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RECENT ADVANCES IN OPTIMIZATION AND MAXIMIZATION OF ARTEMISININ YIELD FOR ARTEMISININ BASED COMBINATION THERAPY

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ABSTRACT

According to WHO artemisinin based combination therapy (ACT) is the only reliable treatment option for multidrug resistant *Plasmodium falciparum* malaria and to control the spread of the disease under its global malaria eradication programmes. Artemisinin is a sesquiterpene lactone endoperoxide isolated from *Artemisia annua* plants belonging to family Asteraceae. Artemisinin and its derivatives are found to be effective against schistosomiasis, breast cancer, human lukaemia, colon cancer and lung carcinomas. Low yield of artemisinin in *A. annua* plants (0.01-0.8%, in some strains 1.5%) is a serious limitation to meet the annual targets of the drug (approximately 115-135 tonnes) for ACT treatments. The present review covers the biosynthesis of artemisinin and various approaches to increase artemisinin content in *A. annua* plants and new researches on heterologous expression systems.

KEYWORDS : ***Artemisia annua*, ACT : artemisinin based combination therapy, *Plasmodium falciparum* malaria, heterologous expression**

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INTRODUCTION

Malarial parasite *Plasmodium falciparum* has become resistant to conventional antimalarial drug chloroquine. Resistance is evolving for sulfadoxine- pyrimethamine that replaced chloroquine as antimalarial therapy. Artemisinin and its derivatives naturally formed in *Artemisia annua* plants, have proved to be effective and safe antimalarial agents, exhibiting excellent activities against *Plasmodium falciparum* parasites that are resistant to commonly used antimalarial drugs. Artemisinin based combination therapies (ACTs) involving two or three drugs are recommended by WHO to control multidrug resistant malaria [1].
The following ACTs are currently recommended by WHO for the treatment of uncomplicated falciparum malaria :

Artemether + lumefantrine,
artesunate + amodiaquine,
artesunate + mefloquine,
artesunate + sulfadoxine–pyrimethamine,
dihydroartemisinin + piperaquine.

Artemisinin and its semisynthetic derivatives are believed to kill asexual and sexual intra erythrocytic stages of *Plasmodium* by interacting with the heme discarded by the proteolysis of ingested hemoglobin [2]. Artemisinin and its derivatives are also found to be effective against Schistosomiasis caused by parasite *Schistosoma japonica, S. mansoni* and *S. haematobia* causing 1.0 -1.5 million disabilities each year [3]. Oral administration of artemisinin has also been found to prevent and delay the development of 7, 12-dimethylbenz (a) anthracene (DMBA) induced breast cancer in rat[4]. Artemisinin reacts with iron and forms cytotoxic free radical. It is selectively more toxic to cancer than normal cells because cancer cells contain more intracellular free iron. Covalent tagging of artemisinin to transferrin is reported to increase its selectivity and toxicity towards cancer cells *in vitro*. Artemisinin-transferrin conjugate significantly retarded the growth rate of breast tumors in rat and could be developed into a potent therapeutic agent of cancer in humans [4]. Artemisinin is also reported to be effective against human leukemia, colon cancer and lung carcinomas [5,6].

DISTRIBUTION OF ARTEMISININ IN PLANTS

Artemisia annua L. (annual wormwood, qinghao) belongs to the tribe Anthemideae of the Asteroideae, a subfamily of Asteraceae. The plant is native to China and Vietnam, but now cultivated in many parts of the world. It is mentioned in the Pharmacopoeia of the People's Republic of China[7] and is widely used indigenous medicinal plant, indicated to relieve fever. The ethnopharmacological applications led to the isolation of artemisinin, as the plant's active principle [8]. *A. annua* is an annual determinate shrub and dies after seed set. It is 1.0 - 2.5 m in height, branches are alternately arranged, leaves are aromatic and dissected and contain biseriate glandular trichomes that sequester artemisinin and essential oil. At the end of vegetative growth, capitula arise in loose panicles containing numerous central bisexual florets and marginal pistillate florets. Both florets and receptacle bear abundant biseriate trichomes. Recent evidence indicates that artemisinin is compartmentalized and sequestered in glandular trichomes present in the leaves [9,10] and flowers [11]. Highest levels of artemisinin are found in flowers and leaves. Content of artemisinin is highly variable and ranges from as low as 0.01% upto 1.5% [12] . Maximum artemisinin content occurs at the time of anthesis, when floral glandular trichomes reach physiological maturity. Artemisinin is not detected in roots, seeds and pollens, structures that do not contain trichomes.

A. annua plant | Leaf with trichomes | Inflorescence

ARTEMISININ

Artemisinin is a sesquiterpene (C15) lactone endoperoxide and contains a 1,2,4- trioxane ring structure. The peroxide chemical bond is believed to be essential for its antimalarial activity. Complexation of heme with the peroxide bond followed by electron transfer generates an oxy radical that evolves to theultimate parasiticidal agent [2]. Artemisinin can be quantified by various analytical procedures including thin layer chromatography, gas chromatography, high performance liquid chromatography with ultraviolet or electrochemical detection[13], radioimmunoassay and enzyme linked immunosorbent assay[14]. Artemisinin and its semisynthetic derivatives such as artemether, arteether, artesunate, artelinic acid and dihydroartemisinin are some of the antimalarials semisynthesized from artemisinin. These are being used as monotherapies or as component drugs in ACTs to treat multidrug resistant *P. falciparum* malaria. The total organic synthesis of artemisinin has been achieved[15,16], but is very complicated and low yields are obtained. The plant is still the only valid source of artemisinin[17].

ARTEMISININ BIOSYNTHESIS IN PLANTS

Enzymes of Artemisinin biosynthesis are localized in the two outer apical cells of the glandular secretory trichomes which comprise of ten cells in total [18]. All the terpenes share the common precursors of five carbon (C5) isoprene units. Terpenes are generated by the condensation of two building block isomers, isopentenyl pyrophosphate (IPP) and dimethylallyl diphosphate (DMAPP) [19] . Biosynthesis of IPP and DMAPP takes place by two pathways, ie., mevalonate (MVA) pathway originating from acetyl CoA in the cytoplasm [20] and deoxyxylulose phosphate (DXP)/ methylerythritolphosphate (MEP) pathway originating from glyceric acid -3-phosphate and pyruvate [21] in the plastids. There exist a crosstalk between the MVA pathway operating in the cytosol and DXP/MEP pathway of plastids [19, 22] Three phases of artemisinin biosynthesis are [23,24]:

Isopentenyl pyrophosphate (IPP) is converted to farnesyl diphosphate by the activity of enzyme farnesyl diphosphate synthase. Conversion of farnesyl diphosphate to amorpha 4-11- diene is the first committed step in the biosynthesis of artemisinin .

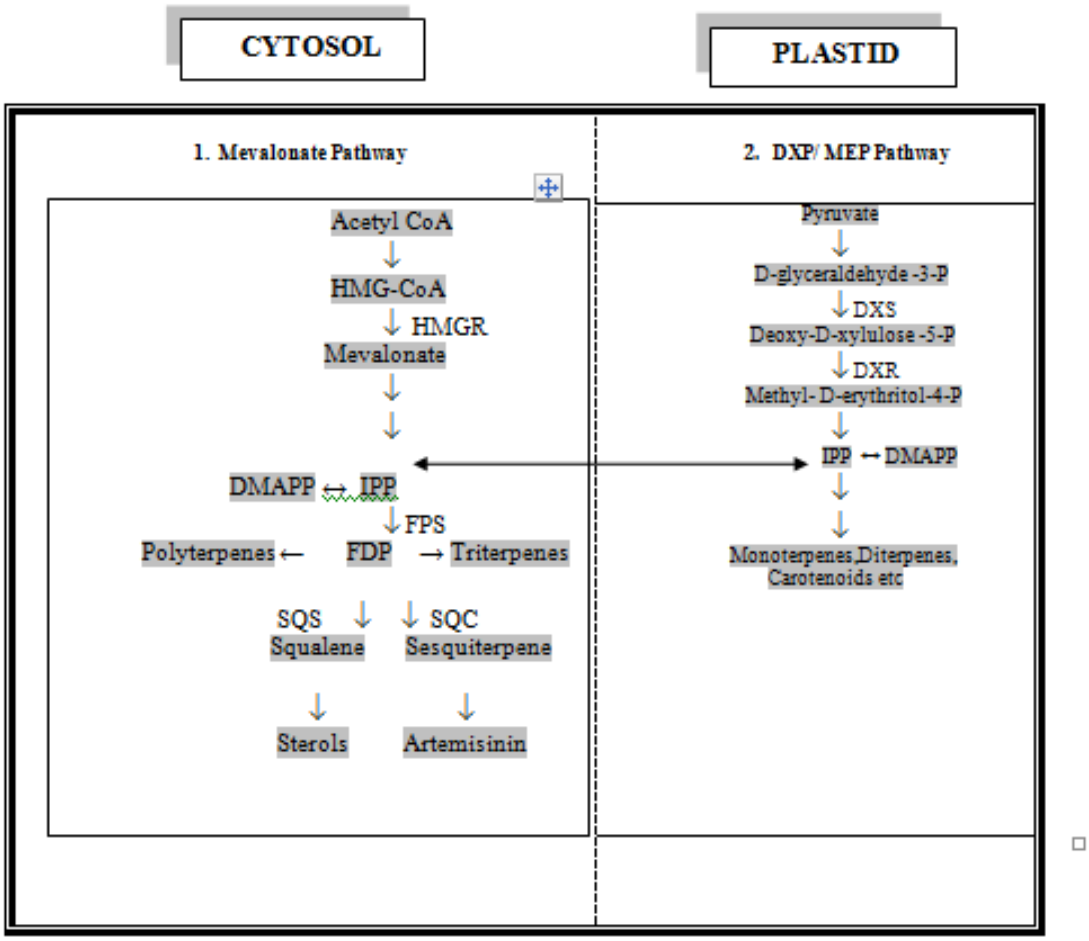
Hydroxylation of amorpha-4,11-diene to artemisinic alcohol is followed by oxidation to artemisinic aldehyde, reduction to dihydroartemisinic aldehyde and then oxidation to dihydroartemisinic acid.

Dihydroartemisinic acid is the true precursor of artemisinin.

Dihydroartemisinic acid forms artemisinin, probably by non enzymatic process within the plant in the presence of light.

TERPENOID BIOSYNTHESIS PATHWAYS

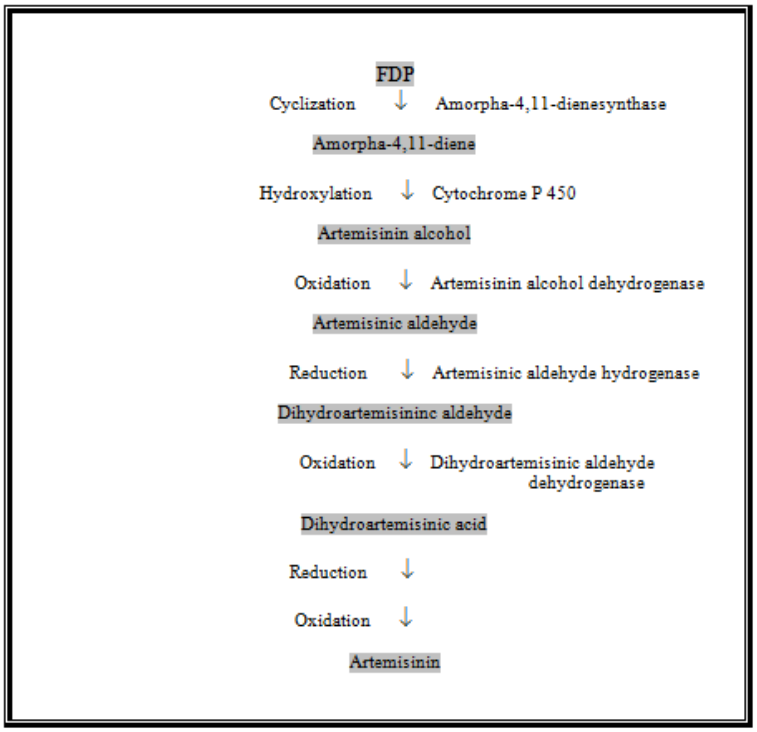
(Adapted from Weathers et.al., 2006 [25])



Some of the regulatory enzymes catalyzing different reactions HMGR, 3-hydroxy-3-methylglutarylcoenzyme A reductase ; FPS, Farnesyl diphosphate synthase; SQC, Sesquiterpene cyclase; SQS, Squalene synthase; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase, IPP, isopentenyl diphosphate; DMAPP, dimethylallyldiphosphate.

PROPOSED PATHWAY FOR THE BIOSYNTHESIS OF ARTEMISININ FROM FARNESYL DIPHOSPHATE (FDP)

(Adapted from Bertea et.al., 2005 [23])



APPROACHES TO INCREASE ARTEMISININ CONTENT

NON TRANSGENIC APPROACH

Efforts have been made to increase the content of valuable drug, artemisinin by improving agronomic practices. Several studies have been published on the formation of artemisinin in *A. annua* plants grown in experimental fields. In order to investigate the possibility of isolating greater amounts of the antimalarial compound artemisinin, plants of *A. annua* were cultivated and analysed at different stages of development. Highest content was found just before flowering and it was also possible to correlate development of the plants with the maximum content of artemisinin[26]. But in Vietnamese plants, highest artemisinin content was found at early developmental stages in five month old plants and not just prior to flowering [27]. The effect of stage of harvest and plant growth regulators on herbage, artemisinin and essential oil yield of *A. annua* were studied and was found that artemisinin content was maximum during early flowering stage of growth and declined at later harvests[26]. Application of GA_3 significantly increased yield and artemisinin content[28]. Population density of 2.22 plants/ hactare yielded 7.4 Kg of artemisinin and 91 Kg of essential oil in North Central India. The plant architecture and canopy structure presumably favored the synthesis and accumulation of artemisinin resulting in artemisinin rich mature plants [29]. High artemisinin producing chemotypes appeared to be associated with tall robust plants with long internodes, open branching, dense leaves and thick stems[30]. Genetic basis of this variation gave evidence of a quantitative inheritance of artemisinin[31]. Additive genetic components were predominant, resulting in a high narrow sense heritability estimate. Thus, good results can be expected from mass selection for the breeding of lines of *A. annua* rich in artemisinin[31]. Dominance variance is also present in the total genetic variability,

indicating that crosses between selected genotypes should generate progenies with particularly high artemisinin content [31]. *Artemisia annua* was introduced in India by Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow and four artemisinin rich cultivars have been developed, namely Jeevanraksha, Jeevanraksha 2, Arogya and Jwarharti, the respective artemisinin content in the young leaves is 0.8, 1.0-1.2 and 1.2%. These cultivars are the polycross products of serial selections [32,33].

Sequencing of transcriptome of *A. annua* was done to identify genes and markers for fast track breeding. Genetic map was prepared with five linkage groups. Replicated field trials resulted in a quantitative trait loci (QTL) map, that accounted for a significant amount of variation in key traits controlling artemisinin yield. Enrichment for positive QTLs in parents of new high yielding hybrids, confirmed that *A. annua* can be developed into an economically promising crop in terms of better artemisinin yield [34]. Selection coupled with *in vitro* cloning should be a promising method for crop and product improvement . Relationship of artemisinin content of tissue cultured, greenhouse grown and field grown plants of *A. annua* was analysed and it was found that artemisinin content of the plants grown under long days in the field was highly correlated with the same clones grown under long days in the greenhouse[35].The high correlation between artemisinin content for greenhouse and field plants indicates that greenhouse evaluation can provide reliable procedure to select high artemisinin containing plants. In the study artemisinin content only weakly correlated with regenerated clones grown in the greenhouse, or even with tissue cultured clones grown two years apart. This suggests that the weak correlation for artemisinin content between tissue culture and greenhouse plants was a tissue culture effect, perhaps due to somaclonal variation. Therefore, selection for artemisinin from tissue culture clone is not advisable[35]. Several tissue culture studies were carried out on *A. annua*[35,36,37] and artemisinin content was found to be highly variable under tissue culture condition, from trace amounts to 0.063%. Although roots do not produce artemisinin, the presence of roots enhances artemisinin production in shoot cultures[38]. However, the role of roots in increasing shoot artemisinin is unclear. The possibility that roots may provide a precursor or promoter of artemisinin production that is translocated to shoots needs investigation.

TRANSGENIC APPROACH

Transformed root and shoot cultures of *A. annua* were established by infection with *Agrobacterium rhizogenes* and *A. tumifaciens*. Transformed hairy root cultures of *A. annua* were obtained by co-culture method using leaf segments of *A. annua* and *A. rhizogenes* strain NCIB 8196 or MAFF 03-01724 [39]. The hairy root clones thus obtained grew vigorously on hormone free medium, showing typical transformed morphology. The genetic transformation of the root was proved by opine assay. Artemisinin concentration in *A. tumifaciens* mediated transformed plant was increased to 3.14 fold (31.4mg/g dry wt) by suppressing the expression of SQS (squalene synthase), a key enzyme of sterol pathway that is competitive to artemisinin biosynthetic pathway. This was done by means of RNA mediated RNAi (RNA interference) technique [40]. The study demonstrates that genetic engineering strategy of RNAi could be an effective method of increasing artemisinin content in the plant. Farnesyl diphosphate synthase (FPS) gene and many more genes of artemisinin biosynthetic pathway such as 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), 1-deoxy-D-xylulose-5-phosphate synthase (DXS), 1-deoxy-d-xylulose-5-phosphate synthase (DXPS) and 1-deoxy-d-xylulose-5-phosphate reductoisomerase (DXPR) have been cloned [41,42,43,44]. Transformed root cultures expressed these genes showing the presence of plastid localized terpenoid biosynthetic pathway [43].

PRODUCTION OF ARTEMISININ USING HETEROLOGOUS SYSTEM

During biosynthesis of artemisinin, sesquiterpene synthases form various sesquiterpenes from farnesyl diphosphate. cDNA clones encoding amorpha-4,11-diene

synthase (ADS), a key enzyme in the artemisinin biosynthesis, and epi-cedrol synthase (ECS), a complex sesquiterpene cyclization synthase, were cloned into Cowpea mosaic virus-based viral vector (pEAQ-*HT*) with Kozak consensus motif and C-terminal histidine tag. The plasmids were transformed into *Agrobacterium* LBA4404 and, agroinfiltrated into *Nicotiana benthamiana* leaves along with vector (pJL3:p19) containing Tomato bushy stunt virus post-transcriptional gene silencing suppressor. The recombinant enzymes ADS and ECS converted farnesyl diphosphate into amorpha-4,11-diene (97 %) and *epi*-cedrol (96 %) [45].

Attempts are in progress to produce artemisinic acid, an intermediate in artemisinin biosynthetic pathway by genetically modifying *Escherichia coli* by transferring the mevalonate pathway genes from *A. annua* to *Escherichia coli*. Synthesis of amorpha -4,11- diene in *E. coli* has been achieved[46,47,48]. Heterologous expression of germacrene A synthase in *E. coli* catalysed the cyclation of farnesyl di phosphate to germacrene A[49]. cDNA clones of epi-cedrol synthase, a sesquiterpene cyclase from *Artemisia annua* L has been isolated. When expressed in *E. coli*, the recombinant epi-cedrol synthase enzyme catalysed the formation of both oleifinic (3%) and oxygenated (97%) sesquiterpenes from farnesyl diphosphate [50]. *Saccharomyces cerevisiae* strains were genetically engineered having mevalonate pathway, amorpha -4,11-diene synthase and a novel cytochrome P 450 monoxygenase (CYP71AV1) from *A. annua.* These engineered yeast strains were capable of producing up to 100 mg/l artemisinic acid . The synthesized artemisinic acid is transported out of the yeast cell and can be purified [51]. Artemisinic acid lacks the endoperoxidebridge, necessary for therapeutic action. Artemisinic acid can be converted chemically to artemisinin in two steps [52]: Reduction of the exocyclic double bond[23] & Photo-sensitized oxidation of the endocyclic double bond to produce the 1,2,4-trioxane ring [24]. By overexpressing every enzyme of the mevalonate pathway in engineered *Saccharomyces cerevisiae* CEN.PK2, production of artemisinic acid was doubled and that of amorpha- 4,11-diene was 10- fold higher than artemisinic acid. By standardization of fermentation process for *Saccharomyces cerevisiae* CEN.PK2, > 40g/L amorpha-4,11-diene was produced which was converted to dihydroartemisinic acid and subsequently to artemisinin by a chemical process [53].

Studies are in progress for the optimization of heterologous expression systems and production of artemisinin precursors for which knowledge of complete biosynthetic pathway and its expression in genetically engineered microbe is required.

CONCLUSION

ACT treatment is needed for up to 500 million *P. falciparum* Malaria cases.

There is currently no licenced vaccine against malaria, *in vivo* artemisinin production is low and cost of chemical synthesis is not economically viable. Researches in the field of plant breeding, tissue culture, transgenics and heterologous systems aim to increase artemisinin content in the plants and to increase the supply of artemisinin and its derivatives for cost effective ACT treatments. Metabolic engineering of microbes like *E. coli* and *Saccharomyces cerevisiae* to produce artemisinin is a novel approach for improving the economics of artemisinin production. Synthetic trioxane molecules with endoperoxidebridge are also being tested for their antimalarial potential. Efforts for maximization and optimization of artemisinin yield in heterologous system and synthetic molecules like trioxanes are in infancy and currently all the artemisinin needed for ACT treatment is obtained from field grown *A. annua* plants. Increase in the yield of artemisinin through various approaches would significantly bring down the cost of Malaria treatment.

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REFERENCES

1 World Malaria Report, Prepared by Roll Back Malaria, World Health Organization, UNICEF (2005).
2 Jefford CW, Why artemisinin and certain synthetic peroxides are potent antimalarials. Current Medicinal Chemistry, 8 (15) : 1803-1826, (2001).
3 Shuhua X, Tanner M, N'Goran EK, Utzinger J, Chollet J, Bergquist R, Minggang C and Jiang Z, Recent investigations of artemether, a novel agent for the prevention of *schistosomiasis japonica, mansoni and haematobia*. Acta Trop, 82:175-181, (2002).
4 Lai H and Singh NP, Oral artemisinin prevents and delays the development of 7,12- dimethylbenz 9a) anthracene (DMBA) induced breast cancer in the rat. Cancer Lett, 231(1) : 43-48, (2006).
5 Efferth T, Dunstan H, Sauerbrey A, Miyachi H and Chitambar GR, The antimalarial artesunate is also effective against cancer. Int J of Oncol, 18: 767-773, (2001).
6 Singh NP and Lai H, Selective toxicity of dihydroartemisinin and holotransferrin towards human breast cancer cells. Life Sci, 70: 49-56, (2001).
7 W Tang and G Eisenbrand , Ed. Chinese drugs of plant origin, Springer-Verlag, Berlin: 159-174, (1992).
8 Klayman DL, Qinghaosu (artemisinin) : an antimalarial drug from China. Science, 228:1049-1055, (1985).
9 Duke SO and Paul RN, Development and the fine structure of glandular trichomes of *Artemisia annua* L. Int J Plant Sci, 154 (1) : 107-118, (1993).
10 Duke MV, Paul RN, El-Sohly HN, Sturtz G and Duke SO, Localization of artemisinin in foliar tissues of glanded and glandless biotypes of *Artemisia annua* L.. Int J Plant Sci, 155: 365-372, (1994).
11 Ferreira JFS and Janick J, Floral morphology of *Artemisia annua* with special reference to trichomes. In J Plant sci, 156 : 807-815,(1995).
12 Abdin MZ, Israr M, Rehman RU, Jain SK, Artemisinin, a novel antimalarial drug: biochemical and molecular approach for enhanced production. Planta Med, 69: 289-299, (2003).
13 Ferreira JFS, Charles DJ, Wood K, Janick J and Simon J, A comparison of GC and HPLC for artemisinin analysis. Phytochem Anal, 5 : 116-120, (1994).
14 Ferreira JFS and Janick J, Immunoquantitative analysis of artemisinin from *A.annua* using polyclonal antibodies. Phytochemistry, 41 : 97-104, (1996).
15 Schmid G and Hofheinz W, Total synthesis of qinghaosu. J Amer Chem Soc,105: 624-625, (1983).
16 Avery MA, Chong WKM and Jennings-White C, Stereo-selective total synthesis of (+) artemisinin, the antimalarial constituent of *Artemisia annua* L. J Am Chem Soc, 114: 974-979, (1992).
17 Woerdenbag HJ, Lugt CB and Pras N *Artemisia annua* L.,a source of novel antimalarial drug. Pharma week Sci, 12: 169-181, (1990).
18 Olsson ME, Olofsson LM, Lindahl AL, Lundgren A, Brodelius M and Brodelius PE, Localization of enzymes of artemisinin biosynthesis to the apical cells of glandular secretory trichomes of *Artemisia annua* L. Phytochemistry, 70 : 1123-1128, (2009).
19 R Croteau , T Kutchan and N Lewis . Natural products (Secondary metabolites). In: Buchanan, B; Gruissem, W; Jones, R. (eds), Biochemistry and molecular biology of plants. American Society of Plant Physiologists,2000, pp. 1250-1318.
20 DE Cane. Sesquiterpene biosynthesis: cyclization mechanisms. In: DE Cane ,

D Barton , K Nakanishi and O Meth Cohn (eds.), Comprehensive natural products chemistry, isoprenoids including carotenoids and steroids, Vol. 2, Elsevier, NY, 1999, pp. 155-200.

21 Newman JD and Chappell J, Isoprenoid biosynthesis in plants: Carbon partitioning within the cytoplasmic pathway. Crit Rev Biochem Mol Biol, 34 : 95-106, (1999).

22 Laule O, Furholz A, Chang H, Szhu T, Wang X, Heifetz PB, Gruissem W and Lange M, Crosstalk between cytosolic plastidial pathways of isoprenoid biosynthesis in *Arabidopsis thaliana*. Proc Natl Acad Sci USA, 100: 6866-6871, (2003).

23 Bertea CM, Freije JR, Van der Woud H, Verstappen FW, Perk L, Marquez V, De Kraker JW, Posthumus MA, Jansen BJM, De Groot AE, Franssen MCR and Bouwmeester HJ, Identification of intermediates and enzymes involved in the early steps of artemisinin biosynthesis in *Artemisia annua.* Planta Med, 71 (1), 40-47,(2005).

24 Brown GD, The biosynthesis of artemisinin (qinghaosu) and the phytochemistry of *Artemisia annua* L. (qinghao). Molecules, 15:7603-7698, (2010).

25 Weathers PJ, Elkholy S and Wobbe KK, Artemisinin: The biosynthetic pathway and its regulation in *Artemisia annua*, a terpenoid rich species. In Vitro Cellular and Developmental Biology, 42 (4) : 309-317, (2006).

26 Liersch R, Soicke H, Stehr C and Tullner HU, Formation of artemisinin in *Artemisia annua* during one vegetation period. Planta Med, 53: 387-390, (1986).

27 Woerdenbag HJ, Pras N, Chan NG, Bang BT and Bos R, Artemisinin related sesquiterpenes and essential oil in *Artemisia annua* during a vegetative period in Vietnam. Planta Med, 60: 272-275, (1994).

28 Farooqi AHA, Shukla A, Sharma S and Khan A, Effect of plant age and GA_3 on artemisinin and essential oil in *Artemisia annua* L. J. of Herbs, Spices and Medicinal Plants. 4 (1): 73-80, (1996).

29 Muni Ram, Gupta MM, Dwivedi, S and Kumar S, Effect of plant density on the yields of artemisinin and essential oil in *Artemisia annua* cropped under low input cost management in North Central India. Planta Medica, 63 (4): 372- 374, (1997).

30 Elhag HM, El-Domiaty MM, El-Feraly FS, Mossa JS and El-Olemy MM, Selection and micropropagation of high artemisinin producing clones of *Artemisia annua* L. Phytotherapy Research, 6 (1) : 20-24, (1992).

31 Delabays N, Simonnet X, Gaudin M, The gentics of artemisinin content in *Artemisia annua* L. and breeding of high yielding cultivars. Current Medicinal Chemistry, 8 (15) : 1795-1801, (2001).

32 Kumar S, Banerjee S, Dwivedi S, Gupta MM, Registration of Jeevanraksha and Suraksha varieties of the antimalarial medicinal plant *Artemisia annua*. J Med Arom Plant Sci, 21: 47-48, (1999).

33 Kumar S and Srivastava S, Establishment of artemisinin combination therapy as first line treatment for combating malaria: *Artemisia annua* cultivation in India needed for providing sustainable supply chain. Current Sci, 89 (7) : 1097-1102, (2005) .

34 Graham IA, Besser K, Blumer S, Branigan CA, Czechowski T, Elias L, Guterman I, Harvey D, Isaac PG, Khan AM, Larson TR, Li Y, Pawson T, Penfield T, Rae AM, Rathbone DA and Reid S,The genetic map of *Artemisia annua* L. identifies loci affecting yield of the antimalarial drug artemisinin .Science, 327 (5963): 328-331, (2010).

35 Ferreira JFS, Simon JE and Janick J, Relationship of artemisinin content of tissue cultured, greenhouse grown and field grown plants of *Artemisia annua*. Planta med, 61 (4): 351-355, (1995).

36 Pras N, Visser JF, Batterman S, Woerdenbag HJ, Malingre TM and Lugt CB, Laboratory selection of *Artemisia annua* L. for high yielding types. Phytochem Anal, 2 :80-83, (1991).

37 Sharma G, Shankar V and Agrawal V, An efficient micropropagation protocol of an elite clone EC- 353508 of *Artemisia annua* L., an important antimalarial plant. International J of Pharma and Biosciences. 2(4) : 205-214, (2011).

38 Ferreira JFS and Janick J, Roots as an enhancing factor for the production of artemisinin in shoot cultures of Artemisia annua L. Plant, Cell, Tissue and Organ Culture , 44: 211-217, (1996).

39 Jaziri M, Shimomura K, Yoshimatsu K, Fauconnier ML, Marlier M and Homes J, Establishment of normal and transformed root cultures of *Artemisia annua* L. for artemisinin production. J Plant Physiol, 145 : 175-177, (1995).

40 Zhang L, Jing F, Li F, Li M, Wang Y, Wang G, Sun X and Tang K, Development of transgenic *Artemisia annua* (Chinese wormwood) plants with enhanced content of artemisinin, an effective antimalarial drug, by hairpin RNA mediated gene silencing. Biotechnol Appl Biochem, 52: 199-207, (2009).

41 Matsushita Y, Kang W, Charlwood BV, Cloning and analysis of cDNA encoding farnesyl diphosphate synthase from *Artemisia annua*. Gene, 172 (2) : 207-209, (1996).

42 Chen D, Liu C, Ye H, Li G, Liu B, Meng Y and Chen X, Ri mediated transformation of *Artemisia annua* with a recombinant farnesyl diphosphate synthase gene for artemisinin production. Plant Sci 155: 179-185, (2000).

43 Souret F, Weathers PJ and Wobbe KK,The mevalonate independent pathway is expressed in transformed roots of *Artemisia annua* and regulated by light and culture age. In Vitro Cell Dev Biol Plant, 38 : 581-588, (2002).

44 Souret FF, Kim Y, Wyslouzel BE, Wobbe KK and Weathers PJ, Scale up of *Artemisia annua* L. hairy root cultures, produces complex patterns of terpenoid gene expression. Biotechnol Bioeng, 83 : 653-667, (2003).

45 Kanagarajan S, Muthusamy S, Gliszczynska A, Lundgren A and Brodelius PE, Functional expression and characterization of sesquiterpene synthases from *Artemisia annua* L. using transient expression system in *Nicotiana benthamiana.* Plant Cell Reports, 31(7), 1309-1319, (2012).

46 Picaud S, Olofsson L, Brodelius M and Brodelius PE, Expression, purification and characterization of amorpha-4,11-diene synthase from *Artemisia annua* L. Arch Biochem Biophys, 436 : 215-226, (2005).

47 Martin VJ, Pitera DJ, Wither ST, Newman JD and Keisling JD, Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. Nature Biotechnol, 21: 796-802, (2003).

48 Mercke P, Bengtsson M, Bouwmeester HJ, Posthumus BA and Brodelius PE, Molecular cloning, expression and characterization of amorpha-4,11-diene synthase, a key enzyme of artemisinin biosynthesis in *Artemisia annua* L. Arch Biochem Biophys, 15; 381(2): 173-180, (2000).

49 Bertea CM, Voster A, Verstappen FW, Maffei M, Beekwilder J and Bouwmeester HJ, Isoprenoid biosynthesis in Artemisia annua : cloning and heterologous expression of germacrene A synthase from a grandular trichome cDNA library. Arch Biochem Biophys, 448 (1-2): 3-12, (2006).

50 Mercke P, Crock J, Croteau R and Brodelius PE, Cloning, expression, characterization of epi-cedrol synthase, a sesquiterpene cyclase from *Artemisia annua* L. Arch Biochem Biophys, 369 (2): 213-222, (1999).

51 Ro DK, Paradise EM, Ouellet M, Fischer KJ, Newman KL, Ndungu JM, Ho KA, Eachus RA, Ham TS, Kirby J, Chang MC, Withers ST, Shiba Y, Sarpong R and Keisling JD, Production of the antimalarial drug precursor artemisinin acid in engineered yeast. Nature, 440: 940-943, (2006).

52 Roth RJ and Acton N, Simple conversion of artemisinic acid into

artemisinin. J Nat Prod, 52 : 1183-1185, (1989).

53 Westfall PJ, Pitera DJ, Lenihan JR, Eng D, Woolard FX, Regentin R, Horning T, Tsuruta H, Melis DJ, Owens A, Fickes S, Diola D, Benjamin KR, Keasling JD, Leavell MD, McPhee DJ, Renninger NS, Newman, JD and Paddon CJ, Production of amorpha diene in yeast, and its conversion to dihydroartemisinic acid, precursor to the antimalarial agent artemisinin. Proc Natl Acad Sci USA, 109 (3) : 111-118, (2012).