ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

IN VITRO ANTI-CANCER EFFECTS OF *ARNEBIA BENTHAMII*AGAINST COLORECTAL CANCER CELL LINE

BY

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ABSTRACT: *The commercial medication known as Gaozaban contains a significant amount of ARNEBABENTHAMII, which possesses antibacterial, antifungal, anti-inflammatory, or wound-healing effects. An inside vitro anticancer activity for Arnebiabenthamii leaf extract was examined in the current study.* Cytotoxicity of theextracts (10–100 µg/mL) was tested on human colorectal cancer cell line, HCT 116.The purpose of this study has been to determine whether a methanolic extract of A. benthamii leaves might beused to treat colon cancer cells from the HCT 116 cell line. GC-MS was used to characterise the extract'sphytochemical properties. The MTT assay was used to detect the extract's effect on cell growth. An annexin VFITC labelling was used to determine the commencement of apoptosis, The potential of cell migrationinhibition was examined using a wound healing assay. Cell proliferation was inhibited by methanolicextract of A. benthamii leaves when examined using MTT cell viability assay in a dosage and timedependent manner. In addition, treatment with methanolic leaf extract of A. benthamii inhibited the cells'capacity to colonise and induced cell cycle arrest. Our results suggest that crude extract from the leaves of A. benthamii plays a significant role in abrogating cancer proliferation by inducing cell cycle arrest &down regulate the metastatic properties in colorectal cancer cells and hence, it may therefore, bedeveloped into potential chemopreventive drug against colorectal cancer in the near future.

Keywords: Colon cancer; HCT 116; Walnut; GC/MS; MTT; Colony Formation; Wound healing.

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INTRODUCTION

Antioxidants have been the focus of a lot of study because of their possible role in warding off chronic illnesses including diabetes, cancer, and heart disease. Cancer and cardiovascular disease, among others, have been linked to free radicals because of their role in cellular degradation. It's possible that free radicals, both those ingested and those produced by the body's regular processes, can do damage. Oxygen radicals can damage biomolecules (lipids, proteins, and DNA), which can result in many chronic illnesses, including cancer, metabolic syndrome, rheumatoid, postischemic perfusion damage, infarction, cardiovascular problems, inflammation, stroke, and septic shock, as well as ageing and other degenerative illnesses [1]. Antioxidant systems, both enzymatic and nonenzymatic, are present in the human body to counteract the damaging effects of oxygen species (ROS) [2]. When faced with severe or persistent oxidative stress, the brain's normal defences may be overwhelmed. Therefore, the human body constantly necessitates specific amounts to exogenous antioxidants to maintain a sufficient level of antioxidants and balance the ROS within the human body. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary-butylhydroquinone are only a few examples of the synthetic antioxidants commonly used in the food industry to prevent oxidation (TBHQ). Food should not contain synthetic antioxidants [3] because of their toxicity and carcinogenicity.

A monocarpic perennial, Arnebiabenthamii matures for reproduction in 3–4 years. It is possible to trade and eat the flowering stalk, leaves, and base of the root. Arnebiabenthamii is present in high concentrations in the commercially available medications known as Gaozaban, which now has antifungal, anti-inflammatory, or wound-healing properties. The plant's roots produce shikonin, a red pigment with several medical applications. Red alkanin, a lipophilic pigment responsible for the plant's colour and medicinal properties, is the plant's major active component. This plant has traditionally been used to cure a wide range of tongue, tongue, fever, heart, and wound-related diseases, and its primary active component, alkanin, is a lipophilic red pigment. The root-derived red pigment shikonin is used to make the dye Ratanjot, which has several beneficial medical properties. The plant is used to treat several problems related to the tongue, throat, fever, or heart, and it is said in folklore that it can heal any wound. The root can be used to add a vibrant red colour to foods, oils, and fats, and it also has medicinal uses as an anthelmintic, antipyretic, or antiseptic. The herb also acts as a stimulant, a tonic, a diuretic, and an expectorant. Sherbet (syrup), jam, as well as other products made from the blossoming branches have been used to successfully treat a wide range of tongue, throat, heat, and heart disorders. The antioxidant and antibacterial properties of this native to Kashmir valley plant have not yet been studied. This initial investigation's primary goal was to assess the defences provided by various Arnebiabenthamii extracts against the harm caused by free radicals in in vitro settings. Cytotoxic effects of Arnebiabenthamii was carried out. GC/MS analysis was carried out to determine the phytochemical constituents of the Arnebiabenthamii leaf extract. MTT assay was carried out

ISSN PRINT 2319 1775 Online 2320 7876

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on HCT116 cell line. Colony formation assay was carried out and Apoptosis was carried out in order to determine the anti metastatic potential on HCT 116 cell line.

2. Materials and methods:

2.1 Research Area

The explorative research was carried out in district Kulgam, Jammu and Kashmir India. Kulgam District is one among 22 Districts of Jammu & Kashmir State, India. Kulgam District Administrative head quarter is Kulgam. It is located 60 km North towards State capital Srinagar, Jammu. Kulgam District population is 422786. It is 12th largest district in the state by population. Kulgam district is sharing border with Anantnag district to the East, Shopian District to the West. It is in the 1671 meters to 1619 meters' elevation range. Northern India is home to this district. During mid- November until the end of April, the Kulgam district experiences harsh weather, with temperatures averaging less than 2.0 degrees Celsius. Summers are mild and brief, lasting from June through August, with only an average temperature around 37.0 degrees Celsius. The yearly rainfall averages 165 cm [11]. The vast majority of the region is covered in dense vegetation [12]. The Gujars& Kashmiris are the two primary ethnic groups in the study region. Kashmiri and Gujri are the most commonly spoken languages[12].

2.2 Chemicals and Reagents

While HIMEDIA supplied MTT, Dulbecco's Modified Eagle Mix (DMEM), Fetal Bovine Serum (FBS), & penicillin-streptomycin, MERCK supplied methanol and dimethyl sulfoxide (DMSO). Merck contributed all of the additional chemicals.

2.3 Extraction process and plant material

In the months of July to September 2020, Arnebiabenthamii was gathered from the Chiranbal garden in district Kulgam, UT of J&K, India; verified by the Centre for Plant Taxonomy in the University of Kashmir's Department of Botany by Mr. AkhterHussain. Under the reference number 4195- KASH, a reference.

Extract preparation

The plant material's leaves were dried inside the shade around 25°C. The plant parts were dried for five days in the shade. Using a mortar and pestle, the material was dried was pounded into a powder & passed through with a sieve with a mesh size of 0.33mm. The powder was extracted for 48 hours using the solvent methanol. In order to obtain the crude extracts of Arnebiabenthamiileaves, soxhlet extraction was performed which works on the double distillation process. The solvents used was methane. The extract was subsequently concentrated under lower pressure using rotary vapour, together with the extract being stored in the fridge for subsequent use.

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2.4 GC-MS Analysis of plant mater

To determine the chemical composition of methanol extracts for Juglansregia leaves, GC-MS analysis was used.

2.5. Cell culture

The colorectal cancer cell line was donated by NCSS Pune. In a 5% CO2 humidified environment, colorectal cancer cells proliferated in DMEM at 37 degrees Celsius.

2.6. Assay for cytotoxicity

in vitro MethanolicArnebiabenthamii leave extract was tested for their cytotoxic effects towards colorectal carcinoma (HCT 116) using MTT assay. After seeding 96-well plates with cells at a density of 10/5, test solution ranging between 500µg/mL - 15.75µg/ml were poured to each well & incubated for 24 hours. The solvent control consisted of cells that had been treated with 0.5 percent DMSO. MTT was applied at concentration of 0.5 mg/mL to cells treated with phosphate buffered saline (PBS) & incubated for 4 hours at 37°C. After removing the medium, DMSO was employed to dissolve the formazan dye crystals. At a wavelength of 545 nm, the absorbance was determined. The absorbance-to-control ratio was used to determine cell viability. The 50% inhibition concentration (IC50), defined as the concentration of extract that inhibits 50percent of cell development, was determined using concentration graphs for 24 to 48 hourand 72 hour exposure intervals. These calculations were based on three independent, duplicate trials.

2.7. Healing of wounds

As previously described by [13,20], a wound healing test was used to determine the ability of Arnebiabenthamii leaves extract to block cell migration. A 6-well plate of HCT 116 cells was planted, & after cellconfluency reached 85-90 percent, Scratches were inserted into the monolayer using a sterile micropipettetip with a capacity of 20 litres, & the detached cells were rinsed twice with Dulbecco's phosphate-bufferedsaline (DPBS). The detached cells were cultured for 48 hours after being supplemented with incompletefresh media containing selected extract (Ic50 dosage). A phase contrast invert microscope was used tostudy and photograph the cells. Cells were graded based on their capacity to heal wounds [20].

2.8. Assay for colony development

HCT 116 cells was planted in 6-well plates and incubated of 500-1000 cells per well & cultured for 24hours before being treated with methanol extract from Juglansregia for 48 hours at 37 degrees. As acontrol, DMSO was utilised. The cells were then cultivated for 10 days after the media was changed withfresh medium. Cells was washed twice with PBS & placed in methanol for 15 min, followed by 30minutes staining using 0.1 percent crystal violet at room temperature. carefully removing all crystal violetstaining and soaking the plate in tap water; plates were also air dried at room temperature [14,15].

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2.9. Assay for apoptosis with Annexin V/FITC

The Annexin V/FITC experiment was used to investigate the ability of plant extract to induce apoptosisusing a commercially available Annexin V kit. In a 6-well plate, cells were planted at a density of 2.4105cells/mL and incubated overnight. The following day, the cells were treated and grown for 24 hours. Thecells were harvested at the appropriate time point throughout the incubation period, and The pellets werethen resuspended in the provided binding buffer. To the sample solution, 5:l FITC Annexin V and 5:lpropidium iodide (PI) were added & left out in the dark for 15 min at room temperature. Flow cytometry was used to examine the labelled cells after that using FACS.

3. RESULTS

In order to obtain the crude extracts from Arnebiabenthamii leaves, soxhlet extraction was performed whichworks on the double distillation process. The solvents used was methane. To prepare the extracts, 80 gArnebiabenthamii leaves were used. The leaves of Arnebiabenthamii weighed 38g following extraction.

3.1 The methanol extract was analysed using GC-MS.

In order to validate the compounds, present in the methanol extract GC-MS analysis was done for themethanolic extract. The sample was dissolved in methanol (1 mg/mL) and analysed using GCMS (Shimadzu GCMS-QP2010S). In this case, the RTX-5 column was used, and its settings was (Length 30 metres; Diameter 0.25 millimetres; Film Thickness 0.25 millimetres). We used a temperature of 250 C for the injection and maintained a temperature of 320 C at the interface. With a flow rate of 1, helium has been used as the carrier gas. For analysis, a 1L sample was injected. The split ratio was set at 1:5, whichmeans 5 times the number of carrier gas that flowing out of split vent compared to the column.Temperature ramping was given from initial 60 °C (hold time 2 min) to 310 °C with 10 °C/min rise. At310 °C hold of 13 min was given.For compound identification and confirmation, an MS spectra was compared to the NIST and WILEYlibraries. The experiment took place at Jawaharlal Nehru University's Advance Instrument ResearchFacility (AIRF) in Delhi, India. GCMS chromatogram of methanol extract is presented in Figure 1A. Thesample extract was injected into the analyzer after proper dissolution of the extract in an appropriatesolvent. The analyzer was run in the required standard conditions. As shown in the gas chromatogram ofthe extract, it contained around 38 compounds in which Yangambin and Methoxybenzodioxol are the majorcompounds whose retention time are 30.050 and 30.162 min respectively.

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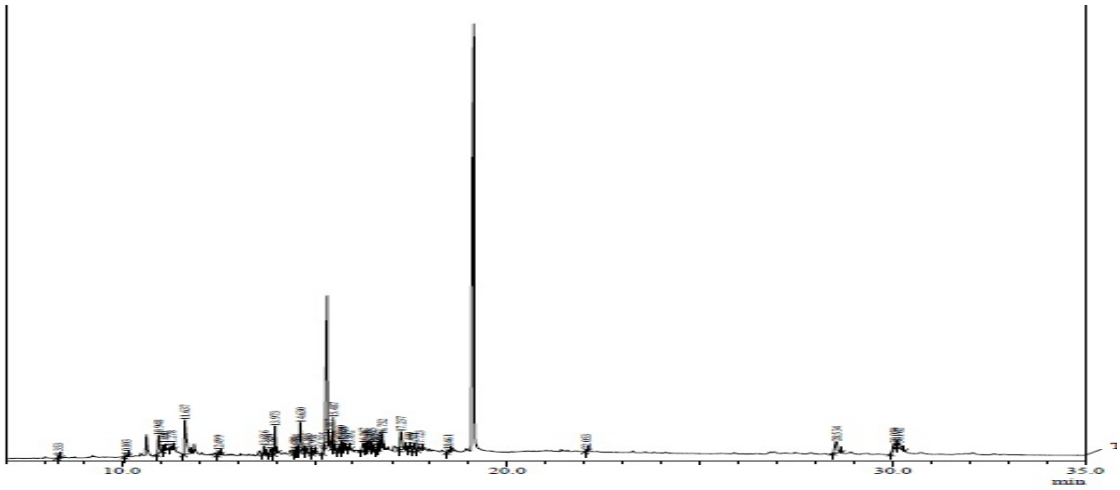

Fig 1. Gas chromatogram of the methnolic extract of Arnebiabenthamii leaves

Table 1: In methanol leaf extract for GC-MS Arnebiabenthamiianalysis confirmed the presence allphytochemical components

Peak	R. Time	Area	Area%	Name
1	8.353	481903	0.71	3-Buten-2-one, 4-(6,6-dimethyl-1-cyclohexen-1-yl)-
2	10.103	499443	0.74	2-HYDROXY-1-ADAMANTANECARBONITRILE
3	10.948	4541872	6.74	1H-Indene, 3-ethyl-1-(1-methylethyl)-
4	11.090	681413	1.01	1-Naphthalenol, decahydro-1,4a-dimethyl-7-(1-methylethyl
5	11.278	800022	1.19	MOME INOSITOL
6	11.637	10382081	15.40	4-Hydroxy-6-methyl-3-(4-methylpentanoyl)-2H-pyran-2-o
7	12.499	957300	1.42	(S,E)-4-Hydroxy-3,5,5-trimethyl-4-(3-oxobut-1-en-1-yl)cy
8	13.686	1293925	1.92	Hexadecanoic acid, methyl ester
9	13.849	1395263	2.07	Gazaniolide
10	13.973	4399431	6.53	Dehydroxy-isocalamendiol
11	14.480	96963	0.14	TRICYCLO[5.4.3.0(1,7)]TETRADECANE-3,6-DIOL, 4-F

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12	14.534	514629	0.76	Gazaniolide
13	14.630	6524623	9.68	1-METHYL-4-METHYLENE-2-(2-METHYL-1-PROPEN
14	14.805	1165275	1.73	3-(1,1-Dimethylallyl)-6-hydroxycoumarin
15	14.958	595523	0.88	2-Isopropenyl-5-methyl-6-hepten-1-ol
16	15.235	379499	0.56	Chrysantenyl 2-methuylbutanoate
17	15.387	1042027	1.55	9,12,15-OCTADECATRIENOIC ACID, METHYL ESTER
18	15.487	4111979	6.10	Phytol
19	15.623	951446	1.41	PENTACOSANOIC ACID, METHYL ESTER
20	15.699	965953	1.43	2-Isopropenyl-5-methyl-6-hepten-1-ol
21	15.740	571077	0.85	8,14-Seco-3,19-epoxyandrostane-8,14-dione, 17-acetoxy-3.
22	15.892	417155	0.62	Methyl (Z)-5,11,14,17-eicosatetraenoate
23	16.267	1951420	2.89	Urs-12-ene
24	16.355	363617	0.54	Achillicin
25	16.403	538296	0.80	(3RS,6R)-3-METHYL-6-(1'-METHYLETHENYL)DEC-9
26	16.475	731931	1.09	1-[2-(2,2,6-TRIMETHYLBICYCLO[4.1.0]HEPT-1-YL)ET
27	16.603	197046	0.29	Acetic acid, [(3-acetyl-5-benzofuranyl)oxy]-, methyl ester
28	16.667	297696	0.44	4-Nitrobenzoic acid, heptadecyl ester
29	16.752	2429149	3.60	TRICYCLO[20.8.0.0E7,16]TRIACONTAN, 1(22),7(16)-D
30	17.237	5700445	8.46	3-PHENANTHRENOL, 4B,5,6,7,8,8A,9,10-OCTAHYDR
31	17.440	348672	0.52	2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-

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				ethylhexyl ester
32	17.571	367771	0.55	4aH-Cycloprop[e]azulen-4a-ol, decahydro-1,1,4,7-tetramet
33	17.723	1162645	1.72	1H-BENZOCYCLOHEPTEN-7-OL, 2,3,4,4A,5,6,7,8-OCT
34	18.461	539792	0.80	Silane, dimethyl(3-ethylphenoxy)heptyloxy-
35	22.053	329256	0.49	2,2-Dimethyl-3-(3,7,16,20-tetramethyl-heneicosa-3,7,11,15
36	28.534	5044836	7.48	Yangambin
37	30.050	2483590	3.68	Yangambin
38	30.162	2159130	3.20	(3R,4R)-3-((7-Methoxybenzo[d][1,3]dioxol-5-yl)methyl)-4
		67414094	100.00	

3.2 In vitro cytotoxicity assay

To evaluate cell viability, the MTT test was used, which has been calculated as a percentage of the controlcell viability. This was checked for the methanolic extract for Arnebiabenthamii mentioned in colorectal cancercell line for the time period of 24 hours as illustrated in the Figure 2 Methanol extract of Arnebiabenthamii showed cell cytotoxicity in a dose and time dependent fashion against HCT 116 cancer cell line (Figure 2).After 24-hour treatment, With an IC50 value of, the methanol extract appears to be extremely effectiveagainst HCT 116 cells 189.35µg/ml.

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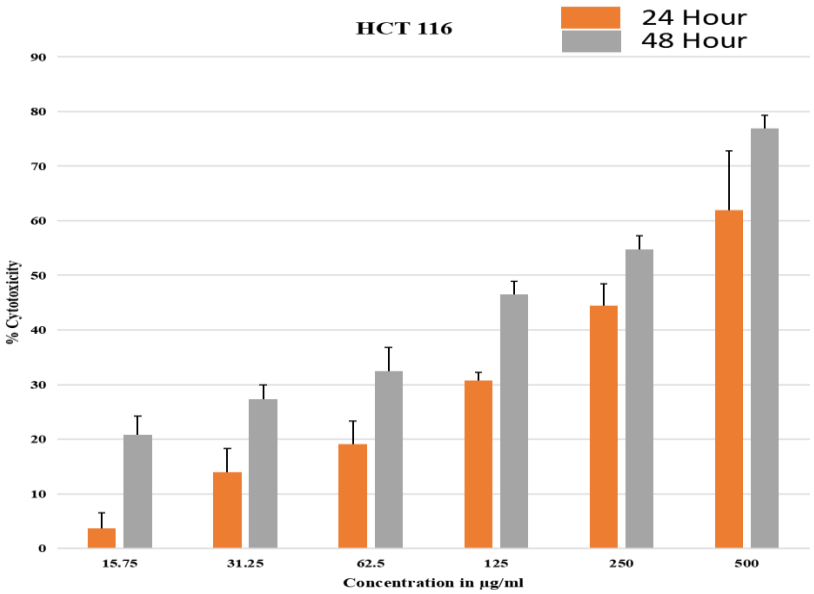

Fig 2.*MTT assay findings for Juglansregia leaf extract in 24 and 48 hours for HCT 116 colorectalcancer cell lines. (2A) HCT 116 cells cultured in Dulbecco's modified Eagle's medium (DMEM) mediumsupplemented with 0.2 percent foetal bovine serum (FBS) 0.2 percent foetal bovine serum (FBS) medium0.2 percentfetal bovine serum (FBS) solutions Medium containing 0.2 percent (FBS) 0.2 % (FBS) medium0.2 percent (FBS). The cells were cultured for 4 hours after receiving 5 litres of MTT-labeling reagentbefore receiving 100 litres of solubilization solution. At least three times, the trials were carried out. Thedata is presented as a mean & standard deviation. **p 0.01, respectively. ***p 0.001 & ***p 0.01,respectively.*

HCT 116	**IC 50 µg/mL**	
A. benthamii leaf extract	**24 HOUR**	**48 HOUR**
	325.64	**189.35**

Table 2.IC50 values of methanolic extract of Arnebiabenthamii against HCT 116 cell line for colorectalcancer.step2 :Adjust For Technical Difficulty.

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3.3 Wound healing assay

After extract was given an IC50doseThe ability for HCT 116 cells to migrate was tested. The treatmentlasted 48 hours, and the wound's width was assessed before and after the procedure.

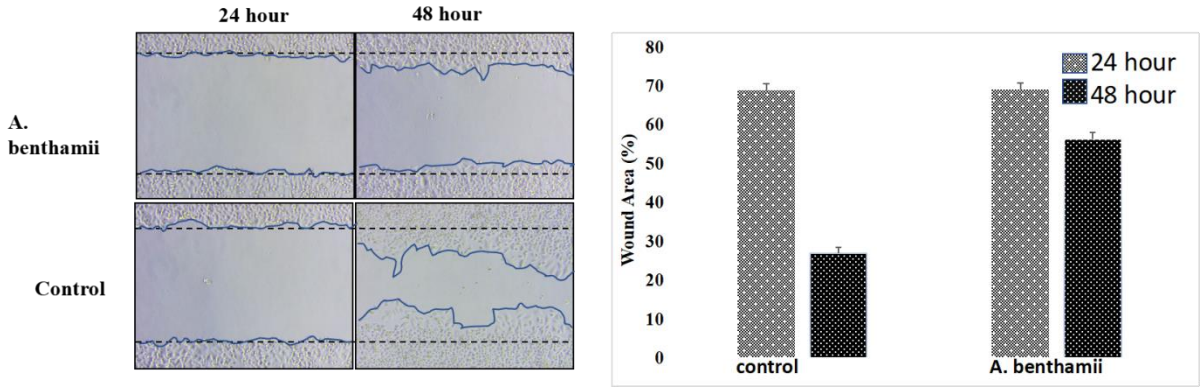

Fig 3Representative pictures (top) & bar graphs quantifying HCT 116 cell movement in thepresence of extract at 24-hour treatment (n=5 separate trials; *P 0.05 vs the untreated cell; bar=200m).

3.4 Assay for colony development

The colonies formation assays [16] is a commonly used tool for evaluating in vitro cellular transformation.This is an in vitro system survival experiment that assesses a single cell's ability to divide forever in apopulation [17,18,19]. According to the findings, the extract inhibited colony formation in a dose dependentway. With increasing concentrations of the extract.

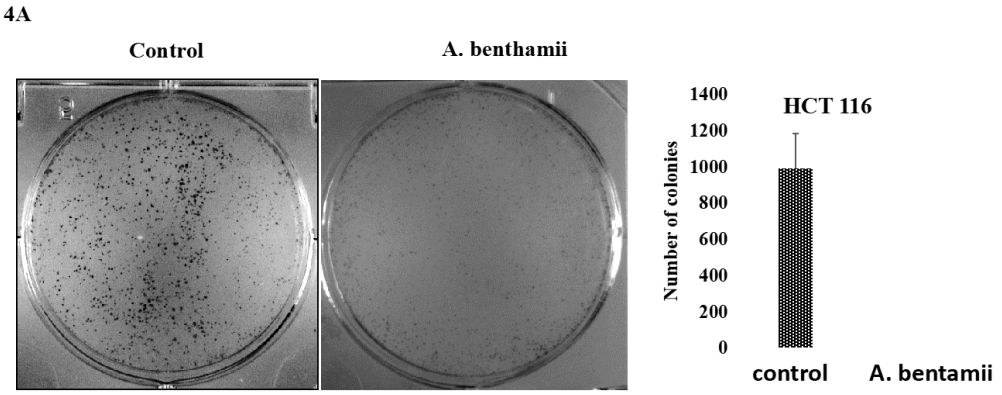

Fig 4A.*On HCT116 cells, the results showed that colony formation was inhibited. Graph 4B. The errorbars in the upper panel indicate the standard deviation of the mean number in visible colonies acquired forjust a cell line across three different experiments.*

3.5 Using annexin V-FITC to label HCT 116 cells for apoptosis analysis

After being treated with a methanolic extract with *Arnebiabenthamii* leaves, HCT 116 cells went into apoptosis.The number of early (Q4) and late (Q2) apoptotic HCT 116 cell were

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considerably higher in comparisonwith the control group, according to flow cytometry examination of apoptotic HCT 116 cells.

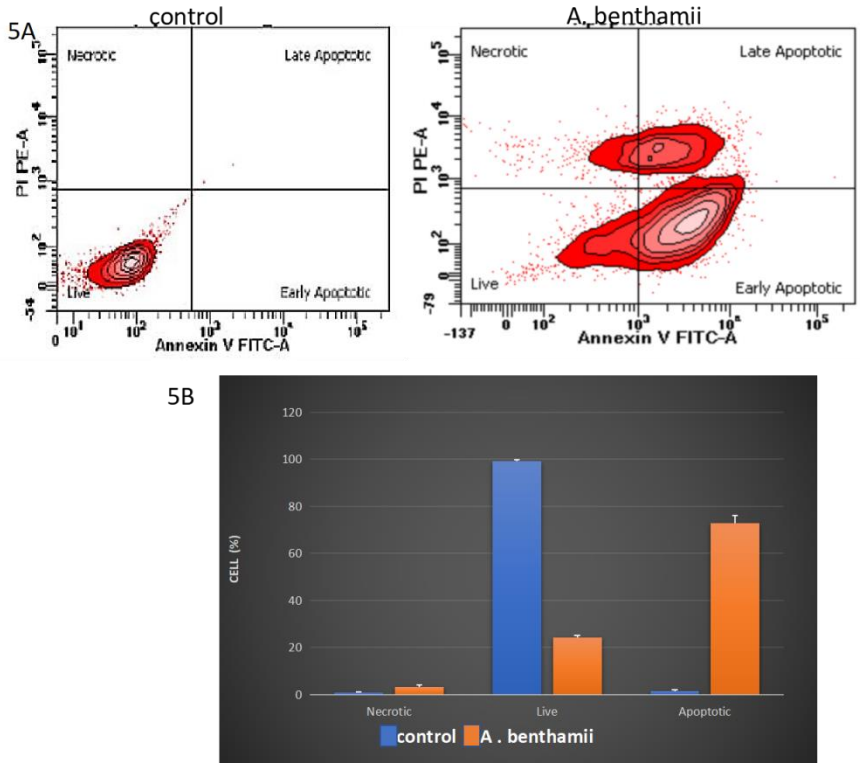

Fig 5A.*Flow cytometry was used to examine apoptosis. (5A) Flow cytometry sorting of HCT 116 cells. Over the course of 24 hours, different concentrations of the extract were added to HCT 116 cells incubated on Medium consisting of Dulbecco's modified Eagle's (DMEM) and foetal bovine serum (FBS) at a concentration of 0.2% (FBS). The cells were collectedand doubly stained using Annexin V-FITC &propidium iodide after being rinsed in cold PBS. Flowcytometry was used to sort the cells. At least three times, the trials were carried out. (5B) Apoptotic HCT116 cell percentage. The percentage of apoptotic cells inside the parts of Q2 & Q4 to the total number ofcells was used to compute the apoptotic HCT 116 cells. Three separate experiments were conducted, and all results are presented as the mean SD (*p 0.05, **p 0.01, ***p 0.001 vs. a untreated control).*

4. DISCUSSION

According to the MTT viability experiment, In a dose- and time-dependent way, Arnebiabenthamii leaf extract suppressed cell proliferation. Additionally, treatment with Arnebiabenthamii whole extract reduced the cells' capacity to form colonies and resulted in cell cycle arrest. Furthermore, the whole extract from Arnebiabenthamii was found to have an anti-metastatic action. The fact that Arnebiabenthamiimethanolic leaf extract inhibited HCT 116 cancer cell proliferation suggests that even a specific phytochemical or even a class of phytochemicals of Arnebiabenthamii leaf extract could be responsible for its anti-proliferative properties. Alternatively, the study's GC/MS data suggests that the combinations

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of elements present in Arnebiabenthamii leaves extract may play a significant role with in final biological activity. Our study suggests that crude extract from the leaves of Arnebiabenthamii plays a significant role in abrogating cancer proliferation by inducing cell cycle arrest & down-regulating the metastatic properties in colorectal cancer cells. Hence, As a result, it is suggested the Arnebiabenthamii could be exploited as a low-cost, easily available source of natural chemopreventive medicines, and that further clinical research into this plant be encouraged.

CONCLUSION

The findings of this study revealed that Arnebiabenthamii is a powerful antioxidant source. In addition, On HCT 116 cells, the menthol extract demonstrated a severe cytotoxic effect. As a result, Arnebiabenthamii extracts methanol merits further research since an active molecule with anticancer capabilities, as it could be used in pharmaceutical goods & functional foods for cancer prevention and therapy.

Observance of Ethical Standards

Possibility of a Conflict of Interest:

There were no inconsistencies interests among authors about the study's substance.

Statements of Data Availability

This published article contains all of the data generated and analysed during this investigation.

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