Fractions of *Hoslundia opposita* Vahl and hoslundin induced apoptosis in human cancer cells via mitochondrial-dependent reactive oxygen species (ROS) generation

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**ABSTRACT**

**Background:** Cancer remains one of the leading causalities of several morbidity and mortality with negative impact on global economy due to low workforce and management/treatment cost. A number of conventional therapies have been explored in the management/treatment of cancer including chemotherapeutic intervention, radiotherapy, and surgery. Among these treatment modalities, chemotherapy remains the most popular first line of intervention in management/treatment of cancer, and natural products have been implicated as the major source of antineoplastic agents with phenomenal efficacy. However, current antineoplastic agents suffer from lack of selectivity and specificity necessitating the need for further research in the search for novel anticancer drug molecules.

**Method:** In this present study, the anticancer activity of *Hoslundia opposita* leaves extracts were tested against a number of cell lines including human hepatoma cell line (HepG2), human breast cancer cell lines (MDA-MB-231), intestinal epithelial cell lines (Caco-2), and human keratinocyte HACAT cell lines. A bio-guided fractionation assay and the structural elucidation of the pure isolate (hoslundin) was conducted by 1D and 2D NMR spectroscopy. The cell viability, colony formation, and apoptotic activities were investigated using MTT assay, clonogenic assay, and caspase – 3 and – 7 kits respectively. Flow cytometry was employed in assessing the altered cell cycle expression. The production of the intracellular reactive oxygen species (ROS) levels and the reduction of the mitochondrial membrane potential (MMP) was determined at the cellular level using fluorescent probe dyes dihydro-fluorescin diacetate (DCFH-DA) and tetramethylrhodamin (TMRE), respectively.

**Results:** The *H. opposita* fractions and its pure isolate (hoslundin) demonstrated a potent cytotoxic activity against the tumorigenic cells (HepG2, MDA-MB-231, Caco-2) at concentration ranging from 25 to 100 \(\mu\)g/mL. The inhibition of the colony formation was significantly observed in HepG2 cell lines. More so, the cellular viability of the normal cells (HaCaT) was relatively unchanged in the presence of *H. opposita* fractions and its isolate proving the selectivity of the compounds towards tumourigenic cells. The *H. opposita* fractions and hoslundin exerted their anticancer activity via cell cycle arrest with the accumulation of the DNA content at the S-phase, activation...
1. Introduction

Cancer remains one of the leading causatives of several morbidity and mortality with negative impact on global economy due to low work force and management/treatment cost [1,2]. According to International Agency for research on Cancer, an estimated 10 million cancer-related deaths was projected in 2020 [1]. Several conventional therapies have been explored in the management/treatment of cancer including chemotherapeutic intervention, radiotherapy, and surgery. Among these treatment modalities, chemotherapy remains the most popular first line of intervention in the treatment of cancer, and natural products have been implicated as the main source of antineoplastic agents with phenomenal efficacy [3,4]. The potential of natural plant products in cancer treatment and their aid in the attainment of therapeutic efficacy has gained considerable attention in recent years [5,6].

Compounds such as curcumin, etoposide, camptothecin, and paclitaxel are plant derived antineoplastic agents with efficacious anticancer dispositions including the disruption of tumourigenic cells growth by apoptosis and the inhibition of cancer cells proliferation [7,8].

Hoslundia opposita Vahl (H. opposita) is an herbaceous perennial shrub with characteristically round yellowish or orange berries which belong to the family of Lamieae with different local names in the different regions of Nigeria and have been utilized for the different treatment of various diseases including venereal diseases, herpes, and skin diseases [9-13].

The pharmacological activities of H. opposita have been extensively studied and reported [11,14-20]. Despite the several studies on its therapeutic actions in the treatment of several disease conditions, its mechanistic role as anticancer agent remains unexplored. This is typical of several plants derived compounds as their pharmacological actions are not fully explored [8].

Two major mechanisms are mostly explored in the anticancer activity of antineoplastic agents including apoptosis (i) and cell cycle arrest (ii). The apoptosis ensures DNA integrity while cell cycle modulates the cell growth. Moreover, the disruption of the cell cycle is known to foster tumor cells production and proliferation. Anticancer agents target the aberrant cell cycle thereby inducing tumor cell death by apoptosis. However, current antineoplastic agents suffer from lack of selectivity and specificity in distinguishing between healthy and abnormal cells leading to drug resistance, adverse drug reaction, alopecia, nausea, and aggravation of cancer pathogenesis. This has necessitated the need for further research in the search for ideal antineoplastic agents with high selectivity and specificity against tumor cells without causing damage to healthy cells [3,4]. This study will seek to investigate the molecular mechanisms of Hoslundia opposita Vahl fractions and its pure isolate (hoslunin) as anticancer agents against several mammalian cell lines including human hepatoma cell line (HepG2), human breast cancer cell lines (MDA-MB-231), intestinal epithelial cell lines (Caco-2), and human keratinocyte HACAT cell lines. A bio-guided fractionation and the structural elucidation of the pure isolates (hoslunin) was conducted by 1D and 2D NMR spectroscopy. The cell viability, colony formation, and apoptotic activities were investigated using MTT, clonogenic and caspase 3/7 assays respectively. Flow cytometry was employed in assessing the altered cell cycle expression. The production of the intracellular reactive oxygen species (ROS) levels and the reduction of the mitochondrial membrane potential (MMP) was determined at the cellular level using fluorescent probe dyes dihydro-fluorescin diacetate (DCFH-DA) and tetramethylrhodamin (TMRE), respectively.

2. Materials and methods

2.1. Chemicals and tumourigenic cells

All chemicals and reagents used in this study were HPLC and analytical grade, procured from Sigma-Aldrich. Hoslundia opposita leaves was obtained from Nigeria. Mammalian cell lines including human hepatoma cell line (HepG2), human breast cancer cell lines (MDA-MB-231), intestinal epithelial cell lines (Caco-2), and human keratinocyte HACAT cell lines were supplied by the University of Western Cape, South Africa. The Dulbecco’s modified eagle’s medium (DMEM) with phenyl-red, Dulbecco phosphate-buffer saline (DPBS), the heat-inactivated fetal bovine serum (FBS, 10% (v/v)), the 100 μg/mL-penicillin; 100 unit/mL-streptomycin-ampicillin-B-mixture (PSA) and neutral red cell proliferation reagent (MTT) were purchased from Lonza® Group Ltd, Verviers, Belgium.

2.2. Plant collection and preparation

The leaves of Hoslundia opposita were obtained from the riverside in Agbara town, Ogun State (Nigeria) at a coordinate of 6°29’59.1”N 3°06’07.4”E. Plant material was identified and authenticated at the department of Botany, University of Lagos, Nigeria. Hoslundia opposita leaves were rinsed, air-dried for 20 days at ambient temperature, and blended into a fine powder using a laboratory blender. The powdered Hoslundia opposita (2.329 kg) leaves was macerated in 5 L of 80% methanol in distilled water at ambient temperature for seven days. The extract was filtered using Whatman N0.1 filter paper and the solvent was evaporated by reduced pressure using rotary evaporator at 40 °C to obtain the methanolic crude extract of H. opposita (HO1, 116.4 g).

2.2.1. Fractionation of the crude extracts

Vacuum liquid chromatography (VLC) was performed with silica gel (0.063–0.2 mm mesh) as described by Olugbuyiro et al. [21]. Silica gel was loaded in hexane, and 80.74 g of the methanolic crude extract was dissolved in 4:1 Hexane/Ethyl acetate and mixed with 150 g of silica gel. The mixture was air-dried on filter paper at ambient temperature, and it was loaded into the VLC column. A gradient elution was employed for the mixture fractionation using solvent systems in order of increasing polarity including hexane/ethyl acetate (90:10, 3 L) HO2, (70:30, 8 L)
HO3, (50:50, 8.5 L) HO4, (30:70, 3 L) HO5; ethyl acetate (100%, 4 L) HO6; ethyl acetate/methanol, 50:50, 3 L) HO7 and methanol (100%, 2.5 L) HO8. HO2–7 represents the obtained fractions by various solvent or solvent systems. Fractions HO2–4 were obtained using same solvent system (hexane/ethyl acetate) in increasing polarity ratio. The collected fractions were concentrated under reduced pressure by rotary evaporator, subsequently dried in a desiccator and stored in the freezer at – 4 °C. Cytotoxicity was conducted on the collected fractions. Fractions HO5 and HO6 were combined based on their cytotoxicity towards brine shrimp, human cancer cells and their thin-layer chromatography (TLC) profile.

2.2.2. Phytochemical screening

2.2.2.1. Qualitative and quantitative phytochemical screening. The preliminary qualitative phytochemical screening to test for alkaloids, flavonoids, saponins, tannins, phenols, steroids, cardiac glycosides and reducing sugar was performed on the methanolic crude extract of H. opposita leaves using the method described by Trease et al., Sofowora et al., Obadoni et al. [22,24,26].

The quantification of the secondary metabolites of the methanolic crude extract of H. opposita (HO1) leaves were achieved as described in the literatures [23–26]. Phenols were determined as described by the method of Waterhouse et al., [23]. Terpenoids, tannins, and steroids were determined as described by Sofowora et al., by the spectrophotometric method [24]. Flavonoids, alkaloids, and saponins were determined by methods described by [25,26].

2.2.2.2. Determination of antioxidant activity. The antioxidant activity of the extract were evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity [27], nitric oxide scavenging activity [28], and the reducing power [29], as previously described by Adisa et al. [30]. The total phenolic compounds, total flavonoids and total antioxidant capacity were determined by a modified Folin–Ciocalteu colorimetric method [9], aluminum chloride colorimetric method [31], and the pH differential spectrophotometric method [32], respectively.

2.2.3. Isolation of hoslundin

The purification of the combined extracts (HO5 and HO6) was performed three times by column chromatography, scheme 1. The combined HO5 and HO6 (6.573 g) was subjected to column chromatography packed with silica gel and gradient elution using solvent system utilized in decreasing polarity. Hexane/ethyl acetate (90:10, 0.8 L; 80:20, 0.8 L; 70:30, 1 L; 60:40, 1 l; 1:1, 1.2 L; 40:60, 1 L; 20:80, 1.2 L); hexane/ethyl acetate 1 L; followed by ethyl acetate/methanol (95:5, 0.6 L; 85:15, 0.8 L; 70:30, 0.8 L; 60:40, 0.6 L; 50:50, 0.5 L; 30:70, 0.4 L). Approximately 20 mL of eluents were collected into 566 vials and then pooled together based on their TLC profile to obtain 9 fractions (F1 – F9) which were concentrated under reduced pressure. Based on cytotoxicity and TLC profile, fraction 6 (2.058 g) was selected and subjected to further purification using column chromatography packed with silica gel and solvent system of DCM/EtOAc (30:70, 0.2 L; 10:90, 2 L; 5:95, 0.6 L), followed by DCM 0.6 L, and then DCM/MeOH (98.2; 0.2 L; 95:5, 1.2 L; 90:10,0.8 L; 85:15, 0.4 L; 80:20, 0.6 L; 50:50, 0.6 L; 30:70, 0.4 L). This purification and isolation yielded 719 vials of 5mLs each, grouped based on their TLC profile to give 9 sub-fractions (F6.1 – F6.9). The third column chromatography was carried out on a Sephadex column using 100% methanol as eluent. Fractions were obtained and combined based

![Scheme 1. Schematic diagram illustrating bio-assay guided fractionation of Hoslinda opposita leaves.](image-url)
Each value of the total phenols, total flavonoids, and total antioxidant capacity represent the mean± standard deviation of triplicate replicate values with *, **, *** a, b, c, d, e, f, g are significantly different at P < 0.05 according to ANOVA/turkey’s multiple-comparison tests. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity; TP- Total Phenol; TAC- Total antioxidant capacity; TF-Total Flavonoids.

2.3. Cytotoxic activity

2.3.1. Cell lines and culture conditions

The human hepatoma cell line (HepG2), human breast cancer cell lines (MDA-MB-231), intestinal epithelial cell lines (Caco-2), and human keratinocyte HACAT cell lines were used in this study. The cell lines culturing and maintenance were conducted as described by Omoruyi et al. [36]. A gradient dose of 25–100 µg/mL of H. opposita and Hoslundin were prepared in supplemented media. IC50 [34] values for H. opposita and Hoslundin was obtained from the base study, and this was subsequently used as dose for the apoptotic assays, clonogenic, microscopic assays, cell cycle, reactive oxygen species (ROS), and mitochondrial membrane potentials (MMP) for 48 h.

2.3.2. Cell viability assay

The proliferation of the cells was determined using the colorimetric dye reduction assay [3-(4, 5-dimethylthiazol-2-yl)—2, 5-diphenyl tetrazolium bromide)] (MTT, Sigma-Aldrich). HepG2, MDA-MB-231, HACAT cell lines were seeded at a density of 5000 cells per well, while Caco-2 was seeded at 3000 cells per well in sterile 96 well plates and allowed to attach for 24 h. Followed by the administration of 100 µL (gradient dose) of H. opposita and Hoslundin against the cells whilst leaving the vehicular and static controls untreated with H. opposita and Hoslundin except for the addition of supplemented media or DMSO in supplemented media. The compound was allowed to incubate with cells for 48 h. After the treatment, the cells were washed with 100 µL of PBS and thereafter, fresh supplemented media was added and 10 µL of MTT solution (5 mg/mL) were added and allowed to incubate for 4 h. The media were carefully removed from each cell and 100 µL of DMSO to solubilize the purple formazan crystals added. Optical density (OD) was read at 570 nm using a microplate reader (BMG Labtech Omega® POLAR Star), and the mean cell proliferation was calculated relative to the control. The IC50 was calculated using Graphpad Prism 6 software (Graphpad®) from triplicate replicate measurements [33].

Percentage(%) cell proliferation = Optical density of treated cells × 100%
Optical density of control cells

2.4. Caspase-3 and -7 activities

Caspase 3 and 7 activities were measured on the cancer cells using a Caspase-Glo 3/7® Assay kit (Promega, Madison, WI, USA). The experimental procedure of Caspase 3/7 activity is described as follows: The cells were plated at a concentration of 5000 cells/well in white-walled 96-well plates. Cells were treated with the fraction of their IC50 solution (5 µg/mL) and incubated at ambient temperature for 30 mins. Luminescence was measured at 520 nm using a microplate reader (BMG Labtech Omega® POLAR Star). The mean apoptotic activity was
Fig. 2. Percentage cell viability of cancer (HepG2, Caco-2 and MDA-MB-231) and normal cells (HACAT) treated with selected fractions of Hoslandia opposita leaves and positive control drug doxorubicin (DOX) in increasing concentrations (0–100 µg/mL) for 48 h. A–E show a dose dependent decrease in cell viability of the cancer cells in relative to their control. Although, A–D show selective growth inhibition against cancer and normal cell line of HO1, HO5, HO6, and F-6 compared to the E and F (Hoslundin and DOX). Results show bars with the mean percentage cell viability ± SEM relative to the control of data obtained from triplicate replicate measurements.
2.5. Clonogenic assay

Cancer cells were plated in 6 cm dishes according to their densities and left for 24 h to attach. They were treated using the IC\textsubscript{50} (Table 4) of the fractions of the plants for 48 h. Cells were trypsinised, re-suspended in 2 mL of culture media, counted, and re-seeded at 500 cells per dish (35 mm). The cells were incubated and monitored using the untreated cells for ten days and stained. Cells were washed in PBS, fixed with methanol and glacial acetic acid solution (3:1), fixed cells were stained with 0.5% crystal violet in methanol, and a final wash with PBS and distilled water was done to get a clear image of the dishes. Images of the dishes were taken, and areas covered by colonies were calculated using Image J software [35] and expressed as a percentage of control set to a hundred percent.

2.6. Microscopy assay

To establish the morphological changes related with the treatments on HepG2, Caco-2 and MDA-MB-231 cell lines. The cell lines were plated on 60 mm dishes and left for 24 h to settle. Growth media was changed and cells were treated with the IC\textsubscript{50} \textit{H. opposita} and hoslundin (Table 4) for 48 h, and changes in their morphology were monitored using an inverted light (Olympus, USA) and photographs were taken with Zeiss Axiocam (Germany) camera [36].

2.7. Cell cycle assay

The cells were seeded at their various densities as described above for 24 h and treated with the IC\textsubscript{50} (Table 4) of the fractions and hoslundin for 48 h on each cell lines. After treatment, media from each well-containing cells were transferred to 15 mL conical tubes. Cells were washed with 1 mL PBS and transferred into the corresponding 15 mL conical tubes containing the floating cells. Cells were trypsinised, incubated at 37 °C for 1 min, and washed twice with PBS (transferred into the 15-mL conical tubes) centrifuged at 3000 rpm for 5 mins. Supernatants were discarded, pellets were re-suspended in 2 mL of cold PBS and fixed in 8 mL of ice-cold 70% ethanol. DNA content of samples was determined by flow cytometry. Following fixation, samples were centrifuged at 3000 rpm for 5 mins, the supernatant was removed, and pellets were re-suspended in 500 µL of PBS. Samples were again centrifuged at 3000 rpm for 5 min, the supernatant was removed, the pellets were re-suspended in 300 µL of FxCycle\textsuperscript{™} PI/RNase (Molecular Probes, Life Technologies, UK) for 30 mins. Cell cycle analysis was done using the FACS Calibur flow cytometer (Beckman Coulter, USA), and data were analyzed using the Cell Quest Pro version 5.2.1. [36].
Fig. 4. (A): Clonogenic view (a) of the fractions of *Hoslundia opposita* leaves and hoslundin. Bar charts (b) of clonogenic analysis on Hep G2 cell lines colony formation. Images of the dishes were taken, and areas covered by colonies were calculated using Image J software. Values with *, ** are significantly different ($P \leq 0.05$) according to ANOVA and Tukey’s multiple comparison tests. (B): Clonogenic view (a) of the fractions of *Hoslundia opposita* leaves. Bar charts of clonogenic analysis on Caco-2 cell lines colony formation (b). Images of the dishes were taken, and areas covered by colonies were calculated using Image J software. Values with ***, **** are significantly different ($P \leq 0.05$) according to ANOVA and Tukey’s multiple comparison tests. (C): Clonogenic view (a) of the fractions of *Hoslundia opposita* leaves. Bar charts of clonogenic analysis on MDA-MB-231 cell lines colony formation (b). Images of the dishes were taken, and areas covered by colonies were calculated using Image J software. Values with ***, **** are significantly different ($P \leq 0.05$) according to ANOVA and Tukey’s multiple comparison tests.
2.8. Assessment of intracellular reactive oxygen species (ROS)

Levels of intracellular ROS were determined using the fluorescent probe Dihydro-fluorescin diacetate (DCFH-DA) with some modifications [37]. Briefly, cell lines (5000 cells/well) were seeded into 96 well plates and treated after 24 h with *H. opposita* and hoslundin for 48 h. Control cells were incubated with 250 mM H$_2$O$_2$, as a positive control for 15 mins. ROS activity in cells was determined by staining the cells with 20 µM DCFH-DA in dark at 37 °C for 60 mins, washed once with PBS, and 100 µL of PBS was added to each well, and fluorescence intensity of DCFH-DA was measured using a POLAR Star Omega BMG.

2.9. Measurement of mitochondrial membrane potential (MMP)

Mitochondrial membrane potential (MMP) was determined using the fluorescent dye, Tetramethylrhodamin (TMRE) according to the methods by Zamzami et al. [38]. Cells were seeded at 2000 per well in 96-well plates, incubated for 24 h, and treated with *H. opposita* and hoslundin for 48 h. Cells were treated with carbonyl cyanide m-chlorophenyl hydrazine (CCP) as positive control for 10 mins, supernatant was removed and washed with 100 µL PBS. Cell pellets were re-suspended in 100 µL PBS, and fluorescent intensity was measured at 544 nm using a microplate reader (BMG Labtech Omega® POLAR Star). The percentage mean was calculated relative to the control using
2.10. Statistical analysis

The data generated in this study were statistically analyzed using GraphPad Prism version 6 software and expressed as mean ± standard error of means (SEM) of the triplicate replicate measurements. The one-way analysis of variance (ANOVA) entails the investigation of the significance of difference between the treated (intra) and control groups and values were considered to be statistically significant at $P < 0.05$.

3. Results

The results of phytochemical screening and anticancer activities of Hoslundia opposita (HO) leaves are presented in this section. Phytochemical screening of the methanolic crude extract of H. opposita leaves (Table 1) demonstrated relatively high quantities of secondary metabolites which could be attributed to the choice of solvent(s) employed for the compound extraction. The order of increasing quantitative estimation of extracts are Tannins > alkaloids > phenols and flavonoids ranging between $275.97 \pm 0.28$ mg/mL and $74.88 \pm 0.83$ mg/mL.
However, quantitative estimation of steroid, cardiac glycosides and reducing sugar were within an average of 17.33 ± 0.23.

The activities of the antioxidant capacities were evaluated using the IC\textsubscript{50} of the antioxidant capacities which corresponds to the anticancer fractions that are able to scavenge 50% of the free radicals in the reaction mixture of the plants. Since low IC\textsubscript{50} indicates a strong antioxidant capacity, fractions HO5 (1.53 µg/mL) and HO6 (1.74 µg/mL) showed a strong DPPH scavenging activity compared to other fractions in relative to their positive control ascorbic acid (1.17 µg/mL). This study also showed a trend that fractions HO5 and HO6 compared to other fractions of the plant have the best scavenging activities.

3.1. Identification and spectroscopic analysis of the purified Hoslundin

Hoslundin was identified as a white amorphous solid. The pure isolate (Hoslundin) showed HR-ESIMS m/z of 407.1130 (M + H) which is consistent to the calculated value 407.1131 corresponding to a molecular formula of C\textsubscript{23}H\textsubscript{18}O\textsubscript{7}. The \textsuperscript{1}H NMR spectrum displayed a characteristic de-shielded hydroxyl proton signal at δH 13.02 (s, 1 H, OH-5) followed by the aromatic signals resonating at 7.91 (dd, J = 7.9, 1.7 Hz, 2 H-2'6'), 7.73 (s, 1 H, H-6''), 7.55 (m, 3 H, H-4', 2 H-3'5'), 6.70 (s, 1 H, H-3), 6.59 (s, 1 H, H-8), as well as two methoxy singlets at 3.91 (s, 3 H, OCH\textsubscript{3}-8''), and 3.88 (s, 3 H, OCH\textsubscript{3}-11), and a methyl singlet at 2.38 (s, 3 H, CH\textsubscript{3}-7'). The \textsuperscript{13}C NMR spectrum (Fig. 1) exhibited 21 carbon
resonances, the ketone functionalities at positions 4 and 4′ resonated at δC 182.4 and 173.3, respectively. It also displayed the same chemical environment for C-3, 5′ at δC 129.1 and C-2,6′ at δC 126.3 with higher peak intensities. The methoxy carbon resonances were observed at δC 60.1 (C-8′), and 56.4 (C-11), and up-field is the sp3 hybridized methyl carbon that resonated at δC 14.8 (C-7″), Table 2. The 2D NMR data (HSQC, HMBC, and COSY) confirmed all the 1H–13C and 1H–1H correlations, affirming the assignments.

Preliminary bioassay using brine shrimp and cancer cell lines was employed in selection of the fractions used for further biological activity (data not included). HO1, HO5, HO6, F-6 and positive control drug (Doxorubicin) and these fractions were chosen based on their phenomenal activity informing their evaluation on Caco-2, HepG2 and MDA-MB-231 using a dose dependent study (Fig. 2 A-F) to establish IC50 dose which was subsequently used for other biological assay including caspase – 3 and – 7 activities, clonogenicity, microscopy evaluation, cell cycle assay, intracellular ROS quantification, mitochondrial membrane potential (MMP) test. IC50 represent the minimum inhibitory concentration of the compound in inhibiting at least half of the tumourigenic cells. All the fractions considered showed significant activity against the tumors except for relative innocuous activity against the normal cells (HaCaT) cell line. The compound activity was compared with doxorubicin, and it was found to afford better efficacy using the current cell lines, Fig. 2A-F.

The IC50 data of the compounds are presented in Table 4. The IC50 data of the fractions were found to outperform the doxorubicin with better efficacy and selectivity. Interestingly, the biochemical effector pathway of HO1, HO5, HO6, F-6 and hoslundin induced apoptosis, were measured using caspase 3/7 caspase Glo kit (Promega), Fig. 3. Fractions HO1, HO5, HO6, and F-6 show significant activation of caspases 3/7 in cancer cell lines. However, a significant decrease was observed in F-6 treated cells of HepG2, while the hoslundin showed a significant increase in HepG2. Hence, these results imply apoptotic induction via the caspase cascade pathway.

The ability of cells to form colonies and survive treatment with the tested fractions were carried out using crystal violet dye staining, Fig. 4 A–C. The colonies population of HepG2 and MDA-MB-231 compared to the Caco-2 cell lines were significantly reduced in the presence of H. opposita fractions, Fig. 4A–C. Specifically, the results presented in Fig. 4A–C demonstrated the possibility of fraction of HO to be irreparable including ability to slow down and prolong the growth of colonies formation. However, the most effective cell growth inhibition was observed on the HepG2 cell lines as shown in Fig. 4A.

Typical apoptosis changes were also investigated as shown in Fig. 5A–C in cancer cells lines after 48 h treatment with the Hoslundia opposita leaves fractions. The H. opposita leaves caused changes in the
morphologies of the employed cell lines as indicated by the morphological parameters (roundish, dark spot and inhibition of growth) as observed in Fig. 5 A–C. More so, the changes in growth inhibition by the fractions of *Hoslundia opposita* can be attributed to the induction of apoptotic cell death in the cancer cell lines under study.

To understand the mechanism of the fractions of *HO* leaves mediated cell growth inhibition, cell cycle distribution was determined in cancer cells in Fig. 6 A–C. The DNA content in HepG2 cells were arrested after treatment with the *H. opposita* fractions and Hoslundin. Moreover, *H. opposita* leaves fractions and Hosludin induced a decrease in the percentage of cells in the G1/G0 phase and increase in the S-phase. The fractions did not show the arrest of MDA-MB-231 cell lines as compared to the control.

To determine whether the observed cell death was due to ROS-induced activity and disruption of MMP, treated cells were stained with florescent dyes for 48 h, Fig. 7. Fractions HO5 and HO6 of *H. opposita* leaves significantly increased ROS activity and caused a reduction in the MMP activity in HepG2, Caco-2 and MDA-MB-231 cells. Although, hosludin caused a significant reduction in ROS and increased MMP activity in HepG2 cell lines.

4. Discussion

Natural anticancer compounds from plants are well recognized to be involved in mechanism-oriented targets that disrupt or mitigate other mechanisms that promote cancer cell proliferation and invasiveness [39, 40]. Plant phytochemicals are adjudged to be effective anticancer agents in different cancer types from research findings. For example, flavonoids that belong to the polyphenols class exhibit effective inhibition of angiogenesis, proliferation, and metastasis through activation of apoptosis, important mechanisms of cancer therapy [41,42]. Plant chemicals act as antioxidant, maintaining redox status in cells by inhibiting oxidative stress, especially in pathological conditions such as cancer. Some of the antioxidants at higher concentrations act otherwise and induce reactive oxygen species in cells, a characteristic pro-oxidant property to promote cell death of cancer [43,44].
Fig. 6. (A): (a) Representative flow cytometry profile of HepG2 cells exposed to 48 h treatment and stained with propidium iodide for 30 mins. Different phases of the cycle are represented by blue (G1 phase), orange (S phase), green (G2 phase) and plots are representation of two individual experiments. (b) Bar graphs of HepG2 cells in the different phases of the cell cycle. Each bar represents the mean ± SEM of two repeated experiments, and their level of significance was measured at $P < 0.05$ between the control and the treated cells. The number of cells was expressed as the percentage of the total number of cells analyzed. (B): (a) Representative flow cytometry profile of Caco-2 cells exposed to 48 h treatment and stained with propidium iodide for 30 mins. Different phases of the cycle are represented by blue (G1 phase), orange (S phase), green (G2 phase) and plots are representation of two individual experiments (b) Bar graphs of Caco-2 cells in the different phases of the cell cycle. Each bar represents the mean ± SEM of two repeated experiments, and their level of significance was measured at $P < 0.05$ between the control and the treated cells. The number of cells was expressed as the percentage of the total number of cells analyzed. (C): (a) Representative flow cytometry profile of MDA-MB-231 cells exposed to 48 h treatment and stained with propidium iodide for 30 mins. Different phases of the cycle are represented by blue (G1 phase), orange (S phase), green (G2 phase) and plots are representation of two individual experiments (b) Bar graphs of MDA-MB-231 cells in different phases of the cell cycle. Each bar represents the mean ± SEM of two repeated experiments, and their level of significance was measured at $P < 0.05$ between the control and treated cells. The number of cells was expressed as the percentage of the total number of cells analyzed.
The bio-guided assay employed in this current study is to ensure that undesirable or antagonist of biological responses are excluded, thus improving bioavailability and pharmacokinetic profiles leading to better anticancer agent activity [45,46]. Hoslundin was previously isolated from *Hoslundia opposita* along with other pyrone-substituted flavonoids, and the spectroscopic data presented in Table 3 agrees with the literature reports [17].

The selectivity strategy achieved in this work revealed that Fractions HO1, HO5, HO6, F-6 and Hoslundin showed a significant dose-dependent decrease in cell viability in cancer cell lines, which usually signifies a high cytotoxic effect, Fig. 2, and Table 4. At the same time, it is less toxic in a normal cell line which is in line with other anticancer-related studies [7,47–49]. This study shows that fractions HO1, HO5, HO6, F-6, and the purified bioactive compound, hoslundin showed a relative safety profile compared with standard drug doxorubicin on HACAT, a normal cell line (Fig. 2, and Table 4).

Findings of this study in Fig. 6A–C also indicates that Fractions HO1, HO5, HO6, and F-6 accumulate in the S phase of the cell cycle, which implies the disruption of DNA synthesis and the G1 phase inhibits the progression of damaged DNA in HepG2 and MDA-MB-231 cell lines. In
addition, all the fractions caused cell cycle arrest at the G1 phase as shown in Fig. 6A–C. However, it was noted that only fraction HO1 induced cell cycle arrest in the S phase in the Caco-2 cell line, indicating that the cell cycle could be anticancer agent-specific. The arrest at various stages in the cell cycle that induces apoptosis has been explored as an essential strategy of natural products of crude and purified compounds as anticancer agents [51] where they promote cancer cell death. The study depicts that the apoptosis-inducing potential of the fractions HO1, HO5, HO6, F-6 are attributed to an array of phytochemicals as shown in Table 1. The previous studies indicated that phenolic acid and some specific phytochemicals from natural products have an apoptotic effect on different cancers [50,52].

The apoptotic activity in Fig. 3 is indicative of the activation of caspases – 3 and 7, catalytic proteases from the treatment of HO1, HO5, HO6, F-6, and hoslundin in different cancer cell lines. The apoptotic events were significant and could be mediated intrinsically via mitochondrial transduction pathway. Furthermore, the results in Fig. 5A–C revealed the characteristics morphology of apoptosis showed patterns of cell death with respect to cell shrinkage, chromatin condensation, cell organelle degradation, and protein cleavage during HO1, HO5, HO6, F-6, and hoslundin treatment in the cell lines. The exhibited characteristics of apoptosis could be attributed to pattern of cell death [7,53].

The reactive oxygen species (ROS) mediated apoptotic death denotes manipulations of ROS level by targeting the redox system, Fig. 7. This vital strategy of anticancer agent selectively kills cancer cells thus allows for the survival of the normal cells [54]. For instance, some ROS inducers that target ROS generating system have been implicated in causing toxicity with reported non-bioavailability. As a result, ROS inducers failing therapeutic intention in cancer cells in clinical settings. The mechanism of ROS in causing the death of cancer cells involved
In conclusion, this study successfully identified hoslundin from a bio-guided fractionation assay with characteristic selectivity and sensitivity to ROS generation and MMP disruption in cancer and normal cell lines and specific to HepG2 cells compared with other cell lines used in this study. The results of this study showed guided fractionation assay with characteristic selectivity and sensitivity to ROS generation and MMP disruption in cancer and normal cell lines and specific to HepG2 cells compared with other cell lines used in this study. The results of this study showed guided fractionation assay with characteristic selectivity and sensitivity to ROS generation and MMP disruption in cancer and normal cell lines and specific to HepG2 cells compared with other cell lines used in this study.

The integrity of the cell is synonymous with mitochondrial potential due to the regulation of mitochondrial protein that determines ATP production and ROS. Hence, changes or alterations in membrane potential lead to cell cycle arrest and apoptosis due to impaired metabolic activity as shown in Fig. 5A-C. The results depict that HO1, HO5, HO6, F-6, and hoslundin are inducers of ROS in cancer cell lines which cause reduced membrane mitochondrial potential via activation of mitochondria/cytochrome c signaling pathway as revealed in increase activity of caspases 3/7, hence regarded as potential anti-cancer agents for clinical settings (Scheme 1).

In conclusion, this study successfully identified hoslundin from a bio-guided fractionation assay with characteristic selectivity and sensitivity in cancer and normal cell lines and specific to HepG2 cells compared with other cell lines used in this study. The results of this study showed anti-proliferative activity of hoslundin and fractions of *Hoslundia opposita* leaves.
opposita leaves via mitochondrial-dependent ROS generation resulting in apoptosis. This work revealed that the treatments could be the most sought-after anticancer therapy that may be adopted as tailor-designed drugs specific for particular cancer types. Thus, the in vivo anticancer effect of purified hoslundin and its underlying molecular mechanism is strongly recommended.

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CRediT authorship contribution statement

Abosede Christiana Ajibare: Conceptualization, most plant extraction and its experiment, most cell culture experiment, Writing – original draft, Writing – review & editing, Data analysis, Visualization, Osaretin Albert Taiwo Ebuwei: Conceptualization, Writing – review & editing, Supervision. Rahmat Adetutu Adisa: Conceptualization, Writing – review & editing, Supervision. Margaret Oluwatoyin Sho-fidiya: most plant extraction and its experiment. Joseph A.O. Olugbeyiro: most plant extraction and its experiment. Kolajo Adedamola Akinseye: most cell culture experiment, Writing – original draft, Visualization. Helen Adeola Iyiola: most plant extraction and its experiment. Yusuf Adeyemi Adegkoe: NMR Data analysis, Writing – original draft, Writing – review & editing. Sylvester Ifeanyi Omoruyi: Conceptualization, most cell culture experiment, Writing – review & editing, Okobi Eko Ekpo: Conceptualization, Writing – review & editing, Supervision. All authors have read and agree to the publishing of this manuscript. All data were generated in-house; no paper mill was used. We are accountable for all aspects of this research attesting to integrity and accuracy.

Author’s declaration

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Conflict of interest statement

All authors, therefore, declare that there is no potential or intending conflict of interest regarding this article.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopharma.2022.113475.

References


