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Evaluation of Anticancer Activity of *Cannabis Sativa* On B-16 Cell Line Induce Skin Cancer Model .

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Abstract

Skin cancer most basic malignancy nowadays. Distinctive remedial methodology for the treatment of skin disease as of now being examined. Cannabinoids delta THC is a dynamic cannabinoid utilized as a part of the different tumor and furthermore in various malady like Parkinson's disorder, AIDS incite emesis. Cannabinoids have two subtypes of cannabinoids receptors CB1 & CB2 we examined the potential utility of these compound in skin tumor in mice by the B-16 cell line. Where both petroleum ether and ethanolic extract of cannabis sativa give better reaction by treatment of tumor-bearing mice. This went with by an impedance of tumor vascularization, as controlled by adjusted vein morphology and diminished articulation of proangiogenic factors (VEGF, placental development factor, and angiopoietin 2). Repeal of EGF-R work was additionally seen in cannabinoid-treated tumors. These outcomes bolster another remedial approach for the treatment of skin tumors.

Introduction

Skin cancer: Skin malignancy are growths that arranged on skin. They can attack or spread to various parts of the body because of the improvement of anomalous cells.[1] Skin disease is the uncontrolled development of irregular skin cells. It happens when unrepaired DNA harm to skin cells[14].

Type: There are three primary kinds of skin tumor: basal-cell skin disease (basal-cell carcinoma) (BCC), squamous-cell skin growth (squamous-cell carcinoma) (SCC) and dangerous melanoma. Basal-cell carcinomas are available on sun-uncovered regions of the skin, particularly the face. They once in a while metastasize and once in a while cause demise. They are effectively treated with surgery or radiation. Squamous-cell skin malignancy are normal, yet significantly less regular than basal-cell diseases. They metastasize more regularly than BCCs. And still, after all that, the metastasis rate is very low, except for SCC of the lip, ear, and in individuals who are immunosuppressed. Melanoma are the slightest successive of the 3 regular skin tumors. They much of the time metastasize, and could conceivably cause demise once they spread[2].

Prognosis: The death rate of basal-cell and squamous-cell carcinoma are around 0.3%, causing 2000 passing for each year in the US. In correlation, the death rate of melanoma is 15– 20% and it causes 6500 passing for every year.[3]:

Cannabis: A specialist who found out about its therapeutic properties while working in British East India Company. Its utilization was accounted for as pain relieving, calming, mitigating, antispasmodic, and anticonvulsant impacts. The principle psychoactive constituent of Cannabis was delta-9-tetrahydrocannabinol (THC). An isomer of engineered delta-9-THC in sesame oil was affirmed and authorized for the treatment of chemotherapy in 1986 related with sickness and retching under the nonexclusive name dronabinol[4]. **Receptors**: Two diverse cannabinoid receptors are: cannabinoid receptor 1 (CB1), initially named 'focal' receptor [5] and CB2, otherwise called 'fringe' receptor [6]. Both the CB1 and CB2 are having a place with the G protein-coupled receptor family [6] the flag transduction pathway of cannabinoid receptors incorporates adenylatecyclise [7]. Though CB1 is essentially in the focal sensory system [5], CB2 communicated in fringe resistant cells [7].

Location: The enactment of CB1 demonstrates the focal and the greater part of the fringe impact. This receptor is mostly present in a few mind regions, at abnormal states in the basal ganglia, hippocampus, cerebellum and cortex, where it demonstrates cannabinoid psychoactive impacts [8]. CB1 receptors are likewise present in fringe nerve terminals, and additionally in additional neural tissues, for example, testis, uterus, vascular endothelium, eye, spleen, ileum and in adipocytes [9]. The CB2 receptor is available in resistant cells primarily in zones enhanced in B lymphocytes, for example, the spleen peripheral zone, the lymph hub cortex[10].

MOA: The cannabinoids did this by encouraging two regular procedures of cell passing known as autophagy and apoptosis. Amid autophagy, cells dismantle any harmed parts they have coasting around within them[11]. Apoptosis can be thought of as cell suicide. Amid apoptosis, the cell starts to separate or break itself into different parts[12]. The parts are then tidied up by different cells in the resistant framework. While autophagy is frequently observed as an approach to fight off apoptosis, cannabinoids like THC and CBD animate both of these procedures in tumor cells when tried in creature models[13].

Material and Method

Drug and drug sample collection:

- Standard: Cannabis Indica were purchased from Bhatia medicos, New Delhi
- Sample: Cannabis Sativa collected

Extraction of Cannabis:

1. Ethanolic extract of cannabis:100g of cannabis add in 1000ml of ethanol Shake at 120 rpm for 1hr. Filter the extract Collect the filtrate then add 1000ml of ethanol to the remaining marc after filter Shake at 120 rpm for 1 hr. & filter Combine the both extract & concentrate on water bath at $1000C^0$ Brown color extract is ready for use.
2. Petroleum ether extract of cannabis: 100g of cannabis add 1000ml of petroleum ether Shake at 120 rpm for 1hr. and Filter the extract Collect the filtrate Add 1000ml of petroleum ether to the remaining marc after filter Shake at 120 rpm for 1 hr. & filter combine the both extract & concentrate on water bath at 1000C Brown color extract is ready for use.

Standardization: Thin-layer chromatography (TLC)

There are a number of TLC methods for the qualitative and semi-quantitative analysis of cannabis, using a variety of different stationary phases (TLC plates) and solvent systems, and slightly different sample preparation and spot visualization techniques. Many of those methods also produce acceptable results but each method that is newly introduced to a laboratory must be validated and/or verified prior to routine use. The following method has been field-tested and is considered fit-for-purpose.

Solvent System: Petroleum ether 60/90 -80% v/v, Diethyl ether -20% v/v

Sample preparation: If the sole purpose of the THC examination is qualitative (i.e. to confirm the micro- or macroscopic evidence that the suspect material is cannabis), homogenization of the herbal material is not necessary Those parts of the cannabis plant known to contain the highest levels of cannabinoids (i.e. the flowering

tops and upper leaves) should be selected for extraction. The sample is extracted with 10 ml of solvent for 15 minutes at room temperature by shaking or in an ultrasonic bath. The extract is filtered and is now ready for chromatography.

Since cannabinoids are easily soluble in most organic solvents, methanol, petroleum ether, n-hexane, toluene, chloroform and solvent combinations, such as methanol: chloroform (9:1) are equally suitable for their extraction. It should, however, be noted, that non-polar solvents such as n-hexane and petroleum ether give a relatively clean extract but will only extract the neutral/free cannabinoids quantitatively, while the other solvents and their combinations give quantitative extractions of the cannabinoid acids as well. For identification, the simplest clean extraction with petroleum ether is enough, while for the purposes of quantitation and total THC determinations other solvents have to be used.

Standard solutions: The standard solutions should be prepared at a concentration of approximately 0.5 mg cannabinoid per ml in methanol and should be stored in a cool, dark place.

Visualization: The plates must be dried prior to visualization. This can be done at room temperature or by use of a drying box, oven or hot air. In the latter cases, care must be taken that no component of interest is decomposed.

Spray reagent: Anis aldehyde

Experimental design: Animal Grouping: Female albino mice weighing 20-22gm were procured from the animal house of DPSRU after the approval of protocol from Institutional ethical committee under protocol no-IAEC/2016/15Animals were housed in polypropylene cages lined with husk. It was renewed every 24 hours under a 12:12 hour light: dark cycle at room temperature.

Model Development: For the study 10-15 days old mice was procured from the animal house of DPSRU at day one injects B-16 skin cancer cell line in amount $2*10^5$/mice by IV route. At 8thweek the skin cancer cell line B-16 was acquired from the NCCS Pune.

Cells were cultivated in RPMI high glucose media (RPMI) supplemented with 10% fetal bovine serum and 1% antibiotic solution administered under 5% CO2-humidified atmosphere at 37°C. Cells growing exponentially in vitro were trypsinised and harvested for tumor implantation. All animal manipulations were performed under sterile conditions. skin cancer models, mice were anesthetized with intra-venous injection of a ketamine/diazepam solution (50 mg/kg ketamine and 5 mg/kg diazepam) and operated on a pre-warmed operation table.

Animals: 10-15 days old Swiss albino male mice was procured from animal house. The mice were grouped, housed in poly acrylic cages and maintained under standard laboratory conditions and were allowed to have free access to standard dry pallet diet and water ad libitum. Treatments were started first days after inoculation with B-16 cells and continued for the next consecutive 21 days. Blood was collected on 22nd day.

Drug Treatment

Number of Group	Groups	Number of Animals	Treatment
1	Control Group	8	Ethanol
2	skin cancer cell line control group (disease control)	8	Placebo (Ethanol)
3	Standard Group Cannabis Indica	8	Standard drug Cannabis Indica (0.02mg/25g/p.o)
4	Skin cancer cell line + petroleum ether extract of cannabis	8	Petroleum ether extract of cannabis (0.01mg/25g/p.o)
5	Skin cancer cell line + petroleum ether extract of cannabis	8	Petroleum ether extract of cannabis (0.02mg/25g/p.o)
6	Skin cancer cell line+ petroleum ether extract of cannabis	8	Petroleum ether extract of cannabis (0.03mg/25g/p.o)
7	Skin cancer cell line+ ethanol extract of cannabis	8	Ethanol extract of cannabis (0.01mg/25g/p.o)
8	Skin cancer cell line+ ethanol extract of cannabis	8	Ethanol extract of cannabis (0.02mg/25g/p.o)
9	Skin cancer cell line+ ethanol extract of cannabis	8	Ethanol extract of cannabis (0.03mg/25g/p.o)

Table 1

Each group involved 8 independent tumor-bearing mice. Body weights, ascites formation, and overall survival were monitored twice weekly. Tumor-bearing mice were sacrificed when they became moribund and the presence of ascetic fluid was recorded for each mouse. At the end of the treatment regimen, all animals were sacrificed and tumors harvested for histological analysis.

Collection of blood: Via retro-orbital route the animal is anaesthetized and a drop of proparacaine topical ophthalmic anesthetic in each eye to minimize discomfort. The animal is held by the back of the neck and the loose skin of the head is tightened with thumb and middle finger to keep the animal stable. The tip of the capillary tube is placed at the medial canthus of the eye under the nictitating membrane. With a gentle thrust and rotation motion past the eyeball the tube will enter the slightly resistant sinus membrane. The eyeball itself remains uninjured. As soon as the sinus is punctured, blood enters the tubing by capillary action. When the desired amount of blood is collected, the tube is withdrawn and slight pressure with a clean gauze pad on the eye is used to ensure hemostasis. Note: Avoid scratching the cornea with the gauze pad.

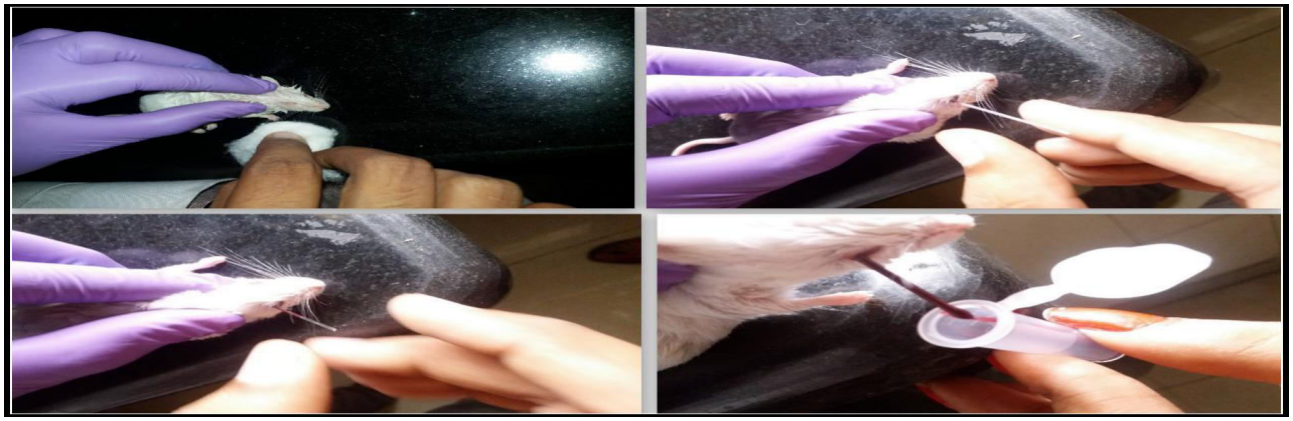


Figure 1: Blood sample collection by retro-orbital route.

Kits used for biochemical estimation:

Estimation of IL-6 level by ELISA method (Krishgen Biosystems)

IL-6 is a potent lymphoid cell growth factor that stimulates the growth and survivability of certain B cells and T cells. IL-6 plays a role in host defense, acute phase reactions, immune response and hematopoiesis. IL-6 is expressed by T cells, B cells, monocytes, fibroblasts, hepatocytes, endothelial cells, and keratinocytes. Recombinant mouse IL-6 is a 21.7 kDa protein containing 187 amino acids.

Determination of TGF-β by ELISA method (Kinesis Dx Cat No: K11- 0779)

Introduction: Transforming growth factor beta (TGF-β) is a multifunctional cytokine belonging to the transforming growth factor superfamily that includes three different isoforms (TGF-β 1 to 3, HGNC symbols TGFB1, TGFB2, and TGFB3) and many other signaling proteins produced by all white blood cell lineages. Activated TGF-β complexes with other factors to form a serine/threonine kinase complex that binds to TGF-β receptors, which is composed of both type 1 and type 2 receptor subunits. After the binding of TGF-β, the type 2 receptor kinase phosphorylates and activates the type 1 receptor kinase that activates a signaling cascade. This leads to the activation of different downstream substrates and regulatory proteins, inducing transcription of different target genes that function in differentiation, chemotaxis, proliferation, and activation of many immune cells.

Estimation of VEGF level by ELISA method (Krishgen Biosystems)

Vasculorgenesis in the embryo is the process by which new blood vessels are generated *de novo* from primitive precursor cells. Angiogenesis is the process of new blood vessel formation from pre-existing vasculatures. It plays an essential role in development, normal tissue growth, wound healing, the female reproductive cycle (placental development, ovulation, corpus luteum) an also plays a major role in various diseases. One of the key molecules for angiogenesis and for the survival of the endothelium is vascular endothelial growth factor (VEGF). VEGF is a heparin binding glycoprotein, secreted as a homodimer of 45kDa by many different cells types..

Results

a) **Thin Layer Chromatography (TLC):**
Rf x 100 values are subject to variation depending on laboratory conditions (temperature, humidity etc.) as well as other parameters (e.g. age and quality of cannabis materials used). It is therefore good practice to run cannabinoid standards along with the sample on the same TLC plate.

Fig .2

Compound	**Ethanolic Extract (Rf x 100 values)**	**Petroleum Ether Extract(Rf x 100 values)**
THC	37	35
CBD	42	39
CBN	33	32
THCA	6	7

Table 2

Body Weight:

An analysis of body weight variations evaluated the adverse effects of the different therapy regiments (Figure 7.2). For oral administration, ethanolic cannabis extract and petroleum ether cannabis extract and vehicle (ethanol) showed favorable results without any obvious body weight. These results indicated that the security of the both cannabis extract was acceptable.

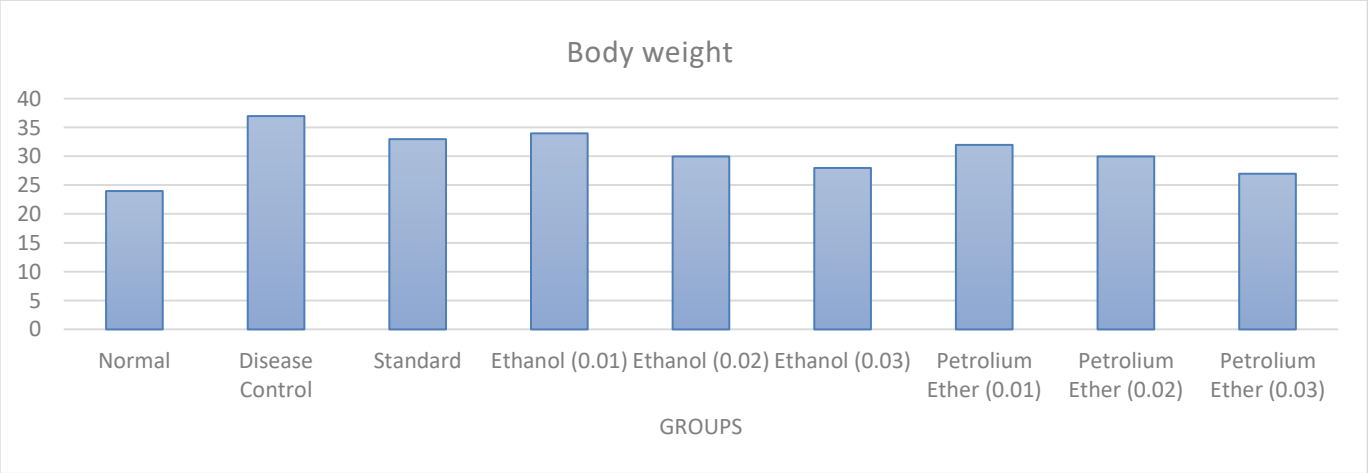


Fig 3: Body weight variations among the groups during study. Considered significant when ($P<0.001$) n=8

The results presented in figure 7.2 are the mean of animals in a group and statistical analysis is done by comparing treatment with control and by applying ANOVA followed by Dunnett's Multiple Comparison Test using Graph pad Prism version 7 software.

Haematological Parameters:

Figure 7.3, 7.4 and 7.5 show the effect of both cannabis extract in controlled and experimental groups of animals. Vehicle group of Liver cancer model has shown significantly decreased Hemoglobin and RBC count (7.3, 7.5) with increased WBC count (7.4) when compared with the low dose and high doses of both cannabis extract , the results has shown significantly rise in RBC and Hemoglobin count with the decrease in WBC count.

Parameter	Group1 Normal	Group2 control	Group3 standard	Group4 E.E 0.01	Group5 E.E 0.02	Group6 E.E 0.03	Group7 P.E 0.01	Group8 P.E 0.02	Group9 P.E 0.03
R.B.C(106)	7.65±0.25	6.25 ±0.23	5.74 ±0.21	6.92 ±0.23	7.01 ±0.25	7.23 ±0.25	6.23 ±0.25	7.83 ±0.25	7.95±0.25
W.B.C	8.3 ±0.39	12.6 ±0.27	9.8 ±0.45	8.69 ±0.27	8.25 ±0.25	7.65 ±0.25	8.65 ±0.25	7.75 ±0.25	6.95 ±0.29
Hb count	13.0 ±1.1	6.0 ±0.62	10 ±0.26	7 ±1.6	8.69 ±1.29	9.65 ±1.23	6.65 ±1.23	7.95 ±1.27	9.99 ±1.25

Table 3: Hematological parameters

Table 4 (RBC count)

Treatment	Control	Normal	Standard Drug	E.E 0.01	E.E 0.02	E.E 0.03	P.E 0.01	P.E 0.02	P.E 0.03
Before starting treatment (ng/ml)	238±26	9.3±1.3	226±25	213±18	232±21	232±21	232±21	232±21	233±21
After treatment	237±24	9.1±1.3	98±12	112±13.2	108±12.3	99±12.2	109±12.2	105±13.3	97±12.2

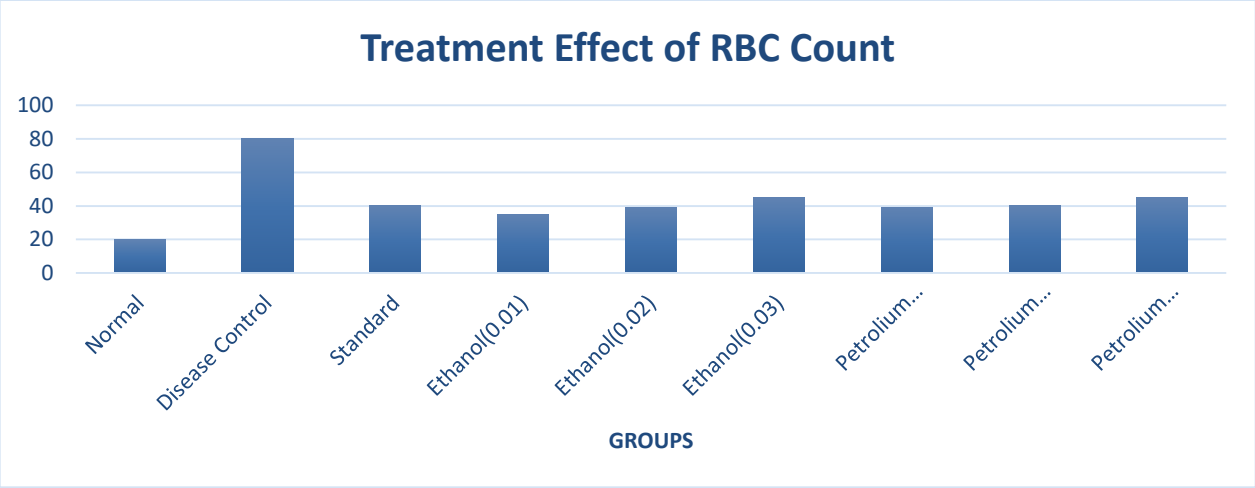
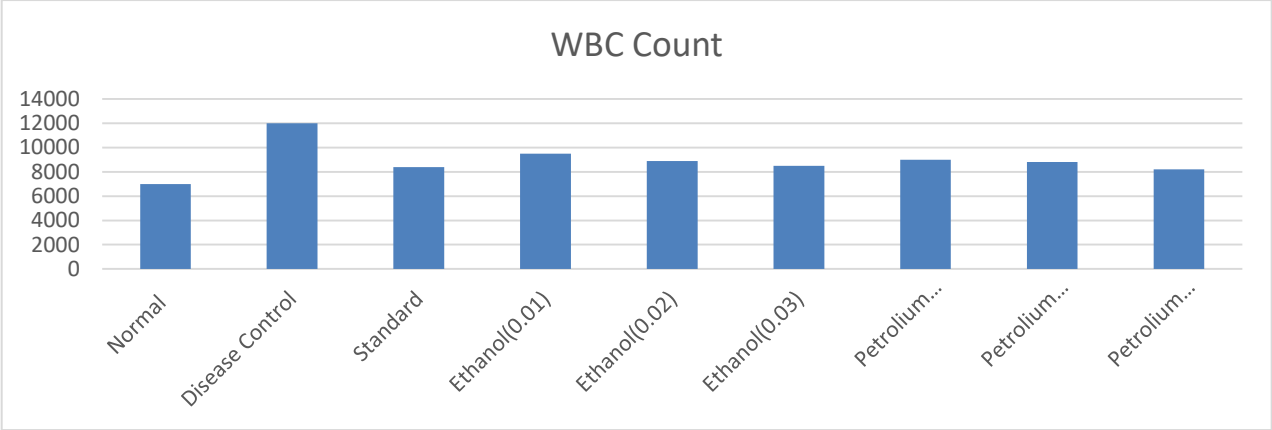


Fig 4 Treatment effect on RBC count p< o.o4



Fi g 5 Treatment effect on WBC count: # p < 0.0123. Normal vs. standard drug $ p < 0.001 normal mice vs control. * P < 0.05. ** P < 0.01. *** P < 0.001 high cannabis extract v/s control.

Tumor necrosis factor: (TNF-alpha):

A significant rise (p<0.001) was observed in the expression of TNF-α levels in Control group (2982±74.166) as compared to normal group(471.66±136.536). However, vehicle treated (2694.33±111.690) and Cannabis treated groups showed significant protection (p<0.001) against the rise in Inflammatory expression of TNF-α in the treatment group as compared to the Control group (Figure 7.6). cannabis treated groups were significantly better.

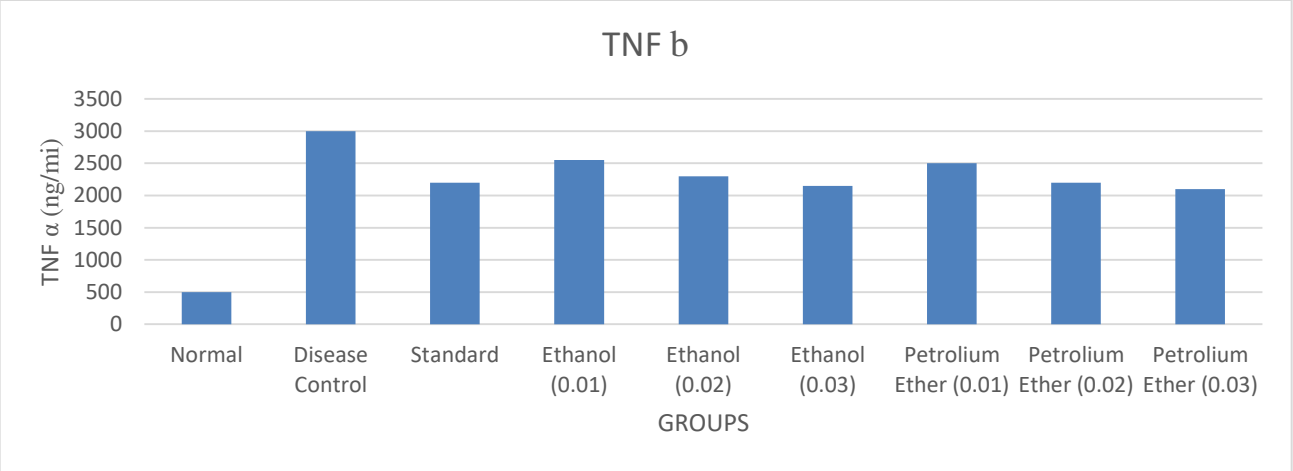


Fig 6 Expression of TNF-alpha in different groups. Effect of cannabis extracts on TNF alpha level in tumor compared to normal and high doses of both cannabis extract treated groups. Each value represents mean ±SEM(n=8) *p <o.o5 vs. normal standard treated, p<0.001 for high dose cannabis extract when compared with the vehicle control group. One-way Analysis of Variance (Dunnett's Multiple Comparision test).

Effect of IL-6 on Cannabis treated group:

A significant rise (p<0.001) was observed in the expression of IL-6 levels in Control group (78±14.166) ng/ml as compared to normal group (32.32±17.536). However, Cannabis treated groups showed significant protection (p<0.01) against the rise in Interleukin level in the treatment group as compared to the Control

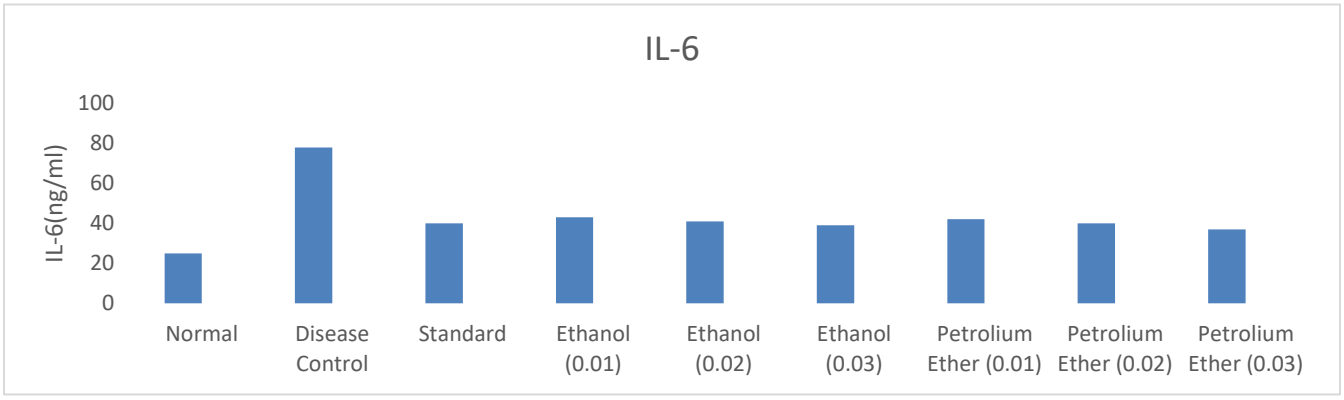


Fig 7

5.Effect of VEGF on both cannabis extract treated group:

A significant rise (p<0.001) was observed in the expression of VEGF levels in Control group (598.32±22.166) ng/ml as compared to normal group (43.3±12.536). However, both cannabis extract treated groups showed significant protection (p<0.001) against the rise in VEGF level in the treatment group as compared to the Control group (Figure 7.17).High dose of both cannabis extract show showed better decrease in VEGF levels (324±43.23) as compared to Low dose of both cannabis extract.

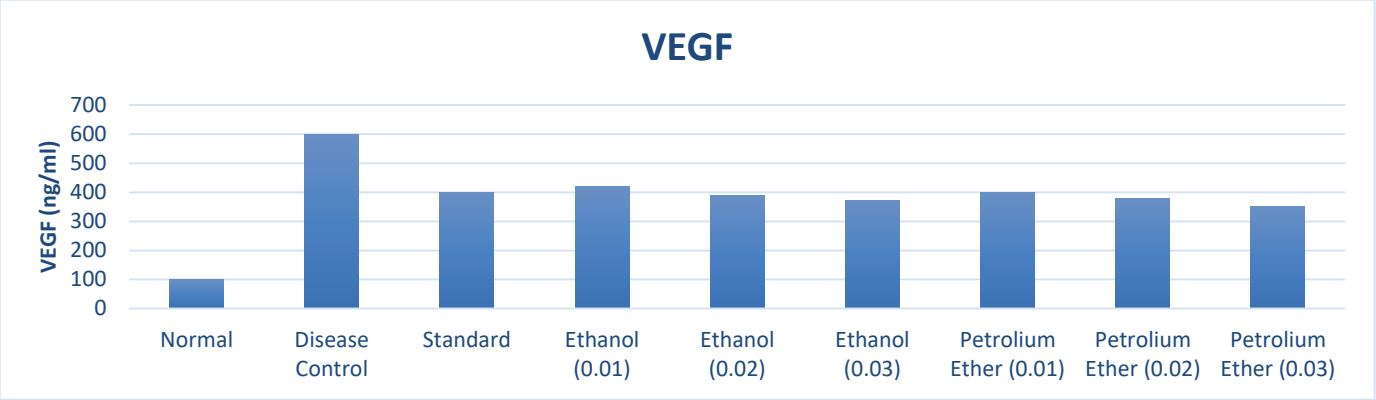


Fig 8: VEGF levels in treatment groups: The results of VEGF has shown significantly decrease in the levels of VEGF in high and low dose cannabis extract when compared with the control group.Effect of both extract of cannabis on VEGF levels in liver compared to Disease control and normal groups. Each value represents mean ± SEM (n=8).* $p<0.001$ vs. Normal group, $p<0.001$ vs. disease control. One Way Analysis of Variance (Dunnett's Multiple Comparison Test)

Macroscopic evaluation:

Fig 9 Skin Cancer Model

Histopathological Parameters:

Hematoxylin and eosin staining: The mice were killed and the tissue samples of the skin tumor were instantly fixed in buffered formalin overnight,

Followed by dehydration with a tissue processor for 16 h. The tissues were embedded by paraffin. The 7 mm sections were prepared for H&E staining and microscopic examination.

Histo morphological observations of mice skin tumor (light microscopy [LM] o.m. ×10). (a) Masson stain. (b) Hematoxylin–eosin stain.

In normal mice (Figure 7.28) light microscopy showed a normal parenchyma. Network sinusoids appeared continuous and interconnected. Necrosis and fibrosis were absent.

Fibrosis was present in uniform septa of connective tissue and the nodules were defined by a thin fibrous capsule. Necrosis areas were present.
In low and high dose of both cannabis extract treated (Figure 7.31 & 7.32 respectively), livers showed recovery to normal parenchymal architecture with the disappearance of nodular shapes. Connective tissue was organized into fibrotic bundles. Necrosis was almost absent.

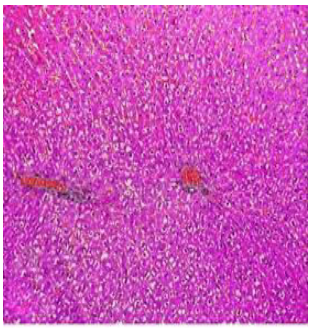
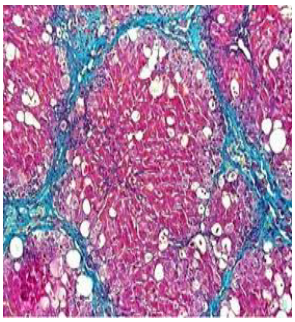
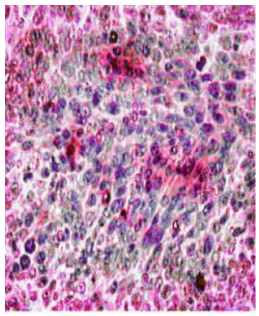


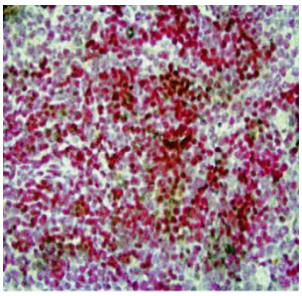
Fig Normal Group



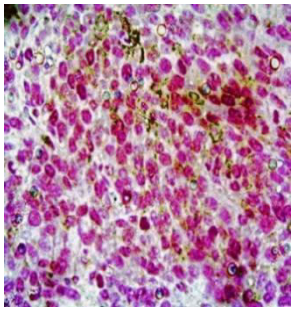
Disease control group:



Standard Drug treated Group



Low dose etanolic group



High dose petroleum ehter group

DISCUSION

Here we report that *cannabis sativa* both ethnolic and petlorium ether extract effective in skin cancer tumour cell that both are funtional in the induction of apoptosis. Here we are report that vesel deloped by cannabinoids treated carcinomas that expression of prangiogenesis factor that of skin cancer in which VEGF plays a pivotal roles. Our data also show that cannabinoid treatment decreases the expression of PIGF (another VEGF family member) and Ang2, and these two proangiogenic factors may act in concert with VEGF because their expression is highly increased since the early stages of tumor development The first phase of slow growth occurs independently of EGF-R function. Later, an angiogenic switch response mediated by the EGF-R seems to be an essential requirement for complete tumor growth, involving high VEGF levels. The first phase of slow growth occurs independently of EGF-R function. Later, an angiogenic switch response mediated by the EGF-R seems to be an essential requirement for complete tumor growth, involving high VEGF levels.

Refrence

1. *"Defining Cancer". National Cancer Institute. Retrieved 10 June 2014.*
2. *Schaffer Library of Drug Policy: The Marihuana Tax Act of 1937: Taxation of Marihuana. Washington, DC: House of Representatives, Committee on Ways and Means, 1937. Available onlineExit Disclaimer. Last accessed January 7, 2016.*
3. *Schaffer Library of Drug Policy: The Marihuana Tax Act of 1937: Taxation of Marihuana. Washington, DC: House of Representatives, Committee on Ways and Means, 1937. Available onlineExit Disclaimer. Last accessed January 7, 2016.*
4. *Sallan SE, Zinberg NE, Frei E 3rd: Antiemetic effect of delta-9-tetrahydrocannabinol in patients receiving cancer chemotherapy. N Engl J Med 293 (16): 795-7, 1975. [PUBMED Abstract]*
5. *Yosef Sarne, Fadi Asaf, Miriam Fishbein, Mikhal Gafni, and Ora KerenThe dual neuroprotective–neurotoxic profile of cannabinoid drugs Br J Pharmacol. 2011 Aug; 163(7): 1391–1401. [PubMed]*
6. *Nephi StellaCannabinoid and cannabinoid-like receptors in microglia, astrocytes and astrocytomasGlia. 2010 Jul; 58(9): 1017–1030.[PubMed]*
7. *Berrendero et al. Endocannabinoids: The Brain and Body's Marijuana and Beyondedited by Emmanuel S Onaivi, Takayuki Sugiura, Vincenzo Di Marzo page no. 193, 1999.[PubMed]*
8. *Maurizio Bifulco,1,* Chiara Laezza,2 Simona Pisanti,1 and Patrizia GazzerroCannabinoids and cancer: pros and cons of an antitumour strategyBr J Pharmacol. 2006 May; 148(2): 123–135.[PubMed]*
9. *Brady K Atwood1,2 and Ken MackieCB2: a cannabinoid receptor with an identity crisisBr J Pharmacol. 2010 Jun; 160(3): 467–479.[PubMed]*
10. *Joy JE, Watson SJ, Benson JA, eds.: Marijuana and Medicine: Assessing the Science Base. Washington, DC: National Academy Press, 1999. Also available onlineExit Disclaimer. Last accessed January 7, 2016.*
11. *Sallan SE, Zinberg NE, Frei E 3rd: Antiemetic effect of delta-9-tetrahydrocannabinol in patients receiving cancer chemotherapy. N Engl J Med 293 (16): 795-7, 1975. [PUBMED Abstract] Preet A, Qamri Z, Nasser MW, et al.: Cannabinoid receptors, CB1 and CB2, as novel targets for inhibition of non-small cell lung cancer growth and metastasis. Cancer Prev Res (Phila) 4 (1): 65-75, 2011. [PUBMED Abstract]*
12. *Felder CC1, Glass M.Cannabinoid receptors and their endogenous agonists.Annu Rev Pharmacol Toxicol. 1998;38:179-200.[PubMed]*
13. *"Skin Cancer Treatment (PDQ®)". NCI. 2013-10-25. Retrieved 30 June 2014.*