Antioxidant activity of *Bougainvillea glabra* stem extract on various *In-vitro* experimental models

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Abstract

The *Bougainvillea glabra* stem extract was screened for phytochemical constituents such as qualitative as well as quantitative analysis and also investigated for its *in vitro* anti-oxidant and *in-vitro* hepatoprotective activity assay. Phytochemical screening of stem extract demonstrated the presence of carbohydrates, glycosides, alkaloids, proteins, amino acids, steroids, flavonoids, tannins, and phenols. Further, through quantitative screening, the total phenolic content, total flavonoid content, total tannin content and total antioxidant capacity, total reducing power, and *in vitro* anti-oxidant assay were analysed. Total antioxidant capacity was found to be R^2 = 0.9946, obtained from standard ascorbic acid estimation. Through *in vitro* 2,2-diphenyl-1- picrylhydrazyl **(**DPPH**)** scavenging activity, the *Bougainvillea glabra* stem extract exhibits an IC_{50} value of 436.7±14.5 µg/ml. Besides, the cell viability of HepG2 cell lines tested with stem extract was found to be 85 %. Nitric oxide, SOD and ROS scavenging activities against H_2O_2 treated HepG2 cell lines anticipated the hepatoprotective activity of the *Bougainvillea glabra* stem extract.

Keywords: *Bougainvillea glabra*; DPPH; Nitric oxide, SOD, ROS; *in vitro* antioxidant assay; HepG2 cell lines

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1. Introduction

Plants are a fundamental part of medicinal sources from the origin of human civilization up to the modern world till today of synthetic medicine. The presence of various efficient synthetic drugs and the usage of medicinal plant material for the maintenance of human health problems have received an enormous importance in the present era (Aye, Aung et al. 2019, Süntar 2020, Yu, Gouvinhas et al. 2021). The worldwide interest in the field of natural and

non-synthetic drugs is obtained from plant materials, due to their cost-effective, non-toxic ability and widespread availability. Human diseases associated with heart problems, atherosclerosis, diabetes, and aging were pathologically implemented by free radical reactions (Burtenshaw, Kitching et al. 2019, Yaribeygi, Sathyapalan et al. 2020, Wang, Li et al. 2021). Presence of several phytochemical constituents such as flavonoids, tannins, phenolic components and many more vital components of the plants contribute to their biomedical properties (Aye, Aung et al. 2019, Farag, Abdel-Latif et al. 2020).

The ongoing research in the field of medicinal plants demonstrated a variety of plants possessing a variety of medicinal properties, one among them is *Bougainvillea glabra*. *Bougainvillea glabra* also known as an ornamental flower plant belongs to the genus *bougainvillea,* a family of *Nyctaginaceae* originate to Brazil (Ogunwande, Avoseh et al. 2019, Saleem, Usman et al. 2021). The genus of *bougainvillea* has 18 species, among them *B. glabra, B. Peruvian* and *B. spectabilis* have gained medicinal importance (Tiwari, Dubey et al. , Umamaheswari, Shreevidya et al. 2008). *Bougainvillea glabra* is a woddy climber having a thin stem with long branches and also smooth leaves with papery bracts, it grows more than 10 meters of height (Mariajancyrani, Chandramohan et al. , Saleem, Usman et al. 2021).

Bougainvillea glabra has variety of colours and is a great for container planting (a camera pictures of the plant are provided as figure 1). The plant can be draped along fences which creates security barrier because of its twiggy growth and thorns (Gunavathy and Sangeetha). *Bougainvillea glabra* body has an antioxidant system that reacts along with reactive species and further neutralizes them. The spontaneous antioxidant system consists of enzymes like glutathione, superoxide dismutase and catalase, which are useful for protecting the body from free radicals and further prevents oxidative stress (Elumalai, Eswariah et al. 2012, Garcia-Caparros, De Filippis et al. 2021). The synthetic antioxidants butylated hydroxyl anisole and butylated hydroxyl toluene are reported to be carcinogenic and need to be replaced by natural ones (Venkatachalam, Singh et al. 2012, Rodrigues, do Valle et al. 2020). The explored antioxidant activities of acetone and hydraulic extracts of *Bougainvillea glabra* has further motivated this work. The present work explores the phytochemical evaluation of extracts of stem of *Bougainvillea glabra*. The in vitro antioxidant and hepatoprotective activity for the same is also explored via DPPH assay, iron chelating assay, nitric oxide inhibition assay, SOD and ROS scavenging assays. The cell viability was also carried out against HepG2 cell lines to explore the toxicity of the considered extract.

Figure 1: The camera pictures of *Bougainvillea glabra*

2. Results and discussion

2.1 Qualitative analysis

2.1.1 Total phenolic content (TPC)

The total phenolic content of the extracted Bougainvillea glabra stem material was expressed in terms of gallic acid equivalence (GAE) mg/g of dry extract. The total phenolic content at various concentrations of extracted *Bougainvillea glabra* stem material was calculated by using the following formula.

$C=cV/m,$

Where, C – refers total phenolic content expressed in terms of gallic acid equivalence (GAE) mg/g of dry extract. c is concentration of gallic acid acquired from calibration curve and is expressed in terms of mg/ml. V is the volume of extracted *Bougainvillea glabra* stem material in ml, m is the mass of the extracted *Bougainvillea glabra* stem material in gram. The R^2 of 0.9924 was obtained from standard gallic acid estimation. The absorbance values obtained at different concentrations of extract are listed in Table 1.

Table 1: Results of total phenolic content

The concentration of *Bougainvillea glabra* stems extract in µg/ml	**Absorbance @ 760 nm**	**TPC as GAE in mg/g**
16	0.158	-1.90
62.5	0.196	0.268

125	0.23	2.211
250	0.33	17.92

2.1.2 Total flavonoid content (TFC)

The total flavonoid content was determined and expressed in terms of rutin equivalent (RE) milligram per gram of sample (RE mg/g). The R^2 value obtained for the calibration curve is 0.9926, considered by rutin estimation and the absorbance values for the same are provided in Table 2.

Table 2: Results for total flavonoid content.

Concentration in µg/ml	Absorbance @ 415 nm	TFC as RE in mg/g
62.5	0.161	2.67
125	0.338	6.23
250	0.558	10.33
500	0.873	17.02

2.1.3 Total tannin content

The total tannin content was expressed in terms of tannic acid equivalent (TAEq) in mg per dry weight of fraction in gram. The blank consisting of all reagent without sample. The value of R^2 is 0.9933, obtained from standard tannic acid estimation. The absorbance values are presented in Table 3.

Table 3: Result for total tannin content

Concentration in µg/ml	Absorbance @760 nm	TAE in mg/g
62.5	0.261	5.53
125	0.316	7.31
250	0.432	11.07
500	0.636	17.67

2.1.4 Total antioxidant capacity

The value of absorbance for individual reaction mixture is expressed in terms of equivalents of ascorbic acid. The total antioxidant capacity was determined by the following equation

$$Antioxidant\ activity = (A_0 - \frac{A_i}{A_0})\ X100$$

Where, A_0 is the value of absorbance of control, A_1 is the absorbance of test sample. A linear plot of the results (Table 4) gave R^2 value of 0.9946

Table 4: Results of total antioxidant capacity.

Concentration in µg/ml	Absorbance @695 nm
125	0.05
250	0.068
500	0.0188
1000	0.161

2.1.5 Total reducing power

Results exhibit that higher the value of absorbance, greater is its reducing power. The results are presented in Table 5. With an increase in concentration of the extract, the absorbance value increased indicating the increased reducing power with higher concentrations.

Table 5: Total reducing power of *Bougainvillea glabra* stem extract

Concentration in µg/ml	Absorbance @ 695 nm
62.5	0.32
125	0.46
250	0.61
500	0.86

2.1.6 *In vitro* antioxidant property

2.1.6.1 DPPH assay

Various compounds or herbal extracts were screened using DPPH scavenging activity and it is well known, convenient and easier to estimate scavenging activity of variety of natural products (Kamble and Gacche 2019, Jain, Chaudhary et al. 2020). The scavenging activity of the compounds depending upon its ability to form stable radicals. The herbal compounds having hydroxyl group are found to be necessary for determining scavenging activity. Herein, vitamin C acts as reference standard. An IC_{50} value of 436.7±14.5 µg/ml is obtained for the extract via DPPH assay and was much greater from the value of reference standard vitamin C, 27.3±2.9 µg/ml.

2.1.6.2 Iron chelating assay

The values of % iron chelating activity of the extract was estimated at varied concentrations of extract and the standard reference, quercetin. The results are elaborated in figure 2. The IC_{50} value for the extract is 80.16 and the same for standard is 50 µg/ml.

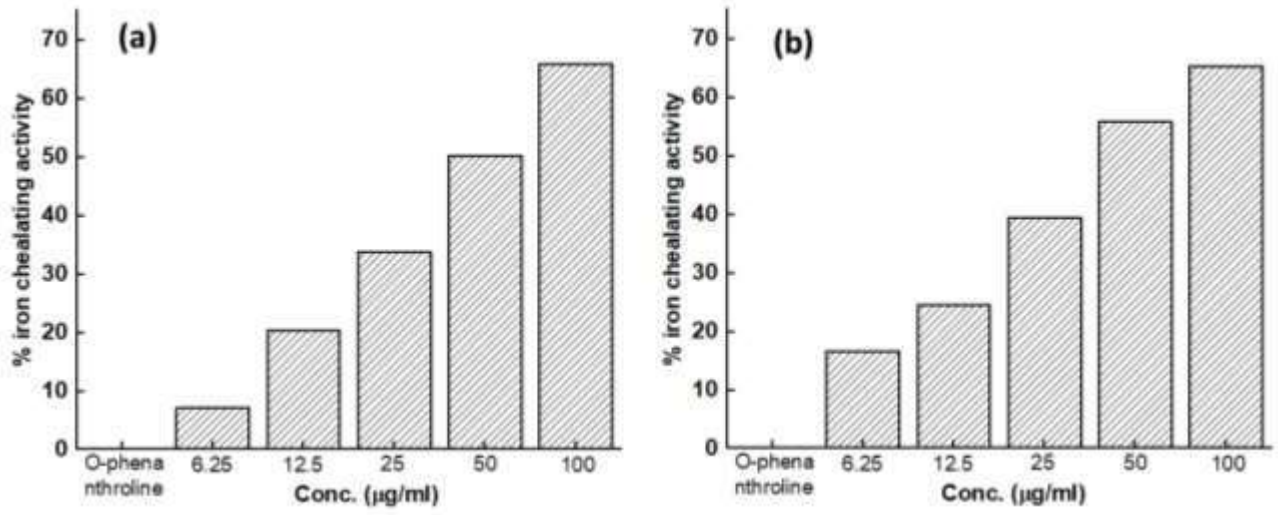

Figure 2: % iron chelating activity obtained for (a) quercetin and (b) extract

The absorbance of ferrozine and ferrous decreases according to dose. The chelating activity increased with increase in concentration from 6.25-100 µg/mL. The chelating activity of extract at 100 µg/mL was found to be 65 %. Whereas the standard one at 100 µg/mL exhibited 66 %. The chelating activity of the extract increases with increase in concentration.

Lesser the IC_{50} value more will be the metal chelating activity of extract. This exhibits the extract was found to be better chelating agent compared to quercetin. The ferrous ion-chelating activity was exhibited by extract and positive control, quercetin having IC_{50} value of 80.16 µg/mL and 50 µg/mL respectively.

2.1.7 *In vitro* hepatoprotective acitvity

2.1.7.1 MTT assay

Before moving on to any assays of in vitro hepatoprotective activity, the toxicity of the extract towards HepG2 cell lines was examined with two models. The first one was the direct treatment of cells with extract and the other model involve treatment of extract with H_2O_2 hepatotoxic induced model. The results are provided as figure 3. At 100 µg/ml the sample was non-toxic to human liver cells lines having 85 % of cell viability. Whereas H_2O_2 hepatotoxic induced model, shows effective hepato-protective potency obtained by retrieving the autophagy/cell death based on a dose-dependent manner the suppressed hepatotoxicity was caused by H_2O_2 till 100 µg/ml concentration. At 100 µM/ml, the H_2O_2 is significantly cytotoxic to the human cell line (HepG2) with 20 % cell viability and that confirms the hepatotoxic potency. Additionally, hepatoprotective efficacy in H_2O_2-induced HepG2 was studied at various concentrations confirming the hepato-protective capability. In addition to that few more studies like apoptosis and ROS were conducted at 100 µg/ml to decide the cytoprotective property of the test sample.

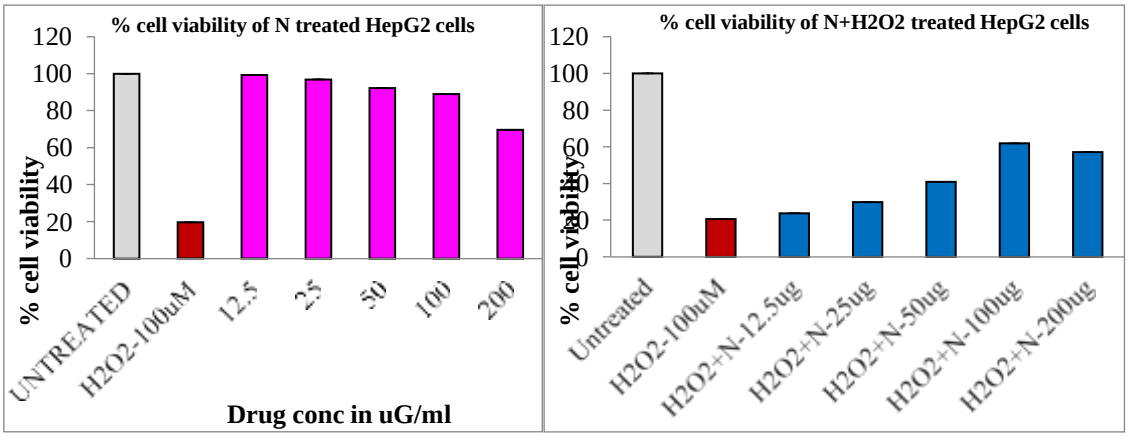

Figure 3: (a) % cell viability of HepG2 cells treated after the incubation period of 24 h. (b) % cell viability of H_2O_2 induced HepG2 cells treated with various concentrations after the incubation period of 24 h.

2.1.7.2 Nitric oxide inhibition assay

The nitric oxide inhibition activity for untreated, H_2O_2 treated and extract treated cells lines was estimated and the % NO inhibition and total NO concentrations were plotted in figure 4. The concentration of NO in untreated cell was found to be 8.53 µM/ml and the concentration of H_2O_2 treated was found to be 310.04 µM/ml. Whereas, the % NO inhibition was found to be 0 at H_2O_2 alone and at minimum concentration of 6.25 µg % NO inhibition was found to be 0.85% whereas the percentage of NO inhibition was found to be maximum at 100 µg.

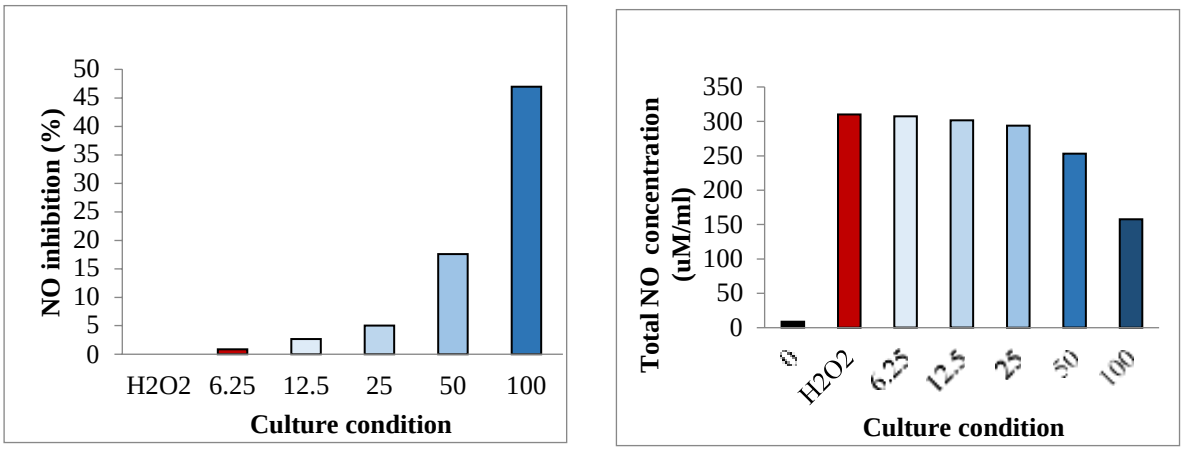

Figure 4: (a) % NO inhibition observed in untreated, H_2O_2 and H_2O_2+extract on HepG2 cells, (b) Total NO concentration secreted in untreated, H_2O_2 and H_2O_2+extract on HepG2 cells.

2.1.7.3 Superoxide dismutase scavenging activity.

The dissolved oxygen derived from superoxide is used to reduce NBT. The amount of oxygen consumed by superoxide anion is calculated by measuring the value of decreased absorbance at 560 nm (Senthilkumar, Amaresan et al. 2021). Figure 5 explains the percentage of superoxide inhibition at various concentrations of extract (6.25, 12.5, 25, 50 and 100 µg). For untreated cells, SOD concentration was 5.39 unit/Min/mg the same has decreased to 1.02 for H_2O_2 alone treated samples, and further the concentration of SOD increased with increase in concentration of extract. Cells treated with maximum amount of extract (100 µg) showed a maximum SOD concentration depicting dose-dependent SOD scavenging activity.

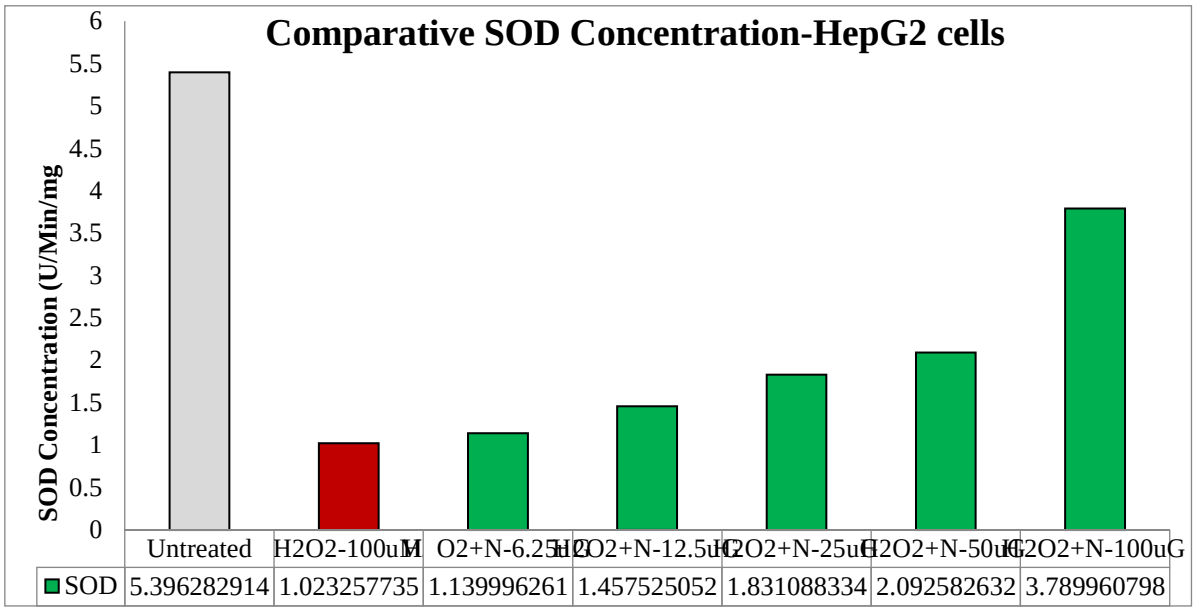

Figure 5: The concentrations of the SOD observed in the untreated, H_2O_2 alone and combination of H_2O_2 with extract- treated HepG2 cell lysates results.

2.1.7.4 Free radical scavenging

ROS production is important for the normal cell functioning. The imbalance between the production of ROS and antioxidants leads to the oxidative stress. With the proven antioxidant activity of the considered extract in the present study, the ROS scavenging assay for the untreated, H_2O_2 treated and H_2O_2 + extract treated HepG2 cells was performed and the results are provided in figure 6 in terms of DCF intensity.

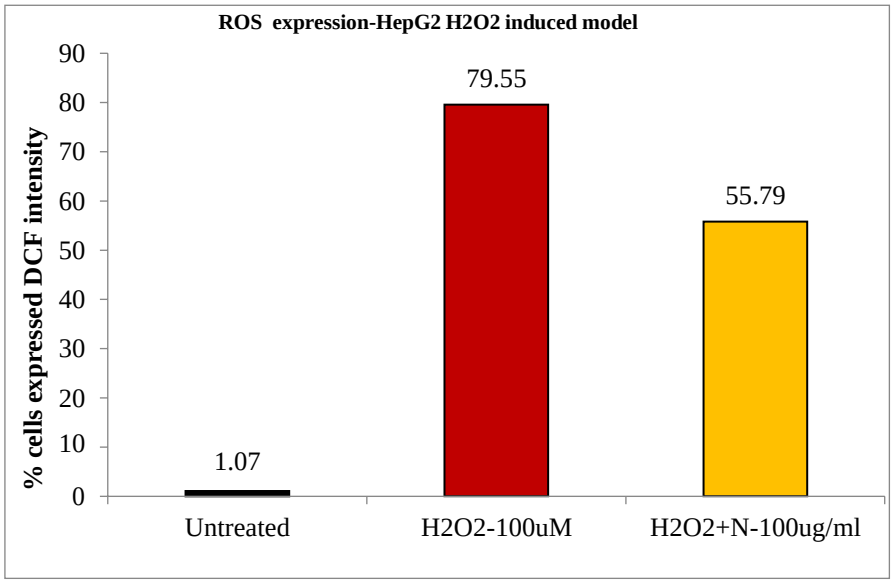

Figure 6: Figure showing the % HepG2 cells expressed DCF intensity in different culture conditions like Untreated, H_2O_2 alone induced and Combination of H_2O_2

In H_2O_2 induced model a significant decrease in the DCF expression was observed compared to H_2O_2 alone treated cells. At a maximum concentration of 100 µg/ml the compound exhibits more than 85% viability. Whereas H_2O_2 alone shows 79.55% of and H_2O_2+N shows 55.79% of cell viability. The above results exhibit the hepato-protective potential of the sample.

3. Materials and methods

3.1 Plant material

Bougainvillea glabra stems were collected in and around the Gurugram city, Haryana. The stems of *Bougainvillea glabra* were dried in shade for about three weeks at room temperature and crushed in a blender, further sieved to afford fine powder.

3.2 Chemicals and reagents

All the solvents, chemicals, and reagents were of analytical grade and used without any prior purification.

3.3 Preparation of plant extracts

The plant extracts were prepared according to the previous literature [16]. The 20 g of fine powdered *Bougainvillea glabra* stem material was successfully extracted with 200 ml of each solvent, pet ether, ethanol, and water according to their increasing order of polarity and 70 % of hydro-alcoholic mixture. Each and every time before using the other solvent, the stem sample was dried completely. The extracts of *Bougainvillea glabra* stem were filtered using Whatman filter paper and further, the extracted sample was combined, filtered, and evaporated to dryness using a hot water bath to attain a yield of 1.5 g and stored in an air-tight container and the same sample is used for analysis. The step wise process is presented in figure 7.

Figure 7: *Bougainvillea glabra* stem extraction process.

3.4 Phytochemical analysis

3.4.1 Qualitative screening of the plant extracts

During qualitative analysis, tests for carbohydrates, glycosides, alkaloids, proteins, amino acids, steroids, flavonoids, tannins, and test for phenols were carried out. The Soxhlet extraction process was carried out for the qualitative phytochemical analysis of *Bougainvillea glabra* stems according to the increasing rate of polarity of pet ether, ethanol, water as well as 70 % of hydro-alcoholic extraction.

3.4.1.1 Test for carbohydrates

The characterization of carbohydrates was carried out by three different tests Molisch's test, Fehling's test and Benedict's test (Chanda and Ramachandra 2019). Initially, a general test called Molisch's test was carried out, and the formation of a violet ring at the junction of two liquids indicated the Molisch's test.

The presence of reducing sugar was examined by Fehling's test and Benedict's test. For Fehling's test, a few drops of the sample was taken to that an equal amount of Fehling's A and B reagents and mixed together. The reaction mixture is allowed to boil, and the formation of brick red precipitate confirmed the presence of carbohydrates in the form of reducing sugars. Additionally, 0.5 ml of Benedict's reagent was added to 0.5 ml of filtrate and the obtained solution was heated for 2 minutes in a water bath and the formation of red precipitate confirmed the presence of carbohydrates as reducing sugar. Barfoed's test was carried out the formation of red precipitate confirmed the presence of monosaccharides.

3.4.1.2 Test for glycosides

The presence of glycosides was carried out by the different sets of tests, glycosides such as cardiac, saponin, cyanogenetic, and anthraquinone were tested (Ali, Nguta et al. 2022). The formation of red colour confirmed Baljit's test, cardiac glycosides presence was confirmed by

Legal's test by the formation of pink colour. The killer killiani test was confirmed by the formation of reddish brown color. The presence of saponin glycoside was directed by foam test, the foam was formed and is stable for about one minute. Grignard reaction were carried out for the confirmation of cyanogenetic glycoside, no filter paper turns to brick red colour confirmed the absence of cyanogenetic glycoside. Borntrager's test and modified Borntrager's tests were carried out for the confirmation of anthraquinone, however, there was no observation of change of ammonical layer to pink color, which indicated the absence of anthraquinone glycosides.

3.4.1.3 Test for alkaloids

The presence of alkaloids was confirmed by five different tests, Dandruff's test results in the formation of orange-brown precipitate, Mayer's test results in the formation of white precipitate, Hager's test results in the formation of a yellow precipitate, Wagner's test results in the formation of reddish brown precipitate, further Tannic acid test was also carried out results in the formation of buff colored precipitate.

3.4.1.4 Test for proteins

Xanthoprotein test confirms the protein presence by the formation of orange colour. The precipitation test was confirmed by formation of white colloidal precipitate by using absolute alcohol, 5 % $CuSO_4$, 5 % lead acetate.

3.4.1.5 Test for amino acids

Ninhydrin test was carried out for the characterization of amino acids, the formation of purple colour confirms the presence of amino acids.

3.4.1.6 Test for steroids

Salkowski test was carried out for the confirmation of steroids, no $CHCl_3$ layer appears that will confirm the absence of steroids.

3.4.1.7 Test for flavonoids

Three different tests were carried out for the confirmation of flavonoids, the Shinoda test was confirmed by the formation orange color. The H_2SO_4 test confirms the presence of flavonoid by the formation of deep yellow color. Furthermore, lead acetate test was carried out and the formation of yellow color confirms the presence of flavonoids.

3.4.1.8 Test for tannins and phenols

The presence of tannins and phenols was carried out by ferric chloride test, the presence was confirmed by the formation of black color. Both gelatin test and lead acetate test forms white coloured precipitate and confirm the presence of tannins and phenols. Bromine water test give the decoloration of bromine water. The presence of Tannins and Phenols was also confirmed by acetic acid test and HNO_3 test by the formation of red colour and reddish yellow colour respectively.

3.4.2 Quantitative phytochemical screening tests.

The extracted Bougainvillea glabra stem material was screened for quantitative analysis for the determination of total phenolic content, total flavonoid content, tannin content, total antioxidant capacity, total reducing power, *in vitro* antioxidant assay.

3.4.2.1 Total phenolic content

Folin-Ciocalteu colorimetric technique (Attard 2013) is used to determine the total phenolic content present in extracted *Bougainvillea glabra* stem material which is based on a redox (oxidation-reduction) reaction. Several concentrations of the gallic acid solution were prepared in methanol (1-10 µg/ml). In each test tube, 300 µL gallic acid of individual concentration was added, and 300 µL of Folin-Ciocalteu reagent (10 %) was added further and made up to 1.5 ml by adding 900 µL of 7 % Na_2CO_3. The obtained blue-colored reaction mixture was incubated at room temperature for 120 minutes. At 760 nm, against a blank, the absorbance value was measured. Triplicate values were taken for accuracy and precision. At various concentrations of gallic acid, the average absorbance value was obtained along with the calibration curve. The extract of various concentrations (serial dilution) were prepared. A routine procedure was followed to measure the absorbance value for individual concentration.

3.4.2.2 Total flavonoid content

Colorimetric assay (Meda, Lamien et al. 2005) was used to measure the total flavonoid content present in extracts of *Bougainvillea glabra* stem material. A final concentration of 1 mg/ml was attained by dissolving the extract in 80 % methanol. The calibration curve was attained by using 0.1-1 ml of sample solution, 2 ml of pyridine, 500 µL of acetic acid and 1 ml of aluminium chloride solution. A final volume of 10 ml was attained using 80 % methanol and the final concentration was of 1-10 µg/ml. To determine the flavonoid content,

0.5 ml of ethanolic extract and 0.5 ml of acetic acid, 1 ml of aluminium chloride, 2 ml of pyridine, 6 ml of 80 % methanol were added. The obtained reaction mixture was incubated at room temperature for 30 minutes and the value of absorbance was measured at 420 nm. The measurements were done in triplicates.

3.4.2.3 Tannin content

Tannin content in the obtained extracts was measured using previously reported literature (Bajaj and Devsharma 1977). 1ml (1-10 µg/ml in distilled water) of standard tannic acid solution was taken in 10 ml of volumetric flask having 7.5 ml of water. 1 ml of sodium carbonate and 0.5 ml of Folin-Denis reagent was added to that and diluted to the mark using distilled water. For the obtained reaction mixture, the absorbance value was measured at 760 nm. A graph of absorbance versus concentration of tannic acid was plotted. Even for samples same procedure was followed whereas 1ml of individual extract (1mg/ml) was taken in place of standard.

3.4.2.4 Total antioxidant capacity

0.1 ml aliquot of individual extract were mixed with 0.3 ml of reagent having 0.6 M H_2SO_4, 4 mM of ammonium molybdate and 25 mM sodium sulphate. The sample container was capped properly, and the obtained reaction mixture was incubated for 90 minutes at 95 ^oC in water bath. The absorbance of all the samples were measured at 695 nm against blank (Pan, Wang et al. 2008).

3.4.2.5 Total reducing power

The total reducing power of the *Bougainvillea glabra* extracts was determined by the addition of 100 µg/ml of extract in 2.5 ml of distilled water, 2.5 ml of 1 % of potassium ferricyanide was added and is mixed with 2.5 ml of phosphate buffer having a pH of 6.6 and concentration of 0.2 M and the obtained reaction mixture was incubated for 20 minutes at 50 ^oC. 2.5 ml of 10 % tricarboxylic acid (TCA) was added and centrifugated for 10 minutes at 1000Xg. The 2.5 ml of upper layer solution was mixed with 2.5 ml of distilled water, 0.5 ml of 0.1 % $FeCl_3$ and absorbance value was measured at 700 nm. (1-10 µg/ml) of ascorbic acid was used as positive control (Kalita, Tapan et al. 2013).

3.5 *In vitro* antioxidant assays

3.5.1 2,2-Diphenyl-1- picrylhydrazyl (DPPH) Assay

In solution, DPPH readily forms free radicals, the maximum absorbance of formed radicals was measured at 515 nm (Jayanti, Ulfa et al. 2021). The free radical scavenger or an antioxidant reduces the DPPH whereas the reduced DPPH lose its colour. The amount of colour reduction is directly proportional to free radical scavenging activity.

The stock solution of 10 mg/ml of *Bougainvillea glabra* stem extract was prepared in distilled water, at first, 100 µl of sample was added to individual row to the 96 well plate serial dilution was made up to last well to attain low dilution, to that 100 µL of DPPH having 0.1 mM concentration was added. The obtained sample was incubated for 30 minutes at room temperature and value of absorbance was measured at 515 nm.

3.5.2 Iron chelating assay

The ferrous/iron chelating assay was resolved in according to Dinis et al (1994) method which is already reported (Md Yusof, Hasan et al. 2013). Stock solution of $FeCl_3$ was diluted upto a concentration of 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL. 50 and 125 µL of O-phenanthroline indicator was added to 25 µL of different concentration of plant extract. By replacing plant extract with quercetin, standard solution was prepared. The blank was the solvent used during reaction. Triplicate readings were taken for better readings. The obtained reaction mixture was incubated for 10 minutes. Furthermore, 100 µL ferrozine having a concentration of 5 mM was added and mixed properly and incubated for 10 minutes in dark condition. The absorbance was measured at 562 nm. The % inhibition of ferrozine-Fe^{2+} complex was calculated by using the absorbance value of test sample and control.

3.5.3 Nitric oxide inhibition assay

In 96 well plates, the HeGP2 cells were cultured with a confluence of 70-80 % were seeded and they have a density of 20,000 cells, further incubated for 24 hours. The spent media was removed and further treated with test compound having various concentration present in H_2O_2 stimulated cells with a concentration of 100 µM/ml acts as negative control excluding untreated and having a concentration of 100 µM/ml behaves as control and later for about 24 hours the cells were incubated. After incubation, the entire supernatant was collected from 96 well plates and later washed with cold phosphate buffer solution and centrifuged at 1000Xg for about 5 minutes. The whole supernatant was collected and transferred into a clean tube during the assay while the culture medium is used as blank. It was retained in ice and later the experiments were carried out based on EZ assay which is provided in nitric oxide estimation

kit. 100 µL of cultured supernatant was taken and mixed with Griess reagent and later plates were incubated for 2 hours at 37 ^{O}C in 5 % CO_2 incubator. Using multimode microplate reader, the absorbance was measured at 580 nm. From sodium nitrate standard curve, the concentration of nitrite was calculated. Standard curve was obtained by preparing a sample of 200 mM sodium nitrite using 320 µM of sodium nitrate to that 16 – 9984 µl of water was added. By following serial dilution 320 µM of $NaNO_3$ in the ratio of 1:1 to attain 160 µM, 80 µM, 40 µM, 20 µM, 10 µM and 5 µM solution.

3.5.4 (4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay

MTT a colorimetric assay is used to determine the cytotoxicity and cell proliferation, which is based on the reduction of yellow-colored tetrazolium dye MTT to formazan crystals (Constante, Rodríguez et al. 2022). The MTT assay was carried out individually as well as combinational treatment, it reduces to insoluble formazan crystal by mitochondrial lactate dehydrogenation which is produced by live cells. The dissolution with an appropriate solvent displays purple color. The intensity of the colour is directly proportional to the number of viable cells and is measured at 570 nm spectrophotometrically.

The seed 200 µl cell suspension in 96 well plates at the required cell density. The cells were allowed to grow for 24 hours. Later, an appropriate amount of test agents was taken. The hepatotoxicity of cells was stimulating cells with a concentration of 100 µM/ml H_2O_2 for 2 hours and desired concentration of test compound was added. The plate was incubated at 37 ^{o}C for 24 hours in a CO_2 atmosphere. After incubation, plates and spent media were removed from the incubator. MTT reagent was added to attain a final concentration of 0.5mg/ml. The plate was wrapped with aluminium foil to avoid sunlight. Further, the plates were incubated for 3 hours. The MTT reagent was removed and 100 µl of solubilisation solution was added. The rate of dissolution was increased by gentle stirring. The absorbance was measured at 570 nm using ELISA reader or spectrophotometer.

3.5.6 Superoxide dismutase (SOD) scavenging assay

The superoxide dismutase scavenging activity of the extract was carried out using HepG2 cells and superoxide dismutase kit. SOD analysis was carried out at room temperature. 1.5 ml of reaction mixture is prepared by taking 107.5 µL of the reagent as constant and 1500-107.5 µL deionized water is used. The reaction mixture was prepared by 60 µL reaction buffer and 7.5 µL of xanthine was added to it and made upto 1500 µL using distilled water. To the

obtained reaction mixture 30 V of nitroblue tetrazolium (NBT) solution was added and mixed properly, further cell lysate was added and for 5 minutes it is vortexed, after 5 minutes the value of absorbance was measured using ELISA reader. In the present study, different concentration of cells with H_2O_2 was estimated and SOD level on HepG2 cell lysates using ELISA.

3.5.7 Reactive oxygen species

Reactive oxygen species are the molecules having peroxides or hydroxyl radicals having unpaired electrons. ROS is produced by healthy aerobic cells as one of the by-products of oxidoreductase, metal-catalyzed oxidation, oxidative phosphorylation at a controlled level. Whereas, ROS can be induced under few stress conditions, particularly when exposed to few drugs and environmental oxidants which leads to oxidative stress. Excess of ROS leads to damage in building block of cells that includes proteins, lipids and DNA and further leads to death of cells. Cell-permeant 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) is a commonly used ROS indicator that converts and oxidizes fluorescent DCF 2,7-dichlorofluorescein by intercellular ROS. Herein, H_2DCFDA was used to indicate intracellular ROS and used to detect the DCF intensity using a fluorescence microscope (Chang, Huang et al. 2013).

The cells were cultured in 6-well plates and incubated for 24 hours at 37 ^{o}C. the cells were induced with H_2O_2 having a concentration of 100 µg/ml for 2 hours in order to induce hepato-toxicity later the control and compounds were added to 1ml of culture medium and further incubated for 24 hours. The one untreated well is considered a negative control and 100 µM/ml of H_2O_2 was considered as a positive control. Finally, the medium was removed and washed with PBS, and 250 µl of trypsin-EDTA was added and incubated for 3 minutes at 37 ^{o}C. The sample was centrifuged for 5 minutes at 25 ^{o}C and the supernatant was washed with PBS decanted. The stock solution was diluted to make a 10 µM working solution. Later the sample was incubated for 30 minutes at 37 ^{o}C and centrifuged for 5 minutes the obtained sample was analyzed using flow cytometer at 488 nm for laser excitation and detection at 535 nm.

4. Conclusions

The 70 % hydroalcoholic extract of *Bougainvillea glabra* stem screened for phytochemical evaluation confirmed the presence of several phytochemical components such as flavonoids, tannins and phenolic compounds. The DPPH scavenging assay provided the antioxidant property of the extract and further ROS scavenging activity also uttered the antioxidation mechanism in H_2O_2 induced hepato activity in HepG2 cell lines. The iron chelating assay provided the IC50 value of 80.16 µg/ml. The MTT assay proved the cell viability upto 85 % for the extract treated cells. The NO scavenging and SOD production were found to be dose dependent and the results are in good agreement with the standard drugs.

Declaration of competing Interest

Authors declare no conflict of interest

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