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Cannabinoids Characterization and the Anti-cancer Activity of *Cannabis sativa* Stem Extracts against Colorectal Cancer Cell

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Abstract

Cannabis sativa is a plant with growing interests in research, and this is because of it therapeutic value associated with its active constituents such as cannabinoids and the terpenes. In this study, we characterized some isolated cannabinoids from the stems of cannabis plant using a JEOL Delta GX 400 MHz FT nuclear magnetic resonance instrument, and also evaluate the anti-cancer activity of the extracts against colorectal cancer cell. The cell viability was assessed using AlamarBlue and readings were recorded in term of fluorescence values using a microplate reader. Two cannabinoids were isolated (cannabinol and tetrahydrocannabinolic acid) from the stems. HCT 116 cancerous cell was used to test the treatment with various concentrations of the extracts (ethanol and hexane) for 24 h, and the % viability ranges from 3.8 % (higher concentration of 600 µg/mL) to 104.30 % (lower concentration of 2.5 µg/mL) for both extracts. The LC_{50} values of ethanol and hexane extracts were found to be 52.30 µg/mL and 51.89 µg/mL for HCT116 cell. Treatments was also carried out on a non-cancerous cell (MCF10A cell), and the % viability for both extracts (ethanol and hexane) ranges from 1.98–113.40 %. The LC_{50} values of ethanol and hexane extracts are 52.40 µg/mL and 50.70 µg/mL for MCF10A cell. Findings from this study showed that *Cannabis sativa* stems contains cannabinoids, and is a promising anti-cancer agent.

Keywords: Cancer, *Cannabis sativa*, Colorectal, HCT116, MCF10

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Introduction

Cancer ranks among the leading causes of death worldwide, accounting for approximately 9.6 million fatalities globally [1]. Recently, new methods such as nano-therapy, neutron capture, and low-intensity electro-resonance therapy have been developed to curb the spread of these deadly diseases. However, all these methods are associated with numerous side effects that negatively impact the patient's health. There is an urgent need to develop cancer drugs from plant materials and apply them in clinical practice, as this approach offers the safest means to reduce the ever-increasing cancer-related mortality rate [2]. Many drugs derived from natural products are currently used to treat cancer, with numerous examples demonstrating their success [3].

Research indicates that a variety of food plants, medicinal herbs, and spices contain both primary and secondary metabolites that can aid in cancer treatment [4]. Research has also demonstrated that plant extracts and their active components, including polyphenols and other bioactive constituents, possess anti-cancer, antibacterial, anti-inflammatory, antioxidant, and immunomodulatory properties [5]. These activities in plant materials ensure their effectiveness in preventing and treating various cancers, including those of the stomach, colon, colorectal, skin, lung, liver, and prostate [6]. Phytochemical analysis of the cannabis plant has revealed the presence of cannabinoids (THC, CBD, CBN, CBG) along with numerous minor constituents, including terpenes and terpenoids, phenols, fatty acids, and flavonoids [7].

However, only a few of these constituents exhibit medicinal properties, and since the plant's extracts vary in composition, cannabis cannot be considered a generic medicine. Active constituents of Cannabis sativa, including THC, CBN, and CBD, are supported by substantial pre-clinical and clinical evidence, particularly regarding their potential in cancer treatment [8]. This study evaluates the efficacy of *Cannabis sativa* stem extracts in preventing and treating colorectal cancer using standard analytical methods. Colorectal cancer, also known as colon, bowel, or rectal cancer, is the development of malignancy in the colon or rectum [9], with signs and symptoms that may include blood in the stool, changes in bowel movements, weight loss, abdominal pain, and fatigue [10]. The primary causes of colorectal cancer are lifestyle factors and genetic disorders [11, 8]. Colorectal cancer may be treated through a combination of surgery, radiation therapy, chemotherapy, and targeted therapy [9]. Although the methods listed above are viable options for treating colorectal cancer, they pose a life-threatening risk due to the toxicity of some of these treatments.

Consequently, the present study was designed to evaluate the efficacy of *Cannabis sativa* stem extracts in the prevention and treatment of a lethal form of cancer. *Cannabis sativa*, a natural product, offers an eco-friendly and safer administration method for cancer treatment compared to other conventional approaches. The stem extract concentrations were profiled, and an analysis was conducted based on these profiles to evaluate their anti-cancer activities and identify both positive and negative modulating effects. The present study demonstrates that *Cannabis sativa* stems possess promising anti-cancer properties, as their extracts inhibit the growth of colorectal cancer cells, and this could be because of cannabinoids isolated in the present study.

Materials and Methods

Sample collection and preparation

The stems were sourced from the National Drugs Law Enforcement Agency in Akwanga Local Government Area, Nasarawa State, and authenticated by a botanist from the Department of Biology at the College of Education, Akwanga. The collected samples were air-dried in the sun for two weeks to ensure they were completely free of moisture. The dried samples were cut into smaller pieces and then ground into a powder using a mortar and pestle.

Extraction

Hexane

The stems sample was extracted using the maceration method. 800 g of ground stem samples were macerated with 800 mL of hexane. The mixture was then filtered using Whatman filter paper. The filtrates were air-dried under ambient conditions. The same procedure was then applied to the marc for ethanol extract production [12].

Fractionation of extracts/isolation of cannabinoids

Extracts were combined based on their similarity in thin-layer chromatography (TLC) and then fractionated and purified using a 50 cm long silica-gel column with an internal diameter of 2 cm. The extracts were dissolved in ethyl acetate, combined with 6 g of silica gel in a beaker, and then allowed to dry under ambient conditions. Silica gel (35 g in hexane and 40 g in ethanol) was mixed with 200 cm^3 of hexane to form a slurry for packing the column. The column was packed with a wet mixture of hexane and ethyl acetate (95:5) and eluted using a gradient of ethyl acetate in hexane, starting at 5 % ethyl acetate and increasing by 5 % increments up to 100 % ethyl acetate, with 10 cm^3 fractions collected to yield fractions 135 and 140 for hexane and ethanol, respectively. The collected fractions were air-dried under ambient conditions to yield a mixture of compounds 1 and 2 (fraction 14) and compound 1 (fraction HH-12) [12].

Nuclear Magnetic Resonance Spectroscopy (NMR)/characterization of cannabinoids

Approximately 6 mg of the isolated and further purified cannabinoid compounds were dissolved in $CDCl_3$ and transferred to NMR tubes for Nuclear Magnetic Resonance (NMR) spectroscopy characterization. Proton NMR spectra were recorded on a JEOL Delta GX 400 MHz FT-NMR instrument at the University of Strathclyde in Glasgow, Scotland, operating at a magnetic field strength of 400 MHz, with 1H signals referenced to the residual protons in $CDCl_3$ [12].

Isolation and collection of cell samples

Cell lines were collected from human tumor tissue using the ascetic effusion method. The collected cell lines were separated by density centrifugation according to their densities.

Cell culture

Both cancerous and noncancerous cell lines were cultured. To evaluate the treatment, the study employed HCT 116 (cancerous) and MCF10A (noncancerous) cell lines. The cell lines were cultured in McCoy's 5a medium supplemented with 10 % fetal bovine serum and 1 % penicillin-streptomycin. All cell lines were cultured in petri dishes at 37 °C within a humidified incubator containing 5 % CO_2. Cultured cells were maintained as a monolayer until reaching 70–90 % confluency and then sub-cultured every two days. Cell division was achieved by removing the media and washing the cells with sterile DPBS. The cells were subsequently treated with trypsin-EDTA and incubated at 37 °C until detachment occurred. The trypsinized cells were subsequently centrifuged at 1500 rpm for 5 min, after which the supernatant was discarded to retain the pellet. The remaining pellet was re-suspended in 15 mL of McCoy 5a medium, and 10 µL of the cell suspension was mixed with 10 µL of trypan-EDTA in preparation for counting. The mixture was placed on a slide, and cell counting was performed using a TC20TM automated cell counter [13].

AlamarBlue analysis

For the AlamarBlue assay, cell lines were seeded in 96-well plates at a density of 2. 4 × 104 cells per well, incubated overnight, and subsequently treated with *Cannabis sativa* stem extracts (hexane and ethanol) at concentrations ranging from 600 to 2.5 µg/mL. Cell viability was assessed using AlarmaBlue, with absorbance readings recorded through a BioTek Synergy HT microplate reader [13].

Cell viability was calculated using the formula

(Absorbance of treated cells – Absorbance of blank) × 100/Total number of cells,

Statistical analysis

The Student t-test was employed to assess the statistical significance of differences in HCT116 and MCF10A expression, as well as cell growth measured by the AlamarBlue assay. A p-value of 0.05 or less was deemed statistically significant.

Results and Discussion

Characterization of HH-12 as cannabinol (compound 1)

The characterization of HH-12 as cannabinol (compound 1) revealed signals corresponding to six doublets, five singlets, and three multiplets. The proton NMR spectrum (Fig. 1) gives signals for five aromatic protons at δH 6. 30 ppm (1H, d, J = 1. 12 Hz), 6. 45 ppm (1H, d, J = 1. 12 Hz), 7. 08 ppm (1H, d, J = 7. 89 Hz), 7. 14 ppm (1H, d, J = 7. 8 Hz), and 8. 15. It also displays one hydroxyl proton at δH 5. 23 (s, 1H), three methyl groups at δH 0. 90 (s, 3H, J = 1. 69 Hz), 1. 59 (s, 6H), and 1. 59 (s, 6H), one methyl proton attached to benzene ring at δH 2. 39 (s, 3H), and three methylene protons at δ_H 1.33 (2H, m), 1.33 (2H, m) and 1.60 (2H, m) (Table 1). The methyl groups at positions 15 and 16 exhibit similar chemical shifts due to the additional aromatic ring, which creates a planar molecular structure where both methyl groups occupy equivalent positions above and below the aromatic plane. By comparing its 1H NMR data with literature reports and those of cannabinol, compound 1 was identified as cannabinol (structure 1, Fig. 2).

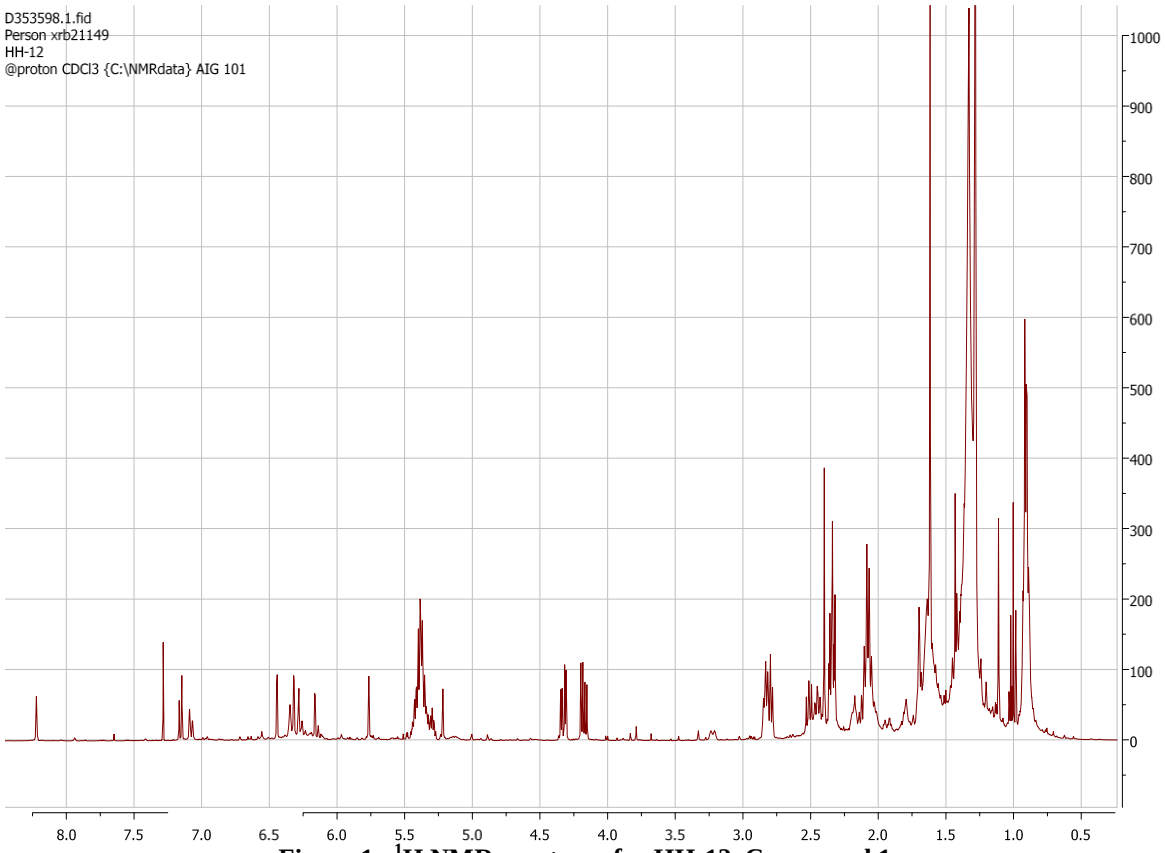

Figure 1: ^{1}H NMR spectrum for HH-12. Compound 1

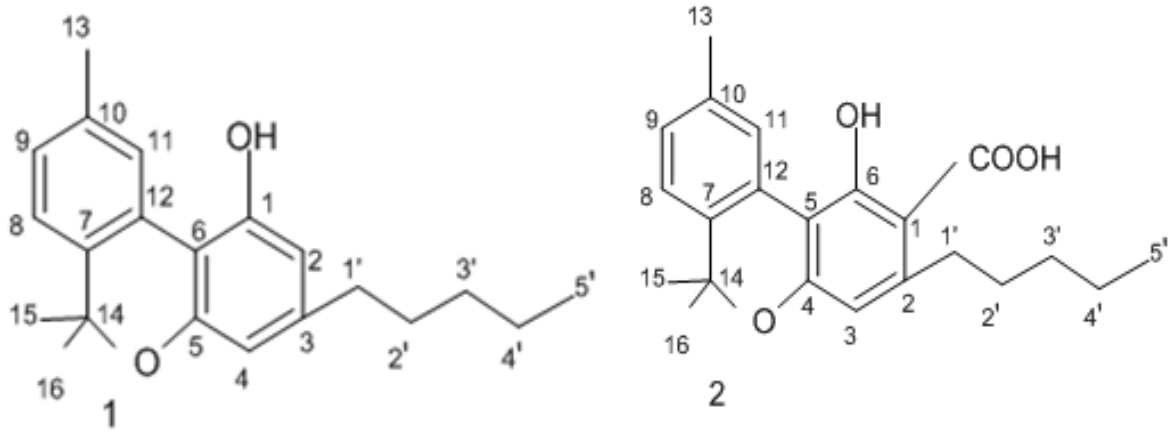

Figure 2: Structures of compound 1 (cannabinol) and compound 2 (tetrahydrocannabinolic acid isolated from *Cannabis sativa* stems

Table 1: ^{1}H NMR data for cannabinol (compound 1)

Position	Experimental ^{1}H (δ ppm Mult)	Literature(ref) cannabinol [14] ^{1}H (δ ppm)
1-OH	5.23(1H, s)	5.23
2	6.30 (1H, d)	6.29
3		
4	7.05 (1H, d)	7.07
5	6.45 (1H, d)	6.44
6		
7		
8		
9	7.14 (1H, d)	7.14
10		
11	8.16 (1H, d)	8.16
12		
13	2.39 (3H, s)	2.38
14		
15	1.59 (6H, s)	1.32
16	1.59 (2H, s)	1.32
1’	2.50(2H, t)	2.50
2’	1.60 (2H, t)	1.61
3’	1.33 (2H, m)	1.32
4’	1.33 (2H, m)	1.32
5’	0.90(3H, s)	0.89

Table 2: ^{1}H NMR data for cannabinol (compound 1)

Position	Experimental ^{1}H (δ ppm Mult)	Literature (ref) cannabinol ^{1}H (δ ppm) [14]
1-OH	5.34 (IH, s)	5.13
2	6.25 (1H, d)	6.29
3		
4	7.09 (1H, d)	7.07
5	6.42 (1H, d)	6.44
6		
7		
8		
9	7.13 (1H, d)	7.14
10		
11	8.16 (1H, d)	8.15
12		
13	2.38 (3H, s)	
14		
15	1.59 (6H,m)	1.32
16	1.59 (6H,m)	1.32
1’	2.50 (2H, t)	2.50
2’	1.61 (2H, m)	1.32
3’	1.31 (2H, m)	1.32
4’	1.31 (2H, m)	
5’	0.09 (3H, t)	0.89

Characterization of HH-14 as a mixture of cannabinol and tetrahydrocannabinolic acid (compound 1 and 2)

Compound 1, showed signals corresponding to five doublets, three singlets, three multiplets and a triplet. The proton NMR spectrum (Fig. 3) displays signals for five aromatic protons at δ_H 6.25 (1H, d), 6.42 (1H, d), 7.09 (1H, d), 7.13 (1H, d) and 8.16 (1H, d). One hydroxyl proton at δ_H 5.34 (1H, s), three methyl protons at δ_H 0.90 (3H, t) 1.59 (3H, s) and 1.59 (3H, s) and three methylene protons at δ_H 1.31 (4H, m), 1.31 (4H, m) and 1.61 (2H, m) (Table 2). A broad hydroxyl signal was also observed at δ_H 5.34 (1H, s) because of the interaction between the proton and the adjacent oxygen atom.

The proton NMR spectrum (Fig. 3) for compound 2 gives signals for two aromatic protons at δ_H 6.25 (1H, s) and 6.42 (1H, s). One methyl proton attached to benzene a ring at δ_H 1.67 (3H, s), three other methyl protons at δ_H 0.90 (3H, t J= 6.9 Hz), 1.13 (3H, s) and 1.46 (3H, s). It also showed signals for four methylene protons at δ_H 1.35 (2H, m), 1.35 (2H, m), 1.58 (2H, m) and 2.95 (1H, m) (Table 3). The splitting pattern of H-2 in compound 2 is a broad singlet as against the doublet observed in compound 1 because of the changes in stereochemistry between H-2 and adjacent proton (H-4) due to the hydroxyl group [14]. The hydroxyl functional group of compound (6-OH) was observed at δ_H 12.19 while that of compound 1 was at δ_H 5.34 (1-OH) and this is because of intra-molecular hydrogen bonding between the OH and ortho-COOH in compound 2. Compound 1 and 2 showed signal for easily recognizable triplet at 0.90 ppm attributed to all cannabinoids [14].

Table 3: ^{1}H NMR data for compound 2 (Tetrahydrocannabinolic acid)

Position	Experimental ^{1}H (δ ppm Mult)	Literature cannabinol [14] ^{1}H (δ ppm)
1-COOH		
2		
3	6.42 (1H, brs)	6.39
4		
5		
6-OH	12.19 (1H, s)	12.23
7		
8		
9		
10		
11		
12	6.25 (1H, s)	6.26
13	1.67 (3H, s)	1.68
14		
15	1.46 (3H, s)	1.46
16	1.13 (3H, s)	
1’	2.95(2H,m),2.79 (1H,m)	2.94, 2.78
2’	1.58 (2H, m)	1.57
3’	1.34 (2H,m)	1.35
4’	1.34 (2H,m)	1.35
5’	0.90 (3H, t)	0.90

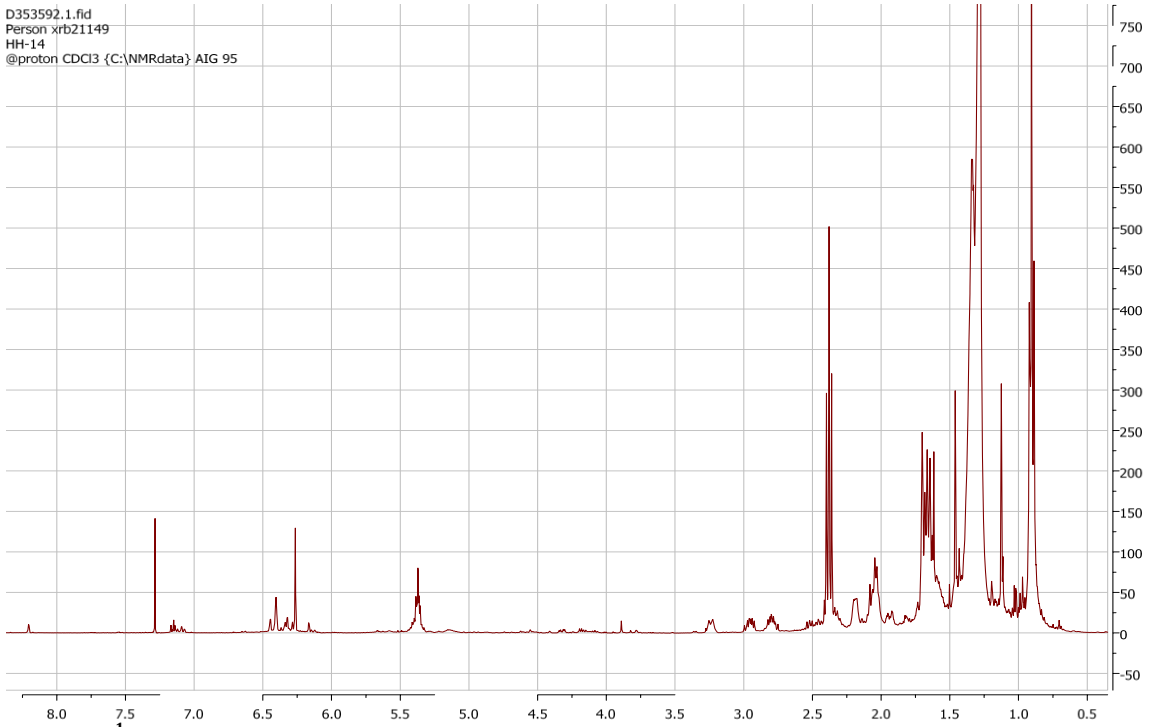

Figure 3: ^{1}H NMR spectrum for HH-14. Compounds 1 and 2

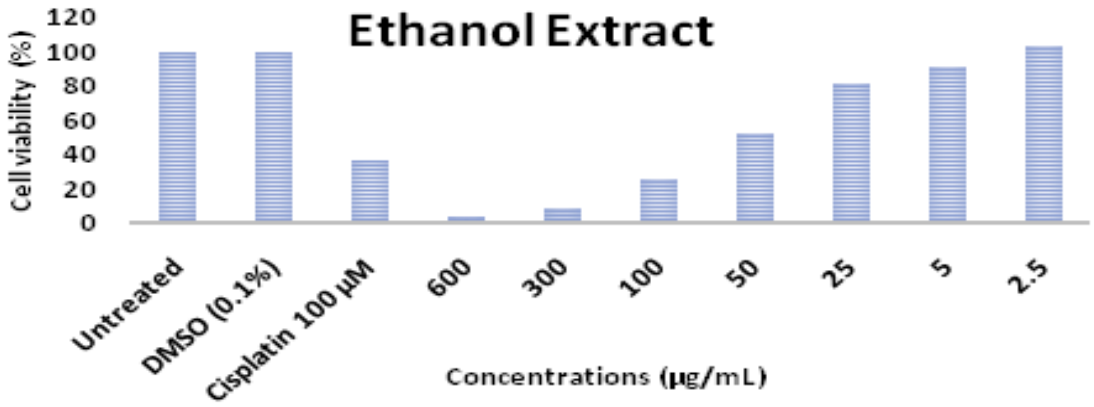

Figure 4: Cell viability of HCT 116 cells at various concentrations of EtOH extract for 24 h (DMSO =Dimethyl sulfoxide)

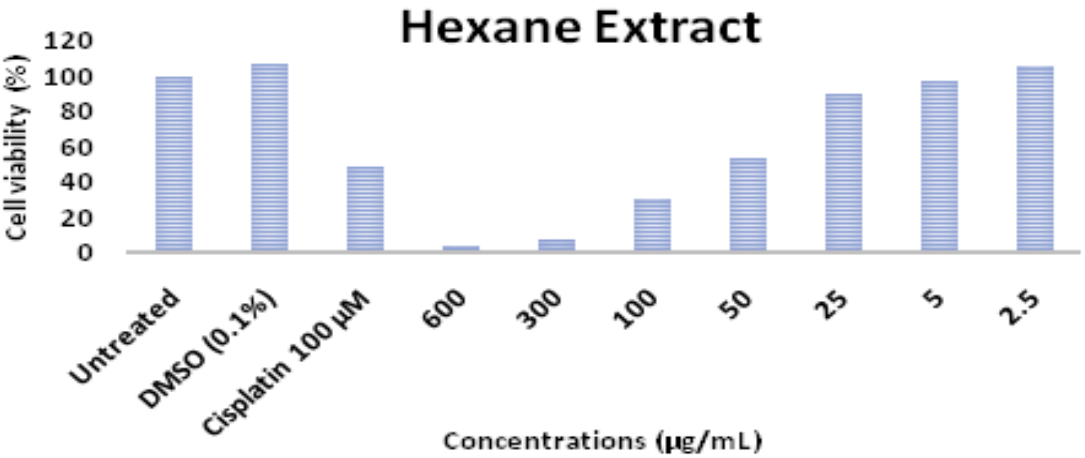

Figure 5: Cell viability assay of HCT116 cells at various concentrations of hexane extract for 24 h (ns= non-significant)

Cytoxicity effect of *Cannabis sativa* stems extract on HCT 116

HCT116 cells were treated with six different concentrations of cannabis stems extracts (Ethanol extract) (2.5, 5, 25, 100, 300 and 600 µg/mL) (Fig. 4), and the results revealed that the cell viability decreases with increase concentrations. Concentration at 600 µg/ml was seen to be highly effective on HCT116 cells, and this can be attributed to the decrease in cell viability (4.01 %), while lower concentrations such as 2.5 and 5 µg/mL were not cytotoxic because they resulted in 103.2 and 91.40 % cell viability respectively. The results also revealed that concentrations such as that of 600 µg/mL was highly significant when compared to the untreated control cells. The negative control (DMSO) produce a cell viability at 99.88 %, which was non-significant. The positive control (Cisplatin treatment) resulted in cell viability of 37.06 %, which was highly significant. A highly significant difference was observed at higher concentrations compared to lower concentrations when HCT116 cell lines was treated with hexane extracts (Fig. 5). Concentration at 600 mg/L was measured as the highest, resulting in cell viability of 3.80 %. Lower concentrations such as 2.5 and 5 µg/mL showed a non-significant difference, with a cell viability of 104.89 and 97.40 % when compared with untreated control. Cisplatin showed cell viability of 48.70 %, which is highly significant, and DMSO had no effect on the proliferation of HCT116 cell lines. 50 % of the cancerous cells were found death at a concentration of 52.30 µg/mL when treated with ethanol extract and 51.89 µg/mL with hexane extract. The effects of these extracts on the cell lines could be attributed to the isolation of cannabinoids such as cannabinol and tetrahydrocannabinolic acid (decomposed into tetrahydrocannabinol over time). Studies have shown that these compounds target key signaling pathways involved in all the hallmarks of cancer [14].

Cytoxicity effect of *Cannabis sativa* stems extract on MCF10A

The treatment of MCF10A cell lines (normal cell lines) with *Cannabis sativa* stems extract (ethanol extract) (Fig. 6) revealed that higher concentrations resulted in lower cell viability. This is because a slightly higher concentration at 300 µg/mL showed that only 9.10 % of the cells are viable. This result is highly significant when compared to the untreated control cells. The results also revealed that lower concentrations showed higher cell viability, with concentration at 2.5 µg/mL producing 113.40 % of viable cells, and this is slightly non-significant. DMSO (negative control) showed non-significant difference in the cell viability of MCF10A cells (105.20 %) when compared to untreated control cell line. However, analysis using cisplatin (positive control) revealed that at a much lower concentration of 3.50 µg/mL, the cell was viable, and this was highly significant.

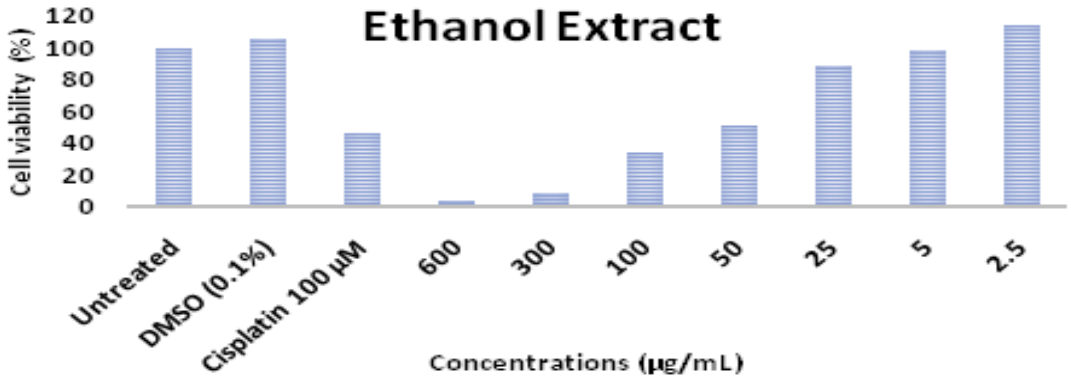

Figure 6: Cell viability of MCF10A cells at various concentrations of EtOH extract for 24 h

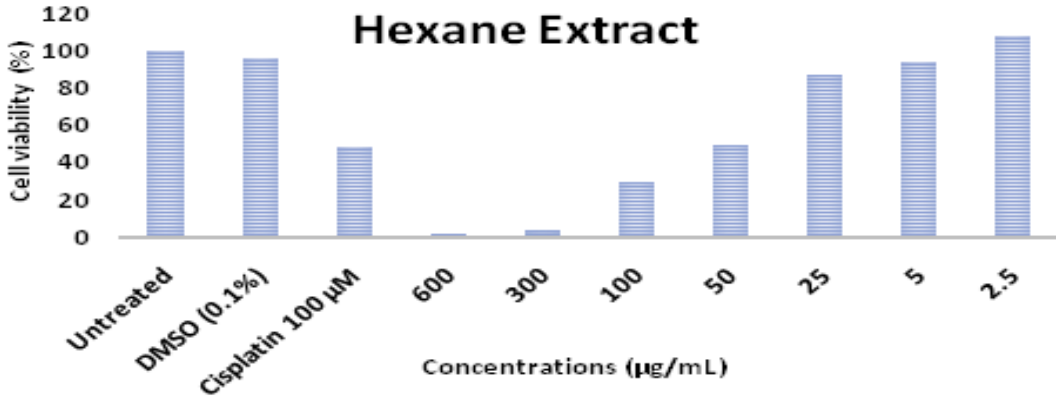

Figure 7: Cell viability of MCF10A cells at various concentrations of hexane extract for 24 h

The treatment of MCF10A cells with hexane extract (Fig. 7) showed that higher concentrations induced very high cell death with 600 µg/mL resulting in 3.45 % viable cells. Lower concentrations such as 2.5 µg/mL showed proliferative effects, and this caused a cell viability of 101.20 %. The results also revealed that higher concentrations were highly significant while lower concentrations were non-significant. DMSO had proliferative effect on MCF10A cell lines resulting in 96.08 % viable cells. Cisplatin gave 48.07 % of death cells when treated on MCF10A cell lines, which is highly significant. 50 % of the cells died at concentrations of 52.40 and 50.70 µg/mL

Conclusion

The findings from this study revealed that *Cannabis sativa* stems contained cannabinoids, and the hexane and ethanol extracts inhibit colorectal cancer cells growth. However the viability of these extracts is dependent on the concentrations of the extracts. Therefore, the extracts are promising anti-cancer agent, and their mechanism of action should be explored.

Conflict of interest: There are not conflicts of interest relating to the publication of this paper.

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