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Macy R. Shirley

University of Arkansas- Fayetteville

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Anti-Cancer and Bioavailability of Arachidin-1 and Arachidin-3 in Colon
Cancer Cells

Macy R. Shirley

University of Arkansas

Acknowledgements

Completing this project and writing the accompanying thesis seemed very intimidating at the onset. I am thankful for the copious hours that Dr. Sun-Ok Lee invested despite her many other commitments in order to patiently guide me in this process. The skills that I have developed through completing this project will be invaluable in my future career in dentistry. I am also very grateful that Mrs. Mechelle Bailey and Dr. Sabrina Trudo were willing to serve on my Honors Thesis Committee. Many other people have also supported me throughout this process, and I would like to thank them as well. I am grateful for Cindi Brownmiller, Wing Shun Lam, Danielle Ashley, and Inah Gu. I could not have completed this project successfully without their patience, guidance, and support. I would also like to thank my family for their support in this process. My parents, Thomas and Kay Shirley, have provided me with unconditional love and support throughout the course of this project. I am very thankful for all of the individuals that have supported me and assisted with this process.

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Abstract

Cancer is a common cause of death in the United States and locally in the state of Arkansas. Modifiable factors such as tobacco use, physical activity, and diet lead to reduced incidence of colon cancer diagnosis. Fruits and vegetables contain phytochemicals that confer health promoting properties. Specifically, peanuts contain phytochemicals known as resveratrol, arachidin-1, and arachidin-3 that have been linked to anticancer activities. However, a few research studies have been done on arachidin-1 and arachidin-3 that could further develop understanding of their health promoting properties or nutraceutical applications. The objectives of this study were to (1) determine the most effective concentration of arachidin-1 and arachidin-3 for inhibiting cell proliferation and (2) to assess the bioavailability of these compounds. Concentrations of 0, 5, 10, and 20 μM arachidin-1 and arachidin-3 were applied and cell viability was measured at 0, 24, and 48 hours. Significant reduction of cell proliferation occurred with treatments of 10 and 20 μM arachidin-1 and 10 and 20 μM arachidin-3 in comparison with the control. Due to the limit of HPLC detection, no transport values were determined when arachidin-1 and arachidin-3 were applied in 50 and 100 μM concentrations. The findings suggest that arachidin-1 and arachidin-3 inhibit cell proliferation in human colon cancer cells. Further research is needed to understand the bioavailability of arachidin-1 and arachidin-3.

Introduction

Cancer is one of the leading causes of mortality in the state of Arkansas and around the United States. Specifically, an incidence rate of 50.1% per 100,000 for males and 37% per 100,000 for females exists for colon cancer in the state of Arkansas (American Cancer Society, 2017). The mortality rate for colon cancer in Arkansas is approximately 18.2% per 100,000 people, which ranks third highest compared with other states in the United States (National Cancer Institute, 2015).

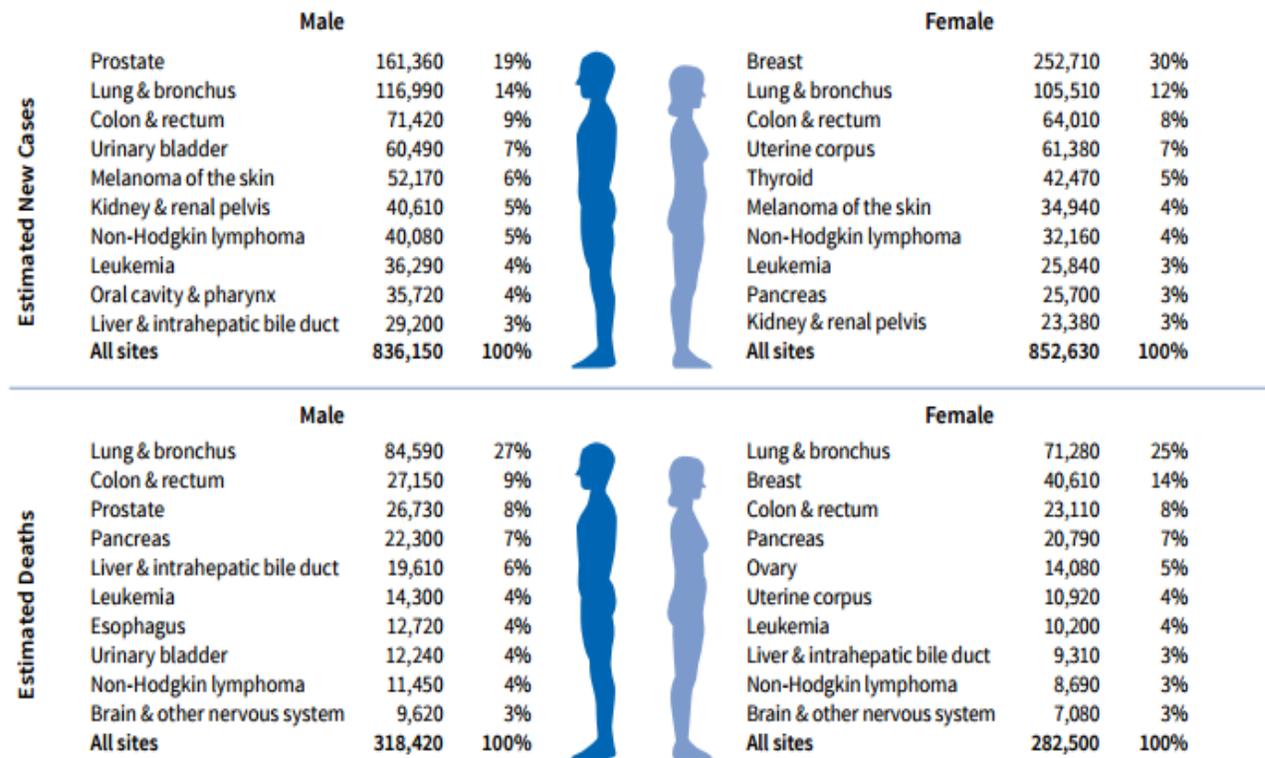
Genetic and lifestyle factors are potentially influential in the development of colon cancer (Burt et al., 2010). Prevention is important in reducing incidence and mortality rates associated with colon cancer (American Cancer Society, 2016). Amin et al. (2009) reported an inverse relationship between a diet rich in fruits and vegetables and diagnosis with colon cancer. This relationship can be attributed to the phytochemicals found in fruits and vegetables (Amin et al., 2009). The peanut plant (*Arachis hypogaea* L.) is a source of phytochemicals known as stilbenoids, which confer cancer preventative properties following extraction from the root or kernel of the peanut plant. Arachidin-1, arachidin-3, and resveratrol are stilbenoids derived from the peanut plant (Huang et al., 2010). While a significant amount of research has been conducted involving resveratrol, there is limited information available about the health-promoting properties of arachidin-1 and arachidin-3 (Chang et al., 2006).

Understanding the bioavailability of phytochemicals is essential for determining their biological and health effects in humans. Therefore, the objectives of the present study were to: 1) determine the effective concentrations of arachidin-1 and arachidin-3 for inhibiting colon cancer cell growth, and 2) assess the intestinal transport of arachidin-1 and arachidin-3 to determine which compound is more bioavailable.

Literature Review

Colorectal Cancer

Cancer remains the second leading cause of death in the United States with a mortality rate of 22.9% (Heron, 2015). Mortality rates related to cancer vary among people of different age, sex, and racial backgrounds (Heron, 2015). A study conducted by Moghaddam et al. (2007) reported that cancer of the colon and rectum contributes to a major portion of the morbidity and mortality occurring globally. Colon and rectum cancer is projected as the third leading type of cancer for estimated new cases and estimated deaths in 2017 (Figure 1, American Cancer Society, 2017).



Estimates are rounded to the nearest 10, and cases exclude basal cell and squamous cell skin cancers and in situ carcinoma except urinary bladder.

©2017, American Cancer Society, Inc., Surveillance Research

Figure 1. 2017 Estimation of Leading Cancer Sites. (American Cancer Society, 2017)

Several factors can influence the development of colorectal cancer. In fact, 30% of colorectal cancer cases stem from mutated genes predisposing individuals for development of the disease. These mutations are diagnosed through genetic testing. Genetic factors may be especially influential in the development of colon cancer if the individual has Lynch syndrome, familial adenomatous polyposis, or several other genetic conditions (Burt et al., 2010). Additional factors affecting colorectal cancer incidence rates include Type II diabetes and a family history of colon cancer (American Cancer Society, 2016).

About 70% of colorectal cancer cases are influenced by major lifestyle factors such as overweight/obesity, physical activity, alcohol consumption, tobacco use, and diet (American Cancer Society, 2016; Burt et al., 2010). These factors can be controlled by the lifestyle choices that individuals make (American Cancer Society, 2016). Precautionary measures such as regular health screenings in addition to consumption of a healthy diet for prevention of obesity can reduce colorectal incidence rates (American Cancer Society, 2016). Overweight or obesity resulting from lack of balance between nutritional intake and physical activity leading to excess weight increases the risk for colorectal cancer. An individual with obesity possesses a risk of 20% greater for developing colorectal cancer than individuals of normal weight (Moghaddam et al., 2007).

Epidemiological studies indicate that diet has implications on the incidence of colorectal cancer (Birt and Philips, 2014; Chan and Giovannucci, 2010; Chen et al., 2015; Kim and Milner, 2007). Diets characterized by large quantities of red meat, alcohol, simple sugars and refined grains are associated with obesity and higher incidence of colorectal cancer (Chen et al., 2015). Conversely, a diet consisting of vegetables, fiber, legumes, whole grains, poultry, fish, and a low amount of dietary fat is associated with lower incidence of colorectal cancer (Chan and

Gioannucci, 2010). Phytochemicals found in fruits and vegetables hold potential for prevention of cancer due to antioxidant activity (Amin et al., 2009).

Phytochemicals in the Peanut Plant

Chemical Structure of Resveratrol, Arachidin-1, and Arachidin-3

Phytochemicals such as resveratrol (trans-3,5,4'-trihydroxystilbene), arachidin-1 [trans-4-(3-methyl-1-butenyl)-3,5,3',4'-tetrahydroxystilbene], and arachidin-3 [trans-4-(3-methyl-1-butenyl)-3,5,4'-trihydroxystilbene] are stilbenoids derived from the peanut plant. Each of these phytochemicals have similar chemical structures. Arachidin-1 and arachidin-3 are the prenylated analogs of resveratrol. Figure 2 demonstrates the structural similarities of these compounds.

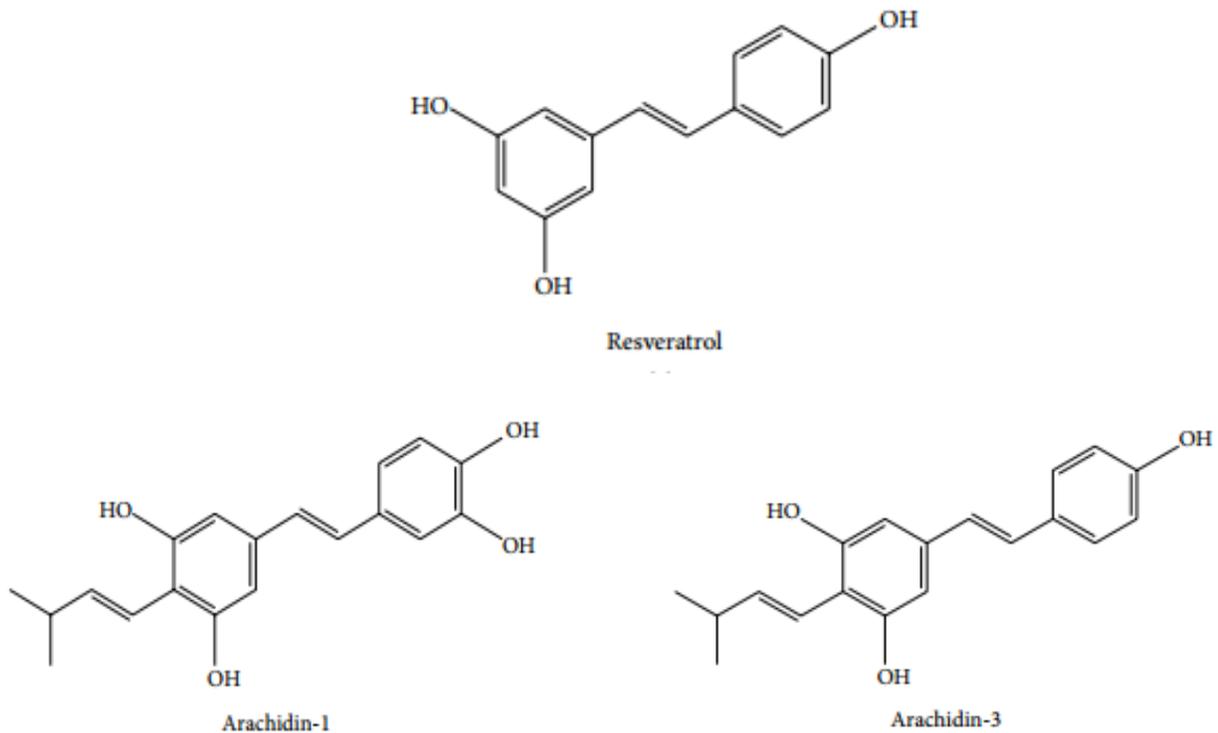


Figure 2. Chemical Structures of Resveratrol, Arachidin-1, and Arachidin-3. (Ball et al., 2015.)

Concentrations of Resveratrol, Arachidin-1, and Arachidin-3 in the Peanut

Resveratrol is found in various parts of the peanut plant in addition to being available in processed foods (Chang et al., 2006). Resveratrol in peanuts prepared by boiling contains 5.1 µg resveratrol per gram of boiled peanut while peanut butter contains 0.3 µg resveratrol per gram. No studies have detected arachidin-1 and arachidin-3 in peanuts. However, arachidin-1 and arachidin-3 are obtained for research purposes via extraction and purification from seeds and roots of the peanut plant, and range from 0.4 to 5.7 nmol/g dry weight (Condori et al., 2010).

Health Effects of Phytochemicals in the Peanut

Anti-Inflammatory Properties

Antioxidant activity of resveratrol has been widely investigated. This stilbenoid has been linked to anti-inflammatory effects through *in-vitro* cell study (Chang et al., 2006; Djoko et al., 2007; Huang et al., 2010). In a particular study, 15 µM arachidin-1, 15 µM arachidin-3, and 15 µM resveratrol were applied to RAW 264.7 mouse macrophage cells to cause inhibition of LPS (lipopolysaccharide)-induced responses (Chang et al., 2006). Macrophage cells are essential for inflammation, infection, and immune response. LPS results in activation of macrophage cells to secrete inflammatory responses such as prostaglandins (PGs) and nitric oxide (NO). The production of these mediators is associated with inflammation within the cell. Application of all three stilbenoids to RAW 264.7 macrophage cells resulted in reduced inflammation activity. Of the three, resveratrol yielded the greatest anti-inflammatory effect. Arachidin-1 yielded greater anti-inflammatory effects than arachidin-3 (Chang et al., 2006).

Nuclear factor kappa B (NF-κB) is a heterodimeric protein consisting of different combinations of transcription factors. NF-κB plays a major role in regulating transcription of

genes for cytokine production and cell survival. Activation of NF- κ B is associated with inflammation, cancer, and autoimmune diseases (Badr et al., 2009).

In a study conducted by Djoko et al. (2007), stilbenoids of similar structure including arachidin-1, piceatannol, and resveratrol were investigated for the reduction of inflammatory effects in RAW 264.7 mouse macrophage cells. The inhibition of LPS-induced NO and PGE₂ production was evaluated to determine their role in immune functions. The NF- κ B activity leading to inflammatory gene expression was also monitored (Djoko et al., 2007). The treatment of stilbenoids including resveratrol, piceatannol, and arachidin-1 correlates with reduced NF- κ B activity in RAW 264.7 macrophage cells. All three compounds inhibited productions of PGE₂ and NO in a dose-dependent manner. Arachidin-1 and resveratrol were applied in concentrations of 3.75, 7.50, 15.0, and 30.0 μ M while piceatannol was applied at 1.88, 3.75, 7.5, and 15 μ M concentrations. Application of arachidin-1, resveratrol, and piceatannol resulted in reduced inflammation responses. This study indicates that these derivatives have potential to contribute to nutraceutical applications by providing anti-inflammatory effects (Djoko et al., 2007). More research is needed to further determine the anti-inflammatory effects of prenylated stilbenoids (arachidin-1 and arachidin-3) since these derivatives have been less researched.

Anti-Cancer Properties of Phytochemicals

Excluding resveratrol, anticancer properties of stilbenoids have been minimally investigated. However, in the studies that have been done, resveratrol, arachidin-1, and arachidin-3 have also been linked to anti-cancer properties (Huang et al., 2010; Amin et al., 2009; Ko et al., 2013; Ball et al., 2015). Resveratrol and arachidin-1 induce anticancer activity by causing programmed cell-death or apoptosis in human leukemia HL-60 cells (Huang et al., 2010). Arachidin-1 was more effective than resveratrol for inducing programmed cell-death

(PCD) when both compounds were applied in concentrations of 0-20 μM . Optimal concentrations for achieving cell death were 4.2 and 17.6 μM , respectively, for arachidin-1 and resveratrol. Programmed cell-death is essential for maintaining equilibrium in cell division patterns. This occurs because caspase-independent pathways are activated in the process of PCD and these pathways lead to apoptosis when cells contain mutations in apoptotic genes (Huang et al., 2010). Apoptosis is important in eliminating mutated cells that can lead to the development of cancer (Badr et al., 2009). Cell exposure to arachidin-1 caused PCD to occur more efficiently than resveratrol due to activation of both caspase-dependent and caspase-independent pathways (Huang et al., 2010).

The results reported in Athar et al. (2009) support that resveratrol suppresses cancer cell growth by influencing cell cycle kinase activity and accumulation of p53 in cells. Cell cycle kinases are involved in the development of colorectal cancer and leukemia by phosphorylating receptors on the cell to affect tumorigenesis. Resveratrol stops transition between cell cycle phases so that cells with mutated DNA either cannot double their DNA or divide (Athar et al., 2009). Resveratrol also increases p53 levels and decreases Bcl-2 levels. P53 is a transcription factor that functions as a tumor suppressor gene and monitors for mutations. Bcl-2 is an anti-apoptosis protein that is expressed less frequently with increased p53 levels present in a cell. As a result, high levels of p53 and low levels of Bcl-2 resulting from exposure of cells to resveratrol can lead to apoptosis of cancer cells (Athar et al., 2009). Since arachidin-1 and other stilbenoids are similar in structure to resveratrol, these stilbenoids have the potential to confer anticancer properties. It is necessary to further investigate the specific roles of arachidin-1 and arachidin-3 in anticancer effects.

Bioavailability of Phytochemicals in Peanut Plant

Bioavailability refers to the rate at which a nutrient is sent to the bloodstream for use in the body (Parada and Aguilera, 2007). Chukwumah et al. (2011) evaluated the transport and absorption of *trans*-resveratrol using a Caco-2 cell monolayer. The preparation of the peanut derivative impacts the rate of transport, or bioavailability. A study examining bioavailability of *trans*-resveratrol used different preparation methods including boiling and roasting the peanuts. Roasted peanuts yielded better transport ability for *trans*-resveratrol than boiled peanuts (Chukwumah et al., 2011).

Bioavailability studies on arachidin-1 and arachidin-3 are not available. However, a strong understanding of bioavailability for arachidin-1 and arachidin-3 is necessary so that knowledge of biological activity by the compounds are available.

Materials and Methods

Materials

Arachidin-1 and arachidin-3 were generously donated by Dr. Medina-Bolivar in their pure form (Arkansas State University, Jonesboro, AR). All reagents and media components were purchased from ThermoFisher (Waltham, MA).

Methods

HPLC Analysis

To measure arachidin-1 and arachidin-3, a System Gold high performance liquid chromatograph (Beckman-Coulter, Fullerton, CA, USA) with autosampler (model 508), dual pump (model 126), photodiode array detector (model 168) with Beckman-Coulter System 32 Karat software (version 8, 2006) was used. Arachidin-1 and arachidin-3 were separated using the method proposed by Abbott et al. (2010) to ensure the compounds being used for the study were pure. Separation of the phytochemicals occurred with the Phenomenex (Torrance, CA) Aqua 5 μ M C18 (250 x 4.6 mm) column containing a binary gradient of 2% formic acid [mobile phase A] and 99:1 (v/v) acetonitrile/mobile phase A [mobile phase B]. The gradient began with 10% B and increased uniformly to 18% for approximately 8 minutes. The gradient was then held at 18% B for 2 minutes before increasing linearly to 25% B over the course of 5 minutes, and increasing to 35% B for 3 minutes. Then the gradient remained isocratic or constant and uniform in the mobile phase for 20 minutes at 35%. B increased uniformly to 60% over the course of 59 minutes before returning to the initial binary gradient of 10%. The peaks of arachidin-1 and arachidin-3 were monitored at 340 nm.

Cell Culture

Caco-2 cells, a human epithelial colorectal adenocarcinoma cell line, purchased from American Type Culture collection (ATCC, Rockville, MD) were incubated at 37°C in an atmosphere containing 5% CO₂. Cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), 1 % nonessential amino acids solution, and 1% antibiotic-antimycotic. Cell cultures were maintained under sterile conditions and incubated at 37°C, 70% humidity, and 5% CO₂ in the incubator (VWR® symphony™, VWR® International LLC, Radnor, PA).

Cell Viability Assay

Caco-2 cells, passage number 36-42, were used for the viability assay. For a standard curve, cells attached to the chambers of a 96-well microplate (Corning Inc., Tewksbury, MA) for 24 hours after being seeded in a range from 0 to 3.2x10³ cells with 100 µL working media (WMEM). For the experiment, cells (2 x 10³) were seeded in wells of a 96 well plate for 24 hours. Three separate plates were prepared for each experiment. A total of five experiments were performed over the course of this study. At the end of the 24 hour period, the media was aspirated and then cells were treated with 100 µL of 0, 5, 10, or 20 µM concentration of arachidin-1 or arachidin-3. These concentrations were chosen based off of results from previous studies evaluating cell viability. Dried arachidin-1 or arachidin-3 was suspended in DMEM in the quantity deemed calculated when the density was converted to moles. The compounds were then suspended in media to make a 100 µM stock solution and then dilutions with media were made accordingly. Each treatment was applied in quadruplicate. The control was treated with

100 μL of 0.1% DMSO. Measurements were obtained at 0 (T0), 24 (T24), and 48 (T48) hours following treatment in order to determine cell viability. When the absorbance was read at T0-T48, 20 μL of cell titer reagent (CellTiter96® Aqueous One Solution Proliferation Assay, Promega Co., Madison, WI) was added to each well. Using a plate reader (Synergy HT Multi-Mode Microplate Reader, BioTek Instruments, Inc. Winooski, VT) the absorbance of the cells were measured at 490nm. This absorbance was converted to the number of viable cells using an equation from the standard curve. Based on the number of viable cells present at T0, a proliferation percentage was calculated for T24 and T48.

Intestinal Transport Assay

Caco-2 cells were seeded at a density of $1 \times 10^5/\text{cm}^2$ cells on polycarbonate membrane inserts (12 mm diameter, pore size 0.4 μM) fitted in bicameral chambers (Corning Inc., Tewksbury, MA). Cell monolayer integrity was confirmed by determining the transepithelial electrical resistance (TEER) values using the Millicell ERS-2 Voltohmmeter (EMD Millipore, Billerica, MA). After seeding, media was aspirated from both the apical and basal chambers. 1 mL of PBS was added to the basal chamber and 0.5 mL of 50 and 100 μM arachidin-1 or arachidin-3 was added to the apical chamber.

For time 0h, transport media containing arachidin-1 (or arachidin-3) was loaded and 0.5 mL of PBS were collected immediately in a vial. Each vial is preserved with 25 μL trifluoroacetic acid (TFA) and 25 μL 70% ethanol. At this point, 0.5 mL of PBS was replaced in the basal chamber. The plate was incubated at 37°C for 30 minutes. For time 0.5h and time 1h, steps performed for time 0 were repeated. The plate was incubated for 1 hour following time 1. At the end of 2 hours, media from the apical was collected in a vial and rinsed with 0.5 mL of

PBS three times. PBS was removed from the basal chamber, collected in a vial, and rinsed with 1 mL of PBS. Each vial was preserved with 25 μ L TFA and 25 μ L 70% ethanol. All aliquots were frozen at -20°C until analysis via HPLC. Apparent permeability coefficients (P_{app}) was calculated using the following equation:

$$P_{app} = \left(\frac{dQ/dt}{C_0 \times A} \right)$$

Where dQ/dt is the rate of permeation across the cell, A is the area of the cell monolayer, and C_0 is the initial concentration of the apical chamber.

Statistical Analysis

All statistical analyses were carried out by JMP software (version 13; SAS Institute Inc., Cary, NC) using a one-way analysis of variance test (ANOVA). ANOVA results were compared with the student's t test. Data was represented as means \pm standard error of mean (SEM).

Statistically significance was accepted at $P < 0.05$.

Results

Identification of Arachidin-1 and Arachidin-3

Arachidin-1 and arachidin-3 used for this experiment were identified as pure compounds at 304 nm. The results are shown in Figure 3.

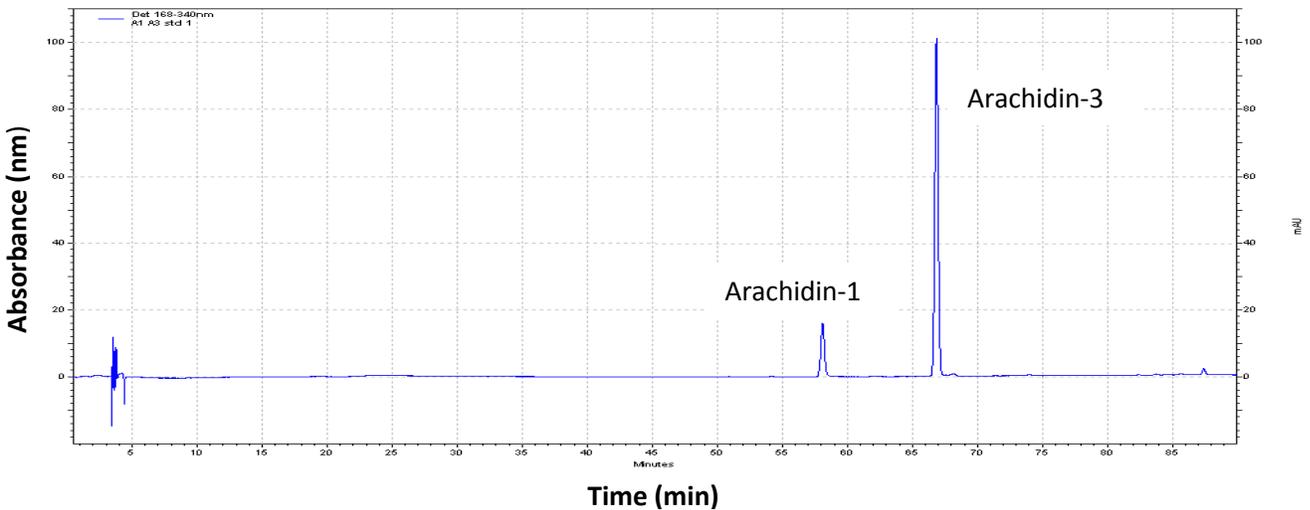


Figure 3. High Performance Liquid Chromatogram of arachidin-1 and arachidin-3 at 340 nm.

Cell Viability after Arachidin-1 and Arachidin-3 Treatment

The data presented in Figures 4 and 5 shows that arachidin-1 and arachidin-3 treatments (10 μ M and 20 μ M) reduced cell viability compared to control.

At the 48 hour time point, 10, and 20 μ M concentrations of arachidin-1 resulted in a significant reduction of cell viability (%) compared to the control ($P < 0.05$) (Figure 4). There was no significant difference of cell viability between treatments and control at time point 24 hours.

The cell viability was significantly reduced in treatments of 10 and 20 μ M arachidin-3 compared to 5 μ M arachidin-3 and the control at time point 48 hours ($P < 0.05$) (Figure 5).

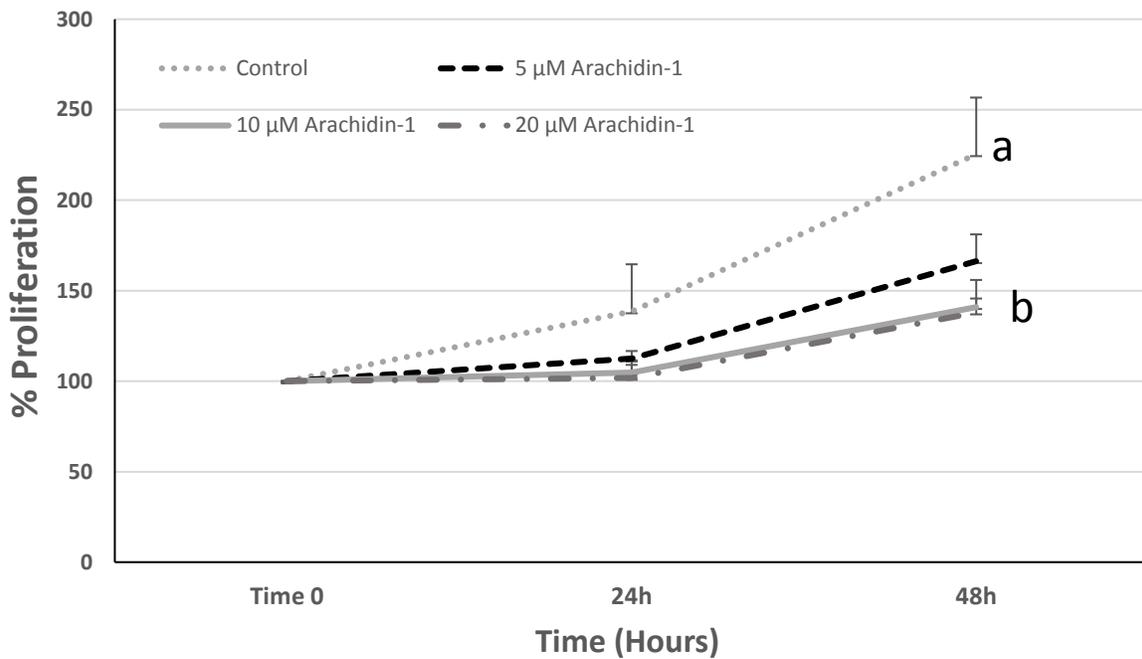


Figure 4. Effect of arachidin-1 on the survival of Caco-2 cells. Values with different letters at the same incubation time are significantly different ($P < 0.05$).

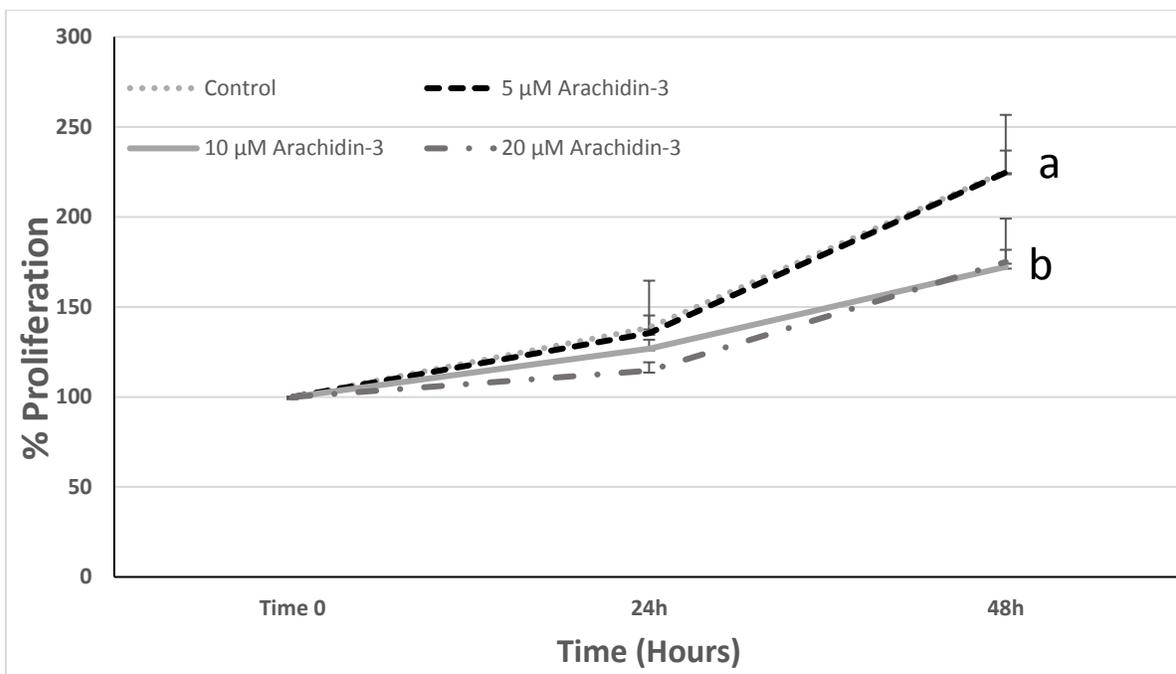


Figure 5. Effect of arachidin-3 on the survival of Caco-2 cells. Values with different letters at the same incubation time are significantly different ($P < 0.05$).

At time point 48 hours, 10 and 20 μM concentrations of arachidin-1 showed the lowest cell viability in comparison with cell viability measured after treatments of 10, or 20 μM arachidin-3 or control ($P < 0.05$).

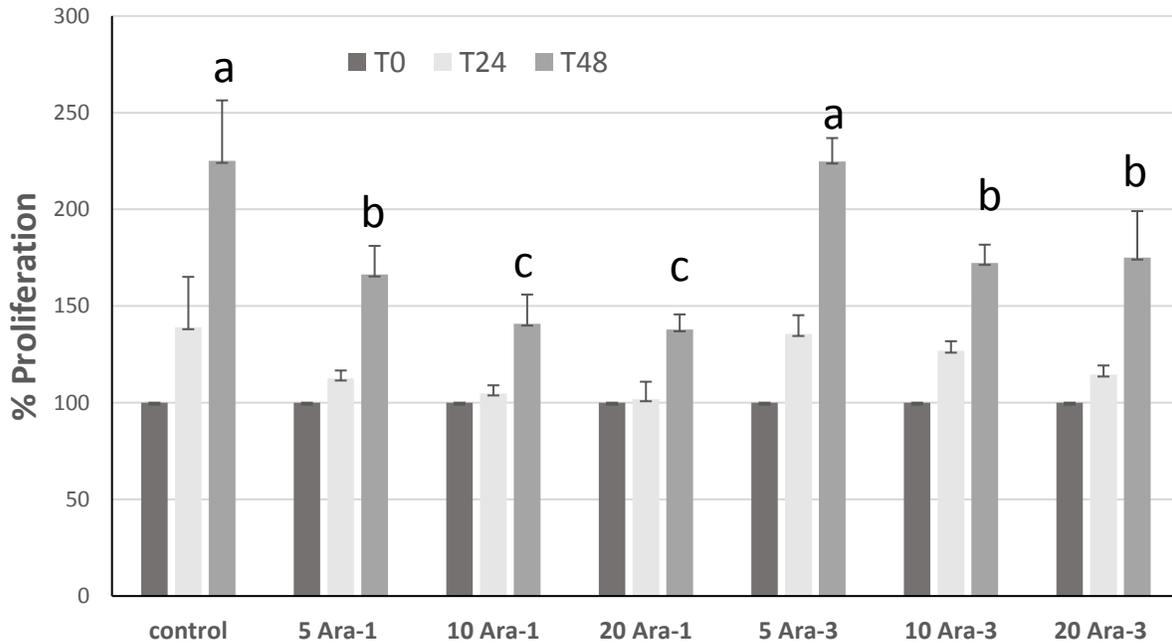


Figure 6. Summarized effects of arachidin-1 and arachidin-3 on the survival of Caco-2 cells. Values with different letters at the same incubation time are significantly different ($P < 0.05$).

Intestinal Transport Assay of Arachidin-1 and Arachidin-3 Treatment

Arachidin-1 was not detected in samples from both apical and basal chambers treated with 50 μM or 100 μM at time point 2 hr (Figure 7). At time point 2 hr, a trace amount of arachidin-3 was detected in apical sample treated with 100 μM (Figure 8). Apparent permeability coefficient (P_{app}) values of arachidin-1 and archidin-3 were not determined.

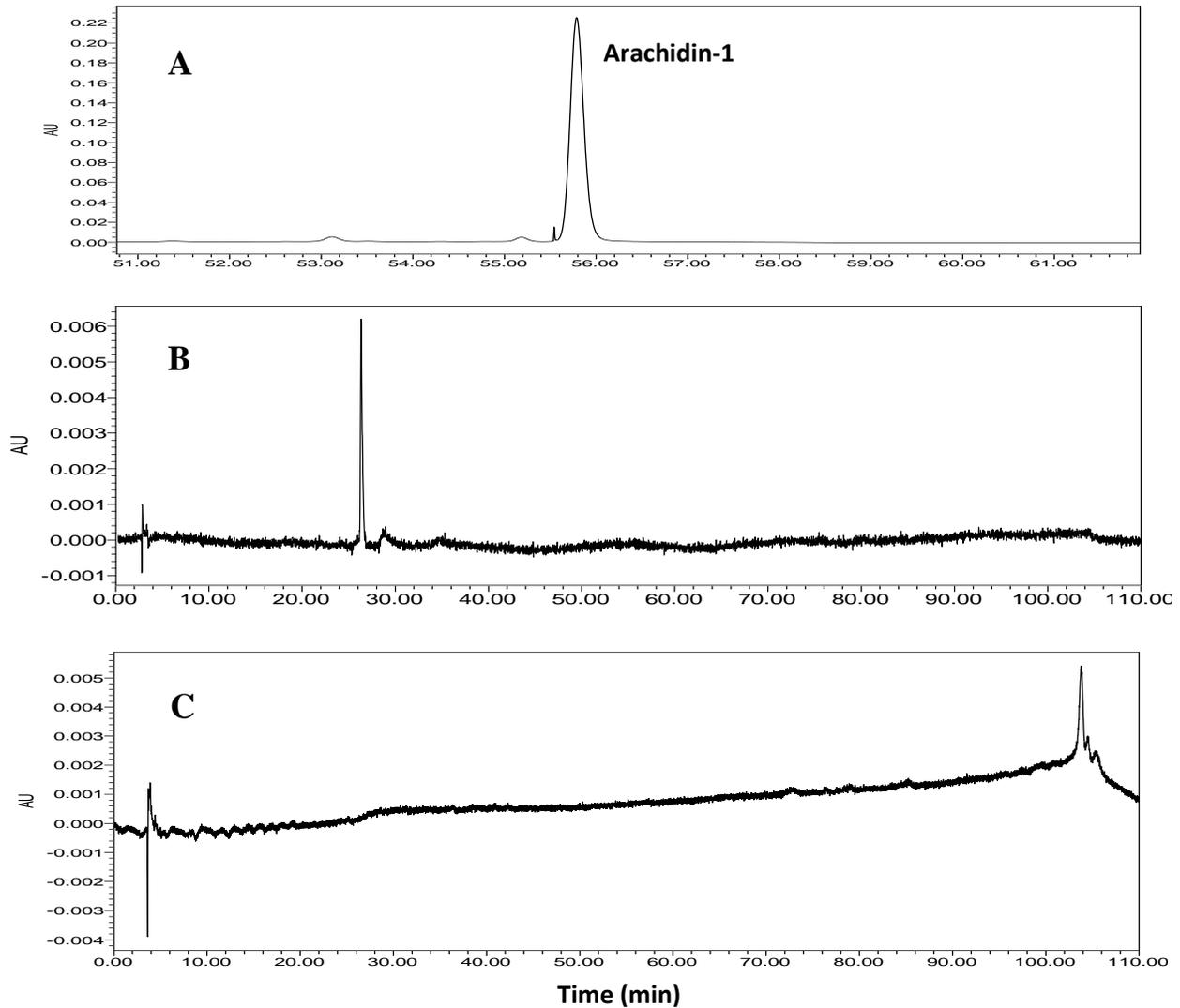


Figure 7. HPLC Chromatogram of (A) arachidin-1 standard, (B) apical sample treated with 100 μM of arachidin-1 and (C) basal sample treated with 100 μM of arachidin-1 at time point 2 hr.

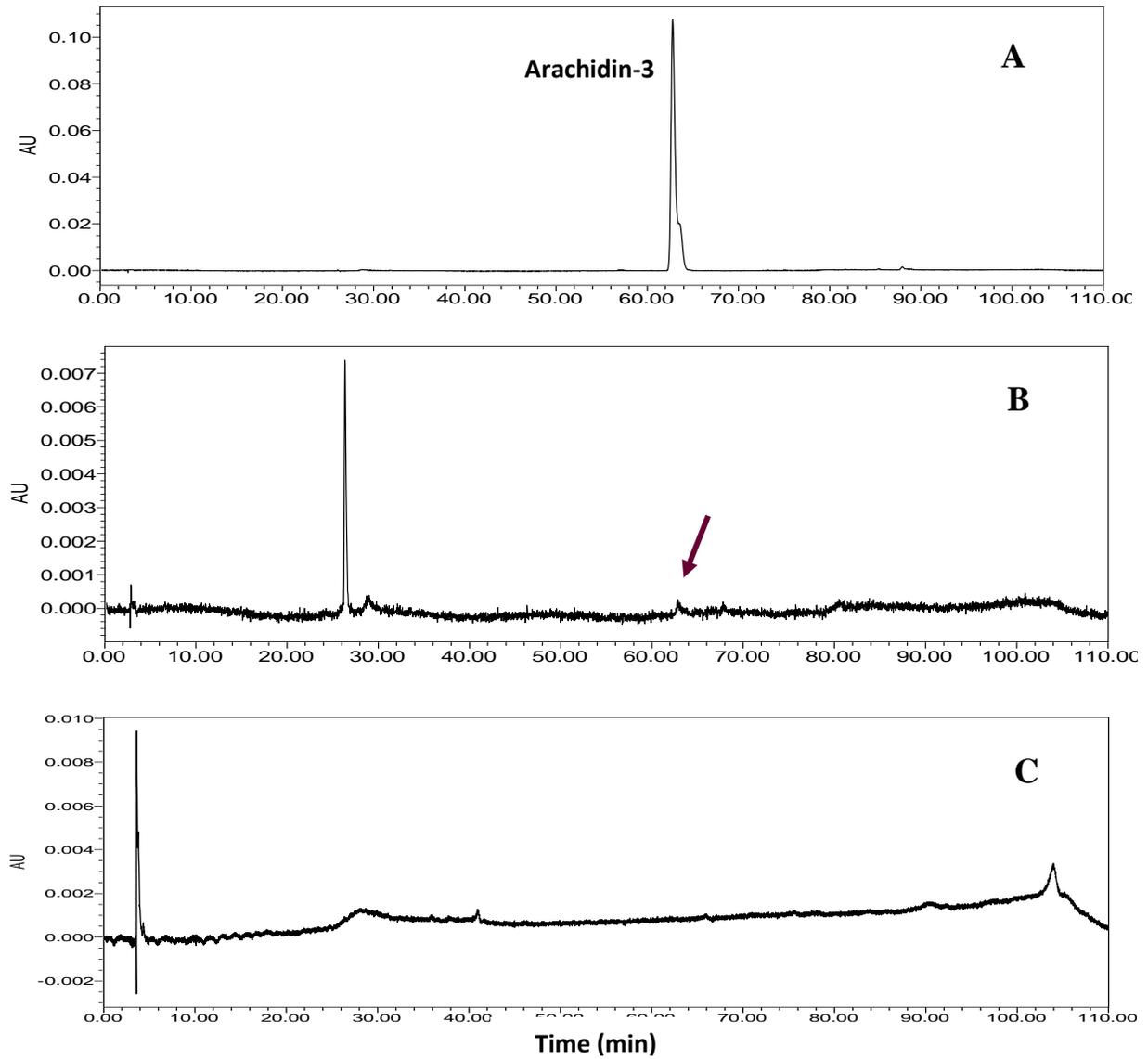


Figure 8. HPLC Chromatogram of (A) arachidin-3 standard, (B) apical sample treated with 100 μM of arachidin-3 and (C) basal sample treated with 100 μM of arachidin-3 at time point 2 hr.

Discussion

The peanut root contains phytochemicals that confer anticancer and anti-inflammatory effects to the body for a nutraceutical approach. Arachidin-1 and arachidin-3, derivatives from the peanut plant, have been involved in limited research relating to their health effects in the body for cancer prevention. Therefore, the anti-colon cancer activity and bioavailability of arachidin-1 and archidin-3 have been investigated in this study.

In order to understand the anticancer properties linked with arachidin-1 and arachidin-3, it is important to evaluate the effect of these compounds on colon cancer cell proliferation. In the present study, both arachidin-1 and arachidin-3 treatments (10 and 20 μM) resulted in reduced cell proliferation rates compared with the control ($P < 0.05$). The most significant reduction of cell proliferation occurred at time point 48 hours when arachidin-1 was applied at concentrations of 10 and 20 μM in comparison with the control or 5, 10, and 20 μM arachidin-3 treatments ($P < 0.05$).

The limited studies have investigated the anticancer properties of arachidin-1 and arachidin-3 in different cell lines with different concentrations of stilbenoids (Huang et al., 2010; Ko et al., 2013; Ball et al., 2015). Cell viability results from a study on the effect of 0 to 20 μM concentrations of arachidin-1, arachidin-3, and resveratrol on human leukemia cell (HL-60) proliferation reported that arachidin-1 resulted in reduced cell viability (Huang et al., 2010). Another study investigated the effects of metformin and arachidin-1 upon dose-dependent application to A549 and H1975 human lung cancer cell lines. Metformin is an anti-diabetic drug that is associated with anticancer properties. Treatments of 0.5, 1, 5, and 10 μM arachidin-1 with 25 μM metformin over the course of a 4-day incubation period resulted in reduced cell viability. (Ko et al., 2013).

10 and 20 μM concentrations of arachidin-1, resveratrol, piceatannol, and arachidin-3 were applied to rotavirus infected HT29.F8 cells from the human adenocarcinoma line of the intestine. These concentrations of stilbenoids did not result in a substantial decrease in cell viability. However, application of arachidin-1 or arachidin-3 resulted in reduced viral replication and can be attributed to antioxidant activity of these compounds (Ball et al., 2015). Additional research must be conducted to understand the effects of stilbenoids from the peanut plant on cell viability.

Previous studies regarding the transport abilities of arachidin-1 and arachidin-3 have not been conducted. Due to the limit of detection in the HPLC system, the apparent transport of arachidin-1 and arachidin-3 were not calculated. Additional research must be conducted with higher concentrations of arachidin-1 and arachidin-3 in order to evaluate bioavailability.

Conclusions

The results of this study indicate that concentrations greater than 10 μM arachidin-1 and arachidin-3 did reduce cell viability in human colon cancer cells in comparison with the control. Arachidin-1 was more effective for inhibiting cell proliferation than arachidin-3. No apparent intestinal transport occurred, so it is likely that concentrations of arachidin-1 and arachidin-3 greater than 100 μM are needed to determine bioavailability. Additional research is needed to establish the chemopreventative or nutraceutical applications of arachidin-1 and arachidin-3.

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