Phytochemical examination and *in vitro* antioxidant, and *in vitro* hepatoprotective efficacy of *Biophytum umbraculum* leaf extract

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Abstract

The plant extracts were found to be the best source of natural antioxidants in comparison to synthetic ones, as their nature is non-toxic, medicinal properties, easy isolation process and mechanism of action. Herein, it is intended to investigate the in vitro antioxidant and in vitro hepatoprotective feature of leaf extract of Biophytum umbraculum. Standard methods were used for the identification of flavonoids, alkaloids, carbohydrates, saponins, tannins, sterols, terpenoids, reducing sugars and amino acids. Further, through quantitative analysis, total flavonoid, phenolic, tannin components, antioxidant feature and total reducing power were analyzed. Additionally, the DPPH free radical scavenging activity was studied with respect to vitamin C as standard, nitric oxide radical scavenging activity, iron chelating assay and superoxide dismutase scavenging activity were also investigated. The Biophytum umbraculum extract was found to be an efficient antioxidant with an IC_{50} value of 576.7±25.2 µg/ml. MTT assay revealed the % cell viability on HepG2 cells as 90.75 (200 µg/mL of Biophytum umbraculum leaf extract alone) and 99.28 (200 µg/mL of Biophytum umbraculum leaf extract on cells induced with H_2O_2) whereas 38.05 % ROS inhibition activity was observed at 100 µg/mL of Biophytum umbraculum leaf extract in H_2O_2 induced HepG2 cells. A dose-dependent response was evident in SOD and NO assays. The antioxidant property of 70% hydro alcoholic leaves extract of Biophytum umbraculum was confirmed and attributed to the phenolic components. The same of other extracts with active chemical constituents could be explored further.

Keywords: *Phytochemical analysis; Biophytum umbraculum; Antioxidant assay; 2,2-Diphenyl-1-Picrylhydrazyl; hepatoprotective; HepG2 cells.*

1. Introduction

From past few decades, many research is being done in the field of free radical scavengers and antioxidants due to the exclusive prevention of pathologies, where free radicals or reactive oxygen species (ROS) are involved.[1] Though molecular oxygen is essential to sustain a life, it is toxic in the form of ROS that includes hydrogen peroxide, hydroxyl, super oxide radicals, and a singlet oxygen moieties which pose initiation as well as progression of several diseases like cancer, cardiovascular diseases (CVD), neurodegenerative disease, reperfusion injury, inflammatory injury and atherosclerosis etc.[2,3,4] Among these, cardiovascular diseases and cancer ranks first and second most common cause of death, worldwide.[5,6,7] There were many attempts to find a brand new anticancer compound which are plant-derived. [8] The extraordinary therapeutic potentiality of medicinal plants is because of their phytochemical components such as flavonoids, alkaloids, tannins, glycosides, saponins, phenolic compounds, volatile oils, steroids and many more.[9,10,11,12,13]

Biophytum umbraculum is an annual plant of Oxalidaceae family and distributed in the tropical region of Africa, across New Guinea to Asia. Especially, Mali and many African countries use the aerial parts of plants for many medicinal purposes.[14] The detailed information regarding the plant was collected by an ethnopharmacological survey and the major medicinal purposes is with the treatment of cerebral malaria,

wounds, fever, stomach ache, colonic ailments and haemorrhoids.[15-17] In addition to this, in Nigeria the same plant is used against urethral stones and gonorrhoea.[14] Other significant reports are found on treating the breathing difficulties, migraine, hypertension, constipation, lack of fertility and also for hypertension. [14,18,19] *In vitro* analysis of *Biophytum umbraculum* extract exhibits its pharmaceutical use on hypertension. Furthermore, the pectic polysaccharide is isolated from *Biophytum umbraculum* which reveals the distinct effect on the immune system.[17] As a part of this, present study explored the phytochemical analysis, antioxidant and *in vitro* hepatoprotective activity and intended to provide an efficiency of *Biophytum umbraculum* plant material that is non-toxic in nature.

2. Experimental

2.1 Plant material

Biophytum umbraculum, plant species belongs to oxalidaceae family. The whole plant is collected from Mou village in Seoni district located at Madhya Pradesh, India, during the flowering stage and the moisture content was removed from it. The leaves of *Biophytum umbraculum* were collected and dried under at room temperature for about three weeks. The dried material was crushed using a blender, furthermore, the crushed leaves were sieved in order to get fine powder.

2.2 Chemicals and reagents

The chemicals and reagents used such as Meyer's reagent, Fehling's A, Fehling's B, Folin-Ciocalteu reagent, potassium dichromate ($K_2Cr_2O_7$), sodium carbonate (Na_2CO_3), ferric chloride, ammonium molybdate, sodium phosphate, $FeCl_3$, K_3 [$Fe(CN)_6$], gallic acid, 2,2-Diphenyl-1- Picrylhydrazyl (DPPH), ascorbic acid, 1, 10-Phenanthroline, Quercetin, FerroZine iron reagent, Griess reagent, methanol, aluminium chloride, methanol, acetic acid, tannic acid, Folin-Denis solution, phosphate buffer solution, ethanol, chloroform, petroleum ether, hydro alcohol, acetone, sulphuric acid (H_2SO_4) were of laboratory grade or analytical grade and used without any prior recrystallization or distillation. Double distilled water is used whenever required.

Materials for *in vitro* studies such as, HepG2- Human Hepatocellular adenocarcinoma cell line was procured from NCCS, Pune. The Dulbecco's Modified Eagle Medium (DMEM) – low glucose, cell culture medium, fetal bovine serum, MTT reagent, EZ assay Nitric Oxide estimation kit, Superoxide dismutase kit, D-Phosphate buffer saline (PBS) were from Himedia, H_2O_2, DMSO were from Sigma, 2',7'-dichlorofluorescein diacetate/ H_2DCFDA from Life technologies, 96-well plate was from Corning, USA, for cell culturing. T25 flask from Biolite – Thermo, centrifuge tubes, serological pipettes, and tips from TARSON.

Equipment used includes Remi -8˚C Centrifuge, inverted binocular biological microscope from Biolinkz, India, biosafety hood from Biobase, China and biosafety hood class level 2 from Thermo Scientific, incubator from Healforce, China, FACS Calibur Flow cytometer was from BD Biosciences equipped with BD CellQuest Pro software 6.0.

2.3 Plant extract preparation

The soxhlet extraction process was followed for preparation of plant extracts, as given in Fig.1. 100 g of finely powdered *Biophytum umbraculum* leaves was subjected to an extraction process with 200 ml of different solvents like ethanol, water, chloroform and petroleum ether and a hydro alcoholic extraction (70%) in the decreasing order of polarity. Every time prior to extracting with the succeeding solvents, the powdered *Biophytum umbraculum* leaf material was dried thoroughly in an oven. The Whatman filter paper, No. 1 was used for extracts filtration. Filtered extract was then evaporated to dryness with the help of a hot water bath to get 1.5 g of crude sample. Obtained sample was refrigerated in an air-tight container until unless used for phytochemical screening tests.

Fig. 1: *Biophytum umbraculum* leaves extraction process**.**

2.4 Qualitative and quantitative analysis of phytocomponents

2.4.1 Qualitative screening of the plant extracts

Under qualitative analysis, tests for alkaloids using Mayer's reagent, for flavonoids *via* Jone's test, carbohydrates using Benedict's reagent, Saponins using Froath test, Tannins using ferric chloride test, terpenoids and sterols *via* Libermann-Buchard test, reducing sugars by Fehling's test and amino acids *via* Ninhydrin test were carried out.[20,21] The details procedure of the same is provided below.

The qualitative phytochemical analysis of different chemical groups of *Biophytum umbraculum* leaves extract was carried out from serial Soxhlet extraction in the decreasing polarity order of solvents as petroleum ether, chloroform, ethanol, water, and a hydro alcoholic extraction (70 %).[3]

2.4.1.1 Tests for Alkaloids

The alkaloids were characterized by using Meyer's reagent. For 1 ml each of sample, few drops of potassium mercuric chloride which is a Meyer's reagent was added. The cream white precipitate formation confirms the presence of alkaloids.

2.4.1.2 Tests for Flavonoids

The characterization of flavonoids was carried out using the Jone's test. A small portion of sample was dissolved in 1 ml of acetone, 2 ml of aq. $K_2Cr_2O_7$ (10%) and 6 ml of H_2So_4 (6M). The blue colour formation indicate the flavonoids content.

2.4.1.3 Test for Carbohydrates

The characterization of carbohydrates was carried out using the Benedict's test. Benedict's reagent was added to 0.5 ml of filtrate, and the resulting reaction mixture was heated in a boiling water bath for 2 minutes. The production of a reddish precipitate verifies the existence of carbohydrates (sugar).

2.4.1.4 Test for Saponins

The characterization of saponins was carried out using the Froth test. 0.05 ml of the filtrate was added to 5 ml of distilled water, stable persistence froth was obtained by shaking reaction mixture vigorously. The froth which persisted on heating confirm the saponins.

2.4.1.5 Test for Tannins

The ferric chloride test was used to identify the tannins. The 2 ml of leaf extract received a few drops of 5% ferric chloride. The development of violet colour indicates the existence of tannins.

2.4.1.6 Tests for Sterols and Terpenoids

The sterols and terpenoids were characterized by using Libermann-Buchard test. Acetic acid (few drops) was added to the sample the obtained solution was boiled and then cooled. Concentrated H_2SO_4 was added along the sides of test tube and the brown ring of two layers was obtained at the junction of test tube, where the above layer turns to green colour indicating steroids and the deep colour formation utters existence of terpenoids.

2.4.1.7 Test for Reducing Sugars

The reducing sugars were characterized by using Fehling's test. Fehling's reagent A and B (equal volumes) were mixed and the sample was added and the obtained reaction mixture should be boiled. The brick red coloured precipitate obtained is of cuprous oxide which confer the existence of sugars.

2.4.1.8 Test for Amino Acids

The amino acids were characterized by using Ninhydrin test. The sample solution of 1 ml was added to acetone solution of ninhydrin (0.1%) and boiled, the violet colour confirms the existence of amino acids.

2.4.2 Quantitative screening of 70 % hydro-alcoholic extract of leaves

Obtained extract was screened quantitatively for total phenolic components, total flavonoid components and tannin components, antioxidant capacity and total reducing power via colorimetric method.

2.4.2.1 Total phenolic components

The Folin-Ciocalteu colorimetric method which is based on redox reaction (both oxidation reduction) was trailed to estimate the total phenolic contents in *Biophytum umbraculum* extract. Different concentration of gallic acid in methanol solution was prepared from 1-10 µg/ml. In each test tube, 300 µl each concentration of gallic acid, 300µl of Folin-Ciocalteu reagent (10 %) and made up to 1.5 ml by adding Na_2CO_3 (7%). The obtained blue coloured solution was mixed well and incubated for 120 minutes at room temperature. Further, the absorbance of obtained solution was noted at 760 nm against blank. The triplicate readings were taken for all the experiments. The calibration curve was obtained by plotting taking average absorbance value at various concentrations of gallic acid.

Preparation of samples:

The serial dilution method was carried out to prepare the samples. The same procedure has been followed as that of standard, absorbance at particular concentration for extract was measured. The total phenolic acid content in extract is stated in terms of mg as gallic acid equivalents (GAE)/gram of dry extract (mg/g).

2.4.2.2 Total flavonoid content

Total flavonoid content was estimated by colorimetric method.[22] 1 mg/ml was attained by dissolving a dried extract in 80% of methanol. A calibration curve was created using 0.1–1 ml of the aliquot of rutin solution, 2 ml of pyridine, 1 ml of aluminium chloride solution, and 500 µl of acetic acid. The resulting volume, which contains 1–10 µg/ml of rutin, was increased to 10 ml using methanol (80 %). The fraction of 0.5 ml of the ethanol extract was transferred to a test tube, along with 5 ml of acetic acid, 2 ml of pyridine, 1 ml of aluminium chloride solution, and 6 ml of 80% methanol in order to count the quantity of flavonoid content that was present. The obtained sample was incubated for 0.5 hours at room temperature, after which the sample's absorbance at 420 nm was determined. Three times the test was run, and the results are expressed in terms of Rutin equivalents (RE) per gramme of sample extract (mg/g).

2.4.2.3 Tannin content

The Tannin content of the samples was estimated by following the colorimetric method.[23] 1 ml of standard tannic acid solution (1-10 µg/ml in distilled water) was added to volumetric flask of 10 ml which consisting of 7.5 ml of water, to that 1 ml Na_2CO_3 solution was added and 0.5 ml of Folin-Denis solution was added as reagent and diluted to the mark using water. The obtained solution was mixed properly and absorbance was noted after 30 minutes at 760 nm. A graph of absorbance was plotted against concentration of tannic acid. Same procedure was followed as that of standard to determine the amount of sample present, in which instead of standard; extract (1 ml, 1 mg/ml) was taken. The total tannin content present is uttered as tannic acid equivalent (TAEq) (mg)/dry weight of fraction (g). The same procedure was followed for blank also and it contains all the reagents without sample.

2.5 *In vitro* antioxidant assays

2.5.1 DPPH Assay

A stock *Biophytum umbraculum* leaf extracts 10 mg/ml was made with water, 100 µl of the sample was added to the first well of each row of the 96 well plates and serial dilution was done till the last well to obtain lower dilutions. Each well received 100 µl of DPPH (0.1 mM in pure alcohol) before being incubated at room temperature for 30 minutes. At 517 nm, the absorbance was noted, wherein, reduced absorbance indicate the free radical scavenging. The concentration of DPPH in reaction medium is then estimated *via* calibration curve, obtained by linear regression.[21]

2.5.2 Iron-chelating assay

The iron (ferrous) chelating feature was investigated according to Dinis et al (1994) method with little modification.[25] A sample stock solution of 1 mg/mL $FeCl_3$ was diluted to final concentrations of 12.5, 25, 50, and 100 µg/mL. 125 µL and 50µL of O-phenanthroline were added to 25 µL of various concentration of given plant extract. The standard solution was prepared by replacing plant extract with quercetin. The solvent used in the reaction is considered as blank. The reaction mixtures were prepared in duplicate and incubated for a period of 10 minutes. Further, ferrozine (100 µL, 5 mM) was added, thoroughly mixed and kept for 10

minutes in the dark condition. The absorbance was noted spectophotometrically at 562 nm. The percent inhibition of formation of ferrozine-Fe^{2+} complex was determined with reference to control sample absorbance and the test samples.

2.6 *In vitro* hepatoprotective assays

2.6.1 MTT assay

To measure cell viability, a trusted colorimetric MTT reduction test was used. Based on the formation of formazan crystals from the reduction of the yellow-colored soluble tetrazolium dye MTT, cell growth and cytotoxicity were assessed. When MTT is dissolved in an appropriate solvent, mitochondrial lactate dehydrogenase from living, viable cells convert it to insoluble formazan crystals that are purple in colour. The intensity of these crystals can be measured spectrophotometrically at 570 nm and is proportional to the number of livings, viable cells [29, 30].

2.6.2 Reactive Oxygen Species (ROS) Assay

ROS are molecules that naturally occur as by-products of oxidative phosphorylation, the oxidoreductase enzyme cycle, or metal catalysed oxidation at a controlled rate and contain hydroxyl radicals or peroxides with unpaired electrons. Overproduction of ROS damages DNA, proteins, and lipids, the building blocks of cells, and finally leads to cell death, which is shown by the cell-permeant H2DCFDA. Fluorescence microscopy was used to measure the intensity of the intracellular ROS that oxidised the reduced non-fluorescent H2DCFDA and converted it to fluorescent 2', 7'- dichlorofluorescein (DCF)[31]. Cells were cultivated and incubated in a CO_2 incubator overnight at 37 °C for 24 h at a density of 0.5 x 106 cells/2 ml. After removing the used media, 1ml of 1 X PBS was used to wash the cells. After inducing hepatotoxicity in the cells with H_2O_2 (100 µM/mL) for 2 h, the necessary quantity of leaf extract was added to 1 ml of culture media, and the mixture was incubated for 24 h. H_2O_2 (100 µM/mL) and untreated cells served as the positive and negative controls, respectively, whereas cells treated solely with H_2O_2 served as the positive control. After incubation, the medium is withdrawn, and 500 µl of PBS is used to wash the cells. 250 µl of trypsin-EDTA solution was then added, followed by the removal of PBS, and incubated at 37 °C for 3–4 minutes. Cells were immediately harvested and placed into 12 x 75 mm polystyrene tubes after the culture liquid had been poured back into each well. The tubes were properly decanted after being centrifuged for five minutes at 300 x g at 25 °C. PBS was used to wash the cells twice, and then it was totally decanted. By mixing H2DCFDA stock solution (4 mM) into DPBS, a working solution of 10 µM was created. At a density of 1 x 10^6 cells/ml, the cells were suspended in the H2DCFDA working solution and incubated at 37 °C for 30 minutes before being centrifuged at 150 x g for 5 minutes. In 400 µl of pre-warmed DPBS, the cells were resuspended after the supernatant was removed. These cells were examined using a flow cytometer with a "488 nm laser" for excitation and a "535 nm laser" for detection (FL1).

2.6.3 Nitric acid-radical scavenging assay

The cultured HepG2 cells upon 70-80% confluency was seeded in 96 well plate with a cell density of 20,000 and subjected to 24 hours of incubation. The spent media was then removed and treated with a test compound with a different concentration in H_2O_2 stimulated cells with 100 µM/ml (negative control) except untreated and with (control) concentration 100 µM/ml and the cells were given an incubation of 24 hours. After which, the supernatant was collected from all the wells and washed with cold PBS and centrifuged for 5 minutes 1,000Xg. The entire supernatant was collected in the assay and is transferred into a clean tube and made sure to use the culture medium as blank. It was kept in ice and further proceeded for experiment which is based on EZ assay nitric oxide estimation kit instruction. 100 µL of supernatant culture was mixed with Griess reagent and incubated to 37 ^oC for 2 hours in a 5 % CO_2 incubator. The absorbance of the obtained solution was measured in Multimode Microplate Reader at 580 nm.[24] The nitrite concentration was calculated from the sodium nitrite standard curve. To prepare the standard curve, 320 µM of sodium nitrate solution was prepared by using 200 mM of sodium nitrate, for that 16 µl to 9984 µl of cell culture grade water was added. Through serial dilution 320 µM of $NaNO_3$ in 1:1 ratio to attain 160, 80, 40, 20, 10 and 5 µM solutions.

2.6.4 SOD scavenging assay

Superoxide anion scavenging activity of the considered extract was performed using Superoxide Dismutase Kit (#7500-100-K, R & D Systems INC, USA) and HepG2 cells. The SOD assay was performed at room temperature. A total reaction mixture of 1.5 ml is prepared by keeping 107.5 µL of the reagent component as constant. The volume of deionized water is equal to 1500 µL-107.5 µL - sample volume µL. Each reagent was immediately vortexed prior to use. To a cuvette, 25X reaction buffer (60 µL) and 7.5 µL of xanthine were added, diluted to 1500 µL using double distilled water and mixed well. To the obtained solution 30 V of NBT solution was added and mixed vigorously, cell lysate was added and vortexed for 5 minutes. 10 µL Xanthine Oxide (XOD) solution was vortexed and added to the cuvette and vortexed again for 5 minutes, after 5-6 minutes the absorbance at 450 nm was noted with the help of ELISA reader. Herein, various concentrations of simulating cells with H_2O_2 were evaluated and to measure SOD level using ELISA on HEPG2 cell lysates.[26-28]

2.7 Statistical analysis

The experiments were all performed in triplicate, and the findings are presented as Mean ± SD (standard deviation). The graph work is done by GraphPad Prism 3.0 software and Dunnett's tests was used to find *P*-values.

3 Results

3.1 Qualitative phytochemical investigations

The qualitative phytochemical screening is significant, as it gives the information on the composition of the obtained extract. Several tests were performed involving either the precipitate formation or the color change, indicating presence or absence of the respective components. The *Biophytum umbraculum* leaf extract obtained from series of solvents depending on the varied polarity was subjected for the phytochemical screening process and the presence or absence of flavonoids, alkaloids, carbohydrates, saponins, tannins, sterols and terpenoids and the absence of reducing sugars and amino acids is provided in Table 1. The 70 % hydroalcoholic extract is observed to present more phytochemicals and hence, the same was considered for all the future studies.

Table 1: The results of phytochemical investigation of *Biophytum umbraculum* leaf extract obtained with different solvents. ('+' indicates the presence of that particular component, '++' indicate the trace amounts and '–' indicate the absence of the same).

Test component	70% Hydroalcoholic extract	Water	Ethanol	Chloroform	Petroleum ether
Alkaloids	+	+	+	–	–
Flavonoids	+	+	+	–	–
Carbohydrates	++	–	–	–	–
Saponins	–	–	–	–	+
Tannis	+	–	–	–	–
Sterols and Terpenoids	+	–	+	+	–
Reducing Sugars	–	–	–	–	–
Amino Acids	–	–	–	–	–

3.2 Quantitative phytochemical screening

The leaves extract of *Biophytum umbraculum* had appreciable amounts of total phenolic compounds, flavonoids and tannins. The results screened for all three components at three different concentrations are provided in Table 2. The total phenolic component is uttered in terms of standard gallic acid estimation as R^2 = 0.9871. The total flavonoid content is expressed in terms of standard rutin estimation as R^2 = 0.9962 and the total tannin content is uttered in terms of standard tannic acid estimation as R^2 = 0.9994. Compared to phenolic and flavonoid content, tannin content was found maximum at 1000 µg/ml plant extract and is 8.66

mg/gm. At the same concentration, the phenolic content was 3.18 mg/gm and flavonoid content was 2.88 mg/gm.

Table 2: Quantitative analysis of leaves extract of *Biophytum umbraculum***.**

Parameter	**Concentration of *Biophytum umbraculum* leaf extract in µg/ml**	**Test components concentration (mg/gm)**
Total phenolic content	125	-3.788
	500	-1.50
	1000	3.18
Total flavonoid content	250	1.57
	500	1.93
	1000	2.88
Total tannin content	125	0.92
	500	4.08
	1000	8.66

3.3 Total antioxidant capacity

The total antioxidant capacity of considered test samples and the standard vitamin C is performed using phosphomolybdenum assay and obtained results are provided in Fig. 2. The test sample with plant extract showed greater absorbance values than that of standard, indicating the potential antioxidant capacity of the plant extract. Phosphate-Mo (VI) is reduced to phosphate-Mo (V) complex under acidic pH. With increasing reduction rate, the absorbance at 695 nm increases and hence, antioxidant capacity increases.

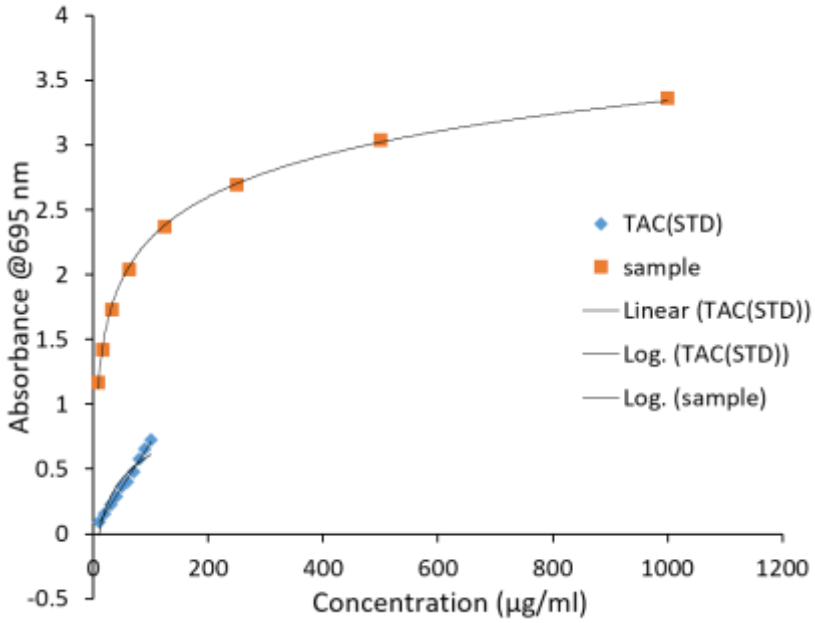

Fig. 2: The alteration in the absorbance values at 695 nm, indicating the antioxidant capacity of plant extract.

3.4 An assay for reducing power

The reducing potency of plant extract and the standard, ascorbic acid is provided in terms of absorbance in the Fig. 3. The absorbance values increased with increased plant extract content, showing the presence of reduced Fe^{2+} ions.

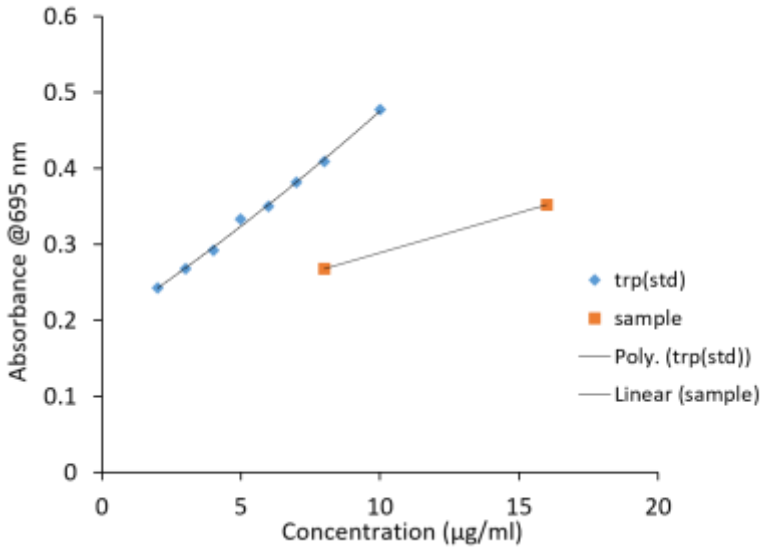

Fig. 3: The reducing power (from Fe^{3+} to Fe^{2+}) of plant extract at different concentration along with standard.

3.5 DPPH radical scavenging assay

In vitro DPPH free radical scavenging assay with respect to vitamin C (standard) was carried out for considered leaf extract and the respective result is provided in Table 3. *Biophytum umbraculum* leaves extract (70 % hydroalcoholic) was found to eradicate more free radical with an IC_{50} value of 576.7±25.2 µg/ml, which exhibits good antioxidant property. Whereas, vitamin C gave IC_{50} value of 27.3±3.1 µg/ml.

Table 3: Result of DPPH radical scavenging activity.

Sl no.	Details	DPPH assay (IC_{50} µg/ml)
1	*Biophytum umbraculum* Leaf Extract	576.7±25.2
2	Vitamin c	27.3±3.1

3.6 Iron chelating assay

The quantitative complex formation between ferrozine and Fe^{2+} ions is the basis for the assay. The existence of chelating complexes would disturb the formation of complexes and result in the reduced intensity in red color. The rate of decrease in the color of the complex is used to estimate the ability of the other chelator. In present work, quercetin was considered as standard, iron chelating ability was investigated for the considered plant extract. The results on iron chelating ability for quercetin and the extract are presented in Fig. 4. From the observations in statistical data of iron chelating activity of test compounds, plant extract and quercetin displayed an IC_{50} concentration of 45.76 and 50 µg/ml, respectively. The plant extract had a significant IC_{50} value in comparison to normal medication and shown satisfactory metal ion chelating activity.

As the concentration of ferrozine and the ferrous complex increased from 12.5-200 g/mL, the absorbance of both substances reduced in a dose-dependent manner. The chelating feature of plant extract at 100 µg/mL was 90 %, at which, standard drug, exhibited 66 %. However, the chelating activity of the extract increased with an increased extract concentration. The compound's or extract's metal chelating activity increases with a lower IC50 value. Comparing the extract to quercetin demonstrates that it is a substantial metal chelating agent with a statistically significant value $p < 0.005$.

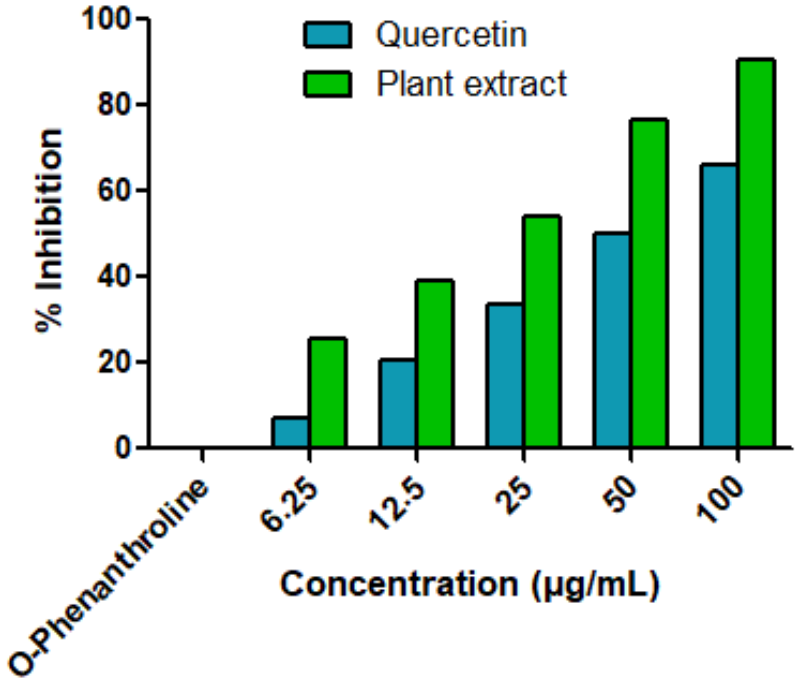

Fig. 4: Iron chelating assay for quercetin and plant extract.

3.7 MTT assay

Leaf extract of *Biophytum umbraculum* by MTT assay suggested the non-toxic and high proliferative efficiency with 93.71% cell viability at 100 µg/mL and revealed dose-dependent protection against H_2O_2-induced hepatotoxicity with 99.28% cell viability at 200 µg/ mL and data is presented in Fig. 5.

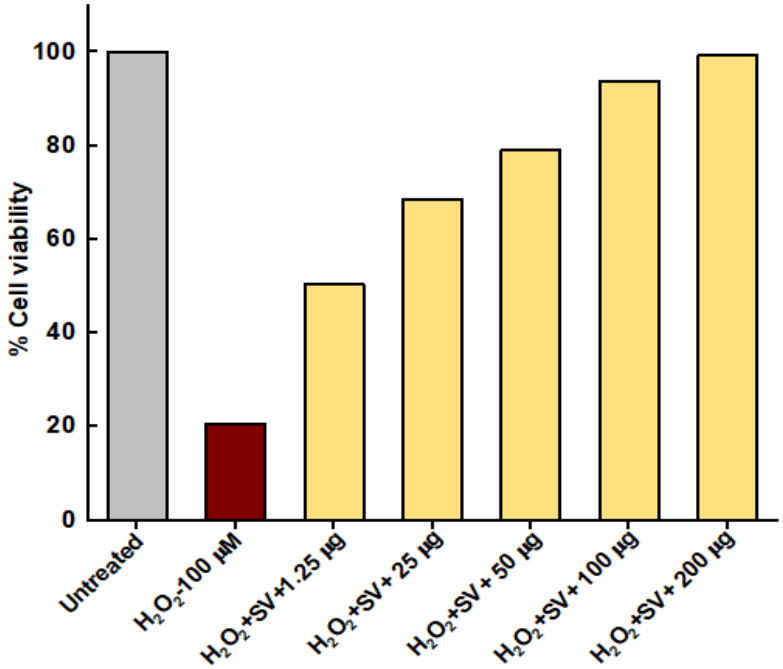

Fig. 5: % cell viability for HePG2 cells treated with H_2O_2 and the plant extract at different concentrations**.**

3.8 Reactive Oxygen Species Assay

Leaf extract of *Biophytum umbraculum* at 100 µg/ml exhibited significant suppression of H_2O_2 toxicity in this study. 81.33% of H_2O_2 alone induced cells exhibited DCF expression and 38.05% of H_2O_2 along with leaf extract at 100 µg/ml treated cells expressed DCF expression and data is presented in Fig. 6.

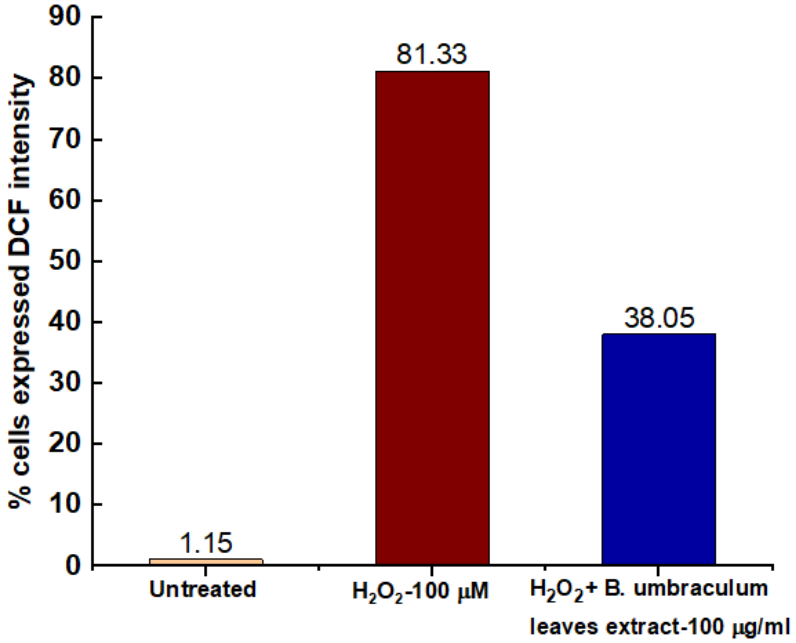

Fig. 6: ROS expression for HePG2 cells treated with H_2O_2 and the plant extract at different concentrations**.**

3.9 Nitric oxide radical scavenging

The percent inhibition of NO and the concentration of NO for untreated, H_2O_2 and H_2O_2 + extract treated HepG2 cells is provided in Fig. 7 (a) and 7 (b). The NO concentration in untreated cells was 8.53 µM/ml and H_2O_2 treated cells, it was 310.04 µM/ml while, % NO inhibition remained 0. At minimal concentration of extract (6.25 µg), % NO inhibition and concentration were 22.75 % and 236.14 µM/ml, respectively. An increased concentration of plant extract, lead to increase in % NO inhibition and decrease in the NO concentration. At maximum concentration of plant extract, 100 µg, maximum NO inhibition of 89.09 % and minimum NO concentration of 20.72 µM/ml was obtained.

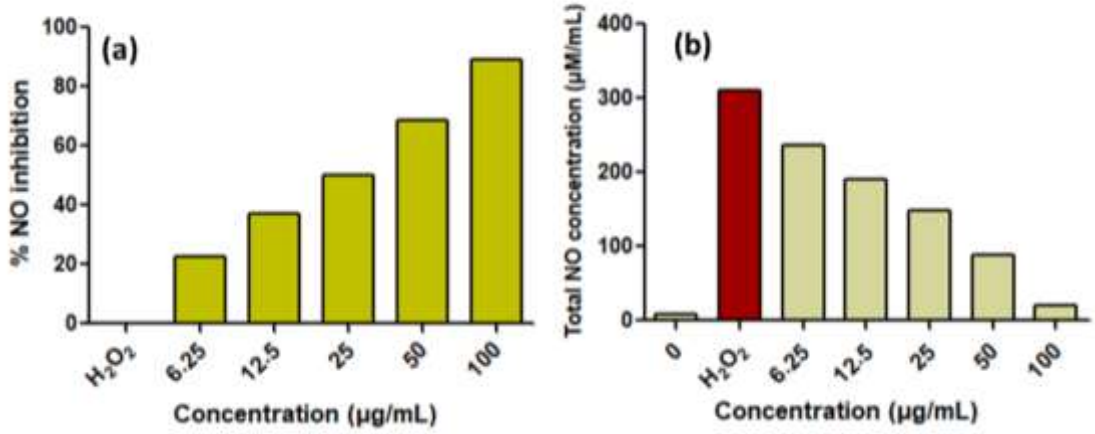

Fig. 7: (a**)** % inhibition of NO and (b) concentration of NO in treated and standard samples.

3.10 Superoxide dismutase scavenging activity

NBT is reduced by the superoxide anion, which is derived from the dissolved O_2. The consumption of superoxide anion is estimated by using a decreased absorbance at 560 nm. The percent superoxide inhibition of 70 % hydroalcoholic plant extract at the concentrations 6.25, 12.5, 25, 50 and 100 µg along with untreated and only H_2O_2 treated test samples is presented in Fig. 6. Dose dependent increase in SOD concentration is obtained. The cells treated with 100 µg of plant extract showed almost equal amounts of SOD as of untreated cells.

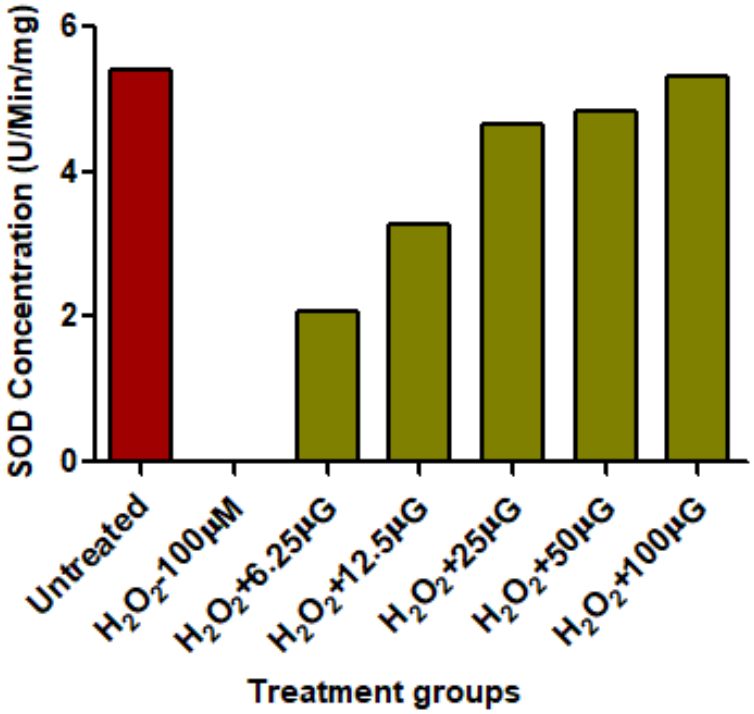

Fig. 8: SOD concentration for HePG2 cells treated with H_2O_2 and the plant extract at different concentrations**.**

4 Discussion

The phytochemical qualitative and quantitative screening of *Biophytum umbraculum* leaf extract in 70 % hydroalcoholic extract confirmed the presence of several biochemical constituents. The tannin content was maximum and is found to be valuable in treating ulcerated and inflamed tissues. Tannins are known for their usage in cancer prevention and its treatment as well.[32] Flavonoids are known to permeate the cell membrane and present their actions. They inhibit the phospholipase A2 and ATPase enzymes which are membrane bound.[33] As the flavonoids contain anion radicals, they promote the health of living beings and could be useful components in stress related medical conditions.[34] Similarly, *Zingiber officinale* extract,[35] ethanolic extract of *Hibiscus rosa sinensis L* [37] and several other plants were examined to possess phenolic components, flavonoids, and tannins and this have exhibited a strong antioxidant activity [38] and potential application in other fields.[9,39]

The reduction of Fe^{3+} to Fe^{2+} in the presence of considered plant extract is termed as the reducing power of the test samples and it serves as major indicator of its probable antioxidant activity, which is confirmed by reducing power assay. A free radical of DPPH is stable radical, which forms a diamagnetic molecule by accepting hydrogen free radical or an electron. The presence of antioxidants reduces the capability of DPPH radicals and is analysed by reduced absorbance at 517 nm. This enables the use of DPPH radicals as a substrate to assess the antioxidant property of antioxidants. Obtained results confirmed the capability of plant extract to donate the H_2 to free radicals that could remove the anomalous electrons accountable for free radical activity.

Nitric oxide (NO) is one of the significant mediators by neurons, macrophages and endothelial cells. NO is involved in several physiological functions of the living system. The imbalance in the NO production is allied with various diseases. Nitric oxide synthase generates NO in the biological tissues while metabolizing arginine to citrulline through a five-electron oxidation reaction. A liner time-dependent NO is produced when $NaNO_3$ is incubated with a buffered saline for 2 h at 25 ^oC. The extraction was allowed to scavenge the generated NO and the reduced absorbance at 550 nm indicated better scavenging activity. Anion radical of superoxide is a strong ROS amongst other generated free radicals.[40] The SOD analysis results indicated the scavenging activity for these radicals and project the same as strong antioxidant agent. The crude leaf extract of *Helichrysum longifolium,* [39] *Nymphaea lotus,*[40] methanolic extract of *Uncaria gambit,* [41] and many have showed the similar results.

The antioxidant assays investigated by means of DPPH assay, NO inhibition, iron-chelating assay and SOD scavenging activity showed the potency of considered extract of leaves of *Biophytum umbraculum*. Nevertheless, the antioxidant activity is attributed to several mechanisms, such as chain initiation, transition metal ion catalysts binding, peroxides decomposition and abstraction of hydrogen. The proven potency of antioxidant ability is dedicated to phenolic compounds and many other chemical constituents in the *Biophytum umbraculum*. The ROS scavenging activity of the extract is proven by above tests indicating [1] its probable applications in biomedical practices.

Conclusions

In conclusion, the 70% hydroalcoholic, water, ethanol, chloroform and petroleum ether extract of *Biophytum umbraculum* plant leaves were screened quantitatively and qualitatively for the presence of phytochemicals. The 70% hydroalcoholic extract showed the presence of maximum phytochemicals and was further chosen for quantitative investigation and antioxidant activity. Free radical scavenging activity was confirmed with the obtained IC_{50} value of 576.7±25.2 µg/ml. The *in vitro* antioxidant activity was further confirmed by iron-chelating assay. *In vitro* hepatoprotective activity was evident in MTT and ROS assays with % cell viability being 99.28 at 200 *µg/mL* and with 38.05 % ROS inhibition activity at 100 µg/mL *Biophytum umbraculum* leaf extract respectively. NO inhibition and SOD assays also showed dose-dependent response. The hepatoprotective efficiency of the plant extract is owed by the phenolic and other phytochemical constituents. However, the exact mechanism and *in vivo* antioxidant activity needs further exploration.

List of abbreviations

Abbreviations: DPPH: 2,2-Diphenyl-1- Picrylhydrazyl; GAE: Gallic Acid Equivalents; TPC: Total Phenolic Content; TFC: Total Flavonoid Content; TAEq: Tannic Acid Equivalent; TAC: Tannic Acid Content; SOD: Superoxide Dismutase. MTT: (3- [4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide); ROS: Reactive Oxygen Species.

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