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Unveiling Novel Compounds and Cytotoxic Activity in Iraqi Guava Leaves: Extracting and Characterizing Essential Oil and Petroleum Ether Fraction

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Abstract

Psidium guajava is a compact tropical tree or shrub belonging to the Myrtaceae family. Originally native to tropical America, The literature survey has unveiled the diverse medicinal properties of guava, which have been documented worldwide through ethnobotanical and ethnopharmacological surveys so it is newly cultivated in Iraq, this research is considered the first of its kind to extract essential oil from guava leaves and fatty acid from petroleum ether fraction of guava leaves newly cultivated in Iraq to identify the compounds present and test the cytotoxic activity of petroleum ether fraction on HEPG2 cell line. The extraction of essential oil (EO) from (Guava) leaves was carried out using the hydro distillation method with the Clevenger) apparatus and the (EO) was subjected to GC/MS and many compounds were identified, some of them only identified in Iraqi guava essential oil leaves. Ultrasonic-assisted extraction (UAE)was used for the extraction of guava leaves and the crude extract after drying suspended with water for fractionation method (3 times) with 100 ml of different solvents polarity in ascending manner(petroleum ether, chloroform, ethyl acetate, and n. butanol) petroleum ether fraction was dried and subjected to GC/MS spectrometry many compounds identified in petroleum ether fraction and large amount of gamma-sitosterol was found in the petroleum ether fraction of Iraqi guava leaves compared to its amount in the rest of the guava leaves in the world. This study investigates the antitumor potential of *P.guajava* petroleum ether fraction on the human hepatoma cell line (HEPG2) and the result shows the inhibition of the HEPG2 cell line in dose dose-dependent manner.

Keywords: Ultrasonic-assisted extraction, essential oil, petroleum ether fraction, gamma sitosterol, Carvone.

Introduction

Since the dawn of life on Earth, a symbiotic relationship has existed between humans, animals, and plants, providing essential elements for survival, including oxygen, sustenance, and medicinal remedies for treating illnesses. As time progressed, through experimentation and experience, humans acquired the knowledge of harnessing the benefits of herbal resources to enhance their quality of life [(1,2)]. Iraq is home to a considerable variety of medicinal and toxic plants, frequently employed in domestic treatments. The examination and research into the active components of these plants hold promising potential for the pharmaceutical sector. The analysis of certain wild plant-based remedies has yielded highly favorable outcomes [(3)] Guava (*Psidium guajava*) is a compact tropical tree or shrub belonging to the Myrtaceae family. Originally native to tropical America, it is widely cultivated for its edible fruits and have many pharmacological activities The literature survey has unveiled the diverse medicinal properties of guava, which have been documented worldwide through ethnobotanical and ethnopharmacological surveys, laboratory experiments, and clinical trials. Furthermore, in addition to establishing the safety of guava, the existing body of literature affirms its effectiveness against a range of health conditions, Guava leaves exhibit a range of pharmacological activities related to the presence of bioactive compounds like phenolic acids, flavonoids, essential oil, terpenoids, glycosides, and saponins. The pharmacological activity of guava such as anti-oxidant [(4)] Antidiabetic [(5)], Anti-inflammatory, Hepatoprotective Antifungal, and antiviral action [(6-9)]. Cancer constitutes a collection of illnesses marked by the emergence of aberrant cells that proliferate uncontrollably and possess the capacity to invade and disrupt normal bodily tissues [(10)]. Globally, it stands as the second most prevalent cause of mortality. The onset of cancer transpires as certain cells within the body undergo unbridled growth, disseminating to diverse regions. The

accelerated proliferation of these cells may lead to the formation of tumors and disturbance of the body's regular physiological activities. The genesis of cancer is attributed to genetic alterations in cells, from either inherited mutations or environmental influences [11]. The utilization of plant extracts in the pursuit of anti-cancer activities has garnered substantial attention in scientific research. Plants are a rich source of diverse bioactive compounds with the potential to impede the growth and proliferation of cancer cells These plant-derived substances, often referred to as phytochemicals, encompass a spectrum of compounds such as alkaloids, flavonoids, terpenoids, and polyphenols. Several studies have explored the anti-cancer properties of guava leaves, revealing promising results [12]. The leaves have demonstrated cytotoxic effects against various cancer cell lines, inhibiting their growth and inducing apoptosis, a programmed cell death crucial for preventing the uncontrolled proliferation of cancer cells [13]. So, Guava newly cultivated in Iraq, this research is considered the first of its kind and aims to extract essential oil from guava leaves and show any new compounds in Iraqi guava leaves, investigate the components in petroleum ether fraction of guava leaves, and if there is differ from compounds previously identified, and test the cytotoxic activity of petroleum ether fraction of these plant leaves on human hepatoma cell line (HEPG2).

Materials and Methods

Plant material collection

In April 2023, *Psidium guajava* leaves were gathered at the end of the flowering stage from Musayyib City in Babylon province. The leaves were washed to get rid of dust, dried in the shade, and then finely powdered using a mechanical grinder.

Equipment and chemicals

In this study, various equipment and chemicals were employed. A rotary evaporator from BÜCHI Rotavapor was used for specific processes. All chemicals utilized in the research were of high purity, and the solvents and chemicals were of analytical grade, sourced from BDH, Ltd. in Poole, England. A Clevenger apparatus, GC mass spectrometry, and the HEPG2 cell line obtained from the Iraqi Center for Cancer Research and Medical Genetics were also instrumental in conducting the experiments.

Extraction of essential oil (EO)

Extraction and isolation of essential oil from (Guava) leaves were carried out using the hydro distillation method with the Clevenger device. The extraction process was conducted as follows: for every 500 grams of dried leaves, 1500 liters of water were used in the Clevenger device for 10 hours. A pale yellow was obtained from guava leaves and dried using anhydrous (sodium sulfate) The yields of the essential oils were calculated by the formula: **Yield of essential oil= Volume of essential oil (g)/ The volume of sample (g) x 100%** [14]. The EO was stored in a dark glass container at approximately (4°C) until analysis by Gas Chromatography/Mass Spectrometry (GC/MS). [15,16]

Initial testing of plant extracts

Chemical showing tests were used for the finding of Sterols, steroids, and terpenoids

Salkowski test

The Salkowski test is a chemical test commonly used to detect the presence of terpenoids in plant extracts. Here is how the test was conducted for detecting terpenoids Sample Preparation: Five milliliters (5 ml) of the plant extract were taken. Chloroform Addition: To 5 ml of plant extract, 1.5 ml of chloroform was added (2 ml) of concentrated sulfuric acid was added carefully to the mixture. The reaction was observed for any color changes. If a reddish-brown color developed, it indicated a positive result for the presence of terpenoids in the plant extract, The formation of a reddish-brown color is a characteristic reaction associated with terpenoids in the Salkowski test. [17]

Liberman-Burchard test

The detection of sterols and steroids in a sample was performed using a chemical test involving acetic anhydride and sulfuric acid. 0.5 grams of the sample extract were placed in a test tube. (2 ml) of (acetic anhydride) was added to the test tube holding the sample Subsequently, 2 ml of concentrated (H2SO4) was carefully added to the mixture. The reaction mixture was observed for any color changes. If a green or blue color appeared in the mixture, it confirmed the presence of steroids in the sample. The development of a green or blue color in this test is indicative of the presence of steroids or sterols in the tested sample. This chemical test is a qualitative method for detecting these specific compounds [17].

Preparation of plant extract

Ultrasonic-assisted extraction (UAE) is a method that employs ultrasonic waves to extract bioactive compounds from various plant materials, including guava leaves. It is known for its speed and efficiency in extracting bioactive constituents. It can accomplish extraction in a relatively short duration. lower temperatures compared to some other extraction methods, which is beneficial for preserving the integrity of heat-sensitive compounds. It requires less energy compared to traditional extraction techniques, making it more environmentally friendly and cost-effective. Utilizes smaller quantities of solvents, reducing the overall environmental impact [18-20]. Powdered leaves of guava (50gm) were extracted with 70 %Ethanol using Ultrasound-assisted extraction with the following parameters(400 min, solid-solvent ratio

1:10gm/ml, temperature 40 C^0, frequency 20 kHz;), then concentrated under reduced pressure and suspended with water for fractionation method (three times) with 100 ml of different solvents polarity in ascending manner(petroleum ether, chloroform, ethyl acetate, and n. butanol) petroleum ether fraction was dried and subjected to Gc mass spectrometry [21,22].

(GC/MS) conditions

GC/MS (Gas Chromatography-Mass Spectrometry) instrument, specifically a SHIMADZU QP-2010 ULTRA, with the following conditions: A capillary column HP-5MS with dimensions of 30 m in length, 0.25mm in inner diameter, and a thickness of 0.25µm was employed for the separation of constituents. The flow rate was set at (1.0 mL/min). The gas was used as the carrier gas. The split ratio was adjusted to 2.0. The injector port temperature was maintained at (250°C). initial oven temperature was 80°C for 1 minute, followed by a programmed increase to 240°C at a rate of 10°C/min, detector temperature was set at 280°C. (EI) mode was employed with an ionization energy of 70e.

MTT assay

The cytotoxic effect of petroleum ether fraction on HepG2 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 placed in an incubator at 37 ℃ for 24 hours until 60% of the well surface was filled, otherwise, more time is needed. On the second day of treatment of cells with different concentrations (31.25–500µg/ml) of petroleum ether fraction for 24 h, After emptying the supernatant of each well by the sampler, 100 µl of each 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 after 24 hours, the medium was removed and 100 µl of MTT solution (concentration 0.5 mg/ml) was added to the plates in the dark and placed in the incubator for 4 hours. Then, the top medium of the wells was removed with a sampler, and 100 µl of DMSO was added to the wells, then placed on a shaker for 20 minutes (at this stage, the container should be covered so as not to be exposed to light). Finally, the intensity of the resulting color was read by a microplate reader (DNM-9602G) at a wavelength of 570 nm [23-25].

Statistical analysis

Statistical analysis was performed using Statistical Package for Social Science (SPSS) version 24.0. The data were presented as mean values with standard deviation (mean± SD). Analysis of the results was carried out using a one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test for multiple comparisons. Statistical significance was determined at a p-value of less than 0.05, denoting a significant difference.

Results and Discussion

Initial testing of plant extracts

This screening was carried out to provide an overview of there is terpenoids and sterols contained in the guava leaves extract, The components contained in the extract were analyzed by their compounds by color test (qualitative) with several reagents like the Salkowski test is commonly used to detect the presence of terpenoids and sterol in plant extracts and Liberman-Burchard test to detect the presence sterols and steroids both Salkowski test and Liberman-Burchard test give positive result as shown in figure 1[17].

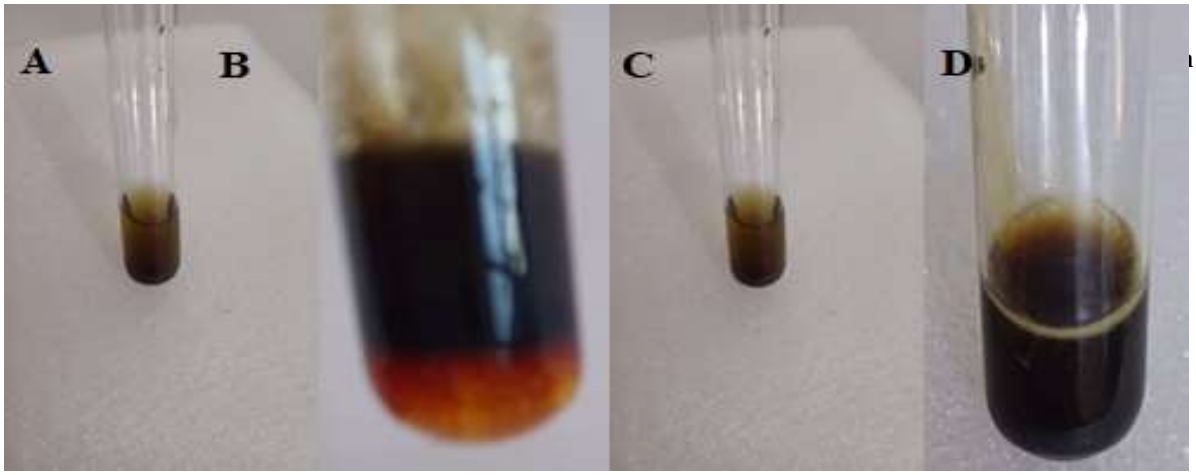

Figure1. A, Guava leaves extract. B, Guava leaves extract after adding Salkowski reagent test. C, Guava leaves extract. D, Guava leaves extract after adding Liberman-Burchard reagent test

GC/MS analysis for (EO).

Yield of essential oil from 500 grams of Iraqi guava leaves is equal to 0.002% [14] .These essential oil constituents GC/MS analysis of guava essential oil identified many phytochemicals as constituents of these, the main constituents are Caryophyllene%22.31, D-Limonene 11%, Aromandendrene % 6.71, Humulene % 3.53, Nerolidol 2 % 8.77, beta.-Bisabolene % 3.87, and many other shown in figure 2 and table 1 compare with previous studies many essential oils firstly extracted from Iraqi guava and not remembered in guava leaves essential oil universally such as((-)-Carvone% 0.12, Anethole% 0.81,(-)-Globulol % 4.17, Verbenol % 0.08, Phytol% 0.13) [26-28]. Globulol is a sesquiterpene compound identified in the fruits of Eucalyptus globulus Labill. Research has investigated its antimicrobial activity and other biological properties [29]. Carvone is a terpenoid compound that can be found in numerous essential oils, with its highest concentrations in the oils extracted from the seeds of caraway, spearmint, and dill. This monoterpene ketone is present in the essential oils of various aromatic and medicinal plants. Additionally, carvone finds applications as a biopesticide, demonstrating its utility in pest management, anti-inflammatory, and anti-diabetic effects [30,31]. Anethole is known to possess a range of biological activities, making it a versatile compound in various applications. Some of its notable biological effects include anti-inflammatory properties, antioxidant activity, and antimicrobial effects. These properties contribute to its significance in the fields of medicine, food science, and beyond [32]. All of these compounds identified for the first time in Iraqi guava give it additional importance for benefiting from them in the medical fields and an attempt to delve deeper into the study of this newly cultivated plant in Iraq. The components of essential oils in Iraqi guava leaves were identified by comparison of their mass spectra with those of NIST and those described by Adams [33]. retention index calculated according to n-hydrocarbons (C6–C22) on the HP-5MS column.

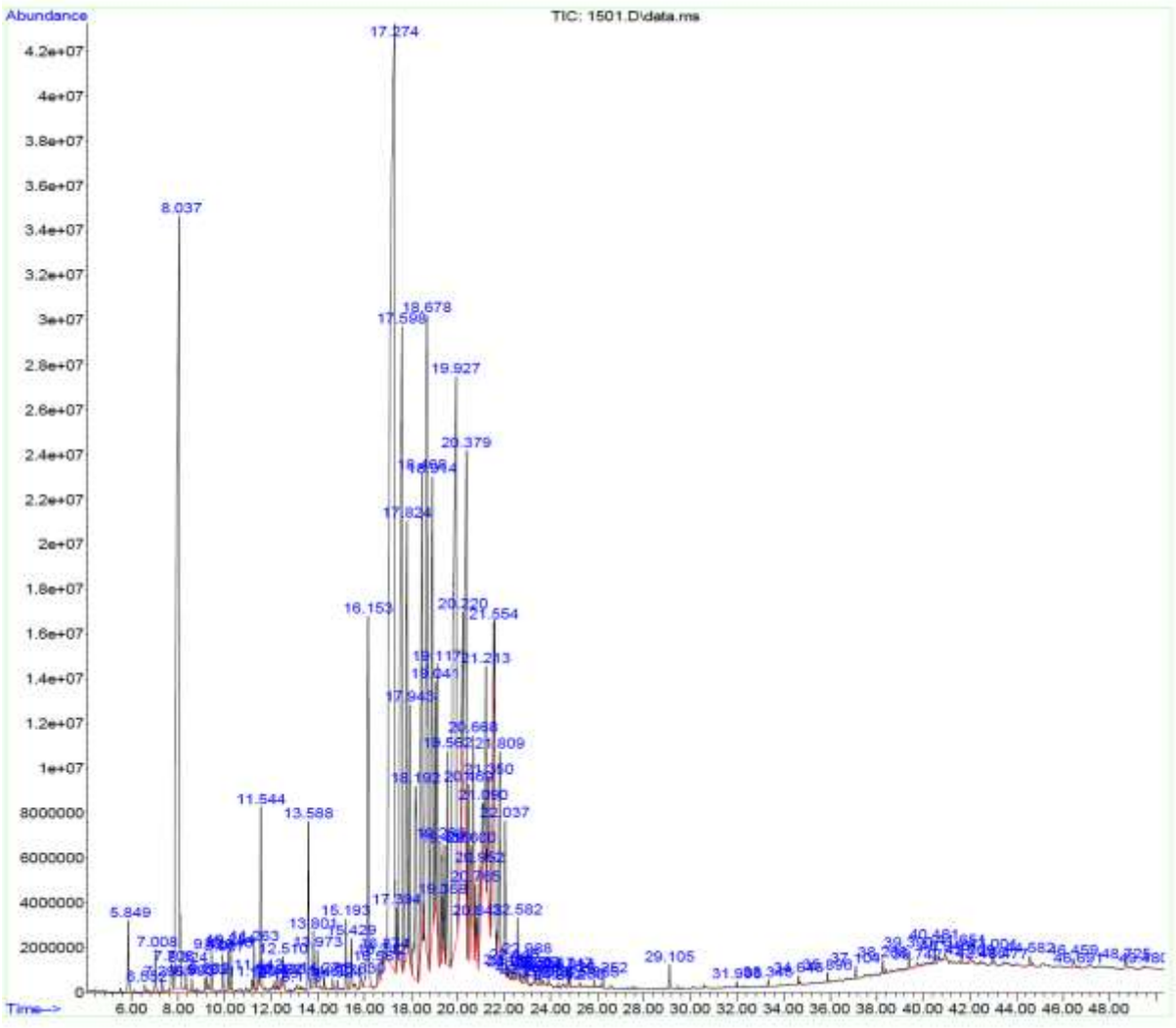

Figure2. GC mass for guava leaves (EO)

Table 1. GC/MS analysis of the essential oil of Guava leaves

Compound name	Retention index	Area %	Retention time in min
Alpha pinean	939	0.3	5.85
Beta myrcene	990	0.23	7.008
o-cymine	1026	0.25	7.708
d-limonine	1031	11.91	8.035
Gamma terpinene	1059	0.05	8.580
linalool	1100	0.15	11.05
Alpha terpineol	1175	0.86	11.543
carvone	1252	0.12	12.508
anethol	1281	0.81	13.587
2-carene	1013	0.39	15.196
Alpha-copaene	1386	2.59	16.156
caryophyllene	1421	22.31	17.276
aromadendrin	1440	6.71	17.598
humulene	1461	3.53	17.826
alloaromadenderen	1458	1.17	17.940
Gamma muurolene	1477	2.12	18.195
Beta bisabolene	1508	3.87	18.916
alphacalocarene	1522	0.17	19.357
Nerolidol	1564	8.77	19.928
Caryophyllene oxide	1583	1.06	20.218
globulol	1580	4.17	20.379
epicubenol	1495	0.53	21.09
copaene	1379	1.15	21.349
Gamma himachalene	1477	1.01	22.034
verbenol	1188	0.08	22.444
phytol	1942	0.13	29.106
eicosan	1999	0.02	31.99

(GC/MS) analysis for petroleum ether fractions

After analyzing the petroleum ether extracts of guava leaves by GC/MS, it was found that they contain many active compounds about 22 compound monoterpenes and sesquiterpenes as shown in Table 2. previous studies, GC-MS analysis revealed the main components of petroleum ether fractions 2-pentadecanol, carbonic acid, eicosylvinyl ester, bis (2-ethylhexyl) phthalate, 14-heptadecanoic acid and 2-methyl tetracosane, D-friedoolean-14-en-3-one, tert- butyl , 1- [4 - (2 ,6 – ditert - butyl-4-methoxy-phenoxy) – 3 – nitro – 4 - oxobutyl] pyrrolidine-2 carboxylate,globulol,4,4,8trimethyltricyclo[6.3.1.0(1,5)]dodecane-2,9-diol and iso aromadendrene epoxide [34,35]. While Iraqi guava leaves petroleum ether fraction revealed other compounds that have high percent area such as caryophyllene (4.53Area%), Cadina-3,5-diene (1.7Area%), Phytol (5.88 Area%), glycerol 1-palmitate (4.43 Area%), gamma. -Sitosterol (30.48 Area%) which is summarized in the following Figures 3 and Table 2. The high amount of gamma sitosterol extracted from Iraqi guava leaves has garnered attention in research due to its various pharmacological activities, which encompass potential anticancer and antidiabetic properties [36-38]. The petroleum ether fraction of guava leaves has been analyzed using (GC-MS), revealing the presence of various phytoconstituents that differ from others in the world and contribute to geographic location can have a significant impact on the formation of phytochemical substances in plants. The phytochemical composition of a given plant species can vary according to geographic region, specific vegetative stages, and other environmental variables such as light intensity, humidity, temperature, soil type, and sun exposure. The production of phytochemicals varies not only between varieties or species but also depends on agricultural practices and post-harvest handling. These properties make it a subject of interest in the study of natural compounds with potential health benefits that are found in Iraqi guava.

Table 2. GC-MS analysis of petroleum ether guava leaves extract

RT (min)	Area (Ab*s)	Area%	Name	Quality
16.996	13346228	4.53	Caryophyllene	99
17.572	5108262	1.73	Cadina-3,5-diene	98
19.674	5208153	1.77	γ-Gurjunene	90
20.135	19029268	6.45	Globulol	99
21.023	9167855	3.11	10,10-Dimethyl-2,6-dimethylenebicyclo[7.2.0]undecan-5-ol	99
21.344	7693392	2.61	Neointermedeol	99
24.8	4692018	1.59	2-Pentadecanone, 6,10,14-trimethyl	96
24.956	11925076	4.04	4,4,8-Trimethyltricyclo[6.3.1.0(1,5)]dodecane-2,9-diol	99
26.123	6968591	2.36	Hexadecanoic acid, methyl ester	99
27.233	3756696	1.27	Hexadecanoic acid, ethyl ester	99
29.101	17364736	5.89	Phytol	99
31.665	3218997	1.09	2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	93
44.377	89883995	30.49	gammaSitosterol	95

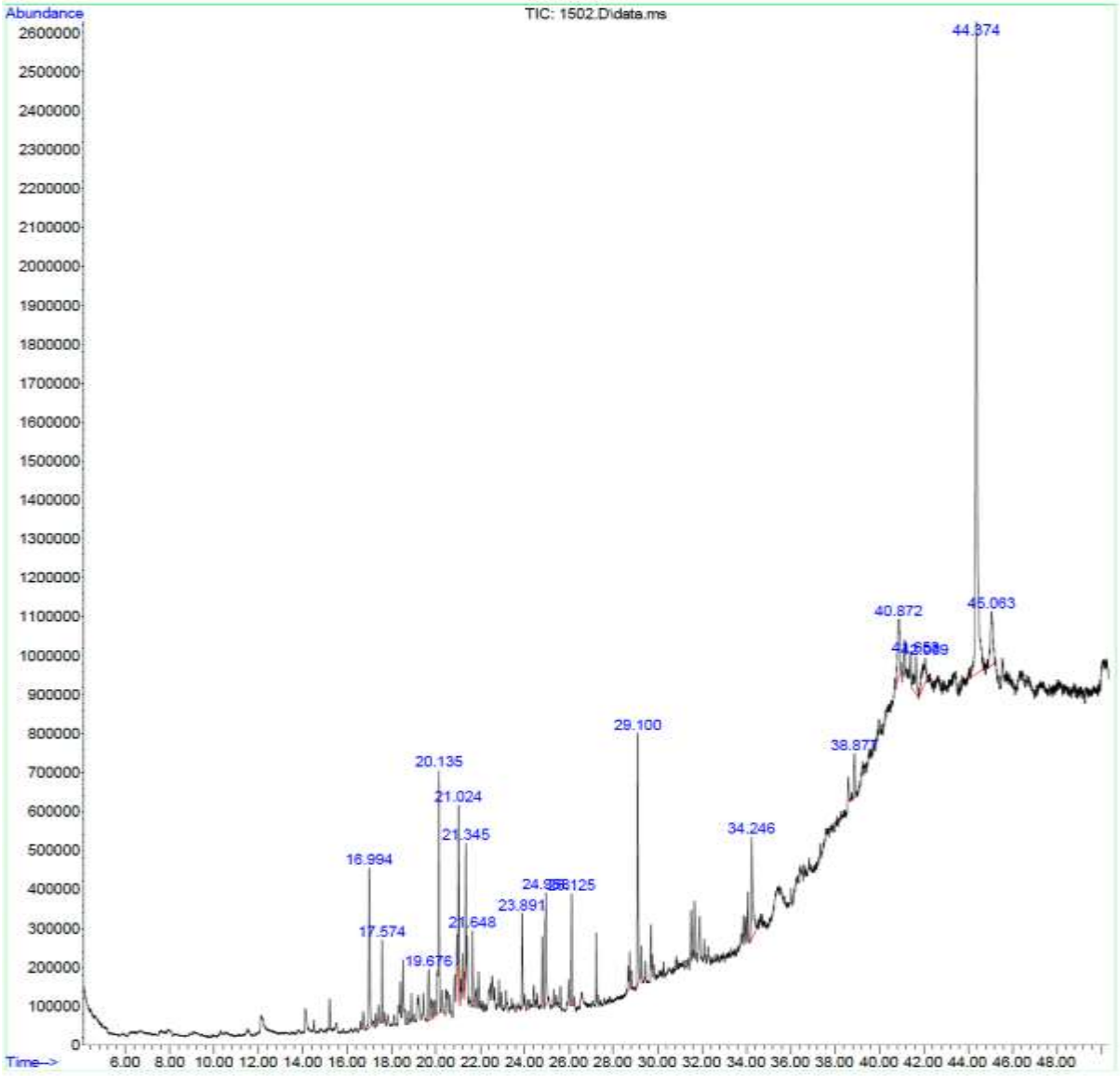

Figure3.GC-MS analysis of petroleum ether guava leaf extract

MTT assay

MTT assay was performed first time to assess the toxicity of petroleum ether fraction of Guava leaves on the HEPG2 cell line in this paper by the MTT test. As shown in Figure 4, petroleum ether fraction showed cytotoxic activity against the HEPG2 cell line with a minimum cytotoxic effect from (31.25-125 µg/mL) and a maximum cytotoxic effect at 500 µg/mL, the (IC50) concentrations inducing (50%) cell growth inhibition for scanned fraction was 656 µg/ml (Figure 5). The change in morphology of the HEPG2 cell line is exhibited in Figure 7. HEPG2 cells line control and those treated with petroleum ether fraction were taken using a microscope at 100x magnification as seen in Figures 6 and 7. The growth of HEPG2cells was not inhibited significantly ($p > 0.05$) by petroleum ether fraction from 31.25 µg/ml to 125µg/ml compared to control. However, treatment with 250 and 500 µg/ml of petroleum ether fraction significantly inhibited ($p < 0.05$) the growth of HEPG2cells as compared to the control group as shown in (Figures 4 and 5), this inhibition was in a dose-dependent manner when increasing concentration led to an increase in cytotoxicity effect on cancer cell line as indicated in previous studies [39]. main components (caryophyllene and gamma sitosterol) found in petroleum ether fraction have been evaluated for their possible antiproliferative activity and showed a viability reduction of HepG2 cells, the previous studies showed that β-caryophyllene evidenced cytotoxicity in a dose-dependent manner [40], the available literature indicates that sitosterol exhibits excellent anticancer activity against several cancer cells like lung, breast, and liver and exhibited the most potent cytotoxic effect on HepG2[41,42]. mechanism of action of petroleum ether fraction was to induce apoptosis in the cells causing a change of morphology with the cells exhibiting nuclear condensation and DNA fragmentation. The fraction also contributed to mitochondrial dysfunction, significantly increasing the cell's reactive oxygen species (ROS) generation and disrupting their mitochondrial membrane potential [43].

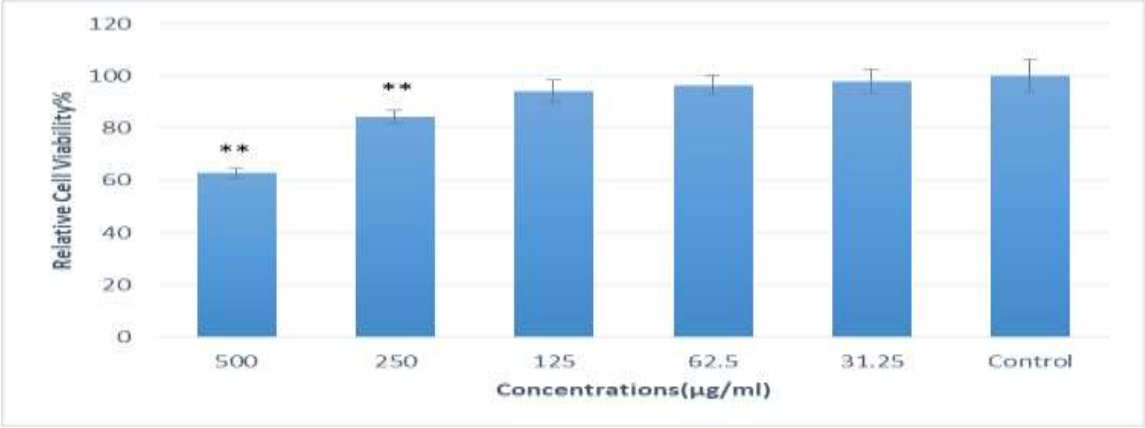

Figure 4. Effect of petroleum ether fraction of Guava leaves on HEPG2, (mean ± SD, n=6), ($^{**}p < 0.05$)

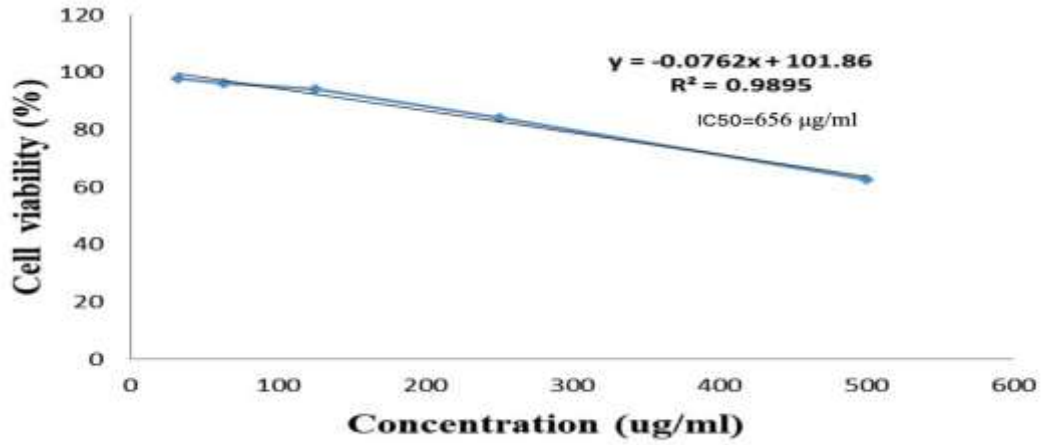

Figure 5. IC50 of petroleum ether fraction of Guava leaves on HEPG

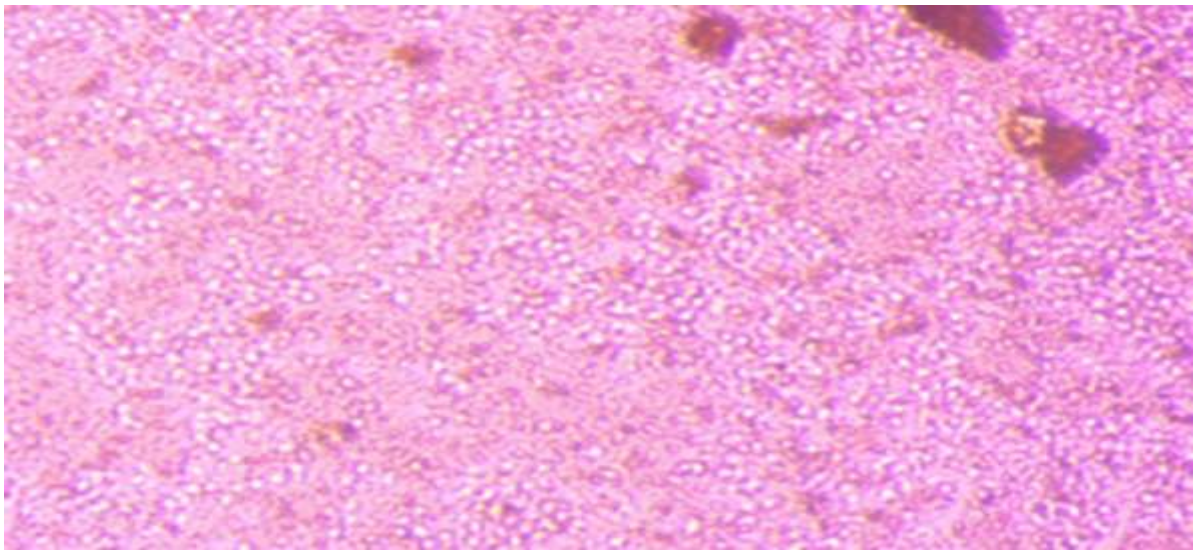

Figure 6. Morphology of control untreated HEPG2

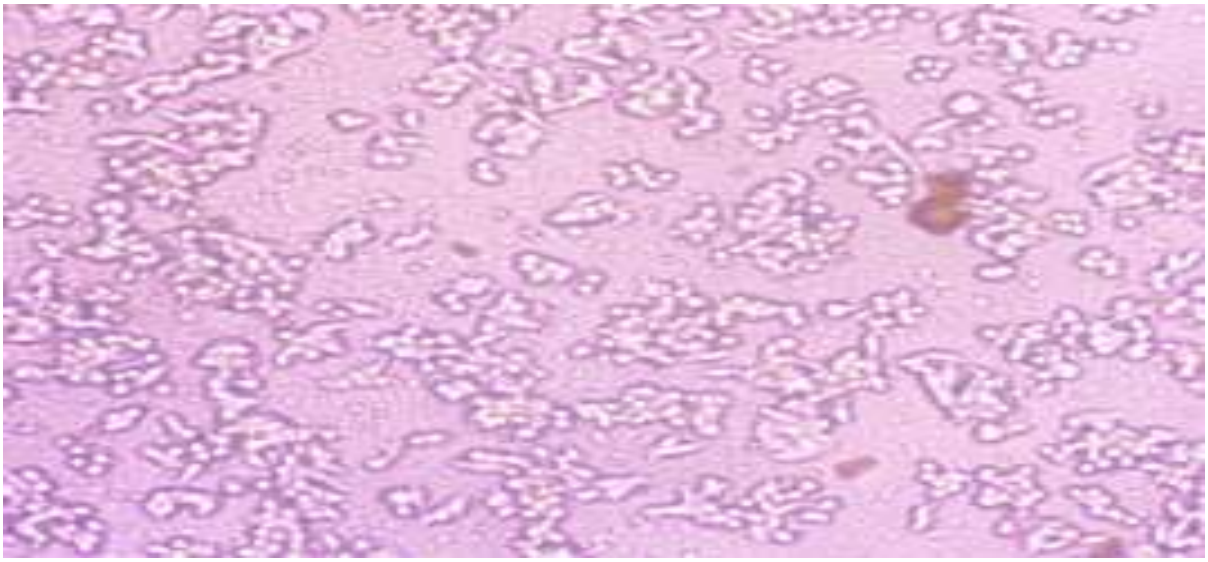

Figure 7. Morphology of HEPG2 cell line after treatment with petroleum ether fraction of Guava leaves at conc. 500 µg/ml.

Conclusion

Essential oil from guava leaves has many phytochemical constituents like d-limonene, nerolidiol and caryophyllene. Petroleum ether fraction has high amount of caryophyllene and gamma sitosterol has been evaluated for their possible antiproliferative activity and showed a viability reduction of HepG2 cells in high concentration 250 and 500 µg/ml. according to the result geographic location can have a significant impact on the formation of phytochemical substances in plants and their cytotoxic activity

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Conflict of interest

The authors declare that there is no conflict of interest.

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Ethics Statements

None

Author contributions

Ashwaq T. Kareem: contributed to data gathering, analysis, practical (follow the procedure) and written parts of the study. Enas J. Kadhim gave final approval and agreement for all aspects of the study, supervision, revision, and rearrangement.

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الكشف عن مركبات جديدة ونشاط سام للخلايا في أوراق الجوافة العراقية: استخلاص وتوصيف جزء "من الزيت العطري والأثير البترولي"

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الخلاصة

الجوافة هي شجرة استوائية او شجيرة تنتمي الى العائلة الآسية. موطنها الأصلي أمريكا الاستوائية، وقد كشفت الدراسات السابقة عن الخصائص الطبية المتنوعة للجوافة، والتي تم توثيقها في جميع أنحاء العالم من خلال المسوحات العرقية النباتية والعرقية الدوائية لذلك تم زراعتها حديثًا في العراق، ويعتبر هذا البحث الأول من نوعه لاستخراج الزيت العطري من الجوافة. أوراق الجوافة والأحماض الدهنية من جزء الأثير البترولي من أوراق الجوافة المزروعة حديثا في العراق للتعرف على المركبات الموجودة واختبار النشاط السام للخلايا لجزء الأثير البترولي على خلايا سرطان الكبد البشري. تم استخلاص الزيت العطري من أوراق الجوافة باستخدام طريقة التقطير المائي بجهاز كليفنجر وتم تعريض الزيت العطري لكروماتوكرافيا الغاز والطيف الكتلي وتم التعرف على العديد من المركبات، تم التعرف على بعضها فقط في زيت اوراق الجوافة العراقية تم استخدام تقنية الاستخلاص بالموجات فوق الصوتية لاستخلاص اوراق الجوافة والمستخلص الخام بعد تجفيفه معلقا بالماء بطريقة التجزئة (٣ مرات) مع ١٠٠ مل من المذيبات المختلفة قطبية تصاعديا (الايثر البترولي ،الكلوروفورم، وخلات الإيثيل، والبيوتانول) تم تجفيف جزء الأثير البترولي وتعريضه لكروماتوكرافيا الغاز والطيف الكتلي ، وتم العثور على العديد من المركبات التي تم تحديدها في جزء الأثير البترولي وكمية كبيرة من غاما-سيتوستيرول في جزء الأثيرالبترولي من أوراق الجوافة العراقية مقارنة بالكمية الموجودة في بقية أوراق الجوافة في العالم، تبحث هذه الدراسة في القدرة المضادة للأورام لجزء الأثير البترولي في الجوافة على خط خلايا سرطان الكبد البشري وتظهر النتيجة تثبيط خط خلايا سرطان الكبد البشري بطريقة تعتمد على تركيز الجرع.

الكلمات الافتتاحية :استخلاص بالموجات فوق الصوتية ،زيوت الطيارة،جزء البتروليوم ايثر، كاماسايتوستيرول ،الكارفون