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IN VITRO CYTOTOXIC POTENTIAL OF MEDICINAL PLANT ALANGIUM SALVIFOLIUM AGAINST CANCER CELL LINES

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Abstract

The use of medicinal plants to treat diseases is the most common remedy. The seeds of Alangium salvifolium are rich in phytochemical components. They exert antioxidant and anticancer effects known for their beneficial effects on human health. The aim of this study was to investigate the antioxidant and cytotoxic potential of Alangium salvifolium seed extracts against human skin melanoma (A375) and human skin carcinoma (A431) cell lines in vitro. Phytochemicals and antioxidant activity were investigated in ethanol and ethyl acetate extracts of Alangium salvifolium seeds using standard methods. In vitro cytotoxicity assays were performed against human skin melanoma (A375) and human skin carcinoma (A431) cell lines using the MTT assay. There was results showed that Alangium salvifolium has antioxidant potential. The observations of the MTT cell cytotoxicity study indicate that the ethanolic and ethyl acetate extract of the plant exhibits significant cytotoxic properties against the A431 cell lines and moderate cytotoxic properties against the A375 cell lines. Overall, the ethanolic extract shows effective antioxidant potential and cytotoxicity against both A431 and A375 cell lines. It appears that Alangium salvifolium seed extracts may prove to be promising anti-cancer cell agents if the ethanolic extract is specific. However, further studies are needed to confirm these results in vivo.

Keywords : cytotoxic potential, Antioxidant, Medicinal plant, cancer, cell line

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1. Introduction

Cancer is the abnormal growth of cells in our body that leads to death. Cancer cells often invade and destroy normal cells. The disease is characterized by the rapid and uncontrolled formation of abnormal cells that may form tumours or develop or multiply throughout the body and begin to grow. If this process is not stopped, it can be fatal to the body[1]. Cancer is not a disease, but a collection of over 100 different disorders. An estimated 11 million people have been diagnosed with cancer worldwide, and this number is expected to increase by 2022[2]. Skin cancer is expected to be the most common malignancy in the near future, especially among Caucasians[3]. Each year, more than one million new cases are registered worldwide. The different types of skin cancer are named according to the cells that develop them and their clinical behaviour. The most common types are basal cell carcinoma (BCC), squamous cell carcinoma (SCC) (also called non-melanoma skin cancer (NMSC)), and malignant melanoma (MM)[4]. Globally, prevalence is increasing at a rate of 10% per year, mainly in older men but also in younger women[5]. There is a constant need for the development of new, cost-effective anticancer drugs. Over the years, various approaches have been used singly or in combination to treat cancer. These include chemotherapy, radiotherapy, surgery, and immunotherapy. However, these approaches have a number of side effects.

Therefore, researchers are constantly looking for alternatives to these treatments. Natural products are the most important element of research. For more than 30 years, the potential of natural products as preventive and therapeutic agents against cancer has been recognized. There is strong evidence that plant-derived compounds, phytochemicals, and antioxidants inhibit various stages of carcinogenesis[6]. Plant species are known sources of drugs used in modern medicine. Medicinal plants are directly and indirectly involved in this. Combating diseases such as cancer is an important part of human society[7]. Plants occupy a special position in the treatment of oncological diseases. It is estimated that over 50% of anticancer drugs are derived from plants[8].

Plant extracts are used to treat various diseases and are the basis for all Indian systems of medicine Natural antioxidants help control the formation of free radicals and reactive oxygen species. Although free radicals play a key role in cellular processes, they pose a threat to cells by damaging DNA, proteins and cell membranes, leading to the onset of cancer. By reducing free radicals and oxidative stress, antioxidants play a role in eliminating DNA damage and reducing the rate of abnormal cell divisions. Antioxidants can be used as dietary supplements and for prophylactic treatment of various diseases and are responsible for reducing oxidative stress in cancer[9,10]. In addition, antioxidants can reduce morbidity associated with the use of various chemotherapeutic agents by reducing chemotherapy and pressure ulcer rates. Because of their great efficiency to reduce chemotherapy and mutagenesis[11]. Induced toxicity, which seems to increase survival rates in cancer patients and improve therapeutic efficiency. Many medicinal plants have anticancer effects on a variety of cell lineages. Phytochemicals have antioxidant antibacterial anticancer effects against a variety of pathogenic organisms[12].

The main objective of this study was to determine the antioxidant and antitumor properties of ethanolic and ethyl acetate extract from Alangium salvifolium seeds. Due to their bioactive compounds (i.e. polyphenols), they possess remarkable biological properties that protect mainly against oxidative stress and related diseases. Alangium salvifolium is a medicinal plant that botanically belongs to the Cornaceae family and is native to the desertic regions of India, especially in the forests of southern India[13]. It grows in tropical Australia, Madagascar, West Africa, islands of the South and West Pacific, and East Asia (China, Malaysia, Indonesia, India). Alangium salvifolium is composed of different types of plant components derived from different parts of the plant. They have been shown to possess nutritional and medicinal properties as they are an excellent source of bioactive products[14]. The most important properties of Alangium salvifolium are attributed to its antioxidant, anticarcinogenic, antidiabetic, anti-obesity and anti-ageing properties. In addition, Alangium salvifolium seeds are an excellent source of

plant secondary metabolites with potent antioxidant and anticancer properties, mainly phytochemical components. Phytochemicals are abundant in the plant Alangium salvifolium It has been reported that Phytochemicals such as flavonoids, alkaloids, glycosides, amino acids, carbohydrates, and proteins are beneficial to human health due to their antioxidant and anticancer effect[15].

2. MATERIALS AND METHOD

2.1. Plant materials

Alangium Salvifolium seed samples were collected from the Herbal Garden of Govt. Ashtang Ayurveda College, Indore, Madhya Pradesh, India. This plant material was identified and authenticated by Dr. Hariom Parihar, Assistant Professor and Head Department of Dravyagun, Indore, M.P., India. All chemicals used in the study were of analytical grade and obtained from Invitrogen, Sigma, Bench top, USA, Biolite Thermos, gibco and HiMedia Laboratories Private Limited, Mumbai, India. The cell line used in the study was A-375 (Human skin malignant melanoma) , A-431 (human skin adenocarcinoma carcinoma) cell line which was procured from the National Centre for Cell Science (NCCS) , Pune, Maharashtra, India.

2.2. Preparation of extracts

The seeds of Alangium Salvifolium were carefully harvested and washed. The washed seeds were air dried and chopped into small pieces. While the leaves were well dried, the seeds were weighed. The powdered Alangium salvifolium seeds were placed in a thimble of the Soxhlet apparatus. The extraction was carried out with different organic solvents, ethyl acetate and ethanol, for 8-10 hours at a temperature of 40-60oC in a heating mantle. After the extraction process, the sample extracts were filtered and evaporated to dryness. The extracts were collected in an airtight container[16].

2.3. Preliminary phytochemical investigation

The extract was subjected to qualitative chemical investigation for the identification of different phytoconstituents like sterols, glycosides, saponins, carbohydrates, alkaloids, flavonoids, tannins, proteins and terpenoids using standard procedures[17].

2.4. In Vitro Antioxidant activity

Based on the examination of preliminary phytochemical screening the ethanol and ethyl acetate extract of Alangium salvifolium plant seeds was used for antioxidant study[18].

2.4.1. Hydrogen peroxide scavenging assay

The ability of the extract to scavenge hydrogen peroxide (H2O2) was tested using the approach. An aliquot of 0.1 mL of the extracts from the seeds of Alangium salvifolium (20-100µg, mL) was transferred to eppendorf tubes and their volume was increased to 0.4 mL with 50 mM phosphate buffer (pH 7.4) , then 0.6 mL of H2O2 solution (2 mM) was added. The reaction mixture was shaken, and absorbance was measured at 230 nm after 10 min of reaction time. Ascorbic acid was used as a positive control. The ability of the extracts to scavenge H2O2 was determined using the following equation.

$$Percent\ Inhibition = \frac{Ab\ of\ Control - Ab\ of\ Sample}{Ab\ of\ Control} \times 100$$

2.4.2. Reducing power assay

In 1.0 ml of deionized water, aliquots of various concentrations of the standard and extracts of Alangium salvifolium seeds (20 to 100 g, ml) were mixed with 2.5 ml of phosphate buffer (pH 6.6) and 2.5 ml of potassium ferricyanide (1%). After cooling, the mixture was incubated in a water bath at 50 °C for 20 min. 2.5 ml (10%) trichloroacetic acid was added to the mixture, which was then centrifuged at 3,000 rpm for 10 minutes. The top layer of the solution was mixed with 2.5 ml of distilled water and 0.5 ml of freshly prepared ferric chloride solution (0.1%). In a UV spectrometer, the absorbance was measured at 700 nm. A blank was prepared without addition of extract. Ascorbic acid at various concentrations (20 to 100µg, ml) was used as a standard.

2.5. In vitro evaluation of anticancer activity

Anticancer activity of Alangium salvifolium extract against A 431 and A 375 cell lines.

Monitoring the behaviour and well-being of cells in culture after exposure to various stimuli often involves tests to measure cell growth, viability, and cytotoxicity[19].

2.5.1. Required Material

Cell lines

- A-375 — Human skin malignant melanoma cell line (From NCCS, Pune)
- A-431 — Human skin adenocarcinoma carcinoma cell line (From NCCS, Pune)

Cell culture medium

- DMEM media with high glucose - (Cat No:2120785, Gibco)
- Adjustable multichannel pipettes and a pipettor (Benchtop, USA)
- Fetal Bovine Serum (#RM10432, Himedia)
- MTT Reagent (5 mg/ml) (# 4060 Himedia)
- DMSO (#PHR1309, Sigma)
- D-PBS (#TL1006, Himedia)
- T25 flask (# 12556009, Biolite — Thermo)

Equipment's

- Centrifuge (Remi: R-8oC).
- Pipettes: 2-10µl, 10-100µl, and 100-1000µl.
- Reader for 96-well ELISA plates (ELX-800, Biotech, USA)
- Inverted microscope (Biolink)
- Incubator at 37 °C with humidified atmosphere containing 5% CO2 (Heal force, China)

2.5.2. MTT Cell Proliferation Assays

MTT (3-[4,5-dimethylthiazole-2-half of 2,5-diphenyltetrazolium bromide; thiazolyl blue) is a tetrazolium salt that dissolves in water to give a yellowish solution when prepared in salt solutions or media without phenol red. Dehydrogenase enzymes cleave the tetrazolium ring. They convert dissolved MTT to an insoluble purple formazan. Isopropanol or other solvents can be used to dissolve the water-insoluble formazan, and the dissolved material is then quantified spectrophotometrically using absorbance as a function of reacted dye concentration.

Procedure

Seed 200 µl of the cell suspension in 96-well plates at the required cell density (20,000 cells per well) without test medium. Allow approximately 24 hours for the cells to grow. Add appropriate concentrations of the indicated test medium. Incubate for 24 hours at 37 °C in a 5% CO2 atmosphere. At the end of the incubation period, remove the plates from the incubator, discard the spent medium, and then add the MTT reagent at a final concentration of 0.5 mg, ml of the total volume. Wrap the plates in aluminium foil to protect them from light. Place the plates back into the incubator and allow to stand for 3 hours. After removing the MTT reagent (DMSO) , add 100µl of the solubilization solution. Shake gently in a gyrator shaker to accelerate dissolution. Pipetting up and down may be necessary to thoroughly dissolve the MTT formazan crystals, especially for dense cultures. Measure absorbance using a spectrophotometer or ELISA reader at a wavelength of 570 nm. Calculate the percent viability of the cells. Below formula:

$$Percent\ Cell\ Viability = \frac{Ab\ of\ Treated\ cells}{Ab\ of\ Untreated\ cells} \times 100$$

The linear regression equation Y = Mx + C was used to calculate the IC50 value, where Y = 50, and the values M and C were derived from the viability figures.

2.5.3. **Morphological changes in A431 and A375 cells**.

A431 and A375 cells were grown in sterile 35-mm cell culture Petri dishes at 37°C in a CO2 incubator and exposed to the seed extracts at the IC50 concentrations. The morphology of A431 and A375 cells was visualized using an inverted phase contrast microscope (Biolink). Untreated cells served as controls, and morphological changes were visualized based on the extent of cell rounding.

3. RESULTS

3.1. Phytochemical screening

The therapeutic or curative effect of plant extracts depends on the type and amount of secondary metabolites they contain. Preliminary phytochemical analysis of ethanol and ethyl acetate extracts revealed the presence of important secondary metabolites such as alkaloids, flavonoids, tannins, glycosides, saponins, phenolic compounds, carbohydrates and proteins. The results of qualitative phytochemical analysis showed the presence of various secondary metabolites such as alkaloids, saponins, sterols, cardiac glycosides, tannins, terpenoids, proteins and sugars in the plant extracts.

Table-1: Preliminary Phytochemical Screening of Various Extracts of Alangium salvifolium

S.No.	Name of the Phytochemical	Name of the test	Ethyl acetate extract	Ethanolic extract
1	Carbohydrates	Molisch's Test	-	+
		Fehling's Test	-	+
		Benedict's Test	-	+
		Bareford's Test	-	+
2	Glycosides	Legal's Test	-	+
		Keller Killiani Test	-	+
		Borntrager's Test	-	+
3	Flavonoids	Lead Acetate Test	+	+
		Alkaline Reagent Test	+	+
		Shinoda Test	+	+
4	Alkaloids	Mayer's Test	-	+
		Hager's Test	-	+
		Wagner's Test	-	+
		Dragendroff's Test	-	
5	Triterpanoids and steroids	Salkowski Test	+	+
		Libermann-Burchard's Test	+	+
			+	+
6	Tanin and Phenolic compound	FeCl3 Test	+	+
		Lead Acetate Test	+	+
		Gelatine Test	+	+
		Dilute Iodine Solution Test	+	+
7	Protein and amino acids	Ninhydrin Test	+	+
		Biuret's Test	+	+
		Million's Test		
8	Saponins	Froth Test	+	-

3.2. **Antioxidant**

The antioxidant activity of Alangium salvifolium seed extract was evaluated in vitro. The overall IC50 results for the hydrogen peroxide scavenging assay and the reducing power assay were determined based on the percent radical scavenging and are shown in the tables and in the figures.

3.2.1. **Hydrogen peroxide scavenging assay**

The overall results on the IC50 values for the scavenging activity of hydroxyl radicals ethyl acetate and ethanolic extract of Alangium salvifolium seeds showed percent inhibition of 57.39% and 78.69%, and the IC50 value was found to be 79.95 and 48.45 µg/ml, respectively. The reducing capacity of a compound may serve as an important indicator of its potential antioxidant activity. A dietary antioxidant such as ascorbic acid was used for comparison (Table 2). Compounds with a reducing capacity indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, allowing them to act as primary and secondary antioxidants. The reducing power of ethanol and ethyl acetate extracts and standard increases with increasing sample and standard concentration [Table 2, 3 and 4]. The reducing power shows a good linear relationship for both the standard and sample extracts [Figures 1, 2 and 3]. Looking at the tables and figures for the two extracts, it can be seen that the ethanolic extract showed a strong reducing capacity compared to the ethyl acetate extract. Figures represent the Percentage Inhibition Vs Concentration of extracts and ascorbic acid.

Table 2 H_2O_2 radical scavenging activity of Ascorbic acid

Concentration (µg/ml)	Absorbance	% Inhibition
20	0.331	41.72535211
40	0.276	51.4084507
60	0.209	63.20422535
80	0.154	72.88732394
100	0.095	83.27464789
Control	0.568	
	IC50 36.16	

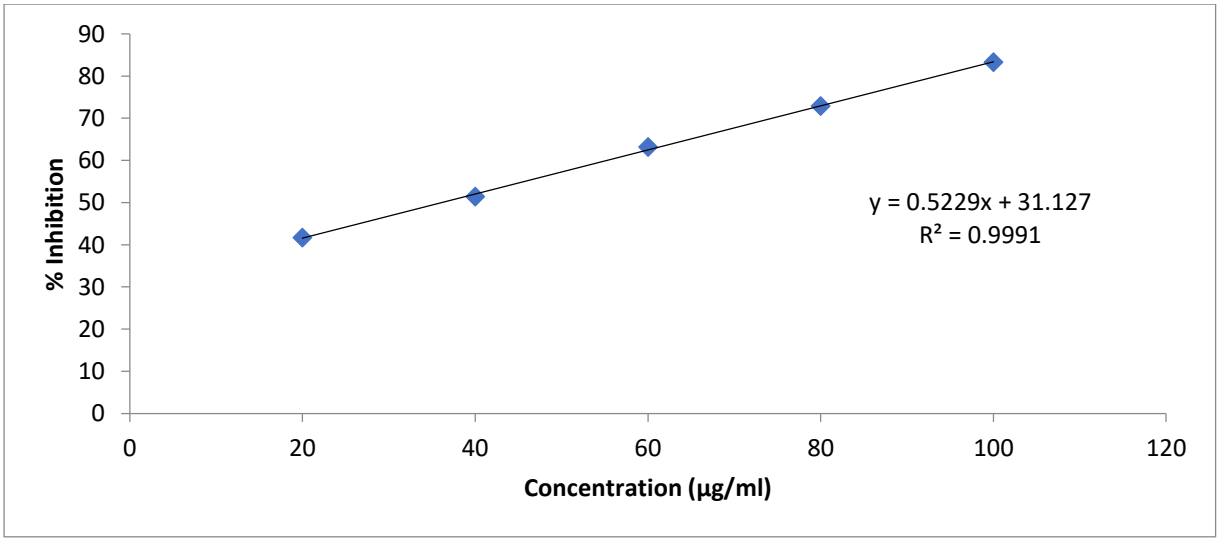

Figure 1 represents the Percentage Inhibition *Vs* Concentration of ascorbic acid

Table 3H_2O_2 radical scavenging activity of Ethyl acetate extract

Concentration (µg/ml)	Absorbance	% Inhibition
20	0.431	24.11971831
40	0.376	33.8028169
60	0.318	44.01408451
80	0.285	49.82394366
100	0.242	57.3943662
Control	0.568	
IC50 79.95		

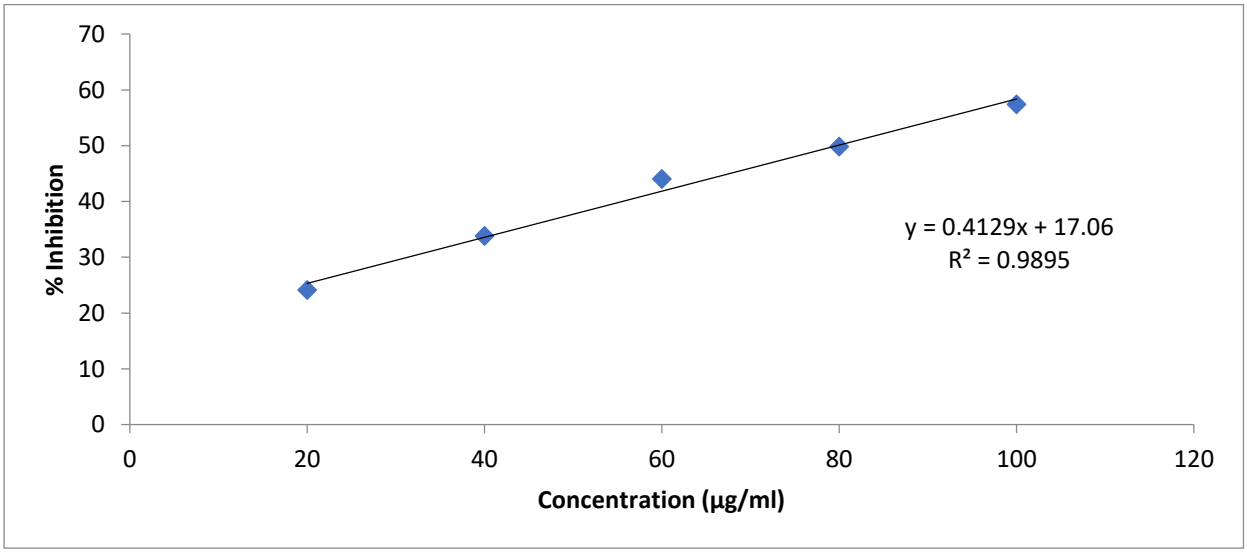

Figure 2 represents the Percentage Inhibition *Vs* Concentration of Ethyl acetate extract

Table 4 H_2O_2 radical scavenging activity of Ethanolic extract

Concentration (µg/ml)	Absorbance	% Inhibition
20	0.378	33.45070423
40	0.301	47.00704225
60	0.244	57.04225352
80	0.196	65.49295775
100	0.121	78.6971831
Control	0.568	
IC50 48.45		

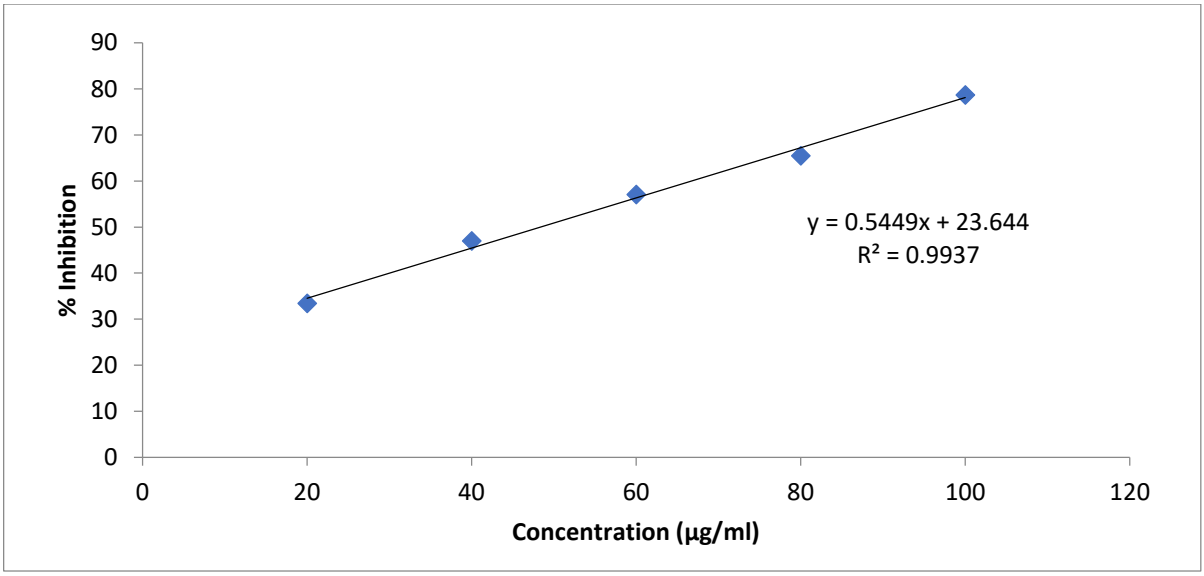

Figure 3 represents the Percentage Inhibition *Vs* Concentration of Ethanolic extract

3.2.2. power scavenging activity

The reducing power of a compound can serve as an important indicator of its potential antioxidant activity. The method for determining the reducing power is based on the principle that substances with reducing potential react with potassium ferricyanide (Fe3+) to form potassium ferricyanide (Fe2+), which then reacts with ferric chloride to form an iron(III) complex that has an absorption maximum at 700 nm. The reducing power of the ethanol and ethyl acetate extracts and the standard increases as the sample and standard concentrations are increased [Table 5,6, and 7]. The reducing power shows a good linear relationship for both the standard and sample extracts [Figures 4,5 and 6]. The ethanolic extract showed a stronger reducing power than the ethyl acetate extract. Dietary antioxidants such as ascorbic acid were used for comparison.

Table 5 Reducing power scavenging activity of Ascorbic acid

Concentration (µg/ml)	Absorbance
20	0.402
40	0.457
60	0.511
80	0.573
100	0.657

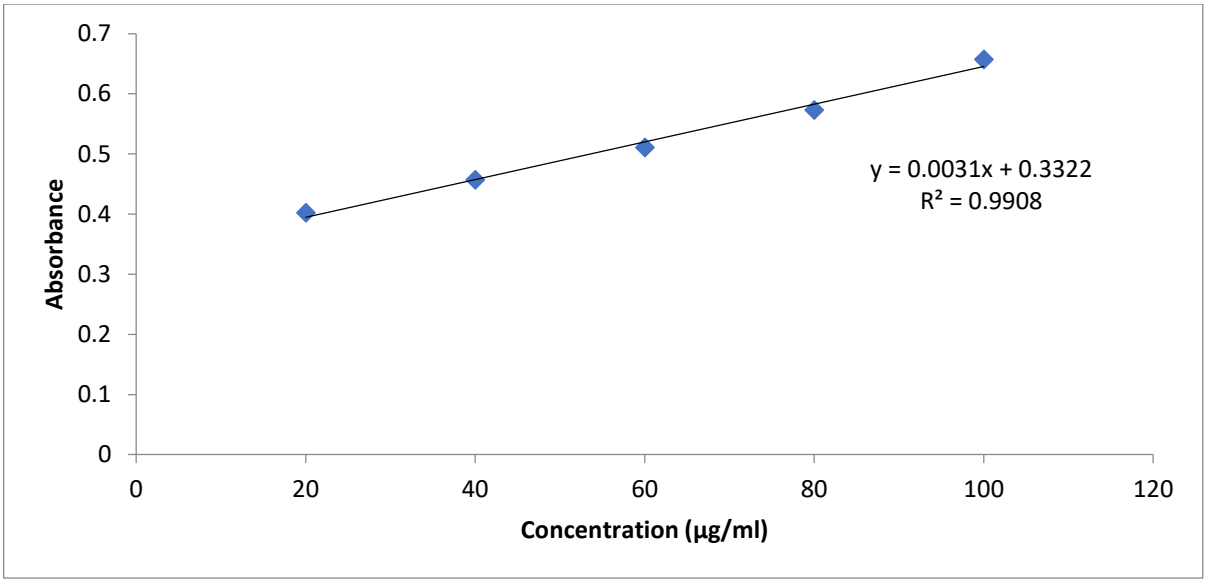

Figure 4 Graph represents the Absorbance *Vs* Concentration of Ascorbic acid

Table 6 Reducing power scavenging activity of Ethyl acetate extract

Concentration (µg/ml)	Absorbance
20	0.043
40	0.096
60	0.125
Saw80	0.159
100	0.196

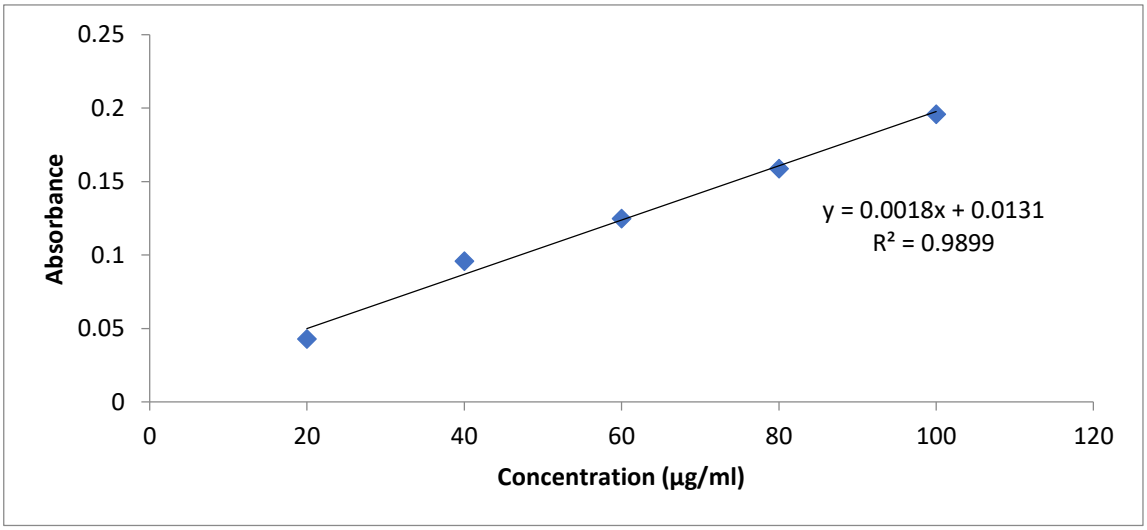

Figure 5 represents the Absorbance *Vs* Concentration of Ethyl acetate extract

Table 7 Reducing power scavenging activity of Ethanolic extract

Concentration (µg/ml)	Absorbance
20	0.176
40	0.211
60	0.278
80	0.315
100	0.356

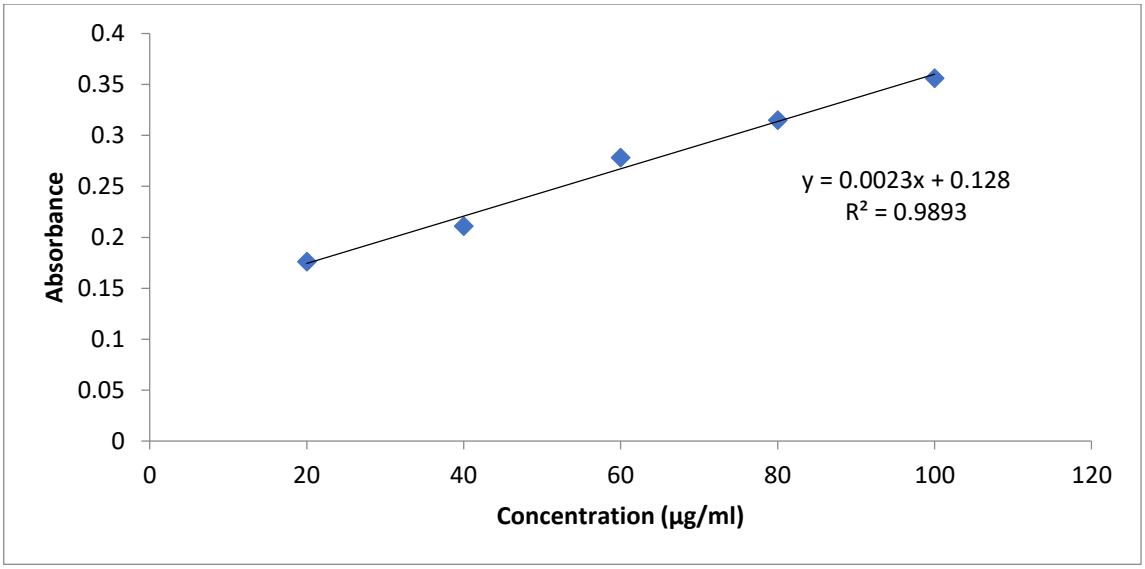

Figure 6 represents the Absorbance *Vs* Concentration of Ethanolic extract

3.3. MTT TEST ASSAY

The extracts induced cell death and may be considered as potent anti-skin cancer and anti-melanoma agent. The results clearly suggested that given extracts are anticancer in nature by significantly inhibiting the % cell viability and having considerable cells inhibitory potential properties against the A 375 and A 431 cell lines. Observations in Statistical data of MTT cell cytotoxicity study suggesting us that against A 431cell lines, EA and EtOH extracts showing significant cytotoxic potential properties with the IC50 concentration at 60.84ug/ml and 47.43ug/ml respectively, A 375 cell lines, EA and EtOH extracts showing moderate cytotoxic potential properties with the IC50 concentration at 90.11ug/ml and 50.71ug/ml respectively. Overall EtOH showing effective cytotoxicity against both A431 and A375 cell lines and may be considered as potent anti-skin cancer and anti-melanoma agent due to its low IC50 values than EA extract. Which shown in table 8. By seeing graph we can notice that the viability of cells are decreasing by increasing in concentration. Extract contains Phyto-constituents for anticancer, it was observed that the crude extract may contain bioactive components showed significant effect of anti-cancer activity.

A431 CELLS

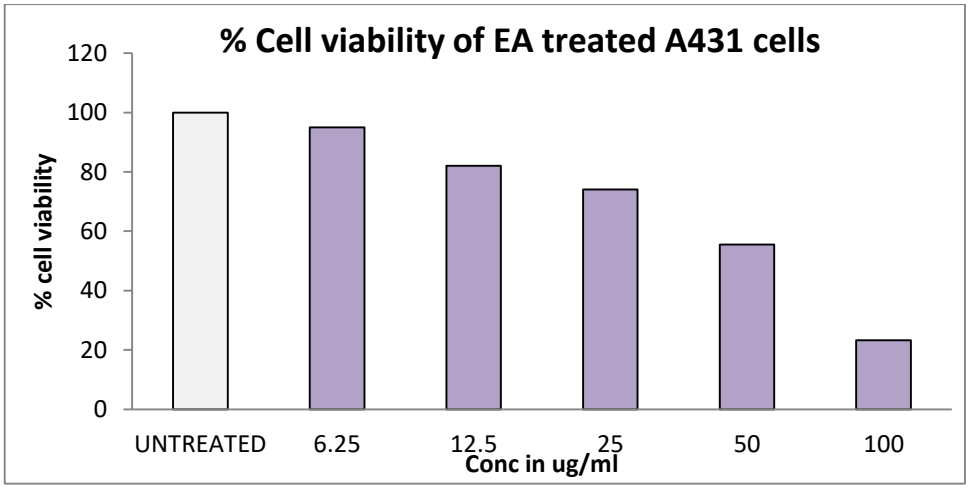

Figure 7 % cell viability of EA treated A431 cells

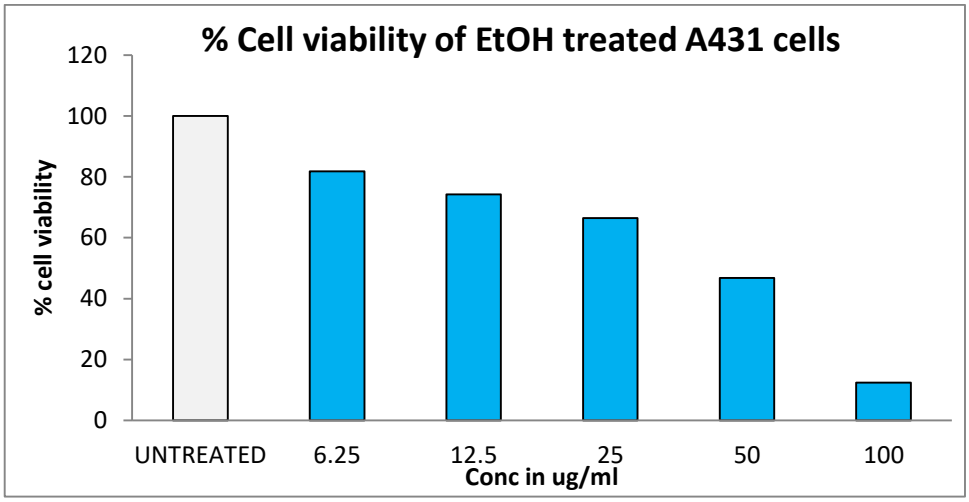

Figure 8 % cell viability of EtOH treated A 431 cells A 375 cells

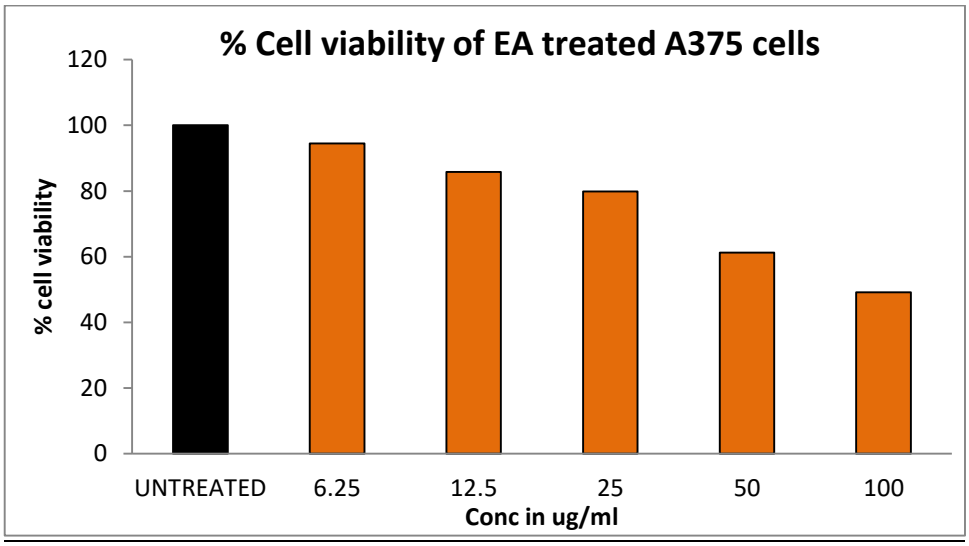

Figure 9 % cell viability of EA treated A375 cells

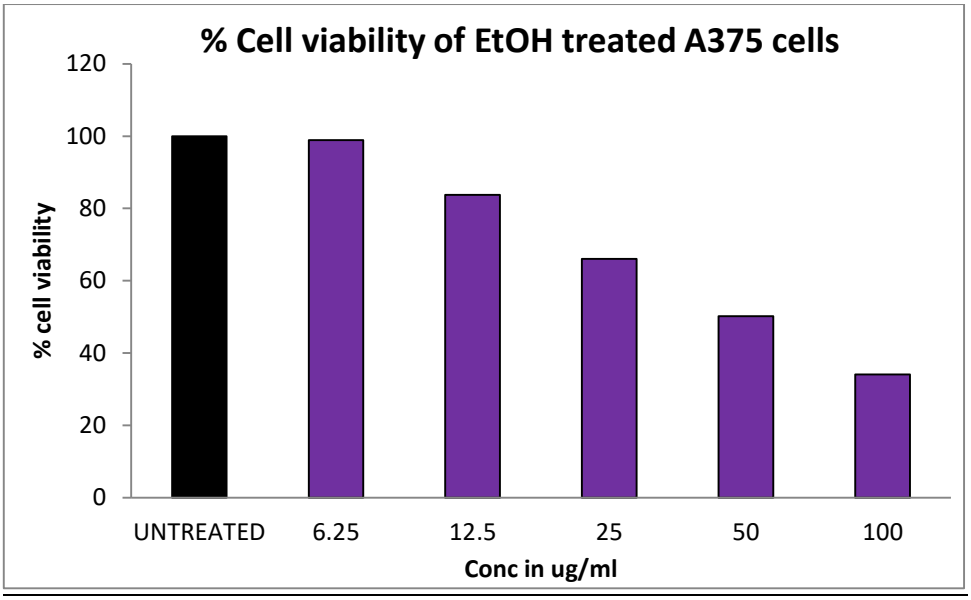

Figure 10 % cell viability of EtOH treated A375 cells

Table 8 Table showing the IC_{50} concentrations of the given Test compounds, EA and EtOH treated A431 and A375cell lines after the incubation period of 24hrs.

S.No.	Sample	A431 IC50 values	A375 IC50 values
1	Ethel acetate	60.84ug/ml	90.11ug/ml
2	Ethanol	47.43ug/ml	50.71ug/ml

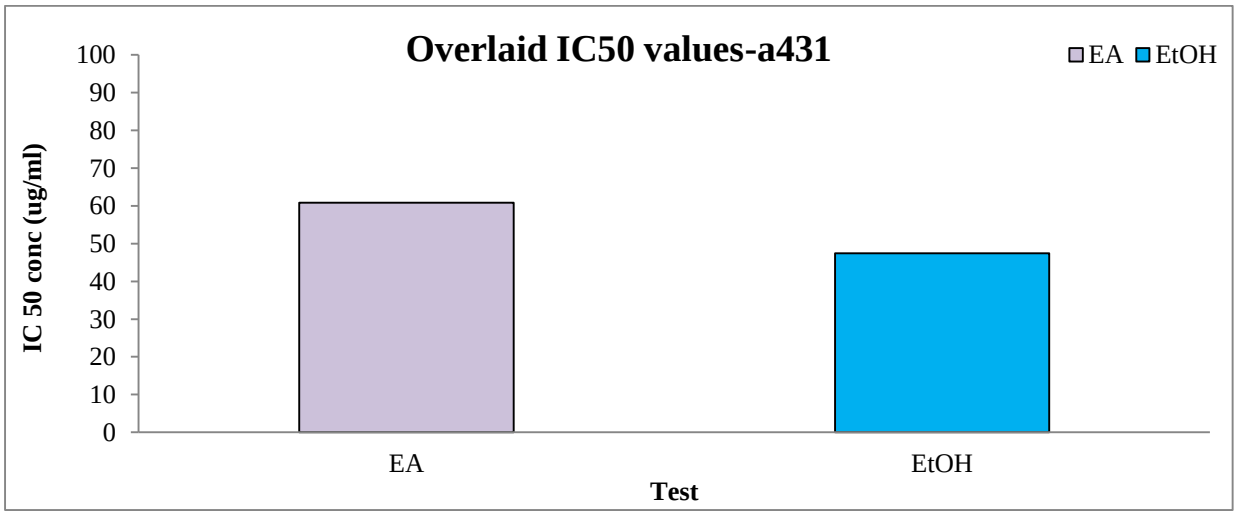

Figure 11 Overlaid Bar graph showing the IC_{50} values of given test compounds, EA and EtOH against the A431cell lines by MTT study.

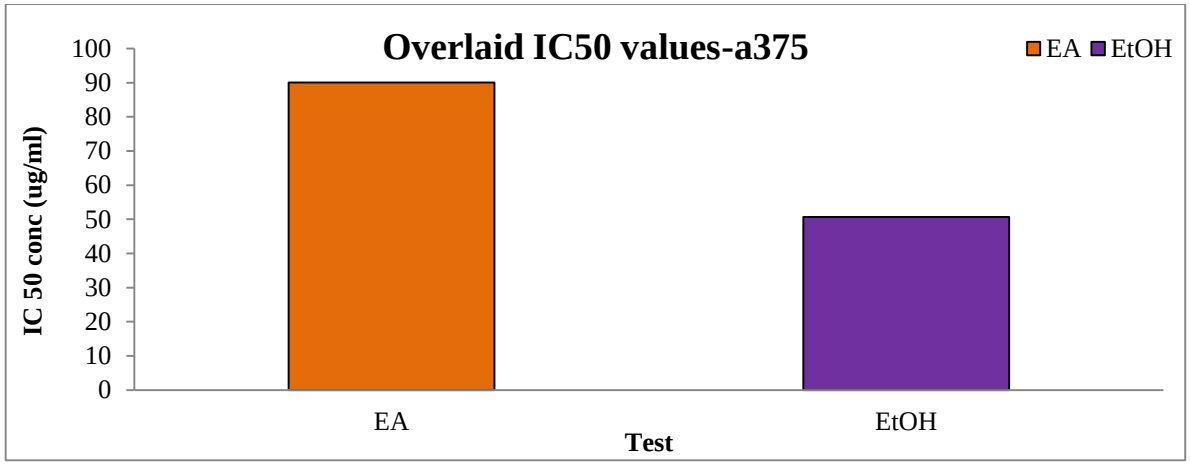

Figure 12 Overlaid Bar graph showing the IC_{50} values of given test compounds, EA and EtOH against the A375cell lines by MTT study.

4. DISCUSSION

The therapeutic effect of plants is mainly due to their biologically active substances. In this study, we investigated the seeds of the medicinal plant Alangium Salvifolium to evaluate their antioxidant activity using H2O2 radical scavengers and reducing power scavengers, and investigated their antiproliferative activity against human cancer cell lines from plants, which remains one of the most important sources of natural products for new drugs. Therefore, it is a straightforward approach to screen plants for bioactivity. It is likely that the antioxidant mechanisms in plants may also have beneficial effects on human health. Phytochemical screening of the extract reveals the presence of flavonoids, sterols, alkaloids, carbohydrates, and saponin glycosides (see Table 1). Considering the current status of cancer research in search of new anticancer drugs, the literature review shows that the toxic chemical agents, therefore, the work was focused on herbal and phytomedicine as the drug of choice for cancer . The incidence of cancer continues to increase due to recent lifestyle changes, which include eating fewer or no fruits and vegetables, lack of exercise, excessive alcohol consumption, and exposure to harmful chemicals.

Therefore, there is an urgent need to find new therapeutic agents or drugs that specifically target cancer cells without affecting normal cells. Here, we investigated the anticancer activity of the ethanolic and ethyl acetate extracts from the plant seeds of Alangium Salvifolium against human cancer cells such as cell lines A-375 (malignant melanoma of human skin) and A-431 (adenocarcinoma of human skin). Cancer cells have a higher baseline level of reactive oxygen species (ROS) due to their higher metabolic rate and other functions that differ from those of normal cells. An elevated level of ROS is essential for cancer cells to grow, proliferate, and metastasize. At the same time, exceeding the required level of ROS can cause cancer cells to oxidative stress and possible death. It is possible that anticancer activity of the extract studied here is due to the presence of compounds that altered the redox balance essential for the survival of the cancer cells. So antioxidant property of alangium salvifolium plant Seeds help in treatment of cancer disease like oxidative stress caused by cancer cell.

The MTT cell proliferation study is performed using EA and EtOH extract to evaluate the cell proliferation potential against human skin melanoma (A 375) and human skin carcinoma (A 431) cells with different incubation times of 24 hours each. The MTT cell cytotoxicity study observations show that EA and the EtOH extract exhibit significant cytotoxic properties against the A 431 cell lines with IC50 concentrations of 60.84 ug/ml and 47.43 ug/ml, respectively. The observations from the MTT cell cytotoxicity study indicate that the extracts of EA and MeOH exhibit moderate cytotoxic potential against the A375 cell lines with IC50 concentrations of 90.11 ug/ml and 50.71 ug/ml, respectively. Overall, EtOH shows effective cytotoxicity against both A

431 and A 375 cell lines and can be considered as an effective agent against skin cancer and melanoma due to its low IC50 values compared to EA extract.

The results clearly indicate that the given MeOH extract has anticancer activity by significantly inhibiting the percent cell viability and exhibiting considerable cell inhibitory potential against A 375 and A 431 cells in a time-dependent manner at 37oC. The Indian medicinal plants studied showed a significant increase in antioxidant capacity and free radical neutralizing ability. These results support the use of these plants in traditional medicine for the treatment of various diseases. Oxidative cell damage is often associated with oxidative stress. We show here that these two properties are present in an ethanolic and ethyl acetate extract of Alangium salvifolium seeds, which exhibits significant antiproliferative and high antioxidant activity. The results presented here are very promising indicators of the potential use of this plant for prophylactic and therapeutic purposes.

5. CONCLUSION

Accordingly, our studies had shown that in vitro treatment with Alangium Salvifolium seed extracts reduces the growth of cancer cell lines A 431 and A 375 by generating cytotoxicity and experimentally demonstrated. The number of viable cells decreases with increasing dose, and herbal extracts are attracting more and more attention due to their lower toxicity and high efficacy. The results also showed that the extract contains bioactive components with potential antioxidant activity. The present study concludes that the extract of Alangium salvifolium plant seeds exhibits antioxidant properties and potent anticancer activity against A431 and A375 cancer cell lines. These anti-cancers and antioxidant potential could make Alangium salvifolium extract the best treatment option for cancer patients. Clearly, research in safer and more effective chemoprevention and chemotherapy needs to continue in order to increase the efficacy of cancer treatment and reduce its cost. In this regard, the fight against cancer with the help of phytochemicals and antioxidant potential is an important research area in recent years.

6. REFERENCES

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