DOI: https://doi.org/10.31351/vol34iss2pp271-281

Isolation and Characterization of Two Flavonoids from Guava leaves Cultivated in Iraq: A Broad Study on Structural Elucidation and Cytotoxic Evaluation

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Received 1/4/2024, Accepted 23/10/2024, Published 25/6/2025

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Abstract

The guava plant (*Psidium guajava*), a member of the Myrtaceae family, was the focus of this study, which aimed to isolate and characterize flavonoids from its leaves recently cultivated in Iraq. The research also evaluated the cytotoxic potential of the ethyl acetate extract against prostate cancer, breast cancer cells, and normal human neonatal dermal fibroblasts. Flavonoid isolation was performed using preparative-layer chromatography and preparative high-performance liquid chromatography. Structural characterization of the isolated flavanoids was carried out using nuclear magnetic resonance and Fourier-transform infrared spectroscopy, allowing for a detailed analysis of two flavonoids: avicularin and guaijaverin, obtained from the ethyl acetate and n-butanol fractions. These practices effectively yielded pure compounds and confirmed their structures. Cytotoxicity assays revealed that the ethyl acetate fraction significantly reduced cell viability, IC50 values of 91.9 µg/mL against PC3 cells and 379 µg/mL against MCF7 cells. In contrast, it showed no toxic effect on normal human neonatal dermal fibroblasts cells, with an IC50 well above 100 µg/mL. These findings provided an in-depth considerate of the chemical profile of the flavonoids demonstrated the selective anticancer potential of guava extracts.

Keywords: Ultrasonic-assisted extraction, Avicularin, Guaijaverin, Guava, HPLC.

Introduction

From olden times, medicinal plants have been employed to cure various disorders [(1)]. Plants possessing a multitude of healing compounds have played a crucial role in promoting human health and welfare throughout history [(2-3)]. The utilization of plants for medicinal points is deeply ingrained in diverse traditional healing systems across cultures, forming a natural pharmacopeia that has supported communities for many generations [(4-6)]. Whether drawing from the ancient insights of Ayurveda in India or the herbal practices of traditional Chinese medicine, the importance of medicinal plants extends beyond geographical and cultural distinctions, over time and through various attempts, humans acquired the knowledge of utilizing herbal materials beneficially for various aspects of their lives which are used in some aspects of traditional medical uses [(7-9)]. The guava plant, scientifically identified as *Psidium guajava* stands as a testament to nature's generosity, offering more than just a flavorful fruit—it also delivers a wealth of medicinal and nutritional advantages. Initiating in America, the guava plant has covered the globe, establishing itself in tropical and subtropical regions, where its robust characteristics empower it to flourish [(10,11)]. Belonging to the Myrtaceae family, this evergreen shrub or small tree commonly referred to as guava, has embedded itself as an essential component of diverse cultures [(12)]. Guava leaves include several phytochemical components that have been found in numerous previous studies to have a wide range of pharmacological effects and medicinal properties. Numerous pharmacological research has confirmed that this plant can disclose effects that are hepatoprotective, antigenotoxic, anti-bacterial, cytotoxic, antispasmodic, antioxidant, cardioactive, anticough, anti-diabetic, and anti-inflammatory. [(13-16)]. Guava leaves contain terpenoids, volatile compounds, polyphenols, tannins, many minerals like the crystals of calcium oxalate, polyphenols such as quercetin, kaempferol, ferulic acid, luteolin, also have resin and tannins present, carotenoids such as leukopenia and leukocyanidins highly found in guava leaves and fruits [(17)]. The World Health Organisation has noted that cancer ranks as the second most common cause of mortality worldwide Thus, there is a pressing need to discover affordable cancer treatment options. We still have access to untapped materials from nature that may be beneficial to our health Among them, guava's numerous components have recently drawn attention due to its anticancer qualities [(18)]. This education centers on isolation, structural characterization , and quantification of phenolic

compounds from the leaves of *Psidium guajava* (guava) recently cultivated in Iraq, along with an evaluation of the cytotoxic effects of the ethyl acetate fraction (F3) on prostate cancer (PC3) and human breast cancer (MCF7) cell lines.

Material and method

Plant collection

In Apr. 2023, leaves of guava were collected from Musayyib City, marking the initial cultivation of this plant in Iraq. Following collection, the specimens were carefully identified and authenticated by a specialist from the University of Baghdad. The leaves were meticulously cleaned, shade-dried to preserve their phytochemical integrity, ground into a fine powder, and properly stored for subsequent extraction and detailed analysis [19,20].

Extraction and Isolation of Plant Material

Approximately 50 grams of finely powdered guava leaves were subjected to extraction using bath sonicator, the process was carried out at a frequency of 40 kHz, with the leaf crush immersed in 150 mL of 70% ethanol and sonicated at thirty °C for fourty minutes. After extraction, the crude extract was filtered and concentrated. The resulting dried extract was then melted in 100 mL of distilled water and sequentially divided with equal volumes (100 mL) of solvents have different polarity. All solvent fractions, with the exception of n-butanol, were dried over sodium sulfate , filtered, and subsequently vaporized to dryness, yielding concentrated fractions for further analysis [23].

Phytochemical assessment

phytochemical screening was conducted to limit the presence or absence of polyphenolic compounds in the Iraqi guava leaf extracts, specifically focusing on the (F3) and (F4). The analysis was performed using the alkaline reagent test, where small amounts of each extract were treated with a few drops of NaOH solution. The attendance of a yellow coloration indicated the manifestation of flavonoids; however, this color disappeared upon the addition of dilute HCL, confirming the presence of polyphenolic constituents. [24].

Examination of the Portions by PLC, HPLC and PHPLC

(F3) and (F4) derived from guava leaves were subjected to analysis and evaluation using preparative layer chromatography (PLC) and high-performance liquid chromatography (HPLC). For the chromatographic procedure, aluminum plates measuring 20 × 20 cm and coated with silica gel GF254 were used. The mobile phase employed for both F3 , F4 consisted of ethyl acetate, toluene, methanol, and formic acid in the ratio of 15:15:1:4. Diluted samples, along with standard compounds (avicularin and guaijaverin), were carefully applied on the baseline of the TLC plate, which was then developed in a TLC chamber. The resulting chromatographic spots were showed under UV light at wavelengths of 254 nm without damaging the plate.A qualitative assessment of the F3 ,F4 was further exploration using HPLC, with comparison against avicularin and guaijaverin standards. Identification of the compounds was confirmed by matching the retention times of the samples with those of the standards. The HPLC analysis was carried out using a Knauer C18 column .The mobile phase comprised 1% aqueous acetic acid and CH_3CN, applied through gradient elution. The flow rate was stay at 1 mL/min, the column temperature was set at 28°C, and the injection volume for each run was 20 µL. Gradient elution progressed in a linear manner throughout the analysis[26,27].

Preparative HPLC (PHPLC)specification

Phytochemical constituents within plant extracts typically occur in trace amounts, requiring highly sensitive and precise instrumentation for their detection and isolation. In this investigation, PHPLC was utilized as an advanced separation technique to isolate phenolic compound in their pure form. The operational parameters and instrumentation specifications for the PHPLC setup adhered to those established in prior research, ensuring consistency and reliability in the isolation process [28,29].

Analysis by (IR)

The IR spectra of the isolated combines were noted using a Shimadzu FTIR, spectrometer, done at the University of Basrah /College of Science [30]. IR spectroscopy analysis of A4 and A5 (Figures 6,7 and Table 5,6), IR spectra of compounds A4 and A5 were matched with standard Avicularin and Guaijaverin standards as shown in prior studies [31,32].

Analysis by NMR

NMR spectra were acquired at 25 °C using a 400 MHz Bruker Avance 6D spectrometer, with DMSO-d6 serving as the solvent, at the University of Basrah /College of Education Department of Chemistry.

Cell Line Maintenance

The method of work was conducted for cell line maintenance according to Freshney RI [33].

MTT assay

F3 cytotoxic effect on PC3and MCF7 was determined by MTT assay was performed as follows: On day one, cells were seeded into a 96-well microplate. Approximately 1×10^4 cells were cultured in each well. The plate was then incubated at 37°C for 24 hours until 60% confluence was reached. On day two, the cells were treated with various concentrations (6.25–400 µg/mL) of the F3 for 24 hours. After removing the supernatant from each well, hundred µL of each dilution was added to the respective wells, with eight replicates per concentration. On day three, hundered µL of MTT

mixture was added to each one. The plate was developed for 4 hours in the dark. After this incubation, the medium was discarded, and hundred µL of DMSO was added to each well. The plate was surprised for 20 minutes, the absorbance of the subsequent color was measured using a DNM-9602G microplate reader at 570 nm [34,35].

Statistical study

The data were analyzed using the (SPSS) version 24.0. Results are presented as (mean ± SD). T -test was performed to assess the outcomes. Statistical importance was considered at a p-value of less than 0.05.

Result and Discussion

Extraction by bath sonicator

The method for lextraction was bath sonicator resulting in a higher percentage yield [36]. 20 %w/w from 50g of leaves allowing to the formula:

$$\textbf{percentage yield} = \frac{\textbf{weight of crude (g)}}{\textbf{weight of plant material (g)}} x100$$

[37].

phytochemical research

Chemical examinations were occured on the guava leaves and displayed the results in Table 1

Table 1. Exploration of guava leaf fractions

Name of fraction	NaOH test
F3	+
F4	+

Check of guava leaves Fractions.

Analysis of F3 by PLC as in Figure 1, describes the isolation of A4 and A5 showing the bands separated match with avicularin and guaijaverin st. Further analysis of the F3 , F4 were managed for leaves using HPLC, as in Figures 2 and 3.

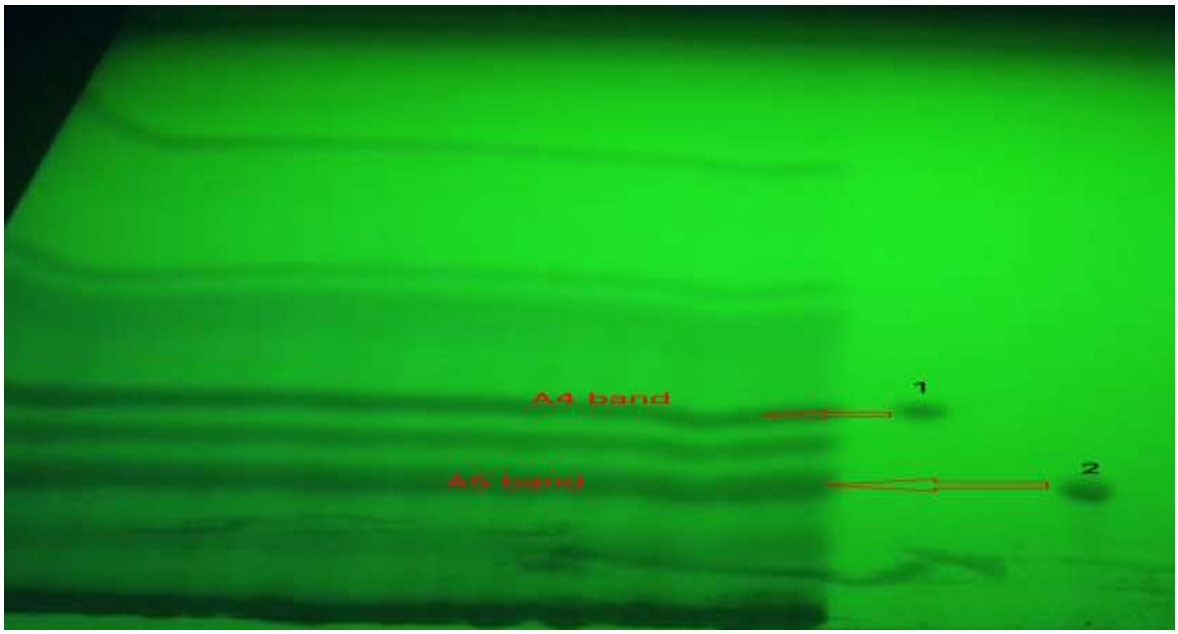

Figure 1. PLC for Standards and Analyzed Ethyl Acetate Fractions where (1) Avicularin St. and (2) Guaijaverin St.

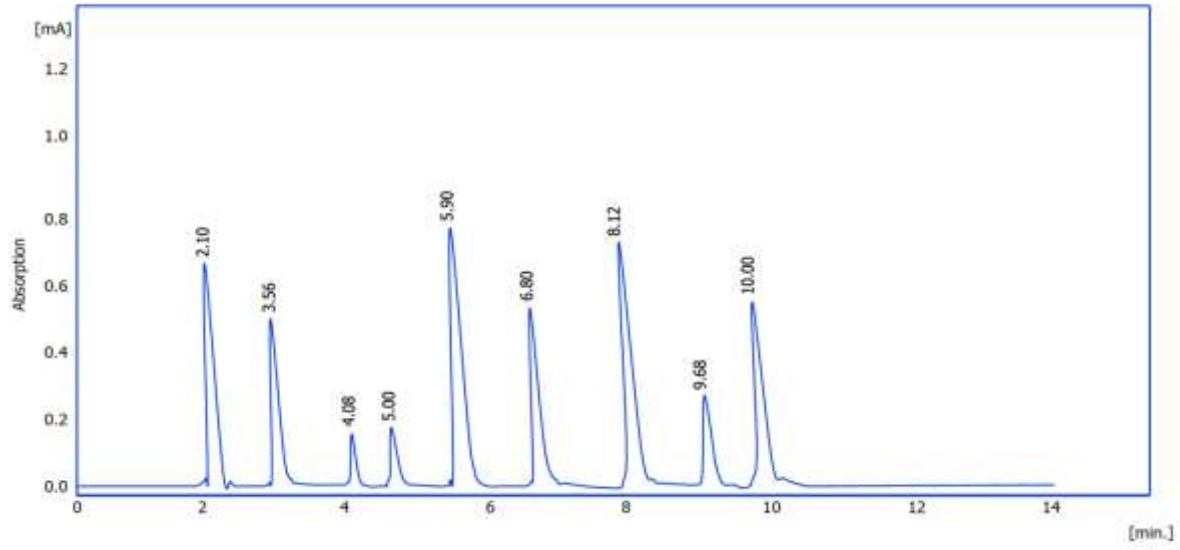

Figure 2. Chromatogram of F3

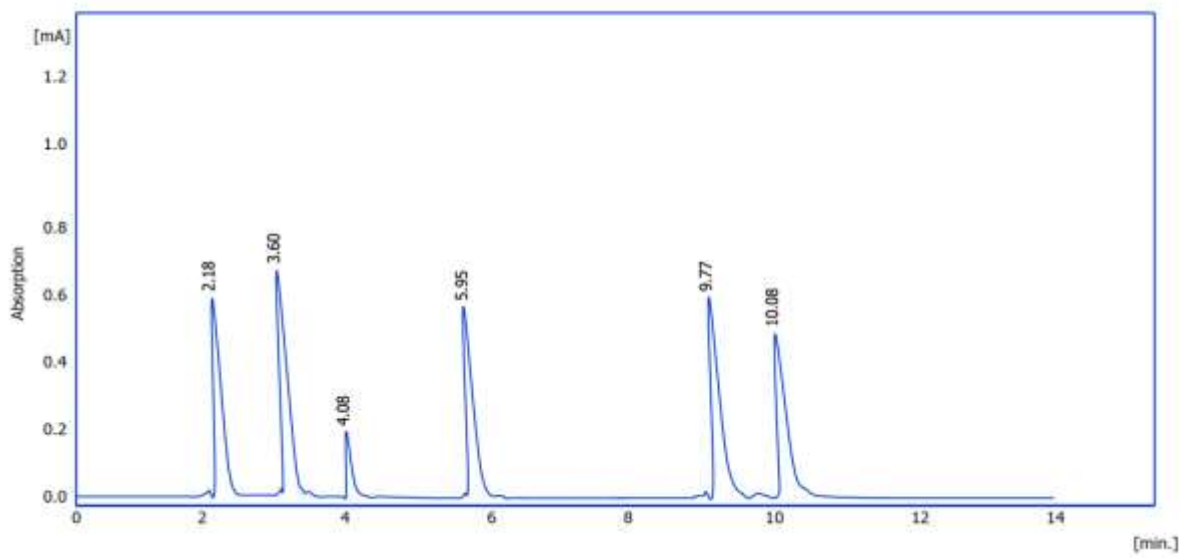

Figure 3. Chromatogram F4

Documentation of the isolated compounds

Documentation of compounds A4 and A5 by PLC

The separation A4 and A5 from the F3 andF4by PLC which reveals close Rf value of Avicularin St. and guaijaverin St. Bands fitted to the Isolated A4 and A5, Rf values 0,27 for avicularin St. and A4,0.18 for guaijaverin St. and A5.

Identification of isolated compounds by HPLC and PHPLC

The improved chromatography column method, a widely employed technique for separating natural materials, was utilized in this study. The amounts of the calculated compound were determined by establishing a calibration curve. This involved plotting the peak area against the mass concentration for each standard at four different concentration values. The straight-line equation of the standard curve was then applied. The outcomes obtained from the conducted HPLC and exploration of the F3, F4 extract from the *Psidium guajava* plant affirmed the retention time of compounds A4 and A5. This retention time closely matched those of real reference standards under the same chromatographic conditions, as demonstrated in Figure 4. These findings affirm the presence of avicularin and guaijaverin, in the F3 and F4 of Guava plant leaves. The quantitative HPLC analysis of the isolated compounds showed elevated concentrations (conc.) of avicularin and guaijaverin in F3 , F4 fractions from guava leaves, as outlined in table 2 and explained in Figure 5.

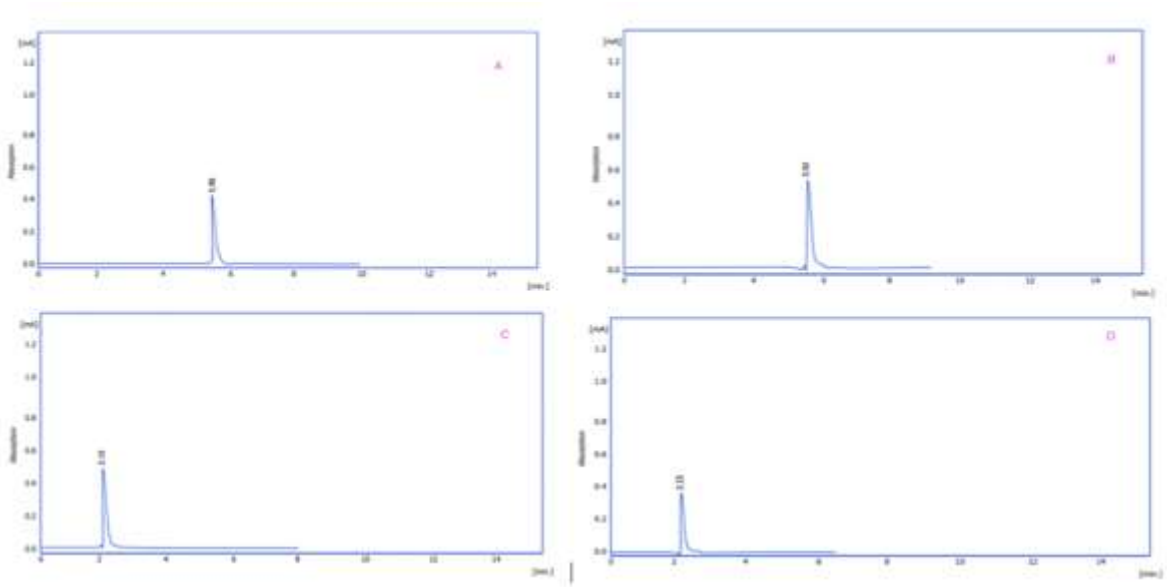

Figure 4. A, HPLC chromatogram of A4. B, HPLC chromatogram of the Avicularin St., C, HPLC of A5. D, HPLC chromatogram of the Guaijaverin St.

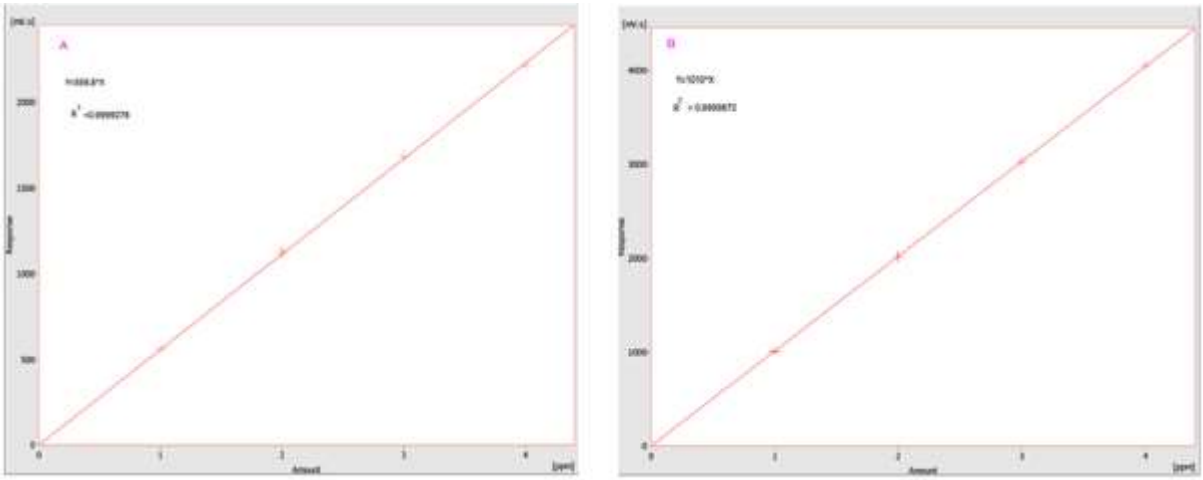

Figure 5. A, Standard curves of Avicularin by PHPLC. B, of Guaijaverin

Table 2. The conc. of A4, A5 in the F3 and F4

Names of compounds	Area under the curve	Con. In (µg/ml)
Avicularin(A4)	25748.99 IN F3 5624.18 IN F4	56.3
Guijaverin(A5)	20547.89 IN F3 9856 IN F4	30.104

Analysis by FTIR

IR spectroscopy examination of A4 as in figure 6 and A5 as in figure 7, IR spectra of A4 exposed the presence of 3319 band referring to phenolic O-H stretching broadband vibration,2960 band referring to C-H stretching vibration of CH2,1651band refer to C=O stretching vibration of α-β unsaturated ketone,1606 band refer to aromatic C=C stretching vibration,827 refer to C-H bending fingerprint of aromatic and A5 IR spectra revealed the presence3448 broadband refer to O-H stretching vibration of phenol, 1654 band refer to α-β unsaturated ketone stretching vibration,875 band refer to the fingerprint of the aromatic ring, So IR spectra of A4 and A5 matched with St. Avicularin and Guaijaverin[31,32].

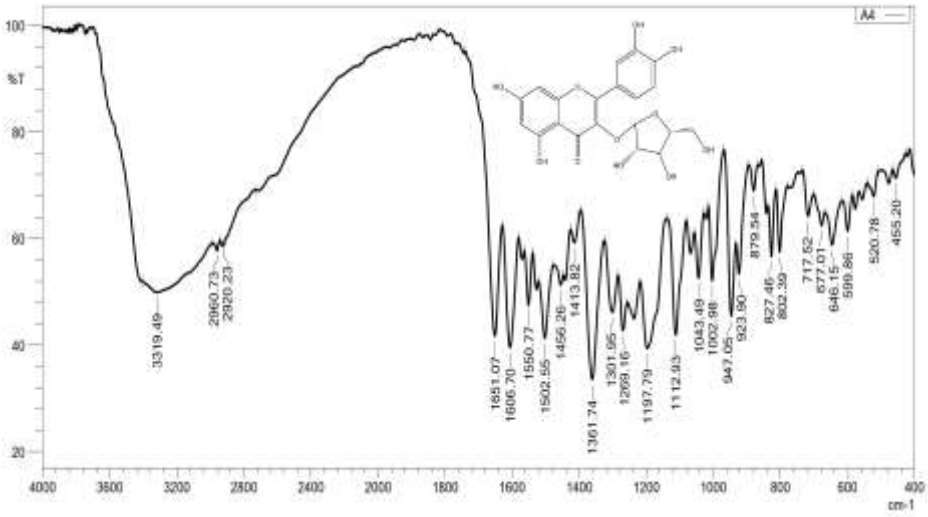

Figure 6. IR spectrum of isolated A4 compound.

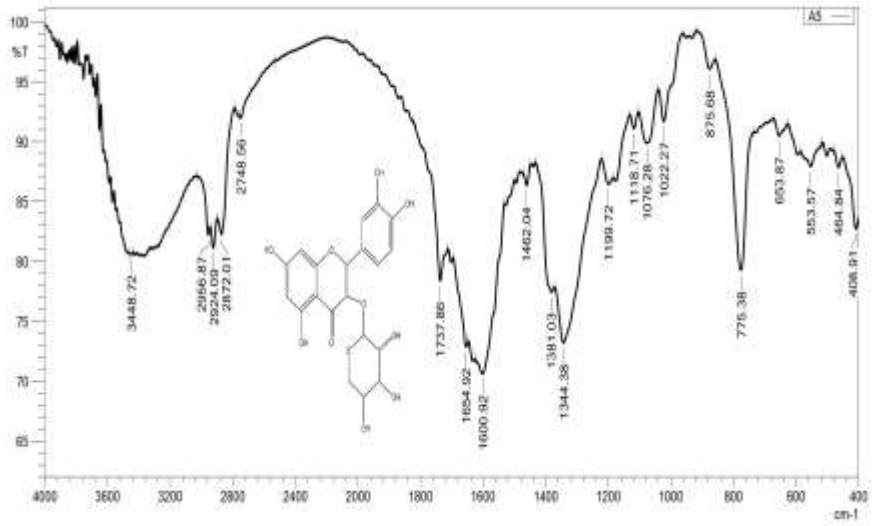

Figure 7. IR spectrum of isolated A5

Analysis of isolated compounds by NMR spectroscopy

The ^{1}H NMR spectroscopy, conducted at 400 MHz in DMSO-d6 was working to analyze the chemical structure of the A4 and A5 compounds. The recorded spectral data for A4 are as follows Figure 8 and 9.^{1}H -NMR spectral data of A4 compound are recorded in NMR spectroscopy using (400 MHz) 12.66 (s, 1H, OH5), 10.91 (s, 1H, OH7), 9.77 (s, 1H, OH3`), 9.31 (s, 1H, OH4`), 7.56 (dd, , 1H, H6`), 7.48 (d, 1H, H8), 6.86 (s, 1H, H2`), 6.42 (d, 1H, H5`), 6.21 (d, 1H, H6),), 5.59 (s, 1H, H1``), 4.15 (d, 1H, H2``), 3.72 (q, , 1H, H4``),3.59(m,1H, H3``) *3.38* (S, 1H, H-5``α, 11.8 Hz), 3.29 (dd, 1H, H-5``β).

^{13}CNMR (100MHz) (177., C4) (164.66, C7) (161., C5) (157, C9) (156, C2) (148, C4`) (145., C3`) (133.82, C3) (122., C1`) (121. C6`) (116.00, C2`) (115.95, C5`) (108.25, C1``) (141, C10) (99., C6) (94.01, C8) (86.24, C4``) (82.59, C2``) (77.35, C3``) (61.03, C5). The NMR spectra of A4 show good compact with described in the nonfiction on Avicularin [38].

^{1}H NMR spectral data of A5 compound 12.67 (s, 1H, OH5), 10.91 (s,OH7), 9.80 (s, 1H, OH3`), 9.25 (s, 1H, OH4`), 7.68 (s, 1H, H2`), 7.52 (d,1H, H6`), 6.85 (d, 1H, H5`), 6.41 (s, 1H, H8), 6.21 (s, 1H, H6), 5.28 (d, 1H, H1``), 4.75(d, 1H, H2``), 4.61(d, 1H, H4``), 3.76(s, 4.9 Hz, 1H, H3``), 3.63 (m,2H, H5``). $^{13}$CNMR (100MHz,) (177.98, C4), (163.63, C7) (161.69, C5) (156.73, C9) (149.05, C2) (145.44, C4`) (134.19, C3`) (122.57, C3) (121.35, C6`) (116.19, C2`) (115.82, C5`) (104.38, C1``) (101.83, C10) (99.13, C6) (93.96, C8) (72.10, C3``) (71.18, C2``) (66.54, C4``) (64.76, C5``). The NMR spectra of A5 show compact with described in the literature as shown in figures 10 and 11 [39,40].

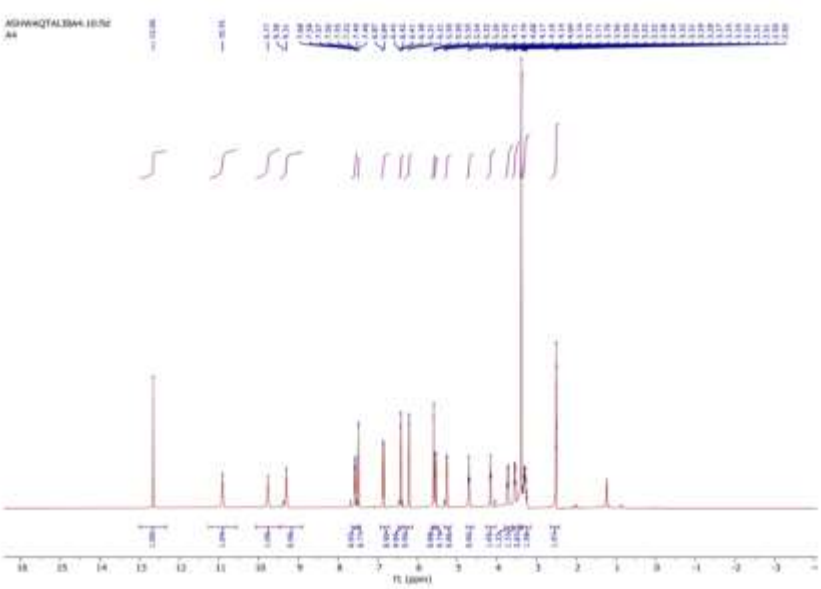

Figure 8. ^{1}H NMR of A4

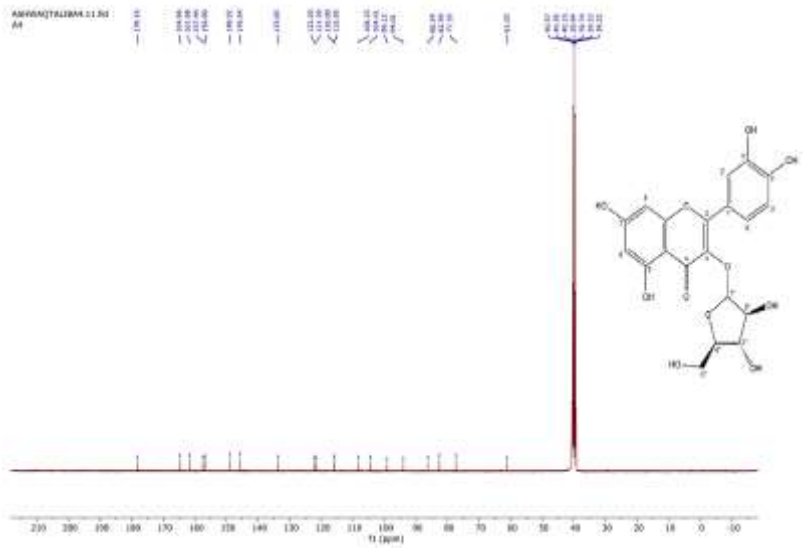

Figure 9. ^{13}C NMR of A4

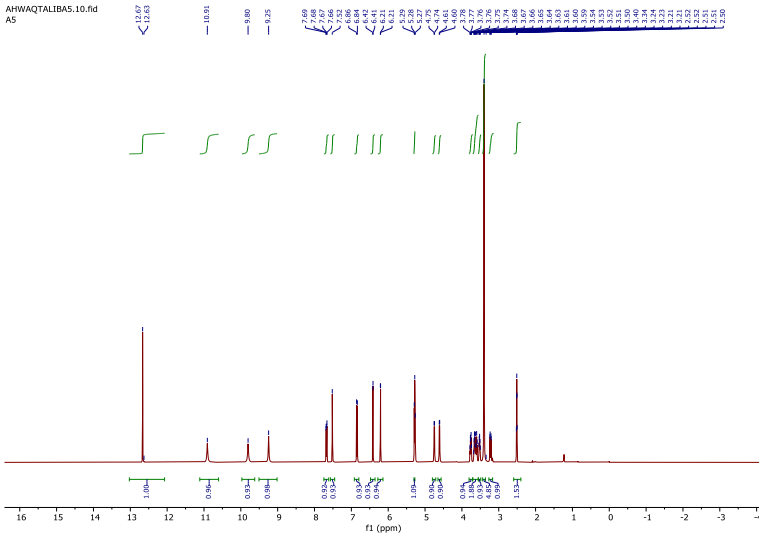

Figure 10. ^{1}H NMR spectrum of A5

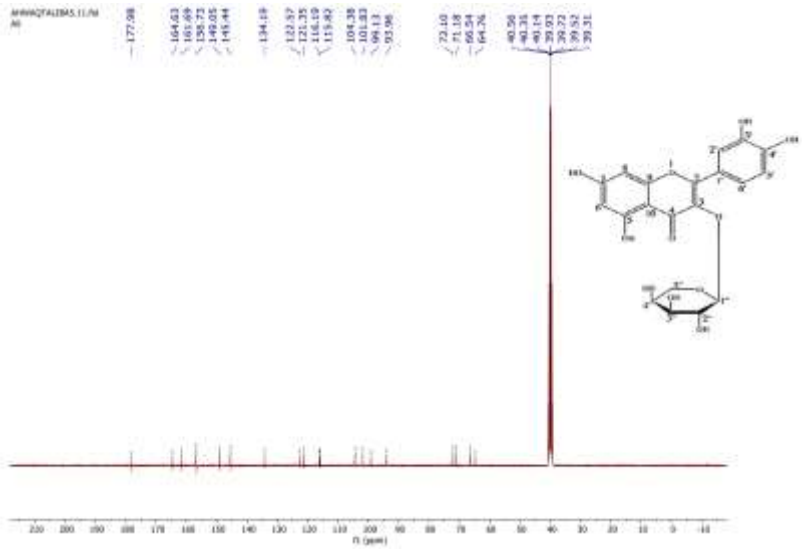

Figure 11. ^{13}C NMR of A5

MTT assay

PC3was exposed to sequential concentrations from (6.25µg /mLto 400 µg /mL) of the F3 fraction leaves of *P. guajava* compared with normal HDFn. The reduction in PC3 cell by F3 fraction after 24-hour incubation, is represented in table 3 and figure 12. It was observed that the growth of cancer cells dropped with a rise in the dose of F3 from 6.25 to 400 µg /mL [41,42]. F3 fraction exhibited IC_{50} 91.9 µg /mL, and this fraction induced noteworthy cell death in 100 and 200 µg /mL ($P < 0.05$, n = 6) cytotoxic activity of F3 fraction attributed to its ability to provoke apoptotic responses. It was observed that the extract hindered the cell cycle progression at G1 phase, leading to cell death [43]. The implicated signaling mechanisms entail the inhibition of the AKT/ (mTOR) ribosomal p70 S6 kinase (S6K1) and the (MAPK) activation pathways. It is noteworthy that an elevation in AKT signaling via the mTOR pathway has been documented in diverse carcinoma cell lines [44,45]. F3 was noncytotoxic to normal HDFn with an IC_{50} value substantially surpassing concentrations of 100 µg/mL (325.3 µg /mL) as in Figure 12. MCF7was exposed to sequential concentrations from (25 µg/mLto1000 µg/mL) of the F3 to calculate its consequences on the viability of cell line as shown in figure12. The reduction in MCF-7 cell viability after 24 hours of treatment with the F3 is illustrated in Chart 4. The F3 fraction demonstrated an IC_{50} value of 379.1 µg/mL. Significant cytotoxic effects were observed at concentrations of 500 µg/mL and 1000 µg/mL ($P < 0.05$, n = 6). F3 exhibited an antiproliferative effect on MCF-7 cells. The strong cytotoxic activity of this fraction may be attributed to its ability to induce cell death and apoptosis in cancer cells by disrupting the cell cycle at the G0/G1 and G2/M phases. Additionally, it may trigger apoptosis by downregulating the mRNA expression of Bcl-2, an anti-apoptotic protein, while upregulating pro-apoptotic gene expression in MCF-7 cells. [46,18]. F3 was noncytotoxic to normal HDFn with an IC_{50} =628,8 µg /mL.

Table 3. Cytotoxicity effect of F3 on PC3 and HdFn

F3 conc. in (µg /mL)	Viable cell counts of PC3 cell line Mean ± S.D.	Viable cell counts of HdFn cell line Mean ± S.D.	*P* value
400	64.15933 ± 8.968532	77.27633 ± 3.653573	0.058
200	63.696 ± 2.122644	86.034 ± 0.853534	0.007
100	77.62333 ± 2.411951	92.12967 ± 1.55742	0.001
50	90.58667 ± 1.801743	95.409 ± 0.37174	0.06
25	94.79167 ± 1.33472	96.18067 ± 0.2315	0.199
12.5	95.332 ± 0.813239	96.798 ± 0.481826	0.184
6.25	95.949 ± 0.463	97.10633 ± 0.5785	0.225

Table 4. Cytotoxicity effect of F3 on MCF7 and HdFn

F3 con. in (µg/ mL)	Viable cell of MCF7cell line,Mean ± S.D.	Viable cell counts of HdFn ,Mean ± S.D.	*P* value
1000	63.619±1.541	77.97067 ± 1.187	0.007
500	71.95233±2.526	86.18833 ± 1.182	0.013
250	86.188±1.428	92.94 ± 1.314	0.089
100	92.78533±0.353	95.409 ± 0.371	0.189
50	94.94567±0.928	96.18067 ± 0.231	0.198
25	95.29333 ± 0.521	96.798 ± 0.4818	0.195

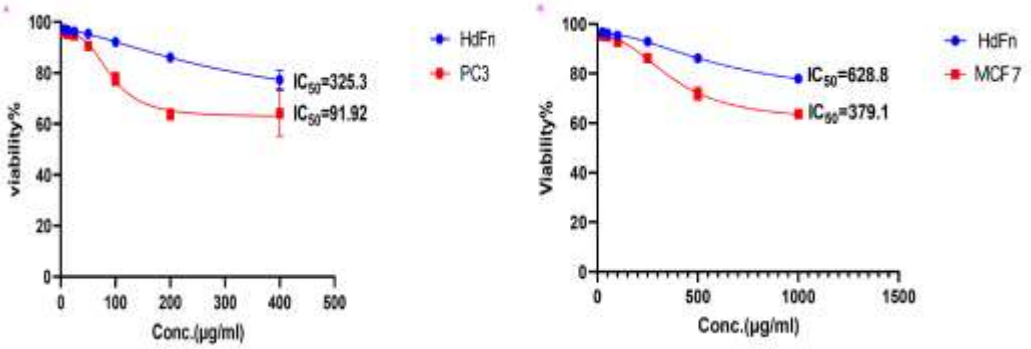

Figure 12. A, IC50 of F3 fraction guava leaves on PC3 cell line. B, IC50 of F3 fraction guava leaves on MCF7 cell line.

Conclusion

The main flavonoids of guava were found to be avicularin and guaijaverin, as indicated by the above results. These two flavonoids were successfully isolated from guava leaves using an extraction method that increased the percentage yield by using an ultrasonic bath sonicator. Techniques like NMR spectroscopy, FTIR, and HPLC were used to identify these flavonoids. The PC3 and MCF7 cell lines were used to test the F3 fraction's cytotoxic potential. Cell viability (%) decreased by the F3 fraction; the IC_{50} values were 91.9 µg/mL for PC3 and 379.1 µg/mL for MCF7. F3 was noncytotoxic to normal HDFn with IC_{50} value significantly exceeding doses of 100 µg/mL. Guava thus exhibits cytotoxic action against PC3 and MCF7 cell lines in a concentration-dependent manner. This guava-based study offers data that might support the plant's therapeutic applications, but further in-depth research in clinical trials is needed before guava can be utilized as a preventative measure or an adjuvant.

Acknowledgment

This education has been supported by the University of Baghdad /College of Pharmacy.

Conflict of Interest

None

Funding

Self-funding

Ethics Statements

In-vitro study does not need ethical approval.

Author contributions

Ashwaq T. Kareem was responsible for data collection, analysis, conducting the experimental procedures, and drafting sections of the manuscript. Enas J. Kadhim provided overall supervision, approved the final version of the study, and contributed to its revision and organization**.**

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عزل وتوصيف اثنين من مركبات الفلافونويد من نبات الجوافة المزروعة في العراق: دراسة شاملة حول التوضيح التركيبي وتقييم السمية الخلوية

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الخلاصة

تعتبر نبتة الجوافة، المعروفة علميًا باسم *Psidium guajava*، تنتمي إلى الفصيلة الآسية، تركز هذه الدراسة على عزل وتوضيح التركيب وحساب تركيز مركبات الفلافونويد من اوراق نبات الجوافة المزروعة حديثاً في العراق وتقييم النشاط السام للخلايا لجزء خلات الإيثيل ضد خلايا سرطان البروستاتا (PC3) وخلايا سرطان الثدي البشرية (MCF7) والخلايا الليفية الجلدية الطبيعية حديثي الولادة (HdFn). تضمنت عملية العزل استخدام كروماتوغرافيا الطبقة الرقيقة وكروماتوغرافيا سائل عالي الأداء الكمي (PHPLC). والتوضيح الهيكلي يتضمن التحليل الطيفي بالرنين المغناطيسي النووي (NMR) وFTIR (مطياف الأشعة تحت الحمراء لتحويل فوريه) للحصول على رؤى هيكلية تفصيلية لاثنين من مركبات الفلافونويد المعزولة، وقد أثبتت تقنيات العزل فعاليتها في الحصول على عينات نقية من الأفيكولارين والجوايجافيرين من أسيتات الإيثيل وأجزاء البيوتانول، وتقنيات التوضيح البنيوي اعطت تفسيرا جيدا لاثنين من الفلافونويدات المعزولة. تم تقييم النشاط السام للخلايا لجزء أسيتات الإيثيل، والذي أظهر تأثيرًا سامًا للخلايا بانخفاض في بقاء الخلية (٪) مع تركيز موافق للتثبيط النصفي ٩١.٩ ميكروغرام / مل ضد خلايا سرطان البروستاتا وانخفاض في بقاء الخلية (٪) مع تركيز موافق للتثبيط النصفي ٣٧٩،١ ميكروغرام / مل ضد سرطان الثدي وجزء خلات الإيثيل غير سام للخلايا الليفية الجلدية الطبيعية لحديثي الولادة، مع قيمة تركيز موافق للتثبيط النصفي تتجاوز بشكل كبير تركيزات ١٠٠ ميكروغرام / مل. قدمت هذه الأساليب التحليلية فهمًا شاملاً للتركيب الكيميائي للفلافونويدات المعزولة وايظا وضحت امتلاك الجوافة نشاطًا سامًا للخلايا ضد بعض أنواع السرطان.

الكلمات الافتتاحية: استخلاص بالموجات فوق الصوتية، افيكيولارين، جوجافيرين، جوافة، كروماتوكرافيا السائل عالي الدقة.