UNVEILING THE ANTICANCER POTENTIAL OF *CANNABIS SATIVA*: A COMPREHENSIVE STUDY ON PURIFIED COMPOUND'S MOLECULAR IMPACT IN COLORECTAL CANCER CELLS

Rohit Kumar[1*], Kamlesh Kumar[2]

Abstract

This study aimed to investigate the potential anticancer properties of *Cannabis sativa* extracts, particularly focusing on the identification and characterization of individual compounds. Additionally, the research aimed to assess the molecular impact of quercetin on colorectal cancer cells. *Cannabis sativa* leaves were collected, and extracts were obtained using different solvents (hexane, chloroform, methanol, and water). Phytochemical analysis, including the detection of alkaloids, flavonoids, phenols, glycosides, tannins, carbohydrates, saponins, and steroids, was conducted. The total flavonoid content was determined using the aluminum chloride colorimetric method. Antioxidant activity was evaluated through DPPH, FRAP, and H_2O_2 assays. The extracts exhibiting anticancer activity were further purified through column chromatography and HPLC. The isolated compounds were tested for their anticancer potential against HCT116 colorectal cancer cells, and IC50 values were calculated. Morphological analysis and gene expression studies were performed to assess the cellular and molecular impact of the identified compounds. The water extract showed the highest extraction yield and a rich phytochemical profile. Methanol and water extracts displayed robust antioxidant activity. Column purification and HPLC led to the isolation of quercetin, which exhibited significant anticancer activity against HCT116 cells with an IC50 of 60.253 µg/ml. Morphological analysis revealed dose-dependent cytotoxicity. Gene expression analysis indicated an upregulation of M-CSF and a slight upregulation of GM-CSF in quercetin-treated cells. This study provides insights into the diverse chemical compositions and biological activities of *Cannabis sativa* extracts. The identified quercetin compound demonstrated promising anticancer properties against colorectal cancer cells, warranting further investigation into its therapeutic potential. The molecular analysis sheds light on the potential mechanisms underlying quercetin's effects, contributing to the understanding of its role in cancer treatment.

Keywords: *Cannabis sativa, quercetin, anticancer, HCT116, gene expression, phytochemical analysis, antioxidant activity.*

[1*,2] Department of biotechnology, OPJS University, Churu Rajasthan 331303
Email:-rohitkumarcanres@gmail.com

***Corresponding Author:** Rohit Kumar
*Department of biotechnology, OPJS University, Churu Rajasthan 331303
Email:-rohitkumarcanres@gmail.com

DOI: 10.53555/ecb/2023.12.7.370

INTRODUCTION

Cancer is a leading cause of death on a global scale, profoundly impacting the life expectancy of populations. Among the various cancer types, colorectal cancer (CRC) is particularly noteworthy, ranking as the third most commonly diagnosed cancer and the second most lethal worldwide [1]. In the year 2020, CRC was responsible for approximately 9.4% of all cancer-related fatalities [2]. Forecasts indicate that the incidence of CRC is set to double by the year 2035, with a substantial increase anticipated in less developed countries [2,3]. Colon cancer, a form of cancer originating in the large intestine and spreading throughout the colon, primarily affects older individuals, although it can afflict people of any age. Typically, it initiates as benign polyps small, noncancerous accumulations of cells developing within the colon. Some of these polyps may progress into colon cancer over time, with small polyps often showing minimal or no symptoms. In cases where colon cancer does develop, there are diverse treatment options available, encompassing surgical procedures, radiation therapy, and pharmaceutical interventions such as chemotherapy, targeted therapy, and immunotherapy. It's noteworthy that colon cancer is often referred to as colorectal cancer, encompassing both colon and rectal cancer, which initiates in the rectum [4]. Traditionally, the identification of colorectal cancer has relied on markers like carcinoembryonic antigen (CEA), carbohydrate antigen (CA 19.9), tissue polypeptide specific antigen (TPS), and tumor-associated glycoprotein-72 (TAG-72). More recently, there has been a growing focus on the use of hematopoietic growth factors (HGFs) and various enzymes for diagnosing and prognosticating colorectal cancer. This includes macromolecule-stimulating factors such as macrophage-macrophage-colony stimulating factor (M-CSF) and granulocyte-macrophage-colony stimulating factor (GM-CSF), along with interleukin-3, interleukin-6, and enzymes like alcohol dehydrogenase and lysosomal exoglycosidases. Research indicates that these markers offer heightened significance and superior diagnostic capabilities compared to earlier markers [5].

Flavonoid and Cancer

In response to various biotic and abiotic stresses, plants undergo physiological and metabolic adaptations, leading to the synthesis of secondary metabolites. These secondary metabolites are of scientific interest due to their distinctive chemical structures and therapeutic attributes. Extensive research has substantiated the antimutagenic and anticancer properties of secondary metabolites, including polyphenols, terpenes, and alkaloids. Among these, flavonoids are prominent examples. Flavonoids have demonstrated potent anticancer properties both in vitro experiments and in animal studies [6]. While there lots of evidence supporting the anticancer potential of various flavonoids, the precise molecular mechanisms underlying their actions remain incompletely understood. For instance, cocoa flavanols, owing to their antioxidant attributes, have been observed to provide protection against colon cancer [7]. In cell models such as MCF-7 and 3T3-L1, the isoflavonoid genistein has been shown to regulate oestrogen receptor expression, adjust the Bax/Bcl-2 ratio, thereby inhibiting cell proliferation, promoting differentiation, and triggering apoptosis [8]. Furthermore, genistein has been reported to suppress various anti-apoptotic factors, including Bcl-2, Bcl-xL, c-inhibitor of apoptosis protein 1 (c-IAP1), survivin, and NF-B, in cell lines such as C200 and A2780 [9], while enhancing caspase-3 activity in HT-29 colon cancer cells [10] and activating the intrinsic apoptosis signalling pathway in HCT-116 and LoVo cells [11]. In a model of colon cancer induced by 1,2-dimethylhydrazine, hesperetin has demonstrated the capacity to reduce indicators of cell proliferation, angiogenic growth factors, and the expression of COX-2 mRNA [12]. Additionally, quercetin has been found to regulate the toll-like receptor 4 (TLR4)/NF-kB pathway, resulting in reduced migration and invasion of human colon cancer cells, particularly the Caco-2 cell line [13].

Marijuana

Cannabis is a genus of flowering plants in the family Cannabaceae. Three species may be recognized: *Cannabis sativa*, *Cannabis indica*, and *Cannabis ruderalis*; *C. ruderalis* may be included within *C. sativa*; all three may be treated as subspecies of a single species, *C. sativa*; or *C. sativa* may be accepted as a single undivided species. The genus is widely accepted as being indigenous to and originating from Asia. FBL-03G, a flavonoid derivative of *cannabis*, has shown considerable therapeutic potential in the treatment of pancreatic cancer [14]. There are number of flavonoids that has been isolated from marijuana and showed anticancer activity. Kaempferol, Apigenin, Cannflavins, Silymarin, Luteolin, Orientin, Vitexin, Isovitexin and Quercetin are some of the flavonoid compounds that has been extracted from *cannabis*. Kaempferol help in decreasing cell viability and proliferation and found to be effective in colon cancer in

combination with 5-FU [15], apigenin reduced the formation of circular chemorepellent-induced defects in the endothelial barriers in colon cancer [16], Luteolin, quercetin is found to be effective in colon cancer [17, 18].

Wild herbs and shrubs may harbour potent anticancer properties, even if they aren't economically significant. While there are reports of marijuana's anticancer effects on colon cancer, they often use common biomarkers for detection. The impact of these botanicals on hematopoietic growth factors (HGFs), M-CSF and GM-CSF, which can detect cancer early, is an area with limited research but substantial diagnostic potential.

MATERIAL AND METHODS

Materials

In November 2021, *Cannabis sativa* leaves, commonly known as marijuana, were gathered from various areas in the Uttarakhand region. These botanical samples were carefully stored in zip-lock bags to preserve them for future reference. The authentication process was conducted by Dr. Anamika Singh of Vardhman College in Bijnor, India, ensuring the precise identification of the plant species.

Chemicals of analytical-grade reagents were used. Mercuric chloride, methanol, ethanol, sodium carbonate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, MTT reagent, and dimethyl sulphoxide (DMSO). The HCT116 cell line used in the study was sourced from the National Centre for Cell Science (NCCS) in Pune.

Methods

Soxhlet extractions of leaves

Leaves were dried under shade, and once dried, they were ground into powder. The Soxhlet extraction method was used with different solvent, following the procedure of Chauhan *et al*., 2021 [19]. Briefly, a 5-gram portion of the dried sample was subjected to extraction with 500 mL of hexane, chloroform, methanol and water for 4 cycles per hour, resulting in a total of 24 cycles. After the extraction process, the obtained extract was subjected to evaporation and weighed. Then, it was further diluted in 20 mL of their respective solvent.

Phytochemical analysis of extracts

The crude samples were screened to detect different phytoconstituents, including Alkaloid, Flavonoids, Steroids, Saponins according to protocol of Vaghasiya *et al*., 2011 [20], while Tannins, and Carbohydrates were examined following the protocol of Bhat *et al*., 2019 [21]. Additionally, the presence of Cardiac Glycosides was assessed using the protocol of Yadav *et al*., 2011 [22].

Total Flavonoid content

The estimation of flavonoid content was conducted using the aluminum chloride colorimetric method [23]. Briefly, 0.5 ml of a solution containing 1.2% aluminum chloride and 1M potassium acetate was added to 0.5 ml of the extract. The reaction mixture was then diluted to a total volume of 3 ml using methanol and left to incubate at room temperature for 30 minutes. After incubation, the absorbance of the solution was measured at a wavelength of 415 nm. A blank sample was prepared by combining all reagents except for the extracts.

Standard solutions of quercetin were prepared within the concentration range of 0.01 mg/ml to 0.150 mg/ml. The Total Flavonoid Content (TFC) was calculated using the equation derived from the standard curve of quercetin and was expressed as milligrams of quercetin equivalent (QE) per 100 grams of dried fruit sample.

Antioxidant assay of crude extract

DPPH assay

To evaluate the crude extract's DPPH free radical scavenging activity, the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was utilized as a marker [24]. The samples were diluted to a concentration of 1 mg/ml. A reaction mixture was prepared by combining 1.0 mL of 0.1 mM DPPH in methanol with 0.1 mL of the extract. This mixture was then incubated in the dark at room temperature for 30 minutes. The extent of DPPH inhibition was measured by monitoring the drop in absorbance at 517 nm. Ascorbic acid was used as the positive control. The radical scavenging activity was calculated as the percentage of free radical inhibition using the formula:

$$\%\ DPPH\ inhibition = \left(\frac{A0 - At}{A0}\right) * 100$$

where A0 represents the absorbance of the control (blank without the sample), and At represents the absorbance in the presence of the sample. All tests were performed in triplicate, and the mean values were used to plot the graph.

FRAP assay (Ferric Reducing Antioxidant Power Assay)

The FRAP test, following the procedure by Benzie *et al*. in 1996 [25], involved diluting the samples to

1 mg/ml. An aliquot (100µL) of the properly diluted extract was added to 3 mL of the standard reaction solution. The absorbance was measured at 593 nm immediately and six minutes later at room temperature. This process was repeated three times. A standard curve was created using $FeSO_4$ in the range of 200–1000 µM. The FRAP values for the standards and samples were expressed in units of µM Fe[II]/g dry wt.

H_2O_2 assay

To determine the H_2O_2 scavenging activity of the diluted crude extract, 40 mM H_2O_2 solution (0.6 mL) was mixed with the diluted extract (0.1 mL) in 2.4 mL of phosphate buffer (0.1 M, pH 7.4). The mixture was vigorously agitated and incubated for 10 minutes at room temperature. After incubation, the absorbance of the reaction mixture was measured at 230 nm. Ascorbic acid was used as the positive control in the experiment. The H_2O_2 scavenging activity was then calculated using the following formula:

$$\%\ H2O2\ \ inhibition = \left(\frac{A0 - At}{A0}\right) 100$$

Where A0 represents the absorbance of the ascorbic acid (positive control), and At represents the absorbance of the sample.

Column purification of crude extracts

Crude extracts were screened for their anticancer activity against HCT116 cell lines. Extract with potent anticancer activity was subjected to column purification following the protocol of [26]. Briefly, the crude extract was dried and packed into a column with silica 60–120 mesh using hexane as the solvent. Carefully applying the crude extract on the silica layer without disturbing it, the purification process utilized a step-wise increase in solvent polarity with varying ratios of chloroform, and methanol (Chloroform (20 ml); Chloroform: Methanol (16:4); Chloroform: Methanol (10:10); Chloroform: Methanol (4:16) and Methanol (20 ml) with a flow rate of 5 ml per min. The eluted fractions were screened for anticancer activity, and subsequently, specific fractions were further purified using HPLC.

HPLC purification and isolation of single compound

The most potent fraction with anticancer properties was chosen for further analysis. To separate its compounds, High-Performance Liquid Chromatography (HPLC) was employed, following the procedure outlined by Khuluk *et al*., 2021 [27]. Standard solutions of various flavonoids (Catechin, Diosmetin, Epicatechin, Quercetin, Rutin, Kaempferol) were prepared at a concentration of 20 µg/ml in high-quality HPLC-grade methanol. The filtered fractions were sonicated at room temperature for 10 minutes. The analysis was conducted using an Agilent 1200 series HPLC system equipped with a dual pump and a PDA (UV/Vis) detector. Separation was achieved using a C18 column with dimensions of 4.6 mm in diameter, 150 mm in length, and a pore size of 5 µm. A consistent flow rate of 1 ml/min was maintained throughout the analysis. The separation process employed a gradient solvent system comprising two solvents: Solvent I (Methanol) and Solvent II (0.2% formic acid). The gradient was programmed as follows:

15-30% solvent I for 0-10 minutes
30-50% solvent I from 10 to 45 minutes
50-80% solvent I from 45 to 50 minutes
80-100% solvent I from 50 to 55 minutes
A constant 100% solvent I from 55 to 60 minutes

This gradient system effectively separated the compounds present in the anticancer fraction. The UV/Vis detector measured the absorbance of the eluted compounds, facilitating their identification and quantification.

Anticancer Activity

After the HPLC purification, the compounds obtained were tested for their potential anticancer activity against HCT116 cell lines. All the purified compounds were dried and then diluted to a concentration of 1 mg/ml. The cell lines were incubated with the maximum concentration of each compound. The % cytotoxicity was then calculated based on the response of the cells to the compounds. Compounds that showed significant cytotoxicity and demonstrated potential anticancer activity were selected for further analysis. These promising compounds were subjected to IC50 (half-maximal inhibitory concentration) calculation.

IC50 estimation for the purified compounds

The compounds were appropriately diluted based on their concentrations in DMSO. Cell lines that had been grown for 24 hours to reach confluence with a cell number of $3*10^5$ were seeded in a 96-well microtiter plate. Different dilutions of the compounds were added to the wells, and the plate was then incubated at 37°C in a 5% CO_2 incubator for 72 hours. After the incubation period, the supernatant was removed, and 25µL of MTT

reagent (5 mg/ml) was added to each well. The plate was further incubated for 2 hours. Following this, 125µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan precipitate, and the wells were stirred for an additional 15 minutes. Absorbance measurements were taken at a wavelength of 490 nm using a spectrophotometer. To provide a baseline for comparison, two negative controls were included: one with only DMSO, and another with only media along with cell controls.

The percentage cell cytotoxicity was calculated using the following formula:

$$\% \text{ Cell cytotoxicity} = \left(\frac{A0 - AS}{A0}\right) * 100$$

Morphological analysis of cells under IC50 treatment

Cells HCT116 cell lines, initially at a concentration of $2*10^5$ cells/ml, were treated with isolated compounds at their IC50 concentrations. They were then incubated for 24, 48, and 72 hours. Control cells without treatment were incubated for the same durations. After each incubation period, cell morphology was observed under a 40X inverted microscope. The cells were subsequently counted using the trypan blue cell exclusion method.

Gene expression analysis

To isolate RNA, an overnight cultured monolayer of HCT116 cells, at an approximate concentration of $2.6*10^4$ cells/ml, was treated with purified compounds at their IC50 concentrations in a six-well microtiter plate. Control wells with untreated cells were also included. Following a 72-hour incubation at 5% CO_2 and 95% humidity in a CO_2 incubator, the cells were trypsinized and pelleted by centrifugation at 2000 rpm for 10 minutes.

The RNA isolation procedure followed the protocol outlined by Rio *et al*., 2010 [28]. Briefly, the cell pellet was resuspended in 1 ml of Trizol, mixed to dissolve the pellet, and then 0.2 ml of chloroform was added and vigorously mixed. After a brief incubation, the mixture was centrifuged, and the upper aqueous phase was transferred to a new tube. Isopropanol was added, followed by centrifugation, and the RNA pellet was obtained. After a series of steps, the RNA pellet was finally suspended in 70 µl of nuclease-free water and stored at -20°C. RNA quality was assessed by gel electrophoresis and its quantity was measured at 260 nm and 280 nm using a UV-Vis spectrophotometer.

The cDNA first strand was synthesized using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit #K1621. The kit components were thawed and centrifuged before use. RNA samples were adjusted to a concentration of 5 µg in a total volume of 20 µl.

The reaction mixture consisted of RNA template (5 µg), oligo (dt)18 primer (1 µl), and nuclease-free water, which were combined in PCR vials to reach a total volume of 20 µl. After incubation at 65°C and chilling on ice, the enzyme mix was added. The reaction proceeded for cDNA synthesis and was terminated at 70°C. The cDNA reaction products were stored at -20°C.

Real-time polymerase chain reaction (PCR) amplification was performed using the ABI PRISM 7000 SDS instrument, adhering to the universal thermal cycling conditions. Each 50 µl reaction mixture comprised 25 µl of Sybr green PCR master mix, 5 µl of cDNA, 2 µl of diluted forward and reverse primer. Stringent quality control measures were implemented through the inclusion of no template controls on each PCR plate to mitigate the risk of PCR contamination. Gene expression analysis was executed utilizing the comparative CT method, with CT values of the target gene being normalized to the endogenous reference gene (GAPDH) and subsequently compared with a designated calibrator sample. The relative expression levels of target genes were quantified using the $2^{-\Delta\Delta CT}$ formula, and results were reported as the ratio of relative gene expression in compound treated cells relative to that in the initial control replicate.

RESULT AND DISCUSSION

Hexane extract exhibited the highest extraction yield of 46% yield. The chloroform and methanol extracts demonstrated equivalent yield of a 38% extraction yield for each. Conversely, the water extract exhibited lower efficiency, yielding a 34% extraction yield. These findings provide valuable insights into the selection of appropriate solvents in *Cannabis sativa* extraction procedures.

Phytochemical analysis of extracts

The phytochemical analysis of various *Cannabis sativa* extracts using different tests revealed distinct qualitative differences in their chemical compositions. Water extract exhibited abundant presence alkaloids, flavonoids, phenols, tannins, carbohydrates, saponins, and steroids. Methanolic extract exhibited moderate presence of several compounds, while hexane extract showed mild presence of alkaloids, flavonoids, and phenols. Chloroform extract, on the other hand, displayed a

lack of most compounds (Table 1). Water extract was found to possesses a rich array of phytochemicals, making it a potentially valuable focus for further exploration and study.

Sample	Alkaloid			Flavonoid	Phenol	Glycosides	Tannins	Carbohydrate			Saponins	steroids
	Mayer's test	Dragendorff's test	Wagner test					Molisch	Fehling's	Benedicts		
CSH	+	+	+	++	+	+	+	++	+	-	-	-
CSC	-	-	-	+	++	-	-	-	-	-	-	-
CSM	-	-	-	++	+	-	+	+	+	-	+	-
CSW	++	++	+++	+	+++	++	++	++	++	+++	+++	++

Table 1: Phytochemical analysis of different extracts of *Cannabis sativa*. CSH: *Cannabis sativa* hexane extract, CSC: *Cannabis sativa* chloroform extract, CSM: *Cannabis sativa* methanol extract, CSW: *Cannabis sativa* water extract, +: mildly present, ++: moderately present, +++: abundant, -: absent

Total Flavonoid content

In the hexane extract, the concentration of QE is 0.034 mg/ml, with a total flavonoid content of 0.819 mg/g. The chloroform extract exhibits values of 0.048 mg/ml for QE and 1.149 mg/g for total flavonoid content. The methanol extract shows a QE concentration of 0.071 mg/ml and a total flavonoid content of 1.697 mg/g. Notably, the water extract demonstrates the highest concentrations, with a QE of 0.123 mg/ml and a total flavonoid content of 2.948 mg/g. Different TFC of each extract may be due to variations in the extraction methods. Flavonoids, known for their antioxidant properties, play a vital role in shielding plants from UV light. Hemp exposed to intense solar radiation and cold temperatures show elevated levels of cannflavin A, indicating its contribution to the plant's resilience in challenging conditions [29].

Antioxidant assay of crude extract

The antioxidant activity of different extracts of *Cannabis sativa* has been assessed using three key parameters: DPPH (% scavenging activity), FRAP value (µM Fe (II)/g dry wt.), and H_2O_2 % scavenging. In the hexane extract, the DPPH scavenging activity was recorded at 49.1%, with a corresponding FRAP value of 400 µM Fe (II)/g dry wt. and an H_2O_2 scavenging of 9.91%. Contrastingly, the chloroform extract exhibited lower antioxidant activity, with a DPPH scavenging activity of 27.45%, a FRAP value of 466.6 µM Fe (II)/g dry wt., and an H_2O_2 scavenging of 4.05%. The methanol extract displayed robust antioxidant potential, with a high DPPH scavenging activity of 76.4%, a significantly elevated FRAP value of 4200 µM Fe (II)/g dry wt., and an H_2O_2 scavenging activity of 36.94%. Similarly, the water extract exhibited significant antioxidant activity with DPPH scavenging activity of 77.941±1.104%, a FRAP value of 3666.67±115.47 µM Fe (II)/g dry wt., and an H_2O_2 scavenging activity of 43.7% (Table 2). The findings highlight the diverse antioxidant capacities of different *Cannabis sativa* extracts, with the methanol and water extracts showing particularly robust antioxidative properties. The findings contribute to our understanding of the potential health benefits associated with *cannabis* extracts, suggesting their utility in combating oxidative stress-related conditions. There is no direct link between the flavonoid content in plant extracts and their antioxidant activity. This implies that the antioxidant activity cannot be solely attributed to the flavonoid content, and other metabolites present in the extracts also play a role in determining the overall antioxidant activity.

Sample	DPPH (% scavenging activity)	FRAP value (µM Fe (II)/g dry wt.)	H_2O_2 % scavenging
CSH	49.106±1.850	400	9.91
CSC	27.450±3.677	466.667±115.47	4.054
CSM	76.376±0.149	4200±200	36.937
CSW	77.941±1.104	3666.667±115.47	43.694

Table 2: Antioxidant activity of different extracts of *Cannabis sativa*. CSH: *Cannabis sativa* hexane extract, CSC: *Cannabis sativa* chloroform extract, CSM: *Cannabis sativa* methanol extract, CSW: *Cannabis sativa* water extract. Data are mean ± std. dev. and n=3.

Purification and isolation of single compound

All crude extracts were screened to assess their potential for anticancer activity, and the extract exhibiting strong anticancer effects was column purified. Only water extract demonstrated anticancer activity against the HCT116 cell line and were selected for subsequent purification. The purification process involved column chromatography of raw materials, resulting in the acquisition of approximately 20 fractions for each

sample. Anticancer activity testing was then conducted on these fractions at their maximum concentrations. Fractions that exhibited positive anticancer activity underwent further purification steps, which included evaporation, quantification of the remaining material through weighing, and dissolution in HPLC-grade methanol. Fractions F19 and F20 displayed significant anticancer activity against HCT116 cell lines. These fractions underwent additional purification using High-Performance Liquid Chromatography (HPLC) to isolate individual compounds.

Anticancer Activity and IC50 estimation for the purified compounds

All the purified compounds were screened at their maximum concentrations against HCT116 cell lines. Two compounds namely Quercetin and a novel compound were purified from F19 and F20 respectively. Only quercetin showed anticancer activity and was proceeded for IC50 estimation. The percentage of cytotoxicity increases as the concentration of quercetin rises, indicating a potential dose-dependent impact on cell viability. At 100 µg/ml, the highest observed concentration, the cytotoxicity reaches 87.85% (Figure 1). The half maximal concentration of the purified quercetin was found to be 60.253 µg/ml against HCT116 cell lines. Quercetin is a natural compound with antiproliferative activity against different cancer cell lines. It is reported to have IC50 of 55µM in MDA-MB-468 breast cancer cell line [30] and 36 µg/ml against HCT116 cell line after 72 hrs. of incubation [31]. The variation in IC50 values between different cell lines may be attributed to differences in cellular characteristics and sensitivity to quercetin.

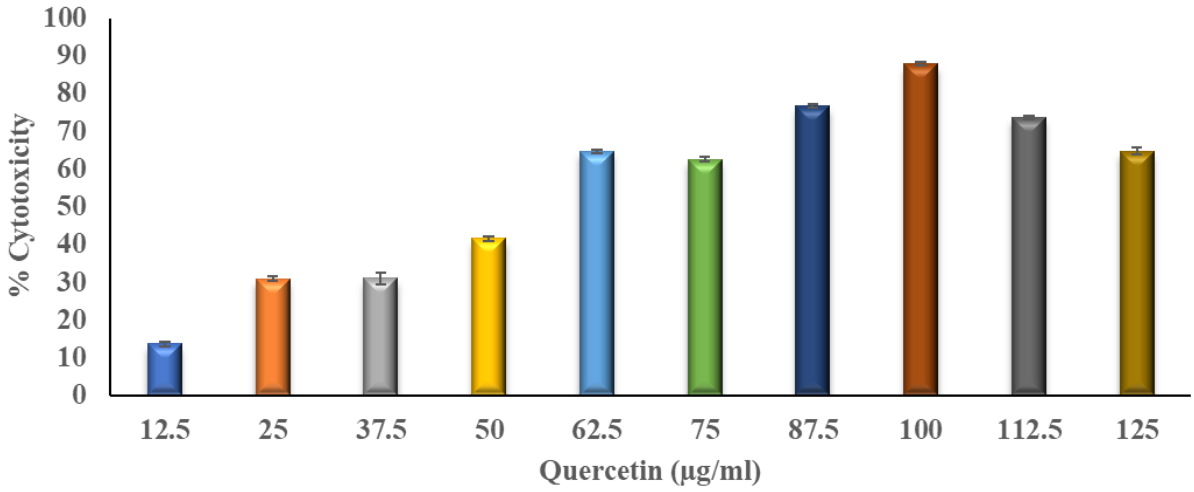

Figure 1: % Cytotoxicity at different concentration of quercetin against HCT116 cell lines. Data are represented as mean ±std dev.

Morphological analysis of cells under IC50 treatment

HCT116 cell lines were incubated with IC50 concentration of quercetin and the cell morphology was assessed at 24, 48 and 72 h intervals, respectively (Figure 2A). No morphological differences were observed in control HCT116 (without treatment) cell line after 72 hrs. of incubation. While cell lines incubated with IC50 concentration of quercetin showed loss of attachment to the substrate and are more circular after 24 hrs. of incubation. A significant cell blebbing can be observed after 48 hrs. of treatment indicating early apoptotic cells. Small circularized cell aggregated indicated late apoptotic or dead cells after 72 hrs. of incubation with quercetin.

In the HCT116 cell line, untreated cells exhibit a typical growth pattern, with the cell population increasing over the 72-hour period. The quercetin-treated HCT116 cells, on the other hand, display a suppressive effect on cell proliferation (Figure 2B). The observed decrease in cell counts at 24, 48, and 72 hours in the presence of quercetin suggests a potential anti-proliferative or cytostatic impact. This could be indicative of quercetin's interference with cellular processes involved in growth or cell cycle progression. Further molecular and mechanistic studies would be necessary to elucidate the specific pathways affected by quercetin and to comprehensively understand its biological impact on HCT116 cells.

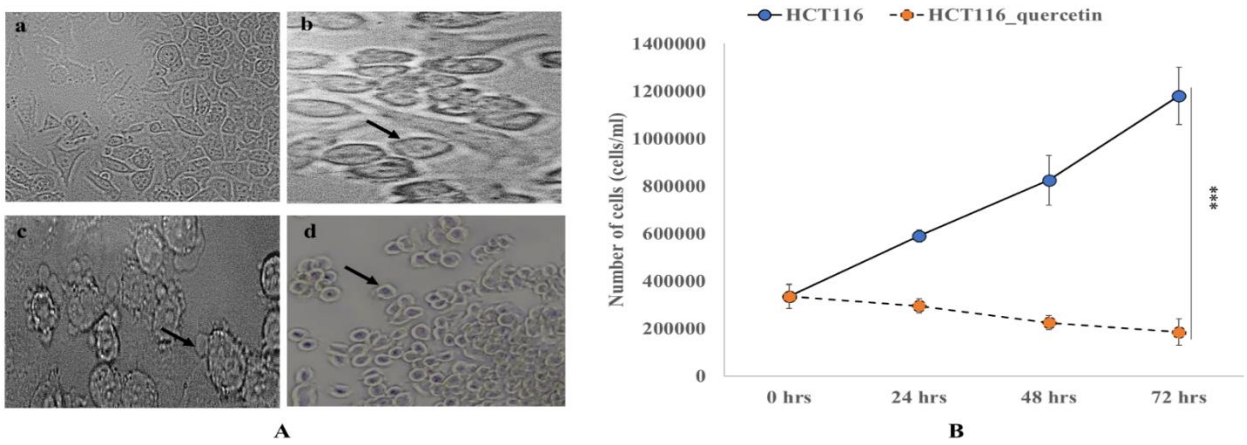

Figure 2: A: Morphological analysis of HCT116 cell lines after 0, 24, 48 and 72 hrs. of incubation with quercetin. B: Number of cells calculated after each period of incubation using trypan blue exclusion method. All data are represented as mean ±std dev, where n=3 and *** indicate p<0.001.

Gene expression analysis

The RNA that was extracted is in good condition with a high level of integrity and purity, ranging from 1.84 to 1.9. The $2^{(-ddCt)}$ method is employed to assess the relative gene expression levels of M-CSF and GM-CSF in two experimental conditions: "HCT116_quercetin" and the reference condition "HCT116." The gene expression of M-CSF is approximately 5.29 times higher in quercetin-treated cell lines than the non-treated, indicating an upregulation in the presence of quercetin (Figure 3). Similarly, the gene expression of GM-CSF is approximately 1.04 times higher in the same condition compared to the non-treated. This suggests a slight upregulation of GM-CSF in the presence of quercetin. The results indicate that quercetin treatment may have a more pronounced effect on the expression of M-CSF compared to GM-CSF in HCT116 cell lines.

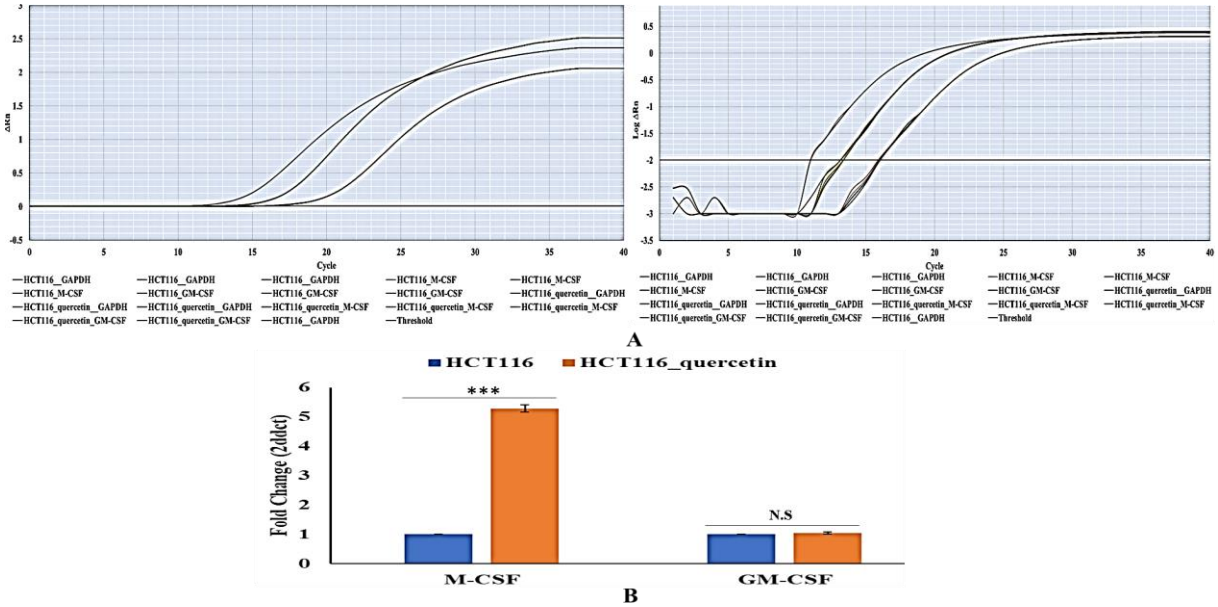

Figure 3: A: Amplification plot (ΔRn and log ΔRn), B: Relative gene expression of M-CSF and GM-CSF. All data are represented as mean ± std dev., where n=3, Significance is denoted by asterisks, with "***" indicating high significance (p<0.001), and "N. S" indicating non-significant differences.

CONCLUSION

The comprehensive investigation into various *Cannabis sativa* extracts and their properties provides valuable insights into their potential applications in medicine and health. The phytochemical analysis highlighted the distinctive compositions of each extract, emphasizing the richness of compounds in the water extract. The antioxidant assays demonstrated varying capacities among extracts, with methanol and water extracts displaying particularly potent antioxidant properties. These findings underscore the potential health benefits associated with *cannabis* extracts, especially in combating oxidative stress-related

conditions. The purification and isolation of compounds from the water extract unveiled the presence of quercetin, a natural compound known for its anticancer properties. The subsequent assessment of quercetin's impact on HCT116 cell lines revealed significant cytotoxicity, suggesting its potential as an anti-proliferative agent. The morphological analysis further supported this, indicating quercetin's influence on cell attachment, morphology, and viability over time.

Gene expression analysis provided insights into the molecular mechanisms underlying quercetin's effects, showing an upregulation of M-CSF and a slight upregulation of GM-CSF. These findings contribute to a better understanding of the potential therapeutic mechanisms of quercetin in cancer treatment. In conclusion, this study sheds light on the diverse chemical compositions and biological activities of different *Cannabis sativa* extracts. The identified quercetin compound, with its promising anticancer properties, opens avenues for further research into the therapeutic potential of *cannabis*-derived compounds in oncology. These findings contribute to the growing body of knowledge surrounding the pharmacological potential of *Cannabis sativa* and its constituents.

REFERENCES

1. Siegel, R. L., Miller, K. D., Fuchs, H. E., & Jemal, A. (2021). Cancer Statistics, 2021. *CA: a cancer journal for clinicians*, *71*(1), 7–33. https://doi.org/10.3322/caac.21654
2. Hossain, M. S., Karuniawati, H., Jairoun, A. A., Urbi, Z., Ooi, J., John, A., Lim, Y. C., Kibria, K. M. K., Mohiuddin, A. K. M., Ming, L. C., Goh, K. W., & Hadi, M. A. (2022). Colorectal Cancer: A Review of Carcinogenesis, Global Epidemiology, Current Challenges, Risk Factors, Preventive and Treatment Strategies. *Cancers*, *14*(7), 1732. https://doi.org/10.3390/ cancers 14071732
3. Papamichael, D., Audisio, R. A., Glimelius, B., de Gramont, A., Glynne-Jones, R., Haller, D., Köhne, C. H., Rostoft, S., Lemmens, V., Mitry, E., Rutten, H., Sargent, D., Sastre, J., Seymour, M., Starling, N., Van Cutsem, E., & Aapro, M. (2015). Treatment of colorectal cancer in older patients: International Society of Geriatric Oncology (SIOG) consensus recommendations 2013. *Annals of oncology : official journal of the European Society for Medical Oncology*, *26*(3), 463–476. https:// doi.org/ 10.1093/ annonc/ mdu253
4. *Colon cancer - Symptoms and causes*. Mayo Clinic. (2021). Retrieved 04 December 2023, from https://www.mayoclinic.org/diseases-conditions/colon-cancer/symptoms-causes/ syc-20353669.
5. Jelski, W., & Mroczko, B. (2020). Biochemical Markers of Colorectal Cancer - Present and Future. *Cancer management and research*, *12*, 4789–4797. https://doi.org/ 10.2147/CMAR.S253369
6. Rodriguez-Garcia C., Sanchez-Quesada C. Dietary Flavonoids as Cancer Chemopreventive Agents: An Updated Review of Human Studies. *Antioxidants*. 2019;8: 137. doi: 10.3390/antiox8050137.
7. Martin M.A., Goya L., Ramos S. Preventive Effects of Cocoa and Cocoa Antioxidants in Colon Cancer. *Diseases*. 2016:4. doi: 10.3390/diseases4010006.
8. Choi E.J., Jung J.Y., Kim G.H. Genistein inhibits the proliferation and differentiation of MCF-7 and 3T3-L1 cells via the regulation of ERalpha expression and induction of apoptosis. *Exp. Ther. Med.* 2014;8: 454–458. doi: 10.3892/etm.2014.1771.
9. Solomon L.A., Ali S., Banerjee S., Munkarah A.R., Morris R.T., Sarkar F.H. Sensitization of ovarian cancer cells to cisplatin by genistein: The role of NF-kappaB. *J. Ovarian Res.* 2008;1: 9. doi: 10.1186/1757-2215-1-9.
10. Shafiee G., Saidijam M., Tavilani H., Ghasemkhani N., Khodadadi I. Genistein Induces Apoptosis and Inhibits Proliferation of HT29 Colon Cancer Cells. *Int. J. Mol. Cell. Med.* 2016;5: 178–191.
11. Qin J., Teng J., Zhu Z., Chen J., Huang W.J. Genistein induces activation of the mitochondrial apoptosis pathway by inhibiting phosphorylation of Akt in colorectal cancer cells. *Pharm. Biol.* 2016;54: 74–79. doi: 10.3109/13880209. 2015. 1014921.
12. Maurya A.K., Vinayak M. Quercetin Attenuates Cell Survival, Inflammation, and Angiogenesis via Modulation of AKT Signaling in Murine T-Cell Lymphoma. *Nutr. Cancer*. 2017;69: 470–480. doi: 10.1080/ 01635581.2017.1267775.
13. Han M., Song Y., Zhang X. Quercetin Suppresses the Migration and Invasion in Human Colon Cancer Caco-2 Cells Through Regulating Toll-like Receptor 4/Nuclear Factor-kappa B Pathway. *Pharmacogn. Mag.* 2016;12: S237–S244.
14. Moreau, M., Ibeh, U., Decosmo, K., Bih, N., Yasmin-Karim, S., Toyang, N., Lowe, H., & Ngwa, W. (2019). Flavonoid Derivative

of *Cannabis* Demonstrates Therapeutic Potential in Preclinical Models of Metastatic Pancreatic Cancer. *Frontiers in oncology*, *9*, 660. https://doi.org/10.3389/fonc.2019.00660

15. Riahi-Chebbi I., Souid S., Othman H., Haoues M., Karoui H., Morel A., Srairi-Abid N., Essafi M., Essafi-Benkhadir K. The Phenolic compound Kaempferol overcomes 5-fluorouracil resistance in human resistant LS174 colon cancer cells. *Sci. Rep.* 2019;9: 195. doi: 10.1038/s41598-018-36808-z.
16. Berenda J., Smöch C., Stadlbauer C., Mittermair E., Taxauer K., Huttary N., Krupitza G., Krenn L. Flavonoids distinctly stabilize lymph endothelial- or blood endothelial disintegration induced by colon cancer spheroids SW620. *Molecules.* 2020; 25: 2066. doi: 10.3390/molecules25092066.
17. Potočnjak I., Šimić L., Gobin I., Vukelić I., Domitrović R. Antitumor activity of luteolin in human colon cancer SW620 cells is mediated by the ERK/FOXO3a signaling pathway. *Toxicol. Vitr.* 2020;66: 104852. doi: 10.1016/j.tiv.2020.104852.
18. Zhou Y., Zhang J., Wang K., Han W., Wang X., Gao M., Wang Z., Sun Y., Yan H., Zhang H., *et al*. Quercetin overcomes colon cancer cells resistance to chemotherapy by inhibiting solute carrier family 1, member 5 transporter. *Eur. J. Pharmacol.* 2020:173185. doi: 10.1016/j.ejphar.2020.173185.
19. Chauhan, O., & Sharma, S. B. (2021). Gene Expression Analysis of Therapeutically Active Antiproliferative Polyphenolic Fractions Extracted from *Rubus Ellipticus* on Human Cervical Cancer Cells (Hela). *Uttar Pradesh Journal of Zoology*, 42(22), 178–194.
20. Vaghasiya, Y., Dave, R., and Chanda, S., (2011). Phytochemical analysis of some medicinal plants from Western Region of India. Research Journal of Medicinal Plants. 5:567–576.
21. Bhat, B. A., Shergojri, F. A., Gaur, M., & Sham, Q. J. (2018). Phytochemical analysis and in vitro antioxidant activity of rubia cordifolia. *Int J Recent Trends Sci Technol*, 26-33.
22. Yadav RNS, Agarwala M. 2011. Phytochemical analysis of some medicinal plants. J Phytol 3 (12): 10-14.
23. Pontis JA, LA Mendonca, AD Costa, SJRD Silva, A Flach (2014) Color, phenolics and flavonoid content and antioxidant activity of honey from Roraima, Brazil. Food Sci Tech 34: 69-73.
24. Blois, M.S. (1958) Antioxidant Determinations by the Use of a Stable Free Radical. Nature, 181, 1199-1200. http://dx.doi.org/10.1038/1811199a0
25. Benzie, I. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical biochemistry*, *239*(1), 70–76. https://doi.org/ 10.1006/ abio.1996.0292
26. Zhang, H., Wang, W., Fu, Z. M., Han, C. C., & Song, Y. (2014). Study on comparison of extracting fucoxanthin from Undaria pinnatifida with percolation extraction and refluxing methods. *Zhongguo Shipin Tianjiaji*, *9*, 91-95.
27. Khuluk, R. H., Yunita, A., Rohaeti, E., Syafitri, U. D., Linda, R., Lim, L. W., Takeuchi, T., & Rafi, M. (2021). An HPLC-DAD Method to Quantify Flavonoids in Sonchus arvensis and Able to Classify the Plant Parts and Their Geographical Area through Principal Component Analysis. *Separations*, *8*(2), 12. https://doi.org/10.3390/separations8020012
28. Rio, D. C., Ares, M., Jr, Hannon, G. J., & Nilsen, T. W. (2010). Purification of RNA using TRIzol (TRI reagent). *Cold Spring Harbor protocols*, *2010*(6), pdb.prot5439. https://doi.org/10.1101/pdb.prot5439
29. Calzolari, D., Magagnini, G., Lucini, L., Grassi, G., Appendino, G. B., & Amaducci, S. (2017). High added-value compounds from Cannabis threshing residues. *Industrial Crops and Products*, *108*, 558-563.
30. Maugeri, A., Calderaro, A., Patanè, G. T., Navarra, M., Barreca, D., Cirmi, S., & Felice, M. R. (2023). Targets Involved in the Anti-Cancer Activity of Quercetin in Breast, Colorectal and Liver Neoplasms. *International journal of molecular sciences*, *24*(3), 2952. https://doi.org/ 10.3390/ijms24032952
31. Mohammed, H. A., Almahmoud, S. A., El-Ghaly, E. M., Khan, F. A., Emwas, A. H., Jaremko, M., Almulhim, F., Khan, R. A., & Ragab, E. A. (2022). Comparative Anticancer Potentials of Taxifolin and Quercetin Methylated Derivatives against HCT-116 Cell Lines: Effects of *O*-Methylation on Taxifolin and Quercetin as Preliminary Natural Leads. *ACS omega*, *7*(50), 46629–46639. https://doi.org/10.1021/ acsomega.2c05565