Influence of spacing, pruning and drying methods on concentration of artemisinin in *Artemisia annua*

by

Mary Leann Maphoto

MINI-DISSERTATION
Submitted in (partial) fulfilment of the
requirements for the degree of

Master of Science

in

Agriculture (Horticulture)

in the

FACULTY OF SCIENCE AND AGRICULTURE
(**School of Agricultural and Environmental Sciences**)

at the

UNIVERSITY OF LIMPOPO

Supervisor : **Dr H.A. Abdelgadir (ARC)**

Co-Supervisor : **Dr A.R. Ndhlala (ARC)**

Co-Supervisor : **Prof P.W. Mashela (UL)**

2017

TABLE OF CONTENTS

DECLARATION

I declare that the mini-dissertation hereby submitted to the University of Limpopo, for the degree Master of Science in Agriculture (Horticulture) has not previously been submitted by me for a degree at this or any other University; that it is my work in design and in execution, and that all material contained herein had been duly acknowledged.

Maphoto, ML (Mrs) **Date**

DEDICATION

To my beloved husband and my son, Favour.

ACKNOWLEDGEMENTS

I would love to pass my sincere gratitude to the Agricultural Research Council (ARC) - Professional Development Programme (PDP), National Treasury Economic Support Competitive Packages (ESCP) and the Land Bank Chair of Agriculture ─ University of Limpopo, for financial support which sustained and materialised this project. Unwavering gratitude to my supervisors Dr H.A. Abdelgadir, Dr A.R. Ndhlala and Prof P.W. Mashela, you have taught me a lot, contributed greatly in building me in various areas and brought out the best in me. I have learnt so much in research even in disciplines that were not of my field of study. I would also love to appreciate the ARC-PDP and Department of Science and Technology (DST) for sponsoring me to present my MSc study at the 42nd Joint South African Association of Botanists (SAAB) - Southern African Society for Systematic Biology (SASSB) conference in January 2016 that was held in Free State, Bloemfontein. I also thank The ARC-PDP for allowing me to present my MSc study and awarding me for the best MSc presentation in 2015. I would also love to acknowledge my colleagues from Green Technologies Research Centre, University of Limpopo and the Agricultural Research Council – Vegetable and Ornamental Plants (ARC-VOP); you created the best working environment and continuously gave me support. Special appreciation to my husband, mom (Grace) for your undying support and my entire family. My sincere gratitude to the Almighty God through the Lord Jesus Christ who has enabled me to work hard and complete this project.

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ABSTRACT

Artemisia annua L. from the family Asteraceae is an annual medicinal plant and has been used to make herbal remedies in Asia for thousands of years. Artemisinin is a sesquiterpene lactone, isolated from aerial parts of *Artemisia annua*, with the highest concentrations being in flowers and leaves. In addition to potent anti-malarial activity, artemisinin possesses anti-cancer, anti-schistosomiatic, anti-hepatitis B, anti-HIV, anti-leishmanial and herbicidal activities. Low artemisinin production (0.01-2%) from *A. annua* is a major constraint in commercialisation of the drug for control of malaria. Worldwide, efforts have been underway to improve the concentration of artemisinin using conventional breeding, biochemical, physiological, molecular and hairy-root culture techniques, however all these methods are not economical. Cultural practices like spacing and pruning have limitation in improving artemisinin concentration and these may help in increasing the concentrations of artemisinin. Study was conducted at the experimental farm of the Agricultural Research Council – Vegetable and Ornamental Plants, Roodeplaat Pretoria. The objective of this study was to determine whether spacing, pruning and their interactions would have any effect on the concentrations of artemisinin, growth and yield of *A. annua* and whether drying methods would have an effect on the concentrations of artemisinin in *A. annua.* Since there was only one field trial, all sub-objectives were addressed at once (Chapter 3). Fresh seeds of *A. annua* were obtained from the ARC-VOP gene bank and sown in seedling trays in September 2014. Uniform eight-week-old seedlings were hardened-off, transplanted in November 2014 in 10 cm deep holes and then pruned ten weeks after transplanting. Treatments for Experiment 1, *viz.*, 3 × 4 factorial experiment were laid out in a randomised complete block design, with four replications (n = 48). The two factors of the experiment were (a) spacing [0.5 × 1 m^2

(standard: 0.50 m^2), 0.5 × 0.7 m^2 (small: 0.35 m^2) 0.5 × 0.5 m^2 (smaller: 0.25 m^2) and 0.3 × 0.7 m^2 (smallest: 0.21 m^2)] and (b) pruning [no pruning (control), removing the apical bud and removing shoots three nodes from the bottom]. The plants were irrigated using overhead sprinklers system for two hours three times per week. Four readings for growth variables (plant height, stem diameter and chlorophyll content) were collected with one week interval. Plants were harvested after 180 days from planting, and leaves, stems and roots were separated weighed and oven dried at 40 ºC for 72 h. In Experiment 2 (drying methods), treatments, namely, 100% sun, 100% shade, 50% shade, freeze and oven drying were arranged in completely randomised design with four replicates (*n* = 20). The treatments were exposed for a week, to full sunlight, 50% shade-drying under a shade net that allows 50% light penetration, 100% shade under enclosed room at ambient (24-25 ºC) temperature, oven drying for 24 h at 40 ºC, and freeze-drying for three days. Freeze-drying had significant effect on artemisinin concentration of 1.941%. It was followed by oven (1.738%) and 100% shade drying (1.657%) and the lowest artemisinin concentration (1.412%) was obtained from 50% shade drying. The smaller spacing of 0.25 m^2 in combination with apical bud removal had a significant effect on artemisinin concentration, producing artemisinin concentration of 0.193%. Spacing had a significant effect on stem diameter, fresh leaf mass and dry leaf mass but had no effect on plant height and chlorophyll content. Pruning had a significant effect on plant height and chlorophyll content and had no effect on stem diameter. The small spacing of 0.35 m^2 had the highest fresh and dry leaf mass of 17.99 and 9.62 t/ha. The interaction of spacing and pruning had no significant effect on the growth and yield of *A. annua.* The results from this study suggested that cultural and processing practices may have direct effects in the concentration of artemisinin, growth and yield of *A. annua*. The results

provided some understanding on how agronomic and processing practices can be used to increase artemisinin content in *A. annua* and understand the interaction between different agronomic practices and thereby allowing the development of economic methods for *A. annua* post-harvest handling. Future work should focus on implementing various pruning techniques to trigger stress and indirectly secondary metabolites.

CHAPTER 1

RESEARCH PROBLEM

1.1 Background

Artemisinin is a sesquiterpene lactone, isolated from aerial parts of *Artemisia annua*, with the highest concentrations being in flowers and leaves (Abdin *et al.,* 2003). Artemisinin, along with its derivatives, is a relatively safe drug with multiple anti-microbial activities, without adverse effects even when consumed by pregnant women (WHO, 2003). In addition to potent anti-malarial activity, artemisinin possesses anti-cancer (Efferth *et al.,* 2001), herbicidal (Chen and Zhang, 1987; Duke *et al*., 1987), anti-hepatitis B (Romero *et al*., 2005), anti-HIV (Jung and Schinazi, 1994), anti-leishmanial (Sen *et al*., 2007) and anti-schistosomiatic activities (Bormann *et al.,* 2001). Consequently, artemisinin is of great interest in various medicinal industries.

1.1.1 Description of the research problem

Low artemisinin production (0.01-2%) from *A. annua* is a major constraint in commercialisation of the drug for control of malaria (Abdin *et al*., 2003; Bertea *et a*l., 2005; Debrunner *et al*., 1996; De Ridder *et al*., 2008). Worldwide, efforts have been underway to improve the concentration of artemisinin using conventional breeding, biochemical, physiological, molecular and hairy-root culture techniques, however they are not economical (Aquil *et al.,* 2009; Dong and Thuan, 2003; Newman *et al*., 2006; Ro *et al*., 2006; Weathers *et al*., 2005; Zeng *et al*., 2007; Zhang *et al.,* 2009).

1.1.2 Impact of the research problem

Globally, artemisinin is widely used in areas where malaria is still a major health problem (Newton and White, 1999; Snow *et al.,* 2005). Currently, artemisinin-based combination therapies (ACTs) are considered the best therapeutic combination against the drug-resistant and cerebral malaria-causing parasite *Plasmodium falciparum* (Newton and White, 1999). The demand for ACTs increased enormously in 2004 and was expected to continue to grow due to lack of its synthetic substitutes (WHO, 2005a; WHO, 2005b). In 2005, WHO (2005a; 2005b) indicated that demand for ACTs increased from 2 million treatment courses in 2003 to 30 million in 2004, an estimated 70 million in 2005, and a projected 130 million in 2006. The number of ACT treatments utilized by the public and private sectors in endemic countries between 2005 and 2013 was 392 million. Artemisinin based combination therapies (ACTs) have been adopted by 56 countries in Africa, Asia and South America as their first or second line antimalarial treatment. This is done wherever the common quinoline and sulphadoxine pyrimethamine based drugs are no longer effective thus increase in demand of ACTs (Olliaro and Taylor, 2004).

1.1.3 Possible causes of the research problem

Plant extracts from *A. annua* remain the sole economical resource for artemisinin. Efforts and attempts to chemically synthesise artemisinin have not been successful. Primarily, there are high production costs which makes all these efforts not economically viable (Aquil *et al*., 2009; Dong and Thuan, 2003; Haynes, 2006; Newman *et al*., 2006; Weathers *et al*., 2005).

1.1.4 Proposed solutions

Photosynthesis is a key supplier of carbon and energy and therefore plays a vital role in the biosynthesis of terpenoids (Singh *et al.,* 1990). Majority of the attempted approaches were not focused on implementing cultural practices like spacing and pruning as mechanisms through which artemisinin could be stimulated and produced as secondary metabolites (Aquil *et al*., 2009; Dong and Thuang, 2003; Newman *et al.,* 2006; Ro *et al*., 2006; Weathers *et al*., 2005; Zeng *et al.*, 2007; Zhang *et al*., 2009). Coupling appropriate cultural practices with high-producing hybrids that promote the accumulation of photosynthates while increasing respiration rates may increase the concentrations of artemisinin.

1.2 Problem statement

The commercialisation of products from *A. annua* is limited by low concentrations (< 2%) of artemisinin. Artemisinin is associated with high respiration rates since the end-product of glycolysis, namely, acetyl CoA, is required to initiate biosynthesis of this material, while acetyl CoA is also required to drive the Krebs cycle. Consequently, any cultural practice which increases respiration and thereby the end-products of glycolysis will inevitably increase concentration of acetyl CoA and by implication, improve the concentration of artemisinin.

1.3 Rationale of the study

Biosynthesis of artemisinin is closely linked with the availability of acetyl CoA at the end of glycolysis during respiration. Coupling appropriate cultural practices that promote the accumulation of photosynthates while increasing respiration rates may

increase the concentrations of artemisinin. The identification of such cultural practices could help in increasing the concentrations of artemisinin.

1.4 Purpose of the study

1.4.1 Aim

The aim of the study was to investigate the effect of cultural practices such as spacing, pruning and drying method on artemisinin concentration in *A. annua.*

1.4.2 Objective

The objective of this study was to determine whether spacing, pruning and their interactions would have any effect on the concentrations of artemisinin, growth and yield of *A. annua* and whether drying methods would have an effect on the concentrations of artemisinin in *A. annua*.

1.4.3 Hypothesis

Spacing, pruning and their interactions would have an effect on the concentrations of artemisinin, growth and yield of *A. annua* and drying methods would have an effect on the concentrations of artemisinin in *A. annua*.

1.5 Reliability, validity and objectivity

In this study, reliability of data were based on statistical analysis of data at the probability level of 5%. Validity was achieved through repeating the experiments in time. Objectivity was achieved by ensuring that the findings are discussed on the basis of empirical evidence, thereby eliminating all forms of subjectivity (Leedy and Ormrod, 2005).

1.6 Bias

Bias was minimised by ensuring that the experimental error in each experiment was reduced through adequate replications. Also, treatments were assigned randomly within the selected research designs (Leedy and Ormrod, 2005).

1.7 Format of mini-dissertation

The mini-dissertation was designed using the Senate-approved technical format of the University of Limpopo. Following the description of the research problem (Chapter 1), the work done and not yet done on the research problem was reviewed (Chapter 2). Since there was only one field trial, all sub-objectives were addressed at once (Chapter 3). Finally, findings would be summarised and integrated to provide the significance of the findings, recommendations with respect to future research and culminated in conclusions (Chapter 4). The Harvard style of citing and referencing was used.

CHAPTER 2

LITERATURE REVIEW

2.1 Work done on artemisinin

2.1.1 Introduction

Medicinal plants are of great importance to human and animal health throughout the world. This is most especially in developing countries due to their availability, affordability and cultural acceptance (Abbiw *et al*., 2002; Brown, 1994). Traditional medical practitioners are also being recognised and there are efforts being made to integrate western and indigenous medicine (Brown, 1994). *Artemisia* is a genus of aromatic small herbs or shrubs spreading in northern temperate zones. It is one of the largest and most widely distributed genera of the Asteraceae family and consists of more than 500 species (Bora and Sharma, 2011). This genus has always been of great botanical and pharmaceutical interest (Teixeira da Silva, 2004; Willcox, 2009).

Many of *Artemisia* species have long been used in traditional medicines for the treatment of a variety of diseases and complaints as antiseptic, antiatherogenic, antihelmintic, tonic, diuretic, antioxidant, anti-inflammatory, bacteriostatic, or fungistatic agents in countries like China, Japan, South Korea, and Turkey *(*Klayman, 1993; Lachenmeier, 2010; Tadesse, 2004; van Wyk and Wink, 2004*). Artemisia* species are frequently utilized for the treatment of diseases such a malaria, hepatitis, cancer, inflammation and infections by fungi, bacteria and viruses (Willcox, 2009). *Artemisia afra (Lengana* in *Setswana, Umhlonyane* in *isiZulu* is found in South Africa and is widely distributed in Eastern Cape, KwaZulu-Natal, Mpumalanga, Free State, Gauteng, North West and Limpopo Provinces and Cederberg mountains in the Western Cape. The plant is currently cultivated in Eastern Cape and Limpopo

Provinces and it's of great interest in the pharmaceutical and perfumery industry. *Artemisia annua* L. is an annual medicinal plant. The common names of the plant are Qinghao (which means green herb), wormwood, Chinese wormwood, sweet wormwood, annual wormwood, annual sagewort, annual mugwort, and sweet sagewort (Ferreira *et al*., 1997). The plant is native to China but had since been naturalised in many countries like Argentina, Bulgaria, France, Hungary, Romania, Italy, Spain, the United States, Brazil, Cameroon, Ethiopia, India, Kenya, Mozambique, Myanmar, Tanzania, Thailand, Uganda and Zambia (Ferreira and Janick, 1996; Ozguven *et al*., 2008). The plant can grow up to 3 m tall; its stem is cylindrical and branched. The leaves are fern-like, alternate, dark green, or brownish green and flowers are small (1–2 mm) pale yellow. Odor is distinctive and aromatic whereas the taste is bitter. The seeds are small and oval in shape (Ferreira *et al*., 1997).

Artemisia annua has been used as a herbal medicine in Asia for thousands of years (Ferreira and Janick, 1996*;* Klayman, 1993; Lachenmeier, 2010 and Tadesse, 2004). The plant is used to treat skin infections, itchy scabs, malign sores and killing lice. Various sources have also described other effects of *A. annua* products as antiseptic, digestive and febrifuge. An infusion of the leaves is used internally to treat fevers, colds and diarrhea. Leaves are put onto nosebleeds, boils, abscesses, contraception, relief of joint pains, deworming, hemorrhoids, immune boosting and cancer treatment. Essentials oils derived from *A. annua* are used in cosmetics and aromatherapy (Muzemil, 2008).

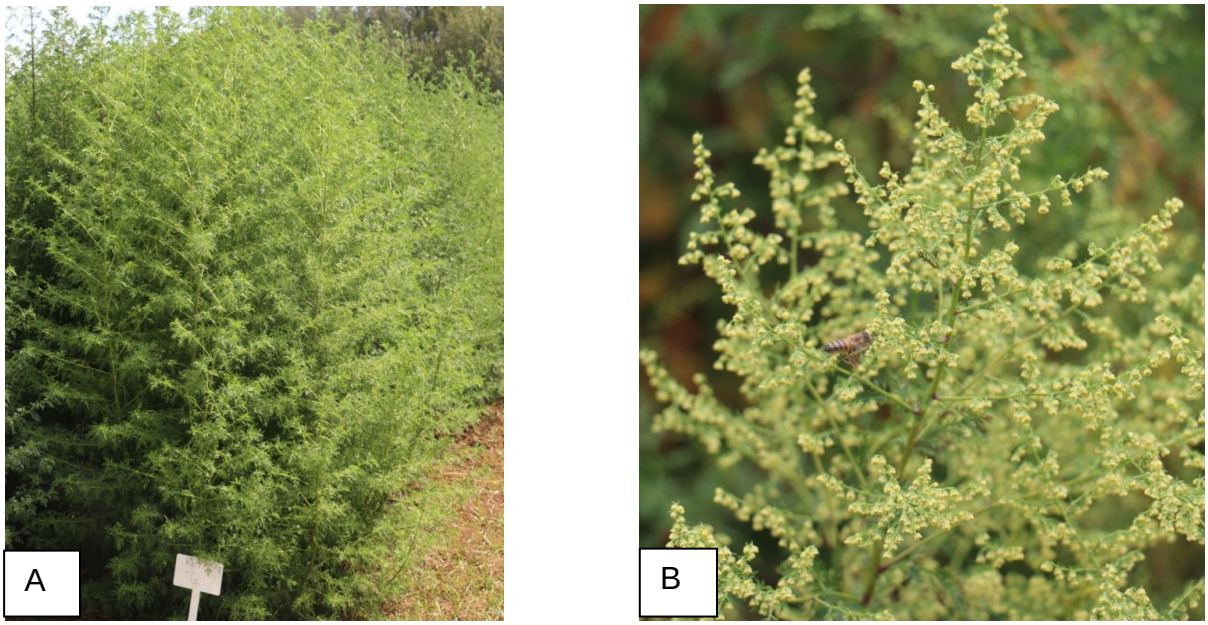

Figure 2.1 *Artemisia annua* plant (A) and *Artemisia annua* plant with flowers (B).

2.1.2 Biosynthesis of artemisinin

The first step in the biosynthesis of artemisinin is the cyclisation of farnesyl diphosphate (FDP) by amorphadiene synthase to amorpha-4, 11-diene at the end of glycolysis in respiration (Bouwmeester *et al*., 1999). Artemisinic acid is required as intermediates in the formation of artemisinin (Abdin *et al.,* 2003; Wallaart *et al*., 1999). However, information on enzymes involved in the conversion of amorpha-4, 11-diene to artemisinic acid is scant (Bertea *et al*., 2005).

Modification of amorpha-4, 11-diene carbon skeleton to produce artemisinic acid involves a cytochrome P450 enzyme, resulting in the production of artemisinic alcohol that could further be oxidised twice by either cytochrome P450 enzymes or dehydrogenases to yield the intermediate acids (Bouwmeester *et al*., 1999). The C11–C13 double bond of artemisinic acid is thought to be reduced to yield artemisinic acid, which is converted non-enzymatically to artemisinin (Figure 2.2) (Abdin *et al*., 2003; Wallaart *et al*., 1999; Weathers *et al*., 2011).

.

.

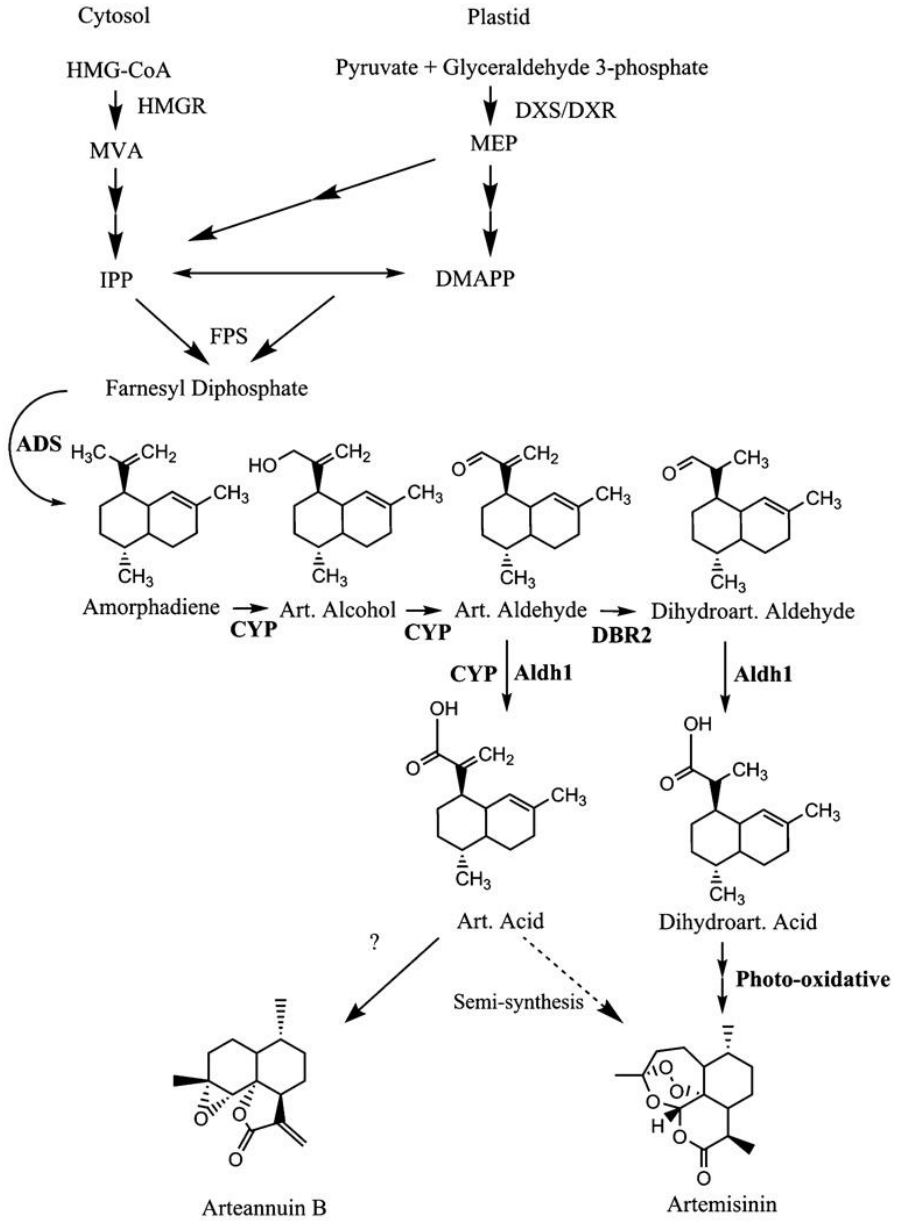

Figure 2.2 Artemisinin structure and simplified biosynthetic pathway. (ADS) amorphadiene synthase, (Aldh1) aldehyde dehydrogenase 1, (CYP CYP71AV1), (DBR2) double bond reductase 2, (DMAPP) dimethylallyl diphosphate, (DXS 1)deoxyxylulose 5-phosphate synthase, (DXR) 1-deoxyxylulouse 5-phosphate reductoisomerase, (HMGR) 3-hydroxy- 3-methylglutaryl-CoA reductase, (IPP) isopentenyl diphosphate, (MEP) methyl erythritol phosphate, (MVA) mevalonic acid (Weathers *et al*., 2011).

2.1.3 Effect of cultivation methods on artemisinin

Crop density and spacing: Previous studies have shown that *A. annua* yield of 1 - 4 t/ha of dried leaf was obtained from low density of 1 plant/m^2 (Maynard, 1985; WHO, 1988) and 2.5 plants/m^2 (Delabays *et al*., 1993). Highest biomass was obtained at the highest density in Indiana, USA, when comparing 3, 7 and 11 plants/m^2 (Simon *et al*., 1990). In another study 1, 5, 10, 15 and 20 plants/m^2 were compared from a Yugoslavian strain in Tasmania, Australia and found that leaf dry matter yield increased up to a density of 20 plants/m^2. Maximum dry leaf yield of 6.8 t/ha was obtained from 10 plants/m^2 however, plant population density had no effect on the concentration of artemisinin (Laughlin, 1993). In Vietnam maximum leaf dry matter yield of 5.3 t/ha was obtained from a high density of 25 plants/m^2 (Woerdenbag *et al*., 1994). Ram *et al*. (1997) recommended that *A. annua* should be cultivated at a high plant density of about 22 plants/m^2.

Artemisia annua planted at the research farm of Centre for Aromatic Plant (HRDI), Selaqui, Dehradun, during 2006/2007 at the spacing of 30 × 45 cm^2 significantly produced maximum dry leaves (3.38 t/ha) as well as oil yield (168.9 kg ha^{-1}) as compared to other treatments of 30 × 60, 45 × 45, 45 × 60 and 60 × 60 cm^2 (Chauhan *et al*., 2008). Uniyal *et al.* (2010) in an experiment of different spacing (45 × 45, 45 × 60, 60 × 30, 60 × 45 and 60 × 60 cm^2) and time of harvesting (0600 h, 1200 h and 1800 h) on vegetative growth and oil yield of *A. annua* found that the growth variables and oil yield per plant were optimum at low plant density (60 × 60 cm^2). Biomass and oil yield per plot were maximum at higher plant density (30 × 60 cm^2).

Abirami *et al.* (2014) reported that no significant differences were observed in artemisinin contents and essential oil content among the different spacing but the yields of essential oil (54.95 kg/ha) and artemisinin (8.88 kg/ha) were found maximum in the spacing of 45 × 45 cm^2. However, they found significant differences in fresh (30.79 t/ha) and dry (18.35 t/ha) leaf yield.

Pruning: Kumar *et al*. (2004) found that multiple harvesting led to higher yield of artemisinin due to high levels of leaf yield accompanied by increased stem and shoot mass. Crop regeneration and production of new branches resulted from intermittent harvesting and led to high artemisinin. It was further observed that high artemisinin concentration occurred in *A. annua* plants which were multi harvested than those harvested once, twice or thrice (Kumar *et al*., 2004). The increase in artemisinin may be due to preservation of photosynthates, redirection to newer leaves and avoidance of losses due to senescence from old branches (Kumar *et al*., 2004).

Growth stage and plant organ: *Artemisia annua* growth cycle comprises of six growth stages namely the seedling, branching, flower-budding, flowering, fruiting and withering phases. Growth period varies with seed source, cultivation techniques and environmental factors (WHO, 2006). Artemisinin content was found to vary per plant part and has been reported to accumulate more in leaves, buds, flowers and seeds (Acton *et al.,* 1985; Liersch *et al.,* 1986; Madhusudanan, 1989; Martinez and Staba, 1988; Zhao and Zeng, 1985). It was found higher in inflorescence than in leaves (Ferreira *et al*., 1997). The main stems and roots contain little to none artemisinin (Woerdenbag *et al*., 1991). Artemisinin content also varies per growth stage and it was found to be highest just before flowering (Acton *et al.,* 1985; Liersch *et al.,* 1986;

Woerdenbag *et al*., 1991; Woerdenbag *et al*., 1993) and at full flowering (Pras *et al*., 1991; Singh *et al*., 1988).

Drying methods: According to Laughlin *et al*. (2002) leaves that were oven dried immediately after harvest and those from whole plants subjected to direct sunlight for 7 days under field conditions had similarities in artemisinin concentration. However, those leaves that were left for 21 days under open shade drying or dried in dark containers and direct sun drying had high artemisinin concentration than the leaves that were oven dried immediately after harvest (Laughlin *et al.,* 2002). However, Charles *et al.* (1993) found that drying of *A. annua under* ambient conditions inside or outside resulted in high artemisinin concentration than sun drying. Shade drying had 30% more artemisinin than oven drying at 40 ºC (Ferreira *et al*., 1992).

2.1.4 Influence of environmental factors on artemisinin

Light: Both quality and intensity of light influence the rate of photosynthesis (Campbell, 1990). As the intensity of light increases, the rate of photosynthesis increases (Taiz and Zeiger, 2006). Photosynthesis is sensitive to higher temperatures because the enzymes involved are denatured at higher temperatures (Taiz and Zeiger, 2006). The biomass and leaf mass, plant height and stem diameter along with artemisinin content of *A. annua* decreased as growth irradiance decreased, but increased as irradiance increased (Hoeft *et al*., 1996; Stuefer and Huber, 1998). Plants exposed to 100% sunlight yielded more artemisinin content (Laughlin *et al*., 2002), suggesting that increased photosynthesis rates could be associated with improved artemisinin content.

Photoperiod: *Artemisia annua* clones subjected to 16 h photoperiod for 55 days when transferred to short photoperiods (8, 10 and 12 h), flowered after two weeks, while those under long photoperiods (16, 20, 24 h) remained vegetative (Ferreira *et al*., 1995). Artemisinin content was found to be higher in plants maintained under short photoperiods at week 6, 8 and 10, but was not significantly higher for week 8. Long photoperiod increased artemisinin content in week 8 than the short photoperiod (Ferreira *et al*., 1995). This observation suggests that increase/decrease in artemisinin content was directly linked to the photoperiod and time of harvesting.

Temperature: High temperature increases the rate of photorespiration in C_3 plants, through making use of the photosynthates produced during photosynthesis (Taiz and Zeiger, 2006). This might indirectly decrease artemisinin content in *A. annua*. Generally, photorespiration rates are high under hot, dry and bright days (Campbell, 1990). In *A. annua*, high temperatures reduce artemisinin content (Ferreira *et al*., 1995), which is in line with the view that high levels of photosynthates are required for the accumulation of high artemisinin content. Singh *et al.* (1988) demonstrated that artemisinin content was higher in plants growing in temperate climates than in subtropical climates (0.10% vs 0.06%).

Water stress: The rate of photosynthesis is reduced with the decreased hydration of leaves (Taiz and Zeiger, 2006). Water deficiency not only limits the amount of available water and the quantity of CO_2 because in response to water deficits the leaves close their stomata in order to curtail transpiration. Artemisinin content was found to be higher in plants subjected to moderate water stress than in irrigated controls (Marchese and Figueira, 2005). Treatments of 38-h water deficit (Ψw -1.39

MPa) and 62 h water deficit (Ψw -2.51 MPa), respectively, resulted in 29% and 13.3% more artemisinin content than in irrigated control plants (Marchese and Figueira, 2005). Significant increases in leaf artemisinin content resulting from treatment with moderate water deficit (38-h WD), which could be attributed to the fact that plant growth ceases under moderate water deficits, while photosynthesis is still continuing due to CO_2 from respiration (Marchese and Figueira, 2005). However, due to oxygen deficits which is required as to find acceptor of electrons during respiration, acetyl CoA accumulates at the end of glycolysis.

Essential nutrients elements and growth hormones: High artemisinin content was obtained from plants that received both N and S in two equal splits, along with the basal dosage of P and K fertilisers, with significant increase in artemisinin content at 120 and 80 N kg/ha (Ahmad and Abdin, 2000). In another study, Kapoor *et al.* (2007) reported that the application of P at 30 kg/ha significantly increased artemisinin content. However, N deficiency or excess decreased artemisinin content (Ferreira, 2007).

According to Liu *et al.* (2003), potassium dihydrogen phosphate (KH_2PO_4) increased plant biomass and artemisinin content (0.05–0.20%) up to 200 mg/L. There was a slight increase in plant biomass above 200 mg/l, but artemisinin content decreased to original levels. Magalhães *et al.* (1996) conducted nitrogen fertiliser trials at 0, 32, 64 and 97 kg N/ha applied as urea. The leaf dry matter yield of 2420 kg/ha increased to 4 690 kg/ha dry leaves whereas artemisinin yield of 26 kg/ha obtained at 0 kg N/ha increased to 41 kg/ha artemisinin at 97 kg N/ha. The rate of 64 kg N/ha resulted in a leaf biomass of 3 880 kg/ha and artemisinin yield of 40.4 kg/ha. In

another study by carried out in Indiana, USA Simon *et al*. (1990), investigated nitrogen levels of (0, 67 and 134 kg N/ha) and three plant densities of (28, 56 and 112 plants/ha. He reported that optimum essential oil of 85 kg/ha and fresh whole plant biomass of 30 t/ha were obtained at 67 kg N/ha at medium plant density (55 plants/ha). The highest plant density (at 67 kg N/ha) provided 35 t/ha of plant biomass, the plants had a lower leaf-to-stem ratio.

Artemisinin content was increased by applications of 50 and 80 mg/L gibberellic acid (GA3) to field grown plants from 0.77 to 1.10 and 0.77 to 1.3% mg/g, respectively. Application of kinetin at 10 and 20 mg/L increased leaf yield and oil content, but decreased artemisinin content (Farooqi *et al*., 1996). Similar results on the beneficial effect of GA3 on artemisinin accumulation were obtained by Zhang *et al.* (2005). Ferreira and Janick (2002) reported under *in vitro* conditions that no other growth regulator, besides GA3, produce plant secondary metabolites and have the most vital class of natural products with different and valuable chemical properties and biological activities.

Salicylic acid (SA) plays an important role in regulating a number of plant physiological and biochemical processes. Four levels of SA (0.00, 0.25, 0.50, and 1.00 mM SA) were applied on the aboveground plant parts of *A. annua*. Salicylic acid at 1.00 mM increased the content and yield of artemisinin by 25.8 and 50.0%, respectively as compared to the untreated control.

2.1.5 Current status of *A. Annua* breeding on artemisinin production

A new hybrid known as Artemis which can produce from 1 to 2% artemisinin on a dry weight basis was produced by the company Mediplant in Switzerland, which produce high yield of artemisinin (De Ridder *et al*., 2008). Apparently, it is the best genetic variety suited for lower latitudes in tropical countries such as Tanzania (De Ridder *et al*., 2008). Many more hybridizations between Chinese and Vietnamese clones were made to create hybrids like Artemis which can generate artemisinin content of up to 1.4% and artemisinin production of 38 kg/ha (Delabays *et al*., 2001). Artemisia plants were bred at the Institute of Materia Medica in Vietnam in order to achieve optimum concentration of artemisinin and high plant biomass (Dong and Thuan, 2003). Mediplant in Switzerland also crossed a late-flowering clone of Chinese origin which is rich in artemisinin of 1.1% with European plants. They produced progenies that contained 0.64% and 0.95% artemisinin, with dry leaf yields between 14 and 21 t/ha (Delabays *et al*., 1993).

Unicamp and Mediplant in Campinas, Brazil, have been growing *A. annua* cultivars (CPQBA) in Teresina, Piaui (58N) and Calabar, Nigeria (58S). The height of the plants from Teresina varied from 40 cm to 120 cm before the Kew Garden strain of *A. annua* was later declared to be a poor grower when compared to the other strain from Europe and USA (Singh *et al*., 1988). Artemis, Anamed-A3 or CPQBA are best clones for high leaf-to-stem dry matter ratio to be grown in climates, such as Africa. General access to these hybrids is unavailable except for research purposes even though high artemisinin and high vigour clones have been developed (Delabays *et al*., 1993). The Chinese and Vietnamese selections with even higher artemisinin (1.0–1.5%) are only available to a limited extent (Debrunner *et al*., 1996). A

Vietnamase strain of *A. annua* was studied in Vietnam near Hanoi (218020N). The plants were sown into field plots at density of 25 plants/m^2. Artemisinin yield of 45.4 kg/ha, artemisinin concentration of 0.86% and dry matter yield of leaves (5.3 t/ha) was obtained (Woerdenbag *et al*., 1994).

Wallaart *et al.* (1999) obtained a tetraploid *A. annua* (2n ¼ 36) with the mitotic inhibitor colchicines by with an efficiency of approximately 20%. It had artemisinin content of 0.46% dry weight in the tetraploid *A. annua* during one vegetation period was 39% higher than in the diploid parental clone of *A. annua* (0.33% artemisinin). The essential oil obtained was 32% lower than in the diploid parental clone. Higher production of artemisinin might be achieved at the expense of the essential oil level. De Jesus-Gonzalez and Weathers (2003) also obtained stable tetraploid *A. annua* root cultures with artemisinin production.

2.2 Work not done on artemisinin

Previous work done on artemisinin was not focused on increasing artemisinin as a secondary metabolite. The pruning techniques in this study were implemented to trigger artemisinin as a result of stress. The production of secondary metabolites is a common phenomenon in stressed plants. All the pruning techniques implemented in this study in relation to *A. annua* have not been published. Work done on interaction between plant density and pruning on artemisinin production is also scant. Plant density has a direct impact on the plant`s ability to absorb nutrients in soil and the plant`s ability to photosynthesise. The objective of using spacing is to determine which spacing will allow the plant to increase its photosynthates.

2.3 Gaps on the research problem

The newly developed hybrids remain inexpensive when compared with synthetic chemical methods. Therefore, cultivation practices in combination with new hybrids that can increase artemisinin should be considered and studied further. Work published concerning plant density and pruning in *A. annua* and their effect on artemisinin concentration is limited. Hence this study focused on comparative effects of different planting densities and their influence on growth and yield in *A. annua* and artemisinin concentration.

2.4 Addressing the gaps

Spacing and pruning were recognised as having the potential to enhance growth, yield and ultimately the artemisinin content of *A. annua*. This study will be beneficial in determining which pruning technique is best to adopt in terms of increasing growth and yield in *A. annua* and artemisinin concentration. Information concerning preserving quality and quantity of artemisinin using different drying methods is limited. This study will determine which drying method between direct sun drying, direct and indirect shade drying, freeze drying and oven drying will preserve artemisinin concentration and quality in *A. annua.* Cultural practices are one of the major factors that affect growth, yield and quality of plants. Coupling appropriate cultural practices that promote the accumulation of photosynthates help in increasing the harvestable concentrations of artemisinin and in developing economic methods for *A. annua* post-harvest.

CHAPTER 3

EFFECTS OF CULTURAL AND DRYING METHODS ON PRODUCTIVITY OF *ARTEMISIA ANNUA*

3.1 Introduction

Artemisinin is the active ingredient in *Artemisia annua* and is predominantly located in the leaves, trichomes and flowers (Abdin *et al*., 2003). High artemisinin concentration is observed just before or during full flowering. Younger leaves contain significantly more artemisinin than mature ones (Charles *et al*., 1990; Laughlin, 1995). This sesquiterpene lactone has curative abilities for cancer, diverse viral and parasitic diseases, including malaria (Romero *et al*., 2005; Utzinger and Keiser, 2004). The major constraint concerning artemisinin availability is its low concentration and its chemical synthesis is expensive. Although WHO (2006) recommends it to be used in combination with other chemical compounds against malaria, its low quantity remains a major setback. Therefore, improved production of artemisinin in *A. annua* plants is vastly sought-after.

Cultural practices like spacing and pruning are key factors influencing growth, biomass, essential oil content and yield in plants. Plant spacing is vital in managing competition for light, conserving water, nutrition and ultimately growth of the plants (Abirami *et al.,*2014). Pruning is an important agronomic technique used in different plants (Sarkka and Erikson, 2003). It is used to control plant growth, enhance branching and rejuvenate plants resulting in greater number of leaves (Satyanarayana and Sharma, 2004). The style of pruning plants the affects the

distribution of nutrient elements. Shoot pruning enables light to penetrate and be allocated efficiently (Admasu and Struikb, 2000).

Post-harvest practices like drying can significantly affect the quality of secondary metabolites. Therefore, such practices must be carried out properly to conserve the quality of the materials. Prolonged drying can result in losses in artemisinin (Charles *et al*., 1993). Different drying methods should be continually assessed to ensure that artemisinin is conserved. The method of drying should also be as affordable and efficient as possible. Field drying may be a way of reducing the cost of antimalarial drugs. However, it is crucial to reassess its role in some inter-tropical regions where *A. annua* is currently grown, categorically with the more incipient high artemisinin culls which have recently been developed (Laughlin *et al*., 2001). Traditionally, sun and shade drying have been used in China and Vietnam (Laughlin *et al*., 2002). However, wilting of *A. annua* in the field after harvesting with exposure to direct sun has in some cases reduced the artemisinin content (Laughlin *et al*., 2002). Oven and freeze drying plant material in immensely large scale is a sumptuous operation but the less wasteful alternative of field drying in the sun is reputed to rigorously reduce artemisinin (Laughlin *et al*., 2002). The objective of this study was to determine whether spacing, pruning and their interactions would have any effect on the concentrations of artemisinin, growth and yield of *A. annua* and whether drying methods would have an effect on the concentrations of artemisinin in *A. annua.*

3.2 Materials and methods

3.2.1 Study location

Experiments were conducted under field conditions at Agricultural Research Council-VOP Roodeplaat (25º 56’ S; 28º 35’ E) in summer (2014) with average rainfall of 461 mm, minimum temperature of 15 ºC and maximum temperature of 29 ºC.

3.2.2 Experimental design, treatments and procedures

Treatments for Experiment 1, *viz.*, 3 × 4 factorial experiment were laid out in a randomised complete block design, with four replications (*n* = 48). The first factor was the pruning treatments, *viz*, no pruning (control), removing the apical bud and removing shoots three nodes from the bottom. The second factor was plant density population (spacing), *viz.*, 0.5 m × 1 m (standard: 0.50 m^2), 0.5 m × 0.7 m (small: 0.35 m^2) 0.5 m × 0.5 m (smaller: 0.25 m^2) and 0.3 m × 0.7 m (smallest: 0.21 m^2).

Figure 3.1 *Artemisia annua* seedlings in seedling trays (A) and at transplanting (B).

The soil samples from each depth per replication were mixed thoroughly to form a composite sample and taken for analysis. *Artemisia annua* seeds were obtained from the ARC-VOP gene bank and were sown in seedling trays in September 2014. Uniform eight-week-old *A. annua* seedlings were each hardened-off for a week and transplanted in November 2014 in 10 cm deep holes with different spacing treatments namely; 0.5 m × 1 m (standard; 0.50 m^2), 0.5 m × 0.7 m (small; 0.35 m^2) 0.5 m × 0.5 m (smaller; 0.25 m^2) and 0.3 m × 0.7 m (smallest; 0.21 m^2). Plants were pruned ten weeks after transplanting according to their respective treatments which were: 1) no pruning (control), 2) removing the apical bud and 3) removing shoots three nodes from the bottom.

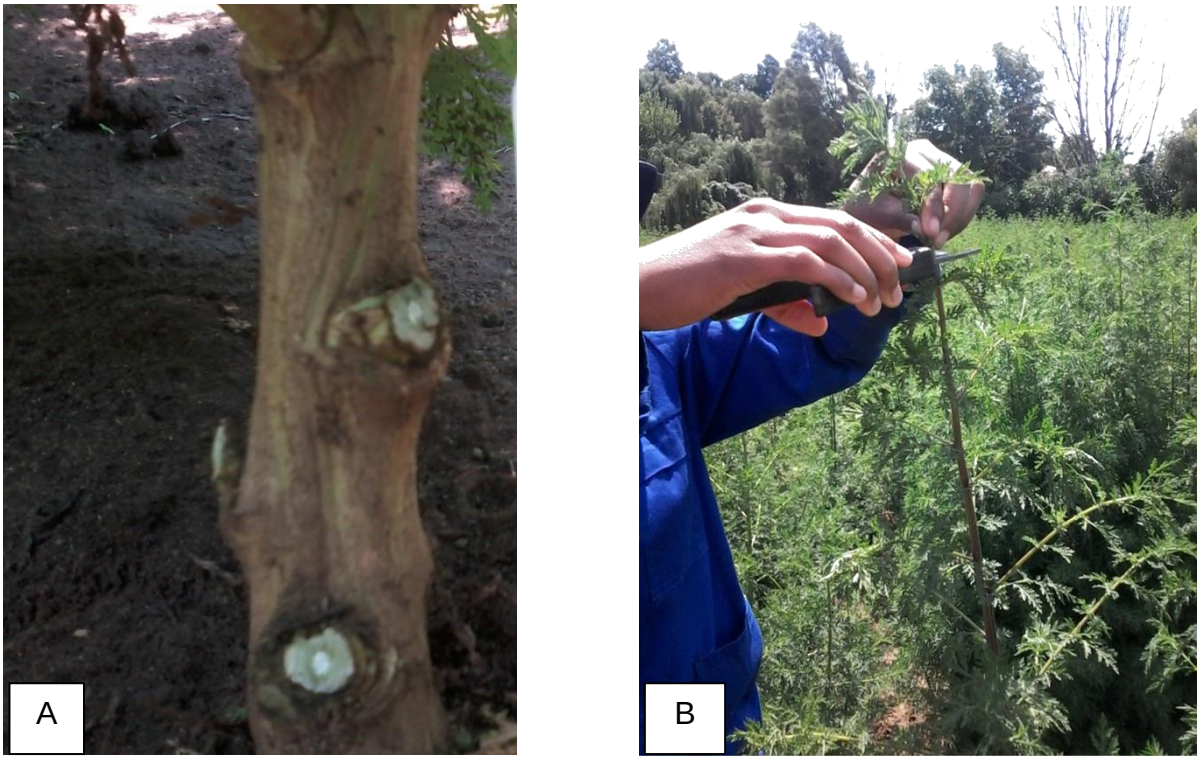

Figure 3.2 Removing shoots three nodes from the bottom (A) and removing the apical bud (B).

The plants were irrigated using overhead sprinklers system for two hours three times per week (22 ml per week). Weeding was done manually. After 180 days from

planting, data was collected, with leaves being separated from stems and roots, put in 1kg brown bags, oven dried at 40 ºC for 72 h and weighed.

Figure 3.3 Artemisia annua during harvesting after 180 days.

The experiment for drying methods was arranged in completely randomised design with four replicates (*n* = 20). The treatments were as follows; freeze drying, oven drying, 100% sun drying, 100% shade drying and 50% shade drying. Samples were collected from the untreated control *A. annua* plants from the field experiment. Each drying method had four replicated samples weighing 750 g.

For sun drying, samples were spread evenly and exposed to full sunlight for a week, for oven drying the samples were put in brown bags (SO16: 196 × 115 × 385 mm) and they were put in an oven for 72 h at 40 ºC, for freeze drying the samples were put in the freeze drier for three days and for 50% shade drying samples were uniformly spread and put under a shade net that allows 50% light penetration for a

week and lastly for 100% shade the samples were homogeneously dispersed and put in an enclosed room for a week.

Figure 3.4 Freeze dryer (A) and oven (B) used as some of the drying instruments for *Artemisia annua* samples in the study.

3.2.3 Data collection

Data were collected every two weeks after pruning; chlorophyll content on flag leaves was measured using a digital chlorophyll meter (Minolta chlorophyll meter SPAD-502). Plant height was measured from the soil surface to the tip of the flag leaf using a measuring tape, leaf area index was measured using digital plant canopy analyser (LAI- 2200C) and stem diameters measured 5 cm above the severed ends using a digital Vernier caliper (KTV150-Major Tech). Leaves were harvested, weighed, and oven-dried at 40 ºC for 72 h and reweighed.

3.2.4 Data analysis

Data were subjected to analysis of variance (ANOVA) using Statistix 10. Significant treatment ($P \leq 0.05$) means were separated using Fisher's Least Significant Difference test and Duncan Multiple Range test. Treatments that were significant at the probability level of 5% were reported.

3.2.5 Extraction of artemisinin

The extraction of artemisinin was done following a method by Shuoqian *et al. (*2008) and Tarsisius *et al.* (2013). One gram of *A. annua* dried ground leaves were weighed and refluxed with hexane (100 ml) at 75 ºC for 1 h in a soxhlet. The plant material to solvent ratio of 1:100 was maintained to prevent solvent saturation in all extractions (Shuoqian *et al.,* 2008). The n-hexane extract was filtered through a Whatman No.1 filter paper and the filtrate was evaporated under vacuum on a rotary evaporator until the materials were dry.

Figure 3.5 Artemisinin extraction from *Artemisia annua* leaf samples.

The residue was again dissolved in 50 ml of n-hexane and the n-hexane phase washed in a separating funnel with 2% NaOH solution to get rid of the impurities, which are soluble in aqueous sodium hydroxide (Tarsisius *et al.*, 2013). The lower layer which contained the alkali solution was discarded, while the upper solution (hexane extract) was washed with equal volume of distilled water two times. The hexane extract was filtered through anhydrous sodium sulphate on a Whatman No.1 filter paper and the filtrate was evaporated to dryness under vacuum at 45 ºC on a rotary evaporator. The dried extract was re-dissolved with 95% aqueous ethanol and then topped to the mark in a 50 ml volumetric flask (final Aliquot). The solution was then ready to measure on a spectrophotometer.

In the standard preparation, artemisinin (Sigma Aldrich) 1.8 mg was accurately weighed and dissolved in 1 ml 95% aqueous ethanol in a pure vial. An aliquot of artemisinin solution of 100 µ/L and 900 µ/L of 95% ethanol was transferred into another pure vial. This solution was coded as Solution-A then sodium hydroxide solution (4 ml, 0.2% w/v) was added and the solution made to react at 50 ºC for 30 min. After, acetic acid solution (0.08 mol/L) was added and filled up to the mark of the 10 ml volumetric flask (Nurgün *et al.*, 2007). During the assay a two-fold serial dilution was done to make four more concentration to construct a standard curve.

The UV spectrophotometric method specified for artemisinin determination by Zhong (2007) was adopted. The extract (10 ml) was pipetted into a 100 ml volumetric flask; diluted to the mark with sodium hydroxide (0.2%), mixed thoroughly, and warmed to 50 °C in a water-bath for 30 min. An aliquot of 1 ml was transferred into a cuvette and absorbance at 290 nm was read on a spectrophotometer (2100 - C). A standard

curve was obtained from five concentration of the standard artemisinin solution. GraphPad Prism v5 software was used to interpolate the artemisinin concentration in the test samples from the standard curve. The percentage of artemisinin content in the absolutely dry leaves of *A. annua* was calculated using Equation 1 below adopted from WHO (2006) with modification because of the different analytical method used.

$$\% \text{ artemisinin} = \left(\frac{A \times V \times d.f}{1000 \times Wt}\right) \times \left(\frac{100}{100 - B}\right) \times 100 \qquad \text{[Equation 1]}$$

Where A = concentration of artemisinin in sample (mg/L), V = initial aliquot volume of reconstituted extract = 50 ml, d.f = dilution factor, Wt = mass of dry leaves of *A. annua* (mg), B = percentage moisture of the dry leaves of *A. annua* which is 13%.

3.3 Results

3.3.1 Effects of spacing and pruning on artemisinin

Spacing and pruning had a significant effect on artemisinin concentration ($P \leq 0.05$) (Appendix 3.1). The smaller spacing of (0.25 m^2) with apical bud removal had more artemisinin concentration, followed by the smallest spacing with no pruning (control) and the smallest spacing (0.21 m^2) with bottom shoot removal. Relative to the standard spacing (0.50 m^2) with the pruning treatments, the smaller spacing (0.25 m^2) with apical bud removal increased artemisinin concentration by 65%, the small spacing with apical bud removal by 21% and the smallest spacing (0.21 m^2) with bottom shoot removal by 20% (Table 3.1).

3.3.2 Effects of drying methods on artemisinin

Freeze drying had a significant effect ($P \leq 0.05$) on artemisinin concentration in *Artemisia annua* (Appendix 3.2). More artemisinin was obtained from freeze drying, followed by oven, 100% shade, sun and 50% shade drying. Relative to the standard (sun drying) according to WHO (2006), freeze drying preserved artemisinin concentration by 23%, oven drying conserved artemisinin concentration by 10% and 100% shade drying conserved by 5%. Shade drying (50%) had a slight decrease in conserving artemisinin of 10% (Table 3.2).

3.3.3 Effects of spacing on growth of *Artemisia annua*

Spacing had a significant effect ($P \leq 0.05$) on stem diameter, fresh leaf mass and dry leaf mass (Tables 3.1, 3.2; Appendix 3.3, 3.4, 3.5,). Smaller spacing of (0.25 m^2) had the biggest stem diameter (Table 3.2) than other spacing treatments and the small spacing (0.35 m^2) had more fresh leaf mass (Table 3.3) and the highest plant height (Table 3.4). Relative to the standard spacing, the small spacing (0.35 m^2) increased stem diameter by 3%, smaller spacing (0.25 m^2) increased stem diameter by 175 and the smallest spacing (0.21 m^2) by 21% (Table 3.2). The small spacing (0.35 m^2) also increased fresh leaf mass by 54%, smallest (0.21 m^2) by 31% and smaller (0.25 m^2) by 10%. Dry leaf mass was increased by 39% by the smaller spacing (0.25 m^2), 31% by both the small (0.35 m^2) and smallest spacing (0.21 m^2) relative to the standard spacing (Table 3.3). The small spacing (0.35 m^2) increased plant height by 7%, the smaller spacing (0.25 m^2) by 5% and the smallest (0.21 m^2) by 2% relative to the standard spacing (Table 3.4).

3.3.4 Effects of pruning on growth of *Artemisia annua*

Pruning had a significant effect (P ≤ 0.05) on plant height and chlorophyll content in *Artemisia annua* (Appendix 3.7, 3.8). Bottom shoot removal had more plant height and chlorophyll content (Table 3.5, 3.6). Relative to the untreated control, removing shoots from the bottom increased plant height by 10% in the last week 4, apical bud removal increased plant height by 1% Table 3.5. Apical bud removal relative to the untreated control increased chlorophyll content by 2% and bottom shoot removal decreased by 1% in week 4 (Table 3.6). The interaction of spacing and pruning had no significant difference on the growth and yield of *A. annua.*

Table 3.1 Relative effects of reducing planting spacing on concentration of artemisinin in *Artemisia annua* under field conditions.

Spacing (m^2)	Pruning	Artemisinin (%)[y]	Relative impact (%)[z]
0.50 (standard)	Control	0.125^{bcd}	−
0.50 (standard)	Apical bud	0.117^{cd}	−6.4
0.50 (standard)	Bottom shoot	0.122^{bcd}	−2.4
0.35 (small)	Control	0.144^{bcd}	15
0.35 (small)	Apical bud	0.141^{bcd}	21
0.35 (small)	Bottom shoot	0.116^{cd}	−5
0.25 (smaller)	Control	0.109^{c}	−13
0.25 (smaller)	Apical bud	0.193^{a}	65
0.25 (smaller)	Bottom shoot	0.118^{cd}	−3
0.21 (smallest)	Control	0.152^{b}	22
0.21 (smallest)	Apical bud	0.125^{bcd}	7
0.21 (smallest)	Bottom shoot	0.147^{bcd}	20

[y]Column means followed by the same letter were not different (P≤ 0.05) according to Duncan Multiple Range test.

[z]Relative Impact = $(\frac{\text{Treatment}}{\text{Standard}} - 1) \times 100$.

Table 3.2 Influence of different drying methods on concentration of artemisinin from *Artemisia annua* plants after growing 180 days under field conditions.

Drying treatments	Artemisinin (%)[y]	Relative impact (%)[z]
Sun-drying (Standard)	1.577[bc]	–
Freeze-drying	1.941[a]	23
Oven-drying	1.738[ab]	10
Shade-drying (100%)	1.657[abc]	5
Shade-drying (50%)	1.412[c]	−10

[y]Column means followed by the same letter were not different (P≤ 0.05) according to Fisher`s Least Significant Difference test.

[z]Relative Impact = $(\frac{\text{Treatment}}{\text{Standard}} - 1) \times 100$.

Table 3.3 Effect of spacing on fresh and dry leaf mass of *Artemisia annua* at 180 days after growing under field conditions.

	FLM (g)		DLM (g)	
Spacing (m^2)	Value[y]	RI (%)[z]	Value[y]	RI (%)[z]
0.50 m^2 (standard)	419[b]	–	292.7[b]	–
0.35 m^2 (small)	646[a]	54	329.7[a]	13
0.25 m^2 (smaller)	378[c]	−10	178[ab]	−39
0.21 m^2 (smallest)	548[ab]	31	201.5[b]	−31

[y]Column means followed by the same letter were not different (P≤ 0.05) according to Fisher`s Least Significant Difference test.

[z]Relative Impact = $(\frac{\text{Treatment}}{\text{Standard}} - 1) \times 100$.

Table 3.4 Relative effect of reducing planting spacing on stem diameter of *Artemisia annua* at weekly interval under field conditions.

	Week 1		Week 2		Week 3		Week 4	
Spacing (m^2)	value[y]	RI (%)[z]	Value[y]	RI (%)[z]	Value[y]	RI (%)[z]	Value[y]	RI (%)[z]
0.50 m^2(standard)	22.287[a]	−	25.732[a]	−	29.330[a]	−	31.647[a]	−
0.35 m^2 (small)	20.884[a]	−6	24.397[a]	−5	27.068[a]	−8	30.784[a]	−3
0.25 m^2 (smaller)	19.738[b]	−11	20.662[b]	−20	22.987[b]	−21	26.357[b]	−17
0.21 m^2 (smallest)	19.167[b]	−13	20.692[b]	−20	22.377[b]	−23	24.209[b]	−24

[y]Column means followed by the same letter were not different (P≤ 0.05) according to Fisher`s Least Significant Difference test.

[z]Relative Impact = $(\frac{\text{Treatment}}{\text{Standard}} - 1) \times 100$.

Table 3.5 Relative effect of reducing planting spacing on plant height of *Artemisia annua* at weekly interval under field conditions.

	Week 1		Week 2		Week 3		Week 4	
Spacing (m^2)	value[y]	RI (%)[z]	Value[y]	RI (%)[z]	Value[y]	RI (%)[z]	Value[y]	RI (%)[z]
0.50 m^2 (standard)	1.643[ab]	−	1.848[ab]	−	1.974[a]	−	2.217[a]	−
0.35 m^2 (small)	1.753[ab]	7	1.917[ab]	4	2.133[a]	8	2.362[a]	7
0.25 m^2 (smaller)	1.583[b]	−4	1.733[b]	−6	2.048[a]	4	2.333[a]	5
0.21 m^2 (smallest)	1.800[a]	10	1.979[a]	7	2.024[a]	3	2.269[a]	2

[y]Column means followed by the same letter were not different (P≤ 0.05) according to Fisher`s Least Significant Difference test.

[z]Relative Impact = $(\frac{\text{Treatment}}{\text{Standard}} - 1) \times 100$.

Table 3.6 Relative effect of pruning different positions on plant height of *Artemisia annua* at weekly interval under field conditions.

	Week 1		Week 2		Week 3		Week 4	
Pruning	Value[y]	RI (%)[z]	Value[y]	RI (%)[z]	Value[y]	RI (%)[z]	Value[y]	RI (%)[z]
Control	1.719[ab]	–	1.894[ab]	–	2.037[ab]	–	2.174[b]	–
Apical bud removal	1.584[b]	−8	1.774[b]	−6	1.922[b]	−6	2.206[ab]	1
Bottom shoot removal	1.781[a]	4	1.941[a]	2	2.176[a]	7	2.388[a]	10

[y]Column means followed by the same letter were not different (P≤ 0.05) according to Fisher`s Least Significant Difference test.

[z]Relative Impact = $(\frac{\text{Treatment}}{\text{Standard}} - 1) \times 100$.

Table 3.7 Relative effect of pruning different positions on chlorophyll content of *Artemisia annua* at weekly interval under field conditions.

	Week 1		Week 2		Week 3		Week 4	
Pruning	Value[y]	RI (%)[z]	Value[y]	RI (%)[z]	Value[y]	RI (%)[z]	Value[y]	RI (%)[z]
Control	16.019[b]	–	27.657[a]	–	31.113[ab]	–	36.662[a]	–
Apical bud removal	21.019[a]	31	28.463[a]	3	32.950[a]	6	37.494[a]	2
Bottom shoot removal	18.631[ab]	15	27.885[a]	1	32.881[a]	6	36.463[a]	−1

[y]Column means followed by the same letter were not different (P≤ 0.05) according to Fisher`s Least Significant Difference test

[z]Relative Impact = $(\frac{\text{Treatment}}{\text{Standard}} - 1) \times 100$.

3.4 Discussion

3.4.1 Effects of Spacing and pruning on artemisinin

The smaller spacing of 0.5 × 0.5 m^2 (0.25 m^2) and removing the apical bud had a significant effect on artemisinin concentration. It was followed by the smallest spacing of 0.3 × 0.7 m^2 (0.21 m^2) and no pruning control. The artemisinin yield ranged from 0.193 - 0.109%. Limited studies have been conducted where plant spacing was used to determine its effect on artemisinin concentration (Abirami *et al.,* 2014; Uniyal *et al.*, 2010). However, results from this study did correspond to some of the few experiments that were conducted. Findings from this study exhibited more artemisinin concentration as compared to a study that was conducted by Abirami *et al.* (2014), who found no significant differences from different plant spacing. Spacing of 0.5 × 0.5 m^2 (0.25 m^2) and removing the apical bud had more artemisinin concentration of 0.193% as compared to the same spacing but with no pruning treatment. This may be due to the pruning effect which triggers secondary metabolites through stress (Satyanarayana and Sharma, 2004). When the apical bud is removed growth tends to focus more on the vegetative part of the branches and leaves.

The effect of pruning was also observed by Kumar *et al.* (2004) who found that multiple harvesting had higher yield of artemisinin through high levels of leaf yield accompanied by increased stem and shoot mass. The photosynthates are channeled to the growth of the leaves and the branches. The other reason for this increase in artemisinin concentration may be due to adequate space for the plants to utilize all the nutrients they need for growth. Abirami *et al.* (2014) discovered that

artemisinin concentration was higher than in the current study. They obtained optimum artemisinin concentration of 8.88 kg/ha. This high concentration of artemisinin may be due to the spacing of 0.45 × 0.45 m^2 that was used as compared to the spacing of 0.5 × 0.5 m^2 (0.25 m^2) which was used in this study. This might prove that artemisinin concentration may be increased by smaller spacing. Our findings however, also show that the spacing of 0.3 × 0.7 m^2 (0.21 m^2) with the no pruning control in terms of pruning had the second highest concentration of artemisinin. This may be due to the plants being densely populated and having more plants per plot. Even though the spacing was not enough for the plants to adequately utilize the nutrients for growth having more plants per plot compensated that loss. More plants produced more herbage yield and hence more artemisinin. This corresponded to what was stated by Ram *et al.* (1997) who recommended that plants should be planted more closely together to increase the yield of artemisinin.

Even though the findings of this study were significant, the artemisinin concentration that was found was less than what others had found in previous studies. Woerdenbag *et al.* (1994) found artemisinin concentration of 0.86%. Delabays *et al.* (1993) found 0.64% and 0.95% artemisinin from the cross of a late-flowering clone of Chinese origin which is rich in artemisinin (1.1%) with European plants. Delabays *et al.* (2001) found artemisinin content of up to 1.4% from the Chinese and Vietnamese clones which were made to create Artemis hybrid. The Chinese and Vietnamese selections with even higher artemisinin (1.0–1.5%) are only available to a limited extent (Debrunner *et al.,* 1996). There are many factors that contributed to high artemisinin from these previous studies. For some it was breeding that improved

concentration of artemisinin and these are not affordable or accessible by most farmers (Debrunner *et al.,* 1996; Delabays *et al*., 1993; Delabays *et al.,* 2001).

3.4.2 Effects of drying methods on artemisinin

Sun drying was chosen as the standard drying according to WHO (2006) in this experiment and it was not significant on artemisinin concentration. Freeze drying had a significant effect on artemisinin concentration producing 1.941% artemisinin. It was followed by oven (1.738%) and 100% shade drying (1.657%) and the lowest artemisinin concentration (1.412%) was obtained from 50% shade drying. However, these results varied from the findings by Ferreira *et al.* (1992) who reported that lowest content of artemisinin was found in freeze-dried leaves as compared to shade and oven-dried leaves. Freeze drying may not be economically viable for farmers due to cost even though it had higher artemisinin.

Results from this study observed high artemisinin in the samples that were oven dried as compared to sun and shade drying; with sun drying having the lowest artemisinin yield. Artemisinin concentration under freeze and oven drying were more than other drying methods and this contradicted the findings of Charles *et al.* (1993) who found that drying under ambient conditions yielded higher artemisinin. The findings from this study on oven drying also contradicted what was reported by Laughlin *et al.* (2002) that there was a trend for sun, shade and dark drying for 21 days to give higher artemisinin than oven drying. The results from this study also contradicted the findings of Laughlin *et al*. (2002) who indicated that shade and sun drying significantly increased artemisinin as compared to oven drying.

The findings of this study correlated with the findings of a study that was conducted in USA where shade drying of *A. annua* under ambient conditions inside gave higher artemisinin than sun drying (Charles *et al*., 1993). However, results contradicted the findings that reported that full shade drying resulted in higher artemisinin than oven drying at 40 ºC (Ferreira *et al*., 1992). Drying *A. annua* plants (either under shade or sun) has been mentioned as a possible way to reduce costs of the *A. annua* crop-drying process (Willcox *et al*., 2004). Ferreira *et al.* (1992) indicated that artemisinin from shade-dried leaves was 30% higher than in oven-dried (40 ºC) leaves. However, in this study oven drying had more artemisinin concentration than shade.

Sun drying was studied by Willcox *et al.* (2004) and Laughlin *et al.* (2001) to increase artemisinin, they found that sun drying decreased artemisinin. Their findings corresponded with our results as samples from sun drying had the lowest artemisinin as compared to other drying methods. Sun drying may be affected by prolonged drying according to Laughlin *et al.* (2001) and the temperature level during exposure.

3.4.3 Effects of spacing on growth of *Artemisia annua*

Plant height: The results of this study showed that plant height increased consistently with maturity and density. Similar findings were reported by Belay (2007) who reported an increase of plant height with increase in age of *A. annua*. Simon *et al.* (1990) also reported an increase in plant height with increased plant density. Spacing treatments of 0.5 x 0.7 m^2 (0.35 m^2) and 0.3 × 0.7 m^2 (0.21 m^2) had the tallest plants as compared to other spacing treatments which were in line with the findings by Simon *et al.* (1990). The observation might have been due to highly populated plants that competed for light. Plants in high densities were more erect,

taller and had less side shoots than lower densities. The plant height values recorded ranged from 1.5 to 2.4 m, which were within the range reported by other researchers. Damtew *et al.* (2011) found minimum and maximum plant height of 1.4 m and 2.0 m respectively. Several factors like different agronomic practices, growing conditions, climatic factors and plant ecotypes may affect the plant height.

Stem diameter: Stem diameter increased as the plant matured. The standard spacing of 0.5 × 1 m^2 (0.50 m^2) and by 0.5 × 0.7 m^2 (0.35 m^2) were not significantly different on stem diameter and both had the biggest stem diameter as compared to the other treatments. This may be due to intermediate spacing between the plants which contributed to good growth and allowed plants to have less competition for water, air, nutrition and light. Uniyal *et al.* (2010) also found that stem diameter increased with lower plant density of 0.6 × 0.6 m^2.

Fresh and dry leaf mass: Spacing had a significant effect on fresh and dry leaf mass from spacing. The spacing of 0.3 × 0.7 m^2 (0.21 m^2) had the highest fresh and dry leaf mass of 17.99 and 9.62 t/ha, respectively. This may be due to increased number of plants producing more leaves as compared to low plant densities. Laughlin (1993) found maximum yield of 6.8 t/ha from 10 plants/m^2. In another study the spacing of 0.3 x 0.6 m^2m produced an average fresh weight of 275 g/plant, as opposed to 430 g/plant from the intermediate spacing of 0.6 x 0.6 $m^2$and 750 g/plant from 0.3 x 0.3 m^2 (Simon *et al*., 1990).

3.4.4 Effects of pruning on growth of *Artemisia annua*

Plant height: There was a significant effect from the pruning treatment which involved removing shoots three nodes from the bottom. The tallest plant which was 2.4 m was obtained from removing shoots three nodes from the bottom as compared to the other pruning treatments. Scientific literature is limited in relation to pruning effect on *A. annua* therefore more studies need to be conducted on the subject. Increased plant height may be due to removal of excess shoots which can channel and reserve plant nutrients for the growth of the plant. During this study it was observed that older branches tend to wilt giving room for new ones to develop. The interaction between spacing and pruning was not statistically significant. However, the tallest plant height of 2.4 m was obtained from the smallest spacing of 0.3 × 0.7 m^2 (0.21 m^2) with removing shoots three nodes from the bottom and the small spacing of 0.5 × 0.7 m^2 (0.35 m^2) with no pruning.

Chlorophyll content: Pruning had a significant increase in chlorophyll content. The treatment of removing the apical bud had more chlorophyll content than other pruning treatments over time and as the plant matured. Removing the apical bud might have channeled photosynthates to focus on the photosynthesis, leaf development and maturity than plant height.

3.5 Conclusion

The smaller spacing of (0.25 m^2) with apical bud removal had more artemisinin concentration, followed by the smallest spacing with no pruning (control) and the smallest spacing (0.21 m^2) with bottom shoot removal. The spacing of 0.5 × 0.5 m^2 (0.25 m^2) with no pruning had the lowest artemisinin concentration. Even though

other hybrids have been developed and produced more artemisinin concentration, it is still vital to consider the affordability and accessibility of high-artemisinin yielding seeds. It is therefore practical to recommend the spacing of 0.5 × 0.5 m^2 (0.25 m^2) with removing the apical bud to obtain more artemisinin concentration. Increased fresh and dry leaf mass were obtained from the spacing of 0.3 × 0.7 m^2 (0.21 m^2) as compared to the other treatments. The smallest spacing of 0.3 × 0.7 m^2 (0.21 m^2) significantly affected plant height whereas the standard spacing significantly affected the stem diameter. Plants that were removed bottom shoots also had the highest plant height as compared to other pruning treatments. Chlorophyll content was higher on plants that were removed the apical bud. The results showed that freeze, oven and 100 % shade drying of *A. annua* plants preserves artemisinin. 100 % shade drying demonstrated that drying *A. annua* leaves for one week in an enclosed room at room temperature can also be an effective way of preserving artemisinin.

CHAPTER 4

SUMMARY, SIGNIFICANCE OF FINDINGS, RECOMMENDATIONS AND CONCLUSIONS

4.1 Summary

Artemisinin is produced in very low amounts and even though there are efforts being made to increase its production through breeding for high yielding plants, the new hybrids remain unaffordable and inaccessible by farmers. This study was done to determine whether cultural practices like spacing and pruning can increase artemisinin concentration. Proper spacing is vital for increased herbage and can aid in improving the concentration of artemisinin. Pruning can be adapted to induce stress on the plant so that artemisinin as a secondary metabolite may be triggered. The study was also initiated to evaluate efficient drying methods that can aid in preserving artemisinin to avoid degrading the secondary metabolite.

4.2 Significance of findings

Spacing and pruning had a significant effect on the artemisinin concentration. The smaller spacing of 0.25 m^2 in combination with apical bud removal had the highest artemisinin concentration, these preserved artemisinin concentration by 65%. Spacing had a significant effect on stem diameter, fresh leaf mass and dry leaf mass but had no effect on plant height and chlorophyll content. Small spacing of 0.35 m^2 increased fresh leaf mass by 54%. Pruning had a significant effect on plant height and chlorophyll content and had no effect on stem diameter. Freeze, oven and 100% shade drying had a significant effect on artemisinin concentration. Freeze-drying preserved artemisinin by 23%.

Drying methods have also showed that they have a direct impact on artemisinin concentration. Freeze and oven dried samples had more artemisinin concentration and 50% shade drying had the lowest concentration. Freeze, oven and shade drying showed that they are efficient drying techniques that can be utilised by farmers.

4.3 Recommendations

The small spacing of 0.35 m^2 could be adopted for increased biomass. However, to obtain more artemisinin concentration smaller spacing of 0.25 m^2 and removing the apical bud may be adopted. Freeze, oven and 100% shade drying may be recommended for drying *A. annua*. Freeze and oven drying on a larger scale may be expensive to implement. Thus, it would be ideal to adapt 100% shade drying which would be more economical. More studies on pruning as a trigger for producing secondary metabolites in combination with spacing treatments should be validated under various conditions.

4.4 Conclusions

Cultural practices (spacing and pruning) showed potential in increasing the concentration of artemisinin, growth and yield of *A. annua*. Different drying methods (freeze, oven and 100% shade drying) also showed positive preserving qualities on artemisinin concentration. The results provided some understanding on how agronomic and processing practices can be used to increase artemisinin content in *A. annua* and understand the interaction between different agronomic practices and thereby allowing the development of economic methods for *A. annua* post-harvest handling.

REFERENCES

Abirami, K., Rana, V.S., Baskaran, V. and S. Maiti. 2014. Effect of plant spacing on herb, essential oil and artemisinin yields in the antimalarial herb (*Artemisia annua*). *In*dian *Journal of Agronomy* 59:163-167.

Abbiw, D., Köhler, I., Jenett-Siems, K., Kraft, C., Siems, K., Bienzle, U. and E. Eich. 2002. Herbal remedies traditionally used against malaria in Ghana: bioassay-guided fractionation of *Microglossa pyrifolia* (Asteraceae). Zeitschrift für Naturforschung. *Journal of Biosciences* 57:1022-1027.

Abdin, M.Z., Israr, M., Rehman, R.U. and S.K. Jain. 2003. Artemisinin, a novel antimalarial drug: Biochemical and molecular approach for enhanced production. *Planta Medica* 69:289-299.

Acton, N., Klayman, D.L. and I.J. Rollman.1985. Reductive electrochemical HPLC assay for artemisinin (qinghaosu). *Planta Medica* 51: 445–446.

Admasu, T. and P.C. Struikb. 2000. Influence of repetitive transplanting and leaf pruning on dry matter and food production of enset (Ensete ventricosum Welw. (Cheesman). *Field Crops Research* 68:61-74.

Ahmad, A. and M.Z. Abdin. 2000. Interactive effect of sulphur and nitrogen on the oil and protein concentrations and on the fatty acid profiles of oil in the seeds of rapeseed (*Brassica campestris* L.) and mustard (*Brassica juncea* L. Czern. and Coss.) *Journal of Agronomy and Crop Science* 185:49-54.

Aquil, S., Husaini, A.M., Abdin, M.Z. and G.N. Rather. 2009. Over expression of HMGCoA reductase gene leads to enhanced artemisinin biosynthesis in transgenic *A. annua* L. plants. *Planta Medica* 75:1-6.

Belay, B. 2007. Assessment of crop growth and artemisinin content of the medicinal plant *Artemisia annua* Anamed in the Gamo highlands of SW Ethiopia. MSc. Thesis, University of Hohenheim, Sttutgart.

Bertea, C.M., Freije, J.R., Van der Woude, H., Verstappen, F.W., Perk, L., Marquez,V., De Kraker, J.W., Posthumus, M.A., Jansen, B.J.M., De Groot, A.E., Franssen, M.C.R. and H.J. Bouwmeester. 2005. Identification of intermediates and enzymes involved in the early steps of artemisinin biosynthesis in *Artemisia annua*. *Planta Medica* 71:40-47.

Bora, K.S. and A. Sharma. 2011. The genus *Artemisia*: a comprehensive review. *Pharmaceutical Biology* 49:101-109.

Bormann, S., Szlezak, N., Faucher, J.F., Matsiegui, P.B., Neubauer, R., Biner, R.K., Lell, B. and P.G. Kremsner. 2001. Artesunate and praziquantel for the treatment of Schistosoma haematobium infections: a double-blind, randomized, placebo-controlled study. *The Journal of Infectious Diseases* 184:1363-1366.

Bouwmeester, H.J., Wallaart, T.E., Janssen, M.H., Van Loo, B., Jansen, B.J., Posthumus, M.A., Schmidt, C.O., De Kraker, J.W., Knig, W.A. and M.C. Franssen. 1999. Amorpha-4, 11-diene synthase catalyzes the first probable step in artemisinin biosynthesis. *Journal of Phytochemistry* 52:843-854.

Brown, G.D. 1994. Secondary metabolism in tissue culture of *Artemisia annua*. *Journal of Natural Products* 57:975-977.

Campbell, N.A. 1990. Biology. The Benjamin/Cummings Publishing Company, INC. Redwood City.

Chauhan, N.K., Gupta, S., Singh, S., Narayan, O. and H. Lohani. 2008. Effect of different plant spacings on biomass and oil yield of *Artemisia annua* L. *Journal of Medicinal Aromatic Plant Science* 30:270-272.

Charles, D.J., Simon, J.E., Shock, C.C., Feibert, E.B.G. and R.M. Smith. 1993. Effect of water stress and post-harvest handling on artemisinin content in the leaves of *Artemisia annua* L. In: Janick, J. and J.E. Simon, New Crops. New York.

Charles, D.J., Simon, J.E., Wood, K.V. and P. Heinstein. 1990. Germplasm variation in artemisinin content of Artemisia annua using an alternative method of artemisinin analysis from crude plant extracts. *Journal of Natural Products* 53:157–160

Chen, F.I. and G.H. Zhang. 1987. Studies on several physiological factors in Artemisinin synthesis in *Artemisia annua* L. *Plant Physiology Communications* 5:26-30.

Damtew, Z., Tesfaye, B. and D. Bisrat. 2011. Leaf, essential oil and artemisinin yield of *Artemisia* (*Artemisia annua* L.) as influenced by harvesting age and plant population density. *World Journal of Agricultural Sciences* 7:404-412.

Debrunner, N., Dvorak, V., Magalhães, P. and N. Delabays. 1996. Selection of genotypes of *Artemisia annua* L. for the agricultural production of artemisinin. Proceedings of the International Symposium on Breeding Research on Medicinal and Aromatic Plants. Quedlinburg, Germany, In: F. Pank Editors.

De Jesus-Gonzalez, L. and P.J. Weathers. 2003. Tetraploid *Artemisia annua* hairy roots produce more artemisinin than diploids. *Plant Cell Reports* 21:809-813.

Delabays, N., Benakis, A. and G. Collet. 1993. Selection and breeding for high artemisinin (qinghaosu) yielding strains of *Artemisia annua*. *Acta Horticulturae* 330:203-207.

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

De Ridder, S., Van der Kooy, F. and R. Verpoorte. 2008. *Artemisia annua* as a self-reliant treatment for malaria in developing countries. *Journal of Ethnopharmacology* 120:302-314.

Dong, N.H. and N.V. Thuan. 2003. Breeding of a high leaf and artemisinin yielding *Artemisia annua* variety (abstract). International Conference on Malaria: Current Status and Future Trends, Chulabhorn Research Institute, Bangkok, Thailand.

Duke, S.O., Vaughn, K.C., Croom, E.M. and H.N. ElSohly. 1987. Artemisinin, a Constituent of annual wormwood (*Artemisia annua*), is a selective phytotoxin. *Journal of Weed Science* 35:499-505.

Efferth, T., Dunstan, H., Sauerbrey, A., Miyachi, H. and C.R. Chitambar. 2001. The anti-malarial artesunate is also active against cancer. *International Journal of Oncology* 18:767-773.

Farooqi, A.H.A., Shukla, A., Sharma, S. and A. Khan. 1996. Effect of in *Artemisia annua* L. plant age and GA3 on artemisinin and essential oil yield. *Journal of Herbs, Spices and Medicinal Plants* 4:73-81.

Ferreira, J.F. 2007. Nutrient deficiency in the production of artemisinin, dihydroartemisinic acid, and artemisinic acid in Artemisia annua L*. Journal of Agricultural and Food Chemistry* 55:1686-1694.

Ferreira, J.F., Charles, D., Simon, J.E. and J. Janick. 1992. Effect of drying methods on the recovery and yield of artemisinin from Artemisia annua L. *Journal of Horticultural Science* 565:27-63.

Ferreira, J.F.S. and J. Janick. 1996. Distribution of artemisinin in *Artemisia annua. Progress in New Crops* 32:579-584.

Ferreira, J.F.S. and J. Janick. 2002. Production of artemisinin from *in vitro* cultures of *Artemisia annua* L. *Biotechnology in Agriculture and Forestry* 51:1–12.

Ferreira, J.F.S., Simon, J.E. and J. Janick .1995. Developmental studies of *Artemisia annua*: flowering and artemisinin production under greenhouse and field conditions. *Planta Medica* 61: 167 – 170.

Ferreira, J.F.S., Simon, J.E. and J. Janick. 1997. *Artemisia annua*: botany, horticulture, pharmacology. *Horticultural Review* 19:319-371.

Haynes, R.K. 2006. From artemisinin to new artemisinin antimalarials: biosynthesis, extraction, old and new derivatives, stereochemistry and medicinal chemistry requirements. *Current Topics in Medicinal Chemistry* 6:509-537.

Hoeft, M., Verpoorte, R. and E. Beck. 1996. Growth and alkaloid contents in leaves of *Tabernaemontana pachysiphon Stapf* (Apocynaceae) as influenced by light intensity, water and nutrient supply. *Oecologia* 107:160-169.

Jung, M. and R.F. Schinazi. 1994. Synthesis and in vitro anti-human Immune deficiency virus activity of artemisinin (Qinghaousu) related trioxanes. *Bioorganic and Medicinal Chemistry Letters* 4:931-934.

Kapoor, R., Chaudhary, V. and A.K. Bhatnagar. 2007. Effects of arbuscular mycorrhiza and phosphorus application on artemisinin concentration in *Artemisia annua* L. *Mycorrhiza* 17:581-587.

Klayman, D.L. 1993. *Artemisia Annua*: From Weed to Respetable Antimalarial Plant. In Human Medicinal Agents from Plants; Kinghorn, A.D. and M.F. Balandrin Editors.; ACS Symposium Series: Washington, DC.

Kumar, S., Gupta, S.K., Singh, P., Bajpai, P., Gupta, M.M., Singh, D., Gupta, A. K., Ram, G., Shasany, A.K. and S. Sharma. 2004. High yields of artemisinin by multi-harvest of Artemisia annua crops. *Industrial Crops and Products* 19:77-90.

Lachenmeier, D.W. 2010. Wormwood (*Artemisia absinthium* L.): A curious plant with both neurotoxic and neuroprotective properties? *Journal of Ethnopharmacology* 131:224-227.

Laughlin, J.C. 1993. Effect of agronomic practices on plant yield and antimalarial constituents of *Artemisia annua* L. *Acta Horticulturae* 331:53-61.

Laughlin, J.C. 1995. The influence of distribution of antimalarial constituents in *Artemisia annua* L. on time and method of harvest. *Acta Horticulturae* 390:67-73.

Laughlin, J.C., Heazlewood, G.N. and B.M. Beattie. 2001. Cultivation of *Artemisia annua* L. In: C.W. Wright Editors, *Artemisia*. Taylor and Francis: London.

Laughlin, J.C., Heazlewood, G.N. and B.M. Beattie. 2002. Cultivation of *Artemisia annua* L. In: Wright CW Editors, *Artemisia.* Taylor and Francis: London.

Leedy, P. D. and J.E. Ormrod. 2005. Practical Research: Planning and Design (8th ed.). Upper Saddle River, NJ: Prentice Hall.

Liersch, R., Soicke, H., Stehr, C. and H.V. Tullner.1986. Formation of artemisinin in *Artemisia annua* during one vegetation period. *Planta Medica* 52:387-390.

Liu, C.Z., Guo, C., Wang, Y. and F. Ouyang. 2003. Factors influencing artemisinin production from shoot cultures of *Artemisia annua* L. *World Journal of Microbiology and Biotechnology* 19:535-538.

Marchese, J.A. and G.M. Figueira. 2005. The use of pre and post-harvest technologies and good agricultural practices in the production of medicinal and aromatic plants. *Brazilian Journal of Medicinal Plants*, 7:86-96.

Madhusudanan, K.P. 1989. Mass spectral studies on artemisinin, dihydroartemisinin and arteether. *Indian Journal of Chemistry* 28:751-754.

Magalhães, P.M. 1996. Seleca˜o, melhoramento, e nutric¸ăo da *Artemisia annua* L. Para cultivo em regia˜o intertropical. PhD Thesis, University of Campinas, Brazil.

Martinez B.C. and J. Staba. 1988. The production of artemisinin in *Artemisia annua* L. tissue cultures. *Advances in Cell Culture* 6:69-87

Maynard, L. 1985. Malaria cure. Pharmacy Report. *The University of Mississippi School of Pharmacy* 7:10-13.

Muzemil, A. 2008. Determination of Artemisinin and essential oil contents of *Artemisia annua* L. grown in Ethiopia and In vivo Anti-malarial activity of its crude extracts against Plasmodium berghei in mice. Medicinal Chemistry Thesis, Addis Ababa University, Addis Ababa, Ethiopia 53

Newman, J.D., Marshall, J., Chang, M.C.Y., Nowroozi, F., Paradise, E., Pitera, D., Newman, K.L. and J.D. Keasling. 2006. High-level production of amorpha 4, 11-diene in a two phase partitioning bioreactor of metabolically engineered *Escherichia coli. Biotechnology and Bioengineering* 95:684-691.

Newton, P. and N. White. 1999. Malaria: New development in treatment and prevention*. Annual Review of Medicine* 50:179-192.

Nurgün, E., Orhan. l., Kartal, M., Adıgüzel, N. and B. Bani. 2007. Determination of Artemisinin in selected *Artemisia L*. Species of Turkey by Reversed Phase HPLC. *Records of Natural Products* 1:36-43.

Olliaro, P.L. and W.R. Taylor. 2004. Developing artemisinin based drug combinations for the treatment of drug resistant *falciparum* malaria: A review. *Journal of Postgraduate Medicine* 50:40.

Ozguven, M., Sener, B., Orhan, I., Sekeroglu, N. I., Kirpik, M., Kartal, M., Pesin, I. and Z. Kaya.. 2008. Effects of varying nitrogen doses on yield, yield components and artemisinin content of *Artemisia annua* L. *Industrial Crops* 27:60-64

Pras, N., Visser, J.F., Batterman, S., Woerdenbag, H.J. and T.M. Malingre. 1991. Laboratory selection of *Artemisia annua* L. for high artemisinin yielding types. *Phytochemical Analysis and Products* 27:60-64.

Ram, M., Gupta, M.M., Dwivedi, S. and S. Kumar. 1997. 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* 63:372-374.

Ro, D.K., Paradise, E.M., Ouellet, M., Fisher, K.J., Newman, K.L., Ndungu, J.M., Ho, K.A., Eachus, R.A., Ham, T.S. and J. Kirby. 2006. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* 440:940-943.

Romero, M.R., Efferth, T., Serrano, M.A., Castano, B.; Macias, R.I., Briz, O. and J.J. Marin. 2005. Effect of artemisinin/artesunate as inhibitors of hepatitis B virus production in an *'in vitro'* system. *Antiviral Research* 68:75-83.

Sarkka, L. and C. Erikson. 2003. Effects of bending and harvesting height combinations on cut rose yield in a dense plantation with high intensity lighting. *Scientia Horticulturae* 98:433-447.

Satyanarayana, N. and V.S. Sharma. 2004. Pruning and harvesting practices in relation to tea productivity in South India. Proceedings of the International

Seminar on Integrated Crop Management in Tea: Towards Higher Productivity Colombo, Sri Lanka, April 26-27, 2004.

Sen, R., Bandyopadhyay, S., Dutta, A., Mandal, G., Ganguly, S. and P. Saha. 2007. Artemisinin triggers induction of cell-cycle arrest and apoptosis in Leishmania onovani promastigotes. *Journal of Medical Microbiology* 56:1213-1218.

Shuoqian, L., Na, T., Zhonghua, L., Jianan, H., Juan, L. and F.S.F. Jorge. 2008. Affordable and sensitive determination of artemisinin in *Artemisia annua L*. by gas chromatography with electron-capture detection. *Journal of Chromatography* 1190:302-306.

Simon, J.E., Charles, D., Cebert, E., Grant, L., Janick, J. and A. Whipkey. 1990. *Artemisia annua* L.: a promising aromatic and medicinal. In: Janick, J. and J. Simon (eds.). Advances in New Crops. Timber Press: Portland, OR.

Singh, A., Vishwakarma, R.A. and A. Husain. 1988. Evaluation of *Artemisia annua* strains for higher artemisinin production. *Planta Medica* 7:475 –476.

Singh, N., Luthra, R. and R.S. Sangwan. 1990. Oxidative pathways and essential oil biosynthesis in the developing lemon grass (*Cymbopogon flexuosus*) leaf. *Plant Journal of Physiology and Biochemistry* 28:703-710.

Snow, R.W., Guerra, C., Noor, A.M., Myint, H.Y. and S.I. Hay. 2005. The global spectral light quality on growth, morphology and development of two stoloniferous *Potentilla* species. *Oecologia* 117:1-8.

Stuefer, J.F. and H. Huber. 1998. Differential effects of light quantity and spectral light quality on growth, morphology and development of two stoloniferous Potentilla species. *Oecologia* 117:1-8.

Tadesse, M. 2004. Asteraceae (Compositae). Hedberg, L., Friis, I.B. and S. Edwards (eds.). Flora of Ethiopia and Eritrea, Addis Ababa, Ethiopia.

Taiz, L. and E. Zeiger. 2006. Plant Physiology. MA Sinauer Associates Inc., Publishers, Sunderland.

Tarsisius, B., Byamukama, R. and N. Muhammad. 2013. Tests confirm suitability of Ugandan soils for commercial growing of *Artemisia annua* Linn. *African Journal of Agricultural Research* 8:4565-4572.

Teixeira da Silva, J.A. 2004. Mining the essential oils of the Anthemideae. African *Journal of Biotechnology* 3:706-720.

Uniyal, S., Nautiyal, M.C., Sah, Yadav, V.K., Ahlawat, V.P., Sehrawat, S.K. and P.S. Partap. 2010. Effect of different spacing and time of harvesting on vegetative growth and oil yield of Artemisia annua L. in Garhwal Himalayas. Haryana *Journal of Horticultural Sciences* 39:202-204.

Utzinger, J. and J. Keiser. 2004. Schistosomiatic and soil-transmitted helminthiasis: common drugs for treatment and control. *Expert Opinion on Pharmacotherapy* 5:263-285.

Van Wyk, B.E. and M. Wink. 2004. Medicinal Plants of the World; Briza Publications: Pretoria, South Africa.

Wallaart, T. E.; Pras, N. and W.J. Quax. 1999. Isolation and identification of dihydroartemisinic acid from *Artemisia annua*: A novel biosynthetic precursor of artemisinin. *Journal of Natural Products* 62:1160-1162.

Weathers, P.J., Arsenault, P.R., Covello, P.S., McMickle, A., Teoh, K.H. and D.W. Reed. 2011. *Phytochemistry Review* 10:173-183.

Weathers, P.J., Bunk, G. and M.C. McCoy. 2005. The effect of phytohormones on growth and artemisinin production in *Artemisia annua* hairy roots. *In Vitro Cellular and Development Biology Plant* 41:47-53.

WHO. 1988. The Development of Artemisinin and its Derivatives. WHO mimeographed document WHO/TDR/ CHEMAL ART 86.

WHO. 2003. *Assessment of the safety of artemisinin compounds in pregnancy*. http://www.who.int/malaria/cmc_upload/0/000/016/323/artem_pregnancy.html, 1–2. Accessed 15 September 2015.

WHO. 2005 a. Meeting on the production of artemisinin and artemisinin-based combination therapies. June 6–7, Arusha, United Republic of Tanzania. Accessed 18 August 2015.

WHO. 2005 b. Roll back malaria. World malaria report: section III global financing, commodities and services delivery. World Health Organization: Geneva. Available at: http://www.rbm.who.int/wmr2005/htm. Accessed 17 February 2015.

WHO. 2006. http://www.who.int/medicines/publications/traditional/Artemisia Monograph. Accessed 9 April 2015.

Willcox, M. 2009. *Artemisia* species: From traditional medicines to modern antimalarial and back again. *The Journal of Alternative and Complementary Medicine* 15:101-109.

Willcox, M., Bodeker, G., Bourdy, G., Dhingra, V., Falquet, J., Ferreira, J.F., Graz, B., Hirt, H.M., Hsu, E., de Magalhães, P.M. and D. Provendier. 2004. *Artemisia annua* as a traditional herbal antimalarial. In: Willcox, M.L., Bodeker, G. and P. Rasoanaivo (eds.). CRC Press: Boca Raton

Woerdenbag, H.J., Pras, N., Bos, R., Visser, J.F., Hendriks, H. and T.M. Malingré. 1991. Analysis of artemisinin and related sesquiterpenoids from *Artemisia annua* L. by combined gas chromatography/mass spectrometry. *Phytochemical Analysis* 2:215-219.

Woerdenbag, H.J., Bos, R., Salomons, M.C., Hendriks, H., Pras, N. and T.M. Malingre. 1993. Volatile constituents of *Artemisia annua* L (*Asteraceae*). *Flavour and Fragrance Journal* 8:131-137.

Woerdenbag, H.J., Pras, N., Chan, N.G., Bang, B.T., Bos, R., Uden, W., Van, Y.P., Boi, N.V., Batterman, S. and C.B. Lugt. 1994. Artemisinin, related sesquiterpenes, and essential oil in *Artemisia annua* during a vegetation period in Vietnam. *Planta Medica* 60: 272-275.

Zeng, Q.P., Qiu, F. and L. Yuan. 2007. Production of artemisinin by genetically modified microbes. *Biotechnology Letters* 30:581-592.

Zhao, S.S. and M.Y. Zeng. 1985. Spektrometrische hochdrck- flussigkeits-chromatographische (HPLC) untersuchungen zur analytic von qinghaosu. *Planta Medica* 51:233-237.

Zhang, Y.S., Ye, H.C., Liu, B.Y., Wang, H. and G.F. Li. 2005. Exogenous GA3 and flowering induce the conversion of artemisinic acid to artemisinin in *Artemisia annua* plants. *Russian Journal of Plant Physiology* 52:58-62.

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

Zhong, Y.C. 2007. Determination of artemisinin in *Artemisia annua* by the ultraviolet spectrophotometric method. *Journal of Chinese Medicine* 30:1257-1259.

Appendices

Appendix 3.1 Analysis of variance for reducing planting spacing and pruning different positions on concentration of artemisinin under field conditions.

Source	DF	SS	MS	F	P
Treatment	11	2.903	0.264	5.581	0.000
Error	24	1.135	0.047		
Total	35	4.038	0.311		

Appendix 3.2 Analysis of variance for drying methods on concentration of artemisinin from *Artemisia annua* plants after growing 180 days under field conditions.

Source	DF	SS	MS	F	P
Treatment	4	0.461	0.115	4.215	0.029
Error	10	0.274	0.027		
Total	14	0.735	0.142		

Appendix 3.3 Analysis of variance for reducing planting spacing (A) and pruning different positions (B) on fresh leaf mass of *Artemisia annua* under field conditions.

Source	DF	SS	MS	F	P
Rep	3	82518	27506		
Spacing (A)	3	543542	181181	3.49	0.026
Pruning (B)	2	34407	17203	0.33	0.720
A×B	6	371794	61966	1.19	0.334
Error	33	1714046	51941		
Total	47	2746307	339797		

Appendix 3.4 Analysis of variance for reducing planting spacing (A) and pruning different positions (B) on dry leaf mass of *Artemisia annua* under field conditions.

Source	DF	SS	MS	F	P
Rep	3	15150	5049.9		
Spacing (A)	3	188730	62909.8	4.60	0.009
Pruning (B)	2	18863	9431.5	0.69	0.509
A×B	6	159739	26623.1	1.95	0.102
Error	33	450841	13661.8		
Total	47	833322	11767.1		

Appendix 3.5 Analysis of variance for reducing planting spacing (A) and pruning different positions (B) on Stem diameter of *Artemisia annua* at weekly interval under field conditions (week 1 and week 2).

		Week 1				Week 2			
Source	DF	SS	MS	F	P	SS	MS	F	P
Rep	3	16.48	5.49			0.71	0.24		
Spacing (A)	3	68.33	22.78	1.35	0.28	241.66	80.55	4.54	0.01
Pruning (B)	2	4.09	2.05	0.12	0.89	36.33	18.17	1.02	0.37
A×B	6	165.15	27.53	1.63	0.17	194.73	32.46	1.83	0.12
Error	33	557.13	16.88			1059.16	17.75		
Total	47	811.18	74.73			1532.59	149.17		

Appendix 3.6 Analysis of variance for reducing planting spacing (A) and pruning different positions (B) on Stem diameter of *Artemisia annua* at weekly interval under field conditions (week 3 and week 4).

		Week 3				Week 4			
Source	DF	SS	MS	F	P	SS	MS	F	P
Rep	3	24.83	8.28			121.60	40.53		
Spacing (A)	3	398.18	132.72	7.13	0.00	454.43	151.477	6.19	0.001
Pruning (B)	2	47.52	23.76	1.28	0.29	84.59	42.294	1.73	0.193
A×B	6	82.15	13.69	0.74	0.62	97.13	16.189	0.66	0.681
Error	33	613.96	18.61			807.39	24.466		
Total	47	1166.63	197.06			1565.14	274.956		

Appendix 3.7 Analysis of variance for reducing planting spacing (A) and pruning different positions (B) on plant height of *Artemisia annua* at weekly interval under field conditions (week 1 and week 2).

		Week 1				Week 2			
Source	DF	SS	MS	F	P	SS	MS	F	P
Rep	3	0.016	0.005			0.055	0.018		
Spacing (A)	3	0.354	0.118	2.09	0.119	0.399	0.133	2.64	0.065
Pruning (B)	2	0.324	0.162	2.88	0.070	0.237	0.119	2.35	0.111
A×B	6	0.235	0.039	0.70	0.654	0.154	0.026	0.51	0.797
Error	33	1.857	0.056			0.166	0.050		
Total	47	2.785	0.38			2.506	0.346		

Appendix 3.8 Analysis of variance for reducing planting spacing (A) and pruning different positions (B) on plant height of *Artemisia annua* at weekly interval under field conditions (week 3 and week 4).

		Week 3				Week 4			
Source	DF	SS	MS	F	P	SS	MS	F	P
Rep	3	0.159	0.053			0.468	0.156		
Spacing (A)	3	0.080	0.027	0.67	0.577	0.018	0.006	0.13	0.944
Pruning (B)	2	0.519	0.259	0.50	0.004	0.227	0.113	2.40	0.107
A×B	6	0.259	0.043	1.08	0.394	0.391	0.065	1.38	0.253
Error	33	01.319	0.039			1.561	0.047		
Total	47	2.336	0.421			2.664	0.387		

Appendix 3.9 Analysis of variance for reducing planting spacing (A) and pruning different positions (B) on chlorophyll content of *Artemisia annua* at weekly interval under field conditions (week 1 and week 2).

		Week 1				Week 2			
Source	DF	SS	MS	F	P	SS	MS	F	P
Rep	3	57.41	19.135			146.711	48.904		
Spacing (A)	3	61.07	20.357	0.80	0.501	113.877	37.959	2.14	0.114
Pruning (B)	2	9.57	4.785	0.19	0.828	99.813	49.907	2.84	0.074
A×B	6	207.12	34.519	1.36	0.259	32.237	5.373	0.30	0.931
Error	33	836.67	25.354			586.277	17.767		
Total	47	1171.83	104.15			978.915	159.91		

Appendix 3.10 Analysis of variance for reducing planting spacing (A) and pruning different positions (B) on chlorophyll content of *Artemisia annua* at weekly interval under field conditions (week 3 and week 4).

		Week 3				Week 4			
Source	DF	SS	MS	F	P	SS	MS	F	P
Rep	3	136.47	45.489			80.02	26.672		
Spacing (A)	3	53.81	17.936	0.93	0.435	89.68	29.892	1.14	0.345
Pruning (B)	2	66.49	33.244	1.73	0.193	200.14	100.068	3.83	0.031
A×B	6	125.36	20.893	1.09	0.389	229.54	38.256	1.46	0.221
Error	33	633.76	19.205			861.77	26.114		
Total	47	1015.87	136.767			1461.14	221.002		