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Preliminary Phytochemical Analysis and *in vitro* Anti-cancer Screening of Ethanolic extract of Roots of *Bauhinia tomentosa* L. against HUH-7 Human Liver cancer Cell lines

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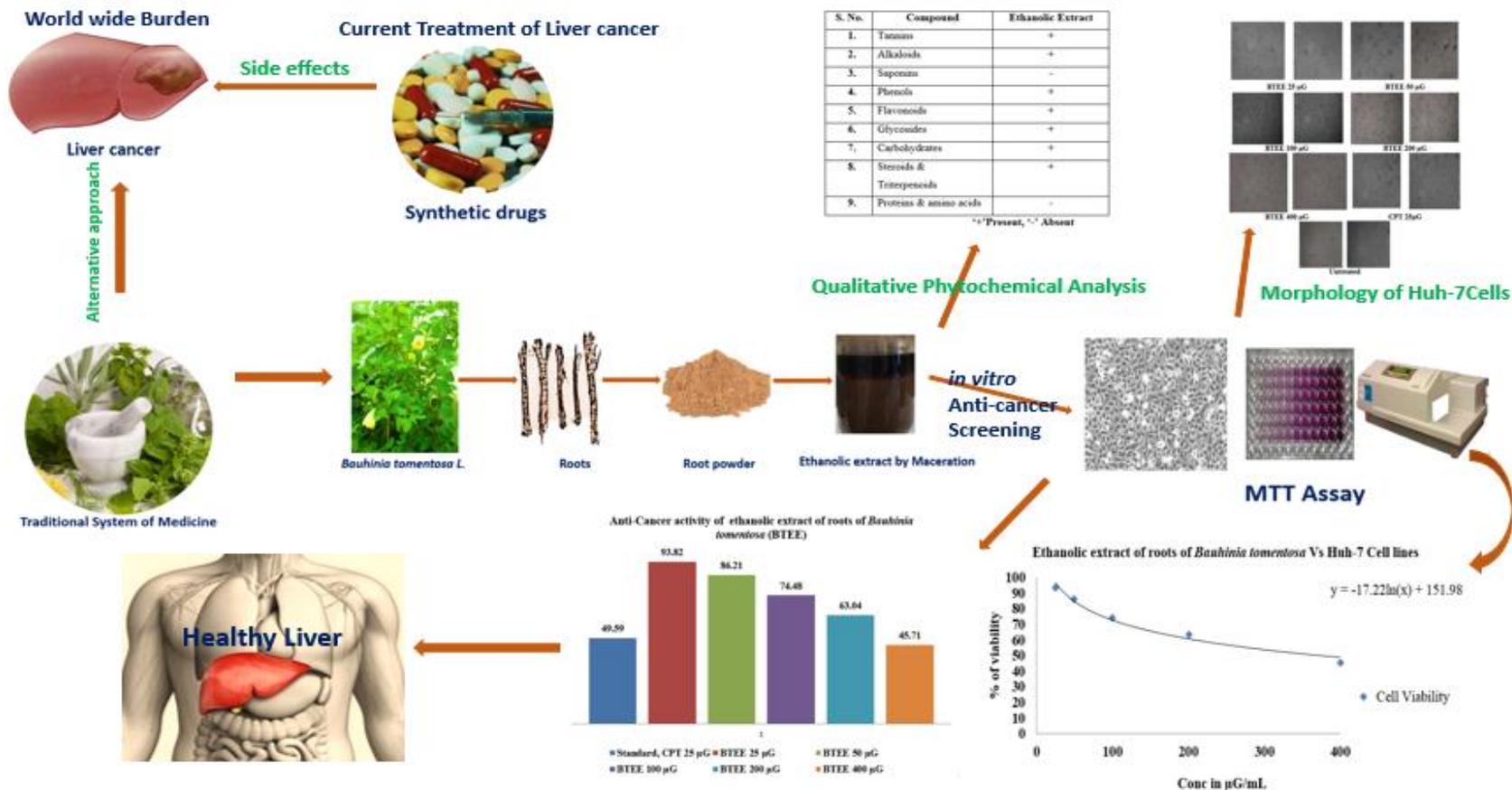
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Preliminary Phytochemical Analysis and *in vitro* Anti-cancer Screening of Ethanolic extract of Roots of *Bauhinia tomentosa* L. against HUH-7 Human liver Cancer cell lines

Graphical Abstract



Abstract:

The most effective way to reduce the worldwide burden of liver cancer is to prevent it from happening in the first place. The current treatment of liver cancer has significant side effects. Hence, there is a need to develop anti-liver cancer agents of plant origin, which are less toxic, more efficacious and cost-effective. The present study has been performed experimentally by *in vitro* to examine the anti-liver cancer activity of roots of *Bauhinia tomentosa* L (Fabaceae). The roots of *B. tomentosa* was tested for its anti-cancer activity against HUH-7 human liver cancer cell lines by MTT assay. The standard used in this assay was Camptothecin (CPT) at 25 μ G concentration. Plant extract was tested at 25 μ g/mL, 50 μ g/mL, 100 μ g/mL, 200 μ g/mL and 400 μ g/mL concentrations. The percent cell viability of standard drug was found to be 49.59% and plant extracts at 25 μ g/mL, 50 μ g/mL, 100 μ g/mL, 200 μ g/mL and 400 μ g/mL concentrations were found to be 93.82%, 86.21%, 74.48%, 63.04%, 45.71% respectively. The cell morphology was observed and recorded under a microscope. The results clearly indicated that *B. tomentosa* shows a dose dependent activity and it was maximum at 400 μ g/mL concentration where it shows 45.71% of liver cancer cell viability and it was comparable to the standard drug where it shows 49.59% of viability.

Keywords: *Bauhinia tomentosa* L, Roots, MTT Assay, HUH-7 human liver cancer cell lines, Anti-liver cancer



Introduction

The main aim of the work done by the author was to carry out the preliminary phytochemical analysis and *in vitro* anti-cancer screening of ethanolic extract of roots of *Bauhinia tomentosa* L (Fabaceae). It has been valued in Ayurveda and Unani system of medication for possessing variety of therapeutic properties. Most of the plants parts are used in traditional system of medicine in India. According to Ayurveda all the part of the plant is recommended in the combination with other drugs for the treatment of snake bite and scorpion-sting. It has also been claimed to use traditionally in the treatment of various ailments including different types of cancer. A decoction of the root bark is prescribed for liver troubles and as a febrifuge. Infusion of the steam bark is useful as an astringent gargle. The leaves constituent an ingredient of a plaster applied to abscesses. The dried leaves, buds an flower are used in dysentery. The fruit is a diuretic. The seed are used as tonic. The wood is used as fuel. The leaves are used as dyes. The bark is externally applied to tumors and wounds.

Literature survey indicated that no published reports on roots of *Bauhinia tomentosa* for anti-cancer activity against Huh-7 human liver cancer cell lines. In view of this, the author was aimed to carry out the extract of roots of *Bauhinia tomentosa* by using solvent ethanol and then plan to study the *in vitro* anti-cancer activity.





Bauhinia tomentosa L.



Preliminary phytochemical examination of ethanolic extract of roots of *B. tomentosa* L.

Cold extraction (Maceration)

The dried powdered plant material (1500g) was allowed to contact with solvent ethanol in a closed vessel and then allowed to macerate with occasional shaking for 7 days. Strain the liquid, press the marc; mix the liquids and finally clarifying by filtration. The extract thus obtained was concentrated under vacuum (50⁰ C) by using Rotary Evaporator, dried completely and weighed. The extract thus collected subjected to preliminary phytochemical analysis and *in vitro* anticancer screening.

Table 1: Details of the Cold Maceration

Plant material	Solvent used	% Yield
Roots of <i>Bauhinia tomentosa</i> L.	Ethanol	42.24



Preliminary Phytochemical Analysis :

The extract was prepared and tested for the type of chemical constituents present by known and standard qualitative tests.

The following tests were carried out on the extract to detect various phytoconstituents present in them.

1. Tests for Alkaloids
2. Tests for Carbohydrates
3. Tests for Glycosides
4. Tests for Saponins
5. Tests for Phenolic Compounds and Tannins
6. Tests for flavonoids

***In vitro* anti-liver cancer screening of ethanolic extract of roots of *Bauhinia tomentosa* L. using huh-7 cancer cell lines**

Test Sample: Ethanolic extract of roots of *Bauhinia tomentosa* L.

Preparation of plant extract:

The samples were prepared with concentrations of 100µg/ml and syringe filtered using 0.22µM sized syringe filtration units to ensure sterility.

Selected Cell Line:

The Huh-7 cell line was compassionately offered by *NCCS, Pune*.

Huh-7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum & 100 IU/ml penicillin & 100 µg/ml streptomycin, at 37°C in an atmosphere of 5% CO₂.



MTT assay for Cytotoxicity

Principle

MTT assay is a colorimetric assay used for the determination of cell proliferation and cytotoxicity, based on reduction of the yellow colored water soluble tetrazolium dye MTT to formazan crystals. Mitochondrial lactate dehydrogenase produced by live cells reduces MTT to insoluble formazan crystals, which upon dissolution into an appropriate solvent exhibits purple color, the intensity of which is proportional to the number of viable cells and can be measured spectrophotometrically at 570nm.

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 25uM of Curcumin)

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.



Procedure for determining Cell Cytotoxicity by MTT Assay

Cell Seeding

1. Seed 200µl cell suspension in a 96-well plate at required cell density (20,000 cells per well), without the test agent. Allow the cells to grow for about 24 hours.
2. Add appropriate concentrations of the test agent (25µg/mL, 50 µg/mL, 100µg/mL, 200 µg/mL and 400µg/mL of plant extract).
3. Incubate the plate for 24 hrs at 37°C in a 5% CO₂ atmosphere.
4. After the incubation period, takeout the plates from incubator, and remove spent media and add MTT reagent to a final concentration of 0.5mg/mL of total volume.
5. Wrap the plate with aluminum foil to avoid exposure to light.
6. Return the plates to the incubator and incubate for 3 hours.

(Note: Incubation time varies for different cell lines. Within one experiment, incubation time should be kept constant while making comparisons.)

Remove the MTT reagent and then add 100 µl of solubilisation solution (DMSO).



7. Gentle stirring in a gyratory shaker will enhance dissolution. Occasionally, pipetting up and down may be required to completely dissolve the MTT formazan crystals especially in dense cultures.
8. Read the absorbance on a spectrophotometer or an ELISA reader at 570nm and 630nm used as reference wavelength.

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.

Cell viability was obtained using the following equation:

$$\text{Percent cell viability} = \frac{\text{Test 570 nm} - 620 \text{ nm}}{\text{Control 570 nm} - 620 \text{ nm}} \times 100$$

$$\text{Mean OD treatment} / \text{Mean OD control} \times 100 = \underline{\quad} \%$$



Results and discussion

The most common liver cancers are hepatocellular carcinoma (HCC), cholangiocellular carcinoma (CCC), and metastatic colorectal cancer. Liver cancer is much more common in countries in sub-Saharan Africa and Southeast Asia than in the US. In many of these countries it is the most common type of cancer. More than 700,000 people are diagnosed with this cancer each year throughout the world. Liver cancer is also a leading cause of cancer deaths worldwide, accounting for more than 600,000 deaths each year.

This year, an estimated 42,220 adults (30,610 men and 11,610 women) in the United States will be diagnosed with primary liver cancer. Since 1980, incidence of liver cancer has tripled, although rates in young adults are starting to decrease. Men are about 3 times more likely than women to be diagnosed with the disease. It is estimated that 30,200 deaths (20,540 men and 9,660 women) from this disease will occur this year. For men, liver cancer is the 10th most common cancer and the 5th most common cause of cancer death. It is also the 8th most common cause of cancer death among women.

Because there are only a few effective ways to prevent or treat liver cancer at this time, there is always a great deal of research going on in the area of liver cancer. Scientists are looking for causes and ways to prevent liver cancer, and doctors are working to improve treatments.

The most effective way to reduce the worldwide burden of liver cancer is to prevent it from happening in the first place. Some scientists believe that vaccinations and improved treatments for hepatitis could prevent about half of liver cancer cases worldwide. Researchers are studying ways to prevent or treat hepatitis infections before they cause liver cancers. Research into developing a vaccine to prevent hepatitis C is ongoing. Progress is also being made in treating chronic hepatitis.

The current treatment of liver cancer has significant side effects. Hence, there is a need to develop anti-liver cancer agents of plant origin, which are less toxic, more efficacious and cost-effective. Previous studies demonstrated that medicinal plants used for centuries against different diseases including for cancer and become a focal point to identify, isolate and purify new compounds to treat diseases. Many traditional medicinal plants and herbs were reported to have strong anti-liver cancer activity and also used for treatment of different liver disorders and some of these include *Silybum marianum* (milk thistle), *Picrorhiza kurroa* (kutkin), *Curcuma longa* (turmeric), *Camellia sinensis* (green tea), *Chelidonium majus* (greater celandine), *Glycyrrhiza glabra* (licorice) etc.

The results of preliminary phytochemical analysis are given in the following table

Table 2: Preliminary Phytochemical analysis of ethanolic extract of roots of *Bauhinia tomentosa* L.

S. No.	Compound	Ethanolic Extract
1.	Tannins	+
2.	Alkaloids	+
3.	Saponins	-
4.	Phenols	+
5.	Flavonoids	+
6.	Glycosides	+
7.	Carbohydrates	+
8.	Steroids & Triterpenoids	+
9.	Proteins & amino acids	-

'+' Present, '-' Absent



Cellular toxicity through MTT Assay:

For the anti-cancer screening of ethanolic extract of roots of *Bauhinia tomentosa* against Huh-7 human liver cancer cell lines, the effect of selected plant extract was determined through MTT cell proliferation assay. The MTT substance is reduced by mitochondrial succinic dehydrogenases in living cells to purple formazan crystals that are not soluble in aqueous water. The absorption of dissolved formazan in the visible region correlates with the number of alive cells (Table 5). The direct microscopical observations of selected plant extract treated images of Huh-7 cell lines by Inverted Biological Microscope after incubation of 24 hours were shown in Figure 5-8.



Table 3: Cytotoxicity Study of ethanolic extract of roots of *Bauhinia tomentosa* (BTEE) against HUH 7 Cell line by MTT Assay:

	Blank	Untreated	STD 25µG	BTEE 25 µG	BTEE 50 µG	BTEE 100 µG	BTEE 200 µG	BTEE 400 µG
Reading 1	0.03	1.092	0.559	1.017	0.932	0.819	0.689	0.516
Reading 2	0.05	1.077	0.557	1.023	0.949	0.817	0.708	0.519
Mean	0.04	1.0845	0.558	1.02	0.9405	0.818	0.6985	0.5175
Mean OD- Mean Blank		1.0445	0.518	0.98	0.9005	0.778	0.6585	0.4775
Standard Deviation		0.010	0.001	0.004	0.012	0.001	0.013	0.002
Standard Error		0.007	0.001	0.003	0.008	0.001	0.009	0.001
Viability %		100	49.59	93.82	86.21	74.48	63.04	45.71
IC 50 VALUE= 374.06 µG/ml								

BTEE: Ethanolic extract of roots of *Bauhinia tomentosa*



Figure 1: Anti-Cancer activity of ethanolic extract of roots of *Bauhinia tomentosa* (BTEE)

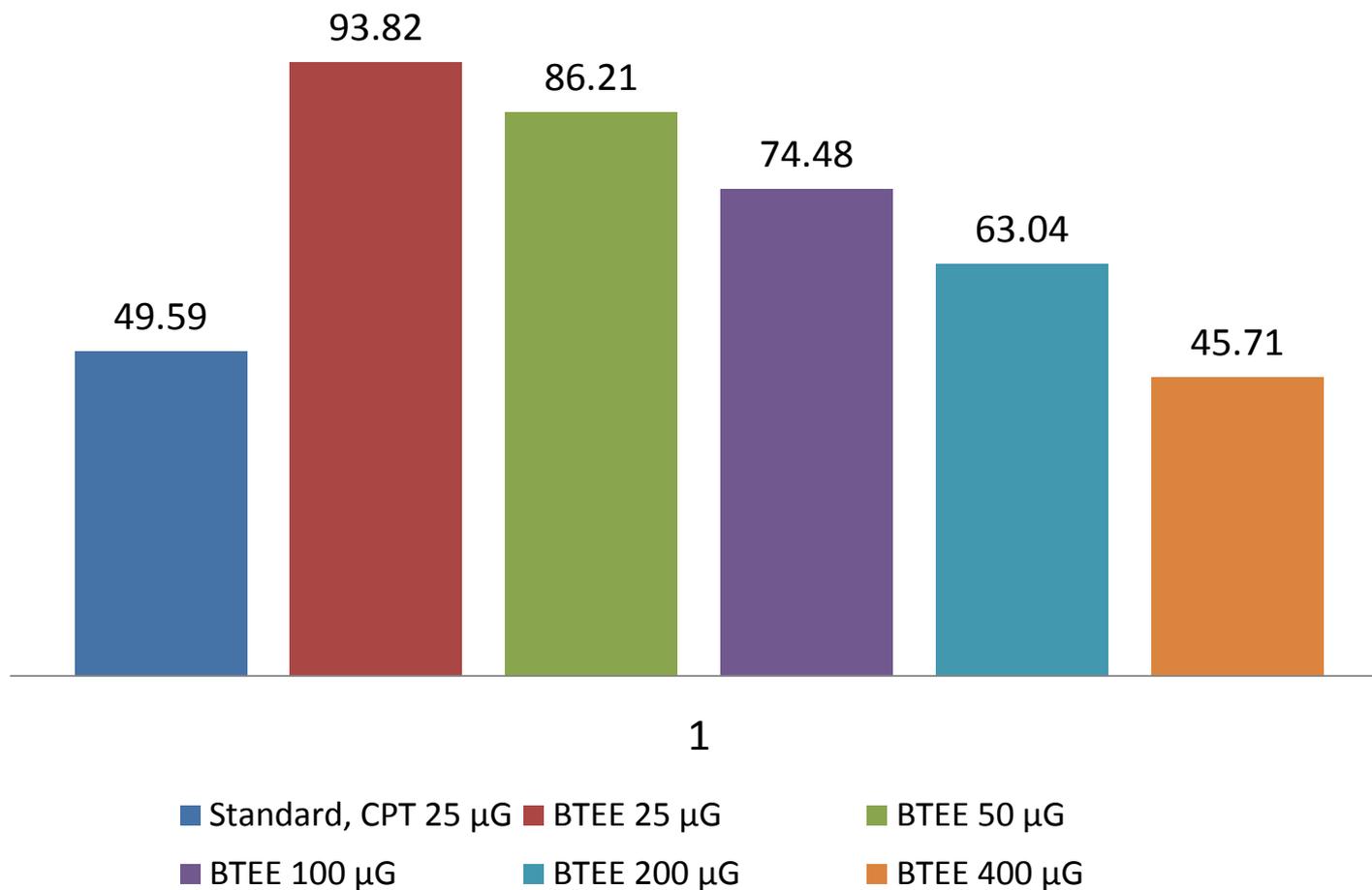
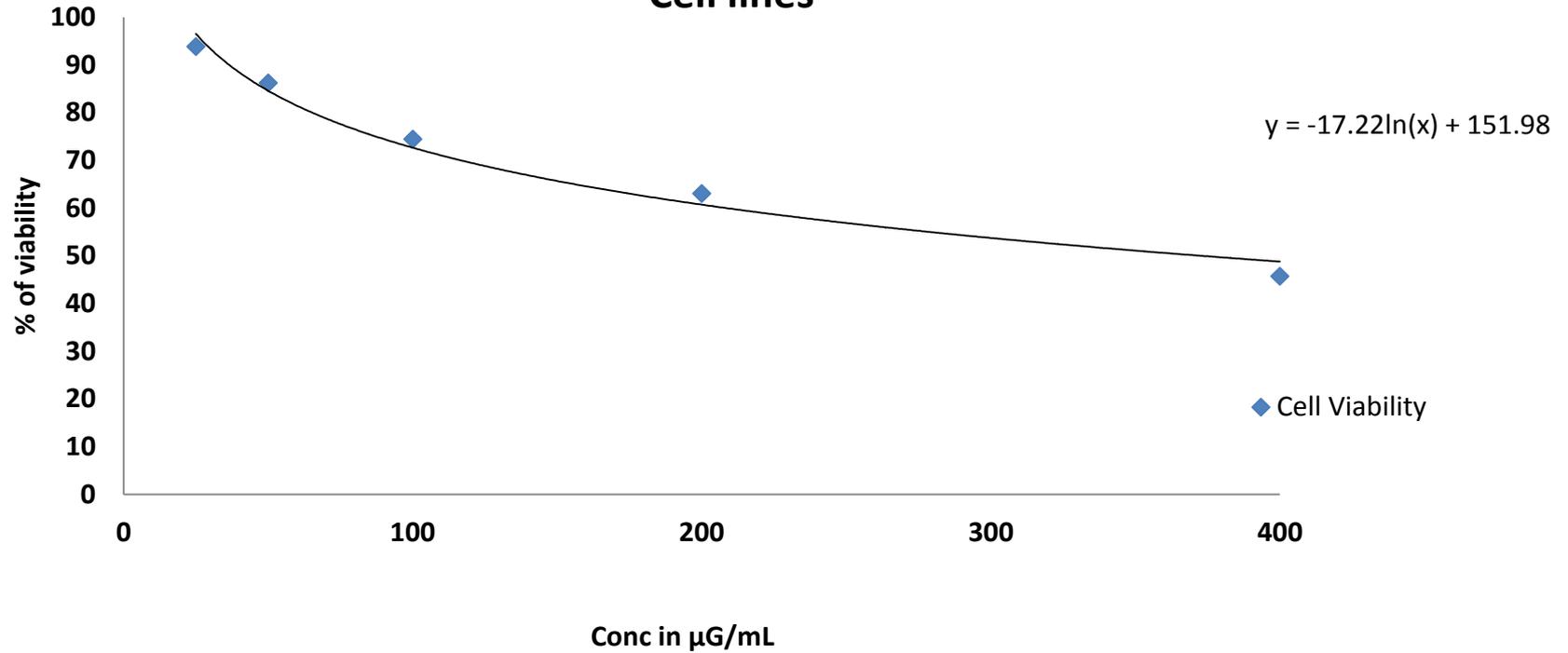
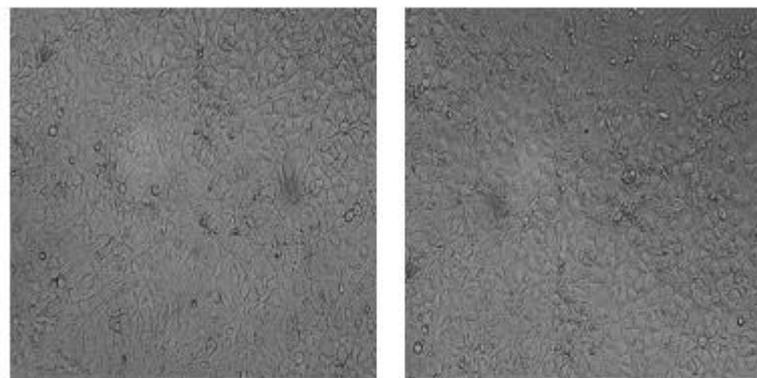


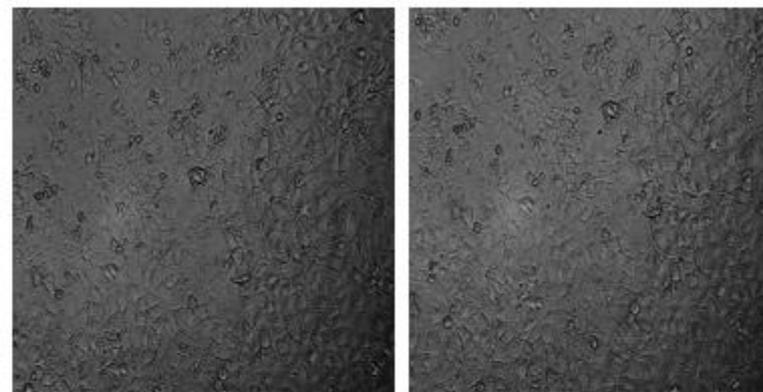
Figure 2: Ethanolic extract of roots of *Bauhinia tomentosa* Vs Huh-7 Cell lines



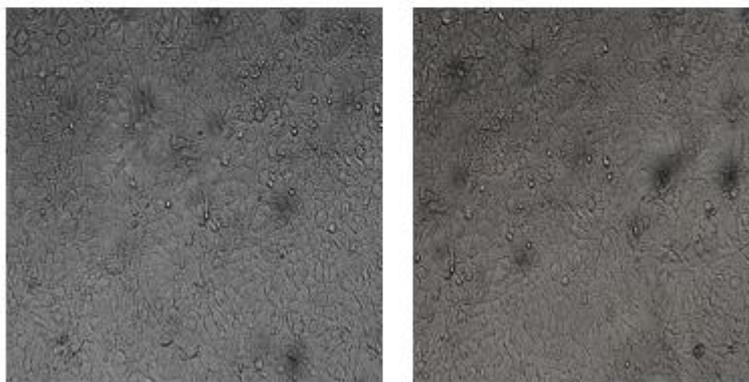
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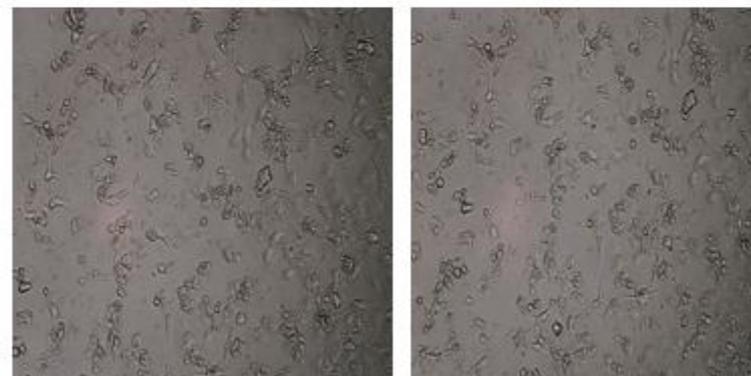
BTEE 25 µG



BTEE 100 µG



BTEE 50 µG



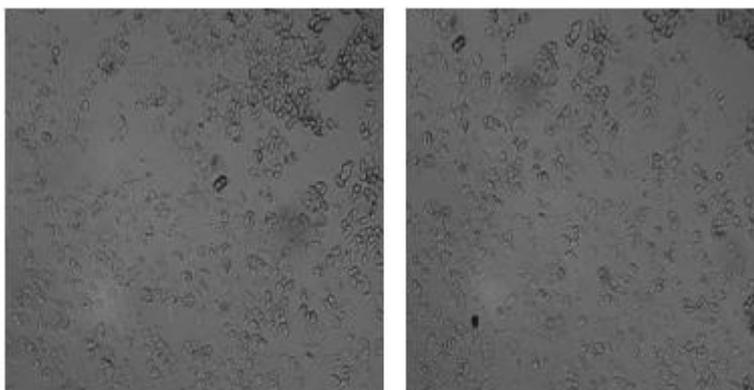
BTEE 200 µG

Figure 3: Photomicrographs of morphology of Huh-7 cells cells





BTEE 400 μ G



CPT 25 μ G



Untreated

Photomicrographs of morphology of Huh-7 cells



In the present study, ethanolic extract of roots of *Bauhinia tomentosa* L. was screened for anti-cancer activity against Huh-7 human liver cancer cell lines through MTT cell proliferation assay. The standard used in this assay was Camptothecin (CPT) at 25 μ G concentration. Plant extract was tested at 25 μ g/mL, 50 μ g/mL, 100 μ g/mL, 200 μ g/mL and 400 μ g/mL concentrations. The percent cell viability of standard drug was found to be 49.59% and plant extracts at 25 μ g/mL, 50 μ g/mL, 100 μ g/mL, 200 μ g/mL and 400 μ g/mL concentrations were found to be 93.82%, 86.21%, 74.48%, 63.04%, 45.71% respectively. The cell morphology was observed and recorded under microscope (Figure 3). The results clearly indicated the selected plant extract shows a dose dependent activity and it was maximum at 400 μ g/mL concentration where it shows only 45.71% of liver cancer cell viability and it was comparable to standard drug where it shows 49.59% of viability.



Conclusions

The study confirms the traditional claim of anti-liver cancer activity of roots of *Bauhinia tomentosa* L. Phytochemical screening of extract reveals the presence of different secondary metabolites like tannins, alkaloids, phenols, flavonoids, glycosides, steroids & triterpenoids. Presence of these compounds in alone or combination may be responsible for the observed anti-liver cancer activity. Further studies need to be carried out to isolate the individual compounds from the crude ethanolic extract, their purification, characterization and pharmacological screening will be an informative tool in revolutionizing the plant based medicine for treating liver cancer.



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