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Research Article

Phytochemical analysis of *Diospyros buxifolia* (Blume) Hiern. bark extract *via* LC-MS/MS and evaluation of its antiproliferative activity against human colon carcinoma cells

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ABSTRACT:

Bioactive phytochemicals have shown a very significant role in pharmacological ailments in humans. The literature review indicates the extensive use of *Diospyros* extracts for the treatment of diseases related to the oral cavity and gastrointestinal disorders. Further, the bark, fruit, and root are the most commonly utilised components in traditional preparations. Due to a lack of awareness about *Diospyros buxifolia* (DB) and its underutilized status, relatively few studies have been conducted on its nutraceutical potential. Hence, the present study aimed to identify bioactive compounds in DB bark extracts using liquid chromatography mass spectroscopy (LC-MS/MS) and evaluate their antiproliferative activity against human colon carcinoma cells. The LC-MS/MS chromatogram showed the presence of various phytochemical compounds such as O-Phosphoryl ethanolamine, Dulcitol, 2-(2-Aminoethyl) pyridine, Alpha-Lipoic acid, 14-(Hydroxymethyl)-5,9-dimethyltetra-cyclo-hexadecan-5-ol, Geranyl acetone, Retinol, Retinoids, Pimelic acid, 3-Methyl-2-oxovaleric acid sodium, and Prolineamide, etc. An extensive literature survey revealed various pharmaceutical properties. The extract demonstrated dose-dependent antiproliferative effects against HT-29 and HCT-116 cells with IC₅₀ values of 43.5 ± 4.2 and 48 ± 3.7 µg/ml, respectively, by MTT assay. Moreover, the extract enhanced the reactive oxygen species (ROS) level, altered cellular morphology, and induced apoptosis as evaluated by fluorescence microscopy. Current findings suggest DB produces significant levels of ROS (1.8-fold), which may cause apoptosis by disrupting the balance between the oxidant and antioxidant enzyme systems to alter the redox balance in the carcinoma cells tested. Interestingly, DB at the highest concentrations significantly enhances the release of lactate dehydrogenase (2.7-fold) in HT-29 and HCT-116 cells. This result suggests the involvement of necrosis in the cytotoxic effect of DB. In conclusion, DB includes strong anticancer agents with notable antiproliferative activity, which makes it suitable for application in medicine and the food industry.

KEYWORDS: Bioactive compounds, *Diospyros buxifolia*, Antiproliferative activity, Reactive oxygen species, Apoptosis, Necrosis.

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INTRODUCTION:

Phytochemical preparations or bioactive phytochemicals play a very significant role in the treatment of diseases in humans. About 40% or more of the therapeutic agents currently used in Western Ghats

by the locals are derived or at least partially derived from natural resources, particularly from medicinal plants. *D. buxifolia*, commonly known as Kunchigana mara, is one such traditional plant notable for its ethnopharmacological values for treating diseases

related to oral cavity health (oral wounds, bad breath, and toothaches) and gastrointestinal disorders. Further, the bark, fruit, and root are the most commonly employed *Diospyros* plant components in traditional medicine [1]. The plant *Diospyros buxifolia* (Blume) Hiern. (Ebenaceae family, **Figure 1**) has a pantropical distribution and includes the genera *Diospyros*, which have around 500 to 600 species. The species of the genus *Diospyros* have long been extensively employed in traditional medicine to treat various illnesses. So far, the reported compounds from *Diospyros* species have been naphthoquinones, triterpenoids, and tannins [2,3].

Cancer is a broad word that refers to a wide range of illnesses that can affect any region of the body. Most of the malignancies originate generally from one aberrant cell. As a result of the aberrant proliferation of cells, there is the formation of tumours (neoplasm). It may be benign or malignant. Malignant tumours conquer local tissue, and there is metastasis and destruction in the tissue of origin. Globally, the increase in cancer incidence and its associated mortality rate is challenging the present cancer care delivery system.

Still, chemotherapy and radiotherapy remain the primary treatments for various types of tumours. Chemotherapy drugs and untargeted radiotherapy kill the rapidly proliferating cancer cells as well as the normal cells of patients, resulting in serious side effects. Therefore, safe, non-toxic anti-tumour therapy is still a long way from reality. The interactions between genetics and physical factors (ultraviolet radiation), chemical factors (tobacco and asbestos), and biological factors (viruses and bacteria) result in these changes. On the other hand, diet and malnutrition are among the major contributing factors for cancer in low and middle-income families. The underprivileged are affected when it comes to receiving evidence-based cancer prevention and treatment. Therefore, stomach and colorectal cancer contribute to the high burden of the disease in developing countries, leading to 17% of mortality. Having these in mind, patients rely on traditional, complementary medicine, which is less costly and widely available. As a result, investigations that reveal the natural, semi-synthetic compounds demonstrating selective cytotoxicity against colon cancer cell lines can be promising drug empirical targets for developing new chemotherapeutics against colon cancer. Therefore, the food and pharmaceutical industries have become increasingly interested in analysing medicinal plants [4-6].

A critical literature analysis has revealed that very few studies have been reported on the biochemical analysis of *D. buxifolia* extract. The scientific literature did not report much work on the traditional effects of this tree's extracts, particularly against gastrointestinal disorders. In addition, the fruits of *D. buxifolia* are consumed as food. Hence, the phytochemicals of extracts need to be explored both qualitatively and quantitatively for biological activities. The studies should be focused on judiciously utilizing the advances in structural chemistry, cellular biology, and molecular pharmacology to validate the efficacy and safety of *D. buxifolia* extracts.

It is essential to figure out the most ideal plant part as a potent future source of herbal drugs. Therefore, the present study was intended to evaluate and compare the phytochemical contents of the bark methanol extract. Further, *D. buxifolia* extract was evaluated for *in vitro* antiproliferative properties specifically on colon cancer cell lines [7,8].

MATERIALS AND METHODS:

Plant material: The stem bark sample of *Diospyros buxifolia* was collected from the Western Ghats, Karnataka, India, surface sterilized, air-dried, and powdered finely (Herbarium no-BOTIDB01).

Soxhlet extraction and preparation of methanol extract for cell culture studies: The powder prepared from *D. buxifolia* 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 $60 \pm 1^\circ\text{C}$ in a rotatory vacuum evaporator (Steroglass, strike 300, Italy) till a solid mass was obtained and stored in an air-tight container in a refrigerator (4°C). The extract was dissolved in dimethyl sulfoxide (DMSO) to obtain a stock solution. Working stocks were prepared in DMEM medium without fetal bovine serum (FBS), keeping the final concentration of DMSO less than 0.4%. These were filter sterilised through $0.22\ \mu\text{m}$ filters [9-11]. The percentage yield of the extract was calculated using the formula.

$$\text{Ey\%} = \frac{w_1}{w_2} \times 100$$

Where;

Ey% = Extract yield in percentage.

W_1 = net weight of extracts (grams)

W_2 = total weight of stem-bark powder (grams).

LC-MS/MS analysis: *D. buxifolia* extract (5 mg) is dissolved in 2 mL of methanol, filtered, and used for LC-MS/MS analysis [12, 13].

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°C [12,13].

Antiproliferative Assay/ MTT assay: The cytotoxicity effect of *D. buxifolia* extract was confirmed through MTT (2,7,3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. [13]

Cell morphology: The effect of *D. buxifolia* on the morphological changes of cells was observed under a microscope. Briefly, cells were seeded in 6-well microplates at a density of 1×10^4 cells/well and incubated overnight. The cells were then treated with *D.*

buxifolia extracts at their IC₅₀ concentration for 24 hr. The changes in the morphology were observed under a brightfield microscope [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. buxifolia* for 24 hr. Cells were treated with DCF-DA (20 μ M) for 30 min at 37 °C in the dark. Relative changes in intracellular ROS were monitored by fluorometric detection of DCF using a fluorescent microplate reader at excitation and emission wavelengths of 485 nm and 530 nm, respectively [12, 13].

LDH release assay: The release of LDH from culture media was quantified using a kit-based assay method (agappe). Colorectal cancer cells were treated with *D. buxifolia* for 24 hr as mentioned above section. Next, the cell supernatant was mixed with the LDH mixture and incubated for 10 min in the dark at room temperature. The absorbance was measured at 490 nm [13].

Acridine orange/Ethidium bromide dual stain: The apoptotic morphology of cells was determined using a double fluorescence stain assay. Following treatment with *D. buxifolia*, HCT-116 cells were stained with an equal volume of a mixture of Acridine orange/Ethidium bromide (AO/EtBr, 100 μ g/mL each) solution and incubated for 10 min in the dark. After incubation, cells were visualised under a fluorescence microscope at an excitation wavelength (400–490 nm) [13].

RESULTS AND DISCUSSION:

Plants are rich sources of natural compounds with extraordinary therapeutic potential, essential for developing novel medications. *Diospyros* species, medicinal plants traditionally used for managing various diseases, represent a promising avenue for further exploration. Due to a lack of awareness about *D. buxifolia* and its underutilized status, relatively few studies have been conducted on its nutraceutical potential [7,8]. The present study aimed to identify bioactive compounds in *D. buxifolia* bark extracts using LC-MS/MS and evaluate their antiproliferative activity against human colon carcinoma cells. The methanol extract obtained following vacuum evaporation from *D. buxifolia* was dark brown, yielding 24.9% w/w [with W₁ of 27.49 gms and W₂ of 110 gms] (Figure 2). The extract showed the presence of 11 phytochemicals in LC-MS/MS analysis. Among which, the compounds such as O-Phosphoryl ethanolamine, Dulcitol, 2-(2-Aminoethyl) pyridine, Alpha-Lipoic acid, 14-(Hydroxymethyl)-5,9-dimethyltetracyclo-hexadecan-5-ol, Geranyl acetone, Retinol, Retinoids, Pimelic acid, 3-Methyl-2-oxovaleric acid sodium, and Prolinamide, etc., via an extensive literature survey, revealed rich pharmaceutical properties. The details of the proposed

secondary metabolite name and reported biological activities are given in Table 1 and Figure 3.

DB inhibits colorectal cancer cell proliferation: *D. buxifolia* extract indicated concentration-dependent inhibition of different molecular subtype human colorectal cancer cell lines (HT-29 and HCT-116). *D. buxifolia* treatment to HT-29 & HCT-116 cells at the concentration range of 5 to 160 μ g/ml inhibits cell proliferation by 8 to 100%, respectively. Further, GraphPad Prism software was used to calculate the half-maximal inhibitory concentration (IC₅₀). Both cell lines showed the (IC₅₀) value at 43.5 ± 4.2 and 48 ± 3.7 μ g/ml, respectively (Figure 4). Thus, it can be concluded that the extract contains phytochemicals that inhibit the proliferation of cancer cells as analysed by MTT assay [11-13].

***D. buxifolia* alters the cellular morphology of HT-29 and HCT-116 cells:** *D. buxifolia*-treated cells exhibited typical characteristics of apoptosis, such as cellular shrinkage, apoptotic bodies, membrane blebbing, and nucleus condensation. Cells treated with higher concentrations of extract were markedly more prominent with these morphological features, with cellular disintegration. These results reinforce the viability data and indicate the dose-dependent cytotoxic effect [11-13].

Elevated production of ROS levels in *D. buxifolia*-treated HT-29 and HCT-116 cells: High fluorescence intensity was observed in extract-treated cells at a concentration of 43.5 and 48 μ g/ml (Figure 5). Comparatively, ROS production levels were slightly increased in HT-29 cells than in HCT-116 cells. Since ROS is a common inducer of mitochondrial pathway-mediated apoptosis, the above observations of anti-proliferative effect and cellular disintegration effect indicate that bioactives present in the extract selectively interfere and prevent cancer cell proliferation by releasing ROS. Further, this may cause apoptosis by disrupting the balance between the oxidant and antioxidant enzyme systems to alter the redox balance in the cell [12].

***D. buxifolia* increases LDH release in HT-29 and HCT-116 cells:** *D. buxifolia* extract at the higher concentrations (2X IC₅₀) significantly enhances the release of LDH as observed in HT-29 and HCT-116 cells when compared to the control (Figure 6). The measurement of LDH releases is one of the accurate methods to monitor cell viability and membrane integrity. Therefore, the elevated levels of LDH release suggest the involvement of necrosis in the cytotoxic effect of *D. buxifolia* [12, 13].

***D. buxifolia* extract induces apoptosis in HT-29 and HCT-116 cells:** Using the acridine orange/ethidium bromide differential staining method, morphological changes caused by *D. buxifolia* were investigated. Uniformly, green live and normal morphology were

observed in untreated HT-29 and HCT-116 cells, which indicates no sign of apoptosis. The colorectal cancer cells treated with *D. buxifolia* exhibit irregular, shrunken, and red/orange fluorescence (**Figure 7**). The apoptotic changes could be due to the presence of phytochemicals such as Dulcitol, amino-ethyl pyridine, alpha lipoic acid and retinol in *D. buxifolia* extract that have been previously reported to have anticancer and antioxidant activities. The changes in apoptotic morphology resulted in nuclear fragmentation and chromatin condensation, which were indicators of the onset of apoptosis. In addition, with increasing concentrations of *D. buxifolia* extract, the number of apoptotic cells increased, leading to elevated levels of LDH [13-18].

CONCLUSION:

D. buxifolia includes strong anticancer agents with notable anti-proliferative activity, such as Dulcitol and 2-(2-Aminoethyl) pyridine. Our results suggest the involvement of apoptosis followed by necrosis due to ROS in the cytotoxic effect of *D. buxifolia*. Therefore, the stem and the edible fruit extracts of *D. buxifolia* are suitable for application in the medicine and food industry.

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CONFLICT OF INTEREST: The Authors declare no conflict of interest.

ETHICAL STATEMENT: As no animals are involved, no ethical concern exists.

FUNDING: No external funding was provided for the present work.

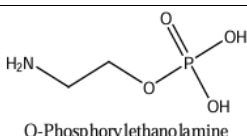
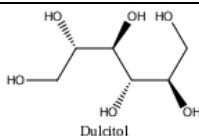
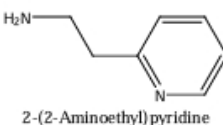
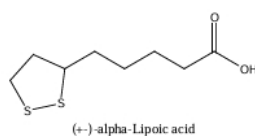
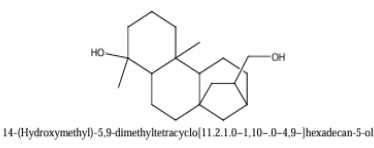
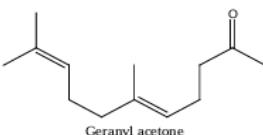
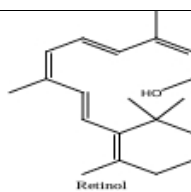
DATA AVAILABILITY: All data are available as part of the article.

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

Sl. No.	Name of the compounds	Structure	Reported biological activity
1.	O-Phosphorylethanolamine	 O-Phosphorylethanolamine	Metabolite of phospholipid metabolism and structural precursor for neurotransmitters such as GABA (Chidley, C <i>et al.</i> , 2016).
2.	Dulcitol	 Dulcitol	Anti-inflammatory, Antioxidant, and potential anti-cancer properties (Liu, Z <i>et al.</i> , 2024).
3.	2-(2-Aminoethyl) pyridine	 2-(2-Aminoethyl)pyridine	Antibacterial, Anticancer, and Anti-inflammatory (Pan, A <i>et al.</i> , 2023).
4.	(+)-alpha-Lipoic acid	 (+)-alpha-Lipoic acid	A potent antioxidant, acting as a free radical scavenger, a metal chelator, and a detoxifying agent (Tripathi, A.K. <i>et al.</i> , 2023).
5.	14-(Hydroxymethyl)-5,9-dimethyltetracyclohexadecan-5-ol	 14-(Hydroxymethyl)-5,9-dimethyltetracyclo[11.2.1.0-1,10-0-4,9]-hexadecan-5-ol	No reported biological activity.
6.	Geranyl acetone	 Geranyl acetone	Bactericidal, fungicidal, and anti-feedant effects, and is used as a fragrance and flavour enhancer (Celuppi, L. C. M <i>et al.</i> , 2023).
7.	Retinol	 Retinol	Anticancer activity, Acts as a ligand for nuclear retinoic acid receptors, regulating gene transcription and impacting cellular growth, differentiation, and other vital functions (Zhao, B., 2024).

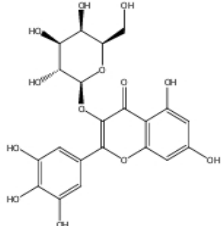
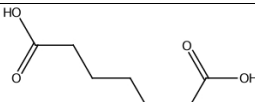
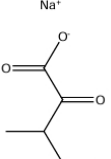
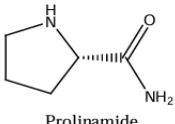
8.	Retinoids	 5,7,4-trihydroxy-3-[(2S,3R,4S,5R,6R)-3,4,5-trihydroxy-6-hydroxymethyl]oxan-2-yl]oxy-2-(3,4,5-trihydroxyphenyl)chromen-4-one	Involved in diverse processes like cell growth, differentiation, and embryonic development, influencing vision, immune function, and more, primarily through interactions with nuclear receptors (Zhao, B., 2024).
9.	Pimelic acid	 Pimelic acid	Direct precursor of biotin and amino acids such as lysine (Mumtaz, A., 2020).
10.	(+)-3-Methyl-2-oxovaleric acid sodium	 (+)-3-Methyl-2-oxovaleric acid sodium salt	A biomarker for Sarcopenia, maple syrup urine disease (MSUD), a metabolic disorder, and is also used as a substrate to study α -keto acid dehydrogenases (Shin, H. E, 2022)
11.	Prolinamide	 Prolinamide	Anticancer property, contributes to protein structure, acts as a stress response agent in plants, and potential role in pathogen interactions and energy production (Osinubi, A, 2020).



Figure 1: Photographs of different parts of the *Diospyros buxifolia* tree.

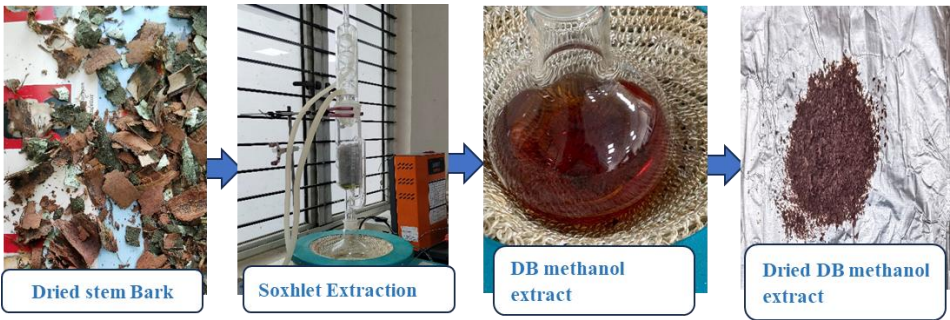


Figure 2: Soxhlet extraction of DB methanol extract.

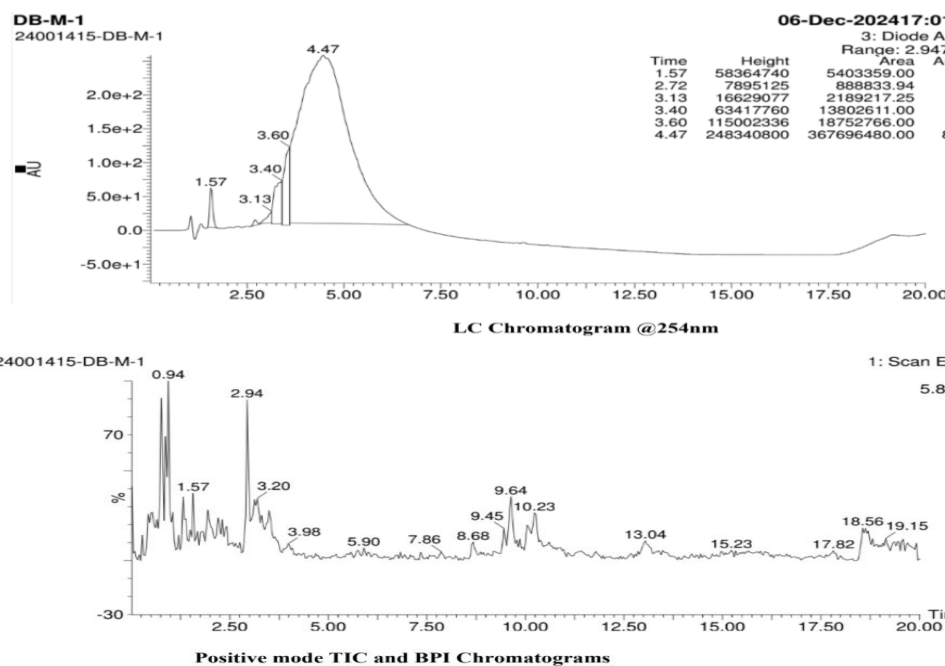


Figure 3: LC-MS/MS metabolic profile of the *Diospyros buxifolia* stem methanol extract.

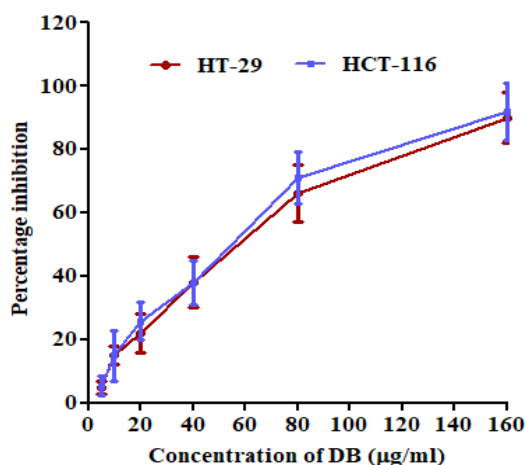


Figure 4: Antiproliferative effect of DB extract on HT-29 and HCT-116 cells.

Cells were treated with different concentrations (5 -160 µg/ml) of DB stem for 24hours and cytotoxicity was

determined by MTT assay. Data are represented in terms of mean $\pm$ SD (n=6).

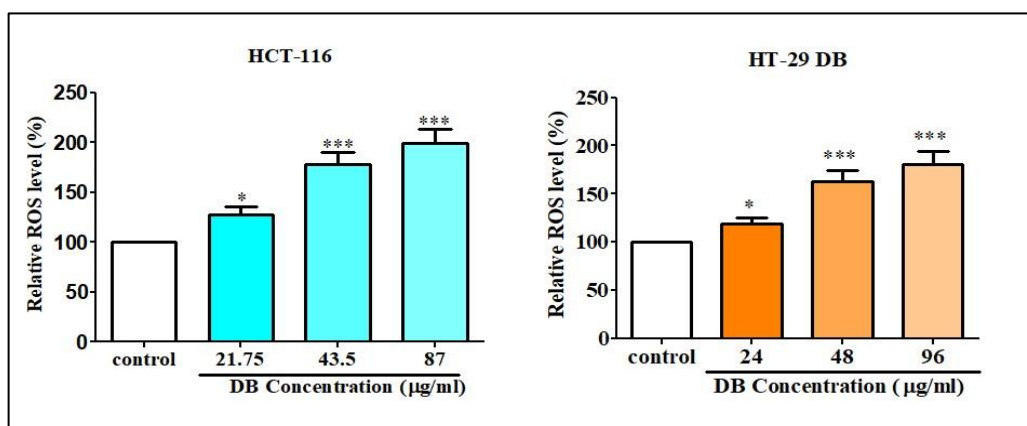


Figure 5: Intracellular ROS level in HT-29 and HCT-116 cells treated with DB extract.

HT-29 and HCT-116 cells were treated separately with indicated concentration for 24 h, and the accumulation of ROS levels was analyzed using a fluorometric

method. Data were represented as mean $\pm$ SD (n=6), * p <0.05, and *** p <0.01 are considered as significant values.

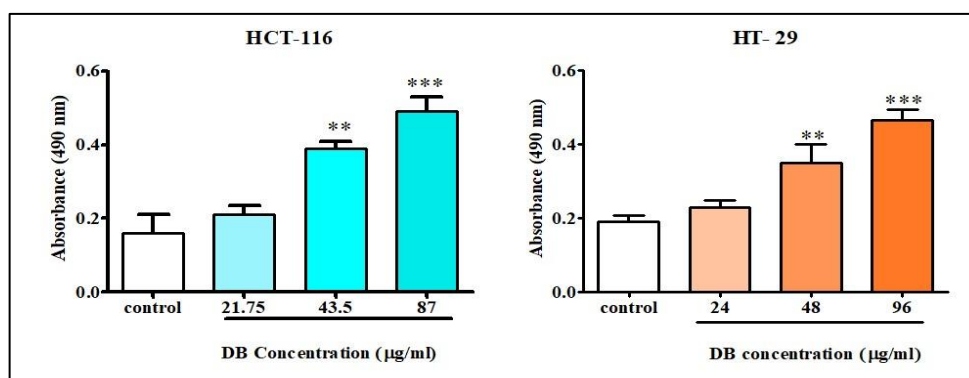


Figure 6: Release of LDH from DB extract-treated HT-29 and HCT-116 cells supernatant.

HT-29 and HCT-116 cells were treated separately with the indicated concentration for 24 h, and the release of LDH was analysed using a kit-based assay. Data were

represented as mean $\pm$ SD (n=6), *** p <0.01 are considered as significant values.

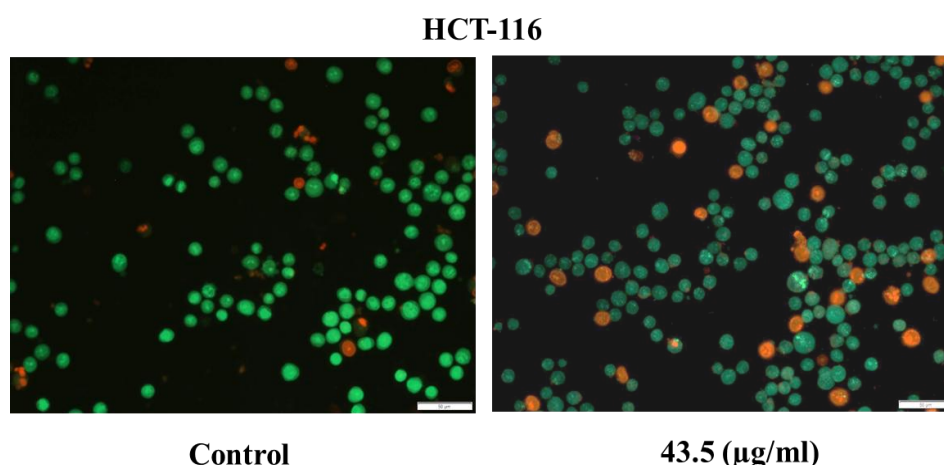


Figure 7: The apoptotic morphology of HCT-116 cells upon treatment with DB.

Apoptotic changes in HCT-116 cells treated with DB (IC₅₀ concentration) were assessed by AO/EtBr dual staining. Untreated cells exhibit green in colour, whereas

apoptotic cells are observed in red/orange colour (Magnification, x100).