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RESEARCH ARTICLE

THE EFFECT OF CANNABIDIOL ON THE GROWTH OF TUMOURIGENIC (MCF-7) AND NON-TUMOURIGENIC (MCF-10) HUMAN BREAST CELLS

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Abstract

Breast Cancer is a crucial disease faced prominently by the female population in the United Kingdom, which reports more than 55 thousand new cases annually (GLOBOCAN, 2014). A variety of treatments have been introduced and tested in different regions of the world; however, breast cancer still causes more than 11 thousand females to lose their lives every year in the United Kingdom. This declares breast cancer as the deadliest of all known cancers among black women and seeks immediate interventions. Currently, cannabinoids treatment has been identified to play analgesic and antiemetic properties that highlight its applicability in palliative medicine. Cannabinoids indicated anticancer properties when tested in the pre-clinical setting for mice model. Cannabinoids are known to exhibit these properties by modulating signal pathways of cancer cells, causing inhibition of cancer cell proliferation, autophagy stimulation, and programmed cell death. The current study compared the possible influence of crude extract of *Cannabis sativa* and its main compound cannabidiol on tumorigenic (MCF-7) and non-tumorigenic (MCF-10) human breast cells proliferation. This study suggests *Cannabis sativa* crude extracts are more effective than cannabidiol to prevent cell proliferation and induce apoptosis in tumorigenic (MCF-7) human breast cell lines while non-cancerous MCF-10 cells survived the cannabidiol treatment, which suggested that CBD prefers to kill cancer cells over normal cells.

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Introduction:-

Cannabis sativa, commonly called marijuana, produce metabolites that are being used widely for both medicinal and recreational purposes. The active compound Cannabis that is the chemical structure of cannabinoids was realized in the 1960s. It has taken decades to deduce the molecular action of cannabinoids, but since after their therapeutic effects were suspected, intensive research has been carried out on this field relating mostly to medical oncology. Currently, the use of cannabinoids has been widely accepted for medicinal purposes due to their proven potency and abundance in cannabis preparations (Choene & Motadi, 2016).

Cannabinoids are of different forms. Tetrahydrocannabinol has pronounced biological effects. It imitates the actions of endogenous forms like 2-arachidonoylglycerol and endocannabinoids anandamide, which have definite plasma membrane cannabinoid receptors. There are major cannabinoid-specific receptors identified, CB1 and CB2, as the

characteristic of mammalian tissue clones. Orphan G protein-coupled receptor 55, transient receptor potential cation channel subfamily V, and member 1 are also identified as endocannabinoid receptors along with CB1 and CB2. Active CB1 receptor causes the effects of cannabinoids in the nerve and other tissues. While the other major cannabinoid-specific receptor, CB2, illustrates its expression in cells, especially in immune system cells (Armaniet al., 2013). Most cancer cells point out the existence of both CB1 and CB2 in them independent of the tissue of their origin. The components of the endocannabinoid system are receptors with their synthetic proteins, transport, and degradation of protein constituents. The endocannabinoid system is responsible for neuromodulation, exerting regulatory body functions, including metabolism, reproduction, immunity, among others. The applicability of cannabinoids in cancer patients results in palliative effects. In most medical applications, cannabinoids are preferred for inhibiting nausea and vomiting induced by chemotherapeutic agents. Other palliative effects of cannabinoids are inhibition of pain.

Cannabinoids have a wide therapeutic potential in oncology, which is not restricted to the above palliative actions. Numerous studies have showcased that Cannabidiol oil and other cannabinoids have anti-tumour effects, as proved by animal cancer models. The observations derived from these experiments have triggered preclinical studies to examine the anti-tumour potential of Cannabidiol.

When used in combination with other medications, CBD is non-psychoactive and may exert a negative reaction. Research has been redirected to analyze cannabinoid-induced cell death signalling mechanisms.

Statement of the Problem

Breast cancer still causes more than 11 thousand females to lose their lives every year in the UK. There are more than 55 thousand new cases of women, which are reported each year, from which more than a quarter of these women dies due to the insufficiency ineffectiveness of available treatments (GLOBOCAN, 2014). Radiation therapy, chemotherapy, and surgery are the most recommended treatments these days. Still, they are limited due to their ineffectiveness or side effects like toxicity. Therefore, it is important to analyze naturally synthesized agents that can contribute to planning a therapy affordable in cost and effective in recovery.

Objectives of the study:-

Common objective

To examine the effects of cannabinoids exposure on the development of tumourigenic (MCF-7) and non-tumourigenic (MCF-10) human breast cells

Specific objectives.

1. To measure the viability of MCF-7 cells and MCF-10 cells by MTT and LDH cytotoxicity analysis.
2. To determine the ratio of dead cells in culture.
3. To assess the ability of cannabinoids to promote caspase 7 dependent apoptosis in MCF-7 cells and MCF-10 cells.

Significance of the study.

More research is to be done on breast cancer since its endemicity has intensified. Over the past decade, the cases of breast cancer have gone viral globally, and the World Health Organization has raised concerns over this global threat. The latest advancements on the potential use of cannabidiol as an anticancer agent is a field of interest for research.

The findings from this research will boost the available knowledge of anticancer agents and may probably serve as complementary to chemotherapy. This justifies the applicability of the research.

Literature Review:-

Historical use of Cannabis:

Cannabis sativa, called marijuana, is an ancient type of plant being used among agricultural societies of Africa, Europe, and Asia. Due to the hallucinogenic effects, it causes, *cannabis Sativa* was typically used by fibre manufacturers, nutritionists, and medical professionals and in religious ceremonies. But the use of *C. Sativa* got vanished in medicine and industry in the early part of the last century. In around three centuries, many medical

research projects have shown that *C. Sativa* causes such compounds that can treat cancer and life-threatening diseases (Abrams, 2016 and Bonini et al., 2018). More than 500 biological and chemical active compounds have been identified in the *C. Sativa* plant, whereby 60 structures of the compounds belong to the family of cannabinoids (CBs) (Hanus et al., 2016). CBs have a lipid structure presenting alkyl resorcinol and monoterpene moieties known as terpenophenols (Abrams, 2016, and Nuutinen, 2018).

Medical marijuana that contains CBD together with THC and other compounds have been still used by cancer patients for many years. Marijuana was firstly legalized by California for medical use in 1996. At the federal level, it is still illegal except 33 states where it is legal for medical use, and it is also approved for use by cancer patients in every country where it is legally authorized (For comparison, medical marijuana is authorized in only 27 states for use by the patients of Crohn's disease). Further, for curing nausea and vomiting in patients receiving chemotherapy, the FDA has permitted two chemically pure drugs based on marijuana compounds.

The two CBs, i.e., delta 9-tetrahydrocannabinol (THC) and cannabidiol (CBD), are being comprehensively examined for pharmacological properties; THC in comparison to CBD is identified to exert potent psychotropic effects. THC/CBD ratio, when is taken in high concentrations, can cause the relaxing, anxiolytic, and euphoric effects to the individual while a high CBD/THC ratio exposure has a comparatively painkilling effect (Fasinu et al., 2016).

C. Sativa generates two main varieties when cultivated from different varieties and amounts of CBs, whereby the discernment from the proportion of THC/CBD classify it into three principal chemotypes. The first chemotype flower has the uppermost THC content around 18–23%, while the second one chemotype II and III flowers contain less than 0.3% THC and CBD heights around 10–12% (Jikomesand Zoorob, 2014). Because of the systematic variations observed about the CB content in plant and the comparative stability levels of CB in different experiments, there is an urgent need for the improved standardization of CB analysis.

Potential benefits of CBD for cancer patients are less studied, but still, there are some studies conducted on it. Research conducted by National Cancer Institute indicates that slowing the growth or reducing the risk of relapsing certain cancers, boosting the potency of different medications, and reducing the side effects of chemotherapy include vomiting, anxiety, and nausea can be achieved by CBD. However, these studies are limited; further research is needed to comprehend how CBD affects humans.

CBs have the ability to prevent inflammation and obstruct cancer cell proliferation. This ability has pushed for synthetic and plant-based CBs to be explored for their uses as antitumor drugs. Many reports have revealed that CBs have different properties of blocking or activating CB receptors (CB-Rs), showing receptor role for CBs in tumour cells and which may be beneficial for the treatment of cancer (Chakravarti et al., 2014 and Velasco et al., 2016).

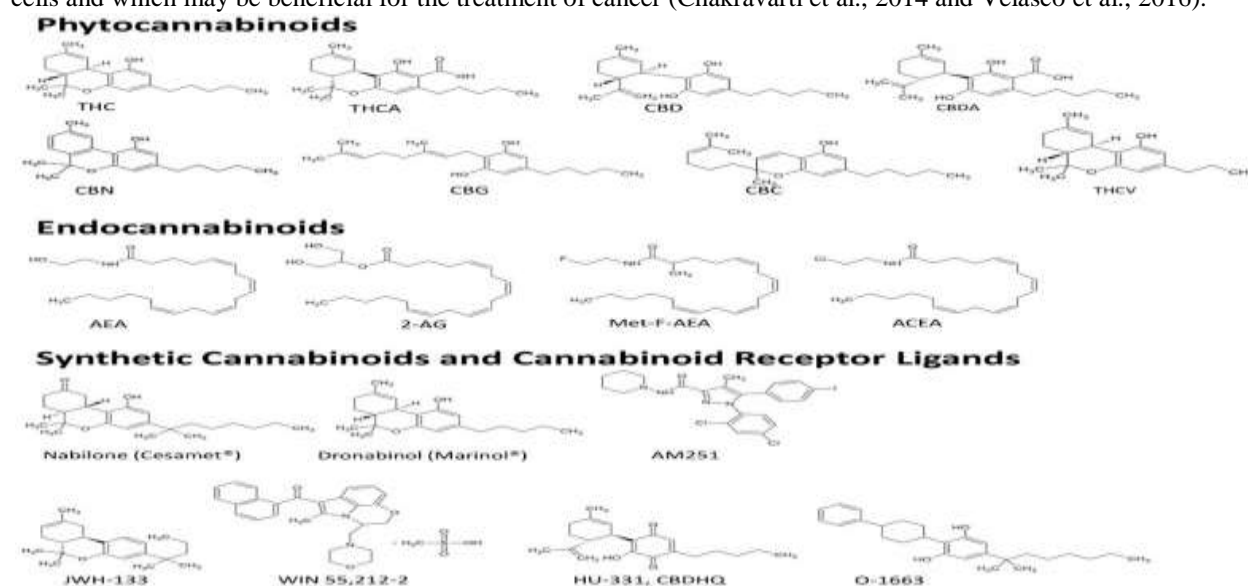


Figure 1:- Structure of CBD compounds.

Mechanism of cannabinoid action

Endocannabinoids are described as a complete signalling system having CB-receptors, along with their endogenous ligands (lipid signalling molecules), precursor molecules, biochemical machinery, enzymes crucial for its synthesis, degradation and transporter proteins. For example, the heat shock protein and protein with fatty acid affinity were originated after the detection of THC membrane receptors and their endogenous ligands (Zou and Kumar, 2016). A lot of endocannabinoid molecules are known to be having a parallel structure. They are natural ligands of CB1-R and CB2-R (the two CB-Rs) that produce a collective number of diseases. Synthetic CBs, along with Plant-derived CBs (Phyto-CBs, phytocannabinoids) interrupts the endocannabinoid system, and certain pharmacological effects of Phyto-CBs (Leyva-Illades and Demorrow, 2013).

arachidonoylglycerol (2-AG) and anandamide (N-arachidonylethanolamine; AEA) are part of the endocannabinoid system as described earlier. The elevation of intracellular calcium levels signals the activation and synthesis of both CB-Rs. AEA was the most initial endocannabinoid identified, and it occurs by N-acyltransferase (NAT) stimulation, conveys a functional acyl group to the membrane phospholipid. So, N-acyl-phosphatidylethanolamine (NAPE) is synthesized. The NAPE-specific phospholipase D produces AEA from NAPE. Sequential hydrolyzes of inositol phospholipids are involved in the biosynthesis of 2-AG through diacylglycerol (DAG). 2-AG and AEA are responsible for performing homeostasis when demanded by the cells (Zou and Kumar, 2016). Both 2-AG and AEA have a little half-life and are immediately cleaved by monoacylglycerol lipase and fatty acid amide hydrolase, respectively. It results in the formation of glycerol and AA from 2-AG and arachidonic acid from AEA. Endocannabinoids cause inhibition of the release of neurotransmitters by retrograde synaptic signalling in the nervous system that permits activation of pre-synaptic CB1-R by crossing the synaptic cleft.

Many physiological functions associated with memory, cellular metabolism, behaviour, temper, reward systems, energy regulation, and addiction have endocannabinoid signalling molecules. These compounds are formed on-demand and degraded immediately after formation due to a little half-life span. Drugs (e.g., inhibitors MAGL of FAAH) that can influence the endocannabinoid system were formulated to treat neuropathic pain and diseases in cancer patients. Although its treatment is practically not safe due to its incidence with phase I in a clinical trial with FAAH inhibitor (Mallet et al., 2016).

CB-Rs (CB1-R and CB2-R) and G-protein coupled receptors (GPRs) exist in the endocannabinoid's mechanism of action. 2-AG is a strong CB1-R agonist, while AEA is partly an agonist of CB1-R and a weak CB2-R agonist. Both CB1-R and CB2-R are members of seven-transmembrane-spanning receptor superfamily. Specialized distribution of CB1-R and CB2-R over the tissue cells allows them to perform specific effects of receptor activation. CB1-R is significantly present in brain areas that perform activities related to anxiety, cognitive functions, pain, visceral perception, memory, motor and sensory coordination, and in endocrine functions. While there is a decreased expression level of CB1-R in the heart, peripheral nervous system, prostate, bone marrow, testicles, small intestine, vascular endothelium, and uterus. Inhibition of forskolin-stimulated adenylyl cyclase is carried out by the activation of CB1-R. It occurs through activation of a pertussis toxin-sensitive G-protein, to inhibit N-, Q- and P-type calcium channels, and activate corrective potassium channels (Grimaldi et al., 2006).

Cells of the immune system, spleen, endothelium, pancreas, lower in glial cells, heart, liver, tonsil, and bones have higher levels of CB2-R. The brain is also found to have a relevant expression of CB2-Rs in various functions (Salort et al., 2017).

Intracellular CB2-R dependent signalling pathways include stimulation of mitogen-activated protein kinase (MAPK), Gi/o-dependent inhibition of adenylyl cyclase, cyclooxygenase-2 (COX-2) signalling, phosphoinositide 3-kinase (PI3K), and activation of de novo ceramide synthesis. Both CB1-R and CB2-R are highly detected in different cancerous cells; CB2-R performs an important role in cancer progression and carcinogenesis. But the exact mechanism of action of CB2-R in cancer progression is still not completely explicated, and now it is a target for emerging therapeutic treatment of cancer (McKallip, Nagarkatti & Nagarkatti, 2005).

Activation and inhibition of different signalling pathways (Figure 2) are mediated by CB-Rs activation at a molecular level that promotes signals of Phyto, endo, and synthetic CBs (Figure 1). The formation of ceramide with rate-limiting enzyme (palmitoyl-transferase) is linked to being stimulated by the transduction pathways regulated by CB-R. Proto-oncogene serine/threonine-protein kinase (RAF1) activation occurs as an effect of prolonged ceramide treatment in cancer patients that in turn causes initiation of apoptosis and activation of p42/p44 MAPK,

demonstrated in the glioma cell line. CB-R agonists, including the endocannabinoids (AEA and 2-AG), THC (by the synthetic CB WIN55,212-2), can block this activation. But the activation of p42/p44 MAPK is short in span and very critical for apoptotic properties due to the shielding features of CBs against the induction of apoptosis by ceramide (Javid et al., 2016).

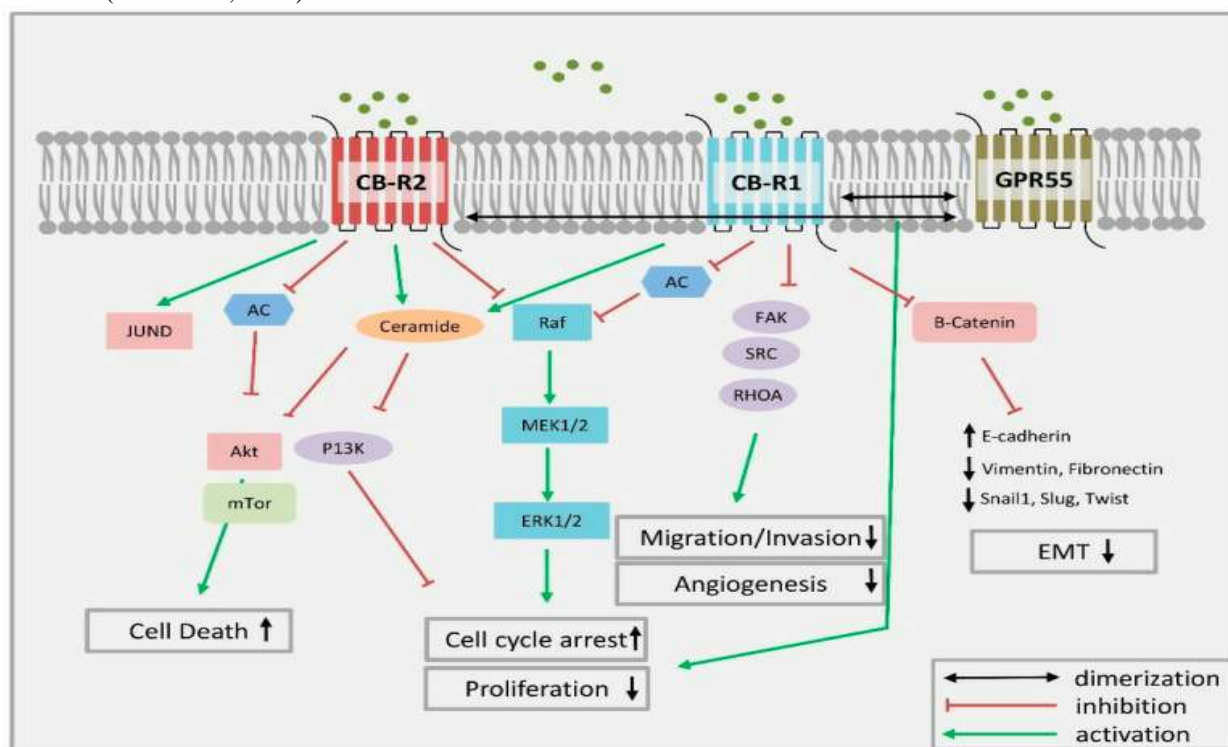


Figure 2:- Mechanism of CB-R-mediated antitumor activity in breast cancer cells.

Different mechanisms block the growth of breast cancer cells when CBs attaches to the receptors, CB1-R, and CB2-R. Cell cycle proceeding is stopped by the activation of CB1-R and CB2-R, which inhibits the cell cycle at two phases, the G1/S and G2/M, respectively. CBs cause the activation of transcription factor jun-D, which causes the destruction of breast cancer cells by apoptosis. They cause inhibition of ERK and Akt signalling, which inhibit cancer cell multiplication in tumours and cultures in HER2-overexpressing breast cancer cells. Angiogenesis and cell migration are also inhibited by them via CB2-R. Inhibition of cell migration is led by blocking the FAK/SRC/RhoA pathway via the activation of CB1-R (Preet et al., 2008). CB2-R activation also causes blocking of cell migration by inhibiting COX-2 and ERK signalling, which is crucial for triple-negative breast cancer. Akt: protein kinase B; MAPK: mitogen-activated protein kinase; GPR: G-protein coupled receptor; SRC: proto-oncogene tyrosine-protein kinase Src; AC: adenylate cyclase; PI3K: Phosphoinositol-3-kinase; ERK: extracellular-signal-regulated kinase; FAK: focal adhesion kinase; RhoA: transforming protein RhoA; HER2: human epidermal growth factor receptor 2; CB-R: cannabinoid receptor; Raf: serine/threonine-protein kinase; EMT: epithelial-mesenchymal transition; mTOR: mammalian target of rapamycin; COX-2: cyclooxygenase-2 (Wright et al., 2016).

Other receptors like GPRs, GPR55, GPR18, and GPR119 can also be bound and activated by CBs. Both endocannabinoids and lysophospholipid 2-AG and AEA activate GPR55, which is a particular receptor of interest. Rho-associated protein kinase (Rock), p38 MAPK, phospholipase C (PLC), extracellular-signal-regulated kinase (ERK), transforming protein RhoA (RhoA) are the main targets of GPR55 (Leyva-Illades and Demorrow, 2013). GPRs (GPR55) and CB-Rs make heterodimers that affect the functions of both the receptors subsequently. CBs also activates other GPRs like 2-alpha adrenoreceptors and acetylcholine receptors as well as angiotensin-, adenosine-, opioid-, prostanoid-, melatonin-, 5-hydroxytryptophan-, dopamine- and tachykinin receptors. Peroxisome proliferator-activated receptors (PPARs) α and γ are also considered as the endocannabinoid receptors (Pertwee et al., 2010).

Cannabinoids from *Cannabis sativa*

Cannabidiol (CBD)

The plant-CBs mainly used in industry are CBs with its precursor Cannabidiol acid (CBDA). CB1-R and CB2-R activity are altered by CBD, which acts as a negative allosteric modulator (Casajuana et al., 2018). They exhibit their pharmacological effects when they bind to each other GPRs and receptors. For example, several GPRs in neuronal cells are thought to confer the effects of CBD like antispasmodic, antiemetic, anticonvulsant, anxiolytic, and neuroprotective effects. CBD shows antagonistic effects to THC and partly agonist effect for GPR55 and GPR18 (Vara et al., 2011)

Pharmacokinetic properties of CBs greatly depend on the route of exposure; however, intersubjective and high internal variability is common in humans. Animal studies, particularly in rodents and dogs' model, a high amount of CBD dose is excreted in intact form or in its glucuronidated form. Hydroxylated 7-carbonyl CBD derivatives are the amplest metabolites which get excreted in unbound or in the form of glucuronides in the urine. Along with metabolites, lipid-soluble CBs are present in blood and urine and can be reserved for several weeks in fat tissues. Usually, CB and its derivatives can be observed in the urine between 60 minutes with high concentration for less than or equal to 4 hours (Sailler et al., 2014).

The 7-carbonyl metabolites are known to promote anti-inflammatory properties in mice models. While in vitro, studies also show reduced nitric oxide (NO) formation and prevention of reactive oxygen species (ROS) production. They are capable of inhibiting the formation of tumour necrosis factor (TNF)- α and other pro-inflammatory cytokines and transcription factors such as nuclear factor (NF)- κ B and interleukin (IL)-1 β , IL-2, IL-6, IL-8. The effects of these metabolites are comparable to that of CBD. The metabolism of AA to leukotriene B4 via 5-lipoxygenase is also known to be inhibited by CBD when it plays its anti-inflammatory properties. Along with the role in reducing the formation of nitric oxide (NO) and reactive oxygen species (ROS) in various cell lines and animal models of inflammation, CBD alternatively also reports to cause induction of ROS formation particularly in cancer cells, which leads to the cytotoxicity of these cells (Shrivastava et al., 2011).

Delta-9-tetrahydrocannabinol (THC)

THC is the main psychotropic component of *Cannabis Sativa*, which is partly an agonist of CB1-R and CB2-R. So, the signalling efficiency of CB-Rs and CB-R expression level initiate its effects when combined with the release of endogenous CBs (Moela et al., 2013 and Scott et al., 2014). One of the psychotropic effects which are seen most frequently is Euphoria, which causes dysphoric reactions like panic reactions and anxiety, and sometimes paranoia is observed. The exposure route also affects the kinetic absorption of THC as other CBs. For example, THC exposure from the nasal cavity that is inhalation causes distribution rapidly in the bloodstream, with usually a peak level observed near 2-10 minutes. Within 30 minutes, the concentration drops rapidly, and 11-hydroxy metabolite formation stops. The half-life of THC is between 20-30 hours, while THC achieves its peak level after 2-4 hours of oral consumption. The bioavailability of orally administered dose of highly lipophilic THC and other CBs is low and variable between 6-20%. THC is primarily metabolized to many inactive hydroxylated metabolites by the hepatic cytochrome p450 system. 11-hydroxy-delta-9-tetrahydrocannabinol (11-OH-THC), a potent psychoactive activity promotor, is the main active metabolite of THC. Further metabolism mostly generates inactive metabolites, which can be identified in urine like 11-nor-delta-9-tetrahydrocannabinol-carboxylic acid. Excretion of THC is crucial and indicates various symptoms like its presence in urine points exposure to THC within the last three days. But increased exposure to THC leads to the chronicity of its use, which may prolong its excretion time in faeces and urine from normal hours to days (McAllister et al., 2011).

Acute toxicity of CBs is uncommon in adults, while THC usually causes toxic effects. If 5-20 mg THC is taken orally, and 2-3 mg dose of THC is inhaled, it can result in reduced memory and attention along with common symptoms of conjunctivitis and executive functioning. Delirium, ataxia, hypotension, anxiety, respiratory depression, and panic symptoms can result when even higher doses (5-300mg) of THC are taken orally in adults and pediatric patients. Prolonged application of THC can also cause compromised attention and memory as well as an inability to interpret complex information. Neurological abnormalities like hyperkinesia and lethargy can be the symptoms of severe toxicity of THC in children. Unborn and newly born babies also get toxic effects when a pregnant and breast-feeding woman has significant exposure to THC by any route. THC can cross the placental barrier as well pass into the breast milk if a mother consumes THC while pregnant or breast-feeding (Hegde et al., 2015).

The central nervous system is affected primarily when it comes to the physiological effects of THC. Trouble in the release of dopamine and gamma-aminobutyric acid/glutamatergic neurotransmission system occurs by THC, which activates CB1-R. The psychotropic effects of THC are determined by the signalling efficiency and expression level of CB1-R.

THC antiproliferative activity in tumour cells affects various signalling mechanisms, which are caused by the activation of CB-Rs (Figure 2). Mechanism of action is the activation of CB2-R, which leads to the down-regulation of cell division control 2 (Cdc2) and arrest of the cell cycle at the G2/M phase, impairing the cell cycle progression. CB2-R causes the activation of transcription factor jun-D, which prevents cell proliferation and induce the death of cancer cells by apoptosis (Munson et al., 2011).

The induction of PPAR γ -regulated pathways in tumour cells occurs by the activation of CB2-R. Through this channel, CBs causes the expression of intercellular adhesion molecule 1. Cancer cell lysis results as this process enhance the adhesion of cancer cells to lymphokine-activated killer cells.

Tumour-promoting GPR55 was observed to be antagonized by THC at both within the CB2-R-GPR55 heterodimers and at the single receptor level. ERK-1/2 pathways and cyclic adenosine monophosphate (cAMP) are modulated by GP55 and CB2-R heterodimers that, in turn, affect the tumour growth (Pellati et al., 2018).

Role of Cannabidiol in cancer

It is suggested that CBD oil can be used in the treatment of cancer because of several benefits associated with it. But it would be too early to predict anything about CBD for treating cancer. But it is sure that it is helpful in dealing with the symptoms of cancer and also the effects of treatment therapies used for cancer (Nahtigal et al., 2016). The majority of evidence shows that cannabis can prove helpful in complementing the treatment of cancer. Here we will discuss the role of Cannabidiol oil in the treatment of breast cancer and how it affects cancerous and non-cancerous human breast cells.

Effect on MCF-7 and MCF-10

MCF-7 and MCF-10 are two cell lines of human breasts, which are tumourigenic and non-tumourigenic, respectively. According to research made by Whiting et al. (2015), cannabidiol compounds are receptors protein in nature that can modify cancer cells MCF-7, their growth, and signal pathways. But the mode of action of cannabidiol molecule is still unclear. The interest to understand the concept of CBD cellular growth inhibitory pathways such as apoptosis and signal pathway shut down in relation to human breast cancer and non-cancer cells, MCF-7 and MCF-10, respectively (Solinas et al., 2012).

In an article by Fasinu et al. (2016), it was explained that CBDs could inhibit cell cycle progression, their growth and also induce programmed cell death of cancer cells through blocking active pro-oncogenic signal transduction mechanisms, such as ECSRP (extracellular-signalling regulated kinase pathway). That, in animal models for breast cancer cells, CBDs have shown to reduce tumour metastases and angiogenesis.

Methodology:-

Various methodologies studied in the literature review are summarised as follows:

For MCF-7 cells

Cell Culture Techniques.

In this research, cannabidiol oil and paclitaxel will be used as the inhibitory agents, with the latter acting as the positive control chemo-agent. The essays explain the cannabidiol oil model, but a similar procedure is applied with paclitaxel.

Cytotoxicity Assays.

Human breast cell lines ATCCMCF-7 were cultivated in High Glucose-DMEM (Millipore-Sigma) comprising 10% fetal bovine serum (FBS) with 100 mg/mL streptomycin 100 U/mL penicillin at specific conditions of 37⁰C and 5% CO₂ atmospheric concentration. The ATCC cell lines, MCF-7, were tested for purity, mycoplasma presence, and contaminants before confirmed for assaying. The ATCC cell lines underwent expansion on delivery with numerous vials of cells subjected to liquid nitrogen preservation. Tocris Bioscience was the source of cannabidiol oil dissolved

in ethanol, while bafilomycin was obtained from Sigma. Capsazepine was purchased from EMD Chemicals(Lu & Anderson, 2017).

MTT and LDH assay.

Prior to the addition of drugs, MCF-7 were planted at 20,000 cells/well in 96-well plates and proliferated for a period of 24 hours. Cannabidiol oil treatment was given to cells in the range of 0–10 μ M with DMSO as the control for 48 hours. The cell viability of the incubated cells for 3 hours at a temperature of 37°C was assessed by using MTT. MTT solvent was then added. Biotek Cytation 3 was used to determine the cellular absorption at 590nm. Cell viability was measured as the quantity of purple formazan. The obtained data of viable cells contrasted with control-treated cells were presented in percentages referenced as 100%.

The percentage cytotoxicity of cells was determined using the following formula:

$$\text{Percentage cytotoxicity} = \frac{\text{Test Sample} - \text{Low Control}}{\text{High Control} - \text{Low Control}} \times 100$$

Cell Proliferation Assay.

Promega, a Cell Proliferation Assay Kit, was taken to analyze the viability of cells according to the instructions of the manufacturer provided. MCF-7 lines were given the treatment of specific concentrations of cannabidiol oil in serum-free DMEM. The incubation of these cells with MTS solution was done after 24 hours, and after that, its absorbance was checked at 490nm. The cells were given pre-treatment of capsazepine for one hour before being incubated with cannabidiol oil to analyze the inhibition factors on cannabidiol treatment (McAllister, Soroceanu & Desprez, 2015).

Apoptosis Assay.

24 hours treatment of abnormal cannabidiol concentrations in DMSO was given to the cell lines grown on well-plated glass coverslips. Annexin (V) apoptosis detection kit was used for the measurement of the apoptosis rate. The assay was passed out as per the manufacturer instructions, which included the harvesting of the cell, washing with PBS followed by its absorption in Annexin (V) assay buffer. Then cells were shaken gradually in Annexin (V)-fluorescein isothiocyanate-conjugated stain in the dark for 30 minutes. The fluorescence microscopy cells were observed by using an Olympus IX81 microscope from different angles. PI excitement and images were captured at 535nm and 617nm, respectively. Following formula was used to measure the rate of early apoptosis (Singer et al., 2015 and Salazar et al., 2013):

$$\text{Rate of apoptosis} = \frac{\text{Number of positively stained Annexin (V) cells.}}{\text{The total number of cells.}}$$

For MCF-10 cells

The methodology involves the extraction of cannabidiol extraction and its effect on the cultures of MCF-10 cells of human breasts, which are non-tumourigenic using different assays and techniques.

Extraction:

The extraction of the cannabidiol oil was done according to the protocol used by Apeks. Two distillation methods were used for the extraction of cannabidiol oil from cannabis, which was short path distillation and winterization.

Cell cultures preparation:

It involved the culture of ATCC MCF-10 in media with selected supplements named Dulbecco Modified Eagle Medium (DMEM). Media contained 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% fetal bovine serum. Environmental conditions given were 5% CO₂ and 37C temperature. Purity, contaminants, and presence of mycoplasma of ATCC cell lines, MCF-7 and MCF-10, was tested before assaying (Pertwee, 2012). For examining the inhibition behaviour of CBD oil capsazepine was integrated. Different assays were used on these cultures to understand the effect of cannabidiol oil.

MTT Cytotoxicity assay

Normal MCF-10 cells were planted at 20,000 cells/well in 96-well plates and were allowed to proliferate before adding cannabidiol oil. With DMSO in range of 0–10 μ M cannabidiol oil cells were treated. It was a control medium for 48 hours. The incubation of cells for 3 hours at 37C was done, followed by the addition of MTT solvent. Using

Biotek Cytation 3 absorption of cells was checked at 590nm. Normal MCF-10 cell's viability was measured as the quantity of purple formazan(Street&Prinsloo, 2013).

Live/dead staining – fluorescent microscope (FDA):

Cell Proliferation Assay kit was used to determine the viability of MCF-10 cells as per kit instructions. Treatment of cells with CBD concentrations (0-10 μ L) is done in serum-free DMEM. Then these cells were given pretreatment with capsazepine prior to incubation. After incubation, the staining of live and dead cells was carried out with suitable reagents. Dead cells presented red-orange fluorescence due to compromised plasma membranes, while live cells presented green colour.

Results:-

Cell Viability.

Paclitaxel is one of the chemotherapeutics commonly used because during migration and cell replication has a strong effect on cytoskeletal arrangement. PR MCF-7 cell lines and paclitaxel -sensitive were used for identifying whether against breast tumour and chemotherapeutic –resistant breast tumour cell lines cannabidiol could be effective. In our studies, two atypical cannabinoids were used due to their importance in certain cancer types. These cannabinoids can mediate their effect through GPR18 and GPR55. We were able to verify cell lines we used in our studies were resistant or sensitive to paclitaxel (470nM). At this concentration, paclitaxel was able to kill nonresistant cells while leaving the resistant cell line was not affected in terms of viability (Figure2A). As suggested by different reports, the cannabinoids can kill cancer cells at the concentration that has no effect on noncancer cell lines. This study was also capable of assessing possible effects of different amounts of Abn-CBD and O-1602 alone, ranging between 0.1 and 10 μ M and along with 470nM paclitaxel for about 2 days (48 hours) on paclitaxel non-resistant breast cancer cell lines. The figure shows the dependent effect of 2C-F in a varying amount of Abn-CBD and O-1602. Paclitaxel could not induce a synergistic effect but was able to induce cell death when added to these cells. The cannabinoid's effect was observed in Pharmacology once Abn-CBD were added concurrently on Taxol-Resistant Breast Cancer Frontiers. Further, we continued to assess the O-1602 effect on the same concentrations alone, as well as 470nM paclitaxel for around 48 hours on cell lines resilient to paclitaxel (Figure 3). No substantial difference was observed between O-1602 curves with or without paclitaxel. Therefore, the response gained from Abn-CBD 0.1 to 10 μ M alone and together with paclitaxel was parallel(Happyana et al., 2013).

The inconsistency of MCF-7 cell lines resilient to paclitaxel stimulated towards the greater effect once shared with paclitaxel at Abn-CBD's low concentration; however, the effect was reduced at Abn-CBD's higher concentrations. The cannabinoids effect was also assessed on the cell line of triple-negative MDA-MB231(Kramer, 2015).

At several concentrations of O-1602 from 0.1 to 10 μ M (Figure3) with and without combining it with 470nM paclitaxel, cells were nurtured. The feasibility of paclitaxel-resistant cells was decreased by O-1602 while the effect was slightly increased without having significant change once paclitaxel was added in this treatment.

In this case, O-1602 decreased viability of paclitaxel-resistant cells, and when paclitaxel was added during treatment, it increased the effect slightly but did not change significantly. From the results, it was suggested, breast tumour cells resistant to paclitaxel were susceptible to the antitumor effect of cannabinoids.

On the feasibility of MDA-MB231 cancerous cells and non-tumorigenic MCF-10 cells, the effects of CBD were also compared. CBD decreased the viability of both cells, but significantly more MCF-10 survived when treated with CBD(Ellert-Miklaszewska, Kaminska & Konarska, 2005).

Receptor Dependency

On the next step, the target responsible for Abn-CBD and O-1602 cytotoxic effects was identified. Two receptors of G protein-coupled, GPR55 and GPR18, are triggered by the association of these cannabinoids. Thus, paclitaxel-resistant MDA-MB-231 was transfected in determining the effect on cannabinoids activity with the help of GPR18 siRNAs and GPR55. Expression of receptors was reduced strongly by siRNA, although for GPR18, this effect was not complete. Cells expressing siRNA were treated with 2 μ M of either Abn-CBD or O-1602 for about 48 hours, and an assay for cell viability was performed on them. Form this assay, then it was confirmed, paclitaxel-resistant MDA-MB-231 cell's feasibility was decreased by 50% in the presence of control scrambled siRNA (Figure 4A). The effects of drugs were blocked partially when either GPR18 siRNAs or GPR55 were expressed alone. In the presence

of siRNAs effects of Abn-CBD and O-1602 were abolished completely for both targets simultaneously. Paclitaxel-resistant MCF-7 cells also showed similar results (Figure 4B).

Apoptosis Assay.

In this essay, it was determined that if cell death was induced through apoptosis using PI and annexin V by various drug treatments. PI was used to stain about 5% of cells during all drugs treatment. In Figure 5A, Abn-CBD or 2 μ M O-1602 treatment's effect on MDA-MB-231 and MCF-7 of paclitaxel-resistant cells tagging annexin V for 24 Hrs. are revealed. Following, vehicle control DMSO about 10% of cells showed positive results for annexin V.; On the other hand, for annexin V, positive results were shown by 40% MDA-MB-231 cells. With regards to Abn-CBD and O-1602, about 25%-30% of MCF-7 cells were labelled. In figure 5B, the treatment of MDA-MB-231 for 24 hrs. With O-1602 or Abn-CBD at 2 μ M slashed proapoptotic protein caspase 3. In several cell lines and involve AMPK, ROS and autophagy, the antitumor activity of cannabinoids have been shown. As demonstrated in Figure 5C, Abn-CBD, and 1602 to a small extent, could induce the production of ROS in breast cancer cell lines resilient to paclitaxel(Gumulec, Balvan, Sztalmachova& Raudeska,2013).

In Vitro Migration and Invasion.

Some important features are associated with triple-negative breast cancer cells. Their aggressiveness was shown with such features of cells regarding migration. For assessing the Abn-CBD and O-1602 effects on cell line migratory properties, we used MDA-MB-231 paclitaxel-resistant cells a performed Transwell assay. As a potent cell migration inducer, the epidermal growth factor was used at 10 ng/ml. In the occurrence of DMSO and several concentrations of Abn-CBD or O-1602 cells were treated with EGF for about six hours. It was indicated in results that the concentration-dependent effect was shown for inhibiting migration by Abn-CBD and O-1602 in comparison to control (Figure 6A). When treated with 100nM of O-1602, a 22% decrease was observed, with 500nM, 49% decrease, and with 1 μ M, 88% decrease was observed. And in the case of Abn-CBD at 100nM, 500nM, and 1 μ M, 14%, 41%, and 80% decrease was observed, respectively. In comparison to vehicle control DMSO, during invasion assay, Abn-CBD and O-1602 decreased invasion level when paclitaxel-resistant MDA-MB-231 cells were used(Elbaz et al., 2015).

In Vivo Toxicity.

Teratogenicity also called as each cannabinoids' developmental toxicity, was also assessed in this research. It was done by exposing the zebrafish embryo to either Abn-CBD or O-1602 between 48 and 120 hpf at various concentrations. Whereby the result showed, the associated test of phenotypic abnormalities with each drug was seeming in a concentration-dependent manner. These abnormalities included a truncated tail, malformations, and developmental delay. For comparing toxicity levels between every drug, EC50 values were determined. For this purpose, the percentage of larvae showing different phenotypic abnormality was measured. With the use of current results, a curve of concentration-response was generated built on each measured concentrations' percentage. Concentrations greater than 2.5 μ M from the graph showed higher toxicity levels to the larvae (Figure 7)(Grimaldi et al., 2006).

In Vivo Cell Viability.

Into yolk sac of zebrafish larvae, Cells labelled with Cm-Dil were injected at 48 hpf, for assessing whether atypical cannabinoids had antitumor effects in vivo or not. In this research, it was shown that the feasibility of inoculated paclitaxel-resistant MDA-MB-231 human cell line reduced when freshwater larvae were nurtured, having 2 μ M of Abn-CBD and O-1602 (Figure 8). The cells' proliferation in the yolk sac and the further body areas in zebrafish was increased permitted by control larvae. (In Figure 8A it is evident as white spots, quantification is shown in Figure 8B); the existence of inoculated cancer cells in larvae of zebrafish was reduced significantly via 50% approximately due to atypical cannabinoids(Carracedo et al., 2006).

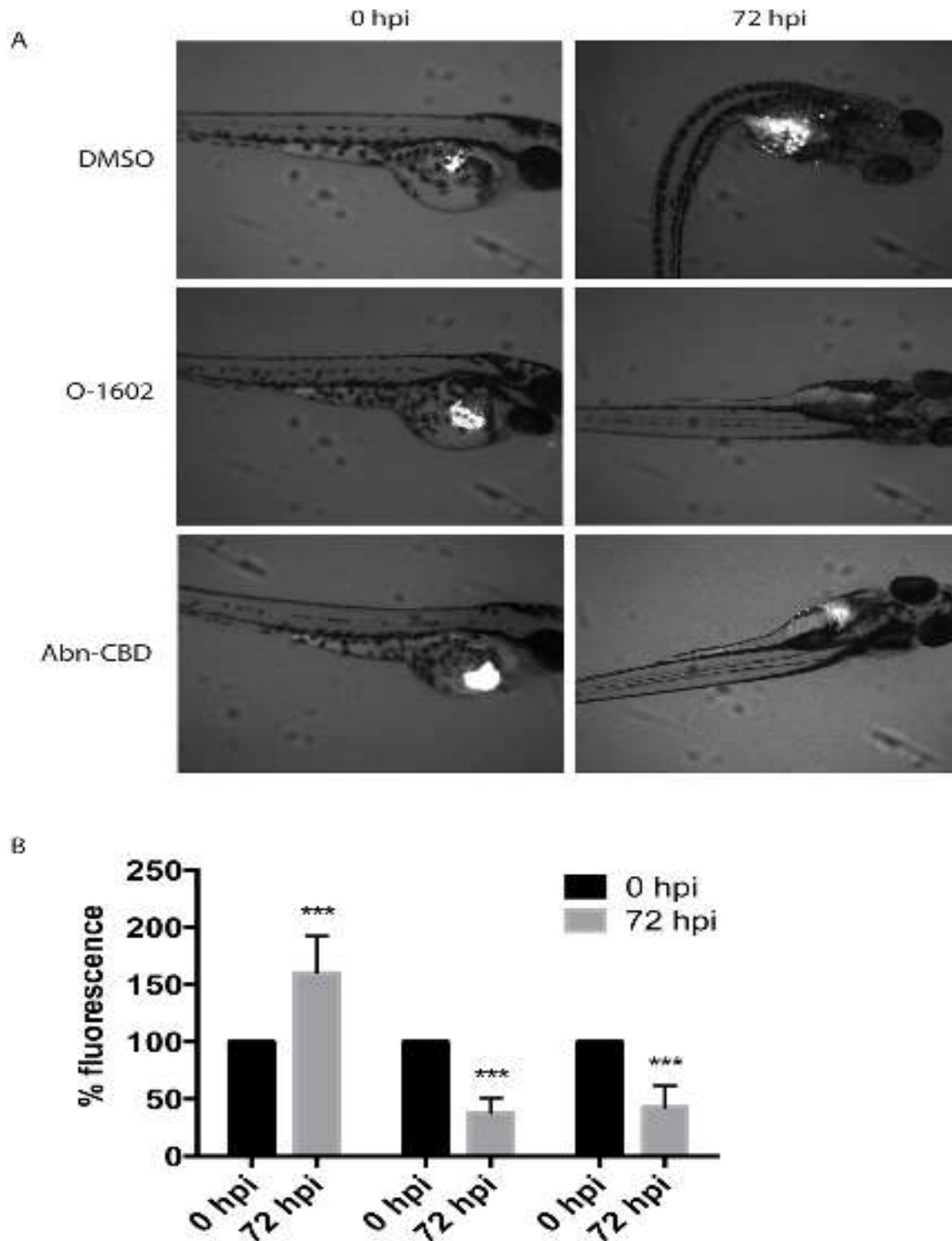


Figure 8:- Inhibition effect of cannabinoids on the ability of paclitaxel-resistant breast cancer cells in vivo.

Discussion And Implications:-

At various concentrations, cancer cells can be killed by Cannabidiol oils without affecting the non-cancer cell lines. The effects ranging from 0.1 to 10 μ M of various concentrations of CBD on breast cancer and normal cells that were not resilient to paclitaxel was also determined by the current study (figure below shows the concentration-dependent effect of CBD). Paclitaxel encourages the death of cells when it is added to these cells; however, when paclitaxel and CBD were simultaneously added, no synergistic effect was observed.

- A. A change in expression levels for G protein-coupled receptors (fix the cannabinoid) has been connected with tumour progression and metastasis. Receptors, e.g., GPR55 expression with intense prognosis is being observed in MCF-7 cell lines as well as in tumours (Andradas et al., 2016). Cholangiocarcinoma model show anandamide (AEA), an endocannabinoid which reticent the growth of cholangiocarcinoma growth by inductive apoptosis. (Velasco et al., 2012).

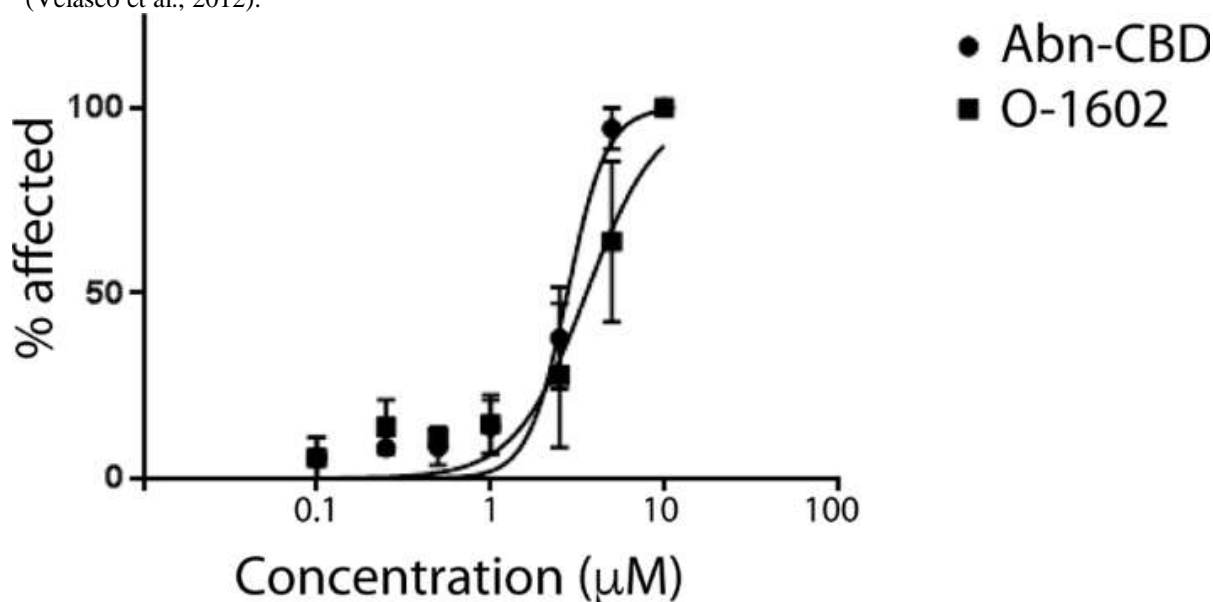


Figure 7.0:- The percentage of cells inhibited by cannabidiol oil in relation to increasing concentration.

As revealed in the studies of in vitro and in vivo, the development of cholangiocarcinoma cells may also be reduced by AEA. This study deduced that cannabidiol oil treatment on breast cell lines MCF-7 caused reduced cell viability of the MCF-7 and greatly inhibited the MCF-7 cell movement towards chemotactic agents. (Romano et al., 2014; and Galluzzi et al., 2015).

Current research signifies cannabidiol oil to effect on breast cancer cell viability is synergized with GPR55 (Ramer et al., 2013). These receptors have unclear impacts on the development of tumours and their progression. More research on this subject may unearth the mechanisms and their signal pathways. These findings are in agreement with some studies which have already projected cannabidiol oil as a cancer therapy adjuvant in model studies. The study proves that WIN55, 212-2, a synthetic cannabidiol agent, integrated the antimyeloma role of melphalan and dexamethasone, consequently decreasing the confrontation of melphalan cell culture. Nabissi et al., (2012) has shown similar findings that Phyto-cannabinoids integrated with chemotherapeutic agents improved the glioblastoma cells sensitivity to the drugs. The cannabinoids induce apoptosis (Ramer et al., 2017) through ROS activation, most likely, although other mechanisms may be involved, such as AMPK and autophagy. These cellular death mechanisms have not been exhaustively studied. Generally, cannabinoids have a great therapeutic prospective in the current anticancer chemotherapy by providing a complementary pharmacological approach to it (Caffarel et al., 2016).

Pérez-Gómez et al., (2018) study shows there's sensitivity to cannabinoids by MCF-7 cells, not limited to the hyperactive triple-negative cancer cell line. Antitumor characteristics of cannabinoids were also shown on different other types of cancer cells, like lung, liver pancreas, skin, and others. Among all the studies conducted, cannabinoids, when used at a specific concentration necessary to inhibit cancer cells and causing apoptosis, shows no documented toxicity on normal cells. The cytotoxic pathway of cannabinoids, when used without specificity, still remains uncovered. The interest of the project was to evaluate growth inhibition caused to cancer cells by these agents, and they are potential-drugs with a unique therapeutic profile both in vitro and in vivo. There are no uncovered evasion mechanisms by cancer cells to the induced inhibition, a factor that earmarks cannabinoid use for therapeutic agents for exploration.

Cannabidiol oil, a pharmaco-agent of cannabis, has proved to hold cellular anti-inflammatory properties, performing through the CB1-R, CB2-R, and CB-Rs. Their receptor-mediated action is, therefore, pertinent in breast cancer cell

signalling. The ability of cannabidiol to stimulate the immune system of the body, the tumour cell microenvironment, the proto-oncogene, and the block COX-2 expression are the keys to decrease inflammation, causing inductive apoptosis, suppress the growth of tumour cell and also autophagy.

Conclusion:-

Cannabinoids are the active agents in cannabis, and their other synthetic formulations have been found to be useful in complementary medicine, displaying chemotherapeutic potential. Cannabinoids have proved to be unique in their mode of action by modulating cell proliferation pathways in targeted cell lines. The effects of cannabinoids vary with cells and tumour models. CB1 and CB2 receptors are expressed differently; overexpression may lead to apoptosis of tumour cells while limited-expression may suppress the antitumor immune response, thus facilitate proliferation and tumour development.

The mechanism of action of cannabidiol is not well explored. Similarly, the safety profile of cannabinoids is not well formulated. The user can be challenged by dose fluctuation regimens and, as a result, compromise the safety of medicinal cannabis. Research focusing on developing cannabinoid delivery systems, guidelines, and safety measures is needed in order to explore this complementary therapy.

Some experiments say that CBD is helpful not only in treating cancer but also in reducing the effects of chemotherapeutic techniques. But alone, it is not enough. Moreover, it is not helpful in preventing people from cancer. But it can prove helpful in dealing with symptoms of cancer and also aids treatments used for cancer. So, although CBD oil is beneficial, there are some safety concerns related to them. They may affect other medications you are taking at that time. So, it would be better to consult your doctor before using CBD oil. So, the end of all discussion is that CBD can be used by cancer patients according to the instruction of the doctor.

However, it should be noted that cannabinoids have been inappropriately used for recreation, unlike the intended medicinal purpose. They have been smoked, and this is a wrong form of administration from the medical angle. The fact that there are no recommended biomarkers to indicate the responses of patients during cannabinoid treatment remains a big challenge in their therapeutic application.

Recommendations:-

The legality and medicinal application of cannabinoids at large will depend on its safety and proved therapeutic potential. In this view, this study recommends that more research should be done to guarantee the safety of cannabinoids. Such researches should unearth the possibility of cannabinoids being cytotoxic to normal cells so as to develop this novel therapeutic anticancer compound. Unlike in this research, studies involving human cancer cell therapy should incorporate normal cells as reference codes to measure the biological effect induced by the target agents.

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