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Effect of Some Natural Products on Hepatocellular Carcinoma

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Abstract: ***The Pharmacological potential, such as antioxidant, anti- inflammatory and antibacterial activities of Ambrosia maritime (Demsisa) and Trigonell afoenumgraecum (fenugreek) leaves and oil are well known so,the present study was designed aiming at highlighting the effect of Ambrosia maritime and Trigonella foenum-graecum leaves and oil on human hepatocellular carcinoma cell line HepG2. HepG2 cells wereexamined and molecular mechanisms were also investigated. Treatment with an apoptosis- inducing concentrations of (Demsisa) and (fenugreek) leaves and oil caused typical morphological changes and apoptotic blebbingin HepG2 cells. The apoptotic process triggered by them involved the upregulation of p53 and the downregulation of Bcl-2 and CDK4.The mRNA and protein expression levels of apoptosis-associated factors Bcl-2, p53and CDK4 were therefore evaluated by qRT-PCR.the coordinate performance of these molecules is crucial for controlling life and death of cell.Our results demonstrated preliminary screening of anticancer activity of Demsisa and fenugreek extracts and oil against HepG2 cells, which can be further used for the development of a potential therapeutic anticancer agent. Demsisa extract was the most virulent one while anti-cancer activity decreased from Trigonella leaves to Trigonella oil respectively.***

Keywords: HepG2 cells -Apoptosis-p53- Bcl-2–cdk4- anticancer activity

1. Introduction

Natural Products, especially plants, have been used for the treatment of various diseases for thousands of years. Terrestrial plants have been used as medicines in Egypt, China, India and Greece from ancient time and an impressive number of modern drugs have been developed from them **(Kaur *et al.*, 2011).** Some medicinal plants are potential hepatogenic/ hepatoprotective agents. It is distinct that the medicinal plants possess versatile antioxidant, immunomodulatory and phagocytic activities, and thereby may be beneficial against liver diseases**(Govind, 2011).**

Ambrosia maritimaL.,Traditionally, the whole plant is used to cure gastrointestinal disturbance,abdominal pain, kidney inflammation and renal colic, whereas the leaves are used for diabetes and blood pressure. In addition, its curative properties extend to include antimolluscicidal, antimalarial and antitumor activities **(Dirar *et al.* 2014).**

*Trigonellafoenum - graecum Linn.*has been used as a medicinal plant since more than 4000 years in various parts of world. Due to this reason, it is regarded as oldest medicinal plant in history of mankind. It has wide therapeutic applications including carminative, and lactation stimulant in women after child birth. Also has many effects e.g. anthelmintic, antinociceptive anticancer, antibacterial, , gastro and hepatoprotective, immunomodulatory, etc**(Bano *et al.*, 2016)**.

Hepatocellular carcinoma (HCC) is one of the most common and malignant diseases worldwide, it is the third most common cause of cancer deaths**(Hussain *et al.*, 2007).** Usually, males are more affected than females and, are most common between the 30-50 years of age **(Farshori *et al.* 2014).**

p53 is a tumour suppressor gene, which is mutated in half of human malignancies. Wild-type p53 has important roles in the cell cycle, DNA repair and apoptosis. In the presence of DNA damage, p53 is activated and initiates cell cycle arrest. In the absence of wild-type p53 mutated cells survive continuing their clonal expansion resulting in tumour progression**(Kudahetti *et al.*, 2009),** so that the tumour behavior can be described using alterations of the p53 gene **(Graur *et al.*, 2016).**

Bcl-2 is an oncogenic protein that acts by inhibiting programmed cell death. The expression of Bcl-2 in some tumor cell types inhibits cell adhesion, spreading, and motility by enhancing actin polymerization **(Zhang *et al.*, 2016).**It not only inhibited apoptosis owing to deprivation of growth factors but also protected cells from a broad range of cytotoxic stimuli, importantly including diverse anti- cancer drugs **(Delbridge *et al.*, 2016).** CDK4 is the key regulator of the G1-S transition. In complex with Cyclin D, CDK4 phosphorylates Rb and drives cell cycle progression. CDK4-cyclin D regulation is perturbed in a large proportion of human cancers**(Dickson, 2014).**

2. Materials and Methods

Ethanolicextract

Leaves of *Trigonella* and *Ambrosia Maritima*plant materials were collected, air dried at room temperature and ground in a Wiley grinderthenextracted with ethanol. The crude ethanolicextract wascondensed using Buchner Rotavapor the resultant yield wassuspended in deionized water and filtrated in 0.22. Millipore filter paper.

Maintenance of HepG2 cells

HepG2 cells (HepG2, ATCC No. 84113001) are cultured in MEM Medium supplemented with 10% heat inactivated fetal calf serum (FCS) (Sigma Aldrich, USA), The culture

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flasks were incubated in 5% CO2 incubator with 95% humidity at 37°C.

Cytotoxicity Study

Cells were seeded in 96-well flat-bottomed microtiterplates (100 µl/well) under complete aseptic conditionand incubated in 5% CO2 incubator at 37˚C for 24 hr toreach complete monolayer. Serial dilutions of testedsamples were titrated in triplicate to the cells; in addition to the control wells that left without extracts. The plate wasthen incubated in 5% CO2 incubator at 37˚C for 24 hr and 72 hr to investigate the LD50 and cytopathic effectof the tested samples.

Antitumor Assay

Sublethal dose of tested samples were added, cellwell plates containing no tested samples were used as control. Cell culture plates are incubated at 37ºC in 5% CO2 incubator for 24 h, each well was accessed by observation under microscope after staining the cells with a 0.5% trypan blue stain.

Quantitative RT-PCR of BCL2, P53 and CDK4 gene expression

Total RNA was isolated with RNA extraction Qiagen kit), and oligo (dT)-primed RNA (1 µg) was reverse-transcribed using iScriptTM One-Step RT-PCR Kit with SYBR® Green. The obtained cDNA was used to determine the mRNA expression levels of Bcl-2, P53and CDK4 by PCR analysis. GAPDH was used as an internal control. The primer sequences used for the amplification of Bcl-2, P53and CDK4 and GAPDH were as follows:

Target Gene	Primer Sequence
Bcl-2	F 5′-TCCGCATCAGGAAGGCTAGA -3′R5′-AGGACCAGGCCTCCAAGCT -3′
P53	F 5′-TGCAGCTGTGGGTTGATTCC-3′ R 5′-AAACACGCACCTCAAAGCTGTTC-3′
CDK4	F 5′ CTGGTGTTTGAGCATGTAGACC -3′ R 5′- AAACTGGCGCATCAGATCCTT -3′
GAPDH	F 5′- TGCACCACCAACTGCTTAGC -3′ R5′- GGCATGGACTGTGGTCATGAG-3′

3. Results

Data obtained from the study showed that the IC50 of *Ambrosia* was 0.001x while*Trigonellla* leaves extract and oil were 0.01 x of acute dose and the anti- antitumor activity decreased from Ambrosia to Trigonella leaves to Trigonella oil respectivly Compared to control as illustrated in the figures1,2,3and 4

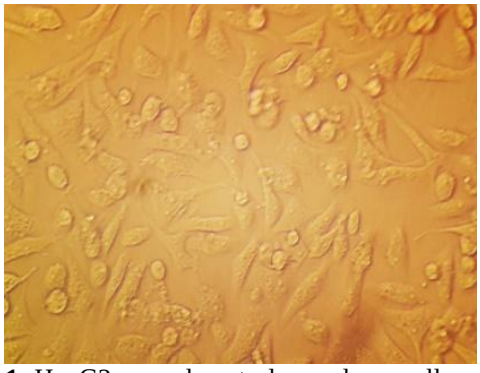

Figure 1: HepG2 normal control monolayer cells untreated showed complete monolayer viable andwell attached cells. Cells proliferation appearingand apoptosis not found.(X:200)

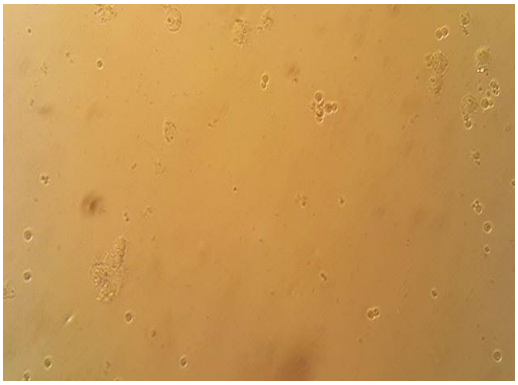

Figure 2: HepG2 cells treated with Ambrosiashowed a highly apoptosis and noproliferated cells were found. (X:200)

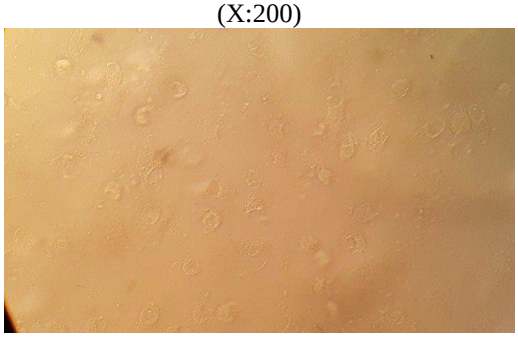

Figure 3: HepG2 cells treated with Trigonellaleaves showed cell apoptosis and no proliferation was found. (X:200)

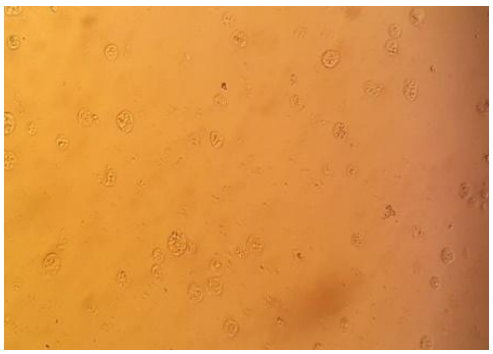

Figure 4: HepG2 cells treated with Trigonella oil, proliferation start to be obvious even apoptosis occurred. (X:200)

The mRNA and protein expression levels of apoptosis-associated factors Bcl-2, p53and CDK4 were therefore evaluated.A reduction in Bcl-2 and CDK4 mRNA

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expression levels andincrease in p53 mRNA expression levels was observed in the followingtreatment with extracts of *Ambrosia*, *Trigonella* leaves and oil respectively compared with untreated control cells, that illustrated in Table1and figure5

Table 1 :Expression levels of Bcl-2, p53 and CDK4

Sample data				Results					
Serial no.	Sample	Cell line	IC50	CDK4 IU/ml		BCL2 IU/ml		P53 IU/ml	
				CT	Conc.	CT	Conc.	CT	Conc.
1	Control	HepG2	---	25.34	48,661	26.16	120,000	32.64	0,950
2	Ambrosia	HepG2	0.001	28.14	6,580	32.46	2,844	25.28	145,329
3	Trigonella	HepG2	0.01	28.95	32,240	31.74	48,274	25.34	92,560
4	Trigonella oil	HepG2	0.01	29.06	39,270	27.50	56,185	26.62	78,192

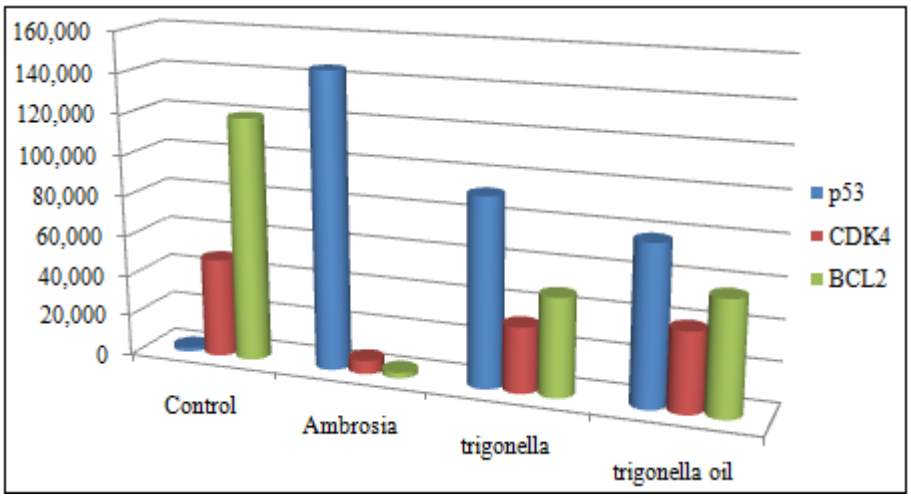

Figure 5: Effects of *Ambrosia* ,*Trigonella* leaves and T oil on the mRNA expression levels of p53, Bcl-2 and CDK4

Respectively. A significant reduction in Bcl-2 and CDK4 mRNA expression levels and increase in p53 mRNA expression levels was observed.

4. Disscusion

Hepatocellular Carcinoma is the most well-known primary liver malignancy and is a major health problem worldwide**(Hamid*et al.*, 2013).**The antitumor effect of medicinal plants has been investigated by several investigators **(Lampronti *et al.* 2003 ; Kviecinski *et al.*, 2008 and Dirar*et al.* 2014).** *Ambrosia maritima L.*, is an important medicinal plant well known for its pharmacological potential, such as antifungal, antibacterial **(Makkawi*et al.*, 2015)**, Antioxidant **(Helal*et al.*,2014; Makkawi*et al* .,2015 and Said *et al.*, 2018), antidiabetic (Helal*et al.*,2014)** activities. Fenugreek was selected for this study since fenugreek have many properties, such as hepatoprotective and antioxidant activity **(Meera*et al.*, 2009; Yadav*et al.*, 2014)**, Antinociceptive activity **(Bhalke*et al.*, 2009**), and anti-inflammatory, antibacterial, antifungal, antiulcer, anticarcinogenic**(Yadav*et al.*, 2014).**

The present study,demonstrated that 0.001 IC50 of *Ambrosia* and 0.01 IC50 of both *Trigonella* leaves and oil decreased the cell viability, cellular shrinkage and blebbing with the increasing concentrations which are a characteristic of apoptosiswere also found. Morphological changes showed growth inhibition of HepG2 cells, which is obvious with Ambrosia and *Trigonella* leaves extracts, while in treatment with *Trigonella* oil, these changes are less observed and cell proliferation started to be obvious even apoptosis occurred.These kinds of alterations in the cellular morphology induced by plant extracts at different concentration have also been reported **(Berrington and Lall, 2012; Al-Oqail*et al.*, 2013, Farshori*et al.*, 2013 and Al-Sheddi*et al.*, 2014).**

In the present study, after 24hour of treated sample, an increase in mRNA expression level of P53 and decrease in mRNA expression levels of both Bcl-2 and CDK4 were noticed when compared to the control. P53 increased with *Trigonella* oil followed by *Trigonella* leaves and Ambrosia leaves extracts, while both Bcl-2 and CDK4 levels decreased with *Trigonella* oil followed by *Trigonella* leaves and Ambrosia leaves extracts when compared to the control. previous study showed changes in the mRNA expression of CCND1 (cyclin D1), CDK4, CDK6, p21, p16, p53, caspase-3, caspase-9, caspase-8, DR4, DR5, Bcl-2, BID, FADD,TRADD, Bax genes relative to GAPDH mRNA expression using Real Time PCR demonstrated thatCCND1 expression was reduced in HepG2 dose the group cells when compared with the control group cells and p53, caspase-3, caspase p21, caspase-8 and expressions were increased in the dose group cells when compared with the control group cells. Other expressions of genes were found statistically insignificant. CCND1, CDK4 and CDK6 expressions were reduced in MIA PaCa-2 dose group cells when compared with the control group cells and p53 expression was increased in the dose group cells when compared with the control group cells. Other expressions of geneswere found statistically insignificant **(Alur*et al.*, 2016).**

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5. Conclusion

In view of the obtained results, it could be concluded that *Ambrosia maritima*, *T. foenum* leaves and oil are able to reduce the cell viability, and altered the cellular morphology of HepG2 cells in a concentration dependent manner. The data also revealed that HepG2 cells were more sensitive to *Ambrosia maritima* leaves than the *Trigonellafoenum-graecum* leaves and oil extracts.

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