Annexure-VIII

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI – 110 002

Final report (01.04.2013 to 31.03.2017) of the work done on the Major Research Project entitled"Enhancement of repellent activity in *Artemisia vulgaris* to control yellow fever mosquito *Aedes aegypti* through *in* vitro precursor and *Agrobacterium rhizogenes mediated* transformation"

1. Project report No. 1st /2nd /3rd/Final : **Final**
2. UGC Reference No : **F. No. 42-922/2013 (SR) Dated. 28.03.2013**
   F. No. 42-922/2013 (SR) Dated. 10/06/2016
3. Period of report : **1st April 2013 to 31st March 2017**
4. Title of research project : **Enhancement of repellent activity in *Artemisia vulgaris* to control yellow fever mosquito *Aedes aegypti* through *in vitro* precursor and *Agrobacterium rhizogenes* mediated transformation**
5. (a) Name of the PrincipalInvestigator : **Dr. B.D.Ranjitha Kumari**
   (b) Department : **Plant Science**
   (c) University/College where work has progressed : **Bharathidasan University Tiruchirappalli, TamilNadu**
6. Effective date of starting of the project : **01.04.2013**
7. Grant approved and expenditure incurred during the period of the report
   a. Total amount approved Rs : **14, 20,800/-**
   b. Total expenditure Rs : **14, 20,800/-**
   c. Report of the work done : **See Annexure-I**

i. Brief objective of the project

- **To standardize protocols for regeneration of *A. vulgaris* by using precursor treatment.**
- **To introduce *A. rhizogenes* gene for hairy root culture for the enhancement of root biomass.**
- **To isolate, quantify and to analyze the enhanced essential oil production.**
- **To test the efficacy of the insecticidal compound against *Aedes aegypti* mosquitoes**.

ii. Work done so far and results achieved and publications

1. **B. Sundararajan and B.D. RanjithaKumari.** Effect of Farnesyl Diphosphate on Beta caryophyllene content in the calli of *Artemisia vulgaris* L. *Int Jew Tech Sci and Eng* 2 (2015): 307-316.
2. **B. Sundararajan and B.D. Ranjitha Kumari.** Novel synthesis of gold nanoparticles using *Artemisia vulgaris* L. leaf extract and their efficacy of larvidical activity against dengue fever vector *Aedes aegypti* L. *J Trace Elements in Medicine and Biology* (2017): http://dx.doi.org/10.1016/j.jtemb.2017.03.008 **(Impact Factor 2.5).**

Presented Research papers in the Conference

- Poster presented in the title of "**COMPOSITION OF LEAF ESSENTIAL OIL OF *ARTEMISIA VULGARIS* L. AND ITS LARVICIDAL AND REPELLENT ACTIVITY AGAINST THE DENGUE FEVER VECTOR *AEDES AEGYPTI* L."** in the XXVI Annual Conference of Indian Association for Angiosperm Taxonomy and International Seminar on Conservation and Sustainable Utilization of Biodiversity Organized by, Department of Botany held on7th & 9th November 2016 at Shivaji Unviersity, Kolhapur.

- Short Invited oral presented in the title of **"NOVEL SYNTHESIS OF GOLD NANOPARTICLES IN LEAF EXTRACT OF *ARTEMISIA VULGARIS* L. AND THEIR EFFICACY ON LARVICIDAL AND REPELLENT ACTIVITY AGAINST DENGUE FEVER VECTOR *AEDES AEGYPTI* L."** in the Fourth International conference on Nano medicine and Tissue engineering Organized by, International and Inter-University Centre for Nano science and Nanotechnology held on12th & 14th August 2016 at Mahatma Gandhi University, Kottayam.

- Oral presented in the title of **"ESTABLISHMENT OF HAIRY ROOTS PRODUCTION THROUGH *AGROBACTERIUM RHIZOGENES*MEDIATED TRANSFORMATION AND ESSENTIAL OIL ANALYSIS OF SECRETED IN BETA CARYOPHYLLENE CONTENT IN *ARTEMISIA VULGARIS* L."** National Conference on Emerging Trends in

Plant Science Organized by, Department of Plant Science held on 10th March &11th 2016 at Bharathidasan University, Tiruchirappalli.

- Poster presented in the title of **"EFFECT OF FARNESYL DIPHOSPHATE ON BETA CARYOPHYLLENE CONTENT IN THE CALLI OF *ARTEMISIA VULGARIS L.*"** in the International Conference on Modern Progress in Biotechnology Organized by, Department of Biotechnolgy held on 9th & 10th October 2014 at Anna University, Tiruchirappalli.

iii. Has the progress been according to original plan of work and towards achieving the objective. if not, state reasons

The progress of the project has been achieved towards objectives

iv. Please indicate the difficulties, if any, experienced in implementing the project

-Nil-

V. If project has not been completed, please indicate the approximate time by which it is likely to be completed. A summary of the work done for the period (Annual basis)may please be sent to the Commission on a separate sheet.

Project work is complete

Vi. If the project has been completed, please enclose a summary of the findings of the study. One bound copy of the final report of work done may also be sent to University Grants Commission.

See Annexure I & II

vii. Any other information which would help in evaluation of work done on the project. At the completion of the project, the first report should indicate the output, such as (a) Manpower trained (b) Ph. D. awarded (c) Publication of results (d) other impact, if any

(a) Manpower trained : 1

(b) Ph. D. awarded : Work completed, Synopsis yet to submit (Thesis not submitted)

(c) Publication of results: 2

(d) Other impact, if any: Nil

Signature of the Principal Investigator

Registrar/Principal (Seal)

Annexure-IX

UNIVERSITY GRANTS COMMISSION
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NEW DELHI – 110 002

PROFORMA FOR SUBMISSION OF INFORMATION AT THE TIME OF SENDING THE FINAL REPORT OF THE WORK DONE ON THE PROJECT

1. Title of the Project	: **Enhancement of repellent activity in *Artemisia vulgaris* to control yellow fever mosquito *Aedes aegypti* through *in* vitro precursor and *Agrobacterium rhizogenes* mediated transformation**
2. Name and address of the Principal Investigator	: **Dr. B.D.RanjithaKumari Professor Department of Plant Science**
3. Name and address of the Institution	: **Bharathidasan University Tiruchirappalli-620024 TamilNadu**
4. UGC approval letter no. and date	: **F. No. 42-922/2013 (SR) Dated. 28.03.2013 F. No. 42-922/2013 (SR) Dated. 10/06/2016**
5. Sate of Implementation	: **01.04.2013**
6. Tenure of the project	: **4 years (01.04.2013 to 31.03.2017)**
7. Total grant allocated	: **Rs.14, 20,800/-**
8. Total grant received	: **Rs. 13, 18,000**/-
9. Final expenditure	: **Rs. 14, 20,800/-**
10. Title of the project	: **Enhancement of repellent activity in *Artemisia vulgaris* to control yellow fever mosquito *Aedes aegypti* through *in* vitro precursor and *Agrobacterium rhizogenes mediated* transformation**

11. Objectives of the project :

- **To standardize protocols for regeneration of *A. vulgaris* by using precursor treatment.**
- **To introduce *A. rhizogenes* gene for hairy root culture for the enhancement of root biomass.**
- **To isolate, quantify and to analyze the enhanced essential oil production.**
- **To test the efficacy of the insecticidal compound against *Aedes aegypti* mosquitoes**.

12. Whether objectives were achieved: **Yes**

13. Achievements from the project : 1) By using precursor Farnesyl diphosphate the β-caryophyllene Content has been enhanced.

. 2) Quantification of β-caryophyllene in hairy root culture was done.

3) The efficacy of β-caryophyllene content on larvicidal and repellent activity on *Aedes aegypti* was preferred**.**

14. Summary of the findings : **See annexure –II**

15. Contribution to the society : **See annexure –III**

16. Whether any Ph.D. enrolled/ Produced out of the project : 1 (Thesis yet to be submit)

17. No. of publications out of the Project : 2

Signature of the Principal Investigator

Registrar/Principal (Seal)

Annexure-I

Title of the Project

Enhancement of repellent activity in *Artemisia vulgaris* to control yellow fever mosquito *Aedes aegypti* through *in vitro* precursor and *Agrobacterium rhizogenes* mediated transformation

1. Introduction

Artemisia vulgaris L. is an important perennial medicinal herb belongs to the family Asteraceae and is a tall aromatic, shrub 0.6-2.4m high and pubescent. The plant grows in the hilly district of India in areas up to 2400 m elevation. The species of *A. vulgaris* native place temperate Europe, Asia, Northern Africa and Alaska and is naturalized in North America. It is commonly called as Mugwort or St. Johns plant (English), Dona (Hindi), Macippaccai (Tamil). In Traditional Medicine, mugwort has been used as an analgesic agent and in conjunction with acupuncture therapy (Yoshikawa 1996), to treat the Neonatal Jaundice (Fok, 2001), Gastric (Ulcers Repetto 2002*)*, (Hepatitis Tan, 1999).

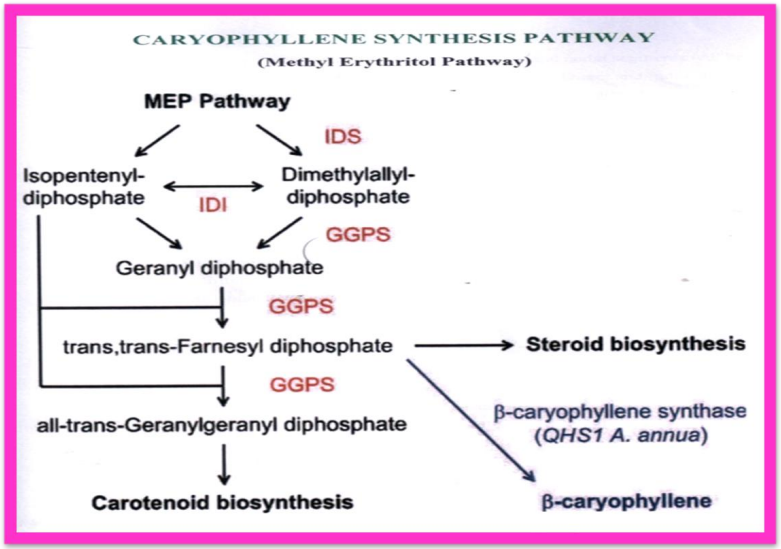
Secondary metabolites present in the Plant exhibit, Anesthetic, Antiseptic, Antispasmodic, CNS stimulant, Cancer preventive, cosmetic, Decongestant, Expectorant, Insect-repellant, Insectifugant, Nematicidal, Pesticidal, Antiasthmatic, Antibacterial, Anti-inflammatory and Sedative activities and are used as flavors and in perfumery (Teixiera Da Silva 2004). The essential oil of the plant was reported to exhibit 90% mosquito repellency against *Aedes aegypti* mosquitos that transmit dengue and Yellow fever (Ram and Mehrotra, 1995). The chief compounds of volatile oils include Camphor, Camphene and Beta-caryophyllene. Phytochemical studies revealed that more than 20 flavonoids are present in mugwort extracts (Lee 1998). Some flavonoids such as acetylenes, coumarins, sesquiterpene lactones, and volatile oil components have already reported in mugwort (Marco et al., 1990).

The advances in Plant Biotechnology and increasing interest in plant derived pharmaceuticals have opened up new ways for medicinal plant improvement and the production of plant drugs (Parr, 1989). Hairy Root Culture is the new route for large scale secondary metabolite production because of their fast and plagiotropic growth, genetic and biochemical stability. Hairy roots of *A. vulgaris* standardized and accumulation of essential oils was

reported by, Sujatha and Ranjitha Kumari, (2007) Sujatha et al., (2013) in *in vitro* micro propagationof *A. vulgaris* using shoot tip and nodal explants Rezvan Karami Borzabad et al., (2010). The callus induction and regeneration protocol was developed from leaf explants of *A. vulgaris*. Propagation through tissue culture offers a viable alternative for multiplication of this species and for the production of essential oils and secondary metabolites.

1.1. Caryophyllene Synthesis Pathway

Terpenoids compound naturally plays significant roles in plant–environment interactions, plant–plant communication and plant–insect and plant–animal interactions (Pichersky and Gershenzon, 2002). Terpenoid biosynthesis involves mostly head to tail addition of isopentenyldiphosphate (IPP, the active C_5 isoprene unit), to its isomer dimethylallyldiphosphate (DMAPP) synthesizing geranyldiphosphate (GPP, C_{10}). Further, condensation of enzyme-bound geranyldiphosphate with additional IPP units forms successively larger prenyldiphosphates for example farnesyl diphosphate (FPP, C_{15}), geranylgeranyldiphosphate (GGPP, C_{20}), that might undergo cyclization, coupling and rearrangement to produce the parent carbon skeleton of sesquiterpenes and diterpenes (Singh et al., 1989, McGarvey and Croteau, 1995), Luthra et al., 1999).



2. Materials and Methods

2. 1. Indirect organogenesis with precursor treatment

Callus induction

A. vulgaris (L.) young leaf explants were collected from 35 days old seedlings. The medium Murashige and Skoog (1962) consisted of containing a) macronutrient at full strength b) micronutrient at full strength c) 3 % sucrose d) 0.8% agar. To induce callus, the sterile leaf explants were inoculated MS medium alongwith various combinations of plant growth hormones 2, 4 D (4-13µM) and NAA (5-16 µM) as Farnesyl diphosphate (1 and 3 µM) and Beta-caryophyllene precursor was added.

Shoot induction and elongation

Shoots from callus was observed at different concentrations of BAP (0.4-8.8µM), BAP+FDP (0.4-8.8 µM+ 1and 3µM), 2iP (0.4-9.8µM), 2iP+FDP (0.4-9.8µM+1and 3µM). Elongation of shoots was also observed on the same medium.

Rooting and Acclimatization

The shoots were excised from the parent cultures and transferred onto MS medium supplemented with different concentrations and combinations of IAA (2.8-17.1µM), IAA+FDP (2.8-17.1µM+ 1- 3 µM).

The P^H of the medium was adjusted to 5.6 - 5.8 before autoclave the media bottles were autoclaved at 121ºC 15 psi pressure for 20 minutes. The explants were inoculated in the media and kept in controlled conditions 16/8 hours of light intensity of 10 µmol m^2 s^{-1}(cool white fluorescent light) and a temperature of 25±20ºC.

2.2. Direct Organogenesis with precursor treatment

Plant Material

A. vulgaris (L.) young nodal explants were collected from 45 days old seedlings1-1.5 cm long.

Shoot induction

The medium Murashige and Skoog (1962) consisted of containing a) macronutrient at full strength b) micronutrient at full strength c) 3 % sucrose d) 0.8% agar. To induce shoot initation, the nodal explants were inoculated MS medium along with various combinations of plant growth hormones BAP (0.4- 4.4µM), KIN (0.4- 4.6µM) and TDZ (0.4 – 4.9µM) and Beta-caryophyllene precursor Farnesyl diphosphate (1 and 3 µM) were added.

Rooting and Acclimatization

The shoots were excised from the parent cultures and transferred onto MS medium supplemented with different concentrations and combinations of IBA (2.6 – 16.1µM), IBA (2.6 – 16.1µM + 1- 3 µM), IAA (2.8 -17.1µM), IAA+FDP (2.8 -17.1µM + 1- 3 µM).

The P^H of the medium was adjusted to 5.6-5.8 before autoclave the media bottles were autoclaved at 120ºC 15 psi pressure for 20 minutes. The explants were inoculated in the media and kept in controlled conditions 16/8 hours of a light intensity of 10 µmol m^2 s^{-1}(cool white fluorescent light) and a temperature of 25±20ºC.

2.3. Hairy Root Culture

Bacterial Culture Preparation

Agrobacterium rhizogenes (A_4 and R_{1000}) strains stored in sterile glycerol at −70°C and was cultured on LB (Luria-Bertani) solid medium for activation of the strain and sub cultured thrice on the same medium. The bacteria were then transferred into LB liquid medium and cultured at 25°C for overnight on orbital shaker (120 rpm). Bacteria exponential growth phase was observed at OD 600 nm. The bacterial suspension was transferred into sterile centrifuge tubes and centrifuged at 5000 rpm for 15 minutes. This suspension was used for the transformation studies.

Induction and Establishment of Hairy Roots

The leaf explants derived from sterile seedlings were wounded with a sterile scalpel and dipped into the overnight grown *A. rhizogenes* culture for the induction of hairy roots. Afterwards, the explants were dried using sterile filter paper and were placed on semi-solid 20 ml of ½ MS medium supplemented with 3 % sucrose and control, 50,100 and 150 µM of Acetosyringone (AS) (Sigma USA). The AS was filter-sterilized using a 0.2 µm syringe filter (Pall, USA) and was added to cooled, autoclaved medium. The experiment was replicated thrice and a minimum of 25 explants was used for each experiment. After co cultivation explants were washed first time with sterile distilled water followed by ½ MS medium, which contained 500 mg/l cefotaxime for root induction under dark condition. The induced transformed roots of more than 1-2 cm length were excised from the explants and transferred to 30 ml ½ MS, ½ MS+B_5Vitamins, ½ MS+FDP (1µM), ½ MS+FDP (3µM), ½ MS+B_5Vitamins + FDP (1µM) and ½ MS+B_5 Vitamins+ FDP (3µM) supplemented with 3 % sucrose and 500 mg/L cefotaxime.

Determination of the Growth Index (GI) of the HR cultures

Experiments were conducted to evaluate the growth rate of the hairy roots using½ MS, ½ MS + B_5Vitamins, ½ MS + FDP1µM, ½ MS + FDP 3µM, ½ MS + B_5Vitamins + FDP1µM and ½ MS + B_5 Vitamins + FDP 3µM. The GI of all four lines were determined by initially inoculating approximately, 20 mg of fresh weight bacteria-free HRs (3 weeks old) in 30 mL of liquid medium in a 250 mL conical flask and grown them for 12 weeks. Non-transformed roots were also cultured in each of the above mentioned media as controls and all the cultures were maintained on a rotary shaker at 120 rpm at dark condition. The *in vitro* HRs was sub cultured on fresh medium with the same constituents at 6 week intervals for further growth. Each treatment consisted of three replicates and each replication per treatment contained 25 explants. The growth index was determined using the formula (Ashraf et al., 2013).

$$\text{Growth Index} = \frac{\text{Final Biomass weight} - \text{Initial Biomass Weight}}{\text{Initial Biomass Weight}}$$

PCR analysis

Integration of the T-DNA responsible for hairy root formation was confirmed by PCR analysis using custom primers (Meta bion International AG, Germany). The primer sequence of *rol A* gene was as suggested by (Kumar et al. 2005) and *rol C* gene by (Lee et al. 2004). Genomic DNA from the bacterial strain, normal and transformed hairy root culture was isolated using the CTAB method (Khanuja et al., 1999). After ethanol precipitation DNA was resuspended in 100 µl of 1X TE buffer (pH.8.0). The DNA was quantified

spectrophotometrically by taking the absorbance at 260 nm. For amplification of the rol A product, the primers used were, forward -5' -AGA ATG GAA TTA GCC GGA CTA-3' and Reverse -5' -GTA TTA ATC CCG TAG GTT GTT T-3' and for rol C, the forward 5' -ATG GCT GAA GAC GAC CTG GT T-3' and reverse 5' -TTA GCC GAT TGC AAA CTT GCT C-3' respectively. The PCR assay was carried out in 25 µL reaction mixture containing 0.2 mMdNTPs, 10 mMTris–HCL,1.5 mM $MgCl_2$, 50 mMKCl, 0.1 % Triton X-100, 1.0 U Taq DNA polymerase (Finzymes, Helsinki, Finland),15 pmol primers (IDT, Coralville, USA) and 50 ng of genomic DNA. The PCR for both rol A and rol C was carried out by amplifying with an initial denaturation at 94° C for 5 min followed by 35 cycles of denaturation at 94° C for 1 min, 1.0 min annealing at 55° C for *rol A* and *rol C* and 1.0 min extension at 72° C with a final extension of 72° C for 10 min using a thermo cycler (Eppendorf ESP-S, Germany).The amplicons were analyzed by electrophorizing on 1.4 % (w/v) agarose gel (Lonza) along with 100 bpDNA marker (New England Biolabs, UK) followed by Ethidium bromide staining. Plasmid DNA isolated from *A. rhizogenes* strain TR105 was used as a positive and DNA from normal root cultures were used as negative controls.

Statistical analysis

All experiments were set up in a completely randomized design and data subjected to Duncan's Multiple Range Tests (DMRT) using SPSS software version 16.0. The significance of differences among means was carriedout using Tukey's test at 5% probability level

2.4. Essential oil isolation and quantification of β-caryophyllene content in precursor treated leaves and hairy root by GC-MS analysis.

Isolation of essential oil

The 10 g of the leaves and hairy root samples were subjected to hydro-distillation for 4 h, using a Clevenger-type apparatus at 60°c. The obtained essential oil was dried over anhydrous sodium sulfate. The oil was filtered, stored at 4°Cfor further experiments.

GC-MS analysis

The extract was cleaned by passing it through a small (Pasteur pipette) column containing anhydrous Na_2SO_4 and silica gel 60 (230-400 mesh, Merck), to dry the sample and remove high molecular weight polar substances that interfere with GC- MS analyses. The qualitative and quantitative analyses of β-caryophyllene standard and essential oil diluted Ethyl acetate were performed using a GC–MS QP- 2010 (Shimadzu, Japan) equipped with AOC-20i auto sampler. One microliter of the extract was injected and the chromatographic separation of β-caryophyllene was achieved with Rtx column (60m £ 0.25 mm I.D £ 0.25 mm thickness (Restek, USA). Helium with a purity of 99.999% was used as the carrier gas at a flow rate of 10 ml/min.

The injector port, interface and ion source temperatures were set at 230°C, 270°Crespectively. The oven temperature was programmed as follows: initial temperature at 150°C for 1 min, then raised to 300°C at 8°C/min and finally to 320°C at 7°C/min, held for 10 min. A solvent delay of six minutes was set to protect the filament from oxidation. The mass spectrometer was operated in electron impact (EI) mode at ionization potential of 70eV and at an emission current of 60 mA. Full scan data were obtained in a mass range of m/z 35–500.Scanning interval and Selected Ion Monitoring (SIM) sampling rate were 0.5 s and 0.2 s, respectively. The quantification was performed in SIM mode by monitoring three mass ions for each β-caryophyllene.

2.5. To test the efficiency of larvicidal and repellent activity of enhanced β-caryophyllene content in essential oil

Larvicidal activity

Aedes aegypti eggs were collected from Center for Research in Medical Entomology, Madurai and maintained in the Department of Plant Science, Bharathidasan University.
The larvae were fed on Brewer's yeast/dog biscuit with 1:3 ratio were maintained at 28±2°C temperature 70-80% Relative Humidity (RH) with a photo period of 12h light and 12 h dark.
The larvae at early third and fourth instar stage were used for larvicidal of the essential oil *A. Vulgaris* evaluated according to World Health Organisation (WHO) standard protocol

(2006).The efficiency of larvicidal activity was carried out with slight modification protocol of (Chellasamy Panneerselvam et al., 2012)and activity was tested with calli essential oil, hairy root essential oil.

Twenty larvae of early third and fourth instar stage treated with different concentrations of essential oil diluted with Ethyl acetate (25, 50, 100. 200 and 400 ppm). Thee replicates were maintained for each concentrations and dead larvae were counted after 12 hrs and 24 hrs. The larval mortality was calculated after 12 and 24 hrs of the exposure. The lethal concentration LC_{50} at which concentration (ppm) 50% of larvae showed mortality, 95% confidence limit of upper and lower confidence levels were calculated by probit analysis (SPSS, version 16.0).

$$\%\ \text{Percentage of mortality} = \frac{\text{Number of dead larvae}}{\text{Number of larvae}} \times 100$$

Histological analysis

The histological test analyze twenty larvae was exposed to higher mortality concentration of the 12 hrs and 24 hrs only live larvae were examined. Briefly, the larvae were fixed in 10 % buffered formaldehyde for 24 h, dehydrated through a graded series of ethanol, and cleared with xylene solutions. They were embedded in a block using melted paraffin at the embedding station. The paraffin blocks were sectioned at 5 µm thicknesses using a rotary microtome, and stained with Harris's hematoxylin and eosin (H&E). The glass slides were examined for abnormalities using the Olympus CX31 light microscope and photographed by a Canon EOS 1100D digital cameras.

Repellent activity

The stock solution of the essential oil were diluted with Ethyl acetate (essential oil) with slight modification different concentrations like 25, 50. 100. 200 and 400 ppm some modified protocol was used (Gabi Baba et al., 2012 and Chellasamy Panneerselvam et al., 2012).For repellent experiment involves counting of the number of mosquitoes biting a volunteer's hands introduced laboratory screen cages (45×45×40 cm) containing 50 laboratory-reared bloods starved adult *Aedes aegypti* female mosquitoes that were between 3 and 10 days old were placed into separate.

Before each test, the forearm and hand of a human subject were washed with unscented neutral soap, thoroughly rinsed and allowed to dry 10 min before extracts application. The different extracts being tested were applied from the elbow to the fingertips. The arm was left undisturbed. An arm treated with Ethyl acetate and DEET served as control. The control and treated arms were introduced simultaneously into the cage. The numbers of bites were counted over 5 min, every 15 min, from 30 to 60 hours. Each test was repeated three times for each concentration. Four different human volunteers were used per test after obtaining their consent to participate in the experiment.

$$\text{Percentange of Biting} = \frac{(\text{No. of bites received by control arm} - \text{No. of bites received by treated arm})}{\text{No. of bites received by control arm}} \times 100$$

3. Results

3.1. Indirect Regeneration

Leaf explants were inoculated on MS medium containing 2, 4 D (4-13µM) and NAA (5-16 µM) with Farnesyl diphosphate precursor (1 and 3 µM) individually or in combination. Among the various concentrations tested, the highest response of callus 99% and 95.2% was achieved in MS medium supplemented with combination of 2,4 D (9.0 µM) + FDP (3 µm) followed by MS medium supplemented with NAA (16.1µM) + FDP (3µM) (Table 1 and Figure 1). The Shoots from leaf derived callus was observed at different concentrations and combinations of BAP (0.4-8.8µM), BAP + FDP (0.4-8.8 µM+ 1and 3µM), 2iP (0.4-9.8 µM), 2iP + FDP (0.4-9.8 µM + 1and 3 µM). The best response of shoot induction BAP 4.4 µM + FDP 3 µM (57.6), 2iP 7.3 µM + FDP 3 µM (56.3) per explants was observed. The elongation was also achieved on the same parental medium (Table 2 and Figure 2, 3).

Roots were not induced during callus induction, shoot formation and shoot multiplication in the cytokinin regime. Individual shoots when implanted in MS medium free from growth regulators, poor and few numbers of roots were elicited with low frequency. MS medium supplemented with various concentrations of precursor and IAA (2.8-17.1µM), IAA + FDP (2.8-17.1µM + 1-3 µM) enhanced the frequency of roots. The Rooting efficiency was observed

maximum in IAA 8.5 µM + FDP 3 µM) individually. A maximum of 100 % rooting was achieved with 35.4 roots. The maximum root length measured was 17.5 cms (Table 3).Survival efficiency of the hardened plants decreased gradually from 10-20 weeks. Inflorescence induction was observed after 14th week of transfer to field conditions. The number of heads ranged from 3.2-9.5 per plant after 14-20 weeks (Table 3).

3.2. Direct Regeneration

Nodal explants of *A. vulgaris* were cultured on MS media containing various concentrations of BAP, KN and TDZ individually for shoot regeneration with precursor treatment. The PGR-free media did not respond well for shoot induction. The incubation of nodal explants on MS media supplemented was found to yield the maximum response of shooting frequency BAP 4.4 µM (92%), BAP 4.4 µM + FDP 3 µM (99%), KN 4.6µM (76%), KN 4.6 µM + FDP 3 µM (98%), TDZ 4.9 µM (91%), TDZ 4.9 µM + FDP 3 (99%) with number of shoots was observed various concentrations such as BAP 4.4 µM (20.5), BAP 4.4 µM +FDP 3 µM (38.5), KN 4.6µM (28.2), KN 4.6 µM + FDP 3 µM (49.0), TDZ 4.9 µM (40.1), TDZ 4.9 µM + FDP 3 (59.6) (Figure 4, 5, 6 and Table 4). The formed multiple shoots at the crown region and across the node. Shoot initiation was observed by day 15, and multiple shoots appeared after 60 days of culture.

Rooting and Acclimatization

Individual shoots were excised from the micro propagated shoots and blotted dry on sterile tissue paper and then transferred immediately to rooting medium augmented with IBA (2.6 – 16.1µM), IBA (2.6 – 16.1µM + 1- 3 µM), IAA (2.8 -17.1µM), IAA+FDP (2.8 -17.1µM + 1- 3 µM). Rooting efficiency was maximum in IBA 8.5 µM (90%), IBA 8.5 µM + 3 µM (99%), IAA 8.0 µM (80%), IAA+FDP 8.0 µM + 3 µM (88%). tested individually. The maximum root length measured was 17.2cms.Survival efficiency of the hardened plants decreased gradually from 10-20 weeks. Inflorescence induction was observed after 14th week of transfer to field conditions. The number of heads ranged from 3.2-9.5 per plant after 14-20 weeks (Table 5).

3.3. Hairy Root Culture

Induction of establishment of hairy roots

Transformation efficiency of the *A. rhizogenes* required different time and duration for the root induction on *A. vulgaris* leaf explants (Table 6 and Figure 7, 8). The higher transformation frequency with direct root induction from the wounds site was observed from the leaf explants infected with Acetosyringone (AS) 50, 100 and 150µM was strains A4 and R1000 after 10- 15 days of culture.

Analysis of Growth Index of the HRs

Among all of the treatments (½ MS, ½ MS + B_5 Vitamins, ½ MS + FDP1µM, ½ MS + FDP 3µM, ½ MS + B_5Vitamins + FDP1µM and ½ MS + B_5 Vitamins + FDP 3µM), the HR culture attained a maximum and minimum of a 30 fold and a 65 fold increase the initial weight of 200 mg (A_4 Strain) and 25 fold and a 55 fold increase the initial weight of 163 mg (R_{1000}) within 12 weeks on ½ MS+FDP1µM, ½ MS+FDP 3µM respectively (Figure 9). At weeks 6, there was no significant difference in the GI % obtained using ½ MS, ½ MS + B_5 Vitamins, ½ MS + FDP1µM, ½ MS + FDP 3µM, ½ MS + B_5Vitamins + FDP1µM and ½ MS + B_5 Vitamins + FDP 3µM. However, at 12 weeks of culture ½ MS, ½ MS + B_5 Vitamins, ½ MS + FDP1µM, ½ MS + FDP 3µM, ½ MS + B_5Vitamins + FDP1µM and ½ MS + B_5 Vitamins + FDP 3µM was most significantly greater than GI % than those using the same tested media HR. Figure (9 and 10) shows that growth index of *A. vulgaris* A_4HRs at 6 and 12 weeks cultivation of the rotary shaking system. At 6 weeks of culture, ½ MS (42.00%), ½ MS+B_5 Vitamins (32.55%), ½ MS + FDP1µM (60.22%), ½ MS + FDP 3µM (70.85%), ½ MS + B_5Vitamins + FDP1µM (61.11%) and ½ MS + B_5 Vitamins + FDP 3µM (65.44%) treatment did not attain a significant growth rate respectively. These result crucial role of the ½ MS + FDP1µM (80.66%), ½ MS + FDP 3µM (97.33%) a showed highest biomass production after 12 weeks same medium with rotary shaking system.

Figure (10) shows that growth index of *A. vulgaris* R_{1000} HRs at 6 and 12 weeks cultivation of the rotary shaking system. At 6 weeks of culture, ½ MS (22.11%), ½ MS + B_5 Vitamins (27.44%), ½ MS + FDP1µM (32.88%), ½ MS + FDP 3µM (45.11%), ½ MS + B_5Vitamins + FDP1µM (52.89%) and ½ MS + B_5 Vitamins+ FDP 3µM (61.00%) treatment did not attain a significant growth rate respectively. These result crucial role of the ½ MS +

FDP1µM (65.00%), ½ MS + FDP 3µM (77.33%) a showed highest biomass production after 12 weeks same medium with rotary shaking system. The hairy root lines were subjected to DNA isolation, and PCR amplified with gene specific primers to show the integration of *rol B* and *rol C* genes into the genome of *A. vulgaris*. The isolated DNA showed *rol B* and *rol C* gene amplification of 540 and 780 bp respectively, while no amplification was observed in non-transformed roots (Figure 11).

3.4. GC-MS analysis

The essential oil was isolated from leaves and hairy root of *A. vulgaris* at a yield of 0.5% (v/w) and oil less than of the water respectively (Figure 12). The quantification of β-caryophyllene in precursor with and without treated hairy root essential oil was achieved 4.4% in without precursor treatment of hairy root culture and 5.7% in with precursor treatment of hairy root culture was observed (Figure 13a, 13b and Table 7).

3.5. Larvicidal activity

The larvicidal activity of essential oil were carried out various concentrations (25 ppm, 50 ppm, 100 ppm, 200 ppm, 400 ppm) against the dengue fever vector (*A. aegypti)* larva third and fourth stage is given in the (Table 8 and 9). The essential oil was observed 12 hours exposure and 24 hours exposure period considerable mortality was evident after the treatment of 12 hours exposure period of 3rd instar larva with highest mortality 400ppm essential oil (68.00 ± 1.52) then 24 hours exposure period 3rd instar larva with highest mortality was observed 400 ppm essential oil (73.33 ± 2.4). The 4th stage instar larva mortality was observed during 12 hrs exposure period with essential oil (62.66 ± 1.5) then 24 hours exposure period 4th stage larva mortality was essential oil (80.00 ± 2.8). The LC_{50} and LC_{90} values were represented as follows: 3rd instar larva after 12h of exposure period were LC_{50} 128.99 LC_{90} 1447.08 ppm (essential oil) and 24 h of exposure period were LC_{50} 111.15 LC_{90} 1441.51 ppm (essential oil). Then 4th instar larva after 12h of exposure period were LC_{50} 136.15 LC_{90} 2223.55 ppm (essential oil) and 24 h of exposure period were LC_{50} 74.22 LC_{90}858.36 ppm (essential oil), respectively.

3.6. Histological analysis

Histological description was performing highest mortality of *A. aegypti* larvae of 3rd and 4th stage of 24 hours exposures period. More over to our knowledge this is the first report of histological analysis of *A. vulgaris* essential oil. In this study, histological alterations were seen in the midgut (mg), digestive tract (dt) and cortex (ct) sometimes distinct protruding ends were observed. Our result supported with earlier reports of where 3rd and 4th larva of *A. aegypti* was treated with essential oil. In this study our data revealed that at highest ppm (400) for 24 hours exposure of *A. aegypti* with essential oil for 3rd and 4th stage larvae was highly affected in the area of midgut, epithelial cells and cortex (Figure 14).

3.7. Repellent activity

The effect of *A. vulgaris* essential oil on repellent activity was observed at various concentrations such as (2.5, 5, 15, 25, and 50 %) essential oil against dengue fever female mosquito's *A. aegypti* are given in (Table 10). All concentration of essential oil were showed considerable difference with chemical compound of N, N-Diethyl-meta-toluamide (DEET). Then essential oil at 50% concentration highest repellent percentage (6.66±1.5) was observed in 60 mins against *A. aegypti* female mosquitoes.

Annexure-I

Summary of the Findings

A. vulgaris extract was used for various diseases such as analgesic and anti-inflammatory activities and also indicated hepatoprotective activity and validated the traditional use of this plant for various liver disorders. The essential oil from *A. vulgaris* is reported to be 90% effective in repelling *Aedes aegypti*, a mosquito strain that transmits dengue and yellow fever.

***In vitro* regeneration**

- *In vitro* regeneration of *A. vulgaris* L. was achieved through indirect and direct organogenesis with and without precursor treatment.

Indirect Organogenesis

- Highest response of green compact callus was obtained from leaf explants on MS medium supplemented with 2, 4 D (9.0 µM) + FDP (3 µm) (99 %) and NAA (16.1µM) + (FDP 3µM) (95%).
- Multiple shoots were obtained from leaf derived callus supplemented with BAP (4.4 µM)+ FDP 3 µM, 2ip µM 7.3 + FDP 3 µM with mean number of 57.6 and 56.3shoots were observed and mean of 35.4 roots were observed on IAA 8.5 µM + FDP 3 µM.

Direct Organogenesis

- MS medium supplemented with BAP 4.4 µM +FDP 3 µM (99%), KN 4.6 µM + FDP 3 µM (98%) and TDZ 4.9 µM + FDP 3 (99%) gave highest response for multiple shoot induction from nodal explants with a mean of 38.4, 49.4 and 59.6 shoots per explants were observed 8 weeks of culture.
- MS medium supplemented with IAA 8.0 µM + FDP 3 µM and IBA 8.5 µM + FDP 3 µM was the most effective for root induction with mean of 11.7 and 17.2 roots was observed.

Hairy Root Culture

- Induction of hairy root from *A. vulgaris* leaf explants with A_4 and R_{1000} was achieved after 11 days of culture. The highest transformation frequency 93.0 ± 0.4 and 85.9 ± 0.2 was observed with co-cultivation of 3 days.
- Further, 20 days of old culture of hairy roots transferred into to various concentrations of liquid medium such as (½ MS, ½ MS + B_5 Vitamins, ½ MS + FDP1µM, ½ MS + FDP 3µM, ½ MS + B_5Vitamins + FDP1 µM and ½ MS + B_5 Vitamins+ FDP 3µM).
- Highest root biomass of 200 mg (A_4 Strain) and 163 mg (R_{1000}) was obtained in the media treated with ½ MS + FDP 1µM, ½ MS + FDP 3µM. In this study growth index of hairy root culture was attained at a maximum and minimum of 30 fold and 65 fold increased in A_4 strain and 25 fold and a 55 fold increased in R_{1000} strain.
- Further, DNA was isolated X and confirmed the transferred hairy root culture by using PCR with *rol A* and *rol C* primers.

GC-MS analysis

- The GC–MS technique was used to analyze the volatile secondary metabolite profile, particularly β-caryophyllene of the *A. vulgaris* essential oil. The results of the qualitative analysis showed β-caryophyllene content was 4.4% in hairy root without precursor treatment whereas 5.7 %s with precursor treatment of hairy roots culture.

Larvicidal activity

The larvicidal activity of essential oil was carried out various concentrations (25 ppm, 50 ppm, 100 ppm, 200 ppm and 400 ppm) against the dengue fever vector (*A. aegypti)* larva second and third stage. Considerable mortality was evident after the treatment of *A. vulgaris* essential oil mortality was increased as the concentration increased, for example in 24 hours exposure period 3rd instar larva with highest mortality was observed 400 ppm essential oil (73.33 ± 2.4) and 24 hours exposure period 4th stage larva mortality was essential oil (80.00 ± 2.8).

Conclusion

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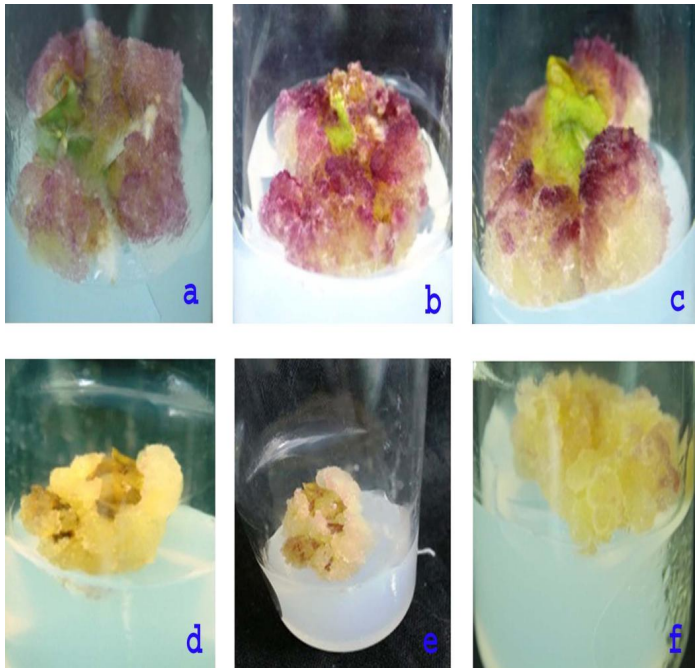
With the above research findings we conclude that enhancement of β-caryophyllene content with precursor treatment via indirect organogenesis, direct organogenesis and hairy root cultures from *A. vulgaris* L. Further confirm the compound through GC-MS analysis and best response was observed in precursor treated hairy root culture compare with indirect organogenesis and direct organogenesis. The identified pharmaceutical important insecticidal compound was tested against *A. aegypti* larvae to cure dengue fever. These findings could encourage and support to search new active natural compounds offering an alternative to synthetic repellents and insecticides after successful clinical trials.

Annexure III

Contribution to the society

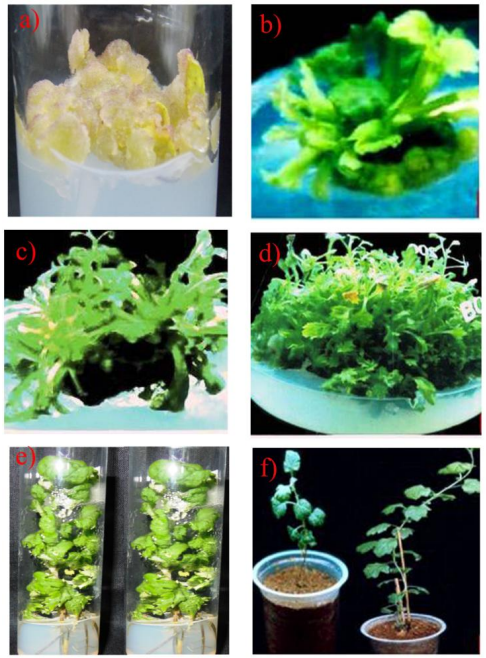
In this work improved mass production of β-caryophyllene content through *in vitro* precursor treatment and it is worthwhile to identify new larvicidal and repellent activity compounds from natural products to act against the *A. aegypti* mosquitoes. Natural compounds of enhanced β-caryophyllene content present in *A. vulgaris* L. essential oil will reduce environmental pollution, diseases and also promote sustainable utilization of locally available bio-resources by marginalized rural communities. This enhanced compound essential oil of *A. vulgaris* L. have the potential to be used as an ideal ecofriendly approach for the vector control programmes.

Figure: 1 *In vitro* **Callus induction from leaf explants of** ***A. vulgaris*** **L.**



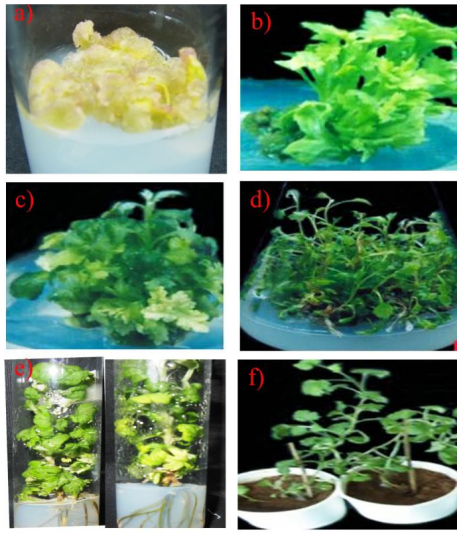
(A) 2,4D 9.0 µM, (B) 2,4D 9.0 µM + FDP 1 µM, (C) 2,4D 9.0 µM + FDP 3 µM, (D) NAA 16.1 µM. (E) NAA 16.1 + FDP 1 µM, (F) NAA16.1 + FDP 3 µM.

Figure: 2 Callus Induction and Plant Regeneration from Leaf Explants of ***A. vulgaris*** **with 2, 4D and Farnesyl Diphosphate**



a. Callus induction from leaf explants cultured on MS medium supplemented with 2,4 D (9 µM) (after 4 weeks)
b. Shoot regeneration from leaf explants cultured on MS medium with 2,4 D + FDP (9 + 3 µM) (2 weeks old)
c. Shoot regeneration from leaf explants cultured on MS medium with 2,4 D + FDP (9 + 3 µM) (4 weeks old)
d. Shoot regeneration from leaf explants cultured on MS medium with 2,4 D + FDP (9 + 3 µM) (8 weeks old)
e. Rooting of *in vitro* raised shoots
f. Hardened plantlets in cups

Figure: 3 Callus Induction and Plant Regeneration from Leaf Explants of *A. vulgaris* with NAA and Farnesyl Diphosphate



a. Callus induction from leaf explants cultured on MS medium supplemented with NAA (16.1 µM) (after 3 weeks)
b. Shoot regeneration from leaf explants cultured on MS medium with NAA+ FDP (16.1 + 3 µM (3 weeks old)
c. Shoot regeneration from leaf explants cultured on MS medium with NAA+ FDP (16.1 + 3 µM (6weeks old)
d. Shoot regeneration from leaf explants cultured on MS medium with NAA+ FDP (16.1 + 3 µM (8 weeks old)
e. Rooting of *in vitro* raised shoots
f. Hardened plantlets in cups

Figure: 4 Effect of hormones KN with Farnesyl Diphosphate on Micropropagation of *A. vulgaris*

a. Shoot initiation from nodal explants cultured on MS medium supplemented with KN 6.9 µM + FDP 3 µM (after 2 weeks)
b. Shoot multiplication from nodal explants cultured on MS medium containing KN 6.9 µM + FDP 3 µM (after 6 weeks)
c. Elongated shoots with roots
d. Hardened plants in cups

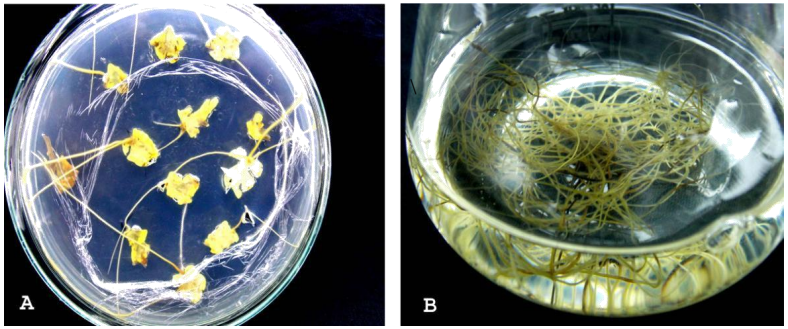
Figure: 5 Effect of hormones TDZ with Farnesyl Diphosphate on Micropropagation of *A. vulgaris*

a. Shoot initiation from nodal explants cultured on MS medium supplemented with TDZ 4.9 µM + FDP 3 µM (after 2 weeks)
b. Shoot multiplication from nodal explants cultured on MS medium containing TDZ 4.9 µM µM + FDP 3 µM (after 6 weeks)
c. Elongated shoots with roots
d. Hardened plants in cups

Figure: 6 Effect of hormones TDZ and Farnesyl Diphosphate on Micropropagation of *A. vulgaris*

a. Shoot initiation from nodal explants cultured on MS medium supplemented with BAP 4.4 µM + FDP 3 µM (after 2 weeks)
b. Shoot multiplication from nodal explants cultured on MS medium containing BAP4.a µM µM + FDP 3 µM (after 6 weeks)
c. Elongated shoots with roots
d. Hardened plants in cups

Figure: 7 Hairy root cultures of *A. vulgaris* L.



A) Hairy root initiation from leaf explant cultured on MS + 500 mg L^{-1} cefotaxime medium (after 2 weeks of co-culture) B) Enhancement of biomass accumulation of hairy roots cultured on ½ MS+FDP 3µM liquid medium after (12 weeks).

Figure: 8 Hairy root inductions in *A. vulgaris* in the presence of different *A. rhizogenes* strains with/without AS in different concentrations

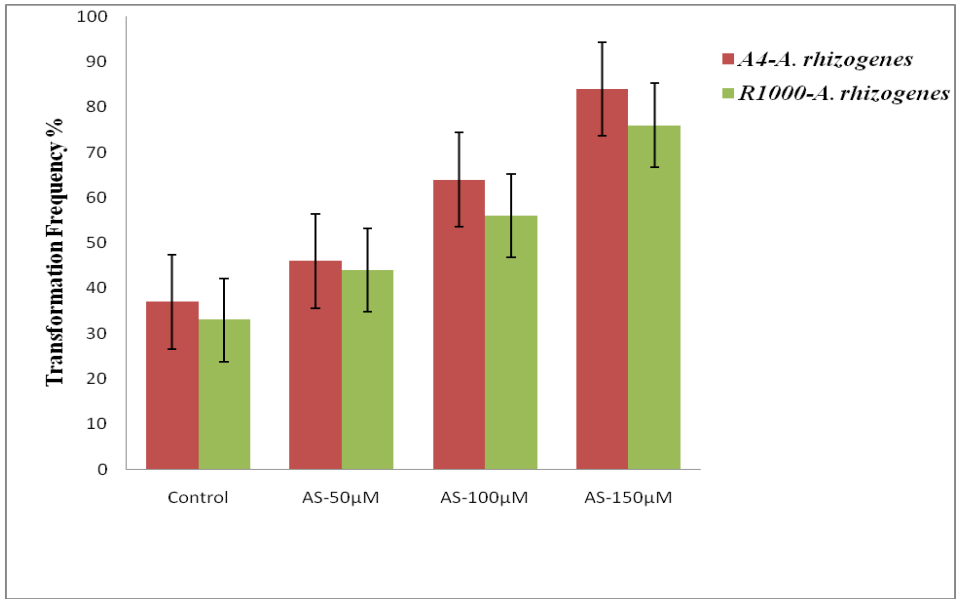


Figure 9 A_4 strain effect on mean growth index of *A. vulgaris* Hairy Root after 6 and 12 weeks

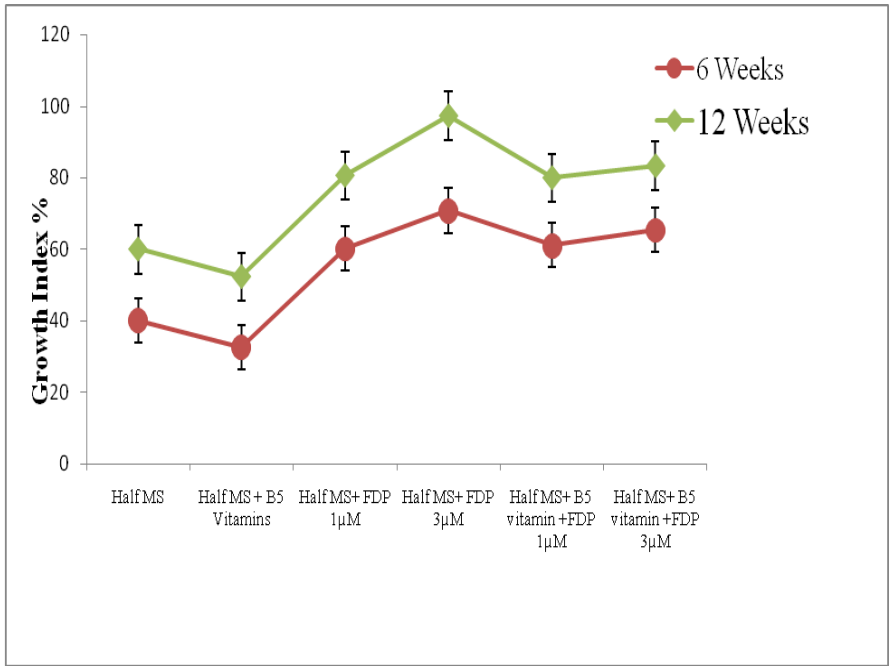


Figure: 10 R_{1000} strain effect on mean growth index of *A. vulgaris* Hairy Root after 6 and 12 weeks

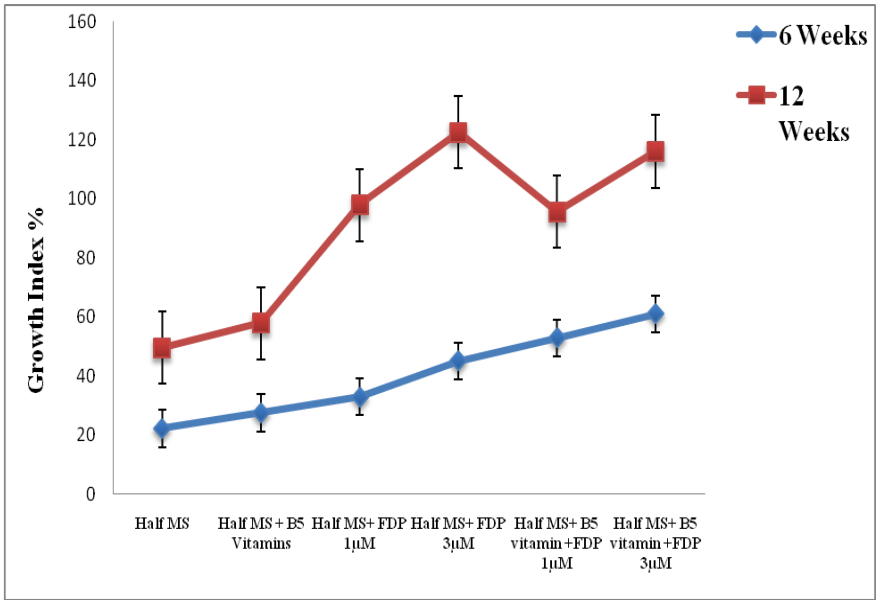
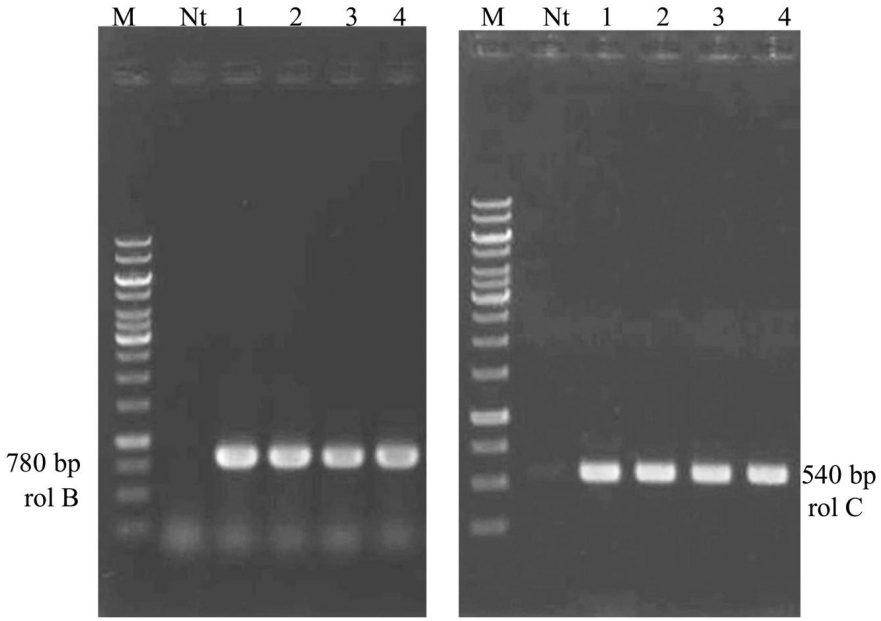


Figure: 11 PCR conformations of transgenic hairy roots (A_4 and R_{1000}) from *A. vulgaris* L. leaf explants



a) rol B gene amplification (780 bp), **b)** rol C gene amplification (540 bp). Lane M: Marker, Nt: Non transgenic root, Lane 1: A_4 Plasmid DNA, Lane 2: R_{1000} Plasmid DNA, Lane 3: Genomic DNA of hairy root culture (A_4 strain), Lane 4: Genomic DNA of hairy root culture (R_{1000} strain).

Figure 12 Isolation of the *A. vulgaris* essential oil through Clevenger type of hydro distillation apparatus

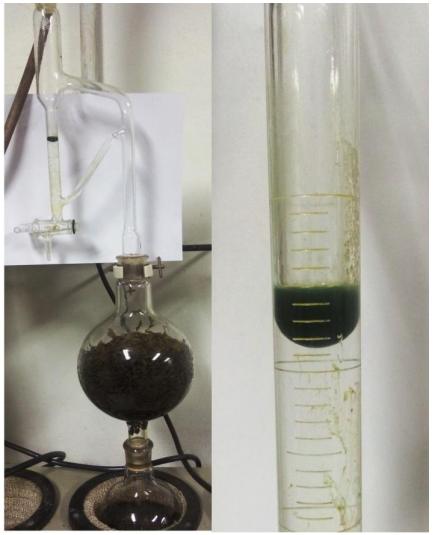


Figure: 13a. Gas-Chromatogram of Non-Transformed Root Essential Oil of *Artemisia vulgaris*

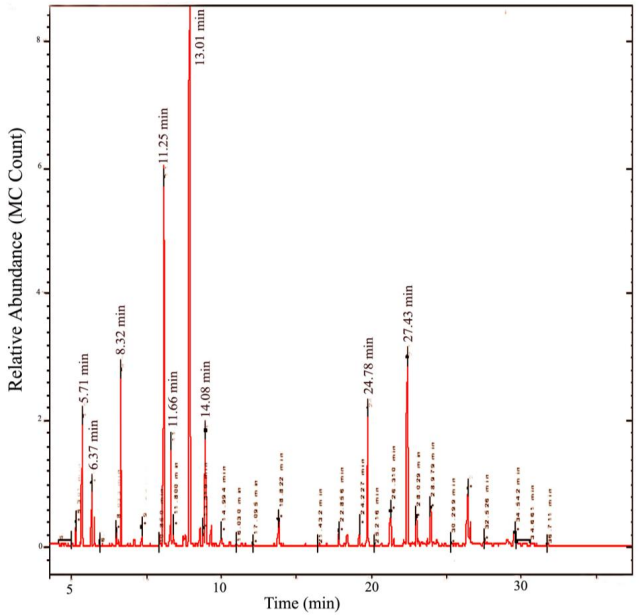


Figure: 13b. Gas-Chromatogram of Transformed Root Essential Oil of *Artemisia vulgaris*

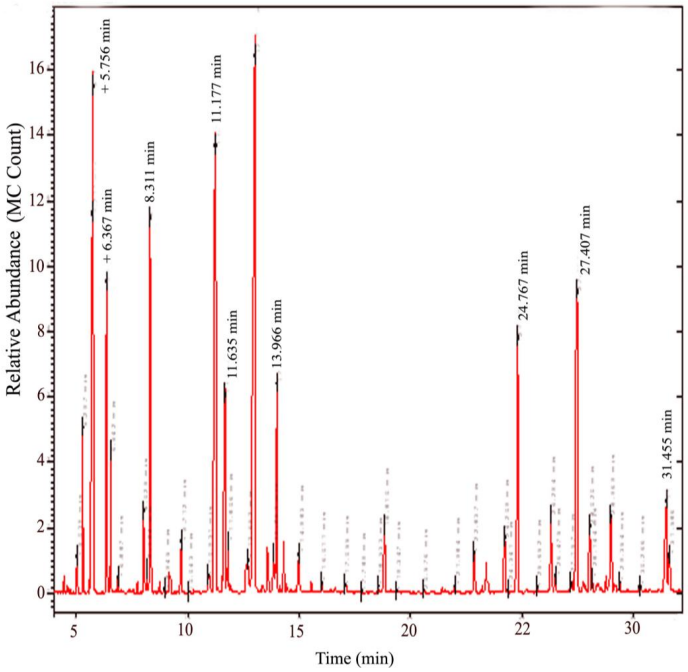


Fig. 14 Histo chemical analysis of *A. aegypti* larvae in a) control 3rd stage larvae b) 24 hours 3rd stage larvae c) control 4th stage larvae d) 24 hours 4th stage larvae essential oil

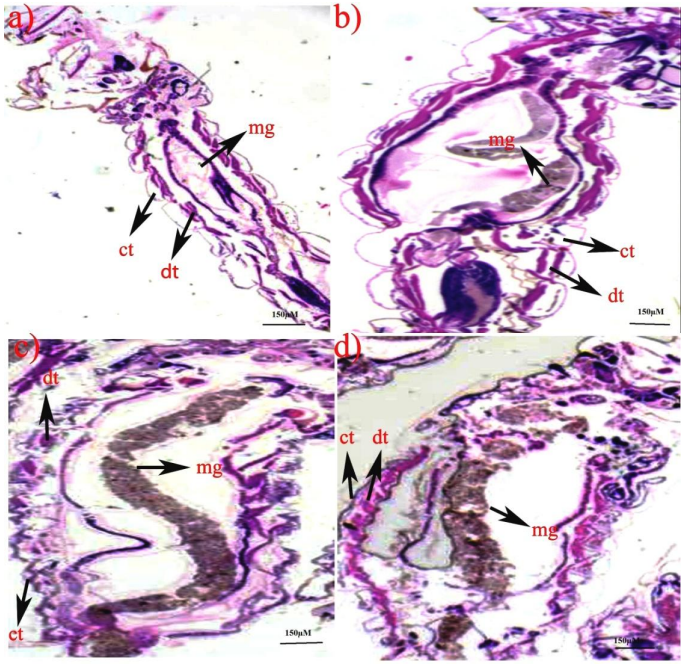


Table. 1 Effect of various concentrations (µM) of growth regulators and precursor on callus induction of *In vitro* raised leaf explants of *A. vulgaris* L.

Auxins concentration (µM)	Leaf	
	Callus Induction	Morphology of Callus
2,4D		
4.5	71.80 ^{h}	Brownish Light Pink Callus
9.0	**92.60** ^{cde}	Pinkish Nodular Callus
13.5	91.00 ^{de}	Yellowish Pink Callus
2,4D+FDP		
4.5+1	78.40 ^{g}	Whitish Pink Callus
9.0+1	**96.80** ^{ab}	Yellowish Pink Callus
13.5+1	93.60 ^{cde}	Yellowish pink Callus
4.5+3	91.20 ^{de}	Pink Callus
9.0+3	**99.00** ^{a}	Yellowish Light Pink Callus
13.5+3	95.40 ^{bc}	Green Compact Callus
NAA		
5.3	81.40 ^{fg}	Yellowish Light Pink Callus
10.7	90.20 ^{e}	Yellowish Friable Callus
16.1	**94.00** ^{cde}	Yellowish Compact Callus
NAA+FDP		
5.3+1	82.20 ^{f}	Yellowish Compact Callus
10.7+1	91.80 ^{cde}	Whitish Yellowish Friable Callus
16.1+1	**94.80** ^{bcd}	Yellowish Compact Callus
5.3+3	84.80 ^{f}	Yellowish Light Pink
10.7+3	92.20 ^{cde}	Yellowish Compact Callus
16.1+3	**95.20** ^{cde}	Yellowish Compact Callus

Treatment means followed by different letters in their superscript are significantly different from each other ($p<0.05$) according to Duncan's multiple range test.

Table: 2 Effect of Cytokinins BA, 2iP and FDP on Shoot Regeneration from Calli of *A. Vulgaris*

Hormone Concentrations (µM)	Shoot Regeneration Frequency (%)	Number of Shoots	Shoot Length (cms)
BAP			
0.4	78.8^{cd}	35.3^{cd}	8.1^{b}
2.2	81.6^{c}	42.2^{ab}	9.7^{ab}
4.4	**90.4**^{a}	**44.5**^{a}	**10.9**^{a}
6.6	88.2^{b}	37.4^{bc}	8.7^{b}
8.8	84.2^{bc}	32.3^{d}	7.9^{c}
BAP+ FDP (1 µM)			
0.4+1	80.3^{bc}	38.7^{b}	7.7^{c}
2.2+1	91.7^{ab}	46.1^{ab}	10.8^{ab}
4.4+1	**92.1**^{a}	**47.3**^{a}	**11.2**^{a}
6.6+1	82.4^{b}	38.5^{b}	10.4^{ab}
8.8+1	76.5^{d}	33.6^{c}	8.5^{b}
BAP+ FDP (3 µM)			
0.4+3	79.6^{d}	41.6^{c}	8.2^{c}
2.2+3	89.5^{bc}	48.3^{b}	10.8^{ab}
4.4+3	**99.9**^{a}	**57.6**^{a}	**11.9**^{a}
6.6+3	93.7^{ab}	48.9^{b}	10.2^{ab}
8.8+3	86.8^{c}	40.5^{cd}	9.4^{b}
2iP			
0.4			
2.4	78.9^{d}	12.4^{d}	4.9^{d}
4.9	87.4^{bc}	20.1^{bc}	5.6^{c}
7.3	90.8^{ab}	26.0^{b}	5.0c
9.8	**91.9**^{a}	**36.9**^{a}	**10.5**^{a}
2iP + FDP (1 µM)	84.4^{c}	21.4^{c}	8.3^{b}
0.4 +1			
2.4 +1	65.7^{d}	25.7^{cd}	7.4^{d}
4.9 +1	71.4^{c}	29.6^{b}	7.8^{cd}
7.3 +1	86.5^{b}	26.9^{bc}	8.1c
9.8 +1	**94.4**^{a}	**31.6**^{a}	**11.9**^{a}
2iP+ FDP (3 µM)	70.3^{cd}	20.6^{d}	9.9^{b}
0.4 + 3			
2.4 + 3	67.4^{d}	26.7^{cd}	8.3^{bc}
4.9 + 3	72.6^{bc}	30.6^{b}	8.1^{bc}
7.3 + 3	88.9b	28.5^{bc}	8.7^{b}
9.8 + 3	**98.3**^{a}	**56.3**^{a}	**15.3**^{a}
	71.0^{c}	21.9^{d}	7.6^{d}

Data recorded after 45 days of culture

Treatment means followed by different letters are significantly different from each other ($p<0.05$) according to Duncan's Multiple Range Test

Table 3: Effect of Different Concentrations of IAA and FDP on Rooting of *in vitro* raised Shoots of *A. vulgaris*.

Hormone Concentration (µM)	% Rooting	Number of Roots/Shoot	Root Length (cms)
IAA			
2.8	69.8^{c}	18.9^{bc}	11.7^{bc}
5.7	82.1^{bc}	19.8^{b}	13.8^{ab}
8.5	**87.8^{a}**	22.7^{a}	15.4^{a}
11.4	81.4^{ab}	16.8^{c}	11.6^{bc}
17.1	62.1^{d}	14.5^{d}	10.2^{c}
IAA+FDP (1 µM)			
2.8	73.2^{c}	19.5^{bc}	2.8^{d}
5.7	85.6^{b}	20.7^{b}	14.2^{ab}
8.5	**99.0^{a}**	**24.6^{a}**	**16.1^{a}**
11.4	84.5^{b}	17.8^{c}	12.4^{bc}
17.1	63.4^{d}	15.9^{cd}	10.3^{c}
IAA+FDP (3 µM)			
2.8	74.2^{d}	25.2^{bc}	3.5^{d}
5.7	87.6^{c}	28.5^{b}	4.0^{c}
8.5	**100.1^{a}**	**35.4^{a}**	**17.5^{a}**
11.4	99.5^{ab}	21.2^{c}	15.4^{ab}
17.1	94.4^{b}	20.1^{c}	12.1^{b}

Data recorded after 2 weeks of culture

Treatment means followed by different letters are significantly different from each other ($p<0.05$) according to Duncan's Multiple Range Test.

Table 4: Effect of Cytokinin on Shoot Proliferation in Nodal Explants of *Artemisia vulgaris* with and without Precursor (Farnesyl Diphosphate) treatment

Cytokinin Concentrations (µM)	Nodal Explants					
	Without Precursor		With Precursor (FDP 1 µM)		With Precursor (FDP 3 µM)	
	Percentage of Response	Number of Shoots per Explant	Percentage of Response	Number of Shoots per Explant	Percentage of Response	Number of Shoots per Explant
BAP						
0.4	63.4^{c}	12.2^{bc}	61.5^{cd}	16.2^{d}	54.2^{d}	12.5^{d}
2.2	81.1^{b}	18.1^{ab}	83.5^{b}	20.8^{b}	62.8^{c}	16.5^{c}
4.4	**92.4^{a}**	**20.5^{a}**	**95.8^{a}**	**33.3^{a}**	**99.9^{a}**	**38.4^{a}**
6.6	76.8^{bc}	14.2^{b}	79.3^{bc}	18.0^{bc}	72.6^{b}	29.1b
KN						
0.4	42.6^{c}	9.6^{cd}	54.5^{d}	16.9^{d}	64.4^{d}	10.7^{d}
2.4	66.2^{bc}	10.8^{c}	64.1^{c}	27.7^{c}	73.6^{c}	15.8^{c}
4.6	**76.9^{a}**	**28.3^{a}**	**92.9^{a}**	**44.4^{a}**	**98.1^{a}**	**49.4^{a}**
6.9	70.9^{b}	22.2^{b}	72.6^{b}	31.7^{b}	80.7^{b}	41.0^{b}
TDZ						
0.4	83.7^{bc}	33.7^{bc}	70.4^{c}	39.0^{c}	60.7^{d}	19.9^{d}
2.4	88.4^{b}	38.6^{ab}	80.5^{b}	48.6^{bc}	75.3^{c}	27.7^{c}
4.9	**91.5^{a}**	**40.1^{a}**	**94.9^{a}**	**53.9^{a}**	**99.0^{a}**	**59.6^{a}**
6.9	73.4^{d}	32.7^{bc}	93.1^{ab}	50.9^{ab}	83.7^{b}	40.3^{b}

Data recorded after 2 weeks of culture

Treatment means followed by different letters are significantly different from each other ($p<0.05$) according to Duncan's Multiple Range Test.

Table 5: Effect of Different Auxins on Rooting of *In vitro* Raised Shoots of *A. vulgaris*.

Auxins Concentrations (µM)	With FDP (1 µM)			With FDP (3 µM)			Without FDP		
	Percentage of Roots	Number of Roots / Shoot	Root Length (cms)	Percentage of Roots	Number of Roots / Shoot	Root Length (cms)	Percentage of Roots	Number of Roots / Shoot	Root Length (cms)
Control	10.4^{e}	4.1^{e}	3.1^{e}	4.0^{e}	3.7^{e}	4.5^{e}	9.7^{e}	3.8^{e}	3.2^{e}
IAA									
2.6	69.6^{bc}	12.6^{c}	7.8^{cd}	47.6^{d}	10.6^{c}	5.5^{d}	63.2^{c}	4.1^{d}	8.3^{c}
5.3	76.2^{b}	13.8^{b}	9.8^{b}	58.8^{cd}	11.2^{b}c	6.5^{cd}	75.3^{b}	7.9^{c}	10.2^{ab}
8.0	**82.1^{a}**	**16.3^{a}**	**12.7^{a}**	**88.4^{a}**	**15.8^{a}**	**16.6^{a}**	**80.2^{a}**	**11.7^{a}**	**11.9^{a}**
10.7	61.9^{cd}	15.3^{ab}	11.2^{ab}	73.2^{b}	13.3^{ab}	8.9^{b}	57.9^{cd}	10.5^{ab}	9.6^{b}
16.1	41.2^{d}	12.2^{bc}	8.2^{c}	67.9^{c}	12.0^{b}	8.1^{bc}	40.4^{d}	9.8^{bc}	7.8cd
IBA									
2.8	75.4^{cd}	19.9^{bc}	11.4^{bc}	65.4^{d}	9.4^{d}	6.7^{d}	72.6^{c}	5.7^{d}	11.6^{bc}
5.7	86.7^{b}	21.6^{ab}	13.3^{ab}	70.4^{cd}	10.5^{c}	8.4^{cd}	84.5^{b}	7.5^{cd}	12.4^{ab}
8.5	**98.2^{a}**	**25.1^{a}**	**15.5^{a}**	**99.2^{a}**	**18.4^{a}**	**17.8^{a}**	**90.5^{a}**	**17.2^{a}**	**14.6^{a}**
11.4	84.1^{bc}	17.7^{cd}	12.6^{b}	82.4^{b}	12.8^{b}	11.9^{b}	83.7^{bc}	12.9^{b}	11.5^{bc}
17.1	62.6^{d}	14.7^{d}	10.9^{c}	76.7^{c}	11.2^{bc}	9.2^{c}	61.4^{d}	10.6^{bc}	10.5^{d}

Data recorded after 2 weeks of culture

Treatment means followed by different letters are significantly different from each other ($p<0.05$) according to Duncan's Multiple Range Test.

Table 6. Frequency of hairy root induction on leaf explants derived from *A. rhizogenes* strains

Bacterial strain	Explants type	Frequency of transformation%	Hairy root induction (days)
A_4	Leaf explants	93.0±0.4	11
R1000	Leaf explants	85.9±.0.2	

Data are means of three replicates and ± is SE

Table 7. The essential oil constituents in *A. vulgaris* L. hairy root with and without precursor treated

Compounds	LRI	Molecular weight	Percentage		Method of identification
			Without Precursor	With Precursor	
(E)-Salvene	867	138	tr	tr	MS, RI
Santolina triene	910	136	tr	0.2 (0.1)	MS, RI
Tricyclene	928	136	0.2 (0.1)	0.5 (0.2)	MS, RI
α-Pinene	941	136	0.8 (0.2)	1.7 (0.2)	MS, RI, ST
α-Fenchene	953	136	2.9 (0.3)	3.7 (0.3)	MS, RI
Camphene	955	136	4.2 (0.2)	5.5 (0.1)	MS, RI, ST
Sabinene	978	136	2.2 (0.2)	3.0 (0.4)	MS, RI, ST
β-Pinene	982	136	1.2 (0.1)	1.7 (0.2)	MS, RI, ST
3-Octanone	988	128	tr	tr	MS, RI, ST
Myrcene	991	136	tr	tr	MS, RI, ST
1,8-Dehydrocineole	993	152	0.2 (0.1)	0.2 (0.0)	MS, RI
3-Octanol	995	130	tr	tr	MS, RI, ST
α-Phellandrene	1007	136	tr	tr	MS, RI, ST
α-Terpinene	1020	136	tr	0.1 (0.1)	MS, RI, ST
p-Cymene	1028	134	tr	1.1 (0.2)	MS, RI, ST
Limonene	1033	136	0.8 (0.2)	0.4 (0.1)	MS, RI, ST
1,8-Cineole	1035	154	0.2 (0.0)	5.8 (0.4)	MS, RI, ST
Lavender lactone	1040	126	5.7 (0.3)	tr	MS, RI
(E)- -Ocimene	1050	136	tr	0.1 (0.1)	MS, RI, ST
Artemisia ketone	1062	152	tr	0.1 (0.0)	MS, RI
γ-Terpinene	1064	136	0.4 (0.1)	0.2 (0.1)	MS, RI, ST
cis-Sabinene hydrate	1070	154	1.0 (0.2)	0.8 (0.2)	MS, RI, ST
Artemisia alcohol	1083	154	tr	tr	MS, RI
Terpinolene	1088	136	0.8 (0.3)	tr	MS, RI, ST
trans-Sabinene hydrate	1098	154	tr	tr	MS, RI, ST
Linalool	1100	154	6.5 (0.5)	0.6 (0.1)	MS, RI, ST
α-Thujone	1104	152	4.0 (0.3)	12.3 (0.6)	MS, RI, ST
β-Thujone	1116	152	1.1 (0.1)	3.4 (0.3)	MS, RI, ST
cis-p-Menth-2-en-1-ol	1123	154	0.2 (0.1)	0.1 (0.0)	MS, RI
Chrysanthenone	1125	150	0.7 (0.1)	1.0 (0.2)	MS, RI
Thujanol (=3-isothujanol)	1138	154	tr	tr	MS, RI
trans-Pinocarveol	1141	152	1.1 (0.2)	0.9 (0.2)	MS, RI, ST
Camphor	1145	152	15.3 (0.5)	20.8 (0.9)	MS, RI, ST
Neoisothujanol (=3-thujanol)	1152	154	tr	0.8 (0.1)	MS, RI
Sabina ketone	1156	138	1.0 (0.2)	tr	MS, RI
Isoborneol	1158	154	1.1 (0.3)	0.8 (0.2)	MS, RI, ST
Borneol	1167	154	5.3 (0.5)	4.0 (0.2)	MS, RI, ST
4-Terpineol	1179	154	tr	0.8 (0.1)	MS, RI, ST
Thuj-3-en-10-al	1183	150	1.0 (0.2)	0.1 (0.1)	MS, RI
p-Cymen-8-ol	1185	150	0.2 (0.1)	tr	MS, RI
Myrtenal	1195	150	tr	0.9 (0.1)	MS, RI, ST
Verbenone	1206	150	1.1 (0.2)	0.3 (0.1)	MS, RI, ST
trans-Carveol	1219	152	0.1 (0.0)	tr	MS, RI, ST

cis-Carveol	1231	152	0.2 (0.1)	0.1 (0.0)	MS, RI, ST
Cumin aldehyde	1241	148	0.2 (0.0)	0.1 (0.1)	MS, RI, ST
Carvone	1244	150	tr	0.1 (0.0)	MS, RI, ST
Perillaaldehyde	1273	150	0.1 (0.1)	0.2 (0.1)	MS, RI
Lavandulyl acetate	1289	196	0.1 (0.0)	1.6 (0.2)	MS, RI, ST
Silphiperfol-5-ene	1329	204	0.2 (0.1)	tr	MS, RI
α-Cubebene	1351	204	0.2 (0.1)	tr	MS, RI
Silphiperfol-4,7(14) diene	1361	202	1.9 (0.2)	0.2 (0.1)	MS, RI
Cyclosativene	1370	204	tr	tr	MS, RI, ST
α-Copaene	1376	204	tr	0.8 (0.2)	MS, RI
β-Bourbonene	1384	204	tr	0.1 (0.0)	MS, RI
β-Cubebene	1390	204	0.8 (0.1)	0.6 (0.1)	MS, RI
β-Elemene	1391	204	tr	0.1 (0.1)	MS, RI
Cyperene	1399	204	0.7 (0.2)	tr	MS, RI
α-Gurjunene	1409	204	1.0 (0.2)	1.0 (0.1)	MS, RI
β-Caryophyllene	**1419**	**204**	**4.4 (0.3)**	**5.7 (0.3)**	**MS, RI, ST**
β-Cedrene	1421	204	–	tr	MS, RI, ST
trans-Alpha-bergamotene	1439	204	–	tr	MS, RI
Aromadendrene	1440	204	–	0.1 (0.0)	MS, RI, ST
-Humulene	1456	204	1.4 (0.1)	1.4 (0.3)	MS, RI, ST
allo-Aromadendrene	1461	204	0.3 (0.2)	0.3 (0.1)	MS, RI
9-epi-(E)-caryophyllene	1467	204	–	tr	MS, RI
γ-Curcumene	1480	204	0.2 (0.1)	0.2 (0.0)	MS, RI
Germacrene D	1482	204	0.2 (0.2)	7.2	MS, RI
β-Selinene	1487	204	0.2 (0.0)	0.2 (0.1)	MS, RI
trans-Muurola-4(14),5-diene	1493	204	7.0 (0.5)	0.2 (0.1)	MS, RI
Bicyclogermacrene	1495	204	0.2 (0.1)	1.3 (0.3)	MS, RI
α-Muurolene	1499	204	0.1 (0.1)	0.3 (0.1)	MS, RI
α-Cadinene	1538	204	1.2 (0.4)	0.2 (0.1)	MS, RI
γ-Cadinene	1511	204	0.2 (0.1)	0.3 (0.1)	MS, RI
δ-Cadinene	1524	204	0.1 (0.0)	1.0 (0.3)	MS, RI
α-Calacorene	1542	204	0.3 (0.1)	tr	MS, RI
trans-Nerolidol	1564	222	2.4 (0.4)	tr	MS, RI, ST
Davanone B	1566	236	0.7 (0.2)	2.4 (0.3)	MS, RI
Ledol	1567	222	–	tr	MS, RI
Caryophyllene oxide	1583	220	–	0.8 (0.2)	MS, RI, ST
Longiborneol (=juniperol)	1594	222	0.1 (0.0)	0.2 (0.1)	MS, RI
Guaiol	1597	222	–	tr	MS, RI, ST
Humulene oxide II	1608	220	0.2 (0.1)	0.1 (0.1)	MS, RI
Caryophylla-4(14), 8 (15)-dien-5-ol	1640	220	–	0.1 (0.0)	MS, RI
T-cadinol	1642	222	0.5 (0.3)	0.2 (0.0)	MS, RI
T-muurolol	1644	222	–	tr	MS, RI
β-Eudesmol	1651	222	0.3 (0.1)	0.1 (0.0)	MS, RI, ST
α-Eudesmol	1654	222	–	0.2 (0.1)	MS, RI

LRI, linear retention indices; tr, trace amounts (<0.1%); –, not detected

Table 8. Larvicidal activity of essential oil against the dengue fever larvae *A. aegypti* 3rd stage 12 and 24hours of exposure period

Name of the mosquito Species	Time	Concentration (ppm)	%mortality	LC_{50}(LCL-UCL)[a]	LC_{90}(LCL-UCL)[a]	X^2 (df=4)[b]
A.aegypti 3rd stage	12 h	Essential oil				
		25	16.66 ± 3.3			
		50	31.00 ± 0.57			
		100	49.00 ± 1.0	128.99 (103.54-164.36)	1447.08 (864.75 -3409.18)	3.45
		200	62.33 ± 1.4			
		400	68.00 ± 1.52			
A.aegypti 3rd stage	24 h	25	22.33 ± 4.5			
		50	35.00 ± 5.9			
		100	47.66 ± 3.7	111.15 (88.11-141.66)	1441.51 (827.05-3486.89)	3.99
		200	62.33 ± 2.2			
		400	73.33 ± 2.4			

LCL lower confidence level, UCL upper confidence level

[a] 95 % Confidence interval

[b] Degrees of freedom; χ^2 chi-square value

Table 9. Larvicidal activity of essential oil against the dengue fever larvae *A. aegypti* 4th stage 12 and 24hours of exposure period

Name of the mosquito Species	Time	Concentration (ppm)	%mortality	LC_{50}(LCL-UCL)[a]	LC_{90}(LCL-UCL)[a]	X^2 (df=4)[b]
A. aegypti 4th stage	After 12 h	Essential oil				
		25	21.66 ± 1.6			
		50	32.33 ± 1.4			
		100	44.00 ± 2.0	136.15	2223.55	3.92
		200	54.00 ± 2.1	(106.11-181.43)	(1136.71-6803.14)	
		400	62.66 ± 1.5			
A.aegypti 4th stage	24 h	Essential oil				
		25	25.00 ± 2.8			
		50	45.00 ± 2.5			
		100	59.33 ± 2.00	74.22	858.36	3.60
		200	68.33 ± 1.20	(57.92-92.56)	(540.95-1755.18)	
		400	80.00 ± 2.8			

LCL - Lower Confidence Level, UCL - Upper Confidence Level

[a] 95 % Confidence Interval

[b] Degrees of freedom; χ^2 chi-square value

Table 10. Repellent activities of the *A. vulgaris* essential oil against dengue fever vector *A. aegypti*

Concentration of *A. vulgaris* L. essential oil (%)	Percentage of repellency ± SD (min)		
	15'	30'	60'
Control (DEET 12%)	11.00±2.8[a]	8.33±2.8[a]	4.33±1.5[a]
2.5	85.00±4.3[d]	60.00±10.0[d]	49.00±1.7[e]
5.0	71.00±1.0[c]	53.66±3.2[d]	39.66±2.5[d]
15	39.00±8.5[b]	35.33±5.5[c]	30.66±2.5[c]
25	33.33±2.8[b]	24.00±3.6[b]	15.66±4.0[b]
50	15. ±5.0[a]	9.00±1.7[a]	6.66±1.5[a]

Means in each column followed by different letters are significantly different (P<0.05, by one-way ANOVA and Duncan's Multiple Range Test)