4.1. **Sample collection.**

Healthy and matured leaves of *A. marina* were collected from Killai backwater mangrove forest (Latitude: 11°.46’20” N and Longitude: 79°.77’60” E), Tamilnadu, India (Fig. 8). Collected leaves samples were kept in the sterile plastic bags and brought to the laboratory.

![Map showing sample collection site Killai, Tamilnadu.](image)

This mangrove region is located between the two prominent estuaries *i.e.* the Vellar in the north and Coleroon estuary in the south. The Vellar and coleroon estuarine complex forms the Killai backwaters on the south east coast of India. This region consists of numerous creeks and rivulets interlacing through dense mangrove jungle with uninterrupted wide connections to the sea. In addition to the thick debris from bushes and shrubs fringing the channels, this backwater receives nutrients through copious flow of freshwater discharges from extensive paddy fields on the hinterlands.
4.2. Isolation of Mangrove Endophytic fungi

Collected leaves of *A. marina* were processed within 24 hours for the isolation of endophytic fungi, based on the method suggested by Julia Kjer *et al.* (2010) with some modifications.

1. Leaves were cut into small segments of ~1 cm × 1 cm and rinsed three times with sterile sea water to eliminate adherent surface debris under laminar air-flow.

2. Leaf segments were immersed in EtOH 70% (vol/vol) for 60–120 seconds for surface sterilization.

   This is very crucial step, if the treatment with EtOH is too short; the sterilization of the outer part is not completed; if the sterilization time is too long, EtOH kills fungi in the inner parts of the tissue, so appropriate time duration has been followed.

3. Immediately after sterilization, the segments were transferred into sterile distilled water/ sea water to stop the sterilization with EtOH, and the leaves were dried on sterile cotton under flow.

4. Surface sterilized leaf segments were placed carefully over the surface of Potato Dextrose Agar medium (PDA) containing Potatoes infusion 200 g, Dextrose 20 g, Chlormphenicol 0.2 g, Agar 15 g and Distilled water 1000 ml, final pH 7.4 to 7.8 and Malt Agar Medium (MAM) containing Malt extract 15 g, Artificial sea salt 10 g, Agar 15 g, Chlormphenicol 0.2 g and Distilled water 1000 ml, pH- 7.4-7.8 with sterilized tweezers, it consider as negative control.

   Note: If fungal growth observed on negative control plates, the positive plates of entire batch were not used for further process. Growth in negative control dishes
denotes that inadequate surface sterilization and phylloplane/ ubiquitous contamination in all pleats. The aim is isolation of true endophytic fungi, so the entire process repeated to get the pure endophytic strains.

5. Leaf segments were cut again at four edges and made into smaller segments with a sterile razor blade.

6. Segments were placed on petriplates containing isolation medium such as PDA and MAM. So that freshly cut edges were directly contact with the surface of medium.

7. Inoculated petriplates were sealed with parafilm, labeled and incubated at 20–25°C under the daylight for 15 day.

8. Different fungal colonies observed on positive plates. The individual strains were isolated by transferring hyphal tips growing out from the edges of cut leaf segments, into fresh PDA/MAM.

9. Fungal morphological characteristic were recorded.

10. The pure fungal strains were maintained with PDA at 4°C and used for further process.

4.3. Analysis of relative frequency

The percentage relative frequency (RF %) of an fungal endophytic species in leaves samples was calculated using following formula (Photita et al., 2001).

\[
\text{Relative frequencies} = \frac{\text{Number of occurrences of a particular fungus}}{\text{Total number of isolates}} \times 100.
\]

4.4. *Hypoxylon* sp.

Endophytic fungus *Hypoxylon* sp isolated from fruits of *Rizophora recemosa* was kindly provided by Prof. Dr. Peter Proksh, Institute of Pharmaceutical Biology
and Biotechnology, Heinerich Heine University, Dusseldorf, Germany, and used for the separation of active bio-molecules.

4.5. **Mass scale fermentation and extraction of secondary metabolites.**

Mass scale fermentation of mangrove endophytic fungi and extraction of secondary metabolites were done according to Julia Kjer *et al.* (2010) method (Flow chart. 1).

- Mass scale fermentation of isolated fungi was carryout by transferring fresh fungal cultures into ten sterile Erlenmeyer flasks (1L) containing 100 ml distilled water 100 g of rice. For small scale 2-3 flasks only used for fermentation.
- The cultures were then incubated at room temperature for 30 days.
- After incubation, 250 mL of EtOAc were added to the cultures and left overnight.
- Culture media were cut into pieces and allow it for complete extraction and left for 3–5 days. Filter the contents under vacuum using a Buchner funnel.
- For optimal extraction of the fungal biomass, the extraction was repeated for three times with EtOAc. If color didn’t change, the extraction was repeated with EtOAc until the color fade.
- All filtrates were combined and washed with distilled water. The aqueous and EtOAc phases had left in a separation funnel until complete separation of the two immiscible liquid phases is achieved.
- The dry residue was obtained from EtOAc extracts were partitioned between \( n\)-hexane and 90% MeOH in the ratio of 1:1 (vol/vol) (~150 ml each).
- Separation of the two immiscible liquid phases is achieved and MeOH phase were dried under vacuum (~200 mbar) using rotary evaporator at 40°C.
- Finally solid residue were obtained and used for further purification.
Flow chart. 1. Schematic illustration of fungal secondary metabolites extraction.
4.6. Extraction of secondary metabolites from the leaves of *Avicennia marina*.

Crude extract from the leaves of *Avicennia marina* was according to the method proposed by Bharathi *et al.* (2011) (Flow chart. 2).

- Collected leaves were washed once with tap water, twice with distilled water and dried under the shadow. Dried leaves were powdered with the help of pestle & mortar, and stored in room temperature for further experiment.

- Twenty grams of leaf powder was taken in 500ml conical flask and 80 ml of MeOH were added (1:4 ratio). Then samples were kept in incubator shaker at 27°C for 35 minutes.

- The extracts were filtered through Whatmann No. 1 filter paper and filtrates were collected. Same procedure was repeated for three times, and combined filtrates were dried under vacuum (~200 mbar) using a rotary evaporator at 40°C.

- The dried residues were subjected into partition between *n*-hexane and 90% MeOH.

- The 90% MeOH phase were dried under reduced pressure under vacuum (~200 mbar) using a rotary evaporator at 40°C and used for further purification.
Materials and methods

Flow chart. 2. Schematic representation of crude extracts preparation from leaves of *A. marina*.

Leaves of *Avicennia marina*

Washed once with tap water and twice with distilled H$_2$O

Powdered

Powder added with MeOH in the ratio of 1:4 & kept in incubator shaker at 27º C for 35 min

Filtered through Whatmann No.1 filter paper.

Filtrate

Evaporate

Residue Obtained

90% MeOH + n Hexane

MeOH Phase

n Hexane Phase

Evaporate

Crude Extract
4.7. Purification and characterization of bioactive compounds from leaves of *Avicennia marina* and endophytic fungi.

The crude extracts of mangrove leaf and endophytic fungi were first fractionated by Vacuum Liquid Chromatography (VLC) using a step gradient with non-polar solvent n-hexane/dichloromethane and increasing polar solvent ethyl acetate/ methanol. All fractions were collected and analyzed by analytic HPLC using a reversed-phase column. Based on chemical profile, VLC fractions were subjected into Sephadex LH-20 with methanol as mobile phase. Collected fractions were run in Thin Layer Chromatography (TLC), fractions which showed similar bands were pooled. Chemical profiles were obtained for all the fractions from analytical HPLC. Based on Ultra violet (UV) pattern and library hits, Sephadex fractions were chosen and loaded into the semi-preparative reversed-phase HPLC for final purification. The mobile phase was combination of MeOH and nanopure H$_2$O with 0.01 % TFA. Purified bioactive compounds obtained from semi-preparative HPLC were characterized through spectroscopic analysis. Spectroscopic values were fed in MarinLit February 2013 and AntiBase February 2013 database to find the compound structures. All the purification and characterization steps and methods were adopted according to Hassan, 2007. The detailed procedure was given below.

4.8. Isolation of secondary metabolites


Vacuum liquid chromatography is a useful method for initial isolation procedure for large amounts of sample. VLC apparatus is a sintered glass about 50-60 cm and inner diameter is 12 cm, with filter funnel. Silica gel (0.04 - 0.063 mm mesh size) was packed as hard cake at a height of 5-10 cm under applied vacuum. The sample was mixed with volatile solvents and silica, resulting mixture was adsorbent
of sample with silica, packed onto the top of the column. Above the sample sand was added for ~2 cm and finally cotton was kept to prevent the column damage (Fig. 9).

Column was run using step gradient elution with non-polar solvents (e.g. \(n\)-Hexane and DCM) and increasing amounts of polar solvents (e.g. EtOAc or MeOH) at 20% interval of each solvent gradient. The successive fractions were collected and obtained chemical profile from analytical HPLC. The flow was produced by vacuum and the column was allowed to run dry after each fraction collected.

![Fig. 9. Vacuum Liquid Chromatography experimental setup.](image)

4.8.2. **Sephadex Column chromatography**

Fractions derived from VLC were subjected into repeated separation through sephadex LH-20 column chromatography. Column was carefully assembled in vertical position. Sephadex L-20 slurry was prepared with MeOH. Mixture was stirred gently to get rid of air bubbles and even slurry is achieved. To pack the column, slurry was carefully poured into column through the plastic funnel. The bottom of the column was opened to allow the solvent to flow through the column. After adding the slurry, the column was tapped very gently for uniform distribution of gel. The gel was
packed ~3/4 of the column. The packed column was washed with mobile phase MeOH until surface level of the gel remains same that means gel was settled and tightly packed; now it is ready to use (Fig. 10). Remaining solvent above the surface have flow down till slightly above the gel.

Before loading, sample was thoroughly mixed with 1 ml of MeOH and centrifuged at 5000 rpm for 5 min and supernatant was collected. Collected supernatant sample was loaded as pure and very concentrated solution from the top of the column without disturbing the surface of stationary phase. Sample was allowed to flow through and enter into the gel, and then column was completely filled with mobile phase MeOH. Solvent was added continuously from reservoir into the column throughout the run. During the process, solvent level was maintained above the gel level.

As elution takes place, narrow band of sample was separated into several bands according to the number of compounds in the mixture, relative polarities and molecular weights. The larger compounds excluded from the interior of the bead and thus elute first. The smaller compounds allowed entering the small sized pores, so they elute at last.

The fractions are started to collect when the first colored fraction is starts to come out of from column. Fractions were collected at every 3 minutes in a separate test tube which placed in sample collector and labeled properly.
4.8.3. Thin layer chromatography (TLC)

Sephadex LH-20 fractions were run thin layer chromatography. TLC was performed on pre-coated TLC plates with silica gel 60 F254 (layer thickness 0.2 mm, E. Merck, Darmstadt, Germany). TLC plates were lightly marked the baseline with
pencil at just 1 cm above the bottom of TLC plate. Each fraction were spotted evenly on the line of origin by using capillary tubes, and dried before placing the sheet into the TLC chamber. TLC was run with different solvent systems of MeOH: DCM (90:10, 80:20, 70:30 and 60:40). Chromatographs were developed about until 1 cm from the top of the sheet. TLC sheets were removed from chamber and marked the solvent front with a pencil. Plates were allowed to dry. The separated bands on TLC were detected under UV lamp at 254 and 366 nm. Chromatography refers to any separation method in which the components are distributed between stationary phase and mobile phase. The separation occurs because sample components have different affinities for stationary and mobile phases. Fractions which showed the similar band patterns were pooled and their chemical profile were obtained from analytical HPLC.

\[
R_f = \frac{\text{Distance from the starting point to the center of the spot on the TLC plate}}{\text{Distance from the starting point to the solvent front}}
\]

### 4.8.4. Semi-preparative High Pressure Liquid Chromatography (HPLC)

This process was used for purification of compounds from fractions previously separated using column chromatographic separation. The machine was configured with Pump: Merck Hitachi L-7100, Detector: Merck Hitachi UV detector L-7400, Column: Knauer (300 × 8 mm, ID), prepacked with Eurospher 100-10 C18, with integrated pre-column. The most appropriate solvent system was determined before running the HPLC separation (Fig. 11). The mobile phase was combination of MeOH and nanopure H₂O with 0.01 % Trifluoroacetic acid, pumped in gradient or isocratic manner depending on the compounds retention time. Each injection consisted of 1-3 mg of the fraction dissolved in 1 mL of the solvent system. The solvent system was pumped through the column at a rate of 5mL/min. The eluted peaks were detected by
the online UV detector and collected separately in Erlenmeyer flasks. The separation column (125 × 4 mm, ID) was pre-filled with Eurospher C18 (Knauer, Berlin, Germany).

Fig. 11. Semi-preparative high pressure liquid chromatography (HPLC) experimental setup.

4.8.5. Analytical High Pressure Liquid Chromatography (HPLC)

Analytical HPLC was used to identify the distribution of peaks either from fractions, as well as to evaluate the purity of isolated compounds. The machine was integrated with Reservoir of mobile phase, Pump: Dionex P580A LPG, Detector: Dionex Photodiode Array Detector UVD 340S, Column thermostat: STH 585, Autosampler: ASI-100T, HPLC Program: Chromeleon (V. 6.3) and Column: Knauer (125 × 4 mm, ID), pre-packed with Eurosphere 100-5 µm C18, with integrated pre-column. The solvent gradient was started with MeOH:nanopure H₂O (10:90), adjusted to pH 2 with phosphoric acid, and reached to 100 % MeOH in 35 minutes. The autosampler injected 20µL per sample. All peaks were detected by UV-VIS photodiode array detector.
4.9. Structure elucidation of the isolated secondary metabolites

4.9.1. Mass spectrometry (MS)

Mass spectrometry was used to find out the difference in mass-to-charge ratio ($m/z$) of ionized molecules to separate them from each other. Mass spectrometry is therefore useful for quantification of atoms or molecules. Also used for determination of chemical and structural information of molecules. A mass spectrometer consists of an ion source, ion detector and mass-selective analyzer. The output of mass spectrometers shows a plot of relative intensity vs. the mass-to-charge ratio ($m/z$).

4.9.2. Liquid Chromatography Mass Spectrometry (LC/MS)

High pressure liquid chromatography is a powerful method for the separation of complex mixtures, especially when many of the components may have similar polarities. If a mass spectrum of each component can be recorded as it elutes from the LC column, quick characterization of the components is greatly facilitated. LC-MS consist of HPLC system: Agilent 1100 series (pump, detector and auto sampler) Finnigan LC Q-DECA, MS-spectrometer: Knauer, (250 × 2 mm, ID), prepacked with Eurosphere 100-5, Column: C18, with integrated pre-column. The samples were dissolved in water/MeOH mixtures and injected to HPLC/ESI-MS set-up. For standard LC/MS measurements, a solvent gradient that started with acetonitrile:nanopure H$_2$O (10:90), adjusted with 0.1 % HCOOH, and reached to 100 % acetonitrile in 35 minutes.

4.9.3. High Resolution Mass Spectrometry (HR-MS)

High resolution is achieved by passing the ion beam through an electrostatic analyzer before it enters the magnetic sector. In such a double focusing mass spectrometer, ion masses can be measured with an accuracy of about 1 ppm. With
measurement of this accuracy, the atomic composition of the molecular ions can be determined. HRESI-MS was measured on a Micromass Qtof 2 mass spectrometer at Helmholtz Centre for Infection Research, Braunschweig. The time-of-flight analyzer separates ions according to their mass-to-charge ratios \( m/z \) by measuring the time it takes for ions to travel through a field free region known as the flight.

4.9.4. **Nuclear Magnetic Resonance spectroscopy (NMR)**

Nuclear magnetic resonance is a phenomenon which occurs when the nuclei of certain atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic field. Some nuclei experience this phenomenon, and others do not, dependent upon whether they possess a property called spin. It is used to study physical, chemical, and biological properties of matter. As a consequence, NMR spectroscopy finds applications in several areas of science. NMR spectroscopy is routinely used by chemists to study chemical structure using simple one dimensional technique. Two dimensional techniques are used to determine the structure of more complicated molecules.

All NMR spectra including Proton NMR \((^1H)\), Carbon NMR \((^{13}C)\), Correlation Spectroscopy (COSY) and Hetronuclear Multiple Bond Correlation (HMBC) were recorded on Bruker DRX 500 & 600 spectrometers at Heinrich-Heine Universität, Düsseldorf, Germany. Spectrums were obtained using the standard Bruker software. All samples were dissolved in CD\(_3\)OD. The residual solvent signals were used as internal standards (reference signal). The observed chemical shift values \((\delta)\) were given in ppm and the coupling constants \((J)\) in Hz.

4.10. **In silico evaluation of purified compounds against cFLIP, HPV 16 E6 and E7**

4.10.1. **cFLIP PDB Protein Structure.**

Crystal structure of cFLIP protein was retrieved from Protein Data Bank (PDB), PDB ID: 3H13 (www.rcsb.org/pdb).
4.10.2. HPV16 E6 and E7 protein structure modeling.

HPV-16 E6 (GenBank ID: AAD33252.1) and HPV-16 E7 (GenBank ID: AHK23257.1) protein sequences were retrieved from NCBI (http://www.ncbi.nlm.nih.gov) (Fig. 12). The protein sequences were submitted in Phyre2 server for modeling the three dimensional structure of targeted protein depicted in Fig. 13 (Kelley and Sternberg, 2009).

Fig. 12. Retrieval of amino acid sequence from NCBI. A) HPV 16 E6 oncoprotein sequence retrieval B) HPV 16 E7 oncoprotein sequence retrieval.
Fig. 13. Submission of amino acid sequence in PHYRE sever for modeling 3D structure. A) HPV 16 E6 protein sequence submission in PHYRE server. B) HPV 16 E7 protein sequence submission in PHYRE server.
4.10.3. Energy minimization and structure validation.

Energy minimization of cFLIP, HPV16 E6 and E7 were done by Yet Another Scientific Artificial Reality Application (YASARA) Energy Minimization Server, for structural refinement (Krieger et al., 2009). YASARA server page has opened and 3D structures of all proteins were loaded in file option, valid email ID has given and structures were submitted for refinement.

Quality of all refined structure of proteins was assessed on line servers. Procheck analysis was done in NIH-Structural Analysis and Verification Server (http://nihserver.mbi.ucla.edu/SAVES/), pdb file of proteins were uploaded using choose file option and clicked on procheck button, and got the Ramachandra plot for each protein (Laskowski et al., 1993).

Protein Structure Analysis is simply called ProSA-web, ProSA-web server page (https://prosa.services.came.sbg.ac.at/prosa.php) (Sippl, 1993; Wiederstein and Sippl, 2007) was opened, then pdb files were uploaded and clicked on Analysis button. Finally Z-score of proteins were obtained.

Protein structure quality (ProQ) (http://www.sbc.su.se/~bjornw/ProQ/ProQ.html) also been assessed by loading pdb files into ProQ server and LG scores were obtained for all refined proteins (Wallner and Elofsson, 2003).

4.10.4. Ligand Preparation.

The chemical structure of isoquercitrin (CID: 5280804) was retrieved from Pubchem database as sdf file. New Hypoxylonol and Aldose reductase inhibitor 3D structures were obtained through Chem3D Pro 12 as cdx files. All sdf and cdx files were converted into pdb format by using Open Babel sorftware.
4.10.5. Hex8.0.0 docking.

First docking studies were performed in Hex 8.0.0 (Ritchie, 2012). Hex window was opened. Receptor and ligand molecules were retrieved from the file menu, docking were activated from option control. And the following docking parameters were used, correlation type – Shape only, Grid Dimension – 0.6, Receptor range – 180, Ligand Range – 180, Twist range – 360, distance Range–40. Finally binding energy (\(\Delta E\)) was obtained and saved carefully. The docking complex was saved separately in pdb format for Ligplot analysis.

4.10.6. Autodock analysis

Autodock 4.2 also used for docking studies, Protein-Ligand docking method was followed (Kumar et al., 2012; Mohd et al., 2013). In brief the receptor molecules were uploaded in Autodock Tools, water molecules were deleted, all hydrogen atoms were added into carbon atoms of the receptor, and Kollman charges were assigned. Ligand molecules also uploaded in ADT, non-polar hydrogens, Gasteiger charges were assigned and torsions degrees of freedom were allocated. The grid box was made to cover entire receptor molecule, then auto grid has run. The Lamarckian genetic algorithm (LGA) was applied to receptors to interact with isoquercitrin, docking parameters 10 independent genetic algorithm, population size 150, 250000 energy evaluations, 27000 maximum generations, rate of gene mutation 0.02 and rate of crossover 0.8 were used for all docking. Autodock was run and docking log file (DLG) files were obtained.
4.10.7. Ligplot analysis

Ligplot was performed to generate schematic diagrams of protein-ligand interface from Hex docked complex file. In Ligplot window, the receptor-protein complex, pdb files was opened. Ligand was selected and clicked the run button (Laskowski and Swindells, 2011). Plot has been generated on screen and binding interfaces were viewed in Pymol.

4.11. Cell culture

All the experiments were performed on human cervical cancer SiHa cell line (National Centre for Cell Science, Pune). Cells were grown in mono-layers cultures in Dulbecco’s Modified Eagle’s Medium (Sigma-Aldrich, St. Louis, MO, USA), containing 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), 100 U/mL penicillin (Sigma Chemical Company (St. Louis, MO, USA), 100 µg/mL streptomycin Sigma Chemical Company (St. Louis, MO, USA) and incubated at 37ºC in atmosphere containing 5% CO₂.

4.12. MTT assay

HPV16 infected SiHa cells were seeded onto 96-well microtiter plates at a density of 1 x 10⁴ cells per well and incubated for 24 h. And culture medium was removed and SiHa cells were treated with drugs at the different concentrations for 48 hours along with medium, medium was added to the control well. At the end of the incubation, 20µl of 3-(4-5 dimethylthiazol-2-yl) 2-5 diphenyl-tetrazolium bromide (MTT) (5 mg/ml) per well was added and incubated in dark at 37°C for 4 hours. The formazan crystals formed after 4 hours, medium were aspirated and solubilized in 100µl of DMSO. The 96-well microtiter plate absorbance of each well was measured by ELISA reader at 570nm. The percentage of growth inhibition was determined
using the formula of growth inhibition = (control Optical Density – sample Optical Density)/control Optical Density X 100. The IC\textsubscript{50} value was defined as the concentration of compound that produced 50% reduction of cell viability.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Name of the drug</th>
<th>Concentrations of the drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Crude extract \textit{Xylaria} sp.</td>
<td>100 µg, 200 µg, 300 µg, 400 µg, 500 µg</td>
</tr>
<tr>
<td>2</td>
<td>Crude extract \textit{Hypoxylon} sp.</td>
<td>100 µg, 200 µg, 300 µg, 400 µg, 500 µg</td>
</tr>
<tr>
<td>3</td>
<td>Crude extract \textit{A.marina}</td>
<td>100 µg, 200 µg, 300 µg, 400 µg, 500 µg</td>
</tr>
<tr>
<td>4</td>
<td>Isoquercitrin</td>
<td>400 µM, 600 µM, 800 µM, 1200 µM, 1400 µM</td>
</tr>
<tr>
<td>5</td>
<td>Hypoxylonal</td>
<td>400 µM, 600 µM, 800 µM, 1200 µM, 1400 µM</td>
</tr>
</tbody>
</table>

Table 1. Concentrations of mangrove derived drugs treated on SiHa cell for MTT assays.

\textbf{4.13. Treatment of SiHa cells with isoquercitrin and rhTRAIL.}

SiHa cells were seeded in six well plates in the density of \((2.5\times10^5)/mL\) per well and incubated for 24 h and culture medium was removed. Well plates are added with fresh medium and cells were treated under three different conditions 1) 980µM of isolated isoquercitrin alone for 48 h treatment 2) 100 ng of rhTRAIL protein (PeproTech Inc. Rocky Hill, NJ, USA) alone for 24h treatment and 3) isoquercitrin (980 µM) for 36 h treatment and then stimulation with rhTRAIL (100 ng) for 12 h.

\textbf{4.14. Apoptosis measurement by Fluorescence-Activated Cell Sorting (FACS)}

FITC Annexin V/PI staining is used to quantitatively determine the percentage of cells within a population that are actively undergoing apoptosis. Flow cytometry is quantifying the levels of detectable phosphatidylserine (PS) on the outer membrane of apoptotic cells, whereas it present in inner membrane of the normal cells. Externalized PS on the outer surface of the cytoplasmic membrane becomes labeled by
fluorescein-labeled annexin V, which has a high affinity for PS-containing phospholipid bilayers. Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. Cells that stain positive for FITC Annexin V and negative for PI are undergoing apoptosis. Cells that stain positive for both FITC Annexin V and PI are either in the end stage of apoptosis, are undergoing necrosis, or are already dead. Cells that stain negative for both FITC Annexin V and PI are alive and not undergoing measurable apoptosis. This assay does not distinguish between cells that have undergone apoptotic death and necrotic pathway.

FITC Annexin V/PI staining was done according to the manufacturer’s procedure. SiHa cells (2.5x10^5/mL) were treated with isolated isoquercitrin and/or TRAIL as mentioned earlier, then washed twice with PBS and resuspended in 1 mL of 1X binding buffer. Five hundred microliters of cell suspension was then incubated with 5µL of annexin V-FITC and 5µL of Propidium Iodide (PI) for 15 min at room temperature in the dark. Finally 400µl of 1X binding buffer were added to each tube and analysis by flow cytometry within 1 hour. The population of Annexin V-positive cells was evaluated by flow cytometry (BD FACScan, BD Biosciences, San Jose, CA, USA).
4.15. Analysis of TRAIL-R1, TRAIL-R2, HPV16 E6 and HPV16 E7 genes expression by quantitative Real Time-PCR.

4.15.1. Isolation of Total RNA from treated cells.

Total RNA from the treated cells were isolated by Trizol reagent method.

1. The lysate with Trizol reagent was incubated at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes.

2. 200 µl chloroform was added per 1 ml Trizol Reagent used. The tube was shaken vigorously by hand for 15 seconds and incubated at room temperature for 5 minutes.

3. The sample was centrifuged at 12,000 × g for 15 minutes at 4°C and transferred ~400 µl of the colorless, upper phase containing the RNA to a fresh RNase–free tube.

4. The RNA was precipitated by adding 500 µl of isopropyl alcohol (per 1 ml of TRIZOL reagent) to colorless solution from the aqueous phase by mixing with isopropyl alcohol. Kept in -20°C for 1 h and centrifuged at 12,000 x g for 10 minutes at 4°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

5. Discarded the supernatant completely and washed the RNA pellet with 75% ethanol, mixed the samples by vortexing and centrifuge at no more than 7,500 xg for 5 minutes at 2 to 8°C. Repeated the above washing procedure once.

6. Air-dried RNA pellet for 30 minutes. Dilute the RNA with DEPC-treated water (1:40 dilution). Isolated RNA was checked in 1% agarose gel.
4.15.2. cDNA synthesis

GoScrip Reverse Transcription kit (Promega, Madison, USA) were used to convert RNA into cDNA. Manufactures procedures were followed.

1. Following components were combined in 200 µl PCR tubes.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental RNA</td>
<td>3 µl</td>
</tr>
<tr>
<td>Primer [Oligo(dT)15</td>
<td>0.5 µg</td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>Final volume</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

2. The PCR tubes were heated in a 70°C heat block for 5 minutes. Immediately PCR tubes were chilled in ice water for 5 minutes and Spin for 10 seconds and stored on ice until reverse transcription mix is added.

3. The meantime following reverse transcription reaction mix 15 µl for each cDNA reaction was prepared on ice.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>GoScript 5X Reaction Buffer</td>
<td>4.0 µl</td>
</tr>
<tr>
<td>MgCl₂ (final conc 1.5–5.0mM)</td>
<td>1.2 µl</td>
</tr>
<tr>
<td>PCR Nucleotide Mix (final conc. 0.5mM)</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Recombinant RNasin Ribonuclease Inhibitor</td>
<td>20 units (0.5 µl)</td>
</tr>
<tr>
<td>GoScript Reverse Transcriptase</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
<td>7.3 µl</td>
</tr>
</tbody>
</table>
| Final volume                             | 15 µl
4. Combined the 15 µl of reverse transcription mix combined with 5 µl of RNA and primer mix.

5. Annealing was performed in a heat block at 25°C for 5 minutes.

6. Extended at 42°C for up to 60 minutes.

4.15.3. Quantitative Real-time PCR.

Real-time RT-PCR was performed by monitoring the increase in fluorescence intensity of the SYBR Green dye with a Rotor-Gene 3000 Real-time PCR apparatus (Corbett Research) according to the manufacturer’s instructions. All measurements were performed in triplicate. Samples were amplified using the following thermal profile 70°C for 2 min, 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 sec followed by annealing (Table 2) and extension at 60 °C for 1 min. Real-time RT-PCR data were represented as Ct values, where Ct was defined as the threshold cycle of PCR when amplified product was first detected. The Ct or threshold value of the target sequence is directly proportional to the absolute concentration when compared with the threshold value of GAPDH. The relative expression level of target gene were plotted as fold change compared to control and determined by the $2^{-\Delta\Delta ct}$ method (Livak 2001), a relative quantification algorithm. The factor X by which the amount of the changed gene can be calculated with the formula: $X=2^{-\Delta\Delta ct}$, where $\Delta\Delta ct = (Ct \text{ of target}) - (Ct \text{ of target x GAPDH})$ sample. Primer sequence was given in table 2. Data are presented as the mean ± SD of results from 3 independent experiments. Statistical significance was assessed using one way ANOVA with Tukey test, the $P<0.05$ values were considered as statistically significant.
### Table 2. Sequences of primers (Forward and Reverse) used for real-time PCR and lengths of amplification products.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Primer name</th>
<th>Primer sequence</th>
<th>PCR Product Size</th>
<th>Reference</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DR4</td>
<td>Forwar d: ACTTTGGTTGTTCCGTTGCT (20)</td>
<td>102 bp</td>
<td>Helge Bertram et al., (2009)</td>
<td>58 ºC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: GAAACACACCCTGTCCATGC (20)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>DR5</td>
<td>Forwar d: GCACCACGACCAGAAA (16)</td>
<td>120bp</td>
<td>Hirokiinoue et al., (2012)</td>
<td>48 ºC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: CACCGACCTTGACCAT (16)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: AAATCCCCAAAGCAGAGATTCA (21)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>HPV-16 E7</td>
<td>Forwar d: TCCAGCTGGACAAGCAGA (20)</td>
<td>84bp</td>
<td>Rosty et al., (2005).</td>
<td>59 ºC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: CACAACCGAAGCGTAGAGTC (20)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>GAPDH</td>
<td>Forwar d: ACACCCAATGCTGTGCCATACC (20)</td>
<td>94bp</td>
<td>Yung-Tsuan Ho et al., (2009)</td>
<td>59 ºC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: TAGCCAAATTCGTTGTCATACC (22)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>