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AN INVITRO ANTI-OXIDANT ACTIVITY OF GENTISIN – AN ACTIVE FRACTION FROM *GENTIANA LUTEA*

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ABSTRACT

This study evaluates the in vitro antioxidant activity of gentisin, a bioactive xanthone derivative isolated from *Gentiana lutea*. The antioxidant potential of gentisin was assessed through several assays: DPPH radical scavenging, nitric oxide and hydroxyl radical scavenging, hydrogen peroxide scavenging, and total antioxidant capacity. Additionally, the MTT assay was conducted to assess cell viability and cytotoxicity in HepG2 cells. The results indicate that gentisin effectively scavenges free radicals and protects cells from oxidative stress-induced damage, supporting its potential as a natural antioxidant and hepatoprotective agent.

KEYWORDS: Gentisin, *Gentiana lutea*, antioxidants, free radical scavenging, HepG2, DPPH assay, nitric oxide, hydroxyl radicals, hydrogen peroxide, total antioxidant capacity, MTT assay.

INTRODUCTION

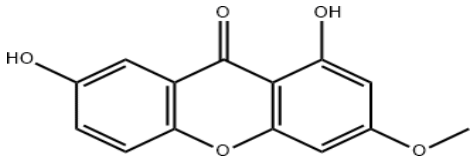
An antioxidant is a molecule that prevents the consumption of oxygen. It is capable of repressing or slowing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electron from a substance to an oxidizing agent.[1] These chemical reactions can produce free radicals and these radicals are capable of initiating wide variety of toxic oxidative reactions such as peroxidation of membrane lipids, direct inhibition of mitochondrial respiratory chain enzymes, fragmentation or random cross linking of molecules like DNA, enzymes and proteins and can commence different degenerative diseases like neurological disorders, cancer, emphysema.[2,3] Free radicals have an unpaired electron that causes them to seek out and capture electrons from other substances in order to neutralize themselves.

Reactive oxygen species (ROS) are described as highly reactive, oxygen containing molecules, including free radicals. They are generated as a by-product of cellular metabolic pathways and play an important role as a critical second messenger in a variety of intracellular signaling pathways. Defect in the anti-oxidant defense system and excessive intracellular generation of ROS resulting in oxidative stress.[4] Different types of ROS produced in cells include the hydroxyl radical, the superoxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical, and various lipid peroxides.[1,5]

The majority of the active antioxidant constituents are flavonoids, isoflavones, flavones, anthocyanins, coumarins, lignans, catechins, and isocatechins. The chosen medicinal plant namely as *Gentiana lutea* belongs to the family Gentianaceae has been proven with the antioxidant activity. Therefore, the present study is to investigate the antioxidant activity of gentisin obtained from *Gentiana lutea* through the antioxidant assays such as DPPH, nitric oxide scavenging, superoxide scavenging, hydroxyl radical scavenging, hydrogen peroxide radical scavenging, and MTT assay.

Gentiana lutea belonging to Gentianaceae family which comprises more than 400 species spread in the mountain areas of central and Southern Europe, America, Australia and New Zealand,etc.[6] The plant is commonly known by other names in Ayurvedic: Traayamaana, Traayanti, Anujaa, Balbhra, Girisaanja, Girijaa.

The dried gentian root contains gentisein, gentisin, iso-gentisin, gentinin and gentiamarin, bitter glycosides, together with gentianic acid (gentisin).[7] The major constituents of Gentisin and isogentisin from Gentiana lutea may potently inhibit monoamine oxidase types A and B. Gentian may interact with antidepressant herbs and supplements, antifungal herbs, monoamine oxidase inhibiting herbs and supplements. Three monoamine oxidase inhibitors isolated from Gentiana lutea have shown competitive MAO inhibition more effectively against MAO-B than against MAO-A.[8]



According to phytochemical and pharmacological studies, G. lutea is rich in compounds that show several biological activities, such as hepatoprotective, antimicrobial, anti-inflammatory, antioxidant, radioprotective/ sensitizing actions, hypoglycemic, choleretic activity, anti- tubercular activity, blood coagulation, atherosclerosis, neuroprotection, synergistic effect, CNS stimulation, gastroprotective, anti- atherosclerotic and immunomodulatory activities.

MATERIALS AND METHODS

Chemicals and Reagents

Gentisin was purchased from sigma Aldrich-Merck, Bengaluru. All the *invitro* activity (MTT assay, DPPH radical scavenging assay, Nitric oxide radical scavenging assay, Hydroxyl radical scavenging assay, Superoxide radical scavenging assay, Hydrogen peroxide radical scavenging assay, Total antioxidants) were done at Center for Research on Molecular and Applied Science Pvt Ltd, Thirumala, Thiruvananthapuram.

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), L-glutamine, sodium bicarbonate, antibiotic mixture containing penicillin, streptomycin and amphotericin B, 0.25% trypsin, phosphate-buffered saline

(PBS), hydrogen peroxide (H_2O_2), dimethyl sulfoxide (DMSO), MTT reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH) prepared in ethanol, sodium nitroprusside, components of Griess reagent including sulphanilamide, phosphoric acid and N-1-naphthyl ethylenediamine dihydrochloride, gallic acid as standard, 2-deoxy-2-ribose, ferric chloride ($FeCl_3$), EDTA, ascorbic acid, potassium dihydrogen phosphate–potassium hydroxide (KH_2PO_4–KOH) buffer, trichloroacetic acid (TCA), thiobarbituric acid (TBA), riboflavin, nitro-blue tetrazolium (NBT), phosphate buffer, sulfuric acid, sodium phosphate, ammonium molybdate, and methanol, which together constituted the chemical systems employed for cell culture, oxidative stress induction, and the various *in vitro* antioxidant assays.

1. *IN VITRO* HEPATOPROTECTIVE EFFECT DETERMINATION BY MTT ASSAY

HepG2 (Liver Hepatic cells) cell line was purchased from NCCS Pune was maintained in Dulbecco's modified eagles' media (HIMEDIA) from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecco's Modified Eagles medium (DMEM) (Sigma Aldrich, USA).

The cell line was cultured in 25cm 2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml).

Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany).The viability of cells were evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.[9]

Cells seeding in 96 well plate

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100µl cell suspension ($5x10^4$ cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator.

Preparation of compound stock

The extract solution was filtered through 0.22µm Millipore syringe filter to ensure the sterility. H_2O_2 (200µM), was added to induce toxicity.

Cytotoxicity Evaluation

After attaining sufficient growth, H_2O_2 (200µM), was added to induce toxicity and incubated for one hour, freshly prepared each compound were added at concentrations of 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, and 6.25 µg/ml of DMEM. Each concentration was added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator. Untreated control cells and H_2O_2 alone treated wells were also maintained.

Cytotoxicity Assay by Direct Microscopic observation

Entire plate was observed after 24 hours of treatment in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observation were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

Cytotoxicity Assay by MTT Method

Fifteen mg of MTT (Sigma, M-5655) was reconstituted in 3ml PBS until completely dissolved and sterilized by filter sterilization. After 24 hours of incubation period, the sample content in wells were removed and 30µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours.

After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution (DMSO) was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 540 nm.[9]

Instruments & Reagents

Table 1.0: Instruments and reagents with their source.

Instruments & Reagents	Source
DMEM media	Sigma Aldrich, USA D5648
Fetal Bovine Serum	Gibco, US orgin-
0.25% Trypsin	Invitrogen, USA 25200-056
Micropipettes	F1 Thermoscientific USA
CO 2 Incubator	Eppendorf, GERMANY
Phase Contrast Microscope	Olympus, JAPAN with Optika Pro 5 Camera
MTT	Sigma Aldrich M5655
ELISA Reader	ERBA, GERMANY
Culture Plates and Flasks	NUNC, Thermoscientific USA
Image Magnification	- 10 X

2. DPPH RADICAL SCAVENGING ASSAY

The radical scavenging activity of different extracts was determined by using DPPH assay according to Chang et al [2001]. The decrease in the absorption of the DPPH solution after the addition of an antioxidant was measured at 517 nm.

PRINCIPLE

1, 1-diphenyl-2-picryl hydrazyl is a stable free radical with pink colour which turns yellow when scavenged. The DPPH assay uses this character to show free radical scavenging activity. The scavenging reaction between (DPPH) and an antioxidant (H-A) can be written as,

$$DPPH + [H\text{-}A] \rightarrow DPPH\text{-}H + [A]$$

Antioxidants react with DPPH and reduce it to DPPH-H and as consequence the absorbance decreases. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability.

REAGENT PREPARATION

0.1mM DPPH solution was prepared by dissolving 4mg of DPPH in 100ml of ethanol.

PROCEDURE

Different concentrations of sample such as 12.5µg/mL- 200µg/mL from stock solution of 10mg/mL were made up to a final volume of 20µl with DMSO were taken and 1.48ml DPPH (0.1mM) solution was added. A control without the test compound, but an equivalent amount of distilled water was taken.

The reaction mixture incubated in dark condition at room temperature for 20 minutes. After 20 minutes, the absorbance of the mixture was read at 517nm (SHIMADZU(UV-1900i) UV-VIS spectrophotometer). 3ml of DPPH was taken as control.[10]

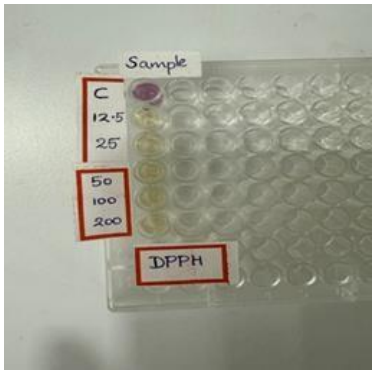


Fig. 1.0: DPPH radical scavenging activity assay showing concentration- dependent antioxidant activity of the sample.

3. NITRIC OXIDE SCAVENGING ACTIVITY

PRINCIPLE

The procedure is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. Large amounts of NO may lead to tissue damage. Nitric oxide scavenging activity was measured spectrophotometrically.

PROCEDURE

1.5mL of Sodium nitroprusside (5mmolL -1 - dissolved in phosphate buffered saline pH 7.4), was mixed with different concentration of sample such as 125µg/mL -2000µg/mL from a stock concentration of 10mg/mL and incubated at 25°C for 60minutes.A control without the test compound, but an equivalent amount of distilled water was taken. After 60minutes, 1.5mL of the incubated solution was removed and diluted with 1.5mL of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% N-1-naphthyl ethylene diamine dihydrochloride).[11]

Absorbance of the chromophore formed during diazotization of the nitrate with sulphanilamide and subsequent coupling with N-1 naphthyl ethylene diamine dihydrochloride was measured at 546nmusing a UV-Visible –light spectrophotometer (SHIMADZU- UV-1900i) and the percentage scavenging activity was measured with reference to the standard gallic acid.[12]

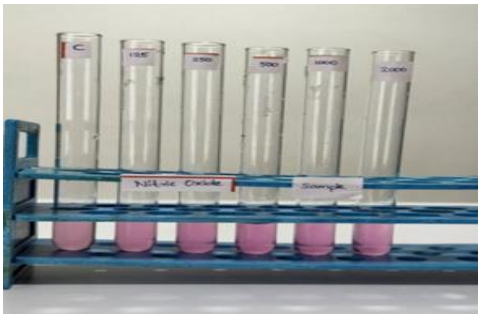


Fig. 1.1: NO scavenging activity of the sample at various concentrations.

4. HYDROXYL RADICAL SCAVENGING ASSAY

Hydroxyl radical is one of the potent reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell (Halliwell and Gutteridge, 1981).

PRINCIPLE

This assay is based on the quantification of the degradation product of 2 deoxy ribose by condensation with TBA (Elizabeth and Rao, 1990). Hydroxyl radical was generated by the Fe^{3+} - ascorbate- EDTA –Hydrogen peroxide (H_2O_2) system (The Fenton reaction).

PROCEDURE

Different concentration of sample such as 125-2000µg/ml from a stock concentration of 10mg/mL were mixed with 500µl reaction mixture ((2 deoxy 2 ribose (2.8mM), $FeCl_3$ (100µM), EDTA (100µM), H_2O_2 (1.0mM), ascorbic acid (100µM) in KH_2PO_4 – KOH buffer (20 mM pH 7.4)) was made up to a final volume of 1 ml .A control without the test compound, but an equivalent amount of distilled water was taken.

After incubation for 1 hour at 37°C, add 1ml of 2.8% TCA, then 1ml 1% aqueous TBA was added and the mixture was incubated at 90°C for 15 minutes to develop the colour. After cooling the absorbance was measured at 532nm (UV-VIS Spectrophotometer- SHIMADZU- UV-1900i) against an appropriate blank solution.[13]

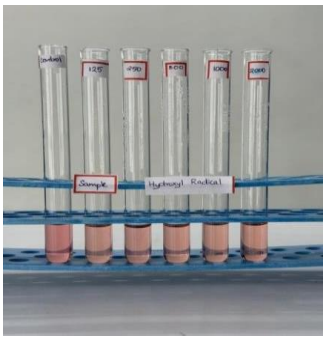


Fig. 1.2: Hydroxyl radical scavenging assay for various concentration of the sample.

5. SUPER OXIDE FREE RADICAL SCAVENGING ACTIVITY PRINCIPLE

Superoxide is biologically important as it can form singlet oxygen and hydroxyl radical. Overproduction of super oxide anion radical contributes to redox imbalance and associated with harmful physiological consequences. Super oxide anion is generated in riboflavin-NADH system by the oxidation of NADH and assayed by the reduction of NBT resulting in the formation of blue formazan product.

PROCEDURE

Different concentration of sample such as 125 - 2000µg/ml from a stock solution of 10mg/ml, 0.05ml of Riboflavin solution (0.12mM), 0.2 ml of EDTA solution [0.1M], and 0.1 ml NBT (Nitro-blue tetrazolium) solution [1.5mM] were mixed in test tube and reaction mixture was diluted up to 2.64ml with phosphate buffer [0.067M]. A control without the test compound, but an equivalent amount of distilled water was taken.[68]

The absorbance of solution was measured at 560nm after illumination for 5 minutes incubation in fluorescent light and also measured after illumination for 30 min. at 560 nm on UV visible spectrophotometer (SHIMADZU-UV-1900i).

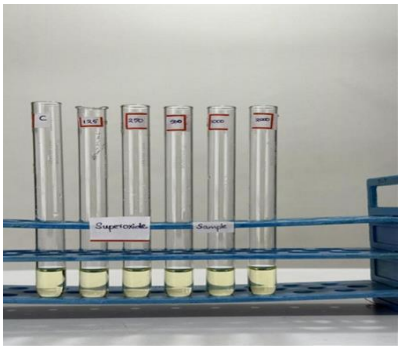


Fig. 1.3: Superoxide radical scavenging assay for various concentration of the sample.

6. HYDROGEN PEROXIDE SCAVENGING ASSAY PRINCIPLE

A hydrogen peroxide (H_2O_2) scavenging assay measures a substance's ability to neutralize H_2O_2, a reactive oxygen species, indicating antioxidant potential, typically by mixing the sample with H_2O_2 in buffer, incubating, and then measuring the remaining H_2O_2 absorbance at 230 nm via UV-VIS spectrophotometry.

PROCEDURE

A 40 mM hydrogen peroxide (H_2O_2) solution was prepared in phosphate buffer (pH 7.4). Various concentrations of the test sample (125–2000 µg/mL) were prepared from a stock solution (10 mg/mL) and added to 0.6 mL of the H_2O_2 solution. A control sample, containing an equivalent volume of distilled water instead of the test compound, was included.[69]

The optical density (OD) was measured at 230 nm using a UV-Vis spectrophotometer (Shimadzu UV-1900i) at 0 minutes and after 10 minutes of incubation. The change in OD (Δ OD) was determined.

Fig. 1.4: Hydrogen peroxide radical scavenging assay for various concentration of the sample.

7. TOTAL ANTIOXIDANT ACTIVITY PRINCIPLE

The principle of total antioxidant activity (TAA) assays measures a sample's ability to neutralize or scavenge free radicals or reduce oxidizing agents, typically through electron or hydrogen atom donation, leading to a measurable color change or fluorescence loss in a chromogenic / fluorogenic probe.

PROCEDURE

Different concentrations of sample such as 125µg/mL -200µg/mL from a stock concentration of 10mg/mL was obtained with 3ml of reagent solution (0.6M H_2SO_4, 28mM sodium phosphate and 4mM ammonium molybdate). The tube containing the reaction solutions were incubated at 95^0C for 90 minutes.

The absorbance of the solution was measured at 695nm (UV-VIS Spectrophotometer- SHIMADZU-UV-1900i) against blank after cooling to room temperature (Methanol 0.3ml) in the place of extract was used as blank. Ascorbic acid (10mg/mL DMSO) was used as reference. The antioxidant activity is expressed as number of gram equivalent of ascorbic acid.[70]

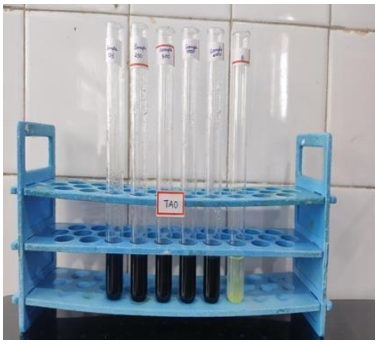


Fig. 1.5: Total antioxidant activity (TAA) assay.

RESULTS AND DISCUSSION

1. *IN VITRO* HEPATOPROTECTIVE EFFECT DETERMINATION BY MTT ASSAY

The absorbance values were measured by using microplate reader at a wavelength of 540 nm The percentage of growth inhibition was calculated using the formula:

$$\% \text{ viability} = \frac{\text{Mean OD Samples}}{\text{Mean OD of Control group}} \times 100$$

Table 1.1: Cytotoxicity Assay by MTT method results.

Sample volume (µg/ml)	OD I	OD II	OD III	Average Absorbance @ 540nm	Percentage Viability
CONTROL	0.7156	0.7328	0.7216	0.7233	100.00
H_2O_2	0.3416	0.3506	0.3523	0.3482	48.14
Sample code: Sample					
6.25	0.3612	0.3741	0.3694	0.3682	50.91
12.5	0.3874	0.3995	0.3871	0.3913	54.10
25	0.4152	0.4308	0.4279	0.4246	58.71
50	0.4576	0.4698	0.4529	0.4601	63.61
100	0.4832	0.4911	0.5064	0.4936	68.24

STATISTICAL ANALYS

Table 1.2 Statistical analysis

Cell line – HepG2									
Sample Code – Sample									
	OD 1	**OD 2**	**OD 3**	**% viability 1**	**% viability 2**	**% viability 3**	**Average**	**Stdev**	**Std error**
Control	0.7156	0.7328	0.7216	100	100	100	100	0	0
H_2O_2	0.3416	0.3506	0.3523	47.7362	47.84389	48.8221	48.134	0.59828	0.34541
6.25	0.3612	0.3741	0.3694	50.4751	51.05076	51.1918	50.9059	0.37966	0.2192
12.5	0.3874	0.3995	0.3871	54.1364	54.51692	53.6447	54.0993	0.4373	0.25248
25	0.4152	0.4308	0.4279	58.0212	58.78821	59.2988	58.7027	0.64304	0.37126
50	0.4576	0.4698	0.4529	63.9463	64.11026	62.7633	63.6066	0.73493	0.42431
100	0.4832	0.4911	0.5064	67.5238	67.01692	70.1774	68.2394	1.69741	0.98

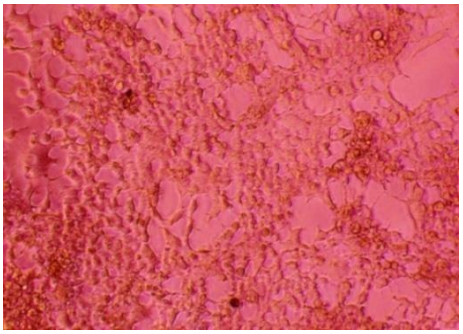


Fig. 1.6: MTT Assay cell viability control group.

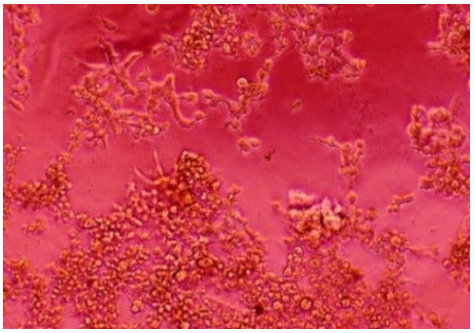


Fig. 1.7: MTT Assay cell viability H2O2 treated group.

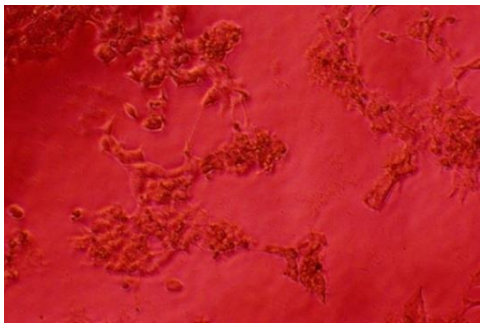


Fig. 1.8: MTT Assay cell viability treatment group at a concentration (6.25µg/ml)

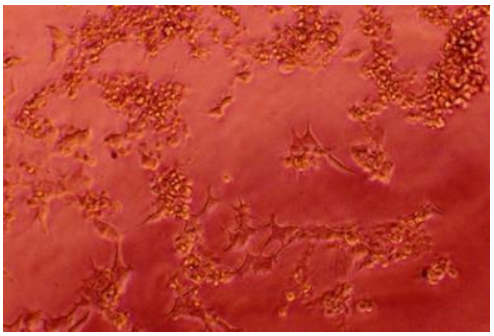


Fig. 1.9: MTT Assay cell viability treatment group at a concentration (12.5µg/ml)

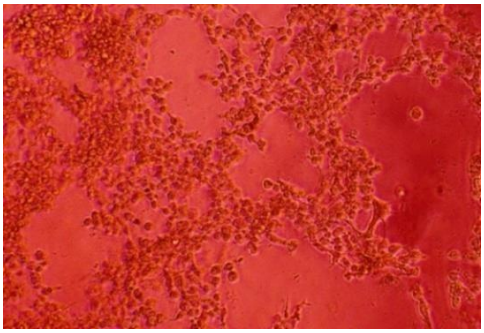


Fig. 2.0: MTT Assay cell viability treatment group at a concentration (25µg/ml)

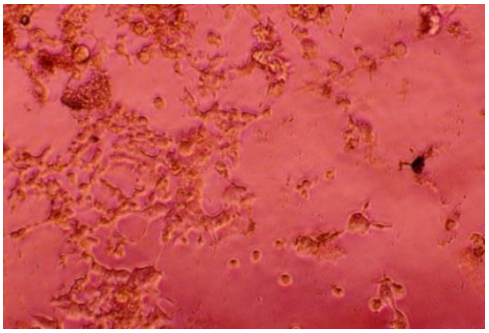


Fig. 2.1: MTT Assay cell viability treatment group at a concentration (50µg/ml)

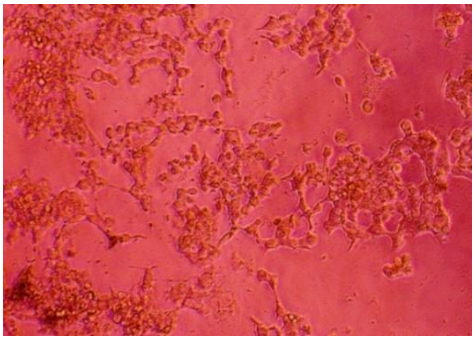


Fig. 2.2: MTT Assay cell viability treatment group at a concentration (100µg/ml)

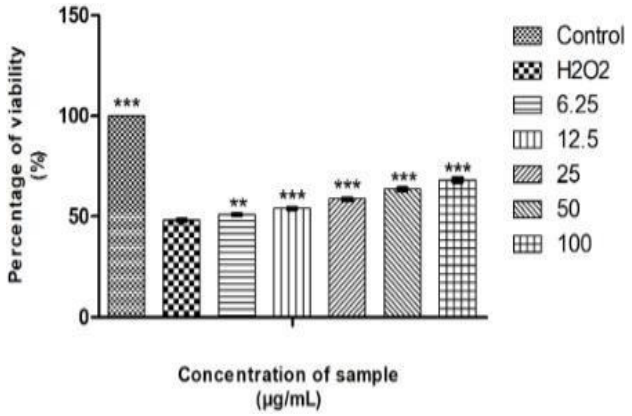


Fig. 2.3: Graphical representation depicting the hepatoprotective effect of sample by MTT assay.

All experiments were done in triplicates and result represented as Mean+/-SE. One-way ANOVA and Dunnets test were performed to analyse data. ***p< 0.0001 compared H_2O_2 exposed group, **p< 0.001compared to H_2O_2 exposed group.

2. DPPH RADICAL SCAVENGING ASSAY

After 20 minutes, the absorbance of the mixture was read at 517nm (SHIMADZU(UV-1900i) UV-VIS spectrophotometer). 3ml of DPPH was taken as control.

CALCULATION

$$\text{Percentage of Inhibition} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

RESULTS

Sample

Table 1.3: IC 50 Value- Sample: 40.185 µg/mL (Calculated using ED 50 PLUS V1.0 Software).

Concentrations (µg/mL)	Absorbance	Percentage of inhibition
Control	0.700	0.000
12.5	0.485	30.714
25	0.41	41.429
50	0.308	56.000
100	0.207	70.429
200	0.135	80.714

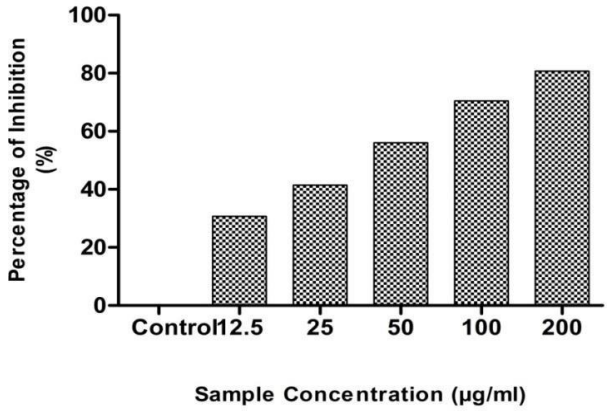


Fig. 2.4: Graphical representation of DPPH Radical scavenging assay in sample.

3. NITRIC OXIDE SCAVENGING ACTIVITY

Absorbance of the chromophore was measured at 546nm using a UV-Visible –light spectrophotometer.

CALCULATION

$$\text{Percentage of Inhibition} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

RESULTS

Sample

Table 1.4: IC 50 Value- Sample: 859.332 µg/mL (Calculated using ED 50 PLUS V1.0 Software)

Concentrations (µg/mL)	Absorbance	Percentage of inhibition
Control	0.532	0.000
125	0.424	20.301
250	0.375	29.511
500	0.314	40.977
1000	0.249	53.195
2000	0.162	69.549

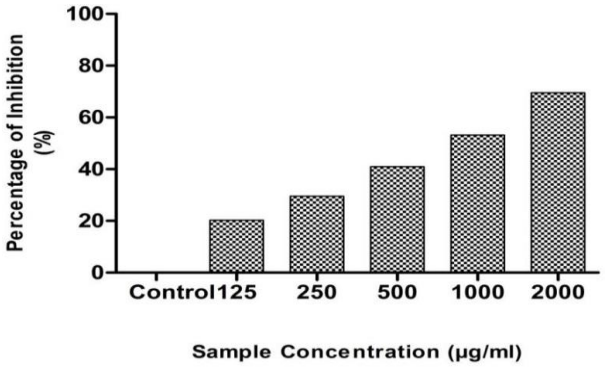


Fig. 2.5: Graphical representation depicting the Nitric oxide scavenging activity of sample.

4. HYDROXYL RADICAL SCAVENGING ASSAY

The absorbance was measured at 532nm (UV-VIS Spectrophotometer- SHIMADZU-UV- 1900i) against an appropriate blank solution.

CALCULATION

The % hydroxyl radical scavenging activity is calculated by the following formula,

$$\text{Percentage of Inhibition} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

RESULT

Sample

Table 1.5: IC 50 Value (Hypothetical)-Sample – 781.2500µg/mL (calculated using ED50 PLUS V1.0 Software).

Concentration (µg/mL)	Absorbance	Percentage of Inhibition
Control	0.7810	0.0000
Sample code – Sample		
125	0.5490	29.7055
250	0.4930	36.8758
500	0.4510	42.2535
1000	0.3450	55.8259
2000	0.2130	72.7273

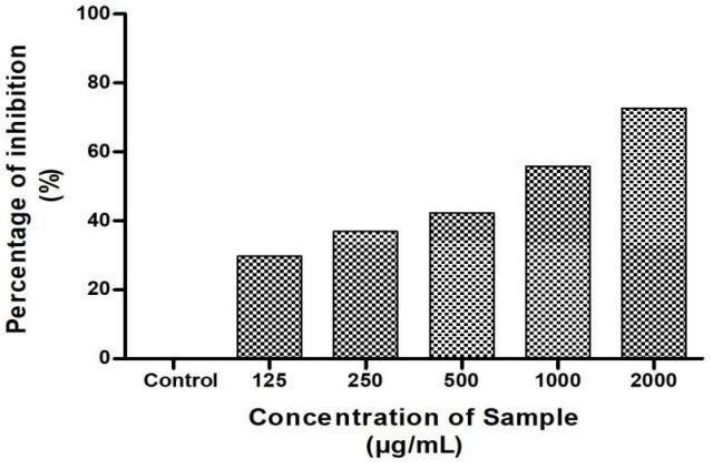


Fig. 2.6: Graphical representation depicting Hydroxyl radical scavenging assay of sample.

5. SUPER OXIDE FREE RADICAL SCAVENGING ACTIVITY

The absorbance of solution was measured at 560nm after illumination for 5 minutes incubation in fluorescent light and also measured after illumination for 30 min. at 560 nm on UV visible spectrophotometer

CALCULATION

$$\% \text{ Inhibition} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

RESULTS

Table 1.6: IC50 Value (Hypothetical)- Sample- 1723.36µg/mL (Calculated using ED50 PLUS V1.0 Software)

Concentration (µg/ml)	OD at 560nm	Percentage of inhibition
Control	0.214	0
Sample code -sample		
125	0.177	17.2897
250	0.159	25.7009
500	0.148	30.8411
1000	0.120	43.9252
2000	0.104	51.4019

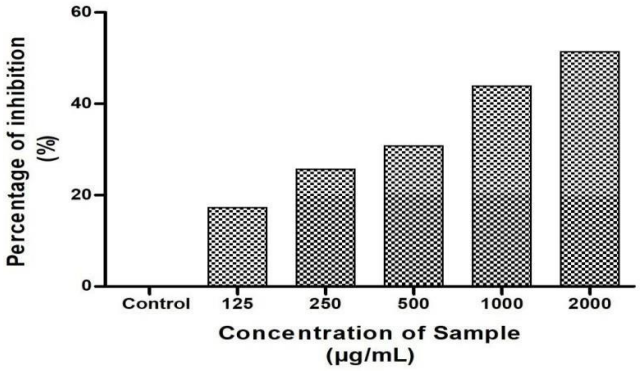


Fig. 2.7: Graphical representation depicting Superoxide activity of sample.

6. HYDROGEN PEROXIDE SCAVENGING ASSAY

The optical density (OD) was measured at 230 nm using a UV-Vis spectrophotometer (Shimadzu UV-1900i) at 0 minutes and after 10 minutes of incubation. The change in OD (Δ OD) was determined.

CALCULATION

$$\text{Percentage of Inhibition} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

RESULTS

Table 1.7: IC50 Value (Hypothetical)- sample-1488.08µg/mL (Calculated using ED50 PLUS V1.0 Software)

Concentration (µg/mL)	Absorbance	Percentage of Inhibition
Control	0.103	0
Sample code -Sample		
125	0.081	21.3592
250	0.071	31.0680
500	0.062	39.8058
1000	0.058	43.6893
2000	0.045	56.3107

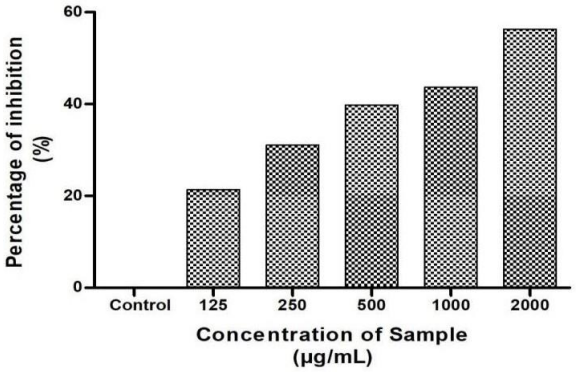


Fig. 2.8: Graphical representation depicting the Hydrogen Peroxide scavenging activity of sample. Along Y axis Percentage inhibition, Along X axis varied concentration of sample.

7. TOTAL ANTIOXIDANT ACTIVITY

The absorbance of the solution was measured at 695nm (UV-VIS Spectrophotometer- SHIMADZU-UV-1900i) against blank after cooling to room temperature (Methanol 0.3ml) in the place of extract was used as blank. Ascorbic acid (10mg/mL DMSO) was used as reference. The antioxidant activity is expressed as number of gram equivalent of ascorbic acid

CALCULATION

Table 1.8: Absorbance values for total antioxidant activity of the sample at different concentrations.

Concentration (µg/mL)	Absorbance
62.5	0.072
125	0.151
250	0.266
500	0.43
1000	0.915
2000	1.668

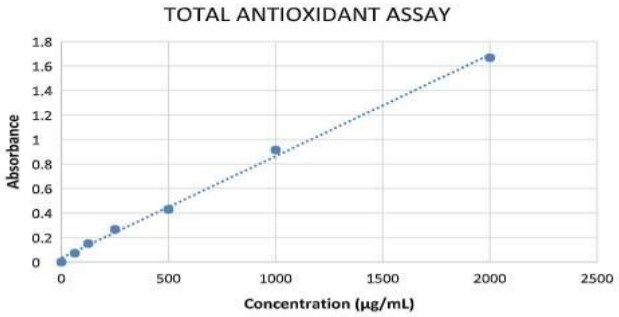


Fig.2.9 Graphical representation depicting the Total Antioxidant activity of sample.

RESULTS

Table 1.9 Total antioxidant activity of the sample expressed as ascorbic acid equivalents at different concentrations.

Concentrations of sample (µg/mL)	Absorbance	c acid equivalent (µg/mL)
125	0.269	294.25
250	0.483	561.75
500	0.610	720.5
1000	0.738	880.5
2000	0.898	1080.5

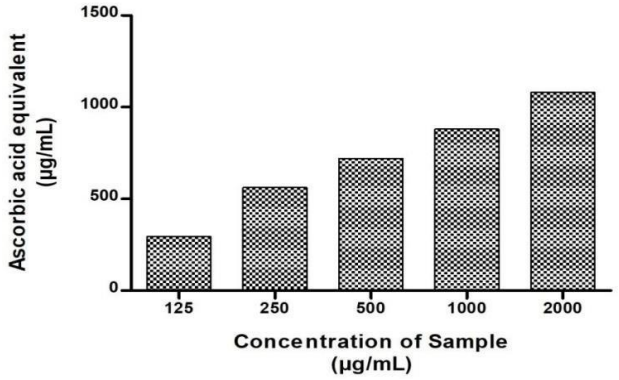


Fig. 3.0: Graphical representation showing total antioxidant activity of sample.

DISCUSSION

The present study evaluated the *in vitro* antioxidant and hepatoprotective potential of gentisin, an active xanthone fraction from *Gentiana lutea*, using multiple radical scavenging assays and an MTT-based HepG2 cell model. Since oxidative stress is a major contributor to liver damage and various degenerative diseases, identifying natural antioxidants with multi-mechanistic action is of significant therapeutic relevance. The results demonstrate that gentisin exhibits concentration-dependent antioxidant activity and provides protection against hydrogen peroxide (H_2O_2)-induced oxidative stress in HepG2 cells.

In the MTT assay, exposure of HepG2 cells to 200 µM H_2O_2 reduced cell viability to 48.14%, confirming oxidative cytotoxicity. Treatment with gentisin significantly improved cell viability in a dose-dependent manner, reaching 68.24% at 100 µg/mL. Statistical analysis (one-way ANOVA with Dunnett's test) showed significant protection (**$p < 0.001$, ***$p < 0.0001$) compared to the H_2O_2-treated group. Microscopic observations supported these findings, as gentisin-treated cells exhibited reduced morphological damage, indicating its cytoprotective effect likely through reduction of intracellular reactive oxygen species (ROS).

In the DPPH assay, gentisin showed strong free radical scavenging activity with an IC_{50} value of 40.185 µg/mL, indicating effective hydrogen/electron-donating ability characteristic of xanthone compounds. This suggests that gentisin acts as a primary antioxidant by directly neutralizing free radicals.

The nitric oxide scavenging assay showed moderate activity (IC_{50} = 859.332 µg/mL), indicating that gentisin can reduce nitrite formation and may help mitigate nitric oxide- mediated oxidative and inflammatory damage. In the hydroxyl radical scavenging assay, gentisin exhibited notable activity (IC_{50} = 781.25 µg/mL), demonstrating its ability to counter highly reactive and damaging ROS generated via the Fenton reaction.

In the superoxide radical scavenging assay, gentisin showed comparatively weaker activity (IC_{50} = 1723.36 µg/mL), yet still demonstrated a dose-dependent inhibitory effect, suggesting a role in maintaining redox balance and preventing secondary ROS formation. The hydrogen peroxide scavenging assay further supported its antioxidant potential (IC_{50} = 1488.08 µg/mL), indicating its ability to neutralize H_2O_2 and indirectly prevent hydroxyl radical generation. Total antioxidant activity (TAA) analysis using the phosphomolybdenum method showed increasing absorbance with concentration, with ascorbic acid equivalent values ranging from
294.25 µg/mL (125 µg/mL) to 1080.5 µg/mL (2000 µg/mL), confirming strong reducing power and overall antioxidant efficiency.

Overall, gentisin exhibited antioxidant activity through multiple mechanisms, including free radical scavenging, reducing ability, and cellular protection. The strongest activity was observed in the DPPH assay, while moderate activity was seen in other radical scavenging assays. The hepatoprotective effect in HepG2 cells adds biological relevance to its chemical antioxidant properties.

CONCLUSION

The present study systematically evaluated the *in vitro* antioxidant and hepatoprotective potential of gentisin, an active xanthone fraction isolated from *Gentiana lutea*, using multiple radical scavenging assays along with an MTT-based HepG2 cell model. The results demonstrated that gentisin exhibits significant and concentration-dependent antioxidant activity across different mechanistic assays, including DPPH, nitric oxide, hydroxyl, superoxide, hydrogen peroxide scavenging, and total antioxidant capacity. Among these, gentisin showed the most potent activity in the DPPH assay with a low IC_{50} value of 40.185 µg/mL, indicating strong free radical scavenging ability. Moderate to appreciable activity was also observed in other radical scavenging models, suggesting its multi-targeted antioxidant mechanism.

Furthermore, gentisin provided substantial protection against H_2O_2-induced oxidative stress in HepG2 cells, as evidenced by improved cell viability in the MTT assay. The dose-dependent increase in cell viability, supported by statistical significance and microscopic observations, confirms its cytoprotective and hepatoprotective potential. These findings indicate that gentisin not only acts as a chemical antioxidant but also confers biological protection at the cellular level by mitigating oxidative damage.

Overall, the study establishes gentisin as a promising natural antioxidant with potential therapeutic relevance in preventing or managing oxidative stress-related liver damage and associated disorders. However, further *in vivo* studies, toxicity assessments, and mechanistic investigations are recommended to validate its safety, bioavailability, and clinical applicability.

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