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Research Article

BIOACTIVE POTENTIAL OF *ALOE BARBADENSIS* ROOT EXTRACT INHIBITING LUNG CANCER ANGIOGENESIS

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ABSTRACT

In India, lung cancer constitutes 6.9 percent of all new cancer cases and 9.3 percent of all cancer related deaths in both sexes. By cutting of the blood supply for tumours, cancer cells would be deprived of nutrients and hence the cancer can be treated to greater extent. Anti-angiogenic strategies aimed at blocking new blood vessel formation are currently emerging. The study reveals that the *Aloe barbadensis* contains variety of bioactive potential compounds such as antioxidant, anti-inflammatory, anti-microbial activity and anti-angiogenetic activity. The Gas chromatography Mass spectrometric analysis reveals that *Aloe barbadensis* contains nearly 276 compounds, based upon the library results. The compounds passing PK studies are allowed to virtual screening and docking studies against Vascular Endothelial Growth Factor (VEGF) receptor. The receptor is allowed for molecular dynamics and energy minimization before the docking, in order to get the stable and clean protein structure. The results show that the phytoconstituents in *Aloe barbadensis* have more binding affinity than the commercial drugs. *Escherichia coli* and *staphylococcus aureus* shows the higher anti-microbial activity compared to the standard antibiotic discs. The extracts are then used for chicken chrioallantoic membrane assay, in order to study the effect of the plant extract over the growing blood vessels of the chicken embryo. This study utilised the *in vitro* angiogenesis assay using human lung cancer explants as sources for angiogenetic vessel development. There is complete inhibition in the case of fractionates and partial inhibition in case of crude extracts. The extracts are then allowed to cytotoxicity studies in A549 Lung cancer cell lines, which shows that there is no migration in the scratched area in Cancer cell lines treated with the plant extracts. The studies reveal that the *Aloe barbadensis* have the anti-angiogenetic potential and can be used to reduce the size and growth of the cancerous cells with the phytoconstituent, in order to reduce the toxicity caused by the chemotherapy agents.

Keywords: *Aloe barbadensis*, Chicken chorioallantoic membrane assay, Fractionation, Vascular Endothelial Growth Factor, Angiogenesis assay

INTRODUCTION

Lung cancer is the most common cause of cancer death worldwide, with most patients dying with metastatic disease. Lung cancer contributes over 160,300 deaths annually in the US, more than cancers of the breast, colon and prostate combined. The incidence of lung cancer varies according to the geographic region and ethnicity and largely reflect the prevalence and smoking patterns. In India, lung cancer contributes about 6.9 per cent of all new cancer cases and 9.3 per cent of all cancer associated deaths in both sexes, with the highest reported incidences from Mizoram in both sexes at rate 28.3 and 28.7 per 100,000 population in both sexes respectively. Anthraquinones are the major secondary metabolites that are abundantly present in the *Aloe barbadensis* extract. *Aloe barbadensis* extracts have been recorded to have many biological activities such as antibacterial, antifungal, anticancer, antioxidant and immune-protective properties. The Elevated interstitial fluid pressure can compromise the delivery of therapeutics to tumors[1]. By blocking vascular endothelial growth factor (VEGF) signalling, there is the decreases in the interstitial fluid pressure by producing a morphologically and functionally "normalized" vascular network. It also improves the integrity and function of the remaining vasculature by enhancing the perivascular cell and basement membrane coverage. EGF is a signalling protein developed by cells that initiates the vasculogenesis and angiogenesis. VEGF induces the proliferation, migration and survival of vascular endothelial cells, and induces the recruitment of bone marrow-derived endothelial progenitor cells to the fresh vasculature. VEGF also improves vascular permeability, contributing to malignant pleural effusion in Non-Small Cell Lung Cancer (NSCLC). Angiogenesis is the formation of new blood vessels from pre-existing vessels involves the migration, growth, and differentiation of endothelial cells, which line the inside wall of blood vessels. The newly formed blood vessels can promote cancer growth by supplying nutrients and oxygen and by removing waste products. By cutting off the blood supply, cancer cells would be deprived of nutrients and hence the cancer can be treated to greater extent. Chemotherapy has lower efficiency and higher toxicity than Phytoconstituent. GROMACS is a package that carries out molecular dynamic simulations, and generates a trajectory of the molecule[3]. A MD simulation generates a sequence of points in a multidimensional space as a function of time. Anti-angiogenesis therapy is an anti-cancer strategy that targets the new vessels that grow to provide oxygen and nutrients to actively proliferating tumour cells. Thus, this study the *in vivo* anti-angiogenic activity of *Aloe barbadensis* extracts evaluated using both In-vitro, In-silico and In-vivo assays. Further, bioactivity-guided fractionation and isolation were carried out to obtain the potential anti-angiogenic chemical constituents from *Aloe barbadensis.*

MATERIALS AND METHODS

Collection of *Aloe barbadensis root*

An identified fresh root of *Aloe barbadensis* Miller plant was deposited at Tamilnadu Agricultural University, Coimbatore, India. The roots are then washed with the running tap water, in order to remove the soil and other debris. They were chopped into small pieces, dried in shade, grinded into a fine powder form and stored in an air tight container.

PHYTOCHEMICAL SCREENING

The freshly prepared extracts were subjected to standard phytochemical analyses for different constituents. Chemical tests were carried out on the aqueous extract using standard procedures of the identified constituents. The series of tests is used to screen the varieties of the phytoconstituents that are present in the extract. Test for alkaloid, steroids, polyphenols, saponins and flavonoids are shown as the positive results. [1]

CHROMATOGRAPHIC SEPARATION

The dried Methanol extract (4 gm) of *Aloe barbadensis* was first dissolved in the methanol and carefully applied by pipette at the top of prepared column. Immediately after application of sample, a gradient of mixture of ethyl acetate and hexane (mobile phase) was used as eluent to collect fractions of methanol extract of *Aloe barbadensis*. The column was run with a gradient of hexane and ethyl acetate (8:2, 7:3, 6:4, 5:5, 4:6, 3:7,2:8) finally 7 fractions (F1-F7) were collected. Thereafter, from all the collected fractions solvent was removed by evaporation at room temperature. The crystalline residues were then retreated with Chloroform and were recovered after filtration. The qualitative analysis of the crude and eluted fractions for the presence of the compounds was done using TLC. TLC was performed on pre-coated 5cm x 10cm silica gel 60 F254 plates. The chromatographic plates were developed using 10ml of developing solvent, hexane: ethyl acetate (40:60) at room temperature in a saturated chamber by the ascending technique. After developing and drying the plates were sprayed in the Liebermann–Burchard reagent and then heated at 120ºC for 5-10 minutes and Retention actor (R_f) was calculated. The presence of the bands at the particular distance from the solute front indicates the mobility of the compounds based upon the polarity.

$$\text{Retention factor} = \frac{Distance\ tavelled\ by\ the\ solute\ front}{Distance\ traveled\ by\ the\ solvent\ front}$$

ANTIOXIDANT STUDY

DPPH (2, 2-diphenyl-1-picylhydrazyl) assay

The antioxidant activity of phenolic compounds is due to their adsorbing and neutralising free radicals, reducing singlet and triplet oxygen, or decomposing peroxides. 0.1 ml of extract was added to 2.9 mL of methanol solution. After centrifugation, the supernatant is collected 50 µmol/L of DPPH solution is added and incubated in the dark for 45 min. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability. [4]

$$\%\ \text{inhibition} = \frac{Absorbance\ (control) - absorbance\ (sample)}{Absorbance\ (control)} \text{ x } 100$$

Hydrogen peroxide (H_2O_2) scavenging assay

A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Extracts (100 µg/mL) in distilled water were added to a hydrogen peroxide solution (0.6 mL, 40mM). 200,400, 600, 800 and 1000µg/ml extracts were added to a 0.6 ml of hydrogen peroxide (40mM) with the already prepared phosphate buffer (pH 7.4). The reaction mixture was placed at room temperature for 10 minutes [5]. The percentage of inhibition of hydrogen peroxide scavenging of both *Aloe barbadensis* extracts and standard compounds were calculated based on the formula,

$$\%\ \text{Scavenged}\ [H_2O_2] = \frac{Abs\ (control) - Abs\ (sample)}{Absorbance\ (control)} \text{ x } 100$$

Ferric Reducing Anti-oxidant Power (FRAP) assay

Plant extracts of varying concentrations were mixed with 2850 μL FRAP solution (25 mL acetate buffer (300 mM, pH 3.6), 2.5 mL 2-4-6 tripyridyl-s-triazine (10 mM in 40 mM hydrochloric), and 2.5 mL hydrated ferric chloride solution (20 mM) equilibrated for 30 minutes in the dark at 37°C. These determinations were performed in triplicate and percentage inhibition is recorded. [6]

$$\%\ \text{inhibition} = \frac{Absorbance\ (control) - absorbance\ (sample)}{Absorbance\ (control)} \text{ x } 100$$

RESULTS AND DISCUSSION

COMPOUND ISOLATION AND FRACTIONATION

Ten grams of powder was extracted by maceration with 100ml of methanol for three days and extracted further with the same quantity of 20% aqueous methanol for another one week. Each of the extracts were concentrated by using rotavapor apparatus collected and stored in a vial for further studies. Totally 18 compounds are present in the crude and fractionates and their bands are clearly shown in the figure 1.

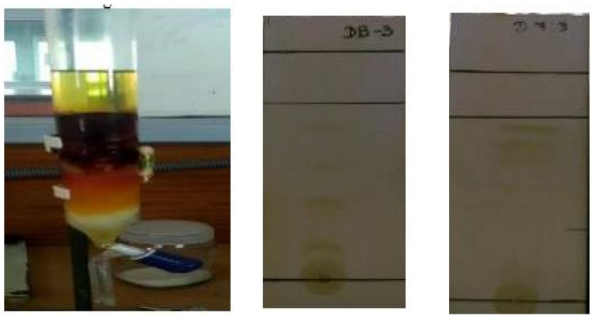

Figure 1: (a) column fractionation of the sample; (b) TLC plate of the crude & (c) TLC plate of fractionate

ANTIOXIDANT ASSAY

DPPH (2, 2-diphenyl-1-picylhydrazyl) assay

The decrease in absorbance of the DPPH radical at 517nm due to the scavenging capability of methanolic extract of *Aloe barbadensis* were recorded against blank using a UV-Vis Spectrophotometer. Ascorbic acid (10mg/ml DMSO) was used as reference. All the extracts showed a rapid decrease in absorbance with methanolic extracts of *Aloe barbadensis* exhibiting the fastest scavenging rate.

Hydrogen peroxide (H_2O_2) scavenging assay

The reaction mixture after the incubation is read at 230 nm against the blank solution with phosphate buffer solution. Absorbance of hydrogen peroxide at 230 nm was determined 10 minutes later against a blank solution containing the phosphate buffer without hydrogen peroxide.

Ferric Reducing Anti-Oxidant Power (FRAP) assay

2.85 ml of freshly prepared FRAP reagent (TPTZ, $FeCl_3$ and acetate buffer) at 37ºC was mixed with 90µl of leaf extract and 270µl of distilled water. Using a blank containing FRAP reagent as reference, absorbance at 593 nm was determined at 10 min.

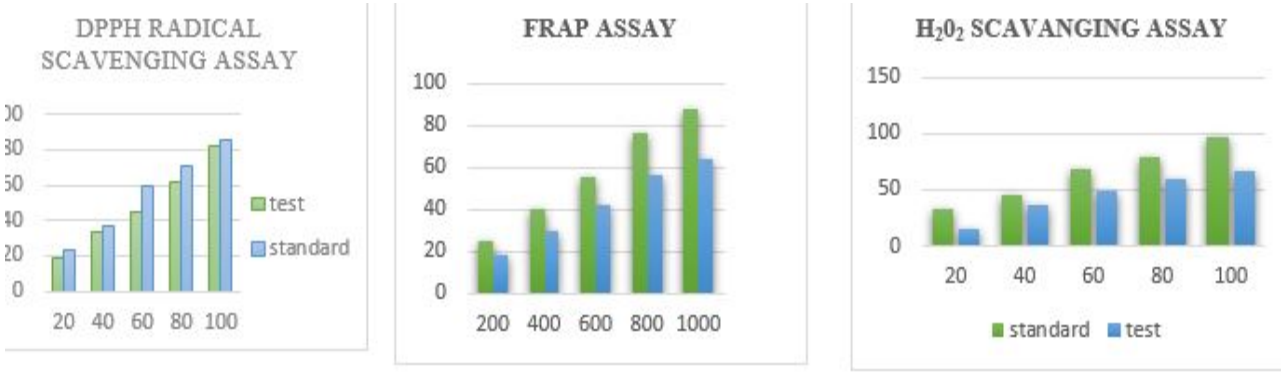

Figure 2: (a) Antioxidant assay using DPPH assay; (b) FRAP assay; (c) H_20_2 scavenging assay

***IN VITRO* ANTI-INFLAMMATORY ACTIVITY**

Membrane stabilization assay

Fresh whole human blood (10ml) was collected and transferred to EDTA coated centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10minutes and were washed with equal volume of normal saline. Samples of the extract used were dissolved in isotonic phosphate buffer solution HRBC suspension (0.1 ml) was added to each of the tubes and mixed gently. 5 ml graded doses of the extracts (200, 400, 600, 800 and 1000 µg/ml) were arranged in triplicate. All the centrifuge tubes containing reaction mixtures were incubated in a water bath at 56ºc for 30minutes in a regulated water bath. The reaction was centrifuged at 2500rpm for 5minutes and the absorbance of the supernatant was taken at 560nm and performed in triplicates. % of membrane stabilization activity was calculated by the formula [7].

$$\% \text{ inhibition} = \frac{Absorbance(control) - Absorbance(treated)}{Absorbance(control)} \times 100$$

Egg albumin denaturation assay

The reaction mixture (5ml) consist of 0.2 ml of egg albumin (from fresh hen's egg), 1% of aqueous solution of bovine serum albumin, 2.8ml phosphate buffered saline (pH: 6.4) and 2ml of varying concentration of plant extracts and the pH of the reaction mixture was adjusted using small amount of 1N Hydrochloric acid(HCL). The sample extracts were incubated at 37±2 °C for 20minutes and then heated to 51ºc for 20 minutes, after cooling the samples the turbidity was measured at 660nm (UV spectrometer). Diclofenac at the final concentration of (1mg/ml) was used as reference drug and treated similarly for determination of absorbance [8]. The experiment was performed in triplicates. %inhibition of protein denaturation was calculated as follows

$$\% \text{ inhibition} = \frac{Absorbance(control) - Absorbance(sample)}{Absorbance(control)} \times 100$$

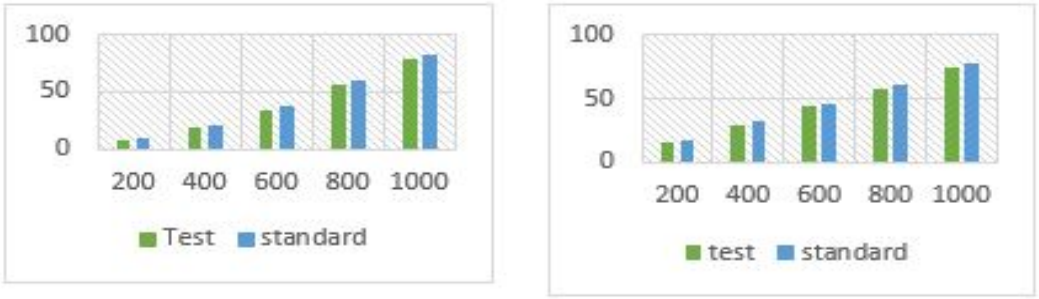

Figure 3: (a) Anti-inflammatory assay using Membrane stabilization assay; (b) Egg albumin denaturation assay

ANTI-MICROBIAL ACTIVITY

The Mueller-Hinton agar medium is used as a medium for the antimicrobial activity. Then the medium and other glass wares are placed in sterilization process at 121ºC for 15 min. After sometimes the medium was taken and pour into 5 sterile petri plates. Three discs were taken for crude, fractionates, and two standard antibiotics (streptomycin and Gentamycin).

The clinical pathogenic strains of *Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, and pseudomonas fluorescens* were used to spread over the Mueller-

Hinton agar plates using separate sterile cotton buds in order to determine the antibacterial activity of the phytoconstituents. The crude extract and fractionates collected are used as samples. The discs were dipped into the specific concentration of the crude

extract and its fractionates. The plates were incubated at 27°C for 16-18 hours in sterile room. All bacterial plates were incubated for 24 h. The diameter of the minimum zone of inhibition was measured in millimetre. For each test, three replicates were performed.

Figure 4: Anti-microbial activity of the phytoconstituents

ENERGY MINIMIZATION

The target protein was solvated and then protein internal energy was minimized in water at 310K to obtain global energy minima because these are necessary to obtain a better model of entire receptor which can mimic the human physiological conditions[3].

The various plots generated during the GROMACS are shown in the figure. The pressure, temperature and potential values of the various protein can be determined by performing GROMACS [9]. The average value and the total drift from the initial position can be visualized using the terminal commands and the Plots generated upon the drift vs time is shown in the figure 7.

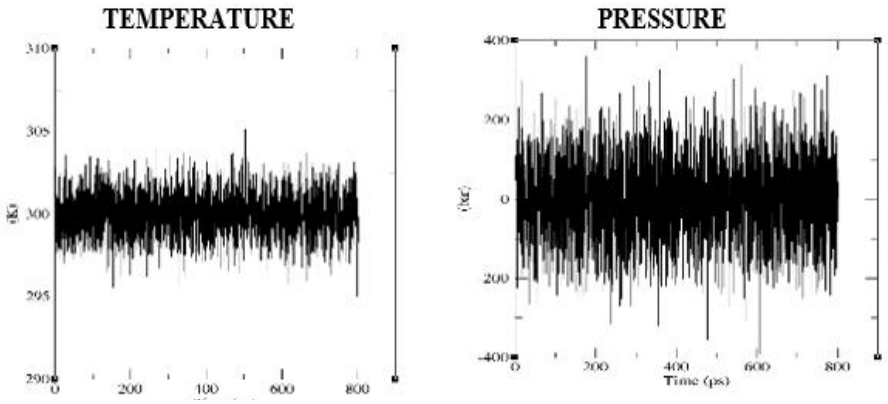

Figure 5: Energy Minimization plot of the receptor

GAS CHROMATOGRAPHY – MASS SPECTROMETRY (GCMS)

GC/MS analysis of the *Aloe barbadensis* extract was performed using a Thermo GC-trace Ultra ver 5.0 system and Gas Chromatograph interfaced to a Mass Spectrometer (GC/MS) equipped with an DB-35 MS capillary standard Non-polar column (30 m × 0.25 mm ID, 0.25 µMdf). For GC/MS detection, an electron ionization system with ionization energy of 70 eV was used.

Helium gas (99.999%) was used as the injection volume of 1 µl was employed (split ratio of 10:1). Injector temperature was 70°C and Ion-source temperature was 260°C. The oven temperature was programmed from 110°C (isothermal for 2 min.), with an increase of 10°C/min, to 200°C, then 5°C/min to 280°C, ending with a 9 min. isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 45 to 450 Da.

Total GC running time was 36 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was a TurboMass Ver5.2.0. Nearly 190 compounds are identified in the extract using Gas chromatography–mass spectrometry (GC-MS). The compounds which satisfies the pharmacokinetic properties and physiochemical properties are selected based upon the Lipinski rule of five.

The peaks in the figure indicates the relative abundance of the compounds injected into the column. The compounds have highest peaks presents highly in the extracts. The compounds are selected based upon the retention time of the compounds inside the column. The library results are recorded based upon the retention time and the molecular mass of the compounds using mass spectrometry.

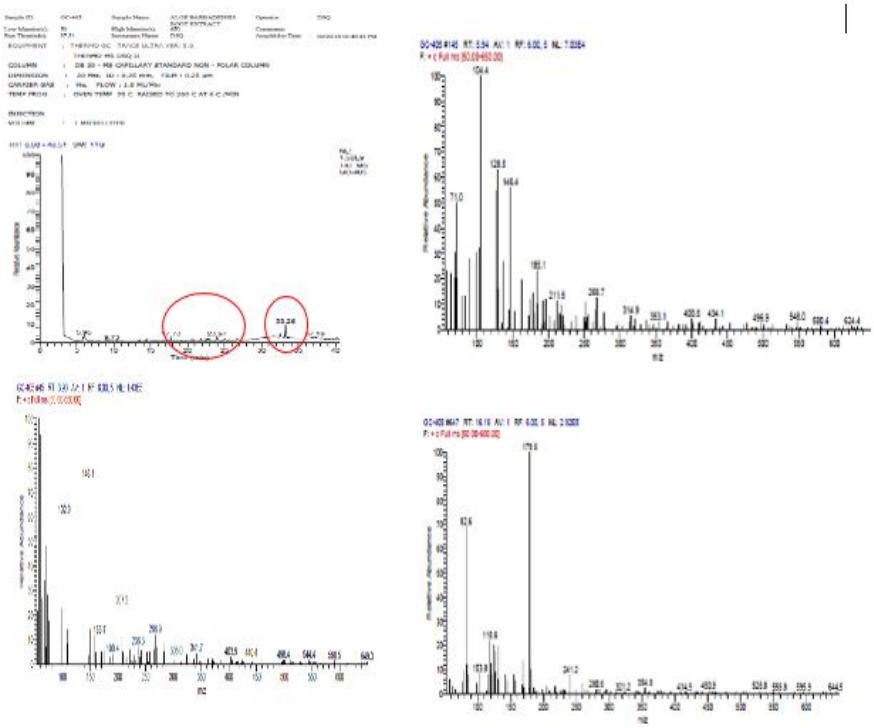

Figure 6: (a &b) GCMS spectrum of the sample

VIRTUAL SCREENING

Autodock is a flexible ligand-protein docking program which basically runs as a two steps procedure, such as calculation of the map of interactions of the binding site and the posing of the ligand. Totally 23 docking scores shows >6 binding affinity. The best drugs are selected based upon the binding affinity over the drug targets. The 6-methyl-2-[(3-methylbenzyl) sulfanyl]-5,6,7,8-tetrahydro[1,6]naphthyridine-3-carbonitrile and 2-[(2-Benzenesulfonylamino-acetyl)-(2-methoxy-phenyl)-amino]-pentanoic acid (furan-2-ylmethyl)-amide are the best drugs which have the binding affinity of -10.3 and -8.2 kcal/mol respectively. The synthetic has less binding affinity compared to phytoconstituents, which shows that phytoconstituents is effective over the angiogenetic drug targets.

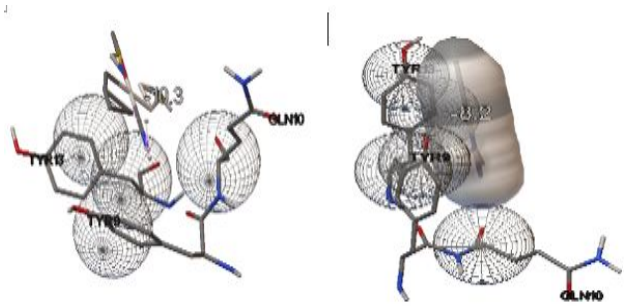

Figure 7: Docked conformation of the ligands

IN VITRO ANTI-ANGIOGENETIC ACTIVITY

Cell viability assay (MTT)

Plate 1×10^4 cells/well in a 96-well plate and incubate with the different concentrations (1 - 100µL) of given sample for 48hrs. Remove medium and wash cells with PBS. Add MTT solution (0.5 mg/ml) made up in medium to a final concentration of 0.5mg/ml. Incubate for 30 minutes to 4 hours at 37°c, until intracellular purple formazan crystals are visible under microscope. Remove MTT and add solubilizing solution and triturate. Incubate at room temperature or 37°c for 30 minutes to 2 hours, until cells have lysed and purple crystals have dissolved. Measure absorbance at 570 nanometres. Absorbance value greater than the control indicates cell proliferation, while lower values suggest cell death or inhibition of proliferation [2]. Untreated sets were also run under identical conditions and served as controls. The IC_{50} Values for the given sample was found to be 9µL (57.67).

$$\% \text{ viable cells} = \frac{abs(sample)-abs(blank)}{abs(control)-abs(bkank)} \text{ x100}$$

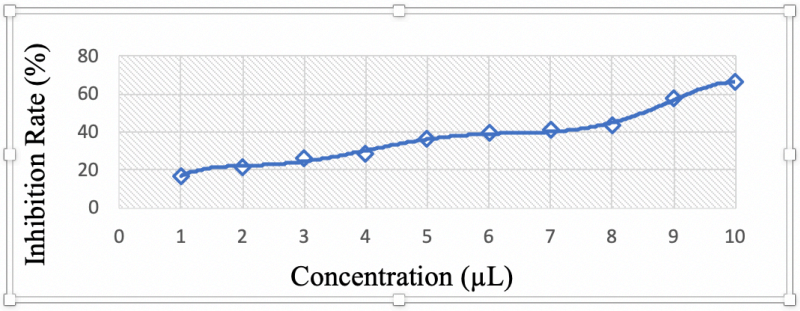

Figure 8 % Cell Inhibition rate of given sample treated A549 cell line by MTT Assay

Cell Migration Assay

The cell is detached from the tissue culture plate using 0.25% Trypsin-EDTA solution. The pelleted cells in a 15 ml conical tube by centrifugation, aspirate the supernatant, and re-suspend cells in culture media. Plate the appropriate number of cells in a 6-well plate for 100% confluence in 24 hours. 12-well or 24-well plates may be used. In a sterile environment use a 200 µl pipette tip to press firmly against the top of the tissue culture plate and swiftly make a vertical wound down through the cell monolayer. Carefully aspirate the media and cell debris. Slowly add enough culture media against the well wall to cover the bottom of the well and avoid detaching additional cells.

Place the tissue culture plate in an incubator set at the appropriate temperature and CO_2 concentration (typically 37 °C and 5% CO_2). At several time point, remove the plate from the incubator and place it under an inverted microscope to take a snapshot picture and to check for wound closure. Depending on the cell type, wound closure time may vary. To analyze the results of snapshot pictures, measure the distance of one side of the wound to the other using a scale bar.

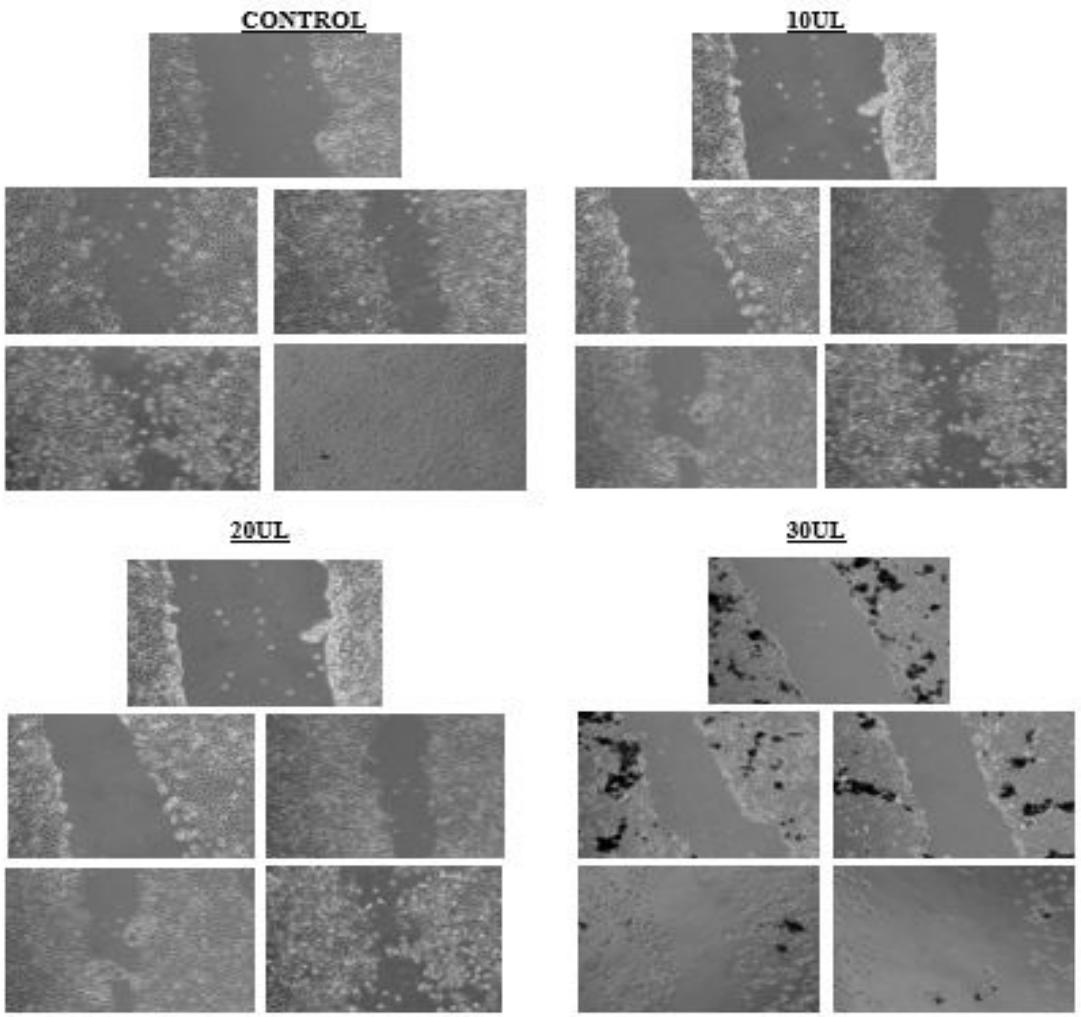

Figure 9: Cell migration assay – scratch assay for different concentrations

IN VIVO ANTIANGIOGENETIC ACTIVITY

Chick Chorioallantoic Membrane Assay

Fertile white Leghorn chicken eggs (*Gallus domesticus*) were obtained from a local hatchery with 5 or 6 days incubation[7]. The eggs are incubated at 37ºC in incubator for 48 hours, 70-80% humidification, placed in horizontal position and rotated several times. The eggs were grouped as per type of extracts and sprayed with 70% ethanol and air-dried to reduce contamination from the egg surface. On 6th day fertilized egg was punctured a small hole in the air sac of the egg, with 26- gauze needle and 2-3 mL of albumen is sucked with the help of sterile syringe and sealed with paraffin. A window was then cut in the shell using a sterile blade and shell is removed with sterile forceps, under laminar air flow. The window is closed with a paraffin tape after capturing the photographs of the embryo. The eggs are returned to the incubator after the injection of 100 micro litres of methanol extracts over the blood vessels of embryo. After 48 hours of incubation on third day of extract added were photographed, then the process continuous until all the blood vessels get vanished. Several photographs of embryos of three-day interval are taken to obtain the image of CAM after treatment with various extracts. At least six eggs are used for each extract dose. There is a control egg which is untreated with extracts and simultaneously observed as for eggs treated with extracts. The eggs are incubated until the blood vessels of the embryo reach inhibition of 100%. [3]

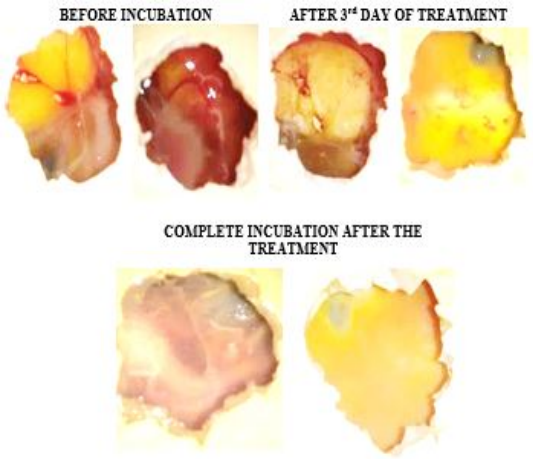

Figure 10: Chicken Chrioallantoic membrane assay

CONCLUSION

Anti-angiogenetic therapy has been effective approach for the treatment of many cancers. Anti-angiogenetic agents are either used alone or in combinations with other cytotoxic and/or chemotherapeutic drugs. Additional laboratory research and well-designed clinical studies are necessary to improve on the existing agents, approaches and combinations of drugs for the use of anti-angiogenic agents for cancer treatment. The study reveals that the *Aloe barbadensis* contains variety of bioactive potentials such as antioxidant, anti-inflammatory, anti-microbial activity and anti-angiogenetic activity. The *Aloe barbadensis* proven to have the polyphenols and flavonoids have higher antioxidant properties. The crude methanolic extracts of *Aloe barbadensis* are allowed to column chromatography, in order to get the purified compounds. The presence of the pure compounds in the fractions are checked using Thin Layer Chromatography (TLC). The bands present in the TLC plates shows the purity of the compounds and they can be compared with the crude extracts. The Gas chromatography Mass spectrometric analysis reveals that *Aloe barbadensis* contains nearly 276 compounds, based upon the library results. The compounds are then allowed for pharmacokinetic studies such as drug likeliness (i.e: Lipinski rule of five, ADME/T and Solubility analysis). Then the compounds are allowed to dock with VEGF receptor and the binding affinity of the compounds are predicted. The binding affinity of the phytoconstituents are compared with the binding affinity of the commercially available drug. The results show that the phytoconstituents have more binding affinity than the commercial drugs. The receptor is allowed for molecular dynamics and energy minimization before the docking, in order to get the stable and clean protein structure. The fractionates are then allowed for the antimicrobial activity by disc diffusion techniques. *Escherichia coli* and *staphylococcus aureus* shows the higher anti-microbial activity compared to the standard antibiotic discs. The extracts are then used for chicken chrioallantoic membrane assay, in order to study the effect of the plant extract over the growing blood vessels of the chicken embryo. The extract was tested over the 3rd and 9th day embryo. There is complete inhibition in the case of fractionates and partial inhibition in case of crude extracts. The extracts are then allowed to cytotoxicity studies. The cell migration assay was done in A549 Lung cancer cell lines, which shows that there is no migration in the scratched area. The results explain that there is no growth or migration of the cancer cell in the area treated with the plant extracts. The studies reveal that the *Aloe barbadensis* have the anti-angiogenetic potential and can be used to reduce the size and growth of the cancerous cells with the phytoconstituent, in order to reduce the toxicity caused by the chemical antibodies.

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