TURKISH REPUBLIC OF NORTH CYPRUS

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CYTOTOXIC EFFECT OF *ORIGANUM ONITES* ESSENTIAL OIL ON HEPATOCELLULAR CARCINOMA (HepG2) CELLS

SUISUMWONI DEBORAH JARED

MASTERS THESIS

BIOCHEMISTRY

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SUPERVISOR

Assoc. Prof. Dr. Eda Becer

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DECLARATION

Hereby I declare that this thesis study is my own study, I had no unethical behaviour in all stages from planning of the thesis until writing thereof, I obtained all the information in this thesis in academic and ethical rules, I provided reference to all of the information and comments which could not be obtained by this thesis study and took these references into the reference list.

Suisumwoni Deborah Jared

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First of all, my appreciation goes to God Almighty for making it possible for me to complete this thesis.

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ABSTRACT

CYTOTOXIC EFFECT OF *ORIGANUM ONITES* ESSENTIAL OIL ON HEPATOCELLULAR CARCINOMA (HepG2) CELL.

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Thesis Supervisor: Assoc. Prof. Dr. Eda Becer

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Origanum onites is a plant commonly found in Greece and Turkey. It has been utilized locally for the treatment of different diseases such as gastrointestinal diseases, diabetes and different cancer. It is commonly called Turkish oregano. The plant is rich in essential oil and the oil is said to have antioxidant and anti-cancer activities due to the presence of carvacrol and thymol in the essential oil. The aim of this study is to determine the cytotoxic effect of the essential oil of *Origanum onites* on HepG2 cell line. Cell viability and cytotoxicity was measured using MTT assay with different concentrations (100 µg/ml- 500 µg/ml) of *Origanum onites* essential oil. There was a significant decrease in cell viability at the 500 µg/ml compared to the 100 µg/ml treated cells after 24hrs, and it further decreased after 48hrs. In conclusion, *Origanum onites* essential oil is cytotoxic to HepG2 cells dose and time dependently and might have the potential to be used for the treatment of hepatocellular carcinoma.

Key words: *Origanum onites,* Hepatocellular carcinoma, HepG2 cell, cytotoxic

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Abbreviations

ACTL6B- Actin-like protein 6B

ALP Alkaline phosphatase

ALT Alanine transaminase

ARID1- AT-rich interactive domain-containing protein 1A

AST Aspartate transaminase

BMP4 - Bone Morphogenetic Protein 4

CCL20 gene - C-C Motif Chemokine Ligand 20

CCND1- Cyclin D1

CDKL2- Cyclin-dependent kinase-like 2

CDKN2A- cyclin-dependent kinase Inhibitor 2A

CTNNB1- Catenin beta-1

DDX11-AS- 1DDX11 Antisense RNA 1

DPPH- 2,2-Diphenyl-1-Picrylhydrazyl

EGF - Epidermal growth factor

EMILIN2 gene- Elastin Microfibril Interfacer 2

FBS- Fetal bovine serum

FGF- Fibroblast growth factors

GSH Reduced glutation

GSTP1- Glutathione S-transferase P

HBV- Hepatitis B virus

HCC - hepatocellular carcinoma

HepG2 - liver hepatocellular cells

HIST1H4F- Histone H4

HULC - highly up-regulated in liver cancer

IRF2- Interferon regulatory factor 2

lncRNA-Dreh - Down-regulated expression by HBx

lncRNA-HEIH - Long noncodingRNA High Expression In HCC

lncRNAs- Long noncoding RNAs

MDA Malondialdehyde

MGMT - Methylguanine-DNA methyltransferase

miRNA - MicroRNAs

MTT- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAT- N-Acetyltransferase

NEFH- Neurofilament Heavy

NFATC1- Nuclear factor of activated T-cells

O.onites- *Origanum onites*

PRPH- Peripherin

RPS6KA3- Ribosomal protein S6 kinase, 90kDa, polypeptide 3,

SMPD3- Sphingomyelin phosphodiesterase 3

TGF - Transforming growth factor (TGF)

TGF β - Transforming growth factor beta

TM6SF1 gene - Transmembrane 6 Superfamily Member 1.

TP53- Tumor protein p53

INTRODUCTION

Origanum (Lamiaceae) is a genus characterized in Turkey by 27 species or 31 taxa (Celep & Dirmenci, 2017). It is found mostly in Turkey, Greece, Aegean islands and Silicy (Baser and Arslan, 2016). Turkey is the leading provider of oregano to the world markets and *Origanum* species are utilized in cooking and in traditional medication and the dried leaves are utilized as seasoning and herbal tea (Baser, 2008, Nazim et al., 2006). Dundar et al., (2008) likewise stated Oregano has been utilized as an energizer, asnalgesic, antitussive, expectorant, narcotic, antiparasitic, and antihelminthic in Turkish traditional treatment, and it is generally utilized for gastrointestinal problem.

Linalool, carvacrol, thymol, linalool p-cymene, terpinen-4-ol and gamma-terpinene are the significant components of *Origanum onites* volatile oil with thymol and carvacrol being the main phenolic constituents and the core source of antioxidant activity (Aysun and Ayşe 2011). The antioxidant result of such essential oils is credited to their most important constituents, carvacrol and thymol, and it is the consequence of different potential mechanisms including free-radical scavenging activity, transition-metal-chelating activity, and additionally singlet-oxygen quenching capacity (Shan et al., 2005).

Essential oil distilled from *Origanum onites* and its two principle components showed antioxidant effect and anticancer properties for HepG2 cells at lesser and greater concentrations respectively. (Aysun and Ayşe, 2011). Curiously, *Origanum onites* essential oil was tried on four human malignant growth cell lines and it indicated a dose dependent antiproliferative impact against all the four cell lines. The most grounded impact was seen in the colon cancer (HT-29) cell line, after which skin melanoma (A375), then breast carcinoma (MCF-7), and finally, hepatocellular carcinoma cells (HepG2) (Spyridopoulou et al., 2019). At minimal concentrations, the essential oil from *Origanum onites* and carvacrol showed antiproliferative and apoptotic activity in hepatocellular carcinoma cells (HepG2) (Hülya and Özlem, 2011). Oral administration of *Origanum onites* essential oil for 13 days fundamentally repressed the development of CT26-tumor colon carcinoma

in BALB/c mice contrasted with control animals that were getting corn oil (vehicle) in a similar dose plot (Spyridopoulou et al., 2019).

Different species, for example, essential oil from *Oregano vulgare* also showed lower cytotoxic action against non-tumor cell line HEK293, particularly within a range of 100 to 800µg/µL, while the cell viability of hepatocarcinoma cell line was altogether diminished dose dependently within the range 25–800 µg/µL (Hazem, 2017). Feyza and Ibrahim (2017) proposed that *O. acutidens* volatile oil might be a promising natural agent for improving the development of HeLa and HT-29 cells lines, as their outcome demonstrated that the oil cytotoxically affected both tumor cell lines, and this impact was because of the existence of carvacrol which is its principle constituent.

Hepatocellular carcinomas (HCCs) is the most widely recognized sort of liver disease and one of the main source of malignant growth related mortality around the world, and its occurrence and death rate is the most increasing contrasted with different kinds of malignant growth (Sia et al., 2017, Ferlay et al., 2012). HCC tumors emerge from a foundation of various incessant pathological conditions, for example, liver cirrhosis and fibrosis which are most as often as possible related with hepatitis B or hepatitis C infections, alcoholic liver maladies or metabolic issues thus they are genetically and phenotypically exceptionally heterogeneous (Castelli et al., 2017). The opposition of HCC to conventional anticancer treatment, for example, clinical resection, liver transplantation and radiotherapy brings about high pace of mortality and poor patient results (Huang et al., 2018).

The utilization of natural products has been given a great deal of consideration lately because of their capacity to provide prevention and remedial impact against numerous malignant growths (Ruhul et al., 2009).

The aim of this study is to determine the cytotoxic effect of *Origanum onites* essential oil on Hepatocellular carcinoma (HepG2) cell line.

GENERAL INFORMATION

2.1 Origanum onites

Origanum onites regularly known as Turkish oregano is a perennial plant with woody stems. It has periodic dimorphism, a typical adaptive approach which empowers the woody plants arising in phryganic environments to endure the late spring dry spell. Appearance of two kinds of leaves; little ones during summer and enormous ones during winter is the principle qualities of this approach. It blooms from May to June (Vokou et al., 1998).

For quite a while, this species has generally been utilized as hot herb due to its fragrance and flavor to improve the flavor of nourishments. *O. onites* has a sharp, peppery flavor and a fragrance like thyme (Figure 2.1). It is one of the flavorings in Turkish enjoyment treat and can be utilized new or dried in tea, servings of mixed greens and meat dishes. Its strong taste complements Greek dishes and meats (Meyers, 2005).

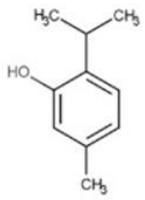
Figure 2. 1 *Origanum onites*. (Hsa, Herb Society of America Archives)

Different preparations are likewise utilized in pharmaceutical and cosmetic enterprises (Bakkali et al., 2008). As indicated by U.S. food and Drug Administration (USDA) (2002), *O. vulgare, O. onites* (pot marjoram), *O. majorana* (sweet marjoram) and *O. dictamnus* (dittany of Crete) are considered GRAS (Generally Recognized as Safe).

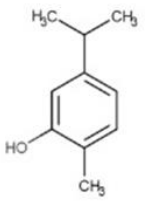
Additionally, according to USDA investigation (2003), one teaspoon of dried marjoram has 2 calories, 0.04g of fat, 36g of starch, 0.08g of protein, 0.2g of fiber, 12mg of calcium, 9mg of potassium and 48 IU of vitamin A, and a variety of different nutrients and minerals. A similar amount of dried oregano is more nutritious with 6 calories, 0.2g of protein, 0.18 grams of fat, and 1.16g of sugar and 0.8g of fiber. It likewise contains 28mg of calcium, 30mg of potassium, and 124 IU vitamin A.

Aromatic herbal parts of *Origanum* species are utilized as condiment or herbal tea. The leaves and blooming portions of the plant comprises oil glands that secrete essential oils and the plant's ability to produce fragrance (Talbert, 2004). Dried *Origanum* species are also utilized for the production of volatile oil (*Origanum* oil) and an aromatic water or hydrosol (*Origanum* water) (Baser, 2002).

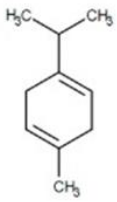
Revealing the principle constituents of the essential oil, their wellbeing and their restorative incentive in ailment treatment will help in drug advancement and medication focusing on treatment. Essential oils are lipophilic in nature which allows them to apply their impact by interacting with intracellular proteins as well as intra-organelle site due its ability to cross the plasma membrane (Ka et al., 2003). The oil from *Origanum onites* contains 6 significant constituents carvacrol, thymol, linalool p-cymene, terpinen-4-ol and gamma-terpinene (Özkan & Erdogan, 2011) (figure 2.2).



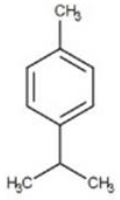
Thymol



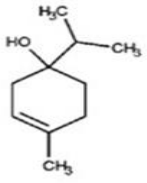
Carvacrol



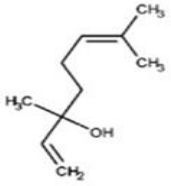
γ-Terpinene



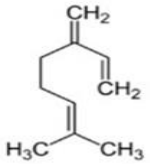
p-Cymene



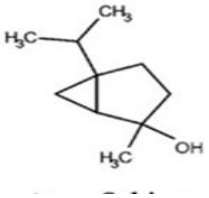
Terpinen-4-ol



Linalool



β- Myrcene



trans-Sabinene hydrate

Figure 2. 2 Some Main Constituents of *Origanum* Essential Oil. (Leyva-López et al., 2017).

Monoterpenes are exceptionally hydrophobic substances present in plant essential oils. Similarly as with numerous herb oils, the significant components of *Origanum onites* essential oil are carvacrol (71.2%) and thymol (6.0%) (Azcan et al., 2000).

Carvacrol (2-methyl-5-(1-methylethyl) phenol), a cyclic monoterpene, is a component of the oil of oregano. The method of activity of carvacrol has received a great deal of enthusiasm from specialists due to its utilization in seasoning, and furthermore as an antibacterial or antifungal operator in food conservation strategies (Baser 2008, Ultee 1997). Thymol (5-methyl 2-(1-methylethyl) phenol) is an isomer of carvacrol, having the hydroxyl group at an alternate area on the phenolic ring. Carvacrol and thymol being hydrophobic interact with the lipids of the cell membrane and mitochondria, rendering them porous and prompting spillage of the cell components (Lambert 2001).

2.1.1 Essential oil

The term 'Essential oils' was first utilized five centuries ago by Paracelsus von Hohenheim, who named the active component of a medication 'Quinta essential', essential oils from aromatic plants, are formed as secondary metabolites of plants. They have since been utilized for their additive and curative properties since olden days (Edris, 2007).

The physical role of secondary metabolites found in plants is to defend plants from microbes, fungi, infections, insects and even against herbivores, by decreasing their craving for these plants, and these oils are separated from plants mostly by refining (Bakkali et al., 2008).

We are able to separate the distinctive taxa of O*riganum* into 3 categories depending on their volatile oil composition.

1. *Origanum* having a fewer oil content below 0.5% (ml/100 g dry weight), for instance the Greek endemic *O. calcaratum* (Karousou. 1995).
2. The oil taxa which contains somewhere in the range of 0.5 and 2%, for instance *Origanum microphyllum* known as 'Cretan marjoram' (Karousou 1995).
3. Rich essential oil of *Origanum* containing over 2% oil, for example the two common industrially utilized 'oregano' plants, *O. vulgare subsp. hirtum* (Greek oregano) and *O. onites* (Turkish oregano) (Vokou et al., 1988, 1993).

2.1.2 Cultivation in Turkey

There are distinctive planting patterns based on inter-row distances and quantity of rows within a plot. Air- dried plant harvest and sum of harvests fluctuated by areas relying upon the measure of nitrogen fertilization applications and planting designs however it did not influence the essential oil content and likewise the chemical compound proportion of the oil constituents, for instance, carvacrol (Ceylan et al., 1994). The cultivation of *O. onites* in the Izmir area started in 1990 as an alternative crop to the ailing tobacco farming industry and has become successful.

Cultivated areas reached 9428 ha in 2012, with a crop yield of 11,598 tons (Baser and Arslan 2016).

2.1.2.1 Propagation

An investigation of *Origanum onites* cultivation reveals it has an annual establishment period, though its economic life expectancy is generally 6 years. The economic life expectancy is determined starting from the time of crop generation to when variable expenses equates gross income. Complete expenditures (from planting to sifting) and all out income are practically proportional to one another in the primary year (Kitiki, 1996).

2.1.2.2 Harvesting and Handling

The preferable planting pattern and most appropriate expanse for automation is 45 cm between rows. The most noteworthy measure of essential oil in *O. onites* is reached when half of the plants in the field have initiated blooming. Reaping is done physically in little fields while mechanical collecting is suggested uniquely for enormous fields. In the wake of collecting, plants should then be air-dried. A 25-cm stack height is favoured during drying activities so as to encourage the aggregation of essential oil content. In spite of the fact that drying under normal conditions is a typical methodology, drying stoves working at 30-35°C can likewise be utilized in business scale generation. A dampness content of at least 7% to at most 12% is necessary (Kitiki, 1996).

2.1.2.3 Threshing

Splitting dried leaves and spike-like inflorescences from stems is carried out physically and joined sifting machines are utilized for large production. Dried leaves ought to be preserved in states of cool and moderately low humidity, as percentage of the oil progressively diminishes following 4-5 months of storage. In Turkey, business

organizations managing oregano are normally well equipped as to appropriate yield handling (Kitiki, 1996).

2.1.3 Medicinal Properties

Studies reported that aerial portions of *O. onites* are been utilized and infusions acquired from these aerial region are successful for treating respirational tract infections, gastro-intestinal illnesses, kidney issues, diabetes, cholesterol, rheumatism, different malignant growth, cardiovascular issues and injury (Sargin et al 2013, Gurdal and Kultur 2013). Decoctions of *Origanum onites* have likewise been utilized for abdominal ache, toothache, headache, diabetes as well as itching (Polat and Satil, 2012). Water concentrate together with and essential oil have similarly been utilized for the treatment of numerous infirmities, for example, convulsive coughs, stomach related issue, menstrual issues, diabetes, and elevated cholesterol (Bostancio et al., 2002).

They are additionally utilized as stimulant, pain relievers, antitussive, expectorant, narcotic, antiparasitic, antihelmintic, topically as a general germicide, astringent, and as an oral antiseptic for gargling (Bakkali 2008, Sharifi-Rad et al. 2018, Baser, 2008).

2.1.4 Phytochemistry

In one study, essential oil from *Origanum onites* was found to have, a sum of 134 terpenoids (twenty five monoterpene hydrocarbons, sixty seven oxygenated monoterpenes, twenty seven sesquiterpene hydrocarbons, and fifteen oxygenated sesquiterpenes), six phenylpropanoids, as well as twenty six different constituents. ten phenolics, three triterpene acids, four sterols, one quinone, 14 flavonoids and associated compounds, seven hydrocarbons, five porphyrins, 14 unsaturated fats, too fourh tocopherols (table 1), including inorganic compounds as well as minerals (B, Ca, Cu, Fe, K, Mg, Mn, N, Na, P, and Zn) was portrayed in *Origanum onites*. Research on Phytochemicals present in *Origanum onites* discloses the extent to

which *Origanum onites* is opulent in terpenoids, particularly monoterpene hydrocarbons and oxygenated monoterpenes (Bektas, 2016)

Table 1 Fatty acids present in *Origanum onites* essential oil (Azcan et al., 2004)

	Seed	
Oil yield	*o. onites* a (%) 20.0	*o. onites* b (%) 14.1
FA	%	%
Caprylic (8:0)	Trace	Trace
Pelargonic (9:0)	Trace	Trace
Capric (10:0)	Trace	Trace
Lauric (12:0)	Trace	Trace
Myristic (14:0)	0.1	0.1
Pentadecanoic (15:0)	0.1	0.1
Palmitic (16:0)	6.5	5.9
(*Z*)-9-Hexadecenoic (16:1)	0.1	0.1
(*Z*)-7-Hexadecenoic (16:1)	0.1	0.1
Margaric (17:0)	Trace	Trace
Stearic (18:0)	2.4	2.1
(*Z*)-9-Octadecenoic (18:1)—Oleic	8.7	8.9
(*Z*)-11-Octadecenoic (18:1)	0.6	0.8
Linoleic (18:2)	21.7	21.5
a-Linolenic (18:3)	56.3	57.0
Arachidic (20:0)	0.2	0.2
11-Eicosenoic (20:1)	0.2	0.2
S Saturated	9.8	9.1
S Unsaturated	87.7	88.6

a From Mugla, Turkey.
b From Antalya, Turkey, Trace <0.1%

2.1.5 Hepatoprotective Effect

Carvacrol acquired from *Origanum onites* essential oil builds the recovery of liver experiencing fractional hepatectomy because liver regeneration (calculated measuring the weights of their liver before and after the hepatectomy), mitotic index and proliferating cell nuclear antigen (PCNA) index increased significantly in rats treated with carvacrol compared to those treated with saline at the 72nd hour after

partial hepatectomy (Uyanoglu et al., 2008). There was a significant difference in AST, ALT, GSH, MDA and CAT levels in the blood of rats treated with carvacrol from *Origanum onites* essential oil compared to those treated with physiological serum and silymarin after being subjected to 45 min long liver ischemia and 60 min reperfusion, implying that Carvacrol secures the liver against defects brought about by ischemia and reperfusion and it's not hepatotoxic at the applied dosage (Canbek et al., 2008).

2.1.6 Antioxidant Capacity

Various research have indicated that phenolic compound existing in essential oil is the primary source of its cancer prevention agent action (Dorman et al., 2000, Ruberto and Barata, 2000). Aydin et al., (2005) additionally detailed that phenolic compound such as thymol and carvacrol, at concentrations beneath 0.2 and 0.1 mM, respectively, decreased harm ensuing from oxidation in human lymphocytes. *Origanum onites* oil has cytoprotective (cancer prevention agent) impacts against hydrogen peroxide-prompted cytotoxicity and membrane damage in hepatocellular carcinoma (Hep G2) cells. It likewise diminished the malondialdehyde (MDA) level (Özkan and Erdogan, 2011). Antioxidant capacity is tried utilizing DPPH radical scavenging activity and the linoleic acid oxidation restraint. It was impressing that *Origanum onites* essential oil indicated high DPPH radical scavenging activity and high linoleic acid oxidation hindrance rate (Özkan and Erdogan, 2010) Aqueous distillate of *Origanum onites* demonstrated gainful impacts on lipid profiles, antioxidant status and endothelial function within patients having gentle hyperlipidaemia (Ozdemir et al., 2008).

2.1.7 Anticancer Activity

O. onites diminished the ability of HepG2 cell to survive at higher concentrations (Özkan and Erdogan, 2010). Zeytinoglu et al. (2003) considered the monoterpenic phenol, carvacrol, distilled from *Origanum onites* and reported that it

repressed the DNA synthesis of N-ras altered mouse myoblast cells CO25 in development medium and ras-initiating medium containing dexamethasone. Since their outcome indicated that the development of myoblast cells was repressed considerably after enactment of transformed N-ras-oncogene, they recommended that carvacrol may find application in malignancy treatment. Ipek et al. (2003) additionally reported that carvacrol from *Origanum onites* could be utilized as an antigenotoxic agent since it did not expand arrangement of sister-chromatid-exchange (SCE) yet diminished the pace of SCE incited by mitomycin C utilizing the in vitro sister-chromatid-trade (SCE) measure on human peripheral leukocytes.

Essential oil of *Origanum* o*nites* and its primary constituent carvacrol have pharmacological significance (Table 3) for the counteraction of malignant growth as it indicated solid inhibition against mutagenicity initiated by 4-nitro-o-phenylenediamine and 2-aminofluorene utilizing Salmonella typhimurium strains TA98 and TA100 (Ipeket al., 2005).

Anti-angiogenic effect of *Origanum onites* oil was reported by Bostancioglu et al. (2012). It was discovered that *Origanum onites* essential oil could extraordinarily restrain cell ability to live and incited cell death of 5RP7(c-H-ras transformed rat embryonic fibroblasts) cells and likewise could obstruct in vitro tube arrangement and movement of RATEC (rodent fat tissue endothelial cells) (Bostancioglu et al., 2012).

Table 2 pharmacological effects of *Origanum onites*

Biological activity	References
Larvacidal	Huseyin et al 2007
Hepatoprotective	Uyanoglu et al., 2008
Antioxidant	Ozdemir et al., 2008
Acaricidal	Coskun et al., 2008
Anti protozoal	Deniz et al., 2019
Antimutagenic	Ipek et al., 2005
Antigenotoxicity	Ipek 2003

Cholinesterase Inhibitory	Orhan et al., 2008
Antimicrobial	Altintas et al., 2013, Orhan et al., 2011
Antifungal effect	Sokovic et al., 2002

2.1.8 Other activities

For over 15 years now, the use of plant as insect repellent has been of incredible interest because of ecological concerns and resistance of insects to chemical compounds. Various effects of plant derivatives on insect have been discovered (Isman, 2000). Huseyin and Atila (2006) detailed about volatile oils distilled from two *Origanum* species, *Origanum onites* and *Origanum minutiflorum*, he recorded that they were very toxic to third/fourth-instar hatchlings of the mosquito, *Culex pipiens L*. (Diptera: Culicidae). The volatile oils of *Origanum onites* and *Satureja thymbra* demonstrated profoundly powerful insecticide effect against mature stored product insects, Mediterranean flour moth *Ephestia kuehniella Zeller* (Lepidoptera: Pyralidae) and the Indian supper moth *Plodia interpunctella Hübner* (Lepidoptera: Pyralidae) with 100% death obtained after 24h of being exposed to essential oil vapours at 9 and 25 µl/l air for both insects. (Ayvaz et al.,2010).

Origanum onites oil and its two primary constituent carvacrol and thymol was larvicidal against the fourth/fifth-instar larvae of pine processionary moth (PPM), *Thaumetopoea wilkinsoni* (Huseyin et al 2007) *T. wilkinsoni* causes solid hypersensitive responses in people and other warm-blooded creatures. This creepy crawly is of therapeutic importance and a significant woodland pest (Bruchim et al., 2005). The *O. onites* oil was tolerably dynamic against *Plasmodium falciparum* and *Leichmania donovani*, yet showed great effectiveness against trypanomastigote types of African trypanosome *Trypanosoma brucei rhodesiense* (Deniz et al., 2019). Essential oil from *O. onites* L. can possibly be used at sensible concentrations to control tick invasions (Coskun et al., 2008). *Origanum onites* essential oil repelled 100% of the ticks tried at 0.103 mg oil/cm^2 of filter paper and 66.7% of the ticks at 0.075 mg oil/cm^2 filter paper. Thymol repelled 66.7% of the ticks compared to 28.7% by carvacrol at that same concentration (John et al., 2017).

2.2 Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is the most widely recognized tumor of the hepatocytes. HCC is a critical reason for disease and mortality. It conveys a negative diagnosis with violent development conduct and a high pace of repeat (Poulou et al., 2015). Bray et al., predicted liver malignancy as the 6th most regularly analyzed malignancy and the fourth driving reason for malignant growth demise around the world, with a yearly passing pace of 782,000 and 841,000 new case and men having a higher occurrence and death rate, making it the second reason for death among men (Freddie et al., 2018).

2.2.1 Risk Factors

Chronic diseases with hepatitis B infection or hepatitis C infection, aflatoxin-polluted food, substantial liquor consumption, obesity, smoking, and type 2 diabetes are the significant causative factors for hepatocellular carcinoma (London et al., 2018) (figure 2.3). It likewise caused from ecological and hereditary variables (Gomes et al., 2013)

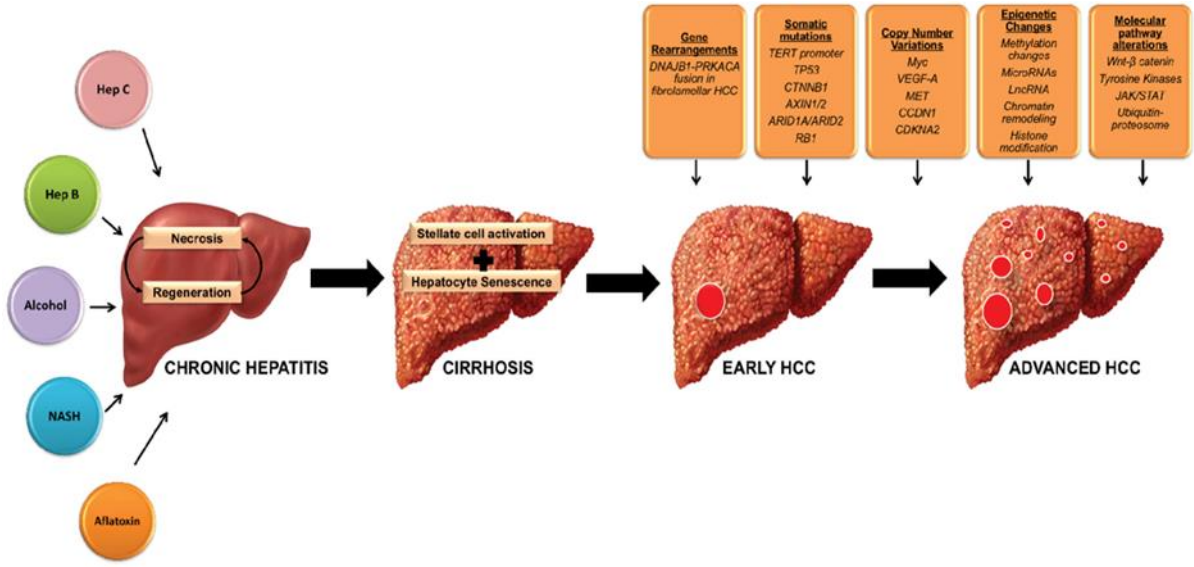


Figure 2. 2 Pathogenesis of Hepatocarcinoma.

Incessant introduction of the liver to damage from viral hepatitis, liquor misuse or NASH causes rehashed hepatocyte harm and sets up an endless loop of cell passing and recovery which in the long run outcomes in cirrhosis. The resulting genomic instability prompts commencement of hepatoma. Step by step accumulation of various hereditary occurrences including gene adjustments, somatic alterations, duplicate number changes, epigenetic changes and growth factor pathway changes in the long run lead to tumor advancement and metastases.

Abbreviations: Hep B, hepatitis B; Hep C, hepatitis C; HCC, hepatocellular carcinoma; NASH, non-alcoholic steatohepatitis.

2.2.1.1 Viral Hepatitis

HCC and liver cirrhosis develops from hepatitis B infection and hepatitis C infection. HBV prompted HCC pathogenesis is assumed to include a various mechanisms including the incorporation of HBV-DNA into host hereditary machinery, DNA methylation, oxidative stress, and hepatitis B X protein (HBx) (Tarocchi et al., 2014). The danger of evolving HCC has been demonstrated to be corresponding to HBV-DNA level in hepatic cells.

Hepatitis B virus passes in into liver cells via a pathway facilitated by a receptor. Perseverance of the infection in the host cells by means of different mechanisms that include contamination of immune system control points, viral inhibition of antigen presentation, selective immune suppression, down-regulation of

viral gene expression and viral alterations that functionally incapacitate virus-specific T cells from recognizing HBV antigen leads to prolonged illness (Ortega-Prieto and Dorner, 2017).

2.2.1.2 Diabetes Mellitus

A chronic ailment, for example, diabetes mellitus and obesity raises the danger of HCC. Diabetes mellitus legitimately influences the liver in view of the fundamental role the hepatocyte plays in glucose digestion. It can prompt ceaseless hepatitis disease, hepatosteatosis, hepatic failure, and cirrhosis of the liver. This condition diabetes is an autonomous risk factor for hepatoma (Gao et al., 2013, Wang et al 2012).

2.2.1.3 Aflatoxin

This is another powerful liver carcinogen. Aflatoxin is made by a mold known as Aspergillus species, commonly seen on grains, corn, peanuts, or soybeans that has been warehoused in warm moist conditions. The threat of hepatoma with aflatoxin relies upon the dosage and length of exposure time. Aflotaxin exposure is increasingly common in provincial United States and it applies a synergistic impact on hepatitis B and hepatitis C instigated hepatoma, contact with aflatoxin alone causes a lower chance of developing hepatoma yet hepatitis B in addition to aflatoxin exposure causes a 30 times more serious risk of developing hepatoma (Liu and Wu. 2010). Expulsion of aflatoxin, AFB1 (best) from nature brought about a decrease of the occurrence of HCC (Chen et al., 2013).

The significant hazard factors change from area to locale. In places like China and Eastern Africa serious hepatitis B virus disease and contact with aflatoxin are the primary risk factor while Hepatitis C Virus is the fundamental driver of hepatocellular carcinoma in nations like Japan and Egypt (Chimed et al., 2017).

2.2.1.4 Excessive Alcohol Intake

Intake of excessive alcohol is a severe medical issue worldwide. Overwhelming alcoholic intake leads to hepatosteatosis, alcoholic steatohepatitis (ASH), cirrhosis, and in the longrun, hepatocellular carcinoma. Alcoholic steatohepatitis (ASH) has been accounted for to advance to HCC at a yearly pace of 3%-10% (Schwartz and Reinus, 2012). Despite the fact that the pathogenetic mechanism underlying alcohol-prompted tumour initiation has been characterized, the pathways associated with tumour advancement and developments are ineffectively comprehended. Incessant alcohol intake instigates Cytochrome p450 2E1 (CYP2E1), a member from the cytochrome p450 blended function oxidase system bringing about introduction of different biologic impacts, for example, increments in alcohol digestion, boosted oxidative stress, expanded hepatotoxicity and interractions with different medications, xenobiotics and carcinogens (Neuman et al., 2015).

Especially, acetaldehyde created by liquor digestion emphatically induces oxidative stress, intensifying liver illnesses (Yoon, 2018). The molecular mechanism for the immediate role of liquor on hepatocarcinogenesis stays hazy. Notwithstanding, an on-going report utilizing exome sequencing examination of 243 liver tumors distinguished mutational marks related with explicit hazard factors and showed that the Catenin beta 1 (CTNNB1) bunch was essentially related with alcohol as a hazard factor for Hepatoma (Schulze et al., 2015).

2.2.2 Staging System in Hepatocellular carcinoma

Staging of hepatocellular carcinoma is essential to decide result and to know the best treatment it also has to do with evaluation of tumor size, level of serum Alpha-fetoprotein, how well the liver is functioning, portal hypertention and performance status (PS). How well the liver is functioning is usually evaluated using Child-Pugh score system which comprises of bilirubin level in serum, albumin level in serum, ascites, prothrombin time as well as hepatic encephalopathy. Performance status (PS) is a scale to rate how well one is (Vogel et al., 2018). Various systems of

staging HCC have being formed which includes selected or the entire aforementioned tests. They include TNM, Okuda, CLIP (Cancer of the Liver Italian Program), JIS (Japanese Integrated Staging) Score and lastly BCLC (Barcelona Clinic Liver Cancer) system.

The BCLC classification was based on outcomes of randomized control trial and cohort studies, it also connects tumor size, how the liver is functioning, symptoms related to cancer as well as Performance status (Table 4) to a confirmed treatment procedure (figure 2. 4). The classification recognizes patients diagnosed as having early hepatocarcinoma (i.e stage 0 and A) and might gain from ablative management, patients diagnosed as having intermediary (i.e stage B) or stage C also known as advanced stage HCC who might gain from intra-arterial or systemic treatments as well as patients having a very low lifespan (i.e stage D). Existence with no treatment is more than five years for early stage HCC, two and half years for intermediate stage, one year for advanced stage also three months for stage D (Forner et al., 2018).

Table 3 BCLC Staging System

STAGES	TUMOUR SIZE	PERFORMANCE STATUS (PS)	THE CHILD-PUGH SYSTEM
0 (Very early stage)	less than 2cm,	(PS 0) patients feel healthy	(Child-Pugh A) The liver is working normally.
A (Early stage)	single tumour of any size, or up to three tumours all less than 3 cm	patients feel healthy and are lively (PS 0)	The liver is working well (Child-Pugh A or B)
B (Intermediate Stage)	many tumours in the liver	patients feel healthy (PS 0)	The liver is working well (Child-Pugh A or B).
C (Advanced stage)	cancer has spread into the blood vessels, lymph nodes or other body organs	patients do not feel well and are not lively (PS 1 or 2)	The liver is still working well. (Child-Pugh A or B).

D		Not healthy and requires assistance in being looked after (PS 3 or 4).	severe liver damage (Child-Pugh C

2.2.3 Treatment

Hepatocellular carcinoma is medically heterogeneous and strangely the histopathology view of hepatoma additionally displays huge heterogeneity. The scope of cell variation stretches out from all around differentiated to ineffectively separated tumors (Dhanasekaran, et al., 2016). Often, clinical diagnoses of hepatocellular carcinomas are quiet until it is all progressed or tumor diameter surpasses 10 cm (Silva and Sherman 2011).

Potential remedial treatment, for example, clinical resection, liver transplantation or local removal altogether increases survival to about 40% to half at five years for patients determined to have a little tumor and no far off spread (i.e stage 0 and A) (El-Serag, et al., 2001), for this reason early recognition and sufficient treatment are vital. At late stage (i.e stage c) survival of patients have successfully been improved using sorafenib (Gomes, et al., 2013) (figure 2.4).

An enormous number of populaces may stay asymptomatic. Hepatocarcinoma patients may display jaundice, swelling from liquid in the abdomen, simple wounding from coagulopathy, lack of hunger, inadvertent weight reduction, sickness, spewing or weakness.

When hepatocarcinoma is diagonized at the beginning period, a multi-year endurance rate can be accomplished with successful treatment, yet at a late stage just a few months of survival are expected, anyway the endurance of patient with hepatocellular carcinoma relies upon the stage of the malignant growth at the time of determination (Forner, et al., 2012, Liu, et al., 2015).

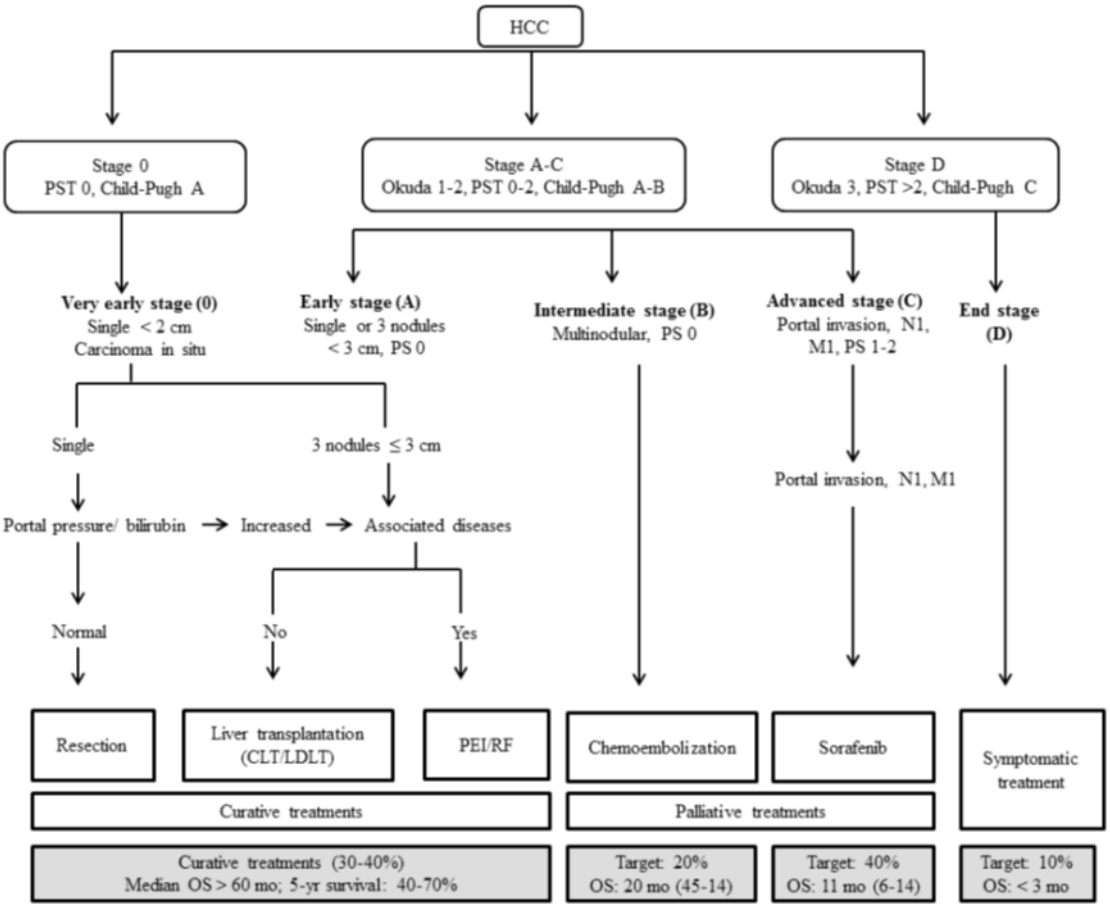


Figure 2. 3 Barcelona Clinic Liver Cancer Staging and Treatment Allocation. (Bruix and Sherman, 2011).

CLT: Cadaveric Liver Transplantation, HCC: Hepatocellular Carcinoma, LDLT: Living Donor Liver Transplantation, M: Metastasis Classification, N: Node Classification, OS: Overall Survival, PEI: Percutaneous Ethanol Injection, PST: Performance Status Test, RF: Radiofrequency.

Biomarkers utilized in hepatoma analysis includes Golgi 73 protein (GP73), Glypican-3(GPC3), microRNAs and a few more (Ba, et al., 2012; Feng, et al., 2014; Bartel, 2004). Alpha-fetoprotein (AFP) is likewise utilized as a biomarker however it is anything but an exact marker as it gives low responsiveness and particularity (Lok, et al., 2010). These markers comprise of any results of either tumor itself or the host in response to tumor's quality that recognizes harmful tissues from nonthreatening and is quantifiable in body liquids or tissues. They rise with dynamic or persistent infection, decline with reaction to treatment, and standardize with reduction (Abdulaziz and AlSalloom, 2016).

2.2.4 Molecular Carcinogenesis in Hepatocellular Carcinoma

Development of hepatocarcinoma (HCC) is unpredictable and happens through a multistep organic procedure of dangerous change of typical hepatocytes in which different variables, for example, hereditary and epigenetic mutations, oxidative stress, irritation, and immunity are included in this steps. Till now, various examinations have depicted the molecular mechanism of hepatoma, however the exact molecular pathogenesis of hepatoma advancement stay unknown (Yoon, 2018). Table 5 summarizes the genetic changes seen in hepatocellular carcinoma.

2.2.4.1 Telomerase Promoter Mutation

Redundant DNA arrangements at linear chromosome ends, securing chromosomes against start to finish merging and harm, giving chromosomal dependability known as telomeres, reduces in length with mitotic cell division, however are kept up by telomerase in cells having high proliferative capability (Calado and Young, 2009).

Genetic causative factors for cirrhosis advancement in people and murine models are loss-of-function alteration in telomere support genes. Telomerase inadequacy incites quickened telomere shortening and malfunction, encouraging genomic instability and oncogenesis (Donaires et al., 2017). Constant degenerative situation related with high cell replication rate elevates telomere shortening. Consequently, association of telomeres and telomerase mutation is by all accounts significant in susceptibility to liver infection advancement towards hepatocellular carcinoma (HCC) (Donati and Valenti, 2016) because cirrhosis is a forerunner to hepatocellular carcinoma, the telomere hypothesis holds that this telomere shortening brings about chromosomal instability that drives malignancy commencement.

A key mechanism of cell immortalization is adjustment of the telomeric DNA through either expanded telomerase articulation or elective systems of telomerase enactment, enabling cells to survive and multiply uncertainly (Stewart and Weinberg,

2000). Mutations in the telomerase reverse transcriptase (TERT) promoter area happen in 30–60% of HCCs (Chen et al., 2014).

Nault et al. found telomerase reverse transcriptase (TERT) promoter alteration in 59% of HCCs as well as in 25% of cirrhotic preneoplastic injuries, proposing it is likely a driver mutation. Strikingly, telomerase reverse transcriptase (TERT) promoter changes are obviously less common in hepatitis B initiated hepatocarcinomas, however these tumors appeared to have intermittent incorporation of hepatitis B Virus sequence into the TERT gene locus, which fills in as a complementary mechanism for telomerase enactment (Ferber et al., 2003).

Table 4 Major Molecular Events in the Pathogenesis Of Hepatocellular Carcinoma.

Genomic alterations	Gene mutations	TERT promoter TP53 CTNNB1 AXIN1, AXIN2 RPS6KA3	Killela et al., 2013 Guichard et al., 2012
		ARID1, ARID2	Fujimoto et al., 2012
	Gene amplification/Deletions	CCND1 FGF19 CDKNA2A, CDKNA2B AXIN1 IRF2 MET	Guichard et al ., 2012 Wang et al., 2013
Growth factor pathway Alterations	Major signaling pathways	Wnt/β–catenin Tyrosine kinase pathways- *EGF,* *HGF/c-MET,* *FGF* *VEGF* IGF HIF 1, TGF β Hedgehog	(Thompson and Monga, 2007) Llovet et al., 2012 Giannelli et al., 2005

2.2.4.2 Copy Number Variations and Gene Rearrangement

These are adjustments in the structure of a genome in which little or huge portions of the chromosome are either intensified (addition of genomic DNA) or erased (loss of genomic DNA). These type of difference in structure stimulate carcinogenesis by expanded expression or initiation of oncogenes and diminished expression or inactivation of tumor silencers (Dhanasekaran et al., 2016). An investigation of two hundred and eightysix Hepatocellular carcinoma patients recognized twentynine intermittently intensified areas and twentytwo repetitively erased regions with a significant level of duplicate number variations.

These areas harbor set up oncogenes and tumor silencers which includes, CCND1 (cyclin D1), MET (hepatocyte growth factor receptor), CDKN2A (cyclin-subordinate kinase inhibitor 2A) and CDKN2B (cyclin-subordinate kinase inhibitor 2B), and numerous different genes associated with liver carcinogenesis that hasn't been accounted for previously (Wang et al., 2013).

Another report likewise indicated areas showing critical duplicate number changes and utilized the investigation as a methodology for recognizing potential hepatoma driver genes (Hyun et al 2009). A few of these changes have known related pathogenic mechanism; for instance, an extent of hepatoma initiates telomerase reverse transcriptase (TERT) by focal enhancement in the telomerase reverse transcriptase (TERT) region, and deletion of AXIN1 is one of the mechanisms mediating Wnt/ß-catenin pathway enactment in hepatocellular carcinoma.

Chromosomal rearrangement is another type of somatic deviation adding to carcinogenesis, which can bring about the combination of two genes by chromosome translocation, reversal, or erasure. As of late, in an achievement study, such a gene combination was depicted in fibrolamellar hepatocarcinoma. This is an alternate kind of hepatoma that emerges in non-cirrhotic livers, typically in youthful people, and has an unmistakable morphology (Graham et al., 2015).

A chromosomal adjustment including a roughly 400-kilobase deletion in chromosome 19 outcomes in the arrangement of a chimeric RNA encoding a protein containing the amino-terminal space of DNAJB1 (a homolog of the atomic chaperone DNAJ), intertwined in outline with PRKACA (the catalytic domain of protein kinase A). This combination appears to be profoundly explicit for fibrolamellar hepatocarcinoma, being recognized in 100% of fibrolamellar hepatocarcinoma, recommending that this genetic change likely adds to tumorigenesis and the extraordinary morphology of this hepatocarcinoma subtype (Honeyman et al., 2014).

2.2.4.3 Wnt-ß-Catenin Signaling Pathway.

Mutations in this pathway have been portrayed in 20 to 40% of hepatocellular carcinoma. Wnt-ß-catenin signalling pathway plays a part in all stages of liver advancement and growth, which include stem cell renewal, zonation, cell adhesion, expansion, separation, liver Major molecular activities in the pathogenesis of hepatocarcinoma revival, and epithelial-mesenchymal change (Lee et al., 2006). hepatocarcinoma happens much of the time through transformations in the N-terminal region of ß-catenin that balances out the protein and grants an increase of constitutive transcriptional enactment by ß-catenin/TCF complexes (Takigawa and Brown, 2008).

The WNT/ß - catenin signaling pathway is involved in embryogenesis, separation, cell expansion, and tumorigenesis and it is among the utmost generally disturbed pathways observed in hepatocarcinoma (Thompson and Monga, 2007). Research has described 19 Wnt ligands and 10 transmembrane frizzled receptors bond to them, prompting either ß-catenin-dependent or ß-catenin-independent Wnt pathway enactment (Zeng et al., 2007).

Gene transformations that enact WNT/ß-catenin pathway are seen in up to half of hepatocarcinomas. The most widely recognized are initiating changes in CTNNB1, which bring about adjustment of ß-catenin. Also, changes in AXIN1 seen in 3–16% of hepatocarcinomas and AXIN 2 seen in about 3% of HCCs which are

both negative controllers of the Wnt pathway, just as inactivating adenomatous polyposis coli (APC) a tumor silencer gene, add to Wnt pathway activation, initiation of this pathway brings about gathering of ß-catenin in the cytoplasm and moving it to the core (nucleus), where it ties to trancription factor TCF/LEF and initiates downstream target genes (Guichard et al ., 2012) (figure 2.5).

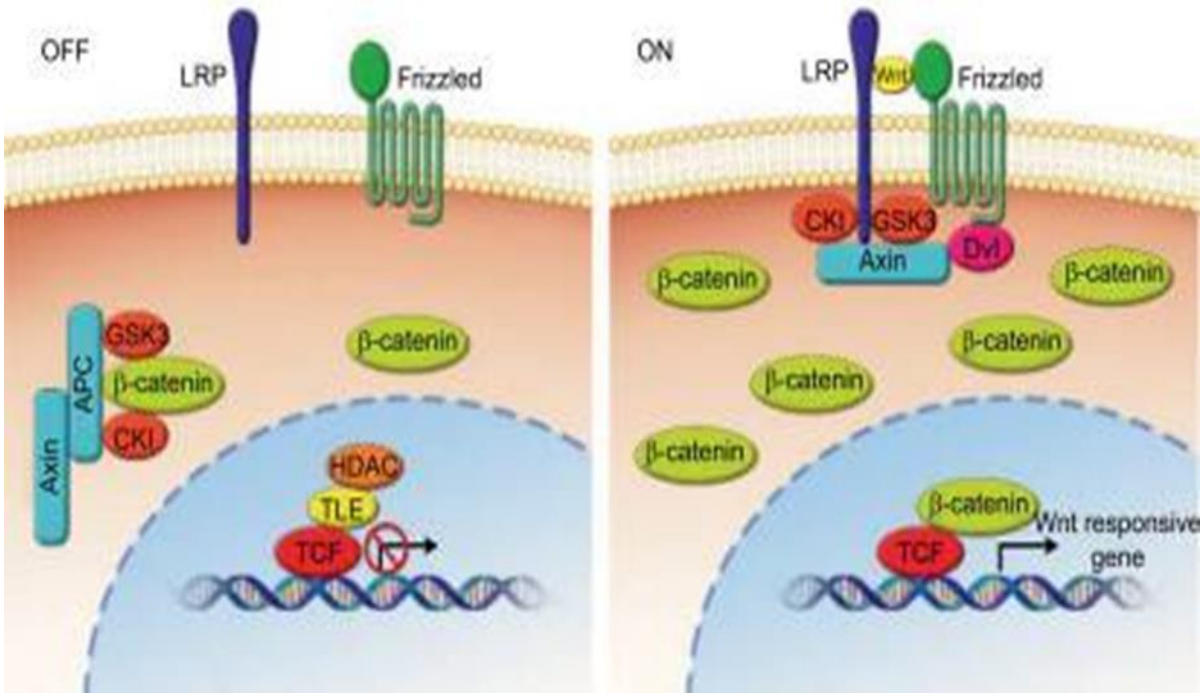


Figure 2. 4 Overview of Wnt/β-catenin signaling.

In the off-state, or nonappearance of Wnt, cytoplasmic β-catenin forms a complex with APC, axin, CKI, and GSK-3 afterward is focused for proteosomal degradation while Wnt target genes are repressed by TCF/TLE and HDAC. In the on-state, or existence of Wnt, a receptor complex forms among Frizzled and lipoprotein receptor-related protein families, which leads to accumulation of β-catenin in the cytoplasm and nucleus, where it functions as a coactivator for T cell-factor proteins to trigger Wnt-responsive genes (MacDonald et al., 2009)

Abbreviations: CKI, cyclin-dependent kinase inhibitor; GSK-3, glycogen synthase kinase 3; TCF/TLE, T cell-factor proteins/transducin like enhancer of split; HDAC, histonedeacetylases; LRP, low-density-lipoprotein receptor-related protein; APC, adenomatous polyposis coli.

2.2.4.4. Activation of Insulin-like Growth Factor Signalling Pathway.

The insulin-like growth factor (IGF) pathway is likewise engaged with incidence of hepatocarcinoma. The insulin-like growth factor system involves two ligands, insulin-like growth factor -I and insulin-like growth factor II; three cell-membrane receptors, insulin-like growth factor -I receptor, insulin receptor (IR), and IGF-II receptor; six high-Affinity IGF binding proteins, insulin-like growth factor bp-1,2,3 to 6 (Samani, et al., 2007).

Silencing of the insulin-like growth factor-binding proteins 1-5 and overexpression of the insulin-like growth factor -1 and -2 receptor lead to cascade of molecular activities, for example, cell expansion, anti- apoptosis and invasive action (Lachenmayer, et al., 2010). Overexpression of insulin-like growth factor -1receptor was distinguished in 33% of hepatocarcinomas and increased initiation of insulin-like growth factor -1receptor was seen in 52% of tumors (Desbois-mouthon, et al., 2009).

2.2.4.5 Tp53 Tumor Suppressor Gene.

Changes in TP53 gene and hepatocarcinomas are emphatically related and considered the utmost consistently mutated tumor silencer gene in hepatocarcinoma. It happens in 30% hepatocellular carcinomas (Villanueva and Hoshida, 2011). In hepatocellular carcinomas, type of TP53 transformations and its recurrence are known to be changing as per the geographical areas of tumors.

Dietary exposure to aflatoxin B1contamination (mostly in Africa and Asia) instigates changes at the 3rd base in codon 249 by transversion of the nucleobase G-T and combines with Hep B Virus infection in causing p53 transformations in hepatocarcinomas (Hussain et al., 2007). Reactive oxygen/nitrogen species that can both harm DNA and transform malignant growth related genes, for example, TP53 can be created because of Chronic disease with Hep B and C Virus infections, and oxyradical issue, for example, hemochromatosis (Hussain et al., 2007). In places

without aflatoxin B1contamination like Western nations TP53mutations are seen in around 20% of patients with HCCs (Zucman-Rossi, 2010).

2.2.4.6 Epigenetic Modifications.

The motivation behind why it is imperative to study epigenetics in the liver is because of the way that it is the one of the organs that is continually adjusting to highly inconstant ecological conditions.

2.2.4.6.1 DNA Methylation and HCC

DNA methylation includes the covalent relocation of a methyl group to the C-5 position of the cytosine ring on a DNA strand. Felsenfield G defined epigenetics as a structural and chemical change of DNA and related administrative proteins (e.g. histones), barring changes to the nucleotide sequences (Aquino et al., 2018).

DNA methylation usually happens at the 5’-C-phosphate-G-3’(CpG) dinucleotides in somatic cells with around 25% happening in a non-CpG way in embryonic stem cells (ESCs). 5’-C-phosphate-G-3’dinucleotides are normally located in "CpG islands", a short CpG-rich areas. CpG islands, which possess over half of the entire promoter, can be methylated during growth and enhance elongated long-term gene silencing (toh et al., 2019) (figure 2.6). DNA methylation is firmly controlled by a group of DNMTs that comprises of DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L (Bestor, 2000). Table 6 summarizes a list of DNA methylation studies in HCC

Adding to genetic mechanisms of deletions or alterations, epigenetic changes can elevate or reduce gene expression through controlling DNA methylation. In HCC, an expanded expression of DNA methyltransferases (DNMTs), enzymes which catalyse epigenetic modifications, happens from the start in the advancement of tumorigenesis. The recurrence of deviant DNA methylation rises from

precancerous injuries to dysplastic nodules lastly HCC, implying their significant role in tumor advancement (Kumar et al 2011).

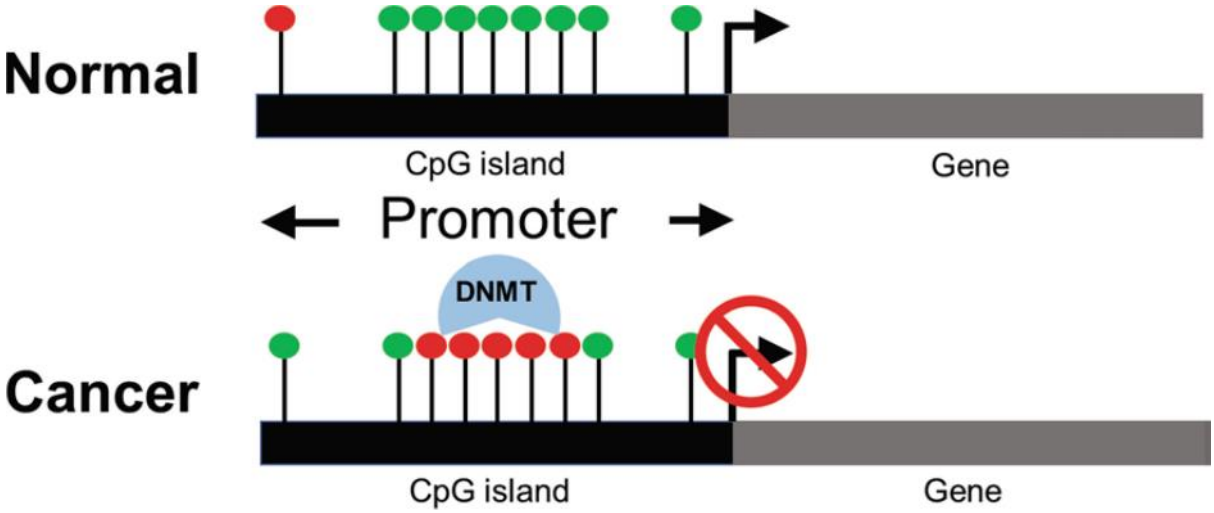


Figure 2. 5 DNA methylation profile in cancer.

Liver cancer cells usually shows DNA hypermethylation at promoter sites of tumor suppressor genes, which results in silencing some of the tumor suppressive genes (Toh et al 2019).

Table 5. Methylated genes in HCC

Type of sample.	Genes Hypo-methylated	Genes Hyper-methylated	Key findings	Implications of study	Refs.
Only hepatocytes derived from HBV positive HCC (HBHC) Tissues	non	EMILIN2, WNK2, TM6SF1, TLX3, HIST1H4F, TRIM58, GRASP	Based on the source of the hepatocyte. 3 groups of Hepatocyte methylation were profiled HCC, nearby tissue and regular liver. 7 different genes were seen to be abnormally	The genes found can be possible unique biomarkers of HBHC as soon as they are confirmed in greater medical cohorts	Tao et al.,s 2011

			methylated in HBHC		
62 paired HCC tumour and NAT	CCL20, AKT3, SCGB1D1, WFDC6, PAX4, GCET2, CD300E, CD1B, FLJ00060, MNDA	DAB2IP, BMP4, ZFP41, SPDY1, CDKN2A, TSPYL5, CDKL2, ZNF154, ZNF540, CCDC37	684 CpG sites significantly hypermethylated in HCC tissues. 5 of these genes (*CDKL2*, *CDKN2A*, *HIST1H3G*, *STEAP4*, *ZNF154*) had obvious hypermethylated DNA in plasma about 63% of patients	Evaluating DNA methylation from the plasma of a patient is likely. group of methylated Genes discovered could be potential biomarkers for early diagnosis	Shen et al., 2012
27 HCC and 20 NAT	NFATC1	BMP4, CDKN2A, GSTP1	Greater universal hypomethylation patterns detected in HCC compared to NAT, with higher incidence taking place in promoter region of CpG islands than CpG shores and shelves	These gives a deeper understanding of different forms of methylation in several gene regulatory regions	Song et al., 2013
71 primary HCC tissues, 8 non-diseased normal tissues, 4 HCC cell lines		ACTL6B, C19orf30, DGKI, DLX1, ELOVL4, LDHB, LRAT, MLF1, NEFH, PPM1 N, PRPH, SLC8A2, SMPD3	13 tumor suppressor genes were found; *NEFH* and *SMPD3* were functionally confirmed in vitro and in vivo. Low levels of SMPD3 were linked with early HCC recurrence	SMPD3 was discovered to be a strong tumor suppressor gene and can be an autonomous prognostic factor for early repetition of HCC	Revill et al., 2013

			after curative surgery in an autonomous patient cohort		

2.2.4.6.2 ncRNAs and HCC

There are 2 sorts of noncoding RNAs (ncRNAs) the short noncoding RNAs; endogenous small interfering RNA (siRNAs) and microRNA (miRNAs) which are under two hundred nucleotides and around eighteen to twenty five bases long also the other one is the Long noncoding RNAs (lncRNAs). Furthermore, the human genome have been accounted for to encode over of a thousand diverse microRNAs each with particular messengerRNA target(s) and is responsible for controlling post-transcriptional gene expression. Consequently, MicroRNAs (miRNAs) denote to a group of significant epigenetic regulators that impact biological reactions. miRNAs are by a wide margin the most well-examined class of epigenetic regulators in liver malignancy (Toh et al 2019). The first report of microRNA dysregulation in liver malignant growth is from Murakami et al. (2006), who revealed a greater expression of 3 microRNAs in hepatocarcinoma samples, to be specific miRNA-20, miRNA-92, and miRNA-18. Thus, various reports on miRNA dysregulation have been accounted for in HCC. A portion of the reliably revealed miRNAs usually expressed differently in hepatoma tumors likened with typical liver tissues are miRNA-21, miRNA-26, miRNA-122, miRNA-199a, miRNA-200a, miRNA-221, miRNA-222, and miRNA-224 (figure 2.7).

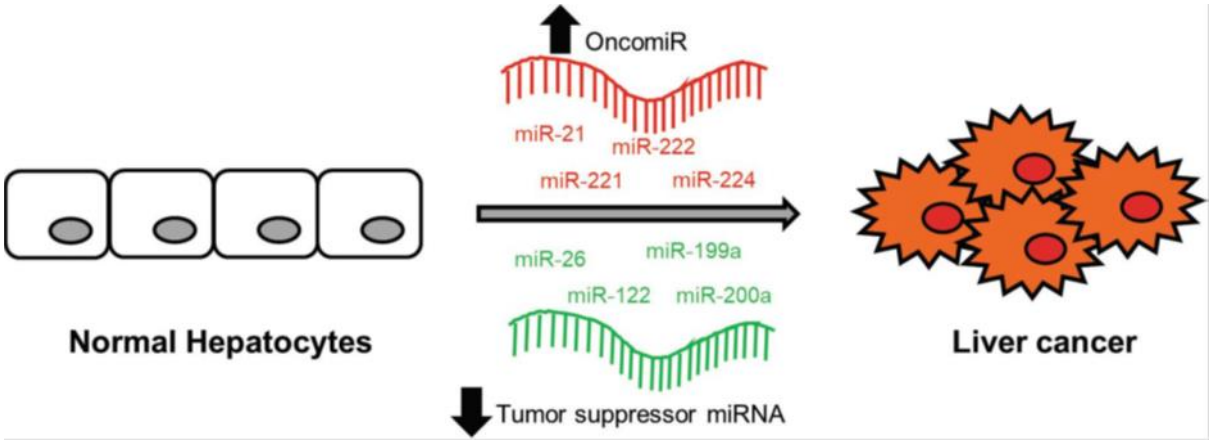


Figure 2. 6 MicroRNAs in cancer.

Elevation of oncogenic miRNAs (oncomiRs) brings about silencing of tumor suppressor genes whereas down regulation of tumor suppressor microRNAs prompts diminished inhibition of oncogenes, therefore lead to the advancement of liver cancer (Toh et al., 2019).

Oncogenic microRNA that drives development of hepatocarcinoma, for example, miRNA-21, miRNA-221, miRNA-222 and miRNA-224 are often seen as upregulated in hepatocarcinoma. For example, miRNA-21 was seen as upregulated in HCC and inhibition of miR-21 brought about a raise in expression of the tumor silencer phosphatase and tensin homolog (PTEN) with complemented decrease in tumor cell multiplication, migratory and invasive capacity (Meng et al., 2007). Moreover, mitogen-activated protein-kinase 3 (MAP2K3) was seen to be an straight target of miRNA-21 whereby MAP2K3 expression, which was subdued in hepatocellular carcinoma tissues, was seen to be contrariwise associated with miR-21 (Xu et al., 2013). miRNA-221 and miRNA-222 have been demonstrated to be overexpressed in hepatoma and the raised degrees of these 2 microRNAs are correlated with tensin homolog (PTEN) and TIMP metsllopeptidase inhibitor 3 (TIMP3) downregulation (Garofalo et al., 2009). Likewise, miRNA-221/222 is up controlled in an early occurrence and has the most elevated expression in hepatocarcinoma tests. It has been found to target CDK inhibitor p27 to stimulate tumor multiplication and its overexpression is associated with more unfortunate diagnosis (Pineau et al., 2010).

miR-224 is also a specifically upregulated HCC microRNA. microRNA-224 was found to advance spread, prevent cell death, movement and raid of hepatoma tumour cells (Zhang et al., 2013). Besides, people diagnosed with early hepatocarcinoma indicated an elevated serum level of miRNA-224 when contrasted with patients having liver cirrhosis and severe HBV subjects, emphasizing that, miRNA-224 has a capability of being a dependable indicator for early recognition of hepatocarcinoma in serum (Lin et al., 2016). Lnc RNAs are additionally significant modulators of HCC development. Because of the developments in genomic systems, long non-coding RNAs roles such as focal regulations in genome guideline and dynamics have started to develop. Parts of the long noncodingRNAs known associated with hepatocellular carcinoma are highly up-regulated in liver cancer, HOTAIR, MEG3 as well as HOTTIP (Toh et al., 2019). Although, the genetic role that DDX11-AS1 plays in hepatocarcinoma isn't well known, Yong et al detailed that appearance of DDX11-AS1 was drastically greater in Hepatocarcinoma tissues and cell lines. Greater DDX11-AS1 appearance anticipated lowly complete existence of affected people. Practically, expansion, cell cycle advancement, movement, also attack on hepatocellular carcinoma cells were repressed by DDX11-AS1 quieting, whereas advanced by ectopic manifestation of DDX11-AS1.

2.2.5 Human Hepatocellular Carcinoma (HepG2)

Hepatocarcinoma cell line is a human liver diseased cell line that was obtained from the hepatic tissue of a multiyear (15 years) old Caucasian male with hepatocarcinoma. (ATCC) This is a never-ending cell line and is non-tumourigenic in naked mice.

The significant plasma proteins discharged by these cells are albumin, transferrin, fibrinogen, alpha-II macroglobulin, alpha-I antitrypsin and plasminogen. Hep-G2 reacts to incitement with human development hormone; no hepatitis B infection surface antigens have been found. The cells are adherent epithelial cells developing as monolayers in little are aggregates, each one containing 55 chromosome sets (ECACC). HepG2 cells are of incredible significance to identify

cytotoxic and genotoxic substances and by expansion cytoprotective, antigenotoxic and cogenotoxic agents (Mersch-Sundermann et al., 2014).

MATERIALS AND METHOD

3.1 Essential oil

The commercial essential oil of *Origanum onites* was acquired from TÜRER Inc. (İzmir, Turkey).

3.2 Simultaneous Gas Chromatography and Gas Chromatography and Mass Spectrometry Analysis

An Agilent 5977B GC-MSD system was used in the Gas Chromatography-Mass Spectrometry (GC-MS) analysis. Helium (0.8 mL/min) was utilized as carrier gas in the Innowax FSC column (L × I.D.= 60 m x 0.25 mm; d_f: 0.25 μm). firstly, The heat of the Gas Chromatography oven was sustained at 60°C for 10 min and then set to 240 °C at a rate of 1 °C/min after which the setting was changed to 220°C at a rate of 4°C/min. The mass spectra were recorded at 70 eV by adjusting the split ratio to 40: 1 and the injector temperature to 250 °C. The added sample (1μL) was dissolved in 10% n-hexane. The relative percentage of the different components was calculated using flame ionization detector (FID) chromatograms having a detector temperature of 300 °C. The same elution sequence was obtained by GC-MS (an Agilent 7890B GC) by simultaneous automatic injection triplicates.

3.3 Identification of Compounds

Relative retention times are compared with those of the authentic samples or comparison of the linear retention indices with the n-alkane series was used to identify the essential oil components. Computer matching (Wiley GC/MS Library, NIST Chemistry Volatile oil constituents of *Origanum* species Web Book, Başer's Library of Essential Oil Constituents), and MS literature were used evaluating genuine compounds and components of known oils (18-21).

3.4 Chemicals

RPMI-1640 (biochrom,FG 1215), fetal bovine serum (FBS), penicillin-streptomycin, glutamine, trypsin- EDTA solution, dimethysulfoxide (DMSO) and MTT solution. All solvents and chemicals were of analytical grade**.**

3.5 Cell culture

Cell lines hepG2 were cultured in RPMI-1640 medium (biochrome FG1215), 10% heat inactivated fetal bovine serum (FBS)(capricon scientific , FBS-11B), 1% penicillin-streptomycin (Biochrom A2213) and 1% glutamine (EMD milipore, K0282) and maintained at 37℃ in a humid atmosphere containing 5% CO^2. They were sub cultured using 0.25% trypsin- EDTA solution (Biocrom, L 2143).

3.6 Cell Viability Assay

Hep cell line were seeded in 96 well culture dishes at a density of 5×10^4/ml cells in each well with 100µl medium and incubated for 24 hours. The essential oil of *Origanum onites* was dissolved in dimethysulfoxide (DMSO, sigma-Aldrich) to 100µm. it was then further diluted to 100 µg/ml, 200 µg/ml ,300 µg/ml , 400 µg/ml and 500 µg/ml in culture medium. Final concentration of DMSO in cell lines was less than 0.05%. The cultured cells were treated with the different concentrations of the essential oil, in triplicates and were incubated for 24 and 48 hours.

The cell viability was estimated by MTT assay. MTT solution (Biotium, 30006) was heated to 37℃ and then 10µl were added to each well. It was incubated for 4 hours after which 200µl DMSO was added to dissolve the formazan salts. The absorbance was measured at 570nm with spectrophotometer (versa Max, Molecular Device, Sunnyvale, USA).

3.7 Morphological Observation

The morphology of the Hep G2 cell line was observed under inverted microscope. The cell lines were initially observed before treatment with *Origanum onites* essential oil.

RESULTS

4.1. Cell morphology

HepG2 cells are epithelial-like cells. After treated with *Origanum onites* essential oil for 24 and 48 hours, HepG2 cells number decreased and vacuoles were detected in the cytoplasm of the cells (Figure 3.1. and Figure 3.2.). Number of the HepG2 cells was dramatically decreased with elevated *Origanum onites* essential oil concentration.

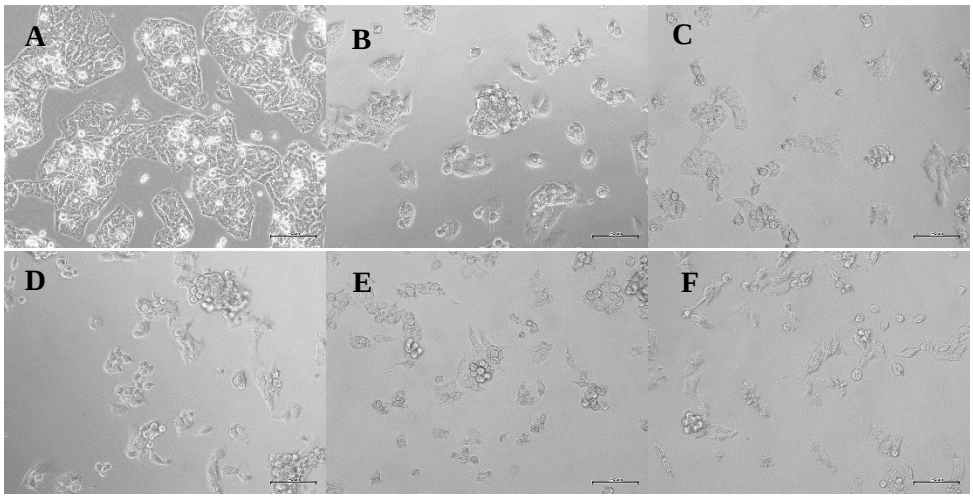


Figure 4. 1 HepG2 cells imaged under the inverted microscope:

(A) HepG2 cells (control group),
(B) 100 µg/ml *Origanum onites* essential oil treated HepG2 cells for 24h,
(C) 200 µg/ml *Origanum onites* essential oil treated HepG2 cells for 24h,
(D) 300 µg/ml *Origanum onites* essential oil treated HepG2 cells for 24h,
(E) 400 µg/ml *Origanum onites* essential oil treated HepG2 cells for 24h,
(F) 500 µg/ml *Origanum onites* essential oil treated HepG2 cells for 24h.
Scale bars= 100 µm.

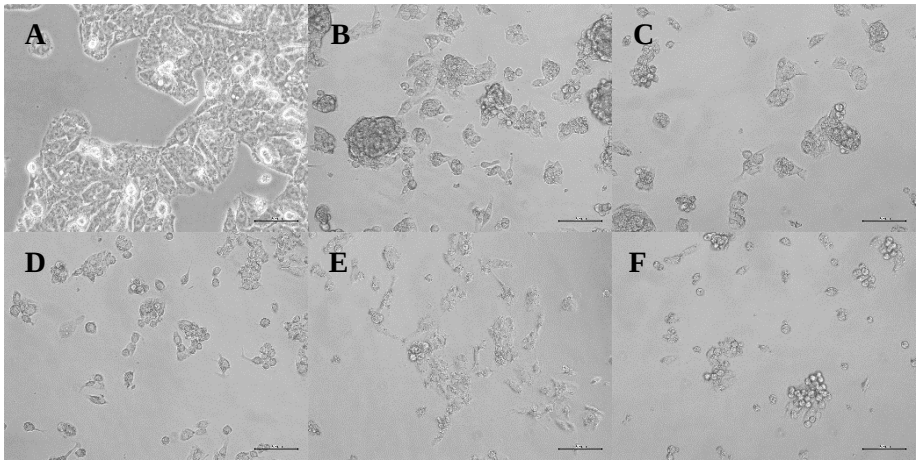


Figure 4. 2 HepG2 cells imaged under the inverted microscope:

(A) HepG2 cells (control group),
(B) 100 µg/ml *Origanum onites* essential oil treated HepG2 cells for 48h,
(C) 200 µg/ml *Origanum onites* essential oil treated HepG2 cells for 48h,
(D) 300 µg/ml *Origanum onites* essential oil treated HepG2 cells for 48h,
(E) 400 µg/ml *Origanum onites* essential oil treated HepG2 cells for 48h,
(F) 500 µg/ml *Origanum onites* essential oil treated HepG2 cells for 48h.
Scale bars= 100 µm.

4.2. Cell viability and cytotoxicity

HepG2 cells were treated with various concentrations of (100-500 µg/ml) *Origanum onites* essential oil for 24 and 48 hours. The cell viability was determined as described below by MTT assay. All concentrations of *Origanum onites* resulted in a dramatic decrease of HepG2 cell proliferation and had toxic effect in a dose and time-dependent manner. We found that, *Origanum onites* at 400 µg/ml concentration was more effective at inhibiting HepG2 cell growth when compared with other concentrations for 48 h incubation period (Figure 4.3.).

The MTT assay suggested that cell viability decreased significantly after treatment with 500 µg/ml *Origanum onites* essential oil (57.5 ± 15.66) compared to 100 µg/ml (118.6 ± 17.07) and 200 µg/ml (110.2 ± 6.687) *Origanum onites* essential oil treated HepG2 cells for 24h ($p=0.0042$ and $p=0.018$, respectively). Furthermore, our results showed that cell viability decreased significantly after treatment with 500 µg/ml (41.02 ± 13.96) and 400 µg/ml (53.13 ± 11.26) *Origanum onites* essential oil

compared to 100 µg/ml (88.21 ± 5.526) *Origanum onites* essential oil treated HepG2 cells for 48h (p=0.028 and p=0.0053, respectively) (Table 7).

Table 6. The cell viability values for HepG2 cells were treated with different concentrations of (100-500 µg/ml) *Origanum onites* essential oil. for 24 and 48 hours.

Concentration	**24 hours**	**48 hours**
100µg/ml	118.6 ± 17.07[a]	88.21 ± 5.526[c,d]
200 µg/ml	110.2 ± 6.687[b]	78.31 ± 8.598
300 µg/ml	91.42 ± 4.723	71.66 ± 5.193
400 µg/ml	86.59 ± 4.292	53.13 ± 11.26
500 µg/ml	57.5 ± 15.66	41.02 ± 13.96

Data are shown as means ± SD. Based on Mann–Whitney U test.

[a] Significant difference from 500 µg/ml concentration treated HepG2 cells for 24h (p=0.0042).

[b] Significant difference from 500 µg/ml concentration treated HepG2 cells for 24h (p=0.018).

[c] Significant difference from 400 µg/ml concentration treated HepG2 cells for 48h (p=0.028).

[d] Significant difference from 500 µg/ml concentration treated HepG2 cells for 48h (p=0.0053).

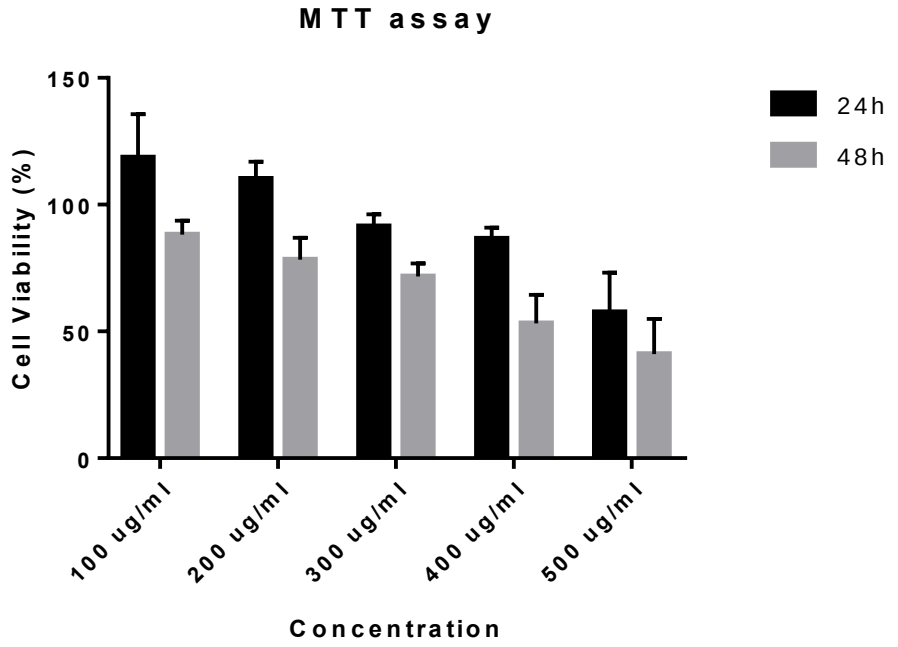


Figure 4. 3 Effect of *Origanum onites* essential oil on cell viability of HepG2 cells.

HepG2 cells were treated with different concentrations of *Origanum onites* essential oil for 24 or 48 h. Viability was quantitated by the MTT assay.

4.3. *Origanum onites* Essential Oil

The *Origanum onites* essential oil chemical content obtained by steam distillation using a library and relative retention indices by gas chromatography and gas chromatography mass spectrometry analyzes are given in Table 8. Carvacrol was found to be a major component of the essential oil as 78.4 %, and *p*-cymene and linalool 4.1 %, and 1.5 % respectively (Table 8).

Table 7. Essential oil composition of *Origanum onites* (TÜRER Inc.)

LRI	Compound Name	Relative percentage amounts (%)
1020	α-pinene	0.4
1024	α-thujene	1.1
1072	Camphene	0.3
1119	β-terpinene	0.1
1172	Myrcene	0.6
1177	α-phellandrene	0.2
1191	α-terpinene	1.3
1213	Limonene	0.2
1222	β-phellandrene	0.2
1260	γ-terpinene	6.9
1287	*p*-cymene	4.1
1298	Terpinolene	0.1
1457	1-octen-3-ol	0.1
1478	*trans*-sabinene hydrate	0.4
1555	Linalool	1.4
1564	*cis*-sabinene hydrate	0.2
1569	Linalyl acetate	0.1
1624	Terpinene-4-ol	0.7
1628	β-caryophyllene	0.5
1638	Aromadendrene	0.1
1717	α-terpineol	0.2
1728	Borneol	0.4
1748	β-bisabolene	1.1
1770	Carvone	Tr
1786	δ-cadinene	Tr
1793	γ-cadinene	0.1
1899	Carvacryl acetate	0.1
2033	Caryophyllene oxide	0.1
2159	Spathunelol	0.1
2205	T-cadinol	0.2
2210	Thymol	0.2
2243	Carvacrol	78.4
	Total	100.0

*LRI: The linear retention indices against the n-alkane series

DISCUSSION

Morphology of the HepG2 cell was observed under inverted microscope and the microphotograph showed changes in the cell morphology such as decreased adhesion and shape of the cells. It also showed a decrease in cell viability dose dependently with HepG2 cells treated with 400 µg/ml and 500 µg/ml of *Origanum onites* essential oil showing the least cell viability compared to control. Likewise there was a time dependent change, as cells treated after 48hrs showed a more significant cell death than those treated for 24hrs.

The possible mechanism of the cytotoxicity effect of either *Oregano* essential oil or its principle single components, particularly carvacrol and thymol, could be because of the activation of Glutathione S-Transferase (GST) in different tissues. GST is thought to assume a significant role in detoxifying cancer-causing agents (Lam and Zheng, 1991).

The results derived from the microscopic morphological observations from this research underlined that the possible mechanism of the cytotoxicity of *Origanun onites* essential oil could be because of the activation of apoptosis, which prompts expanding the cell porousness and henceforth losing a few cytoplasmic organelles.

Ashutosh et al., (2017) indicated a diminishing absorbance at 540 nm in the cells treated with elevating concentration of the medication in contrast with the control cells with no treatment. A diminished absorbance in the cells treated with drugs recommends cytotoxicity. This study agrees with the suggestion stated earlier (Ashutosh et al., 2017) as the result showed a decrease in absorbance with an increase in concentration from 100 µg/ml to 500 µg/ml indicating a significant decrease in cell viability after been treated for both 24 an 48hrs, implying that *Origanum onites* essential oil has a cytotoxic effect on HepG2 cell line.

According to Gautam (2018), MTT measure is a colorimetric test and is one of the best strategy to check the cell viability and cytotoxicity or cytostatic activity of cell line to assess safe, and productive measure of the medication. Under characterized condition, NADPH dependent cell oxidoreductase shows the amount of viable cells. The MTT enters the cells and goes into the mitochondria, the enzyme

dehydrogenase and reductase decrease the tetrazolium salt MTT(3-(4,5 Dimethyl thiazol-2-Yl)- 2,5-Diphenyltetrazolium Bromide) in an insoluble purple colored formazan product. The MTT results indicated that *origanum onites* essential oil was cytotoxic at 500 μg/ml concentration and it significantly increased after being treated for 48 hours. We can say from this result that the effect of *Origanum onites* essential oil on HepG2 cells rises as the concentration increases and if it it being treated for longer period of time the effect increases.

Drugs utilized in malignant growth treatment focus on the disease cell by initiating apoptosis or cell cycle arrest. Thus, natural compounds causing apoptosis in the malignant growth cells are important resources in disease suppression. Apoptosis can happen because of impact on different signalling pathways, hereditary material, and other cellular activities like changes in the proteins at the intracellular level (Gautam et al., 2014). A research on 5RP7 (c-H-ras transformed rat embryonicfibroblasts) cell lines stated that treatment of *Origanum onites* essential oils on the cell line actuated apoptosis (Bostancioglu et al., 2012). In like manner, microscopic morphology observation and MTT assay result demonstrated a significant decrease in cell suitability recommending apoptosis.

From the outcome above carvacrol was seen as the main constituent in the oil which could be the result of low absorbance value and decrease in cell viability from the MTT result as carvacrol has appeared to instigate apoptosis in HepG2 cell line by means of activation of caspases and mitogen-initiated protein kinase (MAPK) pathway (Yin et al., 2012).

5.2 CONCLUSION

Incidence and mortality as a result of hepatocellular carcinoma are of major concern. Hepatocellular carcinoma is resitance to many drugs thereby need for use of phytocemicals from plants is of great importance. In our research invitro cytotoxic effect of *Origanum onites* essential oil on HepG2 cell was investigated and the result showed a significant decrease in cell viability. In conclusion, essential oil from *Origanum onites* having carvacrol as its major constituent is cytotoxic to

hepatocellular carcinoma cell line with increase in concentration and time of treatment.

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Hep G2 , (ECACC 85011430)

Hep G2, American Type Culture Collection.

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