ANTITUMOR EFFECTS OF LEAVES, STEM, AND ROOT EXTRACTS OF *Cichorium intybus IN VITRO* AND *IN VIVO*

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ABSTRACT

Cichorium intybus or Chicory was used in traditional medicine for curing several diseases such as diarrhea, to improve the prostate and other reproductive organs, to treat lung and tobacco disease, cancer, severe headache, and others. We examined the anti-tumor and antimetastatic effects of *C. intybus* extracts in vitro and in vivo. Cytotoxicity of *C. intybus* extracts (leaf, stem, and root) was done on the 4T1 cell line; a high significant inhibition value was detected in the 4T1 cell line at three periods of exposure .In vivo study; For 21 days, tumor-bearing female Mus musculus Balb/c mice were given orally one hundred microliters of leaf extract (1000 mg /kg/BW), whereas the control group received only PBS. Leaf extract induced a significant decrease in tumor size ($P < 0.05$). There was little indication of liver metastases in leaf extract-treated animals relative to the controls, a markedly less of metastases than expected.

INTRODUCTION

Tumors are a category of diseases marked by irregular cell proliferation, invasion, and spread across the body (1). Recent studies showed that about 33% of the community had been encircled by cancer. Despite improvements in healthcare, developed nations struggle to battle dangerous diseases, like cancers. (2) With about 12 million new cases of breast cancer diagnoses and 6.6 million cancer deaths in females per year, today, one of the most frequent causes of cancer cases and mortal outcomes for women worldwide is breast cancer. (3,4**)** A wide range of hereditary and environmental agents plays an essential role in breast cancer incidence (5, 6) These ultimately alter the cells' genetic material that regulates cell growth and survival, relationship with adjacent cells, and the ability to avoid the immune system. These changes included (mutation, gene expression disorder, activation of the tumor promoter gene, inactivation of tumor suppressor genes, etc.) And which has been considered as the most important cause of cancer pathogenesis between normal and tumor cells (7-10).

Scientists have shifted their focus to the usage of natural bioactive compounds in the treatment of a variety of cancer because of the significant resistance to chemotherapies and the adverse impact of pharmaceutical drugs on normal cells, especially in breast cancer patients (11, 12)**.** These treatments are preferred in terms of efficacy, safe, and also inexpensive (13). *Cichorium intybus* is a herb kind that belongs to the Asteraceae family, and it is a biennial or perennial plant and can reach about 170 cm in height. The *Cichorium* species originally arose from the Mediterranean zone. In ancient times, wild chicory and chicory cultivars have been dispersed throughout Europe, Africa, Asia, the Americas, Australia, oceanic islands, and the genus Cichorium comprise from sex different species distributed worldwide (14, 15) Several studies have shown to have high antioxidant content as the main phytochemical component of many *Cichorium* species (16-18) . Phytochemical analysis of various parts of *C. intybus* has helped discover over 100 compounds, including sesquiterpene lactones (especially lactucin, lactucopicrin, 8-deoxy lactucin; guaianolid glycosides, which included chicoroisides B and C, sonchuside C). caffeic acid derivatives, inulin, sugars, proteins, hydroxycoumarins, flavonoids, alkaloids, steroids, terpenoids, oils,

volatile compounds, coumarins, vitamins, etc. Someone else research also found that mouse mammary tumor cells 4T1 that are highly metastatic are mainly can be used to study the ways of helping the successful anticancer treatments, and their effectiveness and molecular targets of therapies that are applied in humans (19, 20). This study is intended to evaluate the effects of utilizing the hydroalcoholic extracts of *C. intybus* against 4T1 cell lines both in vitro and in vivo.

MATERIALS AND METHODS

Preparation of plant extract

Samples of *C. intybus* leaves, stems, and roots were obtained from various places in Basrah, Iraq. Plant parts have been dried at room temperature and finely grinded by the electrical grinder, then 150 g of each plant part were immersed and stirred with 80 % methanol for 48 hours on a magnetic stirrer. After filtration, the extract was evaporated at room temperature and, the crude extracts of each one were weighted. Then, the extracts were kept at -20 until use (21).

Cell Line and Culture Medium

Cell lines 4T1 was kindly provided, By the tissue culture unit/ Faculty of the Nursing / University of Babylon. These cells were preserved with DMEM medium / high glucose supplemented with 10% heat-inactivated fetal bovine serum, 100IU/ml penicillin, and 100 mg/ml streptomycin. The cultured cell was grown in a 5% CO2 humidified incubator at 37 ºC. Subculture was done by trypsinization (0.25%) when 80% of confluence was achieved. The growth medium has changed every three days.

Anticancer effect

Anti-neoplastic properties of extracts were clarified by studying the cell cytotoxicity and morphological changes that occur after treatment on cell lines

Cytotoxicity assay

The impact on total cell behavior was calculated by conducting an MTT assay based on (22). Cells of 4T1 have been seeded in a 96-well plate at a density of 10000 cells/well in 100µl of medium and incubate at 37C in a CO2 incubator for 8- 24 h to get a confluent

monolayer. After 24h, the cells were treated with a serial dilution of each extract (7.812, 15.625, 31.25, 62.5, 125, 250, 500, 1000 µg/ml); three replicates were used for each concentration, the plates were re incubated at 37°C in the CO2 incubator for the selected exposure times (24, 48, and 72 h.). After selected exposure periods, ten µl of MTT reagent (5mg/ml) was added to each well and mixed carefully, and cells were incubated for 2-4 hours. The medium was then decanted; the excess dye was solubilized by adding 100 µl of DMSO per well for 30 min at 37°C in a CO 2 incubator. Finally, the optical density (O.D.) of each well's living cells was read using ELISA Reader at a transmitting wavelength of 490 nm. (22, 23). The percentage of cytotoxicity was calculated as = (OD of Control) - (OD of Treated) / (OD of Control) x100. OD: Absorbance of each well.

Experimental animals

Determination of median lethal dose (LD50) in mice

To check the effect of a crude leaf extract in vivo, *Mus musculus* Balb/ c mice (8 weeks of ages) with bodyweight (20- 25) g. were used. The mouse is reproduced under optimal conditions in the animal's lab. It has 25 C temperatures with relative humidity at 60% and a 12-hour light/ dark cycle. Distilled water and sterilized food for mice were available ad libituium. LD50 can determine by the up&down method was mentioned by Dixon(24). In this method, several mice were used with a different concentration of leaf extract was taken. One mouse was administered orally with 1000mg/kg/BW and showed for 24h. if the animal did not die within 24 h of the given dose, the method used with another mouse and using another dose greater than the previous quantity (a constant increase between one dose and another of 500 mg/kg/BW or 50%of the dose. Thus, the dose processes continue upwards until 5000 mg/kg/BW was reached without any toxic effect on the administered animals.

Establishment of the primary 4T1 tumor mice model

All procedure to induce the primary 4T1 tumor on mice depending on the method was described by Paschall & Liu (25). 4T1 cell was cultured in DMEM. After trypsinization, the sub-confluent cell monolayer was harvested by adding 7ml of media to the plate and centrifugation for 5 minutes, washed twice with PBS, and finally resuspended

in PBS at concentration 2 *106 per ml. 250µl of cell sample was then inserted subcutaneously in the mammary fat bud of female mice.

Therapeutic Experiment

When the cancerous mass had been detected (7-10 days after transplantation), the mice are randomized into control and treatment groups. The experimental groups were treated with 1000 mg /kg/BW daily. The animals were divided into four groups, each one with eight individuals (of each group, four animals as untreated a positive control, and four animals were treated with the leaf extract except group 4 as a negative control. The treated animals in each group were administrated daily by 0.1 ml / 1000 mg/kg/BW by oral gavage, while the positive control was treated with PBS only. The control and treated mice groups were killed after 7, 14, and 21 days from the end of treatment. Tumor size was measured using Vernia calipers by calculating their volume depending on the equation (A * B^2) ^2, where A and B represent length and width, respectively (26). Tumor growth inhibition also was calculated by using the following formula %G1= ((growth untreated tumor group- growth treated tumor group)/ (growth untreated tumor group)) x 100 (27).

Histopathological examination

Tissues were fixed with 10% buffered formalin, embedded in paraffin, and cut into 5 mm sections; then, the sections were stained with hematoxylin and eosin (H & E), Van – Gieson stain, and Periodic acid Schiff's staining method.

Statistical study

Statistical analysis of all data was carried out using the ANOVA test with differences at P -values at levels ($P \leq 0.05$) was considered to be statistically significant. All quantitative data are shown as means ± SD. whereas . the IC50 value was determined using the Graph Pad Prism software.

RESULTS

Cytotoxic assay

The MTT test was performed to identify the cytotoxic effects for different extracts of *C. intybus* on 4T1 cells in vitro. The results found that all kinds of *C. intybus* extracts had a highly significant cytotoxic impact ($P<0.05$) on the growth rate and proliferation of the 4T1 cell line after 24, 48, and 72 hours in a time and dose-dependent way. The root extract has shown more potent cytotoxicity on the 4T1 cell line than the leaf and stems extracts at the three periods of exposure (24, 48, and 72 hours) (Fig.1 A, B, C). Moreover, The half-maximal inhibitory concentration (IC_{50}) of all types of *C.intybus* extracts after 24,

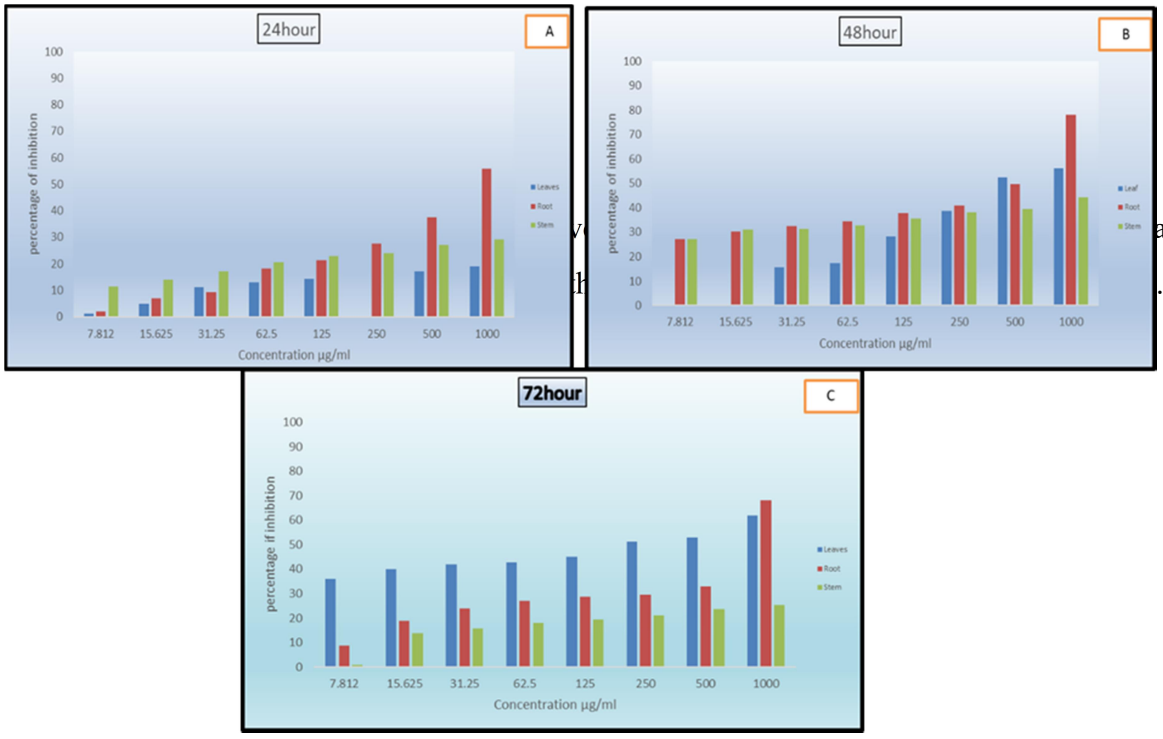


Fig. (1): Effect of leaf, root, and stem extract of *C.intybus* on 4T1cell line. (A) at 24 hours, (B) at 48 hours, (C) at 72 hours. Values were expressed as mean ± SD (n=3).

Table 1: IC_{50} Values of different extract of *C. intybus* on 4T1 cell line Tumor growth inhibition

Extract	**IC_{50} value µg/ml**		
Concentration	**24hrs**	**48hrs**	**72hrs**
Leaf	3263.89	97.82	178.7
Root	202.6	406	278
Stem	2262.89	1315.57	2478.57

One dose and three periods were used for studying the effect of *C. intybus* leaf extract on tumor growth inhibition. The result showed that the tumor growth was significantly decreased ($p<0.05$) in all periods of the treated groups when compared with the control (Table 2). The results revealed that the tumor growth inhibition was 54.2% on the seven days of treatments, contrasting with the untreated control group, as seen in Fig. 2 (A, B, C). At two and three weeks of treatments, the results showed a significant decrease ($p<0.05$) in treated groups with tumor growth inhibition, 70.57 %, and 88.2% respectively compared with the untreated group, Fig.2(D, E)., 2(F, G), 3(A, B, C)

Table (2) represents the tumor volume inhibition in tumor-bearing - mice groups

Periods	Groups	
	Control	treated
(0) weak	125.03 ± 17.43 ns	125.14±13.17ns
(1) weak	246.28±95.94*	112.78±40.75*
(2) weak	255.13±100.14*	75.06±35.34*
(3) weak	361.43±74.78*	42.58±16.83*

(treated and control) treated with C. intybus leaf extract at different periods

*Values are Mean ±SD (n-3).*Represents a significant difference in comparison between treated and control group($p<0.05$).

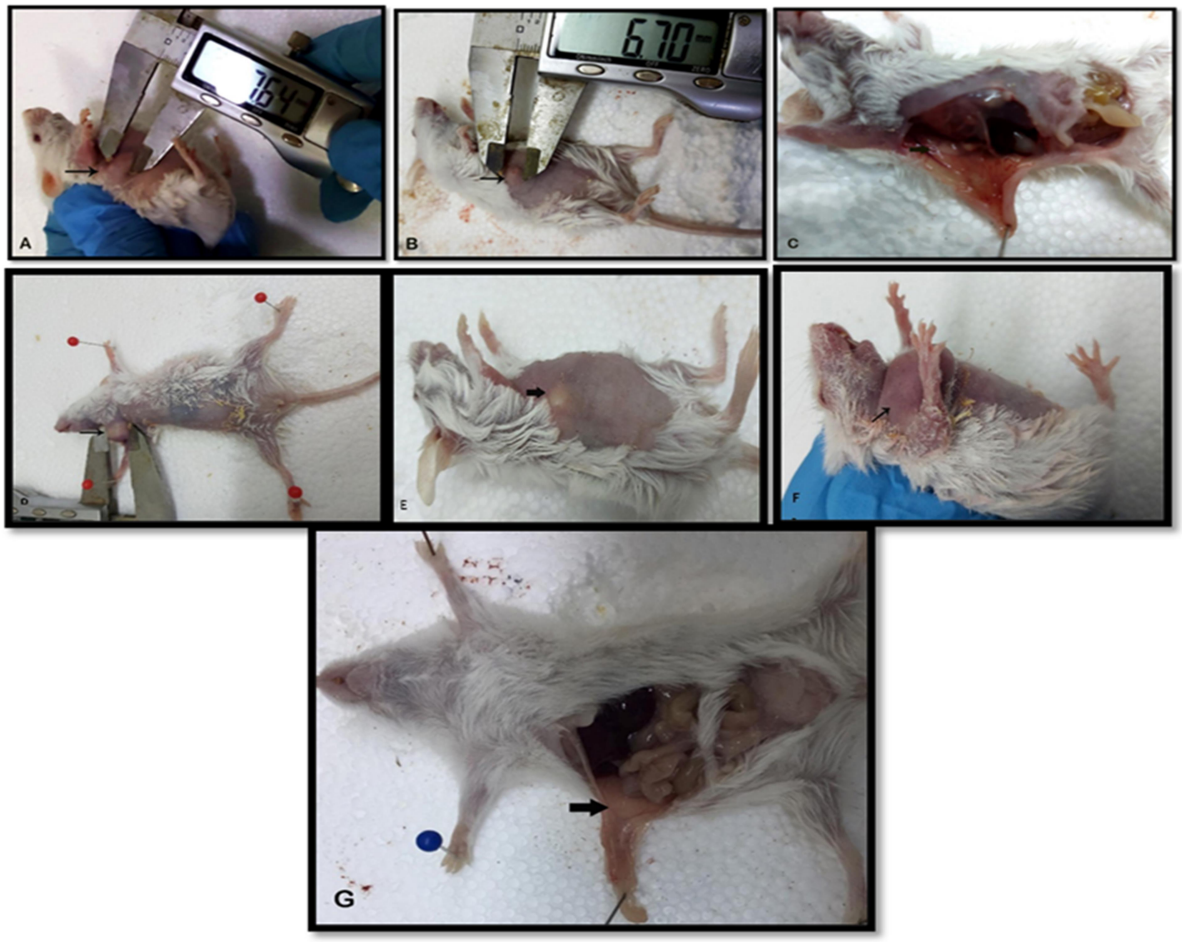


Fig.(2): antitumor activity of leaf extract of *C.intybus* in tumor-bearing mice; (A, B, C) after 1week of treatment ; (D, E) after 2week of treatment and (F, G) after three weeks of treatment, A, control group after one week, (B and C) after one week of treatment, D: control group after two weeks, E: treated group after two weeks, F: control group after three weeks, (F and G) treated group after three weeks of treatment.

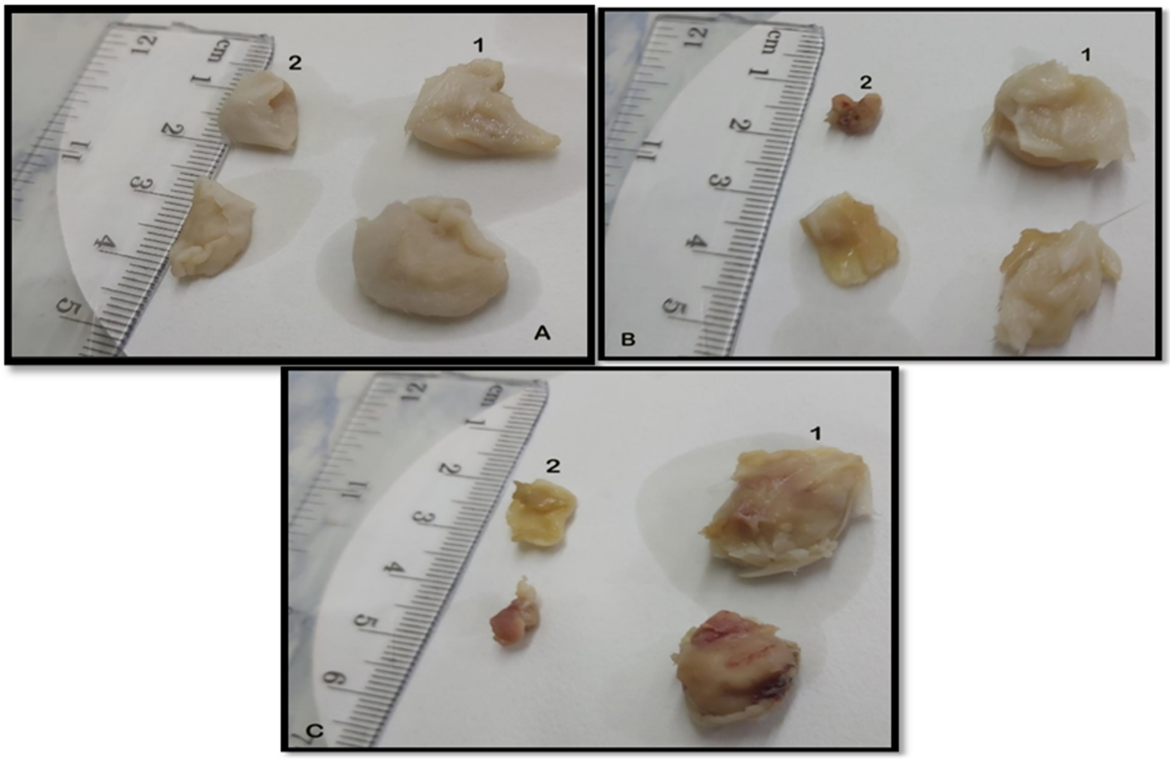


Fig. (3): antitumor activity of leaf extract of *C.intybus* in tumor-bearing mice. (A) After one week, ((B) two weeks, and (C) three weeks, (1) control, (2) treated.

Histopathological Examinations

Histopathological analysis of 4T1 tumor-bearing mice treated with leaf extracts of *C. intybus*

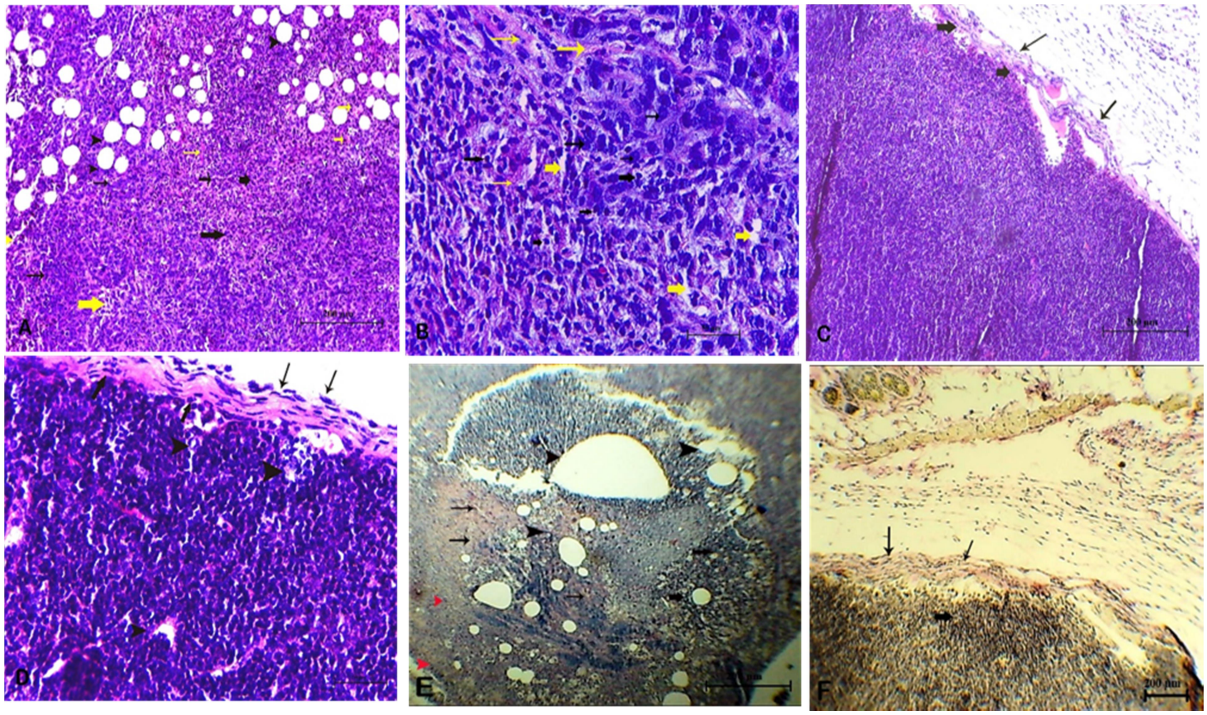
After 1, 2, and 3 weeks of treatment, microscopic samples of the primary tumor and liver from the treated and positive control of transplanted mice were analyzed under a compound microscope. The histopathological analysis of mass tumor in positive control group after one, two and three weeks showed many changes include glandular configuration; tumor cells have appeared with large polymorphic nuclei with dense chromatin stain and tiny amount of cytoplasm, a new vascular form of irregular, expanding vessels with red blood cells' focal

extravasation, the connective fibrous tissue which surrounded the tumor mass became dense and mature with a high number of inflammatory mononuclear cells (macrophages and lymphocytes) with an area of the very aggressive tumor (solid anaplastic tumor) poorly differentiating, a central area of necrosis, which appeared homogenous structures and associated with a few cellular of polymorphonuclear cells and high number of mitotic with high collagen deposited Fig(4).

In contrast histopathological findings after 1, 2, and 3 weeks of treatment showed decreasing in tumor mass tissue with many necrotic areas that appeared as a homogenous structure with a few numbers of polymorphonuclear cells and less number of mitotic when compared with the control sample, high infiltration of inflammatory cells (polymorphic nuclear leucocyte, plasma cell, and mature lymphocyte), new vascularization was observed with more area of necrosis could be seen besides decreasing in tumor mass; vacuolated cells and late apoptosis that could be seen, and degeneration appeared with dilated of the lymphoid nodules was shown in Fig.(5). On the other hand, the histological examination revealed the typical structure of the liver in the negative control group Fig (6, A, B).

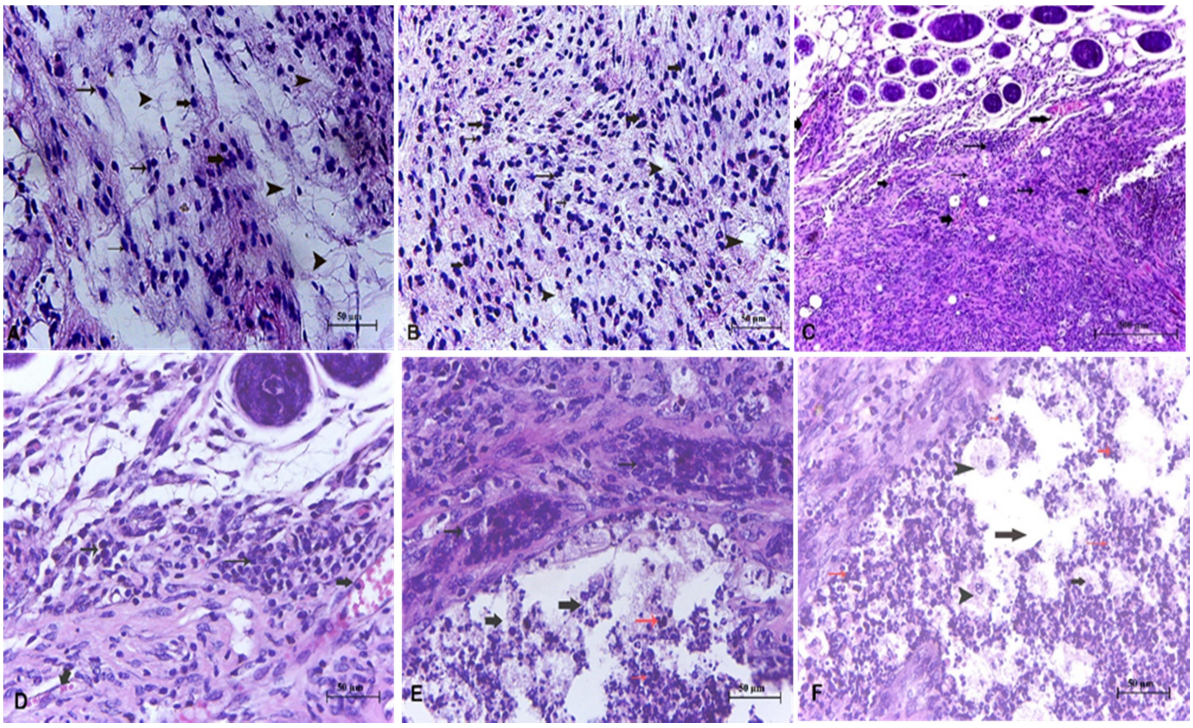
After two and three weeks of leaf extract therapy, the liver of the positive untreated group showed multiple histopathological characteristics, this including inflammation reaction around the portal triads and central vein, very mild infiltrate of small heavy basophilic inflammatory cells, mainly lymphocyte and plasma cells are also present, and the portal vein is enlarged, Congested dilated central vein with dilated sinusoids was observed, the liver undergoes widely hepatocellular necrosis, in which cells appeared more eosinophilic than normal. The histiocytic body was demonstrated around, and near to the central vein, Strong reaction with PAS, an indicator to the glycogen granules deposition, the histiocytic formation was well defined, massive infiltration of tumor cells in the parenchymal liver plate, dilated and bleeding sinusoids were detected with The histiocytic structure was shown. The Gilson capsule's connective tissue was unclear, and the aggregation of tumor cells under the capsule; there are several tumor cells detected as undifferentiated cells with heavy basophilic, rounded nucleus when compared with hepatocyte Fig.(6, C,D, E,F) and Fig(7).

After three weeks, the liver of the treated group developed several histological characteristics, including normal Gilson capsule's connective tissue; no evidence of tumor metastasis occurred. Normal central vein and the number of inflammatory cells in this part is comparatively small, dilated sinusoids in some places and around the central vein; highly PAS's reaction was detected around the central vein and within parenchymal liver plate Fig.(8)

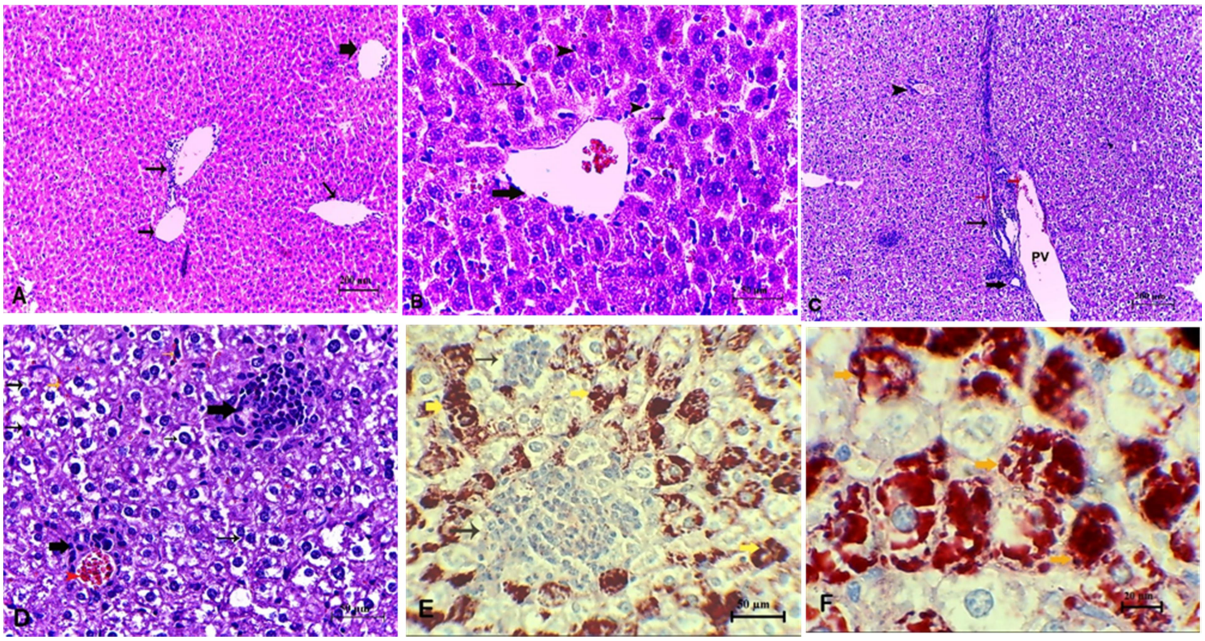


Fig(4): Cross -section of tumor mass of the control group after one , two. and three weeks of implantation; (A and B)after one week showing a glandular structure of tumor (thick arrow), blood vessels formation (yellow thin arrow), necrosis (yellow thick arrow), tumor cells (thin black arrow) and adipocyte (head arrow), and inflammatory cells (thick arrow). (C and D) tumor mass after two weeks in the control group showing thick fibrous connective tissue (thin arrow), a high number of inflammation cells (thick arrow) .(E and F) histological changes after 2 weeks in control group showing the central area of necrosis (head arrow), a high collagen deposited (thin arrow) with poorly differentiated tumor cells (thick arrow), and high numbers of lymphocyte

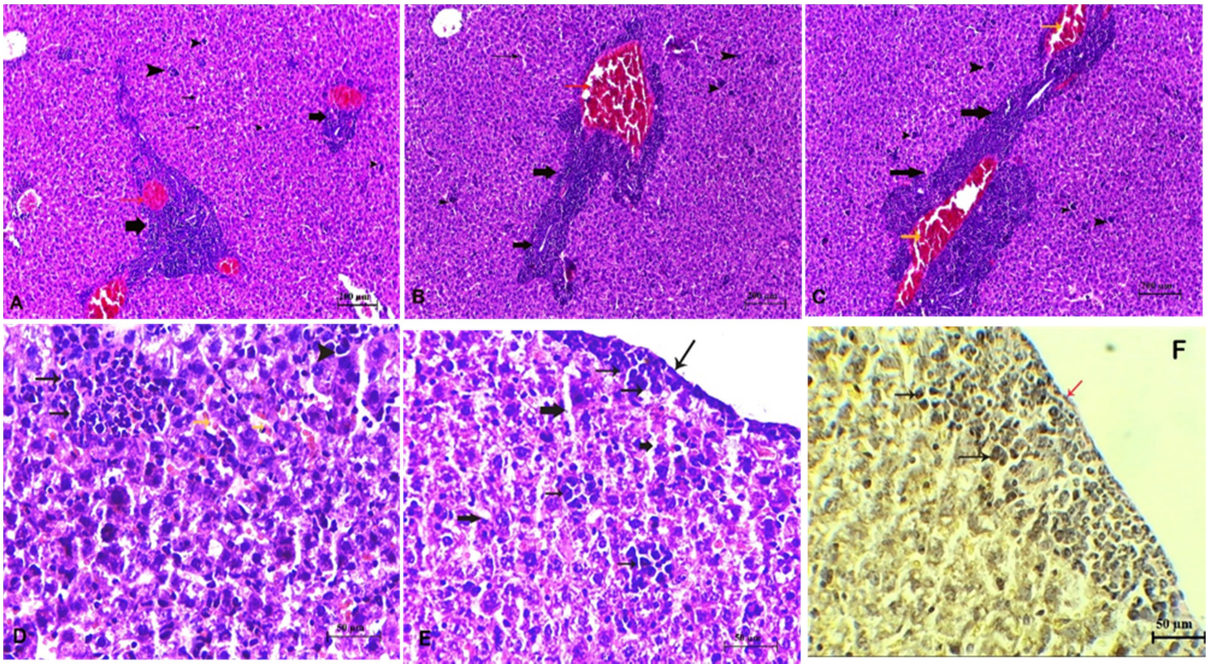
appeared (redhead arrow) with thick fibrous capsule surrounded the tumor mass (thin arrow). A,B,C,D : H&E , E: PAS stain, and F: Van Gieson stain. A,C,E,F : 10X. B and D : X40.



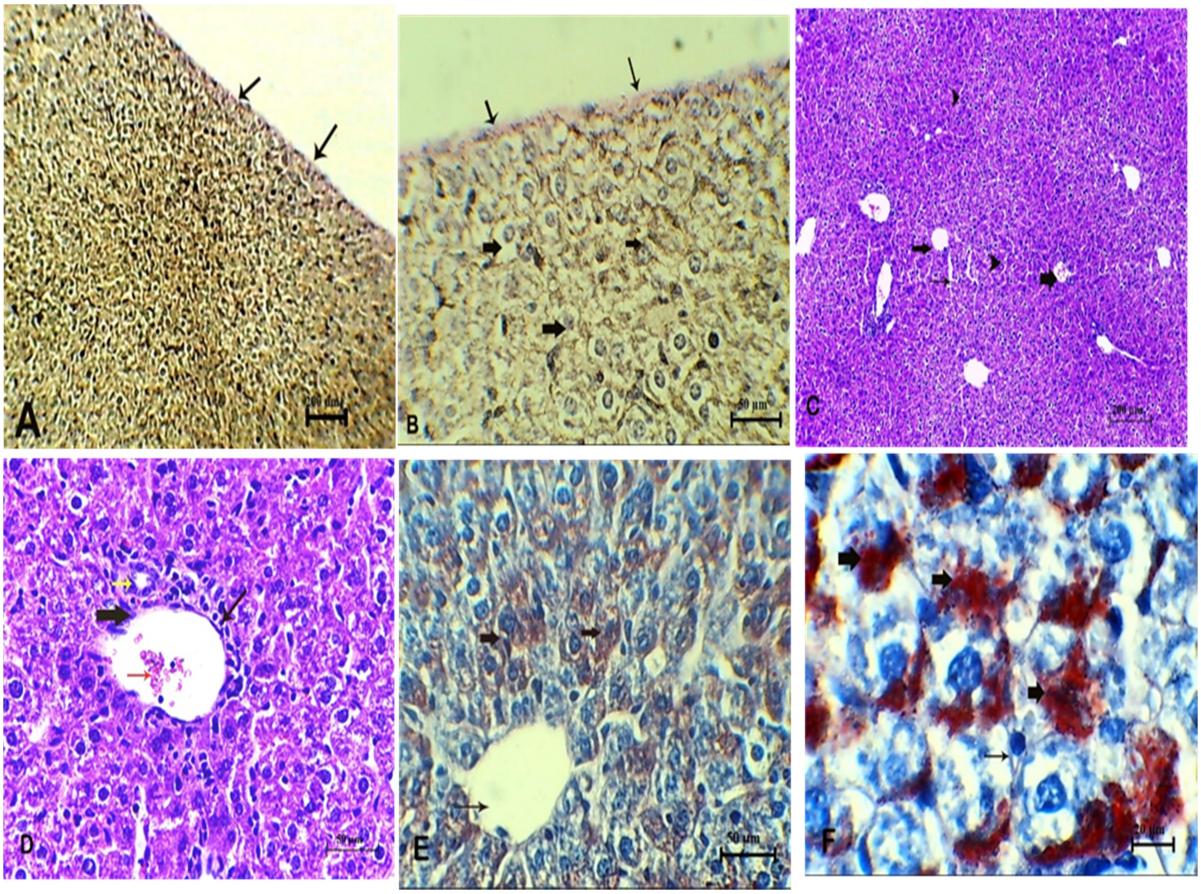
Fig(5): Histological changes after one, two, and three weeks in the treated group (A and B) after one week, showing many necrotic areas (head arrow), few numbers of polymorphous nuclear cells (thin arrow), and fewer mitotic cells (thick arrow).(C and D) Appearance of the tumor mass 2 weeks after the treatment, (A) showing high infiltrations of inflammatory cells (thin arrow) B, high power view from A (40X) showing blood vessels, probably arise through neovascularization (thick arrow), (E and F) 3 weeks of the treated group showing the extensive area of necrosis (thick arrow) with dilated of the lymphoid nodules (thin arrow) and vacuolated cells also detected (head arrow) with late apoptosis in some cells (red thin arrow). H&E stain.



Fig(6): Photomicrograph of the liver section, A and B in negative control group showing the normal structure of hepatic lobules, the central vein (thick arrow) , portal triad (thin arrow.(B) high power view (40X) of the central vein surrounded by endothelial cells (thick arrow) that is continuous with those lining the sinusoid (thin arrow), kupffer cells found in the sinusoid (head arrow).(C ,D) Histological change in the liver of untreated control group after 2 weeks of treatments, appear the high inflammation reaction around the portal vein mainly lymphocyte and plasma cells appeared as deep basophilic cells (thin arrow) ,central vein also surrounded with inflammatory cells (head arrow) with enlarged of the portal vein (PV) and bile duct(thick arrow).(E,F) shows glycogen granules deposition by mean PAS reaction (yellow arrow), histiocytic bodies formation within the hepatocyte plate (thin arrow). (A,B,C,D): H&E, (E,F): PAS stain.



Fig(7): liver section after 3 weeks of treatment in positive control group (A , B, C,D, E,F) shows necrotic liver cells surrounded by inflammatory cells mainly lymphocyte and plasma cells (thin arrow), central vein appears dilated (red arrow). marked less differentiated tumor cells infiltrated the liver parenchyma (head arrow) also detected with high aggregation of the less differentiated tumor cells under and within the Gilson capsule (thin arrow) which appeared as the heavy basophilic, rounded or oval nucleus, unclear connective tissue capsule (red arrow). Many necrotic areas in the hepatocyte plate (thick arrow).as seen in(E and F).(A,B,C,D,E): H&E , (F): Van Gieson stain



Fig(8): Histopathological observation in the liver section in treated group after 3 weeks (A&B) shows normal structure of hepatocyte plate (thick arrow), normal Gilson capsule structure (thin arrow) no evidence of tumor cells infiltrated, (C,D) normal structure of liver plate (head arrow), few lymphocyte infiltrations around the central vein (thick arrow). (B) showing portal vein with less inflammatory cells (red arrow), few lymphocytes around the portal triad (thin arrow), branch of the bile duct (yellow arrow). (E, F) shows extensive PAS reaction (thick arrow), normal central vein (thin arrow).(A, B): Van Gieson stain, (C,D): H&E, and (E,F): PAS stain.

DISCUSSION

Various studies have recently shown the benefits of medicinal plants in treating a broad range of diseases. There are also many natural herbal formulations on the market that have shown to be effective in treating cancer patients. Also, a few of the chemotherapeutic drugs showed cytotoxicity on normal cells, and many more of them lost their efficacy, which resulted in increasing resistance to them. (28, 29). There is more information on the biological roles of *C. intybus* and others such as antitumor, anti-inflammatory, hepatoprotective agent besides antioxidant effect, bringing it to light as a useful candidate for cancer treatment and prevention into light (30-32) . In vitro research has also shown that *Cichorium extracts* are highly toxic to many kinds of cancer cells (33, 34). Besides that, there was a limited amount of evidence supporting Chicory's anticancer properties *in vivo*.

Chicory contains immense therapeutic properties, which are recognized by the existence of secondary metabolites. It has been reported that contains many glycosides and flavonoids, alkaloids, and various terpenoids are found in this herb. These plant phytochemicals have distinctive and different medicinal features (35, 36). In the current study; various *C. intybus* extracts at varying concentrations have been tested for their cytotoxic ability by utilizing an MTT assay on 4T1 cancer cell line, we found that all *C. intybus* extracts were extremely cytotoxic in vitro on the 4T1 cell line. Also , *in vivo* study, we discovered that three weeks of oral administration of *C. intybus* leaf extract resulted in a dramatic reduction in the volume of 4T1 tumors in mice. Mehrandish *et al.* (30) showed that Methanol Extracts of Chicory reduced cell viability of the human breast cancer SKBR3 cell line after three-time exposure. In comparison, 100 µg/ml of chicory leaf hydro alcohol extract isolated is highly efficient toward a cell line of prostate cancer, whereas, the root extract is highly successful effective towards breast cancer cells (MCF-7), amelanotic melanoma cells (C32) and renal adenocarcinoma cells (ACHN) (37) .

Some preliminary evidence suggests that the anticancer effects of *C. intybus* extracts can be due to the levels of polyphenols which play a key role in removing free radicals

produced by cancer cells (38- 40). Hazra *et al*.(41) studied the effect of the root ethanolic extract of *C. intybus* in white mice implanted with Ehrlich ascites carcinoma, as mice were dosed with the extract daily by intraperitoneal at a concentration (300 -700 mg/kg body weight) from the first day.

Results showed that dosing mice with (500 mg/kg body weight) of the extract reduced tumor growth and 70% increase in the lifespan compared to control. In the present study, the histopathological analysis of tumor tissue after one week showed glandular configuration; tumor cells have appeared with large polymorphic nuclei with dense chromatin stain and little cytoplasm, angiogenesis, and inflammatory cells infiltrated with necrosis area, aggregation of lymphocyte. Then, the alteration was more evident after two and three weeks; this includes an area of the very aggressive tumor (solid anaplastic tumor) poorly differentiating, a central area of necrosis with an aggregation of inflammatory cells.

At the same time, the metastasis showed in liver tissue after two weeks in inoculated mice. Several changes also detected in liver tissue included inflammation reaction around the portal triads and central vein, very mild infiltrate of small heavy basophilic inflammatory cell. These tumor cells appeared as big, deeply basophilic stains; this agrees with Kumar *et al*. (42) reported that metastasis occurred approximately 11 days after mice had been inoculated with 4t1 murine breast cancer. Hepatosplenomegaly was demonstrated in cross studies, and many changes in liver tissue also detect with more tumor cells were seen. Fast-growing breast cancer cells, started from the tumor tissue, were noted to be progressive to the liver after 11 days. In conclusion, we conclude that *C. intybus* extracts are more effective on both cell line and animal tumor models through decreasing cell viability and reduce tumor growth besides preventing metastasis to reach other body parts.

التأثيرات المضادة للورم لمستخلصات الأوراق والساق والجذر لعشبة الهندباء في الزجاج وداخل الجسم الحي

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الخلاصة

استخدمت عشبة الهندباء في الطب التقليدي لعلاج العديد من الأمراض مثل الإسهال ، تحسين غدة البروستاتا والأعضاء التناسلية الأخرى ، علاج أمراض الرئة والتبغ ، السرطان والصداع الشديد وغيرها. قمنا بفحص التأثيرات المضادة للورم والانبثاث لمستخلصات عشبة الهندباء في الزجاج وداخل الجسم الحي. تم إجراء اختبار السمية الخلوية لمستخلصات الأوراق والساق والجذر لنبات الهندباء على خط الخلايا **4T1** تم اكتشاف قيمة تثبيط عالية في خط الخلايا **4t1** وعند ثلاث فترات من التعرض. اجريت الدراسة في داخل الجسم الحي لمدة ٢١ يومًا ، أعطيت من خلالها أناث الفئران الحاملة للورم والتي كانت من نوع (**Mus musculus Balb / c**) عن طريق الفم مائة ميكرولتر من مستخلص الأوراق (١٠٠٠ ملغم / كغم / وزن الجسم) ، بينما تلقت المجموعة الضابطة محلول دارى الفوسفات الملحي فقط. تسبب مستخلص الأوراق في انخفاض كبير في حجم الورم (**P <0.05**).مع ظهور القليل من المؤشرات على حدوث الانبثاث الى الكبد في الحيوانات المعالجة بمستخلص الأوراق مقارنةً بالعينة الضابطة ، أو أقل بشكل ملحوظ مما كان متوقعًا.

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