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# Anti-Neoplastic Effects of Extracts from Gnaphalium gracile on Colon, Pancreatic, and Prostate Cancer Cells

Joshua R. Canter

*East Tennessee State University*

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Anti-Neoplastic Effects of Extracts from *Gnaphalium gracile* on Colon,  
Pancreatic, and Prostate Cancer Cells

Thesis submitted in partial fulfillment of Honors

By

Joshua Ryan Canter  
The Honors College  
University Honors Scholar  
East Tennessee State University

February 20, 2015

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Victoria Palau, Ph.D., Faculty Mentor

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Michael Cody, Ph.D., Faculty Reader

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Cerrone Foster, Ph.D., Faculty Reader

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***Abstract***

Over 4,000 flavonoids have been identified, and among these, many of them are known to possess cardioprotective, anti-inflammatory, antimicrobial, and antitumor effects. However, most of these properties have yet to be fully understood. In this study, extracts from *Gnaphalium gracile*, thought to possess a mixture of flavonoids, have been tested for cytotoxic activity on pancreatic (MiaPaca, Panc28), colon (HCT-116, Caco-2), and prostate (PC3, LNCaP), cancer cell lines. Polar extracts from the leaves of *G. gracile* have the most cytotoxic effect on these cancer cell lines, particularly the prostate cancer cell lines PC3 and LNCaP. Evidence suggests the extracts have antineoplastic effects on these cancer cells lines possibly due to differentiation status on pancreatic and colon cancer, but not prostate cancer. Cytotoxic activity is not dependent on tumorigenic potential. Further research is needed to identify the bioactive compounds within these extracts.

## ***Introduction***

In 2014 alone, an estimated 376,250 people will be diagnosed with colon, pancreatic, or prostate cancer [1,2,3]. Characterized by the uncontrolled growth of cells of the respective organ sites, these cancers can be fatal if not diagnosed early. The five-year survival rates of these cancers, with the exception of prostate cancer, are extremely low. For pancreatic cancer, the five-year survival rate is 1-14% depending on how far the cancer has progressed [4]. With survival rates of 6-74% [5], colon cancer survival rates are much more dependent on an early diagnosis. Five-year survival rates of men with prostate cancer are nearly 100% due to the ability to remove the prostate [6], but it drops to 27.8% for metastatic disease [7]. Thus, there is a continued need for newer treatment approaches in order to better control metastatic prostate cancer and improve survival.

Modern medicine has developed three main treatment options for combatting different types of cancer. These options are available for healthcare providers to choose from: surgery, radiation therapy, and chemotherapy.

Perhaps the most effective means to treat cancer is physically removing the malignancy from the body [8]. Although surgery has the best chance of sending patients into remission, there are many detrimental effects to the patient physically and mentally. Frequently, large parts of the organ or the whole organ must be removed to excise all of the cancerous tissue. This leaves the patient with a loss of function, which decreases quality of life in the short and long term. Additionally, it is often difficult to convince the patient to undergo surgery. The idea of a major surgery is daunting for many patients who have never dealt with a similar situation. As a result, families typically suffer

because they watch their loved ones slowly decline in health when there is a treatment option available.

Radiation therapy is defined as the treatment of cancer patients using high-energy particles or waves directed at the site of the malignancy. Radiation works by damaging the DNA of cancer cells. When the DNA is damaged, parent cells can no longer divide and are eliminated from the body, thus halting the growth of the tumor. Although an effective means of destroying cancer cells, radiation therapy also destroys the DNA of normal cells in the body. As a result, healthcare providers must plan radiation treatment so that damage to normal cells is minimized. Because of this, there are many side effects of radiation treatment. Normal actively dividing cells in the body such as those of the salivary gland or epithelial tissue are also especially vulnerable to this type of treatment. Many patients suffer from nausea and vomiting, hair loss, and overall fatigue from radiation therapy [9].

Chemotherapy is a widely used treatment option and is characterized by the use of drugs to combat cancer cells. Chemotherapy targets cells that are actively growing and dividing. However, some agents may also include cells that are not traversing the cell cycle. As mentioned earlier, normal cells of the body such as epithelial tissue are always actively growing and dividing, so they become vulnerable to chemotherapy as well. Chemotherapy is similar to radiation therapy in that the side effects of the treatment occur when normal cells are destroyed in the process of targeting the tumorigenic cells. However, the main difference between the other two main treatment options and chemotherapy is that it has the capacity to work throughout the body whereas surgery and

radiation therapy are limited to specific areas. In this way, chemotherapy may in some cases be a more effective method of treating metastatic tumors [10].

With these treatment options and statistics in mind, it is easy to see the need for more effective means of treating patients with varying stages of cancer that do not result in the physical and psychological damage that is typically seen in these individuals. For example, according to Ashbury et. al., about 14% of cancer patients are prescribed antidepressants [11]. Clinically depressed patients experience a lower quality of life that may have been avoided if current treatment options were able to eliminate the physical and psychological damage that inevitably impact most cancer patients.

To investigate other treatment options that spare normal cells, ethnobotanical studies were considered. For the treatment of cancer, in the Andean regions of South America, there is a group of medicinal plants known as *vira-viras*. They belong to the family *Asteraceae* and to the genera *Gnaphalium*, *Achyrocline*, and *Gamochaeta*. They are annuals or perennials that grow between 2,000 and 3,000 meters above sea level. In this region and other parts of the world, they are used as a hemostatic, to ease inflammation, and to fight infection. In this region of South America, these plants are commonly used to battle cancer [12]. In this study, extracts from *Gnaphalium gracile* were tested for antineoplastic activity on cancer cells from the colon, pancreas, and prostate with varying states of differentiation. Among the bioactive compounds present in these extracts, flavonoids stand out as the molecule with the highest therapeutic potential. Certain flavonoids have been shown to promote defense mechanisms against cancer,



partly due to the counteraction of free radicals in the body [13]. Other flavonoids and flavonoid derivatives [14] have been shown to induce cell cycle arrest, particularly at the G1/S [15] and G2/M [16] phases. In other studies, certain flavonoids have been observed to stimulate DNA repair machinery [17]. In many instances, there is an inverse relationship with dietary flavonoid intake and risk of cancer [18]. The role of these compounds as a potential ingredient in therapeutic drugs has yet to be fully explored. Because of the known properties of flavonoids, it has been hypothesized that the extracts from *Gnaphalium gracile* will demonstrate significant antineoplastic activities against the cancer cell lines.



## ***Materials and Methods***

### **Cell Culture**

Tumor derived cells that originated in multiple tissue sites were obtained from the American Tissue Type Culture Collection (ATCC, Manassas, VA), including colon (Caco-2 and HCT-116), pancreatic (MIA PaCa), and prostate (LNCaP and PC-3), and were maintained according to ATCC instructions. These cell lines, including their differentiation status are highlighted in **Table 1**. The Panc-28 cell line was a gift from Dr. Paul Chiao (University of Texas M. D. Anderson Cancer Center, Houston, TX) and was grown in tissue culture in the same manner as pancreatic cell line MIA PaCa-2, in Dulbecco's modified Eagle's medium with high glucose, supplemented with 10% serum and penicillin/streptomycin (GIBCO Invitrogen, Carlsbad, CA). All cells were seeded in 48 well plates and allowed to reach 75% confluency before treatment.

Cell Line	Differentiation Status
Pancreas - MiaPaca	Poor
Pancreas – Panc28	Better
Colon – HCT - 116	Poor
Colon - CaCo-2	Better
Prostate – PC3	Poor
Prostate - LNCaP	Better

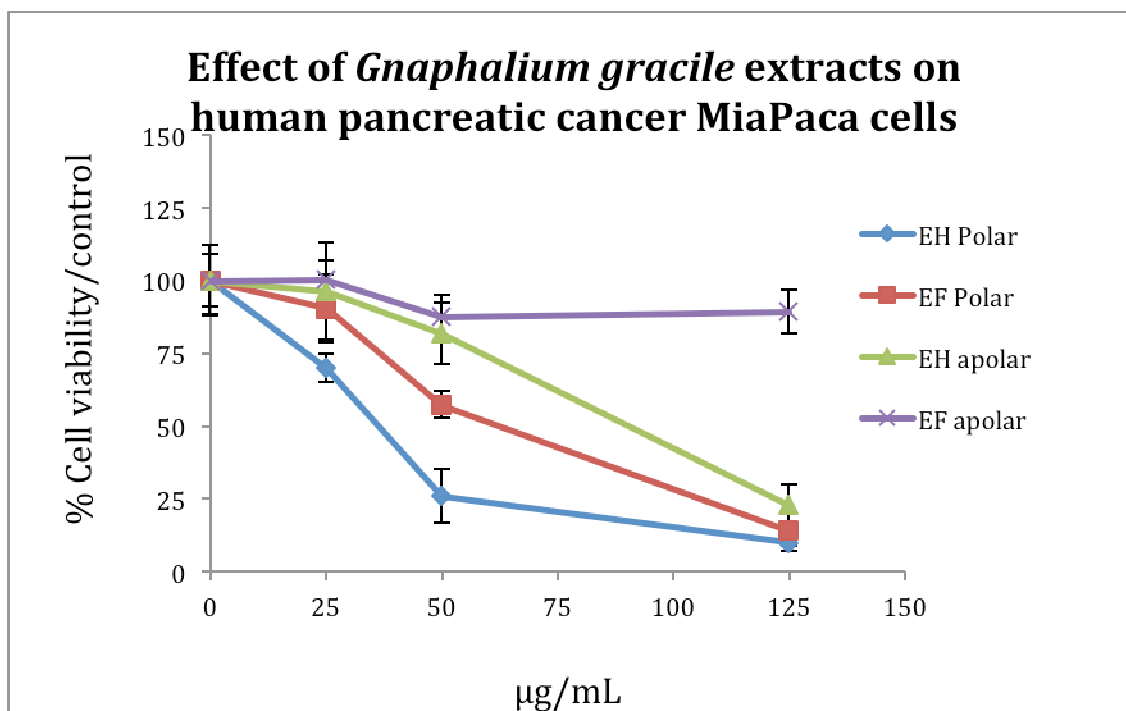
**Table 1: Name and differentiation status of each cell line.** Poorly differentiated cells lines are less similar to their normal cell counterparts. Better-differentiated cell lines are more like normal cells. Differentiation status is used to determine the grade of the cancer and assist in planning treatment options for patients.

## Procedure to Obtain Extracts

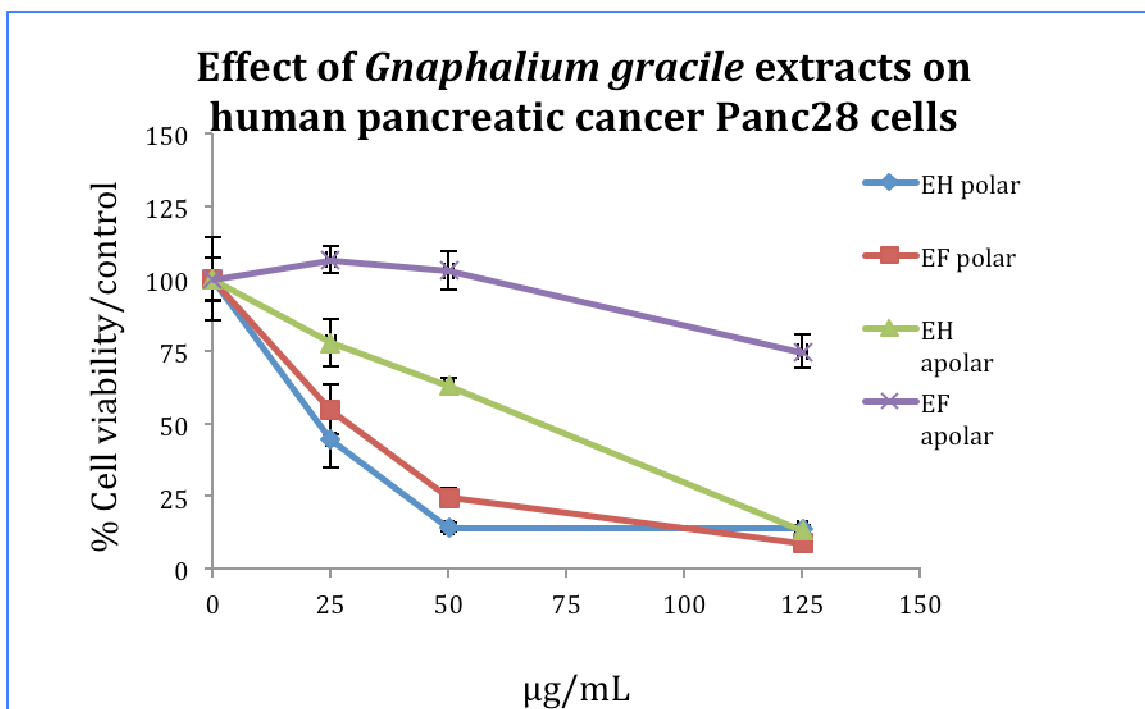
Plants of *G. gracile* HBK were collected during the months of July through September in the area surrounding the Tominé reservoir, in the municipality of Guatavita, in Cundinamarca, Colombia. The specimens were identified at the Colombian National Herbarium. Leaves and flowers were separated, dried in the shade, and soxhlet extracted with AcOEt. From 230g of flowers, 17g of extract was obtained. Following the same procedure, but separately, 70g of extract was obtained from 645g of leaves and stems. 5g of each extract were fractionated sequentially with petrol and ethanol, to obtain the apolar fractions (in petrol) and polar (in EtOH) of leaves and flowers. The fractions were designated as EF apolar, EF polar, and EH polar, EH apolar for flowers and leaves respectively, and were subsequently dried under vacuum. A 10g sample of leaf extract was subjected to column chromatography on SiGel, with mobile phase Petrol: EtOAc 8:2. Fractions of 50 mL were collected and isolated and identified as stigmasterol, dehydrostigmasterol and gnaphaline in fractions 6 and 12 respectively. In the same manner, gnaphalin A was identified after isolation from the flower extracts. It was found in a higher proportion of about 0.3% of dry material. Column chromatography with mobile phase RP18 and EtOH : water 7:3, using the polar extract of leaves or flowers, quercetine, and a mixture of 3- methoxyquercetine and quercetin were found. The compounds were identified by comparison of their spectra with  $^1\text{HNMR}$  and  $^{13}\text{CNMR}$  spectra of authentic standards.

### Cell Survival (MTT) Assay

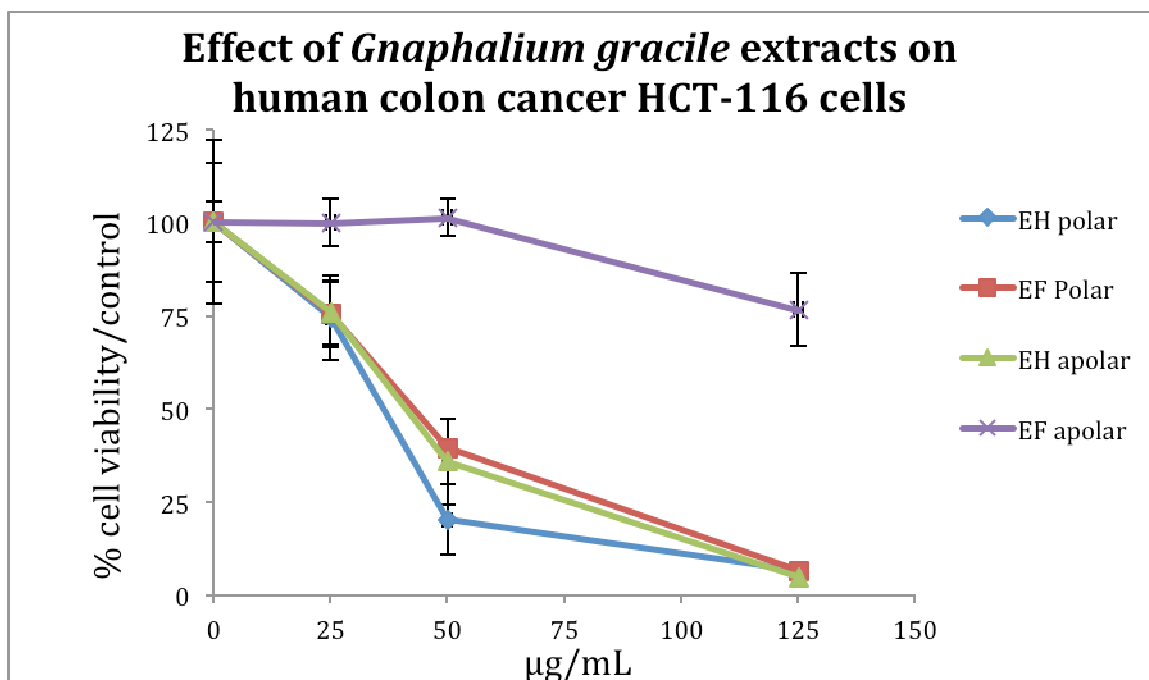
Cells were treated with either EF polar, EF apolar, EH polar, or EH apolar extracts at concentrations of 25, 50, or 125  $\mu\text{g/ml}$ . The dissolution vehicle was dimethyl sulfoxide at a maximum final concentration of 0.3% in the treated well (Sigma- Aldrich, St. Louis, MO) After 24 hours of incubation, 3-(4, 5-methyl-thiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) (Sigma- Aldrich) was added at 500 $\mu\text{g/ml/well}$  for 3 hours. In the mitochondria of cells, NADH-dependent cellular oxidoreductase enzymes, such as NADH dehydrogenase, reflect the number of viable cells. These enzymes are capable of reducing the tetrazolium dye to its insoluble formazan, which is purple in color. Formazan products were solubilized with acidified 2-propanol (0.1N HCl). Assays were quantified by reading optical density at a wavelength of 590 nm using a Biotek PowerWave XS2 plate reader (Winooski, VT) [11]. Statistical analyses were used to determine the significance difference with  $p < 0.05$ . Half maximal inhibitory concentrations were calculated and are shown in **Table 2**.

**Results**

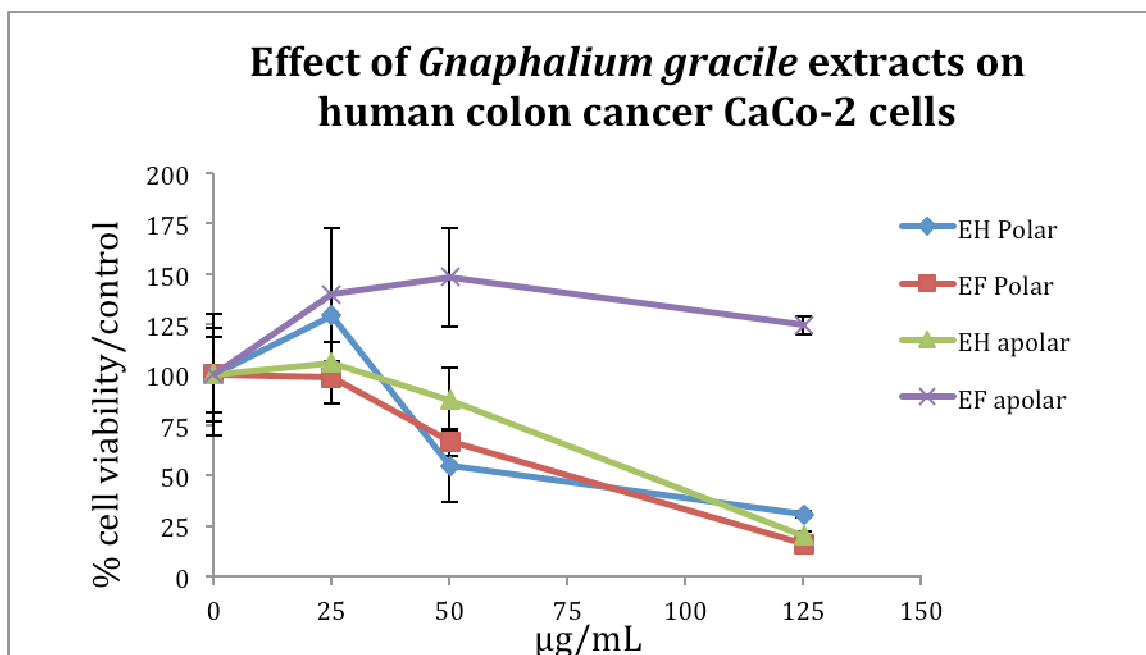
**Figure 1: Effect of EH polar, EF polar, EH apolar, and EF apolar extracts on human pancreatic cancer MiaPaca cells.** MiaPaca cells were dosing with 25, 50, and 125 µg/mL of drug. The control was dosed using dimethylsulfoxide (DMSO). MTT assay was used to determine the percent of cell viability after dosing. Significant difference between the control and each concentration was determined with p values < 0.05.



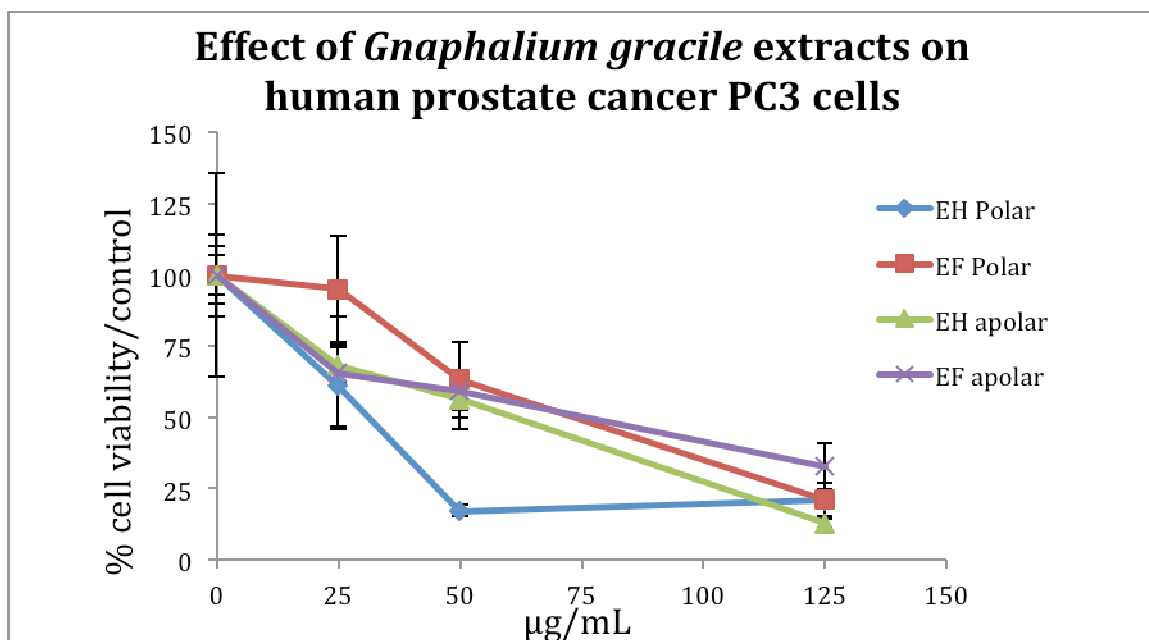
**Figure 2: Effect of EH polar, EF polar, EH apolar, and EF apolar extracts on human pancreatic cancer Panc28 cells.** Panc28 cells were dosed with 25, 50, and 125 µg/mL of drug. The control was dosed using dimethylsulfoxide (DMSO). MTT assay was used to determine the percent of cell viability after dosing. Significant difference between the control and each concentration was determined with p values < 0.05.



**Figure 3: Effect of EH polar, EF polar, EH apolar, and EF apolar extracts on human colon cancer HCT-116 cells.** HCT-116 cells were dosed with 25, 50, and 125 µg/mL of drug. The control was dosed using dimethylsulfoxide (DMSO). MTT assay was used to determine the percent of cell viability after dosing. Significant difference between the control and each concentration was determined with  $p$  values  $< 0.05$ .

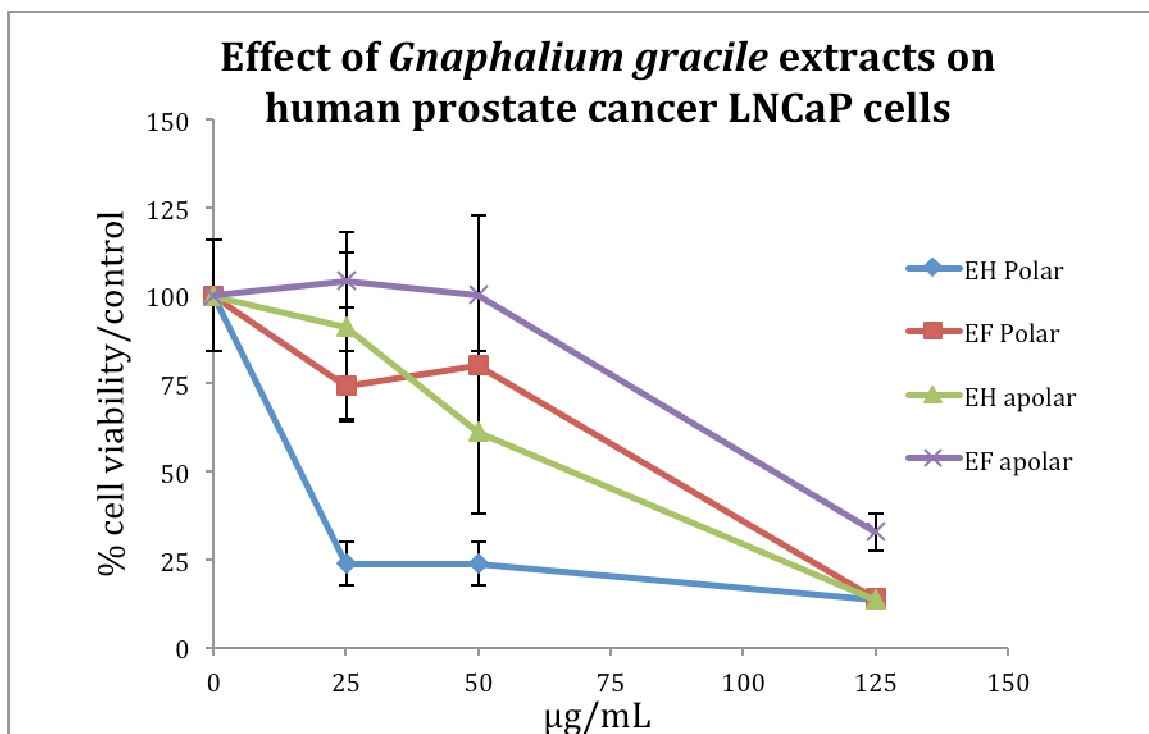


**Figure 4: Effect of EH polar, EF polar, EH apolar, and EF apolar extracts on human colon cancer Caco-2 cells.** Caco-2 cells were dosed with 25, 50, and 125 µg/mL of drug. The control was dosed using dimethylsulfoxide (DMSO). MTT assay was used to determine the percent of cell viability after dosing. Significant difference between the control and each concentration was determined with p values < 0.05.

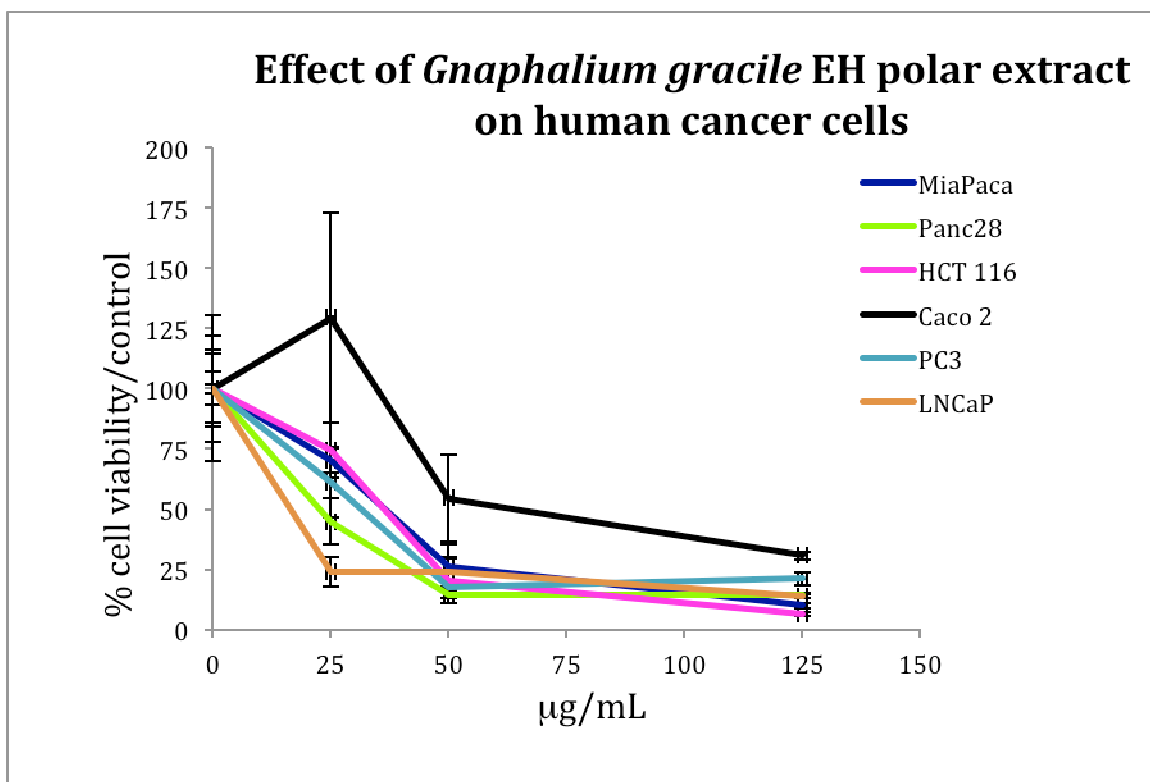


**Figure 5: Effect of EH polar, EF polar, EH apolar, and EF apolar extracts on human prostate cancer PC3 cells.** PC3 cells were dosed with 25, 50, and 125 µg/mL of drug. The control was dosed using dimethylsulfoxide (DMSO). MTT assay was used to determine the percent of cell viability after dosing. Significant difference between the control and each concentration was determined with p values < 0.05.

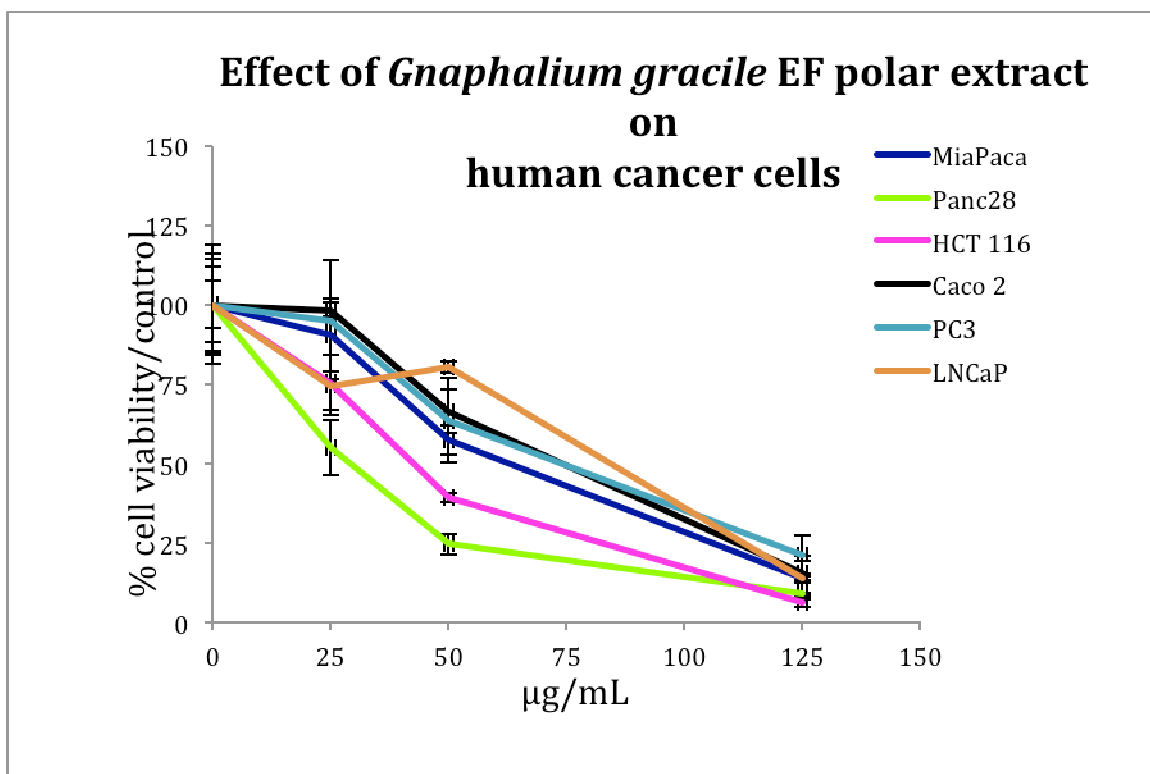




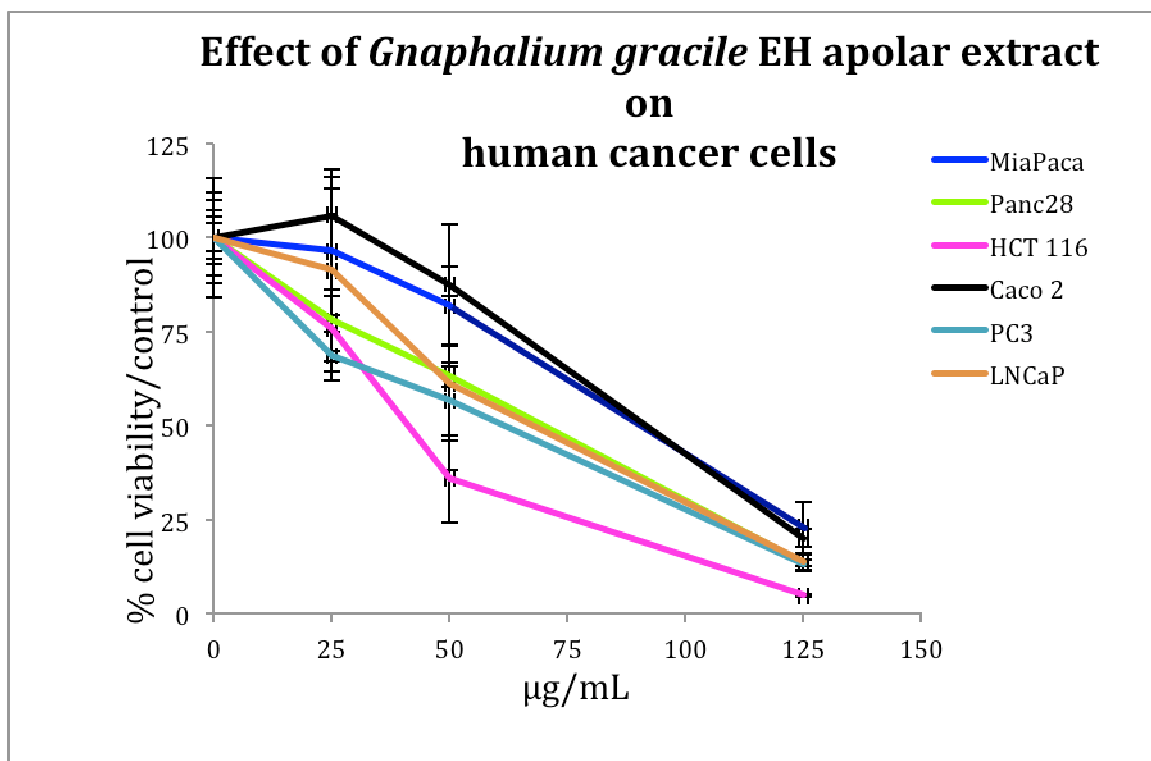
**Figure 6: Effect of EH polar, EF polar, EH apolar, and EF apolar extracts on human prostate cancer LNCaP cells.** LNCaP cells were dosed with 25, 50, and 125 µg/mL of drug. The control was dosed using dimethylsulfoxide (DMSO). MTT assay was used to determine the percent of cell viability after dosing. Significant difference between the control and each concentration was determined with p values < 0.05.



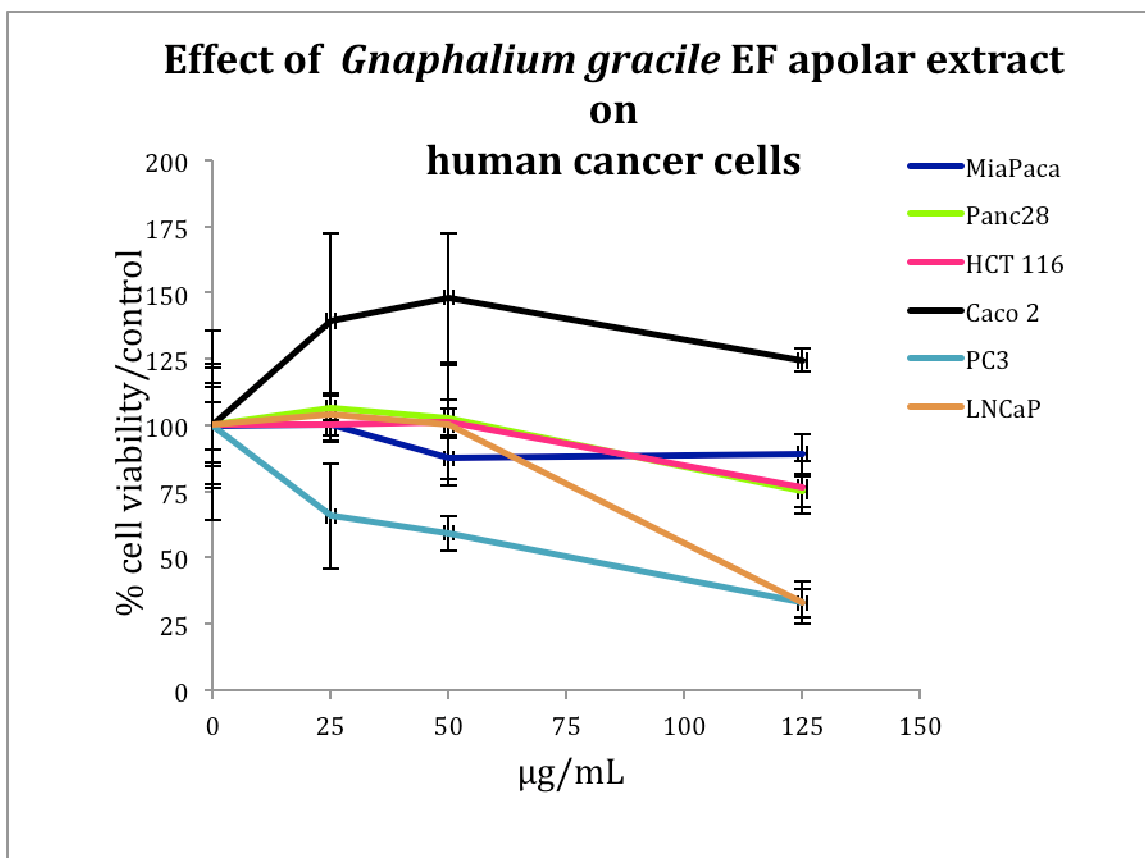
**Figure 7: Effects of EH polar extract on human pancreatic, colon, and prostate cancer cells.** The effects of the EH polar extract on each cancer cell line is shown. Cells were dosed with 25, 50, and 125 µg/mL of drug. The control was dosed using dimethylsulfoxide (DMSO). MTT assay was used to determine the percent of cell viability after dosing. Significant difference between the control and each concentration was determined with p values < 0.05.



**Figure 8: Effects of EF polar extract on human pancreatic, colon, and prostate cancer cells.** The effects of the EF polar extract on each cancer cell line are shown. Cells were dosed with 25, 50, and 125 µg/mL of drug. The control was dosed using dimethylsulfoxide (DMSO). MTT assay was used to determine the percent of cell viability after dosing. Significant difference between the control and each concentration was determined with p values < 0.05.



**Figure 9: Effects of EH apolar extract on human pancreatic, colon, and prostate cancer cells.** The effects of the EH apolar extract on each cancer cell line are shown. Cells were dosed with 25, 50, and 125 µg/mL of drug. The control was dosed using dimethylsulfoxide (DMSO). MTT assay was used to determine the percent of cell viability after dosing. Significant difference between the control and each concentration was determined with p values < 0.05.



**Figure 10: Effects of EF apolar extract on human pancreatic, colon, and prostate cancer cells.** The effects of the EF apolar extract on each cancer cell line are shown. Cells were dosed with 25, 50, and 125 µg/mL of drug. The control was dosed using dimethylsulfoxide (DMSO). MTT assay was used to determine the percent of cell viability after dosing. Significant difference between the control and each concentration was determined with  $p$  values  $< 0.05$ .

Cell line	Mia Paca	Panc 28	HCT116	Caco2	PC3	LNCaP
<b>Extract</b>						
<b>EH Polar</b>	33	70	37	63	31	16
<b>EF Polar</b>	51	28	43	77	75	84
<b>EH apolar</b>	88	21	42	94	61	67
<b>EF apolar</b>	>125	>125	>125	>125	79	106

**Table 2:** Half maximal inhibitory concentrations (µg/mL) of each extract on pancreatic (MiaPaca, Panc28), colon (HCT-116, Caco2), and prostate (PC3, LNCaP).

## ***Discussion***

In this experiment, apolar and polar extracts from *Gnaphalium gracile* were tested for cytotoxic activity on colon, pancreatic, and prostate cancer of varying differentiation status. The evidence supports the hypothesis that these extracts from the leaves and flowers had significant cytotoxic effect on the cells lines based on differentiation status in colon and pancreatic cancer, but not in prostate cancer. Evidence suggests that cytotoxic activities are independent of tumorigenic potential of the cell lines characterized by the expression of Aldehyde dehydrogenase (ALDH) and other genetic markers [12].

A useful piece of information gathered from the data is the half maximal inhibitory concentration (IC<sub>50</sub>). According to the FDA, the calculated IC<sub>50</sub> value is the concentration of a drug in which 50% inhibition occurs *in vitro* [19]. This value is particularly useful because it quantifies the relative effectiveness of the drugs. For example, for EH polar the IC<sub>50</sub> value for MiaPaca is 33 µg/mL while it is 70 µg/mL for Panc28 (**Table 2**). This is evidence that EH polar has more cytotoxic properties towards MiaPaca which is poorly differentiated as compared to Panc 28, but remains uncertain due to the mixture of compounds within this extract.

Overall, EH polar was the most effective at diminishing cancer cell viability. It was effective against 4 of the 6 cell lines tested (**Figure 7**), while its closest competitor was EF polar (**Figure 8**) and EH apolar (**Figure 9**), which were only effective against the well differentiated pancreatic cancer cell line, Panc28.

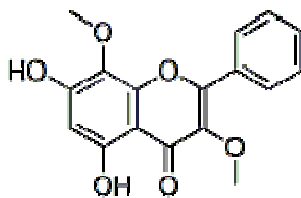
IC<sub>50</sub> values of PC3 and LNCaP prostate cancer of EH polar were 16 and 31 µg/mL, respectively (**Table 2**). These cells lines are characterized largely due to their differentiation status. PC3 is poorly differentiated, which makes it highly metastatic,

relative to LNCaP [20]. Among the mutagenic factors within these cell lines, both contain an overexpression of the oncogene k-ras [21]. This oncogene is responsible for regulating parts of cell division that are vital to growth and development, as well as specialization [22]. When mutated, as in the case of PC3 and LNCaP, this oncogene can lead to the development of a tumor in the prostate. However, it appears that the activity of EH polar is independent of tumorigenic potential. A useful measure of tumorigenic potential is the levels of aldehyde dehydrogenase (ALDH). ALDH has been shown as a marker of tumorigenic potential, especially in PC3 and LNCaP. Both cell lines contain low levels of ALDH [12]. In this way, the extract is effective against the prostate cancer cells lines, independent of tumorigenic potential because they contain similar levels of ALDH, and EH polar shows no preferential effect towards PC3 or LNCaP, with respect to ALDH levels. Additionally, the effects of EH polar on these prostate cancer cells lines is also independent of the tumor suppressor gene p53, the androgen receptor (AR), and the prostate specific antigen (PSA) because LNCaP expresses these genes while PC3 does not express these genes and proliferation is not dependent on androgen [23].

According to **Figure 7**, there were also differential effects of EH polar on human colon cancer cell lines, HCT 116 and Caco 2. These cell lines differ in their expression of several oncogenes, including APC, TP53, and SMAD4. Caco 2 has been shown to have mutations in these oncogenes, but not in k-ras [24]. LNCaP does not express oncogenes in APC, TP53, or SMAD4. This preferential effect of the extracts towards HCT-116 may be due in part to the lack of expression of these oncogenes. As mentioned previously, both prostate cell lines expressed oncogenes and there was no preferential effect towards them. Similarly, the extracts were more effective against the colon cancer

cell line that did not express these oncogenes. This is further evidence to suggest that the extracts had cytotoxic effects due to differentiation status in colon cancer, but not tumorigenic potential.

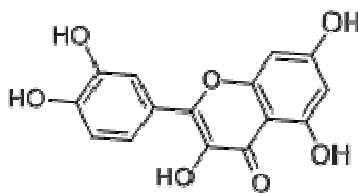
In addition to EH polar, EF polar and EH apolar possessed cytotoxic effects against the better differentiated pancreatic cell line, Panc28, while there was no significant effect against the poorly differentiated pancreatic cell line, MiaPaca (**Figure 8 and 9**). MiaPaca may be resistant to treatment partially due to the overexpression of several oncogenes including serine/threonine kinase-15 (STK15), colony stimulating factor subclass I (CSF-I), and plasminogen activator [25]. EF polar and EH apolar have been shown to contain a high concentration of 5,7-dihydroxy-3,8-dimethoxyflavone (gnaphalin A) [26]. This compound has tracheal smooth muscle relaxant properties [28]



Chemical Structure of Gnaphalin A  
Source: Chemical Book

In a previous study, flavonoids such as quercetin and 3-methoxy-quercetin were identified in polar extracts from the leaves of *Gnaphalium gracile* [26]. These molecules may be responsible for the cytotoxic effects of EH polar. Traditionally sold as a dietary supplement, quercetin has been associated with reduced risk of cancer [27].





Chemical Structure of Quercetin  
Source: Chemical Book

The least effective extract was the apolar extract from flowers (EF apolar) (**Figure 10**). This extract has previously been shown to contain high concentrations of substances such as aromatic monoterpenes, fatty acids, and low concentration of gnapthalin A [26]. This may be the reason for the low cytotoxic effects of this extract on the cancer cells lines, relative to the other extracts.

### ***Conclusion***

These extracts from *Gnaphalium gracile* have shown cytotoxic effects on cancer cell lines from the pancreas (MiaPaca, Panc28), colon (HCT-116, Caco2), and prostate (PC3, LNCaP), which may be due to the difference in differentiation status, but definitely not of tumorigenic potential. The polar and apolar have different activities based on characteristic molecules found within them in both leaves and flowers. The cytotoxic effects of the extracts, particularly EH polar, show selective cytotoxic effects amongst tissue type, thus suggesting a preference for differentiation status.

Since these extracts contain numerous molecules, further research is needed to isolate the bioactive compounds within these extracts. In order to develop an anticancer drug, these molecules must be identified and the mechanism of action must be characterized for the drug to be approved as an antitumor agent. With this research, it is possible that the detrimental treatment options such as surgery, chemotherapy, and radiation may become less frequent as a treatment option for patients with pancreatic, colon, or prostate cancer.

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