IN VITRO HEPATOPROTECTIVE EFFECT OF ETHANOLIC EXTRACT OF *SESBANIA GRANDIFLORA* AGAINST HEPATOTOXICITY INDUCED IN HEPG2 Cell Lines

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ABSTRACT

Background: Sesbania grandiflora (Fabaceae) has been shown to be useful in the treatment of liver disorders and to possess other activities. Other plants such as Mangifera indica, Indigofera tinctoria, Vigno mungo are also used in the treatment of liver disorders
Aim of the study: The purpose of this investigation was to see if ethanolic extract had any hepatoprotective properties. The In vitro hepatoprotective activity of ethanolic extract of Sesbania grandiflora (Fabaceae) by antitubercular drugs inducing method in Hep G2 cell lines. To estimate MTT Assay. To estimate CAT, SOD, GSH
Materials: The hepatoprotective effects were evaluated by antitubercular drugs inducing method in Hep G2 cell lines. The plant material used for the extraction is properly authenticated and identified. The stem of Sesbania grandiflora was collected, washed, dried in shade and pulverized in a grinder- mixer to obtain a coarse powder and then passed through 40 mesh sieves. The powdered drug was subjected to solvent extraction by soxhlet apparatus.
Results: The preliminary phytochemical screening of the ethanolic extract of stem of Sesbania grandiflora revealed the presence of alkaloids, phytosterols, phenolics, terpenoids, tannins and flavonoids.The MTT assay results suggest that the test compound, extract showing moderate cytotoxicity against the Human Liver cancer cells at the 200ug/ml concentration with IC50 value of 190.28ug/ml and remaining concentrations were non toxic in nature.Combination of LOLA and Extract also showed similar results with 11.67 DCF expression. Untreated cells expressed DCF intensity with relative mean fluorescence intensity of 9.25 whereas Ethanol alone and Ethanol conjugated extract showed 93.93 and 20.51 DCF expression.

Keywords: sesbania grandiflora species, Hep G2 cells, LOLA, Hepatotoxicity, antitubercular drugs

INTRODUCTION

Except for Allopathy, herbal medications account up a large portion of all officially recognised health systems in India, including Ayurveda, Yoga, Unani, Sidda, Homeopathy, and Naturopathy.According to the Indian Pharmaceuticals Act, there is currently no special category for herbal drugs or dietary supplements. However, there is a vast experimental-evidence base for many of the natural drugs[1].. Significant basic and clinical research has been carried out on the medicinal plants and their formulations, with the state-of-the-art methods in a number of institutes/universities[2.]

Sesbania grandiflora (Fabaceae) has been shown to be useful in the treatment of liver disorders and to possess other activities. Other plants such as *Mangifera indica, Indigofera tinctoria, Vigno mungo* are also used in the treatment of liver disorders[3].

Fig.1 *Sesbania grandiflora*

The different chemical constituents present in the plant *Sesbania grandiflora* revealed the presence of Alkaloids,Phytosterols,Phenolics,Terpenoids ,Tannins and Flavonoids[4.]

The liver is the second largest organ of the human body after skin and is the largest internal organ, weighs approximately 1500g, and is located in the upper right corner of the abdomen between the organs of the gastrointestinal tract and the heart[5]. Its upper and anterior surfaces are smooth and curved to fit the under surface of the diaphragm; its posterior surface is irregular in outline[6].

Plan of work : Identification & authentification of plant, Drying & powdering, Extraction, Phytochemical Analysis, Toxicology studies, In vitro Hepatoprotective Activity- Evaluation parameters -*Invitro* Anti-oxidant studies, MTT Assay, CAT, SOD, GS

Plant profile: *Sesbania grandiflora*, commonly known as vegetable hummingbird, West Indian pea, agati, or katurai , Avisha, is a small tree in the genus *Sesbania* in the legume family. It has edible flowers and leaves commonly eaten in the southeast Asia and south asia. The bark is used as astringent to cure smallpox. In Philippines, the decoction from the crushed bark is used for the treatment of ulcers in the mouth and alimentary canal[7].

Common name: Sesban, Vegetable Hummingbird, Red Wisteria

Description:

It is a fast-growing tree. The leaves are regular and rounded and the flowers white, red or pink. The fruits look like flat, long, thin green beans. The tree thrives under full exposure to sunshine and is extremely frost sensitive. The flowers are white and 7 to 9 centimeters long. The pods are linear, 20 to 60 centimeters long, 7 to 8 millimeters wide, pendulous, and somewhat curved, and contain many seeds[8].

Identification and Authentication of plant material:

The *Sesbania grandiflora* (*Fabaceae)* stem were identified and authentified by Dr. Madhava chetty, Dept of botany, S.V. University, Tirupati, chittoor (dist).

Ethanolic extraction of *sesbania grandiflora* stem:

The commonly employed technique for the separation of the active constituents from the crude drug is called extraction which involves the use of different solvents. Many of the complex substances metabolized by the plants have therapeutic importance. But these are always found in association with other substances. Therefore in order to study these active constituents alone it has to be separated from other unwanted substances produced[9].

Preparation of extract:

The stem of *Sesbania grandiflora* was collected, washed, dried in shade and pulverized in a grinder- mixer to obtain a coarse powder and then passed through 40 mesh sieves. The powdered drug was subjected to solvent extraction by soxhlet apparatus[10].

Fig.2: Soxhlet apparatus

Procedure:

About 300g of powdered drug was extracted successively with ethanol using soxhlet apparatus. The extraction was carried out for 72 hours until the extract becomes colourless. Then the solvent was completely removed by evaporating in rotatory flask evaporator[11]. The dried extract thus obtained was kept in desicator and was used for further experiment.

Percentage yield:

- Percentage yield of ethanolic extract of stem of *Sesbania grandiflora* was found to be 8.98% w/w

MATERIALS AND METHODS

Preliminary phytochemical screening:

The Ethanolic extract of stem of *Sesbania grandiflora (Fabaceae)* was subjected to preliminary phytochemical screening for the detection of various phytochemical constituents such as carbohydrates, glycosides, alkaloids, saponins, phenolic compounds, gums, mucilages and proteins. The detailed study about the phytochemical test procedure as follows[13].

MTT Assay:

MTT assay is a colorimetric assay used for the determination of cell proliferation and cytotoxicity, based on reduction of the yellow coloured 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 colour, the intensity of which is proportional to the number of viable cells and can be measured spectrophotometric ally at 570nm[14].

ROS Study:

Reactive oxygen species (ROS) are molecules containing hydroxyl radicals or peroxides with unpaired electrons. In healthy aerobic cells, ROS are produced naturally as a biproduct of oxidative phosphorylation, oxidoreductase enzymes, or metal catalysed oxidation at a controlled rate[15]. However, ROS can be induced under some stress conditions especially exposure to environmental oxidants and certain drugs that leads to oxidative stress. Exceed ROS can cause damages in the building blocks of cells including DNA, proteins, and lipids, and eventually results in cell death. Cell-permeant 2', 7'-dichlorodihydrofluorescein diacetate (H2DCFDA) is a widely used ROS indicator. The reduced non-fluorescent fluorescein H2DCFDA can be oxidized and converted into fluorescent 2’, 7’-dichlorofluorescein (DCF) by intracellular ROS. In this protocol, we applied H2DCFDA to label the intracellular ROS and detected the DCF intensity by flow cytometry.

Sl. No.	Sample Name	Concentration ug/ml
1	Extract	100ug/ml
2	Ethanol	100Mm
3	LOLA	0.1Ug
4	Ethanol+Extract	100mM+100uG
5	Standard+Extract	0.1uG+100uG

Tab 1: Details of sample used for the study

Catalase assay:

Catalase Activity Assay is a highly sensitive, simple and direct assay for measuring catalase activity in a variety of biological samples such as cell and tissue lysates or biological fluids. In this assay, the catalase present in the sample reacts with hydrogen peroxide (H2O2) to produce water and oxygen. The unconverted H2O2 reacts with probe to produce a product that can be measured colorimetrically at OD 570 nm or fluorometrically at Ex/Em = 535/587 nm.

Fig.3: Principle of Catalase activity study

$$CAT\ (Unit) = [(Corrected\ Absorbance - \frac{(y - intercept))}{Slope}]$$

Unit definition: 1 Unit Catalase activity = amount of catalase that will decompose 1.0 µmol of H2O2 per minute at pH 4.5 at 25°C.

SOD Study:

Superoxide Dismutase (SOD) catalyses the dismutation of the superoxide radical (O2-) into hydrogen peroxide (H2O2) and elemental oxygen (O2) (Figure 1) and as such provides an important defense against the toxicity of the superoxide radical. In fact, overexpression of SOD protects murine fibrosarcoma cells from apoptosis and promotes cell differentiation (1). SOD also inhibits adriamycin-induced apoptosis in murine peritoneal macrophages (2). In the assay, The assay is free of interference by other catalytic activities and is ideal for assaying SOD in mammalian cell lysates. Each assay requires approximately 5 minutes and after a simple calculation, the percent inhibition of the formation of NBT-diformazan by SOD is converted to the relative activity of the sample.

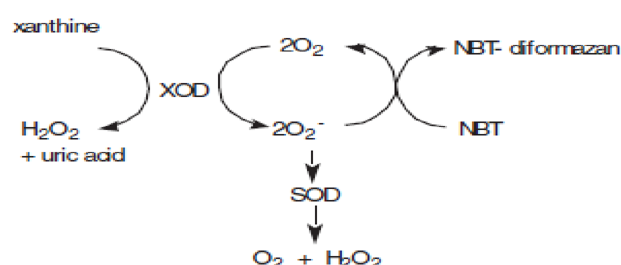


Fig.4: Relationship between XOD, SOD, substrates, products, and the superoxide radical.

Glutathione study:

Glutathione is a key intracellular tripeptide thiol composed of glutamic acid, cysteine, and glycine. Glutathione helps protect cells from free radical damage by acting as an antioxidant. Within cells, glutathione exists in reduced (GSH) and oxidized (GSSG) states. In healthy cells and tissue, more than 90% of the total glutathione pool is in the reduced form (GSH) while less than 10% exists in the disulfide form (GSSG)

RESULTS

The preliminary phytochemical screening of the ethanolic extract of stem of *Sesbania grandiflora* revealed the presence of alkaloids, phytosterols, phenolics, terpenoids, tannins and flavonoids.

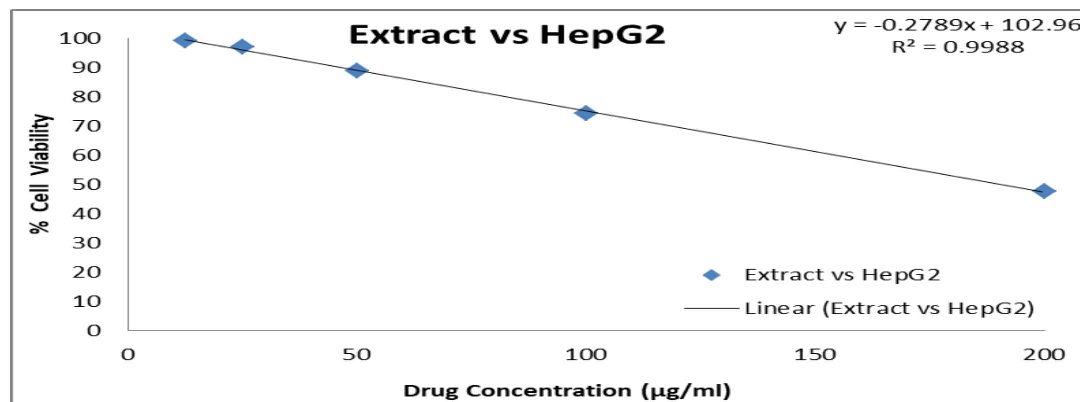


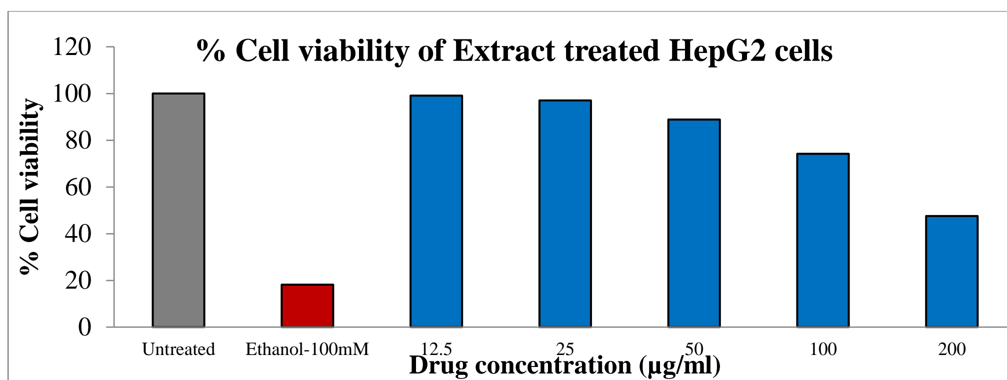
Fig 5: MTT Assay

Tab:2: Different types of concentration and its % Cell Viability

Parameter	**Blank**	**Untreated**	**Ethanol-100Mm**	**12.5**	**25**	**50**	**100**	**200**
Absorbance reading 1	0.025	0.857	0.174	0.849	0.827	0.765	0.648	0.424
Absorbance reading 2	0.031	0.85	0.182	0.843	0.831	0.758	0.633	0.417
Mean abs	0.028	0.8535	0.178	0.846	0.829	0.7615	0.6405	0.4205
Mean Abs (Test-Blank)		0.8255	0.15	0.818	0.801	0.7335	0.6125	0.3925
Std Deviation	0.004243	0.00494975	0.005656854	0.004243	0.002828	0.00495	0.010607	0.00495
Std Error	0.003	0.0035	0.004	0.003	0.002	0.0035	0.0075	0.0035
% Cell Viability	**0**	**100**	**18.17080557**	**99.09146**	**97.0321**	**88.85524**	**74.19746**	**47.54694**

S. NO	**Concentration (ug/ml)**	**TEST PARAMETER-MTT**
		% Cell viability
1	Untreated	100
2	Ethanol-100Mm	18.17
3	12.5	99.09
4	25	97.03
5	50	88.85
6	100	74.19
7	200	47.54

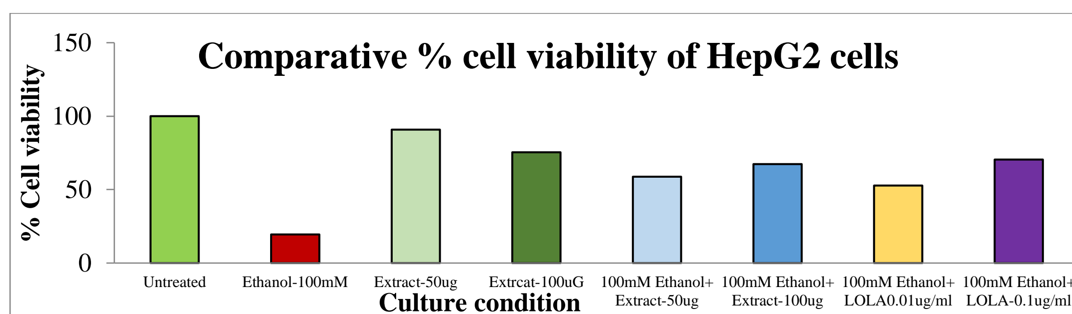
Table 3: Table showing the % cell viability of the Test compound, Extract treated with HepG2 cell lines with different concentrations after the incubation period of 24hrs.



Graph 1: Overlaid bar graph depicting the % cell viability of Extract treated HepG2cell lines by MTT study

S. NO	Culture condition	TEST PARAMETER-MTT
		% Cell viability
1	Untreated	100
2	Ethanol-100mM	19.47
3	Extract-50uG	90.82
4	Extract-100uG	75.4
5	100mM Ethanol+ Extract-50ug	58.78
6	100mM Ethanol+ Extract-100ug	67.36
7	100mM Ethanol+ LOLA 0.01ug/ml	52.76
8	100mM Ethanol+ LOLA 0.1ug/ml	70.45

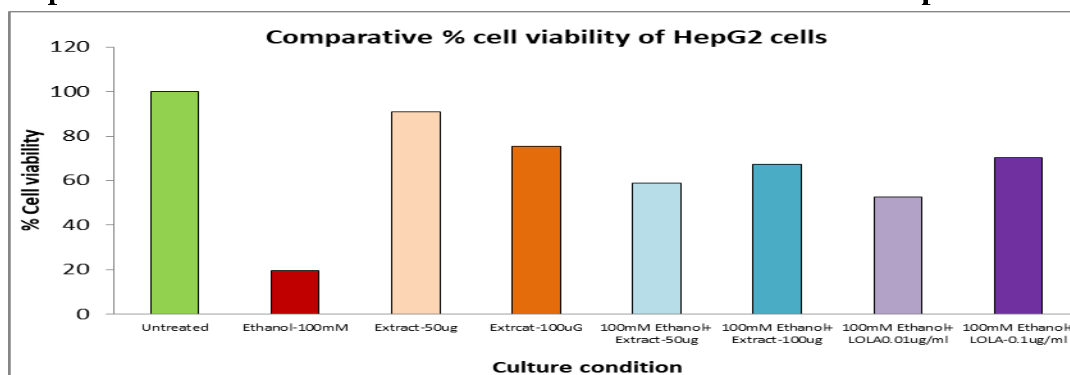
Table 4: Table showing the % cell viability of the HepG2 cell lines treated with various combinations of extract and LOLA conjugated with the ethanol after the incubation period of 24hrs.



Graph 2: Overlaid bar graph depicting the % cell viability of extract and LOLA treated HepG2cell lines which are pre-stimulated with Ethanol-100mM concentration by MTT study.

Group-I	Group-II	Group-III		Group-IV		Group-V	
Untreated	Ethanol-100mM	Extract-50ug	Extrcat-100Ug	100mM Ethanol + Extract-50ug	100mM Ethanol + Extract-100ug	100mM Ethanol+ LOLA0.01 ug/ml	100mM Ethanol + LOLA-0.1ug/ml
0.864	0.204	0.793	0.671	0.518	0.605	0.487	0.631
0.885	0.193	0.802	0.665	0.539	0.596	0.469	0.622
0.8745	0.1985	0.7975	0.668	0.5285	0.6005	0.478	0.6265
0.8395	0.1635	0.7625	0.633	0.4935	0.5655	0.443	0.5915
0.01484924	0.007778175	0.006363961	0.004242641	0.014849242	0.006363961	0.012727922	0.006363961
0.0105	0.0055	0.0045	0.003	0.0105	0.0045	0.009	0.0045
100	**19.4758785**	**90.82787373**	**75.40202501**	**58.78499107**	**67.36152472**	**52.76950566**	**70.45860631**

Table 5: Table showing the % cell viability of the Test compound, Extract treated with HepG2 cell lines with different concentrations after the incubation period of 24hrs.



Graph 3: Overlaid bar graph depicting the % cell viability of extract and LOLA treated HepG2cell lines which are pre-stimulated with Ethanol-100mM concentration by MTT study

S. NO	Culture condition	TEST PARAMETER-MTT
		% Cell viability
1	Untreated	100
2	Ethanol-100mM	19.47
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5	100mM Ethanol+ Extract-50ug	58.78
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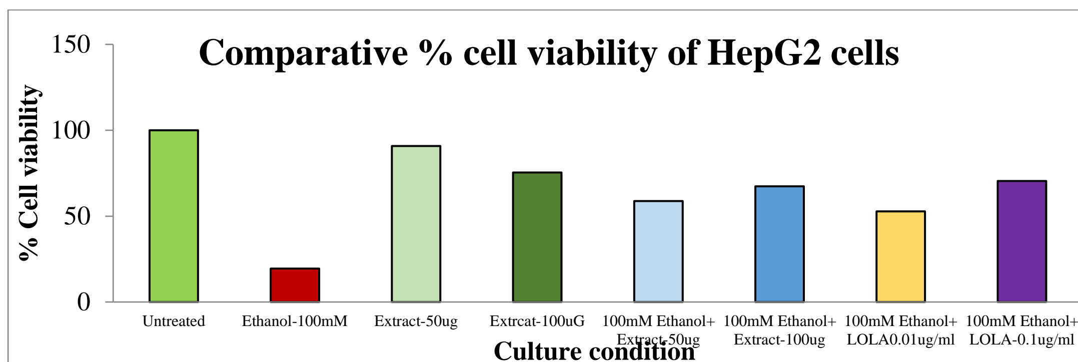
7	100mM Ethanol+ LOLA 0.01ug/ml	52.76
8	100mM Ethanol+ LOLA 0.1ug/ml	70.45

Table 6: Table showing the % cell viability of the HepG2 cell lines treated with various combinations of extract and LOLA conjugated with the ethanol after the incubation period of 24hrs.

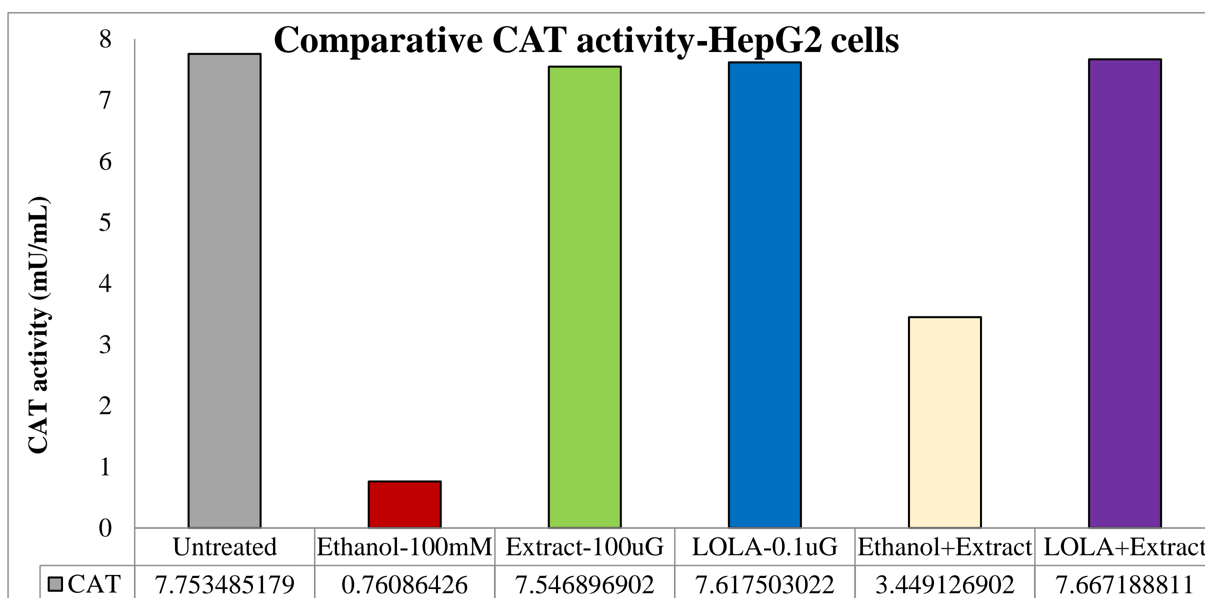


Graph 4: Overlaid bar graph depicting the % cell viability of extract and LOLA treated HepG2cell lines which are pre-stimulated with Ethanol-100mM concentration by MTT study.

Catalase results:

Culture condition	CAT activity (mU/mL)
Untreated	7.75
Ethanol-100mM	0.76
Extract-100uG	7.54
LOLA-0.1uG	7.61
Ethanol+Extract-100uG	3.44
LOLA+Extract-100uG	7.66

Table 7: The Catalase activity observed in the Untreated, Ethanol alone, Extract alone, LOLA alone and combination of ethanol with Extract and LOLA+extract treated HEPG2 pelleted cells and the results as depicted below

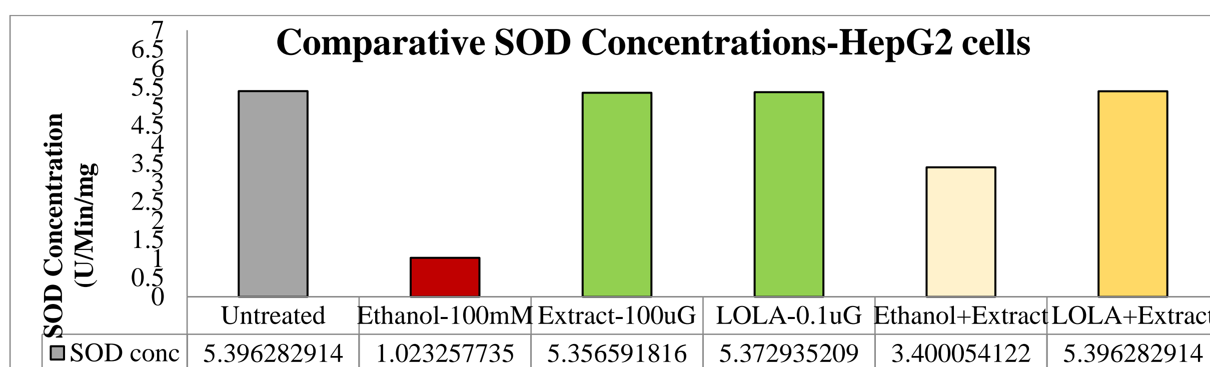


Graph 5: Comparative CAT activity HepG2 cells

Superoxide disulphide results:

Culture condition	SOD Concentration (Unit/Min/mg)
Untreated	*5.39*
Ethanol-100mM	*1.02*
Extract-100uG	*5.35*
LOLA-0.1uG	*5.37*
Ethanol+Extract-100uG	*3.40*
LOLA+Extract-100uG	*5.39*

Table 8: The concentrations of SOD observed in the Untreated, Ethanol alone, Extract alone, LOLA alone and combination of ethanol with Extract and LOLA+extract treated HEPG2 pelleted cells and the results as depicted below



Graph 6: Comparative SOD Concentrations HepG2 cells Note:

1) The Observed absorbance Readings with calculations were enclosed in the separate folder of the report in MS Excel format.

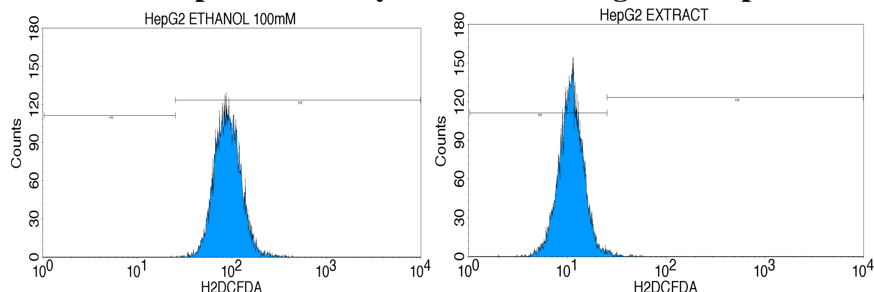
GSH results:

Sample	GSH concentration (uM)
Untreated	0.496
Ethanol-100mM	0.066
Extract-100uG	0.484
LOLA-0.1uG	0.493
Ethanol+Extract-100uG	0.361
LOLA+Extract-100uG	0.494

Table 9: The GSH concentration observed in the Untreated, Ethanol alone, extract alone, LOLA alone and combination of ethanol with Extract and LOLA+extract treated HEPG2 pelleted cells and the results as depicted below

OBSERVATIONS:

1) **H2DCFDA expression study of the Extract against HepG2 Cell line:**



Graph 7: B-Ethanol-100mM/ml **Graph 8:C-Extract-100uG/ml**

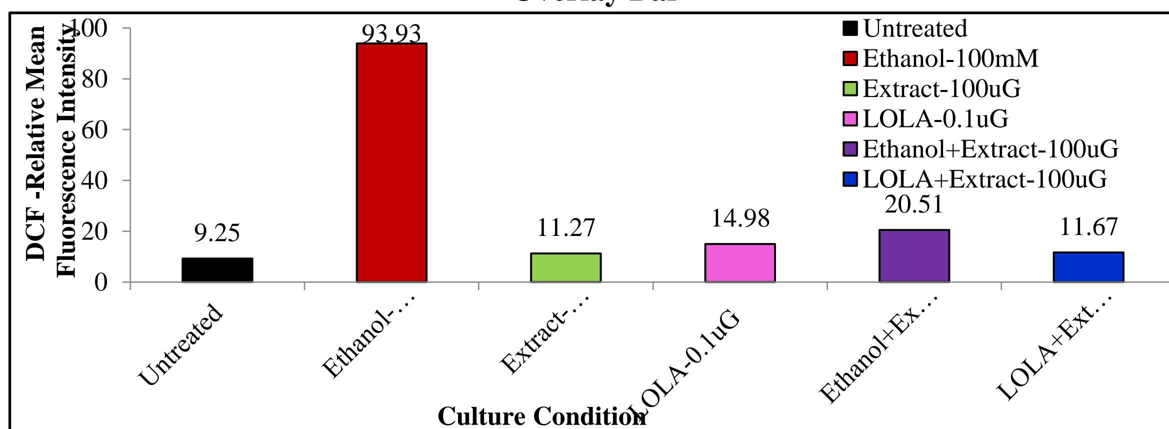
H2DCFDA expression study of the Extract against HepG2 Cell line:

Culture condition	DCF Relative Mean Fluorescence Intensity
Untreated	9.25
Ethanol-100Mm	93.93
Extract-100uG/ml	11.27
LOLA-0.1uG /ml	14.98
Ethanol+Extract-100uG	20.51
LOLA+Extract-100uG	11.67

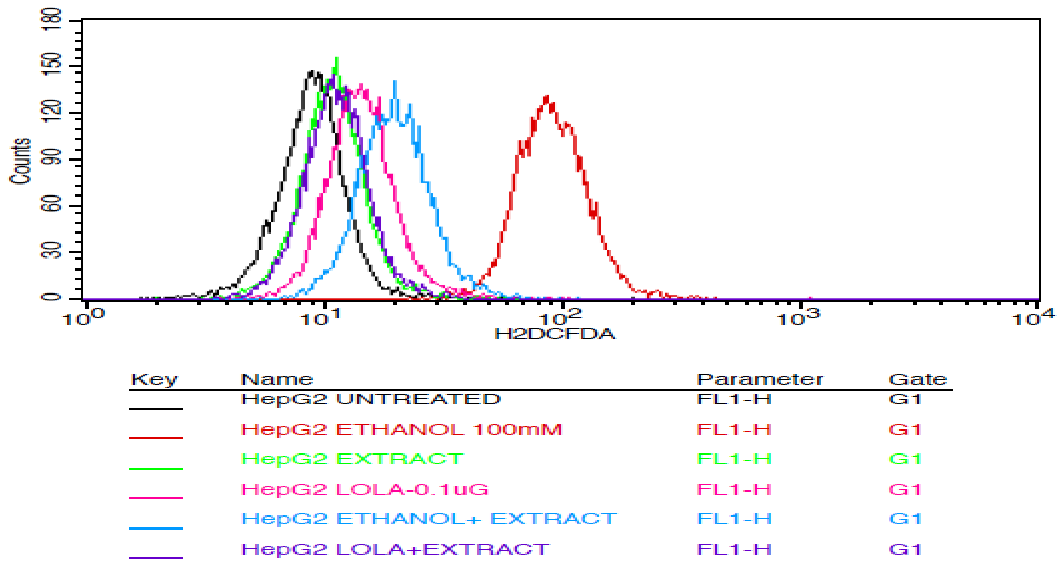
Table 10: Table showing the Relative Mean Fluorescence Intensity of DCF against the Untreated, Ethanol alone, Extract alone, LOLA alone and Combination of Ethanol with Extract and LOLA with extract treated HepG2 cells and overlay of the results plotted in Bar graph as below.

Plant extract significantly suppressed the DCF Mean Fluorescence Intensity in Ethanol stimulated model and confirmed the Hepato-protective potency of extract. Untreated cells expressed DCF intensity with relative mean fluorescence intensity of 9.25 whereas Ethanol alone and Ethanol conjugated extract showed 93.93 and 20.51 DCF expression. Combination of LOLA and Extract also showed similar results with 11.67 DCF expression. Overall observations strongly suggesting us that the extract significantly suppressed the DCF expression in Ethanol induced Liver cells (HepG2) and confirmed the significant hepato-protective potential nature in HepG2 cell lines.

Overlay Bar



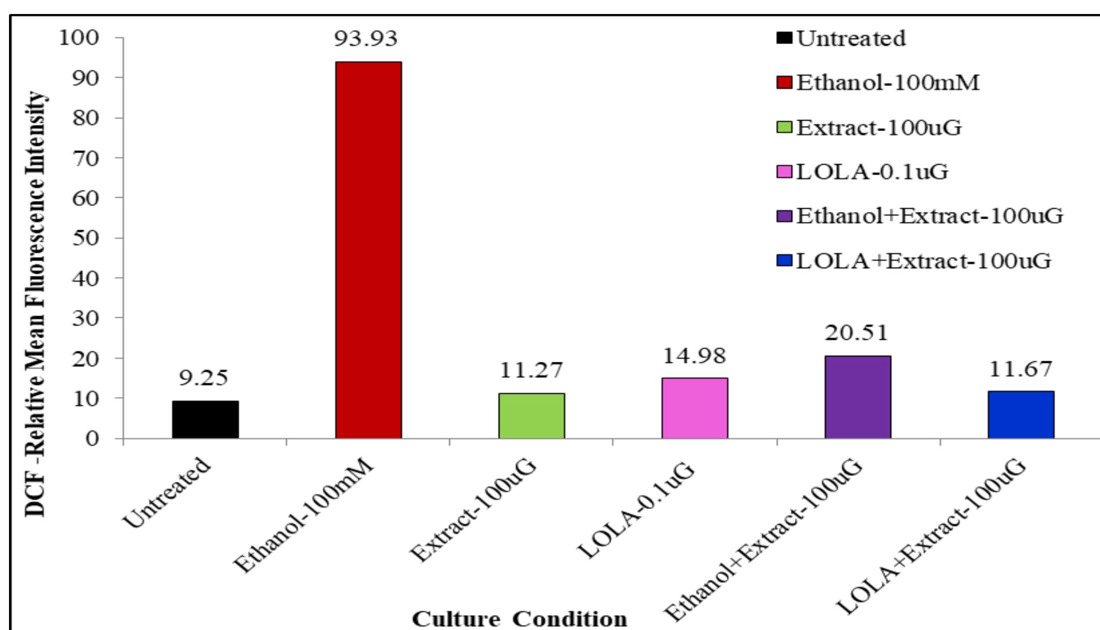
Graph 10: r Graph



Graph 11: Overlaid histograms representing the comparative DCF expression in HepG2 cell lines with different culture conditions.

Table 11: DCF relative mean fluorescence intensity

Culture condition	DCF Relative Mean Fluorescence Intensity
Untreated	9.25
Ethanol-100mM	93.93
Extract-100uG	11.27
LOLA-0.1uG	14.98
Ethanol+Extract-100uG	20.51
LOLA+Extract-100uG	11.67



Graph 12: DCF -Relative mean fluorescence intensity

ROS STUDY

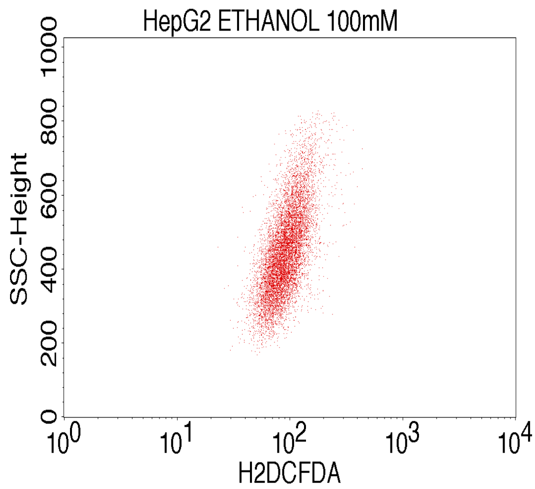


Fig.6 HepG2-ethanol 100mM

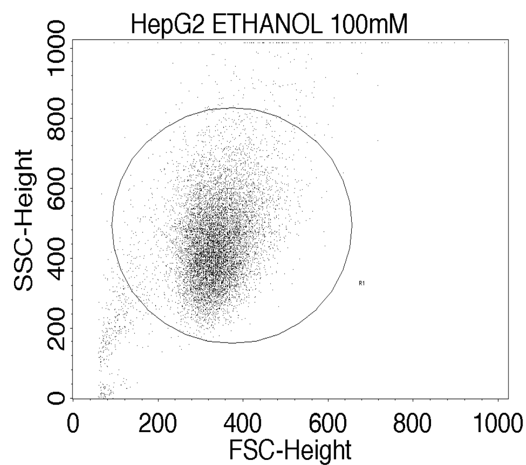


Fig.7 HepG2-ethanol 100mM 2

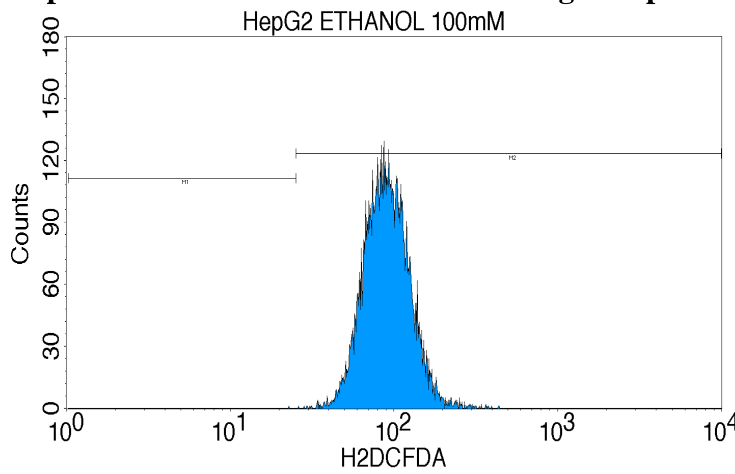


Fig.8 HepG2-ethanol 100mM 3

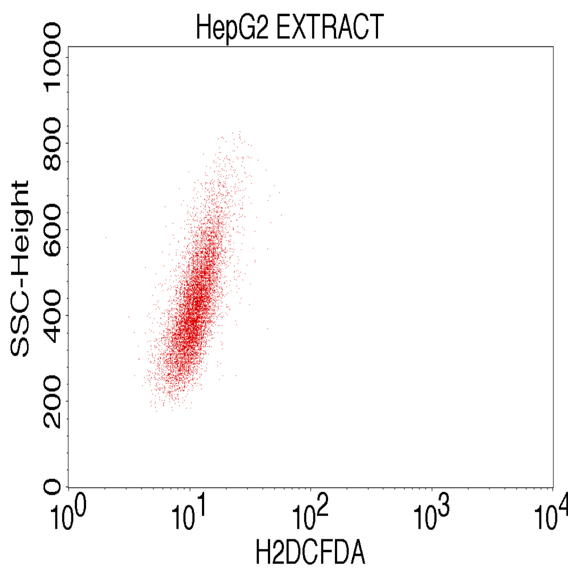


Fig.9 HepG2-Extract

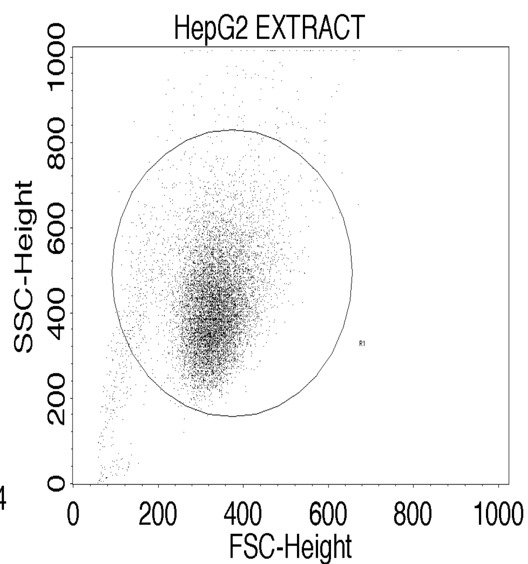


Fig.10 HepG2- Extract 2

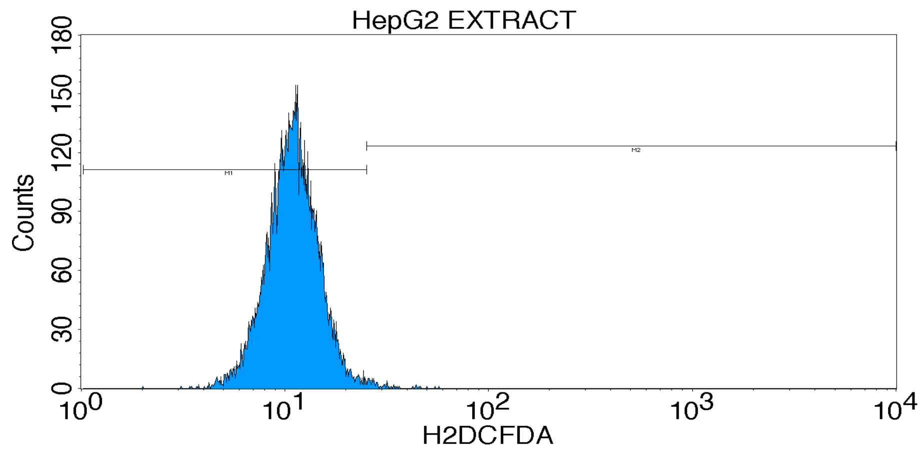


Fig.11 HepG2- Extract 3

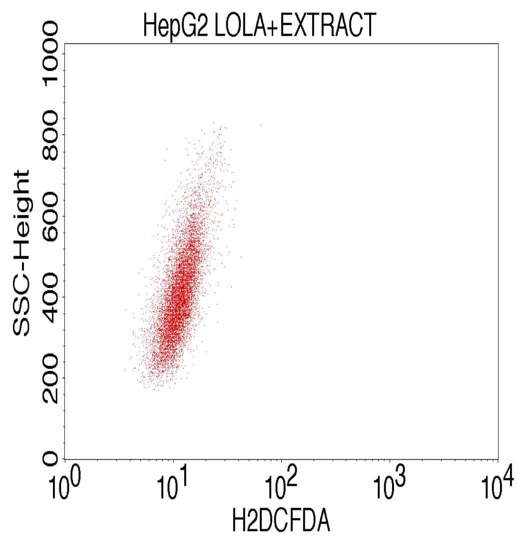


Fig.12 HepG2-LOLA+ Extract

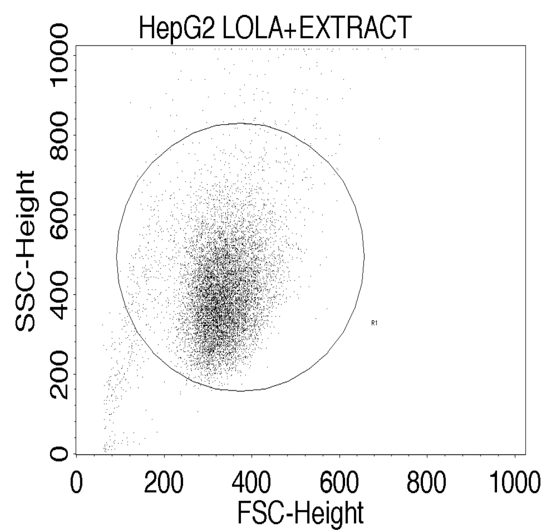


Fig.13 HepG2-LOLA+ Extract 2

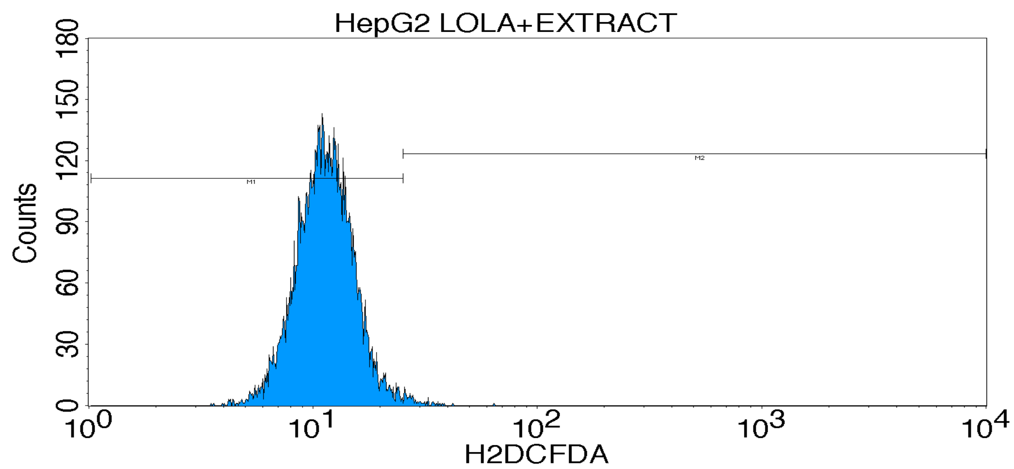
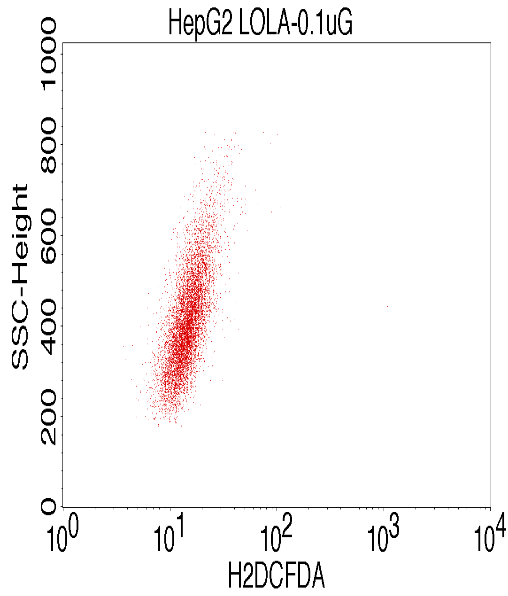


Fig.14 HepG2-LOLA+ Extract 3



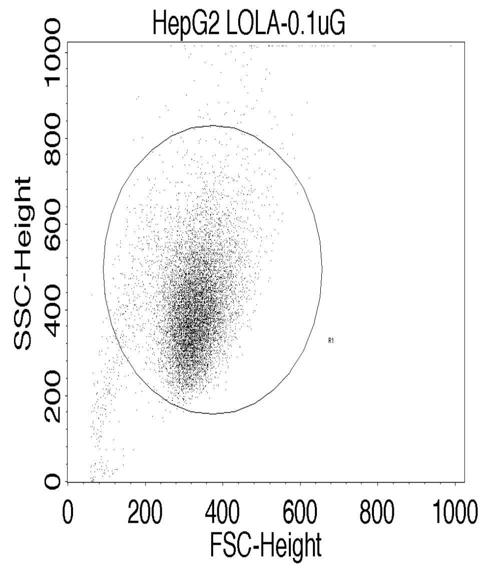


Fig.15HepG2-LOLA-0.1uG **Fig.16 HepG2- LOLA-0.1uG**

2

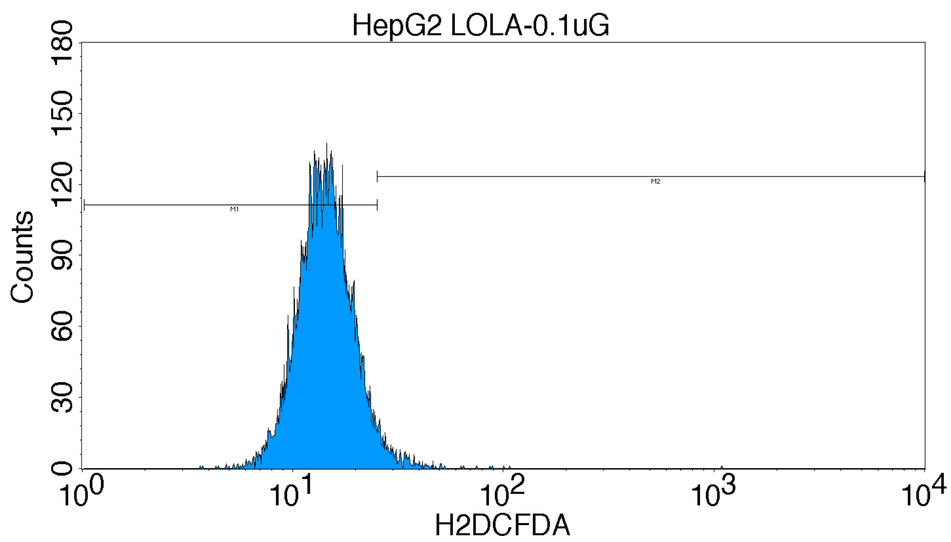
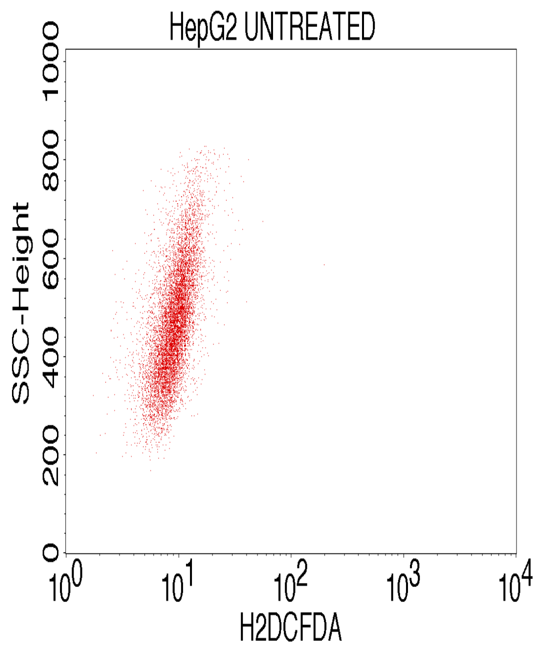


Fig.17 HepG2- LOLA-0.1uG 3



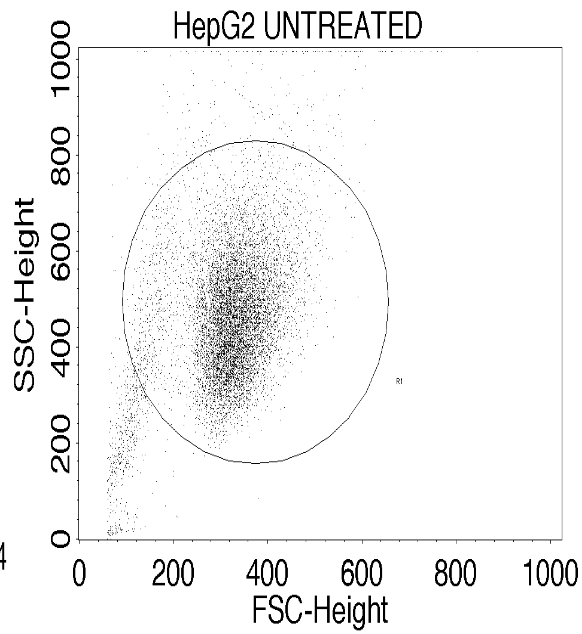
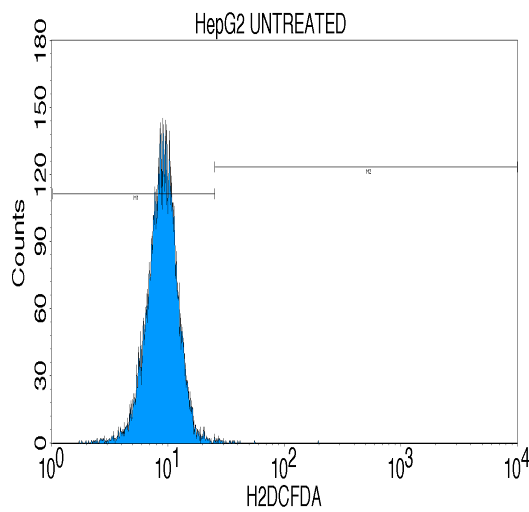


Fig.18 HepG2-Untreated

Fig.19 HepG2- Untreated 2



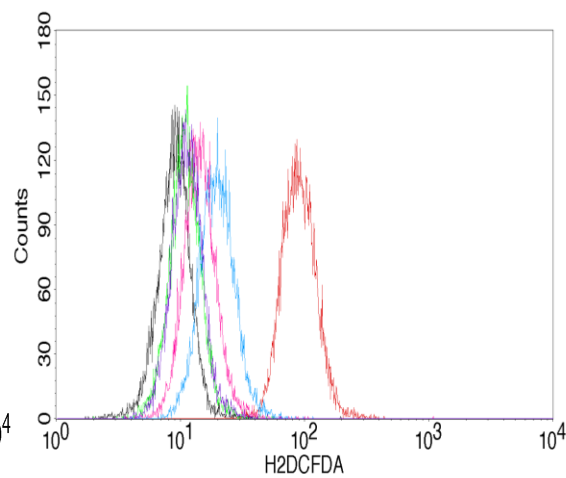


Fig.20 HepG2-Untreated 3

Fig.21 HepG2- overlay

MTT assay :

Combination:

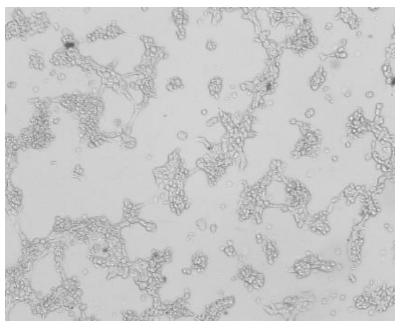


Fig.22 HepGethanol 100mM

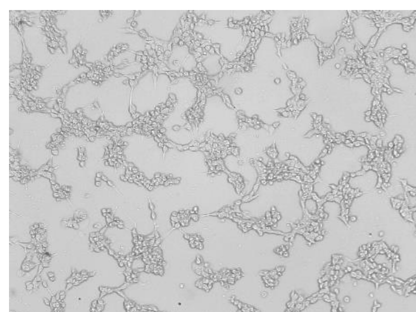


Fig.23 HepG2-ethanol+extract-50uG

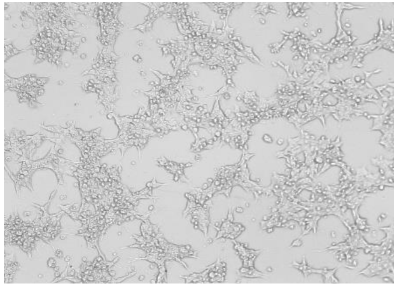


Fig.24 HepG2-Ethanol+extract-100uG

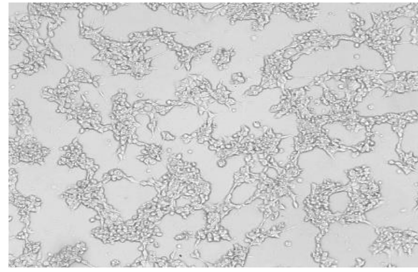


Fig.25 HepG2-ethanol+LOLA-0.01uG

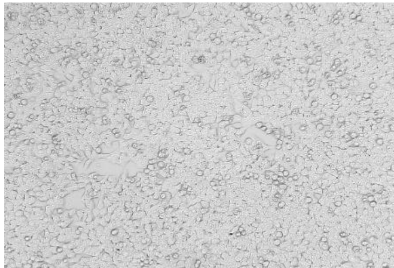


Fig.26 HepG2-Extract-50uG

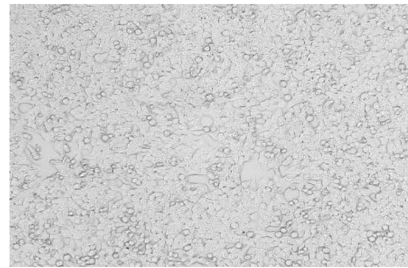


Fig.27 HepG2-Extract-100uG

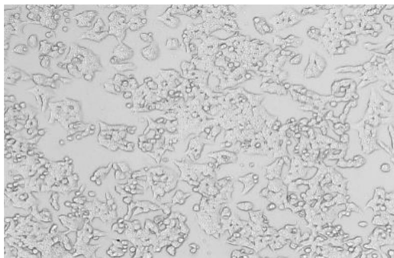


Fig.28 HepG2-LOLA-0.1uG

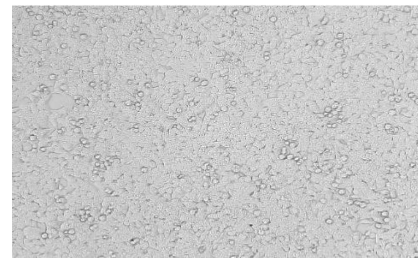


Fig.29 HepG2- Untreated

Extract:

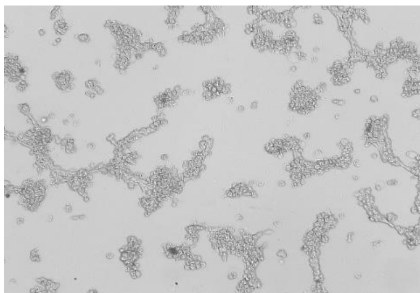


Fig.30 HepG2-ethanol 100mM

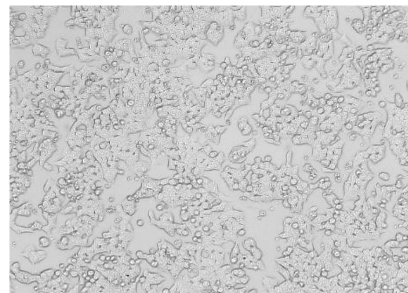


Fig.31 HepG2-extract-12.5uG

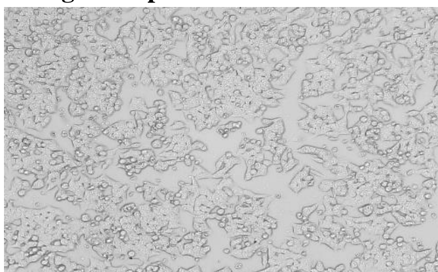


Fig.32 HepG2-extract-25Ug

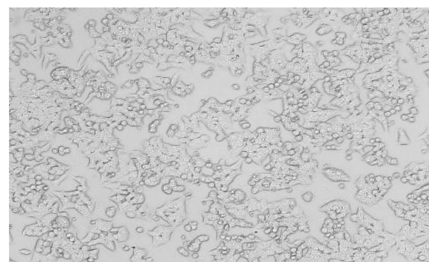


Fig.33 HepG2-Extract-55uG

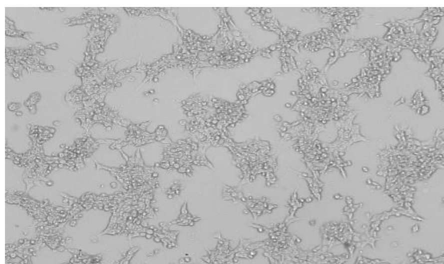


Fig.34 HepG2-extract-200uG

Fig.35 HepG2-extract-100uG

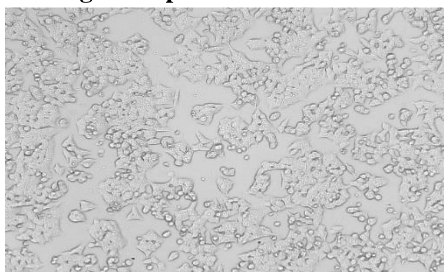


Fig.36 HepG2-Untreated

DISCUSSION

Liver is the major organ of our body. It can be injured by many chemicals and drugs. Here in the present study anti tubercular drugs (combination of isoniazid, rifampicin, pyrazinamide, ethambutol) was used as a toxicant to induce liver damage, since it is clinically very relevant. Anti-tubercular drugs produce a constellation of dose related deleterious effects in liver.

The phytochemical screening of sesbania grandiflora was revealed the presence of Alkaloids, saponins, glycosides, flavanoids, terpenoids and these chemical constituents was responsible for different therapeutic actions. The MTT assay results suggest that the test compound, extract showing moderate cytotoxicity against the Human Liver cancer cells at the 200ug/ml concentration with IC50 value of 190.28ug/ml and remaining concentrations were non toxic in nature. The obtained results clearly confirmed the hepatoprotective potency of Extract on dose dependent manner in ethanol induced cells and showed similar results like LOLA, commercially available hepatoprotective drug by increasing the % cell viability. Combination of LOLA and Extract also showed similar results with 11.67 DCF expression.Overall observations strongly suggesting us that the extract significantly suppressed the DCF expression in Ethanol induced Liver cells (HepG2) and confirmed the significant hepato-protective potential nature in HepG2 cell lines**.**

CONCLUSION:

The results suggest that the chemical constituents present in SG ethanolic extract play a pivotal role in the therapeutics of hepatotoxicity by increasing the body's natural antioxidant defenses with depletion in the Anti-tubercular drugs-induced oxidative stress and reduction in the elevated levels of liver enzymes.In conclusion, the present study demonstrated that the *sesbania grandiflora* Plant extract significantly suppressed the DCF Mean Fluorescence Intensity in Ethanol stimulated model and confirmed the Hepato-protective potency of extract. Untreated cells expressed DCF intensity with relative mean fluorescence intensity of 9.25 whereas Ethanol alone and Ethanol conjugated extract showed 93.93 and 20.51 DCF

expression. Combination of LOLA and Extract also showed similar results with 11.67 DCF expression. Overall observations strongly suggesting us that the extract significantly suppressed the DCF expression in Ethanol induced Liver cells (HepG2) and confirmed the significant hepato-protective potential nature in HepG2 cell lines.

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