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Research Article

Efficacy of Anti-Tumour and Akt Phosphorylation on Cancer Cell Lines, A549 and HepG2, by Extracted *H. polyrhizus* (Red Dragon Fruit)

Alexis M Labrador[1] and Maria Nilda M Muñoz[2,3,4]*

[1]*Department of Biochemistry, Faculty of Pharmacy, University of Santo Tomas, Philippines*

[2]*Biological Control Research Unit, De La Salle University, Metro Manila, Philippines*

[3]*Cagayan State University Andrews Campus, Tuguegarao City, Philippines*

[4]*Center for Marine and Environmental Studies, Ehime University, Ehime, Japan*

***Corresponding Author:** Maria Nilda M Muñoz, Biological Control Research Unit, De La Salle University, Metro Manila and Cagayan State University Andrews Campus, Tuguegarao City, Philippines and Center for Marine and Environmental Studies, Ehime University, Ehime, Japan.

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Abstract

Hylocereus polyrhizus is a climbing cactus that produces red dragon fruit (RDF). It has been frequently employed as a normative foundation for preserving good health such that the pulp and meat of red-skinned dragon fruit have a cancer-fighting effect. Studies *in vitro* have demonstrated that extracted RDF were able to kill cancer cells, however, studies are limited on the mechanisms by which RDF demonstrate its anti-proliferative action on cultured human lung and liver cell lines. The objective of the study is to determine the impact of extracted RDF on inhibiting the cell growth of cultured human lung (A549) and human liver (HepG2) cancer cell lines as well as the underlying mechanism of actions of RDF.

RDF was harvested in Region 1 Burgos, Ilocos Norte, Philippines and authenticated by National Museum. The secondary metabolites, phenols, flavonoids and steroids were analyzed quantitatively from extracted RDF. We identified that the presence of phytosterols was greatest than phenols and flavonoids. Using PrestoBlue cell proliferation assay, the effect of extracted RDF on both cancer cells was assessed on cell viability and cytotoxicity. Treatment of cultured cancer cells with RDF extracts caused a time- and concentration-dependent manner in reduction of cell growth. The minimum inhibitory concentration of RDF was 71.58 ± 3.85 µg/mL against A549 after 16 hr treatment as compared to 58.44 ± 3.15 µg/mL for HEPG2 liver cells.

The mechanism(s) following anti-proliferative effect of RDF were investigated using DNA fragmentation test and immunoblot analysis of key components involved in cell proliferation such as expression of Akt and Caspase-3 protein. RDF caused significant DNA fragmentation, a marker of apoptosis, in both cultured cancer cell types. By Western Blot, application of RDF to cultured A549 and HepG2 cells caused decreased Akt phosphorylation while an increased in Caspase-3 protein expression was observed in both cultured cancer cells. In summary, RDF-induced apoptosis was linked with a) DNA fragmentation of cultured A549 and HepG2 cells, b) downregulation of phosphorylated Akt expression, and c) overexpression of Caspase-3 expression This is the first evidence demonstrating that RDF extracts has an anti-proliferative property in cultured lung and liver cancer cells. Uncovering vast amounts of pharmacological information and establishing the mode of action of these active principles could offer up a new avenue for future drug development efforts, and that the extracted RDF might be used as a possible therapeutic for anticancer medication development.

Keywords: *H. polyrhizus; A549 Cancer Cell Line; HepG2 Cancer Cell Line; Akt; Caspase-3; Apoptosis*

Abbreviations

RDF: Red Dragon Fruit; DNA: Deoxyribonucleic Acid; DOX: Doxorubicin

Introduction

Cancer overtakes stroke and coronary heart disease as the world's leading cause of mortalities in most countries making it as one of the barriers for suppressing increased life expectancy to most people in every part of the world [1,2]. The pronounced increase in cancer incidence and mortality worldwide are being associated to socioeconomic development [1,3,4]. According to GLOBOCAN 2020 report, the top 5 leading cause of cancer deaths worldwide for both sexes are lung (18.0%), colorectal (9.4%), liver (8.3%), stomach (7.7%) and breast (6.9%) cancers [1].

The most fundamental trait of cancer is their ability to sustain limitless cell growth and proliferation [5]. This capability can be achieved by cancer cells in many ways such as the deregulated mitotic signaling [6,7]. Cancer cells can produce their own growth factors and respond to these signals by expressing their own receptors via autocrine proliferative stimulation. Additionally, these cancer cells can send growth factors to neighboring normal cells to convert these cells to become malignant [5,8]. Another important trait of cancer cells is their independence from growth factors which is attributed to the constitutive activation of signaling pathways' components that are regulated by ligand-mediated receptor activation [6,7]. High-throughput DNA sequencing analysis of cancer genomes revealed that somatic mutations ranging from gene amplifications, translocations, and point mutations triggered the predicted constitutive activation events in cell growth signaling pathways leading to carcinogenicity [9]. One of these cells signaling receptor signaling pathway that is disrupted in most cancers is the phosphoinositide-3 kinase (PI3K) signaling including its Akt/PKB (Protein Kinase B) signal transducer [10,11]. PI3Ks are a family of lipid kinases involved in various cell processes such as cell survival, cell proliferation, and differentiation [12]. Different ligands such as growth factors, cytokines, and small molecules can transduce signals to PI3Ks by generating phospholipids that will activate the serine-threonine kinase Akt and other downstream effector pathways. This cell signaling circuit is regulated by negative feedback loop, particularly by the tumor suppressor PTEN (phosphatase and tensin homologue) [13,14]. According to recent cancer genomic studies, germline and somatic mutations are frequently found in many components of PI3K pathways in various types of cancers. These discoveries underscore the importance of exploring the PI3K/Akt component as essential targets in pharmacologic intervention for treating cancer [15].

Treatments to limit its progression include a combination of surgery, radiation, and chemotherapy, as well as hormone treatment. However, these accessible medicines are linked with severe side effects, chemotherapy resistance, and expensive healthcare costs. Many cancer patients are now interested in pursuing complementary and alternative treatment techniques. Although phytochemicals are developing as a supplemental medication in cancer treatment, their increasing demand is exerting strain on plant biodiversity, necessitating appropriate conservation methods [16]. Although the precise mechanism of action of some of these bioactive chemicals is yet unknown, they remain an appealing option for therapeutic potential.

Though not potent enough to replace cancer drugs, it may have a positive health impact by alleviating many of the signs and symptoms associated with cancer. These health issues necessitate further research into alternative medicines or herbal plants as potential sources of natural compounds that could slow the progression of tumor growth and other reoccurring ailments [17,18].

Plant-derived anti-cancer drugs have been demonstrated to display good to excellent anti-cancer activities against a broad range of cancer cell lines. Some of these plant phytochemicals have been studied extensively and serve as reference for the synthesis of novel anti-cancer agents [19-21]. Being useful in overcoming chemotherapy adverse effects intensifies the quest for innovative plant-derived chemotherapeutic drugs. Dragon fruit is a tropical cactus vine that thrives in warmer climates. It is a member of the *Hylocereus* genus and

the *Cactaceae* family. The most often-farmed dragon fruit species are the red variants *Hylocereus undatus* (usually with white meat) and *Hylocereus polyrhizus* (*H. polyrhizus*; less commonly with dark-red flesh) [22]. In the Philippines, *H. polyrhizus* (RDF) is generally used for culinary purposes because the fruit is rich in minerals and vitamins. Farmers and ethnic communities report that the exotic nature of dragon fruit contributes to its wide range of medicinal properties which include its potential as reservoir of potential anti-cancer components. Studies *in vitro* show that extracted RDF offers anti-cancer activity against some cancer cell lines [23]. In addition, the fruit extract also exhibits potent antioxidant activity which is attributed to its diverse bioactive components including phytosterols, phenolic compounds and flavonoids [23-25]. The precise mechanisms by which RDF has an anti-proliferative effect in cancer cell lines, however, have yet to be determined.

The anti-proliferative effects of methanolic extract of red dragon fruit (RDF) on two cultured cancer cell lines, A549 lung cancer cells and HepG2 liver cancer cells, were investigated in this study. To determine the mechanism of anti-proliferation produced by extracted RDF, DNA fragmentation and immunoblotting were used to assess the expression of p-Akt and Caspase -3 in RDF-treated cells. Isolating, structurally elucidating, and establishing the mode of action of these active principles could reveal a wealth of pharmacological information, potentially opening up a new avenue for future drug development efforts.

Materials and Methods

Chemicals and reagents

Solid pellets of NaOH, Na_2CO_3 and $AlCl_{3;}$ Chloroform, ethanol, methanol, and ultra-pure water were purchased from Sigma Aldrich Inc., Singapore. Standards such as gallic acid, quercetin, cholesterol, and doxorubicin were also purchased from Sigma Aldrich Inc., Singapore. Dulbecco Modified Eagle Medium (DMEM) high glucose content, Penicillin-Streptomycin Liquid (PS; 10,000 U/mL), 10% Fetal Bovine Serum, and trypsin-EDTA solution (containing 0.25% trypsin and 0.02% EDTA) were obtained from Invitrogen through Medical Test Systems, Inc. Kits including PrestoBlue cell proliferation assay and DNA fragmentation assay kit were also purchased from Invitrogen through Medical Test Systems, Inc. Caspase-3 antibodies, and Total phosphorylated Akt (Protein Kinase B) horse-radish peroxidase conjugated rabbit monoclonal antibodies were purchased from CBC Link Manila Inc.

Preparation of cell culture

Cancer cell lines (A549, HepG2) and non-cancer C_2C_{12} cell line were purchased from American Tissue Culture Collection (ATCC). These cell lines were stored in liquid nitrogen and aseptically revived prior to use. All cells were maintained in DMEM high glucose containing 10% fetal bovine serum and 1% Penicillin-Streptomycin Liquid. The cells were incubated at 5% CO_2 under 37°C.

Preparation and extraction of RDF fruits

Matured and ripened red dragon fruits were obtained from RefMed plantation farm in Burgos, Ilocos Norte, Philippines, during the fruiting season of June-July (Figure 1). The voucher specimen was submitted for authentication to the National Museum of the Philippines. The fruit was peeled and chopped into pieces before being oven dried at 50°C for three days to remove around 80 to 85% of the moisture. Dried RDF was subjected to Hammermill grinder to obtain powdered RDF; 15g of milled RDF was dissolved in 45 mL of 80% methanol to provide 0.33 g/mL crude extract. The resultant solution was gently heated (50°C) for 1 hr while agitation on a hot plate. The crude extract was filtered using Whatman filter paper No. 1 after cooling for 30 minutes and the filtrate was stored at 4°C overnight. The crude extract mixture was filtered twice using Whatman filter paper no.1 and collected into separate amber vials for storage at -20°C after being refrigerated overnight. The collected extract was placed inside GeneVac Concentrator (Thermo Scientific, Philippines) for 4 hr at 37°C for lyophilization. The lyophilization process turned the liquid extract into powdered form and this was kept at -20°C until use.

Preparation of cultured cancer and non-cancer cell lines

Both types of cells were first cultured in T25 culture flasks in full medium made up of Dulbecco Modified Essential Medium (DMEM) with a high glucose content and 10% Fetal Bovine Serum (FBS). The cells were cultured at 37°C for 2 - 3 days until 80 - 90% confluence was attained. The flasks were then treated with a Trypsin-EDTA solution to remove adherent cells. The supernatant was decanted after centrifugation, and the cell pellet was resuspended in 2 to 3 mL of complete growth media. A 10 mL volume of this cell solution was utilized to test cell viability using trypan blue exclusion dye.

Test for total phenolic content Folin-Ciocalteu method

For standard calibration preparation, increasing concentration (0.0025, 0.0150, 0.0250, 0.0350, 0.0450 mg/mL) of gallic acid solution was prepared as standard. For sample preparation, 0.150 mL of distilled water was added to 0.05 mL of 0.33 g/mL extract. Thereafter, 1 mL of 10% Folin-Ciocalteu's phenol reagents was added to the mixture followed by 0.8 mL of 2% Na_2CO_3. For blank preparation, 0.2 mL of distilled water was added to 1 mL of 10% Folin-Ciocalteu's phenol reagent and 0.8 mL of 2% Na_2CO_3. The standard, sample and blank was incubated for 15 min at room temperature. For absorbance reading, the spectrophotometer was set at 765 nm. The spectrophotometer reading was set to zero or 0.000 using the blank. The standard and sample readings were then recorded. The data was expressed in mg gallic acid equivalent per gram sample (mg GAE/g sample).

Total flavonoid content (TFC)

The total flavonoid content of the RDF crude extracts was determined using the Dowd method. Quercetin was used as the standard flavonoid for the calibration. For sample preparation, 1.0 mL of RDF extracts was aliquoted into test tubes. Into each tube, 990 µL of 2% $AlCl_3$ was added. The mixtures were incubated at room temperature for 10 min. Then, all of the prepared samples and standards were incubated at room temperature for 10 min. The spectrophotometer was set at 415 nm and blank and standards were read accordingly. The assay was conducted in triplicates. Data are expressed as mean ± SEM.

Total steroid (phytosterol) content (TSC)

Estimation of total steroids was performed by Liebermann Burchard reaction. Calibration of the assay was conducted using cholesterol as standard. For sample preparation, 300 µL *of* RDF extracts was aliquoted separately and mixed with 2.50 mL of cholesterol color developer. The resulting mixture was vortexed for 10 sec and the reaction tubes were placed in a 37°C water bath for 10 min. Test tubes were allowed to cool down to room temperature prior absorbance reading. The spectrophotometer was set at 620 nm in which distilled water was used as blank solution. The assay was conducted in triplicates. Data are expressed as mean ± SEM.

Proliferation assay (PrestoBlue assay)

Each well was seeded with 5 x 10^4 prepared cells per well and incubated at 37°C for 24 hr. When the cells were ~80% confluent, increasing concentrations of RDF extracts (25, 50, 75, 100, 250, 500, 750 µg/mL) were added to the test material microwells. Positive controls were cells treated with doxorubicin-HCl, negative controls were cells treated with PBS (+/-) Ca^{+2}/Mg^{+2}, and untreated controls were cells treated with growth media (no RDF extract). The microwell plates were incubated for 16 and 24 hr, respectively. Presto blue reagent (10 µL) was directly applied to all wells, and the plates were incubated at 37°C for 10 min. Plates were read at 560 nm on a microplate reader, and the findings were recorded. Three IC values were calculated to see if the extract has concentration-dependent antiproliferative action.

DNA fragmentation

A549 and HepG2 cancer cell lines were seeded in 6-well plates at a density of 5 x 10^5 cells per well for 24 hr. When the cells achieved ~80% confluence, they were washed with PBS and treated for 16 hr at 37°C with the calculated concentrations of IC_{25}, IC_{50}, and IC_{75} of the RDF extracts. Positive control cells were treated with Doxorubicin-HCl (DOX), a synthetic anti-cancer medication. PBS-treated cells served as the negative control, whereas cells grown in growth media without RDF extracts served as the untreated control. For 16 hr, the plates were incubated at 37°C. After treatment, the plate was centrifuged for 5 min at 1000 rpm. The mixture was centrifuged after being washed with ice-cold PBS. The cell pellet was resuspended in 35 µL Tris-EDTA lysis buffer, and the crude lysate was treated with 5 µL Enzyme A Solution. After ten min, 5 µL Enzyme B Solution was added and incubated for another 30 min in a 50°C water bath. A 5 µL of ammonium acetate solution and 100 µL of 100% Ethanol (kept at -20°C) were separately added to each cell lysate in the combination. Following mixing, the DNA could precipitate at -20°C before being rinsed with 0.5 mL of 70% cold ethanol. DNA pellet was air dried and 30 µL of suspension buffer was added to resuspend the DNA.

Preparation of agarose gel for DNA separation

Each sample was put to a 1.2% agarose gel containing 0.5 µg/mL Gel Red in a total of 10 µL. The DNA sample was transferred onto the gel and was separated for 30 min at 100 V/cm in 1X Tris-Acetate EDTA (TAE) buffer. Transillumination with UV light was used to visualize the gel red-stained DNA, which was subsequently photographed.

Immunoblotting analysis

The Bradford test was used to assess total protein content. In the presence of protease and phosphatase inhibitors, cells from treated A549 and HepG2 cancer cell lines were lyzed with RIPA lysis buffer. Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at continuous voltages of 120 V for 15 min (Stacking Gel) and 200 V for 30 min (Resolving Gel). Ponceau S was used to dye the membranes to ensure full protein transfer. The membrane was then blocked with 1% milk in Tris-buffered saline (TBS, 1x) solution. The membrane was treated overnight at 4°C with continual agitation with primary antibodies (1:2000 dilution; Rabbit polyclonal antibody for Akt and Mouse Monoclonal antibody for Caspase-3). The membrane was incubated for 1 hr at room temperature with a horse radish peroxidase (HRP)-labeled goat anti-rabbit secondary antibody (1:2000). The interaction of the primary and secondary antibodies was observed colorimetrically by treating the membrane with Enhanced Chemiluminescence (ECL). Images were photographed using the Image Quant Detection (GE Bioscience, ChemoScience Inc.) and densitometric analysis was performed on photographed blot using Image J Analysis software.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM) and were analyzed using One-way ANOVA. In comparison of three or more groups, Tukey method was used as post hoc test regarding $p < 0.05$ as significant after analysis of variance (ANOVA). GraphPad Prism Software 9.0 (GraphPad, San Diego, CA) was used for all analyses. A value of $p < 0.05$ was considered statistically significant.

Results and Discussion

Description of *Hylocereus polyrhizus* (Red Dragon Fruit)

Dragon fruit has a striking look, with vivid red skin and noticeable scales (Figure 1). The fruit is either round, elliptical, or pear-shaped. The meat has a mildly flavored sweet or somewhat sourish flavor. The flesh is red, with edible black seeds strewn about.

Figure 1: *Red Dragon fruit. Original Photograph by Alexis M. Labrador (2017; copyright).*

Quantitation of flavonoids, polyphenols, and phytosterols

Baseline amount of flavonoids in the RDF extract was 14.78 ± 0.22 mg quercetin equivalent/g extracts. When compared to standard reference Lagundi leaves, the amount of flavonoids was < 2.7%, less than the value of RDF extracts ($p < 0.01$). Lagundi leaves were chosen since it is known to have substantial amounts of active secondary metabolites as well as anti-inflammatory properties [26]. Flavonoids are the most abundant group of plant phenolics and are broadly dispersed in diets. Flavonoids have been shown to be very useful, serving as antioxidants and offering protection against a variety of inflammatory disorders including cancer [27,28].

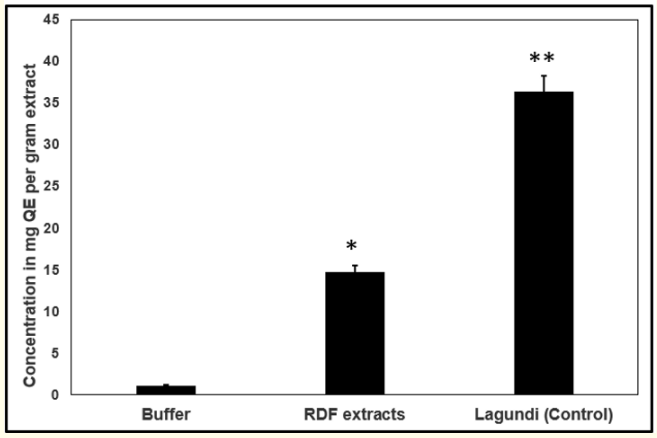

Figure 2: *Total flavonoid content present in extracted RDF. Results are expressed as mg quercetin equivalent (QE) per gram extract as compared to Lagundi leave extract as control. *p < 0.05, buffer vs RDF extracts; *p < 0.05, RDF extracts vs Lagundi; **p < 0.001 Buffer vs Lagundi.*

The measured total phenolic content of RDF fruit was 20.21 ± 0.20 mg gallic acid equivalent (GAE)/gram extracts. Extracted Lagundi leaves yielded 10.26 ± 0.050 mg GAE equivalent/g extract, which was 50.77% lower than RDF ($p < 0.01$ vs. RDF). It has been reported that the phenols are the most abundant group of phytochemicals, accounting for the majority of antioxidant activity in plants or plant products [29].

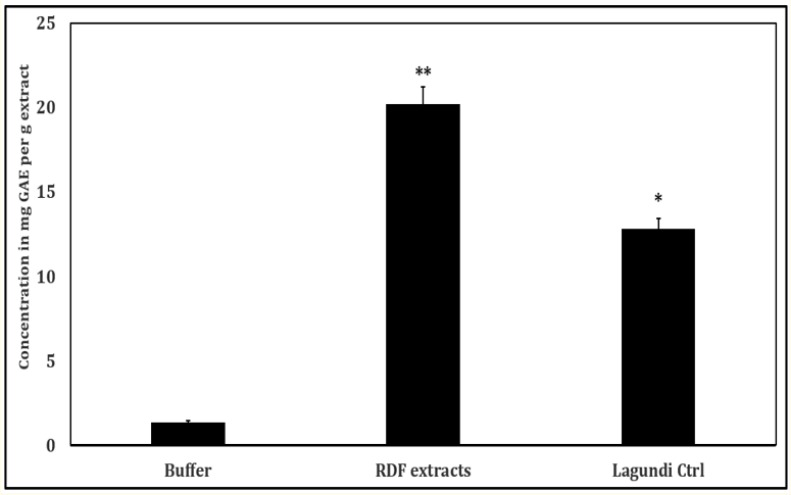

Figure 3: *Total phenolic content present in extracted RDF. Results are expressed in mg gallic acid equivalent (GAE) per gram extract as compared to Lagundi leaves extract as control. **p < 0.01, Buffer vs RDF extracts; *p < 0.05 Buffer vs Lagundi; *p < 0.05 RDF vs Lagundi.*

The overall phytosterol concentration of RDF fruit extracts was 78.75 ± 4.75 mg cholesterol equivalent/gram extract. The amount of phytosterol in Buffer (no extracts) was insignificant while Lagundi leave extracts was 11.99 ± 0.40 mg cholesterol equivalent/g extract ($p < 0.01$; RDF vs. Lagundi). It has been demonstrated that plant sterols have the ability to limit dietary cholesterol absorption in people with cardiovascular disease, a kind of inflammatory disease [30,31]. With these findings, it is presumed that RDF fruits play an important role in radical scavenging and, as a result, can be considered a beneficial plant species for natural antioxidant sources with potential applications in the treatment of many life-threatening inflammatory diseases.

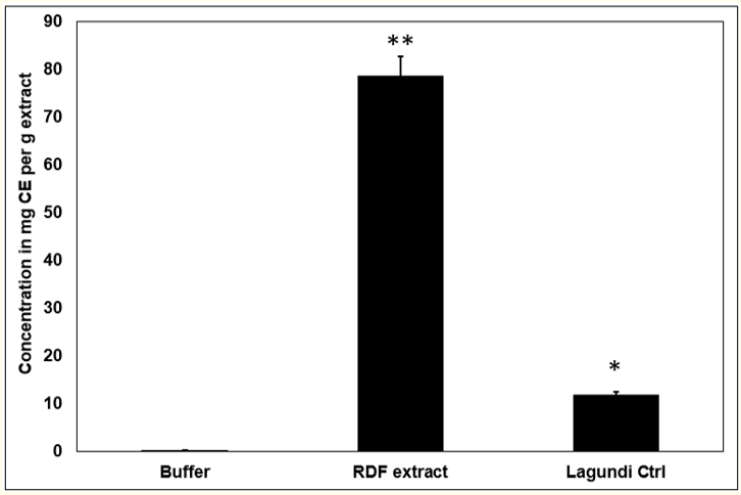

Figure 4: *Total phytosterol content in mg cholesterol (CE)/mg extracted sample. Results are expressed in mg cholesterol (CE)/mg extracted sample as compared to Lagundi leaves extract as control. **p < 0.01, Buffer vs RDF extracts; RDF vs Lagundi; p = NS, Buffer vs Lagundi.*

Moreover, the amount of phenolics and phytosterols in the fruit can be ascribed to the antiproliferative effect of extracted RDF against lung and liver cancer cells [32,33]. One of the most abundant phytochemicals measured in extracted RDF is phenolic acids, which is followed by flavonoids [34]. Phenolic acids and flavonoids have the structural property of possessing C2-C3 double bonds, which is important for their antiproliferative actions [35]. Methanolic extract of RDF shows anti-cancer properties similar to previous reports [34,36]. in which common triterpenoids discovered in the fruit extract displayed promising anti-cancer actions against a range of cancer cell lines. The results provided are consistent with the findings of Li F., *et al*. 2012, who discovered that an ethanolic extract of *H. undatus* and *H. polyrhizus* produces cytotoxicity on HepG2 cells in a concentration and time-dependent manner [37].

Effects of extracted RDF on A549 and HepG2 cancer cell growth by PrestoBlue

Treatment of cultured A549 lung cancer cells with extracted RDF demonstrated anti-proliferative effects in a concentration-dependent manner (Figure 5A). The inhibition of cancer cell growth in A549 cancer cells 16 hr after treatment with extracted RDF ranged from 45% to 90%. The onset of inhibition caused by RDF was observed at 25 µg/mL and peaked at 100 µg/mL. Doxorubicin (DOX), a type of chemotherapy drug, was used as standard reference drug for this study. Treatment with DOX demonstrated comparable blockade of cell growth in A549 lung cancer cells (p = NS). In other separate experiments, the extracted RDF reduced substantially the growth of HepG2 liver cancer cells by >80% even at 25 µg/mL, the lowest concentration used (Figure 5B). It is likely that extracted RDF has cytotoxic activity similar to DOX that blocked the enzyme called topoisomerase 2 activity necessary in stopping the growth of cancer cells through stabilization of DNA topoisomerase II complex [38,39].

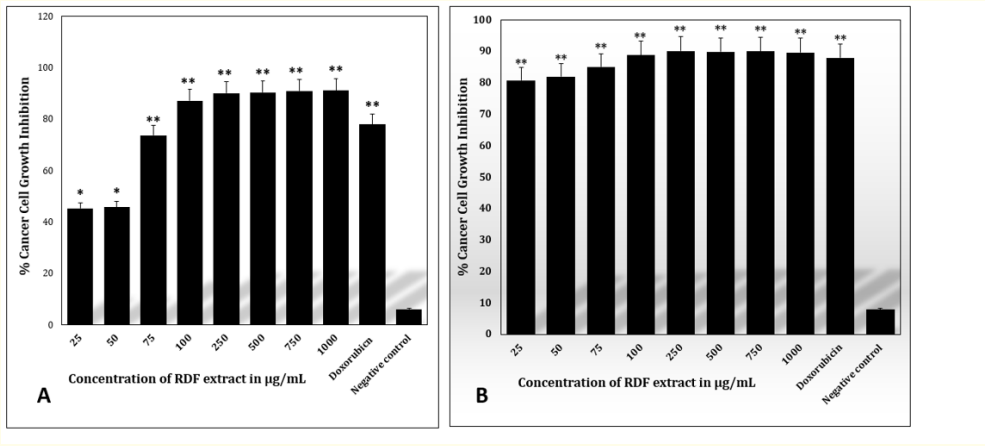

Figure 5: *Anti-proliferative effect of extracted RDF on cultured (A) A549 lung cancer and (B) HepG2 liver cancer cells after 16 hr treatment. Positive control is doxorubicin (DOX) and negative control (NC) is untreated cells. *p < 0.05, NC vs 25 = 50 µg/mL; ** p < 0.001, NC vs concentrations > 75 µg/mL vs DOX; p = NS DOX < 75 µg/mL.*

Incubation of cultured (A) A549 lung cancer and (B) HepG2 liver cancer cells for 24 hr with RDF extracts had no further blocking effect on cell proliferation (Figure 6A/6B) as compared to 16 hr treatment. We observed that > 75 µg/mL extracted RDF is more effective and potent in suppressing the HepG2 liver cancer cell proliferation compared to A549 lung cancer cells and that RDF is equally potent as Doxorubicin. Furthermore, it is highly likely that the extracted RDF could serve as a DNA interchelator that inhibits topoisomerase II thereby, inhibiting cancer cell growth.

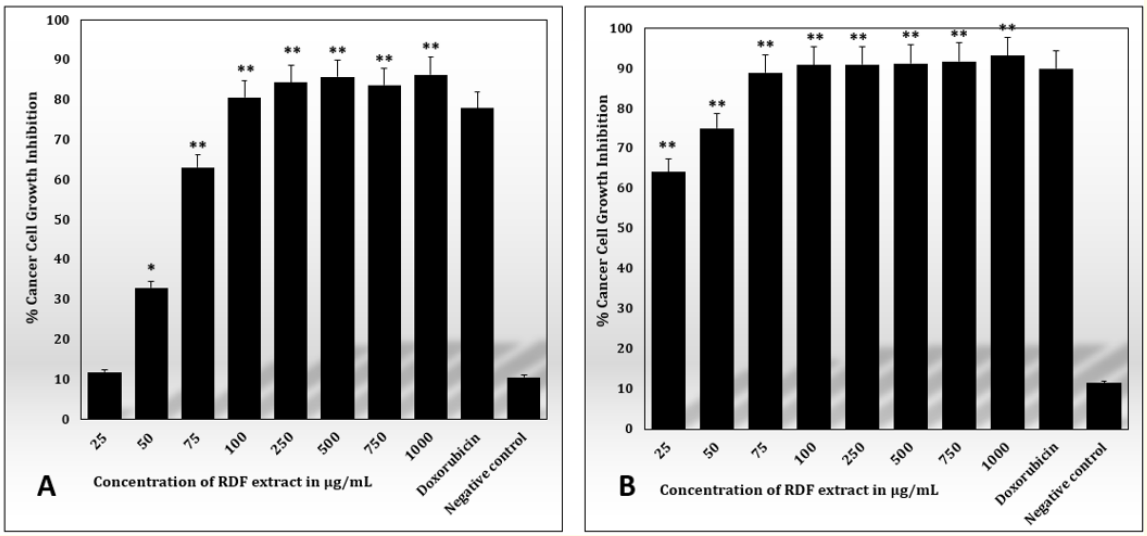

Figure 6: *Anti-proliferative effects of extracted RDF on (A) A549 lung cancer and (B) HepG2 liver cancer cells after 24 hr treatment. Positive control is doxorubicin (DOX) and negative control is untreated cells.*

*P = NS, Control vs 25 ug/mL; *p < 0.05, control vs 50 ug/ mL; **p < 0.001 control vs > 75 ug/mL; p = NS DOX vs > 75 ug/mL.*

Effect of RDF in DNA Fragmentation

The observation that nuclear DNA isolated from apoptotic cells was frequently damaged in an internucleosomal pattern was a defining feature of apoptosis [40]. During apoptosis, DNA cleavage happened at places between nucleosomes, protein-containing structures found in chromatin at 200-bp intervals. This DNA fragmentation was frequently examined using agarose gel electrophoresis, which revealed a "ladder" pattern at 200-bp intervals.

Figure 7 demonstrated the DNA fragmentation of human A549 lung cancer cells (Figure 7A) and human HepG2 liver cancer cells (Figure 7B) treated with increasing concentrations of extracted RDF. DNA fragmentation caused by application of RDF was concentration-dependent. In A549 lung cancer cells, we observed that extracted RDF resulted in 2 distinct bands located at 50-bp and 100-bp, and was consistent with DOX, the positive control. Although lighter in appearance, RDF causes more fragmented DNA bands on HepG2 liver cancer cells than on A549 (Figure 7B). Bands of DNA at > 300-bp were identified and aligned with DOX. The strong band smearing observed beneath the NC (negative control) and UC (untreated cells) lanes was caused by an intact DNA molecule that did not separate during agarose gel electrophoresis. We discovered that RDF had the same impact as doxorubicin in generating DNA fragmentation, implying that the extract has genotoxic effects on cancer cells. The genotoxicity of extracted RDF on cancer cells may be related to the presence of phenolics and phytosterols in the extract, which produce free radicals that interfere with cancer cells' actively dividing DNA [29,41].

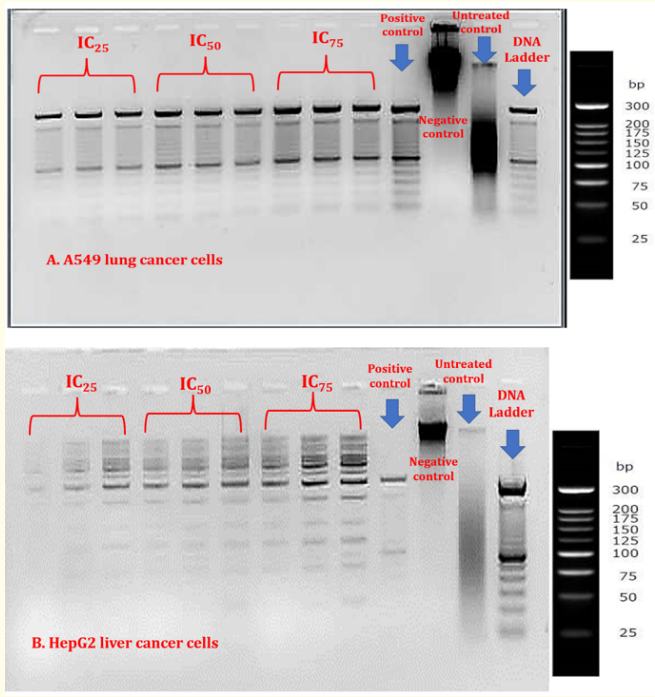

Figure 7: *Appearance of DNA fragmentation on cultured human (A) A549 lung cancer cells and (B) HepG2 liver cancer cells by extracted RDF using agarose gel electrophoresis. IC (Inhibitory concentration); PC (Positive Control, DOX); NC (Negative Control); UC (Untreated cells, no RDF).*

There are some limitations in our DNA fragmentation analysis. We failed to identify strand breaks early in the apoptotic process, however, we presumed that this could be due to an insufficiently sensitive approach or an unintended failure to further damage this sensitive DNA during processing. Whatever the cause, the timing of DNA strand breaks during apoptosis remains unknown. These findings showed that detection of DNA fragmentation should be interpreted with caution, and that more research is needed to identify the nature and timing of DNA alterations that occur during apoptosis.

Protein expression of p-Akt transduction and Caspase-3 activation

Overexpression of Akt protein, a serine/threonine-specific protein kinase, has been reported in several malignancies, including ovarian, lung, and pancreatic tumors, and has been linked to higher cancer cell proliferation and survival [11,15]. As a result, targeting Akt could be a significant technique for cancer prevention and treatment. Overexpression of Akt in cancer delays apoptosis by a variety of mechanisms, including the inactivation of Caspase-3, which inhibits apoptosis [42].

In this study, we measured the 60 kDa Akt protein expression to examinee the putative mechanism(s) of action of anti-proliferative activities of RDF against A549 and HepG2 cancer cells. Using the mAb directed against Akt phosphorylation, we detected that Akt protein was constitutively expressed in untreated (UC) and media-treated A549 lung cancer cells (Figure 8A/8B). Representative immunoblot showed that increasing RDF concentrations (IC_{25}, IC_{50}, and IC_{75}) attenuated substantially the Akt phosphorylation (p-Akt). Collated data

by densitometirc analysis (n-=3 trials), demonstrated that application of IC_{75} RDF attenuated significantly the p-Akt compared to IC_{25} and IC_{50}, respectively ($p < 0.01$ vs IC_{25}; $p < 0.05$ vs IC_{50}). Comparable inhibition of p-Akt was observed when Doxorubicin (DOX) was compared with IC_{25} RDF (p = NS). These findings validated that RDF is as potent as DOX in blocking the proliferation of A549 cell growth. Higher concentration of RDF (>IC_{50}) further suppressed the p-Akt.

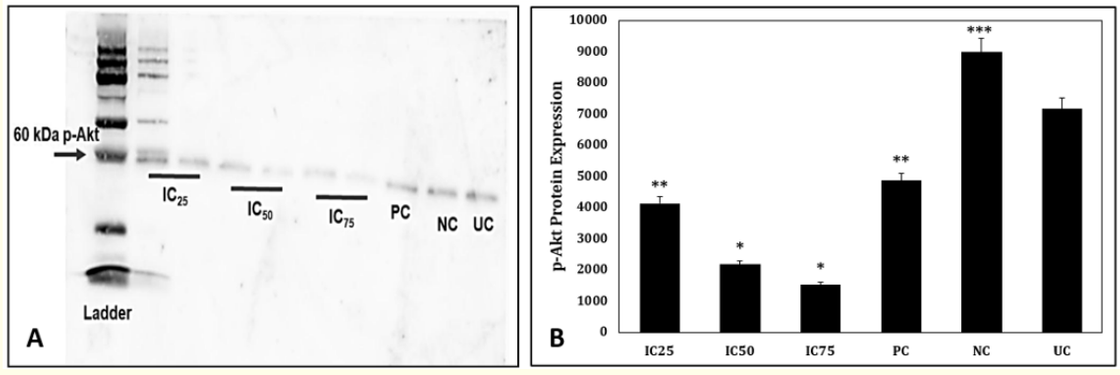

Figure 8: *(A) Representative immunoblot of Akt phosphorylation (p-Akt) on RDF-treated A549 lung cancer cell lines. RDF caused reduction of phosphorylated Akt in concentration-dependent manner. (B) Composite data after densitometric analysis of 60 kDa p-Akt expression on RDF-treated A549 lung cancer cells (n = 3 trials). *p < 0.05, IC_{75} vs IC50; **p < 0.01 IC_{75} vs IC_{25} = PC vs NC; ***p < 0.001, IC_{75} vs NC = UC.*

The pattern of inhibition of p-Akt on HepG2 liver cancer cells (Figure 9A) treated with RDF was parallel to A549 lung cancer cells. There is no statistical difference in baseline Akt expression in A549 lung cancer cells when compared to HepG2 liver cancer cells (p = NS). However, RDF is more effective and potent in blocking the proliferation of HepG2 liver cancer cells for all concentrations of RDF tested (Figure 6B). Inhibition of p-Akt was ~76% at IC_{25}, ~92% at IC_{50}, and ~95% at IC_{75} (Figure 9B). Our findings indicate that RDF is more efficient in suppressing p-Akt in treated HepG2 cells than in treated A549 cells.

There are three different Akt isoforms, namely Akt1, Akt2 and Akt3 [43]. Despite their great similarity (80%), the different Akt isoforms have non-redundant, and in some cases antagonistic effects under healthy and pathological settings [44]. Although numerous suggestions have been proposed as to how isoform specificity is accomplished, the processes underlying isoform-specific effects remain mostly unknown. As a result, more research is needed to have a better understanding of isoform specific Akt signaling in cancer cells and the mechanism underlying it.

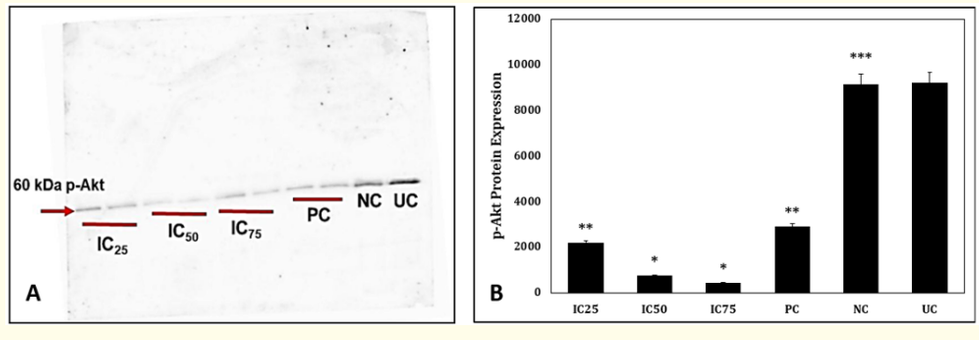

Figure 9: *Expression of 60 kDa p-Akt expression on RDF-treated HepG2 liver cancer cells. By Western Blot analysis, the reduction of p-Akt is concentration dependent (n=3 trials). Reduction of expression of p-Akt in liver cancer cells treated with extracted RDF is significantly different (P < 0.001) compared to PC, NC and UC treated cells. *p < 0.05, IC_{75} vs IC_{50}; **p < 0.01 IC_{75} vs IC_{25} = PC vs NC; ***p < 0.001, IC_{75} vs NC = UC.*

Expression of Caspase-3 in A549 lung cancer cells and HepG2 liver cancer cells

Caspase-3 is classified as an executioner caspase in apoptosis because it coordinates the destruction of cellular structures such as DNA fragmentation or cytoskeletal protein breakdown [45]. Downregulation of Caspase-3 has been associated to the development of breast cancer, prostate cancer, etc. according to growing data [46,47]. Several studies found that caspase-3 expression reduced the risk of getting breast cancer, etc. [48], while others found the opposite [49,50]. This study investigated the predictive relevance of Caspase-3 expression *in vitro* and the effect of RDF in cultured A549 lung cancer cells and HepG2 liver cancer cells.

The expression of Caspase-3 was notably upregulated in A549 lung cancer cells treated by extracted RDF (Figure 10). However, the presence of Caspase-3 in PC (DOX)-treated cells is greatest compared to RDF treated A549 cells ($p < 0.01$ vs RDF). There is minimal expression of Caspase-3 expression observed in NC and UC (Figure 10A/10B). Densitometric analysis from 3 trials showed that Caspase-3 expression was greatest at IC_{50} RDF in lung cancer cells but did not differ significantly when compared with DOX (p = NS).

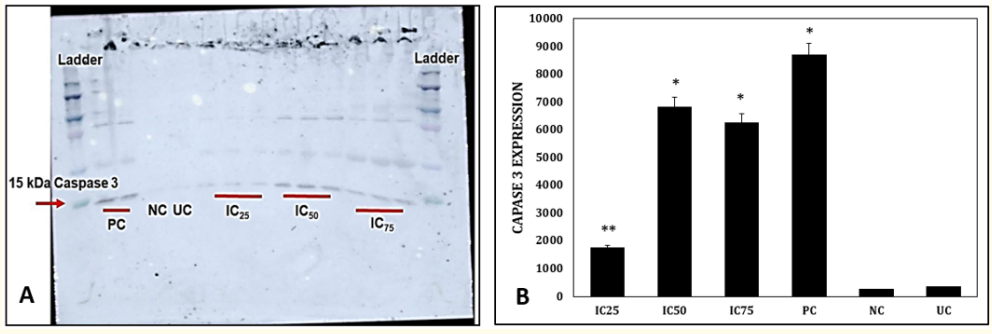

Figure 10: *Caspase-3 expression. (A) Western blot analysis of Caspase-3 expression of treated cultured A549 lung cancer cells. (B) Densitometric analysis of Caspase-3 expression on RDF-treated A549 lung cancer cells (n = 3 trials). **p < 0.01 IC_{25} vs IC_{50} vs IC_{75}; *p < 0.05 IC50 = IC_{75}.*

Figure 11 demonstrates the concentration-dependent increase in Caspase-3 expression mediated by extracted RDF in HepG2 liver cancer cells. RDF stimulates Caspase-3 considerably (P 0.05), which is comparable to the upregulating actions of PC-treated cells (n = 3 trials). Caspase-3 expression was not significantly increased in cells treated with NC or UC.

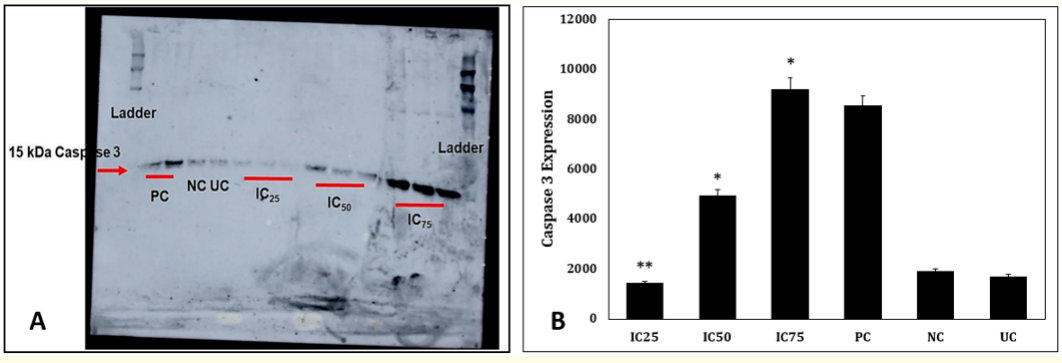

Figure 11: *Caspase-3 expression. (Left) Western blot analysis of Caspase-3 expression of treated cultured HepG7 cells. (Right) Densitometric analysis of Caspase-3 expression on RDF-treated HepG2 liver cancer cells (n=3 trials). **p < 0.01 IC_{25} vs IC_{50} vs IC_{75} vs PC; *p < 0.05 IC50 vs IC_{75} vs PC; p = NS IC_{75} vs PC.*

Summary

In summary, this study is the first to show that RDF is a novel medicinal plant that induces apoptosis in A549 lung cancer cell lines and HepG2 liver cancer cell lines. Fragmented DNA and downregulation of phosphorylated Akt expression with subsequent Caspase-3 activation are responsible for the anti-proliferative effect in both cells. RDF is high in phytosterols, flavonoids, and phenols, all of which have been shown to induce a strong apoptotic death mode in lung and liver cancer cells. RDF is expected to become one of the candidates for a potent anti-cancer agent in the future. RDF's discovery could lead to significant advancements in cancer therapy in the future, as well as significant translational implications in the management of this deadly disease.

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Conflict of Interest

There is no conflict of interest related in this study.

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Volume 10 Issue 3 March 2022