Differential Anticancer Effect of an Apple Extract (Applephenon®), Polyphenols and Isoflavones on Normal Human Keratinocytes and Epidermoid Cancer Cells

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Abstract

Applephenon®, a purified extract prepared from green apples, was examined for its cytotoxicity and inhibitory effects on the proliferation of cultures of normal human keratinocytes and several epidermoid cancer cell lines. Our HPLC studies demonstrated a high content of phenolic compounds (>65%), including catechin, epicatechin, caffeic acid and phloretin as well as polyphenols such as proanthocyanidins. Applephenon® demonstrated a greater cytotoxic effect against HeLa, A431 cancer cell lines and HaCaT, an immortalized keratinocyte cell line than serum-free cultures of proliferating normal human keratinocytes (NHK). Proliferation of NHK was inhibited at concentrations above 0.0013% while concentrations above 0.005% were cytotoxic. By contrast, Applephenon® solutions above 0.00025% killed each of the cancer cell lines. Treated cells displayed increased intercellular separation and evidence of keratinizing stratification. We also tested the effect of epicatechin, and two isoflavonoids, genistein and daidzein, on cancer cell lines. Hela cells were more sensitive to epicatechin and genistein inhibition of cell growth and cytotoxicity than were NHK. Daidzein at these concentrations had little effect on cancer cells. These results indicate that Applephenon® and some of its phenolic components have selective anticancer activity.

Keywords

Applephenon™ (AP-SH Grade), Cytotoxicity, Epicatechin, Epidermal Keratinocytes Epidermoid Cancer Cell Lines (A431, HaCaT, HeLa), Genistein, Polyphenols

1. Introduction

Applephenon™ (AP) is a powdered extract produced from unripe apples [1]. A process to produce a procyanidin and polyphenolic-enriched powders has been patented [2]. For commercial food use, it meets Generally Recognized as Safe (GRAS) status for oral consumption [3]. The many claims made by the manufacturer's source data are antioxidant, hypotensive agent, anti-mutagenic agent, certain physiological activities such as anti-allergenic, hyaluronidase-inhibiting activity, ACE-inhibiting activity and GTPase-inhibiting activity [4]. The toxicology, mutagenicity, and safety of AP polyphenols as food additive were also studied [5]. Nikki Whiskey [1] has also performed various tests to validate the many uses of AP as supplements, cosmetics, confectionaries, beverages, processed foods and deodorants. In cosmetic supplications, it is claimed to restrain allergic diseases, control melanin production, and absorb ultraviolet rays in the UVA and UVB ranges. In Japan, Applephenon is used as a food additive and nutritional supplement [1] [2] [3] [4]. Earlier studies reported that Applephenon is safe in oral administration toxicity test in rats and acceptable based on the Ames mutagenicity tests [5]. The antiproliferative effect of apple polyphenols and procyanidins derived from apples on human metastatic colon carcinoma was investigated [6]. They reported that only the procyanidin fraction inhibited cell growth, intracellular signaling pathways, and trigger apoptosis in tumor cells. The procyanidin fraction also reduces the number of preneoplastic lesions in Wistar rats fed procyanidin dissolved in drinking water compared to controls receiving only water. In another study [7], apple polyphenols and procyanidins inhibited the growth of transplanted B16 mouse melanoma cells and mouse mammary tumor cells. The procyanidins rather than the apple polyphenols specifically had a major effect on cell proliferation. Procyanidins affected their antiproliferative action by way of activation of caspases 3 and 9 within tumor cells. Our HPLC analysis found only 5% - 6% procyanidins indicating that the major effect on inhibition of cell growth and eventual cell death is the result of the phenolic content of commercially available Applephenon®.

Relevant to this report, the chemopreventive properties of AP procyanidins were reported on human colon cancer SW620 cells and in a rat model [6] with significant tumor reduction. Apple polyphenols and procyanidins inhibited the growth of transplanted B16 mouse melanoma cells and BALB-MC.E2 mouse mammary tumor cells. In addition, the procyanidin fraction was more effective than the apple polyphenols including chlorogenic acid, (−)-epicatechin, phloridzin, in inhibiting cell proliferation and apoptosis [7].

Our studies were undertaken to examine the effects of AP-SH, a procyanidin-enriched version of the (standard) AP powders, on the growth and cytotoxicity of normal human keratinocytes (NHK) compared with several different human epidermoid cancer cells (HECC). These studies were aided by use of serum-free culture medium for NHK cultivation and minimal serum concentrations provided for the growth of HECC. Although no isoflavones were found as

components of Applephenon, their role as dietary phytoestrogens in cancer prevention and structural similarities to polyphenols suggest that they merited attention in these studies [8]. We initially conducted HPLC studies to affirm the polyphenolic and procyanidin contents of AP-SH, as a prelude to examining the effect of specific polyphenols including, (−)-epicatechin, and two isoflavones genistein, and daidzein on cell proliferation and cytotoxicity of both NHK and several HECC cells. The results of these studies suggest that AP-SH has a selective and profound growth inhibiting and lethal effect on HECC relative to its effect on NHK.

2. Materials and Methods

Materials: Applephenon™ SH grade Lot/Batch # 050922 was purchased from TOMEN Corp a subsidiary of TOYOTO Tsusho America, Inc. NY, NY. Polyphenolic compounds were obtained as a gift from Dr. Mark Berhow of ARS USDA, Peoria, IL. HeLa, A431, and HaCaT cancer cell lines were a gift from the laboratory of Dr. Robert E Scott on Mayo Medical School, Rochester, MN. Plastic dishwares were purchased from Fisher Lab Products, (St. Louis, MO).

Cell culture: Normal human foreskin keratinocytes (NHK) were obtained from newborn foreskin and were grown in serum-free medium (MCDB 153) basal nutrient medium supplemented with insulin (5 μg/ml) and EGF (5 ng/ml) in a water-jacketed humidified incubator at 37°C as previously described [9]. For the purpose of polyphenolic treatment, HeLa cell cultures were propagated in DMEM medium supplemented with 0.1% fetal bovine serum (FBS). A431 and HaCaT cell cultures were propagated in MCDB 153 medium containing insulin (5 μg/ml) and EGF (5 ng/ml). Stock solution of AP-SH was made with sterile distilled water.

Analytical methodology: HPLC analysis was conducted on a stand-alone Shimadzu 10A HPLC system (SCL-10A system controller, two LC-10A pumps, CTO-10A column oven, and SIL-10A autoinjector). Peaks were monitored using a Hewlet-Packard 1040A photodiode array detector running under the HP Chemstation software version A.02.05. The column used was an Intersil ODS-3 reverse phase C-18 column (5 μM, 250 × 4.6 mm from Varian). For analysis, the initial conditions were 20% methanol and 80% aqueous 0.01 M phosphoric acid, at a flow rate of 1 ml per minute. The effluent was monitored at 280 nm on the PDA. After injection (typically 25 μl), the column was held at the initial condition for 2 minutes, then developed to 100% methanol in a linear gradient over 55 minutes [10].

Experimental treatments: Polyphenolic compounds were prepared as 1000 × stock solution in DMSO:Ethanol (1:10) dilutions were made by micropipetting directly into the cell culture medium. Cell cultures were incubated in a CO_2-humidified incubator for 24 hours unless otherwise indicated. Applephenon-SH powder was dissolved in sterile distilled water and resterilized by passage through a 0.45-micron filter. Stock solutions were diluted by micropipetting

directly into the cell culture medium to obtain the desired final concentration. Test treatment was made triplicate dishes and the results tabulated as means(X) ± their respective standard errors (S.E). Antioxidant properties of AP-SH polyphenolics were verified by the diphenylpicrylhydrazine reagent (DPPH*) assay [11].

3. Results

3.1. HPLC Analyses of Applephenon™: Batch Comparisons

Figure 1 is an HPLC analysis of AP (standard grade) compared to special AP-SH grade as published by Ashai Breweries, LTD [1]. They claim that AP-SH is enriched in total procyanidin components with a much-reduced percentage of chlorogenic acid and phlorizin relative to the standard grade. The predominant phenols were (−)-epicatechin (13% - 15%), phloretin-2'-glucoside (4% - 6%), (+)-catechin (6% - 8%) and caffeic acid (3% - 4%). Total procyanidins were shown at 40% - 45% with procyanidin B1 (2% - 4%) and procyanidin B2 (4% - 8%). Figure 2 presents our HPLC analysis on a commercial batch of AP-SH. Table 1 provides composition of phenolics. In general, we confirm the previous Ashai report based on an mg/g basis using the extinction coefficient of epicatechin [1]. We report the percentage of total eluted phenolics was 65%. The predominant polyphenolics were catechin (11.2%), chlorogenic acid (24.9%), epicatechin (11.3%), ferrulic acid (1.2%), *p*-coumaric acid (1.2%), and phloretin-2-glycoside (8.7%). The HPLC chromatogram shows about 5-6% of proanthocyanidins
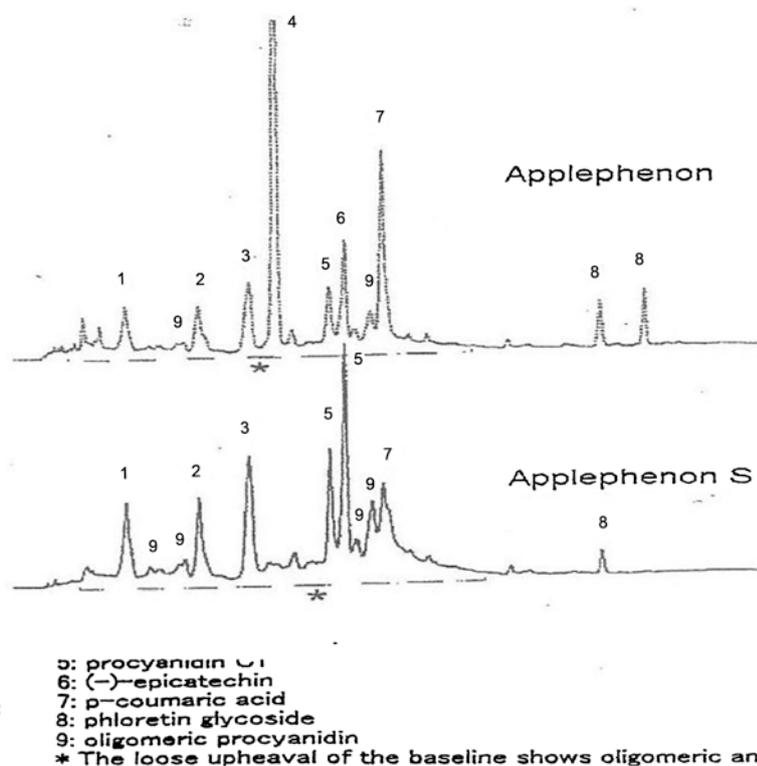

1: procyanidin B1
2: (+)-catechin
3: procyanidin B2
4: chlorogenic acid
5: procyanidin C1
6: (−)-epicatechin
7: p-coumaric acid
8: phloretin glycoside
9: oligomeric procyanidin
* The loose upheaval of the baseline shows oligomeric and polymeric procyanidins

Figure 1. HPLC chromatographic profile of Applephenon™ standard grade and Special Grade (SH). The profiles are reproduced from a promotional brochure for Applephenon published by Ashai Breweries, Nikki Whiskey, Tokyo, JP.
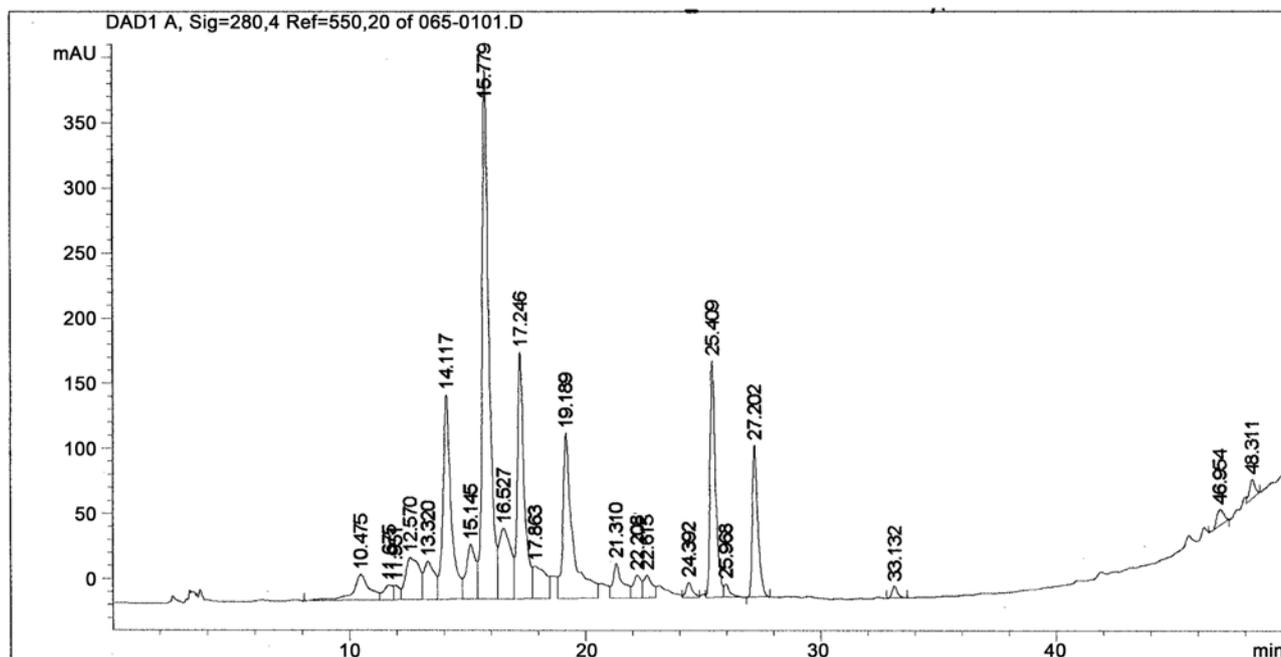
Figure 2. HPLC chromatographic profile of Applephenon™ special grade (SH) from a commercial lot (Batch #: 050922). The elution time or each peak is given as RT (run time). (see **Table 1** for the chemical identity of the main phenolic compounds).

Table 1. Composition of Applephenon-SH by HPLC Analysis (Wille & Berhow).

Peak Components	RT(min)	Mg/g	Phenolic (%)
1	10.475	19	2.9
2	11.675	6	0.9
4	12.57	26	4.0
5	13.32	17	2.6
6 Catechin	**14.117**	**72**	**11.2**
7	15.145	22	3.4
8 *Chlorogenic acid*	**15.779**	**161**	**24.9**
9	16.527	36	5.6
10 Epicatechin	**17.246**	**73**	**11.3**
11	17.863	19	2.9
12	19.189	72	11.2
13	21.310	15	2.3
14 Ferrulic acid	**22.208**	**8**	**1.2**
15 p-Coumuric	**22.615**	**8**	**1.2**
17Phoretin glycoside	**25.409**	**56**	**8.7**
19 Proanthocyanidin (?)	**27.202**	**36**	**5.6**
Total		**646**	**65**

as a trailing minor peak. However, we failed to confirm the reduction of chlorogenic acid in AP-SH relative to standard grade AP.

3.2. Effect of Epicatechin on Normal Human Keratinocytes (NHK) Relative to HeLa Cells

Figure 3 presents a typical photograph of control high cell density NHK cultures (a) and NHK treated with epicatechin (81 µg/ml) for 24 hours (b). Both flasks were fixed and stained with crystal violet (0.2%) and photographed using a Nikon phase inverted microscope equipped with a Cannon camera system. Epicatechin treatment resulted in a modest decrease in growth apparent as reduced cell density relative to untreated control. To explore the lowest dose of epicatechin inhibiting cell growth, we treated NHK cultures with increasing doses of epicatechin ranging from 20 to 80 µg/ml. Figures 4(a)-(c) shows that neither 20 µg/ml nor 40 µg/ml was effective in reducing cell growth in a 24 hour treatment period. Figures 4(d)-(f) presents a typical photograph showing the effect of culturing NHK with 40 µg/ml (e) compared with 81 ug/ml (f) of epicatechin relative to untreated control (d). NHK cell growth was only reduced at 81 µg/ml evident as reduced cell density. Figure 5 is phase contrast photomicrograph showing the effect of (b) epicatechin (81 µg/ml) on cell growth and cell morphology. Note: Clumping of epicatechin-treated cells, absence of intercellular spacing, and loss of dividing cells.

Figure 6 showed the effect of 23 hours treatment with 40 µg/ml epicatechin (b) on HeLa cells compared with untreated control (a). There was an overall reduction in crystal violet blue-stained cells relative to untreated control. Figure 6(c) & Figure 6(d) is a photograph of 6-well dish showing the reduction in cell density in HeLa cells treated for 24 hours with 40 ug/ml (d) of epicatechin relative to untreated control (c). The figure also includes a notation for the concentration of DMSO given the finding that 12.5 µl of DMSO is not toxic to HeLa cells in media containing low FBS (0.1%). Higher concentrations of DMSO in DMED containing 0.1% FBS obscure the effect of epicatechin due to loss of buffering capacity of higher concentrations of FBS. The effect of 40 µg/ml of epicatechin
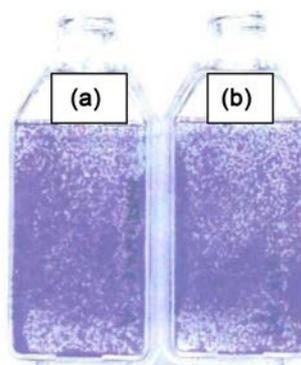

Figure 3. A photograph of two flasks of low density proliferating NHK grown for 5 days in SFM, and then refed fresh medium for 24 hours containing 81 µg/ml of epicatechin (b) or an equal volume of vehicle (a) to serve as a control. The flasks were fixed and stained with 0.2% crystal violet. The image has an enlargement factor of 1/2X.
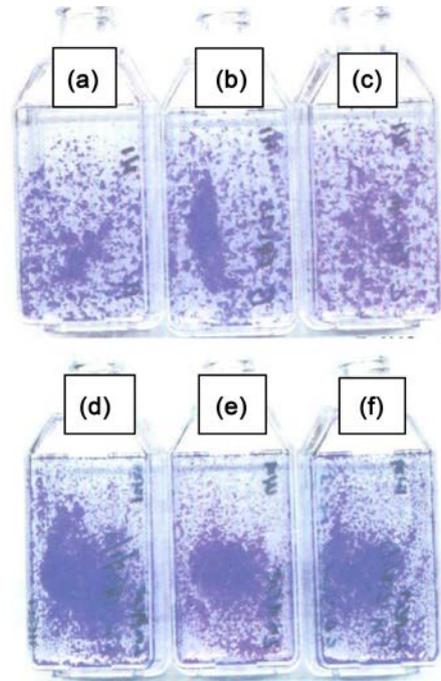
Figure 4. A photograph of three flasks of NHK cells. (a) Untreated, (b) the effect of 20 µg/ml and (c) effect of 40 µg/ml of epicatechin on low density proliferating NHK cultured for 24 hours in SFM. **Figure 4** ((d), (f)) presents three flasks of NHK showing the effect of culturing NHK with 40 ug/ml (e) and 81 ug/ml (f) of epicatechin relative to untreated control (d). Magnification factor, 1/2X.
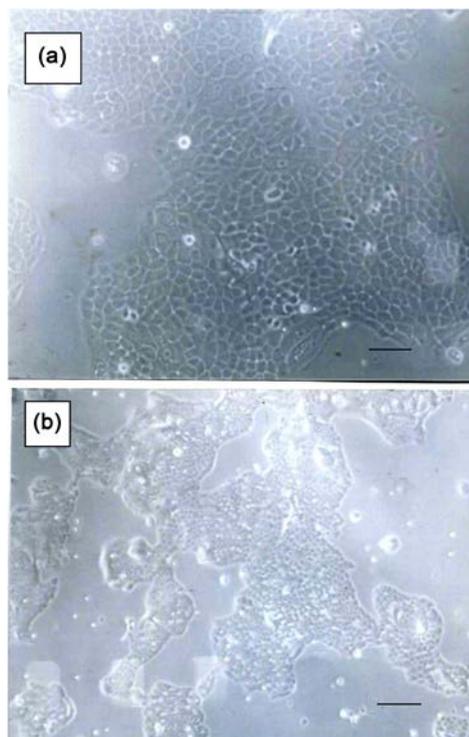

Figure 5. A photomicrograph of (a) untreated NHK cells grown in SFM for 6 days and NHK cells refed SFM containing 81 µg/ml of epicatechin. The black bars on each photograph are 10 micron markers.
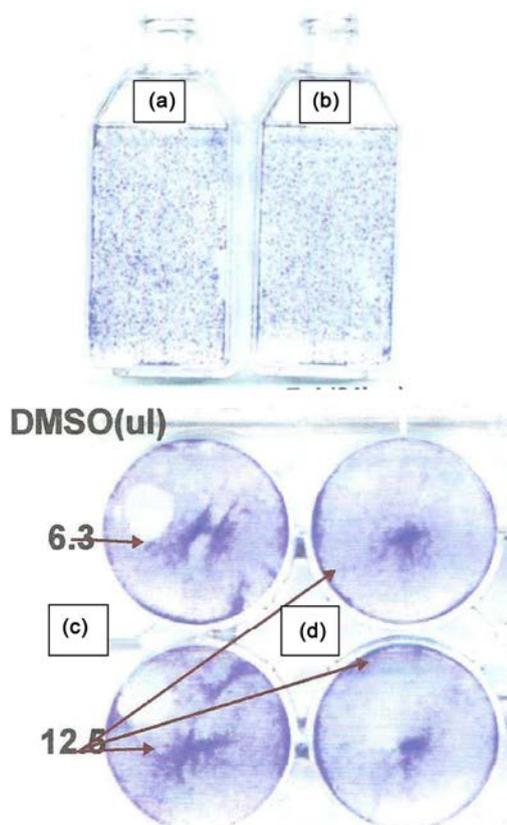
Figure 6. A photograph of four flasks of low density proliferating HeLa cells (a) untreated cells, (b) cells treated with 40 µg/ml of epicatechin, (c) photograph of 6-well culture dish of untreated Hela cells cultured for 24 hours before being terminated, and (d) HeLa cells treated with 40 µg/ml of epicatechin. The culture dishes were fixed and stained with 0.2% crystal violet stain. NB: the arrow designates those wells that received the addition either of 6.3 µl or 12.5 µl of DMSO: Ethanol vehicle. Magnification factor, 1/2X.

on Hela cells (b) was shown with comparison to an untreated control (a) (**Figure 7**). Epicatechin is completely cytotoxic to HeLa cells as seen by the highly refractivity and small cell size. In separate experiments, we tested the effect of two hydroxylated phenolic acid, caffeic and gallic acids. Both were highly toxic to HaCaT cells at concentration above 0.0025% or <25 µg/ml.

3.3. Effect of Two Isoflavones: Genistein and Daidzein on HeLa Cells

Figure 8 is a photograph of three flasks of HeLa cell cultures exposed to 14 µg/ml of genistein (b) compared to the effect of 20 µg/ml of daidzein (c) relative to the untreated control (a). Genistein inhibited HeLa cell growth. By contrast, daidzein had no effect even at a higher concentration. In a separate study, the effect of genistein on HaCat cells had a modest cytotoxic effect at concentrations above 20 µg/ml (data not shown).

3.4. Effect of Applephenon (AP-SH) on NHK

Figure 9 presents a photograph of four flasks of 7-day-old NHK cultures: (a)

untreated controls, (b) 25 μg/ml, (c) 50 μg/ml, and (d) 100 μg/ml of AP-SH treated for 24 hours fixed and stained with crystal violet stain. The results show that AP-SH inhibited cell proliferation and was possibly toxic at 50 μg/ml and 100 μg/ml. Figure 10 presents a composite photomicrograph (a)-(d). AP-SH treatments below 50 μg/ml exhibit only moderate cytotoxicity, while AP-SH
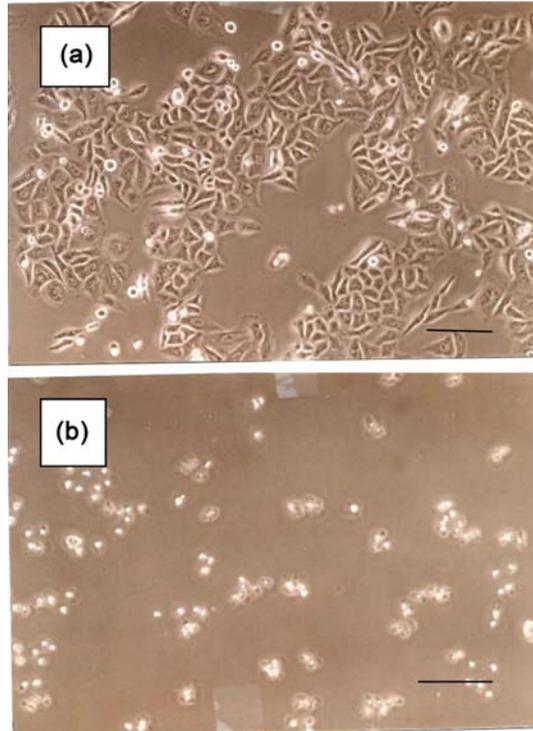

Figure 7. A phase contrast photomicrograph of a low density proliferating HeLa cell cultures (a) untreated, and (b) treated with 40 μg/ml of epicatechin. The black bars denote an image magnification of 10 microns.
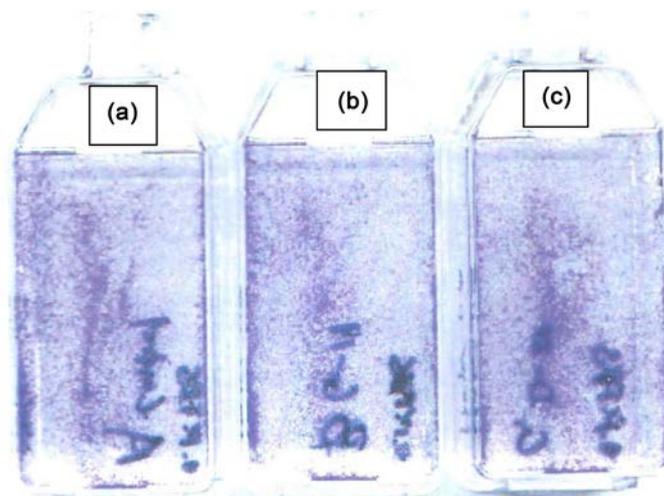

Figure 8. A photograph of three flasks of low density proliferating HeLa cell cultures. (a) Untreated control culture, (b) treated with 14 μg/ml of genistein for 24 hours, and (c) 20 μg/ml of daidzein treated for 24 hours. The blue dots are HeLa cell colonies stained with 0.2% crystal violet. Magnification factor is 1/2X.
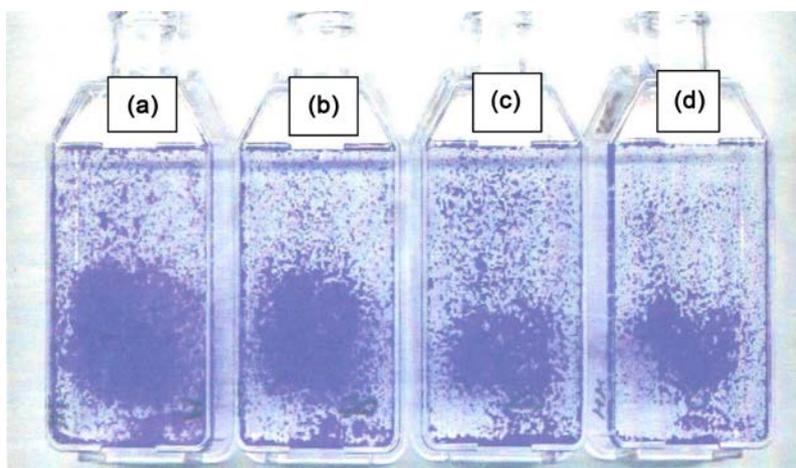
Figure 9. A photograph of four flasks of low density proliferating NHK cultures: (a) untreated control culture, treated with AP-SH for 24 hours with 25 μg/ml, and (b), and (c) and (d) were treated with 50 μg/ml and 100 μg/ml of AP-SH, respectively. The dark blue masses and dots are crystal violet (0.2%) stained NHK colonies. Magnification factor is 1/2X.
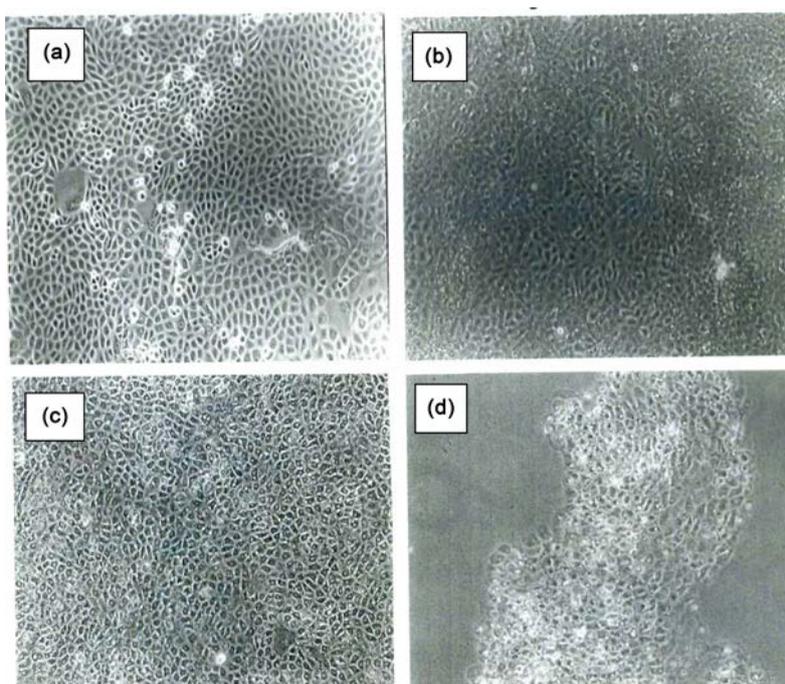

Figure 10. A composite photomicrograph of four low density proliferating NHK cultures. (a) untreated control, (b), (c), and (d) are NHK cultures treated for 24 hours with 25 μg/ml, 50 μg/ml and 100 μg/ml of AP-SH, respectively. The black bars denote image magnification of 10 microns.

treatment at above 50 μg/ml is severely cytotoxic with clumping of colonies, and cell refractility is seen clearly, at 100 μg/ml (d).

3.5. Effect of Applephenon (AP-SH) and Genistein on HeLa

Figure 11 is a photograph of three consecutive 6-well dishes plated with HeLa

cell at 3×10^3 cells/cm^2 in DMEM: 0.1% FBS, and treated with genistein (b)-(e) or AP-SH (f)-(i) with increasing doses ranging from 10, 20, 40, and 80 μg/ml of AP-SH and 4, 8, 13, and 25 μg/ml of genistein. Examination of the dishes reveals a dose-dependent effect for both agents and a minimal cytotoxic dose of 10 μg/ml for genistein and a minimal cytotoxic dose of 20 μg/ml for AP-SH. Separately, daidzein at 25 μg/ml had no effect nor did an extract of green tea at 100 μg/ml. Figure 12 is a phase contrast photomicrograph of HeLa cell culture grown on DMEM: 10% FBS (a) showing many dividers and (b) a culture treated with 0.02% AP-SH for 23 hours. Note the loss of dividers, increased refractivity, and distortion of cell shape indicative of cytotoxicity.
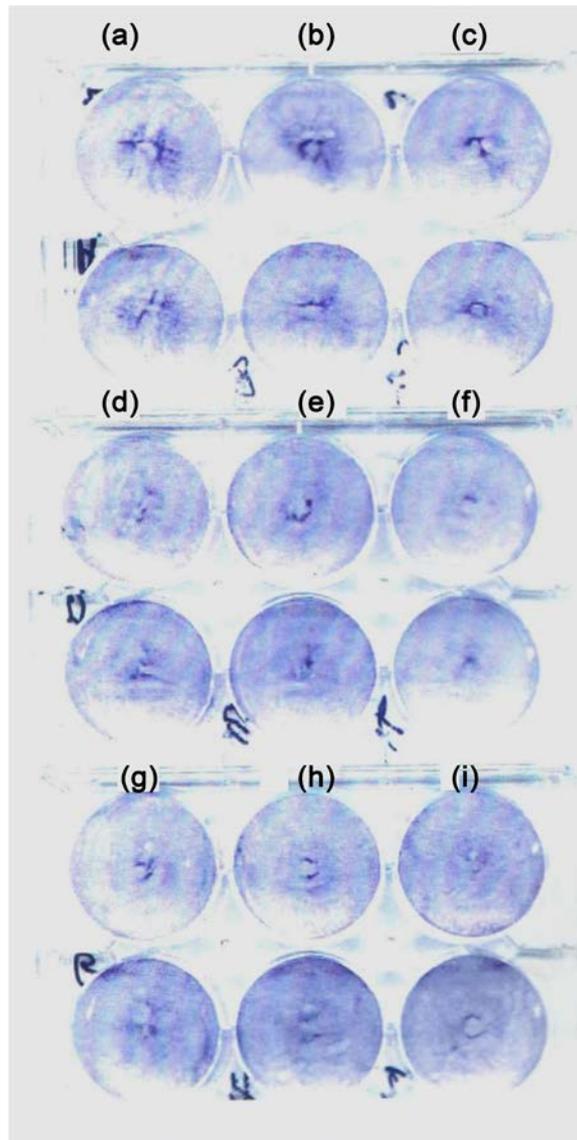

Figure 11. A photograph of three 6-well dishes of low density proliferating HeLa cell cultures plated at 1×10^3 cell/cm^2 and either refed fresh medium (a), or fresh medium containing genistein (b)-(e) at increasing concentrations of 4, 8, 12.5 and 25 μg/ml, respectively, or refed fresh medium with AP-SH (f)-(i) at increasing concentrations of 10, 20, 40, and 80 μg/ml, respectively for 24 hours.
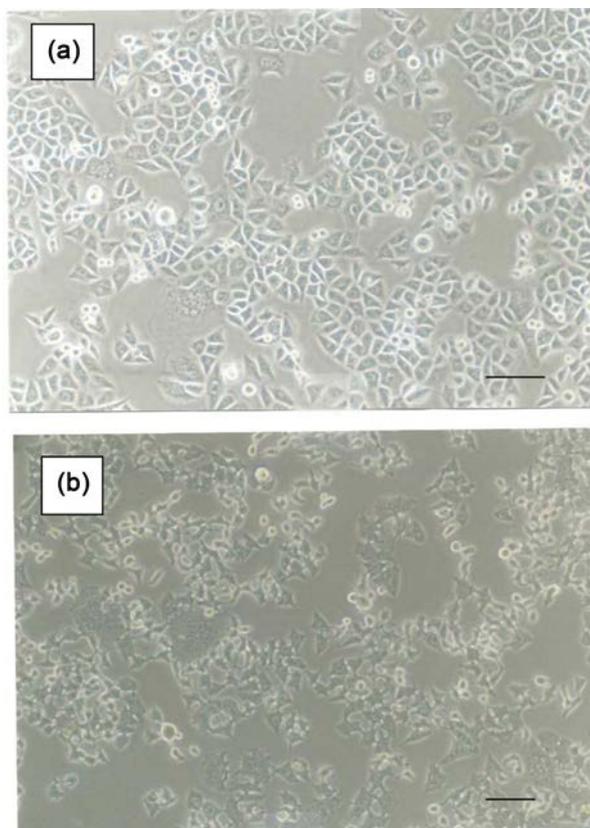
Figure 12. A photomicrograph of untreated low density proliferating HeLa cell culture (a), and HeLa cell culture treated with AP-SH for 23 hours. The black bars denote image magnification of 10 microns.
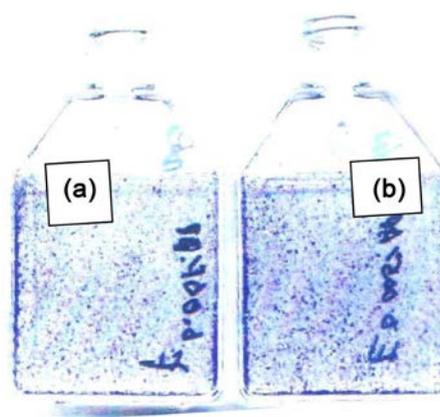

Figure 13. A photograph of two flasks of HaCaT cells treated with (a) 0.001% or 0.005% AP-SH for 23 hours. **Figure 14(b)** shows a darker blue background due to the fixation and flocculation of serum in combination with AP-SH Magnification factor is 1/2X.

3.6. Effect of Applephenon (AP-SH) on HaCaT Cells

Figure 13 is a photograph of two flask ((a), (b)) showing the effect of AP-SH on HaCaT cell grown in DMEM: 1.0%FBS and treated with (a) 0.001% or (b) 0.005% AP-SH. The cell density (blue dots) is reduced in (b) relative to (a). **Figure 14** is a composite photomicrograph of the effect of AP-SH on a culture of
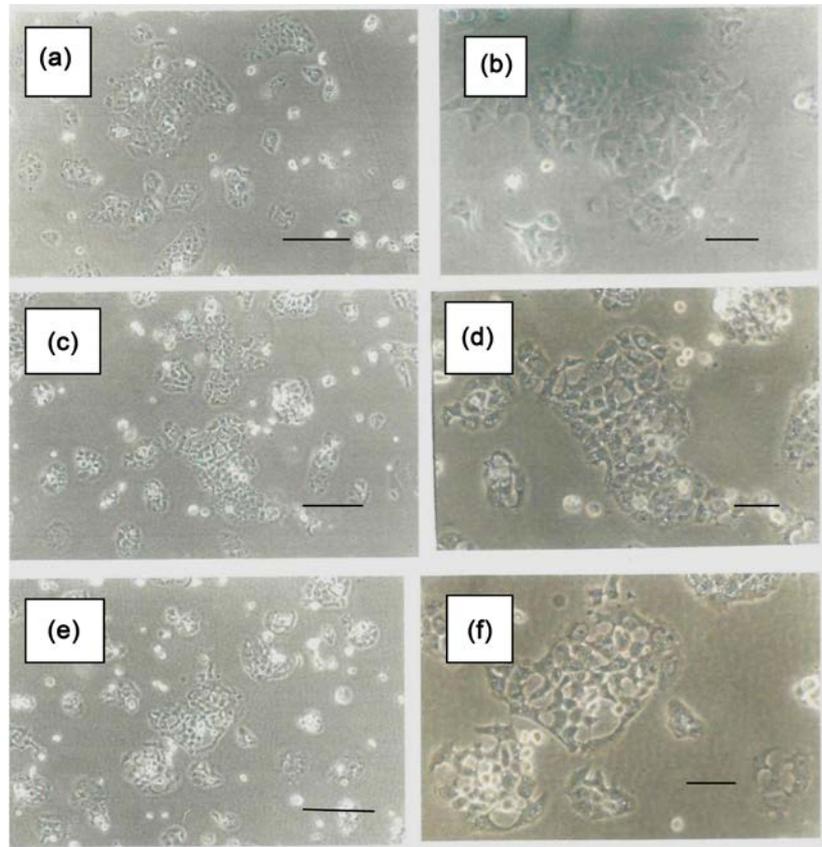
Figure 14. Composite photomicrograph of AP-SH-treated HaCaT cultures. The left three panels ((a), (c), (e)) are magnified 100X and the right three panels ((b), (d), (f)) were magnified 200X both without enlargement factor correction. Figures (a) and (b) are untreated controls, (c) and (d) were treated with 0.0025% AP-SH, and E and F were treated with 0.005% AP-SH. The black bars denote image magnification of 10 microns.

HaCaT. The left three panels ((a), (c), (e)) are magnified 100X and the right three panels ((b), (d), (f)) were magnified 200X both without enlargement factor correction. Figure 14(a) and Figure 14(b) are untreated controls, (c) and (d) were treated with 0.0025% AP-SH, and E and F were treated with 0.005% AP-SH. The latter two treatment show signs of cytotoxicity as seen by ballooning of the intracellular junctions and loss of dividers. The estimated minimal toxic concentration appears to be between 0.0025% or 12.5 µg/ml and severe toxicity at 25 µg/ml.

3.7. Effect of Applephenon (AP-SH) on A431 Cells

Figure 15 presents a photograph of four flasks of cultures of A431 epidermoid carcinoma cells showing a flask of untreated control (a), and three flasks with increasing concentrations of AP-SH: (b) 0.001%, (c) 0.0025%, and (d) 0.005%. The minimal cytotoxic dose appears to be (0.0025%), or 20 µg/ml. Figure 16 is a photomicrograph showing the phase contrast microscopic images of (a) control and (b) a culture treated for 24 hours with 20 µg/ml of AP-SH. The treated cells appear more refractile and have increased intercellular spaces. Table 2 summarizes minimal cytotoxic doses observed for epicatechin (a) and AP-SH (b) treated

NHK (b) relative to the minimal cytotoxic doses for each of the epidermoid carcinoma cell lines.
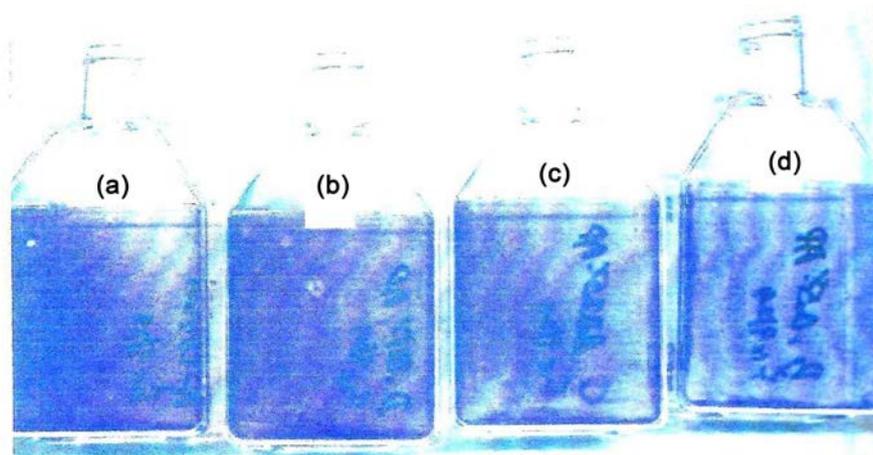
Figure 15. A photograph of four flasks showing the effect of treating A431 cells with increasing concentration AP-SH. (a) untreated control, (b) 0.001%, (c) 0.0025%, and (d) 0.005%. Magnification factor is 1/2X.
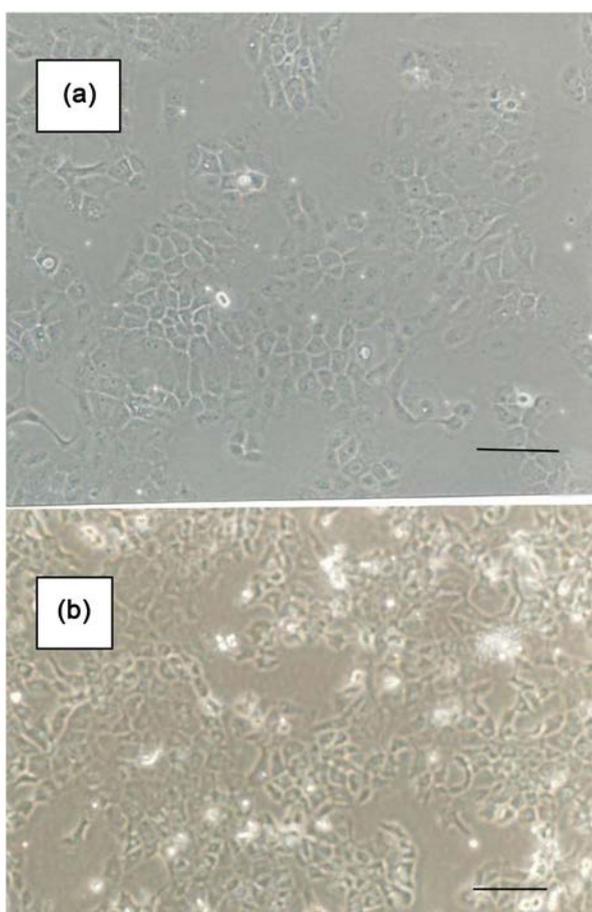

Figure 16. A photomicrograph showing the phase contrast microscopic images of A431 cells treated with 20 μg/ml of AP-SH for 24 hours (b), and (a) untreated control. The black bars denote image magnification of 10 micron.

Table 2. Minimal Cytotoxic Concentration (μg/ml) of Applephenon™ (SH) and Select Polyphenolic Compounds.

CELLS	EPICATECHIN	GENISTEIN	APPLEPHENON
NHK	80 ± 6.8	65 ± 15.5	50 ± 10.2
HaCaT	Nd	25 ± 5.3	13 ± 4.6
HeLa	40 ± 5.2	14 ± 3.4	20 ± 6.3
A431	Nd	Nd	20 ± 5.6

Nd: not done.

4. Discussion

This study examined the phenolic and polyphenolic composition of Applephenon (AP-SH grade) by HPLC analysis. Our results are in general agreement with those published by Nikki Whiskies, Ltd. [4] [5]. Phenolics accounted for 65% of the total identifiable polyphenolics of AP-SH. The commercial lot we procured labelled Special Grade (SH) was claimed to be devoid of chlorogenic acid and enriched in procyanidins. However, our HPLC analysis indicated that chlorogenic acid was present at >24% of phenolics and there was no significant increase in procyanidins relative to standard grade. This might be accounted for by mistakenly receiving the standard grade rather than the SH grade, even though it was accompanied by the AP-SH certificate of analysis, or to differing methodologies of analysis. The role of chlorogenic acid as a single component of AP was not examined, but it is likely that it does contribute to the total cytotoxic activity of AP [12]. By contrast, we focused on the toxic effect of epicatechin which together with catechin constituted > 22% of the total phenolics. We report that low serum cultures of HeLa cells were two times more sensitive than NHK cells to the cytotoxic effects of epicatechin. Phase contrast microscopy of treated NHK colony gave the appearance tight cell clumping. By contrast, HeLa cells were highly dispersed, refractile and reduced in number akin to the action of enzymatic dislodgement by trypsin treatment.

The anti-carcinogenic properties of botanical polyphenolics and of epicatechins (*a flavon*-3-*ol*) are well-documented [13]. The healthy effect of drinking green and black tea extend to the anti-cancer effects of epigallocatechin gallate (ECGC), which binds to the anti-apoptotic protein Bcl-x1 often over-expressed in tumor cells [14]. Earlier studies provided support that procyanidins were cytotoxic to human tumor cell lines. [15] [16] Other in vitro studies have shown that several bioactive food components, including tea polyphenols (catechin, epicatechin, and (−)-epigallocatechin-3-O-gallate, EGCG) and bioflavonoids (quercetin, fisetin, and myricetin), inhibit DNMT-1-mediated DNA methylation in a dose-dependent manner [14]. Dietary and other environmental factors such as AP that induce epigenetic alterations may have important consequences for cancer development [17]. Epigallo-3-gallate is effective in chemoprevention in the multistage carcinogenesis model [18]. The major antioxidative polyphenolic compounds of green tea have been shown to exert growth-inhibitory potential of

various cancer cells in culture and antitumor activity in vivo models [19]. It has been proposed that ECG or EGCG may interact with various molecules like proteins, transcription factors, and enzymes, which block multiple stages of carcinogenesis via regulating intracellular signaling transduction pathways, regulation of cell proliferation and apoptosis effects. The phenolic constituents of epicatechin oligomers longer than trimers have been reported to have anti-cancer activities, but not the catechin counterparts [20]. Epicatechin oligomers have effects on cell cycle distribution as cell cycle arrest in the G2 phase was induced. Also, epicatechin oligomers suppressed significantly the expression of the cancer-promoting gene, *FABP*5, which is related to cell proliferation and metastasis in various cancer cells.

The relationship between cytotoxicity and the antioxidant activity of AP-SH phenolics and polyphenolics was explored to determine whether the sensitivity of normal and cancer cells resides in hydroxylation level of these compounds. As reported above both caffeic acid and gallic acid were highly toxic to HeLa cells relative to epicatechin, which accords with their assessed stronger antioxidant activity. The cytotoxic effects two hydroxylated isoflavones, genistein and daidzein, on HeLa and HaCat cells were tested. Although neither genistein nor daidzein had any appreciable free radical scavenging antioxidant activity, genistein was found to be even more cytotoxic to HeLa cells than epicatechin, By contrast, daidzein was neither cytotoxic nor had any testable antioxidant activity. This confirms an earlier report that genistein but not daidzein inhibits the growth of two human prostate cancer cell lines [21]. The anticancer and chemopreventive property of genistein is thought to be due to its estrogenic effect on breast cancer cells [8]. The mechanism of genistein's antiproliferative activity is its antiapoptotic effect mediated through inhibition of the nuclear transcription factor NF-kB and the AKT signaling pathway [22].

Table 2 summarizes HaCaT, HeLa and A431 epidermoid cancer cells were relatively more sensitive to AP-SH induced cytotoxicity than epicatechin alone. It should be pointed out that strictly speaking the spontaneously immortalized HaCat cells are not tumorigenic [22]. Their immortalized state and ready malignant transformation suggest that HaCaT are a premalignant type of epidermal keratinocyte, *i.e.*, malignant transformation of HaCaT cells can be achieved through processes leading to the deregulation of NF-kB signaling [23].

In summary, our studies showed that both AP-SH and epicatechin were highly effective in inhibiting growth of normal human keratinocytes and epidermoid tumor cells when cultured in either low serum or in serum-free culture media. This is so because serum at concentrations greater than 0.2% blocks the anti-proliferative effect of polyphenols, presumably by binding the free polyphenols. Lastly, we show here that epidermoid tumor cells each were more sensitive to the antiproliferative and cytotoxic effect of epicatechin, genistein and unfractionated AP-SH, containing less than 5% procyanidins, than their normal human keratinocyte counterpart.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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