Myrtus Communis Leaf Extract - A Source of Secondary Metabolites Exhibiting Anticancer and Antimycobacterial Activities

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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 notable therapeutic herb *Myrtus communis,* worldwide utilized in the traditional medication for centuries, is an evergreen aromatic and medicinal plant of the Mediterranean region.

Materials and methods: 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. Annexin V-FITC/PI staining coupled with flow cytometry was used to analyze apoptosis and necrosis of the cancer cells. Western blotting detected the expression of pro- and anti-apoptotic proteins. Zone of inhibition and MIC were determined by well diffusion method and microplate alamar blue assay, respectively. Biofilm formation was studied by crystal violet method. For statistical analysis, a two-tailed Student's *t*-test of GraphPad Prism 6.0 was used.

Results: In this study, the secondary metabolites of *M. communis* leaves extracted in ethanol showed the highest cytotoxicity and thus the greatest anticancer effects against diverse cancer cell lines of the breast (MCF-7), liver (HepG2), cervix (HeLa), and colon (HCT116) (IC_{50}; ranging from 33 to 83 mg/ml). The cancer cells arrested in the G_1 phase of the cell cycle undergo apoptosis. The induction of the latter is mediated by activation of the intrinsic mitochondrial pathway.

Furthermore, the extract 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 that cause tuberculosis and several other mycobacterial infections. The biofilm formation in BSL2 microorganisms, *M. smegmatis* and *S. aureus,* is strongly inhibited by the extract.

Conclusion: These results suggest that *M. communis* leaf extract is a potential source of secondary metabolites, which could be developed further as potential anti-cancer and anti-mycobacterial agents to treat diverse types of cancers and mycobacterial infections.

1. Introduction

Cancer, also called malignancy is an abnormal growth of cells. An estimated 9.6 million deaths were caused by cancer in 2018 [1]. It is the second leading cause of death globally. Breast cancer, the most common cancer among women and prevalent in underdeveloped countries, is one of the leading causes of morbidity and mortality for women worldwide [2]. Hepatocellular carcinoma (HCC), the most common primary malignancy of the liver, is a leading cause of death in people with cirrhosis [3]. Significant progress has been made in the diagnosis and treatment of HCC using multidisciplinary approaches of surgical and non-surgical [4], including systematic chemotherapy [5]. Colorectal cancer is caused by several risk factors including genetic and environmental factors, lifestyle, and gut microbiota. Because of the undesirable side effects and efficacy, a variety of cytotoxic drugs are incompetent. There is a need for novel anticancer compounds of having no undesirable side effects. In eukaryotes apoptosis plays an

essential role in regulating tissue development and maintaining homeostasis [6–8], deregulation of which is one of the hallmarks of cancer [8].

One of the reasons of resistance of many common pathogens to commonly used therapeutic agents, such as antibiotics [9] 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[10]. As a result, biofilms serve as effective physical barriers to antibiotic and nutrient penetration [11]. 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.

There are about 200 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 to immunocompetent individuals [12]. 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 [13].

Myrtus communis or Myrtle (Arabic name: Aas or Hadas), belonging to the Myrtaceae (Saudi Arabia refers it as Mesk Ul-madena), is a notable therapeutic herb. It is an evergreen aromatic and medicinal plant inhabitant of the Mediterranean region, along with other nations such as Iraq, Jordan, the Southern and Eastern provinces of Saudi Arabia [14], and Iran in the Middle East. The various parts of this herb such as its berries, leaves, and fruits have been used extensively as a folk medicine for several centuries. The herb is used traditionally for the treatment of disorders such as inflammation, diarrhea, hemorrhoid, peptic ulcer, pulmonary, 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 [15]. Not much progress has been made on understanding the anticancer activity of *M. communis*, except few studies, wherein mostly essential oils and methanol extract of several medicinal plants have been screened for anticancer activity [16][17].

In the present study, the ethanolic leaf extract of *M. communis* was investigated for its effect on cell viability, cell cycle arrest, and apoptosis of four different cancer cell lines, *viz*, human hepatocellular carcinoma cell line (HepG2), colorectal adenocarcinoma cell line (HCT116), breast adenocarcinoma cell line (MCF-7), and human cervix adenocarcinoma cell line (HeLa). Furthermore, the mechanism underlying apoptosis was also investigated. This is the first report of the anticancer activity of the *M. communis* leaf extract against the above-mentioned cancer cell lines and the underlying mechanism of apoptosis.

Antibacterial activity of the ethanolic leaf extract of *Myrtus communis* was also investigated against diverse mycobacterial strains including laboratory and reference strains, *viz*, *M. abscessus, M. avium, M.*

fortuitum, M. kansasii, M, M. mucogenicum, M. tuberculosis, M. tuberculosis Rif-R, and *M. xenopi*. Furthermore, we investigated the effect of *M. communis* extract on biofilm formation in *S. aureus* and *M. smegmatis*, the latter being a model organism to study *M. tuberculosis*. Collectively, these results indicate that *M. communis* leaf extract is a promising herbal extract, which could be developed further as anticancer and antimycobacterial agents to treat diverse types of cancers, tuberculosis, and other mycobacterial diseases, including *S. aureus* infections. However, further evaluation, active compound isolation, *in-vitro* and *in-vivo* investigations of *M. communis* leaf extract constituents are recommended.

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 [18]. 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 Soxhlet extraction method. 50 grams of the leaf powder were incubated with 200 ml of absolute ethanol for 2 hours in 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 till complete evaporation of ethanol. The dried extract of *M. communis* was re-dissolved in ethanol at desired concentrations and used in assays.

2.2. 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^{o}C (16). Caspase 3, Bax, Bcl2, and β-actin antibodies were purchased from Santacruz biotechnology, Santacruz, CA, USA.

2.3. Cytotoxicity assessment

The cytotoxicity of the extract was tested against human tumor cells using Sulphorhodamine B assay (SRB). Healthy growing cells were cultured in a 96-well tissue culture plate (3,000 cells/well) for 24 hours before treating with the extract to allow attachment of the cells to the plate. Cells were exposed to the five different concentrations of extract (0.01, 0.1, 1, 10, 100, 1,000 µg/ml), dissolved in DMSO. Untreated cells were included as a control. Triplicate wells were included for each concentration of extract. The plate was incubated for 72 hrs at 30^{o}C and subsequently fixed with TCA (10% w/v) for one hour at 4°C. After several washings, cells were stained by 0.4% (w/v) SRB solution for 10 min in dark. 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 which reduces survival to 50%) using SigmaPlot 12.0 software [19].

2.4. Cell cycle distribution using DNA flow cytometry

DNA flow cytometry for cell cycle distribution was performed as described by Shati Ali A *et.al* [20]. 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. 10 ml of 70% ice-cold ethanol was added gently while vortexing. Cells were kept at 4^{o}C for one hour and stored at -20^{o}C 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 37^{o}C 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 data represents the mean ± SD of three replicates.

2.5. Apoptosis assessment using Annexin V-FITC/PI staining coupled with flow cytometry.

To assess the effect of extracts on programmed cell death, apoptotic and necrotic cell populations were determined using Annexin V-FITC apoptosis detection kit as described by Shati Ali A *et. al*. [20] (Abcam Inc., Cambridge Science Park, and Cambridge, UK). Briefly, cells were treated with different concentrations for 48 hr. Cells after treatment were harvested by trypsinization and washed twice with ice-cold PBS. As per the manufacturer protocol, the cells were resuspended for 30 min in 0.5 mL of annexin V-FITC/PI solution in dark. After staining, cells were injected into ACEA Novocyte™ flow cytometer (ACEA Biosciences Inc., San Diego, CA, USA). FL1 and FL2 signal detector were used to analyze FITC and PI fluorescent signals (λex/em 488/530 nm for FITC and λex/em 535/617 nm for PI), respectively. For each sample, 12,000 events were acquired. Quadrant analysis was performed to quantify positive FITC and/or PI cells using ACEA NovoExpress™ software (ACEA Biosciences Inc., San Diego, CA, USA).

2.6. Western immunoblotting:

Western blotting for the detection and estimation of various cellular proteins was performed as described [20]. Cancer cells were treated with IC_{50} concentration of ethanolic leaf extract for 48 hrs and lysed with cell lysis buffer. The total protein concentration was estimated by Coomassie plus protein assay kit (Pierce; Rockford, IL, USA), and 20–40 µg protein from the cell lysate was fractionated with SDS-PAGE followed by transfer to a nitrocellulose membrane. After probing with the respective primary antibodies, HRP–secondary antibodies were added, and the blots were developed using enhanced chemiluminescence (ECL) detection kit. The membranes were stripped off the antibodies and incubated with β-actin antibody at 1:5000 dilutions and redeveloped to detect the β-actin band. Bands specific to Bcl-2, Bax, and Caspase-3 were quantified using Image J (Ver. 1.46, NIH) and then normalized to the intensity of β-actin band. All the experiments were performed in triplicates. For statistical analysis two-tailed Student's *t*-test of GraphPad Prism 6.0 (Jo La USA) was used and the *P*-value o*f* ≤ 0.05 was

considered statistically significant. Caspase 3, Bax, Bcl-2, and β-actin antibodies were purchased from Santacruz Biotechnology, Santacruz, CA, USA.

2.7. Bacterial strains, media, well diffusion method, and microplate alamar blue assay.

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 microorganisms included both slow growing mycobacteria (SGM) and rapidly growing mycobacteria (RGM). These mycobacterial strains are grown and maintained in the TB laboratory (BSL3 facility), at Southern Region Military Hospital, Khamis Mushait, Saudi Arabia. *Staphylococcus aureus* was grown in the Trypticase Soy broth (TSB). For the zone of inhibition and MBC assays, Trypticase Soy Agar (TSA) plates were used

MIC, MBC, and Zone of inhibition were determined as described by Mir *et al*, [18] with slight modification for the zone of inhibition. 0.5 McFarland mycobacterial cultures after diluting 100 times with OADC supplemented Middlebrook7H9 broth were used for streaking on TSA plates. Depending on the growth status of a bacterial strain, the plates were incubated aerobically for 1 - 4 weeks at 37°C. 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) + diameter of a well (6mm).

2.8. 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 [21]. In brief, the above-prepared 0.5 McFarland bacterial suspensions were 100 times diluted in TSB and 190 µl of the diluted cultures were dispensed in the wells of the 96-well plate. 10 µl each of *M. communis* extract to the final concentration of ½ and ¼ of its MIC for each bacterial strain were dispensed in triplicate wells. For the positive control 10 µl of absolute ethanol, a carrier solvent, were similarly dispensed into the triplicate culture wells for each bacterial strain. For blank as a control for the binding of crystal violet to walls of wells, 200 µl of sterile TSB were dispensed in triplicate wells. The plates were incubated either 24 hours (for *S. aureus*) or 48 hours (for *M. smegmatis*) at 37°C under static conditions. The supernatant of each well including blank wells was saved in a sterile 96 well plate, which was later used for growth kinetics experiment. The biofilms were developed as described by Christensen et al. and measured at the absorbance of 570 nm, using FLUOstar® Omega microplate reader featuring BMG LABTECH's proprietary Tandem Technology. The average of the blank corrected values was plotted using excel. The standard deviations (±SD) calculated using Microsoft Excel were plotted on a graph as shown by error bars.

2.9. Growth kinetic assay

In this assay 5 µl from the supernatant of triplicate wells of the blank, 1/2 MIC, 1/4 MIC of extract and control, saved during biofilm assay, were inoculated in triplicate wells containing 195 µl TSB each in of a 96-well plate. 20 µl of alamar blue was also dispensed into each well, and the plate was incubated at 37^{o}C in FLUOstar® Omega microplate reader. The fluorescence readings at excitation and emission maxima of 544 nm and 590 nm, respectively, were taken every 30 min. The readings were taken for a total duration of 4½ and 23 hours for *S. aureus* and *M. smegmatis*, respectively. Every time the plate was shaken for 5 sec at 200 rpm before taking a reading. The average of the blank corrected fluorescence units was plotted against the time using Microsoft Excel software. The standard deviations (±SD) calculated using Microsoft Excel at each time point were plotted as shown by error bars on the graph.

3. Results

3.1. *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 by the treatment with different concentrations of extract for 72 hrs was measured by percentage viability of the cancer cells. The results showed that the viability of cells decreased in a dose-dependent manner (Fig. 1), killing 75% of all cells at the concentration of 100 µg/ml. A gradual increase in the concentration of 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 1) while for other cells it was moderate (IC_{50}; 41.5 - 83.0 µg/ml).

Table 1
Cytotoxic activities (IC_{50}) of *M. communis* leaf extract (µg/ml) against different tumor cell lines. Results were expressed as mean ± SD for three different independent replicates.

Tumor cell line	IC_{50} (µg/ml)	Tumor cell line	IC_{50} (µg/ml)
MCF-7	41.5 ± 0.6	HCT116	83 ± 2.5
HepG2	53.3 ± 0.6	HeLa	33.3 ± 3.6

3.2. M. communis leaf extract caused the cell cycle arrest and induction of apoptosis in cancer cells

One of the major causes of inhibition of cellular growth is the cell cycle arrest. In order to determine whether the growth inhibition of cancer cell lines was due to the 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 2 and figure 2 shows that the significant proportion (≥ 10% more of the control) of all the tumor cell lines was arrested in G_1 phase of the cell cycle with a corresponding drop in

the percentage of cells in 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 of the control) was observed for all cancer cell types except HCT116. These results demonstrate that the extract arrested all the types of cancer cells in G_1 phase of the cell cycle, though the percentage of cells showed a variation.

Table 2
Cell cycle distribution of cancer cells after treatment with *M. communis* leaf extract. Results were expressed as mean ± SD for three independent replicates.

Cancer Cell	Extracts	Cell Phases		
		G_1	S	G_2 / M
MCF-7	Control	49.92 ± 0.74	22.75 ± 0.8	27.33 ± 0.7
	Treated	57.33 ± 0.9	12.09 ± 1.2	30.58 ± 0.81
HepG2	Control	54.23 ± 0.93	23.06 ± 2.01	22.71 ± 1.7
	Treated	70.52 ± 0.79	12.15 ± 1.02	17.33 ± 0.75
HCT116	Control	52.13 ± 1.52	22.75 ± 0.98	25.12 ± 0.75
	Treated	62.22 ± 0.78	10.38 ± 0.58	27.4 ± 2.02
HeLa	Control	53.69 ± 0.87	17.09 ± 0.98	29.22 ± 1.02
	Treated	65.35 ± 1.35	7.25 ± 0.94	27.4 ± 1.31

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. 3) indicated that the major cellular populations of all tumor cell lines tested against leaf extract of *M. communis* were in the early apoptotic stage (57 - 72%). However, a marked percentage (18 – 22%) of all types of cancer cells were in the late stage of apoptosis, while less than 1% of cells have undergone necrosis. Furthermore, cells treated with the extract were 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 (Figure 4). Altogether, these results suggest that the major cellular population of all the four tumor cell lines tested were in the apoptotic stage upon treatment with the leaf extract.

3.3. Apoptosis of cancer cells occurred through the executioner caspase-3

The interruption of apoptosis molecular signaling pathways leads to carcinogenesis. In the programmed cell death pathway (apoptosis) the activation of the executioner caspases-3 and -7 are regulated both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways. The BCL2 family of proteins, including both anti-apoptotic (Bcl-2) and pro-apoptotic (Bax) regulators, is the hallmark of apoptosis regulation.

We, therefore, addressed the potential mechanism by which *M. communis* extract was causing decreased cell viability in all the cancer cell lines tested. For this purpose, all the four cancer cell lines were treated with leaf extract, and the expression level of Bcl-2, Bax, and Caspase-3 was determined in the cell lysates by western blot analysis. The 48 hours treatment with *M. communis* leaf extract decreased the levels of Bcl-2 in all the cancer cells tested (Fig. 5), thus inducing apoptosis. In support of this observation, a higher expression of Bax (pro-apoptotic) protein was observed in the same cells. It has been known that the caspase family activation represents one of the earliest known steps in the programmed cell-death process. Thus, we next explored the expression of caspase-3 in all four types of cancer cells upon their treatment with *M. communis* leaf extract. As shown in figure 5, cancer cells exposed to the leaf extract exhibited the robust activation of caspase-3. These results altogether suggest that the *M. communis* leaf extract induces apoptosis by caspase-3 dependent intrinsic pathway. However, further investigation is needed to find out the role of the extrinsic pathway in the apoptosis of cancer cells, which is not the scope of this study.

3.4. *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 a well-diffusion method. The mycobacterial strains sensitive to the extract produced a clear zone of having no bacterial growth around the well (Fig. 6). The zone of inhibition ranged from 20.3±1.1 mm to 26.3±2.5 mm (Table. 3), 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 alamar blue assay. Alamar blue, a grow indicator, changes the 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 as MIC. As shown in Fig. 6, 4.8/ml is the MIC for *M. kansasii* ATCC35775. MIC of the extract against each bacterial strain was determined and presented in the Table 3. 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.

Table 3
Antimycobacterial activity of *M. communis* leaf extract against pathogenic mycobacterial strains.

Origin	Organism	Zone of inhibition Mean ± SD (mm)	MIC (µg/ml)	MBC (µg/ml)
SGM	*M. avium ATCC25291*	25.1 ± 4.1	19.53	156.2
	M. kansasii	20.3 ± 1.1	9.768	78.14
	M. kansasii ATCC35775	25.6 ± 3.2	4.884	39.07
	M. xenopi	20.5 ± 2.6	19.53	156.2
RGM	*M. abscessus*	20.6 ± 3.2	39.07	312.5
	M. fortuitum ATCC6841	24.3 ± 3.7	156.2	937.2
	M. mucogenicum	22.6 ± 3.5	9.768	156.2
MTB	*M. tuberculosis ATCC25177*	26.3 ± 2.5	78.14	625
	M. tuberculosis RIF-R	22.6 ± 2.5	312.5	1250

From the alamar blue 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 as MBC for the said strain. The MBC ranged from 39.07 µg/ml to 1250 µg/ml for all mycobacterial strains tested (Table 3). It was found that there was a consistent correlation of MIC with MBC for each mycobacterial strain tested. The strain having lower MIC did show 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.

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

Mycobacterial strains are believed to form a biofilm [22], which is the main barrier for antimicrobials to kill the resident microorganism. We attempted to investigate that whether or not *M. communis* leaf extract could affect the biofilm formation of mycobacterial strains. To this end, we chose to use *M. smegmatis* as an ideal model microorganism. *M. smegmatis* is a non-pathogenic and fast-growing microorganism, which shares high similarities with *M. tuberculosis*. The results obtained will be later ascertained on 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.

Both *M. smegmatis* and *S. aureus* strains were individually incubated at 37^{o}C with ½ and ¼ MIC of *M. communis* extract for 24 and 48 hours, respectively. For control, both bacterial strains were individually incubated with carrier solvent ethanol under similar conditions. For blank as a control for nonspecific binding of crystal violet to the walls of wells, growth medium dispensed in triplicate wells of 96-well plate

were also included. After 48 hours of incubation, the biofilm formed by *M. smegmatis* was visible in the positive control, which was significantly affected in wells treated with *M. communis* extract (Fig. 7A). After further development of biofilms by crystal violet method, it is clear from the color intensity of the wells (Fig. 7B) that compared to the control, the biofilm formation of *M. smegmatis* was drastically reduced in the wells treated with ½ MIC of extract than the wells wherein the cells were treated with ¼ MIC of *M. communis* extract. Unlike *M. smegmatis*, which forms the biofilm at the liquid-air interface [23], *S. aureus* forms the biofilm at the solid surface, which was difficult to be photographed.

Quantitative estimation of biofilms formed by *M. smegmatis* at ½MIC and ¼MIC of *M. communis* extract showed 4.3 and 2.2-fold decrease, respectively, that of the biofilm formed in absence of extract (control) (Fig. 8A). The biofilm of *S. aur*eus developed after 24 hours and quantitated by taking absorbance readings at 570 nm showed that the biofilms formed at ½MIC and ¼MIC of *M. communis* extract were drastically reduced by 37.3 and 2.1-fold to that of the control (Fig. 8B). These results suggest that the biofilm formation by *M. smegmatis* as well as *S. aureus* was inhibited by the ethanolic leaf extract of *M. communis.*

One of the possible reasons for the reduction of biofilm formation could be the inhibition of growth of cells, which is the least affecting factor below the concentrations of MIC. To rule out this notion, we performed the growth kinetic assay of the cells of the planktonic phase of the cultures. In brief, before the development of the biofilms, the supernatant of the cultures of *M. communis* treated wells, blank wells, and control wells was saved and used for growth kinetic assay. The supernatant cultures were 40-fold diluted in TSB containing 1x alamar blue in a 96-well plate and allowed to grow at 37^{o}C in FLUOstar® Omega microplate reader. The growth was monitored in real-time by taking fluorescence readings after every 30 min time intervals.

It is clear from the growth kinetic assays (Fig. 8C and 8D) that there is an increase in the growth of cells with the increase in the concentration of extract (compare the growth curve of ¼ MIC with ½ MIC), which correlates well with the fold decrease in the biofilm formation (compare the bar graph of ¼ MIC with ½ MIC). The fold difference in the biofilm formation for each bacterium at ½ and ¼ MIC of extract correlates well with their corresponding difference in the growth of planktonic cells at ½ and ¼ MIC of extract. These results suggest that in both microorganisms of *M. smegmatis* and *S. aureus*, an increase in the concentration of extract resulted in a decrease in the biofilm formation and a corresponding increase in planktonic cellular growth and vice versa. Hence, the kinetic assays ruled out the notion that the inhibition of the biofilm formation is due to the inhibition of the growth of cells.

4. Discussion

Although substantial improvement has 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 additional burden on human health and economy. Unsuccessful treatment of the diseases caused by *Mycobacterium tuberculosis* and NTM by available commercial antibiotics is a growing concern. Thick

cell wall and dormant ability of these bacterial species demands the development of new therapeutic agents.

Natural products extracted in their crude form or as purified compounds used for the treatment of various ailments are obtained from medicinal plants. Because of the chemical diversity of natural products, the demand to identify and isolate the actual effective compound has grown worldwide [24]. Due to the presence of different therapeutic or preventive compounds in the herbal medicine, its treatment provides some advantages over a single purified compound [25]. As a result herbal medicine could be more effective in treating diseases than single products on their own.

In this study, the cytotoxic property of *M. communis* leaf extract was investigated against diverse cancer cells. The crude extract was prepared from dried plant leaves using ethanol as a solvent. The extract prepared showed strong cytotoxic activity against all the 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 cell decreased in or G_2/M phase of the cell cycle compared to control. However a variation was observed in the percentage of cells arrested either in G_1 or G2/M with respect to a particular cell line (Table 2, Figure 2). It has been shown in previous studies that a sequence of events occur 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 [26]. Several distinct pathways lead to apoptosis, which usually occurs as a later event in cell death. As shown in figure 3, 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 [27].

To explore the molecular mechanism responsible for *M. communis* extract-induced apoptosis in cancer cells, *viz*, breast, liver, cervix, and colon, the expression of Bax and Bcl-2 proteins in all the four types of cancer cells was examined. The members of BCL-2 protein family regulate apoptosis by modulating the mitochondrial pathway. The regulatory proteins include anti-apoptotic and pro-apoptotic proteins such as Bcl-2 and Bax, respectively [28]. It is well-known fact that the increased expression of Bax increases the mitochondrial membrane permeability, which causes the release of cytochrome C and other pro-apoptotic molecules, thus activating downstream caspases and ultimately the caspase-3 [29]. Western blots analysis of the Bcl-2 family proteins demonstrated that the expression of Bax increased and of Bcl-2 decreased by 48 hours of leaf extract treatment of all the four cell lines tested (Fig. 5). The increased expression of caspase-3 in the extract-treated cells (Fig. 5) clearly demonstrated that the mitochondrial-mediated caspase activation pathway was involved in the induction of apoptosis in human cancer cell lines of breast, liver, cervix, and colon. This is consistent with the compound Myrtucommulone-A isolated from *M. communis, which* exhibited anti-proliferation activity against certain cancer cell lines through the activation of intrinsic and extrinsic pathways [30].

The other major significance of this study is that the extract showed strong growth inhibition of several NTM strains, including slow and rapid growing (Table 3). Nevertheless, the extract comparatively showed

lesser effect against the *M. tuberculosis* strains. There is one single report wherein the effect of essential oil of *M. communis* on the growth of several strains of *M. tuberculosis* has been studied [31]. The present study is the 2nd report wherein not only *M. tuberculosis* but several NTM strains have been investigated for sensitivity towards the *M. communis* extract, which could potentially extend its application 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* extract. *M. communis* extract resulted in the highest zone of inhibition and lowest MIC against *M. kansasii* ATCC35775 (Table 3) among all the bacterial strains tested.

Multidrug-resistant *M. abscessus* and *M. fortuitum* [32] were significantly inhibited by *M. communis* leaf extract. Furthermore, *M. xenopi*, showed marked susceptibility to *M. communis* extract. Surprisingly, *M. tuberculosis* strains, especially the *Mycobacterium tuberculosis* rifampin-resistance strain showed less sensitivity to the extract (Table 3). It would be interesting to investigate whether or not the sensitivity of the extract against *M. tuberculosis* strains could be enhanced in combination with the commercially available antibiotics, which is not the scope of this study.

Biofilms are one of the impediments to the access of antimicrobials to treat bacterial infections. Biofilms of *S. aureus* are often associated with chronic infections and infected embedded medical devices. The development of phytochemicals to inhibit the formation of biofilm and/or other virulence factors in *Staphylococcus aureus* (*S. aureus*) has been studied to a certain extent [33]. There are a few reports of plant extracts and essential oils being studied for the inhibition of biofilm formation in mycobacteria, including NTM and *M. smegmatis* [34]. We attempted to investigate the effect of the *M. communis* leaf extract on the formation of biofilms by *M. smegmatis* and *S. aureus* (Fig. 7 and 8). Because of *S. aureus* being robust in biofilm formation and frequent pathogen confronted in clinical and laboratory contexts [35], it was included in the biofilm assay. *M. communis* extract showed a strong inhibitory effect on the biofilm formation in both *M. smegmatis* and *S. aureus*. In our previous study, 50 compounds were identified in *M. communis* leaf extract by GC/MS analysis [18]. It would be interesting in the future to isolate and purify the actual compound(s) exhibiting the anticancer and antimycobacterial activities.

Conclusion

The main findings of this study are i) this is the first report of *M. communis* extract showing anticancer activity against diverse types of cancer cell lines. The anti-proliferative activity of the extract against the human cancer cells was due to the accumulation of cells in the G_1 / G_2/M phase of the cell cycle, which eventually led to the induction of apoptosis by the upregulation of pro-apoptotic and downregulation of anti-apoptotic proteins. ii) Furthermore, this is the first report wherein *M. communis* leaf extract not only exhibited strong antibacterial activity against tuberculosis and nontuberculosis strains of slow-growing and rapidly growing mycobacteria but also inhibited the biofilm formation of *M. smegmatis* and *S. aureus*. In conclusion, these findings suggest that *M. communis* leaf extract contains secondary

metabolites that could act not only as potential therapeutic agents against mycobacterial infections but also could be developed to treat breast, liver, cervix, and colon cancers. Further evaluations, active compound isolations, *in-vitro* and *in-vivo* evaluations are recommended for future research on these active ingredients.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials: Not applicable

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

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Figures

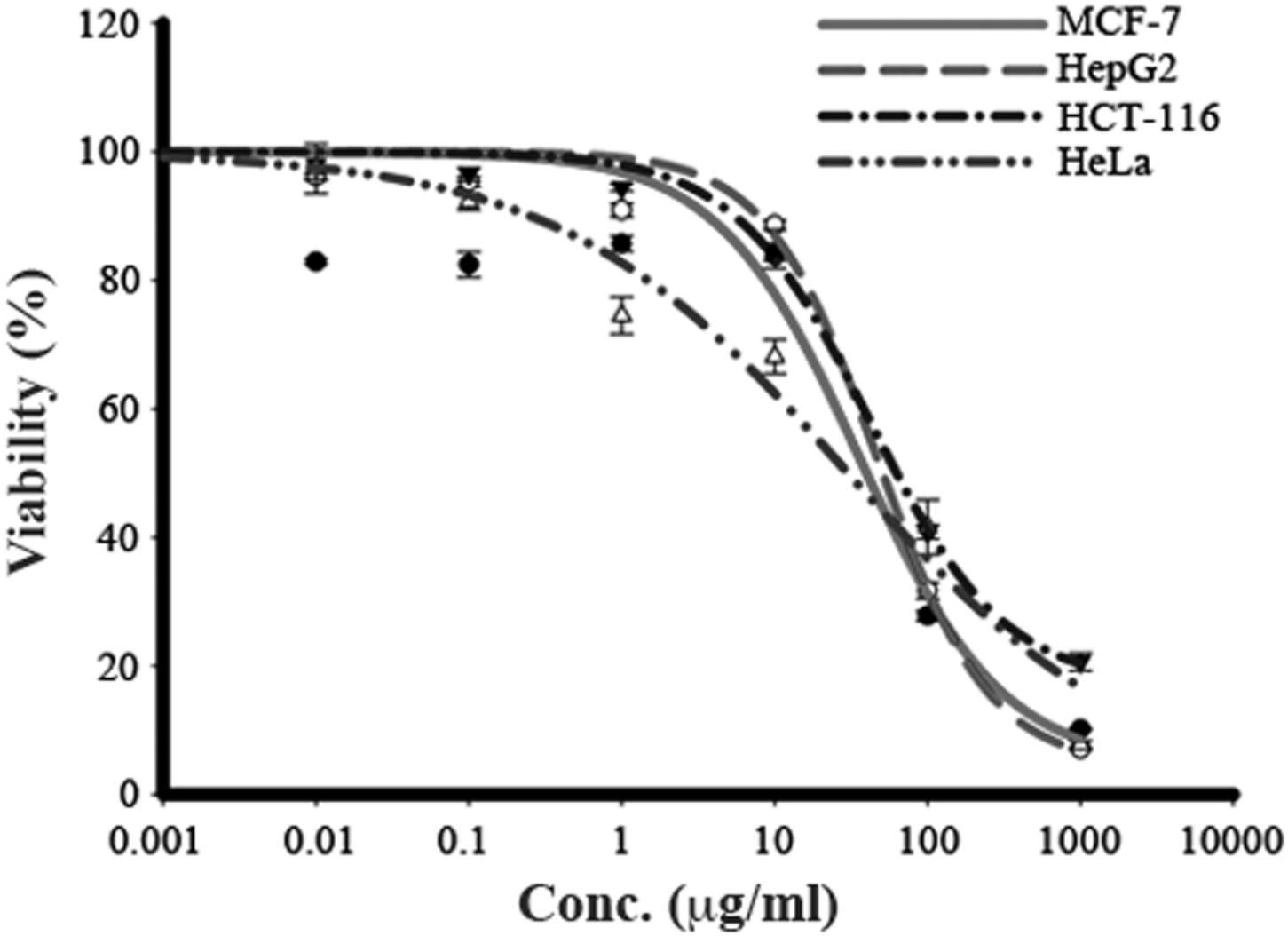

Figure 1

IC50 values of the M. communis leaf extract against cancer cell lines. The cancer cell lines, viz, MCF-7, HepG2, HCT116, and HeLa were individually treated with various concentrations (g/ml) of ethanolic leaf extract. After 72 hours of treatment, the percentage of viable cells of each cell line was plotted against the concentrations of the plant extract used.

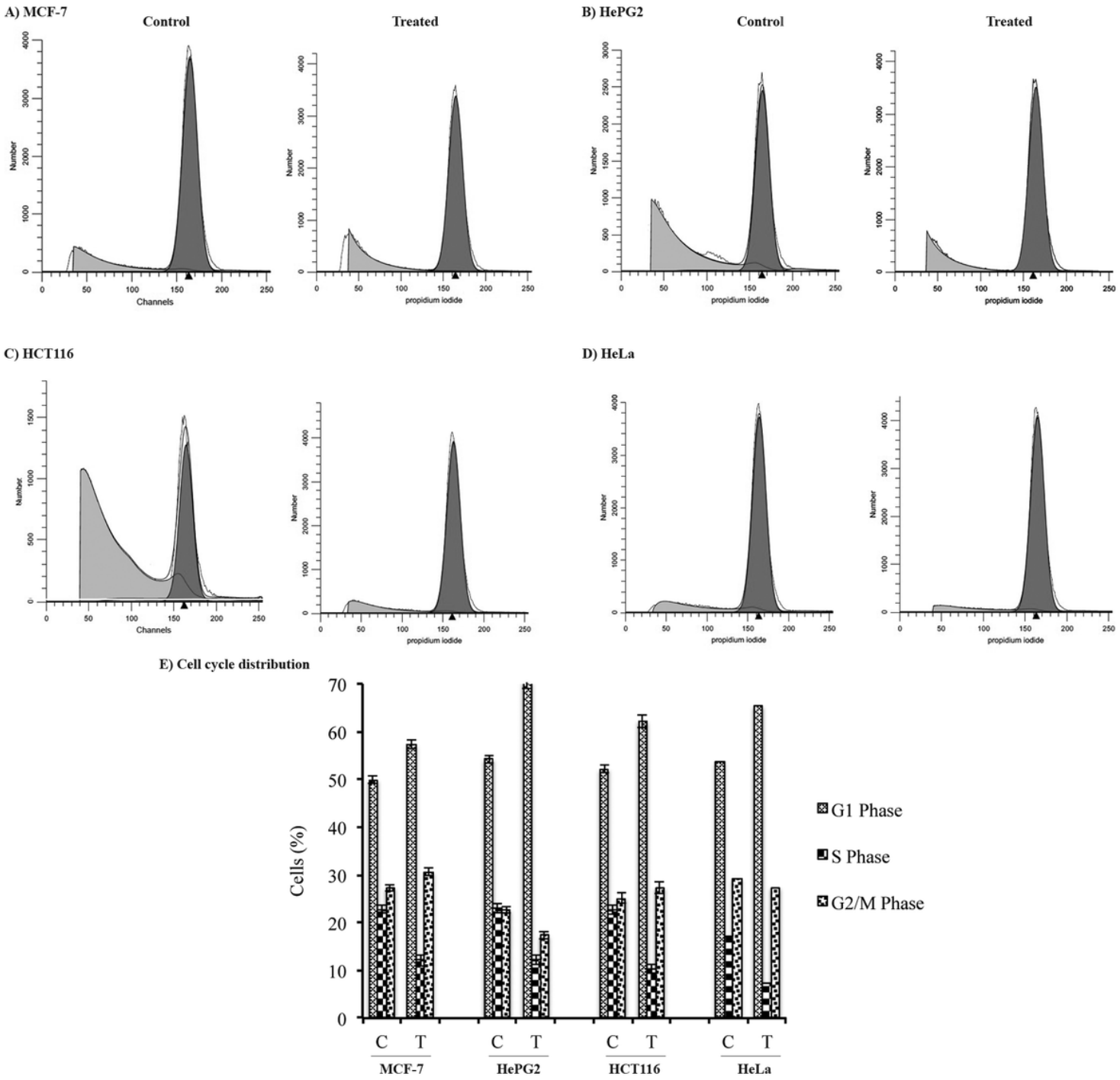

Figure 2

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 cells for 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).

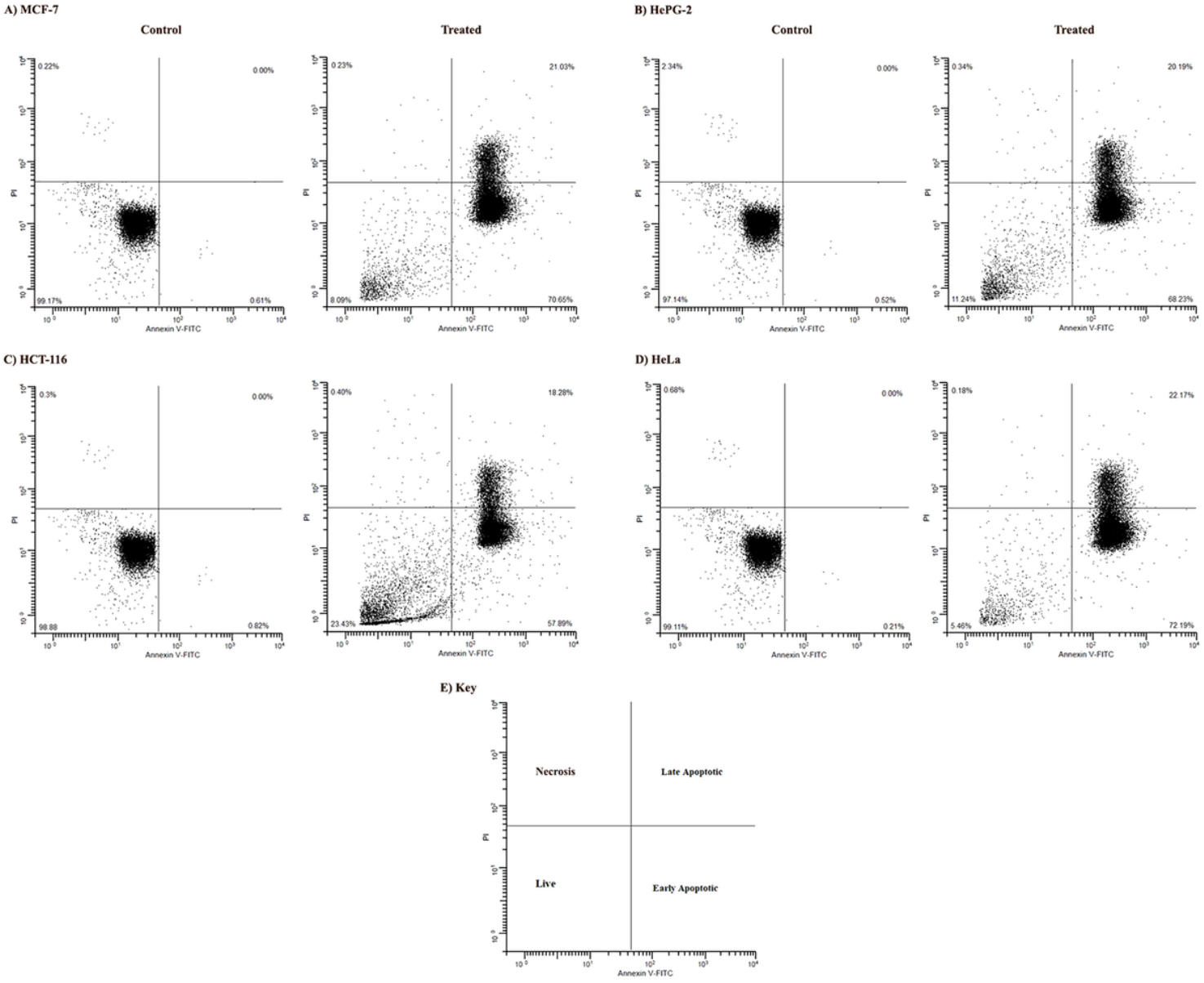

Figure 3

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. (E): Representation of the four quadrants of scatter plot of flow cytometry analysis.

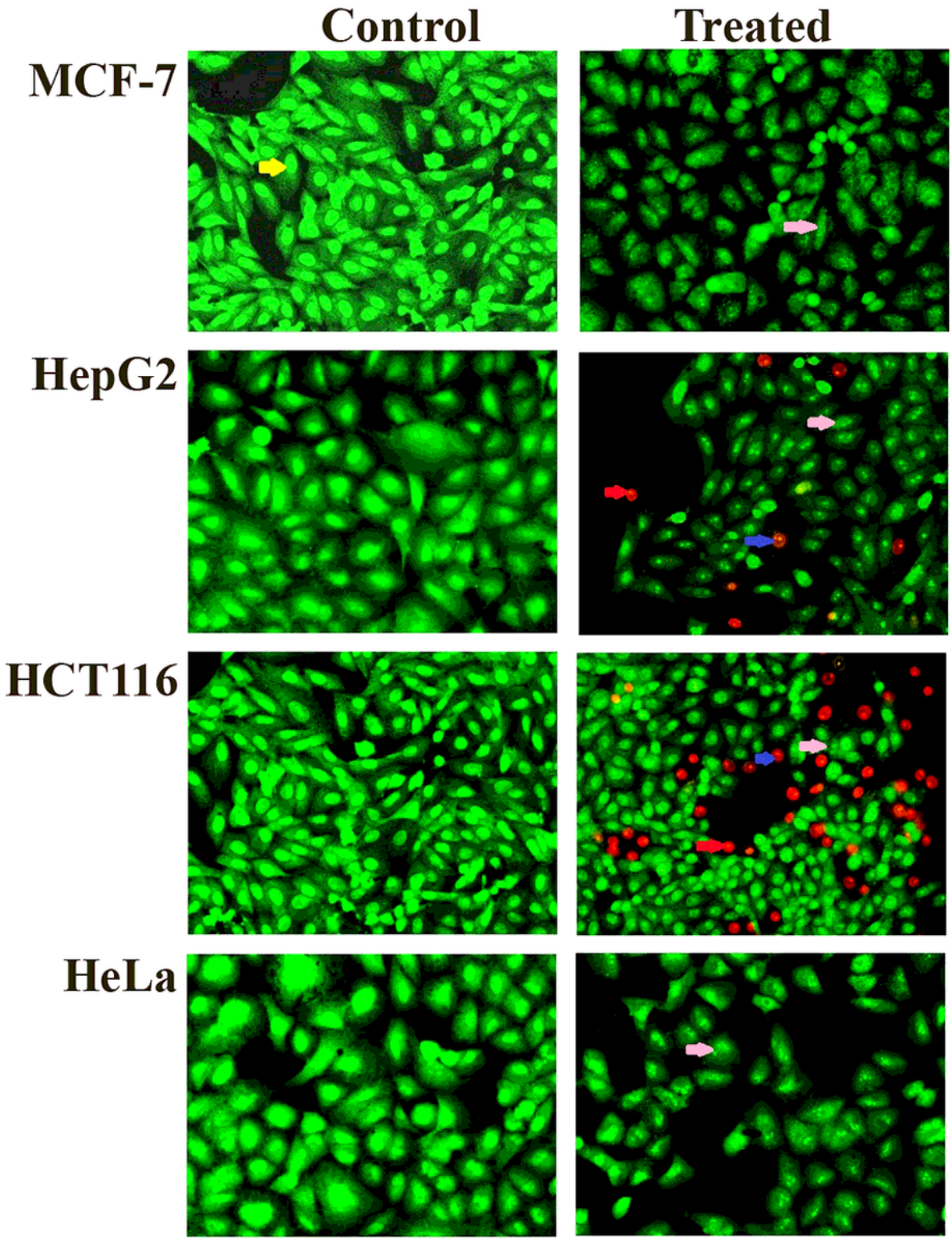

Figure 4

M. communis leaf extract induce apoptosis. The representative fluorescent images of control and M. communis leaf extract treated cancer cell lines of MCF-7, HepG2, HCT116, and HeLa. The cells were treated for 48 hours and stained with AO/EB. Yellow arrow; live cell, pink arrow; early apoptotic, red arrow; necrotic, and blue arrow; late apoptotic.

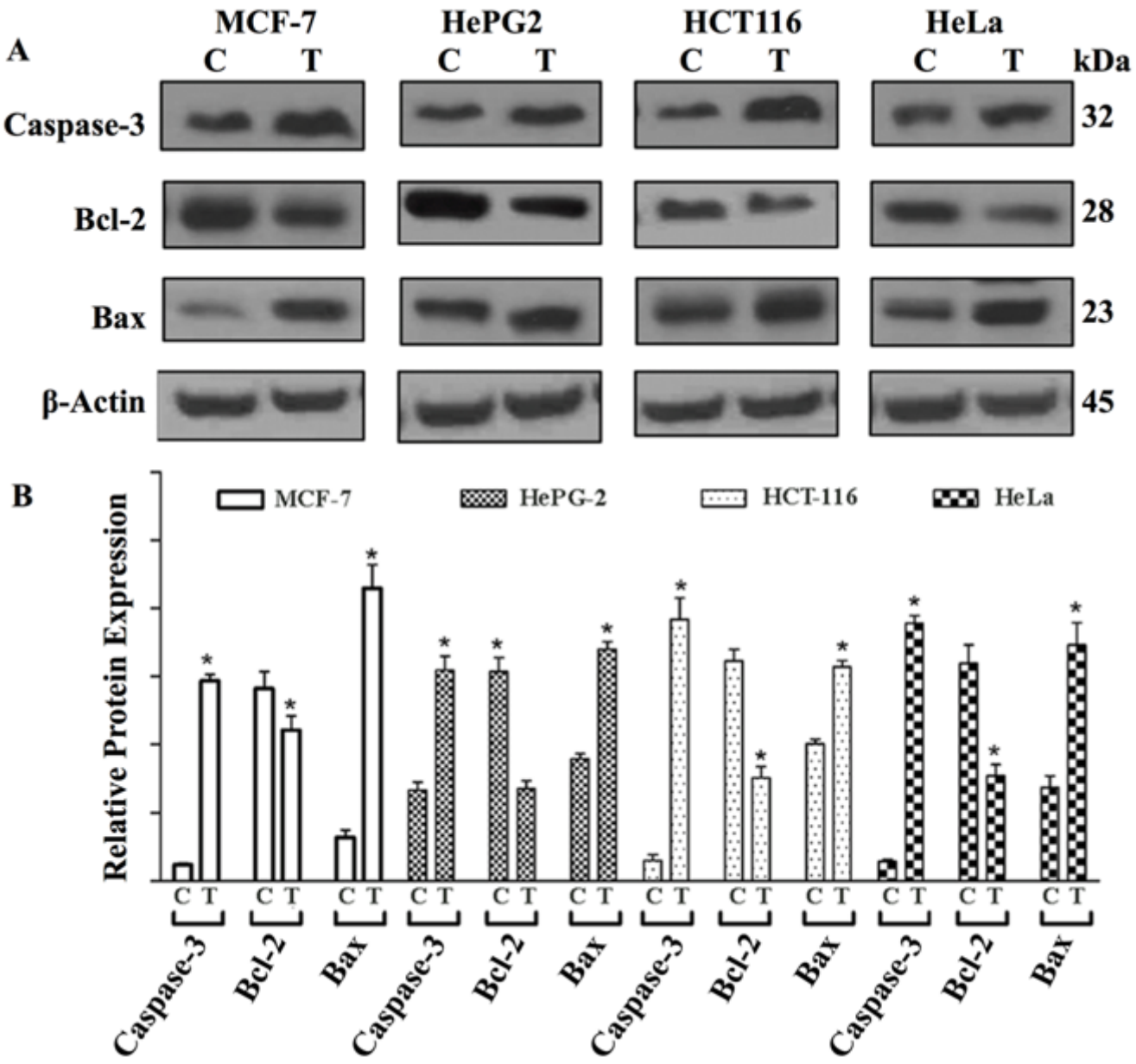

Figure 5

Expression profile of Bcl-2, Bax, and caspase-3. (A) Western blot analysis of Bcl-2, Bax, and Caspase-3 proteins in the cell lysates of MCF-7, HepG2, HCT116, and HeLa cell lines, obtained after 48 hours of treatment with M. communis leaf extract (T) or DMSO (C). The bands specific to a particular protein were cropped, as the lanes were not adjacent. All blots were simultaneously processed and exposed to an X-ray film. (B) Relative protein expression level of Bcl-2, Bax, and caspase-3 in cell lysates of leaf extract (T) or DMSO (C) treated MCF-7, HepG2, HCT116, and HeLa cells. *: $P < 0.05$.

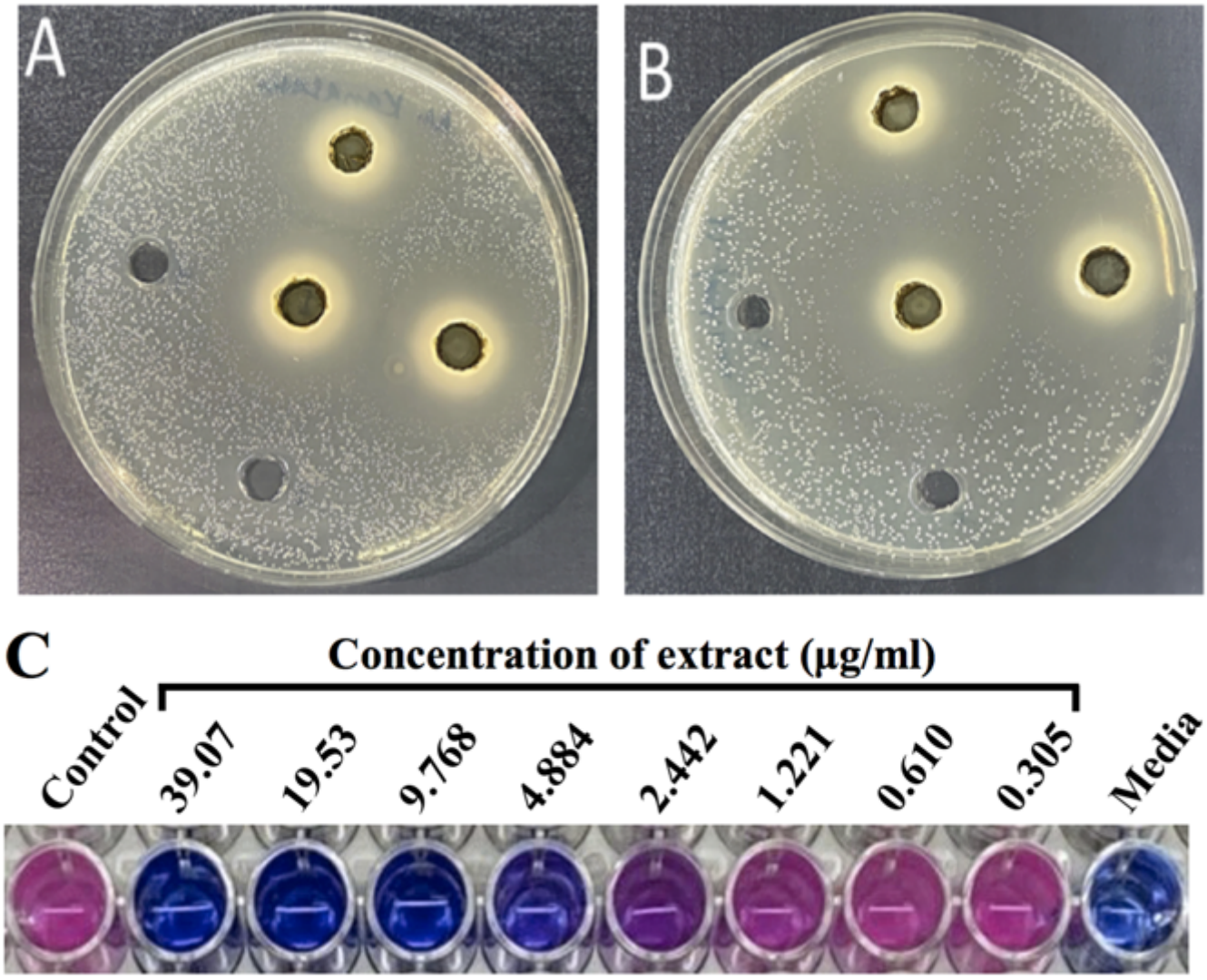

Figure 6

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 the 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.

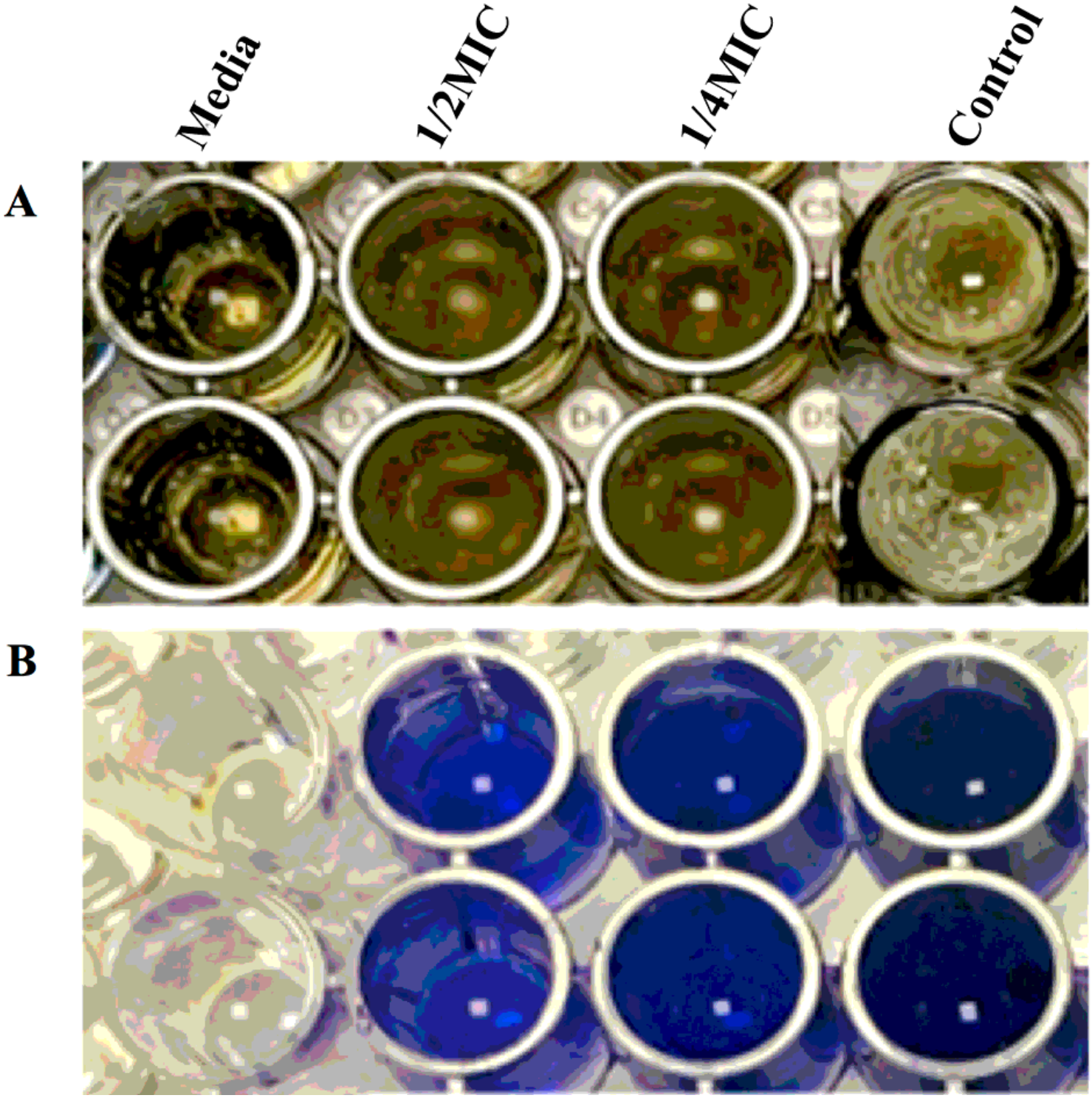

Figure 7

Biofilm formation by M. smegmatis in the presence and absence of M. communis ethanolic. A) Photograph showing biofilm formed at the surface of culture wells. B) Photograph of the wells of subpanel A after development by crystal violet.

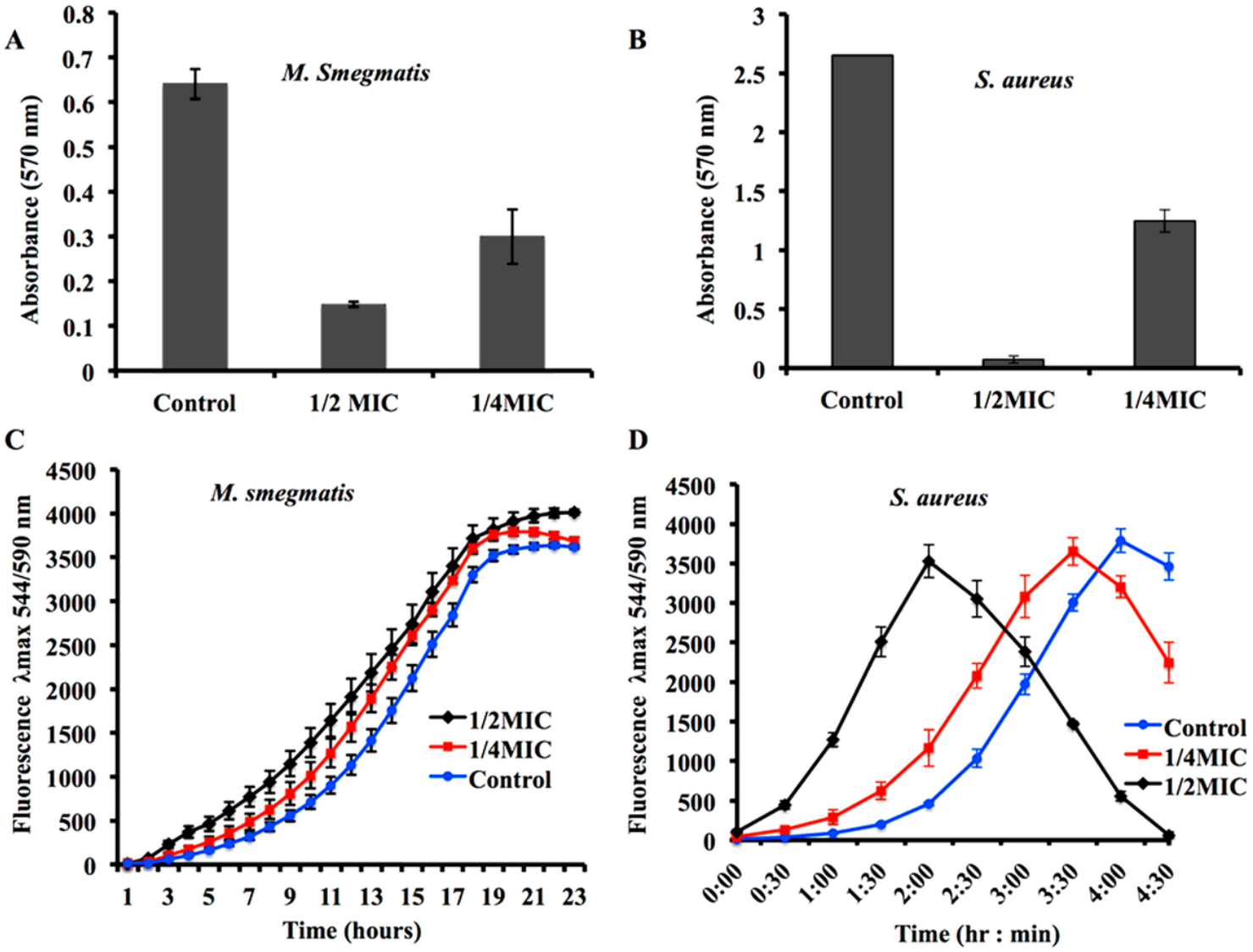

Figure 8

M. communis leaf extract inhibits biofilm formation. A) Inhibition of biofilm formation in M. smegmatis by M. communis leaf extract at its ½MIC and ¼MIC. B) Inhibition of biofilm formation in S. aureus by M. communis leaf extract at its ½MIC and ¼MIC. C) Growth curve of M. smegmatis cells in planktonic phase of the cultures treated with ½MIC and ¼MIC of M. communis leaf extract. D) Growth curve of S. aureus cells in planktonic phase of the cultures treated with ½MIC and ¼MIC of M. communis leaf extract.