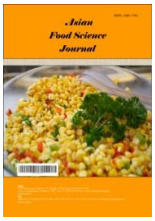

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In-vitro Anticancer and Antioxidant Activity of Green Coffee Beans Extract

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The main objective of this article is to analyze the efficacy of polyphonic compounds, especially Chlorogenic Acid in anti-obesity and anticancer. Overweight and obesity became severe global health issues in all sex and age groups respectively according to WHO, this is the significant risk factors for several chronic illnesses this is increasing from recent decades, due to lifestyle changes intake of food lack of proper healthcare particularly in urban communities. According to the India Council of Medical Research (ICMR), more than 1300 Indians die every day due to cancer, especially Basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and malignant melanoma are primary skin cancers. This research aims to help anti-obese and anticancer by providing health benefits of green Coffee. The ethnobotanical study proved that green coffee beans of *Coffee Robusta* variety have significant medicinal properties, which are essential for the human diet. In vitro results showed that high free radical scavenging activity with IC_{50} towards DPPH 30.99µg/mL ABTS0.83 µg/mL, Alpha-amylase 31.75 µg/mL, MTT assay 101.26 µg/mL and Anti-obesity activity 33.34 µg/mL respectively. In conclusion, more *in vivo* and *in vitro* experimental studies are required to understand the full use of the extract in the prevention of lethal diseases.

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Keywords: Anti-cancer; anti-obesity; overweight; coffee robusta.

1. INTRODUCTION

Coffee origin is Ethiopia, then it came into the north way across the red sea into Yemen in 15thcentury [1].In the 17th century by Dutch in Sri Lanka European first owned the coffee production. The bear fruit of the coffee plant was collected after 3-4 years. This fruit, which bearing seed, is called coffee cherry; initially, it is green in color, then changes its color to a deep red, which is ripped fruit. Coffee fruits are typically harvested in one of three ways: picking, stripping, or mechanical harvesting.

Green coffee beans are purest and rawest form. Raw beans are used to make the extract. Harvested green coffee beans are collected, sorted, and graded to remove immature, over ripped, spoiled, and damaged seeds. Low-quality seeds contain a high fraction of immature seeds with low chlorogenic acid. *Robusta* coffee is more robust and healthier, which is resistant to diseases and pests. It provides a high amount of antioxidant compounds and Caffeine, the presence of high chlorogenic Acid in *Coffee robusta* protects the plant more against the microorganisms, insects, and UV radiation compared to *Coffee arabica*.

In cold, dry conditions, green coffee beans can be stored up to two years in a burlap bag (made of the skin of the jute plant) [2]. Ideal storage conditions for green Coffee are 55 to 80 degrees Fahrenheit, with ambient humidity 60%-75% (to maintain 10-12% bean humidity). Burlap (or cotton) bags are used when there are humidity changes so that the Coffee can "breathe." Mostly, green coffees stay best in conditions that people would also like to sleep in.

Coffee contains many bioactive compounds which are helpful to reduce the risk of Hepatocellular carcinoma [3], Alzheimer' disease [4], and anti-proliferative effect against some human cancer cell lines [5].Caffeine ($C_8H_{10}N_4O_2$) an alkaloid that is chemically known as 1,3,7-trimethylxanthine; it raises levels of physiological or nervous activity in the body.

In Coffee chlorogenic Acid is the primary polyphenolic compound, and this is also known as an antioxidant that decreases the release of glucose into the bloodstream after a meal [6]. CGA is the ester of Caffeic Acid and quinic Acid. Chlorogenic acid is the 5-caffeoylquinic acid; according to the IUPAC-IUB nomenclature [7]. There are different ranges of concentrations in different plant parts, such as root, peel, leaf, fruit pulp, and tuber [8]. Generally, Chlorogenic Acid is high in two main commercial species of *C. arabica* and *C. canephora* showed quantitative and qualitative differences. In GCB (5-Caffeoylquinic Acid), the amount of CGA is 2% DM, 1% DM in 3-caffeoylquinic acid. GCB of *C. arabica* contains less CGA6.5% DM than *C. canephora*10% DM [9]. The amount of Chlorogenic Acid decreases with increasing of roasting till now 85 Chlorogenic Acids have been identified in green beans of *C. canephora* and 45 in *C. arabica.* Compared to *C. arabica, C. canephora* has the highest feruloylquinic and dicaffeoylquinic Acids [10]. Chlorogenic Acid content reduces up to nearly 50% when it is roasted for 230°C to 250°C for 12 to 21 min [11].

Compared to roasted coffee beans, unroasted green coffee beans contain a high amount of Chlorogenic Acid. Many experimental studies proved that CGA has new health benefits in diabetes, metabolic syndrome, hypertension, cardiovascular diseases, neuroprotection [12].

2. MATERIALS AND METHODS

2.1 Sample Collection

Raw Green Coffee Beans average weight of 2kg was collected from Kodagu, Fig. 1 (Coorg) which is a rural district in the southwestern Indian state of Karnataka.

Fig. 1. Coffee cherry

2.2 Sample Preparation

Green Coffee Beans of 500gm were taken and pulverized (one bean 4-5 pieces) with the help of

pastel and mortar. Added 2.5Lit 90% methanol and Extracted for 3 hrs under 60℃, 70rpm with a rotatory evaporator, repeated the above process three times and accumulated all the extracts. Concentrated under reduced pressure and temperature and separated oil layer overnight at room temperature. Chlorogenic acid was extracted by the solvent extraction process.

2.3 Phytochemical Analysis

2.3.1 Solubility test

A pinch of test samples was mixed with a different solvent system. The appearance of a clear solution or turbidity was observed in the tubes.

2.4 Qualitative Phytochemical Analysis

2.4.1 Test for alkaloids

Mayer's Test: To the sample, 2 mL of Mayer's reagent was added; formation of a reddish-brown precipitate indicates the presence of alkaloids.

2.4.2 Test for saponins

1 mL of the sample and 5 mL of water wastakeninto the test tubeand shaken vigorously. Copious lather formation shows the presence of saponins.

2.4.3 Test for tannins

Ferric chloride was added to 1mL of sample; the development of a dark bluecolor or greenish-black color indicates the presence of tannins.

2.4.4 Test for glycosides

Keller-Killani test: 1 mL of the sample, 2 mL of glacial acetic acid containing a drop of $FeCl_3$. An equal volume of conc. Sulphuric Acid (H_2SO_4) was added from the sides of the tube. If a brown color ring appears, it indicates the presence of cardiac glycosides.

2.4.5 Test for flavonoids

Alkaline reagent test: sample was treated with 10% NaOH solution; formation of intense yellow color indicates the presence of flavonoid.

2.4.6 Test for phenols

Lead acetate test: Into the sample, 3 mL of 10% lead acetate solution was added. If bulky white precipitate appears, it indicates the presence of phenolic compounds.

2.4.7 Test for steroids

1 mL sample was dissolved in 10 mL of chloroform &an equal volume of concentrated H_2SO_4 was added from the side of the test tube. The upper layer turns red, and the H_2SO_4coat showed yellow with green fluorescence. This indicates the presence of steroids.

2.4.8 Test for terpenoids

Salkowski test: 5 mL of sample was mixed in 2 mL of chloroform, and concentrated sulphuric acid was carefully added to form a layer. A reddish-brown coloration of the interface indicates the presence of terpenoids.

2.4.9 Test for quinones

Sample was treated separately with Alc. KOH solution. Change in colour ranges from red to blue indicates the presence of quinones.

2.4.10 Test for Proteins

Nin-hydrin test: The sample was taken, and few drops of freshly prepared Ninhydrin reagent were added and heated. If pink colour or purple colour appears, it indicates the presence of proteins, peptides, or Amino Acids.

2.5 Total Phenolic Content

Samples (250 µg) were mixed with 0.5 mL of water (H_2O) and 0.2 mL of Folin-Ciocalteu's phenol reagent (1: 1). After 5 min, 1 mL of saturated Na_2CO_3 sol (8% w/v in water) was taken into the mixture and the volume made up to 5 mL with distilled water. The reaction was kept in the darkroom for 30 min. And the absorbance of blue colour from different samples was measured at 765 nm. It is calculated as Gallic Acid equivalents GAE/g of dry plant material based on a standard curve of Gallic Acid (10-320 µg/mL), $y = 0.0033x - 0.0231, R^2 = 0.9895$.[13]

2.6 Thin Layer Chromatography

The extract was subjected to thin-layer chromatography (TLC) as per the conventional method using silica gel 60F254; 5x3 cm (Merck) was cut using the TLC cutter. Plate markings were made with a soft pencil. Glass capillary

tubes were used to spot the extract in TLC plates. The solvent system was tested for the separation of bioactive components. In the TLC chamber, the solvent system *viz* butanol: acetic acid: water was used. After pre-saturation with the mobile phase for 30 min, the plate was kept inside the chamber, and the elution was performed using the solvent as mentioned above system. After completion of the elution, the plate was dried and subjected to visualize under the UV chamber and sprayed using different spray reagents. R_f values determined by using the following formula:

R_f = Distance travelled by the solute/ Distance travelled by the solvent.

2.7 Antioxidant Activity

2.7.1 DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay)

DPPH radical scavenging assay of test samples GCBE and MRKT were prepared in different concentrations of samples were mixed with 2.5 mL of DPPH solution. The reaction mixture was vortexes thoroughly and kept at room temperature for 30 minutes. It is observed at a rangeof517 nm. For reference, standard Ascorbic Acid was used. The ability of extracts to scavenge DPPH radical and control was calculated from the following formula, which is a modified method described by Perumal *et al*., [14].

% DPPH inhibition = [(OD of control - OD of test)/ (OD of control)] ×100

2.7.2 ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging assay)

ABTS radical scavenging assay of test samples GCBE and MRKT was prepared in different concentrations. ABTS reagent (7 mM, 25 mL in deionized water), a stock solution was prepared with potassium persulfate (140 mM, 440 µL). Different concentrations of test samples and standard (Ascorbic Acid) were mixed with the ABTS working solution (2.0 mL), and the reaction mixture could stand at ambient temperature for 20 minutes; then, the ABTS was observed at 734 nm. ABTS radical scavenging effect was calculated by the following formula, which is a modified method described by Perumal *et al*., [14].

ABTS radical scavenging effect (%) = [(A0 - A1)/A0] ×100. Where, A-0 is the control; A-1 is the test.

2.8 Total CFU

The diluted sample is pipetted on the surface of a solidified agar medium and spread with a sterilized, bent glass rod (glass spreader) for the determination of heterotrophic plate count using the spread–plate method. Make a 10-fold dilution series from a sample: measure 1 g sample into a flask containing 99 mL sterile water (or 1 mL water sample to 99 mL pure water) mix thoroughly with a vortex mixer, pipette 1 mL from this suspension into a test tube containing 9 mL sterile water, mix thoroughly with vortex, pipette 1 mL from this latter suspension into another test tube containing 9 mL pure water, mix thoroughly. Spread 0.1 mL from the given dilution onto the surface of agar plates: pipette 0.1 mL from the appropriate member of the dilution series onto the centre of the agar surface; rinse the glass spreader with alcohol and sterilize the rod by flaming; cooling down the glass spreader by touching the medium surface; spread the liquid evenly over the surface. Incubate Petri dishes at 28°C for 24 and count the number of discrete colonies, in case of parallel plates, average the numbers and calculate the CFU value of the sample. The results of different dilutions should also be averaged. Give the CFU values of the original sample in CFU/mL or CFU/g units [15].

2.9 Alpha-amylase Assay

The α-amylase inhibitory activity of the test samples GCBE and MRKT were carried out according to the standard method with minor modification [16]. 100 µl of α-amylase solution (0.1 mg/mL) were mixed with different concentrations (10, 20, 40, 80, 160, and 320 µg/mL) of test samples, standard (acarbose), and control (without standard/test samples) and pre-incubated at 37°C for 15 min. Then, 100 µl of the starch solution was added to initiate reaction and incubation was done at 37°C for 60 min., then 10 µl of 1 M HCl and 100 µl of iodine reagent were added to the test tubes. It was absorbed and measured at 580 nm. α-amylase inhibitory activity was measured using the formula.

% of Inhibition = [(OD of test - OD of control)/OD of test] x 100

2.10 Anticancer Activity Studies Using MTT

The monolayer cell culture was replanted (well), and the cell count was adjusted to 1.0 x 10^5 cells/mL using respective media containing 10% FBS. To each well of the 96 well microtiter plate, 100µl of the diluted cell suspension (50,000cells/well) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium, and 100µl of different test concentrations of test drugs were added on to the partial monolayer in microtiter plates(well).It is incubated at 37^{o}C for 24hrs in a 5% CO_2 atmosphere. After incubation, the test solutions in the wells were discarded, and 100µl of MTT (5 mg/10 mL of MTT in PBS) was added to each well. It is incubated for 4h at 37^{o}C in a 5% CO_2 atmosphere. The supernatant was removed, and 100µl of DMSO was added, and the plates were gently shaken to solubilize the formed formazan. It is observed at a wavelength of 570 nm [17,18], and the concentration of test drug needed to inhibit cell growth by 50% (IC_{50}) values is generated from the dose-response curves for each cell line.

2.11 Anti-obesity Activity (Pancreatic Lipase Inhibition)

The porcine pancreatic lipase inhibitory assay adapted from Zheng et al., 2010, and Bustanji et al.,[19]it was done by 1 mg/mL plant extract stock solution in 10% DMSO was used, from which five different solutions were prepared with the following concentrations: 10–320 µg/mL. 1 mg/mL stock solution of pancreatic lipase enzyme was prepared immediately before being used. This procedure was carried for the ten studied plant species. A stock solution of PNPB (p-nitrophenyl butyrate) was prepared by dissolving 20.9 mg of PNPB in 2 mL of acetonitrile. 0.1 mL of porcine pancreatic lipase (1 mg/mL) was added to test tubes containing 0.2 mL of the various concentrations (10–320 µg/mL) of plant extract. The resulting mixtures were then made up to 1 mL by adding Tri-HCl solution (pH 7.4) and incubated at 25°C for 15 min. After the incubation period, 0.1 mL of PNPB solution was then added to each test tube. The mixture was again incubated for 30 min at 37°C. Pancreatic lipase activity was determined by measuring the hydrolysis of p-nitrophenyl butyrate to p-nitrophenol at 405 nm using a UV-visible spectrophotometer. The same procedure was repeated for Orlistat (a positive control) using the same concentrations as mentioned above. The established tests were performed in triplicates.

2.12 Statistical Analysis

Measurements were carried out in triplicates, and the obtained data is gathered using the inhibitory concentration (IC50) and calculated using GraphPad Prism version 5.

3. RESULTS AND DISCUSSION

3.1 Solubility Test

After testing of GCBE with different solvent systems, they were soluble in Methanol, DMSO, and partially soluble in Ethanol (Table 1) insoluble in Chloroform and Aqueous, respectively.

3.2 Qualitative Phytochemicals

After testing, the alkaloids were present in GCBE are tannins, glycosides, flavonoids, phenols, steroids, and terpenoids (Table 2), respectively.

3.3 Total Phenolic Content

Total phenol content in the plant extracts GCBE and MRKT using the calibration curve was found to be 211.34, and 134.37 mg of Gallic Acid equivalents/g dry weight of extract (Table 3).

3.4 Thin Layer Chromatography

After performing the experiment on GCBE of TLC the Rf value for GCBE is 1.66; 0.56; 0.73; 0.86; and for MRKT Rf values are 1.66; 0.56; 0.73; 0.86 observed respectively under laminar (Table 4).

3.5 DPPH (2, 2-diphenyl-1-picrylhydrazyl) Radical Scavenging Assay

The IC_{50} values of the given samples GCBE and MRKT were found to be 30.99, and 118.57 µg/mL and the standard drug (Ascorbic Acid) was 19.96 µg/mL, (Table 5) respectively.

3.6 ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-Sulphonic Acid) Radical Scavenging Assay

The IC_{50} values of the given samples GCBE and MRKT were found to be 0.83, and 23.98 µg/mL and the standard drug (Ascorbic Acid) was 4.22µg/mL, (Table 6) respectively.

Table 1. Properties of GCBE in solubility test

Extract	Solvent				
	Chloroform	**Methanol**	**Ethanol**	**Aqueous**	DMSO
GCBE	Insoluble	Soluble	Partial soluble	Insoluble	Soluble
MRKT	Insoluble	Insoluble	Insoluble	Soluble	Soluble

Table 2. Qualitative phytochemicals properties of Green coffee beans extract

	GCBE	**MRKT**
Alkaloids	-	-
Saponins	-	-
Tannins	+	-
Glycosides	+	-
Flavonoids	+	+
Phenols	+	+
Steroids	+	+
Terpenoids	+	-
Quinones	-	-
Proteins	-	-

Where + Present; - Absent

Table 3. Properties of total phenolic content in GCBE

Test sample	concentration	Singlate	Duplicate	Triplicate	Mean OD	TPC
GCBE	250 µg	0.673	0.676	0.674	0.674333	211.3434
MRKT	250 µg	0.421	0.418	0.422	0.420333	134.3737

Table 4. Properties of GCBE in TLC test

Extract	Solvent system	Number of spots	Rf value
GCBE	Butanol: Acetic Acid: Water (4:1:2)	4	1.66; 0.56; 0.73; 0.86
MRKT		4	1.66; 0.56; 0.73; 0.86

Table 5. Properties of DPPH assay in GCBE

GCBE									
Conc.(µg)	**Singlet**	**Duplicate**	**Triplicate**	**Singlet**	**Duplicate**	**Triplicate**	**Mean**	**sd**	**IC50**
5	0.75	0.75	0.75	6.56	5.93	6.31	6.27	0.31	
10	0.57	0.58	0.57	28.48	27.74	28.11	28.11	0.37	
20	0.44	0.44	0.44	44.18	44.55	44.18	44.31	0.21	
40	0.37	0.38	0.38	53.03	52.40	52.65	52.69	0.31	
80	0.15	0.15	0.15	80.81	80.19	80.56	80.52	0.31	
160	0.11	0.11	0.11	85.79	86.29	86.04	86.04	0.24	
320	0.09	0.08	0.09	88.53	89.16	88.66	88.78	0.32	
									30.99

Table 6. Properties of ABTS assay in GCBE

Conc.(µg)	**Singlet**	**Duplicate**	**Triplicate**	**Singlet**	**Duplicate**	**Triplicate**	**Mean**	**Sd**	**IC50**
5	0.30	0.31	0.31	58.73	57.79	58.33	58.28	0.47	
10	0.17	0.18	0.18	76.34	75.67	75.40	75.80	0.48	
20	0.13	0.13	0.13	82.12	81.45	81.72	81.76	0.33	
40	0.028	0.034	0.03	96.23	95.43	95.96	95.87	0.41	
80	0.02	0.025	0.023	97.31	96.63	96.90	96.95	0.33	
160	0.016	0.012	0.015	97.84	98.38	97.98	98.07	0.27	
320	0.005	0.003	0.003	99.32	99.59	99.59	99.50	0.15	
									0.83

Table 7. Properties of CUF in GCBE

Sample name	**Number of colonies (10^{-7})**
	NA
GCBE	-
MRKT	-

where + present; - absent

Table 8. Properties of alpha-amylase inhibitory assay in GCBE

Conc.(µg)	Singlet	Duplicate	Triplicate	Singlet	Duplicate	Triplicate	Mean	Sd	IC50
5	0.06	0.07	0.06	6.46	12.96	7.84	9.09	3.42	
10	0.09	0.09	0.09	32.61	30.37	30.37	31.11	1.29	
20	0.10	0.11	0.10	40.31	43.03	41.43	41.59	1.36	
40	0.18	0.18	0.19	65.75	66.66	67.01	66.48	0.65	
80	0.21	0.22	0.22	71.25	72.02	72.27	71.84	0.53	
160	0.27	0.27	0.27	76.96	76.79	77.29	77.01	0.25	
320	0.32	0.33	0.32	80.95	81.18	80.77	80.97	0.20	
									31.75

Table 9. Properties of MTT assay - HT29 cell lines in GCBE

			Concentration Unit: µG					
GCBE	**Blank**	**Untreated**	**3.12**	**6.25**	**12.5**	**25**	**50**	**100**IC 50
Reading 1	0.004	1.34	1.30	1.20	0.99	0.90	0.86	0.66
Reading 2	0.003	1.35	1.31	1.21	0.98	0.92	0.85	0.65
Reading 3	0.006	1.33	1.31	1.19	1.01	0.91	0.85	0.65
Mean	0.004	1.34	1.31	1.20	0.99	0.91	0.85	0.65
Mean OD-Mean B		1.34	1.30	1.20	0.98	0.91	0.85	0.65
STANDARD DEVIATION		0.011	0.006	0.010	0.015	0.010	0.008	0.009
Viability %		100	97.56	89.55	73.83	67.91	63.69	48.74
								101.26

3.7 Total CFU

The activity of microbial count after incubation the no. of colonies (10^{-7}) formed in GCBE and MRKT is purely absent (Table 7).

3.8 Alpha-amylase Assay

The IC_{50} value of the given samples GCBE and MRKT was found to be 31.75, and 47.77 µg/mL and the standard drug (Acarbose) was10.22 µg/mL (Table 8) respectively.

3.9 Anticancer Activity Studies Using MTT

The IC_{50} value of the given samples GCBE and MRKT is 101.26, and 152.11 µg/mL, (Table 9) respectively, microscopic images Fig. 2 of HT-29 cell lines.

3.10 Anti-obesity Activity (Pancreatic Lipase Inhibition)

The IC_{50} value of the given samples GCBE and MRKT was found to be and 33.34 and 40.48 µg/mL and the standard drug (Acarbose) was 11.12 µg/mL, respectively (Table 10).

From the above results it was clear that Green coffee bean extract (GCBE) contains phenols, flavonoids, glycosides, tannins steroids. Whereas the marketed (MRKT) sample contain flavonoids, Phenols and steroids only. The total phenolic content in GCBE was 211.34mg and in MRKT sample it was 134.37mg. The results of thin layer chromatography showed same Rf values of four spots (Rf values are 1.66; 0.56; 0.73; 0.86). The results of DPPH radical scavenging assay of GCBE and MRKT are as 30.99, and 118.57 µg/mL respectively. The IC_{50} values of ABTS of the given samples GCBE and MRKT were found to be 0.83, and 23.98 µg/mL respectively and the standard drug (Ascorbic Acid) was 4.22µg/mL. The activity of microbial count after incubation the number of colonies formed in GCBE and MRKT were absent. The IC_{50} value of alpha-amylase assay was GCBE and MRKT was found to be 31.75, and 47.77 µg/mL respectively and the standard drug (Acarbose) was10.22 µg/mL. The Anticancer Activity of the given samples of GCBE and MRKT was 101.26, and 152.11 µg/mL, respectively; microscopic images of HT-29 cell lines also supported the above results. The IC_{50} value of Pancreatic Lipase Inhibition for Anti-obesity activity of the given samples GCBE and MRKT was found to be and 33.34 and 40.48 µg/mL.

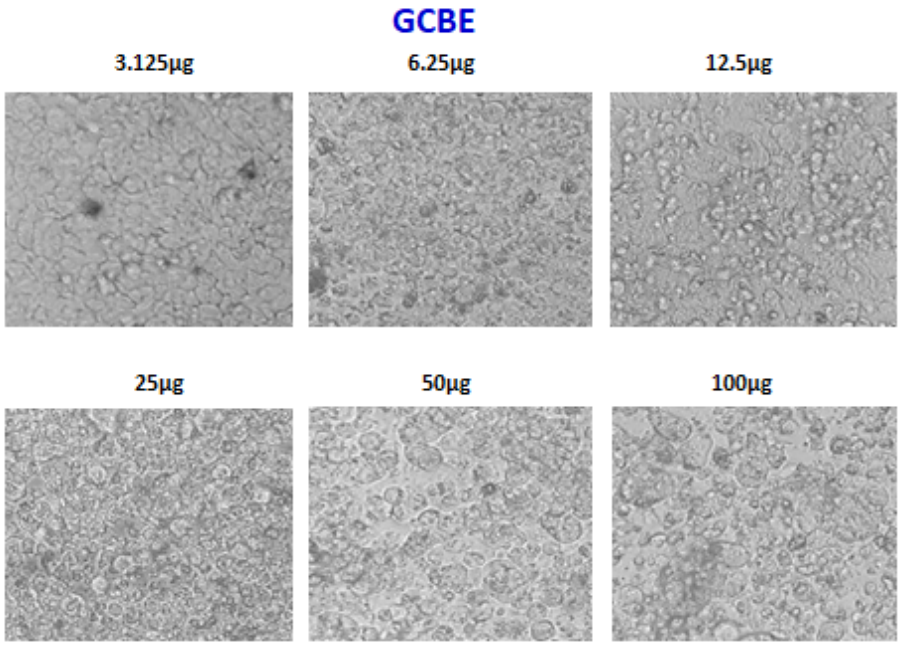

Fig. 2. Microscopic representation of MTT assay-HT29 cell line

Table 10. Properties of anti-obesity in GCBE

Conc.(µg)	Singlet	Duplicate	Triplicate	Singlet	Duplicate	Triplicate	Mean	SD	IC50
10µg	0.87	0.88	0.87	10.76	9.94	11.17	10.62	0.62	
20µg	0.55	0.56	0.55	43.05	42.64	43.87	43.19	0.62	
40µg	0.37	0.36	0.37	61.79	63.02	61.59	62.13	0.77	
80µg	0.22	0.22	0.21	76.97	77.28	77.69	77.31	0.35	
160µg	0.12	0.11	0.12	87.16	87.87	87.36	87.47	0.36	
320µg	0.08	0.08	0.08	91.64	91.13	91.44	91.40	0.25	
									33.34

4. CONCLUSION

Several experimental studies of Green coffee bean extract showed a statistically significant representation, GCBE has brought about a revolution in nutraceuticals marketplace, the outline of this study is about to understand chemistry, antioxidants, bioavailability effects of GCBE and CGA. Therefore, more trials are required to assess the helpfulness of green coffee extract as a health supplement. Notably, according to the effect of green Coffee on increasing energy metabolism, reducing lipogenesis, and additional health benefits over the human body. However, it is very much essential to standardize the extraction technique, quality assurance, and quality control, and clinical studies on the human to substantiate the claims and position of the product in the marketplace.

DISCLAIMER

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of study and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company; instead, it was financed by the personal efforts of the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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