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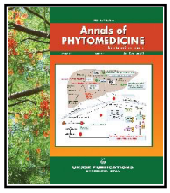

Original Article : Open Access

Phytochemical analysis of *Pithecellobium dulce* (Roxb.) Benth. unripe aril *via* LC-MS/MS and evaluation of its antiproliferative activity against human colon carcinoma cells

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Abstract

The present study aimed to identify the bioactive compounds from the *Pithecellobium dulce* (Roxb.) Benth. unripe aril *via* LC-MS/MS (liquid chromatography-tandem mass spectrometry) and evaluate its antiproliferative activity against human colon carcinoma cells. Unripe aril powder was extracted with methanol and water (70:30). The LC-MS/MS chromatogram showed the presence of 20 different phytochemical compounds such as justicidin B, 2-hydroxyquinoline, hyoscyamine, kaempferol, cyanidin, hypoxanthine, chicoric acid, roemerine, amentoflavone, and cinnamic acid, *etc*. An extensive literature survey revealed the reported anticancer properties of these compounds. PD fruit and bark extracts have already reported cinnamic acid, kaempferol, morin, and catechin B. The extract demonstrated dose-dependent antiproliferative effects against HT-29 and HCT-15 cells with IC_{50} values of 62.5 µg/ml by MTT assay. Moreover, the extract enhanced the reactive oxygen species level, altered cellular morphology, and induced apoptosis as analyzed by fluorescence microscopy. Our current findings suggest PDU produces significant levels of reactive oxygen species (2.5 fold), which may cause apoptosis by disrupting the balance between the oxidant and antioxidant enzyme systems to alter the redox balance in the cell. Interstingly, PDU at the highest concentration (125 µg/ml) tested significantly enhances the release of lactate dehydrogenase (LDH, 4-fold) in HT-29 and HCT-15 cells. This result suggests the involvement of necrosis in the cytotoxic effect of PDU. In conclusion, our results suggest that *P. dulce* unripe aril contains potent anticancer agents that demonstrate significant antiproliferative and apoptotic activity against human colon carcinoma cells. Therefore, the consumption of unripe fruits of *P. dulce* has important gut and digestive health benefits.

1. Introduction

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 treatment 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 (Baskar *et al*., 2014). Therefore, safe, non-toxic anti-tumour therapy is still a long way from reality. During the multistage process of cancer formation, normal cells transform into tumour cells, including the transition from precancerous lesions to malignant tumours (Yu *et al*., 2022). The interactions between genetics and physical factors (ultraviolet radiation), chemical factors (tobacco and asbestos), and biological factors (viruses and bacteria) result in these changes (Woo *et al*., 2022). 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 (Pramesh *et al.,* 2022). Therefore, stomach and colorectal cancer contribute to the high burden of the disease in developing countries leading to 17% of mortality (Xi and Xu, 2021). Having these in mind, patients rely on traditional, complementary medicine, which is less costly and widely available. As a result, findings that reveal the natural, semi-synthetic compounds demonstrating selective cytotoxicity against colon cancer cell lines can be promising drug empirical targets for the development of new chemotherapeutics against colon cancer. Therefore, the food and pharmaceutical industries have become increasingly interested in analyzing medicinal plants.

Pithecellobium dulce (Roxb.) Benth. (*P. dulce*, PD) has been well-recognized in traditional medicine for treating various ailments owing to its curative properties (Saeed Kotb *et al.*, 2022). The bark and pulp possess astringent and hemostatic properties and are used to treat toothache, gum ailments, and bleeding. Furthermore, the bark extracts are used to treat chronic diarrhoea and dysentery. The leaf extract is also used to treat indigestion and wounds. Besides, its powdered seeds are used to treat ulcers. An extensive survey of literature revealed that several phytochemical constituents have been reported from leaf followed by fruit extracts of *P. dulce* using gas

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chromatography-mass spectrometry (GC-MS). Methanol, water, and ethanol or the combination of methanol and water, ethanol and water seem to be the preferred extraction medium. Recent publications by Dhanisha *et al*. (2022) have shown the anticancer effects of ripe fruit ethanolic extracts through modulating TNF-α and proinflammatory cytokines. The same authors and another study by Saeed Kotb *et al.* (2022) are the only investigations to have examined the bioactive intermediates of PD using LC-MS/MS. However, there is not much information available on the phytochemical components and biological activity of the unripe aril/pulp extract of PD. The aril is consumed raw, roasted, cooked, or curried (Nagmoti *et al*., 2012; Rao, 2013). The unripe PD (PDU) aril has exceptional nutritional qualities given its high protein, carbohydrate, polyphenol, and provitamin contents to be a functional food. Our preliminary results indicated that PDU food components have the potential to improve general physiological functions and reduce the risk of developing chronic diseases. LC-MS/MS offers advantages over GC-MS in the ability to identify and measure a broader range of compounds. Therefore, the present investigation was designed to determine the bioactive compounds in the hydromethanolic extract of PDU by LC-MS/MS. Further, since PDU has been widely used traditionally to combat gastric problems, the *in vitro* cytotoxic effect was evaluated against human colorectal carcinoma cells HT-29 and HCT-15.

Figure 1: Images of *P. dulcee* unripe fruit and aril with seeds.

2. Materials and Methods

2.1 Collection, drying, and authentication of *P. dulce* unripe aril samples

The *P. dulce* trees at Kukkrahalli lake, Manasagangotri, University of Mysuru campus were authenticated by Dr. M. S. Sharada, Professor, and Chairman, DOS in Botany, Manasagangotri, Mysuru (UOMBOT21PD03). The unripe arils suitable and appropriate for picking are collected during April end. The unripe aril was identified based on the unopened green round pods containing green aril and dark green seeds (Figure 1). The unripe fleshy aril of PD (1 kg) following removal of seeds was washed thoroughly, cabinet dried, and subjected to size reduction to a coarse powder using a grinder.

2.2 Preparation of hydromethanolic extracts of PD using Soxhlet apparatus

The PD powder prepared above was defatted overnight (n-hexane), subsequently packed into the Soxhlet apparatus (200 g) (Labmatrix, Karnataka, India), and extracted with methanol and water (70:30). The extraction was continued until the solution in the thimble became clear. Insoluble materials were removed by filtration. The extract was concentrated under reduced pressure at 60 ± 1°C in a rotary vacuum evaporator (Steroglass, Strike 300, Italy) till solid to semisolid mass was obtained and stored in an air-tight container in a refrigerator (<4°C) until chemical analysis was performed. The extracts were dissolved in dimethyl sulfoxide (DMSO) to obtain a stock solution. Working stocks were prepared in DMEM medium without foetal bovine serum (FBS), keeping the final concentration of DMSO less than 0.4%. These were filter sterilized through 0.22 µ filters (millipore) to be used for *in vitro* studies.

2.3 LC-MS/MS analysis

PD unripe fruit extract (5 mg) is dissolved in 2 ml of methanol, filtered, and used for LC-MS/MS analysis. The Acquity H-class UPLC (Waters Corporation, Milford, MA, USA) was employed, which had an integrated vacuum degasser, automatic sample manager (Waters Corporation, Singapore), ultra-performance binary solvent manager (Waters Corporation, Singapore), and injection volume range of up to 100 µl with an optional extension loop. A C18 stationary phase (Accucore C18, 50 x 4.6 mm, 2.6 µ) was used for chromatographic separation. A Xevo G2-XS QToF (Waters Corporation, Wilmslow, UK) was employed for mass spectrometric (MS) detection. The mobile phase is made up of 0.1% formic acid in water as an aqueous phase (A) and acetonitrile as an organic modifier (B), and it is delivered at a flow rate of 0.4 ml/min in the following gradient: initially, %B is kept at 5% for 1 min; 1 to 6 min (5 to 50% B) and held at 50% for 4 min, 10 to 12 min (50 to 95% B), held for 4 min, 16 to 17 min (95 to 5% B) and held at 5% for 3 min. The sample was injected in a volume of 5 µl. The column oven temperature was kept at an optimal level throughout the chromatographic run (22°C). For MS detection, a positive polarity electrospray ionization (ESI) source was used. The optimal instrument and acquisition parameters were as follows:

50 l/h. cone gas (nitrogen) flow; 750 l/h. desolvation gas (nitrogen) flow; 450°C probe temperature; 30 V sampling cone voltage; 150°C source temperature; 80 V source offset voltage; the collision energy ramp varies from 6 eV-50eV (Argon, collision gas), and a mass range of 50 to 2000 m/z. To acquire and process data, Waters Corporation's Mass Lynx software (V4.1, Milford, MA, USA) was used.

2.4 Cell lines and culture condition

Human molecular subtype colorectal cancer cells HT-29 and HCT-15 kindly provided by Professor M. Subba Rao, JSS Academy of Higher Education and Research, Mysuru. 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.

2.5 Cytotoxicity assays

The antiproliferative activity of PDU was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Himedia) assay (Soulef *et al*., 2021). After 48 h incubation of HT-29 and HCT-15 cell lines at the density of 1 x 10^4 cells/well cultured in a 96-well plate, cells were treated with different concentrations of PDU (7.8-1000 µg/ml) and cisplatin (positive control, 5 µM). Treated cells were incubated for additional 24 and 48 h. After the incubation period, MTT solutions (20 µl, 5 mg/ml) were added to each well and further incubated for 4 h. The MTT formazan precipitate was then solubilized in 100 µl dimethyl sulfoxide. Absorbance was measured at 570 nm using a microplate reader (Tecan Infinite m200 PRO, Austria). The inhibitory concentration (IC_{50}) value was estimated after 24 h of treatment using GraphPad Prism software.

2.6 Cellular morphology changes of HT-29 and HCT-15 cells following exposure to PDU

The effect of PDU on morphology changes of HT-29 and HCT-15 cells was observed under a microscope. The HT-29 and HCT-15 cells were grown in 6 well plates at the density of 1 × 10^5 cells/well. Following 48 h incubation, cells were treated with two concentrations (62.5 and 125 µg/ml) of PDU and incubated for another 48 h. The changes in the cellular morphology like apoptosis or necrosis characteristics were observed under a brightfield microscope.

2.7 Quantification of intracellular reactive oxygen species

Intracellular ROS production was assessed with 22,72-dichlorodihydrofluorescein diacetate (DCF-DA) as described. In brief, both colorectal cancer cells were cultured separately in 96 well plates at a density of 1 × 10^4 cells/well for 48 h. Further, 48 h incubation of cells with PDU at two concentrations were replaced with Hank's balanced salt solution (HBSS) containing DCF-DA (20 µM) for 30 min at 37°C in the dark. The fluorescence of DCF was monitored using a fluorescent microplate reader at excitation and emission wavelengths of 485 nm and 530 nm, respectively.

2.8 Lactate dehydrogenase release assay

The release of LDH from culture media was quantified using an assay-based kit method (agappe). Colorectal cancer cells were treated with PDU for 48 h as mentioned above (section). Next, cell supernatant was mixed with LDH mixture and incubated for 10 min in the dark at room temperature. The absorbance was measured at 490 nm.

2.9 Cell apoptosis by acridine orange/ethidium bromide dual stain

The apoptotic morphology of colorectal cancer cells was performed using acridine orange (AO) and ethidium bromide (EB) (live/dead) staining assay. In brief, cells were treated with PDU at two concentrations, stained with AO and EB (100 µg/ml each prepared in phosphate-buffered saline), mounted on slides, and incubated for 10 min in the dark. After incubation, stained cells were observed under fluorescence microscope at excitation (400-490 nm).

2.10 Statistical analysis

All experiments were carried out in 3 replicates and were repeated three times. The data were analyzed by one-way ANOVA, followed by the Tukey Test using Prism 5 (GraphPad) software (GraphPad Software Inc. Boston MA, USA). *p<0.05, and ***p<0.01 are considered as significant values.

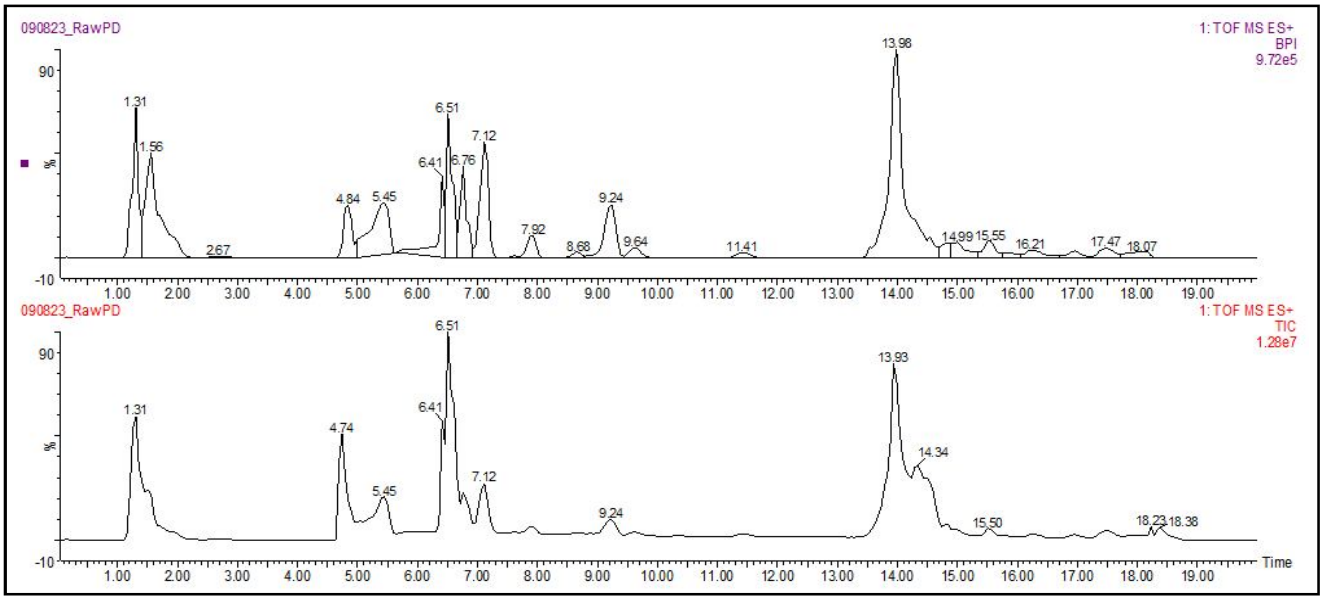


Figure 2: LC-MS/MS metabolic profile of the unripe fruit aril hydromethanolic extract of *P. dulce*.

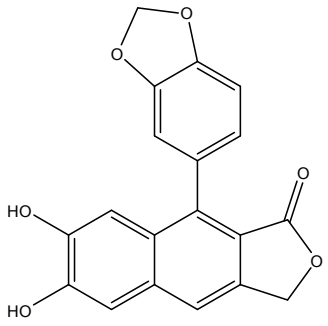
Table 1: Compounds detected in unripe fruit aril hydromethanolic extract of *P. dulce*.

S.No.	Retention time	Proposed metabolite (Chemical composition)	Mol.wt.	Adducts	Ontology	Activity
1	0.093	Justicidin B ($C_{21}H_{16}O_6$)	365.10	[M+H]+	Arylnaphthalene lignans	Anticancer and antiviral
2	1.204	Theophylline ($C_7H_8N_4O_2$)	219.01	[M+H]+	Xanthines	-
3	1.305	Perseitol ($C_7H_{16}O_7$)	230.13	[M+NH4]+	Sugar alcohols	-
4	4.539	2-Hydroxyquinoline (C_9H_7NO)	146.05	[M+H]+	Hydroquinolones	Derivative as anticancer
5	4.943	Hyoscyamine ($C_{17}H_{23}NO_3$)	290.17	[M+H]+	Tropane alkaloids	Antioxidant and anticancer
6	6.509	Kaempferol ($C_{15}H_{10}O_6$)	287.04	[M+H]+	Flavonols	Anticancer and anti-inflammatory
7	6.509	Cyanidin ($C_{15}H_{11}O_6$)	287.05	[M]+	7-hydroxyflavonoids	Anticancer
8	6.509	(-) Catechin ($C_{15}H_{14}O_6$)	291.09	[M+H]+	Catechins	Anticancer
9	6.509	Papaverine ($C_{20}H_{21}NO_4$)	340.15	[M+H]+	Benzylisoquinolines	Antispasmodic
10	6.56	Hypoxanthine ($C_5H_4N_4O$)	273.07	[2M+H]+	Hypoxanthines	Anticancer activity of derived ligand
11	6.56	Biochanin A ($C_{16}H_{12}O_5$)	285.13	[M+H]+	4'-O-methylisoflavones	Anti-inflammatroy
12	6.56	Morin($C_{15}H_{10}O_7$)	303.04	[M+H]+	Flavonols	Anticancer and anti-inflammatory
13	13.832	Isopalmitic acid ($C_{16}H_{32}O_2$)	279.22	[M+Na]+	Long-chain fatty acids	Anticancer
14	13.832	6-Benzoylheteratisine ($C_{29}H_{37}NO_6$)	496.26	[M+H]+	Benzoic acid esters	Na^+ channel activator
15	13.983	Chicoric acid ($C_{22}H_{18}O_{12}$)	497.06	[M+Na]+	Tetracarboxylic acids and derivatives	Anticancer, and anti-inflammatory
16	14.034	Roemerine ($C_{18}H_{17}NO_2$)	302.12	[M+Na]+	Aporphines	Anti-tumour
17	14.084	L-arginine ($C_6H_{14}N_4O_2$)	175.12	[M+H]+	L--amino acids	-
18	14.135	Amentoflavone ($C_{30}H_{18}O_{10}$)	539.08	[M+H]+	Biflavonoids and polyflavonoids	Anticancer
19	14.236	Cinnamic acid ($C_9H_8O_2$)	149.01	[M+H]+	Cinnamic acids	Anticancer
20	15.549	2-thiocyanatopupukeanane ($C_{16}H_{25}NS$)	285.14	[M+Na]+	Sesquiterpenoids	-

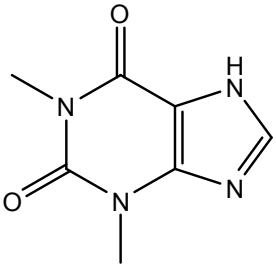
3. Results

3.1 Identification of bioactive compounds

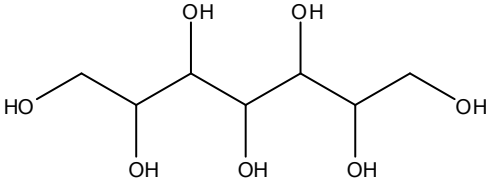
The unripe fruit aril of *P. ducle* is underexplored both in terms of phytochemical and related pharmacological activity. The literature survey indicated methanol and water mixture is a suitable medium for extraction of bioactives for GC-MS and LC-MS/MS characterization. Therefore, we used 70:30, methanol-water mixture for extraction. The hydromethanolic-derived crude extract obtained following vacuum evaporation from PDU was sticky, and light green, with a yield of 13.2% w/w. The LC-MS/MS metabolic profile of the unripe fruit aril hydromethanolic extract of *P. dulce* is given in Figure 2. The unripe fruit extract showed the presence of 20 phytochemicals. The proposed metabolite name, chemical composition, retention time, and m/z ratio is given in Table 1. The ontology and reported biological activity are also given. The chemical structure of the identified phytochemical compounds is provided in Figure 3.



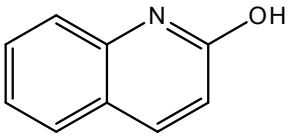
Justicidin B



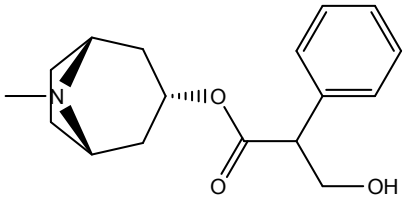
Theophylline



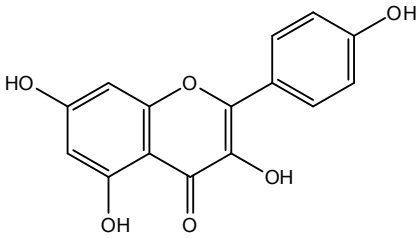
Perseitol



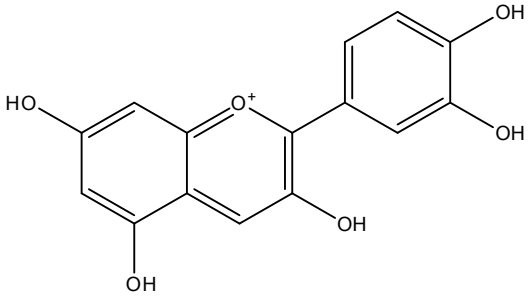
2-Hydroxyquinoline



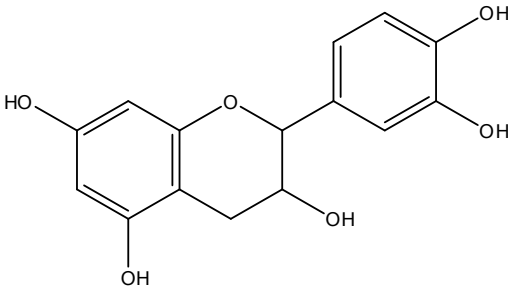
Hyoscyamine



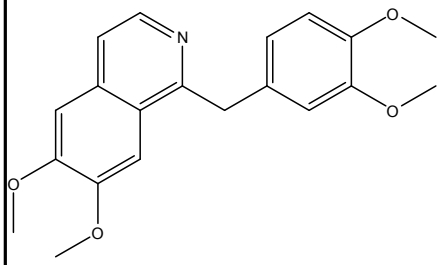
Kaempferol



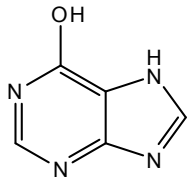
Cyanidin



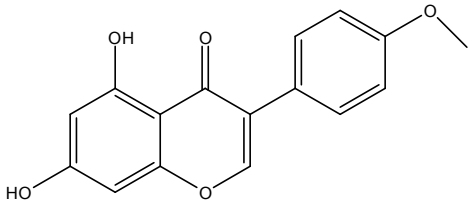
(-) Catechin



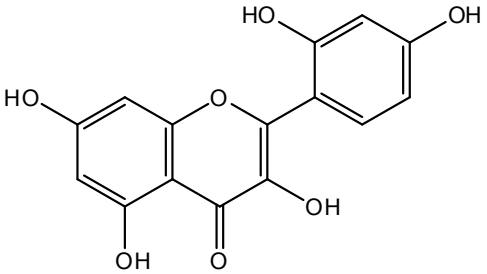
Papaverine



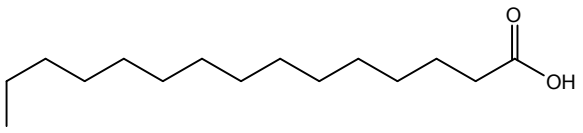
Hypoxanthine



Biochanin A



Morin



Isopalmitic acid

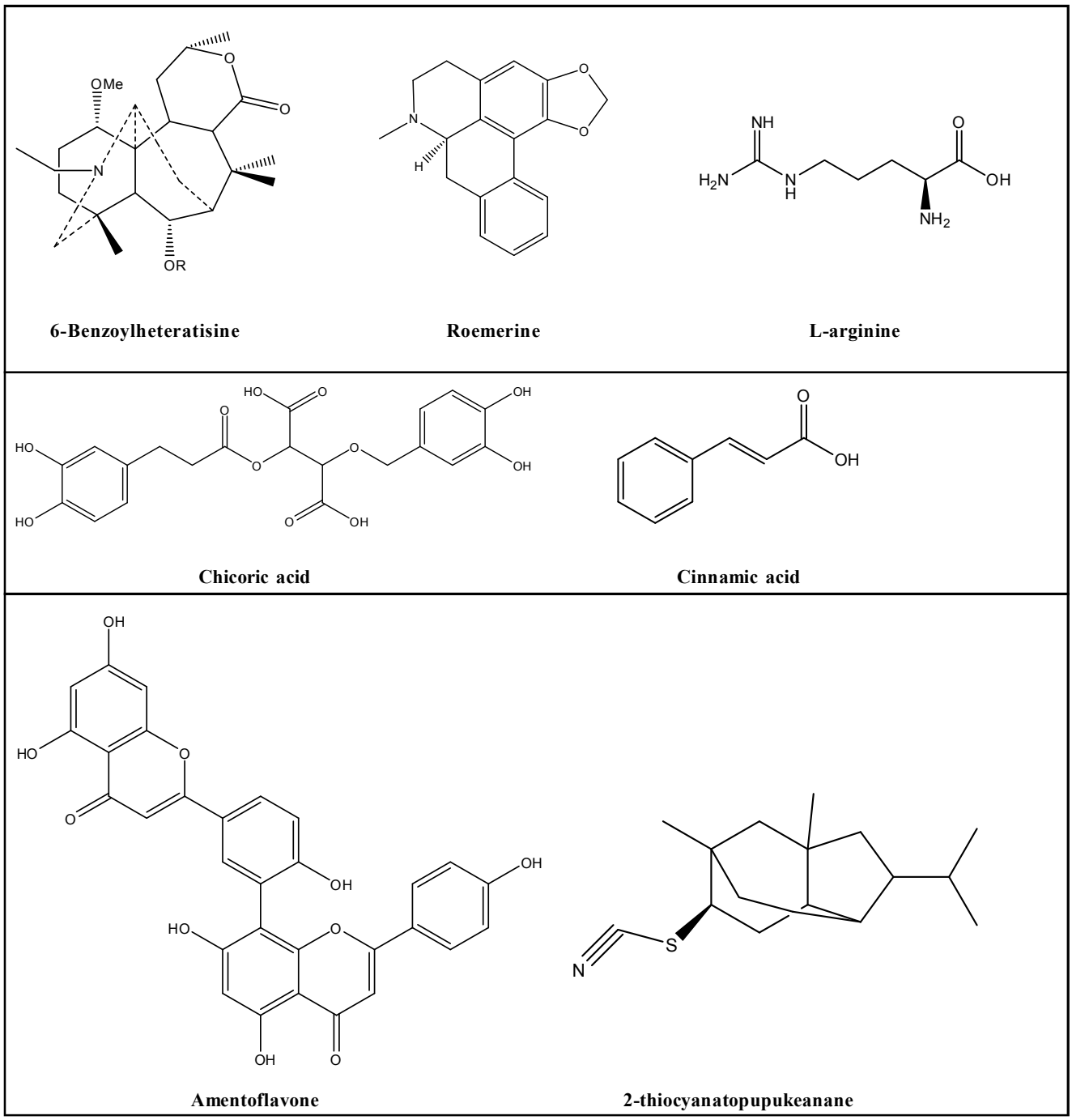


Figure 3: Chemical structures of the identified compounds in *P. dulce* unripe fruit hydromethanolic extract by LC-MS/MS.

3.2 PDU inhibits colorectal cancer cell proliferation

Initially, we evaluated the inhibitory effect of various concentrations of PDU on different molecular subtype human colorectal cancer cell lines (HT-29 and HCT-15) for 24 and 48 h periods using the MTT assay. Results indicated concentration-dependent inhibition of cancer cell proliferation. Comparatively, 48 h treatment of PDU exhibits the highest inhibitory effect than 24 h. PDU treatment to HT-29 cells at the concentration of 7.8, 15.6, 31.25, 62.5, 125, 250, 500, and 1000 µg/ml for 48 h inhibits cell proliferation by 3.7, 10.6, 18.2, 29.0, 51.7, 73.8, 86.7, and 100%, respectively. Similar results were also observed for HCT-15 cells (inhibitory rate 1, 11.6, 23.2, 31.7, 46.5, 64.9, 82.0, and 94.7%). Our results indicate that PDU showed to some extent better cytotoxicity towards HT-29 cells (Figure 4). Further, GraphPad Prism software was used to calculate the half-maximal inhibitory concentration (IC_{50}). Both cell lines showed the (IC_{50}) value at 62.5 µg/ml. Further, two concentrations (62.5 and 125 µ/ml) were chosen for subsequent experiments.

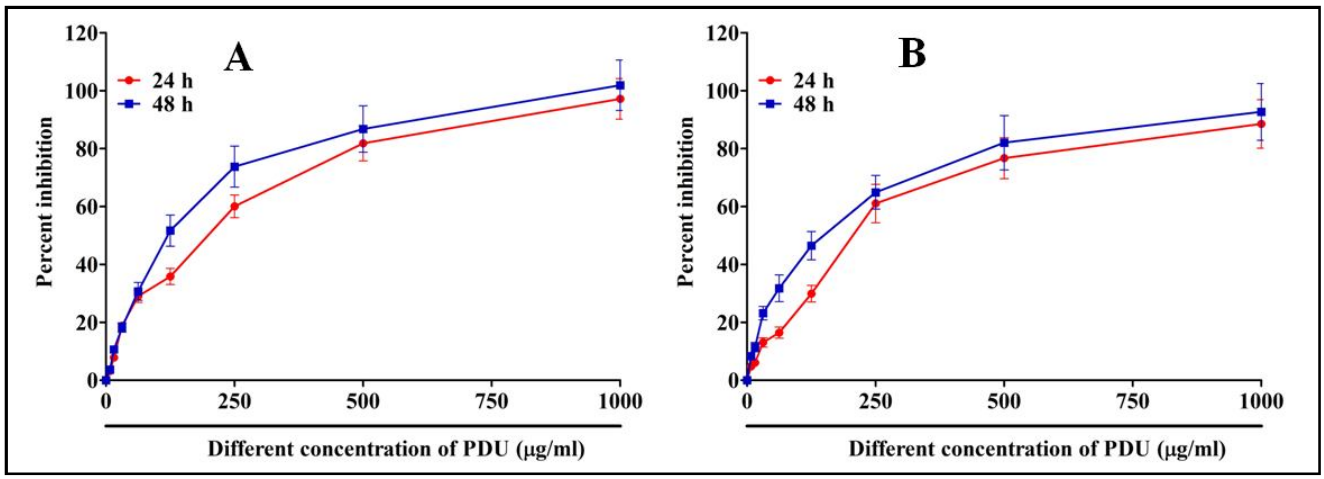


Figure 4: Cytotoxicity effect of unripe hydromethanolic extract of *P. dulce* on HT-29 and HCT-15 cells. Both cells were treated separately with various concentrations of PDU (7.8-100 µg/ml) and cytotoxicity effects were carried out using MTT assay. (A) MTT assay for HT-29 cells, and (B) HCT-15 cells. Data were represented as mean ± SD (n=6).

3.3 PDU alters the cellular morphology of HT-29 and HCT-15 cells

To explore the cellular morphology changes in HT-29 and HCT-15 cells stimulated with PDU were visualized using an inverted brightfield microscope. The results showed that PDU treatment is more sensitive to HT-29 cells than HCT-15 cells. Cultured HT-29 and HCT-15 cells are well attached to the culture plate and their confluence does not appear to be affected. The results of these experiments are in agreement with those of the viability experiments. Interestingly, PDU-treated cells exhibited typical characteristics of apoptosis, such as cellular shrinkage, apoptotic bodies, membrane blebbing, and nucleus condensation (Figure 5). Cells treated with PDU were markedly more prominent with these morphological features. At the highest concentration tested, 125 µg/ml, both HT-29 and HCT-15 cells appeared disintegrated. These results reinforce the viability data and indicate a cytotoxic effect.

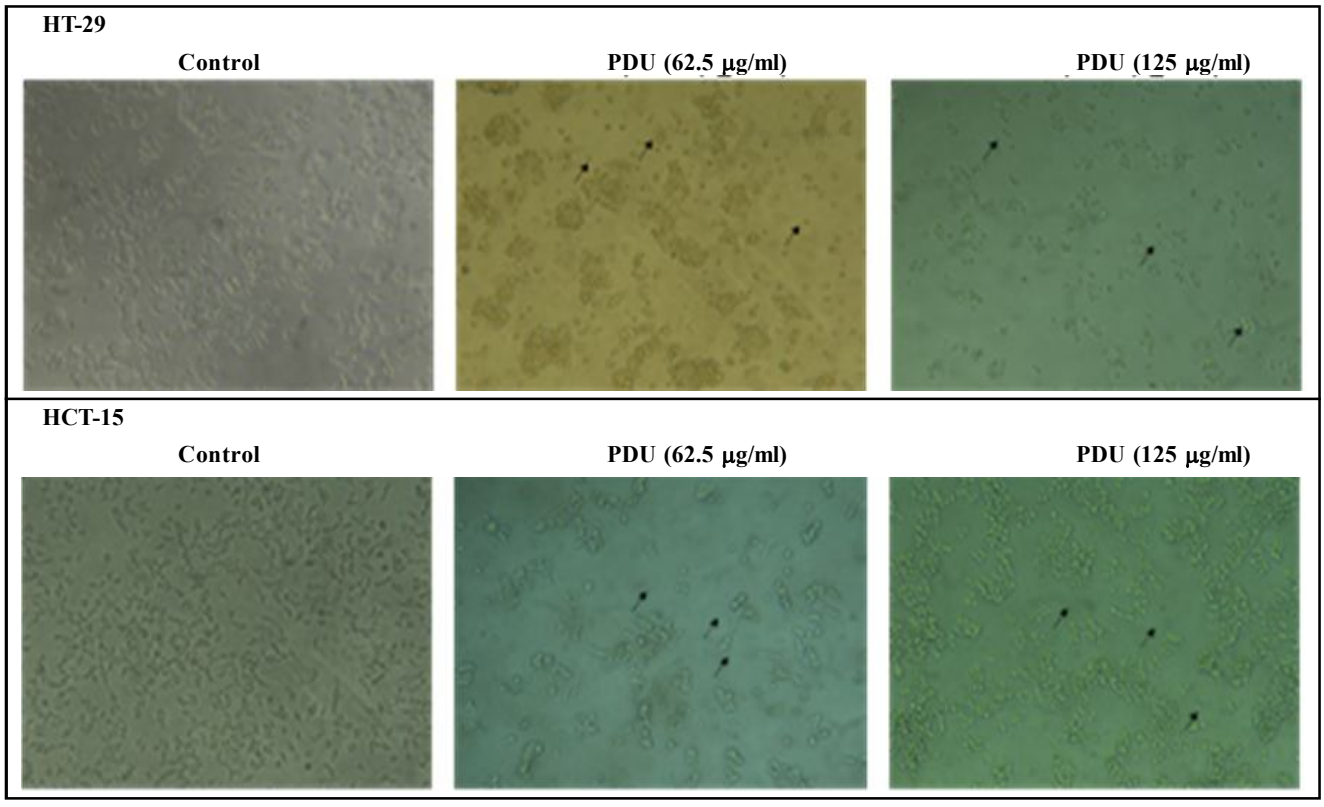


Figure 5: Effect of unripe hydromethanolic extract of *P. dulce* aril on cellular morphology of HT-29 and HCT-15 cells. Cells were treated with two concentrations of PDU (62.5 and 125 µg/ml) and cellular morphology was visualized and captured using an inverted brightfield microscope.

3.4 Production of ROS level in PDU-treated HT-29 and HCT-15 cells

As ROS are capable of causing cell death, DCFDA fluorescence-based fluorometry assay was used to analyze ROS generation in PDU treated HT-29 and HCT-15 cells. High fluorescence intensity was observed in PDU treated cells at a concentration of 125 µg/ml (Figure 6). Comparatively, ROS production levels were slightly increased in HT-29 cells (42, 68, and 110% at the concentrations of 31.125, 62.5, and 125 µg/ml, respectively) than in HCT-15 cells (34, 60, and 97% at the concentrations of 31.125, 62.5, and 125 µg/ml, respectively). Our findings suggest PDU 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 in the cell.

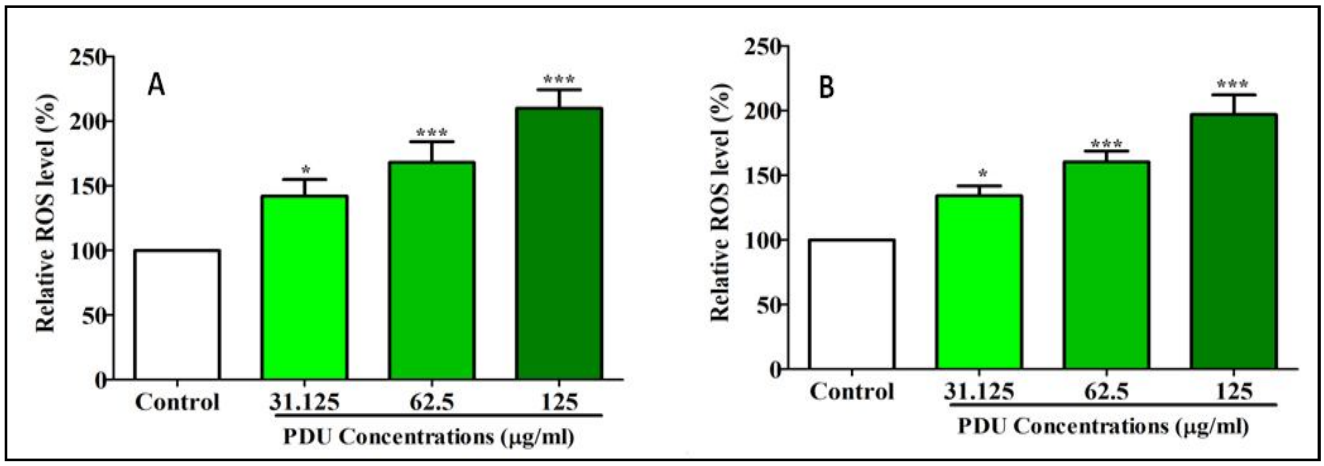


Figure 6: Intracellular ROS level in HT-29 and HCT-15 cells treated with unripe hydromethanolic extract of *P. dulce*. HT-29 (A) and HCT-15 (B) cells were treated separately with indicated concentration for 24 h, and accumulation of ROS levels was analyzed using fluorometric method. Data were represented as mean ± SD (n=6), *$p<0.05$, and ***$p<0.01$ are considered as significant values.

3.5 PDU increases LDH release in HT-29 and HCT-15 cells

LDH release is an accurate assessment of cell viability and membrane integrity. After treatment with PDU for 48 h, the release of LDH in the supernatant of cell medium was measured using kit-based assay. As shown in Figure 7. PDU at the highest concentration (125 µg/ml) significantly enhances the release of LDH in both HT-29 and HCT-15 cells when compared to the control. This result suggests the involvement of necrosis in the cytotoxic effect of PDU.

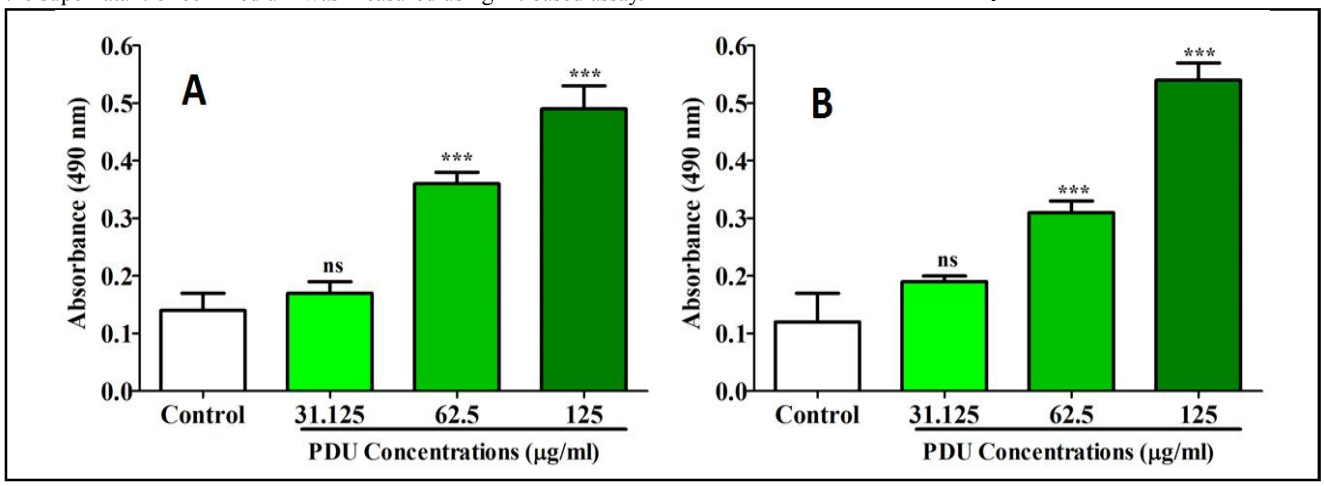


Figure 7: Release of LDH from hydromethanolic extract of *P. dulce* treated HT-29 (A) and HCT-15 (B) cells supernatant. HT-29 and HCT-15 cells were treated separately with indicated concentration for 24 h, and release of LDH were analyzed using kit-based assay. Data were represented as mean ± SD (n=6), ***$p<0.01$ are considered as significant values.

3.6 Apoptosis caused by PDU treatment in HT-29 and HCT-15 cells

Using acridine orange/ethidium bromide differential staining method, morphological changes caused by PDU were investigated. Uniformly, green live and normal morphology were observed in untreated HT-29 and HCT-15 cells which indicates no sign of apoptosis. The colorectal cancer cells treated with PDU exhibit irregular, shrunken, and red/orange fluorescence (Figures 5 and 8). 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 PDU, the number of apoptotic cells increased.

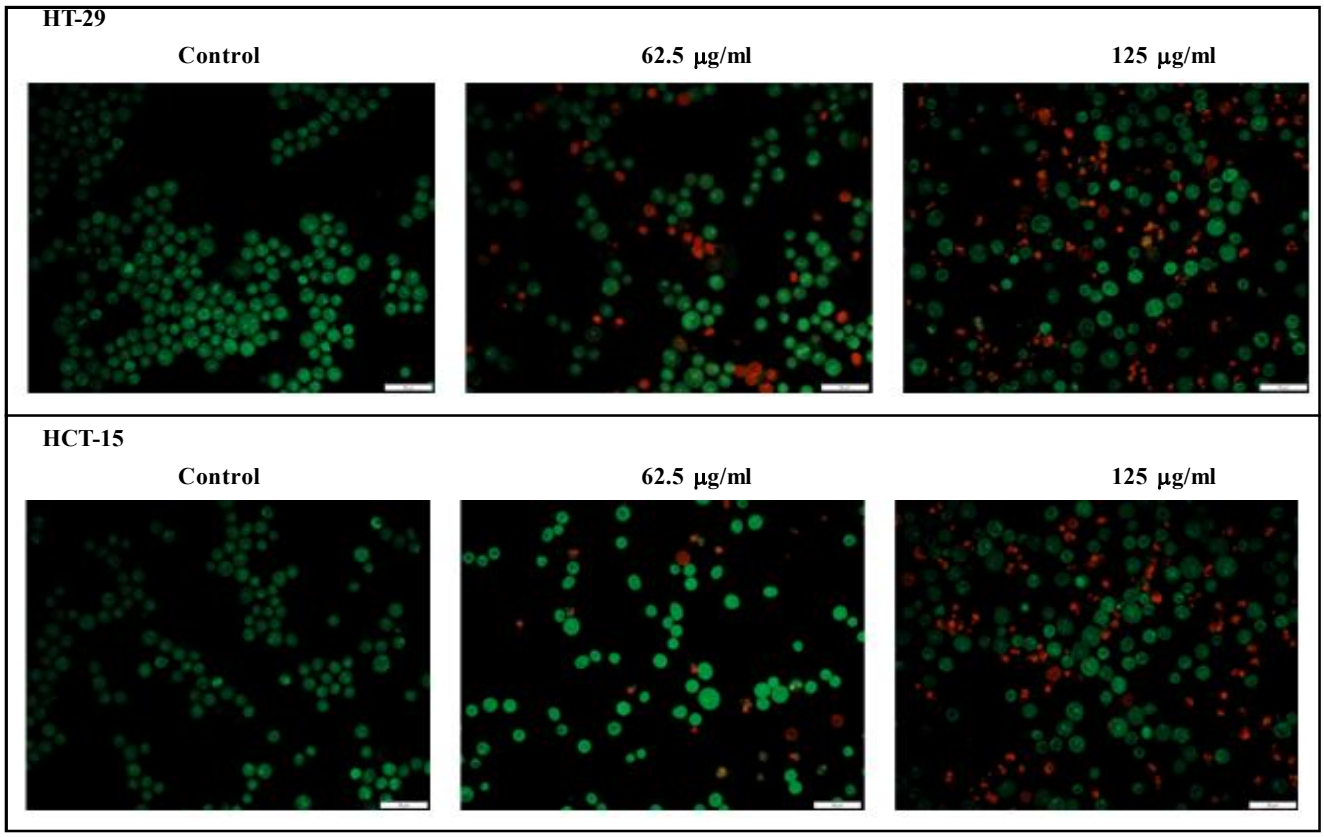


Figure 8: The apoptotic morphology of HT-29 and HCT-15 cells upon treatment with PDU. Apoptotic changes in HT-29 and HCT-15 cells treated with PDU were assessed by AO/EtBr dual staining. Untreated cells exhibit green in color, whereas apoptotic cells are observed in red/orange color (Magnification, x100).

4. Discussion

Cancer is a major disease with millions of patients diagnosed each year with high mortality around the world. Phytochemicals have been considered conducive to cancer prevention (Arif *et al*., 2022). Several preclinical studies have established plant derived dietary substances as suitable candidates for treating various types of cancers. Such phytochemicals can prevent the action of carcinogens thereby suppressing cancer development (Khan and Ahmad, 2021). Hence, the risk of cancer can be repressed by eating more fruits, vegetables, and grains. Several studies show significant evidence for cancer preventive effects with fruit and vegetable consumption as they are good sources of vitamins and fibers (Srivastava *et al*., 2014; Rajashekar *et al*., 2021). Studies have explored the anticancer properties of different fruits, both in terms of their extracts and bioactive ingredients. It has been shown that *Carica papaya* extracts can induce cytotoxicity. Juices of pomegranate and citrus fruits have been specifically found to be effective in preventing colon cancer. Besides, administration of concentrated extracts of *Morinda citrifolia* in tumour containing animals increased the immune response of the animals with a concomitant reduction in tumour burden. Bioactive phenolic components of cherries have also been shown to possess anticancer properties. Recent studies have shown that strawberry fruits possess both cancer preventive and therapeutic values. In particular, fruit ingredients obtained at various stages of ripening are good targets for gastrointestinal cancer agents. Therefore, research on bioactives from underutilized fruits has been rapidly gaining momentum in biological and pharmacological research (Block *et al*., 1992; Sangeeta *et al*., 2023). However, little is known about the anticancer potential of one of the underutilized fruit *P. dulce,* and its bioactives. Therefore, in the present study, we show the bioactive compounds by LC-MS/MS and the *in vitro* cytotoxic effect against human colorectal carcinoma cells HT-29 and HCT-15 (Table 1; Figures 3 and 4).

LC MS/MS analysis of PDU revealed the presence of 20 bioactive compounds which belong to quinolones, alkaloids, flavonoids, isoflavones, and terpenoids. The majority of the bioactives identified by this study have reported anticancer potential. Justicidin B is reported to be present in *Phyllanthus brasiliensis* and its derivatives show anti-viral and anticancer potential by modulating Bax/Bcl-2 in human melanoma cells A375 (Al-Qathama *et al*., 2017). 2-Hydroxyquinoline is reported from *Houttuynia cordata, Aconitum ferox,* and *Glycosmis pentaphylla* and its derivative has anticancer properties (Bindu *et al*., 2019). Hyoscyamine is reported in *Morinda pubescens* leaf hexane extract with antioxidant and cytotoxic effects on human liver cancer cell lines (Kumar and Santhi, 2012).

Kaempferol is abundant in cruciferous vegetables, including broccoli, kale, spinach, and watercress, as well as in herbs like dill, chives, and tarragon. Recent findings have indicated the promise of applying kaempferol in disease prevention due to its potential antioxidant, and antimutagenic properties (Sengupta *et al*., 2022). Kaempferol was recently reported to be present in *P. dulce* fruit ethanol extract by Dhanisha *et al*., (2022). Cyanidin is a type of anthocyanin present in pigmented leaves, fruits, and flowers; distributed widely in berries,

apples, and oranges possesses anticancer activities. Safdar *et al*., (2023) reviewed the potential of using cyanidin to treat various cancer types including breast, liver, lung, prostate, and thyroid cancer. Similarly, studies by Sheng (2020) showed the anticancer effects of catechin flavonoid by targeting mitogen activated protein kinase-extracellular signal-regulated kinase (MAPK/ERK) signaling pathway in human glioma cells mediated *via* autophagy induction, cell cycle arrest, and targeting. Hypoxanthine is reported from extracts of cauliflower and soybean, its derived ligands have been evaluated for *in vitro* anticancer and anti-inflammatory activities (Køikavová *et al*., 2014). Biochanin A is reported in chickpea, red clover, and legume extracts and it exerts anticancer potential against multiple myeloma by targeting the CD38 and cancer stem-like cells by modulating the nuclear factor kappa-B (NF-κB) and MAPK pathways (Jaina *et al*., 2022). Morin is another compound detected by LS-MS/MS that is already reported to be present in *P. dulce* fruit extracts. Morin is a powerful therapeutic candidate for the treatment of HER2 overexpressing breast cancer. It suppresses the human epidermal growth factor receptor (HEGFR) signaling pathway, induces cell death by inhibiting the HEGFR signaling, and suppresses metastatic potential (Lee *et al*., 2021).

Isopalmitic acid is present in many plants including wild bottle gourd (*Lagenaria siceraria*). The alkaloid 6-benzoylheteratisine is reported from *Aconitum tanguticum* and inhibits voltage-gated Na+ channels (Attar and Ghane, 2018). Chicoric acid is studied from *Echinacea purpurea*, Moench, and other plants, which induces autophagy in gastric cancer by promoting the endoplasmic reticulum stress pathway (Sun *et al*., 2019). Roemerine is detected in the extracts of Papaver and Stephania species of plants and reported to have anti-tumour potential against prostate cancer (Ma *et al*., 2017). L-arginine is an α-amino acid, that is present in many plant extracts ubiquitously and reported to have positive effects with potential benefits for chemotherapy (Satoh *et al*., 2020). Amentoflavone is a well-known biflavonoid occurring in many natural plants. This polyphenolic compound has some important bioactivities. Especially, amentoflavone is involved in anticancer activity by mediating various signaling pathways such as ERK, NF-κB, *etc*. (Chen *et al*., 2022). Therefore, amentoflavone is considered to be a promising therapeutic agent for clinical research. Cinnamic acid is an unsaturated carboxylic acid, that occurs naturally in several plants. Cinnamic acid contains a ubiquitous α and β-unsaturated acid moiety presenting potential therapeutic effects in the treatment of cancer. Its derivatives act on cancer cells by diverse mechanisms and serve as critical scaffolds in discovering novel anticancer agents (Feng *et al*., 2022).

The cytotoxic activity of PDU in the present data is mainly attributed to constituents such as justicidin B, hyoscyamine, kaempferol, cyanidin, catechin, morin, chicoric acid, roemerine, amentoflavone, and cinnamic acid which are known cytotoxic compounds. Several of the above agents are reported to promote apoptosis *in vitro* by increasing ROS which is the mechanism shown in this study (Figure 6). Cumulatively, there was a 2-to-2.5-fold increase in ROS in PDU treated HT-29 and HCT-15 cell extract compared to untreated cells. There are various methods of detecting apoptosis e.g., TUNEL, Annexin V assay, *etc*. Assays such as release of lactate dehydrogenase activity assay have been used to detect cell necrosis. In the present study, a double staining method [AO and EtBr (live/dead)] was employed for the detection of apoptosis and necrosis. Apoptotic cells also show morphological changes such as chromatin condensation and nuclear fragmentation. The colorectal cancer cells treated with PDU exhibit irregular, shrunken, and red/orange fluorescence (Figures 5 and 8). The changes in morphology resulted in nuclear fragmentation and chromatin condensation, which were indicators of the onset of apoptosis. Interestingly, we observed that at 125 µg/ml concentration PDU treated cells released four-fold more LDH (Figure 7) indicating a significant necrosis effect which could be attributed to the presence of many cytotoxic and necrotic agents in PDU, however it warrants further scientific investigation.

5. Conclusion

As a result, the hydromethanolic extract of *P. ducle* unripe aril showed 20 bioactive metabolites by LC-MS/MS. Four compounds cinnamic acid, kaempferol, morin, and catechin are already reported from bark and fruit extracts of *P. dulce*. Additionally, 16 new compounds are identified in this study. Many of these compounds have reported anticancer activity. Further, PDU demonstrates a strong anti-proliferative effect against HT-29 and HCT-15 colorectal tumour cells with IC_{50} of 62.5 µg/ml. The extracts also induced apoptosis mediated by ROS generation as assessed by AO/EtBr dual staining. Interestingly, PDU at higher concentrations significantly enhances the release of LDH following treatment compared to the control. This result suggests the involvement of necrosis in the cytotoxic effect of PDU, which requires additional investigation. This study strongly supports the ethnopharmacological use of unripe fruit as a source of potent anticancer drugs and has health benefits.

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Conflict of interest

The authors declare no conflict of interest relevant to this article.

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