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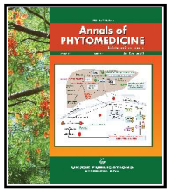

Original Article : Open Access

Comparative evaluation of bioactive phytochemicals and cytotoxic activity of unripe and ripe aril extracts of *Pithecellobium dulce* (Roxb.) Benth.

M. S. Suprada Rao*, P. Pramod Kumar, Nataraju Angaswamy*** and A. C. Sharada**

**Department of Biochemistry, Yuvaraja's College, University of Mysuru, Mysuru-570 005, KA, India*
***Department of Biochemistry, CSIR-Central Food Technology Research Institute, Mysuru-570020, KA, India*
****Department of Studies and Research in Biochemistry, Karnataka State Open University, Mukthagangotri, Mysuru-570 006, KA, India*

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Abstract

The use of folkloric herbal resources as complementary and alternative remedies has been a part of human history for centuries. Researchers have developed drugs from these remedies for many decades. The *Pithecellobium dulce* (Roxb.) Benth. has ethnobotanical records throughout the world, including India. In the present investigation, hydromethanolic extracts of unripe (PDU) and ripe (PDR) aril of *P. dulce* fruits were characterized and compared by GC-MS analysis. Next, we evaluated the anticancer effect of PDU and PDR against human colorectal carcinoma (HCT-116) cells. As a result, GC-MS analysis revealed the presence of 12 similar compounds in both PDU and PDR extracts. In addition, 8 and 31 other compounds were identified in PDU and PDR extracts, respectively. Further, PDU and PDR extracts showed dose-dependent manner anticancer effects against HCT-116 cells with IC_{50} values of 70 and 62.5 µg/ml. Moreover, both extracts enhanced the reactive oxygen species level, altered cellular morphology, and induced apoptosis as analyzed by fluorescence microscopy. Collectively, the present data demonstrated that many phytochemicals present in *P. dulce* unripe and ripe aril display anticancer effects against colon cancer supporting the plant's use primarily for the treatment of gastrointestinal disorders in traditional medicine.

1. Introduction

Worldwide cancer is the second greatest cause of death. Despite tremendous advances in treatment technology, over 10 million deaths are reported due to cancer by WHO. Chemotherapy is effective against diverse types of cancer; the long-term side effects remain a major concern (Nurgali *et al*., 2018). The strive to find drugs that effectively target cancer without harming the normal cells is a hallmark of the cancer. Besides, the pharmaceutical industry failed to develop successive strategies for cancer treatment. Therefore, the exercise for newly developed and improved anticancer agents is a necessary and relentless process. Cancer cells have abnormal cell cycle progression and are apoptosis-resistant. Unhealthy food, free radical exposure, and environmental factors have long been associated with a high risk of the development of cancer (Masdor *et al*., 2022). Numerous cytotoxic agents are known with novel experimental approaches to enhance therapeutic effects by stimulating apoptotic pathways (Singh *et al*., 2022). However, there is still uncertainty about the causes and exact molecular mechanism of cancer development.

Currently, several plant bioactives are used to treat different types of cancer (Esmeeta *et al*., 2022). These natural bioactive compounds have been known for over 50 years to be essential regulators of cellular proliferation and differentiation. Their pharmacological and physiological importance in the regulation of cell death has only been recently recognized. Such bioactives are shown to be less harmful than synthetic drugs. Dietary natural food has gained much attention on the prevention and treatment approach for various cancers (Kamal *et al*., 2022). Fruits, seeds, and grains consumed as food possess bioactive phytochemicals, which cure various ailments. Prospective studies have shown greater intake of fruits and vegetables was associated with a significant reduction in the risk of cancer (Allaqaband *et al*., 2022; Sangeeta *et al*., 2023). Particularly, phytomedicines from fruits are powerful antioxidants with anti-inflammatory potential (Afnan *et al*., 2022). Fruit extracts are rich sources of vital pro-vitamins that are required for normal cellular activities and healthy growth (Rajashekar *et al*., 2021). In the last decade, studies have demonstrated the anti-proliferative potential of various fruit, pulp, peel, and seed phytochemicals (Arif *et al*., 2022). Further, the level of antioxidant, anti-inflammatory, and radical scavenging phytochemicals is greatly affected by the stages of growth, processing, and storage of fruits.

Pithecellobium dulce (Roxb.) Benth. (PD) plant extracts contain various bioactive metabolites with health-promoting potential. Its members are often referred to as Manila tamarind since they closely resemble tamarind. PD fruit has been found to contain a variety of antioxidants that promise therapeutic potential (Dhanisha *et al*., 2021). The presence of glycosides, terpenoids, saponins, alkaloids, tannins, and flavonoids has been reported from root, bark, leaf, and fruit extracts of PD (Dhanisha *et al*., 2021). The specific concentration and types of phytochemical profiles varied based on factors such as age, environmental conditions, and geographical location. The specific health benefits of phytochemicals were complex and depended on various factors. The literature review shows phytochemical and

Corresponding author: Dr. A.C. Sharada
Professor, Department of Biochemistry, Yuvaraja's College, University of Mysuru, Mysuru-570 005, KA, India
E-mail: suprada1212@gmail.com
Tel.: +91-9620697355

anticancer studies on pulp, peel, and seeds of daily consumed fruits from other species (Ganga Rao *et al*., 2018). Various experiments have shown no significant cytotoxicity of PD extracts to untransformed healthy human cells. However, the research on the comparative study of phytochemical content and cytotoxic effect at different stages of fruits of PD is scarce. Hence, the present study was contemplated to evaluate and compare the phytochemical contents and cytotoxic activity in the unripe and ripe aril extracts of PD using GC-MS and human colon cancer cell lines (HCT-116), respectively.

***P. dulce unripe* fruit**

***P. dulce* ripe fruit**

Figure 1: Photographs of *Pithecellobium dulce* (Roxb.) Benth. unripe and ripe fruit.

2. Materials and Methods

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

The *P. dulce* trees at Kukkrahalli lake, Manasagangotri, University of Mysore campus were authenticated by Dr. M.S. Sharada, Professor, and Chairman, DOS in Botany, Manasagangotri, Mysuru (UOMBOT21PD03). The trees flower and bear fruits during April month. The unripe and ripe 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. The well-ripe fruits are identified by the change in the fruit peel color that turns from green to pinkish red or dark red (Figure 1). The unripe and ripe fleshy aril of PD following removal of seeds were washed thoroughly (1 kg, each), 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). Methanol and water are the solvents that can extract more diversity of compounds, which can explain the higher extraction efficiency. The extraction was continued until the solution in the thimble became clear. Insoluble materials were removed by filtration. The extracts were concentrated under reduced pressure at 60 ± 1°C in a rotatory 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 to obtain stock solution. Working stocks were prepared in DMEM medium without 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 GC-MS analysis

The GC-MS analysis of hydromethanolic extracts of *P. dulce* ripe and unripe arils were carried out using an Agilent 5597C system connected with a HP-5 MS fused silica column (5% phenyl methyl siloxane) with dimensions of 30.0 m × 250 µm × 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. Electro ionization mode (70 eV, ion source temperature at 230°C) was used with the quadrupole kept in a 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 was kept at 300 µl/min and 6000 µl/min, respectively, with a pressure of 7.0481 psi. Quad temperature was kept at 150°C, while the oven temperature initially was held at 80°C for 1 min then ramped to 130°C at the rate is 25°C/min, which was again ramped to 220 with the rate of 10°C/min and finally, it reached the temperature of 280°C with the rate of 5°C/min for the last 6 min. Start and end mass 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% of 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.

2.4 Cell culture and maintenance

Human colon cancer cell lines HCT-116 were maintained in DMEM containing 10% fetal bovine serum 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 cytotoxicity effect of PDU and PDR was confirmed through MTT (2.7.3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Trivedi *et al*., 2023). Briefly, cells were plated with 1×10^4 cells/well density, incubated overnight, and treated with PDU or PDR at various concentrations (7.8-1000 µg/ml) and cisplatin (positive control, 5 µM) for 24 h. Following the different treatments, MTT solutions (20 µl, 5 mg/ml) were added and incubated for another 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 m 200 PRO, Austria). The inhibitory concentration (IC_{50}) value was estimated after 24 h of treatment using GraphPad Prism software.

2.6 Cell morphology

The effect of PDU and PDR on morphology changes of HCT-116 cells was observed under a microscope. Briefly, cells were seeded in 6-well microplates at the density of 1×10^5 cells/well and incubated overnight. The cells were then treated with PDU or PDR at their IC_{50} concentration of 62.5 and 70 µg/ml, respectively, for 24 h. The changes in the morphology were observed under a brightfield microscope.

2.7 Quantification of intracellular reactive oxygen species (ROS)

Intracellular ROS measurements were carried out using 22,72 -dichlorodihydrofluorescein diacetate (DCF-DA) method. Briefly, HCT-116 cells at the density of 1×10^4 cells/well were incubated overnight and treated with different concentrations of PDR or PDU for 24 h. Cells were incubated with DCF-DA (20 µM) for 30 min at 37°C in 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.

2.8 Cell apoptosis by acridine orange/ethidium bromide dual stain

The apoptotic morphology of HCT-116 cells was carried out using a double fluorescence stain assay. After treatment with PDU or PDR, the HCT-116 cells was stained with an equal volume of a mixture of acridine orange/ethidium bromide (AO/EtBr, 100 µg/ml each) solution and incubated for 10 min in the dark. After incubation, cells were visualized under fluorescence microscopy at excitation (400-490 nm).

2.9 Statistical analysis

Statistical analyses were performed with Prism 5 (GraphPad) software (GraphPad Software Inc. Boston MA, USA). The results were obtained in triplicate and analyzed by one-way ANOVA followed by the Tukey test. Data were expressed as means ± standard deviation (SD). $^{**}p<0.05$, $^{***}p<0.01$ are considered as significant values.

3. Results

3.1 Identification of bioactive constituents

Previous studies have shown methanol and water can extract more diversity of compounds with higher extraction efficiency compared to water, and methanol alone. 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 PDR aril extract was sticky, and brown, with a yield of 14.8% w/w. GC-MS was carried out in hydromethanolic extract of PDU and PDR. The total ion chromatogram showing the GC-MS profile of the identified compounds is represented in Figure 2 and Figure 3, respectively. About 12 similar compounds were detected (Table 1) both in the hydromethanolic extracts of *P. dulce* unripe (PDU) and ripe (PDR) fruits. The unripe fruit extract showed the presence of an additional 8 phytochemicals. Together a total of 20 compounds were detected in PDU extract (Tables 1 and 2). However, an additional 31 compounds were also present in the PDR extract (Table 3). Together a total of 43 phytochemicals were detected in PDR extract (Tables 1 and 3). Therefore, our GC-MS analysis data reports that 51 bioactive phytochemicals are present in *P. dulce* unripe and ripe aril. The chemical structure, retention time, molecular formula, and molecular weight are tabulated in Tables 1, 2, and 3. The most abundant compounds observed in PDU were maltol, 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl, 5-hydroxymethylfurfural, beta.-D-glucopyranose, and heptanoic acid, 6-oxo representing a peak area of more than 36%. Similarly, the few major compounds found with high percent peak area in PDR were 2-deoxy-D-galactose, O-.alpha.-D-glucopyranosyl-(1.fwdarw.3)-.beta.-D-fructofuranosyl, 5-hydroxymethylfurfural, alpha.-D-glucopyranoside, and d-mannose representing a peak area of more than 39%. Additional compounds with reasonable peak areas are represented in the tables.

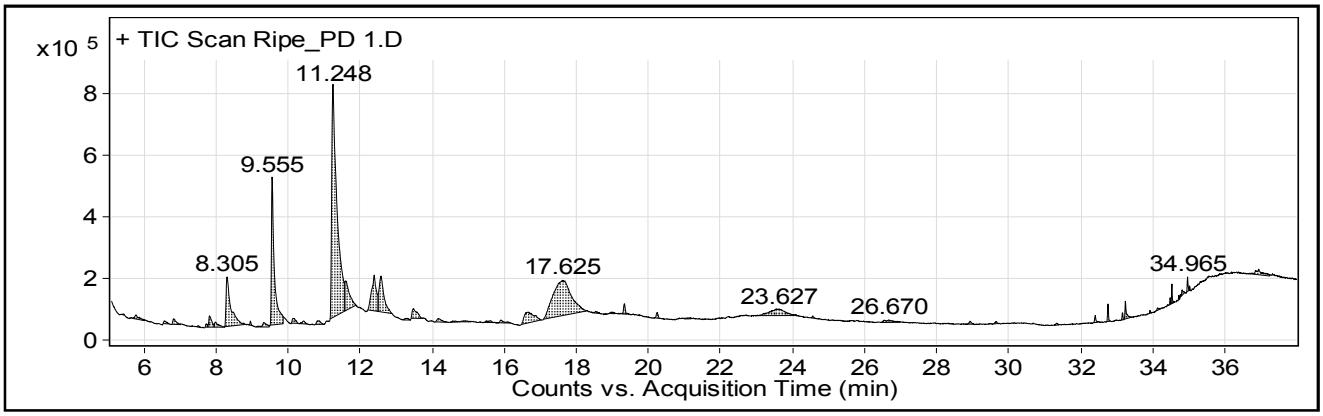


Figure 2: GC-MS chromatogram represents the separated bioactive constituents of hydromethanolic *P. dulce* ripe aril (PDR) extract.

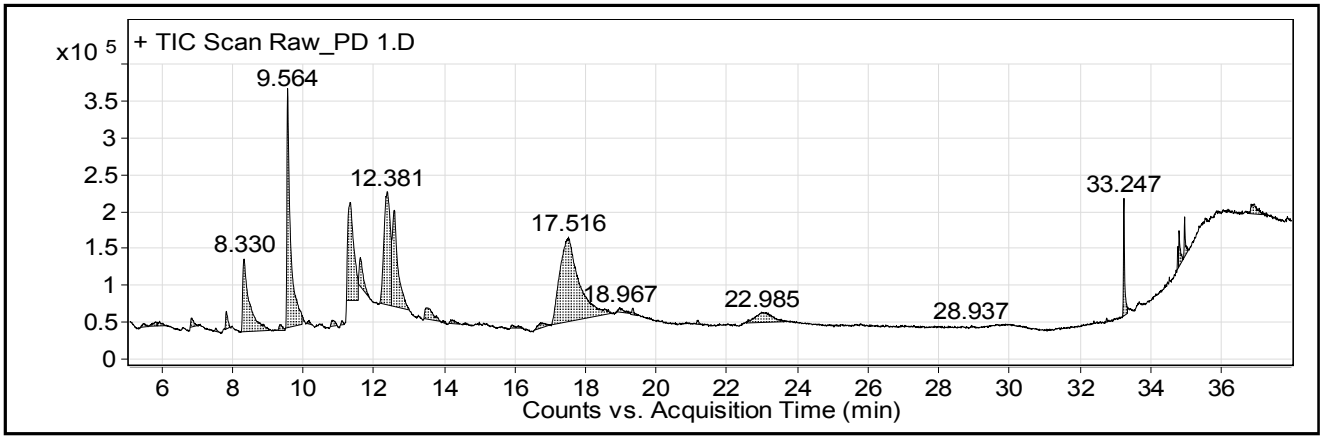
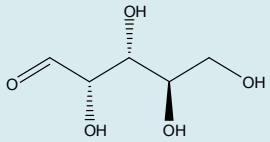
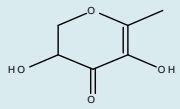
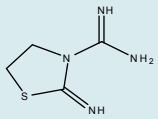
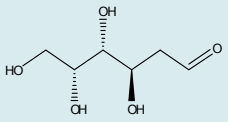
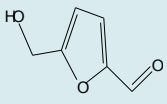
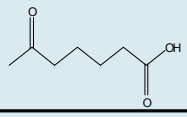
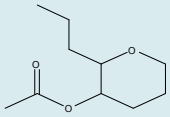


Figure 3: GC-MS chromatogram represents the separated bioactive constituents of hydromethanolic *P. dulce* unripe aril (PDU) extract.

Table 1: Similar compounds detected in both unripe and ripe aril extracts of *P. dulce*

S.No.	Name, molecular formula, and molecular weight of the compounds	Molecular structure of the compounds
1	DL-Arabinose($C_5H_{10}O_5$) , MW.150.1	
2	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- ($C_6H_8O_4$), MW.144	
3	3-Thiazolidinecarboxamidine, 2-imino-($C_4H_8N_4S$), MW.144	
4	Maltol ($C_6H_6O_3$), MW.126	
5	2-Deoxy-D-galactose($C_6H_{12}O_5$), MW.164.1	
6	5-Hydroxymethylfurfural($C_6H_6O_3$), MW.126	
7	Heptanoic acid, 6-oxo-($C_7H_{12}O_3$), MW.144.1	
8	Acetic acid, 2-propyltetrahydropyran-3-yl ester-($C_{10}H_{18}O_3$), MW.186.1	

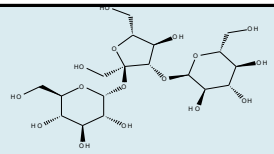
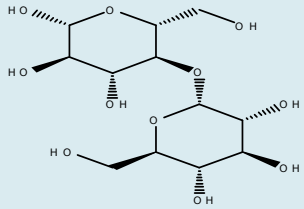
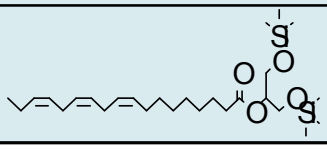
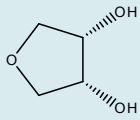
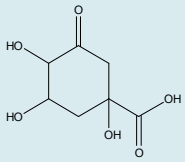
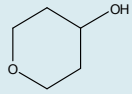
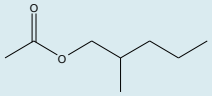
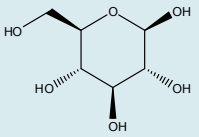
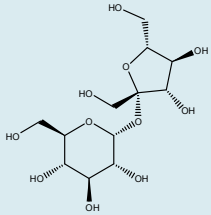
9	alpha.-D-glucopyranoside, O-.alpha.-D-glucopyranosyl-(1.fwdarw.3)-.beta. -D-fructofuranosyl ($C_{18}H_{32}O_{16}$), MW.504.2	
10	n-Hexadecanoic acid($C_{16}H_{32}O_2$), MW.256.2	
11	Maltose($C_{12}H_{22}O_{11}$), MW.342.1	
12	9,12,15-octadecatrienoic acid, 2-[(trimethylsilyl)oxy]-1-[[(trimethylsilyl) oxy]methyl]ethyl ester, (Z,Z,Z)- : ($C_{27}H_{52}O_4S_{12}$), MW.496.3	

Table 2: Compounds detected in unripe aril extract of *P. dulce*

S.No.	Retention time	Name, molecular formula, and molecular weight of the compounds	Molecular structure of the compounds
1	5.13	3,4-furandiol, tetrahydro-, cis- ($C_4H_8O_3$), MW.104	
2	6.64	Cyclohexan-1,4,5-triol-3-one-1-carboxylic acid ($C_7H_{10}O_6$), MW.190	
3	7.56	Tetrahydro-4H-pyran-4-ol ($C_5H_{10}O_2$), MW.102.1	
4	10.17	1-Pentanol, 2-methyl-, acetate ($C_8H_{16}O_2$), MW.144.1	
5	12.38	.beta.-D-Glucopyranose($C_6H_{12}O_6$), MW.180.1	
6	17.49	Sucrose ($C_{12}H_{22}O_{11}$), MW.342.1	

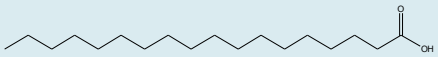
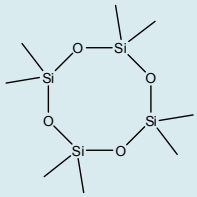
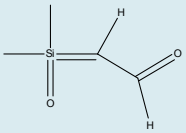
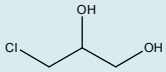
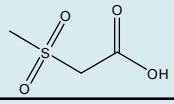
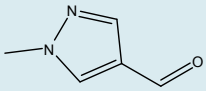
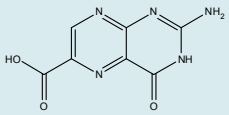
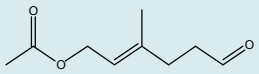
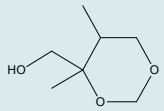
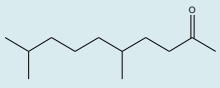
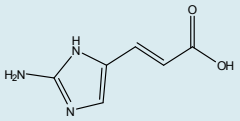
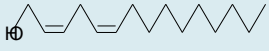
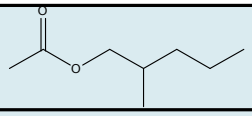
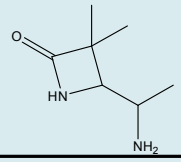
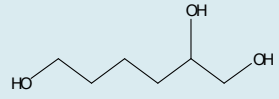
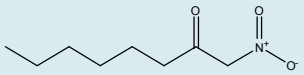
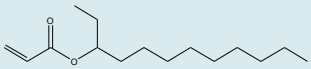
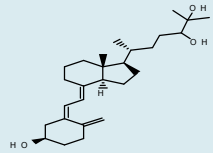
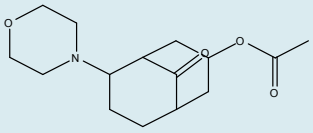
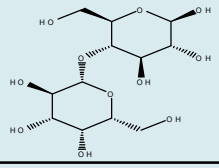
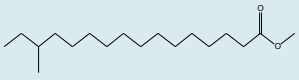
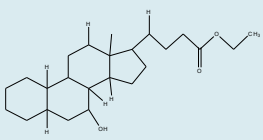
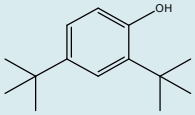
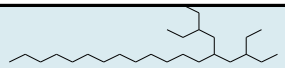
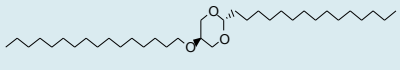
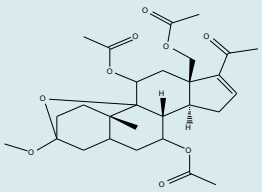
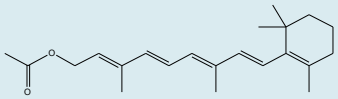
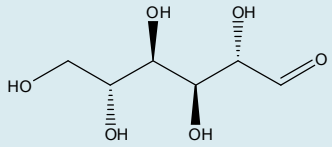
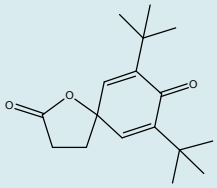
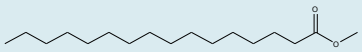
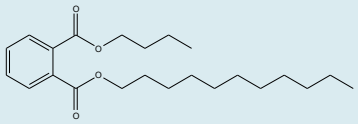
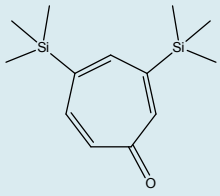
7	34.96	Octadecanoic acid ($C_{18}H_{36}O_2$), MW.284.3	
8	36.94	Cyclotetrasiloxane, octamethyl- ($C_8H_{24}O_4Si_4$), MW.296.1	

Table 3: Compounds detected only in ripe aril extract of *P. dulce*

S. No.	Retention time	Name, molecular formula, and molecular weight of the compound	Molecular structure of the compounds
1	5.43	Dimethylsulfoxonium formylmethylide($C_4H_8O_2S$), MW.120.17	
2	5.76	1,2-Propanediol, 3-chloro-($C_3H_7ClO_2$), MW.110	
3	5.78	Methanesulfonylacetic acid($C_3H_6O_4S$), MW.138	
4	6.56	Pyrazole-4-carboxaldehyde, 1-methyl-($C_5H_6N_2O$), MW.110	
5	7.14	Pterin-6-carboxylic acid($C_7H_5N_5O_3$), MW.207	
6	7.73	Acetic acid, 3-methyl-6-oxo-hex-2-enyl ester ($C_9H_{14}O_3$), MW.170.1	
7	7.82	1,3-Dioxane-4-methanol, 4,5-dimethyl-($C_7H_{14}O_3$), MW.146.1	
8	8.95	2-Decanone, 5,9-dimethyl-($C_{12}H_{24}O$), MW.184.2	
9	9.55	Imidazole, 2-amino-5-[(2-carboxy)vinyl]-($C_6H_7N_3O_2$), MW.153.14	
10	9.82	Z, Z-2,5-Pentadecadien-1-ol ($C_{15}H_{28}O$), MW.224.38	

11	10.15	1-Pentanol, 2-methyl-, acetate($C_8H_{16}O_2$), MW.144.1	
12	10.43	Azetidin-2-one 3,3-dimethyl-4-(1-aminoethyl)- ($C_7H_{14}N_2O$), MW.142.1	
13	10.44	1,2,6-Hexanetriol($C_6H_{14}O_3$), MW.134.17	
14	10.80	2-Octanone, 1-nitro-($C_8H_{15}NO_3$), MW.173.21	
15	10.84	3-(Prop-2-enoyloxy)dodecane($C_{15}H_{28}O_2$), MW.240.38	
16	11.54	9,10-Secocholesta-5,7,10(19)-triene-3,24,25-triol, (3.beta.,5Z,7E) -($C_{27}H_{44}O_3$), MW.416.6	
17	13.55	1-Heptatriacotanol($C_{37}H_{76}O$), MW.537	
18	16.16	Acetic acid, 6-morpholin-4-yl-9-oxobicyclo[3.3.1]non-3-yl ester($C_{15}H_{23}NO_4$), MW.281.35	
19	16.65	beta.-D-Glucopyranose, 4-O-.beta.-D-galactopyranosyl-($C_{12}H_{22}O_{11}$), MW.342.1	
20	18.07	Hexadecanoic acid, 14-methyl-, methyl ester($C_{18}H_{36}O_2$), MW.284.48	
21	19.02	Ethyl isoallocholate($C_{26}H_{44}O_5$), MW.436.6	
22	19.34	Phenol, 2,4-bis(1,1-dimethylethyl)-($C_{14}H_{22}O$), MW.206.2	

23	22.45	Octadecane, 3-ethyl-5-(2-ethylbutyl)-($C_{26}H_{54}$), MW.366.71	
24	23.31	1,3-Dioxane, 5-(hexadecyloxy)-2-pentadecyl-, trans($C_{35}H_{70}O_3$), MW.538.93	
25	24.72	3,9-Epoxypregn-16-en-20-one, 3-methoxy-7,11,18-triacetoxy ($C_{28}H_{38}O_9$), MW.518.6	
26	25.28	Retinol, acetate ($C_{22}H_{32}O_2$), MW.328.5	
27	28.92	D-Mannose ($C_6H_{12}O_6$), MW.180.1	
28	32.4	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione ($C_{17}H_{24}O_3$), MW.276.2	
29	32.75	Hexadecanoic acid, methyl ester($C_{17}H_{34}O_2$), MW.270.3	
30	33.15	Phthalic acid, butyl undecyl ester($C_{23}H_{36}O_4$), MW.376.3	
31	36.92	2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl- ($C_{13}H_{22}OSi_2$), MW.250.1	

3.2 Anticancer effect of PDR and PDU on HCT-116 cells

We evaluated the cytotoxicity effect of PDR and PDU on HCT-116 cells using MTT assay. As shown in Figure 4, cell viability was significantly affected by PDR and PDU in a dose-dependent manner. Treatment with PDR at concentrations of 7.8, 15.6, 31.25, 62.5, 125, 250, 500, and 1000 µg/ml for 24 h resulted in inhibition of cell proliferation by 6.2, 15, 29.5, 51.8, 74, 86, 93, 100%, respectively. Similar results were also observed for PDU (4.58, 11, 18.6, 44.2, 67.2, 73.7, 86.51, 100% inhibition for the same concentrations used for PDR, respectively). Based on the results, PDR showed slightly better cytotoxicity towards HCT-116 cells than PDU (Figure 4). The half maximal inhibitory concentration (IC_{50}) value of PDR on colon cancer was determined at 62.5 µg/ml, and the IC_{50} for PDU was 70 µg/ml. Further, IC_{50} concentrations of PDR and PDU were used for remaining studies.

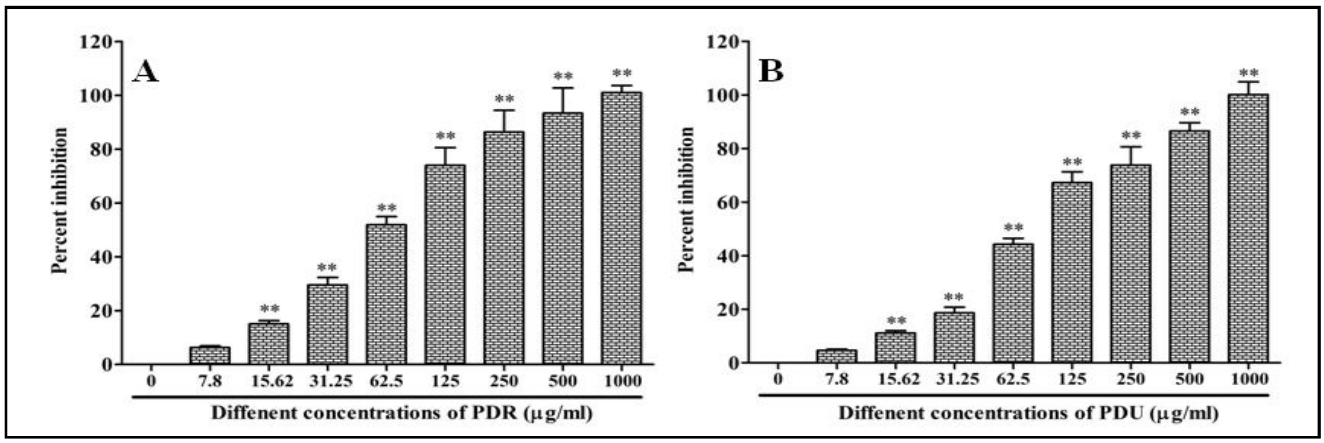


Figure 4: Cytotoxic effect of ripe and unripe hydromethanolic extract of *P. dulce* on HCT-116 cells. Cells were treated with different concentrations (7.8-1000 µg/ml) of either (A) PDR or (B) PDU for 24 h and cytotoxicity was determined by MTT assay. Data are presented in terms of mean ± SD (n=6), ** $p<0.01$ compared with the control group was considered as a statistically significant difference.

3.3 Cellular morphological changes of PDR and PDU treatment on HCT-116 cells

The effect of PDR and PDU on cellular morphology was observed directly using a brightfield microscope. As a result, cultured field of untreated HCT-116 cells had uniform polygonal shapes and were homogeneously distributed. Following treatment with PDR and PDU, various morphological changes were noticed. There was a loss in cell-to-cell contact, reduction in the cell density, floating cells, changes in the cell shapes from polygonal to circular, and bright shrinkage morphology were observed both in PDR and PDU treatment (Figure 5). These morphological changes were observed at the IC_{50} concentration of PDR and PDU. Cell density and morphology of PDU-treated cells were much less affected than those of PDR-treated cells.

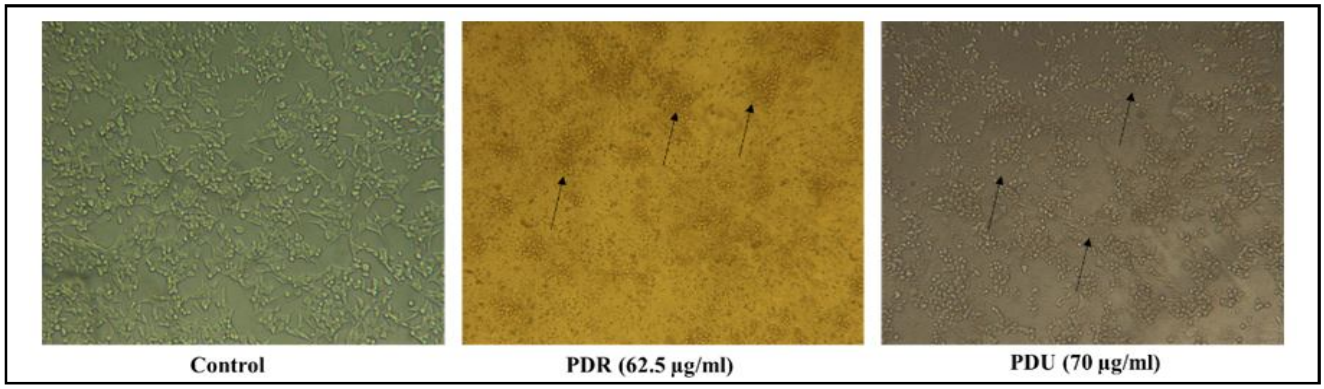


Figure 5: Effect of ripe and unripe hydromethanolic extract of *P. dulce* on HCT-116 cells morphology. HCT-116 cells treated with IC_{50} value of PDR or PDU for 24 h and cell morphology was observed and images were captured using brightfield microscopy (Magnification, x 40).

3.4 PDR and PDU treatment enhance the ROS generation in HCT-116 cells

ROS is a common inducer of mitochondrial pathway-mediated apoptosis. The effect of PDR and PDU on the intracellular ROS level of colon cancer cells was examined by DCF-DA assay. HCT-116 cells were treated with different concentrations of PDR or PDU for 24 h and subjected to ROS analysis. Our study revealed that PDR treatment increases the intracellular ROS generation by 20, 46, and 80% at concentrations of 15.6, 31.25, and 62.5 µg/ml, respectively. Similar results were obtained for PDU treatment (10, 40, and 59% at the concentrations of 17.5, 35, and 70 µg/ml, respectively). The result suggested ripe and unripe hydromethanolic extract of *P. dulce* induces apoptosis in colon cancer cells and is likely mediated by ROS generation.

3.5 PDR and PDU treatment induced apoptosis in HCT-116 cells

We performed an acridine orange/ethidium bromide staining to determine whether enhanced ROS production was functionally relevant in HCT-116 cells treated with PDR or PDU. Untreated viable cells exhibit uniformly pale green. PDU-treated cells stained green to yellow which indicates early apoptosis characterized by loss of membrane integrity and chromatin condensation. Whereas, PDR treatment to HCT-116 cells exhibited late apoptosis with condensed chromatin, membrane blebbing, and necrotic cells showed bright orange-red in appearance (Figure 7).

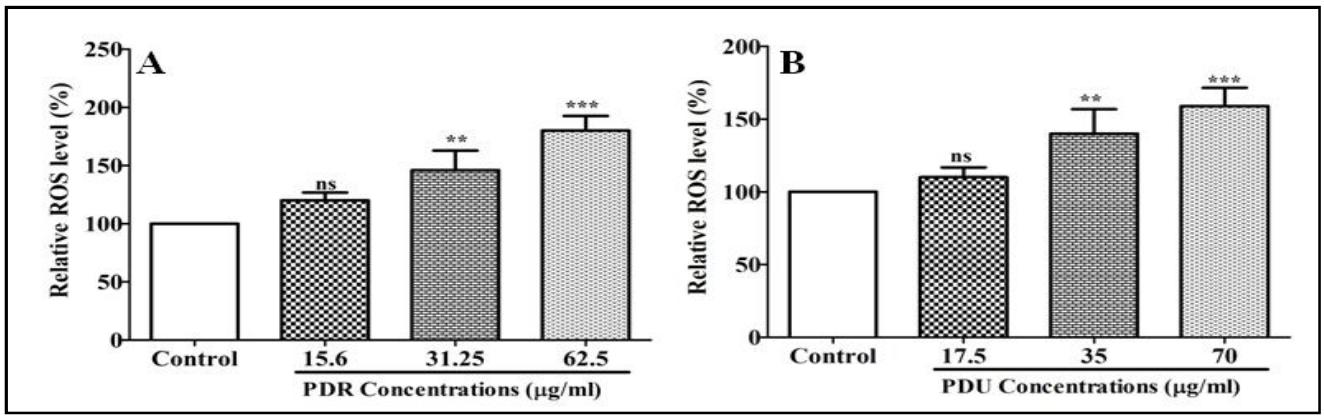


Figure 6: Effect of ripe and unripe hydromethanolic extract of *P. dulce* on ROS accumulation in HCT-116 cells. HCT-116 cells were treated with different concentrations of (A) PDR or (B) PDU for 24 h and subjected to ROS generation assay using fluorometric method. Data are presented in terms of mean ± SD (n=6), ** $p<0.01$, *** $p<0.05$ compared with the control group was considered as a statistically significant difference.

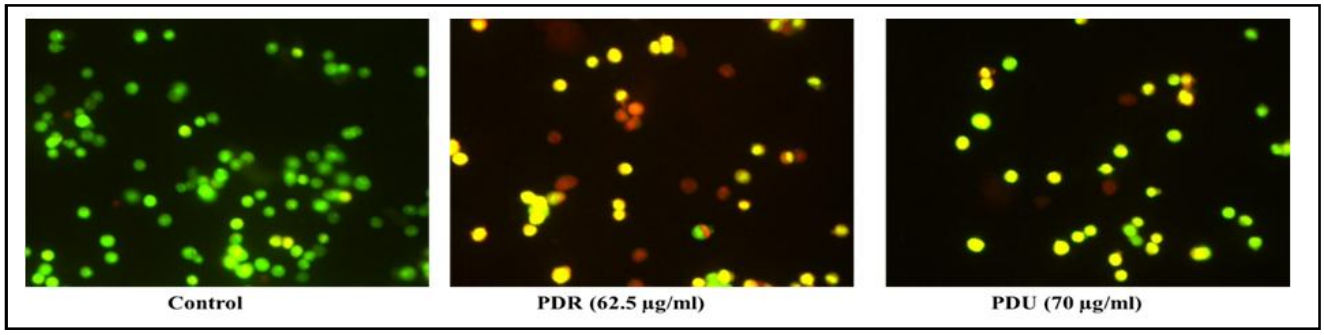


Figure 7: Ripe and unripe hydromethanolic extract of *P. dulce* induces apoptosis in HCT-116 cells. HCT-116 cells treated with IC_{50} concentration of PDR or PDU for 24 h. As a result of the loss of membrane integrity, PDR or PDU-treated cells showed apoptosis which exhibits an orange-red color due to co-staining with ethidium bromide whereas untreated cells were observed as green in color (Magnification, x100).

4. Discussion

Nutrition-conscious consumers are seeking inexpensive plant foods to improve their daily intake of essential nutrients and health-promoting phytochemicals (Chellamal, 2022). Therefore, fruits, vegetables, and multigrain-containing foods are in demand. Particularly, the underutilized fruits consumed by traditional and tribal society are playing a significant hand. Raw and ripe pods of *P. dulce* contain up to 75% fruit aril/pulp rich in protein and carbohydrates. The aril is used to prepare folk remedies, consumed raw, roasted, or as curry, and soup. Wall Medrano *et al*. (2016) reported the presence of strong antioxidant and anti-inflammatory compounds at various stages of fruit ripening. PDR loses 252 mg of ascorbic acid during ripening. Therefore, studies have evaluated the utility of PD fruit in the development of multigrain pasta. Recently, Saha *et al*. (2021) evaluated the nutritional value of PDU and PDR fruit and found elevated levels of protein and reduced sugar in PDR (12.1% and 4.2%, respectively). However, the PDU had a significantly higher content of vitamin C (385 mg /100 g). The above reports established PD could be a more convenient source of specific bioactive compounds and nutraceuticals, if harvested at suitable ripening stages.

A recent study by Dhanisha *et al*. (2022), has shown the usefulness of PD fruit extract in the modulation of pro-inflammatory cytokines and anti-apoptotic genes. The phytochemical constituents of the ripe fruits are evaluated using GC-MS. Our current finding reports the presence of n-hexadecanoic acid, cyclotetra siloxane octamethyl, hexadecanoic acid methyl ester, 9,12,15 octaadecatrienoic acid, octadecanoic acid, and 2 methyl octadecane in consistent with other profiles (Table 2). However, the majority of compounds we have detected are not reported earlier. In addition, a comparative investigation of the unripe and ripe fruit extracts concerning to cytotoxicity and phytochemical constituents correlation is not available. We evaluated the phytochemical composition of PDU and PDR hydromethanolic extract and compared the *in vitro* anticancer activity against HCT-116 cancer cells.

Our GC-MS chromatogram data (Figures 1 and 2) revealed 12 similar compounds present in both PDU and PDR extracts (Table 1). Among them, four phytochemicals maltol, 2-deoxy-D-galactose, 5-hydroxymethylfurfural, and n-hexadecanoic acid have anticancer effects *via* various mechanisms (Han *et al*., 2023; Laszlo *et al*., 1960; Ravi and Krishnan, 2016; Zhao *et al*., 2014). Maltol, a flavor enhancer in the food industry, restricts cancer growth through the downregulation of programmed death ligand-1 (PD-L1) and elicits T cell-mediated anticancer responses (Han *et al*., 2023). The glucose analog, 2-deoxy-D-galactose interferes with glycolysis at the hexokinase reaction to inhibit the growth of solid, ascitic, and systemic transplantable tumors in animal models (Laszlo *et al*., 1960). 5-hydroxymethylfurfural has been found widely in foods through the degradation of hexoses and is elucidated to induce apoptosis and cell cycle arrest by ROS-mediated signaling pathways (Zhao *et al.,* 2014). n-hexadecanoic acid present in plants has a high-affinity interaction with DNA topoisomerase-I to inhibit HCT-116 cell proliferation with an IC_{50} value of 0.8 µg/ml (Ravi and Krishnan, 2016).

Of the 8 compounds present in PDU (Table 3), octadecanoic acid is present in medicinal plants and is represented mostly as oleic and linoleic acids. Octadecanoic acid has an antiproliferative effect against hepatocellular carcinoma (BEL-7402), leukemia (HL-60), and human gastric (SGC-7901) tumor strains (Yu *et al*., 2008). It also functionally and structurally damages the tumor cell membrane and cell ultra-structures leading to apoptosis and G_0/G_1 phase cell cycle arrest.

GC-MS detected 31 metabolites in the PDR extract. Literature survey revealed antitumor activity for 5 compounds pterin-6-carboxylic acid, azetidin-2-one 3,3-dimethyl-4-(1-aminoethyl)-, ethyl isoallocholate, phenol, 2,4-bis(1,1-dimethylethyl)-, and retinol acetate (Bertel *et al*., 2021; Chaiyong *et al*., 2022; Lotan and Nicholson, 1977; Rajaram *et al*., 2013; Thakur and Ahirwar, 2019). Pterin-6-carboxylic acid is a folic acid (vitamin B9) derivative, required for DNA synthesis. Folate derivatives pterin-6-carboxylic acid and 6-formyl pterine are photolytic products that generate ROS responsible for cellular oxidative stress. Therefore, pterin-6-carboxylic acid conjugated gold nanoparticles are used in laser photo-thermal therapy of epithelial carcinoma (Bertel *et al*., 2021). Azetidin-2-one 3,3-dimethyl-4-(1-aminoethyl)- is regularly identified in medicinal plant extracts and is known to show cytotoxicity against the human cholangiocarcinoma cell line KKU-M213 (Chaiyong *et al*., 2022). The ethyl iso-allocholate isolated from plants showed significant caspase 3 cleavage and caspase-dependent apoptosis in A549 lung cancer cells. Further, it demonstrated 55 ± 3% inhibition in liver metastasis (Thakur and Ahirwar, 2019). Phenol, 2,4-bis(1,1-dimethylethyl) is another active component present in PD extract and is known for its anticancer effect on HeLa (cervical cancer) and human hepatocellular carcinoma (HepG2) through matrix metalloproteins and DNA fragmentation (Rajaram *et al*., 2013). Retinol acetate (retinoid) a vitamin A derivative is known to inhibit the growth of untransformed and transformed tumor cell proliferation (Lotan and Nicholson, 1977).

ROS is a common inducer of mitochondrial pathway-mediated apoptosis. Many cancer models have shown that *P. dulce* fruit bioactives selectively kill cancer by releasing ROS. However, little is known about the effects of PD fruit bioactives on intestinal carcinoma. The present study provides evidence that the effects of PDR and PDU on human intestinal cancer cells are more potent and are disrupting ROS homeostasis. In our study, MTT assay revealed that PDR or PDU reduced HCT-116 cell viability (Figure 4), which was accompanied by an alter in the cellular morphology (Figure 5), and an increase in the level of DCFDA fluorescence (Figure 6), and induced apoptosis as analyzed by fluorescence microscopy (Figure 7). The presence of cytotoxic compounds in PDU such as 5-hydroxymethylfurfural, n-hexadecanoic acid, and octadecanoic acid is reported to induce apoptosis through ROS-mediated signaling. Observed DNA damage and structural damage to tumor cell membrane may be the reason for early apoptosis associated with elevated ROS, altered morphology, and chromatin condensation. Similarly, the presence of n-hexadecanoic acid, pterine-6-carboxylic acid, ethyl isoallocholate, and phenol,2,3, bis-(1,1-dimethyl) in the PDR extract explains the significantly higher cytotoxicity and loss in cell to cell contact, changes in cell morphology, and necrosis like symptoms. Since more anticancer compounds are present in PDR compared to PDU, PDR exhibited significantly higher cytotoxicity on HCT-116 cells. Our findings further confirm the utility of not only PDR but PDU aril in traditional medicine, for the treatment of various diseases associated with cancer.

5. Conclusion

The study has investigated the PD unripe and ripe aril hydromethanolic extract and identified twenty and forty-three phytochemicals, respectively. About twelve similar compounds were detected in both extracts. Ten phytochemicals maltol, 2-deoxy-D-galactose, 5-hydroxymethylfurfural, n-hexadecanoic acid, octadecanoic acid, pterin-6-carboxylic acid, azetidine-2-one,3,3-dimethyl-4-(1-aminoethyl), ethyl isoallocholate, phenol,2,4-bis(1,1-dimethyl ethyl)-, and retinol acetate are reported to have anticancer effects *via* various mechanisms. PDU and PDR extracts demonstrated dose-dependent inhibition against HCT-116 cells with IC_{50} of 70 µg/ml and 62.5 µg/ml, respectively. The extracts also induced apoptosis likely mediated by ROS generation. The PDU induced early apoptosis-like symptoms, whereas PDR exhibited late apoptosis. This study supports the ethnopharmacological use of both unripe and ripe fruit extracts as a source of potent anticancer drugs.

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Conflict of interest

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

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