



# Anticancer activity of *Limonia acidissima* L. fruit extract on human Mammary gland adenocarcinoma MDA-MB-21 cells

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## Abstract

The study aimed to reveal the anticancer activity of *Limonia acidissima* L. fruit extract (LAFE) on human Mammary gland adenocarcinoma MDA-MB-21 cells. The ethanolic extract of *Limonia acidissima* L. fruit was prepared by cold-maceration technique. The phytochemical profile of LAFE was revealed by qualitatively and quantitatively. The qualitative analysis showed that the obtained ethanolic LAFE was found to contain a high amount of phenols, flavonoids, and terpenoids. In quantitative analysis, total phenolic content, total flavonoid content, and total tannin content of LAFE were revealed by Folin-Ciocalteu, aluminium chloride, and ferric chloride methods, respectively. In one g of LAFE,  $26.30 \pm 1.08$  mg gallic acid equivalent,  $18.76 \pm 0.68$  mg catechol equivalent, and  $8.14 \pm 0.52$  mg tannic acid equivalent of total phenolics, total flavonoids, and total tannins were present, respectively. The antioxidant potential of LAFE was revealed by ABTS, DPPH, and FRAP assays. The EC<sub>50</sub> value of LAFE (effective concentration of LAFE required to scavenge 50% of free radicals) in ABTS, DPPH, and FRAP assays was determined as  $318 \pm 21.06$ ,  $337.90 \pm 27.88$ , and  $359.43 \pm 17.05$  µg/mL, respectively. The anticancer effect of LAFE was revealed on cancer cells MDA-MB-231 (human mammary gland adenocarcinoma) by MTT, LDH, micro-morphological observation, and live/dead dual staining assays. In the MTT assay, IC<sub>50</sub> (concentration required to inhibit 50% of cell viability compared to control) of LAFE was determined as  $158.03 \pm 4.92$  µg/mL. In LDH analysis, LAFE has dose-dependently escalated the LDH levels and found that LAFE has induced cell death by damaging cell membrane integrity. The effect of LAFE on the micro-morphology of cancer cells was revealed by bright-field inverted microscopy and found that LAFE has affected the viability of the cells by detrimentally inducing micro-morphological changes. In the final part of assessing the anticancer activity of LAFE, a live/dead dual staining assay was considered and revealed that LAFE has affected cell viability and increased the number of dead cells by damaging the cell membrane. The obtained results were in line with MTT, LDH, and micro-morphological observation. The study concluded that LAFE has potential anticancer activity and could be potentially explored as an anticancer agent in the biomedical field.

**Keywords:** Cancer, *Limonia acidissima* L., MDA-MB-21 cells, flavonoids, phenolics, MTT assay, LDH assay, live/dead dual staining.

## I. Introduction

Cancer is a condition when a few of the body's cells grow out of control and spread to other bodily regions. In the millions of cells that make up the human body, cancer can develop practically anywhere. Human cells often divide (via a process known as cell growth and multiplication) to build new cells as the body requires them. New cells replace old ones when they die as a result of aging or damage. Occasionally, this systematic process fails, causing damaged or aberrant cells to proliferate when they shouldn't. Tumors, which are tissue masses, can develop from these cells. Tumors may or may not be malignant. Cancerous tumors can move to distant parts of the body to produce new tumors, invade neighboring tissues, or both (a process called metastasis). Malignant tumors are another name for cancerous tumors. While many malignancies become solid tumors, blood-related cancers like leukemia typically do not. The most prevalent kind of cancer is carcinoma. Epithelial cells, which are the cells that line the interior and exterior surfaces of the body, are responsible for their formation. Epithelial cells come in a variety of types, and when they are magnified under a microscope, they frequently resemble columns. Adenocarcinoma is a type of cancer that develops in mucus- or fluid-producing epithelial cells. Occasionally, glandular tissues are referred to as epithelial

tissues. Adenocarcinomas make up the majority of cases of breast, colon, and prostate cancer (MacMahon *et al.*, 1973; Bailar & Gornik, 1997).

Breast cancer is a condition in which the breast cells proliferate out of control. Breast cancer comes in several forms. Which breast cells develop into cancer determines the type of breast cancer. In 2020, 685 000 people worldwide died and 2.3 million women were diagnosed with breast cancer. The most common cancer in the globe as of the end of 2020 was breast cancer, which had been diagnosed in 7.8 million women in the previous five years. Between the 1930s and the 1970s, there were very minor changes in breast cancer mortality. In nations with early detection programs coupled with various forms of treatment to remove invasive diseases, improvements in survival started in the 1980s. Treatment for breast cancer can be quite successful, especially if the disease is discovered early. To treat microscopic cancer that has spread from the breast tumor through the blood, breast cancer is frequently treated with a combination of surgical removal, radiation therapy, and medication (hormonal therapy, chemotherapy, and/or targeted biological therapy). Such treatment can stop the growth and spread of cancer, saving lives in the process (Ginsburg *et al.*, 2020; Mutebi *et al.*, 2020; Wild *et al.*, 2020).

Long-standing theories suggest that nutrition contributes significantly to the aetiology of cancer. Nutrients are excellent candidates to help prevent cancer due to their biological features. Early epidemiologic cancer studies did seem to support the importance of food (Michels, 2005). Many dietary antioxidants have demonstrated tremendous promise as effective medicines for cancer prevention by lowering oxidative stress, which has been linked to the development of many diseases, including cancer. Therefore, dietary changes, particularly increased consumption of fruits and vegetables rich in antioxidants, are increasingly recommended to lower the risk of cancer. A growing body of research suggests that a variety of dietary components may be utilized either alone or in conjunction with conventional chemotherapeutic medicines to either treat or prevent cancer (Willcox *et al.*, 2004).

There is a wealth of research that suggests fruits may be protective against malignancies. The abundance of bioactive phytochemicals found in fruits, such as polyphenols, stilbenoids, lignans, and triterpenoids, have been linked to the anticancer potential of fruits. In the present study, anticancer activity of *Limonia acidissima* L. fruit extract on human Mammary gland adenocarcinoma MDA-MB-21 cells was explored. *L. acidissima* L. belong to Rutaceae and is a tropical plant species in the Indian subcontinent. Native to India, it was also known there as katha bel, curd fruit, monkey fruit, and elephant apple (Kirtikar & Basu, 1918). The chemical components of *L. acidissima* fruits include flavonoids, triterpenoids, vitamins, saponins, tannins, coumarins, glycosides, carbohydrates, phytosterols, and amino acids (Kangralkar *et al.*, 2010). The woody, rough fruits are used to cure hepatitis, diarrhoea, wounds, heart debility, liver tonic cough, sore throats, and gum diseases. The sour and sweet fruits have been employed in Indian folk medicine, Ayurveda, and Yunani medicine as diuretics and to cure blood impurities and leucorrhoea, respectively (Darsini *et al.*, 2013). Fruit pulp exhibited antipyretic, anti-inflammatory, and analgesic effects, antimicrobial, and larvicidal properties. Additionally, it has been experimentally demonstrated to have the ability to reduce blood glucose levels, making it a potent herbal treatment for diabetes (Gupta *et al.*, 2006).

In the present study, ethanolic extract of *L. acidissima* L. fruit was obtained by cold-maceration technique. The phytochemical profile of *L. acidissima* L. fruit extract (LAFE) was revealed by qualitatively and quantitatively. The antioxidant activity of LAFE was revealed by DPPH, ABTS, and FRAP assays. The anticancer activity of LAFE on MDA-MB-21 cells was revealed by MTT, LDH, micro-morphological, and live/dead dual staining assays.

## II. Materials and Methods

### 2.1. Chemicals and reagents

A live/dead cell staining kit consisting of ethidium homodimer-1 and calcein-AM was obtained from Thermo Fisher Scientific, USA. Fetal bovine serum (FBS), di(chlorohydrofluorescein) diacetate (DCFH-DA), caspase-3 assay kit, Dulbecco's modified Eagle's medium (DMEM), trypsin, amphotericin B, penicillin, streptomycin, Dulbecco's phosphate-buffered saline (DPBS), and lactate dehydrogenase (LDH) assay kit were obtained from Sigma-Aldrich, Hyderabad, India. The other chemicals used in the study were obtained from Merck, Bengaluru, India, and they belonged to the fine grade.

### 2.2 Collection and phytochemical profile of *Limonia acidissima* L. fruit extract

#### 2.2.1 Collection and preparation of *Limonia acidissima* L. fruit extract

The *Limonia acidissima* L. fruit belongs to the family Rutaceae and subfamily Aurantioideae. *Limonia acidissima* L. fruits were collected from the local market, Vijayawada, Andhra Pradesh, India. The fruits were washed with double distilled water and fruit pulp was collected using a sterile knife and dried under the shade for ten days before being crushed into a fine powder with an electrical blender. For the ethanolic extract preparation, the powder was preserved in a sterile amber air-tight container.

Following, 100 g of *Limonia acidissima* L. fruit fine powder was subjected to a cold maceration technique using ethanol as solvent (Chanda & Kaneria, 2012). After a course of three days, the obtained mixture was then filtered through Whatman No. 1 filter paper, and the final filtrate was concentrated using a rotary evaporator with a vacuum at 45 °C and poured into glass Petri dishes, and dried at room temperature. After completely removing the moisture, the obtained *Limonia acidissima* L. fruit extract (LAFE) residue was safeguarded in a sealed amber bottle for future analysis.

## 2.2.2 Qualitative phytochemical profile of *Limonia acidissima* L. fruit extract

### 2.2.2.1 Identification of phenols

The lead acetate test was used to determine the presence or absence of phenols in our sample. This test is one of the most recommended tests to determine the presence of phenols. Briefly, 10 mg of LAFE was dissolved in 10 mL of distilled water, and 0.5 mL of LAFE was mixed with 5 mL of 10% w/v lead acetate. The prevalence of phenolics in LAFE is distinct by the attendance of white precipitate (Tamilselvi *et al.*, 2012).

### 2.2.2.2 Identification of flavonoids

The presence of flavonoids in LAFE was determined using the alkaline reagent assay. A few drops of weak sodium hydroxide solution were added to 0.5 mL of LAFE (10 mg/10 mL), which resulted in a bright yellow color. After adding a drop of sulphuric acid, the liquid turned colorless. The presence of flavonoids was thus established by the bright yellow color that had developed (Lallianrawna *et al.*, 2013).

### 2.2.2.3 Identification of tannins

The ferric chloride test was used to determine the presence or absence of tannins in our sample. This test is one of the most recommended tests to determine whether tannins are present in a sample or not. Briefly, 10 mg of LAFE was dissolved in 10 mL of distilled water and was mixed thoroughly, and added of neutral FeCl<sub>3</sub> (10%) to this mixture. Following, the successful formation of dark blackish blue showed the presence of hydrolyzable tannins (Makkar & Becker, 1993).

### 2.2.2.4 Identification of alkaloids

Mayer's test was used for the identification of alkaloids. The Mayer's reagent contains potassium ion, which will form a coordinate covalent bond with the nitrogen atom of the alkaloid, this results in a creamy yellow complex in the form of precipitates. For that, in this study the LAFE (10 mg/10 mL) was boiled in a boiling water bath with 2% HCl, then filtered and added to Mayer's reagent. The presence of alkaloids was indicated by the yellow precipitation or turbidity in the LAFE solution (Tamilselvi *et al.*, 2012).

### 2.2.2.5 Identification of steroids

Salkowski test was used to identify the steroids. To dissolve the LAFE (10 mg), about 10 mL of chloroform was added, followed by 98% sulphuric acid (1 mL) poured against the LAFE container's wall sides. The presence of steroids is indicated by the visible top layer turning red and the bottom layer of sulphuric acid turning yellow with green color (Patel *et al.*, 2016).

### 2.2.2.6 Identification of carbohydrates

Molisch's test was used for the identification of carbohydrates. Molisch's reagent ( $\alpha$ -naphthol in ethanol) is commonly used to identify carbohydrates based on the formation of furfural compounds from the dehydration of sulphuric acid. A freshly diluted LAFE (10 mg/10mL) was transferred to a glass test tube and added two or three drops of Molisch's reagent. The presence of carbohydrates is indicated by the appearance of a purple thin layer after the addition of 3 mL sulphuric acid into the test tube sample (Tamilselvi *et al.*, 2012).

### 2.2.2.7 Identification of proteins

A Biuret test was used to identify proteins in LAFE. Briefly, 1 mL of 40% sodium hydroxide solution and 1-2 drops of 1% copper sulfate solution (Biuret reagent) were mixed with 1 mL of LAFE (10 mg/10 mL) and boiled under a water bath. The presence of protein is confirmed by the appearance of a blue/purple color (Tamilselvi *et al.*, 2012).

### 2.2.2.8 Identification of terpenoids

Liebermann - Burchard's test was followed to determine the presence of terpenoids in LAFE. Briefly, 2 mL of chloroform was added to the dry LAFE powder (2-3 mg), and followed by 1 mL of acetic anhydride was combined, heated under a water bath, and cooled to dissolve it. Following, the obtained mixture was mixed with 1 mL of concentrated sulphuric acid and resulted in the immediate formation of a red with a violet-colored ring, indicating the presence of triterpenoids (Patel *et al.*, 2016).

### 2.2.2.9 Identification of glycosides

Keller-Killani's test was used to identify the glycosides. Briefly, an equal volume of (2 mL of 10 mg/10 mL) LAFE sample and acetic acid were mixed with a few drops of 2% FeCl<sub>3</sub> solution. Following, 2 mL of sulphuric acid was added dropwise into the test tube and the formation of a brown ring at the junction confirmed the presence of glycoside in the test sample (Tyagi, 2017).

### 2.2.2.10 Identification of saponins

Froth formation test was followed to determine the presence of saponins in LAFE. Briefly, 3 g of LAFE dried fine plant powder was dissolved in 30 mL of sterile distilled water in a conical flask and boiled under a water bath before being filtered. The obtained mixture was vortexed vigorously for up to 15 min to produce a stable persistent froth. 2-4 droplets of olive oil were combined and vigorously agitated to observe the saponins' ability to create an emulsion (Tamilselvi *et al.*, 2012).

### 2.2.2.11 Identification of anthraquinones

Bourn stranger's test was followed to identify the presence of anthraquinones. Briefly, 2 mL of LAFE (10 mg/10 mL) was combined with 5 mL of a 10% ammonia solution. The formation of red color or precipitation is a sign that anthraquinones are present (Bassey *et al.*, 2016).



### 2.2.3 Quantitative phytochemical analysis of *Limonia acidissima* L. fruit extract

#### 2.2.3.1 Estimation of total phenolic content

The Folin-Ciocalteu method is universal, highly applicable, and described in several pharmacopeias for determining total phenolic contents. The core principle behind the approach is that a phosphotungstic-phosphomolybdenum complex reacting with phenols produces blue chromophores; also, the increased absorption of the chromophores depends on the alkaline solution and the amount of phenolic compounds present in the test sample/plant sample (Singleton *et al.*, 1999).

The assay was performed as the methodology of Alabri *et al.*, (2014) with a few minor modifications. Briefly, 0.5 mL of Folin-Ciocalteu reagent (10%, 1N) and 0.8 mL of sodium carbonate (7.5%) were added to the tube along with 1 mL of LAFE solution (various concentrations) and were carefully mixed. The color solution was then incubated by placing the tube in a dark environment for 30 to 40 min. Following, a microplate reader operating at 765 nm was used to measure color intensity (Synergy H1, BioTek, USA). Water served as the blank solution. Gallic acid (GA) served as the study's standard, and the results were expressed using a calibration curve for the standard gallic acid (GA). The total phenolic content of LAFE was calculated as mg GA equivalent per g of a sample using a standard curve.

#### 2.2.3.2 Estimation of total flavonoid content

The colorimetric aluminium chloride method was used to determine the amount of total flavonoid present in the LAFE (Farang, *et al.*, 2020). It is a widely used method in phytochemical screening and antioxidant activity of some medicinal plants' crude juices. The chemical compound aluminium chloride will bind to the compounds like flavones or flavonols at the C-3, 4 & 5 positions and produce the acid stable complexes.

Briefly, 0.25 mL of the LAFE sample was taken at various concentrations, and 1 mL was made using distilled water. To the LAFE mixture, 0.075 mL of a pure (5%, w/w) sodium nitrite solution was added, and the mixture was then left undisturbed at room temperature for 5–6 min. After the incubation period, 0.075 mL of aluminium chloride at 10% was added to obtain the acid-stable complexes. After incubation for 5 min, 1 mL of 4% sodium hydroxide solution was added and the final volume was made up to 2.5 mL with distilled water. The solution was thoroughly mixed and continued for 15 min incubation at room temperature. At the final, the pink color absorbance was measured against the reagent blank (water) at 510 nm using a microplate reader (Synergy H1, BioTek, USA). The standard flavonoid catechol was taken for the study. By referring to the standard catechol the total flavonoid content in the sample was the result as mg catechin equivalence/g of the sample.

#### 2.2.3.3 Quantification of total tannins

The estimation of total tannins in LAFE was determined by the ferric chloride method as per the technique of Hatim & Makhawi, (2019) with a few small modifications. The colorimetric method has been used to determine the amount of red product that is produced when the tannin reacts with a complex of potassium ferricyanide and ferric chloride.

Briefly, one mL of the LAFE sample (different concentrations), 6 mL of 0.1 M of ferric chloride, and 8 M of potassium ferricyanide were added to the test tube before 10 mL of distilled water was added. After that, the aforementioned reaction mixture was incubated at 37 °C for 15 min. The produced red color indicates the presence of tannin, and the absorbance measurement was carried out at 510 nm with the blank reagent (water) using a microplate reader (Synergy H1, BioTek, USA). The optical density was taken into account to calculate the total concentration of tannin content. The calibration curve prepared for standard tannic acid was then used to convert the results into mg of tannic acid equivalent per g of sample.

### 2.3 Antioxidant activity of *Limonia acidissima* L. fruit extract

Antioxidants are small molecules that work as redox buffers that interact with a variety of cellular constituents and regulate processes ranging from cell division and elongation to senescence and death in plants. These small molecules are referred to as "antioxidants." They function by freely dispensing electrons to free radicals without changing into compounds that scavenge electrons. They participate in processes that preserve cell viability and repair DNA (Virgili & Marino, 2008). The majority of plants include substances with antioxidant properties. All parts of the plant, including the wood, bark, stems, leaves, fruit, roots, blossoms, pollen, and seeds, contain polyphenolics which are referred to as antioxidant molecules (Pratt, 1992). In the present study antioxidant activity of LAFE was considered by DPPH and ABTS free radical scavenging assay and FRAP reducing power.

#### 2.3.1 DPPH free radical scavenging assay

The DPPH free radical scavenging activity of LAFE was revealed by the technique of Bhalodia *et al.*, (2013) with a few minor modifications. Briefly, LAFE (different concentrations) was diluted with methanol to the volume of 0.5 mL, and 0.6 mL of DPPH (8 mM in methanol) was then added. The combined solution was left at room temperature for half an hour under dark condition. Methanol alone and methanol with 0.6 mL of DPPH served as the blank and control, respectively. Ascorbic acid was used as the reference standard. Succeeding, a microplate reader (Synergy H1, BioTek, USA) was used to record the purple color changing to reduced yellow at 517 nm (DPPH free radical scavenging). The 50% scavenging (EC<sub>50</sub>) value of the LAFE was determined as the concentration of LAFE required to inhibit 50% of DPPH free radicals. The formula is as follows below,

$$50\% \text{ Inhibition in DPPH free radicals} = \frac{CS_{OD} - TS_{OD}}{CS_{OD}} \times 100$$

Where, CS<sub>OD</sub> denotes the optical density of the control sample. TS<sub>OD</sub> denotes the optical density of the test sample.

### 2.3.2 ABTS free radical scavenging assay

ABTS free radical quantification by LAFE was done by the approach of Gangwar *et al.*, (2014) with a few minor modifications. Briefly, in the first stage to produce the stable radicals, 2 mL of ABTS (7 mM) and 2.45 mM of potassium persulphate (2 mL) were incubated for 14–16 hrs at room temperature and in the dark condition. Then, before beginning the experiment, the incubated mixture was diluted with 95% ethanol to achieve the optical density of  $0.7 \pm 0.02$  at the nanometer of 734. Succeeding, activated ABTS free radicals solution (1 mL) was added to various concentrations of LAFE and incubated for 20–30 min at room temperature in the dark. Following, color disappearance was measured at 734 nm using a microplate reader (Synergy H1, BioTek, USA). While ethanol was used as a blank, and an ABTS free radicals solution combined with ethanol was considered as a control. Ascorbic acid was used as a standard antioxidant. The 50% scavenging ( $EC_{50}$ ) value of the LAFE was determined as the concentration of LAFE required to inhibit 50% of ABTS free radicals. The formula is as follows below,

$$50\% \text{ Inhibition in ABTS free radicals} = \frac{CS_{OD} - TS_{OD}}{CS_{OD}} \times 100$$

Where,  $CS_{OD}$  denotes the optical density of the control sample.  $TS_{OD}$  denotes the optical density of the test sample.

### 2.3.3 FRAP reducing power

The FRAP reducing power of LAFE was revealed by the technique of Bhalodia *et al.*, (2013) with a few minor modifications. Briefly, 0.2 mL of LAFE sample (different concentrations) was combined with 2.5 mL of potassium ferricyanide (1%) and 0.2 M phosphate buffer (pH 6.6) and incubated for 10–20 min at 50 °C in a water bath. The mixture was then left to attain room temperature without being disturbed. The reaction was then inhibited by adding 2.5 mL of trichloroacetic acid (10%) dropwise to the reaction mixture. The TCA incubated mixture was immediately subjected to 10min centrifugation at a speed of 3000 rpm. The top layer (2.5 mL) was then combined with an equal volume of distilled water, followed by added 0.5 mL (0.1%) of ferric chloride, and the optical density was assessed at 700 nm using a microplate reader (Synergy H1, BioTek, USA). Ascorbic acid was used as standard. The reaction mixture alone (without a sample) was used as the control. The 50% scavenging ( $EC_{50}$ ) value of the LAFE was determined as the concentration of LAFE required to inhibit 50% of FRAP radicals. The formula is as follows below,

$$50\% \text{ Inhibition in FRAP radicals} = \frac{CS_{OD} - TS_{OD}}{CS_{OD}} \times 100$$

Where,  $CS_{OD}$  denotes the optical density of the control sample.  $TS_{OD}$  denotes the optical density of the test sample.

## 2.4 Anticancer activity of *Limonia acidissima* L. fruit extract

### 2.4.1 Cell culture and maintenance

The anticancer effect of LAFE was assessed on cancer cell line MDA-MB-231 (human mammary gland adenocarcinoma). The cells were obtained from National Center for Cell Sciences (NCCS), Pune, India. The cells were grown as adherent cultures in a humid atmosphere with 5%  $CO_2$  at 37 °C in Dulbecco's modified Eagle medium (DMEM) with 10% FBS, 100 units/mL of penicillin, and 100  $\mu$ g/mL of streptomycin, and 2 mM L-glutamine. The fresh media was replenished every three days till cells reaches up to 80% confluence. Cells were passaged by trypsinization (phosphate buffer saline, pH 7.4 containing 0.25 trypsin and 0.1% EDTA) and collected by centrifugation at 2,250 rpm for 10 minutes. The obtained cells were re-suspended in a DMEM medium without FBS for further research.

### 2.4.2 MTT assay

Cells treated with LAFE were tested for vitality using the MTT assay. Cellular enzymes convert the yellow dye MTT into the blue substance formazan. Since only living cells can undergo this enzymatic transition, the quantity of purple-blue formazan generated is directly relative to the number of viable cells (Morgan, 1998).

The MTT assay was performed as per the methodology of Shnoudeh *et al.*, (2022) with a few minor modifications. Briefly,  $1.5 \times 10^4$  cells were seeded in 96-well plates in a DMEM medium with 10% FBS and allowed to adhere for 12 hrs. Following, by changing the media in the wells, attached cells were treated with the 100  $\mu$ L of DMEM medium devoid of FBS containing different concentrations of LAFE. The LAFE-treated cells were then cultured in the incubator for a further 24 hrs at various doses. The cells treated with LAFE-free DMEM media with a lack of FBS served as control. Following the incubation period, control and the LAFE treated cells were treated with 100  $\mu$ L of MTT solution (5 mg/mL in phosphate buffer saline, pH 7.4) for 4 hrs. Succeeding, MTT solution was aspirated and formazan crystals were solubilized by 150  $\mu$ L solution of DMSO, acetic acid, and sodium dodecyl sulfate (99.4 mL: 0.6 mL: 10 g). A microplate reader was used to measure the amount of purple-blue formazan formed at 570 nm (Synergy H1, BioTek, USA). The viability of the LAFE treated samples was expressed with respect to LAFE untreated control sample (100%). The standard anticancer agent was cisplatin. The growth inhibition of LAFE was calculated by the following formula,

$$\% \text{ Cell growth Inhibition} = \frac{CS_{OD} - TS_{OD}}{CS_{OD}} \times 100$$

Where,  $CS_{OD}$  and  $TS_{OD}$  refer to the optical density of the sample at 570 nm, respectively.

### 2.4.3 LDH assay

The LDH Cytotoxicity Assay is a colorimetric assay that offers a quick and accurate way to assess the cytotoxicity of cells. LDH is found in the cytoplasm of many distinct cell types. A cytosolic enzyme called lactate dehydrogenase (LDH) is released into the cell culture media when the plasma membrane is damaged (Stoddart, 2011).

In the present study, as directed by the manufacturer, we performed the LDH assay using the kit (Sigma-Aldrich, India). Briefly, in 12-well plates,  $5 \times 10^5$  cells were seeded in each well and given 12 hrs to adhere in DMEM complete media (including 10% FBS). Following, by changing the media in the wells, attached cells were treated with the 500  $\mu$ L of DMEM medium devoid of FBS containing different concentrations of LAFE. The LAFE-treated cells were then cultured in the incubator for a further 24 hrs at various doses. The cells treated with LAFE-free DMEM media with a lack of FBS served as control. The cells treated with 1% Triton X100 served as a positive control. The plates were gradually shaken to homogenize the released LDH in the cell culture medium and supernatant was collected at 10,000 rpm for 15 min at 4 °C. Succeeding, 100  $\mu$ L of supernatant was allowed to react with the substrate solution of the kit and optical density was observed at 340 nm using microplate reader (Synergy H1, BioTek, USA). The LDH release of the LAFE treated samples was expressed with respect to LAFE untreated control sample in terms of folds. The standard anticancer agent was cisplatin.

### 2.4.4 Micro-morphological observation

The detrimental effect of LAFE on the micro-morphology of cancer cells was observed by an inverted bright-field microscope. The detrimental features include the development of rough surfaces on cellular membranes, evidence of cellular debris, and the formation of apoptotic bodies (Chen *et al.*, 2020).

Briefly, in 12-well plates,  $5 \times 10^5$  cells were seeded in each well and given 12 hrs to adhere in DMEM complete media (including 10% FBS). Following, by changing the media in the wells, attached cells were treated with the 500  $\mu$ L of DMEM medium devoid of FBS containing different concentrations of LAFE. The LAFE-treated cells were then cultured in the incubator for a further 24 hrs at various doses. The cells treated with LAFE-free DMEM media with a lack of FBS served as control. Following this, an inverted microscope (Olympus, Japan) was used to take pictures of the cells, and the micro-morphology of the cells was taken into account to assess the growth inhibitory activity of LAFE on cancer cells. The standard anticancer agent was cisplatin.

### 2.4.5 Live/dead dual staining assay

The live/dead dual staining technique was used to evaluate the impact of LAFE on cell viability, the technique is dependent on the integrity of the cell membrane. The live/dead cell dual staining kit consists of calcein AM and ethidium homodimer-1. The majority of eukaryotic cells' viability can be observed by cell-permeant dye calcein AM. In viable cells, intracellular esterases hydrolyze the acetoxymethyl ester to transform the non-fluorescent calcein AM into a green-fluorescent calcein. Ethidium homodimer-1 is a high-affinity nucleic acid stain that is faintly fluorescent until linked to DNA and generates red fluorescence in cells as a cell-impermeant viability indicator. The presence of red luminous cells indicates that the cell membrane is destroyed and that the cells were not viable (Spaepen *et al.*, 2011).

The assay was performed as per the instructions provided by the manufacturer of the live/dead dual cell staining kit (Thermo Fisher Scientific, USA). Briefly,  $1.5 \times 10^4$  cells were seeded in 96-well plates and given 12 hrs to adhere in DMEM complete media (including 10% FBS). Following, by changing the media in the wells, attached cells were treated with the 500  $\mu$ L of DMEM medium devoid of FBS containing different concentrations of LAFE. The LAFE-treated cells were then cultured in the incubator for a further 24 hrs at various doses. The cells treated with LAFE-free DMEM media with a lack of FBS served as control. Following incubation of 24 hrs, cells were gently washed with D-PBS twice and stained with a live/dead dual staining kit as per instructions of the manufacturer. The live cells and dead cells present in the experiment were calculated by applying the recommended formula of manufacture. Also, phase-contrast and fluorescent microscopic images were captured at 100x magnification using EVOS FLC fluorescent microscope (Thermo Fisher Scientific, USA).

## 2.5 Statistical analysis

The investigations were conducted independently three times ( $n = 3$ ), and the results were presented as a mean  $\pm$  standard deviation. One-way ANOVA was used to process the collected data, and the student's t-test was used to determine the statistical significance. The statistical measurements and graphical representations were obtained using GraphPad Prism software, trial version 8, USA.

### III. RESULTS AND DISCUSSION

#### 3.1 Phytochemical profile of *Limonia acidissima* L. fruit extract

##### 3.1.1 Qualitative phytochemical screening of *Limonia acidissima* L. fruit extract

Fruits and vegetables are essential foods for human health since they offer a wide range of flavors and are linked to a higher standard of living. The consumption of fruits and vegetables has a protective effect against the main diet-related chronic diseases, cardiovascular diseases, and cancer. Bioactive substances are derived from natural sources and have positive impacts on human health. Polyphenol compounds are just a few of the nutrients abundant in fruits and vegetables. These chemicals' extraction procedures are influenced by a number of variables, including the extraction method, the raw material, and the organic solvent. Soxhlet, maceration, and hydrodistillation are the three primary traditional extraction methods for bioactive substances (Soquetta *et al.*, 2018).

In the maceration technique, the surface area of the sample ensures a suitable mixing with the solvent, and the sample is ground into smaller particles. Both the increased diffusion and the removal of the concentrated solution from the sample's surface are facilitated by the agitation that occurs throughout the maceration process. This method has been employed for a very long time to maximize bioactive chemicals and essential oils (Soquetta *et al.*, 2018).

In the present study, bioactive compounds from *Limonia acidissima* L. fruit were extracted by cold maceration technique using ethanol as solvent. Following extraction, the preliminary qualitative phytochemical profile of *Limonia acidissima* L. fruit extract (LAFE) was considered. The obtained ethanolic LAFE was found to contain a high amount of phenols, flavonoids, and terpenoids, Also, LAFE was found to contain a medium amount of tannins, alkaloids, carbohydrates, and proteins (Table 1).

**Table 1:** Qualitative phytochemical profile of *Limonia acidissima* L. fruit extract

Qualitative phytochemical analysis	Result
Phenols	+++
Flavonoids	+++
Tannins	++
Alkaloids	++
Steroids	-
Carbohydrates	++
Proteins	++
Terpenoids	+++
Glycosides	+
Saponins	+
Anthraquinones	-

High content: +++, Moderate content: ++, Low content: +, Absent: -

##### 3.1.2 Quantitative phytochemical analysis of *Limonia acidissima* L. fruit extract

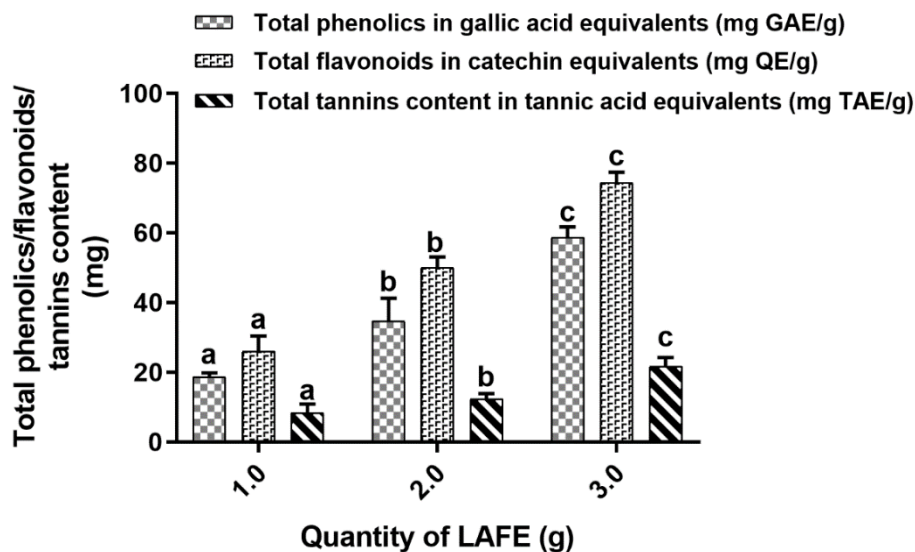
Phenolic compounds are common secondary metabolites found in food and beverages made from plants and plant-based materials. They exhibit a wide range of structural variation, including relatively straightforward compounds (such as vanillin, gallic acid, and caffeic acid), polyphenols like stilbenes and flavonoids, and polymers generated from these distinct groups. For instance, the flavonoid family alone has reported approximately 8,000 compounds, and the number is growing (Fossen & Andersen, 2006; Cheynier, 2012).

The term "polyphenol" should only be applied to molecules with at least two phenolic rings, despite the fact that it is frequently used as a synonym for phenolic compounds. The term "polyphenol" should be used to define plant secondary metabolites derived exclusively from the shikimate-derived phenylpropanoid and/or the polyketide pathway(s), featuring more than one phenolic ring and being devoid of any nitrogen-based functional group in their most basic structural expression (Quideau *et al.*, 2011). While some phenolic chemicals are extremely common, others are only present in specific plant groups, organs, or developmental stages of certain plants. The variety of features that the structures possess are connected to various roles in plants, which accounts for their distinct distribution. Anthocyanins, for instance, are the pigments found in the majority of red and blue plant organs. They are present in ripe fruits and flowers and aid in the pollination and seed dispersal processes by acting as attractants. They are also highly prevalent on young leaves, where they may have a deterrent effect on insects that feed on plants (Gould, 2010).

Flavonoids shield plant tissues from UV radiation, whereas proanthocyanidins (PA, also known as condensed tannins and flavan-3-ol oligomers and polymers) and hydrolyzable tannins (gallotannins and ellagitannins, based on multiple esters of gallic or hexahydroxydiphenic acid, respectively, with a polyol, typically glucose or quinic acid) are present in the majority of plant tissues from early developmental stages and may aid in plant defence against herbivores, fungi, and viruses (Haslam, 2007).



In the present study total phenolic content, total flavonoid content, and total tannin content of LAFE was revealed by Folin-Ciocalteu, aluminium chloride, and ferric chloride methods, respectively. The study showed that LAFE contains phenolics, flavonoids, and tannins. Though, flavonoids were found abundant and followed by phenolics and tannins. In one g of LAFE,  $26.30 \pm 1.08$  mg GA equivalent,  $18.76 \pm 0.68$  mg catechol equivalent, and  $8.14 \pm 0.52$  tannic acid equivalent, respectively. The concentration of total phenolic content, total flavonoid content, and total tannin content was found to be dose-dependent with the quantity of LAFE (Fig. 1).



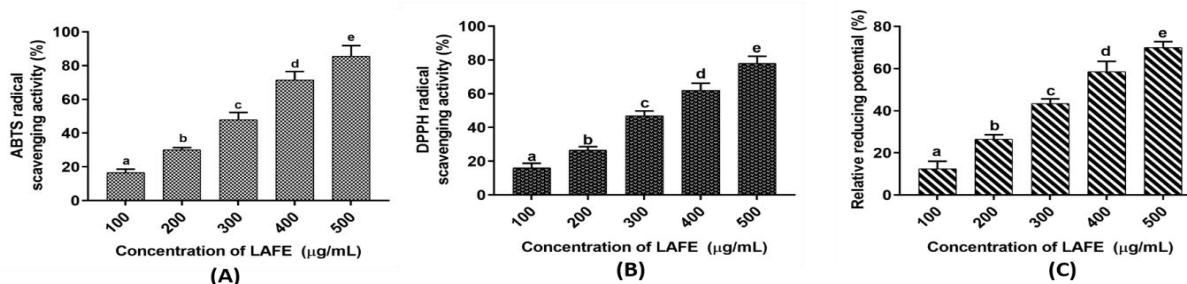
**Figure 1:** Total phenolic, total flavonoid, and tannin content in *L. acidissima* L. fruit extract (LAFE). The analysis was performed three times ( $n = 3$ ), and the findings were presented as mean  $\pm$  standard deviation. Tukey's test was used to establish the statistical significance between the test samples, and a  $p$ -value  $\leq 0.05$  was considered significant. In the specific study group, bar graphs with distinct alphabets indicate that statistically significant.

The obtained results of the study were in accordance with previous reports, Darsini *et al.*, (2013) reported the phenol and flavonoid contents in methanol, chloroform, and ethyl acetate extract. Also, Kumar *et al.*, (2016) assessed the phytochemical profile of LAFE and LC-MS phytochemical analysis revealed the presence of the various important polyphenol phytoconstituents like kinetin, esculin, 3,4,2',4',6'-pentamethoxychalcone, and *N*-desmethylpheniramine. Our study concluded following previous reports concluded that LAFE is a rich source of various highly beneficial compounds, especially phenolics and flavonoids. The LAFE could be highly regarded as a highly beneficial fruit in daily diet.

### 3.2 Antioxidant activity of *Limonia acidissima* L. fruit extract

In the cell, free radicals and reactive oxygen species (ROS) are squelched by several chemical molecules known as antioxidants. One of the most intriguing characteristics in the scientific community nowadays is the antioxidant activity in food and drink. Free radical damage is a major factor in the development of many chronic diseases, including cancer, inflammation, heart disease, anemia, and cardiovascular diseases. These antioxidants protect against this damage. Natural antioxidants are compounds found in food that stop reactions like sourness, color change, and disruption. Natural antioxidants are often produced from plant sources, and the amount of protection they provide depends on the type of plant, its diversity, the extraction or processing method used, and the growing environment (Zehiroglu & Ozturk Sarikaya, 2019).

In the present study, the antioxidant potential of LAFE was revealed by ABTS, DPPH, and FRAP assays. The studies showed that LAFE has potential antioxidant activity (Fig. 2). The LAFE exhibited dose-dependent free radical scavenging potential in ABTS, DPPH, and FRAP assays. The  $EC_{50}$  value of LAFE (effective concentration of LAFE required to scavenge 50% of free radicals) in ABTS, DPPH, and FRAP assays was determined as  $318 \pm 21.06$ ,  $337.90 \pm 27.88$ , and  $359.43 \pm 17.05$   $\mu$ g/mL, respectively.



**Figure 2:** Antioxidant potential of *L. acidissima* L. fruit extract (LAFE). (A) ABTS radical scavenging potential of LAFE. (B) DPPH radical scavenging potential of LAFE. (C) FRAP reducing potential of LAFE. The analysis was performed three times ( $n = 3$ ), and the findings were presented as mean  $\pm$  standard deviation. Tukey's test was used to establish the statistical



significance between the test samples, and a  $p$ -value  $\leq 0.05$  was considered significant. In the specific study group, bar graphs with distinct alphabets indicate that statistically significant.

In support of our study, Darsini *et al.*, (2013) assessed the antioxidant potential of LAFE by DPPH, FRAP, Trolox equivalent antioxidant capacity, and total antioxidant activity (TAA) by phosphomolybdenum method, and revealed that LAFE has good antioxidant potential. Similarly, Pandey *et al.*, (2014) reported the  $IC_{50}$  value of *L. acidissima* L. fruit pulp methanolic extracts by ABTS assay was found to be 0.7 mg/ mL. The present study concluded that LAFE has good antioxidant potential and could be used to overcome oxidative stress-related disorders.

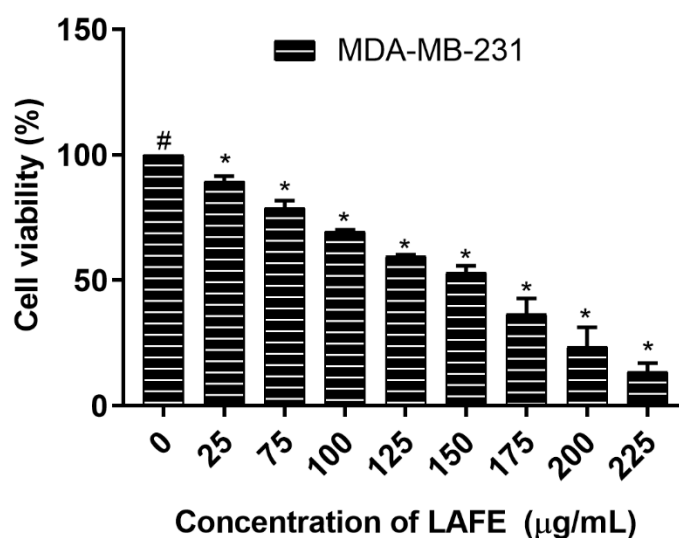
### 3.3 Anticancer activity of *Limonia acidissima* L. fruit extract

Cancer is a disorder in which the body's cells proliferate uncontrollably. Chemoprevention is a broad phrase that refers to the use of outside substances to inhibit or reduce the growth of cancer. In cancer chemoprevention, both synthetic and natural substances are proven to be beneficial. Investigating the causes and risk factors for common types is one of the best ways to find cancer prevention strategies. The type of treatment is chosen based on these variables as well as the patient's health status, cancer stage, and severity. Even though there are effective treatments, cancer continues to be one of the leading killers and serious public health concerns on a global scale. According to research, the majority of malignancies can be avoided and treated, or their onset can be postponed (Ferlay *et al.*, 2021; George *et al.*, 2021; Hanahan, 2022).

It has become standard practice in the treatment of cancer to use plant-based chemicals for chemoprevention. A major objective of anticancer research is the quest for new chemopreventive phytochemicals, which also aid in the identification of novel therapeutic targets. A number of dietary chemicals are effective cancer chemopreventive agents, and it has been demonstrated that eating foods containing these bioactive compounds has both protective and curative effects on a variety of cancer types. Plant-derived polyphenols play an immunomodulatory role in identifying and eliminating cancer cells through antiangiogenic actions. Chemopreventive medications improve the effectiveness of chemotherapy and radiotherapy through various signal transduction pathways. The antioxidant properties of dietary phenolic compounds may be a promising cancer prevention method because oxidative stress is a major factor in the etiology of many malignancies. In plants, many dietary antioxidant phytochemicals belong to various chemical classes and have different effects on tumors. Because of this, chemoprevention through diets high in plant-based antioxidants has a tremendous potential to lower the risk factors for the progression of cancer (Garcia-Oliveira *et al.*, 2021; George *et al.*, 2021).

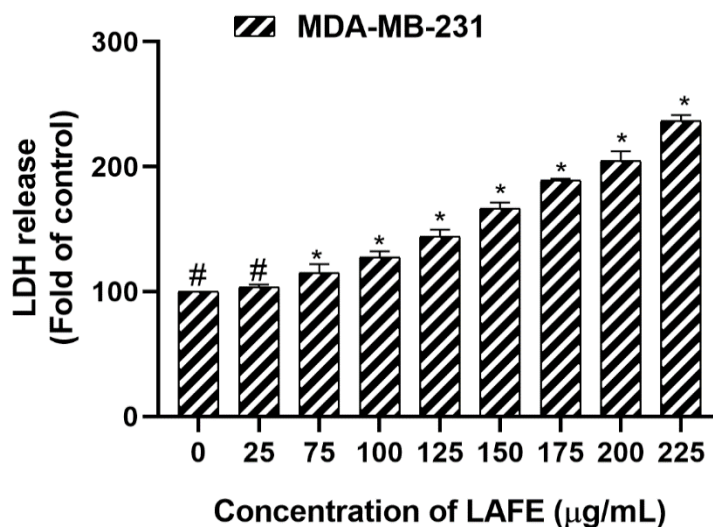
In the present study, the growth inhibitory effect of LAFE was revealed on cancer cells MDA-MB-231 (human mammary gland adenocarcinoma) by MTT, LDH, micro-morphological observation, and live/dead dual staining assays.

MTT assay determines the viability of the cells based on the mitochondria's enzymatic conversion of MTT tetrazolium salt to formazan. In the MTT assay, LAFE has dose-dependently reduced the growth of cancer cells (Fig. 3). In all the tested concentrations, LAFE has a significant ( $p \leq 0.05$ ) difference compared to the control sample. The  $IC_{50}$  (concentration required to inhibit 50% of cell viability compared to control) of LAFE was determined as  $158.03 \pm 4.92 \mu\text{g/mL}$ . The  $IC_{50}$  value of cisplatin was noticed as  $2.89 \pm 0.21 \mu\text{M}$ . The MTT assay showed that LAFE has the potential induced anticancer activity by affecting the function of the mitochondria.



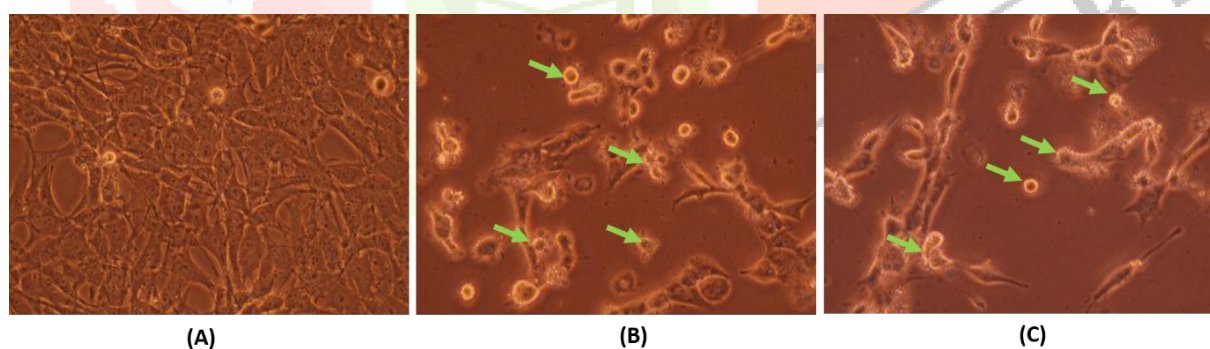
**Figure 3:** Effect of LAFE on MDA-MB-21 cell viability determined by MTT assay. The analysis was performed three times ( $n = 3$ ), and the findings were presented as mean  $\pm$  standard deviation. Dunnett's test was used to establish the statistical significance between the control and test samples, and a  $p$ -value  $\leq 0.05$  was considered significant. The test sample is not significantly different from the control sample, as indicated by the asterisk (#). While the asterisk (\*) indicates that the test sample is significant in comparison to the control.

Succeeding MTT assay, the anticancer activity of LAFE was revealed by LDH assay. The release of the lactate dehydrogenase (LDH) enzyme into the culture media following cell membrane injury is the basis for the LDH leakage assay. In the present study, LAFE has dose-dependently escalated the LDH levels (Fig. 4). The LDH study concluded that LAFE has induced cell death by damaging cell membrane integrity.



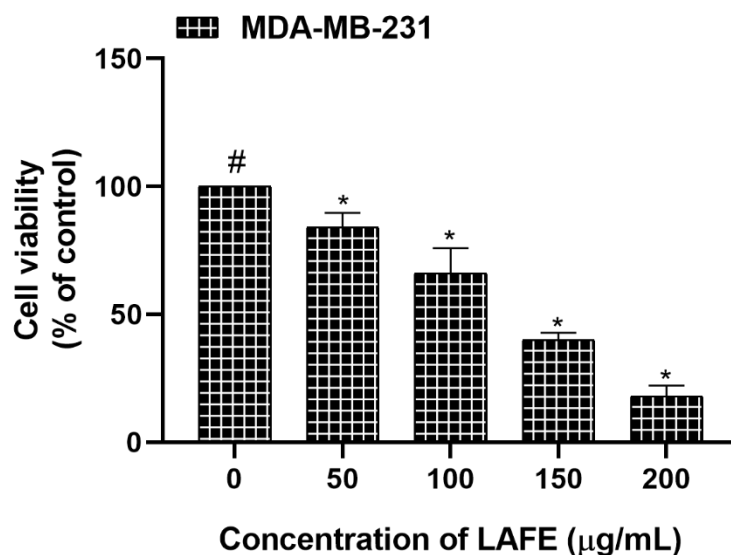
**Figure 4:** Effect of LAFE on MDA-MB-21 cell viability determined by LDH assay. The analysis was performed three times ( $n = 3$ ), and the findings were presented as mean  $\pm$  standard deviation. Dunnett's test was used to establish the statistical significance between the control and test samples, and a  $p$ -value  $\leq 0.05$  was considered significant. The test sample is not significantly different from the control sample, as indicated by the asterisk (#). While the asterisk (\*) indicates that the test sample is significant in comparison to the control.

Succeeding MTT and LDH assays, the effect of LAFE on the micro-morphology of cancer cells was revealed by bright-field inverted microscopy (Fig. 5). The control cells were found to have healthy morphology with a smooth surface and characteristic shape. Whereas, cells treated with  $IC_{50}$  values of LAFE and cisplatin exhibited detrimental changes in the micro-morphology which includes loss of monolayer, the appearance of cell debris, formation of apoptotic bodies, and a decrease in cell count. The study showed that LAFE has affected the viability of the cells by detrimentally inducing micro-morphological changes. The observed results were found in line with MTT and LDH assay.

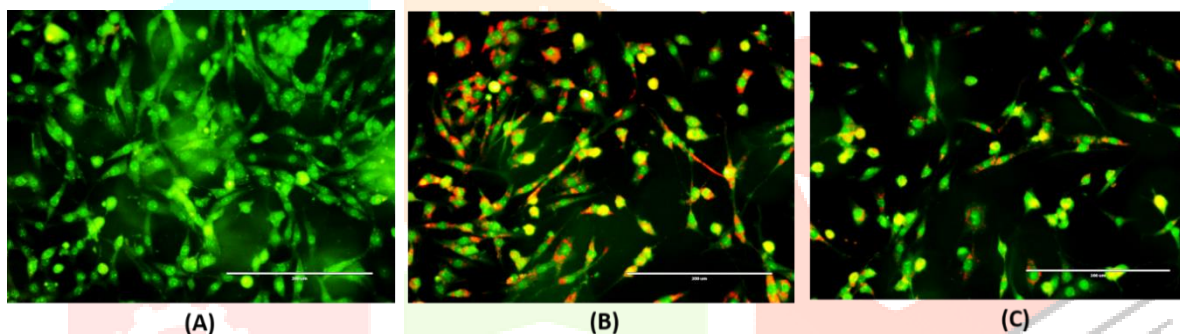


**Figure 5:** Detrimental effect of LAFE and cisplatin on micro-morphology of MDA-MB-21 cells captured by bright-field inverted microscope. (A) Control cells (B) Cells treated with  $IC_{50}$  value of LAFE ( $67.81 \pm 3.05 \mu\text{g/mL}$ ). (C) Cells treated with  $IC_{50}$  value of cisplatin ( $2.89 \pm 0.21 \mu\text{M}$ ). The images were presented at a magnification of 400x.

In the final part of assessing the anticancer activity of LAFE, a live/dead dual staining assay was considered. The stain kit calcein AM and ethidium homodimer-I. The calcein AM stains green color and represents viable cells. The ethidium homodimer-I stains red color and represents dead cells. The live/dead dual staining relies on membrane integrity, membrane damaged/compromised cells appear red and are determined as dead cells. In the present study, LAFE has dose-dependently decreased the viability of cells (Fig. 6). The effect of LAFE on cell viability was captured by fluorescent microscope using GFP and RFP filters, and images were shown in Figure 7. The control samples showed a green color and were determined as viable cells. Whereas, cells treated with  $IC_{50}$  value of LAFE and cisplatin showed a combination of green color and red color, and the red color shows that cells were dead. The assay concluded that LAFE has affected cell viability and increased the number of dead cells by damaging the cell membrane. The obtained results were in line with MTT, LDH, and micro-morphological observation.



**Figure 6:** Effect of LAFE on MDA-MB-21 cell viability determined by live/dead dual staining assay. The analysis was performed three times ( $n = 3$ ), and the findings were presented as mean  $\pm$  standard deviation. Dunnett's test was used to establish the statistical significance between the control and test samples, and a  $p$ -value  $\leq 0.05$  was considered significant. The test sample is not significantly different from the control sample, as indicated by the asterisk (#). While the asterisk (\*) indicates that the test sample is significant in comparison to the control.



**Figure 7:** Effect of LAFE and cisplatin on the viability of MDA-MB-21 cells visualized by live/dead dual staining assay. (A) Control cells (B) Cells treated with  $IC_{50}$  value of LAFE ( $67.81 \pm 3.05 \mu\text{g/mL}$ ). (C) Cells treated with  $IC_{50}$  value of cisplatin ( $2.89 \pm 0.21 \mu\text{M}$ ). The green color represents the viable cells and the red color represents the dead cells. The images were presented at a magnification of 400x.

It has long been known that medicinal herbs or plants can be a valuable source of therapies or curative aid. India is renowned around the world for its ancient expertise in "Ayurveda." Ayurveda knowledge has been used to treat a wide variety of illnesses. Wood apples are very valuable medicinally. The components of the fruit have medicinal value. To lessen throat and gum infection, the fruit pulp is consumed with sugar. Additionally, it eases sore throats. Gum bleeding and poor breath are both treated with it. Regular consumption of pulp in the morning works as a medicine to treat sterility brought on by a lack of progesterone hormones by toning the uterus and lifting the sagging breast. To relieve the discomfort brought on by venomous stings, the ripe pulp is rubbed. The unripe fruits are employed in the Ayurvedic medical system to cure diarrhea, pharyngodynia, and pruritus because they are naturally sour, fragrant, astringent, constipating, and alexipharmic. The unripe fruit is astringent, and the seeds are used to treat cardiac conditions.

In modern medicine, many researchers revealed the anticancer activity of LAFE. Pradhan *et al.*, (2012) assessed the antiproliferative effect of LAFE on human breast cancer cell lines SKBR3 and MDA-MB435 cells and revealed their potential anticancer role. Similarly, Eluru *et al.*, (2015) have demonstrated the antitumor activity of methanolic LAFE in a mice model of Dalton's ascitic lymphoma. In our study, the noticed outcome of the study was found in line with previous reports. The present study and earlier studies revealed that LAFE has potential anticancer activities. However, the in-detailed elemental composition of LAFE and the molecular level of anticancer research are needed to pinpoint the active ingredients that can cause growth inhibition and to demonstrate a potential link between the aforementioned functions of LAFE.

#### IV. Conclusion

The obtained LAFE was found rich in secondary metabolites such as phenolics and flavonoids. The LAFE has exhibited potential antioxidant activity in DPPH, ABTS, and FRAP assays and as a result, LAFE could be used as an antioxidant agent for revealing various oxidative stress-mediated disorders. The LAFE has shown potential anticancer activity on human Mammary gland adenocarcinoma MDA-MB-21 cells. The LAFE exhibited anticancer activity through down-regulation of metabolic process,



damaging cell membrane, and apoptosis. The LAFE could be highly useful as a potential anticancer agent in the biomedical field. However, in-detailed studies should be considered for approval of LAFE as a potential anticancer agent.

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### REFERENCES

- [1]. Alabri, T.H.A., Al Musalami, A.H.S., Hossain, M. A., Weli, A. M., and Al-Riyami, Q. 2014. Comparative study of phytochemical screening, antioxidant and antimicrobial capacities of fresh and dry leaves crude plant extracts of *Datura metel* L. *Journal of King Saud University-Science*, 26(3): 237-243.
- [2]. Bailar, J.C., and Gornik, H.L. 1997. Cancer undefeated. *New England Journal of Medicine*, 336(22): 1569-1574.
- [3]. Basse, E.E., Gwana, A.M., Halima, M.B., Umeh, S.O., Bagudu, B.Y., and Okali, C.M. 2016. Phytochemical analysis and antimicrobial activity of methanolic, ethanolic and acetonitrilic extracts of stem bark and leaf of neem plant (*Azadirachta indica*). *Journal of Diseases and Medicinal plants*, 2: 14-23.
- [4]. Bhalodia, N.R., Nariya, P.B., Acharya, R.N., and Shukla, V.J. 2013. In vitro antioxidant activity of hydro alcoholic extract from the fruit pulp of *Cassia fistula* Linn. *Ayu*, 34(2): 209.
- [5]. Chanda, S.V., and Kaneria, M.J. 2012. Optimization of conditions for the extraction of antioxidants from leaves of *Syzygium cumini* L. using different solvents. *Food Analytical Methods*, 5(3): 332-338.
- [6]. Chen, X., Hu, C., Zhang, Y., Hao, W., He, X., Li, Q., ... and Chen, Y. 2020. Anticancer activity and mechanism of action of kLa-TAT peptide. *International Journal of Peptide Research and Therapeutics*, 26(4): 2285-2296.
- [7]. Cheynier, V. 2012. Phenolic compounds: from plants to foods. *Phytochemistry reviews*, 11(2): 153-177.
- [8]. Darsini, D.T.P., Maheshu, V., Vishnupriya, M., Nishaa, S., and Sasikumar, J. M. 2013. Antioxidant potential and amino acid analysis of underutilized tropical fruit *Limonia acidissima* L. *Free Radicals and Antioxidants*, 3: S62-S69.
- [9]. Eluru, J.R., Taranalli, A.D., and Kawatra, S. 2015. Anti-tumour activity of *Limonia acidissima* L. methanolic extract in mice model of Dalton's ascitic lymphoma. *International Journal of Pharmacognosy and Phytochemical Research*, 7(6): 1094-1100.
- [10]. Farag, R.S., Abdel-Latif, M.S., Abd El Baky, H.H., and Tawfeek, L.S. 2020. Phytochemical screening and antioxidant activity of some medicinal plants' crude juices. *Biotechnology Reports*, 28: e00536.
- [11]. Ferlay, J., Colombet, M., Soerjomataram, I., Parkin, D.M., Piñeros, M., Znaor, A., and Bray, F. 2021. Cancer statistics for the year 2020: An overview. *International journal of cancer*, 149(4): 778-789.
- [12]. Fossen, T., & Andersen, Ø.M. 2006. Spectroscopic techniques applied to flavonoids. *Flavonoids: chemistry, biochemistry and applications*: 37-142.
- [13]. Gangwar, M., Gautam, M.K., Sharma, A.K., Tripathi, Y.B., Goel, R.K., and Nath, G. 2014. Antioxidant capacity and radical scavenging effect of polyphenol rich *Mallotus philippinensis* fruit extract on human erythrocytes: an in vitro study. *The Scientific World Journal*, 2014.
- [14]. Garcia-Oliveira, P., Otero, P., Pereira, A.G., Chamorro, F., Carpena, M., Echave, J., ... and Prieto, M.A. 2021. Status and challenges of plant-anticancer compounds in cancer treatment. *Pharmaceuticals*, 14(2): 157.
- [15]. George, B.P., Chandran, R., and Abrahamse, H. 2021. Role of phytochemicals in cancer chemoprevention: insights. *Antioxidants*, 10(9): 1455.
- [16]. Ginsburg, O., Yip, C.H., Brooks, A., Cabanes, A., Caleffi, M., Dunstan Yataco, J.A., ... and Anderson, B.O. 2020. Breast cancer early detection: A phased approach to implementation. *Cancer*, 126: 2379-2393.
- [17]. Gould, K.S. 2010. Muriel wheldale onslow and the rediscovery of anthocyanin function in plants. *Recent advances in polyphenol research*, 2: 206-225.
- [18]. Gupta, R., Sharma, P., and Saxena, A.M. 2006. Contribution of indigenous anti-diabetic herbs to alternative medicine of diabetes mellitus. *Journal of Medicinal and Aromatic Plant Sciences*, 28: 612-623.
- [19]. Hanahan, D. 2022. Hallmarks of cancer: new dimensions. *Cancer discovery*, 12(1): 31-46.
- [20]. Haslam, E. 2007. Vegetable tannins—Lessons of a phytochemical lifetime. *Phytochemistry*, 68(22-24): 2713-2721.
- [21]. Hatim, M.Y., and Makhawi, M.A. 2019. Phytochemical screening of leaves and roots of *Stylochiton borumensis*: a medicinal plant. *Eart & Envi Scie Res & Rev*, 2(1): 1-5.
- [22]. Kangralkar, V. A., Patil, S. D., Bandivadekar, R. M., Nandagaon, V. S., and Burli, S. C. (2010). Hepatoprotective activity of *feronia elephantum* fruit extract against paracetamol induced hepatic damage in Wistar rats. *International Journal of Pharmaceutical Applications*, 1: 46-9.
- [23]. Kirtikar, K.R., and Basu, B.D. 1918. Indian Medicinal Plants. *Indian Medicinal Plants*.
- [24]. Kumar, C.S., Mahesh, A., Antoniraj, M.G., Vaidevi, S., and Ruckmani, K. 2016. Ultrafast synthesis of stabilized gold nanoparticles using aqueous fruit extract of *Limonia acidissima* L. and conjugated epirubicin: targeted drug delivery for treatment of breast cancer. *RSC advances*, 6(32): 26874-26882.
- [25]. Lallianrawna, S., Muthukumar, R., Ralte, V., Gurusubramanian, G., and Kumar, N.S. 2013. Determination of total phenolic content, total flavonoid content and total antioxidant capacity of *Ageratina adenophora* (Spreng.) King & H. *Science vision*, 13(4): 149-156.
- [26]. MacMahon, B., Cole, P., and Brown, J. 1973. Etiology of human breast cancer: a review. *Journal of the National Cancer Institute*, 50(1): 21-42.
- [27]. Makkar, H. P., and Becker, K. 1993. Behaviour of tannic acid from various commercial sources towards redox, metal complexing and protein precipitation assays of tannins. *Journal of the Science of Food and Agriculture*, 62(3): 295-299.



- [28]. Michels, K.B. 2005. The role of nutrition in cancer development and prevention. *International Journal of Cancer*, 114(2): 163-165.
- [29]. Morgan, D.M. 1998. Tetrazolium (MTT) assay for cellular viability and activity. In *Polyamine protocols*, Humana Press. 179-184.
- [30]. Mutebi, M., Anderson, B.O., Duggan, C., Adebamowo, C., Agarwal, G., Ali, Z., ... and Eniu, A. 2020. Breast cancer treatment: A phased approach to implementation. *Cancer*, 126: 2365-2378.
- [31]. Pandey, S., Satpathy, G., and Gupta, R.K. 2014. Evaluation of nutritional, phytochemical, antioxidant and antibacterial activity of exotic fruit " *Limonia acidissima*". *Journal of Pharmacognosy and Phytochemistry*, 3(2).
- [32]. Patel, M.R., Panchal, H.S., and Saluja, A.K. 2016. Identification of terpenoids and steroidal compounds in *Caryota urens* leaves by column chromatography and various spectroscopic techniques. *World journal of pharmacy and pharmaceutical sciences*, 5(5): 1610-22.
- [33]. Pradhan, D., Tripathy, G., and Patnaik, S. 2012. Screening of antiproliferative effect of *Limonia acidissima* Linn. fruit extracts on human breast cancer cell lines. *African Journal of Pharmacy and Pharmacology*, 6(7): 468-473.
- [34]. Pratt, D.E. 1992. Natural antioxidants from plant material.
- [35]. Quideau, S., Deffieux, D., Douat-Casassus, C., & Pouységu, L. 2011. Plant polyphenols: chemical properties, biological activities, and synthesis. *Angewandte Chemie International Edition*, 50(3): 586-621.
- [36]. Shnoudeh, A.J., Qadumii, L., Zihlif, M., Al-Ameer, H.J., Salou, R.A., Jaber, A. Y., and Hamad, I. 2022. Green Synthesis of Gold, Iron and Selenium Nanoparticles Using Phytoconstituents: Preliminary Evaluation of Antioxidant and Biocompatibility Potential. *Molecules*, 27(4): 1334.
- [37]. Singleton, V.L., Orthofer, R., and Lamuela-Raventós, R.M. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. In *Methods in enzymology*, Academic press. 299: 152-178.
- [38]. Soquetta, M.B., Terra, L.D.M., and Bastos, C.P. 2018. Green technologies for the extraction of bioactive compounds in fruits and vegetables. *CyTA-Journal of Food*, 16(1): 400-412.
- [39]. Spaepen, P., Boodt, S.D., Aerts, J. M., and Sloten, J. V. 2011. Digital image processing of live/dead staining. In *Mammalian Cell Viability* (pp. 209-230). Humana Press.
- [40]. Stoddart, M.J. 2011. Cell viability assays: introduction. *Mammalian cell viability*, 1-6.
- [41]. Tamilselvi, N., Krishnamoorthy, P., Dhamotharan, R., Arumugam, P., and Sagadevan, E. 2012. Analysis of total phenols, total tannins and screening of phytoconstituents in *Indigofera aspalathoides* (Shivanar Vembu) Vahl EX DC. *Journal of Chemical and Pharmaceutical Research*, 4(6): 3259-3262.
- [42]. Tyagi, T. 2017. Phytochemical screening of active metabolites present in *Eichhornia crassipes* (Mart.) Solms and *Pistia stratiotes* (L.): Role in ethanomedicine. *Asian Journal of Pharmaceutical Education and Research*, 6(4): 40-56.
- [43]. Virgili, F., and Marino, M. 2008. Regulation of cellular signals from nutritional molecules: a specific role for phytochemicals, beyond antioxidant activity. *Free Radical Biology and Medicine*, 45(9): 1205-1216.
- [44]. Wild, C., Weiderpass, E., and Stewart, B.W. (Eds.). 2020. *World cancer report: cancer research for cancer prevention*. IARC Press.
- [45]. Willcox, J.K., Ash, S.L., and Catignani, G.L. 2004. Antioxidants and prevention of chronic disease. *Critical reviews in food science and nutrition*, 44(4): 275-295.
- [46]. Zehiroglu, C., and Ozturk Sarikaya, S.B. 2019. The importance of antioxidants and place in today's scientific and technological studies. *Journal of food science and technology*, 56(11): 4757-4774.