

3. Materials and Methods

3.1. Collection of plants

In the present investigation, *E. alba* and *A. barbadensis* plants were collected from the Medicinal Plants garden at Tezpur University, Tezpur, Assam, India. *A. barbadensis* plantlets were cultivated in pots with sand and less water-absorbing soil for a period of 30 days with regular pulverization. *E. alba* plantlets were grown in water absorbing soil for a period of 15 days till plants attained maturity and its seeds were also used for cultivation in higher quantity.

3.2. Morpho-phenological study of the plants

The important morphological characters such as plant height, leaf shape, arrangement, type of inflorescence, flower colour and type of seeds were studied for both the selected plants and recorded. Height and length of the plant, size of leaf were measured using measuring tape and scale was used to measure length petiole, flowers diameter etc. Colour chart was used to describe the flower colour. Data were collected from five sample plants. The phenological data such as flower initiation, flowering period, seed formation etc were studied and recorded.

3.3. Genomic study of the plants

3.3.1. DNA isolation

Fresh young leaves were used for the isolation of DNA from the selected plant

species. The leaves were collected in the morning, washed with distilled water repetitively, placed in between moist tissue papers and then stored in darkness at room temperature. The experiment was done according to the protocol given by Doyle and Doyle²¹⁰ with some modifications.

3.3.1.1. Equipment used

Autoclave

Mortar and pestle

Mettler electronic balance

Micropipettes – (2-20 µl, 20-200 µl and 200-1000 µl) with tips

Polypropylene tube (25 ml)

Microcentrifuge tube (1.5 ml)

Microwave oven

Incubator (37°C)

Sorvall RC 5B Plus centrifuge

Bench top centrifuge (Hettich Zentrifugen, MIKRO 12-24)

Magnetic stirrer

Shaking hot water bath

Speed vacuum (Maxi dry plus, Hoefer Pharmacia Biotech Inc., USA)

Gel Doc system (BIO RAD Gel Doc 1000)

Vertical Gel Apparatus

UV/VIS Spectrophotometer (Beckman DU® 530 Life Sciences)

3.3.1.2. Reagents and chemicals

Tris-Cl pH 8.0 (1.0 M): Tris base 121.1 g was dissolved in 800 ml of dH₂O. The pH was adjusted to 8.0 by adding concentrated HCl. The solution was allowed to cool to room temperature. The volume was adjusted to 1 liter and sterilized by autoclaving. The solution was stored at room temperature.

EDTA pH 8.0 (0.5 M): Na EDTA.2H₂O 186.1 g was dissolved in 700 ml of dH₂O. The pH was adjusted to 8.0 with 10 M NaOH (~ 50 ml). The volume was adjusted to 1 liter and sterilized by autoclaving. The solution was stored at the room temperature.

NaCl (5.0 M): NaCl 292 g was added to 900 ml of dH₂O and the volume was adjusted to 1.0 liter.

CTAB (2.5%): CTAB 2.5 g was added to 80 ml of distilled water and dissolve properly. Volume was adjusted to 100 ml with distilled water.

Chloroform: Isoamyl alcohol (24:1 v/v): Isoamyl alcohol 4 ml was added to 96 ml of chloroform and mix properly.

β -mercaptoethanol (Himedia)

Bromophenol blue (Himedia)

Ethidium bromide (Himedia)

Isopropanol (Merck)

RNase (Bangalore Genei)

Agarose (Himedia)

Hind III digested λ DNA molecular weight marker (Bangalore Genei, India)

3.3.1.3. Buffers

DNA extraction was performed using extraction buffer, high salt TE buffer and TAE buffer.

3.3.1.3.1. Extraction buffers (100 ml)

100 mM Tris-Cl (pH 8.0): From 1M Tris.Cl solution, 10 ml was added to the extraction buffer to make the concentration 100mM.

25 mM EDTA: From the stock EDTA solution (0.5 M), 5 ml was added to the extraction buffer.

1.5 M NaCl: From 5M NaCl solution, 30 ml was added to the extraction buffer to make 1.5M concentration of NaCl.

2.5% CTAB: 2.5 gm CTAB was added to the extraction buffer and dissolve properly.

0.2% β -mercaptoethanol (v/v): 200 μ l of β -mercaptoethanol was added to the extraction buffer just before the experiment.

After adding all the compositions, volume was made upto 100 ml with distilled water.

3.3.1.3.2. High salt TE buffer (100 ml)

1 M NaCl: From the 5M NaCl solution, 20 ml was added to the high salt TE buffer.

10 mM Tris-Cl (pH 8.0): From the 1M Tris-Cl solution, 1 ml was added to the TE buffer to make the Tris-Cl concentration 10mM.

1 mM EDTA: 200 µl of 0.5M EDTA was added to the TE buffer solution to make the final concentration of EDTA as 1mM in the solution.

3.3.1.3.3. TAE buffer

24.2 g Tris-Cl dissolved in 57.1 ml glacial acetic acid and 10.1ml EDTA was added into the solution and pH was adjusted to 8.0. Volume was made upto 100ml with distilled water.

3.3.1.4. Loading dye and fluorochrome

Bromophenol blue and ethidium bromide were used as loading dye and fluorochrome, respectively for DNA visualization during and after electrophoresis.

3.3.1.5. Bromophenol blue (6x, 4.0 ml)

Bromophenol blue 10 mg

Xylene cyanol 10 mg

Glycerol 1.2 ml (autoclaved)

3.3.1.6. Ethidium bromide (10 mg/ml)

Ethidium bromide 100 mg

Sterile dH₂O 10 ml

Stored at 4°C in darkness

3.3.1.7. DNA extraction protocol

The CTAB based DNA isolation protocol described by Doyle and Doyle²¹⁰ was used and standardized with slight modification.

1. Preheated CTAB extraction buffer at 60°C in a water bath.
2. Fresh leaves weighing 1 g of the selected plants was ground into fine powder in liquid nitrogen in a chilled mortar.
3. The powder was transferred directly to a 25 ml polypropylene tube and added 2/3 of freshly prepared preheated extraction buffer and mixed by gentle inversion to slurry.
4. The sample was incubated at 60°C in a water bath for 2 h with occasional mixing to avoid aggregation of the homogenate.
5. Extract once with chloroform-isoamyl alcohol (24:1), mixing gently but thoroughly. This produces two phases, an upper aqueous phase which contains the DNA, and a lower chloroform phase that contains some degraded proteins, lipids, and many secondary compounds. The interface

between these two phases contains most of the "junk"--cell debris, many degraded proteins, etc.

6. The extract was centrifuged at 6,000 rpm in a Sorvall RC-5B Plus centrifuge for 10 min at 25°C to get rid of the junk. The upper aqueous phase was transferred to a clean polypropylene tube and the process was repeated twice to clear the aqueous phase.
7. An aliquot of 3 ml of 5 M NaCl was added to the aqueous phase and mixed properly by gentle inversion without vortexing.
8. An ice cold isopropanol (0.6 volumes) were added to the mixture and the mixture was incubated at 4°C overnight to precipitate the nucleic acid.
9. The sample was centrifuged at 8,000 rpm in the centrifuge for 10 min at 4°C.
10. The supernatant was poured off and the pellet was washed with 80% ethanol and carefully transferred to a clean micro-centrifuge tube. The pellet was again washed with 80% ethanol.
11. The pellet was air-dried and dissolved in 0.5 ml of high salt TE buffer.
12. 5 µl of RNase was added to the sample and incubated at 37°C for 1h.
13. After incubation, the sample was extracted with equal volume of chloroform: isoamyl alcohol (24:1). The aqueous layer was transferred to a fresh 1.5 ml microcentrifuge tube and added 2 volumes of pre-cooled ethanol.
14. The sample was then centrifuged at 7,500 x g (10,000 rpm) for 10 min in a bench top centrifuge (Hettich Zentrifugen, MIKRO 12-24) at room

temperature (25-30°C) to precipitate the DNA.

15. The pellet was rinsed with 80% ethanol, dried in speed vacuum (Maxi dry plus, Hoefer Pharmacia Biotech Inc., USA) and resuspended in 200 µl of high salt TE buffer.

3.3.1. 8. Modification of the protocol

The chloroform: isoamyl alcohol (24:1) washing step was performed twice to clear the aqueous phase of the extract. Before addition of ice-cold isopropanol, 3 ml of 5M NaCl solution was added to the sample to precipitate the DNA.

3.3.1.9. Purity and yields of the isolated DNA from selected plants

The concentration and the purity of the isolated DNA were measured by taking the reading at 260 nm and 280 nm in a UV/VIS spectrophotometer (Beckman DU® 530 Life Sciences) against blank and diluted sample. Isolated DNA sample 5 µl was taken in a quartz cuvette and made up the volume to 1 ml by adding double distilled water. Since 1 OD (optical density) corresponds to 50 µg of double stranded (ds) DNA/ml, the following calculation was done to determine the concentration of DNA:

$$\text{DNA concentration } (\mu\text{g/ml}) = (\text{OD}_{260}) \times (\text{dilution factor}) \times (50 \mu\text{g/ml})$$

The ratio of absorbance of DNA solution at 260 nm/280 nm is a measure of the purity of DNA sample and it should be in between 1.75 to 2.00.

3.3.2. Genome size determination

Genome size of the plants was determined by using flow cytometry according to the procedure described by Otto²¹¹ with minor modifications. Otto I and Otto II buffer solutions were used for the same.

3.3.2.1. Preparation of Otto I buffer

4.2 gm of citric acid monohydrate (0.1 M) and 1ml 0.5% (v/v) Tween 20 was dissolved in 100 ml distilled water. The volume was adjusted to 200 ml with distilled water and kept at 4°C for further use.

3.3.2.2. Preparation of Otto II buffer

28.65 g of Na₂HPO₄, 12H₂O (0.4 M) was dissolved in 100 ml of distilled water. The volume was adjusted to 200 ml with distilled water and kept at 4°C for further use.

3.3.2.3. Stain or flurochrome

Propidium Iodide 50 µg/ml

RNase 50 µg/ml

3.3.2.4. Procedure

1. 20 mg of fully grown young leaves were collected from a selected plant, washed thoroughly and chopped with a razor blade in 0.5 ml of ice cold Otto I buffer in a Petri dish.
2. Then added another 0.5 ml of ice cold Otto I buffer and mixed thoroughly

with a pipette.

3. The suspension was filtered through a 42 μm nylon mesh and incubated the sample for 5 min with occasional shaking.
4. Otto II buffer 2 ml was added to the sample along with the stained solution (200 μl of stock solution) and stored at room temperature for about 15 min.
5. The sample was analyzed in a FACS Calibur flow cytometer (Becton Dickinson, USA) for relative DNA content of isolated nuclei. The instrument was calibrated using FACS COMP software.
6. Garden pea (*Pisum sativum*) was used as the external reference standard. The use of an internal reference standard gave poor reading of results in peak quantities, probably resulting from interference between the staining solutions and the genome of pea and the selected species. For this reason external reference standard was used and controlled every 3 samples to check the calibration of the flow cytometer.
7. The gain of the instrument was adjusted so that G_0/G_1 peak of pea (reference standard) was positioned at channel 200.
8. The nuclear DNA content of the plant samples was estimated according to the equation:
9. $2C$ nuclear DNA content of the sample = $(9.09 \times G_0/G_1 \text{ peak mean of the sample}) / G_0/G_1 \text{ peak mean of pea}$. The means of nuclear DNA content were calculated for each sample and analyzed as a single value.

3.4. Isolation of phyto-compounds from the selected plants

Fresh tender leaves of *E. alba* and *A. barbadensis* were used for the isolation of active compounds. Young leaves from both the plants were collected.

3.4.1. Isolation of phyto-compounds from *E. alba*

3.4.1.1. Preparation of crude extracts

The tender leaves of *E. alba* were washed thoroughly with distilled water and shade-dried. Dried leaves were ground into powder and 400 g of the powder was extracted with 800 ml of methanol in a soxhlet apparatus for 12 h. The solvent was evaporated to dryness from the extract in a rotary evaporator. The procedure yielded 8.23 g of brown methanol extract. The crude extract was partitioned between equal volumes of ethylacetate and methanol. Both the parts (4.08 g each) were concentrated and taken for further analysis.

3.4.1.2. Fractionation of crude extracts

The methanol dissolved fraction was chromatographed with column size 20 cm x 14 mm and the column was packed with silica gel (60-120 mesh size) and methanol. Elution was done with water - methanol in increasing polarity and at 70:30 (v/v) solvent gradients and the compound Ea 1 was collected.

The crude ethylacetate extract was chromatographed with column size 32 cm x 19 mm and it was packed with silica gel (100-200 mesh size) and hexane. The compound was eluted successfully with 0.5-10% ethylacetate in hexane with a flow rate 1ml/min. The fraction eluted with 2% ethylacetate (2:98, v/v) was

afforded as Ea 2.

3.4.1.3. Thin layer chromatography

Compound Ea 1 was also subjected to thin layer chromatography (TLC) using chloroform: toluene (7:3) solvent system followed by Iodine spraying and heating. The R_f value was calculated for Ea 1. The preparative TLC was performed on the TLC plate having size 20 cm x 20 cm and coated with silica gel. TLC plates were visualized by Iodine spray, followed by heating. The iodine visible portions were scraped, then dissolved in the same solvent system and eluted with a column where the silica gel retained and the dissolved compound passed through. After evaporation of the solvent the fraction afforded the compound Ea 1 (56.2 mg).

Similarly, the compound Ea 2 was subjected to thin layer chromatography (TLC) using hexane: ethylacetate (3:7) solvent system followed by anisaldehyde spraying. The R_f value was calculated for the compound. The preparative TLC was performed on the TLC plate having size 20 cm x 20 cm and coated with silica gel. The TLC plates were visualized after spraying with anisaldehyde reagent. The visible portions were scraped, dissolved in the same solvent system and eluted with a column where the silica gel retained and the dissolved compound passed through. After evaporation of the solvent the first fraction afforded the compound Ea 2 (22.5 mg).

3.4.2. Isolation of phyto-compounds from *A. barbadensis*

3.4.2. A) Preparation of crude extracts

Tender leaves of *A. barbadensis* were collected, washed thoroughly with distilled water and shade-dried. Dried leaves were powdered and 500 g of it was extracted with 1000 ml of methanol in a soxhlet apparatus for 20 h. The solvent was evaporated to dryness from the extract in a rotary evaporator. The procedure yielded 6.77 g of dark brown methanol extract.

3.4.2. B) Fractionation of crude extracts

The crude methanol extract was eluted with column size 32 cm x 19 mm and it was packed with silica gel (100-200 mesh size) and hexane. The extract was separated using 0.5-10% ethylacetate in hexane with a flow rate 1ml/min. The fraction eluted with 2% ethylacetate (2:98, v/v), afforded the compound Av 3. From the same extract, another compound Av 4 was eluted by using 4% ethylacetate in column chromatography.

3.4.2. C) Thin layer chromatography

The fraction Av 3 was subjected to TLC using hexane: ethylacetate (3:7) solvent system followed by anisaldehyde spraying. The R_f value was calculated for the compound. The preparative TLC was performed on the TLC plate having size 20 cm x 20 cm and coated with silica gel. TLC plates were visualized by anisaldehyde spray. The anisaldehyde visible portions were scraped, then dissolved in the same solvent system and eluted with a column where the silica gel retained and the dissolved compound passed through. After

evaporation of the solvent the first fraction afforded the compound Av 3 (43.2 mg).

To isolate the compound Av 4, TLC was done using hexane: ethylacetate (3:7) solvent system followed by anisaldehyde spraying and heating and R_f value of the compound was calculated. The compound spotted portions were scraped, then dissolved in the same solvent system and eluted with a column where the silica gel retained and the dissolved compound passed through. After evaporation of the solvent, 51.4 mg of compound Av 4 was collected.

3.5. Identification of isolated compounds

The isolated compounds from the plant species were subjected to high performance liquid chromatography (HPLC), infrared (IR) spectroscopy and mass spectroscopy. Proton and carbon nuclear magnetic resonance (NMR) spectra were also studied for their identification and structure elucidation.

3.5.1. High performance liquid chromatography (HPLC)

Purification of the isolated compounds from *E. alba* and *A. barbadensis* was done in HPLC using Ascentis Reverse Phase-Amide column with the dimension of 10 cm×10 mm I.D. and particle size 10 μ m (Supelco, USA). The HPLC analysis of the compound was performed using liquid chromatography (Waters, model 600E) with a 486 UV variable wavelength detector and Novapack® column C-18 (5 μ m, 150×3.9 mm). The mobile phase consisted of a gradient mixture, methanol/water (70:30, v/v). The solution was degassed in an ultrasound bath and filtered under vacuum through a membrane (Millipore,

PVDF). The flow was 1.0 ml/min and the sensitivity was 0.001 AUFS. The absorption spectra of the compounds were measured at 280 nm for purification purpose. All three compounds were separated using two solvent gradient systems: solvent A, 100% MilliQ water and solvent B, 100% Methanol (HPLC grade). The separated compounds were collected by repetitive injections. The collected compounds were run in the same gradient to confirm their elution time. The solvent gradient used for performing HPLC is shown in Table 3.1.

Table 3.1 HPLC solvent gradient system

| Time (min) | Flow (ml/min) | % A | %B |
|-------------------|----------------------|------------|-----------|
| 0 | 2 | 100 | 0 |
| 5 | 2 | 80 | 20 |
| 10 | 2 | 70 | 30 |
| 15 | 2 | 60 | 40 |
| 20 | 2 | 50 | 50 |
| 25 | 2 | 40 | 60 |
| 30 | 2 | 30 | 70 |
| 35 | 2 | 20 | 80 |
| 40 | 2 | 0 | 100 |
| 45 | 2 | 100 | 0 |
| 48 | 2 | 100 | 0 |

3.5.2. Fourier Transformation Infrared Spectroscopy (FTIR)

FTIR spectra of all the isolated compounds were recorded using KBr pellet in Nicolet Impact 410 FT-IR Spectrometer. From each sample, 5 mg was prepared by dispersing the sample uniformly in a matrix of dry KBr, compressed to form an almost transparent disc. The spectra showing functional groups were used to study the composition of the compounds. IR spectra were collected from 400 – 4,000 wave numbers (cm^{-1}).

3.5.3. Mass spectroscopy

The mass spectra of the compounds were recorded in a Micromass ToF-Spec 2E instrument using nitrogen 337 nm lasers with 4-nanosecond pulse and mass Lynx 4.1 SCN 714 in SAIF, Central Drug Research Institute, Lucknow, India. Each compound was dissolved in methanol (MeOH) was used as the matrix. A minimum of 1-2 mg sample was taken for the mass spectra analysis.

3.5.4. Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectra of the isolated and purified compounds were scanned on Varian Mercury 400 Spectrometer operating at 400 MHz for ^1H and ^{13}C nuclei, respectively. The deuterated chloroform (CDCl_3) was used as solvent and tetramethyl silane (TMS) as the internal standard. For ^1H NMR spectra 5 mg of sample and for ^{13}C NMR spectra, 30-35 mg sample were taken for analysis.

3.6. Biological characterization of the isolated compounds

3.6.1. Antibacterial assay of the isolated compounds

The purified compounds were subjected to antibacterial assay using three pathogenic bacteria and one yeast.

3.6.1.1. Test organisms

The standard strains of Microbial Type Culture Collection (MTCC) were obtained from the Department of Molecular Biology & Biotechnology, Tezpur University, Assam, India. The same was used to assess the antibacterial potential of the plant compounds. Bacterial strains *Bacillus subtilis* (MTCC 619), *Klebsiella pneumoniae* (MTCC 109), *Escherichia coli* (MTCC 739), *Pseudomonas aeruginosa* (MTCC 7815) and *Staphylococcus aureus* (MTCC 737) were used as the test organisms.

3.6.1.2. Media

The bacterial test pathogens were cultured and maintained in nutrient agar (NA) medium. For antibacterial activity test, Mueller-Hinton (MH) agar medium was used. The composition of the medium is presented below:

3.6.1.2.1. Nutrient Agar medium

| | |
|--------------|------|
| Compositions | g/l |
| Peptone | 10.0 |
| Beef extract | 10.0 |

| | |
|-----------------|-----|
| Sodium chloride | 5.0 |
|-----------------|-----|

| | |
|------|------|
| Agar | 12.0 |
|------|------|

pH of the medium was adjusted to 7.3

3.6.1.2.2. Muller Hinton agar medium

| | |
|--------------|-----|
| Compositions | g/l |
|--------------|-----|

| | |
|---------------|-------|
| Beef infusion | 300.0 |
|---------------|-------|

| | |
|-------------------------|------|
| Casein acid hydrolysate | 17.5 |
|-------------------------|------|

| | |
|--------|-----|
| Starch | 1.5 |
|--------|-----|

| | |
|------|------|
| Agar | 17.0 |
|------|------|

pH of the medium was adjusted to 7.2

3.6.1.3. Determination of antibacterial activity

The antibacterial activity of the isolated and purified compounds of *E. alba* and *A. barbadensis* were evaluated by well diffusion method²¹². Stock cultures were maintained at 4°C on nutrient agar medium. Active cultures were prepared by transferring a loop full of cells from each stock culture to test tubes containing Mueller-Hinton broth (MHB) and incubated at 37°C for 24 h without agitation. The disc diffusion method was used to determine the antibacterial activity of the isolated compounds from *E. alba* and *A. barbadensis*. *In-vitro* antibacterial activity was screened by using Mueller Hinton Agar medium. The MHA plates were prepared by pouring 15 ml of

molten media into sterile petri dishes. The plates were allowed to solidify for 20 min and then 200 μl of the test microbes in the log phase of growth (10^6 - 10^8 cells as per McFarland standard) were seeded on the surface of Mueller Hinton agar medium using a micropipette and spreaded all over the medium using a sterile glass spreader. With the help of a sterile cork borer wells having 6 mm diameter each were made on Mueller Hinton agar plates. The tested compounds (Ea 1, Ea 2, Av 3 and Av 4) were dissolved in sterilized DMSO (10% v/v) and introduced into one of the wells. As the 10% DMSO (v/v) had no detectable effect on bacterial growth, compounds at concentrations of 0.1 $\text{g}\cdot\text{ml}^{-1}$ were prepared in 10% DMSO (v/v). Streptomycin sulphate (1 $\text{mg}\cdot\text{ml}^{-1}$) was taken as a positive control and 10% DMSO (v/v) as negative one. After the incubation of the plates at 37°C for overnight period, bacterial growth was determined by measuring the diameter of inhibition zone using a transparent metric ruler.

The microbroth dilution method was performed to determine the minimum inhibitory concentration (MIC). The *E. alba* and *A. barbadensis* isolated compounds were dissolved and diluted in Luria Bertani (LB) broth, seeded in a 96-well culture plate and then inoculated with a fresh bacterial inoculum. Inoculated microplates were incubated at 37°C for 24 h. Each compound concentration was tested in duplicates for each organism. The viability of the treated cells was determined by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium) assay and the absorbance was measured at 570 nm using a microtitre plate reader (Bio-Rad Model 680; Hercules, California) for

bacterial strains. The MIC was determined as the lowest concentration of each purified compounds required inhibiting the growth of each organism. The mean and standard deviation of triplicates for each treatment were calculated.

3.6.2. Antifungal assay of the purified compounds

The compounds from *E. alba* and *A. barbadensis* were subjected to antifungal assay using different fungal strains.

3.6.2.1. Test organisms

The fungal strains used in the present investigation were obtained from the Department of Molecular biology and Biotechnology, Tezpur, Assam, India. The fungal strains are *Candida albicans* (MTCC 227) and *Fusarium oxysporium* (MTCC 284).

3.6.2.2. Media used

Potato Dextrose Agar medium (PDAM)

| Compositions | g/l |
|-----------------|------------------------------------|
| Potato infusion | 4.0 (Infusion from 200 g potatoes) |
| Dextrose | 20.0 |
| Agar | 15.0 |

pH of the medium was adjusted to 7.3

3.6.2.3. Determination of antifungal activity

Fungal cultures were maintained at room temperature in PDAM. Active cultures of the fungal strains were prepared by seeding a loopful of fungi into PD broth and incubated without agitation for 48 h at 25°C. The culture was diluted with PD broth to achieve the optical density corresponding to 2.0×10^5 spores/ml.

The disc diffusion method was also used to screen for antifungal properties. In vitro antifungal activity was screened by using PDA media. The PDA plates were prepared by pouring 15 ml of molten media into sterile petriplates. The plates were allowed to solidify for 10 min and 200 μ l of the test strains were introduced into media and allowed to spread with the help of sterile glass spreader. The plates were incubated at room temperature for 10 min. A sterile cork borer of 5 mm diameter was used to make wells on each plate and filled with 200 μ l of each plant isolated fractions. These were carried out in triplicate for each fungal strain. The plates were incubated at 25°C for 96 h and the resulting zone of inhibition was measured using a transparent metric ruler. Each set of seeded plates were compared for confirmation. Amphitericin (1mg.ml^{-1}) was used as positive while 10% DMSO (v/v) was kept as negative control.

3.6.3. Antioxidant assay

The purified compounds were studied for free radical scavenging as well as antioxidant activity. The free radical scavenging capacity of the isolated

compounds was measured *in-vitro* by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay according to the method described by Brand-Williams *et al.*²¹³.

3.6.3.1. Chemicals and Reagents used

DPPH was purchased from Signa-Aldrich (St. Louis, MO). Ethanol was purchased from Merck Co. (Germany), Mumbai. All the chemicals are of analytical grade and used as received.

3.6.3.2. Determination of free radical scavenging activity

The stock solution was prepared by dissolving 4 mg DPPH in 50 ml absolute methanol and stored in a dark coloured bottle at 4°C until required. An aliquot of 3.0 ml DPPH solution was mixed with 100 µl of each phyto-compound at various concentrations (3.12-100µg/ml). The reaction mixture was incubated in the dark for 30 min at room temperature and absorbance was observed at 517 nm. The absorbance of 3.0 ml DPPH solution as control was measured in each compound. The scavenging activity was estimated based on the percentage of DPPH radical scavenged as per the following equation:

$$\text{Scavenging percentage (\%)} = (\text{Control absorbance} - \text{Sample absorbance}) / \text{Control absorbance} \times 100$$

Compound concentration providing 50% inhibition (IC₅₀) was calculated from the graph plotted as scavenging percentage against compound concentration. Quercetin and gallic acid were taken as standards. All tests were carried out in triplicate.

3.6.4. Assessment of cell cytotoxicity on murine macrophage cell line (RAW 264.7)

Murine macrophage cell line was obtained from the Defence Research Laboratory, DRDO, Solmara, Tezpur, Assam, India. The cells were maintained in Dulbecco's minimum essential medium (DMEM) containing 2 mM.L⁻¹glutamine, 1.5 g.L⁻¹ sodium bicarbonate (NaHCO₃), 0.1 mM non-essential amino acid and 1.0 mM sodium pyruvate, supplemented with 10% (v/v) fetal bovine serum and 1% antibiotic antimycotic solution (1,000 U.ml⁻¹ penicillin G). Cells were maintained at 37°C in a saturated-humidity atmosphere.

To quantitatively measure cell toxicity, MTT (3-[4, 5-dimethylthia-zole-2-yl]-2, 5-diphenyl tetrazolium bromide) dye conversion assay was used (Mossman, 1983). For the MTT viability studies, murine macrophage cell line RAW 264.7 was cultured at a density of 1×10⁴ cells per well in a 100 µl volume of cell culture medium (DMEM supplemented with 10% fetal bovine serum) in a 96-well cell culture plate. After 24 h, cultured cells were treated with a series of different concentrations (20, 40, 60, 80 and 100 µg.ml⁻¹) of Ea1, Ea 2, Av 3 and Av 4 dispersed in 100 µl per well DMEM without serum and phenol red, and incubated further for 4 hours with MTT dye. After incubation 100 µl of dimethylsulfoxide (DMSO) was added to each well to dissolve blue formazan precipitate, and absorbance was measured at 570 nm using a microtitre plate reader (Bio-Rad Model 680; Hercules, California). All experiments were performed in quadruplets. The cell viability was expressed as a percentage of

the control by the following equation: Cell viability (%) = $\frac{\text{Absorbance of control cells} - \text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100$

3.7. Inducing alopecia on animal model using warfarin

The protocol for experimentation was approved by the Central Animal Resources, Defence Research Laboratory, Defence Research and Development Organization, Tezpur, Assam, India. Approval to carry out these studies was obtained from the Institutional Animal Ethics Committee.

3.7.1. Animal husbandry and maintenance

Adult 12 wistar strain albino rats (5–8 weeks, both male and female) weighting 150-180 gm were placed in polypropylene cages with free access to standard laboratory diet (Pranav Agro Industries Limited, Sangli, Maharashtra, India) and provided municipal water ad libitum. Each individual animal was clinically examined and identified by fur marked with picric acid. The females were nulliparous and not pregnant at the time of experiment. Animals were grouped and housed in an environmentally controlled room with temperature of $22^{\circ}\text{C} \pm 3^{\circ}\text{C}$ and 40-70% relative humidity with a 12-hour light–dark cycle and ventilation of 15-21 air changes/h for an acclimatization period of 7 days to laboratory conditions prior to the beginning of the experiment in order to adjust to the new environment and to overcome stresses incurred during their transit. Only healthy animals were assigned for these studies.

3.7.2. Alopecia induction

For the experiment, 18 wistar albino rats were taken and they were subjected to warfarin treatment to induce alopecia. Standardization of warfarin drug was done after various trials to induce alopecia in the rats. The rats were dosed with 3, 2.5, 2, 1.7 and 1.5 mg/kg warfarin to induce hair loss. Among them, warfarin dose 1.7 mg/kg showed patchy hair loss after 2 months of continuous medication without any harmful side-effects or mortality, which lead to severe alopecia in the albino rats. After 2 months of continuous dosing, medication was stopped.

3.8. Application of isolated phyto-compounds on alopecia induced animal model

After inducing hair loss in wistar albino rats by using warfarin drug, all the animals were kept for 1 month in normal food, water and normal temperature to observe if any hair re-growth occurs. The animals were divided into 6 groups, 3 animals in each group. Group I was kept as control, group II was studied with standard drug minoxidil and rest four groups were treated with tested compounds i.e. Ea 1, Ea 2, Av 3 and Av 4. Histopathology was done for the warfarin treated albino rats to check any follicular regeneration. The purified phyto-compounds Ea 1 and Ea 2 from *E. alba* and AV 3 and Av 4 from *A. barbadensis* were dissolved in alcohol to prepare 2% solution and applied topically in the alopecia affected area for 15 days. The standard drug minoxidil solution (2%) was applied on the rats as positive control. Qualitative, haematological, serum biochemistry and histological study were carried out for

both treated and control animal skin to differentiate changes between plant purified compounds and minoxidil treated and control animals in follicular and dermal levels.

3.8.1. Acute dermal irritation study

The acute dermal irritation/corrosion study was carried out in accordance to the OECD Guideline 404 method²¹⁴. Twelve healthy rabbits with intact Primary irritation index (PII) were used. It was then classified according to Draize method using PII scoring as non-irritant (if PII <0.5), slightly irritant (if PII <2), moderately irritant (if PII < 2.5), and severely irritant (if PII >5). For each animal, the dermal response scores (sum of the scores for erythema and edema formation) at 24, 48, and 72 h after the removal of the patches were summed up, and divided by 3 to obtain a mean irritation score per time point. The mean scores at 24, 48, and 72 h were summed and derived the average to obtain the PII.

3.8.2. Qualitative study

Qualitative hair growth was evaluated by observation of two parameters: hair growth initiation time and hair growth completion time i.e. minimum time taken to cover the denuded skin region with new hair completely. Hair growth initiation and completion time were recorded for each group of animals and compared with the positive control minoxidil 2% solution and control. Also the average length and weight of hair was recorded and compared for each group of animals.

3.8.3. Haematological study

All animals were fasted overnight prior to necropsy and blood collection (14th day of topical application of phyto-compound treatment). Blood samples were collected through orbital sinus vein puncture technique from retro orbital sinus of rats by 75 mm heparinized capillary tube (Haematocrit capillary, Himedia Laboratories Private Limited, Mumbai, Maharashtra, India). The blood samples were collected in non-vacuum blood collection tubes containing K3 EDTA (Peerless Biotech Pvt Ltd, Chennai, Tamil Nadu, India) and analyzed within 60 minutes. The hematological parameters including white blood cell (WBC) count, WBC differential counts, like lymphocyte (Lym), monocyte (Mon), neutrophil (Neu), eosinophil (Eo), and basophil (Ba) counts, red blood cell (RBC) count, mean corpuscular volume (MCV), hematocrit (Hct), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), hemoglobin (Hb) concentration, and platelet (Pct) were examined by Automatic Hemato analyzer (MS-4) (Melet Schloesing Laboratories, Osny, France).

3.8.4. Study of serum

Blood for clinical chemistry was placed in the vacuum blood collection tubes (Peerless Biotech Pvt Ltd) devoid of anticoagulant (serum tube) and allowed to clot at room temperature. Blood samples were centrifuged within 60 min at 3,000 rpm (604 g) for 10 min after collection and then the serum was separated. Serum biochemistry parameters including low-density lipoprotein (LDL), high-density lipoprotein (HDL), glucose (GLUC), urea (UREA), total protein (TP),

uric acid (UA), triglycerides (TGL), cholesterol (CHOL), creatinine (CRE), alanine aminotransferase (ALT/SGPT) and aspartate aminotransferase (AST/SGOT) were analyzed by Coralyzer-100 (Tulip Diagnostics Pvt Ltd, Goa, India) with the help of commercially available biochemical kits.

3.8.5. Histological study

3.8.5.1. Chemicals

Formalin, paraffin wax, ammonia, Hematoxyline stain, Eosin stain, Mount DPX etc were purchased from Sigma-aldrich (St.MO), absolute alcohol and xylene were purchased from Merck Co. (Germany). All chemicals were of analytical grade.

3.8.5.2. Preparations of reagents

3.8.5.2.1. Acid-alcohol solution

One ml of concentrated Hydrochloric (HCl) acid was dissolved in 70% alcohol to prepare the acid-alcohol solution.

3.8.5.2.2. Mayer's albumin

Egg albumin 1 part was mixed with 1 part of glycerol and then added water in such a way that eggs get dissolved on shaking. Added thymol as preservative and the total volume of the solution was 2.0 ml.

3.8.5.3. Staining Protocol

1. After harvesting tissue from the control and the treated animals, a part

tissue was collected and washed in 0.9% (w/v) saline (3 times) and put in 10% formalin with 1.0 ml ammonia for 24 h.

2. Formalin was decanted and tissues were kept in 70% alcohol with 1.0 ml ammonia for 2 h.
3. Alcohol 70% was decanted and tissues were kept in 80% alcohol for 2 h.
4. 80% alcohol was decanted after 2 h and the tissues were kept in 90% alcohol for 2 h.
5. 90% alcohol was decanted after 2 h and tissues were kept in 100% alcohol for 2 h.
6. Decanted 100% alcohol and added xylene (100%) till the tissues become transparent and hard in appearance.
7. Infiltration of the tissue was done by putting the cleared tissues in melted paraffin for 2 h by using infiltration cassette. Infiltration was done twice for each tissue.
8. Embedding was done by using melted paraffin in 'O' spare ring which used for block making. Allowed the paraffin to solidify and then put the tissue to get the transverse section. Allowed the block to solidify properly on a cooling plate.
9. After solidification for 2 h, the block was ready for trimming and sectioning.
10. Trimmed cuboid of the block in such a way that on one side, the tissue in

the front which could be the face for sectioning.

11. Took the trimmed block for making sections that were overlaid on water.
12. Sections were cut using automatic microtome instrument.
13. Keep the tissue sections on the slide, and then coated with Mayer's albumin.
14. Once the sections were layered on the slide, they were kept on the slide warmer plate so that the tissue could be stretched properly. Now the slides were ready for staining.

Staining

Slides were placed in coupling jar in the sequence mentioned below:



