Letters in Applied NanoBioScience
Open-Access Journal (ISSN: 2284-6808)

Article
Volume 14, Issue 3, 2025, 149
https://doi.org/10.33263/LIANBS143.149

Bioactivity of *Ficus Racemosa* Leaf Methanol Extract Against Pathogenic Microorganisms and its Antioxidant, Cytotoxicity Activity on Oral Cancer Cell Line

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Received: 15.05.2024; Accepted: 1.01.2025; Published: 6.09.2025

Abstract: According to the alarming statistical analysis, over 10 million people die from cancer worldwide each year, and over 19 million new cases are diagnosed. Oral squamous cell carcinoma (OSCC) is one such cancer that demands new therapeutic approaches. *Ficus racemosa (F. racemosa)* extract has been used in traditional medicine to treat infections. Hence, in this study, *Ficus racemosa* leaves were screened to explore their potential antioxidant, antimicrobial, and anticancer activity. Methanol extract of *F. racemosa* leaves was tested for antioxidant potential (DPPH scavenging activity (65.47 μg/mL), ABTS (56.61 μg/mL). The methanol extract (leaves) of *F. racemosa* was tested on four types of bacteria (two Gram-positive bacteria and two Gram-negative bacteria). From the results, we inferred that high antioxidant and free radical scavenging activities, with relatively strong antioxidant activity, were observed. *In vitro* antibacterial studies confirmed the significant antibacterial effect against both Gram-positive and Gram-negative. Furthermore, the anticancer activity of the leaf extract on KB cells was evaluated by MTT assay. Moreover, the ability of the dual acridine orange/ethidium bromide (AO/EB) staining method was used to detect tumor cell apoptosis. Our results indicate that the methanolic extract of *F. racemosa* leaf extract possesses strong antioxidant, antimicrobial, and cytotoxic activity; evidently, it proves to be an ideal candidate for a pharmacological agent.

Keywords: *Ficus racemosa*; anticancer; antioxidant; dual staining; KB cells.

1. Introduction

Cancer is the leading cause of death among people. According to the World Health Organization (WHO), cancer ranks as the second leading cause of death globally, with about 70% of cases occurring in low and middle-income countries. Any malignant tumour that forms on the tongue, floor of the mouth, inner cheek lining, gums, palate, or lips is classified as oral cancer [1].

In India, oral cavity cancer is the most prevalent type among men, contributing to nearly one-third of the world's cases and deaths associated with oral cancer. Additionally, these cancers are becoming more common among younger age groups in India, unlike in Western countries. This shift poses a serious threat to the nations, which may significantly impact economic productivity.

Squamous cell carcinoma is the most common histologic type of oral cancer, making up 90-95%, which ranks sixth in the world's cancer incidence rates. It is of serious major concern in Southeast Asia primarily because of the widespread oral habits of smoking, chewing betel quid, and alcohol consumption [2,3]. According to the International Agency for Research on Cancer (IARC), cancer cases are expected to increase from 1 million in 2012 to over 1.7 million by 2035. During the same period, cancer-related deaths are predicted to rise from 6,80,000 to 1-2 million [4]. IARC discovered sufficient evidence connecting tobacco use and chewing habits such as betel quid to diseases of the oral mucosa, including leukoplakia, oral submucous fibrosis, and oral cancer [5,6]. In spite of advancements in combination therapy comprising surgery, chemotherapy, and radiotherapy, the oral squamous cell carcinoma (OSCC) 5-year survival rate remains approximately 40 % and has not changed for the past few decades. Research is still being done to discover naturally occurring antiproliferative and chemopreventive substances that could replace chemically synthesized drugs and perhaps be less toxic with fewer side effects. Therefore, developing advanced treatments with plant derivatives that act as potent therapeutic agents with minimal side effects is imperative [7,8]. Hence, *Ficus racemosa*, *a* traditional natural plant, was selected for this research to meet the needs of this study.

Ficus belongs to the Moraceae family and has been known for its diverse number of species, consisting of more than 800 species in the form of trees, shrubs, epiphytes, and hemiphytes [9].

The genus *Ficus* is a significant group of trees found across tropical and sub-tropical areas, mainly in Asia, America, Australia, and Africa. It possesses numerous medicinal properties and plays a significant role in various treatments [10]. Ficus is classified into four species groups: *Ficus racemosa*, *Ficus benghalensis*, *Ficus microcarpa*, and *Ficus religiosa*, etc. [11]. Among these species, the well-known *Ficus racemosa(F.racemosa)* plant is the most popular species of *Ficus*, also known as common fig or cluster fig, native to Southeast Asia. It is often employed to treat wounds and has many medicinal properties. Pharmacological investigations have been conducted on various parts of the plant, including fruit, bark, and leaves [12]. They consist of hepatoprotective, antidiarrheal, anti-inflammatory, antipyretic, antifungal, antibacterial, hypolipidemic, antifilarial, and anticancer properties [13-15].

To the best of our knowledge, limited work has been carried out on the cytotoxic activity of oral cancer. Considering this point of view along with the medicinal significance of the plant *F. racemosa*, this research focused on evaluating the antibacterial, antioxidant, and cytotoxicity activities of methanol extracts of *F. racemosa* leaves against the oral cancer KB cell line and characterizing its phytochemical profile. Furthermore, the total yield of flavonoids and phenolics of the methanol extracts was quantified. Thus, this study suggested that *F. racemosa* leaves could be a great natural source for the development of novel therapeutics, which serve as new entities in the development of drugs derived from plants.

2. Materials and Methods

2.1. Collection, identification, and authentication of plant material.

The leaves of *F. racemosa Linn* were collected from the outfield village of Arakkonam in the Ranipet District. The leaves were washed thoroughly in tap water for 2-3 times, dried in air shade for 2 weeks, finely powdered, and used for the successive extraction method. Plant material was identified/authenticated by Botanist- Prof. P. Jayaraman (Late), Director, Plant Anatomy Research Centre (PARC), Tambaram, Chennai (PARC/2020/3600).

2.2. Preparation of methanolic extract – Soxhlet extraction.

Briefly, 20 g of powdered leaf was extracted using methanol (300 mL) in a Soxhlet apparatus. The extraction was achieved over 72 h. The obtained extract was then filtered through Whatman No. 1 Filter paper. Then the extract was evaporated at a temperature of 45°C using a rotary evaporator [16]. The crude extract was stored in a desiccator in order to prevent moisture and oxidation. The obtained methanolic crude extracts were used for further study.

2.3. Quantitative phytochemical analysis.

2.3.1. Determination of total phenolic content (TPC) assay.

TPC was evaluated according to the methodology of Singleton et al. [17] using Folin-Ciocalteu reagent. This method is based on the reduction of Folin-Ciocalteu reagent by phenols to a mixture of blue oxides with maximum absorption at around 750 nm. About 100 μL of diluted plant extract (100 μg/mL) is placed in test tubes, to this 500 μL of Folin-Ciocalteu's phenol reagent was added (1/10 mL diluted) and kept for incubation at room temperature (RT) for 5 min. After 5 min, 1500 μL (1.5ml of 2% Na_2CO_3) was added to all the test tubes. The tubes are then placed in the dark for 120 minutes at RT. Then the optical density for the obtained dark blue solution was read at 750 nm against a blank [18]. By applying the gallic acid calibration curve as a reference, the total phenolic contents were determined and expressed as milligram gallic acid equivalents. All the experiments were carried out in triplicate.

2.3.2. Determination of total flavonoid content (TFC) assay.

TFC was determined according to the Karadeniz et al. slightly modified method [19]. When flavonoids and aluminium chloride reagent react, a coloured product is produced, which can be detected at 510 nm using spectrophotometry. An aliquot of leaf extract solution (600 μL) was mixed with 1.5 mL (1500 μL) of methanol. 0.3 mL of 5% sodium nitrite was added. Upon vortexing, this solution was allowed to stand for 5 min at RT. 0.6 mL of 10% aluminium chloride was added. Again, the mixtures were allowed to stand for 6 min, then 1 mL of 1M Sodium hydroxide was added to the test tube, and finally, the mixture was diluted with 1 mL of distilled water. The contents were left for 15 minutes, and the absorbance of the reaction mixture was read at 510nm with a UV-Vis spectrophotometer. This test was performed in triplicate, and the TFCs were assessed as milligrams of quercetin equivalents (mg QE/g dry weight).

2.4. In vitro antioxidant methods.

2.4.1. Determination of antioxidant activity by DPPH scavenging assay.

The antioxidant activity of methanolic leaf extract of *F. racemosa* was evaluated by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity method described by Szabo et al. [20]. Briefly, the assay contained 0.5 ml of plant extract at increasing concentrations of (10- 200 µg/mL)), which were mixed with 1 ml of DPPH (0.5 mM in methanol) solution and allowed to react at RT for 60 min. After 30 min, the absorbance values were recorded at 517nm and converted into percentage antioxidant activity using the following equation:

DPPH Scavenging effect (%) = Control OD - Sample OD / Control OD × 100

Quercetin was used as the positive control, and methanol as the blank. Each experiment was carried out in triplicate, and IC_{50} (µg/mL) of the methanolic leaf extract of *F. racemosa* was reported.

2.4.2. Determination of antioxidant activity by $ABTS^{+\bullet}$ scavenging assay.

$ABTS^{+\bullet}$ cation radical scavenging activity of F. racemosa methanolic leaf extract was determined using 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) following the method reported by Re et al [21] and Fatih Uckaya [22], in which 7mM ABTS solution was mixed with 2.45 mM of potassium persulfate and allowed to incubate for 12-16 hr in order to produce $ABTS^{+\bullet}$. Different concentrations of the leaf extracts (10 to 200 µg/mL) were mixed with diluted $ABTS^{+\bullet}$ radical ion solution (160 µL of 7mM ABTS in 40 µL of methanol). The reaction mixture was left in the dark for 6 min, and its absorbance was measured at 734 nm. Ascorbic acid was used as the standard, while methanol was used as the control. $ABTS^{+\bullet}$ radical activity was calculated using the formula:

Inhibition % = [(A control - A sample) / A control] × 100

2.5. Antibacterial activity by well diffusion method.

Agar well diffusion modified method [23] was used to determine the antibacterial activity of methanolic leaf extract obtained from the *F. racemosa*. Four species of bacteria were studied: 2 species of Gram-positive and two species of Gram-negative bacteria. The bacterial cultures (*Escherichia coli* (*E. coli*), Methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus* (*S. aureus*), and *Pseudomonas aeruginosa* (*P. aeruginosa)* were procured from King's Institute Guindy and maintained at Bionyme Laboratories Pvt.Ltd, Chennai. To obtain a fresh culture, the bacteria were subcultured in a nutrient broth. 50 µL of stock culture was mixed with 950 µL of sterile Muller Hinton broth and incubated for 24 h at 37°C. From this 24h incubated culture, 50 µL of bacteria was taken and spread onto the solidified Muller Hinton agar, and wells were made accordingly [24]. The well was filled with varying concentrations of leaf extract (50, 75, and 100 µg/mL), while streptomycin (25 µg/mL)was used as standard positive control and DMSO as negative control. The plates were allowed to diffuse for about 30 minutes at RT and then incubated overnight at 37°C. After incubation, plates were observed for the formation of a clear zone around the well, which corresponds to the antimicrobial activity of the extract against strains. The zone of inhibition (ZOI) was observed and measured in mm. Each assay was repeated three times.

2.6. Cell culture and maintenance.

KB cancer cell lines were procured from the cell repository of the National Centre for Cell Sciences (NCCS), Pune, India. Dulbecco`s Modified Eagle Media (DMEM) was used for maintaining the cell line, which was supplemented with 10% Fetal Bovine Serum (FBS). Penicillin (100 U/ml) and streptomycin (100 µg/ml) were added to the medium to prevent bacterial contamination. The medium with cell lines was maintained in a humidified environment with 5% CO_2 at 37°C.

2.7. MTT Assay for cytotoxicity assessment.

The *in vitro* cytotoxic effect of the methanolic crude extract of *F. racemosa* leaf was evaluated for its antitumor activity against KB cancer cells using MTT (3-(4, 5-DimethylThiazol-2-yl)-2, 5- Diphenyl Tetrazolium bromide) assay [25,26]. A cell count of 1×10^4 cells/ml was seeded in 96 96-well plates in DMEM and incubated for 24 h at 37°C under 5 % CO_2 and 95 % air. Following 24h of cell attachment, plates were washed with 100µl of PBS and further treated with 100 µl of the methanolic leaf extract (*F. racemosa*) samples with concentrations ranging (25, 50, 100, 150, 200, 250 and 300 µg/mL) were incubated further for 24h, and 48 h respectively. After incubation, the plates were washed with 100 µL of PBS and then treated. After incubation, the KB cells were treated with MTT (5 mg/ml, 100 µl) in each well and incubated for another 4 hours. The formazan crystals formed in the cells were replaced with 100 µl of dimethyl sulphoxide (DMSO). A microplate reader was used to measure absorbance at 570 nm. The values of the half maximal inhibitory concentration (IC_{50}) were calculated, and the optimum doses were analyzed over different time periods. Each study was carried out in triplicate.

The morphological characteristics of the KB cell line treated with the methanolic leaf extract were photographed using a fluorescent microscope at a magnification of 20x

2.8. Acridine orange/ethidium bromide (AO/EB) - dual staining assay.

The study of apoptotic cell death by fluorescence microscopy was carried out using the method followed by Baskic et al. [27]. The dual dye AO/EB staining of the cells was examined using fluorescence microscopy (Zone fluorescent cell imager, Biorad).

A 6-well plate was seeded with 5×10^4 oral cancer cells (KB) /well, and the cells were incubated for 48 hr. Following a 48 hr of treatment with control and leaf extract (100 and 150 µg/ml), the cells were detached, cleaned with cold PBS and stained for 5 min at RT using a mixture of AO (100 µg/mL^{-1})/ EB (100 µg/mL^{-1}) ratio (1:1). Using a 20x magnification, the stained cells were examined under a fluorescence microscope. The cells were collected at the end of the treatment and washed thrice with PBS.

The number of cells exhibiting apoptotic features was calculated as a function of the total number of cells present in the area.

2.9. Statistical analysis.

The data were expressed as mean ± SD, and all assays were repeated thrice. One-way analysis of variance (ANOVA) and the Student's t-test were the statistical methods used to analyze the data. P values less than 0.05 were considered significant.

3. Results and Discussion

3.1. Total phenolics and flavonoids total content.

Plants synthesize polyphenolics in large quantities to support growth and provide protection against pathogens and predators. The total phenolics and flavonoids composition (TPC and TFC) in *F. racemosa* methanol extract was determined from a standard calibration curve of gallic acid: R^2 = 0.9964 for TPC and R^2 = 0.9991. For TPC, the methanolic leaf extract of *F. racemosa* showed the highest phenolic concentrations of about 69±0.97 mg GAE/g of dry extract (Table 1). Moreover, for TFC (Table 1), the methanol extract exhibited the highest content of 87±0.82 mg QE/g of dry extract, respectively.

Table 1. Total phenolic content and Total flavonoid content of *F.racemosa* leaf.

Species	Extract	Total phenolic content (mg GAE/g dry extract)	Total flavonoid content (mg QE/g dry extract)
F.racemosa Leaf	Methanol	69 ± 0.97	87± 0.82

Phenolic compounds, which include flavonoids, phenolic acids, tannins, and lignans, are a diverse group of secondary metabolites with a significant role in plants. They possess a wide range of structures, from simple molecules to complex polymers [28,29].

As one of the most significant categories of phenolic compounds, flavonoids assist various functions in plants, such as defense against fungi, bacteria, insects, viruses, and protection against UV radiation [30]. Flavonoid chemical structures are primarily responsible for their biochemical activities.

Phenolic compounds possess significant biological properties such as anti-inflammatory, antimicrobial, antioxidant, and anticancer. These compounds slow down ageing, prevent chronic degenerative disease, and enhance cellular defense [31]. Thus, plants rich in phenolic compounds have considerable pharmacological potential.

3.2. In vitro antioxidant assays.

Reactive oxygen species (ROS) and free radicals contribute to the development of various health conditions, including cardiovascular and metabolic diseases, diabetes, and cancer, as antioxidants are unable to adequately control the excessive levels of ROS [32,33]. The imbalance between free radicals production and antioxidant activity leads to oxidative stress, which can result in the damage of cellular proteins, DNA, membrane lipids, and eventually cell death [33]. Managing oxidative stress could be a key approach to treating diseases such as diabetes and metabolic diseases. Phytochemicals, like phenolic compounds and flavonoids found in plants, possess antioxidant properties and have proven effective in disease management [34]. Thus, the antioxidant efficacy of plants is closely related to the quality and concentration of those phytochemicals [35]. DPPH and ABTS assays are commonly employed to identify the presence of antioxidants.

3.2.1. DPPH scavenging activity.

The antioxidant potential for the methanol extract of *F. racemosa* leaves was evaluated by using two antioxidant assays, namely, DPPH and ABTS scavenging activity. The DPPH assay (Figure 1a) showed that the methanol extract exhibited the highest potential with an IC_{50} value of 65.47 μg/mL. The extract exhibited high concentration-dependent inhibition of DPPH radical, highest at 100 μg/mL. The electrons in the DPPH solution are paired off and turn yellow when they react with an antioxidant. The number of electrons absorbed determines whether DPPH radicals stabilize into the hydrazine form. Therefore, the antioxidant capacity of the methanol extract may be due to the donation of electrons from the phenolic rings of the polyphenols [36,37].

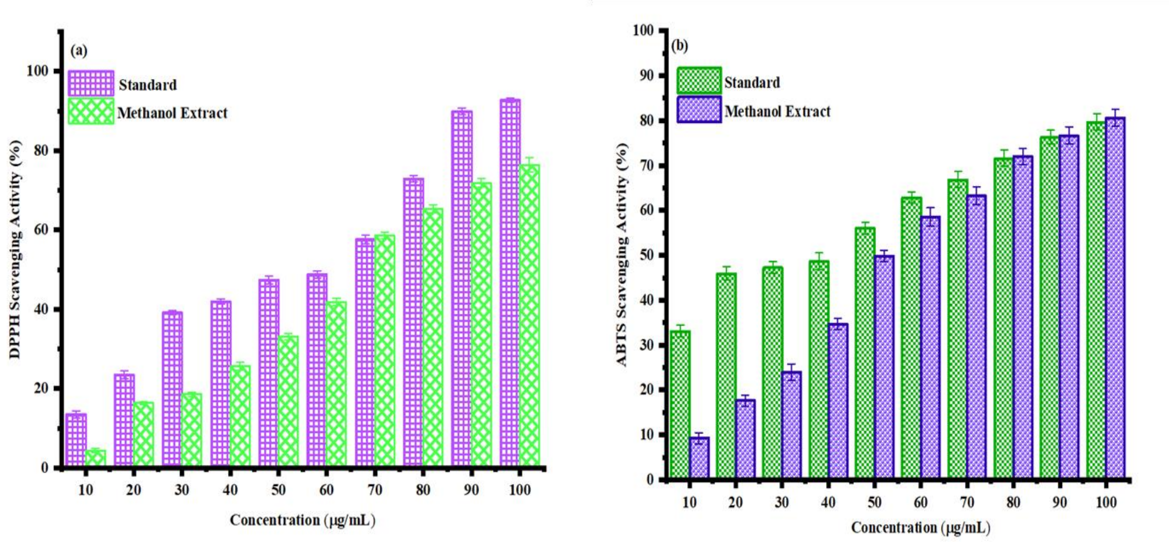

Figure 1. (a) DPPH scavenging activity of *F. racemosa* methanol extract (Leaves); **(b)** ABTS radical scavenging activity of *F. racemosa* methanol extract (Leaves).

3.2.2. ABTS radical scavenging activity.

The well-known $ABTS^{+\bullet}$ method is widely employed in evaluating antioxidant activity. The concentration of the extract was positively correlated with the ABTS free radical scavenging activity. The standard ascorbic acid has been compared with the extract's relative antioxidant activity to scavenge the radical $ABTS^{+\bullet}$. The results showed that $ABTS^{+\bullet}$ activities in methanol extract of *F. racemosa* exhibit good antioxidant activity with the IC_{50} value of 56.61 μg/mL (Figure 1b). This might be due to its richness in phenolic compounds, specifically flavonoids and flavonols [38]. Furthermore, it is well known that the content of phenol compounds has a significant association with the ability to scavenge free radicals [39-41].

Hence, the above antioxidant study (DPPH and $ABTS^{+\bullet}$) revealed that methanol leaf extract of *F. racemosa* possesses an appreciable quantity of antioxidant activity.

3.3. Antibacterial activity.

The antibacterial assay was performed by agar well diffusion method for the methanol extract of *F. racemosa* leaves against four microorganisms, including two Gram-positive bacteria and two Gram-negative bacteria, and depicted the size of the inhibition zones in Table 2 and Figure 2. The methanol extract demonstrated a larger inhibition zone of 10mm against Gram-negative bacteria (*P. aeruginosa*), and 9 mm (*E. Coli*) at a concentration of 100 μg/mL, whereas Gram-positive bacteria showed an inhibition zone of 11 mm against Gram-negative bacteria (*S. aureus*) and 9 mm (MRSA) at a concentration of 100 μg/mL.

From the results, we inferred that Gram-positive bacteria were found to be more sensitive than Gram-negative bacteria. This difference in sensitivity may result from the bacterial peptidoglycan layer in Gram-negative bacteria being surrounded by an outer membrane made of hydrophilic lipopolysaccharides. This membrane acts as a barrier towards macromolecules and limits the diffusion of hydrophobic compounds into the bacterial cytoplasm [42-44]. On the contrary, Gram-positive bacteria's cell wall has many pores and a thinner layer of peptidoglycan, which makes it easier for the entry of foreign substances, which eventually causes cell death by rupturing the membrane and loss of cytoplasmic components [45,46]. It concludes that the methanol extract of *F. racemosa* shows antibacterial activity for the tested organisms. The zone of inhibition varied, pointing to the presence of different phytoconstituents in the leaves and the varied degree of efficacy on the tested organisms.

Table 2. Antibacterial Activity of methanol extract of *F. racemosa* against tested microorganism.

Bacterial species	Zone of inhibition (mm) Concentration(µg/ml)			Antibiotic (Streptomycin) mm
	50µl	75 µl	100 µl	
Staphylococcus aureus	2 ± 0.15	8 ± 0.20	11 ± 0.79	8 ± 0.2
Methicillin-resistant Staphylococcus aureus	-	7 ± 0.64	9 ± 0.44	8 ± 0.22
Escherichia coli	-	6 ± 0.20	9 ± 0.92	7 ± 0.2
Pseudomonas aeruginosa	-	8 ± 0.42	10 ± 0.54	10 ± 0.44

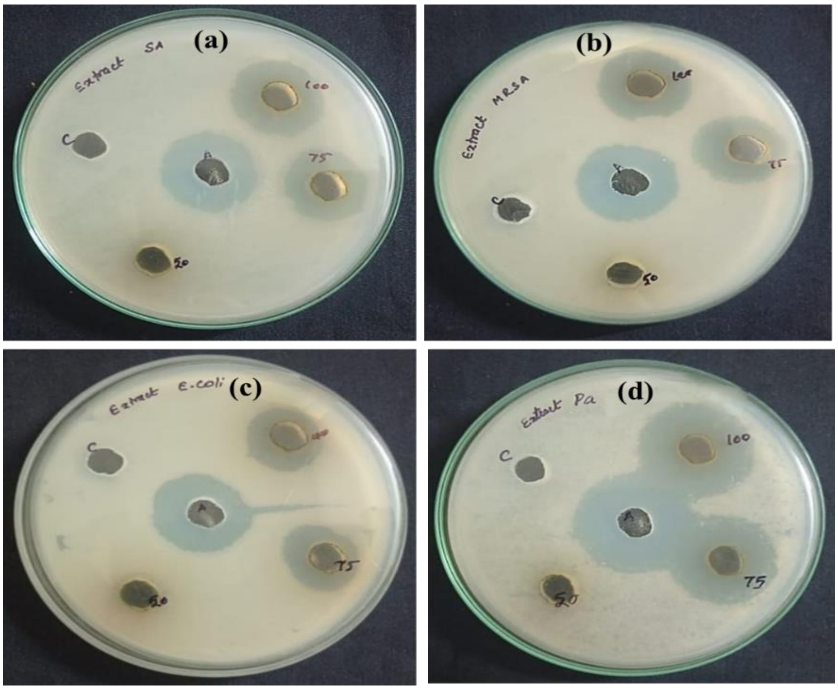

Figure 2. Antibacterial activity *F. racemosa* Methanol extract – **(a)** *Staphylococcus aureus*; **(b)** *Methicillin-resistant staphylococcus aureus*; **(c)** *Escherichia coli*; **(d)** *Pseudomonas aeruginosa*.

3.4. Assessments of cytotoxicity of F. racemosa methanol extract by MTT assay.

To the best of our knowledge, this is the first intensive study on cytotoxicity of *F. racemosa* leaves extract (methanol) against KB cancer cell lines. The cytotoxic response of the methanol extracts of *F. racemosa* leaves against KB cells, as given in Figures 3 and 4, was examined for varying concentrations (25 to 300 µg/mL) over 24 and 48h. It was revealed that the methanol extracts exhibited a concentration-dependent activity on KB cells with IC_{50} equal to 173.85 µg/mL (24h) and 136.53 µg/mL (48h), respectively. The *F. racemosa* leaf extract is promising for the anticancer activities since it showed 82% cell death when it was incubated for 48h at a concentration of 300µg/mL of leaf extract. The MTT assay of methanol extracts on KB cells indicated that the leaf extract was biocompatible with the cell line.

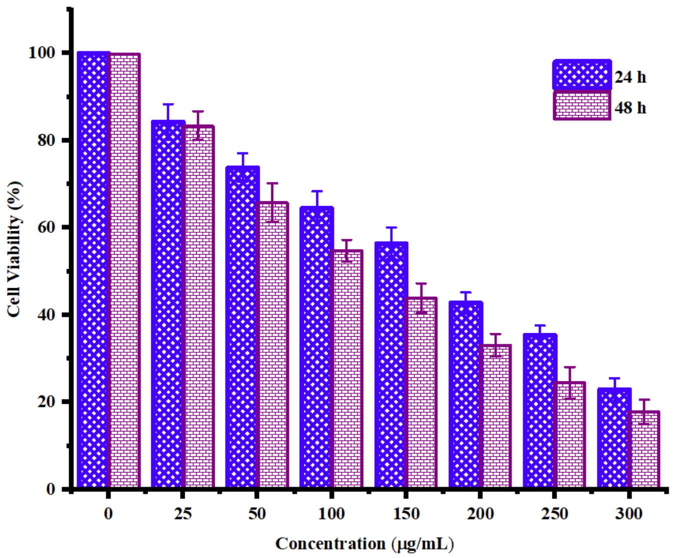

Figure 3. Cell viability of the methanol extract of *F. racemosa* (Leaves) on KB cells.

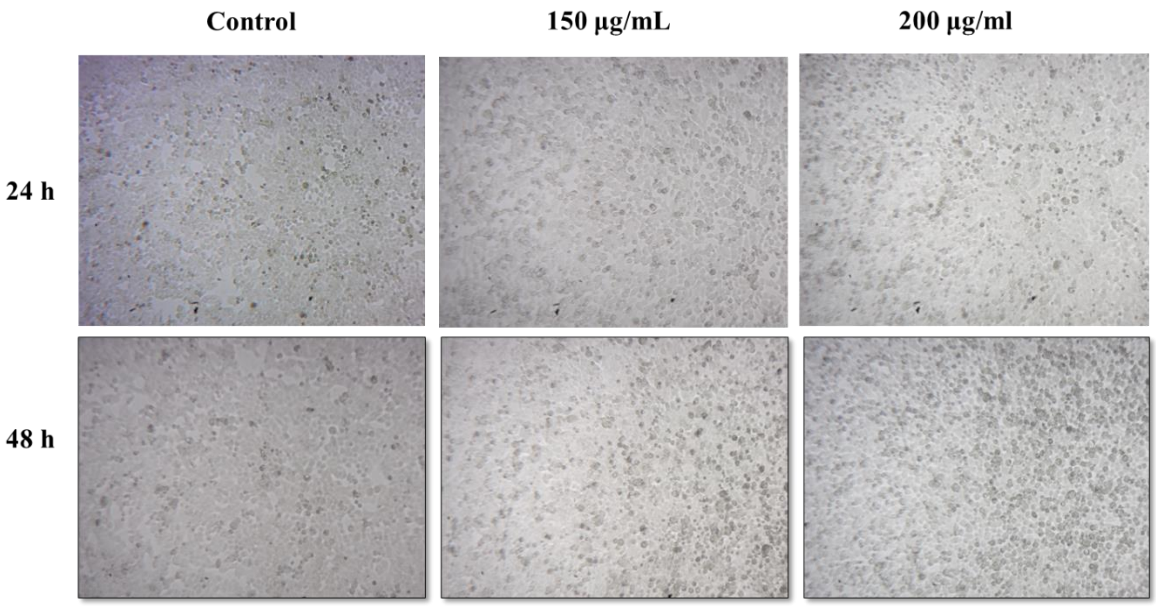

Figure 4. Morphological changes observed in KB cell line when treated with concentrations of *F. racemosa* leaf extract (methanol) along with the control.

3.5. Acridine orange (AO) and ethidium bromide (EB) dual staining assay.

Basic morphological changes in apoptotic cells can be identified using dual AO/EB fluorescent staining. Additionally, it helps in the differentiation of necrotic cells, early and late apoptotic cells, and normal cells. AO/EB staining is therefore a qualitative and quantitative technique to identify apoptosis [47]. AO penetrated through intact membranes of both normal and early apoptotic cells, fluorescing green when bound to DNA. Cells with damaged membranes, such as late apoptotic and dead cells, were penetrated by EB, emitting orange to red fluorescence when attached to concentrated DNA fragments or apoptotic bodies [48].

In order to distinguish between early apoptotic cells, late apoptotic cells, and dead cells, the morphology of cells must be analyzed. In the present study, *F. racemosa* leaves (methanol extract) on KB cells at 48h exhibited orange to red fluorescing nuclei with condensed or fragmented chromatin (Figure 5c)compared to the control, which showed a uniform green fluorescing nuclei with a highly organised structure (Figure 5a). The results from AO/EB dual staining are shown in Figure 5. From the figure, it was clear that the number of viable cells decreased tremendously in support of cell viability studies.

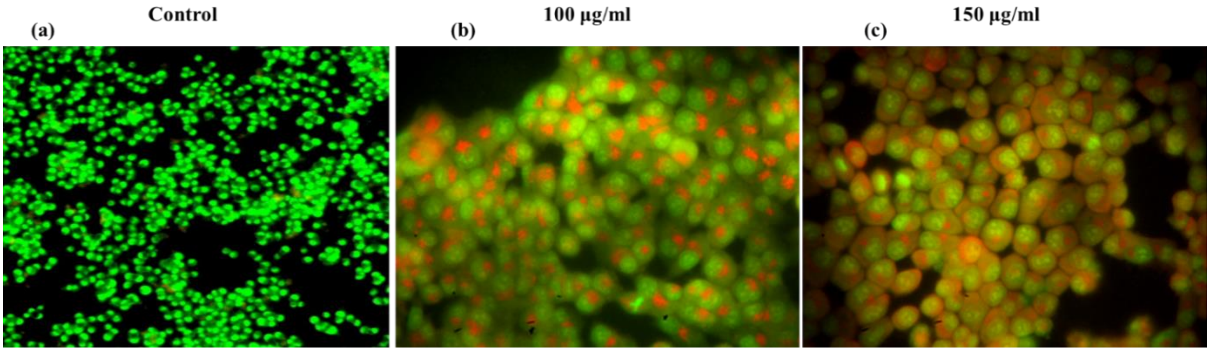

Figure 5. AO/EB dual staining of KB cells at 48h **(a)** Control; **(b)** Extract treated with KB cells at 100 µg/mL; **(c)** Extract treated with KB cells at 150µg/mL. Live cells are uniformly green, early apoptotic condensed or fragmented form of yellow colour nucleus with chromatin, late apoptotic chromatin condensation or fragmentation, orange-stained nuclei, and necrotic cells (uniformly red-stained cell nuclei).

4. Conclusion

This study concludes the phytochemical screening of the methanolic extract of *F. racemosa* leaves along with the quantification of phenolic content, flavonoid content, and evaluation of all bioassays, such as ABTS, DPPH, antibacterial, and cytotoxicity activities on KB cells. This is the first study to report on the cytotoxic properties and apoptosis of the methanol extract of *F. racemosa* on oral cancer (KB) cell lines. The methanolic leaf extract depicted good efficacy in antioxidant, antibacterial, and antitumor activities. Hence, this study comprehensively suggests *F. racemosa* as an alternative therapy for cancer phytomedicine development. The potential impacts on oral cancer treatment and on the synergistic effects of different antioxidants are to be studied at the molecular level in the future.

Author Contributions

S.K. Conceptualization, writing - original draft preparation. R.D. Methodology, Writing-review and editing. S.R. Supervision, investigation, and review.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

Funding

The authors did not receive any external funds.

Acknowledgments

The author (S.K) express sincere gratitude to the management of Ethiraj College for Women, Egmore, Chennai, for their invaluable support and resources. The authors (R.D and S.R) are grateful to the management of Dr. Ambedkar Government Arts College, Vyasarpadi , Chennai and Chennai National Arts and Science College, Avadi, Chennai, for providing infrastructure facilities and support.

Conflicts of Interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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