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ORIGINAL ARTICLE

Evaluating the Anticancer Potential of *Terminalia chebula* extract and Suppresses the Growth and Migration of A-549 (Human lung cancer cell line, in a Zebra fish Xenograft Model

Prasanth S, B.V.Vibala*,
Periyar University, Salem-636011, Tamilnadu, India
*Chief scientific officer, Xcellogen Biotech india pvt Ltd, Thiruvananthapuram- 695035.
Corresponding author's E mail: bala10101990@gmail.com

ABSTRACT
The paper was designed to analyze the anticancer activity of Terminalia chebula aqueous extract. The aqueous extract of the Terminalia chebula helps to identify the main pathway on lung cancer. Anticancer activity was identified by MTT assay towards A549 cell line of lung cancer. The detection of mitochondrial pathway using western blot, which is Bcl-2 family protein-mediated, it is regulated by the action of Terminalia chebula. T. chebula which acting as a natural substance for the treatment of tumor in lungs. This paper is based on the anticancer activity against lung cancer cell lines. For the research of migration of cancer cell of human gastric and tumor growth in In-vitro method, CCK-8 and Transwell assays are used. In xenograft model of zebrafish, the growth of tumor cell via In vivo method were done. Quantification of anticancer activity of T. chebula, depends on inhibition rate of metastasis and growth. Western blotting technique were used to identify the molecular mechanism. In vitro cell migration which is suppressed and reduced proliferation of tumor indicated by the action of T. chebula utilizing Transwell experiments and CCK-8. The in vivo research on the zebrafish xenograft, which explained that the T. chebula blocked the transplanted cancer cells metastasis and growth of xenograft. Phosphorylation inhibition of ERK, GFR and Akt are identified due to the presence of T. chebula by Western blotting technique. Demonstration of signaling cascade regulation of Akt/ERK/EGFR associate with mechanism of antineoplastic. T. chebula considered as antineoplastic agent to prevent the lung cancer cell lines with appropriate antiproliferative and antimetastatic activities.
KEYWORDS: *T. chebula*, aqueous extract, lung cancer (A-549), apoptosis.

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INTRODUCTION

The plant and plant extracts are widely used for the treatment of diseases from prehistoric times. In the world, the herbal pant extracts with medicinal property were used for the treatment of ailments. Majority of the drugs have side effects Side effects of several allopathic drugs and development of resistance to currently used drugs have led to increased emphasis on the use of plant materials as a source of medicines for a wide variety of human ailments. The World Health Organization estimates that 4 billion people (i.e., 80% of the World's population) use herbal medicines in some aspects of primary healthcare, and there is a growing tendency to "Go Natural" [1-5].

Chemotherapeutic drugs against cancer were derived from the resource of plant [6,7]. *Terminalia chebula* (Gaertn.) Retz. (Combretaceae), is a most relevant medicinal plant, which commonly present in the tropical area.It is also used for the production of formulations to treat diseases. It is used in the all the stream of Indian medical systems including Ayurveda, Siddha, Unani, and Homeopathy.[8,9]It includes the creation of excrescences which is in a gall like structure, by plant insect *Dixothrips onerosus* (Thysanoptera) on the leaves, petioles and branches [10]. The galls are also called Karkatshringi, which is a most probable ayurvedic drug used for the production of medicines. Which includes dasamularista, cyavanaprasa, and shringyadi churna. [8,9]. The application of treatment in suppression of heamorrhage

from gums, bleeding from nose and infection in ear of children's were recognized by Karkatshringi. [9-13]

T. chebula extract was identified some properties such as ; antioxidant, anti-inflammatory, antibacterial, anti-tyrosinase, and anti-aging activities [14]. In an earlier study it was found that the aqueous seed extract of *T. chebula* possessed potent anthelmintic activities [15]. Henceforth, in the present study, the cytotoxic potential of extract of *T. chebula* is evaluated to exemplify its further potential use and develop it as an anticancer agent. *T. chebula* were used as a traditional medicine and also used for the treatment of many verity of diseases and infections. Different papers and studies based on *T. chebula* indicate that, it can used as a anticancer agent. Which has the capacity to treat cancer cell lines in prostate, colon, lymph, pancreas, breast, endometrial, cervical and ovarian cancer [16]. *T. chebula,* it has same application in lung cancer treatment, it is not yet reported in the recent studies. So here we are evaluating the application and importance of *T. chebula* in lung cancer cell lines by *in vitro* and *in vivo.*

MATERIALS AND METHODS

Plant material:

T. chebula dried seed were collected from the herbal market at Trivandrum , Kerala, India. The seed taken and cleaned well. The seed were discarded then the pericarp were crushing well and make it as a fine powder with the help of electric grinder.

Preparation of extract:

The fine powder was extracted by water in Soxhlet apparatus for 24 h. The solvent was evaporated under reduced pressure using rotary vacuum evaporator and a portion of the residue was used for the assay.

PHYTOCHEMICAL ANALYSIS:

Alkaloids:

0.2g of extract was re extracted with 1% HCl for 24hours. 2ml portion of the filtrate was taken and tested for alkaloids by adding drops of Meyer's reagent. Alkaloids formed a pieric yellow precipitate with the reagent.

Terpenoids:

To 0.5g of the extract of *T. chebula* were mixed with 2ml of chloroform. 3 ml of concentrated sulphuric acid was added carefully, a layer is formed. A reddish brown colour formation at the interface indicate the positive result (the presence of terpenoids).

Flavonoids:

Dilute Ammonia (5ml) was mixed with the extract then Conc. H_2SO_4 (1ml) was added. Yellow colour turning to colourless is an indication of positive result.

Saponins:

The extract of 0.5 g were mixed in a test tube containing 5 ml of distilled water. The contents in the test tube were shaken well. A persistent froth is formed at the top of the solution. Then 3 drops of olive oil were added to the tube and mixed well, the emulsion formation were observed.

Tannins:

In a test tube containing 10 ml of water, into that add extract of 0.5g and then boiled, filtered. For precipitation of blue-black or brownish green color by adding few drops of ferric chloride having 0.1%.

Coumarins:

Few mg of extract were diluted in 2ml of ethanol and then few drops of alcoholic NaOH solution added and observed for appearance of yellow colour.

Cardiac glycosides (keller-killani test):

0.5 g of extract mixed with 5 ml of distilled water, then it mixed with 2ml of glacial acetic acid with one drop of ferric chloride solution. Then 1 ml of concentrated sulpuric acid added to it, a brown ring is formed at the interface which indicate the presence OD a deoxy sugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring and gradually spread throughout this layer.

Quantitative analysis

Determination of total phenolic content:

The total phenolics content in the aqueous extract of *T. Chebula* seed was determined using method with FC reagent, standard solution is gallic acid. Aqueous extract having 200 µl were make up to distilled water having 3 ml. Then add FC reagent having 0.5 ml. Continue the mixing process for three minutes, then mixed with 20 % of sodium carbonate of 2 ml. Kept it for one hour in dark. At 650 nm the absorbance of the solution were obtained. The outcome of this study were mentioned in the unit mg of dry weight.

Total flavonoid and phenolic content determination:

Total flavonoid content in the aqueous seed extract of *T. Chebula* was determined using the aluminum chloride colorimetric method. 50 microliter of aqueous extract is added to methanol, volume of 1ml. Then mix with distilled water having 4ml and then with solution of 5% Sodium nitrite of 0.3 ml. Then it incubated for 5 minutes, after that 0.3 ml of 10% Aluminium chloride solution added to the mixture, then kept it for 6 minutes. Subsequently mixing 2 ml of one molar Sodium hydroxide to the mixture. Then make up with few ml of distilled water, kept it for 15 minutes. At 510 nm the absorbance of the reaction mixture have to be measured. From the curve of calibration, calculation of total content of flavonoid were done as quercetin. The formation of calibration curve, by forming solutions of quercetin in methanol at concentrations of 12.5–100 mg/ml. Expression of the outcome as mg of QUE/g of dry weight.

Folin Ciocalteu's method used to identify the total phenolic content. 1 ml of aliquots and various concentrations of standard gallic acid including 10 µg/ml, 20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml, 100 µg/ml were taken in the test tubes. Then 5 ml of distilled water and 0.5 ml of Folin Ciocalteu's reagent added and mixed well, kept it for 5 minutes. Added 1.5 ml of 20% Sodium carbonate , then made up to 10 ml with deaminized water. Then it subjected to incubation for 2 hrs at room temperature, a blue colur wee imparted. After the incubation period, the absorbance of the reaction mixture were measured at 750 nm using spectrophotometer, were standard is gallic acid. Plotting the curve of calibration with standard gallic acid (Patel *et al.,* 2010).

In-vitro Anticancer Activity:

From NCCS at Pune, India 549 cell line was procured and the maintenance of the cell line in Eagles medium of Dulbecos modified. Culturing of the medium DMEM containing antibiotics like Penicillin, Streptomycin, and Amphoteracin B, sodium bicarbonate, 10% FBS and L-glutamine in flask of tissue culture having 25 cm2. Keeping of the cell lines which is cultured in a 5% CO2 humidified incubator at 370C. Using phase contrast microscope which is inverted and then followed by assay of MTT, evaluation of cell viability were done via direct observation.

Cell seeding in 96 well plates:

Trypsinization were done with two days old monolayer of cell. These cells placed in 10% culture medium. 100µl cell suspension were seeded in 96 well tissue culture plate. Then it incubated in incubator which humidified with 5% CO_2 at 37 ºC.

Cytotoxicity Assay by MTT Method:

Different concentrations of (0–1000 µg/ml) *T. chebula* extract were introduced and incubated at 72 h. After 72 h, the drug solutions in the wells are discarded and MTT of 50µL in PBS was transfered to each well and to the control. The plate have to be incubated at 37ºC for 4 hours in humidified incubator after the gently shaken the plate. After completion of incubation process, Solubilization Solution of MTT were added when the removal of supernatant were done. And mix the solution by pipetting for formazan crystal solubilization. At 570 nm the absorbance were measured utilizing micro plate reader (Laura B. Talarico *et al.,* 2004). Using the formula cell viability of percentage were calculated.

% cell viability= mean OD treatment/mean OD control *100

In Vitro Cell Growth Assay:

Lung cancer cell lines with density of 1 × 104 cells per well were taken in a 96-well plates incubated about 24 hours for attachment process and it will then cultured with different drug concentrations which is increased such as 0 μg/mL, 50 μg/mL, 100 μg/mL, and 150μg/mL , incubated for 48 hours. Viability of human lung cancer cell line (A-549**)** were obtained by CCK-8 assay based on the procedure denoted by the manufacturer. Microplate reader were used to detect the Optical Density (OD) at 540 nanometer. Inhibition rate were calculated by sing this equation:

Inhibition rate of Growth in percentage = (1 - experimental group Absorbance / control group Absorbance) × 100%.

With the help of 19.0 software of SPSS Statistics, evaluate values of IC50 were done. Based on the instructions of manufacturer, using fluorescence probes of Calcein- AM/Propidium Iodide, there is a observation of cytotoxic activity of *T. chebula* were done.

Assay of Cell Migration in In-Vitro Method:

The migration capacity of A-549 were identified by Transwell Boyden chamber assay. During the culturing of the cell, the cells in the logarithmic growth phase was taken. Them it resuspended in serum-free HDMEM medium. And at 5 × 104 cells per well density, cells were seeded in Transwell plates of 24-well, in upper chamber. Into the lower chamber, normal medium having 10% of FBS and H-DMEM are introduced. Removal remaining cells in upper side of the membrane using swabs of cotton only after 37°C incubation period of 24 hours. At the same time migration of cells to bottom side of well via micropore

membrane and it is then stained with crystal violet of 0.1%. At 630 nm, values of optical density were calculated. The following equation is about the inhibition rate of migration:
Inhibition rate of Migration (%) = (1 – experimental group Absorbance / control group Absorbance) × 100%.

Assay of Western Blot:

Culturing of A-549 cells in Petri dishes of 10 cm and kept for growing process to approximately half confluence. Which is then mixed with extract of T. chebula, in different concentrations for 48hours. Lysis of Cells which is collected in RIPA buffer having cocktail of phosphatase inhibitor and protease. Separation of Cell lysates with the help of Sodium Dodecyl Sulfate –Poly Acrylamide Gel Electrophoresis and it was then transported through a membrane of nitrocellulose. Probing of cell with the help of p-ERK1/2, monoclonal antibodies of p-AKT and p-EGFR, which is then incubated with the presence of secondary antibodies, depends on instructions of manufacturer, enhanced chemiluminescence were used for detection process.

Zebrafish Embryos:

Wild type AB strain of zebrafishes those are adult and under standard particular laboratory environment was maintained and their embryos are multiplied by natural mating pairwisely. Two days post fertilized embryos are anesthetized with sedating compound tricaine and for microinjection process which is then positioned in a Petri plate.

Antitumor Assay in In-Vivo method:

In buffer solution of phosphate, resuspended the cancer cells of human lung and with CM-Dil incubation process were done, having final concentration at 37°C, for four minutes, which is followed that at 4°C for 15 minutes. Loading of cells labeled with fluorescent, into the capillary needles and using injector of nanoliter, cells were injected into embryos of zebrafish, perivitelline space of abdominal. Transfer of embryos having tumor to plate having 24-wells and acclimation in embryo water for 24h at 35°C. Extract of T. chebula is then incubated for 48 hours at 0, 50, 100, and 150 μg/mL. Utilizing fluorescence inverted microscope, observation of metastasis and growth of tumor were done and imaged. With the help of software of ImageJ, analyze the intensity of mean fluorescence (MFI). Calculation of inhibition rate of growth was calculated using the equation:
Rate of Inhibition (%) = (1 - experimental group MFI / control group MFI) × 100%.
The expression of migratory inhibition activity in In-vivo method of T. chebula as metastasis occurrence rate. The inhibition rate of metastasis is calculated using the following equation:
Rate of Inhibition (%) = (1 – experimental group incidence / control group incidence) × 100%.

RESULTS AND DISCUSSION

Plant material/ Chemicals and reagents:

From local herbal market the dried seeds of TC were acquired. All the chemicals and solvents were imported from Merck in Mumbai, which is used in research experiments. Beacon in Gujarat will provide the kits of Biochemical assay which is used in studies of toxicity.

Extraction from TC:

Crush the pericarp of the collected seed only after cleaning and seeds removing process. The pericarp were grinded into thin powder. Using water, extraction of pericarp powder by continuous stirring at 80 °C for one hour. *T. Chebula extraction was prepaid by* aqueous extraction method. With the help of rotary vacuum evaporator under low pressure the solvent of the sample were evaporated. After that the extract solution was checked by TLC, the dry seed and powdered form of pericarp were given in image 1 (a) and (b).

Phytochemical analysis of Terminalia chebula:

Qualitative phytochemical screening of the pericarp, revealed that proteins, flavanoids and tannins were present in aqueous extract of *T.* The result as in table 1 show that these plants rich in bioactive compounds and hence is a potential source of therapeutic properties.
The present study indicated that the T. chebula Retz. contained different types of phytochemicals such as alkaloids, flavonoids, saponin, phenolic compounds, steroids, carboxylic acid, tannin and glycoside. Presence of phytochemicals differed in different type's solvent in the seed extract. Similar result was obtained by different researchers in different plants. Alkaloids reacted with Dragendroffs reagents to produce reddish brown precipitate, indicate the presence of alkaloids in the pericarp extract (Table 1). Ferric chloride reacted with seed extract and formed blue colour precipitate. The above result indicated the presence of tannins in the extract of T. chebula Retz. (Table 1) Glycosides reacted with - napthol and sulpuric acid to form brick red colour. (Table 1) Steroids reacted chloroform, acetic anhydride and concentrated sulphuric acid to produce rosy red colour, indicated the presence of steroids. Saponin

reacted with mercuric chloride to produce white precipitate as positive result (Table1). Flavonoids reacted with ferric chloride to form blackish red colour confirm the presence of flavonoids (Table 1). Quinine reacted with alcoholic NaOH solution to colour change from red to blue (Table 1). Phenolic compounds reacted with neutral.

The previous work of Saleem *et al*.,[19] and Lee *et al*.[18] provide that the species *Terminalia* having compounds of phenolic which are rich group of hydroxy are responsible for the activity of cytotoxicity. In our study, the aqueus extract of the *T. chebula* is also found to be rich in phenolics (439 ± 7.2 mg/g). the several actions of pharmacology such as apoptosis, pro-oxidant toxicity, free radical scavenging and antioxidant were shown by phenolic compounds, which is present in different plants. [20,21] The cytotoxic effects of the phenolic compound against various cell lines were shown to be higher than the gallic acid used.[18] However, some studies have shown that most of the phenolic compounds have same cytotoxicity effect as gallic acid on cancer cell lines.[22,23] Further, it has been demonstrated that the phenolic compounds in polar extracts are shown to have inhibition of proliferation and induce cell death by apoptosis and necrosis.[22] The results of this study show that the phenolic and flavonoid content which is higher in the aqueous extract of gall extracts of *T. chebula* had significant cytotoxic potential, thus could act as lead for the development of anticancer agents. These observations vindicate the relationships that exist between peoples and plants for the exploration of novel bioactive compounds for therapeutic application. The identified quantity of phenol and flavonoid were mentioned in table 2.

Cytotoxicity Assay by MTT Method

Assay of MTT is a colorimetrical method for the determination of cell viability and cell proliferation. It relies on the ability of an enzyme named mitochondrial dehydrogenase from living cells for converting the substrate which is water soluble, MTT into a formazan product having dark blue color, which is insoluble in water. When the aqueous extract of *T. chebula* was screened for their cytotoxic (anticancer) effect against A-549 (Human lung cancer cell line) cell lines, it was found that the extract was potent and effective in inducing cytotoxic effect at increasing concentration of the extract tested [table 3]. The extract was more effective against A549 cell lines.

In Vitro Cell Growth and Migration Assay

CCK-8 and Transwell assay were done to determine the properties of antimigratory and in vitro antitumor activity of *Terminalia chebula*. A dose-dependent growth inhibition of human lung cancer cell line (A-549) with an IC50 value is 84.8±2.87 μg/mL (Figure; 2(a)) were acquired through the quantitative CCK-8 assay. Demonstration of negative relation between ability of A-549 cells migration in vertical and *Terminalia chebula* concentration, using migration assay of transwell (Figure 2(b)).

T. chebula Inhibited Tumor Growth and Metastasis in the Zebrafish Xenogra Model.

Activities of antimetastatic *and in vivo* anticancer *activity* of *T. chebula* were experimented in A-549 cell lines of xenograft model. Using software of image analysis localization of tumor cells which are fluorescence labelled and also their intensities was determined. Fish bodies treated with *T. chebula* had a reducing intensity of fluorescence than the increasing dose of *T. chebula* coorelate positively with control group (Figure; 3 and 4(a)). Merely, with increasing concentration of *T. chebula* a decreasing quantity of metastasis were detected in tumor bearing zebrafishes (Figures 3 and 4(b)). From these results we can conclude that *T. chebula* has the ability of mediation of metastasis and growth of tumor in-vivo dose-dependent manner.

Western bloat assay

In most human cancers functional activation of EGFR occurs. It plays a major part in mediating cancer cells migration and proliferation. Western blotting technique is employed to identify the *T. chebula* effect on signaling pathways of EGFR. After the treatment with different concentrations of *T. chebula* there is a reduction in p-ERK1/2, p-EGFR and p-Akt levels were observed (Figure 5). This shows the EGFR/Akt/ERK signaling pathway suppression by the anticancer activity of T.chebula.

In India and Iran *T. chebula* can be taken as a traditional medicine. *T. chebula* powder is a good dentifrice in loose gums, bleeding and ulceration in gums. It is also used to increase appetite, digestive aid, liver stimulant, gastrointestinal optokinetic agent and mild laxative. *T. chebula* also possess many biological properties like activity of antimutagenic , anticancer, antibacterial, antiviral, antidiabetic and antifungal. Different products from *T. chebula* is also used to inhibit bacterial growth and metastasis. In TCM, the concentrated mixture of *T. chebula* (boiling with water) was primarily used. At low temperature, extract of T. chebula was maked in water for this study. In cell line of lung cancer, extract of *T. chebula* used as a programmed cell death inducer.

Detection of the aqueous solution of extract can inhibit the growth of cancer cells and also cause morphological changes in lung cancer cell lines. Results showed from the assay of MTT, relative cell viability and cytotoxicity was time- and dose-dependent. As narrated in the introduction, *T. chebula* has

the properties that is much safer and should be re comparatively admirable to common chemotherapeutic agents. From this study it is evident that *T. chebula* is safe to use and can be used as an adjunctive and alternative medicine.

The reason behind the effect of synergistic of *T. chebula* is because of the signaling pathways regulation. In association with signaling pathway of ERK/EGFR/Akt , mechanism of anticancer activity were identified. Actually for understanding the T. chebula mechanism in molecular level can be done by further research. In this study, a single cell line is used; therefore, the further studies having an additional cell is needed for validation. Also in this project we done limited number of *in vivo* experiments. Toxicology and pharmacology activity can be well known by doing several studies against to the cancer cell line of human lungs by the *T. chebula* extract. Given that solution of *Terminalia chebula* in treatment of lung cancer play an important role as a medicine of complementary. In vivo and in vitro method in cancer cells of lung, *T. chebula* can act as agent of anticancer having better effects on metastasis and proliferative. Using *T. chebula* extract proliferations of cell were inhibited by making arrest of A549 cell line and programmed cell death prompting. Anticancer property of *T. chebula* is identified from these studies. However, analysis of fundamental mechanism, properties of toxicology and pharmacology were done in future research.

Terminalia chebula	**Extract(Aqueous)**
Alkaloids	Positive
Terpenoids	Positive
Flavonoids	Positive
Saponins	Positive
Tannins	Positive
Coumarins	Positive
Steroids	Positive
Cardiac glycosides (keller-killani test)	Positive

Table 1: Preliminary qualitative phytochemical analysis of aqueous extract of *Terminalia chebula*

Determination of total phenolic and total flavonoid content

Extract	**Total phenolics (mg of GAE/g d.w)**	**Total flavonoids (mg of QUE/g d.w)**
Aqueous	439±7.2	490±3.4

Table 2: quantitative analysis of aqueous extract of *T. Chebula*. Results represent means±SD (*n*=3). SD: Standard deviation; GAE: Gallic acid equivalent; QUE: Quercetin equivalent

Sample Concentration (µg/ml)	**Average OD at 570nm**	**Percentage Viability**
Control	0.5223	-
6.25	0.5177	99.1
12.5	0.5093	97.5
25	0.5001	95.7
50	0.4932	94.4
100	0.4124	78.9

Table 3: *In vitro* anticancer activity of *Terminalia chebula* extract. Cytotoxic activity of aqueous extract of *Terminalia chebula* on A 549 cell lines. Cell viability was determined by the MTT method

Figure 1: (a) dry seeds of Terminalia chebula; (b) powdered form of seed pericarp

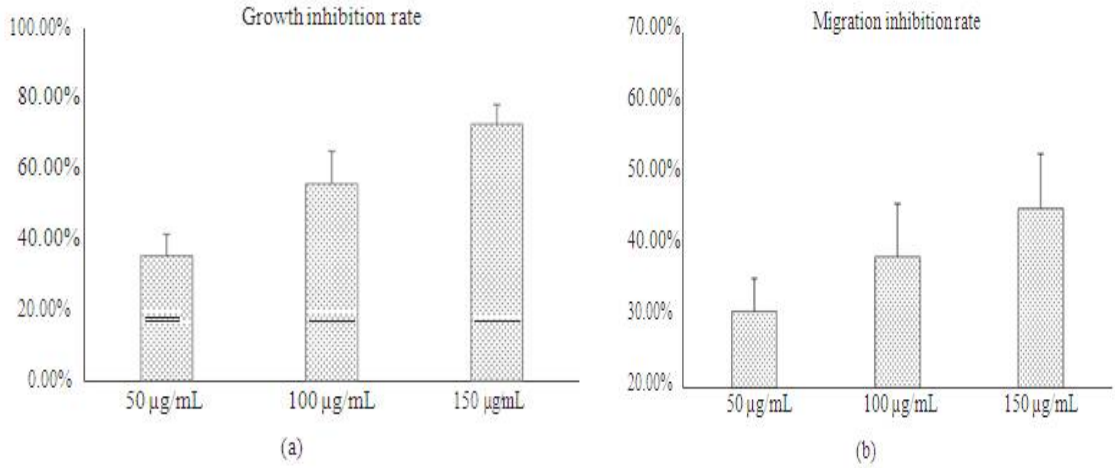

Figure 2: Antitumor activity in *In-vitro* method of *Terminalia chebula* extract in cell line (A-549) of human lung cancer. (a) inhibition rates of Growth of A-459 after exposure of 48 h to *Terminalia chebula* extract, acquired through the quantitative assay of CCK-8. (b)Transwell assay, inhibition rates of Migration of tumor cells with *Terminalia chebula* extract.

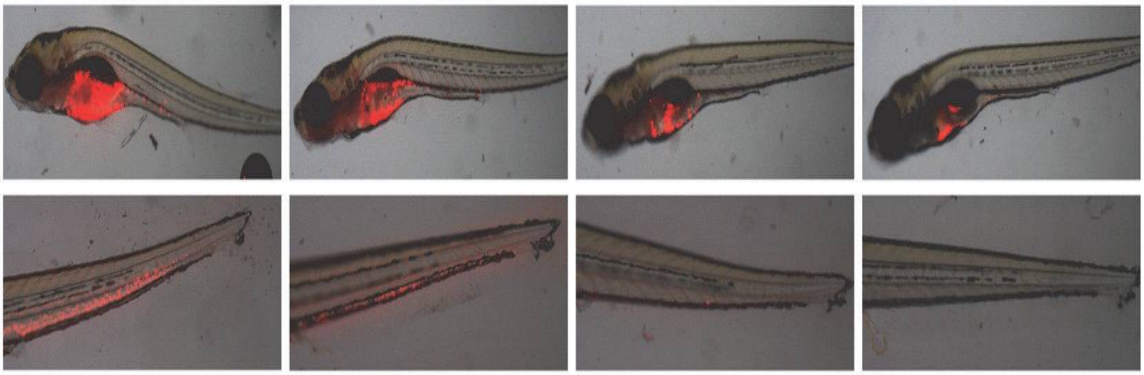

Figure 3: Images of fields of microscopic, cells of lung cancer stained using Calcein-AM/PI after treatment of 48 hours having various concentrations of *T. chebula*

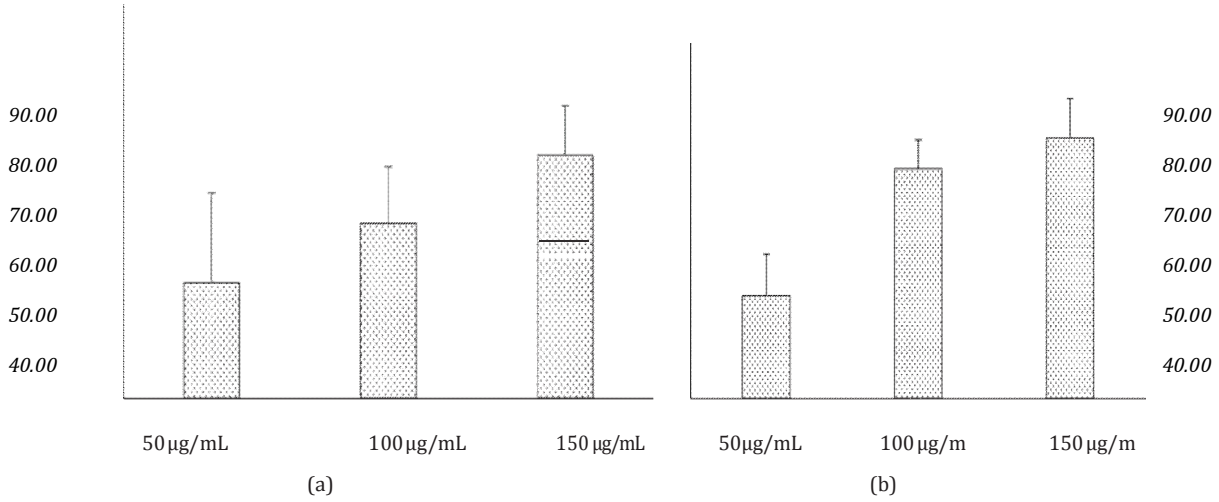

Figure 4: Quantitative CCK-8 assay showing tumor cells growth inhibition rate after 48 h exposure to T. chebula. (c)Transwell assay showing rate of inhibition of migration of tumor cells with administration of *T. chebula.*

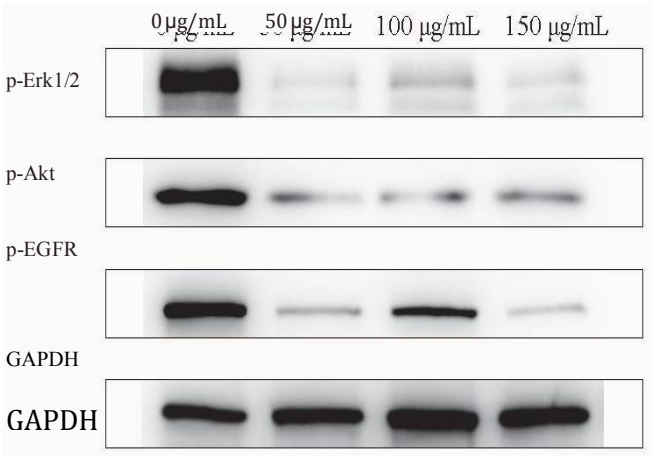

Figure 5: EGFR signaling cascade of *T. chebula*. Comparison of untreated lysates and treated with T. chebula A-549 cells with p- ERK1/2, p-AKT monoclonal antibodies and p-EGFR. For Western blotting an antibody of anti-GAPDH taken as a control.

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