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Evaluation of in-vitro Anticancer Activity of *Vernonia leopoldii* Methanolic Extract on HepG2 Human Cancer Cells, relative to its Phytochemical Contents determined by LC-MS/MS fingerprint

Helmi A. Mokbel[1,4], Seham S. El Hawary[1], Rasha M. Allam[2], Ahmed H. Eldesoky[3], Amira K. El-Motayam[1], Ali M. El Halawany[1*]

[1]*Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Cairo 11562, Egypt*

[2]*Pharmacology Department, Medical and Clinical Research Institute, National Research Centre, Dokki, Cairo, 12622, Egypt*

[3]*Pharmacognosy Department, National Research Centre, 33 El Buhouth Street, Dokki, Giza, 12622, Egypt*

[4]*Ministry of Higher education and scientific research, Yemen*

Abstract

Plants in the Genus *Vernonia* family (Asteraceae) contain physiologically active chemicals, making them ideal for discovering and developing anticancer medicines. Hepatocellular carcinoma (HCC), one of the most recognized cancers in the world, is on the rise, with a high mortality rate and a dismal prognosis. As a result, the treatment of HCC remains a splendid challenge. This study aimed to assess the cytotoxic activity of Yemeni *Vernonia leopoldii* (Sch. Blip. Ex Walp) Vatke extracts on HepG2 cell line. The SRB assay was used to evaluate their cytotoxicity against liver cancer cell line (HepG2), breast cancer cell line (MCF-7) and normal liver cell line (BNL), calculating the half-maximal growth inhibitory concentration. Metabolite profiling of the total methanolic extract of *V. leopoldii* was carried out for the first-time using LC-MS/MS in positive mode to investigate its chemical composition. Moreover, flow cytometry analysis was used to quantify the cell cycle arrest, apoptotic/necrotic, and autophagic cell death in the treated HepG2 cells. A scratch assay was performed to examine the effect of the methanolic extract on HepG2 cell migration. Methylene chloride fraction of the total methanol extract was found to be more cytotoxic against HepG2 cells (IC_{50}= 9.2 ± 0.88 µg/mL) compared to (14.26 ± 0.97 µg/mL) on MCF-7, with less cytotoxicity in BNL cells (IC_{50} = 85.9 ± 2.3 µg/mL). The LC-MS/MS analysis of MeOH extract tentatively identified thirty-five chemical compounds, including one anthocyanin glycoside, eight flavones, one flavanone, five flavonol glycosides, two furanocoumarins, four phenolic acids, one sesquiterpene ester, and thirteen sesquiterpene lactones. The MeOH extract induced G2/M cell cycle arrest along with apoptotic and autophagic cell death in HepG2 cells. Moreover, this extract inhibited HepG2 migration, as shown in the scratch healing assay. These findings provide interesting new information about how *V. leopoldii* induces apoptotic/autophagic, anti-migratory, and anti-proliferative cell death in HepG2 cells, which may be able to lessen the aggressiveness of HCC.

Keywords: *Vernonia leopoldii;* sesquiterpene lactones; flavonoids; LC-MS/MS; metabolomics; apoptosis; wound healing; migration; HepG2 cell line; cytotoxicity

1. Introduction

Hepatocellular carcinoma (HCC) considered as one of the most prevalent types of primary liver cancer (PLC), one of the most dangerous and rapidly growing diseases in the world[1, 2]. Currently, it is one of the top five main causes of cancer-related mortality around the world, and the 5-years survival for most patients is less than 20% [3, 4]. According to prospective epidemiological studies (with a projected incidence), by 2025, one million people will have a liver cancer diagnosis[5]. Over 80% of liver cancer cases have typically been discovered in low- and middle-income nations, where resources for social and medical care are usually inadequate[6, 7]. HCC is the most common frequently disease in Africa and Asia [8, 9]. Only patients with early-stage HCC are now eligible for approved treatments, and even these have limited long-term efficacy. Furthermore, a lot of the current treatments have serious side effects, which means that people with advanced-stage HCC cannot afford them [10]. Therefore, in order to enhance the prognosis and quality of life for patients with HCC, new and more effective treatments are urgently required[11, 12].

Apoptosis is a tightly controlled mechanism of cell death that getting rid of unwanted, senescent, and flawed cells, and reducing inflammation [13]. Apoptosis detection can aid in monitoring disease development and predicting tumor responses to anticancer treatments. However, Apoptosis does not occur in cancer cells because apoptotic signal transmission is

*Corresponding author e-mail: ali.elhalawany@pharma.cu.edu.eg ; (**Ali Mahmoud El Halawany**).
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disrupted. As a result, inducing apoptosis death in cancer cells could be a beneficial approach to develop anticancer therapy [14].

In addition to inducing apoptosis, several different programmed cell death pathways might be employed to eradicate cancer cells. Since autophagy plays a crucial role in carcinogenesis and development, Numerous studies have been conducted on "autophagic cell death," or cell death induced by excessive autophagy[15]. Autophagy, a type II form of programmed cell death, is a double-edged sword in carcinogenesis, acting as a tumor suppressor and a protective aspect for survival of a cancer cell. It is one of the most well-documented pathogenic cellular remodeling in cancer [16]**.** Autophagy's role in HCC initiation and progression has been extensively researched since it may be triggered in hepatocytes, and the liver is a vital metabolic organ. Several studies have correlated autophagy to a tumor suppression role in HCC [17-19]**.**

The major source of natural compounds and anticancer drugs are medicinal plants, they are also possess a significant potential for treating cancer by disrupting the cell cycle, inducing apoptosis, or scavenging free radicals[20, 21].

Reviewing the current literature [4, 6, 10-16]; Several Yemeni medicinal plants showed cytotoxic effect on different cancer cell lines. *Vernonia leopoldii* (Sch. Bip.) Vatke is a member of the Vernonia genus, which considered between the largest subgroups of the Asteraceae family and has over a thousand species (family; Asteraceae, Subfamily; Vernonioideae, tribe; Vernonieae). It is a typical and noticeable plant of Yemen's Escarpment Mountains and is widespread in many rocky settings. It is only recognized outside of Yemen from the mountains of Ethiopia. The plant is employed in Yemeni traditional medicine to cure skin conditions, colic, and cough [22].

Upon reviewing the available literature, it was found that very little information could be found about the chemical components of the plant. Flavonoids, phytosterols, and terpenoids were found via phytochemical screening in a study that assessed several plants of Yemen used in traditional medicine for particular biological properties[23]. Three human cancer cell lines were shown to be significantly inhibited in their proliferation by the methanolic extract of *V. leopoldii* [23], antitrypanosomal activity [24] and mild antimalarial activity [25].

Many phytochemical compounds were isolated and identified from *V. leopoldii* for example two flavonoid glycosides, a new lanostane-type triterpene in addition to two known hirsutinolide-type sesquiterpene lactones **[**22**]**. In spite of the shortage of evidence to support its therapeutic value, *V. leopoldii* was rated one of the commonly utilized anti-cancer traditional medicinal herbs in Ethiopia by a study published in 2022, where they described the isolation and identification of three flavonoids (eriodyctiol, apigenin, and luteolin), three sesquiterpene lactones (vernolepin, vernomenin, and 11ß,13-dihydrovernodalin)**,** these SQL at low concentrations, were shown to be cytotoxic to the breast cancer cell lines JIMT-1 and MCF-7; however, somewhat less toxic to MCF-7 cells compared to JIMT-1 cells **[**26**]**. To the best of our knowledge, *V. leopoldii* has never been assessed as an anti-cancer natural product for treatment of HCC, and the effects of *V. leopoldii* methanolic extract on cell cycle, apoptosis, and migration has not been examined. Because of this, in current study, we investigated whether treatment with *V. leopoldii* methanolic extract and it`s fractions could aid in preventing HCC cell survival and rationalize these effects with the identified chemical compounds in the plant.

2. Methods

2.1 Plant materials

Aerial parts of *Vernonia leopoldii* (Sch. Blip. Ex Walp) Vatke were collected in December 2019 from Aba`ar Almoadem at the east of Jabal Sabr, Taiz. Plant identity was kindly authenticated by Agricultural Engineer Abdulhabeeb Mahyoob Ali, Director of the Pasture and Forests Department in General Authority of Research and Agricultural Guidance, Taiz, Yemen. the aerial parts of the under-investigation *V. leopoldii* plant were separately airdried, coarsely powdered then stored at ambient temperature (25 °C) in firmly closed amber-colored glass containers.

2.2 Chemicals

All extraction and fractionation solvents were supplied from (El-gomhoria Co. for Drugs-Egypt). HPLC grades solvents were supplied from Merck (Darmstadt, Germany).

2.3 Extraction and sample preparations

The dried powdered of *V. leopoldii* aerial parts (100 g) were extracted with methanol 99% several times till exhaustion at room temperature. BUCHI Rotavapor (Switzerland) R-100 was used to filter and evaporate the extracts under vacuum while maintaining a vacuum pressure of 250 mbar and 45 °C.

2.3.1. Sample preparation for LC-MS/MS

Prior to injection, the concentrated residue was precisely dissolved in methanol of HPLC grade to a concentration of 10 mg/mL and filtered via a syringe-filter membrane has a pore size of 0.45 µm.

2.3.2. LC-DAD-ESI/MS for Metabolite Profiling

The analysis of liquid chromatography-photodiode array-electrospray ionization-tandem mass spectrometry (LC-PDA-ESI-MS/MS) was carried out using a Shimadzu LC-20AD solvent delivery system, which was connected to a Bruker amaZon Speed mass spectrometer and had a binary pump, a refrigerated autosampler, a mobile phase degasser, and a column oven.

Freshly manufactured mobile phase was achieved through filtration using a membrane disc filter (0.45 µm). Reversed phase C18 HPLC (Inertsil ODS-3, 4.6 × 150 mm, GL Sciences Inc.) was used for high-performance liquid chromatography separation. It was operated at a flow rate of 0.45 mL/min and an oven temperature of 40 ^{O}C. The sample was eluted using a gradient of two solvents: 0.1% acetic acid in H_2O (solvent A) and 0.1% acetic acid in CH_3CN (solvent B). The following was the gradient program's execution: During the first 12 minutes, 10-100% B, and then 12-150% B. Cone gas flow rate of 50 L/h, cone voltage of 50 eV, capillary voltage of 3 kV, source temperature of 150 °C, desolvation temperature of 350 °C, and desolvation gas flow rate of 600 L/h are the MS interface parameters. Spectra with a resolution of 50–2000 *m/z* were captured. The mass spectra were found in positive mode, retention periods were compared using Compass Data Analysis viewer software, UV spectra from PDAD in the 200–500 nm region, and *m/z* values were determined using MS/MS^2.

2.4. Biological studies

2.4.1. Sample preparation for biological studies

The methanolic extract of *V. leopoldii* was suspended in water and fractionated with methylene chloride. The remaining liquor was chromatographed on Diaion HP-20 (Supelco) column using a methanol gradient that started with 100% water and increased to 50% and then 100% methanol, the remaining aqueous layer was chromatographed on a diaion column. The methanol/water effluents were collected to be evaporated till drying by using rotatory evaporator as described in literature[27], then collected and preserved in refrigerator for further work. All extracts and fractions were dissolved in DMSO at a stock of concentration (50 mg/mL) for biological activity.

Cell culture

Human hepatocellular carcinoma cell line (HepG2), breast cancer cell line (MCF-7) and the hepatocyte cell line (BNL) were supplied by Nawah Scientific Inc. (Mokatam, Cairo, Egypt). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) with 100 µg/mL streptomycin (Lonza GmbH, Köln, Germany), 100 units/mL penicillin (Lonza GmbH, Köln, Germany), and 10% fetal bovine serum (FBS; Gibco, NY, USA) at 37 °C and 5% CO2 [28].

2.4. A Cytotoxicity using SRB assay.

We used the SRB assay for assessment of the cell viability after treating HepG2, MCF-7 and BNL cells with methanolic extract of *V. leopoldii* with its different fractions in 96-well plates for 72 hours. Then, cells were fixed with 10% trichloroacetic acid (Merck), washed five times with distilled water, stained with sulforhodamine (SRB) solution (Sigma-Aldrich) (0.4% w/v) for 10 min in the dark, and washed three times with 1% acetic acid (Chem-Lab). Then, the excess dye was dissolved in Tris pH 10.5 (Chem-Lab) (10 mM), and using a BMG LABTECH®-FLUOstar Omega microplate reader (Ortenberg, Germany), the absorbance was determined at 540 nm. IC_{50} values were reported as mean ± SD [29].

2.4. B. Cell cycle analysis

The cellular DNA content of HepG2 cells (more sensitive cancer cell line) was examined using an ACEA NovocyteTM flow cytometry (ACEA Biosciences Inc., San Diego, CA, USA) after the treatment for 48 hours with the IC_{50} value of most potent *V. leopoldii methanolic extract* fraction (methylene chloride). Following trypsinization, the cells were pelleted, washed twice, and fixed in 60% ethanol in the refrigerator for at least 2 hours. After washing with PBS, the pellets were stained with a staining mixture of (RNase, Sigma-Aldrich) and Propidium Iodide (PI, Sigma-Aldrich)) for 30 minutes in the dark, and the results were assessed using the ACEA NovoExpressTM program (ACEA Biosciences Inc., San Diego, CA, USA)[30].

2.4. C. Annexin V/PI apoptotic/necrotic assay

The impact of *V. leopoldii* methylene chloride fraction on the apoptotic/necrotic cell death of HepG2 cells was investigated using the Annexin V-FITC Apoptosis Staining/Detection kit (ab14085; Abcam, Cambridge, MA). In brief, cells were planted in six-well plates at a density of 1×10^4 cells per well for 24 h and exposed for treatment for another 48 hours to the set IC_{50} values of each extract. After that, the cells were trypsinized and washed twice with PBS. The cells were then resuspended in 500 µL of 1X Binding Buffer and stained with Annexin V-FITC and PI for 30 minutes at room temperature in the dark [31].

2.4. D. Autophagy assessment

To assess autophagy in response to 48 hours of treatment with *V. leopoldii* methylene chloride fraction, HepG2 cells were trypsinized and washed with ice-cold PBS. The staining solution (1 mg/mL acridine orange in PBS) was then added to cells and left at room temperature for 30 minutes, away from light. Using "ACEA NovocyteTM flow cytometry, The NFIs (net fluorescence intensities) were measured using an FL1 signal detector (San Diego, CA, USA)(488 nm excitation/ 530 nm emission) [32].

2.4. E. Scratch/wound healing test

To ensure a confluent cell monolayer, HepG2 cells were seeded in 6-well culture plates at a density of 3×10^5 per well for 24h, then the monolayer was gently scratched with a sterile 200-µL pipette tip to create a single straight cell-free line. Following a PBS wash, cells were exposed to sub-cytotoxic doses of *V. leopoldii* methylene chloride fraction, and scratch healing was measured at 0, 24, 48, 72, and 96 hours using an inverted microscope (Olympus, Japan) at ×100 magnification. The experiments were carried out in triplicate. The wound closure percentage was estimated using the following equation: % wound closure = 100 - [(Dt/D0) × 100], where Dt is the wound width at time t and W0 is the initial width [33].

3. Results

3.1 LC-MS/MS results

The methanolic extract of *V. leopoldii* was evaluated using RP-HPLC-MS-MS. Findings revealed the detection and tentative identification of thirty-five chemical compounds; one anthocyanin glycoside, one flavanone, nine flavones and flavone glycosides, four flavonols and flavonol glycosides, two furanocoumarins, four phenolic acids, one sesquiterpene ester and thirteen sesquiterpene lactone. LC-MS/MS results are recorded and illustrated in Table (1) and figure (1-A and 1-B), the structures of these compounds are illustrated in Figures (1-C and 1-D). Identification of the compounds is discussed below in some details.

- **Anthocyanin Glycosides**

Only one anthocyanin glycoside was identified at Peak 1 at RT=2.82 min. It showed mass spectrum with a precursor ion at *m/z* 596.6 $[M+H]^+$. A product ion *m/z* 434 $[(M+H)\text{-hexose}]^+$ and *m/z* 271 $[(M+H)\text{-2 hexose}]^+$ corresponding to the aglycon moiety (Pelargonidin). From these results and based on food and mass bank data base [34] (website), compound 1 suggested to be Pelargonin.

- **Flavonoids**

A total of 14 flavonoids peaks were assigned including 1 flavanone, 9 flavones and 4 flavonols.

Peak 25 showed mass spectrum with a precursor ion $[M+H]^+$ at *m/z* 305 and a product ion at *m/z* 287 $[(M+H)\text{-}18]^+$ after losing H_2O, *m/z* 259 $[(M+H)\text{-}46]^+$ after further losing CO from previous ion peak, *m/z* 231 $[(M+H)\text{-}74]^+$ by continuous losing CO, *m/z* (195, 179, and 153). From these results and based on foodb [35] and mass bank data base [34] (website) and previous literature **[**36**]** compound **25** was suggested to be Taxifolin (2,3-dihydro-quercetin).

- **Flavones**

Nine flavones and flavone glycosides were identified in our study. The flavones identified in our work were expressed in **tableta (1)**, structures illustrated in **figure (1-C)** at peaks no. (3, 4, 7, 9, 11, 13, 14, 18 and 21). These flavones were identified as Apigenin, Luteolin and their derivatives.

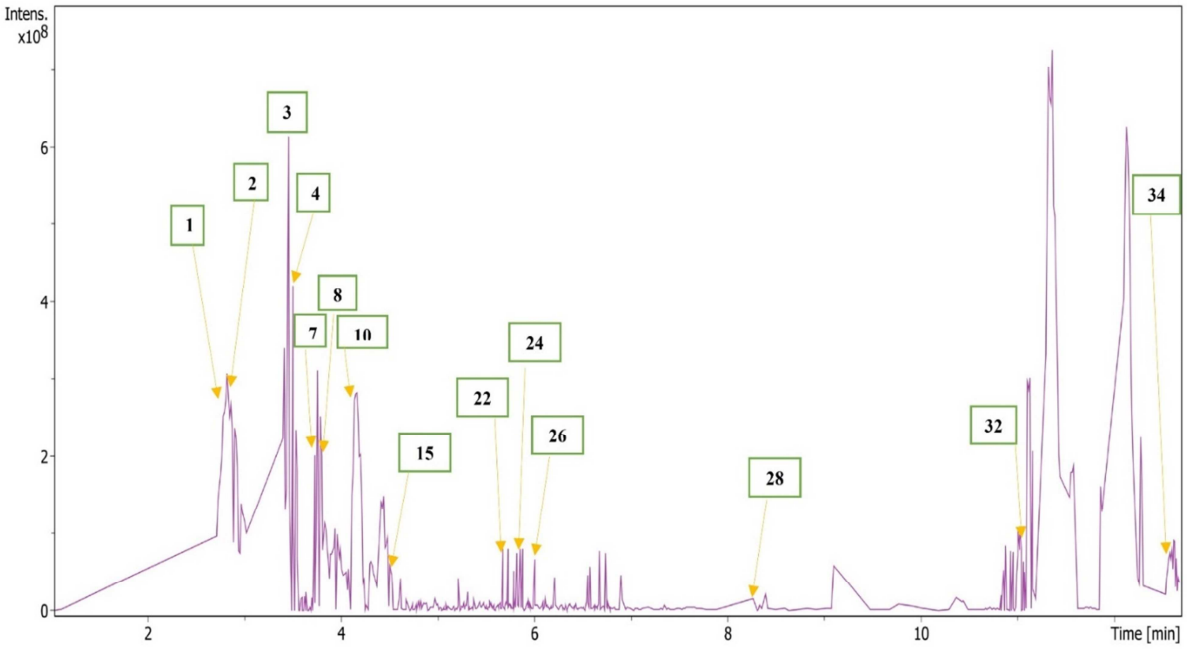

Figure (1-A): Typical Total ion chromatogram (TIC) profile of ***Vernonia leopoldii*** (Sch. Blip. Ex Walp) Vatke methanolic extract

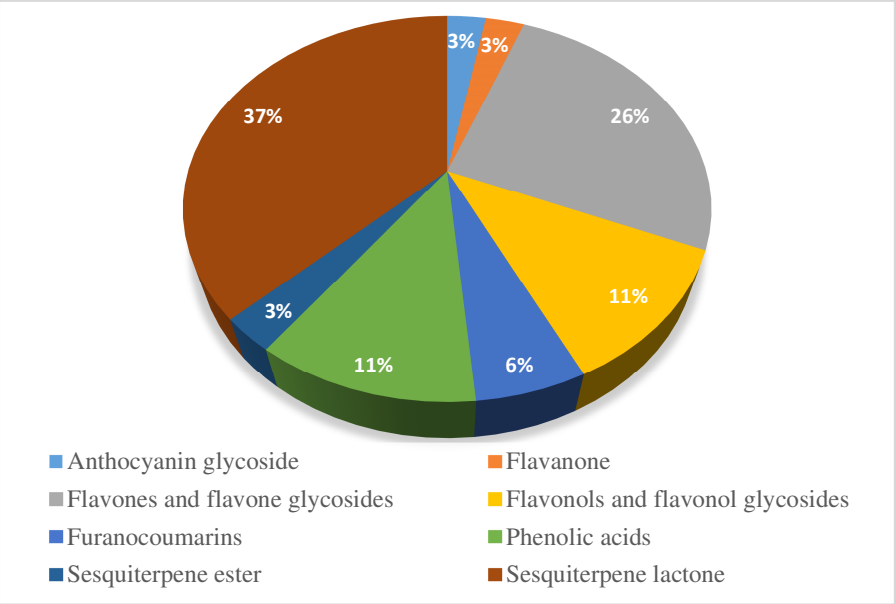

Figure (1-B): Phytochemical classes identified on ***Vernonia leopoldii* (Sch. Blip. Ex Walp) Vatke** methanolic extract by LC-MS/MS

Peak 21 showed mass spectrum with a precursor ion at *m/z* 271 $[M+H]^+$ at RT=5.59 min. and *m/z* 119 was determined previously as Apigenin **[**37**]**, MS^2 spectrum showed product ion at *m/z* 253 $[(M+H)-18]^+$ after the neutral losing of H_2O moiety (18 Da) and *m/z* 225 $[(M+H)-46]^+$ after further loss of CO moiety (46 Da) that confirm presence of flavone compounds **[**36**]**, so, compound **21** was suggested to be Apigenin, this was confirmed by previous identification of Apigenin in *V. leopoldii* **[**38**].**

- **Identification of Apigenin Derivatives**

The compounds' identity as C-glycosylated apigenin derivatives is supported by the occurrence of *m/z* 385 $[Agly+113]^+$ and 355 $[Agly + 85]^+$ ions in the MS^2 spectra for peaks 3, 4, 6, 13, and 14 as well as neutral losses of 60, 90, and/or 120 amu**[**39**]**. The signal at *m/z* 565 [M+H]+ was identical in peaks 3 and 4, and the fragmentation pattern was unique for asymmetric 6,8-di-C-glycosides. Fragments at *m/z* (475, 445, 415, 385, 355, 327, 295) were visible in MS^2. Comparison with an authentic standard used in literatures **[**40**]** and other literatures [41-44] gave the same fragmentation pattern so compounds 3 and 4 are considered as Apigenin-6-C-arabinoside-8-C-glucoside (Isoschaftoside and Schaftoside) respectively, these compounds were previously identified in Asteraceae family **[**40**]**. Also Peak 6 showed *m/z* 535 $[M+H]^+$, showed a product ion at *m/z* 271, $[Aglycone]^+$ which is Apigenin, *m/z* 445 $[(M+H)-90]^+$, 415 $[(M+H)-120]^+$ and 355 $[(M+H)-180]^+$, compound **6** was suggested to be Apigenin-6,8-di-C-pentoside **[**42**].** Peaks 11, 13 and 14 have similar *m/z* value = 433 $[M+H]^+$, The identities of peaks 13 and 14 have fragmentation pattern: *m/z* 413, $[(M+H)-18]^+$, 343 $[(M+H)-90]^+$, 313 $[(M+H)-120]^+$, and 285 $[(M+H)-148]^+$ were confirmed as (Apigenin-8-C-hexose) and (Apigenin-6-C-hexose) respectively, named as Vitexin and Isovitexin **[**41**,** 45**,** 46**]**. Peak 11 *m/z* 433 $[M+H]^+$ with MS^2 fragmentation at 271 $(Aglycone)^+$ which is Apigenin and other ions as described in literatures **[**47**]**. So, compound 11 was confirmed as Cosmosiin (Apigenin-7-O-glucoside) that was identified previously in *V. leopoldii* **[**22**]**.

- **Identification of Luteolin Derivatives**

Peak 18 found to be Luteolin with *m/z* 286.9 $[M+H]^+$ and product ion *m/z* 242.9 compared with mass bank data base[34] (website) and previous literature **[**47**]**, Also peak 9 is related to it because it has *m/z* 449 $[M+H]^+$ and mass fragmentation pattern MS^2 *m/z* 286.9 for Luteolin $[(M+H)-162]^+$after losing the sugar part, so compound 9 was identified as Luteolin-7-O-glucoside. Luteolin was founded within metabolomics in the Genus of *Vernonia* Schreb. [46]. Both Luteolin and Luteolin-7-O-glucoside were identified previously in *V. leopoldii* **[**38**]**.

- **Flavonols**

Peak 2 showed mass spectrum with a precursor ion at *m/z* 597 $[M+H]^+$ and product ion *m/z* 465 after losing apiose, also product ion *m/z* 303 corresponding to quercetin aglycone moiety, after losing apiose and glucose and a product ions at *m/z*

285, 275, 257, 247 after losing of H_2O, CO, H_2O+CO, and 2CO respectively from Quercetin that confirmed presence of flavone compounds [36], so compound **2** was suggested to be Quercetin 3-O-*β*-D-apiofuranosyl-(1→6)-*β*-D-glucopyranoside in comparison to published data **[**48**]** and as determined and identified from *Solidago altissima* Linn. (Family; Asteraceae).

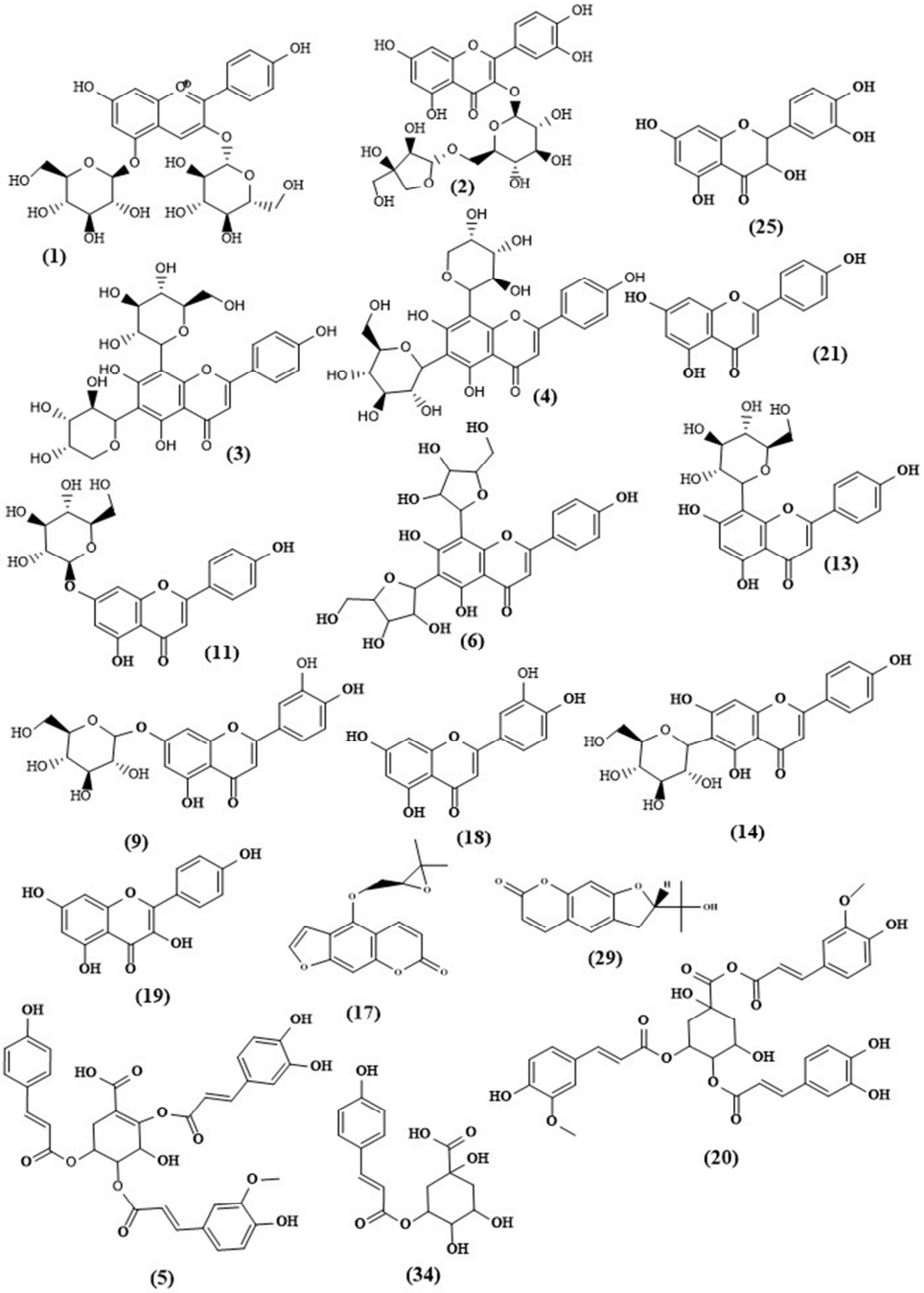

Figure (1-C): Illustrated chemical structures of flavonoids and phenolic compounds identified on ***Vernonia leopoldii*** (Sch. Blip. Ex Walp) Vatke

Peak 10 at *m/z* 449 $[M+H]^+$ showed a product ion *m/z* 303 $[(M+H)\text{-rhamnosyl}]^+$ corresponding to Quercetin aglycon, so compound **10** was suggested to be Quercetin- rhamnoside. **[**49**]**.

Peak 19 showed mass spectrum with a precursor ion at *m/z* 287 $[M+H]^+$ with MS^2 spectrum showed a product ion at *m/z* 269 $[(M+H)\text{-}18]^+$, 259 $[(M+H)\text{-}28]^+$, *m/z* 241 $[(M+H)\text{-}46]^+$, *m/z* 231 $[(M+H)\text{-}56]^+$, and *m/z* 213 $[(M+H)\text{-}74]^+$ after the neutral

loss of H_2O, CO, (H_2O and CO), 2CO and (H_2O and 2CO) respectively that confirm presence of flavone compounds [36]. So, compound 19 was suggested to be Kaempferol. Also Peak 8 with a precursor ion at *m/z* 471 $[M+H]^+$ showed all previous fragmentation ions after product ion *m/z* 287 which identical to kaempferol aglycone after losing of hexosyl moiety. From these results and based on foodb [35] and mass bank data base [34] (website) and previous literatures **[**50**]**, compound 8 was suggested to be Kaempferol-hexoside which was identified previously in *Vernonia amygdalina* **[**51**]**.

- **Furanocoumarins**

Two Furanocoumarins were identified in our study; Peak 17 showed mass spectrum with a precursor ion at *m/z* 287 for $[M+H]^+$ and MS^2 fragmentation of *m/z* 245 $[(M+H)-42]^+$, 227 $[(M+H)-42-18]^+$, 203 $[(M+H)-72]^+$, and 187 $[(M+H)-98]^+$, as described in literature **[**52**]**, so compound 17 was identified as oxypeucedanin. Also another Furanocoumarin was identified at Peak 29 showed mass spectrum with a precursor ion at *m/z* 247 for $[M+H]^+$, and product ion of *m/z* 231 for $[(M+H)-16]^+$, *m/z* 228 for $[(M+H)-19]^+$, *m/z* 202 for $[(M+H)-45]^+$, and *m/z* 187 for $[(M+H)-60]^+$, as described in literature **[**53**]** and HMDB data base[54] (website), so compound 29 was identified as Marmesin.

- **Phenolic Acids**

Four phenolic acids were identified in the methanolic extract of *V. leopoldii*. Peaks 5, 20, 23 and 34 are shikimic and quinic acid derivatives coupled to different cinnamate derivatives. peak 5 showed *m/z* 677 $[M+H]^+$ at RT 3.59 min and a product ion at *m/z* 338 characteristic for Caffeoyl shikimic acid and therefore compound 5 was identified as feruloyl-O-p-coumaroyl-O-caffeoylshikimic acid. Peak 20 showed *m/z* 707 as the molecular ion peak with product ions at *m/z* 531 corresponding to the loss of quinic acid moiety and *m/z* 369 corresponding to the loss of caffeoyl acid moiety. From these data compound 20 was identified as 3,7-O-diferuloyl-4-O-caffeoyl quinic acid. Peak 23 showed *m/z* 779 $[M+H]^+$ at RT 5.7 with product ion at *m/z* 761 as a result from losing of (18 Da) H_2O $[(M+H)-H_2O]^+$ and it was identified as (E)-3-(3,4-diacetoxy-5-methoxyphenyl) acroyl-4-O-p-coumaroyl-5-O-caffeoylquinic acid in comparison to published data **[**55**]**. Peak 34 showed mass spectrum with a precursor ion at *m/z* 338 $[M+H]^+$ at retention time RT=12.6 min., also MS/MS spectrum showed a product ion at *m/z* 320 $[(M+H)-18]^+$, product ion at *m/z* 303 $[(M+H)-35]^+$ and *m/z* 193 $[(M+H)-145]^+$corresponding to the protonated ion of quinate. According to these results and referring to published data **[**56**,** 57**]**, HMDB[54] and mass bank base data[34], so compound **34** was suggested to be 5-coumaroylquinic acid.

- **Sesquiterpene**
- **Sesquiterpene esters**

One sesquiterpene ester was identified in the methanolic extract of *V. leopoldii* as illustrated in **table (1)** and **figure (1-D).** Peak 33 showed mass spectrum with a precursor ion at *m/z* 391 $[M+H]^+$ at retention time RT=12.2 min. its MS^2 spectral showed product ions at *m/z* 373 $[(M+H)-18]^+$ (from loss of H_2O), *m/z* 288 $[(M+H)-103]^+$ after losing $CH_2(OH)C{=}CH_2(CO_2H)$, then losing H_2O to give *m/z* 270 $[(M+H)-121]^+$, then losing ($-CH_2$) *m/z* 256 $[270-14]^+$, *m/z* 228 by losing (-CO), and finally losing $CH_2{=}CH$ to give *m/z* 201 in according to these data, compound 33 was identified as Jugalinone which was previously identified from *Vernonia jugalis* **[**58**]**

- **Sesquiterpene Lactones**

Thirteen compounds from different skeletons of sesquiterpene lactones were tentatively identified in the methanolic extract of *Vernonia leopoldii*. Peak 8 have a precursor ion *m/z* 535 $[M+H]^+$ and MS^2 spectrum showed a product ion at *m/z* 517 $[(M+H)-18]^+$, 474 $[(M+H)-61]^+$, 414 $[(M+H)-121]^+$, and 385 $[(M+H)-150]^+$ after losing H_2O then HOAC then further HOAC also losing CHO respectively similar to published data [59] so, compound 8 was identified as 14-0-Acetylvernocistifolid-8-0-angelat. Peak 12 showed mass spectrum with a precursor ion at *m/z* 577 $[M+H]^+$ at retention time RT=4.26 min. its MS^2 spectrum showed a product ion at *m/z* 559 $[(M+H)-18]^+$ $[(M+H)-H_2O]^+$, and 517 $[(M+H)-60]^+$ $[(M+H)-OAC]^+$., So compound 12 was identifies as 14-O-Senecioylvernocistifolid-8-0-(2,3-epoxyisobutyrat)B both compound 8 and 12 were classified as Vernocistifolide and were previously isolated from different *Vernonia* species**[**59**]**.

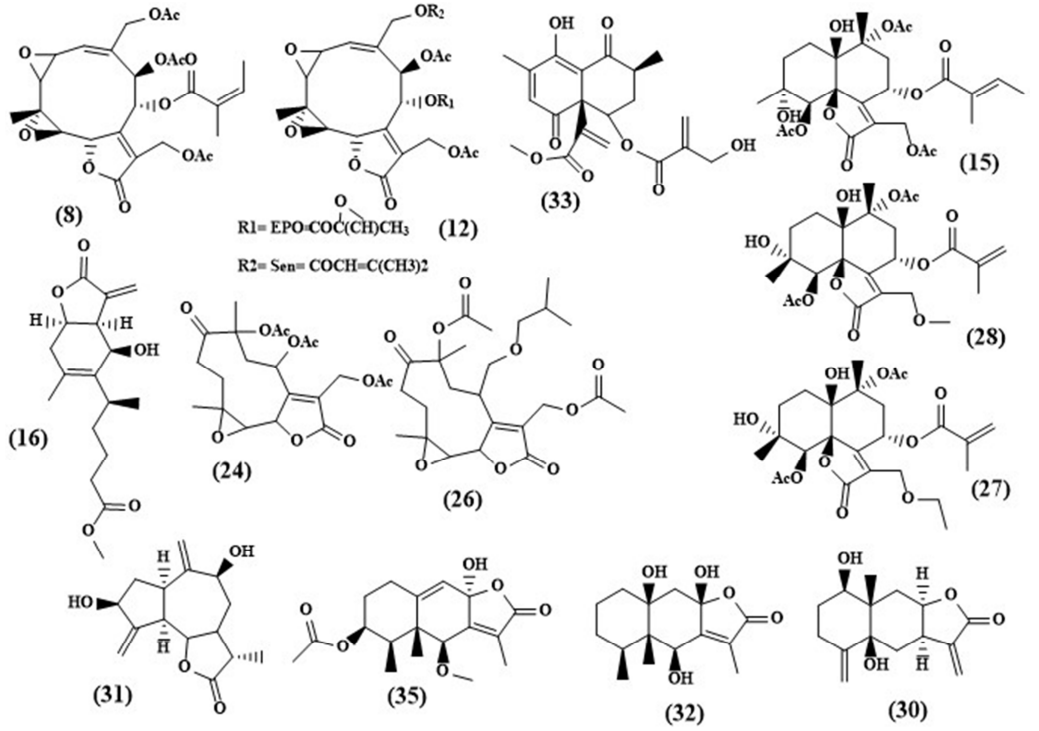

Figure (1-D): Illustrated chemical structures of Sesquiterpene compounds identified on ***Vernonia leopoldii*** (Sch. Blip. Ex Walp) Vatke

Peak 15 with precursor ion at *m/z* 539 $[(M+H)]^+$ and MS^2 spectrum fragments *m/z* (521, 497, 479, 461, 439, 419 and 377). These ions *m/z* were compared to published data **[**60**]** and compound 15 was suggested to be (1S*,4R*,5S*,6S*,8S*,10R*)-1,4-Dihydroxy-5,10,13-triacetoxy8-tiglyloxycadin-7(11)-en-6,12-olide which is a glaucolide previously isolated from *Vernonia nudiflora.*

In otherwise Peak 16 showed mass spectrum with a precursor ion at *m/z* 309 $[M+H]^+$ and product ion at *m/z* 291 $[(M+H)-18]^+$ $[(M+H)-H_2O]^+$, then it lose CH_3 to give *m/z* 276 $[(M+H)-32]^+$, 249 $[(M+H)-60]^+$ $[(M+H)-CH_3COOH]^+$, 231 $[(M+H)-78]^+$ $[(M+H)-H_2O-CH_3COOH]^+$.These data confirmed the identification of compound 16 as 1-O-acetylbritannilactone in comparison to published data [61]**.** Also Peak 30 showed mass spectrum with a precursor ion at *m/z* 265 $[M+H]^+$ at retention time RT=10.88 min..MS^2 spectrum showed its product ions at *m/z* 247 $[(M+H)-18]^+$ and 229 $[(M+H)-36]^+$. From these data and in comparison, to published data, Compound 30 was identified as 5β-Hydroxyasperilin . Both compounds were identified in *Inula Britannica* one of the Asteraceae family members **[**61**].**

Peak 31 has precursor ion at *m/z* 265 $[M+H]^+$ at retention time RT=10.9 min., It showed *m/z* 247 $[(M+H)-18]^+$ and 233 $[(M+H)-32]^+$ as result from losing H_2O and CH_2 and identified as 3*β*-hydroxy-4,15-dehydrograndolide, this compound was previously identified in *Vernonia* plants **[**62**]**.

Peak 22 showed at retention time RT=5.67 min. mass spectrum with a precursor ion at *m/z* 407 $[M+H]^+$ and MS^2 spectrum showed a product ion at *m/z* 393 $[(M+H)-14]^+$, 348 $[(M+H)-59]^+$ $[(M+H)-]^+$, and 321 $[(M+H)-86]^+$ resulted from losing of $C_2H_3O_2$ then $C_4H_6O_2$ as mentioned in literature [22] when they isolated this sesquiterpene lactone isolated from *V. leopoldii* and named as 8*α*-methacryloyloxy-hirsutinolide-13-O-acetate

Peaks 24 and 26 were considered Glaucolides (B and J); Compound 24 contains on C-8 an acetoxy group instead of an isobutyl group in 26. The set of fragments *m/z* (277, 259, 241, and 231) were observed in fragmentation patterns of Glaucolide skeletons regardless 24 or 26. These peaks were estimated as diagnostic ions for the identification of Glaucolide type of sesquiterpene lactone**[**63**]**. Peak 24 showed mass spectrum with a precursor ion at *m/z* 439 $[M+H]^+$ at retention time RT=5.85 min. and MS^2 spectrum showed a product ion at *m/z* 397 $[(M+H)-42]^+$ after losing of ketene moiety (42 Da), *m/z* 309 $[(M+H)-42-60]^+$ after further loss of AcOH (60 Da), 259 $[(M+H)-42-60-50]^+$ and other ion peaks as *m/z* (379, 337, 277, 243, 231, 189,

and 173). Likewise, Peak 26 with *m/z* 467 $[M+H]^+$ at RT= 6.01 min, differs from peak 24 just by 28 units of atomic mass. it showed the fragments at *m/z* 407 after eliminating AcOH (60 Da), *m/z* 379 after eliminating isobutyric acid (iBuOH, 88 Da), *m/z* 365 after eliminating AcOH (60 Da) and ketene (42 Da), *m/z* 337 after eliminating iBuOH (88 Da) and ketene (42 Da), *m/z* 319 after eliminating AcOH (60 Da) and iBuOH (88 Da), *m/z* 295 after eliminating iBuOH (88 Da) with two ketene (84 Da), and *m/z* 277 after eliminating AcOH (60 Da) with iBuOH (88 Da) plus ketene (42 Da) and the diagnostic sesquiterpene lactone ions at *m/z* (259, 241, 231, 213, 189, and 173) were also detected. Taking into account the fragmentation pattern resembling the sesquiterpene lactone of the Glaucolide class, an isobutyroyloxy group at C-8 was proposed for peak 26 instead of an acetate group present in Glaucolide B (peak 24) structure. So compound 24 was identified as Glaucolide B and compound 26 was identified as Glaucolide J; both compounds previously isolated and identified in different species of *Vernonanthura* genus (Vernonieae, Asteraceae)[63].

As shown in **table (1) Peak 27** RT=6.9 min. and **peak 28** RT=6.9 min. both compounds have approximate molecular structure, **since Peak 27** (contains ethoxy group in structure) differ from **peak 28** (contains methoxy group in structure) just by 14 units of atomic mass resulted from (CH2)**.** This illustrated the little difference in mass fragmentation pattern of them as follows**. Peak 27** showed mass spectrum with a precursor ion at *m/z* 511 $[M+H]^+$ and a product ion at *m/z* 482 $[(M+H)-29]^+$, 465 $[(M+H)-46]^+$, 451 $[(M+H)-60]^+$, 447 $[465-18]^+$, 432 $[450-18]^+$, 405 $[465-60]^+$, 379 $[465-86]^+$, 319 $[379-60]^+$, from losing (C_2H_5), (C_2H_5OH), (CH_3COOH), (H_2O), (H_2O), (CH_3COOH), (C_3H_5COOH), and (CH_3COOH) respectively. Also, *m/z* 276 $[319-43]^+$, 259 $[319-60]^+$, **Peak 28** showed mass spectrum with a precursor ion at *m/z* 497 $[M+H]^+$ and MS^2 spectrum product ions at *m/z* 482 $[M+H)-CH_3]^+$, and other previous peaks *m/z* 465, 446, 419, 405, 379, 351, 319, 277 and 259. So **compounds 27** and **28** were identified as **8-(2′-methyl) acryloyloxy-13-ethoxyvernojalcanolide** and **8-(2′-methyl) acryloyloxy-13-methoxyvernojalcanolide** respectively which are previously isolated from ***Vernonia menthaefolia*** **[**62**]**,

Peak 32 showed mass spectrum with a precursor ion at *m/z* 282 $[M+H]^+$ at retention time RT=10.8 min. Also, MS^2 spectrum showed a product ion at *m/z* 265 $[(M+H)-17]^+$ and 247 $[(M+H)-18-17]^+$. Peak 35 showed mass spectrum with a precursor ion at *m/z* 338 $[M+H]^+$ at retention time RT=12.57 min., also has MS^2 spectrum approximately similar to spectrum of peak 32 with little differences, Peak 35 showed a product ion at *m/z* 321 $[(M+H)-17]^+$, *m/z* 307 $[(M+H)-17-14]^+$, 304 $[(M+H)- 17-17]^+$, and *m/z* 303 $[(M+H)-17-18]^+$, 277 $[(M+H)- 61]^+$, 245 $[(M+H)- 61-32]^+$, from literature [64] these data was identical with compounds known as 6, 8, 10 -Trihydroxyeremophil-7 (11)-en-12, 8-olide (compound 32) and 3-Acetoxy-8-hydroxy-6-methoxyeremophila-7(11),9-dien12,8-olide (compound 35) which are sesquiterpene lactones previously isolated from genus *Farfugium* (family; Asteraceae).

Table (A1) Tentatively identified compounds in *Vernonia leopoldii* methanol extract using LC-MS/MS (Positive mode)

Peak no.	Compound name	RT (min)	Mwt	$[M+H]^+$ *m/z*	Mass fragmentation MS^2	References
Anthocyanin Glycoside						
1	Pelargonin	2.82	595.16	596.67	596, 434, 271	[35] [34]
Flavonoids						
Flavanones						
25	Taxifolin (2,3-dihydro-quercetin)	5.9	304.60	305.60	305, 287, 259, 231, 195, 179, 153	[36], [34, 35]
Flavones and Flavone Glycosides						
3	Isoschaftoside	3.46	564.14	565.38	565, 475, 469, 445, 415, 391, 385, 355, 327, 295	[40-44] [35]
4	Schaftoside	3.5	564.14	565.38	565, 475, 469, 445, 415, 391, 385, 355, 327, 295	[41-44] [35]
6	Apigenin-6,8-di-C-pentoside	3.73	534.14	535.40	535, 475, 445, 415, 385, 355, 335, 327, 299, 271	[41-44] [35]
9	Luteolin 7-O-glucoside (Cynaroside)	3.94	448.10	449.13	449, 287 (100), 363, 269, 259, 245, 243	[22, 47]
11	Apigenin-7-O-glucoside (Cosmosiin)	4.23	432.10	433.1	433, 271(100)	[22, 47]
13	Apigenin-8-C-glucoside (vitexin)	4.32	432.10	433.57	433, 413, 313, 285, 271(100)	[45, 46]
14	Apigenin-6-C-glucoside (Isovitexin)	4.37	432.10	433.08	433, 413, 313, 285, 271(100)	[45, 46]

Table (1): Continued

18	Luteolin	5.18	286.04	287.27	287, 269, 259, 245, 243, 226	[38, 47] [35]
21	Apigenin	5.59	270.05	271.01	271, 253, 227, 225, 199, 173, 183, 151, 119	[37, 38, 47]
Flavonol Glycosides						
2	Quercetin 3-O-b-D-apiofuranosyl-(1→6)-b-D-glucopyranoside	2.85	596	597	597, 465, 303, 285, 275, 257, 247	[48]
8	Kaempferol-O-hexoside	3.64	448	449	449, 287, 269, 258	[50] [35]
10	Quercetin-rhmnoside	4.07	448.10	449.31	449, 303	[49]
19	Kaempferol	5.2	286.04	286.91	287, 269, 258,	[36, 106] [35]
Furanocoumarin						
17	Oxypeucedanin	5.23	286.09	287.38	287, 251, 245, 227, 203, 187	[52]
29	(-)-marmesin	11.07	246.08	247.13	247, 231, 229, 201, 187	[53] [35] [54]
Phenolic Acids						
5	Feruloyl-O-p-coumaroyl-O-caffeoylshikimic acid	3.59	676.59	677.59	677, 645, 630, 604, 592, 515, 456, 338, 317, 297	[55]
20	3,7-O-diferuloyl-4-O-caffeoyl quinic acid	5.47	706.22	707.22	707, 689, 665, 647, 562, 544, 531 (-Quinic acid), 485, 409, 369 (-Caffeic acid), 339, 267	[55]
23	(E)-3-(3,4-diacetoxy-5-methoxyphenyl)	5.7	778.26	779.26	779.26, 743,681, 626, 574, 487, 413,	[55]

Table (1): Continued

	acroyl-4-O-p-coumaroyl-5-O-caffeoylquinic acid				367, 286, 241	
34	5-Coumaroylquinic acid	12.6	337	338	338, 320, 303, 193, 165	[55-57]
Sesquiterpene Esters						
33	Jugalinone	12.23	390.09	391.09	391, 373, 288, 270, 256, 228, 201	[58]
Sesquiterpene Lactone						
7	14-0-Acetylvernocistifolid-8-0-angelat	3.80	534.35	535.35	535, 517, 474, 414, 385	[59]
12	14-O-Senecioylvernocistifolid-8-0-(2,3-epoxyisobutyrat)	4.26	576.02	577.02	577, 559, 517, 271	[59]
15	(1S*,4R*,5S*,6S*,8S*,10R*)-1,4-Dihydroxy-5,10,13-triacetoxy 8-tiglyloxycadin-7(11)-en-6,12-olide	4.53	538.88	539.88	521, 497, 479, 461, 439, 419, 377	[60]
16	1-O-acetylbritannilactone	4.7	308	309	309, 291, 277, 249, 231, 217	[61]
22	8α-methacryloyloxy-hirsutinolide-13-O-acetate	5.67	406.22	407.22	407, 393, 348, 321	[22]
24	Glaucolide B	5.85	438.13	439.13	439, 397, 379, 337, 309, 277, 259, 243, 231, 189, 173	[63]
26	Glaucolide J	6.01	466.27	467.27	467, 407, 379, 365, 337, 319, 295, 277, 259, 241, 231, 213, 189, 173	[63]
27	8α-(2′-methyl) acryloyloxy-13-ethoxyvernojalcanolide	6.9	510.59	511.59	511, 482, 465, 451, 447, 433, 405, 379, 319, 277, 259	[62]

Table (1): Continued

28	**8α-(2′-methyl) acryloyloxy-13-methoxyvernojalcanolide**	**8.26**	**496.21**	**497.21**	**497, 482, 465, 446, 419, 405, 379, 351, 319, 277, 259**	**[62]**
30	**5β-Hydroxyasperilin**	**10.88**	**264.17**	**265.17**	**265, 247, 229**	**[61]**
31	**3β-hydroxy-4,15-dehydrograndolide**	**10.9**	**264.08**	**265.08**	**265, 247, 233, 174**	**[62]**
32	**6β, 8β, 10 β-Trihydroxyeremophil -7 (11)-en-12, 8-olide**	**11**	**282.01**	**282.39**	**282, 265, 247, 184, 156, 135**	**[64]**
35	**3β-Acetoxy-8α-hydroxy-6β-ethoxyeremophila-7(11),9-dien12,8-olide**	**12.57**	**338.35**	**338.35**	**338, 321, 303, 307, 305, 277, 245, 187**	**[64]**

3.2 Biological Results

3.2. A. Effect of *V. leopoldii* methanolic extract with its fractions on cancer cells viability

To assess the impact of methanolic extract of *V. leopoldii* and its fractions (Methanolic Diaion, Methanol/Water Diaion and Methylene chloride), on HepG2 and MCF-7 cell lines viability, cells were subjected to escalating doses (0.01-100 µg/mL) for 72 hours. The methanolic extract significantly reduced HepG2 and MCF-7 cells viabilities in a dose-dependent manner Figure (2 A and B), with IC_{50} values of 32.37 ± 1.78 and 54.31± 2.67 µg/mL, respectively, indicating that liver cancer cells are more sensitive to cytotoxic effect of *V. leopoldii* than breast cancer cells. Then, we investigated three fractions of this methanolic extract.

The Methanol/Water Diaion was almost inactive against both cell lines and failed to demonstrate notable cell inhibition activity until 100 µg/mL. Unlikely, the methanolic Diaion fraction showed moderate cytotoxic activity against both cell lines with an IC_{50} value of 43.26 ± 2.91 µg/mL against the HepG2 cell line and 55.51 ± 2.78 µg/mL against MCF-7 cell line. The methylene chloride fraction was the most potent one, showing potent cytotoxic activities with IC_{50} values of 9.32 ± 0.86 µg/mL and 14.26 ± 0.97 µg/mL against HepG2 and MCF-7 respectively, indicating high cytotoxic activity according to the National Cancer Institute classification (IC_{50} value < 20 µg/mL) **[**21**]**. Therefore, it was selected for further biological tests, to investigate its cytotoxic effect in detail.

Inhibiting cancer cell proliferation is a critical stage in tumor management and development [65]. Few investigations found that *V. leopoldii* extracts have an antiproliferative effect on cancer cell lines [38, 66, 67]. Their findings were consistent with ours in that *V. leopoldii* elicited a strong cytotoxic impact; however, we first demonstrated the activity of the methanolic extract in HepG2 cells. The anticancer properties of *V. leopoldii* extract may be attributed to the presence of a variety of bioactive components in the *Vernonia* species[68], including sesquiterpene lactones [69], flavonoids [70], steroids [71], and polysaccharides [72].

Furthermore, the methanolic extract of *V. leopoldii* and its fractions had selective cytotoxicity against liver cancer cells. Exposing normal liver cells (BNL) to the same doses and period resulted in high IC_{50} values of 85.9 ± 2.3 µg/mL for methanolic extract, 281.6 ± 4.32 µg/mL for methanolic Diaion fraction, and 68.25 ± 4.32 µg/mL for methylene chloride fraction (Figure 2BC).

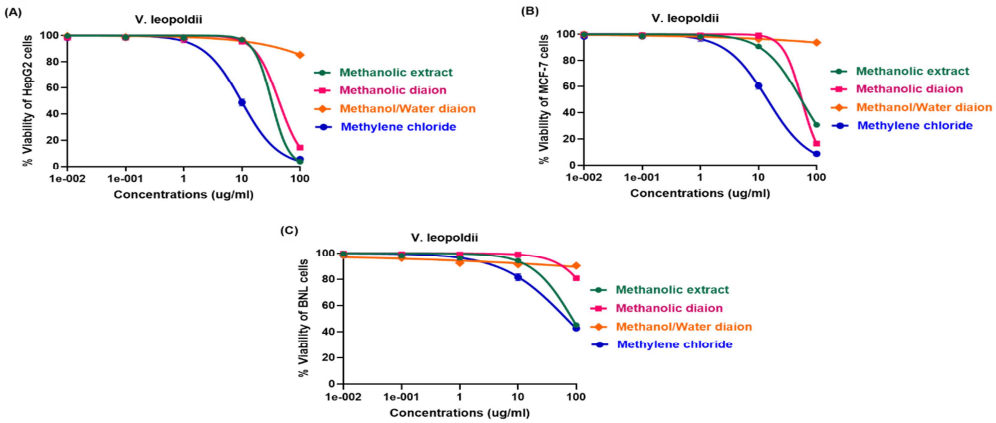

Figure (2): Cytotoxic activity of *V. leopoldii* methanolic extract and its fractions (Methanolic diaion, Methanol/Water diaion and Methylene chloride), on cancerous **(A)** HepG2 **(B)** MCF-7 and **(C)** normal BNL cells. Cells were treated with serial dilution of all for 72 h. Cell viability was evaluated by SRB assay. Data are expressed as mean ± SD (n = 3).

3.2. B. Effect of *V. leopoldii* methylene chloride fraction on the cell cycle distribution in HepG2 Cells

As shown in Figure (3), the cell cycle distributions in HepG2 were analyzed using flow cytometry to determine whether the *V. leopoldii* methylene chloride fraction disturbs the cell cycle progression in HCC. Treatment with the methylene chloride fraction of plant resulted in a significant decline in the G0/G1 phase cell population from 64.09 ± 1.6% to 59.88 ± 2.2% and S phase from 16.65 ± 0.6% to 12.87± 1.3%, compared to control cells. Reciprocally, *V. leopoldii methanolic extract halted the cells in the mitotic phase, represented by a* notable rise in the number of cells *of* the G2/M phase from 19.26± 0.3% to 27.25 ± 0.99%, compared with the control cells without causing a considerable increase in the pre-G1 population, just from 2.04 ± 0.2 % to 5.4 ± 0.9%.

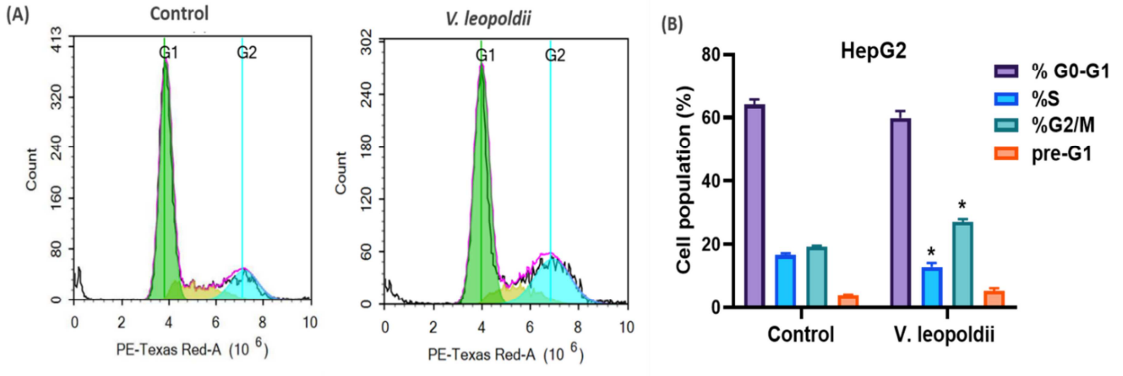

Figure (3): (A) Cell cycle distribution in HepG2 cells after treatment with *V. leopoldii* methylene chloride fraction for 48h, that was determined using DNA cytometry analysis compared with control (untreated cells)**. (B)** Quantification of percentage of cells in each phase of the cell cycle including pre-G1 is depicted as a bar graph of mean proportion of total events ± SD; n = 3. * Statistically significant difference from the control Quantification of the percentage of cells in each phase of the cell cycle is illustrated as a bar graph of mean ± SD; n = 3.

3.2. C. Effect of *V. leopoldii* methylene chloride fraction on the percentage of apoptosis/necrosis in HepG2 Cells

To elucidate the cytotoxic effect of methylene chloride fraction of *V. leopoldii* on HepG2 cells, apoptotic and necrotic cell percentage was detected via Annexin/PI double staining coupled with the flow cytometry. This technique enables the simultaneous reveal of sample dead cells from necrosis and/or apoptosis, as well as apoptotic and intact cells[1]. Figure (4) shows the flow cytometric results, with Q1 representing the intact cells, Q3 representing the necrotic cells, Q2 representing the percentages of late apoptotic cells and Q4 representing the early apoptotic cells[20].

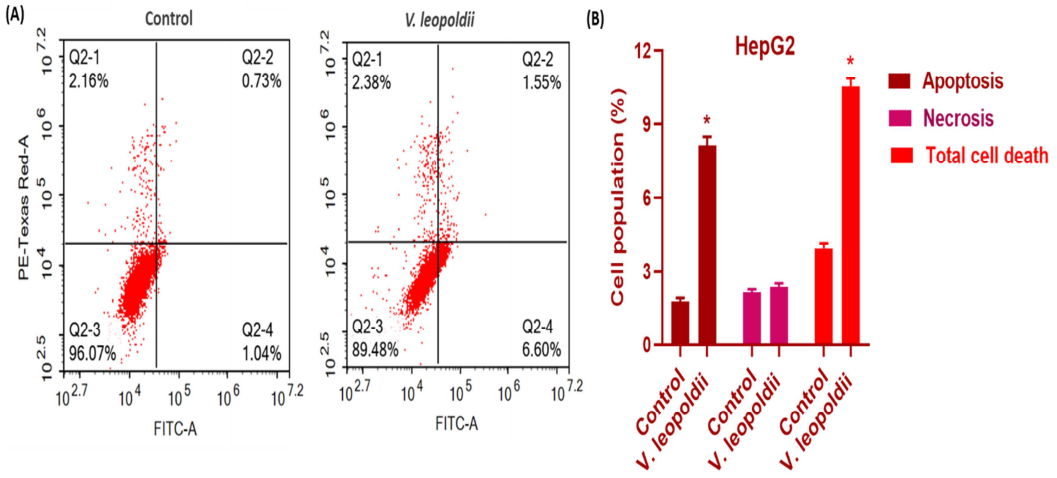

Figure (4): (A) Evaluation of cell death modality (Apoptosis/necrosis) after treatment of HepG2 cells with the *V. leopoldii* methylene chloride fraction for 48 h followed by double staining with Annexin-FITC/PI. **(B)** Different cell populations were plotted as a percentage of total events and presented as mean ± SD; n=3. * Statistically significant difference from the control

Cell cycle regulation necessitates the collaboration of a miscellany of intracellular and extracellular signals; without correct signals, cells cannot pass from one stage to the next, resulting in cell cycle arrest [73]. Hence, targeting cell cycle phases and checkpoints, particularly the two critical G1/S and G2/M boundaries [74], is a relatively new field of oncology that may offer unique prospects and potential for improving cancer treatment, specifically since cancer cells are known to resist pharmacological therapies by modulating the cell cycle phases [75-77]. In our study, the observed G2/M arrest of HepG2 cells after treatment with *V. leopoldii* may present a potential for its application in HCC, one of the most common types of malignancies with a poor prognosis in currently available therapies [78, 79]. This study reinforces the prior findings of targeting G2/M arrest of Genus Vernonia in triggering apoptosis of cancer cells [80-82].

Our findings revealed that treatment with a methylene chloride fraction of *V. leopoldii* caused cell death in HepG2 cells, primarily through apoptosis, compared to control untreated cells. *V. leopoldii* extract at 10 µg/mL reduced the percentage of viable cells to 89.48 ± 3.7% and increased the proportion of apoptotic cells from 1.77 ± 0.16% of untreated cells to 8.15 ± 0.34% Figure (4A). It is also worth noting that cells treated with *V. leopoldii* methylene chloride fraction did not exhibit an increase in the percentage of necrotic cells. Our findings are consistent with previous studies that depicted modulation of apoptosis pathways as an antiproliferative effect of the genus Vernonia on diverse tumor cell lines [80, 83, 84], which is primarily due to the presence of sesquiterpene lactones and flavonoids, the compounds with the highest antitumor potential within the *Vernonia* species [38, 68]. These findings emphasize Vernonia's involvement in fighting HCC.

3.2. D. The effect of *V. leopoldii* methylene chloride fraction on the Autophagy in the liver cancer cells

Here, autophagy was assessed using flow cytometry and acridine orange dye after HepG2 cells were treated with *V. leopoldii* methylene chloride fraction for 48 hours Figure (5A). The findings revealed that treatment with *V. leopoldii* boosted the autophagic signal by about 1.4-fold when compared to untreated control cells. The net fluorescence intensity (NFI) increased from 5.11 x 10^6 to 7.45 x 10^6 Figure (5A,B). Autophagy plays a dual role in tumors, including HCC [15], inhibiting malignancy formation first but promoting cancer maintenance in the long run [85, 86]. Thus, research has suggested that high levels of autophagy can also cause a unique type of autophagy-dependent cell death, termed autosis. Thus, studies have revealed that elevated autophagy levels may also result in autosis, a particular kind of autophagy-dependent cell death[87, 88].

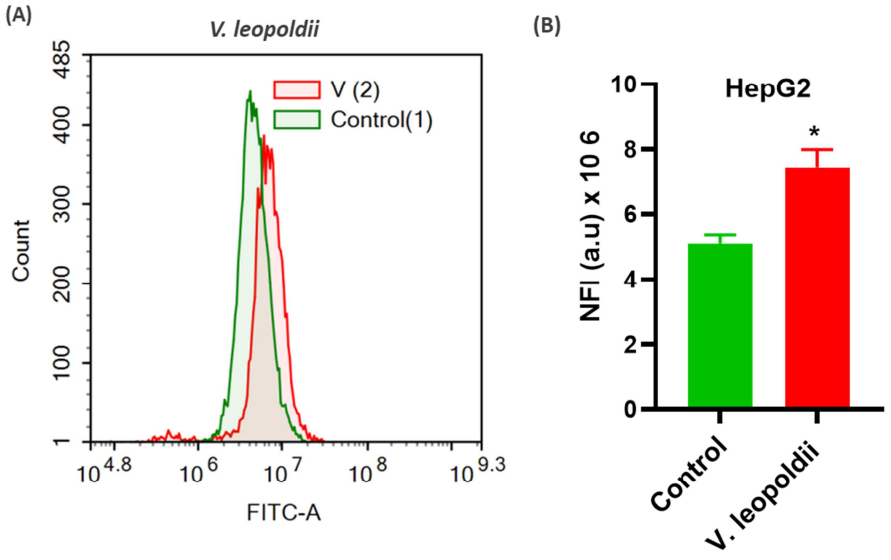

Figure (5): (A) Autophagic cell death was assessed in HepG2 cells after 48 hours of exposure to a methylene chloride fraction of *V. leopoldii*. The cells were stained using acridine orange dye. **(B)** NFI was plotted against the basal fluorescence of untreated HepG2 cells. Data are presented as mean ± SD; n=3. * Significant difference from control.

These data suggested that *V. leopoldii* at the IC_{50} concentration induced autosis in liver cancer cells. In line with our findings, a study on *Vernonia zeylanica*'s anticancer properties showed that it enhanced autophagy and apoptosis in Human Teratocarcinomal (NTERA-2) Cancer Stem-Like Cells [69]. Our findings demonstrated for the first time that *V. leopoldii* is

expected to perform an anticarcinogenic role by modulating autophagy, which has been reported as a smart approach in cancer therapy [89].

3.2. E. Effect of *V. leopoldii* on HepG2 Cell Migration

Invasion and metastasis, as the primary malignant hallmarks linked to lack of cell-cell adhesion, bring severe challenges to HCC treatment [90, 91],

Therefore, *V. leopoldii's* impact on cell migration was noted in the HepG2 cell line. For three days in a row, images were taken every day. Although *V. leopoldii* methylene chloride fraction causes a reduction in HepG2 cell viability and induces apoptosis at higher concentrations, it also has a noticeable effect on cancer cell migration at lower concentrations (5 µg/mL used in the wound healing assay) Figure (6 A), indicating that *V. leopoldii* targets different pathways to varying degrees.

Compared to the untreated HepG2 cells, the percentage of wound closure was significantly lower after treatment with *V. leopoldii* from 34.01 ± 2.87 % to 15.1 ± 1.16% at 24 h, from 62.61 ± 2.78 % to 40.1 ± 2.2% at 48 h and from 100 ± 1.89% to 68.75 ± 3.3 % at 72 h, respectively Figure (6B). In line with the previous results, many substances derived from *Vernonia* species, including Marmesin [47], Kaempferol [92], and Apigenin [93, 94], have the potential to suppress cancer cell migration by altering a variety of cellular signaling pathways.

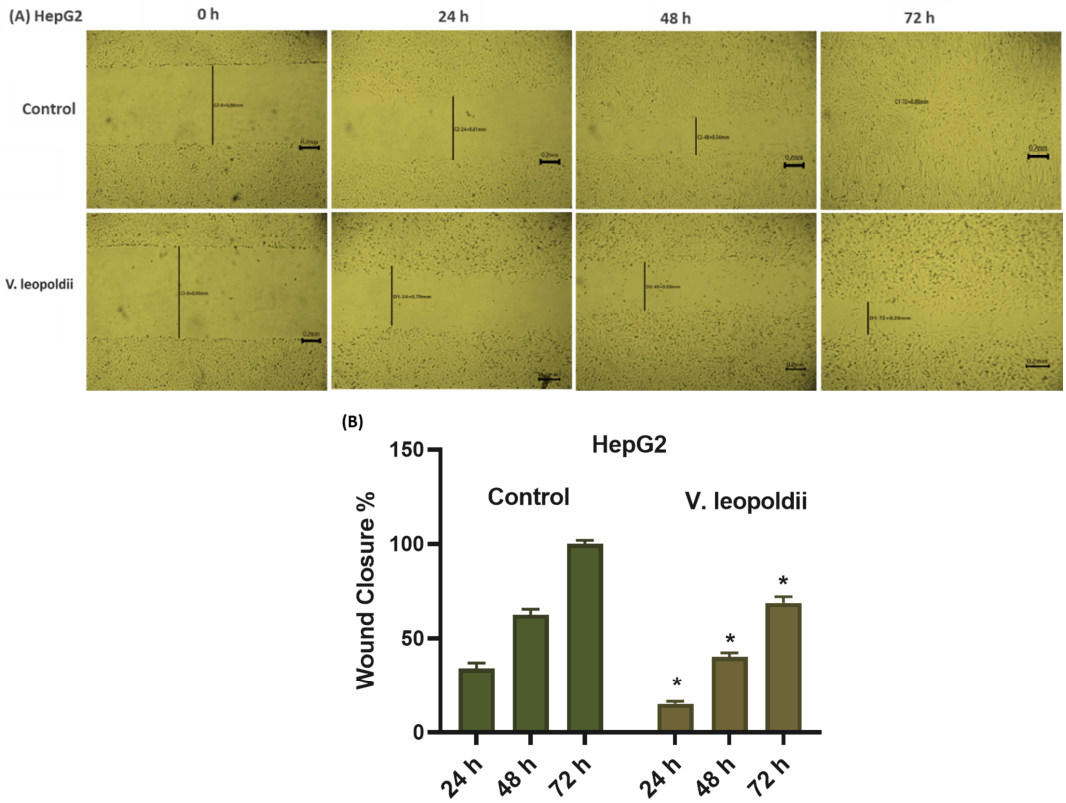

Figure 6: (A) *V. leopoldii* methylene chloride fraction inhibits HepG2 migration. **(B)** The migratory distances were measured daily for 24, 48, and 72 hours. Data were reported as wound closure percentages at each time interval and given in triplicates. *Statistically significant from control at $p < 0.05$

4. Discussion

V. leopoldii **methanolic extract,** showed high anti-cancer activity in both MCF7 and HepG2 cancer cell lines as described in the literature for Yemeni [22, 23] and Ethiopian[38, 95, 96] plants. *V. leopoldii* was found to have a notable growth inhibitory impact on three human cancer cell lines, namely bladder cancer (5637), breast cancer (MCF-7), and lung cancer (A-427) cell lines, in a study conducted by (Mothana et al. 2009)[23]. Another study was done by (Marzouk and Abd Elhalim, 2016)[22] that reported the isolation of five compounds and screened the effectiveness of three of them against four human

tumor cell lines, namely Colorectal carcinoma (HCT-116), Mammary gland carcinoma (MCF-7), hepatocellular carcinoma (HepG-2), and Epidermoid carcinoma of the larynx (HeP-2) of these reported compounds, they were found compound (22) 8α-methacryloyloxy-hirsutinolide-13-O-acetate showed strong activity against HeP-2[22].

Also studies on the *V. leopoldii* plant from Ethiopia (Tuasha etal, 2019) revealed significant activity on breast cancer cell lines [95]. A report by (Tuasha etal, 2020) found that, the nuclear factor-kappa B-induced translocation to the nucleus by tumor necrosis factor-α was greatly reduced by the ethyl acetate portion of *V. leopoldi* (0.87 µg/mL)[96].

These previous studies did not shed light on determining the cytotoxic mechanism of the plant on hepatic cancer cells. Thus, in this study, we analyzed the mechanism of cytotoxic effect of *V. leopoldii* extracts. This activity can be attributed to the metabolites identified by LC-MS/MS, namely; flavonoids and phenolic compounds, coumarins and sesquiterpene lactone.

Numerous anticancer effects of flavonoids have been shown, such as regulating the activity of enzymes that scavenge reactive oxygen species (ROS), taking part in cell cycle arrest, triggering autophagy and apoptosis, and lowering the growth and invasion of cancer cells. Flavonoids have a dual effect on ROS homeostasis; They not only function as antioxidants in healthy conditions but also as potent pro-oxidants in cancer cells, inducing apoptosis and suppressing pro-inflammatory signaling pathways[97]. Most of the identified flavonoids were known to have good inhibitory activity against most cancer types, especially hepatic HEPG2 cancer cell line. For instance, Apigenin (21) is a bioactive substance with numerous biological properties, including anti-cancer effects. By causing apoptosis, cell cycle arrest, and the creation of ROS, Apigenin prevents tumor cells from proliferating and remaining viable, slows angiogenesis and metastasis by changing a number of cellular signaling pathways [98]. Also Apigenin induced G1 phase arrest in HepG2 cells [93].

HepG2 cells treated with taxifolin (25) induced apoptosis and had their liver cancer's growth and migration inhibited[99]. In a dose-dependent mechanism, Kaempferol (19) was discovered to reduce the ability of human liver tumor cells (HepG2) to multiply. In addition, kaempferol and luteolin (18) in a combination slowed cell growth inhibition and hastened cell death in rats with 2-acetylaminofluorene-induced HCC and diethyl nitrosamine. Kaempferol does inhibit cell migration and invasion by stopping the cell cycle and causing cell death at the G2/M stage [92]. Cynaroside (9) can stop carcinogenesis, invasion, and cell proliferation in stomach cancer [100]. Vitexin (13) has anti-proliferative properties that cause human leukemia (U937) cells to undergo apoptosis through the mitochondrial death pathway. In hepatocellular carcinoma (SK-Hep1 and Hepa1-6) cells, studies have revealed the effectiveness of Vitexin in the inhibition of autophagy and the promotion of apoptosis through the JNK signaling pathway [101]. Through downregulating Bcl-2, upregulating the tumor suppressor p53 and its downstream Bax, and downregulating the transcription factor YY1, quercetin caused apoptosis and decreased the growth of human hepatoma cells (HepG2). In HepG2 cells, quercetin may exert its anticancer effects by directly engaging the transcription factor YY1's binding sites, which are situated within the p53 binding area, as well as by changing the conformation of YY10. All YY1 alterations resulted in a disruption of the YY1-p53 connection, which in turn led to p53 activation and polyubiquitination mediated by HDM2, which in turn initiated and promoted the apoptotic signaling cascade. Due to their structural similarity, quercetin derivatives and metabolites, along with rutin, isoquercetin, and other flavonoids, may potentially interfere with YY1 directly to break the link between YY1 and p53 proteins, leading to cell death and consequent anticancer potential[102, 103].

Sesquiterpenoids are organic compounds having a backbone of 15 carbons that are widely distributed in the leaves, fruits, or roots of numerous plants. Sesquiterpene lactones (SLs) are a sesquiterpenoids subclass and are common secondary metabolites in many plant families; they can be found practically in every genus of the sunflower family, Asteraceae (also known as Compositae), particularly *Vernonia* and others. The compounds are lipophilic, bitter, colorless molecules that are less volatile and thermolabile. Over 5000 structurally distinct SLs have been identified to date, with over 3000 of them being isolated from the Asteraceae family [26].

The SLs have been linked to a wide range of biological and pharmacological functions. Numerous SLs have attracted significant interest for their potential use in the treatment of cardiovascular disease and cancer, and their anti-cancer actions have been demonstrated. Numerous of the SLs' pharmacological effects are related to the presence of the methylene-lactone group. The addition Rapid Michael type occurs when cytotoxic (SLs) react with free sulfhydryl groups and other thiols, which are primarily found in the amino acid cystine in proteins, according to studies on structure-activity connections. [26]. In our findings compounds 32 and 35 are sesquiterpene lactones that were identified previously in *Farfugium japonicum* which has an inhibitory effect on

HepG2 [64]. Hirsutinolides and Glaucolides (24, 26) are sesquiterpene lactones isolated from flowers and leaves of *Vernonia scorpioides* with a cytotoxic effect against Ehrlich's tumor, melanoma (B16-F10), sarcoma 180 ascitic tumor, cervical and Hela cancer cells [104].

According to experimental data, furanocoumarins cause malignant cells to undergo apoptosis, autophagy, antioxidant defenses against metastasis, and cell cycle arrest. Furanocoumarins have also been demonstrated to have synergistic chemotherapeutic potential and chemo preventive when used in conjunction with anticancer medications. Marmesin (29) causes Bcl-2 down regulation, Bax upregulation, and an increase in the Bax/Bcl-2 ratio, which prevents colony formation and causes apoptosis in cause human leukemia (U937) cells. In U937, Marmesin inhibits G2/M and cell migration. Oxypeucedanin (17) has been shown to exhibit antioxidant properties and cause cytotoxicity in SK-MEL-2, HCT-15, XF498, A549, and SK-OV-3 cells [105]. All these previously reported data on compounds identified in *V. leopoldii* is in accordance with the current finding of the plant activity against hepatic HepG2 cancer cell line.

5. Conclusion

In conclusion, this study was done for first time on *V. leopoldii* collected from Yemen to determine the phytochemical content using LC-MS/MS. Results showed that it contains potential bioactive anticancer compounds, like; flavonoids, phenolic compounds, coumarins and sesquiterpene lactones, which are readily extractable using methanol/methylene chloride. The methylene chloride fraction of the methanol extract revealed potent cytotoxic activity against HepG2 cancer cell line with IC_{50} of 9.1 µg/mL. Current research offers interesting new information about how *V. leopoldii* induces apoptotic/autophagic, anti-migratory, and anti-proliferative cell death in HepG2 cells, which may be able to lessen the aggressiveness of HCC. This study suggested that *V. leopoldii* may be used for the isolation and development of anticancer drugs.

6. Conflicts of interest

There are no conflicts to declare

7. Formatting of funding sources

None

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9. References

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