ANTICANCER ACTIVITY OF PEACH AND PLUM EXTRACTS AGAINST HUMAN BREAST CANCER IN VITRO AND IN VIVO

A Dissertation

by

GIULIANA DORIS NORATTO DONGO

Submitted to the Office of Graduate Studies of Texas A&M University in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

August 2008

Major Subject: Food Science and Technology
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Approved by:

Co-Chairs of Committee, Luis Cisneros-Zevallos
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ABSTRACT

Anticancer Activity of Peach and Plum Extracts Against Human Breast Cancer *In Vitro* and *In Vivo*. (August 2008)

Giuliana Doris Noratto Dongo, B.S., Universidad Nacional Agraria La Molina, Peru; M.S., Universidad Nacional Agraria La Molina, Peru

Co-Chairs of Advisory Committee: Dr. Luis Cisneros-Zevallos Dr. David Byrne

Commercial varieties of peaches and plums contain a mixture of phenolics that may possess anticancer activity. Our objectives were to evaluate extracts from a commercial variety of yellow fleshed peach ‘Rich Lady’ (RL) and of the red fleshed plum ‘Black Splendor’ (BS) on tumor breast cells *in vitro* and *in vivo*, to elucidate the molecular mechanisms behind the cancer growth-suppression of the phenolics identified in peach and plum extracts for their chemopreventive potential and to evaluate the tumor growth-suppression *in vivo*.

The RL extract preferentially inhibited the proliferation of the estrogen-independent MDA-MB-435 breast cancer cells over the estrogen-dependent MCF-7 or the normal MCF-10A breast cells. Similarly, BS extracts, though less effective than RL extracts, showed greater effects on MDA-MB-435 cells compared to the other cell lines.

Fractionation of RL extracts into different groups of phenolic compounds allowed the identification of a fraction of phenolic acids (F1) with the major components of chlorogenic and neo-chlorogenic acid with potential in chemoprevention because of the relatively high growth-inhibition exerted on MDA-MB-435 and low toxicity exerted on MCF-10A cells. The F1 isolated from RL, and its major components, chlorogenic and neo-chlorogenic acids, triggered the extrinsic and intrinsic apoptotic pathways. The extrinsic death-receptor pathway involved the activation of caspase-8 followed by caspase-6, caspase-7, and PARP cleavage. By targeting the intrinsic pathway, the pro-apoptotic proteins cytochrome c, EndoG and AIF were released from mitochondria. The relatively higher cell-growth inhibition exerted by neo-chlorogenic acid was associated with its ability to inhibit the pro-survival Akt pathway. In contrast, F1 isolated from the
red flesh genotype BY00P6653, induced apoptosis mainly through the intrinsic mitochondrial pathway upon sustained MAPK-ERK1/2 phosphorylation.

The tumor growth-suppression of RL extracts was confirmed *in vivo*. Moreover, a dose-dependent decrease in lung metastasis was found, even at doses that showed no effect in tumor growth-suppression.

These results suggest that peach phenolics may have potential in therapy and chemoprevention of metastatic breast cancer. Specifically chlorogenic and neo-chlorogenic acids, widely distributed among food plants, may be a useful therapeutic tool for targeting multiple cell signaling pathways in the treatment and chemoprevention of metastatic breast cancer.
DEDICATION

To my beloved children Giuliana, Katherine and Gino
To my Father Gustavo Noratto (In memoriam), my mother Doris Dongo and my brothers.
    They constantly encouraged me.
ACKNOWLEDGMENTS

I would like to thank my committee co-chairs, Dr. David Byrne and Dr. Luis Cisneros-Zevallos, and my committee members, Dr. Weston Porter and Dr. Jimmy Keeton, for their guidance and support throughout the course of my graduate career. And thanks to Dr. Cisneros-Zevallos for giving me my start in the Food Science Laboratory.

I am very grateful to Dr. Porter for going out of his way, for all his support, guidance and for providing me a space in his lab and to Dr. Byrne who made this research possible through his active search for funding and collaborations.

Thanks also go to my friends and colleagues at Texas A&M. Thanks to Elizabeth Wellberg, Michelle Boggs, Brian Laffin and Rick Metz, who always gave me a helping hand. I want to extend my gratitude to Dr. Bhimu Patil, director of the Vegetable and Fruit Improvement Center (VFIC) for providing the funding for this project.

Finally, thanks to my children, they are the light of my life.
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CHAPTER I

INTRODUCTION

Breast cancer is the second leading cause of cancer-related deaths in American US women and continues to be the most diagnosed type of cancer. The American Cancer Society has estimated that there will be 182,460 new cases of invasive breast cancer and 67,770 new cases of in situ breast cancer among women in the US during 2008. Unlike the hormone receptor-positive MCF-7 breast cancer in which the use of hormone therapy has achieved a significant reduction in cancer incidence, the estrogen receptor negative MDA-MB-435, which have an inherent capacity to metastasize, are being treated with chemotherapeutics that kill cells indiscriminately.

Death, and most of the complications associated with breast cancer are due to metastasis. It has been reported that approximately 10-15% of patients with breast cancer develop distant metastasis. Because of the lack of accuracy on predicting the risk of metastasis in patients with primary tumors, most of them are being treated with adjuvant therapy to prevent or eradicate the breast tumor cells that might have already spread to distant sites by the time of diagnosis. Therefore, many women who could be cured by local treatment alone, which includes surgery and radiotherapy, are ‘over-treated’ and experience the toxic side effects of chemotherapy unnecessarily.

Cancer

The process of carcinogenesis

Cancer is a disease of deregulation of cell growth, a multistep process that takes place over a period of time due to the accumulation of mutations in a single cell, resulting in gradual phenotypic changes, from a normal to a preneoplastic cell that progress to neoplastic. Cancer occurs through the loss or mutations of genes that cause deregulation of signal transduction pathways, abnormal amplification of growth signals, and aberrant expression of genes that ultimately transform the cells into invasive cancer.

This dissertation follows the style of Journal of Medicinal Food.
The different stages in carcinogenesis are: initiation (days), promotion (5-10 years), and progression (1–5 years).\textsuperscript{4, 5} \textit{Initiation} is irreversible and includes an initial chemical or physical carcinogenic stimulus directly targeting DNA. \textit{Promotion} is usually a relatively slow and reversible process leading to accumulation of pre-malignant cells dividing abnormally. \textit{Progression} is generally irreversible and leads to the final stage of carcinogenesis with tumor growth and acquisition of invasiveness and metastatic potential.\textsuperscript{6} The progression from primary to metastatic tumor implies the acquisition of phenotypes that allow these cell lines to invade, disseminate and colonize distant sites.\textsuperscript{7}

The passage from pre-malignant to malignant cell involves activation of proto-oncogenes and/or inactivation of tumor suppressor genes, resulting in the generation of mutant cells with selective advantages.\textsuperscript{3} The six essential alterations in cell physiology that lead to malignant growth are: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), unlimited replicative potential, sustained angiogenesis, and tissue invasion and metastasis.\textsuperscript{6}

In this context, the goal of cancer preventive therapy, or "chemoprevention," is to eliminate premalignant cells or to block the progression of normal cells into cancerous cells.

\textbf{Chemoprevention}

Dr. Michael Sporn was the first who proposed the approach of chemoprevention to effectively control cancer before the complex series of genetic and epigenetic events that result in invasive and metastatic malignancy have occurred. A more modern and complete definition of chemoprevention includes the use of natural or pharmacological agents to suppress, arrest or reverse carcinogenesis, at its early stages.\textsuperscript{8} Good chemopreventive agents should interfere with one or more of the stages of carcinogenesis. The efficacy of a novel chemopreventive agent is measured following the same procedure applied to a new drug.\textsuperscript{5} It must satisfy the following requirements: (1) primary prevention in high risk healthy individuals; (2) cancer prevention in individuals that already had developed pre-malignant lesions; (3) prevention of secondary forms of cancers in patients already treated for a primary cancer.\textsuperscript{9, 10} The achievement of clinical
evidence for cancer reduction would be the final endpoint of all three aspects of chemoprevention.

Most chemoprevention experiments in animal models are performed in groups of animals that have been treated with a extremely high doses of a carcinogen, or have a genetic defect that result in a short latency period. However, in humans the latency period is long (10-20 years). Effective chemopreventive agents can work to raise the quality of millions of people by extending the latency period for the onset of cancer, often more than doubling this parameter.\textsuperscript{8}

**Targets of chemoprevention**

Cancer chemopreventive agents target specific transcription factors and signal transduction pathways abnormally altered. Deregulation of transcription factors and signal transduction pathways cause altered gene expression that leads to imbalanced proliferation, differentiation, apoptosis, and eventually to malignant transformation.\textsuperscript{3}

**Transcription factors**

Agents that target transcription factors for breast cancer treatment include the selective estrogen receptor (ER) modulators (SERMs). However, about 20–30\% of ER positive and all ER-negative breast cancers fail to be prevented by SERMs.\textsuperscript{11}

**Cell signaling pathways**

Multiple alterations in signal transduction pathways are observed during the process of mammary carcinogenesis. Signaling cascades are mediated by several families of protein kinases that control the phosphorylation process and alters enzymatic activity, resulting in regulation of gene expression, metabolism, proliferation, differentiation, and apoptosis.\textsuperscript{3}

One of the cell signaling pathways that may play an important role in breast cancer disease is the *mitogen activated protein kinases (MAPKs)*. It is upregulated in~30\% of human breast tumors; indeed, modulation of these pathways may be a promising strategy for cancer chemoprevention.\textsuperscript{12}

MAPKs are the family of kinases that transduce signals from the cell membrane to the nucleus in response to a wide range of stimuli including stress.\textsuperscript{13} The MAPK signaling pathways modulate gene expression, mitosis, proliferation, motility, metabolism, and programmed death or apoptosis. MAPKs consist of three family members: the
extracellular signal-regulated kinase (ERK), c–Jun NH2-terminal kinase (JNK); and the p38-MAPK. ERK is generally activated in response to growth stimuli whereas JNK and p38-MAPK are known to be simultaneously activated in response to a variety of cellular and environmental stresses that trigger apoptosis. Although most studies relate ERK activation to cell proliferation, others show that activation of the ERK cascade promotes contrary cellular responses that vary upon cellular context, including cell proliferation, cycle arrest, differentiation, senescence, or apoptosis.

In addition to MAPK, mutation and amplification of the PI3K/Akt cascade is frequent in breast cancer. The PI3K/Akt signaling pathway plays a critical role in breast cancer development and promotes breast cancer cell survival and resistance to treatment. The Akt (Protein Kinase B) is a major downstream effector of PI3Ks that regulates various biological processes such as cellular growth, differentiation, and survival via activating or repressing multiple factors and cofactors. The anti-apoptotic effect of Akt may be mediated through its ability to phosphorylate and inactivate Bad and other death proteins. Akt can also regulate apoptosis at a post-mitochondrial level by inhibiting activation of caspase-9 and -3 by post-translational modification of a cytosolic factor downstream of cytochrome c and before activation of caspase-9. Thus, by targeting the PI3K/Akt signaling, it would be possible to modulate some cellular responses, including cell proliferation and protection from apoptosis.

**Apoptosis**

Apoptosis is referred to as synonymous to “programmed cell death”. A mechanistic “endpoint” or “point of no return.” It occurs when there is a massive caspase activation, loss of mitochondrial transmembrane potential, complete permeabilization of the outer mitochondrial membrane or exposure of phosphatidylinerine residues that emit “eat me” signals to neighboring normal cells. Apoptosis is essential to maintain the balance between cell growth and cell death. It can be induced through the crosstalking extrinsic death receptor pathway and the intrinsic mitochondria pathway.

a. **The death receptor pathway** or Fas-mediated apoptosis may be induced by ligation of the Fas-receptors leading to the activation of caspase-8, an initiator of an apoptotic process that is followed by the activation of effectors caspase-3, caspase-7 and caspase-6 and mitochondrial damage. Caspases are synthesized in normal cells as
inactive proenzymes but they can rapidly be activated by autoproteolytic cleavage or cleavage by other caspases at specific aspartic acid residues. During apoptosis, caspases acting as upstream signal transducers are called “initiator caspases”, whereas the proteolytically activated downstream caspases are called “effector caspases”. The effector caspases act on a variety of substrates resulting in proteolysis of cellular proteins and death by apoptosis. The best characterized caspase substrate is poly-(ADP-ribose) polymerase (PARP), a nuclear protein implicated in DNA repair.

b. **The intrinsic mitochondrial pathway** is controlled by the Bcl-2 family of proteins, which are in turn regulated by several stimuli, including stress by reactive oxygen species (ROS) and cytotoxic compounds. When this pathway is activated, the pro-apoptotic proteins permeabilize the outer mitochondrial membrane resulting in the release of proteins from the intermembrane space. The Bcl-2 family members act as either pro- or anti-apoptotics. The pro-apoptotic members of the Bcl-2 family, Bax, Bak and Bid form channels or regulate preexisting channels in the mitochondrial membrane, whereas the antiapoptotic Bcl-2 and Bcl-xL have the opposite effects on membrane channel formation. Unlike Bax, which exists as a monomer either in the cytosol or loosely attached to the outer mitochondrial membrane, Bak is constitutively associated to the mitochondria. Upon induction of apoptosis, the cytosolic Bax translocates to the mitochondria, and simultaneously with this translocation, Bax oligomerizes into large complexes. Like Bax, Bak homo-oligomerizes during apoptosis. Both have a degree of functional cooperation during the channel formation and trigger the release of pro-apoptotic proteins from the mitochondria.

On the other hand, the antiapoptotic proteins Bcl-xL and Bcl-2, reside in the outer mitochondrial membrane and blocks cytochrome c release that follows the activation of Bax and Bak, thus preventing it from catalyzing caspase activation. Additionally, the crosstalk between extrinsic and intrinsic pathways occurs when caspase-8 is activated by the death receptor pathway and targets the mitochondrial pathway through the cleavage of Bid, another pro-apoptotic Bcl-2 family member.

Several proteins, including cytochrome c, Smac/DIABLO, HtrA2/Omi, endonuclease G (EndoG) and apoptotic inducing factor (AIF), normally sequestered
in the mitochondria induce or promote apoptosis once released into the cytosol.\textsuperscript{23} Cytochrome c, binds Apaf-1 (apoptosis protease-activating factor-1), which permits the oligomerization to form the apoptosome. This allows the recruitment of multiple procaspase-9 molecules to the complex, facilitating their activation. After that, caspase-9 can cleave and activate executioner caspase-3, -6 and -7 as well.\textsuperscript{32}

Endo G, and AIF represent caspase-independent apoptotic pathways initiated from the mitochondria. Once translocated from mitochondria to nuclei in response to apoptotic stimuli, EndoG cleaves chromatin DNA into nucleosomal fragments independently of the caspases.\textsuperscript{33} Likewise, AIF shuttles to the nucleus and induces peripheral chromatin condensation, large-scale fragmentation of DNA and, finally, cytotoxicity.\textsuperscript{34} An overview of the mechanisms that induce apoptosis or regulate the survival pathway is shown in Figure 1.

![Figure 1](image-url)  
**Figure 1.** Overview of molecular targets for chemoprevention.
The use of agents that potentially attack the mitochondria is appealing for cancer treatment since unlike the nuclear genome, the mitochondrial DNA is not protected by histones, which makes it more susceptible to damage, and the DNA repair capacity of mitochondria is limited. Research on natural compounds that may act as novel chemotherapeutics by targeting the mitochondria pathway and induce apoptosis on estrogen receptor negative breast cancer is intense. 

Diet and breast cancer

A diet rich in fruit and vegetables has been recommended for preventing many diseases. Epidemiological and human studies indicate that a lowered risk of cancer is related to antioxidant-rich diets. Indeed, specific dietary factors may contribute to breast cancer prevention.

Likewise, such studies indicate that regional differences in breast cancer incidence are most likely partially attributable to life long dietary habits. Incidence and mortality of breast cancer is up to 5-fold higher in Western countries than in some Asian countries. Dietary patterns in Asian countries include a diet high in n-3 polyunsaturated fatty acid and phytochemicals present in fruit and vegetables. This is consistent with evidence showing that dietary components strongly associated with breast cancer include fat and phytochemicals. Though time of intake appears to be important, and effective protection may be achieved if one is exposed to a dietary factor that lowers breast cancer risk early in life.

In general, there are many biologically plausible reasons why consumption of plant foods might slow or prevent the appearance of cancer. These include the presence in plant foods of potentially anticarcinogenic substances as carotenoids, vitamin C, vitamin E, selenium, dietary fibre (and its components), dithiolthiones, isothiocyanates, indoles, phenols, protease inhibitors, allium compounds, plant sterols, and limonene. The observations on the anticarcinogenic potential of all of these compounds have come from animal and in vitro studies. These phytochemicals have shown to be able to affect every one of the stages of the cancer process. So far, the National Cancer Institute (NCI)
has identified about 40 edible plants whose phytochemicals have potential as chemopreventive agents.\(^5\)

**Role of polyphenols in cancer**

Plant polyphenols have shown potential as natural anticarcinogenic compounds that can act in each one of the steps of the process of carcinogenesis. According to their mechanisms of action may have potential as cancer-blocking and/or cancer-suppressing agents.

*Cancer-blocking agents* act during the initiation stage protecting cellular targets by scavenging reactive oxygen species (ROS) and other oxidative species, enhancing carcinogen detoxification, modifying the carcinogen uptake and metabolism, and enhancing DNA repair. *Cancer-suppressing agents* inhibit the promotion and progression stages after the formation of pre-neoplastic cells by interfering with cell cycle regulation (cyclin dependent proteins), regulation of signal transduction pathways (MAPKs, TGF-\(\beta\) serine-threonine kinase signaling and \(\beta\)-catenin pathways),\(^45\) transcription (NF-\(\kappa\)B activation), and apoptosis (activation of pro-apoptotic genes and pro-apoptotic proteins).\(^45,46\) Plant polyphenols may target one or more of the molecular pathways in cancer cells including the regulation of the angiogenesis process. During progression, tumor angiogenesis is critically important for the growth of solid tumors and is mediated by the initiation of blood vessel formation. Since this is mediated by endothelial cells, it is easy to achieve active concentrations of antiangiogenic agents.

Furthermore, the mixture of phytochemicals, as they are naturally in foods, might be more effective in cancer chemoprevention than single compounds. This is known as “combination chemoprevention” that explains why low doses of chemopreventive agents differing in their mode of action may increase efficacy and minimize toxicity, through synergistic mechanisms.\(^5\) In addition, potential apoptotic inducers from plants have shown to induce apoptosis in neoplastic cells but not in normal cells.\(^47-49\) Therefore, the study of these compounds with respect to their abilities to induce apoptosis and the underlying molecular mechanisms may provide valuable information for their potential application in cancer therapy and chemoprevention.
Phytochemicals in stone-fruits

Commercial varieties of peaches and plums contain a mixture of phytochemicals that include phenolic acids, flavonols, anthocyanins, procyanidins and carotenoids.\textsuperscript{50, 51}

Specifically, compounds identified in peaches and plums are the hydroxycinnamates, chlorogenic acid and neo-chlorogenic acid, the flavan 3-ols catechin, and epicatechin; the flavonols quercetin 3-glucoside, 3-rutinoside, 3-galactoside, 3-xyloside and 3-rhamnoside; the anthocyanins cyanidin 3-galactoside, 3-glucoside, 3-rutinoside, and 3-acetylglucoside and procyanidin dimers and trimers.\textsuperscript{51}

Several \textit{in vivo} and \textit{in vitro} studies have shown that phenolics extracted from plants have potential in chemoprevention and chemotherapy of breast cancer.\textsuperscript{52-57} They may act as \textit{cancer blocking agents} due to their ability to scavenge free radicals, and/or \textit{cancer suppressing agents}, due to their capacity to target different signal transduction pathways and preventing tumor development by inducing tumor cell apoptosis.\textsuperscript{5}

Unlike flavan 3-ols, flavonols, anthocyanins and procyanidins, research on chemoprevention and anticancer activity of the phenolic acids chlorogenic and neo-chlorogenic acids is limited. Even more, when these phenolic acids are compared to the other groups of polyphenols, they appear to be less potent in modulating cell viability, and inducing apoptosis.\textsuperscript{36, 58, 59} However, most studies failed to assess the toxicity of the more potent polyphenols on noncancerous cells.

Chlorogenic acid and neo-chlorogenic acid

Chlorogenic and neo-chlorogenic acids are the esters formed between caffeic acid and quinic acid in position 5 or 3 respectively (Figure 2). These compounds have been subject of many health related claims that include many properties as antioxidant dietary compounds. \textit{In vitro}, they scavenge free radicals,\textsuperscript{60} increase resistance of LDL to lipid peroxidation,\textsuperscript{61} and inhibit DNA damage.\textsuperscript{62, 63} Chlorogenic acid and neo-chlorogenic acid are also present in coffee\textsuperscript{64} and some herbs widely used in traditional Chinese medicine with a wide range of biological activities.\textsuperscript{65} In addition, the role of chlorogenic acid and caffeoylquinic acid derivatives as cancer suppressing agents has been reported for several human cancer cells other than breast cancer.\textsuperscript{66} Alternatively it has been reported that chlorogenic acid could act as cancer blocking agent, protecting against carcinogen-
inducers through its capacity to up-regulate the cellular antioxidant enzymes and suppress the ROS-mediated pro-inflammatory and oncogenic pathways. Likewise, chlorogenic acid may act either by enhancing the intrinsic cellular tolerance against oxidative insults and activating survival/proliferation pathways in non-tumor cells.

![Chemical structure of caffeoylquinic acid derivatives: chlorogenic acid (A) and neo-chlorogenic acid (B).](image)

On the other hand, most reports indicate that the cancer suppressing activity of phenolic compounds is mediated by modulatory actions on intracellular signaling pathways rather than the conventional hydrogen donating properties. Therefore, a clear understanding of the mechanisms of action of these phenolic acids, either as antioxidants or modulators on cell signaling and the influence of their metabolism on these properties are very important for their evaluation as anticancer agents.

So far, the role of chlorogenic acid and caffeoylquinic acids derivatives as chemopreventive agents has been supported by evidence from in vitro studies showing the cell growth suppression and modulation of different cell signaling pathways on leukemia cells, oral tumor cells, brain tumor, and several other human tumor cells. However, each one of these studies reported different molecular mechanisms behind the cancer suppressing properties of chlorogenic acid. This suggests that the molecular targets of chlorogenic acid are particular in each cancer cell line studied.

Likewise, animal studies have shown that chlorogenic acid might provide protection against cancer-induction in the tongue, the colon, the intestine, and the
stomach,\textsuperscript{77} as well as against genotoxicity and DNA breakage in gastric mucosa.\textsuperscript{63} In this context, chlorogenic acid may be acting as antioxidant and/or repressor of metabolic activation of carcinogens rather than as a modulator of intracellular signaling pathways. In addition, the real protection \textit{in vivo} would be dependent on its bioavailability.

\textbf{Bioavailability of chlorogenic acid}

The antioxidant and anticarcinogenic properties of chlorogenic acid and caffeoylquinic acid derivatives have been well established \textit{in vitro} and animal studies. However, these phenolic acids must be absorbed through gastrointestinal tract firstly in order to achieve the chemopreventive and protective effects \textit{in vivo}. Several studies have reported the absorption kinetics of chlorogenic acid after intravenous administration,\textsuperscript{78} though the absorption kinetics through the gastrointestinal tract is rather scarce. It has been reported that after intragastric administration of chlorogenic acid in rats, a rapid absorption and quick distribution is achieved followed by a slow elimination.\textsuperscript{65} Likewise, human studies have shown that chlorogenic acid is differentially absorbed and/or metabolized, with a large inter-individual variation,\textsuperscript{79} and depends largely on its metabolism by the gut microflora.\textsuperscript{80} In addition, neo-chlorogenic acid is rapidly metabolized and/or stored in organs such as the liver.\textsuperscript{79}

Most importantly, the concentration in plasma after drinking a cup of coffee (1-3\textmu mol/L)\textsuperscript{79} is \textasciitilde 14 to 4 fold the active concentrations required to induce apoptosis in malignant cells \textit{in vitro} (0.07-0.7 \textmu mol/L).\textsuperscript{70} Therefore, it is very likely that the anticancer chemopreventive properties of chlorogenic acid can be translated in clinical trials, though it has to be confirmed by using \textit{in vivo} models before application in preclinical models.

\textbf{Mouse models in human breast cancer}

Mouse models have made an important contribution to the understanding of breast cancer progression and metastasis. The main reasons for using laboratory mouse models are: \(i\) the mouse is a mammalian organism that shares many anatomic, physiological and genetic similarities with humans; \(ii\) the mouse germ line can be easily manipulated whereby genes may be over-expressed or inactivated, even in a time or tissue-especific manner.
The mouse models commonly used to investigate breast cancer include xenografts and genetically engineered mice (GEM). The use of GEM allows the study of effects of over-expressed or down-regulated genes that may have significant role in human breast cancer, such as the loss of tumor suppressor genes and the effects of gain of function in oncogenes. In contrast, the growth of breast cancer cell lines as xenografts allows investigation in the \textit{in vivo} environment, which includes the complex tumor–stromal cell interactions that facilitate tumor formation and progression.

**Xenograft model**

Xenografts consist of an injection of cells into a live animal, usually a rodent, in order to study the actions of the cells in an \textit{in vivo} environment. The use of a xenograft model in breast cancer research allows for the examination of cells in the context of the \textit{in vivo} environment, and the modeling of complex multicellular and cell–extracellular matrix interactions, inflammation and angiogenesis that are involved in the initiation and progression of breast cancer.

The model used for tumor transplantation can be orthotopic or the conventional flank subcutaneous model (s.c.). The orthotopic transplantation refers to the delivery of cancer cells to the anatomic location or tissue from which a tumor was derived and responds to the application of “seed and soil” hypothesis. This model resembles more closely human cancers including tumor histology, vascularity, gene expression, responsiveness to chemotherapy and metastatic biology. In addition, this model provides an opportunity to study the metastatic process and many aspects of the metastatic cascade that are bypassed using experimental metastasis models.

In contrast, by using transplantable s.c. tumor models, we can evaluate the rate of primary tumor growth at subcutaneous sites. Such transplantable models are often labeled as ‘non-metastatic’. Even so, both systems will reinforce our ability to select the most appropriate therapeutic agents for recommended use in clinical studies.

**Limitations of xenograft models**

The main limitations when using a xenograft model are the use of cell lines that may not represent the most common types of breast cancer observed in the clinic and the fact that xenografts must be established in immunocompromised mice to prevent immune
rejection. The later eliminates the ability to examine the role of the immune system in tumor development and progression in xenografts.\textsuperscript{85, 86}

In addition, there have been reports that significant differences exist for angiogenesis between transplanted and autochthonous tumors, which may reduce the predictive power of xenografts to clinical tumors.\textsuperscript{87, 88} Furthermore, it has been reported that about one-third of the compounds with an \textit{in vivo} activity tested by xenograft models, had correlation with activity in some phase II clinical trials. These characteristics may potentially reduce the predictive power of xenograft models in pre-clinical testing.\textsuperscript{89} The limitations of xenograft mouse models have opened new opportunities and challenges for the application of novel genetically engineered mouse models that may be used to validate the tumor response, investigate pharmacodynamic markers of drug action, and evaluate toxicity during the development of new drugs.\textsuperscript{90}

\textbf{Application of xenograft models in breast cancer research}

Xenografts studies represent a useful preclinical tool for testing new agents, to test and validate protocols and for further exploration of the biological basis of drug responses.\textsuperscript{91}

When studying breast cancer, the xenograft model allows the use of cell lines that represent all of the sequential stages of the progression in the breast cancer disease.\textsuperscript{82} Likewise, xenograft models are often used in metastasis research, and offer excellent insights into invasion properties and progression studies.\textsuperscript{81} Metastatic capacity is an inherent feature of breast tumors, which develop in regional lymph nodes and in distant organs, including bone, lung, liver, and brain.\textsuperscript{2} Metastasis frequently arises from orthotopic tumors,\textsuperscript{82} in which implantation of cancer cells in the skin or mammary fat pad, precedes the formation of primary tumors and the subsequent formation of metastasis. These steps resemble the multiple stages involved in malignant breast cancer development in patients.\textsuperscript{84}

\textbf{Xenograft models and natural compounds}

Several studies have shown the effectiveness of plant extracts in tumor-growth suppression by using the mice xenograft model, thus showing that the \textit{in vitro} antiproliferative results can be effectively extrapolated to the \textit{in vivo} situation.\textsuperscript{52, 92-95} Furthermore, the specific phenolic compounds identified in peaches and plums\textsuperscript{51} have
been shown to inhibit tumor growth and metastasis with xenograft models. For example, cyanidin 3-glucoside, as component of an anthocyanin rich extract, was reported to inhibit tumor growth \textit{in vivo},\textsuperscript{96,97} and invasion via repression of metalloproteinases.\textsuperscript{97} Proanthocyanidins showed antitumor activity and increased the anti-tumor activity of the chemotherapeutic doxorubibin,\textsuperscript{98,99} quercetin inhibited the growth and exhibited pro-apoptotic activities in xenografted MDA-MB 435 cells into nude mice,\textsuperscript{52} and phenolic acids from propolis induced abortive mitosis and massive necrosis in malignant cells.\textsuperscript{100} The treatment with caffeic acid derivatives of C6 glioma cells grown as xenografts on nude mice induced a significant dose dependent decrease in tumor growth and reduced the number of mitotic cells.\textsuperscript{95}

Finally, all these polyphenols either as components in plant extracts or as isolated compounds have shown potential for tumor growth-suppression \textit{in vitro} and \textit{in vivo}. The use of cell lines is ideal for studies examining signal pathways and gene regulation. While the xenograft model resembles the complex interactions that are involved in breast cancer progression and is an important tool to test and validate novel treatment strategies. However, there are no reports with either \textit{in vitro} or \textit{in vivo}, regarding the breast cancer suppression properties of stone fruit extracts. Therefore the findings from this study will set the basis for chemopreventive and chemotherapeutic recommendations using peaches and plums as source of such novel anticancer compounds.
CHAPTER II

BREAST CANCER CELL GROWTH-INHIBITION OF PHENOLICS IN COMMERCIAL VARIETIES OF PEACHES AND PLUMS

Synopsis

Commercial varieties of peaches and plums have been reported to contain a mixture of phenolics that may exert anticancer activity. Many of these phenolic compounds have been shown to inhibit the proliferation of a number of cancer cell lines. However, there are differences in the amounts and distribution of these phenolics among commercial varieties. Our objective was to evaluate the cancer suppression activity of extracts from a commercial variety of yellow fleshepd peach ‘Rich Lady’ (RL) and of a red fleshepd plum ‘Black Splendor’ (BS) and identify the phenolic fractions that may possess potential as chemopreventive and/or chemotherapeutic natural compounds.

The peach RL extract effectively inhibited the proliferation of the estrogen-independent MDA-MB-435 breast cancer cell line. The concentration to inhibit 50% of cell proliferation (IC$_{50}$) was ~ 42 mg/L for this cell line compared to a IC$_{50}$ of ~130 mg/L and ~ 515 mg/L for the non cancerous breast line MCF-10A and the estrogen dependent breast cancer line MCF-7 respectively. Similarly, BS extracts showed greater effects on MDA-MB-435 cells compared to the other breast cancer or the normal breast cell lines. In general, BS extracts were less effective than RL extracts. Within all RL and BS fractions, Fraction 3 (flavonoids) and Fraction 4 (procyanidins) were the most potent against the three cell lines. The order of potency of RL fractions against MDA-MB-435 was F3 > F4 > F1 > F2.

The antiproliferative activity of pure compounds identified in F3 and F1 confirmed that quercetin 3β-glucoside is the bioactive compound in F3, with the same level of toxicity on the estrogen independent MDA-MB-435 breast cancer and on breast epithelial MCF-10A cells (IC$_{50}$ = 1.9 ± 0.2 and 1.8 ± 0.3 respectively). However, we confirmed that phenolic acids present in F1: chlorogenic and neo-chlorogenic acids have potential as chemopreventive dietary compounds because of the relatively high growth inhibition
exerted on the estrogen independent MDA-MB-435 and low toxicity exerted in the normal MCF-10A cells.

**Introduction**

Breast cancer is the second leading cause of cancer-related deaths in US women and continues to be the most diagnosed type of cancer. The American Cancer Society has estimated that there will be 182,460 new cases of invasive breast cancer and 67,770 new cases of in situ breast cancer among women in the US during 2008. Most deaths from breast cancer are the result of distant metastasis rather than primary tumor burden and chemotherapy is based on the use of non-specific cytotoxic agents that kill cells indiscriminately.

Commercial varieties of peaches and plums have been previously quantified for their antioxidant activity and level of phenolic compounds. They contain a mixture of phytochemicals that include phenolic acids, flavonols, anthocyanins, procyanidins and carotenoids. Several *in vivo* and *in vitro* studies have shown that these groups of phenolic compounds found in plant extracts have potential in chemoprevention and chemotherapy of breast cancer. The chemopreventive activity of phenolic compounds may result from their ability to scavenge free radicals, but they also inhibit the promotion stage of carcinogenesis by targeting different signal transduction pathways and may prevent tumor development by inducing tumor cell apoptosis (programmed cell death). Our goal is to study the anti-proliferative activity of phenolics present in peaches and plums in terms of high chemopreventive potential and low toxicity in normal cells and identify their bioactive compounds.

**Materials and Methods**

**Plant material**

Commercial varieties of yellow-fleshed peach ‘Rich Lady’ (RL) and of red-fleshed plum ‘Black Splendor’ (BS) grown in California, were collected at a mature firm stage and stored at 2-4°C until use (less than 5 days). Upon arrival at Texas A&M University, the fruits were frozen after removal of the stones and kept at -20°C until use.
Extraction of phenolic compounds

100 g of frozen fruit (flesh plus skin) was blended with 300 mL of methanol and left at 4°C overnight. Methanolic extracts were filtered through whatman #1 filter paper and methanol evaporated at 45°C using a rotavapor (Büchi, Switzerland). Aqueous extracts were frozen at -80°C and freeze-dried in a freeze dryer (FTS® Systems, Inc., Stone Ridge, NY) at -50 °C and at 200 mm Hg of pressure. These extracts, designated as “crude extract,” were further used for the cell culture assays by applying different doses based on total phenolic content measured spectrophotometrically by Folin-Ciocalteu colorimetric method. Crude extract was re-suspended in culture medium and sterile filtered for the cell growth proliferation assay.

Fractionation of phenolic compounds

The crude extract was fractionated into phenolic acids (F1), anthocyanins (F2), flavonols (F3) and procyanidins/polymeric anthocyanins (F4) by solid phase extraction using C18 cartridges. Briefly, the aqueous extract was adjusted to pH 7.0 with 5 N NaOH. The extract was loaded in SEP Pack C18 cartridge previously conditioned to pH 7.0 with 50 mL of 100% methanol and 50 mL of nanopure water (pH 7.0). The neutral phenolics were absorbed in the cartridge while the phenolic acids were not. The cartridge was washed with 50 mL of water (pH 7.0). The water from wash was combined with the phenolics that were not adsorbed in the cartridge and adjusted to pH 2.0. This mixture of compounds was loaded into a second cartridge previously conditioned at pH 2.0 with 50 mL 100% methanol and 50 mL nanopure water pH 2.0. Phenolic acids bound to the matrix of the second cartridge (F1) were later eluted with 50 mL 100% methanol. After adjusting the pH to 2.0 in the first cartridge, elution of anthocyanins was accomplished by passing 50 mL of 16% acetonitrile at pH 2.0 (F2). The flavonols were eluted using 50 mL 100% ethyl acetate (F3) and the anthocyanin polymers using 50 mL of 100% methanol (F4). Fractions were evaporated using a speed vac (Savant SC100) (F1, F3 and F4) or a rotavapor (F2) (Büchi, Switzerland) followed by freeze-drying (FTS® Systems, Inc., Stone Ridge, NY) at -50 °C and at 200 mm Hg of pressure and kept under nitrogen gas at –20 °C for use in cell culture.
HPLC-DAD analysis

The RL and BS crude extracts as well as their fractions were analyzed using a Waters (Milford, MA, USA) HPLC system. An AtlantisTM C$_{18}$ 5 µm, 4.6 mm x 150 mm column and a 4.6 mm x 20 mm guard column was used for the separation of phenolic compounds. The mobile phase was composed of solvent A (nanopure water adjusted at pH=2.3 with 2N HCl) and solvent B (acetonitrile HPLC grade). The elution was as follows: isocratic conditions from 0-5 min with 85% A and 15% B; gradient conditions from minute 5 to 30 starting with 85% A and ending with 0%, and starting with 15% B and ending with 100%; then, isocratic conditions from minute 30 to minute 35 with 0% A and 100% B.$^{105}$ The mobile phase was used at a flow rate of 1 mL/min and 20 µL of sample previously dissolved in MeOH and filtered through a 0.2µm PTFE filter was injected. Identification of phenolic compounds was performed by comparison of retention times and UV-visible spectral data of the chromatographic profiles with known standards.

Cell lines

Cell lines used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA). MCF-7, the estrogen-positive human breast cancer cells, were cultured using Dulbecco’s modified Eagle’s medium (DMEM) high glucose, L-glutamine, 25 nM HEPES buffer, pyridoxine hydrochloride, without sodium pyruvate and without phenol red, supplemented with 5 mL insulin (1 mg/mL), 10% (v/v) fetal bovine serum (FBS) and 1% Penicillin-Streptomycin antibiotic mix. MDA-MB-453, the estrogen-negative human breast cancer cells, were cultured using Dulbecco’s modified Eagle’s medium (DMEM) without L-glutamine, 4.5 g/L glucose and without sodium pyruvate, supplemented with 10% (v/v) Fetal Bovine Serum (FBS) and 1% Stripticin-penicillin antibiotic mix. MCF-10A, the breast epithelial cells, were cultured in Dulbecco’s modified Eagle’s medium/F12 (DME/F12) supplemented with 5% (v/v) FBS, 1% Stripticin-penicillin antibiotic mix, 1mL EGF (10µg/mL), 5 mL insulin (1 mg/mL), 250 µl hydrocortisone (1 mg/mL), and 20 µL cholera toxin. Culture media were supplied by Invitrogen (Gibco™, Invitrogen Corp., Grand Island, NY). Cells were maintained at 37 °C with a humidified 5% CO$_2$ atmosphere.
Cell proliferation

Cell growth and the concentration to inhibit the cell growth by 50% (IC\textsubscript{50}) was assessed by using an electronic coulter counter (Z1\textsuperscript{TM} Series, Beckman Coulter, Inc). Cells were seeded and incubated for 24 h to allow cell attachment before exposure to varying concentrations of extracts. A pretreatment number of cells (0-time value) was established and medium was replaced containing the crude extracts or fractions at different concentrations. Total phenolics were quantified by the Folin-Ciocalteau method\textsuperscript{103} and doses were based on their phenolic content and expressed as mg chlorogenic acid equivalent/L. Fresh medium along with compounds, was replaced every 48 h. Crude extracts, F1 and F2 were re-dissolved in culture medium and sterile-filtered before use; whereas F3 and F4 were dissolved in ethanol, all cultures contained 1 mL/L each of ethanol. Each experiment was carried out in triplicate, and results were expressed as means ± SD. The difference in number of cells between final and 0-time represents net growth.

Statistical analysis

Quantitative data represent mean values with the respective standard deviation (SD) or standard error of the mean (SE) corresponding to 3 or more replicates. Data were analyzed by one-way analysis of variance (ANOVA) using SPSS version 15.0 (SPSS Inc., Chicago, IL). Post-hoc Tukey pairwise comparisons were used (p<0.05).

Results and Discussion

HPLC-DAD analysis of phenolic compounds in Rich Lady (RL) and Black Splendor (BS) extracts and fractions

The RL and BS crude extracts and fractions (Figure 3) were analyzed by HPLC-DAD. Identification of phenolic compounds was performed at 280 nm by comparison of retention times and UV-visible spectral data with known standards (Figure 4).
Figure 3. Scheme of fractionation used for RL and BS crude extracts.

The chromatographic profile of RL extract (Figure 4A) indicated the presence of neo-chlorogenic acid (peak 1\textsuperscript{a}, RT = 4 min), cyanidin 3β-glucoside (peak 2, RT = 7.2 min) and quercetin 3β-glucoside (peak 3\textsuperscript{b}, RT = 16.2 min). Chromatograms of RL fractions showed that the main compounds in F1 were neo-chlorogenic acid (Peak 1\textsuperscript{a}, RT = 4 min) and chlorogenic acid (peak 1\textsuperscript{b}, RT = 7 min). The F2 presented cyanidin 3β-glucoside (peak 2, RT = 6.8 min). In F3, flavonols with the typical glycosylation at position 3 were found\textsuperscript{106} in between 15 and 17 min. We identified quercetin 3β-rutinoside (peak 3\textsuperscript{a}, RT = 15.9 min) and quercetin 3β-glucoside (peak 3\textsuperscript{b}, RT = 16.3 min). F4 was composed by procyanidins and traces of cyanidin 3β-glucoside (peak 2).
Figure 4. Chromatograms at 280 nm of RL (A) and BS (B) extracts and the four fractions obtained using solid phase extraction with a C$_{18}$ cartridge. (1$^a$) neo-chlorogenic acid; (1$^b$) chlorogenic acid; (1$^c$) unidentified caffeoylquinic acid; (2) cyanidin 3β-glucoside; (3$^a$) quercetin 3β-rutinoside; (3$^b$) quercetin 3ß-glucoside; (4) catechin derivative.

Chromatograms of BS extract (Figure 4B) indicated the presence of cyanidin 3β-glucoside (peak 2), the flavonols quercetin 3β-rutinoside (peak 3$^a$), quercetin 3ß-glucoside (peak 3$^b$) and based on the spectral characteristics, a catechin derivative was also found (peak 4, RT = 8 min). The BS fractions presented profiles similar to those found for RL fractions: F1 contained neo-chlorogenic acid (Peak 1$^a$), and a caffeoylquinic acid with same UV spectra of chlorogenic acid, though different retention time (peak 1c, RT = 11 min), other minor compounds with the characteristic spectra of
hydroxycinnamic acid derivatives were detected as well. The F2, F3 and F4 presented same phenolic profiles than the found in RL fractions.

In general, our findings regarding the phenolic profile of RL and BS extracts and fractions are in agreement with the previously reported for California peach and plum varieties.\textsuperscript{51} Among them, peaches contain mainly the phenolic acids: chlorogenic and neo-chlorogenic acids; the flavan-3-ols: catechin, catechin derivatives and procyanidins; the flavonols: quercetin and quercetin derivatives; and small amounts of anthocyanins: cyanidin 3β-glucoside and 3 β-rutinoside.\textsuperscript{51, 107} The red fleshed plum varieties contain the same pattern of phenolics combined with high amounts of anthocyanins.\textsuperscript{51, 102} Moreover, the study on California RL peach using HPLC-MS analysis\textsuperscript{51} confirmed the structure of main phenolics identified in our F1, F2 and F3. These phenolics have been the subject of many health-related claims supported by \textit{in vitro} and \textit{in vivo} studies and related to their properties as antioxidants and anti-carcinogenic dietary compounds.\textsuperscript{52, 68, 108, 109}

**Cell proliferation**

The yields of RL and BS extracts after methanol extraction were 5.8% ± 0.8 and 5.6% ± 0.5 (g total phenolics/100g fruit) respectively. The RL and BS extracts exerted cell growth-inhibition against both cell lines, the estrogen dependent MCF-7 and the estrogen independent MDA-MB-435. Within the range of 0-35 mg/L, RL extracts suppressed the proliferation of MDA-MB-435 cells, whereas the MCF-7 estrogen receptor positive cells were several times more resistant to RL extracts (active concentrations in the range 0-500mg/L). A similar pattern of dose and time-dependent cell growth suppression in both cell lines was found for BS extracts although the dose range was higher than the active dose range found for RL (0-70 and 0-1000 mg/L for MDA-MB-435 and MCF-7 respectively) (Figure 5).
Figure 5. Cell proliferation of MCF-7 estrogen receptor positive (A), and MDA-MB-435 estrogen-receptor negative (B) breast cancer cells. Cells were treated with different doses of extracts from RL and BS. Values are mean of three replicates ± SE. Asterisk indicates significant difference between treatments at the same incubation time (days). (*) p ≤ 0.05, (**) p ≤ 0.01.

Several studies have reported the association of fruit and vegetable consumption with reduced cancer risk. 46,110,111 Edible plants, because of their safety and the fact that they are not perceived as “medicine,” are increasingly being considered as sources of natural anticancer compounds. This is supported by evidence that many non-nutrient compounds in plant foods are effective inhibitors of human breast cancer cell proliferation in vitro. 112 The antiproliferative activity of fruit extracts as sources of dietary phytochemicals has
been previously reported for several cancer cell lines and most of the studies have attributed their enhanced antiproliferative activity to interactions of individual phytochemicals suggesting a synergistic or additive effect.\textsuperscript{113-115} However, most of these studies fail to assess the toxicity of these natural compounds in normal cells.

**Cell growth-suppressive activity of Rich Lady (RL) and Black Splendor (BS) extracts against epithelial MCF-10A breast cells**

To evaluate the potential toxicity of RL and BS extracts to non-tumor cells, we added the MCF-10A breast epithelial cells to our study (Figure 6). At 35, 70 and 140 mg/L, RL suppressed the proliferation of MCF-10A by 0\%, 30\% and 93\% respectively. The BS extracts also inhibited the growth of MCF-10A, although to a lesser degree. Within the range 55-220 mg/L, BS extracts suppressed the proliferation of MCF-10A by 5 to 28\%. MCF-10A cells were more resistant to the extract-mediated growth suppression than MDA-MB-435 cells. At concentrations that RL and BS extracts suppressed the growth of MDA-MB-435 cells by 50\% (42 mg/L and 54 mg/L respectively), RL and BS extracts inhibited the growth of MCF-10A by merely \( \sim 15\% \) and 6\% respectively, demonstrating the preferential suppression of these extracts on the growth of MDA-MB-435 breast carcinoma cells. The 50\% inhibitory concentrations (IC\textsubscript{50}s) for RL and BS extracts for the three cell lines used in our study are shown in Table 1.

**Cell growth suppressive activity of RL and BS fractions**

To identify the specific compounds with chemopreventive and chemotherapeutic potential for breast cancer treatment, the four fractions obtained by solid phase extraction were tested for their cell growth inhibition activity. The yields of F1, F2, F3 and F4 for RL were 54 ± 11, 73 ± 23, 40 ± 10, and 12 ± 6 (mg total phenolics/100 g fruit fresh weight), whereas the yields for BS were 30 ± 18, 200 ± 6, 67 ± 15 and 61 ± 11 (mg total phenolics/100 g fruit). The RL fractions exerted a dose-dependent cell growth-inhibition within different range of concentrations. The order of potency against MDA-MB-435 was F4 \( \geq \) F3 > F1 > F2 for RL fractions.
**Figure 6.** A representative evaluation of the concentration-dependent impact of methanolic extracts from the commercial variety of peach RL and plum BS on the net growth of human MDA-MB-435 breast cancer cells and the epithelial MCF-10A breast cells. Values are mean of three replicates ± SE. Asterisk indicates significance difference compared to untreated control (***) p ≤ 0.01.

**Table 1.** The concentration to inhibit the cell growth by 50% (IC\textsubscript{50}) values (mg chlorogenic acid equiv./L) of yellow-fleshed peach Rich Lady (RL) and of red-fleshed plum Black Splendor (BS) extracts for the growth suppression of estrogen dependent MCF-7, estrogen independent MDA-MB-435, and epithelial MCF-10A breast cells.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>IC\textsubscript{50} (mg chlorogenic acid equiv./L)</th>
<th>Rich Lady (RL)</th>
<th>Black Splendor (BS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>515\textsuperscript{2}</td>
<td></td>
<td>925\textsuperscript{1} ± 70</td>
</tr>
<tr>
<td>MDA-MB-435</td>
<td>42\textsuperscript{1} ± 4</td>
<td></td>
<td>54\textsuperscript{1} ± 8</td>
</tr>
<tr>
<td>MCF-10A</td>
<td>130\textsuperscript{1} ± 36</td>
<td></td>
<td>223\textsuperscript{1} ± 103</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Average of three or more independent determinations ± SD. \textsuperscript{2}Average of two determinations.

The active growth-inhibition doses for F3 and F4 were from 5 to 20 mg chlorogenic acid equiv./L, whereas F1 and F2 were less potent with active dose range from 40 to 80 mg chlorogenic acid equiv./L (Figure 7A). A similar pattern was observed for BS fractions; however, F2 (anthocyanins rich fraction) appears more potent than F1 (phenolic acid rich fraction). The estrogen independent MCF-7 cells were more resistant to RL and BS fractions with active growth-inhibition doses reached at levels as high as ~
100-150 mg/L (F3 and F4) or ~ 300-500 mg/L (F1 and F2) (data not shown). This may indicate that the chemopreventive effect of RL and BS polyphenols in breast cancer is not mediated by modulation of estrogen nuclear receptors as has been reported for phyto-oestrogens.\textsuperscript{116}

![Graph](image)

**Figure 7.** A representative evaluation of the concentration-dependent impact of RL and BS fractions on the net growth of human MDA-MB-435 breast cancer cells (A) and the epithelial MCF-10A breast cells (B). Values are mean of three replicates ± SE. Asterisk indicates significance difference compared to untreated control (*) p ≤ 0.05.
We further evaluated the chemopreventive potential of RL and BS fractions by assessing their dose-dependent cell growth inhibition on the breast epithelial MCF-10A cells (Figure 7B). We found that F3 (flavonol) and F4 (procyanidins) were the most potent in inhibiting the growth of MCF-10A within the same range of active concentrations for MDA-MB-435 (5-20mg/L). However, MCF-10A were more resistant to F1 (phenolic acids) and F2 (anthocyanins), as reflected for their higher IC$_{50}$ values (Table 2). These results suggest that phenolic acids present in F1, mainly chlorogenic and neo-chlorogenic acids$^{51}$ may have potential as chemopreventive and chemotherapeutic natural compounds against metastasis cancer since they target preferentially the MDA-MB-435 cancer cells with low toxicity to normal cells.$^5$

Table 2. IC$_{50}$ values (mg chlorogenic acid equiv./L) Rich Lady (RL) and Black Splendor (BS) fractions for the suppression of growth of estrogen dependent MCF-7, estrogen independent MDA-MB-435 and epithelial MCF-10A breast cells.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>IC 50 (mg chlorogenic acid equiv./L)</th>
<th>MCF-7</th>
<th>MDA-MB-435</th>
<th>MCF-10A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RL</td>
<td>BS</td>
<td>RL</td>
</tr>
<tr>
<td>F1</td>
<td>~210</td>
<td>~250</td>
<td>30$^1$ ± 10</td>
<td>62$^1$ ± 9</td>
</tr>
<tr>
<td>F 2</td>
<td>~600</td>
<td>~220</td>
<td>85$^1$ ± 9</td>
<td>56$^1$ ± 14</td>
</tr>
<tr>
<td>F 3</td>
<td>~110</td>
<td>~200</td>
<td>10$^1$ ± 3</td>
<td>16$^1$ ± 2</td>
</tr>
<tr>
<td>F 4</td>
<td>~140</td>
<td>~180</td>
<td>10$^1$ ± 9</td>
<td>12$^1$ ± 5</td>
</tr>
</tbody>
</table>

$^1$Average of three or more independent determinations ± SD. $^2$Average of two determinations.

Previous studies have reported the growth inhibitory effect of plant extracts against different cancer cells lines and identified the most active fraction of polyphenols. However, most of the studies have found compounds other than phenolic acids as the most active. Groups of polyphenols found to possess the highest antiproliferative activity on different cancer cell lines were the anthocyanin fraction from potato extracts,$^{117}$ anthocyanins and flavonols from blueberries,$^{118}$ and procyanidins from apples.$^{109}$ However, these studies failed to assess the toxicity of the most active fractions on non-cancerous cells. Even more, the antiproliferative activity of phenolic acids (i.e.
chlorogenic acid and caffeoylquinic acid derivatives) has been controversial and highly dependent on the cell line. These phenolic acids can enhance the intrinsic cellular tolerance against oxidative insults either by activating survival/proliferation pathways or by increasing antioxidant potential as reported for the human hepatoma cell HepG2,\textsuperscript{68} or by exerting a dose-dependent inhibition of cell proliferation as reported for sweet potato leaf extract\textsuperscript{66} on human cancer cells such as stomach cancer (Kato III), colon cancer (DLD-1), and promyelocytic leukemia cell (HL-60).

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**Cell growth suppressive activity of pure compounds present in F1 and F3**

The main compounds found in F1 and F3, the phenolic acids chlorogenic and neo-chlorogenic acids, and the flavonols quercetin 3 β-glucoside and quercetin 3β-rutinoside were obtained as pure standards from Sigma-Aldrich (St Louis, MO), and tested for their growth-suppressive activity against the estrogen dependent MCF-7, the estrogen independent MDA-MB-435 breast cancer cells and the non cancerous MCF-10A cells. The most potent inhibitor of cell proliferation was quercetin 3β-glucoside, with order of potency: MDA-MB-435-MCF-10A > MCF-7. The active dose range for cell growth-
suppression for MDA-MB-435 and MCF-10A was 2-3 mg/L, whereas the active dose range for MCF-7 was relatively high (~10-25 mg/L, data not shown). On the other hand, the chemically related quercetin 3β-rutinoside did not exert growth inhibition on any of the cell lines tested (Figure 8). In contrast, the phenolic acids chlorogenic and neo-chlorogenic acids induced growth suppression on the estrogen receptor-negative MDA-MB-435 cells with no effect on the growth of breast epithelial MCF-10A cells up to the doses tested (60 mg/L) (Figure 8). Even though, both chlorogenic and neo-chlorogenic acids have been reported to exert almost the same antioxidant activity; they differ in their growth suppressive activity against MDA-MB-435 breast cancer cells (IC\textsubscript{50} = 17 and 10 mg/L for chlorogenic acid and neo-chlorogenic acid respectively) (Table 3). This indicates a structure-activity relationship that may identify neo-chlorogenic acid as a more potent bioactive compound against metastatic cancer. The order of sensitivity of MDA-MB-435 cells to the tested compounds follow quercetin 3β-glucoside > neo-chlorogenic acid > chlorogenic acid. It is interesting to find that chlorogenic and neo-chlorogenic acids, which are widely distributed in fruits and vegetables, may have potential as chemopreventive and chemotherapeutic natural compounds, since they target preferentially the MDA-MB-435 cancer cells with no toxicity on normal cells.
Figure 8. Representative curves showing the concentration-dependent growth suppression of the phenolic acids: chlorogenic acid and neo-chlorogenic acid and the flavonols: quercetin 3β-glucoside and quercetin 3β-rutinoside on the estrogen independent MDA-MB-435 breast cancer cells (A) and the epithelial MCF-10A breast cells (B). Cells were incubated with various concentration of standards dissolved in DMSO and net growth was measured at 3 days using a cell counter. Values are mean ± SE, n=3. Asterisk indicates significance difference compared to untreated control (*) p ≤ 0.05.

Table 3. IC₅₀ values (mg/L) of pure compounds present in F1 and F3 of the yellow fleshed peach RL for the suppression of growth of MDA-MB-435 estrogen independent breast cancer cells and the normal MCF-10A breast cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC 50 (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDA-MB-435</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>17 ± 4</td>
</tr>
<tr>
<td>Neo-chlorogenic acid</td>
<td>10² ± 3</td>
</tr>
<tr>
<td>Quercetin 3β-glucoside</td>
<td>1.9² ± 0.2</td>
</tr>
<tr>
<td>Quercetin 3β-rutinoside</td>
<td>&gt; 60¹</td>
</tr>
</tbody>
</table>

¹Average of three or more independent determinations ± SD. ²Average of two determinations.
Conclusion

Peaches and plums contain a mixture of phenolic compounds that preferentially inhibit the growth of the estrogen independent MDA-MB-435 breast cancer cells over either the estrogen independent MCF-7 breast cancer cells or the breast epithelial MCF-10A cells. In general, RL extracts were more effective than BS extract in inhibiting the proliferation of the three cell lines, with order of sensitivity MDA-MB-435 > MCF-10A > MCF-7. We found that F1 from RL (phenolic acid fraction), may possess chemopreventive and chemotherapeutic potential. The identification of active compounds with chemopreventive potential may lead to the development of natural supplements that exert low or no toxicity to normal cells. The main compounds identified in F1 from RL, chlorogenic and neo-chlorogenic acids did not inhibit the growth of the non-cancerous MCF-10A cells up to the doses tested (60 mg/L, ~5-6 fold the IC$_{50}$ for MDA-MB-435). These results have important clinical implications because chlorogenic acid and neo-chlorogenic acid are widespread among food plants including vegetables and fruits and constitute an integral part of the human diet.
CHAPTER III

PHENOLIC ACIDS OF PEACH RICH LADY FRACTION F1 AND ITS ACTIVE INGREDIENTS CHLOROGENIC AND NEO-CHLOROGENIC ACIDS TARGET MULTIPLE CELL SIGNAL TRANSDUCTION PATHWAYS ON MDA-MB-435 BREAST CANCER CELLS

Synopsis

Several plant polyphenols have been reported to inhibit carcinogenesis. Based on the approach of identifying natural compounds with chemopreventive potential, we found that phenolic acids from peach extracts selectively inhibited the growth of estrogen-independent MDA-MB-435 breast cancer cells. Our objective was to elucidate the molecular mechanisms behind the anticancer activity of a phenolic acid fraction extracted from a commercial peach variety (‘Rich Lady’; RL) and its major components chlorogenic acid and neo-chlorogenic acid on MDA-MB-435. The cell signaling pathways triggered by the tested compounds were assessed with Western blots and immunofluorescence microscopy. The results showed a relatively higher potency of neo-chlorogenic acid in inhibiting cell proliferation that was associated to inhibition of the pro-survival Akt pathway. Whereas all, RL phenolic acid fraction F1, chlorogenic and neo-chlorogenic acid triggered extrinsic and intrinsic apoptotic pathways. The extrinsic death receptor pathway induced the activation of caspase-8 followed by caspase-6, caspase-7, and PARP cleavage. The intrinsic mitochondrial apoptotic pathway mediated the release of pro-apoptotic proteins cytochrome c, EndoG and AIF from the mitochondrial outer membrane. Cytochrome c in the cytosol activated caspase-9 and may later contribute to cleavage of caspase-6 and caspase-7 as well. AIF and EndoG shuttled from mitochondria to nucleus where they might have contributed, at least in part, to DNA condensation and fragmentation, and subsequent cell death. The early and sustained activation of two members of the mitogen-activated protein kinases (MAPKs), ERK1/2, and p38 triggered the up-regulation of the mitochondrial pro-apoptotic Bax, and release of cytochrome c. In addition, crosstalk between both the intrinsic and the extrinsic
apoptotic pathways was confirmed by using the broad spectrum caspase inhibitor zVAD-fmk, which down regulated the mitochondrial pro-apoptotic Bax as well.

Our results are significant because they show how phenolic acids present in F1 and widely distributed among plant extracts that constitute an integral part of the human diet may be used as therapeutic tool for targeting multiple cell signaling pathways in treatment and prevention of metastatic breast cancer.

Introduction

Breast cancer is the second leading cause of cancer death in women in the US and continues to be the most diagnosed type of cancer. The American Cancer Society has estimated that there will be 182,460 new cases of invasive breast cancer among women in the US during 2008.\(^1\)

Our previous study has shown that phenolic compounds from peaches and plums preferentially suppressed the growth of the estrogen receptor negative MDA-MB-435 metastatic breast cancer cells. The phenolic acid fraction from a commercial variety of peach ‘Rich Lady, RL’ and its major components, the caffeoylquinic acid derivatives chlorogenic acid and neo-chlorogenic acid showed chemopreventive and therapeutic potential due to the relatively high cell growth-suppression on MDA-MB-435 and low or no toxicity on the non-cancerous breast cells.

Chlorogenic and neo-chlorogenic acids constitute an integral part of the human diet; they are widespread among food plants with well established antioxidant and free radical scavenger activities. Structurally, chlorogenic acid is the ester formed between caffeic acid and quinic acid in position 5. Isomerisations of the quinic acid in position 3, (3-O-caffeoylquinic acid) constitute the neo-chlorogenic acid (Figure 9). These compounds have been subject of many health related claims that include many properties as antioxidant dietary compounds. In vitro, they scavenge free radicals,\(^{60}\) increase resistance of LDL to lipid peroxidation,\(^{61}\) and inhibit DNA damage.\(^{62,63}\) Chlorogenic acid is also a major phenolic compound in some herbs widely used in traditional Chinese medicine with wide range of biological activities.\(^{65}\) In addition, the growth suppression activity of caffeic acid, chlorogenic acid and caffeoylquinic acid derivatives in different human cancer cells other than breast cancer has been reported.\(^{66}\)
Cancer is a disease of dysregulation of cell growth. The process of carcinogenesis occurs through the loss or mutations of genes that cause deregulation of signal transduction pathways, abnormal amplification of growth signals, and aberrant expression of genes that ultimately transform the cells into invasive cancer. Unlike the hormone receptor-positive breast cancer in which the use of hormone therapy has achieved a significant reduction in cancer incidence, the estrogen receptor negative tumors are being treated with chemotherapeutics that kill cells indiscriminately. Research on natural compounds that may act as novel chemotherapeutics by targeting abnormally altered signaling pathways, decreasing the cell growth and/or inducing apoptosis of metastatic breast cancer is intense.

Apoptosis or programmed cell death is essential to maintain the balance between cell growth and cell death, it can be induced through extrinsic death receptors or through the intrinsic mitochondria pathway, in addition a crosstalk between both pathways occurs. The death receptor pathway or Fas-mediated apoptosis may be induced by ligation of the Fas-receptors, leading to activation of caspase-8, an initiator of an apoptotic process that is followed by activation of the effectors caspase-3, caspase-7 and caspase-6 and mitochondrial damage. The mitochondria pathway is controlled by the Bcl-2 family of proteins. Members of this family act as either pro- or anti-apoptotic. When this pathway is activated, the pro-apoptotic proteins form channels that permeabilize the outer mitochondrial membrane, which results in the release of proteins from the intermembrane.
space. Several proteins, including cytochrome c, Smac/DIABLO, HtrA2/Omi, EndoG and AIF, normally sequestered in the mitochondria induce or promote apoptosis once released into the cytosol.\textsuperscript{23} Several stimuli, including stress by reactive oxygen species (ROS) and cytotoxic compounds have shown to activate this pathway.\textsuperscript{23}

Furthermore, natural plant extracts and phytochemicals appear to target the mitochondrial pathway with the mitogen activated protein kinase pathway (MAPK) key to this targeting.\textsuperscript{14} MAPKs are known to be simultaneously activated in response to a variety of cellular and environmental stimuli including stresses.\textsuperscript{13,120} Chlorogenic acid has been reported to trigger p38 MAPK-dependent apoptosis in leukemic cells.\textsuperscript{70} In addition, caspase-8 activated by the death receptor pathway also targets the mitochondria pathway through cleavage of Bid, another pro-apoptotic Bcl-2 family member.\textsuperscript{23}

Several studies have identified specific cell signaling pathways targeted by natural compounds,\textsuperscript{45,121} yet there are still many unknowns. Recent studies suggest that the chemopreventive effects of phenolic acids may be ascribed to their ability to modulate components of cell signaling pathways. Yet the overall effects of these compounds and specifically, caffeoylquinic acid derivatives in such pathways are largely unknown and differ upon cell context.\textsuperscript{68,122} Therefore, there is a need to study the molecular pathways targeted by these natural compounds that make them effective against the estrogen independent breast cancer MDA-MB-435 cell line and non toxic to the non cancerous cells.

Materials and Methods

Chemicals

The Folin-Ciocalteu reagent, dimethyl sulfoxide (DMSO) and N-acetyl cysteine (NAC) were purchased from Fisher Scientific (Pittsburgh, PA). Ethanol, methanol and ethyl acetate were purchased from VWR International (Bristol, CT). Chlorogenic acid, neo-chlorogenic acid and p38 inhibitor (SB 202190) were obtained from Sigma-Aldrich (St Louis, MO). The general caspase inhibitor zVAD-fmk was purchased from BD Bioscience (San Jose, CA), MEK1/2 inhibitor (U0126), used for ERK1/2 inhibition, and antibodies for phospho-ERK, phospho-p38, cytochrome c, cleaved caspase 3, 6, 7, 8, and
9 were purchased from Cell Signaling Technology Inc. (Beverly, MA) Antibodies for phospho-c-jun, β-actin, and ERK1/2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bcl-2 and Bax were purchased from Fisher (Houston, TX). c-Jun, p38, Bim, Akt, EndoG, and AIF were purchased from eBioscience (San Diego, CA), and phospho-Akt1 was purchased from Millipore (Temecula, CA).

Plant material

The commercial variety of yellow-fleshed peach ‘Rich Lady’, (RL) grown in California, was collected at a mature firm stage and stored at 2-4°C until use (less than 5 days). Upon arrival at Texas A&M University, the fruits were frozen after removal of stones and kept at -20°C until use.

Extraction of RL phenolic acid fraction F1

100 g of frozen fruit (flesh plus skin) was blended with 300 mL of methanol and left at 4°C overnight. The methanolic extract was filtered through whatman #1 filter paper and methanol evaporated at 45°C using a rotavapor (Büchi, Switzerland). The phenolic acid fraction F1 was isolated by solid phase extraction (SPE) using C18 cartridges. Briefly, the aqueous extract was adjusted to pH 7.0 with 5 N NaOH. The extract was loaded in SEP Pack C18 cartridge previously conditioned to pH 7.0 with 50 mL of 100% methanol and 50 mL of nanopure water (pH 7.0). The neutral phenolics were absorbed in the cartridge while the phenolic acids were not. The cartridge was washed with 50 mL of water pH 7.0. The water from the wash was combined with the phenolics not adsorbed in the cartridge and adjusted to pH 2.0. This mixture was loaded into a second cartridge previously conditioned at pH 2.0 with 50 mL 100% methanol and 50 mL nanopure water pH 2.0. The phenolic acid fraction bound to the matrix of the second cartridge was later eluted with 50 mL 100% methanol. This F1 was evaporated using a speed vac (Savant SC100), and kept under nitrogen gas at -20°C. The doses used of F1 in cell culture were calculated based on its phenolic content.

Cell lines

Cell lines used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The estrogen-positive human breast cancer cells MCF-7, were
cultured using Dulbecco’s modified Eagle’s medium (DMEM) high glucose, L-glutamine, 25 mM HEPES buffer, pyridoxine hydrochloride, without sodium pyruvate and without phenol red, supplemented with 5 mL insulin (1 mg/mL), 10% (v/v) fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotic mix. The estrogen receptor-negative MDA-MB-453 human breast cancer cell line, was cultured using Dulbecco’s modified Eagle’s medium (DMEM) with L-glutamine, 4.5 g/L glucose and without sodium pyruvate and supplemented with 10% (v/v) (FBS) and 1% streptomycin-penicillin antibiotic mix. The breast epithelial cells MCF-10A, were cultured in Dulbecco’s modified Eagle’s medium/F12 (DME/F12) supplemented with 5% (v/v) fetal bovine serum (FBS), 1% streptomycin-penicillin antibiotic mix, 1mL EGF (10µg/mL), 5 mL insulin (1 mg/mL), 250 µl hydrocortisone (1 mg/mL), and 20 µL cholera toxin. Culture mediums were supplied by Invitrogen (Gibco™, Invitrogen Corp., Grand Island, NY). Cells were maintained at 37 ºC with a humidified 5% CO₂ atmosphere.

**Cell proliferation**

The concentration to inhibit the cell growth by 50% (IC₅₀) was established with an electronic cell counter (Z1™ Series, Beckman Coulter, Inc). Cells were seeded (1.5 x 10⁴ onto a 24-well plate) and incubated for 24 h to allow cell attachment before exposure to varying concentrations of extracts. A pretreatment number of cells (0-time value) was established and medium was replaced containing the F1, chlorogenic acid or neo-chlorogenic acid dissolved in DMSO. All cultures contained 1 mL/L each of solvent. Each experiment was carried out in triplicate, and the results were expressed as means ± SD. The difference in number of cells between final incubation time (72 h) and 0-time represents net growth.

**Western-blot analysis**

Cells were seeded (0.5 x 10⁶) onto a 6-well culture plate in 10% FBS/DMEM and incubated for 12 h to allow cell attachment. After 12 h of incubation in a serum-free media, cells were treated with F1, chlorogenic acid or neo-chlorogenic acid dissolved in DMSO for different times/doses. Media was discarded and cells were washed with PBS and then removed by scraping using PBS. After centrifugation, cells were lysed with non-denaturing buffer (10 mM Tris-HCl, 10 mM NaH₂PO₄, 130 mM NaCl, 1% (v/v) Triton
X-100, 10 mM sodium pyrophosphate, (pH 7.5), and 1% proteinase inhibitor cocktail (Sigma,-Aldrich) for 30 min in ice. Solid cellular debris was removed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant was collected and stored at -80°C. Protein content was determined using the Bradford assay. For each lane sixty g of protein was diluted with Laemmli’s loading buffer, boiled, and loaded on an acrylamide gel (10%, or 15%) and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis at 100 V for ~2 h. Proteins were transferred by wet blotting onto 0.2 µm PVDF membrane (Bio-Rad, Hercules, CA). Membranes were blocked using 5% milk in TPBS for 30 min and incubated with primary antibody either in fresh 5% milk or 3% bovine serum albumin in TPBS overnight at 4°C with gentle shaking and incubated with the secondary antibody (1:5000) in 5% milk TPBS for 2 h. The concentrations of the primary antibodies ranged from 1:250 to 1:1000. Reactive bands were visualized with an enhanced chemiluminescence (ECL) Plus system (Amersham Corp., Arlington Heights, IL) after 5 min of incubation.

**Immunofluorescence analysis and phase contrast microscopy**

Cell seeded on coverslips (0.5 x 10⁶) onto a 6-well culture plate were incubated for 12 h to allow cell attachment. After 12 h of incubation in serum-free media, cells were treated with different doses of F1, chlorogenic acid or neo-chlorogenic acid (10 mg/L) dissolved in DMSO for 24 h. Monolayers were prepared for immunofluorescence analysis by washing them twice with PBS, followed by fixation with 3.8% paraformaldehyde, and permeabilization in 0.5% Triton X-100. Blocking was performed for 2 hr in PBS containing 5% BSA. Then, primary antibodies (EndoG or AIF) (1:250 dilution) were applied for 2 h in blocking solution. All subsequent steps were performed in the dark. The secondary antibody (Alexa FluorG488 goat anti-rabbit IgG, Invitrogen (Carlsbad, Ca.) (1:1000 dilution), was applied in the same way as the primary for 45 min. After rinsing with PBS, DAPI (4',6-diamidino-2-phenylindole solution, Invitrogen, Molecular Probes (Carlsbad, Ca.) (5µg/mL) was applied for 5 min. Mounting of the coverslip was performed with Prolong Gold Antifade reagent, Invitrogen (Carlsbad, Ca.) according to the manufacturer’s instructions. All microscopic images were taken with a
Zeiss Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) fitted with an Axiocam high resolution digital camera and Axiovision 4.1 software.

**Statistical analysis**

Quantitative data represent mean values with the respective standard deviation or standard error of the mean corresponding to 3 or more replicates. Data were analyzed by one-way analysis of variance (ANOVA) using SPSS version 15.0 (SPSS Inc., Chicago, IL). Post-hoc Tukey pairwise comparisons were used (p<0.05).

**Results and Discussion**

**Cell proliferation**

The net growth of the estrogen independent MDA-MB-435 breast cancer cells was preferentially inhibited by F1, chlorogenic and neo-chlorogenic acid over the estrogen independent MCF-7 or the epithelial MCF-10A breast cells. The order of potency against the three cell lines was MDA-MB-435 > MCF-10A > MCF-7 (Table 4).

The major active ingredients in F1 seem to be chlorogenic acid and neo-chlorogenic acid due to their greater differential effect on cell growth-suppression of the MDA-MB-435 cell line versus the other breast cell lines as compared to the F1 differential effect. Emerging literature reports that plant phytochemicals may induce apoptosis in neoplastic cells but not in normal cells. The study of molecular pathways targeted by these compounds would provide valuable information for their potential application in cancer therapy and chemoprevention.

**Table 4. Concentrations of phenolic acids from yellow-fleshed Rich Lady (F1) and chlorogenic and neo-chlorogenic acids to cause 50% cell-growth inhibition (IC<sub>50</sub>).**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>IC 50 (mg/L)</th>
<th>MCF-7</th>
<th>MDA-MB-435</th>
<th>MCF-10A</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>?210&lt;sup&gt;2&lt;/sup&gt;</td>
<td>30&lt;sup&gt;1&lt;/sup&gt; ± 10</td>
<td>43&lt;sup&gt;1&lt;/sup&gt; ± 8</td>
<td></td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>&gt; 60&lt;sup&gt;2&lt;/sup&gt;</td>
<td>17&lt;sup&gt;1&lt;/sup&gt; ± 4</td>
<td>&gt; 60&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Neo-chlorogenic</td>
<td>&gt; 60&lt;sup&gt;2&lt;/sup&gt;</td>
<td>10&lt;sup&gt;1&lt;/sup&gt; ± 3</td>
<td>&gt; 60&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>Average of three or more independent determinations ± SD. <sup>2</sup>Average of two determinations.
Molecular mechanisms behind the anti-proliferative activity of F1, chlorogenic acid and neo-chlorogenic acid

The process of carcinogenesis results from several cell signaling pathways inappropriately activated or silenced. Recent studies suggest that the chemopreventive effects of phenolic acids may be ascribed to their ability to modulate the components of cell signaling pathways.

The role of RL (F1), chlorogenic and neo-chlorogenic acids on the pro-survival Akt pathway

Akt, also referred to as PKB (Protein Kinase B) or Rac, plays a critical role in controlling survival and apoptosis. We found that treatment with F1, chlorogenic and neo-chlorogenic acids exerted different responses on the Akt pro-survival pathway (Figure 10).

Our results indicate that only neo-chlorogenic acid downregulates the Akt phosphorylation; this is consistent with the increase of basal levels of Akt, whereas chlorogenic acid does not target this pathway. This might explain the relatively higher potency in dose-dependent cell growth inhibition exerted by neo-chlorogenic acid. Akt is
a major downstream effector of PI3K (phosphatidylinositol-3 kinase) that regulates various biological processes such as cellular growth, differentiation, and survival via activating or repressing multiple factors and cofactors.\textsuperscript{3} Mutation and amplification of Akt are frequent in breast cancer.\textsuperscript{16} Akt has antiapoptotic effect by phosphorylating and inactivating several pro-apoptotic targets, including Bad\textsuperscript{124} and caspase-9.\textsuperscript{20}

In addition, Akt stimulates cellular proliferation by regulating cell cycle control proteins,\textsuperscript{125} as well as p38.\textsuperscript{126} However, we did not find changes on the cell cycle kinetics upon treatment with either F1, chlorogenic acid or neo-chlorogenic acid (data not shown).

On the other hand, neither treatment with F1 or chlorogenic acid downregulated the phospho-Akt, in contrast, F1 seems to exert the opposite effect (Figure 10). This highlights the significance of evaluating food extracts instead of pure compounds. The overall effects of such compounds on cell responses are the result of interactions of bioactive ingredients as they are found in the food matrix.

**Role of RL (F1), chlorogenic and neo-chlorogenic acids on the pro-apoptotic pathways**

A mechanistic “endpoint” or “point of no return” occurs in apoptosis or programmed cell death when there is a massive caspase activation, loss of mitochondrial transmembrane potential, complete permeabilization of the outer mitochondrial membrane or exposure of phosphatidylserine residues that emit “eat me” signals to neighboring cells.\textsuperscript{22}

Apoptosis might occur by activation of caspase dependent pathway or alternative pathways that may not involve caspase activation. Depending on the origin of the death stimulus, apoptosis is also classified as being either extrinsic or intrinsic.

**Role of F1, chlorogenic and neo-chlorogenic acids on the extrinsic apoptotic death receptor pathway**

Death ligands have been explored as potential therapeutics in cancer therapy and we found that F1, chlorogenic and neo-chlorogenic acids activate caspase-8, which induces apoptosis through the extrinsic pathway (Figure 11).

The extrinsic cell death pathway is initiated upon ligand-receptor interactions at the cell surface including FAS ligand-FAS/APO1, TNF-TNF receptors, and TRAIL-TRAIL receptors. Upon ligation, the Fas receptor allows the dimerization of caspase-8 monomers, which is a fundamental activation event in the caspase cascade.\textsuperscript{127} Caspases
are the family of proteases that participate in a cascade that is triggered in response to proapoptotic signals and culminates in the cleavage of a set of proteins, and disassembly of the cell.\textsuperscript{128}

**Figure 11. Caspase activation by F1, chlorogenic and neo-chlorogenic acids.** Cells were treated with F1 (30 mg chlorogenic acid equiv./L) (A), chlorogenic acid (17 mg/L) (B), or neo-chlorogenic acid (10 mg/L) (C). These doses were established based on the IC\textsubscript{50} values, control cells (c), cells were cultured with serum free medium for 36 h. Cell lysates were assessed by western blotting using polyclonal and monoclonal antibodies. Total β-actin was detected to show the relatively same amount of protein load. The data represent a typical experiment conducted three times with similar results.

The initiators caspase-8 and caspase-9 were activated by the tested compounds at \textasciitilde{} 0.5 h after treatment. This was consistent with the activation of the downstream executioner caspase-7 and caspase-6. Although most studies indicate that caspase-3 is also activated by the initiator caspases,\textsuperscript{127} we did not find caspase-3 activation by any of the compounds used in our study (data not shown). In contrast, the executioner caspase-6, which has not been extensively studied, seems to play an important role as indicated by its early activation. Furthermore, we speculate that chlorogenic and neo-chlorogenic acids work synergistically to cause the stronger caspase-6 activation seen in the F1-treated cells. A similar pattern for caspase activation has been reported for the chemotherapeutic cisplastin, in which caspase-7 and caspase-6, but not caspase-3 were found to be transcriptional targets of p53 cisplastin-induced expression.\textsuperscript{129}
Caspase-6 and -7 participate in apoptosis as executioner caspases responsible for cellular changes that occur during a programmed cell death. They reorganize the cytoskeleton, shut down DNA replication and repair, interrupt splicing, destroy DNA, disrupt the nuclear structure, induce the cell to display signals that mark it for phagocytosis, and disintegrate the cell into apoptotic bodies.\textsuperscript{128}

We found an increase on the Poly (ADP-ribose) polymerase 1 (PARP) fragment (89kDa), consistent with the time response for caspase activation. PARP is specifically proteolyzed by caspases,\textsuperscript{130} and upon cleavage into its 24kDa and 89kDa fragments, it promotes apoptosis by preventing DNA repair-induced survival.\textsuperscript{131} Alternatively PARP is linked to the intrinsic apoptotic pathway.\textsuperscript{132} When activated by reactive oxygen species (ROS), PARP initiates a nuclear signal that propagates to mitochondria and triggers the release of apoptotic inducing factor (AIF), which in turn translocates to the nucleus and induces peripheral chromatin condensation, large-scale fragmentation of DNA and cell death.\textsuperscript{34}

In addition, the extrinsic pathway targets the intrinsic apoptotic pathway through caspase-8 activation. Caspase-8, as initiator can promote the release of cytochrome c from the mitochondria, which together with Apaf-1 activates caspase-9. This is mediated by its ability to cleave the pro-apoptotic Bcl-2 family member Bid.\textsuperscript{133} Either caspase-8 or caspase-9 can trigger the cleavage and activation of downstream executioner caspase-3, -7 and -6.\textsuperscript{127, 133, 134}

**Role of F1, chlorogenic and neo-chlorogenic acids on the intrinsic apoptotic mitochondrial pathway**

Apoptosis through this pathway is regulated by the Bcl-2 family of proteins. They have the capacity to regulate the permeability of mitochondrial membranes to ions and proteins.\textsuperscript{26} Proapoptotic members of the Bcl-2 family, especially Bax have the ability to form channels and to regulate preexisting channels, whereas antiapoptotic members of this family have the opposite effect.\textsuperscript{26}

We found that F1, chlorogenic and neo-chlorogenic acids trigger apoptosis through modulation of Bcl-2 family of proteins that activated the pro-apoptotic Bax with no changes on levels of antiapoptotic Bcl-2, thus the ratio Bax/Bcl-2 favors apoptosis (Figure 9). However, the time-response for Bax activation differs among the tested
compounds. Neo-chlorogenic acid and F1, exerted an early activation of Bax and cytochrome c release. This may explain the higher potency of neo-chlorogenic acid, therefore the activation of Bax and cytochrome c release time-response exerted by F1 may result mainly due to neo-chlorogenic acid induction, since both showed similar time-response (Figure 12).

Several studies have demonstrated the key role of Bax in counteracting the death repressor activity of Bcl-2 and how the ratio of Bcl-2 to Bax determines survival or death.135

Likewise, the activation of Bim, which interacts with diverse antiapoptotic Bcl-2 family members and promotes apoptosis, seems to be induced by the chlorogenic acid component in F1 (Figure 12). Bim has been related to death stimuli activation and acts as a 'death ligand' which can neutralize certain members of the pro-survival Bcl-2 sub-family.136 The proapoptotic Bim mediates the induction of apoptosis following cytoskeletal perturbations caused by the disturbance on cell adhesion, intracellular transport, or stimulation by growth factors.137, 138 Once dissociated from the cytoskeleton, Bim translocates to the mitochondria where it binds to Bcl-2 and Bcl-xL and sequesters them from proapoptotic proteins such as Bax and Bak. The chemical structure of chlorogenic acid, could represent a specific microtubule-targeting compound for potential application in metastatic cancer, where cell motility and adhesion are critical.138

Unlike chlorogenic and neo-chlorogenic acid treated cells, the anti-apoptotic Bcl-2 in F1-treated cells varied through time with up- and down-regulations. Such pattern may be the result of differences between chlorogenic and neo-chlorogenic acids in the time-response and strength of activation of pro-apoptotic Bcl-2 family members. In general, our results show the up-regulation of proapoptotic Bcl-2 family of proteins and the subsequent efflux of cytochrome c from mitochondria (Figure 12).
Figure 12. Treatment of MDA-MB-435 cells with RL (F1), chlorogenic acid and neo-chlorogenic acid activated pro-apoptotic Bcl-2 family members and induced cytochrome c release from mitochondria. Cells were treated with F1 (30 mg chlorogenic acid equiv./L) (A), chlorogenic acid (17 mg/L) (B), or neo-chlorogenic acid (10 mg/L) (C). These doses were established based on the IC$_{50}$ values, control cells (c), cells were cultured with serum free medium for 36 h. Cell lysates were assessed by western blotting using polyclonal and monoclonal antibodies. Total β-actin was detected to show the relatively same amount of protein load. The data represent a typical experiment conducted three times with similar results.

This intrinsic apoptotic pathway has been shown to be activated by natural compounds in response to a wide range of stimuli including stress that target the upstream activation of mitogen activated protein kinase (MAPK) pathway.\textsuperscript{13, 14, 139} Likewise, several studies have shown the role of MAPKs on the modulation of Bcl-2 family of proteins that control the mitochondrial mediated apoptotic pathway.\textsuperscript{140,142}

\textbf{Role of F1, chlorogenic and neo-chlorogenic acids on the MAPK pathway}

The mitogen-activated protein kinases (MAPKs) pathway is frequently altered in a variety of human cancers and modulation of this pathway by dietary compounds may provide novel strategies for prevention and treatment of cancer.\textsuperscript{143}

MAPKs are the family of kinases that transduce signals from the cell membrane to the nucleus in response to stress, growth factors, cytokines, and other extracellular stimulus.\textsuperscript{13}

We found that F1 and its active compounds chlorogenic and neo-chlorogenic acids target the MAPK pathway by activating the ERK1/2 and p38, but not the JNK (data not shown) in a time and dose dependent manner, though the pattern of activation for ERK1/2 and p38 varied (Figure 13).
Figure 13. RL phenolic acid fraction F1, chlorogenic acid and neo-chlorogenic acid activate MEK/ERK signaling pathway. (Top panel) MDA-MB-435 cells were treated for 0.25, 0.30, 1, 2 6, 12, 24 and 36 h, with F1 (30 mg chlorogenic acid equiv./L) (A), chlorogenic acid (17 mg/L) (B), or neo-chlorogenic acid (10 mg/L) (C). These doses were established based on the IC_{50} values, control cells (C), cells were cultured with serum free medium for 36 h. (Lower panel) MDA-MB-435 cells were treated with different doses of F1 (D), chlorogenic acid (E), and neo-chlorogenic acid (F). All treatments received the same volume of vehicle (DMSO). Positive control (+C) cells received medium containing 10% FBS 6h before harvesting. Cell lysates were assessed by western blotting using polyclonal and monoclonal antibodies. Total β-actin was detected to show the same amount of protein load. The data represent a typical experiment conducted three times with similar results.

The ERK1/2 was phosphorylated by F1, chlorogenic and neo-chlorogenic acids in a similar time-response. A strong and early activation within 1h of treatment was followed by a decrease at 2h and the up-regulation cycle was repeated at ~6h, ~24h, and ~36h. In contrast, the ERK1/2 level of protein differs among the tested compounds, F1 and chlorogenic acid treatments did not alter the basal levels of ERK1/2, whereas in cells treated with neo-chlorogenic acid, ERK1/2 decreased in harmony to the upregulation of phospho-ERK1/2 after 12h. This suggests that neo-chlorogenic acid may exert a stronger ERK1/2 activation, compared to F1 and chlorogenic acid. The ERK1/2 is a well-characterized MAPK family member generally activated in response to growth stimuli. However, the ERK1/2 pro-proliferation effect is likely to occur upon a short duration of
the upstream MEK1/2-MAPK cascade as exerted by growth factors. In contrast, an early and sustained activation of MEK1/2-MAPK has shown to trigger cellular apoptosis. In addition, it has been reported that activation of ERK1/2 pathway may commit the cell to apoptosis when it happens before the activation of p38 and JNK. Previous studies have reported the role of ERK1/2 activation in the anti-proliferative and pro-apoptotic effects of dietary phytochemicals. Oxidants may act usurping growth factor receptor signaling. Studies have shown that many antioxidants can also exhibit prooxidant behavior under certain conditions, thus the phenolic acids used in our study would be acting as pro-oxidants.

Unlike ERK1/2, whose role in the MAPK signaling pathway is controversial, p38 is known to be simultaneously activated in response to a variety of cellular and environmental stresses that triggers to apoptosis. We found p38 MAPK signaling was activated upon treatment with F1, chlorogenic or neo-chlorogenic acid. Although basal levels of p38 increased upon treatment, the time-response and strength varied among the compounds tested. The F1 exerted an increase on p38 levels at~1h of treatment and the activation by phosphorylation occurred relatively late, after~12h. Chlorogenic acid and neo-chlorogenic acid on the other hand, exerted an early expression of p38, at ~0.25h, and it was consistent to the prolonged p38 activation by phosphorylation up to 36h. This may indicate that both of them triggered p38 activation at such magnitude that the basal levels of p38 decreased after~2-6h. On the other hand, even though chlorogenic and neo-chlorogenic acid have been identified as the active ingredients in F1, the effect of both phenolic acids in F1 or the presence of other phenolic acids in this fraction may be antagonist for the activation of p38.

We also found that c-Jun was phosphorylated upon treatment with F1, chlorogenic or neo-chlorogenic acids in a time and dose-dependent manner. The role of c-Jun has been reported to be critical in the induction of apoptosis on MDA-MB-435 cells as a downstream component of three interrelated apoptotic triggering pathways (involving c-Jun amino-terminal kinase (JNK), TGF-β, and Fas). Additionally, the c-Jun phosphorylation and activation may play an important role in induction of apoptosis on multidrug resistance tumor cells. The sustained ERK1/2 activation was found to be upstream of c-Jun phosphorylation (Figure 14). This is in agreement with previous
studies showing the same response of ERK sustained activation on the activation of AP-1 (activating protein-1), consisting of either homo or heterodimers of the Jun and Fos families. The modulation of AP-1 has been proposed as a promising strategy to prevent cancer due its target genes that may control growth, apoptosis, oncogene-induced transformation, and invasiveness.

**Role of F1, chlorogenic acid and neo-chlorogenic acid on the crosstalk extrinsic-intrinsic apoptotic pathways**

We assessed the nuclear relocalization of AIF and EndoG in cells treated with different doses of F1, chlorogenic acid and neo-chlorogenic acid by immunofluorescence microscopy (Figure 15). By using doses equivalent to the IC50 value, this means under similar potency on cell growth-inhibition, we were able to compare the extent of activation of this pathway.

As discussed previously, mitochondrial outer-membrane permeabilization plays a critical role in apoptosis induction mediated by the release of pro-apoptotic proteins cytochrome c, Smac/Diablo and HtrA2/Omi, endonuclease G (EndoG) and apoptotic inducing factor (AIF). However the EndoG and AIF nuclear translocation have been defined as a “caspase-dependent” mitochondria-initiated apoptotic pathway, conserved between mammals and nematodes. This was shown when the broad spectrum caspase inhibitor zVAD-fmk prevented the release of EndoG and AIF but did not affect the efflux of the other mitochondrial pro-apoptotic proteins when used together with pro-apoptotic Bax/Bak drugs.
Figure 14. Inhibition of MEK1/2, the upstream activator of ERK1/2 blocks ERK1/2 activation and decreases c-Jun phosphorylation. MDA-MB-435 cells were treated with 30mg chlorogenic acid equiv./L of RL phenolic acids fraction F1 (A), 17 mg/L of chlorogenic acid (B) or 10 mg/L of neo-chlorogenic acid (C) for 0.5, 1, 2, 6, 12, 24 and 36h, 10µM of U0126 was used for MEK1/2 inhibition, control cells (C) were incubated for 36h with same volume of DMSO as treated cells. Cell lysates were assessed by western blotting. Total β-actin was determined to show the same of protein load for all treatments.
Figure 15. Phenolic acids fraction F1 (A), chlorogenic acid (B) and neo-chlorogenic acid (C) target apoptosis through nuclear translocation of AIF and EndoG from mitochondria. The nucleus is stained blue with DAPI that binds to DNA and AIF and EndoG were tagged with green fluorescent secondary antibody. The images were captured using an Axiocam high152 resolution digital camera. The green brightness provides an estimated level of AIF or EndoG in nucleus. These experiments were repeated for 3 times and the same observations were obtained.
Our results indicate that AIF and EndoG play a critical role on the pro-apoptotic effect of F1, as shown for the dose-response increasing signal of both proteins in the nucleus. In contrast, chlorogenic and neo-chlorogenic acids seem to trigger apoptosis through nuclear translocation of AIF and EndoG to a lesser extent. As discussed previously, it is possible that the release of AIF and EndoG may be dependent on caspase activation as well.\textsuperscript{156}

So far, we have identified key components of the apoptotic machinery that are being triggered by RL phenolic acid fraction F1 and its active ingredients chlorogenic acid and neo-chlorogenic acid. Additionally, evidence of a crosstalk among the activated intracellular pathways has been documented. In general, such evidence includes but is not limited to:\textit{i}) The activation of pro-caspase-9 was found to be regulated by Akt-phosphorylation.\textsuperscript{124}\textit{ii}) Caspase-7 activation was shown to be crucial on the mitochondrial events of apoptosis including AIF and cytochrome c release.\textsuperscript{157}\textit{iii}) Fas activation of p38 MAPK correlated with the onset of apoptosis.\textsuperscript{158}\textit{iv}) Fas-induced apoptosis stimulates the downstream effectors of receptor tyrosine kinases such as ERK1 levels and ERK1 phosphorylation.\textsuperscript{159}

Therefore, we used specific inhibitors to block activation of ERK1/2 (U0126), p38 (SB 202190) or the caspase cascade (zVAD-fmk) in order to evaluate the degree of contribution of each one of the activated cell signaling pathways on the activation of the intrinsic mitochondrial pathway. Bax was used as a marker for mitochondrial permeabilization targeted pathway (Figure 16).

The activation of p38, ERK1/2, and caspase exerts varying effects on the activation of Bax activation. In F1 treated cells, Bax activation seems to be uniformly down-regulated when ERK1/2 and p38 activation is inhibited, whereas cleavage of caspase does not contribute to Bax activation. In chlorogenic acid treated cells, ERK1/2 and caspase activation contribute to Bax activation. In contrast, inhibition of p38 phosphorylation did not affect the level of activated Bax. Finally, in neo-chlorogenic acid treated cells p38 and caspase inhibition in descending targeted the Bax down-regulation, in contrast to the inhibition of ERK1/2, which did not affect the extent of Bax activation.
Figure 16. Bax activation on MDA-MB-435 cells upon activation of different cell signaling pathways. Cells were treated with RL, F1 (30mg chlorogenic acid equiv./L (A), chlorogenic acid (17mg/L) (B) or neo-chlorogenic acid (10mg/L) (C) with and without inhibitors for ERK1/2 phosphorylation (U0126), p38 phosphorylation (SB 202190) or caspase activation (zVAD-fmk) and blocked with anti-Bax. Tested compounds were dissolved in DMSO.

The antiproliferative and pro-apoptotic effect exerted by these phenolic acids on MDA-MB-435 breast cancer cells are likely to be the sum of several cell signal transduction pathways. The proposed model that explains the elucidated cell signaling pathways being targeted by RL phenolic acid fraction F1, chlorogenic and neo-chlorogenic acids is shown in Figure 17. We hypothesize that these compounds are modulating cellular responses to stress and a crosstalk among the activated pathways may contribute to maximize effectiveness and minimize toxicity on non-cancerous cells.
Figure 17. Molecular pathways targeted by RL phenolic acid fraction F1, chlorogenic acid and neo-chlorogenic acid on MDA-MB-435 metastatic breast cancer cells.

Conclusion

Selected phenolic acid fraction F1 from yellow-fleshed peach variety Rich Lady (RL) and its active ingredients chlorogenic and neo-chlorogenic acids target several cell signaling pathways that decrease cell proliferation and induce apoptosis on the MDA-MB-435 estrogen receptor-negative breast cancer cells.

Our study on cell growth-inhibition and molecular mechanisms showed neo-chlorogenic acid as the most potent among the tested compounds. The higher growth-inhibition activity exerted by neo-chlorogenic acid may be related to its unique ability to downregulate the Akt cell survival pathway. However, this effect was masked in F1, probably due antagonist interactions among its components on cell membrane receptors upstream of this pro-survival pathway.
Induction of apoptosis exerted by the tested compounds involved activation of extrinsic and intrinsic pro-apoptotic pathways. The extrinsic pathway was activated by targeting the death receptor pathway, which activates the initiator caspase-8 and the downstream effectors caspase-6 and -7. The intrinsic mitochondrial apoptotic pathway was triggered by activation of the pro-apoptotic Bax, and increasing the ratio Bax/Bcl-2 in favor of outer mitochondrial membrane permeabilization. Although neo-chlorogenic acid was more potent in cell growth-suppression, it failed to activate Bim, another pro-apoptotic Bcl-2 family member. Both the caspase activation and MAPK activation mediated by ERK1/2 and p38 phosphorylation, were shown to play important roles on activation of pro-apoptotic Bax, except on chlorogenic acid treated cells, where p38 phosphorylation did not contribute to Bax activation. Mitochondria permeabilization resulted in the release of the pro-apoptotic proteins AIF, EndoG and cytochrome c. In the cytosol, cytochrome c promotes the formation of the apoptosome, and activates caspase-9, whereas AIF and EndoG shuttle to the nucleus and induces DNA fragmentation and cell death.
CHAPTER IV

APOPTOSIS INDUCTION OF PHENOLIC ACID FRACTION FROM RED-FLESH PEACH BY00P6653 ON MDA-MB-435 CELLS IS MEDIATED BY MITOCHONDRIAL PATHWAY THROUGH MAPK-ERK1/2 ACTIVATION

Synopsis

Phenolic compounds in fruits and vegetables have shown to possess chemopreventive and therapeutic activities by modulating cell signaling pathways and inducing apoptosis on malignant cells. This study investigated the molecular mechanisms involved in anticancer activity of a phenolic acid rich fraction (F1) extracted from the BY00P6653 red-flesh peach genotype on the estrogen receptor negative MDA-MB-435 breast cancer cells. The main phenolics identified in F1 by HPLC were chlorogenic acid, a chlorogenic acid derivative and traces of cyanidin 3-β-glucoside. F1 caused a dose-dependent decrease of cell viability (IC\text{50} = 150 mg chlorogenic acid equiv./L) and activated the mitogen-activated protein kinase (MAPK) pathway through ERK1/2 sustained phosphorylation. Our studies using the MEK1/2 inhibitor (an upstream kinase in ERK1/2 signaling pathway) showed that the intrinsic apoptotic mitochondrial pathway was downstream of ERK1/2 activation. The cascade of events triggered by F1 was ERK1/2 phosphorylation → Bax/Bak activation → mitochondria membrane permeabilization → release of proapoptotic proteins: cytochrome c, apoptotic inducing factor (AIF) and endonuclease G (EndoG). Even though the caspase apoptotic pathway was activated by both cytochrome c release and a delayed activation of death receptor pathway, the intrinsic apoptotic pathway appears to be critical for a F1-induction of caspase-independent apoptosis as assessed when the pan caspase inhibitor (zVAD-fmk) was used. These results suggest that peach phenolics may have potential in therapy and chemoprevention of metastatic breast cancer.
Introduction

Breast cancer is the second leading cause of cancer death in women in the US. The American Cancer Society has estimated that there will be 182,460 new cases of invasive breast cancer among women in the US during 2008.\textsuperscript{1} Whereas localized breast cancer can be cured by surgery, the high mortality rate associated with breast cancer is due to a propensity of the tumor to metastasize when the primary tumor is small or undetectable.\textsuperscript{160}

Multiple factors contribute to the development of human breast cancer of which environmental factors, especially diet, appear to have a great effect. While consumption of a high-fat diet as occurs in industrialized countries may contribute to increase the risk of breast cancer,\textsuperscript{161} a diet rich in fruits and vegetables is one of the factors that may decrease breast cancer risk.\textsuperscript{162} Moreover, some studies have reported greater survival after breast cancer in physically active women with reduced dietary fat and increased fiber, vegetable, fruit, and other nutrient intakes associated with a plant-based diet.\textsuperscript{163, 164}

The process of carcinogenesis involves several cell signaling pathways inappropriately activated or silenced. Research in the signal transduction area has shown that this mechanism is the crucial communication route between membrane-bound proteins and the nucleus. Even though several studies have identified specific cell signaling pathways targeted by natural compounds,\textsuperscript{45, 121} there are still many unknowns and the overall effects of these compounds on breast cancer cells may result from a crosstalk among them. A cell signaling pathway that frequently seems to be altered in a variety of human cancers is the mitogen-activated protein kinases (MAPKs) pathway. MAPKs are the family of kinases that transduce signals from the cell membrane to the nucleus in response to a wide range of stimuli including stress.\textsuperscript{13} The MAPK signaling pathways modulate gene expression, mitosis, proliferation, motility, metabolism, and programmed death or apoptosis. MAPKs consist of three family members: the extracellular signal-regulated kinase ERK1/2, c-Jun NH2-terminal kinase (JNK); and the p38-MAPK. The ERK1/2 is generally activated in response to growth stimuli whereas JNK and p38-MAPK are known to be simultaneously activated in response to a variety of cellular and environmental stresses that triggers apoptosis.\textsuperscript{13} Although most studies relate ERK activation to cell proliferation, others show that activation of ERK cascade promotes
contrary cellular responses that vary upon cellular context, including cell proliferation, cycle arrest, differentiation, senescence, or apoptosis.

In general, apoptosis may occur through the activation of intrinsic mitochondria pathway or extrinsic death receptor pathway. The mitochondria pathway is controlled by Bcl-2 family of proteins which regulate the permeability of mitochondria membranes. The proapoptotic members of the Bcl-2 family, Bax, Bak and Bid form channels or regulate preexisting channels in the mitochondrial membrane, whereas the antiapoptotic Bcl-2 and Bcl-xL have the opposite effects on membrane channel formation. Bak is constitutively associated with the mitochondria, unlike Bax, which exists as a monomer either in the cytosol or loosely attached to the outer mitochondrial membrane. Upon induction of apoptosis, the cytosolic Bax translocates to the mitochondria, simultaneously with this translocation, Bax oligomerizes into large complexes. Like Bax, during apoptosis, Bak homo-oligomerizes and both have a degree of functional cooperation during the channel formation and trigger cytochrome c release from the mitochondria. The antiapoptotic proteins, Bcl-xL and Bcl-2, are located in the outer mitochondrial membrane and blocks cytochrome c release that follows the activation of Bax and Bak, thus preventing it from catalyzing caspase activation. The activation of the death receptor pathway is mediated by Fas-signaling and induced by ligation of the Fas receptors by Fas-ligands or anti-Fas antibodies. When activated, the Fas/Fas-L receptor triggers the recruitment of caspase-8 to the death-inducing signaling complex. Once caspase-8 is activated, it has the ability to activate the executioner caspases 3, 6 and 7. In addition this extrinsic pathway can trigger the mitochondrial pathway through cleavage of Bid, which is further post translationally modified to interact and activate Bax or Bak. The release of cytochrome c, as a cofactor, is a requirement for caspase-9 activation, which is mediated by the formation of the apoptosome, a protein complex in which procaspase-9 is activated into its active form. The active caspase-9 then, may activate downstream executioner caspases. Therefore, this extrinsic pathway works via a crosstalk with the intrinsic mitochondrial pathway.

Plant extracts induce apoptosis in cancer cells by targeting cell signaling pathways that inhibit, reverse or retard tumorigenesis. In this context, the use of plant extracts
containing a mixture of phytochemicals is being intensely researched for their chemopreventive effects.

Peaches contain hydroxycinnamates (chlorogenic acid, neochlorogenic acid), flavan 3-ols (catechin, epicatechin) and flavonols (quercetin derivatives). Many of these phenolics act as antioxidants, antimutagens, and anticarcinogens in vitro and in vivo. Specifically chlorogenic acid and caffeic acid derivatives have been subject of many health related claims. Likewise, emerging literature reports different molecular targets in the chemopreventive effects of caffeoylquinic acid derivatives which differ upon cell context.

Peach varieties with enhanced functional properties (high anti oxidant activity and high phenolic content) have been reported. Our goal is to understand the molecular mechanisms involved in anticancer activity exerted by a fraction rich in phenolic acids (F1) extracted from BY00P6653 red-flesh peach genotype.

Materials and Methods

Chemicals

The Folin-Ciocalteu reagent, dimethyl sulfoxide (DMSO) and N-acetyl cysteine (NAC) were purchased from Fisher Scientific (Pittsburgh, PA). Ethanol, methanol and ethyl acetate were purchased from VWR International (Bristol, CT). Chlorogenic acid was obtained from Sigma-Aldrich (St Louis, MO). The general caspase inhibitor zVAD-fmk was purchased from BD Bioscience (San Jose, CA). Antibodies for pERK, cytochrome c, cleaved caspase-8, -9 and -3 were purchased from Cell Signaling Technology Inc. (Beverly, MA), those for p-c-jun, ERK1/2, Bcl-xL, Bax and AIF were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and antibodies for Endo G was purchased from Prosci (Poway, CA). MEK1/2 inhibitor (U0126) was used for ERK1/2 inhibition (cell signaling Technology, Beverly, MA).

Plant material

The red-fleshed peach selection, BY00P6653, obtained from W. R. Okie who heads the Peach Breeding Program at USDA-ARS (SE Fruit & Nut Research Lab, Byron, GA)
was used for this study. Fruits were collected at the mature firm stage, sent by overnight delivery, and stored at 2-4°C (less than 5 days) until the pits were removed and the fruits were frozen and kept at -20°C until use.

**Extraction of phenolic acid rich fraction (F1)**

100 g of frozen fruit (flesh plus skin) was blended with 300 mL of methanol and left at 4°C overnight. The methanolic extract was filtered through whatman #1 filter paper and the methanol evaporated at 45°C using a rotavapor (Büchi, Switzerland). Phenolic acids (F1) were isolated by solid phase extraction (SPE) using C18 cartridges 104. Briefly, the aqueous extract was adjusted to pH 7.0 with 5 N NaOH. The extract was loaded in SEP Pack C18 cartridge previously conditioned to pH 7.0 with 50 mL of 100% methanol and 50 mL of nanopure water (pH 7.0). The neutral phenolics were absorbed in the cartridge while the phenolic acids were not. The cartridge was washed with 50 mL of water pH 7.0. The water from the wash was combined with the phenolics that were not adsorbed in the cartridge and adjusted to pH 2.0. This mixture of compounds was loaded into a second cartridge previously conditioned at pH 2.0 with 50 mL 100% methanol and 50 mL nanopure water pH 2.0. Phenolic acids bound to the matrix of the second cartridge (F1) were later eluted with 50 mL 100% methanol. This F1 was evaporated using a speed vac (Savant SC100) and kept under nitrogen gas at -20 °C for use in cell culture.

**HPLC analysis of phenolic acids present in F1**

Phenolic acids in F1 were analyzed using a Waters (Milford, MA, USA) HPLC system. An AtlantisTM C18 5 µm, 4.6 mm x 150 mm column and a 4.6 mm x 20 mm guard column was used for the separation of phenolic compounds. The mobile phase was composed of solvent A (nanopure water adjusted to pH= 2.3 with 2N HCl) and solvent B (acetonitrile HPLC grade). The elution was as follows: isocratic conditions from 0-5 min with 85% A and 15% B. Gradient conditions from minute 5 to 30 starting with 85% A and ending with 0%, and starting with 15% B and ending with 100%. Then, isocratic conditions from minute 30 to minute 35 with 0% A and 100% B. The flow rate was 1 mL/min and 20 µL of sample was injected.105 F1 was also analyzed by HPLC after acid hydrolysis with HCl 4N at 100° for 45 min. After cooling, the solution was filtered through a 0.2µm PTFE filter and analyzed by HPLC.
Cell lines

The MDA-MB-435 estrogen receptor negative breast cancer cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained as recommended. MDA-MB-435 were cultured using Dulbecco’s modified Eagle’s medium (DMEM), with L-glutamine and 4.5 g/L glucose without sodium pyruvate, and supplemented with 10% (v/v) fetal bovine serum (FBS), 1% penicillin-streptomycin antibiotic mix. Cells were maintained at 37 °C with a humidified 5% CO₂ atmosphere.

Cell viability

Cell viability was measured by using the MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] assay. Cells were seeded at a density of 7,000 cells per well in a 96-well plate and incubated for 24 h to allow cell attachment. The medium was decanted from each well, and 0.1 mL of fresh medium containing the F1 fraction dissolved in DMSO was added at concentrations based on total phenolic content measured spectrometrically by Folin-Ciocalteu colorimetric method and expressed as mg of chlorogenic acid equiv./L, all cultures contained 1 mL/L each of DMSO. After a 24h incubation period, the culture medium was removed and cells washed with phosphate buffer solution (PBS); 100 µL of MTT (5 mg/ml) were added per well and incubated for 1 h at 37°C. After incubation, MTT was aspirated and 100 µL of DMSO was added to lyse the cells and dissolve the blue formazan crystals. Cell viability was calculated according to the following equation.

\[
\text{Cell viability (\%)} = \left[ \frac{OD_{\text{of cell culture with sample}} - OD_{\text{of the medium}}}{OD_{\text{of the cell culture without sample}} - OD_{\text{of the medium}}} \right] \times 100
\]

OD is the optical density measured by the spectrophotometer with 555 excitation and 520 emission filters.

The role of caspase activation on cell viability was assessed by using the broad spectrum caspase inhibitor zVAD-fmk that prevents apoptotic cell death mediated by caspase activation. Vitamin E succinate (VES) (Sigma-Aldrich, St Louis, MO) was used as a positive control.
**Western-blot analysis**

Cells were seeded (0.5 x 10^6) onto a 6-well culture plate in 10% FBS/DMEM and incubated for 12 h to allow cell attachment. After 12 h of incubation in serum-free media, cells were treated with F1 dissolved in DMSO for different times or at different doses. Media was discarded and cells were washed with PBS and then removed by scraping using PBS. After centrifugation, cells were lysed with non-denaturing buffer (10 mM Tris-HCl, 10 mM NaH$_2$PO$_4$, 130 mM NaCl, 1% (v/v) Triton X-100, 10 mM sodium pyrophosphate, (pH 7.5), and 1% proteinase inhibitor cocktail (Sigma,-Aldrich)) for 15 min in ice. Solid cellular debris was removed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant was collected and stored at -80°C. Protein content was determined using the Bradford assay. For each lane sixty g of protein was diluted with Laemmli’s loading buffer, boiled, and loaded on acrylamide gel (10%, or 15%) and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis at 100V for ~2 h. Proteins were transferred by wet blotting onto 0.2 µm PVDF membrane (Bio-Rad, Hercules, CA). Membranes were blocked using 5% milk in TPBS for 30 min and incubated with primary antibody either in fresh 5% milk or 3% bovine serum albumin in TPBS overnight at 4°C with gentle shaking and incubated with the secondary antibody (1:5000) in 5% milk TPBS for 2 h. The concentrations of the primary antibodies ranged from 1:250 to 1:5000. Reactive bands were visualized with an enhanced chemiluminescence (ECL) Plus system (Amersham Corp., Arlington Heights, IL) after 5 min of incubation.

**Statistical analysis**

Quantitative data represent mean values with the respective standard deviation or standard error of the mean corresponding to 3 or more replicates. Data were analyzed by one-way analysis of variance (ANOVA) using SPSS version 15.0 (SPSS Inc., Chicago, IL). Post-hoc Tukey pairwise comparisons were used (p<0.05).
Results and Discussion

Phenolic profile of F1

Comparison of retention times and UV-visible spectral data of the chromatographic profiles of F1 with known standards revealed the presence of chlorogenic acid (peak 1, RT= 5.8min), chlorogenic acid derivative (peak 1’, RT= 4.2min) and cyanidin glucoside in very low concentration that almost co-eluted with peak 1 (peak 2’, RT= 5.4 min) (Figure 18-A). After acid hydrolysis (Figure 18-B), the ester bond between the caffeic and quinic acid components within chlorogenic acid is broken and caffeic acid is released. Thus, peaks 1 and 1’ were converted into peak 3 (caffeic acid) (RT= 8.9 min) and a less polar caffeic acid derivative with same spectrum but different retention time, probably with an additional methyl group or a more hydrophobic group since it came out ~10 min after the caffeic acid standard on a C18 column (peak 3’, RT= 18.4min). When analyzed the HPLC profile at 520 nm (Figure 18-B1), we identified the aglycon cyanidin as product from acid hydrolysis of cyanidin 3-glucoside (peak 2, RT=14.9min).

Cell viability

The cell viability of MDA-MB-435 breast cancer cells decreased upon treatment with F1 in a dose-dependent manner (Figure 19). Several reports have indicated the
antiproliferative action of caffeic acid, chlorogenic acid and caffeoylquinic acid derivatives in different human cancer cells, evident at low concentrations, comparable with those found in biological fluids after ingestion of foods rich in phenolic acids.\textsuperscript{54, 66, 80} Likewise, the presence of cyanidin 3-glucoside, even in small concentrations may potentiate the anticarcinogenic effect exerted by chlorogenic acid in F1.\textsuperscript{173, 174}

Figure 19. F1 exerts a dose-dependent inhibition on cell viability. Representative evaluation of the concentration-dependent impact of F1 on the viability of human MDA-MB-435 breast cancer cells. MDA-MB-435 cells were incubated with various concentrations of extracts for 24 h and cell viability measured by MTT assay. Values are mean ± SD, n = 4.

Molecular mechanisms behind the anticancer activity of F1 from peach BY00P6653

F1 activates the mitogen-activated protein kinases (MAPKs) pathway

We investigated the role of F1 on the activation of MAPK signaling pathway by western-blot analysis. Our results showed that F1 activated the extracellular signal-regulated kinase (ERK1/2) in a time-dependent and dose-dependent manner (Figure 20). However, the other members of this family, the c–Jun NH2-terminal kinase (JNK), and the p38 MAPK were not activated. A late and weak activation of the transcription factor c-Jun was also observed.
The MAPK cell signaling pathway is frequently altered in a variety of human cancers; therefore modulation of these MAPK pathways by dietary compounds may provide novel strategies for the prevention and treatment of cancer.\textsuperscript{143} The ERK is generally activated in response to growth stimuli, whereas JNK and p38 are known to be simultaneously activated in response to a variety of cellular and environmental stresses that trigger apoptosis.\textsuperscript{13}

On the other hand, several reports have shown that activation of ERK1/2 promotes contrary cellular responses which may vary upon cellular context,\textsuperscript{14, 15, 139} and may commit the cell to apoptosis when it happens before the activation of p38 and JNK.\textsuperscript{144}

In addition, ERK1/2 activation can also contribute to cell death through the suppression of the antiapoptotic signaling molecule Akt.\textsuperscript{175} However, we did not find any effect of F1 treatment on the basal levels of either Akt nor Akt phosphorylation (data not shown).\textsuperscript{175} Key targets of MAPKs include several nuclear transcription factors, and c-Jun was found to be activated downstream of ERK1/2 phosphorylation.\textsuperscript{176} Even though previous studies in MDA-MB-435 cells supported the notion that c-Jun plays a critical role in apoptosis which implicates JNK activation in this process,\textsuperscript{153} we found that F1 induced ERK1/2 prolonged activation and a delayed c-Jun phosphorylation independently of JNK and p38 activation. This was corroborated by blockage of ERK1/2 phosphorylation (Figure 20C). This finding is supported by a previous report indicating that c-Jun activity is enhanced by ERK phosphorylation, which may occur independently from the activation of JNKs.\textsuperscript{177} Additionally, ERK1/2 phosphorylation may trigger the activation of a large number of downstream molecules that may vary under different conditions, cell types and even cellular compartments.\textsuperscript{176} Among the downstream targets of ERK1/2 phosphorylation are the family of Bcl-2 proteins, which regulate apoptosis through the intrinsic mitochondrial apoptotic pathway.\textsuperscript{120, 142}
Figure 20. Peach BY00P6653 phenolic acid fraction F1 activates MEK/ERK signaling pathway. (A) MDA-MB-435 cells were treated for 1, 6, 12, 24 and 36 h, with 150 mg chlorogenic acid equiv./L of F1. Control cells (c), cells were cultured with serum free medium for 36 h. Positive control (+c) cells received medium containing 10% FBS 6 h before harvesting. (B) MDA-MB-435 cells were treated with 25, 50, 100, 150 and 200 mg chlorogenic acid equiv./L of F1, control solvent (s) received same volume of vehicle (DMSO). (C) MEK1/2 inhibitor block F1-induced ERK activation and c-Jun activation. MDA-MB-435 cells treated with 150 mg chlorogenic acid equiv./L for 24 and 36h, 10 µM of U0126 was used for ERK1/2 inhibition. Cell lysates were assayed by western blotting. Total β-actin was detected to show the same amount of protein load for all treatments. The data represent a typical experiment conducted three times with similar results.
**F1 targets the mitochondrial apoptosis signaling pathway**

Several recent reviews have highlighted the role of natural compounds as novel anti-cancer drugs that target mitochondria.\(^{31,178,179}\) We found that F1 activated the pro-apoptotic Bax and Bak with no changes on levels of antiapoptotic Bcl-xL (Figure 21). The pro-apoptotic effects of Bax can be blocked by Bcl-xL, and the ratio of antiapoptotic Bcl-xL and pro-apoptotic Bax in the mitochondria determines survival or death following an apoptotic stimulus.\(^{135}\) The lower heterodimerization ratio of antiapoptotic Bcl-xL to proapoptotic Bax and Bak results in increase of mitochondrial membrane permeability due to Bax/Bak formation channels.\(^{26,180}\) We also found that both cytochrome c release and Bax activation are downregulated by the inhibition of ERK1/2 phosphorylation (Figure 21B). Therefore, these findings suggest that F1 may have proapoptotic effects on MDA-MB-435 mediated by sustained activation of ERK1/2 which targets the mitochondrial apoptotic pathway. Similar effects on targeting the mitochondrial pathway have been reported for natural compounds that can selectively kill malignant cells at concentrations non-toxic to normal cells and tissues.\(^{181}\) Thus, the identification of novel compounds that may target the mitochondrial pathway may be considered in the development of anticancer drugs from natural compounds.

In addition to the critical role that ERK1/2 may play in the mitochondrial apoptotic pathway, the ligand binding death receptor pathway (Fas ligand) may also indirectly trigger the activation of the mitochondrial pathway.\(^{23,26}\)
F1 activates the death receptor pathway

We found that the treatment of MDA-MB-435 breast cancer cells with F1 induced a late activation of caspase-8 and the downstream executioner caspase-3 (Figure 22). This extrinsic pathway comprises a cross-talk with the intrinsic mitochondrial pathway. Our results show that both the initiator caspase-8 and the executioner caspase-3 are activated at ~24h, whereas activation of caspase-9 is correlated with cytochrome c release and PARP (poly ADP-ribose) polymerase-1 cleavage, and occurs ~6 h after F1 treatment.

Figure 21. F1 activates the proapoptotic Bak and Bax while the levels of antiapoptotic Bcl-xL remains constant. (A) MDA-MB-435 cells were treated with F1 at doses: 0, 25, 50, 100, 150 and 200 mg chlorogenic acid equiv./L for 36 hours. (B) Cells treated with 20 mg/L of alfa-tocopheryl succinate (VES) for 24h were used as positive control for Bax activation and cytochrome c release, 10 µM of U0126 was used for MEK1/2 and the downstream ERK1/2 inhibition on F1 treated cells for 24h. Cells were harvested and lysed for Western blot analysis. The data represent a typical experiment conducted three times with similar results.
Indeed, the mitochondria pathway activation seems to be critical in F1-apoptosis induction. Along with cytochrome c release (which activates a caspase dependent cell death), mitochondrial membrane permeabilization also causes the release of pro-apoptotic factors such as apoptosis-inducing factor (AIF) and EndoG, which activate caspase-independent cell death.

![Figure 22. F1 induces early caspase-9 activation and PARP cleavage in a time-dependent manner and delayed caspase-8 and caspase-3 cleavage. MDA-MB-435 cells were treated with F1 (150 mg chlorogenic acid equiv./L) for the indicated times. Cleavage caspase 9, 3, 8 and PARP cleavage were assessed by Western blot analysis. Cells treated with 20 mg/L of α-tocopheryl succinate for 24h (VES) were used as positive control for cleavage caspase 9 and cytochrome c treated Jurkat cell extracts (cell signaling Technology, Beverly, MA ) was used as positive control for caspase 3 and 8 cleavage. Total β-actin was detected to show uniform protein load for all treatments.](image)

**F1 induces apoptosis through a caspase-independent pathway**

So far, we have demonstrated that F1 targets the mitochondria apoptosis pathway through ERK1/2 activation. This is supported by our results showing how cytochrome c release to mitochondria decreases when the MEK1/2 inhibitor was used (Figure 23A), while to a lesser extent the cytochrome c release to the cytoplasm was found in the presence of either the pan-caspase inhibitor of JNK that could be upstream of c-Jun phosphorylation. Furthermore, we found that F1 induced Apoptosis-inducing factor...
(AIF) and Endonuclease G (EndoG) translocation to the nucleus and this event seems to be downstream of ERK1/2 activation (Figure 23B). By inducing mitochondrial membrane permeabilization, several pro-apoptotic proteins such as cytochrome c, AIF, Smac/DIABLO, EndoG, and HtrA2/Omi can diffuse across the mitochondrial inner membrane to the cytosol. The nuclear translocation of AIF and EndoG has been reported to be responsible, at least in part, for DNA condensation and fragmentation, and subsequent cell death. This process may happen upon caspase activation and/or PARP cleavage or just as a result of mitochondrial dysfunction which triggers AIF translocation to the nucleus in a caspase independent pathway.

On the other hand, PARP is a nuclear protein implicated in DNA repair and one of the most utilized for the detection of apoptosis in many cell types. Once PARP is cleaved into its 24kDa and 89kDa fragments, it prevents DNA repair-induced survival. Even though caspase-3 may cleave PARP, the PARP cleavage time-response seems to be more directly influenced by the mitochondria pathway and associated to the release of the proapoptotic factor AIF.

The ERK1/2 sustained phosphorylation seems to play a key role in mitochondria permeabilization. Furthermore, our results showing the time-response for caspase-9 activation, cytochrome c release, and PARP cleavage indicate that apoptosis induction by F1 on MDA-MB-435 is primarily mediated by a caspase independent pathway. Indeed, the contribution of caspases may reflect amplification but not an initiation of the apoptotic process (Figure 23A). Likewise, in Figure 23C we show how cell viability inhibition exerted by F1 was not reversed in the presence of the pan-caspase inhibitor (zVAD-fmk) as it was for the positive control VES (p < 0.05). Caspase inhibitors have been reported to block VES-induced apoptosis of MDA-MB-435 breast cancer cells.
Figure 23. Activation of ERK1/2 by F1 targets the mitochondrial pathway and induces cytochrome c release, AIF and EndoG nuclear translocation, reducing viability in a caspase independent manner. (A) MDA-MB-435 cells were treated with F1 (150 mg chlorogenic acid equiv./L) for 36 h. 10 µM of U0126 was used for MEK1/2 –ERK1/2 inhibition, 20µM of SP6000125 used for JNK-cJun inhibition, and 50 µM of zVAD-fmk for caspase inhibition. The cytoplasm fraction was assessed for cytochrome c by western blotting. (B) Nuclear fractions of cells treated with F1 for 36 h were analyzed by western blots for AIF and EndoG. (C) MDA-MB-435 cells were incubated with the vehicle (DMSO), 50µM of zVAD-fmk for 1 h and then cultured in the absence or presence of F1 (150 mg chlorogenic acid equiv./L ) for 24h. Alfa-tocopheryl succinate (VES) was used as positive control for caspase-dependent apoptosis. Values are mean ± SE, n=4. Different letters indicate significance at the p < 0.05 level.
In general, F1 appears to trigger apoptosis through the induction of mitochondrial changes as a primary intrinsic apoptotic pathway and late activation of caspase-8 as a secondary extrinsic apoptotic pathway. At this level there is a crosstalk between the death receptor pathway and the mitochondrial pathway \(^{23,133}\) (Figure 24).

Figure 24. An overview of the pro-apoptotic mechanism exerted by peach BY00P6653 phenolic acid fraction F1 on MDA-MB-435 cells. Several factors may activate RAS, which then stimulates the Raf-MEK-MAPK pathway upstream of ERK1/2 phosphorylation. This event targets the mitochondrial pathway by Bax and Bak activation and cytochrome c release. The time response for caspase-9 activation and PARP cleavage correlates to ERK1/2 phosphorylation, whereas the delayed caspase-8 and caspase-3 activation suggest a secondary role of F1 induction of apoptosis.
Conclusion

In conclusion, this study showed that phenolic compounds present in F1 of red flesh peach BY00P6653, exerts inhibition of cell viability and induces apoptosis on MDA-MB-435 breast cancer cells by activation of the mitochondrial pathway mediated by sustained ERK1/2 phosphorylation, resulting in mitochondrial membrane permeabilization. The release of pro-apoptotic factors from mitochondria such as cytochrome c, EndoG and AIF, activated caspase-dependent and caspase-independent cell death. The potential applications of these compounds are as potentiators of therapeutic drugs that allow them to be effective at submaximal cytotoxic concentrations, and/or for development of dietary supplements as strategy to inhibit or reverse the development of invasive breast cancer. The in vivo cancer suppression activity of these compounds deserves to be investigated.
CHAPTER V

PEACH EXTRACTS SUPPRESS TUMOR GROWTH AND INVASION OF HUMAN MDA-MB-435 BREAST CANCER CELLS IN VIVO

Synopsis

Phenolic compounds extracted from a commercial variety of the yellow-fleshed peach Rich Lady (RL) were investigated for its tumor suppressive activity in vivo. The xenograft model allowed the assessment the dose-response of tumor growth rate and final weight of MDA-MB-435 transplanted tumors into athymic nude mice fed with RL phenolics. In addition, the anti-metastatic activity and modulation of tumor gene expression by RL phenolics was analyzed by RT-PCR. The results showed that the mixture of phenolics on RL extract when administered to mice at the highest doses (0.8 and 1.6 mg/day) effectively suppressed the palpable tumor-growth rate. Even though, doses lower than 0.8 mg/day appeared not to be effective in delaying the palpable tumor growth, a dose-dependent decrease trend in final tumor weight was found, and most importantly, RL phenolics induced a dramatic decrease in lung metastasis at all doses used. Downregulation of metalloproteinase-2 (MMP-2), metalloproteinase-13 and metalloproteinase-3 gene expression may be some of the molecular targets for anti-metastatic activity of RL phenolics. However the involvement of other members of the metalloprotease family as well as pro-angiogenic and cell motility factors, which may be differentially regulated in situ by RL phenolics deserves further investigation.

Introduction

Breast cancer is the most common malignant disease in Western women. Death, and most of the complications associated with breast cancer are due to metastasis. The American Cancer Society has estimated that there will be 182,460 new cases of invasive breast cancer among women in the US during 2008.\(^1\)

Recently, the rates of metastasis and mortality in breast cancer patients have decreased as a result of early diagnosis by mammographic screening and the implementation of
adjuvant therapy. The adjuvant therapy can help to eradicate breast tumor cells that might have already spread to distant sites by the time of diagnosis. However, chemotherapy has a wide range of acute and long-term side effects that substantially affect the patient’s quality of life. Nowadays more than 80% of the patients diagnosed with primary breast cancer receive adjuvant chemotherapy, although only about 40% of the women really need it. Therefore, women who would be cured by local treatment alone, which includes surgery and radiotherapy, are being ‘over-treated’ and suffer the toxic side effects of chemotherapy unnecessarily.²

Mouse models have made an important contribution to the understanding of breast cancer progression and metastasis. The xenograft model is commonly used to investigate the growth of breast cancer cell lines in an in vivo environment, which resembles the complex interactions that are involved in breast tumor formation and progression.⁸¹ The xenograft models are often used in metastasis research because they offer excellent insights into invasion properties and progression studies,⁸¹ and constitute an important tool to test and validate novel treatment strategies.

A tumor is defined as a local uncontrolled growth of abnormal tissue consisting of transformed cells, as well as other cell types including fibroblasts, macrophages, endothelial cells and connective tissue components known as the stroma.¹⁸⁷ Tumor metastasis is a multistep process involving the invasion of primary tumor cells into the surrounding tissue and their propagation at distal sites. Tumor cells influence and manipulate the stroma in such a way that the latter produces a permissive and supportive environment, which helps facilitate the growth of the carcinoma. To establish a metastasis, tumor cells have to invade their surrounding host tissue, enter the circulatory blood stream, arrest in capillary beds of distant organs, invade the host tissue and proliferate.²,¹⁸⁸ Primary breast cancer cells metastasize through the blood vessels to various distant organs, preferentially, to the lung, liver and bones.² Tumors as small or less than 2 mm in diameter already receive a vascular blood supply,¹⁸⁹ therefore, it is likely that cancer cells have spread throughout the body years before they are first detected.¹⁸⁹

Some of the biomolecules playing a key role in development of breast cancer metastasis are the matrix metalloproteases (MMP), vascular endothelial growth factor
(VEGF) and the transmembrane protein E-cadherin (E-cad). The MMP family of proteolytic enzymes, degrade constituents of the extracellular matrix surrounding invasive breast carcinomas.\textsuperscript{190} The expression of vascular endothelial growth factor (VEGF) has been identified as a key component for tumor growth, metastasis and angiogenesis.\textsuperscript{191,192} Angiogenesis is characterized by migration and proliferation of endothelial cells and the maturation of new blood vessels that would supply oxygen and nutrients to tumor cells in response to local pro-angiogenic factors and activation of matrix metalloproteases.\textsuperscript{193} E-cad is the main component of the adherent cell–cell junction of epithelial cells. Inactivating E-cad \textit{in vivo} results in the disruption of luminal epithelium with the release of individualized cells in the lumen.\textsuperscript{194} Equally, maintenance of E-cad in invasive tumors can block tumor progression in the mouse model.\textsuperscript{195} Repression of the E-cad gene in breast carcinomas has been inversely correlated to expression of Snail or Slug transcription factors that act as transcriptional repressors.\textsuperscript{196}

In this context, the specific phenolic compounds identified in peaches and plums\textsuperscript{51} have shown to inhibit tumor growth and metastasis through xenograft models. For example, cyanidin 3-glucoside, as a component of an anthocyanin rich extract, was reported to inhibit tumor growth \textit{in vivo},\textsuperscript{96,97} and invasion via repression of metalloproteases;\textsuperscript{97} the wine anthocyanins delphinidin and cyanidin inhibited VEGF expression through the p38 MAPK pathway,\textsuperscript{197} proanthocyanidins suppressed the tumor growth and increased the anti-tumor activity of the chemotherapeutic doxorubicin;\textsuperscript{98,99} quercetin inhibited the growth and exhibited pro-apoptotic activities in xenografted MDA-MB 435 cells into nude mice;\textsuperscript{52} treatment with caffeic acid derivatives induced a significant dose dependent decrease in tumor growth and reduced the number of mitotic cells.\textsuperscript{95} Therefore, the potential of peach phenolics as tumor-suppressive and anti-metastatic agents deserves to be investigated. The results from this study could lead to translational research toward a rational application of natural chemopreventive compounds as adjuvant therapy to potentiate the inhibitory effects of non-toxic doses of commonly used chemotherapeutics.
Materials and Methods

Preparation of peach extracts

The commercial variety of yellow-fleshed peach ‘Rich Lady’ (RL) grown in California, were collected at a mature firm stage and stored at 2-4 °C until use (less than 5 days). Upon arrival at Texas A&M University, the fruits after removing the stones were frozen and kept at -20 °C until use. 100 g of frozen fruit (flesh plus skin) was blended with 300 mL of methanol and left at 4 °C overnight. The methanolic extracts were filtered through whatman #1 filter paper and the methanol evaporated at 45 °C using a rotavapor (Büchi, Switzerland). Aqueous extracts were frozen at -80 °C and freeze-dried (FTS® Systems, Inc., Stone Ridge, NY) at -50 °C and at 200 mmHg of pressure. Dry extracts were re-dissolved in sterile water and sterile filtered before use. Doses were calculated based on the content of total phenolics.

Cell culture

The MDA-MB-453estrogen receptor-negative human breast cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured using Dulbecco’s modified Eagle’s medium (DMEM) with L-glutamine, 4.5 g/L glucose, without sodium pyruvate, and supplemented with 10% (v/v) (FBS) and 1% streptomycin-penicillin antibiotic mix. The culture medium was supplied by Invitrogen (Grand Island, NY). Cells were maintained at 37 °C with a humidified 5% CO2 atmosphere.

Animals

Female athymic nude mice (homocystous Nu-/-), aged 3 to 4 weeks, were purchased from Harlan Laboratories (Houston, TX) and maintained in a ventilated rack system. Irradiated food and autoclaved water were provided ad libitum. Experiments were approved by the Institutional Animal Care and Use Committee at Texas A&M University (College Station, TX). The mice were allowed to adjust to their environment for 4 days before initiation of the experiments.
Antitumor efficacy study

This study was performed in two phases. The first phase allowed us to find out the range of active concentrations of peach RL extract and the second phase we tested the most active concentration and went beyond that concentration to find out the threshold of active concentrations. MDA-MB-435 cells (5 x 10^5) suspended in 100µl of DME-50% Matrigel (BD Bioscience, San Jose, CA) were injected subcutaneously into each flank of the nude mice. Injections were carried out 5 days before treatment with peach extracts (day 0). On day 0, mice used in the first phase of the study were randomly divided into four groups (n = 3 for each group) to be fed by oral gavage with sterile water (control) and peach extracts (0.2, 0.4, and 0.8 mg phenolics/day). For the second phase of the study, three groups of xenografted mice (n = 5 for each group) were fed with sterile water (control) and peach extracts (0.8, and 1.6 mg phenolics/day). In addition two groups in which no tumor cells were injected were used as a negative control to evaluate possible toxicity of the highest doses (0.8 and 1.6 mg/L). Tumor volume was measured with calipers every three days. The estimated tumor volume was calculated by the formula: \( a^2 \times b/2 \), where “a” and “b” are the short and the long axis of the tumor, respectively. All procedures were conducted under aseptic conditions in a laminar flow hood. At the end of the study, (day 24), mice were sacrificed following institutional regulations, tumors and lungs were removed, weighed, and frozen for subsequent RNA analysis.

RNA isolation

Tumor and lung samples were mechanically pulverized in liquid nitrogen. RNA was extracted using Trizol (Invitrogen) according to the manufacturer’s protocol. Purification was carried out using the RNeasy mini columns (Qiagen, Clifton Hill, Australia). Quantification of RNA was established based on A260nm and A280nm using an ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE).

cDNA synthesis and quantitative PCR

cDNA was reverse transcribed using 270 ng or 1890 ng of total RNA extracted from tumors or lungs respectively using oligo (dT) and Superscript II Reverse Transcriptase (Invitrogen), following the manufacturer instructions. Real-time, quantitative PCR was done on a 7500 Fast Real-Time PCR System. Briefly, PCR was done using the SYBR
Green PCR Master Mix kit (Applied Biosystems, Foster City, CA), cDNA templates and primers. The reaction volume was 25 µL and all primers were used at a final concentration of 100nmol/L. Thermocycling was initiated with a 10-minute, 95°C enzyme activation step followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Human TBP and mouse β-actin were used as normalizing genes for lungs and tumors respectively. Real-time PCR data was analyzed by the ∆∆CT method.198

**Primers**

Primers encoding human VEGF, SLUG, MMP-2, MMP-1, MMP-3, MMP-9, MMP-13, hß2G, and hTBP and mouse mβ-actin genes were purchased from Integrated DNA Technologies, Inc. (San Diego, CA). Primer sequences, listed in Table 5 were designed using Primer Express® software v2.0 from Applied Biosystems (Foster City, Ca). Product specificity was examined by dissociation curve analysis.

**Table 5. Primer sequences.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
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<tr>
<td>hß2G-RTF1</td>
<td>CGC TCC GTG GCC TTA GC</td>
</tr>
<tr>
<td>hß2G-RTR1</td>
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</tr>
<tr>
<td>hTBP-RTF1</td>
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</tr>
<tr>
<td>hTBP-RTR1</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>MMP-1-RTR1</td>
<td>TGA GCA TCC CCT CCA ATACC</td>
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Statistical analysis

Quantitative data represent mean values with the respective standard deviation (SD) or standard error of the mean (SE) corresponding to 3 or more replicates. Data were analyzed by one-way analysis of variance (ANOVA) using SPSS version 15.0 (SPSS Inc., Chicago, IL). Post-hoc Tukey pairwise comparisons were used (p<0.05).

Results and Discussion

Effect of RL extracts on body weight

The final body weight and weight gain did not differ significantly among the experimental groups over study (Figure 25). Likewise, the appearance of major organs including kidney and liver were not visibly affected. This suggests no toxicity or potential adverse effect on the major organs due to peach phenolics feeding.

Figure 25. The effect of peach extract treatments on weight gain of athymic nude mice. The (-) 0.8 and (-) 1.6 mg/day were the no xenografted mice used as negative controls. Values are average of three or more replicates ± SE of the mean.
Effect of RL extracts on the palpable tumor growth and final tumor weight

The palpable tumor volume (cm$^3$) during the study was visibly lower in the experimental groups fed with 0.8 and 1.6 mg total phenolics/day compared to the other treatments or the control (Figure 26). The weights of tumors at the end showed a dose dependent decrease effect (Figure 26-B). Treatments by oral gavage with RL phenolics at 0.2, 0.4, 0.8 and 1.6 mg/day, decreased the final tumor weight by 9.5, 13 and 23.3 and 23.1% respectively.

Figure 26. Phenolic compounds from Rich Lady (RL) decrease the palpable tumor growth-rate (A), and tumor weight (B). Untreated mouse showing tumor with visible signals of angiogenesis (C). Tumor in mouse treated with 0.8 mg of RL phenolics/day (D). Values represented the mean ± SE (* p< 0.05, n = 5).
Studies have reported the \textit{in vivo} growth inhibitory activity of most of the phenolic compounds present in RL peach extracts.\textsuperscript{52,93,96,98} However, some of these studies used a subcutaneous (s.c.) injection to deliver the bioactive compounds.\textsuperscript{52} Our results are relevant, because the intragastrointestinal delivery resembles the intake of phenolics through a diet rich in fruits and vegetables, or dietary supplements made from plant extracts, especially those plant foods that may contain a phenolic profile similar of to stone fruits.\textsuperscript{51} Indeed, the bioactive compounds can pass through the intestinal barrier and effectively reach the tumors, thus the \textit{in vitro} tumor growth inhibitory activity of RL extracts can be translated to \textit{in vivo} efficacy.

\textbf{Effect of RL phenolic extract on the invasive potential of MDA-MB-435}

The anti-metastatic activity of RL extracts \textit{in vivo} was assessed by analyzing gene expression in the lungs of the sacrificed mice. The mRNA levels of the hβ2G (human β-2 globulin) were normalized to the mβ-actin (mouse β-actin) gene expression in lung tissues and analyzed by RT-PCR (Figure 27).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure27.png}
\caption{Dose dependent effect of RL phenolics on the human β-2 globulin (hβ2G) gene expression on mouse lungs. Dose dependent hβ2G gene expression (A), values are averages ± SE (n ≥ 2). The results from the second phase of the study show the basal level of hβ2G gene expression in negative controls (no xenografted mice) (B), values are averages ± SE (n ≥ 3). Different letters indicate significance at P < 0.05.}
\end{figure}
Treatment with RL extracts significantly decreased the human β-2 globulin (hβ-2G) gene expression on mouse lungs, this suggests that RL phenolics have potential in preventing progression and metastasis in distant organs. Even the lower doses (0.2 and 0.4 mg/day), that appeared not to be effective on tumor growth-rate suppression, showed a significant anti-metastatic activity. This is consistent with previous studies showing the anti-metastatic potential compounds present in peach extracts. Quercetin, was found to potentiate the inhibitory effect of a non-toxic dose of the chemotherapeutic cisplatin and inhibited more effectively then tamoxifen the colonization of the murine melanoma B16-BL6 in the lungs in a dose-dependent manner. Chlorogenic acid was found to be a strong matrix metalloprotease-9 inhibitor, apple polyphenols also containing chlorogenic acid, epicatechin and procyanidins inhibited the growth and the metastasis of AH109A hepatomas in vivo. In general, several reports are currently identifying plant polyphenolics as anti-invasive cancer agents.

This is relevant, because approximately 10-15% of patients with breast cancer develop distant metastasis, due to the inherent metastatic capacity of breast tumors. To reduce the risk of metastasis, patients with primary tumors are sometimes over-treated with adjuvant chemotherapy and suffer the toxic side effects that reduce their quality of life unnecessarily. Therefore, the mixture of phenolics present in RL extracts may constitute a novel chemopreventive tool in the combination therapy when primary cancer is diagnosed.

The effect of RL phenolic extract on the tumor gene expression

There are several proteins associated with invasion and metastasis that can be regulated at the level of gene expression. Among these proteins are the matrix metalloproteinases (MMPs), which are important targets in breast cancer progression due to their role in facilitating tumor invasion and metastasis. Our results showed a dose-dependent decrease on MMP-2 gene expression on tumors of mice treated with RL extracts (Figure 28). The production of MMPs allows for the degradation of surrounding extracellular matrix and eventual development of new blood vessels. Specifically MMP-2 expression, has been reported to be dysregulated in breast cancer invasion and
metastasis.\textsuperscript{204} Therefore, RL phenolics may be promising therapeutic agents that block tumor progression by targeting MMP-2.

**Figure 28.** Dose dependent effect of RL phenolics on the human MMP-2 gene expression on xenografted tumors. In (A) values are averages ± SE (n ≥ 2). The results from the second phase of the study (B) show significance at P < 0.05, values are averages ± SE (n ≥ 5).

Similar results were found in the second phase of the study for MMP-1, MMP-13 and MMP-3 (Figure 29). However, RL treatments apparently did not modulate the gene expression of MMP-9, VEGF and SLUG on xenografted tumors.
Figure 29. Gene expression on xenografted MDA-MB-435 tumors. Phase I of the study (n = 3) (A). Phase II of the study (n=5) (B). Values are means ± SE. Different letters indicates significance at P < 0.05.
However, these biomolecules directly related to tumor growth, degradation of extracellular matrix, generation of new blood vessels and cell motility to sustain survival, proliferation and invasion to distant organs could be localized on the invasive fronts. In addition, MMP activity can be regulated at least at three levels: transcription/translation, proteolytic activation of the zymogen, and inhibition of the active enzyme. Likewise, the tumor-associated stroma has shown to be an important site of VEGF production that is required for growth of new blood vessels and tumor nutrient and oxygen supply. Similar location is expected for the expression of MMP and SLUG as well. These areas could represent invasive fronts linked to local progression in connection with increased invasiveness. The use of techniques such an in situ hybridization or immunohistochemistry may allow the detection of differences on localized gene expression.

**Conclusion**

The in vitro antitumor activity of phenolic compounds extracted from the yellow-fleshed peach Rich Lady (RL) variety on MDA-MB-435 estrogen receptor negative breast cancer cells has been confirmed in vivo. The xenografted tumors of the highly metastatic MDA-MB-435 cells were effectively decreased in weight and volume when mice were fed with RL extracts without weight loss or toxicity to the main organs. The mixture of phenolics extracted from RL decreased the tumor growth rate at only the highest dose (0.8mg/day). At the end of the study, a RL phenolics dose-dependent response was found for tumor weight. Most importantly, the metastasis on lungs was dramatically decreased even by the lowest dose of RL phenolics that appeared not to be effective against tumor growth-rate. The potential targets of RL phenolics for anti-metastatic activity are the metalloproteases (MMPs), specifically MMP-2 was found to be downregulated at the gene expression level on tumors. While the other MMPs analyzed, the VEGF and SLUG were not found to be transcriptional regulated in the homogenized tumor tissue, the post transcription regulation of MMPs as well as the localized gene expression of VEGF, SLUG and MMPs in the tumor-stroma boundaries warrants further investigation. This is the first study to disclose the anticancer activity and potential anti-metastatic application of RL phenolics in vivo. These findings support the potential use of
RL phenolics as chemopreventive and therapeutic novel compounds for metastasis. Careful elucidation of the molecular mechanisms underlying the antimetastatic action of RL phenolics may amplify its clinical applications.
CHAPTER VI
GENERAL CONCLUSIONS AND RECOMMENDATIONS

Peaches and plums contain a mixture of phenolic compounds that preferentially inhibit the growth of the estrogen independent MDA-MB-435 breast cancer cells over either the estrogen dependent MCF-7 breast cancer cells or the breast epithelial MCF-10A cells. When polyphenols were fractionated into different groups, the phenolic acids fraction F1 from the rich Rich Lady (RL) peach and its major components chlorogenic and neo-chlorogenic were found to suppress the growth of MDA-MB-435 cells with no or low toxicity on the non-cancerous MCF-10A cells.

Our study on molecular mechanisms targeted by the RL phenolic acids fraction F1, chlorogenic acid and neo-chlorogenic acid showed that the relatively higher growth-inhibition activity exerted by neo-chlorogenic acid may be related to its unique ability to downregulate the Akt cell survival pathway.

Induction of apoptosis exerted by the tested compounds involved activation of extrinsic and intrinsic pro-apoptotic pathways. The extrinsic pathway was activated by targeting the death receptor pathway, which activates the initiator caspase-8 and the downstream effectors caspase-6 and -7. The intrinsic mitochondrial apoptotic pathway was triggered by the activation of the pro-apoptotic Bax, and increasing the ratio Bax/Bcl-2 in favor of outer mitochondria membrane permeabilization. Both the extrinsic death receptor pathway and MAPK activation mediated by ERK1/2 and p38 phosphorylation, were shown to play important roles on the activation of pro-apoptotic Bax, mitochondria permeabilization and subsequent release of the pro-apoptotic proteins AIF, EndoG and cytochrome c.

In contrast, the fraction F1 extracted from the red flesh peach BY00P6653 genotype, in which chlorogenic acid and cyanidin 3 β-glucoside were identified, induced apoptosis on MDA-MB-435 breast cancer cells through the intrinsic mitochondrial pathway mediated by sustained ERK1/2 phosphorylation as a primary pro-apoptotic mechanism. The late caspase activation indicated that the extrinsic apoptotic pathway plays a secondary role on the F1-induction of apoptosis.
The *in vitro* antitumor activity of RL phenolics were further confirmed *in vivo* by using a xenograft model. The growth of tumors of MDA-MB-435 cells was decreased in weight and volume when nude mice were fed with RL extracts without signals of weight loss or toxicity on main organs. Most importantly, the metastasis on lungs was significantly lower even by the doses of RL phenolics that appeared not to be effective against tumor growth-rate. The RL phenolics may exert their anti-metastatic activity by targeting the metalloproteinases (MMPs), specifically the gene expression of MMP-2 in tumors was found to be downregulated in a dose-dependent manner. We suggest further analysis on the localized gene expression of other MMPs family members as well as the proangiogenic VEGF and the transcriptional cell migration repressor SLUG. These results have important clinical implications because the active compounds in RL extracts, chlorogenic acid and neo-chlorogenic acid are widespread among food plants and constitute an integral part of the human diet. Careful elucidation of the molecular mechanisms underlying the antimetastatic action of RL phenolics may amplify its clinical applications.
REFERENCES


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VITA

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