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Research Article

CORRELATION OF ANTIOXIDANT AND ANTIPROLIFERATIVE ACTIVITY OF AMLA AND GINGER

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ABSTRACT

Objective: Our study focused on evaluating the anticancer property of ascorbic acid and aqueous extract of amla and ginger.

Methods: Antioxidant capacity of ascorbic acid, aqueous extract of amla, and ginger was obtained by 2,2-diphenyl-1-picrylhydrazyl method and total antioxidant capacity (TAC). Vero cell line, PA-1, Cal-27, Cal-27 CisR, and DysMSCTR16 cell lines were treated with antioxidants to evaluate its antiproliferative property using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Colony and spheroid formation assays were also carried out in the presence of extracts to assess its role in anticancer stem cell activity.

Results: Two volumes of amla 5 µl (~0.016 g) and 25 µl (~0.08 g) and ginger 5 µl (~0.02 g) and 25 µl (~0.1 g) showed TAC activity equivalent to 0.25 mM and 2 mM ascorbic acid, respectively. Amla and ascorbic acid showed significant antiproliferative property in normal (Vero) p=0.05, cancer (PA-1, Cal-27) p=0.005, and resistant (Cal-27 CisR) p=0.05 cell lines and ginger extract in Vero and Cal-27 cell lines (p=0.05). In PA-1 and Cal-27 CisR cell line, ginger extract showed proliferative activity (p=0.005). Antioxidants showed no antiproliferative activity in DysMSCTR16 cells. Amla extract and ascorbic acid showed significant inhibitory effect on spheroid (p=0.005) and colony formation capacity (p=0.0005) among dysplastic, cancer, and resistant cell lines. Ginger showed inhibitory effect (p=0.05) only in colony formation capacity.

Conclusion: Overall, we found a strong correlation between antioxidant and antiproliferative activity of ascorbic acid, amla, and ginger. Amla and ascorbic acid proved to be effective in controlling cell proliferation and self-renewal properties of cancer cells. However, ginger was found to have selective and less antiproliferative effect in comparison to amla.

Keyword: Amla, Ginger aqueous extract, Cancer progression, Cancer stem cells, Antioxidant-induced cell death.

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INTRODUCTION

Amla, Indian gooseberry (*Phyllanthus emblica officinalis*) extracts, has been used in traditional medicine to treat symptoms ranging from constipation to cancer treatment for centuries in the Indian system of medicine [1]. *E. officinalis* has been shown to be a potent free radical scavenging agent, thereby preventing carcinogenesis and mutagenesis. A dose of 100 mg/kg body weight has shown to reduce the incidence of tumor by approximately 60% [2,3]. Ginger (*Zingiber officinale* Roscoe) is also another natural dietary component generally used in complementary and alternative medicine (CAM). Various ginger and ginger leaf extracts have been reported positive response in controlling cancer proliferation [4]. Various components of ginger such as gingerols, paradols, shogaols, and gingerones have shown antioxidant, anti-inflammatory, anticancer, and antiangiogenic property [5]. Many studies showed its beneficial effect in lung, ovarian, colorectal, and gastric cancer [6-8]. In addition, dietary supplements containing ascorbic acid, lysine, proline, and green tea extracts are known to reduce the tumor growth *in vivo* studies [9-12]. Micromolar concentration of extracellular Vitamin C kills cancer cells but not normal cells in an H_2O_2-dependent manner [13].

Pro-oxidants are chemicals that induce oxidative stress, either by generating reactive oxygen species or by inhibiting antioxidant systems [14]. Oxidative stress in cancer cells is, however, a dual-edged sword and many cancer therapies rely on using an additional oxidative stressor to selectively drive cancer cells into programmed cell death. The high level of intrinsic oxidative stress expressed by many cancer cells has often been called the "Achilles' heel" of cancer [15]. Many exogenous antioxidants such as Vitamin E (tocopherols and tocotrienols), Vitamin C, green tea polyphenols, beta-carotene, and curcumin are individually as well as in combination have been known for their potential roles in CAM approaches to cancer prevention and treatment [16,17]. For public health professionals, the question of whether or not antioxidants have a safe chemopreventive role is central: It is clearly much more effective to prevent cancer than deal with its consequences including the damaging side effects of many chemotherapeutics. However, evidence is lacking for the effectiveness of most CAM therapies for cancer [18]. In parallel, cancer stem cells (CSCs) have been implicated in cancer progression, recurrence, and thereby poor prognosis due to their inherent quiescence and drug-resistant properties [19,20]. The role of CSCs in carcinogenesis has been investigated with multiple concepts proposed to explain their possible role in initiation of the tumorigenic process, subsequent field cancerization, and recurrence [21].

In this study, we investigate the antioxidant capacity of crude aqueous extracts of amla and ginger in comparison with ascorbic acid and study their effect on CSC in cell lines representing different stages of cancer progression. Normal, dysplastic, squamous cancer cells, and resistant cell lines, with different percentage of CSC population [22,23], were evaluated for the response of antioxidants from the extracts of amla and ginger.

METHODS

Preparation of plant extracts

Ginger extract

Fresh aqueous ginger extract (5 mL) was prepared from 20 g of fresh ginger. Briefly, skin peeled ginger was grated and boiled in deionized water for 5 min and juice was squeezed under sterilized condition. After extraction, the juice was filtered through Whatman filter paper (#Z146374-100EA, Sigma-Aldrich, USA). Sterility check was done by streaking the extract on nutrient agar plate for 3 days. It was stored in sterilized glass container at 4°C until used (5 µl of extract was approximately equivalent to 0.02 g of fresh ginger).

Amla extract

For aqueous extract, surface sterilized amla was peeled, grated, and squeezed with multilayered muslin cloth followed by filtration through Whatman paper-1 under sterilized conditions. Sterility check was done by streaking the extract on nutrient agar plate for 3 days. The extract was stored in sterilized glass container at 4°C until used. 6 mL of fresh amla extract was obtained from 20 g of fresh amla (5 µl extract was approximately equivalent to 0.016 g of amla).

Antioxidant activity test

Determination of total antioxidant capacity (TAC)

The antioxidant activity of the extract was evaluated by the phosphor-molybdenum method according to the procedure describe by Prieto *et al*. [24]. The assay is based on the reduction of Mo (VI)–Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. Fresh extracts of amla and ginger of varying amount (5–100 µl) made to 0.3 mL with water was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). Methanol (0.3 mL) in place of extract is used as the blank. Tubes containing the reaction solution were incubated at 95°C for 90 min. Then, the absorbance of the solution was measured at 695 nm using spectrophotometer (UV-1800, Shimadzu UV-Spectrometer) against blank after cooling to room temperature. The antioxidant activity is expressed as the number of g equivalents of ascorbic acid. Experiments were performed in triplicates**.**

Radical scavenging activity

The free radical scavenging capacity of the extracts was determined using modified 2,2-diphenyl-1-picrylhydrazyl (DPPH) method [25]. Briefly, freshly prepared DPPH solution (0.4%) in 95% methanol was taken in test tubes, and sample extract appropriately diluted in the range of 5–20 µl was added to every test tube so that the final volume was 3 mL and after 10 min, the absorbance was read at 517 nm using a colorimeter (CL-157, ELICO Ltd., Hyderabad, India). Ascorbic acid (Hi-Media laboratory, Mumbai, India) was used as a reference standard. The stock solution was made to 0.1 M, 0.01 M, and 0.001 M using distilled water. 95% methanol was used as blank. Experiments were performed in triplicates and represented as percentage of mean inhibition ± standard error. Percentage scavenging of the DPPH free radical was measured, using following formula:

% Scavenging of the DPPH free radical=(Absorbance of the control–Absorbance of the test sample)/Absorbance of the control) × 100

Cell culture

PA-1 (ovarian teratocarcinoma metastatic) and Vero cell line (monkey kidney cells) were obtained from National Center for Cell Sciences, Pune, India. Head and neck squamous cell carcinoma cell lines and Cal-27 were obtained from Institute of Bioinformatics, Bangalore, and cisplatin-resistant subline (Cal-27 CisR) was developed and characterized in the laboratory (Mazumdar Shaw Centre for Translational research, MSCTR), Bangalore, was used in this study [22]. The monolayer cultures grown in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, Massachusetts, U.S.A) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific). The resistant cell lines were cultured in the presence of the drug (Cisplatin; IC 6.25 each). Mice, dysplastic cell line, DysMSCTR16 developed in the MSCTR, Bangalore, were also maintained in monolayer cultures in DMEM:F12 (Thermo Fisher Scientific, Massachusetts, U.S.A) supplemented with 20% FBS and growth factors (data unpublished, Indian provisional Patent: TEMP/E-1/20383/2017 - CHE).

Cell viability assay

Viability of treated and untreated cells was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as per standard protocols. Briefly, 1×10^4 cells/well were plated for 18 h in 96-well plate before the treatment with different concentrations of amla, ginger extract (5 µl and 25 µl), and ascorbic acids (0.25 mM and 2 mM) for 24 h and 48 h in 100 µl media/well. Cells were then incubated with 20 µl of MTT (5 mg/mL) (#M5655, Sigma, USA) for 4 h (37C) and the formazan crystals formed dissolved in DMSO (100 µl). The plate was read at 570 nm (test) and 690 nm (reference) in Tecan Infinite F200 Pro (Bio-Rad Laboratories, Inc., USA). The percentage viability was calculated using the formula (Mean OD of the test/Mean OD of the control)*100. All the experiments were carried out in triplicates.

Spheroid formation assay

To assess the spheroid forming ability of cells, Cal-27 and Cal-27 CisR cell lines were plated along with amla, ginger, and ascorbic acid. Briefly, a required number of single cells (1000 cells/well) were resuspended in serum free, DMEM: F12 medium supplemented with 1% N2 supplement, 20 ng/mL human basic fibroblast growth factor, 20 ng/mL human epidermal growth factor, 5 mg/mL of insulin, and cultured in 24-well ultra-low attachment plates for 10–12 days. The medium was supplemented with the growth factors every 2 days and the number of spheroids was counted after 12 days. All the experiments were done in triplicates and average number was plotted.

Colony Formation Assay

Colony formation capacities of the DysMSCTR16, Cal-27, and Cal-27 CisR cell lines were evaluated by plating 500 cells in a 24-well cell culture plate and cultured for 14 days in the presence of amla, ginger, and ascorbic acid. Colonies were fixed and stained with Giemsa stain (Hi-Media Pvt., Ltd., Mumbai, India). 50 cells per clone were counted manually. The colony-forming capacity of each cell line was compared with untreated control cells, and results were concluded from triplicate experiments.

Statistical analysis

Statistical significance for MTT assay, colony formation, and spheroid formation assays was determined among different cell lines in comparison to the untreated cell lines. The unpaired t-test was used for calculating significance in all the experiments (GraphPad Prism Software 7.0).

RESULTS

TAC

TAC determines the efficacy of an antioxidant to donate its electrons. TAC was expressed in terms of ascorbic acid equivalents. The TAC of 5 µl of amla and ginger was found be equivalent to 202 µM and 47 µM ascorbic acid, respectively, as given in Table 1.

DPPH radical scavenging activity

DPPH is a free radical donor, which has been used widely in studying the free radical scavenging capability of various compounds. The method is based on the reduction in absorbance at 517 nm in the presence

Table 1: Total antioxidant capacity (µM ascorbic acid equivalents) of amla and ginger

Sample	Average absorbance at 695 nm	Ascorbic acid equivalent (µM)
Amla	0.202±0.01	202
Ginger	0.047±0.02	47

Absorbance=Avg. Mean (n=3)±SE. SE: Standard error

of proton-donating substance, accompanied by a sharp color change from purple to yellow. The crude extracts of amla and ginger showed a significant decrease in both absorbance and the color change.

Every 5 µl of aqueous extract of amla and ginger showed scavenging activity of 16% and 21%, respectively (Fig. 1a). Aqueous extract of amla showed same scavenging activity at both its native (pH 3) and adjusted physiological pH 7. These observations showed that ginger and amla both are good antioxidants. Although ginger showed that the higher radical scavenging capacity and amla have higher TAC.

Effect of antioxidants on normal, dysplastic, cancer, and resistant cell line

Normal kidney cell line, Vero cells showed a significant decrease in percentage viability ($p<0.0001$) by end of 48 h in both low dose 0.25 mM ascorbic acid (55%±0.023) and high dose 2 mM (1%±0.3); amla 5 µl (7%±0.47) and 25 µl (0%±0.001); and ginger 5 µl (67%±0.094) and 25 µl ginger (15%±0.31) as compared to control untreated Vero cells (100%) (Fig. 1b). These results show that the cells are susceptible to the presence of antioxidants in the culture media for longer period.

Dysplastic cell line, DysMSCTR16, showed no significant effect on cell viability at 48 h of treatment with both low and high doses of ascorbic acid as well as extracts of amla and ginger as compared to control untreated DysMSCTR16 cells (100%) (Fig. 1c).

In teratoma cell line PA-1, a 48 h of treatment with ascorbic acid and higher dose of amla were significantly effective in decreasing the cell viability, ascorbic acid 0.25 mM (6.25%±0.034; $p=0.0001$) and 2 mM ascorbic acid (0%±.01; $p=0.0001$); and 25 µl amla (0%±0.62; $p=0.0001$). Low dose of amla and ginger extract failed to inhibit the proliferation in PA-1 cell line as compared to untreated control PA-1 cells (Fig. 1d).

In oral squamous cell carcinoma cell line Cal-27, all the antioxidants ascorbic acid, amla, and ginger extract were significantly effective in controlling the cell proliferation at 48 h. Cell viability was significantly affected at 0.25 mM ascorbic acid (85%±0.28; $p=0.003$) and 2 mM ascorbic acid (86%±0.42; $p=0.02$); 5 µl amla (64.7%±0.02; $p=0.002$) and 25 µl amla (93.5%±0.02; $p=0.004$); and 5 µl ginger (84.2%±0.02; $p=0.04$) and 25 µl ginger (91%±0.02; $p=0.04$) as compared to the untreated control Cal-27 (Fig. 1e).

The percent cell viability of cisplatin-resistant cell line, Cal-27 CisR decreased after 48 h of treatment with high dosage of antioxidants; 2 mM ascorbic acid (30%±0.18; $p=0.04$); amla 25 µl (68%±0.29; $p=0.03$); and 25 µl ginger (52%±0.91 $p=0.04$) (Fig. 1f).

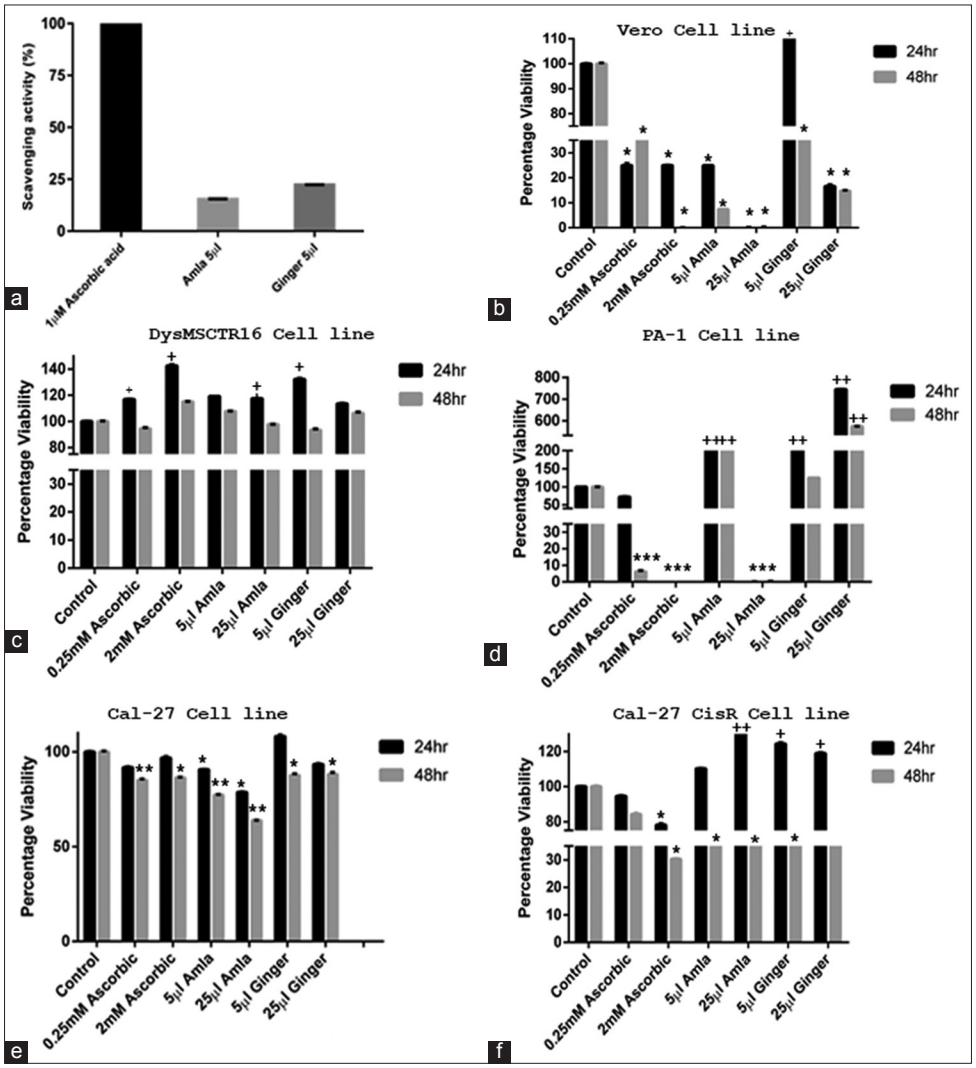

Fig. 1: Represents the percentage scavenging activity of 5 µl of amla and ginger extract as compared to the 1 µM ascorbic acid (a). Proliferation assay confirms the effect of amla, ascorbic acid, and ginger on Vero cells (b), DysMSCTR16 cells (c), PA-1 cells (d), Cal-27 cells (e), and Cal-27 CisR (f) cell line at 24 and 48 h treatment. All of the experiments were performed in triplicates. Results show the mean SE of three independent assays. Statistical significance is also represented (*$p<0.05$, **$p<0.005$, ***$p<0.0005$). (*) represents the inhibitory significant data; (+) represents the anti-inhibitory significant data

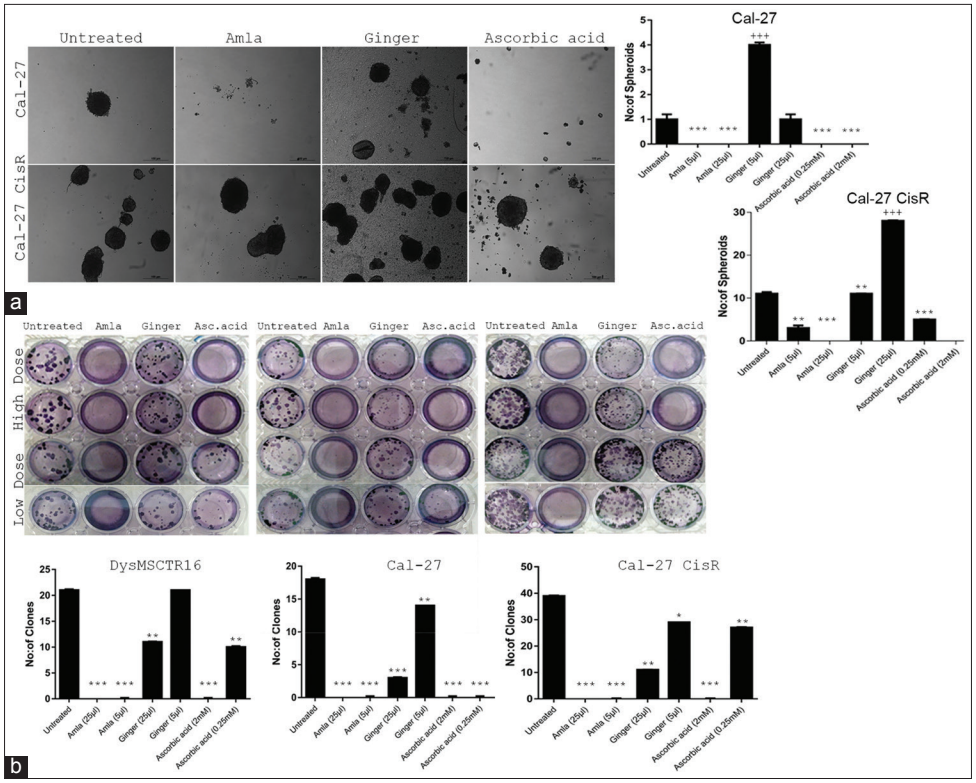

Fig. 2: Functional assay showing the inhibitory action of amla and ascorbic acid on spheroid formation on Cal-27 and Cal-27 CisR cells (a) and anchorage-dependent self-renewal capacity (colony formation capacity) in DysMSCTR12, Cal-27, and Cal-27 CisR cell lines. Ginger extract shows no significant inhibitory effect on cell lines. All of the experiments were performed in triplicates. Results show the mean SE of three independent assays. Statistical significance is also represented (*p<0.05, **p<0.005, ***p<0.0005). (*) represents the inhibitory significance data; (+) represents the anti-inhibitory significant data

Results of the cell viability study suggest that at the chosen dosage of ascorbic acid (0.25 mM and 2 mM), amla extract (5 µl and 25 µl), and ginger extract (5 µl and 25 µl) were significantly effective in controlling cell proliferation of normal kidney cells (non-cancerous cells). Cell proliferation of dysplastic cells DysMSCTR16 was not affected any of the antioxidants used in this study. Cell viability of PA-1 cells was significantly reduced by ascorbic acid and high dose of amla extract only. Proliferation of squamous cell carcinoma cells (Cal-27) was significantly affected by all the studied antioxidants. Proliferation of cisplatin-resistant cells (Cal-27 CisR cell line) was significantly affected only at higher concentrations of the studied antioxidants.

Effect of antioxidants on anchorage-independent self-renewal capacity (spheroid formation) of cancer cells

Spheroid formation capabilities of Cal-27 and Cal-27 CisR cells were analyzed in the presence of crude extracts of amla, ginger, and ascorbic acid. Cal-27 cells showed a significant inhibition in the spheroid formation capacity in the presence of both low and high dose of amla and ascorbic acid (n=0, p<0.0001), whereas, lower dose of ginger (5 µl) showed a significant increase in number of spheroids formed (n=4, p<0.0001) as compared to the untreated control Cal-27 (n=1).

In Cal-27 CisR, 25 µl amla and 2 mM ascorbic acid were effective in inhibiting the spheroid formation capacity (n=0, p<0.0001). Lower dose (5 µl) amla (n=3) and 0.25 mM ascorbic acid (n=5) p<0.0001) could significantly reduce the spheroid formation capacity as compared to the untreated Cal-27 CisR cells (n=11). On the other hand, ginger extract showed an increase in spheroid formation capacity of Cal-27 CisR cell line (n=28, p<0.0001) (Fig. 2a).

Effect of antioxidants on anchorage-dependent self-renewal capacity (colony formation) of cancer cells

DysMSCTR16, Cal-27, and Cal-27 CisR cells were used to evaluate the effect of antioxidants (ascorbic acid and crude extracts of amla and ginger) on colony formation capacity of cancer cells.

In DysMSCTR16 and Cal-27 cells, both amla and ascorbic acid showed a complete inhibition of the colony formation capacity (n=0, p<0.0001), and only high dose (25 µl) of ginger extract reduced the colony formation capacity to 50% DysMSCTR16 (n=11, p<0.0001), Cal-27 (n=3, p<0.0001) as compared to the untreated control DysMSCTR16 (n=21) and Cal-27 (n=11). Low-dose ginger (5 µl) did not show any inhibitory effect in DysMSCTR16 (n=21) (Fig. 2b).

In Cal-27 CisR cells, amla extract and 2 mM ascorbic acid showed full inhibition of colony formation (n=0, p<0.0001) and as compared to untreated control Cal-27 CisR (n=39). Low dose of 0.25 mM ascorbic acid showed low significant reduction in clone formation (n=27, p<0.003). Ginger extract showed least significant reduction in clone formation capacity in both low dose 5 µl ginger (n=29, p<0.05) and 25 µl ginger (n=11, p<0.004) as compared to the untreated control Cal-27 CisR (n=39) (Fig. 2b).

Amla and ascorbic acid showed a significant effect in controlling the self-renewal capacity of cells in various lineages of cancer cell.

DISCUSSION

Accumulating evidence suggests that many dietary factors like antioxidants (polyphenols) are used alone or in combination with traditional/conventional chemotherapy to prevent or treat cancer [26-35]. Crude and processed plant extracts are studied

extensively to evaluate its role in treatment as chemopreventive drug or to use in combination that can synergistically improve the outcome of current chemotherapy and lower the toxicity [36-38]. European Prospective Investigation into Cancer and Nutrition is one such agency that studies the relationship between diet and cancer [29,39-41].

Many chemopreventive therapies utilize polyphenolic components such as curcumin, ginger, Vitamin C, amla, and green tea extracts as cancer therapy [42-46]. The primary mechanism of many chemotherapy and radiotherapy system is generation of reactive oxygen species in the cancer cells to induce apoptosis. Free radicals produced during the therapy causes serious side-effect and toxicity in the patients. For more than two decades, discussion continues on whether antioxidants should be used along with chemotherapy and radiation. Many oncologists now advise patients to discontinue antioxidant type ingredients which may also include fruits and vegetables used in higher quantities than recommended in diet. Apart from the common usage, new studies also highlight on crossover between antioxidant to pro-oxidant property of polyphenols based on the concentration, solubility, formulation, dosage, and combination with metal ions during the intake [42,47-49].

In our study, we highlighted on the use of aqueous extracts of ascorbic acid, amla, and ginger to evaluate its effect on different lineages of cancers. We observed that a low dosage of ascorbic acid (0.25 mM) and low-dose aqueous extract of fresh amla (0.25 g/mL) were effective in controlling the cell proliferation of ovarian tetratomic (PA-1), oral cancer (Cal-27); whereas the cisplatin-resistant (Cal-27 CisR) cell line was effected only at high dose (2 mM ascorbic acid and 25 µl of amla extract). Is the inefficacy due to increasing percentage of CSC population in Cal-27, PA-1, and Cal-27 CisR cells? [22,50]. Dysplasia cells (DysMSCTR16) were not affected by ascorbic acid or the amla and ginger extracts, in regard to cell proliferation. This may be due to slow growth of dysplasia cells as compared to cancer cells or may be even higher concentration of extracts required to trigger cell death. It was also observed that aqueous extract of fresh ginger was not as effective as amla extract and ascorbic acid, in controlling the proliferation of cells even after 48 h of treatment, as observed in dysplastic, teratoma, and cisplatin-resistant cells. To further assess, we looked into the inhibitory effect of extracts on the self-renewal capacity of cells using spheroid and colony formation capacity. We observed that longer exposure of amla and ascorbic acid was able to inhibit both spheroid and colony formation among various lineages of cancer cells including dysplasia cell lines. Ginger extract, on the other hand, showed to increase or insignificant effect in controlling the anchorage-independent self-renewal capacity of spheroid formation capacity of oral squamous cell line and cisplatin-resistant cell line. However, high dose of ginger was able to inhibit anchorage-dependent growth (colony formation capacity) of cancer cells. Although the total antioxidant and scavenging capacity were in the same range between amla and ginger, yet the effects were different in functional aspects. Suggesting that, the observed effects could also be due to the nature, structure and origin of the antioxidants, and only on the antioxidant potential. Inhibition of clone formation in dysplasia cells also indicated toward a longer incubation of extracts to be effective in controlling the cell viability and growth of dysplasia cell line.

Polyphenols are extracted in organic solvents for high yield as compared to the aqueous extraction. Previous studies highlight on anticancer property of amla and ginger extracts and are used as chemopreventive and therapeutic drug in tradition medicines [3,51-55]. Our study focused on evaluating the direct effect of aqueous extract of ginger and amla, in its natural dietary form. Ginger is traditionally used in tea and as "trikatu" as a therapeutic decoction to improve the immunity, metal detoxification, digestion, and fight against the cold inflammation in Indian continent [56-60]. High doses of antioxidants have been shown to inhibit the growth of different rodent and human cancer cells *in vivo* and *in vitro* [61-65]. Dose-dependent effect of antioxidant (pro-oxidant and antioxidant theory) of the known "antioxidants" on normal and cancer cell is still not well established [66]. Nutrients such as carotenoids, tocopherol, or ascorbate derivatives show an antioxidant or pro-oxidant characteristic depending on the redox potential of the individual molecule and the inorganic chemistry of the cell [65,67,68]. We used low and high doses of extracts that relied on scavenging and TOC activity (Fig. 1). This could be due to nature and structure of the phytochemicals present in the ginger extract as compared to the amla extract. Hence, this suggests that the chemical nature and structure of the antioxidant also contributes toward the bioactivity, rather than the antioxidant property as a sole parameter. This is due to aqueous formulation or grated form of fresh ginger is not yet clear. Traditional medicine describes the use of dried ginger or whole ginger crushed decoction form for therapy. Antioxidant activity is highly correlated to total phenols concentration in the extract. The extracting solvent significantly altered the antioxidant property of the extracts [69]. We still have to evaluate, whether the lack of anticancer property of ginger is due to its extraction protocol and loss of anticancer components. There is also possibility of transformation of component during the extraction. Dried ginger and fresh ginger extracts may be maturing components that showed anticancer property. Question now raised is to use ginger aqueous extract in diet during and postchemotherapy or not. There is possibility that during extraction process of grating and boiling, ginger juice may have lost its anticancer components. However, amla seems to be better potential candidate for anticancer activity.

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AUTHOR'S CONTRIBUTIONS

1. Safeena Kulsum: Design of the work; the acquisition, analysis, and interpretation of data, drafting/revising the work.
2. Amritha Suresh: Guidance in cancer stem cell-based assays, draft correction, and final approval for publication.
3. Alka Mehta: Study design, guidance in antioxidant assays, analysis, and interpretation of data, drafting/revising the work draft preparation, final approval for publication.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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