Role of the Red Fruit (*Pandanus conoideus* LAM) Ethyl Acetate Fraction on the Induction of Apoptosis vs. Downregulation of Survival Signaling Pathways in Cervical Cancer Cells

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Authors' contributions

This work was carried out in collaboration among all authors. All authors designed the study, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and managed literature searches. All authors read and approved the final manuscript.

| Original Research Article |

ABSTRACT

Red fruit (*Pandanus conoideus* Lam) has been used as a traditional herbal medicine in Papua and also as an agent against malignant diseases empirically. To elucidate the detailed mechanisms producing such an activity, the characterization and determination of the molecular mechanisms of its antitumor effects were conducted. The inhibitory activities against cervical cancer cell proliferation and the expression levels of corresponding molecules were investigated using human cervical cancer cells treated with the ethyl acetate fraction of red fruit extract (EtOAc RF). The EtOAc RF possessed strong anti-proliferation activities against HeLa and CaSki cells. Furthermore,

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the down regulation of the expression of phosphorylated Akt and mTOR in the cells was induced shortly after treatment with the fraction, followed by the activation of caspase-8, caspase-9, caspase-3, p53 serine 46 and PARP along with the suppression of the expression of Bcl-2 family proteins and Akt–mTOR, leading to apoptotic cell death. Taken together, these results suggest that red fruit extract could be a promising agent against cervical cancer cells.

1. INTRODUCTION

Cervical cancer is the second most common gynecological cancer worldwide, carrying a high mortality rate for women. Death due to cervical cancer is mostly found in third-world countries such as Indonesia, where 90% mortality is found [1]. Of the estimated 500,000 new cases of cervical cancer diagnosed each year, 80% occur in developing countries, with the highest rates occurring in Africa, Asia, and Central and South America [2,3]. Cervical cancer is a major problem in Indonesia. In its early stages, the disease often does not cause symptoms or complaints, so most patients visit the health center already at a later stage [4]. The data show that 60% of patients who visited our center (2007–2011) were diagnosed with cervical cancer out of a total of 11,434 gynecological cancer patients; among them, 72% already had end-stage cervical cancer [5,6].

Red fruit (RF) or *Pandanus conoideus* Lam, commonly known as Buah Merah, is a medicinal plant that originated from Papua (Fig. 1A, B). It is popular among Indonesians due to its wide array of medicinal properties. Some studies have demonstrated that the fruit extracts of RF have properties against degenerative diseases, such as hypertension, diabetes, heart disease and cancer [7]. Moreover, its potency as an anticancer agent has been known for generations. Many studies have been conducted concerning the extraction, isolation, and characterization of RF's bioactive constituents (compounds) as potential sources of anticancer agents [7-9]. In this study, the *Pandanus conoideus* Lam extract prepared from the ethyl acetate fraction of red fruit (EtOAc RF) was investigated *in vitro,* and its antitumor effects on cervical cancer cells were elucidated together with the determination of the molecular mechanisms involved and apoptosis- and survival-signaling pathways. Our results suggest that EtOAc RF, as a natural compound, may provide the possibility of an application for future cancer prevention and therapy.

2. MATERIALS AND METHODS

2.1 Cell Lines and Culture Conditions

Two human cervical cancer cell lines, HeLa and CaSki, were purchased from the American Type Culture Collection (Manassas, VA). HeLa cells were maintained in Eagle's Minimum Essential Medium (EMEM) obtained from Sigma Chemical Co. (St. Louis, MO), supplemented with 2 mM L-glutamine, 1.0 mM sodium pyruvate, and 10% heat-inactivated fetal bovine serum (FBS) obtained from Gibco (BRL, Grand Island, NY). CaSki cells were maintained in RPMI-1640 medium (Gibco) supplemented with HEPES (Sigma) and 10% FBS.

2.2 Plant Materials

Pandanus conoideus Lam or red fruit (RF) was purchased from the Merdey district, Teluk Bintuni, West Papua, Indonesia. The plant species was identified by the laboratory of Plant Taxonomy staff at State University of Papua, Manokwari, West Papua, Indonesia. A voucher of the specimen was deposited at the Herbarium of the Bandung Institute of Technology, Bandung, Indonesia.

2.3 Extraction and Isolation (Fig. 1C)

The fresh fruits of RF (900 gr) was subjected to MeOH extraction. The MeOH extract (600 gr) was partitioned among EtOAc, *n*-hexane and water to afford an active EtOAc extract (16 gr). Four grams of EtOAc was evaporated (Fig. 1D) to yield 3.2 gr of EtOAc, which was then dissolved in 0,05% DMSO. After serial testing, we determined the IC_{50} EtOAc BM for CaSki cells (after 32× dilution from the stock) to be 3.3 mg/ml.

2.4 Inhibition Concentration (IC) Assay

An IC assay for each compound was conducted in the presence of serially diluted compounds as described previously [10-12]. Cell viability was then measured with the aid of a cell counting kit

(Dojin, Tokyo, Japan) according to the manufacturer's instruction. Cell proliferation rate was then determined by measuring the absorbance of the well at 450 nm with the reference wavelength at 650 nm. The IC rate (%) was calculated using the formula: (1 – optical density of the treated cells/ optical density of the untreated cells) x 100. Each sample was assayed in triplicate.

2.5 Protein Extraction and Western Blot Analysis

All of the cells were harvested at approximately 80% confluent growth. The protein concentrations of the cell lysate were determined using a BCA protein assay kit (Pierce, Rockford, IL) and BSA as a standard. Each sample (50 µg proteins/line) was run on a 5-20% Ready Gel (Bio-Rad, Tokyo, Japan), and the gel was then electrotransferred to a Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The apoptosis-related proteins were analyzed using antibodies against Bax, Bcl-2, Bcl-xL, Xiap, p53 serine 46, caspase-3, caspase-8, caspase-9, and PARP (1:1000, Cell Signaling). The survival-related proteins were analyzed using antibodies against p-Akt, Akt total, p-mTOR and mTOR total (1:1000; Cell Signaling). Changes in the expression levels of the corresponding proteins after treatment with the extract were analyzed by Western blotting; β-actin was used as a loading control. The bands on the membrane were detected using an enhanced chemiluminescence detection system, and horizontal scanning densitometry was performed using Photoshop software (Adobe ver. 3.0; Japan) and analyzed using Quantity One (BioRad).
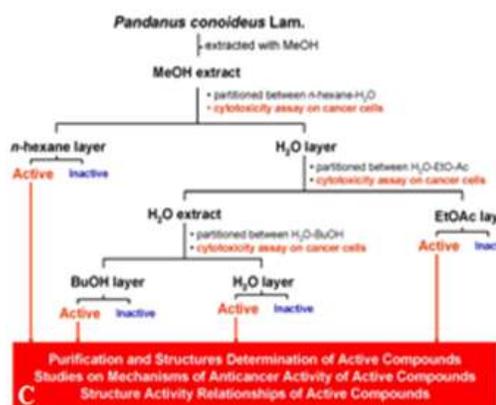

Fig. 1. Red fruit (*Pandanus conoideus* Lam) as a native plant of West Papua and the nearby island. Governor of Papua, Lukas Enembe in white t-shirt, harvesting Red Fruit (A) [27]. Most of the RF extract content oil (B). Schematic flow chart of RF extraction and isolation (C). The RF fraction of EtOAc was evaporated using an evaporator machine (D)

2.6 Statistical Analysis

Statistical analysis was performed using StatView software (ver. 5.0, SAS Inst. Inc., NC). A *P* value < 0.05 was considered to be statistically significant.

3. RESULTS

3.1 Inhibition Concentration of Cervical Cancer Cells by EtOAc RF

The RF's effects on the viability of cancer cells were evaluated at various concentrations. As shown in Fig. 1C, the *Pandanus conoideus* Lam extract prepared from the ethyl acetate fraction of red fruit (EtOAc RF), methanol fraction of red fruit (MeOH RF), *n*-hexane fraction of red fruit and water fraction of red fruit (H$_2$O RF) showed considerable inhibitory activities against the proliferation of cervical cancer cells in a dose-dependent manner. EtOAc RF showed very high activity only at a low concentration in cervical cancer cells.

3.2 Effect of RF Extract on HeLa Cells (Fig. 2)

High concentrations of MeOH RF can induce apoptosis in most HeLa cells, producing 95.3% inhibiting cells, leaving only 4.7% viable cells. The n-hexane RF fraction when given to HeLa cells produces 92.7% inhibiting cells, leaving only 7.3% viable cells. High levels of EtOAc RF produce 86.5% inhibiting cells and 13.5% viable cells. H$_2$O RF leaves a high amount of viable cells (43.6%). When given in low concentrations, at a 512× dilution, the MeOH RF, n-hexane RF and H$_2$O RF treatment groups yielded almost 100% viable HeLa cells; however, EtOAc RF in low concentrations yielded 85% viable cells and 15% inhibiting cells. At IC$_{50}$, 50% of HeLa cells die due to the administration of the RF fraction: (I) H$_2$O RF has a IC$_{50}$ at 1.5× dilution, (II) n-hexane RF has a IC$_{50}$ at 1.75× dilution, (III) MeOH RF has a IC$_{50}$ at 2× dilution, and (IV) EtOAc RF has a IC$_{50}$ at 8× dilution. The EtOAc RF is very effective at a lower dilution rate than other fractions and inhibits HeLa cells at almost any concentration compared with the other fractions.

3.3 Effect of RF Extract on CaSki Cells (Fig. 3)

High concentrations of three fractions of RF (MeOH RF, n-Hexane RF and EtOAc RF) can

inhibit the growth of 95% of CaSki cells, leaving only 5% viable cells. The H$_2$O RF fraction at high concentrations inhibits only 64% of CaSki cell growth, leaving 36% viable cells. At low concentrations, at 512× dilution, the MeOH RF fraction yields 11% viable cells and 89% inhibiting cells. With low concentrations of n-hexane RF and H$_2$O RF, 100% of CaSki cells remain viable. Low concentrations of EtOAc RF can inhibit 30% of CaSki cell growth, leaving 70% viable cells. The IC$_{50}$ of these fractions are as follows: (I) H$_2$O RF has a IC$_{50}$ at 1.5× dilution, (II) n-hexane RF has a IC$_{50}$ at 2× dilution, (III) MeOH RF has a IC$_{50}$ at 3× dilution, and (IV) EtOAc RF has a IC$_{50}$ at 32× dilution. Table 1, shows that EtOAc RF is the fraction with the highest bioactivity against cervical cancer cell lines, achieving a IC$_{50}$ for HeLa cells at 8× dilution and a IC$_{50}$ for CaSki cells at 32× dilution and indicating that, with a higher dilution, the EtOAc RF fraction can still achieve 50% cancer cell death. Due to the above results, in a future experiment, we will use CaSki cells with the 32× dilution of the EtOAc RF fraction because CaSki cells are very sensitive to EtOAc RF.

Table 1. Dilution of red fruit (RF) fractions that inhibited 50% (*IC$_{50}$*) of cervical cancer cell lines, HeLa and CaSki

Red fruit fractions	IC$_{50}$	
	HeLa	**CaSki**
MeOH	2x dil	3x dil
n-hexane	1.75x dil	2x dil
EtOAc	**8x** dil	**32x** dil*
H$_2$O	1.5x dil	1.5x dil

(I) IC$_{50}$: Inhibition Concentration $_{50}$, 50% cell population inhibited after RF treatment.
(II) MTT Assay measured after 24 hours cells treated with RF fractions

3.4 Western Blot Analysis of the *Caspase cascade* and PARP Activation

Caspase signaling pathways consisting of a death receptor-dependent extrinsic pathway and death receptor-independent intrinsic pathway were examined in the cervical cancer cells treated with EtOAc RF. The expression levels of active caspase-8 for the extrinsic pathway, caspase-9 for the intrinsic pathway, and caspase-3 were found to increase in the CaSki cells in a time-dependent manner (Fig. 4). The expression levels of PARP, an important bio-marker of apoptosis, were analyzed in CaSki cells 24 h after treatment with EtOAc RF. The N-terminal fragment of PARP, possessing an 89-

kDa peptide cleaved from full-sized PARP (116 kDa), was detected as early as 2 h in CaSki cells after treatment with EtOAc RF (Fig. 4). These results suggested that EtOAc RF induced apoptotic cell death through both extrinsic and intrinsic pathways.

3.5 Western Blot Analysis of Bcl-2 Family Proteins

The expression levels of Bcl-2 family members, consisting of both pro-apoptotic and anti-apoptotic factors, were then analyzed in the CaSki cells treated with EtOAc RF. Slightly increased expression of Bax (pro-apoptotic) was detected in the CaSki cells in a time-dependent manner, and slightly decreased expression of Bcl-2, Bcl-xL and Xiap (anti-apoptotic) was detected in the same cells (Fig. 5). We also evaluated the expression level of p53 serine 46, an initiator that activates Bax and/or downregulates Bcl-2, Bcl-xL and Xiap. Our results indicated that CaSki cells treated with EtOAc RF showed increased expression of p53 serine 46 in a time-dependent manner (Fig. 5).
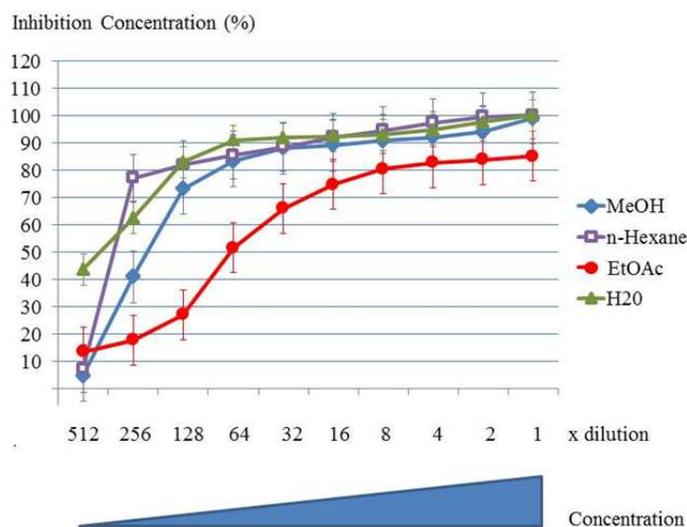

Fig. 2. Effect of RF extract (MeOH, EtOAc, *n*-hexane and water) on the viability of HeLa cells treated with various concentrations of RF extract for 24 h. IC: Inhibition concentration
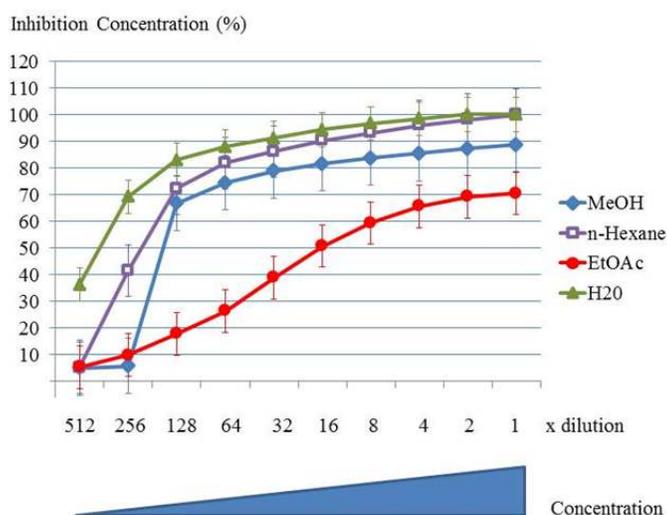

Fig. 3. Effect of RF extract (MeOH, EtOAc, *n*-hexane and water) on the viability of CaSki cells treated with various concentrations of RF extract for 24 h. IC: Inhibition concentration
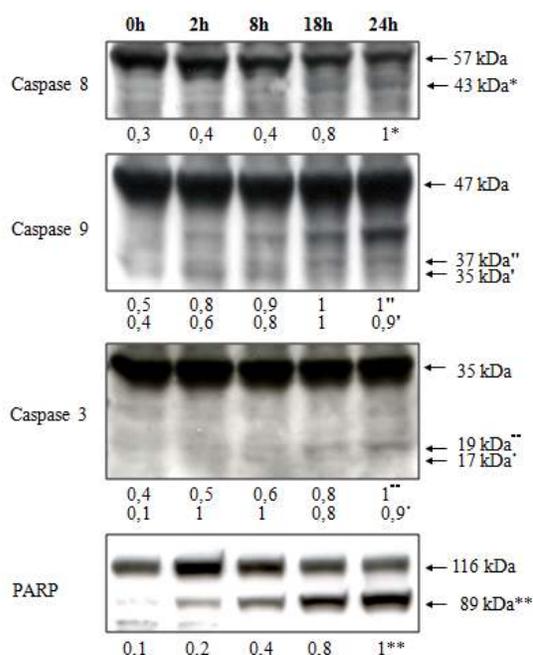
Fig. 4. Western blot analysis of CaSki cells treated with EtOAc RF. The cells were treated with EtOAc RF (3,3 mg/mL) for 24 h, and the values given below the Caspase-8, -9, -3, and PARP figures indicate the calculation of the active form band (41-43kDa, 35-37kDa, 17-19kDa and 89kDa, respectively superscripted) after normalization of its expression to that of β-actin, shown as a percentage compared with the control

3.6 Western Blot Analysis of Survival Pathways

The expression of survival signaling proteins was evaluated in CaSki cells in response to EtOAc RF. The treatment of CaSki cells with EtOAc RF indicated the inhibition of Akt activation and expression of both phosphorylated Akt serine 473 and phosphorylated mTOR serine 2448, the downstream targets of Akt in CaSki cells (Fig. 6).

4. DISCUSSION

Cervical cancer treatment depends on the *Fédération Internationale de Gynécologie et d'Obstétrique* (FIGO) staging. The early stage of cervical cancer is generally treated with radical operations; however, to date, there is no fixed standard therapy for FIGO stage Ib-IIa of cervical cancer. Cancer within these stages can be treated with radical operation, radiotherapy, surgery-radiotherapy combination, surgery-

anticancer drug combination, or chemotherapy, although the protocols for these combination treatments may vary. However, the systemic therapy protocol for this stage of cervical cancer keeps changing. In the past, chemotherapy was used for recurrent and persistent cervical cancer or for cervical cancer with metastasis. Currently, chemotherapy is the primary treatment for cervical cancers with a high risk of recurrence. Meta-analyses have suggested that conventional treatment methods have reached a plateau; therefore, to overcome this problem, finding a novel chemotherapy agent and its adjuvant might be useful.
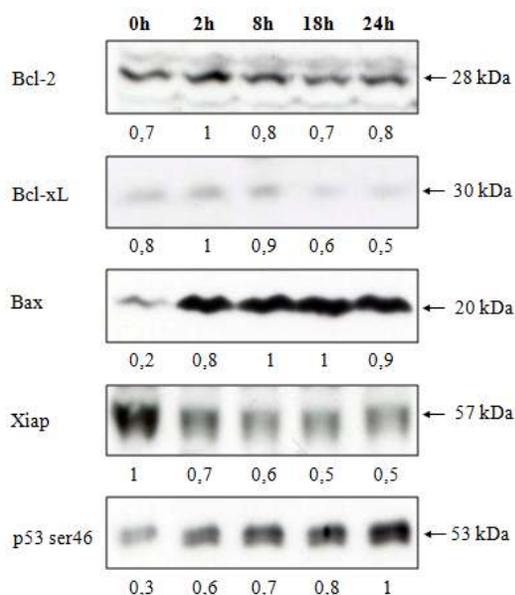

Fig. 5. Western blot analysis of CaSki cells treated with EtOAc RF. Changes in the expression levels of the Bcl-2 family in the CaSki cells are shown. The cells were treated with EtOAc RF (3.3 mg/mL) for 24 h. The values given below the figures indicate the calculation of each band after normalization of the expression to that of β-actin, shown as a percentage compared with the control

In Indonesia, the use of medicinal plants and herbal therapy was practiced long before recorded history. Species of *Pandanus conoideus* Lam, known as red fruit (RF), have a long history of use in traditional medicine. Mahkota Dewa (MaDe) has been used for the treatment of various diseases, including empirical therapy for malignant diseases, such as cancer. There have been several studies supporting the anticancer potential of RF; [7-9] however, scientific evidence concerning the mechanism of

its active compound(s) is very limited. Thus, it is important to screen apoptotic inducers from plants, either in the form of crude extracts or as active isolated components. The people of Papua island consume red fruit as a staple food, along with several types of vegetables (e.g., cassava leaves and papaya leaves). Research has shown that the prominent physical characteristics of people who consume red fruit regularly are resistance to degenerative diseases, such as hypertension, diabetes, coronary diseases and cancer [13]. Red fruit contains many biologically active compounds that have the potential to be used as antioxidants. It is used empirically to treat many diseases, such as AIDS, breast cancer, cervical cancer, hepatic cirrhosis, hepatitis B, stroke and tuberculosis [14]. Research into the tumorigenesis-inhibiting properties of red fruit extract had been performed in vivo in mice with dimethyl-benz[a]anthracene (DMBA)-induced cancers, showing that red fruit has an effect against pulmonary cancer [15]. Other research used *beta cryptoxanthin,* which was isolated from red fruit oil extract and was shown to inhibit the growth of pulmonary cancer cells *in vitro* [9].
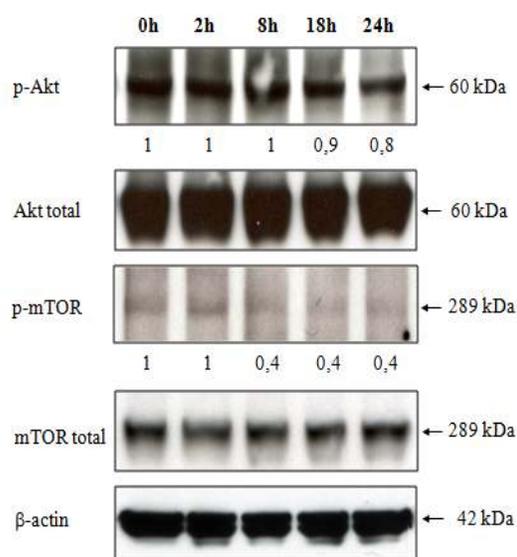

Fig. 6. Western blot analysis of CaSki cells treated with EtOAc RF. Changes in the expression levels of p-Akt and p-mTOR in CaSki cells are shown. The cells were treated with EtOAc RF (3.3 mg/mL) for 24 h. The values given below the figures indicate the calculation of each band after normalization of the expression to that of β-actin, shown as a percentage compared with the p-Akt total and p-mTOR total

In cervical cancer cells treated with EtOAc RF, the activation of the initiator caspases (extrinsic caspase-8 and intrinsic caspase-9) followed by the activation of the executioner caspase (caspase-3) occurred in the CaSki cells after treatment with EtOAc RF. Accordingly, the activation of the cascade involving such caspases induced PARP cleavage, resulting in nuclear fragmentation. Furthermore, the induction of the apoptosis signaling pathway in CaSki cells treated with EtOAc RF appeared to suppress the expression of Bcl-xL and enhance the expression of Bax in anti-apoptotic and pro-apoptotic manners, respectively. Therefore, the induction of apoptosis seemed to be caused by the disruption of the balance between these anti- and pro-apoptosis molecules, as described previously [10-12,16].

One of the most important survival-signaling pathways is mediated by PI3K and its downstream targets, such as Akt and mTOR [17]. Recently, Akt was reported to play an important role in determining the chemosensitivity of many types of cells [18-20]. Recently, p53 has also been revealed to activate autophagy [21]. Several groups have reported the localization of p53 to the outer layer of the mitochondrial membrane and activation of apoptosis through direct binding to the Bcl-2 family members Bax, Bak or Bcl-xL [22,23]. The over-expression of p53 was also reported to increase Bax expression in several cell types followed by the induction of apoptosis [24,25]. The binding of p53 to p53AIP1, which appears to be important for the apoptotic response, is selectively enhanced by the phosphorylation of serine 46 [26]. We also observed that, in fact, p53 at serine 46 was increased in CaSki cells after treatment with EtOAc RF. Our results demonstrated that EtOAc RF inhibits the activation of the Akt/mTOR pathway, as shown by the down regulation of phosphorylated Akt at serine 473 and phosphorylated mTOR at serine 2448. Overall, our results showed that cell death was induced, and the survival signaling pathway was suppressed, by EtOAc RF.

5. CONCLUSIONS

In summary, the activation of apoptosis in human cervical cancer cells induced by EtOAc RF may be involved not only in inducing apoptosis, as we previously demonstrated in cervical cancer cell lines demonstrated herein for the first time, but also in suppressed survival signaling. A growing body of evidence has emerged, based on our

study, suggesting that red fruit extract might be useful in the treatment and prevention of cancer. Understanding the exact mechanism of their actions may provide valuable information for their potential application in cancer therapy and prevention.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENTS

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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