Trop J Nat Prod Res, October 2024; 8(10):8764 - 8773 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

LC-MS Analysis and Biological Potential of the Peel Extracts of *Citrus aurantium* Grown in Jordan

Ali M. Khalid[1], Asma I. Mahmod[2], Hasan M. Rashid[3], Wamidh Talib[2], Eliza Hasen[4], Mohammad Al-Najjar[4], Fatma Afifi[1]*

[1]*Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmacy, Applied Science Private University, Amman 11931-166, Jordan*
[2]*Department of Clinical Pharmacy and Therapeutics, Faculty of Pharmacy, Applied Science Private University, Amman 11931-166, Jordan*
[3]*Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmacy, The University of Mashreq, 10023, Baghdad, Iraq*
[4]*Department of Pharmaceutical Sciences and Pharmaceutics, Faculty of Pharmacy, Applied Science Private University, Amman 11931-166, Jordan*

ARTICLE INFO

Article history:
Received 13 May 2024
Revised 07 August 2024
Accepted 02 October 2024
Published online 01 November 2024

ABSTRACT

Citrus species are well-known for their applications in medicine and cosmetics. *Citrus aurantium* is a promising natural source of biologically active constituents such as phenolics and flavonoids. Water, ethanol, and ethyl acetate extracts, prepared from the fruit peels of *C. aurantium*, were phytochemically and biologically evaluated. Total phenol content (TPC) was determined by the Folin-Ciocalteu method and total flavonoid content (TFC) by the aluminum chloride ($AlCl_3$) colorimetric method. The micro-titer plate dilution method was applied to determine the minimal inhibitory concentration (MIC) and cell viability was assessed utilizing MTT. Ethyl acetate extract exerted the highest TPC and TFC (110.8 mg and 52.1 mg, respectively). Tested cancer cell lines and microorganisms showed high sensitivity to the ethyl acetate extract. Liquid Chromatography-Mass Spectrometry (LC-MS) of this extract revealed the presence of naringin, eriodictyol-7-neohesperidoside and hesperidin as the major constituents. Ethanol and ethyl acetate extracts showed also potent antioxidant capacity against DPPH radicals with respective IC_{50} values of 102.21 µg/mL and 110.89 µg/mL. The findings demonstrated that ethyl acetate extract of the peels of *C. aurantium*, grown in Jordan, possesses substantial antioxidant, anticancer and antimicrobial activities.

***Keywords*:** *Citrus aurantium*, Rutaceae, Anticancer, Antimicrobial, Liquid Chromatography-Mass Spectrometry.

Introduction

Medicinal and aromatic plants have played a remarkable role in new drug discovery, either in the form of their standardized crude extracts or in the form of their isolated and identified pure entities.[1] Many plant species, such as fruits, vegetables and spices with their natural bioactive compounds have long been explored for their cancer preventive and cancer treatment propensities due to their availability and limited or no side effects.[1] The dietary adjustment was suggested to contribute to almost 50% of all cancer prevention while particular diet choices could provide an ideal environment for cancer development.[2]
Citrus aurantium L. (Rutaceae), an evergreen tree commonly known as 'bitter orange' or 'sour orange', is cultivated in many countries such as in Spain, Italy and United States. However, the species is native to southeastern Asia. The most common biologically active flavonoids of this species are quercetin, kaempferol, luteolin, hesperidin and naringenin.[3,4] Also, coumarins, limonoids and biogenic amines were isolated from C. *aurantium*.[5,6] The fruit peel is rich in essential oils. Limonene, a monoterpene, is one of the identified and reported major volatile constituents. A big variety of flavonoids, as aglycons or as glycosides, were identified. *C. aurantium* is usually used as a food flavoring agent.[6]
The pharmacological properties of different organs of *C. aurantium* including flowers, fruits and essential oil have been reported in the literature.[7-10] Due to the abundance of valuable secondary metabolites, *C. aurantium* is utilized for the treatment of numerous illnesses such as anxiety, some types of cancers, gastrointestinal disorders and obesity.[11-17] Furthermore, *C. aurantium* extract appeared to have alcohol-induced liver injury protective ability by Nrf2 and AMPK-related signal regulation.[18]
The present study aimed to investigate the bioactivity and therapeutic potential of the peel extracts of *C. aurantium* grown in Jordan. This was achieved by utilizing different solvents in the preparation of the extracts. For the qualitative and quantitative determination of the phytochemical profile of the *C. aurantium* extract, liquid chromatography-mass spectrometry (LC-MS) was employed. Similar to gas chromatography-mass spectrometry (GC-MS), this technique is considered, nowadays, as an indispensable analytical method in the identification and quantification of secondary metabolites of plant species. This analytical method, known for its superior separation capabilities and high sensitivity, can lead to the characterization of bioactive compounds of medicinal plants used in different civilizations for their therapeutic viabilities [19,20] Inspired by the numerous publications on the anticancer and antimicrobial activities reported for the flowers, fruits and essential oil of this species, the investigations of the present study continued in the determination of antioxidant, antiproliferative and antimicrobial activities of *C. aurantium* peel extract.[7-10] To the best of our knowledge, the present study is the first investigation of *C. aurantium* peels collected from the Mediterranean biogeographic zone of Jordan.

Materials and Methods

Plant samples and preparation
Fresh fruits of *C. aurantium* L. (Rutaceae) were purchased from a local plant nursery (Shafa Badran, Amman, Jordan; 32°02'40.7"N,

*Corresponding author. E mail: *f_afify@asu.edu.jo; fatueafi@ju.edu.jo*
Tel: +962795737352

Citation: Khalid AM, Mahmod AI, Rashid HM, Talib W, Hasen E, Al-Najjar M, Afifi F. LC-MS Analysis and Biological Potential of the Peel Extracts of *Citrus aurantium* Grown in Jordan. Trop J Nat Prod Res. 2024; 8(10): 8764 – 8773. https://doi.org/10.26538/tjnpr/v8i10.19

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

35°54'29.9"E) in May 2022. The plant material was authenticated using descriptive references.[21,22] Voucher specimens were kept in the Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmacy, Applied Science Private University (FMJ-RUTA-CA-P1). Each 10 g fresh peel was extracted with distilled water, ethanol 70% and ethyl acetate (1:10 w/v) separately, by gently heating up to the boiling point. Then, the extracts were kept overnight at room temperature (25 ºC) and filtrated the day after. Solvents were evaporated using rotary evaporator (VV 2000 Heidolph, Germany) at 40 ºC until dryness.

Total Phenol Content (TPC) and Total Flavonoid Content (TFC)
The total phenol content (TPC) of the C. *aurantium* extracts was determined according to the Folin-Ciocalteu (F-C) procedure as reported in the literature.[23] Gallic acid (GA) (Sigma Aldrich, USA) was used as the standard in serial dilutions (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, and 6.125 µg/mL). Extracts were used in concentrations of 1 mg/mL. Results were represented as mg GAE/g dry weight of extracts.
Aluminum chloride ($AlCl_3$) colorimetric method was used to determine the total flavonoid content (TFC) of the extracts as described by Ebrahimi and Rastegar, with slight modifications.[24] Half mL of 1 mg/mL extract was added to 100 µL of 10% $AlCl_3$ (Scharlau, Spain) and mixed. Afterwards, 100 µL of 1 M sodium acetate (JHD, China) and 2.8 mL distilled water were added to the mixture. The absorbance for the resulting solution was measured at 415 nm. Rutin (Alpha Acer, Germany) was used as the standard in serial dilutions (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, and 6.125 µg/mL. The TFC of extracts was expressed as mg rutin equivalent/gm (RE) of the extract.

Antioxidant activity
To assess the radical scavenging activity of the extracts, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical test was employed according to the procedure described by Okafor *et al*.[25] DPPH (Sigma Aldrich, USA) solution 0.004% (w/v) was used as the standard. Serial dilutions were performed to prepare solutions in different concentrations (400 µg/mL, 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.12 µg/mL, 1.56 µg/mL, 0.78 µg/mL, 0.39 µg/mL, 0.19 µg/mL). The percentage of inhibition activity was estimated using the following equation (Equation 1)[25]:

$$Equation\ 1: \%I = \frac{(Ao - A*)}{Ao}\ x\ 100$$

Where Aº represents the absorbance of the control, and A* represents the absorbance of the extract or the standard solution. Calculation of IC_{50} was done by plotting the % inhibition against the concentration.

Cell lines and cell culture conditions
In this study, two breast- (T47D and MDA-MB231), one cervical- (HeLa), one colorectal adenocarcinoma- (Caco-2) and one prostate cancer (PC3) cell lines were used. Human Gingival Fibroblast (HGF) was used as a normal cell line. All cell lines were obtained from The European Collection of Cell Cultures, Salisbury, UK. The cells were cultured and treated as described and reported earlier.[26]

Cytotoxicity and antiproliferative activity assay
Cell viability was assessed utilizing MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] assay kit (Bioworld, UK) according to the procedure in literature.[27] The cells were treated with a serial dilution of each extract (5-0.015 mg/mL), followed by 10 µL of the addition of MTT solution. Microsoft Excel software was employed to estimate the percentage of survival cells and calculate the IC_{50} values, where OD is optical density as presented in equation 2.[27]

$$Equation\ 2: Percentage\ of\ Cell\ Viability\ (\%) = \frac{(OD\ of\ treated\ cell)}{(OD\ of\ control\ cell)}\ x\ 100$$

Antimicrobial assay
Different *C. aurantium* extracts (100 mg) were dissolved in (40 µL) 1% dimethyl sulphoxide (DMSO) and Oxoid Nutrient Broth was added up to l mL and assessed for their antibacterial and antifungal activities. The bacterial strains used in this study were gram-negative bacteria (*Escherichia coli* (ATCC®14169™) and *Pseudomonas aeruginosa* (ATCC®27853™)), gram-positive bacteria (*Staphylococcus epidermidis* (ATCC®12228™) and *S. aureus* (ATCC®25923™)) in addition to yeast *Candida albicans* (ATCC®90028™). The micro-titer plate dilution method was applied to establish the minimal inhibitory concentration (MIC).[28] Gentamycin and amphotericin B were used as positive controls. Both positive controls were prepared at concentrations of (7.8-1000 µg/mL).

Liquid Chromatography-Mass Spectrometry (LC-MS)
Chromatographic evaluation of the ethyl acetate extract was done as described by Mterin *et al*. using a Bruker Daltonik Impact II ESI-Q-TOF System with a Bruker Daltonik Elute UPLC system (Bremen, Germany).[29]

Statistical analysis
Data from independent trials were introduced as the mean ± SEM (Standard Error of Mean) obtained by utilizing the SPSS statistical package version (version 21). SPSS one-way Analysis of Variance (ANOVA) test was used to determine the statistical significance between the groups. IC_{50} was estimated by applying non-linear regression analysis. The significant *P*-value was (< 0.05).

Results and Discussion

In the present study *C. aurantium* peel was extracted using water, ethanol and ethyl acetate as solvents and the obtained extracts were quantified after complete evaporation of the solvents. Afterwards, all three extracts were submitted to biological evaluation to estimate their antiproliferative and antimicrobial potential. The phytochemical composition of the biologically most potent extract was determined using LC-MS.
The highest percentage yield of *C. aurantium* peel extract was obtained from the water extract (19.8%). Ethanol and ethyl acetate extracts yielded 14.34% and 3.22%, respectively. The TPCs and TFCs were also determined for the different extracts in addition to the evaluation of their antioxidant potentials (Table 1). The highest total phenol and total flavonoid contents were obtained for the ethyl acetate extract (Table 1). These high TPC (110.8 mg GAE/g) and TFC (52.1 mg RE/g) values for the ethyl acetate extract obtained in the present study agree with the literature data.[30] Standard curves for the phenolics and flavonoids are given in Figure 1. Also, estimation curves for the antioxidant activity of the ascorbic acid standard and of the three prepared extracts are given in Figure 2. Studies showed that the peels of bitter orange contain higher amounts of phenolic compounds-including flavonoids-than in the fruit itself.[31,32] Accordingly, the potential use of *C. aurantium* peel as a source of antioxidant agents in the food and medical industries was suggested.[6,33] Researchers from Algeria also revealed high antioxidant activity in the peels of different *C. aurantium* species.[34,35]

Liquid Chromatography-Mass Spectrometry (LC-MS) analysis
The phytochemical composition of the ethyl acetate extract was determined by LC-MS based on the potent antiproliferative and antimicrobial activities of this extract as well as its high TPC, TFC and antioxidant values (Figures 3 and 4). The analysis revealed the presence of thirteen compounds with naringin as the major constituent (65.15%), followed by eriodictyol-7-neohesperidoside (17.26%) and hesperidin (12.18%). The molecular formula, molecular weight, retention times and the percentages of the identified compounds are given in Table 2. The identification of high percentage of phenolics, especially of flavonoids (naringin, eriodictyol-7-neohesperidoside and hesperidin), in the LC-MS analysis supports the high TPC and TFC values obtained for this extract.
The major compound of the ethyl acetate extract, namely naringin, has remarkable pharmacological activities including anti-inflammatory and

anti-cancer activities. Naringin has been shown to exert promising therapeutic efficiency in metabolic syndrome, oxidative stress, CNS diseases and bone regeneration.[36-42] Eriodictyol, the aglycon of the second major detected component eriodictyol-7-neohesperidoside (neoeriocitrin), has protective antioxidant and anti-inflammatory properties against oxidative stress as well as possessing anti-diabetic activities.[43-47] In addition, immunomodulatory activities of eriodictyol (such as phosphorylation of mitogen-activated protein kinase (MAPK) or production of pro-inflammatory cytokines) indicate its promising therapeutic use as a cancer protective agent.[48,49]

Hesperidin, detected as the third major flavanone in *C. aurantium* ethyl acetate extract, possesses a wide range of pharmacological activities such as antiatherosclerosis, neuro-protective, antiallergic, antiviral, antimicrobial, antioxidant, anti-inflammatory and antiproliferative activities.[50-52] Our findings are in concordance with these published data.

Previous reports suggested that the Mediterranean diet, which includes plentiful amounts of citrus species, was associated with lower breast-, lung-, colon-, prostate- and cervical cancer incidences.[14,53] Several studies provide evidence for the anticancer properties of the secondary metabolites of *C. aurantium*.[16,54,55] Cholestatic liver fibrosis-induced mice treated with *C. aurantium* peel extract showed anti-apoptotic and anti-inflammatory properties. Ethyl acetate extract of this plant was investigated on HT-29 colon cancer cells. The result suggested promising outcomes such as cancer cell cycle arrest.[56]

Table 1: TPC, TFC and antioxidant activity of *C. aurantium* peel extracts. Data are shown as an average of triplicates ±SD, n=3.

Extract	Phenolics (mg/g)*	Flavonoids (mg/g)**	Antioxidant (µg /mL)***
Water	40.55±0.19	20.39±0.20	162.23± 10.99
Ethanol	75.03±0.74	23.72±0.60	102.21±21.61
Ethyl acetate	110.82±0.37	52.06±0.79	110.89±15.05
Ascorbic acid	-	-	2.78± 0.12

*Total phenolic contents (mg GAE/g of extract); **Total flavonoid contents (mg RE/g of extract), ***IC_{50}'s compared to the IC_{50} of the reference ascorbic acid.

Table 2: LC-MS analysis of *C. aurantium* ethyl acetate extract.

Name	Molecular Formula	Molecular Weight	Retention Time [min]	% of the Identified Compounds
Succinic acid	$C_4H_6O_4$	118.03	0.96	0.54
Chlorogenic acid	$C_{16}H_{18}O_9$	354.09	2.87	0.12
Syringic acid	$C_9H_{10}O_5$	198.05	3.1	0.07
Scopoletin	$C_{10}H_8O_4$	192.04	4.95	0.25
Eriodictyol-7-neohesperidoside	$C_{27}H_{32}O_{15}$	596.17	5.73	17.26
6,7,3',4'-Tetrahydroxyflavanone	$C_{15}H_{12}O_6$	288.06	5.73	2.63
Diosmin	$C_{28}H_{32}O_{15}$	608.17	6	0.09
3-O-Neohesperidoside Kaempferol	$C_{27}H_{30}O_{15}$	594.16	6.05	0.09
Benzoic acid	$C_7H_6O_2$	122.04	6.3	0.08
Naringin	$C_{27}H_{32}O_{14}$	580.18	6.61	65.15
4-Methylumbelliferone	$C_{10}H_8O_3$	176.05	6.86	0.074
Hesperidin	$C_{28}H_{34}O_{15}$	610.19	7.24	12.18
Resveratrol	$C_{14}H_{12}O_3$	228.08	7.55	1.46

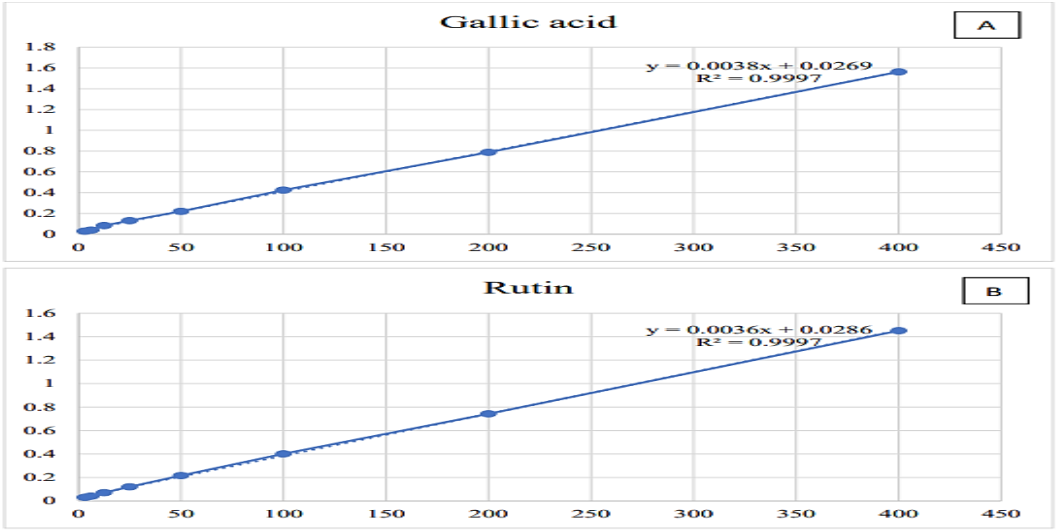

Figure 1: Standard curves for phenolics (A) and flavonoids (B).

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

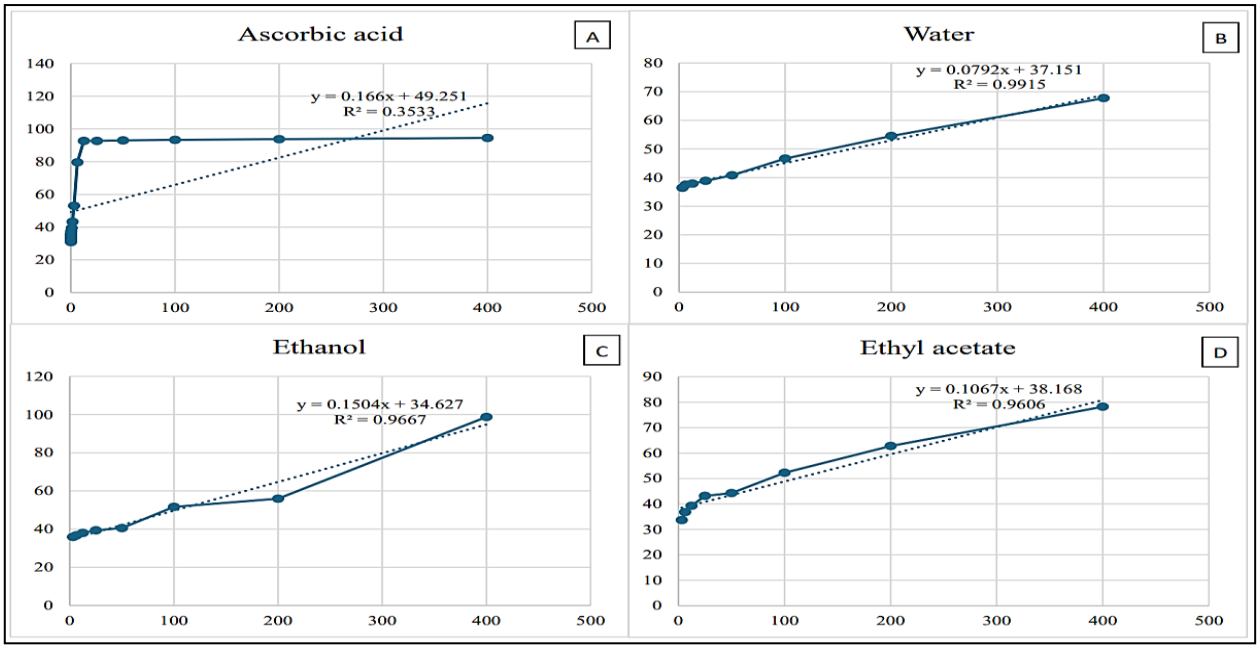

Figure 2: Estimation curves for the antioxidant activities of: ascorbic acid standard (A), aqueous extract (B), ethanol extract (C), ethyl acetate extract (D)

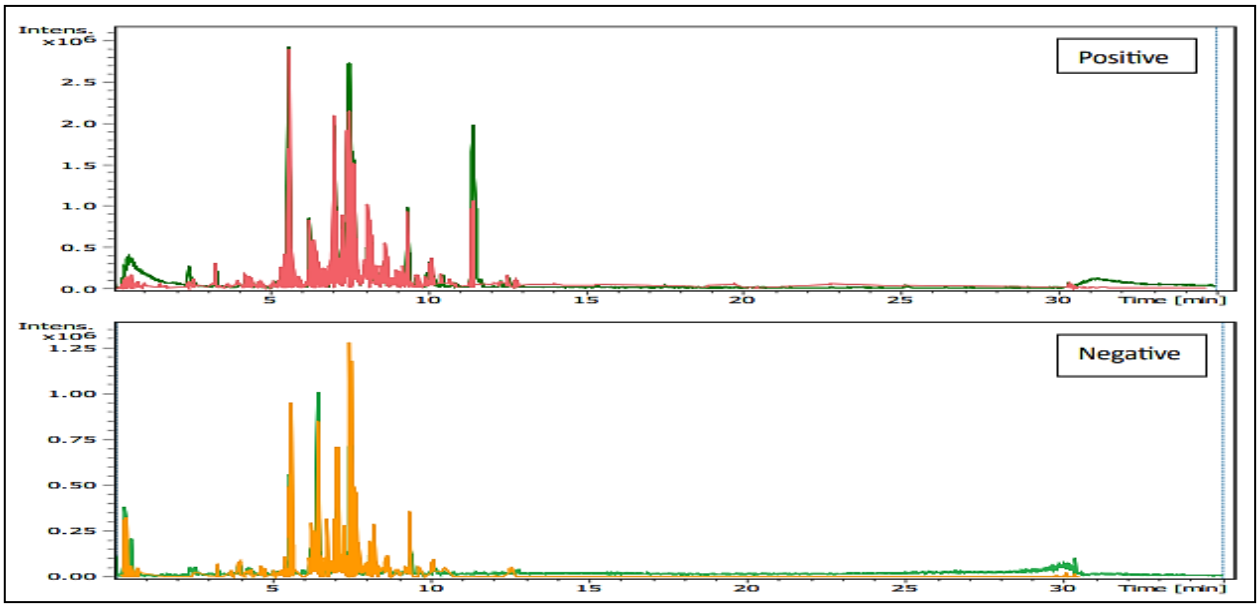

Figure 3: LC-MS results of the ethyl acetate extract

In vitro antiproliferative activity assay

The effects of the three *C. aurantium* extracts on the proliferation of the used cell lines are shown in Table 3 and Figure 5. Ethyl acetate extract revealed promising cytotoxic activity against all used cancer cell lines and especially against Caco-2 and PC3 cell lines with IC_{50} values of 0.58 mg/mL and 0.57 mg/mL, respectively. This extract also showed considerable activity against T47D, MDA-MB231 and HeLa cell lines. Ethanol extract showed some activity against PC3 cell lines and weak activity against the remaining cell lines. The water extract was inactive against all the investigated cell lines.

On the other hand, *C. aurantium* water, ethanol and ethyl acetate extracts did not exhibit any toxicity to normal Human Gingival Fibroblast (HGF) cells (Figure 6).

In the present study, ethyl acetate extract exhibited potent antiproliferative activity against all tested cell lines. Naringin, eriodictyol-7-neohesperidoside and hesperidin are probably the main sources of the anti-cancer effect. Naringin has suppressing and blocking abilities on cancerous cells and displayed antiproliferative and apoptotic-inducing effects in tumor cells; breast-, human cervical- and bladder cancer cells. In triple-negative breast cancer (TNBC) cells, naringin-induced G1-phase cell cycle arrest. In addition, naringin caused a significant decrease in breast cancer cell growth and improved survival by increasing the p21 level through β-catenin pathway inhibition.[57] Naringin also demonstrated apoptotic cell death in human cervical cancer cells alongside DNA fragmentation and generating morphological changes. It increased the expression of many death receptors such as caspases, p53, Bax and Fas.[58] Another study revealed that naringin might act as an anti-migration agent in chondrosarcoma by enhancing the expression of miR-126.[39] Another example of naringins' capabilities as an anticancer agent is its suggested potential to modulate gene expression. Naringin was found to cause DNA

hypomethylation in a prostate cancer cell line and induced G1 arrest of bladder cancer cells by upregulation of p21.[59,60]

Zhang *et al.* demonstrated the potent anticancer effect of eriodictyol against A549 based on multiple activities of this flavonoid.[61] Also, eriodictyol induced apoptosis of Glioma cells *via* PI3K/Akt/NF-κB signaling pathway.[62] Luo *et al.* identified neoeriocitrin as one of the flavonoids in the *C. aurantium* fruit extract and showed the extracts' efficacy by inhibiting the proliferation of HepG2 cells by blocking the rate-limiting enzymes in the glycolysis.[63]

Hesperidin has anti-cancer therapeutic effects due to its anti-inflammatory and antioxidant properties.[64] It has an inhibition ability on the xanthine oxidase (XO) enzyme, which is crucial in pathological conditions such as cancer since it is associated with the purine catabolism pathway.[65] Al-Rikabi *et al*. reported that hesperidin reduces breast cancer cell proliferation and colony formation while increasing nuclear condensation and apoptosis.[66] The same study revealed its anti-inflammatory effect in mice by reducing IL-33 and TNF-*α* following lipopolysaccharide (LPS) stimulation. The study suggested that hesperidin has a promising future in cancer treatment in addition to anti-inflammatory activities. Many preclinical studies suggested its role against malignant developments through various pathways affecting the cellular signaling altering tumor cell mechanisms such as survival, division and cell death.[67,68]

Antimicrobial assay

Water and ethanol extracts lacked antimicrobial activity against the tested microbial species while ethyl acetate extract exerted antibacterial activity against *E. coli*, *P. aeruginosa*, *S. aureus* and *S. epidermidis*. The calculated MICs were 12.5 mg/mL for each of them. On the other hand, ethanol and ethyl acetate extracts exerted potent antifungal effect against *C. albicans* with MIC values of 6.25 and 3.15 mg/mL, respectively (Table 4). Previous investigations with C. *aurantium* extracts showed potent antimicrobial activity.[69,70] To the best of our knowledge, the present study is the first to examine *C. aurantium* peel extracts antimicrobial activities. Different detected phenolic compounds are responsible for this activity.

Naringin had antimicrobial properties against gram-positive, gram-negative bacteria and fungi. In a previous study, naringin was used as an element of titanium implant coating and the controlled release of naringin provided protection against *S. aureus*.[71] Also, encapsulated naringin in gold nanoparticles revealed 21.98 µg/mL MIC against *Bacillus subtilis*, where the pure naringin showed MIC of 68.43 µg/mL.[72] On the other hand, many studies showed low levels of detectable gram-negative bacterial inhibitory ability for naringin.[73,74] Different properties of naringin such as reducing the biofilm formation, toxin production and inhibiting the growth of classic resistant bacteria (MRSA) made it a potential therapeutic agent.[75] Similarly, hesperidin showed MIC against *Proteus mirabilis* and *S. aureus* 12 times lower than chloramphenicol.[76,77] Another investigation established antibacterial effects against Gram-positive and Gram-negative bacteria for naringin and hesperidin found in grapefruit ethanol extract.[78] Naringin exhibited significant antifungal activity against *C. albicans*, *C. parapsilosis*, *Trichosporon beigelii*, *Trichophyton rubrum* and other species of fungi.[79] The present study could suggest that naringins' antifungal activity is due to the apoptosis process, mediated by mitochondrial dysfunction, although the exact mechanism is not clear.[79] Hesperidin, on the other hand, displayed a range of antifungal effects on species such as *Aspergillus parasiticus*, *A. flavus*, *Fusarium semitectum* and *Penicillium expansum*.[71,80] However, no remarkable activity was discovered against *C. albicans*. Indeed, recent studies focused on enhancing hesperidin antimicrobial properties based on several methods such as β-cyclodextrin and metal complexations or biotransformation.[81-83]

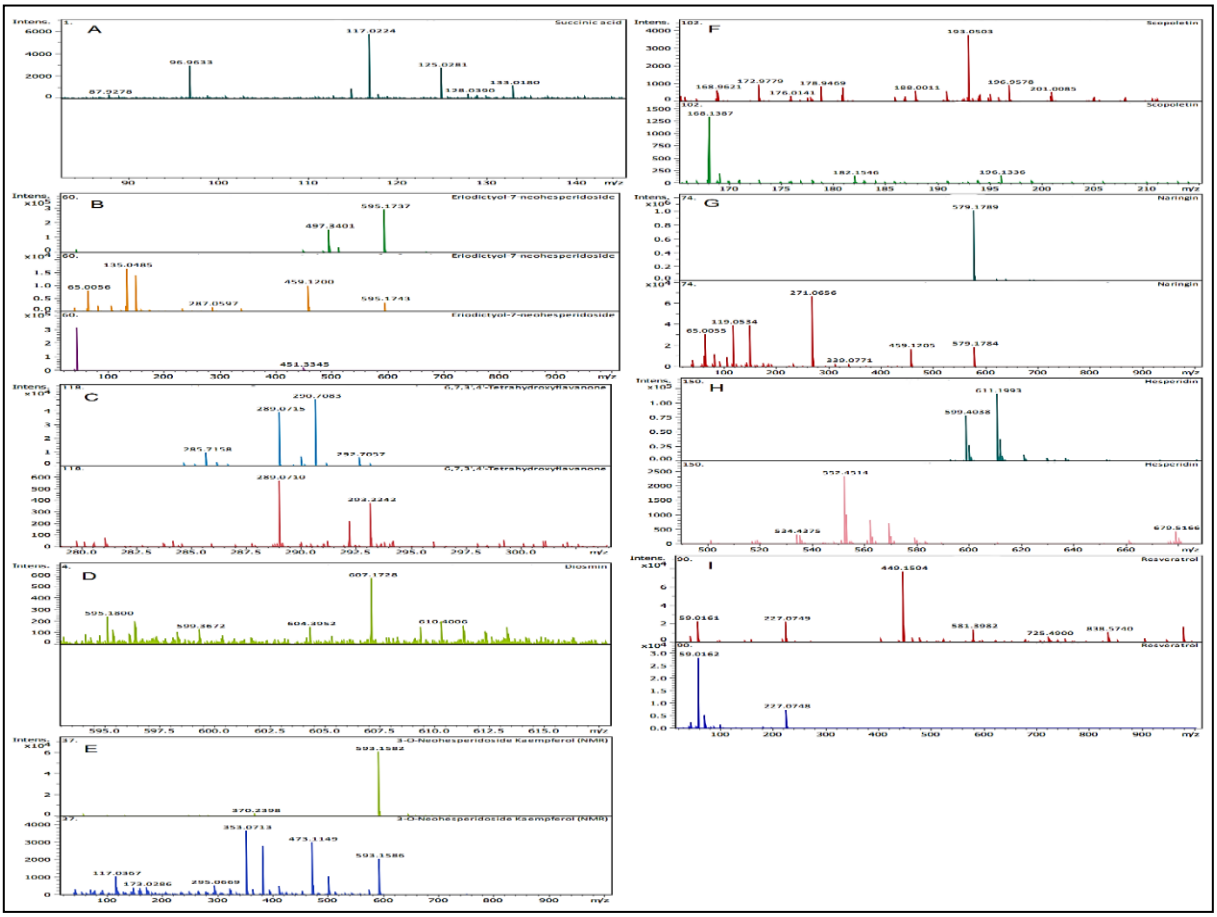

Figure 4: Compounds identified in the ethyl acetate extract by LC-MS: A; Succinic acid, B; Eriodictyol-7-neohesperidoside, C; 6,7,3',4'-Tetrahydroxyflavanone, D; Diosmin, E; 3-O-Neohesperidoside Kaempferol, F; Scopoletin, G; Naringin, H; Hesperidin, I; Resveratrol.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 3: Effect of the *C. aurantium* extracts on the proliferation of selected cancer cell lines and on the normal Fibroblast cell line with their IC_{50} values (mg/mL) as mean ± SEM, n=3

Extracts	T47D	MDA-MB231	HeLa	Caco-2	PC3	Fibroblast
Water	4.87±0.5	>5	>5	>5	>5	>5
Ethanol	1.82±0.17	4.9±0.8	2.98±0.04	3.6±0.14	1.07±0.09	>5
Ethyl acetate	0.6±0.08	0.62±0.1	0.79±0.05	0.58±0.2	0.57±0.16	>5

Table 4: Minimum inhibitory concentration (MIC) of *C. aurantium* extracts against various microorganisms in mg/mL, (n=3). (NA = No Activity).

The tested microorganism	*C. aurantium* extracts			Positive control
	Water	Ethanol	Ethyl acetate	Gentamicin & Amphotericin B*
E. coli	NA	NA	12.5	0.11 ±0.02
S. epidermidis	NA	NA	25	0.01±0.03
P. aeruginosa	NA	NA	12.5	0.062±0.008
S. aureus	NA	NA	12.5	0.15 ± 0.06
C. albicans	NA	6.25	3.15	0.76± 0.007*

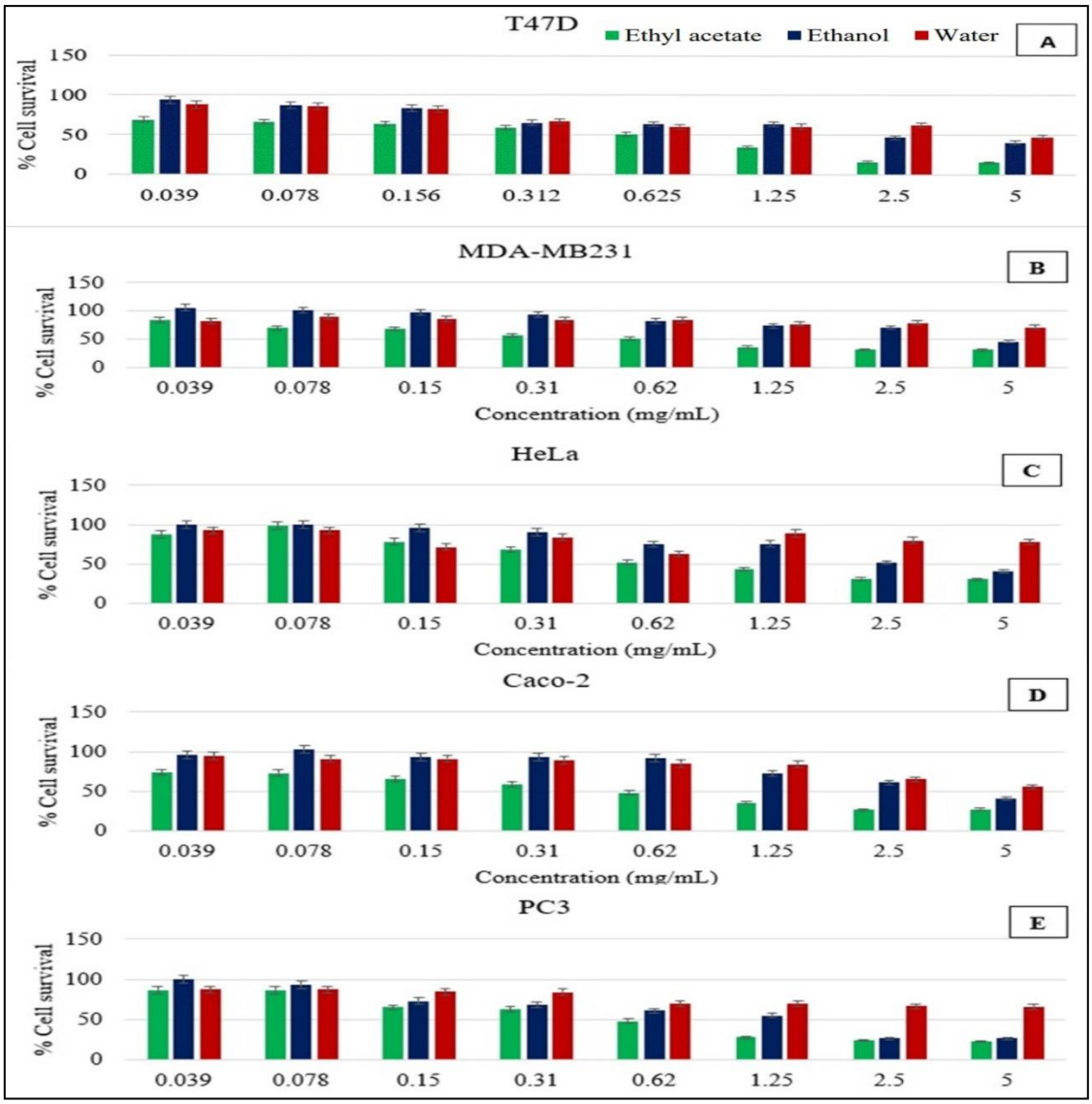

Figure 5: The antiproliferative activity of *C. aurantium* extracts against: (A) T47D, (B) MDA-MB231, (C) HeLa, (D) Caco-2, (E) PC3 cancer cell lines. Results are displayed as means of 3 independent experiments ± S

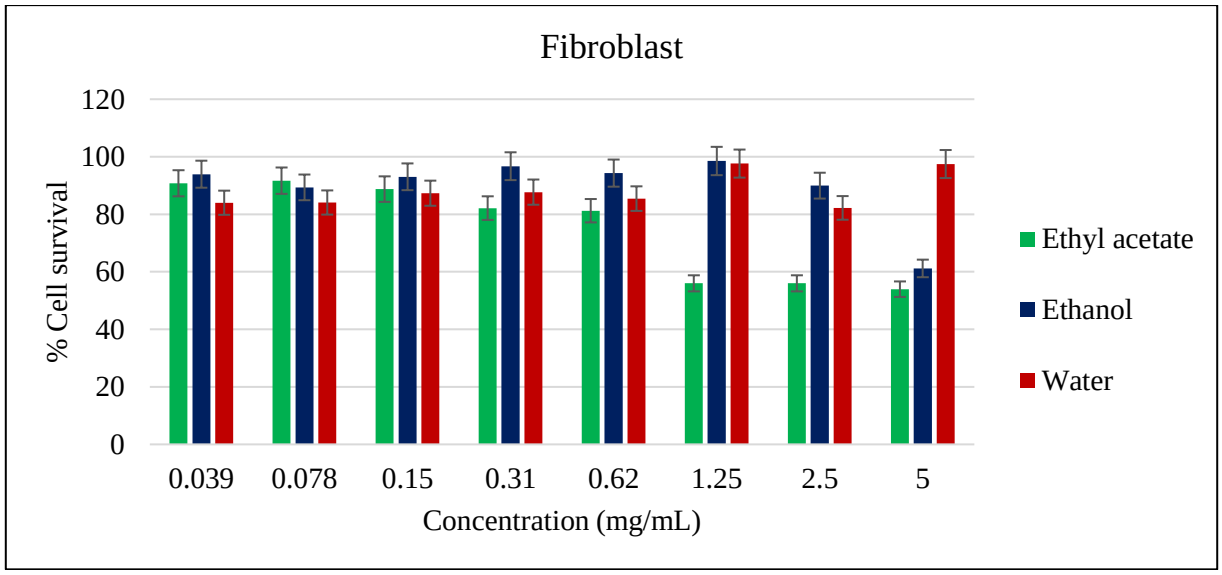

Figure 6: The cytotoxic activity of *C. aurantium* extracts against Human Gingival Fibroblast (HGF) cell line. Results are displayed as means of 3 independent experiments ± SEM.

Conclusion

The present study demonstrated that ethyl acetate extract of *C. aurantium* peels obtained from plants grown in Jordan is a rich source of biologically active components. Ethyl acetate peel extract is rich in compounds such as naringin, eriodictyol-7-neohesperidoside and hesperidin. This extract was shown to have antioxidant, antiproliferative and antimicrobial activities. Moreover, other minor detected components may have contributed to these effects through synergistic or additive effects. However, further studies are needed to confirm the valuable properties of this species by isolation of the beneficial components and evaluation of their possible synergy. The present study can be further expanded through *in vivo* experiments. In conclusion, the pharmacological activities observed in the present study with the *C. aurantium* peel extracts may lead to the development of novel natural drugs with possible prophylactic or therapeutic activities.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

References

1. Khan N, Afaq F, Mukhtar H. Cancer chemoprevention through dietary antioxidants: progress and promise. Antioxid Redox Signal. 2008; 10: 475-510.
2. WHO Report on Cancer. [Online] Setting Priorities, Investing Wisely and Providing Care for All. 2020. [cited 2023 Oct 15]. Available from: https://www.who.int/publications-detail/who-report-on-cancer-setting-priorities-investing-wisely-and-providing-care-for-all.
3. Kang SR, Park KI, Park HS, Lee DH, Kim JA, Nagappan A, Kim EH, Lee WS, Shin SC, Park MK, Han DY, Kim GS. Anti-inflammatory effect of flavonoids isolated from Korea *Citrus aurantium* L. on lipopolysaccharide-induced mouse macrophage RAW 264.7 cells by blocking of nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signalling pathways. Food Chem. 2011;129: 1721-1728.
4. Maksoud S, Abdel-Massih RM, Rajha HN, Louka N, Chemat F, Barba FJ, Debs E. *Citrus aurantium* L. active constituents, biological effects and extraction methods. An updated review. Molecules 2021; 26(19): 5832.
5. Khan IA, Abourashed EA. Leung's Encyclopedia of Common Natural Ingredients: Used in Food, Drugs and Cosmetics. (3rd ed.) New York: John Wiley & Sons; 2009. 477 p.
6. Suntar I, Khan H, Patel S, Celano R, Rastrelli L. An overview on *Citrus aurantium* L.: Its functions as food ingredient and therapeutic agent. Oxid Med Cell Longev. 2018; 7864269.
7. Jiang Z, Li Z, Wang F, Zhou Z. The protective effects of sour orange (*Citrus aurantium* L.) polymethoxyflavones on mice irradiation-induced intestinal injury. Molecules 2022; 27(6): 1934.
8. Kang P, Ryu KH, Lee JM, Kim HK, Seol GH. Endothelium- and smooth muscle-dependent vasodilator effects of *Citrus aurantium* L. var. *amara*: Focus on Ca2+ modulation. Biomed Pharmacother. 2016; 82: 467-471.
9. Mejri H, Wannes WA, Mahjoub FH, Hammami M, Dussault C, Legault J, Saidani-Tounsi M. Potential bio-functional properties of *Citrus aurantium* L. leaf: Chemical composition, antiviral activity on *Herpes simplex* virus type-1, antiproliferative effects on human lung and colon cancer cells and oxidative protection. Int J Environ Health Res. 2023; 8:1-11.
10. Oulebsir C, Mefti-Korteby H, Djazouli ZE, Zebib B, Merah O. Essential oil of *Citrus aurantium* L. leaves: Composition, antioxidant activity, elastase and collagenase inhibition. Agronomy 2022; 27(6): 1934.
11. Fugh-Berman A, Myers A. *Citrus aurantium*, an ingredient of dietary supplements marketed for weight loss: Current status of clinical and basic research. Exp Biol Med. 2004; 229: 698-704.
12. De Moraes Pultrini A, Galindo LA, Costa M. Effects of the essential oil from *Citrus aurantium* L. in experimental anxiety models in mice. Life Sci. 2006; 78: 1720-1725.
13. Mannucci C, Calapai F, Cardia L, Inferrera G, D'Arena G, Di Pietro M, Navarra M, Gangemi S, Spagnolo EV, Calapai G. Clinical pharmacology of *Citrus aurantium* and *Citrus*

sinensis for the treatment of anxiety. Evidence-based Complement Altern Med. 2018: 3624094.
14. Moraes TM, Kushima H, Moleiro FC, Santos RC, Rocha LRM, Marques MO, Vilegas W, Hiruma-Lima CA. Effects of limonene and essential oil from *Citrus aurantium* on gastric mucosa: Role of prostaglandins and gastric mucus secretion. Chem Biol Interact. 2009; 180: 499-505.
15. Suryawanshi JAS. An overview of *Citrus aurantium* used in treatment of various diseases. African J Plant Sci. 2011; 5: 390-395.
16. Park KI, Park HS, Kim MK, Hong GE, Nagappan A, Lee HJ, Yumnam S, Lee WS, Won CK, Shin SC. Flavonoids identified from Korean *Citrus aurantium* L. inhibit non-small cell lung cancer growth *in vivo* and *in vitro.* J Funct Foods. 2014; 7: 287-297.
17. Yao L, Zhang X, Huang C, Cai Y, Wan CC. The effect of *Citrus aurantium* on non-small-cell lung cancer: A research based on network and xperimental pharmacology. Biomed Res Int. 2023: 6407588.
18. Choi B, Kim T, Lee D, Jung W, Lim J, Jung J, Yang SH, Suh J. A polymethoxy flavonoids-rich *Citrus aurantium* extract ameliorates ethanol-induced liver injury through modulation of AMPK and Nrf2-related signals in a binge drinking mouse model. Phytother Res. 2015; 29:1577-1584.
19. Healthcare T. PDR for Herbal Medicines. (4th ed.). Toronto: Thomson Reuters; 2004. 86 p.
20. El Sayed AM, Basam SM, El-Naggar EBA, Marzouk HS, El-Hawary S. LC-MS/MS and GC-MS profiling as well as the antimicrobial effect of leaves of selected *Yucca* species introduced to Egypt. Sci Rep. 2024; 10:17778.
21. Bouhalla AM. Benabdelmoumene D, Dahmouni S, Bengharbi Z, Hellal K, Qadi WSM, Al-Olayan E, Moreno A, Bekada A, Buzgaia N, Aziz H, Mediani A. Comparative LC-MS-based metabolite profiling, antioxidant, and antibacterial properties of *Bunium bulbocastanum* tubers from two regions in Algeria. Sci Rep. 2024; 14:21719.
22. Vandebroek I, Picking D. Popular Medicinal Plants in Portland and Kingston, Jamaica. (1st ed.), Berlin: Springer; 2020. 79 p.
23. Mahmod AI, Oqal M, Khalid AM, Afifi FU, Talib WH. Phytochemical analysis, antioxidant, and antitumor activity of *Ligustrum ovalifolium* leaves grown in Jordan: an *in vitro* and *in vivo* study. Pharmacia 2024; 71: 1-10.
24. Khalid AM, Mahmod AI, Talib WH, Afifi FU. LC-MS analysis and antioxidant, antimicrobial and antiproliferative investigations of *Leucaena leucocephala* flowers growing in Jordan. Farmacia 2024; 72(2): 337-345
25. Okafor CE, Ijoma IK, Igboamalu CA, Ezebalu CE, Eze CF, Osita-Chikeze JC, Uzor CE, Ekwuekwe AL.. Secondary metabolites, spectra characterization, and antioxidant correlation analysis of the polar and nonpolar extracts of *Bryophyllum pinnatum* (Lam) Oken. BioTechnologia, 2024; 105(2):121-136.
26. Alobaedi OH, Talib WH, Basheti IA. Antitumor effect of thymoquinone combined with resveratrol on mice transplanted with breast cancer. Asian Pac J Trop Med. 2017; 10: 400-408.
27. Rashid HM, Mahmod AI, Afifi FU, Talib WH. Antioxidant and antiproliferation activities of lemon verbena (*Aloysia citrodora*): An *in vitro* and *in vivo* study. Plants 2022; 11: 785.
28. Al Safi, MA, Rashid HM, Afifi FU, Talib WH. Gaz Alafi: A traditional dessert in the Middle East with anticancer, immunomodulatory, and antimicrobial activities. Front Nutr. 2022; 9: 900506.
29. Al-Mterin MA, Aboalhaija NH, Abaza IF, Kailani MH, Zihlif MA, Afifi FU. Chromatographic analysis (LC-MS and GC-MS), antioxidant activity, total phenol and total flavonoid determination of *Ononis natrix* L. grown in Jordan. Jordan J Chem. 2021; 16:31-39.
30. Bocco A, Cuvelier ME, Richard H, Berset C. Antioxidant activity and phenolic composition of citrus peel and seed extracts. J Agric Food Chem. 1998; 46: 2123-2129.
31. Abeysinghe DC, Li X, Sun C, Zhang W, Zhou C, Chen K. Bioactive compounds and antioxidant capacities in different edible tissues of citrus fruit of four species. Food Chem. 2007; 104:1338-1344.
32. Gorinstein S, Martín-Belloso O, Park YS, Haruenkit R, Loje A, Ĉíž M, Caspi A. Comparison of some biochemical characteristics of different citrus fruits. Food Chem. 2002; 74: 309-315.
33. Rekha C, Poornima G, Manasa M, Abhipsa V, Devi JP, Kumar HTV, Kekuda TRP. Ascorbic acid, total phenol content and antioxidant activity of fresh juices of four ripe and unripe citrus fruits. Chem Sci Trans. 2012; 1: 303-310.
34. Ghasemi K, Ghasemi Y, Ebrahimzadeh MA. Antioxidant activity, phenol and flavonoid contents of 13 citrus species peels and tissues. Pak J Pharm Sci. 2009; 22: 277-281.
35. Lagha-Benamrouche S, Madani K. Phenolic contents and antioxidant activity of orange varieties (*Citrus sinensis* L. and *Citrus aurantium* L.) cultivated in Algeria: Peels and leaves. Ind Crops Prod. 2013; 50: 723-730.
36. Zhao ZH, Ma XL, Zhao B, Tian P, Ma JX, Kang JY, Zhang Y, Guo Y, Sun L. Naringin-inlaid silk fibroin/hydroxyapatite scaffold enhances human umbilical cord-derived mesenchymal stem cell-based bone regeneration. Cell Prolif. 2021; 54(7):e13043.
37. Chen R, Qi QL, Wang MT, Li QY. Therapeutic potential of naringin: an overview. Pharm Biol. 2016; 54: 3203-3210.
38. Hassan RA, Hozayen WG, Abo Sree HT, Al-Muzafar HM, Amin KA, Ahmed OM. Naringin and hesperidin counteract Diclofenac-induced hepatotoxicity in male Wistar rats *via* their antioxidant, anti-Inflammatory, and antiapoptotic activities. Oxid Med Cell Longev. 2021; 2021:9990091.
39. Chen R, Gao S, Guan H, Zhang X, Gao Y, Su Y, Song Y, Jiang Y, Li N. Naringin protects human nucleus pulposus cells against TNF-α-induced inflammation, oxidative stress, and loss of cellular homeostasis by enhancing autophagic flux via AMPK/SIRT1 activation. Oxid Med Cell Longev. 2021; 2021:7655142.
40. Bin-Emran T, Islam F, Nath N, Sutradhar H, Das R, Mitra S, Alshahrani MM, Alhasaniah AH, Sharma, R. Naringin and naringenin polyphenols in neurological diseases: understandings from a therapeutic viewpoint. Life 2023; 13(1): 99.
41. Stabrauskiene J, Kopustinskiene DM, Lazauskas R, Bernatoniene J. Naringin and naringenin: Their mechanisms of action and the potential anticancer activities. Biomed. 2022; 10(7):1686.
42. Chen J, Qin X, Chen M, Chen T, Chen Z, He B. Biological activities, molecular mechanisms, and clinical application of naringin in metabolic syndrome. Pharmacol Res. 2024; 202:107124.
43. Bucolo C, Leggio GM, Drago F, Salomone S. Eriodictyol prevents early retinal and plasma abnormalities in streptozotocin-induced diabetic rats. Biochem Pharmacol. 2012; 84: 88-92.
44. Johnson J, Maher P, Hanneken A. The flavonoid, eriodictyol, induces long-term protection in ARPE-19 cells through its effects on Nrf2 activation and phase 2 gene expression. Invest. Ophthalmol Vis Sci. 2009; 50: 2398-2406.
45. Narváez-Mastache JM, Novillo F, Delgado G. Antioxidant aryl-prenylcoumarin, flavan-3- ols and flavonoids from *Eysenhardtia subcoriacea.* Phytochemistry 2008; 69: 451-456.
46. Zhang X, Hung TM, Phuong PT, Ngoc TM, Min BS, Song KS, Seong YH, Bae KH. Anti-inflammatory activity of flavonoids from *Populus davidiana.* Arch Pharm Res 2006; 29: 1102-1108.

47. Zhan WY, Lee JJ, Kim Y, Kim IS, Han JH, Lee SG, Ahn MJ, Jung SH, Myung CS. Effect of eriodictyol on glucose uptake and insulin resistance *in vitro*. J Agric Food Chem. 2012; 60:7652-7658.
48. He J, Fu H, Li C, Deng Z, Chang H. Eriodictyol inhibits breast carcinogenesis by targeting circ_0007503 and repressing PI3K/Akt pathway. Phytomed. 2022; 102:154159.
49. Lee SE, Yang H, Son GW, Park HR, Park CS, Jin YH, Park YS. Eriodictyol protects endothelial cells against oxidative stress-induced cell death through modulating ERK/Nrf2/ARE-dependent heme oxygenase-1 expression. Int J Mol Sci.2015; 16: 14526-14539.
50. Aggarwal V, Tuli HS, Thakral F, Singhal P, Aggarwal D, Srivastava S, Pandey A, Sak K, Varol M, Khan M.A. Molecular mechanisms of action of hesperidin in cancer: Recent trends and advancements. Exp Biol Med 2020; 245: 486-497.
51. Ahmadi A, Shadboorestan A. Oxidative stress and cancer; the role of hesperidin, a citrus natural bioflavonoid, as a cancer chemoprotective agent. Nutr Cancer 2016; 68: 29–39.
52. Alshatwi AA, Ramesh E, Periasamy VS, Subash-Babu P. The apoptotic effect of hesperetin on human cervical cancer cells is mediated through cell cycle arrest, death receptor, and mitochondrial pathways. Fundam Clin Pharmacol. 2013; 27: 581-592.
53. Al-Ataby IA, Talib WH. Daily consumption of elmon and gingerherbal infusion caused tumor regression and activation of the immune system in a mouse model of breast cancer. Front Nutr. 2022; 13(9): 829101.
54. Han MH, Lee WS, Lu JN, Kim G, Jung, JM, Ryu CH, Kim GY, Hwang HJ, Kwon TK, Choi YH. *Citrus aurantium* L. exhibits apoptotic effects on U937 human leukemia cells partly through inhibition of Akt. Int J Oncol. 2012; 40: 2090-2096.
55. Shen CY, Yang L, Jiang JG, Zheng CY, Zh W. Immune enhancement effects and extraction optimization of polysaccharides from *Citrus aurantium* L. var. *amara*. Engl Food Funct. 2017; 8: 796-807.
56. Lim SW, Lee DR, Choi BK, Kim HS, Yang SH, Suh JW, Kim KS. Protective effects of a polymethoxy flavonoids-rich *Citrus aurantium* peel extract on liver fibrosis induced by bile duct ligation in mice. Asian Pac J Trop Med. 2016; 9: 1158-1164.
57. Li H, Yang B, Huang J, Xiang T, Yin X, Wan J, Luo F, Zhang L, Li H, Ren G. Naringin inhibits growth potential of human triple-negative breast cancer cells by targeting β-catenin signaling pathway. Toxicol Lett. 2013; 220: 219-228.
58. Ramesh E, Alshatwi AA. Naringin induces death receptor and mitochondria-mediated apoptosis in human cervical cancer (SiHa) cells. Food Chem Toxicol. 2013; 51: 97-105.
59. Kim DI, Lee SJ, Lee SB, Park K, Kim WJ, Moon SK. Requirement for Ras/Raf/ERK pathway in naringin-induced G 1-cell-cycle arrest *via* p21WAF1 expression. Carcinog. 2008; 29:1701-1709.
60. Lewinska A, Siwak J, Rzeszutek I, Wnuk M. Diosmin induces genotoxicity and apoptosis in DU145 prostate cancer cell line. Toxicol Vitr. 2015; 29: 417-425.
61. Zhang Y, Zhang R, Ni H. Eriodictyol exerts potent anticancer activity against A549 human lung cancer cell line by inducing mitochondrial mediated apoptosis, G2/M cell cycle arrest and inhibition of m-TOR/PI3K/Akt signaling pathway. Arch Med Sci. 2020; 16(2): 446-452.
62. Li W, Du Q, Li X, Zheng X, Lv F, Xi X, Huang G, Yang J, Liu S. Eriodictyol inhibits proliferation, metastasis and induces apoptosis of Glioma cells *via* PI_3K/Akt/NF-κB signaling pathway. Front Pharmacol. 2020;11: 114.
63. Luo X, HeJ, Bao Y, Wang S, Li T, Leng J, Meng X, Zhang YA. Preliminary study on *Fructus aurantii* extract against hepatocarcinoma *via* glycolysis and PD-1/PD-L1 pathway. Pharmacol Res-Mod Chin Med. 2020; 2: 10005.
64. Madureira MB, Concato VM, Cruz EMS, de Morais JMB, Inoue FSR, Santos NC, Gonçalves MD, de Souza MC, Scandolara TB, Mezoni MF, Galvani M, Seiva FRF, Panis C, Miranda-Sapla MM, Pavanelli WR. Naringenin and hesperidin as promising alternatives for prevention and co-djuvant therapy for breast cancer. Antioxidants 2023; 12: 586.
65. Li C, Schluesener H. Health-promoting effects of the citrus flavanone hesperidin, Crit Rev Food Sci Nutr. 2017; 57: 613-631.
66. Al-Rikabi R, Al-Shmgani H, Dewir YH, El-Hendawy S. *In vivo* and *in vitro* evaluation of the protective effects of hesperidin in lipopolysaccharide-induced inflammation and cytotoxicity of cell. Molecules 2020; 25(3): 478.
67. Ahmadi A, Shadboorestan A, Nabavi SF, Setzer WN, Nabavi SM. The role of hesperidin in cell signal transduction pathway for the prevention or treatment of cancer, Cur Med Chem. 2015; 22: 3462-3471.
68. Roohbakhsh A, Parhiz H, Soltani F, Rezaee R, Iranshahi M. Molecular mechanisms behind the biological effects of hesperidin and hesperetin for the prevention of cancer and cardiovascular diseases. Life Sci. 2015; 124: 64-74.
69. Meléndez PA, Capriles VA. Antibacterial properties of tropical plants from Puerto Rico. Phytomedicine 2006; 13: 272-276.
70. Oliveira SAC, Zambrana JRM, di Iorio FBR, Pereira CA, Jorge AOC. The antimicrobial effects of *Citrus limonum* and *Citrus aurantium* essential oils on multi-species biofilms. Braz Oral Res. 2014; 28: 22-27.
71. Yu M, You D, Zhuang J, Lin S, Dong L, Weng S, Zhang B, Cheng K, Weng W, Wang H. Controlled release of naringin in metal-organic framework-loaded mineralized collagen coating to simultaneously enhance osseointegration and antibacterial activity. ACS Appl Mater Interfaces. 2017; 9: 19698-19705.
72. Rao K, Imran M, Tabri T, Ali I, Perveen S, Ahmed S, Shah MR. Gum tragacanth stabilized green gold nanoparticles as cargos for naringin loading: A morphological investigation through AFM. Carbohydr Poly. 2017; 174: 243-252.
73. Adamczak A, Ożarowski M, Karpiński TM. Antibacterial activity of some flavonoids and organic acids widely distributed in plants. J Clin Med. 2019; 9: 109.
74. Pereira RMS, Andrades NED, Paulino N, Sawaya ACHF, Eberlin MN, Marcucci MC, Favero GM, Novak EM, Bydlowski SP. Synthesis and characterization of a metal complex containing naringin and Cu, and its antioxidant, antimicrobial, antiinflammatory and tumor cell cytotoxicity. Molecules. 2007; 12: 1352-1366.
75. Duda-Madej A, Stecko J, Sobieraj J, Szymańska N, Kozłowska J. Naringenin and its derivatives health-promoting phytobiotic against resistant bacteria and fungi in humans. Antibiotics. 2022; 11: 1628.
76. De Gregorio AC, Garcıa-Domenech R, Gálvez J, Ros MJ, Wolski S, Garcıa MD. Molecular topology: a useful tool for the search of new antibacterials. Bioorg Med Chem Lett. 2000; 10 :2033-2036.
77. Iranshahi M, Rezaee R, Parhiz H, Roohbakhsh A, Soltani F. Protective effects of flavonoids against microbes and toxins: The cases of hesperidin and hesperetin. Life Sci. 2015; 137: 125-132.
78. Cvetnic Z, Vladimir-Knezevic S. Antimicrobial activity of grapefruit seed and pulp ethanolic extract. Acta Pharm. 2004; 54: 243-250.
79. Kim H, Lee DG. Naringin-generated ROS promotes mitochondria-mediated apoptosis in *Candida albicans*. IUBMB Life 2021; 73: 953-967.
80. Salas MP, Céliz G, Geronazzo H, Daz M, Resnik SL. Antifungal activity of natural and enzymatically-modified flavonoids isolated from citrus species, Food Chem. 2011; 124: 1411-1415.

81. Corciova A, Ciobanu C, Poiata A, Mircea C, Nicolescu A, Drobota M, Varganici CD, Pinteala T, Marangocim N. Antibacterial and antioxidant properties of hesperidin: β-cyclodextrin complexes obtained by different techniques. J Incl Phenom Macrocycl Chem. 2015; 81:71-84.
82. Daoud S, Afifi FU, Al-Bakri AG, Kasabri V, Hamdan II. Preparation, physicochemical characterization and biological evaluation of some hesperidin metal complexes. Iran J Pharm Res. 2014;13: 909.
83. Hao Y, Wei Z, Wang Z, Li G, Yao Y, Dun B. Biotransformation of flavonoids improves antimicrobial and anti-breast cancer activities *in vitro.* Foods. 2021;10: 2367.