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Dose and Time Dependent Response of HL - 60 Cells Against Newly Isolated Phenolic Compound from *Pandanus odoratissimus*

Abhaykumar Kamble*, Chinnappa Reddy, Amitkumar Kulkarni

Department of Biotechnology, Gulbarga University, Gulbarga,
Karnataka - 585106, India

*E-mail address: abhay.gene@gmail.com

ABSTRACT

Bioassay guided chromatographic purification of methanolic extract of Pandanus odoratissium leaves afforded new phenolic compound. The isolated compound was identified as 4-(4-(3,4-dimethoxyphenyl) hexahyddrofuro[3,4-c]furan-l-yl)-2-methoxyphenyl acetate by the spectroscope methods. The puropose of this investigation was to check the effect of the phenolic compound 4-(4-(3, 4-dimethoxyphenyl)hexahyddrofuro[3,4-c]furan-l-yl)-2-methoxyphenyl acetate isolated pure compound, the human leukemia (HL-60) cell line as model to evaluate the cytoxicity of compound on the MTT assay. The results obtained from the parameter indicates the compound has significantly reduced the viability of HL-60 cells shows LD_{50} values of 21.24 ± 0.5 µg/mL, 10.25 ± 0.3 µg/mL and 5.2 ± 0.6 µg/mL, upon 12, 24 and 48 hours of exposure respective indicates a dose and dependent response relationship. The present study indicate the compound is highly cytotoxic to human leukemia (HL-60) cells supports it as an effective therapeutic agent against leukemia.

***Keywords*:** Phenolic compound; HL- 60; Cytotoxicity; MTT assay

1. INTRODUCTION

Leukemia is one of the most common malignancies causing death worldwide. Although chemotherapy is the standard methods of treatment for patients, it has no been fully effective. Therefore development of new agents is still important to reduce the rate of mortality [1]. The HL-60 cell lines, derived from a single patient with acute promyelocytic leukemia (APL), provides a unique in vitro model system for studying the cellular and molecular events involved in the proliferation and differentiation of normal and leukemic cells of the granulocytes/monocytes/macrophage lineage [2].

APL (Acute Promyelocytic leukemia) is subtype of acute mylocytic leukemia with most cases carrying the characteristic chromosomal translocation that results in the PML-RARa fusion protein [3]. HL-60 cells express the transferring markedly decreases as the cells are induced to differentiate terminally [4,5].

Moreover, monoclonal antibodies to the transferring receptor inhibit HL-60 cell proliferation [6]. Similarly, insulin receptors are also displayed by HL-60 cells and insulin receptor expression decreases with granulocytic differentiation of HL-60 [7].

The *invitro* proliferation of normal myeloid progenitor cells is stimulated by the colony – stimulating factor (CSF) family of compounds, certain CSF also stimulate the proliferation of HL-60 cells [8-10]. Although the postulated leukemia specific growth factor was never reproducibly identified, this experimental approach proved fruitful in a number of unexpected ways.

The important characteristics of this unique cell line, with particular emphasis on HL-60 proliferation, differentiation and cellular oncogenes expression [11]. HL-60 cells also exhibit genetic abnormalities in cellular oncogenes and these abnormalities most likely also play a role in the unusual ability of these myeloid leukemic cells to proliferate continuously *in vitro* [12].

The aim of the present study was to evaluate the cytotoxicity of the compound to human leukemia (HL-60) cells, with a special emphasis on the assessment of time and dose response relationships.

The pure phenolic compound was isolated from MeOH extract of *pandanus odoratissimus* Linn and identified as 4-(4-(3, 4-dimethoxyphenyl) hexahydrofuro [3,4-c]furan-1-yl)-2-methoxyphenyl acetate. Pandan is said to be a restorative, deodorant, indolent and phylactic, promoting a feeling of wellbeing and acting as a counter to tropical lassitude.

It may be chewed as a breath sweetener or used as a preservative on foods. It is also said to have healthful properties antitumor [13]. There is no earlier report on this compound, as it mentioned that it is isolated newly.

2. MATERIAL AND METHODS

Preparation of extract

The dried material of pandanus odoratissimus was taken for extraction of bioactive compound with methanol using soxhlet apparatus. The solvent was removed under reduced pressureand a semisolid mass was obtained.

Column chromatography (Wilson and Walker, 1995)

Preparation of column

A clean and dry 500 ml capacity column of about 60 cm length was filled with slurry of silica gel- H of mesh size 60-120 µ (Himedia, Mumbia) to 45 portion using hexane. Due care was taken to avoid air bubbles while packing the column with stationary phase. Then the column was run through twice with the solvent system contains hexane to make the column air tight and compact one.

Loading

10 gm of methanolic extract of *Pandanus odoratissimus* was ground well with a small amount of silica gel loaded on to the top of the silica gel. Column that was 45 cm in height. The column was eluted with solvent of increasing polarity (Hexane, Chloroform and Methanol).

Collection of fraction

Totally 35 fractions with each 100 ml were collected, as they came off the column in a series of conical flasks (100 ml). thin layer chromatography was done with these fractions. Based on the results similar fraction were pooled together and concentrated to vacuo to isolate the active principle.

There was no mixture of other compounds, isolated single pure compound and 12 % of yield was obtained after the extraction and further used for elucidation of the compound.

2. 1. Physio-chemical Elucidation of Compound

The purified compound of *P. odoratissimus* isolated from leaf were subjected to IR, NMR and Mass Spectroscopy, spectroscopic studies and obtained spectral data, which is of immense use in the detection of the functional groups and further to elucidate their structrue (Yamaguchi 1970).

2. 2. IR spectroscopy

The infrared spectra of purified compound were obtained by KBR discs on Perkin elmer RX_1 spectrometer in the wave number (cm^{-1}) in the range of 2300-200 cm^{-1} was recorded as the inverted peaks.

2. 3. NMR Spectroscopy

The pure isolated compound spectra were recorded in JEOL Model GS x 400 spectrophotometer $CDCl_3$ (denatured methanol) was the solvent. NMR was recorded in the Bruker AMX 400 NMR Spectrometer using TMS (Tetramethyl silane) as an internal standard at 500. 124 MHz (^1H) and 27 ºC or 300 K. the chemical shift were recorded in δ (ppm) based either on δ TMS = 0 and the coupling constants or in hertz.

2. 4. Mass Spectroscopy

The fast atom bombardement – Mass spectra of the puirified compound were recorded on a Schimadzu instrument at 70 *eV* by direct in let method. The room temperature (27 ºC), M-nitrobenzyl alcohol (NBA) was used as the matrix unless specified otherwise. The matrix peaks may appear at m/z 136, 137, 154, 289 and 413 in the absence of any metal ions. If metals ions such as Na^+ are present, these peaks may be shifted accordingly. The positive FAB- MS spectra were obtained m/z verse relative abundance after taking 2-4 scans.

Based on the bioactive compounds present in the methanolic extract by observing significant result obtained in phytochemical and spectral elucidation studies.

2. 5. Growth media

Growth medium RPMI 1640 containing 1mmol/L L-glutamine was purchased from Gibco BRL products India pvt. Ltd. Fetal bovine serum (FBS), Antibiotics (Penicillin and streptomycin), Phosphate buffer saline (PBS) and MTT assay kit were obtained from sigma chemical company, Mumbai. 4-(4-(3, 4-dimethoxyphenyl) hexahydrofuro [3,4-c]furan-1-yl)-2-methoxyphenyl acetate isolated pure compound.

2. 6. Tissues culture

The HL-60 promyelocytic leukemia cell line was purchased from the Amercian type Culture collection ATCC (Manassas, VA). This cell line has been derived form peripheral blood cells of a 36 year old Caucasian female with acute promyelocytic leukemia (APL). The HL-60 cells grow as a suspension culture. The predominant cell population consists of neutrophilic promyelocteds. [14,15].

Cells were stored in the liquid nitrogen until use. They were next thawed by gentle agitation of their containers (Vails) for 2 minutes in a water bath at 37 ºC. after thawing, the content of each vial of cell was transferred to a 25 cm^2 tissue culture flask, diluted with up to 10mL of RPMI 1640 containing 1 mmol/L L-glutamine(GIBCO/BRL, Gaithersburg MD) and suspplemented with 10% (v/v) fetal bovin serum (FBS), 1% (w/v) penicillin/streptomycin.

The 25 cm^2 culture flasks (2X106 viable cells) were observed under the microscope, followed by incubation in a humidified 5 % CO_2 at 37 ºC. Three times a week, they were diluted under same conditions to maintain a density of 5X 105/mL and harvested in the exponential phase of growth.

The cell viability was assessed by the trypan blue exclusion test and manually counted using a hemocytometer.

2. 7. Cytotoxicity/ MTT Assay

The preliminary cytotoxic analysis was done by the MTT assay on all the extracts. A mitochondrial enzyme in the living cells namely succinate dehydrogenase, cleaves the tetrazolium ring, converting the methyl tetrazolium (MTT) to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells.

The assay detects living, but not dead cells and the signal generated is dependent on the degree of activation of the cells. This method was therefore used to measure cytotoxicity, proliferation or activation.

The colorimeteric assay that measured the reduction of 3-(4, %-dimethylthiasol-2-yl)-2,4-diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase.

The MTT enters the cells and passes into the mitochondria where is reduced to an insoluble, colored, formazan product. The cells are then solubilised with an organic solvent (DMSO or isoproponal) and the released, solubilized formazan reagent is measured spectrophotometrically [16,17].

Human leukemia (HL-60) cells were maintained in RPMI 1640 containing 1 mmol/L L-glutamine, supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (w/v) penicillin/Streptomycin, and incubated at 37 ºC in humidified 5% CO_2 incubator. To 180 µL aliquots in six replicates of cell suspension (5x 105/mL) seeded to 96 well polystyrene tissue culture plates, 20 µL aliquots of compound 4-(4-(3, 4-dimethoxyphenyl) hexahydrofuro [3,4-c]furan-1-yl)-2-methoxyphenyl acetate solutions (1.25, 2.5, 5.0, 10, and 20 µg/mL) were added to each well using distilled water as solvent.

Cells incubated in culture medium alone served as a control for cell viability (untreated wells). All chemical exposures were carried in 96 well tissue culture plates for the purpose of chemical dilutions. Cells were placed in the humidified 5% CO_2 incubator at 37 ºC for 12 h, 24 h and 48 hours respectively.

After incubation, 20 µL aliquots of MTT solution (5mg/mL in PBS) were added to each well re-incubated for 4 hour at 37 ºC, followed by low centrifugation at 800 rpm for 5 minutes. Then, the 200 µL of supernatant culture medium were carefully aspirated and 200 µL aliquots of dimethylsulfoxide (DMSO) were added to each well to dissolve the formazan crystals, followed by incubation for 10 minutes to dissolve air bubbles.

The culture plate was placed on a Biotex Model micro-plate reader and the absorbance was measured at 550 nm. The amount of colour produced is directly proportional to the number of viable cell. All assays were performed in six replicates for each concentration and means ± SD values were used to estimate the cell viability. Cell viability rate was calculated as the percentage of MTT absorption as follows

$$\% \text{ survival} = \frac{\text{Mean experimental absorbance}}{\text{Mean control absorbance}} \times 100.$$

2. 8. Statistical Analysis

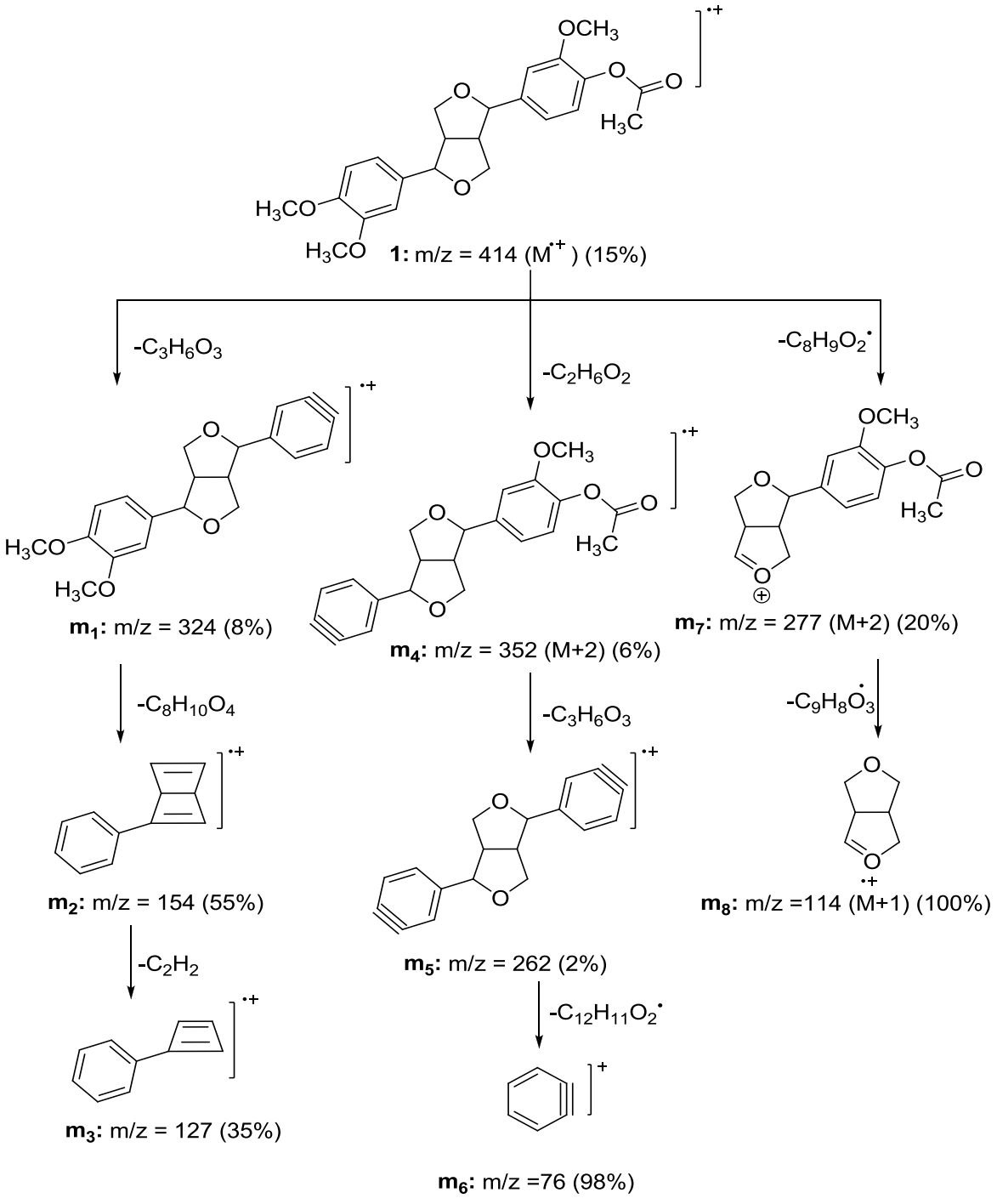
SPSS (Version 18.0, Chicago, IL) was used for all statistical analyses. Mean value and standard deviation were calculated using descriptive statistics. IC_{50} values were calculated by Probit regression.

Comparison of means was performed by one-way analysis of variance with Tukey's posttest. Comparison of assays was made by correlation and linear regression analysis. Differences were considered significant if $P < 0.05$.

3. RESULTS

3. 1. Physio-chemical Elucidation of Compound

4-(4-(3, 4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate:

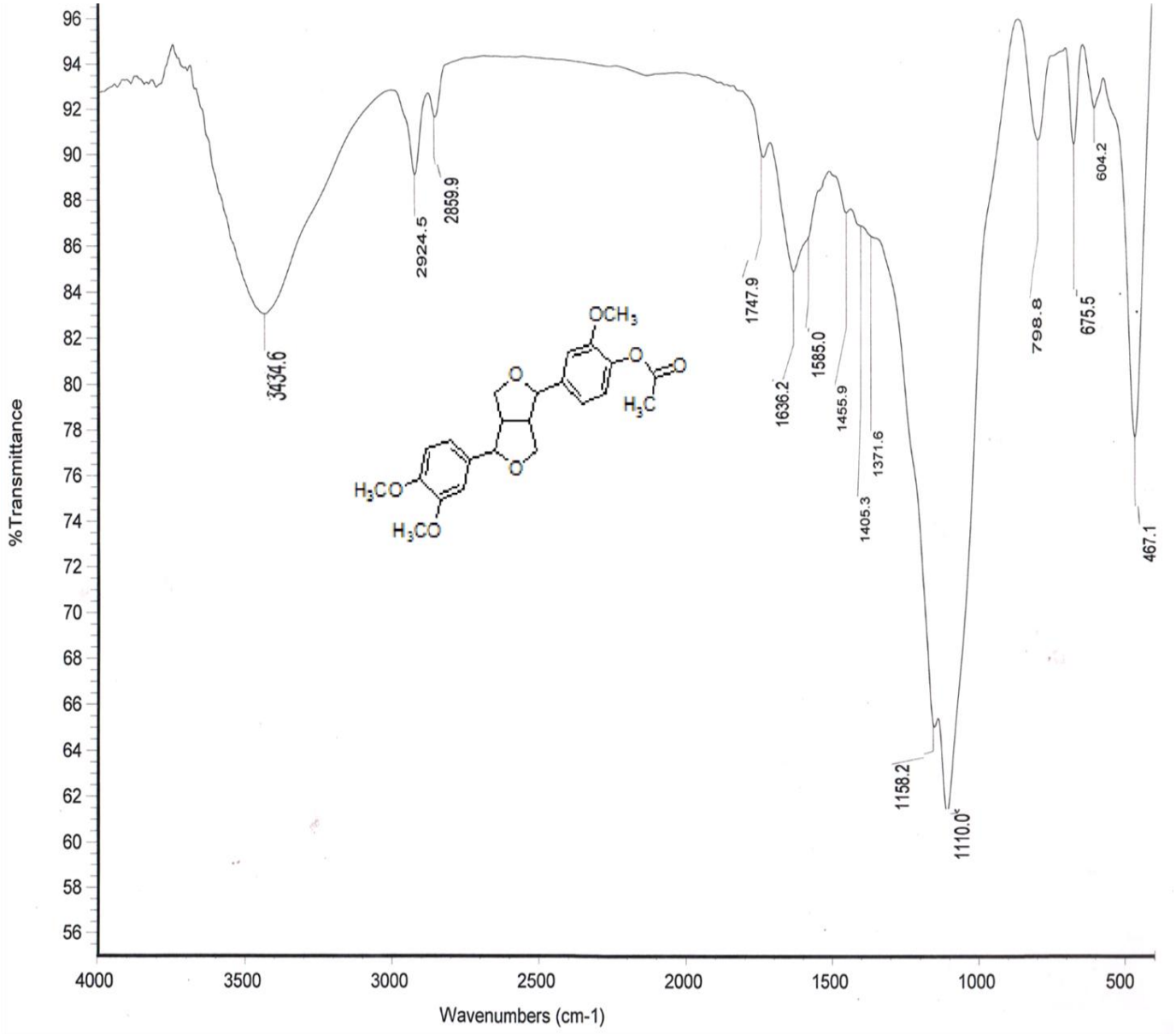


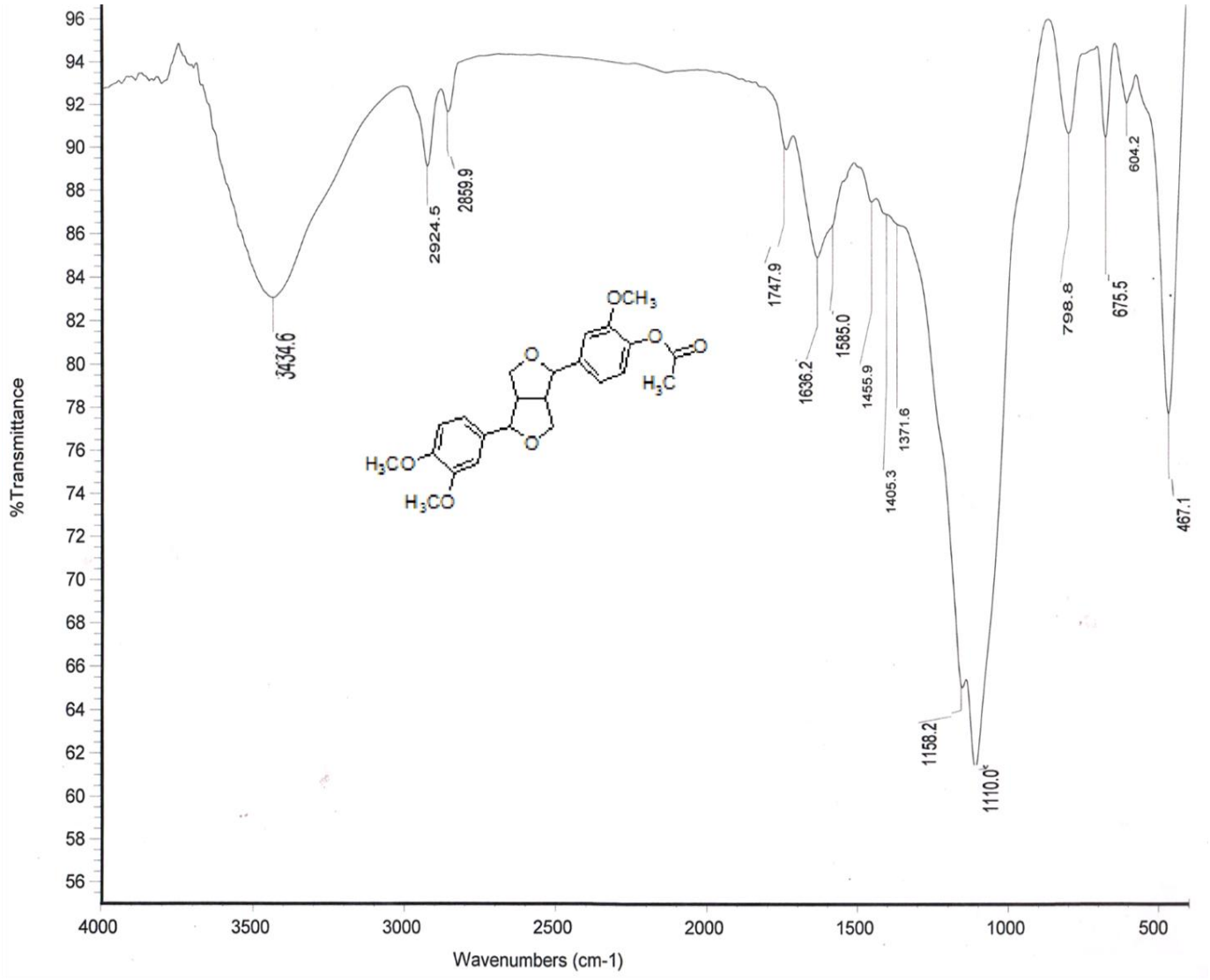
(Scheme-1)

3. 2. Infra Red Spectrum (IR)

The infra Red Spectrum of compound showed peaks at 3434, 2924, 2859 cm^{-1} due to OCH_3 OCH_3 and OCH_3 near to ester group respectively. The band exhibited at 1747 cm^{-1} due to C=0 group of esters. The compound was identified as 4-(4-(3,4-dimethoxyphenyl) hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate by comparing the IR spectrum of (Ting-Ting Jong and Shang-Wang Chau 1998) (Spectra - 1).

The spectra represents IR 1.



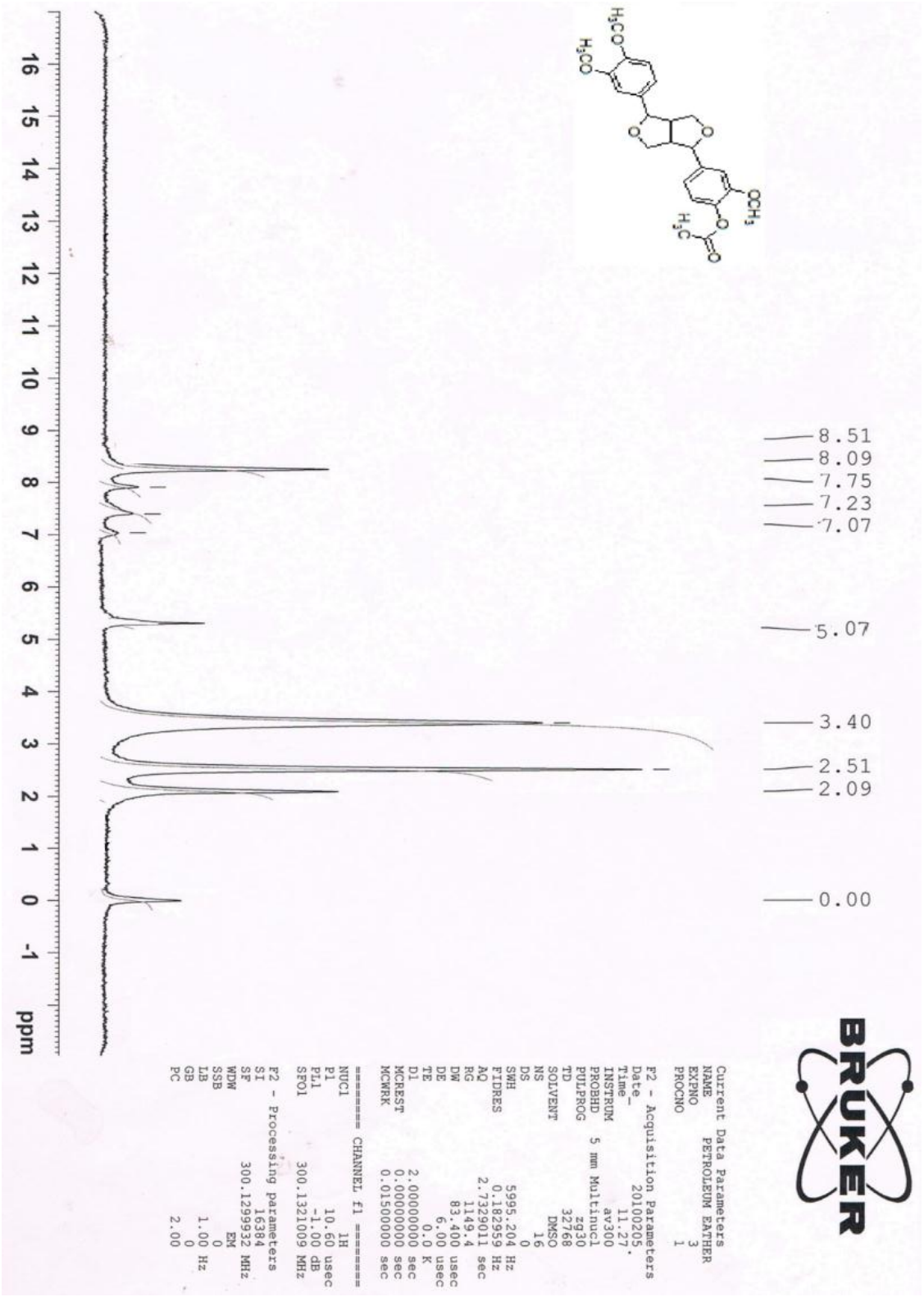


3. 3. ^{1}H Nuclear magnetic resonance spectrum (^{1}H NMR)

^{1}H NMR Spectrum showed five singlets at 7.07, 7.23, 7.75, 8.09, 8.51 δ (ppm) resonated to multiple of five protons of aromatic systems. The δ 5.07 two protons of furan rings. The δ 3.40 has single furan compound. The δ 2.51 resonated to singlet of one proton resonated to ester group.

The compound singlet peak at δ 8.51 (1*H*, *br s*) was identified as Phenolic hydroxy. The identification was further confirmed by ^{1}H NMR spectral data available in literature. (Ting-Ting Jong and Shang-Wang Chau (1998) (Spectra - 2).

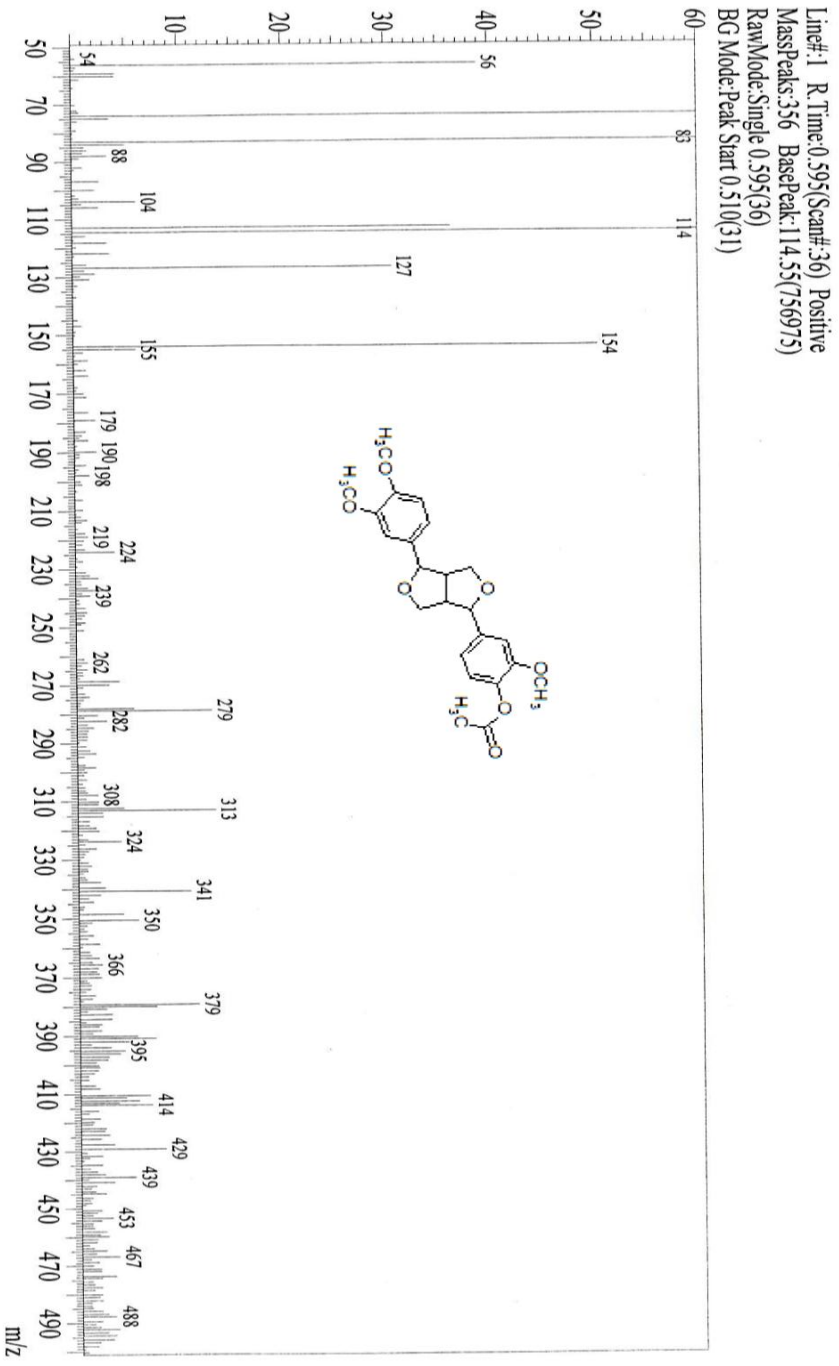
The spectra represents NMR 2



3. 4. Mass Spectrum (MS)

The mass spectrum disiplayed a molecular ion [M^+] peak at m/z 414 (15%) corresponding to molecular formula ($C_{23}H_{26}O_7$) together with fragmentation of at m/z 324 (8%), m/z 154 (55%) and m/z 127 (35%) (Spectra - 3).

The spectra represents Mass spectra. 3



3. 5. MTT assay

The MTT assay to examine the cytotoxic effect of phenolic compound 4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate on HL-60 cells for 12 h, 24 h and 48 hours, respectively.

Data generated form these studies clearly indicates that the compound 4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate exposure significantly reduced the viability of HL-60 cells. After 12 h, 24 h and 48 hours of exposure, the compound exerted a significant cytotoxic effect on HL-60 cells, showing LD_{50} values of 21.24 ± 0.5 µg/mL for 12 h (Fig - A), 10.25 ± 0.3 µg/mL for 24 h (Fig- B), and 5.2 ± 0.6 µg/mL for 48 h (Fig - C).

The compound 4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate, effect indicating was a dose and time dependent response.

The time response with regard to the cell viability in different doses of compound 4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate was compared with control (Fig - D). Similar, result were observed with response to the lethal median dose (LD_{50}) (Fig - E).

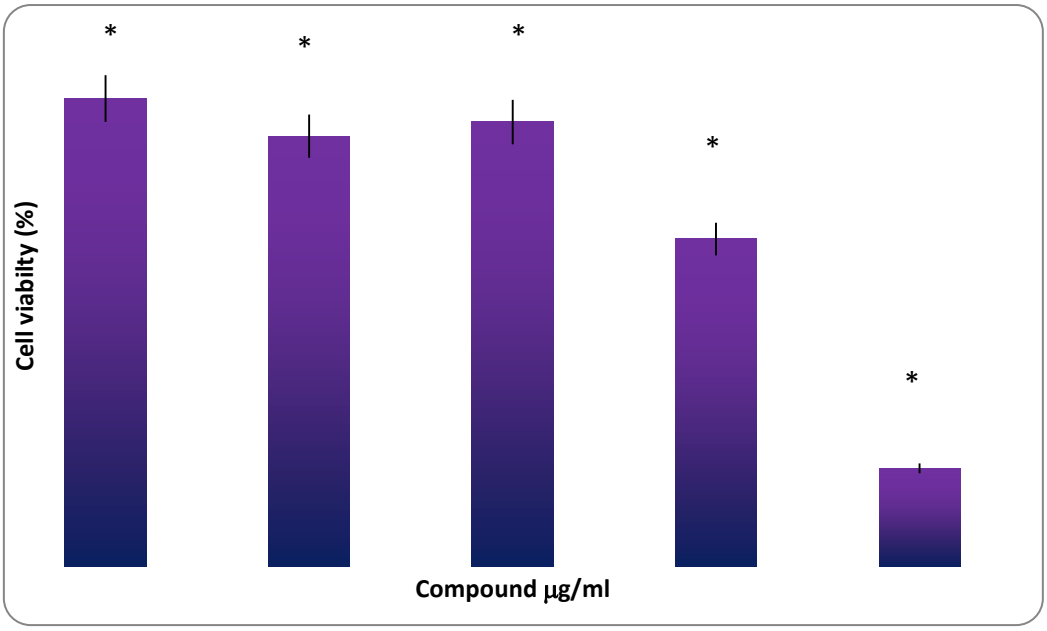


Fig - A: Toxicity of compound (4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate to human leukemia (HL-60) cells. HL-60 were cultured with different doses of compound for 12 hours as indicated in the materials and methods. Cell viability was determined based on the MTT assay. Each point represents a mean value and standard deviation of 3 experiments with 6 replicated per dose. Cell viability in 10 and 20 µg/ml are significantly different ($p < 0.05$) compared to the control according to ANOVA Dunnett's test.

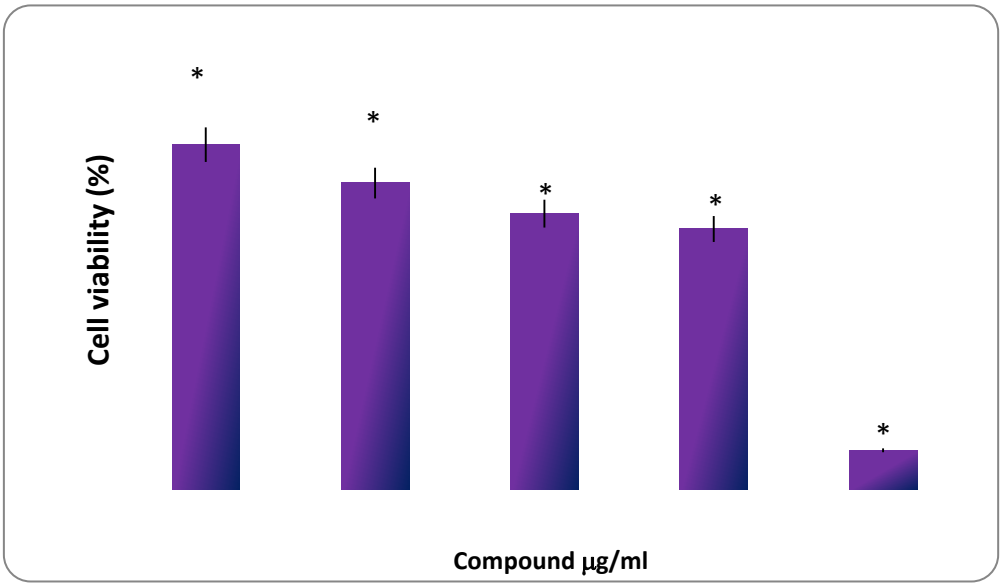


Fig - B: Toxicity of compound (4-(4-(3, 4-dimethoxyphenyl) hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate to human leukemia (HL-60) cells. HL-60 were treated with different doses of compound for 24 hours as indicated in the materials and methods. Cell viability was determined based on the MTT assay. Each point represents a mean value and standard deviation of 3 experiments with 6 replicated per dose. Cell viability in 5, 10 and 20 µg/ml are significantly different ($p < 0.05$) compared to the control according to ANOVA Dunnett's test.

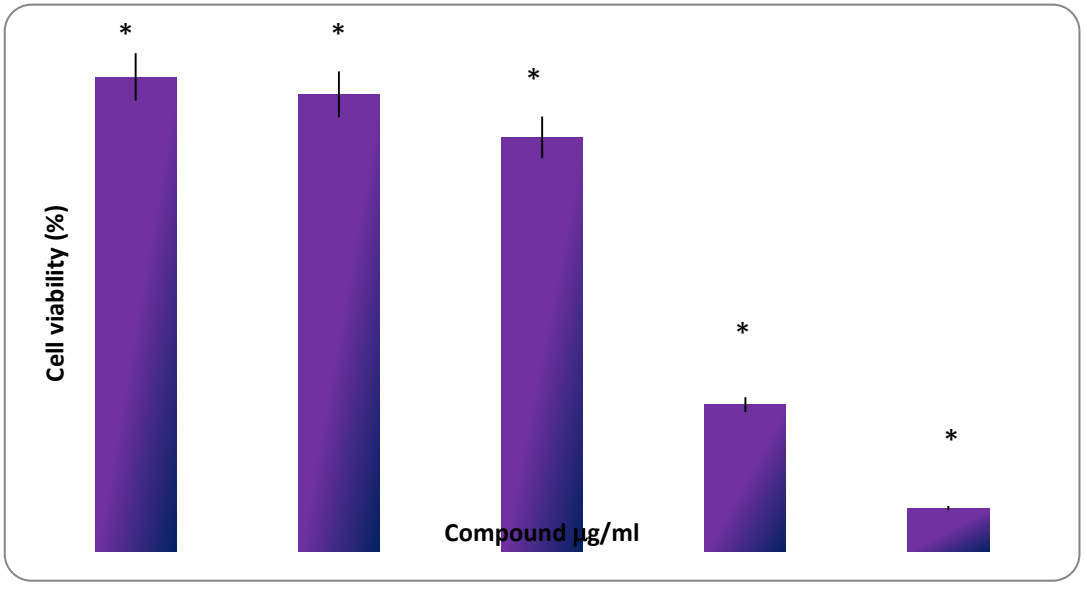


Fig - C: Toxicity of compound (4-(4-(3, 4-dimethoxyphenyl) hexahydrofuro [3,4-c]furan-1-yl)-2-methoxyphenyl acetate to human leukemia (HL-60) cells. HL-60 cells were cultured with different doses of compound for 48 hours as indicated in the materials and methods. Cell viability was determined based on the MTT assay. Each point represents a mean value and standard deviation of 3 experiments with 6 replicated per dose. All values are significantly different ($p < 0.05$) compared to the control according to ANOVA Dunnett's test.

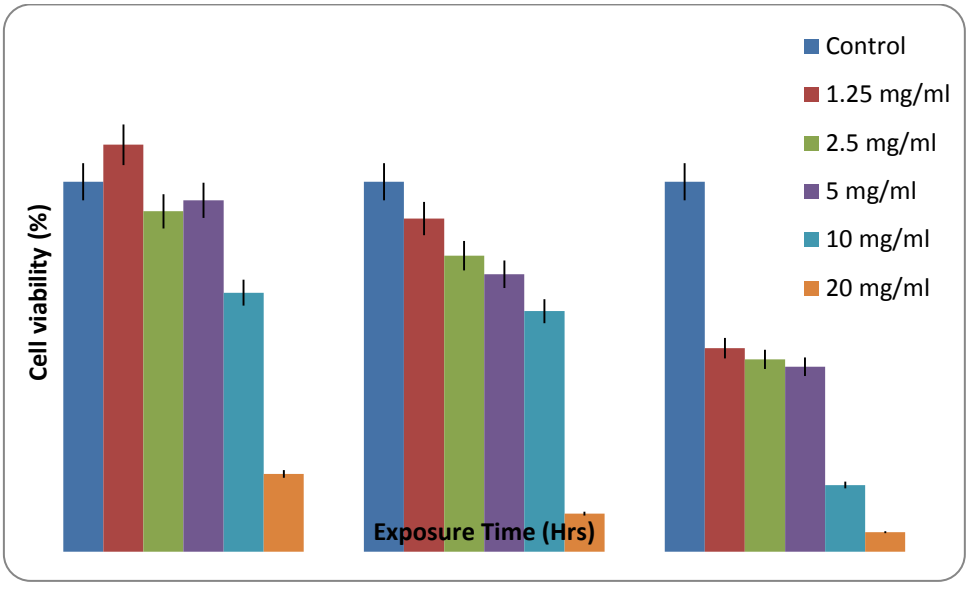


Fig - D: Time response relationship with regard to the cytotoxicity of compound (4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate to human leukemia (HL-60) cells. HL-60 cells were cultured with different doses of compound for 12,24 and 48 hours repectively. Cell viability was determined based on the MTT assay. Each point represents a mean ± SD of 3 experiments with 6 replicates per does.

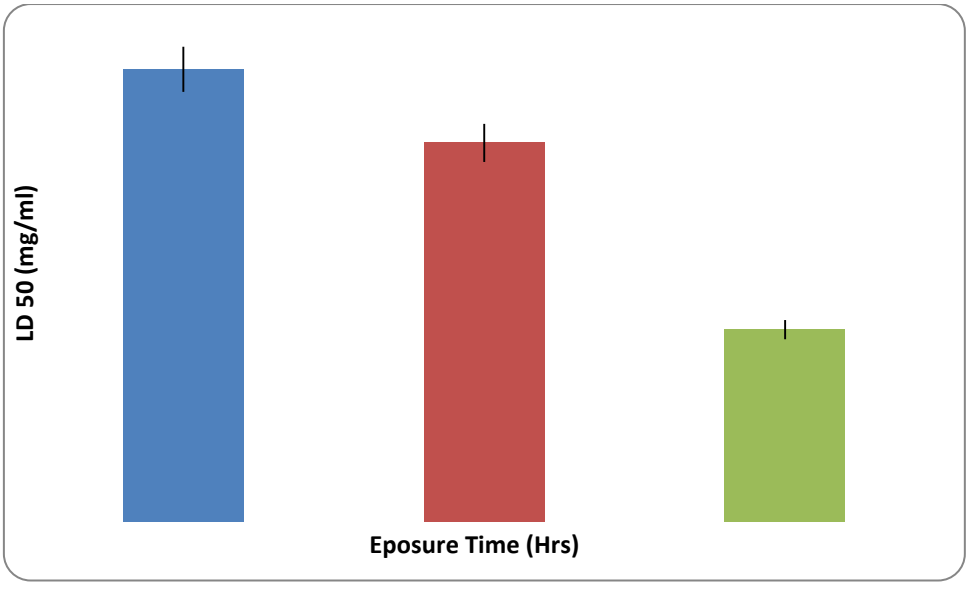


Fig - E: Time response relationship with regard to the LD_{50} values of compound (4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate (1) to human leukemia (HL-60) cells. LD50= 12.24 ± 0.5µg/mL for 12 hrs: 10.25 ± 0.3 µg/mL for 24 hrs: and 5.2 ± 0.6 µg/mL for 48 hrs of exposure.

4. DISCUSSION

The IR Spectrum of exhibited absorption band at 3434, 2924 and 2859 cm^{-1} due to OCH_3, OCH_3 and OCH_3 group near to Ester group respectively. The band exhibited at 1747 due to C=0 group of Ester. The 'H NMR spectrum δ 7.07-8.51 resonated to multiplet of five protons of aromatic systems. The δ 5.07 resonated due to two protons of furan rings. The δ 3.84 due to single furan compound. The δ 2.51 resonated to singlet of one proton resonated to ester group. The mass spectrum compound has displayed molecular weight at M/Z 414 (15%) corresponding to molecular weight.

Cytotoxicity has been defined as the cell killing property of a chemical compound independent from the mechanism of death. Assessment of a compound's toxicity to various cell types can be made using *in vitro* cytotoxicity tests, which are available and widely used. The inclusion of an *in vitro* cytotoxicity assay in early discovery efforts provides an important advantage in identifying potentially cytotoxic compounds [19].

In the present study, we have examined the cytotoxic effect of phenolic compound on HL-60 cells. Data from this study clearly indicates that the phytochemical compound isolated from methanolic extract is highly cytotoxic to human leukemia (HL-60) cells, showing LD_{50} values of 12.24 ± 0.5 μg/mL , 10.25 ± 0.3 μg/mL and 5.2 ± 0.6 μg/mL for 12 h, 24h and 48 hours of exposure, respectively. Recently, it is observed that the compound is cytotoxic to human liver carcinoma (HepG2) cells, showing a LD_{50} of 8.55 ± 0.58 μg/mL after 48 hours of exposure [20]. We found that low dose of compound has induced minimal toxicity in HL-60 cells upon 48 hours of exposure. Interestingly, such doses are similar to the therapeutically effective concentrations of compound which have been shown to induce remission in APL patients with minimal toxicity. Clinically, the standard dose for the treatment of patients with APL is 0.15 mg/kg per day which yields a maximum dose of 2-3 μM of compound in the plasma. High levels of compound (5 μg/mL and higher for 48 hrs) induce more than 50% of cell mortality [21].

In the present study, the results indicate that high level of phenolic compounds exposure of HL-60 cells have inhibited cell proliferation and induced mortality in a dose and time-dependent manner. Such effects have been observed with other test models, as well as clinical studied [22,23]. During the research work we proved the isolated compound has potentiality to act as anticancerous agent.

5. CONCLUSION

The column chromatography study has indicated that there is a great diversity in the distribution of secondary metabolites in leaf of *Pandanus odoratissimus*. The spectral data of the compound obtained from the IR, ^1H NMR and MS- spectrophotometric revealed that there is a phenol skeleton.

The compound has checked for the cell viability in duration of 12 h, 24 hand 48 hrs, in which the 48 hrs duration has showed a significant inhibition of cell viabiltity. Thus the inhibition of cancer after the treatment of pure extract of phenolic compounds 4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate on the HL-60 cell line indicates that the compound is a potent drug for the cancer treatment.

Acknowledgement

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