FIRAT UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
TÜRKİYE

INVESTIGATION OF CYTOTOXIC ACTIVITIES OF *TERFEZIA* AND *PICOA* EXTRACTS AND MOLECULAR EFFECTS ON CANCER CELL LINES

Kochar Khasro SALEH

Doctoral Thesis

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Author
Kochar Khasro SALEH

Supervisor
Prof. Dr. Sevda KIRBAĞ

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This thesis, which was prepared according to the thesis writing rules of the Graduate School of Natural and Applied Sciences, Fırat University, was evaluated by the committee members who have signed the following signatures and was unanimously approved after the defense exam made open to the academic audience.

Signature

Supervisor:	Prof. Dr. Sevda KIRBAĞ Firat University, Faculty of Science	Approved
Chair:	Prof. Dr. Mustafa KARATEPE Firat University, Faculty of Science	Approved
Member:	Assoc. Prof. Dr. Mehmet AKYÜZ Bitlis Eren University, Faculty of Science	Approved
Member:	Assoc. Prof. Dr. Yaşar KIRAN Firat University, Faculty of Science	Approved
Member:	Assist. Prof. Dr. Burak BİRCAN Osmaniye Korkut Ata University, Vocational School of Health Services	Approved

This thesis was approved by the Administrative Board of the Graduate School on

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Signature

Prof. Dr. Kürşat Esat ALYAMAÇ
Director of the Graduate School

DECLARATION

I hereby declare that I wrote this Doctoral Thesis titled " Investigation of Cytotoxic Activities of *Terfezia* and *Picoa* Extracts and Molecular Effects on Cancer Cell Lines " in consistent with the thesis writing guide of the Graduate School of Natural and Applied Sciences, Firat University. I also declare that all information in it is correct, that I acted according to scientific ethics in producing and presenting the findings, cited all the references I used, express all institutions or organizations or persons who supported the thesis financially. I have never used the data and information I provide here in order to get a degree in any way.

01 June 2022

Kochar Khasro SALEH

PREFACE

Desert truffles are a type of edible hypogeous macrofungus with no stalks or gills that forms a mycorrhizal association with Cistaceae annual plants. They have spread over Africa and Asia, originating in the Middle East. They were once considered a marvel of nature and were consumed as customary nourishment in the ancient world.

Terfezia and *Picoa* are two of the most popular desert truffles in the world. They have been documented to engage in a wide range of biological activities. Despite its bioactivities and nutritional worth, *Terfezia* and *Picoa*'s impact on molecular pathways of cancer cells has received insufficient attention. The current study investigated the anticancer activities of *Terfezia* species (*Terfezia claveryi, T. boudieri, and T. olbiensis*), and *Picoa* species (*Picoa juniperi* and *P. olbiensis*) discovered in Turkey environments.

This study is designed to determine the impacts of *Terfezia* and *Picoa* on apoptotic mechanisms in three different cancer cell lines (PANC-1, MDA-MB 231, A549). Apoptotic pathway has a great role in the regulation of cell growth by contact with apoptotic genes. Therefore, need to establish an effective evaluation technic to determine the exact effects of *Terfezia* and *Picoa* extracts on apoptotic mechanism in cancer cell lines (PANC-1, MDA-MB 231, A549). As part of this effort, we initiated this study to determine the new line of study in this field to estimate the roles of fungi as a resource of cancer treatment.

First and initially, praise and appreciation to Allah, the Almighty, for His showers of blessings during my study effort, which enabled me to successfully complete the research.

I am very grateful to my supervisor, Professor Dr. Sevda KIRBAĞ for providing me with an opportunity to work on this research. Her guidance, support, and constant help have made this dissertation possible, thank you so much for your countless help. Also, I would like to thank Dr. Semih DALKILIÇ for his support. In addition, I would like to thank Assoc. Prof. Dr. Mehmet AKYÜZ for his guides.

I am extremely grateful to my parents for their love, prayers, care, and sacrifices for me. Thank you dear Bahra Radhaa HAMARASHID for your love, understanding, encouragement, and continuing support. Also, I express my thanks to my sister, and brothers, for their support and valuable prayers. My Special thanks go to my friend Dr. Şule İNCİ. I appreciate all my friends in the laboratory especially Mr. İsmail KORKMAZ, and Mr. Gökhan AKAY for the interest shown to complete this study successfully.

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TABLE OF CONTENTS

ABSTRACT

Investigation of Cytotoxic Activities of *Terfezia* and *Picoa* Extracts and Molecular Effects on Cancer Cell Lines

Kochar Khasro SALEH

Doctoral Thesis

FIRAT UNIVERSITY
Graduate School of Natural and Applied Sciences

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June 2022, Page: x + 72

The cytotoxic and apoptosis-inducing activities of *Terfezia* and *Picoa* species were investigated on three different cancer cell lines. Human pancreas adenocarcinoma cell line (PANC-1), human breast adenocarcinoma cell line (MDA-MB 231), and human lung adenocarcinoma cell line (A549) were used. MTT assay was applied for detecting the cytotoxic activity of water, methanol, and acetone extracts, with exposure of each cancer cell line to concentrations of 400, 200, 100, and 50 µg/well. Hoechst 33342 (HO) and Propidium Iodide (PI) stains were applied for differentiation between necrotic and apoptotic cancer cells. Four target genes; *BAX, Bcl-2, P21, P53*, and one reference gene; *GAPDH* was included. Quantitative real-time PCR (QRT-PCR) was performed to analyze gene expression. In the results, cytotoxic effects of water, methanol, and acetone extracts from *Terfezia claveryi*, *T. boudieri*, *T. olbiensis*, *Picoa juniperi*, and *P. lefebvrei* were determined. The impacts were observed in a dose-dependent manner (400, 200, 100, and 50 µg/ml). HOPI double-stains revealed that the extracts effectively induced apoptosis rather than necrosis in the treated cell lines. *p21, p53*, and *BAX* genes were significantly ($p<0.001$) up-regulated in treated cancer cells by more than 3-fold changes. While the *Bcl2* gene was significantly ($p<0.001$) down-regulated by more than 0.3-fold changes when compared to untreated cancer cells. Finally, the inhibitory effects of *Terfezia* and *Picoa* water extracts on the PANC-1 cell line, MDA-MB 231 cell line, and A549 cell line can be considered functional natural products.

Keywords: *Terfezia*, *Picoa*, Desert truffles, Cytotoxic activity, Apoptosis, QRT-PCR

ÖZET

Terfezia ve *Picoa* Ekstraktlarının Sitotoksik Aktivitelerinin ve Kanser Hücre Hatları Üzerindeki Moleküler Etkilerinin Araştırılması

Kochar Khasro SALEH

Doktora Tezi

FIRAT ÜNİVERSİTESİ
Fen Bilimleri Enstitüsü

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Terfezia ve Picoa mantarlarının sitotoksik ve apoptozu indükleyen aktiviteleri üç farklı kanser hücre hattı üzerinde araştırıldı. İnsan pankreas adenokarsinomu hücre hattı (PANC-1), insan meme adenokarsinomu hücre hattı (MDA-MB 231) ve insan akciğer adenokarsinom hücre hattı (A549) dahil kullanıldı. MTT tahlilitest, su, metanol ve aseton ekstrelerinin sitotoksik aktivitesini saptamak için her kanser hücre hattının 400, 200, 100, 50 ug/kuyucuk konsantrasyonlarına maruz bırakılmasından sonra yapıldı. Nekrotik ve apoptotik kanser hücrelerini ayırt etmek için Hoechst 33342 (HO) ve Propidium Iodide (PI) boyaları uygulandı. Dört hedef gen *BAX, Bcl-2, P21, P53* ve bir referans gen *GAPDH* dahil edilmiştir. Gen ekspresyonunu analiz etmek için kantitatif gerçek zamanlı PCR (QRT-PCR) yapıldı. Sonuçlarda, *Terfezia claveryi, T. boudieri, T. olbiensis, Picoa juniperi* ve *P. lefebvrei*'den su, metanol ve aseton ekstraktlarının sitotoksik etkileri doza bağlı (400, 200, 100, and 50 µg/ml) arıttıgı tespit edildi. HOPI çift boyamaları, ekstraktların uygulanan hücre hattılarında nekrozdan ziyade apoptoza etkili bir şekilde neden olduğunu ortaya çıkardı. *p21, p53* ve *BAX* genleri, uygulanan hücrelerde 3 kattan fazla değişiklikle önemli ölçüde ($p<0,001$) yukarı regüle edildi. *Bcl2* geni, uygulanmamış hücrelere kıyasla 0.3 kattan fazla değişiklikle önemli ölçüde ($p<0,001$) aşağı regüle edildi. Son olarak, *Terfezia* ve *Picoa* su ekstraktlarının PANC-1 hücre hattı, MDA-MB 231 hücre hattı ve A549 hücre hattı üzerindeki inhibitör etkileri, fonksiyonel doğal ürünler olarak kabul edilebilir.

Anahtar Kelimeler: *Terfezia, Picoa,* çöl trüfleri, Sitotoksik aktivite, Apoptoz, QRT-PCR

LIST OF FIGURES

Page

LIST OF TABLES

Page

SYMBOLS AND ABBREVIATIONS

Symbols

$^{2-\Delta\Delta}$CT	: 2ˆ (–delta delta cycle threshold)
%	: Percent
ºC	: Centigrade
ml	: Milliliter
µl	: Microliter
ml	: Milligram
µg	: Microgram
g	: Relative centrifugal force

Abbreviations

DNA	: Deoxyribonucleic Acid
RNA	: Ribonucleic Acid
cDNA	: Complementary Deoxyribonucleic Acid
PCR	: Polymer Chain Reaction
CDK	: Cyclin-Dependent Kinase
DMSO	: Dimethyl sulfoxide
FBS	: Fetal Bovine Serum
gDNA	: genomic DNA
PANC-1	: Human Pancreatic cancer cell line
A549	: Human lung cancer cell line
Bp	: Base pair
HO	: Hoechst 33342
PI	: Propidium Iodide
HOPI	: Hoechst 33342 and Propidium Iodide
IA	: Immunogenic Apoptosis α
PCSCs	: Pancreatic Cancer Stem Cells
FDA	: Food and Drug Administration
EMA	: European Medical Agency
miRNA	: Micro RNA
RTK	: Protein Tyrosine- Kinase
TNF	: Tumor Necrotic Factor
EDTA	: Ethylenediaminetetraacetic acid
TP53	: Tumor Protein P53
BAX	: BCL2 Associated X, Apoptosis Regulator
BCL2	: BCL2 Apoptosis Regulator
PNET	: Pancreatic Neuroendocrine Tumor
MOMP	: Mitochondrial Outer Membrane Permeabilization
MDM2	: Mouse Double Minute 2 homolog
PCD	: Programmed Cell Death
PC-3	: Prostate Cancer cell
PCSCs	: pancreatic cancer stem cells

1. INTRODUCTION

Macrofungi are highly consumed in daily diets due to their highly delectable flavor and aroma, and their nutritional and medicinal properties have long been recognized. Because of their distinct flavor and texture profiles, they are a popular food in many cultures. Edible mushrooms are also well-regarded due to their nutritional effects, and health benefits [1, 2], which have been widely used in middle east countries [3].

Among all edible mushrooms, desert truffles are regarded as one of the oldest food products known for their nutritional value, particularly when compared to fish and meat. They have become a supplement for meat and are eaten in large amounts due to their very nice taste and musky scent, which is one of the oldest types of food [4]. They contain adequate amounts of ascorbic acid, fibers, fats, proteins, and carbohydrates [5]. One of the main factors as to why the world's food products are attractive to truffles is their distinctive aroma. In the Chinese, Greek and Egyptian, and other communities desert truffles were used as a medical food, also in Mesopotamia named the natural miracle [4, 6, 7].

Truffles are hypogeous fruit bodies produced by fungi belonging to the Ascomycetes class. These fungi's hypogeous ascocarps are known as truffles. Forest and desert truffles (arid or semi-arid truffles) are the two types of truffles [8]. Desert truffles are seasonal and important ectomycorrhizal fungi that grow naturally in many countries worldwide. They are an edible type of hypogeous macrofungus that grows without stalks or gills and forms a mycorrhizal symbiosis with annual *Cistaceae* plants, which have been widely used in Middle East Countries [9]. To discover this form of truffles is typically collected by experts who have experiences. The most recognized species of desert truffles are *Terfezia, Delastreopsis, Phaeangium Picoa,Balstonia, Delastria, Leucangium, Tuber,tiramania* and *Mattirolomyces* .

Terfezia species, along with others such as especially *Picoa* and *Tirmania,* are known as desert truffles or locally recognised as "turmas, terfass, al-kamaa, al-fag'a, keme, kumi, domalan, duvberon etc.'' and can be found in many Mediterranean countries [5, 7]. They are common in semi-arid marl-gypsum soils, where it forms mycorrhizal symbioses with several annual and perennial *Helianthemum* species [10]. They are highly valued for their traditional beneficial effects and significant nutritive and medicinal compositions. There are many physiological activities associated with truffles, including antivirus, bacteriostasis, anti-inflammation, anticancer, antioxidation, liver defense, and many more [11, 12]. These biological functions make it possible for truffles to have huge potential for therapeutic strategies [13]. Desert truffles are one of the critical foods, they contain the main significant components, to kill cancer cells and can be used as an essential treatment for cancer therapy [14].

Cancer is the second cause of human death worldwide and the sixth most common disease [15]. It is a condition characterized by uncontrolled cell division, as well as cell growth and proliferation problems [16]. Cancer therapy and diagnosis have long been challenges for humans. Despite collected knowledge and information, cancer therapy and diagnosis are full of difficulties. The obstacles to cancer treatment are evolving in response to novel kinds uncovered by researchers. Each level of progress has resulted in novel cancer therapy strategies, Natural products, especially traditional foods, have been studied in need of more efficient and less toxic new medicines in recent decades, and are seen as an appealing option for pharmaceutical anti-cancer exploration [10]. This technique exemplifies the most recent iteration of cancer cell treatment [11, 12].

Pancreatic cancer remains one of the notable malignancies in the world [17, 18]. The fourth and tenth cause of death in the Unites States and Europe is pancreatic cancer, respectively [19-21]. Also, it represents the seventh leading cause of death in the world, and more than 300,000 deaths per year [22]. It has the worst survival rate, less than 30% in one year. This is because the symptoms of pancreatic cancer do not appear in the beginning [23]. Pancreatic cancer is more likely to grow in men than in women [19, 24]. Even though the exact cause and etiology of pancreatic cancer are not identified, some risk factors are strongly related to it, such as smoking, diet, and particular gene mutation [20, 25-27]. The pancreas is a gland consisting predominantly of exocrine and endocrine cells [28]. Which are giving rise to two main tumor types. The endocrine tumor arises from the portion of endocrine cells, such as pancreatic neuroendocrine tumor (PNET). Exocrine tumors arise from the portion of exocrine cells, such as pancreatic exocrine neoplasms [29, 30]. PDAC is a widespread and lethal malignancy that causes more than 250,000 deaths worldwide annually. It is more than 95% of all pancreatic cancers [31]. Despite intensive attempts, treatments have only given minimal effectiveness for patients with PDAC [32].

Breast cancer, along with lung and colon cancer, is one of the three most common cancers in the world [33]. Breast cancer affects one out of every eight to ten women at some point in their lives . Breast in females is more common than in males, and women show more severe symptoms in breast cancer cases [34]. Sex hormones or gender variations can play significant functions in cancer pathophysiology. At least one of the gender disparities in cancers may be attributed to the different biobehavioral responses to stress [32].

A possible target for cancer therapy is apoptosis, the cell's death machine. The apoptotic progression may be initiated by a wide range of circumstances, together with DNA injury or unnecessary growth. Both intracellular and extracellular signals trigger the apoptotic process [35, 36]. A multitude of strategies is used by cancer cells to avoid apoptosis. Disruption from the typical routes might result in mutated genes, while not designated to boost their expression. Proapoptotic genes, on the other hand, may function as cancer suppressors. Inhibitors and activators were discovered in cancer cell lines outside of their typical spectrum of expression. *BCL-2* expression,

for example, is raised in about half of all human malignancies [37]. To destroy cancer cells, the vast majority of classical anticancer treatments rely on *BCL-2/BAX*-dependent processes. If this system is altered in any manner, medicines trouble and develop inherent chemoresistance. Furthermore, apoptotic faults boost the threshold for chemotherapy or radiation, leading to resistance to such treatments [38, 39]. Apoptotic cell death is caused by two major molecular signing mechanisms. The initial is the intrinsic route, which is triggered from within the cell by (*BCL-2*) protein family members and downstream mitochondrial signals. The extrinsic route, which is initiated from external the cell by proapoptotic ligands that connect with particular cell surface receptors, is the second (DRs) [40]. The extrinsic pathways for apoptosis are defined by the receiving of an extracellular signal that causes PCD in the cell. This route can be used to eradicate damaged or undesirable cells. This intricate route may be separated into parts, where particular chemicals or gene expression can trigger downregulation or overexpression of various steps [41]. Injury or some other factors activates the intrinsic apoptotic path. Oxygen deficiency, DNA damage, and other stressors all have a detrimental impact on the cell's capacity to fulfill its functions. As a result, a stressed cell is determined to cease its life. As a result, a subset of proteins as BH3-proteins becomes activated. This BH3-proteins are a protein family that includes both anti and pro-apoptotic proteins. The activation of these proteins dictates whether or not the apoptotic pathway is triggered. Activating *BAX* and *BAK,* a condition known as Mitochondrial Outer Membrane Permeabilization (MOMP) is induced [42].

Autophagy is a type two planned cell death to perform critical functions in cancer. To date, the dual function of autophagy in cancer growth and inhibition has remained debatable [43]. Nowadays, we have an additional understanding of the autophagy process at the molecular level [44-47]. A set of genes linked to autophagy-related gene (Atg) have been discovered in fungi, plants, and mammals [48, 49]. Analysis of messenger RNA and proteins is commonly used to evaluate gene expression patterns between different types of cells or tissues and under different situations, such as normal and malignant cells [50]. *P53,* the tumor suppressor, plays a vital function in determining cell destiny in normal cells and has been dubbed the "guardian of the genome". The result of *P53* activation is essentially determined by its participation in the transcriptional control of several genes implicated in these responses [51].

In this research cytotoxic activities of *T. claveryi, T. boudieri, T. olbiensis, Picoa juniperi,* and *P. lefebvrei* against PANC-1, MDA-MB 231, and A549 have been studied. In addition, the possibility of inducing apoptosis was investigated in vitro in three different cancer cell lines (PANC-1, MDA-MB 231, A549).

2. SUMMARY OF LITERATURE REVIEW

Hussain et al. (2021) noted the impact of *T. claveryi* extracts on four cancer cells (PC3, MCF-7, HT 29, and U-87) was confirmed [52, 53]. The extracts greatly inhibited the human carcinoma cell lines; this may due to the potency of many bioactive constituents and peptide antibiotics found in *T. claveryi*. Six serial concentrations were applied from 3.125 µg/ml to 100 µg/ml for each cell line and IC50 were showed a significant values (50.3 µg/ml, 72.6 µg/ml, 109.2 µg/ml, and 136.2 µg/ml) respectively for (PC3, MCF-7, HT 29, and U-87) cell lines [9].

Elsayed et al. (2019) investigated the different solvent extracts of *Tirmania nivea* and *Terfezia claveryi* had cytotoxic activity against HepG2 and L929 cancer cell lines [52, 54]. Various solvents were used to extract from various desert truffles in the most recent study (*T. nivea and T. claveryi*) against HepG2 and L929 cancer cell lines, all of the extracts tested showed dose-dependent cytotoxic activity. The toxicity of *T. nivea* and *T. claveryi* hexane extracts was 91.2, and 92.7 percent, respectively, at 1 mg/ml, whereas *T. claveryi* ethyl acetate extract was 94.5 at 1 mg/ml [55].

Cytotoxic properties of *Terfezia* and *Picoa* species (*T.olbiensis*, and *P. lefebvrei*) on (H1299) cells have confirmed in the study of Dahham et al. (2017). The anticancer effects of the species may be due to anticancer properties of *Terfezia* and *Picoa* species. [56]. While methanol extracts of *Terfezia* and *Picoa* species showed the least cytotoxic effects 4.66%, and 12.04% respectively on HUVEC cells [57].

Fidan et al. (2022) showed that MCF 7 cells were more sensitive to *T. boudieri* methanol extracts than *P. lefebvrei and P. juniperi* extracts. *T. boudieri* has been shown to have cytotoxic activity at concentrations of 10–100 ug/ml, about 40%–70%, respectively. The extracts of *P. lefebvrei* and *P. juniperi* had the least cytotoxic effects overall [58].

Ehrlich's ascites carcinoma (EAC) cell line is very susceptible to polysaccharides derived from *Terfezia claveryi*. The cytotoxic activity of *T. claveryi* polysaccharides extracts was 77.6 g/mL after 24 hours and 47.6 g/mL after 48 hours of treatment, respectively, at 20 µg/mL [59].

Other researchers' findings suggested that silver nanoparticles generated by *Terfezia claceryi* have potent anticancer capabilities. At low concentrations of 10 µg/ml, they cause 50% of cancer cell death [60].

Extracts of *T. boudieri* with four different solvents (acetone, aqueous, chloroform, and methanol) were subjected to six tumor cell lines: MCF7, CACO2, HCT116, HEPG2, HELA, and HEP2. The aqueous extracts shown strong antitumor activity against four cell lines: HEP2, HCT116, and MCF7 at 10 µg/ml in 35%, 49%, 64%, and 75%, respectively [61].

A recent study about the benefits of polysaccharide production from edible *Tuber melanosporum* has shown this product act as a significant anticancer agent against A549, and

HepG2 cell line at the concentration (0.005, 0.05, and 0.5 mg/mL) in the inhibition ratio of 59.2% and 92%, respectively [62].

The anti-cancer and anti-inflammation properties of *Tuber magnatum* and *Tuber aestivum* were demonstrated in a study conducted by Ivana N. Beara and her colleagues. It is because of the phenolic and flavonod compounds found in black and white truffles. Cytotoxic activity against tumor cell lines (HT-29, MCF7, and HeLa) was demonstrated at serial doses ranging from 0.1 to 1000 ug/mL, with IC50 values of (34.5 g/mL, 447.4 g/mL, and 34.6 g/mL, respectively) [63].

Polysaccharides were extracted from *Tuber fermentation* (TF)and *Tuber fruiting bodies (TFB)*. TF exhibited a strong antitumor activity against HepG2, A549, and HCT-116 cell lines than those from TFB. The percentage of inhibition were 26.5%, 49.2%, 58.9% respectively at concentration 200 ug/ml [64].

The aqueous extracts of *Terfezia gennadii* have potent anticancer properties. The inhibition of cytotoxicity against three cell lines (HeLa, HepG2, and MCF-7) was confirmed at six different concentrations (400, 200, 100, 50, 25, 12.5 µg/mL)with IC50 ranges of 19–78, 33–301, 83–321 and 102–321 g/mL, respectively [65].

Proteins, fibers, fatty acids, minerals, amino acids, and carbohydrates are abundant in desert truffles. Furthermore, they are high in anti-bacterial, anti-cancer, and anti-oxidant compounds that benefit human health [66]. The study was conducted to evaluate the immunomodulatory, and anticancer effects of *Terfezia boudieri* (desert truffle) with methanol solvent at concentrations of 25–0.78 mg/ml against two cell lines MCF-7 and MDA-MB231 with IC50 0.78 mg/ml and 25 mg/ml, respectively [14].

Clitocybe alexandri ethanolic extract, *Lepista inversa* methanolic extract, and *Suillus collinitus* methanolic extract all showed a strong growth inhibitory effect in the examined human tumor cell lines (NCI-H460) by triggering an S-phase cell cycle arrest and raised the rate of apoptotic cells [67].

Flammulina velutipes and *Coprinus comatus* extracts revealed strong anticancer activity on two cancer cell lines. MCF-7, MDA-MB 231. The promoted apoptosis pathway has inhibitory effects on ER-negative and positive breast cancer cells [68].

Another study found that extracts from *Terfezia claveryi* truffle inhibited brain cancer cells (U87 MG) with an IC50 of 50ug/ml by strongly promoting cell apoptosis via the mitochondrial channel and DNA breakage [69].

A novel agent called grifolin was discovered in mushrooms. Grifolin is a physiologically active natural substance isolated from the fresh fruiting bodies of the *Albatrellus confluens*, with the ability to restrict tumor cell growth by induction of apoptosis. Grifolin dramatically inhibited the growth of tumor cell lines, including CNE1, HeLa, and MCF7 cells [70]

3. MATERIALS AND METHODS

3.1. Materials

Wild samples of fresh *Picoa lefebvrei* (Pat.) Maire, *Picoa juniperi Vittad*., *Terfezia boudieri Chatin*, *Terfezia claveryi Chatin* and *Terfezia olbiensis*. were collected from Malatya and Elazığ, Turkey (N 38° 19°- 43° E 038° 19 °- 51°) with an altitude of 690-1375 m, the beginning of March to the end of May (rarely continue until mid-July). The specimen was identified by examining their macroscopic and microscopic features [71-78] (Figure 3.1).

Figure 3.1. Desert truffles "*Terfezia* and *Picoa* species" were collected from different places around the cities of Elazig and Malatya from January to February 2020, in Turkey

3.1.1. Extractions

The *Terfezia* and *Picoa* samples were then mechanically ground using the maceration method [9] after drying in a 35°C oven. To determine the total yield, 10 grams of sample was extracted with 50 ml of solvents (water, methanol, acetone). The solvent was evaporated to isolate and concentrate the extracts. For further study, the extracts were kept at -20°C (Figure 3.2).

Figure 3.2. Different species of *Terfezia claveryi, T. boudieri, T. olbiensis, Picoa juniperi* and *P. lefebvrei* were mechanically ground using the maceration method

3.1.2. Cell lines and culture conditions

The PANC-1, MDA-MB 231, and A549 cell lines were obtained from Inönü University and were cultivated in DMEM (1% Penicillin-Streptomycin, 2 mM L-Glutamine), and (10% heat-inactivate FBS) in 25cm^2 culture flasks. Maintained at 37ºC in a humidified 5% CO_2 (Table 3.1).

Table 3.1. Cancer cell lines

Cell line	Origin	Species	Morphology	Ploidy
PANC-1	Pancreas	Human	Epithelial	Hypertriploid
MDA-MB-231	Breast	Human	Epithelial	Aneuploid
A549	Lung	Human	Epithelial	Hypotriploid

3.2. Methods

The target point of this investigation was to look into the anti-proliferative effects of *Terfezia* and *Picoa* truffle species such as *T. claveryi, T. boudieri, T. olbiensis, Picoa juniperi,* and *P. lefebvrei* , and its impact on the apoptotic genes in (PANC-1), (MDA-MB 231), and (A549).

3.2.1. Design of the study for cytotoxic activity

This section of the study is designed to conduct a comprehensive investigation of the anti-proliferative properties of *Terfezia* and *Picoa* species belonging to *T. claveryi, T. boudieri, T. olbiensis, Picoa juniperi* and *P. lefebvrei* on the PANC-1, MDA-MB 231, and A549 cells (Figure 3.3, Figure 3.4). Cell viability assay: the MTT assay (Thermofisher, Cat. No. M6494) was used to assess cell viability as a previous protocol [79, 80]. In detail, 100 µl aliquots of the pancreatic cancer

cells (PANC-1), (MDA-MB 231), and (A549) suspension (1x 10^5 cells/well) were transferred to 96-well microplates. For 24 hours at 37°C in a humidified 5% CO_2 atmosphere were incubated.

Water, methanol, and acetone extracts were prepared from powders of *Terfezia and Picoa truffles*. *Terfezia claveryi, T. boudieri, T. olbiensis, Picoa juniperi* and *P. lefebvrei* extracts were arranged in DMEM, and the final concentrations were adjusted to 400, 200, 100, and 50 µg/well. All cell lines were incubated with the extracts for 72 hours at 37ºC. MTT was applied at the end of the incubation, and the plate was incubated for another 4 hours. After that, the supernatant was removed, and diluted DMSO (1/4µl) was added to each well. An ELISA multiple reader (KHB ST-360) set to 570 nm was used to calculate the optical density values. The concentration of *Terfezia claveryi, T. boudieri, T. olbiensis, Picoa juniperi* and *P. lefebvrei* extracts that are responsible for 50% inhibition of cancer cell growths (IC_{50}) was determined. The percent live-cell value was calculated according to the following formula:

$$\%\ \text{Live cells} = A_{570}\ \text{of treated cells} / A_{570}\ \text{of control cells} \times 100$$

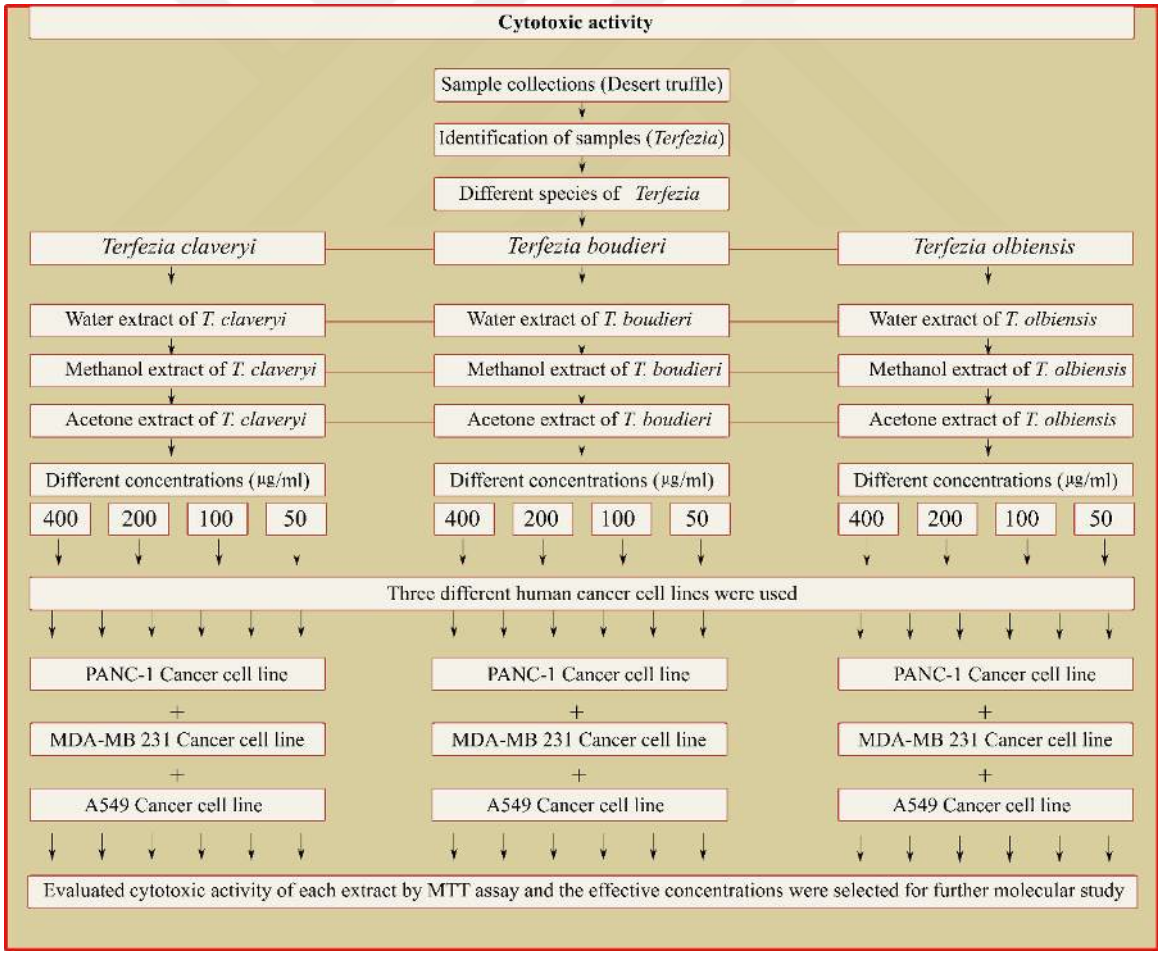

Figure 3.3. Three different extracts were prepared from powdered *Terfezia*, the potency of each extract for cytotoxic activity was observed, and the ability of inhibition against three different cancer cell lines was detected, further, the IC_{50} concentration of water extract was selected

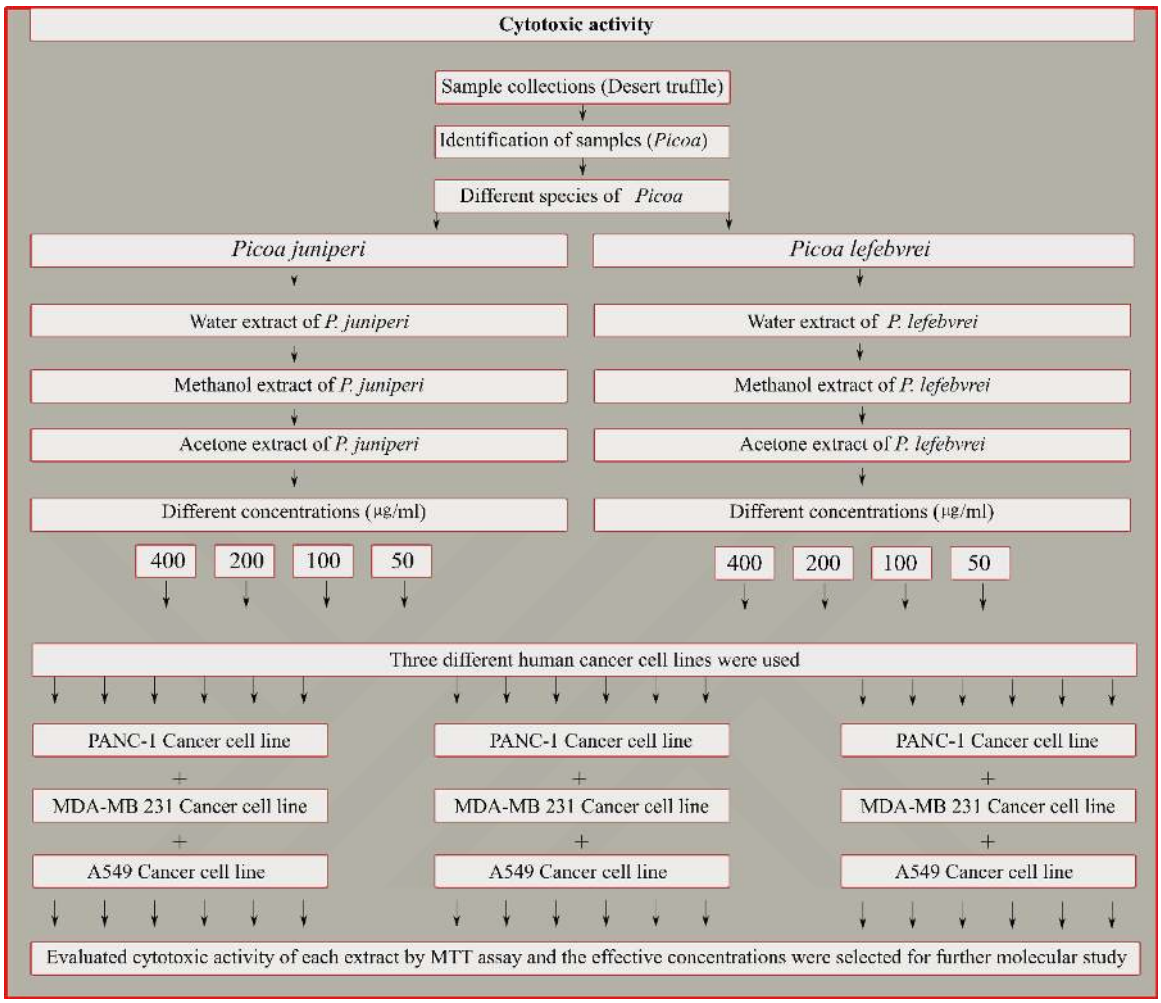

Figure 3.4. Three different extracts were prepared from powdered *Picoa*, the potency of each extract for cytotoxic activity was observed, the ability of inhibition against three different cancer cell lines was detected, further, the IC_{50} concentration of water extract was selected for molecular study

3.2.2. Design of the study for apoptosis and necrosis

This section of research aimed to look into the apoptosis and necrosis induction properties of *Terfezia* and *Picoa* truffle species such as *T. claveryi, T. boudieri, T. olbiensis, Picoa juniperi,* and *P. lefebvrei* on the PANC-1, MDA-MB 231, and A549 cancer cells (Figure 3.5, Figure 3.6).

The molecular processes of apoptosis and necrosis, two primary types of cell death, have been widely explored. Although these two mechanisms of cellular death were once assumed to be mutually incompatible, current results show cellular settings that necessitate a balanced interaction between them [81]. During the progressions of transformation, embryogenesis, pathogenesis, and tissue turnover, undesirable cells are eliminated. Cell death is usually caused by two mechanisms: planned cell death and necrosis [82]. Apoptotic and necrotic activities of extracts on pancreatic cancer cell line (PANC-1), MDA-MB 231, A549 cells were evaluated using the double staining

Hoechst 33342 (Cat. No.: HY-15558A/CS-7568), and Propidium Iodide (Cat. # HY-D0815/CS-7538) technique according to the standard methods [83, 84].

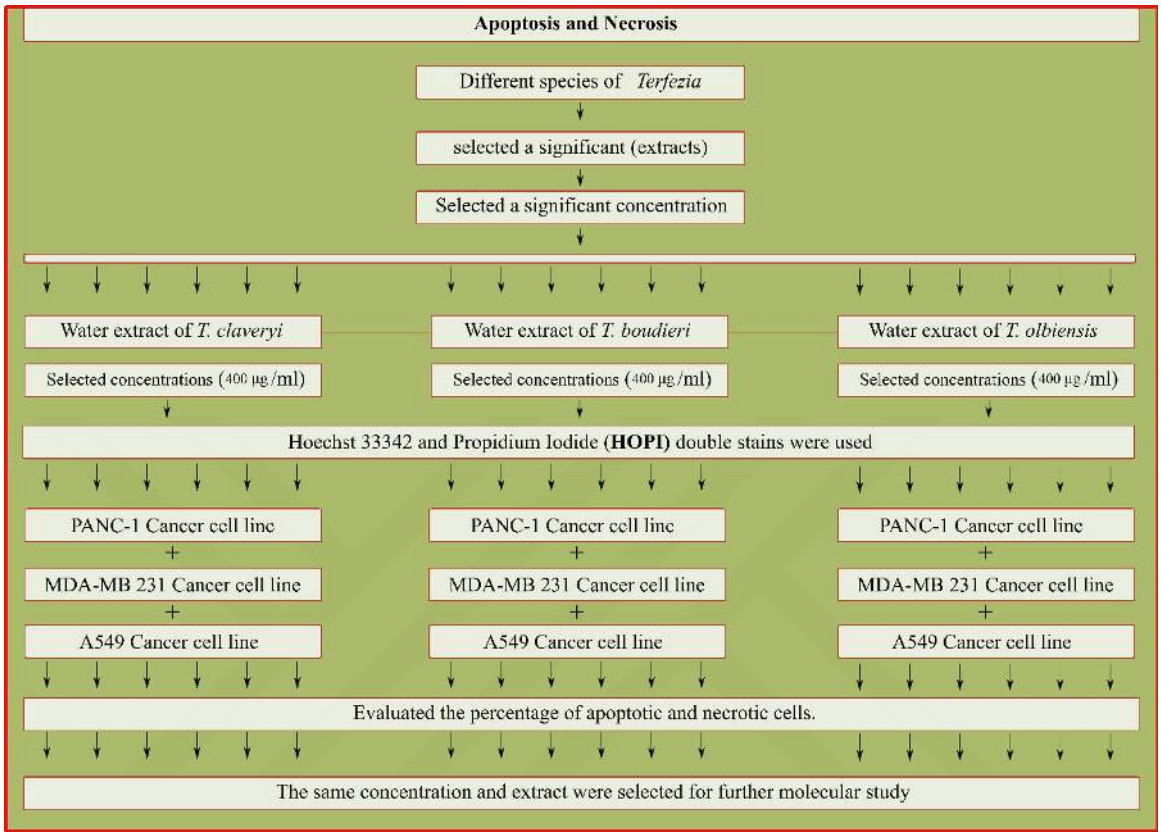

Figure 3.5. Water extract from *Terfezia claveri, T. boudieri,* and *T. olbiensis* was selected for double staining (HOPI), the potency of water extract for cytotoxic activity was showed a high ability of inhibition against (PANC-1, MDA-MB 231, A549) at a specific concentration

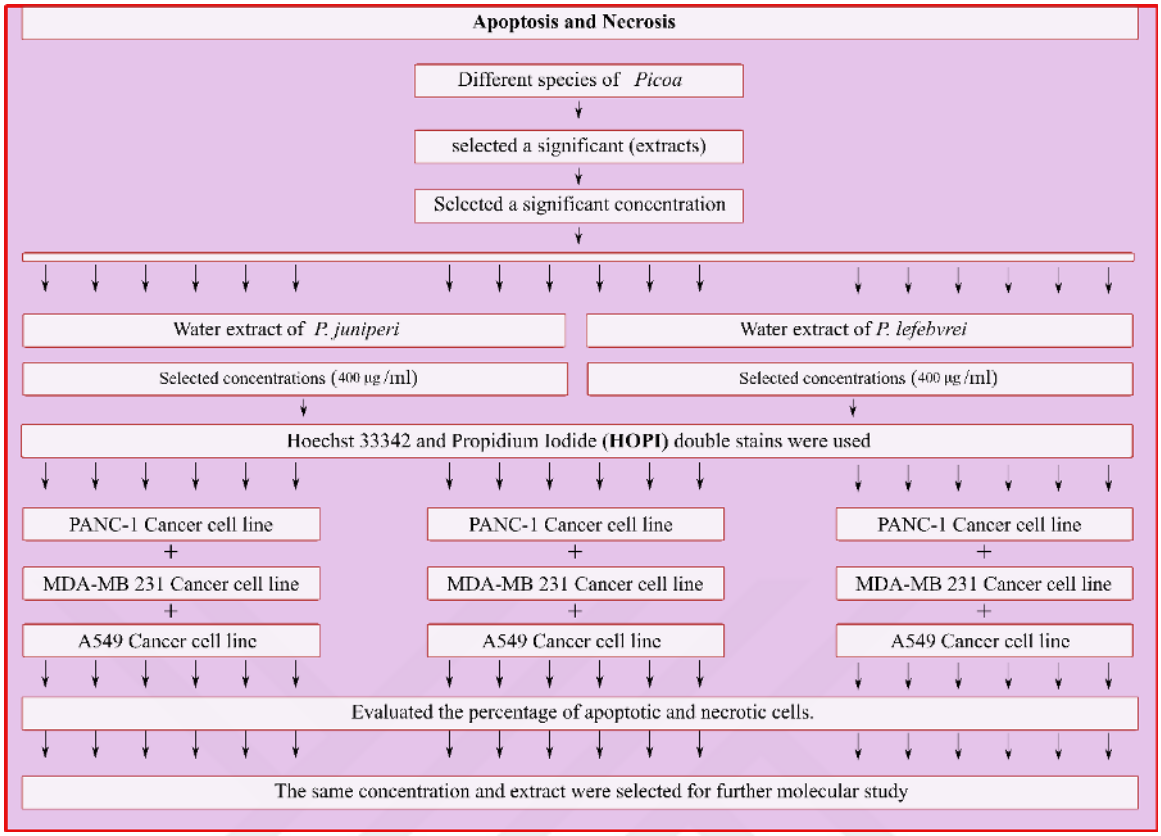

Figure 3.6. Water extract from *Picoa juniperi,* and *P. lefebvrei* was selected for double staining (HOPI), and the potency of water extract for cytotoxic activity showed a high ability of inhibition against three (PANC-1, MDA-MB 231, A549) at a specific concentration

3.2.3. Design of the study for gene expression

In this section, the effects of *Terfezia* and *Picoa* species belonging to *T.* In this section, the effects of *Terfezia* and *Picoa* truffle species belonging to *T. claveryi, T. boudieri, T. olbiensis, Picoa juniperi,* and *P. lefebvrei* on gene expression in the PANC-1, MDA-MB 231 and A549 cells (Figure 3.7, Figure 3.8). The design of the study started by collecting *Terfezia* and *Picoa* samples then cytotoxic activities were evaluated on the three different cancers: human pancreatic, breast, and lung cancer cells. For evaluation of the impacts of extracts on the gene expression, the first RNA was extracted from cancer cell lines by a special kit. The extracted RNA was measured by running gel electrophoresis with Qubit measurement. cDNA was done from the collected RNA samples after that gene expression was done by quantitative RT-PCR.

Total RNA extraction was obtained from (PANC-1, MDA-MB 231, A549 cells) using RNA Mini Kit (Geneaid-ISO 9001) according to the manufacturer's protocol. Our previous study [85], determined the good quality of RNA. The yields of extracted RNA were evaluated by Qubit® 2.0 Fluorometer, Qubit™ RNA HS Assay kit (REF: Q32852, LOT 1913928), and gel electrophoresis, by analysis image system (iBright™750). cDNA was synthesized from total RNA using reverse

transcription (Applied Biosystems High-Capacity cDNA Reverse Transcription Kit, Lot 00754387, Lithuania).

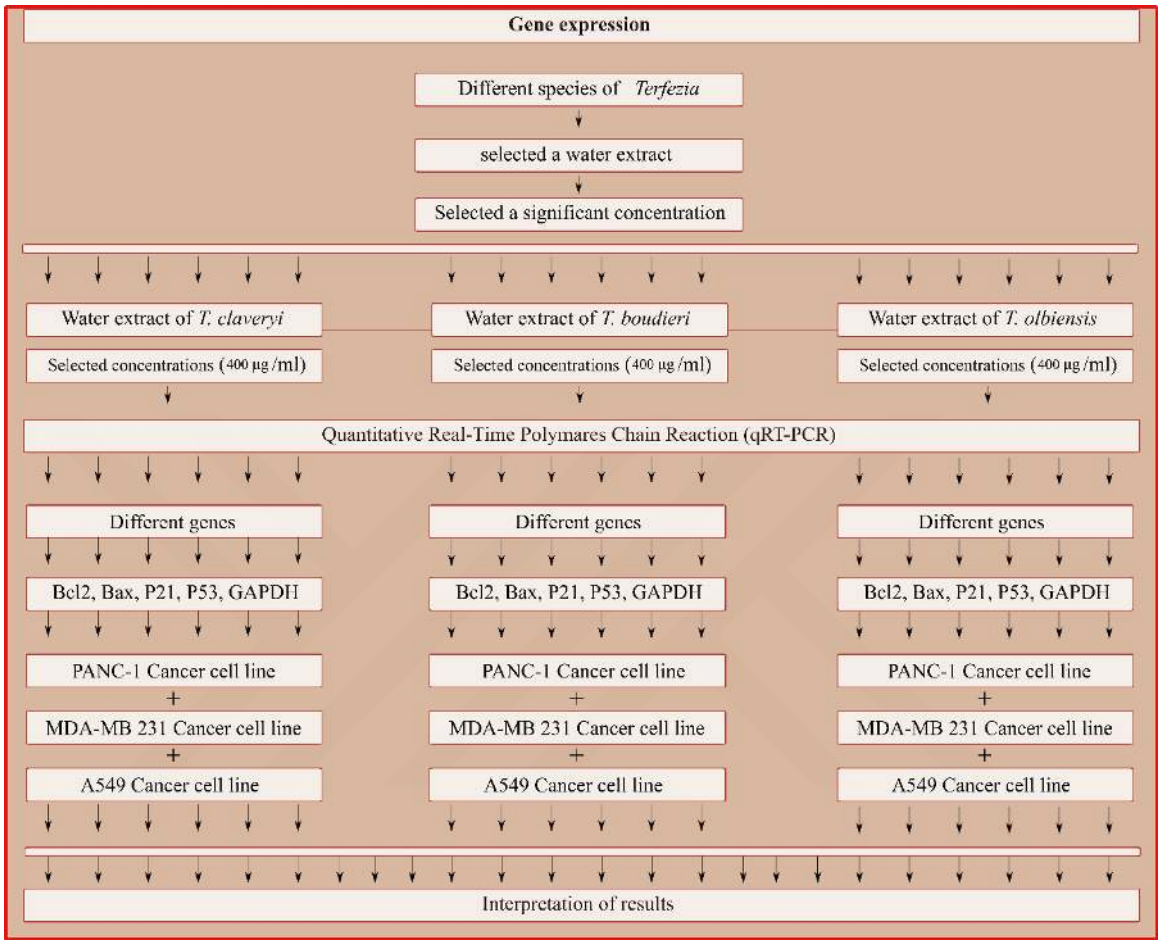

Figure 3.7. Water extract and a significant concentration from *Terfezia claveri, T. boudieri,* and *T. olbiensis* were chosen for observation of its effects on molecular mechanisms in (PANC-1, MDA-MB 231, A549)

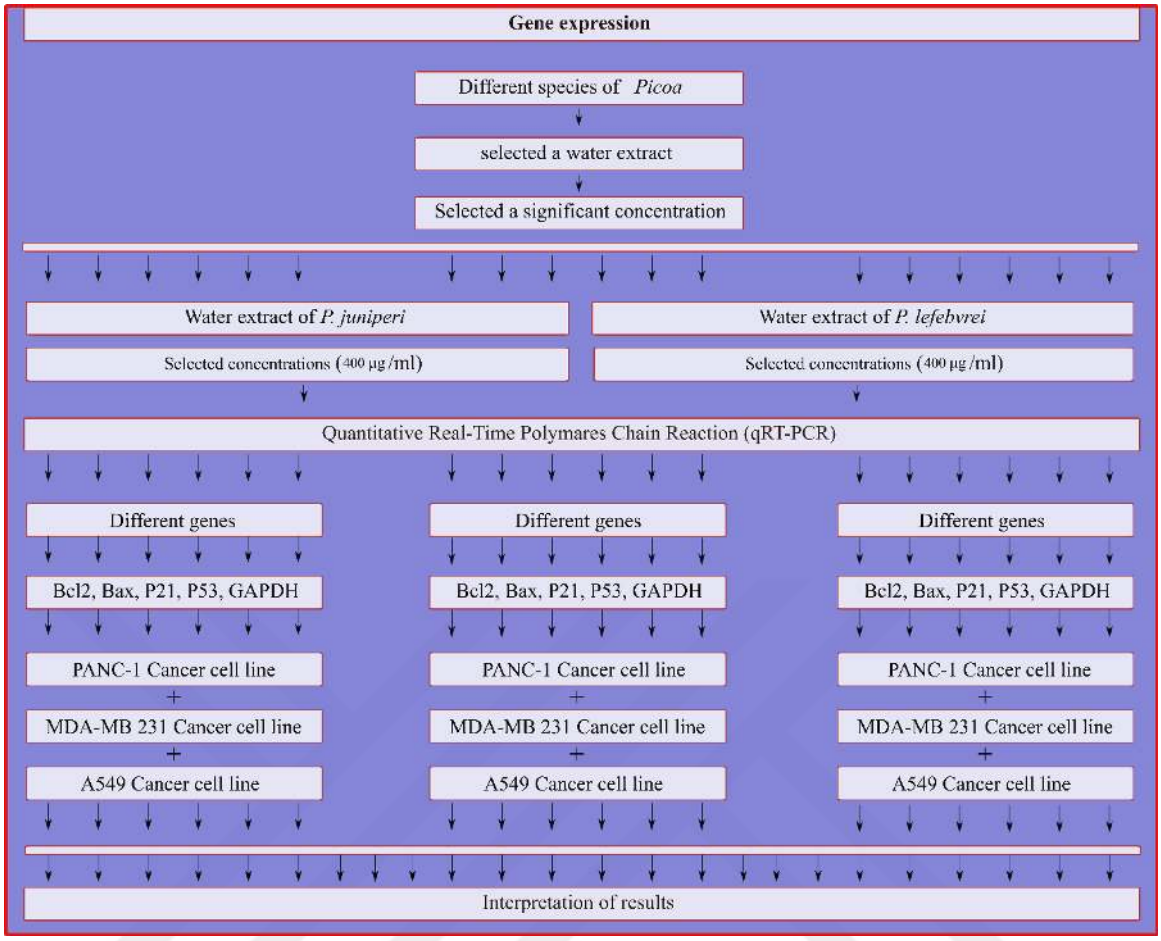

Figure 3.8. Water extract and a significant concentration from *Picoa juniperi,* and *P. lefebvrei* were chosen for observation of its effects on molecular mechanisms and gene expression in (PANC-1, MDA-MB 231, A549)

3.3. Quantitative real-time PCR

QRT-PCR was performed using the SYBR Green master mix (AM02-020) to regulate the expression levels of four target genes: *BAX, Bcl-2, P21,* and *P53*. The StepOnePlus® Real-Time PCR System (Applied Biosystems, Singapore) was carried out in 10 µl, including 5 µl of 2x Amplifyme SG Universal Mix), 0.3µl forward primer, 0.3µl reverse primer, 0.2µl 50X High ROX Solution, 2.2µl PCR grade water, and 2 µl cDNA sample. The program was set to run for one cycle at 95°C for 3 min, followed by 40 cycles of the following protocol: 95°C for 5s, 60°C for 10s, and 72°C for the 20s, as shown in Table 3.6, Table 3.7 and Table 3.8. *GAPDH gene* was used as an internal control, and untreated cancer cell lines were used as external control. Agarose gel electrophoresis was used to confirm the accuracy of PCR amplification. The primer sequences are shown in Tables 3.2. The standard $2^{-\Delta\Delta CT}$ technique was used for data analysis.

Table 3.2. The sequence of genes primers for qRT-PCR

Gene name	Primer sequences	
Bcl2	Forward (5` to 3`)	GAA GGT TTC CTC GTC CCT GG
	Reverse (5` to 3`)	CTG TGT TGA AAC AGG CCA CG
Bax	Forward (5` to 3`)	CCC CGA TTC ATC TAC CCT GC
	Reverse (5` to 3`)	GAG CTA GGG TCA GAG GGT CA
P21	Forward (5` to 3`)	GCT TCA TGC CAG CTA CTT CC
	Reverse (5` to 3`)	CCC TTC AAA GTG CCA TCT GT
P53	Forward (5` to 3`)	GCT GCT CAG ATA GCG ATG GTC T
	Reverse (5` to 3`)	CAT CCA AAT ACT CCA CAC GCA A
GAPDH	Forward (5` to 3`)	GAA GGT GAA GGT CGG AGT C
	Reverse (5` to 3`)	GAA GAT GGT GAT GGG ATT TC

3.3.1. Agarose Gel Electrophoresis

Following the collection of genomic RNA, Agarose gel electrophoresis was used to validate the amount and integrity of the extracted RNA. As seen in Figure 3.9 and Table 3.3.

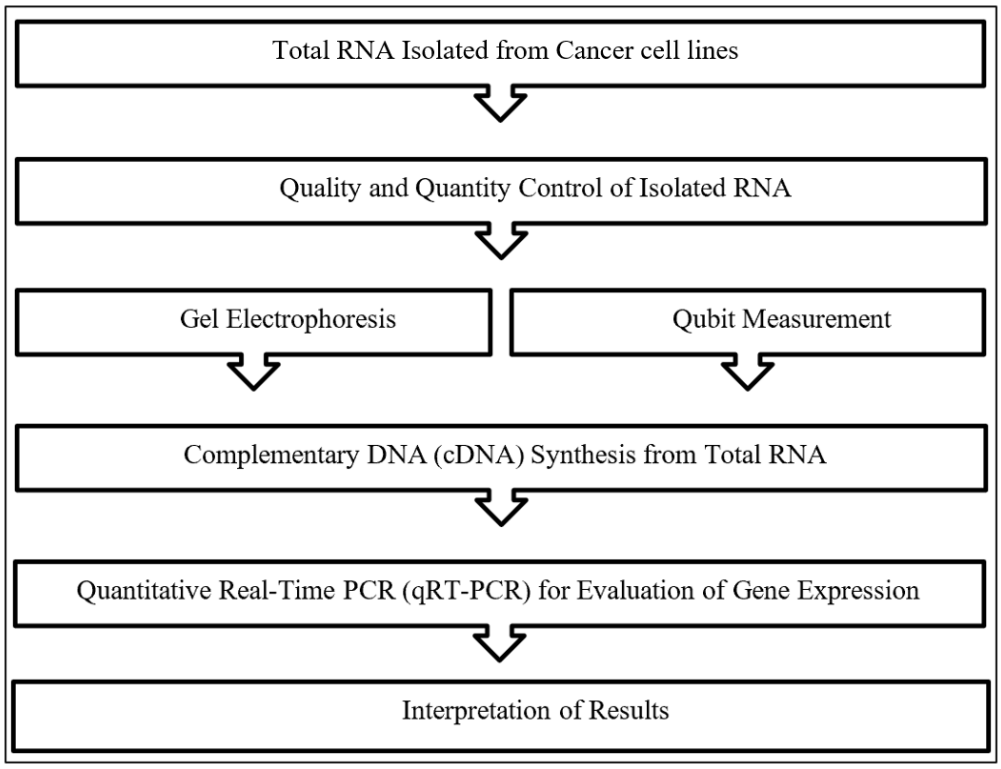

Figure 3.9. The quality and quantity of total RNA extracted from cancer cells (PANC-1, MDA-MB 231, A549) were evaluated by gel electrophoresis and qubit measurement

Table 3.3. Materials for gel electrophoresis

Materials	Amounts
Agarose	1.0 gr
Tris/Boric Acid/EDTA (1xTBE) loading buffer	100 ml
Red Safe	8 µL
6× loading dye	2 µL
RNA Ladder Marker (1kb)	3 µL

3.3.2. Preparation of Agarose gel

Weight 1.0 g Agarose powder by electron balance and put in a conical flask, then 100ml 1 × TBE buffer was added. Swirled gently and put in microwave for 2-3 minutes until the Agarose has dissolved. Allowed the Agarose solution to cool under running the tap water, and then the gel apparatus was constructed.

3.3.3. RNA Loading and Electrophoresis Running

On the paraffin paper, 2µl 6× loading dye was put and 5µl of RNA samples were added with mixing well by pipette. The mixture was loaded into the gel with a 3 µl control ladder of 1kb. The voltage machine was set at 90 V and run for 30 min. After running ended, the gel electrophoresis machine used a UV transilluminator given the photo of the gel. Then the result of RNA running was analyzed.

3.3.4. Qubit RNA Concentration Measurement

For standards and samples, 0.5 ml tubes were set with labeling all-tube lids according to the samples The Qubit® RNA HS Assay requires 2 standards. For each standard tube 190 µL of Qubit working solution was added and 10 µL of each Qubit standard to the appropriate tube, then mixed by vortexing for 2–3 seconds. For samples tubes, 198 µL of the working solution was added with 2 c of the RNA sample. the correct volume of Qubit® working solution with samples RNA became 200 µL. After vortexing for a few seconds allowed all tubes to incubate for two min at room temperature. Finally, standards and samples were read

3.3.5. cDNA Reverse Transcription

cDNA synthesis from total RNA extracted from (PANC-1, MDA-MB 231, A549) cells by cDNA Reverse Transcription Synthesis Kit (Cat. No.4368814). The volumes were determined according to the lowest RNA Molar concentration amount between the samples (10 ng/ µl). The

calculations were done and the RNAs were adjusted to (10 ng/ µl) final volume. At first 2X RT master mix was prepared according to sample number. For each sample the cDNA was done like this: Nuclease-free H_2O (4.2 µl), 10X RT Random Primers (2.0 µL) were added then 10X RT Buffer (2.0 µL), 25X dNTP Mix (100 mM) (0.8 µL) and MultiScribe™ Reverse Transcriptase (1.0 µL) was added to the RNA sample. The total volume is 20 µL per every 0.5 tube. As shown in Table 3.4 and Table 3.5.

Table 3.4. Component of the cDNA Reverse Transcription

Component	**Reaction Volume Without RNase inhibitor**
10X RT Buffer	2.0 µL
25X dNTP Mix (100 mM)	0.8µL
10X RT Random Primers0	2.0 µL
MultiScribe™ Reverse Transcriptase	1.0 µL
Nuclease-free H2O	4.2 µL
Total per reaction	10µL

Table 3.5. Program of the thermal cycling conditions

Setting	**Step1**	**Step 2**	**Step 3**	**Step.4**
Temperature	25°C	37 °C	85°C	25°C
Time	10 minutes	120 minutes	5 minutes	∞

Table 3.6. Quantitative RT- PCR reaction mixture content

Reagent	**Suggested amount per reaction**
2x AMPLIFYME SG Mix	10µ
Forward primer (10µM)	0.6 µM (0.3 µM)
Reverse primer (10µM)	0.6 µM (0.3 µM)
50x ROX solution	0.4µl
DNA or cDNA template	1 – 100 ng
PCR-grade water	fill up to 20 µl

Table 3.7. Three-step thermal cycling profile

Step	Temperature	Time	Cycle
Activation and denaturation	95°C	180 s	
Denaturation	95°C	5 s	35 – 45 cycles
Annealing	60°C	10 s	
Extension/Fluorescence Detection	72°C	5 – s	
Melt curve analysis	according to the qPCR instrument manual		

Table 3.8. Two-step thermal cycling profile

Step	Temperature	Time	Cycle
Activation and denaturation	95°C	180 s	
Denaturation	95°C	5 s	35 – 45 cycles
Annealing/Extension/ Fluorescence Detection	60°C	15 – 30 s	
Melt curve analysis	according to the qPCR instrument manual		

3.4. Statistics analysis

GraphPad Prism (version 8), Inkscape (version 0.92.4), and SPSS (version 22) software were applied. A student t-test was performed, and values of $p < 0.01$ were considered significant.

4. RESULTS AND DISCUSSION

The results of this dissertation are divided into three sections, each of which has been discussed independently. The results of cytotoxic activities of different extracts (water, methanol, and acetone) from the distinct species of *Terfezia* (*T. claveryi, T. boudieri,* and *T. olbiensis*) and *Picoa* (*P. juniperi* and *P. lefebvrei*) have been explained in the first section of this chapter; in the second section, the possibility of water extracts from all species of *Terfezia* and *Picoa* for inducing apoptosis and necrosis in PANC-1, MDA-MB 231, and A549 cancer cell lines has been discussed; in the third section, the impacts of *Terfezia* and *Picoa* extracts on genes associated apoptosis have been discussed. In addition, some further analysis was performed, and the results were compared to the literature reviews.

4.1. Cytotoxic activity

Traditional medicinal mushrooms are often used to cure ailments for centuries in local societies. Nonetheless, it is now used as a primary health strategy for around eighty-five percent of the world, and as a basis of drug detection, with eighty percent of all synthetic drugs coming from traditional medicine [86]. To our knowledge, few studies have shown that *Terfezia* and *Picoa* have therapeutic properties, and no research has been done to establish the effects of *T. claveryi, T. boudieri, T. olbien*sis, *P. juniperi.* and *P. lefebvrei* extracts on genes associated with apoptosis in human cancer cell lines. In this study, anticancer activities of *T. claveryi, T. boudieri, T. olbien*sis, *P. juniperi.* and *P. lefebvrei* extracts were investigated. The impacts of water extract on the relative expression of selected genes associated with apoptosis in the PANC-1, MDA-MB 231, and A549 cell lines were observed.

4.1.1. Cytotoxic activity of *Terfezia*

One of the key qualities for evaluating the therapeutic properties of desert truffles is cytotoxic activity. The determination of cytotoxic activity is a common method for assessing the biological activity of natural products and *Terfezia* [9, 87]. To assess the impact of *T. claveryi*, *T. boudieri*, and *T. olbiensis* extracts on the apoptotic pathway in the human cancer cells, first the cytotoxic activities of *T. claveryi, T. boudieri,* and *T. olbiensis extracts* were evaluated. The cytotoxic activity of water, methanol, and acetone extracts from each species of *Terfezia* (*T. claveryi, T. boudieri,* and *T. olbiensis*) was determined using the MTT assay as described previously [79].

PANC-1 cells were treated for 72 hours with different concentrations of *T. claveryi* water, methanol, and acetone extracts (from 400 to 50 µl//ml). The cytotoxic effects of extracts on PANC-1 cells have been confirmed. The results, as shown in Figure 4.1. Water, methanol, and acetone of

T. claveryi extracts were shown to have a dose-dependent effect on the survival of pancreatic cancer cells. Extracts at concentrations of 400 µg/ml, and 200 µg/ml had a high and significant cell death rate. The extracts had a mild impact at the 100 µg/ml concentrations, while cell damage at the doses of 50 µg/ml did not significantly occur. The half-maximal inhibitory concentration (IC_{50}) values of water, methanol, and acetone extract from *T. claveryi* on PANC-1 cells have shown 156.5 µg/ml, 246.8 µg/ml, and 216.7 µg/ml, respectively as shown in Table 4.1.

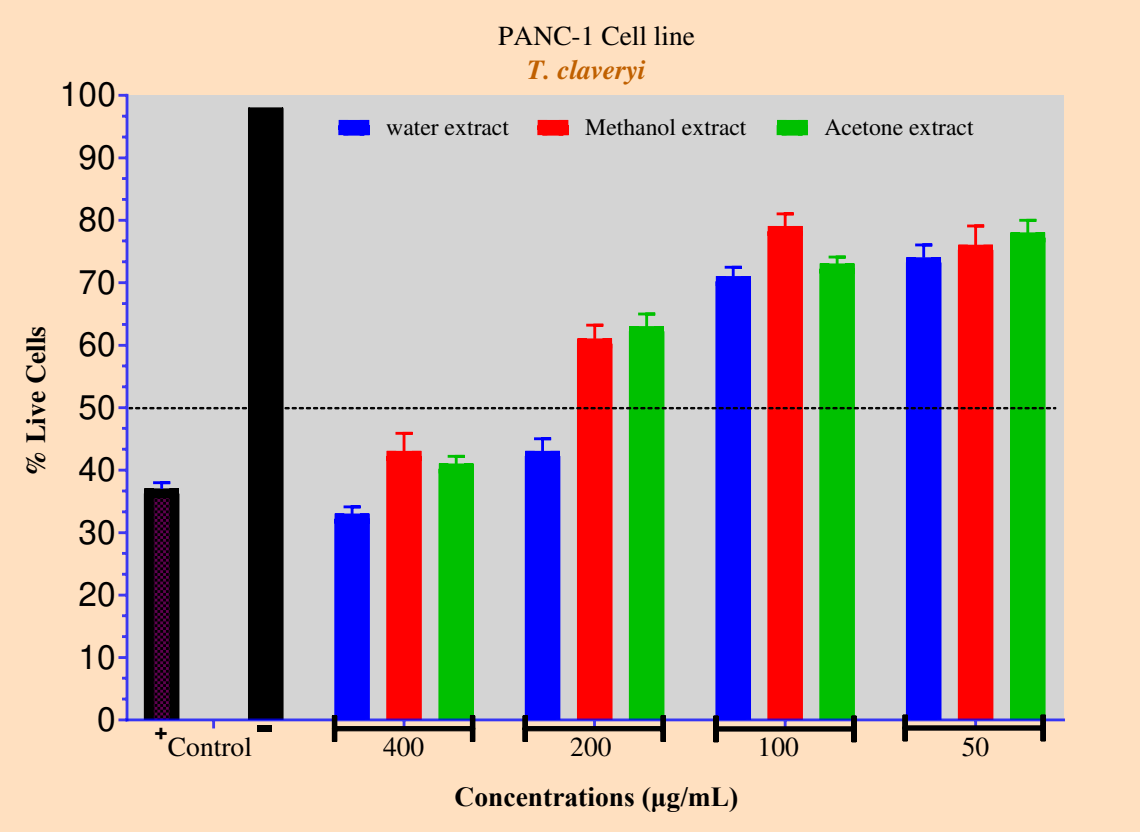

Figure 4.1. Cytotoxic effects of water, methanol, acetone extracts of *Terfezia claveryi* on (PANC-1). Results were obtained by using an MTT assay with four different concentrations of extracts to observe their impacts on pancreatic cancer cells. Controls included untreated (-) and treated (+) cancer cells. Data are expressed as mean±SD

Table 4.1. IC_{50} values of cytotoxic activity of *Terfezia* (*T. claveryi*/PANC-1).

Agent	PANC-1 cells		
	Terfezia Desert truffles		
	T. claveryi		
	Water extract	Methanol extract	Acetone extract
IC_{50}	156.5 µg/ml	246.8 µg/ml	216.7 µg/ml

MDA-MB 231 cells were incubated for 72 hours to various doses of *T. claveryi* extracts in water, methanol, and acetone (from 400 to 50 µl/ml). The cytotoxic effects of extracts on MDA-MB 231 cells have been confirmed. The results, as shown in Figure 4.2. Water, methanol, and acetone extracts of *T. claveryi* could decrease the viability of MDA-MB 231cells in a dose-dependent manner. Extracts at concentrations of 400 µg/ml, and 200 µg/ml had a high and significant cell death rate. The extracts had a mild impact at the 100 µg/ml concentrations, while cell damage at the doses of 50 µg/ml did not significantly occur. The half-maximal inhibitory concentration (IC_{50}) values of water, methanol, and acetone extract from *T. claveryi* on MDA-MB 231 cells have shown 146.9 µg/ml, 226.2 µg/ml, and 236.7 µg/ml, respectively as shown in Table 4.2.

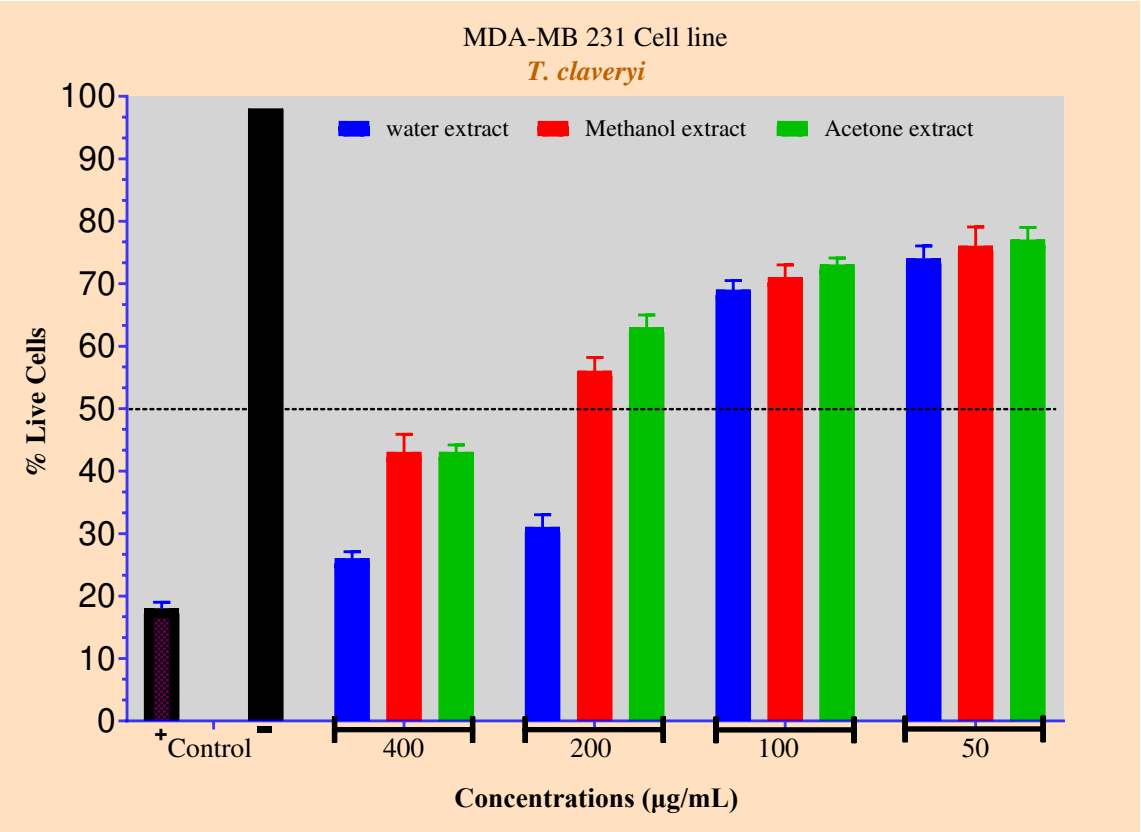

Figure 4.2. Cytotoxic effects of water, methanol, acetone extracts of *Terfezia claveryi* on MDA-MB 231 cells. Results were obtained by using an MTT assay with four different concentrations of extracts to observe their impacts on breast cancer cells. Controls included untreated (-) and treated (+) cancer cells. Data are expressed as mean±SD

Table 4.2. IC_{50} values of cytotoxic activity of *Terfezia* (*T.claveryi*/MDA-MB 231)

	MDA-MB 231 cells		
	Terfezia Desert truffles		
Agent	*T. claveryi*		
	Water extract	Methanol extract	Acetone extract
IC_{50}	146.9 µg/ml	226.2 µg/ml	236.7 µg/ml

Water, methanol, and acetone extract from *T. claveryi* (from 400 to 50 µl/ml for 72 h) were applied to A549 cells at different doses. The cytotoxic effects of extracts on A549 cells have been

confirmed. The results, as shown in Figure 4.3. Extracts of *T. claveryi* could inhibit lung cancer cell viability in a dose-dependent style. Extracts at concentrations of 400 µg/ml, and 200 µg/ml had a high and significant cell death rate. The extracts had a mild impact at the 100 µg/ml concentrations, while cell damage at the doses of 50 µg/ml did not significantly occur. The half-maximal inhibitory concentration (IC_{50}) values of water, methanol, and acetone extract from *T. claveryi* on A549 cells have shown 144.3 µg/ml, 243.4 µg/ml, and 234.8 µg/ml, respectively as shown in Table 4.3.

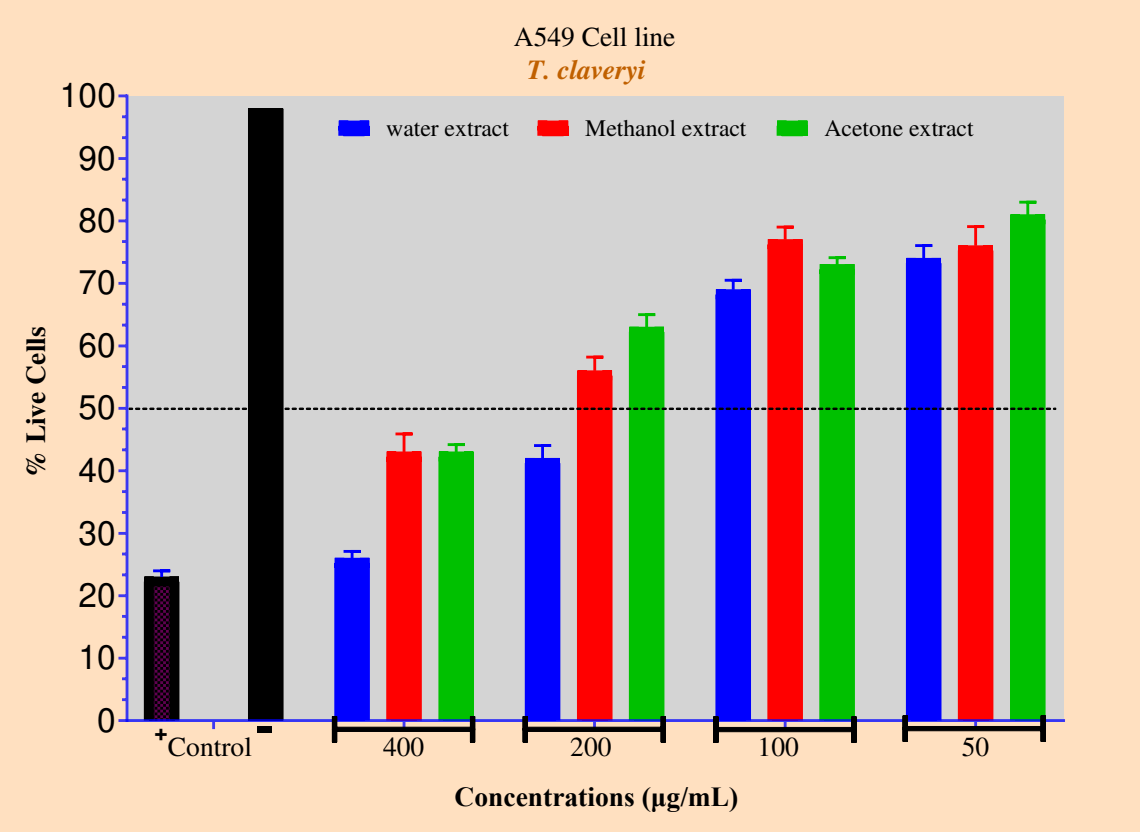

Figure 4.3. Cytotoxic effects of water, methanol, acetone extracts of *Terfezia claveryi* on the human lung cancer cell line (A549). Results were obtained by using an MTT assay with four different concentrations of extracts to observe their impacts on lung cancer cells. Controls included untreated (-) and treated (+) cancer cells. Data are expressed as mean±SD

Table 4.3. IC_{50} values of cytotoxic activity of *Terfezia* (*T. claveryi*/A549)

Agent	A549 cells		
	Terfezia Desert truffles		
	T. claveryi		
	Water extract	Methanol extract	Acetone extract
IC_{50}	144.3 µg/ml	243.4 µg/ml	234.8 µg/ml

The PANC-1 cells were exposed to different concentrations of water, methanol, and acetone extracts from *T. boudieri* (from 400 to 50 µl/ml for 72 h). The cytotoxic effects of extracts on PANC-1 cells have been confirmed. The results, as shown in Figure 4.4. Extracts of *T. boudieri* could reduce the growth of PANC-1 cells. Extracts at concentrations of 400 µg/ml, and 200 µg/ml had a high and significant cell death rate. The extracts had a mild impact at the 100 µg/ml concentrations, while cell damage at the doses of 50 µg/ml did not significantly occur. The half-maximal inhibitory concentration (IC_{50}) values of water, methanol, and acetone extract from *T. boudieri* on PANC-1 cells have shown 139.9 µg/ml, 251.3 µg/ml, and 232.5 µg/ml, respectively as shown in Table 4.4.

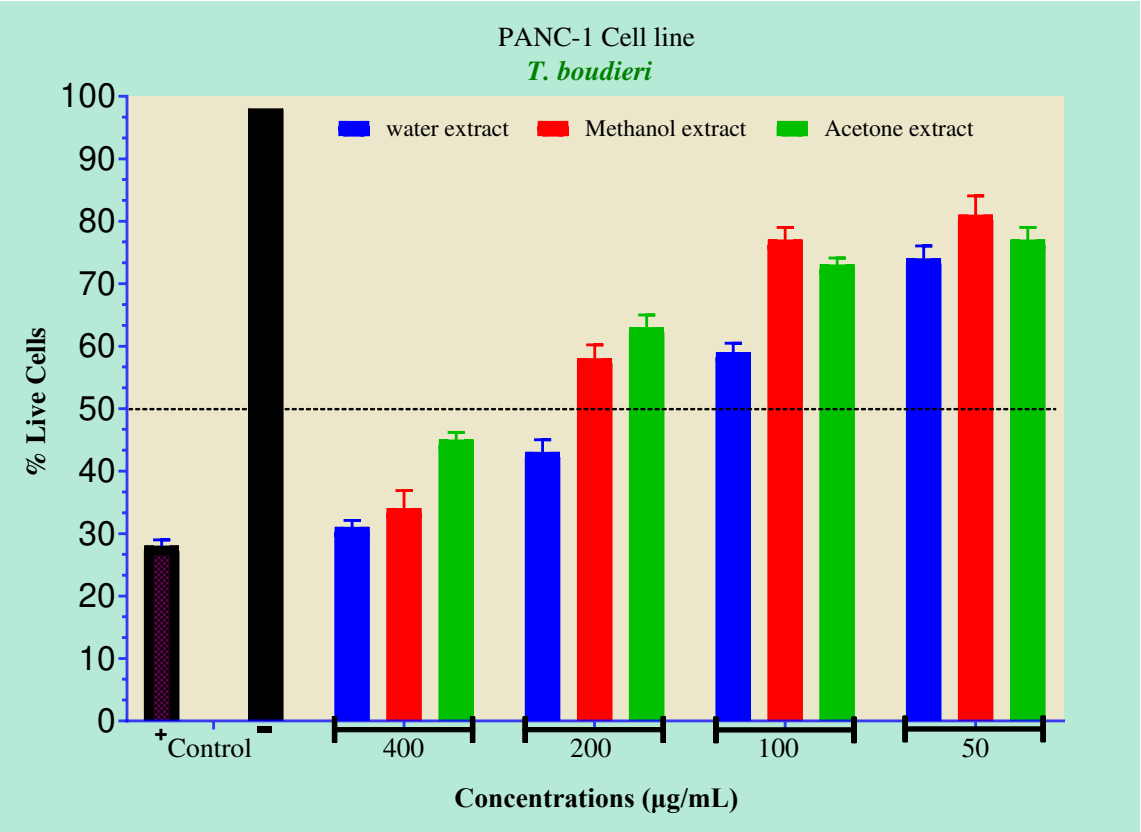

Figure 4.4. Cytotoxic effects of water, methanol, and acetone extracts of *Terfezia boudieri* on (PANC-1). Results were obtained by using an MTT assay with four different concentrations of extracts to observe their impacts on pancreas cancer cells. Controls included untreated (-) and treated (+) cancer cells. Data are expressed as mean±SD

Table 4.4. IC_{50} values of cytotoxic activity of *Terfezia* (*T. boudieri*/ PANC-1)

	PANC-1 cells		
	Terfezia Desert truffles		
Agent	*T. boudieri*		
	Water extract	Methanol extract	Acetone extract
IC_{50}	139.9 µg/ml	251.3 µg/ml	232.5 µg/ml

T. boudieri extracts were given to MDA-MB 231 cells at different concentrations of water, methanol, and acetone (from 400 to 50 µl/ml for 72 h). The cytotoxic effects of extracts on PANC-

1 cells have been confirmed. The results, as shown in Figure 4.5. *T. boudieri* extracts can diminish breast cancer cell proliferation on a dose-dependent basis. Extracts at concentrations of 400 µg/ml, and 200 µg/ml had a high and significant cell death rate. The extracts had a mild impact at the 100 µg/ml concentrations, while cell damage at the doses of 50 µg/ml did not significantly occur. The half-maximal inhibitory concentration (IC_{50}) values of water, methanol, and acetone extract from *T. boudieri* on MDA-MB 231 cells have shown 140.5 µg/ml, 204.3 µg/ml, and 198.5 µg/ml, respectively as shown in Table 4.5.

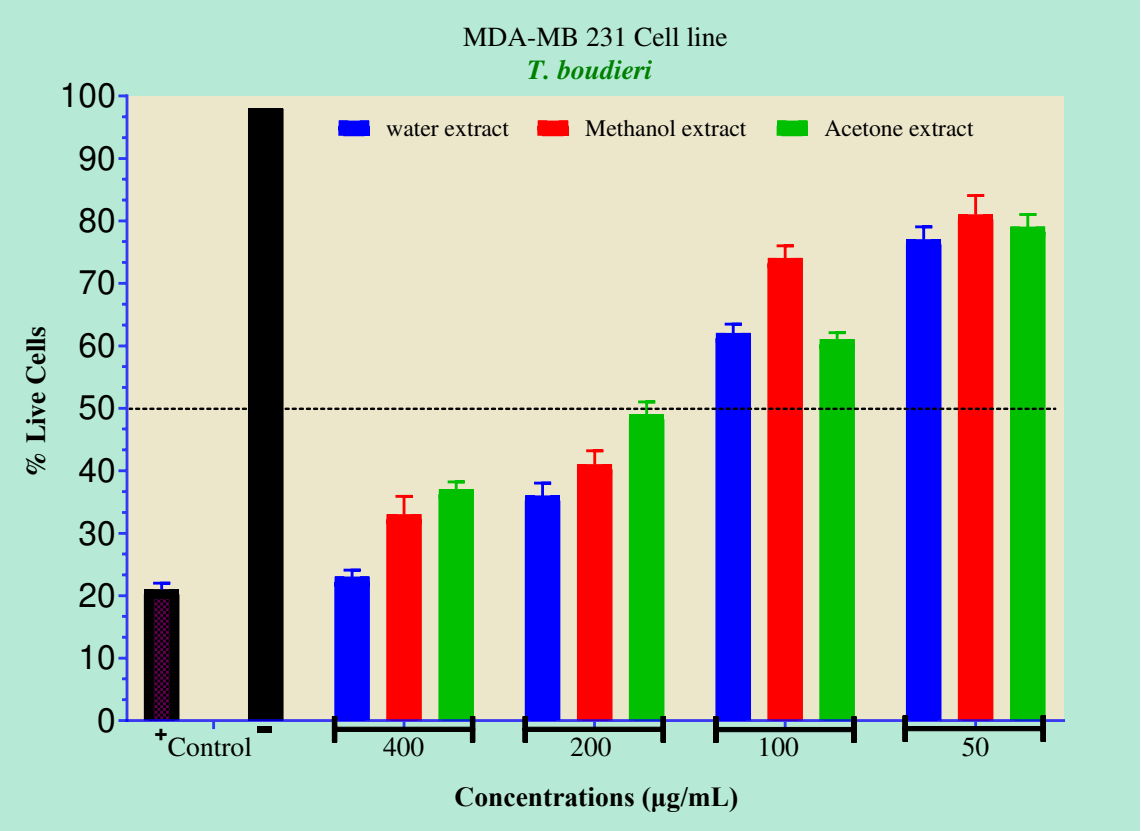

Figure 4.5. Cytotoxic effects of water, methanol, and acetone extracts of *Terfezia boudieri* on (MDA-MB 231). Results were obtained by using an MTT assay with four different concentrations of extracts to observe their impacts on breast cancer cells. Controls included untreated (-) and treated (+) cancer cells. Data are expressed as mean±SD

Table 4.5. IC_{50} values of cytotoxic activity of *Terfezia* (*T. boudieri*/MDA-MB 231)

Agent	MDA-MB 231 cells		
	Terfezia Desert truffles		
	T. boudieri		
	Water extract	Methanol extract	Acetone extract
IC_{50}	140.5 µg/ml	204.3 µg/ml	198.5 µg/ml

The A549 cells were presented to different concentrations of water, methanol, and acetone extracts from *T. boudieri* (from 400 to 50 µl/ml for 72 h). The cytotoxic effects of extracts on A549 cells have been confirmed. The results, as shown in Figure 4.6. Extracts of *T. boudieri* decrease the growth of A549 cells in a dose-dependent manner. Extracts at concentrations of 400 µg/ml, and 200 µg/ml had a high and significant cell death rate. The extracts had a mild impact at the 100 µg/ml concentrations, while cell damage at the doses of 50 µg/ml did not significantly occur. The half-maximal inhibitory concentration (IC_{50}) values of water, methanol, and acetone extract from *T. boudieri* on A549 cells have shown 143.7 µg/ml, 214.6 µg/ml, and 188.1 µg/ml, respectively as shown in Table 4.6.

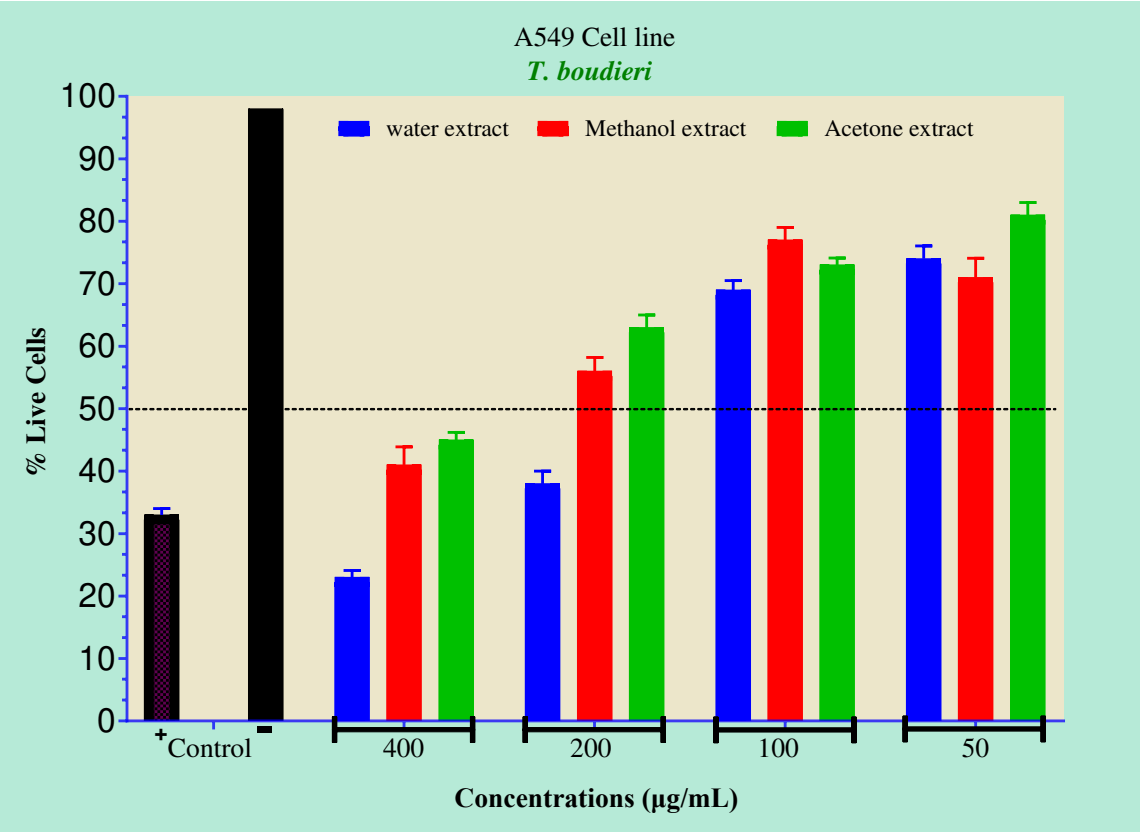

Figure 4.6. Cytotoxic effects of water, methanol, and acetone extracts of *Terfezia boudieri* on the human lung cancer cell line (A549). Results were obtained by using an MTT assay with four different concentrations of extracts to observe their impacts on lung cancer cells. Controls included untreated (-) and treated (+) cancer cells. Data are expressed as mean±SD

Table 4.6. IC_{50} values of cytotoxic activity of *Terfezia* (*T. boudieri*/A549)

	A549 cells		
	Terfezia Desert truffles		
Agent	*T. boudieri*		
	Water extract	Methanol extract	Acetone extract
IC_{50}	143.7 µg/ml	214.6 µg/ml	188.1 µg/ml

The PANC-1 cells were treated with four concentrations of water, methanol, and acetone extracts from *T. olbiensis* (from 400 to 50 µl/ml for 72 h). The cytotoxic effects of extracts on

PANC-1 cells have been confirmed. The results, as shown in Figure 4.7. Methanol, water, and acetone extracts of *T. olbiensisi* can limit the viability of pancreatic cancer cells in a dose-dependent manner. Extracts at concentrations of 400 µg/ml, and 200 µg/ml had a high and significant cell death rate. The extracts had a mild impact at the 100 µg/ml concentrations, while cell damage at the doses of 50 µg/ml did not significantly occur. The half-maximal inhibitory concentration (IC_{50}) values of water, methanol, and acetone extract from *T. olbiensis* on PANC-1 cells have shown 139.2 µg/ml, 231.3 µg/ml, and 212.6 µg/ml, respectively as shown in Table 4.7.

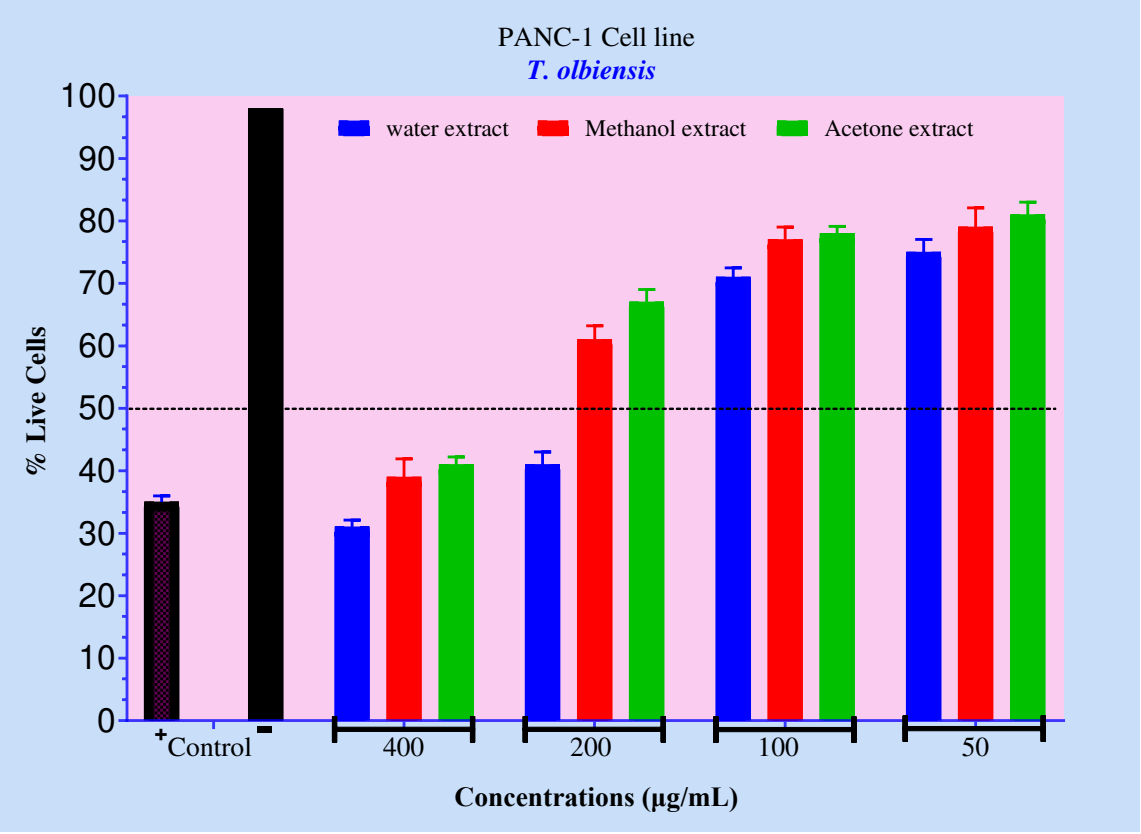

Figure 4.7. Cytotoxic effects of water, methanol, and acetone extracts of *Terfezia olbiensis* on (PANC-1). Results were obtained by using an MTT assay with four different concentrations of extracts to observe their impacts on pancreas cancer cells. Controls included untreated (-) and treated (+) cancer cells. Data are expressed as mean±SD

Table 4.7. IC_{50} values of cytotoxic activity of *Terfezia* (*T. olbiensis*/PANC-1)

Agent	PANC-1 cells		
	Terfezia Desert truffles		
	T. olbiensis		
	Water extract	Methanol extract	Acetone extract
IC_{50}	139.2 µg/ml	231.3 µg/ml	212.6 µg/ml

The MDA-MB 231 cells were exposed to various concentrations of water, methanol, and acetone extracts from *T. olbiensis* (from 400 to 50 µl/ml for 72 h). The cytotoxic effects of extracts on MDA-MB 231 cells have been confirmed. The results, as shown in Figure 4.8. Water, methanol, and acetone extracts of *T. olbiensisi* could reduce the viability of MDA-MB 231cells in a dose-dependent manner. Extracts at concentrations of 400 µg/ml, and 200 µg/ml had a high and significant cell death rate. The extracts had a mild impact at the 100 µg/ml concentrations, while cell damage at the doses of 50 µg/ml did not significantly occur. The half-maximal inhibitory concentration (IC_{50}) values of water, methanol, and acetone extract from *T. olbiensis* on MDA-MB 231 cells have shown 149.9 µg/ml, 231.3 µg/ml, and 207.5 µg/ml, respectively as shown in Table 4.8.

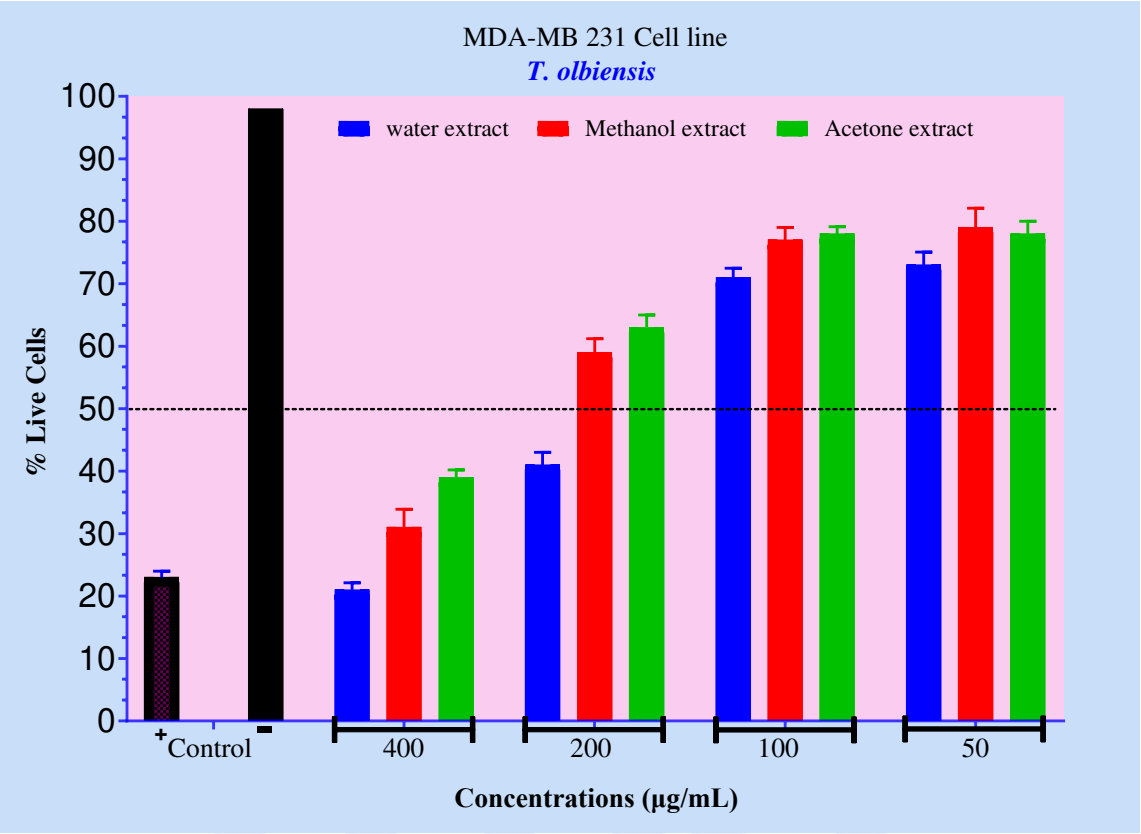

Figure 4.8. Cytotoxic effects of water, methanol, and acetone extracts of *Terfezia olbiensis* on (MDA-MB 231). Results were obtained by using an MTT assay with four different concentrations of extracts to observe their impacts on breast cancer cells. Controls included untreated (-) and treated (+) cancer cells. Data are expressed as mean±SD

Table 4.8. IC_{50} values of cytotoxic activity of *Terfezia* (*T. olbiensis*/MDA-MB 231)

	MDA-MB 231 cells		
	Terfezia Desert truffles		
Agent	*T. olbiensis*		
	Water extract	Methanol extract	Acetone extract
IC_{50}	149.9 µg/ml	231.3 µg/ml	207.5 µg/ml

The A549 cells were subjected to different amounts of water, methanol, and acetone extracts from *T. olbiensis* (from 400 to 50 µl/ml for 72 h). The cytotoxic effects of extracts on A549 cells

have been confirmed. The results, as shown in Figure 4.9. Water, methanol, and acetone extracts of *T. olbiensis* reduce lung cancer cell vitality in a dose-dependent manner. Extracts at concentrations of 400 µg/ml, and 200 µg/ml had a high and significant cell death rate. The extracts had a mild impact at the 100 µg/ml concentrations, while cell damage at the doses of 50 µg/ml did not significantly occur. The half-maximal inhibitory concentration (IC_{50}) values of water, methanol, and acetone extract from *T. olbiensis* on A549 cells have shown 131.5 µg/ml, 211.5 µg/ml, and 231.1µg/ml, respectively as shown in Table 4.9.

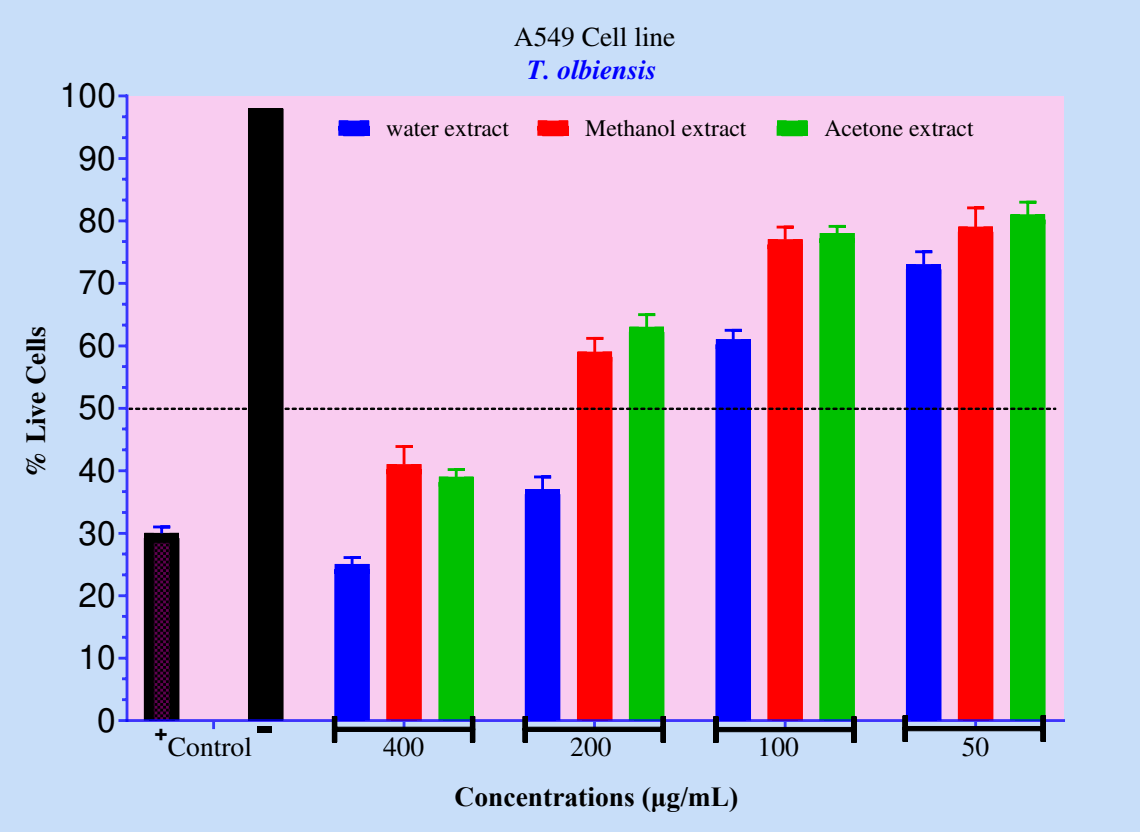

Figure 4.9. Cytotoxic effects of water, methanol, and acetone extracts of *Terfezia olbiensis* on the human lung cancer cell line (A549). Results were obtained by using an MTT assay with four different concentrations of extracts to observe their impacts on lung cancer cells. Controls included untreated (-) and treated (+) cancer cells. Data are expressed as mean±SD

Table 4.9. IC_{50} values of cytotoxic activity of *Terfezia* (*T. olbiensis*/A549)

Agent	A549 cells		
	Terfezia Desert truffles		
	T. olbiensis		
	Water extract	Methanol extract	Acetone extract
IC_{50}	131.5 µg/ml	211.5 µg/ml	231.1 µg/ml

In the present study, significant cytotoxic activities of methanol, water, and acetone extract from *T. claveryi, T. boudieri,* and *T. olbien*sis were observed for PANC-1, MDA-MB 231, A549 cell lines. Our findings showed a promising growth inhibitor effect of the *T. claveryi, T. boudieri,* and *T. olbien*sis extracts. These findings agree with the results of the current previous study, it has shown the cytotoxic effects of ten truffle species that contain phenolic acids and tocopherols [53]. In another previous study, the impact of *T. claveryi* extracts on four cancer cells (PC3, MCF-7, HT 29, and U-87) was confirmed, which supports our findings. The extracts greatly inhibited the human carcinoma cell lines; this may due to the potency of many bioactive constituents and peptide antibiotics found in *T. claveryi* [9]. In addition, apoptosis and/or growth inhibition may lead to decreased cell numbers in treated cancer cell lines. Several apoptosis mechanisms have been clarified, but further studies are still required, particularly in cancer cells. Finally, in the PANC-1, MDA-MB 231, and A549 cell lines, an obvious inhibition in viabilities was observed as early as 48 hours, but IC_{50} was achieved at 72 hours of incubation. The active extract and concentration were water extracts and 400 µg/ml. These extract, concentration, and incubation periods were chosen for further molecular studies.

4.1.2. Cytotoxic activity of *Picoa*

The cytotoxic activities of *P. juniperi* and *P. lefebvrei* extracts were first investigated in PANC-1, MDA-MB 231, and A549 cancer cell lines to assess their effects on the apoptotic pathway. The cytotoxic activity of water, methanol, and acetone extracts was determined using the MTT assay as described previously [79]. For 72 hours, PANC-1, MDA-MB 231 and A549 cancer cells were exposed to different concentrations of *P. juniperi* and *P. lefebvrei* extracts, ranging from 400 to 50 µl/ml.

The PANC-1 cells were subjected to four concentrations of water, methanol, and acetone extracts from *P. juniperi* (from 400 to 50 µl/ml for 72 h). The cytotoxic effects of extracts on PANC-1 cells have been confirmed. The results, as shown in Figure 4.10. Water, methanol, and acetone extracts of *P. juniperi* could inhibit the growth of PANC-1 cells in a dose-dependent style. Extracts at concentrations of 400 µg/ml, and 200 µg/ml had a high and significant cell death rate. The extracts had a mild impact at the 100 µg/ml concentrations, while cell damage at the doses of 50 µg/ml did not significantly occur. The half-maximal inhibitory concentration (IC_{50}) values of water, methanol, and acetone extract from *P. juniperi* on PANC-1 cells have shown 150.5 µg/ml, 241.8 µg/ml, and 226.7 µg/ml, respectively as shown in Table 4.10.

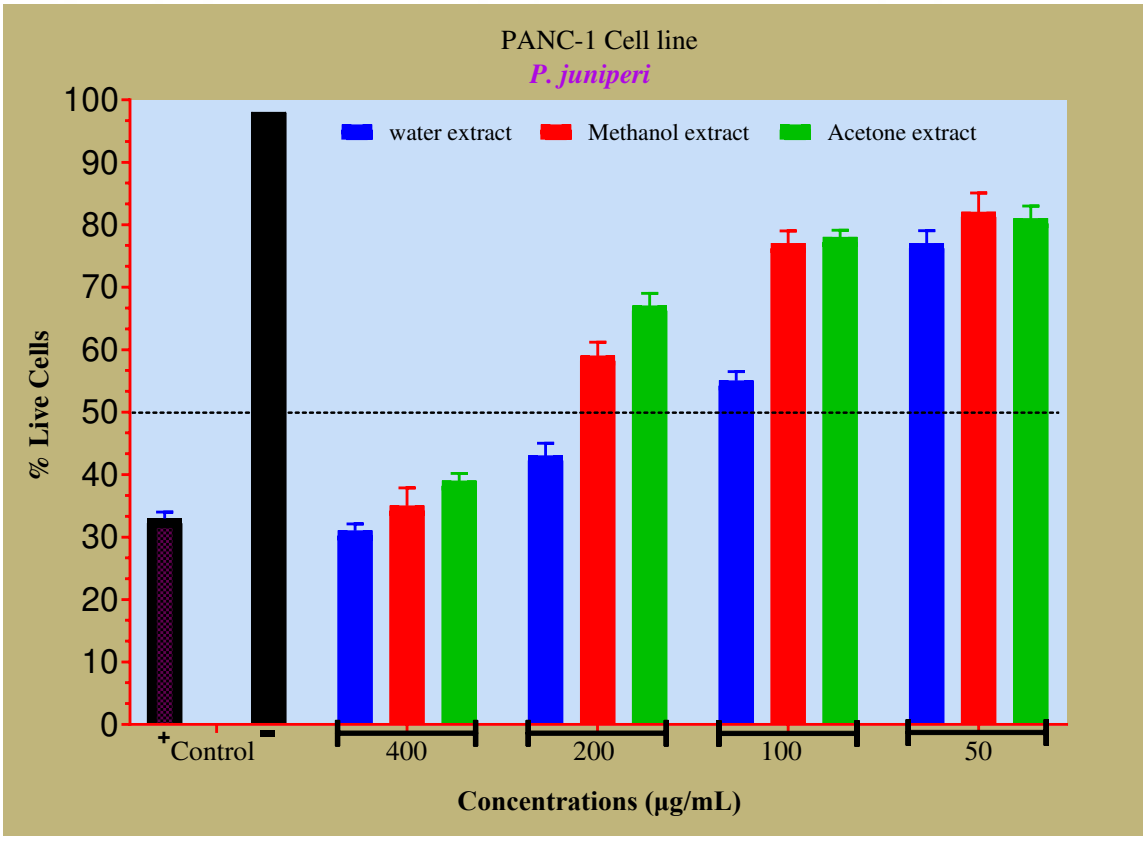

Figure 4.10. Cytotoxic effects of water, methanol, and acetone extracts of *Picoa juniperi* on (PANC-1). Results were obtained by using an MTT assay with four different concentrations of extracts to observe their impacts on pancreas cancer cells. Controls included untreated (-) and treated (+) cancer cells. Data are expressed as mean±SD

Table 4.10. IC_{50} values of cytotoxic activity of *Picoa* (*P. juniperi*/PANC-1)

Agent	PANC-1 cells		
	Desert truffles		
	P. juniperi		
	Water extract	Methanol extract	Acetone extract
IC_{50}	150.5 µg/ml	241.8 µg/ml	226.7 µg/ml

The MDA-MB 231 cells were given to various concentrations of water, methanol, and acetone extracts from *P. juniperi* (from 400 to 50 µl/ml for 72 h). The cytotoxic effects of extracts on MDA-MB 231 cells have been confirmed. The results, as shown in Figure 4.11. Water, methanol, and acetone extracts of *P. juniperi* could decrease the viability of MDA-MB 231 cells in a dose-dependent manner. Extracts at concentrations of 400 µg/ml, and 200 µg/ml had a high and significant cell death rate. The extracts had a mild impact at the 100 µg/ml concentrations, while cell damage at the doses of 50 µg/ml did not significantly occur. The half-maximal inhibitory concentration (IC_{50}) values of water, methanol, and acetone extract from *P. juniperi* on MDA-MB 231 cells have shown 136.5 µg/ml, 240.8 µg/ml, and 217.7 µg/ml, respectively as shown in Table 4.11.

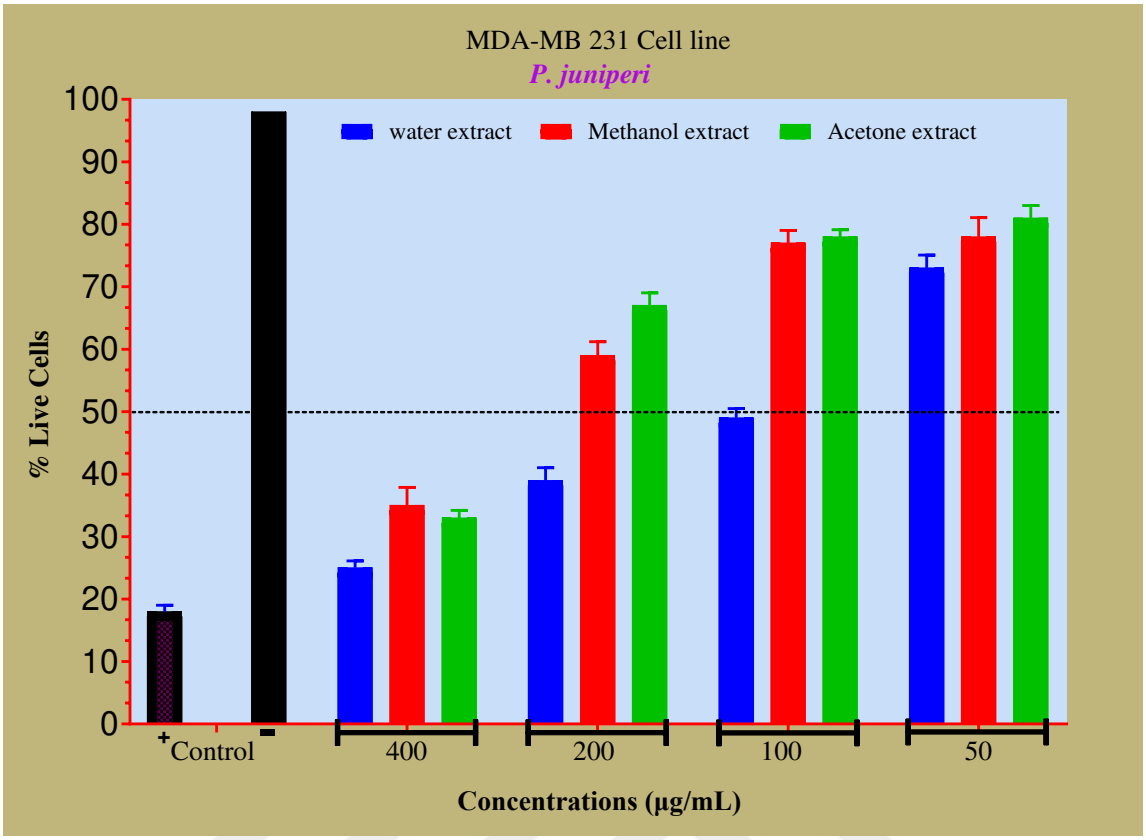

Figure 4.11. Cytotoxic effects of water, methanol, and acetone extracts of *Picoa juniperi* on the human breast cancer cell line (MDA-MB 231). Results were obtained by using an MTT assay with four different concentrations of extracts to observe their impacts on breast cancer cells. Controls included untreated (-) and treated (+) cancer cells. Data are expressed as mean±SD

Table 4.11. IC_{50} values of cytotoxic activity of *Picoa* (*P. juniperi*/MDA-MB 231)

	MDA-MB 231 cells		
	Desert truffles		
Agent	*P. juniperi*		
	Water extract	Methanol extract	Acetone extract
IC_{50}	136.5 µg/ml	240.8 µg/ml	217.7 µg/ml

The A549 cells were exposed to four concentrations of water, methanol, and acetone extracts from *P. juniperi* (from 400 to 50 µl/ml for 72 h). The cytotoxic effects of extracts on A549 cells

have been confirmed. The results, as shown in Figure 4.12. Water, methanol, and acetone extracts of *P. juniperi* could inhibit the viability of A549 cells in a dose-dependent manner. Extracts at concentrations of 400 µg/ml, and 200 µg/ml had a high and significant cell death rate. The extracts had a mild impact at the 100 µg/ml concentrations, while cell damage at the doses of 50 µg/ml did not significantly occur. The half-maximal inhibitory concentration (IC_{50}) values of water, methanol, and acetone extract from *P. juniperi* on A549 cells have shown 155.5 µg/ml, 226.8 µg/ml, and 198.7 µg/ml, respectively as shown in Table 4.12.

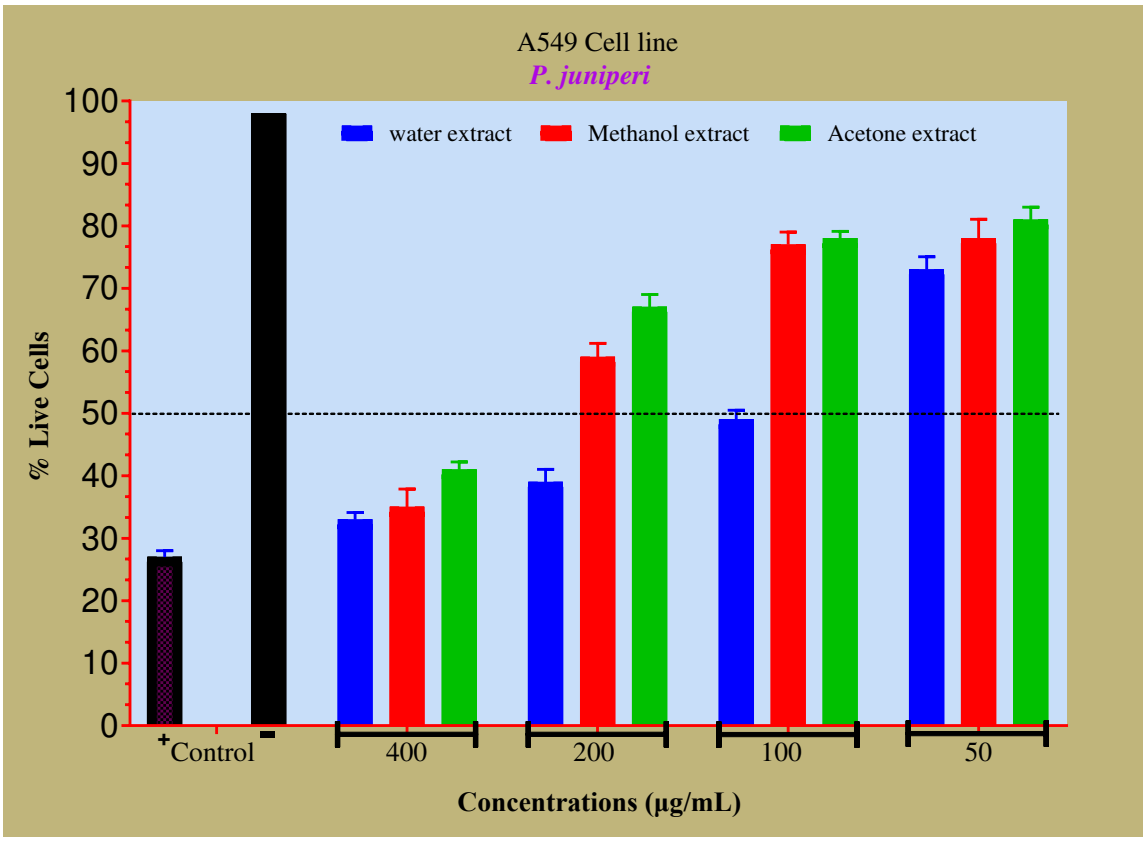

Figure 4.12. Cytotoxic effects of water, methanol, and acetone extracts of *Picoa juniperi* on the human lung cancer cell line (A549). Results were obtained by using an MTT assay with four different concentrations of extracts to observe their impacts on lung cancer cells. Controls included untreated (-) and treated (+) cancer cells. Data are expressed as mean±SD

Table 4.12. IC_{50} values of cytotoxic activity of *Picoa* (*P. juniperi*/A549)

Agent	A549 cells		
	Desert truffles		
	P. juniperi		
	Water extract	Methanol extract	Acetone extract
IC_{50}	155.5 µg/ml	226.8 µg/ml	198.7 µg/ml

The PANC-1 cells were presented to different concentrations of water, methanol, and acetone extracts from *P. lefebvrei* (from 400 to 50 µl/ml for 72 h). The cytotoxic effects of extracts on PANC-1 cells have been confirmed. The results, as shown in Figure 4.13. Water, methanol, and acetone extracts of *P. lefebvrei* could diminish the activity of PANC-1 cells in a dose-dependent manner. Extracts at concentrations of 400 µg/ml, and 200 µg/ml had a high and significant cell death rate. The extracts had a mild impact at the 100 µg/ml concentrations, while cell damage at the doses of 50 µg/ml did not significantly occur. The half-maximal inhibitory concentration (IC_{50}) values of water, methanol, and acetone extract from *P. lefebvrei* on PANC-1 cells have shown 151.1 µg/ml, 252.8 µg/ml, and 203.7 µg/ml, respectively as shown in Table 4.13.

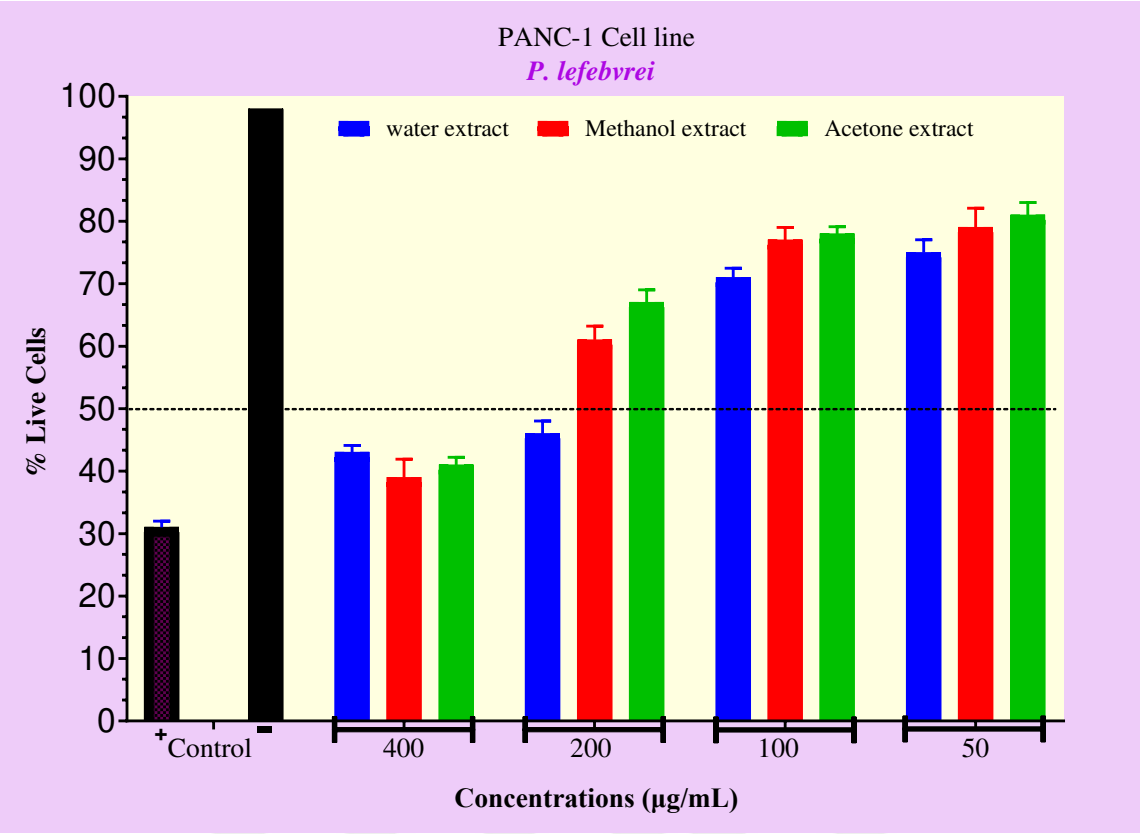

Figure 4.13. Cytotoxic effects of water, methanol, and acetone extracts of *Picoa lefebvrei* on the human pancreatic cancer cell line (PANC-1). Results were obtained by using an MTT assay with four different concentrations of extracts to observe their impacts on pancreas cancer cells. Controls included untreated (-) and treated (+) cancer cells. Data are expressed as mean±SD

Table 4.13. IC_{50} values of cytotoxic activity of *Picoa* (*P. lefebvrei*/PANC-1)

	PANC-1 cells		
	Desert truffles		
Agent	*P. lefebvrei*		
	Water extract	Methanol extract	Acetone extract
IC_{50}	151.1 µg/ml	252.8 µg/ml	203.7 µg/ml

The MDA-MB 231 cells were subjected to various concentrations of water, methanol, and acetone extracts from *P. lefebvrei* (from 400 to 50 µl/ml for 72 h). The cytotoxic effects of extracts

on MDA-MB 231 cells have been confirmed. The results, as shown in Figure 4.14. Water, methanol, and acetone extracts of *P. lefebvrei* can diminish the growth of MDA-MB 231 cells in a dose-dependent aspect. Extracts at concentrations of 400 µg/ml, and 200 µg/ml had a high and significant cell death rate. The extracts had a mild impact at the 100 µg/ml concentrations, while cell damage at the doses of 50 µg/ml did not significantly occur. The half-maximal inhibitory concentration (IC_{50}) values of water, methanol, and acetone extract from *P. lefebvrei* on MDA-MB 231 cells have shown 130.9 µg/ml, 218.3 µg/ml, and 224.1 µg/ml, respectively as shown in Table 4.14.

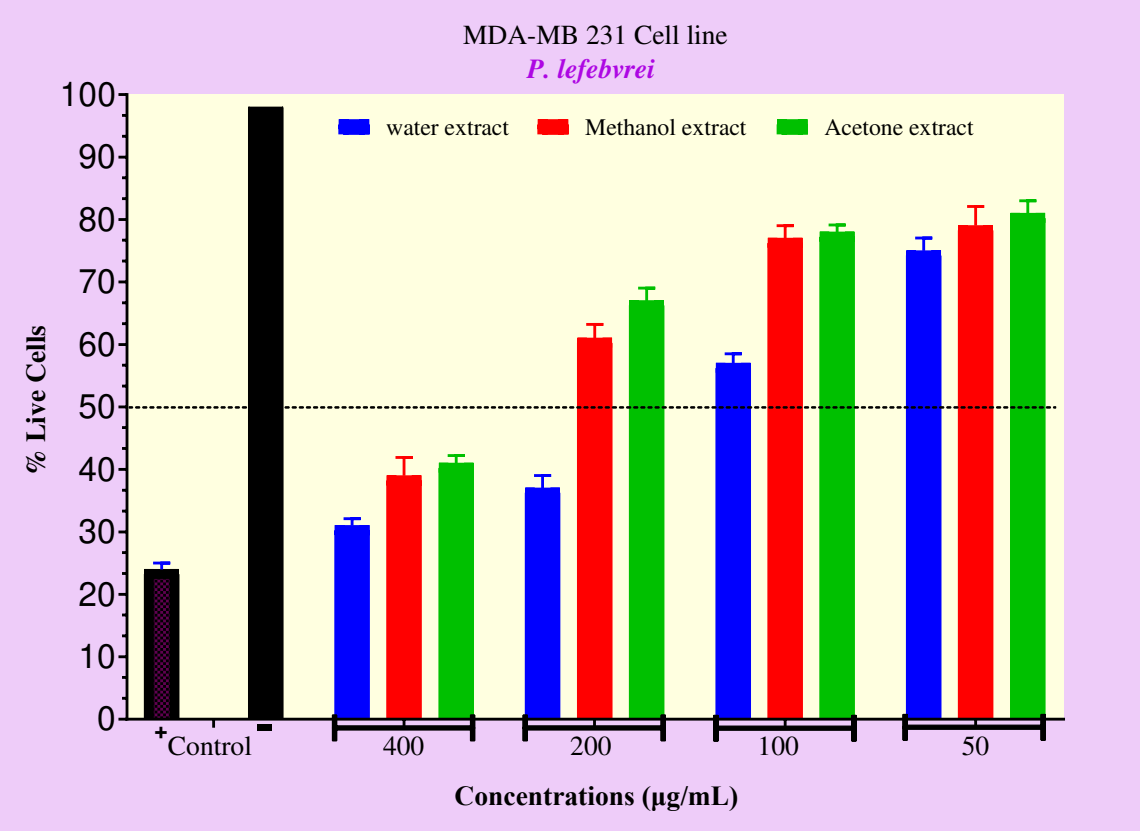

Figure 4.14. Cytotoxic effects of water, methanol, acetone extracts of *Picoa lefebvrei* on the human breast cancer cell line (MDA-MB 231). Results were obtained by using an MTT assay with four different concentrations of extracts to observe their impacts on breast cancer cells. Controls included untreated (-) and treated (+) cancer cells. Data are expressed as mean±SD

Table 4.14. IC_{50} values of cytotoxic activity of *Picoa* (*P. lefebvrei*/MDA-MB 231)

Agent	MDA-MB 231 cells		
	Desert truffles		
	P. lefebvrei		
	Water extract	Methanol extract	Acetone extract
IC_{50}	130.9 µg/ml	218.3 µg/ml	224.1 µg/ml

The A549 cells were given various doses of water, methanol, and acetone extracts from *P. lefebvrei* (from 400 to 50 µl/ml for 72 h). The cytotoxic effects of extracts on A549 cells have been confirmed. The results, as shown in Figure 4.15. Water, methanol, and acetone extracts of *P. lefebvrei* could destroy the viability of A549 cells in a dose-dependent aspect. Extracts at concentrations of 400 µg/ml, and 200 µg/ml had a high and significant cell death rate. The extracts had a mild impact at the 100 µg/ml concentrations, while cell damage at the doses of 50 µg/ml did not significantly occur. The half-maximal inhibitory concentration (IC_{50}) values of water, methanol, and acetone extract from *P. lefebvrei* on A549 cells have shown 135.6 µg/ml, 226.8 µg/ml, and 199.7 µg/ml, respectively as shown in Table 4.15.

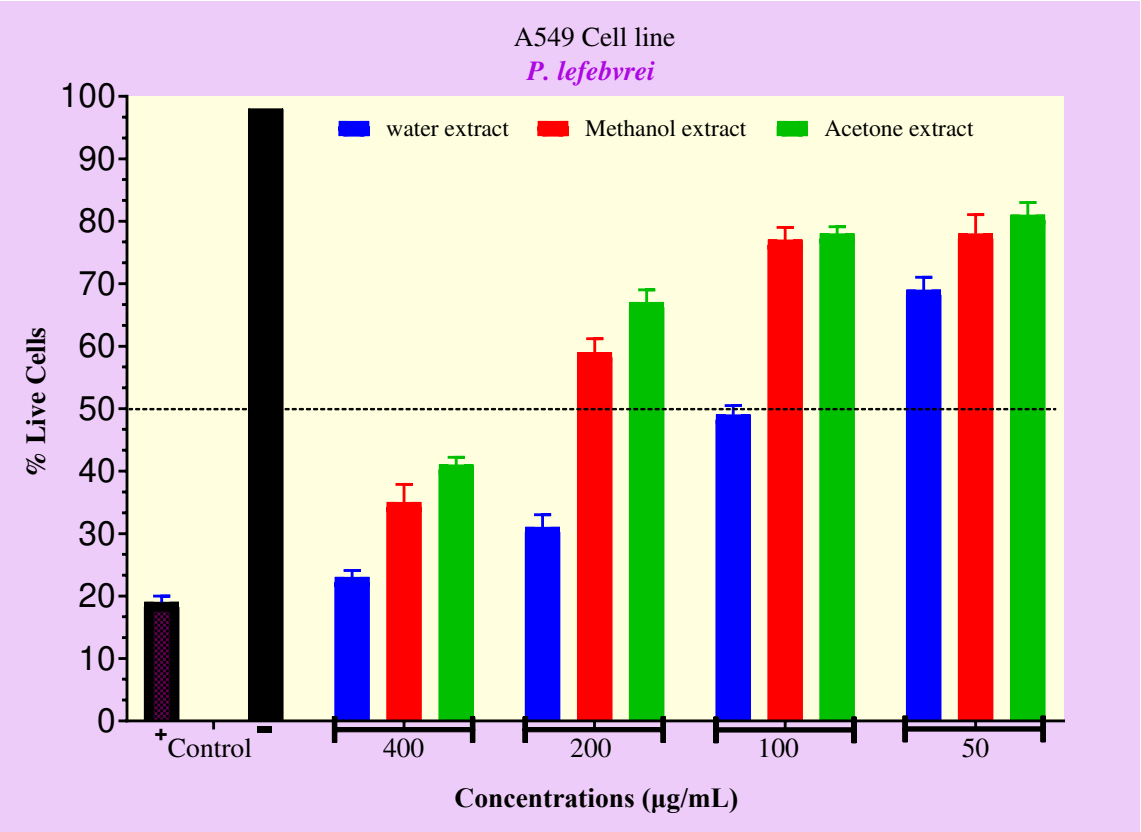

Figure 4.15. Cytotoxic effects of water, methanol, acetone extracts of *Picoa lefebvrei* on the human lung cancer cell line (A549). Results were obtained by using an MTT assay with four different concentrations of extracts to observe their impacts on lung cancer cells. Controls included untreated (-) and treated (+) cancer cells. Data are expressed as mean±SD

Table 4.15. IC_{50} values of cytotoxic activity of *Picoa* (*P. lefebvrei*/A549)

	A549 cells		
	Desert truffles		
Agent	*P. lefebvrei*		
	Water extract	Methanol extract	Acetone extract
IC_{50}	135.6 µg/ml	226.8 µg/ml	199.7 µg/ml

The cytotoxic activities of *P. juniperi* and *P. lefebvrei* on three cancer cells revealed strong anticancer properties. The ability of growth inhibitions of methanol, water, and acetone extracts of

P. juniperi and *P. lefebvrei* against (PANC-1, MDA-MB 231, A549) were confirmed, these findings are in agreement with the recent study [88]. All figures and (IC_{50}) values from this investigation showed that extracts at concentrations of 400 and 200 ug/ml had a large and substantial cell death rate, and had a modest influence at 100 g/ml, while cell damage at 50 g/ml was not significant. At the same time, the Figures show that *P. juniperi* and *P. lefebvrei* extracts can suppress cancer cell activities in a dose-dependent manner. Similar results have been shown in the previous study [89]. Finally, an apparent inhibition in viability was observed in cell lines as early as 48 hours, but IC_{50} was achieved after 72 hours of incubation. Water extracts had a significant effect at 400 μg/well. Water extract, 72 h of incubation, and concentration (400 g/ml) were chosen for the more molecular investigation (apoptosis/necrosis).

4.2. Apoptosis and necrosis inductions

The cytotoxic activities of water extracts from *T. claveryi, T. boudieri, T. olbiensis, P. juniperi* and *P. lefebvrei* on PANC-1, MDA-MB 231, and A549 cells were clearly evident. Reduces of cancer cell growths may result in initiation of apoptosis and/or necrosis. Therefore, the nuclear morphological changes were investigated.

4.2.1. Apoptosis and necrosis inductions by *Terfezia*

The effects of aquous extracts from the *T. claveryi, T. boudieri,* and *T. olbiensis* on PANC-1, MDA-MB 231, A549 cells for nuclear chromatin condensation were evaluated using HOPI stains. The cancer cell lines were treated with the extracts (400 μg/ml) and analyzed at 48h. DMEM (10% FBS with 1% Penicillin-Streptomycin) and doxorubicin (2.5 μg/ml) were used. A fluorescent EVOS FLC digital microscope was used to evaluate nuclear morphological changes. Apoptotic cells were those fragmented and/or condensed, the cells were imaged at 20 magnifications. When untreated cells were compared to cells treated with *T. claveryi, T. boudieri,* and *T. olbiensis* extracts at a concentration of 400 μg/ml for 48 hours, the number of apoptotic and necrotic cells increased significantly ($p<0.001$). The morphological properties of apoptotic cells were represented by condensed and fragmented nuclei as shown in Figure 4.16, Figure 4.18 and Figure 4.20**.** Furthermore, the percentages of apoptotic and necrotic cells in the cancer cell line were demonstrated, as shown in Figure 4.17, Figure 4.19 and Figure 4.21. The apoptotic indexes of doxorubicin (positive control), and extracts of *T. claveryi, T. boudieri,* and *T. olbiensis* were 73.1±1.3, 79.7±1.5, 60.1±1.9, and 76.6±1.7, respectively. While necrotic indexes for doxorubicin (positive control), and extracts of *T. claveryi, T. boudieri,* and *T. olbiensis* were 28.8±1.9, 33.6±1.7, 20.2±1.7, and 22.9±1.2, respectively. This discovery demonstrated that aqueous extracts of *T. claveryi, T. boudieri,* and *T. olbiensis* had significant apoptotic effects on the PANC-1, MDA-MB

231, A549 cells rather than necrotic, which are similar to the effect of doxorubicin. These findings suggest that the apoptotic pathway acting the main role in the suppression of cancer cells utilizing *T. claveryi*, *T. boudieri*, and *T. olbiensis* extracts.

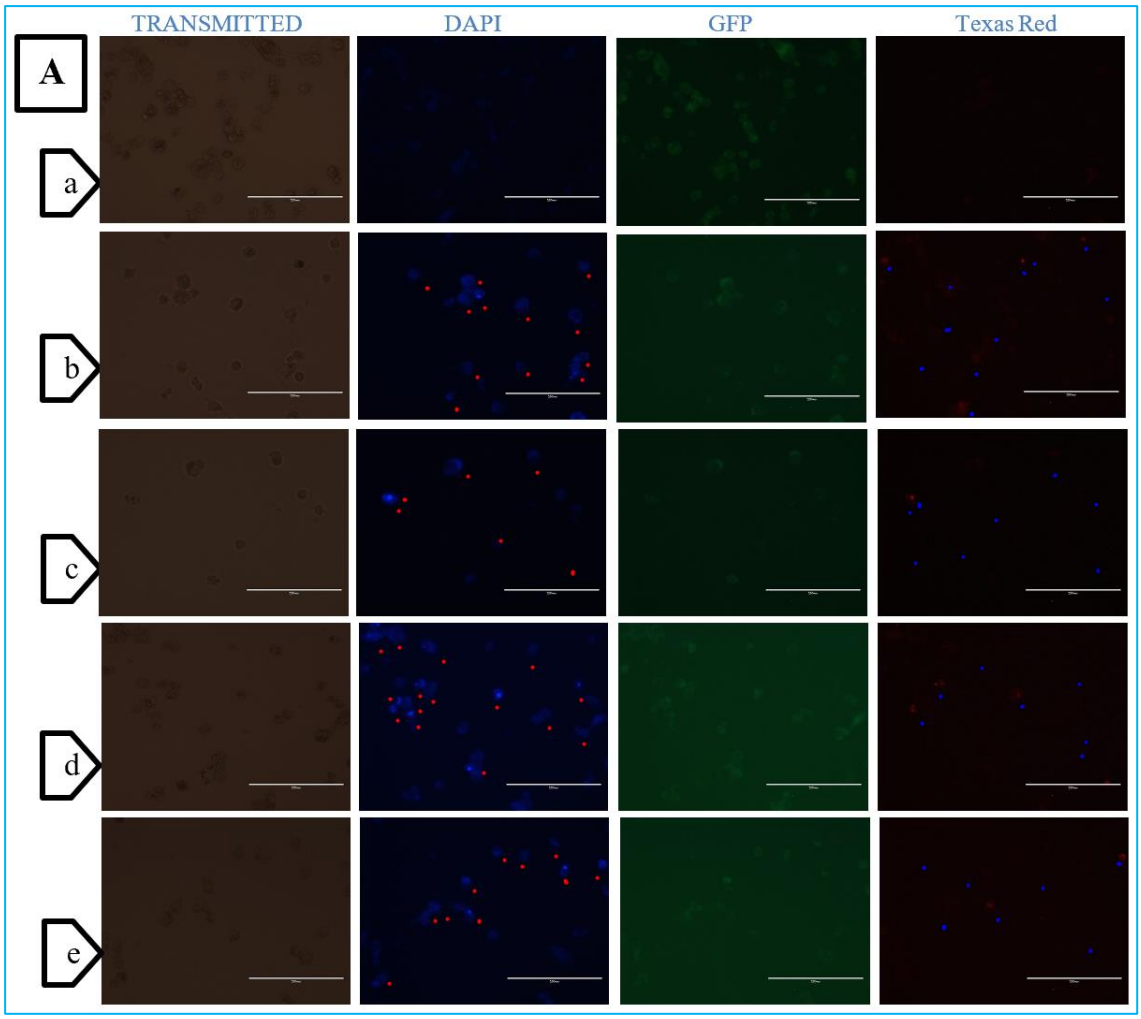

Figure 4.16. (A) Shows the effects of water extracts of *T. claveryi, T. boudieri,* and *T. olbiensis* on the nucleic morphology of human pancreatic cancer cells (PANC-1). (a) Negative control was untreated cells (b) Positive control was treated cells with 2.5 µg/ml of doxorubicin (c) Treated cells with 400 µg/ml of *T. claveryi* (d) Treated cells with 400 µg/ml of *T. boudieri,* and (e) Treated cells with 400 µg/ml of *T. olbiensis*

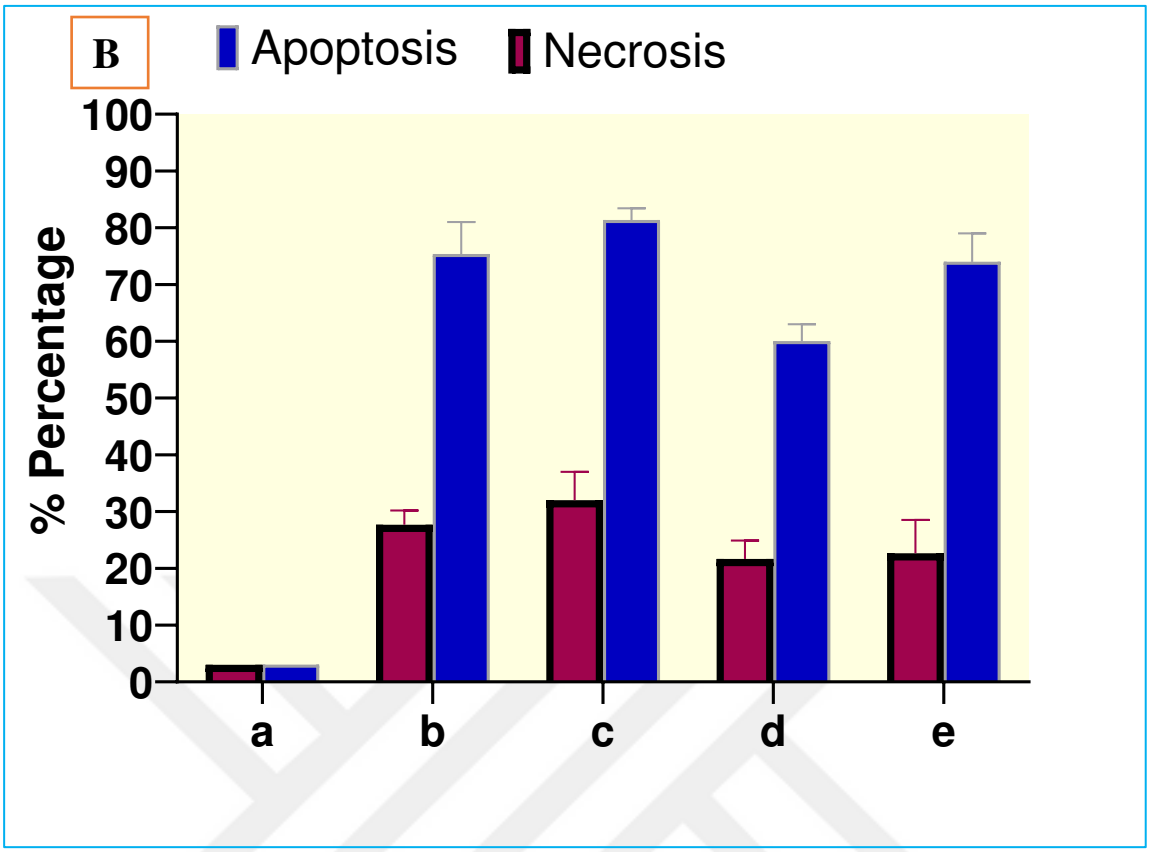

Figure 4.17. (B) Shows the percentage of apoptotic and necrotic indexes in ten different microscopic fields, the apoptotic and necrotic indexes were expressed as a percentage ratio of the number of apoptotic and necrotic cells to the total number of cancer cells. The mean and standard deviation are used to express the values. *$p<0.001$ versus the negative control group. effects of water extracts of *T. claveryi, T. boudieri,* and *T. olbiensis* on the nucleic morphology of pancreatic cancer cells (PANC-1), (a) Negative control was untreated cells (b) Positive control was treated cells with 2.5 µg/ml of doxorubicin (c) Treated cells with 400 µg/ml of *T. claveryi* (d) Treated cells with 400 µg/ml of *T. boudieri,* and (e) Treated cells with 400 µg/ml of *T. olbiensis*

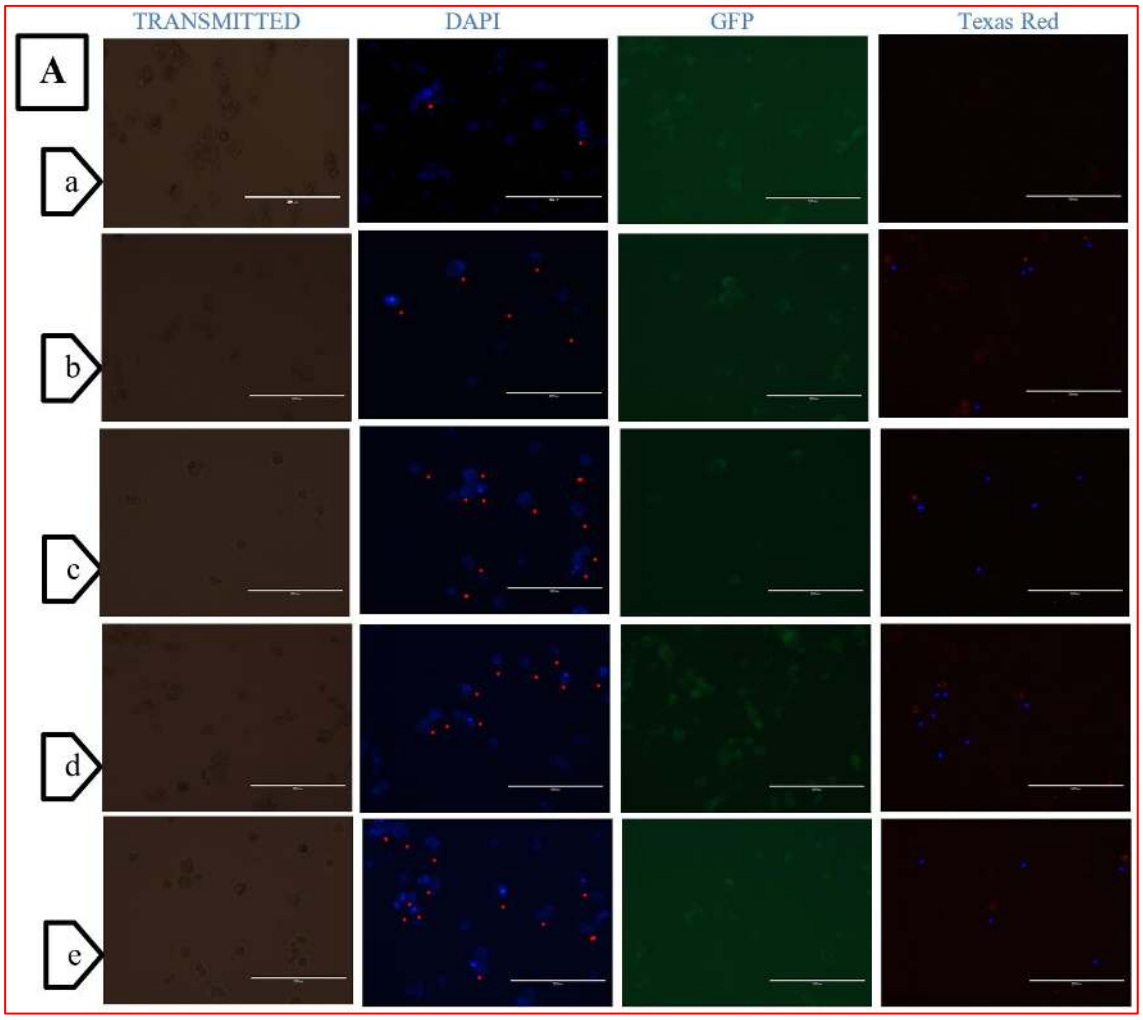

Figure 4.18. (A) Shows the effects of water extracts of *T. claveryi, T. boudieri,* and *T. olbiensis* on the nucleic morphology of human breast cancer cells (MDA-MB 231), (a) Negative control was untreated cells (b) Positive control was treated cells with 2.5 µg/ml of doxorubicin (c) Treated cells with 400 µg/ml of *T. claveryi* (d) Treated cells with 400 µg/ml of *T. boudieri,* and (e) Treated cells with 400 µg/ml of *T. olbiensis*

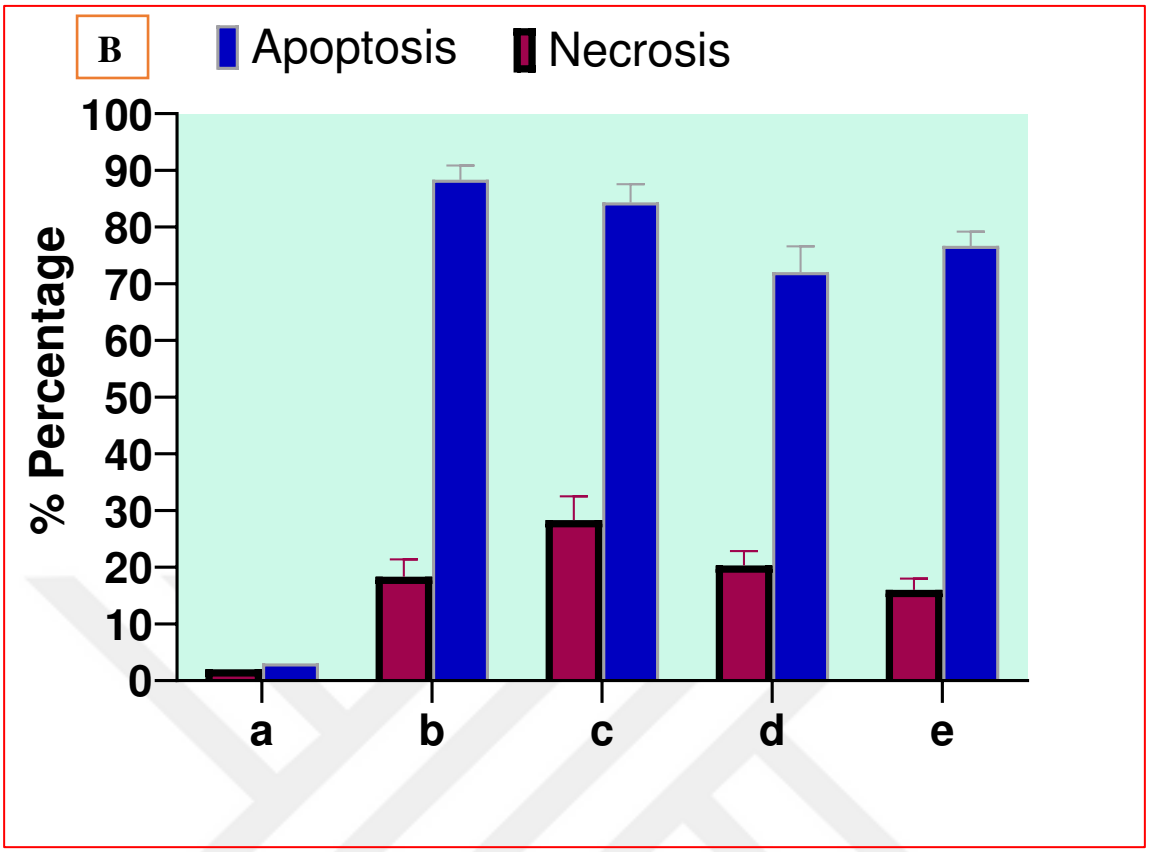

Figure 4.19. (B) Shows the percentage of apoptotic and necrotic indexes in ten different microscopic fields, the apoptotic and necrotic indexes were expressed as a percentage ratio of the number of apoptotic and necrotic cells to the total number of cancer cells. The mean and standard deviation are used to express the values. *$p<0.001$ versus the negative control group. effects of water extracts of *T. claveryi, T. boudieri,* and *T. olbiensis* on the nucleic morphology of breast cancer cells (MDA-MB 231), (a) Negative control was untreated cells (b) Positive control was treated cells with 2.5 µg/ml of doxorubicin (c) Treated cells with 400 µg/ml of *T. claveryi* (d) Treated cells with 400 µg/ml of *T. boudieri,* and (e) Treated cells with 400 µg/ml of *T. olbiensis*

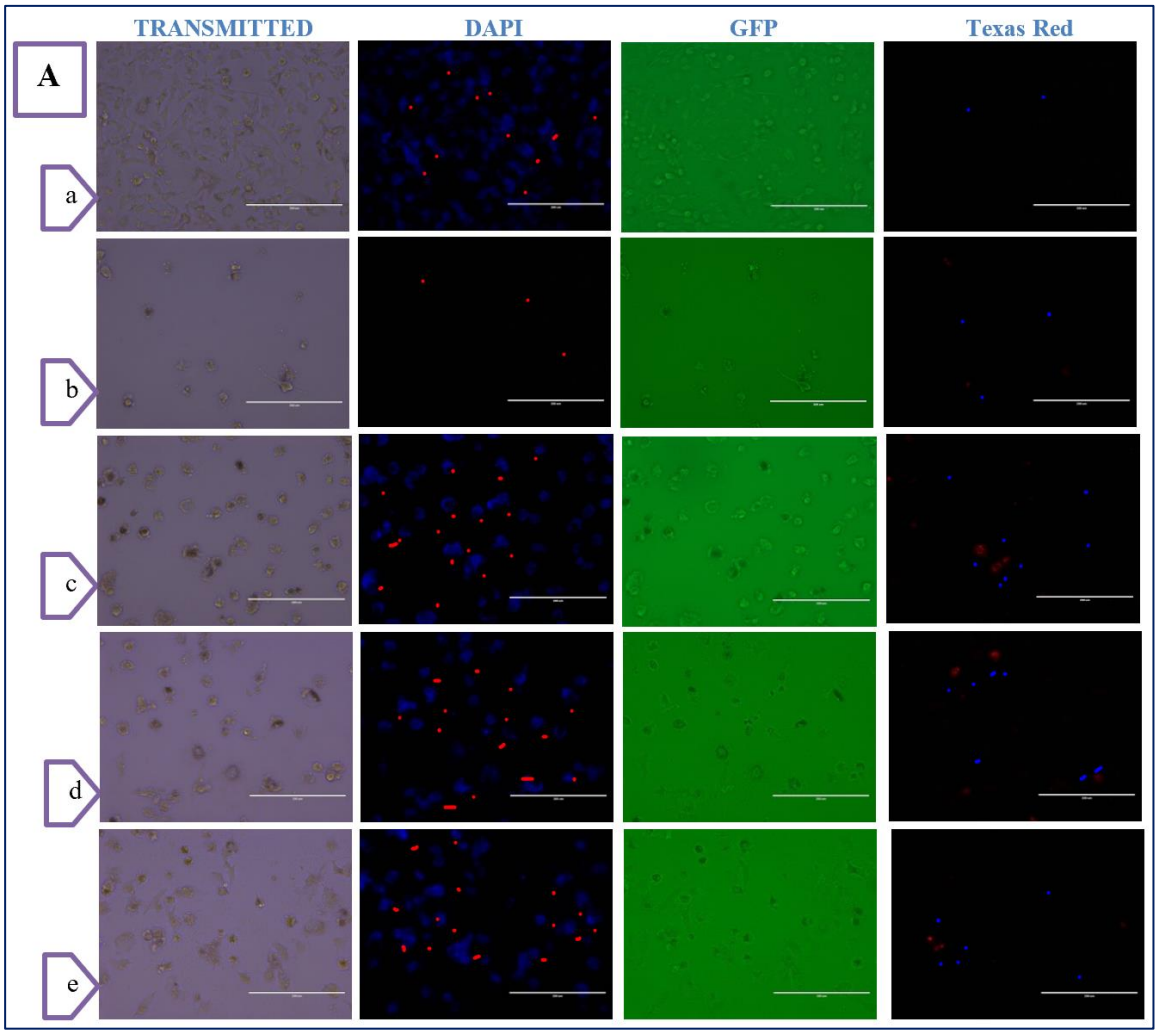

Figure 4.20. (A) Shows the effects of water extracts of *T. claveryi, T. boudieri,* and *T. olbiensis* on the nucleic morphology of human lung cancer cells (A549), (a) Negative control was untreated cells (b) Positive control was treated cells with 2.5 µg/ml of doxorubicin (c) Treated cells with 400 µg/ml of *T. claveryi* (d) Treated cells with 400 µg/ml of *T. boudieri,* and (e) Treated cells with 400 µg/ml of *T. olbiensis*

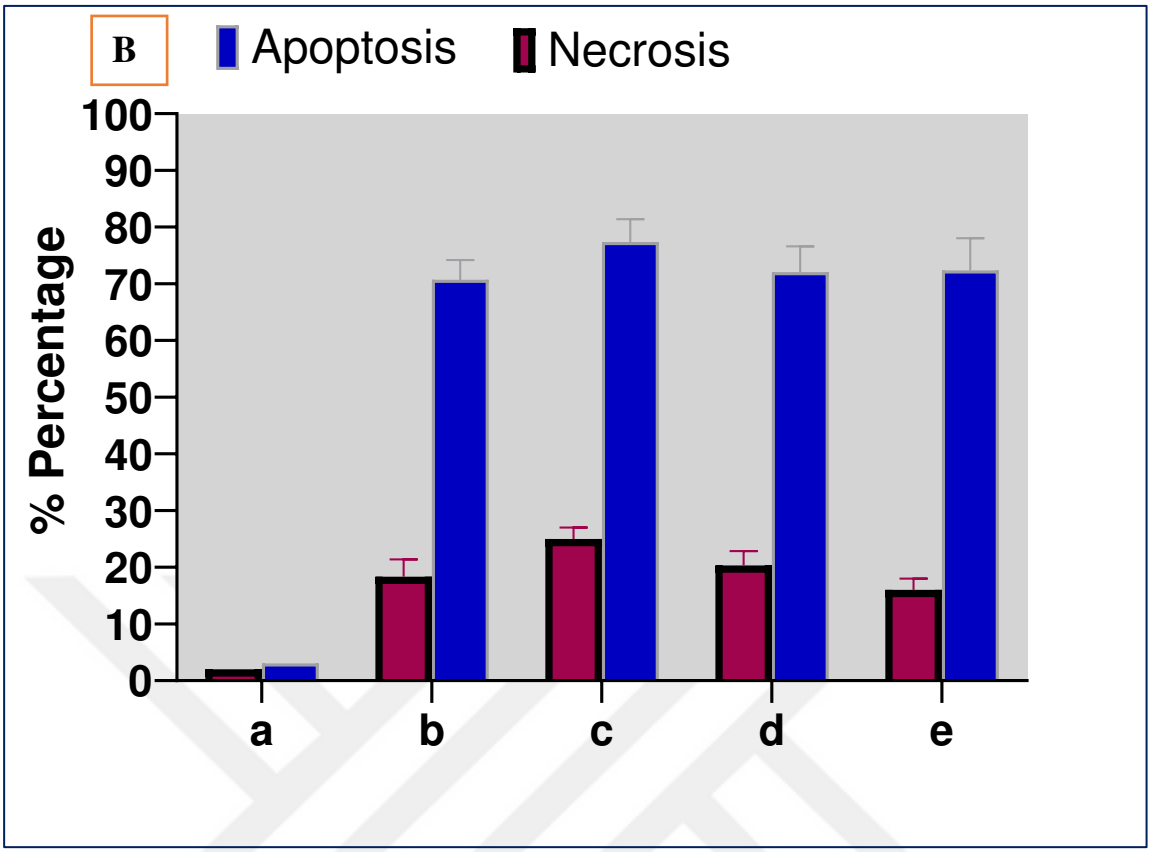

Figure 4.21. (B) Shows the percentage of apoptotic and necrotic indexes in ten different microscopic fields, the apoptotic and necrotic indexes were expressed as a percentage ratio of the number of apoptotic and necrotic cells to the total number of cancer cells. The mean and standard deviation are used to express the values. *$p<0.001$ versus the negative control group. effects of water extracts of *T. claveryi, T. boudieri,* and *T. olbiensis* on the nucleic morphology of lung cancer cells (A549), (a) Negative control was untreated cells (b) Positive control was treated cells with 2.5 µg/ml of doxorubicin (c) Treated cells with 400 µg/ml of *T. claveryi* (d) Treated cells with 400 µg/ml of *T. boudieri,* and (e) Treated cells with 400 µg/ml of *T. olbiensis*

4.2.2. Apoptosis and necrosis inductions by *Picoa*

In the present study, water extracts' inhibitory properties of *P. juniperi* and *P. lefebvrei* on PANC-1, MDA-MB 231 and A549 cell lines were evident and confirmed. Inhibition of cancer cell proliferation may result in apoptosis and/or necrosis induction. Therefore, the roles of aquous extracts from *P. juniperi* and *P. lefebvrei* in the apoptotic and necrotic pathways were investigated. The morphological properties of apoptotic cells were represented by condensed and fragmented nuclei as shown in Figure 4.22, Figure 4.24 and Figure 4.26**.** Additionally, the percentages of apoptotic and necrotic cells in the cancer cell lines (PANC-1, MDA-MB 231, A549) were demonstrated, as shown in Figure 4.23, Figure 4.25 and Figure 4.27. The apoptotic indexes of doxorubicin (positive control), and extracts of *P. juniperi* and *P. lefebvrei* were 73.1±1.3, 72.3±1.1, and 68.1±1.5, respectively. While necrotic indexes for doxorubicin, and extracts of *P. juniperi* and *P. lefebvrei* were 28.8±1.9, 31.7±1.3, and 21.4±1.4, respectively. This finding demonstrated that

aqueous extracts of *P. juniperi* and *P. lefebvrei* had significant apoptotic effects rather than nectotic on the PANC-1, MDA-MB 231, A549 cancer cell lines.

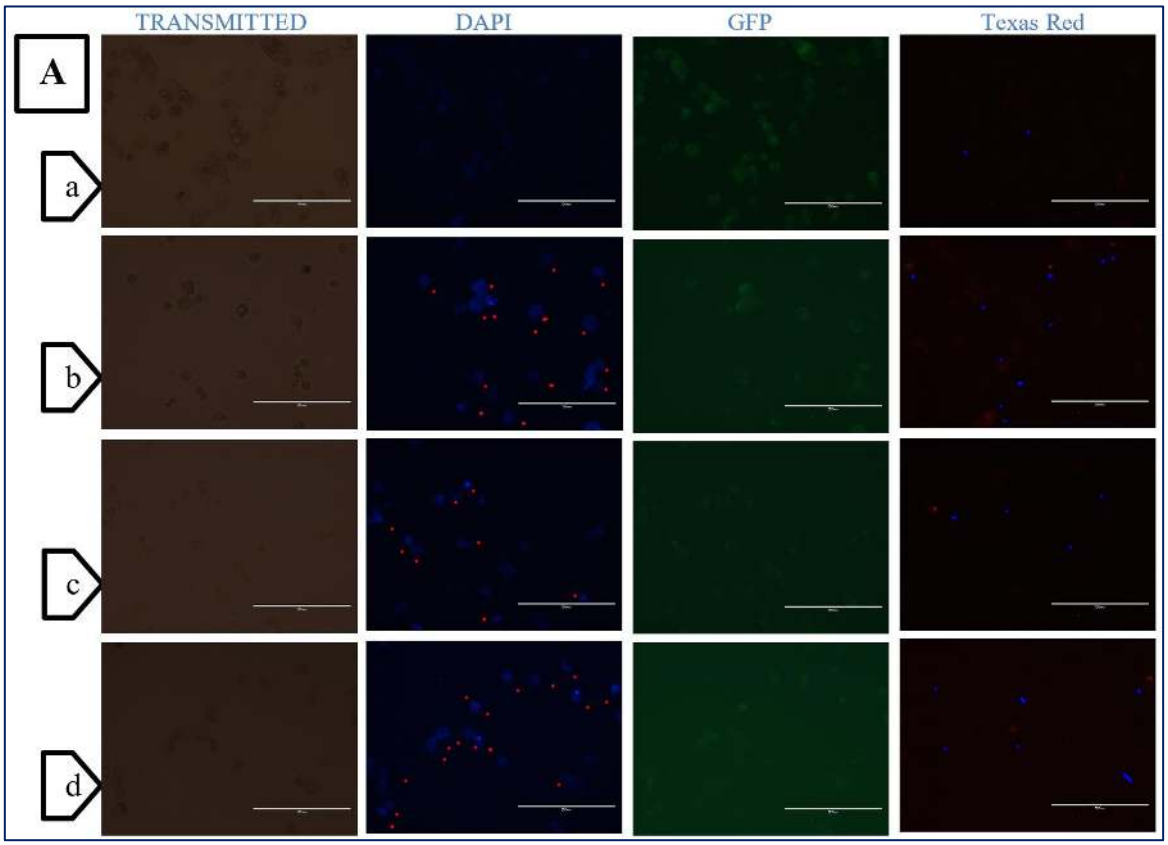

Figure 4.22. (A) The effects of water extracts of *P. juniperi* and *P. lefebvrei* on the nucleic morphology of PANC-1 cancer cells were demonstrated under TRANSMITTED, DAPI, GFP, and Texas Red filters of EVOS digital microscope. To determine apoptotic and necrotic morphology, untreated and treated cells were stained with Hoechst 33342, and Propidium Iodide. (a) Negative control was untreated cell line (b) Positive control was treated cell line with 2.5 µg/ml of doxorubicin (c) Treated cell line with 400 µg/ml of *P. juniperi* (d) Treated cell line with 400 µg/ml of *P. lefebvrei*

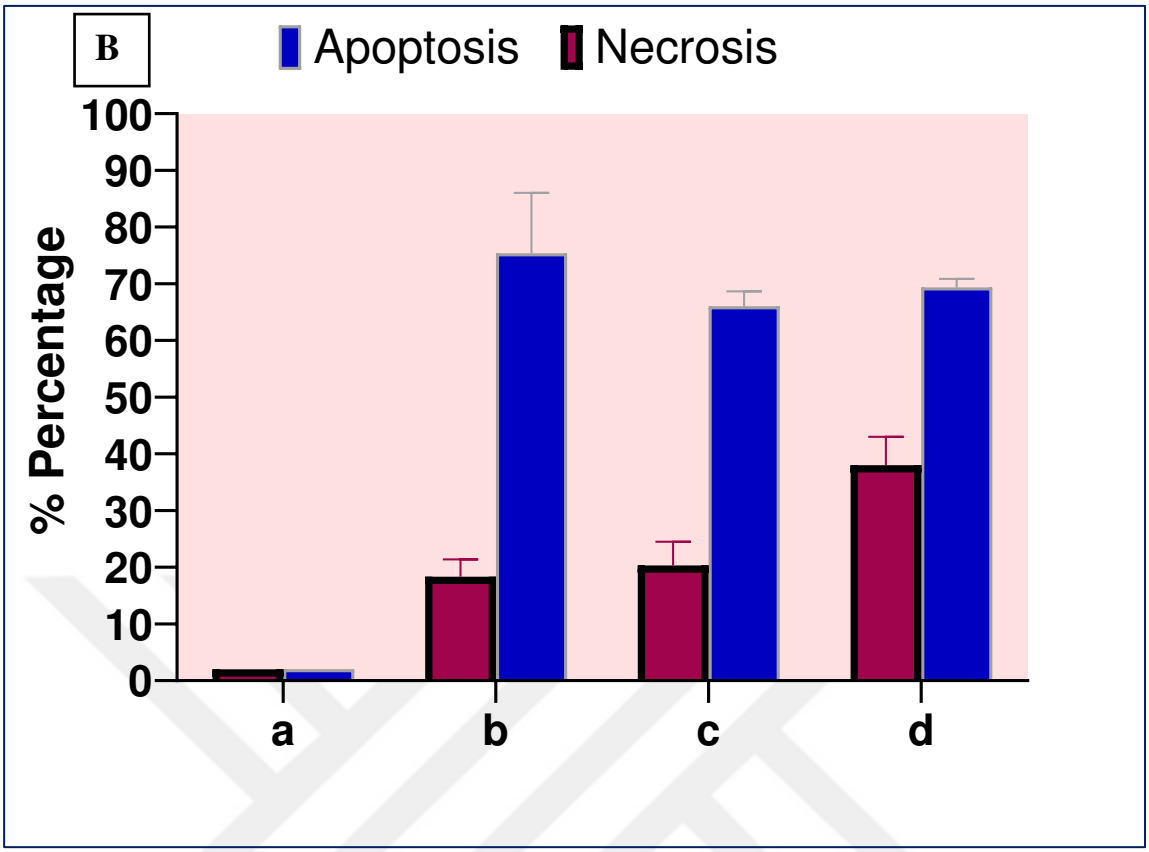

Figure 4.23. (B) Shows a graphical representation of the percentage of apoptotic and necrotic markers for PANC-1 cancer cells. In ten different microscopic fields, the apoptotic and necrotic indexes were expressed as a percentage ratio of the number of apoptotic and necrotic cells to the total number of cancer cells. The mean and standard deviation are used to express the values. *$p<0.001$ versus the negative control group. To determine apoptotic and necrotic morphology, untreated and treated cells were stained with Hoechst 33342, and Propidium Iodide. (a) Negative control was untreated cell line (b) Positive control was treated cell line with 2.5 µg/ml of doxorubicin (c) Treated cell line with 400 µg/ml of *P. juniperi* (d) Treated cell line with 400 µg/ml of *P. lefebvrei*

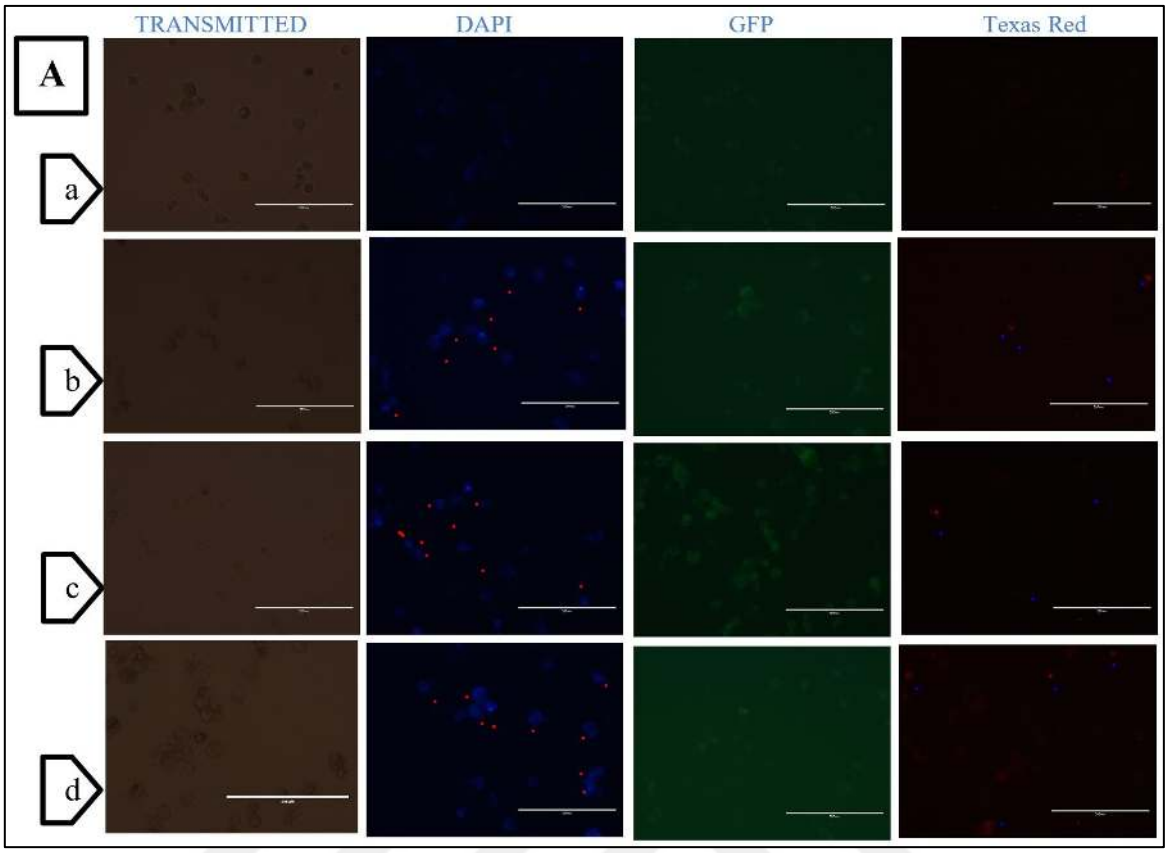

Figure 4.24. (A) The effects of water extracts of *P. juniperi* and *P. lefebvrei* on the nucleic morphology of MDA-MB 231 cancer cells were demonstrated under TRANSMITTED, DAPI, GFP, and Texas Red filters of EVOS digital microscope. To determine apoptotic and necrotic morphology, untreated and treated cells were stained with Hoechst 33342, and Propidium Iodide. (a) Negative control was untreated cell line (b) Positive control was treated cell line with 2.5 µg/ml of doxorubicin (c) Treated cell line with 400 µg/ml of *P. juniperi* (d) Treated cell line with 400 µg/ml of *P. lefebvrei*

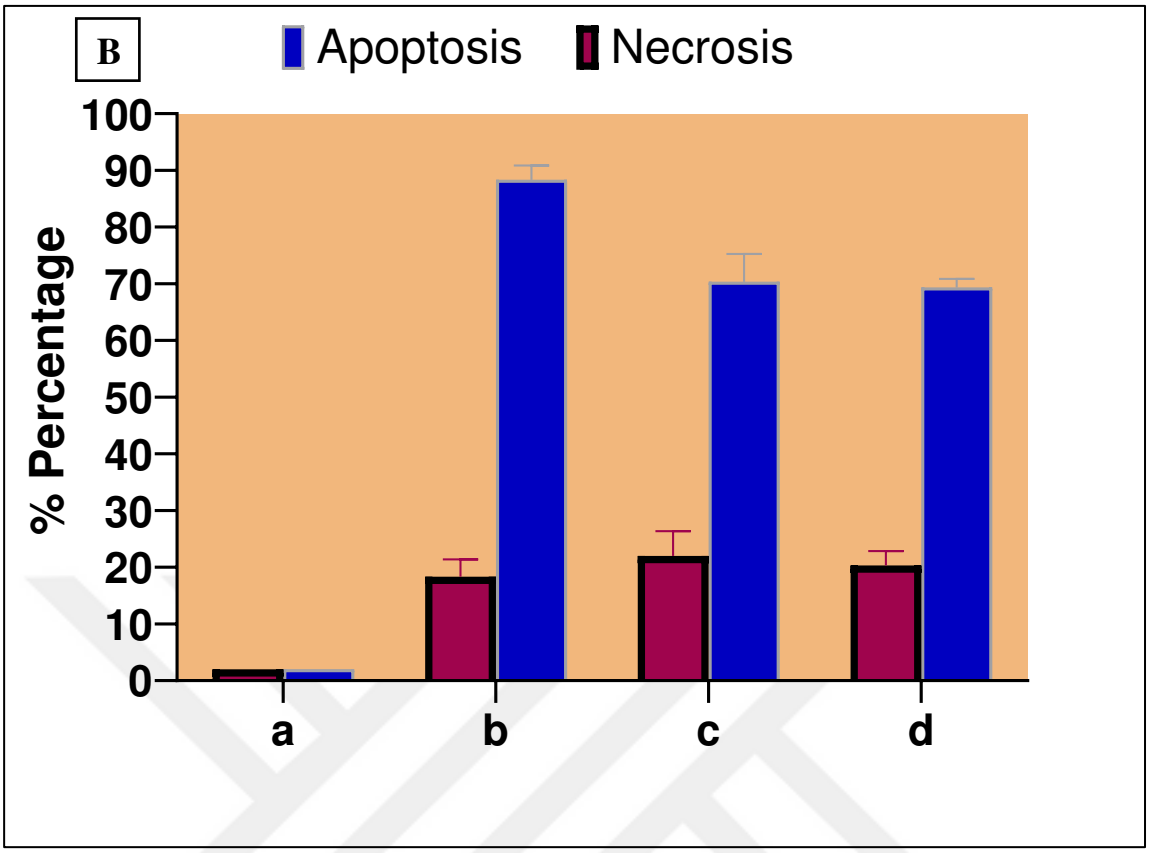

Figure 4.25. (B) Shows a graphical representation of the percentage of apoptotic and necrotic markers for MDA-MB 231 cancer cells. In ten different microscopic fields, the apoptotic and necrotic indexes were expressed as a percentage ratio of the number of apoptotic and necrotic cells to the total number of cancer cells. The mean and standard deviation are used to express the values. *$p<0.001$ versus the negative control group. To determine apoptotic and necrotic morphology, untreated and treated cells were stained with Hoechst 33342, and Propidium Iodide. (a) Negative control was untreated cell line (b) Positive control was treated cell line with 2.5 µg/ml of doxorubicin (c) Treated cell line with 400 µg/ml of *P. juniperi* (d) Treated cell line with 400 µg/ml of *P. lefebvrei*

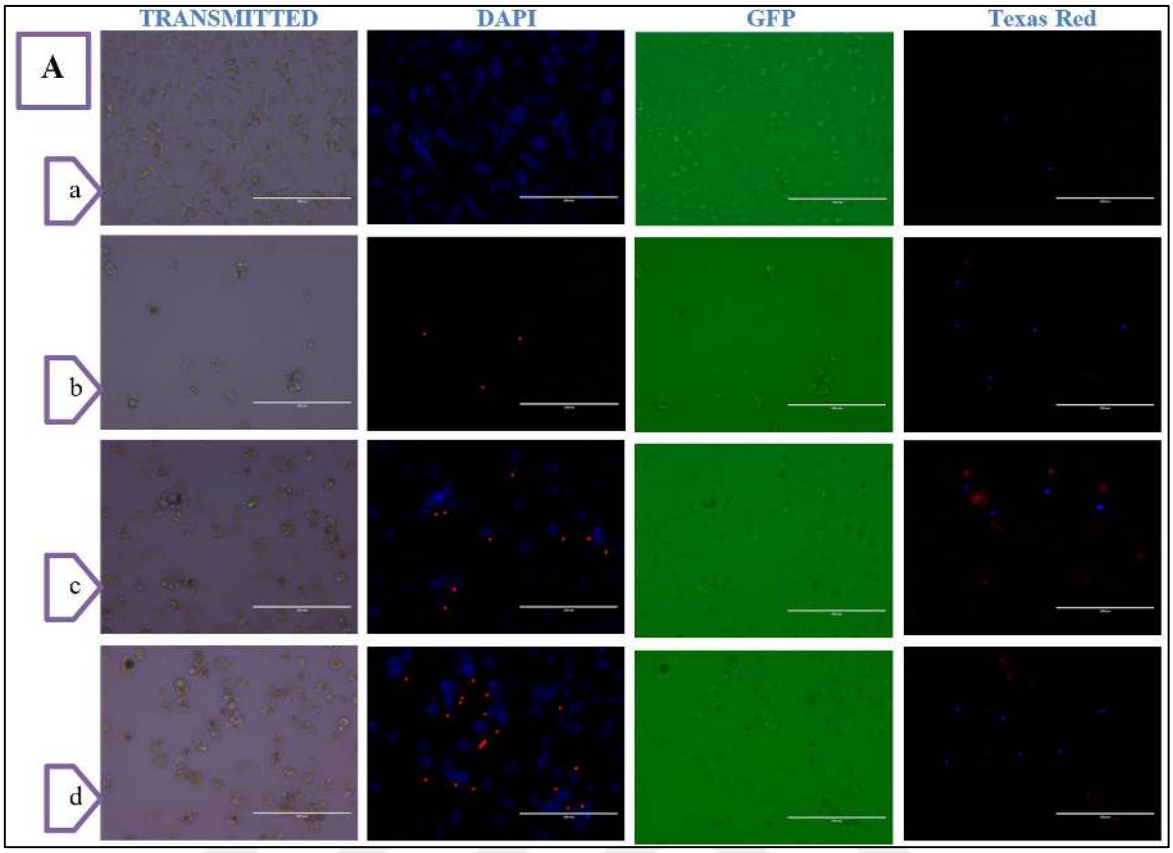

Figure 4.26. (A) The effects of water extracts of *P. juniperi* and *P. lefebvrei* on the nucleic morphology of A549 cancer cells were demonstrated under TRANSMITTED, DAPI, GFP, and Texas Red filters of EVOS digital microscope. To determine apoptotic and necrotic morphology, untreated and treated cells were stained with Hoechst 33342, and Propidium Iodide. (a) Negative control was untreated cell line (b) Positive control was treated cell line with 2.5 µg/ml of doxorubicin (c) Treated cell line with 400 µg/ml of *P. juniperi* (d) Treated cell line with 400 µg/ml of *P. lefebvrei*

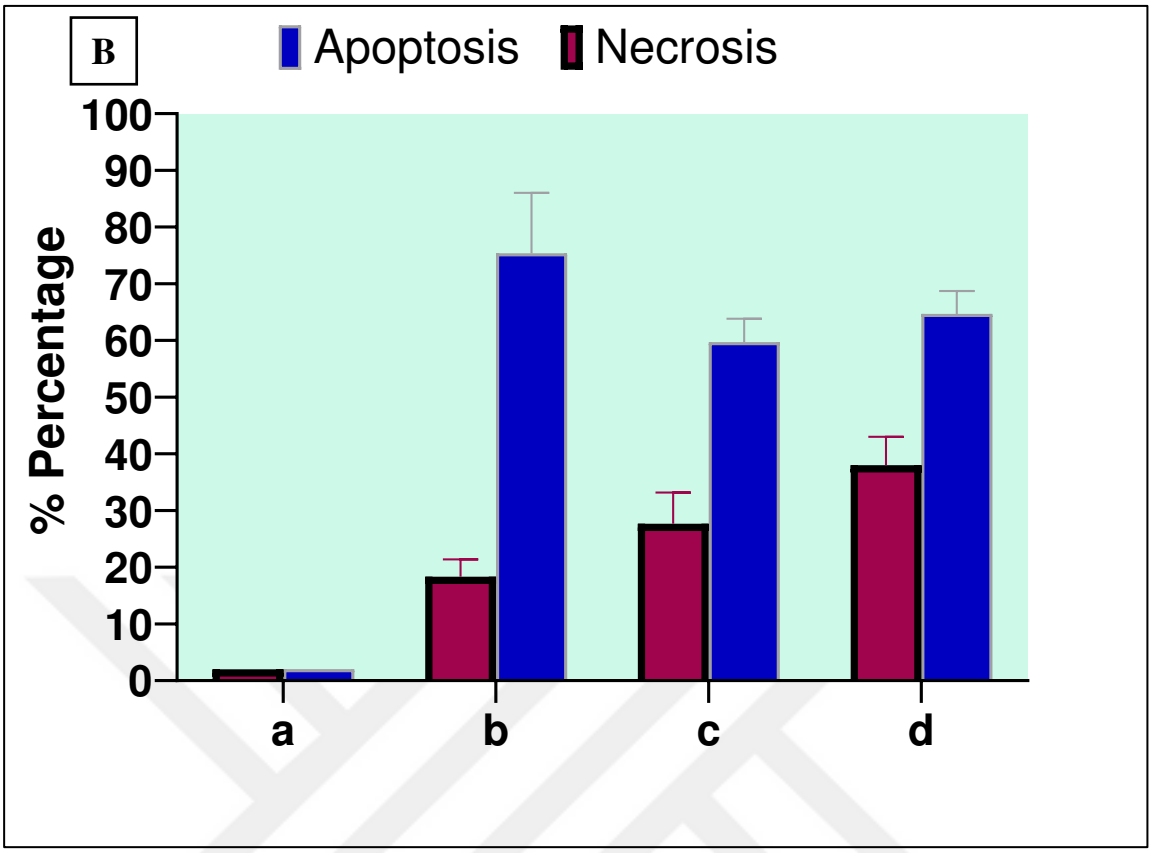

Figure 4.27. (B) Shows a graphical representation of the percentage of apoptotic and necrotic markers for A549 cancer cells. In ten different microscopic fields, the apoptotic and necrotic indexes were expressed as a percentage ratio of the number of apoptotic and necrotic cells to the total number of cancer cells. The mean and standard deviation are used to express the values. *p<0.001 versus the negative control group. To determine apoptotic and necrotic morphology, untreated and treated cells were stained with Hoechst 33342, and Propidium Iodide. (a) Negative control was untreated cell line (b) Positive control was treated cell line with 2.5 µg/ml of doxorubicin (c) Treated cell line with 400 µg/ml of *P. juniperi* (d) Treated cell line with 400 µg/ml of *P. lefebvrei*

Apoptotic cell death is a tightly controlled process defined by different cellular structures and a variety of metabolic alterations [90]. Other forms of cell death include necrosis, which is produced by a rapid abrupt and expel their contents [91]. To accurately diagnose and distinguish different types of cell death, HOPI double staining techniques were applied in this study. The findings of the present study showed that water extracts of *T. claveryi, T. boudieri, T. olbiensis*, *P. juniperi* and *P. lefebvrei* induced apoptosis rather than necrosis in the PANC-1, MDA-MB 231, and A549 cell lines. The current previous studies support these findings [9, 59]. The cancer cells are losing their membrane potential first, according to Marchetti et al., [92], as a reaction to tumor necrosis factor, and subsequently, these cells experience late-apoptotic modifications like DNA condensation and fragmentation. The ability of fluorescent dyes to attach to DNA to make the chromatin and hence the nucleus of the cell visible is the basis of this technique. The dye Hoechst 33258 (HO) can bind to DNA and thus pass through the cell membrane. It is used to stain the nuclei of both living and dead cells (apoptotic/necrotic). Propidium iodide (PI) dye is only taken up by

cells with compromised membrane integrity, allowing for the detection of (late apoptotic/necrotic) cells. Apoptotic cells are distinguished by the presence of a smaller (pyknotic) and/or fragmented nucleus relative to normal cells, whereas necrotic cells have a little larger nucleus and less staining [93].

4.3. Gene expression

The MTT assay and HOPI double staining results demonstrated that the extracts have cytotoxic and apoptosis-inducing properties. To confirm these findings, the role of certain genes involved in apoptotic pathways was explored. At first, to get clear conclusions about gene expression, high-quality total RNA is required. There are numerous methods for isolating RNA, in the present research, the quality control for total RNA extraction from three different cancer cell lines by special kits was evaluated by gel electrophoresis as shown in Figure 4.28, the product was shown clear RNA bands (28s and 18s). The quantity evaluations of total RNA samples by Qubit measurement, the normal Qubit values (ng/ml) for each sample. Total RNA Mini Kit (GENEAID) was performed to found an optimized and standard amount of total RNA

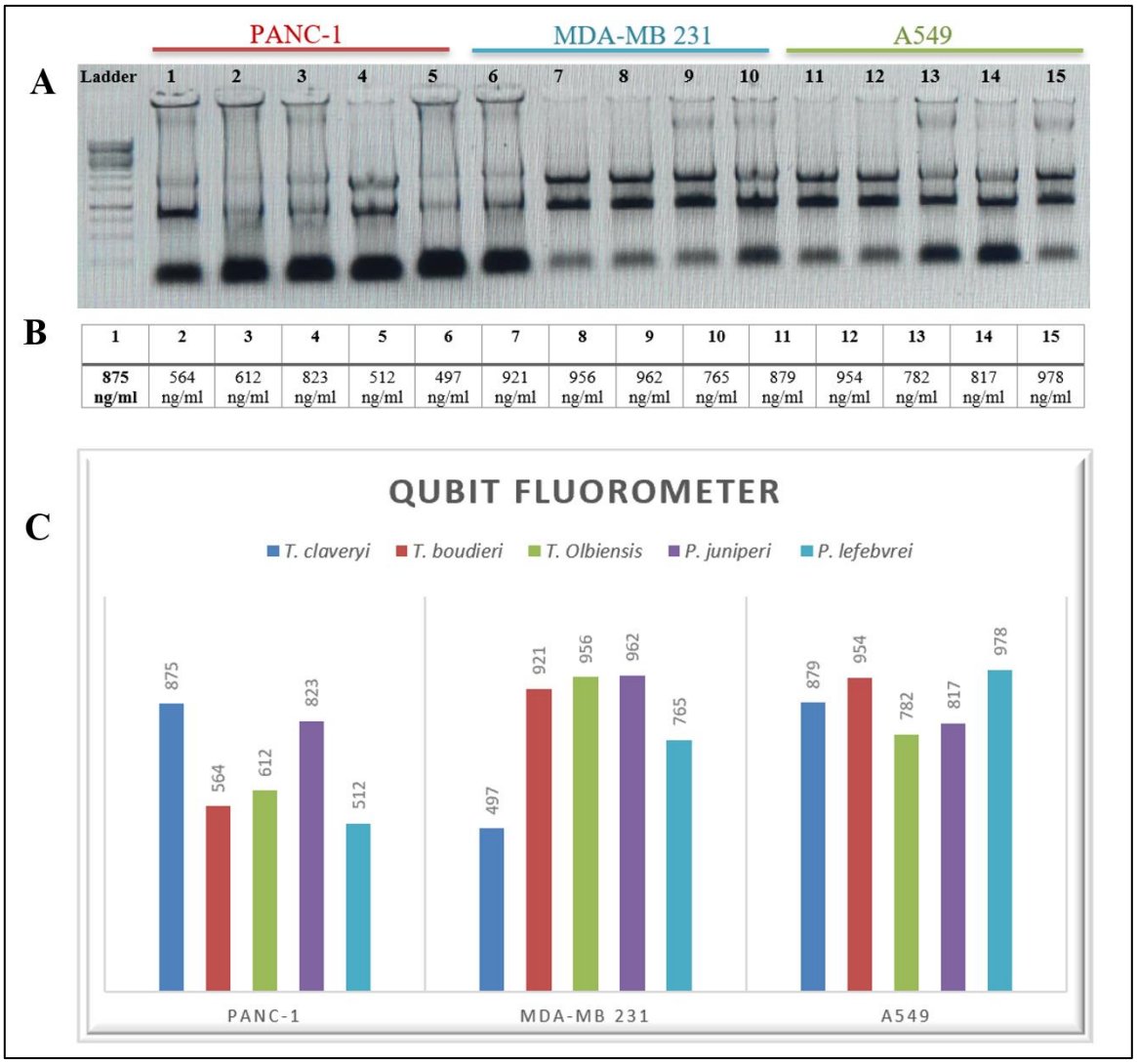

Figure 4.28. Assessment of quality and quantity of total RNA extraction from PANC-1, MDA-MB 231, and A549 cancer cells. **A:** agarose gel electrophoresis, and analysis image system iBright^{TM}750, (a) RNA samples were run on a 1% agarose gel for 30 minutes, ladder (1 kb). **B:** Qubit **C:** Qubit values of same RNA samples arranged from 1,2,3, to 13,14,15). PANC-1 included (1,2,3,4,5), MDA-MB 231 included (6,7,8,9,10), and A549 included (11,12,13,14,15)

4.3.1. The effects of *Terfezia* on gene expression

In this study, the effects of significant concentrations of water extracts of *T. claveryi, T. boudieri,* and *T. olbiensis* on the *p53, p21, Bax*, and *Bcl2* genes were investigated in PANC-1, MDA-MB 231, and A549 cell lines. The *p53, p21, Bax*, and *Bcl2* genes were amplified and normalized to the GAPDH gene by using qRT-PCR.

When compared to the untreated PANC-1 cells, expression levels of the *p21* gene in pancreatic cells treated with 400 µg/well of water extracts of *T. claveryi, T. boudieri, and T. olbiensis* for 72 hours were significantly (p<0.001) up-regulated by 6.2, 5.9, and 5.8-fold, respectively. The expression level of *p53* was significantly (p<0.001) up-regulated in pancreatic

cells treated with *T. claveryi, T. boudieri,* and *T. olbiensis* extracts by 6.3, 6.8, and 6.3-fold, respectively, in 400 µg/wells when compared to untreated cells. *Bax* gene expression was significantly (p<0.001) increased by 7.0, 7.3, and 6.8-fold, respectively, in PANC-1 cells treated with 400 µg/well *T. claveryi, T. boudieri,* and *T. olbiensis* extracts. Regarding the *Bcl2* gene expression, its level was significantly (p<0.01) down-regulated by 0.6, 0.5, and 0.53-fold, respectively, in pancreatic cells treated with *T. claveryi, T. boudieri,* and *T. olbiensis* extracts at 400 µg/well, when compared to untreated cells (Figure 4.29).

The expression levels of the p21 gene in breast cancer cells treated with 400 g/well water extracts of *T. claveryi, T. boudieri,* and *T. olbiensis* for 72 hours were dramatically (p<0.001) up-regulated by 6, 5.7, and 5.6-fold, respectively, as compared to untreated MDA-MB 231 cells. The expression level of *p53* was significantly (p<0.001) up-regulated in MDA-MB 231 cells treated with *T. claveryi, T. boudieri,* and *T. olbiensis* extracts by 6.1, 6.6, and 6.2-fold, respectively, in 400 µg/wells when compared to untreated cells. *Bax* gene expression was significantly (p<0.001) increased by 6.9, 7.2, and 6.6-fold, respectively, in MDA-MB 231 cells treated with 400 µg/well *T. claveryi, T. boudieri,* and *T. olbiensis* extracts. Regarding the *Bcl2* gene expression, its level was significantly (p<0.01) down-regulated by 0.7, 0.6, and 0.55-fold, respectively, in MDA-MB 231 cells treated with *T. claveryi, T. boudieri,* and *T. olbiensis* extracts at 400 µg/well, when compared to untreated cells (Figure 4.30).

The expression levels of the p21 gene in lung cancer cells treated with 400 g/well water extracts of *T. claveryi, T. boudieri,* and *T. olbiensis* for 72 hours were intensely (p<0.001) up-regulated by 6.3, 6, and 5.9-fold, respectively, as compared to untreated A549 cells. The expression level of *p53* was significantly (p<0.001) up-regulated in A549 cells treated with *T. claveryi, T. boudieri,* and *T. olbiensis* extracts by 6.4, 6.9, and 6.5-fold, respectively, in 400 µg/wells when compared to untreated cells. *Bax* gene expression was significantly (p<0.001) increased by 6.8, 7.1, and 6.5-fold, respectively, in A549 cells treated with 400 µg/well *T. claveryi, T. boudieri,* and *T. olbiensis* extracts. Regarding the *Bcl2* gene expression, its level was significantly (p<0.01) down-regulated by 0.7, 0.6, and 0.55-fold, respectively, in A549 cells treated with *T. claveryi, T. boudieri,* and *T. olbiensis* extracts at 400 µg/well, when compared to untreated cells (Figure 4.31).

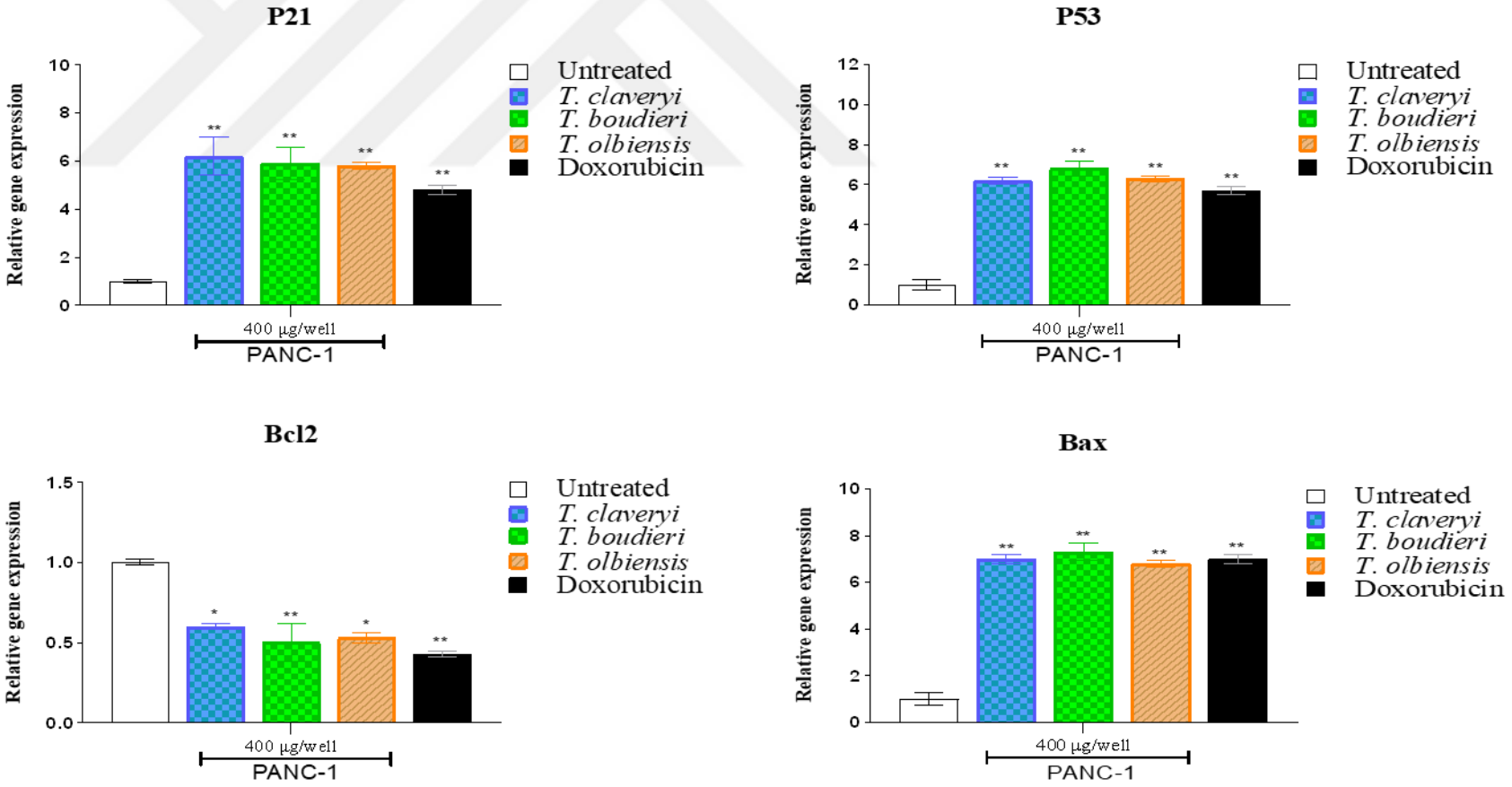

Figure 4.29. Shown the impacts of water extract of *Terfezia* on *p53, p21, Bax*, and *Bcl2* genes in the pancreatic cancer cell line (PANC-1), relative gene expressions in cells treated with IC_{50} concentration for 72 hours were compared to untreated cells. As a positive control, doxorubicin was used. Significant differences were analyzed by a one-way ANOVA test. Bars represent mean±SD

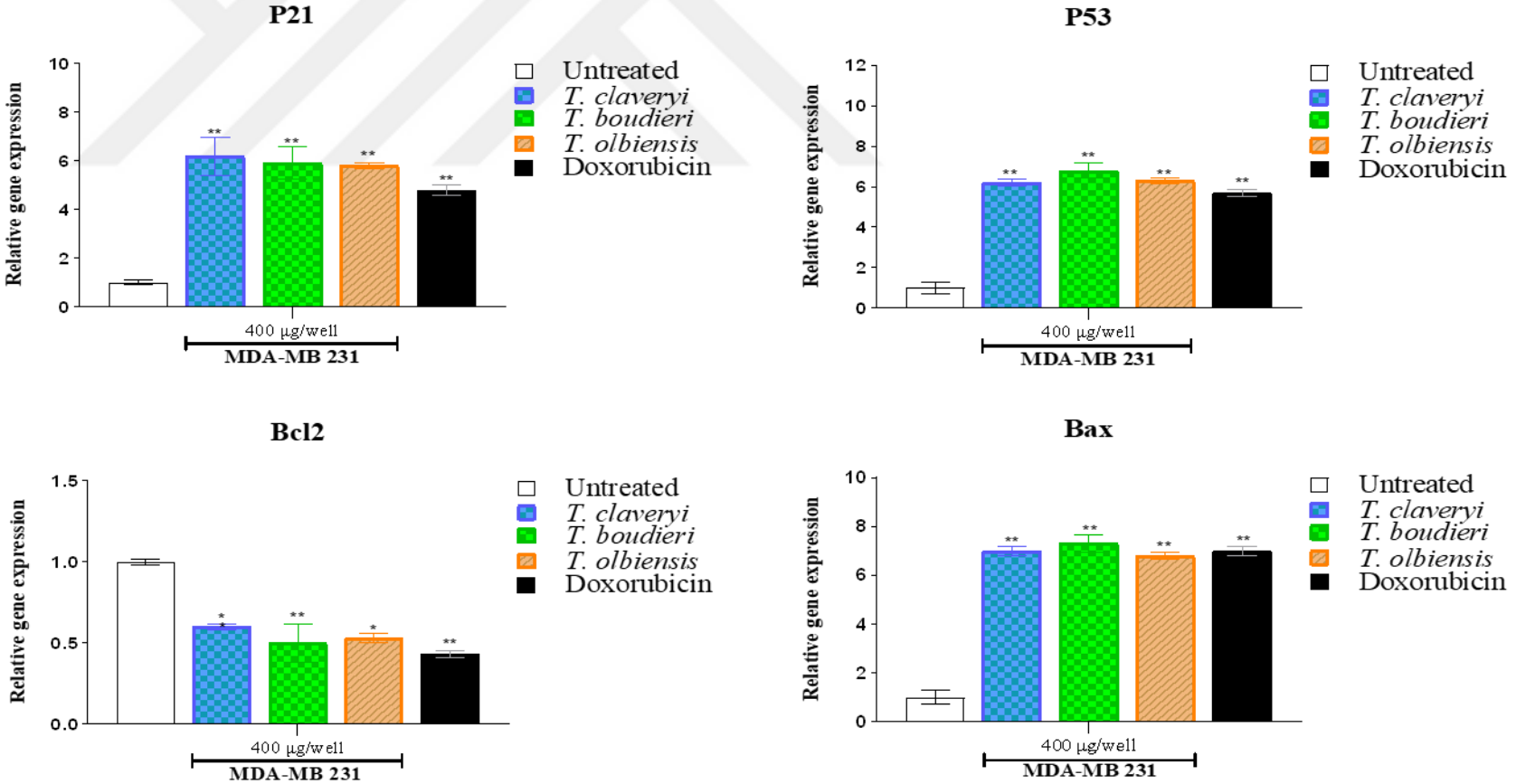

Figure 4.30. Shown the impacts of water extract of *Terfezia* on *p53, p21, Bax*, and *Bcl2* genes in the (MDA-MB 231), relative gene expressions in cells treated with IC_{50} concentration for 72 hours were compared to untreated cells. As a positive control, doxorubicin was used. Significant differences were analyzed by a one-way ANOVA test. Bars represent mean±SD

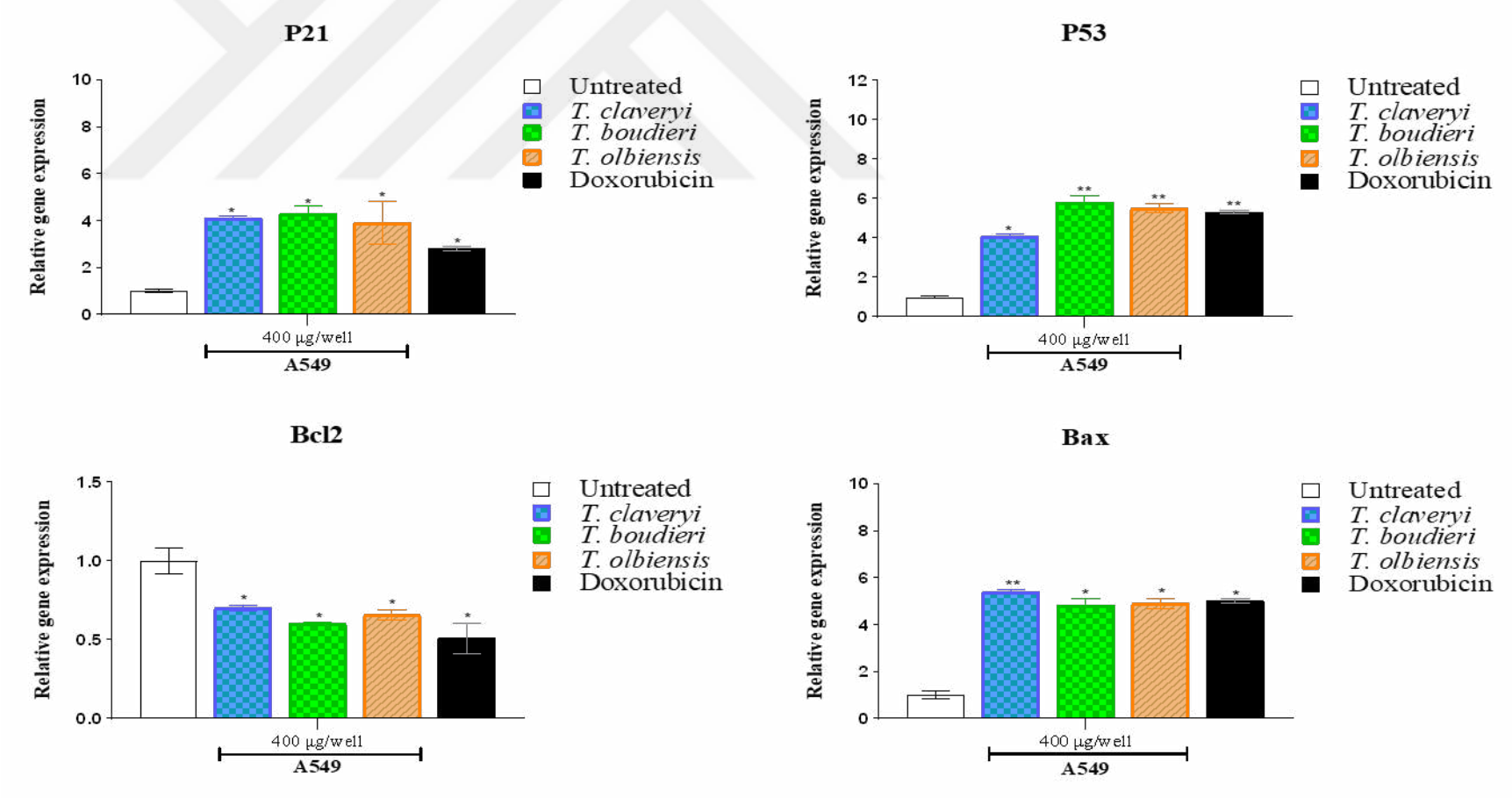

Figure 4.31. Shown the impacts of water extract of *Terfezia* on *p53, p21, Bax*, and *Bcl2* genes in the (A549), relative gene expressions in cells treated with IC_{50} concentration for 72 hours were compared to untreated cells. Doxorubicin was used as a control. Significant differences were analyzed by a one-way ANOVA test. Bars represent mean±SD

4.3.2. The effects of *Picoa* on gene expression

In this study, the effect of different concentrations of water extracts of *P. juniperi* and *P. lefebvrei* for 72 hours on the expression of *p53, p21, Bax*, and *Bcl2* genes were investigated. *p21, p53, Bcl2,* and *Bax* genes were amplified and normalized to the GAPDH gene by using qRT-PCR.

In the PANC-1 treated cells with water extracts of *P. juniperi* and *P. lefebvrei*, *p21* gene expressions were significantly (p<0.001) up-regulated by 4, and 4.2-folds respectively in treated cells with 400 µg/well. *p53* gene expression levels were significantly (p<0.001) increased in PANC-1 treated cells with extracts by 4.6, and 5.2-folds respectively in 400 µg/well when compared to untreated cells. *Bax* gene expressions were significantly (p<0.001) increased in PANC-1 treated cells with *P. juniperi* and *P. lefebvrei* extracts by 5.0, and 4.6-folds respectively in 400 µg/well when compared to untreated cells. Regarding the *Bcl2* gene expressions, its level was significantly (p<0.001) decreased in PANC-1 treated cells with *P. juniperi* and *P. lefebvrei* extracts by 0.77, and 0.61-folds respectively in treated cells with 400 µg/well (Figure 4.32).

When compared to the untreated MDA-MB 231 cancer cells, expression levels of the *p21* gene in MDA-MB 231 cells treated with 400 µg/well of water extracts of *P. juniperi* and *P. lefebvrei* were significantly (p<0.001) increased by 5.3, and 5.7-folds respectively. *p53* gene expression levels were significantly (p<0.001) increased in MDA-MB 231 treated cells with *P. juniperi* and *P. lefebvrei* extracts by 5.8, and 6.4-folds respectively in 400 µg/well when compared to untreated cells. *Bax* gene expressions were significantly (p<0.001) increased in MDA-MB 231 treated cells with *P. juniperi* and *P. lefebvrei* extracts by 6.7, and 7.1-folds respectively in 400 µg/well. Regarding the *Bcl2* gene expressions, its level was significantly (p<0.01) decreased in MDA-MB 231 treated cells with *P. juniperi* and *P. lefebvrei* extracts by 0.62, and 0.48-folds respectively in treated cells with 400 µg/well, when compared to untreated cells (Figure 4.33).

In the A549 treated cells with water extracts of *P. juniperi* and *P. lefebvrei*, *p21* gene expressions were significantly (p<0.001) increased by 3.9, and 4.1-folds respectively in treated cells with 400 µg/well. *p53* gene expression levels were significantly (p<0.001) increased in A549 treated cells with extracts by 4.5, and 5.1-folds respectively in 400 µg/well when compared to untreated cells. *Bax* gene expressions were significantly (p<0.001) increased in A549 treated cells with *P. juniperi* and *P. lefebvrei* extracts by 5.1, and 4.7-folds respectively in 400 µg/well when compared to untreated cells. Regarding the *Bcl2* gene expressions, its level was significantly (p<0.001) decreased in A549 treated cells with *P. juniperi* and *P. lefebvrei* extracts by 0.75, and 0.59-folds respectively in treated cells with 400 µg/well (Figure 4.34).

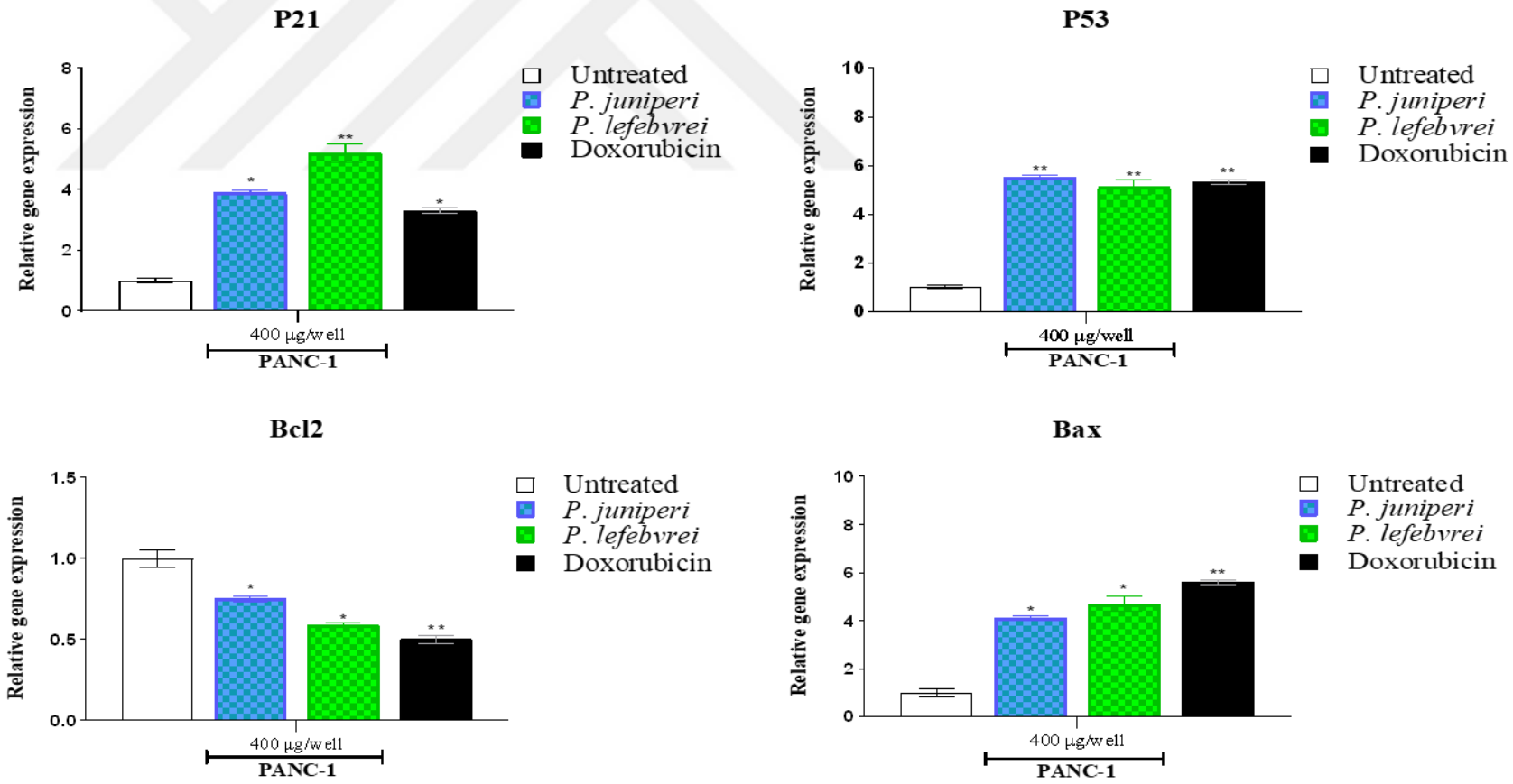

Figure 4.32. Shown the impacts of water extract of *Picoa* on *p53, p21, Bax*, and *Bcl2* genes in the (PANC-1), relative gene expressions in cells treated with IC_{50} concentration for 72 hours were compared to untreated cells. Doxorubicin was used as a control. Significant differences were analyzed by one-way ANOVA test. Bars represent mean±SD

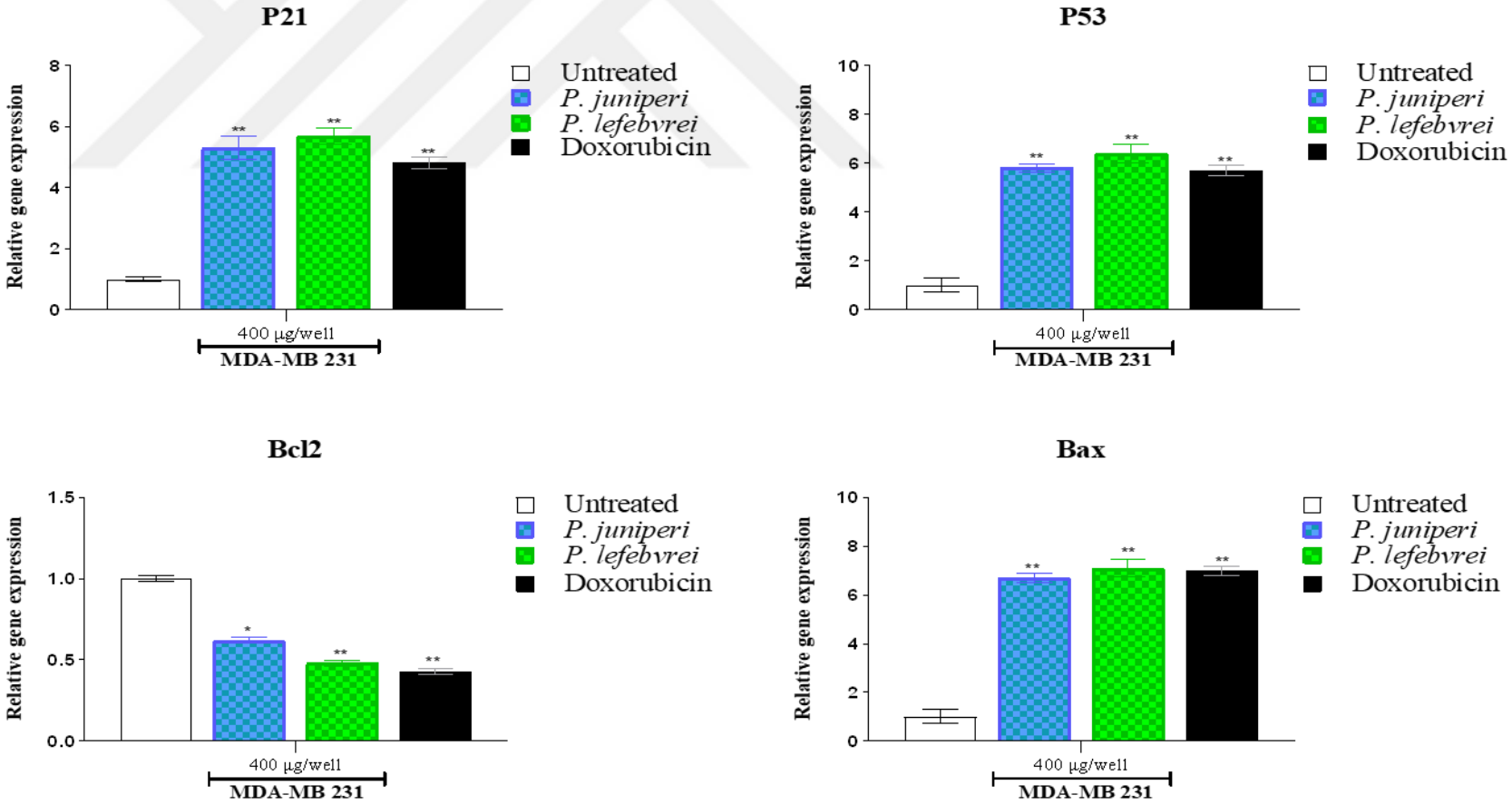

Figure 4.33. Shown the impacts of water extract of *Picoa* on *p53, p21, Bax*, and *Bcl2* genes in the (MDA-MB 231), relative gene expressions in cells treated with IC_{50} concentration for 72 hours were compared to untreated cells. Doxorubicin was used as control. Significant differences were analyzed by one-way ANOVA test. Bars represent mean±SD

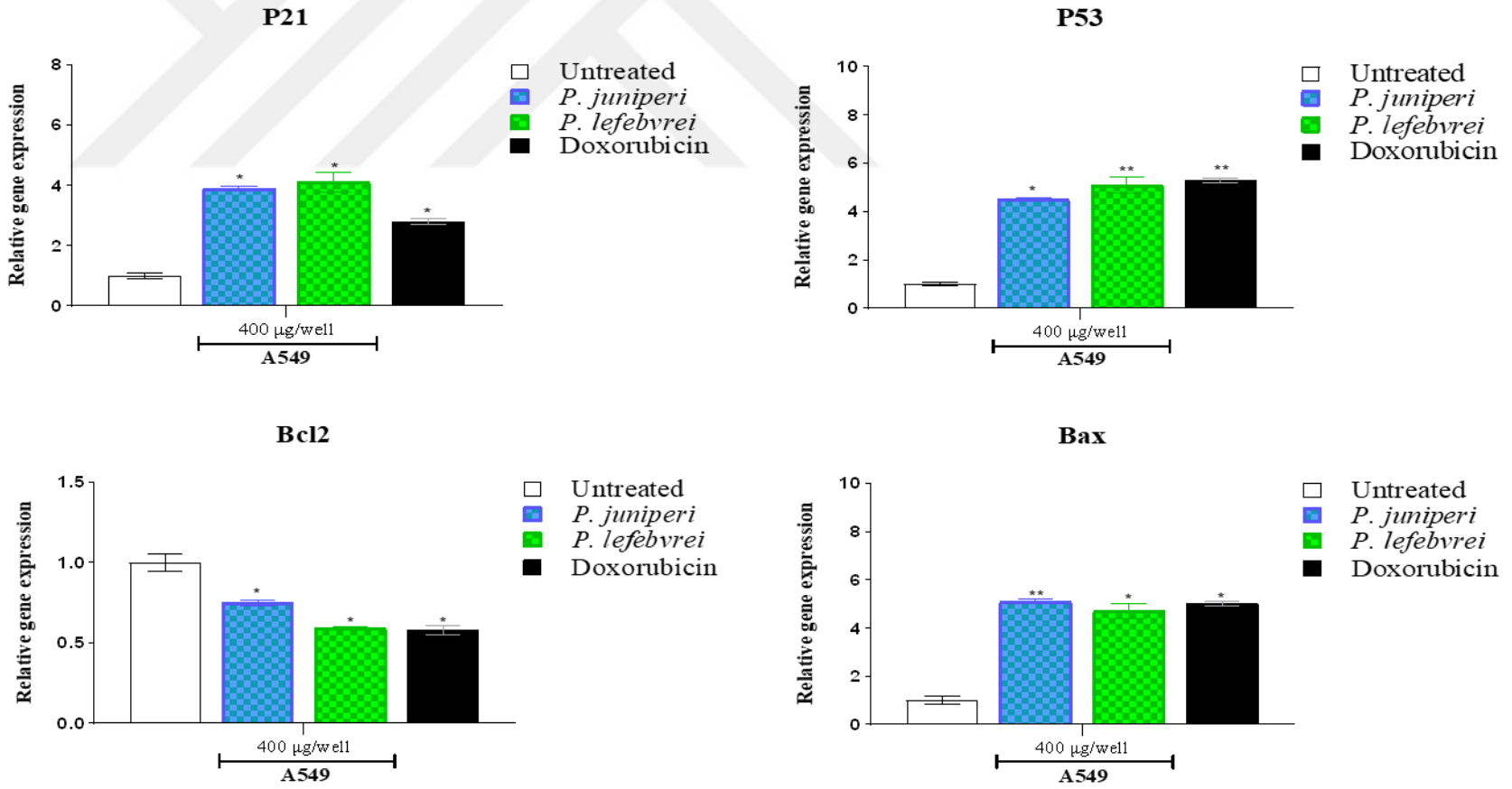

Figure 4.34. Shown the impacts of water extract of *Picoa* on *p53, p21, Bax*, and *Bcl2* genes in the (A549), relative gene expressions in cells treated with IC_{50} concentration for 72 hours were compared to untreated cells. Doxorubicin was used as control. Significant differences were analyzed by one-way ANOVA test. Bars represent mean±SD

5. CONCLUSIONS

First, several extracts were produced using different solvents (water, methanol, acetone) and tested for cytotoxic activity and other biological characteristics (inducing apoptosis and effects on gene expression) in three distinct human cancer cell lines (pancreatic cancer PANC-1, breast cancer MDA-MB 231, lung cancer A549). To begin, all extracts were chosen to be evaluated for cytotoxicity on the PANC-1, MDA-MB 231, and A549 cell lines. The series of concentrations for each extract (water extract, methanol extract, acetone extract), including (400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml). *Terfezia claveryi, T. boudieri, T. olbiensis, Picoa juniperi,* and *P. lefebvrei* were identified utilizing differential scanning of microscopical, macroscopical, and morphological investigations using conventional mycological procedures. Using the data of the MTT reaction and an ELISA multiple readers, certain basic calculations were conducted, such as half-maximal inhibitory concentration (IC_{50}) and GraphPad prism.

Cytotoxic activity is one of the most important characteristics to consider when evaluating the medicinal effects of desert truffles. Significant cytotoxic activity of extracts from *T. claveryi, T. boudieri, T. olbiensis, Picoa juniperi,* and *P. lefebvrei* on PANC-1, MDA-MB 231, and A549 cell lines were detected in our investigation. T. *claveryi, T. boudieri, T. olbiensis, Picoa juniperi,* and *P. lefebvre*i extracts were found to have a promising growth inhibitor effect. The inhibitory activities of water extracts of *T. claveryi, T. boudieri, T. olbiensis, Picoa juniperi,* and *P. lefebvrei* on PANC-1, MDA-MB 231, and A549 cell lines were verified in this work. Inhibiting cancer cell growth might result in the activation of apoptosis and/or necrosis. As a result, the involvement of nuclear condensations and fragmentation in apoptotic and/or necrotic pathways was studied. Then, one of the effective extracts and concentrations from water, methanol, and acetone extracts of *T. claveryi, T. boudieri, T. olbiensis, Picoa juniperi,* and *P. lefebvrei* was chosen to explore its apoptosis-inducing capabilities and gene expression impacts. Water extracts and the initial concentration (400 µg/well) were chosen for further molecular research.

The apoptotic and necrotic activities of water extract on PANC-1, MDA-MB 231, and A549 cell lines were assessed using the standard double staining Hoechst 33342 and Propidium Iodide (HOPI) model. The cell lines were treated with truffle extracts (400 µg/well) and evaluated 48 hours later. As negative and positive controls, DMEM (10% FBS with 1% Penicillin-Streptomycin) and doxorubicin (2.5 µg/ml) were utilized, respectively. Nuclear morphological alterations were assessed using a fluorescent EVOS FLc digital microscope. The results showed that water extract of T. *claveryi, T. boudieri, T. olbiensis, Picoa juniperi,* and *P. lefebvrei* exhibited substantial apoptotic rather than necrotic effects on the PANC-1, MDA-MB 231, and A549 cell lines, comparable to doxorubicin's impact (positive control). These findings imply that the apoptotic pathway is important in the inhibition of cancer cells.

Subsequently, the MTT analysis and HOPI double staining findings confirmed that the water extracts are cytotoxic and induce apoptosis. The involvement of specific genes involved in apoptotic pathways was investigated to clarify these findings. Four target genes were included: *BAX*, *Bcl-2, P21,* and *P53*, as well as one reference gene, *GAPDH*. To examine gene expression, quantitative real-time PCR (QRT-PCR) was used. According to the findings, the p21, p53, and BAX genes were substantially ($p<0.001$) up-regulated in treated cells by more than 3-fold. When compared to untreated cells, the Bcl2 gene was dramatically ($p<0.001$) down-regulated by more than 0.3-fold. Finally, the inhibitory effects of *Terfezia* and *Picoa* water extract on the PANC-1, MDA-MB 231, and A549 cell lines can be regarded as functional natural products.

RECOMMANDATIONS

Our findings will be useful to other studies that want to look at more genes linked to apoptosis to see if they can identify more effective genes for different biological pathways. We urge that the investigator extend the sample of truffles and include other groups. We anticipate that incorporating these two types of mushrooms will aid researchers in detecting genetic issues and determining the gene expression biomarker more precisely for cancer treatment. Desert truffles of several species were explored in this study. The primary goal was mostly to enhance the medicinal characteristics of *Terfezia* and *Picoa* species by evaluating cytotoxic activities, apoptosis induction, and effects on molecular pathways in cancer cell lines. Other gene families such as caspases, an inhibitor of apoptosis proteins, and tumor necrosis factor (TNF) receptor gene superfamily are recommended. To identify particular protein molecules related to gene expressions, western blot methods are recommended.

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CURRICULUM VITAE

Kochar Khasro SALEH

[redacted]

ACADEMIC ACTIVITIES

Paper:

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