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Phytochemical Investigation and Cytotoxic Effect against A459 Cell Line via Iraqi *Portulacaria afra* (f. *Protulacaea*) Ethyl Acetate Whole Plant Extract

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Abstract

The coniferous plant extracts have recently been found effective against the global prevalent lung tumors disorders. *Portulacaria afra Jacq.* (Spekboom or Pork bush) is a medicinal plant belonging to the family of *Didieraceae*, which contains seven genera and twenty species.The plant's historical advantages and mythical significance include its usage as a diuretic and for sores, persistent rash, heartburn, and rheumatism. Additionally, it has been claimed that *P. afra* exhibits antibacterial, antifungal, antioxidant anti-inflammatory, and cancer-prevention. In the former total extract phytochemical studies was investigated but screening the ethyl acetate fraction was only discussed at limited range., the main aim of this study is to detect the presence of different polyphenolic constituents in the plant ethyl acetate extract by reverse phase-high performance liquid chromatography (RP-HPLC) and evaluate the cytotoxic effect of ethyl acetate extract of *P. afra* on human lung cancer (A549) cell line using the 3-[4,5- dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Phytochemical analysis of the *Portulacaria afra* ethyl acetate extract identified five polyphenolic constituents designated as phenolic acids According to the result, the ethyl acetate extract that contains the high concentration of polyphenolic constituents demonstrated the highest activity as cytotoxic against lung cancer A549 cell line with IC50 value of 0.47 µg/ml.

Keywords: Ethyl acetate extract, Ferulic acid, Myricetin, Sinapic acid, A549 cell line.

Introduction

About Two million new cases of cancer are diagnosed each year worldwide, with lung cancer being one of the most prevalent cancers. 1.7–1.8 million deaths were caused by lung cancer[(1)]. Numerous natural products have been studied for their anticancer activity against various cancer types both in *vivo* and in *vitro* because they represent an important source of biologically active compounds that could be useful in the treatment of cancer [(2,3)]. *Portulacaria afra* is an evergreen shrub with one-meter-long, stem is erected 30-80 cm from ground and succulent leaves previously belonging to the Portulacaceae family, but due to morphological and anatomical research as well as RNA sequences and geographic distribution, *Portulacaria afra* was classified as part of the Didieracea family [(4)]. Due to its high water content and sour, acidic flavor, spakboom is edible and used as vegetables in South Africa [(5)]. In South Africa, *P. afra* is used as a folk remedy for a variety of ailments, including sores, chronic rashes, dermatitis, increased milk production in nursing mothers, fever, heartburn, quenching thirst in dehydration hypertension, rheumatism, and as diuretic [(6)]. The plant is distributed widely in, East Africa, Southern Africa and the semi-arid area of Madagascar [(7)]. It is indigenous to the following countries: Norway, Mexico, India, New Caledonia, Germany, Sweden, Eswatini, Australia, Italy, Malta, Kenya, Mozambique, Morocco, USA, El Salvador, and Sweden[(8)]. The plant is known by several names, including Spekboom, elephant food, and pig bush. In Iraq, it is known as dumooa Altefel [(9)]. The plant is replicated through cuttings as opposed to seeds and has reddish stems, tiny oval green leaves, and small pink flowers that are rarely in bloom [(4)]. Additionally, *P. afra* has a variety of phytoconstituents that influence its pharmacological capabilities, such as its antibacterial, antioxidant, antifungal, anti-inflammatory, and anticancer effect [(10, 11)] . Secondary metabolites in the Spekboom includes fatty acids, vitamins, minerals, steroids (stigmasterol, β-sitosterol), phenolics, such as (P-Hydroxybenzoic acid, Protocatechuic acid) glycosides (vitexin, isovitexin), and flavonoids like (Mearnsetin, rutin) are responsible for the biological

activity [12,13]. To the best of our knowledge, few papers on *Portulacaria afra* were recorded so far, which generated interest in doing scientific research on the polyphenolic compositions and cytotoxic properties of Iraqi *Portulacaria afra* ethyl acetate leaves extract.

Figure 1. Photo of Iraqi *Portulacaria afra* plant cultivated in Iraq shows the shape of the plant's aerial parts.

Material and method

All the investigations conformed to the ethics of research and the study was approved by the Ethical Committee of the University of Baghdad.

Plant material collection

The whole of *Portulacaria afra Jacq* plant (cultivated in Iraq) was collected from a medicinal plants garden in Department of pharmacognosy and Medicinal plants, collage of pharmacy, University of Baghdad in April 2021. The plant was identified and authenticated by Baghdad University Herbarium of the Department of Biology, College of Science at the University of Baghdad registered at BUH No. 19147. The plant was rinsed, dried under shade, and pulverized in a mechanical grinder for extraction purposes [14].

Preparation of Portulacaria afra Jacq extract

Portulacaria afra Jacq powder (25 g) was macerated in 250 ml of n-hexane for a day before being filtered. The remaining plant components were extracted using a soxhlet device for 12 hours with 250 ml of aqueous ethanol 85%. The crude extract was filtered and concentrated under vacuum using rotary evaporator. As depicted in Figure 2, the crude extract was suspended in water and fractionated with 100 ml of ethyl acetate twice was then saved for later analysis[15,16].

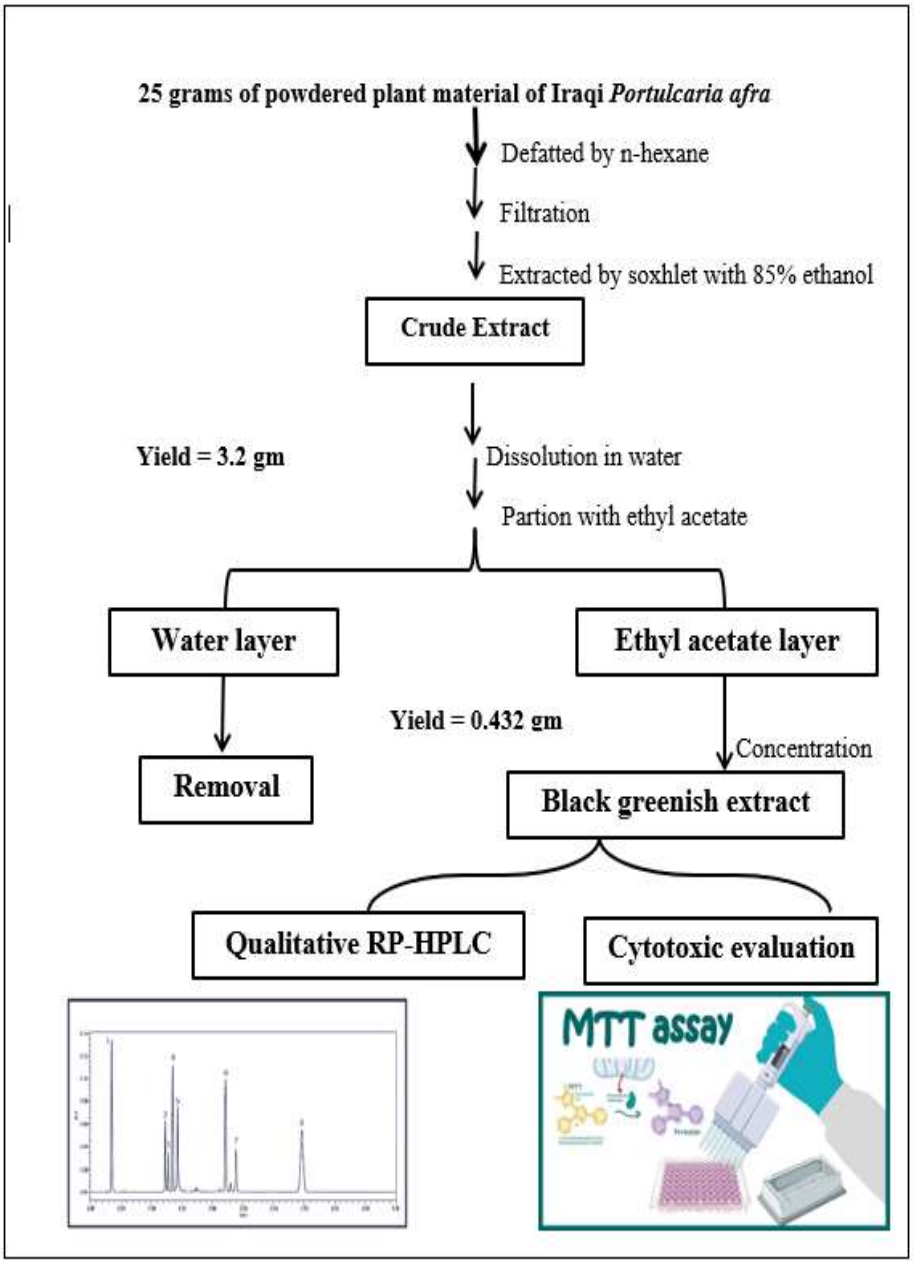

Figure 2. Scheme of extraction of polyphenolic compounds using 85% ethanol, Fractionation with100 ml ethyl acetate solvent , Qualitative analysis of ethyl acetate extract by (RP-HPLC), and cytotoxic evaluation of Iraqi Portulacaria afra ethyl acetate extract using MTT assay(17)

Qualitative analysis of, ethyl acetate extract, by Revers phase high-performance liquid chromatography (RP-HPLC)

High-performance liquid chromatography, or HPLC, is a widely utilized technique for qualitative analysis and the separation of mixture ingredients. The Ministry of Industry and Minerals at Al-Jadriyah, Baghdad, carried out the HPLC analysis. SYKAM and the eluted peak from liquid chromatography were observed using a UV-Vis 10A-SPD spectrophotometer.

Validated HPLC conditions for ethyl acetate extract

- **Mobile phase:** Linear gradient elution A (0.1% formic acid) B (acetonitrile) the linear ratio as follows (0-15 min, 95%A; 15-35 min, 88% A; 35-55 min, 75% A; 55-65 min, 60% A).
- **Column:** SYKAM LC C18 (250 mm x 4.6 mm, 5 µm particle sizes).
- **Sample:** ethyl acetate extract
- **Standards:** Sinapic acid (E1), Ferulic acid (E2), Caffeic acid (E3), Quercetin, (E4),Myricetin (E5),
- **Flow rate:** 1 ml/min
- **Injection volume:** 2ml

• **Detection:** UV Detector at λ 230 nm [17,18].

Cytotoxic Evaluation of the ethyl acetate extract of Portulacaria afra

The methodology described here is to investigate the effects of ethyl acetate extract of *Portulacaria afra* on the viability of human lung cancer cell line(A549) compared with Erlotinib (FDA approved) as control and its effect on the human lung cancer cell line by3-(4,5-Dimethylthiazol-2-yl)-2, 5 Diphenyl tetrazolium Bromide(MTT)colorimetric assay which is a colorimetric assay for assessing cell metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT, to its insoluble formazan, which has a purple color .

Chemicals

MTT 3-(4,5-Dimethylthiazol-2-yl)-2, 5 Diphenyltetrazolium Bromide was purchased from Promega (USA), while Erlotinib was supplied by Shanghai Roche Pharmaceuticals Ltd, China. Erlotinib was dissolved in dimethyl sulfoxide (DMSO) (ZHENGZHOU MEYIA CHEMICAL PRODUCTS CO. LTD.), while ethyl acetate extract (EAE) was reconstituted in distilled water. The MTT was diluted in Phosphate buffer saline (PBS) at a rate of 5 mg/ml to create the MTT stock solution. Filtration was used with a 0.22 µm filter unit to sterilize this solution. DMSO solvent was used to solubilize the formazan crystals in percentage 0.1% v/v in each well microtiter plates. Antibiotics (100U/ mL penicillin and 100 g/mL), RPMI -1640 medium and fetal bovine serum (FBS) were purchased from Euro clone. (Italy's Euro clone). All compounds were filter sterilized using 0.22 µM Millipore syringe filter.

Cell Culture

The American Type Culture Collection ATCC (USA) provided the human lung non-small cell carcinoma cell line A549. These cells were then collected and stored in the cell bank of the tissue culture research center of AL- Mustansiriyah University/College of Pharmacy. The fetal bovine serum (FBS) 10 %, 2 M of L-glutamine, and 1% penicillin/streptomycin were added to the standard RPMI 1640 medium to complement it. A humidified 5% CO2 atmosphere was used to sustain the cell culture at 37 °C. A logarithmic growth phase was maintained for the cells. Mycoplasma contamination in cells was routinely checked.

Cell Viability Assay

The MTT assay was used to assess the cytotoxic effects of ethyl acetate extract of *Portulacaria afra* on human lung carcinoma cell line A549. Cells were seeded in 96-well plates at a density of 5x 10^3 cells per well and cultured in a CO_2 incubator for 24 hr., 48 hr., and 72 hrs. Following overnight culture, cells were then treated with ethyl acetate extract (tested compound) and Erlotinib. The cells were exposed to each compound for 48 hrs. before 10 µL of MTT reagent (Sigma Aldrich) was added to each cell. The medium was removed after 4 hrs. of incubation (37 ℃ , 5% CO_2) to generate formazan crystals, which were then dissolved in 100 µL of DMSO solution. The optical density of each well was measured at 530 nm using Glomax microplate reader (PromegaUSA.). Each individual experiment was achieved in 3 replication.

The cell viability was calculated using the following formula: Cell viability (%) = {(absorbance of the treated cells) / (absorbance of the untreated cells)} × 100. The IC50 (the concentration required for 50 % cell inhibition) was calculated using the GraphPad Prism 9.2 program (GraphPad Software Inc., USA) [19, 20].

% Inhibition rate of cells = 100 − (Test OD/Non-treated OD) x 100) OD: optical density [19]

Morphological Changes Study

The morphology of A549 cells was studied under an inverted microscope with 40x magnification (Optika, Italy) to view and record the morphological changes of the apoptotic cells. Apoptotic characteristics were identified after 48 hours of incubation by the appearance of cell shrinkage and/or the presence of membrane-bound cellular bodies.

Statistical analysis

The MTT assay data were expressed using the mean and standard deviation (mean±STD). The IC50 values for each were calculated using GraphPad Prism 9.2 software (GraphPad Software Inc., USA), The following equation used to estimate IC50 values [19] .

$$\boldsymbol{Y = Bottom + (Top - Bott0m)/1 + 10\text{^}((Log\ IC50 - X) * HiII\ Slope)}$$

Y: Response

X: Log of concentration

Top and Bottom: Plateaus in same units as Y

HiII Slope: Slope factor, unit less

Results and Discussion

The whole plant was extracted using the Soxhlet process, which is heat dependent and allows solvent to penetrate through the walls of the plant pieces. Differences in polarities were used as a critical separation step in order to analyze interconnected chemicals in extracts preceded by different polarity n-hexane and ethyl acetate fractionation solvents, The yield of the concentrated ethyl acetate extract was 0.432 gm and had a percentage of 1.732%.

Qualitative determination of phenolic compounds using RP-HPLC

HPLC is a flexible, stable, and sensitive instrument that can provide a lot of information about the content of the extract [20, 21]. The main

purpose of HPLC method validation is to examine the methodology of the analysis which are performing and ensure that works well with the goal of the experiment—be that quantifying compounds, purifying them, or any other intention. Therefor the pre-validated HPLC qualitative estimation of phenolic acids and flavonoids in the *Portulacaria afra* ethyl acetate extract in comparison to authentic samples such as, (Sinapic acid, Ferulic acid, Caffieic acid , Quercetin , Myricetin) was carried out according to Flandez et al. [22] .The retention times of the sample components were compared with the authentic ones at identical chromatographic condition.

The RP-HPLC results demonstrated the presence of phenolic acids (Sinapic acid, Ferulic acid, and Caffeic acid) , flavonoids (Quercitin,and Myricetin), whose retention times matched those of their standards, as shown in Figure 3, and Table. 1

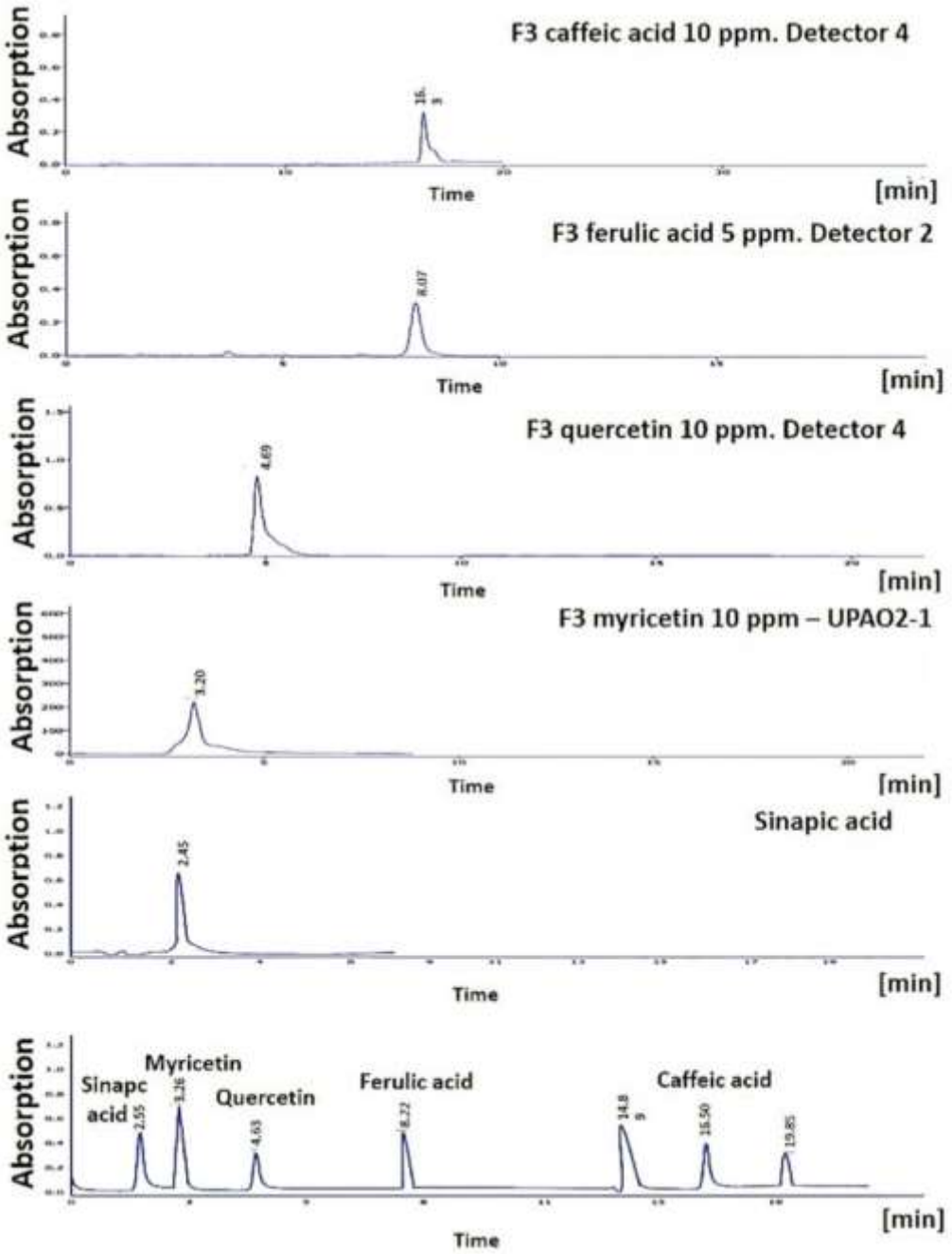

Figure 3. HPLC chromatograms of polyphenolic standards (caffeic acid, Sinapic acid, ferulic acid, quercetin, and myricetin), and EAE of Iraqi *Portulacaria afra* using SKYAM HPLC by linear gradient elution ,Mobile phase: A (0.1% formic acid) B (acetonitrile) .

Table 1. Table shows the retention time of polyphenolic standards as compared with EAE sample in min under same chromatographic condition.

Name of standard	R_t of standards min.	R_t of sample min.
Ferulic acid	**8.07**	**8.22**
Caffieic acid	**16.3**	**16.5**
Sinapic acid	**2.45**	**2.55**
Myricetin	**3.20**	**3.26**
Quercetin	**4.69**	**4.63**

Cytotoxic effect of Iraqi Portulacaria afra against lung cancer cells.

The cytotoxicity results are illustrated in Figure (4,5) and Table .2 showing an inhibitory concentration (IC50) for Erlotinib when measured after 24hr., 48hr. and 72hr. were (33.5, 13.9, 0.8755 µg/dl) respectively these concentration were lower as compared to *Portulacaria afra* extract in which, it found that inhibitory concentration (IC50) for this extract when measure after 24hr, 48 hr. and 72 hr. were(610.5, 244.7, 47.01µg/dl) respectively . According to the results for Erlotinib and plant extract, the cytotoxic activity have been decreased as the time was increase which give an indication that cytotoxic activity was time dependent. In MTT assay, the plant extract showed significant toxicity ($p< 0.05$) toward the Human lung non-small carcinoma cell line A 549 mainly at the concentration 1000mg/ml with no significant differences comparing to control drug (Erlotinib) after 72 hour as in Fig 5.

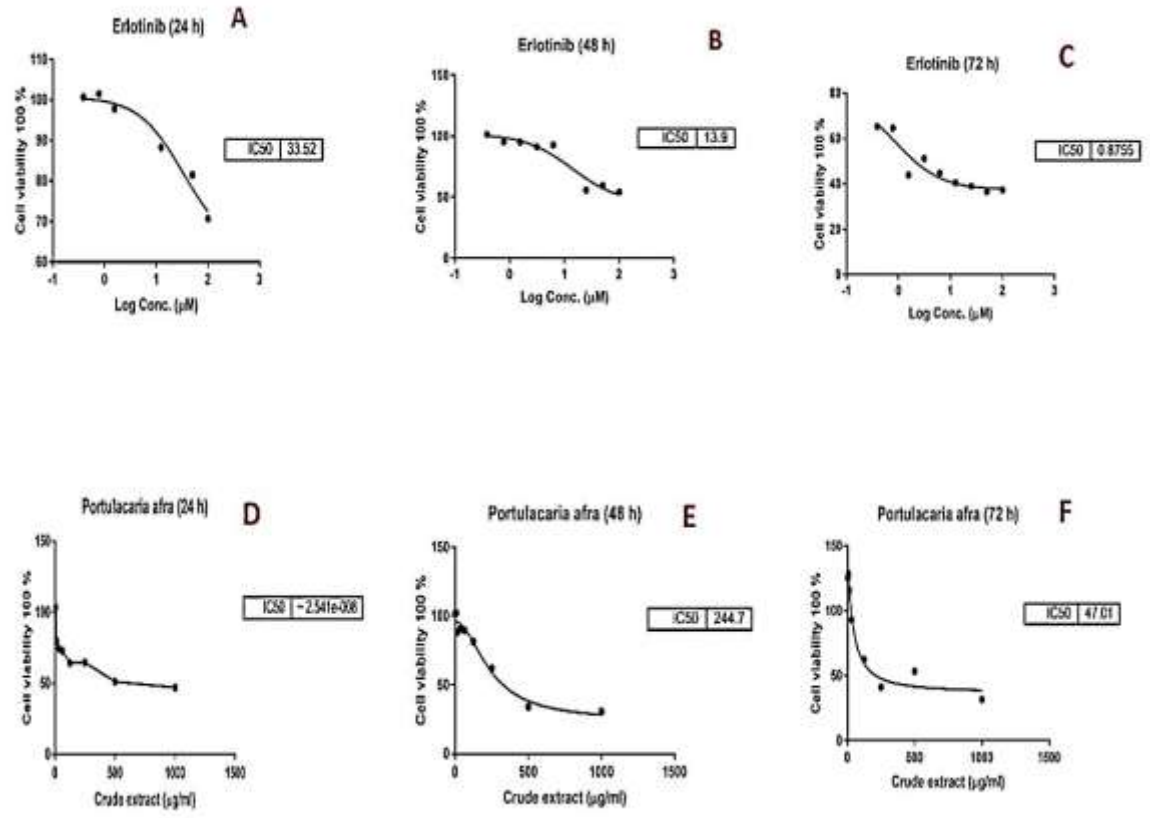

Figure 4 . Dose-response curves of IC50 for assessment the effect of *Portulacaria afra* extract &Erlotinib against A549 cancer cells. Fig. A-C represents IC_{50} of positive control Erlotinib on A549 cell line. Fig. D-F represent IC50 of EAE of *Portulacaia afra* on A549 cell line after 24 hr., 48hr. ,and 72hr .

Table2.The IC50 for *Portulacaria afra* extract and Erlotinib on A549 cell line after 24 hr., 48 hr., and 72hr.

Sample	IC 50 after 24hr	IC 50 after 48hr	IC 50 after 72hr
Erlotinib (positive control) (µg/ml)	33.5	13.9	0.8755
Portulacaria afra extract (µg/ml)	610.5	244.7	47.01

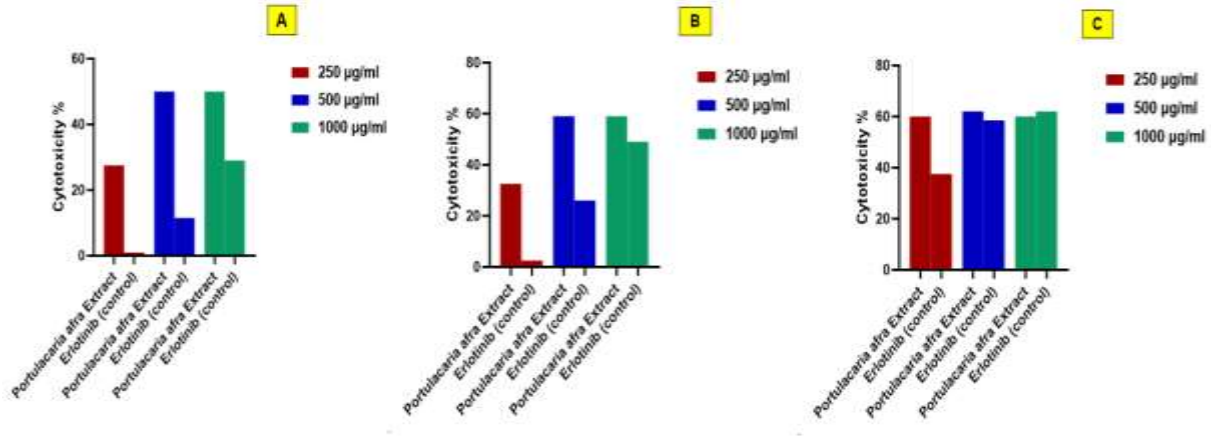

Figure 5. Cytotoxicity effects of the different concentration (250 mg/100ml, 500 mg/ml, 1000 mg/ml) of Extract of *Portulacaria afra* compared to Erlotinib against Human lung non-small carcinoma cell line A 549 after 24 hours (A), 48 hours (B) and 72 hours (C).

Morphological Comparison of lung cancer cells A549 upon exposure to Erlotinib and Portulacaria afra extract

Studies on cell morphology have also demonstrated that cells undergo morphological alterations as cell de-attachment and cell separation as well as a considerable inhibition of cell proliferation as shown in Figure . 6.

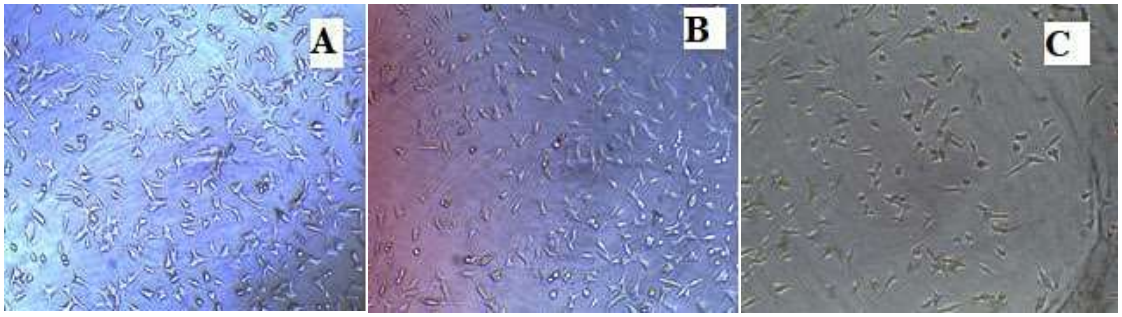

Figure 6. Effect of EAE and Erlotinib on the Morphology of lung cancer cells (A549cell) A: before treatment, B: After treatment with erlotinib C: After treatment with *Portulacaria afra* ethyl acetate extract. Compared with the control group, treated cells exhibited cell de-attachment and cell separation as well as a considerable inhibition of cell proliferation.

The phytochemical analysis by RP-HPLC demonstrated the presence of flavonoids and phenolic acids in the *Portulacaria afra* ethyl acetate extract which is one of the significant classes of substances known as phenolic compounds that function as antioxidants [23].

Tyrosine kinase receptors (RTKs) are essential for regulating the cell division cycle. There are currently over 50 RTKs described, grouped into various subfamilies. The inhibition of these enzymes has emerged as a significant research topic. Compounds that reduce the activity of these enzymes are likely to have anti-proliferative effects [24]. Since the U.S. Food and Drug Administration (FDA) approved Erlotinib for the treatment of locally advanced non-small cell lung cancer(NSCLC) in the United States in 2004 , So use it as a model for comparison with my plant extract as preliminary study [25]. Erlotinib is an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor used to treat non-small cell lung cancer, pancreatic cancer, and various other forms of cancer. It is commonly marketed under the brand name Tarceva. Erlotinib interacts to the epidermal

growth factor receptor (EGFR) tyrosine kinase in a reversible manner at the receptor's ATP binding site [26].

The primary mechanisms of *Portulacaria afra* extract's cytotoxicity against cancer cells may be connected to its nitric oxide scavenging activity, suppression of hydroxyl radicals, metal chelating activities, peroxides breakdown, and oxygen quenching of phenolic compounds[27,28]. The ability of herbal extracts and natural compounds to inhibit cellular viability, growth, proliferation, and colony formation can be determined as an indication of cytotoxicity, which is widely used in drug discovery and basic research to screen and identify toxic compounds and provide guidance for the design and development of in vivo tests. Natural cytotoxic substances can block cellular attachment, induce noticeable morphological changes, inhibit cell cycle and DNA replication, and dramatically reduce cell viability [29]. Other expected mechanism for flavonoids serve as RTK inhibitors by attaching to the ATP-binding sites of many kinases. The most reasonable current idea for how these chemicals function on kinases is that the flavonoid's chromenone moiety mimics the adenine moiety of ATP, the receptor co-factor, as related to the Erlotinib action [24] .

Myricetin has been identified as having anti-cancer potential through modulation of a variety of cell signaling molecules and pathways, including inflammation, apoptosis, cell cycle, PI3K/Akt, angiogenesis, transcription factor/components, and also induced sub-G1 phase aggregation of cells and reduce in the fraction of cells incoming the S as well as subsequent phase, as well as by arresting the progression of cell cycle and ROS–dependent mitochondria-mediated mortality in cancer A549 lung cancer cells and it would be useful to develop as a drug candidate for lung cancer therapeutics. [30, 31] .

In human lung carcinoma A549 cells, researchers demonstrated that quercetin appreciably suppresses cell invasion and migration. It inhibits the activity and expression of MMPs-2 in a dose-time dependent manner. It also increases the expressions of nm23-H1 and TIMP-2 and inhibits the protein expression of MMP-2. GW9662, a PPAR-γ antagonist [32-34].

Plant phenolic acids are secondary metabolites that have gained importance as potential anti-cancer compounds. Their potency as anticancer compounds is primarily attributed to their antioxidant activity; being strong radical scavengers, metal chelators, modifiers of endogenous defense mechanisms as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPx), enhancers of glutathione (GSH) redox status, and regulators of diverse proteins and transcriptional factors such as nuclear factor erythroid related factor (NRF2). Moreover, their anticarcinogenic effects is associated with their ability to inhibit cell proliferation (extracellular signal-regulated kinase (Erk)1/2, D-type cyclins, and cyclin-dependent kinases (CDKs)), angiogenic factors (vascular endothelial growth factor (VEGF) and MIC-1), oncogenic signaling cascades (phosphoinositide 3-kinase (PI3K) and protein kinase B (Akt)), inducing apoptosis, and preventing cellular migration and metastasis [35-38] .

Treatments using herbal medications may raise major concerns regarding their toxicity profiles in the lack of scientific data ,Thus, the toxicity of herbal medications must be evaluated in an animal model to ensure their safety profile for human use. The acute toxicity test was carried out on albino rats in accordance with OECD recommendations. On the oral administration of PAME at a dose of 5000 mg/kg to albino rats, no significant weight variation or changes in the behavioral pattern were noticed in the body weight of animals when treated groups were compared to the control group of animals [39-41].

Conclusion

According to the findings of this study, the phytochemicals present in the leaves extract of Iraqi *Portulacaria afra* can be regarded as a significant source of medicine and as being essential for good health. Five polyphenolic chemicals have been discovered in this study's leaves extract of *Portulacaria afra*, several of them for the first time. The outcome demonstrates that the RP-HPLC method can be used for qualitative measurements of phenolic acids, and flavonoids in the dried leaf extract of *Portulacaria afra*. Additionally, the ethyl acetate extract of the dried leaves of this plant was effective against the lung cancer A549 cell line with an IC50 value of 0.4701 µg/ ml, and the presence of polyphenolic chemicals and other related substances is mostly responsible for the plant's good cytotoxic properties.

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Conflicts of Interest

There is no conflict of interest regarding the publication of my manuscript.

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Ethics Statements

The manuscript did not include human and/or animal studies, so ethical approval is not needed for this research.

Author Contribution

The author (**Nabaa M. Ibrahim**) conceived and planned the experiments, carried out the sample preparation and extraction process, analyzed the extraction sample, evaluated the cytotoxic activity, and doing the statistical analysis. The author also wrote the manuscript.

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التحقيق الكيميائي النباتي والتأثير السام للخلايا لمستخلص دمعة الطفل العراقية من خلاصة إيثيل أسيتات

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الخلاصة

تبين مؤخرا أن مستخلصات النباتات الصنوبرية فعالة ضد اضطرابات أورام الرئة المنتشرة عالميا. دمعة الطفل (سبيكبوم أو شجيرة لحم الخنزير) هو نبات طبي ينتمي إلى عائلة ديديراسيا، التي تحتوي على سبعة أجناس وعشرين نوعا. تشمل المزايا التاريخية للنبات وأهميته استخدامه كمدر للبول ولعلاج القروح والطفح الجلدي المستمر وحرقة المعدة والروماتيزم. بالإضافة إلى ذلك، يُزعم أن دمعة الطفل يُظهر مضادات للبكتيريا والفطريات ومضادات الأكسدة المضادة للالتهابات والوقاية من السرطان. في الدراسات الكيميائية النباتية للمستخلص الكلي السابقة، تم التحقيق ولكن فحص جزء أسيتات الإيثيل تمت مناقشته فقط في نطاق محدود. الهدف الرئيسي من هذه الدراسة هو اكتشاف وجود مكونات بوليفينولية مختلفة في مستخلص إيثيل أسيتات النبات عن طريق سائل عالي الأداء في الطور العكسي. تحليل كروماتوغرافي وتقييم التأثير السام للخلايا لمستخلص أسيتات إيثيل دمعة الطفل على خط خلايا سرطان الرئة البشري (A549) باستخدام مقايسة ٣ - [٤،٥- ثنائي ميثيل ثيازول -٢-يل] -٢،٥-بروميد ثنائي فينيل تيترازوليوم (MTT). حدد التحليل الكيميائي النباتي لمستخلص أسيتات إيثيل دمعة الطفل خمسة مكونات بوليفينولية تم تصنيفها على أنها أحماض فينولية (حمض الكافيين، وسينابيك، والفيروليك)، والفلافونويدات (كيرسيتين وميريستين). وفقا للنتيجة، أظهر مستخلص أسيتات الإيثيل الذي يحتوي على تركيز عال من مكونات البوليفينول أعلى نشاط كسام للخلايا ضد سرطان الرئة خط الخلايا A549 بقيمة IC50 البالغة ٠،٤٧ ميكروغرام / مل

الكلمات المفتاحية: مستخلص خلات الإيثيل، الأحماض الفينولية، الفلافونويد، HPLC، خط الخلايا A549.