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ORIGINAL ARTICLE **OPEN ACCESS**

Anti-Cancer Activity of the Leaves Extract of *Bauhinia acuminata* Linn.

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ABSTRACT

This research compared the cytotoxicity of aqueous and ethanolic extract of Bauhinia acuminata Linn. using a Brine Shrimp Lethality (BSL) assay, MTT assay and Trypan blue exclusion assays. In order to measure cytotoxicity, the LC_{50} value was used (lethality concentration). Bauhinia acuminata Linn aqueous and ethanolic extract LC_{50} values against brine shrimp were 104.79(µg/ml) and 63.05 (µg/ml), respectively. Their cytotoxicity was then tested in HepG2 and L929 normal and cancer cells using the MTT assay. The aqueous and ethanolic leaf extracts were found to be selectively cytotoxic in vitro to (HepG2 & L929 cell lines) with IC_{50} values 206.43 (µg/ml) and 96.544 (µg/ml) on HepG2 cell line and IC_{50} values 2416.31 (µg/ml) and 1937 (µg/ml) on L929 cell line respectively, while it had no cytotoxic effect on normal cells. In tryphan blue assay IC_{50} values 186.78 (µg/ml) and 137.90 (µg/ml) on HepG2 cell line of aqueous and ethanolic extract and IC_{50} values 2864.07 µg/ml and 1998.19µg/ml on L929 cell line of aqueous and ethanolic extracts. This finding provided evidence that this plants contain bioactive components that may account for their pharmacological effects.. Results from the tests showed that the ethanol extract had the greatest anticancer properties.

Keywords: *Brine shrimp, MTT, Trypan blue, HepG2, L929*

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INTRODUCTION

Cancer treatment is a major international health concern [1,2]. The rising rates of both its incidence and mortality in both developing and developed nations have been documented [3,4]. With a predicted doubling of cancer deaths by 2030, the cancer epidemic is steadily growing. In 2008, it was responsible for 7.6 million deaths [5]. Many countries have not seen a significant increase in cancer patient survival despite substantial investment and advances in chemotherapy [6]. Multiple sources agree that there is currently no cancer treatment that is both highly effective and completely risk-free. Therefore, there is still an absolute requirement for the discovery of new drugs that will lead to effective anticancer agents to overcome the difficulties associated with chemotherapeutics [7].Half of the roughly 200 new chemical compounds approved over the past few decades to fight cancer have their structural origins in naturally occurring products or have been modified from these structures to be safe and advantageous [8,9]. Scientists from all over the world are investigating the potential role of antioxidant activity in cancer treatment, specifically looking into whether or not antioxidants extracted from plants act as cancer suppressors [10]. Organic molecules (such as alkaloids, lignin, Saponins, terpenes, flavonoids, vitamins, glycosides, oils, and other secondary metabolites) play an important role in selective inhibition of proliferation and induction of cancerous cell death due to their structural diversity. [11,12]

Although the *Bauhinia acuminata* Linn plant has been touted as a powerful medicinal, little is known about its cytotoxicity against liver cancers or how different cell lines react to its extract. The purpose of this research was to determine whether or not ethanol and aqueous extract had cytotoxic and apoptotic properties.

MATERIAL AND METHODS

Determination of Acute Oral Toxicity (AOT)

Toxicology tests for acute effects were performed on female Albino Wistar rats following OECD Protocol No. 423. (OECD 423, 2001)[13].

PLANT MATERIAL

Collection and preparation of plant material:

Bauhinia acuminata Linn fresh leaves are used in the research the plants used in the study are sourced from the Botanical Department at Shivaji University in Kolhapur, Maharashtra State, India and authenticated by the Botanical Survey of India, Pune, Maharashtra. (BSI/WRC/IDEN.CER./2019/H3 Dated: 19.08.2019).

The leaves of *Bauhinia acuminata* Linn remained shade-dried at 26 ± 2^0 C, the dried plant material (500 g) each was finely powdered. It was extracted by hot percolation method (Soxhlet extraction) with ethanol as solvents. The Cold maceration was used to prepare Aqueous extract.

Brine Shrimp Lethality Assay:

Material:

Artemiasalina Eggs: The eggs of the brine shrimp, *Artemiasalina*, were purchased from the Central Institute of Fisher Education in Mumbai. The eggs were a tiny size and a brownish grey colour. Eggs were stored at room temperature in an air tight container. The 100 mg of eggs roughly contain 2.5 to3.0 thousands of eggs. They have a very long shelf life in these conditions.

Hatching Chamber: Meyer et al. (1982) served as a blueprint for the design of the hatching chamber. An aluminium cover rested atop the glass container that formed the chamber. A fibre sheet served as the divider between the room's two sections, creating an imbalanced setup. One part was illuminated with a light, and other was kept darkened. Provide the aeration to both the chamber.

Method

Sample preparation: A stock solution of 1000 µg/ml was made by dissolving ten milligrams of extract in 20 µl of DMSO and bringing the volume up to 10 ml with distilled water. Extract of *Bauhinia acuminata* Linn was prepared at concentrations ranging from 20 µg/ml to 500 µg/ml from this stock. Each dose was studied with three separate sets of data. The distilled water in the control vials was measured to be the same volume as the test solution. A standard drug preparation has a concentration of 20 µg/ml.

Sea Water: The Central Institute of Fisher Education's senior scientist in charge of fish nutrition, Dr. Munil Kumar Sukham, provided formula for preparing sea water for the hatching of Brine shrimp. The 25 g of crude sea salt is dissolved in 1000 ml distill water & add 6 mg of dried yeast in this solution which acts as the food of brine shrimp. Then filtered before use.

Hatching of Brine shrimp eggs: The hatching chamber was filled with the five and a half litres of sea water that had been prepared. Brine shrimp eggs, weighed to be 20 mg, were washed in water and then scattered in the hidden area. Make sure the storage area has adequate ventilation. After 48 hours, the nauplii were collected and put through a bioassay.

Bioassay: Meyer et al. (1982) provided a protocol which was followed for this bioassay experiment. [14] Each sample vial contained 4.5 ml of brine solution, and 10 shrimp nauplii were taken in a capillary along with water. Twenty to five hundred micrograms per ml of brine solution were used in each experiment, with 0.5 ml of plant extract added. We put 4.5 ml of seawater and 0.5 ml of distilled water into the control vial. Add 20 µg/ml of the standard drug concentration. The vials were displayed in a well-lit area. Using a black or white background, researchers counted the number of surviving nauplii after 24 hours. Dose response was used to determine the fatality rate and LC_{50} value. [15]

TRYPAN BLUE EXCLUSION ASSAY (Short-term *in-vitro*cytotoxicity assay)

Principle: A cell membrane has properties to restrict the entry of dyes like Trypan blue into the cell. Dye cannot cross the cell membrane of a living cell, so it remains unstained; in contrast, a dead cell absorbs the dye, which allows for easy differentiation from a living one.

Procedure:

1. Cells were counted and found to be 1.8 x 10^6cells/ml in the stock cell suspension. An initial 0.1 ml cell suspension was plated in microwells from this stock.
2. We used 0.1 ml of DMSO (0.2%v/v with PBS) as a negative control in the first well.
3. In the next wells, 0.1ml 20µg/ml5-FU (5-Flurouracil) added and considered as oppositive control.
4. In the next wells, 0.1ml aqueous extract of *Bauhinia acuminata*Linn. added at concentration 20, 40, 80. 160, 340,640 µg/ml in the respective cells.
5. In the next wells, 0.1ml ethanolic extract of *Bauhinia acuminata* Linn. added at concentration 20, 40, 80. 160, 340,640 µg/ml in the respective cells.
6. The cells were incubated at 37°C and 5% CO_2 for 3hrs in a CO_2 incubator.
7. After the exposure, 0.1 ml of Trypan blue was added as well as mixed well.
8. The percentage of live cells was determined by counting the number of cells in each quadrant of a hemocytometer. [16]

Viable Cells: A×B×10^4
Dead Cells: C×B×10^4
Where,
A=Mean number of unstained cells
B=Dilution factor in Trypan blue
C=Mean number of dead/stained cells
10^4=Conversion of0.1 mm^3 to ml
%Viability= (Viable cell count / Total cell count) x 100

MTT Assay:

Principle:

This method is based on the principle of the colorimetric assay in which measures the reduction of yellow dye (MTT) in to the formazan product (dark purple) by using mitochondrial succinate dehydrogenases enzymes.

Sample preparation:

The extracts were made into stock solutions by dissolving 100 mg of extract in 20 µl of DMSO and then filling the remaining volume to 10 ml with phosphate buffer saline (PBS). Various concentration of extract were prepared like 640 µg/ml, 320 µg/ml, 160 µg/ml, 80 µg/ml, 40 µg/ml, and 20 µg/ml from stock solution. Each dosage was prepared in triplicate.

Standard drug solution preparation:

Standard. drug prepared by liquefying 100mg of 5-fluorouracil in 20µl of DMSO & dilute to 10ml with PBS to give a concentration of 10000 µg/ml. Of these, 640, 160 & 40 µl were taken out and adjusted to 10ml with PBS. Final concentrations are 640, 160, & 40 µg/ml.

PROCEDURE

The IC_{50} value was calculated for use in assessing the cytotoxicity of various leaf extracts. IC_{50} values were determined to assess the cytotoxicity of various leaf extracts. "Dulbecco's Modified Eagle Medium/(DMEM)" medium supplemented with 10% heat-inactivated (FBS) & 1% antibiotics was used to cultivate cell lines. Antifungal 100X solution. Cells were left seeded in 96-well flat-bottom microplates at a density of approximately 5 x 103 cells/well and maintained overnight at 37°C in 95% humidity, 5% CO_2. Different concentrations of samples (640, 320, 160, 80, 40 and 20 µg/ml) were prepared. Cells were incubated for an additional 24 hrs. After washing the cells again with PBS, MTT solution (20 µl) (5 mg/ml in PBS) was introduced to each & the plates were incubated at 37°C in an incubation chamber. After 4 hrs. 100 µl DMSO was added to both wells to dissolve the formazan crystals. Microplate reader was used to detect absorption at 570 nm & record the results.(Stockert, et al., 2012). [17]

RESULTS AND DISCUSSION

ANTI-CANCER ACTIVITY

Bauhinia acuminata Linn. has powerful antioxidants in its leaves, which prevent cell and tissue damage from reactive oxygen species. Hydrogen peroxide scavenging and the diphenyl-2-picrylhydrazyl (DPPH) assay were used to measure the extracts' free radical scavenging abilities in a previous study on the antioxidant properties of the aqueous and ethanolic extracts of *Bauhinia acuminata* Linn (Md. Reyad-Ul-Ferdous, et al, 2014) [18] There has been no formal scientific assessment of this plant in relation to cancer research so far. The cytotoxicity of aqueous and ethanolic extracts of *Bauhinia acuminata* Linn. leaves was tested using the Brine Shrimp Lethality Assay (BSLA). Table no.1 provides a brief overview of the BSLA findings.

In this bioassay, death is determined by the absence of coordinated forward motion over the course of 30 seconds. For every concentration and the control, the percentage of nauplii that died was determined. Determine the percentage of mortality by tallying the number of dead and alive nauplii in each tube.

$$\textbf{\% Death} = \frac{\textbf{Number of dead nauplii}}{\textbf{Number of dead nauplii} + \textbf{Number of live nauplii}} \times \mathbf{100}$$

As a first step in evaluating cytotoxic activity in plants, the lethality bioassay in brine shrimp has seen extensive use. The brine shrimp lethality bioassay is a useful tool for identifying compounds with anti-tumor activity because it is simple and cheap to conduct [19]

Toxicological classification of crude extracts and pure substances according to the method of Meyer et al. (LC_{50} value< 1000 µg/ml) and non-toxic (LC_{50} value>1000 µg/ml) [20]

The anti-cancer efficacy of *Bauhinia acuminata* Linn.leaf aqueous and ethanolic extracts was evaluated in a Brine shrimp lethality assay. Shows LC_{50} values 104.79 and 63.05 µg/ml respectively. (Table 1 and

Figure 1 & 2). Extracts with an LC_{50} value of less than 250 µg/ml were used for further research (Pisutthanan et al., 2004) [21].

Bauhinia acuminata Linn.leaf ethanolic extracts contain alkaloids, tannins, and flavonoids. Its cytotoxic effects may have this explanation. The exact mechanism by which an ethanolic extract of *Bauhinia acuminata* Linn.leaves inhibits cancer growth needs more research. Additional research is needed to determine whether or not the extract's inhibitory effect is due to the presence of toxic compounds; these approaches to improve gradually with the increase in extract concentration.

Brine Shrimp Method:

Table 1: The results of a comparison of *Bauhinia acuminata* Linn. Leaf extracts prepared in aqueous and ethanolic extract.

Treatment	Conc. (µg/ml)	% Mortality			Mean % Mortality (Mean ± SEM)	LC_{50} (µg/ml)
		T1	T2	T3		
5-FU (Std.)	20	50	50	60	53.33±3.33	**15.86 (µg/ml)**
	50	70	60	60	63.66±3.33	
	100	90	80	80	83.33±3.33	
	200	100	100	100	100.00±0.00	
	500	100	100	100	100.00±0.00	
AQ BA	20	0	0	0	0.00±0.00****	**104.79 (µg/ml)**
	50	20	30	30	26.66±3.33****	
	100	60	50	50	53.33±3.33****	
	200	80	70	80	76.66±3.33****	
	500	90	90	80	86.66±3.33**	
ETH BA	20	20	30	30	26.66±3.33****	**63.05 (µg/ml)**
	50	40	40	50	43.33±3.33****	
	100	60	60	60	60.00±0.00****	
	200	70	70	80	73.33±3.33****	
	500	90	100	100	96.66±3.33ns	

Values are expressed as (mean±SEM). n=3 ****P<0.0001 statistically significant when compared with control group by ANOVA followed by Dunnett's test.

Triplicate tests were run for each concentration in the bioassay.

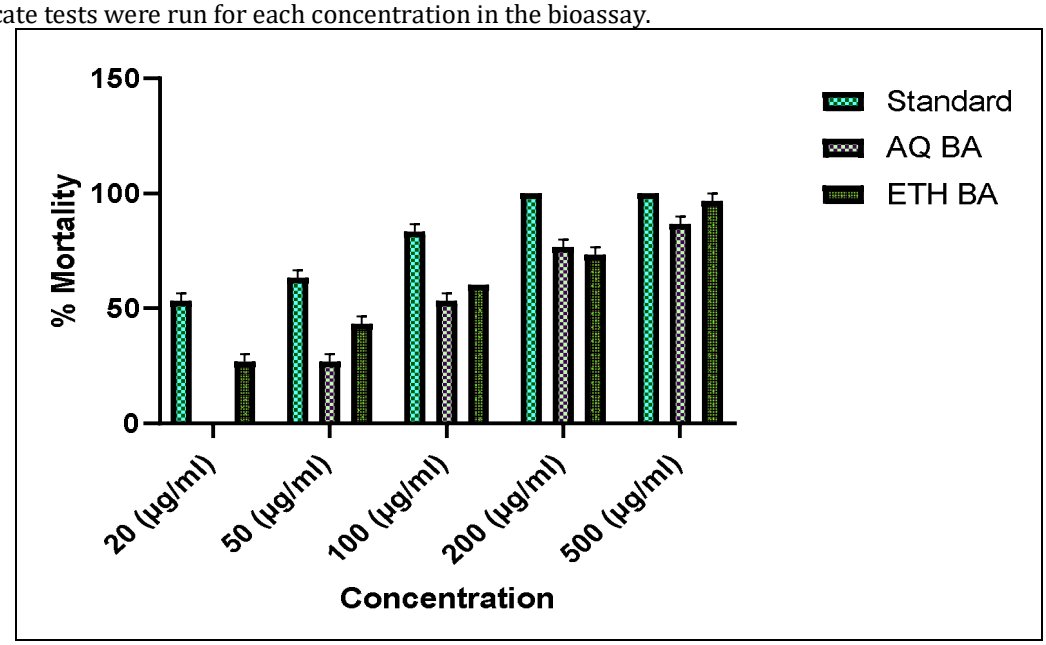

Figure 1: Effect of extracts of leaves of aqueous and ethanolic extract of leaves of *Bauhinia acuminata* Linn. and Standard (5-flurouracil) in brine shrimp lethality bioassay.

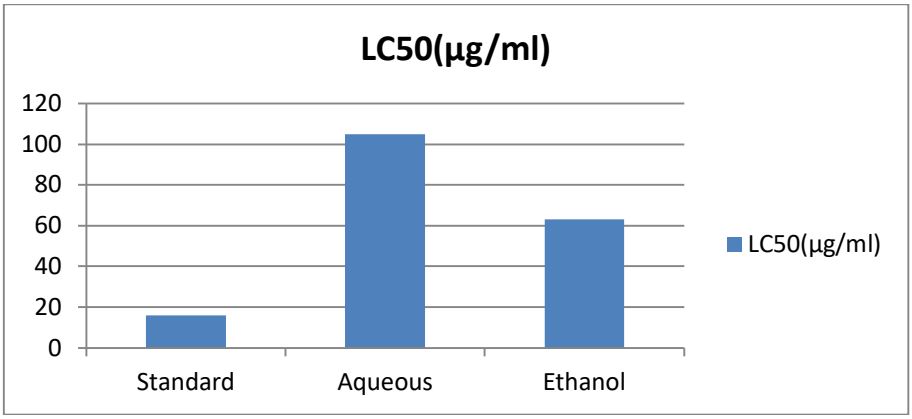

Figure 2: Comparison of LC_{50} values of aqueous and ethanolic extract of leaves of *Bauhinia acuminata* Linn with Standard

MTT ASSAY

Table 2: Result of anti-cancer effect of aqueous and ethanolic extract of leaves of *Bauhinia acuminata*Linn. On HepG2 cell lines by MTT Assay.Cell Line:- Hep G2 [Liver Cancer Cells]

Sr. No.	Treatment	Concentration (µg/ml)	% Cell Viability (Mean±S.E.M)	IC_{50} (µg/ml)
1	Normal Control	---	100%	---
2	Standard 5FU	640	10.58±0.35****	1.66
		160	20.25±0.43****	
		40	28.87±0.56****	
3	Aqueous Extract B A	640	42.34±0.47****	206.43
		320	47.03±0.57****	
		160	51.33±0.57****	
		80	54.84±0.54****	
		40	61.25±0.46****	
		20	70.62±0.43****	
4	Ethanol Extract B A	640	42.03±0.56****	96.544
		320	45.15±0.53****	
		160	48.43±0.45****	
		80	50.15±0.48****	
		40	53.28±0.48****	
		20	57.96±0.56****	

Values are expressed as (mean±SEM). n=3 ****P<0.0001 statistically significant when compared with control group by ANOVA followed by Dunnett's test.

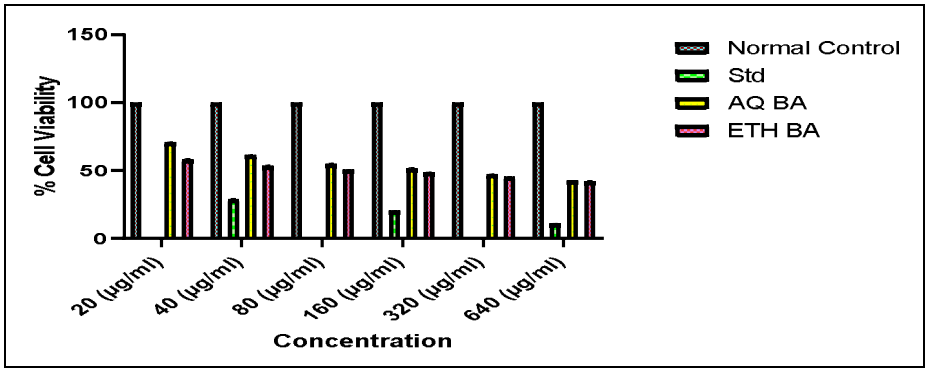

Figure 3: Effect of extracts of leaves of aqueous and ethanolic extract of leaves of *Bauhinia acuminata* Linn. Standard (5-flurouracil) by MTT Assay

Table 3: Result of anti-cancer effect of aqueous and ethanolic extract of leaves of *Bauhinia acuminata* Linn. On Normal cell lines by MTT Assay.Normal Cell Line: - L929

Sr. No.	Treatment	Concentration (µg/ml)	% Cell Viability (Mean ± SEM)	IC_{50} (µg/ml)
1.	Normal Control	--	100	---
2.	Aqueous Extract B A	640	59.65±0.47****	2416.31
		320	64.68±0.42****	
		160	70.50±0.45****	
		80	74.33±0.40****	
		40	79.76±0.54****	
		20	83.99±0.22****	
3.	Ethanol Extract B A	640	58.46±0.40****	1937
		320	63.75±0.56****	
		160	70.89±0.52***	
		80	74.20±0.47****	
		40	80.95±0.55****	
		20	84.52±0.44****	

Values are expressed as (mean±SEM). n=3 ****P<0.0001 statistically significant when compared with control group by ANOVA followed by Dunnett's test

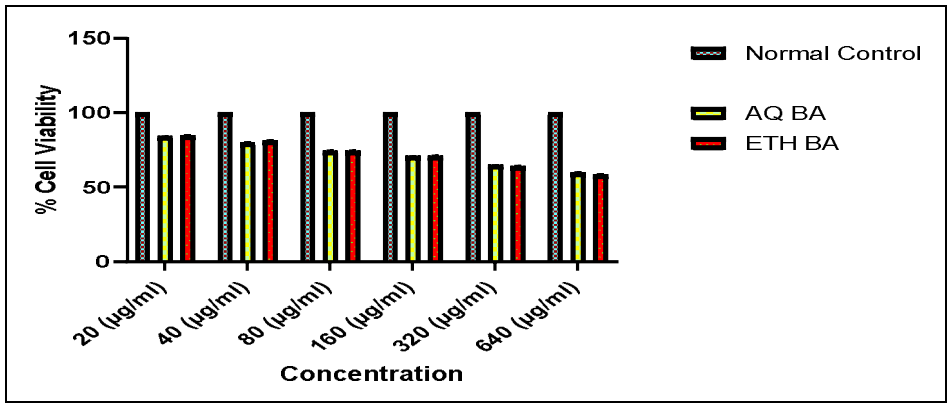

Figure 4: Effect of extracts of leaves of aqueous and ethanolic extract of leaves of *Bauhinia acuminata* Linn. Normal Cell lines by MTT Assay

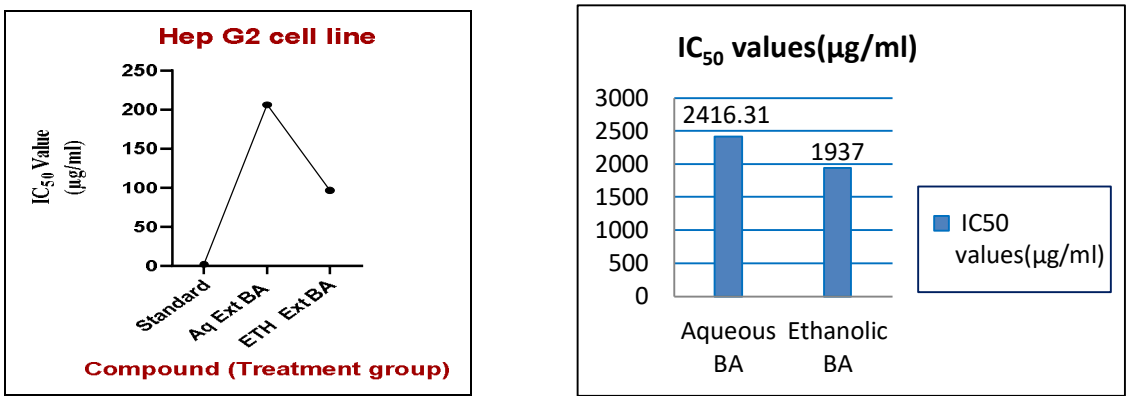

Figure 5: IC_{50} determination using MTT assay for aqueous & ethanolic extracts of leaves of *Bauhinia acuminata* Linn.HepG2and L929 cell line IC_{50} value were calculated based on MTT assay, graph showing the IC_{50} values of various leaves extracts.

TRYPHAN BLUE ASSAY

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Table 4: Result of anti-cancer effect of aqueous and ethanolic extract of leaves of *Bauhinia acuminata* Linn. on HepG2 cell lines by Tryphan blue assay method.Cell line – HepG2

Treatment	Concentration (µg/ml)	% Cell viability	IC_{50}
Standard (5-FU)	640	11.20±0.22****	1.91 µg/ml
	320	21.33±0.23****	
	160	29.56±0.48****	
Aqueous Extract B A	640	30.45±0.40****	186.78 µg/ml
	320	35.20±0.52****	
	160	50.06±0.39****	
	80	66.57±0.50****	
	40	83.70±0.21****	
	20	89.04±0.28****	
Ethanol Extract B A	640	29.52±0.37***	137.90 µg/ml
	320	34.55±0.30****	
	160	38.45±0.38*****	
	80	53.09±0.32*****	
	40	80.15±0.25****	
	20	85.21±0.44****	

Values are expressed as (mean±SEM). n=3 ****P<0.0001 statistically significant when compared with control group by ANOVA followed by Dunnett's test

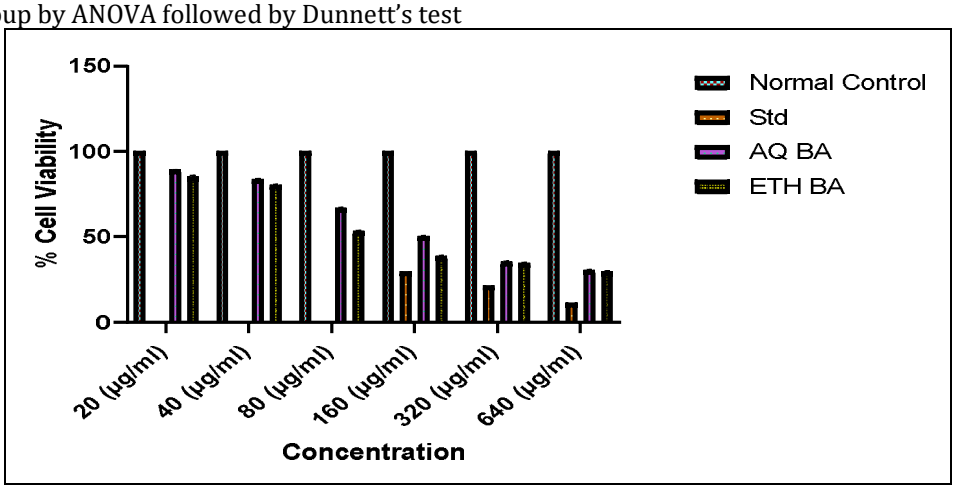

Figure 6: Effect of extracts of leaves of aqueous and ethanolic extract of leaves of *Bauhinia acuminata* Linn. Standard (5-flurouracil) by Trypan Blue Assay

Table 5: Result of anti-cancer effect of aqueous and ethanolic extract of leaves of *Bauhinia acuminata* Linn. On L929 cell lines by Tryphan blue assay method.Cell line – L929.

Treatment	Concentration (µg/ml)	% Cell viability (Mean ± SEM)	IC_{50} value
Normal Control	--	100	**---**
Aqueous Extract BA	640	61.28 ±0.39****	2864.07 µg/ml
	320	64.23±0.20****	
	160	69.87 ±0.51****	
	80	75.89 ±0.55****	
	40	81.84 ±0.54****	
	20	83.67 ±0.45****	
Ethanol Extract BA	640	55.2±0.52****	1998.19µg/ml
	320	58.33 ±0.49****	
	160	62.61 ±0.38****	
	80	64.23 ±0.56****	
	40	67.45 ±0.54****	
	20	71.89 ±0.55****	

Values are expressed as (mean±SEM). n=3 ****P<0.0001 statistically significant when compared with control group by ANOVA followed by Dunnett's test

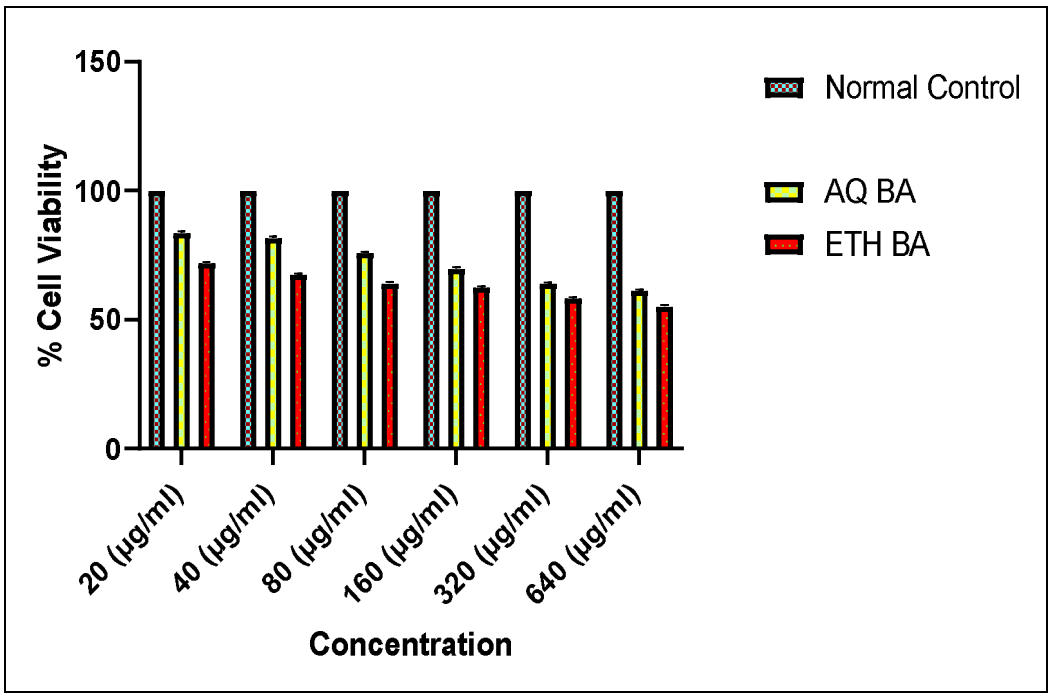

Figure 7: Effect of extracts of leaves of aqueous and ethanolic extract of leaves of *Bauhinia acuminata* Linn. Normal Celline by Trypan blue Assay

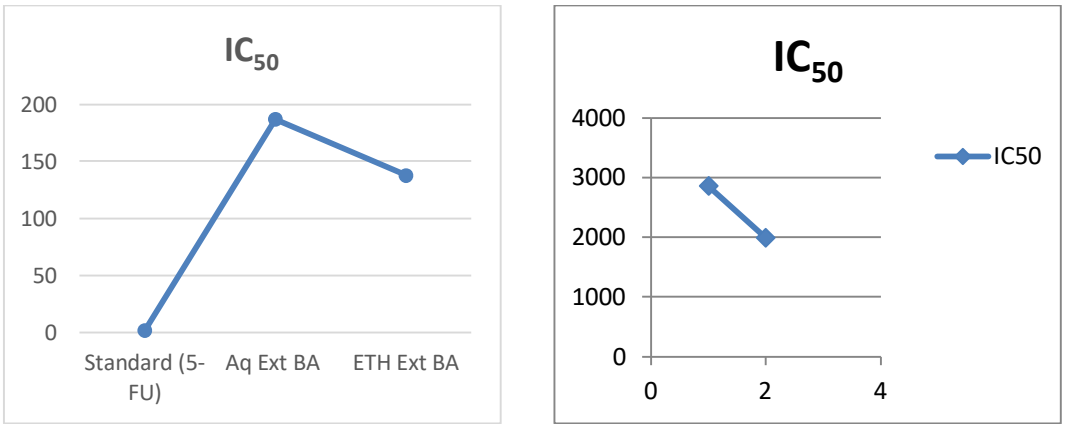

Figure 8: IC_{50} determination using Tryphan blue assay for aqueous & ethanolic extracts of leaves of *Bauhinia acuminata* Linn.HepG2and L929 cell line IC_{50} value were calculated based on Tryphan blue assay, graph showing the IC_{50} values of various leaves extracts.

Reduced yellow 3-(4,5-dimethylthiazole-2-yl)2,5-diphenyltetrazolium bromide (MTT) is an indicator of mitochondrial succinate dehydrogenase activity in the MTT assay. When MTT reaches a cell's mitochondria, it undergoes a reduction to an insoluble complex dye called form azan, which is a deep purple colour. The metabolically active cell is responsible for the reduction of MTT. The cell's health can be estimated from its activity level. Effect of extracts with Aqueous and Ethanolic extracts of leaves of *Bauhinia acuminata* Linn. On HepG2 & L929 cell line was assessed using MTT assay. The aqueous and ethanolic leaf extracts were found to be selectively cytotoxic in vitro to (HepG2 & L929 cell lines) with IC_{50} values 206.43 (µg/ml) and 96.544 (µg/ml) on HepG2 cell line and IC_{50} values 2416.31 (µg/ml) and 1937 (µg/ml) on L929 cell line respectively, while it had no cytotoxic effect on normal cells

Table 2 and Table 3 shows the IC_{50} (50% inhibitory concentrations) values of the extracts on the HepG2 & L-929 cell line. The Ethanolic extract of leaves of *Bauhinia acuminata* Linn.(IC_{50}: 96.544 µg/ml) in Figure 3. Showed the most potent cytotoxic effects against HepG2 cells; and the Aqueous Extract (IC_{50}, 206.43µg/ml).

The use of trypan blue as a stain for the detection of necrotic cells is essential. Cell membranes that are still functioning properly prevent the dye from penetrating living cells and tissues. It is able to pass

through the membrane of dead cells, though. As a result, under the microscope, dead cells appear a distinct shade of blue. Treatment of cancer cell lines with aqueous and ethanolic extracts of leaves of *Bauhinia acuminata* Linn. Results in a dose-dependent inhibitory effect, as measured by the Trypan blue exclusion assay [21].This method helped to determine the percentage of viable and dead cells in the aqueous & ethanolic extract of leaves of *Bauhinia acuminata* Linn. The extracts were tested on the HepG2 and L-929 cell lines, and their IC_{50} (50% inhibitory concentrations) values are shown in Tables 4 and 5. The results of Trypan blue assay of aqueous & ethanolic extracts of leaves of *Bauhinia acuminata* Linn. On HepG2 and L929 cancerous cell lines were represented in Figure no. 6 and 7 respectively.

CONCLUSION

The cytotoxic effect of leaves of *Bauhinia acuminata* Linn extract showed the concentration-dependent effect in the culture of HepG2 and L929 cells. The Ethanolic extract of leaves of *Bauhinia acuminata* Linn. Showed the most potent cytotoxic effects against HepG2 cells than Aqueous Extract. The use of some herbs has attracted a great deal of attention as one of the alternative cancer therapies from the point of less toxicity and cost benefits. The result of the present study concluded that leaves of *Bauhinia acuminata* Linn showed effective anticancer activity in a dose-dependent manner by BSL assay method , MTT and Trypan blue assay.

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