Complementary/Alternative Medicine Strategies for Prevention and or Cure of Breast Cancer: A Review

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Abstract
This article reviews the current state of art in the field of phytochemicals and cancer. Various phytochemicals impacting cancer have been catalogued and their possible mode of action has been reviewed. In addition, synergistic action of combination of phytochemicals has been reviewed, with particular reference to our previous work using a ‘super cocktail’ of six phytochemicals in breast cancer cells.

Introduction
Breast cancer is the most common cancer in women according to the American Cancer Society. Once diagnosed, therapies include hormone antagonists, surgical removal of the tumor tissue (if well localized), chemotherapy and radiation therapy. The latter two have undesirable side effects as they are not specific enough for the tumor cells. Further, there is no therapy for cancer metastasized to other organs. In a small percentage of cancers (about 10-15%) which develop due to mutations in the tumor suppressor genes BRCA1 and BRCA2, the only available treatment is surgical removal of both breasts. Recent evidence suggests that such mutations can also lead to ovarian cancer and thus even surgical removal of both breasts may not be sufficient to completely eradicate the cause of cancer. In addition, a primary cause for recurrence is due to presence of stem cells which may evade therapy. In view of these facts, there is an urgent need for development of alternative strategies for successful treatment of breast cancer. Fortunately a number of antioxidant compounds present in plants, fruits and herbs have been shown to kill breast cancer cells without any toxic effect on normal cells. However a main problem is they are effective only at high levels, not achievable when administered orally, due to their relative insolubility in aqueous medium and they are soluble in lipids. In addition, antioxidants have both unique as well as a shared common spectrum of activity. Also they have been shown to synergize when given together and rarely, antagonize in certain combinations. In this review we will document the progress in this field of complementary and alternative medicine strategies for prevention and cure of breast cancer.

Phytochemicals that have been shown to be active against breast cancer cells

Curcumin
This is the yellow pigment present in turmeric root, used as a component of curry paste used in indian cooking. It inhibits proliferation of breast cancer cell lines by down regulation of cell cycle regulators such as cyclin D and NF-kB, and inhibits metastasis of the triple negative breast cancer cell line MDA-MB-231 [1,2]. It has also been shown to induce apoptosis of breast cancer cells by inhibiting survivin, the survival protein and modulate BRCA1 protein [3]. Excellent reviews are available on the various actions of curcumin in breast cancer [4,5].

Quercetin
This flavonoid is widely present in fruits such as apples, strawberries, vegetables such as onions and in tea [6]. It suppresses Bcl-2 protein family (anti-apoptotic) and upregulates Bax and TRAIL which cause apoptosis. These actions result in fragmentation of DNA resulting in cancer cell death [7,8].

Resveratrol
This is a polyphenol present in red wine, grape seeds and skin. It causes cell cycle arrest by acting on cyclin E and D1, up-regulates tumor suppressor genes p53, p21Cip1/WAF1, and induces Bax, the pro-apoptotic family of proteins. It also down regulates anti-apoptotic proteins Bcl-2, Bcl-XL and survival protein Survivin [9-11]. These activities inhibit cancer cell proliferation and survival. We demonstrated that Resveratrol synergizes with Indole-3-carbinol to kill ovarian cancer cells in tissue culture and this will be dealt with in detail later in this review [12].

Genestin
It is an isoflavone from soybeans. It has been shown to sensitize cancer cells to chemotherapeutic drugs. It causes expression of pro-apoptotic proteins, inactivates NF-kB, causes cell cycle arrest [13-15]. It has been shown to protect against prostate cancer, breast tumors and small cell lung cancer. Interestingly it has been shown to kill BRCA1 mutation bearing breast cancer cells at high concentrations [16].

C-Phycocyanin
This is a blue pigment present in the blue-green algae, Spirulina. It has been shown to inhibit growth of MCF7 breast cancer cells, and hepatocellular carcinoma cells [17]. We have demonstrated it to inhibit rat liver toxicity and carcinogenesis induced by dibutyl nitrosamine (DMB) precursors [18]. Thus it has potent chemopreventive effects on hepatocellular carcinoma.
In combination with selenium (which has anti-cancer activity by itself), it induces cell cycle arrest at G1 stage, inhibits cyclin D1, D3 and cyclin dependent kinases CDK4 and CDK6. Similar to resveratrol, it also increases levels of tumor suppressor p53, p21Cip1/WAF1, up-regulates expression of pro-apoptotic proteins Bax, Caspase-8, Caspase-9. It also causes cleavage of DNA repairing enzyme poly ADP ribose polymerase (PARP). These actions result in acceleration of DNA fragmentation and apoptosis. It has been shown to have anti-inflammatory activity.

We have conducted extensive in vitro and in vivo investigations on the effects of C-phycocyanin and spirulina [19]. We utilized MCF7 breast cancer cells for in vitro studies. Treatment with a spirulina extract inhibited cancer cell proliferation with no effect on normal cells. Tumor suppressors p53, p21 and proapoptotic Bax were all elevated 24 to 48 hrs after treatment. In contrast, the antiapoptotic Bcl2 was down regulated at this time. In vivo experiments were done utilizing the dimethyl benzanthracine treated rat model, which develops extensive mammary and liver tumors. Treatment with 1% spirulina extract fed in the diet starting one week before administration of DMBA 9 (7,12-Dimethylbenz[a]anthracene) reduced the mortality from tumors to 13% from 87% in the group of rats treated with DMBA only. This is a whopping 74% reduction in incidence of tumor formation. This clearly illustrates the chemopreventive effect of SP against DMBA induced rat mammary tumorigenesis. Immunohistochemical analysis revealed that SP supplementation reduced expression of both Ki-67 (tumor marker) and estrogen receptor alpha levels. Spirulina treatment induced a desquamation of the neoplastic cells leading to eradication of the tumors. A normal epithelial layer formed once the tumor was eradicated [19]. In conclusion, SP exhibited remarkable antitumor activity by inhibiting mammary tumor cell proliferation induced by DMBA in rats.

In contrast to our studies in the rat, we observed that C-Phycocyanin treatment by itself did not inhibit proliferation of human breast cancer cells (MCF-7 and MDA-MB-231) over a 6 day period, at the dose used [20]. However, it was useful as part of a super cocktail of six phytochemicals in inhibiting proliferation of the human cancer cells, by participating in a synergistic reaction with the 5 other phytochemicals. This illustrates possible differences in susceptibility of murine cancer cells as compared to human cancer cells to spirulina treatment. Clearly a combination of several phytochemicals seems to be superior to treatment with any single phytochemical for chemoprevention of breast cancer in women.

**Indole-3-Carbinol**

This powerful antioxidant is present widely in cruciferous plants such as broccoli, cabbage, cauliflower and Brussels sprouts. It has been shown to sensitize breast cancer cells to taxotrex, a chemotherapeutic drug used to treat breast cancer [21]. It also has been shown to inhibit bone metastasis of MDA-MB-231 breast cancer cells, in a mouse model [22].

**Epigallocatechin-3-gallate**

This is the active compound found in green tea. It inhibits growth of MCF7 breast cancer cells by increasing tumor suppressor protein p53, p21, as well as reducing insulin like growth factor binding protein 2. It also caused increased cell death of another breast cancer cell line, MDA MB-231 which is estrogen receptor negative. Further it has been shown to increase sensitivity to tamoxifen treatment [23]. Wang et al showed that EGCG potentiates the effect of curcumin in causing growth inhibition and inducing apoptosis of MCF 7 breast cancer cells. This was in part due to induction of caspase dependent pathways as well as increased curcumin uptake by cells due to P-glycoprotein pump function [24].The anticancer properties of this polyphenol have been reviewed by Butt et al [25].

**Piperine**

This is the compound found in black pepper. It has been shown to act in the digestive tract to increase absorption of other active compounds, thus modifies the bioavailability of several other polyphenols acting on breast cancer cells. In addition, in the human triple negative breast cancer cells such as MDA MB 231, piperine has been shown to enhance TRAIL dependent apoptosis [26]. Together with curcumin, it has been shown to inhibit mammosphere formation breast cancer stem cells [27].

**Carotenoids**

A number of carotenoids have been studied for their effects on breast cancer. Prominent among them are lycopene, beta carotene, zeaxanthin and astaxanthin. All four have been shown to inhibit proliferation of MCF 7 human breast cancer cells. The first two were most potent. They acted through blocking cell cycle progression at G2M phase and inhibiting Bcl2 antiapoptotic protein expression [28].

**Silymarin**

Silymarin is an extract from the medicinal plant Silybum marianum, and its major constituent, Silibinin, with isosiliibin, silichristin, sildianin, are flavonoids. They have traditionally been used for the treatment of liver diseases. These orally active, flavonoid agents have also been shown to exert significant anti-neoplastic effects in skin, breast, lung, colon, bladder, prostate and kidney carcinomas. The major constituent of silymarin is siliibin. It has multiple mechanisms of action during inhibition of growth and proliferation of breast cancer cells. It induced apoptosis in MCF 7 and TD47 breast cancer cells thru activation of caspase 8 pathway [29]. It inhibits global protein synthesis by targeting rapamycin signaling pathway, in a concentration dependent manner, leading to inhibition of proliferation of transformed cells [30]. Decreased Wnt/Beta catenin signaling in breast cancer cells, contributing to growth inhibition [31]. It induces cell death through reactive oxygen species dependent down regulation of notch-1/ERK/AKT signaling in human breast cancer cells [32]. Further it inhibits metastasis by multiple mechanisms such as inhibition of cell adhesion/migration molecules CD44 and beta1-integrin expression [33,34]. It shows synergistic activity with conventional chemotherapeutic agents such as Doxorubicin, and displatin in human breast carcinoma cell lines MCF7 and MDA MB 460 cell lines [35].

**Ellagic acid**

This is a compound present mainly in pomegranate fruit. Like other polyphenols described above, it has been shown to inhibit proliferation of breast cancer cells by multiple mechanisms. It modulates TGF beta signaling [36], NFkB activity and cancer cell migration [37,38]. It also inhibits angiogenesis by modulating VEGFR-2 signaling pathway and induced apoptosis of MCF7 breast cancer cells by decreasing the antiapoptotic Bcl2 and increasing the pro-apoptotic Bax [39,40].

The above list of compounds is by no means complete; it is given in order to illustrate the extensive distribution of such antioxidants in the plant kingdom. The vibrant colors of fruits, berries, many vegetables and flowers is due to a class of compounds called anthocyanins, which are also powerful antioxidants. Daily consumption of 3 servings of fruits and vegetables have been associated with a reduced risk of cancers.

**Synergistic Interactions amongst Dietary Antioxidants**

While the antioxidants are widely distributed, they are mostly
Figure 1: Effects of each of the phytochemicals alone or their combination on cell proliferation of MCF7 cells (Top panel) and the highly invasive MDA-MB-231 cell line (Bottom panel) assayed with Alamar-Blue dye. Cells were treated over a six day period and Alamar-Blue assay was performed daily. The data is expressed as percent of growth ± SEM, as compared to the negative control mesenchymal stem cells (MSCs) (100%), unless noted otherwise. Level of significance is denoted as follows: *, p <0.05; **, p<0.01; ***, p<0.001.
lipophilic and are not easily absorbed from the food. Thus their ‘bioavailability’ is low. They are effective at high concentrations in circulation, which cannot be achieved by ingestion of these purified compounds. However, they exhibit a synergistic activity when combined together. Extensive reports of synergism between two or three antioxidants in killing cancer cells have been reported. We demonstrated that Indole-3-carbinol, when combined with resveratrol is much more efficient in killing ovarian cancer cells [12]. The combination showed synergism in up-regulating tumor suppressor p21 and inhibiting cell cycle protein Rb (retinoblastoma protein). Survivin, the survival protein for cancer cells was almost completely inhibited by the combination. Similarly, quercetin combined with EGCG or 1,2,3,4,6-penta-o-galloyl-B-D-glucose (a derivative of EGCG) was much more efficacious in arresting cell cycle and causing apoptosis of human breast cancer cells [41]. A combination of curcumin with arctigenin, and EGCG increased chemopreventive effects against breast and prostate cancer cells [42]. Wang et al inferred that the low bioavailability of most flavonoids limits their application as anti-carcinogenic agents in humans and treatment with a mixture of bioactive compounds that share molecular anti-carcinogenic targets may enhance the effect on these targets at low concentrations of individual compound, thereby overcoming the limitations of reduced bioavailability. They achieved strongest effects on cell cycle arrest and apoptosis by combining all three compounds in these cell lines. The combination treatment significantly increased the ratio of Bax to Bcl-2 proteins, decreased the activation of NFκB, PI3K/Akt and Stat3 pathways and cell migration compared to individual treatment. They concluded that such results warrant in vivo studies to confirm the efficacy of this novel regimen by combining Arc and EGCG with Cur to enhance chemoprevention in both prostate and breast cancer. They concluded that a novel approach of treatment with a mixture of bioactive compounds that share molecular anti-carcinogenic targets may enhance the effect on these targets at low concentrations of individual compound, thereby overcoming the limitations of reduced bioavailability. Kakrala et al [27] demonstrated that curcumin synergizes with piperine in inhibiting mammosphere formation of breast cancer cells.

In summary, there has been reported innumerable instances of two or three different antioxidants synergizing to kill cancer cells including breast cancer cells, and molecular pathways responsible for cell cycle arrest, survival and metastasis, were shown to be augmented by such combinations. We extended this principal and hypothesized that combinations of several such bioactive compounds can be developed to a degree where the synergism results in the bioavailable levels becoming effective levels to kill 100% of cancer cells. We demonstrated this by combining 6 phyto- antioxidants at their bioavailable levels and investigating their effects on breast cancer cells [20]. We chose six phytochemicals to be included in this combination of a ‘super cocktail’: Indole-3-carbinol (from broccoli), resveratrol (from grape seeds and skin), curcumin (from turmeric root), quercetin (from apple peel), C-phycocyanin (from spirulina) and genestin (from soy beans). They were used at bioachievable levels and MCF-7 and MDA-MB-231 breast cancer cells were exposed for up to six days. This resulted in progressive inhibition of proliferation of these cells as judged by the alamar blue proliferation assay (Figure 1). This inhibition was preceded by contraction, blebbing and lifting of the treated cells from the substratum by day 2 of culture (Figure 2).

Figure 2: Effect of the phytochemical combination on MCF7 and MDA-MB-231 cell morphology. MCF7 and MDA-MB-231 cells at day 0 exhibited a smooth epithelial cell pattern with prominent nuclei. In contrast the cells treated with the 6-combination start to lose cell-cell contact and attain more rounded shape at day 1. By day 2, cells cluster together, demonstrate membrane blebbing, and start to detach from the dish (original magnification, 100X).
These changes were accompanied by apoptosis as judged by flow cytometry (Figure 3). Further it resulted in inhibition of migration of these cells in a Boyden chamber assay (Figure 4). In Western blot analysis we found that expressions of proteins involved in several pathways including control of cell cycle (CDK4, Rb), proliferation (PCNA), survival (SVV), tumor metastasis (CD44) and apoptosis (Bcl2) were all severely reduced in both MCF7 and MDA-MB-231 breast cancer cell lines. The expression of tumor suppressor gene product, p53 was interesting in these two cell lines. MCF7 has a wild type p53 which functions as a tumor suppressor and its expression was highly upregulated by treatment with super cocktail. In contrast, MDA-MB-231 has a mutated p53 which has lost its tumor suppressive function and is in fact oncogenic. Its expression was severely down-regulated by the same treatment with the super cocktail (Figure 5). Thus we demonstrated a differential effect of the super cocktail in these two cancer cell types. The knockdown of wild type p53 has been shown to cause resistance to chemotherapy [43]. The induction of Wt p53 would sensitize the breast cancer cells for chemotherapy by suppressing the transcription of breast cancer resistance protein (BCRP) via NF-kB pathway as shown previously [44]. Expression of both Wt P53 and mutated p53 are regulated by the same promoter in these two cancer cell types. Thus the observed differential effects in these two cell types by the same super cocktail treatment is most likely caused by a post-translational modification through regulation of hyper-methylation or histone de-acetylation enzymes.

Microarray analysis revealed that expression levels for Bcl-2, SVV, CD44, mutant p53, CDK4 and Rb were highly down regulated at the transcription level, after treatment with super cocktail for 24 hrs (Table 1). In addition to these observed effects, we have identified new targets for up-regulated genes by the super cocktail treatment. These were ARC (activity regulated cytoskeleton associated protein, 13.5 fold), MYLIP (myosin regulated light chain interacting protein, 18.3 fold), GADD45B (growth arrest and DNA...
Figure 4: Determination of the phytochemical effect on the invasiveness of MCF-7 and MDA-MB-231 cell lines. Cell invasiveness is demonstrated by Boyden chamber invasion assay. Images of Boyden chamber membranes (bottom panels) represent the number of invaded cells, illustrating the difference in invaded cell numbers. Note the reduction in invasion capability of MCF-7 and MDA-MB-231 cell after 6-combination treatment. All data were performed in triplicate and in three independent experiments. Black bars represent MCF-7 cells invasion, while the grey bars represent MDA-MB-231 cell invasion (student's two-tailed t-test, *P<0.05; **P<0.01, ***P<0.001).

Figures 5: Molecular mechanisms of the 6-combination inhibitory actions on cell migration, invasion and induction of cell apoptosis in MCF-7 and MDA-MB-231 cell lines. Cells were treated with the 6-combination for 48 hrs, protein lysates were collected and examined by western blot analysis as described under methods. All bands were quantified and normalized against β-Actin that was used as loading Control. Top panel: Synergistic down-regulation of cell proliferation marker PCNA and cell cycle regulators Rb, CDK4. Bottom panel: Down regulation of anti-apoptotic Bcl-2, SVV and the cell metastatic marker cell adhesion molecule CD44 (marker of cell metastasis and BC stem cell marker) in both cell lines after 48hr from cell treatment with the six phytochemicals combination. Down-regulation of the MDA-MB-231 mutant P53 and up-regulation of P53 wild type in MCF7 was interestingly analyzed.
damage-inducible-beta, 18.5 fold) and CDKN1C (cyclin dependent kinase inhibitor 1C p57, Kip2, 16.9 fold). These genes are involved in regulation of cell motility, apoptosis, cell cycle and survival. Such up-regulated genes have the potential for being used as markers to follow efficacy of therapies. Finally there was a marked decrease in expression of CD44 following super cocktail treatment. CD44 is a metastasis initiating factor and also serves as a marker for breast cancer stem cells. Further, our preliminary results (unpublished) suggest that the super cocktail could be effective in killing breast cancer cells harboring BRCA1 mutations.

### Clinical Trials with Phytochemicals

Numerous clinical trials have been conducted with each one of the above mentioned phytochemicals. The clinical trials include indications for several types of cancers, inflammatory diseases, cardiovascular disease, and diabetes, to mention a few. As this review is on breast cancer, we will limit the discussion to clinical trials in breast cancer.

Curcumin was used in phase I clinical trial in combination with docetaxel and found to be tolerated in daily doses of up to 6 gms, in 14 women [45]. The authors concluded that some improvements were observed in biological and clinical responses to the treatment in most patients. A Phase II Study of Curcumin vs Placebo for Chemotherapy-Treated Breast Cancer Patients Undergoing Radiotherapy is currently in progress at Emory University, Atlanta [46]. No final results have yet been released. There has been no systematic double blind randomized clinical trial of curcumin in breast cancer to date. Similarly a survey of pub med for use of resveratrol for clinical trial in breast cancer did not show any publications; Chow et al, in a pilot study of resveratrol (1gm/day) in 40 obese post-menopausal women showed an increase in sex hormone binding globulin [47], which implies a beneficial effect in estrogen positive breast cancer patients, since SHBG (Sex hormone-binding globulin) will sequester estrogens and make it less available to breast cancer tissue. A phase I trial of indole-3-carbinol in 17 women from a high risk breast cancer cohort revealed elevation of cytchrome P450 1A2 in 94% of women [48]. This correlated with a 66% increase in excreted 2-hydroxyestrone/16alpha-hydroxyestrone ratio in urine. These authors suggest this as a biomarker for chemoprevention of breast cancer and therefore a 400 mg daily dose of Indole 3 carbinol will elicit a maximal protective effect in preventing breast cancer. In a placebo-controlled, double blind dose-ranging study of 60 women at increased risk for breast cancer who took different doses of I3C orally [49], the urinary estrogen metabolite ratio of 2-hydroxyestrone to 16 alpha-hydroxyestrone, as determined by an ELISA assay served as the surrogate endpoint biomarker (SEB).

The results in this study suggest that I3C at a minimum effective dose schedule of 300 mg/day is a promising chemopreventive agent for breast cancer prevention. The authors concluded that a larger study to validate these results and to identify an optimal effective dose schedule of I3C for long-term breast cancer chemoprevention will be necessary. Regarding clinical trials of genistein, quercetin, silymarin, ellagic acid, c-phycocyanin and many other phytochemicals, while there is a plethora of information in tissue culture studies and animal models, no clinical trials in breast cancer patients have been reported.

### Conclusions

This review does not include entire previous work done in this area. We had to choose significant observations and highlight our own work in this area. It can be concluded from this review of work done on effects of phytochemicals on breast cancer in vitro and in vivo, that a viable alternative strategy for prevention and treatment of breast cancer is feasible. It is highly significant to note that these effects of phytochemicals were highly selective and specific to cancer cells and there was no effect on normal cells. This translates into development of treatments devoid of toxic effects. Indeed we have brought out the super cocktail as a nutritional supplement named “Breast Safeguard-Susthana” in collaboration with Protegene Corporation. It is available on the web site www.protegenicorporation.com. or www.breastsafeguard.com. There is an immediate need for clinical trials in women in order to evaluate the effectiveness of this treatment for both prevention as well as cure of this disease.

### References


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**Table 1:** Microarray data of the 6-combination targeted genes, showing a significant down-regulation at mRNA levels of anti-apoptotic proteins Bcl-2 and SVV, cancer cell migratory protein CD44, mutant Tp53, cyclin cell cycle regulatory proteins CDK4 and Rb. (Negative values refer to fold change of down-regulated genes, the positive values refer to the fold change of up-regulated genes after the 6-combination treatment).


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