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RESEARCH ARTICLE

ANTI-CANCER ACTIVITY IN HEPATOCELLULAR CARCINOMA CELL LINE HEPG2 FROM FOLKLORE PLANT

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ABSTRACT

Hepatocellular carcinoma (HCC) is a primary malignancy of the liver and occurs predominantly in patients with chronic liver disease and cirrhosis. A better knowledge of molecular Hepato carcinogenesis provides today the opportunity for targeted therapy. Most of the existing anti-cancer produces side effects such as fever, hallucinations, low blood pressure, bone marrow problems, paralysis, coma, liver problems, and kidney problems. The present study aimed**.** The herbal extract were prepared in solvents like ethanol and screened for ant cancer activity. 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 Spectro- photo metrically at 570nm. Viability of cell decreasing with increasing dose and 450 ug/ml of the extract shows 60% viability count and shows good anticancer activity compared to standard drug,

Aim: The present study was aimed to investigate Pharmacognostical, Phytochemical screening and Anti-cancer activity in hepatocellular carcinoma cell line HepG2 from folklore plant

Materials and Methods

Assay controls:

- Medium control (medium without cells)
- Negative control (medium with cells but without the experimental drug/compound)
- Control (medium with cells treated with a known drug, Metformin; 5uM)

$HepG_2$ 200 µl cell suspension without the test agent. Allow the cells to adhere to the culture plate for about 24 hours. Add test agent incubate 37°Cin a 5% CO_2 atmosphere. Add MTT reagent incubates for 3 hours. Then add solubilisation solution (DMSO) in an amount equal to the culture volume. 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. Read the absorbance on a spectrophotometer or an ELISA reader at 570nm.

Results and Conclusion: Folk plant extract after treated with $HepG_2$ cell Viability decreasing with increasing dose and 450 ug/ml of the extract shows 50% viability count and shows good anticancer activity compared to standard drug.

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INTRODUCTION

HCC is the most common primary liver cancer. The annual number of new cases of HCC worldwide is over one million, making it the 5th most common cancer worldwide (Hepatocellular Carcinoma, 2012). Hepatocellular carcinoma (HCC) is a primary malignancy of the liver and occurs predominantly in patients with underlying chronic liver disease and cirrhosis. The cell(s) of origin are believed to be the hepatic stem cells; Current evidence indicates that during Hepato carcinogenesis, two main pathogenic mechanisms prevail: (1) cirrhosis associated with hepatic regeneration after tissue damage caused by hepatitis infection, (2) mutations occurring in single or tumor suppressor genes. These pathways

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are of interest from a therapeutic perspective, because targeting them may help to reverse, delay or prevent tumor genesis (Gaetano Bertino *et al*., 2014). The stomata are anisocytic with three subsidiary cells around the guard cells in the lower epidermis, The Trichomes covering is unicellular with a conical bulbous to the epidermis (Yadav *et al*., 2010). Thin-walled parenchymatous cells were seen on powder microscopy. On an average, the vein islet number is 20–23, stomata index is 15–20 and palisade ratio is 7–8 in upper epidermis and 5–6 in lower epidermis (Zarena *et al*., 2014). To our knowledge, anticancer effect of folk plant Sesbania on HepG2 cell has not been explored. Herbal plant has been used for the cure of several human diseases and is gaining more attention due to less toxicity and high efficacy. Sesbania has been reported to be rich in flavonoids steroids, alkaloids, sugars, stigma sterols (The wealth of India Vol-iv; Wallis TE. 15th ed. London, 1985; Kokate *et al*., 2005).

MATERIALS AND METHODS

Chemicals and plant

Adjustable multichannel pipettes (Thermo Scientific, USA), MTT Reagent (5 mg/ml) (Himedia), DMSO (Himedia), D-PBS (Invitrogen), 96-well plate for culturing the cells (Corning, USA), 96-well ELISA plate reader or spectrophotometer capable of measuring the absorbance (Bio tek), 37°C incubator with humidified atmosphere of 5% CO_2 (Heal force), All used in Stellixir laboratory, Sesbania was obtained from plant farm and surroundings of the Raghavendra Institute of Pharmaceutical Education and Research (RIPER).

Plant extraction process

Plant material was collected and shade dried, powdered to get coarse powder. The powder was extracted with Ethanol at 60-70^{0}c by hot percolation using Soxhlet apparatus. Then extract was concentrated in the desiccator using anhydrous lime as dehydrating agent. Residue was stored at 4^{o}c until use (Somasekhar Reddy *et al*., 2015).

Preliminary Phyto-chemical screening

The chemical tests for various Phyto constituents in the ethanolic extract were carried out Alkaloids, Glycosides, Flavonoids, Carbohydrates, Tannins, Resins, Fixed oils, Terpenoids, proteins (Veerabhadrappa *et al*., 2015; Veerabhadrappa *et al*., 2014) Etc

Physico chemical parameters

Determination of foreign matter

Weigh 100 to 500gm of the drug sample to be examined or the minimum quantity prescribed in the monograph and spread it out in a thin layer. The foreign matter should be detected by inspection with unaided eye or by the use of lens (6 xs). Separate and weigh it and calculate the percentage present.

Determination of bulk density

A sample of about 50gm which is previously has been passed through sieve no: 20 introduced in a 100ml graduated cylinder. The cylinder is dropped at 2 sec intervals into a hard wooden surface 3 times from a height of 1 inch. The final bulk volume is determined and bulk density calculated.

Determination of total ash

2 to 3 gm powder weighs accurately and placed on crucible at temperature not exceeding 450^{0}c cool and weigh. If a carbon free ash cannot be obtained. Exhaust the charred mass with hot water, collect residue on an ash less filter paper. Add the filtrate, evaporate to dryness and ignite at a temperature not exceeding 450^{0}c. Calculate percentage of total ash.

Determination of acid insoluble ash

The ash obtained in above step is boiled for 5minutes with 25ml of dilute hydrochloric acid. Collect the insoluble matter in ash less filter paper, wash with hot water and ignite to constant weight. Calculate percentage of acid insoluble ash.

Extractive values

Powder was extracted in alcohol 500 ml by hot maceration method and their extractive values are determined as per the method given in Ayurveda pharmacopoeia of India.

Alcohol soluble extractive

Macerate 5gms of the coarsely powdered drug with 100ml of alcohol in a closed flask for 24hours, shaking frequently during 6hours and following to stand for 18hours. Filter rapidly taking precautions against loss of solvent, evaporate 25ml of the filtrate to dryness in a tare flat bottomed shallow dish and dry at 105^{0}to constant weight and weigh. Calculate percentage.

Water soluble extractive

Macerate 5gms of the coarsely powdered drug with 100ml of water and add 5ml of chloroform in a closed flask for 24hours, shaking frequently during 6hours and following to stand for 18 hours. Filter rapidly taking precautions against loss of solvent, evaporate 25ml of the filtrate to dryness in a tare flat bottomed shallow dish and dry at 105^{0}to constant weight and weigh. Calculate percentage.

Loss on drying

Place about 10gms of drug after accurately weighing. Evaporating dish dry at 105^0 for 5hours. Continue the drying and weighing at one hour interval until difference between 2 successive weighing corresponds to not more than 0.25%. Constant weight is reached when 2 consecutive weightings after drying for 30 minutes and cooling for 30 minutes in a desiccators, NMT 0.01g difference.

Foaming index

1g of drug weigh accurately and transfer to a 500ml conical flask containing 100ml of boiling water and maintain at moderate boiling for 30 minutes, cool and filter into a 100ml volumetric flask and add sufficient water through the filter to dilute the volume to 100ml. Place the above decoction into 10 stoppered test tubes (height 16cm and diameter 16mm) in a series of successive portions of 1,2,3,4 up to 10ml and adjust the volume of the liquid in each tube with water to 10ml.stopper the tubes and shake them in a length wise motion for 15 seconds, 2 frequencies per second. Allow to stand for 15 minutes. Note 1cm height of the foam and calculate foaming index (Veerabhadrappa *et al*., 2014).

Foaming index=1000/a [Tascilar *et al*., 2006]

Experimental design

Procedure for determining cell Cytotoxicity

1. Cell Seeding Seed 200µl cell suspension in a 96- well plate at required cell density (25,000 cells per well), without the test agent. Allow the cells to adhere to the culture plate for about 24 hours. Add appropriate concentrations of the test agent 2. Incubate the plate for required period at 37°Cin a 5% CO_2 atmosphere 3. After the incubation period, remove the plates from incubator and add MTT reagent to a final concentration of 10% of total volume. This volume should be same as the volume used while determining optimum cell density 4. Wrap the plate with aluminum foil to avoid exposure to light 5. 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.) 6. For adherent cells, aspirate the culture medium without disturbing the monolayer. Then add solubilisation solution (DMSO) in an amount equal to the culture volume 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.

Histological examination

Thin transverse section was taken observed under microscope after treated chloral hydrate with and HCL solution for histological processing. Section were stained with Phloroglucinal and glycerin before observed under microscope at x10 magnification.

RESULTS

Preliminary Phyto-chemical screening of herbal extracts revealed the presence of alkaloids, glycoside, proteins, Flavonoids. Macroscopic characters include blackish brown color, taste is acrid, and odor is aromatic. Presence of Microscopic characters like epidermis, large vessels, Starch grains, phloem fibres, stomata-anisocytic, and cluster calcium oxalate crystals (Trease and Evans, 1987), Phyto-chemical screening of extract shows the presence of flavonoids, sterols, stigma sterol, alkaloids, carbohydrates, saponin glycosides. Physico-chemical parameters includes Water soluble extract (10.34%), Alcohol soluble extract (6.0%), Loss on drying (8.9%), Total ash (0.5%), Acid insoluble ash (0,51%), foam index (4.29 mm), foreign matter (NMT 1%), swelling index, (Khandelwal 3rd edition, Harbone 3rd edition) values are showed in the Table (2).

Table 1. Preliminary photochemical screening of Sesbania

S. No.	Particulars	Alcohol extract
1.	Alkaloids	+
2.	Gum	+
3.	Carbohydrates	_
4.	Proteins	+
5.	Terpenoids	-
6.	Saponin	+
7.	Glycoside	+
8.	Fixed oil	+
9.	Flavonoids	+
10.	Amino acids	+

+ = present, - = absent

Physico chemical characteristics

Table 2. Physico chemical and their test values

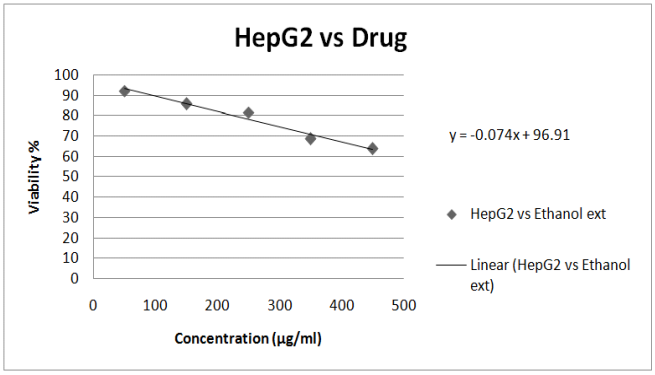
S. No.	Parameter	Lead value (percentage)
1.	Total ash	0.51
2.	Acid insoluble ash	0.15(%w/w)
3.	Alcohol soluble extract	5.76%
4.	Loss on drying	8.90gms
5.	Water soluble extract	10.34%
6.	Extraction weight	6gms
7.	Foreign matter	NMT 1%
8.	Bulk density	NMT 1%

Table 3. Foam index

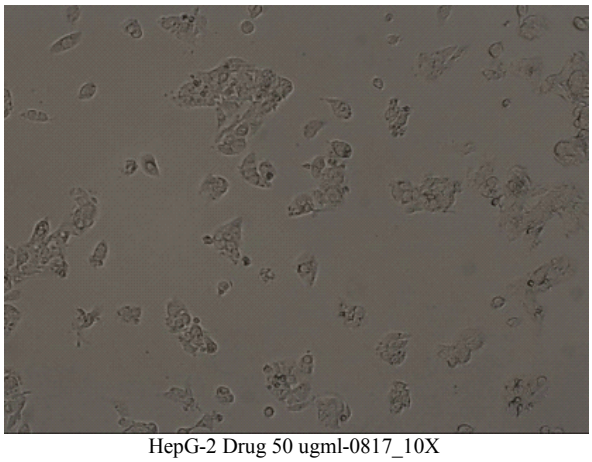
S. No.	1	2	3	4	5	6	7	8	9	9
Foam in mm	4	6	6.5	7	5	1.1	1.3	4	3.5	4.5

Table 4. Results anticancer activity IC50= 553.516 µg/ml

Concentration UNIT: µG/ML	Blank	Untreated	Camptothecin (60µM)	50	150	250	350	450
Test 1	0.004	0.744	0.436	0.610	0.520	0.512	0.450	0.432
Test 2	0.005	0.766	0.4485	0.744	0.746	0.732	0.588	0.566
Mean	0.0045	0.755	0.44225	0.697	0.648	0.616	0.519	0.483
Mean OD-Mean B	NA	0.7505	0.43775	0.6925	0.6435	0.6115	0.5145	0.4785
STANDARD DEVIATION		0.015556349	0.008838835	0.066468	0.138593	0.164049	0.097581	0.11738
STANDARD ERROR		0.011001661	0.006250944	0.047007	0.098015	0.116018	0.06901	0.083013
Viability %	NA	100	58.32778148	92.27182	85.74284	81.47901	68.5543	63.7575

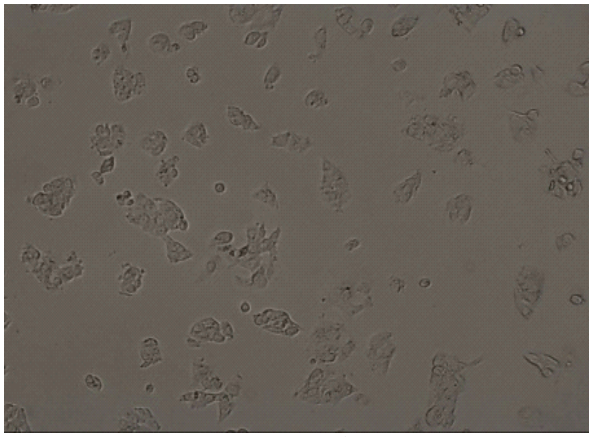


Graph 1.



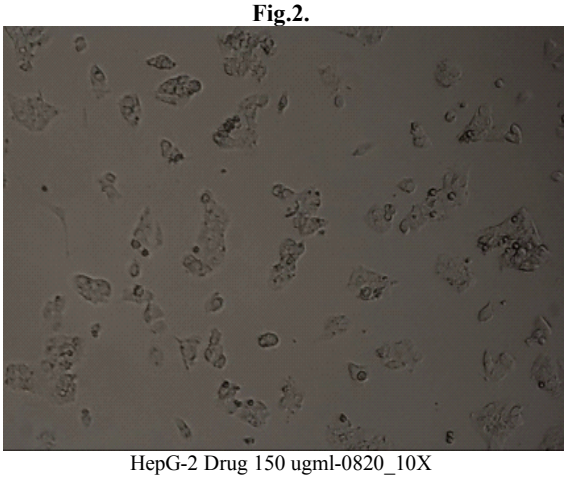
HepG-2 Drug 50 ugml-0817_10X

Fig.1.



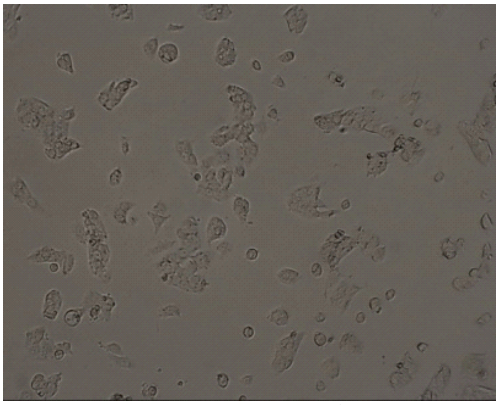
HepG-2 Drug 100 ugml-0818_10X

Fig.2.



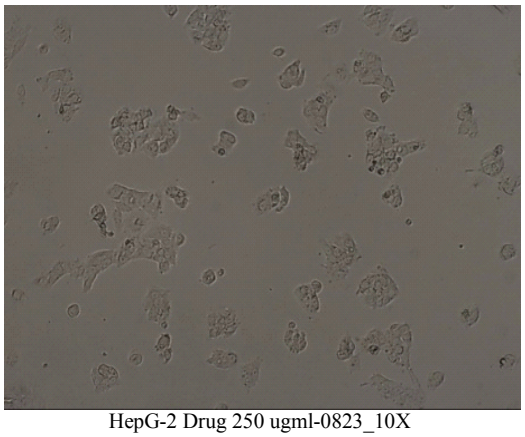
HepG-2 Drug 150 ugml-0820_10X

Fig.3.



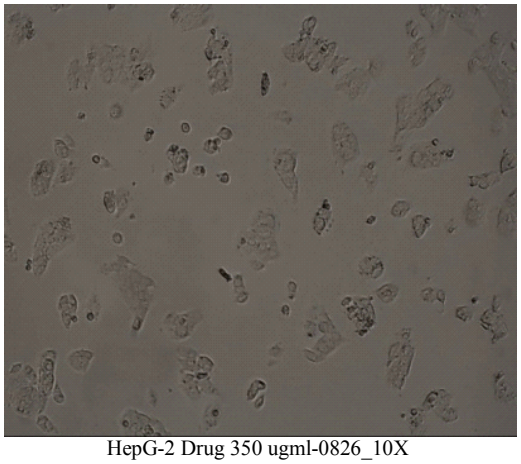
10XHepG-2 Drug 150 ugml-0820

Fig.4.



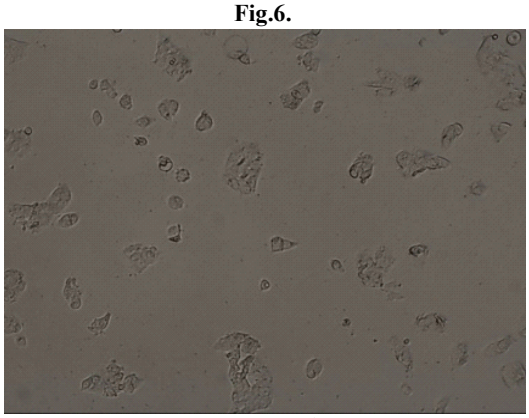
HepG-2 Drug 250 ugml-0823_10X

Fig.5.



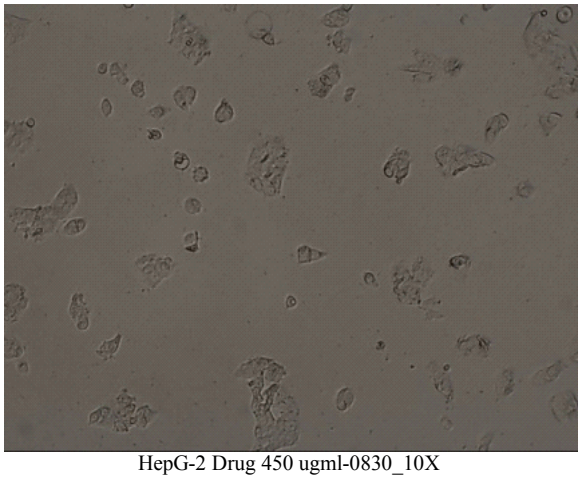
HepG-2 Drug 350 ugml-0826_10X

Fig.6.



HepG-2 Drug 450 ugml-0830_10X

Fig.7.



HepG-2 Drug 450 ugml-0830_10X

Fig.8

In view to the current status anticancer activity research approach in search of new anti-cancer drug, the literature survey reveal the toxicity of chemical agent, hence the work has been directed towards the herbal and Phyto medicine as choice for cancer (Tascilar *et al*., 2006), By seeing graph we can notice that the viability of cells are decreasing by increasing in concentration of dose different concentration like 100,150,250, 350,450 ugml extract shows viability 90, 80, 85, 70, 55(%) respectively. Viability of cell decreasing with increasing dose maximum at 450ugml shows 60% viability count and shows good anticancer activity compared to standard drug, and extract contain Phyto-constituents for anticancer, it was observed that the crude extract may contain alkaloid showed significant effect of anti-cancer activity, Further need of research.

DISCUSSION

Macroscopic characters include blackish brown color, taste is acrid, and odor is aromatic. Presence of powder microscopic characters like epidermis, large vessels, Starch grains, phloem fibers, stomata-anisocytic, and cluster calcium oxalate crystals (Trease and Evans, 1987; Ashok Kumar, 3rd edition). Phyto-chemical screening of extract shows the presence of flavonoids, sterols, stigma sterol, alkaloids, carbohydrates, saponin glycosides. Physico-chemical parameters includes water soluble extract, alcohol soluble extract, loss on drying, Total ash, acid insoluble ash, foam index, foreign matter, swelling index, LOD (Khandelwal, 3rd edition; Harbone 3rd edition) values are showed in the table (2). In view to the current status anticancer activity research approach in search of new anti-cancer drug, the literature survey reveal the toxicity of chemical agent, hence the work has been directed towards the herbal and Phyto medicine as choice for cancer (Tascilar *et al*., 2006; Josep M. Llovet and Jordi Bruix, 2003; Anticancer Activity of *Nigella sativa* (Black Seed) 2011). By seeing graph we can notice that the viability of cells are decreasing by increasing in concentration of dose different concentration like 100,150,250, 350,450 ugml extract shows viability 90, 80, 85, 70, 55(%) respectively. Viability of cell decreasing with increasing dose maximum at 450 ugml shows 60% viability count and shows good anticancer activity compared to standard drug, and extract contain Phyto- constituents for anticancer, it was observed that the crude extract may contain alkaloid showed significant effect of anti-cancer activity, Further need of research.

Conclusion

From the present study it was concluded, that herbal extract exhibit anticancer activity, which is proven experimentally. Viability count decreases with increasing dose dependent manner anticancer activity by confirming by assay method, viability of cell decreasing up to 50 % at 450 ugml of dose. Compared to standard drug extract exhibits similar activity, always herbal extracts gaining more attention due to less toxicity and high efficacy

Conflict of interest

The authors declare that have no conflict of interest

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