ESTABLISHMENT OF CALLUS AND CELL SUSPENSION CULTURES OF *Artemisia annua* L. OF MONGOLIAN ORIGIN FOR THE PRODUCTION OF ARTEMISININ

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TABLE OF CONTENTS

APPENDICES

LIST OF CONFERENCES

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

%	Percent
^{o}C	Degree Celsius
cm	Centimetre
g	Gram
mL	Millilitre
L	Litre
g/L	Gram per litre
mg/g	Microgram per gram
rpm	Revolution per minute
mM	Millimolar
$\mu mol\ m^{-2}\ s^{-1}$	Micromole per square metre per second
2,4-D	2,4-dichlorophynoxyacetic acid
ANOVA	Analysis of variance
BA	6-Benzylaminopurine
KNO_3	Potassium nitrate
KH_2PO_4	Potassium phosphate
LED	Light emitting diode
MS	Murashige and Skoog
NAA	Napthaleneacetic acid
PGR	Plant growth regulator
TDZ	Thidiazuron
WHO	World Health Organisation

LIST OF APPENDICES

PEMBANGUNAN KULTUR KALUS DAN SEL AMPAIAN *Artemisia annua* L. YANG BERASAL DARI MONGOLIA BAGI PENGHASILAN ARTEMISININ

ABSTRAK

Artemisia annua L. adalah sejenis tumbuhan ubatan yang digunakan sejak berkurun lama untuk merawat pelbagai penyakit disebabkan oleh kandungan sebatian semulajadi, artemisinin. Walau bagaimanapun, kandungan artemisinin dalam tanaman ini adalah rendah, lebih kurang 0.01-1.4% daripada berat keringnya bergantung pada variasi tumbuhan dan tertakluk kepada faktor persekitaran. Oleh itu, pokok *A. annua* L. yang berasal dari Mongolia telah dikaji sebagai usaha untuk meneroka sumber variasi tumbuhan baharu untuk penghasilan artemisinin. Kajian ini melaporkan protokol yang dioptimumkan untuk menghasilkan anak pokok secara *in vitro*, mengaruh kalus, dan membangunkan kultur ampaian sel *A. annua* L. untuk menghasilkan artemisinin. Didapati bahawa 0.1 mg/L thidiazuron (TDZ) menghasilkan percambahan pucuk secara langsung dari eksplan daun. Sejumlah 46.67% pucuk dengan purata 4.47 pucuk per eksplan diperolehi selepas 6 minggu. Sejumlah 80% daripada anak pokok yang diperolehi berakar pada media MS yang mengandungi 0.5 mg/L IBA dan 0.5 mg/L tiamin. Semua anak pokok berjaya diaklimatisasi. Media MS dengan pertambahan 0.5 mg/L 2,4-D dan 0.5 mg/L TDZ mengaruh kalus kehijauan dari eksplan daun. Proliferasi kalus yang optimum diperolehi dengan menggunakan kalus saiz 0.1g sebagai inokulum permulaan dikultur dalam media MS beragar dengan kekuatan penuh menggunakan sukrosa 30 g/L, dan tambahan 0.5 mg/L 2,4-D, dan 0.5 mg/L TDZ. Kultur kalus dapat dikekalkan dengan indeks pertumbuhan sebanyak 7 - 35 untuk 10 kitaran subkultur pada setiap 4 minggu untuk satu kitaran. Kultur ampaian sel berjaya dibangunkan daripada kultur kalus. Masa optimum subkultur adalah 30 hari. Indeks pertumbuhan sebanyak 6 dan ke-atas diperhatikan

ketika subkultur sel ampaian secara berterusan untuk 10 kitaran. Biojisim sel kultur ampaian maksimum diperolehi daripada permulaan media pada pH 6.2 dalam 25 mL media cecair yang diemparkan pada 90 rpm. Tiada perbezaan secara signifikan penghasilan biojisim diantara sel yang ditapis (< 850 µm, > 850 µm) dan tidak ditapis. Kepekatan garam bukan organik dan spektrum cahaya LED yang berlainan menunjukkan kesan yang signifikan terhadap biojisim sel dan kandungan artemisinin. Media dengan kepekatan 1.56 mM KH_2PO_4 dan 19 mM KNO_3 menghasilkan biojisim sel yang tertinggi. Medium tanpa KH_2PO_4 dan KNO_3 menghasilkan kandungan artemisinin yang tinggi. Penghasilan sebanyak 70 kali ganda kandungan biojisim diperhatikan di bawah pancaran LED merah dan gabungan LED biru dan merah dengan nisbah 1: 1. Tahap artemisinin maksimum sebanyak 0.45 mg/g dilaporkan di bawah pancaran LED merah. Penghasilan ampaian sel *A. annua* L. berskala kecil dapat dilaksanakan dengan menggunakan kelalang Erlenmeyer saiz 500 mL. Kajian semasa ini berjaya mewujudkan protokol mudah untuk membangunkan anak pokok *A. annua* L. secara *in vitro* yang dapat digunakan untuk penghasilan klon tanaman yang seiras antara satu sama lain. Kultur kalus dan ampaian sel boleh digunakan sebagai bahan kajian dalam meningkatan penghasilan artemisinin.

ESTABLISHMENT OF CALLUS AND CELL SUSPENSION CULTURES OF *Artemisia annua* L. OF MONGOLIAN ORIGIN FOR THE PRODUCTION OF ARTEMISININ

ABSTRACT

Artemisia annua L., a medicinal plant used for centuries to treat a broad range of diseases because of its natural compound, artemisinin. However, the content of artemisinin in the plant is low, about 0.01-1.4 % of its dry weight, depending on the plant variety and subjected to environmental factors. Thus, *A. annua* L. plant originated from Mongolian was studied to explore a new source of plant variety as a source of artemisinin. The present study reported an optimised protocol to produce *in vitro* plants, induce callus, and establish the cell suspension culture of *A. annua* L. to produce artemisinin. It was found that 0.1 mg/L of thidiazuron (TDZ) resulted in direct plantlets regeneration from the leaf explants. A total of 46.67 % shoots with an average of 4.47 shoots per explant was obtained after 6 weeks. A total of 80 % of the plantlets obtained were rooted in MS medium with 0.5 mg/L IBA and 0.5 mg/L thiamine. All the plantlets were successfully acclimatised. MS medium supplemented with 0.5 mg/L of 2,4-D and 0.5 mg/L TDZ induced greenish callus from leaf explants. The best callus proliferation was obtained using an initial inoculum size of 0.1 g of callus cultured in full strength MS agar medium using 30 g/L sucrose and supplemented 0.5 mg/L of 2,4-D, and 0.5 mg/L TDZ. Callus cultures could be maintained with a growth index of 7 - 35 for 10 subculture cycles with 4 weeks intervals per cycle. A successful cell suspension culture was established from the callus induced. The optimum time of the subculture was 30 days. A growth index of 6 and more was observed during the continuous subculturing of cells in liquid suspension for 10 cycles. Maximum cell biomass was obtained from an initial media pH of 6.2 cultured in 25 mL liquid medium

rotated at 90 rpm. There were no significant differences in the cell biomass between sieved (< 850 μm, > 850 μm) and non-sieved cells. Different concentrations of inorganic salt and LED light spectra showed significant effects on cell biomass and artemisinin level. Medium with concentrations of 1.56 mM KH_2PO_4 and 19 mM KNO_3 produced the highest biomass content. Medium devoid of KH_2PO_4 and KNO_3 was high in artemisinin. A 70-fold yield in the accumulation of biomass was observed under red and mixed LED with a ratio of 1:1 blue and red. A maximum artemisinin level of 0.45 mg/g was reported in red LED. Flask-scale production of cell suspension of *A. annua* L. could be conducted using 500 mL size of Erlenmeyer flasks. The present study successfully established a simple protocol for *in vitro* plant regeneration for *A. annua* L. that can be utilised for the production of many plants that are clones of each other. Callus and cell suspension cultures provide useful material to increase the production of artemisinin.

CHAPTER 1

INTRODUCTION

Artemisia annua L. is an ancient medicinal plant applied by Chinese medical practitioners over 2,000 years to treat symptoms associated with fever. The author of the Chinese Handbook of Prescriptions for Emergency Treatments, Ge Hong prescribed the use of *Artemisia* to treat fevers in A.D. 340 (de Magalhães et al., 2016). A large variety of compounds were found in *A. annua* L., for example, coumarins, phenolics, purines, steroids, sesquiterpenoids, aliphatics, flavonoids, lipids, triterpenoids, and artemisinin. The active compounds in *A. annua* L. are claimed to have antiviral, anti-plasmodial, anti-inflammatory, anti-microbial, anti-convulsant, and anti-cholesterolemic properties (Kim et al. 2015). Recently, it has been brought to our attention again in Madagascar as an infusion drink containing *A. annua* L. extract and other herbs for treatment against coronavirus disease (Septembre-Malaterre et al., 2020).

Various physiochemical factors will influence the regeneration efficiency of the growth of a plant, as well as secondary metabolites production (Ali et al., 2013). These include the quality of light (LED), photoperiod, concentrations of the macro and micronutrients of the medium, pH, the explants type, and the composition of plant growth regulators (Ali et al., 2013). Many researchers claimed that *in vitro* regenerated plants are superior to those grown from seeds in terms of yield, content, and uniformity of artemisinin (Hussain et al., 2012; Espinosa-Leal et al., 2018; Wetzstein et al., 2018). Tissue culture for the mass clonal propagation of various plants has been established for field cultivation, rooting, shoot regeneration, and acclimatisation.

The biosynthesis of artemisinin is almost completed previously (Nguyen et al., 2011), and the full chemical production of artemisinin was attained by Hofheinz and

Schmid in 1983. Although significant advances have been made in the laboratory for total synthesis, the process is complicated, uneconomical, and the total yield is relatively low (Wang et al., 2014). Microbial genetic engineering from transgenic or genetically modified microbes is actively deployed as an alternative strategy to produce artemisinin to meet global needs. Cloning and expression of artemisinin biosynthetic genes from *Saccharomyces cerevisiae* and *Escherichia coli* have led to the production of artemisinin precursors such as amorpha-4,11-diene and artemisinic acid. However, the added costs for later chemical synthesis to convert the precursor chemically to artemisinin are a detracting factor (Zeng et al., 2008; Ikram & Simonsen, 2017).

The global supply of artemisinin is largely depending on the extraction derived from *A. annua* L. plant. Cultivation of *A. annua* L. takes about 14 months before the drugs can be produced (Kayani et al., 2018). Moreover, natural disasters such as floods could become limiting factors leading to a shortage of artemisinin supply and fluctuation in its price. The fluctuating and unpredictable demand leads to highly variable costs between USD 200 to 400 per kilogram from 2007 to 2011 (Jolliffe & Gerogiorgis, 2016). Since most developing countries are affected by malaria epidemics, an affordable and constant supply of artemisinin is deemed desirable. Although semi-synthetic production of artemisinin is in progress, field production of *A. annua* L. is still the main commercial source of the compound. *A. annua* L. plant remains the only helpful source for the large-scale production of artemisinin (Hommel, 2008; Kayani et al., 2018). Therefore, the optimised production of artemisinin tissue culture of *Artemisia* species is highly desirable (Grech-Baran & Pietrosiuk, 2012).

A. annua L. is quickly grown under a wide variety of conditions. Cultivation of this plant requires a minimum of 6 months, and extraction, processing, and manufacturing of the final product require at least 2 - 5 months, depending on the

product formulation. The plants are usually harvested at the early stage of flower budding as the content of artemisinin decreases during the flowering stage (WHO, 2006). In order to ensure that *A. annua* L can produce the optimum amount of artemisinin, harvesting at the right timing is important. Low artemisinin production from Artemisia plants of lower than one percent of the total dry weight of the plant leads to a shortage of global supply. The yield of dry leaf per hectare varies from 1.5 to 2 tons per hectare in field-grown plants, and 6 -14 kg of artemisinin per hectare can be expected from well-managed plantations (Wetzstein et al., 2018). The life of artemisinin and derivatives is short, not exceeding a few weeks, depending on the type of packaging and storing temperature (Houzé et al., 2007). Artemisinin yield depends upon many factors, including harvesting time, geographical conditions, temperature, plant population density, soil type, plant age, fertilizer application, and the duration of preserving the leaves as well as the variety of *A. annua* L. (Damtew et al., 2011; Thu et al., 2011; Omer et al., 2013; Jelodar et al., 2014; Das & Sharma, 2015).

A. annua L. is now naturalised in many countries far from its native land, China. Although few differences in morphology were shown in *A. annua* L. collected from different geographical in China, significant differences were reported in artemisinin content (Zhang et al., 2017). Previous research reported that germplasm from North America, Europe, East Africa and Australia has lesser artemisinin contents compared to that from China (Huang et al., 2010). Li et al. (2017a) reported that wild *A. annua* L. from the northern part of China contains lower artemisinin content than that from the southern region. Thus, the distribution of *A. annua* L. may play a vital role in selecting a high yielding superior genotype rich in artemisinin.

Currently, the most effective drug for treating malaria is artemisinin. This active compound was first discovered by Tu Youyou, a Chinese scientist in 1971 (Su & Miller,

2015). Malaria is a life-threatening disease, especially in warmer climate countries. It is a curable disease at the early stage of detection and timely treatment. Increasing severity and death rates resulting from malaria are compounded by continuous infestation and resistance to chloroquine, antifolates, and some other significant classes of antimalarials drugs (Hyde, 2007). To overcome this problem, the World Health Organisation (WHO) had introduced artemisinin, a potent antimalarial drug derived from *A. annua* L., to replace the conventional ineffective medicines. It is now the first-line antimalarial treatment regimens worldwide. Artemisinin, in combination with other antimalarials drugs is recommended in artemisinin combination therapies to treat mild attacks to delay and reduce the risk of resistance (Maude et al., 2010).

This study attempts to study *A. annua* L. samples, which was originated from Mongolia, systematically to establish a protocol for the production of *in vitro* plant. The establishment of cell suspension and callus culture could further enhance desirable secondary metabolites from optimising culture medium composition, the medium pH, the inoculum density, temperature, light, agitation, and many others. The wide variety in the geographical distribution of the samples is an unexplored treasure from which improved mode of artemisinin extraction can potentially be found. Furthermore, the study of the content of artemisinin in *A. annua* L. originated from Mongolia, as reported in the literature, is scarce. As such, the systematic experimental study was embarked by this study on *A. annua* L. from an exotic geographical region, Mongolia, to develop an efficient plant propagation protocol as well as to establish callus and cell suspension cultures.

1.1 Objectives

The objectives of the present study are:

I. To produce *Artemisia annua* L. Mongolian origin plantlets via *in vitro* culture technique,

II. To establish a callus culture of *A. annua* L. and optimize the growth for maximum cell biomass production,

III. To initiate and study the effect of physical and chemical factors on the growth of cells in the cell suspension culture of *A. annua* L.,

IV. To study the effect of different wavelengths of LED light and selected macronutrients on cell biomass and artemisinin production,

V. To study the effect of flask size on the production of *A. annua* L. cells.

CHAPTER 2

LITERATURE REVIEW

2.1 *Artemisia annua* L.

2.1.1 Biology of Artemisia annua L.

Artemisia annua L. Figure 2.1(A) also known as Chinese wormwood, annual wormwood, sweet wormwood, wormwood, annual sagewort, annual mugwort, Qing Hao 青蒿 (Chinese) or sweet sagewort (Bilia et al., 2014). It is an annual herbaceous plant with a sweet aromatic odour and bitter taste. This crop can grow to a height of 1-3 metre and 1 metre in width. It is a large shrub with a single main stem and smaller alternate branches that spirally arise from the side. The plant has bright green fernlike leaves, which range from 2.5 - 5.0 centimetre in length. It has a short taproot and aggressive fibrous root. Floral panicles are always found at the end of the upper stems. The loose panicles consist of a few branches of raceme with small drooping flower heads Figure 2.1(B). The flowers are yellowish and tiny, about 2-3 millimetres in diameter. Florets of *Artemisia* contain little nectar and compose of many tiny flowers called florets. There are two types of florets: marginal pistillate florets and central bisexual florets (Ferreira et al., 1997; WHO, 2006).

Upon maturity, each floret is replaced by a tiny seed that is small enough to be cross-pollinated by wind and insect. The seeds are about one millimetre long, yellow-brownish with a lustrous surface marked by vertical furrows, oblong, and a fatty creamy white endosperm (Smitha et al., 2014). Trichomes are appendages developed from epidermal cells of leaves, stems, as well as flowers and occur in a variety of shapes and sizes. Examples are unicellular or multicellular, branched or unbranched, and glandular or non-glandular. Trichomes are remarkable features of the plant, which allow it to

synthesize, store, and secrete many specialised metabolites (Schilmiller et al., 2008). Two types of trichomes, namely filamentous 5-celled non-glandular T-shaped trichomes and 10-celled biseriate glandular trichomes, are commonly found in *A. annua* L. (Xiao et al., 2016).

Figure 2.1: ***Artemisia annua*** **L. (A) plant, and (B) flowers.** (Scale bar = 1 cm)

2.1.2 Habitat and distribution

Being a native to China, *A. annua* L. is found commonly as a weed in the wild. *Artemisia* is generally found at altitudes of 1500 m throughout the country. However, it can be found below 1500 m in the eastern regions, 2000-3000 m above sea level in the north and southwestern regions, and at 3650 m above sea level in Tibet. It is growing by the roadside, grasslands, forest margins, hillsides, wastelands, dry river valleys, forest meadows, rocky slopes and semiarid climates. The plant has been collected wild in China since the late 1970s when artemisinin, the active ingredient of *A. annua* L., was first identified to be effective against malarial parasites (Faurant, 2011). The plant cultivated in China and also Vietnam produces about 80 % of the global supply; whereas the remaining 20 % is from east Africa (Shretta & Yadav, 2012).

The plant is now widely dispersed throughout the world, far from its native land, growing in a wide range of subtropical and temperate environments. During the Vietnam war, *Artemisia* plantations were established in North Vietnam by the Vietnamese Communists, Vietcong using seeds supplied by the Chinese Government to combat a rapid rise in malaria cases among army personnel (Ellman, 2010). In India, *A. annua* L. was introduced by the Central Institute of Medicinal and Aromatic Plants in 1986, from the Royal Botanical Gardens, Kew, England. It is now cultivated in different states like Uttar Pradesh, Bihar, Madhya Pradesh, Uttarakhand, and Gujarat. Cultivation by Indian farmers has led to enhanced incomes in the rural sector (Kumar et al., 2015). In the United States, the herb is cultivated on a small scale as a material source for aromatic wreaths and craft trade (Simon et al., 1990). In Eastern Europe, Romania and Bulgaria in particular, it was used by the perfume industry as a source of essential oils. This plant is increasingly grown for this purpose (Heemskerk et al., 2006).

In Australia, Turkey, Afghanistan, and Iran, *Artemisia* is also cultivated in a large scale (Bhakuni et al., 2001).

Geographical range with temperate latitude is the main criteria for determining the area to cultivate *A. annua* L. Some studies proposed the speculation that *A. annua* L., being a short-day plant, is not as easily cropped in tropical regions due to the possibility of premature flowering, which in turn leads to inadequate leaf biomass production (Ferreira et al., 1997, Ferreira et al., 2005). However, some varieties may have been adapted by breeding at a lower latitude. For example, a high artemisinin level of 0.86 % of dry weight was detected in the leaves of native grown genotypes *A. annua* L. originated from Langson, Vietnam. The short harvesting period of 5 months at the early development stage after sowing enables the plants to cultivate twice a year (Woerdenbag et al., 1994).

Successful trials were also performed to address the influence of climate conditions near the equator to increase biomass yield and artemisinin content. For the cultivation of *A. annua* L. in the humid tropics, soil moisture, planting season, and seed origin are the three important factors to consider (Brisibe et al., 2012). In humid tropics lowland, the successful cultivation of *A. annua* L. has been owed to the use of non-photoperiodic hybrid lines. They reported a high fresh leaf biomass yield of 30 tonnes/ha and artemisinin up to 1.0975 % of dry weight using a high yield hybrid line originated from Brazil. The hybrid plant was cultivated during the rainy season and irrigated intermittently to avoid drying the soil under relentless heat. In East Africa, irrigation on a large scale is applied to overcome the agricultural risks on seasonality to get a more constant *Artemisia* biomass yield (Thomas & Ying, 2007).

However, Thu et al. (2011) revealed that variations in artemisinin contents in *A. annua* L. plants were attributed more strongly to environmental factors than genetic factors. Seeds of two selected high-yielding clones of *A. annua* L. were planted in two different locations in the southern part of Vietnam. The high temperatures and low elevation in Thù Đúc Province led to slow vegetative growth and low artemisinin contents for all the elite plants from the two different clones.

2.2 *Artemisia*

Artemisia is vast; an assorted group of plants contains about 500 species worldwide (Bora & Sharma, 2011b). It belongs to the family Asteraceae or sometimes is recognised as a sunflower or daisy family. It is a big family that consists of about 1000 genera and over 20000 species (Nigam et al., 2019). This family is the most prominent family of flowering plants in terms of the number of species (Gao et al., 2010).

The *Artemisia* genus is distributed worldwide across the temperate zones of the Northern Hemisphere, mainly Asia, Europe, and North America. Some species reaching the Arctic, but a few species can also be found in the Southern Hemisphere. These species are mostly considered as small shrubs, or sometimes herbs. They are biennial, annual, and perennial (Septembre-Malaterre et al., 2020). Asia has the most significant *Artemisia* species population, with 30 species in Italy, 35 in Iran, 150 in China, about 50 in Japan, and 174 in the former Soviet Union (Nigam et al., 2019).

2.2.1 Uses of *Artemisia* species

Artemisia species has a long and varied history of diverse uses. In western countries, some species of *Artemisia* was collected or cultivated for culinary purposes. The distinctive bittersweet flavour of the dried plant of *A. dracunculus* L. (Tarragon) is particularly suited to enhance the flavour of poultry (chicken and pork). It is the main flavouring component of many sauces, cream soups, and some popular sweet non-alcoholic drink in countries likes Armenia, Azerbaijan, Georgia, and Russia. (Obolskiy et al., 2011). *A. ludoviciana* commonly known as silver wormwood or grey sagewort, was grown for its attractive silvery-white foliage. It makes a beautiful foliage for more colourful flowering plants in flower arrangement and ornamental crops (Dana & Lerner, 2003). The bitter and aromatic properties of *A. absinthium* L. make it a vital flavouring agent in the food industry to prepare alcoholic beverages such as wine, vermouth, bitters, and other spirits (Altunkaya et al., 2014). It is the main component of absinthe, the most popular high alcohol spirit drink in Europe during the nineteenth century (Lachenmeier et al., 2006).

In Iran, *A. absinthium* is traditionally known as an orexigenic herb. It is used to stimulate appetite for the patient with anorexia, an eating disorder characterised by malnutrition and weight loss (Taraghdari et al., 2015). Incense is one of the items of Puja, Buddhism worship in Nepal. It is comprised of various parts of plants from 31 species. The dried leaves and twigs of *A. dubia*, *A. indica*, and *A. japonica* are used as the ingredients for making the incense (Manandhar, 2004). A yellow dye extracted from the branches of *A. abrotanum* L. is used for colouring wool in the olden day of India. Its strong, sharp, scent dried leaves are hung in the closet to keep moths and other insects away from wardrobes (Shah, 2014). *A. herba-alba*, commonly known as desert wormwood or wormwood, greyish in colour, is an aromatic perennial dwarf shrub found

in Western Asia, Arabian Peninsula, and Northern Africa. It is essential as a fodder for sheep and livestock for it grows abundantly in the plateau regions of Algeria (Mahomoodally, 2013).

Many *Artemisia* species are used as a traditional medicine in many parts of the world because it possesses a wide range of health benefits. In China, Ge Hong (284-363 AD), in his text titled “Emergency Prescriptions Kept Up One’s Sleeve”, recommended soaking fresh *A. annua* L. plant in water and take the juice to treat fever and chills (Hsu, 2006). Whereas in Pakistan, the leaves and inflorescence of *A. brevifolia* Wall. ex DC. is grounded to form a powder (phaki) that used to treat gastric problems (Hayat et al., 2009). In Turkey, *A. vulgaris* is commonly used to relieve cough and remove pain in the stomach (Dulger & Gonuz, 2004). *A. pallens*, also known as "davanam" in Tamil, is native to the southern part of India (Ruikar et al., 2009). It is widely used in Indian folk medicine to treat diabetes mellitus (Pavithra et al., 2020; Ruikar et al., 2009). *A. campestris,* or field sagewort is growing wild in the south of Tunisia and used for several purposes (Akrout et al., 2010). Some native North American Indian tribes used it as an abortifacient to terminate complicated pregnancies (Akrout et al., 2010). A poultice of the crushed leaves can treat sore eyes (Dib et al., 2017). An infusion of the roots has been used as a hair tonic to treat scalp infection, especially in children (Akrout et al., 2010). It was also taken internally to promote urination and bowel movements (Akrout et al., 2010).

2.2.2 Medicinal properties of *Artemisia* species

Recently, many attempts have been made to characterise the valid therapeutic properties of *Artemisia* species and to enhance the production of the valuable

compounds found in the selected chemotypes. Many useful chemical compounds can be derived from *Artemisia*. Over 800 non-volatile and volatile chemical compounds were identified in different genus species. The major categories of these compounds include phenolics, terpenes, flavonoids, fatty acids, phenylpropanoids, hydrocarbons, fatty esters, lignans, and, sterols (Koul & Khatr, 2020).

At present, consensus holds that artemisinin is the most effective drug against drug-resistant malaria (WHO, 2018). The content of artemisinin in *A. annua* L. usually ranges from 0.01 to 2% dry weight (Namuli et al., 2018). Although artemisinin is also reported in many *Artemisia* species, *A. annua* L. remains the natural and feasible artemisinin source (Czechowski et al., 2019). Numonov et al. (2019) reported the presence of artemisinin in 40 *Artemisia* species, and the content of artemisinin of seven *Artemisia* species found in Tajikistan ranged between 0.07% and 0.45% based on the dry weight. Singh and Sarin (2010) found that the content of artemisinin was higher in the aerial parts of the *A. scoparia* (0.015%) plant compared to callus culture (0.001%). According to Mannan et al. (2008), the artemisinin level was higher in the hairy roots culture of *A. dubia*, and *A. indica* transformed by two *Agrobacterium rhizogenes* strains 9402 and 8196 than those cultured in the liquid medium.

Besides, Nahrevanian et al. (2010) reported that the herbal extract of Iranian *A. khorassanica* has antimalarial effects on the early decline of *Plasmodium berghei* parasitaemia infected mice. The significant components detected, including chrysanthenone (7.8%), palmitic acid (7.4%) and, cis-thujone (5.8%). Some researchers suggested *A. vulgaris* is a potential cheap source of plant-based antimalarial in the future because it is an invasive weed abundantly available in Sri Lanka and it is not toxic (Kodippili et al., 2011; Bamunuarachchi et al., 2013).

A total of 20 compounds were identified in the essential oil of *A. annua* L.. Three major components include camphor (18.00 %-23.30 %), 1,8-cineole (9.00 %-10.39 %), and artemisia ketone (28.30 %-37.15 %) (Şenkal et al., 2015). The variation in the percentage of components in the essential oils depended very much upon the harvest stage. The amounts of the three most significant components were recorded when the plants were harvested throughout the entire flowering stage. The essential oil derived from *A. annua* L. can remarkably inhibit the growth of a diverse range of fungal and bacterial strains (both gram-positive and gram-negative) (Juteau et al., 2002, Bilia et al. 2014). Hence, these findings explore the possible use of oil in the food, medical, cosmetic, and aromatherapy industries. Kordali et al. (2005) also reported that the essential oils of *A. dracunculus*, *A. absinthium*, *A. santonicum*, and *A. spicigera* exhibited a potent antifungal activity on the growth of agricultural pathogenic fungi at a broad spectrum. It also exhibited antibacterial activity against 64 bacterial strains of the plant, food, and clinic origins.

The possible uses of *A. annua* L. extracts for the treatment of dental diseases were studied by Kim et al. (2015). It was found that acetone, ethanol, water or methanol extracts of *A. annua* L. had antimicrobial effects against the periodontopathic bacteria, such as *Fusobacterium nucleatum* subsp. *polymorphum*, *Prevotella intermedia*, *Fusobacterium nucleatum* subsp. *animalis*, *Aggregatibacter actinomycetemcomitans*, that caused oral disease.

Artemisia species are considered as a safer alternative to some commercially available toxic fumigants available in the market. The essential oils derived from the aerial parts of *A. mongolica* and *A. capillaris* are found to produce intense insecticidal activity in maize weevil, *Sitophilus zeamais* (Motsch). Hence, it is a possible natural, nontoxic fumigant for the control of stored product insects (Liu et al., 2010). Tripathi

et al. (2000) discovered that the essential oil of *A. annua* L. has repellent action and inhibitory activities against the development of two economically crucial stored grain beetles *Tribolium castaneum* and *Callosobruchus maculatus*. Besides, Mojarab-Mahboubkar et al. (2015) found that the compound in the essential oil of *A. annua* L. has a toxic effect on the activity of macromolecules, digestive enzymes, and detoxifying enzymes on 4th instar larva of cotton bollworm *Helicoverpa armigera* (Hübner), a harmful pest against field crops. Moreover, as discovered by Cheah et al. (2013), the crude extract of *A. annua* L. could be used as vector control for it increases the mortality of the larvae, reduce oviposition and the number of eggs hatched from mosquitoes (genera *Aedes*, *Anopheles*, and *Culex*) that transmit various fatal diseases.

Due to the rich in phenolic compounds, *Artemisia* species are also a potent antioxidant remedy to prevent the generation of free radicals that may lead to damage and death of cells (Nimse & Pal, 2015). According to Lobo et al. (2010), the scavenging activities of antioxidants promote the decomposition of free radicals as a preventive measure against atherosclerosis, inflammatory disease, certain cancers, and the process of ageing. Besides, synthetic antioxidants such as butylated hydroxytoluene and butylated hydroxyanisole are known to present a dangerous threat to human health. Hence, sourcing for a nontoxic natural antioxidant has been intensified in recent years. The radical scavenging activity was reported in many *Artemisia* species that is *A. vulgaris* (Temraz & El-Tantawy, 2008), *A. annua* L. (Chukwurah et al., 2014), *A. absinthium* (Bora & Sharma, 2011a), *A. rutifolia* (Ashraf et al., 2017) and *A. nilagirica* (Gul et al., 2017).

In recent years, *Artemisia* species have gained increasing attention for their anticancer properties. Lang et al. (2019) reported that the momundo extract of an artemisinin-deficient *A. annua* L. herbal preparation exhibits potent anticancer activity

against MDA-MB-231 triple-negative breast cancer, a highly aggressive form of breast cancer. The active ingredients with potential anticancer activity identified were arteannuin B, casticin, chrysosplenoal D, arteannuic acid 6,7-dimethoxycoumarin. It was also reported that iron plays a crucial role in the cytotoxicity of artemisinin against cancer cells by forming reactive oxygen species, a cancer-killing free radical. However, the addition of iron as supplementation is only necessary for patients with iron-deficient conditions (Efferth, 2015; Saeed et al., 2020).

The antiproliferative effect of dichloromethane, n-hexane, methanol, and ethyl acetate extracts of some *Artemisia* species from Iran was reported by Taghizadeh Rabe et al. (2011). All the seven tested *Artemisia* extracts displayed inhibitory effects in cultured cancer cell lines. Dichloromethane extract from *A. ciniformis* showed the highest overall inhibitory effect on the human gastric and colon adenocarcinoma cancer cell lines, human breast carcinoma, and human cervix carcinoma cell line. In more recent evidence, Efferth (2017) also highlighted the synergizing effect of combination therapy regimens rather than monotherapy in combining cancer. *Artemisia* seems to work quite well when combined with other promising cancer drugs that have already shown success in inhibiting cancer and associated symptoms. A clinical trial of using artesunate form *A. annua* L. to treat advanced non-small-cell lung cancer patients was reported by Zhang et al. (2008). Artesunate, in combination with chemotherapy regimens such as vinorelbine and cisplatin, was shown to lengthen the progression time and improve the short-term survival rate of cancer patients without extra side effects.

Several studies in the literature reported that extracts of *Artemisia* species produced significant hypoglycemic effects in humans and diabetic animals' model. Water extraction of *A. annua* L. can greatly reduce glucose level and lessen the metabolic abnormalities in alloxan-induced diabetes in male albino rats (Helal et al.,

2014). Another study by Li et al. (2015) showed that the use of *A. absinthium* significantly reduced the serum glucose level in type II diabetes patients age between 30-60 of both sexes. The patients were given capsules, which contained finely grounded dried leaves of *A. absinhium* of 1 g/day dosage twice a day, and it was found that the level of high-density lipoproteins (HDL) was reduced by 3 % and low-density lipoproteins by 6 % in 30 days.

The extract of *Artemisia* plants is also effective and safe as a potential treatment for hypertension. Conventional Western medicine for treating hypertension could pose a high risk of cardiovascular complications and kidney diseases. Using an animal model, Esmaeili et al. (2009) proved that oral consumption of *A. Persia* aqueous extract grown in the Kerman province of Iran reduced the blood pressure in hypertensive male rats much more than enalapril, a conventional anti-hypertensive drug. Besides, moxibustion could be an alternative way to treat hypertension in combination with antihypertensive drugs. It involves igniting moxa containing dried *A. vulgaris* that formed a small cone or cigar-like shape and then applying it to a specific point on the body. Encouraging results have been reported on randomised trials indicating the efficacy of moxibustion (Yang et al., 2014; Siddiqui et al., 2017).

Many researchers also report other health benefits and ethnomedical uses of *Artemisia* species. Remberg et al. (2004) reported that the nasal spray preparation *A. abrotanum* L. ("southernwood") could help patients with allergic rhinitis and conjunctivitis as well as other upper airway allergies to reduce ongoing symptoms as well as to prevent the development of nasal congestion, sneezing, and rhinorrhea. The nasal spray formulation contains essential oils (4 mg/mL) and flavonols (2.5 μg/mL) of *A. abrotanum* L. Besides, a study on behavioural assays in rodents suggested using the essential oil and crude ethanolic extract of *A. annua* L. as a depressor on the central

nervous system. The sedative effects of *A. annua* L. with intense depressant activity on the central nervous system could be used as a herbal medicine for sleep management and related problems (Perazzo et al., 2008; Emadi et al., 2011).

Meanwhile, Zhao et al. (2017) reported that artemisinin from *A. annua* L. has excellent clinical application potential to treat liver injury induced by alcohol. They demonstrated that treatment with a high dose of artemisinin could prevent liver damage in mice caused by chronic alcohol by suppressing the release of pro-inflammatory cytokines and activation of NF-κB. However, further studies are still needed to explore the possible clinical application to treat liver injury by alcohol in human patients.

Drăgan et al. (2014) suggested the potential use of *A. annua* L. leaf powder to treat *Eimeria ten Ella* infection in chickens. It was reported that chickens infected with a high dose of *E. tenella* (10,000 oocysts) had a 100 % survival rate after feeding them with 1.5 % *A. annua* L. powdered leaf in their daily feed. Besides, those treated chickens were found to gain much larger body weight than those infected/untreated chickens.

2.2.3 Artemisinin

Artemisinin, a sesquiterpene lactone containing an endoperoxide bridge, can be isolated from the aerial part of the plant. It is a compound of colourless and odourless, forming crystals that melt around 156 - 157 °C. Clinical use of artemisinin is safe with no evidence for serious adverse effects (Meshnick, 2002; Efferth & Kaina, 2010). It was reported that artemisinin derivatives did not present apparent safely treat to pregnant women (Dellicour et al., 2007).

It was initially postulated that artemisinin was produced and sequestered in glandular trichomes on the adaxial and abaxial sides of the leaves, flowers, and stem (Duke & Paul, 1993; Duke et al., 1994). According to Ferreira and Janick (1995),

artemisinin was secreted from a saclike structure that developed at the apex of the glandular trichomes that raptured during anthesis and flew into the subcuticular space around apical cells. Olsson et al. (2009) reported that artemisinin was biosynthesised in the outer pair of apical cells from the glandular trichomes. Recent evidence suggested that glandular secretory trichomes of *A. annua* L. are the 10-celled biseriate translucent structure comprised of two stalk cells, two basal cells, four subapical cells, and two apical cells (Xiao et al., 2016). During the vegetative growth of *A. annua* L., the trichome number increased per unit area on the adaxial leaf surface until the leaf expansion stopped, then the trichome began to collapse and dropped in number (Lommen et al., 2006). Although trichomes are the sites of synthesis of artemisinin, Salehi et al. (2018a) reported no significant correlation between artemisinin content with the glandular trichome density based on the five *Artemisia* species collected from different parts of Iran. A recent finding showed that the calli and glandless leaves of inbred *A. annua* L. plants mutant L. produce arteannuin B, artemisinic acid, and artemisinin. This might be an alternative way to produce artemisinin in the future (Judd et al., 2019).

The production yield of artemisinin from *A. annua* L. is meager and varies widely depending on the plant variety from different geographical locations. Typical seed-propagated *Artemisia* plants produce less than 1 % of dry weight artemisinin (Wetzstein et al., 2018). Artemisinin production and crop yield can be enhanced via clonal propagation of superior genotypes. Bayarmaa and de Zorzi (2011) reported that the artemisinin content of a sample of *A. annua* L. collected in the vicinity of Ulaanbaatar city, Mongolia, in August 2006 was 0.29-0.85 % of its dry weight. Huang et al. (2010) reported that germplasm from Australia, North America, East Africa and Europe has a lower content of artemisinin than those from China. Generally, wild *A.*

annua L. in the southern part of China has a higher artemisinin content than that from the northern part. The highest amount of artemisinin content was identified in those found in the north of Hainan, Guangdong, and Guangxi (Li et al., 2017a). Li et al. (2017b) had collected 102 *A. annua* L. plant samples from 12 areas in China. They found that *A. annua* L. plant from Qinling Mountains-Huaihe River Line southern part had a higher artemisinin content (1.52 %) than those plant from the northern part.

Outside of China, high-yielding clones of *A. annua* L. reaching 1.47 % of dry weight were reported in Hanoi, Vietnam (Thu et al., 2011). Misra et al. (2014) reported 0.776 % artemisinin concentration of a high-yielding clone "Jeevanraksha" from the Central Institute of Medicinal and Aromatic Plants, Lucknow, India. *A. annua* L. from Italy was reported to contain only about 0.04 % to 0.05 % artemisinin dry weight. Other European origins were reported to range from 0.03 % to 0.22 % (Thu et al., 2011), while those found in Iran were low in artemisinin content (Salehi et al., 2018b). The extremely high-yielding variety might be a new promising germplasm resource for future *A. annua* L. breeding. Cockram et al. (2012) identified a strong growth line with the competence to flower under long-day photoperiods and contained more than 2 % artemisinin after screening 70 *A. annua* L. plants representing 14 diverse germplasm accessions around the world. A high artemisinin content of 4.85-4.90 % was also reported for the first time by El-Naggar et al. in 2013 for *A. annua* L. planted in the Egyptian desert.

Artemisinin content also exhibits significant variations depending on plant parts and different stages of vegetative growth. By comparing the *Artemisinin* content in the other organs (leaves, branches, main stem, and roots), Misra et al. (2014) found that the leaves have the most artemisinin by side branches and main stem, but none was detected in the roots. The content of artemisinin in the leaves of the same plant varies along with the location on the stem. Yadav et al. (2015) reported the artemisinin level was

significantly greater by about 10-20 % in the leaves of the upper third of the plant than those in the lower two-thirds for two cultivars, Jeevanraksha and CIM-Arogya.

Ferreira et al. (1995) reported that artemisinin levels were 4 to 11-fold higher in inflorescences than in leaves during the flowering stage. However, Mannan et al. (2010) reported that artemisinin concentration in leaves (0.44 ± 0.03 %) and flowers (0.42 ± 0.03 %) of *A. annua* L. were about the same during flowering. According to Towler and Weathers (2015), artemisinin content is the highest in mature leaves (3031.2 μg g^{-1} of fresh weight) during floral budding. A study by Woerdenbag et al. (1994) reported that the highest artemisinin level was in the leaves (0.86 % of dry weight) of an early development stage (5-month-old) *Artemisia* plant. It seems that the contrasting outcomes reported on the inconsistencies in artemisinin content may be cultivar specific. Nevertheless, leaves remain the primary commercial source of artemisinin, and 89 % of total plant artemisinin comes from the leaves (Charles et al., 1990).

It was suggested that future selection and plant breeding should focus on chemotypes low in artemisinic acid but high in dihydroartemisinic acid and artemisinin. It was found that artemisinic acid is competing with dihydroartemisinic acid, the primary precursor of artemisinin in a divert biosynthetic pathway during artemisinin production (Ferreira et al., 2018). In another study, Ferreira and Luthria (2010) added that the final conversion of dihydroartemisinic acid into artemisinin is non-enzymatic and only requires photooxidation. This is why sun-dried shoots had significantly higher concentrations of artemisinin than shade, freeze, and oven-dried shoots.

2.3 *In vitro* culture technology

2.3.1 Plant tissue culture

Plant tissue culture broadly refers to growing plants or any part of a plant like organs, cells, tissues or other components on artificial media free from bacterial and fungal diseases. The aseptic condition is performed under filtered air. The system of tissue culture provides water, energy, and nutrients required for the growth of the plant via the media (Mukta et al., 2017). The person who first comes up with the idea of tissue culture was Haberlandt, a German scientist around 1902 (Thorpe, 2007). Compared to conventional methods, the *in vitro* culture system has an obvious advantage because it is not subjected to the constraints imposed by environmental, geographical, or seasonal variations. Despite the possibility of a large variety of media formulations, two fundamental types of plant cultures can be distinguished, namely liquid and solid culture systems. To ensure that the cultures are constantly kept in a state that is actively growing, they are subcultured to a new media from time to time. To promote growth, optimised temperature settings, humidity, lighting, and air circulation are provided by controlled incubation conditions (Wang et al., 2017).

Plant and tissue culture are a profound biotechnological tool for producing valuable plant-derived secondary metabolites for pharmaceutical, food additives, flavours, fragrances, and biopesticides. However, separation and purification of such metabolites plant-derived natural products are sometimes quite challenging due to their low yield (Atanasov et al., 2015). Recently, this technique is increasingly being employed to produce high-quality and rare bioactive compounds from medicinal plants following the rediscovery of the efficacy and the safety of medicinal plants for modern medication (Cardoso et al., 2019).

Apart from their use for the production of secondary metabolites, plant tissue culture techniques have in recent years become very important for the production of more predictable, consistent, and healthier crops free from diseases. The techniques have been used increasingly as an adjunct to traditional methods to create genetic improvement of various crop plants with a higher yield, better quality, and enhanced resistance to pests and diseases (Espinosa-Leal et al., 2018).

Besides, plant tissue culture has been used for the conservation of endangered, threatened, and rare plant species that comes to extinction due to human activities or changes in habitat. It is a preservation method that enables plant genetic resources to be conserved safely and cost-effectively. Affected species in such instances include numerous cacti (Pérez-Molphe-Balch et al., 2015), carnivorous plants, orchids, and others (Sarasan et al., 2006).

These plant species have successfully grown and conserved by short and medium-term conservation, known as slow growth and long-term conservation, also known as cryopreservation. Short and medium-term conservation is usually achieved by reducing the growth of *in vitro* cultures by applying minimal media and growth retardant or storage at low temperatures resulting in prolonged intervals between subcultures. In contrast, cryopreservation is the preservation of cells and tissue by freezing the plant genetic resources at an ultra-low temperature in liquid nitrogen (–196ºC) or in the vapour phase (Oseni et al., 2018; Chauhan et al., 2019).

2.3.2 Callus and cell suspension cultures

The callus is an unspecialised, unorganised growing, and dividing the mass of cells produced from an explant because of wounding. An explant can be a piece or