

Evaluation of the anticancer potential of coffee beans: An *in vitro* study

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The aim of the present study was to evaluate the anticancer activity of the ethanol extract of raw coffee beans and identification of its active component. It was found that coffee bean ethanol extract, at concentration of 0.1 µg/ml, has potent anti-proliferative effect against HeLa and PA-1 cell lines with decrease in percentage viability to less than 30% after 72 hrs of incubation. Partially purified green TLC fraction was also found to be cytotoxic to HeLa and PA-1 cell lines with reduction in percentage viability to 26.8 % and 13.6%, respectively, after 72 hrs of incubation. The extract and the fraction were able to induce apoptosis and nuclear fragmentation in the cells as evidenced by DNA fragmentation assay and fluorescence microscopy. Flow cytometry analyses confirmed the ability of the green fraction to arrest the cells at G₀/G₁- phase. Its non-cytotoxicity to human peripheral lymphocytes indicates its safety towards humans.

Keywords: Antiproliferative, Coffee bean, Bioactive fraction, Cervical cancer, Ovarian cancer

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In the traditional systems of medicine practiced in India, such as *Unani* and *Ayurveda*, drugs of herbal origin have been used since ancient times. The plant derived drugs offer a stable market worldwide and also plants continue to be an important source for novel drugs¹. The use of natural products proves to be more effective when the preparations are derived from dietary sources like vegetables, fruits and beverages such as coffee and tea.

Coffee beans are isolated from berries of plant of genus *Coffea*. Unroasted coffee is one of the most traded agricultural commodities in the world². The use of coffee as a beverage all over the world dates back to 1700's. Caffeine is a naturally occurring chemical in coffee and is referred to as an "ancient wonder drug" for its potential physiological effects³. Caffeine is an alkaloid found in coffee, tea, cola drinks and cocoa⁴. Until recent reports on health benefits of coffee, it was believed that coffee consumption has negative effects on health. But, due to more sophisticated research on the mechanism of action of coffee and its effects, a more balanced view has emerged on its beneficial effects on health. The coffee contents are of good quality, are safe, effective on continued use and are consistent on a day-to-day

basis⁵. Coffee consumption was shown to exert greater influence on reduction of colorectal cancer. It was also shown that there is a decrease in the incidence of hepatocellular carcinoma in a case control study with moderate coffee consumption⁶. Coffee is prepared from roasted beans of coffee and so far several studies were conducted with coffee and roasted coffee beans. The aim of our current study was to check whether the ethanol extract of raw coffee beans has any anticancer effects on the cervical cancer cell line HeLa and the ovarian cancer cell line PA-1. Also, normal human lymphocytes were used to analyse the safety aspects on humans.

Methodology

Collection and authentication of the herb

The two main species of coffee sold in the world are *Coffea arabica* and *Coffea robusta*. The beans of *Coffea arabica* are the highest quality beans, generally referred to as gourmet coffee, whereas the beans of *Coffea robusta* are of lower grade, bitter and less flavoured than that of arabica. A coffee bean is a seed of the coffee plant, and is the source for coffee. It is the pit inside the red or purple fruit which is called as a cherry. Even though the coffee beans are seeds, they are referred to as 'beans' because of their resemblance to true beans.

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The fruits - coffee cherries or coffee berries - most commonly contain two stones with their flat sides together. Coffee refers to the beans (seeds) collected from plants of the genus *Coffea* belonging to the family Rubiaceae and to the beverage brewed from it.

For the current study, coffee beans from *Coffea arabica* were collected from local Farmers of Chikmagalore district, Karnataka and were authenticated at National Ayurveda Dietetics Research Institute, (NADRI), Bangalore, with the authentication number: RRCBI- 12873. The specimen sample of the same was preserved at the herbarium of Jain University, Bangalore (JUH-29).

Preparation of the extract

The coffee beans were dried under shade and finely ground in a kitchen blender. Fifty gm of the powder was weighed and packed in a Whatmann filter paper and extracted with ethanol using Soxhlet apparatus. The extract thus prepared was concentrated using a rotary evaporator, dried and stored at 4 °C for further use. Stock solution of 1mg/ml of the extract was prepared with DMSO and later diluted to required concentrations with Phosphate Buffered Saline (PBS) just before use⁷.

Cell culture

HeLa and PA-1 cell lines were procured from NCCS, Pune. HeLa cells were grown in Dulbecco's Modified Eagles Medium (HIMEDIA Laboratories Pvt Ltd) and PA-1 cell lines were grown in Minimum Essential Medium, both supplemented with 10% fetal bovine serum (HIMEDIA Laboratories Pvt Ltd). Cells were maintained below passage 20 and used in experiments during the linear phase of growth. Both the cell lines were used for the cytotoxicity tests.

Isolation of lymphocytes

Lymphocytes were isolated from the blood of few healthy male and female individuals, about 20 yrs of age, apparently free from infection by pathogenic agents and had not been under any treatment for the last six months. HiSep medium (HIMEDIA, India) was used for the isolation. Cells were suspended in complete RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (HIMEDIA, India), 5 mg/ml phytohemagglutinin (PHA) and maintained at 37°C in a 5% CO₂ humidified incubator. Lymphocytes were used as control cells to assess the cytotoxicity of the extracts.

MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide] assay

The assay was performed according to the standard protocol⁸. Briefly, 2×10^4 cells/well were seeded in 96 well microplates and incubated for 24 hrs at 37 °C. Then various concentrations of the extract (0.1, 1, 2.5, 5, 10, 15, 20 and 25 µg/ml) were added, and the microplates were further incubated for 24, 48, and 72 hrs in the same conditions. One column of each microplate was used as negative control (containing no extract). After specified time of incubation, the cell survival was analysed by the addition of 20µl of MTT solution (5 mg MTT dissolved in 1ml PBS) and incubated in the dark for 3 hrs. After the incubation period, the media was removed gently by aspiration and 200 µl of DMSO was added to each well and mixed to dissolve the insoluble formazan crystals. The absorbance was read at 540nm and cell survival was calculated. The percentage inhibition was then calculated using the formula:

$$\text{Inhibition (\%)} = (1 - \text{ODs} / \text{OD}) \times 100$$

Where, ODs = Optical Density of the sample, OD = Optical Density of the control.

Bioassay guided fractionation by thin layer chromatography (TLC)

Thin layer chromatography (TLC) is used to identify compounds present in a given mixture. TLC plate (TLC silica gel 60 F₂₅₄) was procured from Merck specialties private limited Mumbai. Different combinations of the solvents like Toluene: ethyl acetate (1:1 v/v and 9:1 v/v), Toluene: formic acid (9:1 and 5: 5 v/v), Butanol: Acetic Acid: Water (7:2:1 v/v), Toluene: ethyl acetate: formic acid (2.5:1:1 v/v), were used to separate the fractions. The fractions obtained were again tested for cytotoxicity with MTT assay and the fraction with highest cytotoxic property was further subjected to LC/MS analysis.

DNA fragmentation assay

HeLa and PA-1 cells (1×10^6 cells/ml) were cultured in 25cm² tissue culture flasks for 24 hrs. Different concentrations of coffee bean ethanol extract and its green fraction were added and incubated again for 24 hrs. A control was maintained with only cells. Cells were washed with PBS, trypsinized, centrifuged and the pellet obtained was lysed in buffer containing 10mM Tris HCl, 10mM EDTA, 0.5% Triton X 100. To avoid RNA and Protein contamination, 200µg/ml of RNase and 200µg/ml proteinase K were added.

DNA was precipitated with ice cold ethanol and suspended in Tris-EDTA solution. Samples were resolved using 0.8% agarose gel and visualised under UV Transilluminator⁹.

Caspase 9 activity assay

The assay was performed using caspase 9 apoptosis detection, colorimetric bioassay kit (G-Biosciences) according to the manufacturer's protocol. HeLa and PA-1 cells were treated with 5µg/ml of coffee bean green fraction for 24 hrs and a control was maintained. The cells were trypsinized, pelleted and resuspended in 50µl of lysis buffer (supplied with the kit). The suspension was centrifuged and the supernatant was collected. Fifty µl of caspase assay buffer (1M) containing DTT (dithiothreitol) was added to the lysate. Five µl of AFC (7-amino-4-trifluoromethyl coumarin) conjugated substrate (50µM final concentration) was added and the mixture was incubated at 37 °C for 2 hrs. Readings were noted down at 405nm for every half an hour and caspase activity was calculated as:

Percentage increase in caspase 9 activity by the sample

$$= \frac{(\text{OD}_{\text{control/sample}} - \text{OD}_{\text{blank}})}{\text{OD}_{\text{blank}}} \times 100$$

Flow cytometry for cell cycle analysis

The effect of coffee bean bioactive fraction on cell cycle was determined by Flow cytometry with PI Staining¹⁰. The treated HeLa and PA-1 cells were harvested by trypsinisation and centrifugation. Cells with a final concentration of 1-2×10⁶ cells/ml were fixed in chilled 70% ethanol at 4°C overnight. After incubation, cells were centrifuged again at 5000 rpm for 10 minutes and washed twice with PBS. Cells were resuspended in 1 mL of PBS and in ribonuclease (100 µg/ml). Then cells were re-suspended in staining solution (50 µg/ml propidium iodide, 30 units/ml RNase, 4 mM/l sodium citrate, and Triton X-100, pH 7.8) and incubated at 37 °C for 15 min. After incubation in the dark, fluorescence-activated cells were sorted in a FACS can flow cytometer (equipped with a 488 nm argon laser), and the data were analysed using MACS Quant analyser.

LC-MS analysis

HPLC coupled with mass spectrometry (LC-MS) is one of the newest and sensitive methods of molecular analysis that yields information on

the molecular weight as well as the structure of analytes. The advantage of LC-MS is the ability to determine selected metabolites in a few minutes from only a small amount of material and with a simple sample preparation¹¹. The TLC fractions which have shown cytotoxicity were collected, dissolved in methanol and centrifuged. The supernatant was collected in a pre-weighed eppendorf tube and air dried. The dried sample was used for LC - MS analysis. HPLC analysis was performed using Waters HPLC system with 2487 dual λ UV detector, 1525 binary pump, and C-18 octadecylsilane (ODS) column (150 × 4.6 mm) with 5 µm particle size. The separation was performed using isocratic elution with Methanol and water in a ratio of 1:2 as solvent system and at a flow rate of 1 ml/min. The sample and the solvent system were filtered through 0.22 µm polyvinylidene fluoride (PVDF) filters prior to injecting to the column¹². After this, Electro spray Ionisation Mass Spectrometric (ESI-MS) analysis was performed using a single quadrupole mass spectrometer (Hewlett Packard HP 1100 MSD series). Spectra were acquired over the mass range of 50-1500 m/z.

Statistical analysis

All experiments were carried out in triplicates. The results were calculated as mean ± standard error (SE) Values. Statistical significance was calculated using one way analysis of variance (ANOVA) and Dunnett's Multiple comparison test. The values < 0.05 were taken as significant.

Results and discussion

Coffee bean ethanol extract and its green fraction inhibit the viability of HeLa and PA-1 cell lines and are nontoxic to humans

When HeLa and PA-1 cells were treated with increasing concentrations of coffee bean extract, it was found that the percentage viability of HeLa was inhibited maximum by the lowest concentration of 0.1µg/ml with percentage viability of 44% and this was further decreased to 33.3% and 26% after 48 and 72 hrs, respectively (Fig. 1). But as the treatment concentration was gradually increased, at 25µg/ml, the percentage viability was 64% and as the duration of exposure further increased to 72hrs, it was found to decrease to 48%. Here it appears as though the other ingredients present in

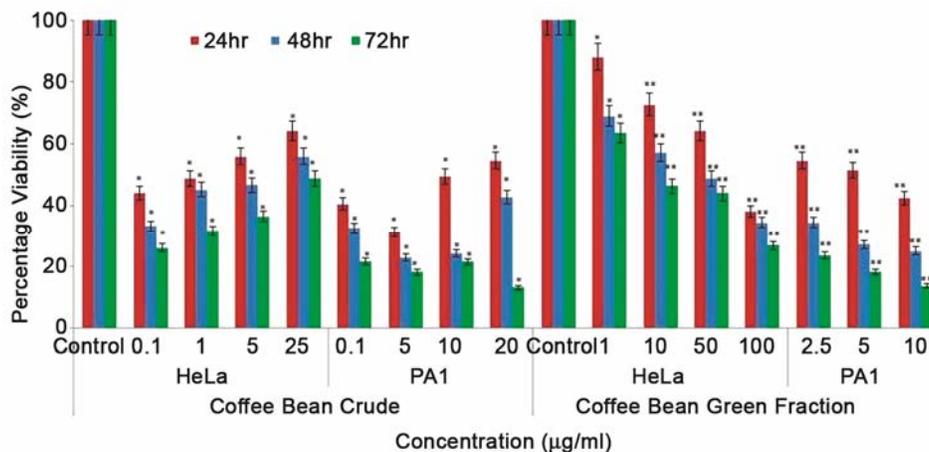


Fig. 1—Effect of different concentrations of coffee bean ethanol extract and its green fraction on HeLa and PA1 cells treated for 24, 48 and 72 hours. * Indicates P<0.01 compared with control.

this crude extract were interfering with the anticancer activity of the bioactive component. Even on PA-1 cells, a similar tendency of highest inhibition at lower concentrations was noticed. Bioactivity guided fractionation by TLC was performed to identify the fraction with anticancer potential. The solvent combination Toluene: Ethyl acetate: Formic Acid (2.5: 1: 1) was found to be the best, as it separated 11 distinct fractions. The fractions were visualised under UV-transilluminator (254-366nm). MTT assay of all the fractions with different concentrations was performed and the green fluorescent fraction with R_f value of 0.7 was found to exhibit highest cytotoxicity against HeLa and PA-1 cells. When HeLa and PA-1 cells were treated with the green fraction for 24, 48 and 72 hrs at different concentrations, there was a decrease in percentage viability of the cells at a dose and concentration dependent manner. This again indicates that there were other factors in the crude extract which counteracted the anticancer activity of the green fraction. The safety of the extract and its fraction on humans was evidenced by the results of MTT assay on lymphocytes (Results not shown), where the percentage viability remained approximately 100% at all the tested concentrations.

Flavonoids in coffee bean ethanol extract was cytotoxic

By phytochemical screening, the green fraction of coffee bean was found to be a flavonoid (results not shown). This is the first report of any flavonoid from coffee bean being anticancerous in nature. Other flavonoids from coffee with antibacterial activity were reported¹³.



Fig. 2—DNA Fragmentation pattern of the HeLa cells treated with Coffee Bean ethanol extract and Coffee Bean Green Fraction. Lanes - 1- Control HeLa Cells, 2 – HeLa cells treated with Coffee Bean Green Fraction (5µg/ml), 3- Control PA1 Cells, 4- PA1 cells treated with coffee bean green fraction (5 µg/ml).

Coffee bean green fraction leads to fragmentation of DNA in HeLa and PA-1 cells

DNA fragmentation is one of the characteristics of apoptotic cells. Hence, we next proceeded to analyse the DNA fragmentation pattern of the cells treated with the fraction using agarose gel electrophoresis. A ladder like DNA smear was observed in the cells treated with the extract. It was also observed that there is an increase in inter- nucleosomal DNA fragmentation with low concentration of the extract. The DNA pattern in the control cells was found to be a distinct band (Fig. 2).

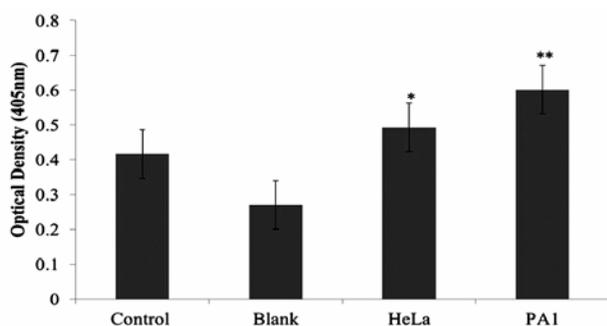


Fig. 3—Caspase 9 activity in HeLa and PA1 cells treated with coffee bean Green Fraction. * Indicates $P < 0.01$ compared with control. ** indicates high significance $P < 0.001$.

Coffee bean green fraction is responsible for elevated caspase 9 activity and induces cell damage in HeLa and PA-1 cell line

Caspase 9 is an initiator caspase that activates all the procaspases thereby bringing about apoptosis in the cells. Hence, we have studied the activity of caspase 9 in the cells treated with the green fluorescent fraction of coffee bean ethanol extract and also in the control cells. There was significant increase in caspase levels in treated cells in comparison to the controls (Fig. 3).

Coffee bean green fraction arrests the cells at G_0/G_1 phase

It was found that when cells were treated with coffee bean green fraction for 24 hrs and analysed for cell cycle phases, a greater number of cells were found at G_0/G_1 phase showing the arrest of the cells at the stage whereas, a lesser number of cells were found in S and G_2/M phase in comparison to control (Fig.4a, b).

LC- MS analysis

When the coffee bean green fraction was subjected to LC-MS analysis, in the HPLC chromatogram we could observe 3peaks, the first peak with a retention time (RT) 5.00min, second peak with RT 9.21min and a third peak with RT 17.48 min (Fig. 5a). The mass spectrophotometric results of the second peak revealed the presence of a compound with a mass to charge ratio of 599.67 (Fig. 5b). Any other anticancer compound with this molecular weight is not reported earlier in the database¹⁴ or in literature about anticancer compounds from coffee beans¹⁵. Hence, this flavonoid green fraction of coffee beans appears to be a novel one, which needs further characterization.

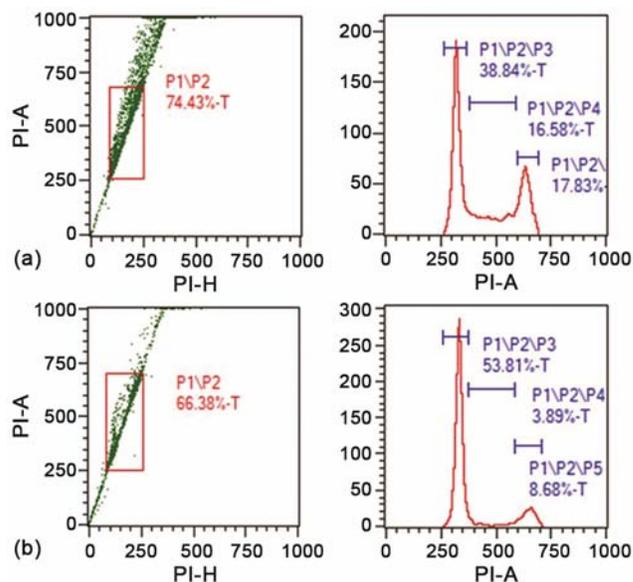


Fig. 4—Flow cytometry analyses. 4a Control HeLa cells. 4b. HeLa cells treated with coffee bean green fraction

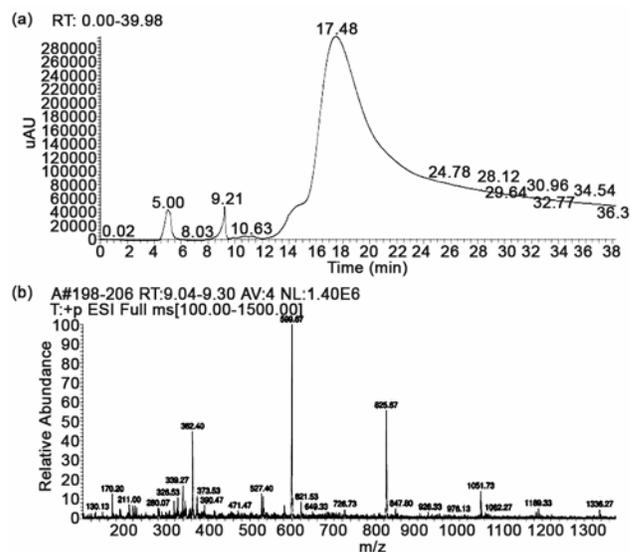


Fig. 5—LC-MS analysis of coffee bean green fraction. 5a: HPLC chromatogram of coffee bean green fraction showing 3 major peaks and 5b: Mass spectrum of coffee bean green fraction indicating the m/z value of 599.67 for the molecular ion fragment with 100% relative abundance

Traditional significance of study to the coffee growers/researchers/society

Coffee (*Coffea arabica* and *Coffea robusta*) has been characterized in the Chinese traditional system as to have taste and therapeutic actions. It belongs to the Rubiaceae family of plants, which is a traditional source of several Chinese medicinal herbs. Coffee also has been analyzed as a medicinal

herb by the Chinese system. According to the Chinese medical-dietary system, the green bean of coffee was classified as an herb which regulates liver qi (energy), which is its therapeutic route to strong energy stimulation, probably due to its caffeine content. In the Indian Materia Medica of 1908, several health benefits of coffee were reported¹⁶. According to some recent studies, moderate coffee drinking may lower the risk of colon cancer by about 25%, gallstones by 45%, cirrhosis of the liver by 80%, and Parkinson's disease by 50% to as much as 80%¹⁷. But all these were case studies. Hence, we took up this study to validate these earlier reports, and we found that the green fraction isolated from unroasted coffee beans was indeed responsible for the anticancer property. As coffee is a very important agricultural commodity and also one of the most popular beverages, this study is of greater significance to coffee growers in India and outside. Millions of small farmers in developing countries make their living by growing coffee¹⁸. As we could provide a scientific basis for the health benefits (anticancer property) of coffee beans through this study, the coffee growers and the people who enjoy consuming it can be greatly benefited as this would enhance the demand for this particular agricultural commodity. This study paves way for further research towards full characterization of the bioactive compound involved.

Conclusion

Our study provides evidence to the belief that only moderate consumption of coffee is beneficial to health rather than its over consumption. The present study further suggests that raw coffee bean is beneficial to health rather than coffee as a beverage which may have negative effects due to multiple bioactive components. The green fraction, if further purified, can be a source of a novel anticancer lead molecule.

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