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Research Article

Extraction and identification of Golder (*Otostegia persica*) root extract phytochemicals and evaluating their anticancer activity

Zahra Forghani[a,b], Seyed Mohammad Hashem Hosseini[a], Paul Van der Meeren[b], Mohamad Taghi Golmakani[a], Fakhraddin Naghibalhossaini[c], Mehrdad Niakousari[a] *

[a] Department of Food Science and Technology, School of Agriculture, Shiraz University, Shiraz, I. R. Iran
[b] Particle & Interfacial Technology Group, Department of Green Chemistry and Technology, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium
[c] Department of Biochemistry, School of Medicine, Shiraz University of Medical Sciences, Shiraz, I. R. Iran

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ABSTRACT- *Otostegia persica* (commonly known as Golder) is a medicinal plant traditionally used for remedial properties. The aerial parts of Golder have been investigated for their phytochemical composition and anticancer potential. This study aimed to extract and identify bioactive compounds from Golder roots to evaluate their anticancer activity against colon cancer cell lines using the thermal reflux method with hexane, chloroform, and methanol solvents. The Golder root and its extracts were analyzed to identify bioactive compounds using various techniques, including energy-dispersive X-ray (EDX) analysis, Fourier-transform infrared (FTIR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, ultraviolet-visible (UV-Vis) spectroscopy, and gas chromatography/mass spectrometry (GC/MS). The results showed that the hexane extract had the lowest extraction efficiency due to its non-polar nature. Also, compared to the methanol extract, the chloroform extract contained fewer carbohydrates which helped to identify other bioactive compounds. The chloroform extraction yielded several compounds with potential anticancer activity. These compounds were squalene, naphthalene derivatives, 1H-indole-2-carboxylic acid, vanillin, benzyl alcohol, and cicillin. The in vitro anticancer activity was evaluated using the MTT assay (3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide assay) on HCT116, SW480, SW48, and SW742 cell lines. The inhibition of cell proliferation was significantly dose-dependent, with IC50 values of 394.9, 563.3, 721.7, and 565.1 µg/mL, respectively. This study provides new insights into the potential use of Golder root extracts as anticancer agents since the chloroform extract induced morphological changes in cancer cells, thus reducing their viability by approximately 50% when the extract was used at 400 µg/mL.

INTRODUCTION

Medicinal plants are valuable sources of new remedies with fewer complications than synthetic drugs. These plants are rich in diverse phytochemical compounds and exhibit a wide range of biological activities (Salehi et al., 2019). The growing interest in replacing synthetic pharmaceuticals with plant-based products is driven by their renewability, availability, ecological friendliness, and safety for human health (Moradnia et al., 2023; Süntar, 2019). *Otostegia persica* (Burm. f.) Boiss., commonly known as Golder, belongs to the Lamiaceae family, which comprises about 33 distinct species. These species thrive particularly well in Mediterranean regions. Iran hosts unique habitats where three species of the Otostegia genus — *Otostegia aucheri*, *Otostegia michauxi*, and *Otostegia persica* — are found. *Otostegia persica* (Golder) is most commonly found in Iran's dry, tropical, and subtropical areas. Traditionally, the aerial parts of this plant have been used for their antispasmodic, antihistaminic, and antiarthritic properties (Sadeghi et al., 2014; Toori et al., 2015). Additionally, studies reported its antibacterial activity against certain Gram-positive bacteria (Alaklabi et al., 2016; Asghari et al., 2007). Plants in the Lamiaceae family are known for their antioxidant properties, which may contribute to the prevention or reduction of heart disease and cancer (Kagawa et al., 2019). Golder root extract (GRE) has also been reported to possess antidiabetic properties comparable to metformin, a widely used antidiabetic drug (Bagherzade et al., 2014). The components of GRE, therefore, present a promising avenue for further research on diabetes and other diseases. Despite the extensive research on the aerial parts of *Otostegia persica*, little is known about the potential anticancer effects of its root extracts. To address this gap, this study focuses on investigating the influence of polar, semi-polar, and non-polar solvents — methanol, chloroform, and hexane — on the extraction of bioactive compounds from Golder root. Advanced analytical

* Corresponding author: Professor, Department of Food Science and Technology, School of Agriculture, Shiraz University, Shiraz, I. R. Iran
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techniques, including energy-dispersive X-ray (EDX) analysis, Fourier-transform infrared spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, ultraviolet-visible (UV-Vis) spectroscopy, and gas chromatography/mass spectrometry (GC/MS), were employed to analyze the Golder root and its extracts. For the first time, the in vitro anticancer activity of GRE was assessed using the MTT cytotoxicity assay against malignant colon cancer cell lines. This investigation aims to identify potential bioactive compounds from the root extract and to evaluate their efficacy on inhibiting cancer cell growth, thereby providing novel insights into the potential application of GRE as an anticancer agent.

MATERIALS AND METHODS

Materials

All solvents were made by Merck (Damstadt, Germany). Deuterated solvents were obtained from Armar (London, United Kingdom) and utilized in their original state. Fetal bovine serum (FBS) and Roswell Park Memorial Institute (RPMI) medium were obtained from Gibco (New York, NY). Penicillin and the streptomycin used were manufactured by Bioidea (New York, NY).

Plant collection and extraction

Otostegia persica (Golder) was collected in April 2022 from the Fars Agricultural Research Center, located in the Kharameh region of Shiraz, Fars province, Iran. The plant was assigned the herbarium voucher number 14445 at the Fars Agricultural Research Center, Shiraz, Iran. The freshly collected roots were cleaned thoroughly and shade-dried under ambient air conditioning for seven days. Once dry, the roots were stored in black polyethylene bags under refrigerated conditions to preserve their integrity prior to extraction. The dried roots were then ground into a fine powder using a Moulinex mill (model 8000, SEB Group Company, France). The extraction process was carried out using the heat reflux method with three different solvents: hexane, methanol, and chloroform. The weight ratio of Golder root powder to solvent was maintained at 1:10. The mixture of root powder and solvent was refluxed for 2 hours at 70 °C using a condenser flask. Following reflux, the solution was strained and concentrated. The final concentrated extract was dried at 40 °C under vacuum using a rotary evaporator (model RV 10, IKA Company, Germany) (Lin et al., 2016).

EDX spectroscopy

To determine the mineral elements of the plant, the root powder sample was used and heated in a furnace at 750 °C for 2 hours. After calcining the root powder, the calculated ash percentage was 1.4% and then the resulted ash was subjected to elemental analysis (Sadeghi et al., 2014).

FTIR spectroscopy

To determine the functional groups in the samples, the GRE was identified via FTIR spectroscopy with KBr discs where a thin layer of extracted sample was placed on a KBr tablet, followed by spectrometry at an ambient temperature. Utilizing the TENSOR II instrument manufactured by Bruker in Germany. The FTIR spectrum of the samples was obtained in a range of 400-4000 cm^{-1} (Kumar Sahu et al., 2019).

NMR spectroscopy

A 400 MHz AVANCE Ultra Shield NMR device (Bruker Company, Germany) was used for identifying the molecular structure of the compounds. Deuterated solvents of chloroform and methanol were used for dissolving the extracted samples. Sampling was done at an ambient temperature. Sixty-four scans were used with a delay time of 6 seconds and a rotation angle of 30° for ^{1}HNMR spectroscopic setup. This was followed by 16,000 scans, 2 s delay time, 90° flip angle, and a power gate pulse program for ^{13}CNMR spectroscopic setup. For the DEPT spectroscopy setup, we used 16,000 scans, 2 s delay time, and a DEPT135 pulse program (Ekeocha et al., 2021).

UV-Vis spectrophotometer

To validate the compounds identified by NMR and FTIR analysis, a UV-Vis spectrophotometer (LAMBDA 365 model, PerkinElmer, Waltham, MA) was used for obtaining the UV-Vis spectra. This device measures the transmission and absorption of liquid and film samples within the wavelength range of 190 to 1100 nm. For the analysis, the extracted samples were dissolved in chloroform, and spectrometry was conducted at room temperature (Conceição et al., 2019).

GC/MS technique

To identify low molecular weight compounds, a GC/MS (model 5973N, Agilent Technologies, Santa Clara, CA) equipped with a mass-selective detector operating in electron impact mode (70 eV) was used. Helium gas (99.99% purity) served as the carrier gas. For the analysis of GRE, the plant powder was dissolved in chromatographic-grade chloroform. The solution was then concentrated before being injected into the instrument's capillary column. The capillary column measured 30 meters in length, 0.25 mm in internal diameter, and had a film thickness of 0.25 µm (Golmakani & Rezaei, 2008).

Anticancer assay

Cell culture

Four colorectal cancer (CRC) cell lines were obtained from the National Cell Bank of Iran (NCBI, Pasteur Institute, Tehran). We cultured these cells in RPMI, supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL). These cells remained at 37 °C in an environment containing 5% CO_2 and saturated humidity. The replacement of the medium occurred at regular intervals of two to three days.

Cell viability and proliferation

The effects of GRE on the proliferation and viability of four CRC cell lines (HCT116, SW480, SW48, and SW742) were investigated. Cells at the logarithmic growth phase were

collected via trypsin digestion and seeded at a density of 5000 cells per well in 96-well plates containing RPMI medium. Following a 24-hour incubation period, the cells were exposed to increasing concentrations of GRE (0, 200, 400, 600, and 800 µg/mL) in the culture medium. Control wells contained cells without GRE, while blank wells contained culture medium only, without cells. After 48 hours of treatment, cell proliferation was assessed using the MTT assay. Cells were incubated in 200 µL of culture medium containing 20 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (5 mg/mL) for 4 hours. Following incubation, the medium was aspirated, and 150 µL of dimethyl sulfoxide (DMSO) was added to each well. The plate was agitated to ensure complete dissolution of the blue-purple formazan crystals formed during the MTT reaction. Absorbance (A) at 570 nm was measured using a microplate reader (OPT Imax, Molecular Dynamics, Sunnyvale, CA).

$$\text{Cell viability (\%)} = (A_{treatment} - A_{blank})/(A_{control} - A_{blank}) \times 100$$

The experiment was conducted in triplicate, with each assay performed in duplicate, and the data are presented as mean ± standard deviation (SD). The dose-response curve was analyzed using GraphPad PRISM software (version 5.00, San Diego, CA) to calculate the IC50 value (Deng et al., 2013).

RESULTS AND DISCUSSION

Elemental analysis of Golder root

A widely recognized and essential initial step in the development of botanical products is the authentication and identification of raw materials. However, intrinsic chemical heterogeneity is inevitable in any botanical specimen. To ensure accurate identification and characterization of the Golder root and its extract (GRE), a combination of analytical techniques was employed, including EDX analysis, FTIR spectroscopy, NMR spectroscopy, UV-Vis spectroscopy, and GC/MS. The elemental composition of the Golder root, as determined by EDX analysis, is presented in Table 1. The analysis revealed that potassium was the most abundant element in the Golder root, while iron was present in the lowest concentration. These findings align with previous studies conducted on the aerial components of *Otostegia persica* in southern Iran, which also reported the presence of similar mineral elements (Sadeghi et al., 2014).

Table 1: Total content of some inorganic elements in Golder root using energy dispersive x-ray analysis

Element	Na	Mg	P	S	K	Ca	Fe
Amount (mg/kg)	396	1225	1492	334	2308	1734	17

FTIR spectroscopy analysis

Fig. 1 illustrates the FTIR spectra of methanol, chloroform, and hexane extracts of Golder root. While some peaks appear in all three spectra, their intensities differ, reflecting the influence of solvent polarity on the extraction process. The FTIR spectrum of the methanol extract shows prominent broad bands in the 2400–3400 cm^{-1} range, indicative of O-H stretching vibrations, which are characteristic of hydroxyl groups typically found in cellulose (Hossain et al., 2022). This peak is notably more intense in the methanol extract than in the chloroform and hexane extracts, highlighting the higher concentration of cellulose, saccharides, hemicellulose, and related polar compounds in the methanol extract (Fig. 1a). The peak at 3041 cm^{-1} corresponds to the C-H stretching of olefin groups. Peaks in the 2850–3000 cm^{-1} range are attributed to the C-H stretching vibrations of methine, methylene, and methyl groups. Additionally, strong absorption bands at 1739 cm^{-1} and 1711 cm^{-1} are linked to the C=O stretching vibrations of esters, aldehydes, ketones, and carboxylic acids. A peak at 2726 cm^{-1} corresponds to the stretching vibration of aldehyde hydrogen. Other significant peaks include those at 1595 cm^{-1} (C=C stretching vibration), 1464 cm^{-1} (CH_2 bending vibration), 1377 cm^{-1} (CH_3 bending), and peaks in the 1000–1300 cm^{-1} range corresponding to C-O stretching vibrations. The peaks at 913 cm^{-1} and 742 cm^{-1} are attributed to out-of-plane C-H bending vibrations of olefin groups (Kumar Sahu et al., 2019; Stuart, 2004). The intensity of the hydroxyl (OH) stretching vibration (3400 cm^{-1}) varies significantly among the three extracts, with methanol displaying the strongest peak and hexane the weakest. This indicates that methanol extract contain a higher concentration of hydroxyl-bearing compounds such as cellulose, saccharides, and hemicellulose, whereas such compounds are present in much lower amounts in the hexane extract. A similar trend is observed for the O-H stretching of carboxylic acid groups, which appears in the 2400–3400 cm^{-1} region. This peak is the most intense in the methanol extract and the weakest in the hexane extract, indicating that polar solvents like methanol extract a higher concentration of carboxylic acid groups (Hossain et al., 2022; Indran & Raj, 2015; Kumar Sahu et al., 2019; Seki et al., 2013). One of the most significant features shared by all three extracts is the C=O stretching peak observed in the 1740–1700 cm^{-1} range. This peak is stronger in the chloroform and hexane extracts compared to the methanol extract, reflecting differences in the polarity of the extracted compounds. The strong C=O signal in the chloroform and hexane spectra suggests a higher concentration of fatty acids and esters, as these nonpolar compounds are more soluble in less polar solvents. A closer inspection of the C=O peak reveals subtle distinctions between the chloroform and hexane spectra. The C=O stretching in the hexane extract shows characteristics typical of free fatty acids, while the chloroform extract indicates the presence of esterified fatty acids. The slight shift in the carbonyl absorption band position between the two extracts supports this distinction. This difference highlights the polarity-dependent extraction efficiency of hexane and chloroform, with hexane favoring free fatty acids and chloroform favoring esterified fatty acids (Hameed et al., 2015; Pramila et al., 2012).

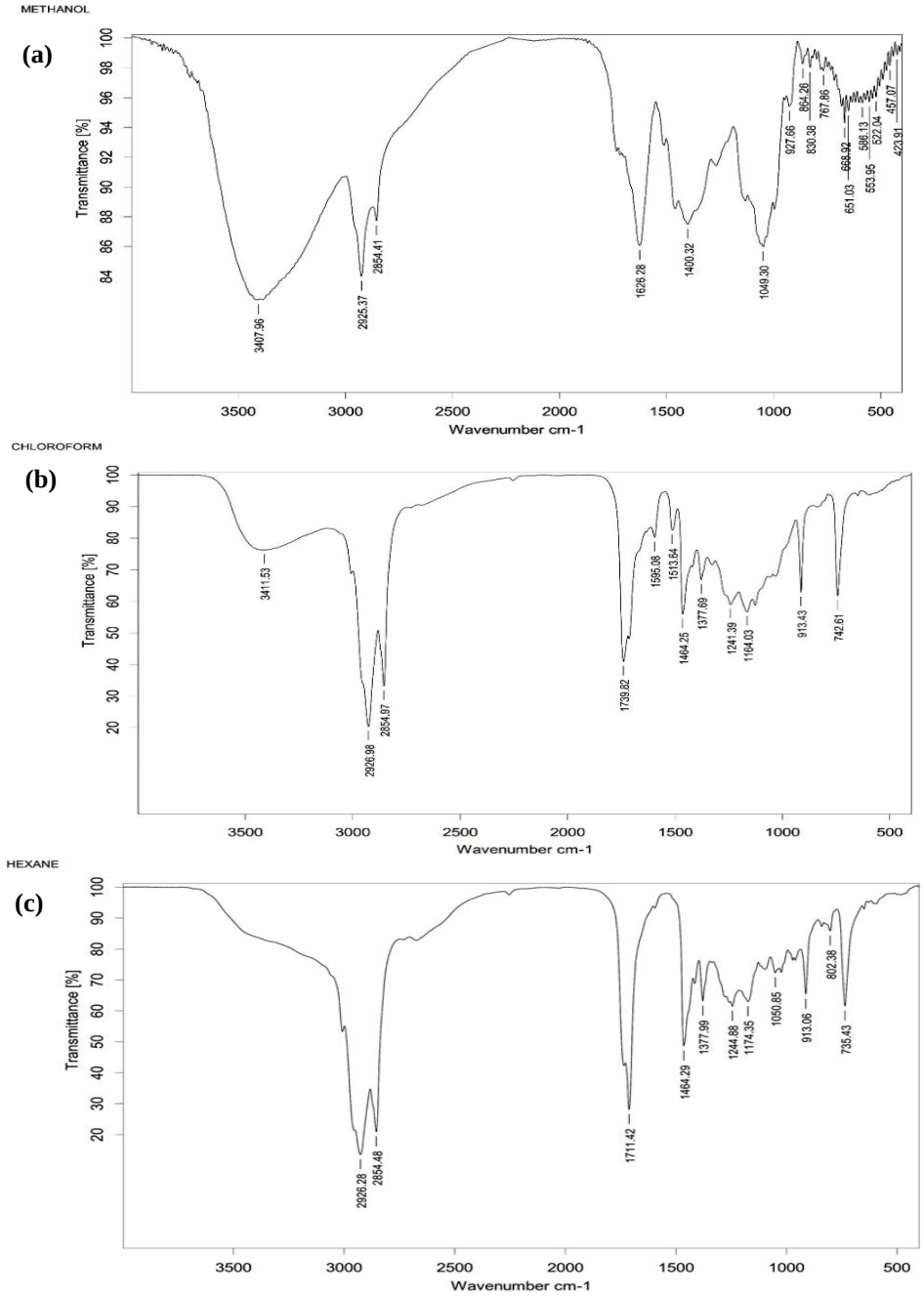

Fig. 1. FTIR spectra of the Golder root extract obtained using (a) methanol, (b) chloroform, and (c) hexane.

NMR spectroscopy analysis

^{1}H-NMR, ^{13}C-NMR, and DEPT135 spectroscopy are widely used for the detection and structural elucidation of a broad range of compounds. Their non-invasive, fast, and sensitive nature allows for the analysis of complex mixtures without the need for sample pretreatment (Saeidian et al., 2013). In this study, high-resolution ^{1}H-NMR, ^{13}C-NMR, and DEPT135 spectroscopy were employed to characterize the chemical composition of GRE obtained using methanol, chloroform, and hexane as solvents (Fig. 2, Fig. 3, and Fig. 4). The NMR spectra revealed notable differences in the chemical profiles of the methanol, chloroform, and hexane extracts. A striking similarity was observed between the NMR peaks of the hexane and chloroform extracts, suggesting a comparable chemical composition of the materials extracted by these two solvents. In contrast, the spectrum of the methanol extract displayed a distinctly different profile, with a higher prevalence of polysaccharides and carbohydrate-related compounds. These observations are consistent with prior studies (Wang et al., 2021), which reported the preferential extraction of polysaccharides by polar solvents like methanol. The ^{1}H-NMR spectrum of the chloroform extract (Fig. 2b) exhibited characteristic peaks corresponding to free fatty acids. Further analysis using ^{13}C-NMR confirmed the presence of fatty acids, most of which were in the form of esters. Fatty acid esters are formed through esterification, where long-chain fatty acids react with alcohols. According to Table 2, several additional compounds, including alcohols like beta-sitosterol and stigmasterol, were identified in the chloroform extract. The presence of these alcohols aligns with earlier studies (Sahu et al., 2020) and reinforces the role of chloroform as an effective solvent for extracting non-polar lipid-soluble compounds from Golder root. The ^{13}C-NMR

spectra (Fig. 3) revealed significant distinctions in the chemical composition of the three extracts. Methanol-extracted compounds exhibited a higher abundance of saccharide-related compounds, as evidenced by the presence of characteristic carbon peaks for carbohydrates. Conversely, the chloroform and hexane extracts contained relatively fewer saccharide-related signals, with the chemical profiles dominated by non-polar compounds such as fatty acids and esters. The DEPT135 analysis (Fig. 4) provided further insights into the types of carbon atoms present in the extracts. The analysis highlighted a predominance of CH_2 carbons in the hexane and chloroform extracts, characteristic of the long hydrocarbon chains found in fatty acids. In contrast, the methanol extract showed a greater concentration of CH and CH_3 carbons, which are commonly associated with saccharide structures. The absence of saccharide-related carbon signals in the chloroform and hexane extracts underscores the selective extraction efficiency of these solvents for non-polar and less polar compounds. Among the three solvents, chloroform was selected for subsequent tests due to its ability to selectively extract non-polar compounds while excluding polar substances like carbohydrates and saccharides. This selective extraction allows for a clearer identification of non-polar bioactive compounds. In contrast, the hexane extract yielded an insufficient quantity of material, rendering it unsuitable for further analysis. The methanol extract, while rich in saccharides and polysaccharides, was not selected due to its limited ability to isolate other target bioactive compounds.

UV-Vis spectroscopy analysis

The UV-Vis absorption spectrum of the chloroform extract, recorded in the wavelength range of 250 to 800 nm, is presented in Fig. 5. A prominent absorption peak at 280 nm is observed, which corresponds to the presence of double bonds found in free fatty acids (Conceição et al., 2019). The spectrum also exhibits a distinct peak around 330 nm, further supporting the existence of various conjugated double bonds within the sample (Conceição et al., 2019). In some studies, an absorption peak at approximately 280 nm is associated with proteins and peptides due to the presence of aromatic amino acids, such as tryptophan, tyrosine, and phenylalanine (Simonian, 2002). However, the contribution of proteins or peptides to the peak observed in the chloroform extract is considered minimal. Members of the Lamiaceae family, to which Golder (*Otostegia persica*) belongs, are generally known to contain low levels of proteins, peptides, and amino acid-derived compounds (Sadeghi et al., 2014). Additionally, several studies have reported that certain phytochemicals, particularly unsaturated fatty acids, exhibit absorption near 280 nm due to their conjugated double-bond systems (Conceição et al., 2019; Zhao et al., 2019a). Our NMR and FTIR analyses confirmed the presence of fatty acids and fatty acid esters as the primary compounds in the chloroform extract of Golder root. No evidence of protein-related structures was detected in these analyses. Therefore, it is reasonable to attribute the 280 nm absorption peak primarily to free fatty acids, rather than to proteins or peptides. Another noteworthy feature of the spectrum is a sharp peak observed just below 280 nm, which may be attributed to the UV cutoff area (COA) of the chloroform solvent. The UV cutoff refers to the wavelength below which the solvent absorbs UV radiation, potentially affecting the spectrum of the analyte despite the use of blanking experiments. Chloroform's UV cutoff begins around 245 nm, and prior studies have reported a distinct absorption peak for chloroform at 254 nm (Pratiwi & Nandiyanto, 2022).

The GC/MS analysis of the chloroform GRE revealed the presence of various bioactive compounds, as illustrated in Fig. 6. The identified peaks corresponded to triterpenes such as squalene, beta-sitosterol, stigmasterol, and other compounds with similar molecular weights. However, certain non-volatile compounds that could not undergo evaporation or traverse the GC column were subjected to further scrutiny and identification using NMR spectroscopy. A total of 67 compounds were identified in the chloroform extract (Table 3), encompassing a diverse range of chemical classes, including terpenoids, hydrocarbons, alcohols, saturated and unsaturated fatty acids, and polycyclic aromatic compounds, steroids, and organic acids. These findings align with prior reports on the chemical composition of other Lamiaceae family plants. Among the terpene-terpenoid compounds, the most abundant were squalene (8.28%), stigmasterol (1.48%), and beta-sitosterol (1.34%). Benzyl alcohol (0.21%) and vanillin (0.80%) emerged as the principal single-ring aromatic compounds, while naphthalene derivatives (3.87%) and 2,3,6,7-tetramethylanthracene-9,10-dione (1.91%) were the predominant polycyclic aromatic hydrocarbons. Noteworthy carboxylic acids included 1H-indole-2-carboxylic acid (2.06%). Saturated fatty acids were well represented in the chloroform extract, with the most prominent being hexadecanoic acid (10.28%), followed by hexanoic acid (1.65%) and octadecanoic acid (0.97%). The unsaturated fatty acids, oleic acid (8.04%) and linoleic acid (4.56%), were also identified as significant components, corresponding to omega-9 and omega-6 fatty acids, respectively. Previous investigations on Lamiaceae species have documented a similar fatty acid composition, with oleic acid being notably abundant, as reported in *Salvia microphylla* (Hazrati et al., 2021; Moshari-Nasirkandi et al., 2023; Serrano et al., 2023). Previous studies have also highlighted the anti-proliferative properties of Lamiaceae species against cancer cells (Kagawa et al., 2019; Kilinç et al., 2022; Nikolić et al., 2014). Notably, numerous anti-cancer compounds were identified in the chloroform GRE through GC/MS and NMR analysis. In our study, these included squalene (Preedy & Watson, 2020), naphthalene derivatives (Kedderis et al., 2014), 1H-indole-2-carboxylic acid (Cui et al., 2020), vanillin (Bezerra et al., 2016), benzyl alcohol (Chang et al., 2011), and cicillin (Tasi et al., 2008).

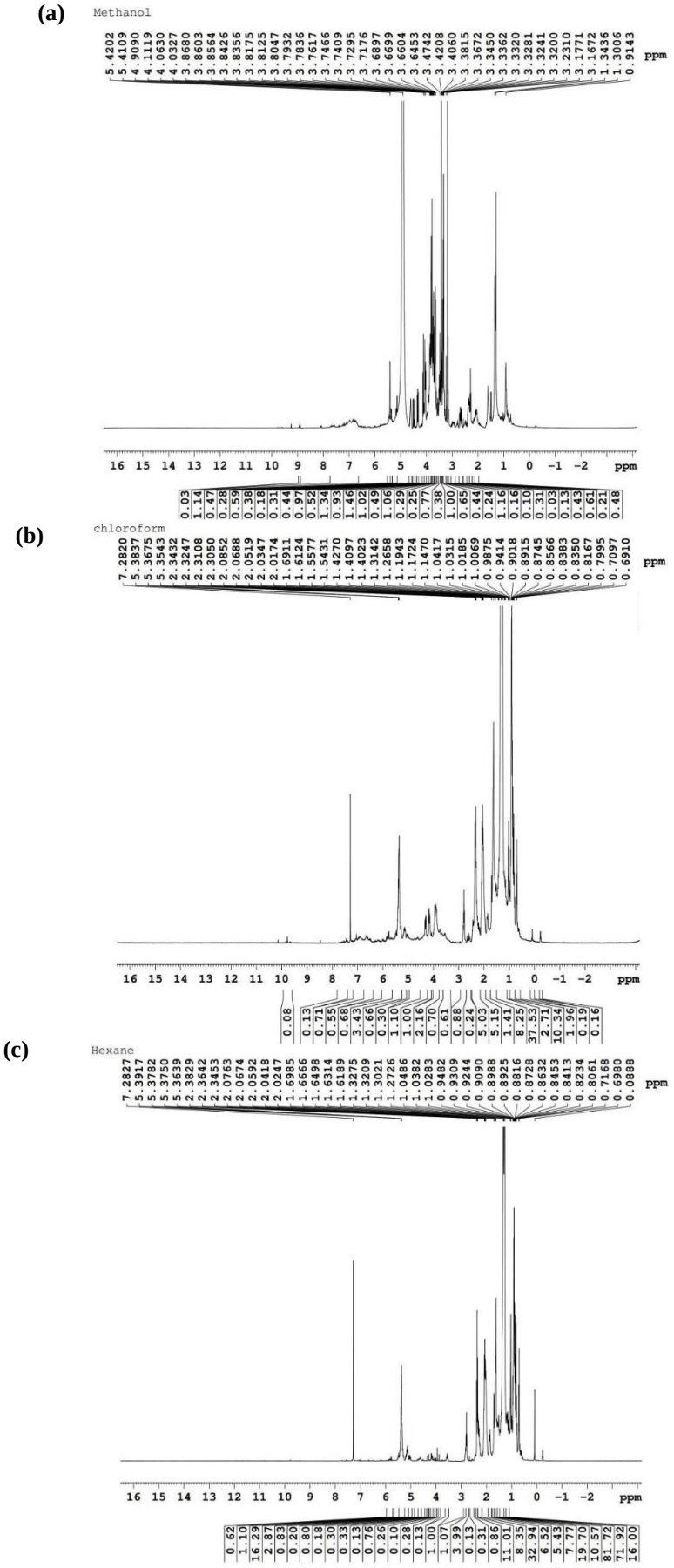

Fig. 2. ^{1}H NMR spectra of the Golder root extract components. (a) methanol extract in CD_3OD, (b) chloroform extract in $CDCl_3$, and (c) hexane extract in $CDCl_3$.

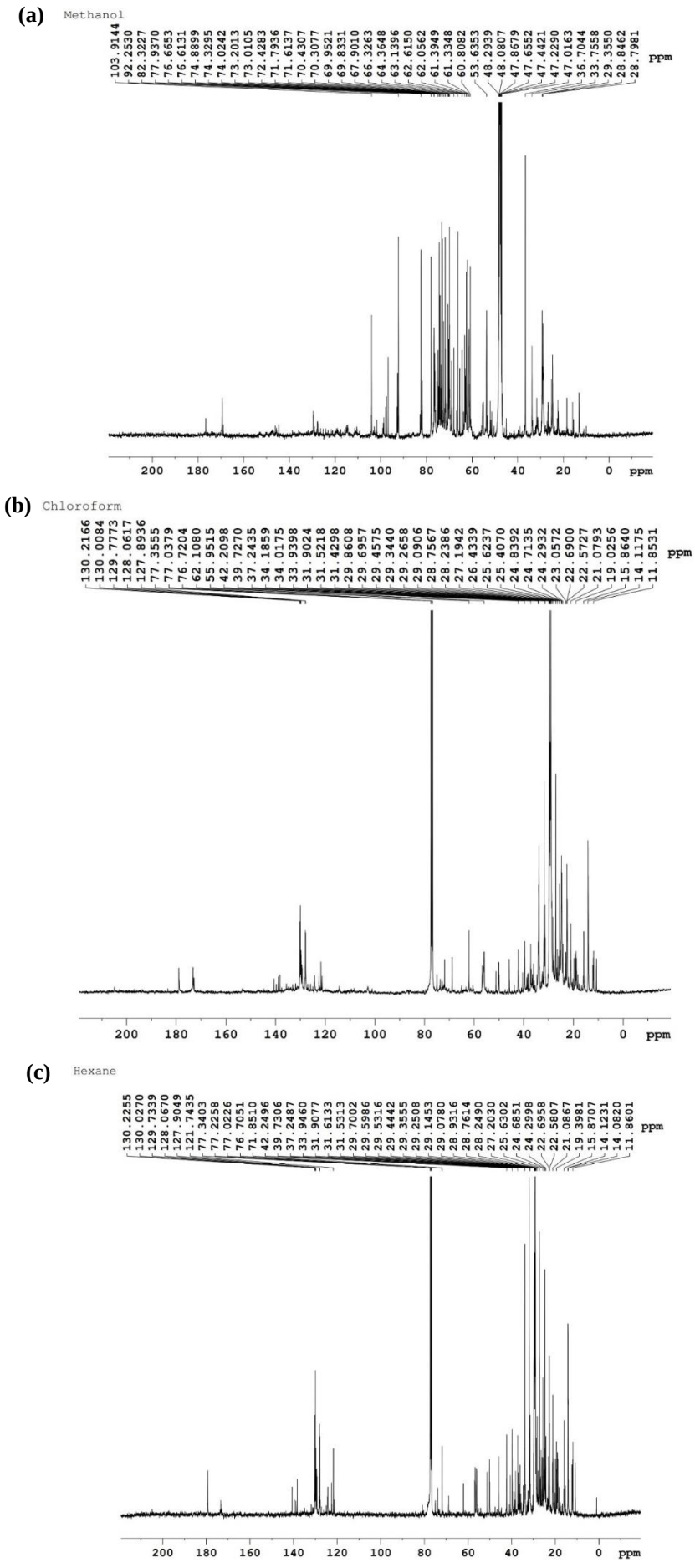

Fig. 3. ^{13}CNMR spectra of Golder root extract components. (a) methanol extract in CD_3OD, (b) chloroform extract in $CDCl_3$, and (c) hexane extract in $CDCl_3$.

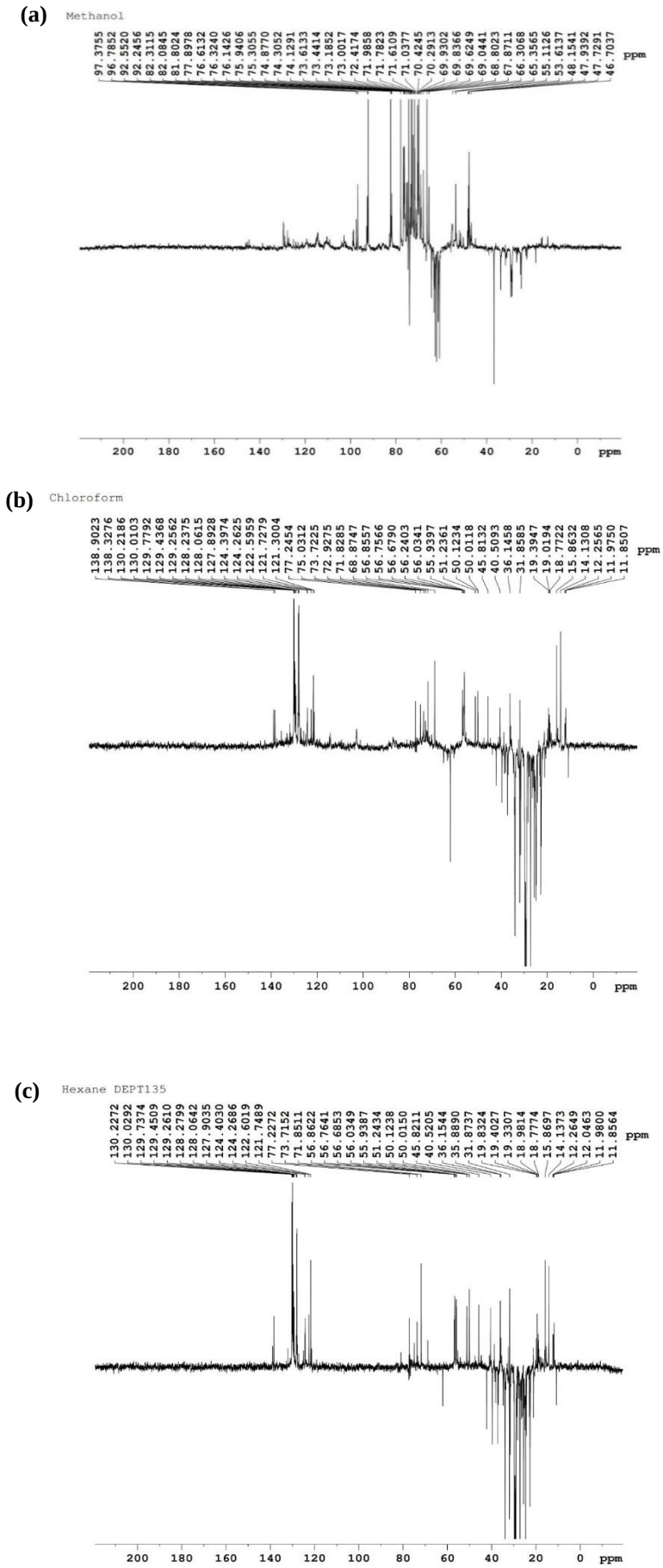

Fig. 4. DEPT135 spectra of Golder root extract components. (a) methanol extract in CD_3OD, (b) chloroform extract in $CDCl_3$, and (c) hexane extract in $CDCl_3$.

Table 2. Identification of functional groups in chloroform-extracted compounds from Golder root using ^{1}HNMR analysis

NO.	Compounds	Chemical formula	Chemical shift (ppm)
1	Methyl proton of triterpenes	$-CH_3$	0.69-0.72
2	All fatty acids (except ω3)	$-CH_2-CH_3$	0.89-0.90
3	All fatty acids (ω3)	$-CH=CH-CH_2-CH_3$	1.01-1.03
4	Methylene main chain	$-(CH_2)_n-$	1.27-1.31
5	Acyl chain, methyl of squalene	$-CH_2-CH_2-COO-$, $-CH=C(CH_3)-$	1.60-1.66
6	Unsaturated fatty acid and squalene	$-CH_2-CH=CH-$, $-CH_2-CH=C(CH_3)-H$	2.01-2.08
7	Unsaturated fatty acid	$-CH_2-COO-$	2.30-2.36
8	Methylene between two olefinic groups	$-CH=CH-CH_2-CH=CH-$	2.75-2.82
9	Alkoxy proton	$-COO-CH_x-$	3.48-4.02
10	Triglycerol methylene	$-CH_2-OCO-$	4.11-4.34
11	Unsaturated proton of squalene	$-CH=C(CH_3)-$	5.10-5.18
12	Triglycerolmethine	-CH-OCO-	5.26-5.30
13	Unsaturated fatty acid	-CH=CH-	5.31-5.46
14	Olefinic protons	-C=CH-	5.48-7.01
15	Aromatic protons	-Ar-H	6.80-8.47
16	Aldehyde and carboxylic acid protons	-CHO, -COOH	9.63-10.16

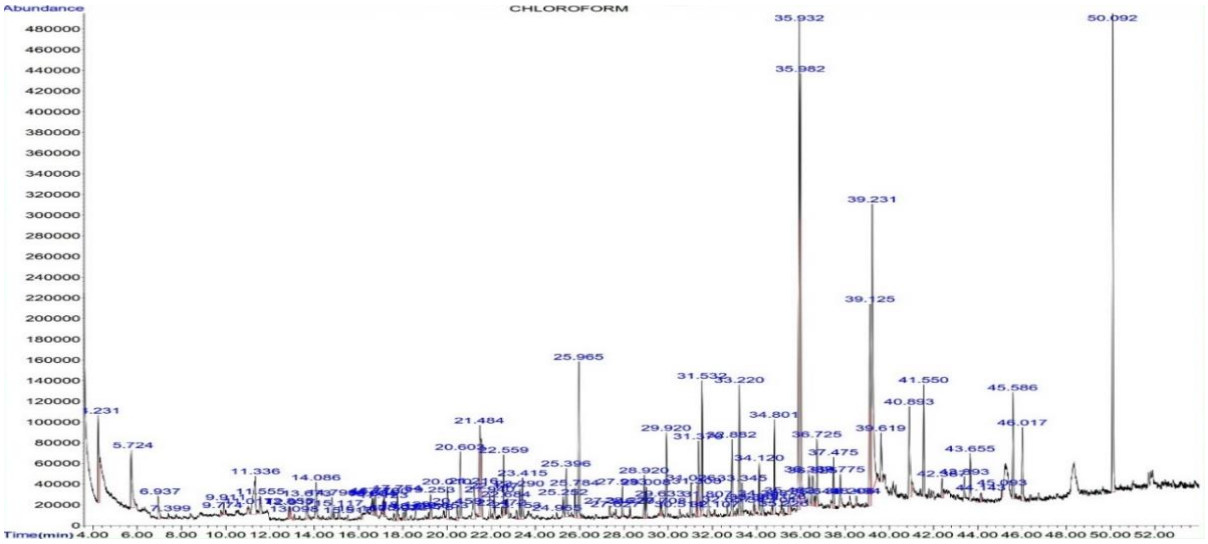

Fig. 6. Chemical composition of chloroform Golder root extract analyzed by GC/MS.

Table 3. Identified compounds of Golder root extracted with chloroform via GC/MS

NO.	Compound	RT (min)	Area (%)	Reference
1	Hexanal	5.724	1.51	(Manandhar et al., 2019)
2	4-hydroxy-4-methyl-2-pentanone	6.937	0.47	(Morteza-Semnani et al., 2005)
3	Hexanoic acid	11.336	1.65	(Guillén & Manzanos, 1999)
4	2-pentylfuran	11.555	0.44	(Miyazawa et al., 2016)
5	Benzyl alcohol	12.917	0.21	(Zhao et al., 2019b)
6	1,5-decalindiol	13.098	0.11	(Ohashi et al., 2023)
7	1-dodecene	13.673	0.44	(Barragan Ferrer et al., 2016)
8	Isophytol	14.798	0.37	(Judzentiene et al., 2015)
9	Pentacosane	15.117	0.21	(Rosselli et al., 2019)
10	5,6,8,9-tetramethoxy-2-methylpepero (3,4,5-jk)-9,10 dihydrophenanthracene	16.685	0.32	(Leong et al., 1997)
11	2-nonenal	16.773	0.32	(Tao et al., 2021)
12	Butyl stearate	17.123	0.18	(Tshilanda et al., 2014)
13	5-decanone	17.160	0.27	(Mekni et al., 2018)
14	3-decanone	17.597	0.16	(Peng et al., 2019)
15	2-decanone	17.754	0.47	(Talavera-Bianchi et al., 2010)
16	4-methyl-3-undecene	18.022	0.16	(Hossain et al., 2011)
17	8-hydroxyoctadecanoic acid methyl ester	18.585	0.14	(Goren et al., 2003)
18	Undecanoic acid	19.853	0.15	(Demirtas et al., 2013)
19	5-undecanone	20.010	0.82	(Mathur & Kamal, 2012)
20	3-undecanone	20.459	0.25	(Afolabi et al., 2022)
21	2-undecanone	20.603	1.02	(Lin et al., 2022)
22	2,4-decadienal	21.216	0.97	(Caboni et al., 2012)

Table 3. Continued

NO.	Compound	RT (min)	Area (%)	Reference
23	7-ethyl-4-nonanone	22.147	0.48	(Saini et al., 2012)
24	Ethyl 2-(oxan-2-yloxy) propanoate	22.472	0.15	(Lucas et al., 2007)
25	6-dodecanone	22.684	0.18	(Aati et al., 2023)
26	3-dodecanone	23.153	0.21	(Tucker et al., 1991)
27	Vanillin	23.415	0.80	(Guillén & Manzanos, 1999)
28	9-oxononanoic acid	25.396	1.04	(Emmanuel et al., 2021)
29	Pentacosane	28.277	0.16	(Guillén & Manzanos, 1999)
30	Isopropyl laurate	28.920	0.68	(Yuan et al., 2012)
31	Benzophenone	29.008	0.53	(Casu et al., 2010)
32	Syringaldehyde	29.708	0.17	(Colaric et al., 2005)
33	3,4-dihydroxymandelic acid ethyl ester	29.920	1.35	(Süntar et al., 2018)
34	Tricosylic acid	30.514	0.14	(Sivanesan et al., 2016)
35	4-hydroxy-2-methoxycinnamaldehyde	31.308	0.49	(Savitskaya & Grinshpan, 2021)
36	Coniferyl alcohol	31.370	1.55	(Son, 2019)
37	1H-indole-2-carboxylic acid	31.532	2.06	(Wang et al., 2018)
38	Myristic acid	31.807	0.42	(Sivanesan et al., 2016)
39	Aristolone	32.107	0.11	(Tian-Shung et al., 2005)
40	Tetracosane	32.657	0.18	(Rosselli et al., 2019)
41	5-hydroxycalamenene	32.882	1.43	(Bett et al., 2016)
42	Isopropyl myristate	33.220	2.08	(Xia et al., 2015)
43	Benzoquinone	33.345	0.53	(Ndontsa et al., 2012)
44	Diisobutyl phthalate	34.120	0.85	(Huang et al., 2021)
45	2,4-dimethyl-2-decene	35.126	0.23	(Pande et al., 2010)
46	Octadecanoic acid, methyl ester	35.226	0.12	(Goren et al., 2003)
47	18-nonadecenoic acid	35.482	0.54	(Praveena et al., 2012)
48	Hexadecanoic acid	35.932	10.28	(Wang et al., 2018)
49	Dibutyl phthalate	35.982	6.50	(Iqbal et al., 2022)
50	7-hydroxycadalene	36.369	0.78	(Thomas et al., 2018)
51	4-ethoxy-2,5-dimethoxybenzaldehyde	36.525	0.73	(Uritu et al., 2018)
52	2-naphthaldehyde, 3-hydroxy-8-isopropyl-5-methyl	36.725	1.22	(Thomas et al., 2018)
53	Acetic acid, octadecyl ester	38.206	0.14	(Zhang et al., 2006)
54	Linoleic acid	39.125	4.56	(Sivanesan et al., 2016)
55	Oleic acid	39.231	8.04	(Sivanesan et al., 2016)
56	Octadecanoic acid	39.619	0.97	(Mamadalieva, et al., 2017)
57	Methyl linolelaidate	40.893	2.16	(Roopa et al., 2020)
58	2,3,6,7-tetramethylanthracene-9,10-dione	41.550	1.91	(Shah et al., 2020)
59	2-propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	42.387	0.42	(Siswadi & Saragih, 2021)
60	4-(p-methoxyphenyl)-3, 4-dimethylnaphthalen1 (4H)-one	43.393	0.33	(Das & Banerji, 1988)
61	Seselin	44.143	0.21	(Tsai et al., 2008)
62	Stigmastrol	45.261	1.48	(Zlatanov & Antova, 2004)
63	Dicyclohexyl phthalate	45.586	1.87	(Sharififar et al., 2007)
64	bis(2-ethylhexyl) phthalate	46.017	1.28	(Huang et al., 2021)
65	β-sitosterol	48.342	1.34	(Wang et al., 2018)
66	Squalene	50.092	8.28	(Guillén & Manzanos, 1999)
67	brassicasterol acetate	51.829	0.44	(Zlatanov & Antova, 2004)
	Total Identification (%)		80.06%	

Cell viability and proliferation

Chloroform GRE at concentrations of 0-800 µg/mL affected malignant colon cancer cells. GRE affected the HCT116, SW480, SW48, and SW742 cellular lines. Following a 48-hour exposure to the GRE, the viability of the cells was significantly reduced upon treatment with the chloroform GRE at a concentration of 400 µg/mL in comparison with the negative control (0 µg /mL) (Fig. 7).

The chloroform GRE exhibited a dose-dependent inhibitory effect on the growth of CRC cell populations. The IC50 values for the HCT116, SW480, SW48, and SW742 cell lines were determined to be 394.9, 563.3, 721.7, and 565.1 µg/mL, respectively. As shown in Fig. 8, GRE-induced cytotoxicity prompted notable morphological changes in the cells, causing them to shift from their typical epithelial shape to a more rounded, oval form. After 48 hours of exposure to GRE, approximately 50% cell viability was observed at a concentration of 400 µg/mL. Notably, further increases in GRE concentration beyond 400 µg/mL did not result in a substantial reduction in cell viability. This plateau in cytotoxic response could be attributed to several potential factors, including the saturation of active sites, specificity of action, or the inherent heterogeneity of cancer cell populations. Tumors and cancer cell lines are composed of diverse subpopulations of cells that may differ in genetic, epigenetic, and molecular functional characteristics. Such heterogeneity can influence the sensitivity of specific cell subpopulations to antiproliferative agents. As a result, some cells within a population may exhibit greater resistance to GRE-induced cytotoxic effects, thereby contributing to the observed limit in cell viability reduction at higher concentrations (Pribluda et al., 2015; Proietto et al., 2023).

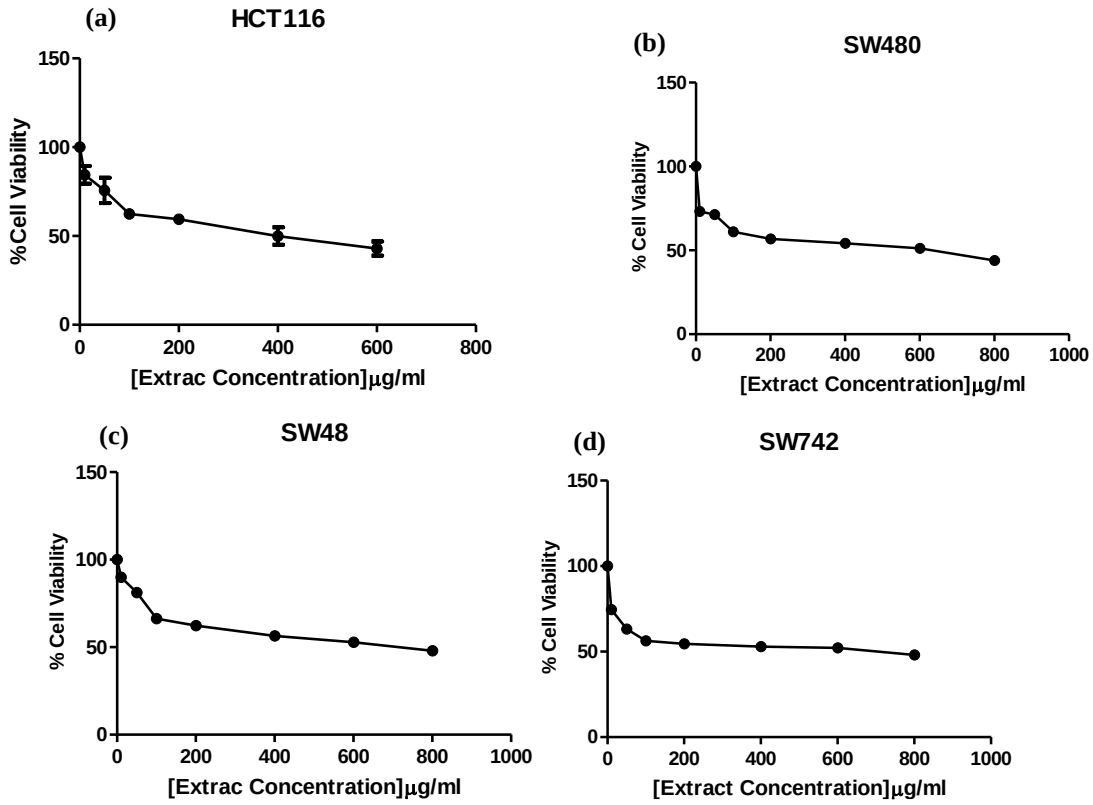

Fig. 7. Inhibitory effect of chloroform Golder root extract on the cell viability of malignant colon cancer cells: (a) HCT116, (b) SW480, (c) SW48, and (d) SW742 cells.

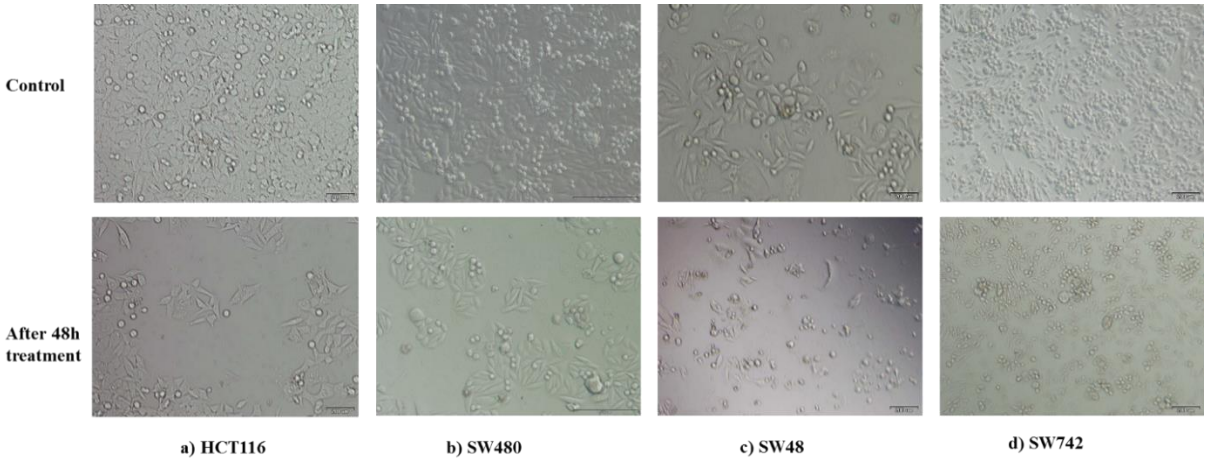

Fig. 8. Effect of chloroform Golder root extract (at a concentration of 400 µg/mL) on the morphology of malignant colon cancer cells after 48 h of exposure. (a) HCT116, (b) SW480, (c) SW48, and (d) SW742 cells. The pictures illustrate that the cell viability decreased by more than 50% after 48 h.

CONCLUSION

This study offers novel insights into the identification of anticancer compounds within GRE and the evaluation of its effects on colon cancer cells for the first time. A unique extraction approach was introduced, employing hexane, chloroform, and methanol as solvents to target non-polar, semi-polar, and polar compounds, respectively. This comprehensive extraction strategy facilitated the identification of bioactive compounds, including those with anticancer potential, using a combination of advanced analytical techniques such as EDX, FTIR, NMR, UV-Vis, and GC/MS spectroscopy. Among the solvents used, chloroform proved to be the most effective for isolating anticancer compounds from Golder root. The chloroform GRE was further tested for its cytotoxic effects on four malignant colon cancer cell lines (HCT116, SW480, SW48, and SW742) using the MTT assay. The results demonstrated significant inhibition of cell proliferation, highlighting the antiproliferative potential of the chloroform GRE. Additionally, morphological changes were observed in fibroblast cells, which shifted from their typical epithelial shape to an oval form, further underscoring the cytotoxic effects of GRE.

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AUTHOR CONTRIBUTIONS

Conceptualization: Zahra Forghani and Mehrdad Niakousari; Methodology: Paul Van der Meeren; Software: Zahra Forghani; Validation: Seyed Mohammad Hashem Hosseini; Formal analysis: Zahra Forghani; Investigation: Zahra Forghani and Mehrdad Niakousari; Data curation: Zahra Forghani; Writing—original draft preparation: Zahra Forghani; Writing—review and editing: Zahra Forghani and Mehrdad Niakousari, Mohamad Taghi Golmakani and Fakhraddin Naghibalhossaini; Supervision: Mehrdad Niakousari, Seyed Mohammad Hashem Hosseini and Paul Van der Meeren; Funding acquisition: Mehrdad Niakousari.

DECLARATION OF COMPETING INTEREST

The authors declare that they do not have any conflict of interest.

ETHICAL STATEMENT

This article has not undergone clinical trials or testing on humans or animals.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

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