2. MATERIAL & METHODS

Agronomical studies were carried out in three regions of India: Gujarat, Madhya Pradesh and Uttarakhand with the objective and planned experiments were carried out on the high yielding plant variety "Jeevan Raksha" of *A.annua* to optimize the agrotechnological process to maximize the yield of herbage and the artemisinin in a single season in one harvest. So that the method, sequence and optimized scheduling approach can be developed crop with varying season.

Experiments for optimizing spacing distance in the field, fertilizer treatment, application of micro nutrient, growth hormones and stimulants to increase yield were carried out. Cultivation techniques included nursery preparation, field preparation, transplantation, weed control, fertilizer application, irrigation, harvesting time and post harvest techniques. Tissue culture studies were performed for callus induction, shoot induction, shoot multiplication, shoot elongation and induction of roots.

The study is categorized in three parts:

I. Agronomical studies (*in vivo*)
II. Post harvest studies
III. Tissue culture studies (*in vitro*)

I. Agronomical studies (*In vivo*)

Three field experiment were conducted on Agrotechnique to enhance the productivity of *A. annua* L. in vertisol of Madhya Pradesh, sandy soil of Gujarat and loamy soil of Uttrakhand, under irrigation condition during Rabi Season of 2009-10, and 2010-11 at Ratlam (Madhya Pradesh),Vadodara (Gujarat) and Dehradun (Uttarakhand).

Crop and Variety

An improved high yielding variety of *A. annua* L. "Jeevan Raksha" was used for the present study.

The studies on effect of fertilizer dose, spacing, density, growth regulators and stress and planting time were done at all the three sites. The details of technique adopted in the present investigation are described below.

Experiment sites

The experiments were conducted in farmer's field at Ratlam (site 1), Vadodara (site 2) and Dehradun (site 3).

Site 1. Ratlam, Madhya Pradesh

Geographically, Ratlam is Situated at 23º 31` North latitude and 75º 07` East Longitude with an altitude of 480 Meter above mean sea level. In Madhya Pradesh, Ratlam is located in the west part of M.P. having sub tropical climate with hot dry summer and cool in winter. Ratlam falls in Malwa agro climatic Zone of Madhya Pradesh.

Ratlam, like most of Madhya Pradesh, has humid subtropical climate zone. Three distinct seasons are observed: summer, monsoon and winter. Summers starts in mid-March and can be extremely hot from April through June. Temperature can reach 112 °F (44 °C), although the humidity is extremely low. The monsoon season starts in late June, with temperatures averaging around 100 °F (38 °C), with sustained, torrential rainfall and high humidity. The average rain fall is 37 inches (937 mm). Winters start in mid-November and are dry, cool and sunny. Temperatures average about 39–46 °F (4–8 °C), but can fall close to freezing points on some nights. Ratlam gets moderate rainfall of 35 to 38 inches (890 to 970 mm) from July through September, due to the southwest monsoon.

Site 2. Vadodara, Gujarat

Geographically, Vadodara is situated at 22º 49` North latitude and 77º 51` East Longitude with an altitude of 37.5 Meter above mean sea level. In Gujarat, Vadodara Is located in the

middle part of Gujarat having sub tropical climate with hot dry summer and cool in winter. Vadodara falls in Middle Gujarat agro climatic Zone of Gujarat.

Vadodara features a tropical savanna climate under Koppen's Climate classification. There are three main seasons: Summer, Monsoon and winter. Aside from the monsoon season, the climate is dry. The weather is hot through the months of March to July – the average summer maximum is 40 °C (104 °F), and the average minimum is 23 °C (73 °F). From November to February, the average maximum temperature is 30 °C (86 °F), the average minimum is 15 °C (59 °F), and the climate is extremely dry. Cold northerly winds are responsible for a mild chill in January. The southwest monsoon brings a humid climate from mid-June to mid-September. The average rainfall is 37 in (930 mm).

Site 3. Dehradun, Uttarakhand

Geographically, situated at 30º 19` North latitude and 78º 03` East Longitude with an altitude of 636 Meter above mean sea level. In Uttrakhand, Dehradun is located in Garhwal region with hot dry summer and cool in winter. Dehradun falls in West Himalayan Region (1) agro-climatic Zone.

The climate of Dehradun is humid subtropical, although it varies from tropical: from hot in summers to severely cold, depending upon the season and the altitude. The nearby hilly regions often get snowfall during winter. Temperature in Dehradun can reach below freezing during severe cold snaps. Summer temperatures can reach up to 44 °C for a few days and hot wind called Loo blows over North India, whereas winter temperatures are usually between 1 and 20 °C and fog is quite common in winters like plains. During the monsoon season, there is often heavy and protracted rainfall. Dehradun and other plains areas of Uttarakhand see almost as much rainfall as coastal Maharashtra and more than Assam. Average rain in 2208mm (86.9 in).

a. Propagation method

The plants were raised through seeds, which were sown in month of September, October, November & December. The seeds were germinated in nursery beds. They were transplanted to field when they attain a height of 10-15 cm or bearing 8 to 10 whorls of leaves.

b. Nursery preparation

For raising the seedlings, seeds were sown in the raised nursery beds. The nursery beds were made as raised bed, so that they are not flooded any time. Assured irrigation facility was provided.

The beds were 1.0 m – 1.25 m. wide to facilitate hand weeding and watering with rose cans. While preparing bed, well-decomposed farmyard manure was mixed with top layer of the soil. Seeds were sown on the surface of these beds. The beds were kept moisten until the seeds germinate. The seeds germinated in 8-10 days. About 40 to 50 g. seeds were sufficient for raising the seedlings required, for planting in one hectare of land. The seedlings were transplanted after 45 to 55 days depending upon site.

c. Field preparation

The land was ploughed and diced three times, to produce a fine tilth before the seedling was transplanted after applying the required dose of manure & fertilizers. For convenient management the field was laid out into ridge and furrow method, the ridge and furrows were made in 'V' shape above 60 cm apart and about 20-25 cm deep.

d. Transplantation

The seedlings were transplanted in rows that are 60 cm. Plant-to-plant distance vary from 45-60 cm depending upon the soil and climate condition. Seedlings were planted in late

October, November to early December (Ratlam & Vadodara) and mid January to February (Dehradun). Field was first irrigated one day prior to planting and after the seedlings were transplanted the field was again irrigated.

e. Field management: Weed control

The field was kept free of weeds by manual weeding or by mechanical weeder. 2-3 weeding were required at early stage of plant growth.

f. Harvesting Schedule

The duration of the crop is 4 months.. Only one cutting was taken after 110 to 120 days from the date of transplanting. Plants were cut once they reach a height of 150-180 cm.

g. Drying and storage

The harvested material of *A.annua* was dried in shade and leaves were separated manually. The dried leaves were stored in 'Jute' bags. The dry leaves having moisture of 8-10 % were stored in dry place.

h. Harvesting, Threshing and sieving

Before harvest different observation were recorded. The crops were harvested 110 to 120 Days after transplanting. The bundle weight i.e. biological yield of net plot was recorded with the help of electronic balance. Then cache bundle was dried and threshed sparsely with the help of bamboo stick.

i. Parameters studied

Five plants were tagged at random in each plot area for recording observation on various growth and yield components at 30, 60, 90 and 120 days after transplanting (DAT) at harvest.

These selected plants were tagged as per treatment. All pre harvest observation was recorded from these selected plants.

a. Plant height (cm)

Height of 80 randomly selected plants for each treatment was measured from base of the plant to tip of the plants. Average of height was worked out from the observations. This observation was recorded at 30, 60, 90, and 120 DAT at harvest days after transplanting

b. Fresh Weight/ Fresh shoot weight

Weight of the shoot (from base of the plant to growing tip) was taken soon after harvesting and was converted in tons / ha. For dry leaves weight, plant was separated by cutting at the base of ground and air dried for 5 days then the leaves were separated. From this dry leaves yield tons per hectare was calculated.

c. **Stalk yield (Tons ha-1)**

The stalks (after removing the leaves) from each net plot area were dried completely in the sun for a week and the weight was recorded. Based on the stalk yield form net plot area, the stalk yield per hectare was calculated and expressed in tons per hectare.

d. Dry leaf yield (Tons ha-1)

It is the yield obtained from each plot and converted in to yield per hectare. Selected plants in the net plot were harvested and dried in shade separately. After drying, dry leaves were remove from stalk and sieved. Then dry leaf yield per hectare was estimated using the factor and expressed as tons per hectare.

e. Total Artemisinin content %

Artemisinin content was determined from dry leaves of each plot. Composite dry samples were taken and crude extract was extracted with n-hexene. Then Artemisinin content was analysed and quantified by HPTLC (Shimatzu) from crude extract according to procedure given by (Gupta *et al*., 1996) and expressed in w/w dry basis.

Estimation of artemisinin in leaves by high perfomance thin layer chromatograpghy (HPTLC)

f. Extraction of artemisinin

Extraction of artemisinin was carried out using the microwave assisted method. 100 mg oven dried plant material was taken for extraction of artemisinin. It was extracted with 20 ml hexane in microwave at 200 mV for 2 min. After 2 min, solvent was decanted and pooled and 20 ml of hexane added again and this step was repeated 3 times. Hexane fraction pooled and concentrated under reduced pressure. Residues were dissolved in 1 ml of methanol.

g. Chromatographic conditions

Chromatographic estimations were performed under the following conditions: stationary phase, precoated silica gel 60 F254 aluminium sheets (20 × 20 cm), which were immersed in methanol for 1 h to remove impurities, the plates were then dried in an oven at 100 ±°C for 3 min and stored in a desiccator until needed; mobile phase, hexane: ethyl acetate (9:1 v/v); chamber saturation time, 40 min; detection wavelength, 540 nm; scanning speed, 20 mm s-1; data resolution, 100 µm step-1 and slit dimensions, 5 × 0.2 mm. The following spotting parameters were used: bandwidth, 4 mm; space between two bands, 6 mm. Experimental conditions; temperature 30 ± 3 °C, relative humidity 55 ± 2%.

h. Preparation of calibration curve

1 mg of standard artemisinin dissolved in 1 ml of HPLC-grade methanol to make the stock solution. The calibration curve consisted of five different concentrations of artemisinin over the range 500–1700 ng. Aliquots of 0.5µl, 0.8µl, 1.1µl, 1.4µl and 1.7µl of standard solutions were spotted on a precoated TLC (20 × 10 cm) plate, using a semiautomatic spotter under a flow of nitrogen gas. The plate was air dried and developed with hexane: ethyl acetate (9:1 v/v) as the mobile phase in a Camag twin-trough chamber previously saturated with the mobile phase for 40 min. The plate was removed from the chamber and dried in air. The dried plate was sprayed with freshly prepared anisaldehyde reagent (0.5 mL anisaldehyde in 10 mL of glacial acetic acid, 85 mL absolute ethanol and 5 mL of 97% of H2SO4), followed by heating for 5 min at 110ºC. The densitometric determination of artemisinin was carried out after derivatization. Scanning and quantification of spots was performed at 540 nm in absorbance/reflectance mode with Camag TLC scanner 3 using Win CATS 1.3.2 software. The curve was prepared by plotting the artemisinin peak area versus its concentration (ng spot-1) corresponding to each spot.

i. Estimation of artemisinin

Methanolic extracts of each sample were applied on a TLC plate under a flow of nitrogen gas using a semiautomatic spotter. The TLC plate was developed and analysed as described under the chromatographic conditions. The amount of artemisinin present in the sample solutions were determined by fitting the area values of peaks corresponding to artemisinin into the equation of the line representing the calibration curve of artemisinin. All determinations were performed in triplicate. Content of artemisinin was expressed as % on dry weight basis.

j. Dry leaf Stalk Ratio/ Leaf: stem ratio (w/w)

It was calculated on basis of weight of leaves and stem recorded from dry plants in each treatment.

k. Harvest index

The harvest index (HI) or sink coefficient is the ratio between economic produce to the above ground bio-mass at harvest and is expressed in percentage.

Harvest Index (%) = Leaves yield x 100/ Biological yield.

Artemisinin yield (Kg/ ha)

It is the yield obtained from harvestable net plot and converted in to yield per hectare basis on Artemisinin content and leaf biomass.

$$\text{Artemisinin Yield (Kg/ha)} = \frac{\text{Artemisinin Content (w/w) X Dry Leaf Yield (Kg/ha)}}{100}$$

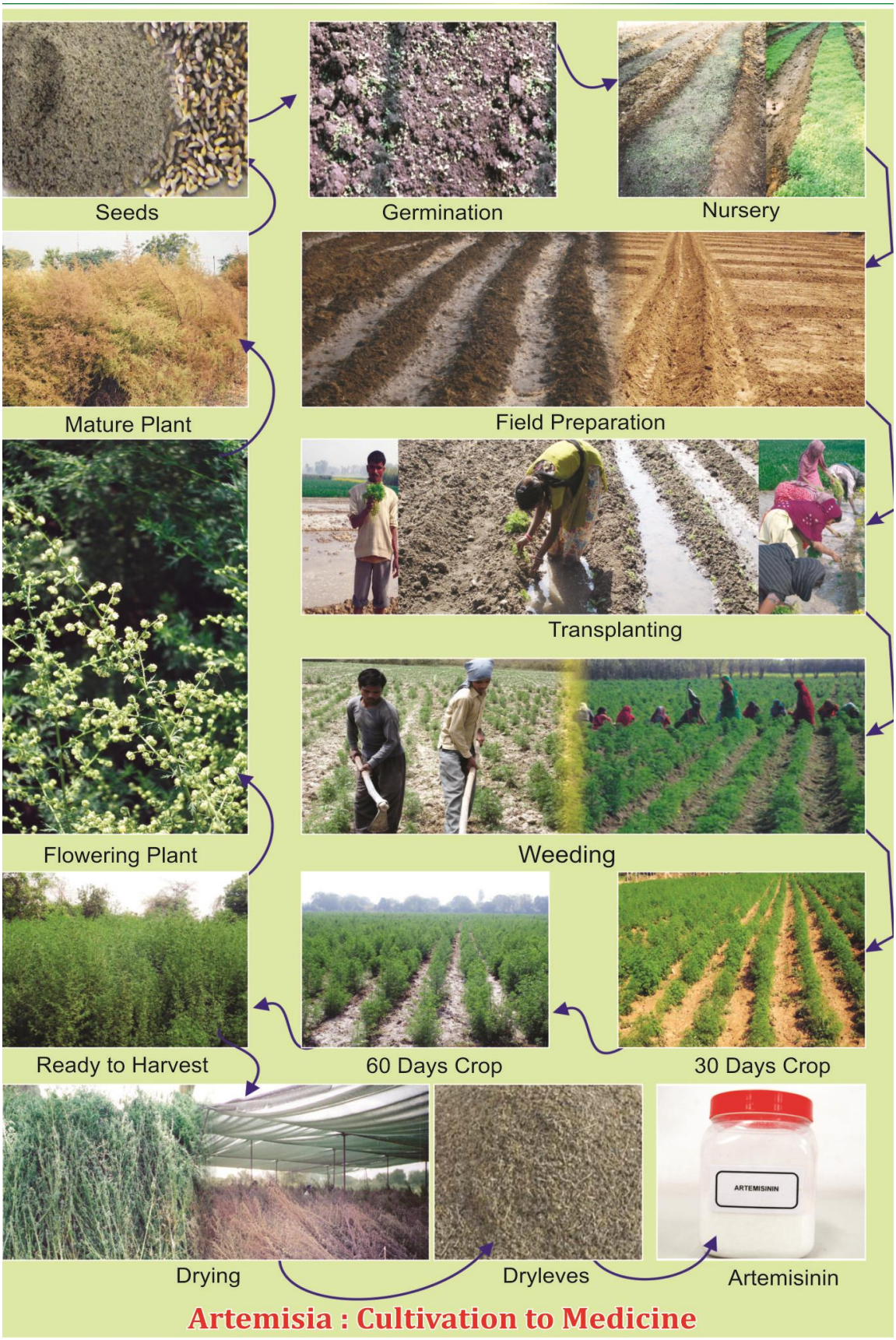

Fig 2.1: Methodology of cultivation of *A.annua*

Experiment 1: Effect of fertilizer treatments on different parameters of *A.annua*.

The crop was provided with proper nutrients in the form of inorganic fertilizers.

Manures and Fertilizers

CAN, S.S.P., M.O.P and Sulphur (agriculture grade) were used as source of nitrogen, phosphorus, potash and Sulphur and applied treatment wise total fertilizer application

Nitrogen	3 splits
Phosphorus	01 time
Potash	01 time
Sulphur	01 time.

Experiment was laid out in randomized block design. The following treatment was applied four times in 16 plots. Plot size was 4.5 x 4.5 = 20.25 m^2 (15Lines). Spacing was kept 03m row to row and 0.3m plant to plant (to maintain 111111Plant /ha.).

(1) T1= control (no fertilizer use)

(2) T2= NPK: 100:60:40 Kg/ha

(3) T3= NPKS: 100:60:40:40 Kg/ha

(4) T3= NPKS: 80:40:40:40 Kg/ha

Height, fresh herb yield, dry stem yield, dry leaves yield, % artemisinin, dry leaf/ stem ratio, leaf harvest index and artemisinin yield were measured for observing the impact of fertilizers.

Experiment 2: Effect of plant population density on different growth parameters of *A.annua.*

Experiment was laid out in randomized block design and plan of layout is depicted in table 2.1 below. Height, fresh herb yield, dry stem yield, dry leaves yield, % artemisinin, dry leaf/ stem ratio, leaf harvest index and artemisinin yield were measured for observing the impact of the density.

Sr.no	Treatment	Spacing	Number of plant per hectare	No of plants m^2
1	T1	100X100	10,000	1
2	T2	75 X 75	17777	2
3	T3	60 X 60	27777.77	3
4	T4	45 X 45	49382.71	5
5	T5	30 X 30	111111	11
6	T6	50 X 30	66666.66	7

Table 2.1: Layout plant for plant population density

Experiment 3: Effect of growth regulators and stress on different growth parameters of *A. annua*.

1. **Field studies:** Experiment was laid out in randomized block design and treatments and dose details are given in table 2.2 below. Height, fresh herb yield, dry stem yield, dry leaves yield, % artemisinin, dry leaf/ stem ratio, leaf harvest index and artemisinin yield were measured for observing the effect of different elicitors. The treatments were applied 12 times with 4 replications on 48 plots. Plot size was 4.5 x 4.5 = 20.25 m^2 (15Lines), Distance between plots was kept 1m. Spacing between two rows was kept 0.3m and plant to plant space was also kept 0.3 m to maintain 111111Plant /ha.

Treatment	Elicitors	Dose
T1	Abscisic Acid	10µM/lit
T2	Gibberlic Acid	10µM/lit
T3	Gibberlic Acid+ Indole Acetic Acid	10µM/lit +10µM/lit
T4	Chitosan	150mg/lit
T5	Methyl Jasmonate	4.85µM/lit
T6	Acetyl Salicylic Acid	1mM
T7	Lead acetate	500µM/lit
T8	Sodium Chloride	160mM
T9	Nanozime	1ml/lit
T10	Pushak	1ml/lit
T11	Fantac	1ml/lit
T12	Control	(No Spray)

Table 2.2: Dose details of treatments applied.

2. Micromorphological studies: Leaf micromorphology was studied to observe the effect of different elicitors on trichome.

Light microscopy - Leaves collected from treated and control plots both, were kept in 50% Jeffrey's solution for one day. The epidermal layers were separated carefully using sharp needles, stained with 1% safranin and mounted in 50% glycerine. The sections were observed under light microscope. Microphotograph was taken on (Leica DME) microscope.

Scanning electron microscopy - Fresh samples of leaves was examined under ESEM (Environmental scanning electron microscope) without any processing and photographs were taken.

Artemisinin content. - Sample consisted of leaves in vegetative stage were collected from the apical portion of the 50 randomly selected plants. For extraction, the samples of leaves were shade dried and pulverized. Hexane extracts were prepared from 0.1g of each sample. Concentration of artemisinin was determined by HPTLC method described by Gupta *et. al*, (1996).

Experiment 4: Effect of planting time on growth parameters of *A.annua.*

The treatments consisted of date of transplanting of *A.annua* at monthly intervals over a period of different cropping seasons. Seedlings were transplanted when they were 45 days old. Transplanting was done in plots of 3m x 2m replicated thrice in a randomized blocked design at a spacing of 50cm x 30cm. the recommended dose of compound fertilizers, N:P:K at 50:50:50 kg/ha, respectively was applied. Data collection for field experiment was carried out by taking random sample of net plots, ten plants were randomly selected. These selected plants were tagged as per treatment. All pre harvest observation was recorded from these selected plants. Height of ten randomly selected plants for each treatment was measured from base of the plant to tip of the plants. Average of height was worked out from the observations. This observation was recorded at 30, 60, 90 and 120 days after transplanting. The AMS% (Artemisinin) was checked at regular intervals of 15 days. It was extracted with 20 ml Hexane fraction pooled and concentrated under reduced pressure. Residues were dissolved in1 ml of methanol. Scanning and quantification of spots was performed at 540 nm in absorbance/ reflectance mode with Camag TLC. Methanolic extracts of each sample were applied on a TLC plate. The TLC plate was developed and analyzed for artemisinin present in the sample solutions. All determination was performed in triplicate. Content of artemisinin was expressed as % on dry weight basis.

II. Post harvest studies

Experiment 5: Effect of shade drying and oven drying on artemisinin content of *A.annua.*

Artemisinin is unstable due to its endoperoxy group and the chemical analysis is difficult. Most secondary products can be altered by the environmental factors and the post harvest handling practices, yet little is known about the stability of artemisinin when subjected to either pre- or post harvest environmental changes. The objective of this study was to examine the influence of drying techniques on the retention of artemisinin.

Two drying treatments were examined that is shade drying and oven drying at 40◦c. To reduce error from interplant variation, composite sample consisting of branches from same plants were used for both treatments. Leaves from each treatment were evaluated for moisture and artemisinin content. All artemisinin concentrations reported were based on the harvest of all leaves from whole plants.

Fresh leaves of *A.annua* which were used in research were harvested from the field. The harvested leaves were randomly divided into three set of 100g each. Then they were stored in a refrigerator at a temperature of 4±0.5˚C before the drying experiments. To measure the initial moisture content, the leaves were dried using an oven (60˚C) until there was no change in weight between the two consecutive measurements. This process was repeated five times.

1. Drying methods: the drying was carried out until reaching moisture content of about 9% on the wet basis in all drying treatments; the weight loss was measured using an analytical balance. All treatments were replicated 3 times.

1.1 Shade drying: this method was performed in a shade and dry room with appropriate ventilation. The temperature of the room was 25±2˚C while relative air humidity varied within the range of 22-27%

1.2 Oven drying: A convection oven, capacity: 45kg, relative humidity: 38±4%. 60˚C was used to dry samples.

Experiment 6: GC-MS studies

Green leaves of *Artemisia annua* L. were collected from 10 different geographical places in India: A-1 (Uttar Pradesh), A-2 (Jaora, Madhya Pradesh), A-3 (Uttarakhand), A-4 (Baroda, Gujarat), A-5 (Andhra Pradesh), A-6 (salinity experiment performed at Karakhadi, Gujarat). Field preparation like nursery preparation, transplantation, irrigation, drainage and harvestingwas done after 120 days from date of transplanting in field for all the locations. Therefore, the phonological stage of the leaves was maintained to be same in all the locations. Hence the variable for the present study was only the geographical location. Collected leaves were shade dried and pulverized to get powder of leaves. The powdered leaves were stored in a close-tight container until used for extraction.

a. Chemicals

All the solvents used in this study were of analytical grade and were purchased from S. D. Fine Chemicals, Mumbai (India).

b. Extraction of dry leaves samples

Extraction of leaves powder (1g) performed by soxhlet extraction by using n-hexane as per the reported method [13], obtained n-hexane extract was concentrated to dryness under vacuum on a rotary evaporator. The same procedure was adopted for the extraction of all the six leaves samples collected from different geographical locations of India. The sample extracts thus prepared were stored in sealed vials at 4°C until GC-MS analysis.

c. Sample preparation for GC-MS analysis

Extracted samples, stored under refrigeration condition, were taken out and allowed to get normalized with the atmospheric temperature. A part of extract was dissolved in chloroform and 1µl volume was injected for GC-MS analysis.

d. Instrument and gas-chromatographic-mass spectrometric conditions

GC-MS analysis was carried out using a Perkin-Elmer Auto System XL gas chromatograph coupled to a Turbo Mass spectrometer Detector (MSD). Volatiles were separated on fused silica capillary column (30m length, 250 µm diameter, 0.5µm thickness). The oven temperature was set at 70°C and hold for 5 minutes, then raised to 280°C at the rate of 10°C/minute and hold at this temperature for 20 minutes. The on-column injector (injection volume 1µl) was heated up to 250°C. The detector's EI source temperature was 220°C and helium gas was used as a carrier gas at rate of 1ml/min. 1µl volumes were injected in split less mode. Peak areas and retention times were measured through electronic integration. The EI-energy was 70 eV. EI-mass spectra were recorded in the 20–620 amu range.

e. Identification and quantification of components

Components detected were identified by comparing mass spectral data of samples with those of NIST and NBS standard database hits provided with GC-MS analysis. The name, molecular weight and structure of the compounds present in the test sample extracts were ascertained by comparing the mass spectra, mass fragmentation pattern with the known compounds either using computer searches on NIST and NBS library or with the help of published literature. The quantitative estimation of each peak obtained in GC was made by computer attached with GC-MS instrument.

Experiment 7: Studies on utilizing *Artemisia* leaves after extraction of artemisinin.

Large quantity of dry leaves after extraction of artemisinin is produced as waste material. The potential usage of these extracted leaves was studied for its beneficial value. Waste produced after extraction of artemisinin can be used in several ways. In present study, it was used to make Bio-briquette. Along with *Artemisia* leaf waste, other wastes obtained from industries like soybean waste and coal waste were also used in making briquettes. They can be used in many industries as a replacement of coal to run boilers. Other use of the waste biomass was estimating a highly resistible secondary metabolite from *Artemisia* leaves known as Scopoletin.

A. Briquette making

Generally 3 types of briquetting presses:

1. Mechanical presses

2. Hydraulic presses

3. Screw presses.

In the production of briquettes for pilot testing, the material used was *Artemisia* leaves, soybean waste, agriculture waste and wooden waste.

Process

Preparation of the raw material: raw material is collected, assembled in a central location for processing.

Size reduction: the raw material was first reduced in size by chopping, crushing, breaking, rolling, hammering, milling, grinding, cutting, etc, until it could pass through a screen or reaches a suitably small and uniform size.

a .Drying: it was done in the sun, with a heater or by using heated air and a rotating drum.

b. Preparation of feed stock: raw material was mixed with the binder to produce feed stock. Correct proportion of raw material to binder was maintained.

c.Carbonization: a biomass material is heated to very high temperature, this process produces charcoal. The briquettes can be made using charcoal finds or ash as part of the feed stock, or the briquettes can be compacted with any of the feed stock.

d.Compaction: The next step, a supply of prepared feed stock is loaded into a chamber, the chamber is covered with a close fitting top, and pressure is applied to compress the feed stock. The pressure applied was 0.5 to 1200 kg/sq cm.

B. Extraction of Scopoletin

A Camag TLC system equipped with Camag Linomat V, an automatic TLC sample spotter, Camag glass twin trough chamber (20 X 10 cm) was used for the analysis. Chromatography was performed using pre-activated (60 oC for 5 min) silica gel 60F254 TLC (20 X 10 cm; layer thickness 250 μm) (Merck, Darmstadt, Germany). Samples and standards were applied on the 6 mm wide bands with an automatic TLC sampler under a flow of N2 gas, 10 mm from the bottom and 10 mm from the side and the space between two spots were 15 mm. The linear ascending development was carried out in a Camag twin trough chamber saturated with 20 ml mobile phase (chloroform: methanol: toluene, 8:1:1, v/v/v) for 20 min at room temperature (25 ± 2 oC and 40% relative humidity). They were developed up to 8 cm under chamber saturation conditions. Subsequent to the development, TLC were dried in current air with the help of a hair dryer. Evaluations of were performed with Camag scanner 3 (win CATS 4.0 integration software). Densitometric scanning was performed at 360 nm (absorption-reflection mode), using a slit width of 6 X 0.45 mm, data resolution 100 μm step and scanning speed 20 mm/s with a computerized Camag TLC scanner. Peak areas were

recorded and the amount of Scopoletin was calculated using the calibration curve. Quantification was done by using external standard method.

a. Preparation of extract

Accurately weighed 5 g of previously extracted Artemisia sample was again extracted with methanol (4 X 50 ml) under reflux (30 min each time). The combined extracts were filtered, concentrated and transferred to a 50 ml volumetric flask and the volume was made up with same solvent.

b. Preparation of standard Scopoletin solution

A stock solution of Scopoletin (Himedia Laboratories Pvt. Ltd., Mumbai, India) (5 mg/50 ml) was prepared in methanol. Working solutions were prepared by appropriate dilution of the stock solution with the same mixture of solvents.

c. Calibration curve for Scopoletin

Standard scopolentin solution in the range of 200 to 700 ng spot-1 was applied on TLC for preparation of calibration curve of peak area versus concentration.

III. Tissue culture studies (In vitro)

a. Plant Materials

The seeds and leaf explants of A. annua L. were collected from the experimental field of Ipca Laboratories Ltd. Ratlam, M.P., India. The seeds were germinated on solidified half strength MS medium under controlled environmental conditions to raise mother plants as source of explants to establish and optimize regeneration and multiplication protocols. The leaf

explants collected from the experimental field, regenerated directly on optimized culture medium.

b. Chemicals

All chemicals and hormones were procured from Himedia Laboratories (India) and Sigma-Aldritch (USA). Sucrose and agar were procured from Himedia Laboratories, India.

c. Nutrient media

The composition of different culture medium is given in annexure I.

Major components	Stock solution (mg/L)	20X (g)
NH4NO3	1650	33
KNO3	1900	38
CaCl2. 2H2O or CaCl2	440	8.8
MgSO4. 7H2O	370	7.4
KH2PO4	170	3.4
Minor components	Stock solution (mg/L)	100X (mg)
CoCl2.6H2O	0.025	2.5
CuSO4.5H2O	0.025	2.5
H3BO3	6.2	620
KI	0.83	83
MnSO4.4H2O	22.3/16.9	2230
Na2Mo.O4.2H2O	0.25	25
ZnSO4.7H2O	8.4	840
FeEDTA	Stock solution (mg/L)	100X (g)
FeSO4	27.8	2.78
Na2EDTA.2H2O	37.3	3.73
Vitamins	Stock solution (mg/L)	100X (mg)
Myo-inositol	100	10000

Nicotinic acid	0.5	50
Thiamine HCl	0.1	10
Pyridoxine HCl	0.5	50
Glycine	2	200
Sucrose	30 g/L	
pH	5.8	
Agar	8 g/L	

The medium was poured in culture vessels and then culture vessels were sterilized at 15 lbs/inch2 and 121°C for 20 minutes in an autoclave.

Table 2.3: Composition of Murashige and Skoog's basal medium (1962)

d. Methods

1. Culture Conditions

All the aseptic manipulations were carried out under laminar air flow chamber. Usual precautions were taken to maintain aseptic conditions inside the cabinet. After inoculation, all the culture vessels were kept in culture room at 25+2ºC and 55% humidity.

2. Selection and sterilization of explant from *A. annua* L.

The leaf and shoot tips of A. annua L. were selected as explant for callus and shoot induction. They were washed under running tap water for 30 min and surface sterilized in 5% solution of teepol for 5-10 min. After surface sterilization the explants were washed with 1% solution of bavistin (an antifunfal agent) for 5-7 min. Thereafter, the explants were washed with 0.25% solution of streptomycin (an antibacterial agent) for 3-5 min and then these were washed 2-3 times with sterile DDW for 2 min. The explants also washed with 5-10% solution of hypochloride for 5-7 min and 0.1% solution of HgCl2 for 5 min and then these were

washed with 70% ethanol for 1 min. Finally, the explants were washed with sterile DDW for 3-5 times. After sterilization, these explants were transferred on regeneration medium.

3. *In vitro* seed germination and preparation of explants of *A.annua* L.

Seeds of high artemisinin yielding strain of *A.annua* L. (approx. 1% artemisinin) were surface sterilized for 5 min in 0.1% solution of mercuric chloride. The seeds were thereafter, washed four times in sterile distilled water and germinated in culture tubes containing half strength MS Basal medium. The pH was adjusted to 5.8 with NaOH before the addition of agar. The medium was autoclaved at 121ºC for 20 min. Germination started within 2 to 3 days. After one week, seedlings were transferred onto full strength MS basal medium. Leaves from 30 days old plantlets were used as explants for regeneration. The cultures were maintained at 25±2ºC, light intensity of 2300 lx and 16hr light and 8hr dark periods.

4. Regeneration of *A. annua* L. plants

A. annua L. plants were regenerated via callus or direct shoot induction from leaf explants.

e. Callus induction

To optimize the culture medium for high frequency callus induction, the whole leaves were cultured with their adaxial surface touching the callus induction medium containing MS salts, vitamins, sucrose (3%), agar (0.8%) and supplemented with different concentrations and combinations of BAP (0.5-1.5 mg/l), NAA (0.5-1.5 mg/l) and Kn (0.5-1.5 mg/l). The explants were maintained under culture room conditions (temperature, 25± 2ºC; light intensity, 2300 lux; photoperiod, 16/8 h day/ night).

f. Shoot induction

One-month old plantlets that were maintained under in vitro conditions on MS basal medium were taken as source of leaf explants or leaf and shoot tip taken from experimental field as explant for direct shoot induction. The explants were derived from fully expanded green leaves of plantlets maintained under culture room conditions (temperature, 25± 2ºC; light intensity, 2300 lux; photoperiod, 16/8 h day/ night). To optimize the culture medium for high frequency shoot regeneration, the whole leaves were cultured with their adaxial surface touching the shoot induction medium containing MS salts, vitamins, sucrose (3%), agar (0.8%) and supplemented with different concentrations and combinations of amino acids, BAP (0.5-2.0 mg/l) and NAA (0.01-0.05 mg/l). The pH of the medium was adjusted to 5.8 with the help of 0.1 N NaOH and 0.1 N HCl before adding agar. The medium was poured in culture vessels and sterilized at 15 lbs/inch2 for 20 minutes in an autoclave. After inoculation, the culture vessels were kept in culture room at 25±2oC under 16 hr photoperiod.

g. Shoot multiplication

After obtaining shoots, the shoots were separated and transferred to shoot multiplication medium (MS + BAP (0.5-2.0 mg/l + NAA (0.01-0.08 mg/l). After three weeks in shoot multiplication medium, the shoots were separated and sub-cultured on half strength MS medium for elongation of shoots.

h. Shoot elongation and induction of roots

After obtaining desired number of shoots, the shoots were separated and transferred to shoot elongation medium (half strength of MS basal medium). After three weeks in shoot elongation medium, the shoots were separated and sub-cultured on medium fortified with various concentrations of NAA (0.1, 0.5, 1.0 mg/L) for root induction.

Optimization of acclimatization of the tissue culture grown plantlets of *A. annua* L.

After proper in vitro development, the plantlets were taken out of the flasks in such a way that no damage was caused to their root system. The roots were washed gently under running

tap water to remove adhering medium, and thereafter, rooted plantlets were transferred into glass bottles containing Hogland's solution. The transplanted plantlets were maintained at 25 ± 2ºC and 50-70µE m-2 s-1 light intensity for 16 hours photoperiod. After two weeks, when plantlets became acclimatized under sucrose deficient medium, they were transferred in pots containing autoclaved soil, sand and peat moss in ratio of 3:3:1. The pots were covered with polythene bags. The humidity was 100% initially, but it was gradually reduced from 90%, 80% and 50%. Some lower leaves were dried during this process. After one month the plantlets were transplanted into pots filled with field soil and kept in open environment. The plants were periodically added with Hogland's solution to provide nutrients in the soil.