Chapter 16

Production of Gymnemic Acid from Cell Suspension Cultures of *Gymnema sylvestre*

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Abstract

Gymnema sylvestre R. Br. is a popular herbal medicine. It has been used in ayurvedic system of medicine for thousands of years. It is popularly called as "Gur-mar" for its distinctive property of temporarily destroying the taste of sweetness and is used in the treatment of diabetes. The leaves of gymnema possess antidiabetic, antimicrobial, anti-hypercholesterolemic, anti-sweetener, anti-inflammatory, and hepatoprotective properties and have traditional uses in the treatment of asthma, eye complaints, and snake bite. The leaves contain triterpene saponins such as gymnemic acid which is an active ingredient of *Gymnema*. Since the cultivation of *G. sylvestre* is a very slow process and the content of gymnemic acid depends on the environmental factors, cell suspension culture is sought as an alternative means for the production of *Gymnema* biomass and to enhance the gymnemic acid content. In this chapter, the methods employed for the induction of callus and subsequent establishment of cell suspension cultures for the production of biomass and analysis of gymnemic acid using high performance liquid chromatography are described.

Key words Antioxidant activity, Cell suspension cultures, *Gymnema sylvestre*, Gymnemagenin, Gymnemic acid, Herbal medicine, Triterpenoid saponin

1 Introduction

Gymnema sylvestre R.Br., a woody climber of the Asclepiadaceae family, has been known for many years for its medicinal value and it has a key place in ayurvedic medicine. It is popularly called as "Gurmar" for its distinctive property of temporarily destroying the taste of sweetness and is used in the treatment of diabetes [1]. It possesses potent antidiabetic properties and has traditional use in the treatment of asthma, eye complaints, and snake bite. It also possesses antimicrobial, anti-hypercholesterolemic, and hepatoprotective properties [2]. The leaf extract of this plant is used as stomachic, stimulant, laxative, diuretic, anti-sweetener [3], and possesses antiviral and anti-inflammatory [4] activities. The leaves of the species contain triterpene saponins such as gymnemic acid, deacyl gymnemic acid, gymnemagenin [5, 6], 23-hydroxylnogispinogenin, and

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gymnestrogenin [7, 8] belonging to the oleanane and dammarene classes, the former being the gymnemic acid and gymnemasaponins, and the latter gymnemaside. The aglycone of gymnemic acid is known as gymnemagenin.

The antidiabetic components of the plant are identified as a group of closely related gymnemic acids following their successful isolation and purification from the leaves [9]. Recently, gymnemic acid formulations have been found to be useful against obesity [1]. In folk medicine, these compounds are obtained as extracts or infusions from *G. sylvestre* wild or cultivated plants, causing a drastic decrease in this plant population. The production of gymnema by field cultivation is a very slow process and needs 4–6 years from planting to the harvesting stage. Further, its production is hindered by environmental factors, pests, and diseases, and the quantity of gymnemic acid, the active principle in *Gymnema* leaves, is, however, variable among accessions from different ecoclimatic regions [10]. A way to mitigate this problem is by developing plant tissue culture techniques for this species, since plant cell/organ cultures are not limited by environmental, ecological, and climatic conditions, and thus, cells/organs can be preferred to proliferate at higher growth rates than whole plant in cultivation [11]. Therefore, in vitro cell suspension cultures have become an alternative source for the production of Gymnema biomass and gymnemic acid. Additionally, there is possibility for year round production of biomass with reduced cost and time. In the present chapter, the relevant methods employed for the induction of callus from leaf explants of in vitro grown *G. sylvestre* seedlings using Murashige and Skoog (MS) medium supplemented with various growth regulators and subsequent proliferation of the callus in the MS medium are described, and the establishment of cell suspension cultures for the production of biomass and analysis of gymnemic acid using high performance liquid chromatography are discussed.

2 Materials

2.1 Induction of Callus from Leaf Explants of In Vitro Grown Seedlings and Establishment of Suspension Cultures

1. *Gymnema sylvestre* R. Br. seeds were collected from the botanical garden of Karnatak University campus, Dharwad, Karnataka, India (Fig. 1a).
2. Murashige and Skoog (MS) [12] medium stock solutions (MS stock I, II, III, and IV) (Table 1). Store in the cold room or in the refrigerator at 4 °C (*see* **Note 1**).
3. Stock solutions (10 mg/10 ml) of 2,4-dichlorophenoxy acetic acid (2,4-D) and kinetin. Store in the deep freezer at −20 °C.
4. Agar-agar to be used at 8 g/L.

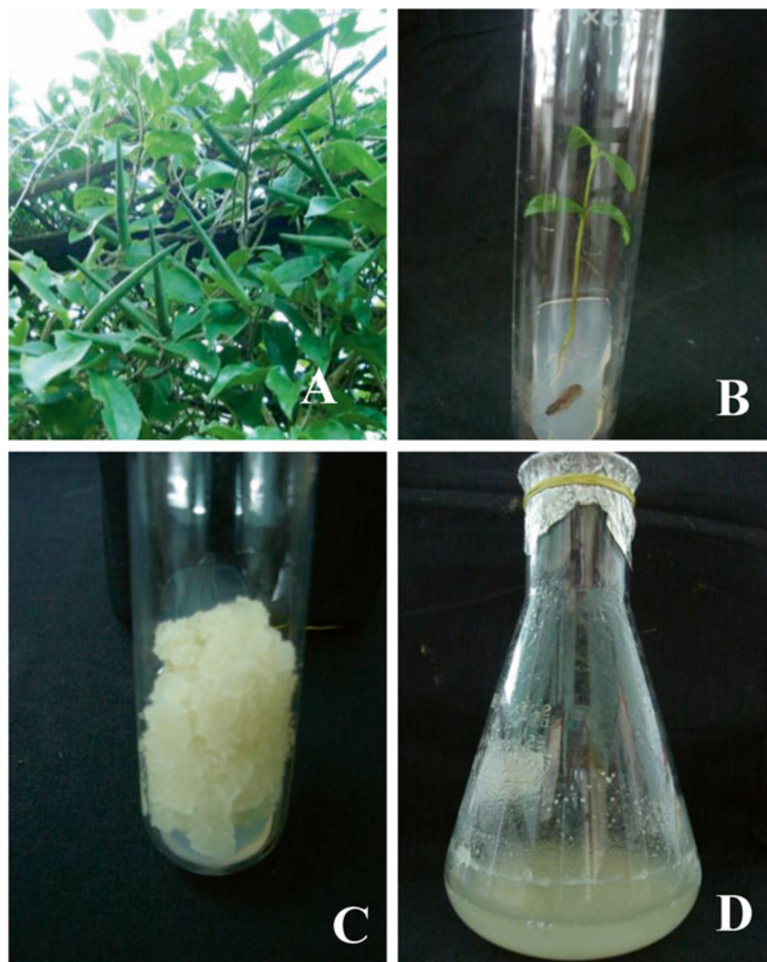

Fig. 1 Habit of the *Gymnema sylvestre* plant with fruits (**a**), 4-week-old in vitro grown seedling (**b**), callus developed from gymnema leaf on MS medium supplemented with 30 g/L sucrose, 2.0 mg/L 2,4-D, and 0.1 mg/L kinetin (**c**), proliferation of cell suspension in liquid medium (**d**)

2.2 Drying of Gymnema Cell Suspension Culture and Extraction of Gymnemic Acid

1. Hot air oven.
2. Heat reflux extraction unit.
3. Extraction solvent (1:1 volume of methanol–water).
4. Potassium hydroxide (11 %).
5. Concentrated HCl.
6. Nylon filter (0.45 μm).

2.3 Estimation of Total Phenols

1. 0.2 N Folin–Ciocalteu (FC) reagent. Store at 4 °C.
2. 1000 ppm gallic acid stock solution. Store at 4 °C in dark condition (*see* **Note 2**).
3. 15 % sodium carbonate solution. Store at 4 °C.
4. 80 % methanol, HPLC grade.

Table 1
Chemical composition of MS medium[a]

Chemical constituents	Concentration (mg/L)	Volume per liter (ml)
Major inorganic nutrients		
NH_4NO_3	33,000	50
KNO_3	38,000	
$CaCl_2 \cdot 2H_2O$	8800	
$MgSO_4 \cdot 2H_2O$	7400	
KH_2PO_4	3400	
Minor inorganic nutrients		
KI	166	5
H_3BO_3	1240	
$MnSO_4 \cdot 2H_2O$	4460	
$ZnSO_4 \cdot 7H_2O$	1720	
$Na_2.MoO_4 \cdot 2H_2O$	50	
$CuSO_4 \cdot 5H_2O$	5	
$CoCl_2 \cdot 6H_2O$	5	
Iron source		
$FeSO_4 \cdot 7H_2O$	5560	5
$Na_2EDTA \cdot 2H_2O$	7460	
Organic supplements		
myo-Inositol	20,000	5
Nicotinic acid	100	
Pyridoxine·HCl	100	
Thiamine·HCl	100	
Glycine	400	
Carbon source		
Sucrose	30 g/L	

[a]Dissolve all the stock solutions of appropriate volume in distilled water and make it up to 1 L, adjust the pH to 5.8 (add 8 g/L agar for semisolid medium) and autoclave for 20 min at 121 °C

2.4 Estimation of Total Flavonoids

1. 1000 ppm quercetin stock solution. Store at 4 °C in the dark condition (*see* **Note 3**).
2. 10 % aluminum chloride solution. Store at 4 °C.
3. 1 M potassium acetate solution. Store at 4 °C.

2.5 Radical Scavenging Effect on 2,2-Diphenyl-1-Picrylhydrazyl (DPPH)

1. 2,2′-diphenyl-1-picrylhydrazyl (DPPH).
2. Ultraviolet (UV)–visible spectrophotometer.

2.6 Total Reducing Power Ability

1. 200 mM sodium phosphate buffer (pH 6.6).
2. 1 % potassium ferricyanide.
3. 10 % trichloroacetic acid.

4. 0.1 % ferric chloride.
5. Deionized water.
6. 1000 mg/L vitamin C.
7. Ultraviolet (UV)–visible spectrophotometer.

2.7 Phosphomolybdenum Activity

1. Reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate).
2. Ultraviolet (UV)–visible spectrophotometer.

2.8 HPLC Analysis of Gymnemagenin

1. HPLC-grade acetonitrile (*see* **Note 4**).
2. HPLC-grade water (*see* **Note 4**).
3. Standard gymnemagenin.

3 Methods

3.1 Induction of Callus from Leaf Explants

1. Wash *Gymnema sylvestre* seeds thoroughly under running tap water for 30 min followed by several rinses in distilled water (5×).
2. Surface-sterilize seeds in 2 % sodium hypochlorite solution containing few drops of Tween 20 detergent for 20 min.
3. Carry out the final step of sterilization in a horizontal laminar air flow chamber by rinsing the seeds in sterile distilled water (2×), followed by 0.5 % mercuric chloride solution for 5 min.
4. Finally rinse seeds several times (5×) in sterile distilled water.
5. Inoculate seeds onto MS basal medium (Murashige and Skoog [12]) for germination.
6. Inoculate leaf explants from 4-week-old germinated seedling (Fig. 1b) on MS medium containing 30 g/L sucrose and medium supplemented with 2.0 mg/L 2,4-D and 0.1 mg/L kinetin (KN), pH 5.8.
7. Incubate the cultures at 25±2 °C, with a 16 h photoperiod (40 mmol/m^2/s) provided with 40-W white fluorescent lamps. Within 4 weeks callus will develop from the leaf explants (Fig. 1c).
8. Culture the callus obtained on semisolid MS medium containing 30 g/L sucrose, 2.0 mg/L 2,4-D, and 0.1 mg/L kinetin (KN), pH 5.8 for proliferation.
9. Transfer the cultures to a fresh medium at 2-week intervals. Use actively growing cell line as explants for further experiments.

3.2 Proliferation of Cell Suspension in the Liquid Medium

1. Inoculate actively growing friable cell lines from the semisolid cultures (5 g/L fresh biomass) into a 250 ml Erlenmeyer flask containing 50 ml MS liquid medium supplemented with 30 g/L sucrose, 2.0 mg/L 2,4-D, and 0.1 mg/L kinetin (KN) (Fig. 1d).
2. Incubate cultures at 16-h photoperiod of cool-white fluorescent light (40 μmol/m^2/s) and 25 ± 2 °C and agitate at 110 rpm. Maintain cultures by regular subculturing at 2-week intervals.

3.3 Cell Suspension Cultures for the Production of Gymnemic Acid

1. Collect the cells at exponential growth phase and initiate the suspension cultures for the production of gymnemic acid.
2. Inoculate 5 g/L fresh cell biomass in a 250 ml Erlenmeyer flask containing 50 ml MS medium supplemented with 30 g/L sucrose, 2.0 mg/L 2,4-D, and 0.1 mg/L kinetin (KN).
3. Incubate cultures at 16-h photoperiod of cool-white fluorescent light (40 μmol/m^2/s) and 25 ± 2 °C and agitate at 110 rpm on a rotary shaker. The cells at these conditions will grow and multiply at a faster rate.
4. After 4 weeks of culture, assess the growth of cell suspensions in terms of fresh weight, dry weight, growth ratio, and amounts of phenolics, flavonoids, and gymnemic acid content.

3.4 Estimation of Cell Biomass

1. Filter the cell suspensions through a stainless steel sieve of pore size 0.45 μm to separate cells from the culture medium.
2. Wash the cells thoroughly in sterile water and blot excessive surface water. Record the fresh weight of cells.
3. Record the dry weight after drying the cells at 50 °C for 24 h in a hot air oven to attain a constant weight.
4. Growth ratio can be determined by using the formula: GR = harvested dry biomass (g) − inoculated dry biomass (g)/ inoculated dry biomass (g).

3.5 Preparation of Cell Extract for Analyzing Bioactive Compounds

1. Dry the cells in a hot air oven unit at 50 °C for 24 h. The dried cells can be stored at room temperature (20–35 °C) for a longer duration in the vacuum desiccator.
2. Take 2 g dried powder and perform the extraction at 40 °C for 6 h using 25 ml of 80 % methanol (rotary shaker).
3. Filter the extract through a double layer of Whatman No. 42 filter paper.
4. Re-extract the residue as in **steps 2** and **3** and mix the extracts.
5. Evaporate the solvent using vacuum rotary evaporator.
6. To the residue add 10 ml of 80 % methanol.

3.6 Estimation of Total Phenolic Content in Cell Culture Extract

The amount of total phenol extracted from cell culture extract is analyzed spectrophotometrically by using Folin–Ciocalteu reagent.

1. Mix 500 μl methanolic extract with 2.5 ml 0.2 M freshly prepared Folin–Ciocalteu reagent. Mix the contents well and allow to stand for 6 min.
2. After 6 min, add 2.0 ml 20 % sodium carbonate solution. After incubation at room temperature for 2 h the purple color will develop.
3. Working standards of 20, 40, 60, 80, and 100 ppm of gallic acid are used to plot the standard calibration graph.
4. The absorbance of test solutions is detected at 760 nm on UV–visible spectrophotometer. The readings are compared with the standard graph for gallic acid. The results are expressed as mg of gallic acid equivalent per 100 g dry weight. All the experiments should be carried out in triplicates and each assay should be repeated at least 2 times.

3.7 Estimation of Total Flavonoid Content in Cell Culture Extract

The amount of total flavonoid present in the cell culture extract can be analyzed spectrophotometrically by following the aluminum chloride method.

1. Mix 500 μl methanolic extract with 0.1 ml of 10 % aluminum chloride, 0.1 ml of 1 M potassium acetate, and 4.3 ml of deionized water and incubate at room temperature for 30 min.
2. After incubation, the absorbance of the test solutions is measured on a UV–visible spectrophotometer at 430 nm.
3. Working standards of 20, 40, 60, 80, and 100 ppm of quercetin are used to plot the standard calibration graph.
4. The readings are compared with the standard graph for quercetin. The results are expressed as mg of quercetin equivalent per 100 g dry weight. All the experiments should be carried out in triplicates and each assay should be repeated at least 2 times.

3.8 Radical Scavenging Activity on 2,2-Diphenyl-1-Picrylhydrazyl (DPPH)

1. For the analysis of the antioxidant activity, mix 100 μl aliquots of the extract and make up the volume to 1.0 ml with methanol.
2. To this sample solution add 2.0 ml of 0.06 M methanolic solution of DPPH.
3. Incubate the sample solutions in dark at room temperature for 30 min and assay the reaction mixture at 517 nm using UV–visible spectrophotometer.
4. For eliminating the interference with the DPPH reaction by extract, a blank sample has to be assayed. For the blank (negative control), 1.0 ml of methanol has to be taken, to which add

2.0 ml of 0.06 M methanolic solution of DPPH and incubate as mentioned above. Carry out all the experiments in triplicate and each assay should be repeated at least 2 times.

5. Record the decrease in percentage of absorbance at 517 nm for the extracts and calculate the percentage of radical scavenging activity of DPPH on the basis of observed decrease in the radical. The inhibition percentage or radical scavenging activity can be calculated by using the following formula:

$$\%\text{Inhibition or }\%\text{Radical scavenging activity} = \left[\left(\text{Ab} - \text{As}\right) / \text{Ab}\right] \times 100$$

where Ab is the absorbance of blank (has the highest value), As is the absorbance of sample (has the lowest value). Curves showing inhibition percentage/μl of the extract are used to determine the concentration at which 50 % radical scavenging occurs (IC_{50}).

3.9 Total Reducing Power Ability

1. Mix 100 μl aliquots of the extract and make up the volume to 1.0 ml with methanol.
2. Then, add 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6), and 2.5 ml of 1 % potassium ferricyanide and incubate at 50 °C for 20 min.
3. After incubation, add 2.5 ml of 10 % trichloroacetic acid and centrifuge at $2000 \times g$ for 10 min.
4. Mix the upper layer in each tube (2.5 ml) with 2.5 ml of deionized water and 0.5 ml of 0.1 % ferric chloride.
5. Measure the absorbance at 700 nm against a blank. Carry out all the experiments in triplicate and repeat each assay at least 2 times.
6. The reducing power increases with the increase in absorbance. The total reducing power of the extract is compared to vitamin C as a positive control and the results are expressed as vitamin C equivalent (mM).

3.10 Phosphomolybdenum Activity

1. Mix 100 μl aliquots of the extract combined with 1 ml of the reagent solution.
2. Cap the tubes tightly and incubate in boiling water bath at 95 °C for 90 min.
3. After 90 min, cool the tubes to room temperature.
4. Prepare a blank solution using 1 ml of reagent solution instead of sample.
5. Measure the absorbance of the sample and the blank at 693 nm. Carry out all the experiments in triplicate and repeat each assay at least 2 times.

3.11 HPLC Analysis of Gymnemic Acid

1. Weigh 500 mg dried sample powder and add 50 ml extraction solvent (1:1 volume of methanol–water) and 10 ml of 11 % potassium hydroxide solution in a 500 ml round bottom flask. Reflux the mixture for an hour; add 9 ml HCl and reflux again for 1 h. Cool the mixture to room temperature, filter the extract through 0.45 μm nylon filter and make the volume to 100 ml with extraction solvent and use the clean supernatant for HPLC analysis.
2. Initially wash the chromatographic equipment consisting of degasser, pump, auto sampler, thermostated column compartment, and diode array detector (DAD) with HPLC grade water twice.
3. Carry out the analysis of the gymnemic acid using C18 (5 μm) column. Use acetonitrile–water (80:20) as the mobile phase. The column temperature should be maintained at 27 °C. The injection volume should be 20 μl. The detection of the eluent is measured at 210 nm. Maintain a running time of 30 min and a flow rate of 1 ml/min. For analysis, use three separate injections of each sample.
4. The gymnemagenin can be identified on the basis of the retention time values and the absorbance of the UV spectra in comparison with the standard gymnemagenin (Fig. 2). The confirmation of the peak can also be performed by spiking the extract with the pure standard gymnemagenin.
5. Eluted samples can be detected with a variable dual wavelength detector coupled to the HPLC system, by comparing the UV spectra of the peak with those of authentic reference samples.
6. Prepare standard stock solutions of gymnemagenin as follows: weigh 10.0 mg standard gymnemagenin accurately and dissolve in 10 ml of HPLC grade methanol in 10 ml volumetric flask. Then, dilute stock solutions with methanol to prepare a series of standard solutions with concentrations of 25, 50, 100, and 150 μg/ml for linearity validation.

   The conversion of gymnemagenin to gymnemic acid is done using the formula:

   Molecular weight conversion of gymnemagenin to gymnemic acid (809.0/506.7) = conversion.

4 Notes

1. Prepare stock solutions of major, minor inorganic nutrients, iron source, vitamins, and individual plant growth regulators (Table 1). Store stock solutions at 4 °C, except vitamins which is stored at −20 °C. Avoid storage of the stock solutions for longer durations (not more than 2 months) as they may get

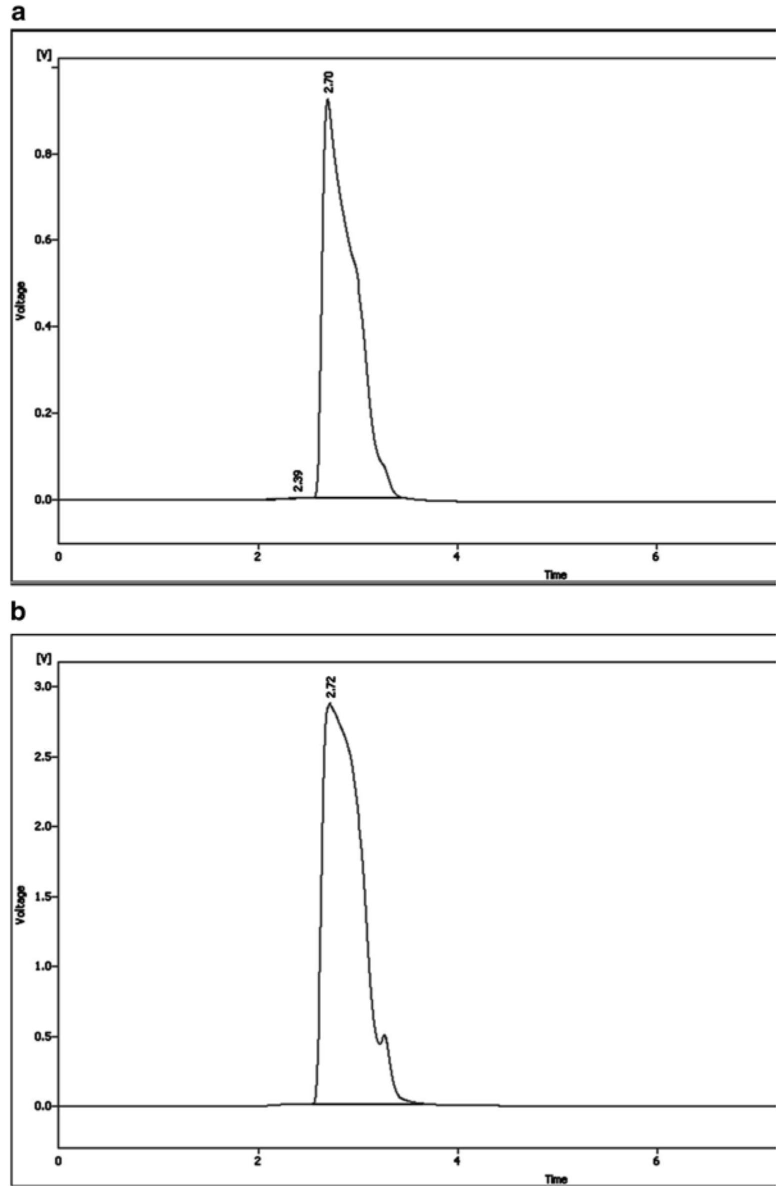

Fig. 2 HPLC profiles of standard gymnemagenin (**a**), cell suspension culture (**b**)

contaminated or precipitated. Prepare fresh plant growth regulator solutions every time. Any color change in the stock solutions may be due to precipitation which can seriously affect the growth of cultures. Alternatively, stock solutions of macro-elements, micro-elements, vitamins, and the ready-to-use MS medium are commercially available.

2. Standard gallic acid is prepared by weighing 1000 mg gallic acid powder and by dissolving it in 1 L distilled water. The addition of 1 ml DMSO or ethanol will readily dissolve the

compound. Store the stock solution in an amber-colored bottle at 4 °C.

3. Quercetin is prepared by weighing 1000 mg quercetin powder and dissolving in 1 L deionized distilled water. Add 1 ml DMSO or ethanol to make the quercetin powder to dissolve readily. Store the stock solution in an amber-colored bottle at 4 °C.
4. Prepare fresh solution of 0.06 M methanolic solution of DPPH and store in darkness at 4 °C in an amber-colored bottle (DPPH reacts with light). All the solutions should be prepared freshly and stored in the refrigerator at 4 °C. All the solvents used for the HPLC analysis should be of HPLC grade and filtered through 0.45 μm polytetrafluoroethylene (PTFE) filters before use.

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