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# EVALUATION OF INVITRO CYTOTOXIC EFFECTS OF AMARANTHUS ROXBURGHIANUS EXTRACT ON LEUKEMIA CELL LINE

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**ABSTRACT**

Leukemia different from most other cancers.Leukemia cells do not always form a tumor. This cancer starts in bone marrow .The bone marrow is thick ,spongy liquid inside our bones where new blood cells are made.Leukemia starts in early forms of blood cells , often white blood cells which help fight infections . When a person is infected with leukemia they may have lost the ability to fight with infections and the white blood cells lost their originality and the person may develops tumors. Leukemia cells travel in the blood and may go all over the body However, it is important to consider that the appropriate use of cell lines needs to follow established guidelines for guaranteed data reproducibility and quality , and to prevent the occurrence of detrimental events those (that are linked to cross contamination and mycoplasma contamination ).

**Keywords**: Leukemia, Chemotherapy, Extraction, Amaranthus Roxburghianus Linn, Tetrazolium.

## INTRODUCTION

Cancer is the second leading cause of death worldwide. Although great advancements have been made in the treatment and control of cancer progression, significant deficiencies and room for improvement remain. A number of undesired side effects sometimes occur during chemotherapy. Natural therapies, such as the use of plant-derived products in cancer treatment, may reduce adverse side effects.[1] Currently, a few plant products are being used to treat cancer. However, a myriad of many plant products exist that have shown very promising anti-cancer properties in vitro, but have yet to be evaluated in humans. Further study is required to determine the efficacy of these plant products in treating cancers in humans. This review will focus on the various plant-derived chemical compounds that have, in recent years, shown promise as anticancer agents and will outline their potential mechanism of action.Cancer starts when cells in the body change (mutate) and grow out of control.[2] Our body made up of tiny building blocks called cells. normal cells grow when our body needs them and they die when our body doesn't need them. In most types of cancers , the abnormal cells grow to form lump or mass called tumor.

### Rationale of Study

Invitro cell viability and cytotoxicity assays with cultured cells are widely used for cytotoxicity tests of chemicals and sample screening.Applications of these assays has been of increasing interest over recent years.Currently,theses assays are also used in ontological researchers to evaluate both compound toxicity and tumour cell growth inhibition during drug development[1].Because they are rapid , inexpensive and do not required the use of animals.Furthermore,they are useful for testing large number of samples.[3]

Cell viability and cytotoxicity assays are based on various cell functions such as cell membrane permeability,enzymeactivity, celladherence, ATP production, Co-enzyme production, nucleotide uptake activity.By doing assays like Dye Exclusion assay,Calorimeteric assay,Fluorometric assay and Luminometric assays we can determine the cell viability and cytotoxicity effect in any plant extract.Natural products of plants possess several biological activities including antioxidant activity.[4].

## METHODOLOGY

### Materials and chemicals used:

70% ethanol, chloroform, Diethylether, Acetic acid, Ethylacetate, Formic acid,N-hexane, Fehlingsreagent,Dragondroff'sreagent,Ninhydrinreagent,Brominewater,Hager'sreagent, Wagner's reagent , Mayer's reagent , zincdust , concentrated sulphuric acid Acetic anhydride, ferric chloride, Hydrogen peroxide, 0.2M sodium hydroxide , pottasium dihydrogen phosphate , k-562-Human acute leukemia cell line, cell culture media: RPMI 1640 media adjustable multichannel pipettes, and pipettor,

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Fetal bovine serum,MTS reagent, DMSO, Doxorubicin,D-PBS, reflux condenser, Test tubes, Glass slides, Beakers , Aluminum foils, Round bottomed flask, UV spectrophotometer, centrifuge, pipettes:2 -10 microliter ,10-100 microliter, 100-1000 microliter, Inverted microscope, 37°C incubator with humidified atmosphere of 5%CO2 ,ELISA plate reader.[5]

**Plant extraction:**

The collected aerial parts of the plant was washed under the running water then air dried after drying aerial parts of plant was powdered and kept for extraction by using 70% ethanol and plant extract was stored in refrigerator.The extraction was the first step to separate desired natural products from the plant raw materials.Extraction methods include solvent extraction,distillationmethod,pressing and sublimation according to the extraction principle.The most widely used method was solvent extraction method.[6]

The extraction of the plant parts through the following stages:

1. The solvent penetrates into the solid matrix
2. Solute dissolves in the solvents
3. Solute was diffused out of the solid matrix
4. Extracted solutes are collected

The selection of solvents was a crucial role for the solvent extraction.Selectivity,solubility,cost and safety should considered in selection of solvents. Selection of solvents was based on the law of similarity and inter miscibility,solvents with a polarity value near to the polarity of solute are likely to perform better and viceversa.Alcohols(ethanol and methanol) are the universal solvents in solvent extraction for phytochemical screening of the extract.Generally, the fine particle size will give the better result in the extraction achieves. The extraction efficiency will be enhanced by the small particle size due to the enhanced penetration of solvents and diffusion of solutes. The extraction efficiency increases with increase in extraction duration in a certain time range. Increasing time will not affect the extraction after equilibrium of the solute was reached inside and outside the solid material [7]. The methodology that done was the powdered plant extract was kept for extraction by using 70% ethanol. All preliminary phytochemical analysis was performed by standard AOAC methods.

**The extraction was done by using the hot soxhelation method.**

The Soxhlet extraction process

the thimble of solid. The condenser ensures that any solvent vapour cools, and drips back down into the chamber housing [8].

**Preliminary phytochemical screening:**

The crude ethanolic extract of leaves was tested for the presence of alkaloids, steroids, tannins, saponins and glycosides. The qualitative results are expressed as presence and absence of phytochemicals.

**Test for alkaloids:**

Few mg of extract was taken for following tests that are involved in the test for alkaloids.

**A.Dragendorff's test:**

To small amount of the extract, Dragendorff's reagent (Potassium bismuth iodide solution) (1 mL) was added; an orange red precipitate shows the presence of alkaloids

**B.Mayer's test***:*

To small amount of extract, Mayer's reagent (Potassium mercuric iodide solution) (1 mL) was added. Formation of cream colored precipitate gives an indication of the presence of alkaloids. [22-24]

**C.Wagner's test**:

Potassium iodide (2 g) and iodine (1.27 g) were dissolved in distilled water (5 mL) and the solution was diluted to 100 mL with distilled water. Few drops of this solution was added to extract; a brown colored precipitate indicates the presence of alkaloids.

**Test for Saponins:**

The crude extract (0.5 g) was separately shaken with distilled water (10 mL) in a test tube. The formation of frothing, which persists on warming in a water bath for 5 min, shows the presence of saponins.

**Tests for steroids and terpenoids :**

**A.Salkowski test***:*

The crude extract (about 100 mg) was separately shaken with chloroform (2 mL) followed by the addition of concentrated $H_2SO_4$ (2 mL) along the side of the test tube, a reddish brown coloration of the interface indicates the presence of terpenoid . b.Liebermann-Burchard test: Each extract (100 mg) was shaken with chloroform in a test tube; few drops of acetic anhydride was added to the test tube and boiled in a water bath and rapidly cooled in ice water. Concentrated $H_2SO_4$ (2 mL) was added to the test tube. Formation of a brown ring at the junction of two layers and turning the upper layer to green shows the presence of steroids while formation of deep red color indicates the presence of triterpenoids [28-30].

**Test for tannins:**

Crude extract was separately stirred with distilled water (10 mL) and then filtered. A few drops of 5% ferric chloride were then added. Black or blue-green coloration or precipitate was taken as positive result for the presence of tannins [31-33].

**Thin layer chromatography:**

In the present study the TLC profiling of all the plant extracts again revealed the presence of different metabolites such asalkaloids,flavonoids, phenols,tannins

and steroids.Thin layer chromatography(TLC) is usually done for a better identification of the bioactive compounds. By calculating the Rf value of each TLC plate we can know which metabolites are present in the plant extract.

**Preparation of TLC plates for spotting the sample:**

TLC is a chromatographic technique which is used for the separation of mixture of compounds. TLC was performed on a sheet of aluminium foil which was coated with a thin layer of adsorbent silica gel.The moisture free TLC plates (5×10) cm were taken .Each solvent extract which was diluted with approximate solvent was loaded 1cm away from the base of the TLC plates using capillary tubes and the samples were allowed to dry .Different solvent systems were used as mobile phase they are methanol, chloroform, Ethylacetate,hexane in different ratios . The tlc plates were developed in a closed chamber containing the mobile phase after which the plates were dried thoroughly dried. The plates were observed under the white light and uv lamp (254nm ,366nm wavelengths)[9]

**Developing systems :**

The following six different solvent systems were used as developing systems: ethyl acetate:aceticacid:formic acid and water (5:0.5:0.5:1.35) for flavonoids.n- hexane :diethylether :acetic acid (13:7:5) for steroids.chloroform:ethyl acetate: formic acid:phenols(5:4:1) for phenols.After the preparation of mobile phases the activated plates which are loaded with the minute sample on the samplespotting are kept in the respective mobile phase and closed with a aluminium foil.The travelled sample was kept aside for drying.Flavonoids,steroids and phenols were determined under the ultraviolet chamber . The relative front (*Rf*) of each fraction was calculated [10].

**Statistical analysis:**

All analysis was repeated thrice and the result was presented as mean+SD

**Invitro antioxidant activity:**

In humans many diseases are associated with the accumulation of radicals antioxidant can scavenge free radical and minimize their impact.

Here,we performed in-vitro antioxidant activity of *Amaranthus roxburghianus Linn* by using hydrogen peroxide radical scavenging assay.

Buffer preparation for hydrogen peroxide scavenging assay as follows:

1. 0.2M of sodium hydroxide was prepared as per the indian pharmacopeia as per 1996standards.
2. 50ml of potassium dihydrogen phosphate was placed in200ml volumetric flask and 39.1ml of 0.2M sodium hydroxide solution was added and finally volume was made up to 200ml with distilled water to prepare phosphate buffer(pH 7.4).
3. 50ml of phosphate buffer solution was added with equal amount of hydrogen peroxide to generate free radicals and solution was kept aside at room temperature for 5minutes to complete the reaction.
4. Extract(1ml)in distilled water were added to 0.6ml of hydrogen peroxide solution and the absorbance measured at 230nm in a spectrophotometer against blank solution containing phosphste buffer solution without hydrogen peroxide.
5. Concentration selected for extract were ranging from 20mg/ml to 100mg/ml.
6. Absorbic acid was used as a standard.The experiment were repeated of scavenging oh $H_2O_2$ of extract was measured using the following equation:

Percentage (%) of $H_2O_2$ radical scavenging activity

$$=(A0-A1)\div A0\times 100$$

Where ,A0 is the absorbance of the control and A1 is the absorbance of the sample [11].

**MTS assay:**

MTS Cell Proliferation Assay is a colorimetric method for sensitive quantification of viable cells in proliferation and cytotoxicity assay. The method is based on the reduction of MTS tetrazolium compound by viable cells to generate a colored formazan product that is soluble in cell culture media. This conversion is thought to be carried out by NAD(P)H-dependent dehydrogenase enzymes in metabolically active cells. The formazan dye produced by viable cells can be quantified by measuring the absorbance at 450nm [12]. The assay can be used for the measurement of cell proliferation in response to growth factors, cytokines, mitogens, and nutrients, etc. It can also be used for the analysis of cytotoxic compounds like anticancer drugs and many other toxic agents and pharmaceutical compounds. MTS assay is performed by adding the reagent directly into the cell culture media without the intermittent steps, which are required in the routine MTT assay. In addition, this high-throughput assay requires no washing or solubilization step and can be performed in 96-well microtiter plate [13]

**Assay controls:**

I. Medium control (medium without cells)
II. Negative control (medium with cells but without the experimental drug/compound)
III. Positive control (medium with cells and 10uM of Doxorubicin)

Note: Extracellular reducing components such as ascorbic acid, cholesterol, alpha-tocopherol, dithiothreitol present in the culture media may reduce the MTT to formazan. To account for this reduction, it is important to use the same medium in control as well as test wells.

Steps followed [14].

1. Seed 100μl cell suspension in a 96-well plate at required cell density (20,000 cells per well),and incubate the cells for 12-24hrs.
2. After 24hrs, add 100ul Media with test agent in required concentrations and incubate.
3. The plate for 24 hrs at 37°C in a 5% $CO_2$ atmosphere.
4. After the incubation period, takeout the plates from incubator and add 20 uL of MTS reagent according to the manufacturer's instructions.
5. Wrap the plate with aluminium foil to avoid exposure

to light.

6. Return the plates to the incubator and incubate for 3 hours.

Gentle stirring in a gyratory shaker will enhance dissolution of the MTS reagent.

1. Read the absorbance on a spectrophotometer or an ELISA reader at 450nm and 630nm used as reference wavelength.
2. The IC50 value was determined by using linear regression equation i.e. Y =Mx+C. Here, Y = 50, M and C values were derived from the viability graph.
3. % of cell viability is calculated using below formula:
4. % of cell viability=[Absorbance of treated cells/Absorbance of Untreated cells] × 100.[15].

## RESULTS AND DISCUSSION

**Percentage yield:**

Percentage yield= (W1)÷(W2)×100 = (6.7)/(100)×100 = 6.7%W/W

W1 is termed as Weight of extract
W2 is termed as Weight of total powder taken

The percentage yield of plant was found to be 6.7%w/w

By performing preliminary phytochemical screening we found that the presence of steroids,phenols,tannins and carbohydrates. And absence of fixed oils and glycosides [16]

The above figures are for the(1) flavonoids,(2)phenols and (3)steroids.

1. **Flavonoids-** Mobilephasemixture Ethylacetate: Aceticacid: Formicacid:Water(5:0.5:1.35)
2. **Phenols**-mobile phase mixture-chloroform:Ethylacetate:Formic acid(5:4:1)
3. **Steroids-** mobile phase mixture-N-heptane:diethylether:aceticacid(8.5:1.5:0.1) By performing TLC we concluded that our plant extractcontains bio active compounds like steroids,flavonoids and phenols [17]

From the graph we conclude that the concentration of the drug increases may decrease the percentage of cell viability close to standard cell viability

The results of cytotoxicity study performed by MTS assay suggests that the given test compounds, S1 were signficantly cytotoxic in nature against K562 cells with IC50 values at 120.09ug/ml respectively. We can consider S1 as anti-leukemia in nature on K562 cells..The direct microscopic observations of drug treated images of K562 cell line after 24hours of incubation [19]

MTS assay reveals that the plant extract has the mild cytotoxicity and anti leukemic activity [20].

**Table 1. Preliminary phytochemical screening:**

| S.NO | Chemical constituents | Presence/absence |
|---|---|---|
| 1. | Alkaloids | + |
| 2. | Saponin | + |
| 3. | Glycosides | - |
| 4. | Carbohydrates | + |
| 5. | Tannins | + |
| 6. | Flavonoids | + |
| 7. | Steroids and triterpenoids | + |
| 8. | Proteins | + |
| 9. | Fats and fixed oils | - |

Note: (+) - indicates the presence of chemical constituents (-) - indicates the absence of chemical constituents.

**Table 2. MTSAssay Results**

| Parameter | Blank | Untreated | Std Control | 12.5 | 25 | 50 | 100 |
|---|---|---|---|---|---|---|---|
| Absorbance Reading 1 | 0.041 | 0.865 | 0.458 | 0.827 | 0.786 | 0.674 | 0.508 |
| Absorbance Reading 2 | 0.047 | 0.859 | 0.442 | 0.834 | 0.793 | 0.685 | 0.503 |
| Mean abs | 0.044 | 0.862 | 0.45 | 0.8305 | 0.7895 | 0.6795 | 0.5055 |
| Mean Abs (Test- Blank) | 0 | 0.818 | 0.406 | 0.7865 | 0.7455 | 0.6355 | 0.4615 |
| Std Deviation | 0.004243 | 0.00424264 | 0.011313708 | 0.00495 | 0.00495 | 0.007778 | 0.003536 |
| Std Error | 0.003 | 0.003 | 0.008 | 0.0035 | 0.0035 | 0.0055 | 0.0025 |
| **% Cell Viability** | **0** | **100** | **49.63325183** | **96.14914** | **91.13692** | **77.68949** | **56.41809** |

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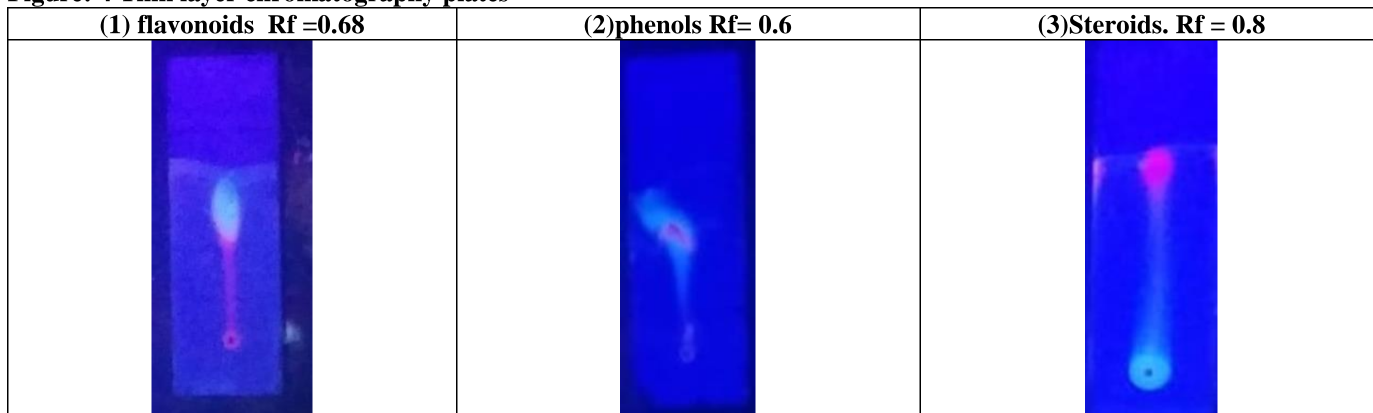
**Thin layer chromatography**

**Rf =Distance travelled by solute Distance travelled by solvent**

**Figure: 4 Thin layer chromatography plates**

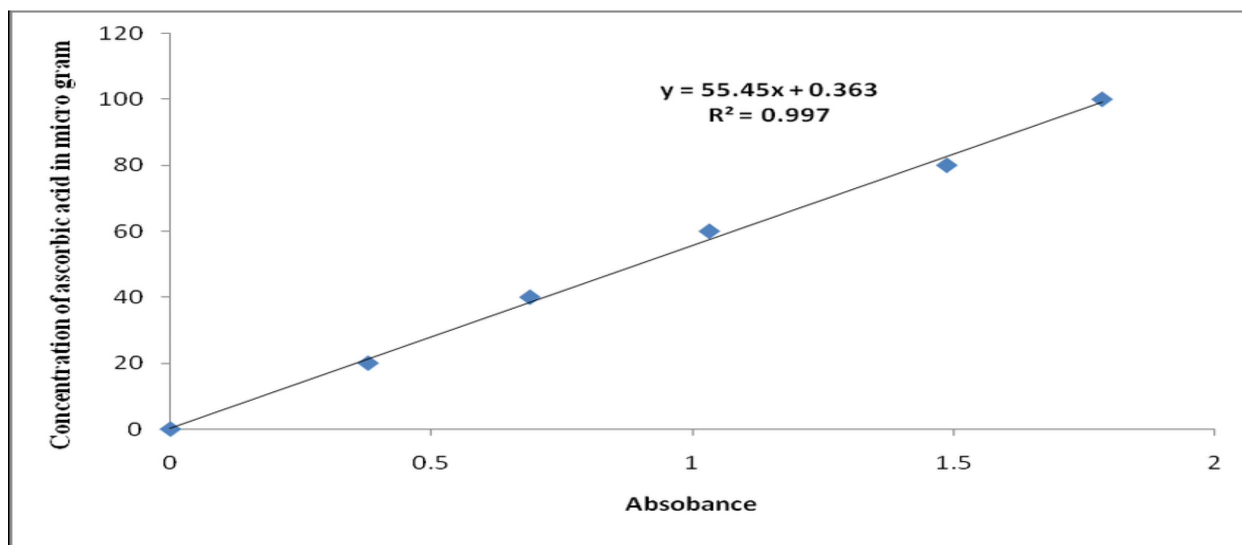


| (1) flavonoids Rf =0.68 | (2)phenols Rf= 0.6 | (3)Steroids. Rf = 0.8 |
|---|---|---|

**ANTIOXIDANT ACTIVITY BY H2O2 :**

**Standard calibration curve for ascorbic acid**

**Figure 1. Standard calibration curve for ascorbic acid.**




**Calibration curve of antioxidant activity by H2O2**

**Figure 2. Calibration curve of antioxidant activity by H2O2**

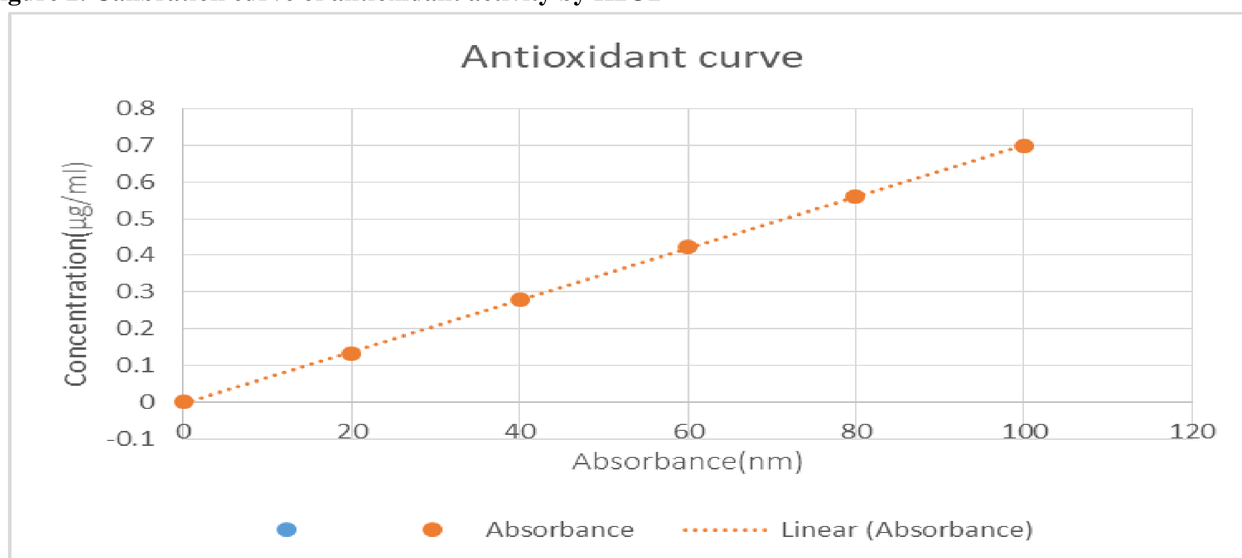


**Figure 3. Percentage of cell viability and drug concentration graph**

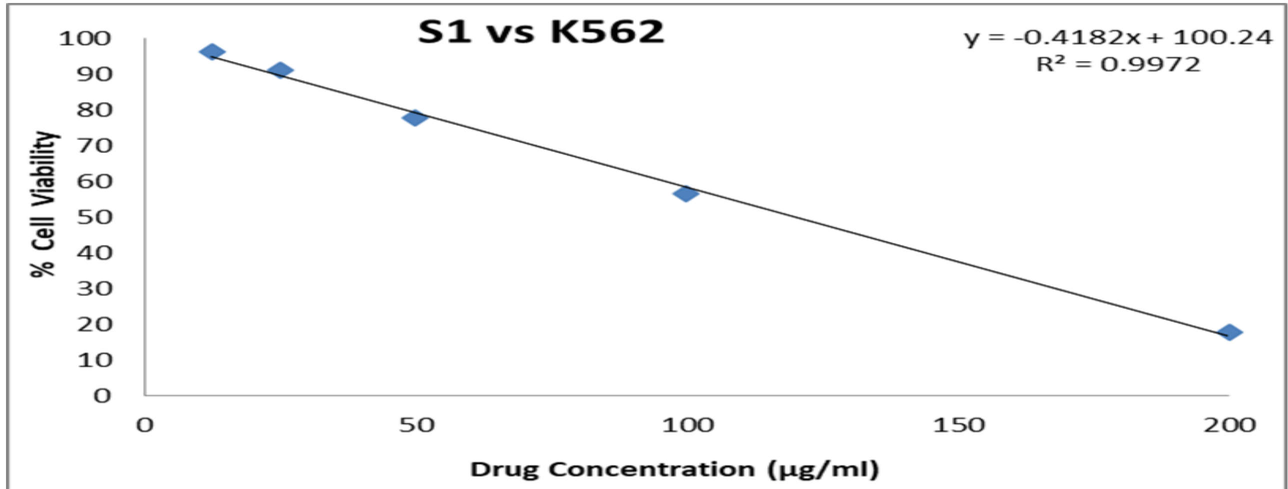
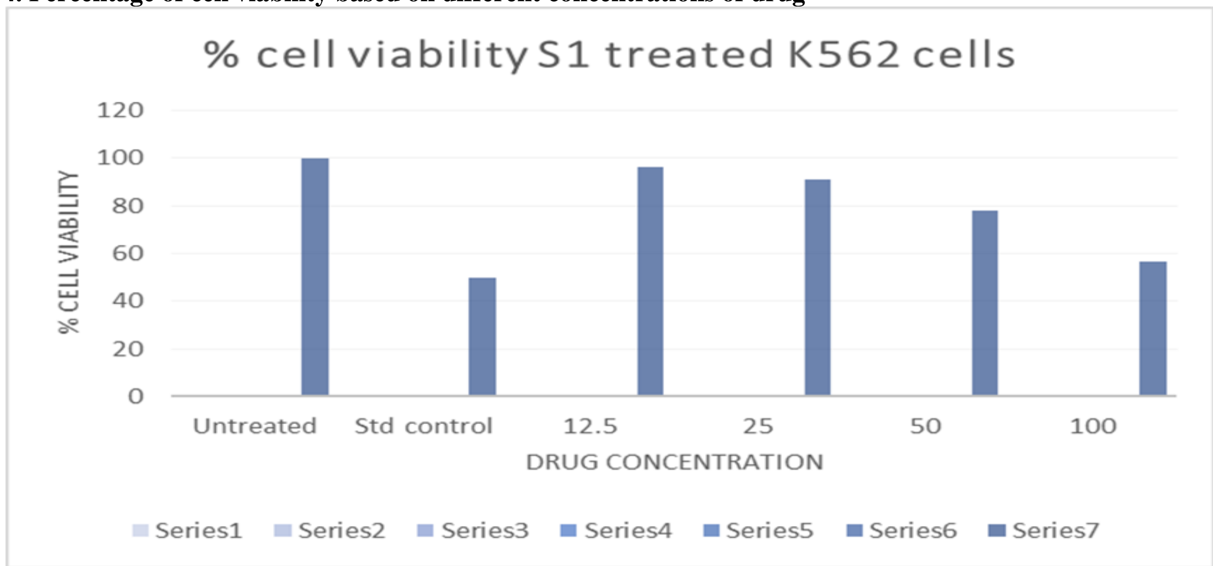
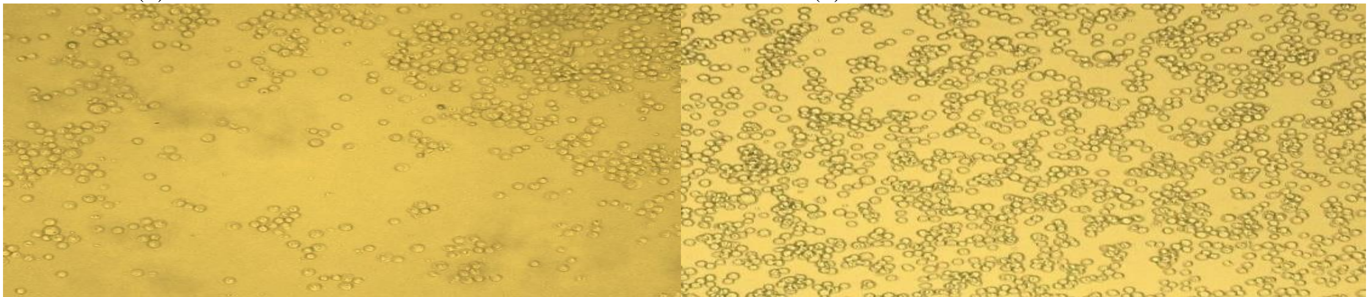


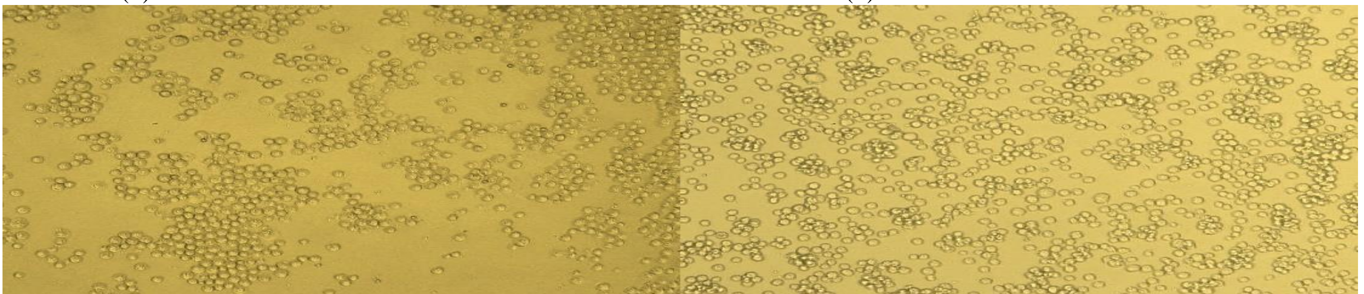

**Figure 4. Percentage of cell viability based on different concentrations of drug**



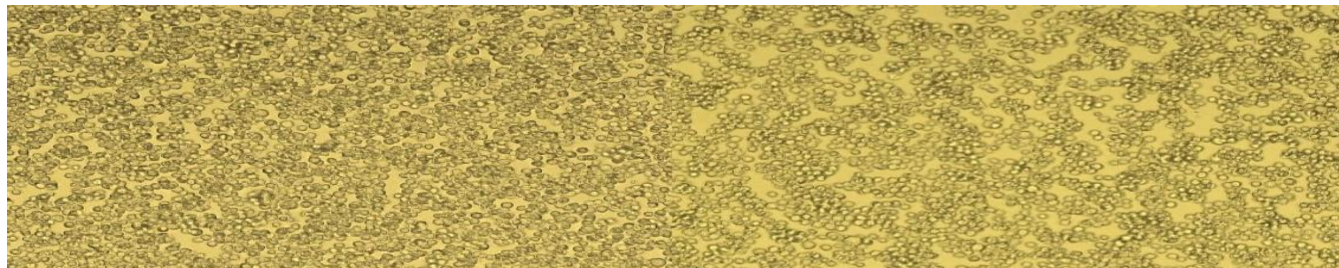

Std control(1) untreated(1)



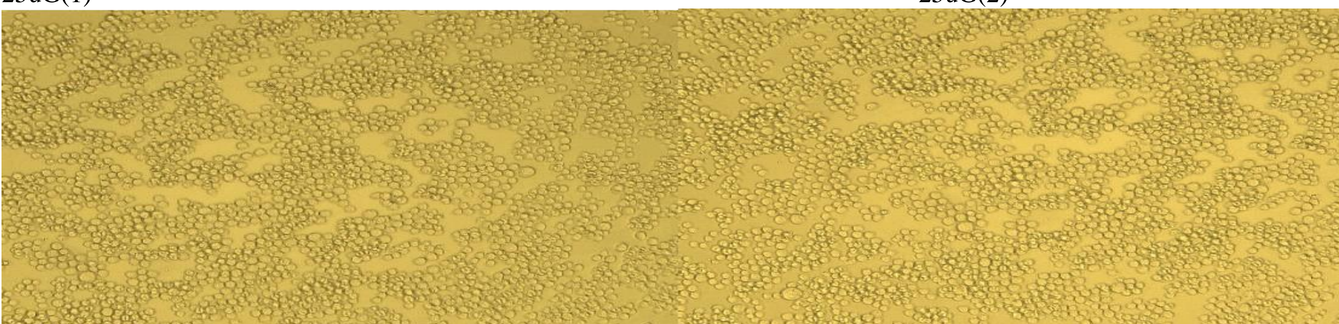
Std control(2) untreated(2)



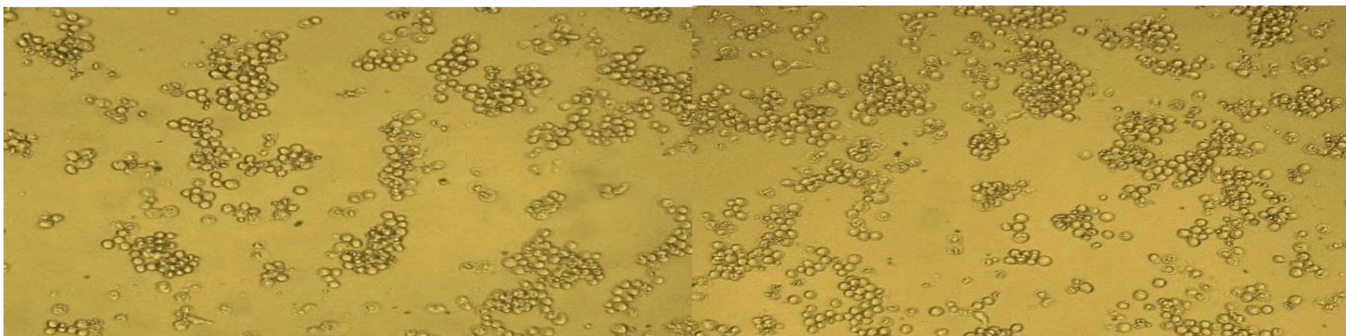
12.5uG(1) 12.5uG(2)



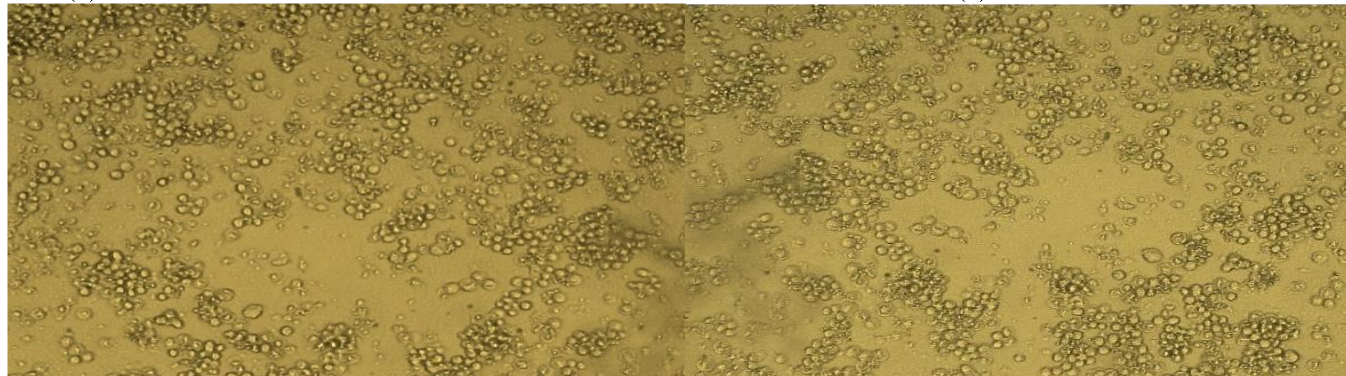
25uG(1) 25uG(2)

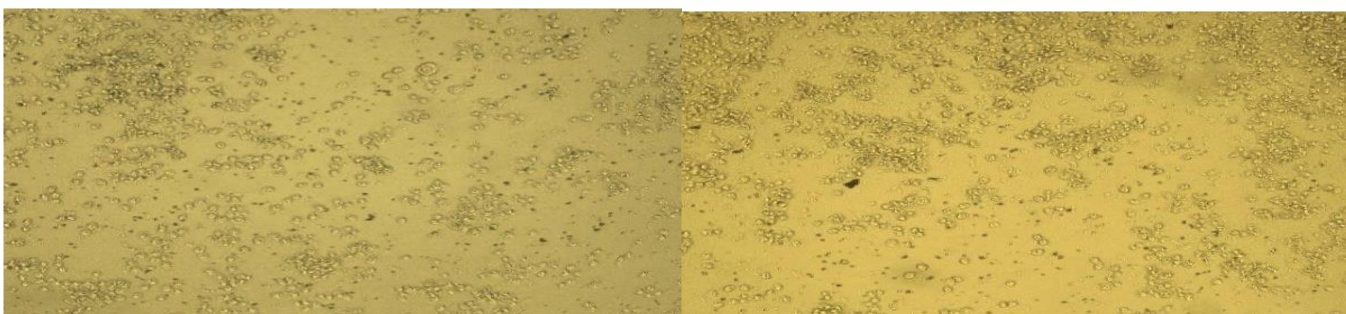


25uG(1) 25uG(2)



50uG(1) 50uG(2)





100uG(1) 100uG(2)

200uG(1) 200uG(2)

## CONCLUSION

Plant serve as a source of rich phytochemical diversity that pocesses important biological and pharmacological activity amaranthus is a important traditional herb widely used in folk medicine for centuries current scientific opinion advocates the consumption of whole plant instead of isolated part,the way the nature prepared it with full compliments of naturally occurring synergistic phytonutrients to attain wellbeing. The result of proximal composition has concluded that Amaranthus roxburghianus could also serve as a good source of nutritional supplement in the diet of animal. Amaranthus roxburghianus aerial part extract have rich phytochemical profile, the phytochemical profiling provides a promising area of research in natural therapeutics and further studies are to be carried out to find the components responsible for its various pharmacological activities. The present study also concludes that the plant can be used as a good antioxidant. The TLC profiling may conclude that the components responsible for various pharmacological activities of the plant. The present data of MTS assay suggest that the plant Amaranthus roxburghianus Linn ethanolic extract is anti- leukemia in nature and showed cytotoxicity potency against Human acute leukemia cells(K562) and further studies need to be conducted to determine the molecular mechanism behind anti- leukemia properties of Amaranthus roxburghianus which is beneficial therapy in future.

## ACKNOWLEDGEMENT
Nil

## CONFLICT OF INTEREST
No Interest.

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