3

MATERIALS AND METHODS

The experimental procedure pertaining to the present study entitled **"Validating the potential of *Plectranthus amboinicus* against lung cancer - *In silico*, *in vitro* and *in vivo* approaches"** is discussed under the following headings.

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PHASE I

3.1 PHYTOCHEMICAL SCREENING OF *P. amboinicus* LEAF EXTRACTS

3.1.1 Collection and preparation of leaf extracts

P. amboinicus leaves were collected from Malappuram (11.041°N 76.083°E), Kerala, India (Fig.7). The collected leaves were cleansed with sterile water and shade dried at room temperature for 10 days. Using a pulverizer the dried leaves were ground into fine powder and stored at 4°C in an airtight container until further use. The plant sample was authentically identified from Kerala Forest Research Institute, Peechi, Thrissur, Kerala (Appendix 1).

Scientific classification	
Kingdom	Plantae
Order	Lamiales
Family	Lamiaceae
Genus	*Plectranthus*
Species	*amboinicus*

Fig. 7 *Plectranthus amboinicus* - The candidate plant

For the preparation of aqueous extract, about 10g of the leaves were homogenised with 10ml of hot water using mortar and pestle, further 90ml of hot water was mixed with the residue and stirred for 30min. The finely pooled extract was centrifuged at 10,000rpm for 15min at 4°C. The collected supernatant was concentrated using rotary evaporator and used for further analysis. While, for the methanol and petroleum extraction, 10g of the dried leaf powder was added to 100ml of the respective solvent and extraction was performed by cold maceration method for 72h. After extraction, it was filtered using Whatman filter paper and the solvent was evaporated to dryness under vacuum using a rotary evaporator. The crude extract was weighed, dissolved in a predetermined volume of dimethyl sulphoxide and the extraction yield was calculated.

$$\text{Extraction yield (\%)} = \frac{\text{Weight of the dry extract (g)}}{\text{Weight of the sample used for the extraction (g)}} \times 100$$

3.1.2 Preliminary phytochemical screening in the leaf extracts

The petroleum ether, methanol and aqueous extracts of *P. amboinicus* leaves were subjected to preliminary phytochemical tests namely carbohydrates, proteins and aminoacids, phenols, sterols, glycosides, quinones/anthroquinones, alkaloids, tannins, anthocyanin, flavonoids, terpenoids, saponins and leucoanthocyanin as per the standard method (Sofowora, 1993) and the protocol is comprehended in Appendix 2.

PHASE II

3.2. DETERMINATION OF FREE RADICAL SCAVENGING ACTIVITY IN THE METHANOLIC EXTRACT OF *P. amboinicus* LEAVES

The free radical scavenging activity of methanolic leaf extract of *P. amboinicus* was determined under *in vitro* conditions against a series of radicals namely DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonuc acid)), H_2O_2 (Hydrogen peroxide), superoxide and nitric oxide.

3.2.1 Determination of DPPH radical scavenging assay

The ability of methanolic leaf extract of *P. amboinicus* to scavenge DPPH radicals was determined using the method of Mensor *et al*. (2001), and the detailed protocol is described in appendix 3.

3.2.2 Determination of ABTS radical scavenging activity

The ABTS (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonicacid) radical cation decolourisation assay proposed by Shirwaikar *et al.* (2006) to assess the radical scavenging effect of methanolic leaf extract of *P. amboinicus* was given in appendix 4.

3.2.3 Determination of hydrogen peroxide scavenging assay

The scavenging of hydrogen peroxide by methanolic leaf extract of *P. amboinicus* was ascertained by the method of Ruch *et al*. (1989) and the detailed procedure is given in appendix 5.

3.2.4 Determination of inhibition of super oxide radical generation

The ability of the methanolic leaf extract of *P. amboinicus* to prevent the *in vitro* formation of superoxide was examined using Winterbourne *et al*. (1975) method, which is conferred in appendix 6.

3.2.5 Determination of inhibition of nitric oxide radical generation

The method developed by Green *et al*. (1982) was employed to test the inhibition of *in vitro* generation of nitric oxide radical by the methanolic leaf extract of *P. amboinicus* as detailed in appendix 7.

PHASE III

3.3 *IN SILICO* DOCKING OF PHYTOCOMPOUNDS FROM *P. amboinicus* WITH APOPTOTIC TARGETS

3.3.1 Identification of phytocompounds in *P. amboinicus by* GC-MS analysis

The phytocompounds present in the methanolic extract of *P. amboinicus* was identified using GC-MS analysis. The GC-MS system was equipped with a flame ionization detector and capillary column of HP-5 (5 per cent phenyl methyl

siloxane, film thickness 0.25µm). At a flow rate of 2.5 ml/min, nitrogen was employed as the carrier gas, with a split injector (split ratio 50:1) and a split flow of 60 ml/min. The oven temperature was programmed from 90°C for 2 min, increased to 90°C - 200°C at the rate of 8°C per min and additionally with an increase of 200°C-250°C at the rate of 3°C per min. Temperatures for the injector and detector were set to 280°C and 250°C, respectively. The sample, methanolic extract (0.1µL) was injected into the GC-MS instrument for its analysis. Ion source temperatures were maintained at 200°C and the mass spectra was taken at 70eV with a total run time of 32.4 min (Hossain *et al.*, 2014).

3.3.2 Ligand generation

The 2D structures of the identified phytocompounds (ligand molecules) from *Plectranthus amboinicus* leaf extract through GC - MS analysis were drawn in ACD – Chemsketch (ACD/ ChemSketch Freeware, Version11, 2006) and their SMILES notation was obtained. The SMILES notation was submitted to online SMILES converter and structure file generator and further converted to 3D PDB file format (Weininger 1988). The obtained 3D PDB files and SMILE notations of ligands were utilized for further study (Thirumalaisamy *et al.*, 2018).

3.3.3 Drug likeness and pharmacokinetic properties

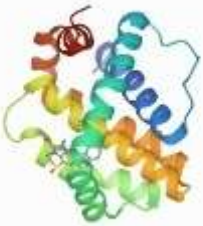
Drug likeness nature of the selected phytocompounds was analyzed using SWISS ADME online server and also examined based on the violations of rules such as Lipinski, Ghose, Veber, Egan, and Muegge. The phytocompounds pervading zero violations were subjected to next level of *in silico* virtual screening. To predict the various pharmacokinetic properties associated such as Adsorption, Distribution, Metabolism, Excretion and Toxicity (ADMET), the phytocompounds were evaluated through web based applications namely preADMET and pkCSM.

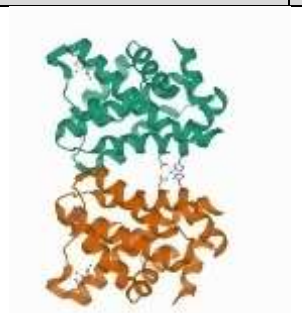
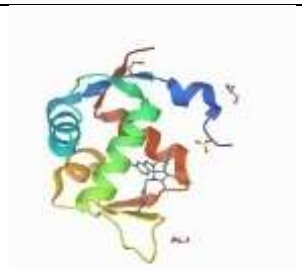
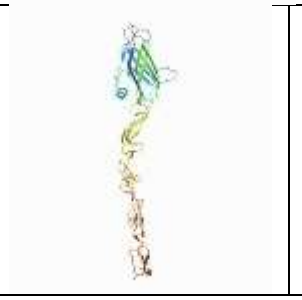
3.3.4 Receptors preparation and molecular docking

To determine the cytotoxic activity of the phytocompounds identified from *P. amboinicus* leaf extract, the crystal structure of seven apoptosis regulator protein molecules namely MCL1 (PDB ID: 3MK8), Bax (PDB ID: 3PK1), NF-Kb (PDB ID: 4IDT), Bcl-2 (PDB ID: 4ZBF), Bak (PDB ID: 5FMI), MDM2 (PDB ID:

5LAY) and Notch (PDB ID: 5MW7) which shows the essential role in lung cancer were chosen for docking study and their three dimensional structures were retrieved from Protein Data Base (PDB www.rcsb.org) (Berman *et al*., 2000). Table 1 depicts the three dimensional structure of the lung cancer protein targets.

Table. 1 Details of the apoptosis regulator proteins for molecular docking

S.No.	Target Protein-Apoptosis regulators	Receptor Molecule (PDB)	Image	Grid Size (A°)
1	MCL1 (Myeloid leukemia 1)	3MK8		X - 39.95 Y - 37.71 Z - 47.51
2	Bax (BCL2-Associated X Protein)	3PK1		X - 57.15 Y - 72.70 Z - 58.57
3	NF-kB (Nuclear Factor kappa-light-chain-enhancer of activated B cells)	4IDT		X - 89.86 Y - 52.17 Z - 59.76
4	Bcl-2 (B-cell lymphoma 2)	4ZBF		X - 95.92 Y - 122.08 Z - 144.89

S.No.	Target Protein-Apoptosis regulators	Receptor Molecule (PDB)	Image	Grid Size (A°)
5	Bak (Bcl-2 antagonist killer 1)	5FMI		X - 40.87 Y - 43.49 Z - 36.40
6	MDM2 (Mouse double minute 2 homolog)	5LAY		X - 73.58 Y - 83.35 Z - 81.35
7	Notch	5MW7		X - 58.09 Y - 40.95 Z - 144.56

AutoDockVina was used to probe the binding affinities between the receptors and ligands (Trott and Olson 2010). The grid map and grid size was calculated using autogrid to represent the protein binding size for docking. The grid size of 40x38x48 points in each dimension was set for 3MK8, the grid size for 3PK1 was set to 57x73x59 points in each dimension for 4IDT the grid size was 90x52x60, the grid size for 4ZBF was set to 95x122x144, for 5FMI the grid size is 41x43x36, the grid size for 5LAY was set to 74x83x81 for each dimension and the grid size of 58x41x145 was set to 5MW7. The spacing of 0.375Å was fixed between the grid points by Autogrid for the apoptotic regulatory proteins and Gasteiger charges were calculated using autodock tools. Assessment of docking (~ 100 times), size of population (150), energy evaluation (maximum number 250,000), generations (maximum number 27,000), rate of mutations (0.02), rate of

cross-over (0.8), value of elitism (1) and other parameters as default values were established using the autotors utility of the AutoDock tool to know the probable torsions of ligand molecules for docking. The docking pose with the enhanced binding affinity score (kcal/mol) is graded as the top orientation of each ligand against each receptor and the binding interaction studies were analysed. The results of molecular docking studies and their molecular protein target-drug interactions were visualized and analyzed using receptor-ligand interaction options in Discovery Studio v2.5 software (Rathinavel *et al*., 2021).

3.3.5 Density Functional Theory analysis

To determine the molecular stability and reactivity of a compound, the energy gap between highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) is predicted through Density Functional Theory (DFT) analysis. HOMO energy illustrates the donating electron while LUMO energy represents the accepting electron ability. The DFT calculations were performed for the phytoconstituents using a hybrid functional B3LYP (Becke's three parameters exchange potential and Lee–Yang–Parr correlation functional) with standard triple split valence basis set 6-3IG** using Gaussian software 09. The vital parameters namely HOMO, LUMO, ionization potential, electron affinity, electronegativity, electronic chemical potential, molecular hardness, softness and electrophilicity index were calculated to reveal the stability and chemical reactivity of the phytocompounds (Anitha *et al*., 2020).

3.3.6 Bioactivity score prediction

The drug score value reveals a compound's overall potential as a drug candidate. The bioactivity score of the phytocompounds against human receptors such as GPCRs, ion channels, kinases, nuclear receptors, proteases and enzyme inhibitors were calculated using Mol inspiration software version 2011.06 (Zhao *et al*., 2002).

From the results of *in silico* study we have inferred that the phytocompound of interest, ***syringic acid*** has significant binding efficacy and molecular stability. Based on this we have attempted to identify and isolate the phenolic acid, syringic acid from the methanolic extract of *P. amboinicus*.

PHASE IV

3.4 IDENTIFICATION OF BIOACTIVE PRINCIPLES IN *P. amboinicus*

In phase IV, effective analytical tools such as Fourier Transform Infrared Spectroscopy (FT-IR) and Nuclear Magnetic Resonance (NMR) were employed to analyse the presence of bioactive principles in the methanolic extract of *P.amboinicus*. Further the crude methanolic extract was subjected to fractionation by column chromatography to identify the presence of syringic acid fraction which was further confirmed by UV-Visible spectroscopy, High Pressure Liquid Chromatography (HPLC) and High Performance Thin Layer Chromatography (HPTLC) due to their high sensitivity, speed and excellent specificity.

3.4.1. FT- IR analysis in the methanolic extract of *P. amboinicus*

The methanolic extract of *P. amboinicus* was scanned in the infrared region (4000-750cm^{-1}) using Fourier Transform Infrared Spectroscopy (FT-IR) (Shimadzu, IR Affinity 1, Japan). To determine the functional groups present in the sample, the various modes of vibration acquired were compared with the reference chart.

3.4.2 NMR analysis in the methanolic extract of *P. amboinicus*

The magnetic characteristics of certain atomic nuclei, particularly the nucleus of the hydrogen atom and proton, are studied using NMR. The presence of syringic acid in the methanolic extract of *P. amboinicus* was investigated using 1H NMR. The samples were dissolved in deuterated methanol and 1H NMR spectra was recorded at 600 MHz. The chemical shifts are represented as parts per million on the δ scale. Since the NMR was performed on the crude extract, the NMR data for synrigic acid obtained from the NIST web book was compared to the obtained results.

3.4.3 Extraction of syringic acid fraction by bioassay guided fractionation

The most useful technique for the isolation of bioactive principles from the crude plant extract is column chromatography. The finely powdered *P. amboinicus* leaves were suspended in methanol for 48 hours at 25°C, and the extract was filtered and the filtrate was concentrated using rocker evaporator and the

remaining residues were removed with the help of freeze-dryer (Lyophilizer-Lark, India). Further, the extract was passed through sodium sulphate for the removal of aqueous matter if present and the dried residue was dissolved in ethyl acetate. For activation, about 500g of silica gel was placed in oven for 1hr at 105˚C and chilled in a desiccator for 30 minutes. The ethyl acetate extract was mixed well and dried by continuous stirring with activated silica gel of mesh size 60-120. A column length of 60cm and diameter of 1.5cm was packed up to two – third portions with the previously activated silica gel G by wet packing protocol. The upper part was then packed with the dried extract mixed with the silica gel and further covered by cotton to avoid the disturbance while pouring the solvent. The column was then eluted with a combination of Toluene: Ethyl acetate in various ratios ranging from 8:2 to 4:6. A total of 12 fractions were collected and the similar fractions were pooled together based on the R_f value in TLC (Thin Layer Chromatography) profile and under reduced pressure the solvents were removed. Further the fraction was subjected to detect the presence of phenolic content as stated in appendix 8. The fraction which revealed high amount of phenolic content was further subjected to UV- Vis spectral, HPLC and HPTLC analyses for further confirmation and compared with the standard syringic acid (Stalikas, 2007).

3.4.4 UV-Vis spectral analysis in the isolated fraction

The UV-Vis absorption spectral analysis was carried for the isolated fraction and compared with the standard syringic acid using Biospec-nano (Shimadzu, Japan) at 200nm to 800nm.

3.4.5. HPLC analysis in the isolated fraction

HPLC is a sensitive and accurate technique used for the separation of organic and inorganic compounds. Prior to use, the samples (the isolated fraction, syringic acid) and the mobile phases were filtered through 0.2µm nylon membrane filter and degassed in ultrasonic bath. The dried residue and standard syringic acid were dissolved in suitable volume of methanol (HPLC grade). At 25ºC, with a total flow of 1ml/min and a detection wavelength of 280nm at 1000psi, the samples (20µl) were injected onto a C18 reverse phase analytical column of an HPLC system (Shimadzu, Japan) equipped with a photo diode array detector.

Employing the mobile phase, acetonitrile and water the peaks displayed were recorded and the retention time obtained at chromatographic conditions for the samples were compared with the standard, syringic acid.

3.4.5 HPTLC analysis in the isolated fraction

The isolated fraction from *P. amboinicus* leaf extract and syringic acid were dissolved in methanol (HPLC grade). Using a Hamilton syringe, the isolated fraction and the standard were loaded on 10×10cm silica gel 60 F_{254} TLC plate and HPTLC was performed in CAMAQ Linomat 5. Toluene: Ethyl acetate: Formic acid in the ratio of 7:2.5:0.5 was used as a mobile phase to develop the plate. The TLC twin trough developing chamber (20×10 cm) was saturated with the mobile phase, and the plates loaded with the samples were placed in it and developed up to 70mm. To evaporate the solvents from the plates the developed plates were dried and placed in photo-documentation chamber (CAMAG, Reprostar 3) and further the plates were placed in densitometry TLC scanner 3 with winCATS software and the absorption measurement was recorded at UV 254 nm. The densitogram of the isolated fraction was compared with the standard syringic acid to confirm its presence in the fraction (Mondal *et al.,* 2010).

Based on the results, we probed to assess the anticancer activity of SAF against A549 lung cancer cell lines and compared with the commercial SA and the standard drug, paclitaxel.

PHASE V

3.5 CYTOTOXIC ACTIVITY OF SYRINGIC ACID FRACTION AGAINST A549 HUMAN LUNG CANCER CELLS - AN *IN VITRO* APPROACH

3.5.1 Cell lines and culture conditions

The A549 human non-small lung adenocarcinoma cell line was obtained from the National Center for Cell Sciences (NCCS), Pune, India and cultured in DMEM supplemented with 10% (v/v) FBS and pencilin-streptomycin (100U/mL) at 37°C under 95% humidity and 5% CO_2. The cancer cells were detached by trypsinization when they reach 80% confluence and subjected to further analysis.

3.5.2 Treatment and experimentation

The cytotoxicity was assessed by treating A549 cell lines with different concentrations (20 to 100µg) of SAF using MTT assay. Based on the available literature the concentration of syringic acid (30 µM) (Karthik *et al*., 2014) and paclitaxel (0.05 µM) (Liebmann *et al*., 1993) was used for the study. The anticancer activities of SAF against A549 cells were evaluated by fluorescence microscopy, flow cytometry, Western blotting, RT- PCR and the results were compared with the commercial syringic acid and the standard drug, paclitaxel.

3.5.3 Evaluation of cytotoxicity by MTT dye reduction assay

The cytotoxic potential of the SAF was examined against the A549 cells by MTT assay according to Igarashi and Miyazawa (2001). Briefly, 100µL of A549 cells with a density of approximately 1×10^5 cells/well was seeded into each well of 96-well microtitre plates and incubated at 37°C for 24h with 5%CO_2 (v/v) in a humidified incubator. After incubation, the DMEM was removed and gently washed with PBS. The A549 cells were subjected to different concentrations of SAF, syringic acid and paclitaxel and incubated at the above mentioned conditions. The medium was removed after 24h incubation and MTT (75µL/well) was added to each well of the microtitre plate and incubated again at the above mentioned conditions for 4h. To this, acid-propanol solution (200µl) was added and incubated in dark overnight. A microtitre plate reader was used to measure the absorbance at 570 nm (Bio-rad, USA). Using GraphPad Prism version 9.2.0 (Windows, San Diego, California), the concentration required to reduce cell viability by half-maximal inhibitory concentration (IC_{50}) was computed using non-linear regression for log (inhibitor) versus normalised response.

The A549 cells were treated with half-maximal inhibitory concentration (IC_{50}) of SAF, syringic acid and paclitaxel and incubated at 37°C for 24h with 5% CO_2. After incubation the treated cells were fixed with ethanol : acetic acid (3:1) and placed on the glass slide covered with cover slips (1×10^5 cells/cover slip) and examined under a bright field inverted light microscope (40X) to observe the morphological changes.

3.5.4 Detection of nuclear changes associated with apoptosis

The nuclear changes of A549 cells during apoptosis was determined using AO/EtBr fluorescence dual staining (Kumar *et al*., 2014), DAPI staining (Rashmi *et al*., 2003) and propidium iodide staining (Sarker *et al*., 2000). A549 cells were seeded at a density of 1×10^5 cells/well in a plate and incubated under appropriate conditions. After incubation, the cells were detached from the plate surface and treated with the IC_{50} concentration of SAF, SA and paclitaxel and incubated at 24h. As a positive control, paclitaxel was used. Both live and dead cells were collected after incubation by centrifugation at 4500rpm for 5min. The collected pellet was washed with PBS (Phosphate-buffered saline) until the complete medium was removed and stained with a 10µL reaction mixture (AO/EtBr-1:1). For DAPI staining, the treated cells were fixed with 3% paraformaldehyde (50l) and kept at room temperature for 10 minutes. The fixed cells were permeabilised for 10 minutes at room temperature with 0.2% Triton X-100 (50 L). Further 10µl of DAPI was added and the cells were incubated for 3 min with a coverslip over them to ensure uniform staining. Similarly, the treated cells were fixed with 50µL of acetone: methanol (1:1) and incubated at -20°C for 10 minutes for propidium iodide staining. Each slide was stained with propidium iodide (PI), covered with a coverslip, and incubated in the dark at 37°C for 30 minutes. The apoptotic cells were identified under a fluorescence microscope (Olympus BX-51, Tokyo, Japan) to analyse the nuclear fragmentation.

3.5.5 Determination of apoptosis by Annexin V / FITC flow cytometry

Syringic acid fraction-induced apoptotic effect on A549 cells was detected by Annexin V / FITC (Fluorescein Isothiocyante) and propidium iodide dual staining using flow cytometer according to Tripathi *et al*. (2020). A549 cells treated with IC_{50} concentration of SAF, syringic acid and paclitaxel were seeded at a density of 1×10^5 cells/well on a six well plate and incubated overnight at 37°C for 24h with 5% CO_2. The cells were removed from the plate surface after incubation and centrifuged at 4000rpm for 5min before being rinsed with PBS and further treated with trypsin / EDTA solution. To the cell pellet, 50µL of binding buffer, Annexin V / FITC (5µL) staining solution, PI (10µL) were added and incubated for

15 min at 25°C. The apoptotic effect of the cells was determined in a flow cytometer (FACSVerse, BD Bioscience, USA) and the fluorescence signal intensity was recorded and analysed by Cell Quest and Modifit.

3.5.6 Analysis of cell cycle arrest by flow cytometry

The cell cycle (G0/G1, S, and G2/M) arrest was examined using a flow cytometer by staining with PI according to Abaza *et al*. (2008). The A549 cells, treated with IC_{50} concentration of SAF, syringic acid and paclitaxel were seeded in a 6 well plate at a cell density of 1×10^5 cells/well and incubated at 37°C for 18h. After incubation, the cells were detached or harvested from the plate surface, centrifuged at 4000 rpm for 5 min, and washed with PBS (0.01M, pH 7.4). The cells were permeabilized using cell-membrane permeabilizing agent (1% Triton X-100), followed by PI (50µg/ml) and RNAase (25µg/ml). The stained cells were incubated at 37°C for 30 min in dark and cell cycle distribution was determined using a flow cytometer. Cell Quest and Modifit were used to monitor and analyse the fluorescence signal intensity.

3.5.7 Apoptotic protein expression by Western blotting

The regulation of apoptotic and anti-apoptotic proteins in the treated and untreated cells were determined by Western blotting to assess according to (Hussain and Sivanandhan, 2021). The A549 cells were seeded at a density of 1×10^5 cells/well in a 6 well plate and incubated at 37°C for 24h before being treated with SAF, SA and paclitaxel. The cells were detached with trypsin-EDTA, washed twice with PBS, and centrifuged at 3000rpm for 5min after treatment. The supernatant was collected and used as cell protein extract after centrifugation at 10,000rpm for 5min at 4°C. On a 12% Sodium Dodecyl Sulphate - Polyacrylamide gel electrophoresis, a known amount (50 µg) of protein from each sample was added. The proteins were transferred onto a nitrocellulose membrane and blocked for 1 h using a blocking buffer containing 10% skimmed milk in water. After blocking, the proteins were washed thrice in PBS containing 0.1% Tween-20 and primary antibodies against Caspase 3, Cytochrome C, p53, Bcl-2 (B-cell lymphoma 2) and β-actin were added at a ratio of 1:1000 (v/v). The primary antibodies were rinsed with PBS after 24h incubation at 4°C followed by addition

of secondary antibodies and incubated for an hour at room temperature and the protein bands were visualized with photographic films.

3.5.8 Apoptotoic gene expression by Reverse Transcription-Polymerase Chain Reaction (RT- PCR)

The gene expression of SAF, SA and paclitaxel treated A549 cells were assessed by RT-PCR. Briefly, the total RNA from the treated and control cells was isolated using TRIzol reagent (Invitrogen, USA). The RNA was reverse transcribed, cDNA was isolated and amplified by one-step quantitative RT-PCR using specific protocol. The reaction mixture (20µL) containing 1µL of forward and reverse random primer pair (0.5µL each) were used for RT-PCR analysis against caspase 3, cytochrome C, Bcl-2 and β-actin (Control). Along with this, 10X reaction buffer (10µL) containing master mix (25 mM/l $MgCl_2$, 10 mM/l dNTPs, Taq polymerase 2.5U), cDNA as template (2µl) and remaining volume (7µl) without nuclease dH_2O was added. The amplification cycles consist of denaturation 94°C for 1 min, primer annealing at 55°C for 40 sec and extension at 72°C for 1 min (for a total of 32 cycles) and extension at 72°C for 10 min (Zeenath and Santhy, 2021).

Following the results of *in vitro* cytotoxic study, SAF was tested against B(a)P induced Swiss albino mice as experimental models.

PHASE VI

3.6 ANTICANCER ACTIVITY OF SYRINGIC ACID FRACTION IN BENZO(A)PYRENE INDUCED EXPERIMENTAL MICE – AN *IN VIVO* APPROACH

The *in vivo* study was carried out to confirm the role played or evoked by the drug / compound on the physiological systems which influence the biological response (Lipinski and Hopkins, 2004). The study was performed to evaluate the efficacy of syringic acid fraction extracted from *P. amboinicus* leaves on lung cancer induced Swiss albino mice.

3.6.1 Selection and maintenance of the animals

Healthy female Swiss albino mice weighing 20 to 30g (8 weeks old) were obtained from the Avinashilingam Institute for Home Science and Higher

Education for Women, Coimbatore, Tamil Nadu, India. The experiments were performed according to the Institutional Animal Ethical Committee's guidelines (AIW:IAEC.2017: ZOO:03) (Appendix 9). Mice were placed in clean polypropylene cages and acclimatized for a period of ten days with a relative humidity of 60% and temperature of 25 ± 2°C and maintained in a restricted environmental circumstance with 12h light/dark cycle. The animals were acclimatized for a week by feeding them with commercially available mice pellet and water (Stanko *et al*., 2016).

3.6.2. Determination of acute toxicity study

The acute toxicity test assesses the adverse effects that occur in a short time after the administration of a single high dose of a drug/ compound to rodents at the beginning of the development of a new drug/ compound to provide information about its potential toxicity. Acute toxicity study solely gives information about the determination of median lethal dose (LD_{50}), therapeutic index and the degree of safety of a pharmacological agent (Chambers, 2008). Healthy Swiss albino mice, were divided into seven groups and allowed to starve overnight prior to drug administration having access only to water. The mice were orally administered with different concentrations of syringic acid fraction (0, 50, 100, 150, 200, 250 and 300 mg/kg body weight). The mice were observed for first 2h for any change in the behavioural, neurological and autonomic profile or any other symptoms of toxicity and continued for next 6h periodically to observe any lethality or mortality (Ghosh, 1984). The dose was selected based on the $1/5^{th}$ and $1/10^{th}$ of the lethal dose (LD_{50}) as per OECD guidelines. Based on the available literature the concentration of commercial syringic acid (25mg/kg of body weight) and the standard drug, paclitaxel (5mg/kg body weight) was administered to the mice to assess the toxicity.

3.6.3 Induction of tumor in experimental mice

Benzopyrene (B(a)P), a powerful carcinogenic agent has been explored for its potential to induce tumor in the experimental mice. The initial body weight of the mice selected for the study was recorded. Tumor was induced in the mice twice per week with B(a)P (50 mg/kg body weight dissolved in corn oil) orally for

4 weeks. The control mice were fed with commercially existing mice pellet (Shreelatha *et al*., 2011).

3.6.4 Experimental design

The experimental animals were randomly divided into seven groups comprising 6 mice in each group.

Group I : Mice were administered orally with corn oil twice per week for 16 weeks which served as control

Group II : Mice were administered orally with B(a)P (50mg/kg body weight dissolved in corn oil) twice per week for 4 weeks and left for 12 weeks to induce lung cancer

Group III : Mice were administered orally with B(a)P as in Group II along with paclitaxel (5mg/kg body weight dissolved in corn oil) daily for 12 weeks

Group IV : Mice were administered orally with B(a)P as in Group II along with syringic acid fraction (25mg/kg body weight in corn oil) daily for 12 weeks

Group V : Mice were administered orally with B(a)P as in Group II along with syringic acid fraction (50mg/kg body weight in corn oil) daily for 12 weeks

Group VI : Mice were administered orally with B(a)P as in Group II along with syringic acid (25mg/kg body weight in corn oil) daily for 12 weeks

Group VI : Mice were orally administered with syringic acid (25mg/kg body weight in corn oil) alone for 16 weeks to evaluate the toxicity (if any) induced by syringic acid (Shoja *et al.,* 2016).

At the end of the experimental period, the mice were allowed to fast overnight and were anasthetized mildly and sacrificed. The blood sample was collected by tail vein puncture into a heparinized centrifuge tube for hematological parameters and the serum was separated immediately by centrifugation and used for various biochemical analyses. The tumor growth responses in the

experimental animals were recorded at the initial and final day (Parasuraman *et al*., 2010).

3.6.5. Evaluation of syringic acid fraction on tumour growth response in experimental mice

3.6.5.2 Mean survival time

Mean survival time is used to calculate the efficacy of the drug / compound induced in the experimental animals. It can be defined as the average length of time (days) to diagnose a disease using the formula given below,

$$\text{Mean survival time (Days)} = \frac{\text{Total number of animals survived}}{\text{Total number of animals in the group}}$$

(Islam *et al*., 2012)

3.6.5.2 Increased life span

Increased life span reveals the percentage increase in life span of the B(a)P induced experimental mice and is calculated using the formula,

$$\text{Increased life span (\%)} = \frac{\text{Mean survival of the treated groups}}{\text{Mean survival of the control groups}} - 1 \times 100$$

(Sunil *et al*., 2013)

3.6.5.3 Body weight analysis

Throughout the trial, a meticulous record of the body weight of all the animals in the control and treatment groups was maintained. The mice body weight was measured at the start of the experiment and again before they were sacrificed. The net body weight of the experimental animals were calculated using the formula given below,

Net body weight (g) = Final body weight - Tumor weight

(Barhoi *et al*., 2020)

3.6.5.4 Relative lung weight

At the end of the study, lungs were excised from the mice, washed in normal saline and the relative lung weight was calculated using the formula,

$$\text{Relative lung weight (\%)} = \frac{\text{Recorded lung weight}}{\text{Final body weight}} \times 100$$

(Hassan *et al*., 2019)

3.6.6. Analysis of haematological and biochemical parameters

The hematological parameters namely haemoglobin, Red Blood Corpuscles (RBC) count, total and differential counts of White Blood Corpuscles (WBC), platelet count, selected biochemical parameters namely Aspartate transaminase (AST), Alanine transaminase (ALT), Alkaline phosphatase (ALP), urea, uric acid, creatinine and tumor marker enzymes namely Gamma glutamyl transferase (GGT), Lactase dehydrogenase (LDH) and Adenosine deaminase (ADA) were estimated in both the experimental and control mice. Table 2 represents the details of the haematological and biochemical parameters assessed in the experimental mice.

Table 2. Details of the haematological and biochemical parameters analysed

Parameters	Method of analysis	Reference	Appendix Number
HAEMATOLOGICAL PARAMETERS			
Haemoglobin	Haemoglobinometer method	Drabkin and Austin (1932)	10
Red blood corpuscles	Diluting fluid method	Sanderson and Phillips (1981)	11
Platelets	Diluting fluid method	Sanderson and Phillips (1981)	12
White blood corpuscles	Truck's fluid method	Sanderson and Phillips (1981)	13
Differential leukocyte count	Staining technique	Sanderson and Phillips (1981)	14
LIVER FUNCTION TESTS			
Aspartate transaminase	Spectrophotometry-DNPH method	Reitman and Frankel (1957)	15

Parameters	Method of analysis	Reference	Appendix Number
Alanine transaminase	Spectrophotometry-DNPH method	Reitman and Frankel (1957)	16
Alkaline phosphatase	Spectrophotometry	King and Armstrong (1934)	17
KIDNEY FUNCTION TESTS			
Urea	Spectrophotometry	Natelson *et al*. (1957)	18
Uric acid	Uricase method	Caraway (1955)	19
Creatinine	Spectrophotometry	Owen *et al*. (1954)	20
TUMOR MARKER ENZYMES			
Gamma Glutamyl Transferase	Spectrophotometry	Persijn and Van der Slik (1978)	21
Lactate dehydogenase	Spectrophotometry	King (1965)	22
Adenine deaminase	Spectrophotometry	Giusti and Galanti's (1984)	23

3.6.7 Histopathological studies in the lung tissues of experimental animals

The mice were sacrificed by cervical dislocation and the lung tissues were dissected and fixed in 10% formalin for an hour. Further the lung tissues were washed thoroughly in running water to remove formalin. Dehydration of the fixed tissue was performed by three changes of acetone (each 100ml). The tissues were cleansed from acetone by giving three changes of xylene (each 500ml) for about 3h. The processed lung tissues were incubated in melted paraffin (two changes) for 3-4h and maintained at 58 - 60ºC in an incubator. The lung tissues were embedded in paraffin wax and the microtome sections of 1-3µm thickness were prepared on glass slides and further cleaned by immersing in xylene. Further the sections are stained with haematoxylin and eosin stain to assess the morphological and pathological observations (Culling, 1979).

3.6.8. Statistical analyses

The results of radical scavenging activity were expressed in mean ± standard deviation. The IC_{50} of the antiproliferative cells using MTT assay was determined by Graphpad 8.01 (Graphpad Software, San Diego, CA, USA). The *in vivo* studies were assessed using one way ANOVA where the p value < 0.05 was considered statistically significant.

The results obtained for the various parameters analyzed in all the six phases and the salient observations made during the study are presented in the next chapter.