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In-silico and *in-vitro* studies of two cannabinoids of *Cannabis sativa* against prostate cancer

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ABSTRACT

The phytochemical profiling of different extracts revealed the presence of high concentration of CBD in 80:20 hydroalcoholic extract, and that of THC in 60:40 hydroalcoholic extract. The MTT assay of combination of CBD and THC highlighted the extracts as potent cytotoxic agents against prostate cancer (PC3) cells, with IC50 values of 1292 ng/ml, 953.3 ng/ml and 1134 ng/ml, respectively for HCP-CO4, HCM-CO1 & HCZ-CO1. The molecular docking study revealed a good binding of androgen receptor (PDB ID, 2am9) with CBD and THC possessing binding affinity energy of -7.1 and -7.2 kcal/mol respectively. Based on the amino acid residual interaction of CBD and THC within the 2am9 receptor, THC reported additional hydrogen bonds as compared to CBD, suggesting it to be more potent antagonist in comparison to CBD. The present study highlighted the potential of CBD and THC as a therapeutic agent for treatment of prostate cancer.

Keywords: Prostate cancer; CBD; THC; MTT assay; Molecular docking.

INTRODUCTION

Globally, prostate cancer is the second most common cause of cancer related deaths. The range of occurrence of prostate cancer increases in older men. Even though the mechanism and etiology of development of prostate cancer is still unknown, there are various factors which are linked with development of disease such as lifestyle related factors, including smoking, diet; high levels of testosterone, family history and age [1] . Numerous treatment options which are currently available for prostate cancer includes radiation therapy, cryotherapy, chemotherapy, high intensity focused ultrasound, laparoscopic prostate surgery, radical prostatectomy, endocrine treatment and androgen deprivation therapy (ADT) [2] . The initial approach for management of pain in most cases is ADT, followed by systemic chemotherapy involving docetaxel and mitoxantrone. However, these treatment options might result in toxicity and is often associated with drug resistance [3] . Therefore, there is a huge need of natural compounds for treatment of various ailments including prostate cancer.

Cannabis sativa have been in use since time immemorial for various purposes such as in medicine and textile industry. Currently, it is widely used as recreational drug possessing various impacts such as prophylactic, anti-asthmatic, analgesic, oxytocic, appetite stimulant, antidepressant, antiepileptic, hypnotic, antibiotic, tranquilizer, anticancer and topical anesthetic. It contains many secondary metabolites such as cannabinoids, flavonoids and terpenes[4] . The cannabinoids are further classified into three main groups: endocannabinoids, phyto-cannabinoids and synthetic cannabinoid. Although around 100 cannabinoids have been identified, the major ones are Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD). The major psychoactive component of cannabis sativa is THC which strongly binds to the CB1 receptors in the central nervous system exhibiting antiemetic, euphoric and analgesic properties[5,6,7] . Various literatures have been reported which demonstrate the use of THC in treatment of prostate cancer[8,9,10] . CBD, on the other hand, has low affinity towards CB1 receptors with no psychoactive properties, but instead binds to other physiological targets in the body. Due to the non-intoxicating properties of CBD, it is of great concern for the researchers. Till date, many literatures have highlighted the use of CBD as anticancer. De Petrocellis et al. 2013[9] reported the use of CBD and inhibition of prostate cancer cells via induction of apoptosis. The current study reports the cytotoxic potential of combination of CBD and THC present in varied concentration in extracts following their docking studies.

MATERIALS & METHODS

Plant Materials

The plant species Dried Cannabis (*Cannabis sativa*) leaves were sourced from Dept of Excise, Odisha and stored at Hempcann warehouse in ambient temperature till further use. (Voucher no: HCS/RM/HLHP/001). The materials (plant extract) were collected from R&D of Hempcann Solutions Pvt. Ltd., Bhubaneswar, Orissa, India. HPLC grade methanol and acetonitrile were obtained from S.D. Fine Chemical Pvt. Ltd., India. All other chemicals used in the experiment were of analytical grade. The leaves were washed, cleaned and dried at room temperature. The leaves were then pulverized and then grinded to fine powder and stored in air tight container.

Quantification of THC and CBD by HPLC

Dried extract of cannabis leaf (1mg) was dissolved in 10 ml of methanol: chloroform (9:1 v/v) by sonicating for 15 min, followed by vortex mixing for 5, 10 and 15 minutes. The extract was then centrifuged and subjected to filtration using 0.45 µm filters and was stored for further analysis. A Shimadzu HPLC system equipped with quaternary pumps, a UV/VIS detector with variable wavelength, an SPD column oven and SCL system controllers was used for analysis. Chromatographic column equipped with reverse phase C18 column (250 x 4.6 mm x5 µm) was used for analysis. The mobile phase was selected based on assay parameters such as peak separation, shape, sensitivity , the cost and the amount of time required for analysis and consists of acetonitrile: water in the ratio of 80:20. The flow rate was maintained at 1ml/min.

Assays were performed by serial dilution method using various concentrations of standard in methanol and comparison was made with blank reference solvent at the wavelength of 220 nm. By using the equation obtained from the standard plot, the concentration of each sample was calculated based on the relationship between concentration and absorbance, and R2 was calculated for each standard.

MTT Assay

MTT (3'- (4, 5 – dimethylthiazol- 2-yl)- 2,5- diphenyl tetrazolium bromide) assay was conducted in PC3 cells for evaluation of *in vitro* cytotoxic activity of CBD and THC. Prostate cancer (PC3) cells were grown in DMEM, supplemented with 1% ampicillin and 10 % fetal bovine serum. The cells were maintained at 37^0C in an incubator with 5% CO_2. Subculturing of cells in 96 well culture plates was done at a density of 2.6 x 10^6 cells/ml. The cells were then treated with different dilutions of extracts. After 24 and 48 hours of incubation, MTT solution was added to each well, followed by 4 hours of incubation and addition of DMSO for terminating the reaction. The absorbance was read against 540 nm. The formation of formazan crystals is directly proportional to the number of live cells.

Molecular Docking

The in-silico docking of CBD and THC with the androgen receptor (PDB ID: 2am9) was performed using Auto dock vina version 112, Pymol version 2.5.1, Open babel version 3.1.1, Ligplot version JDK8-U51, Discovery Studio version 20.1 and UCSF Chimera version 1.10. The calculated binding free energies were compared with acarbose molecules.

RESULTS

Macroscopic, Microscopic and physio-chemical parameter study of plant identification is caried out **(Table 1).**

Table 1: Macroscopic, microscopic, and physio-chemical parameter study of Dried Cannabis (*Cannabis sativa*) leaves

Voucher Specimen No:	HCS/RM/HLHP/001
Common name:	Cannabis, Vijaya
Genus:	*Cannabis*
Species:	*Sativa*
Family Name:	Cannabaceae
Sourced from:	Department of excise, Govt. of Odisha
Date of collection:	03.03.2020
Part of Plant:	Dried leaf
Macroscopic:	The leaves are palmately compound with linear, lanceolate and slightly acrid leaflets, growing up to 20 cm long, pointed, narrow at base. The upper surface is dark green and rough, whereas the lower surface is pale, downy, with a strong and characteristic odor.
Microscopic:	On the dorsiventral surface of leaves and bracts, the upper epidermis is composed of unicellular, pointed, curved, conical trichomes with enlarged bases containing calcium carbonate cystoliths. In the mesophyll, calcium oxalate clusters are located in many cells consisting of usually one layer of palisade cells with a spongy fabric.

Identification	Specification	Result
Foreign matter	NMT 2%	1.82%
Total Ash	NMT 15%	12.67%
Alcohol-soluble extractive	NLT 10%	62.34%
Water-soluble extractive	NLT 13%	42.30%
Image of the Dried Leaf		

The *cannabis sativa lin.* leaves were extracted with hydro-alcoholic solvent. All the extracts were further subjected to quantification of CBD and THC using HPLC. The method was developed using a suitable mobile phase of acetonitrile: methanol (80:20). Repeated analyses of analytes during experimentation and storage of solutions at laboratory conditions and in refrigerator were conducted to evaluate stability of analytes in solution during analysis. The standard plot was constructed for CBD and THC. The leaf extracts were designated with THC & CBD content. Such as High CBD &THC; High CBD & Low THC; High THC & Low CBD (Table 2) The HPLC chromatograms of all the extracts with chromatograph peak is shown in Figure 1.

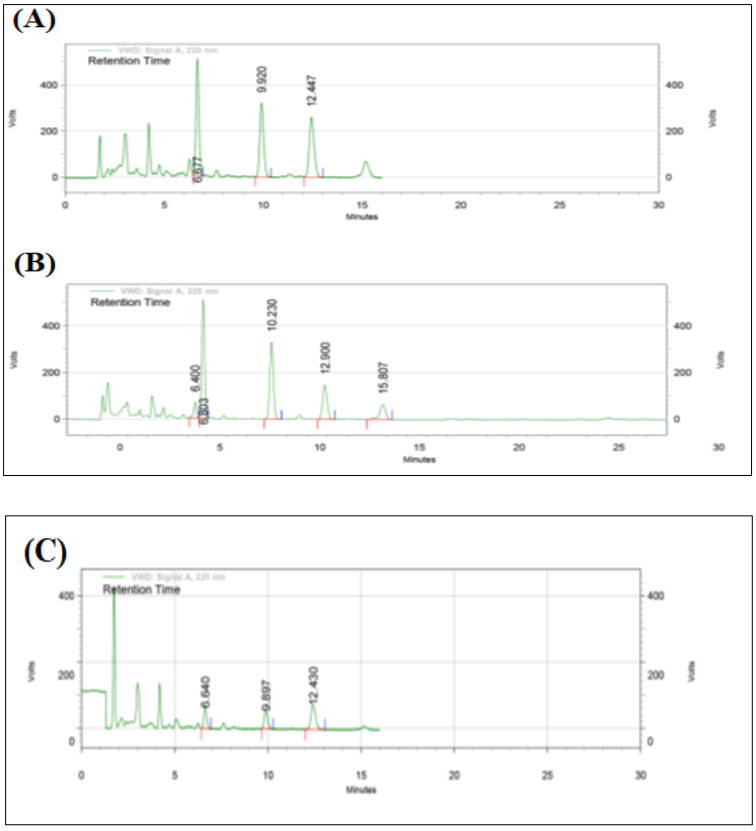

Figure 1: The HPLC chromatograms of all the extracts HCP-CO4 (Cannapain) (A), HCM-CO1(Cannaflam) (B) & HCZ-CO1 (Cannaron) (C) with chromatograph peak.

Table 2: The Leaf extracts with code and brand name as supplied by Hempcann Solutions Pvt. Ltd., Bhubaneswar, Orissa, India

No.	Extract code	Brand Name	THC & CBD Content per ml	Remark
1	HCP-CO4	Cannapain	20 mg CBD & 20 mg THC	High CBD & High THC
2	HCZ-CO1	Cannaron	10 mg CBD & 20 mg THC	Low CBD & High THC
3	HCM-CO1	Cannaflam	20 mg CBD & 10mg THC	High CBD & Low THC

To access the cytotoxic potential of THC and CBD, cell viability assay was performed using MTT assay. Different concentrations (50 ng/ml, 100 ng/ml, 200ng/ml, 400ng/ml, 800ng/ml, 1ug/ml, 2ug/ml and 5ug/ml) of samples (CBD + THC) were evaluated against PC3 cells, compared to paclitaxel (Figure 2).

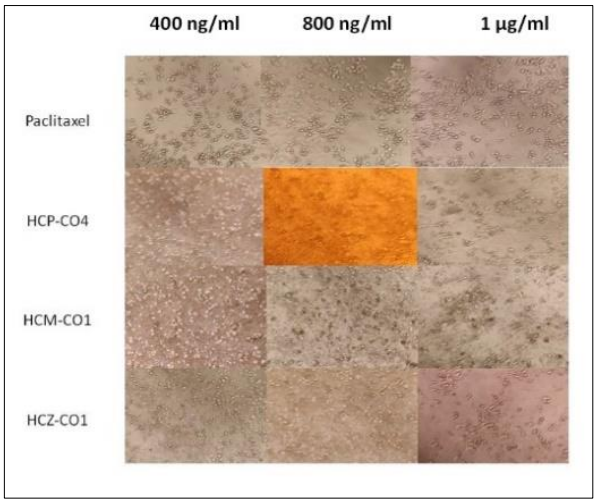

Figure 2: The cytotoxic potential of HCP-CO4, HCM-CO1, HCZ-CO1 & Paclitaxel

Results demonstrated that HCP-CO4, HCM-CO1 & HCZ-CO1 extracts had a significant *in-vitro* effect on human cancer cell line (PC3) at all concentration except 2 and 5 µg/ml. Higher concentration (5 µg/ml) of extracts were found to be too concentrated. Interestingly, all the three extracts (HCP-CO4, HCM-CO1 AND HCZ-CO1) exhibited stronger inhibition with an IC50 of 1292 ng/ml, 953.3 ng/ml and 1134 ng/ml, respectively (Figure 3). This present study confirmed the effectiveness of cytotoxic activity of *cannabis sativa lin.* leaves.

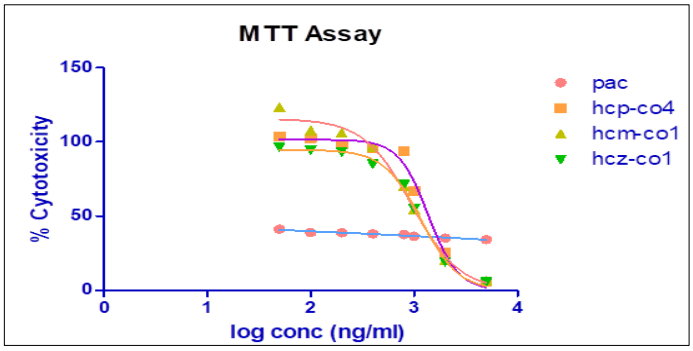

Figure 3: Cell cytotoxicity of different extracts of *cannabis sativa* with comparison to paclitaxel

The docking study was carried out to predict potential binding of CBD and THC with human androgen receptor ligand (PDB ID: 2am9). The binding affinity energy of 2am9 with CBD and THC was found to be -7.1 and -7.2 kcal/mol respectively. The residual amino acid interaction of CBD and THC shows a difference in amino acid residues of both the compounds (Figure 4). This indicates that there is a difference in the activity of both compounds. However, binding of THC with the receptor results in additional hydrogen bonds with key amino acids residues of the androgen receptors (Table 3). This result suggests that THC is more potent antagonist of androgen receptor than CBD. The combined binding of CBD and THC with 2am9 is shown in supplementary material (Figure 5). The present study suggests that CBD and THC proves to be promising candidate for treatment of prostate cancer.

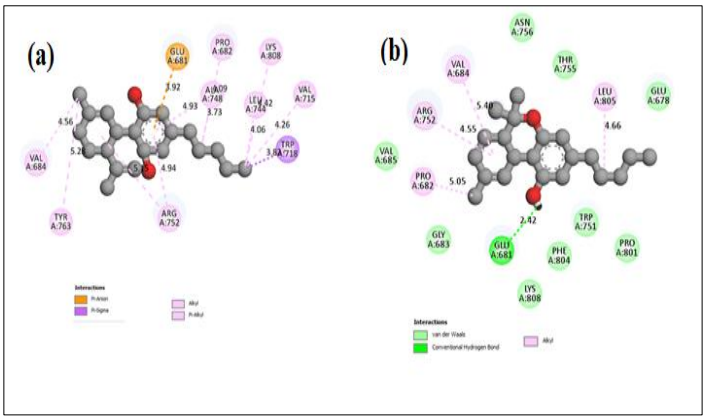

Figure 4: Residual amino acid interaction of (a) CBD with 2am9; (b) THC with 2am9

Table 3: Residual amino acid interaction of CBD and THC with 2am9

Parameter	Value	
	CBD	THC
Residual amino acid interaction	681-Glu**	678-Glu****
	682-Pro*	681-Glu****
	684-Val*	682-Pro*
	715-Val*	683-Gly****
	718-Trp***	684-Val*
	744-Leu*	685-Val****
	748-Ala*	751-Trp****
	752-Arg*	752-Arg*

	763-Tyr*	755-Thr****
	808-Lys*	756-Asn****
		801-Pro****
		804-Phe****
		805-Leu*
		808-Lys****

*Alkyl/Pi-alkyl Interaction; **Pi-anion; ***Pi-sigma; ****Hydrogen BondConclusions

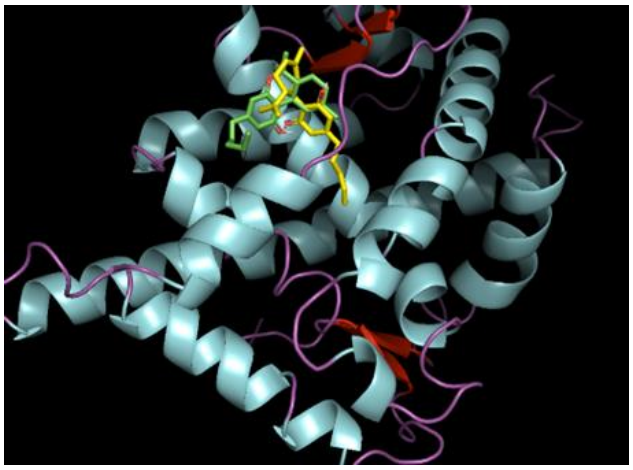

Figure 5: Combined binding pose of CBD and THC with 2am9

DISCUSSION

Researchers have extensively studied the therapeutic effects of cannabinoids on cancer cells in recent years, as well as their potential anticancer effects. In addition, they affect tumor progression and development by interfering with the components of the endocannabinoid system. Our results demonstrate that cannapain, cannaflam, and cannaron tends to inhibit the growth of PC3 prostate cancer cells in a synergistic manner. In a similar study, Scott et al. [11] used neutral forms of cannabinoids in leukemia cells to study combinations of CBD, cannabigerol (CBG), and cannabigevarin (CBGV). The study demonstrated that CBD does not act antagonistically with other cannabinoids to reduce cell number, and that cannabinoid activity is affected by drug combinations and treatment schedules. Similarly, another study suggests that the level of each compound required to affect carfilzomib was reduced when THC and CBD were used in combination, indicating potential synergistic effects of the two cannabinoids together. There has also been evidence that multiple compounds, including cannabinoids, terpenes, and flavonoids, interact synergistically with chemotherapeutics, resulting in a reduction in dosage of each agent required to produce a therapeutic effect, decreasing the number of adverse effects patients may suffer from [12]. Hence, the results of combining low CBD and high THC proves to be promising in reducing cell viability, thereby ruling out cell multiplication.

Furthermore, binding of CBD and THC with human androgen receptor was explored in this study. It was found that 2am9 had binding affinity energies of -7.1 and -7.2 kcal/mol for CBD and THC, respectively. As the residual amino acid interaction between CBD and THC shows a difference in amino acid residues, it indicates that they have different activity levels. Various molecular docking simulation studies have been performed in relation to *Cannabis sativa L*. A study confirmed the binding of eight cannabinoids having higher binding affinities to their respective protein targets for exhibiting the pharmacological effect of the disease [13]. Another study identified cannabinoids as binding partners of DLC1 RhoGAP domain in liver cancer using molecular docking calculations. The calculations indicate that cannabichromene and cannabidiolic acid have a high affinity for binding to GTPase-activating proteins. As a result of non-covalent interactions involving important amino acids, these compounds remain within the active site through hydrogen bonds, for example [14]. Although CBD has been the subject of several invitro and in-silico studies, further studies are urgently necessary to gain a complete understanding of its functions.

The anticancer properties of cannapain, cannaflam, and cannron have been shown to be attributed to their ability to shrink prostate cancer cells. Aside from THC and CBD, there are additional compounds in total cannabis extracts that must also be identified and analyzed to determine their effectiveness as anti-tumor agents. Further studies are needed to determine which cannabinoid compounds are more effective and in what combination. To quantify these results, more controlled and longer-term *in vitro* and *in vivo* studies are necessary.

The combined effect of CBD and THC inhibits the prostate cancer (PC3) cells with IC50 value of 1292, 953.3 and 1134 ng/ml respectively for HCP-CO4, HC-C01 and HCZ-CO1). The molecular docking study revealed that CBD and THC are best fitted into the receptor and shows a good binding affinity to the receptor. The present study may offer important insight for designing a therapeutic dosage for treatment of prostate cancer. Furthermore, toxicity study will be carried out to find the toxic profile of the extracts.

Conflict of interest statement

There is no conflict of interest in publishing the research work.

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