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Phytochemical Evaluation and in Vitro Anti Cancer Study of Cupressus Semperviren Linn. Leaves Extracts

Mr Harshvardhan Suryawanshi, Miss Vishva Kanjiya, Miss Ravina Mali, Mr Dipak Phalle
Nootan College of Pharmacy, Kavathemahankal, Maharashtra, India

Abstract: *Herbal have a long traditional history or use of conventional medicine. It is becoming more main stream as improvements in analysis and quality control along with advances in clinical research show the value of herbal medicine in the treating and preventing disease. The Cupressus Semperviren Linn. Garden plant belong to family cupressaaceae. The plant contain different chemical constituents like such as Glycosides, Essential oil, Phenols, Flavonoids etc. The main aim of the study was to extract the plant material by using pet ether as a solvent by soxhelt extraction method. From the phytochemical analysis the plant was showed chemical constituents. The phytochemical constituent was isolation and separation by using thin layer chromatography and column chromatography. each chemical constituent were identified by using physical and chemical test .Isolated chemical constituent were subjected to further study that is it Spectral analysis like IR, NMR, And Mass spectroscopy the pet ether extact of plant was used for Pharmacological study such as Anticancer activity. Show anticancer activity of plant was done by using MTT Asasay and trypan blue test.*

Keywords: Cupressus Semperviren Linn, Anti cancer activity

I. INTRODUCTION

Since 5,000 years the use of herbal medicine are documented. Herbal medicines are widely utilized as effective remedies for the prevention and treatment of multiple health conditions from centuries.[1] Many of medicinal plants have important role in the development of human culture. Aspirin like modern medicines are produced indirectly from medicinal plants. Many medicinal plant especially food crops have medicinal effects, example garlic. Many medicinal plants are resources of new drugs. The medicinal plant evaluated more than 250, 000 flower plant species. Medicinal plants helps to understand plant toxicity and also help to protect human and animals from natural poisons. Cultivation and preservation of medicinal plants helpful to protect biological diversity for example metabolic engineering of plants. The medicinal effects of plants are due to metabolites especially secondary compounds produced by plant species. Plant metabolites classified primary metabolites and secondary metabolites. medicinal Phytotherapyis the use of plants or plant extracts.[2]Long history and strong base for Ayurveda in india. Used traditional herbal medical system. Herbal plants used in preventing and treating of human diseases. In thousand of year People have been using traditional medicine .[3]The great importance of Natural products and traditional medicines. Ayurveda, Kampo, traditional Korean medicine, and Unani system of medicine used in some areas of world[4] According to World Health Organization (WHO) 70 % of the world's population proof on plants used primary health care and some 35,000 to 70,000 species medicament has been used, some 14-28% of the 250,000 plants species evaluated to occur around the world and in world-wide equivalent to 35-70% of all species[5] Harbal medicine are not used in emergency condition like accident, serious illnesses as compared Modern medicine treats sudden and serious illnesses and accidents. Herbal medicine to treat serious trauma, such as a Heart attack[6]. The crude extract of harbal plants may be used as medicaments and then Further processes the isolation and identification of the active ingradients and elucidation of active moiety[7]Indian herbal drugs have been therapeutic uses are evaluated successfully and many of new medicinal plants are discovered.[8]

Plant Name-*Cupressus sempervirens Linn.*

Family –Cupressaceae

Synonym- Mediterranean cypress, Italian Cypress

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Taxonomic Classification [1]

Kingdom	**Plantae**
Division	Pinophyta
Class	Pinopsida
Order	Pinales
Family	Cupressaceae
Genus	Cupressus

Table no 1

II. MATERIAL AND METHOD

Preparation of Extract

The fresh leaves of cupress sempervarin L.were collected from local rregion of tasgoan Authentication of leaves was done by department of bonty Smt. Kasturbai walchand,sangli.

The collected leaves were washed with tap water and air dried under shead in house for 25-30 days after complete drying powder by mixture grinder to obtain fine powder

sThe 100 gm of dried powder extracted with petroleum ether (40-600) in soxhelt extractor at temperature(40-600) . The extraction was continued until the solvent in the thimble become a clear. After each extraction the solvent was recovered and extract was concentrated by usin rotary evaporator at 600 temperature. After concentrated extract was stored in desiccators.

Phytochemical evaluation:-

The Pet ether extract (40-60^0) extract of cupressus semperviren. were used of qualitative chemical investigation to check various chemical constituent in the extract.

Test for glycoside +

Test for Tannin/ phenolic compound +

Test for Volatile oil +

Test for Flavonoids +

Phytsiochemical evaluation of Extract:-

Physiochemical evaluation of extract was performed by different method such as the total ash value and acid insoluble ash value, water soluble ash value and total moisture content. this method was used to remove the adulteration from the extract.

Isolation of pet ether extract of cupresus sempervarin

Isolation of cupreesus sempervarin extract carried out by using column chromtography Mobile phase used as petroleum ether (40-60^0) :Ethyl acetate :Methanol (5:1:1) The isolated fraction were collected dried and used for further study isolated fraction identified by phytochemical TLC.

Physical Test for Isolated Fraction.

Physical test		
Test	**Observation**	**Inference**
Small test	Characteristics Pungent Test	Essential oil present.

Chemical test		
Test	**Observation**	**Inference**
Solubility test	Soluble in water	Essential oil present.
Filter paper test	Oil spot disappear	Essential oil present.

Table 2: Physical Test for Isolated Fraction

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Experimental

Brine Shrimp lethality bioassay is a rapid and comprehensive bioassay for the bioactive compounds of natural and synthetic origin. In this method, natural product extracts, fractions as well as the pure compounds can be tested for their bioactivity. The method utilizes in vivo lethality in a simple zoological organism (Brine nauplii) as a convenient monitor for screening

and fractionation in the discovery of new bioactive natural products. Brine toxicity is closely correlated with (human nasopharyngeal carcinoma) cytotoxicity. ED50 values for cytotoxicities are generally about one-tenth the LC50 values found in the Brine Shrimp test. it is possible to detect and then monitor the fractionation of cytotoxic. The active extracts using the Brine lethality bioassay. The Brine Shrimp assay has advantages of being rapid (24 hours), inexpensive, and simple. It easily utilizes a large number of organisms for statistical validation and requires no special equipment and a relatively small amount of sample (2-20 mg or less).Furthermore it does not require animal serum as is needed for cytotoxicities.

Preparation of seawater

63 gm sea salt (without iodine) was weighed, dissolved in two liter of distilled water and filtered off to get clear solution.

Hatching of Brine Shrimp

Artemia salina (brine shrimp eggs) collected from pet shops was used as the test organism. Seawater was taken in the small tank and shrimp eggs were added to one side of the tank and then this side was covered. Two days were allowed to hatch the shrimp and to be matured as nauplii. Constant oxygen supply using ariator was carried out through the hatching time. The hatched shrimps are attracted to the light and so nauplii free from egg shell was collected from the illuminated part of the tank. The nauplii was taken from the tank by using pipette and diluted in fresh clear sea water to increase visibility and 10 nauplii was taken carefully by using pipette.

Preparation of test solutions with samples of experimental plants

- 10 mg test Sample of the extract were prepared by dissolving in 20 micro liter of DMSO and volume make up 10ml with distill water, to get 1000µg/ml stock solution.
- From this stock. 100µl, 250µl, 500µl, 1000µl and 2500µl were taken and volume was made up to 5ml with distill water to get the final drug conc. 20µg/ml, 50µg/ml, 100µl/mg, 200µl/mg and 500µl/ml
- Three replicates were prepared from each dose lavel. Control vials were prepared by adding equal volume of distilled water.

Counting of nauplii

- After 24 hours, the test tube were inspected using a magnifying glass against a black background and the number of survived nauplii in each tube was counted. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration[6]

Concentration (µg/ml)	Extract Solution	Sea water containing 10 nauplii	Final volume
100µl	2.5 ml	2.5ml	5ml (20µl/ml)
250µl	2.5ml	2.5ml	5ml (50µl/ml)
500µl	2.5ml	2.5ml	5ml (100µl/ml)
1000µl	2.5ml	2.5ml	5ml (200µl/ml)
2500µl	2.5ml	2.5ml	5ml (500µl/ml)

Table no 3 Calculation of drug dilution

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Effect of *Cupressus Sempervire*n *L.* Leaves Extract on Brine Shrimp Lethality bioassay

Treatment	Dose µg/ml	Mean % death after 24hrs (mean ± SEM)	LC50 µg/mg
Extract A	20	16.22 ± 3.33	228.31
	50	30.67 ± 3.33	
	100	40.33 ±3.33	
	200	53.23 ± 3.33	
	500	100 ±00	

p<0.01 Statistically Significant when compared with control group by ANOVA followed by Dunnett test

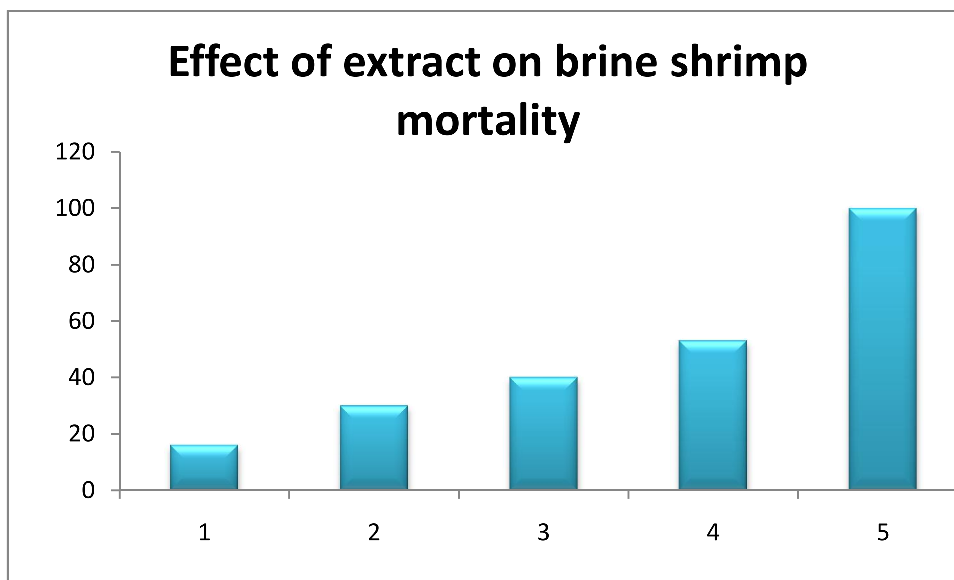

Fig no 1 :-Effect of extract on brine shrimp mortality

Determination Of LC_{50} :-

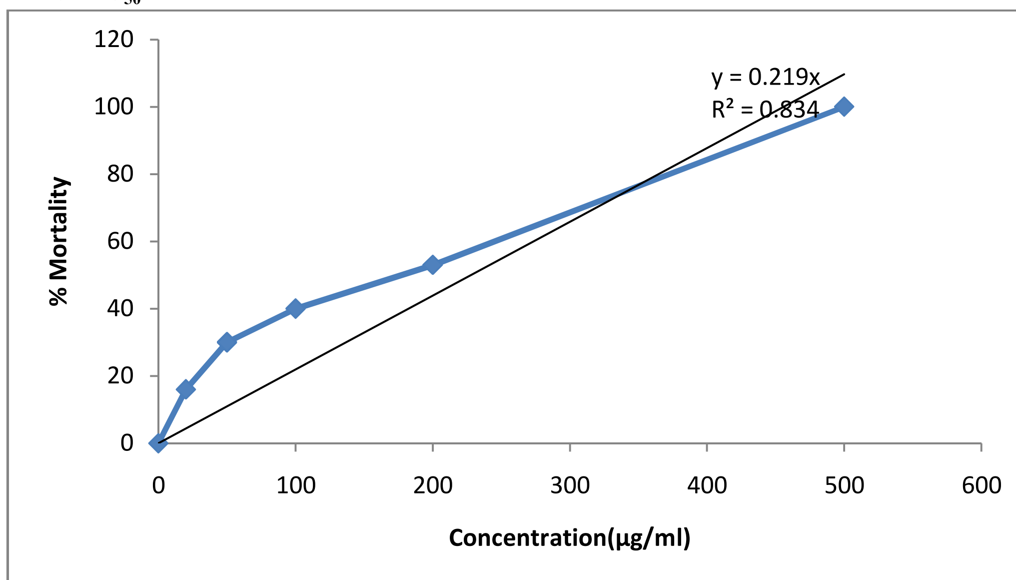

Fig no 2 :- Effect of % Mortality of Brine shrimp and calculation of LC_{50}

The Brine shrimp Lithality bioassay, Trypan blue Exclusion assay and MTT assay are represent such techniques, which can play a significant role in preclinical cytotoxic screening. In present study pet ether extract of *Cupressus sempervire*n *L.* leaves showed significant anti cytotoxic activity. Petroleum Ether Extract of *Cupressus sempervire*n *L.*

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at Conc. 20,50,100,200,500µg/ml administered to brine shrimp. After 24hr counting the brine shrimp nauplii. The result showed that at 200µg/ml the 50% brine shrimp nauplii dead so its showed significant cytotoxic effect as compared to other concentration and showed LC50 was 228.31

Invitro Anticancer :-

Cell lines are used for invitro activity:-

Sr. No.	Cell line	Sources
1	HEp2 cell line	NCCS, Pune.
2	VOCAR-3 Cell line	NCCS Pune.

Table no 5:- cell lines used in vitro activity

Methods:-

1) Cell Viability Testing with Trypan Blue Exclusion Method:-

The Trypan Blue dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue whereas dead cells do not. When a cell suspension is prepared simply mixed with the dye. A viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm. Periodic cell viability assessment provides an early indicator of the quality of your fresh cells prior to freezing. Viabilities of greater than or equal to 95% are excellent.

Procedure-Trypan blue – 100mg Trypan blue was dissolved in 100 ml PBS and store in 4^0c .

Sample Preparation:-

Sample of the extracts were prepared by dissolving 10 mg of extract in 20 µl of DMSO and volume make up to 10 ml with phosphate buffer saline (PBS), to get 1000µl/ml stock 100µl, 1000µl, and 5000µl, were taken and volume was made up 10ml (in 10ml vial capacity) with PBS final drugs conc. 10µg/ml, 100µg/ml, and 500µg/ml. Three replication were prepared for each level. Control vial were prepared by adding equal volume of 0.2% DMSO with PBS.

Standard drugs solution preparation:-

Sample of standard prepared by dissolving 10mg of 5-Fluorouracil in 20µl of DMSO and volume make up to 10 ml with Phosphate buffer saline (PBS),to get 1000µg/ml stock solution

From this stock 10µl was took and volume was made up to 10ml (in 10 ml vial capacity) with PBS Solution to get the final drug conc. 10µl/ml, 100µl was taken and Volume upto 10ml Given final drug conc. 100µg/ml, 500µl was taken and upto volume 10ml and final drug conc 500µg/ml.

Procedure:-

1. In the stock cell suspension cell count determination and cell were found 1.2×10^6 cell/ ml. From this stock cell suspension 0.1 ml of suspension was taken in micro wells.
2. Added only 0.1 ml of DMSO (20% v/v with PBS) and considered as negative control.
3. In the next wells, 0.1 ml of extract (extract A and extract) added at conc. 10 to 500µg/ml in respected micro well and 0.1 ml of 20µg/ml 5-FU added in respective well. 5-FU considered as positive control.
4. The cells were incubated at 37^0C and 5% CO_2 for 3hr in CO_2 incubator.
5. After the exposure 0.1 ml of trypan blue was added and mixed well.
6. The total number of living cells in the four corner squares of the chamber were counted
7. using heamocytometer and the percentage visibility/cytotoxicity was calculated.

$$\%Viability = \frac{\text{Viable cell count}}{\text{Total cell count}} \times 100$$

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2) MTT Assay Method:-

This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent (eg. isopropanol) and the released, solubilised formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells

Procedure:-

Pre incubate cells at a concentration of 1×1006 cells/ml in culture medium for 3h at 37^0C and 6.5% CO2. The cells were added at a concentration of 5×104 cells/well in 100µl culture medium and incubated at 37^oC in 5% CO2 incubator for 24hrs. After 24hours, when the monolayer formed, the supernatant was flicked off and added previously diluted with media of 100µl of different concentrations of test extract in microtitre plates and kept for incubation at 37^oC in5 % CO2 incubator for 72 hours, and cells were periodically checked for granularity, shrinkage, swelling. After 72 hours, the sample solution in wells was flicked off and 10 µl of MTT dye was added to each well. The plates were gently shaken and incubated for 4 hours at 37^oC in 5% CO2 incubator. The supernatant was removed and 100 µl of Isopropanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at 590nm with a reference filter of 620nm.

$$\%\text{Viability} = \frac{\text{Absorbance of Control} - \text{Absorbance of Test}}{\text{Absorbance of Control}} \times 100$$

MTT Assay:-

Treatment	**Dose µg/ml**	**%Mean cell viability (mean±SEM)**	
		Cell Line	
		HEp2	**OVCAR-3**
5-FU (Standerd)	10µg/ml	91.66±0.3203	94.32±0.1234
	100 µg/ml	80.98±1.233	85.26±0.234
	500µg/ml	91.06±0.508	80.09±0.096
Extract	10µg/ml	79.13±0.098	91.82±0.546
	100 µg/ml	66.78±.0.232	45.72±0.548
	500 µg/ml	85.90±0.566	53.93±0.089

Table :- 6 MTT Assay

p<0.01 Statistically Significant when compared with Standerd group by ANOVA followed by Dunnett test

Tryphan Blue exclusion assay:-

Treatment	**Dose µg/ml**	**% Mean cell Viability(mean ± SEM)**	
		Cell Line	
		HEp-2	**OVCAR-3**
5- FU (Standerd)	10 µg/ml	60.08±0.034	60.45±1.030
	100 µg/ml	70.45±1.034	70.34±1.413
	500 µg/ml	79.30±1.120	80.45±1.560
Extract	10 µg/ml	47.7±0.103	45.22±1.023
	100 µg/ml	52.77±0.685	52.77±0.0320
	500 µg/ml	63.27±0.543	57.55±0.0432

Table 7: Tryphan Blue exclusion assay

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p<0.01 Statistically Significant when compared with Standerd group by ANOVA followed by Dunnett test.

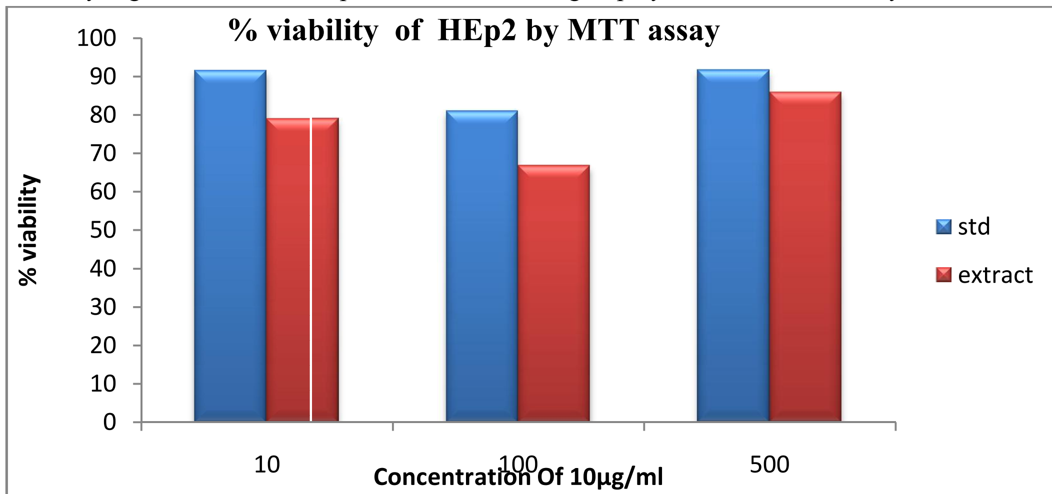

Fig no 3 :- Effect of cytotoxic activity on HEp-2 by MTT assay

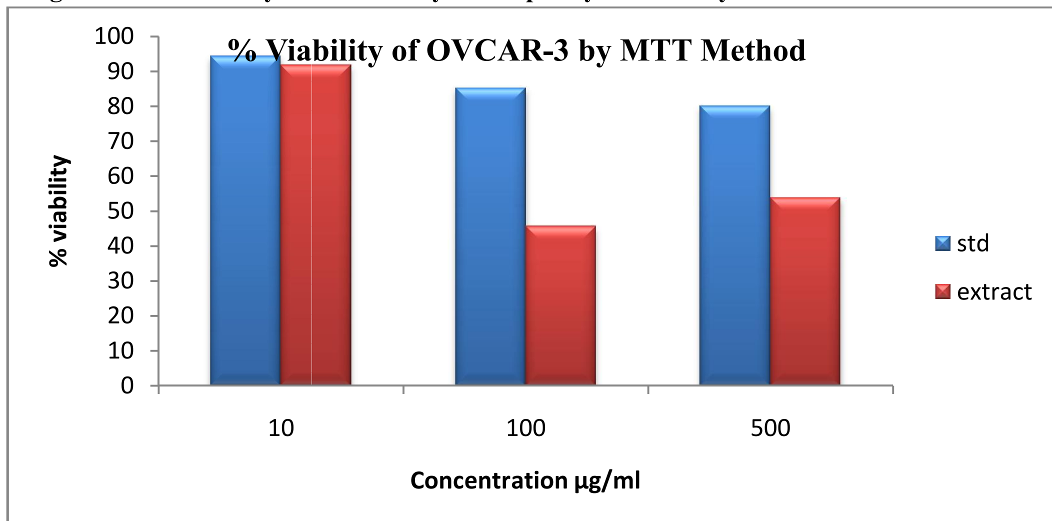

Fig no 4 :- Effect of cytotoxic activity on OVCAR-3 cell by MTT assay

These Identification of cytotoxic activity used two models MTT viability assay and Tryphan blue exclusion assay. Pet ether extract of *Cupressus sempervirens L.* study. In- vitro cytotoxic activity using MTT cell viability assay. The cell line used HEp2 and OVCAR-3. . 500 µg/ml conc. possess significant inhibition of cell as compared to other conc. in HEp-2 cell line and 10µg/ml conc of drug showed more inhibition of cell in OVCAR-3 cell line.

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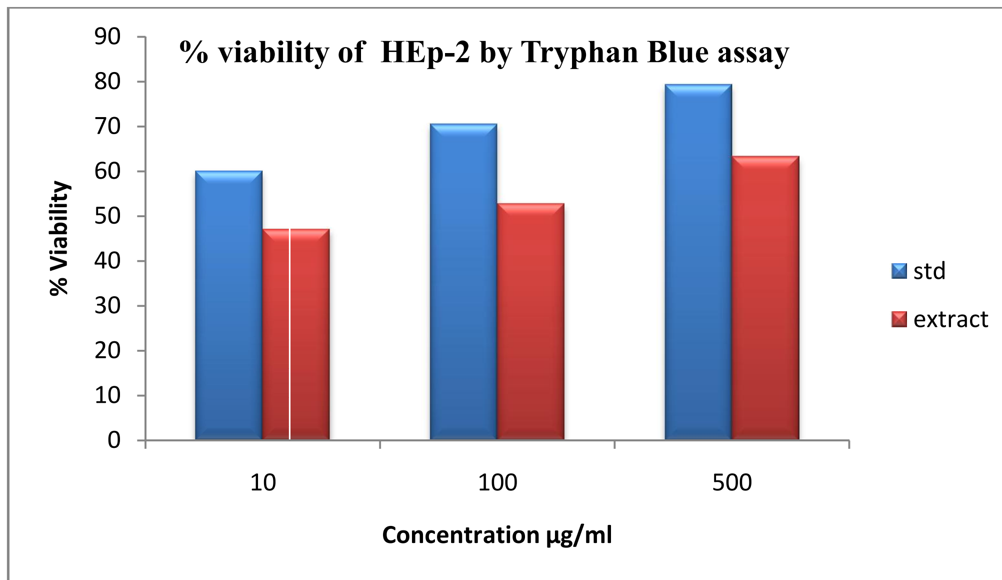

Fig no 5:- Effect of cytotoxic activity on HEp-2 by Tryphan blue assay

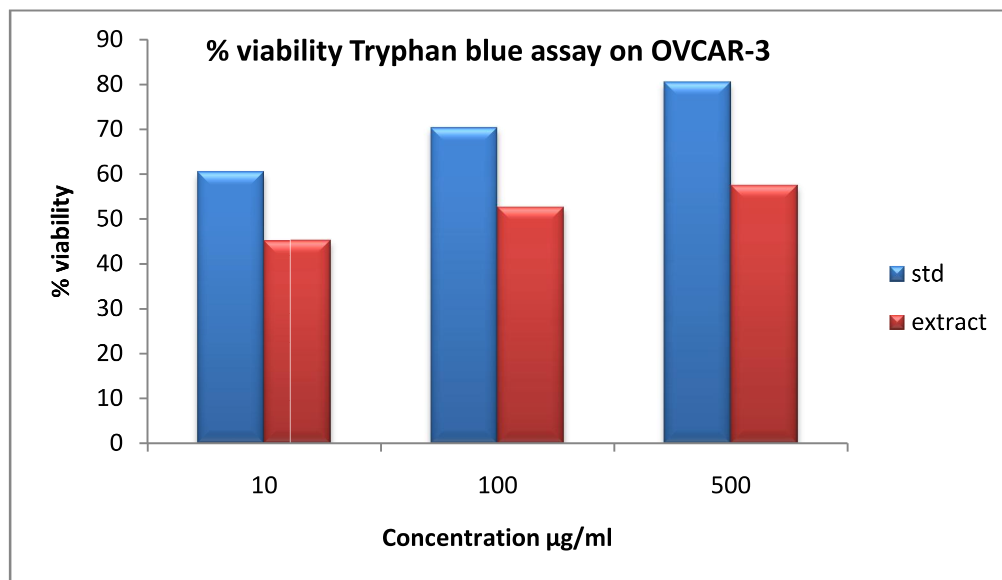

Fig no 6:- Effect cytotoxic activity on OVCAR-3 by Tryphan blue assay

In- vitro cytotoxic activity using Tryphan Blue exclusion assay. The cell line used HEp2 and OVCAR-3. Tryphan blue assay 500 µg/ml conc possess significant inhibition of cell as compared to other conc. in HEp-2 cell line and 500µg/ml conc of drug showed more inhibition of cell in OVCAR-3 cell line.

III. RESULT

The plant leaves extract showed better cytotoxic activity. Extract showed LC_{50} 228.31 using brine shrimp method.
The in-vitro cytotoxic activity HEp-2 and OVCAR -3 cell line used. These cell line showed better cytotoxic activity its showed 85% cell inhibition in MTT assay on HEp-2 cell and 95%cell inhibition on OVCAR-3. and second method used in Tryphan Blue method showed 40% cell inhibition in both cell line

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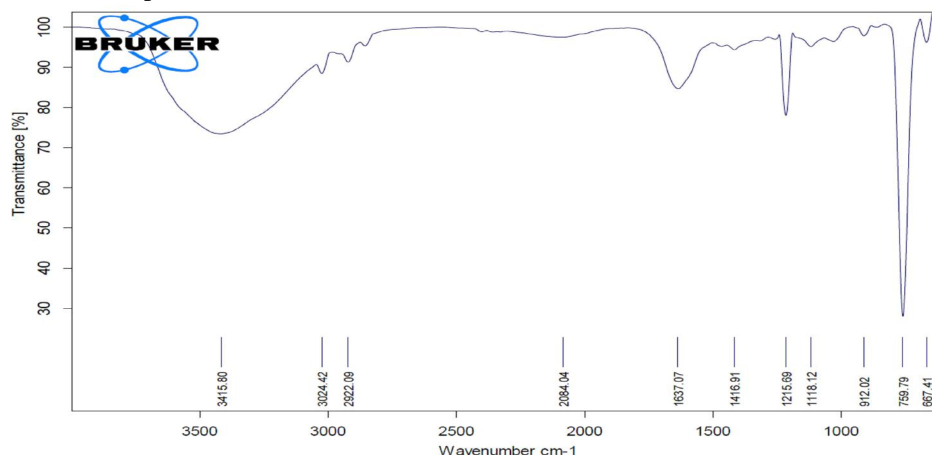
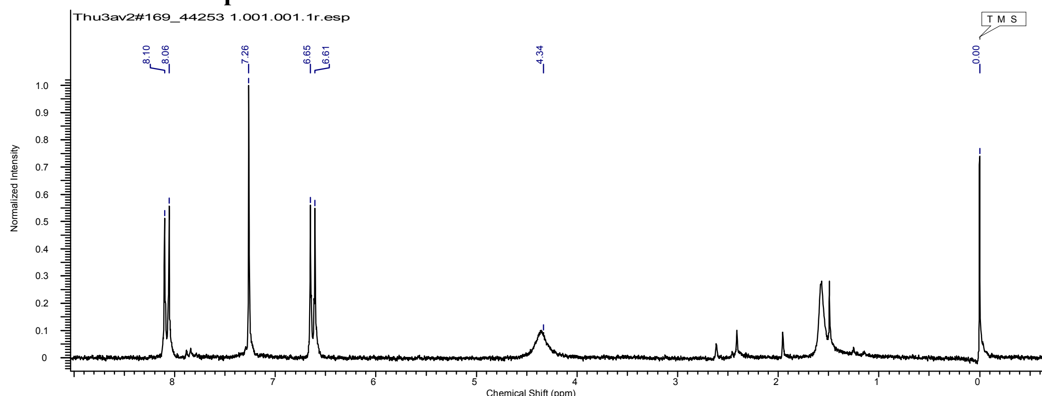
RESULT AND DISSCUSION

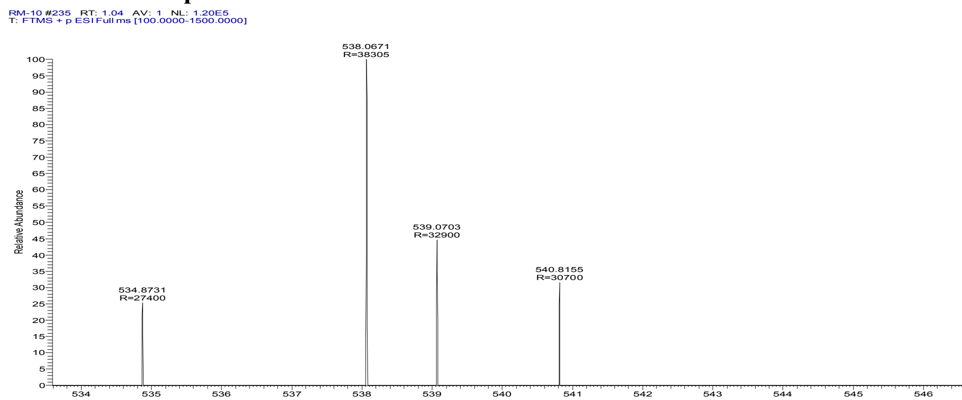
IR Spectra of Isolated Compound



NMR Spectra of isolated compound



Mass spectra of isolated compound



The IR spectra of compound shows absorption band of O-H str.at 3415.80,3024.42,2922. the C-H str.at 756.79, C=O str.at 1642.09 and C-O at 1215.69. All these absorption band shows presence of Flavonoid . The NMR spectra shows chemical shift at 6.65 shows presence of aromatic OH , also chemical shift at 8.18 shows presence of aromatic ring. The Mass spectra of obtained compound showed the molecular weight 538.46 and molecular ion peak 538.067. From these spectral analysis we were concluded that the resulting compound was Cupressuflavone which was Flavonoid in nature.

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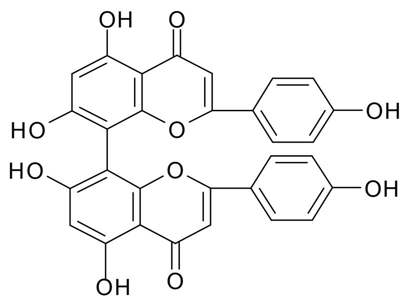

Cupressuflavone