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The potential therapeutic effect of Luteolin against Diethyl nitrosamine induced hepatocellular carcinoma in rats.

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A B S T R A C T

Hepatocellular carcinoma (HCC) is one of the main causes leading to cancer mortality. Luteolin derived from celery and has anti-cancer activity. Cisplatin is used as a chemotherapeutic agent derived from celery and has anti-cancer activity. Cisplatin is used as a chemotherapeutic agent for the treatment of HCC. A major problem with Cisplatin is the development of Cisplatin chemo resistance. This study aims to evaluate the role of Luteolin in HCC treatment and as a chemo-sensitizer for Cisplatin treatment of HCC in rats. Sixty rats were divided in to sex groups, 10 rats each, group 1: normal control group, group 2: cisplatin (1.5 mg/Kg b.wt) for four weeks and group 3: received the Diethyl nitrosamine (DEN) (20 mg/kg b.wt), group 4: received DEN as in group 3 and then treated with cisplatin, group 5: received DEN as in group 3 and then treated with Luteolin (0.2 mg/kg b.wt) and group 6: received DEN as in group 3 and then treated with cisplatin and Luteolin. After 10 weeks animals were sacrificed and liver tissue were removed for Histopathological and tissue parameters examination. A significant increase in serum serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) activity, a significant decrease in serum albumin concentration. Also a significant increases in liver transforming growth factor-beta 1 (TGF-b1), B-cell lymphoma 2 (Bcl-2) and nuclear factor kappa β (NF-kβ) concentrations and a significant decrease in Caspase.3 activity were recorded in DEN-treated rats while treatment with Luteolin and Cisplatin reduced the severity of HCC and enhanced histopathological findings, these result suggesting the efficacy of Luteolin supplementation as an anti-HCC

Keywords: *HCC, Luteolin, TGF-b1 and Caspase.3.*

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1. INTRODUCTION

The incidence of liver cancer is one of the highest in the world. It is up to 90% of all liver malignancies. In Egypt, HCC constitutes a significant public health problem. Where it is responsible for 33.63% and 13.54% of all cancers in males and females respectively. Hepatocellular carcinoma occurs in a number of preexisting conditions that commonly includes hepatitis C and B, alcoholic and nonalcoholic cirrhosis. It has a poor prognosis after discovery. This had been strongly linked to the hepatitis C virus epidemic that affected around 10-15% of the Egyptian

population during the last 3 decades, and was reported as the highest prevalence of HCV in the world (Elghazaly et al., 2019).

Diethyl nitrosamine (DEN) is a strong hepatocarcinogenic substance that causes disturbances in nucleic acid repair mechanisms and also generates reactive oxygen species (ROS) leading to oxidative stress (Fazal et al., 2017).

Cisplatin, cisplatinum, or cis-diamminedichloro-platinum (II), is a well-known chemotherapeutic drug. It has been used for treatment of numerous human cancers including bladder, head and neck, lung, ovarian, and testicular cancers. It is effective against various types of cancers, including carcinomas, germ cell tumors, lymphomas, and sarcomas. Its mode of action has been linked to its ability to crosslink with the purine bases on the DNA; interfering with DNA repair mechanisms, causing DNA damage, and subsequently inducing apoptosis in cancer cells. However, because of drug resistance and numerous undesirable side effects such as severe kidney problems, allergic reactions, decrease immunity to infections, gastrointestinal disorders, hemorrhage, and hearing loss especially in younger patients. Furthermore, combined treatment with other sensitizing agents is an effective strategy to overcome cisplatin resistance (Shaloam et al., 2014).

Luteolin, 3′,4′,5,7-tetrahydroxyflavone, belongs to a group of naturally occurring compounds called flavonoids that are found widely in the plant kingdom in celery, thyme, green peppers, and chamomile tea (Yong et al., 2008) . Humans absorb a large amount of flavonoids orally. Epidemiological studies have revealed that the risk of certain types of cancer, particularly cancers of the breast, digestive tract, skin, prostate and certain hematological malignancies, is inversely correlated with intake of flavonoids (Rui et al., 2017). Luteolin, an important flavonoid, exhibits a wide spectrum of pharmacologic properties including anticancer properties (Manju et al., 2007). It has been well studied that luteolin is capable of inducing cell cycle arrest or apoptosis in various human cancer cells (Lim et al., 2006).it was reported previously that luteolin is capable of sensitizing apoptotic cell death induced by tumor necrosis factor or tumor necrosis factor–related apoptosis-inducing ligand in various human cancer cells (Shi et al., 2005), suggesting the potential therapeutic value of luteolin in cancer therapy.

The aim of this study to evaluate the using of luteolin for treatment of HCC and its ability to reduce the side effect of cisplatin.

2. MATERIALS AND METHODS

2.1. Experimental animals:

Sixty male albino rats (170-220 g), aged about 4 weeks, and were used in this study. Animals were obtained from the National Centre for Radiation Research and Technology (NCCRT), Cairo, Egypt. Animals were housed in cages and maintained under standard conditions of ventilation, temperature and humidity. Animals received standard food pellets and water ad libitum. All animal treatment procedures conformed to the National Institutes of Health (NIH) guidelines.

2.2. Chemical and antioxidant:

Luteolin was purchased from Sigma Company, Cisplatin was purchased from Tarshouby Pharmacy (Mansoura, Egypt), diethyl nitrosamine (DEN) used in this study was purchased from Sigma Chem. Co., (St. Louis, U.S.A) and all other chemicals were of analytical grade and were obtained from standard commercial suppliers.

2.3. Preparation and administration of dosage:

Hepatocellular carcinoma was induced by administration of (20mg/kg b.wt/day) orally of DEN dissolved in 0.9 saline five time per week for six weeks according to (Balamurugan et al., 2012).

Luteolin was administered at a dose level of (0.2 mg/kg b.wt/ i.p /day) from the seventh week to the end of tenth week (Balamurugan et al., 2012).

Cisplatin was administered daily at a dose level of (1.5 mg/kg b.wt/ i.p) from the seventh week until the end of tenth week (Abass et al., 2018).

2.4. Experimental design

Rats were divided randomly into 6 groups, 10 each, G1 (Control): Normal healthy control animals, G2 (Cisplatin): animals received cisplatin for four weeks, G3 (diethyle

nitrosamine): animals received DEN orally for six weeks, G4 (diethyle nitrosamine + cisplatin): animals received DEN then treated with cisplatin, G5 (diethyle nitrosamine + Luteolin): animals received DEN then treated with Luteolin and G6 (diethyle nitrosamine + Cisplatin + Luteolin): animals received DEN then treated with cisplatin and Luteolin.

2.5. Sampling:

2.5.1. Blood samples:

Blood samples were collected by heart puncture in dry, clean test tubes and allowed to clot for 30 min and serum was separated by centrifugation at 3000 r.p.m for 15 min. The serum was separated by automatic pipette and received in dry sterile tubes, processed directly for ALT , AST (Reitman et al., 1957), and Albumin (Lowry, 1951) determination. After blood samples collection rats were decapitated to collect liver samples.

2.5.2. Tissue specimens (liver):

2.5.2.1.Biochemical analysis:

Liver samples for preparation of tissue homogenates where 100 mg tissue was rinsed with 1X PBS, homogenized in 1 ml of 1X PBS and stored overnight at -20°C. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 x g, 2 - 8°C. The supernatant was removed and assayed immediately for estimation of:

TGF-b1 (Rat TGF-b1 ELISA kit purchased from MyBiosource, Inc. (USA), catalog no. MBS824788), BCL2 (Rat BCL2 ELISA kit purchased from MyBioSource, Inc. (USA), catalog no. MBS704330), NF-kβ (Rat NF-kβ ELISA kit purchased from MyBioSource, Inc. (USA), catalog no. MBS043224), and Caspase.3 (rat caspase-3 ELISA kit purchased from MyBio-Source, Inc. (USA), catalog no. MBS700575).

2.5.2.2.Histopathological examination:

The liver tissue were rapidly dissected and excised, rinsed in saline solution for histopathological investigation. Following fixation of the livers with 4% paraformaldehyde, paraffin-embedded sections were subjected to standard hematoxylin and eosin staining and then studied by light microscopy. Hepatic steatosis was measured by staining of 8-mm thick frozen sections with Oil-Red-O (Sigma-Aldrich).

2.6. Statistical analysis

The SPSS (version 25) was used in data analysis. Data were analyzed with one-way analysis of variance (ANOVA) followed by a post hoc test (Duncan alpha) for multiple comparisons. The data were expressed as mean ± standard error (S.E). P values < 0.05 were considered to be statistically significant.

3. RESULTS-

A significant increase in Serum alanine amino-transferase (ALT) and aspartate aminotransferase (AST) activity, a significant increase in liver transforming growth factor-beta 1 (TGF-b1, B-cell lymphoma 2 (BCL-2) and nuclear factor kappa β (NF-kβ) concentration and a significant decrease in serum Albumin concentration and liver caspase.3 activity were observed in DEN-induced HCC in rats when compared with control normal group. Intraperitoneal administration with Cisplatin to rats resulted in a significant decrease in Serum ALT and AST activity, a significant decrease in liver TGF-b1, BCL2 and NF-kβ concentration however a significant increase in serum ALB concentration and liver caspase.3 activity when compared with control normal group. Treatment with Cisplatin and Luteolin to DEN rats resulted in a significant decrease in Serum ALT and AST activity, a significant decrease in liver TGF-b1, BCL2 and NF-kβ concentration while a significant increase in serum albumin concentration and liver caspase.3 activity when compared with control DEN non-treated group.

3.1. Histopathological findings

A liver of rat receiving DEN showing Patchy inflammatory infiltrate associated with dysplastic changes, dilated blood vessels and Perivascular inflammatory infiltrate associated with carcinoma formation, all were recorded in (Fig.3). While treatment with Cisplatin or Luteolin showed hepatocellular carcinoma with moderate degenerative changes, scattered

inflammatory infiltrate all were recorded in fig (4,5), but a liver of rat treated with cisplatin + luteolin after receiving DEN showing marked degenerative changes tumor tissue necrosis all were recorded in fig (6).

Table1. Effect of luteolin and/or cisplatin administration on serum ALT, AST activities and albumin concentration in diethyl nitrosamine –induced HCC in rats.

Experimental groups	ALT (U/L)	AST (U/L)	ALB (g/dl)
G1: Control	43.13 ± 2.25 ^{f}	38.0 ± 7.5 ^{f}	4.11 ± 0.044 ^{a}
G2: Cisplatin	167.33 ± 5.61 ^{b}	253.6 ± 7.93 ^{c}	3.63 ± 0.033 ^{b,d}
G3: diethyl nitrosamine	209.33 ± 10.48 ^{a}	334.6 ± 6.88 ^{a}	2.89 ± 0.052 ^{e}
G4: diethyl nitrosamine + Cispaltin	116.33 ± 5.90 ^{d}	237.66 ± 9.14 ^{c}	3.91 ± 0.066 ^{a,b}
G5: diethyl nitrosamine + Luteolin	87.33 ± 3.71 ^{e}	132.5 ± 4.26 ^{e}	3.82 ± 0.062 ^{a,b}
G6:diethyl nitrosamine + Cispaltin + Luteolin	96.00 ± 5.13 ^{e}	299.7 ± 5.78 ^{b}	3.65 ± 0.029 ^{b}

Data are presented as (Mean ± S.E).S.E = Standard error.
Mean Value with different superscript letters in the same column are significantly different at (P≤ 0.05).

Table (2): Effect of luteolin and/or Cisplatin administration on liver tissue TGF-b1, BCL-2, NF- kβ concentrations and Caspase-3 activity in diethyl nitrosamine -induced HCC in rats.

Experimental groups	TGF-B1 (pg/ml/g. tissue)	BCL2 (ng/ml/g. tissue)	NFKb (pg/ml/g. tissue)	Caspase.3 (ng/ml/g. tissue)
G1: Control	63.5± 2.64 ^{e}	33.93±1.47 ^{e}	54.66±0.775 ^{f}	2.14±0.08 ^{d,e}
G2: Cisplatin	87.36 ±13.15 ^{c,d}	31.26 ± 1.18 ^{e}	60.87±5.42 ^{f}	4.62±0.09 ^{c}
G3: diethyl nitrosamine	200.7± 10.15 ^{a}	127.4±2.99 ^{a}	166.7±3.873 ^{a}	1.54±0.178 ^{e}
G4: diethyl nitrosamine + Cispaltin	107.8 ± 3.84 ^{b,c}	80.9±5.1 ^{b,c}	110.66±1.61 ^{b,c}	6.54±0.18 ^{b}
G5: diethyl nitrosamine + Luteolin	122.03±3.15 ^{b}	92.93±1.26 ^{b}	116.7±1.436 ^{b}	3.36 ± 0.721 ^{c,d}
G6:diethyl nitrosamine + Cispaltin + Luteolin	89.4±1.33 ^{c,d}	46.1±1.61 ^{d}	94.63±0.77 ^{d}	11.63 ± 0.75 ^{a}

TGF-b1: liver transforming growth factor-beta 1, BCL-2: B-cell lymphoma 2, NF-kβ: nuclear factor kappa β and Caspase.3.
Data are presented as (Mean ± S.E).S.E = Standard error.
Mean Value with different superscript letters in the same column are significantly different at (P≤ 0.05).

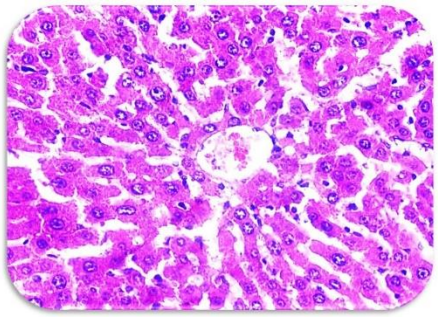

Figure (1) A normal rat liver tissue (H&E x400)

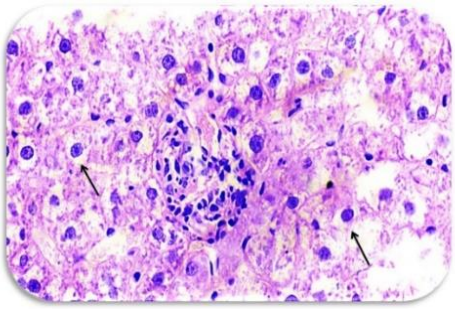

Figure (2): A liver of rat receiving CIS showing degenerative changes, dissolution of hepatic cords (→) and portal inflammatory infiltrate (H&E x400).

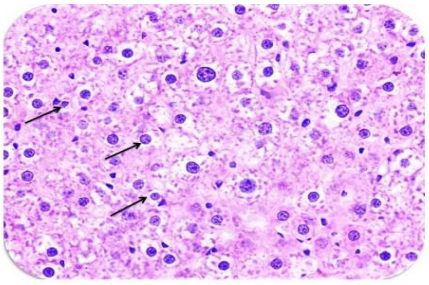

Figure (3): A liver of rat receiving DEN showing hepatocellular carcinoma (note the cellular and nuclear pleomorphism (→), irregular nuclear membranes, coarse chromatin and nuclear inclusions) (H&E x400)

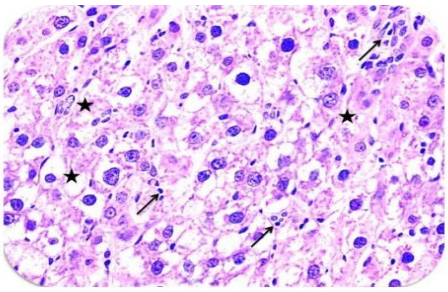

Figure (4) A liver of rat treated with CIS after receiving DEN showing hepatocellular carcinoma with moderate degenerative changes (★), scattered inflammatory infiltrate (→) (H&E x400)

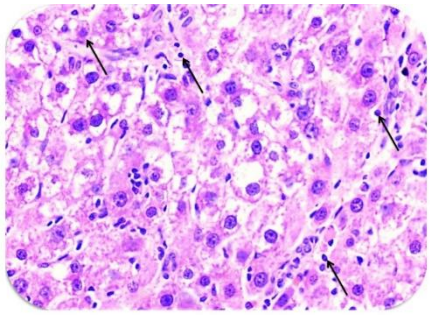

Figure (5): A liver of rat treated with LUT after receiving DEN showing hepatocellular carcinoma with degenerative changes, scattered inflammatory infiltrate and apoptotic figure (→) (H&E x400)

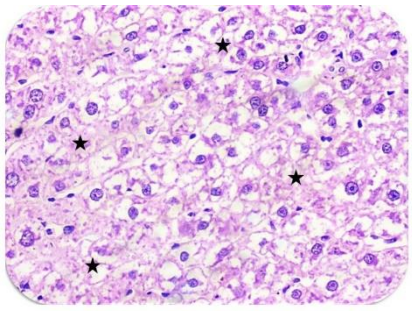

Figure (6): A liver of rat treated with CIS + LUT after receiving DEN showing hepatocellular carcinoma with marked degenerative changes (★) (H&E x400).

4. DISCUSSION

Diethyl nitrosamine (DEN) is a strong hepato-carcinogenic substance that causes disturbances in nucleic acid repair mechanisms and also generates reactive oxygen species (ROS) leading to oxidative stress (Fazal et al., 2017). As such in the present study, we utilized DEN to develop the rat model of HCC.

Both AST and ALT are hepatocyte-predominant enzymes (Giannini et al., 2005), advanced liver disease is associated with mitochondrial injury, a feature that can substantially increase the release of AST. Moreover, elevation of AST with

progression of liver fibrosis is caused by reduced AST clearance and mitochondrial injury with increased release of AST relative to ALT (Okuda et al., 2002). Moreover, serum AST/ALT activity is associated with remnant liver inflammatory necrosis (Tarao et al., 2002), which facilitates the invasion and recurrence of HCC (Wang et al., 2015).

In the present study a significant decrease in Serum albumin concentration was observed in DEN-induced HCC in rats when compared with control normal group. This agree with Bağırsakçı et al., 2017 who reported that larger HCCs are associated with lower albumin level.

Consequently, study emphasizes the existence of HCC due to DEN administration which is in agree with (Moawad et al., 2018 & Faisal et al., 2018) The effects of HCC stages on development of fibrosis were evaluated by quantifying TGF-b1 expression and percentage of fibrosis (%).TGF-b1was significantly increased in all rats with precancerous lesions. This result may suggest that the TGF-b1 is first activated in the early stage of HCC. Due to this activation, stellate cells (HSC) respond with intense deposits of fibrosis observed in the late stage of HCC (Andrea et al., 2015). TGF-b1 is a well-known developmental factor involved in regulation of cell proliferation, differentiation, invasion, and inflammation. In mammals, the TGF-b family regulates many cellular functions, plays an important role in cell growth, differentiation, apoptosis, extracellular matrix (ECM) production, immunization, and even embryonic development (Akhurst et al., 2012). TGF-b1 plays an important role in the pathogenesis of various liver diseases, such as fibrosis, cirrhosis, and HCC (Fabregat et al., 2016). For example TGF-b1 regulates liver cancer development by inducing Treg cell polarization (Shen et al., 2015). Faisal et al., 2018, study on DEN-HCC group showed the most significantly increased Bcl2, TGFβ1 and NFκB.

In accordance with this study, (Rajasekaran et al., 2011) reported that DEN HCC models registered significantly higher expression of apoptotic regulators, such as Bcl2 (anti-apoptotic) in addition (Faisal et al., 2018) reported elevated expression of Bcl2 in DEN-induced HCC rats.

Amina et al., (2017), found that BCL-2 level in liver tissue revealed significant increase in DEN group.

The obtained results, NF-kβ elevated in DEN-induced HCC rats, Nuclear Factor kappa B (NF-kβ) was identified by David Baltimore and Coworkers in 1986 as a factor in the nucleus of B cells that binds to the enhancer of the kappa light chain of immunoglobulin. NF-kβ is a transcription factor that serves as a master switch for turning on certain immune and inflammatory responses. NF-kβ alters cell behavior in many ways; it inhibits apoptosis (programmed cell death), increases cell proliferation and increases inflammatory and immune response. Recent evidence suggests that activation of NF-kβ contributes to the development of several types of human cancer (Mamatha et al., 2016).

This study showed significant down regulation in Casp-3 activity in DEN group compared to normal animals as reported before this is in accordance with Amina et al., 2017. Also, the decrease in caspase -3 expression was confirmed by Soha, et al., 2018, who concluded that caspase -3 activity expression in tumor tissues were significantly lower than those in non-tumor tissue.

This study showed significant decrease in Caspase-3 level in DEN group compared to normal animals as reported before (Amina et al., 2017). Treatment with cispaltin resulted in up regulation in Caspase-3 level compared to DEN rats. These results are in consonance with previous studies, which indicates that both treatments induced apoptosis as reflected by increase in Caspase-3 level (Hemieda et al., 2016).

Cisplatin administration to DEN rats result in decline in NF-kβ concentration these results agree with (Rui et al., 2017) who found several approaches that have been proposed to maintain cisplatin efficacy, including up regulating reactive oxygen species production and inhibiting NF-kβ and antiapoptotic proteins (He et al., 2012).

In this study treatment with Luteolin result in up regulation in caspase -3 expression as reported with (Wei et al., 2017).

NF-kβ and MAPK are two major pathways that

are involved in macrophage activation and in responses of tissue epithelial and stromal cells to inflammation mediators such as TNFα and ILs. Suppression of these pathways by luteolin underlies the main mechanism of its inhibitory effect on both acute and chronic inflammation. The suppression of inflammatory cytokine-induced signaling is at least partly on the level of receptor, because accumulation of lipid rafts, which is the critical step for receptor signaling, was blocked by Luteolin (Yong et al., 2008).

Luteolin can suppress NF-kβ, thus activating TNF-α-induced apoptosis. A possible mechanism for this process is through its ability to mediate the release of reactive oxygen species, which suppresses NF-kβ, stimulating cancer cells to undergo TNF-α-induced apoptosis (Ju et al., 2007).

In this study Luteolin injection to DEN rats showed a significant decrease in Bcl-2 these results agreed with (Ding et al., 2014) who reported that luteolin can trigger both intrinsic and extrinsic apoptosis pathways in a variety of human cancer cells, inhibits cell proliferation by arresting the cell cycle at the G1/S phase, enhancing the level of Bax and reducing the level of antiapoptotic protein Bcl-2, leading to apoptosis. Also (Lee et al., 2012; Pandurangan et al., 2013) both agreed with our result. Kyoung et al., 2019 reported that Luteolin up regulated expression of active caspase-3, while it down regulated the expression of the anti-apoptotic protein Bcl-2. On the other hand, several scientists reported that when luteolin acts on HepG2 cells it (a) suppresses proliferation, (b) induces higher apoptotic cell death and typical apoptotic morphological changes, (c) causes cell cycle arrest at G1/S stage, (d) decreases mitochondrial membrane potential (e) increases the Bax and caspase-3 expression, and (f) reduces anti-apoptotic protein Bcl-2 level which results in the activation of caspase-3 enzyme (Xu et al., 2016).

In this study, histopathological finding revealed that liver of DEN rats showed fibroblastic cell proliferation dividing the degenerated, necrotic, and dysplastic hepatocytes into nodules. This result correlates with others (Amina et al., 2017) and they found that liver tissue of DEN-treated rats showed hydropic degeneration, and focal areas of necrosis, portal inflammation, and hepatocytes showed partial loss of architecture and significant tumor nests. Cisplatin treatment showed histopathological improvement. All sections showed more or less normal architecture. The present study showed that treatment of Cisplatin and Luteolin significantly reduced HCC as evidenced by enhancement of the Histo-pathological finding and improvement of liver functions.

5. CONCLUSION

The current data indicated the efficacy of Luteolin supplementation as an anti-HCC in addition to its ability as a chemo-sensitizer for cisplatin treatment. This is mediated by intracellular pathways, involving improvement of the alterations in liver functions, the suppression of oxidative stress and modulation of antioxidant defense mechanisms. Thus, supplementation with Luteolin may help in the safe application of cancer technology in medicine, as well as in many other aspects of everyday life. Fractionation guided evaluation could help in the development of an ideal anticancer treatment in the near future.

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