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Antimycobacterial and anticancer properties of *Myrtus communis* leaf extract

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Abstract: Background: Plant-derived products or extracts are widely used in folk/traditional medicine to treat several infections, ailments, or disorders. A well-known medicinal herb, *Myrtus communis* is an evergreen fragrant plant native to the Mediterranean region that has been used for ages in traditional medicine around the world. **Materials and methods:** The microplate alamarBlue assay and the well diffusion method were used to evaluate the zone of inhibition and MIC, respectively. Double disc diffusion method was used to investigate the synergy between antibiotics and the extract. Crystal violet method was used to investigate the biofilm development. The SulphoRhodamine-B assay and DNA flow cytometry were used to investigate the proliferation and subsequent distribution of cells among different phases of the cell cycle. The apoptotic and necrotic phases of the cancer cells were examined using flow cytometry in conjunction with Annexin V-FITC/PI labeling. Using the IBM SPSS statistical program, a one-way ANOVA with Tukey's post-hoc test was employed for statistical analysis. **Results:** The ethanolic leaf extract of *M. communis* showed a strong growth inhibitory effect (zone of inhibition: 20.3±1.1 - 26.3±2.5 mm, MIC: 4.88 - 312.5 µg/mL, and MBC: 39.07 - 1250 µg/mL) against several rapid and slow-growing mycobacterial strains in a dose-dependent manner. Damage to the cell wall of bacterial cells was determined to be the cause of the antimycobacterial action. The extract inhibited the biofilm formation (MBIC of 9.7 µg/mL) and eradicated the already formed mature and ultra-mature biofilms of *M. smegmatis* with MBEC of 78 µg/mL and 156 µg/mL, respectively. Additionally, the extract exhibited potent anticancer effects against diverse cancer cell lines of the breast (MCF-7), liver (HepG2), cervix (HeLa), and colon (HCT116) (IC_{50} for HCT116: 83 ± 2.5, HepG2: 53.3 ± 0.6, MCF-7: 41.5 ± 0.6, and HeLa: 33.3 ± 3.6) by apoptosis after arresting the cells in G_1 phase of the cell cycle. **Conclusion:** These results suggest that *M. communis* leaf extract is a potential source of secondary metabolites that could be further developed as potential anti-cancer and anti-mycobacterial agents to treat diverse types of cancers and mycobacterial infections.

Keywords: *Myrtus communis*; anti-cancer; caspases; apoptosis; anti-bacterial; biofilm.

1. Introduction

Myrtus communis or Myrtle (Arabic name: Aas or Hadas), belonging to the Myrtaceae (Saudi Arabia refers it as Mesk Al-Madena), is a notable therapeutic herb. It is an evergreen aromatic and medicinal plant inhabitant to the Mediterranean region, along with other nations such as Iraq, Jordan, the Southern and Eastern

provinces of Saudi Arabia [1,2], and Iran in the Middle East. The various parts of this herb, such as its berries, leaves, and fruits, have been extensively used as folk medicine for several centuries. The herb is traditionally used for the treatment of disorders such as inflammation, diarrhea, hemorrhoids, peptic ulcers, pulmonary diseases, and skin diseases. It possesses a broader spectrum of pharmacological and therapeutic effects, such as anti-diabetic, antioxidative, antiviral, antibacterial, antifungal, anticancer, hepatoprotective, and neuroprotective activity [3]. Not much progress has been made in understanding the anticancer activity of *M. communis*, except in a few studies, wherein mostly essential oils and methanol extracts of several medicinal plants have been screened for anticancer activity [4][5].

One of the reasons for the resistance of many common pathogens to commonly used therapeutic agents, such as antibiotics [6], is the formation of biofilms. Biofilms are surface-attached microbial communities in which microbial cells are entrenched in extracellular polymeric substances (EPS) formed by the cells themselves [7]. As a result, biofilms serve as effective physical barriers to antibiotic and nutrient penetration [8]. For example, *S. aureus,* a Gram-positive bacterium and causative agent of nosocomial infection, causes significant morbidity and mortality in hospitalized patients because of its ability to adhere to the surfaces of indwelling medical devices. Therefore, there is a need to develop new drugs to treat bacterial infections, for which natural compounds are appropriate.

There are about two hundred species of mycobacteria, including *M. tuberculosis* and other non-tuberculosis mycobacteria (NTM) species that cause several types of diseases in humans. Tuberculosis, caused by *M. tuberculosis*, is a leading cause of 1.5 million deaths each year and a major contributor to antimicrobial resistance. Non-tuberculosis Mycobacteria (NTM), once thought to be harmless environmental saprophytes and dangerous to immunocompromised and lung-defective individuals, are now causing a spectrum of diseases that include tuberculosis (TB)-like pulmonary and extrapulmonary disease, visceral and disseminated disease, and cervical lymphadenitis in immunocompetent individuals [9]. In Saudi Arabia and other Gulf countries, *M. fortuitum* and *M. abscessus* were found to be approximately 36% and 21% of the clinical isolates of mycobacterium [10].

Due to the emergence of drug resistance and reduced rate of development of new antibacterial agents, the treatment of bacterial infections is strained. Therefore, strategies like targeting virulence factors with natural compounds and using bacteriophages are needed to combat emerging infections. Moreover, treating microbial infections would prevent the occurrence and prognosis of non-infectious diseases like cancer because various epidemiological studies have substantiated the link between infection and cancer development [11–13]. Viruses do take advantage of host cells for their infection cycle and activate a large number of signaling pathways and cellular genes involved in oncogenesis, proliferation, inflammation, and immune responses, which eventually cause the cells to divide uncontrollably and become malignant. Although bacteria do not

induce genetic instability like viruses, pathogenic bacteria have evolved a wide variety of outer surface modifications (lipopolysaccharides, peptidoglycans) or virulence factors production (e.g. toxins) that ensure immune escape to afford significant survival opportunities. The surface-associated or secreted molecules can manipulate host cell signaling pathways and compromise the integrity of the host cell, which could result in tumorigenesis. Therefore, targeting the pathogenic bacteria or their virulence factors would not only cure infectious but also non-infectious diseases.

Cancer, also called malignancy, is an abnormal growth of cells. An estimated nearly ten million deaths were caused by cancer in 2020 [14]. It is the second-leading cause of death globally. One of the leading causes of morbidity and mortality for women worldwide is breast cancer, which is most prevalent in underdeveloped countries [15]. The leading cause of death in people with cirrhosis [16] is hepatocellular carcinoma (HCC), which is the most common primary malignancy of the liver. Using multidisciplinary surgical and non-surgical approaches [17], including systematic chemotherapy [18], significant progress has been made in the diagnosis and treatment of HCC. Colorectal cancer is caused by several risk factors, including genetic and environmental factors, lifestyle, and gut microbiota. Because of their undesirable side effects and efficacy, a variety of cytotoxic drugs are incompetent. There is a need for novel anticancer compounds with no undesirable side effects. In eukaryotes, apoptosis plays an essential role in regulating tissue development and maintaining homeostasis [19–21], deregulation of which is one of the hallmarks of cancer [21].

In the current study, the ethanolic leaf extract of *M. communis* was examined for its impact on cell viability, cell cycle arrest, and apoptosis of four different cancer cell lines. This is the first report of *M. communis* leaf extract's anticancer efficacy against the aforementioned cancer cell lines.

The leaf extract was also tested for its antibacterial properties against a variety of mycobacterial strains, including reference and laboratory strains. Additionally, we looked at the effects of *M. communis* extract on the development of biofilms in *S. aureus* and *M. smegmatis*, the latter of which acts as a model organism for *M. tuberculosis* pathogenesis.

2. Materials and Methods

2.1. Preparation of plant extract

The method used for the preparation of *M. communis* leaf extract is described in detail [22]. The specimen has already been identified and confirmed as described [19]. In brief, the dry leaves of the medicinal plant *M. communis* native were grinded into a fine powder. The extract was prepared in ethanol by the Soxhlet extraction method. Fifty grams of the leaf powder were incubated with 200 ml of absolute ethanol for 2 hours in the Soxhlet extractor. The extract was filtered through Whatman-1 paper, and the filtrate thus obtained was poured in petri dishes. The Petri dishes were left open at room temperature until complete evaporation of ethanol. The dried extract of *M. communis* was redissolved either in ethanol or DMSO at the desired concentrations and used in assays.

2.2. Bacterial strains and media

Ten mycobacterial strains, including laboratory and reference strains, viz. *M. abscessus, M. kansasii, M. mucogenicum, M. xenopi, M. tuberculosis Rif-R, M. kansasii* ATCC35775*, M. tuberculosis* ATCC25177/*H37Ra, M. avium* ATCC25291*,* and *M. fortuitum* ATCC6841, were grown in Middlebrook 7H9 broth supplemented with OADC. The mycobacterial strains, including both slow-growing (SGM) and rapidly growing mycobacterial (RGM) strains, were maintained in the TB laboratory (BSL3 facility) at Southern Region Military Hospital, Khamis Mushait, Saudi Arabia. Trypticase Soy Broth (TSB) supplemented with 2% glucose was used for *M. smegmatis* and *S. aureus* in their growth and other assays, such as TEM, biofilm inhibition, and antibacterial assays. Antibiotic discs, polymyxin B (50 mg) and bacitracin (130 mg), were from Bio-Rad. Discs of vancomycin (30 mg) and clarithromycin (15 mg), were purchased from Oxoid Ltd., Hampshire, England, while discs of colistin (10 mg), gentamicin (10 mg), ofloxacin (5 mg), chloramphenicol (30 mg), and tetracycline (30 mg) were purchased from Abtek Biologicals Ltd., UK.

2.3. Zone of inhibition

Agar well diffusion method is widely used to evaluate the antimicrobial activity of plant extracts. The zone of inhibition was determined as described [23] with slight modifications. Wells of 6 mm diameter were punched aseptically on TSA plates with a sterile tip. Using aseptic techniques, inoculums of 0.5 McFarland were prepared in normal saline. Subsequently, the bacterial suspension was diluted 100-fold in OADC-supplemented Middlebrook 7H9 growth media. The diluted bacterial suspensions were spread over the agar plate's surface using a sterile cotton swab to obtain uniform microbial growth on the plates. Then, 20 µL of 0.4 g/ml of *M. communis* extract was dispensed into the wells of the plate, while, for control, 20 µL of ethanol was dispensed into separate wells. Depending on the growth status of a bacterial strain, the plates were incubated aerobically for 4 days to 4 weeks at 37°C. A ruler was used to measure the diameter (mm) of a clear zone around the well. The actual zone of inhibition was determined by the equation: Zone of inhibition = (average diameter of the zone of inhibition by extract - average diameter of the zone of inhibition by ethanol) + the diameter of a well (6mm). For double disc diffusion method, the antibiotic and the extract discs were kept at half sum of their zone of inhibitions. The bridging between the zones of inhibition of the antibiotic and extract indicates their synergy.

2.4. Minimum Inhibitory Concentration (MIC)

MIC was determined by the broth dilution method as described [23] with slight modifications. In brief, 0.5 McFarland bacterial cultures in normal saline were 100-fold diluted in OADC-supplemented Middlebrook 7H9 media containing 1x alamarBlue. 190 µL of diluted culture was dispensed into the wells of a 96-well plate having periphery wells filled with 200 µL of distilled water to avoid evaporation during the incubation period. The 11th column was filled with 200 µL of OADC-supplemented Middlebrook 7H9 media for the control of the sterility of the growth medium. Wells of the column were reserved for control, to which 10 µL

of ethanol was added. For each bacterial strain, three wells per dilution of extract were used. The plates were incubated for 4 days to 4 weeks. The lowest concentration of the compound at which no visible growth (no color change) was observed is considered the MIC for the bacterial strain tested. For *M. smegmatis* and *S. aureus*, the cultures grown in TSB (trypticase soy broth) were supplemented with 2% glucose.

2.5. Minimum Bactericidal Concentration

The minimum bactericidal concentration (MBC) is the lowest concentration of an antimicrobial at which a microorganism is completely killed. A sterilized loop was used to streak the cultures from the wells of MIC up to the wells of the highest concentration of extract on TSA plates. The concentration of extract where no growth was detected is considered MBC for the bacterial strain tested.

2.6. Microplate AlamarBlue Assay (MABA)

The Microplate AlamarBlue® assay (MABA) is a sensitive, rapid, and nonradiometric method that evaluates cellular health and offers the potential for screening large numbers of antimicrobial compounds. MABA was performed as described [23] with a slight modification. In brief, 190 µL of the 100-fold diluted culture of a bacterial strain in 2% glucose supplemented TSB containing 1× alamarBlue were dispensed into the wells of a 96-well plate. Then, 10 µL of extract at its MIC, 2×MIC, and 4xMIC were dispensed in triplicate culture wells. For positive control and medium sterility, 10 µL of ethanol was added to triplicate wells of culture and growth media, respectively. The periphery wells were filled with 200 µL of sterile water to avoid evaporation of the well content. The plate was sealed with breathable sealing film (Merck) to allow the exchange of gases. The plate was incubated in a FLUOstarr® Omega microplate reader. The fluorescent readings (excitation/emission maxima at 544/590 nm) were recorded every 30 min and one hour for *S. aureus* and *M. smegmatis*, respectively. The average values of blank-corrected fluorescence units were plotted against time using Microsoft Excel software. Standard deviations were calculated, and graphs were constructed using Microsoft Excel software, version 16.66.1.

2.7. Crystal-violet biofilm assay

A few bacterial colonies of *S. aureus* and *M. smegmatis* mc^2155 grown on TSA plates were suspended in normal saline to obtain 0.5 McFarland (10^8 CFU/ml) bacterial suspensions, which were later used in the biofilm assay as described [24]. In brief, the above-prepared 0.5 McFarland bacterial suspensions were 100 times diluted in TSB supplemented with 2% glucose, and 190 µL of the diluted cultures were dispensed in the wells of the 96-well plate. 10 µL each of *M. communis* extract with a 2-fold increase in a final concentration ranging from 1/2MIC to 4xMIC for each bacterial strain were dispensed in triplicate wells. For the positive control, 10 µL of absolute ethanol, a carrier solvent, was similarly dispensed into the triplicate culture wells for each bacterial strain. As a control for the binding of crystal violet to the walls of wells, 200 µL of sterile TSB were dispensed in triplicate wells. The plates were incubated for either 24 hours (for *S. aureus*) or 48

hours (for *M. smegmatis*) at 37°C under static conditions. The biofilms were developed as described by Christensen et al. and measured at an absorbance of 570 nm using a FLUOstar® Omega microplate reader featuring BMG LABTECH's proprietary tandem technology. The percentage of biofilm inhibition was calculated using the formula "percentage of biofilm inhibition = (1 - average OD_{570} of sample/average OD_{570} of control) x 100". Percentage biofilm inhibition was plotted against the extract concentration using Microsoft Excel software, version 16.66.1. The standard deviations (±SD) shown by error bars were plotted on the graph.

2.8. Transmission electron microscopy (TEM)

The *M. smegmatis* cells were treated with 10xMIC of extract for 12 hours at 37°C in a shaking incubator at 200 rpm. To rule out the effect of carrier solvent ethanol on cell morphology, the final concentration of ethanol was maintained at a minimum concentration of 1.6%. For control, the cells were treated with the same concentration of 1.6% ethanol under similar conditions.

Both control and extract-treated *M. smegmatis* cells were harvested at 5000g. The cell pellet was immediately resuspended in 2.5% paraformaldehyde-glutaraldehyde fixative for 2 hours at 4°C. The samples were further centrifuged at 5000g, and pellets were washed twice with sodium cacodylate buffer. The samples were adsorbed for 1 min onto a formvar-coated 200-mesh copper grid. The adsorbed samples were washed two times in sodium cacodylate buffer, and negative staining was accomplished by immersing coated grids for 20 seconds in 1% uranyl acetate, followed by destaining in sterile Milli-Q water and air drying. Imaging was performed at 80 Kv using a JEM-1011 transmission electron microscope (JEOL Co., Tokyo, Japan) in the EM-Unit, College of Medicine, King Khalid University. TEM images were recorded at magnifications of 15,000x.

2.9.Cell culture

Human hepatocellular carcinoma cell line (HepG2), colorectal adenocarcinoma cell line (HCT116), breast adenocarcinoma cell line (MCF-7), and human cervix adenocarcinoma cells (HeLa) were obtained from the American Type Culture Collection (ATCC). Cells were maintained in RPMI-1640 supplemented with 100 µg/mL penicillin and heat-inactivated fetal bovine serum (10% v/v) in a humidified, 5% (v/v) CO_2 atmosphere at 37°C.

2.10. Cytotoxicity assessment

The cytotoxicity of the extract was tested against human tumor cells using the Sulphorhodamine B assay (SRB). Healthy-growing cells were cultured in a 96-well tissue culture plate (3,000 cells/well) for 24 hours before being treating with the extract to allow attachment of the cells to the plate. Cells were exposed to five different concentrations of extract (0.01, 0.1, 1, 10, 100, and 1,000 µg/mL), dissolved in DMSO. For control, the cells were treated with DMSO alone. Triplicate wells were included for each concentration of extract. The plate was incubated for 72 hours at 30°C and subsequently fixed with TCA (10% w/v) for one hour at 4°C. After several washings, cells were stained with 0.4% (w/v) SRB solution for 10 min in the dark. The excess

stain was washed with 1% (v/v) glacial acetic acid. After drying overnight, the SRB-stained cells were dissolved in tris–HCl, and absorbance was measured in a microplate reader at 540 nm. The linear relation between the viability percentage of each tumor cell line and extract concentration was analyzed to get the IC_{50} (dose of the drug that reduces survival to 50%) using SigmaPlot 12.0 software [25].

2.11. Cell cycle distribution using DNA flow cytometry

DNA flow cytometry for cell cycle distribution was performed as described by Shati Ali A *et al.* [26]. In brief, the cells were treated with the IC_{50} of leaf extract for 48 h, and collected by trypsinization, washed with ice-cold PBS, and re-suspended in 0.5 ml of PBS. Ten ml of 70% ice-cold ethanol were gently added while vortexing. Cells were kept at 4ºC for one hour and stored at -20ºC until analysis. Upon analysis, fixed cells were washed and resuspended in one ml of PBS containing 50 mg/mL RNase A and 10 mg/mL propidium iodide (PI). After incubating the cells at 37ºC for 20 minutes, they were analyzed for DNA content by FACSVantageTM (Becton Dickinson Immunocytometry Systems, San Jose, CA). For each sample, 10,000 events were acquired. CELLQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA) was used to calculate the cell cycle distribution. Each treatment was repeated three times, and the data represent the mean ± SD of three replicates.

2.12. Apoptosis analysis

Cells treated with extract for 48 hours were trypsinized and washed twice with PBS. Apoptosis assessment was done using the Annexin V-FITC/PI Apoptosis Detection Kit, Cell Signaling Technology (CST), as stated by the manufacturer. In Brief, cells were resuspended in 0.5 ml of binding buffer containing 5 µL of Annexin V-FITC and 5µL of PI (staining solution). The suspension was incubated at room temperature with gentle mixing for 15 minutes in a dark place. Finally, the cells were subjected to FACS analysis using a Cytek® Northern Lights 2000 spectral flow cytometer (Cytek Biosciences) using SpectroFlo™ Software version 2.2.0.3 (Cytek Biosciences), USA

2.13. Statistical Analysis

Data were statistically analyzed using IBM SPSS statistical software, version 21. The values were interpreted as the mean standard deviation (SD) for all the data sets, which were collected in triplicate from more than one biological replicate. To evaluate the difference between treated and untreated samples, a one-way ANOVA was employed with Tukey's post-hoc test. The $p < 0.05$ was considered statistically significant.

3. Results

3.1. M. communis leaf extract strongly inhibited the growth of mycobacterial strains

To determine the antimycobacterial activity of the *M. communis* leaf extract, all ten mycobacterial strains were uniformly streaked individually on TSA plates. The zone of inhibition of growth by the extract for each strain was determined using the well-diffusion method. The mycobacterial strains sensitive to the

extract produced a clear zone of no bacterial growth around the well (Fig. 1). The zone of inhibition ranged from 20.3±1.1 mm to 26.3±2.5 mm (Table 1), suggesting that all the mycobacterial strains, including tuberculosis and non-tuberculosis (SGM and RGM), were strongly inhibited by the extract.

The MIC of *M. communis* extract against each mycobacterial strain was determined by microplate alamarBlue assay. AlamarBlue, a growth indicator, changes color from blue to pink during the growth of cells. The bacterial strains were treated with 2-fold dilutions of *M. communis* leaf extract ranging from 0.02 g/mL to 3 µg/mL in the 96-well plate. The lowest concentration of the extract at which no change of color occurs from blue to pink is considered the MIC. As shown in Figure 1, 4.8 µg/mL is the MIC for *M. kansasii* ATCC35775. The MIC of the extract against each bacterial strain was determined and presented in Table 1. It is clear from the table that MIC ranged from 4.8 µg/mL to 312.5 µg/mL. Only two mycobacterial strains, *M. fortuitum ATCC6841* (MIC: 156.2 µg/mL) and *M. tuberculosis* RIF-R (MIC: 312.5 µg/mL), were comparatively less sensitive to the extract.

From the alamarBlue assay plate, the cultures of each strain from the wells having extract concentration above the MIC were streaked on TSA plates. The concentration of the extract at which no visible growth of bacterial colonies was detected for a particular strain was considered MBC for the said strain. The MBC ranged from 39.07 µg/mL to 1250 µg/mL for all mycobacterial strains tested (Table 1). It was found that there was a consistent correlation between MIC and MBC for each mycobacterial strain tested. The strain with a lower MIC did show a lower MBC, and vice versa. Consistent with the MIC, *M. fortuitum ATCC6841* (MBC: 937 µg/mL) and *M. tuberculosis* RIF-R (MBC: 1250 µg/mL) showed comparatively higher MBC.

3.2. Growth Kinetics of Bacterial Strains in the Presence of Extract

MABA, an adequate method for testing antimicrobial activity, was used to monitor the growth of extract-susceptible bacterial strains in the continued presence of different concentrations of the extract. AlamarBlue, a growth indicator, is a fluorescent dye that changes its color from blue to pink in response to the reduced environment of the growing cells. 0.5 McFarland bacterial cultures diluted hundred times in trypticase soy broth containing 1x alamarBlue were incubated with extract at its MIC, 2xMIC, and 4xMIC. For control, the cultures were incubated with ethanol. Growth media containing 1x alamarBlue were used as a blank for their sterility. The growth at regular intervals of 30 min for *S. aureus* and one hour for *M. smegmatis* was monitored in the FLUOstar® Omega microplate reader over time. Blank-corrected average fluorescence units (Ex_{560}/Em_{590}) plotted against the time for each strain (Fig. 2) showed that both strains tested had a normal pattern of growth in the presence of ethanol. However, a decrease in growth was observed for both the two strains tested, with a corresponding increase in the concentration of extract from MIC to 4xMIC. These results suggest that the growth-inhibitory effect is dose-dependent.

3.3. M. Communis leaf extract damages the bacterial cell wall

To investigate the effect of the extract on cellular morphology, *M. smegmatis* cells were treated with the extract. For control, cells were treated with the carrier solvent ethanol under similar conditions. After fixation and negative staining, the cells were observed under the transmission electron microscope. The tomographic images demonstrated that the extract-treated cells have a damaged cell boundary compared to control cells (Fig. 3). This suggests that the extract targeted the cell wall, which is consistent with our earlier observation [22].

To further explore the cell wall damaging effect of the extract, the *M. smegmatis* cells in presence of extract were treated with several commercial antibiotics including cell wall-targeting vancomycin and bacitracin. The antibiotic discs such as gentamycin, tetracycline, ofloxacin, vancomycin, chloramphenicol, polymyxin B, colistin, and bacitracin were impregnated with extract and tested for zone of inhibition. For control, the antibiotic discs were impregnated with ethanol and tested under similar conditions. It was found that the zone of inhibition of vancomycin, colistin, bacitracin, and chloramphenicol in presence of extract was ≥5 nm higher than that of the antibiotic alone. However, no change in the size of inhibition zone was observed for polymyxin B. On the contrary, the extract exhibited negative effect on gentamycin, clarithromycin, ofloxacin, and tetracycline (Table 2).

To further explore the interaction between the extract and antibiotics, the double disc diffusion method was employed, wherein the discs of antibiotic and extract were kept at half sum of their inhibition zone sizes. As shown in figure 4, clear bridging was observed between the inhibition zones of vancomycin, bacitracin, and the extract. However, no such bridging was observed for the remaining antibiotics tested (Fig. 4 and Supplemental S1–Fig. 1). Bacitracin and vancomycin target the cell wall of the bacteria by blocking the synthesis of the cell wall component peptidoglycan, which is abundant in *Mycobacteria*. The synergistic action between the extract and antibiotics vancomycin and bacitracin, and the scarring of bacterial cell wall as shown by SEM indicated that the extract's bio-actives targeted the cell wall of *M. smegmatis.*

3.4. M. communis leaf extract inhibits the biofilm formation of M. smegmatis and S. aureus

Mycobacterial strains are believed to form a biofilm [28], which is the main barrier for antimicrobials to kill the resident microorganism. We attempted to investigate whether *M. communis* leaf extract could affect the biofilm formation of mycobacterial strains. *M. smegmatis* was chosen as an ideal model microorganism because it is a non-pathogenic and fast-growing microorganism that shares high similarities with *M. tuberculosis*. The results obtained will be later ascertained for the mycobacterial strains, which is not the aim of the present study. For a control, we explored the effect of the extract on *S. aureus*, which is a robust biofilm-forming bacterium.

Before investigating the effect of the extract on biofilm formation, the MIC of the extract for both strains was determined by the microdilution method. 0.5 McFarland cultures of *S. aureus* and *M. smegmatis* were individually treated with 2-fold diluted *M. communis* leaf extract, ranging from a final concentration of 10

mg/mL to 0.6 µg/mL. The MIC of the extract for *M. smegmatis* and *S. aureus* was found to be 39 µg/mL and 19.4 µg/mL, respectively. To study the effect of the extract on biofilm formation, *S. aureus* and *M. smegmatis* strains were individually treated with increasing 2-fold *M. communis* extract concentrations ranging from 4.85 µg/mL (1/4MIC) to 77.6 µg/mL (4xMIC) and 9.75 µg/mL (1/4MIC) to 156 µg/mL (4xMIC), respectively. Subsequently, the biofilms formed were developed by the crystal violet staining method. The percentage of biofilm inhibition calculated at each concentration of extract for *S. aureus* (Fig. 5A) and *M. smegmatis* (Fig. 5B) was plotted. It was found that the biofilm formation of *S. aureus and M. smegmatis* was inhibited by 51% and 70% at 9.7 µg/mL of the extract, respectively. Therefore, the minimum biofilm inhibition concentration (MBIC) of the extract for both strains was considered to be 9.7 µg/mL. MBIC was defined as the minimum concentration of extract that exhibits the highest biofilm inhibition without affecting growth.

We further evaluated the effect of the extract on already-formed mature and extra-mature biofilms. For mature biofilm formation, the bacterial cultures of *S. aureus* and *M. smegmatis* were incubated at 37°C under static conditions for 24 and 48 hours, respectively. For extra-mature biofilm formation, the *S. aureus* and *M. smegmatis* cells were incubated under similar conditions for 48 and 72 hours, respectively. The biofilms formed were washed off the planktonic cells and further treated with the extract for 24 hours. Biofilms retained after extract treatment were stained and quantitated by the crystal violet method. Under similar conditions, the biofilms formed in control wells were treated with ethanol only and subsequently stained and quantitated by the crystal violet method. It is clear from figures 6A and 6B that the mature and extra-mature biofilms in *M. smegmatis* were eradicated by 100% and 90% at 78 µg/mL and 156 µg/mL, respectively. Therefore, the minimum biofilm eradication concentration (MBEC) of extract for mature and extra-mature biofilms of *M. smegmatis* was considered to be 78 µg/mL and 156 µg/mL, respectively. Contrarily, for *S. aureus*, the MBEC for mature and extra-mature biofilm was found to be 77.6 µg/mL. MBEC was defined as the minimum concentration of extract that exhibits the highest eradication of already-formed biofilms. At respective MICs of the extract for *M. smegmatis* and *S. aureus*, 80-88% and 35-45% of their already-formed biofilms were eradicated, respectively. These results suggested that the mature biofilms of *M. smegmatis* were comparatively more susceptible to the extract.

3.5. M. communis leaf extract inhibited the proliferation of cancer cell lines

To investigate the effect of leaf extract of *M. communis* on the proliferation of different types of cancer cells, *viz,* breast, liver, colon, and cervix, an SRB assay was carried out. The cell toxicity of the treatment with different concentrations of extract for 72 hours was measured by the percentage viability of the cancer cells. The results showed that the viability of cells decreased in a dose-dependent manner (Fig. 7), killing 75% of all cells at a concentration of 100 µg/mL. A gradual increase in the concentration of the extract resulted in a gradual increase in the growth inhibition of all four types of tumor cells upon their independent treatment with

the extract. The cytotoxic activity of the extract was strong for HeLa cells (IC_{50}: 33 µg/mL) (Table 3) while for other cells it was moderate (IC_{50} for HCT116: 83 ± 2.5, HepG2: 53.3 ± 0.6, and MCF-7: 41.5 ± 0.6).

3.6. M. communis leaf extract caused cell cycle arrest and the induction ofapoptosis in cancer cells

One of the major causes of inhibition of cellular growth is cell cycle arrest. To determine whether the growth inhibition of cancer cell lines was due to cell cycle arrest at a particular phase, the cell lines were treated individually with the ethanolic leaf extract at their IC_{50} concentrations. After the treatment, the cell cycle profile of each cell line was determined by PI staining followed by DNA flow cytometry analysis. Table 4 and Fig. 8 showed that a significant proportion (~ 10% more than the control) of all the tumor cell lines was arrested in the G_1 phase of the cell cycle, with a corresponding drop in the percentage of cells in the S-phase upon treatment with the ethanolic leaf extract of *M. communis*. Moreover, a marked increase in the percentage of cells in the G_2/M phase (3%-5% more than the control) was observed for all cancer cell types except HCT116. These results demonstrate that the extract arrested all types of cancer cells in the G_1 phase of the cell cycle, though the percentage of cells showed a variation.

To further investigate the extract-induced inhibitory effect, cells treated with extract were analyzed in a flow cytometer after Annexin V-FITC/PI staining. Analysis of the percentage of cells detected in different stages of apoptosis (Fig. 9, Sup. 2, and Sup. 1-Fig. 2) indicated that the major cellular populations of MCF-7 (97.15%) and HeLa (97.38%) cell lines were in late apoptotic stage, while as 71.32% of HCT-116 cells were found in early stage of apoptosis. However, significant proportions of HepG2 cells were found in both early (50.6%) and late (47.8%) stages of apoptosis. Less than 1% cells of all cancer cells were found in necrotic stage of apoptosis apart from HCT-116, wherein a significant proportion of cells (17.66%) were found in necrotic stage of apoptosis. The cells treated with the extract were further analyzed under a fluorescent microscope for nuclear morphological changes (apoptosis or necrosis) after acridine orange/ethidium bromide staining. As usual, the major hallmarks of apoptotic cell death are DNA fragmentation and loss of membrane asymmetry. The extract of *M. communis* induced morphological changes, DNA fragmentation, nuclear shrinking, etc., which are characteristics of various stages of apoptosis, *viz*. early or late phase apoptosis or necrosis (Sup.1-Fig. 3). Altogether, these results suggest that the major cellular population of all four tumor cell lines tested was in the apoptotic stage upon treatment with the leaf extract.

4. Discussion

Although substantial improvements have been made in medical technology, there is no cure for almost any cancer around the globe. In addition to non-communicable diseases like cancer, communicable diseases are an additional burden on human health and the economy. Failure to the treat the diseases caused by *M. tuberculosis* and NTM with available commercial antibiotics is a growing concern. The thick cell wall and dormant ability of these bacterial species demand the development of new therapeutic agents.

Natural products extracted in crude or pure form and used for the treatment of various ailments were obtained from medicinal plants. Though herbal medicine has some advantages over a purified constituent compound [29], there is a growing global demand to identify and purify the constituent effectively.

The other major significance of this study is the extract's strong growth inhibition of several NTM strains, including slow and rapid growing (Table 1). The present study is the first report wherein *M. communis* leaf extract inhibited the growth of *M. tuberculosis* and several NTM strains, which is consistent with the growth inhibitory effect of *M. communis* leaf essential oil against several strains of *M. tuberculosis* [30]. These results suggest the potential use of the extract for the treatment of tuberculosis and non-tuberculosis diseases. *M. kansasii,* known to cause pulmonary disease in immunocompromised individuals or those with underlying pulmonary diseases such as silicosis, was strongly inhibited by *M. communis* leaf extract. Among all the bacterial strains tested, *M. communis* extract exhibited the highest zone of inhibition and lowest MIC against *M. kansasii* ATCC35775 (Table 1). *M. abscessus* and *M. fortuitum* [31] were significantly inhibited, while *M. xenopi* showed marked susceptibility to *M. communis* extract. Surprisingly, *M. tuberculosis* strains, especially the *M. tuberculosis* rifampin-resistance strain, showed less sensitivity to the extract (Table 1).

We believe that the cell wall of the susceptible bacteria was the probable target of the extract [19], we performed scanning electron microscopy of the extract-treated *M. smegmatis cells*. The tomographic images of extract-treated cells (Fig. 3) showed that the cell wall of *M. smegmatis* was damaged, which could be one of the leading causes of cell content leakage and subsequent death of bacterial cells. The bridging of inhibition zones between antibacterial indicates their synergistic action [27]. Synergistic antibacterial activity of extract in individual combination with bacitracin and vancomycin (Fig. 4) validates the cell wall as a target of action.

Biofilms are one of the impediments to the access of antimicrobials for the treatment of bacterial infections. Biofilms of *S. aureus* are often associated with chronic infections and infected embedded medical devices. Phytochemicals to inhibit the formation of biofilm and/or expression of other virulence factors in *S. aureus* have been studied to a certain extent [32]. Plant extracts and essential oils of a few medicinal plants have been investigated for the inhibition of biofilm formation in mycobacteria, including NTM and *M. smegmatis* [33–35]. In this study, we attempted to investigate the effect of the *M. communis* leaf extract on the biofilm formation and eradication of already formed biofilms of *M. smegmatis* and *S. aureus* (Figs. 5 and 6). *M. smegmatis* is a model organism to study human pathogens like *M. tuberculosis* and NTM. *S. aureus,* being robust in biofilm formation and a frequent pathogen confronted in clinical and laboratory contexts [36], were included in the biofilm assays. Our results suggest that the *M. communis* extract exhibited strong biofilm inhibition and eradication of already-formed biofilms in both *M. smegmatis* and *S. aureus*. In the future, it would be interesting to isolate and purify the actual compound(s) from *M. communis* leaf extract [22] that are responsible for anticancer and antimycobacterial activities.

In addition to antibacterial property, the cytotoxic property of *M. communis* leaf extract was investigated against diverse cancer cells. The ethanol extract showed strong cytotoxic activity against all four cancerous cell lines tested (IC_{50}: 33.3 ± 3.6 - 83 ± 2.5 μg/mL). Cell cycle analysis showed that the leaf extract arrested all types of cancer cells in the G_1 and G_2/M phases of the cell cycle, except HepG2 and HeLa cell lines, wherein the population of cells decreased in the G_1 or G_2/M phase of the cell cycle compared to the control. However, a variation was observed in the percentage of cells arrested either in G_1 or G2/M for a cell line (Table 4, Fig. 8). It has been shown in previous studies that a sequence of events occurs as the damaged cells proceed through their arrest into G_1 or G_2/M phases. Eventually, after passing through aberrant mitosis, they subsequently undergo apoptosis [37]. Several distinct pathways lead to apoptosis, which usually occurs as a later event in cell death. As shown in Figure 9, a significant population of cells was found in the early and late phases of apoptosis. This is in agreement with the fact that anticancer molecules arrest cells in the growth phase of the cell cycle and subsequently induce apoptosis, which leads to cell death [38].

It is believed that the bacterial infection as well as the dysbiosis lead to the development of cancer [39]. For example, *Fusobacterium nucleatum* has been implicated in various types of cancers, including colorectal cancer, esophageal cancer, and gastric cancer [40]. Similarly, *H. pylori* has a role in gastric tumorigenesis [41]. Dysbiosis, which refers to compositional and functional alterations of the gut microbiome, also contributes to the development of various pathological conditions including, neurodegeneration, obesity, and cancer. Therefore, the anti-bacterial and anti-cancer properties of the extract need to be investigated in a mouse model of infection. Moreover, it needs to be examined whether the extract has any effect on normal flora. We envisage its use in treating mycobacterial infections and possibly being preventive of lung cancers in chronic TB patients, which require further in vivo studies for bioavailability, stability, and cytotoxicity of normal flora and host cells. Although the epidemiological link between chronic tuberculosis and lung cancer substantiates *Mtb* infection a risk factor for lung cancer [42]. The present study suggests that extract contains compounds that not only inhibit the growth of infectious bacterial strains but also exhibits anticancer activity in vitro.

5. Conclusion

The main findings of this study are i) Here we reported for the first time that *M. communis* leaf extract exhibited strong antibacterial activity against both tuberculosis and non-tuberculosis species of mycobacteria. The extract exhibited antibacterial activity by disrupting the bacterial cell wall. iii) The extract exhibited biofilm inhibition and eradication of mature and extra-mature biofilms of *M. smegmatis* and *S. aureus*. iii) This is the first report of *M. communis* extract showing anticancer activity against diverse types of cancer cell lines. The antiproliferative activity of the extract against human cancer cells was due to the accumulation of cells in the G_1 / G_2/M phases of the cell cycle, followed by apoptosis.

In conclusion, these findings suggest that *M. communis* leaf extract's secondary metabolites could be not only potential therapeutic agents against mycobacterial infections but could also be developed to treat breast,

liver, cervix, and colon cancers. Further evaluation, active compound isolation, and *in-vitro* and *in-vivo* evaluations are recommended for future research on these active ingredients.

Declaration: Ethics approval and consent to participate: All methods, including the collection of leaves, complied with institutional guidelines.

Data Availability: The data generated or analyzed during this study are included in this published article.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: Conceptualization, MMA, and MLA; Methodology, MMA, ESE, MLA, and MFS, Software, MMA, MFS, and ESE; Validation, MMA and MLA; Formal Analysis, MMA, MLA, and BN; Investigation, ESE, MMA; Resources, SAA, AMY, MFS, and ASA; Writing, MMA; Original Draft Preparation, MMA; Writing – Review & Editing, MMA and AAM; Visualization, BN, AI, and AMA; Supervision, MMA.; Funding Acquisition, MMA and BN.

Abbreviations:

Hepatocellular carcinoma (HCC)

Extracellular polymeric substances (EPS)

Non-tuberculosis mycobacteria (NTM)

Minimum inhibitory concentration (MIC)

Minimum bactericidal concentration (MBC)

Minimum biofilm inhibitory concentration (MBIC)

Minimum biofilm eradication concentration (MBEC)

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Figures

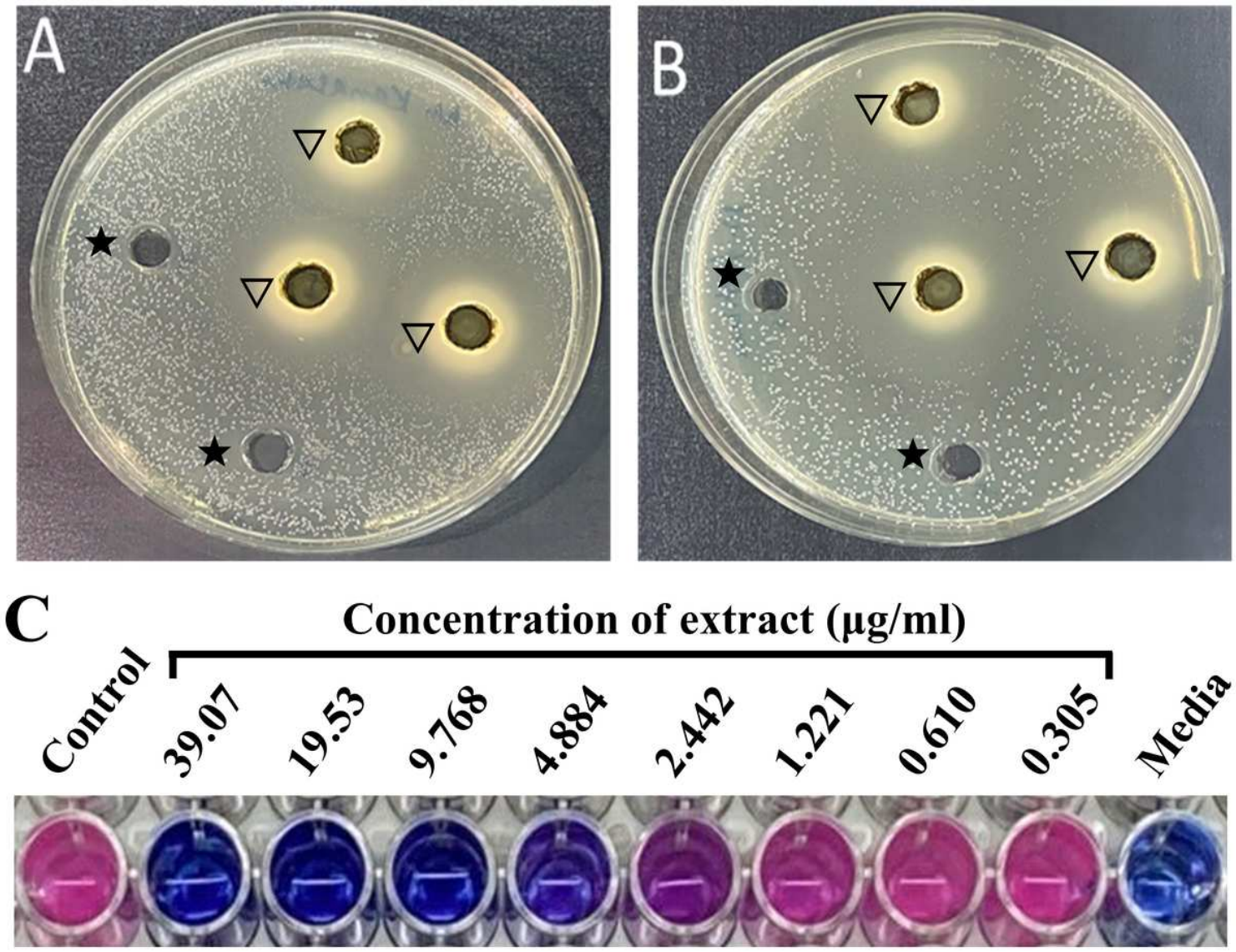

Figure 1

Zone of inhibition and MIC of M. communis leaf extract. A) Zone of inhibition of *Mycobacterium kansasii*. B) Zone of inhibition of *Mycobacterium fortuitum* ATCC6841. C) Two-fold dilutions of the extract (µg/mL) used in the culture wells showed inhibition of growth of *M. kansasii* ATCC35775 at 4.884 µg/mL. The pink color of the well indicates growth, while the blue color indicates inhibition of the growth of bacteria. : well contains ethanol, : well contains extract.

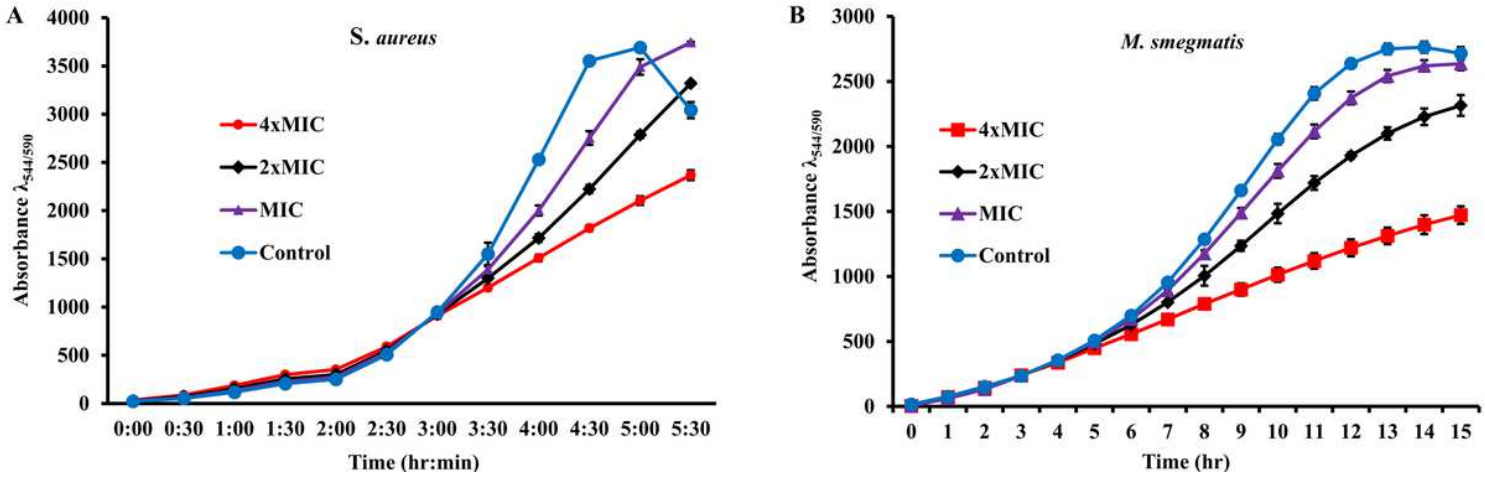

Figure 2

Growth of *S. aureus* and *M. smegmatis* in the presence of extract: The growth of (**A**) *S. aureus* and (**B**) *M. smegmatis* was monitored in the presence of MIC, 2x MIC, and 4x MIC of extract by recording fluorescence readings at 30 min (*S. aureus*) and one-hour (*M. smegmatis*) intervals, respectively, using alarmBlue as a growth indicator.

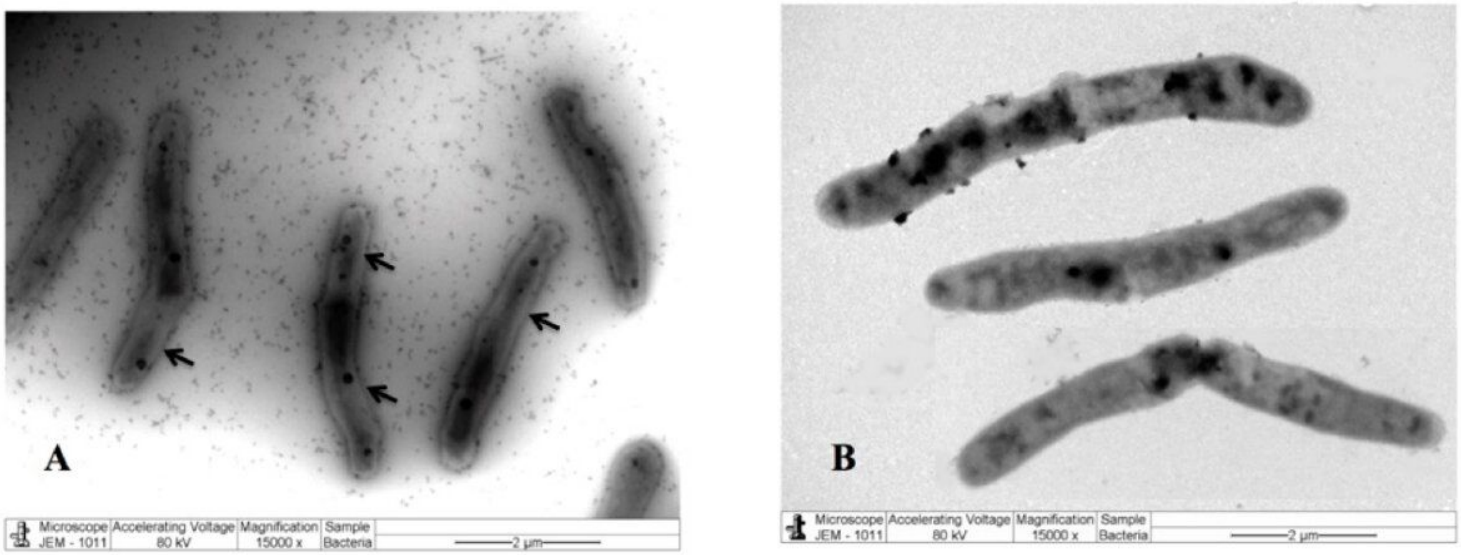

Figure 3

TEM of *M. smegmatis* cells: Tomographic images of untreated (A) and treated (B) *M. smegmatis* cells. Arrows show that the cell wall is intact in untreated cells but damaged in treated cells.

Figure 4

Synergy between antibiotics and extract; Bacitracin and vancomycin exhibited bridging of the zone of inhibition in combination with the extract against *M. smegmatis.*

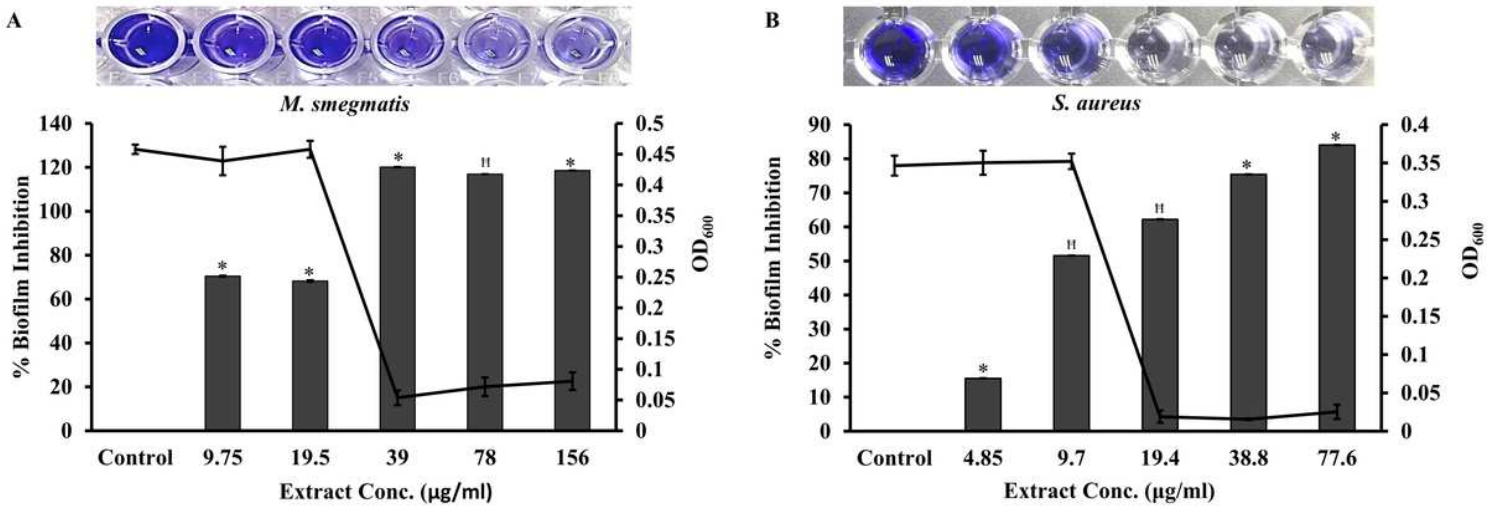

Figure 5

Inhibition of biofilm formation by *S. aureus* and *M. smegmatis* by *M. communis* leaf extract. The growth of A) *M. smegmatis* and B) *S. aureus* in the presence of different concentrations of extract was measured by optical density at 600 nm. The biofilms formed at these concentrations were estimated by crystal violet absorbance at 570 nm. The inlets show the crystal violet-stained biofilms of one of the three replicate wells. *: $p < 0.002$, : $p < 0.006$.

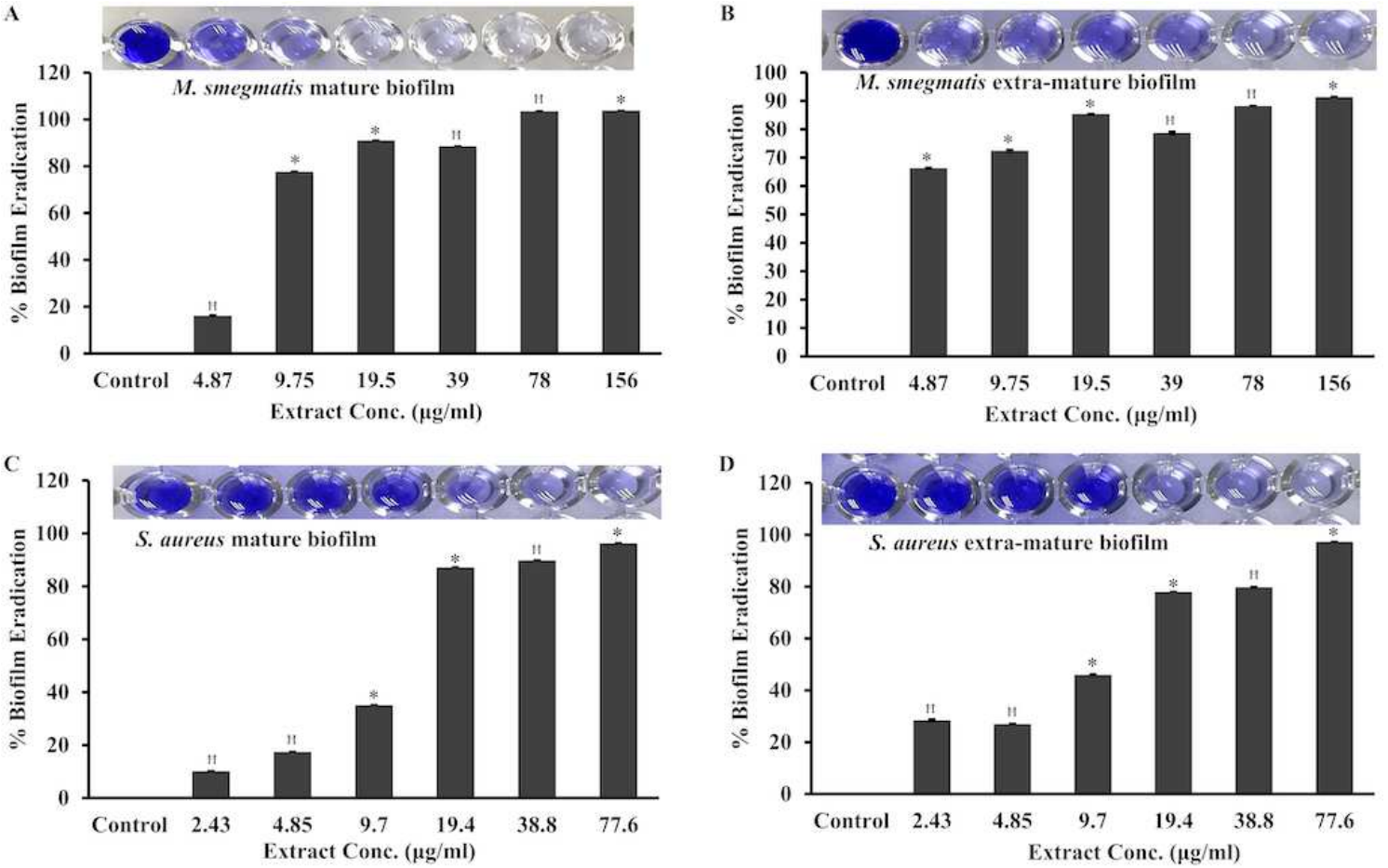

Figure 6

Eradication of mature biofilms by *M. communis* leaf extract. Mature and extra-mature biofilms of *M. smegmatis* (A & B) and *S. aureus* (C & D) were subjected to different concentrations of extract treatment. The biofilms that remained after treatment were developed by the crystal violet method and estimated by absorbance at 570 nm. The percentage of biofilms eradicated was calculated and plotted against the extract concentrations. The inlets show the crystal-violet stained biofilms in one of the three replicate wells. *: $p < 0.002$, : $p < 0.006$.

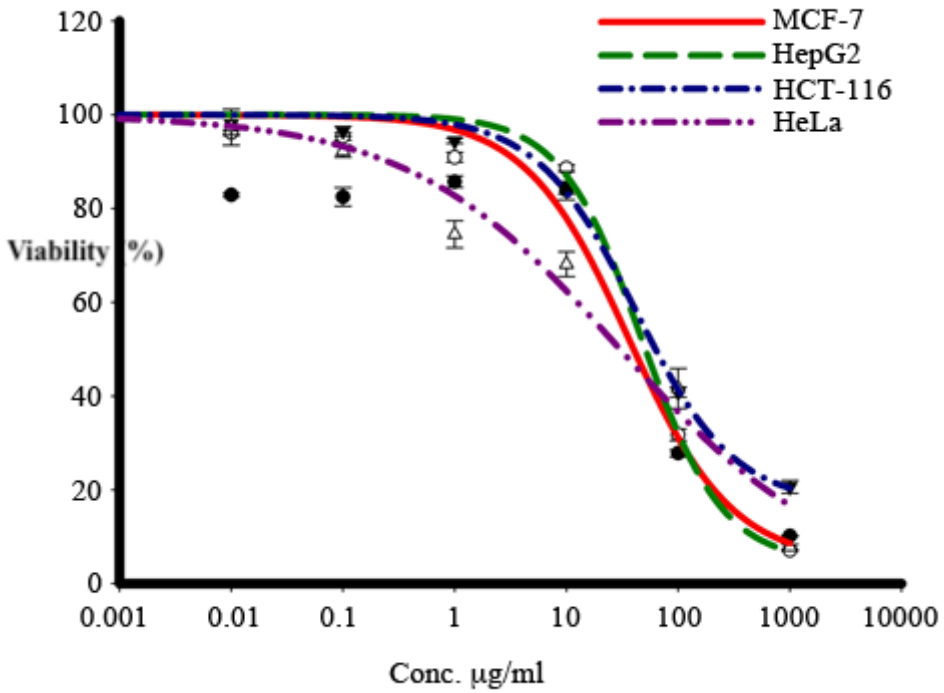

Figure 7

IC_{50} values of the *M. communis* leaf extract against cancer cell lines. The percentage of viable cells after 72 hours of treatment with various concentration of extract.

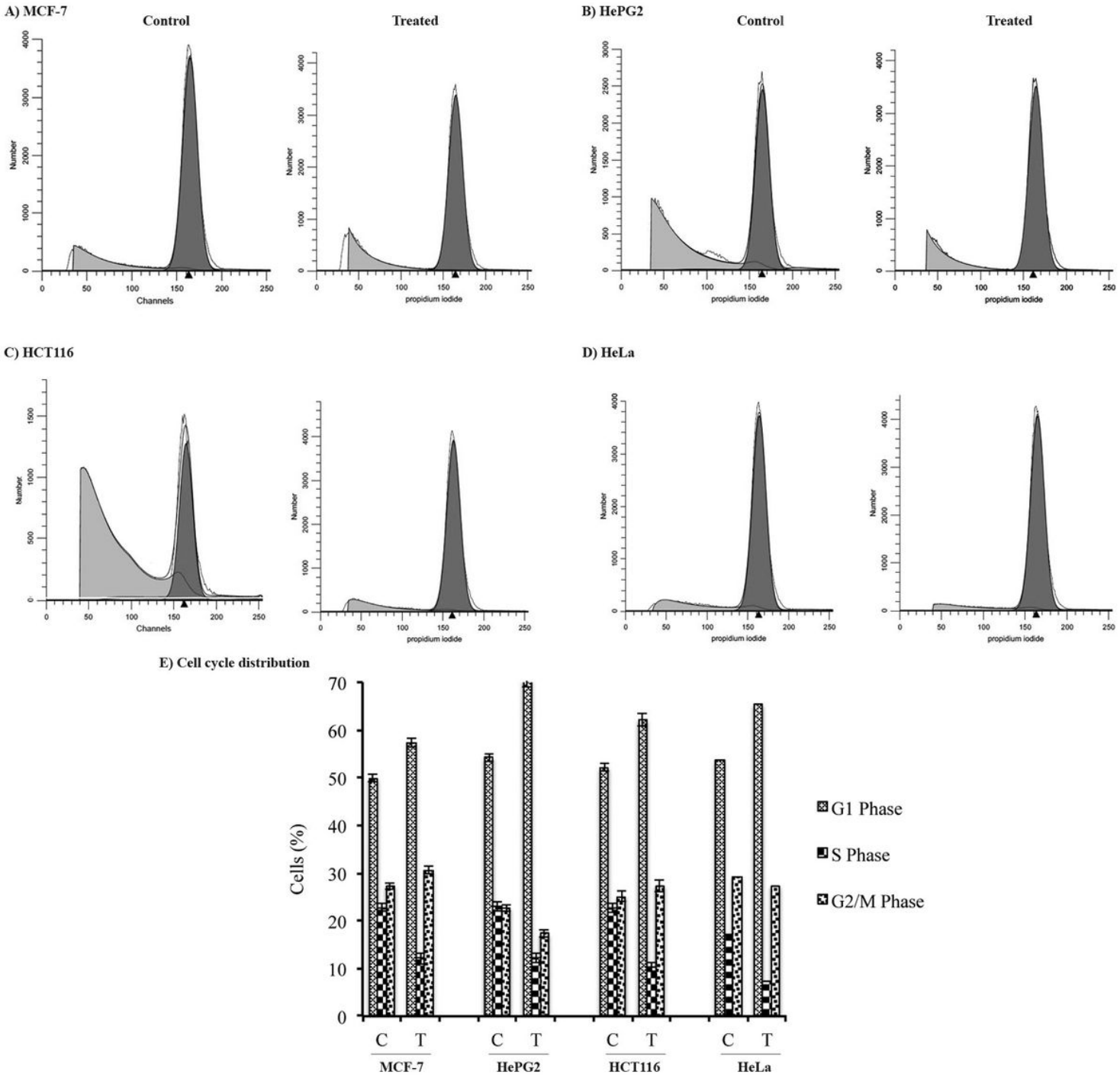

Figure 8

Cell cycle analysis of cancer cell lines after *M. communis* leaf extract treatment. (A-D): The cell cycle distribution of cells of MCF-7 (A), HepG2 (B), HCT116 (C), and HeLa (D) analyzed by DNA flow cytometry after their individual treatment with a plant extract (treated). Similarly, the cell cycle distribution of all cancer cell lines was analyzed after their treatment with DMSO only (control). (E) A bar graph showing the percentage of cells for each cell line in different phases of the cell cycle after their treatment with the plant extract (T) or DMSO (C).

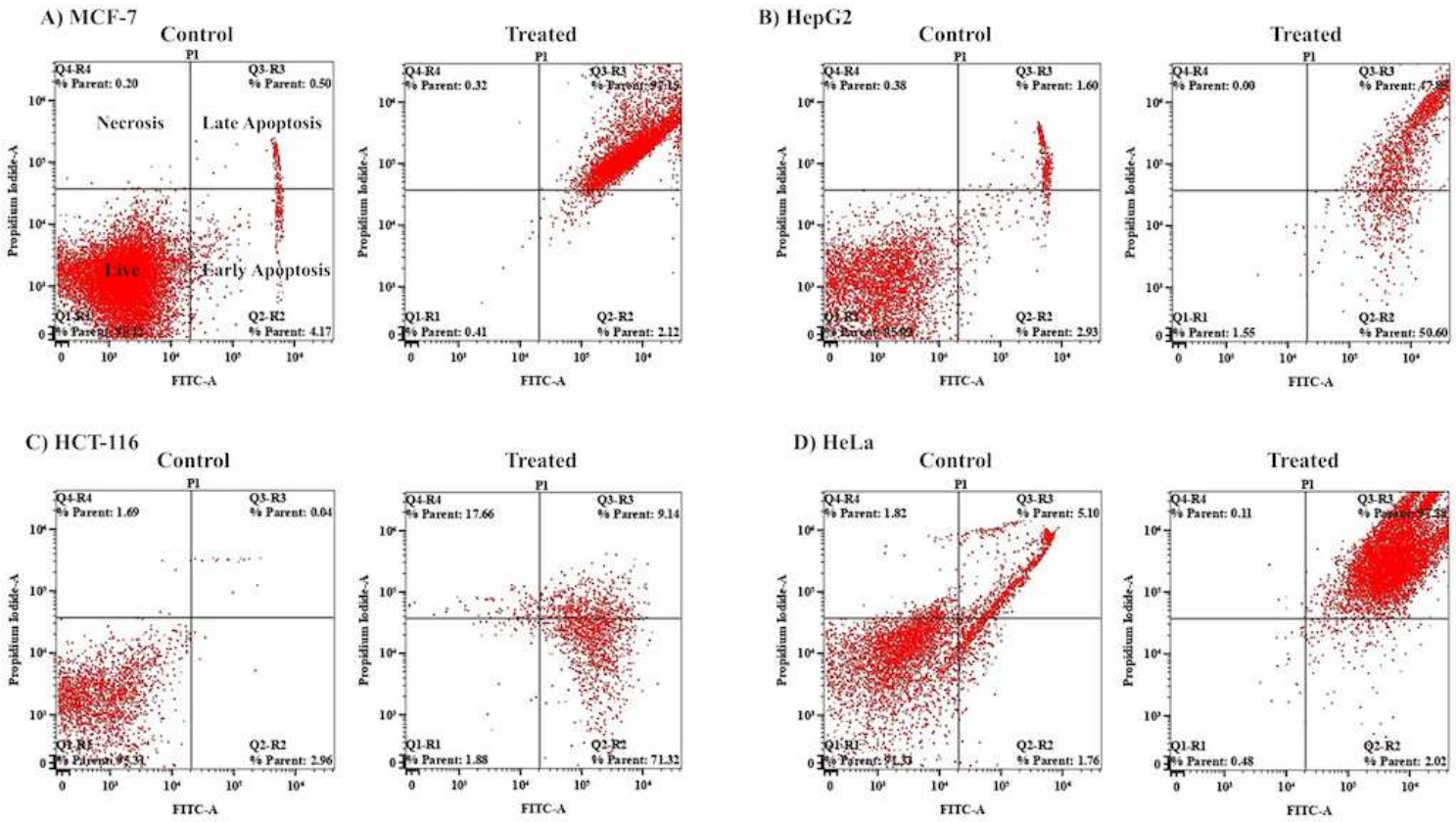

Figure 9

***M. communis* leaf extract induces apoptosis.** (A-D): Scatter plots of flow cytometry analysis showing the distribution of cells of MCF-7 (A), HepG2 (B), HCT116 (C), and HeLa (D) into early and late phases of apoptosis, and necrosis after their treatment with plant extract (treated) or DMSO (control) and subsequent staining with Annexin V-FITC/PI. The representation of the four quadrants is given in the top left corner scatter plot

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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