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Characterization of new pyrone-containing flavones from *Helichrysum petiolare* Hilliard & B.L. Burtt

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ABSTRACT

Helichrysum petiolare Hilliard & B.L. Burtt. (Asteraceae), native to the Cape Floral Region of South Africa, is traditionally used in the management of wounds, respiratory complaints, and diabetes. Although extensive studies on its extracts, few have focused on the bioactivity of the individual phytochemicals. In this study, chromatographic processing of the *H. petiolare* leaf total extract led to the isolation and identification of ten compounds **C1–C10**) using NMR, FTIR, and HRESIMS spectroscopy. Two pyrone-containing flavones (**C1** and **C2**) are reported for the first time from natural sources. The known compounds identified included flavonoids (**C3–C5**), phenolics (**C6–C9**), and a sterol (**C10**). The MTT assay revealed dose-dependent cytotoxicity of **C1–C4** only against MDA-MB-231 (breast cancer), HeLa (cervical cancer), and HEK-293 (non-cancerous kidney) cell lines after 24 h exposure. Notably, **C4** demonstrated selective activity toward HeLa cells (45 % viability reduction at 100 μM; $IC_{50} = 89.19$ μg/mL). This is the first report of compounds **C1–C10** from *H. petiolare* and their in vitro cytotoxic profile. Despite moderate activity, this work provides a foundation for further pharmacological investigations of derivatives of these metabolites in cancer management.

1. Introduction

The Cape Floral Region, located in the southwestern part of South Africa's Western Cape province, is recognized as one of the world's richest areas in terms of plant biodiversity and endemism (Grobler and Cowling, 2021). Its distinctive Mediterranean-type climate supports a highly specialized and diverse flora. *Helichrysum petiolare* Hilliard & B.L. Burtt., for example, belongs to the genus *Helichrysum* (Asteraceae), which represents over 245 indigenous species, many of which are endemic to the Cape region (Hilliard and Leistner, 1983; Lourens et al., 2004). This perennial shrub typically grows 0.5 to 1 m in height and spreads to approximately 1 m in width, with leaves densely covered in silver-grey hairs that give it a distinctive appearance (Goldblatt and Manning, 2000). In traditional medicine, *H. petiolare* is extensively used to treat various ailments. The leaves are commonly applied in wound healing (Serabele et al., 2021; Scott et al., 2004), managing respiratory conditions (Scott et al., 2004), and treating diabetes (Lourens et al., 2008). The plant's bioactivities have been previously investigated. Lourens et al. (Odeyemi and Bradley, 2018), reported that a chloroform-methanol (1:1) extract from the aerial parts exhibited moderate cytotoxicity effects against breast cancer (MCF-7), glioblastoma (SF-268), and transformed human kidney epithelial (Graham) cells at 0.1 mg/mL concentration. Aladejana et al. (Lourens et al., 2011) demonstrated cytotoxicity effects of the whole plant ethanolic extract on L6 myocytes cells and HepG2 (C3A) hepatocytes at 100 μg/mL concentration. The genotoxic and cytotoxic effects of the methanolic extract on Vero cells, B16F10, and MeWo skin melanoma cells were also reported by Sagbo and Otang-Mbeng (Aladejana et al., 2021). In addition, the plant has shown antidiabetic (Lourens et al., 2011; Sagbo and Otang-Mbeng, 2020), antibacterial (Odeyemi and Bradley, 2018;

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Akinyede et al., 2022; Aladejana et al., 2020), antioxidant (Rosa et al., 2017; Adewinogo et al., 2022), and anti-tyrosinase activities (Adewinogo et al., 2022). Although extensive studies have investigated the extracts of *H. petiolare*, very few have focused on the bioactivity of the individual phytochemicals. Nevertheless, several compounds have been isolated from the plant including flavonoids with an unsubstituted B-ring (Bohlmann et al., 1978). One such compound, 7,8-dimethoxy-5--hydroxyflavone, has demonstrated antiviral activity with an IC_{50} value of 2.32 μg/mL against MDCK epithelial cells (Wu et al., 2010). Other classes of compounds identified from the plant include simple sesquiterpenes such as spathulenol, ledol, α-humulene, and caryophylle oxide (Akinyede et al., 2022; Bohlmann et al., 1978; Bohlmann and Suwita, 1979; Bohlmann and Mahanta, 1979; Jakupovic et al., 1976; Jakupovic et al., 1989), which have demonstrated antioxidant (do Nascimento et al., 2018), anti-inflammatory (Chavan et al., 2010), and anticancer (Legault and Pichette, 2007). Recently, Kubínová et al. (Kubínová et al., 2021) reported that dicaffeoylquinic acid isolated from the aerial parts of the plant displayed moderate antibacterial activity against *Escherichia coli* at a concentration of 512 μg/mL. Additionally, pinostrobin chalcone was reported by Mohammed et al. (Mohammed et al., 2019) to exhibit cytotoxic effects against HT-29 colon cancer cells ($IC_{50} = 22.51$ μg/mL) and MDA-MB-231 breast cancer cells ($IC_{50} = 20.42$ μg/mL) cancer cells. Therefore, this study investigates the cytotoxic effects of leaf-derived metabolites isolated from *H. petiolare*.

2. Materials and methods

2.1. Plant material

Helichrysum petiolare were collected on 31 August 2018 from Kirstenbosch National Botanical Gardens, South Africa, Cape Town (−33° 59′ 13.19″ S, 18° 25′ 29.39″ E) and identified by the curator of the Herbarium.

2.2. Extraction of H. petiolared leaves

Air-dried and powdered H. petiolare leaves (264 g) were soaked in 80 % methanol (3 L) and kept at room temperature for 48 h (x3). Combined filtrates were concentrated at 45 °C under vacuo and freeze-dried. A total of 39.0 g of freeze-dried material was dispersed in water and sequentially partitioned with hexane (**H**), dichloromethane (**DCM**), ethyl acetate (**EtOAc**), and butanol (**BuOH**). The resulting fractions were dried by evaporation and kept in the fridge until further analysis.

2.3. Isolation of the metabolites

The **H-extract** (3.42 g) was chromatographed on silica gel and eluted with a H:EtOAc gradient (100:0→0:100, v/v), yielding five main fractions. Compound **C5** (36.0 mg) was isolated as white crystals from fractions **H-5** of the main column. While **C4** (15.7 mg) was obtained from fractions **H-4** by flash silica gel column using isocratic chloroform: EtOAc (90:10, v/v). Fractions **H-3** afforded **C6** (32.0 mg) following further purification by silica gel column using gradient H:EtOAc (100: 0 → 70:30, v/v). The **DCM-extract** (1.80 g) was fractionated using a chloroform:EtOAc gradient (100: 0→50: 50, v/v), resulting in the collection of seven main fractions. Compounds **C1** (44.0 mg) and **C3** (9.1 mg) were obtained as yellow crystals from fractions **DCM-2** and **DCM-3**, respectively. Fraction **DCM-2** was further purified by flash silica gel column using isocratic chloroform:EtOAc elution (50: 50) to yield **C2** (24.0 mg). The **EtOAc-extract** (1.30 g) yielded **C10** (50.9 mg) as clear crystals from fraction **EtOAc-2**, eluted with an EtOAc:methanol gradient (100:0→50:50, v/v). The **BuOH-extract** (6.90 g) was fractionated with a DCM:methanol gradient (100:0→50:50, v/v), producing eight main fractions. Fraction **BuOH-8** was subjected repeatedly to Sephadex LH-20 column with isocratic ethanol:water elution (90:10, v/v) to afford **C7** (32.0 mg). Fractions **BuOH-2** and **BuOH-4** were purified by a silica gel column using a DCM:methanol gradient (95:5 →90:10, v/v) and isocratic EtOAc:methanol (90:10, v/v), yielding **C8** (36.0 mg) and **C9** (14.2 mg), respectively.

2.4. Spectroscopic characterization

2.4.1. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra (^{1}H, ^{13}C, DEPT-135, COSY, HSQC, and HMBC) were recorded on a Bruker Avance 400 MHz NMR spectrometer (Bruker, Rheinstetten, Germany) at 400 MHz (^{1}H) and 100 MHz (^{13}C). Samples (5 mg/mL in $CDCl_3$ or CD_3OD or DMSO-d_6) were analyzed in 5 mm NMR tubes. Chemical shifts (δ) are reported in parts per million (ppm) relative to the internal standard TMS, while coupling constants (*J*) in Hz.

2.4.2. Liquid chromatography-mass spectrometry (LC-MS)

High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) was utilized to analyze the purified compounds using a Waters Synapt G2 mass spectrometer equipped with a BEH C18 column (2.1 × 100 mm, 1.7 μm; Waters). The instrument was operated at a cone voltage of 15 V, and data were collected in both positive and negative ionization modes via direct injection.

2.4.3. Fourier transform infrared (FTIR) spectroscopy

Attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectra were recorded on an Agilent 5500 Series compact FTIR spectrometer (Agilent Technologies, USA) over the 400–4000 cm^{-1} range.

2.5. Human cell lines and culturing for cytotoxicity bioassay

MDA-MB-231 (breast cancer), HeLa (cervical cancer), and HEK-293 (human embryonic kidney) cell lines were cultured in 25cm^2 flasks using Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, South Africa) supplemented with 10 % fetal bovine serum and 1 % penicillin (100 IU/mL)–streptomycin (100 μg/mL). Cells were incubated at 37 °C in a humidified atmosphere under 5 % CO_2.

2.5.1. Cytotoxicity effects on MTT assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described in (Wisitpongpun et al., 2020), with minor modifications. Initial cytotoxic screening was performed at 0.1, 10, and 100 μg/mL concentrations. Compounds showing activity were further evaluated at 12.5, 25, 50, and 100 μg/mL. Cells (5 × 10^3 cells/well) were seeded in 96-well polystyrene plates with 100 μL of complete medium and incubated for 24 h before compound treatment. After 24 h exposure, 10 μL of MTT was added and plates were incubated for 4 h at 37 °C. The Medium was removed, formazan crystals were dissolved in 200 μL DMSO, and absorbance was measured at 570 nm using a Labtech LT-4000 microplate reader (Lasec, Cape Town, South Africa). Viability was reported as a percentage of untreated controls and calculated using Eq. (1):

$$Percentage\ (\%)\ viability = \frac{Absorbance\ (sample)}{Absorbance\ (control)} x100 \quad (1)$$

2.6. Statistical testing

All data were recorded and analyzed using MedCalc (v19, MedCalc Software, Mariakerke, Belgium) and GraphPad Prism (v8.4.3, GraphPad Software, San Diego, USA). Normality was assessed, followed by an independent *t*-test, repeated measures ANOVA, or one-way ANOVA. A p-value of < 0.05 was considered statistically significant. Dose-response curves and IC_{50} values were calculated using GraphPad.

3. Results and discussion

3.1. Chemical characterization

Ten constitutes (**C1–C10**) were isolated from the air-dried and powdered *H. petiolare* leaves. **C1** and **C2** are new (Fig. 1). In-depth interpretation of the spectroscopic data allowed their identification (Table 1, supplementary). For the known compounds (**C3–C10**), identification was based on side-by-side evaluation of their spectral data (supplementary) against those documented in existing scientific reports.

C1 was isolated as yellow needle-like crystals. The HR-ESI-MS data (supplementary) revealed a molecular ion peak $[M–H]^-$ at *m/z* 449.1232 (calculated for 450.1232, $C_{25}H_{22}O_8$). Notable fragmentation ions (supplementary) include the *m/z* 283.0605 and *m/z* 268.0375, which are due to the loss of $C_9H_{13}O_3$ and CH_3 units, respectively. These fragment ions serve as diagnostic markers in its molecular profiling. The IR and UV data (supplementary) showed evidence of hydroxyl groups and conjugated carbonyl systems. Analysis of the ^{1}H NMR spectrum (Table 1) of **C1** showed well-defined signals at δ_H: 1.05 (3H, *t, J* = 7.5 Hz, H-8"), 1.86 (3H, *s*, H-9"), and 2.43 (2H, *q, J* = 7.5 Hz, H-7"). The splitting pattern of these signals was characteristic of the 6"-ethyl-4"-hydroxy-5"-methyl-α-pyrone moiety, which is common in *Helichrysum* species (Bohlmann et al., 1978). Further, the aromatic region featured resonances at δ_H 7.57 (3H, H-3′/4′/5′) and 8.10 (2H, *brd, J* = 6.6, H-2`/6`), suggestive of a free B-ring framework (Bohlmann et al., 1978; Akaberi et al., 2019). Additional singlets signals at δ_H 12.65 (1H, *s*, 5-OH), 6.22 (1H, *s*, H-6) and 3.80 (5H, *s*, 3-OM/ H-10`) supported the structural assignment.

The ^{13}C NMR and DEPT-135 spectra displayed twenty-five carbon signals, which included methyls groups at δ_C 10.2 (C-9"), 11.9 (C-8"), and 60.0 (C-3, OMe), methylene carbons at δ_C 17.8 (C-10"), and 24.1 (C-7"), six methines at δ_C 98.8 (C-6), 128.9 (C-2′/4′), 129.1 (C-3′/5′), and 131.3 (C-4′). Fourteen quaternary carbon peaks were also present (Table 1), including a de-shielded resonance at δ_C 164.3 that was assigned to C-2". The NMR data of **C1** align with those attributed to obstusifolin, a reference compound previously isolated from *Pseudognaphalium obtusifolium* (Hänsel et al., 1970), except for a variation in the substitution motif of the flavonoid framework. Specifically, **C1** bears a methoxy group at C-3, a feature lacking in obstusifolin. Furthermore,

Table 1
^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of **C1** and **C2** in DMSO-d_6.

	C1 (petiolactone A)		C2 (petiolactone B)	
Position	δ_H, *mult.* (*J* in Hz)	δ_C, type	δ_H, *mult.* (*J* in Hz)	δ_C, type
2	–	155.8, C	–	155. 3, C
3	–	138.7, C	–	138.9, C
4	–	178.8, C	–	178.6, C
5	–	159.4, C	–	160.0, C
6	6.22, *s*	98.8, CH	6.24, *s*	98.4, CH
7	–	163.0, C	–	163.6, C
8	–	105.1, C	–	100.3, C
9	–	154.6, C	–	155.4, C
10	–	104.6, C	–	104.7, C
1′	–	130.8, C	–	130.7, C
2′, 6′	8.10, *brd* (6.6)	128.9, CH	8.21, *brd* (6.6)	128.8, CH
3′, 5′	7.57[a], *brt**	129.1, CH	7.56[a], *brt**	129.1, CH
4′	7.57[a], *brt**	131.3, CH	7.56[a], *brt**	131.5, CH
2"	–	164.3, C	–	104.0[a], C
3"	–	100.3, C	–	104.0[a], C
4"	–	166.2, C	–	201.6, C
5"	–	106.6, C	–	107.1, C
6"	–	159.6, C	–	186.4, C
7"	2.43, *q* (7.5)	24.1, CH_2	2.22, *q* (7.7)	21.7, CH_2
8"	1.05, *t* (7.5)	11.9, CH_3	0.75, *t* (7.7)	9.9, CH_3
9"	1.86, *s*	10.2, CH_3	1.41, *s*	5.6, CH_3
10"	3.80[a], *s*	17.8, CH_2	3.21, *d* (13.8) 3.15, *d* (13.8)	29.7, CH_2
5-OH	12.65, *s*	–	12.65, *s*	–
3-OMe	3.80[a], *s*	60.0, CH_3	3.81, *s*	60.0, CH_3

[a] Overlapped signals in the same column. * Multiplicity unresolved due to signal overlapping.

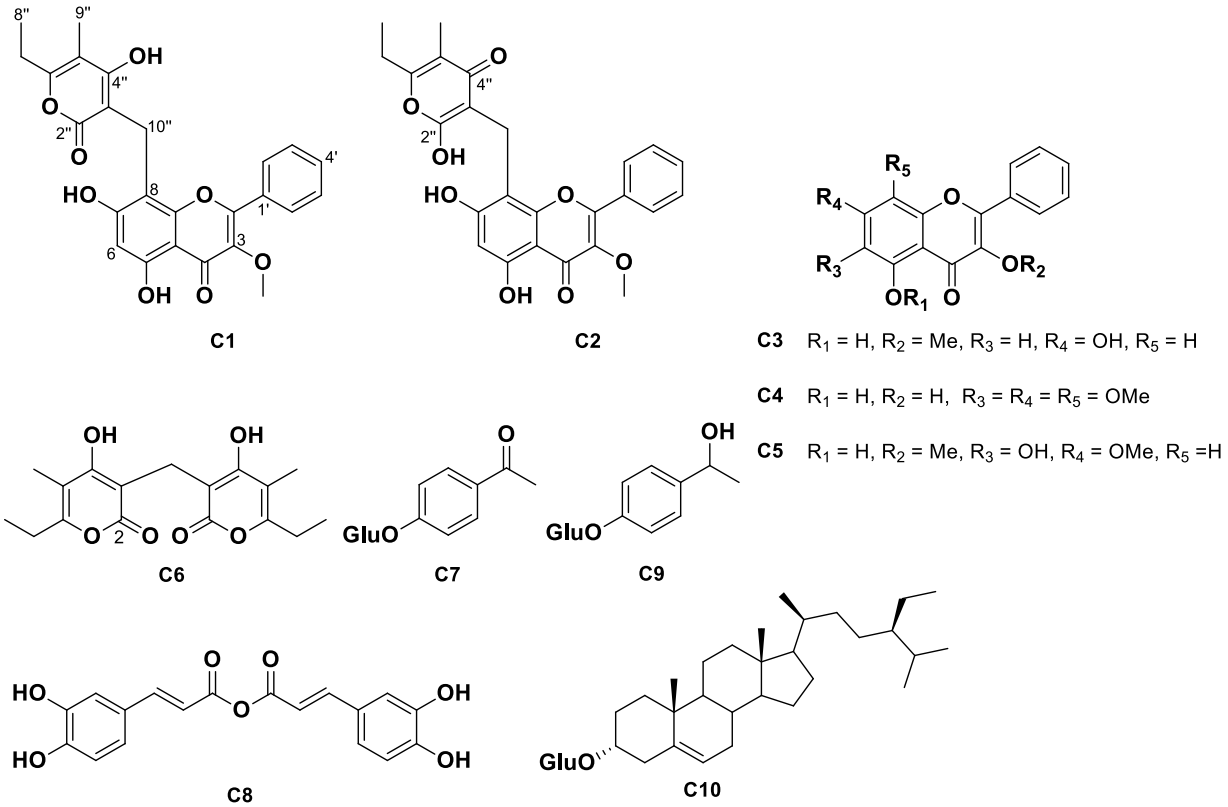

Fig. 1. Molecular structures (**1**–**10**) of the isolated constituents from *H. petiolare*.

extensive interrogation of the HMBC experiment (supplementary, Fig. 2) revealed important correlations (^2J and ^3J), which further assisted with assigning the resonances and supported the attachment of the α-pyrone unit at C-8 of the flavonoid framework. Of importance was the correlation between the methylene at H-10" (δ_H 3.80[a]) and C-3", C-8, C-2", C-4", and C-9, as well as between H-6 (δ_H 6.22) and C-8 and C-10. Therefore, the combined spectroscopic information and comparison to the known compound allowed the correct identification of **C1**. The compound is herein designated as petiolactone A.

C2 was recovered as a yellow amorphous powder. The negative mode HR-ESI-MS spectrum (supplementary) exhibited a fragment ion peak at *m/z* 437.1215 corresponding to $[(M + 2H) - 15]^-$ (calculated for $C_{25}H_{22}O_8$). The UV, IR, ^{1}H, and ^{13}C NMR spectra (supplementary, Table 1) of **C2** showed similarity to those of **C1**. However, the ^{1}H and ^{13}C signals of the pyrone moiety differed significantly. The proton signals appeared upfield at δ_H 0.75 (3H, *t, J* = 7.6 Hz, H-8"), 1.41 (3H, *s*, H-9"), and 2.22 (2H, *q, J* = 7.7 Hz, H-7"). Similarly, the associated carbon signals were observed upfield at δ_C 5.6 (C-9"), 9.9 (C-8"), 21.7 (C-7"), and 29.7 (C-10"). Other key differences include the^{13}C signal of the carbonyl group, which resonated at δ_C 201.6 (this appeared at δ_C 164.3 in **C1**). Additionally, the methylene proton signals at δ_H 3.21 (1H, *d, J* = 13.8 Hz) and 3.15 (1H, *d, J* = 13.7 Hz), which appeared as doublets (these were singlets in compound **1**). The above data clearly support the presence of a γ-pyrone (4-pyrone) heterocyclic framework in **C2**. To corroborate this, the HMBC spectroscopy (supplementary, Fig. 2) was employed. Thus, key HMBC correlation includes the methyl protons at H-9" and C-5" (δ_C 107.1), C-4" (201.6), and C-6" (186.4). Other correlations are between the methylene protons H-10" and C-3" (104.0[a]), C-8 (100.3), C-2" (104.0[a]), C-4" (201.6), C-7 (163.6), and C-9 (155.4). Therefore, the structure of **C2** was unequivocally elucidated and is herein designated as **petiolactone B**. Remarkably, the absence of the fragmentation ion with *m/z* 283.0605 in the HRESIMS spectrum of **C2**, which is observed in **C1**, may allude to its structural stability that may result from a hydrogen bonding between the 7-hydroxyl and 4"-carbonyl group, thereby forcing the molecule to adopt a flat configuration. Additionally, the ability to observe the two doublets of the methylene protons (H-10") in the ^{1}H NMR of **C2** (this is a singlet in **C1**) may further support this explanation.

Interrogation of the spectroscopic data (supplementary) of the known compounds **C3–C10** followed by comparison with the available literature allowed for their identification as galangin-3-methyl ether (**C3**) (Xin et al., 2017), 3,5-dihydroxy-6,7,8-trimethoxyflavone (**C4**) (Wollenweber et al., 1993), 5,6-dihydroxy-3,7-dimethoxyflavone (**C5**) (Wollenweber et al., 1993), helipyrone (**C6**) (Ali et al., 1982), caffeic anhydride (**C7**) (Elabbaraa et al., 2014), picein (**C8**) (Dou et al., 2018), *p*-(1-hydroxyethyl)-phenyl-β-d-Glucopyranoside (**C9**) (Socolsky et al., 2008), and β-sitosterol-3-*O*-glucoside (**C10**) (Peshin and Kar, 2017). Additionally, the ^{13}C NMR spectroscopic data for **C5** is provided for the first time.

3.2. Cytotoxicity effects of the isolated compounds

The study investigated the cytotoxic effects of isolated compounds (**C1-C10**) from H. petiolare against MDA-MB-231 (breast cancer), HeLa (cervical cancer), and HEK-293 (non-cancerous kidney) cell lines after 24 h of exposure using the MTT assay. Among them, **C1** displayed a dose-dependent cytotoxic effect against MDA-MB-231 cells, with an IC_{50} value of 107.8 μg/mL over 24 h (Fig. 3). No activity was observed for **C2** – its structural isomer–under the same conditions. Additionally, **C1–C4** induced a statistically significant decrease in cell viability against HeLa cells, with IC_{50} values of 103.5 μg/mL, 132.5 μg/mL, 102 μg/mL, and 89.19 μg/mL, respectively. Notably, **C4** demonstrated selectivity to HeLa cells, while exhibiting no cytotoxicity against HEK-293 cells. Overall, based on the criteria by the National Cancer Institute (NCI) and Geran protocol for evaluating plant-derived anticancer compounds (Geran et al., 1972; Vieira et al., 2007; Rizeq et al., 2020), each compound may be classified as moderately cytotoxic.

Considering the unique structural features of **C1**–**C4**, along with the observed cytotoxicity results, it may be postulated that the position of hydroxyl groups, whether on the pyrone moiety or flavonoid backbone, plays a pivotal role in their bioactivity. Han et al. (Han et al., 2021) previously reported that **C3** displayed moderate cytotoxic activity against several cancer cell lines, with IC_{50} values exceeding 100 μM. Similarly, Thomas et al. (Thomas et al., 2012) found that **C4** demonstrated cytotoxic activity against colon (HCT116), pancreatic (MIA PaCa), and breast (SK-BR3) cancer cells, with EC_{50} values of 37.50, 33.18, and 11.06 μM, respectively. This activity was attributed to the presence of hydroxyl groups at 3- and 5-positions. To the best of our knowledge, this is the first report describing the cytotoxic effects of **C1**–**4** on MDA-MB-231 and HeLa cells.

4. Conclusions

In this study, ten compounds were successfully isolated and characterized from the leaves of H. petiolare, among which two pyrone-containing flavones (**C1** and **C2**) were reported for the first time from natural sources. The ^{13}C NMR data of **C5** was also reported for the first time. Additionally, cytotoxic effects of the compounds were evaluated against MDA-MB-231, HeLa, and HEK-293 cell lines after 24 h of exposure using the MTT assay. It was found that **C1** induced the cell viability of both MDA-MB-231 and HeLa cells at 100 μM over 24 h but also exhibited cytotoxicity to HEK-293 cells at the same concentration. Notably, **C4** demonstrated selective activity toward HeLa cells, while showing no toxicity against HEK-293 cells. Overall, the compounds may

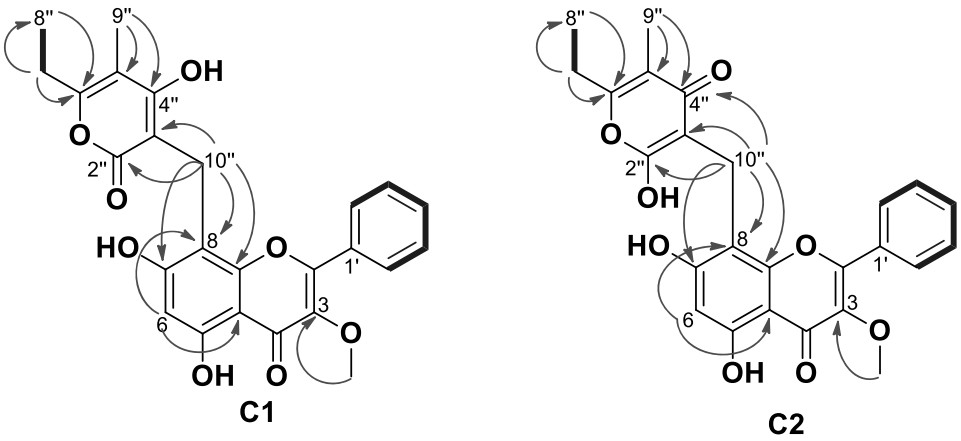

Fig. 2. Key COSY (blue bonds) and HMBC (red arrows) correlations for **C1** and **C2.**

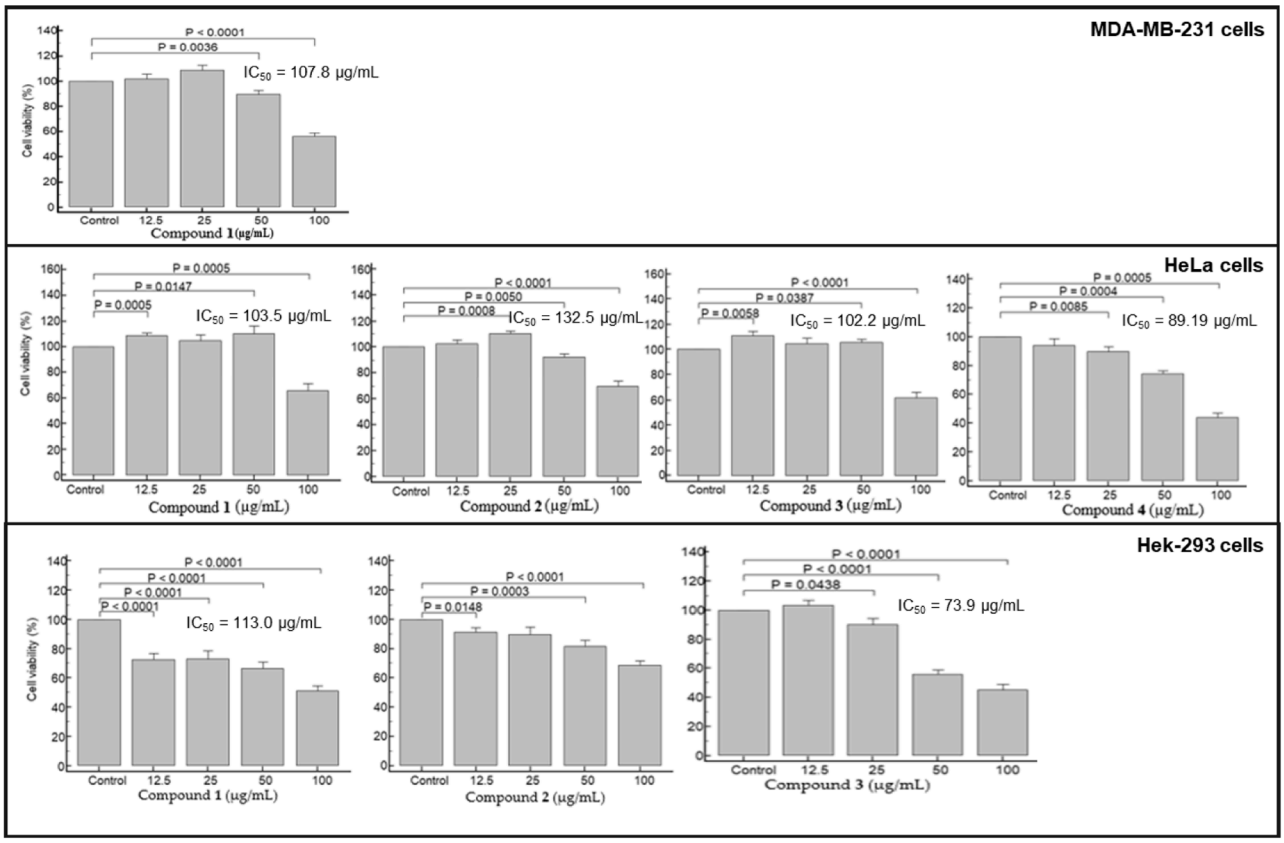

Fig. 3. Cell viability of MDA-MB-231, HeLa, and Hek-293 cell lines treated with **C1–4** for 24 h, as measured by MTT assay.

be classified as moderately cytotoxic and could serve as pharmacophores scaffolds for the development of anticancer agents targeting breast and cervical cancers. However, further investigations into other types of cancer cells is recommended.

CRediT authorship contribution statement

Masixole Makhaba: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Masande Yalo:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Keenau Pearce:** Methodology, Investigation, Data curation. **Mkhuseli Koki:** Methodology. **Ndikho Nako:** Investigation, Data curation. **Rajan Sharma:** Methodology, Data curation. **Wilfred T. Mabusela:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Mongi Benjeddou:** Resources. **Ahmed A. Hussein:** Writing – review & editing, Supervision, Resources, Project administration.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sajb.2025.09.025.

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