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Prevention of liver cancer by standardized extract of *Melissa officinalis* L. in a rat model of hepatocellular carcinoma: Its potential role as a chemopreventive agent

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ABSTRACT:

Introduction: Hepatocellular carcinoma (HCC) is a primary malignancy of the liver and the third most common cause of cancer-related death worldwide. *Melissa officinalis* L. (*M. officinalis* L.), known as lemon balm is a medicinal plant, which has a wide range of pharmacological properties. This study was aimed to assess the chemopreventive effect of aqueous extract of *M. officinalis* (AMO) against diethyl nitrosamine (DEN)-induced hepatocellular carcinoma (HCC) in rats.

Methods and Results: The model of hepatocellular carcinoma was induced by a single intraperitoneal injection of DEN (200 mg/kg) as an initiator and after two weeks was followed by daily oral administration of 2-acetylaminofluorene (30 mg/kg) as a promoter for two weeks. Lemon balm-treated rats were pretreated with AMO intragastrically at three different doses two weeks prior to DEN injection. At the end of the experiment, the marked reduction of serum biomarkers of liver damage and cancer, including alfa-fetoprotein (AFP), gamma glutamyl transpeptidase (GGT), alanine transaminase (ALT), and aspartate transaminase (AST) were observed in AMO complemented rats compared to DEN-treated animals. Furthermore, the extract exhibited *in vivo* antioxidant activity by elevating GSH concentration and preventing lipid peroxidation in the liver tissues of HCC rats. The relative weight of liver was also reduced in lemon balm-treated rats as a prognostic marker in HCC.

Conclusion: Our findings demonstrated that *M. officinalis* has a chemopreventive effect against HCC in rats and can be suggested as a potential agent for the prevention of primary liver cancer.

Keywords: *Melissa officinalis*; Lemon balm; Chemoprevention; Carcinogenesis; Liver cancer.

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1. Introduction

Hepatocellular carcinoma (HCC) or primary liver cancer is an aggressive tumor that frequently occurs following liver inflammation and cirrhosis [1, 2]. It has been extensively reported that viral infections [hepatitis B virus (HBV) or hepatitis C virus (HCV)], diabetes, non-alcoholic steatohepatitis (NASH) along with some genetic diseases such as Wilson's disease and hemochromatosis are the most common causes of this deadly cancer. Industrial chemicals, environmental pollutants, tobacco smoking, food additives, and aflatoxin-B1 are other risk factors that cause hepatocarcinogenesis [2-5].

Diethyl nitrosamine (DEN) or N-Nitrosodiethylamine is a representative environmental carcinogen with the potential to induce tumors in diverse organs especially in the liver. DEN is extensively reported to be found in processed meats, tobacco products, cheese, soybean, cosmetics, drinking water, alcoholic beverages, and a variety of workplaces [6-8]. It has been widely used as a carcinogen in cancer investigation to induce HCC or different kinds of benign and malignant tumors in experimental animal models. It is strongly believed that DEN is metabolized in the liver by cytochrome P450 enzymes and is transformed to a reactive metabolite. Eventually, these active free radicals attack DNA and produce oxidative damages,which result in the formation of HCC [9].

Presently, there are no proven effective chemotherapeutic or surgical intervention for HCC and main focus is on cancer prevention strategies as a suitable method for controlling this aggressive cancer. Natural products have progressively become attractive agents in cancer prevention and treatment [10].

Melissa officinalis L., commonly called lemon balm is a medicinal herb of Lamiaceae, which has been traditionally used for the management of different diseases and as a food additive since about 2000 years ago [11].

Pharmacological studies show that this valuable plant has many biological activities such as: antioxidant, anti-inflammatory, antiviral, antibacterial, hypoglycemic, hypolipidemic, antidepressant, antianxiety, and neuroprotective effects [12-18]. In addition, *M. officinalis* possesses hepatoprective effect and significantly attenuates hepatotoxicity in hyperlipidemic rats [19]. Lemon balm was used in Iranian traditional medicine for the treatment of different types of cancer and there are several reports on the cytotoxic activity of the plant and its components in different cancer cell lines [11, 20-22]. Also, recent studies have pointed out the antigenotoxic and antiangiogenic activities of lemon balm [23, 24]. Therefore, this valuable plant could be efficacious in cancer prevention and treatment, however, the chemopreventive effect of *M. officinalis* have not yet been studied. Thus, the present investigation is aimed to evaluate the preventive activity of aqueous extract of *M. officinalis* (AMO) against DEN-induced HCC in rats.

2. Materials & Methods

2.1. Chemicals

All chemicals were purchased from Sigma- Aldrich Co. (Taufkrichen, Germany) with the highest commercial grade available.

2.2. Plant and extract preparation

The aerial parts of *M. officinalis* obtained from local medicinal herb shops, Tehran, Iran, and were authenticate by Mr. Mohammad Kamalinejad, a qualified botanist at the Department of Botany, Shahid Beheshti University of Medical Sciences (8003, voucher specimen in Shahid Beheshti University of Medical Sciences Herbarium, Tehran, Iran). The plant was cleaned and the extraction was carried out by the maceration of 100 g plant with 900 mL distilled water for 30 min. The resulting extract was evaporated by placing in water bath 90°C. Finally, the extract was filtered and was kept at –20°C until use. The extract was dissolved in distilled water to receive desired concentrations just before use. The moisture level of the extract was determined by weight loss after placing 2 g of the final extract in an oven at 60–65°C for 72 h. The final extract contained 24% water [25].

2. 3. Standardization of Extract

Total polyphenol content was determined by spectrophotometry, using gallic acid as the standard based on the Folin–Ciocalteu method [26]. Total phenolic content was 64.89 ± 2.33 mg gallic acid equivalents (GAE) per gram of AMO (mg of GAE/g of plant extract). Total flavonoid content was measured with the aluminum chloride colorimetric assay [27]. Quercetin was used as the standard and flavonoid contents were expressed as mg of quercetin equivalent per gram of AMO. Flavonoid contents were 36.25 ± 1.17mg quercetin equivalents (QE) per gram of AMO (mg of QE /g of plant extract).

2. 4. Animals

Male Sprague-Dawley rats weighing 180 to 200 g were housed in ventilated plastic cages over PWI 8-16 hardwood bedding. There were 12 air changes per hour, 12 h light photoperiods, an environmental temperature of 21–23°C, and a relative humidity of 50–60%. The animals were fed a standard normal chow diet and given tap water ad libitum. Principles of laboratory animal care (NIH publication No. 85-23, revised 1985) were followed.

2. 5. Hepatocarcinogenesis model

The experimental model of HCC was induced in rats by DEN as the initiator and 2-acetylaminofluorene (2-AAF) as the promoter of hepatocarcinogenesis according to the protocol described previously [28]. Briefly, rats were fasted for 96 h and then were refed for 24 h as a proliferative stimulant. Afterward, rats were injected only a single intraperitoneal (i.p.) dose of DEN (200 mg/kg body weight) for initiating

hepatocarcinogenesis. After two weeks, liver cancer development was promoted with daily dose of oral 2-AAF (30 mg/kg body weight) for two weeks. HCC was confirmed by histopathological evaluations and the measurement of liver damage and cancer markers, including alfa-fetoprotein (AFP), gamma glutamyl transpeptidase (GGT), alanine transaminase (ALT), and aspartate transaminase (AST) [29].

2. 6. Experimental design

As shown in Fig. 1, rats were randomly divided into six different groups of six rats each (n = 6). Group 1 (Control) animals were fed standard diet and served as a normal control, and were injected with the single dose of saline. Group 2 was normal animals that were treated only with daily oral dose of AMO (400 mg/kg body weight) from the beginning of the experiment. Group 3 (HCC) rats served as untreated HCC animals as described before. Test group animals (Groups 4- 6) were pretreated with the increasing doses of AMO (100, 200, and 400 mg/kg body weight, respectively) two weeks prior to DEN injection that continued until the end of the study. The different concentrations of the extract were selected based on previous studies and were administered orally once daily using an intragastric tube for 8 weeks [30].

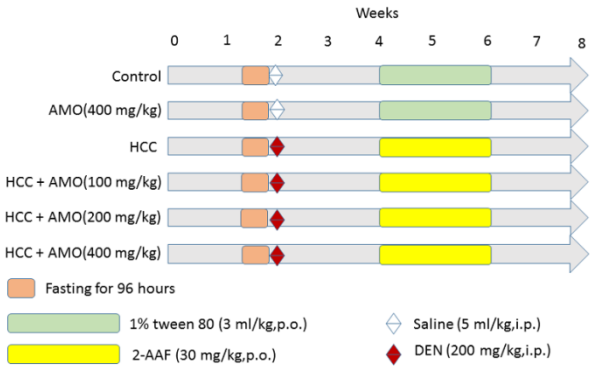

Figure 1. Experimental design. i.p.: intraperitoneal.

2. 7. Sample preparation

At the end of the experiment, animals were anaesthetized by ether inhalation and blood samples were collected from the orbital sinus and centrifuged at 1500×g for 20 minutes at 4°C to obtain serum for biochemical analysis. In addition, body weights of the animals were recorded and rats were sacrificed. Then, the liver tissues were quickly collected, weighed, and frozen for the determination of hepatic oxidative stress markers. Frozen liver samples were homogenized in ice-cold Tris- HCL buffer (150 mM, pH 7.4). The relative liver weight was calculated as the percentage ratio of liver weight to the body weight.

2. 8. Evaluation of biochemical parameters

AFP level is widely used clinically as a tumor marker for HCC and is measured by Calbiotech AFP ELISA Kit [31]. The activities of biochemical markers of liver damage, including GGT, ALT, and AST were determined by commercially available enzyme kits (Pars Azmoon, Tehran, Iran) [32]. The assays were performed according to the protocols supplied with the kits.

2. 9. Determination of protein concentration

The concentration of liver protein was measured by the Coomassie blue protein-binding method using BSA as the standard [33].

2. 10. Determination of hepatic oxidative stress markers

Hepatic GSH contents were estimated in liver homogenate by a spectrophotometric method using dithiobis-2-nitrobenzoic acid (DTNB) as the indicator of GSH and expressed as µg/mg protein. The intensity of the yellow color produced in the samples was recorded at 412 nm with a UV spectrophotometer (Infinite M200, TECAN) [34].

The level of malondialdehyde (MDA) as a reliable marker of lipid peroxidation was estimated in liver homogenate by reading the absorbance of the supernatant layer at 532 nm with an ELISA reader instrument (Infinite M200, TECAN). MDA levels were presented as µg/mg protein [35].

2. 11. Statistical analysis

The homogeneity of variances was tested using Levene's test. The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD as the post hoc test. The data were presented as the mean±SD (n=6). The results with level of significance ($P<0.05$) were regarded as significant.

3. Results

3.1. Effects of AMO on serum biochemical parameters

Serum AFP is one of the most frequent diagnostic markers for HCC, which was significantly enhanced in this investigation in HCC rats compared to normal rats ($P < 0.001$). As can be seen in Fig. 2, the oral pretreatment of different concentrations of AMO to HCC rats caused a significant reduction in AFP. In addition, our results showed that the activities of GGT, ALT, and AST were clearly increased in DEN-treated rats compared to normal animals at the end of the experiment

(Fig. 3-5). The elevations in the serum markers of liver injury, including GGT, ALT, and AST were effectively decreased in AMO-treated rats. Besides, it was shown that the treatment of normal rats (Group 2) with AMO (400 mg/kg body weight daily) did not alter the biochemical markers (Fig. 2-5).

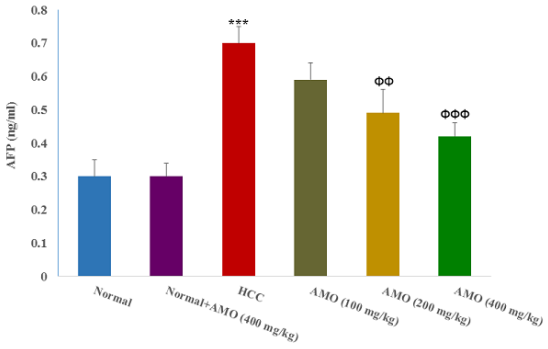

Figure 2. Effects of the lemon balm extract (AMO) on AFP. Values are presented as mean±SD. *** P<0.001 compared to normal group; ΦΦ P<0.01, ΦΦΦ P<0.001 compared to HCC rats.

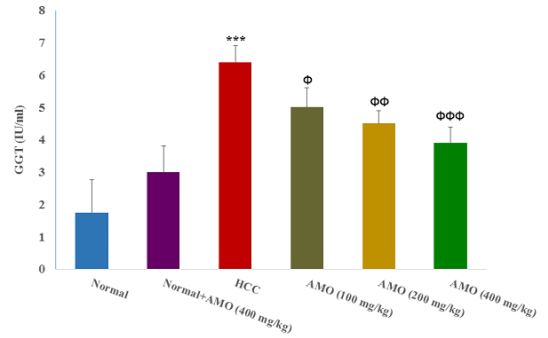

Figure 3. Effects of the lemon balm extract (AMO) on) GGT. Values are presented as mean±SD. *** P<0.001 compared to normal group; Φ P<0.05, ΦΦ P<0.01, ΦΦΦ P<0.001 compared to HCC rats.

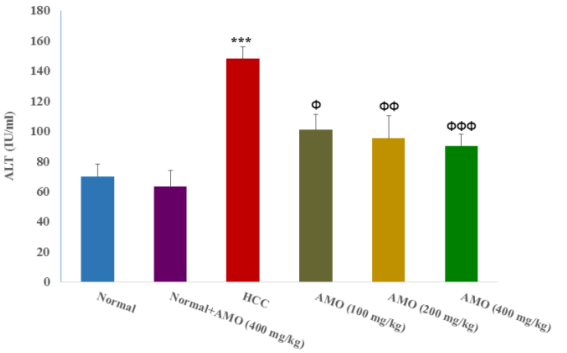

Figure 4. Effects of the lemon balm extract (AMO) on ALT. Values are presented as mean±SD. *** P<0.001 compared to normal group; Φ P<0.05, ΦΦ P<0.01, ΦΦΦ P<0.001 compared to HCC rats.

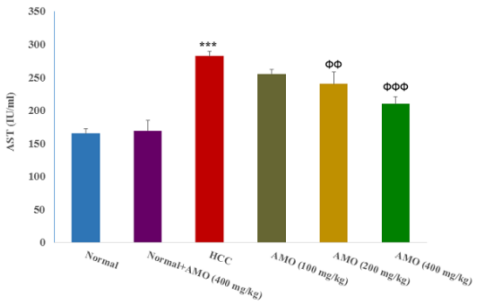

Figure 5. Effects of the lemon balm extract (AMO) on AST. Values are presented as mean±SD. *** P<0.001 compared to normal group; ΦΦ P<0.01, ΦΦΦ P<0.001 compared to HCC rats.

3.2. Effects of AMO on the hepatic markers of oxidative stress

GSH and its related antioxidant defense system are required to neutralize free radicals such as electrophilic carcinogens and to counteract oxidative stress. As indicated in Fig. 6, hepatic GSH levels were decreased in DEN-treated rats compared with normal rats ($P < 0.001$). Moreover, MDA levels as one of the important markers of oxidative stress were increased in HCC rats compared to normal animals ($P < 0.001$) (Fig. 7). In this study, the pretreatment with the lemon balm extract to HCC rats caused an elevation in GSH concentrations along with a reduction in lipid peroxidation (Figs. 6 and 7). It can be also seen that the oral administration of AMO (400 mg/kg body weight/daily) in normal rats did not change the oxidative stress markers (Figs. 6 and 7).

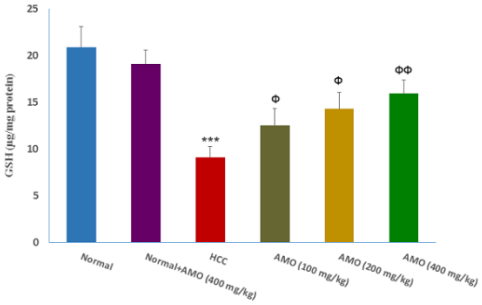

Figure 6. Effects of the lemon balm extract (AMO) on hepatic GSH contents. Values are presented as mean±SD. *** P<0.001 compared to normal group; Φ P<0.05, ΦΦ P<0.01 compared to HCC rats.

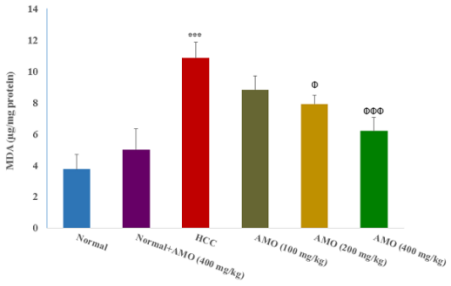

Figure 7. Effects of the lemon balm extract (AMO) on liver lipid peroxidation. MDA formation as the marker of lipid peroxidation was expressed as µg/mg protein. Values are presented as mean±SD. *** P<0.001 compared to normal group; Φ P<0.05, ΦΦΦ P<0.001 compared to HCC rats.

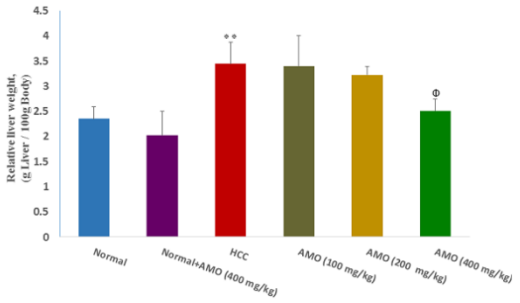

Figure 8. Effects of the lemon balm extract (AMO) on relative liver weight. Values are presented as mean±SD. ** P<0.01 compared to normal group; Φ P<0.05 compared to HCC rats.

3.3. Effect of AMO on relative liver weight

As presented in Fig. 8, the relative liver weight of HCC rats was improved as compared to the normal animals ($P < 0.01$). Our results indicated that the oral administration of AMO to DEN-treated rats caused a significant decrease in relative liver weight ($P < 0.01$). Again, the pretreatment of normal rats (Group 2) with AMO (400 mg/kg body weight daily) did not affect the relative liver weight (Fig. 8).

4. Discussion and Conclusion

The incidence and mortality of primary liver cancer or HCC are rising throughout the world and unfortunately, it is often diagnosed at advanced stages. In the absence of proven effective chemotherapy and surgical intervention for HCC treatment, chemoprevention is needed for decreasing the risk or delaying the onset of this progressive cancer especially in high-risk individuals. Recently, more attention has been focused on medicinal herbs and phytochemicals as an effective chemopreventive agents because of their preventive activity against common cancers like HCC [28].

M. officinalis commonly known as lemon balm is considered as a potential agent for cancer prevention and treatment because of its cytotoxic and antiproliferative effects in different tumor cell lines [20-22]. Also, it has been reported that lemon balm presented apoptosis-inducing activity as well as antigenotoxic, and antimutagenic effects in different *in vitro* and *in vivo* models [24, 36]. In the present study, we investigated the chemopreventive effect of *M. officinalis* against DEN-induced HCC in rats at the end stage of hepatotoxicity. Our results clearly demonstrated that lemon balm possesses a predominant chemopreventive activity in the rat model of HCC which is evident from reduction in the serum markers of liver damage and cancer.

Serum α-fetoprotein or AFP level is considered an important diagnostic marker for HCC [37]. Elevated AFP serum levels are only seen in certain tumors (e.g. HCC), non-tumoral conditions (e.g. cirrhosis), and maternal serum during pregnancy. As shown in Fig. 2, the increased concentration of AFP was effectively reduced by the lemon balm extract. In addition, GGT, ALT, and AST are the most widely used HCC tumor markers. GGT expression in tumor cells provides a selective advantage to the cells during tumor promotion and enables them to preserve elevated levels of intracellular GSH. Therefore, it has been suggested that GGT is an independent prognostic indicator in patients with HCC [38]. As shown in Fig. 2, the activities of GGT, ALT, and AST were excellently decreased by the lemon balm extract.

Glutathione and glutathione-related enzymes play a pivotal role in the metabolism and detoxification of free radicals and carcinogenic compounds. They are also involved in the regulation of carcinogenic mechanisms and cancer cell death [39]. It has been reported that GSH levels and the activities of GSH-dependent enzymes in the liver tissue of HCC patients were diminished because of the deficiency of antioxidant defense system [40]. Our results clearly point out that the lemon balm extract counteracted DEN-induced oxidative stress and restored GSH concentrations in rat livers treated with lemon balm.

Lipid peroxidation, as an important marker of oxidative stress, is a well-defined mechanism of cellular damage in different pathological conditions [41, 42]. MDA is the end product of lipid peroxidation that is involved in the promotion and progression stages of carcinogenesis [43, 44]. In this study, the oral administration of lemon balm to DEN-treated rats decreased MDA levels and liver lipid peroxidation. Finally, our results indicated that the extract prevented any increase in relative liver weight caused by DEN treatment.

It is strongly believed that inflammation-mediated processes and oxidative stress contribute to the development and progression of hepatocarcinogenesis through different mechanisms [45]. Regarding the antioxidant and anti-inflammatory as well as hepatoprotective and anti-cancer activities of lemon balm, it can be suggested that these biological activities are responsible for the chemopreventive of the plant.

Recently, there has been a great interest in natural polyphenols potentially used as chemopreventive agents. Many *in vitro* and *in vivo* studies have suggested that these natural compounds are promising candidates for HCC prevention by reversing, suppressing or preventing the different steps of hepatocarcinogenesis through various mechanisms [46]. The different kinds of phenolic compounds, including phenolic acids, flavonoids, and tannins have been found in *M. officinalis* in addition to many different valuable compounds [11, 47-48]. Phenolic acids such as gallic acid are the main components of the plant, which has been also reported that have many beneficial effects

from the anti-cancer point of view [49]. In the present study, more than 6% of the total weight of the extract related to polyphenolic compounds. So, it can be suggested that the preventive activity of lemon balm against HCC may be mediated through the antioxidant, anti-inflammation, and anti-carcinogenic effects of polyphenols. However, further studies to elucidate underlying mechanisms of the effect would be complementary to this study. In addition, the authors believe that additional experiments are necessary to explain which active constituents are responsible for the chemopreventive effect of lemon balm against liver cancer and to detect synergy among different compounds of this valuable fruit.

For conclusion, our findings obviously showed that *M. officinalis* has a preventive effect against DEN-induced liver cancer by decreasing serum biomarkers of liver damage and cancer. Besides, *M. officinalis* displayed *in vivo* antioxidant activity by elevating GSH contents in addition to prevent lipid peroxidation in the liver tissues of HCC rats. The relative weight of liver was similarly diminished in the extract-treaded rats.

Acknowledgements

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Conflict of interest

There is no conflict of interest.

Ethics

All experiments were conducted according to the ethical standards and protocols approved by the Committee of Animal Experimentation of Zanjan University of Medical Sciences, Zanjan, Iran (protocol approval number: ZUMS.REC.1394.313).

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