UNIVERSITI PUTRA MALAYSIA

ANTIOXIDANT AND ANTICANCER PROPERTIES OF CRUDE EXTRACTS FROM *DEDALU* (*Helixanthera parasitica lour.)*

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Chair : Professor Rusea Go, PhD
Faculty : Science

Helixanthera parasitica Lour, recognized as a hemiparasitic mistletoe from the family Loranthaceae is a mystique plant that has been utilized ethnobotanically as medicinal plant in Southeast Asia countries. Up to now and to the best of our knowledge very scarce research has been conducted on this species to exploit its further medicinal potential particularly on exploring its antioxidant capacity and towards one of the leading non-communicable disease (NCD), that has remained difficult to treat but preventable, the breast cancer. The present study was designed to prepare different extracts from the leaves and stem of *H. parasitica* and to determine their total phenol, flavonoids content, antioxidant activity as well as selecting the best extracts towards anticancer assay by applying established methods. Different extracts were prepared by maceration technique with powdered leaves and stem sample. Extraction of both plant part were performed separately using solvents of different polarity, namely, distilled water (dH_2O), methanol (70% and 100), acetone (70% and 100%) and petroleum ether. The parameters targeted on the plant extracts were determined using Total Phenolic Content (TPC)/ Folin-Ciocalteu reagent (FCR), Total Flavonoid Content (TFC)/ aluminium chloride ($AlCl_3$), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and MTT (for anticancer assay) method. Highest extract yield was obtained from leaf extracts (679.13 ± 9.60 mg/g DW) and the yields of extraction by various solvents decreased in the following order: 70% acetone > 70% methanol > 100% methanol > 100% acetone > dH_2O > petroleum ether. Leaf extract from pure acetone solvent was found to exhibit highest TPC (99.45 ± 12.87 mg GAE/g DW), TFC (17.57 ± 0.63 mg QE/g DW) and DPPH (81.61 ± 1.22 %) with no significant difference found between leaf and stem evaluated from TPC and DPPH. This extract also showed slightly lower FRAP value (6.73 ± 0.36 mg TE/g DW), but without significant difference compared to stem. In terms of 50% inhibition concentration of DPPH (IC_{50}), the lowest value was retrieved from 100% acetone stem extract (42.66 ± 4.82 µg/mL), however this value is insignificant ($p > 0.05$) with the top scoring extract, 100% acetone leaf (45.21±4.91 µg/mL) which are both almost similar to the potent standard antioxidant Trolox (48.22 ± 0.79 µg/mL) with

insignificant difference. A correlation study of the extracts demonstrated very strong significant positive correlation between phenolic content (TPC) and antioxidant potential especially with DPPH (r = 0.928) and followed by FRAP (r = 0.901). Crude extracts of *H. parasitica* was found to be highly cytotoxic or antiproliferative to Michigan Cancer Foundation-7 breast cancer cells (MCF-7) when tested for MTT IC_{50} particularly with selected 100% acetone stem extract at 97.47 ± 2.87 µg/mL, while 5-fluorouracil used as positive standard revealed an IC_{50} of 3.31 ± 0.15 µg/mL. Interestingly, all the 3 tested extracts (100% acetone, leaf; 100% acetone, stem and 100% methanol, leaf) had no cytotoxic effect up to the maximum extract concentration tested on normal dermal fibroblast cells (IC_{50} > 500 µg/mL). Current study suggested that extraction solvents and plant parts (for Objective 1 and 3) played significant effect on extractability which depends to the preference of the research, either in terms of targeting maximal extract yield or active phytocompounds. This study proposed the presence of water with intermediate polar solvent (70% acetone) and leaf part could provide increased extraction yield while 100% acetone is suitable for bioactive compound recovery. Not forgotten, *H. parasitica* also revealed to be a potent source exhibiting anticancer activities but further research need to be done to purify and characterize the extract to identify the potent polar therapeutic compound or candidate against breast cancer. Meanwhile, this report is the first in describing total phenols, flavonoids content, extended assessment of antioxidant activity and anticancer property against breast cancer cell line from extracts of *H. parasitica* especially on species from Malaysia which may account for some of the medical claims attributed to this plant. Hence, this research and outcome may become a follow up from previous discontinued studies on the exploration of this potent source and lead for new nutraceutical and pharmaceutical approach.

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

CIRI ANTIOKSIDAN DAN ANTIKANSER PELBAGAI EKSTRAK MENTAH *DEDALU* (*Helixanthera parasitica* Lour.)

Oleh

THIRUBUVANESVARI DURAIVELU

Mac 2019

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Helixanthera parasitica Lour sejenis pokok dedalu hemiparasit berasal daripada famili Loranthaceae dikenali sebagai tumbuhan unik yang digunakan secara meluas bagi tujuan ethnobotani ataupun sebagai tumbuhan ubatan di negara Asia Tenggara. Berdasarkan pengetahuan kami, terlalu sedikit penyelidikan saintifik yang dijalankan terhadap spesies ini terutamanya untuk mengeksplorasi potensinya sebagai sumber antioksidan dan keupayaannya untuk menentang salah satu punca utama kematian populasi dunia iaitu penyakit kanser payu dara yang kekal sebagai penyakit yang sukar diubati tetapi mampu dicegah. Dalam kajian ini, pelbagai ekstrak berasaskan daun dan batang *H. parasitica* telah diuji bagi mengetahui jumlah sebatian fenolik, flavonoid, aktiviti antioksidan dan juga pemilihan ekstrak terbaik bagi tujuan ujian antikanser dengan mengaplikasikan teknik yang sah. Pelbagai ekstrak mentah telah disediakan menerusi teknik rendaman dengan menggunakan serbuk daun dan batang. Proses pengekstrakan dijalankan dengan mengguna pakai pelarut yang berbagai kepolaran, iaitu air suling (dH_2O), metanol (70% dan 100%), aseton (70% dan 100%) dan petroleum eter, sekiraya disusun pada kadar polariti yang menurun. Teknik Jumlah Kandungan Fenolik (TPC), Jumlah Kandungan Flavonoid (TFC), 2, 2-difenil-1-pikrilhidrazil (DPPH), Kuasa Antioksidan Penurunan Ferik (FRAP) dan MTT (ujian antikanser) telah digunakan bagi memenuhi objektif kajian yang disasarkan. Hasil ekstrak tertinggi diperoleh daripada ekstrak daun 679.13 ± 9.60 mg/g DW dan pelarut pengekstrak terbaik dapat disusun pada kadar menurun iaitu 70% aceton > 70% metanol > 100% metanol > 100% aceton > dH_2O > petroleum eter. Aceton tulen (100%) dapat digelar sebagai pelarut terbaik dari segi Jumlah Kandungan Fenolik (99.45 ± 12.87 mg GAE/g DW), Jumlah Kandungan Flavonoid (17.57 ± 0.63 mg QE/g DW) dan peratusan 2, 2-difenil-2-pikrilhidrazil (81.61 ± 1.22 %) dengan tiada perbezaan signifikan yang dilihat dari segi daun dan batang. Ekstrak ini juga menunjukkan nilai Kuasa Antioksidan Penurunan Ferik (6.73 ± 0.36 mg TE/g DW) yang kurang sedikit berbanding batang, tetapi nilai tersebut juga tidak mempunyai perbezaan yang signifikan. Daripada sudut kepekatan ekstrak yang membawa kepada pengurangan 50% DPPH (IC_{50}), nilai atau kepekatan terendah diperoleh dengan

menggunakan ekstrak batang di dalam pelarut 100% aseton (42.66 ± 4.82 µg/mL), tetapi nilai ini tidak mempunyai perbezaan signifikan jika dibandingkan dengan ekstrak 100% aseton daun (45.21 ± 4.91 µg/mL) yang mana kedua-dua nilai ekstrak ini hampir sama dengan antioksidan piawai Trolox (48.22 ± 0.79 µg/mL) yang turut tidak menunjukkan perbezaan signifikan. Kajian korelasi bagi ekstrak *H. parasitica* memaparkan korelasi sigifikan positif yang sangat tinggi di antara kuantiti sebatian fenolik (TPC) dan aktiviti antioksidan terutamanya dengan DPPH (r = 0.928) dan diikuti dengan FRAP (r = 0.901). Ekstrak mentah *H. parasitica* menunjukkan aktiviti sitotoksik/antiproliferatif yang tinggi terhadap sel kanser payu dara Michigan Cancer Foundation-7 (MCF-7) semasa diuji dengan asai MTT di mana IC_{50} terendah diperoleh apabila ekstrak 100% aseton batang (97.47 ± 2.87 µg/mL) digunakan, sementara 5-fluorouracil yang dijadikan piawai positif merekodkan nilai $IC_{50,}$ 3.31 ± 0.15 µg/mL. Kesan ekstrak ke atas sel normal fibroblas pula tidak menunjukkan kematian sel sehingga pada kepekatan ekstrak maksimum yang diuji (IC_{50} > 500 µg/mL). Kajian ini mencadangkan pelarut pengekstrakan dan bahagian pokok (untuk Objektif 1 dan 3) memainkan peranan daripada segi kuantiti ekstrak dan jenis hasil yang diperoleh yang bergantung kepada objektif penyelidikan, sama ada untuk mendapatkan hasil ekstrak mentah maksimum atau menyasarkan sebatian bioaktifnya. Sehubungan dengan itu, kajian ini menunjukkan campuran komponen air bersama dengan pelarut sederhana polar berupaya menghasilkan jumlah ekstrak mentah yang maksima manakala pelarut tulen aseton adalah sesuai untuk mengekstrak sebatian bioaktif pokok *H. parasitica.* Tambahan lagi, ekstrak pokok ini juga menunujukkan potensi yang tinggi sebagai sumber antiploferatif sel kanser payudara, dan penyelidikan tambahan perlu dijalankan untuk mengenalpasti agen bioaktif spesifik yang membawa kepada kesan sitotoksik ini dan seterusnya menjadi calon antikanser yang berpotensi. Selain itu, laporan ini merupakan manuskrip saintifik pertama yang menerajui jumlah sebatian fenolik, flavonoid, penambahan penilaian antioksidan dan aktiviti antikanser terhadap kanser payu dara bagi ekstrak mentah *H. parasitica* khususnya bagi spesies yang berasal dari Malaysia yang mana selari dengan pengetahuan perubatan tradisional yang dikaitkan dengan pokok ini. Di samping itu, kajian dan keputusan yang ditunjukkan mampu mengusul semula usaha untuk menerajui potensi perubatan pokok ini kepada penemuan nutraseutikal dan farmaseutikal.

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

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ANNOVA	Analysis of variance
BC	Breast cancer
CE	Catechin equivalent
CGM	Complete growth medium
ºC	Degree celsius
DIM	Diindolylmethane
DMSO	Dimethyl sulfoxide
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide
DPPH	2,2-diphenyl-1-picrylhydrazyl
dH_2O	Distilled water
DW	Dry weight
DMEM	Dulbecco's modified eagle medium
EGCG	Epigallocatechin gallate
EMT	Epithelial-mesenchymal transition
E2F	Eukaryote transcriptional factor
FRAP	Ferric reducing antioxidant power
FBS	Fetal bovine serum
5-FU	5-fluorouracil
GC	Guanine-Cytosine
GAE	Gallic acid equivalent
G2/M	Gap 2/mitotic phase
g	Gram
100% Ace	Hundred percent acetone
100% MeOH	Hundred percent methanol
IC_{50}	Inhibition concentration 50%
MCF-7	Michigan Cancer Foundation-7
µL	Microliter
mg	Milligram
mL	Milliliter
%	Percentage
Obj	Objective
PtEth	Petroleum ether
PBS	Phosphate buffer solution
QE	Quercetin equivalent
Rb	Retinoblastoma
rpm	Revolution per minute
70% Ace	Seventy percent acetone
70% MeOH	Seventy percent methanol
S	Synthesis phase
TA	Thymine-Adenine
TPTZ	2,4,6-tripyridyl-s-triazine
TFC	Total flavonoid content
TPC	Total phenolic content
TE	Trolox equivalent
w/v	Weight over volume

CHAPTER 1

INTRODUCTION

1.1 Overview of study

In the course of recent decades (over 30 years) there has been a developing substantial shift in disease burden from communicable to multiple non-communicable chronic diseases (NCDs) among world population (Murray et al., 2012). Nearly two-thirds of deaths recorded worldwide accounts due to NCDs (World Health Organization, 2013). Cancer or malignant growth is one of the leading major cause of death under NCDs which is generally described as an abnormal growth of tissue and cell arising from uncontrolled proliferation autonomously, ensuing in a progressive rise in the magnitude of cell divisions. Among them, breast cancer is the most frequent and number one leading cause of cancer among women that hits high death rate globally, particularly in non-developing nations because of deffered diagnosis and populace upsurge (Nordin, Kadir, Zakaria, Abdullah, & Abdullah, 2018). Oxidative stress is one of the phenomenon often associated with pathways related to carcinogenesis. Reactive free radical species like oxygen and nitrogen is crucial to mediate certain essential roles under a controlled regulation by the human body, but the risk arises when overproduction or an unregulated release is manifested in the form of various life-threatening disorders predominantly cancer.

There is no denial on the availability of existing natural and synthetic compounds which have the power to quench or scavenge these free radicals providing a great scope for correcting such oxidative imbalances. However, usage of synthetic antioxidants like Butylated Hydroxyanisole (BHA), butylated hydroxy toluene (BHT), propyl gallate (PG) and tert-butylhydroquinone causes an alarming concern on its toxicity and carcinogenicity emerged due to their extensive use which necessitates the venture to search for better alternatives.

Additionally, problem with combating against cancer also turns very challenging and requires strenuous effort, particularly in the development of treatments for aggressive and rapidly multiplying tumours. The ability of breast cancer cells inducing metastasis and being invasive as well as relapses as a result of having resistant disease (Early Breast Cancer Trialists' Collaborative Group, 2005) are often blamed as the factors liable for the mortality allied with this disease. Eventhough, existing chemotherapy has implicated relevant outcome on cancer treatment, it exihibits low specificity and is restricted by dose-limiting toxicity (Khandanlou, Murthy, Saranath, & Damani, 2018) and the worst complication arises when drug resistance phenomenon takes over. Even, some analogs that have given a major renewable natural source and mostly utilized in breast cancer treatment was then known to be unproductive in some cases after a period of time which further urge an extensive exploration from natural source.

Alternative traditional and complementary treatment using medicines derived from herbal and natural products such as Ayurveda and Chinese medicine have been used widely in every culture across different times and civilization since ancient immemorial times globally. Asian countries specifically Thailand, India, Indonesia, China, and Malaysia are exceptionally well-known for precious ethnobotanical knowledges. Moreover, Malaysia sets its name to be one of the 17 mega biodiversity countries in the world, enriched in biodiversity as a potential source of both primary and secondary therapeutic metabolites like phenols and flavonoids which exhibit myriad biological properties. Emergence of increasing evidences which indicates that medicinal plant-derived products inhibit tumorogenesis and related to inflammatory processes at various stages emphasizes the significance of these products in chemotherapy and also as therapeutic agents targeted for free radical induced pathologies and disease prevention. Approximately 65% of all the approved drugs used in the treatment namely chemotheraphy or prevention of cancers originates from natural products or natural product derivatives (Nurhanan et al., 2008), of which plants contribute around 25% (Balunas & Kinghorn, 2005).

Natural antioxidants or drugs apart from being safe and causing minimal adverse effect, these compounds receive great attention because of its economic affordability as well as being more efficient than their synthetic counterparts. In addition, not only a plethora of drugs are of plant origin but also the entire plant extracts in some cases could be used as promising therapeutic modalities taking into consideration their bioactivity as well as target specificity (Bhat, Gul, Lohamror, Qureshi, & Ghazi, 2018). Other than that, the use of plant extracts may impart an advantage over using one isolated compound as drugs, due to the availability of various components possessing numerous possible intracellular targets which may work individually or synergistically to combat diseases induced by oxidative stress and improving health (Nordin et al., 2018). Furthermore, complementary practises such as consuming vitamins or herbs for cancer treatment or to relieve its side effects is not something neoteric.

Therefore, this has invoked the quest for continuous search targeting wild species of plant exihibiting unique behavior such as parasitism, for naturally occurring novel drugs providing high efficacy to drug resistant tumors with minimal side effects.

1.2 Problem statements

Mistletoe, a group of parasitic plant species used as medicinal herbs are not sufficiently explored and studied in Malaysia for their therapeutics and pharmacological properties. Mistletoes are often and typically utilized by traditional healers as traditional medicines without any written proof who need to hide the formulas, plant types and treatments from the public (Kwanda, Noikotr, Sudmoon, Tanee, & Chaveerach, 2013). Additionally, parasitic plants are potential traditional medicine through fatal to their hosts. Keeping this view in mind, the current attempt was directed on therapeutic exploration on Malaysian mistletoe, *Helixanthera parasitica* Lour. which is known to be a hemiparasitic shrub in the family of Loranthaceae.

This shrub is chosen due its ethnopharmacological significance, identified therapeutic property and phytochemicals discovered, apart from other potent antioxidative compounds. However, the information gathered on this potential mistletoe shrub remains scarce due to very few scientific researches conducted. It is important to notify that, the last study performed on *H, parasitica* dates 15 years ago in Thailand and another significant study on its phytochemistry dates back in 1994 originating from China and no publication is revealed from Malaysia. However, those limited studies focused on scientific experimentation of its therapeutic and phytochemistry without considering extract preparation and plant parts. Selection of a suitable extraction solvent plays a role for the standardization of herbal products as it is utilized in the removal of desirable soluble constituents, leaving out those not required. Further, different plant part of *H. parasitica* were evaluated for phytochemical values in order to valorize all the plant as functional source of extraction. Optimization of parameters are critical for upscaling purposes to bring *H. parasitica* from bench scale to pilot plant level and subsequently to be adapted readily in clinical research program. Hence, post-harvest treatment variability namely extraction solvents and profiling the composition of different plant part extracts are applied in this research taking into consideration of its great significance towards obtaining a maximum yield of phytocompounds especially antioxidants that correspond to the therapeutic potential of local *H. parasitica.*

Additionally, thus far, no empirical submission has been reported on the antioxidative levels thoroughly and cytotoxic properties of any *Helixanthera* species against breast cancer cell. Other than being used for folkhore benefits, scientific reports exposing the therapeutic potential of *H. parasitica* on anticancer responses and newly discovered compound prompted us to hypothesize that *H. parasitica* plant may exhibit vast range of antioxidant and antiproliferative activities. With this background and in the view of exploration for an alternative medicine, *H. parasitica* was tested to fulfil the imperative need to discover the real potential of the local herbs through its naturally occurring phytocompounds for antioxidant and anti-breast cancer activity. It is important when looking at the side effects of current treatment, damages upon healthy cells and rising therapeutic resistances. Current research also taken to support ideas of using plants synergistically with the available modern drugs to subsequently improve the effect on pharmacological, toxicological and prognosis of treatment if this research and extended studies proves it to be safe and effective. This effort will demonstrate a scientific documentary on post-harvest prep and therapeutic properties of one of the Malaysian mistletoe *H. parasitica,* having a potential medicinal value, that could lead towards drug discovery and being handful in human healthcare.

1.3 Objectives of study

Thus, the objectives of this study are:

1. To determine the best extraction solvent and plant part based on optimum extract yield obtained from *H. parasitica.*
2. To determine the best extract based on extraction solvent and plant part by quantifying phenolic and flavonoid compounds as well as antioxidant potential.

3. To determine the anticancer potential and cytotoxicity effect on breast cancer (MCF-7) and healthy fibroblast cell lines using selected extracts of *H. parasitica* based on phenolic quantification, flavonoid contents and antioxidant activity.

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BIODATA OF STUDENT

The student was born in 1992, January 27th at Hospital Lumut, Perak, Malaysia. She attended her primary education at S.K. Toh Indera Wangsa Ahmad, Batu Gajah, and followed by secondary education at S.M.K. Toh Indera Wangsa Ahmad, Batu Gajah. Her interest in pursuing higher studies enabled herself to enroll into Foundation in Agricultural Program at Universiti Putra Malaysia and right away continuing her degree program in Bachelor Science (Honours) Major in Biology from 2011 to 2015.

The student has ample of experiences conducting research related to algae namely Spirulina, dinoflagellates, genetic related studies, plant sciences and several other fields. One of her biggest involvement in research is through her participation in researches conducted at University of Chungnam, Daejeon, South Korea. Candidate has huge interest and passion in performing biology related studies and now her interest is focused to healthcare and plant ethnobotany. She has equipped herself with knowledge on biochemistry and cell handling through participation in classes and coach from postgraduate collegues. Looking at her interest and capability, the author pursued her studies in degree of Master in Science under the field of Cancer Biology and together related to Biodiversity and Conservation of Natural Resources in 2016. With her studies focused on postharvest treatments, antioxidantive properties and anticancer responses of wild medicinal mistletoe plant, the author has completed her Master of Science program with a Thesis entitled “Antioxidant and Anticancer Properties of Crude Extracts from *Helizanthera parasitica* Lour. (Dedalu)”.

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