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Natural Phytochemicals Induces Apoptosis in Cervical Cancer Cell Line: *A Comparative Preliminary Study*

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Abstract:

Cervical malignancy is the most common female cancer around the world, and it remains a challenge to manage pre-invasive and invasive injuries. There is an incredible requirement for agents capable of inhibiting cervical cancer. Here, we report that various phytochemicals and natural compounds like Lycopene, Rutin, Quercetin, Tangeritin, Proanthocyanidins and Beta-carotene which are a dietary phyto-compound possess potentially important anticancer effects. Caski cell line was separately co-cultured for 24 hr (0-50 ug/ml) with Lycopene, Rutin, Quercetin, Tangeritin, Proanthocyanidins and Beta-carotene. Cell proliferation was measured by MTT assays. The transcendent method of cell death in Caski cell lines was apoptosis, as showed by caspase-9, - 3 activation by caspase activity assay. The results of this study revealed that Lycopene, Rutin, Quercetin, Tangeritin, Proanthocyanidins and Beta-carotene were potential inhibitors of cervical cancer cell growth.

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Key words: Cervical Cancer, Reactive Oxygen species, Z-LEHD-FMK, Z-DEVD-FMK

INTRODUCTION:

Cervical cancer is the fourth most basic malignancy in women around the world, and second for women in ages between of 15 to 44, as is evident from the fact that more than 528,000 new cases and more than 266,000 deaths occurred in 2012 only [1-3]. This number is predicted to only rise by 2030 [4]. Reports indicate that surgery is the standard treatment for cervical cancer alongside taxol chemotherapy, which unfortunately, regularly brings about serious myelotoxicity and neurotoxicity, and that, chemotherapy in cervical cancer is hampered by drug resistance [5, 6]. Thus, there is an urgent need for an effective treatment of cervical cancer to be developed, thereby leading to shifting of research focus on probing various safe and economical natural phytochemicals/compounds.

Modern phytotherapy is occupied with the production of cures from materials got from plants and their utilization in useful and safe therapy [7]. Polyphenol. Polyphenols are basic supplement antioxidants, mostly got from natural products, vegetables, coffee, tea, cocoa, beverages and mushrooms conventional medicative herbs [8]. Polyphenols are potential substances against malignancies and cardiovascular, metabolic [9], and neurodegenerative diseases [10] through their capacities of antioxidation and antimutation. Therefore, we have attempted to carry out comparative preliminary studies with phytochemicals like lycopene, rutin, quercetin, tangeritin, proanthocyanidins on cervical cancer cell lines.

Quercetin, is a naturally occurring phenolic compound that are generally found in dietary plants and natural foods (for example, vegetables and fruits) and have been accounted for to have numerous useful effects including anti-oxidative, anti-

inflammatory, anti-hypertensive, anticancer, neuroprotective, antiviral, hepatoprotective, anti-diabetic activities, and have been proposed to boost the life expectancy of model life forms and reduce cell senescence of normal cells [11, 12].

Next, rutin (5,7,3',4'- OH,3-rutinose), the glycosidic type of quercetin, is a flavonol, having vascular-defensive, antitumor, antioxidant, antiviral, antiinflammatory, myocardial defensive, and anti-diabetic properties [13]. Rutin posses significant activity against breast cancer cells [14], and that, its DNA defensive effects against procarcinogens in HTC hepatic cells are well documented [15].

Another phytochemical employed was proanthocyanidins, which are a heterogeneous group of flavan-3-ol or flavan-3,4-diol oligomers found in different fruits and vegetables. Oligomeric subset of proanthocyanidins, have been reported to have powerful anti-tumorigenic properties [16-19]. Reports indicate its beneficial effects in wound healing, and that, it possesses antiinflamatory and antibacterial activities [17-19]. Proanthocyanidins are getting an increasing consideration in prostrate tumor prevention and hormonal malignancies prevention [20-22]. Reports indicate proanthocyanidins to have pro-apoptotic effects by controlling the expression of MAP kinase, PI-3 kinase, and AP-1 proteins as well as anti-metastatic effects [23, 24].

Next, beta-carotene, found in organic products, fruits, vegetables and entire grains, has been reported to be a powerful natural cancer prevention agent as well as skin photoprotector [25, 26].

Another phytochemical of interest is lycopene which is a carotenoid found in tomatoes, apricots, guavas, pink grapefruits, rosehips, or watermelons. Major dietary sources of lycopene are tomato items. It possesses antioxidant activities, anti-inflammatory properties, inhibits cell proliferation, and induces apoptosis, therefore protecting against cancer and

giving medical advantages to cancer patients by being considered as an anticarcinogenic and chemopreventive agent. Intake of lycopene was related with a decreased risk of prostate cancer [27]. Reports indicate that the major role of lycopene is in the modulation of redox activity, enzyme detoxification, inhibition of cell proliferation and apoptosis induction [28]. Lycopene are known to increase some defensive enzymes, by bringing down the generation of reactive oxygen species and in turn, reduce oxidative stress [29].

Next, tangeretin (4',5,6,7,8 pentamethoxyflavone) is a natural characteristic polymethoxyflavone present in the peel of citrus natural products. It is known to possess anticancer properties against a variety of carcinomas (30-34).

To the best of our understanding, the anti-cancerous effects of the above mentioned phytochemicals have been reported in a variety of cancers but not in cervical cancer cell lines. Therefore, in the present study, we have attempted to carry out comparative preliminary studies with phytochemicals like lycopene, rutin, quercetin, tangeritin, proanthocyanidins on cervical cancer cell lines. It is hoped that the preliminary data may throw some light for further in-depth studies on such phytochemicals, which in turn, may provide a valuable tool in using them as adjuncts in the management of cervical cancer.

2. MATERIALS AND METHODS:

2.1. Chemicals and reagents.

Commercially available Lycopene, Rutin, Quercetin, Tangeritin, Proanthocyanidins and Beta-carotene were employed in the study. Dimethyl sulfoxide (DMSO), MTT, trypan blue and propidium iodide (PI) were of Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) (v/v), 100 U/ml penicillin and 100 U/ml streptomycin were from Gibco Life Technologies

(Carlsbad, CA, USA). Caspase colorimetric kit was of R&D Systems Inc., Minneapolis, MN, USA. Caspase-3 inhibitor (z-DEVD-FMK) and Caspase-9 inhibitor (z-LEHD-FMK) was of BD PharMingen (San Diego, CA, USA). HeLa cell line was obtained from National Centre for Cell Science (NCCS) Pune, India.

2.2. Cell culture.

Human cervical carcinoma cell lines (HeLa) were cultured in DMEM medium (supplemented with 10% FBS, 2 mM glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin) in 96-well plates at 37°C with 5% CO_2 and air humidity of 95%. Exponentially, growing cells were used for experiments.

2.3. MTT assay.

The cytotoxic effect of Lycopene, Rutin, Quercetin, Tangeritin, Proanthocyanidins and Beta-carotene on cervical cancer Caski cells was determined by MTT assay as described by us earlier [35, 36]. Exponentially growing Caski cell lines ($5x10^4$ /well) were seeded onto 96-well culture plate and allowed to attach overnight, followed by treatement with various doses of the above said respective phytochemicals (10, 20, 30, 40 and 50 µg/ml) for 24 h. 10µl MTT (5 mg/ml) was added to each well and incubated for another 4 h at 37°C. Dimethyl sulfoxide (DMSO) was added to each well and thoroughly mixed to dissolve the purple crystals. The absorbance of each well was measured on a microplate reader, using a wavelength of 570 nm and a reference wavelength of 650 nm and the cell survival was expressed as the percentage (%) over the untreated control.

2.4. Caspase Activity Assay.

$5x10^6$ cells (Caski) in 75-T flasks were treated with or without Lycopene, Rutin, Quercetin, Tangeritin, Proanthocyanidins and Beta-carotene (0-50 ug/ml for each), then incubated for 12 h to

detect the activity of caspase-9 and -3 and assessed according to manufacturer's instruction of the Caspase colorimetric kit (R&D Systems Inc., Minneapolis, MN, USA). Cells were collected and lysed in 50 µl lysis buffer containing 2 mM DTT, for 10 min. After centrifugation, the supernatant containing 200 µg protein were hatched with caspase-9 and - 3 substrates in reaction buffer. Then all samples were incubated in a 96 well flat bottom microplate at 37˚C for 1 h. Levels of discharged pNA were measured with ELISA reader (Anthos Labtec Instruments GmbH, Salzburg, Austria) at 405 nm wavelength [37].

2.5. Statistical Analysis.

The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett test, considering $p<0.05$ as significant.

3. RESULTS:

3.1. Cytotoxity versus Phytochemicals in Caski Cervical Cancer cell Lines:

Human Papilloma Virus positive Caski cervical cancer cell lines were cultured with and without varying doses of a variety of phytochemicals, and that, their cell proliferation or viability was assessed by MTT assay. The inhibitory concentration (IC_{50}), i.e. the concentration resulting in inhibition of 50% of cell viability in comparison to control, was measured with these assays. The phytochemicals employed were: Lycopene, Rutin, Quercetin, Tangeritin, Proanthocyanidins and Beta-carotene.

A dose response effect of Lycopene (0-50 ug/ml) in MTT assay failed to show any adverse effect till 30 ug/ml, whereas doses above 30 ug/ml proved to be cytotoxic ($n=3$; $p<0.001$; Fig. 1A). Next, a dose response effect with Rutin (0-50 ug/ml) in MTT assay failed to show any adverse effect till 15 ug/ml, whereas doses above 15 ug/ml proved to be cytotoxic ($n=3$;

p<0.001; Fig. 1B). Further, a dose response effect with Quercetin (0-50 ug/ml) in MTT assay failed to show any adverse effect till 15 ug/ml, whereas doses above 15 ug/ml proved to be cytotoxic (n=3; p<0.001; Fig. 1C). Thereafter, dose response effect with Tangeritin (0-50 ug/ml) in MTT assay failed to show any adverse effect till 40 ug/ml, whereas doses above 40 ug/ml proved to be cytotoxic (n=3; p<0.001; Fig. 1D). Next, a dose response effect with Proanthocyanidins (0-50 ug/ml) in MTT assay failed to show any adverse effect till 20 ug/ml, whereas doses above 20 ug/ml proved to be cytotoxic (n=3; p<0.001; Fig. 1D). Finally, a dose response effect with Beta-carotene (0-50 ug/ml) in MTT assay failed to show any adverse effect till 25 ug/ml, whereas doses above 25 ug/ml proved to be cytotoxic (n=3; p<0.001; Fig. 1F). Thus, the toxicity effect of phytochemicals in Caski cell lines employed in the present study were of the order of Quercetin = Rutin > Proanthocyanidins > Beta-carotene > Lycopene > Tangeritin.

3.2. Phytochemicals exhilarate the activities of caspase-9 and -3 in Caski cervical cancer cell lines during apoptosis.

The cell growth inhibition by the above said phytochemicals strongly induced caspase-9 and -3 activities in Caski cells. This was evident from data of co-culturing for 24 hr separately of Caski cells with 50 ug/ml of respective phytochemicals i.e. Quercetin, Rutin, Proanthocyanidins, Beta-carotene, Lycopene and Tangeritin (Fig. 2). Activation of caspase-9 and -3 in Caski cells treated with the above phytochemicals clearly indicated the involvement of intrinsic or mitochondrial pathway during AITC induced programmed cell death. As evident from the data depicted in Fig. 2, the magnitude of activation of caspase 9 and caspase 3 was of the Quercetin = Rutin > Proanthocyanidins > Beta-carotene > Lycopene > Tangeritin. This is in accordance to our toxicity data depicted.

Furthermore, in order to ascertain that whether the activation of caspase-9 and -3 is required for the induction of cell death by the above said phytochemicals, Caski cells were pretreated with cell permeable caspase inhibitors, Z-LEHD-FMK (caspase-9 inhibitor), Z-DEVD-FMK (caspase-3 inhibitor), followed by treatment for 24 hr with 50ug/ml of the above said respective phytochemicals. Subsequently caspase activities and cell viabilities were measured. Our results showed that pre-treatment with Z-LEHD-FMK and Z-DEVD-FMK abolished caspase-9 and caspase-3 activities respectively in Caski cells induced by the above mentioned phytochemicals, and that, respective caspase activities were found to be similar to the control value (no significant difference) (Fig. 2).

4. DISCUSSION

A wide spectrum of studies has suggested that a high utilization of dietary natural compounds present in vegetables and fruits is related with a reduced risk of numerous cancers [25-29] as they are thought to be rich sources of chemopreventive efficacy compounds having low lethality [30]. Different studies have shown phytochemicals to exhibit antiproliferative and apoptotic properties against various human cancer cells *in vitro* [30].

To the best of our understanding, we report for the first time a comparative apoptotic effect of phytochemicals like lycopene, rutin, quercetin, tangeritin, proanthocyanidins and beta-carotene on cervical cancer Caski cell lines. The most striking finding of the present study was the disturbance in cell viability and induction of apoptosis in cervical cancer Caski cell lines at different doses of phytochemicals employed in the study as revealed by dose response MTT and Caspase assays. It is noteworthy that most of the naturally occurring agents induce cell apoptosis through caspase-dependent and -independent

pathways [37, 38], and that, effect of biologically active phytochemicals in human health is well documented [39]. Our results revealed that the phytochemicals employed in the present study promoted caspase-3 and -9 activities leading to apoptosis.

Therefore, in summary, the data in the present study has generated interest in exploring the complete molecular mechanism of induction of apoptosis by such phytochemicals for further in-depth studies. This would help to ascertain that whether such compounds could be employed as safe and economical valuable tools for their usage as adjuncts in the management of cervical cancer or not.

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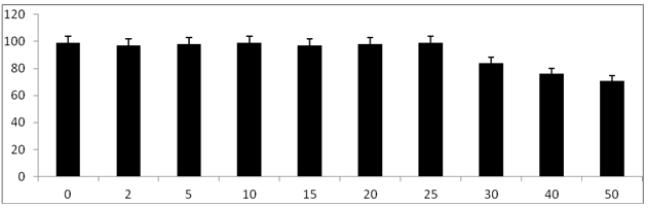
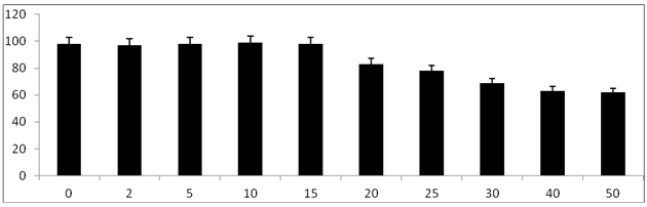
Conflict of Interest:
There is no conflict of interest.

Figures:

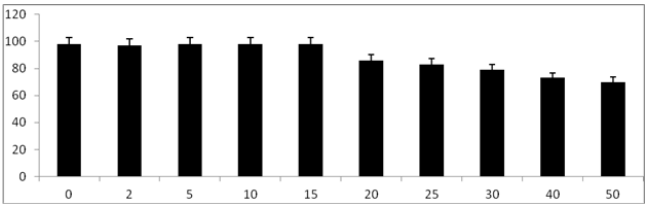


Concentration of Lycopene (ug/ml)
Figure 1A: MTT Cell viability assay of caski cells with Lycopene



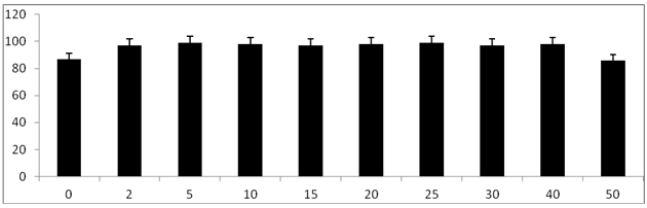
Concentration of Rutin, (ug/ml)

Figure 1B: MTT Cell viability assay of caski cells with Rutin



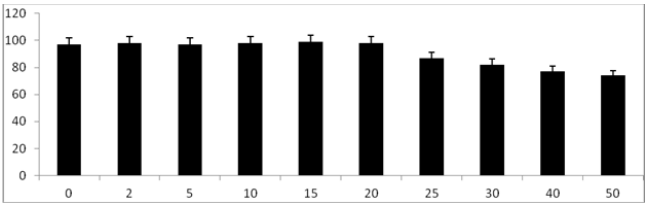
Concentration of Quercetin (ug/ml)

Figure 1C: MTT Cell viability assay of caski cells with Quercitin



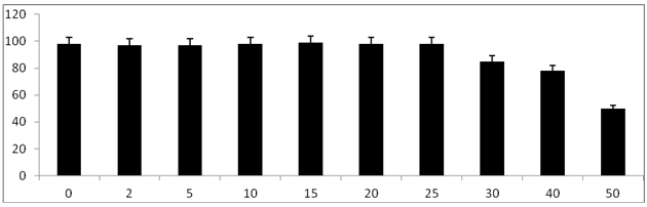
Concentration of Tangeritin (ug/ml)

Figure 1D: MTT Cell viability assay of caski cells with Tangeritin



Concentration of Proanthocyanidins (ug/ml)

Figure 1E: MTT Cell viability assay of caski cells with Proanthocyanidins



Concentration of Beta-carotene (ug/ml)

Figure 1F: MTT Cell viability assay of caski cells with beta carotene.

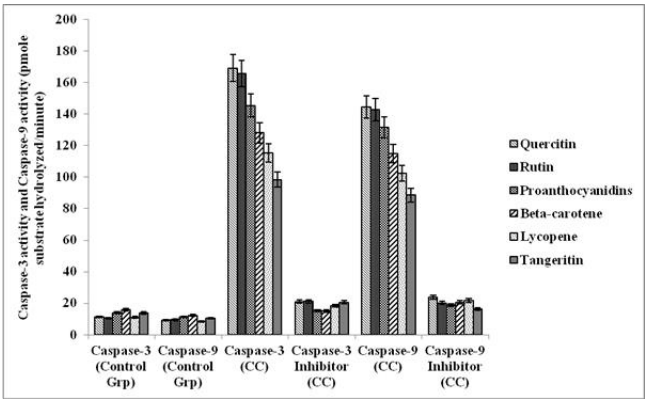


Figure 2: Activation and Suppression of caspase-3 and -9 in Different Phytocompounds treated HeLa cells; HeLa cells were treated with different Phytocompounds for 24 h for Caspase activity and then pretreated with caspase-3 and -9 inhibitor (Z-DEVD-FMK and Z-LEHD-FMK respectively) for Caspase inhibition. Cells were harvested and lysed in lysis buffer. Enzymatic activity of caspase-3 and -9 was determined by incubation of 50 µg of total protein with substrate DEVD-pNA and LEHD-pNA for 2 h at 37 °C. The release of p-NA was monitored spectrophotometrically at 405 nm. Data represent mean ± S.E.M. of three independent experiments. $p<0.001$ was considered significant.

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