plant with benefits, such as chemical communication, transportation of metal and antagonism to parasites and competitors (Demain & Fang 2000). Interestingly, many secondary metabolites have medicinal effects, and therefore are or could be used for the treating various diseases and health conditions (Vaishnav & Demain 2011).

In vitro micropropagation provides an effective, aseptic way of multiplicating plants (Bhatia 2015). Addition of a secondary metabolite precursor into culture media (often referred to as "precursor feeding") can significantly increase the amount of the respective secondary metabolites produced in a plant. For example, precursor feeding of L-tryptophan lead to a 5-fold increase in quinoline alkaloid production in *Cinchona calisaya* cultures (Fusion 1982).

2. Literature review

2.1. Pseuderanthemum palatiferum

2.1.1. Taxonomy

2.1.1.1. Acanthaceae

Acanthaceae is a family of dicotyledonous plants (Kavitha et al. 2016), comprising of more than 200 genera (Scotland & Vollesen 2000; Wood et al. 2002; Kavitha et al. 2016). With more than 4,000 species (Wood et al. 2002; McDade et al. 2008; Kavitha et al. 2016), it is within the 12 most diverse flowering plant families (Scotland & Vollesen 2000). The vast majority of Acanthaceae's species are either herbs, shrubs or undershrubs, while some species in the form of vines or trees are also known (Kavitha et al. 2016). Approximately 172 species of Acanthaceae are currently endangered or critically endangered, according to The International Union for Conservation of Nature ("The IUCN Red List of Threatened Species" 2020).

Acanthaceae is comprised of 11 major lineages: the tribes Acantheae, Ruellieae, Justicieae, Whitfieldieae, Andrographideae and Barlerieae, the genera *Lankesteria* and *Neuracanthus* (all the taxa mentioned in this paragraph so far are part of the subfamily Acanthoideae), the genus *Avicennia* (the only genus in the Avicennioideae subfamily), and the subfamilies Nelsonioideae and Thunbergioideae (McDade et al. 2008). Correspondingly, Acanthaceae can be divided into the subfamilies Acanthoideae, Avicennioideae, Nelsonioideae and Thurbergioideae, while Acanthoideae is the only subfamily further partitioned into tribes, each of which is one of the major lineages mentioned above (McDade et al. 2008; Olmstead et al. 2016).

Justicieae is a tribe within the subfamily Acanthoideae, and it is the taxonomically largest of Acanthaceae's major lineages. It contains approximately 2000 species (Darbyshire et al. 2019; Mcdade et al. 2020).

2.1.1.2. *Pseuderanthemum*

Pseuderanthemum is a genus of herbs and shrubs, present in both Old World and New World tropics. The exact number of species it entails is unclear. According to Olmstead

et al. (2016) there are 60 species. Contrarily, there are 97 unique species listed on the online database Tropicos.org (2020) as part of *Pseuderanthemum*, and according to Ndjateu et al. (2018) there are 160 species.

Phylogenetical and taxonomical research of Justicieae (and Acanthaceae as a whole) is still ongoing and scientific knowledge of this issue is steadily evolving over time (McDade et al. 2008; Joshua 2019). Thusly, the taxonomy of *Pseuderanthemum* can be expected to be partially restructured in the coming years, due to propositions that it is not fully monophyletic (Gâteblé et al. 2019).

2.1.1.3. *Pseuderanthemum palatiferum*

Pseuderanthemum palatiferum (Nees) Radlk. Ex Lindau is a herbaceous tropical plant. It has various unofficial or outdated taxonomical synonyms and homonyms, which rarely are used instead of the correct latin name. The most commonly used synonym is *Pseuderanthemum latifolium*, which has even been used by some researchers, such as by Truong et al. (2019) and Wood et al. (2002). Other synonyms include *Eranthemum palatiferum* (Wallich et al. 1832; Wood et al. 2002; “Tropicos.org” 2020), *Justicia palatifera* (Wood et al. 2002; “The Plant List” 2013; “Encyclopedia of Life” 2020; “Tropicos.org” 2020), *Justicia latifolia* (Forsskål & Vahl 1790; Wood et al. 2002; “The Plant List” 2013; “Encyclopedia of Life” 2020) and others.

Additionally, *P. palatiferum* has several local vernacular names, such as “hoan-ngoc” and “xuan-hoa” in Vietnam (Chayarop et al. 2011a; Buncharoen et al. 2018), “phaya wanon” (Chayarop et al. 2011b; Buncharoen et al. 2018) or “payawanorn” (Chayarop et al. 2011a; Inchab et al. 2018) in Thailand, “longlamak” or “laungla-mak” in India (Quattrocchi 2012) and “shan ke gu” or “山壳骨” in China (Wood et al. 2002).

2.1.2. Origin and geographical distribution

Dieu et al. (2005) and other researchers (mostly citing Dieu et al.), such as Nguyen & Eun (2011), Kavitha et al. (2016) and Inchab et al. (2018), claim that *P. palatiferum* was first discovered in northern Vietnam sometime between the years 1995 and 2000. This is factually incorrect, as *P. palatiferum* was first described in 1790 by Martin Vahl under the name "*Justicia latifolia*", who determined eastern India as its habitat (Forsskål & Vahl 1790). It was subsequently described as "*Justicia palatifera*" by Nathaniel Wallich in 1832, who came across *P. palatiferum* in eastern India (Wallich et al. 1832). Although *P. palatiferum* was first discovered in eastern India, it remains unclear whether India is its exact point of origin.

P. palatiferum is native to Southeast Asia and some of its surrounding areas. It can be found in Vietnam (Wood et al. 2002; Buncharoen et al. 2018; Huy et al. 2019), Laos (Newman et al. 2007; Huy et al. 2019), Cambodia (Wood et al. 2002; Buncharoen et al. 2018), Thailand (Wood et al. 2002; Buncharoen et al. 2018), peninsular Malaysia (Hooker 1872; Turner 1995; Wood et al. 2002), southern China (Wood et al. 2002; Huy et al. 2019), Myanmar (Wood et al. 2002; Kress et al. 2003) and north-east India (Kanjilal et al. 1934; Wood et al. 2002; Choudhary et al. 2012). It has also been introduced to Sri Lanka, where it is widely cultivated (Van Der Poorten & Van Der Poorten 2012).

2.1.3. Morphology

Pseuderanthemum palatiferum is an erect (Hooker 1872), perennial (Wood et al. 2002) plant. Visual representation of *P. palatiferum*, as described by (Wallich et al. 1832) is shown in Figure 1. The habitus of *P. palatiferum* was described by most researchers as a shrub (Wallich et al. 1832; Hooker 1872; Kress et al. 2003; Inchab et al. 2019), while some researchers described it as a herb (Turner 1995; Wood et al. 2002; Choudhary et al. 2012), or undershrub (Kanjilal et al. 1934). According to Wood et al. (2002) it can reach a height of 1 meter, while according to Chayarop et al. (2011) and Pamok et al. (2012) it can be up to 2 meters tall. In a botanical study conducted by Dieu et al. (2005), 135 days old *P. palatiferum* plants had 700-1,000 leaves per plant and a leaf production of 500-700 kg of leaves per 1,000 m^2.

Leaves are decussately situated in opposite pairs on the terete, usually glabrous stem (Hooker 1872). Each leaf pair is horizontally rotated compared to the previous pair – most often by 90 degrees (Wallich et al. 1832; Hooker 1872). The leaves, therefore, face all radial directions, which perhaps is what some researchers mean to express by saying that *P. palatiferum* leaves grow in whorls (Supaporn Pamok 2012; Khumpook et al. 2013).

P. palatiferum leaves are petioled, glossy and green with lighter-green bands along veins (Supaporn Pamok 2012; Choopan et al. 2018). The petioles are 1-2.5 cm long (Dieu et al. 2005). The leaves are longer than in other species of *Pseuderanthemum* (Choopan et al. 2018), with a length of 10-17 cm and width of 3.5-5 cm (Hooker 1872; Dieu et al. 2005). Leaf blades are elliptic and acuminate in both base and apex (Kanjilal et al. 1934; Wood et al. 2002), with edges ranging from entire to obscurely crenulate (Hooker 1872). Additionally, some *P. palatiferum* plants have been reported to have slightly oblique leaf bases (Kanjilal et al. 1934). The leaves are of bifacial nature, as evidenced by the different shape of cells in the upper epidermis layer and the lower epidermis layer. Furthermore, though both tissues contain lithocyst cells and glandular trichomes, warty-walled multicellular trichomes are exclusive to the upper layer (Chayarop et al. 2011).

Figure 1 - Illustration of a flowering *P.palatiferum* plant (Source: Wallich et al. 1832)

The inflorescence of *P. palatiferum* is present in the form of subulate, terminal spikes. These bracts are glandular-pubescent, slightly recurved and can both be and not be branched at the base. Moreover, they are 10-17 cm long and many-flowered, with fascicled flowers in regularly spaced fascicles (Hooker 1872). The base of the corolla tube is covered by sepals, which are linear, acuminate and pubescent. The corolla is

composed of a cylindrical corolla tube, which is terminated in a bilabiate limb (Hooker 1872). The corolla tube is pubescent and about 3 cm long (Kanjilal et al. 1934; Hansen 1989). The limb's upper lip is split into 2 obtuse-oblong lobes, and the lower lip is divided into 2 obtuse-oblong lobes and 1 larger, circular lobe (Hooker 1872). The colour of the corolla is most often described as white to light-purple or light-pink with pronounced dark violet dots on the middle lobe of the lower lip (Kanjilal et al. 1934; Supaporn Pamok 2012; Khumpook et al. 2013; Choopan et al. 2018). Contrarily, Hooker (1872) described the corolla as a scarlet or lilac limb with a yellow spot on the lower lip. Perhaps the specimen described by Hooker (1872) belonged to some local variety or subspecies of *P. palatiferum*, which would explain the stark difference in colours. The anthers are blue (Hooker 1872). *P. palatiferum* blooms after 3 years of cultivation, and only if its leaves are not being continually harvested (Dieu et al. 2005).

Seeds are 2.5 cm long capsules, with a rugose, glabrous surface (Kanjilal et al. 1934; Wood et al. 2002).

2.1.4. Assumed physiological effects of *Pseuderanthemum palatiferum*

Pseuderanthemum palatiferum is one of many plants used in Vietnamese traditional medicine, which still plays an important role in Vietnam, despite the growing presence of modern western medicine (Nguyen et al. 2006; Padee et al. 2010; Adorisio et al. 2016). It is also widely used as a medicinal plant in Thailand (Padee et al. 2010; Khonsung et al. 2011; Komonrit & Banjerdpongchai 2018). In both countries, *P. palatiferum* is used to treat a wide spectrum of different illnesses and diseases, and its believed remedial effects have led to the plant being referred to by some as a "miracle" plant (Dieu et al. 2005). However, certain people may have ulterior motives in praising *P. palatiferum*'s medicinal capabilities, as the plant and products made from it are commercially sold both locally and internationally via the internet (Mai et al. 2011).

The human diseases and symptoms treated with *P. palatiferum* include diarrhea, stomach-ache, hypertension, nephritis, arthritis, inflammation, constipation, hepatitis, peptic ulcers, hemorrhoids, hearth muscle anemia, flu, diabetes mellitus and cancer (Dieu et al. 2005, 2006; Padee et al. 2009, 2010; Chayarop et al. 2011; Khonsung et al. 2011; Adorisio et al. 2016; Komonrit & Banjerdpongchai 2018; Truong et al. 2019). In most cases, *P. palatiferum* is administered to the patient in the form of orally dosed, freshly cut, whole leaves or alternatively as a water infusion made from leaves (Padee et al. 2010; Chayarop et al. 2011; Mai et al. 2011; Komonrit & Banjerdpongchai 2018).

Furthermore, *P. palatiferum* leaves are used in Vietnam and Thailand to treat or prevent diseases in agricultural and household animals. Based on the results of a questionnaire survey, responded to by residents of Cantho city in 2001, examples of this practice include treatment of diarrhea in pigs and dogs, as well as fowl cholera in chickens and ducks (Dieu et al. 2005).

2.1.5. Scientifically investigated physiological effects of *Pseuderanthemum palatiferum* and its extracts

The use of *Pseuderanthemum palatiferum* in traditional medicine raises questions about whether *P. palatiferum*, or a product made from *P. palatiferum*, could be used as a physiologically apt alternative to conventional medication for various diseases. As one of the first steps towards investigating such a possibility, the cytotoxicity of *P. palatiferum* was studied by several researchers. Dieu et al. (2006) found no sign of adverse effects of *P. palatiferum* consumption on piglets, which were fed *P. palatiferum* leaves for the duration of up to 27 days. Additionally, piglets exhibited normal levels of physical activity and had normal body temperature levels. Padee & Nualkaew (2009) tested the cytotoxicity of *P. palatiferum* in Vero (green monkey liver) cells, as well as acute toxicity in rats. According to their results, no cytotoxicity was found. Similarly, Khumpook et al. (2013) reported that no sign of toxicity or mortality was detected in rats treated with an ethanolic extract of *P. palatiferum*.

The results of a Ames mutagenicity test performed by Pamok (2012) in *Salmonella typhimurium* exposed to an ethanolic extract of *P. palatiferum* and aqueous extract of *P. palatiferum*, suggest that neither of these extracts possesses a mutagenic effect.

Buncharoen et al. (2018) evaluated long-term effects of aqueous *P. palatiferum* extracts on the reproductive organs of male rats. This series of scientific experiments revealed that, while no clinical sign of cytotoxicity was detected, the exposure to the extracts had various detrimental consequences on the fertility of the subjects. In all *P. palatiferum* treated rats, the amount of sperm abnormality was significantly higher than in the control group and the epididymal sperm density and progressive motility were significantly lowered. Furthermore, the diameter of seminiferous tubule, as well as the epithelial thickness of prostate gland, were significantly decreased. These reportings suggest that a continuous long-term consumption of *P. palatiferum* (e.g. the use of *P. palatiferum* in traditional medicine) could potentially cause infertility in male – not only in rat, but presumably also in human.

The antibacterial and antifungal capabilities of *P. palatiferum* extracts have been tested on several specific species of bacteria and fungi, respectively. Strong antibacterial activity of an ethyl acetate *P. palatiferum* extract against *Salmonella typhi*, *Escherichia coli* and *Shigella flexineri* was observed. Furthermore, this extract had significant antifungal

effects on *Candida albicans* and *Candida stellatoides* (Giang et al. 2005). Betulin and luteol, two triterpenoid secondary metabolites isolated from *P. palatiferum*, were proven to have selective inhibitory properties towards *Staphylococcus aureus*, while showing no sign of inhibition of *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* and *Bacillus subtilis* as part of the same study (Mai et al. 2011). An ethanolic extract made from powdered leaves of *P. palatiferum* displayed not only minor, statistically insignificant inhibition of *Toxoplasma gondii*, but more importantly also a moderate, significantly high inhibition of *Plasmodium falciparum* (Leesombun et al. 2019).

Based on *P. palatiferum*'s antimicrobial effects and its beneficial nutritional composition, *P. palatiferum* could potentially prove to be a valuable alternative to the use of antibiotics in industrial livestock production systems. For this reason, Dieu et al. (2006) conducted three experiments involving suckling and/or weaned piglets, fed with various doses of *P. palatiferum* fresh or powdered leaves. In all three experiments, the number of cases of diarrhea among piglets threated with *P. palatiferum* was significantly lower than among the piglets of the respective control groups. In addition, average body weight gain of threated piglets was significantly higher than the average body weight gain of control group piglets. The treated piglets were reported to have improved blood physiology – i.e., higher volumes of erythrocytes and hemoglobin. In another experiment conducted by Diệu & Nga (2018), 10 days-old Cherry Valley ducks had *P. palatiferum* leaf powder added to their feed for the duration of 30 days. The ducks which had their feed supplemented with *P. palatiferum* had greater average body weight gains and a higher survival rate than the ducks of the control group.

P. palatiferum possesses anti-oxidant and anti-inflammatory properties, presumably due to the content of specific secondary metabolites. This is evident from the results of Sittisart & Chitsomboon (2014), which evaluated the anti-oxidant and anti-inflammatory capabilities of water extract of *P. palatiferum* leaves and ethanolic extract of *P. palatiferum* leaves, *in vivo* in macrophage cells. Similarly, Padee et al. (2010) found *P. palatiferum* to have high antioxidant activity against hydrogen peroxide radicals. Additionally, Sittisart et al. (2016) reported ethanolic extract of *P. palatiferum* leaves to inhibit the transcription of proinflammatory cytokines, and Khumpook et al. (2013) tested the effect of ethanolic extract of *P. palatiferum* on the acute ear edema and chronic granuloma in rats, finding the extract to significantly inhibit the inflammations to a degree

comparable to phenylbutazone and diclofenac (which are conventional nonsteroidal anti-inflammatory drugs), respectively.

Several studies have shown that aqueous and ethanolic extracts from *P. palatiferum* leaves have hypoglycemic and anti-diabetic physiological effects by stimulating insulin secretion, lowering fasting plasma glucose levels and postprandial plasma glucose levels (Padee et al. 2010; Chayarop et al. 2011b; Pulbutr et al. 2016). More specifically, the extracts have been observed to significantly inhibit the activity of α-amylase and α-glucosidase *in vitro*, as well as the activity of α-amylase *in vivo* in rats (Pawitra et al. 2014; Pulbutr et al. 2016). Furthermore, *P. palatiferum* helps to prevent complications stemming from diabetes mellitus and can improve the function of liver and kidneys (Padee et al. 2010).

Extracts from *P. palatiferum* selectively inhibit the growth of human lung cancer cells and can even cause apoptosis of such cells. This is likely, at least in part, a result of the ability of *P. palatiferum* extracts to increase the intracellular production of reactive oxygen species, disrupt mitochondrial metabolic activity and increase the caspase-3 activity in the effected cells (Kongprasom et al. 2019). *P. palatiferum* can also cause the apoptosis and necrosis of breast cancer cells by raising the levels of Ca^{2+} in the cytosol, disrupting mitochondrial membrane potential and inducing various other stresses (Komonrit & Banjerdpongchai 2018). Similarly, *P. palatiferum* extracts inhibit the proliferation of human colon cancer cells (Supaporn Pamok 2012). Both palatiferin A and betulin, compounds isolated from *P. palatiferum* roots, amounted a significant cytotoxicity against human liver cancer cells and human cervix cancer cells, while betulin was also cytotoxic towards human breast cancer cells. Furthermore, *P. palatiferum* extracts were proven to significantly inhibit human liver cancer cells by Huy et al. (2019), but significant inhibition of human leukemia cancer cells was not observed as part of the same experiment.

Truong et al. (2019) isolated a protease from *P. palatiferum* leaves and investigated its ability to hydrolyze fibrin, the results of which indicate that *P. palatiferum* could have anti-thrombotic capabilities. A polysaccharide was isolated from *P. palatiferum* leaves by Bắc et al. (2018) and subsequently applied to mice, which thereafter exhibited raised levels of white blood cells.

Moreover, *P. palatiferum* has hypotensive and bradycardic effects, as was established in an experiment, in which normotensive and hypertensive rats were exposed to a water extract of *P. palatiferum* (Khonsung et al. 2011). In a different experiment, water extract of *P. palatiferum* administered orally to rats caused inhibition of acetylcholinesterase activity in the hippocampus (Buncharoen et al. 2010), which is the desired effect of many synthetic drugs used to treat Alzheimer's disease. Furthermore, *P. palatiferum* extracts have analgesic effects, but do not seem to have antipyretic effects, as was tested on rats (Inchab et al. 2019). Finally, consumption of *P. palatiferum* in form of a water extract can help prevent the creation of gastric ulcers, as its presence in the digestive tract supports gastric mucus levels (Inchab et al. 2018).

So far there has been no scientific evidence published that would support *P. palatiferum*'s utilisation in traditional medicine against arthritis, viral infections (such as influenza), stomach-ache (apart from stomach pain caused by gastric ulcers), acute or chronic constipation, hepatitis and anemia.

2.1.6. Secondary metabolites of *Pseuderanthemum palatiferum*

Various secondary metabolites are present in *Pseuderanthemum palatiferum*, which are likely the cause of its physiological effects.

The leaves of *P. palatiferum* contain terpenoid compounds (Chayarop et al. 2011a; Petsangkrit & Kittipongpatana 2016; Buncharoen et al. 2018), including saponins (Petsangkrit & Kittipongpatana 2016; Buncharoen et al. 2018; Huy et al. 2019), sterols (Khonsung et al. 2011; Buncharoen et al. 2018) and cardiac glycosides (Chayarop et al. 2011a; Buncharoen et al. 2018). Specific terpenoids isolated from *P. palatiferum* leaves include phytol (Khonsung et al. 2011), β-sitosterol (Dieu et al. 2006; Pulbutr et al. 2016; Sittisart et al. 2016), stigmasterol (Pulbutr et al. 2016) and megastigmatrienone (Khonsung et al. 2011). *P. palatiferum* leaves also contain phenolic compounds, such as flavonoids (Sittisart & Chitsomboon 2014; Buncharoen et al. 2018; Kongprasom et al. 2019), coumarins (Khonsung et al. 2011; Petsangkrit & Kittipongpatana 2016) and tannins (Petsangkrit & Kittipongpatana 2016; Buncharoen et al. 2018). Specifically, kaempferin, rutin, isoquertecin, quertecin, catechin, gallic acid (Sittisart et al. 2016) and apigenin (Supaporn Pamok 2012; Sittisart & Chitsomboon 2014; Komonrit & Banjerdpongchai 2018) were detected. Furthermore, alkaloids were found in *P. palatiferum* leaves, as well as lactams 2-piperidinone and 2-pyrrolidinone (Khonsung et al. 2011).

Additionally, triterpenoids epifriedelanol, betuline, lupeol, lupenone and pomolic acid were isolated from roots of *P. palatiferum*, as well as phenolic compounds dipeptide asperglaucide, palatiferin A and palatiferin B (Mai et al. 2011).

P. palatiferum callus was reported by Petsangkrit & Kittipongpatana (2016) to contain terpenoids, flavonoids, tannins, coumarins and alkaloids.

2.2. *In vitro* cultivation

2.2.1. *In vitro* culture research in Acanthaceae

Micropropagation of *in vitro* cultures is an effective method for vegetative plant multiplication and a potential method for regeneration of plants (Murashige 1974). In addition, an established *in vitro* culture opens up possibilities for researchers to study various fields of interest, such as the metabolite contents of a specific plant, the effects of polyploidy, germplasm preservation, study of plant physiology, and others (Bhatia 2015).

Complete micropropagation protocols are available for numerous plants of Acanthaceae, serving as both a proof of concept of effective aseptic multiplication and a replicable methodology for the culture establishment, optimal cultivation and root induction of the given plants, and their following acclimatization for transfer to *ex vitro* conditions. For example, Acanthaceae species, in which successful *in vitro* culture multiplication has been achieved, include *Barleria prionitis* (Lone et al. 2011), *Phlogacanthus thyrsiflorus* (Hassan et al. 2011), *Andrographis lineata* (Mohammed et al. 2016) and *Strobilanthes tonkinensis* (Srikun 2017).

Callogenensis of *in vitro* cultures in Acanthaceae plants have also been reported by many studies. In most cases, using calli regeneration can be a way to multiply plants faster than via plantlet micropropagation or other conventional methods. Alternatively, callus can be used for intensive production of secondary metabolites (Shalaka & Sandhya 2009; Rashmi et al. 2012). Analysis of secondary metabolite contents in *Aphelandra sinclairiana* (Nezbedová et al. 1999), plant multiplication in *Asteracantha longifolia* (Panigrahi et al. 2007) and production of specific flavones from *Andrographis paniculata* (Jalal et al. 1979) are examples of Acanthaceae plants from which callus cultures were previously established and the uses of said callus cultures for which they were previously utilized.

Furthermore, cell suspension cultures were utilized for example by Henry et al. (1987) in *Hygrophila erecta* and by Hayashi et al. (2009) in *Avicennia alba*. Moreover, transgenic hairy root culture has been utilized by Singh et al. (2014) in *Phlogacanthus thyrsiflorus*.

Research of *in vitro* cultures of plants belonging to the Justicieae tribe have also been reported. Vegetative propagation, *in vitro* rooting of shoots and transfer to field conditions

was conducted for example in *Justicia beddomei* (Sudha & Seeni 1994), *Justicia gendarussa* (Dennis Thomas & Yoichiro 2010) and *Justicia adhatoda* (Shalaka & Sandhya 2009). Callus culture was induced from roots of *Rhinacanthus nasutus* (Cheruvathur et al. 2015) for the purpose of rhinacanthin production and from leaves and nodal explants of *Justicia gendarussa* (Agastian et al. 2006) for the purpose of multiplication. The cell suspension culture method was utilized by Shalaka & Sandhya (2009) in *Justicia adhatoda* in order to increase concentrations of alkaloidal secondary metabolites and a transgenic hairy root culture was employed by Cheruvathur et al. (2015) in *Rhinacathus nasutus* for the production of rhinacanthin.

2.2.2. *In vitro* culture research in *Pseuderanthemum palatiferum*

To date, only one scientific article reporting on *P. palatiferum in vitro* tissue culture research has been published. Petsangkrit & Kittipongpatana (2016) reported on their experimental work, the objective of which was the investigation of secondary metabolite production from *P. palatiferum* callus. Their methodology includes surface sterilization of *P. palatiferum*, subsequent establishment of *P. palatiferum* callus culture and optimization of callus growth using the presence of plant growth regulators in the culture media. They designated Murashige and Skoog culture medium (Murashige & Skoog 1962) supplemented with 1 $mg.l^{-1}$ NAA to be the most suitable medium for callus initiation, and they designated MS medium containing 1 $mg.l^{-1}$ NAA and 0,01 $mg.l^{-1}$ BAP as the optimal medium for maximising callus growth.

There has been no published research about plantlet *P. palatiferum in vitro* cultures, nor about precursor feeding of *P. palatiferum*.

2.2.3. *In vitro* use of aromatic amino acids as precursors

In the plant cell, phosphoenolpyruvate and D-erythrose 4-phosphate can be converted by the processes of the shikimate pathway (through intermediate compounds such as shikimate or chorismate) into L-tryptophan, L-phenylalanine or L-tyrosine (Maeda & Dudareva 2012). Tyrosine can then be metabolized into various secondary metabolites, e. g. catecholamine compounds, such as tyramine and dopamine (Kulma & Szopa 2007). It can therefore be assumed that a significant increase in the amount of tyrosine available to plant cells, which can be achieved by supplementing culture media with dissolved

tyrosine, is likely to increase the amount of catecholamine compounds biosynthesised by the plant cells.

The practice of supplementing *in vitro* culture media with a precursor, in order to influence the secondary metabolite profile of plants or cells, is often referred to as "precursor feeding". The beneficial effect of precursor feeding was shown in practice for example by Watcharatanon et al. (2019) in *Bacopa monnieri* where supplementation with phenylalanine raised the concentrations of triterpenoid saponin glycosides up to 2.5 times as compared to control, and by Zare et al. (2014) in cell suspension cultures of *Papaver bracteatum* where supplementation with tyrosine increased the concentration of thebaine up to twentytwo-fold. The dopamine levels in cells of *Mucuna pruriens* suspension cultures were significantly augmented when tyrosine was made more abundant (Raghavendra et al. 2011).

Addition of L-tyrosine into MS culture media was previously carried out by Fadzliana et al. (2017) in order to improve betalain concentration in callus of *Hylocereus polyrhizus* and by Jawahar et al. (2018) to boost colchicine accumulation in *Gloriosa superba*. Both of these experiments were successful (with at least some of the experimental variants) in significantly increasing the amount of the respective secondary metabolites in the plants.

3. Aims of the thesis

The main goal of this thesis was to supplement *Pseuderanthemum palatiferum in vitro* culture with one or more specific chemical compounds and to investigate the effect of this supplementation on the profile of secondary metabolites in *Pseudereanthemum palatiferum*.

This goal can be divided into the following steps:

- Optimization of *Pseuderanthemum palatiferum* propagation and sufficient multiplication of *Pseuderanthemum palatiferum in vitro* culture.
- Identification of secondary metabolites in *Pseuderanthemum palatiferum.*
- Investigation of the possibility of modifying the secondary metabolite profile of *Pseuderanthemum palatiferum* by supplementation of culture media with plant growth regulators, precursors or elicitors.

4. Materials and methods

4.1. Plant material

Aseptic *P. palatiferum* plantlets were obtained from Department of Plant Science, Faculty of Natural Resources Prince of Songkla University, Hat Yai Campus, Thailand (7°1′N100°28′E). It was authenticated by Dr. Tassanee Khawniam.

4.2. Methodology

4.2.1. *In vitro* plant multiplication

P. palatiferum plantlets were kept aseptically on MS culture medium in Erlenmeyer flasks and were micropropagated using apical and nodal segments at a regular interval of 3-4 weeks.

MS medium used was supplemented with 30 $g.l^{-1}$ sucrose, 100 $mg.l^{-1}$ myo-inositol and 8 $mg.l^{-1}$ of agar and the pH level was adjusted to 5.8 prior to sterilization in an autoclave set to 121°C, 1.1 atm and 20 minutes. Micropropagation was carried out in sterile conditions, provided by the interior of a laminar flow cabinet. The main objective of micropropagation was to multiply the plants sufficiently for the purposes of experiments mentioned in further subchapters of this chapter. Each micropropagated plantlet was separated into individual nodal segments using a sterile scalpel. The leaf pair in the upper part of each nodal segment was shortened into (approximately) one third length of each leaf, in order to both modify the center of gravity of each plantlet and thusly preventing it from falling over into a horizontal position on the medium, and to promote apical growth or growth from axillary meristems. Plants and calli located within an Erlenmeyer flask struck with contamination were discarded. In case of larger plantlets which span more than 3 nodal segments, the last one or two nodal segments tend to be noticeably less vital than the other nodal segments, and for that reason such nodal segments were not used for the purposes of micropropagation.

The Erlenmeyer flasks containing *Pseuderanthemum palatiferum* plants and calli were kept in a cultivation room at all times, providing a constant temperature of 25/23°C and

a light/dark regime consisting of 16 hours of 36 μmol $m^{-2}.s^{-1}$ fluorescent light alternating with 6 hours of dark.

4.2.2. Callus induction and proliferation

Callus was induced from roots and leaves excised from *P. palatiferum* plantlets, which were placed onto MS medium supplemented with 1 $mg.l^{-1}$ BAP. The leaf blades of leaves used for this purpose were intentionally wounded using a scalpel, in order to maximise the exposure of cells to BAP. The successfully initiated calli were cultured on MS supplemented with 0.5 $mg.l^{-1}$BAP and 0.5 $mg.l^{-1}$ NAA. The callus was micropropagated every 3-4 weeks and the significantly darkened parts of the callus were discarded.

4.2.3. Secondary metabolite content analysis of *Pseuderanthemum palatiferum*

Ten plantlets of *P. palatiferum* spanning 3 to 4 nodal segments were selected and cut apart into roots and non-root parts (leaves and stems).

Harvested plants were oven-dried in 40°C for the duration 24 hours. The dried samples were pulverized using mortar and pestle and were subsequently extracted with methanol, vortexed and sonicated. Chloroform was added and the tube was shaken; subsequently, water was added and the tube was vortexed and centrifuged. Polar and lipid phases were transferred separately to clean micro vials and dried. The samples were derivatized by employing a modification of the protocol described by Fiehn et al. (2000) and Hutschenreuther et al. (2012). The derivatized sample was transferred to a GC autosampler glass vial and subjected to GC-MS analysis.

Peak deconvolution was accomplished using AMDIS 2.65 (Stein, 1999). The retention indices (RIs) were automatically calculated according to the retention time (RT) of the alkane mixture. A custom library adopting RI values was created using NIST14 (National Institute of Standards and Technology, Gaithersburg, MD, USA). The measurements were performed in three to six replicates. Excel 2013 (Microsoft, Redmond, Washington, DC, USA) and MetaboAnalyst 3.0 Web application (http://www.metaboanalyst.ca) were used for data processing and normalization procedures, principal component analysis (PCA) and creation of figures (Xia and Wishart, 2016). The data processing included peak

area normalization to the median of all areas within a chromatogram, generalized logarithm transformation, and mean centered data scaling.

4.2.4. L-tyrosine precursor feeding of *Pseuderanthemum palatiferum* callus

A control and four experimental treatments, each with a different concentration of tyrosine present in the medium, were used to establish this experiment. Each group consisted of forty 50 ml Erlenmeyer flasks, each holding 25 ml of the given medium. L-tyrosine was diluted in molar solution of NaOH and added into MS medium using a syringe filter immediately after autoclaving. The concentrations of tyrosine in the four different media (each used exclusively for one of the experimental groups) were 25, 50, 100 and 200 $mg.l^{-1}$, while the medium used for the control group contained no tyrosine.

Twenty of the flasks in each group contained a standardised piece of callus, while the other twenty enclosed a plantlet. To standardise, each plantlet used was the second most-apical nodal segment of a *P. palatiferum* plant and only vital plants with 3 or more total developed nodal segments were used. Only vital-looking (i. e. light-coloured and friable) callus was used, and each used piece of callus was cut down to a volume of approximately 1 cm^3.

After the establishment of the experiment, the plants and calli were cultivated under the aforementioned conditions for 42 days. After that point, the plant material was cut apart and separated into callus, root, leaves and stems (internodal segments). The samples were then oven-dried in 40°C for the duration of 24 hours. The dried samples were pulverized in mortar and pestle and were subsequently extracted with methanol, vortexed and sonicated. Chloroform was added and the tube was shaken; subsequently, water was added and the tube was vortexed and centrifuged. Polar and lipid phases were transferred separately to clean micro vials and dried. The samples were derivatized by employing a modification of the protocol described by Fiehn et al. (2000) and Hutschenreuther et al. (2012). The derivatized sample was transferred to a GC autosampler glass vial and subjected to GC-MS analysis.

5. Results

5.1. Multiplication of *Pseuderanthemum palatiferum* and optimisation of its propagation

P. palatiferum was successfully cultured on MS medium. Originally the plantlets were propagated every 3 weeks. It later became clear that *P. palatiferum* plantlets can be propagated after 4 weeks without a reduce in vitality, despite an apparent change in colour, which is caused by hyperhydricity. After approximately 5 weeks of no subculturing, however, leaves start to become darker and eventually blacken completely, which is almost certainly a sign of one or more nutrients being exhausted in the medium.

In some cases, a recently micropropagated plantlet would fall into a horizontal position on the culture medium, or its basal part would be raised above the culture medium, due to the growth of its downward faced leaves (which became partially embedded into the medium). For this reason, a specific micropropagatin method was employed, which lead to the elimination of these issues. When the leaf pair in the upper part of each nodal segment is shortened into (approximately) one third length of each leaf during micropropagation, the plantlet's center of gravity is modified in such a way, that the plantlet is prevented from falling over and the leaves do not "uproot" the plantlet.

When a vital *P. palatiferum* plantlet is not micropropagated for 4 weeks, it has grown in that time to contain 3-6 nodal segments. Theoretically, each of those nodal segments can be used for propagation. However, it has been observed that the basal-most (physiologically oldest) of those nodal segments is usually the least vital, and after propagation exhibits minimal growth and eventually starts to brown or blacken.

Formation of callus on the roots and leaves excised from *P. palatiferum* could in most cases be observed after 5-14 days, though not all of the explants formed callus.

Initially, the periodically micropropagated callus was cultured on MS medium supplemented with 1 $mg.l^{-1}$ BAP. After 3 to 4 weeks, parts of the callus regularly started becoming dark brown to completely black in colour, while other parts of the same body of callus displayed various lighter colours, including white-ish, light brown and medium brown. The darkest parts of any *P. palatiferum* callus exhibited minimal growth and

proved to be of minimal vitality and therefore also of no use in the process of callus multiplication. When left unpropagated for 5 weeks, the callus blackened completely. In addition, the culture medium under such callus became noticeably yellowed.

After some time, a differently supplemented Ms medium was used, in accordance with the research of Petsangkrit & Kittipongpatana (2016), who suggested MS medium supplemented with 1 $mg.l^{-1}$ NAA and 0,01 $mg.l^{-1}$ BAP as the optimal medium for the cultivation of *P. palatiferum* callus. Taking inspiration in this suggestion, the *P. palatiferum* callus was from that point on cultured on MS medium supplemented with 0.5 $mg.l^{-1}$ BAP and 0.5 $mg.l^{-1}$ NAA. Based on my observations, callus "matures" quicker on this medium than on the medium supplemented only with 1 $mg.l^{-1}$ BAP, meaning that it grows faster and the aforementioned changes in colour seem to occur on average 3-5 days earlier. This means that the callus had to be propagated more often than callus on the previous medium, therefore the speed of the multiplication process was increased.

5.2. Identification of secondary metabolites in *Pseuderanthemum palatiferum*

Data obtained from GC-MS analysis of *P. palatiferum* plants is compiled in Table 1. In total, 63 chemical compounds were identified from the plant material. Furthermore, 23 other compounds were detected, but could not be identified.

Table 1 - Results from GC-MS analysis of *P. palatiferum*

No.	Chemical compound	RT	RI	MW (m/z)
1.	Unknown	10,7094	1142,5	147
2.	Unknown	11,0741	1161	147
3.	Oxalic acid	11,429	1179,1	148
4.	DL-Valine	11,6905	1192,4	144
5.	Ethanolamine	12,1535	1216	174
6.	Glycerol	12,4394	1230,5	147
7.	Unknown	12,757	1246,7	147
8.	DL-Leucine	12,9137	1254,6	158
9.	L-Isoleucine	13,4278	1280,8	158
10.	Glycine	13,6496	1292,1	174
11.	L-Serine	13,7698	1298,2	234
12.	Phosphoric acid	13,9655	1308,2	299
13.	L-Proline	14,1637	1318,3	142
14.	Urea	14,2652	1323,4	147
15.	Unknown	14,383	1329,4	98
16.	DL-Glyceric acid	14,4772	1334,2	147
17.	Alanine	14,7436	1347,8	188
18.	L-Serine	14,9572	1358,6	204
19.	Succinic acid	15,1935	1370,7	147
20.	DL-Threonine	15,3535	1378,8	218
21.	Fumaric acid	15,4372	1383,1	245
22.	β-Alanine	16,359	1427,5	248
23.	DL-Homoserine	16,8496	1450,4	218
24.	Putrescine	17,3881	1475,6	174
25.	DL-Malic acid	18,2165	1515,6	147
26.	Erythronic acid	18,3424	1522	292
27.	trans-4-hydroxy- DL-Proline	18,4992	1529,9	230
28.	4-amino-Butyric acid	18,6275	1536,4	174
29.	L-Aspartic acid	18,8614	1548,3	232
30.	Threonic acid	18,9426	1552,4	292
31.	DL-Asparagine (detected at 3 different RTs)	19,7662 22,5577 25,5937	1594,2 1737,8 1906,2	188 116 188
32.	Unknown	20,4931	1631,1	245
33.	Unknown L-5-oxoproline derivative	20,749	1644,1	156
34.	DL-Glutamic acid	20,8611	1649,8	246

35.	Unknown putrescine derivative	21,2225	1668,1	174
36.	DL-Phenylalanine	21,7431	1694,6	218
37.	Unknown	22,215	1719,5	292
38.	Unknown	22,4416	1731,6	147
39.	Unknown	22,764	1748,9	174
40.	DL-Ornithine	23,3634	1780,9	142
41.	DL-Glycerol-3-phosphate	23,5169	1789,1	357
42.	D(-)-Quinic acid	23,7086	1799,4	345
43.	D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime	23,7785	1803,3	103
44.	Shikimic acid	24,0075	1816,2	358
45.	D-Glucose (detected at 2 different RTs)	24,2187 24,5322	1828,2 1845,9	319 319
46.	Citric acid	24,2731	1831,2	273
47.	Glyceric acid-3-phosphate	24,7807	1859,9	357
48.	Lysine	25,1551	1881,1	174
49.	L(+)-Galactonic acid-1,4-lactone	25,2851	1888,4	217
50.	trans-4-hydroxy-Cinnamic acid	25,6043	1906,8	293
51.	Unknown	25,7277	1914,2	204
52.	D-Glucose	25,8609	1922,2	204
53.	Tyramine	26,0591	1934,1	174
54.	Allantoin	26,1688	1940,6	331
55.	Glucuronic acid	26,2597	1946,1	333
56.	Galacturonic acid	26,6756	1971	292
57.	Unknown	26,7925	1978	305
58.	DL-Tyrosine	26,8689	1982,6	218
59.	Saccharic acid	26,9412	1986,9	333
60.	Unknown	27,4001	2015,2	232
61.	Unknown 4-coumaric acid derivative	27,9662	2051	293
62.	Unknown	28,0994	2059,5	245
63.	trans-Ferulic acid	28,1042	2059,8	338
64.	Dopamine	28,3024	2072,3	426
65.	n-Hexadecanoic acid	28,464	2082,5	313
66.	DL-Noradrenaline	28,9156	2111,1	174
67.	Unknown	29,3347	2137,6	204
68.	trans-Caffeic acid	30,3662	2203,2	219
69.	n-Octadecanoic acid	31,4237	2276,1	341

70.	Unknown L-Tryptophan derivative	32,1863	2328,7	202
71.	Unknown	32,3179	2337,8	217
72.	Unknown	33,468	2418,4	204
73.	Unknown sucrose derivative	34,3533	2484,3	361
74.	Unknown	35,5415	2572,6	254
75.	Unknown	36,7785	2669,2	284
76.	Unknown	36,7817	2669,5	284
77.	Unknown	36,9263	2681	361
78.	alpha,alpha'-D-Trehalose	37,7954	2750,2	361
79.	Galactinol nonakis(trimethylsilyl) ether	38,3282	2792,7	204
80.	Unknown	39,4059	2878,6	361
81.	cis-3-Caffeoyl-quinic acid	40,5934	2973,2	345
82.	trans-3-Caffeoyl-quinic acid	41,7686	3066,8	345
83.	trans-4-Caffeoyl-quinic acid	42,6905	3140,3	307

5.3. Modification of the secondary metabolite profile of *Pseuderanthemum palatiferum*

No contamination occurred in any of the control or experimental treatments. All of the plantlets in the control treatment developed (one or two) roots by the end of cultivation. Approximately a third of these plantlets (7 out of 20) exhibited little to no apparent growth (apart from the formation and growth of roots), though none of them showed specific signs of a loss of vitality, such as blackening or yellowing. The remaining 13 out of 20 plantlets have grown to a size of 2 to 4 nodal segments.

The majority of the calli samples used (17 out of 20) in the control treatment underwent a several-fold increase in size, while 3 out of 20 of them did not exhibit apparent growth during the cultivation and had also become completely black in colour. The mentioned vital calli had also changed colour since micropropagation, however only 3 of them had become mostly completely dark with medium dark parts, and the remaining 14 had pronounced dark part, but still encompassed some light-coloured parts, as well as medium dark parts.

Eight of the plantlets cultivated on MS medium supplemented with 50 $mg.l^{-1}$ of tyrosine exhibited little to no perceivable growth and the tissues of 3 plantlets from among them yellowed or blackened. The 12 other plantlets of this treatment featured apical growth as well as apparent vitality and 7 of these plantlets also developed one or more roots.

All the callus samples in the 50 $mg.l^{-1}$ of tyrosine MS medium treatment exceeded their original size at least by 50 %, although the size of some of them was raised by 250 % or more. Four of the calli used in this treatment were blackened completely by the end of cultivation, while the remaining 16 callus samples retained light-coloured and medium-coloured parts.

Minimal to no growth was observed in 8 of the plantlets culture on MS medium supplemented with 100 $mg.l^{-1}$ of tyrosine. Two of those plantlets with minimal growth developed a root, while the other 6 did not form roots. The other twelve of the plantlets used in this treatment had grown since the beginning of cultivation and they all developed one or more roots.

Seven of the callus samples used in the 100 $mg.l^{-1}$ tyrosine treatment had not noticeably grown during cultivation and became dark black in colour, while 14 other callus samples of this treatment remained vital, increased in size and were partly light or medium in colour even at the end of cultivation.

The number of vital and non-vital plant and callus samples in the 25 $mg.l^{-1}$ tyrosine and 200 $mg.l^{-1}$ tyrosine treatments was not evaluated during cultivation or at end of cultivation.

At the time of writing, the GC-MS analysis of the control and 4 experimental treatments has not yet been conducted and therefore, the results of this analysis are not included in this thesis.

6. Discussion

The achieved growth of callus on MS medium supplemented with NAA and BAP is in accordance to the performance of the one that Petsangkrit & Kittipongpatana (2016) designated as most effective for this purpose, despite the different concentrations of NAA and BAP were used. Future research into the effect of different concentrations of these plant growth regulators would allow for further optimisation of *P. palatiferum* callus multiplication and culturing.

The observed changes in colour and growth rate of callus could be caused by oxidation of phenolic compounds released from callus cells, which can even lead to cell death (Petsangkrit & Kittipongpatana 2016). This theory is further supported by my observations of medium on which callus which was not subcultured for 5 weeks and thus became fully blackened. According to my hypothesis, the observed yellowing of medium under such callus suggests the release of phenolic compounds due to cell death of callus cells.

The presence of catecholamines (specifically dopamine and noradrenaline) in *P. palatiferum* was not previously reported. These catecholamines may be the active compounds behind one or more of *P. palatiferum*'s physiological effects, which could be the subject matter of future research. Furthermore, the anti-oxidant effects of *P. palatiferum* could be (either in part or completely) due to the presence of caffeic acid, which has anti-oxidant effects (Lafay et al. 2005).

Several other compounds detected in the GC-MS analysis were not previously reported to be present in *P. palatiferum*, such as D-trehalose, putrescine and trans-ferulic acid. The detection of various phenolic compounds, is in accordance with previous studies reporting on *P. palatiferum* secondary metabolite contents (Buncharoen et al. 2018; Kongprasom et al. 2019).

7. Conclusions

Pseuderanthemum palatiferum was successfully cultured on MS medium. Callus was induced from excised *P. palatiferum* leaves and roots on MS medium supplemented with 1 $mg.l^{-1}$ BAP and was successfully cultured on MS medium supplemented 0.5 $mg.l^{-1}$BAP and 0.5 $mg.l^{-1}$ NAA. The optimisation of these processes provides an effective methodology for rapid *in vitro* multiplication and long-term cultivation of *Pseuderanthemum palatiferum*, which was not previously published.

Twenty-five unique chemical compounds were detected and identified from various parts of *P. palatiferum* plants. Some of the found compounds were not previously reported to be present in *P. palatiferum*, such as noradrenaline and dopamine. These catecholamine compounds could play a major role in the physiology of some of *P. palatiferum*'s remedial effects, therefore this information could be of importance to future research.

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