ESTABLISHMENT OF *in vitro* PLANTLETS OF *Artemisia annua* L. FOR THE ANALYSIS OF ARTEMISININ BIOSYNTHETIC GENE (*CYP71AV1*) AND TRICHOME INITIATION GENE (*GL3*)

by

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TABLE OF CONTENTS

Acknowledgments	ii
Table of Contents	iii
List of Tables	xiii
List of Figures	xv
List of Abbreviations	xx
Abstrak	xxii
Abstract	xxiv
CHAPTER ONE - INTRODUCTION	1
1.1 Objectives of study	8
CHAPTER TWO LITERATURE REVIEW	9
2.1 Malaria	9
2.1.1 Types of malaria	9
2.1.2 Antimalarial drug resistance	11
2.1.3 Malaria in Malaysia	16
2.2 Asteraceae	17
2.2.1 Characteristics of Asteraceae family	17
2.2.2 Genus Artemisia	19
2.2.3 *Artemisia annua* L.	21
2.2.3.1 Botany of *Artemisia annua* L.	21
2.2.3.2 Trichomes of *Artemisia annua* L.	22
2.2.3.3 Chemical constituents of *Artemisia annua* L.	24
2.2.4 Artemisinin	25
2.2.4.1Artemisinin biosynthesis	25
2.2.4.2 Current issues of artemisinin	28
2.3 *In vitro* culture technology	33
2.4 Mutagenesis	39
2.4.1 Types of mutations	39
2.4.2 Mutagenesis in plant breeding	40
2.4.3 Chemical mutagenesis	43

2.4.3.1 Ethyl methanesulfonate (EMS) as mutagen 43
2.4.3.2 Sodium azide (NaN_3) 44

CHAPTER THREE MATERIALS AND METHODS 46
3.1 Establishment of *in vitro* and greenhouse plants of *Artemisia annua* L. from seeds 46
3.1.1 Plant material 46
3.1.2 Establishment of seed derived *in vitro* plantlets 46
3.1.3 Establishment of greenhouse *A. annua* L. seedlings 48
3.1.3.1 Effect of water imbibitions duration on seed germination 48
3.1.3.2 Effect of substrate on *A. annua* L. seed germination 48
3.2 Effect of *in vitro* and greenhouse conditions on growth, leaf trichomes density and artemisinin detection of *Artemisia annua* L. plants 49
3.2.1 Effect of *in vitro* and greenhouse conditions on growth of *A. annua* plants 49
3.2.2 Effect of *in vitro* and greenhouse conditions on leaf trichome density of *A. annua* plants 51
3.2.3 Effect of *in vitro* and greenhouse conditions on artemisinin production 51
3.3 Construction of cDNA library for *in vitro Artemisia annua* L. plant 53
3.3.1 Total RNA extraction and measurement of RNA quality 53
3.3.1.1 Standard CTAB plus glacial acid method 53
3.3.1.2 Modified CTAB method 55
3.3.2 Isolation of poly (A) mRNA from RNA and measurement of RNA quality 56
3.3.3 Synthesis of first strand cDNA 57
3.3.4 Synthesis of second strand to form duplex cDNA and attachment of linker-adaptor to cDNA termini 57
3.3.5 Screening of cDNA library 59
3.3.5.1 Preparation of calcium chloride ($CaCl_2$) bacterial competent cells 59

3.3.5.2 Ligation of cDNA into a plasmid DNA vector, pUC18 plasmid 61
3.3.5.3Transformation of plasmid DNA into host cell and blue/white colony screening 64
3.3.5.4 Isolation of pUC18 plasmid from JMJ 109*E. coli* 64
3.3.5.5. Endonuclease digestion of the pUC18 plasmid with restriction enzyme, *Eco*R1 65
3.3.5.6 DNA sequencing analysis 66
3.4 Deep sequencing of *in vitro Artemisia annua* L. plant cDNA and *de novo* assembly of 454 transcriptomic data 66
3.4.1 Total RNA extraction and measurement of total RNA quality 66
3.4.2 cDNA library preparation and sequencing on 454 GS FLX Roche sequencer 68
3.4.3 Sequencing raw data pre-processing and transcriptome assembly 68
3.4.4 Functional annotation 69
3.4.5 Primer designing 71
3.5 Determination of mutation density and mutation rate of sodium azide and ethyl methanesulfonate at different concentrations on in vitro *Artemisia annua* L. clones 71
3.5.1 Plant material 71
3.5.2 Treatment of *A. annua* clones with different concentration of sodium azide and ethyl methanesulfonate 71
3.5.3 Total RNA extraction and cDNA synthesis 72
3.5.4 PCR amplification of artemisinin biosynthetic gene (*CYP71AV1*) using *CYP71AV1*USM primers and determination of mutation density and mutation detection rate 74
3.6 Analysis of artemisinin biosynthetic gene (*CYP71AV1*) and trichome initiation gene (*GL3*) expression levels for mutant and normal clones of *Artemisia annua* L. 75
3.6.1 Analysis of effect of different concentrations of EMS on *Artemisia annua* L. clones plantlets initiated from seeds, 75

shoot tips, nodal segments and callus on expression of artemisinin biosynthetic gene (*CYP71AV1*) and trichome initiation gene (*GL3*)

3.6.1.1 Analysis of effect of different concentrations of EMS on *Artemisia annua* L. clones plantlets initiated from seeds on expression of trichome initiation gene (*GL3*) 75

3.6.1.2 Analysis of effect of different concentrations of EMS on *Artemisia annua* L. clones plantlets initiated from nodal segments on expression of trichome initiation gene (*GL3*) 76

3.6.1.3 Analysis of effect of different concentrations of EMS on *Artemisia annua* L. clones plantlets initiated from shoot tips on expression of trichome initiation gene (*GL3*) 76

3.6.1.4 Analysis of effect of different concentrations of EMS on *Artemisia annua* L. clones plantlets initiated from seeds on expression of artemisinin biosynthetic gene (*CYP71AV1*) 77

3.6.1.5 Analysis of effect of different concentrations of EMS on *Artemisia annua* L. clones plantlets initiated from nodal segments on expression of artemisinin biosynthetic gene (*CYP71AV1*) 77

3.6.1.6 Analysis of effect of different concentrations of EMS on *Artemisia annua* L. clones plantlets initiated from shoot tips on expression of artemisinin biosynthetic gene (*CYP71AV1*) 78

3.6.1.7 Analysis of effect of different concentrations of EMS on *Artemisia annua* L. clones plantlets initiated from callus on expression of artemisinin biosynthetic gene (*CYP71AV1*) 78

3.6.2 Analysis of effect of different concentrations of sodium azide (NaN_3) on *Artemisia annua* L. clones initiated from seeds, shoot tips, nodal segments and callus on expression of 79

trichome initiation gene (*GL3*) and artemisinin biosynthetic gene (*CYP71AV1*)

3.6.2.1 Analysis of effect of different concentrations of NaN_3 on *Artemisia annua* L. clones plantlets initiated from seeds on expression of trichome initiation gene (*GL3*) 79

3.6.2.2 Analysis of effect of different concentrations of NaN_3 on *Artemisia annua* L. clones plantlets initiated from nodal segments on expression of trichome initiation gene (*GL3*) 79

3.6.2.3 Analysis of effect of different concentrations of NaN_3 on *Artemisia annua* L. clones plantlets initiated from shoot tips on expression of trichome initiation gene (*GL3*) 80

3.6.2.4 Analysis of effect of different concentrations of NaN_3 on *Artemisia annua* L. clones plantlets initiated from seeds on expression of artemisinin biosynthetic gene (*CYP71AV1*) 80

3.6.2.5 Analysis of effect of different concentrations of NaN_3 on *Artemisia annua* L. clones plantlets initiated from nodal segments on expression of artemisinin biosynthetic gene (*CYP71AV1*) 80

3.6.2.6 Analysis of effect of different concentrations of NaN_3 on *Artemisia annua* L. clones plantlets initiated from shoot tips on expression of artemisinin biosynthetic gene (*CYP71AV1*) 81

3.6.2.7 Analysis of effect of different concentrations of NaN_3 on *Artemisia annua* L. clones plantlets initiated from callus on expression of artemisinin biosynthetic gene (*CYP71AV1*) 81

3.6.3 Analysis of expression level of trichome initiation gene (*GL3*) on *Artemisia annua* L. plants initiated from seeds, nodal segments and shoot tips treated with 1% EMS mutagen. 82

CHAPTER FOUR RESULTS 83
4.1 Establishment of *in vitro* and greenhouse plants of *Artemisia annua* L. from seeds 83
4.1.1 Establishment of seed derived *in vitro* plantlets 83
4.1.2 Establishment of greenhouse *A. annua* L. seedlings 85
4.1.2.1 Effect of water imbibitions duration on seed germination 85
4.1.2.2 Effect of substrate on *A. annua* L. seed germination 85
4.2 Effect of *in vitro* and greenhouse conditions on growth, leaf trichome density and artemisinin detection of *Artemisia annua* L. plants 87
4.2.1 Effect of *in vitro* and greenhouse conditions on growth of *A. annua* plants 87
4.2.2 Effect of greenhouse and *in vitro* conditions on leaf trichome density of *A. annua* plants 87
4.2.3 Effect of greenhouse and *in vitro* conditions on artemisinin production 91
4.3 Construction of cDNA library for *in vitro Artemisia annua* L. plant 95
4.3.1 Total RNA extraction and measurement of total RNA quality 95
4.3.1.1 Standard CTAB plus glacial acid method 95
4.3.1.2 Modified CTAB method 95
4.3.2 Isolation of poly (A) mRNA from total RNA and measurement of RNA quality 97
4.3.3 Synthesis of first strand cDNA 99
4.3.4 Synthesis of second strand to form duplex cDNA and attachment of linker-adaptor to cDNA termini 99
4.3.5 Screening of cDNA library 102
4. 4 Deep sequencing of *in vitro Artemisia annua* L. plant cDNA and *de novo* assembly of 454 transcriptomic data 102
4.4.1 Total RNA extraction and measurement of total RNA quality 102

4.4.2 cDNA library preparation and sequencing on 454 GS FLX Roche sequencer 104
4.4.3 Sequencing raw data pre-processing and transcriptome assembly 106
4.4.4 Functional annotation 109
4.4.5 Identification of genes related to trichome morphology and artemisinin biosynthesis and primer designing 114
4.5 Determination of mutation density and mutation rate of sodium azide and ethyl methanesulfonate at different concentrations on *in vitro Artemisia annua* L. clones 114
4.6. Analysis of artemisinin biosynthetic gene (*CYP71AV1*) and trichome initiation gene (*GL3*) expression of mutant and normal clones of *Artemisia annua* L. 117
4.6.1 Analysis of effect of different concentrations of EMS on *Artemisia annua* L. clones plantlets initiated from seeds, shoot tips, nodal segments and callus on expression of artemisinin biosynthetic gene (*CYP71AV1*) and trichome initiation gene (*GL3*) 123
4.6.1.1 Analysis of effect of different concentrations of EMS on *Artemisia annua* L. clones plantlets initiated from seeds on expression of trichome initiation gene (*GL3*) 123
4.6.1.2 Analysis of effect of different concentrations of EMS on *Artemisia annua* L. clones plantlets initiated from nodal segments on expression of trichome initiation gene (*GL3*) 126
4.6.1.3 Analysis of effect of different concentrations of EMS on *Artemisia annua* L. clones plantlets initiated from shoot tips on expression of trichome initiation gene (*GL3*) 127
4.6.1.4 Analysis of effect of different concentrations of EMS on *Artemisia annua* L. clones plantlets initiated from seeds on expression of artemisinin biosynthetic gene 131

(*CYP71AV1*)

4.6.1.5 Analysis of effect of different concentrations of EMS on *Artemisia annua* L. clones plantlets initiated from nodal segments on expression of artemisinin biosynthetic gene (*CYP71AV1*) 132

4.6.1.6 Analysis of effect of different concentrations of EMS on *Artemisia annua* L. clones plantlets initiated from shoot tips on expression of artemisinin biosynthetic gene (*CYP71AV1*) 136

4.6.1.7 Analysis of effect of different concentrations of EMS on *Artemisia annua* L. clones plantlets initiated from callus on expression of artemisinin biosynthetic gene (*CYP71AV1*) 137

4.6.2 Analysis of effect of different concentrations of sodium azide (NaN_3) on *Artemisia annua* L. clones initiated from seeds, shoot tips, nodal segments and callus on expression of trichome initiation gene (*GL3*) and artemisinin biosynthetic gene (*CYP71AV1*) 141

4.6.2.1 Analysis of effect of different concentrations of NaN_3 on *Artemisia annua* L. clones plantlets initiated from seeds on expression of trichome initiation gene (*GL3*) 141

4.6.2.2 Analysis of effect of different concentrations of NaN_3 on *Artemisia annua* L. clones plantlets initiated from nodal segments on expression of trichome initiation gene (*GL3*) 142

4.6.2.3 Analysis of effect of different concentrations of NaN_3 on *Artemisia annua* L. clones plantlets initiated from shoot tips on expression of trichome initiation gene (*GL3*) 146

4.6.2.4 Analysis of effect of different concentrations of NaN_3 on *Artemisia annua* L. clones plantlets initiated from seeds on expression of artemisinin 147

biosynthetic gene (*CYP71AV1*)
4.6.2.5 Analysis of effect of different concentrations of NaN_3 on *Artemisia annua* L. clones plantlets initiated from nodal segments on expression of artemisinin biosynthetic gene (*CYP71AV1*) 149
4.6.2.6 Analysis of effect of different concentrations of NaN_3 on *Artemisia annua* L. clones plantlets initiated from shoot tips on expression of artemisinin biosynthetic gene (*CYP71AV1*) 152
4.6.2.7 Analysis of effect of different concentrations of NaN_3 on *Artemisia annua* L. clones plantlets initiated from callus on expression of artemisinin biosynthetic gene (*CYP71AV1*) 154
4.6.3 Analysis of expression of trichome initiation gene (*GL3*) on *Artemisia annua* L. plants initiated from seeds, nodal segments and shoot tips treated with 1% of EMS mutagen 158

CHAPTER 5 DISCUSSION 160
5.1 Establishment of *in vitro* and greenhouse plants of *Artemisia annua* L. from seeds 160
5.2 Effect of *in vitro* and greenhouse conditions on growth, leaf trichome density and artemisinin detection of *Artemisia annua* L. plants 164
5.3 Construction of cDNA library for *in vitro Artemisia annua* L. plant 166
5.4 Deep sequencing of *in vitro Artemisia annua* L. plant cDNA and *de novo* assembly of 454 transcriptomic data 174
5.5 Determination of mutation density and mutation rate of sodium azide and ethyl methanesulfonate at different concentrations on *in vitro Artemisia annua* L. clones 176
5.6 Analysis of artemisinin biosynthetic gene (*CYP71AV1*) and trichome initiation gene (*GL3*) expression of mutant and normal clones of *Artemisia annua* L. 180

6.0 CONCLUSION 189
6.1 Conclusion of study 189
6.2 Suggestions for future work 190

REFERENCES 191
LIST OF PUBLICATIONS 241

LIST OF TABLES

		Page
Table 3.1	Soil substrate combination for seed germination	50
Table 3.2	Mixture for ligation of cDNA into a pUC18 vector A: control ligation reaction, B: ligation reaction with linker-adaptor ligated duplex cDNA	63
Table 3.3	Endonuclease digestion of *Eco*R1 of plasmid with DNA insert	67
Table 3.4	Plant segments, clones and mutagen types and concentrations used in analysis of artemisinin biosynthetic gene (*CYP71AV1*) and trichome initiation gene (*GL3*) expression levels	73
Table 4.1	Effect of surface-sterilization methods on establishment of aseptic seeds and seed germination of different clone of *A. annua* L.	84
Table 4.2	Effect of different water imbibitions duration on seed germination and contamination of *A. annua* L. seeds of different clone	86
Table 4.3	Effect of different substrate combination on germination of *A. annua* L. seeds	86
Table 4.4	Effect of greenhouse and *in vitro* condition on the production of dry extract from *A. annua* L. plants	94
Table 4.5	Yield of RNA and absorbance ratios from three different clones of *A. annua* L. using the standard CTAB plus glacial acetic acid method	96
Table 4.6	Yield of RNA and absorbance ratios from three different clones of *A. annua* L. using the modified CTAB method	98
Table 4.7	Basic assembly metrices for three tested assemblers	108
Table 4.8	Genes' function and designed forward and reverse primers and length	115
Table 4.9	Types and percentage of changes in *CYP71AV1* gene in TC2 plantlets with 3 mM sodium azide treatment after first and fourth subculture	118
Table 4.10	Gene *CYP71AV1* screen results after the fourth subculture of *A. annua* L. plants treated with sodium azide	119

Table 4.11 Types and percentage of changes in gene *CYP71AV1* EMS treatment after first and fourth subculture 120

Table 4.12 Gene *CYP71AV1* screen results after the fourth subculture of *A. annua* L. 122

LIST OF FIGURES

		Page
Figure 2.1	World map showing countries with endemic malaria transmission phase, malaria elimination phase and malaria free countries (Shetty, 2012)	13
Figure 2.2	Timeline of malaria resistance to commonly administered drug (Phyo *et al.*, 2012)	15
Figure 2.3	Proposed biosynthetic pathway for biosynthesis of artemisinin from farnesyl diphosphate (Arsenault *et al.*, 2010).	27
Figure 3.1	Ligation of *Eco*R1 adaptor to cDNA. The letters in bold represent the recognition site for *Eco*R1 restriction enzyme.	60
Figure 3.2	Ligation of adaptor linked duplex cDNA to vector (Sambrook & Russell, 2002)	63
Figure 3.3	Pipeline for transcriptomic raw data assembly and analysis	70
Figure 4.1	Growth pattern of *A. annua* L. plantlets (A: TC1; B: TC2; C: Highland clones) in *in vitro* and greenhouse conditions. Bars represent mean ± standard error.	88
Figure 4.2	Height of *in vitro* and greenhouse *A. annua* L. plants after eight weeks of seed germination. Mean height values for each clone followed by different alphabet were significantly different (Student's T-test, $p \leq 0.05$). Bars represent mean ± standard error.	89
Figure 4.3	Two types of trichomes observed under scanning electron microscope (SEM) on *A. annua* L. leaf surfaces: a: glandular trichome; b: non-glandular/T-trichome.	92
Figure 4.4	Effect of greenhouse and *in vitro* conditions on number of glandular and non-glandular trichomes on adaxial and abaxial surfaces of leaves of *Artemisia annua* L. clones (300µm x 300 µm area). A: TC1; B: TC2; C: Highland clones. Mean values of *in vitro* and greenhouse plantlets for each clone of each parameter with the same alphabet on top of each bar were not significantly different (Student's T-test, $p \leq 0.05$). Mean value of each parameter (glandular and non-glandular, adaxial glandular and abaxial glandular,	93

adaxial non-glandular and abaxial non-glandular) for greenhouse or *in vitro* plantlets with the same alphabet at base of bar were not significantly different (Student's T-test, $p \leq 0.05$).

Figure 4.5 Thin layer chromatography (TLC) plate showing artemisinin (in pink) from *in vitro* and greenhouse *A. annua* L. plantlets. S: standard artemisinin; 1: greenhouse TC1 clone; 2: greenhouse TC2 clone; 3: greenhouse Highland clone, 4: *in vitro* TC1 clone; 5: *in vitro* TC2 clone; 6: *in vitro* Highland clone. 94

Figure 4.6 Ethidium bromide-stained total RNA samples of *A. annua* L. clones on 1 % agarose gel. M: Lambda DNA/ *Hind* 111 marker (Thermoscientificbio); 1-2: TC1; 3-4: TC2; 5-7: Highland clones. 96

Figure 4.7 Ethidium bromide-stained total RNA samples of *A. annua* L. clones on 1 % agarose gel. 1: TC1; 2: TC2; 3: Highland clones. 98

Figure 4.8 Ethidium bromide-stained RNA and mRNA samples on a 1 % agarose gel. Lane 1-3: total RNA; lane 4-6: mRNA fraction; lane M: RiboRuler High Range RNA ladder (Thermoscientific). 100

Figure 4.9 Ethidium bromide-stained cDNA sample of *A. annua* L. on 1% agarose gel with λ/*Hind* 111 marker (Thermoscientific). M: marker, 1: cDNA. 100

Figure 4.10 Ethidium bromide-stained second strand cDNA sample of *A. annua* L. on 1% agarose gel with 1 kb marker (Thermoscientific). M: marker, 1: cDNA. 101

Figure 4.11 Purified plasmid with insert. M, 1kb DNA molecular marker; A-I, digested plasmid with inserts 103

Figure 4.12 Ethidium bromide-stained total RNA samples of TC2 clone of *A. annua* L. on a 1 % agarose gel. M: RiboRuler High Range RNA ladder (Thermoscientific). 105

Figure 4.13 Read distribution based on read length generated by deep sequencing 105

Figure 4.14 A histogram of trimmed reads that were cleaned using SeqClean software tool. 108

Figure 4.15 BLAST hits for identification of the assembled contigs 111

Figure 4.16 Classification of the assembled contigs based on their 111

function in plant

Figure 4.17 Contigs classification according to three gene ontology terms to define gene function. A: biological processes; B: cellular component; C: molecular function. 112-113

Figure 4.18 Agarose gel electrophoresis after PCR mediated screening using *CYP71AV1* gene. The sodium azide treated samples were chosen randomly to run on gel electrophoresis for each treatment in each cultivar to confirm PCR amplification of the gene. M- 100 bp DNA ladder (Promega); 2-5: TC1 control, TC1 1M, TC1 3M, TC1 4M, TC1 5M; 6-8: TC2 control, TC2 1M, TC2 3M; 9-13: H control, H 1M, H 2M, H 3M, H 5M. Agarose gel electrophoresis was run on 2% gel at 10V/cm 118

Figure 4.19 Sequence alignment of TC2 mutant (screening at fourth subculture) which is the only treatment showing mutation after treatment with 3 mM sodium azide and TC2 control for gene *CYP71AV1* 119

Figure 4.20 Agarose gel electrophoresis after PCR mediated screening using *CYP71AV1* gene. The EMS treated and control samples were run on gel electrophoresis for each treatment in each cultivar to confirm PCR amplification of the gene. M: 1 Kb DNA Plus ladder (Promega); 1-6: TC1 control, TC1 0.25 %, TC1 0.50 %, TC1 1.0 %, TC1 1.5 %, TC1 2.0 %; 7-12: TC2 control, TC2 0.25 %, TC2 0.50 %, TC2 1.0 %, TC2 1.5 %, TC2 2.0 %; 13-18: H control, H 0.25 %, H 0.50 %, H 1.0 %, H 1.5 %, H 2.0 %. Agarose gel electrophoresis was run on 2% gel at 10V/cm 120

Figure 4.21 Agarose gel electrophoresis after PCR mediated screening using *CYP71AV1* gene. The EMS treated and control samples were run on gel electrophoresis for each treatment in each cultivar to confirm PCR amplification of the gene. M: 1 Kb DNA Plus ladder (Promega); 1-6: TC1 control, TC1 0.25 %, TC1 0.50 %, TC1 1.0 %, TC1 1.5 %, TC1 2.0 %; 7-12: TC2 control, TC2 0.25 %, TC2 0.50 %, TC2 1.0 %, TC2 1.5 %, TC2 2.0 %; 13-18: H control, H 0.25 %, H 0.50 %, H 1.0 %, H 1.5 %, H 2.0 %. Agarose gel electrophoresis was run on 2% gel at 10V/cm 121

Figure 4.22 Sequence alignment of TC1 mutant (screening at fourth subculture) which shows mutation after treatment with 0. 5% EMS and TC1 control for gene *CYP71AV1* 121

Figure 4.23	Fold change in gene *GL3* expression of *A. annua* L. seeds (A: TC1; B: TC2; C: Highland cultivar) treated with various concentration of EMS. Scatter plot represent mean ± standard error.	125
Figure 4.24	Fold change in gene *GL3* expression of *A. annua* L. nodal segment (A: TC1; B: TC2; C: Highland cultivar) treated with various concentration of EMS. Scatter plots represent mean ± standard error.	128
Figure 4.25	Fold change in gene *GL3* expression of *A. annua* L. shoot tips (A: TC1; B: TC2; C: Highland cultivar) treated with various concentration of EMS. Scatter plots represent mean ± standard error.	130
Figure 4.26	Fold change in gene *CYP71AV1* expression of *A. annua* L. seeds (A: TC1; B: TC2; C: Highland cultivar) treated with various concentration of EMS. Scatter plot represent mean ± standard error.	133
Figure 4.27	Fold change in gene *CYP71AV1* expression of *A. annua* L. nodal segments (A: TC1; B: TC2; C: Highland cultivar) treated with various concentration of EMS. Scatter plots represent mean ± standard error.	135
Figure 4.28	Fold change in gene *CYP71AV1* expression of *A. annua* L. shoot tips (A: TC1; B: TC2; C: Highland cultivar) treated with various concentration of EMS. Scatter plots represent mean ± standard error.	138
Figure 4.29	Fold change in gene *CYP71AV1* expression of *A. annua* L. callus (A: TC1; B: TC2; C: Highland cultivar) treated with various concentration of EMS. Scatter plots represent mean ± standard error.	140
Figure 4.30	Fold change in gene *GL3* expression of *A. annua* L. seeds (A: TC1; B: TC2; C: Highland cultivar) treated with various concentration of NaN_3. Scatter plots represent mean ± standard error.	143
Figure 4.31	Fold change in gene *GL3* expression of *A. annua* L. nodal segments (A: TC1; B: TC2; C: Highland cultivar) treated with various concentration of NaN_3. Scatter plots represent mean ± standard error.	145
Figure 4.32	Fold change in gene *GL3* expression of *A. annua* L. shoot tips (A: TC1; B: TC2; C: Highland cultivar) treated with various concentration of NaN_3. Scatter plots represent mean ± standard error.	148
Figure 4.33	Fold change in gene *CYP71AV1* expression of *A. annua*	150

L. seeds (A: TC1; B: TC2; C: Highland cultivar) treated with various concentration of NaN_3. Scatter plots represent mean ± standard error.

Figure 4.34 Fold change in gene *CYP71AV1* expression of *A. annua* L. nodal segments (A: TC1; B: TC2; C: Highland cultivar) treated with various concentration of NaN_3. Scatter plot represent mean ± standard error. 153

Figure 4.35 Fold change in gene *CYP71AV1* expression of *A. annua* L. shoot tips (A: TC1; B: TC2; C: Highland cultivar) treated with various concentration of NaN_3. Scatter plots represent mean ± standard error. 155

Figure 4.36 Fold change in gene *CYP71AV1* expression of *A. annua* L. callus (A: TC1; B: TC2; C: Highland cultivar) treated with various concentration of NaN_3. Scatter plots represent mean ± standard error. 157

Figure 4.37 Scatter plots of gene *GL3* expression level of three clones; TC1, TC2, H cDNA samples of *Artemisia annua* L. initiated from seeds, shoot tips and nodal segment treated with 1% EMS. 159

LIST OF ABBREVIATIONS

AA	Artemisinic acid
AB	Artennuin B
ACT	Artemisinin combined therapy
ADS	Amorpha-4, 11-diene synthase
ALDH1	Aldehyde dehydrogenase
ANOVA	Analysis of variance
cDNA	Complementary deoxyribonucleic acid
Cq	Cycle of quantification
CTAB	Cetyl trimethylammonium brommide
CYP71AV1	Artemisinin biosynthetic gene
DBR2	Double bond reductase
DHAA	Dihyroartemisinic acid
DNA	Deoxyribonucleic acid
DW	Dry weight
FPP	Farnesyl diphosphate
GL3	Trichome initiation gene
GST	Glandular secreting trichome
MEP	Non-mevalonate pathway
MS	Murashinge and Skoog
MVA	Mevalonate pathway
NGT	Non-glandular trichome
NIH	National institute health
PCR	Polymerase chain reaction

Qpcr	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
rpm	Rotation per minute
TLC	Thin Layer Chromatography
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organization

PEWUJUDAN POKOK *Artemisia annua* L. *in vitro* UNTUK ANALISIS EKSPRESI GEN BIOSINTETIK ARTEMISININ (*CYP71AV1*) DAN GEN INISIASI TRIKOM (*GL3*)

ABSTRAK

Artemisia annua L. ialah sejenis tumbuhan herba yang terkenal untuk metabolit sekundernya iaitu, artemisinin. Artemisinin digunakan sebagai ubat antimalaria tetapi pengunaannya terhad disebabkan oleh hasil artemisinin yang rendah di ladang. Untuk menghasilkan artemisinin menggunakan teknik *in vitro,* kultivar *in vitro* yang menghasilkan artemisinin tinggi perlu dipilih terlebih dahulu. Untuk mendapatkan anak benih aseptik, biji benih tiga klon terpilih *Artemisa annua* L. disterilkan permukaannya dengan menggunakan 10% (v/v) Clorox® selama lima minit dan diikuti dengan 70% (v/v) etanol selama lima minit. Protokol pensterilan ini menghasilkan 96.7 % biji benih aseptik untuk klon TC1 dan TC2, dan 86.7 % bagi klon Highland. Peratus biji benih yang bercambah untuk ketiga-tiga klon *A. annua* L. adalah dalam lingkungan 13.3 – 36.7 %. Ujian imbibisi yang dijalankan untuk kesemua biji benih klon *A. annua* L. mendapati imbibisi sebelum percambahan tidak diperlukan. Kombinasi substrat yang paling sesuai untuk percambahan biji benih ketiga-tiga klon *A. annua* L. ialah kombinasi pasir: tanah hitam (1:2). Kesan keadaan *in vitro* dan rumah hijau terhadap pertumbuhan anak benih didapati keadaan *in vitro* menghasilkan anak benih *A. annua* L. yang lebih tinggi dibandingkan dengan rumah hijau. Dari segi bilangan trikom berkelenjar dan tidak berkelenjar, tiada kelainan didapati untuk daun anak benih daripada keadaan *in vitro* dan rumah hijau. Perhimpunan dan analisis perpustakaan transkriptom didapati menghasilkan 10, 647

urutan gen. Daripada 10, 647 gen, 306 gen unik dikenal pasti terlibat dalam proses fungsi sel, proses biologi, dan fungsi molekul. *CYP71AV1* and *GL3* yang terlibat dalam laluan sintesis artemisinin dipilih untuk kajian ekspresi antara anak pokok kawalan dan *A. annua* L mutan. Kepadatan mutasi disebabkan oleh etil metanesulfonat (EMS) didapati 1 dalam 408 kb nukleotida berbanding 1 dalam 816 kb untuk natrium azida. Kadar pengesanan mutan untuk EMS adalah 2.4 manakala untuk natrium azida adalah 1.2 dalam setiap 1000 kb nukleotida. Pucuk untuk ketiga-tiga klon *A. annua* L. yang dirawat dengan 1% EMS menunjukkan tahap ekspresi gen *GL3* yang lebih tinggi dan konsisten apabila dibandingkan dengan pucuk pokok kawalan. Pokok-pokok yang dirawat dengan natrium azida juga tidak menunjukkan tahap ekspresi tinggi yang konsisten berbanding pokok kawalan. Oleh itu, gen *GL3* didapati lebih sesuai sebagai gen penanda artemisinin dalam pokok *A. annua* L. dan cultivar yang meghasilkan artemisinin yang tinggi boleh didapati daripada pucuk yang dirawat dengan 1% EMS.

ESTABLISHMENT OF *in vitro* PLANTLETS OF *Artemisia annua* L. FOR THE ANALYSIS OF ARTEMISININ BIOSYNTHETIC GENE (*CYP71AV1*) AND TRICHOME INITIATION GENE (*GL3*)

ABSTRACT

Artemisia annua L. is an herb known for its secondary metabolite, artemisinin. Artemisinin is used as antimalarial drug but its availability is limited by low yield in plantation. To produce artemisinin using *in vitro* technique, a high yielding *in vitro* cultivar must first be selected. For the establishment of aseptic seedlings of three selected clones of *Artemisia annua* L., the seeds were surface sterilized with 10% (v/v) Clorox® for five minutes followed by 70% (v/v) ethanol for five minutes. This sterilization protocol enabled the establishment of 96.7 % aseptic seeds for TC1 and TC2 clones, and 86.7 % for the Highland clone. The percentage of seed germinated for all the clones were found to be in the range of 13.3 to 36.7 %. Imbibitions test on the three clones of *A. annua* L. seeds indicated imbibitions before seed germination was not required. The best substrate combination for seed germination of all the three clones of *A. annua* L. was sand: black soil (1:2) combination. Effect of *in vitro* and greenhouse condition on *A. annua* L. plantlets growth indicated *in vitro* growth condition produced taller plantlets than greenhouse conditions. There were no differences in number of glandular and non-glandular trichome on *in vitro* and greenhouse grown leaves of *A. annua* L. The assembly and analysis of transcriptome library using next generation sequencing technology produced 10, 647 gene sequences. Of the 10, 647 genes identified through BLAST software, 306 unique genes of *A. annua* L. were classified to be involved in cellular function, biological

processes and molecular function. Of the 306 genes, there were 14 unique genes that were identified to be involved in metabolite biosynthesis pathways. *CYP71AV1* and *GL3* genes that were involved in artemisinin biosynthesis were chosen for expression study of control and mutant plantlets. The mutation density due to ethyl methanesulfonate (EMS) treatment using CYP71AV1 promoter gene was 1 in 408 kb of nucleotides compared to sodium azide induced mutation with 1 in 816 kb. The mutation detection rate for EMS-induced was 2.4 whereas for sodium azide-induced was only 1.2 mutations in every 1000 kb of nucleotides. Shoot tips of all the three clones of *A. annua* L. treated with 1% EMS showed consistently higher expression level for *GL3* gene than in control plantlets. The other plantlets treated with sodium azide were not found to have consistently higher expression level than the control plantlets. *GL3* gene expression was found to be a suitable marker in indicating artemisinin yield in *A. annua* L. initiated from treated shoot tips with 1% EMS.

CHAPTER ONE

INTRODUCTION

Malaria is a deadly disease but it is a treatable and preventable disease. Currently it is affecting 104 countries around the world. This mosquito-borne disease mostly affects children under five years old (WHO, 2012). According to WHO estimates in 2010, 219 million cases of malaria and an approximate 660 000 deaths were reported with about 90% of all deaths occurred in African regions. In Malaysia, 5306 cases reported were confirmed of malaria with 16 deaths recorded (WHO, 2012). Previously in 1995, 60 000 cases of malaria were reported and there was a drastic reduction in malaria prevalence mainly due to strategic control efforts such as distribution of insecticide-treated nets and constant indoor residual spraying to areas with high malaria risk together with early detection and diagnosis of malaria symptoms (West *et al.*, 2013). These efforts of eradication of malaria transmission put Malaysia in malaria pre-elimination phase as of December 2012, which saw a plummet in reported cases of malaria especially in Sabah, Sarawak and Pahang (WHO, 2012).

Malaria was caused by protozoa species namely *Plasmodium falciparum, P. ovale, P. vivax* and *P. malariae* with *P. falciparum* being the most deadly species of Plasmodium and *P. vivax* being more widespread (Atroosh *et al.*, 2011). Predominantly, these protozoa were hosted by *Anopheles maculatus*, a mosquito species found in tropical jungles of Malaysia. With early detection of these *P. falciparum* and *P. vivax* in Malaysia, primary intervention efforts and effective health

care system with full subsidy of quinoline based antibiotics foresee the decrease in malaria cases. However, since 2010, a sudden rise in malaria cases was reported in Malaysia primarily due to the increasing number of knowlesi malaria cases or commonly known as simian malaria (Jiram *et al.*, 2012; Rajahram *et al.*, 2012). *P. falciparum* and the other three *Plasmodium* species mentioned above were the known human infecting protozoa but currently a fifth *Plasmodium* species strain was found infecting humans (White, 2007). This malaria parasite was known to find host in long tailed macaques (*Macaca fascicularis*) and pig tailed macaques (*Macaca nemestrina*) (Bronner *et al.*, 2009) and infects another macaque through mosquitoes; *Anopheles hackeri* (found in Peninsular Malaysia), *A. latens* (Sarawak) and *A. balabacencis* (Sabah) (Vythilingam *et al.*, 2005). The parasite seemed to have found a more stable host in human where the cases of *P. knowlesi* malaria were reported in Kapit district, Sarawak (Singh *et al.*, 2004; Bronner *et al.*, 2009). The transmission could be by mosquito bite which previously has bitten macaque with *P. knowlesi*. The pattern of knowlesi malaria infections in Malaysia was observed in regions with abundant mosquito due to ineffective malaria eradication programme, for example, in Sabah and Sarawak where logging activity is on the rise with loggers often found camping deep in jungle for days. Singh *et al.* (2004) reported *P. knowlesi* accounted for one in five malaria cases in Kapit district which previously mistaken for *P. malariae* and all the reported cases were found in adults. In Sabah, a study done in rural area of Kudat district found that children below 15 years old were also infected with non-severe knowlesi malaria with uncomplicated disease symptoms. Children below 15 years old made up 14% of the malaria cases admitted in Kudat Hospital (William *et al.*, 2013). This consistent emergence of knowlesi malaria dominance in Malaysia Borneo (Sabah and Sarawak) could be due to a few factors; namely the

increasing deforestation in these states, increasing exposure of humans to monkeys and vectors and hampering of inter species competition due to effective control of *P. falciparum* and *P. vivax* malaria prevalence (Rajahram *et al.*, 2012). Such a rise in knowlesi malaria is worrisome especially at the time when Malaysia is fighting to aiming at total elimination of malaria by 2020 and hence total malaria eradication effort (William *et al.*, 2011).

With the ever increasing cases of malaria in endemic districts of Sabah and Sarawak, artemisinin-based combination therapy (ACT) was suggested by WHO in 2001 as the first line treatment for malaria (WHO, 2001). Before 1960s, quinine-based antibiotics treatments effectively controlled malaria. However, emergence of multi resistant strain *P. falciparum* malaria caused alternative treatment to be widely sought. The year 1971 saw the discovery of artemisinin compound from traditionally used Chinese herb, *Artemisia annua* L. Artemisinin is a sesquiterpene lactone with a unique endoperoxide structure which is complicated, difficult and expensive to be synthetically synthesised (Kindermans *et al.*, 2007). The underlying mechanism in antimalarial activity of artemisinin is still unclear but the importance of endoperoxide bridge in artemisinin after iron activation in specifically and selectively inhibiting the endoplasmic reticulum Ca^{2+} ATPase in *P. falciparum* is well documented (Teoh *et al.*, 2006). Artemisinin's rapid therapeutic efficacy, short half life and low toxicity in human have been recommended to be used in combination with other commonly used antimalarial drugs such as lumefantrine and sulfadoxime/pyrimethamine in antimalarial therapy now widely known as artemisinin-based combination therapy (ACT).

The genus Artemisia (Asteraceae) consists of more than 500 species and till to date *A. annua* L. is the only known species with this secondary metabolite, artemisinin (Paddon *et al.*, 2013). However, artemisinin yield often influenced by environmental factors such as local temperature, humidity and soil acidity which cause inconsistent artemisinin availability in commercial market. Mostly planted as crop in Vietnam and China, concentration of artemisinin ranges from 0.01 to 0.8% of the plant dry weight (Zhang *et al.*, 2009; Brown, 2010). To date, 400 million courses of ACT are required throughout a year especially in poor countries and only about 100 million courses of ACT were able to be fulfilled in 2011 (WHO, 2012). Insufficient supply of artemisinin makes artemisinin relatively an expensive drug for countries in poor regions of the world.

In Malaysia, the above scenario was also observed in which quinine-based antibiotics were still used to alleviate malaria despite recommendations from WHO in 2001 to use ACT. High price of this therapy resulted in assignment of different regiments of quinine-based drug to patients in Malaysia which took longer period of time for the parasite clearance in patients (Barber *et al.*, 2011). ACT is only used as the last alternative in Malaysia to fight malaria as a result of high price and low supply in local hospitals. With unsuitable environment for *A. annua* L. growth in Malaysia as a crop plant, Malaysia was left to seek an alternative approach in producing artemisinin locally to meet the drug's growing demand and to combat the rise of new malaria strain (*P. knowlesi*) in Malaysia. *In vitro* culture technology can be the alternative to solve these problems.

Micropropagation is one of the *in vitro* culture techniques for plantlet multiplication under aseptic environment with controlled temperature, nutrients and

light intensity (Zhou & Brown, 2006). *In vitro* culture techniques include few techniques such as callus culture (Bhojwani & Radzan, 1996), cell suspension culture (Bhojwani & Radzan, 1996; Fowler *et al.*, 1998), somatic embryogenesis (Zimmerman, 1993; Radzan, 2002) and protoplast cultures (Bhojwani & Radzan, 1996). These techniques were introduced as an alternative tool for *in vitro* study and production of plant secondary metabolites (Sidhu, 2010). By using *in vitro* culture system, different strategies such as manipulation of growth conditions (McChesney, 1999), medium formulations (Misawa, 1985; Stafford *et al.*, 1986), usage of plant growth regulators (Misawa, 1985) and elicitators (Roberts & Shuler, 1997) have been employed to study and improve production of plant secondary metabolites.

Till date, many medicinal plants with commercially valuable secondary metabolites have been researched and produced in *in vitro* system, for example *Corydalis yanhusuo*, *Dioscorea doryophora*, *Pinellia ternate*, and *Salvia miltiorrhiza* (Tsay & Agarwal, 2005), *Charybdis nunidica* (Jour & Fourr) Speta (Kongbangkard *et al.*, 2005), *Stemona tuberose* Lour. (Montri *et al.*, 2009) and *Dendrobium huoshanense* C. Z. Tang et S. J. Chang (Luo *et al.*, 2009). However, secondary metabolites production in plants often involves complicated biosynthesis pathways. Due to this setback, only certain amount of these compounds can be derived using *in vitro* system, for example *A. annua* L. grown *in vitro* in USM contains 90 - 300 µg/g (DW) of artemisinin compared to 440 µg/g of artemisinin content from *A. annua* L. leaves grown in Vietnamese field (Thu *et al.*, 2011). This low level of secondary metabolites in *in vitro* culture system can be attributed to the influence by temporal (temperature) and spatial (environment) factors and vary in production from cell to cell (Sidhu, 2010). Heterogeneity of plants and morphological characteristics difference of each plant must be addressed and eliminated (Ferreira *et al.*, 2005). One

of the solutions that can be used to increase the *in vitro* production of secondary metabolites is by selecting and using high yielding clones in initiation of *in vitro* system by which all the plants will have similar high quality traits and high yield of secondary metabolites.

Classical breeding involves manual crossbreeding of crops and choosing the economically valuable qualities that are viable such as bigger fruits, plants that are resistance to disease and seedless fruits (Rommens *et al.*, 2007). This technique of crop breeding has been in practice for hundreds of years. One of the setbacks of this manual selection is that it takes practically long time span and high number of workmanship in large scale breeding process (Turan *et al.*, 2012). The potential to improve medicinal plants property and productivity largely depend not only on classical breeding methods but also on plant biotechnology techniques. The integration of plant breeding and plant biotechnology enables selection of useful genotypes, isolation and cloning of commercially important traits and development of organisms that are of high quality traits (Tanksley & McCouch, 1997). Thus, integration of classical breeding method and plant biotechnology not only shortens breeding and selection cycles but also increases productivity. There are many plant biotechnology techniques available for the manipulation of DNA, one of it is chemical mutagenesis. Chemical mutagenesis can be defined as induction of random mutations either by point mutation, deletion, insertion, transversion or transition in plant DNA sequences with the use of chemical mutagens (Koorneef, 2002). Though some of the mutations created by chemical mutagenesis can be deemed as lethal in plants, beneficial new proteins could also be created and studied through this technique (Al-Qurainy & Khan, 2009). Mutational specificity can be determined at two levels; phenotype and genotype level. Mutational effect occurs not only at DNA

stage but also at nucleotide stage (Kodym & Afza, 2003). The inheritable changes can be observed at phenotypic level and affects the expression of the given allele (Rauf *et al.*, 2010). Morphological studies accompanied by genotypic examination of the studied gene provide a better view on the effect of mutagen on the gene (Kostov *et al.*, 2007). Through random mutation induced by chemical mutagens, cultivars with high expression of selected genes and favourable morphological characteristics which also in turn increases productivity of secondary metabolites in *A. annua* L. could be chosen as a starting material for micropropagation of high yielding *A. annua* L. in Malaysia.

Glandular trichome in *A. annua* L. has been identified as the site for synthesis, storage and sequestration of artemisinin (Wagner *et al.*, 2004). Lommen *et al.* (2006) reported that increase in glandular trichome density can in turn increase the level of artemisinin. Thus, selection and establishment of *A. annua* L. cultivar with high density of glandular trichome on the leaves as the starting material for *in vitro* culture techniques could result in high *in vitro* artemisinin production. Gene *GL3* has been identified to be involved in the development of glandular trichome and gene *CYP71AV1* is involved in the artemisinin biosynthesis pathway (Teoh *et al.*, 2006; Caro *et al.*, 2007). Induction of point mutations via chemical mutagens could offer an alternative in improving the genotypes of *A. anuua* L. Changes in genes *GL3* and *CYP71AV1* mRNA levels due to chemical mutagen treatments could be measured using real time PCR. The changes in the transcripts of these two genes after the treatment with chemical mutagens will also reveal the co-relation between trichome density and artemisinin content in *A. annua* L. Consequently, a high yielding clone with high density of glandular trichome would be chosen as a starting material for in *in vitro* culture technique.

1.1 Objectives of study

Hence the objectives of this research are:

1. To investigate and compare the growth of *in vitro A. annua* L. clones and those maintained at greenhouse condition
2. To identify artemisinin related biosynthetic gene and morphology related gene from *A. annua* L. transcriptome cDNA library of *in vitro* leaf tissue
3. To detect the mutation rate and mutagenic density in *A. annua* L. plants caused by ethyl methanesulfonate and sodium azide
4. To detect the expression of *CYP71AV1* and *GL3* genes of mutated *A. annua* L. induced from callus, seeds, shoot tips and nodal segment

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria

2.1.1 Types of malaria

Malaria is an infectious disease caused by protozoa of the genus *Plasmodium*, which is carried by female mosquitoes of the genus *Anopheles* (Bouwmeester *et al.*, 2006). The term malaria was derived from the Italian term for disease (mala=bad, aria=air) (Goldsmith, 2010). A group of Italian researchers in 1898 reported the transmission of malaria in human was conclusively by anopheline mosquitoes. In 1948, another group demonstrated the development of malaria parasites in human liver before entering the blood stream. The presence of dormant stages of this parasite in liver was only reported in 1982 (Cox, 2010). For more than hundred years, malaria and its parasites were actively researched to understand the mechanism of transmission and symptoms in human.

WHO has named five species of *Plasmodium* that were afflicted to malaria with *Plasmodium falciparum* being rated as the most life threatening species and *P. knowlesi* as the most recent finding in malaria-causing parasite (WHO, 2012). The malaria parasite, *Plasmodium*, is a small and single celled organism (protozoan) that lives as a parasite in man and in specific genus of mosquito known as *Anopheles*. There are five different strains of malaria parasite; *P. falciparum* and *P. knowlesi* are the causes of fatal malaria (Barber *et al.*, 2011) while *P. vivax, P. ovale* and *P. malariae* cause more benign types of malaria. Falciparum and knowlesi malaria can kill, but the other forms were less likely to prove fatal (Sandhosam & Thomas, 1982;

William *et al.*, 2011). There are several stages in the life cycle of the parasite as reported by National Institute of Health (NIH) and all are the same for the five types (Morrow, 2001).

Symptoms commonly associated with malaria caused by all the parasite types are bouts of chills (ague) and fever lasting several hours for three or four days, couple with muscle ache, headache, diarrhea and vomiting. Any delayed treatment will cause spleen and liver enlargement, causing anemia to develop and clogging of the vessels of cerebral tissues followed by coma and eventually death (Sandhosam & Thomas, 1982). In Africa, over 90% of life threatening cases were reported to occur in children. Severe malaria is mainly reported in areas with stable endemicity in which children with age range of few months to five years old and symptoms such as severe anemia, respiratory distress in relation to metabolic acidosis or cerebral malaria are frequently recorded (William *et al.*, 2011; WHO, 2012). The severity of malaria lowers in older children and adults as a result of increasing partial immunity in their immune system. Conversely, in lower endemicity areas, adults and older children also inflicted with severe malaria as well as non-immune travelers and migrant workers (Barber *et al.* 2011). Severe malaria is defined as inability to swallow tablets, high parasite counts in bloods and evident vital organ dysfunction which increases risk of dying. However, the stated risks depend on the degree of abnormality, age, background immunity and access to appropriate treatment (WHO, 2001). The symptoms of severe knowlesi malaria and multi organ failure experienced by patients were reported to be comparable to that of severe falciparum malaria reported in adult patients in areas with low transmission rate and unstable endemicity (William *et al.*, 2013).

A common parasite between human and primate, *P. knowlesi*, has a 24 hours erythrocytic cycle that is likely to accelerate the development of complications. Respiratory distress is the frequently recorded complication (Cox-Singh *et al.*, 2008; Daneshvar *et al.*, 2009). Clinical relapses were also reported to occur in weeks or months after the first infection for both *P. vivax* and *P. ovale.* This recurrence was accounted to arise even after the patients have left the endemic area which is caused by dormant liver form of parasite known as hypnozoites. This form is absent in *P. falciparum* and *P. malariae* and special treatment targeted at this specific stage in liver is required for complete cure (WHO, 2012).

2.1.2 Antimalarial drug resistance

For decades, malaria is still considered as one of the three deadliest diseases affecting 104 countries in developing and poor regions of world. Alongside HIV and tuberculosis, malaria is causing poorer countries especially sub-Saharan Africa where *P. falciparum* is the main cause of malaria to lose billions a year due to this infectious disease (Ferreira *et al.*, 2005; WHO, 2012). There were an estimated 219 million cases of malaria and 660 000 deaths reported in 2010. Of the 104 countries with malaria, 80% of deaths estimated to occur in only 14 countries with the Democratic Republic of the Congo and Nigeria accounted for over 40% of malaria deaths worldwide in 2010. There were five malaria eradication phase as outlined by WHO, of the 104 countries, 79 countries were classified in malaria control phase, 10 in pre-elimination phase, 10 in elimination phase and the final five countries which were free of ongoing transmission were classified in the prevention of re-introduction phase (Figure 2.1) (WHO, 2012).

Ferreira *et al.* (2005) stated that in the early 1960s, *P. falciparum* malaria began to show resistance against quinine-derived drugs. This rapid emergence of resistance further complicated malaria control and treatment as few cheap and alternative drugs to chloroquine were available especially in African region (D' Alessandro & Buttiens, 2001). Sulphadoxine/pyrimethamine and mefloquine resistant strains of falciparum malaria have spread in 1953 and in 1980s -1990s, Southeast Asia region countries have reported resistant strain that have spread quickly in this region. Cambodia-Thailand border is one the endemic area that harbors world's most widespread multidrug-resistant falciparum malaria (Figure 2.1) (Price *et al.*, 2004; Song *et al.*, 2011).

In 1969, mefloquine was synthesized by US army in Antimalarial Control Centre in search of new drug for chemoprophylaxis and then in late 1970s, its efficacy against chloroquine resistant falciparum malaria was discovered (Wongsrichanalai *et al.*, 2004; Dassonville-Klimpt *et al.*, 2011). The long half-life of mefloquine and robust efficacy against falciparum malaria enables it to be used widely in malaria prevention drug regimen (Croft & Herxheimer, 2002). Since then, approximately 14.5 million malaria patients especially from Brazil have been prescribed with this drug (Santelli *et al.*, 2012). In Southeast Asia, Thailand was the first region to administer mefloquine in combination with sulfadoxine and pyrimethamine in large scale after successful small scale efficacy trial for non-complicated falciparum malaria. This combination of mefloquine and other drugs was established following reports of mefloquine resistance in non-immune Thai marine recruit in the year 1982. In the following years, mefloquine resistance became widespread to Thai-Cambodian border which is the point of mefloquine resistance to other parts of the region.

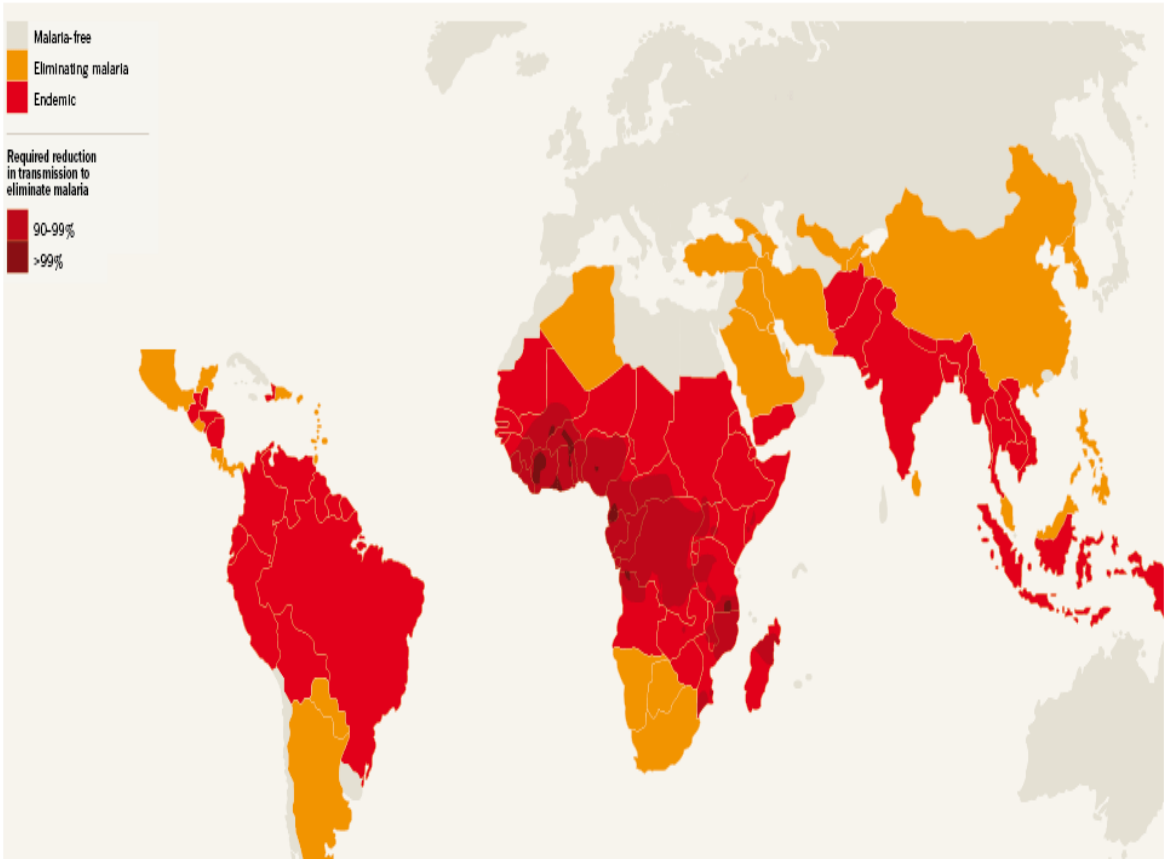

Figure 2.1: World map showing countries with endemic malaria transmission phase, malaria elimination phase and malaria free countries (Shetty, 2012)

Following the isolation and discovery of artemisinin potential in malaria eradication, WHO (2004) introduced combination of artemisinin and other quinine known as ACT as the first line therapy for severe and non-complicated falciparum malaria (Fairhurst *et al.*, 2012). ACT is a treatment using combination of artemisinin or its derivative (artesunate, artemether, dihydroartemisinin) and other quinine-based drug (amodiaquine, mefloquine, piperaquine, lumefantrine). This was recommended as artemisinin has markedly rapid onset of action followed by short half-life in the bloodstream where complete elimination of parasite in blood require one to two weeks. Thus, to avoid losing artemisinin's potency and development of resistance in falciparum malaria, monotherapy of artemisinin was prohibited by WHO (Nosten *et al.*, 2000). Drug pressure was cited as one the main factor involved in selection of the resistant strain when the drug is misused or used alone extensively (Wernsdorfer & Payne, 1991). ACT has been adopted as first line treatment by 79 countries and territories by 2011 for falciparum malaria (WHO, 2012). However, many malaria endemic areas continued to use artemisinin monotherapy for treatment of uncomplicated malaria (Dondorp *et al.*, 2009). In 2006, there have been reports of declination in efficacy of artemisinin-based combination and artesunate monotherapy in western Cambodia followed by resistance of falciparum malaria in Thai-Cambodia border (Denis *et al.*, 2006; Alker *et al.*, 2007; Phyo *et al.*, 2012). Till date, four countries of Greater Mekong subregions (Cambodia, Myanmar, Thailand and Vietnam) have reported parasite resistance to artemisinin and its derivatives (Figure 2.2) (WHO, 2012).

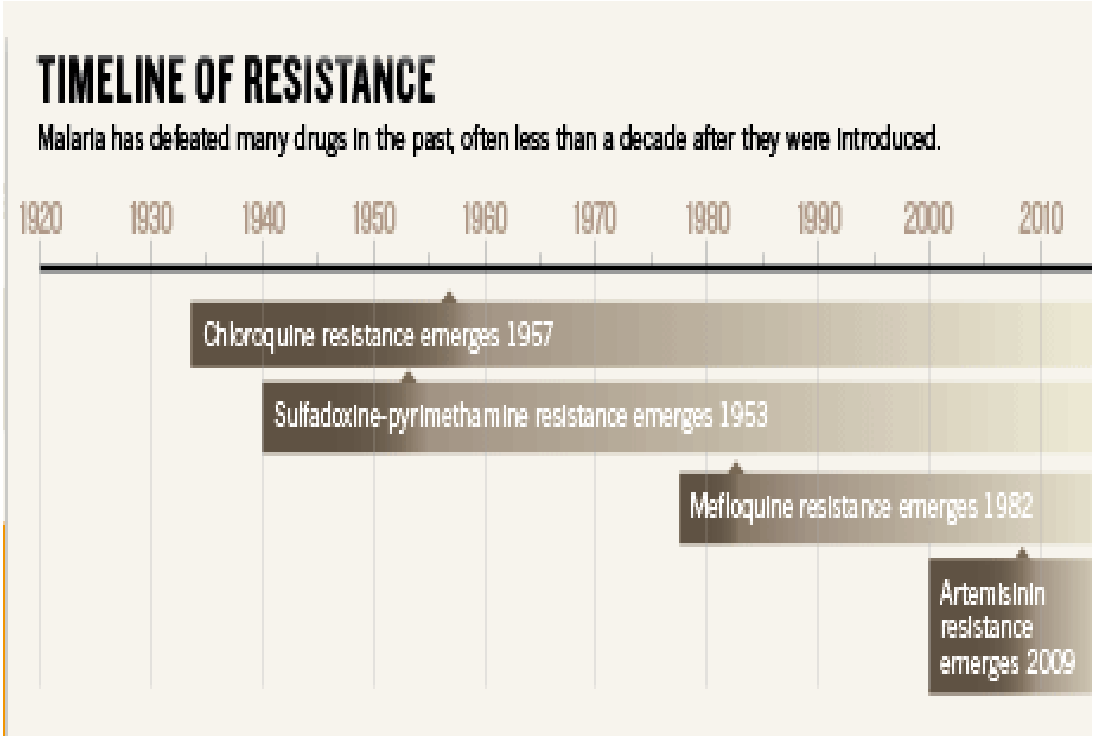

Figure 2.2: Timeline of malaria resistance to commonly administered drug (Phyo *et al.*, 2012)

2.1.3 Malaria in Malaysia

In Malaysia, malaria have been under control and WHO (2012) have categorised Malaysia under pre-elimination phase. Most of the cases reported were from interior parts of Sabah with *P. falciparum* and *P. vivax* being the more predominant species over recent years. Malaysia reported the first case of chloroquine resistant in 1966 and combination drugs of sulphodoxine-pyrimethamine (SDX/PYR) was used as first line treatment to replace chloroquine in Peninsula Malaysia and Sabah in the 1970s (Dondero *et al.*, 1975). As the parasites also began to show resistance against SDX/PYR combination, artemisinin-based combination therapy (ACT) was used as first line drug in East Malaysia (Cox *et al.*, 2003). However, in East Malaysia, chloroquine and SDX/PYR were still used for uncomplicated malaria instead of ACT in treatment policy although previous study have shown 63.6% of *in vivo* study of vivax malaria patients unresponsive to chloroquine (Atroosh *et al.*, 2011). High cost, low supply and treatment policy of Malaysian government for uncomplicated malaria reduces import and usage of artemisinin as drug of choice in Malaysia (Barber *et al.*, 2011). Malaysian falciparum malaria cases was the highest in 1994 with 33 153 cases and dropped to 605 cases in 2011. Whereas, vivax malaria cases reported in 1995 was 15 857 and decreased to 628 cases in 2011 (WHO, 2012). The reduced malaria cases was attributed to elimination and control steps carried out by Malaysian Health Ministry with the application of rapid diagnosis, appropriate and timely treatment, surveillance, residual spraying of indoor and circulation of insecticide-treated nets at risk targeted area (Barber *et al.*, 2011). However, the suppression of *P. falciparum* and *P. vivax* in Malaysia caused an increase in *P. knowlesi* infection especially in Sabah and Sarawak regions. *P. knowlesi* caused

malaria constituted only one percent of the reported malaria infections in Malaysia but in 2011, it was then suddenly increased to 35 % and this trend hampered the elimination of malaria from Malaysia.

P. knowlesi is a zoonotic parasite predominantly found in long tailed (*Macaca fascicularis*) and pig tailed macaques (*M. nemestrina*) (Bronner *et al.*, 2009). Eventually through mosquito bites particularly by *Anopheles* group, this simian parasite transmitted to human. Currently, *P. knowlesi* is identified as the most common cause of human malaria in Sabah and Sarawak (Bruce *et al.*, 2000; Cox-Singh *et al.*, 2008; Daneshvar *et al.*, 2009). Mosquito vectors in Sabah were identified as *A. balabacensis* and *A. donaldi* whereas in Sarawak, *A. latens* was discovered to carry this *P. knowlesi* protozoon (Vythilingam *et al.* 2005). Several factors were attributed to this rise in *P. knowlesi* cases such as rapid deforestation activity in rural area of Sabah and Sarawak which correlated to the reported cases in Kudat District Hospital (KDH) where most of the patients were involved in timbering. Suppression of *P. falciparum* and *P. vivax* was reported to pose less competition for *P. knowlesi* to widespread (Barber *et al.*, 2011; Joveen-Neoh *et al.*, 2011).

2.2 Asteraceae

2.2.1 Characteristics of Asteraceae family

Asteraceae or commonly known as Compositae usually referred to aster, daisy or sunflower representing one of the largest family of flowering plants in order Asterales, covering more than 1600 genera and 24 000 species of herbs, shrubs and

trees that are distributed throughout the world except in Antarctica. Asteraceae is economically important for its many garden ornamentals, such as ageratums, asters, chrysanthemums, cosmos, dahlias, marigolds, wormwood, sunflower and dandelion (Funk *et al.*, 2009). As a monophyletic family, Asteraceae is represented by all its members with common morphological characters such as its floral structure which is characterized by the aggregation of the flowers into capitula and production of fruits typical to this family, the achenes (cypsela), usually present with a pappus (Mucciarelli *et al.*, 2002; Funk *et al.*, 2009). Ironically, in spite of their uniform characters, Asteraceae can be found on every continent except Antarctica, occupying a wide range of habitat types such as open areas, lowland forests and high elevated grasslands but they are not commonly found in tropical wet forests. The members of this family vary in the habit as some of the members in this family are true epiphytes, perennial and annual herbs, shrubs, vines and trees (Cronquist, 1977; Funk *et al.*, 2005). The pollinations were assisted by bee and fly although some Lepidoptera and birds also facilitate the pollinations of the mostly yellow or white flowers. Usually the floral heads can consist up to 1000 florets and for species with wind as pollination agent, the appearance of the floral heads could be different from most of the groups in the family. The chromosome numbers of Asteraceae members range from n=2 to n=114 which is a high level of polyploidy (Funk *et al.*, 2005).

The Anthemideae is the seventh largest tribe in the Asteraceae. It is monophyletic and reported to date to be composed of 109 genera and 1740 species (Bremer & Humpries, 1993). The members of this tribe are mostly herbs, sub-shrubs or shrubs without latex and with characteristic aromatic leaves. These herbs and shrubs are mostly perennial and some are annual with mostly found in Southern

Africa, central Asian and the Mediterranean regions (Tutin & Pearson, 1976; Funk *et al.*, 2009). The genus *Artemisia* is one of the largest and reported to be the most widely distributed with nearly 100 genera in the tribe Anthemideae of Asteraceae (Compositae) (Funk *et al.*, 2009).

2.2.2 Genus *Artemisia*

Under the Anthemideae tribe, genus *Artemisia* is the largest with over 500 species that are very diverse in terms of ecology, morphology and chemical constituents (Watson *et al.*, 2002; Riggins *et al.*, 2012). This genus is mainly found in the temperate zones of Europe, Asia and North America (Watson *et al.*, 2002; Mehrdad *et al.*, 2007; Bora & Sharma, 2011). Most of the species are perennial and only ten species are biennial and annual herbs or small shrubs (Valles *et al.*, 2003; Bora & Sharma, 2011). They are at present categorized into five main groups; *Artemisia*, *Absinthium* (Mill.) Less., *Dracunculus* (Besser) Rydb., *Seriphidium* Besser, and *Tridentatae* (Rydb.) McArthur (Tabur *et al.*, 2011). The general morphology of the members of the genus *Artemisia* is described to have strong aroma, alternate leaves, small capitula that are usually in racemouse, paniculate or capitates inflorescence, obovoid achenes and pappus which is either absent or present as a small scarious ring (Mucciarelli & Maffei, 2002). Many species of this genus are important medicinal plants and some are important to the locals as food, forage and ornamental plants. Although some species are found to be soil stabilizers in arid or semi arid habitats, some are found to be toxic or allergic causing whereas some are insidious weeds

which affect the yield in crop plantations (Tan *et al.* 1998; Hayat *et al.*, 2009; Tabur *et al.*, 2011).

WHO (2004) reported that 80% of world population depend on non-conventional medicine especially on herbal medicine as their first line healthcare. Considerable amount of demand for herbal medicines increases the search for traditionally used medicinal plants and their use in treating various ailments in humans. Following this globally growing trend, genus *Artemisia* members are also researched for its useful medicinal property (Tan *et al.*, 1998; Patil *et al.*, 2011; Abad *et al.*, 2012). Phytochemical constituent of genus *Artemisia* mainly depended on various secondary metabolites secretion of the plants. Secondary metabolism in plants plays a crucial role as chemical defence against predators, disease and as an attractant for pollinators (Tan *et al.*, 1998). Analysis of chemical composition of essential oils of genus *Artemisia* displayed a range of phytochemicals; mainly terpenoids, cumarins, acetylenes, flavonoids, sterols, and caffeoylquinic acids (Bora & Sharma, 2011).

A significant variation in terpene constituents of essential oils in intraspecific species level indicated involvement of few factors such as environment and genetics (Ferreira *et al.*, 1995). Plants growing at different altitudes or plant ontogeny influences essential oil compositions, whereas some plant secondary metabolites content and quality varies according to fertilizers and pH of soils, location, chemotype or subspecies, harvesting season, plant part harvested, methods of drying, plant growth stage and extraction method (Gupta *et al.*, 2002). High concentrations of volatile terpenes in leaves and flowers of some plants in genus *Artemisia* gives the plants their distinct pungent and aromatic smell. Plants species like *A. annua, A.*

maritime, A. absinthium, *A. afra*, and *A. scoparia* (Waldst et Kit) are especially high in terpenoids (Hayat *et al.*, 2009; Bora & Sharma, 2011).

2.2.3 *Artemisia annua* L.

2.2.3.1 Botany of *Artemisia annua* L.

One of the very important members of genus *Artemisia* known for its biologically active compounds is *Artemisia annua* L. *A. annua* L. is a highly aromatic annual herb found mainly in Vietnam and China in the Asia region. It is native to Asia and is commonly known as *qinghaousu* in China. This annual wormwood, as it is known in European region, occurs naturally as part of the steppe vegetation in the northern parts of Chahar and Suiyuan provinces (40ºN, 109ºE) in China, at 1000 – 1500 m above sea level (Bouwmeester *et al.*, 2006). The plant naturalizes in many countries such as Argentina, Bulgaria, France, Hungary, Romania, Italy, Spain, the United States and the former Yugoslavia (Klayman, 1989).

Ferreira *et al.* (1995) stated that *A. annua* L. is a short day plant with a critical photoperiod of 13.5 hour. This vigorous weedy annual shrub is usually single stemmed with height reaching about two meter with alternate branches and aromatic leaves which are also alternate ranging from 2.5 to 5.0 cm in length. A noticeable morphology of *A. annua* L. is its tiny yellow nodding flowers (capitula) which is only 2 or 3 mm across and are displayed in lose panicles containing numerous, greenish or yellowish, bisexual central (disc) florets containing little nectar and pistillate marginal (ray) florets.

2.2.3.2 Trichomes of *Artemisia annua* L.

Trichome in plants is identified as key anatomical feature used in plant taxonomy (Duke & Paul, 1993). Plant trichome is defined as protruding plant epidermis or also known as plant appendages that varies in height/weight ratio with multitude appearance which is distinguishable in plant species of Asteraceae family (Kjaer, 2012). There are two distinct biotypes of trichome; glandular secreting trichome (GST) and non glandular trichome (NGT) (Duke, 1994; Duke *et al.*, 1994). Basically both these biotypes of trichomes do not differ from each other except for the absence of epicuticular sac in non glandular trichome (Wagner *et al.*, 2004). NGT are non secreting and have protuberance stalk that appear as hairs, hooks, umbrellas and plates with various roles such as cold/heat insulator, assisting seed dispersal, preventing desiccation and deterring from herbivore (Monteiro *et al.*, 2001; Kjaer, 2012). On the other hand, there are two variation in GST biotype; capitates and peltates. The only difference between these two biotypes is the presence of stalked protuberance in peltates trichome which lacks in capitates trichome (Carpenter, 1999; Hayat *et al.*, 2009).

The presence of trichomes (GST and NGT) and their mechanical and morphological features are very influential in determining the plants ecological distribution and physiological properties (Wagner *et al.*, 2004; Rusydi *et al.*, 2013). Trichome type, size, hair orientation, shape and surface texture are some important structural keys in determining the ability of plants to adapt and survive in arid habitats and harsh environment (Huang *et al.*, 2008). NGT in *A. annua* L. is proposed to be formed at any stage of plant organ maturation and senescence could occur before or after the maturation of plant organ and some are even not shed and remain

on the plant until plant senescence (Ascensao *et al.*, 1997; Wagner *et al.*, 2004; Kjaer, 2012). The live and dead NGT protect the flower bud from pest, assist water absorption and regulate plant temperature (Levin, 1973; Werker *et al.*, 1994).

The surfaces of leaves, floral buds, florets and receptacles of *A. annua* L. bear abundant 10-celled biseriate glandular trichomes (Arsenault *et al.*, 2010). These biseriate glandular trichomes sequester artemisinin as well as other highly aromatic volatile oils (Ferreira & Janick, 2009). Three apical cell pairs signifies the secretory head or also known as epicuticular sac which consists of a pair of secretory cells with non-photosynthetic amoeboid plastids and two pairs of cells with amoeboid chloroplasts indicate the presence of photosynthetic thylakoids (Duke & Paul, 1993; Duke *et al.*, 1994; Wagner *et al.*, 2004). Whereas, the stalk cell plastids contains starch grains and in basal cells, thylakoids are occasionally present (Tellez *et al.*, 1999). These specialized chloroplasts in GST were assisting in artemisinin production. The glandless trichome lacks these specialized chloroplasts hence do not produce artemisinin or its derivatives (Duke & Paul, 1993). The GST was found to be the hub for synthesis, storage and sequester of unwanted products known as secondary metabolites that could be toxic to the plant itself (Levin, 1973). Although NGT lacks secreting sacs, evidence showed the presence of some monoprenoids in cells that were able to function as pest deterrent phytochemicals. Comparative study of NGT and GST biotypes showed that both were capable of synthesis and sequester of secondary metabolites. However, amount and types of secondary metabolites accumulated are much less in glandless biotype compared to glanded biotype. This indicated that glandless trichome was able to sequester some compounds of secondary metabolites which did not include artemisinin and its derivatives (Duke &

Paul, 1993; Wagner *et al,* 2004). Thus, GST (capitates and peltates) of *A. annua* L. is the sole storage, synthesis and sequester site for artemisinin and its derivatives along with other secondary metabolites (Duke, 1994). Similar findings were also reported and confirmed that glandular trichomes contain necessary biosynthetic enzyme for the synthesis of flavonoid, isoprenoid and other terpenes (Tellez *et al.*, 1999).

2.2.3.3 Chemical constituents of *Artemisia annua* L.

Ferreira and Janick (2009) reported that traditionally *A. annua* L. plant was used to treat fevers and hemorrhoids. This plant has been used to alleviate high fevers in traditional Chinese herbal medicine. It was also used in the crafting of aromatic wreaths, as a flavoring for spirits such as vermouth and also as a source of essential oils for the perfume industry (Hu *et al.*, 1993). Woerdenbag *et al.* (1994) stated that the essential oils of *A. annua* L. contain at least 40 volatile compounds and several non-volatile sesquiterpene, of which artemisinin and other derivatives (dihydroartemisinic acid, artemisinic acid and dihydroartemisinic aldehyde) are the most important due to their antimalarial properties. Artemisinin is an important natural sesquiterpene lactone with an internal peroxide moiety which causes antimalarial effect against susceptible and multi-drug resistant *Plasmodium* (Paniego & Guilietti, 1996). The produced artemisinin accumulates mainly in leaves and flowers buds in concentrations that range from 0.01% to 0.86% dry weight (DW) depending on the variety (Paniego & Guilietti, 1996).

A comparative study of essential oil content in glanded and glandless biotype of trichome showed that there were 78 compounds identified in glanded biotype