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Phytochemical Screening, Chromatographic Determination and Antioxidant Profiling of Hydroethanolic Extract of Green Coffee Bean Extract

Mamta Saini[1,2], Saurabh Gupta[3], Sushma Devi[1*] and Thakur Gurjeet singh[1]

[1]*Chitkara College of Pharmacy, Chitkara University, Rajpura, Punjab 140401, India.*
[2]*Swami Devi Dyal College of Pharmacy, Golpura, Barwala, 134118, India.*
[3]*Chmaeli Devi Group of Institutions, Indore, Umrikheda, Madhya Pradesh, 452020, India.*

[*]sushma.mehla@gmail.com(Corresponding Author)

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ABSTRACT

Background: Green coffee beans are rich in bioactive compounds, particularly chlorogenic acids, which are known for their health benefits. Phytochemical screening and chromatographic techniques help identify and quantify these compounds. This research explores its phytochemical profile and antioxidant efficacy, contributing to its therapeutic relevance.

Purpose: The current work was conducted to inspect the chromatographic and spectroscopic profile, phytochemical constituents, and antioxidant activity of the hydroethanolic extract of green coffee Arabica.

Methods: The hydroethanolic extract of green coffee beans was extracted from shade-dried beans of green coffee Arabica using the Soxhlet extraction method. The bioactive compounds were identified using HPTLC. An analysis of the antioxidant profile of hydroethanolic GCBE and standards was carried out with the help of 2,2-Diphenyl-1-picryl-hydrazyl (DPPH), 2,2'-casino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS), nitric oxide radical assay, superoxide radical test, and lipid peroxidation.

Results: The qualitative phytochemical analysis displayed that the hydroethanolic extract of green coffee beans consisted of carbohydrates, phenolic compounds, saponins, alkaloids, and flavonoids. The presence of chlorogenic acid and trigonelline (RF value 0.32 and 0.16) was confirmed by comparing it with standards (RF value 0.35 and 0.17), respectively. The total phenolic and flavonoid contents of GCBE were found to be 225.6 ± 0.37 & 42.53 ± 0.34 mg /g, respectively. The extract showed significant antioxidant activity.

Conclusion: The hydroethanolic extract of green coffee beans had significant antioxidant activity and confirmed the presence of trigonelline, caffeic acid, chlorogenic acid, and quinic acid. It was concluded that due to the presence of a substantial amount of phenolic compounds, it showed considerable antioxidant activity.

1. Introduction

Plants are utilized as medicine in treating various disorders for being the source of many bioactive components. They are rich in secondary metabolites, which have different structures and physical and chemical properties, and excellent biological activities that are suitable for various applications and treatments (Pagare *et al.*, 2015). Coffee is the most popular beverage consumed worldwide, with a total consumption of approximately 8 million tons per year or 255 kg per second. It belongs to the family Rubiaceae and the genus Coffea. There are more than 80 species of coffee known to exist worldwide, but only two are significant from an economic and medicinal viewpoint, named Coffea arabica and Coffea Canephora var. robusta (Clarke, 2003). Globally, the most famous coffee in the world market is Coffea arabica (International Coffee Organization, 2011, Caldarelli *et al.*, 2019). Coffee is the main crop planted in Brazil, Vietnam, Colombia, and Indonesia, with Brazil being the leading producer worldwide (Moreira, 2013) (Damatta & Ramalho, 2006). More than 700 compounds are present in coffee, which are responsible for its aromatic and unique flavors (Gupta *et al.*, 2020). Altitude and temperature conditions affect the growth of coffee. Arabica coffee grows well at the altitude of 1000 to 2100 m with an ideal temperature range of 18-22°C, and Robusta coffee grows at the altitude of 100 to 1000 m and needs a hotter

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temperature ranging between 22° and 26°C (Toledo *et al.*, 2016; Bertrand *et al.*, 2012). Alcohol is the common solvent used to extract green coffee extract from green coffee beans. The marketed formulations of the green coffee extract are "coffee slender" and "Svetol", which are famous for weight loss (Belviso & Barbosa-Pereira, 2019; Onakpoya *et al.*, 2011). It has also decreased the danger of cancer, diabetes, and liver ailments owing to its antioxidant activity. Green coffee extract is also reported to be used against Parkinson's disease. It reduces blood pressure and is also found to modify hormone secretions and glucose tolerance in humans (Dos Santos *et al.*, 2024; Masek *et al.*, 2020; Satish *et al.*, 2021).

Chromatographic and spectroscopic analyses have emerged as the key techniques in recent years in pharmaceutical and biomedical investigations. Worldwide, analysts have utilized various plant bioactive compound screening techniques, such as mass spectroscopy and high-performance liquid chromatography (Siddiqui *et al.*, 2017; Masood *et al.*, 2021). Green coffee has a variety of elements, the most important of which are carbohydrates, proteins, polyphenols, phenolic species (such as caffeine and chlorogenic acid), polysaccharides, lipids, melanoids, and minerals. Green coffee beans contain various elements, including calcium, magnesium, potassium, iron, copper, and manganese (Saud & Salamatullah, 2021). Green coffee oil is obtained by pressing unroasted green beans using cold pressing. Green coffee oil is abundant in lipids such as triacylglycerol, kahweol, cafestol, sterols, tocopherols, and derivatives of diterpenes. In addition to caffeine, theophylline, chlorogenic acid, trigonelline, and theobromine, the green coffee bean extract (GCBE) contains a significant amount of these compounds. 4-O-caffeoylquinic acid, 3-O-caffeoylquinic acid, and 5-O-caffeoylquinic acid are the three chlorogenic acids that are discovered in green coffee beans that are considered to be the most important (Fischer *et al.*, 2011; Lombo *et al.*, 2022; Kasahun *et al.*, 2024).

2. Materials and Methods

2.1. Plant Collection

The unroasted green coffee beans of *Coffee Arabica* were collected from Ambala City, Haryana, India, which was confirmed by Sri Venkateshwara University, Tirupati, India.

2.2. Extraction

The shade-dried beans of green coffee Arabica were powdered and passed via a sieve of 40 mesh size. The coarse powder was put through the Soxhlet apparatus, and extraction was done for 12 hours at 70°C by subjecting 1.5 kg of the drug to ethanol and water (70:30 v/v) (Cordoba *et al.*, 2020). The suspension was filtered through Whatman no. 4 filter paper. The resulting hydroethanolic solutions were exposed to distillation to remove the solvent at a temperature of 60°C using a rotary evaporator. The semi-solid product obtained after distillation was dried in a vacuum desiccator, and its properties and yield were recorded. The extract was stored at 4°C in a refrigerator. Further, to assess *in vitro* assays, this extract was dissolved in a solvent.

2.3. Preliminary Phytochemical Screening

The standard procedure was followed to perform the phytochemical screening and identify the active phytoconstituents found in the hydroethanolic extract of green coffee beans (Nortjie *et al.*, 2022).

2.4. HPTLC Quantification of Chlorogenic Acid and Trigonelline in GCBE Extract

The obtained extract was subjected to HPTLC analysis to determine chlorogenic acid and trigonelline quantitatively. Before moving to HPTLC, the first optimization of the solvent system for the GCBE was done using precoated silica gel 60F254. Test sample preparation (weigh 100 mg of sample green coffee extract accurately in an iodine flask). After adding 8 milliliters of methanol to the mixture, it refluxed for ten minutes. Following the cooling and filtering of the sample through Whatman No. 1 paper into a volumetric flask with a capacity of 10 milliliters, the sample is then filled to the mark with methanol to hydrolyze undesirable sticky compounds that interfere with the performance of HPTLC. The solvent system, which consisted of ethyl acetate, dichloromethane, formic acid, acetic acid, and water in the proportions of 10:2.5:1.0:1.0:1.1 proportions by volume, was optimized. Through RF determination and comparison with the standard, HPTLC quantification was utilized to verify the existence of chlorogenic acid and trigonelline in the extract to confirm their presence (Chaudhary *et al.*, 2023).

2.4.1. Preparation of Standard and Test Solution Preparation

Took 2 mg of standard chlorogenic acid/trigonelline in a 100 ml volumetric flask. Then, about 1 ml of methanol was dissolved in the standard and filled to the line marked with methanol. Using a syringe, filter the standard solution. The obtained standard solution is used for HPTLC quantification. Carefully weighed 100 mg of green coffee extract in the iodine flask. After adding 8 milliliters of methanol, the liquid refluxed for 10 minutes. Once the sample has cooled, it should be filtered through Whatman No. 1 filter paper, transferred to a volumetric flask with

a capacity of 10 ml, and the volume should be adjusted using methanol. HPTLC quantification was performed using the test solution produced as a consequence.

2.4.2. HPTLC Specifications

Aluminum precoated silica gel HPTLC plates 60 F_{254} (10 cm × 10 cm with 0.2 mm thickness) were activated at 1050°C for one hour, were used as a stationary phase, and were subjected to chromatography. The samples and standards were applied to the stationary phase by using the semiautomatic applicator Linomat V (Camag, Switzerland) and were monitored under densiometric scanning, which was controlled by software win CATS Planar Chromatography Manager, version 1.4.4.6337 (CAMAG).

2.5. Quantitative Estimations

GCBE extracts were tested for total phenolic and total flavonoid content. The samples were tested using a solution of 1 mg ml^{-1} concentration.

2.5.1. Total Phenolics Estimation

The total phenolic content of extracts was determined using the Folin-Ciocalteu reagent. 10 mg of the extract was mixed with 10 ml of methanol (1 mg/ml). Take 0.4 milliliters of this extract and combine it with 2 milliliters of Folin-Ciocalt reagent and 1.6 milliliters of sodium carbonate in a separate container. The absorbance at 750 nm should be recorded after the reaction mixture has been allowed to stand for two hours. All measurements were made in triplicate. Express the total phenolic content as gallic acid equivalent in mg/g or weight per cent of extract weight (Madaan *et al.*, 2011).

2.5.2. Total Flavonoids Estimation

The extent of total flavonoids was determined by the aluminum chloride colorimetry method. A 1 mg/ml solution was prepared by dissolving 10 mg of the extract in 10 ml of methanol. 0.5 ml of this extract was taken out and dissolved in one milliliter of aluminum chloride with a concentration of ten percent, one milliliter of potassium acetate with a concentration of one million, one and a half milliliters of methanol, and two and a half milliliters of distilled water. At a wavelength of 415 nm, the absorbance of the mixture was measured after it had been incubated for thirty minutes at room temperature. All measurements were accomplished in triplicate. Total flavonoid content was expressed as the extract's rutin equivalent (mg/g or % w/w) (Madaan *et al.*, 2011).

2.6. Antioxidant Activity

The extract's in vitro antioxidant activity was investigated using standard methods. *In vitro* methods are based on the inhibition of free radicals. Inhibition of free radical activity is evaluated after adding the free radical system to the sample. The inhibition is related to the model's antioxidant activity. The different techniques are remarkably consistent concerning the radicals produced, the reproducibility of the starting method, and the endpoint estimated for detection. Although *in vitro* procedures specify a valuable suggestion of antioxidant actions, facts achieved from *in vitro* techniques are challenging to employ in living systems, and there is no need to essentially forecast similar *in vivo* antioxidant action. Since every method has its advantages and disadvantages, measuring antioxidant capacity just once is typically insufficient. Several techniques were utilized in the current study to effectively evaluate the in vitro antioxidant activity of a particular molecule or biological fluid. The absorbance was determined using spectrophotometry about the matching blank solution. The formula used to determine the % inhibition is:

$$\%\,\text{inhibition} = \frac{\left[\text{Absorbance of Control} - \text{Absorbance of Test}\right] \times 100}{\left[\text{Absorbance of Control}\right]}$$

The IC50 values represent the intensity at which 50% of free radicals were assessed (Munteanu & Apetrei, 2021).

2.6.1. DPPH (2, 2-Diphenyl-1-Picryl-Hydroxyl) Assay

The antioxidant activity of plant extracts against DPPH was determined using the proposed method (Re *et al.*, 1999). It produced a methanolic dilution of DPPH at a concentration of 1×10^{-4} M. One milliliter of hydroethanolic extract of green coffee extract, at varying quantities, should be collected and then diluted in two milliliters of DPPH in methanol. The absorbance of the mixture was determined using a UV spectrophotometer at a wavelength of 517 nm after it had been stored in the dark at room temperature for thirty minutes. Blanks were made by diluting DPPH with methanol during preparation (Re *et al.*, 1999). For this experiment, methanol was used as both the standard and the experimental blank. A determination was made regarding the level of scavenging activities.

2.6.2. ABTS Scavenging Assay

According to the methodology described in the previous article, the ABTS inhibitory activity of the hydroethanolic extract of green coffee beans was evaluated. In a nutshell, precisely 37.5 milligrams of potassium persulfate were dissolved in one milliliter of purified water. To make the ABTS solution, a total of 44 microliters of this solution was added to 9.7 milligrams

of ABTS that had been dissolved in 2.5 milliliters of distilled water. The ABTS solution was analyzed after roughly fifteen hours of being left in the dark at room temperature. The working solution combined 88 milliliters of forty-five percent ethanol with one milliliter of ABTS solution. After mixing a total of 25 µl of extract with 250 µl of ABTS working solution, the mixture was allowed to sit for 4 minutes. To determine the absorbance at a wavelength of 734 nanometers, an ultraviolet-visible spectrophotometer was taken into consideration. The experiment's results are reported in terms of equivalents of ascorbic acid, which was used as the standard for the experiment.

2.6.3. Nitric Oxide Radical Assay

The measurement of nitric oxide's inhibitory activity was carried out per the method formerly reported (Marcocci *et al.*, 1994). The mixture of hydroethanolic extract from green coffee beans and sodium nitroprusside salt at a concentration of 5 mM in phosphate-buffered saline was then incubated at a temperature of 25 degrees Celsius for 150 minutes. Griess reagent, which consisted of 1% sulfanilamide, 2% H_3PO_4, and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, was then added to the sample and mixed thoroughly. This is accomplished through the absorption of the chromophore produced by the diazotization of nitrite with sulfanilamide and the subsequent coupling with NED. It was measured using a UV-VIS spectrophotometer at a wavelength of 546 nm. Inhibition of nitric oxide production for standard potassium nitrite was determined similarly using Griess reagent. Results were expressed as potassium nitrite equivalents used as a standard (Marcocci *et al.*, 1994).

2.6.4. Superoxide Radical Assay

A modified approach was utilized to evaluate the inhibition level of the superoxide anion radical (O_2). In the reaction mixture, there was 0.2 mL of NBT that was dissolved in DMSO at a concentration of 1 mg/mL, 0.6 mL of extract, and 2 mL of alkaline DMSO that was composed of 1 mL of DMSO that contained five mM NaOH in 0.1 mL of H_2O. Additionally, the total volume of the mixture was 2.8 ml. When the wavelength was 560 nm, a UV-VIS spectrophotometer was used to record the amount of absorption happening. Instead of alkaline DMSO, the blank contained DMSO in its purest form. Ascorbic acid is employed as a standard, and the results are given as ascorbic acid equivalents (Pisoschi *et al.*, 2016).

2.7. Statistical Analysis

All values were presented as mean ± standard error of the mean (SEM). Statistical analysis was conducted using one-way ANOVA, followed by the relevant post hoc test, either Dunnett's or Tukey's multiple comparison test, as appropriate. P values less than 0.0001 were deemed statistically significant. The study was conducted utilizing GraphPad Prism version 8.0.2 software.

3. Result and Discussion

The present investigations' results on the plant *GCBE* are given below.

3.1. Extraction of Green Coffee Bean

The percentage yield obtained for the GCBE was 10.56% w/w.

3.2. Qualitative Phytochemical Screening

In evaluating the components and their superiority in plant extracts, phytochemical screening is utilized. Additionally, the search for bioactive components that may be useful in creating therapeutic agents is also carried out. In the current investigation, a qualitative phytochemical analysis of the hydroethanolic extract of green coffee beans was carried out, and the results are presented in Table 1. The extract contained glycosides, alkaloids, carbohydrates, tannins, and phenolic compounds. All of these substances were found. The presence of these phytochemicals in the green coffee bean extract may be responsible for the medicinal potential of the extract (Table 1).

Table 1: Phytochemical Screening of *GCBE*

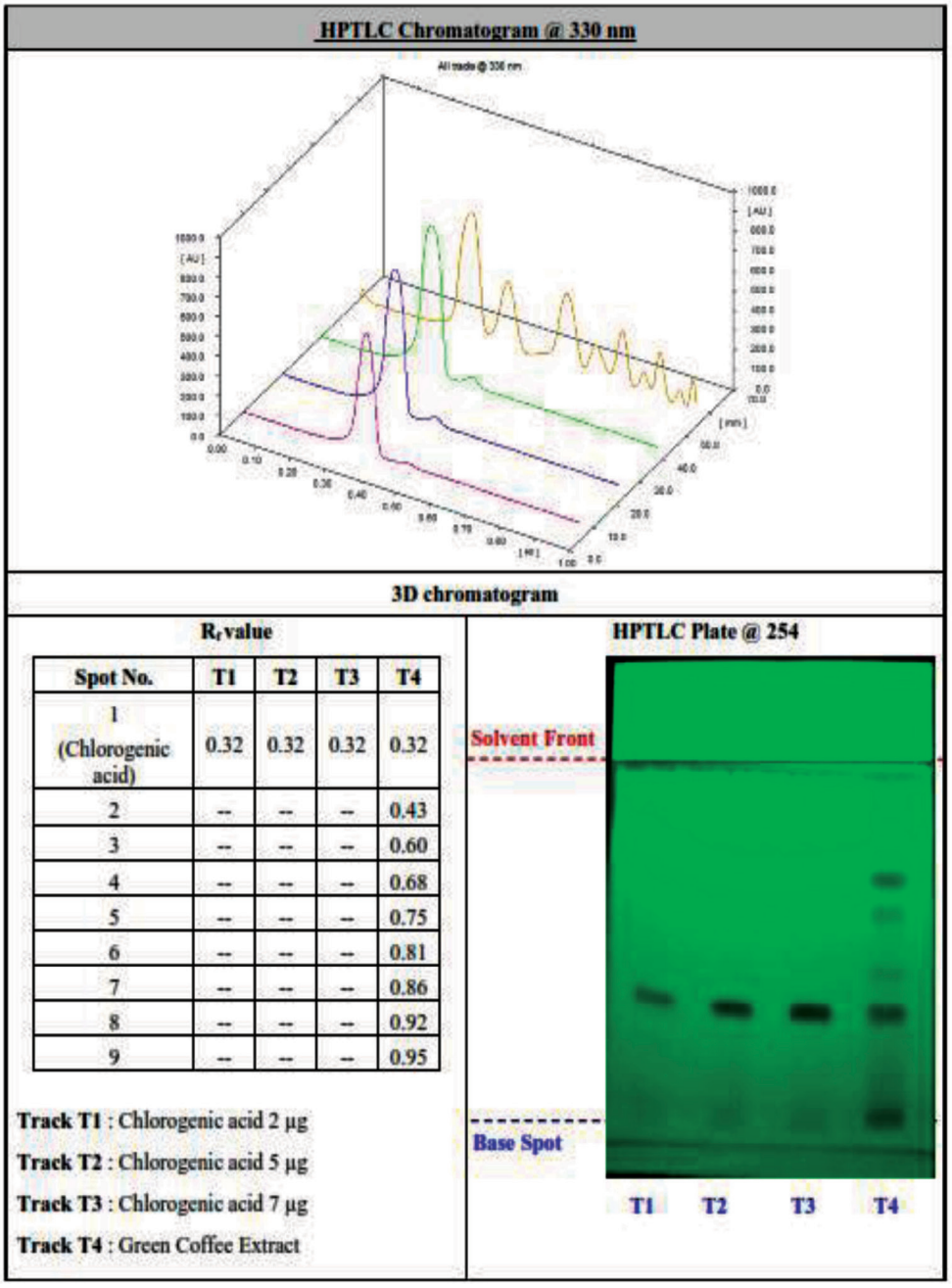
S.No	Phytochemicals	GCBE
1.	Phenolic compounds	+++
2.	Carbohydrates	+
3.	Saponins	+++
4.	Phytosterol	-
5.	Glycosides	++
6.	Alkaloids	+++
7.	Tannins	+++
8.	Amino Acid	-
9.	Proteins	-

Note: (+)signifies presence, (−) signifies absence of Phytoconstituents.
(+) < (++) < (+++) designates the colour intensity in screening procedures.

3.3. Quantitative Estimation of GCBE Marker Compound under HPTLC

High-performance thin-layer chromatography (HPTLC) is a valuable technique for analyzing the chemical composition of green coffee bean extract (GCBE).

Using the optimised solvent system for the HPTLC, the fingerprinting of the extract was carried out, and from this, the quantitative analysis of chlorogenic acid and trigonelline was done. HPTLC results confirmed the presence of chlorogenic acid and trigonelline with R_f of 0.32 and 0.17, respectively, in the green coffee extract compared to the standard with R_f of 0.35 and 0.17. The standard concentrations taken for both compounds were 2, 5, and 7 µg/ml, and the concentration taken for the sample was 100.7 mg/ml. The percentage yield for chlorogenic acid and trigonelline was found to be 4.41% w/v and 0.75% w/v, respectively. The chromatographic conditions and chromatogram for chlorogenic acid are presented in Figure 1 &Table 2 .



Spot No.	T1	T2	T3	T4
1 (Chlorogenic acid)	0.32	0.32	0.32	0.32
2	--	--	--	0.43
3	--	--	--	0.60
4	--	--	--	0.68
5	--	--	--	0.75
6	--	--	--	0.81
7	--	--	--	0.86
8	--	--	--	0.92
9	--	--	--	0.95

Figure 1: HPTLC Chromatogram of Standard Chlorogenic Acid (2 µg/ml), (5 µg/ml) and (7 µg/ml); Hydroethanolic Extract (100.7 mg).

Table 2: Chromatographic Conditions for Chlorogenic Acid

HPTLC Quantification Report				
Chromatographic Conditions				
Application Mode	CAMAGL inomat5– Applicator			
Filtering System	Whatman filter paper No. 1			
Stationary Phase	MERCK-TLC/HPTLC Silicagel60 F254 on Aluminum sheets			
Application (Y-axis) Start Position	10 mm			
Development End Position	80 microplate base			
Sample Application Volume	10 µL sample and 2,5 and 7µL of Standard			
Distance Between Tracks	16.6 mm			
Development Mode	CAMAGTLC Twin Trough Chamber			
Chamber Saturation Time	30 minutes			
Mobile Phase(MP)	Ethylacetate: Dichloromethane: Formic acid: Acetic acid: Water (10:2.5:1.0:1.0:1.1v/v)			
Visualisation	@330			
Parameters	**Standard Chlorogenic acid**			**Green Coffee Extract**
Weight	2 µg	5 µg	7 µg	100.7mg
Rf Value	0.35	0.32	0.32	0.32
AUC	22789.8	30951.8	34910.2	29003.0
% Chlorogenic acid	--	--	--	4.41 %

The chromatographic conditions and chromatogram for trigonelline are presented in Table 3 & Figure 2.

Table 3: Chromatographic Conditions for Trigonelline

HPTLC Quantification Report	
Chromatographic Conditions	
Application Mode	CAMAGL inomat5– Applicator
Filtering System	Whatman filter paperNo. 1
Stationary Phase	MERCK-TLC/HPTLC Silicagel60 F254 on Aluminum sheets
Application (Y-axis) Start Position	10 mm
Development End Position	80 microplate base

Sample Application Volume	10 µL sample and 2,5and 7µL of Standard			
Distance Between Tracks	16.6 mm			
Development Mode	CAMAGTLCT win Trough Chamber			
Chamber Saturation Time	30 minutes			
Mobile Phase (MP)	Ethylacetate: Methanol: Water: Acetic acid (5.4:2.3:1.5:0.8v/v)			
Visualisation	@267			
Parameters	**Standard Trigonelline**			**Green Coffee Extract**
Weight	2 µg	5 µg	7 µg	100.7mg
Rf Value	0.16	0.17	0.17	0.16
AUC	6812.4	11981.0	14349.9	2778.3
% Trigonelline	--	--	--	0.75 %

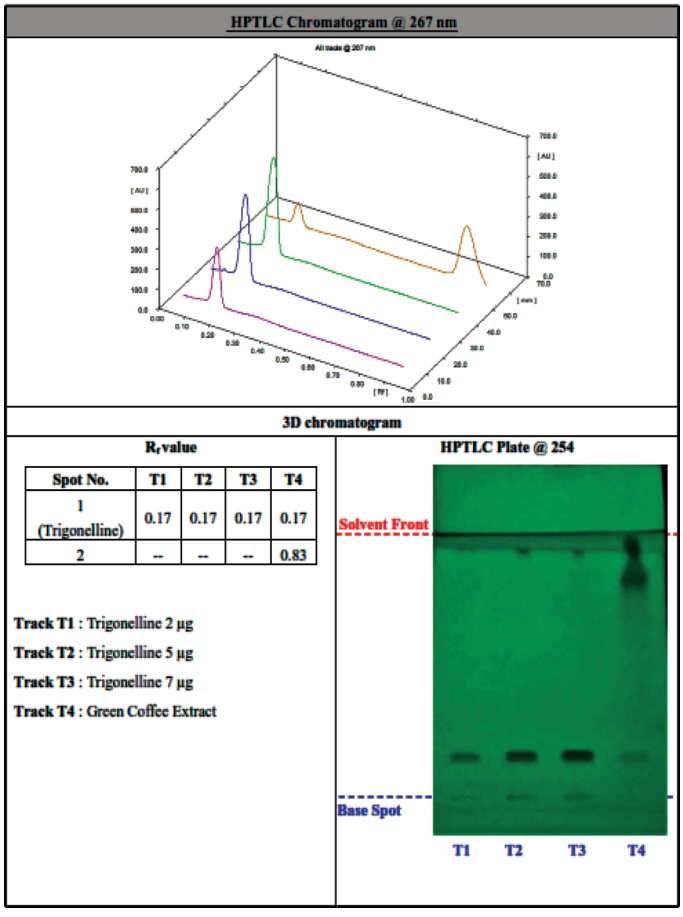


Figure 2: HPTLC Chromatogram of Standard Trigonelline (2 µg/ml), (5 µg/ml) and (7 µg/ml) Hydroethanolic Extract (100.7 mg)

The calibration curve of the marker compounds chlorogenic and trigonelline was calculated using linear regression. The linear calibration curve was found over the concentration ranges used with a standard deviation of 2.0, a correlation coefficient of 0.993 within the range of 0-8 µg/ml for chlorogenic acid and a correlation coefficient of 0.990 for the range of 0-8 µg/ml for trigonelline.

3.5. Quantitative Estimation of Total Phenolic and Flavonoid

The phenolic and flavonoid contents of natural products play an essential role in the whole physiological process and are therefore critical criteria for the quantitative evaluation as well as the biological potency of the extract. The total phenolic and flavonoid content of hydroethanolic *GCBE* was assessed by following the standard procedure. The findings of the studies are outlined below in Table 4. The total phenolic and flavonoid content present in GCBE was 225.6 ± 0.37 & 42.53 ± 0.34 mg /gm, respectively. A wide variety of biochemical activities may be attributed to phenols. These activities include the ability to change gene expression and antioxidant, antimutagenic, and anticancer abilities. By blocking the development of mitochondrial adhesions, flavonoids are active components that perform various biological activities. These actions include resistance to microbial, wound, arthritis, angiogenesis, and cancer illnesses. Flavonoids are also known as flavonaceous compounds.

Table 4: The Total Phenolic and Total Flavonoid Content of Extracts

Plant Extract	Total Phenolic Content	Total Flavonoid Content
Hydroethanolic *GCBE*	225.6 ± 0.37	42.53 ± 0.34

Note: Table values were obtained by calculating the average of three experiments ± S.E.M.

3.6. Antioxidant Studies

The antioxidant efficacy of hydroethanolic GCBE and standards was evaluated through their scavenging effects on stable 2,2-Diphenyl-1-picryl-hydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS), nitric oxide radical assay, superoxide radical assay, and lipid peroxidation (LPO) methods. The GCBE had a significant antioxidant profile across all experiments. The results of the in vitro antioxidant experiments are presented below in Table 5.

Table 5. *In vitro* antioxidant analysis of Hydroethanolic *GCBE*

Extract	DPPH	ABTS	Nitric Oxide	Super Oxide
Hydroethanolic *GCBE*	71.5±0.3	103.3±0.7	70.7±0.4	65.4±0.5
Standard				
Ascorbic acid	5.9±0.5	13.2±0.2	48.2±0.6	-
Catchin	-	-	-	44.6±0.3

Note: Table values were obtained by calculating the average of three experiments ± S.E.M

4. Conclusion

The present study highlights the phytochemical screening, chromatographic profile, and significant antioxidant potential of the hydroethanolic extract of green coffee beans. Phytochemical screening revealed the presence of bioactive compounds, including phenolics, flavonoids, and alkaloids, which are well-known for their therapeutic properties. The chromatographic analysis confirmed identifying and quantifying key bioactive markers, such as chlorogenic acid, trigonelline, etc., contributing to the extract's antioxidant properties. The extract's potential to treat conditions linked to oxidative stress was highlighted by its antioxidant capability, which showed potent free radical scavenging activity. These findings support the use of green coffee bean extract in nutraceutical formulations and functional foods that promote health and prevent chronic diseases. Additional *in vivo* and clinical research is necessary to clarify this extract's therapeutic effectiveness and molecular pathways in treating diseases driven by oxidative stress.

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Authorship Contribution

Mamta Saini and Saurabh Gupta: Methodology, Investigation, Writing-Original draft, Writing-Review and Editing, Sushma Devi and Thakur Gurjeet Singh: Formal Analysis, Supervision, Project Administrations.

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Conflict of Interest

The authors declare no conflict of interest, financial or otherwise.

Ethical Approvals

No ethical approvals were required for this study.

Declaration

It is an original data and has neither been sent elsewhere nor published anywhere.

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