

Anticancer potential of Indian propolis against HT29 colon cancer cells

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Abstract. Indian propolis is known to have a lot of different bioactive compounds with a wide array of biological effects, but it's still unknown how it works against colorectal carcinoma model. In this study, we assessed the in vitro anticancer efficacy of a 70% ethanolic extract of Indian propolis against HT29 colon cancer cells. The extract was tested for its physical and chemical properties as well as its phytochemical constitution. It was found to be high in phenolics and flavonoids, which are known to have strong antioxidant effects. Indian propolis decreased the viability of HT29 cells in a dose-dependent manner, exhibiting minimal toxicity towards HEK293 cells. Flow cytometric analysis and caspase-3/7 activation showed that the treated cells had the shape and nuclear features of apoptosis. Indian propolis also stopped HT29 cells from forming colonies, cell migration, and growing in 3D spheroids. In general, these results show that Indian propolis changes important cellular processes that are linked to the progression of colorectal carcinoma under in vitro conditions.

1 Introduction

Colorectal carcinoma (CRC) is the 3rd highest occurring cancer, and the 2nd chief cause of cancer associated deaths worldwide. It constitutes approximately 10% of annual cancer diagnoses [1]. Even though chemotherapy and targeted therapy for CRC have come a long way, the prognostic scenario for CRC is still not good because of the presence of cancer stem cell populations and chemoresistance to treatment [2]. Therefore, there exists a need for therapeutic agents with improved efficacy and reduced toxicity as a novel candidate for CRC treatment.

Natural products have historically served as a source of potential anticancer agents, with bee-derived substances employed since antiquity. Honeybees make propolis by mixing their saliva and beeswax with plant exudates. It is a sticky substance. It has a lot of effects on living things, like being an antioxidant, an antimicrobial, and an anticancer agent [3]. Propolis has a wide range of phytochemicals, such as flavonoids, phenolic acids, and terpenoids, that make it more useful for healing [4].

Many studies have shown that propolis and its principle phytoconstituents, such as caffeic acid phenethyl ester (CAPE) and galangin, trigger apoptosis, stop cell division, and stop cell migration in a wide range of cancer cell lines [5,6]. While the anticancer properties of propolis are well-established, there is a lack of research examining the effects of Indian propolis on the advancement of colon cancer using 3D spheroid and migratory models. This study examines the anticancer properties of Indian propolis on HT29 colon cancer cells, emphasizing its capacity to induce apoptosis, inhibit migration, and suppress 3D spheroid formation,

given the scarcity of data regarding Indian propolis in colorectal cancer, especially through 3D tumor spheroids and migration-based functional assays.

2 Materials and Methods

2.1 Chemicals and reagents

Indian propolis was collected from local Indian apiaries and grinded using liquid nitrogen. The resulting powder was dissolved in 70% ethanol with overnight shaking and filtered using Whatman no. 1 filter paper (Borosil, India). The resulting solution was further filtered through a 0.22 µm syringe filter before use.

Cell culture reagents including Dulbecco's Modified Eagle's Medium (DMEM), penicillin–streptomycin–amphotericin B (PSA), trypsin–EDTA, and phosphate-buffered saline (PBS) were sourced from HiMedia (Mumbai, India), while fetal bovine serum (FBS) was procured from Gibco (Thermo Fisher Scientific, USA). The following analytical reagents were purchased from Sigma-Aldrich (USA): 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), acridine orange, ethidium bromide, DAPI, and 2',7'-dichlorofluorescein diacetate (DCFDA). 5-fluorouracil (5-FU), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin–Ciocalteu phenol reagent, and Bradford reagent were obtained from Sisco Research Laboratories (SRL), India. The Annexin V-FITC/Propidium Iodide kit was supplied by Invitrogen (USA), and the Caspase-Glo® 3/7 assay system was acquired from Promega (Madison, WI, USA). Unless otherwise specified, all

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additional chemicals and solvents of analytical grade were obtained from SRL (India).

2.2 Physicochemical properties of Indian propolis and phytochemical characterization through liquid chromatography-mass spectrometry (LC-MS)

The different physicochemical properties of Indian propolis such as total phenolic content, total flavonoid content, DPPH free radical scavenging activity, protein content, ash content, moisture content, and pH were evaluated using previously published protocols by Saini et. al, 2025. [7] The LC-MS profiling of the phytochemical constituents of Indian propolis was done by following a previously published protocol. [8]

2.3 Cell culture

HT29 cell line was obtained from the National Centre for Cell Science (NCCS, Pune, India) and was maintained in DMEM high glucose supplemented with 10% FBS and 1% PSA solution. Cells were kept at 37 °C in a incubator supplied with 5% CO₂. For all tests, cells were seeded at 70–80% confluency, and the final concentration of ethanol in all groups was kept below 0.1%. To match the highest ethanol content in the treatment wells and account for any effects of the solvent, equal amounts of ethanol were given to the solvent control group.

2.4 Cell viability assay (MTT)

HT29 cells (10,000 cells/well) were placed in 96-well plates and given 70% ethanolic Indian propolis extract in increasing concentrations for 24 h. MTT (5 mg/mL; 20 µL/well) was added after the treatment period and incubated for 4 h. Fluostar optima (BMG Labtech, Germany) microplate reader was used to check the absorbance at 570 nm after dissolving the formazan crystals in Dimethyl sulfoxide (DMSO). The percentage of cell viability was compared to that of the untreated control.

2.5 Morphological and nuclear assessment

Microscopic examination was used to look at morphological alterations. For AO/EB labeling, treatment and control cells were incubated with a 1:1 mixture (100 µg/mL each) for 5 min in the dark and then looked at under a fluorescence microscope (Evos fluid, Thermofisher, USA). To stain cells with DAPI, they were fixed with 4% formaldehyde and exposed to DAPI (1 µg/mL) in PBS, and then looked at under UV fluorescence microscope to see nuclear condensation and fragmentation.

2.6 Colony forming assay and transwell migration assay

The cells were subjected to 70% ethanolic extract treatment of Indian propolis for 24 h. Then, they were

trypsinized and replated at a low density of 500 to 1,000 cells. After 10 to 14 days of incubation, the generated colonies were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet and let to dry in the air. Colonies were counted using image analysis tool, and the surviving percentage was assessed as compared to control based on plating efficiency.

Cell migration was determined using 8 µm pore-size Transwell inserts (Corning, USA). For this, the HT29 cells (5 × 10⁴/well) were allowed to attach in the upper chamber containing serum-free medium, while complete medium with 10% FBS was placed in the lower chamber to act as a chemoattractant. After 24 h, non-migrated cells from the upper layer were wiped off, and migrated cells in the lower side of the membrane were fixed in 4% formaldehyde, stained with 0.1% crystal violet, and counted microscopically.

2.7 Flow cytometric analysis of apoptosis and Caspase-Glo® 3/7 activity assay

Apoptotic populations were quantified following the source protocol of the kit. The cells were pelleted after trypsinization and washed two times with cold PBS. Then the pellet was dissolved in binding buffer and 5 µL of Annexin V-FITC and PI were added to it. After holding for 15 min in the dark, BD FACSCLyric™ flow cytometer (BD Biosciences, USA) was used to analyze the samples. Using FlowJo software (version 10.9), the percentage of living, early apoptotic, late apoptotic, and dead cell population was calculated.

After 12 and 24 h of treatment, the same amount of Caspase-Glo® reagent was applied to each well according to manufactures protocol. With gentle mixing, samples were subjected to incubation for 30 min in dark. Luminescence was measured with a BioTek Synergy HTX luminometer. The values were normalized with protein levels to reflect cell viability.

2.8 3D spheroid formation assay

To test the anti-spheroid potential, a 3D spheroid model was made by making a 1% agarose coating on 96-well U-bottom plates to stop cells from sticking to the surface. HT29 cells (1 × 10³ cells/well) were seeded and permitted to develop dense spheroids for 72 h. Indian propolis extract was treated on mature spheroids for 72 h. An inverted microscope (RADICAL, India) was used to observe the spheroid's diameter and shape, and ImageJ 1.53t software (NIH, USA) was used to measure the spheroid's area.

2.9 Statistical analysis

Statistical evaluation was performed using GraphPad Prism (version 8). Differences among groups were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was defined at $p < 0.05$.

3 Results and discussion

3.1 Physicochemical characterization and phytochemical profiling of Indian propolis by LC-MS

Table 1 shows a summary of the Indian propolis extract's basic physical, chemical, and biological properties. The extract had a significant amount of total phenolic content (TPC) and total flavonoid content (TFC), which means that polyphenolic compounds constitute a high part of the overall extract. The extract showed strong free radical scavenging activity in the

DPPH assay, which is in line with the observation that is reported in literature showing antioxidant activity. We also found measurable amounts of protein, which is consistent with earlier reports that propolis is a complex mixture of plant-derived resins and bee-derived proteins and enzymes. The amount of ash showed the content of minerals in the extract, and the amount of moisture was within an acceptable range, thus indicating the sample to be stable. The extract was a little acidic, which is typical of natural propolis and may help it work in the body [3].

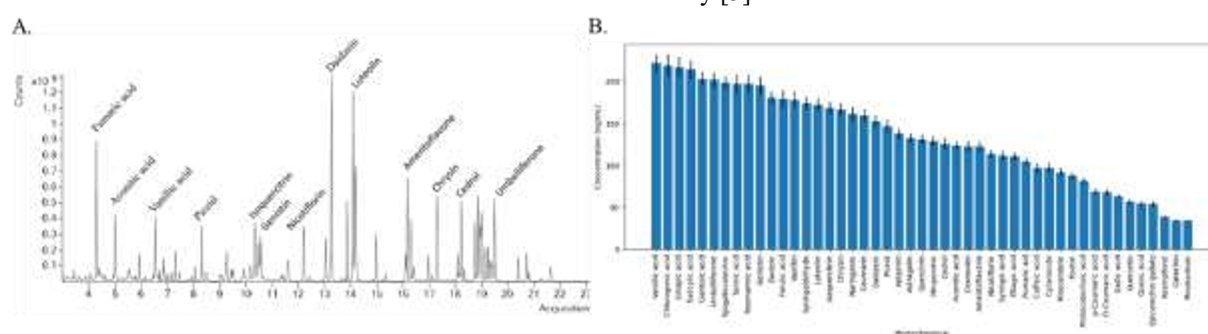


Figure 1. Phytochemical profiling of Indian propolis through LC-MS. A) Representative chromatogram of LC-MS analysis of Indian propolis. B) Quantitative representation of constituents present in the Indian propolis sample; Data are the mean \pm SD of three independent analyses of samples.

Table 1. Physicochemical properties of Indian propolis

Parameter	Values (Mean \pm SD)
Total Phenolic Content (TPC) (mg GAE/g extract)	182.4 \pm 6.8
Total Flavonoid Content (TFC) (mg RE/g extract)	98.6 \pm 4.3
DPPH Radical Scavenging (IC ₅₀ , μ g/mL)	42.3 \pm 3.5
Protein Content (mg/g)	12.4 \pm 1.8
Ash Content (%)	2.7 \pm 0.2
Moisture (%)	4.1 \pm 0.3
pH	4.6 \pm 0.1

Next LC-MS was used check the phytochemical makeup of the Indian propolis extract. The extract had a chemically diverse profile that included phenolic acids, flavonoids, isoflavones, coumarins, and other bioactive compounds. The compounds that were found were spread out over a wide range of concentrations, which indicated the collective nature of all the components rather than one or few major components. There were dominance of phenolic acids, such as chlorogenic acid, salicylic acid, ferulic acid, and caffeic acid. Along with coumarin derivatives like umbelliferone and other substances like cedrol, a number of flavonoids and polyphenolic compounds were found. These include quercetin, luteolin, chrysin, apigenin, genistein, daidzein, and amentoflavone. This phytochemical profile is mostly in line with those reported earlier, but it also showed some compositional differences that

could be due to differences in the floral types and geographical variations [3].

The LC-MS chromatogram (Figure 1A) shows that the extract is chemically complex because there are a number of peaks during the acquisition period. Figure 1B shows the relative abundance profile of some phytochemicals. The data showed that the components are spread out over time rather than being dominated by one compound. This pattern of composition supports the idea that the biological effects seen in later tests are probably caused by the combined actions of several components in the crude extract [9]. Several of the compounds that were found have been shown in the past to have anticancer effects, such as causing apoptosis, changing oxidative stress, and inhibiting cells from growing or migration in different cancer models. These effects have been linked to flavonoids like quercetin, luteolin, chrysin, and apigenin, as well as isoflavones like genistein and daidzein in colorectal and other cancers. Caffeic and ferulic acid are two examples of phenolic acids that are known to affect redox and inflammatory responses [10]. In this study, though, these links are based on previously existing literature, and direct mechanistic links were not tested in the lab.

The LC-MS analysis shows that Indian propolis is a complicated mixture of many different types of bioactive phytochemicals. The analysis being both qualitative and semi-quantitative it provides chemical context to the observed apoptotic, anti-proliferative, and anti-migratory effects in vitro and sets the stage for potential fractionation and studies to identify underlying mechanism.

3.2 Anticancer activity of Indian propolis extract in HT29 cells

Treatment of HT29 cells with Indian propolis extract caused a clear, dose-dependent decline in cell viability (Figure 2A). The IC_{50} value after 24 h was about 400 $\mu\text{g/mL}$. Normal HEK293 cells didn't had any significant cytotoxicity in the tested concentrations, which suggests that the extract has selective anticancer activity with low toxicity towards non-cancerous cell line. When HT29 cells were treated with propolis, they appeared to be reduced in size, rounded, and detached from each other, while the control group cells stayed the same shape (Figure 2B). DAPI staining further corroborated nuclear

condensation and fragmentation, signifying apoptotic cell death. AO/EB dual staining confirmed these results, revealing heightened orange/red fluorescence indicative of late apoptotic and necrotic populations in a dose-dependent manner, akin to the effect of the reference drug 5-fluorouracil (5-FU) (Figure 2C). After treatment with propolis, colony formation and cell migration were greatly reduced. Both low (200 $\mu\text{g/mL}$) and high (400 $\mu\text{g/mL}$) doses significantly inhibited clonogenic survival and motility compared to controls (Figure 2D). These findings collectively demonstrated that Indian propolis extract exerts potent anti-proliferative and anti-metastatic effects against HT29 colon cancer cells.

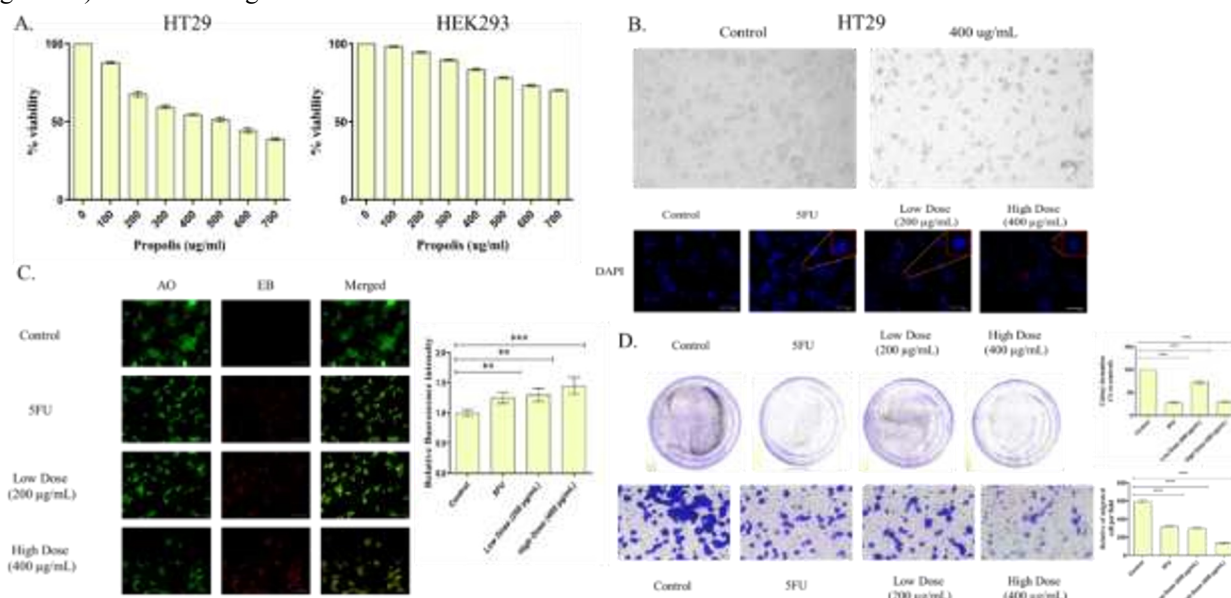


Figure 2: Anticancer effects of Indian propolis on HT29 colon cancer cells. (A) MTT assay showing dose-dependent cytotoxicity of Indian propolis extract with $IC_{50} \approx 400 \mu\text{g/mL}$ in HT29 cells and minimal effect on HEK293 normal cells. (B) Representative morphological and nuclear assessment of HT29 cells showing apoptotic features after propolis treatment compared to control and 5-FU (positive control). (C) Representative AO/EB dual staining demonstrating dose-dependent apoptosis induction by propolis. (D) Representative inhibition of colony formation and migration by propolis at low (200 $\mu\text{g/mL}$) and high (400 $\mu\text{g/mL}$) doses. Data are three independent experiments mean \pm SD; **** $p < 0.0001$ vs control.

3.3 Apoptosis induction by Indian propolis extract

Flow cytometric analysis using Annexin V/PI staining exhibited that Indian propolis extract triggered apoptosis in HT29 cells in a dose-dependent manner (Figure 3A). After the treatment of 24 h, both low (200 $\mu\text{g/mL}$) and high (400 $\mu\text{g/mL}$) doses markedly increased the fraction of late apoptotic populations, comparable to the 5-fluorouracil (5-FU) positive control. Caspase-3/7

activity assays further confirmed apoptotic activation (Figure 3B). A significant rise in caspase-3/7 activity was observed at 12 h post-treatment, which declined by 24 h, indicating early activation of the caspase cascade followed by progression into the late apoptotic phase or potentially into necrosis. These results indicate that Indian propolis extract activates a caspase-dependent apoptotic pathway, commencing early caspase activation and resulting in late-stage apoptosis in a time- and dose-dependent fashion, consistent with previous reports in colorectal cancer models [11].

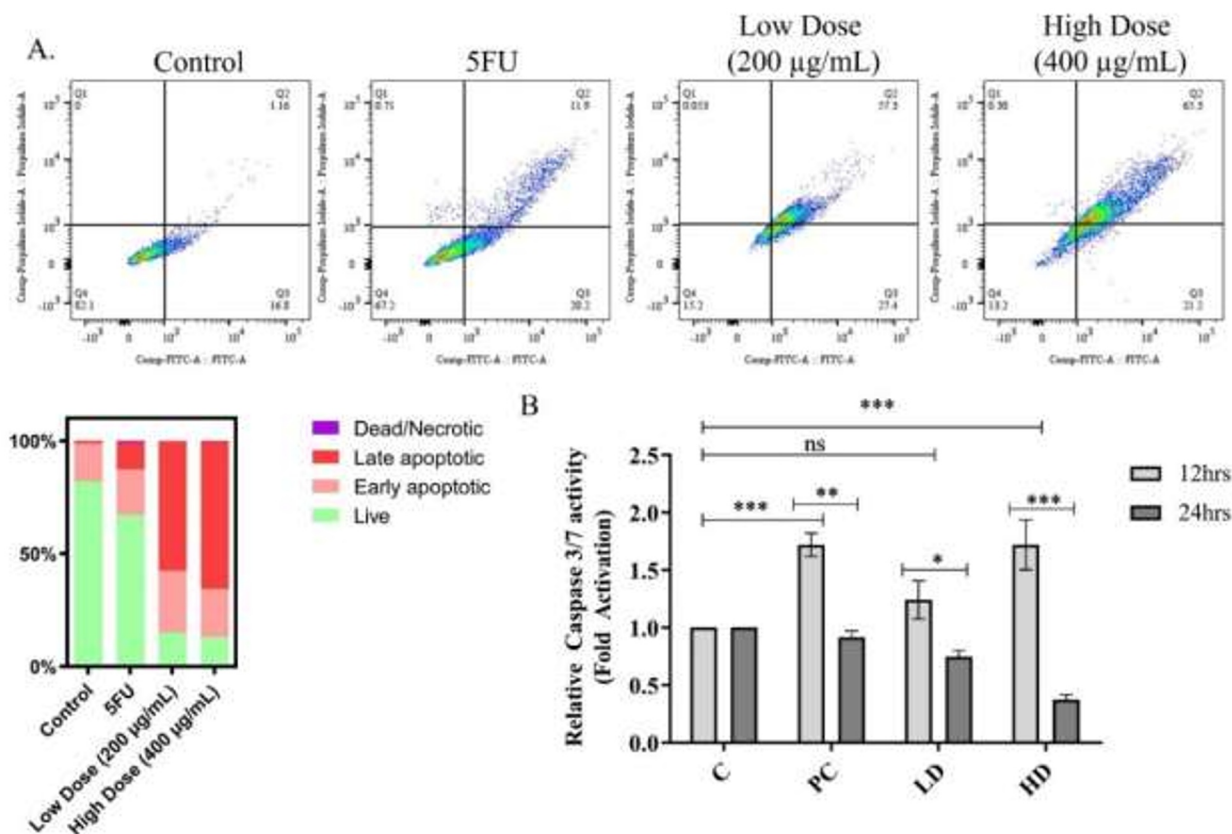


Figure 3. Induction of apoptosis by Indian propolis extract in HT29 cells. (A) Representative annexin V/PI flow cytometry assay showing dose-dependent increase in late apoptotic population after 24 h of treatment with propolis compared to control and positive control drug 5-FU. (B) Caspase-3/7 activity measured at 12 h and 24 h demonstrating early activation of caspase-mediated apoptosis at 12 h, followed by decline as cells transition to the late apoptotic phase. Data are represented as three independent experiments mean \pm SD (n = 3); *p < 0.05, **p < 0.01, ***p < 0.001 vs control.

3.4 Anticancer activity of Indian propolis extract in HT29 cells

To assess the impact of Indian propolis extract using a three-dimensional (3D) tumor model, HT29 colospheroids were cultivated on agarose-coated plates

and administered low (200 µg/mL) and high (400 µg/mL) doses of the extract. Figure 4 shows that the treated groups had much smaller and denser spheroids than the untreated control. Quantitative analysis indicated a notable reduction in the mean spheroid area, akin to that observed in the 5-fluorouracil (5-FU)-treated positive control.

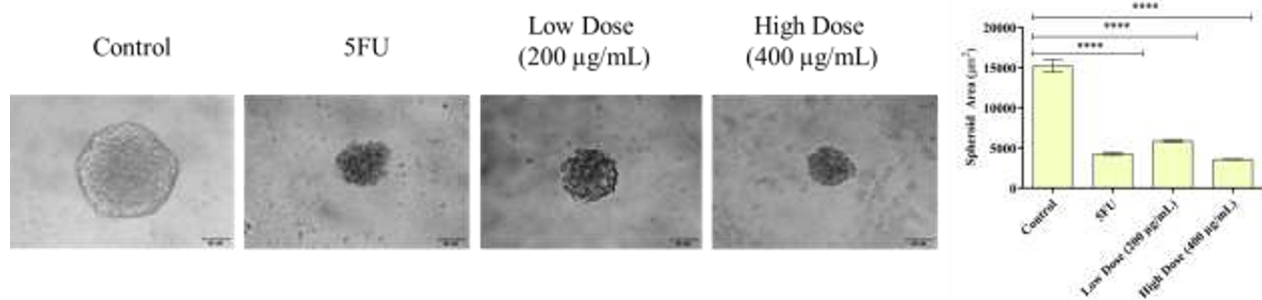


Figure 4: Inhibition of HT29 colospheroid growth by Indian propolis extract. Representative micrographs showing dose-dependent reduction in spheroid size after treatment with Indian propolis extract compared to control and 5-FU reference treatment. The bar graph shows a significant decrease in spheroid area (µm²) quantified from multiple fields. Data represented as three independent experiments mean \pm SD (n = 3); ****p < 0.0001 vs control.

4 Conclusion

The present study demonstrates that an ethanolic extract of Indian propolis exhibits multiple anticancer effects on HT29 colon cancer cells in vitro. Indian propolis

reduced cell viability, induced apoptotic cell death, and inhibited clonogenic survival, migration, and three-dimensional spheroid growth in a dose-dependent manner. Alterations in morphology, flow cytometric assessment of apoptosis, and temporal variations in caspase-3/7 activity supported these effects.

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Physicochemical analysis and LC–MS profiling confirmed that the extract is rich in phenolic and flavonoid compounds, clarifying the chemical foundation for the observed biological effects. This study provides preliminary *in vitro* evidence that Indian propolis can affect essential cellular processes associated with colon cancer progression, thereby supporting the need for additional mechanistic and *in vivo* investigations.

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