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# Anti-cancer Effect of Acetanilide and Para-nitroacetophenone in K562 Cells

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**Keywords**

Anti-tumor effect,
Acetanilide,
Para-nitroacetophenone

**Abstract**

Leukemia is a particular type of cancer characterized by the failure of cell death or disability in differentiation of hematopoietic cells. Chronic myelogenus leukemia (CML) is the most studied kind of this cancer. In this study, anti-cancer effect of acetanilide and Para-nitroacetophenone derivatives were investigated in the human leukemia K562 cells. K562 cell line was cultured by initially seeding 1×106 cells per milliliter in RPMI 1640 medium. Cell viability was investigated using trypan blue exclusion and MTT assays. Cell death in cancer and normal cells was quantified using propidium iodide (PI) and acridine-orange (AO) double staining. The one-way analysis of variance (ANOVA) and the Excel software were used for data analysis. Acetanilide and Para-nitroacetophenone derivatives had strong fatal and concentration-dependent effect on K562 cells and caused cell death mainly through induction of apoptosis. Statistical analysis of cells under fluorescence microscope revealed significant difference in apoptotic cells populations between treated and untreated cells. The results of this investigation clearly indicated that Acetanilide and Para-nitroacetophenone derivatives have cytotoxic effects in the K562 cell line. This information revealed also that these compounds may prepare a new therapeutic approach for the treatment of leukemia.

## 1. Introduction

The search for new anticancer agents continues to be an active area of research at many research centers (1). Acetaminophen has been suggested to be a prostaglandin formation inhibitor,(2) and earlier studies of laboratory rats that were fed the carcinogen N-2-fluorenylacetamide showed that acetanilide, a parent compound of acetaminophen, inhibited liver tumor formation and increased survival time (3, 4). In a study, Yamamoto et al (3) demonstrated that the acetanilide inhibited the toxicity and prevented the carcinogenesis from N-2-fluorenylacetamide. In another study, Grantham et al (5) showed the reduction in toxicity and carcinogenicity of N-2-fluorenyl-acetamide by the simultaneous administration

of acetanilide. With the purpose of evaluating whether Acetanilide and Para-nitroacetophenone would be an option for patients with CML, we incubated K562 cell line with these compounds.

## 2. Material and method

The K562 cell line (Pasture Institute, Iran) and peripheral blood mononuclear cells (PBMC) was used in this study. Cells were cultured by initially seeding $1\times10^6$ cells per milliliter in RPMI 1640 medium (GIBCO-BRL) containing 10 % heat-inactivated fetal bovine serum (GIBCO-BRL), 100 U/ml penicillin, and 100 mg/ml streptomycin (Bioidea) in a humidified incubator at 37 °C with 5 % CO2. The number of viable cells was determined by trypan blue dye exclusion using a hemocytometer. Cells with viability greater than 95% were used for the experiment. MTT assay test was performed to determine the effect of Acetanilide and Para-nitroacetophenone derivatives in K562 cells and IC50 value was obtained. Briefly, appropriate numbers of cells (10,000 cells) were cultured in 96-well plates. After 24 hours incubation, different doses of compounds (50, 100,150,200 and 250 µg/ml) were added to each well. At least three wells were allocated for each concentration. After 48 hours, MTT test was performed. When MTT solution is absorbed by living cells a formazan product that has an absorbance maximum at 450 nm is produced, thus optical density (OD) will be the number of cells per well. The absorbance (optical density, OD) of formazan was measured using spectrophotometry. IC50 value was calculated as the sample concentration that caused 50% cell death as compared to the control cells (no drug added).

### 2.1 Quantification of apoptosis using propidium iodide and acridine orange double staining

cell death in cancer and normal cells was quantified using propidium iodide (PI) and acridine-orange (AO) double staining according to standard procedures and examine under fluorescence microscope (6). Briefly, treatment was carried out in a 25 mL culture flask. K562 cells were plated at concentration of $1\times 10^6$ cell/mL, and treated with pyrimidine derivatives at $IC_{50}$ concentration. Flasks were incubated in atmosphere of 5% $CO_2$ at 37 for 24, 48, and 72 hours. The cells were then spun down at 1000 rpm for 10 minutes. Supernatant was discarded and the cells were washed twice using phosphate buffer saline (PBS) after centrifuging at 1000 rpm for 10 minutes to remove the remaining media. Ten microliters of fluorescent dyes containing acridine orange (AO, 10 µg/mL) and propidium iodide (PI, 10 µg/mL) were added into the cellular pellet at equal volumes of each. Freshly stained cell suspension was dropped into a glass slide and covered by coverslip. Slides were observed under UV-fluorescence microscope within 30 minutes before the fluorescence color starts to fade. The percentages of viable, apoptotic, and

necrotic cells were determined in more than 200 cells. Acridine orange (AO) and propidium iodide (PI) are intercalating nucleic acid specific fluorochromes which emit green and orange fluorescences, respectively, when they are bound to DNA. Viewed by fluorescence microscopy, viable cells appear to have green nucleus with intact structure while apoptotic cells exhibit a bright-green nucleus showing condensation of chromatin as dense green areas. Late apoptotic cells and necrotic cells will stain with both AO and PI. Comparatively, PI produces the highest intensity emission. Hence, late apoptotic cells exhibited an orange nucleus showing condensation of chromatin whilst necrotic cells display an orange nucleus with intact structure. This assay provides a useful quantitative evaluation and was done three times *(n = 3).*

### 2.2 Statistical analysis

Data were analyzed statistically using the $SPSS_{16}$ software. The one-way analysis of variance (ANOVA) is used to determine whether there are any significant differences between the means. A probability level of $p<0.05$ was considered statistically significant reference.

## 3. Results

Effects of Acetanilide on cell viability: The K562 cell line and PBMC were incubated with various concentrations of acetanilide for 24, 48, and 72 h. The impact of acetanilide on cell viability was quantitated 48 h post-exposure by the MTT assay. Exposure of K562 cells to acetanilide significantly inhibited growth in a dose- and time-dependent manner ($p<0.05$). While there was no significant effect from the low concentration of acetanilide after 24 hours (0.3%), there were significant decreases in viability at concentrations of 50, 100, and 200 µg/ml. Treatment of acetanilide cells for 48 and 72 h resulted in significant reductions in the number of viable cells (Fig. 1 and 2). The IC50 dose for acetanilide cells was determined to be 10.705± 0.423 µg/ml and 11.5±0.439 µg/ml at 48 and 72 h, respectively (Table1). IC50 value of K562 cells compared to the IC50 of PBMC was lower. So acetanilide had greater cytotoxicity against cancer cells.

Effects of Para-nitroacetophenone on cell viability: The K562 cell line and PBMC was incubated with various concentrations of Para-nitroacetophenone for 24, 48, and 72 h. The cytotoxicity of para-nitroacetophenon on cell viability was quantitated by MTT assay. After 24 h of exposure to para-nitroacetophenon, significant inhibitory effects on proliferation were only observed for the highest concentration (200 µg/ml). However, there were significant decreases in viability after 48 and 72 h (Fig. 3 and 4). The IC50 dose was determined to be 5.34 ± 0.145 µg/ml and 7.56 ± 0.172 µg/ml at 48 and 72 h, respectively

(Table 2). IC50 value of K562 cells compared to the IC50 of PBMC cells was lower. So Para-nitroacetophenone had greater cytotoxicity against cancer cells compared to normal cells.

### 3.1 Quantification of apoptosis Using Propidium Iodide and Acridine Orange Double Stainin

Apoptotic, necrotic, and viable K562 cells were scored under fluorescence microscope. This is also included the control cells (untreated) whereby, 200 cells were randomly and differentially counted. The study revealed that acetanilide and para-nitroacetophenon triggered morphological features that relate to apoptosis in a time-dependent manner (Figures 5a, b and c).Whereby; early apoptosis is obvious by intercalated AO within the fragmented DNA. In contrast, untreated cells were observed with a green intact nuclear structure. At 48-hour treatment with acetanilide and para-nitroacetophenon, blebbing and nuclear margination were noticed (moderate apoptosis). Differential scoring of treated acetanilide and para-nitroacetophenon cells (200 cells population) showed that there is a statistical significant (P< .001) difference in apoptosis positive cells, which indicates clearly that acetanilide and para-nitroacetophenon have a time-dependent apoptogenic effect.

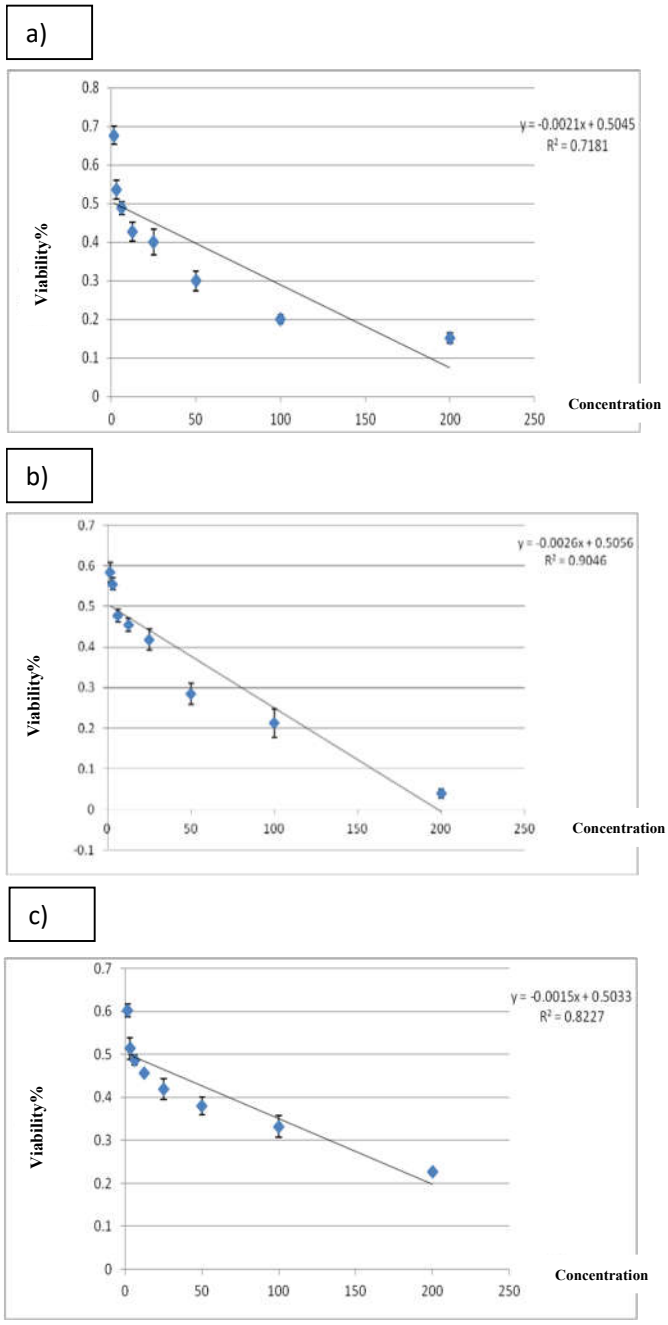



**Fig 1**. Effect of acetanilide on cell viability of K562 cells. Cells were treated with different concentrations of acetanilide for 24 (a), 48 (b), and 72 (c) hours. Viability was quantitated by MTT assay. Results are mean ± SEM.

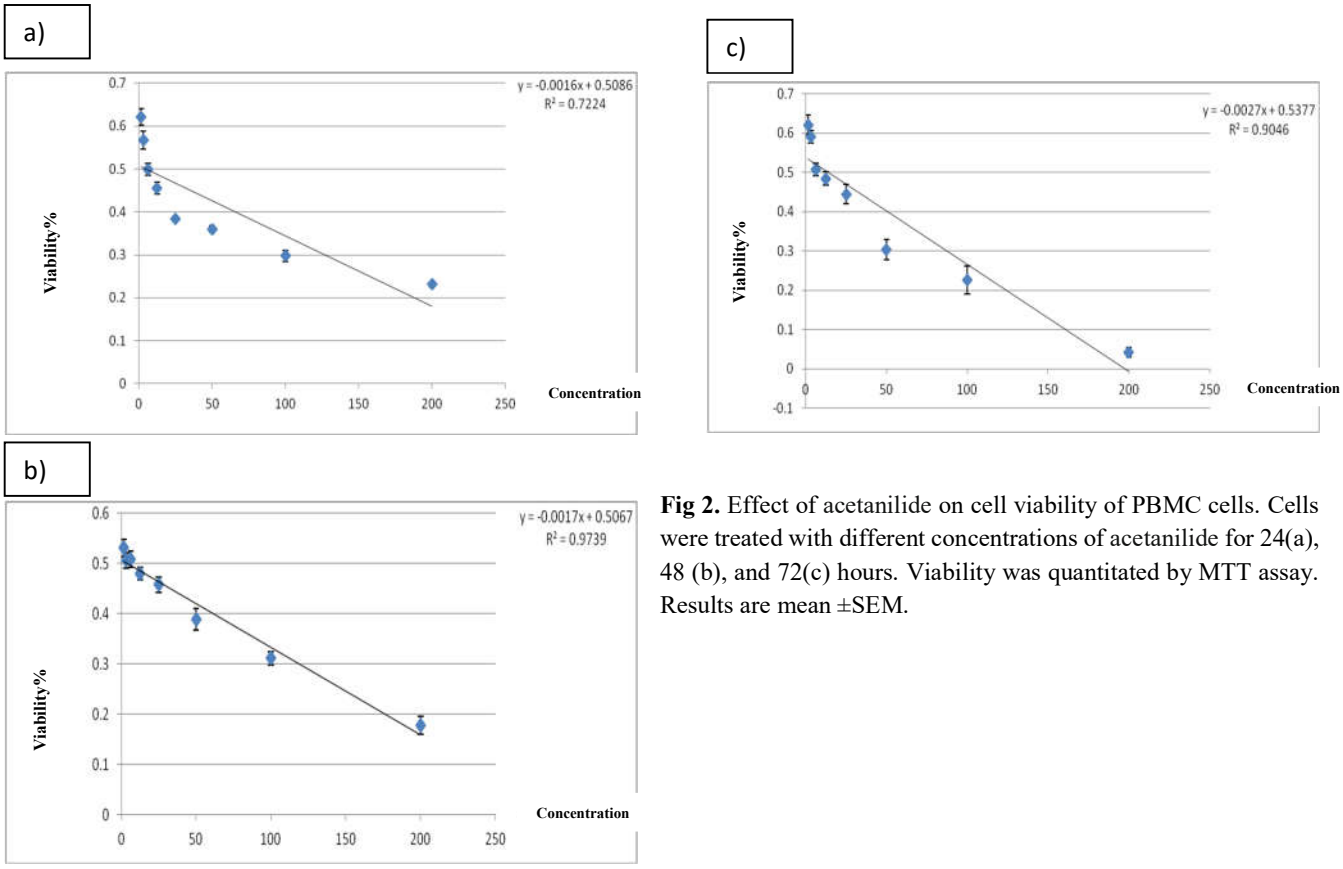



**Fig 2.** Effect of acetanilide on cell viability of PBMC cells. Cells were treated with different concentrations of acetanilide for 24(a), 48 (b), and 72(c) hours. Viability was quantitated by MTT assay. Results are mean ±SEM.

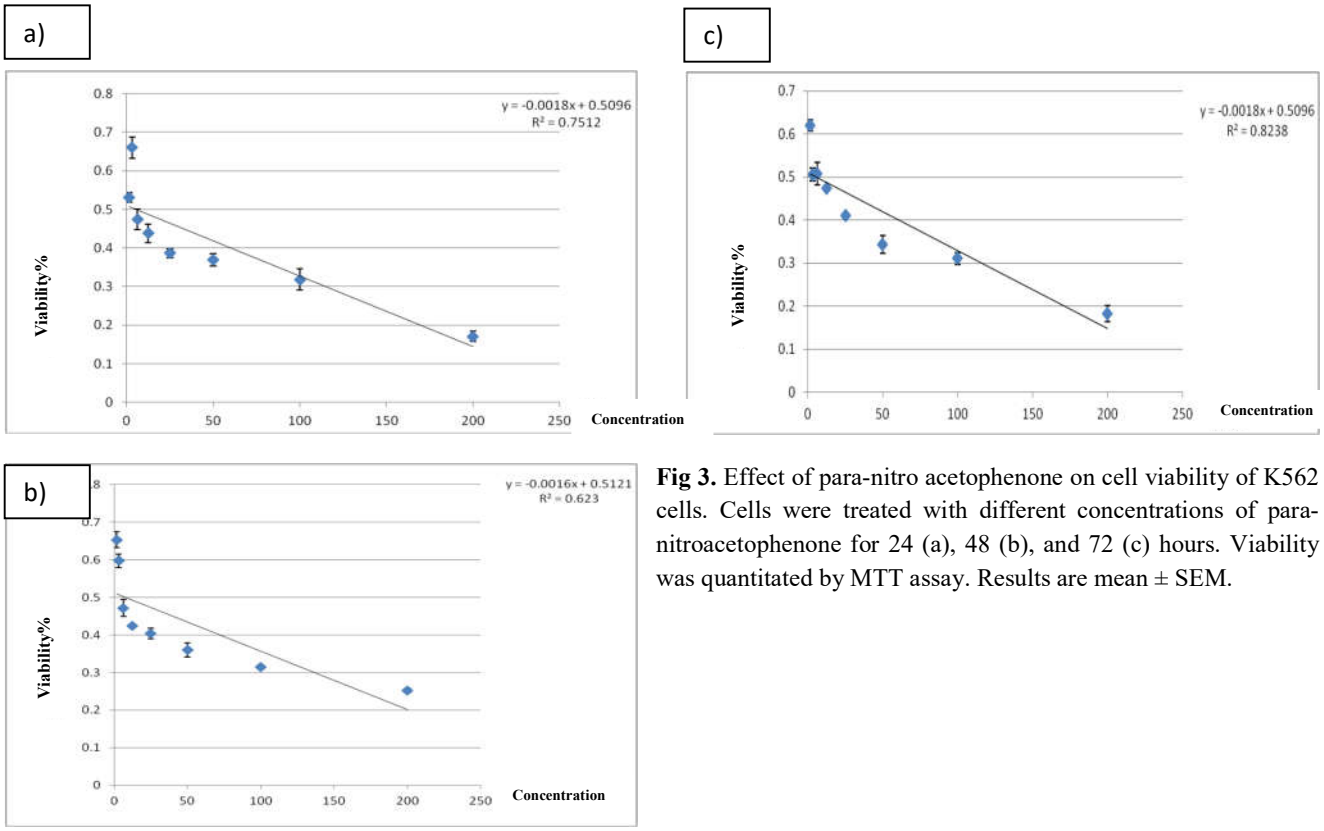
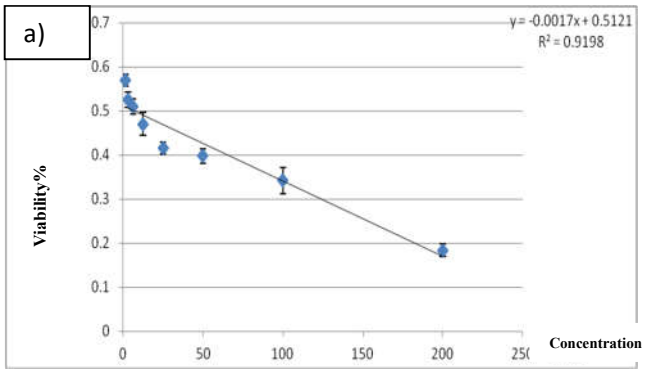
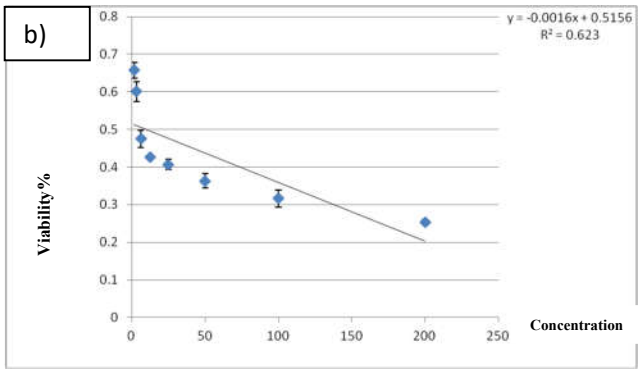



**Fig 3.** Effect of para-nitro acetophenone on cell viability of K562 cells. Cells were treated with different concentrations of para-nitroacetophenone for 24 (a), 48 (b), and 72 (c) hours. Viability was quantitated by MTT assay. Results are mean ± SEM.



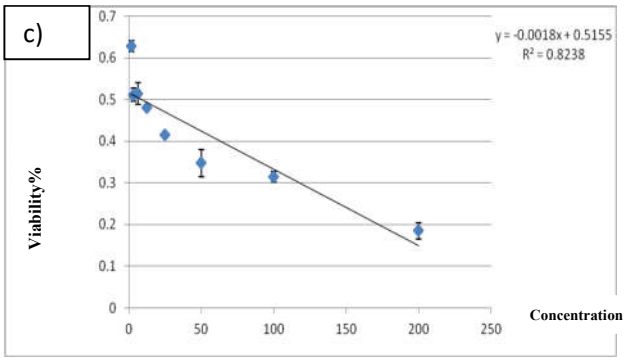




**Fig 4.** Effect of para-nitro acetophenone cell viability of PBMC cells. Cells were treated with different concentrations of para-nitro acetophenone for 24 (a), 48 (b), and 72 (c) hours. Viability was quantitated by MTT assay. Results are mean ± SEM.

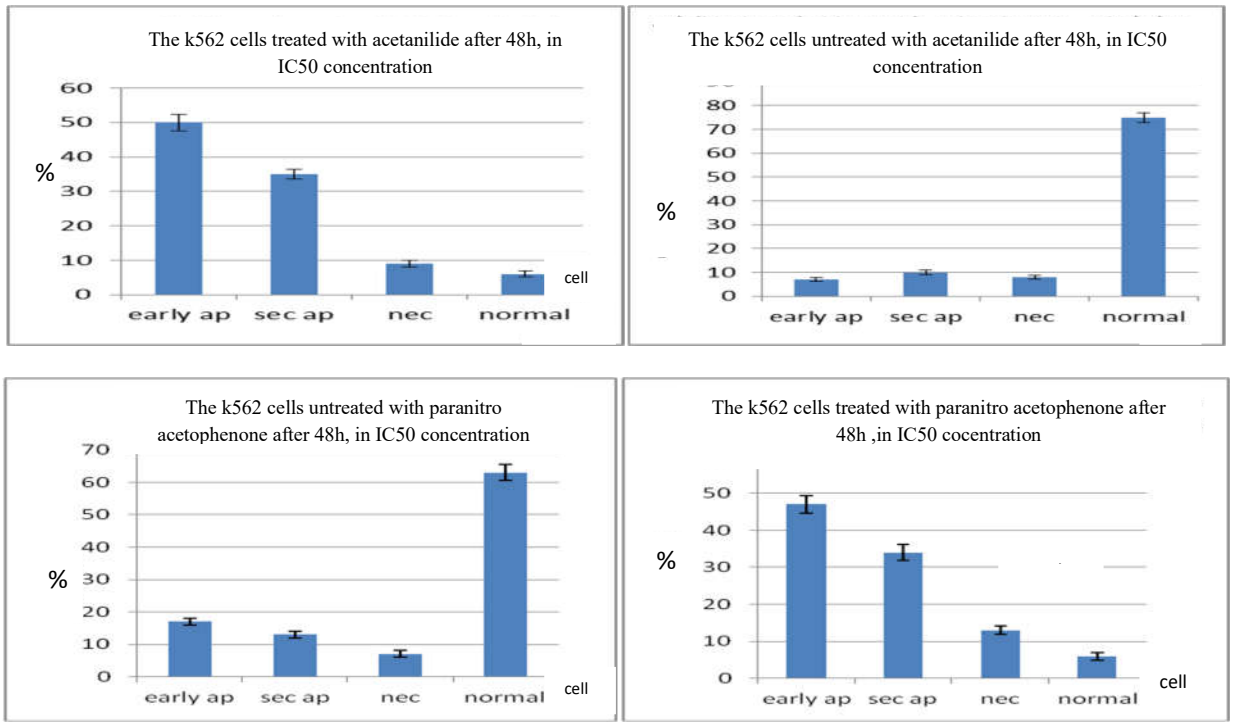



**Fig 5a:** Assessment of apoptosis by propidium iodide (PI) and acridine-orange (AO) double staining.

## 4. Discussion

In the present study, exposure of K562 cells to acetanilide significantly inhibited growth in a dose- and time-dependent manner ($p<0.05$). About para-nitroacetophenon , after 24 h of exposure, significant inhibitory effects on proliferation were only observed for the highest concentration (200 µg/ml). However, there were significant decreases in viability after 48 and 72 h. In addition, our results revealed that acetanilide and para-nitroacetophenon triggered morphological features that relate to apoptosis in a time-dependent manner. Both acetanilide and Para-nitroacetophenone had greater cytotoxicity against cancer cells compared to normal cells. These results are in accordance with other reports about similar compounds. A study showed that one effect of acetanilide is to reduce significantly the amount of the active carcinogenic intermediate, N-hydroxy-N-2-fluorenylacetamide(5). It is also demonstrated that herbicide propachlor (alpha-chloro-N-isopropyl-acetanilide) treatment causes L1210 mouse leukemia cells to accumulate in the G1 phase. This effect of propachlor is dose-dependent with more than 90% of G1 cells accumulating at 10 microM.

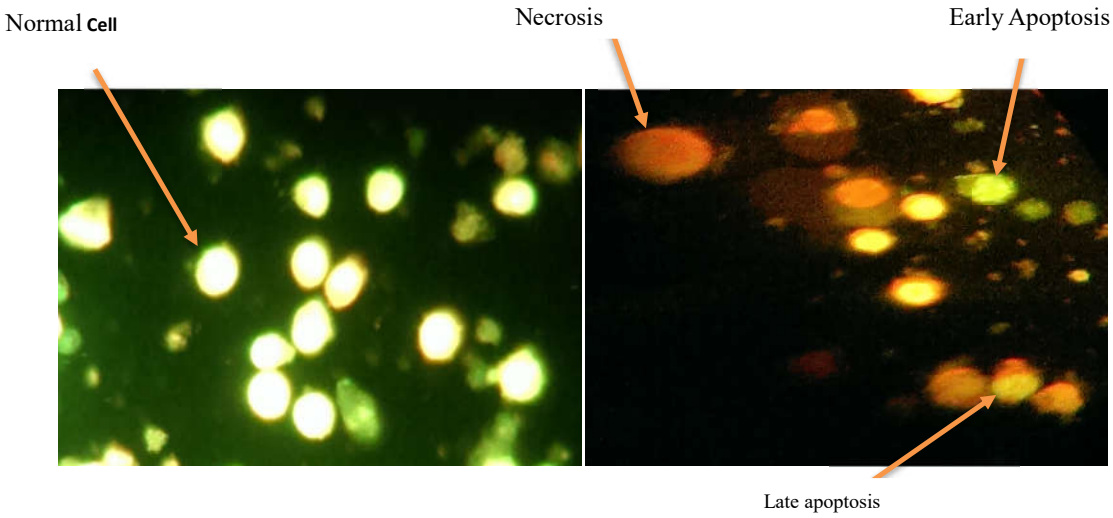



**Fig 5b**. Fluorescent micrograph of acridine orange and propidium iodide double-stained K562. K562 was treated at $IC_{50}$ of acetanilide at time-dependent manner. Cells were cultured in RPMI 1640 media (25 mL flask) maintained at 37 and 5% $CO_2$. (a) Untreated cells showed normal structure without prominent apoptosis and necrosis. (b) Late apoptosis was seen in 48 hours incubated cells, whereby positive staining with orange color represents the hallmark of late apoptosis (magnification 400X).

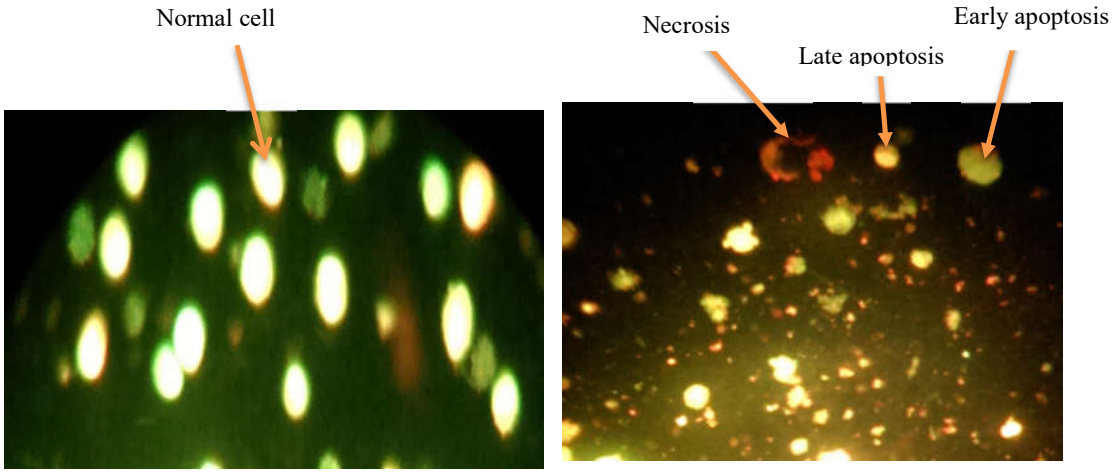



**Fig 5c.** Fluorescent micrograph of acridine orange and propidium iodide double-stained K562. K562 was treated at $IC_{50}$ of para-nitroacetophenone at time-dependent manner. Cells were cultured in RPMI 1640 media (25 mL flask) maintained at 37 and 5% $CO_2$. (a) Untreated cells showed normal structure without prominent apoptosis and necrosis. (b) Late apoptosis was seen in 48 hours incubated cells, whereby positive staining with orange color represents the hallmark of late apoptosis (magnification 400X).

**Table 1.** Doses of acetanilide that inhibited cell growth by 50% (IC50) in the Leukemia cancer cell line K562.

| | 24h | | 48h | | 72h | |
|---|---|---|---|---|---|---|
| Para-nitroacetophenon | IC50 (µg/ml) ±S.E | SI | IC50 (µg/ml) ±S.E | SI | IC50 (µg/ml)±S.E | SI |
| K562 | 5.33 ±0.183 | | 5.34 ±0.145 | | 7.56 ±0.172 | |
| PBMC | 7.118±0.216 | 133 | 8.62±0.211 | 1.615 | 9.75±0.183 | 1.29 |

**Table 2.** Doses of para-nitroacetophenon that inhibited cell growth by 50% (IC50) in the Leukemia cancer cell line K562.

| | 24h | | 48h | | 72h | |
|---|---|---|---|---|---|---|
| Acetanilide | IC50 (µg/ml) ±S.E | SI | IC50 (µg/ml) ±S.E | SI | IC50 (µg/ml) ±S.E | SI |
| K562 | 11.56 ±0.541 | | 10.705 ±0.423 | | 11.5 ±0.439 | |
| PBMC | 13.93±0.22 | 1.21 | 13.16±0.314 | 1.229 | 13.53±0.234 | 1.177 |

Kinetic experiments revealed that the accumulation of cells in G1 starts in about 10 hours, and increased for up to about 44 hours of incubation with 10 microM propachlor. Treated cells can be revised to a normal DNA distribution by removing propachlor (7). A new class of 1-acetanilide-4-aminopyrazole-substituted quinazoline Aurora kinase inhibitors has been discovered possessing highly potent cellular activity. The combination of potent cell activity and high free-drug exposure led to pharmacodynamic changes in the tumor at low doses, indicative of Aurora B-kinase inhibition and a reduction in tumor volume(8). Aurora kinases including Aurora A, B, and C are serine and threonine kinases that function as key regulators of the mitosis process. Aurora A and B are overexpressed in several human cancers including non-small cell lung cancer, upper gastrointestinal adenocarcinomas, and glioblastomas, (9). Because studies has shown that acetanilide is fairly well tolerated (3), thus the combined data of these experiments can be interpreted to imply that the administration of acetanilide and Para-nitroacetophenone may be a useful agents for preventing cancer.

## 5. Acknowledgements

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