Journal of Medicinal Plants Studies
www.PlantsJournal.com

ISSN (E): 2320-3862
ISSN (P): 2394-0530
Impact Factor (RJIF): 5.94
www.plantsjournal.com
JMPS 2025; 13(5): 102-112

Received: 05-05-2025
Accepted: 07-06-2025

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Exploring the antiproliferative activity and bioactive constituents of the petroleum ether fraction from *Syzygium malaccense* peel

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DOI: https://www.doi.org/10.22271/plants.2025.v13.i5b.1938

Abstract
Syzygium malaccense, an underutilized tropical fruit of the *Myrtaceae* family, is recognized for its broad spectrum of biological activities. Despite these promising attributes, its anticancer potential across various human cancer types remains unexplored. This study aimed to evaluate the antiproliferative potential of *S. malaccense* fruit and identify the potential compounds responsible for this effect. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was employed to determine the antiproliferative activity of acetone extracts of the peel and flesh of SM fruit on colon (HCT-15), cervix (HeLa), and prostate (PC-3) cancer cells. Additionally, solvent partitioning of the bioactive acetone peel extract was performed using solvents of different polarities. The most active fraction was further examined for morphological alterations and apoptosis. Compounds identification was performed using gas chromatography-mass spectrometry (GC-MS). The results indicated that the extracts and fractions induced selective inhibition of cell proliferation with a dose-dependent response. The petroleum ether fraction (PEF) derived from the acetone extract of SM peel exhibited the lowest IC_{50} value of 97.47 ± 8.63 µg/mL in the HCT-15 cells. Microscopic examination revealed that HCT-15 cells treated with the PEF fraction displayed pronounced morphological characteristics consistent with apoptosis. Apoptosis induction validated through DNA fragmentation analysis confirmed PEF-induced apoptotic cell death in HCT-15 cells. GC-MS analysis of the PEF fraction identified 20 distinct compounds, with the phenolic compound 3-pentadecylphenol emerging as the major constituent. These findings suggest that SM fruit represents a rich and valuable source of bioactive constituents and can be regarded as a promising candidate for the development of functional foods with chemopreventive potential.

Keywords: *Syzygium malaccense*, Peel, Antiproliferative activity, Petroleum ether fraction, Apoptosis, Bioactive compounds

Introduction
Cancer is a dreadful disease marked by aberrant, unregulated cellular proliferation with the capacity to spread and damage surrounding tissue [1]. In many regions of the world, cancer has overtaken cardiovascular disease as the leading cause of death, accounting for one in eight deaths globally [2]. The GLOBOCAN 2022 database revealed 20 million new cancer cases, 9.7 million cancer deaths annually, and 5-year prevalent cases of 53.5 million. It is projected that by the year 2045, the global cancer burden will rise to 32.6 million new cases, with an estimated 16.9 million deaths attributable to cancer-related causes [3]. Notably, more than two-thirds of these new cases are expected to occur in low- and middle-income countries, which currently experience the lowest cancer survival rates [4].

Colon, prostate, and cervix cancers are among the top 10 cancers with high incidence and mortality rates [3]. More than 30% of cancers are caused by varying environmental and behavioral risk factors, such as alcohol and tobacco use, lack of regular fruit and vegetable consumption, dietary factors, physical inactivity, overweight, obesity, and chronic infections [5]. Currently, chemotherapy is the first treatment approach to treat cancers despite its severe side effects [6]. Numerous clinical studies have highlighted the benefits of combining herbal remedies with conventional treatments, particularly in enhancing the quality of life, immune function, and survival rates of cancer patients [7]. According to the World Cancer Research Fund (WCRF), one of the ways to reduce cancer risk is to maintain a healthy weight throughout life by consuming a balanced diet, and most diets that are protective against cancer are foods of plant origin that contain non-nutritive chemical compounds known as

phytochemicals [2]. Natural compounds are currently seen as potential agents in cancer chemoprevention due to the synergistic protective effects of their bioactive constituents and their ability to target multiple pathways of carcinogenesis [8].

Syzygium malaccense (SM) is an underutilized medicinal plant, and each part has different chemical characteristics and functional effects. The plant's primary product is the fruit, which is valued for its rich nutrients and diverse bioactive compounds with promising biological functions [9]. Its antidiabetic, hypoglycemic, antimicrobial, anti-inflammatory, and antioxidant properties are noteworthy [10]. S. *malaccense* is found in Malaysia, Nigeria, Brazilian forests, and tropical and subtropical regions of the world. Several *in vitro* studies involving the anticancer properties of SM have mainly concentrated on crude extracts with different effects on cancer cells. For example, Rabeta *et al.* [11] showed that SM methanolic extract had anticancer activity against hormone-dependent breast cancer cell line MCF-7 (IC_{50}: 632.3 µg/mL) but not against non-hormone-dependent breast cancer cell line MDA-MB-231. Vuolo *et al.* [12] reported that the ethanolic extract of SM peel inhibited HepG2 cells with an IC_{50} value of 40.92 mg/mL. At a concentration of 2 mg/mL, the aqueous extract of the fruit did not exhibit any measurable IC_{50} value against CP-H460 cells [13]. Also, the dried peel and aqueous extract of *S. malaccense* showed 38% and 16.08% reduced cell viability on HT-29 cells at 1 mg/mL [14].

Extracts from various plant parts might affect cancer cells in diverse ways [7]. Omojufehinsi *et al.* [15] observed that aqueous SM flesh extract exhibited an anticancer effect against prostate cancer (PC-3) cells (IC_{50}: 522.124 µg/mL) and its aqueous peel extract against colon cancer (HCT-15) cells (IC_{50}: 837.59 µg/mL). The anticancer activity of SM may be caused by its unidentified bioactive compounds and may also be impacted by the part of the plant used, the solvent, and the extraction type. Bioactive compounds in plants have been extracted and isolated using a variety of solvents, and the class of compounds extracted or isolated is highly dependent on the solvent used [16].

Bioactive compounds in plants have been extracted and isolated using a wide range of solvents, with the nature and class of compounds obtained significantly influenced by the characteristics of the solvent used. Fractionation of the SM crude extracts would aid in identifying the compounds responsible for its antiproliferative activity. Thus, this study aimed to evaluate the antiproliferative activity of the crude extract and fractions of *S. malaccense* fruit and to identify the compounds responsible for its effect.

Material and Methods

Materials

The cell lines used in this study (HCT-15, HeLa, PC-3, and PNT-2) were obtained from the Department of Clinical Pathology, Noguchi Memorial Institute for Medical Research, Ghana. All chemicals and reagents employed were of analytical grade and sourced from Sigma-Aldrich (St. Louis, USA).

Sample preparation

Fresh, ripe *S. malaccense* fruits were obtained from home gardens in Isefun, Ayobo, Lagos State, Nigeria. The fruits were rinsed with water and separated manually into peels, flesh, and seeds. The fruit peel and flesh were dried separately at ambient temperature to a constant weight, and the seeds were discarded. The dried materials were then pulverized using an electric mill and stored in airtight bags at room temperature until use.

Extraction of *S. malaccense* peel and flesh

As described in a recently published paper [17], the dried peel and flesh of S. *malaccense* were extracted separately with 70% acetone. The homogenates were filtered and concentrated to dryness using a rotary evaporator and lyophilization. The extracts were preserved at -20 °C until further analysis.

Fractionation of *S. malaccense* acetone peel extract by liquid-liquid fractionation

The acetone peel extract of S. *malaccense* was subjected to liquid-liquid fractionation. Briefly, 20.93 g of acetone peel extract was dissolved in 100 mL of hydroethanolic solution (1:1, v/v), poured into a separating funnel, allowed to equilibrate, and slowly fractionated with petroleum ether and subsequently with ethyl acetate to produce distinct fractions with varying polarities: petroleum ether fraction (PEF), ethyl acetate fraction (EAF), and hydroethanolic fraction (HEF). Fractionation was conducted in triplicate. The fractions were concentrated using rotary evaporation, while the remaining aqueous phase of HEF was freeze-dried. They were stored at −20 °C until further use [18]. The fraction yield was calculated using the following formula:

$$\%\ Yield = \frac{Weight\ of\ fraction}{Weight\ of\ acetone\ peel} \times 100$$

Cell viability assay

The antiproliferative activity of the *S. malaccense* extracts and fractions was evaluated using the tetrazolium-based colorimetric assay. (MTT) [19]. The assay relies on the ability of viable cells to reduce the yellow MTT tetrazolium compound to an insoluble purple formazan precipitate, which is solubilized in acidified isopropanol to yield a homogenous solution. Absorbance measurement was done at 570 nm to depict the number of viable cells [20]. Viable HCT-15, HeLa, PC-3, and PNT-2 cells were seeded in triplicate into 96-well plates at a density of 1×10^4 cells per well (100 µL/well) in RPMI 1640 culture medium supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and penicillin-streptomycin antibiotic solution. The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were treated with varying concentrations (0-1000 µg/mL) of the extracts and fractions, as well as with the positive control, curcumin (2-38 µg/mL), all diluted in 1% DMSO, and incubated for 72 hours. Wells containing cells treated with 1% DMSO alone served as the negative control. Following the 72-hour incubation period, 20 µL of MTT solution (2.5 mg/mL in PBS) was added to each well, and the plates were further incubated for 4 hours. The reaction was stopped by the addition of 150 µL of acidified isopropanol to each well. The plates were then incubated in the dark at room temperature overnight to ensure complete solubilization of the formazan crystals. Absorbance was measured at 570 nm using a fluorescence microplate reader, from which percentage cell viabilities were determined using the described formula.

$$Cell\ viability\ = Mean\ absorbance\left(\frac{treated\ cell - blank}{untreated\ cell - blank}\right) \times 100$$

The concentrations of extracts or fractions required to reduce cell viability by 50% (IC_{50}) were derived from the generated dose-response curves. The extract/fraction exhibiting the lowest IC_{50} value was selected for subsequent analysis.

Morphological analysis

Morphological changes in HCT-15 cells following treatment with the selected fraction were examined using a phase-contrast microscope (Zeiss Primo Vert, Zeiss, Germany) and a high-content fluorescence imaging system (Axio Vert.A1, Zeiss AG, Oberkochen, Germany). Viable HCT-15 cells were seeded at a density of 1×10^6 cells in 6 mL of culture medium in sterile Petri dishes and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air for 24 hours to allow the formation of a confluent monolayer. Subsequently, the cells were treated with the selected fraction at concentrations of 50 µg/mL, 100 µg/mL, and 200 µg/mL and incubated for an additional 24 hours. The cells were immediately visualized using a phase contrast microscope.

For fluorescence microscopy, following 24 hours of incubation with the selected fraction, the treated cells were harvested by centrifugation at 1000 rpm for 5 minutes, and the supernatant was discarded. The resulting cell pellets were resuspended in 1 mL of phosphate-buffered saline (PBS), transferred to 1.5 mL Eppendorf tubes, and centrifuged again under the same conditions. The pellets were subsequently fixed with 200 µL of 1% glutaraldehyde and incubated at room temperature in the dark for 30 minutes. After fixation, the glutaraldehyde solution was discarded, and 50 µL of PBS along with 5 µL of Hoechst dye solution were added to each tube. The mixture was gently mixed to ensure even distribution of the stain prior to fluorescence imaging [21].

DNA fragmentation and agarose gel electrophoresis

DNA fragmentation analysis and agarose gel electrophoresis were performed based on the protocol outlined by Uto *et al.* [21]. HCT-15 cells were seeded in sterile Petri dishes at a density of 1×10^6 cells in 6 mL of culture medium and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air for 24 hours to facilitate monolayer formation. After incubation, the cells were treated with the selected fraction at concentrations of 50 µg/mL, 100 µg/mL, and 200 µg/mL and incubated for an additional 24 hours. Curcumin served as the positive control, while a 1% ethanolic solution was used as the negative control. Following treatment, the cells were rinsed with ice-cold phosphate-buffered saline (PBS) and collected by centrifugation. The resulting cell pellets were resuspended in a lysis buffer composed of 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 1% Triton X-100. Following centrifugation, the supernatant was discarded, and the cell pellets were incubated with RNase A (1 mg/mL) at 50 °C for 30 minutes. Next, the cells were treated with proteinase K (0.1 mg/mL) and incubated overnight at 50 °C. DNA was extracted and separated on a 2% agarose gel, followed by staining with ethidium bromide. The DNA bands were visualized under ultraviolet light.

Phytocompound identification by Gas chromatography-mass spectrometry (GC-MS)

The selected fraction was analyzed using an optimized gas chromatography-mass spectrometry (GC-MS) method, with slight modifications according to the method described by Haron *et al.* [7]. The fraction was dissolved in acetonitrile at a 1:20 (w/v) ratio, filtered through a 0.45 µm polytetrafluoroethylene (PTFE) membrane filter, and subsequently injected into the GCMS-QP2010SE system (SHIMADZU, JAPAN). An Agilent DB-5MS capillary column (30 m × 250 mm × 0.25 µm) was used for seperation. The injector was set to 325 °C in split mode (with a 25:1 ratio) and a 1 µL injection volume. The oven temperature was initially set at 70 °C for 2 minutes, then increased at a rate of 5 °C per minute to a final temperature of 280 °C, where it was held for 5 minutes. Helium was used as the carrier gas at a constant flow rate of 1.2 mL/min. The mass spectrometer was operated in electron impact ionization mode at 70 eV, with mass spectra acquired over a scan range of m/z 40 to 700. Identification of compounds was achieved by comparing the observed retention times and mass spectra with those available in the National Institute of Standards and Technology (NIST) spectral database.

Statistical analysis

The data were analyzed statistically using GraphPad Prism software, version 8.0.2 (GraphPad
Software Inc., San Diego, CA, USA) and expressed as the mean±standard deviation (SD). Multiple comparisons were performed using Dunnett's test to assess differences between the treatment and control/untreated groups. A p-value less than 0.05 was considered statistically significant.

Results

Antiproliferative activity of *S. malaccense* fruit extracts

The anti-proliferative/cytotoxic effects of *S. malaccense* acetone extracts on the viability of HCT-15, HeLa, PC-3, and PNT-2 cells are presented in Fig. 1 and 2. The results demonstrated that the acetone extracts exhibited different cytotoxicity towards the cancer lines. Acetone peel (A_cP) extract exhibited significant anti-proliferative activity against HCT-15 cells, showed low cytotoxicity toward the PC-3 cell line, and did not affect HeLa cells, even at 1000 µg/mL. In contrast, acetone flesh (A_cF) exhibited greater activity against HeLa and PC-3 cell lines but did not affect HCT-15 cells. Based on the IC_{50} values provided in Table 1, A_cP extract inhibited 50% of HCT-15 cell growth at 476.023±27.945 µg/mL but showed IC_{50} values greater than 1000 µg/mL in other cancer cell lines. Similarly, A_cF presented low cytotoxicity against all cancer cell lines with IC_{50} values exceeding 1000 µg/mL but was cytotoxic toward the normal cell line, with an IC_{50} value of 631.538±5.86 µg/mL. A_cP crude extract had an SI value greater than 2.

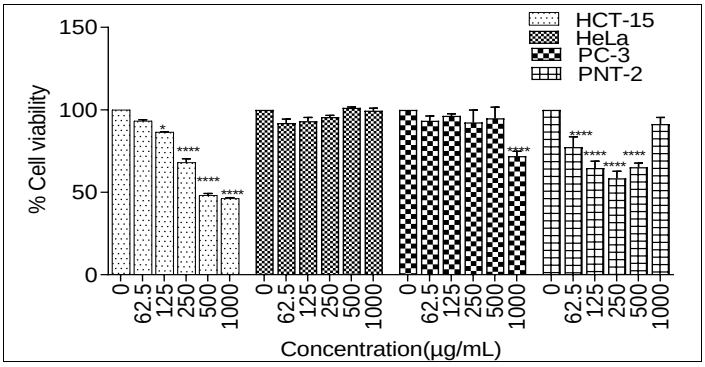


Fig 1: Effect of crude extracts of S. *malaccense* acetone peel on the viability of HCT-15, HeLa, PC3, and PNT-2 cells. Values shown are mean±SD of three different experiments. Compared with negative control, *P ≤ 0.05 and ****P≤ 0.0001

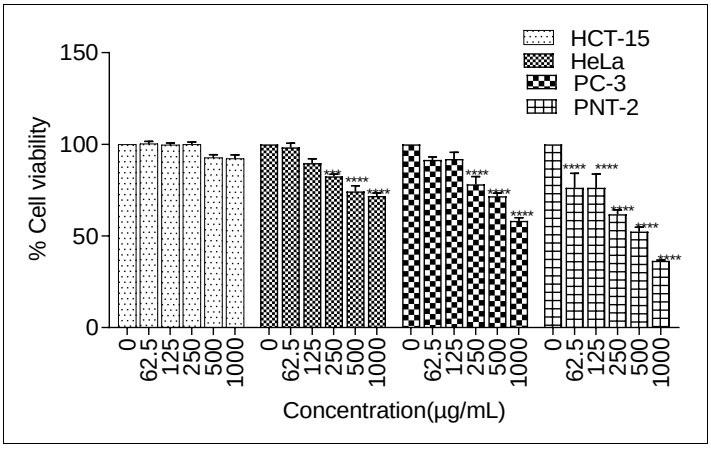


Fig 2: Effect of crude extracts of S. *malaccense* acetone flesh on the viability of HCT-15, HeLa, PC3, and PNT-2 cells. Values shown are mean±SD of three different experiments. Compared with negative control, ***P ≤ 0.0005 and ****P≤ 0.0001

Table 1: Antiproliferative IC_{50} (µg/mL±SD) values and selectivity indices (in parentheses) of acetone extracts of S. *malaccense* peel and flesh on HCT-15, HeLa, PC-3, and PNT-2 cells

Treatment	HCT-15	HeLa	PC-3	PNT-2
A_cP	476.02±27.94 (>2.10)	>1000	>1000	>1000
A_cF	>1000 (<0.63)	>1000 (<0.63)	>1000 (<0.63)	631.54±5.86

Fractions yield

The percentage yield of the fractions from *S. malaccense* acetone peel are presented in Table 2 in increasing order. PEF showed the lowest yield of 6.15% (1.29g) followed by EAF and HEF with values of 27.96% (5.85g) and 64.84% (12.94g) respectively.

Table 2 Percentage yield of the organic solvent fractions of acetone extract of S. *malaccense* peel

Fraction (solvent)	Weight obtained (g)	% Yield of extraction
Petroleum ether	1.29	6.15
Ethyl acetate	5.85	27.96
50% ethanol	12.94	64.84

Antiproliferative activity of the fractions of *S. malaccense* acetone peel

Fig. 3-5 showed the antiproliferative activities of the fractions of *S. malaccense* acetone peel extracts on HCT-15, HeLa, and PC-3 cells. In this study, only the petroleum ether fraction (PEF) exhibited cytotoxic effects across all cancer cell lines, with IC_{50} values of 97.47±8.63, 191.04±7.37, and 100.02±0.75 µg/mL for HCT-15, HeLa, and PC-3 cells, respectively (Table 3). In contrast, no cytotoxic effect was observed for ethyl acetate fraction (EAF) and hydroethanolic fraction (HEF) on any of the tested cell lines, even at a concentration of 1000 µg/mL. PEF was cytotoxic toward the normal cell line, with an IC_{50} value of 113.99±5.16 µg/mL and SI value less than 2. Also, cytotoxicity was not detected in the untreated cell lines (0 µg/mL), and the positive control (curcumin) effectively inhibited cell proliferation across all the treated cell lines.

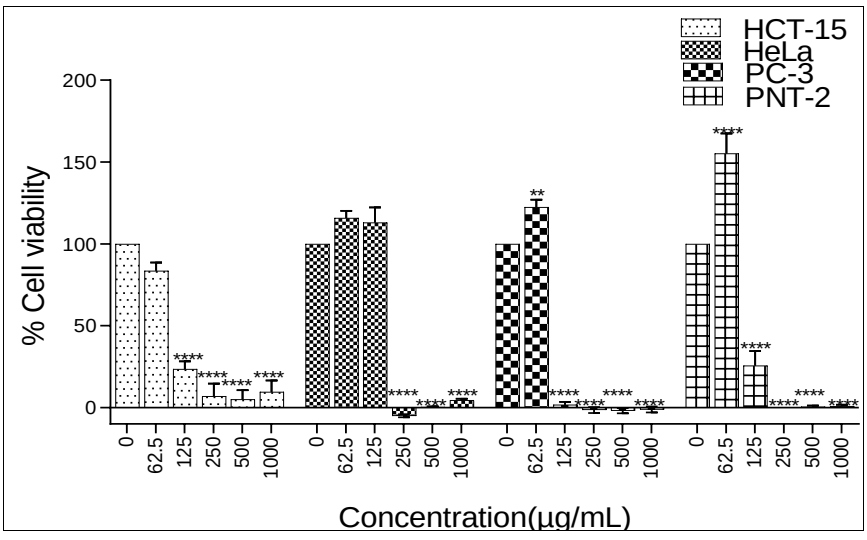


Fig 3: Effect of petroleum ether fraction of S. *malaccense* acetone peel on HCT-15, HeLa, PC3, and PNT-2 cell viability. Values shown are mean±SD of three independent experiments. Compared with negative control, **P ≤ 0.005 and ****P≤ 0.0001

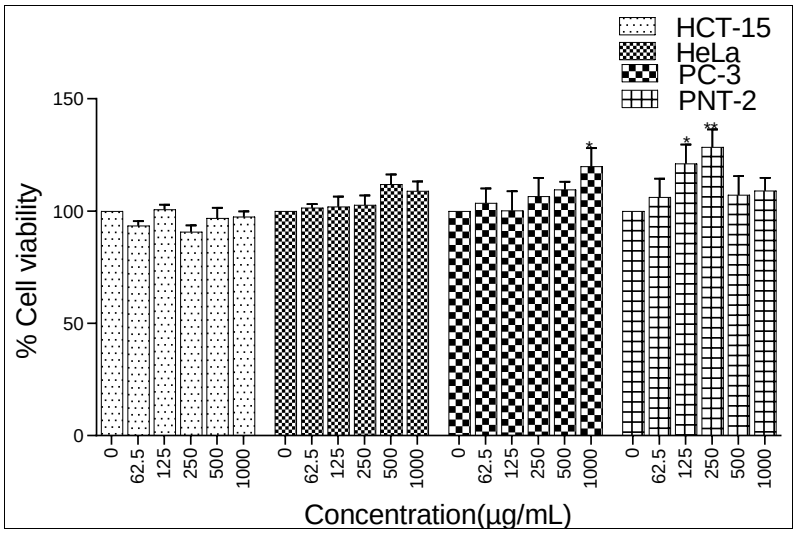


Fig 4: Effect of ethyl acetate fraction of S. *malaccense* acetone peel on HCT-15, HeLa, PC3, and PNT-2 cell viability. Curcumin was used as a positive standard. Values shown are mean±SD of three independent experiments. Compared with negative control, *P ≤ 0.05 and **P ≤ 0.005

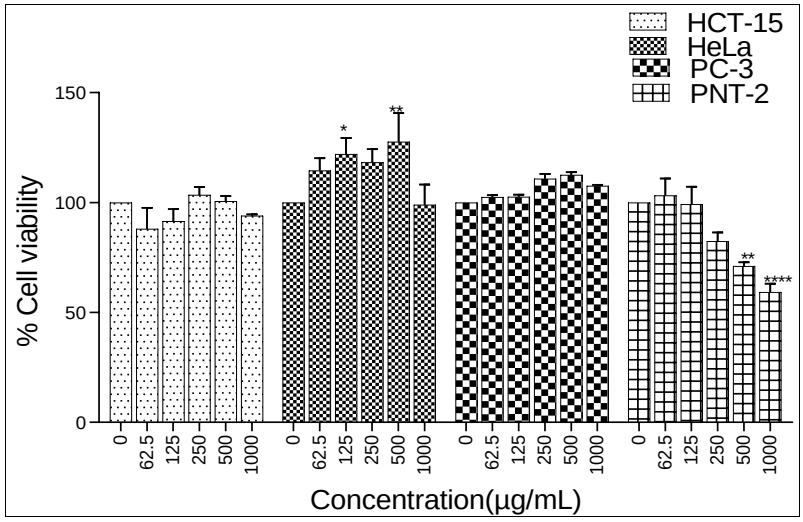


Fig 5: Effect of hydroethanolic fraction of S. *malaccense* acetone peel on HCT-15, HeLa, PC3, and PNT-2 cell viability. Values shown are mean±SD of three independent experiments. Compared with negative control, *P ≤ 0.05, **P ≤ 0.005 and ****P≤ 0.0001

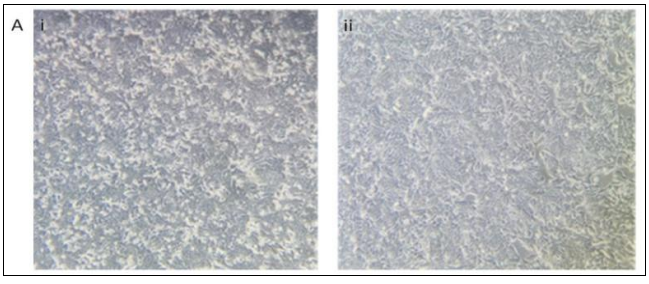
Table 3: Antiproliferative IC_{50} (µg/mL±SD) values and selectivity indices (in parentheses) of fractions of S. *malaccense* acetone peel, and Curcumin on HCT-15, HeLa, PC-3, and PNT-2 cells

Treatment	HCT-15	HeLa	PC-3	PNT-2
PEF	97.47±8.63 (1.17)	191.04±7.37 (0.57)	100.02±0.75 (1.14)	113.99±5.16
EAF	>1000	>1000	>1000	>1000
HEF	>1000	>1000	>1000	>1000
Curcumin	4.30±0.67 (1.25)	4.43±0.04 (1.21)	2.21±0.44 (2.42)	5.87±1.46

Morphological assessment of apoptosis in HCT-15 cells following treatment with PEF of SM

HCT-15 cells treated with 50, 100, and 200 µg/mL of the PEF of SM exhibited decreased cell viability and notable morphological alterations in a dose-dependent manner, compared to untreated cells, as evident from Fig. 6A (i-iv). After 24 hours of treatment with 50 µg/mL of PEF of SM, no visible morphological change was observed, but the cells began to lose adherence. Treatment of cells with 100 µg/mL of SM for 24 hours showed the cells becoming almost non-adherent. Cells exposed to 200 µg/mL of the extract exhibited significant morphological alterations.

Fluorescent microscopy with Hoechst staining showed the induction of apoptosis triggered by the PEF fraction in HCT-15 cells (Fig. 6B i-iv). Untreated HCT-15 cells appeared rounded with large nuclei that were uniformly stained with a vague blue color, as healthy cells typically show restricted Hoechst staining to the chromatin. However, cells treated with 50 µg/mL of the fraction exhibited reduced cell viability with apoptotic body formation, indicating apoptosis induction as visualized by Hoechst staining. Cells treated with 100 µg/mL of the fraction exhibited apoptotic morphological features, showing that apoptosis induction was dose-dependent. At 200 µg/mL, many cells exhibited intense blue fluorescence, with greater intensity than the untreated cells.



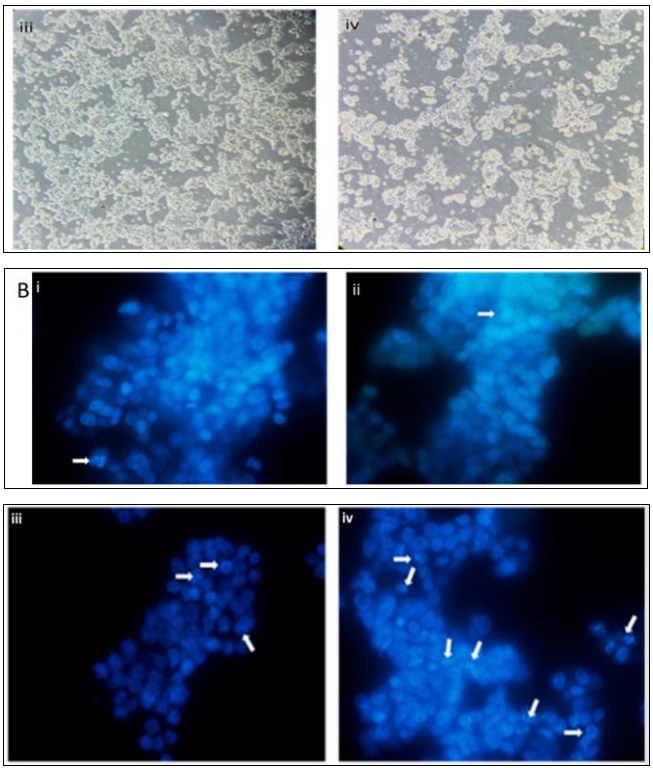


Fig 6: Representative images showing morphological alteration of HCT-15 cells detected by (A) phase contrast microscope and (B) fluorescent microscope: (i) Untreated HCT-15 cells, (ii, iii, iv) HCT-15 cells treated with petroleum ether fraction of SM acetone extract at 50, 100, and 200 µg/mL, respectively.

Induction of DNA fragmentation in HCT-15 cells following treatment with SM PEF

To determine if PEF could induce apoptosis or necrosis, DNA was extracted from HCT-15 cells treated with various concentrations of PEF of SM, electrophoresed, and photographed as depicted in Fig. 7. A DNA ladder pattern was observed in the cells treated with curcumin (positive control). In contrast, DNA from untreated cells showed no signs of fragmentation, neither as a DNA ladder nor as smearing. Cells treated with 50 µg/mL of PEF of SM showed no signs of DNA fragmentation. However, at 100 µg/mL of PEF, a less distinct DNA ladder was seen, with interspersed smearing in the lanes. A mild ladder-like pattern of DNA fragmentation was observed in PEF-treated cells at 200 µg/mL.

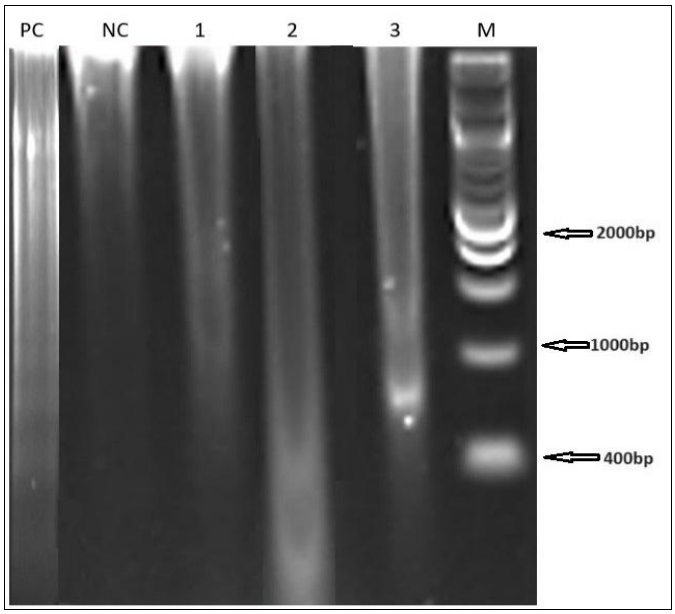


Fig 7: DNA fragmentation pattern. From left: PC (curcumin), NC (untreated cell), 1-3 (treated cells with different concentrations (50, 100, and 200 μg/mL) of PEF of SM, and M (DNA marker).

GC-MS analysis of compounds present in SM PEF

GC-MS analysis was carried out to identify the compounds present in the petroleum ether fraction of *S. malaccense* as shown in Fig. 8. The compounds were identified by matching their mass spectral data with reference spectra in the NIST database, using a minimum similarity index threshold of 80%. Table 4 revealed the presence of 20 distinct compounds in the petroleum ether fraction of *S. malaccense* representing 100% of the total sample. The identified compounds include lipophilic compounds such as terpenes, fatty acids, fatty acid methyl esters, fat-soluble phenolic compounds, phytosterols, fatty acid esters of phytosterols, and fat-soluble vitamins. 3-pentadecylphenol was the predominant component, while hexadecanoic acid, 9,12-Octadecadienoic acid methyl ester, (E, E), trans-13-Octadecenoic acid, and β-sitosterol were present in moderate concentrations.

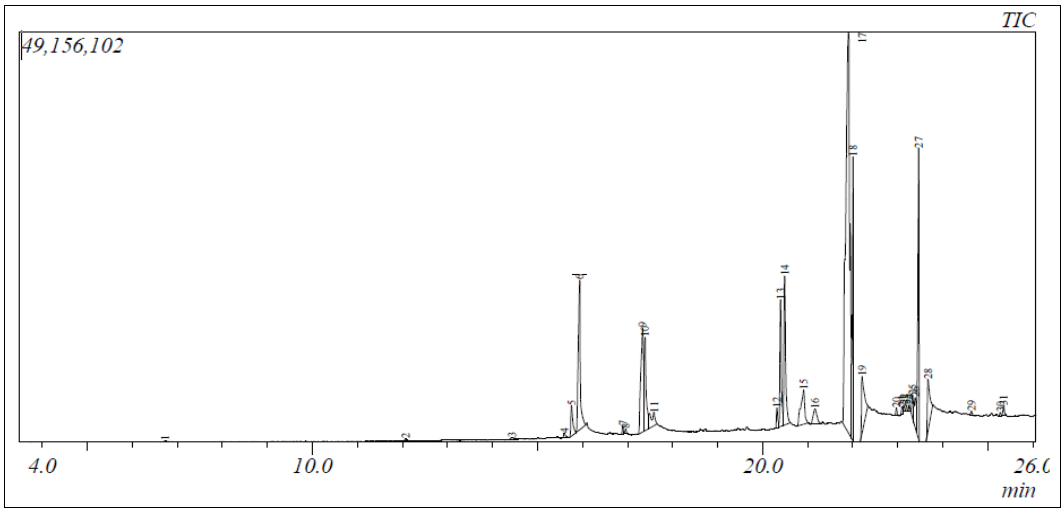


Fig 8: Gas Chromatography-Mass Spectrometry spectrum of the petroleum ether fraction of *S. malaccense*.

Table 4: Chemical composition of the petroleum ether fraction of S. *malaccense* using GC-MS

Peak	Retention Time	Compound Name	Molecular Formula	Molecular Weight (g/mol)	Peak Area (%)	Nature of Compound
1	6.734	D-Limonene	$C_{10}H_{16}$	136.23	0.07	Monoterpene
2	12.070	2,4-bis(1,1-dimethyl ethyl)- phenol	$C_{14}H_{22}O$	206.32	0.12	Phenol
3	14.442	Tetradecanoic acid	$C_{14}H_{28}O_2$	228.37	0.25	Fatty acid
4	15.596	Hexadecanoic acid, methyl ester	$C_{17}H_{34}0_2$	270.5	0.25	Fatty acid methyl ester
5	15.756	Hexadecenoic acid, Z-11-	$C_{16}H_{30}O_2$	254.41	1.46	Fatty acid
6	15.938	n-Hexadecanoic acid	$C_{16}H_{32}0_2$	256.42	7.53	Fatty acid
7	16.894	11,14-Octadecadienoic acid, methyl ester	$C_{19}H_{34}0_2$	294.5	0.24	Fatty acid
8	16.960	trans-13-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296.49	0.15	Fatty acid
9	17.335	9,12-Octadecadienoic acid, methyl ester, (E, E)-	$C_{19}H_{34}0_2$	294.5	6.44	Fatty acid
10	17.389	trans-13-Octadecenoic acid	$C_{18}H_{34}0_2$	282.5	4.59	Fatty acid
11	17.592	Octadecanoic acid	$C_{18}H_{36}0_2$	284.5	1.48	Fatty acid
12	20.319	3-pentadecyl- Phenol	$C_{12}H_{36}O$	304.5	0.65	Phenolic lipid
13	20.398	3-pentadecyl- Phenol	$C_{12}H_{36}O$	304.5	4.39	Phenolic lipid
14	20.489	3-pentadecyl- Phenol	$C_{12}H_{36}O$	304.5	5.87	Phenolic lipid
15	20.911	β-Sitosterol	$C_{29}H_{50}O$	414.7	3.14	Phytosterols
16	21.162	24-propylidene(3β)-Cholest-5-en-3-ol	$C_{30}H_{50}O$	426.7	1.28	Phytosterols
17	21.908	3-pentadecyl- Phenol	$C_{12}H_{36}O$	304.5	38.88	Phenolic lipid
18	22.006	3-pentadecyl- Phenol	$C_{12}H_{36}O$	304.5	5.36	Phenolic lipid
19	22.209	3-pentadecyl- Phenol	$C_{12}H_{36}O$	304.5	3.71	Phenolic lipid
20	22.971	Supraene	$C_{30}H_{50}$	410.7	0.21	Triterpene
21	23.099	1-(9-borabicyclo (3.3.1) non-9-yl)oxy-2-phenyl-ethane	$C_{16}H_{23}BO$	242.2	0.27	Hydrocarbon
22	23.133	1,2-bis (9- borabicyclo (3.3.1) non-9-yloxymethyl)-benzene	$C_{24}H_{36}B_2O_2$	378.2	0.15	Hydrocarbon
23	23.193	3-pentadecyl- Phenol,	$C_{12}H_{36}O$	304.5	0.16	Phenolic lipid
24	23.254	3-pentadecyl- Phenol	$C_{12}H_{36}O$	304.5	0.15	Phenolic lipid
25	23.327	3-pentadecyl- Phenol	$C_{12}H_{36}O$	304.5	0.96	Phenolic lipid
26	23.386	3-pentadecyl- Phenol	$C_{12}H_{36}O$	304.5	1.35	Phenolic lipid
27	23.464	3-pentadecyl- Phenol	$C_{12}H_{36}O$	304.5	7.14	Phenolic lipid
28	23.671	3-pentadecyl- Phenol	$C_{12}H_{36}O$	304.5	3.11	Phenolic lipid
29	24.635	δ-Tocopherol	$C_{28}H_{48}O_2$	416.7	0.18	Fat-soluble vitamin
30	25.276	Stigmast-5-en-3-ol, Oleate	$C_{47}H_{82}O_2$	678	0.11	Fatty acid ester of phytosterols
31	25.349	Vitamin E	$C_{29}H_{50}O_2$	430.7	0.36	Fat-soluble vitamin

Discussion

S. malaccense fruit is a rich source of bioactive constituents with potential applications in dietary strategies for disease prevention and the regulation of oxidative stress [10]. Studies conducted in Malaysia and Brazil have demonstrated the antiproliferative effects of *S. malaccense* on breast and liver cancer cell lines [11, 12]. However, data on the antiproliferative potential of *Syzygium* species native to Africa remain scarce in the global literature [22], and the exact bioactive compounds responsible for the anticancer activity of *S. malaccense* fruit remain unknown [9]. Therefore, *S. malaccense* fruit was studied for its antiproliferative activity on colon, cervix, and prostate cancer cell lines to identify the bioactive compounds.

It is well established that different cell lines can show varying sensitivities to cytotoxic compounds, making it essential to use more than one cell line when screening for bioactive compounds [23]. The three cell lines used in this study originated from different sources, each exhibiting unique morphology and tumorigenic potential [24]. The *in vitro* anti-proliferative effects of the acetone extract of *S. malaccense* peel and flesh were assessed on three cancer cell lines and one normal cell line using the MTT assay. The results showed that A_cP and A_cF extracts exhibited distinct anti-proliferative activities on the cancer cell lines, suggesting that different plant parts may have distinct effects on cancer cells. These findings are consistent with previously observed anticancer effects of aqueous peel extract of *S. malaccense* on HCT-15 cells and aqueous and ethanol extracts of the flesh on HeLa and PC-3 cells [15]. To evaluate the ability of the extracts to target cancer cells, selectivity indices (SI) were calculated from the IC_{50} values of the extracts in the three cancer cell lines. An SI value greater than 2.0 was considered selective for cancer cells with therapeutic potential, while values below 2.0 suggested non-selectivity with potential toxicity [25]. PNT-2, an immortalized human prostate cell line, was used as the normal cell line to determine the SI of the extracts. A_cP crude extract showed selectivity against the normal cells. In contrast, A_cF inhibited PNT-2 cell growth. This indicates that A_cP selectively targeted HCT-15 cells, while A_cF inhibited cancer and normal cells. The low IC_{50} value and selective toxicity of A_cP suggest that HCT-15 cells are more susceptible to the cytotoxic effects of *S. malaccense* peel, thereby showing therapeutic potential against HCT-15. This is consistent with some epidemiological studies that suggest diets abundant in fruits and vegetables may contribute to a reduced incidence of colon cancer due to their phytochemical content [26].

To further concentrate and improve the extract purity while eliminating interfering components, the acetone peel extract was subjected to sequential fractionation with different solvents. The petroleum ether fraction, which had the lowest yield, indicated that *S. malaccense* peel contained a low amount of lipid-soluble compounds. In contrast, hydroethanolic fraction with the highest yield contained a

higher concentration of dissolvable cellular components, soluble fibers, and perhaps some high molecular weight polysaccharides. This result supported previous works which showed that *S. malaccense* peel contains higher carbohydrate, protein, and fibre contents than lipid content [10, 17]. The differences in fraction yields were attributed to the varying polarities of the compounds found in the fruit peel, and similar variations have been documented in the literature for certain medicinal plants [27].

The decision to perform fractionation was informed by previous findings indicating that specific fractions of plant crude extracts may possess greater inhibitory activity compared to the unfractionated extracts [6]. Yadav *et al*. [28] reported that the ethyl acetate fraction of *S. cumini* seeds crude ethanolic extract demonstrated more antiproliferative activity on A2780, MCF7, PC-3, and H460 cancer cell lines than the crude extract. Likewise, the hexane fraction of 80% ethanolic extract from *Moringa oleifera* was found to have enhanced antiproliferative effects against cancer cells [29]. The low IC_{50} value of PEF against HCT-15 cells buttresses the susceptibility of the cell to the cytotoxic effects of *S. malaccense* peel. Hence, the ability of *S.malaccense* fruit to lower the risk of colon cancer. The inactivity of EAF and HEF was attributed to their IC_{50} values exceeding 1000 µg/mL in all cell lines. PEF had the lowest yield but still showed the highest bioactivity. This indicates that higher efficiency in solute extraction is not directly related to the biological activity of the plant. The observed differences in biological activity could be due to the differential solubility of bioactive constituents in the plant. Furthermore, the variations in cell line sensitivity and IC_{50} values observed before and after fractionation may indicate the elimination of inhibitory compounds that counteract antiproliferative effects or the isolation of distinct components with varying activity levels. Thus, the antiproliferative effect of *S.malaccense* differs depending on the compounds present and the cell type. Batista *et al*. [10] reported that *S. malaccense* peel might contain carotenoids and other lipids, warranting the highest lipophilic antioxidant capacity. Interestingly, this study showed that the petroleum ether fraction of *S. malaccense* fruit, which is the lipophilic fraction, exhibited the highest antiproliferative effect against all cell lines tested in this study. Based on the cytotoxicity threshold established by the American National Cancer Institute ($IC_{50} < 30$ µg/mL for crude extracts), neither the crude extracts nor the fractions of *S. malaccense* fruit met the criteria for cytotoxicity [6]. The selectivity index (SI) for PEF was below 2, indicating that its cytotoxic effects were not selective for cancer cells. This is the first study to report the antiproliferative activity of the non-polar bioactive fraction derived from *S. malaccense* fruit.

The induction of apoptosis represents a critical and preferred mechanism through which chemotherapeutics exert their tumoricidal effects. Apoptosis is a highly controlled process essential for regulating physiological homeostasis, frequently occurring in cells actively advancing through the cell cycle. Apoptosis is an energy-dependent process characterized by distinct biochemical and morphological alterations, including nuclear fragmentation, chromatin condensation, membrane blebbing, vesicle formation, loss of intercellular adhesion, cellular shrinkage, and ultimately, DNA fragmentation [30]. By contrast, necrosis is typically triggered by severe toxic stress and characterized by a rapid depletion of ATP levels, damage to intracellular organelles, and the initiation of an acute inflammatory response. The early and late stages of apoptosis can be detected through nuclear staining techniques, and the late-stage apoptosis is typically visualized as a characteristic DNA laddering pattern on standard agarose gel electrophoresis, resulting from internucleosomal DNA fragmentation [31, 32].

Morphological changes induced by the *S. malaccense* petroleum ether fraction are consistent with established hallmarks of apoptosis, supporting its pro-apoptotic activity in treated cells [33]. Although equal cell densities were seeded for both control and treated groups, a significant reduction in cell number was observed in the treated cells. Viable, non-apoptotic cells typically exhibit nuclei with a normal, spherical morphology. Phase contrast microscopy revealed that untreated HCT-15 cells remained adherent, showing an epithelial-like morphology [34]. Treatment of cells with PEF of SM caused a marked change in morphology, loss of adherence to the cell substratum, and cellular shrinkage with membrane blebbing. Fluorescent microscopy with Hoechst staining showed marked nuclear condensation, cell shrinkage, apoptotic bodies, and other alterations of apoptotic cells observed in the HCT-15 cells treated with PEF compared to control cells. This suggested that PEF mediated the induction of apoptosis in the cells and not necrosis. These alterations may likely interfere with normal cellular processes, ultimately resulting in cell death. Similar morphological changes in HCT-15 cells have been reported in response to treatment with various other compounds [34].

DNA fragmentation serves as a crucial biochemical indicator of apoptotic cell death, typically manifesting as a ladder-like arrangement on agarose gel electrophoresis [35]. DNA degradation in terms of DNA laddering observed, with interspersed smearing in the lanes, indicates the presence of post-apoptotic necrotic cells [36]. In addition, other studies have shown the DNA laddering effect of natural products [37, 38]. Therefore, DNA ladder formation indicated that the antiproliferative activity of *S. malaccense* caused inhibition in the growth of HCT-15 cells through apoptosis.

To identify the active compounds responsible for the antiproliferative activity of *S. malaccense*, the petroleum ether fraction was analyzed for the presence of bioactive compounds by GC-MS due to its marked difference in antiproliferative activities. Many of these compounds have been previously identified in *Syzygium* species [9, 39, 40] and are recognized for their anticancer properties. Limonene, a monoterpene, is known for its anticancer properties, as it promotes apoptosis by enhancing pro-apoptotic factors while suppressing anti-apoptotic factors [41, 42]. It has also been shown to effectively inhibit the promotion or progression stage of carcinogenesis in rat mammary tumors [11]. This study is the first to report the presence of D-Limonene in *S. malaccense* fruit, which has been identified in its leaves [43]. 3-pentadecylphenol (Cardanol) is commonly found as a component of a mixture of related phenolic lipids, including anacardic acids, cardols, and/or urushiols. Cardanol, the decarboxylated derivative of anacardic acid, is structurally analogous and exists as a mixture of structurally related phenolic compounds with alkyl side chains of either 15 or 17 carbon atoms. These alkyl chains may be saturated or contain varying degrees of unsaturation, resulting in a complex combination of saturated and unsaturated molecules. The exact combination may depend on the plant species [44], potentially accounting for the presence of multiple distinct peaks observed in the chromatographic analysis. They possess hydrophilic and hydrophobic regions, which facilitate their integration into cellular membranes, leading to structural and functional modifications of biomembranes and influencing the

activity of various membrane proteins [45]. However, despite their chemoprotective capacity, they tend to induce DNA damage [46]. It has previously been identified in *S. jambos* and *S. cumini* [39, 47, 48]. However, to the best of our knowledge, this study validates the presence of cardanol in *S. malaccense*. β-sitosterol is an active phytosterol naturally occurring in plant cell membranes, possessing a chemical structure akin to cholesterol found in mammalian cells. It is a vital micronutrient, widely distributed in higher plants, and is obtained by animals through dietary intake [49]. In its purified form, β-sitosterol has demonstrated anticancer effects against various cancer types, including colon, prostate, leukemia, lung, stomach, breast, and ovarian cancers. Additionally, research in animal models of colon cancer has highlighted its antioxidant properties and chemopreventive potential [50]. It has been reported to be found in *S. aqueum*, *S. cumini*, and *S. jambos* [51, 52]. The compound, 24-propylidene(3β)-Cholest-5-en-3-ol is a bioactive phytosterol known for its antioxidant and antibacterial activity [53]. There is no report about its anticancer properties, but it is found in several plants with such properties, including *Ajuga bracteosa*, *Turbinaria ornata*, *Spondias mombin*, *Bixa Orellana*, and *Rauvolfia serpentina* [54, 55, 56]. Numerous studies have shown that other compounds found in the petroleum ether fraction of S. *malaccense* hold great potential for cancer prevention and treatment. These include 9,12-octadecadienoic acid methyl ester, vitamin E (α-tocopherol), tetradecanoic acid, hexadecenoic acid [53], 2,4-bis(1,1-dimethyl ethyl)-phenol [57], supraene (also called squalene) [58]. δ-tocopherol [59] and stigmast-5-en-3-ol-oleate [60]. Studies have indicated that the health benefits associated with fruit and vegetable consumption can be attributed to the synergistic and cumulative effects of various phytocompounds, which cannot be fully replicated by dietary supplements alone [61]. Thus, the anti-proliferative properties of the petroleum ether fraction of *S. malaccense* fruit may be linked to the presence of 3-pentadecylphenol or in synergy with other bioactive compounds present.

Conclusion

This study is the first to report the antiproliferative activity of the bioactive fraction derived from *S. malaccense* fruit. The findings revealed that the petroleum ether fraction derived from the acetone peel extract exhibited higher antiproliferative activity than the crude extract, thereby highlighting the significance of fractionation in enhancing bioactivity. The findings showed that various extracts and fractions of *S. malaccense* demonstrated distinct cytotoxic effects on cancer cell lines. These differences are likely due to variations in phytochemical composition and the specific mechanisms these compounds act on individual cancer cell lines. Based on cell morphology and apoptosis studies, the antiproliferative activity of *S. malaccense* may be attributed to the most abundant compound, 3-pentadecylphenol, or the synergistic interaction of the compounds present. The findings of this study demonstrate that *S. malaccense* possesses significant potential for development as a functional food and represents a promising candidate for cancer prevention. Additional investigation is required to isolate and characterize the specific bioactive constituents responsible for the observed antiproliferative effects.

Conflict of Interest

The authors declare that they have no competing interests.

Acknowledgement

The corresponding author thank the African-German Network of Excellence in Science (AGNES) for the intra-Africa mobility grant awarded her which funded part of this study.

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