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Research Article
Apoptotic Effects of *Annona reticulata* Leaves Extract in HT-29 Cell Lines

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Abstract

Background and Objective: *Annona reticulata* or custard apple belongs to family Annonaceae. It is traditionally used to treat various human ailments. However, there are no studies on the cytotoxicity or apoptosis-inducing properties of the leaf extracts on HT-29 cell line. Hence, the present study aimed at screening the anti-cancer potential of *Annona reticulata* leaf extract through various *in vitro* studies. **Materials and Methods:** The methanolic leaf extract of *Annona reticulata* (ARM) was subjected to quantification of flavonoids by UPLC/MS; further analyzed for its cytotoxic effect by MTT assay, cell cycle analysis, apoptotic potential by Annexin V-FITC staining assay and morphological study by phase contrast microscopy. **Results:** The ARM showed significant dose-dependent cytotoxicity towards HT-29 cell lines with IC_{50} of 76.76 µg mL^{-1}. In cell cycle analysis, ARM 160 µg mL^{-1} exhibited significant ($p<0.001$) increase in the percentage of cells at S phase, indicating the induction of apoptosis. Further, apoptosis induction was confirmed by Annexin V-FITC assay and morphological evaluation. The results showed that the percentage of late apoptotic cells were found to be higher in ARM 160 µg mL^{-1} treated cells (82.53%) compared to untreated (0.71%) cells. Also, ARM 160 µg mL^{-1} showed similar activity as colchicine treated cells (82.18%). Under morphological evaluation, the formation of apoptotic bodies was found to be more evident in ARM compared to colchicine. **Conclusion:** These findings suggest ARM as a potent anti-cancer agent and also provide a basis for further studies validating ARM as an adjuvant in cancer therapy.

Key words: Colorectal cancer, rutin, anti-cancer, cell culture, herbal medicine

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Colorectal cancer is the most common and prevalent cancer after stomach cancer in India[1]. The treatment of cancer involves expensive drugs that have adverse side effects or toxicity complications. To overwhelm the existing cancer treatments that have adverse side effects on normal cells, it is needful to establish a therapy with minimal side effects, targeting the apoptosis mechanism of cancer cells without destroying normal cells. Thus, discovering a novel or non-toxic anti-cancer agents from natural products that selectively destroy the cancer cells or act as an adjuvant in the treatment of colorectal cancer, particularly for terminal stage patients, has become a decisive approach in cancer therapy[2].

Annona reticulata is commonly known as Bullock's heart or Custard apple in English, Ramaphala in Kannada, is one of the traditionally important plant used for the treatment of various ailments and also possess several medicinal properties such as analgesic, anti-inflammatory, anti-hyperglycemic, anthelmintic, anti-ulcer wound healing and anti-cancer[3]. It belongs to family Annonaceae[4]. It is widely distributed in tropical and subtropical regions. The plant is indigenous to the West Indies. It is widely cultivated in west Bengal and southern regions of India, as a fruit consuming plant and deciduous tree[3]. Different parts of *Annona reticulata* have several phytoconstituents. Stem bark contains tannins, alkaloid and phenolic compounds. Leaves contain alkaloids, amino acids, carbohydrates, steroids, flavonoids, proteins, tannins, glycosides and phenolics. Root has acetogenin, alkaloid, carbohydrates, proteins, flavonoids, tannins. The plant also found to be rich in minerals[3] viz., Ca, P, K, Mg, Na, Cl, S, Mn, Zn, Fe, Cu, Se, Co, Ni and Cr. A study conducted by Mondal *et al.*[5] reported cytotoxic effect of *Annona reticulata* leaves in Caco-2, Hep G2, HEK cell lines. The roots of *Annona reticulata* also exhibited *in vivo* anticancer activity against melanoma cells in mice[6] and *in vitro* cytotoxic activity on MDA-MB-435 human melanoma cells[7]. Some biological activities such as DPPH free radical scavenging activity and anti-bacterial and antifungal activity of leaf extract of *Annona reticulata* have been demonstrated[8]. However, there are no studies on the cytotoxicity or apoptosis-inducing properties of the leaf extracts of *Annona reticulata* on human colorectal cancer (HT-29) cell line. Also, the preliminary screening of methanolic extract of *Annona reticulata* leaves has exhibited better anti-oxidant properties compared to aqueous and ethanol extracts. Thus, the present study aimed to determine cytotoxic activity, cell cycle arrest and apoptosis-inducing potential by methanolic leaf extract of *Annona reticulata* in HT-29 cell lines.

MATERIALS AND METHODS

Experimental site: The experiment was conducted at Institution of Excellence, Vijnana Bhavan, Manasagangotri, University of Mysore, Mysuru, Karnataka, India (12°18'56.4"N 76°37'36.5"E) from May-October, 2017.

Cell lines and culture: Colon cancer cells (HT-29) (PN 70 dated 28.06.2017, Job No.: 1363) were obtained from National Centre for Cell Science, Pune, India. The cells were maintained in the Dulbecco's Modified Eagle's Medium (DMEM) medium containing 10% Fetal Bovine Serum (FBS), 1 mM of sodium bicarbonate, L-glutamine (200 mM), streptomycin (10 mg mL^{-1}), Glucose (25 mM) and penicillin (10,000 units) (DMEM complete media) at 37°C in a humidified 5% CO_2 atmosphere. The culture medium was replaced twice in a week. For the experiments, confluent cells were trypsinized and plated in 6, 12 and 96 well plates. All cell culture operations were carried out in a model New Brunswick Galaxy 48 R CO_2 incubator from Eppendorf.

Chemicals: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), L-glutamine penicillin-streptomycin solution, Triton X-100, Phosphate Buffered Saline (PBS), Colchicine and HPLC Standards viz, Rutin and Quercetin were purchased from Sigma-Aldrich, USA. Trypan blue dye, Propidium iodide, 0.25% Trypsin-EDTA solution, RNase A solution (20 mg mL^{-1}), Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS) were purchased from HIMEDIA chemicals, India. Annexin V-FITC apoptosis kit was purchased from Invitrogen, USA. All other chemicals used were of analytical grade.

Plant material: Fresh leaf samples of *Annona reticulata* (AR) (Reference No: Tree reg. Vol. 1. page No. 2 annona 10.) were identified and supplied by Dr. G.S.K. Swamy from College of Horticulture, Mysuru, Karnataka state, India during July, 2016.

Preparation of methanolic leaf extract: The leaves of AR were cleaned, washed and dried in the oven at 40°C overnight, powdered, passed through 60 mesh sieve and stored at 4°C until further use. About 15 g of powdered leaf sample was extracted with 150 mL of absolute methanol (1:10 w/v) on a mechanical shaker, for 24 h at room temperature. After 24 h, the solvent mixture was filtered and the supernatant was evaporated to dryness at 40°C under reduced pressure in a rotary evaporator (Superfit, India). The

dried extract was stored in an airtight container at 4°C until further use. The total yield of dry extract obtained from 15 g of powdered leaf sample was 2.86 g (19.06%). A stock solution of the extract was prepared by dissolving in dimethyl sulfoxide (DMSO) at a concentration of 32 mg mL^{-1}. Further, it was serially diluted into 320, 160, 80, 40, 20 and 10 μg mL^{-1} in DMEM complete media.

Phytochemical screening of annona reticulate: Qualitative screening of phytochemicals in methanolic leaf extract of AR were tested for constituents such as saponins, terpenoids, alkaloids[9], tannis[10], flavonoids[11], phenols[10] and phytosterols[10] by using standard methods. The qualitative results are expressed as (+) present in low concentrations, (++) present in moderate concentrations, (+++) present in high concentrations and (-) for the absence of constituents.

Quantification of flavonoids by UPLC/MS: Quantification of flavonoids viz, rutin and quercetin in methanolic leaf extract of AR was determined by using a Waters (Acuity UPLC, USA) system coupled to an Q-TOF (Quadrupole time of flight) mass spectrometer (Synapt G2, USA) equipped with an electrospray ionization (negative mode) source and ion source temperature of 100°C. The column used for the separation was acquity UPLC BEH C18 column (1.7 μm 1.0×50 mm). The UPLC/MS method was followed with slight modification. The test sample was dissolved in 80% methanol and 2 μL of sample was injected into a column at a flow rate of 0.3 mL min^{-1}. A gradient program was used for the elution and 0.1% Formic acid in water was Solvent A and acetonitrile was Solvent B. Initially, Solvent B concentration was 2% and increased to 98% at 4 min and maintained up to 6 min and finally at 8 min B concentration was reduced to 2%. The mass spectrometer was operated in [M-H] negative ion mode. Capillary voltage was set at 2.5 kV and the cone voltage was optimized for each compound. Molecular species were identified within the mass to charge ratio (m/z) range 50-1500. Data acquisition was carried out by MassLynx Software (Version: 4.1). The concentration of flavonoids was calculated from the peak area using the calibration curves.

Trypan blue dye exclusion assay: The HT-29 cell lines were seeded at a density of 5×10^5 (3 mL/well) in 6 well plates and incubated at 37°C in a humidified 5% CO_2 atmosphere for 24 h to form a cell monolayer. After 24 h, the growth medium was gently aspirated and treated with varying concentrations of methanolic extract (0, 40, 80, 160 and 320 μg mL^{-1}) and colchicine (320 μg mL^{-1}) for 24 h. After treatment, the adherent cells were trypsinized with 0.25% Trypsin-EDTA solution and centrifuged at 1800 rpm for 8 min. After centrifugation, the supernatant was discarded and the cell pellet was washed twice with PBS. Further, the cell pellets were resuspended in fresh medium from which a 20 μL aliquot was mixed with an equal volume of 0.4% trypan blue dye and then loaded into a haemocytometer. This experiment was done in triplicates and the viable and non-viable count was recorded using an inverted microscope. The percentage of viability was calculated based on the equation:

$$\text{Viable cells (\%)} = \frac{\text{Total No. of viable cells (mL}^{-1}\text{) of aliquot}}{\text{Total No. of cells (mL}^{-1}\text{) of aliquot}}\times100$$

MTT assay: The HT-29 cell lines were seeded at a density of 5×10^4 (100 μL/well) in 96 well plates and incubated at 37°C in a humidified 5% CO_2 atmosphere for 24 h to form a cell monolayer. After 24 h, the growth medium on the monolayer was aspirated and treated with 100 μL of various concentrations (0, 10, 20, 40, 80, 160 and 320 μg mL^{-1}) of AR methanolic leaf extract and colchicine (320 μg mL^{-1}). After 24 h treatment, cytotoxicity was tested by MTT (10 μL/well containing 100 μL of cell suspension, 5 mg mL^{-1} of stock in PBS) solution and the plates were incubated at 37°C for 4 h in a 5% CO_2 atmosphere. The supernatants were aspirated from the wells and washed thrice with PBS. About 100 μL of DMSO was added to each well and incubated for 15 min. After incubation, the plates were gently shaken to solubilize the formazan crystals and absorbance was measured at 590 nm using multimode plate reader (Varioskan Flash Top, Thermo Fisher Scientific, Finland). The percentage of inhibition (%) was calculated using the formula below and IC_{50} values were calculated from log dose-response curves using GraphPad Prism software version 6 for Windows (GraphPad Software, USA):

$$\text{Inhibition (\%)} = 100 - \frac{\text{Test absorbance at 590 nm}}{\text{Untreated control absorbance at 590 nm}}\times100$$

Cell cycle analysis: The HT-29 cell lines were seeded at a density of 5×10^5 (3 mL/well) in 6 well plates and incubated at 37°C in a humidified 5% CO_2 atmosphere for 24 h to form a cell monolayer. After 24 h, the growth medium was aspirated and treated with AR methanolic leaf extract (80 and 160 μg mL^{-1}) and colchicine (320 μg mL^{-1}) for 24 h. After treatment, the cells were washed, trypsinized and

centrifuged at 1800 rpm for 8 min. After centrifugation, the supernatant was discarded and the cell pellet was washed twice with PBS. Further, the cells were resuspended in PBS (300 µL) and fixed with 100% ethanol (700 µL) at -20°C for 1 h. After fixing, the cells were washed with cold PBS and centrifuged at 4000 rpm for 10 min at 4°C. The cells were resuspended in 1 mL of PBS containing PI (0.05 mg mL^{-1}), RNase A (0.05 mg mL^{-1}) and Triton X-100 (0.1%) and incubated for 30 min in the dark at room temperature. Finally, the cells were sorted in a flow cytometer (Cell Lab Quanta™, SC, Beckman Coulter, USA).

Annexin V-FITC staining assay: The apoptotic cells were quantified by flow cytometry using the Annexin V-FITC apoptosis detection kit (Invitrogen, USA). The HT-29 cell lines were seeded at a density of 5×10^5 (3 mL/well) in 6 well plates and incubated at 37°C in a humidified 5% CO_2 atmosphere for 24 h to form a cell monolayer. After 24 h, the growth medium was aspirated and treated with AR methanolic leaf extract (80 and 160 µg mL^{-1}) and colchicine (320 µg mL^{-1}) for 24 h. After treatment, the cells were washed, trypsinized and centrifuged at 1800 rpm for 8 min. After centrifugation, the supernatant was discarded and the cell pellet was washed twice with cold PBS. The cell pellet was resuspended in 100 µL binding buffer containing 10 µL Annexin V-FITC and incubated at 4°C for 30 min. Further, 10 µL of PI in 100 µL binding buffer was added to each of the tubes and incubated for 5 min at room temperature in the dark. Finally, the cells were analyzed by flow cytometer (Cell Lab Quanta™, SC, Beckman Coulter, USA).

Morphological study by phase contrast microscopy: HT-29 cells were seeded in a T-25 flask at a density of 2×10^5 cells/flask and grown for 24 h. After seeding, the cells were treated with AR methanolic leaf extract (160 µg mL^{-1}) and colchicine (320 µg mL^{-1}) for 24, 48 and 72 h, respectively. After various incubation periods, cell morphology was evaluated using phase contrast inverted microscope with digital imaging (Axiovert A1, Zeiss, Germany).

Statistical analysis: Statistical analysis was performed using the statistical analysis program (SPSS, 16.0, International Business Machines, USA). Comparisons between groups (control and treated) were performed by one-way ANOVA with Tukey's HSD post hoc test. Statistical significance was accepted at p-value lower than 0.05. The IC_{50} values were calculated from log dose-response curves using GraphPad Prism software version 6 for Windows (GraphPad Software, USA).

RESULTS

Phytochemical screening of *Annona reticulata* leaf extract: The results of qualitative phytochemical screening of *Annona reticulata* methanolic leaf extract (ARM) have been depicted in Table 1. The extract exhibited the highest concentration of terpenoids, tannins, flavonoids, phenols and phytosterols among which flavonoids showed stronger presence. However, low concentrations of alkaloids and absence of saponins were observed in ARM. Based on these results, flavonoids such as rutin and quercetin were further subjected to quantification in ARM by UPLC/MS method.

UPLC/MS analysis: The anti-cancer and apoptosis-inducing potential of flavonoid compounds such as rutin and quercetin is well documented in several studies[12-14]. A study conducted by Santos and Salatino[15] has reported the presence of rutin and quercetin in different species of Annonaceae family. Therefore, the methanolic leaf extract (ARM) were analyzed for rutin and quercetin by UPLC/MS method. In this study, UPLC chromatogram of ARM (Fig. 1) and a mass spectrum (Fig. 2e) indicated the presence of rutin at a concentration of 61.256 mg g^{-1} extract, which was confirmed by comparing with rutin standard chromatogram (Fig. 2a) and mass spectra (Fig. 2b). However, in this study, quercetin was not detected as shown in ARM chromatogram (Fig. 1) on comparison with standard quercetin chromatogram (Fig. 2c) and mass spectra (Fig. 2d).

Cell viability test by trypan blue dye exclusion assay: Viability test of HT-29 cells using trypan blue is shown in Fig. 3. The cell lines were exposed to doses of 40-320 µg mL^{-1} of extract and there was a significant difference ($p<0.001$) observed between the extracts when compared with the control. The viability (%) of HT-29 cell lines of ARM and positive control-Colchicine (PC) at 320 µg mL^{-1} were comparable with each other but with a significant difference ($p<0.05$).

Cytotoxic effect of ARM on HT-29 cell line: The cytotoxic effect of ARM on HT-29 cell line was evaluated through MTT assay. Dose dependent concentrations of ARM (10-320 µg mL^{-1}) were used and the half maximal inhibitory concentration (IC_{50}) were calculated from dose-response curve. The results of ARM cytotoxicity are shown in Table 2 and there was a significant difference ($p<0.001$) observed

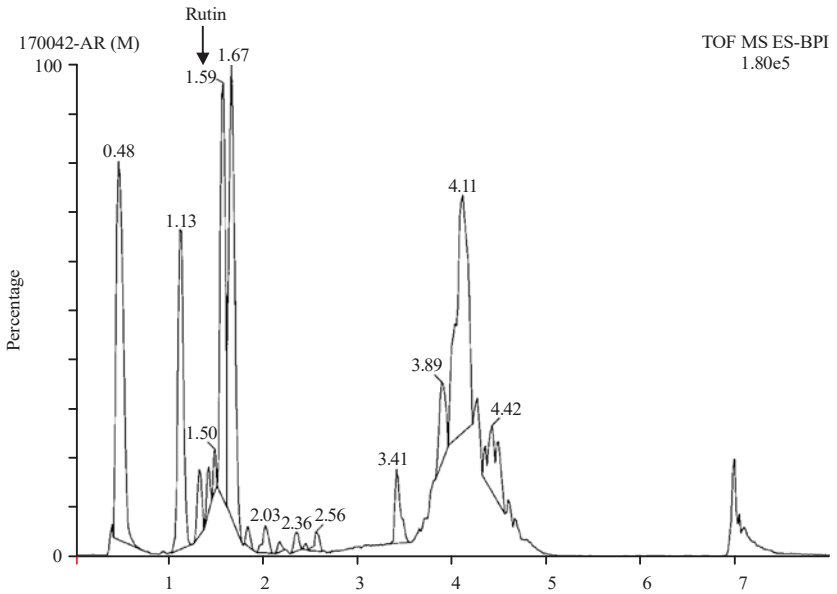

Fig. 1: UPLC chromatogram of Rutin in ARM extract at 1.59 retention time

Table 1: Phytochemical constituents of *Annona reticulata* leaf extract

Phytoconstituents	*Annona reticulata* methanolic leaf extract
Saponins	-
Terpenoids	+++
Alkaloids	0
Tannis	+++
Flavonoids	+++
Phenols	+++
Phytosterols	+++

+++: High concentrations, -: Absent

Table 2: *In vitro* cytotoxicity study of ARM against HT-29 cell line by MTT assay

Samples	Concentration (μg mL^{-1})	HT-29 (Inhibition (%))	IC_{50} (μg mL^{-1})
ARM	10	6.18±0.18^{a}	76.76
	20	20.52±0.53^{b}	
	40	38.08±0.41^{c}	
	80	56.58±1.18^{d}	
	160	62.28±0.19^{e}	
	320	78.11±1.39^{g}	
PC (Colchicine)	160	68.27±0.46^{f}	N/A
	320	75.44±0.14^{g}	

N/A: Not applicable, ARM: *Annona reticulata* methanolic extract, all values are expressed in means±standard deviation (n = 3), values containing different superscript letters a, b, c..., g differ significantly (p<0.001)

between the extracts of ARM and PC at all the doses except for the dose 320 μg mL^{-1}, there was no significant difference observed between ARM and PC suggesting similar cytotoxic activity at higher concentration. The IC_{50} value of ARM on HT-29 cell line after 24 h exposure time was found to be 76.76 μg mL^{-1} and was comparatively lower to a study conducted Sathiyamoorthy and Sudhakar[16] on the similar cell line and exposure time using *F. hispida* leaves extract with IC_{50} value of 125 μg mL^{-1}. Another study conducted by Suresh *et al.*[7] also reported the antiproliferative effect of ethanol extract of *Annona reticulata* roots on A-549, K-562, HeLa and MDA-MB cancer cell lines.

Effect of ARM on cell cycle of HT-29 cells: Based on IC_{50} values from MTT assay, two doses of ARM viz., 80 and 160 μg mL^{-1} were further analyzed for cell cycle arrest and apoptosis-inducing potential in HT-29 cell line. Many anti-cancer agents arrest the cell cycle at one particular phase and then induce apoptosis. The results depicted in Fig. 4 illustrate the effect of ARM on cell cycle mechanism of HT-29 cell line. After 24 h treatment, the dose ARM 160 μg mL^{-1} exhibited significant ($p<0.001$) increase in the percentage of cells at S phase i.e., from 4.46±0.11-15.50±0.12% and 6.36±0.04-15.50±0.12% as compared to control and PC, respectively (Fig. 4e). The proportion of cells in G0/G1 decreased significantly ($p<0.001$) in a dose-dependent manner when compared to control (Fig. 4e).

Apoptotic effect of ARM on HT-29 cell line: The apoptosis-inducing potential of ARM were analyzed by flow cytometry following Annexin V-FITC staining. The quantification of apoptotic and necrotic cells after treatment with ARM (80 and 160 μg mL^{-1}) for 24 h are depicted in Fig. 5. Results demonstrated a statistically significant induction

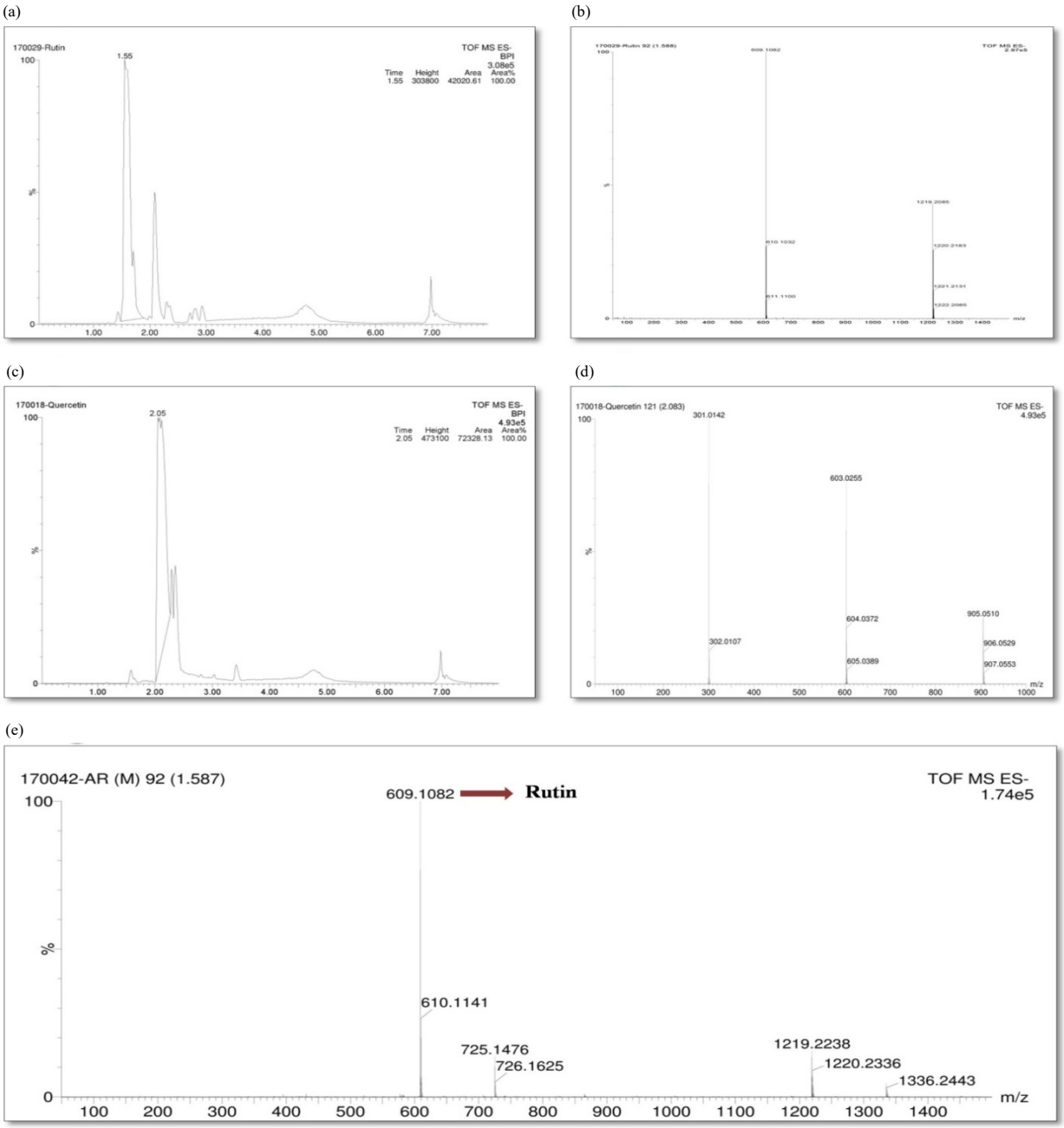

Fig. 2(a-e): UPLC chromatogram and mass spectra of standards viz., Rutin and Quercetin and mass spectra of ARM (a) UPLC chromatogram of standard rutin at 1.55 retention time, (b) Mass spectra of standard rutin ([M-H]-, m/z 609.1082), (c) UPLC chromatogram of standard quercetin at 2.05 retention time, (d) Mass spectra of standard quercetin ([M-H]-, m/z 301.0142) and (e) Mass spectra of rutin in ARM at 1.587 retention time and ([M-H]-, m/z 609.1082)

($p<0.001$) of 90.12±0.01% early apoptosis in HT-29 cells treated with ARM 80 µg mL^{-1} extract (Fig. 5e). The percentage of late apoptotic cells were found to be higher in ARM 160 µg mL^{-1} treated cells (82.53%) compared to untreated (0.71%) cells. Also, ARM 160 µg mL^{-1} showed similar activity as PC 320 µg mL^{-1} treated cells (82.18%). However, there was a significant difference ($p<0.001$) observed between ARM and colchicine treated cells. Based on these results, ARM 160 µg mL^{-1} and PC 320 µg mL^{-1} were further studied for morphological changes caused due to apoptosis.

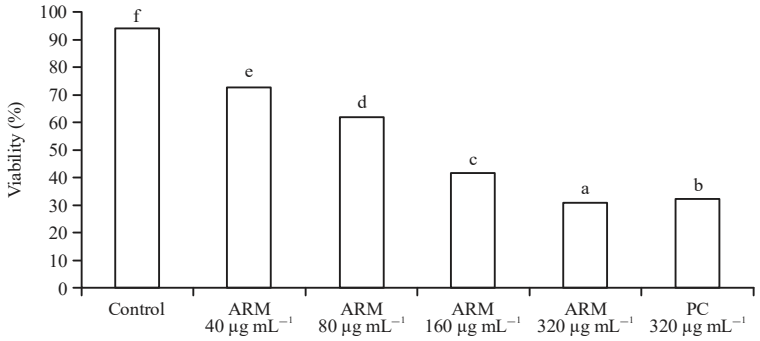

Fig. 3: Effect of ARM on the viability of cell lines (HT-29)

All values are expressed as mean of triplicates (n = 3), Mean values containing different superscript letters a, b, c...,f differ significantly (p<0.001, 0.05)

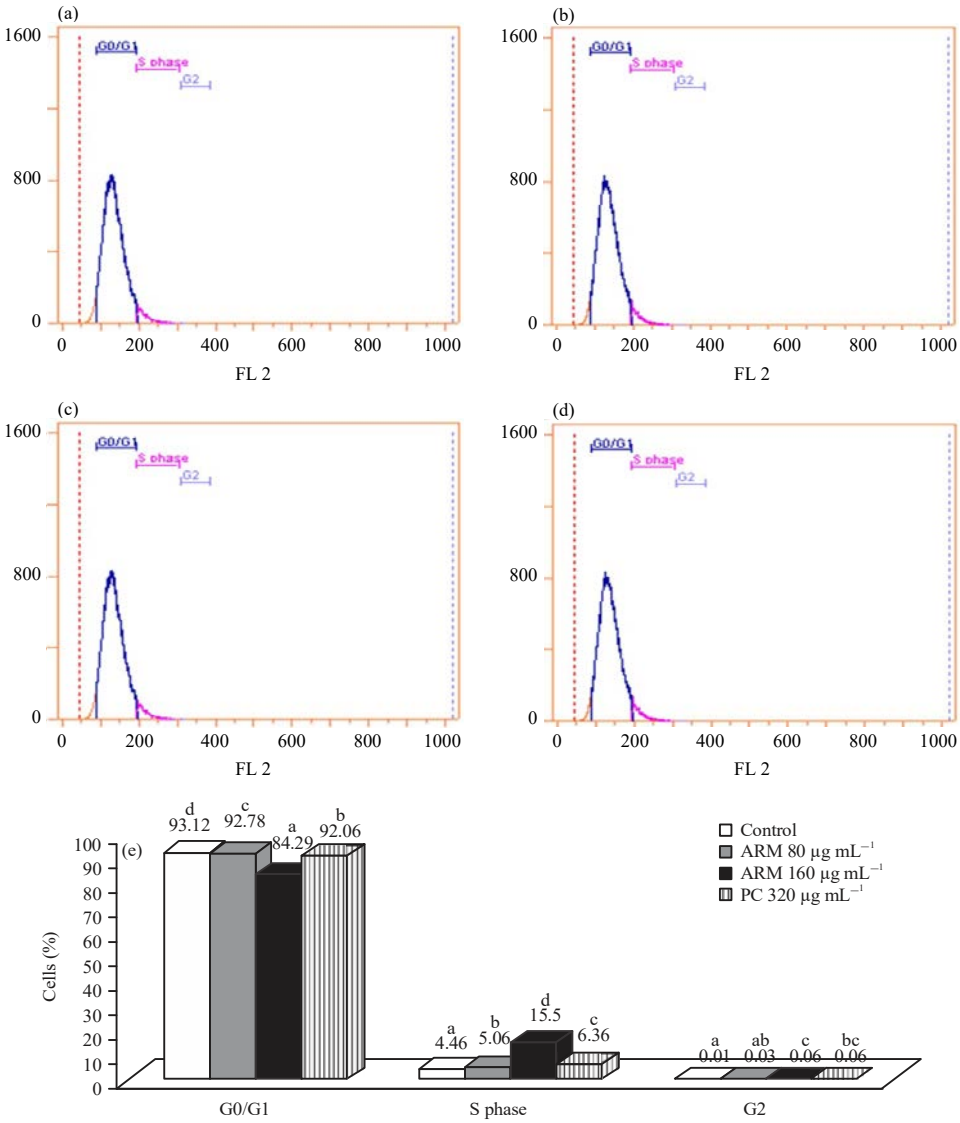

Fig. 4(a-e): Cell cycle analysis of ARM on HT-29 cells after 24 h, (a) Control, (b) ARM 80 µg mL^{-1}, (c) ARM 160 µg mL^{-1}, (d) PC 320 µg mL^{-1} and (e) Cell distribution of HT-29 cells after 24 h treatment

All values are expressed as mean of triplicates (n = 3), Mean values containing different superscript letters a, b, c..., d differ significantly (p<0.001, 0.05)

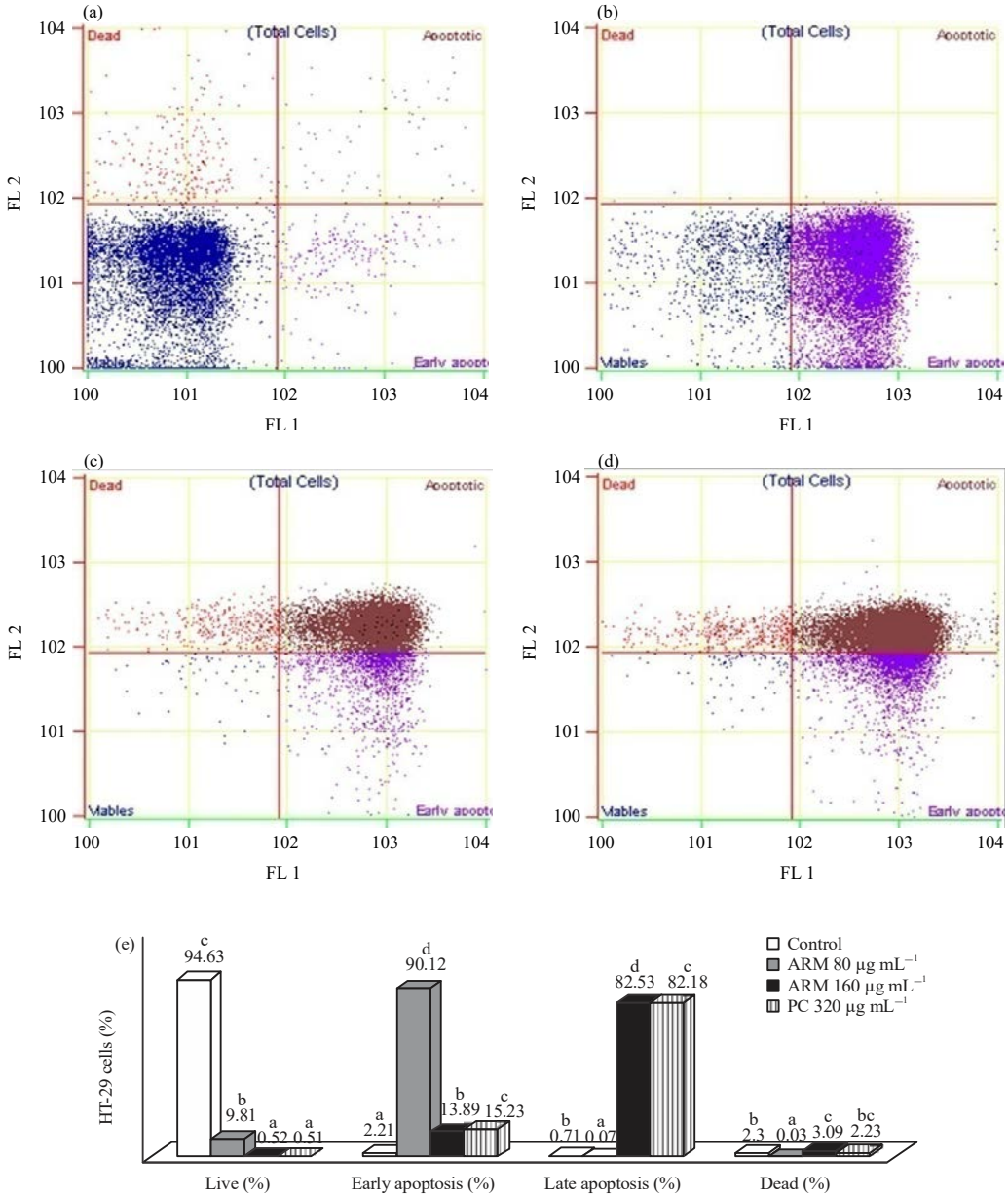

Fig. 5(a-e): Apoptosis assessment of ARM on HT-29 cells after 24 h, (a) Control, (b) ARM 80 µg mL^{-1}, (c) ARM 160 µg mL^{-1}, (d) PC 320 µg mL^{-1} and (e) The percentage of cell population (HT-29 cells) in different stages (live, apoptotic and dead) after 24 h treatment

All values are expressed as mean of triplicates (n = 3), Mean values containing different superscript letters a, b, c...,d differ significantly (p<0.001)

(p<0.001) of 90.12±0.01% early apoptosis in HT-29 cells treated with ARM 80 µg mL^{-1} extract (Fig. 5e). The percentage of late apoptotic cells were found to be higher in ARM 160 µg mL^{-1} treated cells (82.53%) compared to untreated (0.71%) cells. Also, ARM 160 µg mL^{-1} showed similar activity as PC 320 µg mL^{-1} treated cells (82.18%). However, there was a significant difference (p<0.001) observed between ARM and colchicine treated cells. Based on these results, ARM 160 µg mL^{-1} and PC 320 µg mL^{-1} were further studied for morphological changes caused due to apoptosis.

Morphological changes induced in HT-29 cell line by ARM: The HT-29 cells were treated with ARM 160 µg mL^{-1} and PC 320 µg mL^{-1} at exposure periods of 24, 48 and 72 h, respectively. After these exposure periods (24, 48 and 72 h), the morphological characteristics of apoptosis, such as

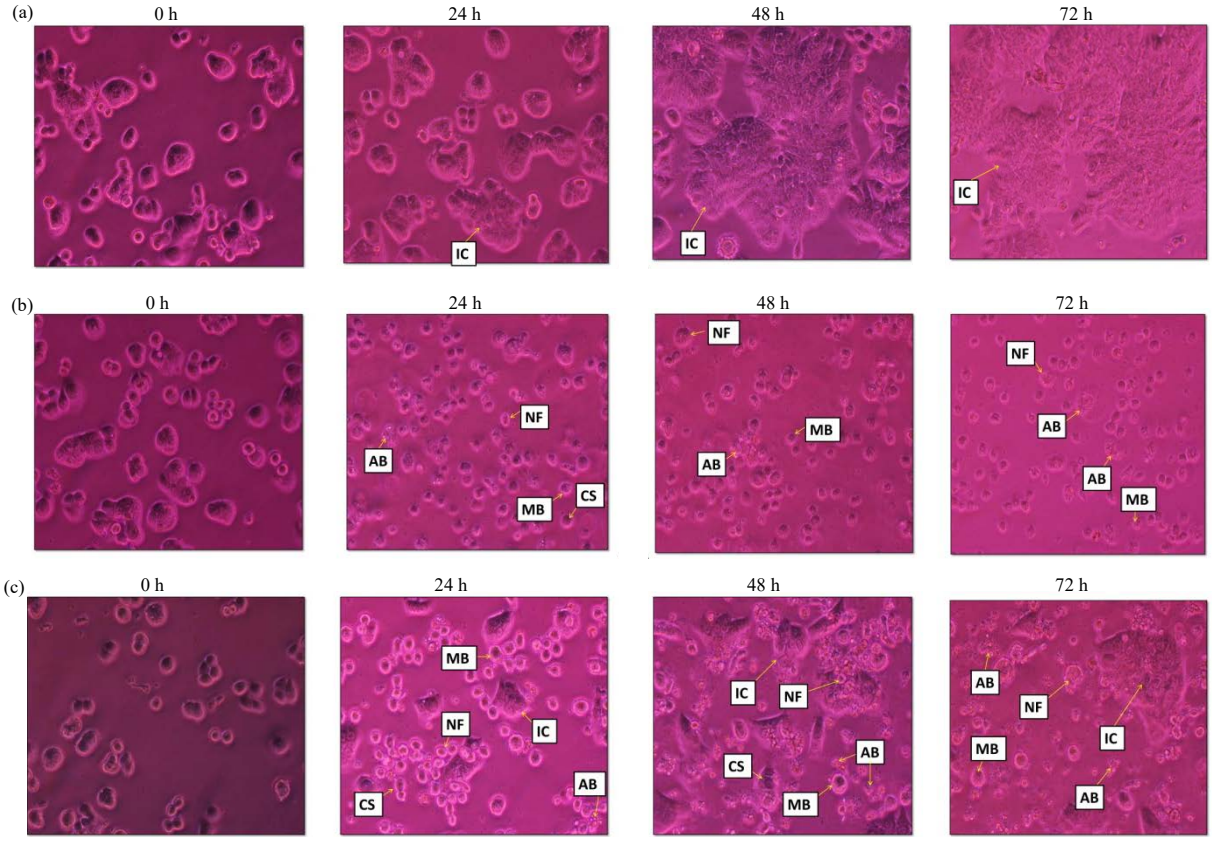

Fig. 6(a-c): Morphological changes in HT-29 cell line as observed under phase contrast inverted microscope at 0, 24, 48 and 72 h, (a) Untreated cells, (b) ARM 160 µg mL^{-1} and (c) PC 320 µg mL^{-1}

Cells demonstrated characteristics of apoptosis, such as cellular shrinkage (CS), membrane blebbing (MB), nuclear fragmentation (NF) and apoptotic bodies (AB), intact colonies (IC) (magnification 20X)

Cellular Shrinkage (CS), Membrane Blebbing (MB), Nuclear Fragmentation (NF) and Apoptotic Bodies (AB) were examined using phase contrast inverted microscope (Fig. 6). At all exposure periods, apoptosis was induced more evidently in ARM 160 µg mL^{-1} compared to PC 320 µg mL^{-1} which had few Intact Colonies (IC) along with Apoptotic Bodies (AB). Thus, indicating that ARM had better apoptosis inducing-potential than Colchicine.

DISCUSSION

Medicinal plants have been utilized in human therapeutic medicine since ancient time[17]. Recently, significant attention has been focused on identifying medicinal plants with potent apoptosis-inducing properties and cell growth inhibition potential. Anti-cancer drugs from natural sources having minimum side effects, low toxicity and high cytotoxicity to cancer cells, is an important criterion in cancer research[18]. About 60% of medicinal drugs are derived from natural sources, including anti-cancer drugs[19].

The study evaluated the anti-cancer potential of ARM, which belongs to the Annonaceae family, a family is known for its secondary metabolites[20]. It is well-known that crude extracts might have some bioactive components that function against human cancer cell lines. In this regard, ARM was screened for the presence of chemical components such as terpenoids, saponins, tannins, flavonoids, phenols, etc. by phytochemical screening. Results of phytochemical screening showed higher concentrations of terpenoids, tannins, flavonoids, phenols and phytosterols among which flavonoids showed a stronger presence. A study conducted by Pumiputavon *et al.*[21] reported the presence of tannins and flavonoids in all Annonaceae plants, as observed in this study. Flavonoids have apoptosis-inducing potential, obstruct the cell cycle[22] by destroying the structure of the spindle fiber and also, inhibit angiogenesis[23,24]. Based on flavonoid's anti-cancer nature, ARM was further subjected to quantification of flavonoids such as rutin and quercetin by UPLC/MS method. Results depicted the presence of rutin as an active compound in ARM. However, quercetin was not detected in this study.

Rutin has been reported as an inhibitor of proliferation in HT-29 cell lines[25] and also, has ability to induce apoptosis in murine leukemia WEHI-3 cells *in vitro* and human leukemia HL-60 cells *in vivo*[26]. To our knowledge, this is the first report showing rutin in the leaf-derived crude extract of *Annona reticulata*.

In this study, ARM demonstrated cytotoxic activity on HT-29 cell lines with IC_{50} value of 76.76 µg mL^{-1} after 24 h exposure time. At higher concentration, both ARM and PC had similar cytotoxic activity suggesting ARM as a potent anti-cancer agent. According to United States National Cancer Institute (NCI) plant screening program, a crude extract is considered to be cytotoxic, if the IC_{50} value following exposure period between 48 and 72 h, is less than[27] 20 µg mL^{-1}. In this study, since the exposure time of ARM was limited to 24 h only, the IC_{50} value was greater than the recommended IC_{50} value i.e., less than[27] 20 µg mL. However, a higher exposure time could have possibly reduced the IC_{50} value.

Cell cycle progression is a major biological event, with controlled regulation in normal cells, which almost becomes deregulated in transformed and neoplastic cells[28]. A study reported that the ability of molecules/drugs to arrest the cell cycle in G2/M or S phase was related to their sensitivity and increased with cell resistance[29].

So, in the present study, cell cycle analysis was performed to confirm whether the ARM mediated any alteration of a specific phase in cell cycle progression. The results showed that there was a significant increase in accumulation of cells at the S phase in a concentration-dependent manner when compared to control and PC. The cell cycle arrest at S phase is well-known to be controlled by CDKI, CDK and cyclins[30]. However, gene expression analysis was not investigated in this study. The following mechanism could be responsible for cell cycle arrest at S phase. Briefly, ARM acts by triggering disruption of mitochondrial membrane to arrest cells in S phase and inhibit cell proliferation. This effect of ARM on cell cycle progression may be due to its phytochemical constituents such as flavonoids.

To further elucidate the apoptotic activity of ARM, Annexin/PI flow cytometric assay was conducted. Annexin V binds specifically to phosphatidylserine on the external surface of the plasma membrane and this event of phosphatidylserine flipping is generally accepted as one of the apoptotic biomarkers[31]. Unlike necrosis, Apoptosis is a very tightly programmed cell death, which is a vital physiological process to eliminate selectively unnecessary and unwanted cells, to maintain the healthy balance between cell survival and cell death[32]. However, cancer cells show resistance to apoptosis in order to sustain their uncontrolled proliferation[33,34]. In this study, Annexin/PI assay confirmed the ability of ARM to induce early and late apoptosis. At higher concentration, ARM showed similar activity as Colchicine but with a significant difference, suggesting ARM as an anti-cancer agent with apoptosis-inducing potential. Further, the mechanism of ARM in inducing apoptotic cell death in HT-29 cells could be evaluated by conducting apoptosis-related gene expression studies. Apoptosis involves certain morphological changes such as cellular shrinkage, membrane blebbing, nuclear fragmentation and apoptotic bodies. Induction of apoptosis is the basic criteria to approve a plant product as an anti-cancer agent[35,36]. The morphological evaluation was carried out to validate the findings on cytotoxicity, cell cycle arrest and apoptosis-inducing potential; and positive results were obtained, with ARM showing better activity at all exposure periods than Colchicine.

CONCLUSION

Annona reticulata exhibits anti-cancer effects on HT-29 cells by inducing loss of cell viability, cytotoxicity, apoptosis, cell cycle arrest and morphological changes. Moreover, leaf extract of *Annona reticulata* provides a new source for rutin, which is capable of inducing apoptosis in cancer cells. Thus, the data suggest that the leaf extract of *Annona reticulata* may be utilized as an adjuvant in treating colorectal cancer. Further studies are required to validate the leaf extract as an adjuvant in cancer therapy.

SIGNIFICANCE STATEMENT

This is the first report showing rutin in a leaf-derived crude extract of *Annona reticulata.* There are very limited or no studies on the cytotoxicity or apoptosis-inducing properties of the *Annona reticulata* leaf extracts on HT-29 cell line. Thus, this study provides baseline data for researchers to explore the anti-cancer activity of the plant and its mechanism of action.

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