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# "EVALUATION OF INVITRO ANTICANCER ACTIVITY OF *OXALIS LATIFOLIA KUNTH* AGAINST MCF – 7 AND HT – 29 CELL LINES"

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## ABSTRACT

Cancer is a disease characterized by uncontrolled proliferation of cells that have transformed from the normal cells of the body. The medicinal properties of plant have been investigated in the light of recent scientific developments throughout the world, due to their potent pharmacological activities, low toxicity and economic viability. Hence in our present study, ethanolic and aqueous extracts of *Oxalis latifolia Kunth* have been tested for its invitro anticancer activity by Tryphan blue method and MTT assay against MCF-7 and HT-29 cell lines. Both EEOL and AEOL showed significant activity against MCF-7 & HT-29 cell lines in a dose dependent manner. These results provide promising baseline information for the potential use of ethanolic and aqueous extract of whole plants of *Oxalis latifolia Kunth* in the treatment of cancer.

**KEYWORDS:** Cancer cell lines, *Oxalis latifolia Kunth*, Tryphan blue, MTT Assay.

## INTRODUCTION

Cancer is a group of diseases in which cells are aggressive (grow and divide without respect to normal limits), invasive (invade and destroy adjacent tissues), and/or metastatic (spread to other locations in the body). These three malignant properties of cancers differentiate them from benign tumors, which are self-limited in their growth and do not invade or metastasize (although some benign tumor types are capable of becoming malignant). Cancer may affect people at all ages, even fetuses, but risk for the more common varieties tends to increase with age. Cancer causes about 13% of all deaths. Apart from people, forms of cancer may affect animals and plants.

### Human Cancer Cell Lines

Human immortal cancer cell lines (residents of cells from a multi-cellular organism which would normally not proliferate indefinitely but, due to mutation, have evaded normal cellular senescence and instead can keep undergoing division) have aggregated an accessible, easily usable set of biological models with which to examine cancer biology and to analyze the inherent efficacy of anticancer (natural, synthetic) drugs. Drug resistance is one of the hefty hindrances to chemotherapy of cancer. Studies with cell lines can serve as an initial screen for agents that might regulate drug resistance. Large quantities and volumes of cells may be propagated to create high-throughput studies. Cell lines are exceptionally versatile in the types of studies they may be used in. Not only can they build in-vitro but can also be injected into mice to form xenograft models of prostate cancer progression. They can be transformed and reviewed over time to dispose sequential events that occur as a result of specific stimulus. As well as the products produced from the cells such as their secretome can be analyzed readily.

**Types of Human Cancer Cell Lines**
**List and Description of NCI**

| Name | Species | Organ of Origin | Disease |
|---|---|---|---|
| BT549 | Human | Breast | Ductal Carcinoma |
| MCF7 | Human | Breast | Adenocarcinoma |
| SF539 | Human | CNS | Glioblastoma |
| SNB-75 | Human | CNS | Astrocytoma |
| HCC 2998 | Human | Colon | Carcinoma |
| HT29 | Human | Colon | Colorectal adenocarcinoma |
| A498 | Human | Kidney | Adenocarcinoma |
| ACHN | Human | Kidney | renal cell adenocarcinoma |
| CCRF-CEM | Human | Leukemia | acute lymphoblastic leukemia |
| K562 | Human | Leukemia | chronic myelogenous leukemia |
| A549 | Human | Lung | Adenocarcinoma |
| HOP-92 | Human | Lung | Large Cell, Undifferentiated |
| NCI-H322M | Human | Lung | Small Cell Bronchioalveolar Carcinoma |
| MALME-3M | Human | Melanoma | malignant melanoma |
| MDA-MB-435 | Human | Melanoma | Adenocarcinoma |
| IGROV1 | Human | Ovary | Cystoadenocarcinoma |
| OVCAR-3 | Human | Ovary | Adenocarcinoma |
| DU145 | Human | Prostate | Carcinoma |
| PC-3 | Human | Prostate | grade IV, adenocarcinoma |

**MCF-7 Cells: (human breast adenocarcinoma) cell lines**

MCF-7 is a cell line that was first isolated in 1970 from the breast tissue of a 69-year old Caucasian woman. Of the two mastectomies she received, the first revealed the removed tissue to be benign. Five years later, a second operation revealed malignant adenocarcinoma in a pleural effusion from which was taken cells for MCF-7. The woman was treated for breast cancer with radiotheraphy and hormonotherapy. MCF-7 cells are useful for in vitro breast cancer studies because the cell line has retained several ideal characteristics particular to the mammary epithelium. These include the ability for MCF-7 cells to process estrogen, in the form of estradiol, via estrogen receptors in the cell cytoplasm. This makes the MCF-7 cell line an estrogen receptor (ER) positive control cell line.

**HT-29: (Human Colorectal Adenocarcinoma) Cell Lines**

**Source**

This cell line was established in 1964 from the primary tumor of a 44-year-old Caucasian female with colorectal adenocarcinoma.

**Description**

HT-29 is a human colorectal adenocarcinoma cell line with epithelial morphology. These cells are sensitive to the chemotherapeutic drugs 5-fluorouracil and oxaliplatin, which are standard treatment options for colorectal cancer. In addition to being a xenograft tumor model for colorectal cancer, the HT-29 cell line is also used as an *in-vitro* model to study absorption, transport, and secretion by intestinal cells. Under standard culture conditions, these cells grow as a non polarized, undifferentiated multilayer. Altering culture conditions or treating the cells with various inducers, however, results in a differentiated and polarized morphology, characterized by the redistribution of membrane antigens and development of an apical brush-border membrane.

**Aim of the Present Study**

The imbalance between oxidants and antioxidants in the body leads to oxidative stress that is being suggested as the root cause of ageing and various life threatening disease like cancer.

Based upon ethno pharmacological survey, the whole plants of *Oxalis latifolia Kunth* has been selected to prove scientifically its in-vitro anticancer activity.

**MATERIALS AND METHODS**

**Collection and Authentication of Plant Material**

The whole plants of *Oxalis latifolia Kunth* were collected from the foot hills Yercaud, Salem district in the month of June – 2018. The plant was then taxonomically identified and authenticated by the Botanist Dr. Kumaresan, The authenticated plant material was used for preparation of extracts.

**Preparation of the Extract**

The whole plants of *Oxalis latifolia Kunth* were collected and air dried under shade condition. The dried whole plants were coarsely powdered using mechanical grinder. The powder was then passed through were sieve no.40 and stored in an airtight container for further extraction.

The collected, cleaned and powdered material of whole plants of *Oxalis latifolia Kunth* were used for extraction purpose above 800gms of powdered material was evenly packed in a soxhlet apparatus. It was then extracted with various solvents from non-polar to polar such as

petroleum ether, ethanol and aqueous successively. The solvents used were purified before use. The extraction method used was continuous hot percolation and carried out with various solvents, for 72 Hrs. The aqueous extraction was carried out by cold-maceration process.

**Petroleum ether extract of whole plants of *Oxalis latifolia Kunth***

The shade dried coarsely powdered whole plants of *Oxalis latifolia Kunth* (800gm) was extracted with petroleum ether (60-80˚C), for 72 hrs. After completion of extraction, the defatted extracts were filtered while hot through whatmann filter paper (No.10) to remove any impurities if present. The extract was concentrated by vacuum distillation to reduce the volume to 1/10; the concentrated extract was transferred to 100ml beaker and the remaining solvent was evaporated on a water bath. Dark greenish brown colored extract was obtained. The concentrated extract was then kept in a desiccator to remove the excessive moisture. The dried extract packed in air tight glass container for further studies.

**Ethanolic extract of whole plants of *Oxalis latifolia Kunth***

The main marc left after pet ether extraction was dried and then extracted with ethanol 95% v/v (75-78˚C), for 72 hrs. After completion of extraction, the solvent was removed by distillation. Dark greenish colored extract was obtained. The extract was then stored in a desiccator to remove the excessive moisture. The dried extract was then packed in an air tight glass container for further studies.

**Aqueous extract of whole plants of *Oxalis latifolia Kunth***

The marc left after ethanol extraction was again dried and then macerated with distilled water in a 2 litres round bottom flask for 72 hrs and 10 ml of chloroform was added to avoid fungal growth. After completion of extraction, it was filtered and the solvent was removed by evaporation to dryness on a water bath. Brown coloured extract was obtained and it was stored in a desiccator to remove the excessive moisture. The dried extract was packed in air tight glass container for further studies. The percentage yields of the above extracts were expressed in Table no.1.

**Table No. 1: Extractive Values of Whole Plant of *Oxalis Latifolia Kunth.***

| Plant name | Parts used | Method of extraction | Yield in percentage | | |
|---|---|---|---|---|---|
| | | | Petroleum Ether | Ethanol | Aqueous |
| *Oxalis latifolia Kunth* | Whole parts of the plant | Continuous Hot Percolation and Cold Maceration | 5.6 | 9.3 | 15.6 |

**Preliminary Phytochemical Studies**

The extracts obtained (petroleum ether, Ethanol and Aqueous) were subjected to the following preliminary phytochemical studies.

**Test for Alkaloids**

a. **Dragendorff's test:** To 2mg of the extracts, 5ml of distilled water was added; 2M Hydrochloric acid was added until an acid reaction occurs. To this 1ml of Dragendorff's reagent was added. Formation of orange or orange red precipitate indicates the presence of alkaloids.
b. **Hager's test:** To 2mg of the extracts were taken in a test tube, a few drops of Hager's reagent was added. Formation of yellow precipitate confirms the presence of alkaloids.
c. **Wagner's test:** 2mg of extract were acidified with 1.5% v/v of hydrochloric acid and a few drops of Wagner's reagent were added. A yellow or brown precipitate indicates the presence of alkaloids.
d. **Mayer's test:** To a few drops of the Mayer's reagent, 2mg of extracts were added. Formation of white or pale yellow precipitate indicates the presence of alkaloids.

**Test for Carbohydrates**

a. **Anthrone test:** 2mg of extracts were shaken with 10ml of water, filtered and the filtrate was concentrated. To this 2ml of anthrone reagent solution was added. Formation of green or blue color indicates the presence of carbohydrates.
b. **Benedict's test**: 2mg of extracts were shaken with 10ml of water, filtered and the filtrate was concentrated. To this 5ml of Benediet's solution was added and boiled for 5minutes. Formation of brick red colored precipitate indicates the presence of carbohydrates.
c. **Fehling's test:** 2mg of extracts were `shaken with 10ml of water, filtered and the filtrate was concentrated. To this 1ml mixture of equal parts of Fehling's solution A and B were added and few minutes. Formation of red or brick red colored precipitate indicates the presence of reducing sugar.
d. **Molisch's test:** 2mg of extracts were shaken with 10ml of water, filtered and the filtrate as concentrated. To these 2 drops of freshly prepared 20% alcoholic solution of α – naphthol was added. 2ml of conc. sulphuric acid was added so as to form a layer below the mixture. Red –violet ring appear, indicating the presence of carbohydrates which disappear on the addition of excess of alkali.

**Test for Flavonoids**

a. **Shinoda's test:** 2mg of extracts were dissolved in 5ml of ethanol and to this 10 drops of dilute hydrochloric acid followed by a small piece of magnesium were added. Formation of pink, reddish, or brown color indicates the presence of flavonoids.
b. **With conc. sulphuric acid test:** Yellow orange color (anthocyanins), yellow to orange color (flavones) and orange to crimson (flavonones).

**Test for Glycosides**

a. **Molisch's test:** 2mg of extracts were shaken with 10ml of water, filtered and the filtrate was concentrated. To these 2-3drops of molischs reagent was added, mixed and 2ml of conc. Sulfuric acid was added carefully through the side of the test tube. Reddish violet ring appears, indicating the presence of glycosides.

**Test for Proteins and Free Amino Acids**

Small quantites of the extracts were dissolved in few ml of water and treated with following reagents.

a. **Million's reagent:** Appearance of red color shows the presence of protein and free amino acid.
b. **Ninhydrin test:** Appearance of purple color shows the presence of proteins and free amino acids.
c. **Biurets test:** Equal volumes of 5% sodium hydroxide solution and 1% copper sulphate solution was added. Appearance of pink or purple color shows the presence of proteins and free amino acid.

**Test for Gums and Mucilages**

**Precipitation with 95% alcohol:** Small quantities of the extract were added separately to 25ml of absolute alcohol with constant stirring and filtered. The precipitate was dried in air and examined for its swelling properties for the presence of carbohydrates.

**Test for Saponins**

**Foam test:** In a test tube containing about 5ml of extracts, a drop of sodium bicarbonate solution was added. The test tube was shaken vigorously and left for 3 minutes. Formation of honeycomb like froth indicates the presence of saponins.

**Test for Sterols**

a. **Liebermann- Burchard's test:** 2mg of dry extracts were dissolved in acetic anhydride, heated to boiling, cooled and then 1 ml of concentrated sulphuric acid was added along sides of the test tube. Formation of green color indicates the presence of steroids.
b. **Salkowski Reaction:** 2 mg of dry extracts were shaken with chloroform, to the Formation of red color indicated the presence of steroids.

**Test for Fixed Oils**

**Spot test:** Small quantities of various extracts were separately pressed between two filter papers. Appearance of oil stain on the paper indicates the presence of fixed oil. Few drops of 0.5N alcoholic potassium hydroxide were added to a small quantity of various extracts along with a drop a phenolphthalein. The mixture was heated on a water bath for 1-2 hours. Formation of soap partial neutralization of alkali indicates the presence of fixed oils and fats.

**Test for Phenolic Compounds and Tannins**

Small quantities of the extracts were taken separately in water and test for the presence of phenolic compounds and tannins was carried out with the following reagents.

- Dilute ferric chloride solution (5%)-violet color.
- 1% solution of gelatin containing 10% sodium chloride-white precipitate.
- 10% lead acetate solution-white precipitate

The results were presented in Table.No.2

**Table No. 2: Preliminary Phytochemical Studies of Extracts of Whole Plant of *Oxalis Latifolia Kunth.***

| S. No | Constituents | Tests | Petroleum Ether | Ethanol Extract | Aqueous Extract |
|---|---|---|---|---|---|
| 1. | ALKALOIDS | Mayer's test | - | + | + |
| | | Dragondroff's test | - | + | + |
| | | Hager's test | - | + | + |
| | | Wager's test | - | + | + |
| 2. | STEROLS | Libermann's Burchard test | - | + | + |
| | | Salkowski's test | - | + | + |
| 3. | CARBOHYDRATES | Molish reagent | - | + | + |
| | | Fehlings reagent | - | + | + |
| | | Benedict's reagent | - | + | + |
| | | Anthrone test | - | + | + |
| 4. | FIXED OILS AND FATS | Spot test | + | - | - |
| 5. | PHENOLIC COMPOUNDS | $FeCl_3$ | - | + | + |
| | | Gelatin test | - | + | + |
| | | Lead acetate test | - | + | + |

| | | | | | |
|---|---|---|---|---|---|
| 6. | PROTEIN AND AMINO ACIDS | Biuret test | + | - | - |
| | | Ninhydrin test | + | - | - |
| | | Xanthoprotein test | + | - | - |
| | | Millon's reagent | + | - | - |
| 7. | SAPONINS | Foam test | - | + | + |
| 8. | TANNINS | Gelatin test | - | + | + |
| | | $FeCl_3$ | - | + | + |
| 9. | GUM AND MUCILAGE | Precipitation with 95% alcohol | + | - | - |
| 10. | FLAVONOIDS | Shinoda's test | - | + | + |
| | | Conc. $H_2SO_4$ | - | + | + |
| 11. | GLYCOSIDES | Molisch's test | - | + | + |

**IN-Vitro Anti Cancer Studies**

Various *in-vitro* models were used to evaluate the anti cancer activity. Among those we have undergone with Tryphan Blue, MTT Assay methods.

**Tryphan Blue Exclusion Assay**

The cell lines MCF-7 & HT-29 were grown at 37˚c at humidified 5% $CO_2$ in RPMI 1640 medium. The RPMI 1640 medium was filtered using 0.45µm membrane filter. The cancer cells were them seeded at a density of $2\times10^4$ cells/well for the cytotoxicity assay. Different concentrations of the extracts (12.5-200 µg/ml) were added to each tubes and the final volume was adjusted to one ml with normal saline. Control tubes were kept with the saline, tumor cells and without the drugs.

All the tubes were incubated at 37˚C for 3hours. After incubation 0.1ml of 0.4% tryphan blue dye in isotonic saline was added to each tube and the number of viable (unstained) and dead (stained) cells were counted using haemocytometer.

$$\% \text{ Dead cells} = \frac{\text{Total cells counted} - \text{total viable cells}}{\text{Total cells counted}} \times 100$$

The results were presented in Table No. 3 & 4.

**Table No. 3: Effect of Ethanolic & Aqueous Extracts of *Oxalis Latifolia Kunth* on Mcf-7 Cell Lines by Tryphan Blue Exclusion Assay.**

| S.No | Treatment | Concentration µg/ml | Cell viability | | % of cell death |
|---|---|---|---|---|---|
| | | | Live cells | Dead cells | |
| 1 | EEOL | 12.5 | 199 | 45 | 19.23 |
| | | 25 | 197 | 67 | 27.46 |
| | | 50 | 184 | 98 | 36.30 |
| | | 100 | 168 | 114 | 42.70 |
| | | 200 | 135 | 135 | 57.45 |
| 2 | AEOL | 12.5 | 189 | 35 | 15.26 |
| | | 25 | 167 | 56 | 23.04 |
| | | 50 | 145 | 78 | 34.98 |
| | | 100 | 132 | 99 | 42.86 |
| | | 200 | 124 | 112 | 47.46 |

**Table No. 4: Effect of Ethanolic & Aqueous Extracts of *Oxalis Latifolia Kunth* on Ht-29 Cell Lines by Tryphan Blue Exclusion Assay.**

| S.No | Treatment | Concentration µg/ml | Cell viability | | % of cell death |
|---|---|---|---|---|---|
| | | | Live cells | Dead cells | |
| 1 | EEOL | 12.5 | 195 | 40 | 17.02 |
| | | 25 | 191 | 67 | 25.97 |
| | | 50 | 179 | 85 | 32.20 |
| | | 100 | 167 | 124 | 42.61 |
| | | 200 | 120 | 134 | 52.76 |
| 2 | AEOL | 12.5 | 190 | 35 | 14.89 |
| | | 25 | 184 | 42 | 22.03 |
| | | 50 | 187 | 81 | 30.22 |
| | | 100 | 158 | 114 | 41.19 |
| | | 200 | 130 | 125 | 49.02 |

**MTT ASSAY 3-(4, 5 - Dimethylthiazole-2-yl)-2, 5 – Diphenyltetrazolium Bromide**

**Principle**

This is a colorimetric assay that measures the reduction of yellow MTT by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilized with an organic solvent (eg. isopropanol) and the released, solubilized formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.

**Formazan-based viable cell mass assay (MTT assay)**

MCF-7&HT-29 cancer cell lines cells were treated with EEOL & AEOL and 5-FU (1 - 0.039µM). Cell viability was determined at 24 h based on MTT assay. Briefly, the cells were seeded in a 96-well plate at a density of 4×103 cells/well and allowed to adhere overnight. After removing the medium, 200 µL fresh medium per well, containing 10 mmol/L HEPES (pH 7.4), was then added. Then, 50 µL MTT was added to the wells and the plate incubated for 2 - 4 h at 37°C in the dark. The medium was removed, and 200 µL DMSO and 25 µL Sorensen's glycine buffer were added to the wells. Absorbance was measured using an ELISA plate reader at 570 nm.

The results were presented in Table No. 5 & 6.

**Table No. 5: Effect of Ethanolic & Aqueous Extracts of *Oxalis Latifolia Kunth* on Mcf-7 Cell Lines by Mtt Assay.**

| S.No | Concentration µg/ml | % Cell inhibition | | |
|---|---|---|---|---|
| | | EEOL | AEOL | Tamoxifen |
| 1 | 12.5 | 4.50 | 4.05 | 11.7 |
| 2 | 25 | 25.80 | 17.50 | 35.0 |
| 3 | 50 | 32.20 | 28.52 | 68.5 |
| 4 | 100 | 38.15 | 35.60 | 80.6 |
| 5 | 200 | 50.25 | 47.20 | 88.5 |

**Table No. 6: Effect of Ethanolic & Aqueous Extracts of *Oxalis Latifolia Kunth* on Ht-29 Cell Lines by Mtt Assay.**

| S.No | Concentration µg/ml | % Cell inhibition | | |
|---|---|---|---|---|
| | | EEOL | AEOL | 5-Flurouracil |
| 1 | 12.5 | 3.80 | 3.32 | 25.8 |
| 2 | 25 | 21.25 | 20.10 | 38.2 |
| 3 | 50 | 30.12 | 28.15 | 60.4 |
| 4 | 100 | 39.34 | 37.19 | 78.5 |
| 5 | 200 | 48.7 | 45.6 | 88.6 |

**RESULTS AND DISCUSSION**

Based on ethano pharmacology literature, the plant *Oxalis latifolia Kunth* was collected from in and around the foot hills Yercaud. The collected plant was identified and authenticated by a Botanist.

The shade dried coarsely powdered whole plants of *Oxalis latifolia Kunth* was extracted by using different solvents of increasing polarity by continuous hot percolation process using soxhlet apparatus and aqueous extracts by cold maceration method. Extractive values were presented in table no: 1.

The phyto constituents present in the various extracts were identified by performing chemical tests and the results were showed in Table No: 2. From the above stated extracts, ethanolic and aqueous extracts showed the presence of same type of constituents. Hence, both ethanolic (EEOL) and aqueous (AEOL) extracts were selected for the pharmacological evaluation.

**Evaluation of Invitro-Anticancer Activities**

**Tryphan Blue Exclusion Assay**

Tryphan Blue is a blue acid dye that has two azo chromophore groups. Trypan Blue is an essential dye, used in estimating the number of viable/ dead cells present in a population. Tryphan blue is a vital stain used to selectively colour the dead tissues. It is a diazo dye. Live cells or tissues with intact cell membranes are not coloured as trypan blue is not absorbed; however, it traverses the membrane in a dead cell and are shown as a distinctive blue colour under a microscope. Since live cells are excluded from staining, this staining method is also described as a dye exclusion method. Staining facilitates the visualization of cell morphology.

The antitumor effect of EEOL & AEOL against MCF-7 & HT-29 cell line produced a concentration dependent cytotoxic effect which was indicated by the increase in number of dead cells with increasing concentrations of EEOL & AEOL. The 12.5µg/ml concentration of EEOL showed 19.23% of dead cells where as in higher concentration of 200µg/ml EEOL 57.45% of dead cells.

The results showed that MCF-7 & HT-29 tumor cell proliferation was significantly inhibited by EEOL & AEOL, with an $ED_{50}$ value i.e 50% of tumor cell death at 200µg/ml respectively and were shown in Table no: 5 & 6.

**MTT ASSAY 3-(4, 5-Dimethylthiazole-2-yl)-2, 5-Diphenyltetrazolium Bromide**

The percentage of survived cells was calculated by measuring the absorbance of respective incubated cells in the 96 wells plate. The effect of ethanolic & aqueous extracts on MCF-7 & HT-29 cancer cell lines is significant and comparable to the standard drugs Tamoxifen & 5-Flurouracil. The extracts have shown the activity even at the lowest concentration of 12.5µg/ml. The extracts have shown concentration dependent and significant activity.

## CONCLUSION

The present work confirms that EEOL & AEOL have potent inhibitive effects on MCF-7 & HT-29 cell lines which was studied by performing MTT based cytotoxic assay and tryphan blue dye exclusion assay. The extract treated cell lines proved its dose dependent activity. These results provide promising baseline information for the potential use of ethanolic and aquoeus extract of whole plants of *Oxalis latifolia kunth* in the treatment of cancer especially mammarian and colon cancer. However further in-vivo investigations are essential for the isolation of the active constituents of EEOL & AEOL and to detail its mechanism of action.

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