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Extraction of Chlorogenic acid from green coffee beans: characterization using HPLC, phytochemical and radical scavenging analysis

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Abstract

Coffee is a popular beverage that is widely consumed around the world. It is high in the phenolic compound Chlorogenic Acid (CGA). CGA is primarily found in green coffee and is lost after roasting. The particle size distribution of green coffee beans was determined using two widely used mathematical models by Rosin-Rammler (RR model) and Gates-Gaudin-Schumann (GGS model). The RR model and the GGS model were used to evaluate the experimental data, and both the models were compared. The GGS model produced better results The impact of different extraction methods and solvents on the yield of green coffee bean extract was also studied. The effect of water, methanol, and propanol on the yield of the extract was observed using two different extraction techniques namely, cold and hot extraction. The phytochemicals found in the extract were studied. Tannins, flavonoids, terpenoids, alkaloids, steroids, and phenols are phytoconstituents found in green coffee bean seeds. The antioxidant activity of the extract was determined using the 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) method of antioxidant testing and the total phenolic content of the extract was estimated with its IC50 of ABTS antioxidant activity. The quantification of CGA's in green coffee extract was also a goal of this study. All seven major CGA isomers (CQA and di-CQA) were quantified.

Keywords: Chlorogenic acid, Green coffee bean extract, Antioxidant activity, Phytochemicals

1.Introduction

Coffee is one of the most popular beverages across the globe. Unroasted coffee beans, known as green coffee beans, are a rich source of antioxidants such as chlorogenic acids (CGA) that are phenolic in nature [1]. It is worth noting that chlorogenic acids in Coffea arabica range from 3.5 percent to 7.4 percent (wt/wt of dry matter), but CGA's in Coffee Canephora range from 7.2% to 14.1% (wt/wt of dry matter) [2]. When compared to conventional roasted coffee beans, levels of CGA's in coffee beans which are not roasted are high. It has the ability to reduce weight, boost fat metabolism in liver cells, and minimise fat storage in the body without affecting caloric restriction [3]. Chlorogenic Acid also aids reducing renal oxidative sress and inflammation which is proved by reduced expression of CA-induced histology improvement paralleled the reduction of renal oxidative stress and inflammation, as demonstrated by the reduced expression of TNF-α (tumor necrosis factor-α) and COX-2 (cyclooxygenase-2) [4]. It exhibits therapeutic action towards Hepatitis-B virus infection, three isomers of chlorogenic acid proved as suppressant on Hepatitis-B Virus [5]. CQA, di-CQA and FQA are the three important groups of CGA's present in green coffee beans, accounting for roughly 81% of the total CGA's. Water, fibre, proteins, minerals, CGA, caffeine, carbs, amino acids, organic acids, lipids and different volatile components etc are the components that make up green coffee beans. Approximately 100 different volatile compounds have been reported in green coffee among the various biomolecules and compounds recorded, also sulphur, furans, pyrazines, ketones, aldehydes, hydrocarbons, esters and alcohol compounds. CGA's are a type of phenolic compound which are derived from esterification reaction of ferulic, caffeic and P-coumaric with quinic acid.

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The esters are formed with the hydroxyl of carbon-5, -3, and -4, depending on the number of cinnamic substituents and the esterification position in the quinic acid's cyclohexane ring. The major CGA subclasses are caffeoylquinic acids, dicaffeoylquinic acids, and feruloylquinic acids; however, the less abundant subclasses include p-coumaroylquinic acids and caffeoylferuloylquinic acids. Only caffeoylquinic acids account for 80% of total CGA. 5-caffeoylquinic acid accounts for approximately 60% of caffeoylquinic acids. The compound 5-caffeoylquinic acid (5-CQA) was discovered first, and it is the isomer that has received the most attention. Figure 1, demonstrates the structure of CGA [6, 7]. CGA is in charge of the bitterness and acidity in coffee brew. Caffeine, CGA, fibre (soluble), trigonelline and terpenes are major contributors for the flavour of coffee after roasting [8].

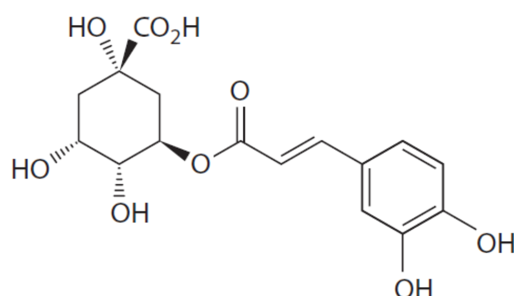

Figure 1. Structure of Chlorogenic acid

2.Materials

Methanol (Denatured), Propan-2-diol, Hydrochloric acid, Ferric chloride, Sodium Hydroxide, Chloroform, Sulphuric acid, Mayer's Reagent, Acetic acid, Copper Sulphate, Benedict's reagent, ABTS, Potassium persulphate, Methanol, Acetonitrile, Ascorbic acid, Sodium Carbonate, Folin's reagent. All the chemicals are analytical grade manufactured by SRL Pvt. Ltd.

3.Experimental Methods

3.1.Collection of green coffee beans

Arabica green coffee beans were procured from a local store in Bangalore, Karnataka, India. Since 1935, they are into the cultivation of fine coffee in different parts of Karnataka state mainly in Sakleshpura which is situated in Western ghats of India. For growing of coffee, Sakleshpura is the best place having average elevation of 950 meters, 3115 feet above sea level.

3.2.Grinding and particle size distribution of green coffee beans

Grinding of green coffee beans is carried out using flour mill (1HP, 4AMP 220/250 V, 960 RPM Amar Industries Ahmedabad). The fine powder was packed and sealed at the earliest in pouches which is made up of polyethylene and was incubated in refrigerator at 4°C for further studies.

For particle size distribution, the ground coffee beans were dried in an oven for a duration of 5-6 minutes in order to obtain dried sample. The sieves were arranged in the ascending order of their sieve number and known quantity of sample was loaded onto the top sieve. Particles are separated based on their size using sieves shaker. The weight of sample retained on every sieve was recorded.

3.3.Cold extraction of green coffee beans

20g of dried powder of green coffee beans added to 100mL wate. Stirred for 48 hours at room temperature. Filtered using Whatman No. 1 filter paper. Filtrate obtained is evaporated at room temperature. Dried extract is weighed and stored at 4°C. Extraction percentage is calculated using Equation 1. Similar procedure is followed to get the extract using methanol and isopropanol as solvent.

$$\text{Extraction percentage} = \frac{W1}{W2} * 100 \qquad (1)$$

Where, W1 = Weight of extract obtained, g

W2 = Weight of sample taken, g

3.4.Hot extraction of green coffee beans

20g of dried powder of green coffee beans added to 100mL water. Incubated in hot water bath for 4 hours at with constant stirring. Filtered using Whatman No. 1 filter paper. Solvent is evaporated at its respective boiling point. Dried extract is weighed and stored at 4°C. Similar procedure is followed to get the extract using methanol and isopropanol as solvent.

3.5.Phytochemical Analysis

The qualitative chemical tests for various phytoconstituents were carried out for the extract of green coffee beans. Experimental methods for phytochemical analysis is followed similar to Fadri et al studies [9].

3.5.1.Test for Alkaloids

The extract was dissolved in dilute HCl and filtered. The filtrate was treated with potassium mercuric

iodide solution (Mayer's reagent). Formation of a whitish yellow or cream-colored precipitate indicated the presence of alkaloids.

3.5.2.Test for Tannins (Braymer's test)

0.2ml of plant extract was mixed with 2ml of water and heated on water bath for 10 minutes. The mixture was filtered and ferric chloride was added to the filtrate and observed for dark green solution which indicates the presence of tannin.

3.5.3.Test for Terpenoids (Salkowki's test)

0.2 ml of plant extract added to 0.2ml of chloroform. Concentrated Sulfuric acid was added carefully through the wall of test tube to form a layer. Presence of reddish-brown color at the interface would show the presence of terpenoids.

3.5.4.Test for Glycosides

0.2ml of sample was mixed with 0.2ml of chloroform. 0.2ml of acetic acid was added to this solution and the mixture was cooled on ice. Concentrated Sulfuric acid was added carefully and the color change from violet to blue to green indicates the presence of steroidal nucleus.

3.5.5.Test for Steroids (Lieberman Burkhardt test)

0.2 ml of sample was mixed with 0.2ml of chloroform. To this 0.2ml of concentrated sulphuric acid was added. The appearance of red color in the lower layer of chloroform indicates the presence of steroids.

3.5.6.Test for Saponins:

0.2 ml of extract and 0.6ml of water was mixed in a test tube. The mixture was shaken vigorously and observed for the formation of persistent foam that confirms the presence of saponins.

3.5.7.Test for Flavonoids

The extract was treated with few drops of sodium hydroxide (NaOH) solution. Generation of yellowish red color on addition of dilute HCl turns colorless which confirms the presence of flavonoids.

3.5.8.Test for Phenolic compounds:

The extract was treated with few drops of ferric chloride solution and the appearance of blackish blue color confirms the phenol's presence in the extract.

3.5.9.Test for Reducing Sugars (Benedict's Test)

The extract was filtered and the filtrate was subjected to treatment with Benedict's reagent and was kept for boiling in thermostatic water bath for 6 minutes. Appearance of orangish red precipitate indicates the reducing sugar's presence in the extract.

3.9.10. Test for proteins (Biuret Test)

The test solution was treated using few drops of copper sulphate (2%) solution. Then it was mixed with 1mL of methanol. Then potassium hydroxide pellets were added in excess, appearance of pink color confirms the presence of proteins in the extract.

3.6.Total Phenolic Content (Folin Ciocalteau method)

100 µL of green coffee bean extract was taken in a test tube. 100 µL of serial dilution of the standard gallic acid (1mg/mL) are taken in different test tubes. 300 µL of Folin Ciocalteau (0.1%) reagent was added to the test tubes containing the bean extract and the standard, followed by the addition of 2000 µL of Sodium carbonate (7.5%) solution. The reaction mixtures were incubated in dark for 1 hour. Using spectrophotometer, the absorbance was recorded at 760nm. Total phenolic content is determined by standard graph.

3.7.ABTS Assay – Antioxidant Assay

The ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical cation decolorization assay was used to determine the free radical scavenging activity of plant samples. The ABTS+ i.e., cation radical was created by reacting 7 mM ABTS in water with 2.45 mM potassium persulfate (1:1) and storing it in the dark at room temperature for 12-16 hours before use. After diluting the ABTS+ solution with methanol, the absorbance at 734 nm was calculated to be 0.7. 5 µL of plant extract is added to 4 mL of diluted ABTS+ solution, absorbance was measured 30 minutes after the addition. Percentage of ABTS+ scavenging activity is calculated using equation 2:

$$\text{ABTS+ scavenging effect (\%)} = \frac{(AM-AS)*100}{AM} \quad (2)$$

Where:

AM - absorbance of ABTS radical + methanol
AS - absorbance of ABTS radical + sample extract/standard

3.8.Characterization using High Performance Liquid Chromatography (HPLC)

HPLC is an accurate technique for characterizing a large group of samples. HPLC (Manufacturer : Thermo Fischer Scientific) analysis is carried out for separation of compounds in a mixture.

4.Results and Discussion

4.1.Particle size distribution

Table 1 depicts the size range of particles against their fraction present in the total sample. It is found that particle size range of 375 to 425μm has the highest fraction among the total sample subjected to sieve analysis. Cumulative weight fraction curve from figure 2 follows the trend of increasing as the particle size increases. Similar results are observed in the study carried out by C F Mora et al [10].

Table 1. Particle size distribution of green coffee bean powder

Particle Size(μm)	Sieve Size (μm)	M, Mass of sample retained (g)	Fraction retained on each sieve	Z_r, Cumulative Weight retained
< 63	63	3.1	0.031	0.031
63-125	125	4.26	0.0426	0.0736
125-250	250	14.63	0.1463	0.2199
250-375	375	20.32	0.2032	0.4231
375-425	425	23.56	0.2356	0.6587
425-500	500	18.71	0.1871	0.8458
500-575	575	11.32	0.1132	0.959
575-625	625	4.1	0.041	1
		100 (Total)		

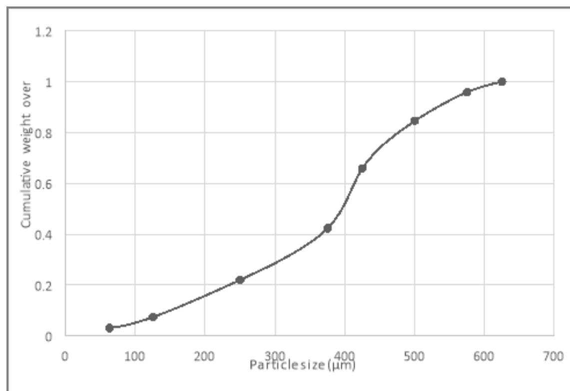

Figure 2. Particle size distribution curve for Green coffee bean powder

4.2. Mathematical Model for particle size distribution

4.2.1.Rosin-Rammler-Bennett (RRB) model

General equation for Rosin-Rammler-Bennett model is given by equation 3 which gives relation between percentage mass retained against size of the particle. From figure 3, it is observed that Co-efficient of Determination [R^2] for the regression was found to be 0.9564. The uniformity index $n^|$ is 2.03, indicating more uniformity of particle size distribution.

$$F(x) = 1 - \exp(-(x/x_r)^n) \quad (3)$$

Rewritten as,

$$\ln \ln (1/1-F(x)) = n \times \ln x - n \times \ln x_r$$

where
$F(x)$ – distribution function (cumulative mass)
x – particle size (μm)
x_r – mean particle size (μm)
n – measure of the spread of particle sizes
Parameters x_r and n are adjustable parameters characteristic for the distribution.

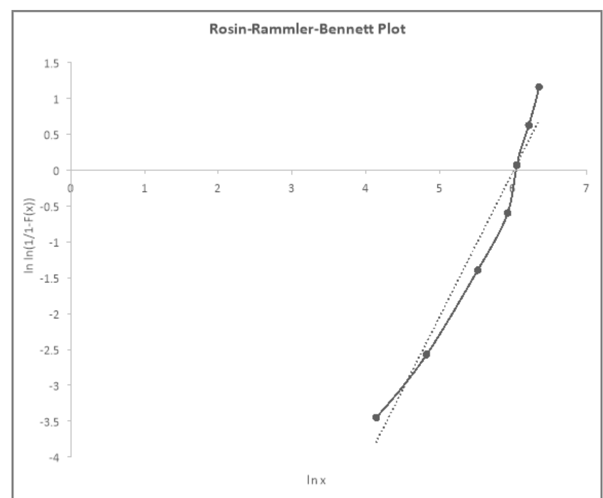

Figure 1. Plot for Rosin-Rammler-Bennett model

4.2.2. Gates Gaudin - Schumann (GGS) model

General equation for GGS model is given by equation 4, which gives relationship between cumulative mass percentage and size of particle. Distribution of particle size plot obtained by GGS model is depicted in figure 4. Co-efficient of Determination [R^2] for the regression is found to be 0.9923 with the uniformity index of 1.60. Based on the R^2 values of the fitted equations, the Gates-Gaudin-Schumann model fits the particle size distribution data which gives more clarity than the Rosin-Rammler-Bennett model on particle size.

$$y = 100 * \left(\frac{x}{k}\right)^a \quad (4)$$

where
y = cumulative % passing
x = particle size
k = size modulus

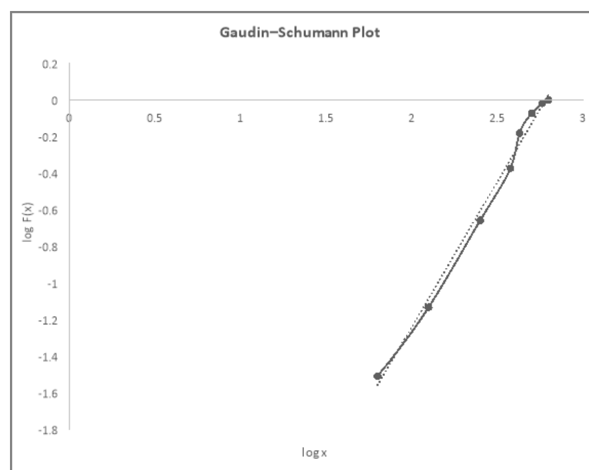

Figure 4. Plot for Gates Gaudin Schumann model

Table 2. Comparison of type pf extraction with yield percentage

Sl. No.	Solvent used	Type of Extraction	Yield	
			g	%
1	Water	Hot Extraction	2.20	11
		Cold Extraction	1.83	9.15
2	Methanol	Hot Extraction	1.87	9.35
		Cold Extraction	1.14	5.7
3	Propanol	Hot Extraction	1.13	5.65
		Cold Extraction	0.97	4.85

Table 3. Results for phytochemical analysis

Sl No	Tests	Distilled Water extract	Methanol Extract	Propanol extract
1	Tannins	+	+	+
2	Flavonoids	+	+	+
3	Saponins	-	-	-
4	Terpenoids	-	+	+
5	Alkaloids	+	-	-
6	Glycosides	-	-	-
7	Steroids	-	+	-
8	Phenols	+	+	+
9	Proteins	-	-	-
10	Reducing sugar	-	-	-

4.3. Extraction of Chlorogenic Acid

Extract of green coffee bean was produced using Propanol, Methanol and Distilled Water as solvents. For extraction, two different extraction methods were used: hot and cold extraction. Percentage of extraction or yield of extraction of CGA against each solvent is tabulated in table 2. It is found that percentage yield is highest with water as solvent in hot extraction with 11% and lowest with propanol as solvent in cold extraction with 4.85%.

4.4. Phytochemical Analysis

Standard tests for phytochemicals for all the three GCB extracts (distilled water, methanol and propanol) showed the presence of tannins, phenols and flavonoids. Saponins, proteins, reducing sugar and glycosides are absent in all the three extracts. Steroids are present with water and propanol extract, alkaloids are present with methanol and propanol extract. The results are tabulated in table 3.

4.5. ABTS Assay – Antioxidant assay

From figure 5, it is depicted that GCB extract showed inhibition of ABTS with IC50 value of 57.81 µg/mL against the standard Ascorbic acid with inhibition of ABTS with IC50 value of 1.76 µg/mL. Samples with IC50 less than 50 µg/mL is said to be very strong antioxidant, 50-100 µg/mL as strong antioxidant, 101-150 µg/mL as average antioxidant and above 150 µg/mL as weak antioxidant [11]. Thus GCB extract exhibits strong antioxidant property.

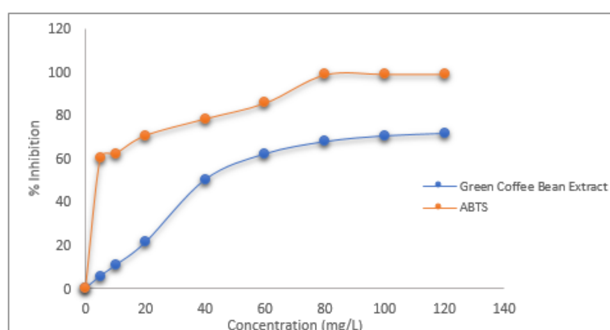

Figure 5. ABTS scavenging activity of GCB extract

4.6.Characterization of Chlorogenic acid using HPLC

The peak profiles were consistent for three different trials and followed the same pattern. Seven main CGA isomers were clearly identified, including 3-CQA, 5-CQA, 4-CQA, 5-FQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA. The major abundant chlorogenic acid isomer is 5-CQA characterized at retention time of 7.2mins. Comparable result is obtained with respect to work done by Oteef et al [12].

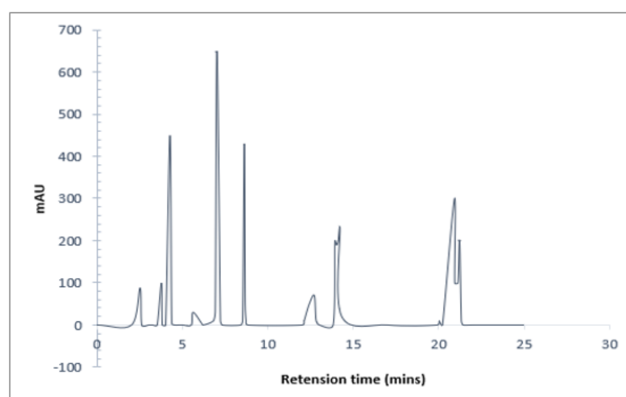

Figure 6. HPLC chromatogram of polyphenols for green coffee bean extract

5.Conclusion

Green coffee beans were ground using a flour mill and the powder obtained was modelled for its particle size distribution using two models namely Rosin Rammler and Gaudin Schumann method. Gaudin Schumann method best fits the particle size distribution with Coefficient of Determination (R^2) of 0.99. Green coffee bean extract was obtained by using three different solvents namely, Distilled Water, Methanol and Propanol and by two methods namely, Cold and Hot extraction. Higher yield of the extract was obtained when Hot extraction was performed using Distilled water as solvent. The yield was found to be around 11%. It was seen that yield of the extract decreased with decreasing polarity of the solvent. i.e., Propanol < Methanol < Distilled water. Phytochemical screening of the extract was performed and the presence of tannins, flavonoids, terpenoids, alkaloids, steroids and phenols was confirmed. Total phenolic content in the extract was estimated using Folin – Ciocalteu method using UV spectrophotometry and the content was found be 1154.212 µg/mL equivalent of Gallic acid. Antioxidant potential of the green coffee bean extract was tested by ABTS assay with IC50 value of 57.81 µg/mL equivalent of Ascorbic acid. The presence of chlorogenic acid in the extract was confirmed by characterizing using High Performance Liquid Chromatography (HPLC).

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Declaration of Competing Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Conflict of Interest: Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest in terms of financial and funding agency support.

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