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Isolation and identification of hyperin and naringenin from guava cultivated in Iraq and evaluation of cytotoxic activity of hyperin

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Abstract

The guava plant, *Psidium guajava* L., serves as proof of the abundant donations of nature, providing a delicious guava fruit; this plant is rich in groups of medicinal and nutritional benefits. Guava belonging to the Myrtaceae family, many previous studies reported many phytochemical constituents in its leaves that have many pharmacological activities and medicinal properties; this study focuses on the isolation, structural elucidation and calculation concentration of flavonoids, assessment of the cytotoxic activityof hyperin from *Psidium guajava* leaves newly cultivated in Iraq. The isolation process involved the use of thin-layer chromatography (TLC) and preparative high-performance liquid chromatography (PHPLC) and structural elucidation involved NMR (nuclear magnetic resonance spectroscopy) and FTIR (Fourier transform infrared spectrometer) for detailed structural insights into 2 isolated flavonoids. The isolation techniques proved effective in obtaining pure samples of hyperin and naringenin from the ethyl acetate and n.butanol fractions and structural elucidation techniques gives a good explanation for 2 isolated flavonoids. Evaluate cytotoxic activity of hyperin flavonoid against prostate cancer cell line (PC-3), human breast cancer cell line (MCF-7) and normal dermal fibroblast neonatal (HdFn), hyperin flavonoid exhibited a decrease in cell viability (%) with IC_{50} 58.9 µg /mL against the prostate cancer cell and IC_{50} 90.58 µg /mL against human breast cancer and noncytotoxic to HDFn, with an IC_{50} value substantially surpassing concentrations of 100 µg/mL. These analytical approaches provided a comprehensive understanding of the chemical composition of the isolated flavonoids and interpreted that guava has cytotoxic activity against some cancers, depending on a concentration-dependent mode.

Keywords

ultrasonic-assisted extraction; hyperin; naringenin; guava; NMR spectroscopy

Introduction

Psidium guajava L. of the Myrtaceae family and it is a popular fruit in tropical areas like Indonesia, India, Pakistan and South America. Many previous studies exhibited that leaves of the guava have many health benefits that are related to their phytochemical constituents, such as quercetin, apigenin, avicularin, kaempferol, myricetin, gallic acid, epicatechin, caffeic acid and chlorogenic acid. Also, guava leaves are studied for their biological effects, such as anticancer, antioxidant, antidiarrheal, lipid-lowering and

hepatoprotection activities (1, 2). The previous studies revealed that guava has several medicinal properties, which have been reported from across the globe in the form of ethnobotanical/ ethnopharmacological surveys; leaves of guava are traditionally used to treat several illnesses like gastrointestinal infections, respiratory challenges, wounds, fevers and rheumatism (3, 4). Guava chemical constituents include compounds such as tannins, phenols, polyphenols, saponins, sterols and terpenoids. It is important to know that the kind and amounts of phytochemicals can vary according to soil conditions, plant tissue and seasonal changes (5). Newly advanced techniques used to extract phenolic constituents from guava, like ultrasonication, were a good process to extract phenolic compounds from guava leaves (6). Breast cancer is widely distributed among women and liver cancer is most commonly found in third-world countries and is one of the causes of death, so alternative medicines are needed in cancer management (7, 8). Many previous studies have evaluated the cytotoxic activity of guava extract and fractions against prostate cancer cell lines (PC-3). In this study, we want to isolate and identify flavonoids from guava leaves and evaluate the cytotoxic activity of isolated hyperin flavonoids against prostate cancer cell line (PC-3), human breast cancer cell line (MCF-7) and normal dermal fibroblast neonatal (HdFn).

Materials and Methods

Preparation of plant material

In spring 2023, *P. guajava* leaves were collected from Musayyib city and were first planted in Iraq. After the collection, the plant specimens underwent identification and authentication conducted by expert Dr. Sukaena Abass from the College of Sciences. The plant components were thoroughly dried in the shade and, finally, ground and stored to use for further extraction and analysis (9).

Extraction of guava leaves

Approximately 50 g of finely ground *P. guajava* leaves underwent extraction using an ultrasonic bath sonicator, capitalizing on the cavitation principle to enhance the release of plant compounds. The ultrasonic waves, operating at 40 KHz, 50 g of leaf powder in 750 mL of 70 % ethanol at 30 °C for 40 min. Following this, the crude ethanol extract underwent filtration and concentration under condensed pressure using a rotary evaporator. The dried extract obtained was liquified in 500 mL of distilled water and underwent successive partitioning with 500 mL aliquots of petroleum ether, chloroform, ethyl acetate and n-butanol. This partitioning process was repeated 3 times for each solvent in a separatory funnel. All fractions, except n-butanol, underwent drying over anhydrous sodium sulfate, followed by filtration and subsequent evaporation with a rotary evaporator until complete dryness (10).

Preliminary phytochemical assessment

Initial chemical examinations were carried out to decide the incidence or lack of polyphenolic compounds in Iraqi *P. guajava* achieved for extracting leaves ethyl acetate fraction (F3) and butanol fraction (F4). On examination done by Alkaline reagent tests, a few amounts of extracts plus drops of sodium hydroxide and a yellow colour appeared, but it appeared colourless by adding drops of dilute HCL (11).

Analysis of Guava Fractions by HPLC (high-performance liquid chromatography) and TLC (thin-layer chromatography)

Examination of the (F3) and (F4) for guava leaves and the assessment of these 2 fractions attained through TLC and HPLC. The chromatographic analysis involves a Stationary phase consisting of silica, readily available as a 20 × 20 cm aluminum sheet covered with silica gel 60 GF254, with a thickness of 0.25 mm. This silica gel is activated by heating at (110 °C for 10 min) to eliminate moisture absorbed by the air; for the (F3) and (F4), the mobile phase (S1) comprises ethyl acetate: toluene: methanol: formic acid: (15:15:1:4). Samples and standards (Hyperin and Naringenin) are organized by dissolving them in a few of methanol.

They are then functional in diluted form along the baseline of the plate, which is advanced in a TLC jar hidden with a glass lid. The separated spots on the TLC plate can be non-destructively visualized using ultraviolet (UV) light with 254 nm and 365 nm, facilitated by a Desaga UV light source from Germany (12). A qualitative check through HPLC was performed on the (F3) and (F4) fractions of leaves, comparing them to the hyperin standard and naringenin standard. Sample preparation involved dissolving (F3) and (F4) fractions in methanol. This solution underwent filtration using a filter membrane (0.4 µm) before inoculation into the HPLC column. Identical between samples and standards was achieved based on observed retention times. The conditions for the analysis of the (F3) and (F4) fractions by (HPLC) were conducted using a stationary phase on (Knuaer, Germany) C18 column (5 µm particle size, 250 x 4.6 mm) with a Dionex Ultimate 3000 liquid chromatograph. The mobile phase consisted of a 1 % aqueous acetic acid solution and acetonitrile utilizing gradient elution. The rate of flow was maintained at 0.7 mL/min. The column was thermally regulated at 28 °C and an inoculation volume of 20 µL was employed. The gradient elution proceeded linearly, transitioning from 10 % to 40 % of the component (13, 14).

Phytochemical ingredients in plant extracts are often present in small quantities, necessitating a sensitive instrument for recognition and isolation. In this study, preparative high-performance liquid chromatography (PHPLC) was employed for the isolation of a pure number of flavonoids, PHPLC apparatus specification and conditions as in previous studies (15, 16).

Examination by Fourier-Transform Infrared Spectroscopy (FTIR)

(FTIR) spectra of isolated compounds were recorded in the Shimadzu FTIR spectrometer in the wave number range 500 to 4000 cm^{-1} and operated in the transmittance mode (17).

Analysis by NMR

NMR spectra were captured at a temperature of 25 °C employing a 400 MHz Bruker Avance 6D spectrometer and utilizing DMSO as the solvent.

Cell Line Maintenance

The method of work was conducted for cell line maintenance according to Freshney RI (18).

MTT assay

Hyperin flavonoids cytotoxicity effect on PC3 and MCF7 cells was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay based on the detection of mitochondrial dehydrogenase activity in living cells, first day poring the cells in a 96-well microplate by counting cells using trypan blue, about 1 x 10^4 cells were cultured in each well. After the cells were cultivated, the 96-well plate was sited in an incubator at 37 °C for 24 h until 60 % of the well surface was filled; otherwise, more time was needed. On the second day of treatment of PC-3 cells with different concentrations (6.25–400 µg/mL) of hyperin flavonoids for 72 h and treatment of MCF-7 cells with different concentrations (15.75–1000 µg/mL) of Hyperin flavonoids also for 72 h, after emptying the supernatant of each well by the sampler, 100 µL of every dilution was added to the wells. The pouring pattern was drawn and eight wells were considered for each dilution. On the third day, Adding MTT dye 24 h later, the medium was rid and MTT solution 100 µL (0.5 mg/mL) was added to the plates in the shady and put in the incubator for 4 h. Then, the top medium of the wells was detached with a sampler and DMSO 100 µL was added to the wells and placed on a shaker for 20 min (at this stage, the container should be hidden so as not to be exposed to light). Finally, the intensity of the resulting color was read by a (DNM-9602G) microplate reader at 570 nm (19, 20).

Statistical analysis

Using the Statistical Package for Social Science version 24.0. The data were articulated as mean values accompanied by standard deviation (mean ± SD). One-way analysis of variance was utilized to evaluate the outcomes and after that, Dunnett's post hoc test was conducted. Statistical significance was to get to know at a p-value less than 0.05, representing a significant difference.

Results and discussion

Extraction by Ultrasonic bath sonicator

The selected method for leaf extraction was based on ultrasound-assisted extraction (UAE), resulting in a higher % yield (21) of approximately 20 %w/w from 50 g of guava leaves according to formula: % =weight of crude (g)/weight of plant material (g) x100 (22). This method relies on the principle of cavitation, which enhances the release of compounds from the plant material. The conditions for UAE included ultrasonic waves at 40 KHz, utilizing 50 g of leaf powder in 750 mL of 70 % ethanol at 30 °C for 40 min. Subsequently, the crude ethanol extract underwent filtration and concentration under reduced pressure using a rotary evaporator.

Preliminary phytochemical investigation

Chemical tests were done on the guava leaves and the results in Table 1 indicating the presence of flavonoids in F3 and F4 fractions.

Table 1. Investigation of guava leaf extract phytochemical compounds

Name of fraction	Alkaline reagent test
F3	+
F4	+

Examination of the guava leaves Fractions by TLC and HPLC

Analysis of (F3) and (F4) fractions by (TLC) and (HPLC) analysis of the F3 and F4 fraction from *P. guajava* leaves using TLC involved the application of a mobile phase S1. A sample of the F3 and F4 dissolved in methanol was applied in diluted form on the baseline of a glass TLC plate (22 cm x 22 cm x 7 cm) within a glass TLC jar enclosed with a glass

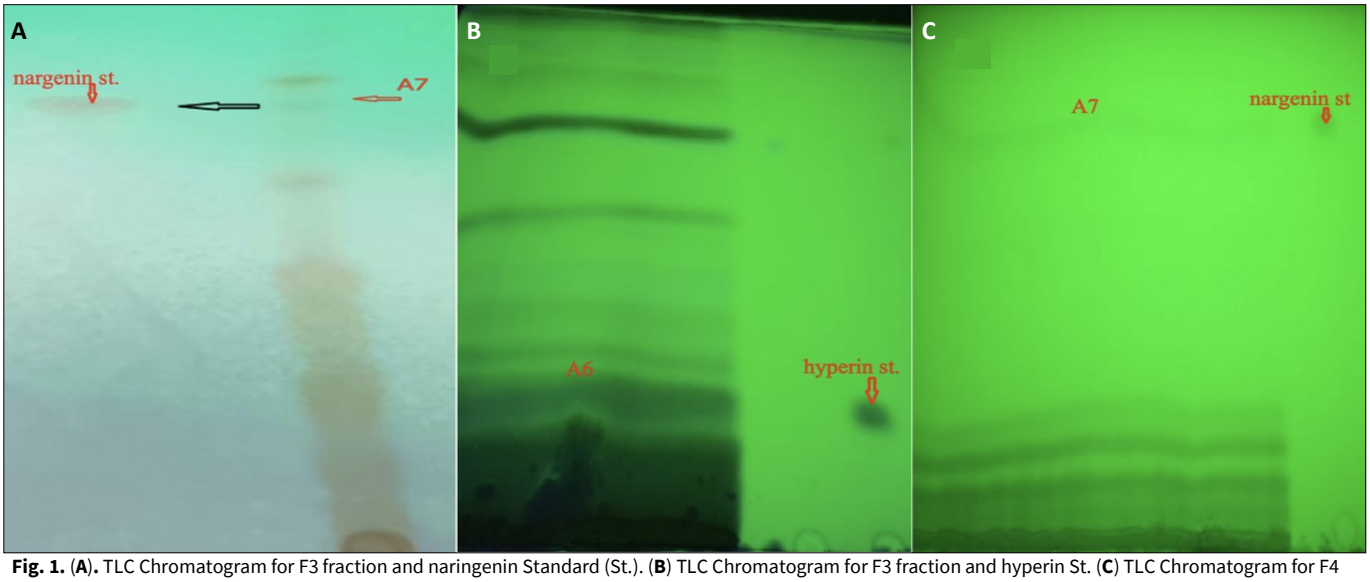

Fig. 1. (**A**)**.** TLC Chromatogram for F3 fraction and naringenin Standard (St.). (**B**) TLC Chromatogram for F3 fraction and hyperin St. (**C**) TLC Chromatogram for F4 fraction and naringenin St. in Toluene: Ethyl acetate: Formic acid: Methanol in a ratio of 30:30:8:2 detection Under UV Light 254 nm.

cap. The separated spots on the TLC plate were imagined under ultraviolet (UV) light, as illustrated in Fig. 1(A). The subsequent isolation method from the leaves utilized a glass plate of 0.5 mm, employing the identical solvent system S1: (15:15:1:4). depicts The isolation of A6 and A7, showcasing the separated bands is given in Fig. 1(B). Further analysis of the F3 and F4 fractions was conducted for leaves using HPLC, as depicted in Fig. 2.

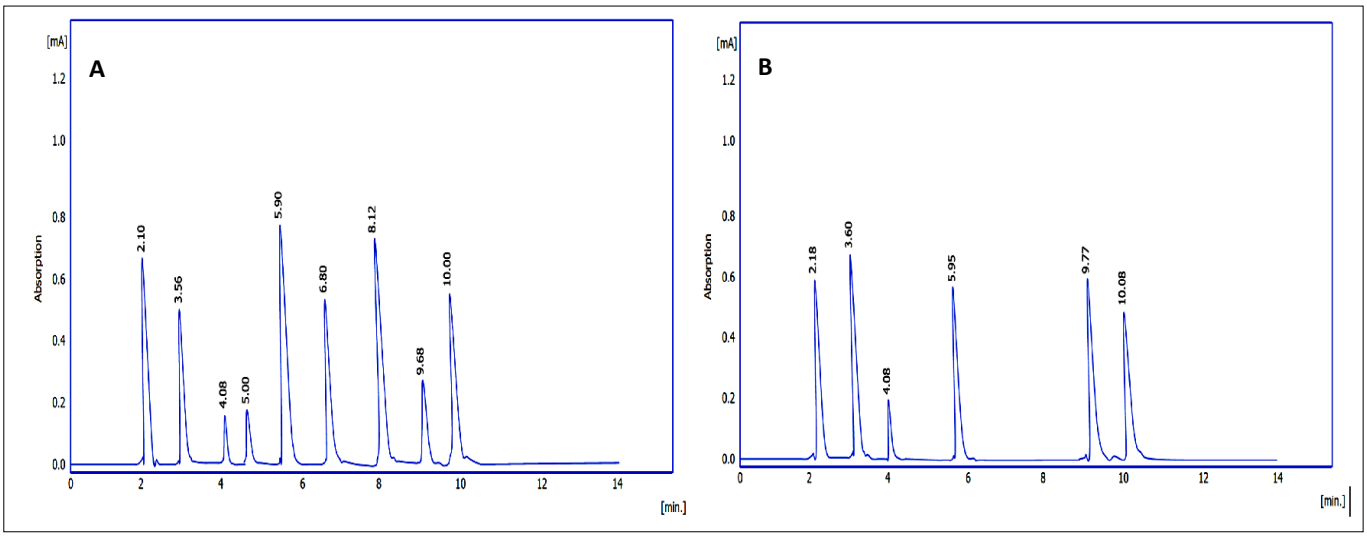

Fig. 2. (**A**)**,** HPLC of guava leaves F3 fraction. (**B**), HPLC of guava leaves F4 fraction.

Documentation of Compounds A6 and A7 by TLC, HPLC and PHPLC

The isolation procedure for compounds A6 and A7 from the (F3) and (F4) fractions of leaves, following the TLC, HPLC examination, indicates a remarkably close Rf value of hyperin standard and nargenin standard spots matched to the A6 and A7 as depicted in and Table 2 and close retention time in HPLC between hyperin standard and nargenin standard matched to the A6 and A7 as depicted in Fig. 3 & 4.

Table 2. Rf value of isolated compounds and standards.

Isolated compounds	RFvalue	Standards (St.)	RFvalue
A6	± 0.1	Hyperin St.	± 0.1
A7	± 0.62	Naringenin St.	± 0.62

Phytochemical compounds in plants are in limited quantities, so used highly accurate instruments for detection and isolation, which is PHPLC (23). The quantitative PHPLC analysis of the isolated compounds indicates that hyperin and naringenin in F3 and nargenin in F4 with high concentrations, as shown in Table 3 and Fig. 5.

Analysis by (FTIR)

(FTIR) spectra of isolated compounds were documented in the FTIR spectrometer (Shimadzu) in the range of wave number 500 to 4000 cm^{-1} (17). IR spectroscopy analysis of Compound A6 and A7 (Fig. 6), IR spectra of compound A6 revealed the presence of 3491 bands referring to broad intermolecular phenolic O-H stretching band, 3216 refer to O-H bending of phenol, 1656 refer to C=O stretching of carboxylic acid, 1608 refer to aromatic C=C stretching band and A7 IR spectra revealed the presence of 3263 bands refer to O-H stretching of phenol, 1627 band refer to C=C stretching of the aromatic ring, 831 band refer to fingerprint of aromatic ring, So IR spectra of compound compounds A6 and A7 matched with standard naringenin and

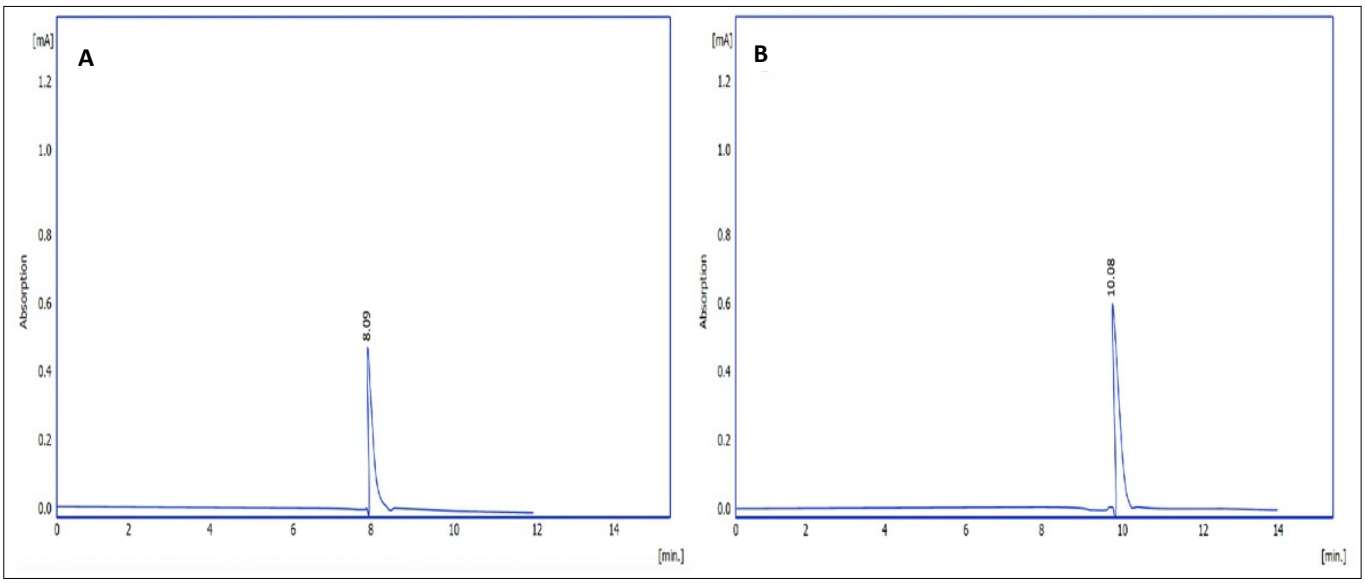

Fig. 3. (**A**), HPLC of the isolated A6 compound. (**B**), HPLC of the isolated A7compound.

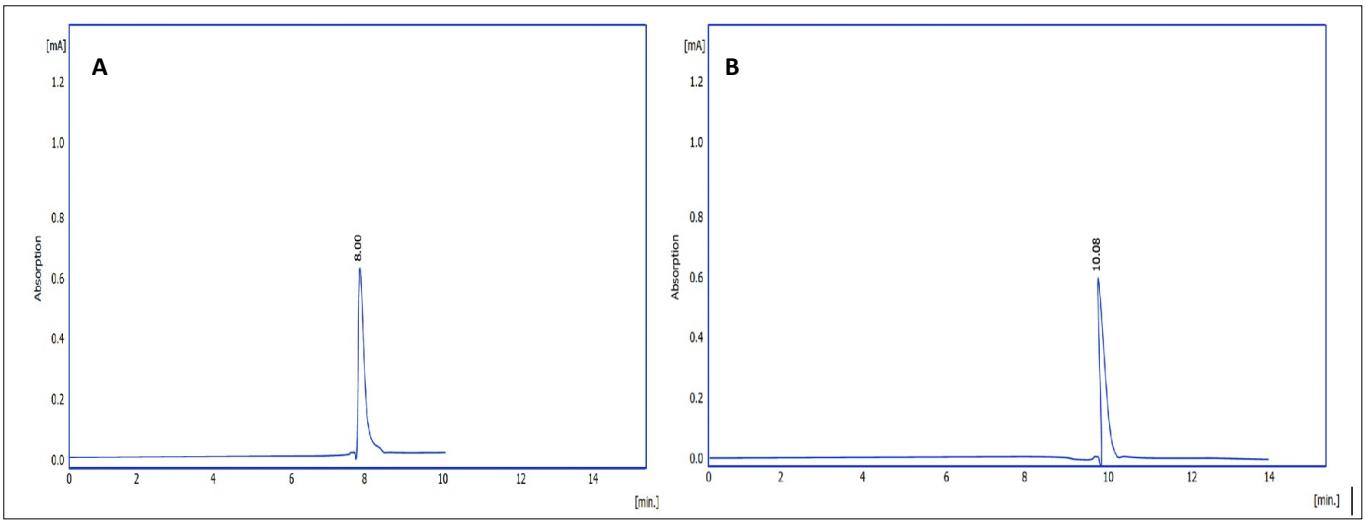

Fig. 4. (**A**), HPLC of hyperin standard. (**B**), HPLC of the nargenin standard.

Table 3. The concentration of isolated compounds in the F3 and F4 of Iraqi *Psidium guajava* leaves plant

Flavonoids names	Area under the curve	Total conc. (µg/mL)
Hyperin	19652.36 in F3	16.459 in F3
Naringenin	1874.64 in F35414.71 in F4	4.6986 in F313.572 in F4

hyperin standards as shown in previous studies (24, 25).

Identification of isolated compounds by NMR spectroscopy

^{1}H NMR spectral data of A6 (Fig. 7) δ 12.65 (s, 1H,OH5), 10.90 (s, 1H,OH3), 9.77 (s, 1H,OH7), 9.19 (s, 1H,OH3`), 7.68

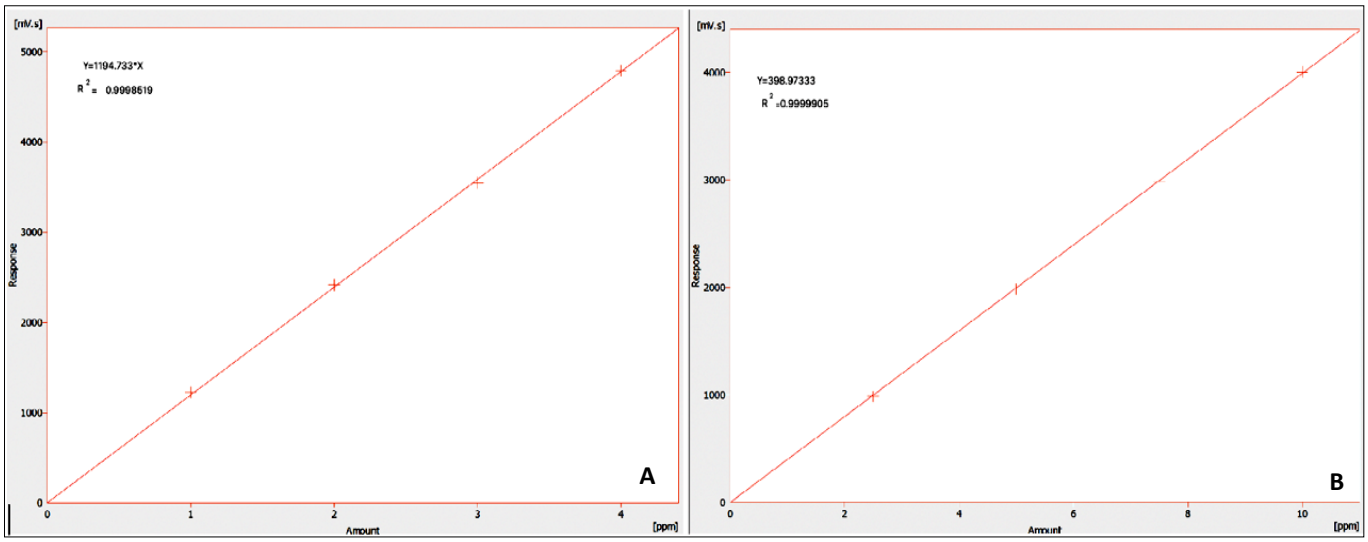

Fig. 5. (**A**), Standard curves of hyperin. (**B**), Standard curves of naringenin by PHPLC.

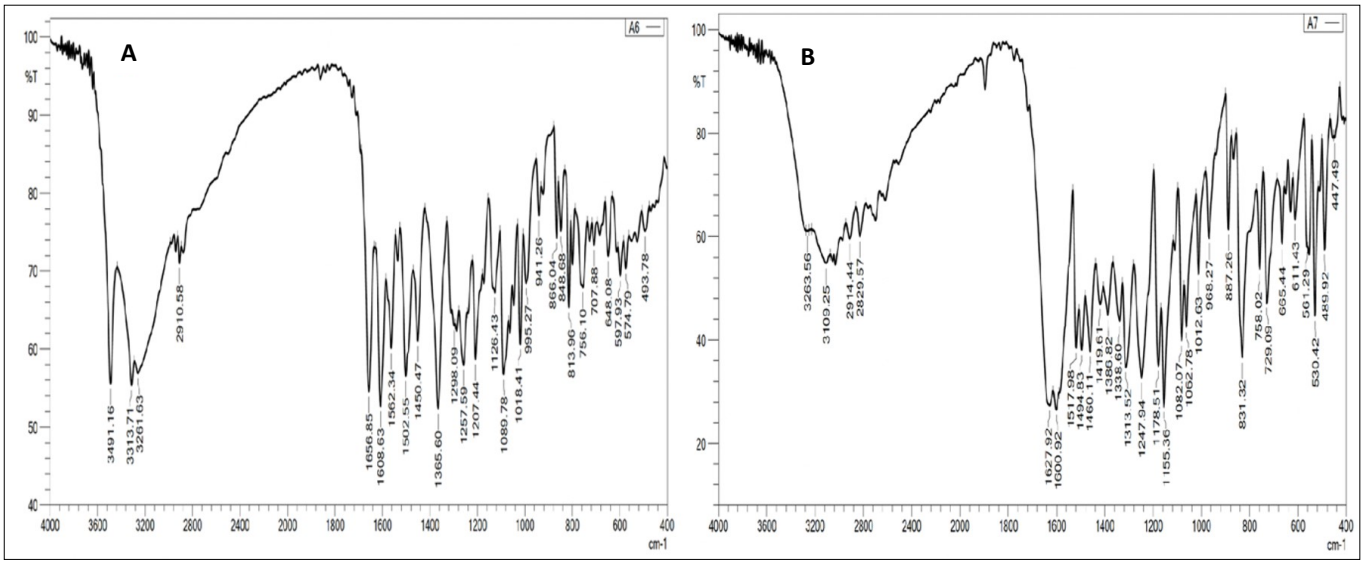

Fig. 6. (**A**), Spectrum of FTIR for A6 compound. (**B**) Spectrum of FTIR for A7 compound.

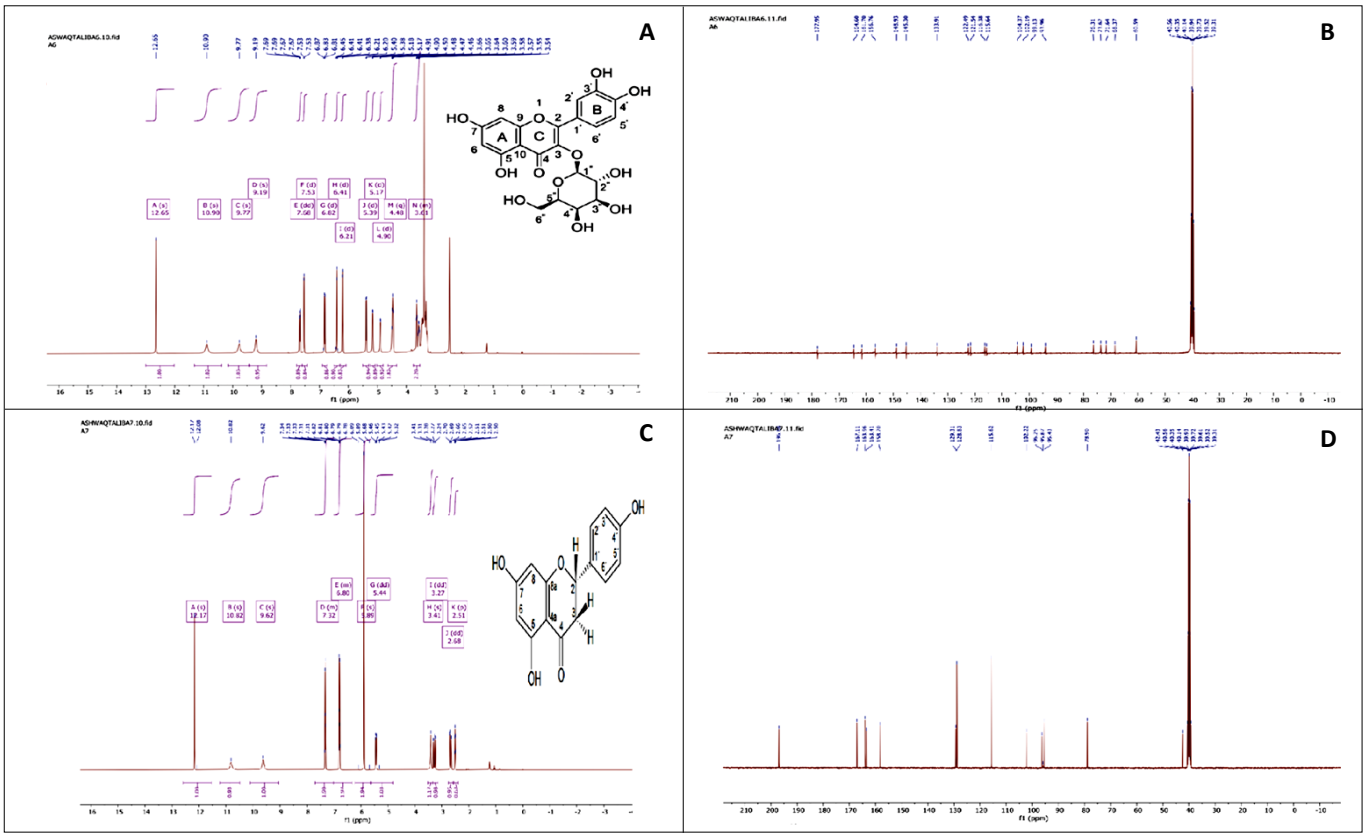

Fig. 7. (**A**),^{1}H NMR of A6 compound. (**B**),^{13}CNMR of A6 compound. (**C**),^{1}H NMR of A7compound. (**D**),^{13}CNMR of A7 compound.

(dd, *J* = 8.5, 2.2 Hz, 1H,H2`), 7.53 (d, *J* = 2.2 Hz, 1H,H6`), 6.82 (d, *J* = 8.5 Hz, 1H,H5`), 6.41 (d, *J* = 2.1 Hz, 1H,H8), 6.21 (d, *J* = 2.1 Hz, 1H,H6), 5.39 (d, *J* = 7.6 Hz, 1H,H1``), 5.17 (d, *J* = 4.6 Hz, 1H,H3``), 4.90 (d, *J* = 5.5 Hz, 1H,H2``), 4.48 (q, *J* = 5.1 Hz, 2H,OH2``, OH3``), 3.61(d, *J* = 3.6Hz, 2H,H6``).^{13}CNMRofA6(Fig.7)_(101MHz,DMSO)δ (177.96,C4)(164.60,C7)(161.70,C5)(156.76,C2)(156.68,C9) (148.93,C4`)(145.30,C3`)(133.92,C3)(122.49,C6`) (121.54,C1`)(116.38,C5`)(115.64,C2`)(104.37,C10) (102.20,C1``)(99.13,C6)(93.97,C8)(76.31, C5``) (73.62,C3``)(71.64,C2``)(68.37,C4``)(60.59,C6``). The NMR spectra of A6 show good agreement with those reported in the literature of Hyperin (26). We identified the chemical structure of each compound using NMR spectroscopy, ^{1}HNMR spectrum compound A7 (Fig. 7) and ^{13}C NMR spectrum (Fig. 7), shows that was a single compound. The interpretation of the ^{1}HNMR,^{13}C-NMR are summarized as follows ^{1}H NMR (400 MHz, DMSO) δ 12.17 (s, 1H,OH5), 10.82 (s, 1H,OH3), 9.62 (s, 1H,OH7), 7.71 – 7.01 (m,2H,H2`,H6`), 7.01 – 6.33 (m, 2H,H3`,H5`), 5.89 (s, 2H,H6,H8), 5.44 (dd, *J* = 12.9, 2.9 Hz, 1H,H2), 3.41 (s, 1H,H3 ALPHA), 3.27 (dd, *J* = 17.1, 12.9 Hz, 1H,H3BETA). C NMR (101 MHz, DMSO) δ 196.88(C4), 167.11(C7), 163.96(C5), 163.41(C8A), 158.20(C4`), 129.31(C1`), 128.83(C3`, C6`), 115.62(C2`, C6`), 102.22(C4A), 96.25(C6), 95.87(C8), 78.90 (C2), 42.43(C3) The NMR spectra of A7 show good agreement with those reported in the literature of Naringenin (27).

MTT assay

The anticancer effect of hyperin flavonoid isolated from *P. guajava* leaves against PC3 and MCF7 human cancer cell line was weighed by MTT assay, PC3 was exposed to sequential concentrations (6.25 µg/mL, 12.5 µg/mL, 25 µg/

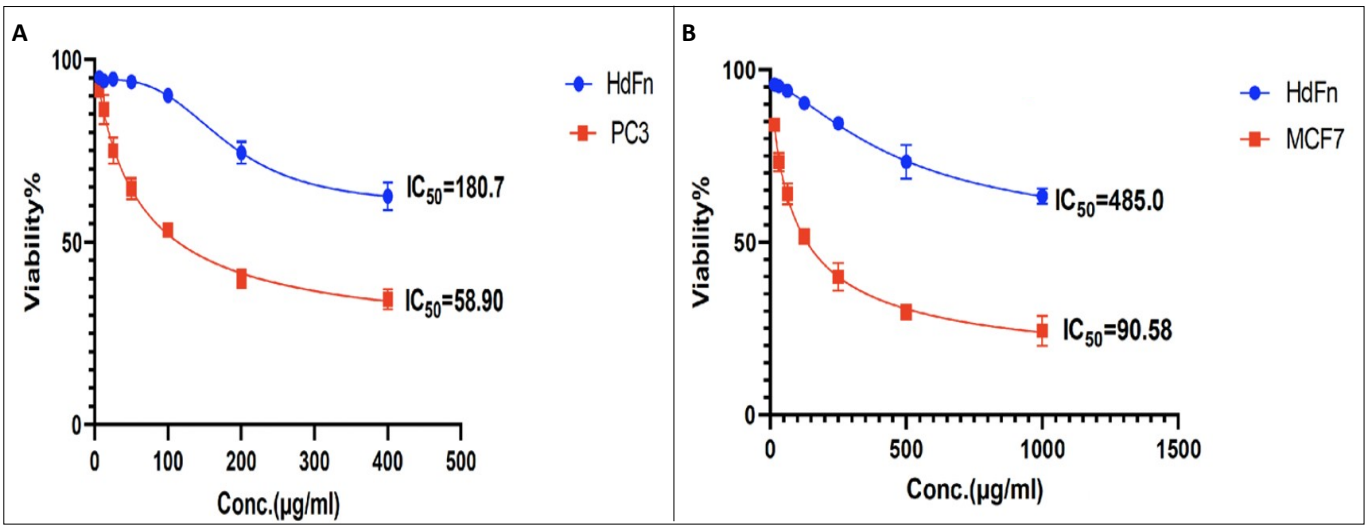

Fig. 8. (**A**), IC_{50} of hyperin isolated from *P. guajava* leaves on PC3. (**B**), IC_{50} of hyperin isolated from *P. guajava* leaves on MCF-7cell lines.

mL, 50 µg/mL, 100 µg/mL, 200 µg/mL, 400 µg/mL) of the hyperin isolated from *P. guajava* to assess its effects on the viability of cell line as shown in Fig. 8. The decrease in PC3

Table 4. Cytotoxicity effect of hyperin isolated from *P. guajava* leaves on PC-3 and HdFn cell after 72 h of incubation at 37 °C

	HdFn		PC-3	
Conc.	**mean**	**SD**	**mean**	**SD**
400	62.53867	3.741931	34.37533	2.736582
200	74.49867	3.076421	39.96933	2.552461
100	90.08467	1.049913	53.318	1.571414
50	93.86533	1.104054	64.66033	2.845406
25	94.63733	0.481986	75	3.599024
12.5	94.097	1.252097	86.26533	4.033667
6.25	95.02333	0.2315	91.58933	1.100001

cell viability (%) by hyperin after 72 h incubation is represented in Table 4 and Fig. 8. Hyperin exhibited a decrease in cell viability (%) with IC_{50} values of 58.9 µg /mL. This extract induced significant cell death began at 25 mg/mL, cell death was significant from 25 to 400 µg /mL ($P < 0.05$, n = 6), hyperin has an antiproliferative effect against PC-3 cells in a concentration-dependent mode. MCF-7 was exposed to sequential concentrations (15.75 µg/mL, 31.25 µg/mL, 62.5 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL, 1000 µg/mL) of the hyperin isolated from *P. guajava* to assess its effects on the viability of cell line as shown in Fig. 8. The decrease in MCF-7 cell viability (%) by hyperin

Table 5. Cytotoxicity effect of hyperin isolated from *P. guajava* leaves on MCF-7 and HdFn cells after72 h of incubation at 37 °C.

	HdFn		MCF7	
Conc.	**mean**	**SD**	**mean**	**SD**
1000	63.387	2.215092	24.30567	4.319746
500	73.26367	4.887475	29.82267	2.077898
250	84.49067	1.334569	39.969	3.936065
125	90.31667	0.928419	51.62033	2.124547
62.5	93.866	0.926	64.08167	3.159664
31.25	95.216	0.371747	73.22533	2.715021
15.75	95.71767	0.834962	84.14333	0.834408

after 72 h incubation, is represented in Table 5 and Fig. 8. hyperin exhibited a decrease in cell viability (%) with IC_{50} values of 90.58 µg/mL. This extract induced significant

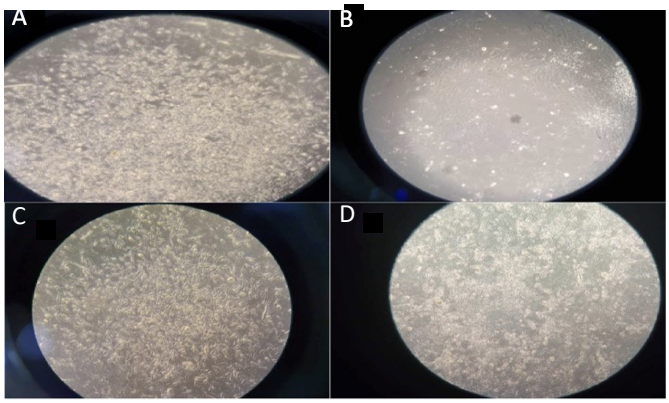

Fig. 9. (**A**), Morphology of control. (**B**), Morphology of PC3cell line after management with hyperin flavonoid, (**C**), Morphology of control. (**D**), Morphology of MCF-7cell line after management with hyperin flavonoid.

cell death began at 31.25 µg/mL and cell death was significant from 31.25 to 1000 µg/mL ($P < 0.05$, n = 6). Hyperin also has morphological alteration on MCF-7 and PC-3 cell lines, as shown in Fig. 9. Hyperin has an anti-proliferative effect against MCF7 cells in a concentration-dependent mode. The maximum cytotoxic activity of Hyperin might be due to its capacity to form cytotoxic and apoptotic activities within cancer cells by dropping in G0/G1 and G2/M phases and activating apoptosis by inhibition of the mRNA expression of Bcl-2, a cellular protein that decreases apoptosis while activating the pro-/anti-apoptotic mRNA expression in the cancer cells (28-31). Hyperin was noncytotoxic to normal HDFn with an IC_{50} of more than 100 µg/mL, as in Fig. 8.

Conclusion

The isolation of 2 flavonoids, Hyperin and Naringenin, from guava leaves proved successful, and an extraction method was employed, using an ultrasonic bath sonicator to enhance the percentage yield. The identification of these flavonoids was carried out through techniques such as NMR spectroscopy, FTIR and HPLC. The cytotoxic activity of Hyperin isolated from guava leaves was assessed on a specific cell line (PC3 and MCF-7), which exhibited a decrease in cell viability (%) with IC_{50} 58.9 µg/mL on PC3 and IC_{50} values of 90.58 µg/mL on MCF7 cell lines and noncytotoxic to normal HDFn, with an IC_{50} value concentration of more than 100 µg/mL.

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Authors' contributions

ATK contributed to data gathering, analysis, practical (follow the procedure) and written parts of the study. EJK gave final approval and agreement for all aspects of the study, supervision, revision and rearrangement.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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