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Antiproliferative potential of *Ficus auriculata* fruit extract on A549 cell lines: Insights from gene expression analysis

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Abstract
Ficus auriculata fruit have gained attention for their potential health benefits, particularly their antioxidant and anticancer properties. The study was aimed to analyse anticancer activity of these fruits. Three different solvents (hexane, methanol, and water) were used for extraction. Phytochemical analysis, antioxidant activity and anticancer potential was evaluated on A549 and L132 cell lines. DNA damage patterns were analysed using the comet assay, and gene expression of epigenetic modulators was examined via qRT-PCR. The study found that the methanolic extract had a high phenolic content and strong antioxidant activity, effectively scavenging free radicals. Both the methanolic and water extracts demonstrated potent anticancer effects on A549 cells. DNA damage analysis indicated potential mechanisms of their anticancer action, with observed double-strand DNA damage in A549 cells. Gene expression analysis revealed intriguing decreased epigenetic modulators except HAT. *Ficus auriculata* fruit extracts, particularly the methanolic and water extracts, exhibit promising antioxidant and anticancer properties.

Keywords: *Ficus auriculata*, A549, antioxidant assay, cell viability assay, gene expression analysis, Genotoxicity

Introduction
Changes in epigenetic processes, which contribute in the conversion of normal cells to malignant cells, are a fundamental cause of mortality and a substantial hurdle to prolonging life expectancy, despite considerable breakthroughs in therapies and preventative drugs. According to the World Health Organization (WHO) cancer is the leading cause of death before the age of 70 in the maximum number of countries [1]. With an expected 19.3 million new cases and 10.0 million cancer deaths in 2020, cancer is the leading cause of morbidity and mortality globally [2]. Lung cancer is the leading cause of cancer death followed by colorectal and liver cancer [3]. Changed lifestyle and high fresh fruit and vegetable intake can greatly decrease the cancer death rate [4]. Insufficient or low intake of fruit and vegetables is one of the top risk factors of global mortality [5]. A bioactive substance is one that affects a living organism, tissue, or cell in some way. Bioactive substances are different from primary nutrients. Bioactive chemicals are not required for an organism's existence because the body can function correctly without them or because nutrients provide the same goal. These compounds are of animal and plant origin. Bioactive and antioxidant compounds such as phenols, flavonoids, tannins, and alkaloids are found naturally in fruits and vegetables [6]. Fruits and vegetables build high levels of phytochemicals, which may protect against free radical damage. Plants that possess valuable phytochemicals can help complement human needs by functioning as natural antioxidants [7]. Fruit and vegetable ingestion has been linked to a variety of health advantages like reduced risk of chronic disease, lowers the cardiovascular diseases, body weight management and in some cancers due to their high therapeutic characteristics and high nutritional content that includes bioactive compounds, antioxidants, high fibre, vitamins and minerals [8, 9]. Lung cancer is the second highest cause of death among cancer patients, and it is one of the most fatal types of cancer [10]. Lung cancer patients have a poor prognosis owing to late-stage diagnosis and the ineffectiveness of existing treatment drugs. As a result, identifying and researching alternative medicine for lung cancer therapy and prevention is critical. Lung cancer is of two main types on the basis of their histological, clinical, neuroendocrine characteristics and molecular or genetic differences. These are, namely non-small cell lung cancer (NSCLC) comprising ~85% of all lung cancer types while ~20% of lung cancer is of small cell lung cancer (SCLC).

Non-small cell large cells are further subtypes on the basis of their histology and molecular characteristics into adenocarcinoma, squamous carcinoma, large cell carcinoma, and bronchoalveolar lung cancer. Small cell lung cancer is divided into small cell carcinoma comprising 95% of SCLC type and combined small cell carcinoma consisting of 5% of SCLC (Figure 1) [11].

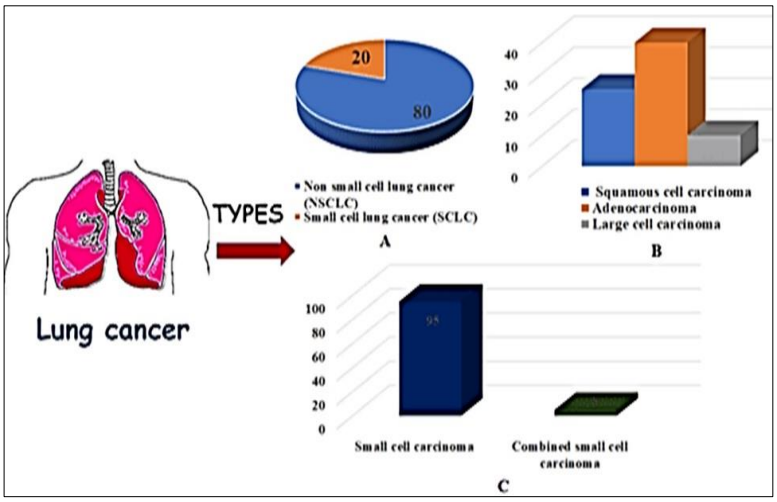


Fig 1: Types of lung cancer on the basis of their histology, molecular and genetic differences.

In cancer, widespread epigenetic changes, including DNA hypomethylation, collaborate with genetic mutations to drive tumour development. These changes affect gene regulation through DNA methylation, histone modifications, and chromatin remodelling. Epigenetic alterations can induce genetic mutations, and vice versa. Epigenetic therapies, effective for certain cancers, aim to reverse these changes and are gaining traction in solid tumour treatment [12].

Natural product alternatives are one of the most promising sources of therapy or prevention. *Ficus* is a fruit that has been utilised as a cancer therapy in numerous civilizations throughout history. *Ficus auriculata* is one of Uttarakhand most prominent ethno-medicinal plants. Timli, Timul, Timla and Timula are some of its local names. The Roxburgh fig, F. auriculata Lour. (family Moraceae), is a significant medicinal plant in the state. Plants can be found between 1800 and 2600 metres above mean sea level in temperate, tropical, and sub-tropical climates. The plant is dioecious and grows to be around 4-10 metres tall. White latex can be seen in abundance throughout the plant. *Ficus auriculata* leaf extract was found to be beneficial in preventing cell adhesion and migration, as well as regulating carcinogenic chemicals [13]. *Ficus auriculata* fruits contain a variety of phenolics that have anti-diabetic, anti-cancer, antioxidant, and anti-inflammatory characteristics [14]. Secondary metabolites such as myricetin and quercetin-3-o-ß-D-glucopyranoside have been identified from *Ficus auriculata* fruit [14]. The primary phytoconstituents of *Ficus auriculata* fruit are glycosides and saponins, which have been linked to a variety of medicinal actions [15].

The objective of this study is to understand the chemical composition of *Ficus auriculata* fruit extracts, evaluate their potential health benefits, especially antioxidant and anticancer properties and identify modes of action, by evaluating effect of extract on DNMT, HAT, MBD and HAT gene by gene expression analysis.

Material Method

Ficus auriculata fruits were collected from Sangarh village, District Bageshwar, Uttarakhand, during their ripening season in June, 2020. To ensure the accuracy of the plant's identity, the plant was authenticated by Dr. Anamika Singh from the Department of Botany at Vardhman College in Bijnor, Uttar Pradesh. A549 cell line and normal cell line L132 were obtained from the National Centre for Cell Science (NCCS) in Pune.

Methods

The collected fruits were sterilised by using a 0.1 percent $HgCl_2$ solution. After sterilisation, the fruits were dried in the shade for a period of two to three weeks. Once dried, the fruits were powdered, and 5 grams of the powder were subjected to Soxhlet extraction. The extraction process involved three cycles per hour, with each cycle lasting for 4 hours. This resulted in a total of 12 cycles. The extraction was carried out using 500 ml of hexane, methanol, and water as the solvents. After completion of the extraction process, the extracts were dried and weighed. They were then diluted in their respective solvents, up to a final volume of 20 ml.

Preliminary phytochemical screening of extracts

Fruits were pre-screened for their phytoconstituents like Alkaloid [16], flavonoid [17], tannins [18], cardiac glycosides [19], steroids (Liebermann-Burchard reaction), saponins [20].

Total Phenolic content (TPC)

The Folin-Ciocalteu reagent was used to determine the total phenolic content of the sample [21]. Briefly, 1 ml of the crude samples were combined with 0.5 ml of FC reagent and left to incubate at room temperature for 15 minutes followed by addition of 5 ml of 7.5% Na_2CO_3. Reaction mixture was then incubated for 1.5 hrs. at room temperature. The absorbance was measured at 765 nm. To create a standard curve, dilutions of gallic acid ranging from 0.2 to 11 µg/ml were prepared. The phenolic content was then calculated using the linear equation derived from the standard graph of gallic acid and was represented as mg of gallic acid equivalent (GAE) per 100 gm of dried fruit sample.

Total Flavonoid content (TFC)

Flavonoid content was estimated by Total flavonoid was estimated by aluminium chloride colorimetric method [22]. Briefly 0.5 ml of each of 1.2 % aluminium chloride and 1M

potassium acetate was added to 0.5 ml of fruit extract. Reaction mixture was diluted to 3 ml by methanol and incubated at room temperature for 30 min. Absorbance was taken at 415 nm. Blank was set by adding all reagents except extracts. Standards of quercetin were prepared in the range of 0.01 mg/ml to 0.150 mg/ml. TFC was calculated by using the standard curve equation obtained from standard curve of quercetin and was represented as mg of quercetin equivalent (QE) per 100 gm of dried fruit sample.

Antioxidant activity of crude extracts

For antioxidant assay all the crude extracts were diluted to 1 mg/ml concentration.

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

To evaluate the crude extract's DPPH free radical scavenging activity, the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was utilised as a marker [23]. A reaction mixture was prepared by combining 1.0 ml of 0.1 mM DPPH with 0.1 ml of the extract. This mixture was then incubated in the dark at room temperature for 30 minutes. The extent of DPPH inhibition was measured by monitoring the drop in absorbance at 517 nm. Ascorbic acid was used as the positive control. The radical scavenging activity was calculated as the percentage of free radical inhibition using the formula:

$$\%DPPH\ inhibition = \left(\frac{A0 - At}{A0}\right) * 100$$

where *A0* represents the absorbance of the control (blank without the sample), and *At* represents the absorbance in the presence of the sample. All tests were performed in triplicate, and the mean values were used to plot the graph.

2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay

The ABTS assay was conducted following the protocol outlined by Pellegrini *et al.*, 1958 [24]. A solution containing 7 millimolar (mM) of ABTS in water and 2.45 mM of potassium persulfate was mixed in a 1:1 ratio and incubated in the dark for 12-16 hours. The resulting mixture was then diluted to achieve an absorbance of approximately 0.7 at a wavelength of 734 nm. To conduct the experiment, 0.1 ml of an extract were combined with 3.9 ml of the reagent and incubated in the dark for 10 minutes. Absorbance readings were taken at 734 nm. A control sample was prepared by measuring the absorbance of ABTS and methanol. Simultaneously, a standard curve of ascorbic acid was generated using a range of standard dilutions ranging from 1 to 5 µg/ml. The $ABTS^{+}$ scavenging effect (%) was calculated using the following equation.

$$ABTS + \ scavenging\ activity\ (\%) = \left(\frac{Ab - Aa}{Ab}\right) * 100$$

Where, *Ab*=Absorbance of ABTS reagent, *Aa*=Absorbance of extracts.

Ferric reducing antioxidant power (FRAP)

The FRAP assay was estimated according to the protocol of Benzie *et al.*, 1996 [25]. To conduct the FRAP assay, 100 µl of an extract with a concentration of 1 mg/ml was added to a 3 ml FRAP reaction solution. The mixture was then incubated at room temperature for 6 minutes. Absorbance readings were taken at 593 nm for each sample. To establish a standard curve in the range of 200-1000 µM, $FeSO_4$ was used. The FRAP values for both the standard solutions and the samples were determined and expressed as µM Fe[II]/g dry weight.

Hydrogen peroxide (H_2O_2) radical scavenging activity

Hydrogen peroxide scavenging activity of crude extracts were estimated according to the method of Ruch *et al.*, 1989 [26]. In order to determine the H_2O_2 scavenging activity, the following procedure was conducted. Initially, a 0.6 ml aliquot of a 40 mM H_2O_2 solution was mixed with 0.1 ml of a diluted crude extract. To this mixture, 2.4 ml of phosphate buffer (0.1 M, pH 7.4) was added, and the resulting mixture was vigorously shaken and allowed to incubate at room temperature for 10 minutes. Subsequently, the absorbance of the reaction mixture was measured at a wavelength of 230 nm. As a reference, ascorbic acid was utilised as a positive control. The H_2O_2 scavenging activity was quantified using the following formula:

$$\%H_2O_2\ inhibiton\ = \frac{A1\text{-}A2}{A1} * 100$$

where *A1* represents the absorbance of the control sample (containing H_2O_2 without the extract), and *A2* denotes the absorbance of the test sample (containing H_2O_2 with the extract).

Thin Layer Chromatography of crude extracts

TLC was used to analyse flavonoids, alkaloids, and phenolic compounds. Silica gel plates were activated at 100 °C for an hour, and crude extracts were spotted 1 cm above the plate's edge. After air drying, plates underwent TLC with different solvent ratios: Meth: Chlo: Hex: Water (3:1:3:3) for flavonoids, ethyl acetate: formic acid: acetic acid: methanol (7:1.1:1.1:2.6) for phenolics, and n-butanol: acetic acid glacial: methanol (2:2:6) for alkaloids. Reagents were sprayed – 1% ethanolic $AlCl_3$ for flavonoids, Dragendorff for alkaloids, and $FeCl_3$ (2.7% in 2M HCl) for phenolics. Plates were dried and viewed under visible, short UV (254 nm), and long UV (366 nm) light.

Screening of anticancer activity of crude extracts

Cells were trypsinized and pelleted, followed by subculturing in DMEM media with 10% FBS and incubating in a CO_2 incubator at 5% CO_2, 95% humidity, and 37 °C till they reached 70–80% confluency. One ml of each extract was evaporated and dissolved in 0.5 ml of DMSO. Monolayer cells of L132 and A549 were trypsinized, and 0.1 ml of cells were seeded in a 96-well microtiter plate. Cells were incubated with 0.1 ml of each extract for 24 hours. Two wells were set as cell controls and did not receive any extract. The supernatant was discarded, and 25 µl of MTT (5 mg/ml) was added to each well, followed by 2 hours of incubation at 37^0 C in the CO_2 incubator. Each well received 100 µl of DMSO and was agitated for 15 min, followed by taking absorbance at 490 nm. The extracts with positive anticancer activity and no to minimal toxicity towards normal cells were selected for further IC50 estimation.

Cell viability assay and IC50 estimation by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The Mosmann procedure (1983) [27] was used to measure cell viability and IC50. Briefly, each well contained 0.1 ml of A549 or L132 cells. Concentrations of crude extracts ranged

from 0.128 to 4.48 mg/ml. Doxorubicin was diluted to quantities ranging from 5 to 40 µg/ml separately. Cells were combined with various dilutions of crude and doxorubicin and cultured for 24 hours in a CO_2 incubator at 37°C with 5% CO_2 and 95% humidity. Two wells were set as cell controls and did not receive any extract. The supernatant was discarded, and 25 µl of MTT (5 mg/ml) was added to each well, followed by 2 hours of incubation at 37^0 C in the CO_2 incubator. Each well received 100 µl of DMSO and was agitated for 15 min, followed by taking absorbance at 490 nm. % of cell viability was estimated by using following formula:

$$\%\, Cell\ viability = \left(\frac{Cell\ treated}{Cell\ ctrl}\right) * 100$$

IC50 was calculated from a log curve equation obtained by plotting a graph between log of conc. of extracts and % cell viability.

Morphological analysis of cells under IC50 treatment

Cells from the L132 and A549 cell lines were introduced to a six-well microplate, starting with an approximate initial cell count of $2.6*10^6$ cells/ml. Extracts and doxorubicin at IC50 concentrations were administered, and the cells were subjected to incubation periods of 24, 48, and 72 hours. Simultaneously cells without treatment were also set for each respective incubation duration. Following a specific day of incubation, the cells were observed using an inverted microscope Magnus INVI at 40X to detect any changes in their morphology. Subsequently, the cells were trypsinized to estimate cell numbers by trypan blue cell exclusion method and conduct comet assay analysis.

Genotoxicity assay or comet assay to estimate DNA damage under treatment

Comet assay was performed for genotoxicity analysis using the protocol of Lu *et al*., 2017 [28]. Briefly, the comet slides were prepared by coating a microscopic slide with a 1% medium melting point agar, which was then dried at 50°C until a thin layer formed. These prepared slides were stored at 37 °C. The cells (IC50 treated with Dox and FAW and incubated for 72 hrs.), after trypsinization, were mixed with a 1% low melting point agarose at a ratio of 1:10 (v/v). The mixture was carefully mixed by pipetting up and down, and then 30 µl of the cell-agarose mixture was loaded onto the prepared slide. The slides were incubated at 40 °C for 20-30 minutes in the dark. Following the incubation, the slides were immersed in a lysis solution containing 2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris-Cl, 0.2 M NaOH, 1% SDS, and 1% Triton X-100, pH 10. The slides were incubated overnight in the dark at 40 °C. For the alkaline comet assay, the slides were immersed in a cold alkaline electrophoresis solution consisting of 0.2 M NaOH and 0.001 M EDTA, pH 13 for 1 hour at 40 °C in the dark. Subsequently, the slides were electrophoresed at 15V for 30 minutes in the chilled alkaline electrophoresis solution. For the neutral comet assay, the slides were dipped in a cold neutral electrophoresis solution containing 0.1 M Tris-Cl, 0.3 M sodium acetate at pH 9 for 30 minutes at 40 °C in the dark. The slides were then electrophoresed at 15V for 30-45 minutes in the chilled neutral electrophoresis solution. After the electrophoresis, the slides were gently removed and immersed in a DNA precipitation solution composed of 1.005 M ammonium acetate in absolute alcohol for 30 minutes at room temperature. Following this, the slides were immersed in 70% ethanol for 30 minutes at room temperature. For both the alkaline and neutral comet assays, the slides were dried at 37 °C for 10-15 minutes in the dark. The comets were stained with 100 µl of Sybr Green nucleic acid stain for 15 minutes. Finally, the slides were washed with distilled water and visualised under a fluorescence microscope Magnus MX21iLED 40X with blue and green filters. The resulting images were analysed using Casp 1.2.3b1 software.

Gene expression analysis

RNA isolation

An overnight grown monolayer of L132 and A549 cells with a cell number of approximately $2.6*10^4$ cell/ml was treated with the IC50 concentrations of extracts and positive drug doxorubicin in a six wells microtiter plate. Two wells were seeded with only L132 and A549 cells without any treatment. The cells were then incubated for 72 hours at 5% CO_2 and 95% humidity in a CO_2 incubator. After incubation, the cells were trypsinized and pelleted by centrifugation at 2000 rpm for 10 minutes. The cell pellets were then processed for RNA isolation following the protocol described by Rio *et al.*, 2010 [29]. Briefly, the pellet was resuspended in 1 ml of Trizol and mixed properly by inverting to dissolve the pellet followed by addition of 0.2 ml of chloroform and vigorously mixed for 15 seconds, The mixture was incubated for 2-3-minute at room temperature. The mixture was then centrifuged at 10,000 rpm for 10 minutes at room temperature. The upper aqueous phase was carefully transferred to another microcentrifuge tube, and 500 µl of isopropanol was added to the aqueous phase. The mixture was vigorously mixed and allowed to rest for 10 minutes. Subsequently, the mixture was centrifuged at 10,000 rpm for 10 minutes at 4 °C, and the supernatant was discarded. The resulting pellet was immediately suspended in 1X SDS solubilization buffer (20mM Tris-Cl, 1mM EDTA, pH 8.0). To the solution, 3M sodium acetate was added to a final concentration of 0.3M. Then, double the volume of PCI was added, and the mixture was vortexed for 1 minute. After centrifugation for 5 minutes at 10,000 rpm at room temperature, the aqueous layer was carefully transferred to another tube. Double the volume of CIA was added, vortexed, and centrifuged at 10,000 rpm for 2 minutes. The aqueous phase was once again transferred to another tube. To the aqueous phase, double the volume of absolute ethanol was added, and the mixture was centrifuged at 10,000 rpm for 5 minutes. The supernatant was discarded, and the RNA pellet was air-dried. Finally, the pellet was suspended in 70 µl of nuclease-free water and stored at -20 °C until further use. RNA quality was estimated on 1% TBE gel run for 30-40 minutes at 60V in cold conditions, while RNA purity and quantity were measured at 260 nm and 280 nm, respectively, using a Helios alpha UV-Vis spectrophotometer.

cDNA first strand synthesis

The cDNA first strand was successfully synthesised using the Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit #K1621. Initially, all the kit components were thawed and centrifuged at 2000 rpm for 2 minutes. The RNA

concentrations of all samples were optimised to 5 µg in a total volume of 20 µl. Reaction mixture was set up as follows: RNA template (5 µg), oligo (dt)18 primer (1 µl), and nuclease-free water were combined in PCR vials to reach a total volume of 20 µl. The reaction mixture was gently mixed, briefly centrifuged, and incubated at 65 °C for 5 minutes using a thermal cycler. Following the initial incubation, the mixture was chilled on ice, spun down, and returned to ice. The enzyme mix, comprising 5X reaction buffer (4 µl), RiboLock RNase Inhibitor (1 µl), 10 mM dNTP mix (2 µl), and RevertAid M-MuLV Reverse Transcriptase (1 µl), was added to the reaction mixture. After gentle mixing and brief centrifugation, the reaction was incubated for 60 minutes at 42 °C to allow cDNA synthesis. The reaction was terminated by heating the mixture at 70 °C for 5 minutes. Finally, the cDNA reaction products were stored at -20 °C for future use.

DNMTs, MBDs, HDAC and HAT gene expression analysis

Genes were quantified following the MIQE guidelines. The primers for target genes were designed using Primer Express software, while the primers for the selection of the reference gene were obtained from previous studies (Table 1) [30].

Table 1: List of Primers synthesized for target gene and reference gene.

List of genes for PCR	Forward sequence	Reverse sequence	Product/amplicon size
HAT	GAGAAGAAACTGGCAGAG	GTCGTTCAATAACACGCC	1192
HDAC1	GGGTCATGACTGTGTCCT	TCAGGCCAACTTGACCTC	875
HDAC2	AGCCTCATAGAATCCGCA	CTTCGACCTCCTTCTCCT	1204
HDAC3	CATTAACTGGGCTGGTGG	ATCTCCACATCGCTTTCC	906
HDAC8	TTATGACTGCCCAGCCACTG	TCTTTGCATGATGCCACCCT	140
HDAC11	TGAACATAGGTAGCCGGG	AATGAACACAAGTCCCGT	296
MBD1	ATGGCTGAGGACTGGCT	TCTGTTCCCGGTTGAAGG	505
MBD2	ATTCCAAGGGCTCGGTTACG	TTTGATTGAGAGGATCGTTTCGC	751
MBD3	CCTGAGCACCTTCGACTTCC	TCCTCCTCGTCTTCCTCGT	~657
DNMT1	CCCATAAATGAATGGTGGATCACTG	TCCATCAGAATGTATTCGGCAAATG	92
DNMT3A	GGTCACGCAAAACAGAACCC	CCTTGGTGAAACCCTTTGCG	293
DNMT3B	AAGAGTTTAATTTTTGAAAACTGGCTACTG	AAAGACATATACAACATTTTCTCTTCTGCT	143
BACT	GAAGATCAAGATCATTGCTCCT	TACTCCTGCTTGCTGATCCA	111 [30]
GAPDH	ATGGGGAAGGTGAAGGTCG	GGGGTCATTGATGGCAACAATA	108 [30]
PGK1	GCCACTTGCTGTGCCAAATG	CCCAGGAAGGACTTTACCTT	102 [30]
18s rRNA	GTGGAGCGATTTGTCTGGTT	AACGCCACTTGTCCCTCTAA	115 [30]
PPIA	TCCTGGCATCTTGTCCAT	TGCTGGTCTTGCCATTCCT	179 [30]

The specificity of the primers was verified using the NCBI Primer-BLAST tool. To assess the PCR efficiency, the cDNA was diluted in a 10-fold series. To select a stable housekeeping gene, five reference genes, GAPDH, 18s, ACTB, PPIA and PGK1 were assessed. The Ct values or data obtained from these housekeeping genes were analysed using RefFinder, which utilises algorithms such as Best Keeper, geNorm, and Norm Finder for comparative analysis. The selection of the most stable reference gene was based on the least standard deviation observed across different samples. This comprehensive approach ensures that the chosen reference gene is stable and reliable for normalisation. B-actin (ACTB) was chosen as a stable reference gene for further analysis due to its consistent stability across different samples, resulting in the least standard deviation. For gene expression analysis, the cDNA concentration was optimised to 50 ng in all samples. The PCR reaction mixture consisted of 2X Kappa Sybr Fast Master Mix with ROX as a passive reference dye (25 µl), forward primer (2 µl), reverse primer (2 µl), cDNA (5 µl), and nuclease-free water (16 µl). The PCR program included UNG activation at 50 °C for 2 minutes, DNA polymerase activation at 95 °C for 10 minutes (1 cycle), and amplification with denaturation at 95 °C for 15 seconds followed by annealing at 60 °C for 1 minute for 40 cycles on in gene quantification experiments. All PCR reactions were performed using an ABI Prism 7000 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA).

Result and Discussion

The extraction procedure produced yields of 17.81%, 18.02%, and 18.502% when utilising hexane, methanol, and water solvents in that order. Employing different extracts did not notably alter the percentage yields. The percentage yield is markedly influenced by the drying technique, extraction method, and the specific solvent employed. Other factors that can also affect the percentage yield include the temperature and duration of the extraction process. Additionally, variations in the quality and quantity of the starting material can also impact the overall yield.

Phytochemical analysis of crude extracts

The analysis of various *Ficus auriculata* fruit extracts revealed that flavonoids were consistently more abundant than phenolics. Among the different extracts studied, it was observed that the hexane extract contained the lowest phenolic content but a notable amount of flavonoids (Table 2).

Table 2: Phytochemical screening of *Ficus auriculata* hexane, methanol and water extracts. +ve: mildly present, ++ve: moderate, +++ve: abundant, -ve: absent.

Fruits	Extract	Phenol	Alkaloid			Flavonoid	Tannins	Steroids	Saponins
			Mayer; s test	Dragendorff test	Wagner's test				
Ficus auriculata	Hexane	+ve	-ve	+ve	+ve	+ve	-ve	-ve	-ve
	Methanol	++ve	+ve	+ve	+ve	+ve	++ve	-ve	-ve
	Water	+++ve	-ve	-ve	-ve	+++ve	+++ve	-ve	-ve

The water extract demonstrated the greatest flavonoid concentration, containing 7.98 mg of QE per gram of dried sample. In contrast, the methanolic extract displayed the highest phenolic content, recording 33.37 mg of GA equivalents per gram of dried fruit sample (Figure 2).

The phenolic content in the methanolic extract closely aligns with the findings of Shrestha *et al*. in 2023 [31], where they determined the phenolic and flavonoid content of *Ficus auriculata* fruit methanolic extract as 27.59 mg GAE/g and 4.07 mg QE/g, respectively.

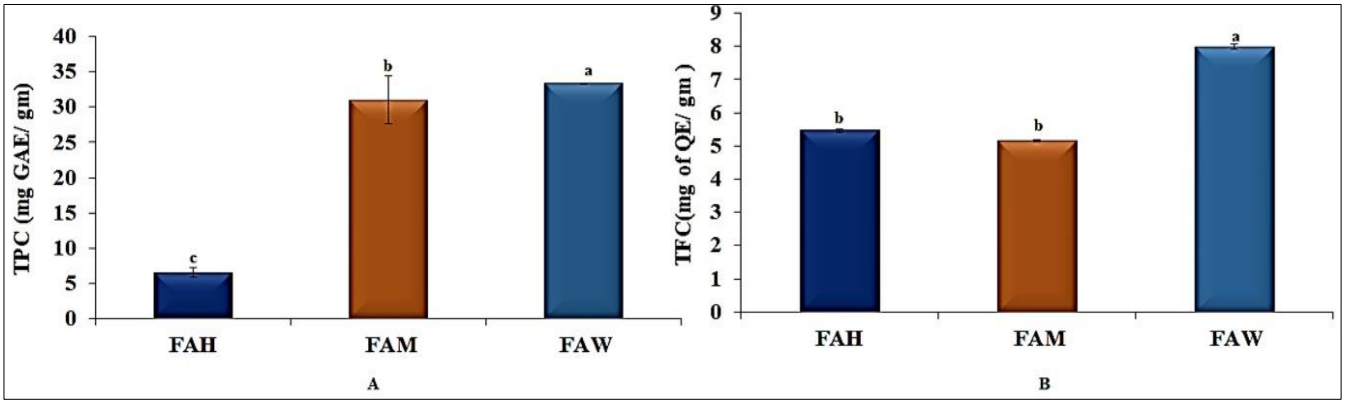


Fig 2: A: Total phenolic content (TPC) and B: Total flavonoid content (TFC) of *Ficus auriculata* fruit extracts. All the data are represented as mean ± Std dev. where, a, b, c indicate level of significance within the samples.

Different TPC and TFC of extract may be due to variations in the extraction methods or the maturity of the fruit at the time of extraction. Additionally, environmental factors such as soil composition and climate could also contribute to differences in phenolic content. Statistical examination of extract TFC and TPC content indicated substantial variations in TPC content amongst extracts, demonstrating the influence of solvent selection on extract phenolic content. While there is no statistically significant difference in flavonoid concentration between methanolic and hexane extracts, there is a significant difference between water extracts and the other two extracts.

Antioxidant activity of *Ficus auriculata* extracts

At a dosage of 1 mg/ml, antioxidant tests of *Ficus auriculata* fruit extracts indicated that the methanolic extract was more effective at decreasing DPPH than ABTS free radicals. Both methanolic and water extracts demonstrated significant antioxidant activity against DPPH and ABTS radicals. Methanolic extract inhibited DPPH by 98.6%, which is equivalent to ascorbic acid's DPPH inhibition action at the same dose. While water extract was more active against ABTS, with an ABTS inhibition percentage of 88.4%. Methanolic extract has a FRAP value of 3028.5 and an H_2O_2 scavenging activity of 91.48% (Figure 3), making it a powerful antioxidant agent with therapeutic potential.

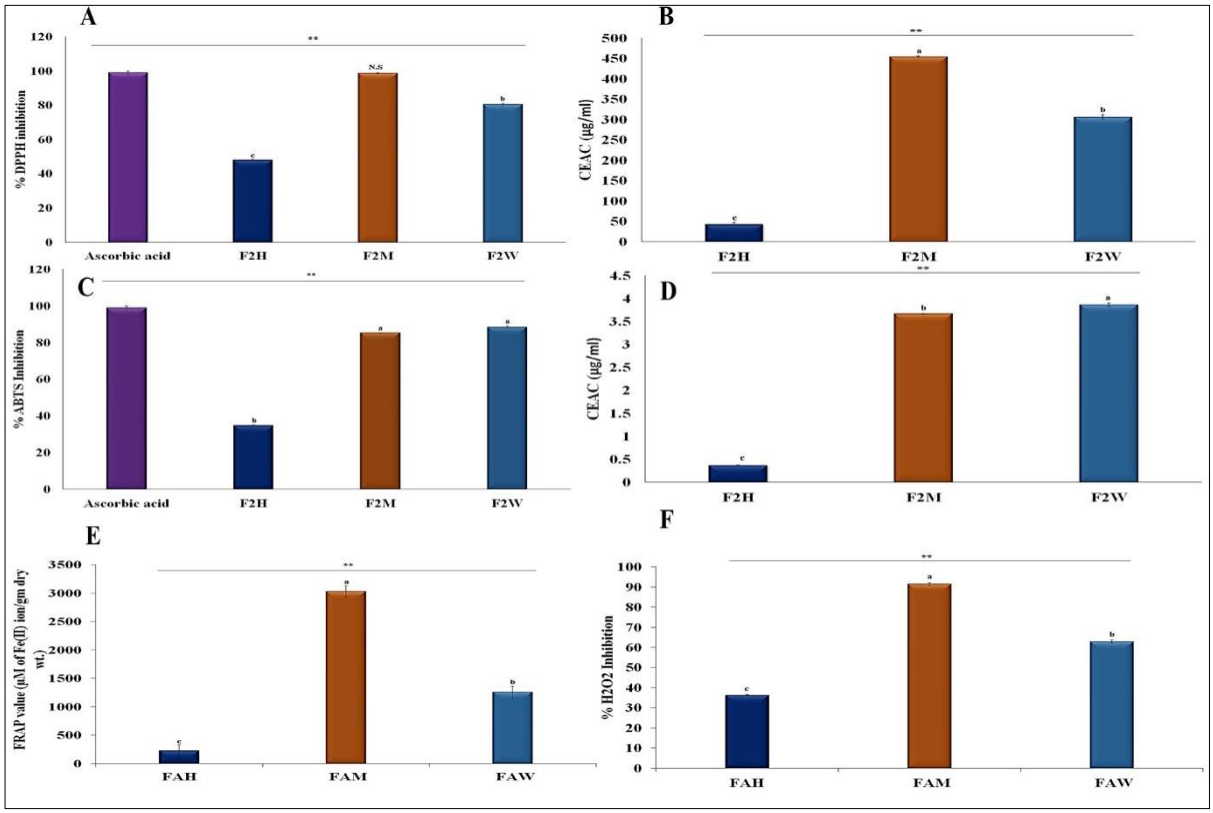


Fig 3: Antioxidant activity of *Ficus auriculata* extracts.

A: % DPPH inhibition, B: Vitamin C equivalent Antioxidant capacity by DPPH assay (CEAC) (µg/ml), C: % ABTS inhibition D: Vitamin C equivalent Antioxidant capacity by ABTS assay (CEAC) (µg/ml), E: Ferric reducing antioxidant power (FRAP) value in µM of Fe(II) ion/gm dry wt. Of extracts, F: % H_2O_2 scavenging activity of each extract. All the data are represented as mean ± std. Dev. where n=3, letters a,b,c indicate level of significance within the group and ** indicate, $p<0.0005$.

Correlation between Antioxidant activity, TPC and TFC of *Ficus auriculata*: The research conducted showed a strong and positive connection between the phenolic content in the extracts and their antioxidant activity. This link was evidenced by the standard curve equation and the coefficient of determination (R^2), which were calculated as 0.9502 for the correlation between total phenolic content (TPC) and DPPH activity, 0.9641 for TPC and ABTS activity, and 0.8455 for TPC and H_2O_2 activity (Figure 4). The antioxidant activity was expressed as the percentage of inhibition. Moreover, the relationship between TPC and the Ferric Reducing Antioxidant Power (FRAP) assay was also determined, resulting in an R^2 value of 0.5291 (Figure 4).

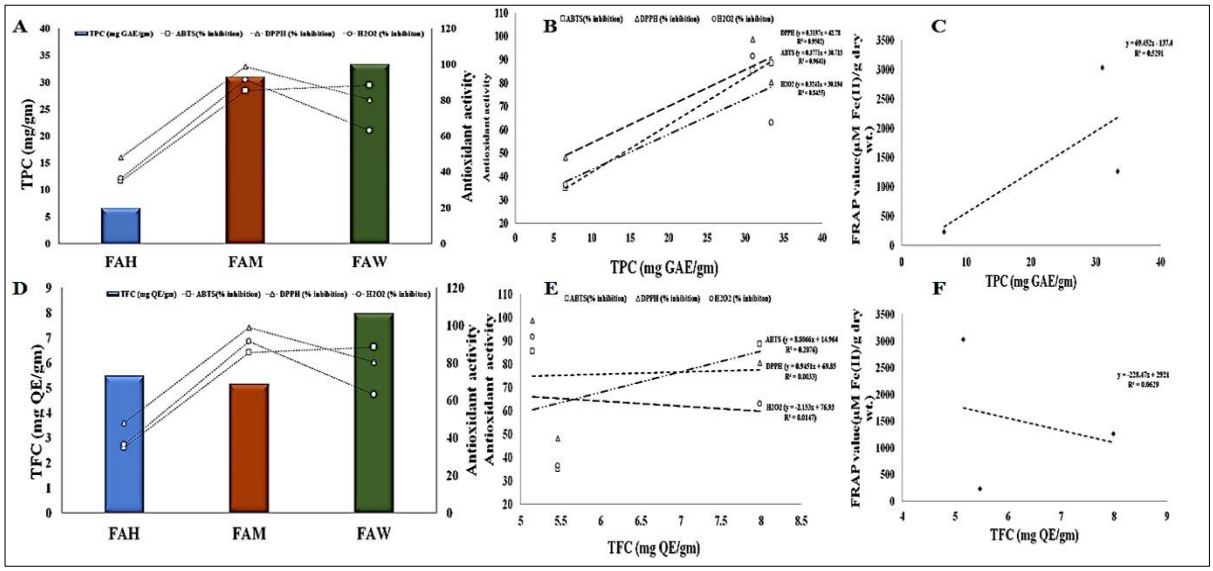


Fig 4: Correlation between Total phenolic content and Total flavonoid contents with extracts antioxidant activity. A: Correlation between TPC, DPPH, ABTS and H_2O_2 % inhibition for *Ficus auriculata* fruit extracts, B: Standard curve graph between TPC and ABTS, DPPH and H_2O_2. C: Standard curve between FRAP value and TPC of extracts. D: Correlation between TFC, DPPH, ABTS and H_2O_2 % inhibition for *Ficus auriculata* fruit extracts, E: Standard curve graph between TFC and ABTS, DPPH and H_2O_2. F: Standard curve between FRAP value and TFC of extracts.

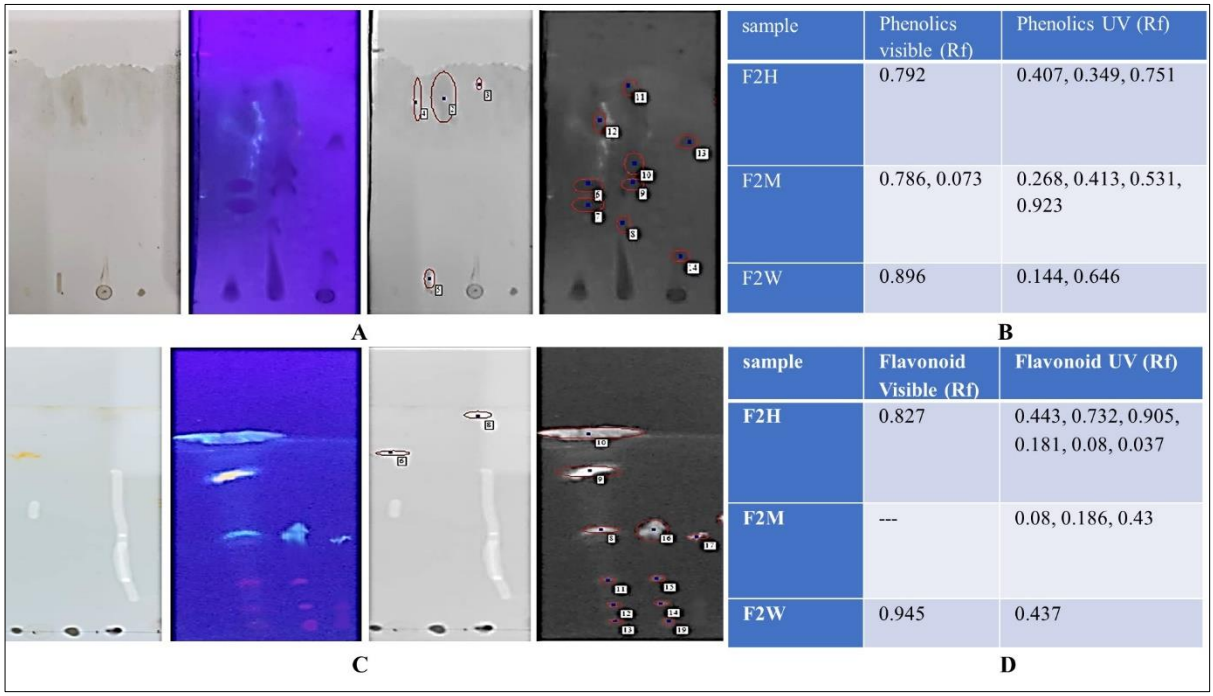
Although this value indicates a moderate correlation, it signifies that while TPC contributes to FRAP results, other factors also play a role. On the other hand, the total flavonoid content (TFC) in the extracts did not exhibit a significant correlation with the outcomes of the antioxidant assays. This finding suggests that the primary reason of the observed antioxidant activity in the extracts is predominantly the phenolic content rather than flavonoids. The result emphasises the central role of phenolic compounds in influencing the antioxidant potential of the extracts.

Thin Layer Chromatography of Extracts

Thin layer chromatography for flavonoid and phenolic compounds exhibited a maximum of four phenolic compounds (spots) in methanolic extract under visible and UV light, whereas hexane extract of the *Ficus auriculata* fruit revealed a maximum of six flavonoids. The methanolic extract had no yellow spots (flavonoid), but it did include three fluorescent spots under UV light (Figure 5). This shows that the phenolic chemicals in *Ficus auriculata* fruit methanolic extract are more abundant than flavonoids. The appearance of luminous patches under UV light shows the existence of fluorescent phenolic chemicals.

Screening, Cell viability and IC50 estimation of crude

All the three hexane, methanol and water extracts of *Ficus auriculata* were screened at their maximum concentrations against L132 and A549 cell lines. Only methanolic and water extract was found to have cell cytotoxic effect. Methanolic and water extracts along with doxorubicin were further diluted to estimate half maximal inhibitory concentration of each extract. The methanolic extract from *Ficus auriculata* demonstrates anticancer effects on A549 cells but also shows cytotoxicity towards normal L132 cells. This suggests its potential as an anticancer agent, though further research is needed to grasp its mechanisms and effects on normal cells. Notably, the estimated IC50 of the methanolic extract is 1678 µg/ml. Interestingly, this finding aligns closely with a study by Purnamasari *et al.*, 2019 [32], where the methanolic extract of *Ficus carica* fruit exhibited an IC50 of more than 2000 µg/ml on Huh7it cells. They also discovered that *Ficus carica* leaves had even stronger anticancer activity, with an IC50 of 653 µg/ml. IC50 of positive drug doxorubicin was found to be 2.664 µg/ml and have no effect on normal cells. *Ficus auriculata* water extract was found to be a very potent anticancer potential with IC50 of 50.68 µg/ml and minimal effect on normal cells (Figure 6).



A

B

sample	Phenolics visible (Rf)	Phenolics UV (Rf)
F2H	0.792	0.407, 0.349, 0.751
F2M	0.786, 0.073	0.268, 0.413, 0.531, 0.923
F2W	0.896	0.144, 0.646

C

D

sample	Flavonoid Visible (Rf)	Flavonoid UV (Rf)
F2H	0.827	0.443, 0.732, 0.905, 0.181, 0.08, 0.037
F2M	---	0.08, 0.186, 0.43
F2W	0.945	0.437

Fig 5: Thin layer chromatography of hexane, methanol and water extracts of *Ficus auriculata* fruit. A: TLC image under visible, at long wavelength (365 nm) UV light and Just TLC image output for phenolic compounds. B: Table indicating retention factor (Rf) of spots under visible and long wavelength (UV). C: TLC image under visible, at long wavelength (365 nm) UV light and JustTLC image output for flavonoid compounds. D: Table indicating retention factor (Rf) of spots under visible and long wavelength (UV).

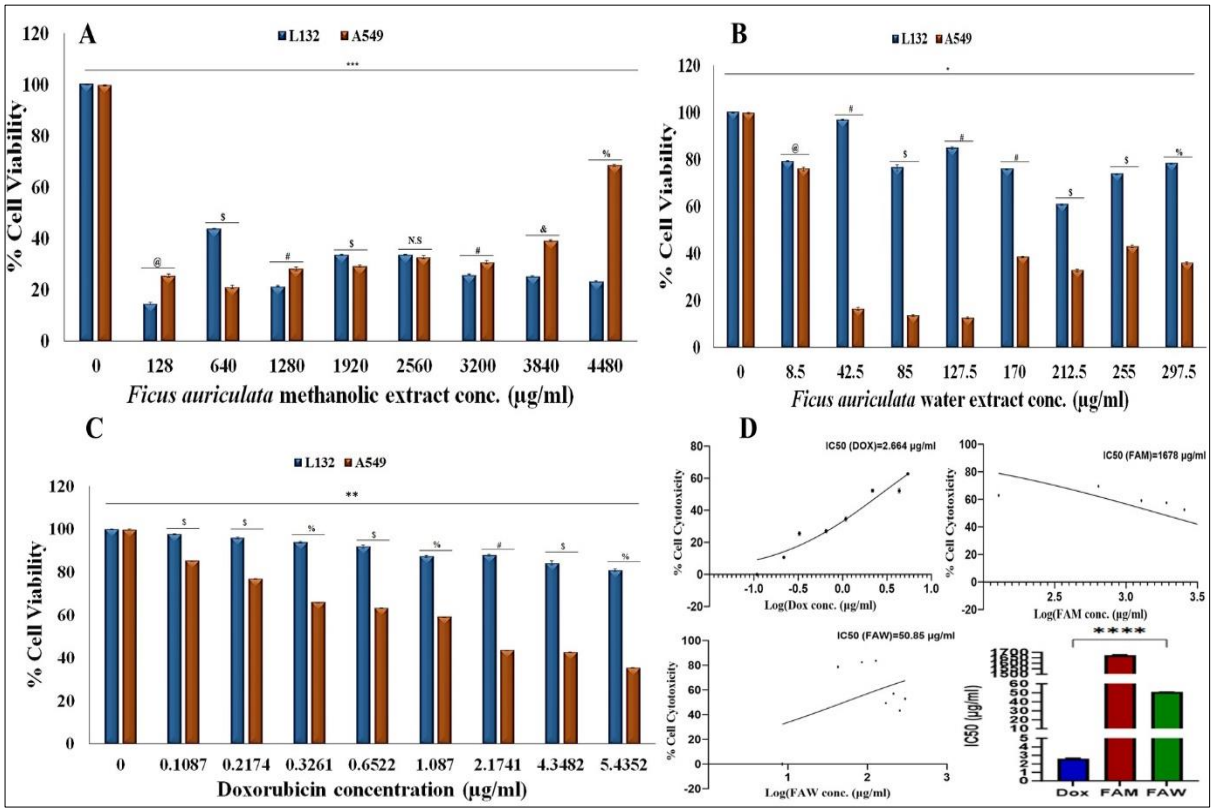


Fig 6: Cell viability assay and IC50 estimation of crude and doxorubicin. A: Cell viability assay of *Ficus auriculata* methanolic extract. All data are represented as mean± Std dev (n=3). Letters @, $, #, &, % level of significance within the group where @ = p<0.005, $ = p<0.0005, # = p<0.01, & = p<0.001 and % = p<0.0001, *** indicate p<0.0005. B: Cell viability assay of *Ficus auriculata* water extract. All data are represented as mean± Std dev (n=3). Letters @, $, # and % indicate level of significance within the group where @ = p<0.05, $ = p<0.0005, # = p<0.00005, and % = p<0.0001, * indicate p<0.05. C: Cell viability assay of Doxorubicin. All data are represented as mean± Std dev (n=3). Letters $, #, % level of csignificance within the group where $ = p<0.0005, # = p<0.00005, and % = p<0.0001, ** indicate p<0.005. D: IC50 graph of crude extracts and doxorubicin where **** indicate p<0.000.

Morphological analysis of cell under treatment and their cell counts after days of incubation: After 72 hours of incubation, morphological examinations of control A549 cells demonstrated adherent and normal cell growth. A drop in cell population and morphological alterations were detected in all treated A549 cells (DOX and FAW IC50 treatment) compared

to the control, although it was more noticeable in the Dox treated group at 72 h of treatment with a very small number of cells and a round shape. After 72 hours of incubation, all treated groups showed cell rounding and detachment (Figure 7).

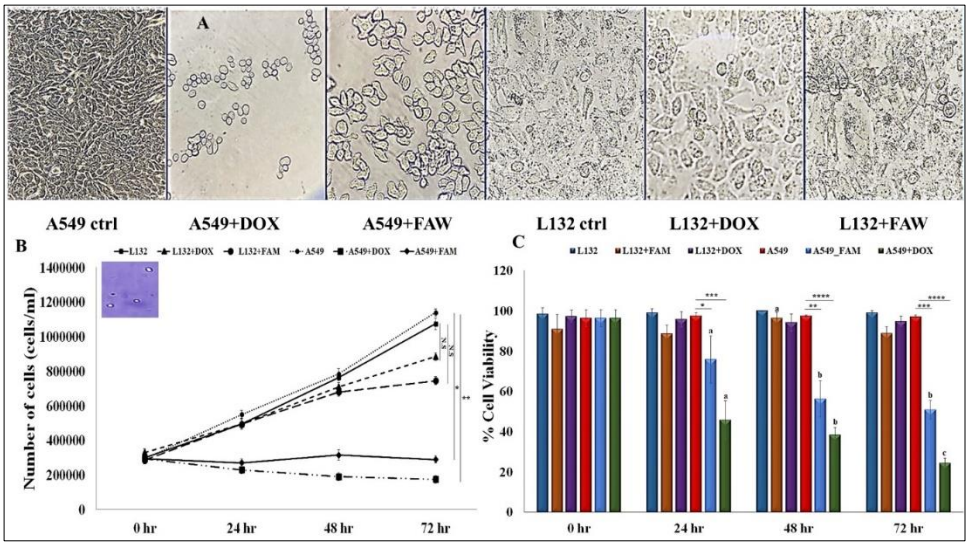


Fig 7: Assessment of Morphology and Cell Viability Over Various Incubation Times. A) Morphological examination of A549 cells with and without treatment using IC50 dosage of doxorubicin and FAW. B) Cell count analysis utilising trypan blue. Asterisks denote significance levels: * ($p< 0.05$), ** ($p< 0.01$). C) Percentage cell viability evaluation after 24, 48, and 72 hours of incubation with IC50 concentrations. Alphabets (a, b, c) indicate significance levels within the group: a ($p< 0.05$), b ($p< 0.001$), c ($p< 0.001$); and asterisks () denote significance levels: * ($p< 0.05$), ** ($p< 0.005$), *** (p < 0.0001), **** ($p< 0.00005$).

These findings imply that FAW IC50 treatments have a considerable influence on A549 cell proliferation and morphology. The observed cell rounding and detachment suggest that these treatments may have a cytotoxic effect on A549 cells. In L132 cells treated with DOX and FAW, only minor morphological alterations in the form of cell rounding were seen. Apoptosis is manifested by a reduction in cell population, cell rounding, and detachment. L132 and A549 cell lines showed unstimulated growth. Exposure to doxorubicin and *Ficus auriculata* water extract, on the other hand, resulted in growth suppression or reduced cellular proliferation during the incubation period, both at their respective IC50 values. The viability of A549 cells decreased significantly after a 24-hour incubation following IC50 treatment, with a more significant decrease observed after 72 hours, with doxorubicin and *Ficus auriculata* extract reporting 24% and 55% viability, respectively. These results imply that doxorubicin and a *Ficus auriculata* extract have significant anti-proliferative actions on A549 cells. The observed drop in cell viability over time suggests that these therapies have a long-term effect on cell growth and survival.

DNA damage pattern of A549 with DOX and *Ficus auriculata* water extract IC50 concentration after 72 hrs of incubation

The comet assay was employed to evaluate the potential impact of IC50 concentrations of doxorubicin (dox) and aqueous extract derived from *Ficus auriculata* (FAW) on the induction of single- and double-strand DNA breaks. The analysis involved the assessment of approximately 20 to 25 individual comets, with image processing and analysis by the Casp software. Intriguingly, the assessment revealed the absence of discernible evidence pertaining to single-strand DNA damage in not only the L132 cells and A549 cells but also in the L132 cells treated with IC50 doses of both dox and FAW. This observation suggests that the single-strand DNA integrity remained largely unaffected under these experimental conditions. However, a distinctive observation emerged in the A549 cells subjected to the same IC50 concentrations of dox and FAW (Figure 8).

In these cells, the single DNA structures exhibited a certain degree of deformation, indicating a notable alteration in their structural conformation. Of particular significance was the pronounced and substantial manifestation of double-strand DNA damage in the A549 cells treated with IC50 doses of both dox and FAW. This observation underscores the potential of these treatments to induce more profound disruptions in the DNA double-strand structure within the A549 cellular context.

Gene expression analysis of DNMT, MBD, HDAC and HAT in A549 cells under Dox and FAW treatment

The RNA that was extracted is in good condition with a high level of integrity and purity, ranging from 1.87 to 2.0. RefFinder was used to evaluate the stability of housekeeping genes in diverse samples, employing various approaches for selecting reference genes. The gene with the minimal standard deviation was chosen to represent the highest stability. ACTB emerged as the most stable gene, closely followed by GAPDH. The hierarchy of gene stability progression was observed as ACTB>GAPDH>PGK1>18s>PPIA according to comprehensive gene stability assessment, the delta Ct method, and NormFinder. Across all these methodologies, ACTB was found to be the most stable gene, while PPIA exhibited the least stability. However, results from Genorm displayed discrepancies by suggesting that both GAPDH and ACTB were the most stable genes, and PPIA was the least stable. Contrarily, BestKeeper's outcome pointed to PGK1 as the most stable gene and PPIA as the least stable. For further analysis B-actin was selected as a reference gene (Figure 9). PCR efficiency of all genes was evaluated by standard curve method. PCR efficiencies were assessed for genes like B-actin, HAT, HDACs, DNMTs, and MBDs, yielding efficiency values between 94.32% and 102.28%. Efficiencies were

confirmed by amplification factors. A slope range of -3.6 to -3.1 was considered for qRT-PCR quality, with -3.32 indicating 100% efficiency (Figure 9). Control and target gene efficiencies were compared using the ΔΔCt method. All gene cDNA quantities were normalised.

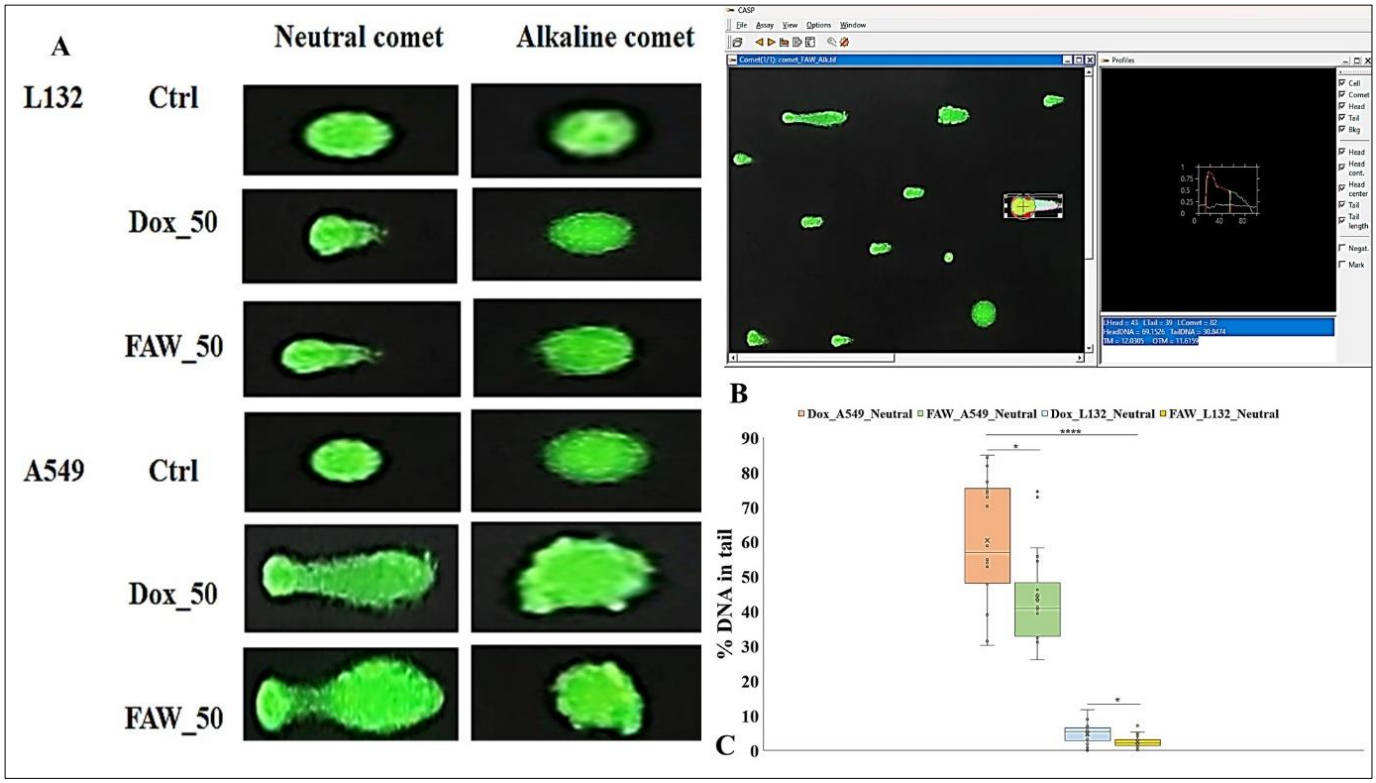


Fig 8: Alkaline and Neutral comet assay after 72 hrs. in incubation with IC50 concentration of Dox and FAW. A: comets of A549, L132 cells after incubation of 72 hrs. B: Casp 1.2.3.1b output. C: % DNA in tail graph, * indicate $p<0.05$ and **** indicate $p<0.00001$.

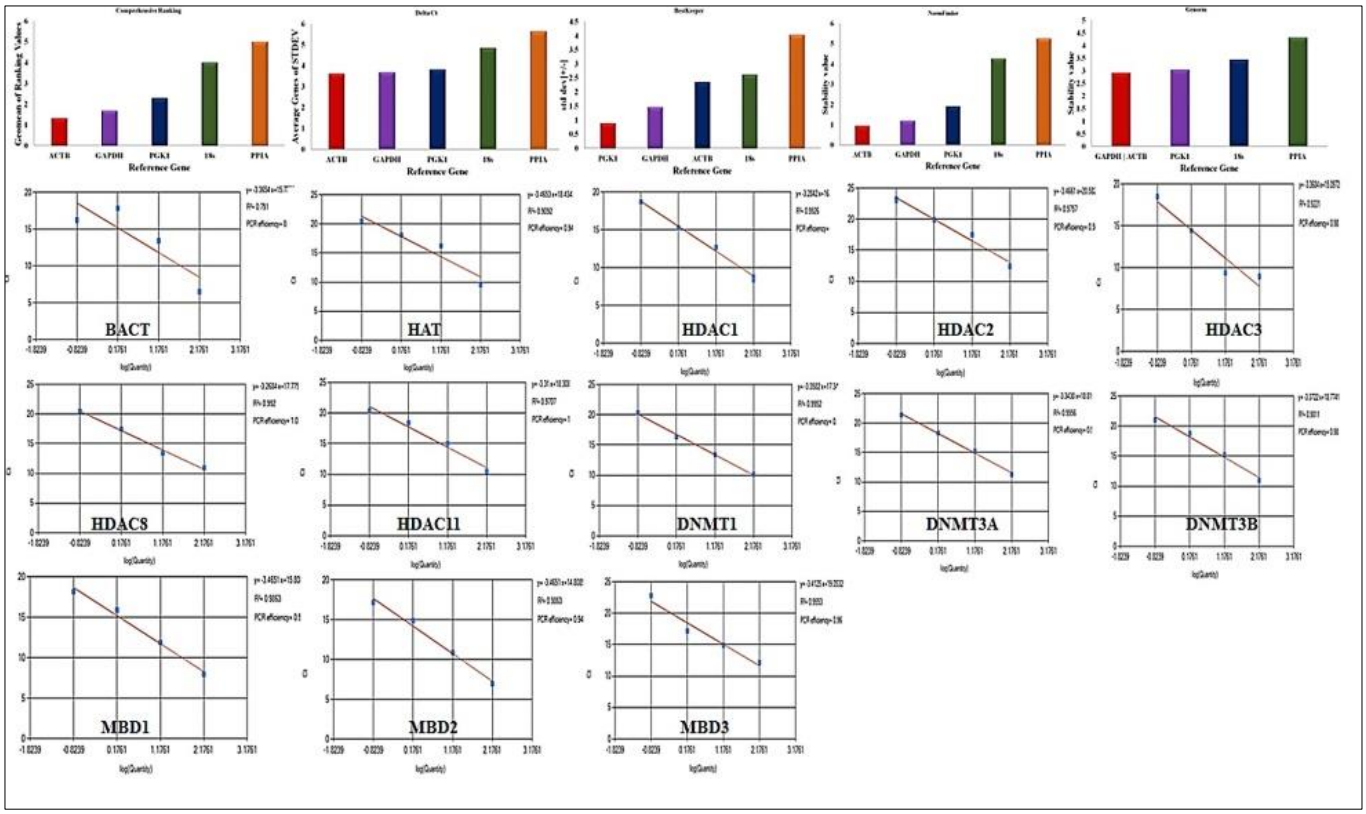


Fig 9: Selection of Reference gene using Ref Finder and Standard curve of all the gene.

The melt curve analysis illustrates the thermal properties of several gene amplicons. The presence of separate melting peaks around 86-88 °C for the HAT, HDAC1, HDAC2, and HDAC3 genes confirms the specificity of PCR amplification. A strong peak at 82 °C suggests effective gene amplification in the case of HDAC8. HDAC11, on the other hand, shows complicated behaviour with several peaks, which may be attributable to structural complications. Similarly, the melting

patterns of MBD1 and MBD2 amplicons are complicated, with many peaks, indicating sophisticated secondary structures. Finally, MBD3 exhibits complicated behaviour with many peaks, indicating the existence of various structural components inside its DNA sequence. B-actin served as an endogenous control, while L132 cell lines treated with IC50 doses of DOX and FAW served as biological controls. Dox treatment markedly reduced the expression of every gene, whereas IC50 treatment with FAW in A549 cells reduced the expression of every gene with the exception of HAT, which had a 2.1-fold increase in gene expression. In the A549 cell line, the expression levels of each gene after DOX treatment were substantially different from those after IC50 treatment with FAW extract (Figure 10). Despite being a widely used anticancer drug, DOX has more effectiveness when it comes to suppressing the expression of epigenetic modulators. This implies that the FAW extract could have a special mode of action that targets the HAT gene in particular, which might be further researched for possible therapeutic uses.

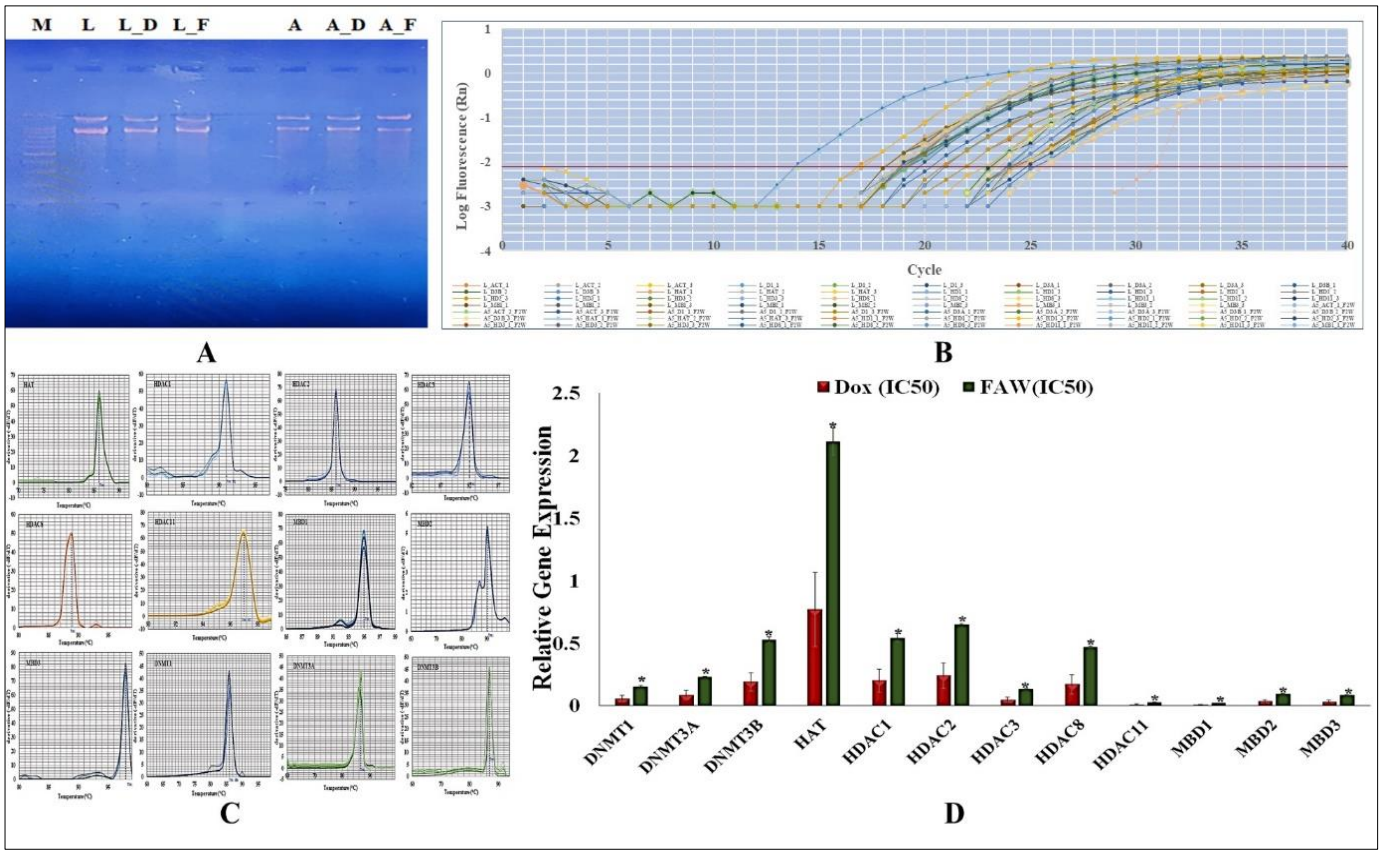


Fig 10: Gene expression analysis of HAT, DNMT, MBD and HDAC genes after 72 hrs. of incubation of L132 and A549 cell line with and without treatment. A: Qualitative analysis of RNA: M-100 bp marker, L-L132 cell without treatment, L_D-L132 cells incubated with dox IC50 concentration, L_F-L132 cell incubated with FAW IC50 concentration, A-A549 cells without any treatment, A_D-A549 cell treated with dox IC50 concentration, A_F-A549 cell treated with FAW IC50 concentration. B: Amplification plot of all genes (Log of fluorescence (Rn), C: Melt curves of HAT, DNMT, MBD and HDAC genes. D: Relative gene expression analysis of DNMT, HAT, MBD and HAT gene. Data are represented as mean, where n=3 and are statistically significantly different with * indicating $p<0.05$.

Conclusion

The investigation into *Ficus auriculata* fruit extracts unveils a promising array of health benefits associated with these natural compounds. The extracts exhibit a diverse phytochemical profile, with notable concentrations of phenolics and flavonoids, known for their antioxidant and health-promoting properties. The methanolic extract emerges as a potent antioxidant, effectively combating harmful free radicals. Furthermore, the extracts demonstrate significant potential as anticancer agents, particularly the methanolic and water extracts, which exhibit cytotoxic effects on A549 lung cancer cells while sparing normal L132 cells to some extent. The observed DNA damage patterns in cancer cells suggest intriguing mechanisms underlying their anticancer effects. Additionally, gene expression analysis hints at unique modes of action, with the *Ficus auriculata* water extract showing an increase in the expression of the HAT gene, warranting further exploration. These findings underscore the significance of investigating natural compounds as potential therapeutic agents, shedding light on their roles in cancer treatment and antioxidant therapy. Future research endeavours hold promise for harnessing the full potential of these extracts in healthcare and cancer management.

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Conflict of interest

The authors declare no conflict of interest.

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