

Research Article

Phytochemical Screening of *Diospyros malabarica* Bark Extracts via GC-MS/MS and Assessment of *In Vitro* Antiproliferative Activity Against Human Colon Carcinoma Cells

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ABSTRACT

Diospyros malabarica bark extract is used in Ayurvedic medicine, particularly to reduce gastrointestinal motility and diarrhoea. This study aims to identify the bioactive compounds of *D. malabarica* using GC-MS/MS and to assess antiproliferative activity against human colon carcinoma cells (HT-29 and HCT-116). The GC-MS/MS chromatogram indicated the presence of 16 phytochemicals. A comprehensive review indicated that 8 bioactives (Hydroquinone(1,4-dihydroxybenzene), 3-Methoxycatechol, 2-Methoxyresorcinol, 2,6-Dimethoxyphenol (Syringol), 1,2,3-Benzenetriol (Pyrogallol), 2,4,6-Trihydroxybenzoic acid, 3,4,5-Trimethoxyphenyl acetate, and Glycerol 1-palmitate) possess anticancer properties. The extract demonstrated dose-dependent *in vitro* antiproliferative effects against HT-29 and HCT-116, with IC₅₀ values of 53 ± 4.2 and 58 ± 5.3 µg/ml, as determined by the MTT assay. The extract increased ROS levels (2.6-fold, $p < 0.01$), altered cellular morphology, and triggered apoptosis, as revealed by fluorescence microscopy. Results indicate that *D. malabarica* generates substantial ROS to promote apoptosis by disrupting the balance between oxidant and antioxidant enzyme systems, thereby shifting the cellular redox balance. Notably, at higher concentrations, *D. malabarica* significantly elevated lactate dehydrogenase (3.2-fold), suggesting late apoptosis and consequent cytotoxicity. Overall, our findings suggest that *D. malabarica* contains strong anticancer agents that exhibit marked antiproliferative and apoptotic activity against human colon carcinoma cells; hence, extracts may offer benefits for gastrointestinal disorders.

Keywords: Antiproliferative Activity, *Diospyros malabarica*, Human Colon Carcinoma Cells, Lactate Dehydrogenase, Reactive Oxygen Species.

INTRODUCTION

Cancer is the second leading cause of death in the United States, with over 1.7 million newly diagnosed cases [1]. Patients receiving chemotherapy and other cancer treatments are at greater risk for treatment-related side effects, such as appetite loss, oral mucositis, nausea, vomiting, dysgeusia, xerostomia, and dysphagia, which can in turn modify dietary habits [2]. Despite these findings, little research has examined the specific interactions between food and beverage intake and the usefulness of traditional medicinal extracts frequently used for digestive and oral

disorders, particularly regarding their effectiveness in cancer treatment or in reducing treatment-related toxicities.

D. malabarica is an evergreen tree with strong antioxidant activity. The various parts of *D. malabarica*, like leaves and fruits, have historically been used to treat high blood pressure and atherosclerosis, owing to their anti-inflammatory and antiproliferative attributes [3]. As a rich source of pharmacologically active bio-constituents, *D. malabarica* could be anticipated for its use as a phytomedicine [4]. It has been reported previously that all organs of this plant,

especially fruits, bark, and leaves, are used in medicinal preparations. Moreover, the leaf extracts are used to treat burning, diabetes, atherosclerosis, and intermittent fever. The extract reflects potent antidiabetic, antimicrobial, anti-inflammatory, and antipyretic activities.

The methanol extract of *D. malabarica* ripe fruits has shown antibacterial, antioxidant, hepatoprotective [5], antidiabetic, and anti-diarrhoeal activities [6]. In addition, the antihyperglycemic, antibacterial, and anti-urolithiatic activities are described [7]. The traditional uses of *D. malabarica* have been reported, particularly for the reduction of gastrointestinal motility and diarrhoea. Further, the alcoholic extract of the stem revealed antidiarrheal and anticancer activities [6, 7].

Recently, *D. malabarica* fruit preparation has been shown to increase the expression of type 1-specific (TH1) cytokines but also augments tumour suppression, modulating various epigenetic markers to evoke tumour protective immunity in non-small cell lung cancer [8]. Another preliminary report on silver nanoparticles prepared from the fruit extract of *D. malabarica* against human primary glioblastoma cells, tested using 10–100 µg/mL, showed an antiproliferative effect at IC₅₀ of 58.63 ± 5.74 µg/ml. Importantly, the extract did not demonstrate any toxicity against normal cells [9, 10].

In our previous publication [11], we demonstrated that the *D. malabarica* stem methanol extract was non-toxic to mice even at a dose of 2000 mg/kg. The doses of 500, 750 and 1000 mg/kg body weight did not cause any mortality or toxic effects in the sub-acute toxicity study on mice. No significant clinical, haematological, or histopathological changes were observed in treated animals versus controls in a 28-day oral sub-acute toxicity study. *D. malabarica* stem bark methanol extract at the given doses did not produce any significant toxic effect and is safe to be considered for use in animal studies and as a constituent of medicinal and food preparations. These findings provide valuable preliminary information on the toxicological profile of *D. malabarica* [11].

Nutrition-conscious consumers are seeking inexpensive plant foods to improve their daily intake of essential nutrients and health-promoting phytochemicals. Therefore, plants with edible fruits, vegetables, and multigrain-containing foods are in demand. Particularly, the underutilised plant parts (such as leaves,

stems and fruits) employed by traditional and tribal societies are playing a significant role [12]. Plants are rich sources of natural compounds with extraordinary therapeutic potential, essential for developing novel medications. *D. malabarica* is one such traditional plant notable for its ethnopharmacological values for treating diseases related to gastrointestinal disorders. The scientific literature did not report much work on the traditional effects of this tree's extracts, particularly against gastrointestinal disorders [13]. In addition, the phytochemicals of the extracts need to be explored for biological activities. It is essential to demonstrate the most ideal plant part as a potent future source of herbal drugs. Therefore, the present study was intended to evaluate the phytochemical contents of the bark methanol extract and to evaluate the antiproliferative properties specifically using human colon cancer cell lines [12].

MATERIALS AND METHODS

Plant Material: Fresh stem bark of *D. malabarica* was collected from the Western Ghats, Chikkamangalore region, Karnataka, India, and was authenticated by Prof. Niranjan Raj S, Chairman and a taxonomist at the Department of Botany, Mukthagangotri, Karnataka State Open University, Mysore-06. A voucher specimen (Herbarium no- KSOU BOTIDM02) was deposited in the department ledger for further reference and records. The stem bark of *D. malabarica* was collected and washed under running tap water to remove soil debris and dirt. The bark were surface-sterilised, air-dried, sliced into small pieces using a knife and hammer, finely powdered, and stored at 4 °C until further use. All the chemicals, reagents and staining dyes used in the present experiment were of analytical grade.

Soxhlet Extraction and Preparation of Extract for Cell Culture Studies:

The powder prepared from *D. malabarica* stem bark was defatted overnight (n-hexane), followed by methanol extraction using the Soxhlet apparatus (Labmatrix, Karnataka, India). The extract was concentrated under reduced pressure at 60 ± 1°C in a rotatory vacuum evaporator (Steroglass, strike 300, Italy) till a solid mass was obtained and refrigerated (4 °C) in an airtight container until further use.

The extract was dissolved in a minimum amount of dimethyl sulfoxide (DMSO) to obtain

a stock solution. Working stocks were prepared in DMEM without fetal bovine serum (FBS), keeping the final concentration of DMSO less than 0.4%. The prepared extract was filter-sterilised through 0.22 μm filters before use [12-15].

GC-MS/MS Analysis: Phytochemical profiling of the methanolic extract of *D. malabarica* was carried out using Gas Chromatography-Mass Spectrometry (GC-MS/MS), using an Agilent 5597C system connected with an HP-5 MS fused silica column (5% phenyl methyl siloxane) with dimensions of 30.0 m $\times$ 250 μm $\times$ 0.25 μm film thickness, interfaced with 5675C Inert MSD with Triple-Axis detector. Pure Helium gas was used as a carrier gas with a flow rate of 0.9 ml/min. Electrospray ionisation mode (70 eV, ion source temperature at 230 $^{\circ}\text{C}$) was used with the quadrupole kept in scanning mode (which ranged from m/z 29 to 500 at a scan rate of 3 scan/s). The solvent wash draw speed and solvent wash dispenser speed were kept at 300 $\mu\text{l}/\text{min}$ and 6000 $\mu\text{l}/\text{min}$, respectively, with a pressure of 7.0481 psi. The quad temperature was kept at 150 $^{\circ}\text{C}$, while the oven temperature initially was held at 80 $^{\circ}\text{C}$ for 1 min, then ramped to 130 $^{\circ}\text{C}$ at the rate is 25 $^{\circ}\text{C}/\text{min}$, which was again ramped to 220 with the rate of 10 $^{\circ}\text{C}/\text{min}$ and finally, it reached the temperature of 280 $^{\circ}\text{C}$ at a rate of 5 $^{\circ}\text{C}/\text{min}$ for the last 6 min. Start and end masses were kept at 30 and 400, respectively, with the threshold of 150 and scan speed of 781 (N=3). Compounds were identified by comparison of their respective mass spectra, retention indices (Kovats index), and above 40% relative abundance of acceptance match criteria with those of standards and by comparing with the NIST mass spectral data system/library. Each compound's molecular formula and weight data were confirmed from the ChemSpider (<http://www.chemspider.com>) and PubChem (<https://pubchem.ncbi.nlm.nih.gov>) databases [12].

Cell Lines and Culture Conditions: Human molecular subtype colorectal cancer cells HT-29 and HCT-116 were kindly provided by Dr. Deepti Saini (Protein Design Private Ltd., Bengaluru). Cells were maintained in DMEM containing 10% FBS and 1% antibiotic solution (100 U/ml penicillin and 100 mg/ml streptomycin) in a humidified atmosphere containing 95% oxygen and 5% CO_2 at 37 $^{\circ}\text{C}$.

Antiproliferative Assay / MTT Assay: The cytotoxicity effect of *D. malabarica* was confirmed through MTT (2,3,5-trimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, cells were plated at a density of 1×10^4 cells/well, incubated overnight, and treated with *D. malabarica* at various concentrations and doxorubicin (positive control, 5 μM) for 24 h. Following the different treatments, MTT solution (20 μl , 5 mg/ml) was added and incubated for another 4 h. The MTT formazan precipitate was then solubilised in 100 μl DMSO. Absorbance was measured at 570 nm using a microplate reader (Tecan Infinite m 200 PRO, Austria). The inhibitory concentration (IC_{50}) value was estimated after 24 h of treatment using GraphPad Prism software [12, 13, 16].

Cell Morphology: The effect of *D. malabarica* on the morphological changes of cells was observed under a microscope. Briefly, cells were seeded in 6-well microplates at a density of 1×10^5 cells/well and incubated overnight. The cells were then treated with *D. malabarica* extracts at their IC_{50} concentration for 24 hr. The changes in morphology were observed under a brightfield microscope [12, 13].

Quantification of Intracellular Reactive Oxygen Species (ROS): Intracellular ROS measurements were carried out using the 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) method. Briefly, cells at a density of 1×10^4 cells/well were incubated overnight and treated with different concentrations of *D. malabarica* for 24 hr. Relative changes in intracellular ROS were monitored by a fluorescent microplate reader at excitation and emission wavelengths of 485 nm and 530 nm, respectively [12, 13, 17].

LDH Release Assay: The released LDH was quantified using an assay-based kit method (agappe). Colorectal cancer cells were treated with *D. malabarica* for 48 h as mentioned above (section). Next, the cell supernatant was mixed with the LDH mixture and incubated in the dark at room temperature for 10 min. The absorbance was measured at 490 nm [12, 13].

Statistical Analysis

Statistical analyses were performed using Prism 5 (GraphPad). The results were obtained in triplicate and analysed by one-way ANOVA followed by the Tukey test. Data were expressed as means $\pm$ standard deviation (SD).

p<0.05, *p<0.01 are considered as significant values.

RESULTS AND DISCUSSION

Previous studies have shown that methanol can extract a greater diversity of plant compounds with higher extraction efficiency. Therefore, the *D. malabarica* bark powder was Soxhlet extracted with methanol as a solvent. The extract obtained after vacuum evaporation was reddish-brown, non-sticky, and powdery, with a yield of 22.5 % w/w (Figures 1 and 2).

The total ion chromatogram showing the GC-MS/MS profile of the identified compounds is represented in Figure 3. The extract showed the presence of 16 phytochemicals following GC-MS/MS analysis. The proposed metabolite name, structure and previously reported biological activity from the literature are given in Table 1. The analysis identified several notable metabolites, including 2-Methoxyphenol (Guaiacol), Catechol(1,2-dihydroxybenzene), Hydroquinone(1,4-dihydroxybenzene), 3-Methoxycatechol, 2-Methoxyresorcinol, 2,6-Dimethoxyphenol (Syringol), 2,3-Dimethoxyphenol, 1,2,3-Benzenetriol (Pyrogallol), 1,3,5-Benzenetriol (Phloroglucinol), 2,4,6-Trihydroxybenzoic acid, 3,4,5-Trimethoxyphenol, 3,4,5-Trimethoxyphenyl acetate, 4-Hydroxy-3-methoxybenzenepropanol (Coniferyl alcohol), Tetradecanoic acid (Myristic acid), n-Hexadecanoic acid (Palmitic acid), Glycerol 1-palmitate. These compounds are well-documented for their antioxidant, antimicrobial, anti-inflammatory, neuroprotective, antispasmodic, and radioprotective activities. Interestingly, 8 of the above-identified phytochemicals have reported anticancer activity, including Hydroquinone (1,4-dihydroxybenzene), 3-Methoxycatechol, 2-Methoxyresorcinol, 2,6-Dimethoxyphenol (Syringol), 1,2,3-Benzenetriol (Pyrogallol), 2,4,6-Trihydroxybenzoic acid, 3,4,5-Trimethoxyphenyl acetate and Glycerol 1-palmitate (Monopalmitin). Hydroquinone is a hydroxylated benzene metabolite with various biological activities, including antioxidative, neuroprotective, immunomodulatory, and anti-inflammatory functions. Hydroquinone is present in leaves and berries in the form of arbutin. Hydrolysis of arbutin gives rise to hydroquinone. Arbutin is also found in coffee beans, tea extracts, broccoli, pear tree, red wine, and wheat germ. Further, the anti-cancer activity of hydroquinone is well understood. The *in vitro* and *in vivo* anti-cancer activity of

hydroquinone has been investigated in various cancer cells and tumour-bearing mouse models. Hydroquinone significantly induced the death of A431, SYF, B16F10, and MDA-MB-231 cells. Reports also highlight that Hydroquinone suppressed angiogenesis and prevented lung metastasis of melanoma cells in mice in a dose-dependent manner without toxicity or adverse effects to normal cells [18]. 3-Methoxycatechol exists as a minor plant metabolite and is structurally related to lignans, which are major components of wood and plant cell walls. It has been identified as a natural product in various plants, including the Norway spruce (*Picea abies*). 3-Methoxycatechol is known to inhibit tumour growth in a rat multiorgan carcinogenesis model and can modify phenolic antioxidant-induced cell proliferation and/or carcinogenesis, particularly in the upper digestive tract [19].

Similarly, 2-Methoxyresorcinol is a naturally occurring phenolic compound derived from syringyl lignin units present in higher plants, particularly hardwood species. It has been widely reported as a lignin-related phytochemical and is commonly detected in plant tissues and lignocellulosic materials. 2-Methoxyresorcinol is a metabolite that is formed by the oxidation of resorcinol and has been shown to inhibit the growth of tumour cells *in vitro* and *in vivo*. It also inhibits tumour cell growth by decreasing protein synthesis and cell division [20].

The compound 2,6-Dimethoxyphenol identified in our extract has been reported as a natural product occurring in many plants, including *Peltophorum africanum*. 2,6-Dimethoxyphenol is reported to induce oxidative stress-related apoptotic effects in HEK-293 cells in a concentration-dependent manner (0.5–2 mg/L) [21].

Another GC-MS/MS-identified phytocompound, 1,2,3-Benzenetriol/pyrogallol, is a common naturally occurring phenolic compound found in various plants, including *Embllica officinalis*, *Jatropha neopauciflora*, tea, coffee, avocado, and apricot [22]. Pyrogallol acts as a novel anticancer agent to enhance sensitivity to cisplatin, a platinum-based chemotherapy medication used to treat various tumours, including testicular, ovarian, bladder, and lung cancers. It works by binding to DNA, resulting in the perturbation of genetic material, preventing cancer cells from dividing, leading to cell death. Pyrogallol prevents ovarian cancer cells by inducing upregulation of miR-15a, leading to apoptosis [23]. Further, 2,4,6-

Trihydroxybenzoic acid is a promising phenolic compound capable of preventing colorectal cancer due to antioxidant and anti-inflammatory properties. 2,4,6-Trihydroxybenzoic acid and its isomer 2,3,4-THBA are plant metabolites present in several fruits and vegetables, including Adzuki beans [24]. This bean variety is a major cultivar in Southeast Asia, known to inhibit Cyclin Dependent Kinase (CDK) activity and cancer cell proliferation in both *in vitro* and *in vivo* cancer models [25].

The other two compounds identified with anticancer potential were 3,4,5-Trimethoxyphenyl acetate and Glycerol 1-palmitate (Monopalmitin). The derivatives of the former are known to inhibit the growth of the hepatocellular carcinoma (HepG2) cell line with an IC_{50} of 1.38 to 3.21 μ M compared to podophyllotoxin as a reference compound. 3,4,5-Trimethoxyphenyl acetate was initially identified as a natural trace constituent in certain plants, such as *Eucalyptus globulus*. These compounds are potent inhibitors of β -tubulin polymerisation, with cell cycle disturbance at the G2/M phase and a significant increase in Annexin-V-positive cells. Further investigation demonstrated decreased levels of MMP and Bcl-2, as well as boosted the levels of p53 and Bax, compared with untreated HepG2 cells [26]. Monopalmitin is a naturally occurring monoacylglycerol found in plants. It occurs as an intermediate in lipid biosynthesis and degradation and has been reported in various plant-derived oils and extracts. This is the principal active constituent of *Mougeotia nummuloidea* and *Spirulina* and has been reported to possess anticancer properties. Monoacylglycerol significantly inhibited A549 and SPC-A1 cell proliferation, induced G2/M arrest and caspase-dependent apoptosis. Moreover, it suppressed the protein expression of inhibitors of apoptosis proteins, providing novel options for lung cancer treatment [27]. The above findings demonstrate the anticancer potential of the extract, and the presence of the compounds highlights the phytochemical richness and pharmacological implications of *D. malabarica* bark extract.

D. Malabarica Bark Extract Dose-Dependently Inhibits Colorectal Cancer Cell Proliferation: We evaluated the cytotoxic effect of *D. malabarica* on different molecular subtype human colorectal cancer cell lines (HCT-116 and HT-29) using an MTT assay. As shown in Figure 4, cell viability was significantly

affected by *D. malabarica* in a dose-dependent manner. Concentrations of 5.2, 7.8, 15.6, 31.25, 62.5, and 125 μ g/ml for 24 hr resulted in an average inhibition of cell proliferation by 16.2, 18.0, 33.5, 58.6, and $90.2 \pm 6\%$, respectively. Our results showed slightly greater inhibition of HT-29 cells than of HCT-116 cells. However, the difference in inhibition was not significant. The average half-maximal inhibitory concentration (IC_{50}) for both cell lines was determined using GraphPad Prism software and was found to be around 52-60 μ g/ml. The $\frac{1}{2} IC_{50}$, IC_{50} , and $2IC_{50}$ concentrations of *D. malabarica* were used for further studies. The inhibition of proliferation of the cancer cells observed in the study may be due to the presence of cytotoxic and anti-proliferative compounds such as Hydroquinone [18] and 3-Methoxycatechol [19]. Further, 2,6-Dimethoxyphenol [21], 1,2,3-Benzenetriol [23] and 2,4,6-Trihydroxybenzoic acid [25] present in the extract are known to induce oxidative stress-related apoptosis, bind to DNA, perturb genetic material to prevent cancer cell division and inhibit cyclin-dependent kinase activity, respectively.

D. Malabarica Alters the Cellular Morphology of HCT-116 and HT-29 Cells: To explore the cellular morphology changes following *D. malabarica* treatment, cells were stimulated with the IC_{50} concentration of *D. malabarica* extract and were visualised using an inverted bright-field microscope. As shown in Figure 5, HCT-116 cells exhibited typical characteristics of apoptosis, such as cellular shrinkage, apoptotic bodies, membrane blebbing, and nuclear condensation. Cells treated with a higher concentration of *D. malabarica* were markedly more prominent with these morphological features, with cellular disintegration. These results are in agreement with the dose-dependent inhibition data and reinforce the cytotoxic effect following *D. malabarica* treatment. Furthermore, compounds present in the *D. malabarica* extract, such as 3,4,5-Trimethoxyphenyl acetate [26] and Glycerol-1 palmitate [27], are known to induce cell apoptosis and inhibit β -tubulin polymerisation, leading to cell cycle disturbance at G2/M phase arrest and caspase-dependent apoptosis.

Elevated ROS Levels In D. Malabarica-Treated HCT-116 and HT-29 Cells: ROS induce cell death. DCFDA fluorescence-based

fluorometry assay was used to analyse ROS generation in *D. malabarica* extract-treated HCT-116 and HT-29 cells. High fluorescence intensity was observed in *D. malabarica* extract-treated cells at IC₅₀ and 2X IC₅₀ concentrations (Figure 6). ROS production levels were similar in both cell types. Our findings suggest *D. malabarica* produces significant levels of ROS, which may cause apoptosis by disrupting the balance between the oxidant and antioxidant enzyme systems to alter the redox balance and leading to cellular disintegration and death. The anticancer phytochemicals identified in our extract, such as 3-Methoxycatechol [19] and 2,6-Dimethoxyphenol [21], are known to induce stress-related apoptosis and modify antioxidant-induced cell proliferation. The presence of the above compounds may be the reason for the elevated ROS-mediated apoptosis and tumour cell membrane damage observed.

D. Malabarica Increases LDH Release in HCT-116 and HT-29 Cells: Release of LDH is one of the accurate methods to monitor viability of the cells and the integrity of the cell membrane. *D. malabarica* at the higher concentrations (110 to 120 µg/ml) significantly enhances the release of LDH in the supernatant of the *D. malabarica* extract-treated HCT-116 and HT-29 cells when compared to the untreated control in a kit-based assay. This result suggests that late apoptosis may be followed by necrosis in the cytotoxic effect of *D. malabarica* (Figure 7). As discussed earlier, the presence of anticancer compounds in the extract has been documented to induce cytotoxicity and anti-tumour growth effects in various cell culture studies. Our results further confirm the above observations following identification of anticancer compounds in our extract.

D. Malabarica Treatment in HCT-116 and HT-29 Cells Induces Apoptosis: Morphological changes caused by any agent in cell culture can be monitored using acridine orange/ethidium bromide. Uniformly, green live and normal morphology were observed in the majority of untreated HCT-116 cells with no sign of apoptosis. A significant increase in the number of apoptotic cells with irregular, shrunken, and red/orange fluorescence was observed following treatment with *D. malabarica* extract (Figure 8). A 3-fold increase in apoptosis compared to the control was recorded. The changes in morphology resulted in nuclear

fragmentation and chromatin condensation, which were indicators of the onset of apoptosis. In addition, with increasing concentrations of *D. malabarica*, the number of apoptotic cells increased. The GC/MS-MS identified phytochemicals Hydroquinone (1,4-dihydroxybenzene) [18], 3-Methoxycatechol [19], 2-Methoxyresorcinol [20], 2,6-Dimethoxyphenol (Syringol) [21], 1,2,3-Benzenetriol (Pyrogallol) [23], 2,4,6-Trihydroxybenzoic acid [25], 3,4,5-Trimethoxyphenyl acetate [26] and Glycerol 1-palmitate (Monopalmitin) [27] are known to induce apoptosis and prevent cancer cell proliferation through various mechanisms, without interfering with the growth of normal cells. A similar observation is recorded in our current study, which further encourages the usefulness of *D. malabarica* extract in anticancer studies both in animal models and pre-clinical settings.

CONCLUSION

D. malabarica contains potent anticancer compounds with significant anti-proliferative activity, including Hydroquinone (1,4-dihydroxybenzene), 3-Methoxycatechol, 2-Methoxyresorcinol, 2,6-Dimethoxyphenol (Syringol), 1,2,3-Benzenetriol (Pyrogallol), 2,4,6-Trihydroxybenzoic acid, 3,4,5-Trimethoxyphenyl acetate, and Glycerol 1-palmitate. The *D. malabarica* extract showed dose-dependent suppression of HCT-116 and HT-29 human colorectal cancer cell growth *in vitro*, with IC₅₀ values of 53 ± 4.2 and 58 ± 5.3 µg/ml. Our findings indicate late apoptosis, followed by cell death driven by ROS in the cytotoxic action of *D. malabarica*, as determined by AO/EtBr dual staining and LDH release assays. Overall, the study reinforces the ethnomedicinal use of *D. malabarica* extracts as a source of strong anticancer drugs with gastrointestinal health benefits.

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Conflict of Interest

No conflict of interest.

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Figure 1: Representative images of *D. malabarica* tree and stem



Figure 2: Representative images of *D. malabarica* stem bark, stem bark powder and the methanol extract.

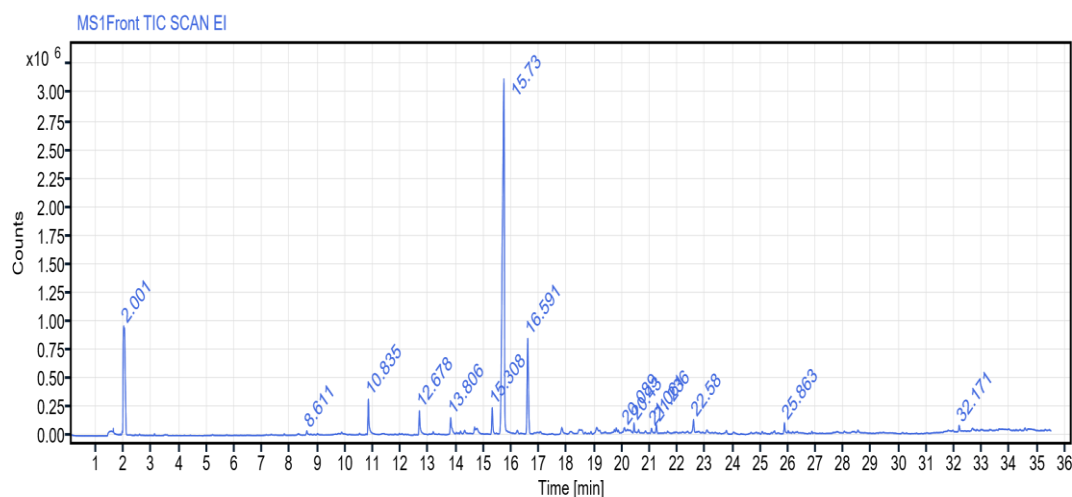
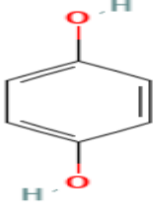
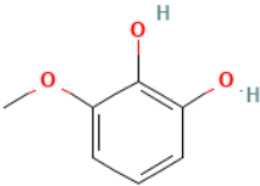
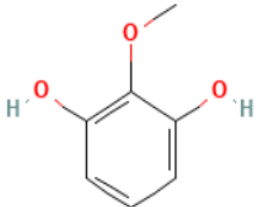
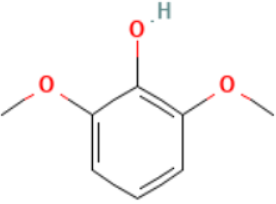
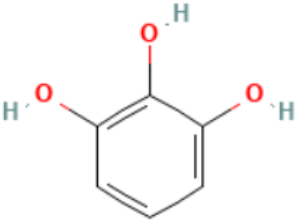
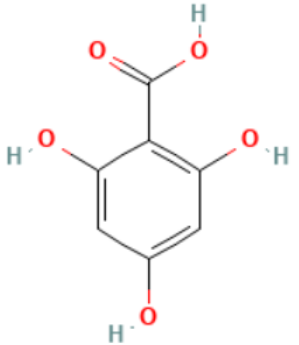
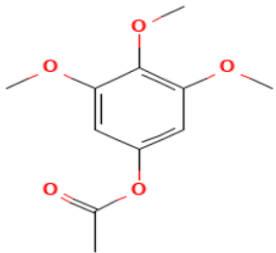
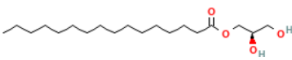
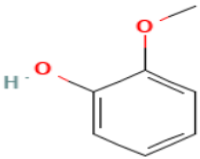
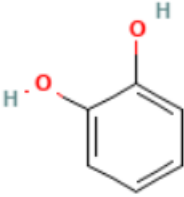
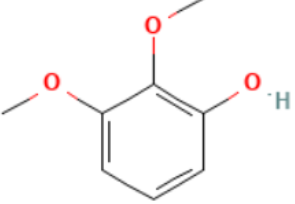
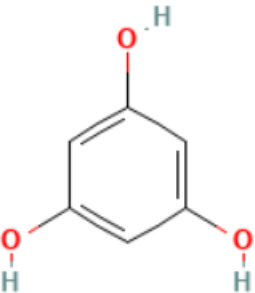
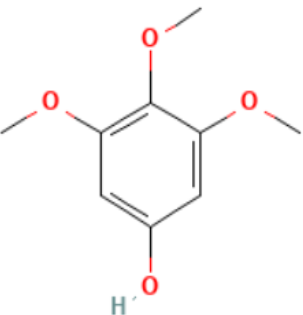
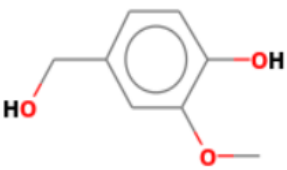
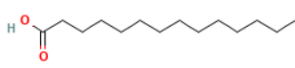
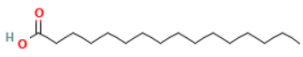


Figure 3: GC-MS/MS metabolic profile of the *D. malabarica* stem methanol extract

Table 1: List of compounds detected in the *D. malabarica* extract by GC-MS/MS.

SL.No.	Compound Name	Structure	Reported Biological Activity
1	Hydroquinone (1,4-dihydroxybenzene)		Cytotoxic, inhibits tumour growth (dose-dependent) [18]
2	3-Methoxycatechol		Antioxidant, potential anticancer [19]
3	2-Methoxyresorcinol		Reported antiproliferative activity [20]

4	2,6-Dimethoxyphenol (Syringol)		Antioxidant, inhibits cancer cell growth [21]
5	1,2,3-Benzenetriol (Pyrogallol)		Potent antioxidant, antimicrobial, and anticancer properties [23]
6	2,4,6-Trihydroxybenzoic acid		Acts as a potent CDK inhibitor, exhibiting anti-proliferative and anti-cancer properties, and demonstrating moderate antioxidant capacity [25]
7	3,4,5-Trimethoxyphenyl acetate		anticancer, anti-tubulin, and central nervous system (CNS) agents [26]
8	Glycerol 1-palmitate		Antitumor & apoptotic activity, anti-inflammatory properties [27]
9	2-Methoxyphenol (Guaiacol)		Prominent antioxidant, antimicrobial, anti-inflammatory, and phytotoxic effects [28]
10	Catechol (1,2-dihydroxybenzene)		A critical player in plant metabolism, bacterial biodegradation, toxicology, and pharmacology [29]

11	2,3-Dimethoxyphenol		Antioxidant properties, Act as a neuroprotective agent, utilised as a chemical tool to study mutagenesis and DNA damage. [30]
12	1,3,5-Benzenetriol (Phloroglucinol)		Acts as a potent anti-inflammatory and antitumor agent, antioxidant, antispasmodic, radioprotective agent, and promising therapeutic lead in neurodegenerative and oncological research [31]
13	3,4,5-Trimethoxyphenol		Antioxidant, utilised as a chemical building block for drug discovery [32]
14	4-Hydroxy-3-methoxybenzenepropanol (Coniferyl alcohol)		Potent antioxidant activity, Anti-inflammatory properties, Antimicrobial effects [33]
15	Tetradecanoic acid (Myristic acid)		Protein modification (myristoylation), antimicrobial/anti-virulence properties, neurological health support, and its role as a precursor to Omega-5 fatty acids [34]
16	n-Hexadecanoic acid (Palmitic acid)		Anti-inflammatory effects, Antimicrobial properties, Antioxidant activity, Metabolic and cellular signalling molecule [35]

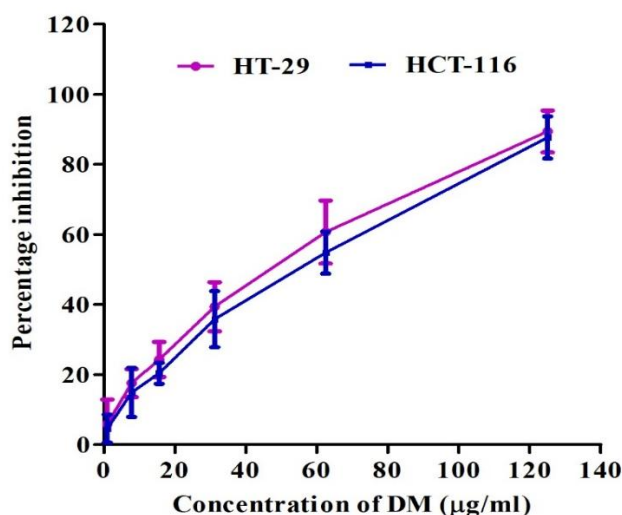


Figure 4: Dose-dependent inhibition curve: HCT-116 and HT-29 cells were treated with increasing concentrations (7-125 µg/ml) of *D. malabarica* extract. The percentage survival of the cells was determined by MTT assay. Data are represented as the mean $\pm$ SD (N=6) for a 24-hr cytotoxicity assay.

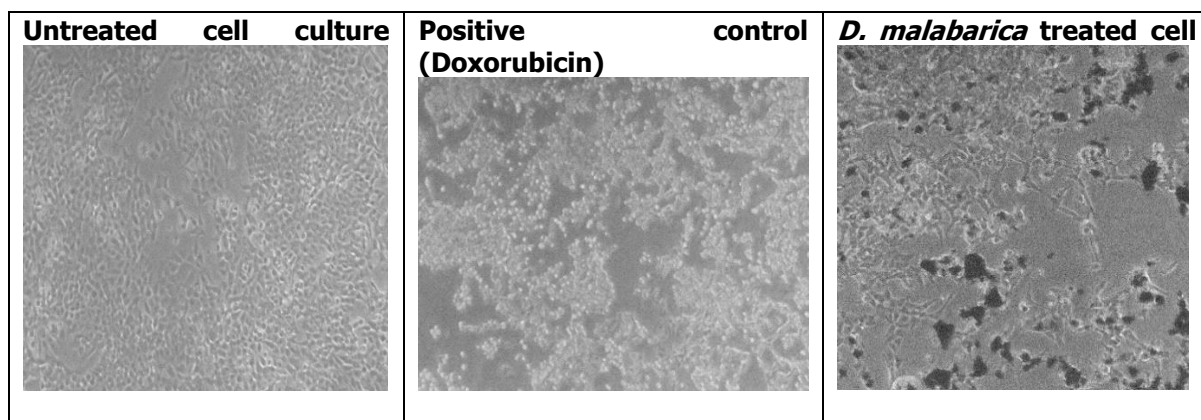


Figure 5: Representative cell morphology images of HCT-116 cells treated with *D. malabarica* extract at IC₅₀ concentration (52-60 µg/ml).

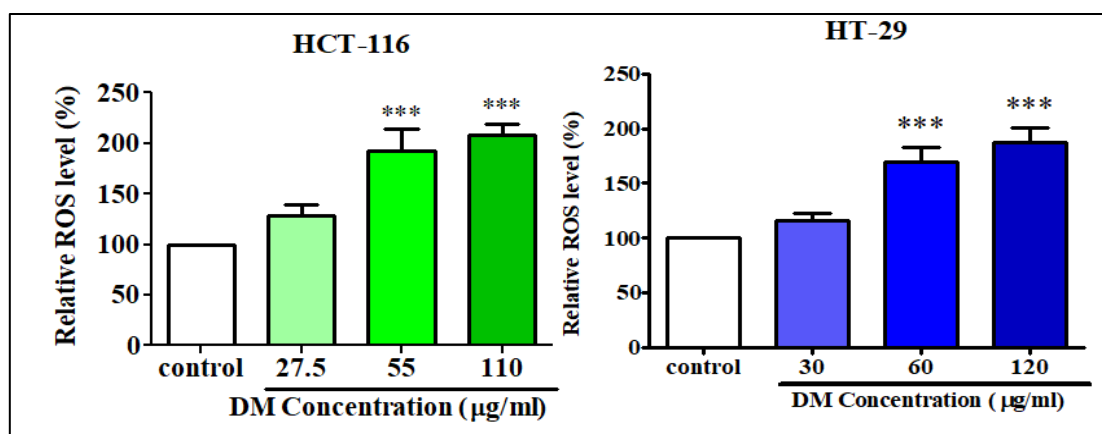


Figure 6: Intracellular ROS level in HCT-116 and HT-29 cells treated with *D. malabarica* extract. HCT-116 and HT-29 cells were treated separately with the indicated concentration for 24 hr, and accumulation of ROS levels was analysed using a fluorometric method. Data were represented as mean $\pm$ SD (N=6); ***P<0.01 are considered significant values.

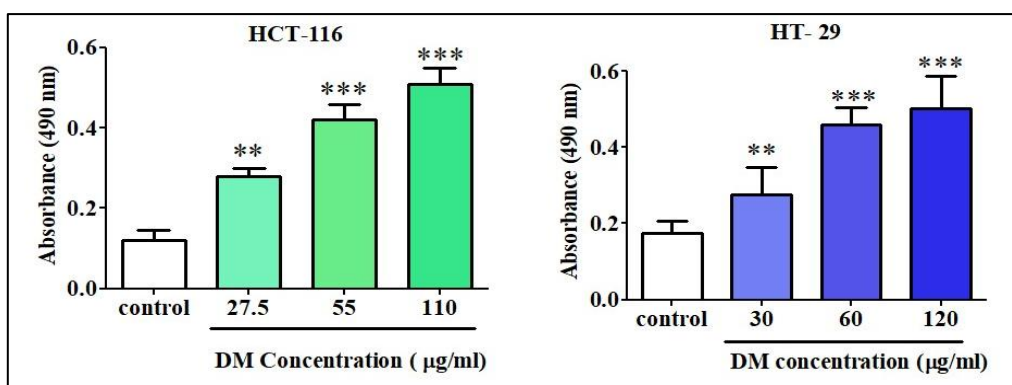


Figure 7: Release of LDH from *D. malabarica* extract-treated HCT-116 and HT-29 cell supernatant. HCT-116 and HT-29 cells were treated separately with the indicated concentration for 24 hr, and release of LDH was analysed using a kit-based assay. Data were represented as mean $\pm$ SD (N=6); ***P<0.01 is considered a significant value.

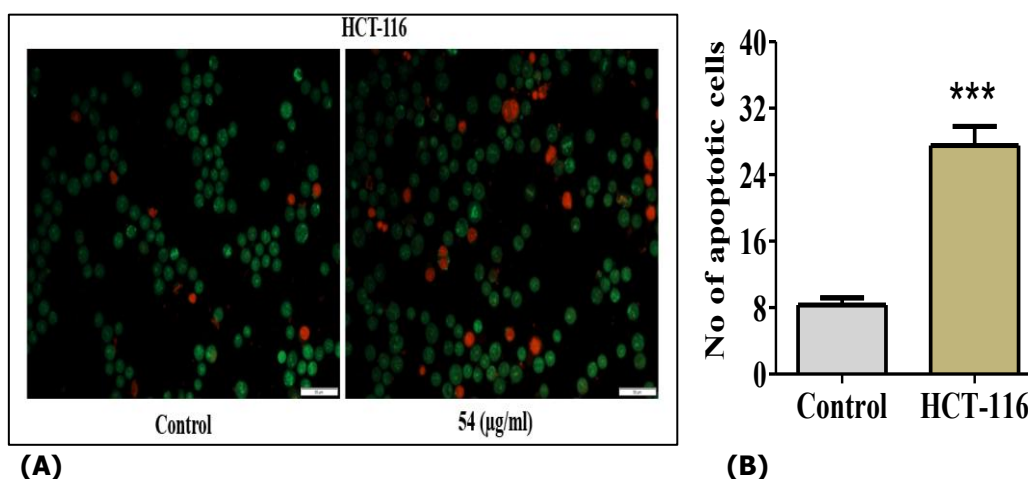


Figure 8 (A) and (B): Representative apoptotic morphology of HCT-116 cells upon treatment with *D. malabarica*. Apoptotic changes in HCT-116 cells treated with *D. malabarica* (IC₅₀ concentration) were assessed by AO/EtBr dual staining. Untreated cells are shown in green, whereas apoptotic cells are observed in red/orange colour (Magnification, X100). (B): Average number of apoptotic cells observed in 5 fields by an independent observer blinded to the study.