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Original Research

Cytotoxicity and antiproliferative activity of *Arctium lappa* extract on an *in vitro* model of human colorectal cancer

Amal I. Hassan[1,*], Ibrahim I. Bondouk[2], Mohamad Taha Abdelrahman[1], Hosam M. Saleh[1]

ABSTRACT

Background: Natural remedies are an excellent source for screening innovative and safe anticancer medicines. This study is intended to explore the potential toxicity of burdock seed extract, *Arctium lappa* (*A. lappa*), as well as its anti-regenerative and anti-proliferative effects on tumor cells *in vitro*. **Methods**: The antiproliferative effect of *A. lappa* ethanol extract (EtOH) was evaluated on human colorectal carcinoma (H-COLO-205) cells and normal skin fibroblast (HSF) cell lines using the SRB assay. Matrix metalloproteinases (MMP-2 and MMP-9) and apoptotic gene expressions (*e.g.*, BAX, BCL-2, P53) were analyzed using Western blot and qRT-PCR, respectively. **Results**: *A. lappa* inhibited H-COLO-205 cell growth with an IC_{50} of 11.80 μg/mL while sparing normal HSF cells (IC_{50} = 1485 μg/mL). The extract significantly increased pro-apoptotic gene expression, with upregulation of *caspase-3* (4.07-fold), *caspase-9* (3.66-fold), *P53* (2.69-fold), and *BAX* (22.44-fold), and downregulated the anti-apoptotic gene *BCL-2* by 13.33-fold. Migration assays showed that *A. lappa* reduced MMP-2 and MMP-9 protein levels by 1.36-fold and 1.34-fold, respectively. The extract also downregulated NF-κB expression and significantly decreased the levels of inflammatory cytokines IL-6, IL-1β, and TNF-α. Furthermore, *A. lappa* enhanced DNA fragmentation, with a 4.36% increase in comet tail migration and an 18.88 tail moment at a 100 μg/mL concentration. Molecular docking revealed that arctigenin, a major compound in *A. lappa*, forms stabilizing hydrogen bonds with TGF-βR1, inhibiting its kinase function and associated cancer pathways. **Conclusion**: These results indicate that *A. lappa* may serve as a promising plant-derived anticancer medication for colon cancer.

Key words: Cancer cells, antiproliferative, burdock, anticancer, apoptosis

[1]*Department of Radioisotopes, Nuclear Research Centre, Egyptian Atomic Energy Authority, Egypt*

[2]*Physics Department, Faculty of Science, University of Tanta, Tanta, Egypt*

Correspondence

Amal I. Hassan, Department of Radioisotopes, Nuclear Research Centre, Egyptian Atomic Energy Authority, Egypt

Email: virtualaml@gmail.com

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INTRODUCTION

Colorectal cancer poses a significant global health concern, with more than 1.5 million new cases reported worldwide in 2018. This number is projected to rise by 60% by the year 2030[1]. The effectiveness of chemotherapy in treating cancer is limited due to issues such as toxicity and tumor resistance. However, *A. lappa* seeds offer a promising solution as they contain essential oils, lignans, chlorogenic acid, and vitamins, including the potent antitumor compound arctigenin[2,3]. Recent research demonstrates that several plant extracts, such as alkaloids, flavonoids, and polyphenols, which have a high molecular weight, could prove effective in the therapy of human multiple myeloma (HMM)[4] and bone marrow (HBM8-k562) cells[5]. These compounds also showed antitumor activity against aggressive malignancies of the liver (Huh-7), oral cavity (HTB-43), and bladder (ECV304)[6].

In this study, we aim to find out the effects of *A. lappa* extract on human colorectal cancer cells, specifically the cell line Colo-205. This research specifically aims to determine (1) the cytotoxicity of A. lappa extract on Colo-205 cells, (2) its effect on cell proliferation and migration, and (3) to explore the molecular mechanisms behind its antiproliferative and pro-apoptotic effects using *in vitro* assays and molecular docking techniques.

METHODS

Chemicals and Reagents

The chemicals and reagents used in this research were of high purity. Ethanol ($\geq$ 99.8%), methanol ($\geq$ 99.9%), and acetonitrile (HPLC grade, $\geq$ 99.9%) were from Sigma-Aldrich, St. Louis, MO, USA. Potassium persulfate, trichloroacetic acid (TCA), aluminum chloride, sodium carbonate, and Folin-Ciocalteu reagent were from Merck, Darmstadt, Germany. Trypan blue dye (0.4%) and phosphate-buffered saline (PBS) were from Gibco (Thermo Fisher Scientific, USA). All solvents used in HPLC analysis were $\geq$ 99.8% pure and from Fisher Scientific (Waltham, MA, USA), including ethyl acetate and ethyl ether. The HPLC standards, which include cinnamic acid, benzoic acid derivatives, and gallic acid, came from Sigma-Aldrich with a purity of $\geq$ 98%.

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The chemicals used for preparing proteins and for Western blotting, such as acrylamide, bis-acrylamide, SDS, TEMED, ammonium persulfate, and Tris-HCl, were purchased from Bio-Rad, Hercules, CA, USA. ELISA kits for cytokine studies were from R&D Systems, Minneapolis, MN, USA. All chemicals used for the comet assay, including ethidium bromide, Triton X-100, and sodium lauroyl sarcosinate, were from Sigma-Aldrich.

Plant Seeds Collection

In April 2019, *A. lappa* seedlings were obtained from Tanta University's medicinal plant garden in Tanta, Egypt. Ethanol extraction was performed on powdered *A. lappa* seeds, yielding dark brown residues.

Phenolic Acids Profile in *A. Lappa* by HPLC

A 1 g sample was transferred into a conical flask equipped with a quick-fit system, followed by the addition of 20 mL of 2 M NaOH. The flask was flushed with nitrogen gas, sealed, and shaken for 4 hours at room temperature. The pH was adjusted to 2 using 6 M HCl. After centrifugation at 5000 rpm for 10 minutes, the supernatant was removed. Phenolic compounds were extracted in two stages using 50 mL of a 1:1 solution of ethyl acetate and ethyl ether. The organic layer was isolated and evaporated at 45°C, and the residue was dissolved in 2 mL of methanol[7]. An Agilent 1260 series HPLC system was utilized for analysis, employing a Zorbax Eclipse Plus C8 column. The mobile phase consisted of acetonitrile (solvent A) and a 2% acetic acid solution in water (v/v) (solvent B). The flow rate was maintained at 0.8 mL/min over a 70-minute run. The gradient program was as follows: 100% solvent B reduced to 85% over 30 minutes, then to 50% over the next 20 minutes, followed by a drop to 0% in 5 minutes, and finally returned to 100% B in the last 5 minutes. A 50 μL injection volume was used, and peaks for cinnamic acid and benzoic acid derivatives were monitored concurrently at 280 nm and 320 nm. Samples were filtered through a 0.45 μm syringe filter before injection. Peaks were identified by matching retention times and UV spectra with standards.

Total Flavonoid Content (TFC) in *A. Lappa* Ethanolic Extract

To assess the total flavonoid content, the aluminum chloride assay was utilized[8]. Samples (500 μL in tubes) at a 1 mg/mL concentration were prepared, followed by the addition of 0.1 mL of 1 M potassium acetate solution, 0.1 mL of 10% aluminum chloride solution, 1.5 mL of methanol, and 2.8 mL of distilled water. After a 30-minute incubation at room temperature, absorbance was measured at 415 nm using a spectrophotometer.

Total Phenol Content in *A. Lappa* Ethanolic Extract

The Folin-Ciocalteu reagent was employed to quantify the phenol content in the samples, following a previously established method[9]. In our procedure, 1 mL of the extract was mixed with 5 mL of tenfold-diluted Folin–Ciocalteu reagent and 4 mL of sodium carbonate solution (75 g/L). After incubating the mixture for 30 minutes at room temperature, the absorbance was measured at 765 nm using a spectrophotometer. Gallic acid was used as the standard for calibration, and the total phenolic content was expressed in milligrams of gallic acid equivalents (GAE) per gram of extract.

Free Radical Scavenging

The DPPH assay was conducted to measure the antioxidant capacity of *A. lappa* extract[10]. Samples (at 6–200 μg/mL) were mixed with 100 μL of 1,1-diphenyl-2-picrylhydrazyl (DPPH) to achieve a final DPPH concentration of 100 μM. Absolute methanol served as the negative control. After incubation at 37°C, absorbance was measured at 512 nm using a UV-Vis Spectrophotometer 2401PC (Shimadzu, Japan). Ascorbic acid (Vitamin C) was used as a standard.

ABTS·$^+$ [2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] Radical Scavenging Activity

The ABTS test was used to determine the radical scavenging activity of *A. lappa* extract[11]. This method involves the generation of the ABTS radical cation (ABTS·$^+$) by the oxidation of ABTS with an oxidizing agent, typically potassium persulfate. The resulting ABTS·$^+$ is a stable, blue-green chromophore that absorbs light at a wavelength of approximately 734 nm. In this study, the ABTS assay was employed to determine the radical scavenging capacity of *Arctium lappa* extract. The extract was introduced to the ABTS·$^+$ solution, and its antioxidant compounds reduced the radical cation, leading to a decrease in absorbance at 734 nm. This reduction is directly proportional to the antioxidant activity of the sample. The results were likely quantified as a percentage of radical scavenging activity or compared to a standard antioxidant, such as Trolox, to calculate the Trolox Equivalent Antioxidant Capacity (TEAC).

The method is valued for its sensitivity and ability to measure the antioxidant capacity of both hydrophilic

and lipophilic compounds, making it suitable for diverse biological extracts like *A. lappa*.

Reducing Power (RP) of *Arctium lappa* Ethanol Extract

The RP test was conducted to determine the reducing power of *A. lappa* extract [12]. This method works on the principle that antioxidants can reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}), which then form a colored complex with ferricyanide. The intensity of this color is directly proportional to the sample's reducing power and can be quantified spectrophotometrically.

To assess the reducing power of the ethanol (EtOH) extract of *Arctium lappa*, the assay was conducted using standard reagents and procedures. Phosphate buffer (0.2 M, pH 6.6) was prepared to maintain optimal pH conditions for the reaction, and potassium ferricyanide [$K_3Fe(CN)$] was used as the electron acceptor. Trichloroacetic acid (TCA, 10%) was employed to terminate the reaction, while ferric chloride ($FeCl_3$, 0.1%) was added to detect the presence of ferrous ions. Varying concentrations of the *A. lappa* ethanol extract (*e.g.*, 20–100 μg/mL) were mixed with 2.5 mL of phosphate buffer and 2.5 mL of potassium ferricyanide solution. These mixtures were incubated at 50°C for 20 minutes to allow for the reduction of ferricyanide to ferrocyanide.

After the incubation, the reaction was halted by adding 2.5 mL of TCA, and the mixtures were centrifuged at 3000 rpm for 10 minutes to separate the supernatant. A 2.5 mL aliquot of the supernatant was combined with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride solution. This mixture was allowed to stand for 10 minutes to develop the characteristic color of the reaction. The absorbance of the resulting solution was measured at 700 nm using a UV-Vis spectrophotometer. A higher absorbance indicated greater reducing power, which reflects the antioxidant capacity of the *A. lappa* extract.

The assay included ascorbic acid as a positive control for comparison and a blank sample, containing all reagents except the extract, as a negative control. The reducing power of the *A. lappa* ethanol extract was expressed as absorbance values at 700 nm. Results were plotted as a function of extract concentration, with a higher slope indicating stronger reducing activity. Furthermore, the reducing power of the extract was compared to ascorbic acid to evaluate its relative antioxidant capacity.

The results of the RP assay demonstrated that the ethanol extract of *Arctium lappa* possesses notable electron-donating properties, indicative of its potential as a natural antioxidant source. These findings suggest that *A. lappa* extract has promising therapeutic applications due to its antioxidant properties.

Cell Culture

This study utilized COLO-205 (human colorectal cancer) and normal skin fibroblast cell lines (HSF), both obtained from Nawah Scientific Inc. (Egypt). All experiments were conducted in accordance with institutional biosafety guidelines in a certified laboratory facility. As this research employed only commercially available cell lines and plant extracts, no specific ethical approval for human or animal subjects was required.

The cells were cultured within RPMI medium with streptomycin, penicillin, and fetal bovine serum at 37°C within a CO_2-rich atmosphere. All cell testing was conducted in a laminar flow hood, with only sterilized instruments coming into direct contact with the cells. After the cells had achieved 80-90% confluence for 2 days, the culture was renewed. For splitting, the media was aspirated, and the cells underwent three washes utilizing phosphate-buffered saline with a pH of 7.4. PBS was removed from the flasks, and 0.025% trypsin-EDTA was added. Flasks were incubated for 3-5 minutes in the incubator before cells were released. Cells were diluted to the desired concentration and cultured in fresh flasks.

Cell Count

Trypan blue staining and hemocytometer counting were performed to determine cell viability and density [13]. This method is widely used to assess the health of cells in culture by distinguishing live cells from dead cells based on membrane integrity. Trypan blue selectively stains dead cells with compromised membranes, while viable cells remain unstained.

The process began with the preparation of materials and reagents. Trypan blue dye (0.4%) was used for staining, while phosphate-buffered saline (PBS) was employed for washing the cells before counting. A hemocytometer (Neubauer chamber) and a light microscope were used for manual cell counting. Cells were harvested from the culture flask using appropriate methods. Adherent cells were detached enzymatically using trypsin-EDTA while suspension cells were directly collected. The cell suspension was centrifuged at 300 g for 5 minutes, and the supernatant was discarded. The pellet was resuspended in 1 mL of fresh culture medium or PBS.

For staining, 10 μL of the cell suspension was mixed with 10 μL of Trypan blue dye to create a 1:1 dilution. The mixture was gently pipetted to ensure even distribution of the dye and incubated at room temperature

for 1–2 minutes. A cleaned hemocytometer was prepared by placing a coverslip over the counting grid, and 10 μL of the stained cell suspension was carefully loaded into one of the chambers, ensuring no bubbles or overfilling occurred.

The hemocytometer was placed under a light microscope at 10× magnification. The total number of live and dead cells was recorded. Cell concentration was calculated using the formula:

Cell concentration (cells/mL) = (Average number of cells per quadrant × Dilution factor × 10) / Volume of quadrant (0.1 mm^3)

The percentage of cell viability was determined by dividing the number of viable cells by the total number of cells and multiplying by 100. The results included the total number of live, dead, and total cells per mL, along with the viability percentage, providing essential data on the health of the cell culture. These metrics guided experimental planning, such as determining the seeding density for further applications.

Cytotoxicity Assay

The sulfate-reducing bacteria (SRB) test was conducted to assess cell survival following exposure to *A. lappa* extract[14]. In 96-well plates, 100 μL of a cell suspension (5×10^3 cells) was added to the complete medium and incubated for 24 hours. A second aliquot of 100 μL of media containing *A. lappa* methanolic seed extract was applied to the cells. Cisplatin (CP) was used as a reference drug at varying concentrations (0.01, 0.1, 1, 10, and 100 μg/mL). After 72 hours of treatment, the cells were harvested by replacing the culture media with 150 μL of 10% trichloroacetic acid (TCA) and incubating at 4 °C for 1 hour. Following TCA removal, the cells were washed five times with distilled water.

Next, 70 μL of 0.4% (w/v) SRB solution was added, and the cells were incubated in darkness at room temperature for 10 minutes. The plates were washed three times with 1% acetic acid and allowed to air dry overnight. The bound SRB stain was dissolved in 150 μL of TRIS buffer (10 mM), and absorbance was measured at 540 nm using a BMG LABTECH® FLUOstar Omega microplate reader (Ortenberg, Germany).

Western Blotting of MMP-2 & MMP-9

Western blotting was conducted to analyze the expression levels of MMP-2 and MMP-9. Protein concentrations were determined using the Bradford method[15]. Standard Western blotting procedures were followed. Proteins (30 μg per lane) were separated on a 10% SDS-PAGE gel using a non-continuous buffer system. The separating gel contained 10% acrylamide-bisacrylamide (30:0.8 ratio), 0.1% SDS, 0.05% TEMED, 0.05% ammonium persulfate, and 375 mM Tris-HCl (pH 8.8). The stacking gel (4%) was formulated with 0.5 M Tris-HCl (pH 6.8). Electrophoresis was performed at 75 V through the stacking gel and 125 V through the resolving gel for approximately 2 hours. A PageRuler™ Unstained Broad Range Protein Ladder (Thermo Scientific™, Catalog No. 26630) was used as the molecular weight marker. Following electrophoresis, proteins were transferred to Hybond™ nylon membranes (GE Healthcare) and blocked with 2–5% nonfat dry milk in Tris-buffered saline (TBS, pH 7.4, containing 25 mM Tris, 150 mM NaCl, and 0.1% Tween® 20 [TBST]) for 1 hour at room temperature. The membranes were incubated overnight at 4 °C with primary antibodies against MMP-2 (ab86607, Abcam, 1:1000 dilution, 73 kDa) and MMP-9 (ab228402, Abcam, 1:1000 dilution, 78 kDa).

After three washes with TBST, the membranes were treated with horseradish peroxidase (HRP)-conjugated secondary antibody (0.1–0.5 μg/mL) for 1 hour at room temperature. β-actin was used as a loading control. Enhanced chemiluminescence (ECL) reagents (Amersham Biosciences) were used to detect the immunoreactive bands. The detected bands were quantified using Totallab analysis software (Version 1.0.1), with band intensities normalized to β-actin and expressed as a percentage of the control.

Finally, the protein bands were visualized and quantified using the ChemiDoc XRS imaging system (Bio-Rad Laboratories, USA) after three washes in TBST. Tween is a registered trademark of ICI Americas.

Gene Expression of *BCL2*, *BAX*, and *P53*

Total RNA was extracted from cell homogenates using the RNeasy Purification Kit (Qiagen, Valencia, CA, USA). Briefly, the cell homogenates were prepared by lysing the cells in the lysis buffer of the kit. The buffer contains guanidine isothiocyanate that enhances RNA protection and protein denaturation, yielding a high RNA output in purity and quantity. The homogenized lysate was transferred into a spin column containing a silica-based membrane that securely bound the RNA molecules. Packaged RNA was washed a number of times by using the kit's included washing machine in order to get rid of contaminants like proteins, DNA, and lipids. DNase digestion had been performed on the column during the operation in order to prevent contamination with genomic DNA. Lastly, the purified RNA was eluted

from RNase-free water and then pooled into sterile tubes for subsequent analyses. The quality and concentration of RNA extracted were checked by two different methods. First, RNA integrity was checked by gel electrophoresis, and the presence of distinct 28S and 18S rRNA bands confirmed a positive RNA class. RNA concentration and purity were measured spectrophotometrically using a Gene Quant 1300 spectrophotometer (Uppsala, Sweden). The absorbance at 260 nm (A260) was used to quantify RNA concentration, while the A260/A280 ratio provided an estimate of RNA purity, with values between 1.8 and 2.0 indicating minimal protein contamination. These steps ensured that the RNA samples were of sufficient quality and quantity for downstream applications such as cDNA synthesis and gene expression.

cDNA Synthesis

First-strand cDNA was synthesized from 4 μg of total RNA as a template, with an oligo (dT)1200 primer and SuperScript^{TM} II RNase H reverse transcriptase (Life Technologies, Breda, The Netherlands) This method targets a poly-A tail a mature upon mRNA did so, ensuring the selection of cDNA from mRNA transcripts. RNA, primer, dNTPs, first-strand buffer, dithiothreitol (DTT), and reverse transcriptase enzyme, prepared in the absence of RNase, in a reaction mixture with a total volume of 20 μL, to prevent RNA degradation or degradation. Initially, the RNA and primer were modified at 65°C for 5 min and then rapidly chilled on ice to minimize secondary products that could interfere with reverse transcription. The reverse transcription reaction was performed at 42°C for 60 min, allowing the enzyme to synthesize complementary DNA. After the reaction, the enzyme was inactivated by heat treatment at 70°C for 15 min. The resulting cDNA was stored at −20°C for subsequent gene expression studies, such as quantitative PCR (qPCR). This approach resulted in high-quality and specific cDNA synthesis, providing reliable templates for downstream analysis.

Real-time Quantitative Polymerase Chain Reaction (RT-PCR)

RT-PCR amplification was accomplished utilizing 10 μl amplification solutions comprising Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 300 nM primers, and 8 ng of reverse-transcribed ribonucleic acid. An ABI PRISM 7900 HT detection platform was employed to perform the reactions (Applied Biosystems). Data from PCR reactions that were run at 95°C for 10 minutes (1 cycle), 94°C for 15 seconds, and 60°C for 1 minute (40 cycles) were resolved using the ABI Prism sequence detection system software and the PE Biosystems v17 Sequence Detection Software (Foster City, CA) was employed for quantification. We computed the relative gene expression using the comparative threshold cycle technique. Furthermore, the results were standardized to the glyceraldehyde-3-phosphate dehydrogenase enzyme. The primer sequences are listed in **Table 1**.

IL6, IL1β, and TNF-α

ELISA kits were employed to measure cytokine release from COLO-205 cells. The cells were seeded in six-well plates and allowed to adhere for 24 hours. Following this, the cells were treated with *A. lappa* extract (100 μg/mL) for 48 hours. The levels of caspase 3, 9, IL-6, IL-1β, and TNF-α in the cell culture supernatants were quantified using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. A chromogenic substrate was used to develop the color reaction, and the optical density (OD) was measured at 495 nm using a microplate reader.

Comet Assay

The comet assay was conducted to detect DNA damage resulting from treatments[16]. After flushing the cells with 3 mL of PBS, the cell solution underwent filtration with a 150-μm bolting cloth. Following the combination of 25 μL of cell suspension with 250 μL of melted low-melting-point agarose in a 1:10 ratio, 75 μL of the resulting mixture was applied evenly onto the slides. Once the gel had set for 20 minutes at 4.0°C, the slides were placed within a tank containing a lysis solution (0.1 M EDTA, 2.5 M NaCl, 10.0 mM Tris base, 1% Triton X-100, and 1% sodium lauroyl sarcosinate) and left for 1 hour at 4.0°C in darkness. After applying three washes with neutralization solution (0.4 M Tris, pH 7.5) to the slides, they were placed at room temperature for 30 minutes in a new alkaline buffer (1.0 mM EDTA and 0.3 M NaOH, pH >13) to facilitate DNA unwinding. Electrophoresis was performed at ambient temperature for 30 minutes utilizing a chilled alkaline buffer (1V/cm; 300 mA). The slides were then carefully rinsed three times in a neutralizing buffer for 5 minutes each before being subjected to 70% ethanol for 5 minutes. After allowing the slides to dry at room temperature, 25 μL of ethidium bromide solution (20 μg/ml) was applied for staining, and the slides were then covered.

Table 1: The oligonucleotide primers sequence of studied genes

Gene	Forward primer (5' —— 3')	Reverse primer (5' —— 3')
BCL2	AGGAAGTGAACATTTCGGTGAC	GCTCAGTTCCAGGACCAGGC
BAX	GTTGCCCTCTTCTACTTTG	AGCCACCCTGGTCTTG
P53	TAACAGTTCCTGCATGGGCGGC	AGGACAGGCACAAACACGCACC
NF-κB	TCTCCTCGCTGGAAAAAGAA	AATGTGCTGGCTGTGCTTTA
GAPDH	TGCTGGTGCTGAGTATGTCG	TTGAGAGCAATGCCAGCC

Molecular Docking

The X-ray crystal structure of the Homo sapiens transforming growth factor receptor (TGF-βRI) was obtained from the protein databank depository via http://www.rcsb.org (PDB id: 3FAA) to be docked with the compound under study (Arctigenin) which was acquired from the PubChem database (PubChem CID: 64981) via https://pubchem.ncbi.nlm.nih.gov. The protein structure was ready for docking upon eliminating water molecules and the co-crystallized inhibitor (2-aminoimidazole). Hydrogen was added, and protein structure was stored into a PDB format employing Biovia Discovery Studio 2021. The arctigenin was docked against TGF-βRI (PDB id: 3FAA) within a cavity space defined by the coordinates (x: 75.1939, y: 28.8968, z: 92.6845). The docking was executed utilizing Autodock Vina with the PyRx 0.8 graphical user interface software.

Statistical Analysis

Data were expressed as the mean ± standard error of the mean (SEM) and analyzed using one-way analysis of variance (ANOVA) to determine statistical significance. For multiple comparisons, Tukey–Kramer's post hoc test was employed to identify significant differences between groups. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant. All statistical analyses were performed using the Statistical Analysis System (SAS) software, version 9.0.

RESULTS

Phytochemical Estimation in *A. lappa*

The phytochemical analysis of *Arctium lappa* revealed strong antioxidant properties. The ethanol extract exhibited a significant IC_{50} value of 41.17 ± 1.84 μg/mL for DPPH scavenging activity and an IC_{50} value of 51.65 ± 1.91 μg/mL for ABTS scavenging activity. Additionally, the extract contained high levels of total flavonoids (118.35 ± 0.62 mg QE/g DW) and total phenolics (124.45 ± 0.88 mg GAE/g DW), indicating a rich antioxidant profile. HPLC analysis identified various polyphenolic and flavonoid compounds in the *A. lappa* extract. The most abundant compounds were chlorogenic acid (1568.14 μg/g), rutin (1265.52 μg/g), and gallic acid (899.86 μg/g). Significant amounts of caffeic acid (774.56 μg/g) were also detected, along with moderate levels of ellagic acid (291.88 μg/g) and rosmarinic acid (115.32 μg/g). These results highlight that *A. lappa* is rich in potent antioxidant compounds, particularly chlorogenic acid, rutin, and gallic acid (**Table 2** and **Figure 1**).

Effects of *A. lappa* on the Propagation of Colorectal Cancer Cells *in vitro*

The results from the SRB assay demonstrated that *A. lappa* extract exhibited a strong inhibitory effect on the growth of Colo-205 colorectal cancer cells following treatment for 24, 48, and 72 hours (**Figure 2**). The ethanolic extract of *A. lappa* showed significant anti-proliferative activity, with an IC_{50} value of 11.80 μg/mL, as depicted in **Figure 2A–E**. Notably, a dose-dependent decrease in Colo-205 cell viability was observed with increasing concentrations of *A. lappa* extract (**Figure 2 A–D**). In contrast, *A. lappa* did not induce cytotoxicity in normal human skin fibroblasts (HSF), with an IC_{50} value of 1478 μg/mL, suggesting a degree of selectivity for cancer cells. For comparison, the reference anticancer drug cisplatin demonstrated an IC_{50} value of 8.40 μg/mL against Colo-205 cells, while showing much higher cytotoxicity toward normal cells, with an IC_{50} value of 645.7 μg/mL (**Figure 2F**). These findings highlight the potential of *A. lappa* as a promising anticancer agent with selective cytotoxicity against colorectal cancer cells while sparing normal cells. The results showed that *A. lappa* has a strong inhibitory effect on Colo-205 human colorectal cancer cells but no toxicity or effects on proliferation in normal cells (HSF) (**Figure 2 A-H**).

MMP-2 and MMP-9 Expression

Western blot analysis specified markedly elevated expressions of MMP-2 and MMP-9 proteins in COLO-

Table 2: Quantification of Polyphenol and Flavonoid Compounds in *Arctium lappa* Extract by HPLC

Compound	Peak Area	Concentration (μg/mL)	Concentration (μg/g)
Gallic acid	721.37	44.99	899.86
Chlorogenic acid	729.89	78.41	1568.14
Coffeic acid	615.31	38.73	774.56
Syringic acid	30.70	1.65	32.91
Rutin	506.19	63.28	1265.52
Ellagic acid	147.15	14.59	291.88
Vanillin	88.60	2.59	51.89
Naringenin	44.38	3.21	64.30
Rosmarinic acid	69.72	5.77	115.32
Daidzein	46.78	2.31	46.20
Querectin	6.95	0.46	9.20
Cinnamic acid	2.67	0.04	0.76
Kaempferol	9.34	0.52	10.43
Hesperetin	24.77	0.94	18.75

Note: Concentrations are presented in both μg/mL and μg/g for comprehensive analysis.

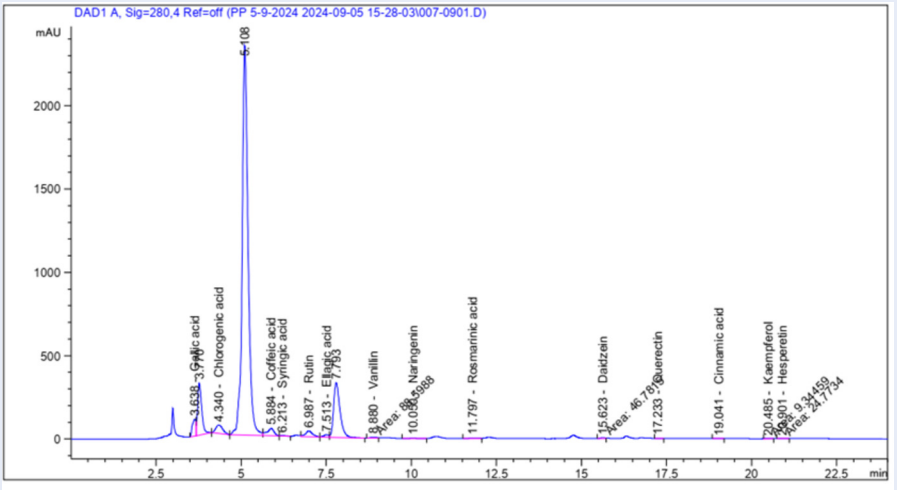

Figure 1: **HPLC analysis of phenolic and flavonoid compounds in *A. lappa* extract**. The chromatogram shows the retention times and peak areas of major phenolic and flavonoid compounds in the extract. **Abbreviation**: **HPLC**: High-Performance Liquid Chromatography

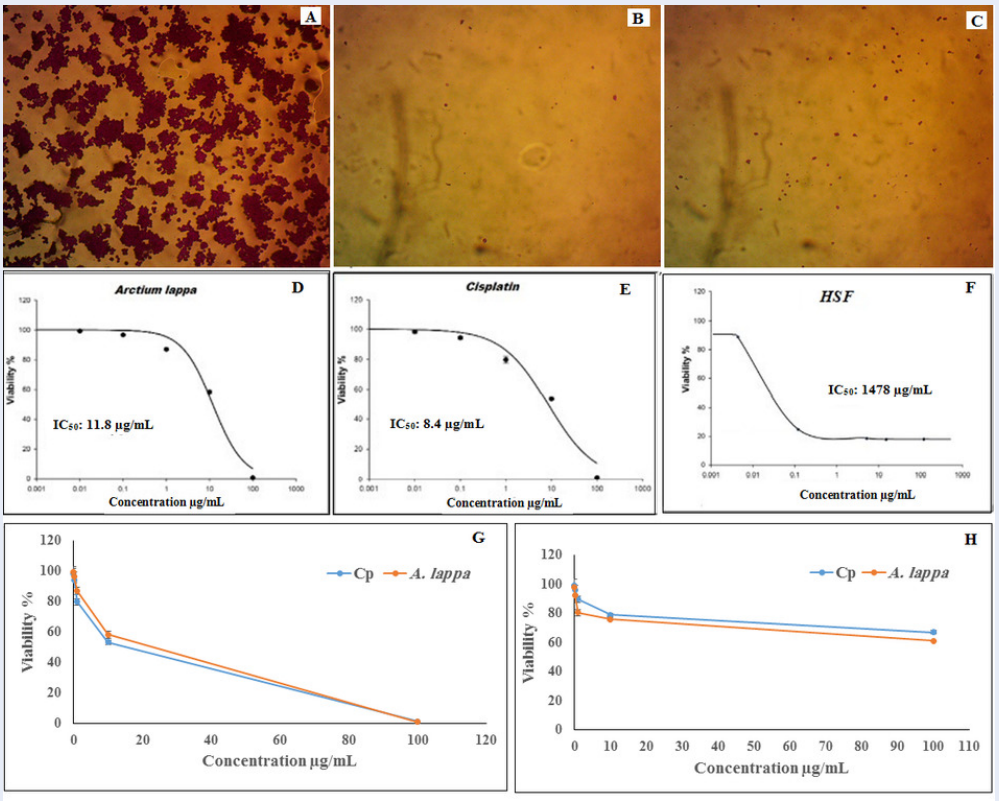

Figure 2: **Effects of *A. lappa* extract on the growth of Colo-205. A)** Untreated Colo-205 cells (Control). Baseline cell viability of Colo-205 cells with no treatment, serving as the control group (100% viability). **B**) Colo-205 cells treated with Cisplatin. Dose-dependent inhibition of Colo-205 cell viability following treatment with varying concentrations of cisplatin for 72 hours. **C**) Colo-205 cells treated with *A. lappa* extract. Dose-dependent inhibition of Colo-205 cell viability following treatment with varying concentrations of *A. lappa* ethanolic extract for 72 hours. **D**) IC_{50} values of *A. lappa* extract and **E**) cisplatin on Colo-205 cell viability, with cisplatin exhibiting an IC_{50} value of 8.40 μg/mL and *A. lappa* extract showing an IC_{50} of 11.80 μg/mL, **F**) *A. lappa* showed with an IC_{50} of 1485 μg/mL for normal HSF, **G**) Comparison of cytotoxicity between cisplatin and *A. lappa* extract on Colo-205 cells. *A. lappa* showed selective cytotoxicity against Colo-205 cells, **H**) *A. lappa* showed with a higher IC_{50} for normal HSF than for Colo-205 cells. **Abbreviations**: **HSF**: human skin fibroblasts, **IC_{50}**: Half Maximal Inhibitory Concentration

205 cells compared to HSF cells. However, when treated with burdock extract, the cells exhibited signs of recovery, as shown in (**Figure 3**). Western blot analysis was accomplished to appraise the expressions of MMP-2 in COLO-205 cancer cells under treatment conditions (**Figure 3**). Densitometric analysis revealed that control cells exhibited baseline MMP-2 expression (3.28%), with minimal change observed in the *A. lappa*-treated control group (3.47%). Notably, cancer induced a substantial upregulation of MMP-2 expression to 13.94%, corresponding to an approximate 4.25-fold increase relative to control conditions. Treatment with *A. lappa* resulted in MMP-2 expression levels of 10.23%, reflecting an approximate 1.36-fold reduction in MMP-2 expression compared to untreated conditions. This indicates that *A. lappa* effectively mitigates the elevated MMP-2 levels associated with cancer.

Western blot analysis was performed to examine Matrix Metalloproteinase-9 (MMP-9) expression levels in COLO-205 cancer cells under treatment conditions (**Figure 3**). Densitometric analysis normalized to β-actin revealed baseline MMP-9 expression in control cells (5.08%). Treatment with *A. lappa* alone showed a slight increase in MMP-9 expression (6.16%), representing a 1.21-fold increase compared to HSF. Notably, cancer induced a substantial upregulation of MMP-9 expression (15.06%), corresponding to a 2.96-fold increase relative to control conditions. Treatment with *A. lappa* resulted in MMP-9 expression levels of 11.24%; this reflects an approximate 1.34-fold reduction in MMP-9 expression compared to untreated conditions.

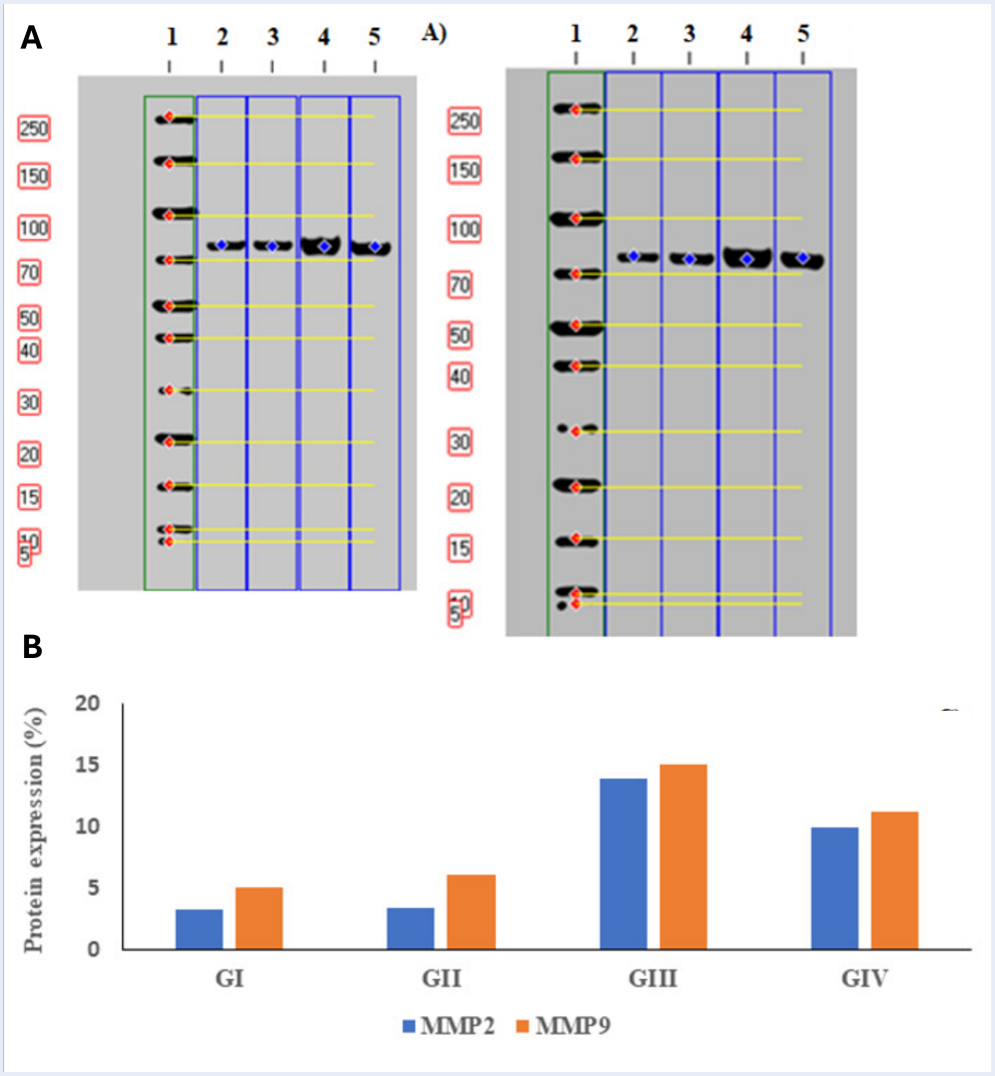

Figure 3: **Western blot analysis showing the expression of MMP-2 in Colo-205 cells treated with varying concentrations of *A. lappa* extract for 72 hours**. The bands corresponding to MMP-2 were detected, and the expression level was quantified relative to GAPDH. **A**) Western blot analysis showing the expression of MMP-9 in Colo-205 cells treated with *A. lappa* extract. The bands corresponding to MMP-9 were observed, with quantification against the housekeeping protein for normalization. **B**) Calculation of MMP-2 and MMP-9 levels based on their molecular weights. Densitometric analysis was performed to quantify the expression of MMP-2 and MMP-9 in treated cells, with values normalized to the loading control. The relative expression of MMP-2 and MMP-9 is shown as fold changes compared to untreated (control) cells. **Abbreviations**: **GAPDH**: Glyceraldehyde-3-Phosphate Dehydrogenase, **MMP**: Matrix Metalloproteinase, **GI, GII, GIII and GIV**: Group I, II, III and IV

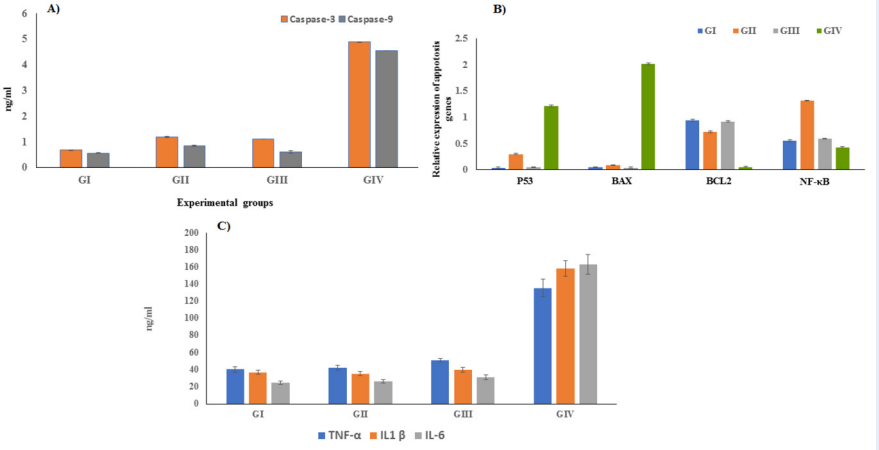

Figure 4: ELISA analysis showing the expression levels of (**A**) Caspase-3 and Caspase-9 in Colo-205 cells treated with *A. lappa* extract. **B**) Gene Expression analysis of apoptosis-regulatory genes *P53, BAX, BCL-2,* and *NF-κB* in *A. lappa*-treated Colo-205 cells. The expression levels of pro-apoptotic (BAX, P53) and anti-apoptotic (BCL-2) proteins, as well as NF-κB, were assessed. **C**) Quantification of inflammatory cytokines (TNF-α, IL-1β, and IL-6) in the cell culture supernatants from Colo-205 cells treated with *A. lappa* extract, measured by ELISA. The levels of these pro-inflammatory cytokines were quantified and compared to untreated controls. Cytokine concentrations are expressed in pg/mL. **Abbreviation**: **ELISA**: Enzyme-Linked Immunosorbent Assay

Caspase-3 and 9

To further investigate the effects of *A. lappa* treatment, we measured the mRNA levels of pro-apoptotic and anti-apoptotic genes using qRT-PCR in COLO-205 cells, after 24 hours of treatment with *A. lappa* at IC_{50} concentrations. The caspase-3, caspase-9 (**Figure 4A**), P53, and BAX expressions (**Figure 4 B**) were upregulated by 4.07, 3.66, 2.69, and 22.44-fold, respectively, following *A. lappa* treatment. In contrast, the mRNA expression of the anti-apoptotic protein BCL-2 was downregulated by 13.33-fold in COLO-205 cell lines after *A. lappa* treatment, as depicted in (**Figure 4 B**).

A. lappa Inhibited COLO-205 Proliferation through NF-κB *In Vitro*

To gain a greater understanding of the mechanisms behind the suppressive effects of *A. lappa* on COLO-205 cell growth and migration, we investigated the levels of the NF-κB signaling pathway. The findings revealed a dose-dependent reduction in NF-κB expression following *A. lappa* treatment, suggesting a potential connection between *A. lappa*'s actions on COLO-205 cells and the NF-κB pathway (**Figure 4 B**).

TNF-α, IL-1β, and IL-6

A. lappa induced a significant reduction in the inflammatory cytokines (IL-6, IL-1β, and TNF-α) of the cell supernatant, as shown (**Figure 4 C**). These findings showed that *A. lappa* might decrease inflammatory cytokine release from the human COLO-205.

Comet Assay

The comet assay provided critical insights into DNA damage induced by *Arctium lappa* ethanol extract. At a concentration of 100 μg, the extract significantly altered DNA structural integrity in Colo-205 cells, demonstrating its potent genetic impact. Quantitative analysis revealed a marked increase in DNA migration within comet tails. The tail moment escalated dramatically from 2.03 $\pm$ 0.03 in the negative control to 18.88 $\pm$ 0.86 after extract treatment. Similarly, DNA migration in comet tails increased from 1.33 $\pm$ 0.05% to 4.36 $\pm$ 0.33% (**Table 3** and **Figure 5A-D**).

Molecular Docking Experiment

In the current study, arctigenin from *Arctium lappa* demonstrated suppressed cancer development *in vitro*. This finding is supported by the *in silico* study results, where arctigenin could bind to the TGF-βR1 cavity similar to its co-crystallized inhibitor, as shown in (**Figure 6 & Table 4**). Arctigenin formed two hydrogen bonds (residues Glu245 and Lys232 with bond lengths of 2.63 Å and 2.89 Å) within the receptor cavity, stabilizing arctigenin. It also exhibited other interactions like van der Waals and π-π interactions with residues resembling those involved with the co-crystallized inhibitor, such as Leu278, Leu260,

Table 3: Effects of *Arctium lappa* on Genomic DNA in HSF and COLO-205 Cell Lines

Groups	Normal (GI)	*A. lappa* (GII) on normal cells	COLO- 205 (GIII)	Treatment with *A. lappa* (GIV)	F value
Tail DNA (%)	1.00 ± 0.02^{c}	0.90 ± 0.02^{b}	1.33 ± 0.05^{ab}	4.36 ± 0.33^{a}	9.148*
Untailed (%)	96.74 ± 0.52^{a}	95.82 ± 0.75^{a}	92.77 ± 0.59^{b}	78.30 ± 0.89^{c}	71.133*
Tail length (μm)	1.03 ± 0.03^{c}	1.00 ± 0.01^{c}	1.30 ± 0.02^{b}	5.90 ± 0.37^{a}	27.594*
Tail moment	1.26 ± 0.04^{c}	1.54 ± 0.06^{c}	2.03 ± 0.03^{b}	18.88 ± 0.86^{b}	101.39*

Data are expressed as mean ± SE. Different superscript letters (^{a}, ^{b}, ^{c}) indicate statistically significant differences at $P < 0.05$, determined by one-way ANOVA followed by Tukey–Kramer post hoc test. F value indicates statistical significance ($P < 0.05$).

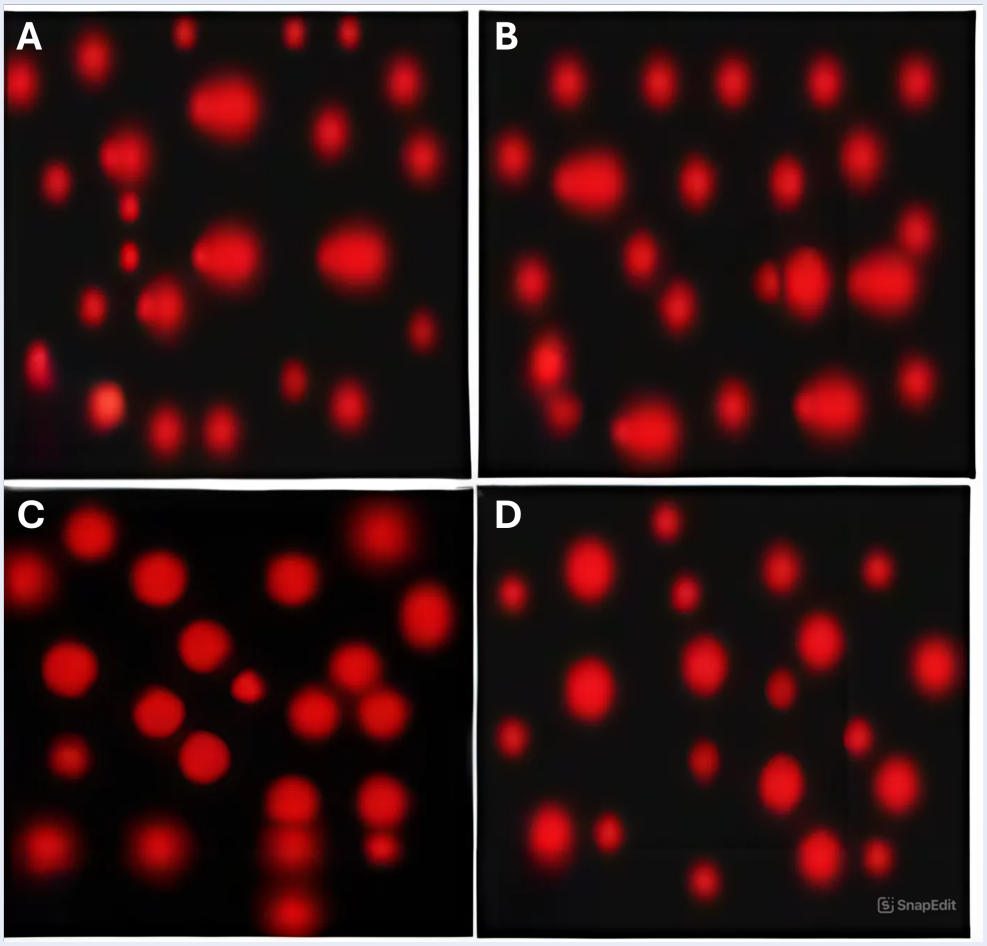

Figure 5: **Comet assay for experimental groups**. **A**) Normal cells (human skin fibroblasts), showing baseline DNA integrity, **B**) H-Colo-205 cells, demonstrating baseline DNA damage in colorectal cancer cells; **C**) Effect of *A. lappa* on H-Colo-205 cells, showing DNA fragmentation indicative of apoptosis or genotoxicity induced by the extract; **D**) Effect of *A. lappa* on HSF cells, showing minimal DNA damage in normal cells, indicating selective toxicity toward cancer cells.

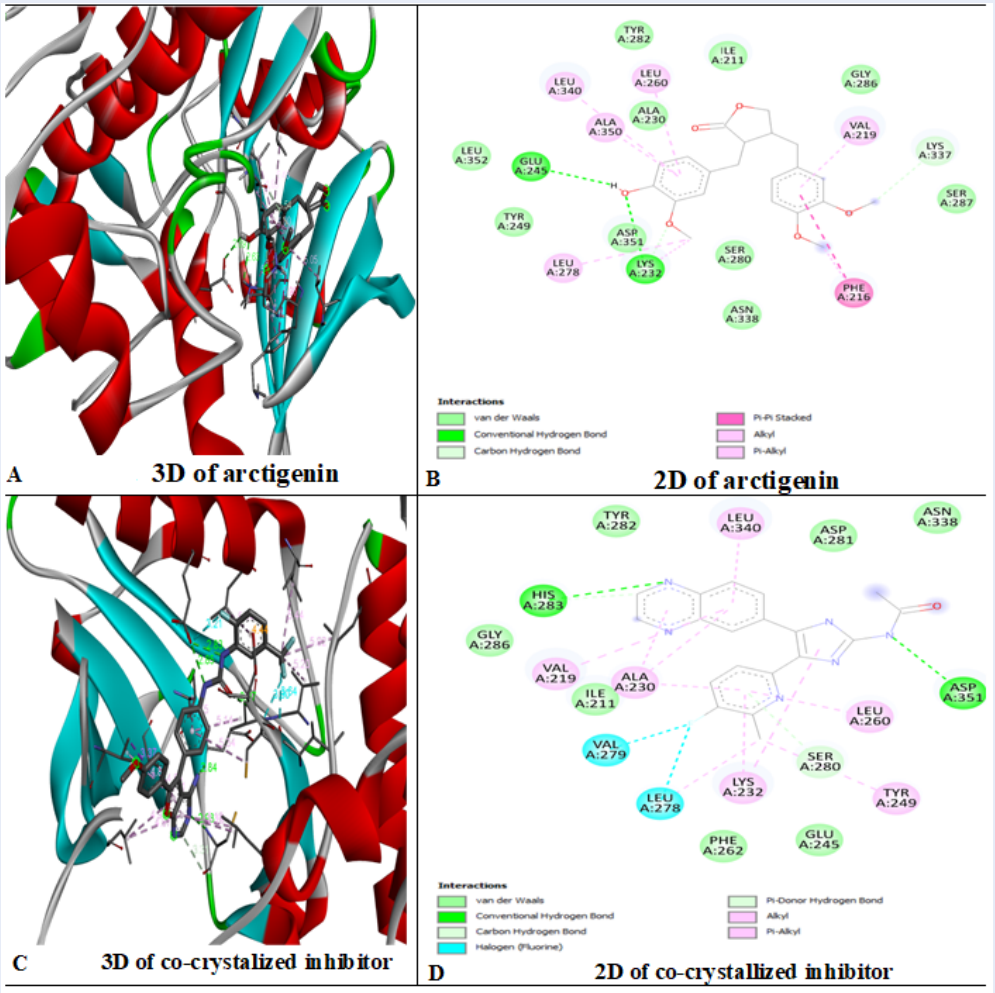

Figure 6: **Molecular docking experiment showing the interactions between arctigenin (the active compound from *A. lappa*) and TGF-βRI.** 2D and 3D depictions of the binding interactions between arctigenin and the co-crystallized inhibitor with TGF-βRI, illustrating the potential binding sites and affinity of arctigenin as a therapeutic agent targeting the TGF-β receptor. The docking results highlight key interactions that may contribute to the anticancer and anti-inflammatory properties of *A. lappa*.

Table 4: Molecular Docking Interactions of Arctigenin with TGF-βRI

Compound	Binding Energy (kcal/mol)	Hydrogen Bonds (Residues)	Residual Interactions
Arctigenin	-8.5	2 (Lys232, Glu245)	Val219, Leu340, Phe216, Leu278, Leu260, Lys232
Co-crystallized Inhibitor	-11.3	2 (Asp351, His283)	Val219, Leu340, Ala230, Leu260, Tyr249, Ser280, Leu278, Val279

Note: Binding energies and interactions were determined through molecular docking studies.

Val219, and Lys232. These hydrogen bonds and other interactions collectively contribute to stabilizing arctigenin within the cavity of TGF-βR1, inhibiting its kinase function and subsequently suppressing the signal pathway that induces other genes involved in cancer proliferation.

DISCUSSION

The study conducted provides supporting evidence for the potential effectiveness of *Arctium lappa* in combating cancer. The ethanol extract of *A. lappa* demonstrated noteworthy inhibition of COLO-205 cell growth and proliferation, indicating its potential as an antiproliferative agent. These results are consistent with previous research that has highlighted the anticancer properties of *A. lappa*. Particularly promising is the selective cytotoxicity of *A. lappa* towards cancer cells, as it exhibited no cytotoxic effects on normal HSF cells, suggesting its specificity in targeting cancerous cells. This selective action is crucial in the development of effective treatments while minimizing harm to healthy cells. Notably, Machado [17] discovered that the ethanol (EtOH) extract from *A. lappa* and its ethyl acetate fraction demonstrated a greater suppression of HSF growth compared to other extracts. Similarly, Taleb Agha *et al.* [18] indicated that the ethyl extract of *A. lappa* substantially reduced MCF-7 proliferation and exhibited antimutagenic activity with an IC_{50} of 50.18 ± 3.66 μg/ml. These outcomes agree with prior research on the anticancer potential of A. lappa.

In our study, the IC_{50} value of the *A. lappa* ethanol extract was determined, and it was found that compounds such as lignans, terpenoids, and sterols have varying pharmacological effects on cancer and pathological angiogenesis [18]. Phytosterols, such as stigmasterol and β-sitosterol, have been shown in numerous investigations to possess anticancer properties through diverse modes of action, including reducing cancer cell development and triggering tumorigenesis or cancer cell death [19]. Therefore, these chemicals could contribute to the anti-proliferative effect of *A. lappa*. Additionally, the fractionation of polyphenol compounds using HPLC of *Arctium lappa* by Ionescu *et al.* [20], exhibited the occurrence of compounds like caffeic acid, ferulic acid, chlorogenic acid, cinaric acid, and cichoric acid, as well as flavone compounds like rutin, quercetin, quercitrin, luteolin, and apigenin.

The study also delved into the underlying mechanisms through which *A. lappa* exerts its anticancer effects. The observed downregulation of MMP-2 and MMP-9 expression in COLO-205 cells suggests a potential role of *A. lappa* in suppressing tumor metastasis and invasion. MMPs play a critical function in these processes, and inhibiting their activity can be a promising strategy for preventing cancer spread [21]. Moreover, regulating the NF-κB signaling pathway by *A. lappa* further supports its anticancer potential. The reduction in NF-κB expression and subsequent decline in inflammatory cytokines (IL-1β, TNF-α, and IL-6) suggest that *A. lappa* may possess anti-inflammatory properties [22,23].

While genotoxic effects were observed in the comet assay, indicating that the *A. lappa* extract induces DNA damage in COLO-205 cells, it is important to note that genotoxicity is desirable in cancer cells as it can contribute to their growth inhibition and destruction [24]. Nevertheless, additional investigations are necessitated to clarify the specific mechanisms of DNA damage induced by *A. lappa*. The presence of arctigenin, a bioactive compound found in *A. lappa*, enhances its potential as an anticancer agent. Arctigenin has been associated with antioxidant, anti-inflammatory, and anticancer properties [25]. Molecular docking experiments have verified arctigenin's capacity to inhibit the TGF-βR1 signaling pathway, which is involved in cancer proliferation [26,27]. This suggests that arctigenin is crucial for the overall anticancer properties of *A. lappa*. Furthermore, the upregulation of pro-apoptotic genes like *P53*, *caspase-3*, *caspase-9*, and *BAX*, along with the downregulation of the anti-apoptotic gene *Bcl-2*, further supports the capacity of *A. lappa* to promote apoptosis in COLO-205 cells. The regulation of apoptosis is a critical process for controlling cancer cell growth and survival [28,29].

Even though our current *in vitro* study strongly suggests that *Arctium lappa* extract may help fight cancer, we are aware of the limitations of cell-based research. We need more research to fully understand the extract's healing properties, even though the controlled cellular environment was helpful for early mechanistic studies.

Future research should prioritize *in vivo* testing to validate the current studies and ensure their potential clinical utility. To figure out how the *Arctium lappa* extracts fight cancer, we will have to use experimental animals and the newest molecular methods. In particular, we focus on looking at dose-response relationships, possible interactions with other drugs, and carrying out an overall pharmacological assessment.

In this context, our study is an important first step in identifying the potential of *Arctium lappa* as an effective anticancer drug. Since there is room for more research, our goal is to lay the groundwork for future studies that can use the favorable results *in vitro* to

focus on cancer therapies in particular. This strategy preserves the scientific rigor of the investigation, acknowledges its clinical relevance and scope for its reproduction, points out the significance of the present results, and prescribes mechanisms for further advancement of the area.

CONCLUSIONS

The findings suggest that *A. lappa* has significant antitumor activity and could be a good source of anticancer compounds. Additional investigations are necessary to explore the underlying mechanisms and optimize the use of *A. lappa* for anticancer therapies.

ABBREVIATIONS

ABTS: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), **ANOVA**: Analysis of Variance, **BAX**: BCL2-Associated X Protein, **BCL-2**: B-cell lymphoma 2, **cDNA**: Complementary DNA, **CO_2**: Carbon dioxide, **CP**: Cisplatin, **DMSO**: Dimethyl sulfoxide, **DPPH**: 1,1-diphenyl-2-picrylhydrazyl, **DTT**: Dithiothreitol, **ECL**: Enhanced Chemiluminescence, **EDTA**: Ethylenediaminetetraacetic acid, **ELISA**: Enzyme-Linked Immunosorbent Assay, **EtOH**: Ethanol, **FBS**: Fetal Bovine Serum, **GAE**: Gallic Acid Equivalents, **HBM8-k562**: Human Bone Marrow Cells (K562 leukemia cell line), **H-COLO-205**: Human Colorectal Carcinoma Cells (Colo-205 cell line), **HMM**: Human Multiple Myeloma, **HPLC**: High-Performance Liquid Chromatography, **HRP**: Horseradish Peroxidase, **HSF**: Human Skin Fibroblast, **HTB-43**: Human Oral Cavity Cancer Cells, **Huh-7**: Human Hepatocellular Carcinoma Cells, **IC_{50}**: Half Maximal Inhibitory Concentration, **IL-1β**: Interleukin-1 Beta, **IL-6**: Interleukin-6, **MMP-2**: Matrix Metalloproteinase-2, **MMP-9**: Matrix Metalloproteinase-9, **NF-κB**: Nuclear Factor Kappa B, **OD**: Optical Density, **P53**: Tumor Protein 53, **PBS**: Phosphate-Buffered Saline, **PCR**: Polymerase Chain Reaction, **PDB**: Protein Data Bank, **qRT-PCR**: Quantitative Reverse Transcription Polymerase Chain Reaction, **RP**: Reducing Power, **SAS**: Statistical Analysis System, **SDS**: Sodium Dodecyl Sulfate, **SEM**: Standard Error of the Mean, **SRB**: Sulforhodamine B, **TBS**: Tris-Buffered Saline, **TBST**: Tris-Buffered Saline with Tween 20, **TCA**: Trichloroacetic Acid, **TEAC**: Trolox Equivalent Antioxidant Capacity, **TEMED**: Tetramethylethylenediamine, **TFC**: Total Flavonoid Content, **TGF-βR1**: Transforming Growth Factor Beta Receptor 1, **TNF-α**: Tumor Necrosis Factor Alpha, **UV-Vis**: Ultraviolet-Visible

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AUTHOR'S CONTRIBUTIONS

Ibrahim I. Bondouk and Hosam M. Saleh prepared the extract of *Arctium lappa*. Ibrahim I. Bondouk and Hosam M. Saleh and Mohamed Taha Abdelrahman performed the *in vitro* experiment on the extract, analyzed the extract phenols, and flavonoid content, and determined the *in vitro* antioxidant characteristics. Mohamad Taha Abdelrahman performed the molecular docking experiment. Amal I. Hassan performed the biological experiment on the cells and the statistical data. All authors were involved in the data management, statistics, writing, and reviewing of the manuscript. All authors read and approved the final manuscript.

FUNDING

None.

AVAILABILITY OF DATA AND MATERIALS

Data and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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