

In vitro antioxidant potential of ethanolic extract of *Hypericum hookerianum* and its glycosidic flavonoid enriched extract

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Abstract

Hypericum hookerianum in current scenario have been targeted for the treatment of neurodegenerative diseases. This study was undertaken to assess the *in vitro* antioxidant potential of ethanolic extract of *H. hookerianum* and its glycosidic flavonoid enriched extract.

Dried aerial parts of *H. hookerianum* were extracted with ethanol using soxhlet apparatus. Glycosidic flavonoid enriched extract (GFHH) was separated by acid hydrolysis from ethanolic extract of *H. hookerianum* (EEHH) and performed thin layer chromatography (TLC). Total flavonoid content was determined spectrophotometrically in EEHH and GFHH. *In vitro* antioxidant potential of EEHH and GFHH were evaluated by various free radical scavenging methods like 1,1-Diphenyl- 2-picrylhydrazyl (DPPH), 2, 2'-azino bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), Super oxide (O_2^-), Nitric oxide(NO), 2,2-azo bis (2- amidino propane) di hydro chloride (AAPH), hydroxyl radicals ($OH\cdot$) assays and compared with standard quercetin.

Flavonoids (quercetin and rutin) present in the plant extracts was confirmed by TLC. Total flavonoid content (TFC) was quantified in both the extracts and the concentration of flavonoid was high in GFHH when compared with EEHH extract. In DPPH assay IC_{50} values for EEHH and GFHH were 23.03 $\mu\text{g/ml} \pm 0.21$ and 26.45 $\mu\text{g/ml} \pm 0.42$ respectively. EEHH and GFHH exhibited potent scavenging effects against ABTS with an IC_{50} values of 8.68 \pm 0.65 $\mu\text{g/ml}$ and 7.38 \pm 0.72 $\mu\text{g/ml}$ which are comparatively equivalent to that of standard quercetin IC_{50} (7.17 \pm 0.76 $\mu\text{g/ml}$). In SO assay, IC_{50} values for EEHH and GFHH was found to that 48.42 $\mu\text{g/ml} \pm 0.45$ and 29.48 $\mu\text{g/ml} \pm 0.45$ respectively, in NO scavenging assay IC_{50} values for EEHH and GFHH were 7.75 $\mu\text{g/ml} \pm 0.45$ and 7.3 $\mu\text{g/ml} \pm 0.21$ respectively, in AAPH assay IC_{50} values for EEHH and GFHH were 8.002 $\mu\text{g/ml} \pm 0.45$ and 7.3 $\mu\text{g/ml} \pm 0.21$ respectively, in HRSA assay IC_{50} values for EEHH, GFHH and standard were 17.17 \pm 0.82 $\mu\text{g/ml}$, 15.18 \pm 0.92 $\mu\text{g/ml}$ and 12.17 $\mu\text{g/ml}$ respectively. In all *in vitro* assays, the potential of the radical scavenging is as follows: EEHH < GFHH < quercetin.

Results obtained in the present study have confirmed the antioxidant potential of EEHH and GFHH due to its flavonoidal constituent. These findings support the use of *H. hookerianum* in traditional medicine for treating neurodegenerative diseases and oxidative stress.

Keywords: antioxidants, *Hypericum hookerianum*, flavonoids, free radicals.

Introduction

Oxygen is necessary to all living species for the production of energy to stimulate biological processes. Oxygen consumption in cellular development leads to the formation of a series of reactive oxygen species (ROS) [1]. ROS mainly includes free radicals (super oxide anion radicals- O_2^- , hydroxyl radicals- $OH\cdot$) and non free radicals (Hydrogen peroxide $-H_2O_2$ and singlet oxygen $-^1O_2$) [2]. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced by oxidative metabolism are capable of damaging cellular components through molecular modifications to a poly unsaturated membrane's lipids, proteins, and nucleic acids [3,4]. Free radicals are known to be the key cause of various persistent and degenerative diseases, including aging, coronary heart disease, inflammation, strokes, diabetes mellitus and cancer [5].

Antioxidants can defend the living organisms from free radicals and ROS effects. They slow down the progress of many chronic diseases as well as lipid per oxidation [6,7]. Recently there has been an increasing interest in the study of traditional plants for pharmaceutical applications because of its low toxicity and economic viability. In the past, a range of plant phyto chemicals *viz* phenolic compounds, flavonoids and tannins reported to possess major antioxidant activity against a wide variety of free radicals [8][9]. These active compounds can be isolated and developed as natural drugs for avoidance and treatment of free radical related disorders [10]. Flavonoids are broadly existed in the plant kingdom, with a listing of 10,000 known structures [11-13]. The most commonly-consumed flavonoids are quercetin, luteolin and apigenin. They show mainly in the glycosidic forms with residues such as: D-glucose, L-rhamnose, galactose, arabinose (eg., quercetin-3- glucoside, quercetin-3-rhamnoside, quercetin-3-

galactoside, quercetin-3-rutinoside (rutin) [14]. Flavonoids are of big concern for their bioactivities, such as neuro protective, anti-inflammatory effects, anti-cancer, anti-genotoxic, anti- Alzheimer's disease, and antiglycative activity [15,16][17] which are principally correlated to their antioxidant properties [18].

The genus *Hypericum* encompasses various species used in traditional medicine worldwide [19]. In neuro psychopharmacology researchers mainly focused on *Hypericum* species due to their plenty of health benefits by synergistic antioxidant activity of phyto constituents. *Hypericum hookerianum* Wight and arnot is a well known ornamental plant among the 20 different species of *Hypericum* found in India.

Previous studies on *H. hookerianum* already reported the antibacterial [20], antitumor [19], anxiolytic [21] and wound healing [22,23] properties.

Perusal of literature revealed that there are no studies conducted so far regarding the comparative antioxidant activity of aerial parts of ethanolic extract of *H. hookerianum* and its glycosidic flavonoids enriched extract. Therefore, the current study investigated the antioxidant potential of ethanolic extract of *H. hookerianum* and its glycosidic flavonoids by various *in vitro* free radical scavenging methods.

Materials and Methods

Collection and validation of samples

The aerial parts of *H. hookerianum* were collected from the Nilgiris, Western Ghats of Tamil Nadu, India. The plant was authenticated by Dr. S. Rajan, Field Botanist, Survey of Medicinal Plants and Collection Unit, (Central Council for Research in Homoeopathy), and Department of AYUSH, Ooty, Tamil Nadu, India. The collected aerial plant parts were subjected to shade drying for about 5 weeks. The dried *H. hookerianum* was further crushed to powder mechanically by pulverizer, sieved and stored in airtight container for further analysis.

Chemicals

All the chemicals used in this study were of analytical grade and were purchased from Sigma Life Sciences, Mumbai, India.

Preparation of plant extract

The shade dried aerial parts of *H. hookerianum* was extracted with pet ether, chloroform and ethanol successively by the soxhlation method at room temperature and concentrated over water bath and evaporated under reduced pressure. The ethanolic extract obtained was filtered and the solvent was evaporated at 50°C under reduced pressure, and then lyophilized.

Phytochemical screening

For preliminary screening of phytoconstituents, EEHH was subjected to different qualitative tests [24-25].

Separation of flavonoidal glycosides enriched extract by acid hydrolysis method

About 25 g of ethanolic extract of *H. hookerianum* (EEHH) was dissolved in 30 mL - 2N HCl: MeOH (1: 1 v/v), sealed in a screw-cap bottle and heated on a steam bath for 30 min. The mixture was extracted with an equal volume of ethyl acetate; the upper organic layer was collected separately and subsequently evaporated to dryness under reduced pressure [26]. The residue was dissolved in ethanol and simultaneously the presence of flavonoids was analyzed by thin layer chromatography. The aqueous layer was analyzed for sugar using Fehling's solution, which was found to be positive and confirmed the presence of glycosidic derivatives.

Thin layer chromatography

For thin layer chromatographic studies of flavonoids (mainly quercetin and rutin), precoated silica gel 60 F₂₅₄ aluminum plates of size 20x20cm were used. EEHH and GFHH were dissolved in respective solvents (ethanol) and the spots were applied with the help of fine capillary tubes. Mobile phase of Toluene: Ethyl acetate: Formic acid with a ratio of 50:40:10 (V/V/V) was performed to improve the separation and identification of quercetin and rutin in the samples. Ascending development of the plates was performed at room temperature (25°C ±2°C) with the solvent system in a Camag twin trough chamber, formerly saturated with the mobile phase for 30 min. The average development time was 15min and after the development, the plates were air dried and the spots were detected. The color and R_F values were recorded using with the UV chamber with the range of UV_{254 nm} and UV_{366 nm}. The detection limit of the samples was 10µg [27].

Determination of total flavonoid content (TFC)

Total flavonoid content was determined with aluminium chloride (AlCl₃) method [28], quercetin as the reference standard. EEHH and GFHH (0.1ml) extracts were diluted to 0.3ml with double distilled water individually and 0.03ml solution of 5% NaNO₂ was added, after the 5 minutes later, 0.2ml of 1mM NaOH was added to the reaction mixture. At the end level, the reaction mixture was diluted to 1ml with water and absorbance was measured at 510 nm by spectrophotometer and the experiments were performed in triplicates and the values are recorded as mean ±SD. This total flavonoid content in EEHH and GFHH was determined by quercetin standard curve and is expressed as % equivalent of quercetin.

Antioxidant activity by free radical scavenging assays

DPPH radical scavenging activity

The free radical scavenging activity of EEHH and GFHH was determined using DPPH radical method [29]. 0.1mM concentration of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) in ethanol was prepared and 1.0 mg/ml of this solution was added to 3.0 ml of EEHH and GFHH (10-50 µg/ml). After 30 minutes, the absorbance was

measured at 517 nm using spectrophotometer. The capacity to scavenge the DPPH radical was calculated using the following equation:

$$\% \text{ inhibition of EEHH} = [(Abs \text{ control} - Abs \text{ sample } 1)] / (Abs \text{ control})] \times 100$$

$$\% \text{ inhibition of GFHH} = [(Abs \text{ control} - Abs \text{ sample } 2)] / (Abs \text{ control})] \times 100$$

Where Abs control is the absorbance of DPPH and Abs sample (1, 2) is the absorbance of the DPPH radical + sample extract and quercetin was used as a standard. The half maximal inhibitory concentration (IC_{50}) values denoted the concentration of sample required to scavenge 50% of DPPH free radicals. All the experiments were carried out in triplicates and the values are recorded as mean \pm SD [29].

ABTS Radical Scavenging Activity

The ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging activity of EEHH and GFHH were determined by Rice-Evans method [30]. ABTS is a cation radical ($ABTS^{\cdot+}$) developed by reacting ABTS solution (7 mM) with 2.45 mM ammonium per sulphate and the mixture was allowed to stand in dark at room temperature for 12-16 hrs before use. Different concentrations (10-50 μ g/ml) of EEHH, GFHH and standard quercetin (0.5 ml) were added individually to 0.3 ml of ABTS solution and the end volume was made up to 1 ml. The absorbance was read at 745nm using spectrophotometer and the % inhibition was calculated and quercetin was used as a standard. The experiments were performed in triplicates and the values are recorded as mean \pm SD. The ABTS scavenging activity was calculated according to the following formula,

$$\% \text{ inhibition of EEHH} = [(Abs_{\text{control}} - Abs_{\text{sample } 1}) / (Abs_{\text{control}})] \times 100$$

$$\% \text{ inhibition of GFHH} = [(Abs_{\text{control}} - Abs_{\text{sample } 2}) / (Abs_{\text{control}})] \times 100$$

Where Abs_{control} is the absorbance of ABTS and methanol and Abs sample (1, 2) is the absorbance of the ABTS radical + sample extract.

Superoxide Anion Scavenging Assay

The scavenging activity of samples (EEHH, GFHH) towards superoxide anion radicals were determined by the method of Nishimiki, 1972 [31]. About 1ml of Nitro blue tetrazolium solution (NBT) (156 μ M in 100 mM phosphate buffer, pH 7.4), 1 ml nicotine amide adenine dinucleotide (NADH) solution (468 μ M in 100 mM phosphate buffer, pH 7.4) and 0.1ml of different concentrations of EEHH, GFHH and quercetin in water were mixed individually. The reactions for all three mixtures were initiated by adding 100 μ l of phenazine metho sulphate (PMS) solution (60 μ M) in 100 mM phosphate buffer, (pH 7.4) to the containing test tubes. The reaction mixtures were incubated at room temperature for 5 min and the absorbance was read at 560 nm against reagent blank

using spectrophotometer. The superoxide anion scavenging activity was calculated according to the following formula:

$$\% \text{ inhibition of EEHH} = [(Abs \text{ sample } 1 - Abs \text{ control})] / (Abs \text{ sample } 1)] \times 100$$

$$\% \text{ inhibition of GFHH} = [(Abs \text{ sample } 2 - Abs \text{ control}) / (Abs \text{ sample } 2)] \times 100$$

Where Abs control is the absorbance of reaction mixture without test substances and Abs sample (1,2) is the absorbance of the reaction mixture + sample extracts (EEHH/GFHH/standard (quercetin)).

Nitric oxide Scavenging potential

Sodium nitro prusside SNP (10 mM) in phosphate buffer saline (PBS) was mixed with different concentrations (10-50 μ g/ml) of EEHH and GFHH and then incubated at 25 C. The samples were added to the Greiss reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophore (colored substance) formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine was read at 546 n and referred to reagent as a positive control. The experiments were performed in triplicates and the values are recorded as mean \pm SD. The percentage of inhibition was measured by the following formula:

$$\% \text{ inhibition of EEHH} = [(Abs \text{ control} - Abs \text{ sample } 1)] / (Abs \text{ control})] \times 100$$

$$\% \text{ inhibition of GFHH} = [(Abs \text{ control} - Abs \text{ sample } 2)] / (Abs \text{ control})] \times 100$$

Where Abs control is the absorbance of only the reaction chromophore without test samples and Abs sample (1, 2) is the absorbance of the reaction mixture + sample extract/standard (quercetin) [32].

Per oxy radical scavenging activity (AAPH assay)

An azo initiator, AAPH, was used to produce peroxy radicals, and the scavenging activity of the extracts was examined by spectrophotometric analysis[33]. The initiation of DCF (2,2'-dichlorofluorescein-diacetate) reaction was achieved by addition of DCF (3.41 μ l of 50 μ g/ml solution) and NaOH (1.75 ml of 0.01N solution) and allowed the mixture to stand for 20 min before the addition of 18.25 ml of sodium phosphate buffer (25 mM, pH 7.2). The reaction mixture contained 10 μ l of extract (diluted to final concentrations of 10, 20, 30, 40 and 50 μ g/ml), 170 μ l activated DCF solution and 20 μ l of 600 mM AAPH (adjusted to a final concentration of 60 mM). The reaction was initiated by the addition of AAPH solution. After 10 min, the absorbance was read at 490 nm using a Spectrophotometer. Percentage of inhibition and IC_{50} value was calculated for EEHH, GFHH extracts and the values are compared with standard quercetin. The experiments were performed in triplicates and the values are recorded as mean \pm SD. The percentage of inhibition was measured by the following formula:

% inhibition of EEHH = [(Abs control – Abs sample 1)] / (Abs control)] 100

% inhibition of GFHH = [(Abs control – Abs sample 2)] / (Abs control)] 100

Where Abs control is the absorbance of reaction mixture without test substances and Abs sample (1, 2) is the absorbance of the reaction mixture + sample extracts (EEHH/GFHH/standard (quercetin)).

Hydroxyl radical scavenging activity (HRSA)

Hydroxyl radical scavenging (OH \cdot) activity was carried out by measuring the contest between deoxyribose and the EEHH, GFHH for hydroxyl radicals generated from the Fe $^{3+}$ /ascorbate/EDTA/H $_2$ O $_2$ method[34]. Hydroxyl radical formed from above reagents combined with sugar moiety of DNA i.e., deoxy ribose led to the formation of TBARS (thio barbituric acid-reactive substances) and it was measured by Ohkawa *et al*/method [35]. The EEHH and GFHH extracts were added to the reaction mixture containing 2.8 mmolL $^{-1}$ deoxyribose, 100 μ mol L $^{-1}$ FeCl $_3$, 104 μ mol L $^{-1}$ EDTA, 100 μ mol L $^{-1}$ ascorbic acid, 1mmol L $^{-1}$ hydrogen peroxide and 230 mmol L $^{-1}$ phosphate buffer (pH 7.4), making a final volume of 1.0 mL . One mL of thiobarbituric acid TBA (1%) and 1.0 mL trichloroacetic acid (TCA 2.8%) were added to the test tube and incubated at 100 C for 20 min. The above mixture was cooled and absorbance was measured at 532 nm against a blank containing deoxyribose and buffer without extracts. The reaction mixture was incubated at 37 C for 1 h. The experiments were performed in triplicates and the values are recorded as mean \pm SD. The HRSA activity of EEHH, GFHH extracts was calculated by the following formula:

% inhibition of EEHH = (1- absorbance of sample 1 /absorbance of control) x 100

% inhibition of GFHH = (1- absorbance of sample 2 /absorbance of control) x 100

Where Abs control is the absorbance of reaction mixture without test substances and Abs sample (1, 2) is the absorbance of the reaction mixture + sample extracts (EEHH/GFHH/standard (quercetin)).

Statistical analysis

All the values are expressed as mean \pm SD from three separate experiments for all the scavenging assays. For in vitro antioxidant assays one way ANOVA test followed by Turkey's test (P < 0.05) was used to analyze the differences among IC $_{50}$ values of EEHH, GFHH and quercetin for all the *in vitro* antioxidant assays. The IC $_{50}$ Values were determined using the Graph Pad Prism 5 software.

Results

Phytochemical screening of EEHH showed the presence of phenols, tannins, flavonoids, terpenoids antraquinones, reducing

sugars, saponins and glycosides. GFHH extract mainly showed the presence of flavonoids, reducing sugars, glycosides (Table 1).

Table 1: Phytochemical analysis of EEHH and GFHH

Phytochemicals	EEHH	GFHH
Phenols	++	-
Tannins	++	-
Flavonoids	++	++
Terpenoids	++	-
Antraquinones	++	-
Reducing sugars	++	++
saponins	++	-
glycosides	++	++

TLC analysis of EEHH and GFHH

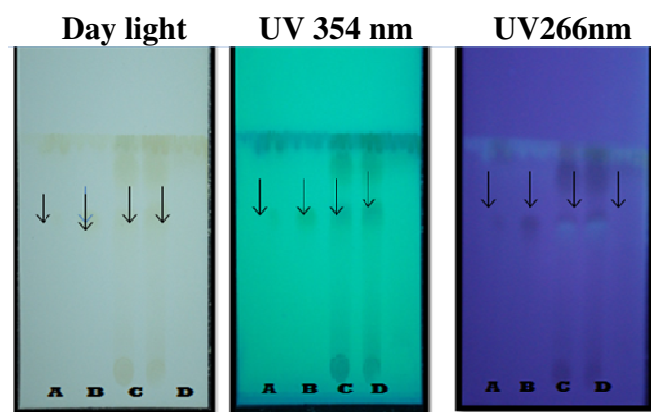


Figure 1.Thin layer chromatography of EEHH and GFHH in various lights .The extracts dissolved in suitable solvents were chromatographed in solvent system showing Toluene: Ethyl acetate: Formic acid with a ratio of 50:40:10 (V/V/V). Arrows indicates that the presence of flavonoids.

Table 2: R $_f$ Values of quercetin and rutin on TLC of EEHH and GFHH

Sample	bands	R $_f$ values
Quercetin(A)	1	0.49
Rutin (B)	1	0.51
EEHH (C)	2	0.48,0.51
GFHH(D)	2	0.48,0.51

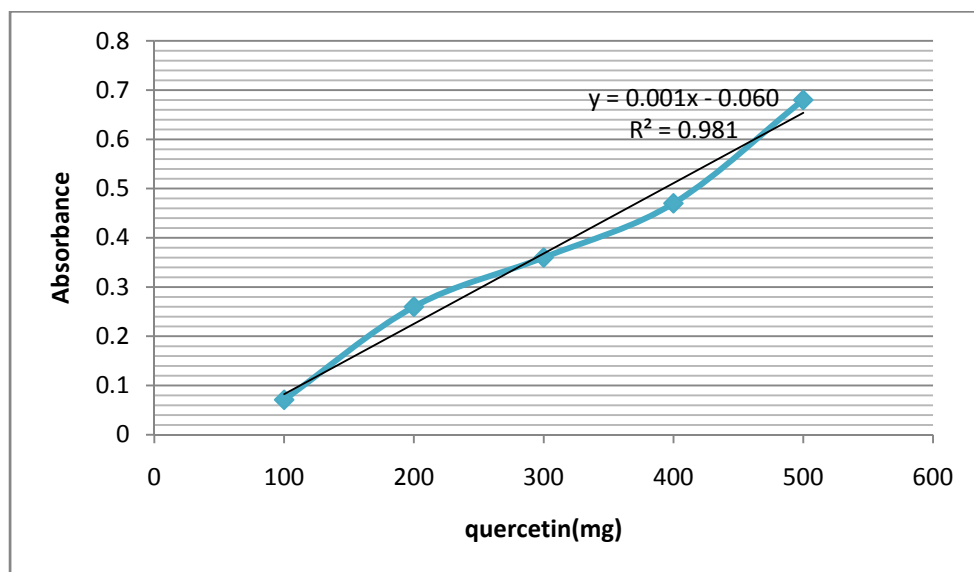


Fig 2. Determination of Total flavonoid content

EEHH and GFHH were subjected to thin layer chromatography with the suitable solvent system for the flavonoids and the bluish bands indicating the high polar soluble nature of flavonoids in both the extracts. The R_f value for quercetin(A) = 0.49, R_f value for rutin (B) = 0.51, R_f value for EEHH(C) = 0.48, 0.51, R_f value for GFHH (D) = 0.48, 0.512. The R_f values of EEHH and GFHH comparable with the standards of quercetin and rutin, indicates the presence of these flavonoids in both the extracts.

Determination of Total flavonoids contained in EEHH and GFHH

There was a significant difference in total flavonoid in EEHH and GFHH which the content was 29.97 ± 0.67 % mg Eq of quercetin and 51.62 ± 0.29 % Eq of quercetin. The acid hydrolysis method has been used to recover more flavonoid from plant extract with the removal of other insoluble compounds.

Free radical scavenging assays

Figure 3 (a), 3 (b), 3 (c), 3(d), 3(e), 3(f) depicts the antioxidant potential of EEHH and GFHH by scavenging potential of various free radicals like DPPH, ABTS, Super oxide, Nitrous oxide, AAPH assay, Hydroxyl radical assays.

DPPH radical scavenging activity

The dose-response curve of DPPH radical scavenging activity of the EEHH and GFHH were compared with quercetin (Figure 3 (a)). In this study IC_{50} values obtained for EEHH and GFHH were $26.45 \mu\text{g/ml} \pm 0.21$ ($r^2=0.9780$; $P<0.0001$), $23.03 \mu\text{g/ml} \pm 0.42$ ($r^2=0.9959$; $P<0.0001$) respectively and the values of both the extracts were

found to be comparable with the quercetin standard ($20.17 \pm \mu\text{g/ml} \pm 0.29$).

ABTS radical scavenging activity

The scavenging activity of ABTS of plant extracts was increased in a dose dependent manner as illustrated in figure 3(b). EEHH exhibited potent scavenging effects against ABTS with an IC_{50} value of $8.68 \pm 0.65 \mu\text{g/ml}$ ($r^2=0.9071$; $P<0.0001$) and GFHH shown that IC_{50} value of $7.38 \pm 0.72 \mu\text{g/ml}$ ($r^2=0.9868$; $P<0.0001$) which is almost equivalent to that of standard quercetin IC_{50} value ($7.17 \pm 0.76 \mu\text{g/ml}$).

Superoxide radical scavenging activity

In super oxide scavenging activity, decrease in the absorbance at 560 nm for both the extracts indicated the consumption of free radical ions (O_2^-). EEHH and GFHH were showed strong scavenging potential against super oxide ion with the IC_{50} values of $48.42 \mu\text{g/ml} \pm 0.45$ ($r^2=0.9751$; $P<0.0001$) and $29.48 \mu\text{g/ml} \pm 0.45$ ($r^2=0.9868$; $P<0.0001$) respectively. The IC_{50} value of standard quercetin for this assay was $6.77 \mu\text{g/ml} \pm 0.27$.

Nitric oxide scavenging activity

In the present study, EEHH and GFHH exhibited potent nitric oxide radical scavenging activity, which competes with oxygen to react with nitric oxide and thus inhibits the generation of nitrite. The IC_{50} values of EEHH and GFHH were $7.75 \mu\text{g/ml} \pm 0.45$ ($r^2=0.977$; $P<0.0002$), and $7.13 \mu\text{g/ml} \pm 0.21$ ($r^2=0.9854$; $P<0.0032$) respectively. These values were significantly identical with that of standard quercetin ($6.76 \mu\text{g/ml} \pm 0.27$).

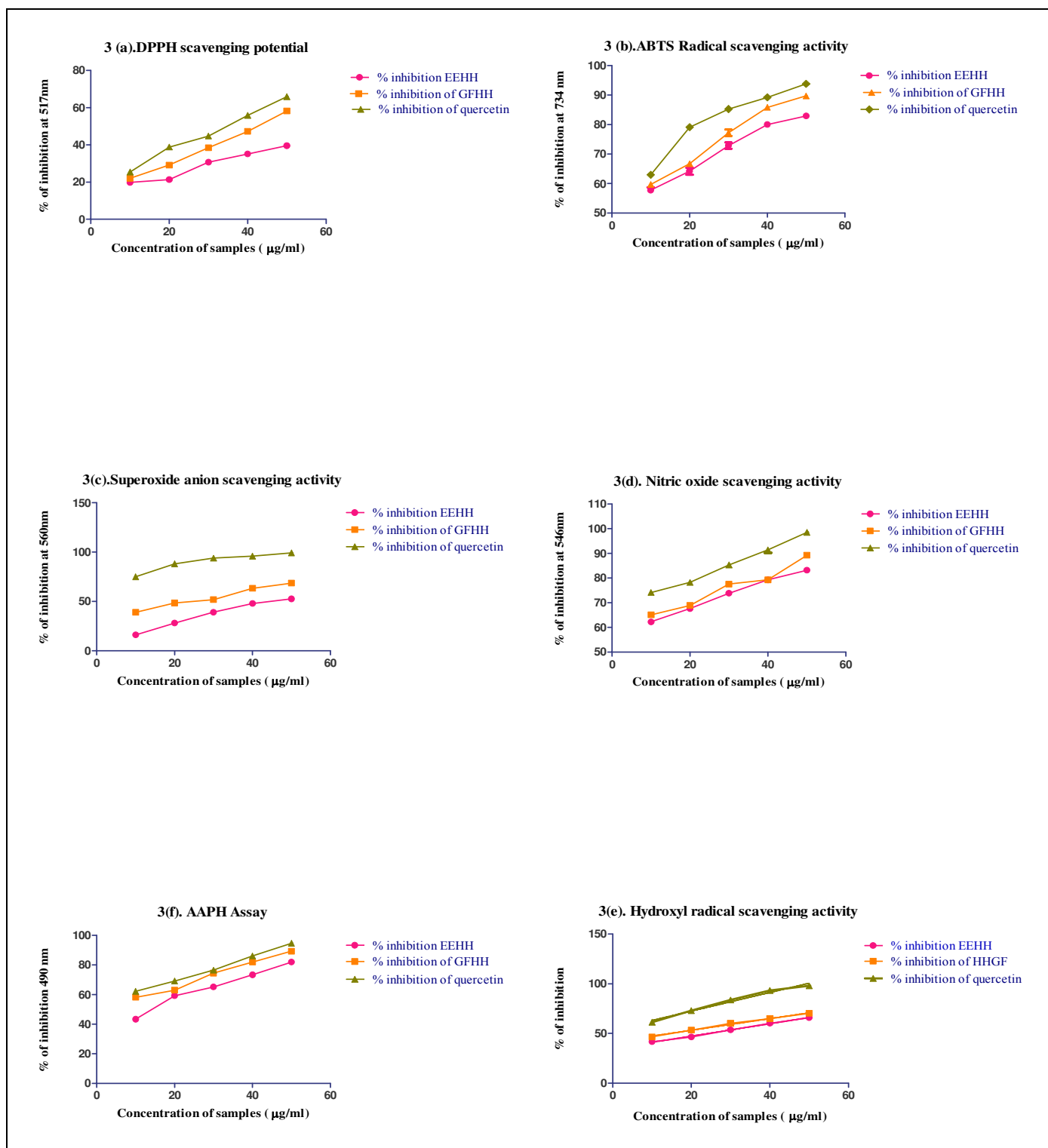


Figure 3: Scavenging activity

AAPH / DCF assay

The azo scavenging activity of EEHH and GFHH were shown by dose dependent manner. In this assay, both the extracts exhibited more scavenging activity and they were comparable with the standard quercetin. The IC₅₀ values for EEHH and GFHH were 8.002 µg/ml ± 0.45 ($r^2 = 0.9996$; $P < 0.0020$), and 7.3 µg/ml ± 0.21 respectively ($r^2 = 0.975$; $P < 0.0005$) and the values were comparatively identical with the standard quercetin (7.03 µg/ml ± 0.27).

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was calculated by measuring the inhibition of the degradation of 2- deoxyribose by the free radicals generated by the Fenton reaction. The IC₅₀ values of the EEHH, GFHH and quercetin were 17.17 ± 0.82 µg/ ml ($r^2 = 0.9912$; $P < 0.0001$), 15.18 ± 0.92 µg/ ml ($r^2 = 0.9856$; $P < 0.0001$), 12.17 ± 0.629 µg/ml respectively.

Discussion

Currently free radicals have aroused considerable interest in research and this has led to exploit therapeutic potential of natural antioxidants. Antioxidants wrestle against free radicals and guard us from different diseases. They exert their action either by scavenging the reactive oxygen species or shielding the antioxidant protection mechanisms [36].

The electron donation capability of natural phytochemicals can be measured by 2, 2'-diphenyl-1- picryl hydrazyl radical (DPPH) purple-colored solution bleaching [37], the method is based on scavenging of DPPH through the addition of a radical species or antioxidant that fades the DPPH solution. The degree of color change is directly proportional to the concentration and scavenging potency of the antioxidants. In the present study both the EEHH and GFHH showed significantly higher inhibition percentage and positively correlated with total flavonoid content. The results of this study recommend that the plant extracts contain phytochemical constituents like flavonoids confirmed by qualitative, quantitative and TLC analysis and they are capable of donating hydrogen to a free radical to scavenge the possible damage.

ABTS radical scavenging is an example for proton radical scavenging potential of antioxidants [38]. The protonated radical ABTS has characteristic absorbance maxima at 734 nm, which decreases with the scavenging of proton radicals. Even in the present study the scavenging activity of the ABTS radical by the EEHH and GFHH were found to be significant. This implies that these plant extracts may be helpful for treating free radical-related pathological damage (particularly at a higher concentration).

Hydroxyl radical is one of the powerful reactive oxygen species in the living organisms. It reacts with PUFA (Poly unsaturated fatty acid) moieties of cell membrane phospholipids and causes injury to cell. The hydroxyl radical is regarded as a harmful agent in patho physiological conditions and capable of damaging nearly every molecule of biological system and leads to carcinogenesis, mutagenesis and cyto toxicity [39]. Hydroxyl radical scavenging capacity of both the extracts was directly proportional to its

antioxidant activity which leads to fading of red color [40]. Here both the extracts of *H.hookerianum* when added to the reaction mixture energetically scavenged the hydroxyl radicals and prohibited the degradation of 2-deoxyribose present in the reaction mixture.

Nitric oxide was generated from sodium nitro prusside (SNP) and calculated by the Greiss reaction. SNP in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be determined by use of Greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide [41]. In nitrous oxide scavenging activity GFHH showed more scavenging potential than EEHH and the values are comparable with the standard quercetin.

Superoxide anion is also very dangerous to cellular mechanism [42]. Flavonoid as natural antioxidants from plant extracts or natural resources due to their chemical interactions has been reported by Robak and Glyglewski [43]. In this study both the plant extracts EEHH and GFHH showed significant scavenging potential of superoxide anions due to their flavonoidal constituents. GFHH exhibited more scavenging potential against superoxide anion than EEHH which is attributable of its higher concentration of flavonoids.

AAPH scavenging method is also known as Total Radical trapping Antioxidant Parameter (TRAP) and mainly used to measure the Oxygen radical absorbance capacity (ORAC) of the samples for commercial purpose [44,45]. In this assay EEHH and GFHH extracts exhibited strong scavenging activity against AAPH and the activity due to their flavonoidal constituents and the values similar with standard quercetin.

The genus *Hypericum* encompasses many species used in conventional medicine in many countries against neurodegenerative diseases [46]. This group has already been proven as well-known antioxidants [47]. *Hypericum perforatum* is a subspecies of *H.hookerianum* and its different standardized extracts showed well pronounced antioxidant activity which correlate with the constituents of flavonoids like quercetin, rutin and hyperoside [48]

Flavonoids referred as potent antioxidants and these are the chief source of plant derived secondary metabolites of poly phenols [49]. Flavonoids are naturally occurring in plants and are thought to have constructive effects on human health. Studies on flavonoidic derivatives have shown a broad range of antibacterial, antiviral, anti inflammatory, anticancer, and anti-allergic activities [50,51].

Flavonoids have been shown to be greatly efficient scavengers of most oxidizing molecules, including singlet oxygen, and different free radicals concerned in numerous diseases [52].

Flavonoids have been reported to be partly responsible for antioxidant activity, as they act on enzymes and pathways involved in anti-inflammatory processes. Furthermore, the hydrogen-donating substituents (hydroxyl groups) attached to the aromatic ring structures of flavonoids enable them to undergo a redox reaction [53].

In this study GFHH showed more scavenging potential in all performed assays than EEHH comparatively correlation with more concentration of flavonoids in GFHH.

Conclusion

The present study clearly gave evidence about presence of quercetin and rutin in ethanolic extract of *H.hookerianum* and glycosidic flavonoid enriched extract of *H.hookerianum*. This

investigation confirms the high *in vitro* antioxidant potential of GFHH than EEHH with respect to its higher concentration of flavonoidal constituents. The results of the present study indicated that both (EEHH and GFHH) might be proposed as a dietary supplement or drug for the treatments of various neuro degenerative diseases. A comprehensive work is being undertaken to explore its neuro antioxidant activity by *in vivo* models.

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