**1,8 Cineole and Ellagic acid inhibit hepatocarcinogenesis via upregulation of MiR-122 and suppression of TGF-β1, FSCN1, Vimentin, VEGF, and MMP-9**

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**Abstract**

Hepatocellular carcinoma (HCC) is one of the most burdened tumors worldwide, with a complex and multifactorial pathogenesis. Current treatment approaches involve different molecular targets. Phytochemicals have shown considerable promise in the prevention and treatment of HCC. We investigated the efficacy of two natural components, 1,8 cineole (Cin) and ellagic acid (EA), against diethylnitrosamine/2-acetylaminofluorene (DEN/2-AAF) induced HCC in rats. DEN/2-AAF showed deterioration of hepatic cells with an impaired functional capacity of the liver. In addition, the levels of tumor markers including alpha-fetoprotein, arginase-1, alpha-L-fucosidase, and ferritin were significantly increased, whereas the hepatic miR-122 level was significantly decreased in induced-HCC rats. Interestingly, treatment with Cin (100mg/kg) and EA (60mg/kg) powerfully restored these biochemical alterations. Moreover, Cin and EA treatment exhibited significant downregulation in transforming growth factor beta-1 (TGF-β1), Fascin-1 (FSCN1), vascular endothelial growth factor (VEGF), matrix metalloproteinase-9 (MMP-9), and epithelial-mesenchymal transition (EMT) key marker, vimentin, along with a restoration of histopathological findings compared to HCC group. Such effects were comparable to Doxorubicin (DOX) (2mg/kg); however, a little additive effect was evident through combining these phytochemicals with DOX. Altogether, this study highlighted 1,8 cineole and ellagic acid for the first time as promising phytochemicals for the treatment of hepatocarcinogenesis via regulating multiple targets.

**Introduction**

Hepatocellular carcinoma (HCC) is the fourth main cause of cancer-related mortality, creating a large global cancer burden [1, 2]. Unfortunately, HCC is characterized by rapid growth, high invasive potential, and early metastasis [3, 4]. Specifically, epithelial-mesenchymal transition (EMT) is partially considered as a critical step for HCC progression via mediating early
metastasis, migration, and invasion of tumor cells and acts as a potential therapeutic target in 
HCC treatment [5]. EMT is the process of cell remodeling that is characterized by upregulated 
expression of mesenchymal markers like vimentin, being one of its most important molecular 
features [6]. Inducers of EMT include several growth factors like vascular endothelial growth 
factor (VEGF) [7] and transforming growth factor beta-1 (TGF-β1) [8]. TGF-β1 is one of the 
pivotal factors regulating EMT, responsible for its initiation and maintenance in HCC [9]. 
TGF-β1 initiation is further activating multiple cellular responses. Fascin-1 (FSCN1), an actin-
binding protein involved in the invasion and migration of a variety of tumors, was verified as a 
direct target of TGF-β1 activation in HCC, and its overexpression was correlated with vimen-
tin upregulation [10]. In another response, several matrix metalloproteinases (MMPs) are 
overexpressed during EMT and because of TGF-β1 activation [11]. From all MMPs, MMP-9 
showed elevated expression and is considered an essential factor in HCC being a promoter of 
tumor metastasis and angiogenesis as well [12]. Thanks to MMP-9 proteolytic activity, it pro-
motes extracellular matrix (ECM) stored growth factors mobilization, including VEGF, thus 
favoring HCC angiogenesis. VEGF, another EMT inducer, is the most potent known angio-
genic factor, and the treatment strategies of HCC targeting VEGF have become a hotspot [13]. 

Ellagic acid (EA) is a natural phenolic constituent present in berries, walnuts, grapes, pome-
granates, black currents, and dried fruits [14]. EA is readily absorbed through the GIT to act 
on sub-cellular components and activate signaling transduction in target tissues and cells [15]. 
EA is considered as one of the most promising and applied chemopreventive agent against sev-
eral tumors without causing toxicity to normal cells [16]. However, little is known about its 
molecular mechanisms in HCC [17]. 1,8-Cineole (also known as eucalyptol), a monoterpen 
found naturally in essential oils of several plants, including eucalyptus, rosemary, cardamom, 
and sweet basil. It has been widely reported for its anti-inflammatory, antimicrobial, antiseptic, 
and antioxidant bioactivities [18-20] and, to a much lesser extent, as an anticancer compound 
with poorly understood underlying mechanisms [21, 22].

We aimed to assess the potential anticancer effect of EA and Cin alone and when combined 
with the standard chemotherapeutic agent, Doxorubicin, in an in-vivo model of rat HCC. Fur-
thermore, we initially shed light on their possible effects on EMT and its regulators.

Materials and methods

Drugs and chemicals

Diethylnitrosamine (DEN) (#bN0756), ellagic acid (#bE2250), doxorubicin hydrochloride 
(#bD1515), 1,8 cineole (#bC80601), acetylaminofluorene (2-AAF) (#bA7015) were purchased 
from Sigma-Aldrich Co.

Animals and experimental design

All procedures in this work were conducted consistently with the ethical standards and proto-
cols of the Guide for the Care and Use of Laboratory Animals and with the Animal Experiment-
ation Ethical Committee of NRC under approval no. of 16/383. The animals were 
acclimatized for one week under laboratory conditions, and then rats were randomly divided 
into seven groups (n = 10). Group 1 (Normal) rats were fed with a standard diet, i.p. injected 
onece weekly with saline, and served as a negative control. Group 2 (Induced-HCC) rats with 
DEN/2-AAF as described below and served as untreated HCC animals. Groups 3–5 (EA, Cin, 
and Dox) were DEN/2-AAF induced HCC rats treated with daily administration of ellagic 
acid (60 mg/kg p.o.) [23] or daily administration of 1,8 cineole (100 mg/kg p.o.) [24] or with a 
weekly injection of doxorubicin hydrochloride (2 mg/kg i.p.) [25], respectively for the last four 
weeks after HCC induction. Group 6 and 7 (EA/Dox and Cin/Dox) were DEN/2-AAF
induced HCC rats administered in combination with the same doses as each alone. The general health conditions of animals concerning food consumption, water intake, and body weight were observed during all the experimental time besides signs of distress including respiratory changes, body temperature, or even unusual behavior were also observed. We did not notice a significant variation in the previous parameters between the experimental groups except for the DEN/2-AAF group that showed a modest weight loss compared with the others.

**Induction of hepatocarcinogenesis**

HCC was experimentally induced in rats by a single i.p. injection of 200 mg/kg diethylnitrosamine (DEN) as the initiator. After eight weeks, liver cancer development was promoted with a once-weekly administration of 2-acetylaminofluorene (2-AAF) 30 mg/kg p.o. for another three weeks according to the protocol previously described [26] with few modifications. Treatment was started in the last 4 weeks of the experiment. Throughout the initiation phase of DEN treatment (first eight weeks), only one rat was dead. After starting 2-AAF administration (promotion phase), another two rats from the DEN/2-AAF induced HCC group died.

**Sample preparations**

At the end of the experiment, rats were lightly anesthetized, and blood samples were collected from the orbital sinuses and centrifuged to obtain sera for biochemical analysis. Then rats were killed by cervical dislocation under light ethyl ether anesthesia. The liver tissues were quickly taken and divided for further analyses. Serum and liver tissues were stored at −80˚C until they were analyzed.

**Determination of serum liver biomarkers**

The enzyme activities of alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) as well as albumin and total protein concentrations were determined in the serum by commercially available reagent kits (Biodiagnostic Co., Cairo, Egypt). The assays were done according to the supplied protocols.

**Determining HCC tumor markers**

Alpha-fetoprotein (AFP), Alpha-L-Fucosidase (AFU), Arginase-1(Arg-1), and ferritin serum levels were determined using ELISA kits obtained from Glory Science Co., Ltd., USA. The assays were performed as mentioned by the supplied protocols.

**MiR-122 expression using quantitative RT-PCR technique.** Total RNA was extracted from liver tissue with the RNeasy kit; Qiagen, Valencia, CA, USA as prescribed by the manufacturer. Isolated RNA was instantly archived into a cDNA library via the high-capacity cDNA reverse transcription kit, Applied Biosystems, Foster City, CA, USA. Real-time PCR was performed using the SYBR Green mix (BioRad) and the Rotor-Gene 1.7.87 RT-PCR system consistent with the manufacturer’s protocol. Relative gene expression was normalized to U6 small nuclear RNA and β actin, respectively, and was calculated using the 2^{ΔΔCT} method. **MiR-122 primer sequence was** F: 5’-GGCTGTGGAGTGTGACAATG -3’ and R: 5’-GAGGTA-TTCGACCAGAGGA -3’ [27].

**Western blot analysis of vimentin, TGF-β1, FSCN1, and MMP-9**

For the analysis of protein expression, liver homogenates were extracted for their protein contents by ice-cold RIPA buffer supplemented with phosphatase and protease inhibitors followed by centrifugation at 13,000 ×g for twenty min. The protein concentrations were quantified by
Bradford assay. Equal protein amounts (50 µg) were loaded in 10% SDS-PAGE and transferred to an activated PVDF membrane after electrophoresis. The membranes were blocked with 5% bovine serum albumin (BSA) solution for overnight at 4°C. Immunoblotting was performed using monoclonal antibodies to vimentin, TGF-β1, FSCN1, and MMP-9 as described previously [28]. Afterward, the incubation with the primary antibody for three h followed by the incubation with the secondary antibody (labeled with alkaline phosphatase) for two h was done at 25°C. Bands were visualized via the NBT-BCIP solution. The relative protein levels were determined after normalization with β-actin protein and analyzed by GS-700 imaging densitometer using V3 Western Workflow™ Complete System software; version 1.5, Bio-Rad Laboratories, Hercules, CA, USA.

Determining VEGF2
Its level in liver homogenates was determined using ELISA kits, Glory Science Co.

Histopathological evaluation
The liver tissues were separated instantly, washed with ice-cold saline, and fixed in 10% formalin. After fixation, tissues were dehydrated in ascending grade of ethanol and embedded in Paraffin wax followed by sectioning at 5 µm to be mounted on slides. Then slides were oven-dried, deparaffinized, and stained with H & E staining to be viewed using a light microscope.

Statistical analysis
The statistical analysis of data was performed with one-way ANOVA and Tukey’s post-hoc test using Graph pad prism software version 6. Data are presented as mean ± SEM, n = 10. The value of (P < 0.05) was considered as significant. ‘a’ vs. the normal group, ‘b’ vs. DEN/2-AAF-induced HCC group, and ‘c’ vs. DOX treated group.

Results
1,8 cineole and ellagic acid protection against DEN/2-AAF induced liver damage
The induction of HCC with DEN/2-AAF resulted in a dramatic change in the functional activity of the liver represented by a significant (P<0.05) increase in the serum activities of ALT, AST, and ALP that was accompanied by a significant decrease in the serum concentrations of total protein and albumin when compared to normal rats as displayed in (Table 1). Treatment with 1,8 cineole (100 mg/kg) and ellagic acid (60 mg/kg) for four weeks showed a significant (P<0.05) decrease in the serum activities of ALT, AST, and ALP compared with HCC untreated group. The ALP activity was noticeably decreased in the DOX-treated group compared with HCC untreated group.

The concentration levels of total proteins and albumin were significantly (P<0.05) increased in treated HCC rats with 1,8 cineole and ellagic acid compared with untreated HCC rats. Notably, the combination treatment of 1,8 cineole and ellagic acid with Doxorubicin did not show significant improvement in the levels of liver biomarkers when compared to the DOX-treated group.

Effect on serum HCC tumor markers
Alpha-fetoprotein (AFP) is the main golden marker for HCC detection. Rats administered DEN/2-AAF showed significant (P<0.05) elevation in AFP level to 716.6 ± 60.5 ng/ml as compared to the control group (270 ± 10 ng/mL). All treatment groups almost normalized the AFP
The level of AFP was decreased to 243.3 ± 20.1 ng/mL, 286.6 ± 21.8 ng/mL and 266.6 ± 8 ng/mL in rats treated with DOX (2 mg/kg), Cin (100 mg/kg) and EA (60 mg/kg), respectively. No significant difference in AFP levels was observed between groups compared to the Dox group, including combination groups (Fig 1A).

Serum ferritin, another biochemical marker of HCC and the major cellular storage protein for iron, was elevated to about two folds (234 ± 4 ng/ml) after DEN/2-AAF induction of HCC compared to normal rats (112 ± 3.5 ng/mL). This elevation was opposed by all treatment

### Table 1. Effect of 1,8 cineole (Cin), ellagic acid (EA), doxorubicin (DOX) and combinations on serum liver markers of DEN/2-AAF-administered rats.

| Groups       | ALT (U/L) | AST (U/L) | ALP (U/L) | Total protein (g/L) | Albumin (g/L) |
|--------------|-----------|-----------|-----------|---------------------|---------------|
| Normal       | 21±0.16   | 66.05±3   | 180.00±8.2| 8.83±0.28           | 5.05±0.1      |
| HCC          | 44.1±1.6 * | 135.7±1.7 * | 567.50±8.5 * | 5.68±0.27 *        | 2.88±0.26 *   |
| HCC+DOX      | 35.57±0.39 * | 132.59±4.2 * | 355.60±9 *   | 4.89±0.16 *        | 2.07±0.3 *    |
| HCC+EA       | 30.05±0.7 b | 107.5±2.7 b  | 401.40±5.3 b | 7.41±0.3 b         | 3.71±0.16 b   |
| HCC+EA+DOX   | 31.9±0.63 b | 96.22±7.6 b  | 380.20±5.1 b | 6.66±0.32          | 4.22±0.21 b   |
| HCC+Cin      | 30.17±0.1 b | 111.3±4.1 b  | 381.25±4.7 b | 7.39±0.17 b        | 3.68±0.13 b   |
| HCC+Cin+DOX  | 28.67±1.8 b | 92.59±5.6 b  | 258.00±9.4 b | 7.08±0.29          | 3.81±0.24 b   |

Data are presented as mean ± S.E.M, n = 10.

*a* P<0.05 vs. the normal group and

*b* P<0.05 vs. DEN/2-AAF-induced HCC group using one-way ANOVA followed by Tukey’s post-hoc test.

ALT (Alanine transaminase); AST (Aspartate transaminase); ALP (Alkaline phosphatase).

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![Fig 1. Effect of 1,8 cineole (Cin), ellagic acid (EA), doxorubicin (DOX), and combinations on HCC tumor markers.](https://doi.org/10.1371/journal.pone.0258998.g001)
groups (Fig 1B). Interestingly, serum ferritin concentrations in Cin (70.5±0.71 ng/mL), Dox+Cin (65±0.71ng/mL), EA (75.5±3ng/mL), and Dox+EA (64±1.4 ng/mL) treated groups were significantly lower than Dox-treated HCC group (122.3 ±3ng/mL).

**Alpha-L-fucosidase (AFU),** has been anticipated as a promising tumor marker in the diagnosis of HCC. The administration of DEN/2-AAF produced a significant increase in serum AFU level (58.6 ± 0.27 U/L) when compared to normal rats (43.1±0.41U/L). The level of AFU was decreased to (47.4± 0.66 U/L), (48.9± 0.73 U/L), and (44.4± 1.6) in rats treated with DOX, 1,8 cineole, and ellagic acid, respectively. Combination treatments of DOX+CIN (42.9± 0.32 U/L) and DOX+EA (41.7± 0.83U/L) showed a lower level of serum AFU compared to that of DOX alone (Fig 1C).

**Arginase-1 (Arg-1),** a recent marker for HCC, is involved in the urea cycle leading to polyamines production and tumor cell proliferation. The serum level of arginase-1 in DEN/2-AAF-administered rats showed a significant increase to (358.6±8 U/L) as compared to normal rats (253.2±5.3 U/L). Treated HCC rats with DOX, Cin, EA along with their combinations significantly decreased serum level of Arg-1 in a range from 286.9± 2.9 U/L to 299.5± 0.71 U/L as compared to the HCC group. Treatment combinations did not show any statistically different reduction in arginase-1 level as compared to the Dox group (Fig 1D).

**1,8 cineole and ellagic acid reduced MiR-122 expression**

MiR-122, a liver-specific miRNA, is a sensitive and specific marker of HCC. Therefore, the expression level of miR-122 in liver tissue was measured by qRT-PCR analysis (Fig 2) after the DEN/2-AAF induction of HCC. The expression of miR-122 was significantly down-regulated in the HCC group (0.2 ± 0.03) compared to the control group (1.01 ± 0.01). Treatment groups with Cin (0.72±0.03), EA (0.66±0.08), and their combinations (0.58±0.05 and 0.58±0.01), respectively with Dox were resulted in a dramatic upregulation of its expression as compared to the HCC group. However, combination treatments did not show any significant improvement in its expression compared to the Dox group (0.72 ± 0.06).

**Effect on EMT and its regulators**

The levels of EMT key marker, vimentin, and EMT regulators, including TGF-β1, FSCN1, MMP-9, and VEGF were measured in liver tissue after DEN/2-AAF administration. Results from the western blot analysis indicated that DEN/2-AAF induced about 6.5, 4.8, 6- and 7.1-fold increases in vimentin, TGF-β1, FSCN1, and MMP-9 protein expression, respectively (Fig 3A). Similar behavior was observed at their levels in all treatment groups showing significant downregulation as compared to the HCC group. Nevertheless, combination treatments did not show any improvement as compared to the Dox group. Comparable results were obtained after determining the level of another EMT inducer, VEGF using ELISA kits. DEN/2-AAF induced a 2.5-fold increase in the VEGF level (761±5.57 pg/mL) compared to the normal group (299 ± 6.24 pg/mL). All treated groups showed a significant reduction in the VEGF level compared to DEN/2-AAF administered group. However, there was no significant difference in its level between combination groups as compared to Dox treated group (284.67 ± 6.11 pg/mL) (Fig 3B).

**Histopathological findings**

The liver of control rats showed the hepatic lobules with the typical hexagonal architecture together with a normal central vein, radiating hepatocytes showing regular cellular and nuclear size (Fig 4A). Normal rats showed the ordinary hexagonal architecture of the hepatic lobules with the normal portal tract that includes portal artery, portal vein, and bile duct (Fig 4B).
Fig 2. MiR-122 expression using the qRT-PCR technique of DEN/2-AAF-induced HCC rats. Rats were treated with 1,8 cineole (Cin), ellagic acid (EA), doxorubicin (DOX), and combinations. Data are presented as mean ± S.E.M, n = 10. "a" P<0.05 vs. normal group, "b" P<0.05 vs. DEN/2-AAF-induced HCC group and "c" P<0.05 vs. DOX treated group using one-way ANOVA followed by Tukey’s post-hoc test.

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Fig 3. EMT and its regulators in DEN/2-AAF-induced HCC rats. (A) Representative Western Blot of Vimentin, TGF-β1, FSCN1, and MMP-9 after treatment with 1,8 cineole (Cin), ellagic acid (EA), doxorubicin (DOX), and combinations. Chemiluminescence analysis; optical densities were normalized to β-actin levels and expressed in arbitrary units. The data shown are representative of three independent experiments with comparable results. (B) VEGF concentration in liver homogenate using ELISA kits. Data are presented as mean ± S.E.M, n = 10. "a" P<0.05 vs. normal group, "b" P<0.05 vs. DEN/2-AAF-induced HCC group, and "c" P<0.05 vs. DOX treated group using one-way ANOVA followed by Tukey’s post-hoc test.

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Microscopic examination of DEN+2-AAF administered rats showed aggregation of inflammatory cells in the portal areas (Fig 4C), multinucleated hepatocytes, loss of radiating hepatocytes (Fig 4D), enlargement of hepatocytes, and nuclear size as compared with normal control (Fig 4E) and micro/macro-vesicular steatosis (Fig 4F). The Dox-treated rats showed foci of enlarged cells were seen (Fig 5A), and the portal areas exhibited mild inflammatory infiltration (Fig 5B). On the other hand, rats treated with Cin or EA showed the ordinary structure of the hepatic lobules and portal areas (Figs 5C, 5D, 6C and 6D), respectively. The combined treatments of Cin or EA with Dox showed the ordinary structure of the hepatic lobule (Figs 5E and 6A), respectively. However, mild inflammatory infiltrations were observed in the portal areas of combined Cin treatment (Fig 5F), and congested portal areas associated with moderate inflammatory infiltration were observed in the portal areas of EA combined treatment (Fig 6B).

Discussion

Hepatocellular carcinoma (HCC) is a very pervasive malignant disorder with multifaceted molecular pathogenesis. Studies proved that hepatocarcinogenesis had been linked with variations in several important cellular responses. Targeting molecules during the multistep process of hepatocarcinogenesis was of interest from the therapeutic perception because this may aid in a coup, delay, or even prevent tumorigenesis [29, 30]. Phytochemicals were shown to control HCC via interference with all phases of carcinogenesis, including initiation, promotion, and progression [31, 32]. Therefore, plant-derived compounds attract a lot of consideration because of their definite therapeutic effectiveness in the treatment of cancers [33, 34].
Fig 5. Photographs of liver sections stained with H & E. (A and B) Doxorubicin-treated rats showing a focal of enlarged cells with mild inflammatory infiltration, respectively. (C and D) 1,8 Cineole-treated rats were showing the ordinary structure of the hepatic lobule and portal area, respectively. (E and F) Dox+Cin treated rats showing the ordinary structure of the hepatic lobule with mild inflammatory infiltration, respectively.

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Fig 6. Photographs of liver sections stained with H & E. (A and B) Dox+EA treated rats showing the ordinary structure of the hepatic lobule and congested portal area (arrows) that were associated with moderate inflammatory infiltration (arrowheads). (C and D) Ellagic acid-treated rats showing the ordinary structure of the hepatic lobule and portal area, respectively.

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Accordingly, the current work demonstrated for the first time the multi-target effects of two phytochemicals, 1,8 cineole (Cin) and ellagic acid (EA), against DEN/2-AAF-induced rat hepatocarcinogenesis.

DEN is the most used carcinogen to investigate hepatocarcinogenesis in rodents [35–37]. In models of experimentally induced HCC, initiation and promotion are essential steps. The initiation with DEN followed by using 2-AAF as a promoter was reported to produce successful development of experimentally induced HCC model [38, 39]. In the present work, DEN/2-AAF administration resulted in liver damage that was showed by an elevated level of liver function enzymes (ALT, AST, and ALP) along with impairment in the protein biosynthesis capacity. These abnormalities were observed remarkably in hepatoma and could be attributed to the hepatic lesions produced by DEN [35, 38, 40]. Treatment with 1,8 cineole and ellagic acid protected the liver from the effects of DEN plus 2-AAF on the previously mentioned markers of liver damage. These results are in agreement with an earlier study by our group showing the hepatoprotective effects of 1,8 cineole and ellagic acid [41] and demonstrated their ability to protect the liver from carcinogens by maintaining the cell membrane and functional integrity, thereby mitigating the progression of carcinogenesis.

Alpha-fetoprotein (AFP), alpha-L-fucosidase (AFU), arginase-1 (Arg-1) are promising diagnostic tumor markers of HCC, and their measurements increased the detection sensitivity and specificity for HCC [42–44]. In the current study, disease progression was further confirmed by the elevated levels of these tumor markers in DEN/2-AAF administered rats, as well as the existence of multinucleated cells and steatosis that are common in early HCC [45, 46]. The marked decrease in levels of AFP, AFU, and Arg-1 along with the improvement of histopathologic features that were observed after treatment with 1,8 cineole and ellagic acid strongly suggested their anticancer activity against HCC.

One of HCC manifestations is iron overload demonstrated by elevation of serum ferritin and correlated with elevated serum aminotransferases proposing that ferritin came from damaged cells and was released into the serum [47, 48]. In accordance with these studies, we showed a significant elevation of serum ferritin after DEN/2-AAF that was overturned by treatment providing evidence for 1,8 cineole and ellagic acid protection against disturbances of iron metabolism in HCC. In addition, circulating microRNAs (miRNAs) have been implicated in hepatocarcinogenesis [49]. More importantly, microRNA-122 (miR-122) is a liver-specific miRNA constituting 70% of the total hepatic miRNAs, acting as a tumor suppressor, and its downregulation has been reported and correlated with HCC progression [50, 51]. In this investigation, our data showed for the first time that DEN/2-AAF induced a marked decrease in miR-122 expression while the therapeutic administration with 1,8 cineole and EA showed a significant improvement signifying the potential use of 1,8 cineole and EA in liver cancer treatment as multi-target anticancer agents.

The development of HCC is associated with the activation of epithelial-mesenchymal transition (EMT) that has been reported from both clinical and experimental observations [52]. Vimentin over-expression, a molecular characteristic of EMT, has been reported in cancers and correlated with increased tumor growth, invasion, and poor prognosis. Consequently, studies provided vimentin as an available targeted cancer therapy [53]. The decreased expression of vimentin in this study indicated for the first time that 1,8 cineole and ellagic acid might have a suppressive role on EMT in hepatocarcinogenesis. This finding comes to an agreement with previous reports displaying the anti-EMT effect of EA that was observed in breast cancer [54] and pancreatic cancer [33].

The activation of transforming growth factor-beta 1 (TGF-β1) and subsequent triggering of EMT is also critical in the development of HCC. It has been observed that TGF-β1-enriched cells showed an increase in the vimentin expression level [55]. There is also evidence
suggesting that FSCN1, an actin-binding protein involved in the invasion and migration, is overexpressed in response to TGF-β1 activation in HCC. Moreover, studies have demonstrated that FSCN1 also promoted EMT in cancers including HCC, and as predictable its suppression significantly suppressed vimentin expression, a key marker of EMT [10]. DEN was reported to induce hepatocarcinogenesis in rats via upregulating TGF-β1 signaling pathway [56]. In our study, we found a pronounced expression of TGF-β1, FSCN1, and vimentin in DEN/2AFF treated rats. These data suggested that TGF-β1 and, for the first time, FSCN1 and vimentin are essential for the DEN/2-AFF induced hepatocarcinogenesis. Interestingly, our work showed primarily that 1,8 cineole and ellagic acid suppressed the expression of TGF-β1, FSCN1, and vimentin in this model providing new targets for both phytochemicals in HCC.

Elevated levels of matrix metalloproteinases (MMPs), especially MMP-9, are considered as an important factor in hepatocarcinogenesis, being a promoter of tumor invasion and angiogenesis as well [12, 57]. MMP-9 is overexpressed during EMT because of TGF-β1 activation [7]. Moreover, studies have demonstrated the functional interplay between MMP-9 and vascular endothelial growth factor (VEGF), another EMT inducer, in HCC, favoring tumor angiogenesis [12]. This can somewhat be explained by studies that demonstrated a positive correlation of MMP-9 and VEGF expression with the progression and recurrence of HCC [57, 58]. Angiogenesis has an important role in HCC progression and aggressiveness, being a part of its multifaceted molecular pathogenesis [13]. The potent suppressed expression of MMP-9 and VEGF was first observed in this study after 1,8 cineole treatment providing these two parameters as important targets of 1,8 cineole in combating HCC. Ellagic acid also decreased MMP-9 and VEGF expression. These findings were in line with earlier studies demonstrating the suppressive activity of EA on VEGF and MMP-9 [28, 59]. Convincingly, these results added potential for the role of 1,8 cineole and ellagic acid in chemoprevention and treatment of hepatocarcinogenesis.

Surprisingly, the different observed effects of 1,8 cineole and ellagic acid in the whole study were comparable to the effect of the potent chemotherapeutic agent, Doxorubicin, which is hindered by toxicity to normal tissues and tumor resistance [60]. However, the combination of both phytochemicals with Dox showed no additive effect regarding the investigated parameters.

Conclusions
In conclusion, the anticancer effects of 1,8-cineole and ellagic acid were demonstrated for the first time through 1) Conserving of liver functions 2) Lessening of HCC tumor markers; alpha-fetoprotein, ferritin, arginase-1, and alpha-L-fucosidase. 3) Improvement in histopathologic features induced by DEN/2-AAF. 4) Upregulation of tumor suppressor microRNA; miR-122. 5) Declining of hepatocarcinogenesis mediators including EMT (vimentin) and EMT regulators (TGF-β1, FSCN1, MMP-9, and VEGF). These results highlighted the multiple-target effects of 1,8 cineole and ellagic acid in the treatment of HCC as potential therapeutic candidatures.

Supporting information
S1 Graphical abstract.
(TIF)
S1 Raw images.
(ZIP)
Author Contributions

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References

1. Zhang Y, Luo X, Lin J, Fu S, Feng P, Su H, et al. Gelsolin Promotes Cancer Progression by Regulating Epithelial-Mesenchymal Transition in Hepatocellular Carcinoma and Correlates with a Poor Prognosis. Journal of Oncology. 2020; 2020:1–10. https://doi.org/10.1155/2020/1980368 PMID: 32377190
2. Ferlay J, Colombet M, Soerjomataram I, Mathers C, Parkin DM, Pineros M, et al. Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. Int J Cancer. 2019; 144 (8):1941–53. Epub 2018/10/24. https://doi.org/10.1002/ijc.31937 PMID: 30350310.
3. Wang H, Rao B, Lou J, Li J, Liu Z, Li A, et al. The Function of the HGF/c-Met Axis in Hepatocellular Carcinoma. Front Cell Dev Biol. 2020; 8:55. Epub 2020/03/03. https://doi.org/10.3389/fcell.2020.00055 PMID: 32117981
4. Yu L, Xu F, Gao L. Predict New Therapeutic Drugs for Hepatocellular Carcinoma Based on Gene Mutation and Expression. Front Bioeng Biotechnol. 2020; 8:8. Epub 2020/02/13. https://doi.org/10.3389/fbioe.2020.00008 PMID: 32047745
5. Jo H, Lee J, Jeon J, Kim SY, Chung JJ, Ko HY, et al. The critical role of glucose deprivation in epithelial-mesenchymal transition in hepatocellular carcinoma under hypoxia. Sci Rep. 2020; 10(1):1538. Epub 2020/02/01. https://doi.org/10.1038/s41598-020-58124-1 PMID: 32001727
6. Du QY, Yao JH, Zhou YC, Xu LJ, Zhao FY, Yang Y. High STRN Expression Promotes HCC Invasion and Migration but Not Cell Proliferation or Apoptosis through Facilitating Epithelial-Mesenchymal Transition. Biomed Res Int. 2020; 2020:6152925. Epub 2020/04/14. https://doi.org/10.1155/2020/6152925 PMID: 32280692
7. Yan L, Xu F, Dai CL. Relationship between epithelial-to-mesenchymal transition and the inflammatory microenvironment of hepatocellular carcinoma. J Exp Clin Cancer Res. 2018; 37(1):203. Epub 2018/08/31. https://doi.org/10.1186/s13046-018-0887-z PMID: 30157906
8. Hao Y, Baker D, Ten Dijke P. TGF-beta-Mediated Epithelial-Mesenchymal Transition and Cancer Metastasis. Int J Mol Sci. 2019; 20(11). Epub 2019/06/15. https://doi.org/10.3390/ijms2012767 PMID: 31195692
9. Bai X, Li YY, Zhang HY, Wang F, He HL, Yao JC, et al. Role of matrix metalloproteinase-9 in transforming growth factor-beta1-induced epithelial-mesenchymal transition in esophageal squamous cell carcinoma. Onco Targets Ther. 2017; 10:2837–47. Epub 2017/06/28. https://doi.org/10.2147/OTT.S134813 PMID: 28652766
10. Zhang Y, Lu Y, Zhang C, Huang D, Wu W, Zhang Y, et al. FSCN1 increases doxorubicin resistance in hepatocellular carcinoma through promotion of epithelial-mesenchymal transition. Int J Oncol. 2018; 52 (5):1455–64. Epub 2018/03/24. https://doi.org/10.3892/ijo.2018.4327 PMID: 29568938
11. Gonzalez-Avila G, Sommer B, Mendoza-Posada DA, Ramos C, Garcia-Hernandez AA, Falfan-Valencia R. Matrix metalloproteinases participation in the metastatic process and their diagnostic and therapeutic applications in cancer. Crit Rev Oncol Hematol. 2019; 137:57–83. Epub 2019/04/25. https://doi.org/10.1016/j.critrevonc.2019.02.010 PMID: 31014516.

12. Roomi MW, Kalinovsky T, Bhanap B, Niedzwiecki A, Rath M. In Vitro Effect of Cytokines, Inducers, and Inhibitors on the Secretion of MMP-2 and MMP-9 in Hepatocarcinoma Cell Line SK-Hep-1. Integrative Cancer Therapies. 2019;18. https://doi.org/10.1177/153475419889155

13. Pan Z, Zhuang J, Ji C, Cai Z, Liao W, Huang Z. Curcumin inhibits hepatocellular carcinoma growth by targeting VEGF expression. Oncol Lett. 2018; 15(4):4821–6. Epub 2018/03/20. https://doi.org/10.3892/ol.2018.7988 PMID: 29552121

14. Chen J, Yang H, Sheng Z. Ellagic Acid Activated PPAR Signaling Pathway to Protect Ileums Against Castor Oil-Induced Diarrhea in Mice: Application of Transcriptome Analysis in Drug Screening. Front Pharmacol. 2019; 10:1681. Epub 2020/02/23. https://doi.org/10.3389/fphar.2019.01681 PMID: 32082169

15. Qiu Z, Zhou J, Zhang C, Cheng Y, Hu J, Zheng G. Antiproliferative effect of urothlein A, the ellagic acid-derived colonic metabolite, on hepatocellular carcinoma HepG2.2.15 cells by targeting Lin28a/let-7a axis. Braz J Med Biol Res. 2018; 51(7):e7220. Epub 2018/05/10. https://doi.org/10.1590/1414-431x20187220 PMID: 29742265

16. Zhao J, Li G, Bo W, Zhou Y, Dang S, Wei J, et al. Multiple effects of ellagic acid on human colorectal carcinoma cells identified by gene expression profile analysis. Int J Oncol. 2017; 50(2):613–21. Epub 2017/01/20. https://doi.org/10.3892/ijo.2017.3843 PMID: 28101576.

17. Sepúlveda L, Laredo-Alcalá E, Buenrostro-Figueroa JJ, Ascacio-Váldes JA, Genisheva Z, Aguilar C, et al. Ellagic acid production using polyphenols from orange peel waste by submerged fermentation. Electronic Journal of Biotechnology. 2020; 43:1–7. https://doi.org/10.1016/j.ejbt.2019.11.002

18. Linghu KG, Wu GP, Fu LY, Yang H, Li HZ, Chen Y, et al. 1,8-Cineole Ameliorates LPS-Induced Vascular Endothelium Dysfunction in Mice via PPAR-gamma Dependent Regulation of NF-kappaB. Front Pharmacol. 2019; 10:178. Epub 2019/04/02. https://doi.org/10.3389/fphar.2019.00178 PMID: 30930772

19. Cutillas AB, Carrasco A, Martínez-Gutiérrez R, Tomas V, Tudela J. Salvia officinallis L. Essential Oils from Spain: Determination of Composition, Antioxidant Capacity, Antienzymatic, and Antimicrobial Bioactivities. Chem Biodivers. 2017;14(8). Epub 2017/05/10. https://doi.org/10.1002/cbdv.201700102 PMID: 28477412.

20. Sobreira Dantas Nobrega de Figueirêdo FR, Monteiro AB, Alencar de Menezes IR, Sales VDS, Peticcia do Nascimento E, Kelly de Souza Rodrigues C, et al. Effects of the Hyptis martiusii Benth. leaf essential oil and 1,8-cineole (eucalyptol) on the central nervous system of mice. Food Chem Toxicol. 2019; 133:110802. Epub 2019/09/08. https://doi.org/10.1016/j.fct.2019.110802 PMID: 31493462.

21. Privitera G, Luca T, Castorina S, Passanisi R, Ruberto G, Napoli E. Anticancer activity of Salvia officinallis essential oil and its principal constituents against hormone-dependent tumour cells. Asian Pacific Journal of Tropical Biomedicine. 2019; 9(1). https://doi.org/10.4103/apjtb.apjtb_2221_16 PMID: 31014576.

22. Rodenak-Kladniew B, Castro A, Starkel P, Galle M, Crespo R. 1,8-Cineole Promising Targets in Suppressing Hepatocarcinogenesis. J Cancer Res Ther. 2017; 13(1):62–8. Epub 2017/05/17. https://doi.org/10.1007/s12284-016-0324-5 PMID: 28508835.

23. Rocha Caldas GF, Oliveira AR, Araujo AV, Lafayette SS, Albuquerque GS, Silva-Neto Jda C, et al. Gastroprotective Mechanisms of the Monoterpene 1,8-Cineole (Eucalyptol). PLoS One. 2015;10(8):e0134558. Epub 2015/08/06. https://doi.org/10.1371/journal.pone.0134558 PMID: 26244547

24. Gao Y, Shen JK, Choy E, Zhang Z, Mankin HJ, Hornicek FJ, et al. Pharmacokinetics and tolerability of NSC23925b, a novel P-glycoprotein inhibitor: preclinical study in mice and rats. Sci Rep. 2016; 6:25659. Epub 2016/05/10. https://doi.org/10.1038/srep25659 PMID: 27157103

25. Amereh Z, Hatami N, Shirazi FH, Gholami S, Hosseini SH, Noorbahari M, et al. Cancer chemoprevention by oleaster (Elaeagnus angustifolia L.) fruit extract in a model of hepatocellular carcinoma induced by diethylnitrosamine in rats. EXCLI J. 2017; 16:1046–56. Epub 2017/01/20. https://doi.org/10.17179/excli2017-389 PMID: 28477412.

26. Lardizabal MN, Nocito AL, Daniele SM, Ornella LA, Palatnik JF, Veggi LM. Reference genes for real-time PCR quantification of microRNAs and messenger RNAs in rat models of hepatotoxicity. PLoS One. 2012; 7(5):e36323. Epub 2012/05/09. https://doi.org/10.1371/journal.pone.0036323 PMID: 22563491
28. Das U, Biswas S, Chattopadhyay S, Chakraborty A, Dey Sharma R, Banerji A, et al. Radiosensitizing effect of ellagic acid on growth of Hepatocellular carcinoma cells: an in vitro study. Sci Rep. 2017; 7(1):14043. Epub 2017/10/27. https://doi.org/10.1038/s41598-017-14211-4 PMID: 29070894

29. Elsonbaty SM, Zahrani WE, Moawed FS. Gamma-irradiated beta-glucan modulates signaling molecular targets of hepatocellular carcinoma in rats. Tumour Biol. 2017; 39(8):1010428317708703. Epub 2017/08/16. https://doi.org/10.1007/s13277-017-1563-8 PMID: 28810822.

30. Jiang X, Hao Y. Analysis of expression profile data identifies key genes and pathways in hepatocellular carcinoma. OncoLett. 2018; 15(2):2625–30. Epub 2018/02/13. https://doi.org/10.3892/ol.2017.7534 PMID: 29434983

31. Khan T, Ali M, Khan A, Nisar P, Jan SA, Afridi S, et al. Anticancer Plants: A Review of the Active Phytochemicals, Applications in Animal Models, and Regulatory Aspects. Biomolecules. 2019; 10(1). Epub 2020/01/02. https://doi.org/10.3390/biom10010047 PMID: 31892257

32. Abdel-Hamid NM, Abass AA, Mohamed AA, Muneam Hamid D. Herbal management of hepatocellular carcinoma through cutting the pathways of the common risk factors. Biomed Pharmacother. 2018; 107:1246–58. Epub 2018/09/28. https://doi.org/10.1016/j.biopha.2018.08.104 PMID: 30257339

33. Choudhari AS, Mandave PC, Deshpande M, Ranjekar P, Prakash O. Phytochemicals in Cancer Treatment: From Preclinical Studies to Clinical Practice. Front Pharmacol. 2019; 10:1614. Epub 2020/03/03. https://doi.org/10.3389/fphar.2019.01614 PMID: 32116665

34. Ahmed OM, Ahmed AA, Fahim HI, Belge-Kurutas E. Experimental Hepatic Carcinogenesis: Oxidative Stress and Natural Antioxidants. Open Access Maced J Med Sci. 2017; 5(5):686–91. Epub 2017/09/22. https://doi.org/10.3889/ oamjms.2017.101 PMID: 28932315

35. Gani SA, Muhammad SA, Kura AU, Barahuie F, Hussein MZ, Fakurazi S. Effect of protocatechuic acid-laminiflourine-induced hepatocarcinogenesis in Wistar rats: the roles of oxidative stress, inflammation and cell apoptosis. Drug Chem Toxicol. 2019:1–12. Epub 2019/11/02. https://doi.org/10.1080/01480545.2019.1683187 PMID: 31665932.

36. Mansour DF, Abdallah HMI, Ibrahim BMM, El Awdan SA, Allam RM, Selim MS, et al. Protective effect of some natural products against chemotherapy-induced toxicity in rats. Heliyon. 2019; 5(5):e01590. Epub 2019/05/14. https://doi.org/10.1016/j.helip.2019.e01590 PMID: 31141523

37. Hegazy RR, Mansour DF, Salama AA, Abdel-Rahman RF, Hassan AM. Regulation of PKB/Akt-pathway in the chemopreventive effect of lactoferrin against diethylnitrosamine-induced hepatocarcinogenesis in rats. Pharmacoel. 2019; 71(5):879–91. Epub 2019/08/24. https://doi.org/10.1016/j.pharep.2019.04.015 PMID: 31442665.

38. Ahmed OM, Ahmed AA, Fahim HI, Zaky MY. Quercetin and naringenin abate diethylnitrosamine/phenobarbital-induced hepatocellular carcinoma in mice. PLoS One. 2019; 14(5):e0217009. Epub 2019/05/30. https://doi.org/10.1371/journal.pone.0217009 PMID: 3141523

39. Timek DT, Shi J, Liu H, Lin F. Arginase-1, HepPar-1, and Glypican-3 are the most effective panel of markers in distinguishing hepatocellular carcinoma from metastatic tumor on fine-needle aspiration specimens. Am J Clin Pathol. 2012; 138(2):203–10. Epub 2012/08/21. https://doi.org/10.1093/ajcp/kjr137 PMID: 22904131.

40. Zhang J, Chen G, Zhang P, Zhang J, Li X, Gan D, et al. The threshold of alpha-fetoprotein (AFP) for the diagnosis of hepatocellular carcinoma: A systematic review and meta-analysis. PLoS One. 2020; 15(2):e0228857. Epub 2020/02/14. https://doi.org/10.1371/journal.pone.0228857 PMID: 32053643.

41. Abbas SA, Mohamed AA, Muneam Hamid D. Herbal management of hepatocellular carcinoma through cutting the pathways of the common risk factors. Biomed Pharmacother. 2018; 107:1246–58. Epub 2018/09/28. https://doi.org/10.1016/j.biopha.2018.08.104 PMID: 30257339

42. Mansour DF, Abdallah HMI, Ibrahim BMM, Hegazy RR, Esmail RSE, Abdel-Salam LO. The Carcinogenic Agent Diethylnitrosamine Induces Early Oxidative Stress, Inflammation and Proliferation in Rat Liver, Stomach and Colon: Protective Effect of Ginger Extract. Asian Pac J Cancer Prev, 2019; 20(8):2551–61. Epub 2019/08/28. https://doi.org/10.31557/APJCP.2019.20.8.2551 PMID: 31450931.

43. Abdel-Hamid NM, Abass AA, Mohamed AA, Muneam Hamid D. Herbal management of hepatocellular carcinoma through cutting the pathways of the common risk factors. Biomed Pharmacother. 2018; 107:1246–58. Epub 2018/09/28. https://doi.org/10.1016/j.biopha.2018.08.104 PMID: 30257339

44. Abdel-Hamid NM, Abass AA, Mohamed AA, Muneam Hamid D. Herbal management of hepatocellular carcinoma through cutting the pathways of the common risk factors. Biomed Pharmacother. 2018; 107:1246–58. Epub 2018/09/28. https://doi.org/10.1016/j.biopha.2018.08.104 PMID: 30257339
46. Caldero J, Ziol M, Paradis V, Zucman-Rossi J. Molecular and histological correlations in liver cancer. J Hepatol. 2019; 71(3):616–30. Epub 2019/06/14. https://doi.org/10.1016/j.jhep.2019.06.001 PMID: 31195064.

47. Song A, Eo W, Kim S, Shim B, Lee S. Significance of serum ferritin as a prognostic factor in advanced hepatobiliary cancer patients treated with Korean medicine: a retrospective cohort study. BMC Complement Altern Med. 2018; 18(1):176. Epub 2018/06/09. https://doi.org/10.1186/s12906-018-2240-7 PMID: 29879960.

48. Wu SJ, Zhang ZZ, Cheng NS, Xiong XZ, Yang L. Preoperative serum ferritin is an independent prognostic factor for liver cancer after hepatectomy. Surg Oncol. 2019; 29:159–67. Epub 2019/06/15. https://doi.org/10.1016/j.suronc.2019.05.013 PMID: 31196483.

49. Peng C, Ye Y, Wang Z, Guan L, Bao S, Li B, et al. Circulating microRNAs for the diagnosis of hepatocellular carcinoma. Dig Liver Dis. 2019; 51(5):621–31. Epub 2019/02/13. https://doi.org/10.1016/j.dld.2018.12.011 PMID: 30744930.

50. Dai M, Li L, Qin X. Clinical value of miRNA-122 in the diagnosis and prognosis of various types of cancer. Oncol Lett. 2019; 17(4):3919–29. Epub 2019/03/19. https://doi.org/10.3892/ol.2019.10024 PMID: 30881509.

51. Pan C, Wang X, Shi K, Zheng Y, Li J, Chen Y, et al. MiR-122 Reverses the Doxorubicin-Resistance in Hepatocellular Carcinoma Cells through Regulating the Tumor Metabolism. PLoS One. 2016; 11(5): e0152090. Epub 2016/05/04. https://doi.org/10.1371/journal.pone.0152090 PMID: 27138141.

52. Yokomichi N, Nishida N, Umeda Y, Taniguchi F, Yasui K, Toshima T, et al. Heterogeneity of Epigenetic and Epithelial Mesenchymal Transition Marks in Hepatocellular Carcinoma with Keratin 19 Proficiency. Liver Cancer. 2019; 8(4):239–54. Epub 2019/10/12. https://doi.org/10.1159/000490806 PMID: 31602368.

53. Oh HR, Jo HY, Park JS, Kim DE, Cho JY, Kim PH, et al. Galactosylated Liposomes for Targeted Co-Delivery of Doxorubicin/Vimentin siRNA to Hepatocellular Carcinoma. Nanomaterials (Basel). 2016; 6(8). Epub 2016/01/01. https://doi.org/10.3390/nano6080141 PMID: 28335269.

54. Wang N, Wang Q, Tang H, Zhang F, Zheng Y, Wang S, et al. Direct inhibition of ACTN4 by ellagic acid limits breast cancer metastasis via regulation of beta-catenin stabilization in cancer stem cells. J Exp Clin Cancer Res. 2017; 36(1):172. Epub 2017/12/05. https://doi.org/10.1186/s13046-017-0635-9 PMID: 29197410.

55. Strouhalova K, Prechova M, Gandalovicova A, Brabek J, Gregor M, Rosel D. Vimentin Intermediate Filaments as Potential Target for Cancer Treatment. Cancers (Basel). 2020; 12(1). Epub 2020/01/17. https://doi.org/10.3390/cancers12010184 PMID: 31940801.

56. Feng T, Dzieran J, Yuan X, Dropmann A, Maass T, Teufel A, et al. Hepatocyte-specific Smad7 deletion accelerates DEN-induced HCC via activation of STAT3 signaling in mice. Oncogenesis. 2017; 6(1): e294. Epub 2017/01/31. https://doi.org/10.1038/oncsis.2016.85 PMID: 28134936.

57. Thieringer FR, Maass T, Meyer E, Schirmacher P, Longeric H, et al. Liver-specific overexpression of matrix metalloproteinase 9 (MMP-9) in transgenic mice accelerates development of hepatocellular carcinoma. Mol Carcinog. 2012; 51(6):439–48. Epub 2011/06/18. https://doi.org/10.1002/mc.20809 PMID: 21681821.

58. Li T, Zhu Y, Han L, Ren W, Liu H, Qin C. VEGFR-1 activation-induced MMP-9-dependent invasion in hepatocellular carcinoma. Future Oncol. 2015; 11(23):3143–57. Epub 2015/11/10. https://doi.org/10.2217/fon.15.263 PMID: 26551737.

59. Ceci C, Lical PM, Tentori L, De Martino MG, Miano R, Graziani G. Experimental Evidence of the Antitumor, Antimetastatic and Antiangiogenic Activity of Ellagic Acid. Nutrients. 2018; 10(11). Epub 2018/11/18. https://doi.org/10.3390/nu10111756 PMID: 30441769.

60. Al-Malky HS, Osman AM, Damamhouri ZA, Alkreathy HM, Al Aama JY, Ramadan WS, et al. Modulation of doxorubicin-induced expression of the multidrug resistance gene in breast cancer cells by diltiazem and protection against cardiotoxicity in experimental animals. Cancer Cell Int. 2019; 19:191. Epub 2019/08/02. https://doi.org/10.1186/s12935-019-0912-0 PMID: 31367189.