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Caspase dependent apoptotic activity of *Rubus fairholmianus* Gard. on MCF-7 human breast cancer cell lines



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ABSTRACT

Developing plant derived chemopreventive agents that can divert the carcinogenic process and inhibit tumour progression may greatly reduce serious health consequences of cancer. This study investigated the antiproliferative and apoptosis inducing ability of *Rubus fairholmianus* root acetone (RFRA) on MCF-7 cells. RFRA treatments showed an increase in the *in vivo* antioxidant levels such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione (GSH) in mice. The extract showed significant antitumour activity with 76.57% increase in life span of ascites tumour bearing mice and 1.94 cm³ reductions in solid tumour volume at 250 mg/kg. The cytotoxicity spectrum analysis indicated that RFRA is a potent cytotoxic agent with varying levels of toxicity (IC₅₀: 57.14–29.36 µg/ml). A significant dose dependent decrease in viability, proliferation and increase in cytotoxicity, caspase 3/7 activity were observed in MCF-7 cells. RFRA (20 µg/ml) induced apoptosis in MCF-7 cells, which was observed by morphology; DNA ladder formation and increased apoptotic population in flow cytometry analysis. These findings strongly suggest the use of *R. fairholmianus* as natural antioxidant with profound antitumour activities. It can act as a potent cytotoxic agent, which is able to induce apoptosis in MCF-7 cells via caspase 3/7 upregulation.

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Introduction

Cancer is one of the leading causes of death and plant derived drugs have been used for the treatment of cancers due to the presence of various potent chemical moieties with pharmacological properties (Kim et al., 2005). Oxidative stress and free radicals harm the cells and leading to severe diseases

including cancer through the damage to DNA at the higher levels. The cancer chemoprevention is a major challenge in health care field globally. However, many bioactive compounds with profound antioxidant activities exhibit potent cytotoxicity by reducing the levels of elevated free radicals and leading to the cancer cell death (Bagchi et al., 2014). Thus the discovery of safe and less toxic drug of plant origin is necessary to fight against the dreaded disease.

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Rubus a diverse genus comprises around 750 species (Finn, 2008). Various pharmacological properties of *Rubus* species have been reported based on the ethnobotanical research (George et al., 2013a, 2013b, 2014a; Jeong et al., 2010). The leaves of *Rubus fairholmianus* have insecticidal characteristics and also been used in the treatment of pain, fruits are edible with stimulating activities (Barukial and Sarmah, 2011). The anti-inflammatory and wound healing properties of *R. fairholmianus* owing to the analogues of quercetin like polyphenolic compounds (George et al., 2014a,b). We have also reported chemical characterization of root acetone extract of *R. fairholmianus*. The preliminary analysis showed that the extract contains phenolics and flavonoids in abundance. The root acetone extract of *R. fairholmianus* showed remarkable total phenolic (63.53 g Gallic acid equivalents/100 g extract), tannins (30.67 g Gallic acid equivalents/100 g extract) and flavonoid contents (981.33 mg Rutin equivalents/g extract) (George et al., 2013a). These reports showed that the extract is rich source of natural phenolics. The evaluation of phenolics is an important concept of phytochemical studies as they are responsible for numerous biological properties due to their free radical-scavenging activities. Since oxidative stress, inflammation, and cancer are interrelated on the basis of the previously found strong antioxidant and pharmacological activity we have selected this plant for the present study (Balkwill and Mantovani, 2012). The literature survey showed that the *in vitro* anticancer properties of this plant have not been well documented. With this background, this study was carried out to evaluate the antitumour properties of *R. fairholmianus* root acetone extract to develop a potent anticarcinogenic agent.

Material and methods

Plant identification and extract preparation

R. fairholmianus Gard. (Syn. *R. moluccanus* L.) was identified by Botanical Survey of India (Voucher No. BSI/SRC/5/23/2010-11/Tech. 1657). The powdered root was extracted in Soxhlet using acetone (50%) following the hot percolation method. The extraction was done at a temperature of 40 °C till the distilled solvent seems decolorized when in contact with the sample in extractor and finally dried to yield the acetone extract.

Thin layer chromatography (TLC)

TLC profiling was done on pre-coated silica gel 60F₂₅₄ TLC plate (Merck, India). After the several trials, mobile phase was standardized as toluene, ethyl acetate and methanol in the ratio of 8:2:0.5. The chromatogram was developed in a saturated chromatographic chamber. The developed plate was visualized under UV at 254 nm and 366 nm.

In vivo studies

Acute toxicity

All the animal experimental protocol was subjected to scrutiny of institutional animal ethics committee for experimental clearance (KMCRET/PhD/03/2011). Acute oral toxicity was performed using three doses of extract (500, 1000 and

2000 mg/kg) as per organization for economic co-operation for development guidelines 423 (OECD, 2001).

In vivo antioxidant activities

Swiss albino mice were treated one month with different doses of RFRA dissolved in carboxy methyl cellulose (CMC – 0.1%). Groups I and II were normal (untreated) and control (treated with 0.1% CMC). The experimental groups were treated with 50, 100 and 200 mg/kg b.wt. RFRA. After 30 days the cytosolic liver homogenate was prepared in ice-cold Tris–HCl buffer (0.1 M, pH 7.4), by centrifugation at 10,000 rpm for 30 min at 4 °C. The following antioxidant enzymes such as superoxide dismutase (SOD) (McCord and Fridovich, 1969), catalase (CAT) (Aebi, 1974), glutathione (GSH) (Moron et al., 1979) and glutathione peroxidase (GPX) (Hafeman et al., 1974) were examined.

Effect of RFRA extract on solid and ascites tumour development
Daltons Lymphoma Ascites (DLA) and Erlich Ascites Carcinoma (EAC) cell lines once developed in the peritoneal cavity of mouse, the cells were aspirated, washed and the viability was checked before injected in to mice for tumour development.

The subcutaneous injection of DLA cell lines (10⁶ cells/animal) to the right hind limb of Swiss albino mice lead to solid tumour development (Rajeshkumar et al., 2002). The tumour control group received CMC (0.1%). Another group was treated with cyclophosphamide (10 mg/kg b.wt.). The experimental groups were treated with 50, 100 and 250 mg/kg b.wt. RFRA. Treatments were started 24 h after inoculation of cells and sustained for 10 days. Initial diameter of the limb was measured and tumour volume was noted every 3rd day for 30 days using digital vernier caliper. The tumour volume (V) = $\frac{4}{3}\pi r_1^2 r_2$, where r_1 and r_2 are the radii of tumours at two different planes.

Swiss albino mice were injected with EAC cell lines (10⁶ cells/animal) intraperitoneally for ascites tumour development (Rajeshkumar et al., 2002). After 24 h of EAC cells inoculation the animals were treated with RFRA (50, 100 and 250 mg/kg b.wt.) and cyclophosphamide (10 mg/kg b.wt.) for 10 days. The ascites tumour developments were observed and calculated the increase in life span (ILS). The percentage of ILS = $(T - C) / C \times 100$, where 'T' and 'C' are the average number of days the experimental (treated) and control animals survived.

In vitro studies

Cell lines used and cytotoxic screening of RFRA

HeLa (Cervical Cancer), MCF-7 (Breast Cancer) and Vero (Monkey Normal Kidney) cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) medium complemented with Foetal Bovine Serum (FBS) 10%, benzyl penicillin and streptomycin (100 µg). JURKAT (Human Leukemic T cells) cells were maintained in Rosewell Park Memorial Institute (RPMI)-1640 medium with FBS 10% antibiotics benzyl penicillin and streptomycin (100 µg). All cells were incubated at 37 °C with 5% CO₂ and 80% humidity. Cells were seeded in microtiter plates and incubated overnight at 37 °C. Cytotoxic screening was performed using 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay by following the method of Campling et al. (1991).

Effect of RFRA on MCF-7 cell line

Cell morphology-inverted microscope. The cellular morphological changes were carefully noticed using Wirsam, Olympus CKX 41 inverted light microscope after 24 h of incubation with RFRA (5, 10, 20 and 40 $\mu\text{g/ml}$) and DMSO (0.5%).

Cell viability, proliferation and cytotoxicity. The Trypan blue dye exclusion method (Sigma–Aldrich T8154) is used to determine the percentage viability. The cell suspension in Hank's Balanced Salt Solution (HBSS) was mixed with Trypan blue (0.4%) in 1:9 ratio and the cells were counted in using Countess™ Automated Cell Counter, Invitrogen.

The CellTiter-Glo¹ luminescent assay (Promega, G7571, Anatech Analytical Technology, Bellville, South Africa) was used for determination of cell proliferation and ATP quantification. Fifty microliters of reconstituted reagent and cell suspension were incubated at room temperature for 10 min in dark to stabilize the luminous indicator. The luminescence was read using the Victor³ (Perkin-Elmer, Separation Scientific).

Cyto-Tox96 X assay (Anatech, Promega G 400) was used to estimate LDH content in the culture media. The cytosolic enzyme LDH will be released due to membrane damage. Fifty microliters of reconstituted reagent and culture medium was incubated for 30 min in dark. The colorimetric complex was measured at 490 nm using Victor³ (Perkin-Elmer, Separation Scientific).

Analysis of programmed cell death

Analysis of caspase 3/7 activity. Activity of caspases 3/7 in apoptotic process was analysed using the Caspase-Glo 3/7 luminescent assay (Promega G8091, Whitehead Scientific, Bracken fell, South Africa). Fifty microliters of treated cells and equal volume of Caspase reagent were seeded in 96-well luminous plate (Scientific Group Adcock Ingram, Midrand, South Africa, BD354651) and incubated for 3 h at room temperature. Luminescent signal was read using the Victor³ (Perkin-Elmer, Separation Scientific).

Apoptosis determination by DNA fragmentation. To evaluate whether the cytotoxic effect observed upon the treatment was due to the induction of apoptosis, the RFRA extract (10, 20 and 40 $\mu\text{g/ml}$) were added to the cultured MCF-7 cells and incubated overnight. The cells were collected after the treatment and washed with PBS. The DNA was isolated by phenol-chloroform extraction procedure (Herrmann et al., 1994), dissolved in TE buffer and run in 1.5% agarose gel to observe the ladder formation.

Cell death analysis- Annexin V-FITC/PI staining. The Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Becton Dickinson, 556570, Scientific Group, Randburg, South Africa) was used to detect apoptotic cells by fluorescence activated cell sorting (FACS). The treated cells were resuspended in $1 \times$ binding buffer at a concentration of $1 \times 10^6/\text{ml}$. One hundred microliters of the cell suspension was stained with 5 ml of FITC Annexin and 5 ml of propidium iodide (PI). The cells were gently vortexed and incubated for 15 min at room temperature and protected from light. After the addition of 400 μl of $1 \times$ binding buffer

to each tube the samples were run in FACS Aria flow cytometer (Becton Dickinson) by running 20,000 events and analysed with Cell Quest Software (BD bioscience).

Statistical analysis

The values were expressed as mean \pm SEM and analysed using one-way ANOVA. The statistical significance of difference was determined using SigmaPlot version 12.0. The cell lines between 15 and 20 passages were used. Statistical significances between control and treated groups are shown at the significance level $2\alpha = 0.05$.

Results and discussion

Thin layer chromatographic analysis

The preliminary chemical profiling was done using TLC profiling. The major compounds were found at Rf 0.52, 0.59, 0.66, 0.75 and 0.83. The compound corresponds to the Rf 0.75 (blue fluorescence at 366 nm) showed maximum band intensity (Fig. 1). The authentication and identification of herbal medicines can be accurately conducted using chromatographic fingerprints. It is very important to obtain reliable chromatographic fingerprints that represent pharmacologically active and chemically characteristic component of the herbal drug (Sulaiman et al., 2014). The root acetone extract of *R. fairholmianus* was chemically standardized with thin layer chromatographic profiling.

In vivo studies

Acute toxicity

The RFRA extract administration did not produce any toxic sign or death and this designates the safety of drug up to 2000 mg/kg b.wt.

In vivo antioxidant activities of RFRA extract

The GSH, GPX, CAT and SOD activities were effectively increased in groups treated with 100 and 200 mg/kg b.wt. RFRA extract compared to control animals (Table 1). GSH level significantly increased in both 100 and 200 mg/kg b.wt. ($P < 0.01$ and $P < 0.001$) treated groups. GPX level also increased significantly in 100 and 200 mg/kg b.wt. ($P < 0.05$ and $P < 0.001$), whereas a significant increase of CAT and SOD levels was found in 200 mg/kg b.wt. ($P < 0.05$).

There are numerous methods present in the body to fight against and neutralize oxidative free radicals among which the most significant mechanism is antioxidants. The metalloprotein SOD converts superoxide radicals into hydrogen peroxide. CAT – a homotetrameric ferri heme containing enzyme and/or GPX – a selenium dependent enzyme eliminates the H_2O_2 . GSH is a substrate for GPX, which act by quenching free radicals (Firdous et al., 2010). Therefore increased levels of these antioxidant systems after the treatment with *R. fairholmianus* may have the potentiality to quench the excess free radicals generated in body and thereby effective against free radical generated disorders.

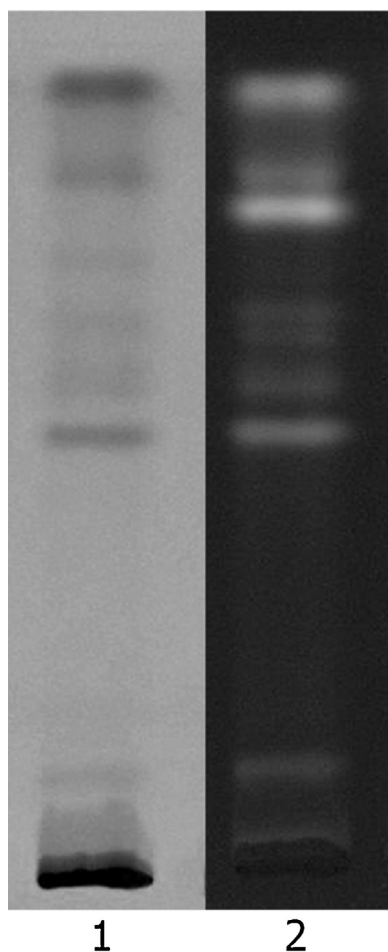


Fig. 1 – Preliminary phytochemical screening of *R. fairholmianus* root acetone extract using thin layer chromatography. The developed plate was visualized under UV at 254 nm (Lane 1) and 366 nm (Lane 2).

Activity of RFRA extract on solid and ascites tumours

The RFRA treated mice showed a significant reduction in tumour volume. The control, 0.1% CMC and 10 mg/kg cyclophosphamide administrated animals on 38th day presented 4.06, 3.85 and 1.01 cm³ of tumour volumes. However tumour volume of RFRA (250,100 and 50 mg/kg b.wt.) treated groups was 1.94, 2.53 and 3.06 cm³ respectively (Fig. 2).

Table 2 – Effect on *R. fairholmianus* root acetone extract on ascites tumour development.

Groups	Mean survival days	Increase in lifespan (%)
Control (tumour alone)	19.16 ± 0.85	
Vehicle control (0.1% CMC)	20.18 ± 0.92	
Cyclophosphamide (10 mg/kg)	35.17 ± 1.12*	83.56
RFRA (250 mg/kg)	33.83 ± 1.99*	76.57
RFRA (100 mg/kg)	30.72 ± 2.31*	60.33
RFRA (50 mg/kg)	27.11 ± 2.09*	41.50

Values are expressed as mean ± SEM (n = 6). RFRA: *R. fairholmianus* root acetone; CMC: carboxy methyl cellulose.
* Significant as compared with control.

The untreated animals of ascites tumour model died of EAC growth by 19.16 days. Administration of RFRA significantly ($P < 0.001$) reduced ascites tumour development. All the treated groups were significantly increased the life span of the tumour animals. Though, cyclophosphamide (10 mg/kg) was most active with increase in the life span by 83.56% ($P < 0.001$) while RFRA 250, 100 and 50 mg/kg showed 76.57, 60.33 and 41.50%, respectively (Table 2).

Raspberries have shown many bioactivities including anti-proliferative against human liver, breast, colon and prostate cancer cells (Seeram et al., 2006). The extracts of *Rubus ellipticus* (George et al., 2013b) and *Phyllanthus amarus* (Rajeshkumar et al., 2002) have reported for its anticarcinogenic and immunomodulatory activities, all these plants have also shown better antioxidant potentials. These observations suggest the ability of RFRA extracts to inhibit the incidence and progression of tumour, which is essentially due to the antioxidant property. The results showed the RFRA extract treated groups showed an increase in life span by inhibiting the ascetic fluid accumulation and decreasing the volume of solid tumour. These clearly demonstrated the antitumour effect of RFRA on tumour cells by a direct cytotoxic effect.

In vitro studies

Cytotoxic screening of RFRA extract

RFRA extracts decreased the cell viability and the IC₅₀ value ranged between 57.14 and 29.36 µg/ml for various cell lines (Fig. 3). Our finding highlights the varying levels of antiproliferative activity of acetone extracts against Jurkat, MCF-7 and

Table 1 – Effect of *R. fairholmianus* root acetone extract on antioxidant systems in liver.

Group	Glutathione (nmol/mg protein)	Glutathione peroxidase (U/mg protein)	Catalase (U/mg protein)	Superoxide dismutase (U/mg protein)
Normal	10.76 ± 1.08	9.73 ± 1.50	3.64 ± 1.66	0.87 ± 1.23
Control (0.1% CMC)	9.33 ± 1.22	10.28 ± 1.82	3.55 ± 1.03	0.91 ± 2.62
RFRA (100 mg/kg b.wt.)	13.74 ± 2.17*	15.59 ± 3.02*	4.4 ± 3.12	1.10 ± 2.18
RFRA (200 mg/kg b.wt.)	14.7 ± 2.01*	135.16 ± 2.38*	6.51 ± 1.22*	1.54 ± 1.09*

Values are expressed as mean ± SEM (n = 6). RFRA: *R. fairholmianus* root acetone; CMC: carboxy methyl cellulose.

* Significant as compared with control.

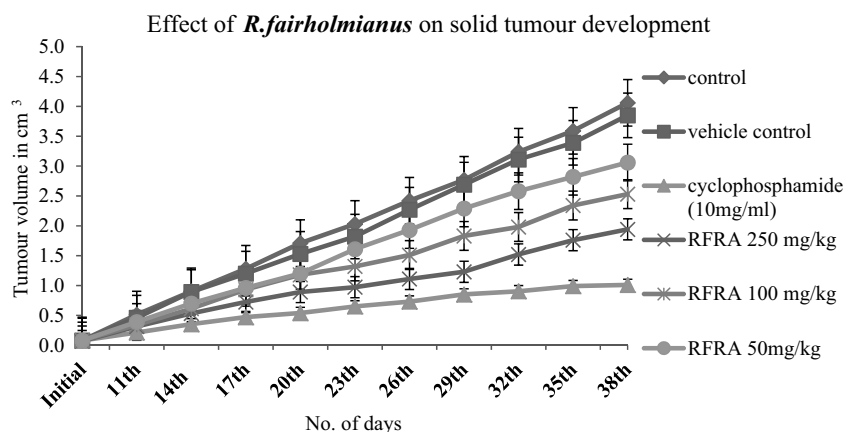


Fig. 2 – Effect of *R. fairholmianus* root acetone extract on Daltons Lymphoma Ascites cells induced solid tumour development in mice. RFRA: *R. fairholmianus* root acetone.

HeLa cells. The plant derived antioxidant active principles (phenolics, flavonoids and tannins) displayed toxicity towards tumour cells (Bhandari, 2015). The present study revealed that RFRA extract is a strong antioxidant and cytotoxic towards human cancer cell lines such as Jurkat, MCF-7 and HeLa cells. The RFRA extracts were more toxic to the MCF-7 breast cancer cell lines (IC_{50} – 29.36) as compared to the normal Vero cell line (IC_{50} – 57.14) and other cancer cell lines that may be due to the strong antioxidant potential of the extract.

Effect of RFRA on MCF-7 cell line

Cell morphology-inverted microscope. The morphology of the MCF-7 cells changed significantly compared to control cells. The extract treated cells appeared less uniform with the loss of membrane integrity, although still intact at lower concentrations (5 and 10 μ g/ml). However the higher concentrations (20 and 40 μ g/ml) showed notable difference (Fig. 4). The significant changes such as loss of intact membrane, karyopyknosis, and cell detachment from the plate were evident when compared to untreated cells. The most detectable morphological features of apoptosis such as detaching from the culture plate, cytoplasmic condensation, cell shrinkage, chromatin accumulation and loss of contact inhibition (Monga et al., 2013) were observed in RFRA

treated cells. Yet the untreated cells appeared normal and confluent.

Cell viability, proliferation and cytotoxicity. Trypan blue viability test is energy-dependent dye exclusion method; the dye is being excluded from live cells. The viability percentages of MCF-7 cells treated with RFRA are presented in Table 3. The control cells showed 96.50% viability, whereas RFRA treated cells displayed a decreased viability such as 85.75, 78.25, 59.26 and 31.25% for 5, 10, 20 and 40 μ g/ml, respectively. The decrease in percentage of viability becomes significant ($P < 0.001$) with all different concentrations of RFRA. The net loss of viability in Trypan blue assay indicated the cytotoxic effect of RFRA towards MCF-7 cells. No other studies have been published previously revealing the influence of RFRA on viability of MCF-7 cell lines. The weak acid Trypan blue has more affinity towards basic proteins and therefore the higher nuclei uptake produced blue intensity, while the cytoplasm remains weakly stained (Grankvist et al., 1977).

The cell membrane damage of MCF-7 cells after the RFRA treatment was measured by the release of LDH by following CytoTox96¹ Assay. The RFRA treatments triggered a higher release of LDH activity than the non-treated cells. A significant ($P < 0.001$) dose dependent rise in LDH release was observed (Table 3). LDH, a cytoplasmic enzyme released to the culture medium when the cellular membrane ruptures. The upregulation of LDH by intracellular release is a measure of cell death due to cell membrane damage and induction of apoptosis (Saad et al., 2006).

The CellTiter-Glo¹ luminescent assay was performed to evaluate the proliferating potential of MCF-7 cells by measuring the cellular ATP content. The energetic level in MCF-7 cells persisted higher, which was evident from the increased ATP level (Table 3). The RFRA (5, 10, 20, and 40 μ g/ml) treated cells resulted in a dose dependent substantial decrease ($P < 0.001$) in cellular proliferation compared control cells. ATP is a biomarker for cell viability and proliferation, and present in all metabolically active cells. The increased levels of ATP can induce cell proliferation, differentiation and apoptosis. The actively dividing cells produce ATP, the main production

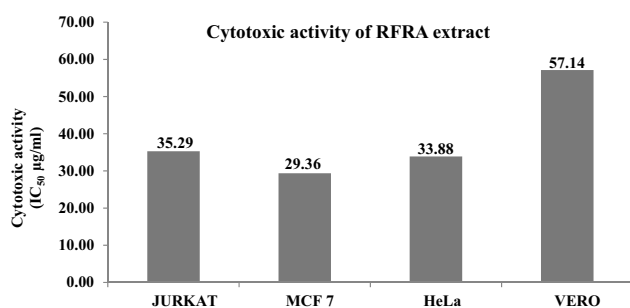


Fig. 3 – Cytotoxic screening of *R. fairholmianus* root acetone extract against various cell lines. RFRA: *R. fairholmianus* root acetone.

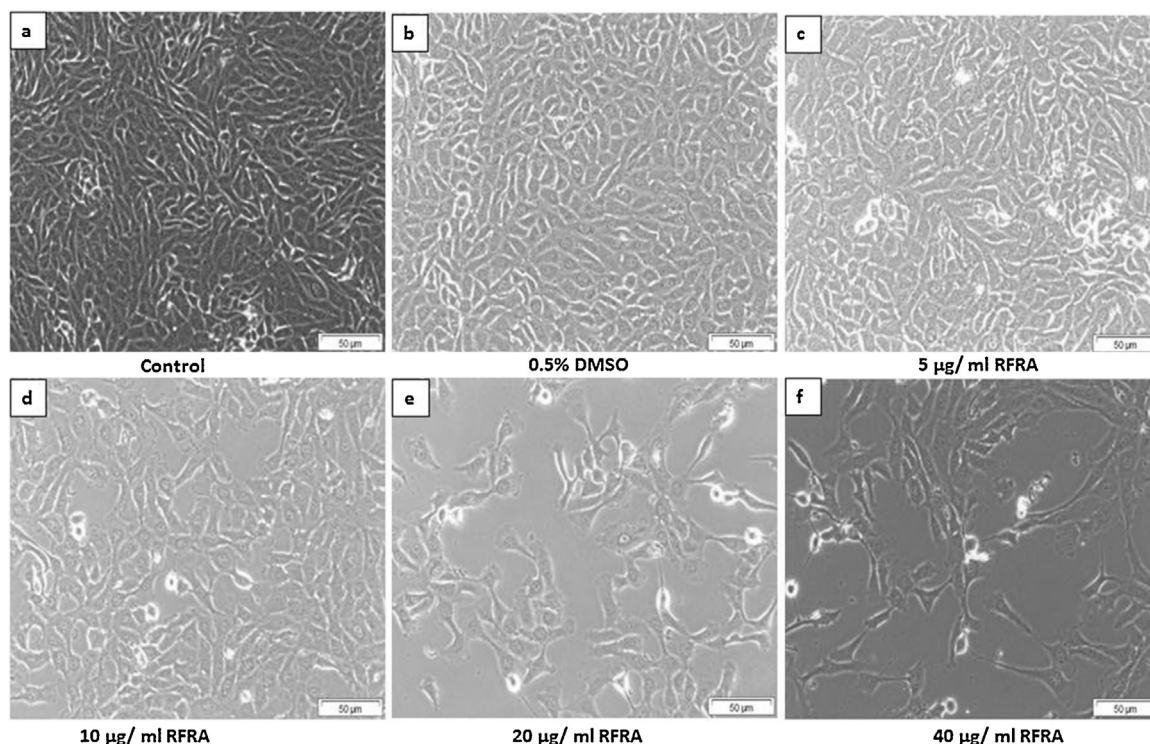


Fig. 4 – Morphological changes of *R. fairholmianus* root acetone extract treated MCF-7 breast cancer cells. Morphology of MCF-7 cells: (a) control cells, (b) 0.5% DMSO treated cells and (c–f) 5, 10, 20 and 40 µg/ml RFRA extract treated cells. RFRA: *R. fairholmianus* root acetone.

occurs in mitochondria and the depletion in ATP correlated with the decreased cell proliferation rate (Vidau et al., 2009). Similar effects were also observed in *R. occidentalis*, *R. jamaicensis*, *R. rosifolius*, *R. racemosus*, *R. acuminatus* and *R. idaeus*; they suppressed the proliferation of colon, prostate, oral, gastric, lung and breast cancer cells and reduced the cell

viability (Jeong et al., 2010; Seeram et al., 2006) by depleting the ATP levels thereby preventing the cell proliferation.

Analysis of programmed cell death

Quantification of caspase 3/7 activity. Caspase 3/7 activity in the cells is a direct mean for the determination of caspase dependent apoptosis. A significant ($P < 0.001$) dose dependent increase of caspase 3/7 activities observed in RFRA treated cells (Fig. 5). Low levels of activity were noticed in the control and 0.5% DMSO treated cells, which was presumably due to the small amount of apoptotic cells present in the growing cell population. Caspases, a family of cysteine-aspartic proteases are vital mediators of apoptotic-signalling (Earnshaw et al., 1999). Caspase 3 and 7 were well established as the major executioner (effector) caspases and have an important role in the execution phase of intrinsic and extrinsic pathways by cleaving many cellular proteins eventually leads to cell death. This cleavage accelerates disassembly of cell, which is evident from the morphological changes including DNA fragmentation (Elmore, 2007). These morphological changes were also observed from the RFRA treated cells and strongly suggested the caspase mediated apoptotic cell death in MCF-7 cells. The previous studies also showed caspase 3/7 dependent apoptotic activities of *R. fairholmianus* root acetone extract on human colorectal cancer cell lines (George et al., 2015). Kim and co-workers (2005) reported the caspase 3/7 activity and apoptosis inducing ability of *R. coreanum* a closely related species of *R. fairholmianus* on colon cancer cells (HT-29).

Table 3 – Effect on *R. fairholmianus* root acetone extract on MCF-7 cells.

Groups	Trypan blue viability (%)	LDH cytotoxicity – membrane integrity ($A_{490\text{ nm}}$)	ATP proliferation (luminescence-relative light unit)
Control	96.50 ± 0.65	0.4250 ± 4.13	574,932.67 ± 5361.46
0.5% DMSO	95.25 ± 0.63	0.4308 ± 3.32	568,694.66 ± 4330.82
5 µg/ml RFRA	85.75 ± 1.38*	0.5033 ± 2.88*	457,607.00 ± 2162.94*
10 µg/ml RFRA	78.25 ± 0.85*	0.5588 ± 2.92*	343,302.68 ± 5917.80*
20 µg/ml RFRA	59.25 ± 1.60*	0.5899 ± 4.31*	326,756.00 ± 6884.78*
40 µg/ml RFRA	31.25 ± 1.94*	0.7881 ± 3.08*	295,452.33 ± 5482.55*

Values are mean ± standard error of six independent assays performed in duplicates. RFRA: *R. fairholmianus* root acetone extract; LDH: lactate dehydrogenase; ATP: adenosine 5' triphosphate; DMSO: dimethyl sulphoxide.

* Significant as compared with control.

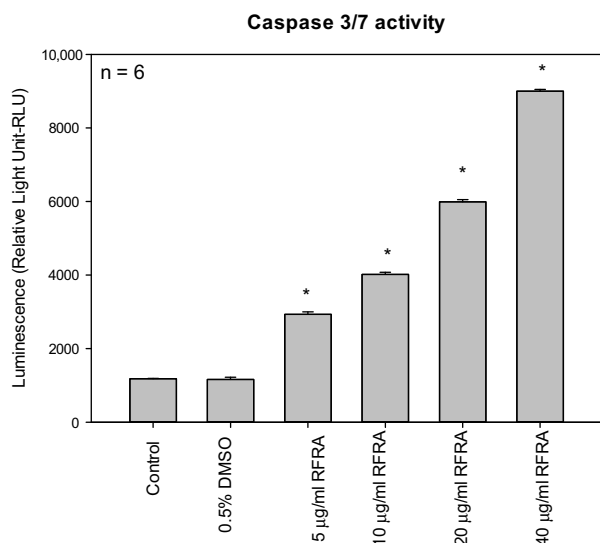


Fig. 5 – Caspase 3/7 activity determined as a function of caspase dependent apoptosis in MCF-7 cells after the treatment with RFRA extract. There is a significant dose dependent increase in caspase 3/7 activity after RFRA treatments (* $P < 0.001$) compared to control and 0.5% DMSO treated cells. RFRA: *R. fairholmianus* root acetone.

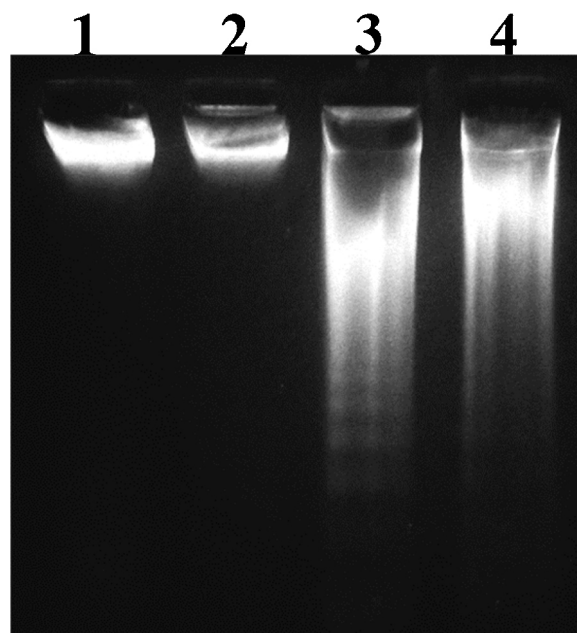


Fig. 6 – DNA isolated from *R. fairholmianus* root acetone extract treated MCF-7 breast cancer cells. DNA isolated from RFRA treated MCF-7 cells; Lane 1: control cells; Lanes 2, 3 and 4: 10, 20 and 40 µg/ml RFRA extract treated cells. RFRA: *R. fairholmianus* root acetone.

DNA laddering. The RFRA (20 µg/ml) treated MCF-7 cells showed an induction of apoptosis with significant ladder formation. However, the higher dose (40 µg/ml) showed a smear but not a clear ladder (Fig. 6). The cells treated with lower dose (10 µg/ml) were not able produce apoptosis by DNA ladder formation. Antitumour activity of plant extracts is either through induction of apoptosis or by inhibition of angiogenesis (Ruby et al., 1995). It has been recommended that apoptosis has a major part in tissue homeostasis and cancer development. Therefore the modulation of apoptosis has become an exciting target for both therapeutic and preventive approaches to cancer. Morphological changes, production of apoptotic bodies and damage to genetic material, which produces cellular destruction, occur during apoptosis. The bioactive compounds isolated from *R. fairholmianus* showed substantial *in silico* anticancer properties against breast cancer target proteins such as BRCA1 and BRCA2. The outcomes of this study furthermore agree and support the previous report and confirm that the antiproliferative activity of RFRA against human breast cancer cells is due to the presence of the 1-(2-hydroxyphenyl)-4-methylpentan-1-one and 2-[(3-methylbutoxy) carbonyl] benzoic acid. In a previous study authors isolated the bioactive compounds from the *R. fairholmianus* root acetone extract via activity guided isolation using chromatography techniques (George et al., 2014b). Our results strongly suggest that the antitumour activity of RFRA extracts is due to induction of apoptosis and a flow of events occurring in the cells producing an eventual cell death and observed by caspase activity, morphological examination and DNA laddering; therefore RFRA could be an external agent for cancer prevention and therapy.

The Annexin V-FITC/PI staining. The Annexin V-FITC apoptosis detection kit was used to distinguish the apoptotic and necrotic cells using flow cytometry. To further understand whether the decrease in cell viability observed was due to apoptosis, we examined the behaviour of cells after treatment using Annexin V/PI staining. The percentage of apoptotic cells increased with the increase in concentration of RFRA. The control and 0.5% DMSO treated cells showed no significant changes in percentage of cell population, most of the cells lined in the live cells range during flow cytometry analysis. The RFRA treated cells showed an increase in the percentages of early and late apoptotic cells. The non-apoptotic or necrotic cells concentration in the treated extracts found to be very low compared with the control group. Fig. 7 shows the results of Annexin V-PI staining after the treatment with RFRA. In the control groups, the population of early and late apoptotic cells were less (4.0 and 0.4%). The 5, 10, 20 and 40 µg/ml RFRA treated groups showed 1.1, 24.4, 42.9 and 50.8 percentages of late apoptotic cells population. When MCF-7 cells were treated with the RFRA, the apoptotic population increased concurrently and induced a decrease in the viable cell (Annexin V-/PI-) population. Although the percentage of late apoptotic and necrotic cells are high in RFRA treated cells, the presence of these cells with early apoptotic cells suggests that such dead cells resulted from the apoptosis rather than necrosis (Hansakul et al., 2014). These findings confirmed that apoptosis may be the possible mechanism by which the RFRA triggers cell death in MCF-7 cells. The low percentage of cell population in quadrant (Annexin V-/PI+) of the RFRA treated MCF-7 cells ruled out necrosis (Naselli et al., 2014).

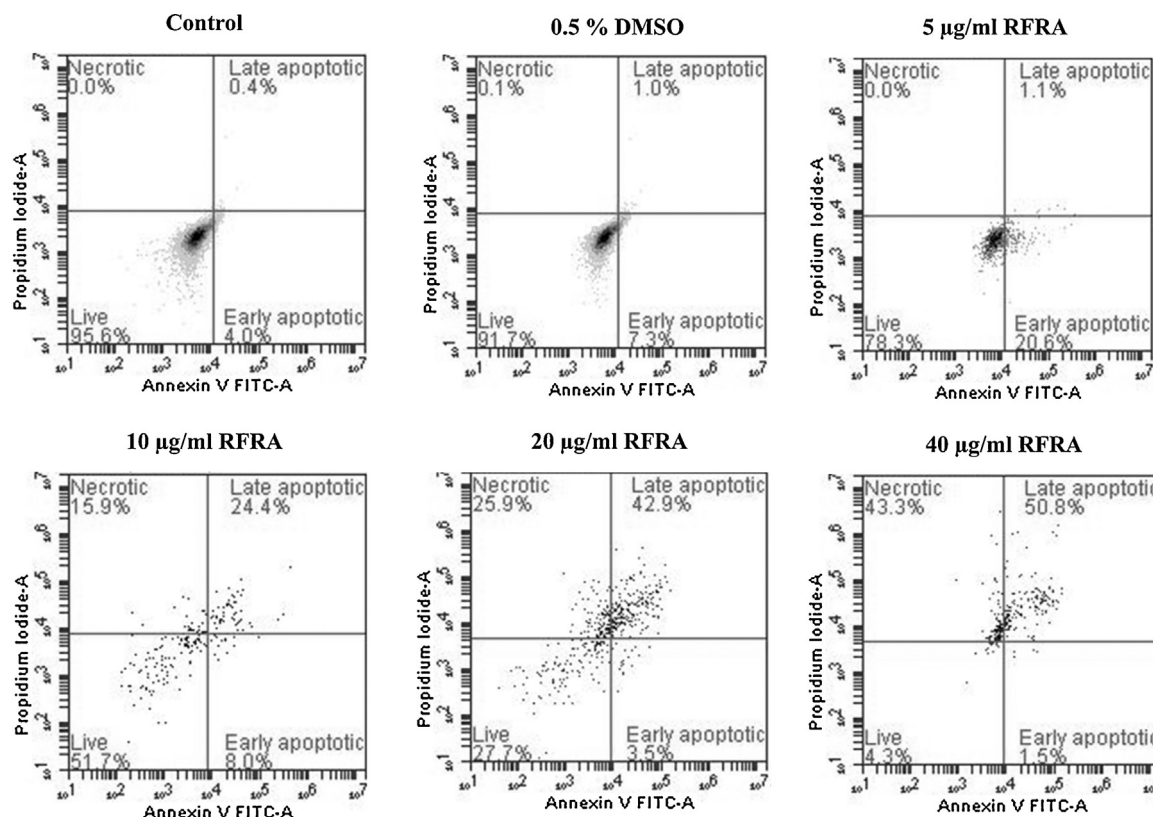


Fig. 7 – Annexin V-FITC/PI staining to assess the cell death mechanism. RFRA (*R. fairholmianus* root acetone) treated MCF-7 cells showed an increased percentage of early and late apoptotic cells population after 24 h incubation. The population of early and late apoptotic cells in the untreated group found to be lower (4.01 and 0.39%) compared with treated groups.

Conclusion

Our findings support the previous literatures related to the pharmacological activities of different closely species of *Rubus*. *R. fairholmianus* also have the potentiality to treat free radical associated disorders including cancer because of its significant antioxidant and reported pharmacological properties. This is the first evidence about the *in vivo* antioxidant and cytotoxic effect on various cancer cell lines, and the antitumour as well as apoptotic activities of *R. fairholmianus* root acetone extract. Further work is wanted to reveal the details of the bioactive compounds which are responsible for the cell death mechanism via caspase 3/7 induced apoptosis. The Annexin V/PI staining supports the apoptosis induced cell death. These results have important clinical implications as it has commendable antiproliferative activities against human breast cancer cells (MCF-7) and it can be considered as an effective adjuvant therapeutic agent after the clinical trials.

Conflict of interest

The authors declare that there is no conflict of interests.

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