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EVALUATION OF BIOLOGICAL ACTIVITY OF TAR EXTRACTED FROM *PINUS BRUTIA* AND *CEDRUS LIBANI* FROM TURKEY

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Abstract

Tar is an example of an herbal product in folk medicine obtained from *Pinus brutia* and *Cedrus libani*. To obtain tar, old trees with thick trunks that are suitable for kindling are preferred. Tar is used not only in traditional medicine but also in antitumor and antimicrobial studies. This study aims to investigate the apoptotic, cytotoxic and antimicrobial - activity of tars obtained from different tree species in Turkey/Karaman, located in the Central Anatolian Region of Turkey**.** This study used pine tar consisting of *C. libani*, natural tar formed by its solidification, and wood tar extracts obtained from the *P. brutia* tree. Their cytotoxic activity on MDA-MB-231, MCF 7, and HepG2 cell lines was determined for the first time by the MTT method and double staining on cell lines, and their antimicrobial and antioxidant activities were also investigated.

The data of the study revealed that these extracts exhibited remarkable cytotoxic effects on cell lines with varying degrees. Also, whether cells undergo apoptosis or necrosis was examined by double staining. In this respect, it is predicted that tar extracts are strategically important and may be used for the treatment of cancer.

Key words: Cytotoxic activity, Apoptosis, Antimicrobial activity, Antioxidant activity, Tar extracts.

Introduction

Cancer is an essential pathological condition threatening humanity (Sudhakar, 2009). It is a major public health problem (WHO; TUIK, NCI), ranking second on the list of fatal diseases worldwide, caused approximately 10 million mortality in 2020 (Sung *et al*., 2021). The most frequently diagnosed cancers worldwide are breast cancer in women and lung cancer in men (Krusinska *et al*., 2018). There are many reasons why the effects of cancer are fatal, the most important of which is the inadequacies in treatment. Despite the significant progress and achievements in cancer treatment by the scientific world, the intended level of success and treatment outcomes for cancer patients has not been reached yet (ASCO). The expense of cancer therapy remains a significant burden, and the adverse effects of chemotherapy and radiotherapy methods present significant challenges for patients receiving treatment (Erkurt *et al*., 2009; Prasad *et al*., 2017; Turkoglu *et al*., 2018). Moreover, surgical treatments may not every time be viable, as they can pose significant risks and the achieved the rate of survival is often not at the intended level. These factors contribute to the high mortality rate associated with cancer as a disease (Siegel *et al*., 2012). As a result of these challenges, researchers are dedicating extensive efforts towards exploring modern diagnostic techniques for cancer, identifying novel molecules with potential as drug candidates and discovering target molecules or molecular pathways that could be addressed by drugs.

In response to the rising incidence of infections caused by antibiotic-resistant microorganisms, there has been a growing trend towards the discovery of new antimicrobial extracts in recent years. One commonly utilized method for identifying biologically active substances is the systematic screening of microorganisms or plants, which have historically been a rich source of many useful therapeutic agents (Sağdıç *et al*., 2002). The efficacy of antimicrobial activity relies on various factors including the type of plant, its components, the structure of the target microorganism, as well as processing and storage conditions of the food. Additionally, variations in protein-lipid, salt, and pH ratios within the plant can also impact its antimicrobial activity (Faydaoğlu & Sürücüoğlu, 2013; Sağdıç *et al*., 2002).

In Turkey, there are five different species of Pinus trees, which are *Pinus nigra*, *Pinus brutia*, *Pinus pinea*, *Pinus halepensis* and *Pinus sylvestris* (Arı *et al*., 2014). *Juniperus* spp. *Cedrus libani* grows mainly in the southern and inner parts of Turkey, and the yellow tar obtained from it is known as pine tar (P.tar). P.tar obtained from the *C. libani* tree is heated and solidified, resulting in a pitch called natural tar (N.tar) in Turkey. Tar obtained from the *P. brutia* tree is defined as wood tar (W.tar) in our country (Alptekin, 2019).Tar types are used in the classification of bituminous materials; it is included in the P.tar member group of the tar genus at the pyrogenic distillations class, and it is included in the pyrogenic residues class under the name of the P.tar, tree tar member group of the pitches genus (Kolaç *et al*., 2017). The usage areas of these substances are pretty broad; they are used in the treatment of pain, haemorrhoids, insect bites or related poisonings, skin diseases, diarrhoea, and respiratory tract diseases. In addition, it is seen that it maintains its place among the people in treating pets, such as treating skin diseases in animals, foot and mouth, fractures, pinworms, colds, wounds, and poisoning (Alptekin, 2019). It is known that mixtures prepared with P.tar are also suitable for skin diseases. (Gevers *et al*., 2019). Coal tar, on the other hand, is formed by heating the coal in an airtight environment and the gases coming out of it are left to cool and turn into a brown-black liquid that was also used in scabies, sarcoidosis, psoriasis, and neurodermatitis in the past years (Paghdal & Schwartz, 2009). Some studies have shown the

carcinogenicity of tar. However, when used topically, epidemiological studies do not confirm similar results (Athanasiou & Lillis, 1982).

The use of chemotherapeutic drugs in cancer treatment is limited due to the presence of both anticancer properties and side effects. Thus, it is crucial to conduct studies aimed at identifying new herbal sources of anticancer compounds that can selectively target cancer cells. This study aims to shed light on the anticancer effect mechanism of *C. libani* and *P. brutia* tars on some cancer cell lines and whether the cells go into apoptosis or necrosis.

Material and Methods

Obtaining the material: The components W.tar, P.tar, and N.tar used in this study were commercially obtained from the local population residing in the Ermenek district of the Karaman province in Turkey. They were collected in the summer season, August 2020.

Preparation of the extract: After weighing the substances on a precision balance, 1 g was taken, and three separate extractions were made with 10 ml of methanol, chloroform, and acetone (Merck, Darmstadt) from each. The dried samples were subjected to 72 hours of shaking at 37˚C in an oven. Subsequently, the obtained extracts were solubilized in 10 ml of DMSO and then filtered through a filter paper of Whatman No.1. Extracts were then stored at +4˚C for future use. (Guzeldag *et al*., 2014). Afterward, it was passed via 0.45 µm milipore filter (Oxoid), and the extracts were subjected to evaporation using a 40˚C rotary evaporator for a period of 4-6 hours (Dalkilic *et al*., 2020a). The efficiency of the extracts was calculated as a percentage (Popielarska-Konieczna *et al*., 2006). The resulting extract was then prepared at four different concentrations. (Dalkılıç *et al*., 2020).

Determination of antimicrobial activity

Microorganisms: This study contains gram-negative (*Escherichia coli* ATCC 25322, *Klebsiella pneumoniae* ATCC700603) and gram-positive (*Staphylococcus aureus* ATCC 25923, *Bacillus megaterium* DSM32) bacteria and *Candida albicans* ATCC 90028 as fungus were used. These microorganisms were provided from Fethi Sekin City Hospital of Health Sciences University, Elazığ, Turkey. Before experimental work, bacteria and fungus were cultivated in Nutrient Broth (Biolife Lot: HE2602) and Mueller-Hinton Agar (Merck Lot: VM779137).

Agar well technique: The agar well technique was utilized to examine the inhibitory effects of the extracts on bacterial strains. (Balouiri *et al*., 2016). This technique is commonly employed to assess the antimicrobial activity of plants, fungi, and extracts (Kakar *et al*., 2020). To achieve a dilution of 1:10 to 10^7 CFU/ml, the Mc Farland setting was adjusted to 0.5 turbidity. Next, 25 ml of agar was added to the petri dishes. Müeller Hinton Agar from the oven was poured into petri dishes and stored so that the medium solidified utterly. After transferring the bacterial cultures from the broth, they were spread onto the Müeller Hinton Agar using a loop and shaken well. After inoculating the petri dishes, they were allowed to solidify at room temperature for 10-20 minutes. A cork borer was utilized to form a well of the appropriate diameter on the solidified Müeller Hinton Agar. Before each use, it was sterilized by flaming it in a Bunsen burner and dipping it in alcohol. Using the sterilized cork borer, four wells were created in each Petri dish, except for the negative and positive controls, of the appropriate size. Each well was filled with 100 µL of samples prepared at four different concentrations. After inoculation, the Petri dishes were incubated at 37°C for 24 hours in an incubator. To serve as positive controls, Clindamycin 2 mcg (Bioanalyse Lot: 171127A) and Gentamicin 30 mcg (Bioanalyse Lot: 190522G) were used. As for the negative control, 100% DMSO was added to the wells. Following the incubation period, the radius of the inhibition zones was measured with a ruler and then recorded (Guzeldag *et al*., 2014).

Determination of minimum inhibitory concentration (MIC): The MIC value is the minimum concentration of the antimicrobial factor that can inhibit the observable growing of the microorganism being tested and is typically measured in µg/ml or mg/L. Various standards have been established for diluting antimicrobial agents to test their effectiveness against fastidious or slow-growing bacteria, yeasts, and filamentous fungi. Micro or macro dilution is a commonly used method for researching antibacterial activity (Buwa & van Staden, 2006). This process takes place within a liquid growth medium and involves creating a dual dilution in tubes or 96-well plates with a minimum volume of 2 ml or less (Arikan, 2007). Sterilized 96-well plate was fulled with 100 µL of nutrient broth, followed by serial dilution of 100 µL of extract starting from the first well to the H12 well, covering wells 1 to 12. Next, 4 µL of pre-inoculated broth from different bacteria was put to the wells, resulting in a final capacity of 100 µL. Incubation of the plate was carried out at 37°C for a period of 24 hours.

Determination of minimum bactericidal concentration (MBC): MBC refers to the minimum concentration of an antimicrobial agent necessary to eliminate 99.9% of the starting inoculum at the end of a 24-hour incubation period. After incubating on the surface of a non-selective agar plate, microbial growth can be assessed by subculturing from the wells to determine the number of viable cells (CFU/mL) that have survived after 24 hours (Arikan, 2007). It is accepted that there is no growth in the culture and MBC (Dalkılıç *et al*., 2020b). To determine the MBC, 3 µL portion of the liquid from each well of the MIC plate was taken and incubated on nutrient agar at 37°C for 24 hours (Gatta *et al*., 2003). Following subcultivation, MBC was decided as the minimum concentration of the antimicrobial agent that did not exhibit any observable bacterial growth.

Determination of antioxidant activity with 2, 2-diphenyl-1-picrylhydrazil (DPPH) radical scavenger: Goldsmith and Renn investigated the DPPH radical (2,2-diphenyl-1-picrylhydrazyl, DPPH or DPPH-R) in 1922 (Goldschmidt & Renn, 1922). Due to its stability and high

redox potential, DPPH has been employed to assess the antioxidant capacity of biological samples by oxidizing typical natural antioxidants (Blois, 1958). The DPPH radical reacts with an antioxidant and is reduced by the addition of a hydrogen atom or electron to its center, leading to the formation of 2,2-diphenyl-1-picrylhydrazine (DPPH-H) (Flieger & Flieger, 2020). The DPPH was put in 0.001 g, resolved in 25 ml of methanol, and vortexed. A1-A12 was arranged to blank and 40 methanol and 160 DPPH were added to these wells. 40 of methanol and 40 of samples were added to B1-C1-D1 wells, and 40 serial dilutions were made parallel to B1-B12, C1-C12, and D1-D12; resulting in 160 DPPH addition. Next, the samples were incubated for 30 min in the dark at room temperature and their absorbance towards the methanol-containing chloride was detected at 517 nm. The percentage of inhibition values was then analysed (Dalkilic *et al*., 2020b).

$$\% \text{ DPPH radical scavenging activity} = \frac{\text{A control - A sample}}{\text{A control}} \times 100$$

Cytotoxic activity: The MTT assay is a commonly used technique to evaluate cell viability, proliferation, and cytotoxicity. The principle of this method is based on the ability of viable cells to reduce active mitochondria. A tetrazolium salt can enter the cell membrane and accept electrons, which then transforms it into a purple, water-insoluble formazan (Ninich *et al*., 2021). After incubation, the effect of the extracts on cell survival was measured by MTT method. Extracts were prepared at four different concentrations: 125, 250, 500, and 1000 µg/µl. The cytotoxic effect was investigated in human breast cancer MDA-MB-231 and MCF-7 cell lines by applying it for 72 hours. These cell lines were obtained from the Fırat University Department of Molecular Biology and Genetics. These cells in DMEM (25 mM L-Glutamine, 1% penicillin-streptomycin, and 10% fetal bovine serum) were cultured in 25cm^2 on flasks at 37°C in 5% CO_2 atmospheric under these conditions.

After reaching 90% confluence, they were removed from the oven and rinsed with 5 ml of a sterile PBS solution. The vials were treated with 1 ml Trypsin-EDTA, followed by an incubation of 2 minutes at a temperature of 37°C in 5% CO_2. After removing the cells from the surface, 5 ml of medium was put to the vials to inactivate Trypsin-EDTA. Following this, centrifugation was performed at 1300 rpm for 5 minutes to separate the cells from the supernatant. Finally, the cell pellets were dissolved in 1 ml of complete medium. The cells were counted, and then diluted with 10 ml of medium. Based on the calculations, approximately 2000-3000 cells per well were seeded in 96-well plates using 100 µl of cell suspension. After incubation, the extracts and DMEM were added and incubated at 37°C with 5% CO_2 for a period of 72 hours. This study was repeated six times. For the positive control, Doxorubicin at a concentration of 2.5 g/ml was employed, while the negative control consisted of only the medium (Meerloo *et al*., 2011). After incubation, the MTT test was performed. First, 20 µl of MTT solution (5 mg/ml) prepared in a sterile phosphate buffer (pH: 7.2) (Thermo Fisher, USA) was added to the wells containing cells and incubated in a dark medium involving 5% CO_2 at 37°C in for 4 h.

Following the incubation, the medium was removed, and the formazan crystals were dissolved using 100 µl of DMSO (dimethyl sulfoxide). After 5-10 minutes of incubation, absorbance values of wells were detected at 540 nm wavelengths with a microplate reader (Synergy HT USA). The absorbance values obtained from the control wells were considered as 100% viable cells. Percent viability values were calculated by comparing the absorbance values got from the sample wells with the control absorbance value. The resulting data were then transferred to a figure (Klymchenko *et al*., 2001).

Determination of apoptotic/necrotic activity (double staining (Hoechst 33342 and Propidium Iodide)): To evaluate apoptotic and necrotic activity, the dual staining technique (Hoechst 33342 and propidium iodide) was used. This method relies on the capability of fluorescent dyes to link to DNA, thereby rendering the chromatin and the nucleus of the cell apparent (Avci, 2023). Hoechst 33342 is a dye with the ability to bind to DNA and pass the cell membrane, thereby staining the nuclei of both live and dead cells (apoptotic/necrotic). Propidium Iodide (PI) dye is only taken up by cells that have a impaired membrane integrity, enabling the identification of cells in the late stages of apoptosis or necrosis (Dalkılıç *et al*., 2022). Hoechst 33342 and PI dyes was utilized to investigate both nuclear morphology and membrane integrity (Tunc *et al*., 2017). In the presence of pycnotic or fragmented nuclei: cells were observed with Hoechst 33342 (+) / PI (+) stain; It was thought that it might be late apoptotic or secondary necrotic. Hoechst 33342 dye is blue and PI dye is red, and in this sense, apoptotic/necrotic activity was determined (Baran *et al*., 2017; Grusch *et al*., 2002). In double staining, MCF-7 and HepG2 cell lines were seeded with 10x103 cells in 100 µl on sterile coverslips placed in a 6-well plate and incubated for 4-5 hours for cells to adhere to the coverslip surface. Then, 2 ml of DMEM (1% L-Glutamine, 1% Penicillin-Streptomycin, and 10% FBS (Fetal Bovine Serum) was added to the wells and incubated for 24 hours at 37˚C, 5% CO_2 atmospheric conditions. It was incubated for 48 hours. Following the incubation period, a solution containing Hoechst 33342 and PI dyes (dye solution 1x PBS (without Ca/Mg)) was added to the wells, with a final concentration of 5-10 µg/ml and 1 µg/ml, respectively. The plate was incubated at 37°C, in the absence of light and with 5% CO_2, for 30 minutes. After the incubation period, the cell morphologies were compared with the control groups, which consisted of non-treated cells as the negative control and Doxorubicin-treated cells at a concentration of 2.5 µg/ml as the positive control. The cells were evaluated using a fluorescence microscope (Çapan *et al*., 2020).

Statistical analysis

The data obtained from the different concentrations of the test extracts were analysed by using a one-way analysis of variance (ANOVA) and calculated with the least significant difference (LSD) test, at a significance level of 5%. These statistical analyses were conducted utilizing the SPSS statistical program for windows (Version 22, SPPS Inc., Chicago, IL, USA)

Results

Extract efficiency: As shown in (Table 1), percent efficiency calculations were made while extracting extracts; thus, yield losses were prevented.

$$\% \text{ Efficiency calculation} = \frac{\text{Amount remaining after extraction (g)}}{\text{Dry amount before extraction (g)}} \times 100$$

Table 1. Percent efficiency results of extracts.

Extract	Petri empty weight (g)	Petri full weight (g)	Yield result (%)
W.tar + Methanol	9,0056	9,68299	67,73
P.tar + Methanol	9,6745	10,3678	69,33
N.tar + Methanol	8,9979	9,6462	64,83
W.tar + Chloroform	8,9953	10,03479	101,47
P.tar + Chloroform	9,4522	10,8174	136,52
N.tar + Chloroform	9,4409	11,1126	165,21
W.tar + Acetone	23,3663	24,3105	94,42
P.tar + Acetone	23,0372	23,7735	73,63
N.tar + Acetone	48,4595	49,4102	95,07

Antimicrobial effect: This study investigated the antimicrobial activity of nine different extracts on five microorganisms (Fig. 1). The obtained extracts' antibacterial properties were analysed according to the width of the inhibition zone, as shown in Table 2. DMSO was used as negative control (Kakar *et al*., 2020) (Table 2). The results were compared with the control as Clindamycin and Gentamicin for antibacterial activity. Extracting W.tar in different solutions showed activity against all bacteria with 14-16mm zone diameter at 1000 mg/ml concentration (Fig. 2). On the contrary, the extract of W.tar in different solvents demonstrated efficacy to *S. aureus*, *C. albicans*, *E. coli*, and *K. pneumonia* with the lowest (9-11 mm) zone diameter at 250mg/ml concentration. P.tar extracts in different solvents indicated the highest effect against *S. aureus*, *K. pneumonia*, and *C. albicans* (18-20 mm) zone diameter at 1000mg/ml concentration. In addition, P.tar displayed less activity against all bacteria with (9-11 mm) zone diameter at 250 mg/ml concentration in different solvents. It was determined that N.tar had the highest effect (20-22 mm) zone diameter against *E. coli*, *B. megaterium*, and *C. albicans* at 1000 mg/ml concentration in different solvents. Extracts in different solvents of N.tar at 250mg/ml concentration (9-11 mm) with zone diameter were found to have a lower effect against all bacteria (Fig. 3). Each concentration has its standard deviation, and these values are shown in (Table 2). According to the MIC results, methanol, chloroform, and acetone extracts of W.tar showed the highest activity (1/32 µg/ml) on *E. coli*, whereas N.tar extract prepared with chloroform and acetone and P.tar extract prepared with acetone showed a lower (1/64 µg/ml) effect (Table 3). While W.tar extract prepared with methanol showed the highest MIC and MBC values (1/32 µg/ml) against *K. pneumoniae*, N.tar extract prepared with acetone showed the lowest value. (Figs. 4-5).

Samples MIC and MBC, ranging from 1/16 µg/ml to 1/256 µg/ml, were determined against four bacteria and one fungus. MIC values varied depending on the test extracts (Table 3). Upon evaluation of the MIC results, it was observed that W.tar extract prepared with methanol showed the highest effect against *E. coli* and *K. pneumoniae* with 1/32 µg/ml (Fig. 3). It was determined that N.tar and P.tar extracts prepared with acetone had the lowest MBC value among the extracts at 1/256 µg/ml. (Table 3). According to our results, Gram (-) bacteria showed more effect than Gram (+) bacteria in MIC and MBC tests (Table 3). *B. megaterium* was the most sensitive in MBC results (Fig. 5). According to MIC and MBC, it was determined that W.tar extract showed the highest activity against *B. megaterium* (Table 3).

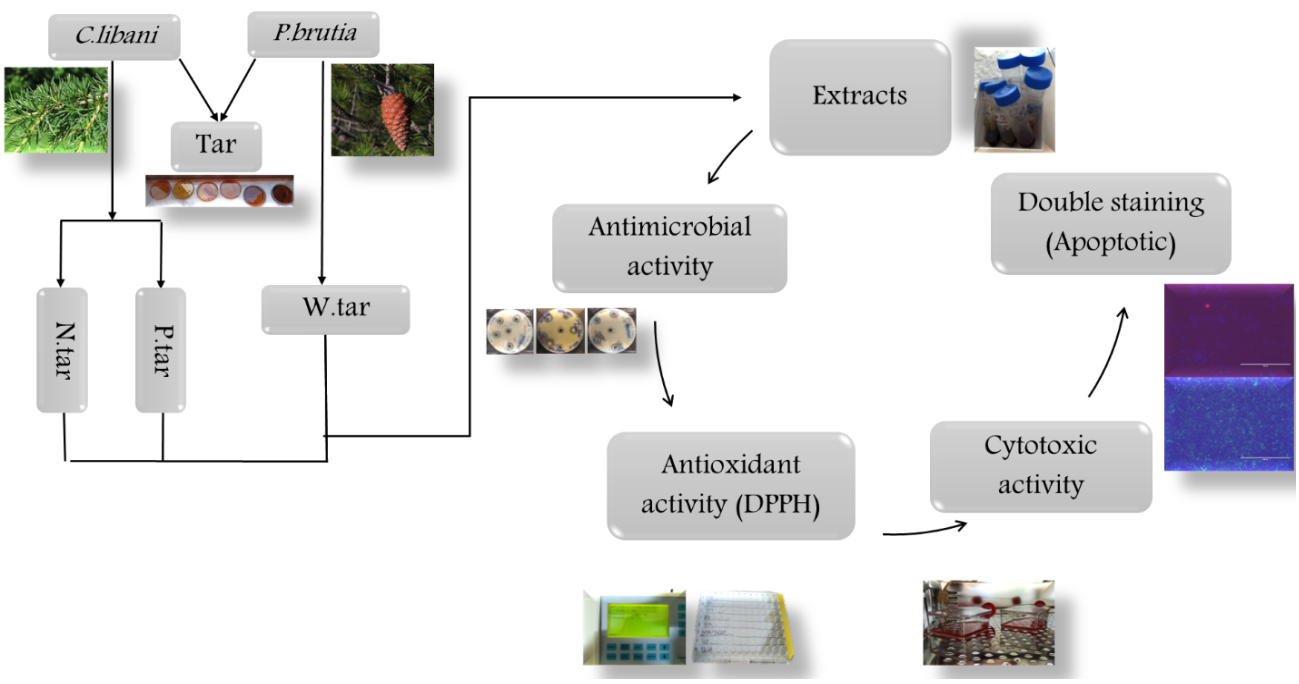

Fig. 1. Experimental flow diagram of the study. W.tar, P.tar, and N.tar, three different extracts were prepared from tar substances, the ability of each extract to scavenge free radicals was observed, and its inhibition ability against microorganisms and cancer cells was determined.

Table 2. Results of antimicrobial effects (Zone diameters in mm).

Extracts		*E. coli*				*S. aureus*				*B. megaterium*				*K. pneumoniae*				*C. albicans*			
Concentrations		**250 µg/ml**	**500 µg/ml**	**750 µg/ml**	**1000 µg/ml**	**250 µg/ml**	**500 µg/ml**	**750 µg/ml l**	**1000 µg/ml**	**250 µg/ml**	**500 µg/ml**	**750 µg/ml**	**1000 µg/ml**	**250 µg/ml l**	**500 µg/ml**	**750 µg/ml**	**1000 µg/ml**	**250 µg/ml l**	**500 µg/ml**	**750 µg/ml**	**1000 µg/ml**
1.	W. tar + Methanol	10 ± 2.44	10 ±2.4	12 ±2.32	15 ±2.6	11 ±2.4	12 ±2.8	13 ±2.5	14 ±2.78	10 ±2.78	13 ±2.35	14 ±3.07	16 ±3.3	10 ±2.2	10 ±3.28	12 ±2.72	13 ±2.72	9 ±2.92	10 ±2.68	12 ±3.21	14 ±3.65
2.	P. tar + Methanol	16 ± 2.44	15 ±2.4	16 ±2.32	17 ±2.6	15 ±2.4	18 ±2.8	18 ±2.5	19 ±2.78	13 ±2.78	11 ±2.35	15 ±3.07	15 ±3.3	14 ±2.2	19 ±3.28	20 ±2.72	20 ±2.72	16 ±2.92	17 ±2.68	19 ±3.21	19 ±3.65
3.	N. tar + Methanol	14 ± 2.44	17 ±2.4	19 ±2.32	20 ±2.6	15 ±2.4	15 ±2.8	16 ±2.5	18 2.78±	18 ±2.78	18 ±2.35	19 ±3.07	20 ±3.3	15 ±2.2	16 ±3.28	16 ±2.72	17 ±2.72	15 ±2.92	16 ±2.68	20 ±3.21	22 ±3.65
4.	W. tar + Chloroform	12 ± 2.44	12 ±2.4	12 ±2.32	13 ±2.6	*	12 ±2.8	14 ±2.5	16 ±2.78	10 ±2.78	11 ±2.35	12 ±3.07	13 ±3.3	10 ±2.2	*	*	*	10 ±2.92	16 ±2.68	12 ±3.21	12 ±3.65
5.	P. tar + Chloroform	10 ± 2.44	16 ±2.4	*	14 ±2.6	10 ±2.4	9 ±2.8	11 ±2.5	10 ±2.78	10 ±2.78	14 ±2.35	12 ±3.07	12 ±3.3	14 ±2.2	16 ±3.28	15 ±2.72	17 ±2.72	9 ±2.92	12 ±2.68	14 ±3.21	16 ±3.65
6.	N. tar + Chloroform	9 ± 2.44	12 ±2.4	13 ±2.32	11 ±2.6	10 ±2.4	12 ±2.8	14 ±2.5	15±2.78	11 ±2.78	13 ±2.35	16 ±3.07	16 ±3.3	10 ±2.2	10 ±3.28	11 ±2.72	11 ±2.72	9 ±2.92	11 ±2.68	12 ±3.21	14 ±3.65
7.	W. tar + Acetone	14 ± 2.44	14 ±2.4	14 ±2.32	14 ±2.6	9 ±2.4	9 ±2.8	10 ±2.5	12±2.78	9 ±2.78	11 ±2.35	9 ±3.07	9 ±3.3	11 ±2.2	11 ±3.28	15 ±2.72	16 ±2.72	9 ±2.92	10 ±2.68	11 ±3.21	10 ±3.65
8.	P. tar + Acetone	*	11 ±2.4	15 ±2.32	17 ±2.6	*	12 ±2.8	14 ±2.5	15±2.78	10 ±2.78	11 ±2.35	11 ±3.07	11 ±3.3	11 ±2.2	13 ±3.28	16 ±2.72	15 ±2.72	13 ±2.92	13 ±2.68	13 ±3.21	13 ±3.65
9.	N. tar + Acetone	11 ± 2.44	15 ±2.4	15 ±2.32	15 ±2.6	12 ±2.4	14 ±2.8	16 ±2.5	16±2.78	13 ±2.78	11 ±2.35	11 ±3.07	12 ±3.3	15 ±2.2	15 ±3.28	15 ±2.72	15 ±2.72	14 ±2.92	14 ±2.68	14 ±3.21	14 ±3.65
Clindamycin		19 ± 0.7				19				18				18 ± 1.41				22 ± 4.24			
Gentamicin		18 ± 0.7				19				18				20 ± 1.41				16 ± 4.24			

W: W.tar; P: P.tar; N: N.tar; M: Methanol; C: Chloroform; A: Acetone

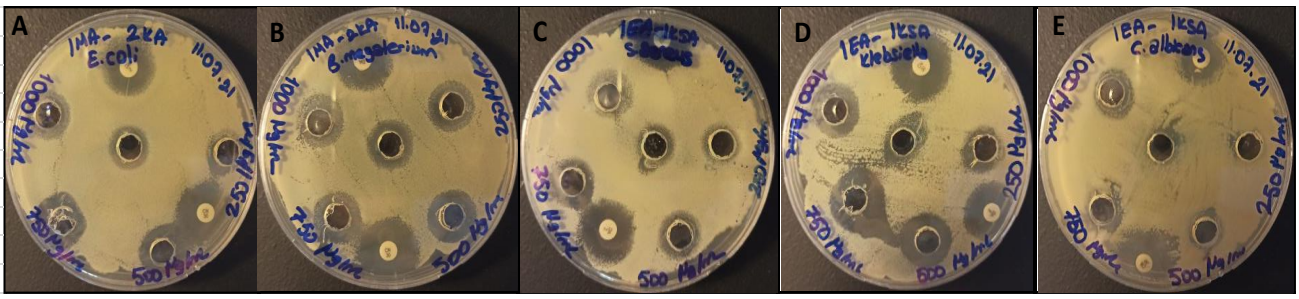

Fig. 2. Zone diameters showing the effects of extracts, Gentamicin(CN), and Clindamycin(DA) controls on microorganisms **A)** *E. coli* ***B)*** *B. megaterium* **C)** *S. Aureus* **D)** *K. pneumoniae* **E)** *C. albicans.*

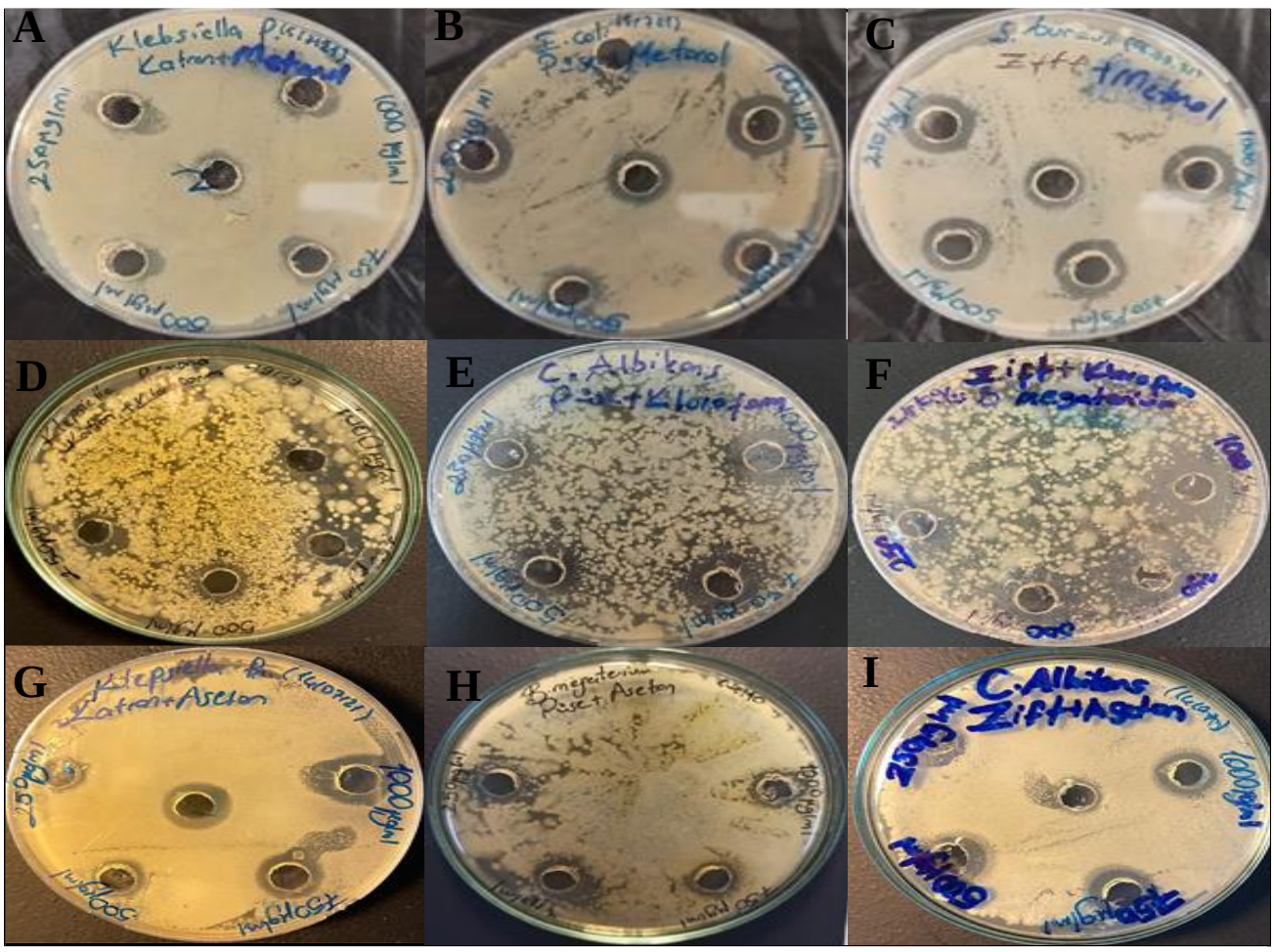

Fig. 3. Inhibition zones of the prepared extract on microorganisms. **A.** Inhibition zone of W.tar + Methanol extract on *K. pneumoniae* **B.** Inhibition zone of P.tar + Methanol extract on *E. coli* **C.** Inhibition zone of N.tar + Methanol extract on *S. aureus* **D.** Inhibition zone of W.tar + Chloroform extract on *K. pneumoniae* **E.** Inhibition zone of P.tar + Chloroform extract on *C.albicans* **F.** Inhibition zone of N.tar + Chloroform extract on *B. megaterium* **G.** Inhibition zone of W.tar + Acetone extract on *K. pneumoniae* **H.** Inhibition zone of P.tar + Acetone extract on *B.megaterium* **I.** Inhibition zone of N.tar + Acetone extract on *C. Albicans.*

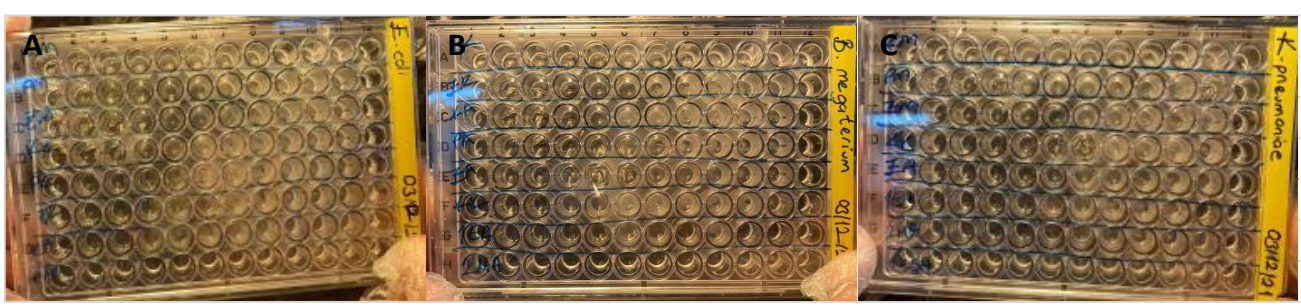

Fig. 4. Effects of samples viewing the results of the MIC test for **A.** *E. coli* **B.** *B. megaterium* **C.** *K. pneumonia.*

Fig. 5. The effects of the samples were determined in the MBC test for **A.** *E. coli* **B.** *B. megaterium* **C.** *K. pneumoniae.*

Table 3. Minimal inhibitory concentration and minimum bactericidal concentration (MBC) values were evaluated according to their bioactivity.

Organism	*E. coli*	MBC	*B. megaterim*	MBC	*K. pneumoniae*	MBC
Samples	MIC		MIC		MIC	
WM	1/32	1/32	1/128	1/16	1/32	1/32
PM	1/32	1/64	1/64	1/32	1/32	1/64
NM	1/32	1/64	1/64	1/32	1/32	1/64
WC	1/32	1/32	1/32	1/32	1/64	1/64
PC	1/64	1/64	1/32	1/64	1/128	1/64
NC	1/64	1/256	1/64	1/64	1/256	1/64
WA	1/32	1/32	1/32	1/32	1/128	1/64
PA	1/64	1/256	1/64	1/64	1/256	1/64
NA	1/64	1/256	1/64	1/256	1/128	1/256

W: W.tar; P: P.tar; N: N.tar; M: Methanol; C: Chloroform; A: Acetone

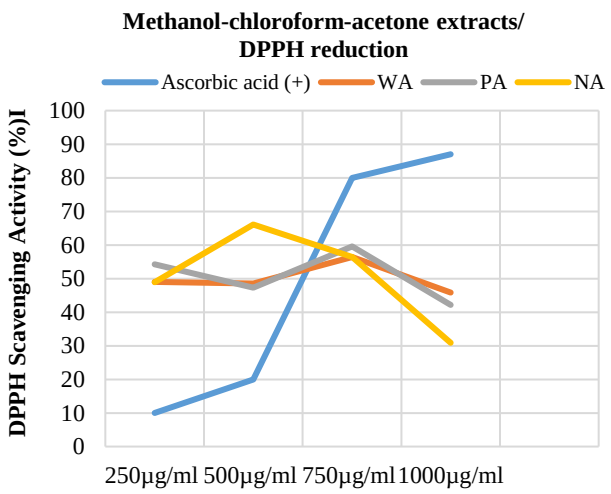

Fig. 6. The antioxidant activity results of the samples extracted with chloroform, methanol, and acetone by the DPPH Radical Scavenging Capacity Method were at 517 nm. W: W.tar; P: P.tar; N: N.tar; M: Methanol; C: Chloroform; A: Acetone.

Antioxidant activity: The percentage of inhibition of DPPH radical of different concentrations of methanol, chloroform, and acetone extract of W.tar, P.tar, and N.tar were shown in (Fig. 6). Optimization of incubation time to test the DPPH method, the antioxidant activities of ascorbic acid were calculated. The variation of sweeping activity over time is presented in Fig. 5. It is probable to measure different concentrations for the 50% radical scavenging activity of ascorbic acid at any period. According to the results obtained, it was observed that among the extracts prepared with methanol, N.tar had the highest DPPH radical scavenging effect with 47.53 µg/ml, while P.tar and W.tar are 40.87 µg/ml and 35.42 µg/ml, respectively. Also, between the extracts prepared with chloroform, the best DPPH radical scavenging effect was seen in the N.tar extract with a value of 48.67 µg/ml, while the minimum DPPH radical scavenging effect was seen in the extract of P.tar with a value of 39.36 µg/ml. However, the DPPH radical scavenging effect of W.tar, P.tar, and N.tar extracts prepared with acetone was the highest compared to those prepared with other solvents, which were 49.93 µg/ml, 50.82 µg/ml, and 50.58 µg/ml, respectively. As a result of the DPPH radical scavenging measurement made with W.tar, P.tar, and N.tar extracts, it was observed that the highest effect was the N.tar extract prepared with Acetone at 500 µg/ml concentration and the lowest effect was the P.tar extract prepared with chloroform at 750 µg/ml concentration.

Cytotoxic effect: Among the seven extracts tested, the extracts generally exhibited significant cytotoxic effect to the cell lines. P.tar, N.tar, and W.tar extracts prepared with methanol showed high cytotoxic activity against the MDA-MB-231 cell line in 1000 µg/µl concentration, which affected of surviving cell count approximately 3%, 4%, and 6% at 540nm wavelength. Extracts prepared with chloroform at the same concentration and wavelength also showed high cytotoxic activity, and the remaining viable cell count was nearly 19% for W.tar, 5%, and 4% for P.tar. It was observed that all extracts were less effective against the MDA-MB-231 cell line at low concentrations, depending on the concentration. W.tar 17%, P.tar 12%, and N.tar 17% of the extracts prepared with methanol at a concentration of 250 µg/µl and a wavelength of 540nm affected viable cells. Similarly, extracts prepared with chloroform had a 14%, 7%, and 13% effect on living cells, respectively (Fig. 7). All results generated from the cytotoxic activity tests were evaluated and shown in Figs. 7-8. Among the extracts, the extracts prepared with methanol showed maximum the cytotoxic activity towards MCF-7 cell line. Figure 8 shows that W.tar 15%, P.tar 44%, and N.tar 22%, prepared with a concentration of 1000,

remained viable cells, thus having a high effect. It was understood that the cytotoxic effect of the extracts prepared with acetone and chloroform against the MCF-7 cell line was not very high. Among the tested extracts, W.tar prepared with chloroform and N.tar prepared with methanol were demonstrated considerable cytotoxic activity to all the cell lines. The results obtained from experiments of cytotoxic activity and values of calculated cytotoxic activity are shown in (Table 4). The cytotoxic activity of nine different extracts was evaluated and the maximum inhibitory concentration (IC50) value was calculated for four concentrations (1000, 750, 500, and 250 µg/ml) using the results obtained (Table 5). The IC50 values of the tested cytotoxic activity for P.tar, W.tar, and N.tar extracts against two different cell lines are presented in Table 5, and the obtained results varied depending on the cell line and the extract used.

Apoptotic/necrotic activity detection: Only damaged and degraded cell membranes are stained with the PI staining technique, while the Hoechst staining technique stains all living and dead cells. At the same time, these staining techniques are used to determine the cell number, reveal the membrane's nuclear physiology, and show the morphological structure. One of the most critical features of apoptosis is the decrease in cell size with the condensation of chromatin and cytoplasm. Likewise, pigmentation of cells indicates apoptosis. Contraction occurs in cells undergoing apoptosis due to water loss. It means that cells die by apoptosis when they are not stained with PI dye or when they are stained much less. Necrotic cells can be stained with both PI and Hoescht stains. However, PI dye has high discrimination in necrotic cells.

Both cancer cell lines (MCF-7, HepG2) and the healthy control group were studied in double staining. In (Fig. 9A) the cells in the negative control group were MCF-7 cell lines; their numbers increased, and membrane integrity was preserved. MCF-7 cells in Fig. 8B have been applied doxorubicin, and these cells were stained with Hoechst dye. The PI dye stained damaged and membrane-disrupted cells (Fig. 9B). As seen in (Fig. 9C), the extract of P.tar prepared with methanol was tested to the MCF-7 cell line and stained with Hoechst and PI dye, and it was determined that the structure of the cancer cells was impaired. Both dyes were applied on the extract of P.tar prepared with chloroform and the effect on MCF-7 cell line was observed. As a result, it was determined that the number of cancer cells decreased and cell structures were disrupted (Fig. 9D). The significant decrease in the number of cells in the positive control group treated with doxorubicin indicated that the cells went into necrosis. This was realised by complete disintegration of the cells and their nuclei. Despite being generally necrotic, cells undergoing apoptosis were relatively common in this group (Fig. 9A-B). In (Fig. 9E), the cells in the negative control group are HepG2 cell lines with prominent cell nuclei and high density. In the HepG2 cell line, the density of necrotic cells was higher than in the positive control. Besides these cells, a significant amount of cells with dispersed nuclei were observed (Fig. 9F). P.tar extract prepared with methanol showed a different morphology against the HepG2 cell line comparing to the other cell line and was colonized more closely and overlapping. In this cell group, both necrotic and apoptotic cells were detected. In addition to the density of apoptotic cells was found to be higher (Fig. 9G). As observed in (Fig. 9H), the influence of P.tar extracts prepared with chloroform against the HepG2 cell line was determined by double staining. It was observed that there was a decrease in the number of cells and that the cells were scattered. It was determined that these cells were found to be early apoptotic or secondary necrotic cells.

Table 4. Cytotoxic effects of some extracts on different cell lines.

Antitumor effects of extracts on the cell lines			
		Cell lines (viability %)	
Extracts	**Dose (µg/ml)**	**MDA-MB-231**	**MCF-7**
WM	125	16	186
	500	17	12
	750	*	10
	1000	5	15
PM	125	11	112
	500	4	14
	750	*	24
	1000	3	44
NM	125	117	169
	500	2	80
	750	5	13
	1000	4	22
WC	125	13	186
	500	21	180
	750	23	190
	1000	18	131
PC	125	7	162
	500	*	11
	750	*	17
	1000	5	24
NC	125	12	106
	500	0	12
	750	4	18
	1000	4	38
WA	125	10	11
	500	9	13
	750	*	20
	1000	*	35
PA	125	*	160
	500	*	40
	750	*	20
	1000	*	44
NA	125	*	31
	500	*	21
	750	*	35
	1000	*	93
Doxorubicin	2,5 ng/ml	37	24
Control cell	-	100	100

Mean with a different letter in curves is significant at $p<0.05$ (ANOVA followed by LSD test) * no results observed.

W: W.tar; P: P.tar; N: N.tar; M: Methanol; C: Chloroform; A: Acetone

Table 5. The IC_{50} value of cytotoxic activity on cells. (The result indicated by * could not be found).

Agent	WM	PM	NM	WC	PC	NC	WA	PA	NA
					IC 50				
MDA-MB-231 cell line	*	*	*	257 µg/ml	*	347 µg/ml	*	*	*
MCF-7 cell line	114 µg/ml	112 µg/ml	244 µg/ml	1146 µg/ml	115 µg/ml	113 µg/ml	931 µg/ml	183 µg/ml	602 µg/ml

W: W.tar; P: P.tar; N: N.tar; M: Methanol; C: Chloroform; A: Acetone

Discussion

A study revealed that a subspecies of *C. atlantica* used in tar production did not display any activity against the MCF-7 cell line, and the IC_{50} value was 143.13±14.6. MIC and MBC values of *C. atlantica* were 62.5 and 125 µg/ml on *S. aureus*. Our study determined that the MIC values of tar species against *E. coli* were between 1/32 µg/ml and 1/64. It was revealed that the MIC values of tar species against *E. coli* were between 1/32 and 1/64, while MBC values were between 1/32 and 1/256, while MBC values were between 1/32 and 1/256 (Belkacem *et al*., 2021). In another study, it was found that *A.cilicica* leaves prepared with chloroform and acetone formed a 16-18mm inhibition zone against *E.coli* (Dığrak *et al*., 1999). This study determined that all tar species prepared with different solvents formed an inhibition zone of 11-20 mm against *E. coli*. The antimicrobial properties of *P. brutia* tar were examined on *S. aureus, C. albicans,* and *K. pneumoniae and* were determined using the disc diffusion method. As a result, they found 13.33mm and 9mm inhibition zones, respectively. However, inhibition zones against *P. pinea* were determined as 8.66 and 9.66, respectively. They found that both plants did not positively affect *K. pneumonia* (Ulukanli *et al*., 2014). In our study, in all cultivars of tar prepared with different solvents, 12-18mm against *S. aureus* and 10-19mm against *C. albicans* and *K. pneumoniae*, inhibition zones of 11-20 mm were detected. It was determined that the inhibition zone of *C. libani* tar against *E. coli* increased depending on the concentration, and the inhibition zone was 15mm at 100% concentration (Takci *et al*., 2021). This study showed parallelism with this study, and it is seen in Table 2 that the inhibition diameter increases depending on the concentration of tar in different solvents, with a zone diameter of 20mm. The potential anticancer properties of cedar tar were investigated in various cancer cell lines, including colorectal adenocarcinoma (HT-29, HCT-116, DLD-1), breast adenocarcinoma cells (MDA-MB-231, MCF-7), prostate carcinoma cells (DU-145, PC-3), umbilical vein endothelial normal cells (HUVEC), breast epithelium normal cells (CRL-8798), endometrium adenocarcinoma cells (ECC-1), cervical adenocarcinoma cells (HELA), mammary epithelial normal cells (CRL-4010), gastric carcinoma cells (HGC-27), bone osteosarcoma cells (U2OS), human lung carcinoma cells (A549), and kidney cells (HEK-293, PNT-1). The remaining viable cell numbers were found to be 30.48 and 65.3 µg/ml in some of the tested cell lines. Our study revealed that the number of viable cells of W. tar, P.tar and N.tar extracts against MDA-MB-231 cell line was between 12% and 17%, while the cytotoxic activities of these extracts against MCF7 cell line were between 15% and 44% (Temiz *et al*., 2022).

In a study, various cancer cells and normal cells were studied to determine the anticancer effect of cedar tar, and it was determined that the most potent cytotoxic impact against cancer cells was exhibited by cedar tar, particularly against colon cancer cells (HCT-116, IC50: 30.4 µg/ml). Additionally, it was noted that the toxic effect of cedar tar on normal cells (HUVEC, IC50: 74.07 µg/ml) was lower than on cancer cells. Also, the rice in NFR-2 and HO-1 gene, which correlates with the intracellular ROS level, confirmed the increase in ROS (Temiz *et al*., 2022). Researchers compared the antioxidant capacities of essential oils obtained from W.tar and sawdust of *Cedrus atlantica* and showed that essential oils produced from W.tar (IC_{50} = 0.126 mg/ml ve 0.143 mg/ml) had more antioxidant activity than those produced from sawdust (IC_{50} = 15.6 mg/ml ve 16.3 mg/ml) (Jaouadi *et al*., 2021). In our results, the DPPH radical scavenging effect of methanol extract of tar and its derivatives changed depending on increasing concentrations, and it was determined that the best DPPH radical scavenging effect of N. tar with methanol was at 250 µg/ml concentration (IC: 244 µg/ml). According to a study, cedar tar had a small amount of phenolic ingredient (0.85±0.06 mg GAE/). It was found that the level of flavonoids, which are low molecular weight phenolic extracts, was 0.068±0.02 mg RE/g. It has been stated that this low secondary metabolite content adversely affects Tars' mutagenic and antioxidant activity. In the conventional production of tar, the firing process, carried out at an average temperature of 300ºC, could result in the depletion of a significant portion of secondary metabolites in the cider (Mercimek Takcı, 2019). As a result of such results, the antioxidant activity determination of tar and its derivatives was determined by DPPH radical scavenging capacity technique.

Contrary to all this, rats investigated the toxicity of the water-soluble fraction of pyrolysed W.tar aerosols in a study. W.tar aerosols induced increased inflammatory and oxidative stress responses. In addition, increased cell death with apoptotic properties was observed in the bronchial epithelial cells of mice (Pardo *et al*., 2020). Similar to this study, exposure to the water-soluble W.tar fraction increased the generation of total ROS in cells, reduced the mitochondrial membrane potential (MMP), and caused oxidative damage and cell death, possibly via apoptosis (Pardo *et al*., 2021). According to a study, Cedar W.tar did not show any antioxidant activity in relation to DPPH radical scavenger (Takci *et al*., 2019). Another study stated that the essential oil obtained from the *C. libani* tree showed a high antioxidant scavenging effect with a value of 1532 mg/ml. In the same study, as a result of testing the in vitro cytotoxic effect of essential oils got from the berries of *P. pinea* tree and *C. libani* tree against human MDA-MB 231 breast adenocarcinoma, IC_{50} values were 107.7 mg/ml and 54.13 mg/ml, respectively. Again, it was stated that the type of oils used in the antimicrobial result of the same study showed remarkable activity against *C. albicans* as in our study (Venditti *et al*., 2022). The researchers tested W.tar oil for its antibacterial activity. They found that the W.tar oil extract had a more significant effect on the tested bacterial strains (*B. subtilis*, (Methicillin-resistant *S. aureus*), *Micrococcus luteus, S. aureus, E. coli, P. aeruginosa, and K. pneumonia*) than the Streptomycin antibiotic and W.tar oil vapor. Also, *P. aeruginosa* was a more susceptible bacterium than the *Micrococcus luteus* strain. The mean inhibition zones of the oil ether extract shown in the tested bacterial strains were 46 - 45.33 mm in diameter, respectively (Gumgumjee, 2020). Another study with tar showed that tar oil had antifungal effects against the strains studied. A higher rate was obtained than our study, showing a zone diameter of 31.33 mm against *C. albicans*. In addition, W.tar oil, *Olea europaea*, has been reported to give 79.10% higher DPPH radical scavenging activity compared to ascorbic acid (Gumgumjee *et al*., 2018).

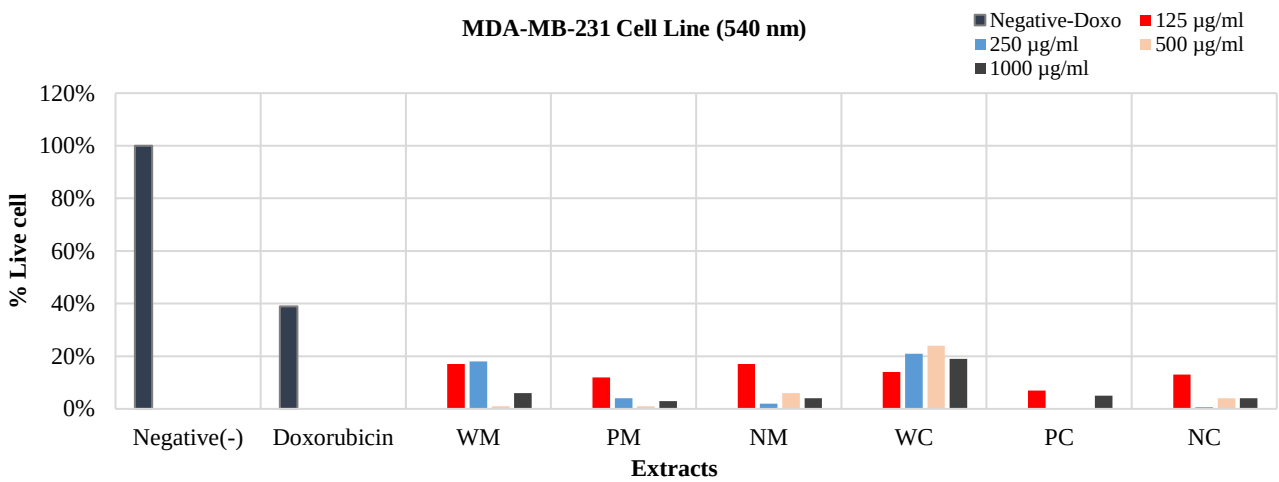

Fig. 7. Measurement results of the cytotoxic activity on the MDA-MB-231 cell line at Wavelength 540nm values. W: W.tar; P: P.tar; N: N.tar; M: Methanol; C: Chloroform; A: Acetone.

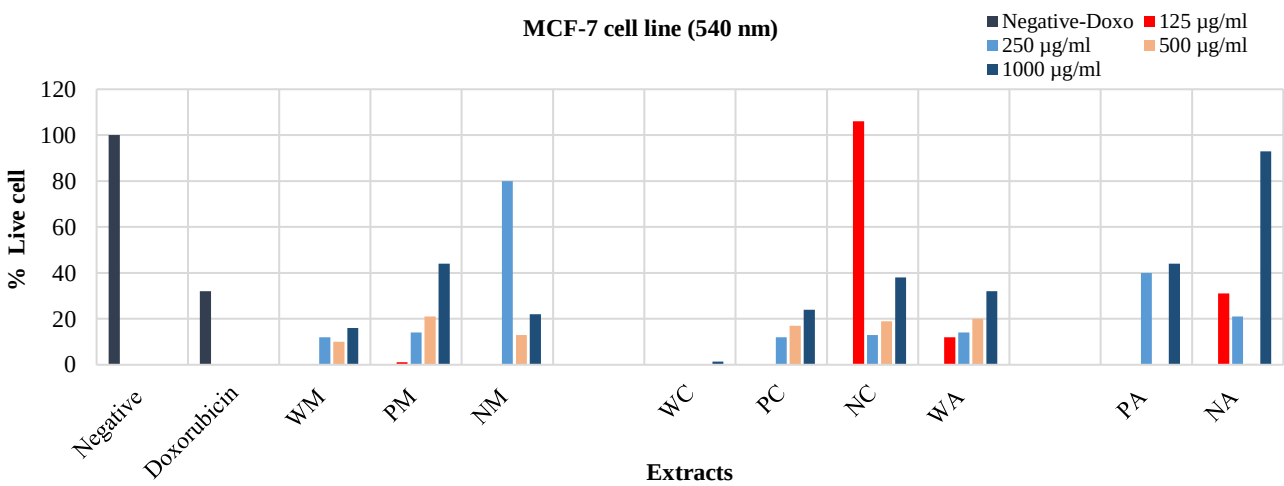

Fig. 8. Measurement results of cytotoxic activity on MCF-7 cell line at 540 nm. W: W.tar; P: P.tar; N: N.tar; M: Methanol; C: Chloroform; A: Acetone.

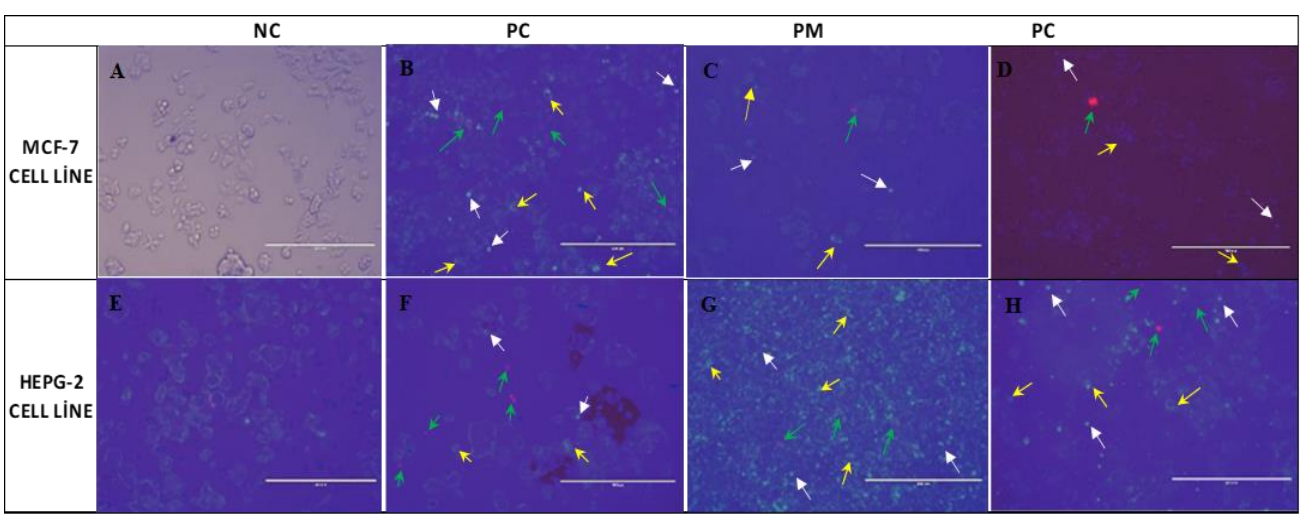

Fig. 9. Detection of apoptotic/necrotic activity by dual staining (Hoechst 33342 and Propidium Iodide) on MCF-7 and HepG2 cell lines. Images were taken with a Fluorescent Inverted Microscope at 10x magnification. Cells were stained with Hoechst 33258 (blue) and PI to visualize cell death. White arrows indicate pycnosis, yellow arrows decomposed nuclei, and green arrows are necrotic cells. **A.** MCF-7 cell line negative (-) control **B.** MCF-7 cell line positive (+) control **C.** Application of P.tar + Methanol extract on MCF-7 cell line **D.** P.tar + Chloroform extract on MCF-7 cell line application of the extract. **E.** HepG2 cell line negative (-) control **F.** HepG2 cell line positive (+) control **G.** Application of P.tar + Methanol extract on HepG2 cell line **H.** P.tar + Chloroform cell line on HepG2 cell line application of the extract.

Conclusion

Double staining showed cytotoxic activity of wood tar, pine tar, and natural tar on MDA-MB-231 MCF-7 cell lines (Hoechst 33342 and Propidium Iodide) method in MCF-7 and HepG2 cell lines. Determination of apoptotic/necrotic activity, investigation of antimicrobial activity of *E. coli*, *K. pneumoniae*, *S. aureus*, *B. megaterium*, and *C. albicans* fungus by hollow agar method, MIC and MBC methods, antioxidant activity determination study with the same extracts was investigated for the first time as far as we know. Antioxidant, antimicrobial and cytotoxicity experiments have shown that W.tar and its derivatives are a promising candidate to be potent antioxidant, antibacterial and anticancer agents at certain concentrations that deserve further investigation.

The antioxidant, antimicrobial, and cytotoxicity experiments showed that W.tar and its derivatives are a promising source of a powerful antioxidant, antibacterial, and anticancer agents at specific concentrations that deserve further investigation.

W.tar and its derivatives resulted in W.tar substance exhibiting solid antioxidant power. The antibacterial evaluation conducted on four bacterial strains and one fungus demonstrated that both W.tar and its fractions exhibited bactericidal effects. To conclude, the extracts displayed a significant cytotoxic impact on the MDA-MB-231 and MCF-7 breast cancer cell lines. At the same time this present study contributes to an improved understanding of W.tar, P.tar, and N.tar. We encourage further investigations into the isolation and characterization of pure compounds that could be crucial pharmaceutical agents.

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