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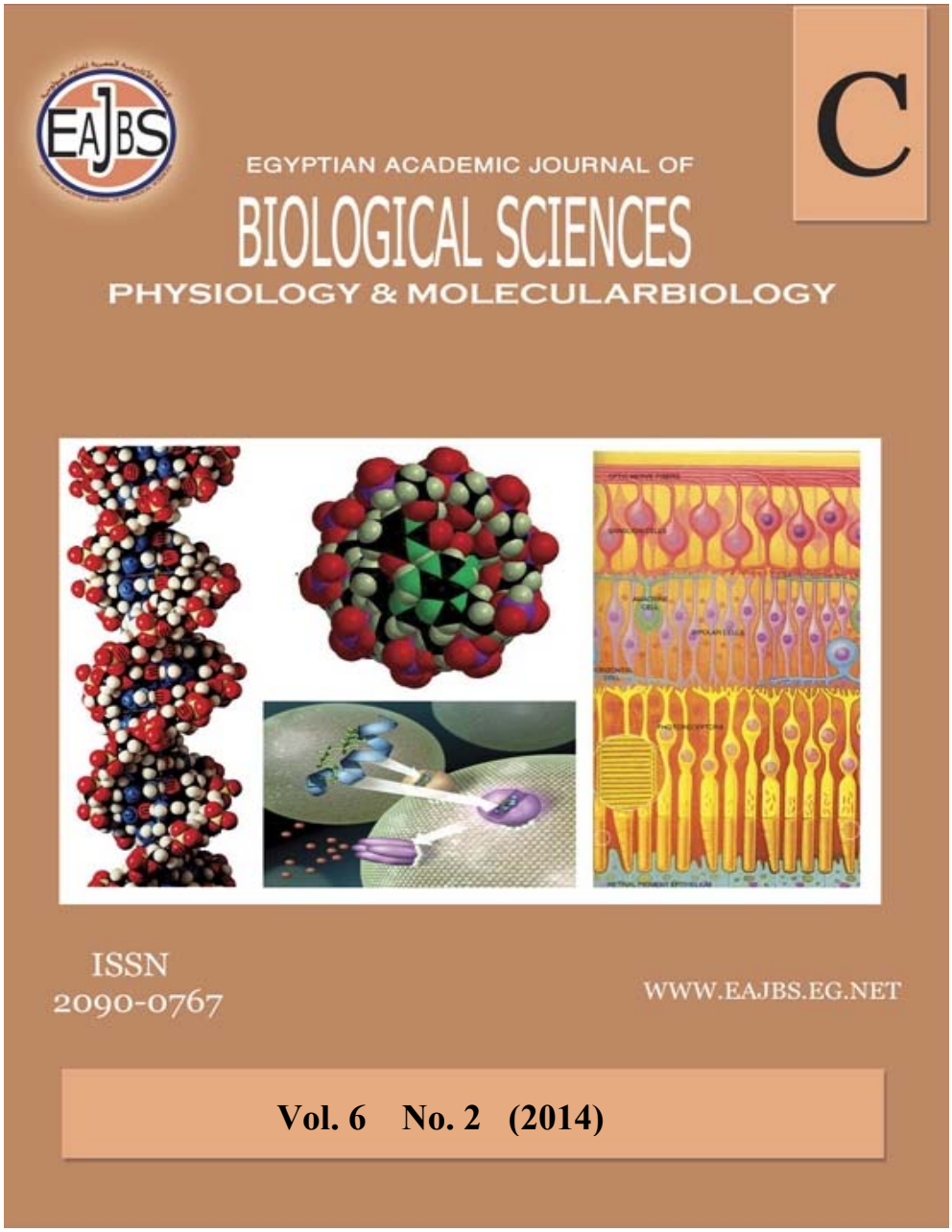

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The Anti-Proliferative Effect of Hesperidin on Hepatocarcinoma Cells HepG2

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ABSTRACT

This study investigates the antiproliferative effect of hesperidin on human hepatocarcinoma HepG2 cell line. Different concentrations of hesperidin 25, 50 *et al.* 100 µM were used for different time intervals 24, 48 *et al.* 72 hrs, while untreated cells were served as control. The effects of different concentrations of hesperidin on HepG2 cell viability, nuclear factor kappa-B (NF-kB) gene expression *et al.* alpha-fetoprotein (AFP) secretion from the cells were measured. The results of the cell viability assays showed that hesperidin concentrations exhibited a highly significant inhibition effect on HepG2 cell proliferation which was evidenced by reduction in viable cells count. The results were confirmed by microscopical observations of cell morphology. Furthermore, hesperidin suppressed the activity of NF-kB gene expression of HepG2 cells compared to the control. Also it caused a highly significant depression in the level of AFP in HepG2 cells compared with that of controls in time dependent manner. Therefore, the conclusion was that hesperidin inhibits the growth of HepG2 cells through suppressing the activity of NF-kB gene expression *et al.* modulates the biochemical marker AFP.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common form of liver cancer; most cases of HCC (approximately 80%) are associated with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infections. Variations in the age-, sex-, *et al.* race-specific rates of HCC rates in different geographic regions are likely to be related to differences in the prevalence of hepatitis viruses in the populations, as well as the timing of the spread of the viral infection *et al.* the age of individuals at the time of the infection. According to the International Agency for Research on Cancer, liver cancer is the fifth most common cancer in men worldwide (523,000 cases per year,7.9% of all cancers) *et al.* the seventh in women (226,000 cases per year, 6.5% of all cancers). Liver cancer has high mortality; the geographic distribution of mortality is similar to that of incidence. Most of the burden of liver cancer is in developing countries, where almost 85% of the cases occur (El-Serag, 2012).

The burden of HCC has been increasing in Egypt with a doubling in the incidence rate in the past 10 years. This has been attributed to several biological (e.g. HBV *et al.* HCV infections) *et al.* environmental factors (e.g. aflatoxin).

Other factors such as cigarette smoking, occupational exposure to chemicals as pesticides, *et al.* endemic infections in the community, such as schistosomiasis, may have additional roles in the etiology or progression of the disease (Anwar, *et al*., 2008). Although substantial advances have been made in HCC chemotherapy regimens, the efficacy of anti-cancer drugs is often limited by adverse side effects. Accordingly, new approaches are needed to further the development of an effective therapy against HCC. So, focus has shifted to materials from natural sources that aid in treating cancer progress *et al.* metastasis, while minimizing adverse side effects (Chun, *et al*., 2011).

Hesperidin, a flavanone glycoside found abundantly in citrus fruits, possesses a wide range of pharmacological properties including potential anti-inflammatory *et al.* anticancer effects (Ou, 2002; Benavente-Garcia & Castillo, 2008). Hesperidin induces cell growth arrest *et al.* apoptosis in a large variety of cells including colon *et al.* pancreatic cancer cells (Park *et al*., 2008; Patil *et al*., 2009). Hesperidin has non-toxic activity in normal cells, but it is reported that hesperidin suppresses cell proliferation in several cancer types. Hesperidin, such as flavonoid is known to have anti-inflammatory, anti-viral, UV protecting, antioxidant, proapoptotic, anti-proliferative *et al.* anti-tumor properties *et al.* protective effects against cerebrovascular disease *et al.* diabetes mellitus (Garg *et al*., 2001; Knekt *et al*., 2002; Choi, 2007; Akiyama *et al*., 2010; Ghorbani *et al*.., 2012; Kamaraj *et al*., 2011; Lee *et al*., 2011). In addition, hesperidin as a radioprotective *et al.* chemoprotective therapeutic agent is expected to prevent invasion or metastasis of human cancers (Lee *et al*., 2010). Anti-cancer effects of hesperidin was studied in tumor implanted animal models or culture cell lines of several cancer types, including colon cancer, bladder cancer, hepatocarcinoma cancer, *et al.* breast cancer, but the mechanism of this compound was not understood (Park *et al*., 2008; Choi, 2007; Lee *et al*., 2010; Tanaka *et al*., 1997; Yang *et al*., 1997; Yeh *et al*., 2009) .

MATERIALS AND METHODS

Chemicals *et al.* reagents

Hesperidin (Sigma Aldrich, Germany), dimethyl sulfoxide (DMSO) (SERVA,USA), cell culture medium; RPMI 1640, heat-inactivated fetal bovine serum (FBS), penicillin-streptomycin ,amphotricin B *et al.* trypsin-EDTA were purchased from Lonza Bio Whittaker (USA).

Cell Culture

HepG2 cells, derived from a human hepatocelluar carcinoma (obtained from the holding company for biological products VACSERA, Egypt), were seeded in 25 cm^2 tissue culture flasks at 1×10^5 cell/cm^2 in RPMI medium supplemented with 10% fetal bovine serum, 2% penecillin *et al.* streptomycin, 1 % amphotricin B at 37°C in a humidified atmosphere of 95% air *et al.* 5% CO_2.

Treatment of HepG2 with hesperidin

Hesperidin was dissolved in DMSO (stock concentration 1 mM) *et al.* sterilized using (0.22 µm) syringe filter then stored at 4°C until use. Cells 1×10^5 /cm^2 were incubated with media containing hesperidin of varying concentrations (25, 50 *et al.* 100 µM) for 24, 48 *et al.* 72 hrs, untreated cells served as control.

Cell viability assay

1×10^5 cells/cm^2 were treated with hesperidin 25, 50 *et al.* 100 µM. After treatment for 24, 48, *et al*. 72 hrs cells were trypsinized, cell viability was counted under the light microscope (100x) using trypan blue dye (0.04%).

Real-Time PCR (RT-PCR) amplification of NF-kB

HepG2 cells were cultured *et al.* treated as described above. The cell pellets were collected after being trypsinized *et al.* centrifuged. Total RNA was extracted using the RNeasy mini kit (Qiagen, USA). Total RNA was transcribed into c-DNA using the reverse transcription system kit high capacity RNA-to-cDNA master mix (Applied

Biosystems, USA) according to the manufacturer's instructions. The resultant cDNA was diluted 10 times in double distilled water *et al.* kept at -20 °C for RT-PCR analysis. Then diluted cDNAs were amplified with the following primer pairs, mouse ß-actin was amplified as the control, using quantifast SYBR Green PCR kit (Qiagen, USA) in a 10 µl final volume according to the manufacturer's instructions: NF-kB 5'-gcgtacacattctggggagt- 3' *et al.* 5'-ccgaagcaggagctatcaac-3'; ß-actin 5'-agccatgtacgtagccatcc-3' *et al.* 5'-gctgtggtggtgaagctgta-3' (A total of 45 cycles were performed, 94°C for 15 seconds, 60°C for 25 seconds, *et al.* 72°C for 20 seconds). The expression differences between the control *et al.* treated cells were calculated by normalizing with ß -actin gene expression according to the following formula (Livak & Schmittgen, 2001):

Fold change = $2^{-\{[Ct\ (control)\ gene\ X - Ct\ (control)\ actin] - [Ct\ (activated)\ gene\ X - Ct\ (activated)\ actin]\}}$.

Assay of AFP synthesis

After treatment of 1×10^5 cells/cm^2 of HepG2 with 25, 50 *et al.* 100 µM of hesperidin, medium that had been in contact with cells for 24, 48 *et al.* 72 hrs was removed *et al.* stored at −20°C to prevent AFP degradation. The amounts of AFP were determined by microparticle enzyme immunoassay (AFP AXSYM Abbott, Abbott Diagnostics, USA) (Mazure *et al.*., 2002).

Data Analysis

The statistical analysis of the data was carried out using ANOVA followed by Tukey's test. Values are expressed as mean ($\bar{x}$) ± st*et al.*ard deviation (SD):

-Insignificant difference from corresponding control (P>0.05).

-Significant difference from corresponding control (P<0.05).

-Highly significant difference from corresponding control (P<0.001).

RESULTS

Hesperidin inhibited the proliferation of HepG2cells

Cell viability assay was done to determine the effect of hesperidin on HepG2 growth rate, this study shown that hesperidin significantly inhibits HepG2 cell growth *et al.* its effects were proportional with increasing the concentration *et al.* the time of exposure (Fig. 1). Microscopic observations of cell morphology show that hesperidin induced morphological changes in HepG2 cells towards more mature forms of hepatocytes using an inverted light microscope (Fig. 2).

Hesperidin inhibited the activity of nuclear factor kappa B gene expression of HepG2 cells. The study showed a time dependent inhibition in the activity of NF-kB gene expression of HepG2 cells treated with 25, 50 *et al.* 100 µM of hesperidin which was evident by depression in the fold change of NF-kB compared with the control (Fig. 3).

Hesperidin inhibited AFP secretion in HepG2 cells culture medium

The AFP level in the culture medium of hesperidin-treated groups was significantly lower than that of the control group at different time intervals. 25µM hesperidin concentration inhibited AFP secretion by 23.8, 31.9 *et al.* 35.5% compared to untreated group after 24, 48 *et al.* 72 hrs, respectively. 50µM hesperidin inhibited the secretion by 45.5, 48.2 *et al.* 53.9%. AFP secretion in 100 µM hesperidin treated group was reduced by 43.4, 51.6 *et al.* 62.3% after 24, 48 *et al.* 72 hrs, respectively.

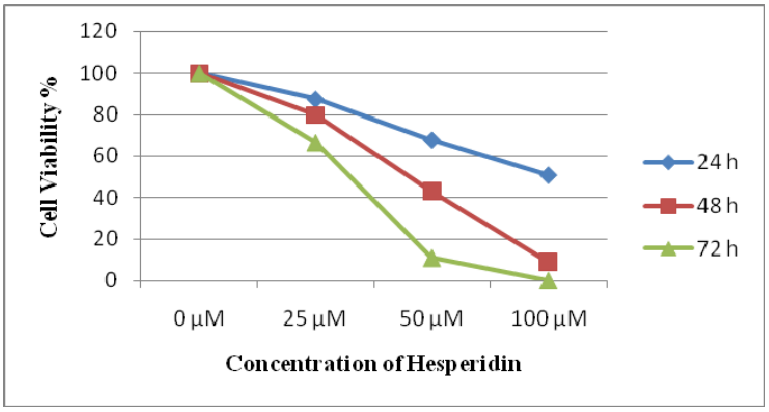

Fig. 1: Time course responses of hesperidin concentrations on HepG2 cell viability (%) : This figure showed a highly significant inhibition (P<0.001) of the proliferation of HepG2 cell line using 25, 50 *et al.* 100 µM of hesperidin as the percentage of cell viability have significantly decreased with the progress of time 24, 48 *et al.* 72 hrs.

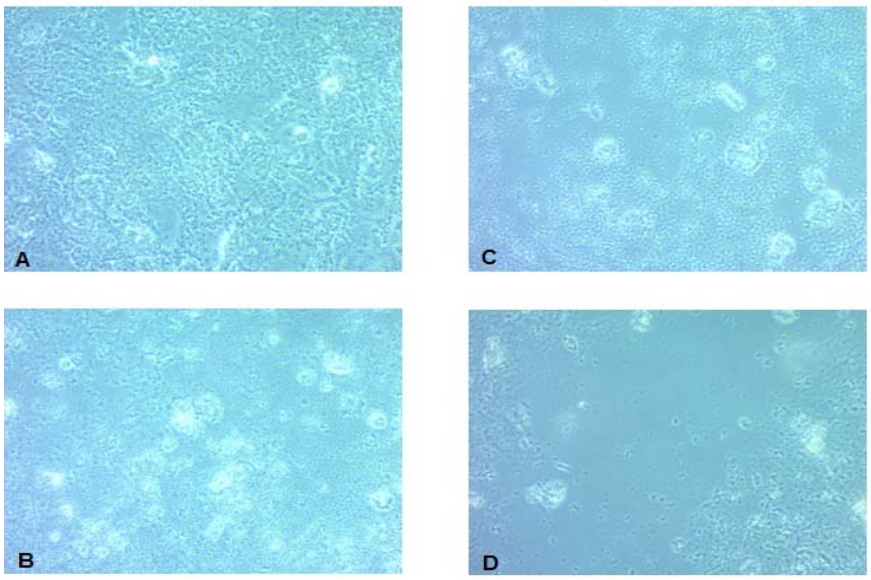

Fig. 2: HepG2 cells as shown under inverted light microscope after hesperidin treatment: A: Control untreated cells, B: 25 µM hesperidin after 72 hrs, C: 50 µM hesperidin after 72 hrs, D: 100 µM hesperidin after 72 hrs.

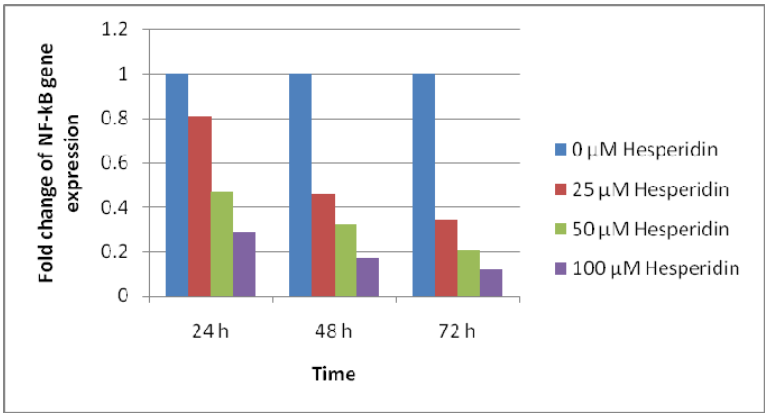

Fig. 3: Fold change of NF-kB gene expression after treatment of hesperidin: NF-kB gene expression was decreased in Hep2 cells treated with 25, 50 *et al.* 100 µM of hesperidin for 24, 48 *et al.* 72 hrs.

DISCUSSION

Flavonoids are members of polyphenolic compounds that are categorized, according to their chemical structure into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins *et al.* chalcones (Beecher, 2003). They are mainly found in fruits, vegetables *et al.* natural beverages. Flavonoids have recently arisen considerable interest because of their potential beneficial effects on human health (Del Rio *et al.*, 2010). It has been reported that they have antiviral, antiallergic, antiplatelet, anti-inflammatory *et al.* antioxidant activities as well as antitumor properties (Barnes, 2008).

Hesperidin is a flavanone glycoside found abundantly in citrus fruits such as lemons *et al.* oranges. According to in vitro studies, it acts as an antioxidant (Wilmsen *et al*., 2005). In human *et al.* animal studies, it contributes to the reduction of cholesterol, blood pressure (Yamamoto *et al*., 2008) *et al.* bone density loss (Chiba *et al*., 2003). Hesperidin has been reported to inhibit cell cycle progression in human pancreatic cells (Patil *et al*., 2009). It also reduces the generation of reactive oxygen species *et al.* triggers caspase-dependent apoptosis in human polymorphonuclear neutrophils in vitro (Zielinska- Przyjemska *et al.* Ignatowicz, 2008).

It has been shown that hesperidin possesses cancer chemopreventive effect on some tumor cells. Its lack of toxicity makes it apotentially attractive agent for use in conjunction with chemotherapy (Aranganathan & Nalini, 2009). Hesperidin has dose-dependent cytotoxic effects on human colon cancer cells, accompanied by DNA fragmentation caspase-3 activation (Park *et al*., 2008). However, in spite of many years of research *et al.* speculation, the exact mechanisms underlying hesperidin-induced apoptosis have not been clearly understood (Nazari *et al*., 2011).

HCC is one of the most common malignancies worldwide. Despite many therapeutic approaches, the long-term prognosis of HCC is poor because of higher relapse *et al.* intrahepatic metastasis. Tumor cells to evadeprogrammed cell death are a major characteristic that enables their uncontrolled growth (Jeong *et al*., 2010).

HCC cells are useful in underst*et al.*ing both phenotypes of liver cancer *et al.* phenotypes of hepatocytes relevant to healthy *et al.* diseased states. Among human HCC cell lines, HepG2 are probably the best characterized (Burley & Roth, 2007). HepG2 cells serve a variety of functions in the laboratory, including assessment of the toxicology of compounds, such as antiviral drugs, genotoxicants *et al.* antigenotoxic agents (Hongo *et al*., 2005). HepG2 cells are also employed to study a range of physiological *et al.* pathophysiological conditions, including liver disease, gene expression *et al.* transcription in cancer, inflammatory response, *et al.* blood coagulation. Furthermore, HepG2 cells are being developed as a potential cell source for bioartificial liver devices. Their versatility *et al.* function make them extremely useful as a model system for liver function (Wolfe *et al*., 2008).

In the present study hesperidin was dissolved in DMSO to prepare different concentrations 25, 50 *et al.* 100 µM, HepG2 cells ware treated with these concentrations for different time intervals to evaluate the anti-proliferative effects of hesperidin on cells. The results of cell viability assays showed that all concentrations exhibited a highly statistically significant ($P<0.001$) inhibition on HepG2 cell proliferation which were evidenced by reduction in viable cells count *et al.* many cancer cells became detached *et al.* floated in the medium. Cell detachment means cell death because HepG2 cells only grow when attached. 100µM of hesperidin had much stronger inhibitory activities than other concentrations as survival rate was zero % (100 % dead cells) after exposure for 72 hrs.

The present study showed that hesperidin exerts a strong growth inhibitory activity against HepG2 cells. Morphological

observations showed that hesperidin-treated cells were clearly discerned by their rounded shapes compared to the polygonal shapes of untreated cells. Increased apoptosis was observed in HepG2 cells, generally, cell bodies shrunk, chromatin condensed, *et al.* plasma membranes preserved continuity bleb (Kai *et al*., 2010). The growth-inhibitory effects of hesperidin were accompanied by cell shrinkage, membrane blebbing *et al.* more intracellular spaces took place between the treated cells suggesting possible growth-arresting *et al.* apoptosis-inducing effects of hesperidin.

The accumulated pieces of evidence show that flavonoids have multiple modes of anti-cancer activities (Benavente-Garcia & Castillo, 2008; Fresco *et al*., 2006). Several mechanisms have been postulated for the tumor growth-inhibitory effects of flavonoids, including, but not limited to, the inhibition of NF-kB signaling pathway (Sarkar *et al*.., 2009; Xu *et al*., 2008).

The NF-kB family of transcription factors is involved in multiple cellular processes, including cytokine gene expression, cellular adhesion, cell cycle activation, apoptosis *et al.* oncogenesis (Cao *et al*., 2011). NF-kB plays an essential role during inflammation *et al.* immune responses as well as in other physiological functions such as cell growth, apoptosis, *et al.* developmental processes (park *et al*., 2012). Recent studies have shown that inactivation of the NF-kB in the hepatic compartment inhibits liver tumor formation through induction of cell death *et al.* inhibition of compensatory proliferation. Furthermore, mounting evidence has illustrated a major role of NF-kB in inducible chemo-resistance of HCCs (Wang *et al*., 2007).

RT-PCR analysis showed that hesperidin suppressed the constitutive activity of NF-kB gene expression of HepG2 cells with treatment of hesperidin which effectively induced tumor growth when compared to the control.

This result was in agreement with Lee *et al*., (2010) who reported that that hesperidin inhibits TPA-induced cell invasiveness *et al.* MMP-9 expression in human cancer cells by suppressing both the AP-1 *et al.* NF-kB signaling pathways. These data suggested that hesperidin more likely reduced apoptotic effects by suppressing NF-kB activity *et al.* NF-kB regulated transcription of genes involved in cell survival.

AFP is a liver specific glycoprotein that expresses primarily at the foetal liver *et al.* the placenta, *et al.* since it overexpresses in 60%–70% of hepatocellular carcinoma cells, is used as a tumor marker in hepatocarcinoma diagnosing (Li *et al*., 2011). Hepatoma cells can synthesize various tumor-related proteins, such as AFP (Yao *et al*., 2007). Specifically, AFP, which is synthesized by transformed hepatocytes with high-grade malignancy, is known to be present in HepG2 cells (Cid *et al*., 2012).

As regard to AFP results in present study, showed a highly significant depression ($P<0.001$) in the levels of AFP in HepG2 cells treated with 25, 50 *et al.* 100 µM respectively compared with that of controls. Also these results showed that 100 µM concentration of hesperidin induced higher activity than other concentrations.

The content of AFP in the supernatant of cultured HepG2 cells when significantly reduced indicated that, it may be a result of growth inhibition *et al.* apoptosis of the HepG2 cells (Liu *et al*., 2004).

In conclusion, the present study demonstrates the effects of hesperidin on the proliferation of human hepatocellular carcinoma cells. It shows that hesperidin inhibit the growth of HepG2 cells through suppressing the activity of NF-kB gene expression *et al.* modulates the biochemical marker AFP.

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ARABIC SUMMARY

تأثير الهيسبيريدين كمضاد لتكاثر خلايا الكبد السرطانية

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سرطان الخلايا الكبدية هو أحد الأورام الخبيثة الأكثر شيوعا في جميع أنحاء العالم، مع ارتفاع معدل انتشاره بما يقرب من مليون حالة سنويا، ومعدل البقاء على قيد الحياة ضئيل جدا من ٣-٥٪. و يعد سرطان الكبد من أكثر الأورام مقاومة للعلاج الكيميائي و هو ثالث أكثر سبب شيوعا للوفاة في العالم من السرطان و يسود في البلدان النامية.

أصبح معدل الإصابة بسرطان الكبد في مصر في حالة زيادة مطردة و يرجع ذلك إلى عدة اسباب بيولوجية مثل التهاب الكبدي الفيروسي ب و ج و كذلك عوامل بيئية مثل الأفلاتوكسين وغيرها، و عوامل أخرى مثل تدخين السجائر والتعرض المهني للمواد الكيميائية مثل مبيدات الآفات، و الأمراض المتوطنة في المجتمع مثل البلهارسيا.

على الرغم من إحراز تقدم كبير في نظم العلاج الكيميائي لسرطان الخلايا الكبدية، غالبا ما تكون فعالية الأدوية المضادة للسرطان محدودة نتيجة آثارها الجانبية الضارة. وفقا لذلك، هناك حاجة إلى نهج جديد لتعزيز تطوير علاج فعال ضد سرطان الكبد و اتجه التركيز في الآونة الأخيرة إلى المواد الطبيعية و التي تساعد في علاج تقدم السرطان مع التقليل من الآثار الجانبية السلبية.

و قد اختير فلافونويد مستخلص من الموالح هو الهيسبيريدين - و الذي يعمل كمضاد للأكسدة و الالتهاب و يساعد على خفض ضغط الدم و وجد أيضا أن له تأثير مضاد للعديد من الخلايا السرطانية – لدراسة تأثيره على خلايا الكبد السرطانية (HepG٢).

لقد تم إذابة الهيسبيريدين في محلول (DMSO) وتم تخفيفه بواسطة وسط نمو الخلايا (IMPR) لمعالجة الخلايا بالتركيزات المختلفة من الهيسبيريدين و ٥٠ و١٠٠ميكرومول بينما يتم معاملة الخلايا الغير معالجة كعينة قياسية ثم فحص و عد هذه الخلايا باستخدام صبغة (trypan) الزرقاء في فترات زمنية مختلفة ٢٤ و ٤٨ و ٧٢ ساعة.

بعد ذلك تم قياس نشاط جين (NF-kB)باستخدام تفاعل انزيم بلمرة التسلسل (TR-RCP) للخلايا بعد تجميعها في نهاية كل فترة زمنية و تم ايضا قياس بروتينات الألفا فيتو (AFP) في الوسط المحيط بالخلايا بعد تجميعها أيضا في نهاية كل فترة زمنية حيث تم معاملة الخلايا الغير معالجة كعينة قياسية.

و من خلال الدراسة الإحصائية انتائج هذا البحث تبين فعالية استخدام الهيسبيريدين كمضاد لتكاثر خلايا الكبد السرطانية (HepG٢) حيث أظهرت النتائج الآتي:

- تناقص ذو دلالة إحصائية لأعداد الخلايا المعالجة بالهيسبيريدين و الذي يتضح من انخفاض عدد الخلايا الحية و قد تم تأكيد ذلك بالفحوصات المجهرية.
- تناقص ذو دلالة إحصائية ملحوظة لنشاط جين (NF-kB) مقارنة بالخلايا القياسية.
- كذلك تناقص ذو دلالة إحصائية لإفرازخلايا (HepG٢) لبروتينات الألفا فيتو (AFP) في الوسط المحيط بالخلايا مقارنة بالخلايا القياسية.