REPUBLIC OF TURKEY
ADANA ALPARSLAN TÜRKEŞ SCIENCE AND TECHNOLOGY UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF NANOTECHNOLOGY AND ENGINEERING

A STUDY ON CYTOTOXIC EFFECTS OF HYPERICUM PERFORATUM ON HUMAN CANCER CELL LINES

FERDA TANACI

MASTER OF SCIENCE

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SUPERVISOR

ASSOC. PROF. DR. DİLEK GÖKTÜRK

ADANA 2020

I hereby declare that all information in this thesis has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all information that is not original to this work.

[Signature]

Ferda TANACI

ABSTRACT

A STUDY ON CYTOTOXIC EFFECTS OF HYPERICUM PERFORATUM ON HUMAN CANCER CELL LINES

Ferda TANACI
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Supervisor: Assoc. Prof. Dr. Dilek GÖKTÜRK
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St. John's wort which is abbreviated as (SJW), denoted as Hypericum perforatum by botanists, is a yawning, foliaged plant that rises in nearly all of the world's mild areas. Utilization of this type as a galenic cure for interior and exterior diseases dates back to archaic times. From these times, SJW is one of the favorite cures for anxiety, depression, incisions, and scalds. Researchers suggest that SJW has active role in the cure of several illnesses, like cancer, diseases related with inflammation, and illnesses caused by bacterias and viruses, and as an anti-oxidant and neuro-protective factor.

In this study the cytotoxic effects of Hypericum perforatum infusion solution on U-87 MG Glioblastoma cancer cell lines was examined when it is applied with and without etoposid. The results demonstrate that 1/25 diluted ethanol extract of Hypericum perforatum without etoposid tend to show the best cytotoxic effect.

Keywords: Hypericum perforatum, St. John’s Wort, U87 MG, Glioblastoma

ÖZET

SARI KANTARON OTU (HYPERICUM PERFORATUM)'NUN İNSAN KANSER HÜCRELERİ ÜZERİNDEKİ SİTOTOKSİK ETKİLERİ HAKKINDA BİR ÇALIŞMA

Ferda TANACI

Nanoteknoloji ve Mühendislik Bilimleri Anabilim Dalı

Danışman: Doç. Dr. Dilek GÖKTÜRK

Nisan 2020, 38 sayfa

Botanik alanında Hypericum perforatum olarak bilinen kantaron otu, yayılarak büyüyen, neredeyse Dünyanın tüm ılıman bölgelerinde yetişebilen yapraklı bir bitkidir. Bu bitkinin harici ve dâhili bir ilaç olarak kullanılması çok eski zamanlara dayanmaktadır. O zamanlardan günümüze kadar anksiyete, depresyon, kesikler ve yanıklar için kullanılan en gözde tedavi yöntemlerinden olmuştur. Araştırmacılar, kantaron otunun kanser, ateşli hastalıklar, virüs ve bakterilerden kaynaklanan hastalıklarda aktif bir rolü olduğunu düşünmektedirler. Ayrıca kantaron otu geleneksel ve alternatif tedaviler arasında bir köprü olarak kabul edilmektedir.

Bu çalışmada, kantaron otu infüzyon solüsyonun, bir kemoterapi ilacı olan etoposid ile birlikte ve etoposid olmadan uygulandığında U 87 MG Glioblastoma kanser hücreleri üzerindeki etkisinin incelenmesi amaçlanmıştır. Etanol ile hazırlanan ve 1/25 oranında seyreltilen sarı kantaron otu çözeltisinin, etoposid uygulanmadan kullanıldığı durumlarda en etkili sitotoksik etkiyi gösterdiği görülmüştür.

Anahtar Kelimeler: Hypericum perforatum, Sarı kantaron otu, U87 MG, Glioblastoma

For my lovely family

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NOMENCLATURE

µm	: Micrometer
UV-VIS Spectrophotometer	: Ultraviolet visible spectrophotometer
PBS	: Phosphate Buffer Saline
FBS	: Fetal Bovine Serum
DMEM	: Dubelco's Modified Eagle's Medium
MTT	:3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
GC-MS	: Gas chromatography mass spectrometer

1. INTRODUCTION

Hypericum perforatum is a plant which has yellow flowers whose shapes are like stars and have five petals which is growing throghout Europe, North and South America, Australia, New Zealand, and Eastern Asia. The plant needs sunlighted and appropriately draining zones to grow. Plant can be 50 to 100 cm in lenght (Nathan, P. J, 2001).

It is thought that Hypericum perforatum is named as the St. John's by the first Christians, who discovered that the herb flowers nearly on the last week of June, which is the day Saint John's-the Baptist's was born. Scientists think that, the St. John's wort shows an activity not less than amitryptilline, fluoxetine and maprotiline, and is absolutely more active than placebo. It is also observed that the St. John's wort has therapeutic effects against diseases of today, like cancer, AIDS and hepatitis. In conformity with what is believed, the St. John's wort is regarded as bridge between the ancient and the medication of today. (C.I. Istikoglou, et al., 2010)

Hypericum perforatum has been used as medication for anxiety, depression, injury and burns from the archaic periods. Researchers suggest that Hypericum perforatum could play an active role in the treatment of many illnesses like cancer, inflammation-related illnesses, bacteria and viruses, and as an antioxidant and neuroprotective factor.

Below, in the Figure 1 Hypericum perforatum full plant, seedlings, fruit and blossom are seen. (Ernst E., 2003)

a) **b)**

c) **d)**

Figure 1: Hypericum perforatum a) Full plant, b) Seedlings, c) Fruit and d) Blossom (Ernst E., 2003 pp. 3-6)

The cells that make up our bodies cluster to form tissues, and these tissues cluster to form organs. All cells, which form the building blocks of the organism, proliferate at a certain rate and in a controlled manner.On the other hand, aging cells are collapsing at a certain rate. Based on this information, cancer is defined as the cell proliferation in an uncontrolled manner. During proliferation, structural differences takes place in cancer cells compared to ordinary cells, and contrasts in functions occur. In some cases the cell performs a few unused functions. Proliferated cells invade the tissues and organs blocking the tasks of these occupied organs or tissues (Tezer, K et al., 2001). In brief, cancer is a complex genetic disease that results in the transformation of human healthy cells into tumors after multiple stages of development (Zhang, X et al., 2012). The global deaths caused by cancer, among non contagious diseases, have been incremental in recent years (Bray, F. et al, 2018).

Glioblastoma (GBM) is the most frequent brain tumor in adults (Yılmaz, A et al., 2012). Despite significant progress in the treatment of the disease, the median survival of patients diagnosed with GBM remains limited for 12 to 15 months (Mohamad Mohanad Al Sayed 2019). In spite of GBMs occur approximately entirely in the brain, it may be seen in the brain stem, cerebellum, and spinal cord. Primary gliomas takes up 61% among other types of GBMs occur in all four lobes of the brain: frontal (25%), temporal (20%), parietal (13%), and occipital (3%) (Mary Elizabeth Davis, 2016). At first, GBMs were regarded to be determined exclusively from glial cells; in any case, prove proposes that they may emerge from numerous cell sorts with neural stem cell - like characteristics. The cells are at discrete steps of specialization from stem cell through neuron to glia, with phenotypic

differentiations observed, in huge portions, by molecular modifications in signaling pathways rather than by variations in source of cell sorts (Phillips et al., 2006).
GBMs are seen at an average age of 64 years on the other hand can be present at any age, covering childhood (Thakkar et al., 2014). Frequency is marginally excessive in men than women (1.6:1) and in Caucasians in relation with different ethnicities (Ellor, Pagano-Young, & Avgeropoulos, 2014). GBMs are sorted as primary, emerging without a precursor that is known; or secondary, where a low-grade tumor changes during time into GBM. Primary GBMs are seen more frequently, and the patients are older aged. Treatment for patients that have primary GBM gives less successful results than patients suffering from GBMs of secondary type (Wilson, Karajannis, & Harter, 2014).

In this study, the cytotoxic impacts of ethanol and isopropanol extracts of Hypericum perforatum in Glioblastoma U-87 MG (ATCC® HTB-14 ™) cancer cell line alone and simultaneously with the etoposide were investigated.

2. LITERATURE REVIEW

2.1. Chemical Constituents of Hypericum Perforatum:

It is necessary to have the information about components of hypericum perforatum to fully understand the effect of the plant.
Constituents of Hypericum perforatum are as follows:

- Flavonoids (e.g. epigallocatechin, rutin, hyperoside, isoquercetin, quercitrin, quercetin , amentoflavone, biapigenin, astilbin, myricetin, miquelianin, kaempferol, luteolin)
- Phenolic acids (e.g. chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, p-hydroxybenzoic acid, vanillic acid)
- Naphthodianthrones (e.g. hypericin, pseudohypericin, protohypericin, protopseudohypericin)
- Phloroglucinols (hyperforin and adhyperforin)
- Tannins
- The main volatile compounds (e.g.2-methyloctane, nonane, 2-methyldecane, undecane, α-pinene, geraniol, myrcene, limonene, caryophyllene, humulene)
- Saturated fatty acids (isovaleric acid , myristic acid, palmitic acid, stearic acid)
- Alkanols (e.g. 1-tetracosanol, 1-hexacosanol)
- Vitamins & their analogues (e.g. carotenoids, choline, nicotinamide, nicotinic acid)
- Others constituents of the plant (pectin, β-sitosterol, hexadecane, triacontane, kielcorin, norathyriol) (https://en.wikipedia.org/wiki/Hypericum_perforatum Access date : 27.05.2020)

The naphthodianthrones hypericin and pseudohypericin along with the phloroglucinol derivative hyperforin are considered to be amongst the various effective ingredients. Essential oils that the plant contains mainly of sesquiterpenes are also among the constituents of hypericum perforatum. Chemical structures of some effective constituents of hypericum perforatum are Hypericin, pseudohypericin and hyperforin which are shown in figures 2, 3 and 4. (https://en.wikipedia.org/wiki/Hypericum_perforatum Access date : 27.05.2020)

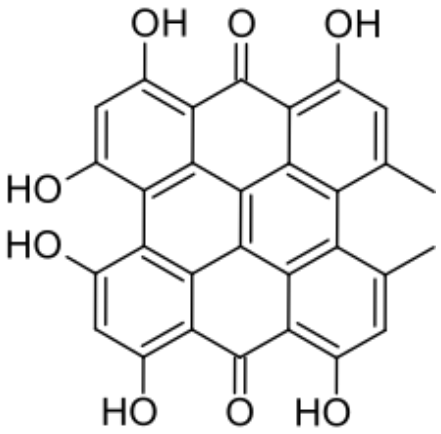

Figure 2 : Chemical structure of hypericin .
(https://en.wikipedia.org/wiki/Hypericum_perforatum Access date : 27.05.2020)

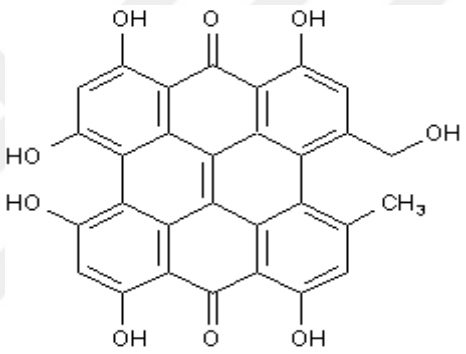

Figure 3 : Chemical structure of pseudohypericin .
(https://en.wikipedia.org/wiki/Hypericum_perforatum Access date : 27.05.2020)

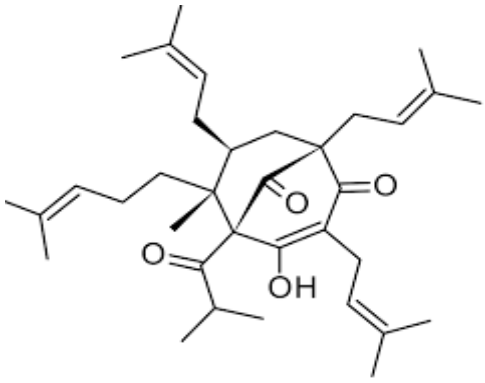

Figure 4: Chemical structure of hyperforin
(https://en.wikipedia.org/wiki/Hypericum_perforatum Access date : 27.05.2020)

2.2. Glioblastoma:

Glioblastoma is regarded as the most fatal and most common brain tumor. Despite all measures, the average survival of Glioblastoma patients is estimated for about 15 months under cure (Balça-Silva et al, 2018). Later progresses in genomic innovation have driven to a stronger understanding of key atomic modifications that underlie glioblastoma (GBM) (Aldape et al., 2015).

Most of the glioblastomas are IDH wild-type. (Isocitrate dehydrogenase is abbreviated as IDH). IDH 1 or 2 seem to describe molecularly the tumors which are related with young patients and more affirmative results. Molecular alterations caused by glioblastoma include mutations in genes organizing receptor tyrosine kinase. Medical therapies used for glioblastoma involves surgery, radiotherapy and chemotherapy with alkylating effect. Present therapeutic advances are based on attacking the molecular features which propel the malignant phenotype, including altered signal transduction and angiogenesis, and, lately, multiple immunotherapy strategies (Wirsching HG et al., 2016).

2.3. Effect of Therapeutic Herbs on Cancer Cells

There are many studies in the literature which supports the therapeutic effects of medicinal herbs on cancer cells. For example in a recent study it has been determined that Rosmarinus Officinalis, that is a natural compound, can help to get over toxicity caused by etoposide when it is used in combination. The cytotoxicity of etoposide on Glioblastoma cell cultures were not influenced by Rosmarinus Officinalis while on the other hand, induced proliferation and reduced the toxic effect of etoposide for MEF cell cultures. As a result, it can be regarded that Rosmarinus Officinalis protects the healthy cells against the side effects of etoposide without reducing its impact on the Glioblastoma cells (Ozdemir, Göktürk, 2017). A developing assortment of proof backings that curcumin is a promising anticancer medication. In preclinical models, curcumin has been appeared to have anticancer impacts, both alone and in blend with other anticancer medications (e.g., gemcitabine, 5-fluorouracil, and oxaliplatin), through the balance of an assortment of atomic targets. The poor bioavailability of curcumin has been the significant test to its clinical application. This issue has now been fathomed by the improvement of exceptionally bioavailable types of curcumin, which can incite higher plasma curcumin levels without expanded poisonousness. Further clinical preliminaries will be important to test the helpful uses of this promising operator in patients with pancreatic disease (Masashi Kanai, 2014). Ginger (Zingiber officinale) has been

demonstrated to be a significant restorative plant utilized in folkloric medication to fix different maladies in South Asia and the Middle East. Steady with this thought, the aftereffects of the study demonstrated that the ethanol extract of ginger intensely repressed, in a portion and time-subordinate way, the expansion of MCF-7 (ER-positive bosom malignancy cells) what's more, MDA-MB-231 (ER-negative breast malignant growth cells). The aqueous extract of ginger showed, though to a less degree, same probability. Subsequently, outcomes of the study show that ginger not just has a ground-breaking inhibitory impact on the expansion of ER-positive (MCF-7) bosom malignancy cells however likewise essentially restrains the development of ER-negative ones (MDA-MB-231). This end holds guarantee for additional in vitro and in vivo sub-atomic objective arranged examinations to analyze the chemoprotective viability of ethanol extract of ginger, especially for ER-negative breast tumors, which have a poorer prognosis and shorter endurance (Ayman I. Elkady et al.,2012).

2.4. One important Therapeutic Herb: Hypericum perforatum:

Exploratory conventions have been too in advance on restorative activity of hypericum perforatum against maladies of nowadays, like cancer, AIDS and hepatitis. In conformity with what is broadly supported, Hypericum perforatum is regarded as a link between the conventional and the alternative medicine. This herb has an unordinary conjunction of safe, effective treatment, with a broad scale of profits, without important side-effects and with a low cost (Istikoglou CI, Mavreas V, Geroulanos G., 2010).

One of the fundamental constituents of Hypericum is called hypericin that is a naphthodianthrone, an anthraquinone derivative. It is regarded that hypericin has a role as an antibiotic, antiviral and not specificied kinase inhibitor. Hypericin is considered as a medication candidate for cancer, it is reported that hypericin slowed down the increase in number of cells derived from a diversity of neoplastic tissues, including glioma, neuroblastoma, adenoma, mesothelioma, melanoma, carcinoma, sarcoma, and leukemia (Fox et al. 1998). In the presence of light and oxygen, hypericin plays a role like a simple photosensitizer, producing superoxide radicals which create peroxide or hydroxyl radicals, or singlet oxygen molecules which destroys cancer cells. By that method, it is thoudgt that hypericin has a role as a part of photodynamic therapy (PDT), (Agostinis et al. 2002). Initially, PDT is utilized in treatment only for treatment of lesions formed on skin, also nowadays it is enhancing progressively regarded as a medication for different sorts of cancers (Kenneth M. Klemow et al., 2011).

In a study the anti-proliferative and pro-apoptotic impacts of St John's Wort (SJW) were studied in human breast cancer (MCF-7) cells. MCF-7 cells were treated with several concentrations of SJW ethanol infusion solution (SJWE). Beside this MCF-7 cells were cultured with or without SJWE or hypericin, the active component of the herb, and after that viability and proliferation of the cells were analyzed. Neither hypericin nor SJWE displayed obvious toxicity in MCF-7 cells processed firstly for 24 h. A reduction is observed in proliferation depending on dosage of cells processed with SJWE for five days period. In comparison the development repressing impact of hypericin with which of SJWE in MCF-7 cells processed with hypericin and SJWE for five days period without photo-activation. It was seen that the proliferation suppression of hypericin was insignificant and poorer than that of SJWE (Mi-Kyoung You et al., 2018).

Jakobs D et al. aimed to test the effects of discrete ingredients of St John's wort infusion solutions on glioblastoma cells. Discrete components of St John's wort infusion solutions were analysed for potential impacts on the β1 AR density and a consequent alteration in downstream signalling in rat C6 glioblastoma cells. The results showed that process of C6 gliobastoma cells by using hyperforin and hyperoside deduces in a diminished β1 AR density in the plasma membrane and a subsequent diminished downstream signaling (Jakobs et al., 2013).

It was observed that hyperforin which is one of the principal active constituents of Hypericum had a decreaing impact on breast cancer cells in cell culture. Hyperforin induced releasing of cytochrome c from mitochondria and repressed tumor development in vivo. Researchers regarded that hyperforin had an important antitumor activity and didn't show toxic effects in vivo. So hyperforin was considered to be an interesting novel antineoplastic agent (Schempp et al. 2002). Different in vitro investigations demonstrated which hyperforin in junction with polyphenolic procyanidin B2 effectively suppressed the development of leukemia K562 and U937 cells, brain glioblastoma cells LN229, and natural human astrocytes (Hostanska, 2003).

3. MATERIALS AND METHODS

In this study it was intended to investigete the cytotoxic impacts of Hypericum perforatum infusion solution on U-87 MG cell line by using the materials and methods as clarified below.

3.1. Materials

Chemicals, consumables and instruments used in the study are given below.

3.1.1 Chemicals:

Chemicals utilized in the investigation is listed below.

- Phosphate Buffer Saline (PBS) (Sigma Aldrich P4417)
- Fetal Bovine Serum (FBS) (Life Technologies, Cat No:10082- 147)
- Dubelco's Modified Eagle's Medium (DMEM) (Life Technologies, Cat No: 22320-030)
- 3-4 5-DIMETHYLTHIAZ (MTT) (Sigma Aldrich 88417)
- Trypsin-EDTA (Sigma Aldrich T2605)
- Ethanol (Merck 818760)
- Isopropanol (Merck 109634)
- Distilled water

3.1.2. Consumables

Consumables used in this study are listed below.

- Petri dish
- Serological plastic pipettes, 5 ml
- Serological plastic pipettes, 10 ml
- Serological plastic pipettes, 25 ml
- Filter, nylon, sterile, 0.22 µm

3.1.3. Instruments

Instruments utilized in the investigation are listed below.

- Incubator (Nuaire)
- Biosafety cabinet (Nuaire)
- UV-VIS Spectrophotometer (Shimadzu)

3.2. Methods

The methods used in this study are shown in the Figıre 5 respectively.

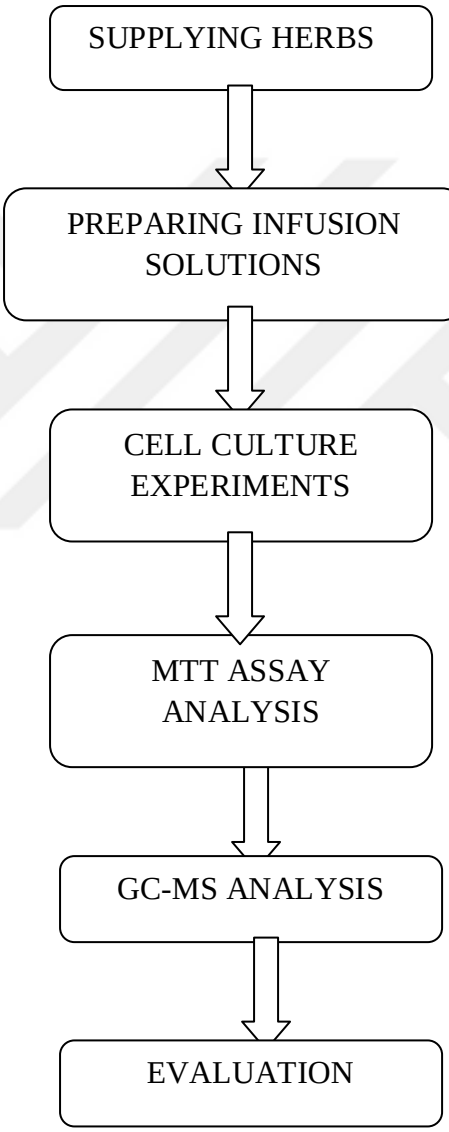

Figure 5: The methods used in this study

3.2.1. Preparing of Infusion Solutions:

The dried Hypericum perforatum was homogenized by grinding. 1g ± 0.01 of ground Hypericum perforatum was weighed and kept in 100 ml of at boiling point of water, ethanol and isopropanol for 10 minutes seperately. Boiling points of water, ethanol and isopropanol are 100 ºC, 78 ºC and 82,5 ºC respectively. The solutions were filtered through a roughing fitler paper. Sterilization is provided by filtering through sterile 0.22 µm filter.

3.2.2. Cell Culture Experiments

Experiments were executed in the human glioblastoma cell line U87MG (ATCC® HTB-14 ™). Cells are cultured in Dulbecco's Modified Eagle Medium (DMEM) with stable L-glutamine extended with 10% fetal bovine serum without antibiotics with incubation conditions, at 37°C and 5% CO_2 (Gokturk et al., 2018).

3.2.2.1. Cell Culture Passaging:

1. The proliferation, growth and surface coating of the cells in the Petri dish were checked under a microscope. For cells that grew and covered 80% or 90% of the surface of petri dish, a passaging procedure was initiated.
2. The materials neede were placed in the Class II cabinet and the materials were disinfected.
3. Disinfectant was added to the waste container and placed in the cabinet.
5. Cells were attached to surface of the petri dish. The old medium of the cells in the Petri Dish was removed by pipette.
6. 2.5 ml of PBS into the petri dish was added and washed the entire surface. PBS also helps to remove old culture medium (serum and culture medium) of cell culture. Because FBS inhibits trypsin activity, prevents the cells from getting up from the surface they hold, and allows the cells to adhere to the surface of the ground.
7. After washing with PBS, trypsinization was applied to cut off the contact of cells with the surface to which they are attached. 1.5 ml of trypsin / EDTA was added to the Petri Dish, the Petri Dish is waited in the incubator for 3-5 minutes.
8. The condition of the cells removed from the incubator in Petri dish was examined under a microscope. If necessary, mechanical shaking was applied by hand to remove adsorbed cells.

9. Growth medium (10% FBS + 90% DMEM) was added to the Petri Dish to diminish trypsin activity.
10. With the help of pipetting, the cells / cell assemblies were separated from the ground surface and from each other.
11. After pipetting, half of the solution in the petri dish was replaced with new medium (10% FBS + 90% DMEM) was added to the cells.
12. The Petri Dish was placed in the incubator and the cells in the new medium are expected to grow, proliferate and adequately coat the surface.
In Figure 6, GBM cells are seen before passage. In figure 7, GBM cells are seen after passage.
The photos in figure 6 and 7 are taken during laboratory studies.

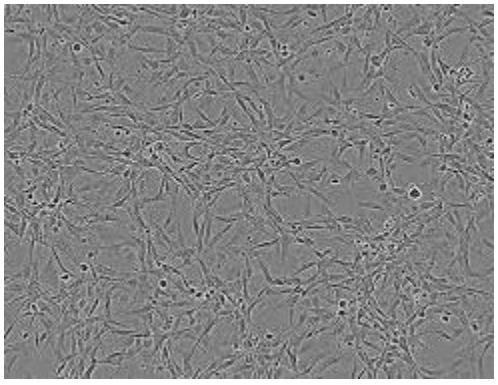

Figure 6: Glioblastoma (U-87 Mg) Cells before passaging

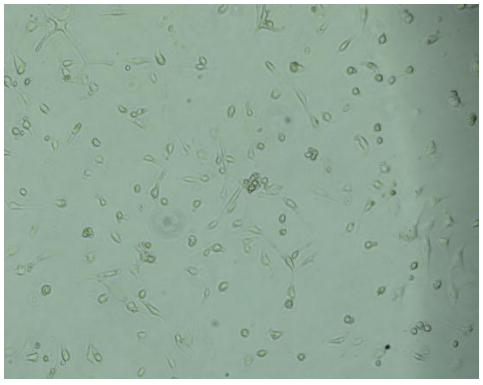

Figure 7: Glioblastoma (U-87 Cells after passaging

Ethanol and isopropanol extracts were added to Glioblastoma U-87 MG (ATCC® HTB-14 ™) cells developed in 24-well plates individually and simultaneously with 50 µM etoposide and incubated for six days.
After incubation period, cell viability was determined by MTT analysis. Cell viability percentages were calculated according to the control groups. Samples were studied in 3 parallel and using control groups.

3.2.3. MTT Assay

The basics of MTT assay is to degree cell practicality and multiplication for different in vitro measures of a cell population's reaction to outside variables. The reduction of tetrazolium salts is generally considered as a conscientious approach to test cell proliferation. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium

bromide) is diminished by metabolically dynamic cells, mostly by the activity of dehydrogenase proteins, to deliver decreasing reciprocals. Purple formazan shaped in cells is solubilized and evaluated by spectrometric strategies, like UV-VIS spectrophotometer.

For the MTT test, MTT is dissolved in PBS at the concentration of 0, 25 mg/ml. After the incubation period, removal of medium, 500 µL of MTT solution was transferred to each well. Cells were incubated for approximately 2 hours until purple MTT formazan crystals became visible (Figure 8). After the purple precipitate formed, 1000 µL of isopropanol was added to each well, and the purple precipitate was incubated until completely dissolved.
Absorbance values of the samples were measured by UV-VIS Spectrophotometer at 540 nm. The analyzes were conducted in 3 parallel sets.

Figure 8 Purple formazon crystals during MTT assay

UV/Vis spectrophotometer is the instrument used to measure the absorbance of the samples that are solubilized purple formazon crystals. Simplified schematic of UV–visible spectrophotometer is seen in Figure 9. It estimates the concentration of light after passing through a test and compares it to the concentration of light some time recently it goes through the test. As The Beer–Lambert law appears that the absorbance of a solution is straightforwardly corresponding to the concentration of the retaining species within the solution and the way length. Therefore, for a stable path

length, UV/Vis spectroscopy is utilized to assign the concentration of the absorber in a solution (Skoog, Douglas A et. Al, 2007).

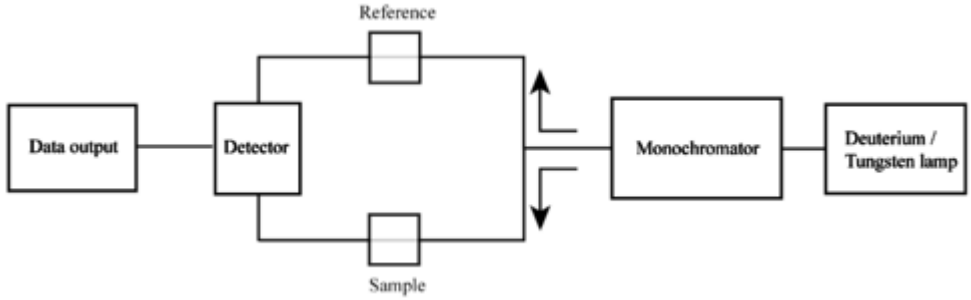

Figure 9 : Simplified schematic of UV–visible spectrophotometer (Skoog, Douglas A et. Al, 2007)

3.2.4. Experimental Set Up

In the table 1 and table 2 experimental set up is shown.

Table 1 Experimental Set Up – Ethanol Extract

	Plate 1		
Cell Line+Medium	Cell Line+1/10 EtOH	Cell Line+1/10 EtOH	Cell Line+1/10 EtOH
Cell Line+Medium	Cell Line+1/10 Extract	Cell Line+1/10 Extract	Cell Line+1/10 Extract
Cell Line+Medium	Cell Line+1/25 EtOH	Cell Line+1/25 EtOH	Cell Line+1/25 EtOH
Control Group	Cell Line+1/25 Extract	Cell Line+1/25 Extract	Cell Line+1/25 Extract
Control Group	Cell Line+1/100 EtOH	Cell Line+1/100 EtOH	Cell Line+1/100 EtOH
Control Group	Cell Line+1/100 Extract	Cell Line+1/100 Extract	Cell Line+1/100 Extract
	Plate 2		
Cell Line+Medium	Cell Line+1/10 EtOH +Etoposide	Cell Line+1/10 EtOH +Etoposide	Cell Line+1/10 EtOH +Etoposide
Cell Line+Medium	Cell Line+1/10 Extract +Etoposide	Cell Line+1/10 Extract +Etoposide	Cell Line+1/10 Extract +Etoposide
Cell Line+Medium	Cell Line+1/25 EtOH +Etoposide	Cell Line+1/25 EtOH +Etoposide	Cell Line+1/25 EtOH +Etoposide
Control Group	Cell Line+1/25 Extract +Etoposide	Cell Line+1/25 Extract +Etoposide	Cell Line+1/25 Extract +Etoposide
Control Group	Cell Line+1/100 EtOH +Etoposide	Cell Line+1/100 EtOH +Etoposide	Cell Line+1/100 EtOH +Etoposide
Control Group	Cell Line+1/100 Extract +Etoposide	Cell Line+1/100 Extract +Etoposide	Cell Line+1/100 Extract +Etoposide

Table 2 Experimental Set Up – Isopropanol Extract

	Plate 1		
Cell Line+Medium	Cell Line+1/10 PRO	Cell Line+1/10 PRO	Cell Line+1/10 PRO
Cell Line+Medium	Cell Line+1/10 Extract	Cell Line+1/10 Extract	Cell Line+1/10 Extract
Cell Line+Medium	Cell Line+1/25 PRO	Cell Line+1/25 PRO	Cell Line+1/25 PRO
Control Group	Cell Line+1/25 Extract	Cell Line+1/25 Extract	Cell Line+1/25 Extract
Control Group	Cell Line+1/100 PRO	Cell Line+1/100 PRO	Cell Line+1/100 PRO
Control Group	Cell Line+1/100 Extract	Cell Line+1/100 Extract	Cell Line+1/100 Extract
	Plate 2		
Cell Line+Medium	Cell Line+1/10 PRO + 25 µM.Etoposide	Cell Line+1/10 PRO + 25 µM.Etoposide	Cell Line+1/10 PRO + 25 µM.Etoposide
Cell Line+Medium	Cell Line+1/10 Extract + 25 µM.Etoposide	Cell Line+1/10 Extract + 25 µM.Etoposide	Cell Line+1/10 Extract + 25 µM.Etoposide
Cell Line+Medium	Cell Line+1/25 PRO + 25 µM.Etoposide	Cell Line+1/25 PRO + 25 µM.Etoposide	Cell Line+1/25 PRO + 25 µM.Etoposide
Control Group	Cell Line+1/25 Extract + 25 µM Etoposide	Cell Line+1/25 Extract + 25 µM. Etoposide	Cell Line+1/25 Extract + 25 µM.Etoposide
Control Group	Cell Line+1/100 PRO + 25 µM. Etoposide	Cell Line+1/100 PRO + 25 µM.Etoposide	Cell Line+1/100 PRO + 25 µM.Etoposide
Control Group	Cell Line+1/100 Extract +Etoposide	Cell Line+1/100 Extract + 25 µM.Etoposide	Cell Line+1/100 Extract + 25 µM. Etoposide

1/10 EtOH or PRO : Ethanol or Isopropanol is added to petri dish as it will be one tenth of total volume of culture medium.
1/25 EtOH or PRO : Ethanol or Isopropanol is added to petri dish as it will be one in twenty fifth of total volume of culture medium.
1/100 EtOH or PRO : Ethanol or Isopropanol is added to petri dish as it will be one percent of total volume of culture medium.

1/10 Extract : Extract is added to petri dish as it will be one tenth of total volume of culture medium.
1/25 Extract: Ethanol or Isopropanol is added to petri dish as it will be one in twenty fifth of total volume of culture medium.
1/100 Extract: Ethanol or Isopropano is added to petri dish as it will be one percent of total volume of culture medium.

Concentration of commercial Etoposide used in this study is 50mg/2,5 ml. As molecular weight of Etoposide is 588 g, concentration of the solution is calculated as 34mM. During the study Etoposide is added 7,5 µl of 34mM solution for each 10 ml of culture medium. The final consantration of etoposide in culture medium was 25 µM.

3.2.5. Content Analysis of Infusion Solutions

In order to analyze the content, gas chromatography-mass spectrometry (GC-MS) analysis of ethanol and propanol extracts were carried out using Agilent 7000 Series Triple Quad GC / MS apparatus by liquid-liquid extraction with dichloromethane. The GC-MS conditions are listed in Table 3.

About GC-MS

The Gas Chromatography/Mass Spectrometry (GC/MS) instrument isolates chemical blends (the GC component), categorizes and distinguishes the constituents at an atomic level (the MS component). The principle of GC is that a blend will be isolated into seperate substances when turned into gas phase. The heated gases are moved and transferred to a column by the help of an inert gas (such as helium). After the isolated components passes through the column, they stream into the MS. Mass spectrometry discriminates compenents by measuring the weight of the analyte atom. A

library of accepted mass spectra, counting a large number of components, is conserved on a computer. In figure 10 simplified schematic of GC-MS is given (Shubin Wu et. al, 2012).

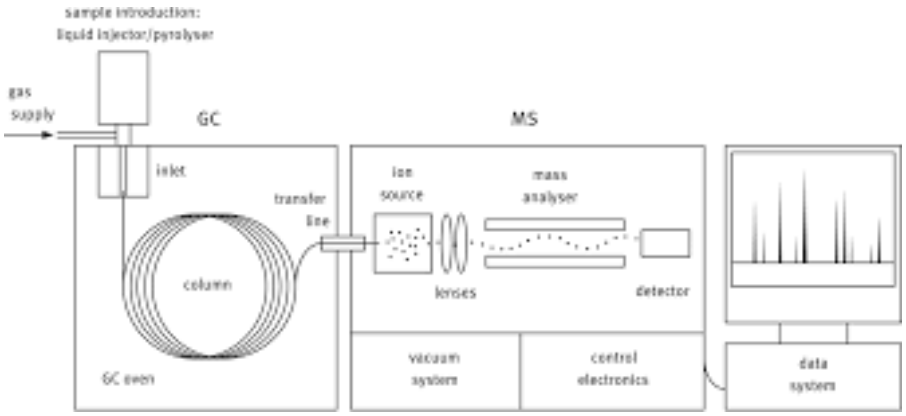

Figure 10: Simplified schematic of GC-MS (Shubin Wu et. al, 2012)

Table 3. GC-MS Conditions

System	Agilent 7000 Series Triple Quad GC-MS
Column	HP 5MS 30 m x 0,25 mm x 0,25 µm (5% phenyl methyl siloxane)
Column Temperature Programme	İnitial Temperature : 50 ºC Final Temperature : 240 ºC by acceleration 3 ºC / min
Injection System	Gerstel multipurpose sampler
Injection Volume	1 µl
Injection Temperature	250 ºC
Carrier Gas	Helium (Flow Rate : 1 ml/min.)
Split Flow	40 ml/min
Split Ratio	20
Dedector Temperature	290 ºC
Electron Energy	70 eV
Mass Spectrum	50-600 m/z

4. RESULTS AND DISCUSSIONS

The findings obtained from MTT tests are given in the graphs in Figures 11 and 12. It was observed that the cytotoxic effect of Hypericum perforatum extracts on the U87 Mg Glioblastoma cell line was dose, incubation period and solvent dependent. At the end of the 6-day incubation period of ethanol extract, 1/100 and 1/25 dilutions reduced the number of glioblastoma cells and also 1/25 dilution increased the cytotoxic effect of the etoposide. However, meaningful cytotoxic effect was observed for applied propanol extract dilutions (Figures 11 and 12).

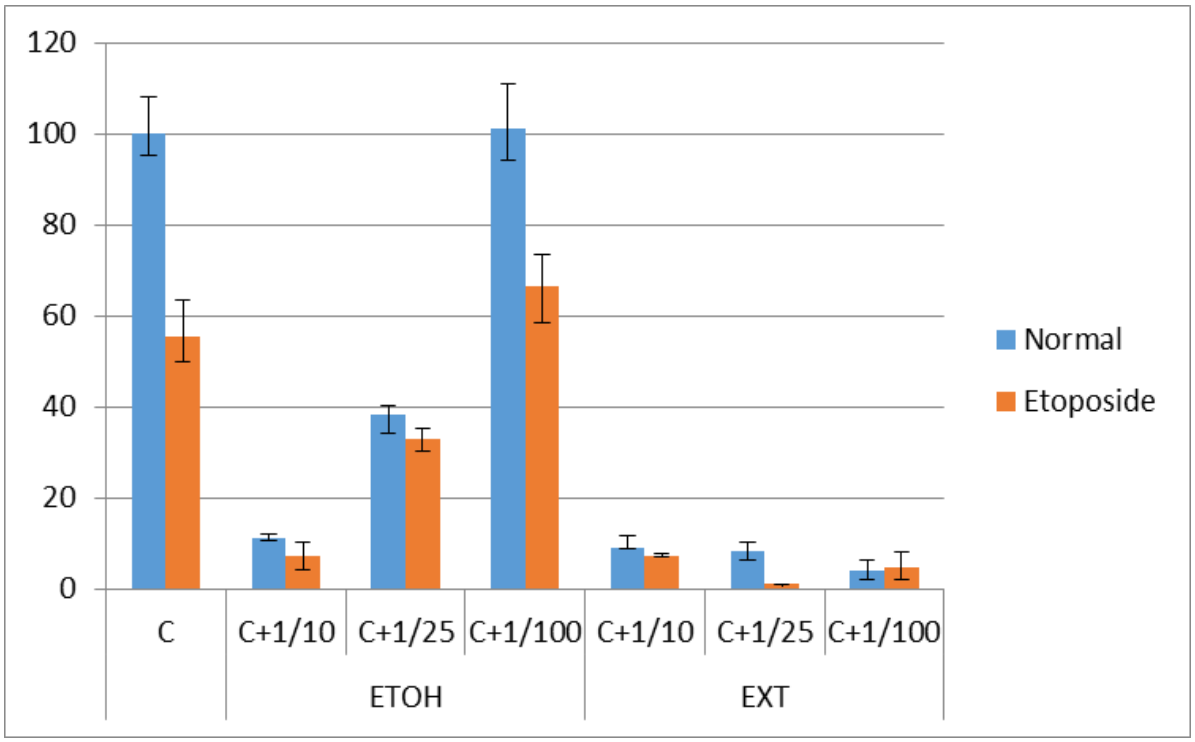

Figure 11: Effects of Etoposide and Hypericum Perforatum Ethanol Extracts on Glioblastoma U87 Mg Cells

C: Control, ETOH: ethanol, EXT: Extract. 1/10, 1/25 and 1/100: Dilution rates in medium

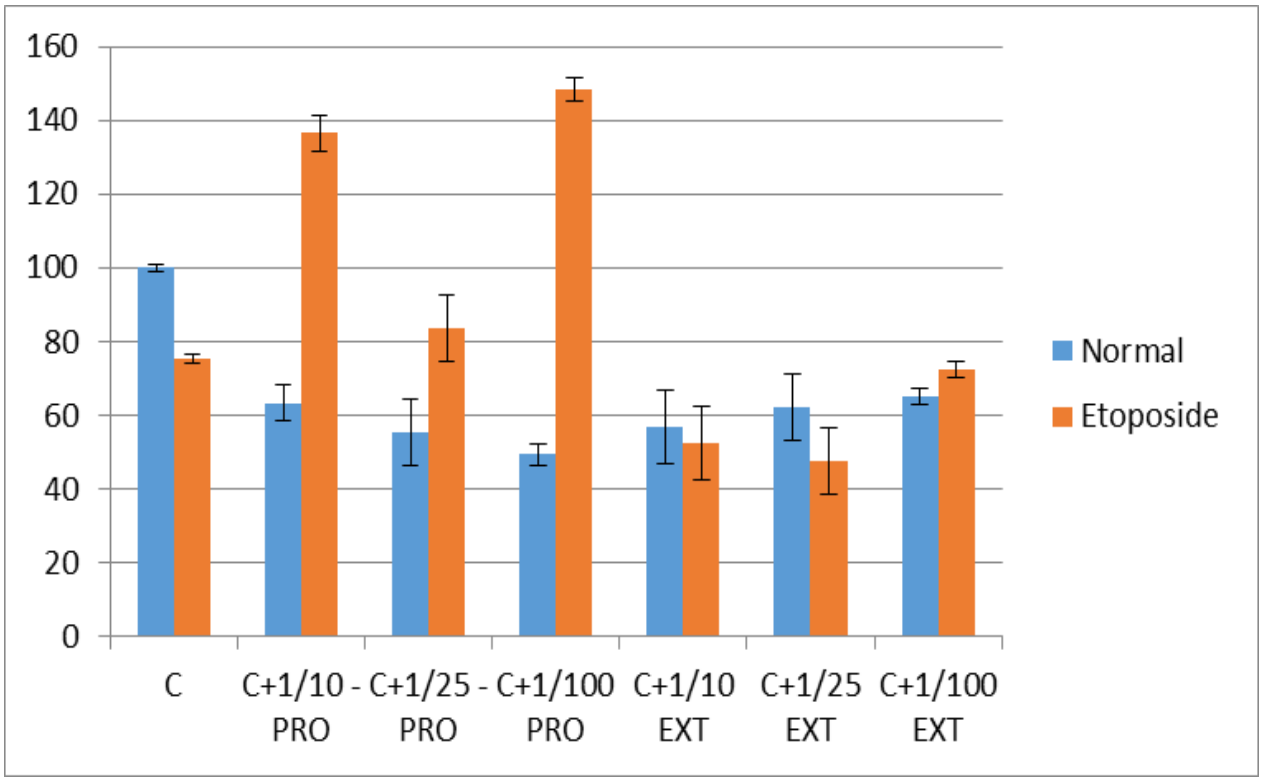

Figure 12: Effects of Etoposite and Hypericum Perforatum Isopropanol Extracts on Glioblastoma U87 Mg Cells

C: Control, PRO: Propanol, EXT: Extract. 1/10, 1/25 and 1/100: Dilution rates in medium

According to MTT assays, it was observed that 1/25 diluted ethanol extract tend to show the most effective results, decreasing the number of glioblastoma cells and increasing the cytotoxic effect of the etoposide.

The ethanol and isopropanol extract contents determined by GC-MS analysis are given in Tables 4 and 5. The main components of ethanol extract detected by GC-MS analysis are 1R-α-Pinene (11.79%), Astaxanthin / Tricyclo [5.4.0.0 (2,8)] undec-9-ene, 2,6,6,9-tetramethyl- (1R, 2S, 7R, 8R) (10.47%), Caryophyllene (9.81%), 9-Octadecenamide (49.28%) and 9,12,15-Octadecatrienoic acid, 2-phenyl-1,3-dioxan- 5-yl ester (10.22%), the major components of the isopropanol extract 1R-α-Pinene (11.79%), Astaxanthin / Tricyclo [5.4.0.0 (2.8)] undec-9-ene, 2,6 , 6,9-tetramethyl-, (1R, 2S, 7R, 8R) (10.47%), Caryophyllene (9.81%), 9,12,15-Octadecatrienoic acid, 2-phenyl-1,3-dioxane -5-yl ester (10.22%) and 9-Octadecenamide (49.28%).

Table 4: Hypericum perforatum GC-MS Analysis Results of Ethanol Extract

Component	Retention Time (min.)	Score	Score (Lib)	Height	Area	% Area
1R-α-Pinene	6,79143	88,56	88,56	679259	1575640	11,79
Astaxanthin/Tricyclo[5.4.0.0(2,8)]undec-9-ene, 2,6,6,9-tetramethyl-, (1R,2S,7R,8R)-	23,55102	81,39	81,39	360726	1399949	10,47
β Carotene	24,6471	69,61	69,61	49251	138365	1,03
Caryophyllene	26,40308	81,28	81,28	326495	1311752	9,81
1H-Cyclopenta[1,3]cyclopropa[1,2]benzene, octahydro-7-methyl-3-methylene-4-(1-methylethyl)-,[3aS (3a.alpha.,3b.beta.,4.beta.,7.alpha.,7aS*)]	28,88343	71,54	71,54	192802	768653	5,75
R-(-)-Cyclohexylethylamine	42,8213	72,42	72,42	49046	219296	1,64
9,12,15-Octadecatrienoic acid, 2-phenyl-1,3-dioxan-5-yl ester	51,13473	78,38	78,38	234577	1366733	10,22
9-Octadecenamide, (Z)	60,41898	80,12	80,12	633683	6588251	49,28

Table 5: Hypericum perforatum GC-MS Analysis Results of Isopropanol Extract

Component	Retention Time (min.)	Score	Score (Lib)	Height	Area	% Area
1R-α-Pinene	6,79927	81,14	81,14	303235	687178	13,12
Methyl (2E)-2-[16-(acetyloxy)-11-hydroxy-4,8,10,14-tetramethyl-3-oxogonan-17-ylidene]-6-methyl-5-heptenoate /R-(-)-Cyclohexylethylamine	7,46298	69,83	69,83	36646	118071	2,25
3'H-Cycloprop(1,2)cholesta-1,4,6-trien-3-one, 1'-carboethoxy-1'-cyano-1β,2β-dihydro-	17,96105	69,05	69,05	65340	325285	6,21
Zeaxanthin	23,56285	70,33	70,33	96012	345112	6,59
R-(-)-Cyclohexylethylamine	45,8221	73,24	73,24	66122	320100	6,11
9,12,15-Octadecatrienoic acid, 2-phenyl-1,3-dioxan-5-yl ester	51,12045	78,78	78,78	81583	326931	6,24
Octadecane, 3-ethyl-5-(2-ethylbutyl)-	56,05465	71,69	71,69	126455	1404764	26,83
Tertbutyloxyformamide, N-methyl-N-[4-(1-pyrrolidinyl)-2-butynyl]-	58,6905	72,56	72,56	143396	1709179	32,64

The different components of ethanol exract from the isopropanol extracts are listed below:

- Astaxanthin/Tricyclo [5.4.0.0(2,8)]undec-9-ene, 2,6,6,9-tetramethyl-(1R,2S,7R,8R)
- β Carotene
- Caryophyllene

- H-Cyclopenta [1,3]cyclopropa[1,2]benzene, octahydro-7-methyl-3-methylene-4-(1-methylethyl) [3aS(3a.alpha.,3b.beta.,4.beta.,7.alpha.,7aS)]

Molecular structures of these compounds are given below.

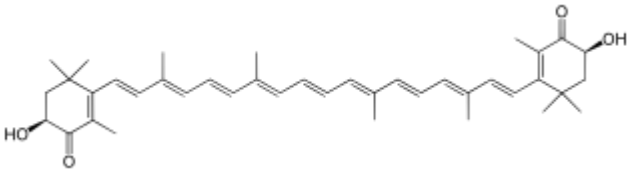

Figure 13 : Molecular structure of Astaxanthin (https://en.wikipedia.org/wiki/ Astaxanthin Access Date:22.05.2020

Figure 14 : Molecular structure of β Carotene (https://en.wikipedia.org/wiki/ β Carotene Access Date:22.05.2020)

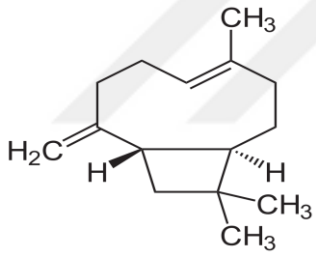

Figure 15 : Molecular structure of Caryophyllene (https://en.wikipedia.org/wiki/Caryophyllene Access Date:22.05.2020)

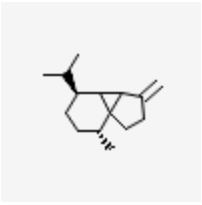

Figure 16 : Molecular structure of H-Cyclopenta

(https://www.ncbi.nlm.nih.gov/pcsubstance/?term=% 221H- Cyclopenta %5B1%2C3%5Dcyclopropa%5B1%2C2%5Dbenzene%2C%20octahydro-7-methyl-3-methylene-4-(1-methylethyl)-%2C%20%5B3aS- (3a.alpha.%2C3b.beta.%2C4.beta .%2C7.alpha.%2C7aS*) %5D-%22[CompleteSynonym]%20AND%206432083 [StandardizedCID] Access Date : 22.05.2020)

It is thougt that in our experiments the reason cytotoxic effect of ethanol exract was arised from these components. In literature our hypothesis is supported by several studies that these components show anti-cancer effects.

Astaxanthin (ASX) is known as a keto-carotenoid belonging to a broader group of chemical class called terpenes **(**Choi, Seyoung; Koo, Sangho, 2005). ASX has been used for treatment of Alzheimer's disease, Parkinson's disease, stroke, high cholesterol, liver illnesses, age-related macular degeneration (age-related vision loss), and preventing cancer **(**Dhankhar et al., 2012). ASX likewise demonstrates preclinical anti-tumor efficacy in vivo and also in vitro in different cancer models. It is demonstrated that Astaxanthin has anti-cancer effect in different sorts of cancer, that includes oral cancer, bladder carcinogenesis, colon carcinogenesis, leukemia and hepatocellular carcinoma. The anti-cancer impacts of ASX are evidently referred to its influences on the pathological technique of cancer cells through different processes with the inclusion of apoptosis, inflammation and cell junction. **(**Li Zhang and Handong Wang, 2015) The hidden reason for cancer is regarded damage to DNA, which is oxidative in nature. These oxidative procedures, the processes of which not completely comprehended, occur during the special phase of carcinogenesis. In this approach, it is conceivable that cell reinforcements might have the option to meddle with the metabolic enactment of compound cancer-causing agents, because relapse of pre-malignant lesions or hinder their improvement into cancer. ASX could have a significant role in decreasing cancer depending on its inflammation and oxidative stress decreasing effects. Its restricted bioavailability might decrease the effectiveness of the supplement. ASX's restricted bioavailability reduceses the ease of consolidation into the body. Applying an emulsification-evaporation technique, ASX was incorporated into a delivery system, which is named as nanosystems, to direct the bioavailability complication. These nanosystems enhance bioavailability by ascending the dissolution rate of ASX and the saturation solubility by decreasing size and ascending surface area. ASX has also been encapsulated for delivery via high-pressure homogenization and microchannel emulsification. Although bioavailability complication, the capability to analyze ASX as a supplement related with cancer like different illnesses is considerably improved (McCall et al., 2018)

Beta-Carotene is a natural retinol (vitamin A) precursor procured from several agricultural products with possible antineoplastic and chemopreventive functions. As beta carotene is an anti-oxidant, playing a significant role for inhibition of free-radical damage to DNA. (https://pubchem.ncbi.nlm.nih.gov/compound/beta-Carotene Access date: 27.05.2020)

Lycopene and beta-carotene are carotenoids generally dispersed in agricultural products, with possible anticancer effect. In a study human breast cancer cells were treated with carotenoids in concentrations between 0.5-10 μM for 2 days and 4 days period. To check cell viability MTT technique was utilized. Results indicated a critical reduction in the quantity of breast cancer cells which are treated with carotenoids. An expansion in apoptosis was seen in cells when treated with carotenoids (Nathalie Fonseca Gloria et al., 2014).

Caryophyllene which can also be named as β-caryophyllene, is a normally occuring bicyclic sesquiterpene which can be assumed as an ingredient of several essential oils. Caryophyllene is one of the components that are contributing to the aroma of black pepper. (https://en.wikipedia.org/wiki/Caryophyllene Access date: 27.05.2020) In a study it was mentioned that beta form of caryophyllene strengthens the anticancer impact of alpha-humulene, isocaryophyllene and paclitaxel. Beta-caryophyllene is a sesquiterpene broadly dispersed in essential oils of several plants. Some biological functions are credited to beta-caryophyllene, as an example, calming, anti-toxin, anticarcinogenic and sedative functions. In a study, the potentiating impact of beta-caryophyllene on the anticancer action of alpha-humulene, isocaryophyllene and paclitaxel against MCF-7, DLD-1 and L-929 human tumor cell lines were assesed. A non-cytotoxic centralization of beta-caryophyllene fundamentally extended the anticancer action of alpha-humulene and isocaryophyllene on MCF-7 cells (Legault J, Pichette A, 2007). Natural bicyclic sesquiterpenes, β-caryophyllene (BCP) and β-caryophyllene oxide (BCPO), can be found in most types of plants around the world. Both BCP and BCPO (BCP(O)) possess significant anticancer activities, impacting the development and proliferation of several types of cancer cells. On the other hand, their antineoplastic impacts have been studied in vivo. Additionally, all components potentiate the classical drug efficacionusness by augmenting the concentrations in the cells. The mechanisms causing the anticancer functions of the sesquiterpenes are weakly defined. BCP is a phytocannabinoid with potent inclination to cannabinoid receptor type 2 (CB 2), but not cannabinoid receptor type 1 (CB 1). In opposition, BCP oxidation derivative, BCPO, does not exhibit CB 1/2 binding, so the mechanism of its function is not in relation with endocannabinoid system (ECS) machinery. It is regarded that BCPO changes different key pathways for cancer development, like mitogenactivated protein kinase (MAPK), PI3K/AKT/mTOR/S6K1 and STAT3 pathways. Additionally, processing with the component decreases the expression of procancer genes/proteins, ascending the amounts of those with proapoptotic effects (Fidyt et al., 2016).

H-Cyclopenta[1,3]cyclopropa[1,2]benzene,octahydro-7-methyl-3-methylene-4-(1-methylethyl) - [3aS(3a.alpha.,3b.beta.,4.beta.,7.alpha.,7aS*)] also named as Beta-cubebene is a tricyclic sesquiterpene, a constituent of the leaf oil cubebene obtained from a variety of types of flowering plant. The component plays a role as a plant metabolite. It is a sesquiterpene and a carbotricyclic compound (https://pubchem.ncbi.nlm.nih.gov/compound/beta-Cubebene #section =ChemID plus Access date : 27.05.2020). In literature, beta-cubenene is not mentioned as a compound used for treatment of any diseases.

5. CONCLUSIONS

In this study the effect of infusion solutions of Hypericum perforatum in ethanol and isopropanol on Glioblastoma cell culture were investigated. In conclusion, the findings obtained in our study show that Hypericum perforatum ethanol extract components; Astaxanthin, Beta-Carotene, Caryophyllene and Beta-cubebene have the potential to be new, anti-proliferative agents against Glioblastoma. On the other hand isopropanol extract components are not regarded to be effective in treatment of Glioblastoma.

6. RECOMMENDATIONS

Despite the findings of our study is showed that Hypericum perforatum ethanol extract components: astaxanthin, beta-carotene, caryophyllene and beta-cubebene have a potential to be used in treatment of Glioblastoma, further research is needed to detect the molecular mechanism and detailed active substance interactions of the extract.

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Kurttepe Anatolian High School - (1993 - 2000)

Instruments That I am Able To Use

LC-MSMS (Applied Biosystem, Waters, Agilent)

GC-MS (Shimadzu, Agilent and Perkin Elmer)

GC (NPD, ECD, FID)

HPLC (DAD, UV-VIS, Pickering)

AAS (Perkin Elmer)

Spectrophotometer

General Laboratory Equipments

Certificates :

Health and Safety Engineer

Training:

No	Subject of Training	Trainer or Place	Duration of Training	Date
1	Analysis of Pesticides	Ministry of Agriculture	5 days	17-21.07.2006
2	ACQUITY UPLC TQ DETECTOR LC-MS/MS User's Training	LiKrom (Distributor Company)	1 day	07.07.2008
3	Fundamentals Of GC-2010 (SHIMADZU)	Ant-Teknik (Distributor Company)	2 days	10.11-09.2008
4	LECO FP528 Nitrogen-Protein Instrument User's Training	Dolunay (Distributor Company)	1 day	15.09.2008
5	Method Validation	Uzman Counseling Company	2 days	25-26.09.2008
6	Measurement Uncertainty	Uzman Counseling Company	2 days	10-11.01.2009
7	Basic Statistics For Food Analyses, Validation of methods and data processing, Significance tests, Statistical evaluation and interpratation of quality control in pesticide residue analysis, Estimation of uncertainty of measurement results	Prof. Dr. Arpad Ambrus	4 days	17-20.08.2009
8	Nitrite-Nitrate Analyses by using HPLC	Ministry of Agriculture	1 day	02.06.2010
9	MWSFour Microwave Digestion System User's Training	Ministry of Agriculture	1 day	03.06.2010
10	General Metrology	KAL-MER Calibration Laboratory	1 day	01.07.2010

11	Aanalyst 800 AAS (Perkin-Elmer) User's Training	TETRA (Distributor Company)	1 day	14.07.2010
12	Clarus 600 GC-MS (Perkin-Elmer) User's Training	TETRA (Distributor Company)	1 day	14.07.2010
13	Varian Tandem Gold LC-MS User's Training	DOLUNAY (Distributor Company)	8 days	14-21.07.2010
14	Fundamentals Of HPLC (SHIMADZU)	Ant-Teknik (Distributor Company)	2 days	16-17.07.2010
15	Method Validation, Quality Control and Accreditation	Songün DEMİREL - Consultant	3 days	27-29.07.2010
16	Agilent 6460 LC-MSMS User's Training	SEM (Distributor Company)	5 days	03-07.10.2011
17	Calculating Measurement Uncertainty for Chemical Analyses	Songün DEMİREL -Consultant	2 days	28-29.09.2011
18	Training of Pool Water	Akdeniz University	1 day	04.12.2011
19	ISO/IEC 17025 General requirements for the competence of testing and calibration laboratories	Sanilab Food Control Laboratory (Necati ALTINDİŞ)	1 day	11.01.2012
20	SGS Annual Integrity Training	SGS / Asım YAZICIOĞLU	1 day	15.01.2012-06.02.2013-17.04.2013
21	Practical Seminar Related to Quality	Dr. Günter LACH /	1 day	09.07.2012

	Assurance Issues	Silke BRUNS		
22	Spectrophotometer User's Training	Thermomed (Distributor Company)	1 day	11-12.07.2012
23	Agilent GC-MS User's and Application Training	SEM (Distributor Company)	3 days	16-19.07.2012
24	Practical Internal Audit Training	Necati ALTINDİŞ	3 days	07-09.08.2012
25	Sampling of Water and Waste Water	Ministry of Environment	3 days	17-19.12.2012
26	First Aid Training	Antalya First Aid Educational Centre	2 days	23-24.05.2013
27	Method Validation and Measurement Uncertainty	Ing-Dr.Hans Thomas MULLER	5 days	01-05.07.2013

EXPERIENCE (Total: 14 yrs)

June.2018-...	Wavin Plastics Company Health and Safety Engineer (24 months) Adana, Turkey
April 2016 - June 2018	Local Health and Safety Consultancy Company Health and Safety Advisor (25 months) Adana, Turkey
09.2014-03.2016	Kahramanmaraş Feed Analysis Laboratory Laboratory Team Leader (7 yrs) Kahramanmaraş, Turkey
07.2006-12.2013	SGS Supervise Ltd. Laboratory Team Leader (7 yrs) Mersin&Antalya, Turkey

Foreign Languages:

English (Advance Level)

German (Beginning Level)

Italian (Beginning Level)

Computer Skills

MS Office

References

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