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2004

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PART I: EVALUATION OF RUSSIAN SYNTHETIC COMPOUNDS AS A POTENTIAL SOURCE OF NEW DRUG LEADS AGAINST BREAST AND COLON CANCER.
PART II: ISOLATION OF β-AMYRIN FORMATE FROM EUCALYPTUS VIMINALIS LABILL AND INVESTIGATION OF ITS COLON CANCER ACTIVITY

by

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B.S. University of Pitesti, 1997

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
in the Department of Chemistry
in the College of Arts and Sciences
at the University of Central Florida
Orlando, Florida

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# ABSTRACT

Although modern medicine made great steps toward curing most diseases considered deadliest, yet cancer remains one of the major public health problems. The first part of this thesis investigates a new source of selective compounds that are potential candidates against cancer. Fifteen Russian compounds were tested in order to establish their efficiency against two types of cancer: human breast SK-Br-3 and colorectal carcinoma HT-29. The bioassay results show that seven of the new synthetic Russian compounds can be considered new drug leads, based upon their low toxicity and efficacy in slowing the growth of human breast cancer and colon cancer cells.

The goal of the second part of the thesis was to isolate pure compounds that inhibit the growth of cancer cells from the methylene chloride extract of Eucalyptus viminalis Labill. This plant was selected for investigations since a preliminary screening of plants from Russia indicated that had activity against cancer. The result of this work was the isolation of a pure compound which has been analyzed using different spectroscopic techniques such as MS, HPLC, $^{1}$H-NMR, DEPT, $^{13}$C-NMR. The extracted compound was β-amyrin formate, which was previously reported by Malhotra[49], from Canarium strictum Gum in 1987. Also, the bioassay results indicated that β-amyrin formate might be considered a possible drug lead against colon cancer and can be recommended for further investigations. This is the first report of isolation of β-amyrin formate from Eucalyptus viminalis Labill and the first test of the activity of this compound against colon cancer.

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# TABLE OF CONTENTS

LIST OF TABLES ........ vi

LIST OF FIGURES ........ vii

LIST OF ABREVIATIONS ........ viii

PART I: EVALUATION OF RUSSIAN SYNTHETIC COMPOUNDS AS A POTENTIAL SOURCE OF NEW DRUG LEADS AGAINST BREAST AND COLON CANCER ........ 1

1. Introduction ........ 1
2. Results and Discussion ........ 13
    2.1. Goal of study ........ 13
    2.2. Bioassay of Russian synthetics ........ 15
    2.3. Inhibition of human breast cancer cells ........ 17
    2.4. Inhibition of human colon cancer cells ........ 22
    2.5. Conclusion ........ 27
3. Experimental ........ 29
    3.1. Cell lines, chemicals and reagents ........ 29
    3.2. Procedure ........ 29

PART II: ISOLATION OF β-AMYRIN FORMATE FROM EUCALYPTUS VIMINALIS LABILL AND INVESTIGATION OF ITS COLON CANCER ACTIVITY ........ 31

1. Introduction ........ 31
2. Results and Discussion ........ 35
3. Experimental ........ 49

3.1. General experimental procedure ........ 49

3.2 Extraction, isolation, characterization and cancer bioassay........ 49

LIST OF REFERENCES........ 51

# LIST OF TABLES

Table 1. Russian synthetics ........................................................................................... 13

Table 2. Variation percent of breast cancer cells ........................................................................ 18

Table 3. Variation percent of colon cancer cells .......................................................................... 23

Table 4. The structure of previously isolated compounds ........................................................... 33

Table 5. Column chromatography (I) of Eucalyptus viminalis Labill .......................................... 36

Table 6. Column chromatography (II) of Eucalyptus viminalis Labill ....................................... 37

## LIST OF FIGURES

Figure 1. Effect of pyrrolone derivatives relative to the growth of breast cancer cells ............... 19

Figure 2. Effect of 3-acylsubstitute derivatives relative to the growth of breast cancer cells ...... 20

Figure 3. Effect of substitute pyruvates relative to the growth of breast cancer cells ................. 21

Figure 4. Effect of 3F-16, 3F-20 derivatives relative to the growth of breast cancer cells .......... 22

Figure 5. Effect of pyrrolone derivatives relative to the growth of colon cancer cells................ 24

Figure 6. Effect of 3-acylsubstitutes relative to the growth of colon cancer cells ........................ 25

Figure 7. Effect of substitute pyruvates relative to the growth of colon cancer cells.................. 26

Figure 8. Effect of 3F-16, 3F-20 relative to the growth of colon cancer cells............................. 27

Figure 9. UV spectrum of β-amyrin formate ............................................................................ 38

Figure 10. IR spectrum of β-amyrin formate ............................................................................ 39

Figure 11. Gas Chromatogram of β-amyrin formate ................................................................. 40

Figure 12. HPLC of β-amyrin formate ..................................................................................... 41

Figure 13. Mass spectrum of β-amyrin formate ........................................................................ 42

Figure 14. Scheme summarizing the mass fragmentation pattern of β-amyrin formate.............. 43

Figure 15. Structure of β-amyrin formate ................................................................................. 44

Figure 16. $^{1}$H-NMR spectrum of β-amyrin formate ................................................................ 45

Figure 17. $^{13}$C-NMR spectrum of β-amyrin formate ............................................................... 46

Figure 18. $^{13}$C-NMR DEPT spectrum of β-amyrin formate ...................................................... 47

Figure 19. Effect of β-amyrin formate relative to the growth of colon cancer cells.................... 48

## LIST OF ABREVIATIONS

| | |
|---|---|
| °C | Celsius degree |
| d | doublet ($^1$H-NMR) |
| DEPT | distortionless enhancement by polarization transfer |
| DQCOSY | double quantum correlation spectroscopy |
| Fig. | figure |
| GC | gas chromatography |
| g | grams |
| HMQC | heteronuclear multiple quantum correlation |
| hrs | hours |
| $^1$H-NMR | proton magnetic resonance |
| HPLC | high performance liquid chromatography |
| Hz | hertz |
| IR | infrared |
| m | multiplet |
| Me | methanol |
| mg | milligrams |
| mL | milliliters |
| MS | mass spectrum |
| µL | microliters |
| $cm^{-1}$ | reciprocal centimeters |

| | |
|---|---|
| $M^+$ | molecular ion |
| m/z | mass to charge ratio |
| mmol | millimoles |
| nm | nanometers |
| NMR | nuclear magnetic resonance |
| ppm | parts per million |
| q | quartet ($^1$H-NMR spectrum) |
| s | singlet ($^1$H-NMR spectrum) |
| t | triplet ($^1$H-NMR spectrum) |
| UV | ultraviolet |
| $t_r$ | retention time |
| $\lambda_{max}$ | maximal wavelength |

# PART I: EVALUATION OF RUSSIAN SYNTHETIC COMPOUNDS AS A POTENTIAL SOURCE OF NEW DRUG LEADS AGAINST BREAST AND COLON CANCER

## 1. Introduction

This study is dedicated to a search for new sources of active and selective compounds against cancer. Fifteen Russian synthetic compounds were tested as inhibitors against two cancer cell lines: human breast and colorectal carcinoma. The relentless attack by cancer on humans continues to demand a devastating toll. On a world-wide basis the devastation caused by cancer is staggering to contemplate. Fortunately, the increased use of well-established anticancer drugs combined with overall advances in cancer treatment has markedly increased survival time for most cancer patients. The nation's investment in cancer research is making a difference. Yet cancer remains a major public health problem.[1]The majority of most useful and curative anticancer drugs continue to be derived from plant and animal sources. Doubtlessly, anticancer drugs of biosynthetic origin will continue to be of accelerating importance in improving cancer treatment and overall survival rates.

Breast cancer is a significant health problem for women in the United States and throughout the world. Although advances have been made in detection and treatment of the disease, breast cancer remains the second leading cause of cancer-related deaths in women, affecting more than 180,000 women in the United States each year. For women in North America, the life-time odds of getting breast cancer are now one in eight. No vaccine or other universally successful method for the prevention or treatment of breast cancer is currently available. Management of the disease

currently relies on a combination of early diagnosis (through routine breast screening procedures) and aggressive treatment, which may include one or more treatments, such as surgery, radiotherapy, chemotherapy and hormone therapy.[2] The most recent studies were focused on developing new and modern methods to assist patients with breast cancer. One method analyzes the correlation between immunohistochemical analysis and fluorescence in situ hybridization (FISH) in HER-2 status and studied the effect of dual-color (D-FISH) versus single-color FISH (S-FISH) scoring on the assignment of tumors to amplified or nonamplified categories.[3] Recent advances in radiation methods provide a new technique which is called accelerated partial breast irradiation (APBI) after breast-conserving surgery. APBI has very recently come to the forefront as a potential local treatment option for women with breast cancer.[4] The use of modern techniques in radiotherapy planning is recommended to minimize excessive normal tissue exposure, particularly to the cardiac and pulmonary structures. A new method related to this issue is loco regional post-mastectomy radiotherapy (PMRT). The optimal sequencing of PMRT and systemic therapy is currently unclear.[5]

Hormone therapy is a very important part in breast cancer treatment. Many drugs have been mentioned in the BCCA Cancer Drug Manual and other sources as being potentially applicable to breast cancer. The mechanism of action is the following: Aromatase catalyzes the final and rate-limiting step in the conversion of androgens to estrogens in peripheral tissues. This occurs mainly in adipose tissue, but also in normal and malignant breast tissues, and provides the main source of estrogen in postmenopausal women. The goal of hormone therapy in breast cancer is to deprive tumor cells of estrogens, which are implicated in the development or progression of tumors.[11,12]

Aminoglutethimide (Cytadren)[6] is a simple chemical derivative of the sedative gluthethimide. It was originally introduced as an anticonvulsant, but it was found to cause adrenal insufficiency. It blocks adrenal steroidogenesis by inhibiting the enzymatic conversion of cholesterol to pregnenolone.[7,8,9,10]

(1) p-Aminoglutethimide

Anastrozole (Arimidex)[6] is a reversible, nonsteroidal aromatase inhibitor.

(2) Anastrozole

Tamoxifen (Nolvadex)[6] was first approved in the United Kingdom in 1973, and in 1977 gained approval in the United States. It is an estrogen antagonist, structurally related to the synthetic estrogen diethylstilbestrol. The precise mechanism of action is uncertain.

(3) trans-Tamoxifen

Gemcitabine[15] is a pyrimidine analog which is metabolized intracellularly to two active metabolites, gemcitabine diphosphate and gemcitabine triphosphate.

(4) Gemcitabine

Tibolone has estrogenic, progestogenic and androgenic activity. There is great concern regarding the effects that hormone replacement therapy (HRT) might have on the risk of breast cancer. This product has emerged as an alternative to conventional HRT in the treatment and prevention of the effects of estrogen deficiency in postmenopausal women. Preclinical data suggest that tibolone behaves mainly as a progestin in breast cell lines, and does not promote tumor development in tumor models. Tibolone appears to be a safe and acceptable alternative to HRT. However, further studies are required in order to achieve more reliable conclusions.[16]

(5) Tibolone

Vinorelbine (Navelbine)[6] is a semisynthetic vinca alkaloid derived from vinblastine. Vinca alkaloids are originally derived from periwinkle leaves (vinca rosea)[17].

(6) Vinorelbine

The call for the discovery of more effective agents to treat cancer has become urgent. Recently, new compounds called "diazenes" were synthesized. It was demonstrated that several of them inhibited the growth of tumor cells, but their biological activity was relatively low. Therefore, new compounds have been synthesized to improve biological activity. The most active diazenes and their derivatives were tested also on human breast carcinoma.[18]

The incidence of hormone-dependent cancers, such as those of the breast and prostate, is much lower in Eastern countries such as China and Japan in comparison with the Western world. Diet is believed to have a major effect on disease risk and one group of compounds, the phytoestrogens, have been implicated in cancer protection. The phytoestrogens are comprised of two main groups: the isoflavone and lignans. Of the isoflavones, genistein and daidzein have been the most widely studied. These compounds have been shown to possess anticancer properties; however their precise mechanism of action remains to be elucidated[19]. In comparison, few studies have investigated the effects of lignans in breast and prostate cancer[19]. In vitro studies have shown that genistein exerts biphasic effects on cancer cell growth, stimulating

growth at low concentrations (<10μM) and inhibiting growth at high concentrations (>10 μM), which suggest that low phytoestrogen levels may stimulate cancer growth in vivo.

(7) Genistein

(8) Daidzein

An important class of compounds which show anti-tumor activity is represented by pyrrole derivatives. Thirty nine[35] derivatives of the 2,5-dihydropyrrole-2,5-dione were synthesized and tested. The 1-benzyl-3-chloro-4-(3-hydroxyanilino)-2,5-dihydro-1H-2,5-pyrroledione was selected after pre-screening and tested on 56 cell lines of human tumors and exhibits anti-tumor activity against breast cancer. Also, 4-oxo-butenoic acids and related compounds were prepared, tested and claimed useful for the treatment of breast carcinoma.[36]

(10) 2-Butenoic acid, 4-(4-ethoxyphenyl)-2-(9H-fluoren-9-ylidenehydrazino)--4-oxo-, methyl ester

Various active compounds (or their semi-synthetic derivatives) derived from medicinal plants have been assessed for their efficacy and tolerability in the treatment of breast cancer. Some of these plant species, including Taxus baccata (docetaxel, see compound 9), Podophyllum peltatum (etoposide), Camptotheca acuminate (camptothecin) and Vinca rosea (vinblastine, vinorelbine, see compound 6) have well recognized anti-tumor activity in breast cancer, and have been evaluated in clinical trials. Docetaxel (Taxotere)[6] is a semi-synthetic drug derived from a precursor extracted from the needles of the European yew tree, Taxus baccata[13]. It acts by disrupting the microtubular network that is essential for mitotic and interphase cellular functions.

(9) Docetaxel

A recent study considers interaction between Trastuzumab, Carboplatin, and Docetaxel highly synergistic, even at very low drug concentrations.[14] For example, results from recent Phase II /III trials have established docetaxel as the most active single agent in the treatment (first or second-line) of advanced metastatic breast cancer. For other plant species, such as Panax ginseng and Allium sativum, anti-tumor activity has been evaluated in experimental studies using cultured cells and animal models, but the therapeutic potential in patients remains to be determined. Anti-tumor activity derived from medicinal plants may produce results via a number of mechanisms, including effects on cytoskeletal proteins, which play a key role in mitosis (paclitaxel), inhibition

of activity of topoisomerase enzymes I (camptothecin) or II (etoposide), stimulation of the immune system (Viscum album), or antiprotease-antioxidant activity. Medicinal plant-derived antineoplastic agents may be used in single agent or in combinational therapies, and have been used in first-line or second-line (including anthracycline-refractory patients) treatment of localized or metastatic breast cancer[20].

Colorectal cancer is the second leading cause of cancer-related deaths and is responsible for about 12% of cancer deaths in the United States. The mortality rate of newly diagnosed large bowel cancer approaches 50% and there has been little improvement over the past 40 years. Most of this mortality reflects local, regional, and distant metastases. About thirty percent of patients with colorectal cancer have unresectable disease at presentation and about 40% develop metastases during the course of their disease. Surgery is the mainstay of treatment for colorectal cancer but recurrence is frequent.[21] Colorectal cancer has proven resistant to chemotherapy, although limited success has been achieved.

Traditional therapies for colorectal cancer include surgery, radiation therapy and chemotherapy with 5-fluorouracil and levamisole, being the preferred chemotherapeutic agents for colorectal adenocarcinoma.[22]. Fluorouracil[6] was developed in 1957, based on the observation that tumor cells utilized the base pair uracil for DNA synthesis more efficiently than normal cells of the intestinal mucosa. It is a fluorinated pyrimidine that is metabolized intracellularly to its active form, fluorodeoxyuridine monophosphate (FdUMP). The active form inhibits DNA synthesis by inhibiting the normal production of thymidine. Fluorouracil is cell cycle phase-specific (S- phase).[23,24]

(11) Fluorouracil

In 1971, it was reported that Levamisole, a drug that had been originally designed for anthelminthic properties, had immunostimulatory properties in vivo. Levamisole is a synthetic imidazothiazole derivative that has been widely used in treatment of worm infestations in both humans and animals. It may function as a modulating agent to increase the action of fluorouracil. Its mechanism of action as adjuvant therapy in cancer has not been determined.[7,9]

(12) Levamisole

Many compounds were tested against human tumor cell lines. One of them is 2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl) adenine (Cl-F-ara-A). The compound showed selectivity in vivo, with excellent activity being demonstrated against human colon tumors.[25]

(13) Cl-F-ara-A

Several nonsteroidal anti-inflammatory drugs (NSAIDs) which have antiproliferative activity in colon cancer cells, are carboxylate compounds forming acyl glucuronide metabolites. It was demonstrated that acyl glucuronides (and isomers) of the carboxylate NSAIDs diflunisal, zomepirac and diclofenac had antiproliferative activity on human adenocarcinoma HT-29 cells in

culture.[26] Ammonia, produced by bacterial degradation of unabsorbed and edogeneous nitrogenous compounds, is found to be present at millimolar concentrations in the colon lumen. From in vivo animal experiments, this metabolite has been shown to alter colonic epithelial cell morphology and to increase compensatory cell proliferation when present in excess. Ammonia was not metabolized by HT-29 cells into carbamoyl-phosphate and citrulline, indicating that ammonia was likely acting on cells by itself. This agent was shown to significantly reduce cellular ornithine decarboxylase (ODC) activity, resulting in a threefold decrease in the capacity of HT-29 cells to synthesize polyamines, these latter metabolites being strictly necessary for cell growth. The unexpected finding that ammonia is acting as an antimitotic agent against tumoral HT-29 colonic cells may be related to the inability of these cells to metabolize this compound.[27] The in vitro cytotoxic activity profile of nine novel phenylarsonic acid compounds against 17 human cancer cell lines, including colon cancer and breast cancer, was determined. The leads compounds, 2-methylthio-4-[(4'-aminophenylazo)-phenylarsonic acid] pyrimidine (PHI-370) and 2-methylthio-4-(4'-phenylarsonic acid)-amino pyrimidine (PHI-380) caused apoptotic death in all 17 cancer cell lines at low micromolar concentrations. PHI -380 was also tested and found to be very active against primary tumor cells isolated from surgical biopsy specimens of 14 patients with therapy-refractory breast cancer, colon cancer, lymphoma or hepatoblastoma as well.[28]

(14) PHI 370

(15) PHI 380

Quinazoline compounds have been suggested as useful compounds in the treatment of cell growth and differentiation characterized by activity of the human epidermal growth factor receptor type2 (HER2) (see Myers et.al., U.S. Pat. No. 5,721,237). A method of preventing the development or recurrence of colorectal cancer in a mammal is administering an effective cancer preventative amount of 4-(4’-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline or a pharmaceutically acceptable salt thereof to the mammal.[31]

(16) Janex 1

Methods for the prevention and treatment of colorectal cancer are provided. Specifically, the method relates to the administration of an effective adenoma or microadenoma preventing amount of 6-fluoroursodeoxycholic acid (6-FUDCA) or a pharmaceutically acceptable conjugate

thereof to a mammal in need of such treatment. The methods find general use in the prevention of the formation of secondary bile acids, the reduction of deoxycholic acid and the protection against cytotoxic effects of other bioacids and carcinogens.[29]

(17) 6-FUDCA

Another method for colon cancer chemoprevention or chemotherapy is administering a pharmaceutical composition comprising of an effective amount of a 2-hydroxy-5-phenylazobenzoic acid derivative or an ester or an active metabolite or an oxidation product of an active metabolite thereof to an individual suffering from colon cancer or at risk to develop colon cancer.[30]

(18) 5-Hydroxy-2-phenylazobenzoic acid methyl ester

Immunotherapy uses different ways to stimulate the immune system and stop cancer cells for growing. Immunotherapy biological extracts may be useful as adjuvant therapy in treating patients who have had surgery for breast cancer, colon cancer, or melanoma.[34]

## 2. Results and Discussion

### 2.1. Goal of study

The main goal of this work was to test new synthetic compounds with different functional groups against breast and colon cancer. The compounds chosen had pyrrolone, pyruvic acid and aryl fluoride moieties, which are found in many nitrogen heterocyclics with demonstrated biological activity as mention previously. The results of this study will be the selection of new drug leads, based upon their low toxicity and efficacy in slowing the growth of human breast cancer and colon cancer cells. Also, one can establish whether they have potential to be considered for further investigations related to the mechanistical mode of action. The structures of the synthetic Russian compounds are shown in Table 1.

Table 1. Russian synthetics

| General Structure | Radicals | Notation |
| --- | --- | --- |
| $R_1$-CO, OH, HO, $H_3COOC$, N, O, $R_2$ | $R_1$ = p-$CH_3C_6H_4$<br>$R_2$ = $C_6H_4$Br-p | 3F-1 |
| | $R_1$= p-$C_2H_5OC_6H_4$<br>$R_2$= $(CH_3)_2C_6H_4$ | 3F-2 |
| | $R_1$= $C_6H_5$<br>$R_2$= $(CH_3)_2C_6H_4$ | 3F-3 |
| | $R_1$= $C_6H_5$<br>$R_2$ = p-$CH_3C_6H_4$ | 3F-4 |
| | $R_1$= p-$C_2H_5OC_6H_4$<br>$R_2$= p-$CH_3C_6H_4$ | 3F-5 |
| | $R_1$= $(CH_3)_3C$<br>$R_2$= p-$CH_3C_6H_4$ | 3F-6 |
| | $R_1$= $C_6H_5$<br>$R_2$= $C_6H_5COOC_2H_5$ | 3F-7 |

| | | |
|---|---|---|
| $R_1$—CO—C—CO—$R_2$<br>‖<br>$NNHC_6H_5$ | $R_1$= p-$H_3OC_6H_4$<br>$R_2$ = COOH | 3F-10 |
| | $R_1$= p-$ClC_6H_4$<br>$R_2$ = $C_6H_5$ | 3F-11 |
| | $R_1$= p-$C_2H_5OC_6H_4$<br>$R_2$ = $COOCH_3$ | 3F-12 |
| F F<br>$H_3CO$ C COOC$H_3$<br>O OH<br>F F | | 3F-13 |
| F F<br>$H_3CO$ C COOC$H_3$<br>O O<br>F F Cu/2 | | 3F-14 |
| p-$CH_3C_6H_4CO$ OH<br>OH<br>N O<br>N<br>$C_6H_5$ | | 3F-16 |
| F O<br>F<br>$CH_3O$ O COOC$H_3$<br>F | | 3F-18 |

| | | |
|---|---|---|
| p-$ClC_6H_4$—CO—CH=C—$COOCH_3$ (C bearing N(H)—N=C($C_6H_5$)—$C_6H_4Br$-o) | | 3F-20 |

## 2.2. Bioassay of Russian synthetics

Both cell lines (human breast carcinoma SK-Br-3 and colorectal HT-29) were ordered from American Type Culture Collection and they were derived independently from different patients. All cells were cultured in the same medium, with identical composition and they were split and fed by the same procedure. The actual bioassay was performed on both cell lines and the compounds were tested in the same way. Five plates were used and they were seeded in the same time with 3 mL of medium each (initial dose). For the first two intervals of time (0-48 hrs and 48-96 hrs), one plate was chosen each time to be read, which means counting the number of cells. All other plates were only fed with 3 mL of medium each. The third interval of time (96-120 hrs) is considered critical for observations and counting number of cells; that is why the plates were not fed and only one plate was read. The last two intervals of time (120-144 hrs and 144-168 hrs) were similar to the first two intervals: one plate was read each time and the remaining ones were only fed. Total volume needed of drug and medium for the entire assay is 39.0 mL, but 50 mL were used for variations in pipeting. Initial screening of any compound,

tested solely for activity, was done by using 10 μg/mL concentration of compound and the sample calculation for 50 mL of a compound weighed out at 0.0018 g is as following:

(1800 μg/mL) X = (10 μg/mL) (50.0 mL)

X = 0.278 mL

The drug solution was prepared by adding 280 μL of drug to 49.720 mL of medium.

The final step on the bioassay test was to count the variation in number of cells for each compound and compare them to a control sample (a sample without any drug added). The counts were done by using a device called a hemacytometer. Once all the counts were finished for all periods of time, all numbers were added up, then they were divided by 24 and the result was multiplied by $10^4$. This represents the hemacytometer formula. The results were recorded, and the logarithm from number of cells/mL was plotted versus time. The variation percent of breast and colon cancer cells was calculated for two intervals of time: 48-96 hrs and 96-120 hrs. Also, the average of the variation percent for both intervals of time was determined. The variation percent for the first time interval is given by the following formula:

$$I(\%) =[S(96h) - S(48h) / C(96h) - C(48h)] \times 100$$

Where S (96h) and S(48h) represent the number of breast or colon cancer cells counted at 96 and 48 hours respectively for each synthetic derivative.

C(96h) and C(48h) represent the number of breast or colon cancer cells counted at 96 and 48 hours respectively for control wells (no drug added). Also, the same formula was applied for the second time interval, 96-120 hours. A negative number represents an inhibition of growth of cancer cells. That means the number of cancer cells is decreasing in time, as opposed to the number of control cells (cells with no drugs added). A positive number represents a growth of

cancer cells. That means the number of cancer cells is increasing in time, in a similar way to the control cells. In order to be considered a possible drug lead, one compound must have a percent inhibition of 15-30% for the considered interval of time. A very high inhibition indicates that the effect of the drug is undesirable because it could be toxic to normal and cancer cells[35]. On the contrary, a very high growing rate, which corresponds to a positive number in the table, is also not valuable because of its failure in suppressing the growth of cancer cells.

### 2.3. Inhibition of human breast cancer cells

The results for testing the compounds against breast cancer cells are shown in Table 2. As we have mentioned previously, two intervals of time were considered, 48-96 hrs and 96-120 hrs. Also the average of the normalized variation of breast cancer cells for both intervals of time was calculated.

Table 2. Variation percent of breast cancer cells

| Comp. | 48-96 (hours) | 96-120 (hours) | Avg. |
|---|---|---|---|
| 3F-1 | 13 | -29 | -8 |
| 3F-2 | -13 | 55 | 21 |
| 3F-3 | -45 | 75 | 15 |
| 3F-4 | -39 | 29 | -5 |
| 3F-5 | -31 | 13 | -9 |
| 3F-6 | -16 | 103 | 44 |
| 3F-7 | -24 | -15 | -20 |
| 3F-10 | -76 | 5 | -36 |
| 3F-11 | -25 | 23 | -1 |
| 3F-12 | -44 | -42 | -43 |
| 3F-13 | -9 | -66 | -38 |
| 3F-14 | -6 | -43 | -25 |
| 3F-16 | -5 | -6 | -6 |
| 3F-18 | -4 | 5 | 1 |
| 3F-20 | 7 | -22 | -8 |

From Table 2 and Figure 1 (pyrrolone derivatives) one can conclude that for the first interval of time (48-96 hours) the 3F-1 compound shows a growth in cell number (13 %). All others show inhibition of cancer cells and the most valuable are considered to be 3F-5 (-31%), 3F-7 (-24%) and 3F-6 (-16%). 3F-2 (-13%) did not show a very significant inhibition. There is a toxicity problem associated with the 3F-3 ( -45%) and 3F-4 ( -39%) compounds. For the second interval of time (96-120 hrs), 3F-1 (-29%) and 3F-7 (-15%) show inhibition of cancer cells. All other pyrrolone derivatives cannot be considered inhibitors of breast cancer cells.

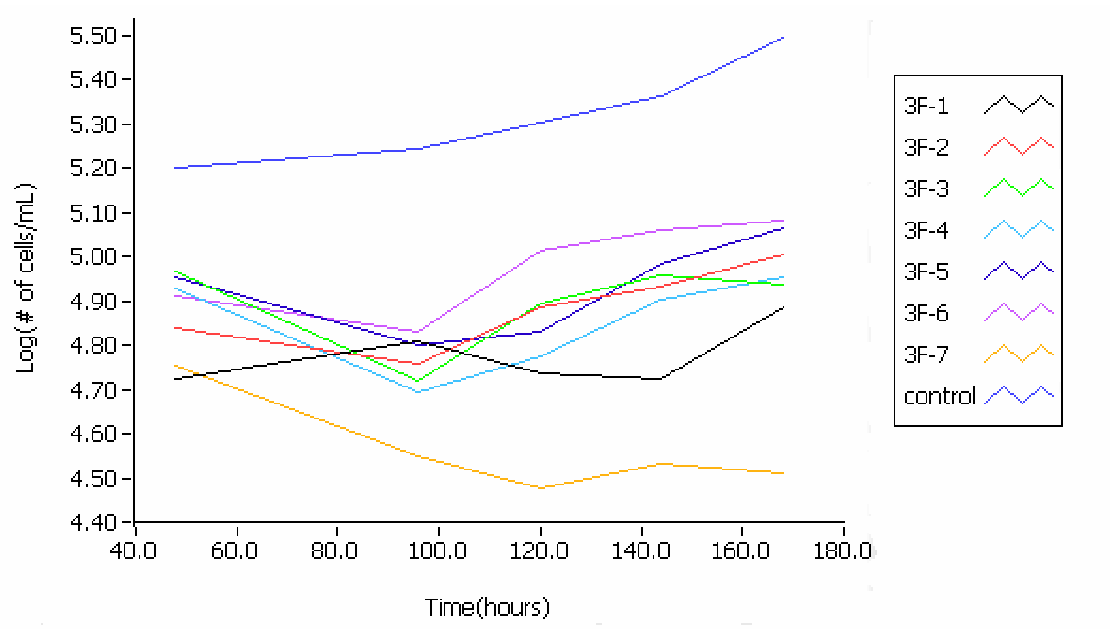

Figure 1. Effect of pyrrolone derivatives relative to the growth of breast cancer cells

From Table 2 and Figure 2, one can conclude that 3-acylsubstitute derivative 3F-10 ( -76%) is the most toxic compound, besides 3F-12 ( -44%), that means they cannot be used for more tests. The 3F-11 (-25%) indicates a good inhibition of cancer growth. None of them presents interest for further studies for the second interval of time.

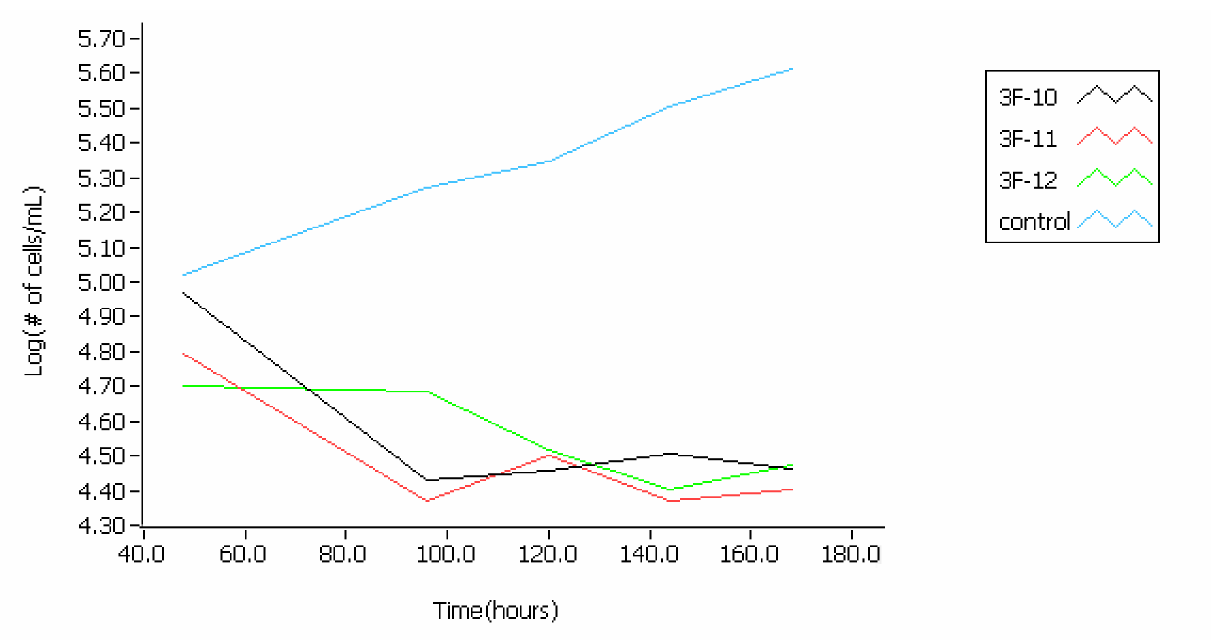

Figure 2. Effect of 3-acylsubstitute derivatives relative to the growth of breast cancer cells

Based on Table 2 and Figure 3, for the first interval of time, substitute pyruvates 3F-13, 3F-14, and 3F-18 show an inhibition of -9%, -6% and -4% respectively. These results cannot be considered significant for future studies. For the second interval of time 3F-13 and 3F-14 show toxicity and the inhibitions are -66% and -43% respectively. 3F-18 shows a growth in cancer cells (5%).

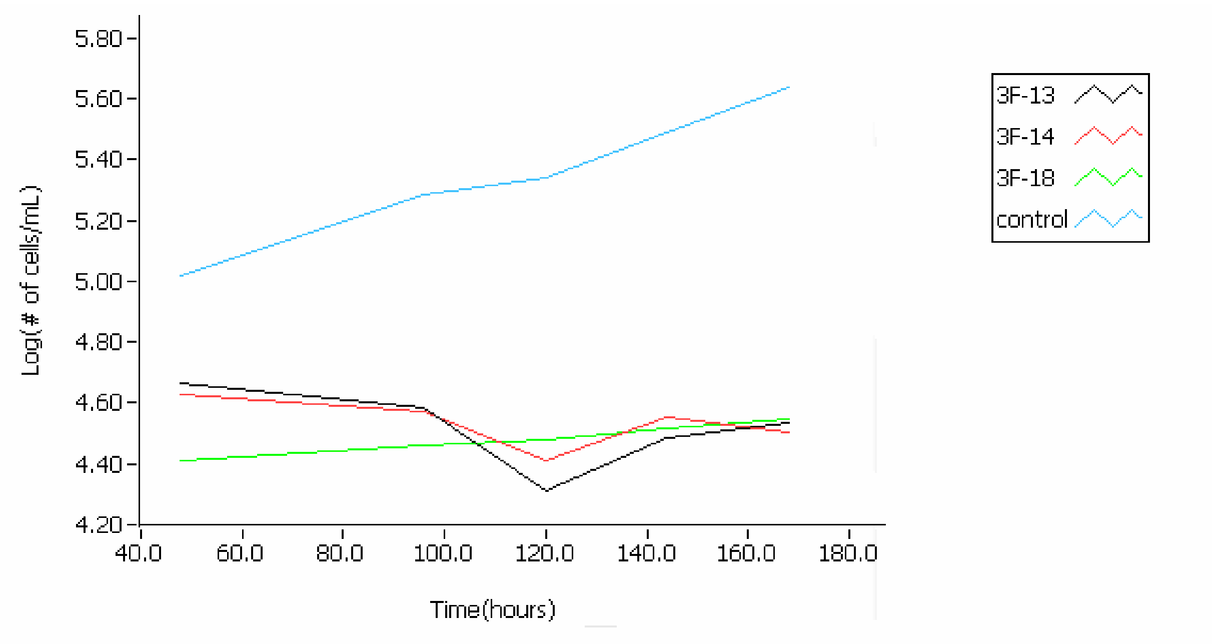

Figure 3. Effect of substitute pyruvates relative to the growth of breast cancer cells

Based on Table 2 and Figure 4, compounds 3F-16 (-5%) and 3F-20 (7%) show no interest for further investigations on breast cancer, because the former has a very low inhibition and the latter shows no inhibition of growth. For the second interval of time, 3F-20 (-22%) might be considered as inhibitor of breast cancer cells.

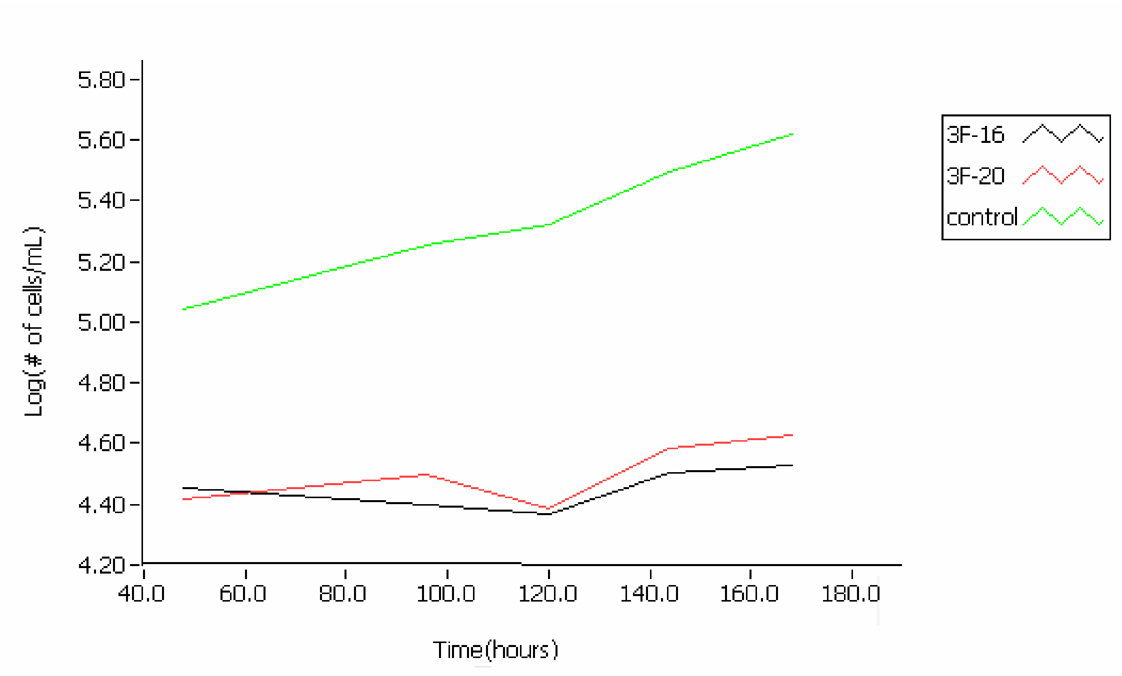


Figure 4. Effect of 3F-16, 3F-20 derivatives relative to the growth of breast cancer cells

Thus, for the first interval of time, the only compounds which might be considered for further investigation are 3F-5, 3F-6, 3F-7 and 3F-11 compounds. For the second interval of time 3F-1, 3F-7 and 3F-20 compounds might present interest for further studies.

### 2.4. Inhibition of human colon cancer cells

The results for testing the compounds against colon cancer cells are shown in Table 3. As we have mentioned previously, two intervals of time were considered, 48-96 hrs and 96-120 hrs. Also, the average of the normalized evolution percent of breast cancer cells for both intervals of time was calculated.

Table 3. Variation percent of colon cancer cells

| Comp. | 48-96 (hours) | 96-120 (hours) | Avg. |
|---|---|---|---|
| 3F-1 | 50 | 26 | 38 |
| 3F-2 | 20 | 10 | 15 |
| 3F-3 | 17 | 13 | 15 |
| 3F-4 | 25 | 26 | 25 |
| 3F-5 | 31 | 13 | 22 |
| 3F-6 | 35 | 25 | 30 |
| 3F-7 | 0 | 44 | 22 |
| 3F-10 | 55 | -17 | 19 |
| 3F-11 | 5 | -67 | -31 |
| 3F-12 | -23 | -45 | -34 |
| 3F-13 | 1 | 44 | 23 |
| 3F-14 | 12 | 40 | 26 |
| 3F-16 | 86 | -66 | 20 |
| 3F-18 | 49 | 31 | 40 |
| 3F-20 | 66 | 13 | 40 |

From Table 3 and Figure 5, one can see that pyrrolone derivatives did not present significant results related to the inhibition of growth of colon cancer cells. For both intervals of time all normalized variations are positive values, which mean no inhibition of colon cancer cells.

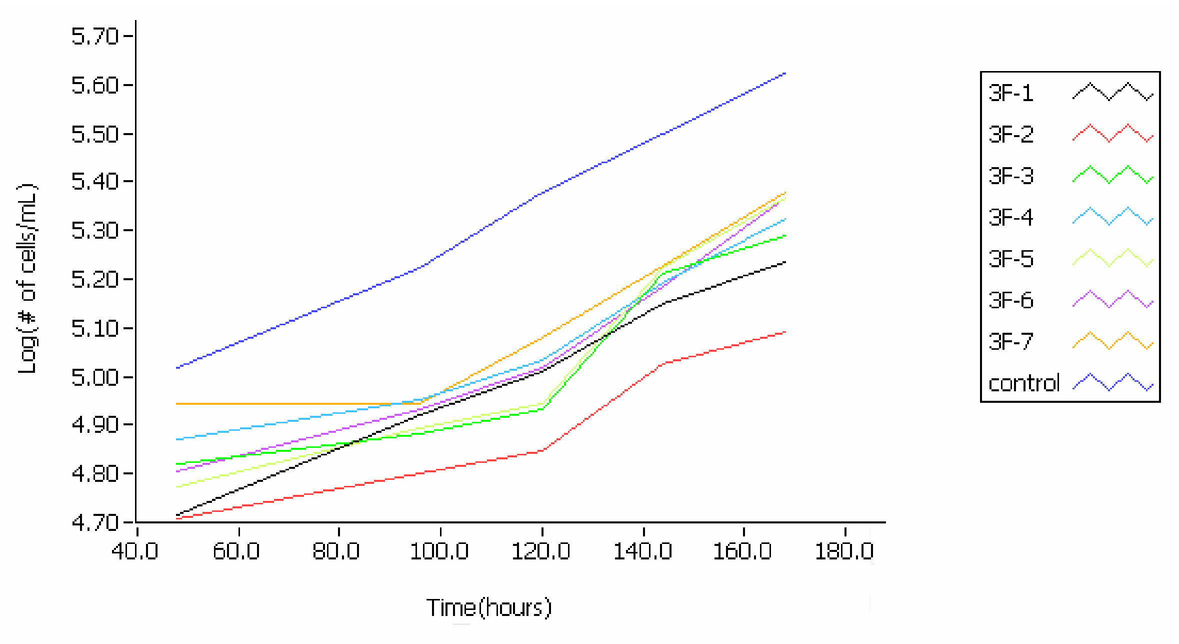

Figure 5. Effect of pyrrolone derivatives relative to the growth of colon cancer cells

Based on Table 3 and Figure 6, 3-acylsubstitute 3F-10 (-17%) shows an inhibition of growth of cancer cells for the second time interval (96-120 hours). 3F-11 (-67%) is the most toxic compound for both types of cells, besides 3F-12 (-45%). 3F-10 (55%) and 3F-11 (5%) show no inhibition of colon cancer cells for the first interval of time.

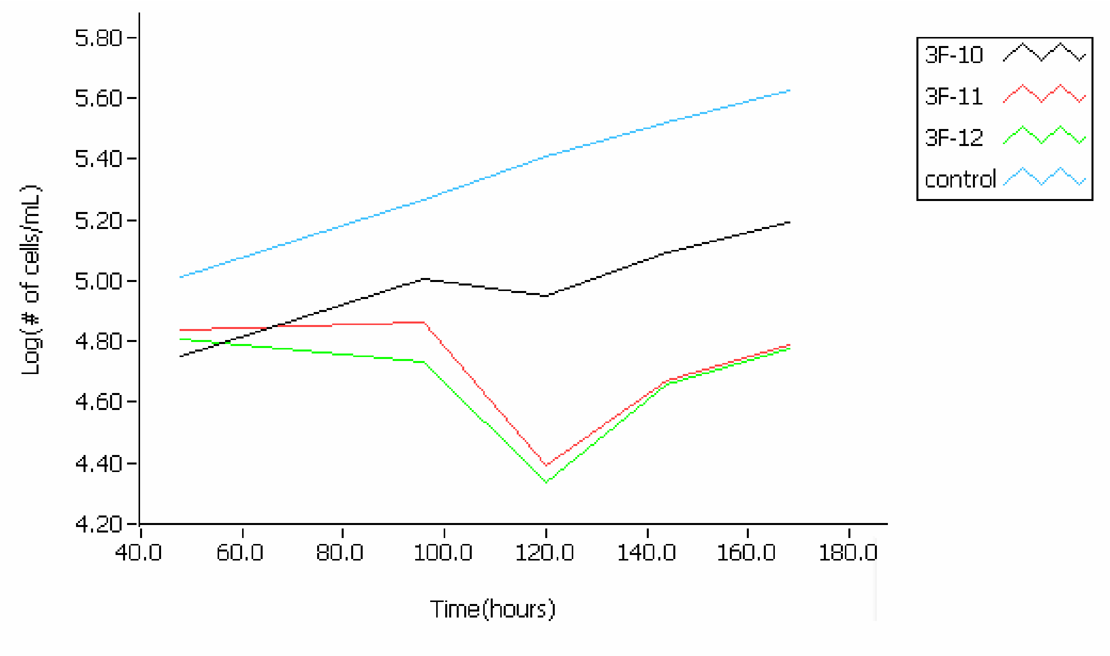


Figure 6. Effect of 3-acylsubstitutes relative to the growth of colon cancer cells

Based on Table 3 and Figure 7 substitute pyruvates did not show significant results related to the inhibition of growth of colon cancer cells (all numbers show no inhibition of colon cancer cells for both intervals of time). These compounds cannot be taken in consideration for development of new drug leads against colon cancer.

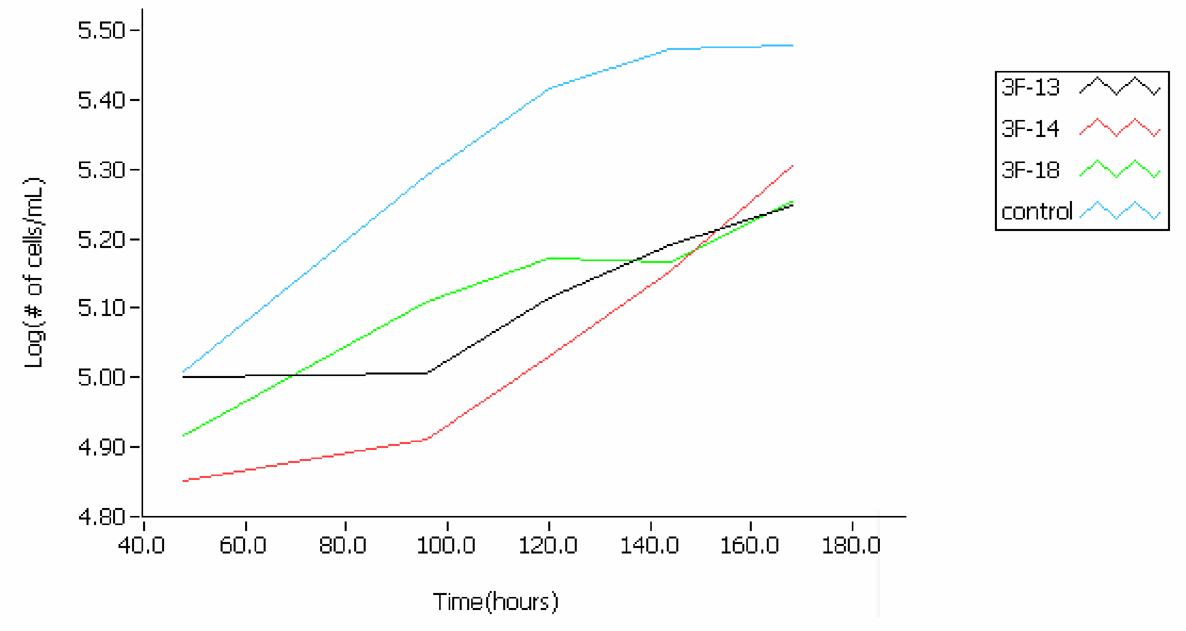


Figure 7. Effect of substitute pyruvates relative to the growth of colon cancer cells

Table 3 and Figure 8 show a toxicity problem for the second interval of time (96-120 hours) for 3F-16 (-66%). For the first interval of time there is no significant inhibition of colon cancer cells for 3F-16 and 3F-20 compounds.

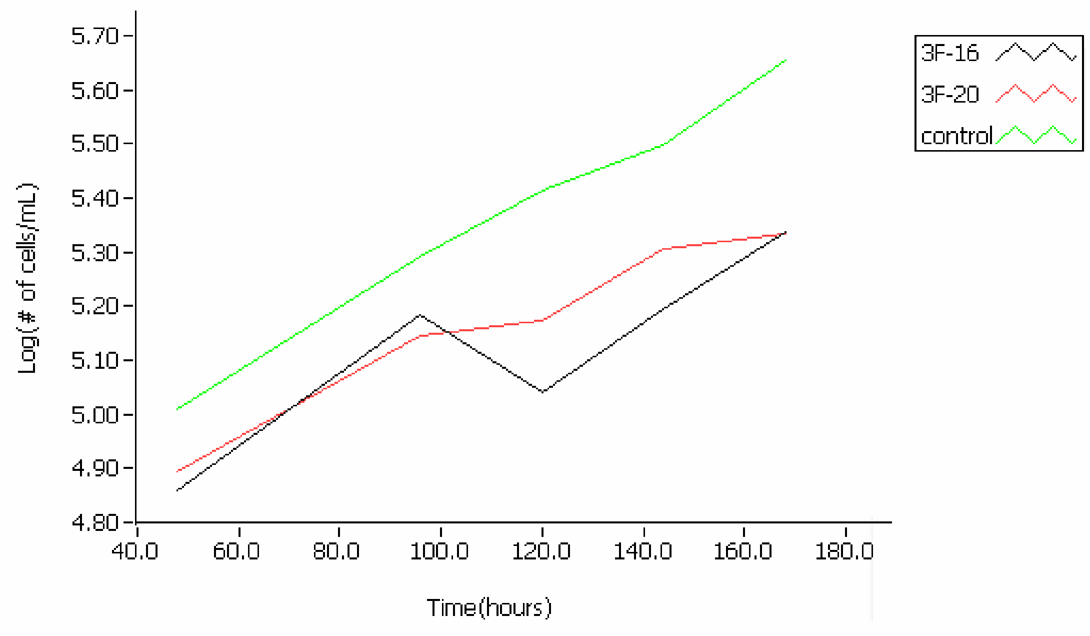


Figure 8. Effect of 3F-16, 3F-20 relative to the growth of colon cancer cells

## 2.5. Conclusion

In conclusion, considering the relation between the structure of derivatives and the possible activity, the following general conclusions can be formulated: Further investigations related to the inhibition of breast cancer cells might be useful for pyrrolone derivative 3F-5 (-31%), 3F-6 (-16%) and 3F-7 (-24%) for the first interval of time (48-96 hrs). Also 3F-7 can be considered for investigations on the second interval of time. 3F-11 (-25%), which is a 3-acylsubstitute derivative, can also be taken in consideration for the first interval of time against growth of breast cancer cells. 3F-20 (-22%) can be considered for the second interval of time against growth of cancer breast cells. From experiments related to colon cancer cells 3-acylsubstitute derivative 3F-12 (-23%) and 3F-10 (-17%) show inhibition for the first interval of time and the

second interval of time respectively. Thus, further investigations might be useful for 3-acylsubstitutes against colon cancer, focusing on studies primarily in the second interval of time (96-120 hours). Thus these synthetic compounds represent potential candidates as new drug leads and might be used in the treatment of breast and colon cancer. All these studies have the purpose to choose the most selective drug leads and to analyze their mechanism of action.

# 3. Experimental

## 3.1. Cell lines, chemicals and reagents

Human breast carcinoma cell line (SK-Br-3) and colorectal cell line (HT-29) were obtained from American Type Culture Collection (ATCC, Manassas, VA). Both cell lines were derived independently from different patients. All cells were cultured in McCoy's 5 a medium with 1.5 mM L- glutamine, fetal bovine serum (FBS) 10%, and antibiotic/antimicotic solution. Trypsin-EDTA solution was used for splitting cells. Other materials used were: specific bioassay glassware (ex: pipettes, flasks, centrifuge tubes, etc.), microscope, hemacytometer, centrifuge instrument, incubator, refrigerator, liquid nitrogen container. The protocol was presented in ATCC (American Type Culture Collection) catalog.

## 3.2. Procedure

The standard operating procedure has two steps: maintenance of cancer cells and bioassay on cancer cells.

A. Maintenance of cancer cells.

A McCoy's 5a medium was mixed with 1.5mM L- glutamine, 90%; fetal bovine serum 10% and antibiotic/antimicotic and represent the medium for growing cells. The cells were split with 1.0 mL of Trypsin/EDTA solution and placed into the incubator for at least 10 minutes to help the cells dislodge. The flask was removed from the incubator, and medium was added to a total volume of 5.0 mL. 1.0 mL of the mixture was added to a new flask containing fresh medium 1:5

split (ATCC recommends a sub cultivation ratio of 1:3 to 1:8). Freezing cells: This step was done during a normal split procedure using a centrifuge at 4000 rpm for 20 minutes, until the cells form a pellet. The supernatant was removed and discarded. 5.0 mL of 95% culture medium and 5% DMSO were centrifuged. The content was transferred into cryovials and placed into liquid nitrogen container.

B. Performing bioassay on cancer cells: Preparation of stock drug.

A mass of 0.0018 g of compound was weighted and dissolved in 1.0 mL of DMSO.

Preparation of drug and medium for maintenance of cells in bioassay:

This protocol was based on running two series of plateau at 5 plates per series. The plates were on the standard feeding schedule of every 48 hours. The compounds were tested in three wells per plate.

Seeding of plates for bioassay: A standard 1:10 split procedure of the flask designated for bioassay was performed. From that, another 1:50 split was performed into three 50.0 mL centrifuge tubes, containing 49.0 mL of medium each.

Each well to be used in the assay was seeded with 1.0 mL of the 1:500 split cells and was placed into the incubator. After 48 hours, the plates were dosed.

Dosing of plates: The old medium was removed from the plates and 1.0 mL of the compound and medium combination that was previously prepared was added to each designated well.

Reading the plates: The old medium was removed and a small amount of Trypsin/EDTA was added to wash away Trypsin inhibitors in fetal bovine serum. Exactly 1.0 mL of Trypsin was added to each well and placed into the incubator. 40 μL of sample was used to load the hemacytometer and perform the counts.

# PART II: ISOLATION OF β-AMYRIN FORMATE FROM EUCALYPTUS VIMINALIS LABILL AND INVESTIGATION OF ITS COLON CANCER ACTIVITY

## 1. Introduction

The goal of this work was to isolate pure compounds that inhibit the growth of cancer cells from the methylene chloride extract of Eucalyptus viminalis Labill. This plant was selected for investigations, since a preliminary screening of plants from Russia indicated that this plant had activity against cancer[36].

While this plant material was collected in Russia, Eucalyptus is native to Australia, and the genus Eucalyptus contains about 600 species. Eucalyptus is a fast-growing tree that is utilized to manufacture paper. For this reason, there has been extensive overseas forest plantation of Eucalyptus trees. When trees are cut for biomass resources, the usage of leaves surplus is considered to be an important research subject. The Aborigines (native Australians) have traditionally used Eucalyptus leaves to heal wounds and fungal infections. The extracts of Eucalyptus leaves have been approved as food additives, and the extracts are also currently used in cosmetic formulations. Recently, attention has been focused on the medicinal properties of these extracts. Research data has demonstrated that the extracts exhibit various biological effects, such as antibacterial, antihyperglycemic (Gray and Flatt 1998)[37] and antioxidant (Lee and Shibamoto 2001)[38] activities. The antimicrobial activities of leaf extracts from Eucalyptus viminalis significantly inhibited the growth of six Gram-positive bacteria (Staphylococcus aureus, MRSA, Bacillus cereus, Enterococcus faecalis,

Alicyclobacillus acidoterrestris, Propionibacterium acnes), and of a fungus (Trichophyton mentagrophytes), but they did not show strong antibacterial activity against Gram-negative bacteria (Escherichia coli, Pseudomonas putida)[39].

Previously, several compounds have been isolated from Eucalyptus viminalis, including ursolic acid[40], maslinic acid[41], 11,12-dehydroursolic acid lactone, eucalyptol[42],α-pinene[42],α-terpenyl acetate[42], grandinin[43], pterocarinin A[44]. The structures of these compounds are shown in Table 6. Ursolic acid is a triterpenoid which occurs especially in the waxy coatings of the leaves and on fruits, such as apples and pears, and may serve as insect repellant and antimicrobial agent[44].Although triterpenoids have rather limited medicinal use, recent studies indicate their great potential as drugs[45]. Ursolic acid has shown significant cytotoxicity in the lymphocytic leukemia cells P388 (ED50= 3.15 mg/mL) and L1210 ($ED_{50}$= 4.00 mg/mL), as well as the human lung carcinoma cells A-549 ($ED_{50}$= 4.00 mg/mL)[46]. The antitubercular activity of ursolic acid has also been reported[47].

Table 4. The structure of previously isolated compounds

| General structure | Compound name |
|---|---|
| OAc<br>C— Me<br>Me<br>Me | Terpinyl acetate |
| Me<br>Me<br>Me | α- pinene |
| Me<br>Me<br>O<br>Me | Eucalyptol |
| Me Me R S S H S $CO_2H$ Me R Me S R H R Me S R HO Me Me H | Ursolic acid |

| | |
|---|---|
| | Maslinic acid |
| | 11, 12-Dehydroursolic acid lactone |
| | Quecimelin |

## 2. Results and Discussion

Eucalyptus viminalis was selected for this investigation because the methylene chloride extract was reported to possess activity against lung cancer. The plant material( dried leaves) were collected in Russia and extracted first with methylene chloride and then with methanol. Methylene chloride extract was chromatographed on silica gel and then eluted with mixed solvents of increasing polarity (hexane, methylene chloride, chloroform, and methanol) to obtain 81 fractions. The eluent fractions were collected and monitored by TLC on silica gel with different solvent systems: hexane to methylene chloride=20%:80% or hexane to methylene chloride=50%:50%. The hexane/methylene chloride fractions 1-32 from the first column were further chromatographed to obtain 12 fractions. Fractions 3-6 were mixed, chromatographed in a small liquid chromatography column. From the resulting fraction a pure compound was isolated, which was proved to be β-amyrin formate, with a jelly consistency and light yellow color. The chromatography results are given in Table 5 and Table 6.

Table 5. Column chromatography (I) of Eucalyptus viminalis Labill

| Fractions | Eluent | Weight ( grams ) |
|---|---|---|
| F 1-5 | 100% hexane | 0.56 |
| F 6-11 | 20%$CH_2Cl_2$ in hexane | 0.68 |
| F 12-15 | 30%$CH_2Cl_2$ in hexane | 1.59 |
| F 16-23 | 40%$CH_2Cl_2$ in hexane | 1.83 |
| F 24-31 | 50%$CH_2Cl_2$ in hexane | 2.83 |
| F 32-42 | 60%$CH_2Cl_2$ in hexane | 7.31 |
| F 43-50 | 70%$CH_2Cl_2$ in hexane | 4.74 |
| F 51-58 | 80%$CH_2Cl_2$ in hexane | 3.57 |
| F 59-70 | 100%$CH_2Cl_2$ | 3.70 |
| F 71-78 | 50% $CH_2Cl_2$ in $CHCl_3$ | 1.32 |
| F 79-81 | 100%$CH_3OH$ | 1.89 |

Table 6. Column chromatography (II) of Eucalyptus viminalis Labill

| Fractions | Eluent | Weight ( grams ) |
|---|---|---|
| F 1-2 | 100% hexane | 0.30 |
| F 3-4 | 20%$CH_2Cl_2$ in hexane | 0.40 |
| F 5-6 | 50%$CH_2Cl_2$ in hexane | 0.31 |
| F 7 | 75%$CH_2Cl_2$ in hexane | 0.48 |
| F 8 | 100%$CH_2Cl_2$ | 0.49 |
| F 9 | 50% $CH_2Cl_2$ in $CHCl_3$ | 2.13 |
| F 10 | 100% chloroform | 2.00 |
| F 11-12 | 50% $CHCl_3$in $CH_3OH$ | 4.17 |

The UV spectrum of β-amyrin formate shows the following absorption maxima $\lambda_{max}$: 200 nm, 276 nm, and 340 nm, suggesting that the compound has double bonds, methyl and other substituents attached to the rings. The UV spectrum is given in Figure 9.

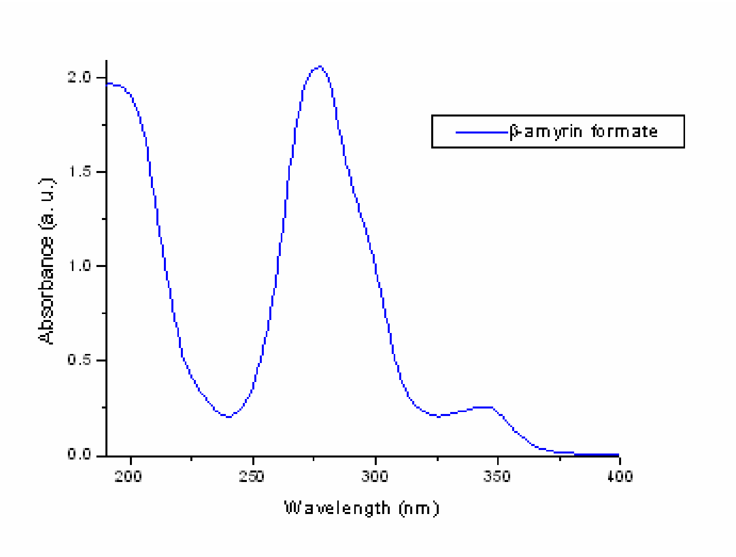


Figure 9. UV spectrum of β-amyrin formate

The IR spectrum (Figure 10) indicated absorption maxima $\lambda_{max}$ at 2851 $cm^{-1}$ (C-H stretch absorptions), 1730 $cm^{-1}$ (aldehydic stretch), 1649 $cm^{-1}$ (double bond six membered ring), 1471, 1380 $cm^{-1}$ (C-O stretch), 1174 $cm^{-1}$ (C-O stretch), 850 $cm^{-1}$ (C-H out-of-plane bend) , which are consistent with the values of the previously isolated compound.

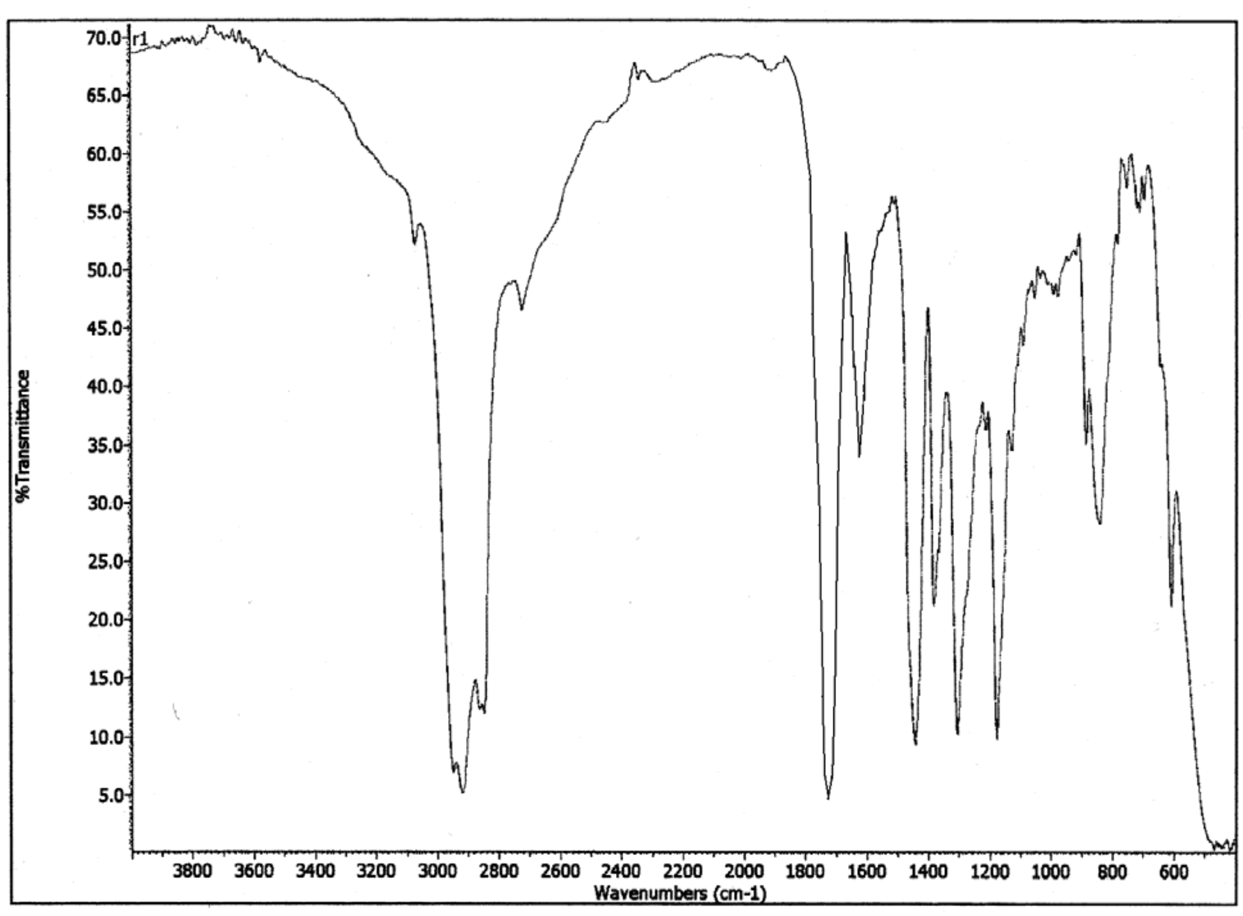


Figure 10. IR spectrum of β-amyrin formate

Another spectroscopic method used to check the purity of isolated compound was gas chromatography. The retention time is the time between injection of a sample and the appearance of a solute peak at the detector of a chromatographic column. The gas chromatogram (Figure 11) shows a single peak with retention time $t_r$ = 14.17 min.

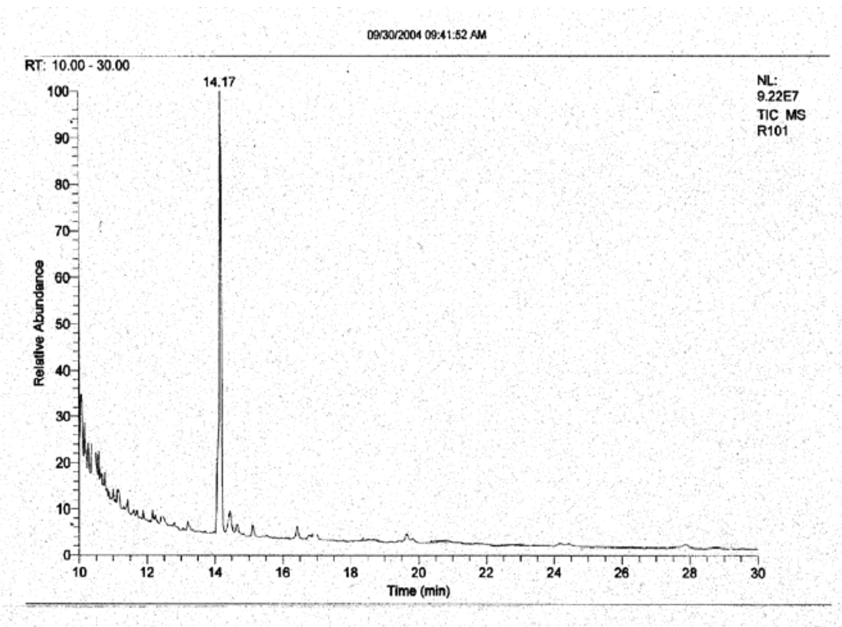


Figure 11. Gas Chromatogram of β-amyrin formate

High-performance liquid chromatography (HPLC) is used for separating and determining species in different materials. Normal phase HPLC was performed utilizing several solvent systems that are normally used and the best results were obtained with hexane and methylene chloride (20%:80%). The chromatogram is presented in Figure 12.

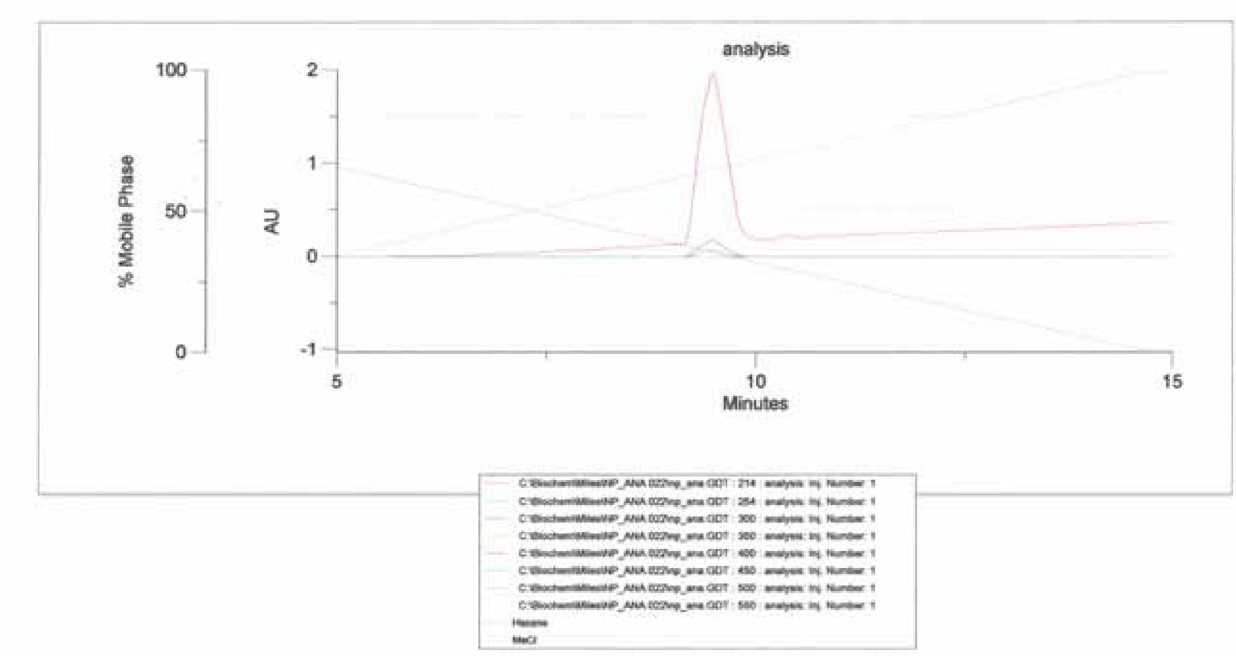


Figure 12. HPLC of β-amyrin formate

Upon mass spectral analysis (Figure 13) β-amyrin formate gave an M peak at m/z 454.7226 which corresponds to the molecular formula $C_{31}H_{50}O_2$. Amyrin series are characterized by the presence of a 12-13 double bond. This feature is being recognized by mass spectrometry, since the molecular ion undergoes the equivalent of a retro-Diels-Alder fragmentation to provide a very characteristic peak due to an ion with a mass of 218.2044.

The fragmentation pattern of the β-amyrin formate consists of molecular ion M= 454.7226, M-COOH= 409.3875, a characteristic signal for triterpenes M-236.5182= 218.2044.

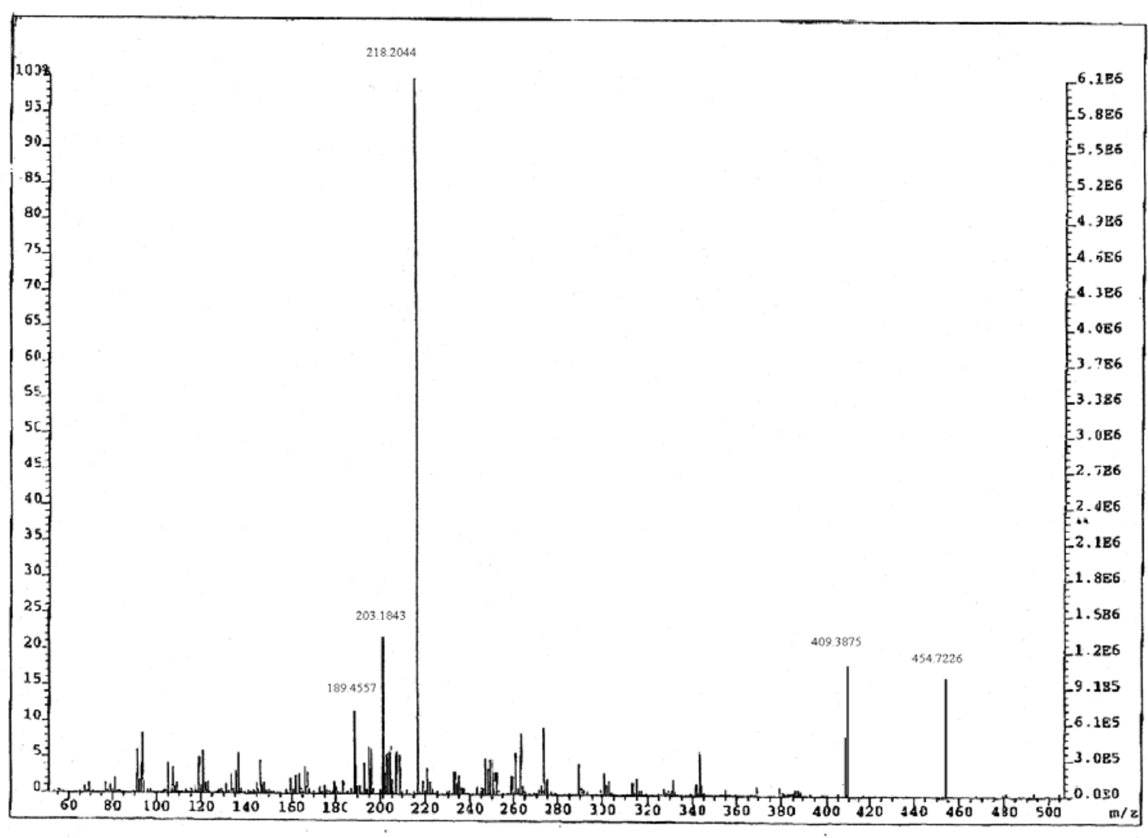


Figure 13. Mass spectrum of β-amyrin formate

The basic fragmentation pattern of the proposed structure is illustrated in Figure 14. The dominant fragmentation cleavage of the molecular ion m/z 454 bond takes place directly, to yield the triterpenyl ion $C_{30}H_{49}$, m/z 409.[48] This is followed by fragmentation of the triterpadiene moiety.

β- Amyrin formate, $M^+$
m/z 454

H—C(=O)—O

OH
C=O

+

$C_{30}H_{49}$ , m/z 409

m/z 408

• $CH_3$

+

$C_{15}H_{23}$ , m/z 203

$C_{16}H_{26}$ , m/z 218

$C_{14}H_{21}$, m/z 189

Figure 14. Scheme summarizing the mass fragmentation pattern of β-amyrin formate

The occurrence of the isolated compound (β-amyrin formate) was reported first time by S. Malhotra and al. in 1987, who extracted it from Canarium strictum gum.[49] This structure is shown in Figure 15.

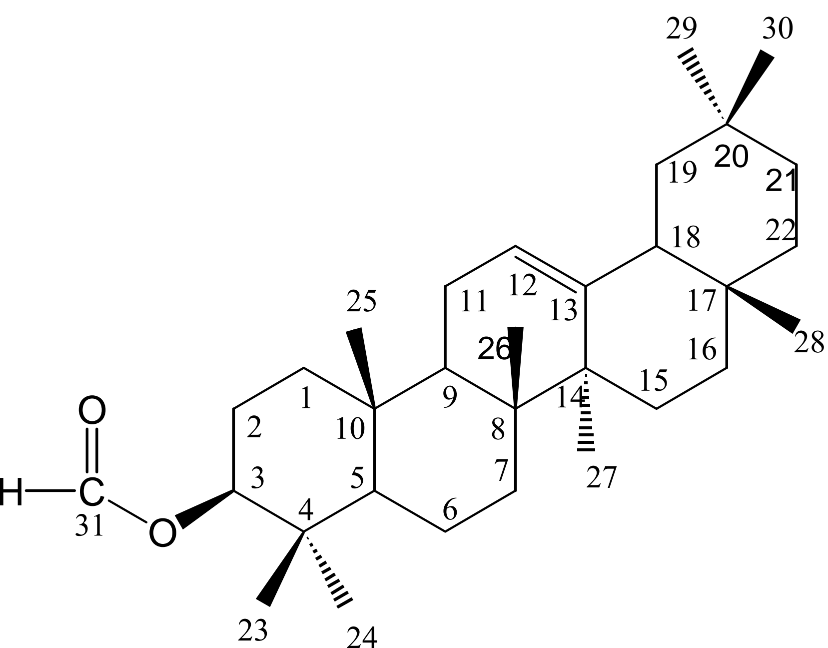


Figure 15. Structure of β-amyrin formate

The parent β-amyrin is a widely occurring triterpene and was identified in the extract by a combination of MS, IR, $^{1}$HNMR, $^{13}$CNMR, and DEPT experiments. The spectra were run in deuterated acetone and deuterated chloroform. The assignments of the $^{13}$C signals confirmed those made by Knight in 1974, based on the analysis of the chemical shifts in a series of triterpenoids and their derivatives.[50] In order to confirm the proposed structure, the $^{13}$C-NMR, $^{1}$H-NMR spectra of β-amyrin formate were obtained. The $^{1}$H-NMR spectrum revealed two peaks

at δ 4.51 ppm (t) and δ 9.81 ppm (s). The former signal was assigned to the methine proton on C3 (Figure 15) and the latter was ascribed to that of formyl proton (C31, Figure 15). The single hydrogen attached to the C-12 gave a peak at 5.2 ppm. Also, the peaks between 0.6 and 1.2 ppm, correspond to methyl groups ($CH_3$ - 8 groups), and the peaks between 1.2 and 2.9 ppm correspond to the methylene groups ($CH_2$ - 10 groups). The $^1$H-NMR spectrum (Figure 16) did not show a good separation of the peaks (between δ 0.00 ppm and δ 2.00 ppm), thus the proton peaks are not resolved. That is why the $^{13}$C-NMR was chosen for the final structure confirmation, besides MS and IR. However, the spectral assignments could be precisely obtained by using HMQC and DQCOSY spectroscopy.

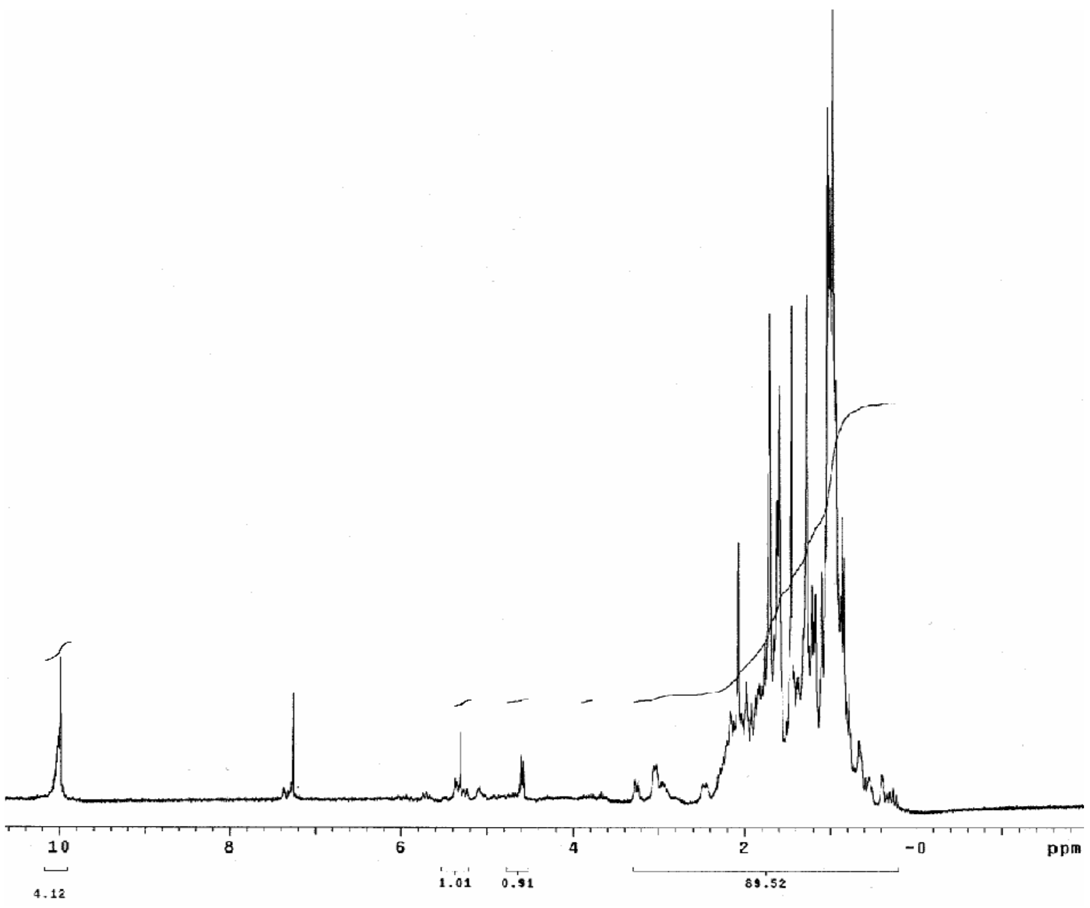


Figure 16. $^1$H-NMR spectrum of β-amyrin formate

The DEPT $^{13}$C-NMR spectrum (Figure 18) revealed the methyl carbons appear at δ 23.81 (C-23 and C-24), δ 15.76 (C-25), δ 16.22 (C-26), δ 25.64 (C-27), δ 28.73 (C-28), δ 28.93 (C-29 and C-30). Methylene carbons appear at δ 22.53 (C-2), δ 38.25 (C-1), δ 18.27 (C-6), δ 32.91 (C-7), δ 26.81 (C-15), δ 26.95 (C-16), δ 37.92 (C-22), δ 33.94 (C-21), δ 47.52 (C-19), δ 23.91 ppm (C-11). Methyne carbons appear at δ 83.13 (C-3), δ 47.73 (C-18), δ 57.92 (C-5), δ 121.63 (C-12), δ 47.53 (C-9), δ 41.74 ppm (C-14). Quaternary carbons appear at δ 42.82 (C-4), δ 38.52 (C-8), δ 41.74 (C-14), δ 32.83 (C-17), δ 30.11 (C-20), δ 145.22 (C-13), δ 37.75 (C-10) and δ162.11 ppm (C-31). A general $^{13}$C-NMR spectrum is shown on Figure 17.

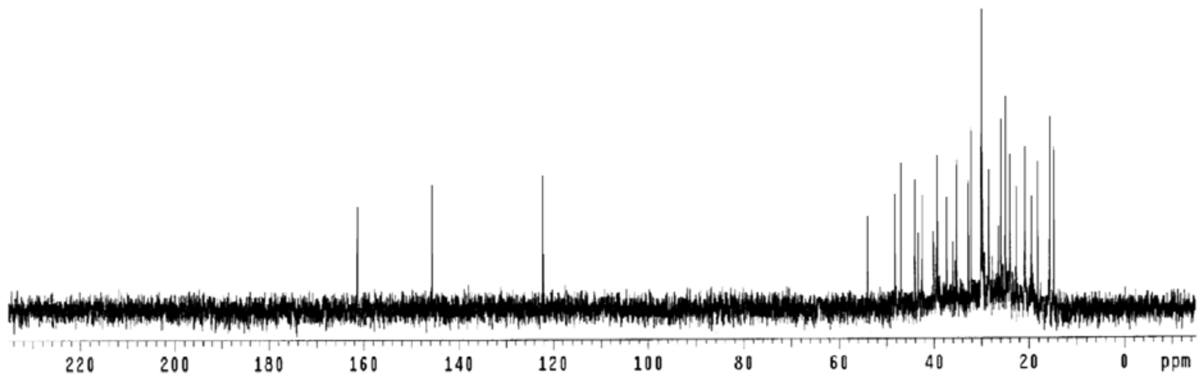


Figure 17. $^{13}$C-NMR spectrum of β-amyrin formate

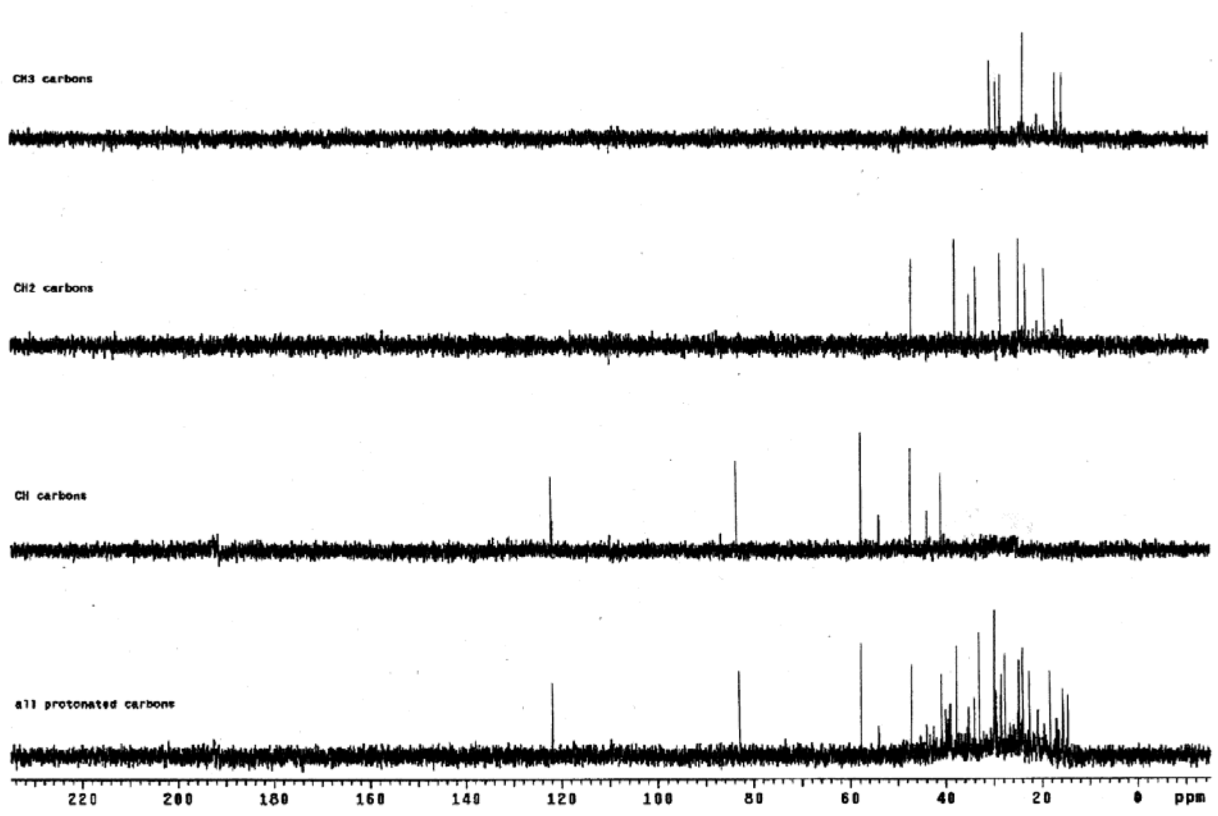


Figure 18. $^{13}$C-NMR DEPT spectrum of β-amyrin formate

The MS, IR, $^{1}$H-NMR, $^{13}$C-NMR data confirms the structure of β-amyrin formate. This pure compound was also tested against colon and breast cancer. The bioassay revealed that β-amyrin formate has -15% inhibition against colon cancer, but did not possess activity against breast cancer. The results are shown in Figure 19. Based on bioassay tests, this compound might be considered for studies related to the mechanism of action. The procedure and interpretation of data were presented in the first part of this thesis.

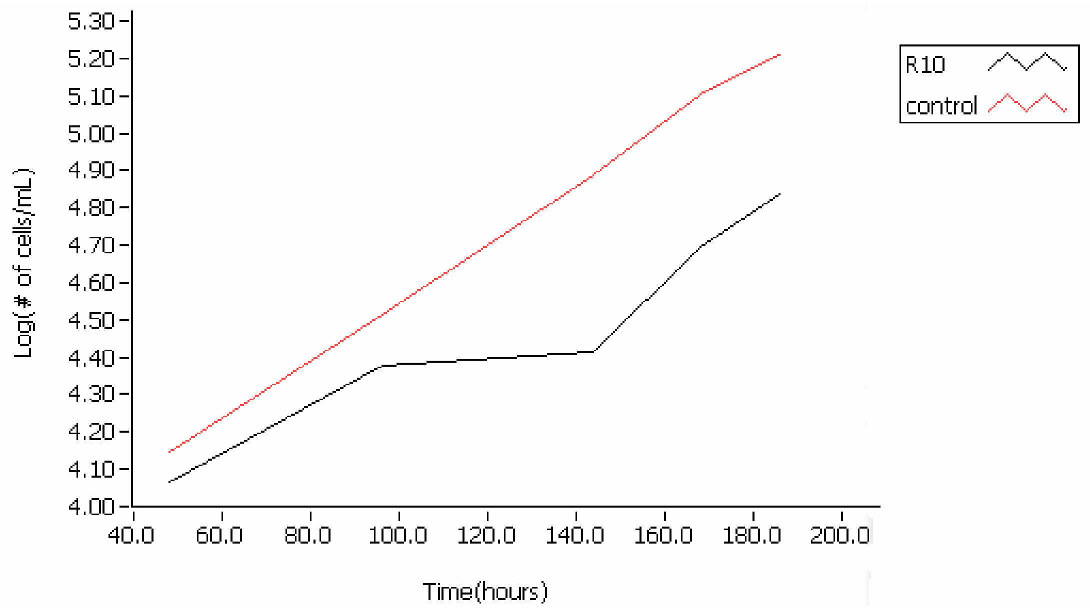


Figure 19. Effect of β-amyrin formate relative to the growth of colon cancer cells

The conclusion of this study is represented by the extraction, isolation and structure confirmation of a known pure compound, β-amyrin formate, which was isolated from dried leaves of Eucalyptus viminalis Labill. Also, it is presented like a possible drug lead against colon cancer and can be recommended for further investigation. However, this is the first report of isolation of β-amyrin formate from Eucalyptus viminalis Labill and the first test of this compound against colon cancer.

# 3. Experimental

## 3.1. General experimental procedure

UV spectrum was obtained in hexane with a Carry 500 scan UV-NIR-spectrophotometer and the absorption maximum is given in nm. IR spectrum was recorded on a Perkin Elmer Spectrum One FT-IR spectrometer. The NMR experiments were run on a Varian 300 MHz and the solvents used were deuterated chloroform and acetone. GC-MS spectra were recorded on Trace GC/Trace DQC from Finnigan. A capillary column of RTX-5MS with a length of 15 m and 0.25 mm ID was used. The oven temperature was initially set up to 80°C for 5 minutes than programmed to reach 250°C. HPLC was run on Gilson equipment, using a silica-gel column normal phase and the solvent used was a mixture of hexane and methylene chloride.

## 3.2 Extraction, isolation, characterization and cancer bioassay

The plant material, Eucalyptus viminalis, was received from Russia. The dried leaves (2.00 Kg) were extracted in methylene chloride and methanol using a Soxhlet device. After extraction in methylene chloride 156.00 grams of material were obtained. Column chromatography was carried out on silica gel (E. Merk, 70-230 mesh). Pre-coated preparative silica gel GF-254 plates (20×20 cm, 0.5 mm thick, E. Merk) were used for thin layer chromatography (TLC).

Spectrum of β-amyrin formate shows the following absorption maxima $\lambda_{max}$: 200 nm, 276 nm, and 340 nm, The IR spectrum indicated absorption maxima $\lambda_{max}$ at 2851, 1730, 1649, 1471, 1380, 1174, 850 $cm^{-1}$.

GC ($t_r$ 14.17 min, Figure 11), HPLC ($t_r$ 9.53), MS m/z: M = 454.7226, 409.3875, 218.2044. $^{13}C$ NMR chemical shifts from C1 to C31 :δ38.25, 22.53, 83.13, 42.82, 57.92,18.27,32.91, 38.52, 47.53, 37.75, 23.91, 121.63, 145.22, 41.74, 26.81, 26.95, 32.83, 47.73, 47.52, 30.11, 33.94, 37.92, 23.81 (C23, C24), 15.76, 16.22, 25.64, 28.73, 28.93 (C29, C30), 162.11 ppm.

In the cancer bioassay, 0.0002g of compound was used and the procedure was described on the first part of this thesis.

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