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Original article

Bioguided isolation of potential antitumor agents from the aerial parts of cultivated cardoon (*Cynara cardunculus* var. *altilis*)

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the leading causes of mortality worldwide; therefore, searching for an effective treatment for this illness is of great importance. In the present work, *in vitro* cytotoxic activity of the ethanol extract of the aerial parts of *Cynara cardunculus* L. against human liver carcinoma cells (Hep G2) was tested. Additionally, the antitumor activity of the extract was confirmed using chemically induced rat liver carcinogenesis with diethylnitrosamine (DEN). Moreover, bioguided fractionation and column chromatographic separation of the active compounds were carried out. The extract of *C. cardunculus* showed a promising cytotoxic activity according to the protocols of the National Cancer Institute. Bioguided chromatographic separation of the ethanol extract of *C. cardunculus* led to the isolation of seven secondary metabolites including two sesquiterpene lactones as the principal active components of the methylene chloride soluble fraction, grosheimin (IC_{50} = 7.49 µg/mL) and cynaropicrin (IC_{50} = 13.9 µg/mL). The compounds were characterized by different spectroscopic techniques such as EI-MS, IR and NMR. Additionally, *in silico* analysis of the two active compounds revealed their ability to bind with caspase-3 via hydrogen bonds interactions to initiate apoptosis of cancer cells. The results shed the light on the significance of *C. cardunculus* as a potential source of antitumor agents.

1. Introduction

Persisting hepatitis infection, alcoholism, metabolic disorders, autoimmune diseases and liver tumors are the ultimate causes of growing incidence of acute and chronic liver failure (Fausto and Riehle. 2005). Liver cancer is the fifth most common cancer in the world and its incidence rate is two to three times higher in developing countries than developed countries (El-Serag. 2012; Organization. 2020).

The treatment of HCC remains a challenge, with one to three years survival rates. Despite the great revolution in HCC therapies, the outcomes are still limited (Befeler and Di Bisceglie. 2002). There are some obstacles to the implementation of these treatments as a loss of hepatic function. Moreover, most patients are diagnosed at late stages which reduce the response to the available treatments. Therefore, an urgent need exists for new therapies to ensure cancer patient live longer with better quality of life (Villanueva et al. 2013).

Asteraceae (ex Compositae) is the largest family of the flowering plants (Funk and Robinson. 2009). *Cynara cardunculus* var. *altitis*. "cultivated cardoon" is an edible Mediterranean plant with valuable medicinal applications; sometimes it is used for ornamental purposes and allelopathic potential against cosmopolitan weed species (Pandino and Mauromicale. 2019). The plant is rich in phenolic acids, flavonoids, inulin and anthocyanins (Pandino et al. 2022). According to literature, the methanol extract of the plant flowers showed remarkable antitumor activity in mouse skin carcinogenesis (Yasukawa et al. 2010). Previous screening of cardoon revealed that the lipophilic leaves and florets extracts, and their

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major compounds, namely cynaropicrin and taraxasteryl acetate had antiproliferative effects on triple-negative breast cancer (TNBC) (Ramos-Silva et al. 2017). Moreover, lupeol and pseudotaraxasterol, isolated from *C. cardunculus*, showed moderate cytotoxic activities against HeLa and MCF-7 cell lines (Ramos et al. 2014). Another report demonstrated that the defatted methanolic extract of edible parts of *Cynara cardunculus* var. *scolymus* (globe artichoke) reduced liver tissue lesions when damaged by Thioacetamide.

HCC can be induced experimentally by several models, one of these methods is a chemical induction using diethylnitrosamine (DEN) (Chakraborty et al. 2007). DEN is known for its potent hepatotoxic and carcinogenic properties which upon administration produces reproducible tumors and thus, it becomes a recommended model for the induction of HCC in experimental animals (Tolba et al. 2015). DEN is converted to its reactive ethyl metabolites that ethylate N7 atom of guanines in DNA (Verna et al. 1996). Additionally, these metabolites cause oxidative stress that contributes to cytotoxicity, mutagenicity and carcinogenicity. The damage induced by DEN starts by fibrosis which proceeds to cirrhosis and HCC (Qi et al. 2008). Signs of DEN induced liver injury are elevated serum aminotransferase activities and increased levels of the tumor marker serum α-fetoprotein (AFP). Besides, the histological changes which are characterized by prominent neutrophilic infiltration, extensive centrilobular necrosis, fibrosis and bridging of fibrosis (Tolba et al. 2015).

The current study aimed to evaluate the antitumor activity of the ethanolic extract of *C. cardunculus* var. *altilis* when administered after HCC induction in rats. The isolation of the cytotoxic components from the extract is another objective of this study. This was achieved by examining the cytotoxic activity of each fraction and to choose the most active fraction to proceed with further purification of individual compounds of that fraction (bioguided fractionation and chromatographic separation). Additionally the study included *in silico* analysis of the bioactive compounds to predict their mechanism of action.

2. Materials and methods

2.1. Plant material

Aerial parts of *C. cardunculus* var. *altilis*, including leaves and floral stems, were collected in September during the flowering period from Faculty of Agriculture, Mushtuhur, Benha University. The collected plant was identified by Dr. Ahmad Saeed, Prof. of Plant Horticulture, Faculty of Agriculture, Mushtuhur, Benha University, Egypt. Reference voucher specimens (CC10) were deposited in Pharmacognosy Department herbarium at Faculty of Pharmacy, Zagazig University, Egypt.

2.2. Extraction of plant material

Three kilograms of fresh aerial parts of *C. cardunculus* were extracted by cold maceration at room temperature using 80 % ethanol till complete exhaustion (3 × 10 L). The combined extracts were evaporated under reduced pressure at 45–50 °C to afford 200 g of total extract. The extract of *C. cardunculus* was successively fractionated with petroleum ether (60–80 °C) then methylene chloride and finally with ethyl acetate, (3 × 6 L) for each. The three soluble fractions were separately dried over anhydrous sodium sulphate and concentrated under reduced pressure at 45–50 °C to afford a greenish brown viscous residue of 30 g, a brown viscous residue of 13 g and a dark orange viscous residue of 20 g, for petroleum ether, methylene chloride and ethyl acetate, respectively.

Samples of the ethanol extract and its fractions (20 mg each) were screened for their cytotoxicity against Hep G2 cell line using MTT-assay.

2.3. Cytotoxic activity

Human hepatocellular carcinoma (Hep G2), obtained from VACSERA Tissue Culture Unit, were used to evaluate the cytotoxic effect of the different extracts, fractions and isolated compounds using cell viability MTT assay (Gomha et al. 2015; Mosmann. 1983). Percentages of relative viability as well as 50 % inhibitory concentrations (IC_{50}) were calculated for the cell line.

Linear regression was performed for the calculation of IC_{50} in case of *in vitro* assay. The Microsoft EXCEL 365 program was used for data analysis and to draw the figures. Experiments were performed in triplicates and data were presented as mean ± SD.

2.4. In vivo antitumor study

2.4.1. Animals

Male Wister rats weighing 100–150 g were obtained from the Modern Veterinary Office for Laboratory Animals, Giza, Egypt. The animals were housed in plastic cages and left to acclimatize for one week at the animal facility of the Faculty of Pharmacy, Cairo University (Egypt). Rats were kept under constant temperature (23 ± 2 °C) and a 12-hour light/dark cycle as well as constant relative humidity throughout the experimental period.

All animals were allowed free access to a standard diet and water and libitum during the investigation period. The experiment complies with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 2011) and was approved by the Ethics Committee for Animal Experimentation at Faculty of Pharmacy, Cairo University PT(2986).

2.4.2. Preparation of plant extract and drugs

The dried ethanol extract of aerial parts of *C. cardunculus* was suspended in 0.5 % Tween-80 solution (ADWIC, Egypt). The concentrations were adjusted according to the rats' weights so that each 0.1 mL of the prepared suspension contains 2 mg of the plant extract. The dose selected for the present work was 150 mg/kg body weight. DEN (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) was prepared as a solution in normal saline and injected intraperitoneally.

2.4.3. Experimental design and samples collection

The antitumor activity of *C. cardunculus* extract using chemically-induced rat liver carcinogenesis with diethylnitrosamine (DEN) was studied as previously described by authors. (Elgazar et al. 2022).

Briefly, after a period of adaptation, animals were allocated into three groups of 15 animals each. The first group represents the negative control and included rats that received saline by i.p. injection, five times weekly for 6 consecutive weeks. The second group (DEN control) received DEN (20 mg/kg) five times weekly for 6 consecutive weeks to induce HCC. The third group received DEN (20 mg/kg) five times weekly for 6 consecutive weeks to induce HCC followed by administration of *C. cardunculus* extract (150 mg/kg; p.o.) daily starting from the 6th week and till the 10th week.

At the end of experimental period (10 weeks), blood and liver samples were collected and rapidly frozen at −20 °C. Alanine transaminase (ALT) and Aspartate transaminase (AST) activities were measured in blood samples. Additionally, 10 % tissue homogenates were prepared from liver samples for the estimation of liver α-fetoprotein while the other part was fixed in 10 % formalin

for 24 h, specimens were processed by paraffin embedding and 5 μm sections were prepared for histopathological examination.

2.4.4. Liver index calculation

The liver index was calculated using the following formula: (liver weight/body weight × 100).

2.4.5. Biochemical determinations

Liver function tests: alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were assessed using Biodiagnostic kits (Egypt). Procedures were performed according to manufacturer's instructions and results were expressed as U/l.

Alpha-fetoprotein (AFP): Hepatic AFP content was assessed using a rat AFP ELISA kit (eBioscience, San Diego, USA). The procedure of the used kit was performed according to the manufacturer's instructions, and the results were expressed as ng/mg protein.

2.4.6. Histopathological examinations

Parts of the liver were obtained from different levels (base, middle and apex), rinsed in ice-cold saline and immediately fixed in 10 % formalin for 24 h. Specimens were then processed for paraffin embedding.

2.4.7. Statistical analysis

Data were expressed as means ± standard error (SEM) and percentage of normal. Analysis of the results was done using one-way-analysis of variance test (Lota et al. 2001) followed by Tukey's post-hoc multiple comparison's test. GraphPadPrism® software package, version 6 (GraphPad Software, Inc., USA) was used to carry out all statistical tests.

2.5. Bioguided column chromatography for isolation

2.5.1. Bioguided column chromatographic isolation of petroleum ether soluble fraction of C. Cardunculus

About 28 g of petroleum ether soluble fraction was subjected to silica gel 60 (0.063–0.200 mm, Merck) column chromatography (80 × 5 cm, 950 g) packed in petroleum ether; the polarity was increased gradually using methylene chloride then methanol. Resulted fractions were examined by TLC (silica gel precoated, kieselgel 60 F254, silica 0.25 mm, Germany) using solvent systems (Petroleum ether: Ethyl acetate, 10:0.25), (Petroleum ether: Ethyl acetate, 10:0.5), (Petroleum ether: Ethyl acetate, 10:0.75) and (Ethyl acetate, 100 %). Developed chromatograms were sprayed with anisaldehyde/sulphuric acid reagent. The similar fractions were pooled, crystallized to afford five compounds, **1** (0.3 g), **2** (1 g), **3** (0.5 g), **4** (0.9 g), and **5** (0.85 g).

For all the isolated compounds, melting point (electro-thermal ltd, England), IR (FT/IR-6100typeA (JASCO- Germany), EI-MS (ISQ LT, USA), ^{1}H - and ^{13}C- NMR analyses (Bruker, Switzerland; 100 and 400 Hz, respectively) were performed.

2.5.2. Bioguided column chromatographic isolation of methylene chloride soluble fraction of C. cardunculus

About 12 g of methylene chloride soluble fraction was subjected to silica gel 60 (0.063 – 0.200 mm, Merck) column chromatography (2.5 × 80 cm, 320 g) packed in petroleum ether; the polarity was increased gradually using methylene chloride then methanol. Resulted fractions were examined by TLC (silica gel precoated, kieselgel 60 F254, silica 0.25 mm, Germany) using solvent system (Methylene chloride: methanol, 9:1). The similar fractions were pooled, crystallized to afford two compounds, **6** (0.2 g) and **7** (0.3 g),

2.6. Molecular docking study

Molecular docking for the active compounds was performed using Molecular Operating Environment 2009 (MOE) as previously described (Nagah et al. 2021). Briefly, the compounds were constructed in 3D structure; their energies were minimized and saved to MDB file. Caspase-3 (responsible for Hep G2 apoptosis) X-ray crystallographic structure coded as 2 J30 was downloaded from the Protein Data Bank (R.P.D. Bank, RCSB PDB: Homepage, 2020. https://www.rcsb.org/ (accessed 2 December 2020). Hydrogens were added to the protein structure and missed connections and their types were corrected automatically. The receptor and its atoms were fixed. The active site of the enzyme was determined based on a co-downloaded natural ligand using surfaces and maps.

The 3D constructed compounds were docked against caspase-3 using the following parameters: placement, triangle matcher; scoring, London dG with ten retains; refinement, forcefield. The resulted poses were investigated based on their energy, root-mean square deviation (rmsd) and formed interactions (bonds).

3. Results

In this study, the dried ethanol extract of *Cynara cardunculus* var. *altitis* was screened for its cytotoxic activities against Hep G2 cell line using MTT assay. The extract showed strong cytotoxic activity against Hep G2 cell lines with IC_{50} = 14.7 μg/mL. Consequently, *C. cardunculus* was subjected to detailed phytochemical and biological investigation.

3.1. In vivo antitumor study:

The high cytotoxic activity of *C. cardunculus* extract and some fractions and components against Hep G2 cells encouraged us to examine the extract effect deeply using *in vivo* model.

3.1.1. Effect of C. cardunculus on liver integrity and plasma aminotransferases activities in DEN-induced liver HCC in rats.

Following DEN (20 mg/kg, i.p.) administration, HCC was manifested as an increase in liver index (liver weight/ body weight %) as well as an elevation in plasma ALT and AST levels. On the other hand, treatment with *C. cardunculus* (150 mg/kg/day, p.o.) succeeded to decrease liver index and suppress the plasma levels of ALT and AST when compared to DEN group (Tables 1-2 and Fig. 1).

3.1.2. Effect of C. cardunculus on liver alpha-fetoprotein content in rats with DEN-induced HCC

Induction of HCC by DEN (20 mg/kg, i.p.) significantly elevated the liver contents of AFP. Administration of *C. cardunculus* (150 mg/kg/day, p.o.) to diseased rats significantly reduced the AFP content in liver compared to the DEN group (Table 3 and Fig. 1).

3.1.3. Effect of C. cardunculus on hepatic histological changes in rats with DEN-induced HCC

Normal control samples demonstrated normal histological features of rat liver parenchyma with many apparent intact well organized hepatocellular architecture with intact subcellular details (arrow), intact hepatic vasculatures (star) as well as hepatic sinusoids (Fig. 2). Induction of HCC by DEN (20 mg/kg, i.p.) showed many records of multiple areas of focal hepatocellular alteration and dysplasia with significant cytomegally, hyperchromatic binucleated cells with prominent nucleoli and many mitotic figures (red arrow), accompanied with moderate dilatation of hepatic blood vessels (black star) and hyperplasia of bile ducts (red star). Abundant mononuclear inflammatory cells infiltrate in perivascular areas (yellow arrow). Significant fibroblastic activity was

Table 1
Effect of *C. cardunculus* ethanol extract on liver index in rats with DEN-induced hepatocellular carcinoma (HCC).

Groups	Liver index (%)
Normal	2.86 ± 0.17
DEN (20 mg/kg)	5.23 ± 0.24*
DEN + CC (150 mg/kg)	3.73 ± 0.12*#

Each value represents the mean of 5 experiments ± S.E.M. Statistical analysis was done using One way ANOVA followed by Tukey's post-hoc test. *$p < 0.05$ *vs* normal, #$p < 0.05$ *vs* DEN. CC: *C. cardunculus*; DEN: diethylnitrosamine.

Table 2
Effect of *C. cardunculus* extract on plasma aminotransferases activities in rats with DEN-induced HCC.

Groups	ALT (U/l)	AST (U/l)
Normal	23.00 ± 1.15	34.67 ± 4.33
DEN (20 mg/kg)	121.00 ± 1.15*	135.00 ± 5.03*
DEN + CC (150 mg/kg)	64.33 ± 4.05*#	61.00 ± 2.08*#

Each value represents the mean of 5 experiments ± S.E.M. Statistical analysis was done using One way ANOVA followed by Tukey's post-hoc test. *$p < 0.05$ *vs* normal, #$p < 0.05$ *vs* DEN. CC: *C. cardunculus*; DEN: diethylnitrosamine.

observed with moderate pseudolobulation of hepatic lobules by newly formed collagen fibers. Administration of *C. cardunculus* (150 mg/kg/day) showed significant decrease of focal areas of hepatocellular alteration sizes with persistence hepatocellular cytomaegally records as model samples with vacuolar degenerative changes records (red arrow). Moderate dilatation and congestion of hepatic blood vessels (black star).

Table 3
Effect of *C. cardunculus* extract on alpha-fetoprotein liver content in livers of rats with DEN-induced HCC.

Groups	Alpha-fetoprotein (ng/mg protein)
Normal	6.40 ± 0.57
DEN (20 mg/kg)	18.37 ± 0.86*
DEN + CC (150 mg/kg)	12.93 ± 0.50*#

Each value represents the mean of 5 experiments ± S.E.M. Statistical analysis was done using One way ANOVA followed by Tukey's post-hoc test. *$p < 0.05$ *vs* normal, #$p < 0.05$ *vs* DEN. CC: *C. cardunculus* and DEN: diethylnitrosamine.

3.2. Bioguided fractionation and column chromatography isolation of active constituent from C. cardunculus extract

C. cardunculus extract was subjected to fractionation using petroleum ether, methylene chloride and ethyl acetate. The fractionation procedure and cytotoxicity of resulted fractions against Hep G2 cell line are shown in scheme 1. According to the protocols of the National Cancer Institute NCI (Ateya et al. 2014; Schmitz et al. 1993), the petroleum ether and methylene chloride extracts of *C. cardunculus* were considered as active fractions against Hep G2 cell line with IC_{50} 18.1 and 25.3 µg/mL, respectively. So, column chromatographic separation was carried out for the components of these fractions.

3.2.1. Structural elucidation of isolated compounds from petroleum ether soluble fraction of C. cardunculus

Column chromatographic separation of petroleum ether soluble fraction resulted in the isolation of five compounds. The isolated compounds were identified as lupeol acetate (**1**), taraxasteryl acetate (**2**), pseudotaraxasterol (**3**), β-sitosterol (**4**) and β-sitosterol glucoside (**5**) (Fig. 3).

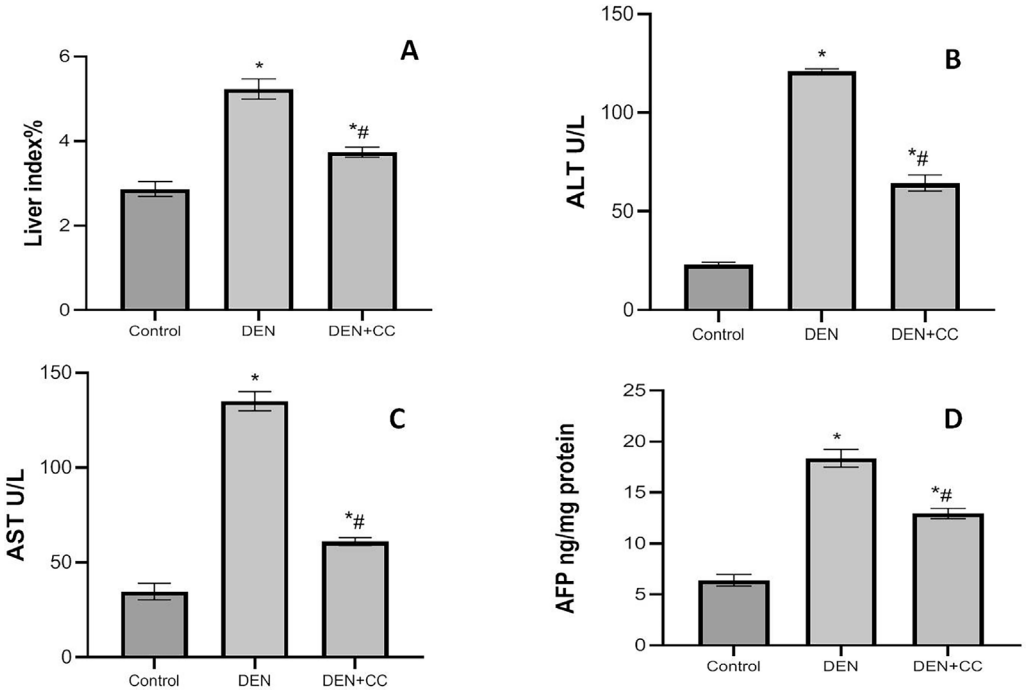


Fig. 1. Effect of *C. cardunculus* extract on liver index and some biochemical markers in rats with DEN-induced HCC. DEN (20 mg/kg, i.p.) was injected five times weekly for 6 consecutive weeks to induce HCC. *C. cardunculus* extract was administered daily from the 6th to the 10th week in a dose of 150 mg/kg o.p. Each value represents the mean of 5 experiments ± S.E.M. Statistical analysis was done using One-way ANOVA followed by Tukey's post-hoc test. A: Effect on liver index; B and C: Effect on plasma aminotransferases activities; D: Effect on alpha-fetoprotein liver content.*p < 0.05 *vs* normal, #p < 0.05 *vs* DEN; CC: *C. cardunculus*.

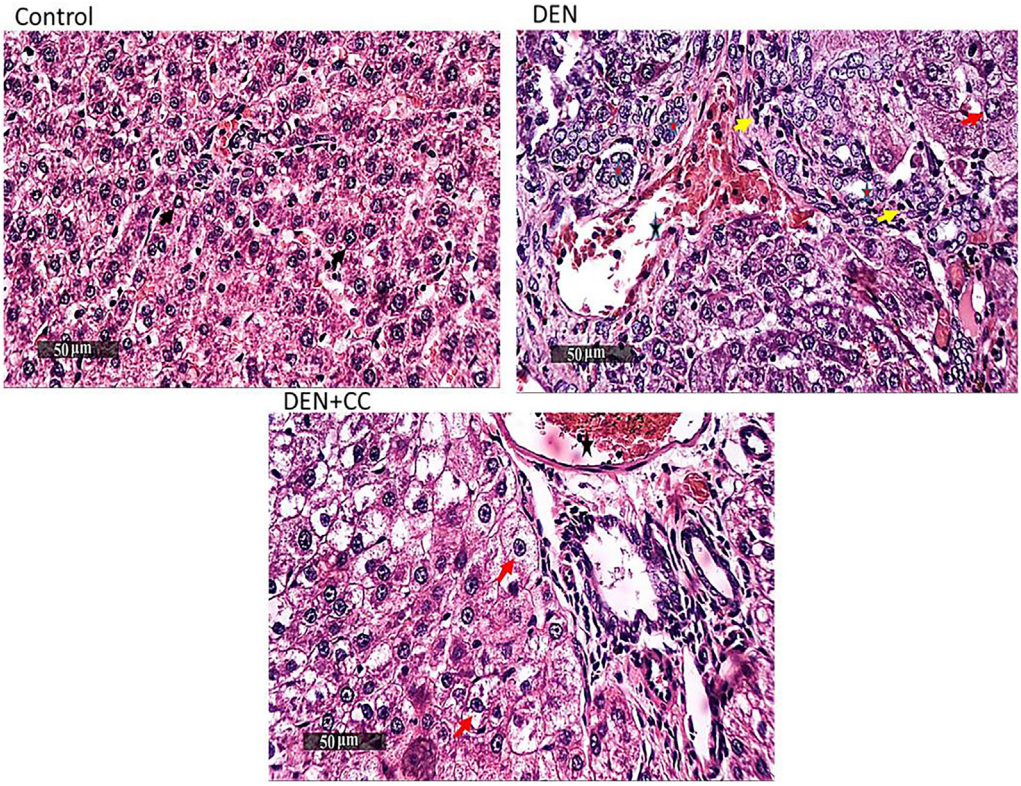
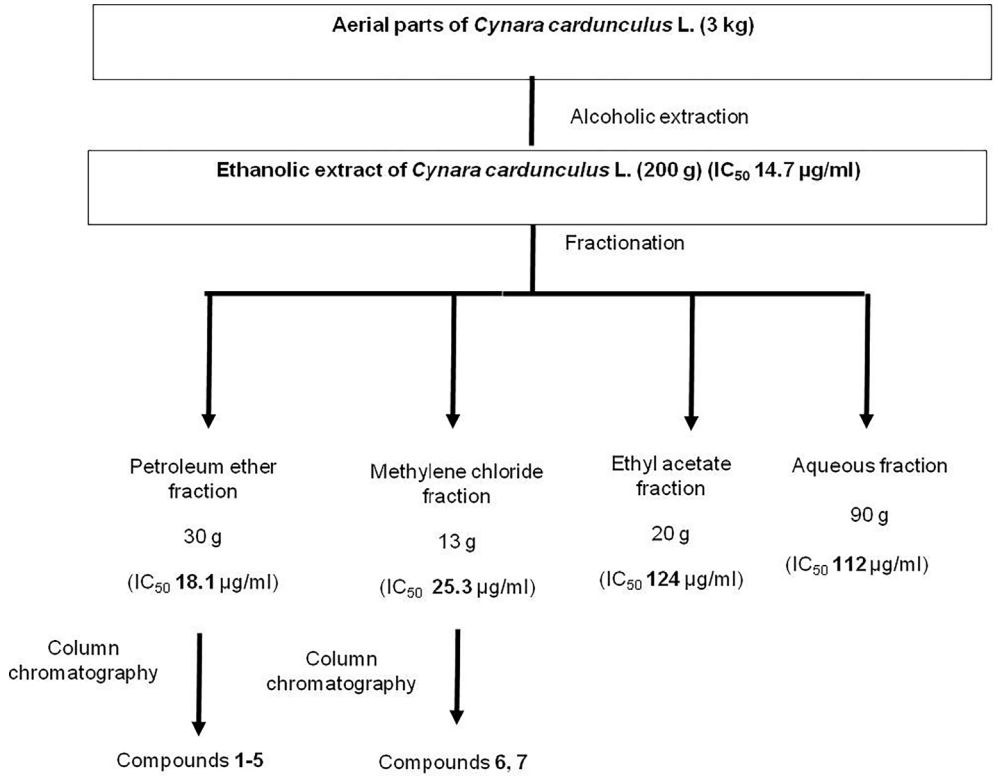


Fig. 2. Effect of *C. cardunculus* on hepatic histological changes in rats with DEN-induced HCC. (H and E staining) (Magnification x40). CC: *C. cardunculus*; DEN: diethylnitrosamine.



Scheme 1. Extraction and bioguided fractionation of *C. cardunculus.*

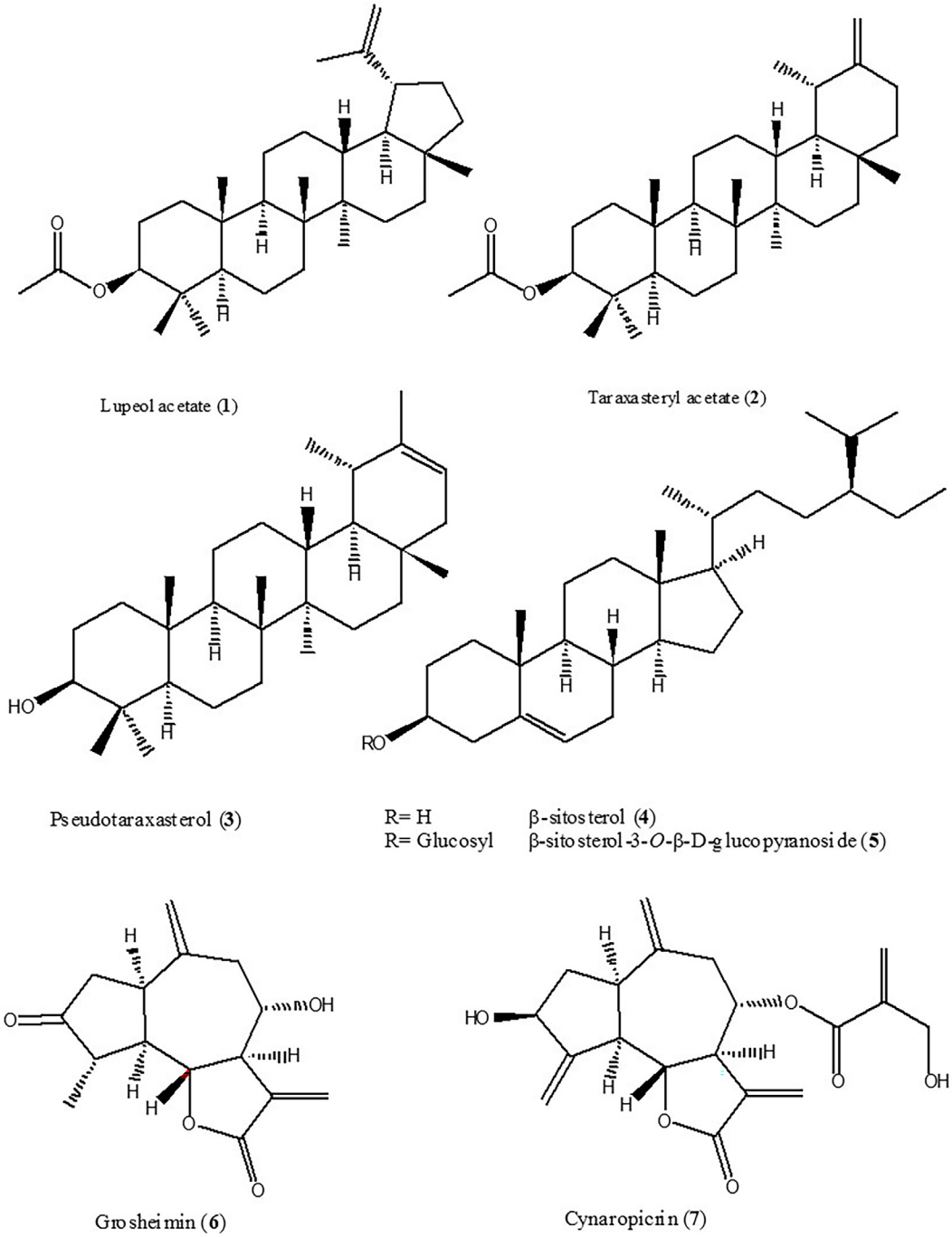


Fig. 3. Isolated secondary metabolites from *C. cardunculus* active fractions.

Lupeol acetate (**1**) m.p. 216–218 °C. ^{1}H NMR (δ, $CDCl_3$, 400 MHz): 1.57 (m, 2H), 1.55 (m,2H), 4.49 (dd,1H), 0.79 (m), 1.50 (m), 1.29 (m), 1.28 (m), 1.50 (m), 1.58 (m), 0.98 (m), 1.66 (m), 1.44 (m), 1.29 (m), 2.36 (m), 1.20 (m), 2.40 (m), 0.87 (s), 0.81 (s), 0.84 (s), 1.03(s), 0.92 (s), 0.78 (s), 4.57 (s), 4.68 (s), 1.64 (s), 2.18. ^{13}C NMR(δ, $CDCl_3$, 100 MHz): 38.14, 23.51, 80.01, 38.13, 55.49, 19.42, 34.79, 40.13, 50.69, 37.44, 21.15, 25.83, 38.38, 43.96, 27.49, 35.47, 43.12, 48.74, 48.07, 150.69, 30.43, 40.13, 28.18, 16.49, 16.18, 14.85, 18.13, 19,45, 110.52, 19.34, 20.91, 174.58. IR spectrum: (ν_{max} KBr cm^{-1}): 2921and 2852 (–CH2), 1730 (C═O), 1620 (C═C) and 1462 (C—O). EI-Mass spectrum: *m/z* (% relative abundance): 468 (M^+) (14), 408(12), 204(13), 190(40) and 189 (50), in comparison with the reported data of lupeol acetate (Yam-Puc et al. 2013) are shown in supplementary data Figures S. (1–4).

Taraxasteryl acetate (**2**) m.p. 256–257 °C. ^{1}H NMR (δ, $CDCl_3$, 400 MHz): 1.66, 0.97 (m, 2H), 1.69,1.68 (m,2H), 4.52 (t, 1H), 0.87 (t, 1H), 1.63,1.32 (t, 2H), 1.32, 1.3 5 (t, 2H), 1.32 (t, 1H), 1.48, 1.21 (m, 2H),1.63, 1.08 (m,2H),1.56 (m, 1H), 1.63, 0.94 (t, 2H), 1.32, 1.17 (t, 2H), 0.99 (m,1H), 2.15 (m,1H), 2.34, 2.23 (m, 2H), 41,1.41(m, 2H), 0.87 (s, 3H), 0.92 (s, 3H), 0.87(s, 3H), 0.97 (s, 3H), 0.94 (s, 3H), 0.99 (s, 3H), 1.04 (s, 3H),4.60, 4.52 (d, 2H), 2.08 (s, 3H). ^{13}C NMR (δ, $CDCl_3$, 100 MHz): 39.00, 23.82, 81.12, 38.57, 55.52, 18.23, 34.08, 40.98, 50.48, 37.18, 21.41, 25.75, 39.52, 42.17, 26.78, 39.00, 34.66, 48.78, 37.94, 154.8, 25.62, 39.29, 28.07, 16.02, 15.94, 16.47, 14.86, 19.6, 26.28, 107.28, 21.59,

171.17. IR absorption: (ν_{max} KBr cm^{-1}): 2926 and 2855 (–CH2), 1727 (C=O), 1641 (C=C) and 1451 (C—H bending). EI-Mass spectrum: *m/z* (% relative abundance): (M^+) 486 (56), 316 (15), 258 (12), and 189 (90) (Khalilov et al. 2003) are shown in supplementary data Figures S. (5–8).

Pseudotaraxasterol (**3**) m.p. 217–219 °C. ^{1}H NMR (δ, $CDCl_3$, 400 MHz): 3.12 (1H, m), 5.27(1H, d, *J* = 8 Hz), 0.95 (3H, s), 1.74 (3H, s), 0.88 (3H, s), 1.13(3H, s), 0.97 (3H, s), 0.70 (3H, s), 0.95 (3H, d, *J* = 6.2 Hz), 1.59 (3H, s). ^{13}C NMR (δ, $CDCl_3$, 100 MHz): 39.01, 27.5, 79.1, 38.9, 55.4, 18.4, 34.3, 42.3, 50.5, 37.2, 21.7, 27.5, 39.3, 42.4, 27.1, 36.8, 34.5, 48.8, 36.4, 140, 119, 42.3, 28.04, 15.5, 16.1, 16.4, 14.8, 17.8, 22.6, 21.7. IR spectrum: (ν_{max} KBr cm^{-1}): 3300 (OH), 2918 and 2853 (–CH2), 1641 (C=C) and 1452 (C—O). EI-Mass spectrum: *m/z* (% relative abundance): 426 (M^+) (30), 357(15), 316(10), 257(20), 189 (80) (Ma et al. 2008) are shown in supplementary data Figures S. (9–12).

β-sitosterol (**4**) m.p. 136–140 °C. ^{1}H NMR (δ, $CDCl_3$, 400 MHz): 1.18 (m, 2H), 1.25 (m, 2H), 3.51 (m, 1H), 2.30 (m, 2H), 5.34 (bs, 1H),1.67 (m, 2H),1.23 (m,1H), 1.25 (m, 1H), 1.45(m, 2H), 1.51 (m, 2H), 1.23 (m, 2H), 1.83 (m, 2H), 1.99 (m, 2H), 1.79 (m, 2H), 0.65 (s, 3H), 0.91 (s, 3H), 1.45 (m, 1H), 0.80 (d, 3H,), 1.79 (m, 2H), 1.78 (m, 2H), 1.13 (m, 1H), 2.23 (m, 1H), 0.76 (d, 3H, *J* = 7.5 Hz), 0.75 (d, 3H, *J* = 1.7 Hz), 1.25 (m, 2H), 0.91 (t, 3H, *J* = 6.8 Hz). ^{13}C NMR (δ, $CDCl_3$, 100 MHz): 37.30, 29.74, 71.37, 42.33, 140.93, 121.70,31.84, 31.90, 50.07, 36.69, 21.07, 38.79, 42.33, 56.65, 24.34, 28.27, 55.90, 12.15, 19.50, 35.95, 20.19, 33.81, 25.94, 45.61, 29.17, 19.14, 19.14, 23.07, 12.26. IR spectrum absorption: (ν_{max} KBr cm^{-1}): 3420 (—OH), 2933 and 2864 (—CH2), 1641 (C=C) and 1463 (C—O). EI-Mass spectrum: *m/z* (% relative abundance): 414 and peak at *m/z* 399 ($M-CH_3$) for molecular formula $C_{29}H_{50}O$. Other fragments at *m/z* 396 (M^+- H_2O), 273, and 231 (Khatun et al. 2012) are shown in supplementary data Figures S. (13–16).

β-sitosterol glucoside (**5**) m.p. 272–274 °C. ^{1}H NMR (δ, $CDCl_3$ and CD_3OD, 400 MHz):1.46 (m, 2H), 1.49 (m, 2H), 3.06 (m, 1H), 2.88 (m, 2H), 5.46 (bs, 1H), 1.78 (m, 2H), 1.23 (m, 1H), 1.25 (m, 1H), 1.49 (m, 2H), 1.46 (m, 2H), 1.23 (m, 2H), 1.80 (m, 2H), 1.78 (m, 2H), 1.79 (m, 2H), 0.65 (s, 3H), 0.93 (s, 3H), 1.46(m, 1H), 0.85 (d, 3H, *J* = 6.5 Hz), 1.79 (m, 2H), 1.78 (m, 2H), 1.13 (m, 1H), 2.88 (m, 1H), 0.76 (d, 3H, *J* = 7.5 Hz), 0.75 (d, 3H, *J* = 1.7 Hz), 1.25 (m, 2H), 0.76 (t, 3H, *J* = 6.8 Hz), 4.85 (d, 1H, *J* = 7.9 Hz), 3.06 (m, 1H), 3.06 (m, 1H), 3.14 (m, 1H), 3.06 (m, 1H), 2.88 (m, 1H). ^{13}C NMR (δ, $CDCl_3$ and CD_3OD, 100 MHz): 37.30, 29.72, 70.51, 42.33, 140.93, 121.70, 31.84, 31.90, 50.07, 36.69, 21.07, 38.78, 42.33, 56.65, 24.34, 28.27, 55.90, 12.15, 19.58, 33.81, 33.81, 25.90, 45.61, 29.17, 19.09, 19.35, 23.07, 12.26, 101.24, 73.91, 77.24, 70.58, 77.24, 61.58. IR spectrum: (ν_{max} KBr cm^{-1}): 3420 (—OH), 2933 and 2864 (—CH2), 1641 (C=C) and 1463 (C—O). EI-Mass spectrum: *m/z* (% relative abundance): (M^+–glucose), 414 (20 %), 369 (100), and 273 (20 %) (Khatun et al. 2012) are shown in supplementary data Figures S. (17–20).

3.2.2. Structural elucidation of isolated compounds from methylene chloride soluble fraction of C. cardunculus

Column chromatographic separation of methylene chloride soluble fraction resulted in isolation of two compounds. The isolated compounds were identified as grosheimin (**6**) and cynaropicrin (**7**) (Fig. 3).

Grosheimin (**6**) m.p. 193–195 °C. ^{1}H NMR (δ, $CDCl_3$, 400 MHz): (3.25, m), (2.57, m), (2.36, m), (2.32, m), 4.11 (dd, *J* = 9.3, 8.9 Hz), 3.31 (dd, *J* = 9.1, 3.4 Hz), 3.80 (dd, *J* = 9.8), 5.8, 9.0 Hz, 2.83 (dd, *J* = 5.7,13 Hz), 2.3,m, 6.32 (dd, *J* = 3.1, 1,0 Hz), 6.26 (dd, *J* = 3.0, 1,2 Hz), 5.08bs, 4.80 bs, 1.24 (d, *J* = 7.0 Hz). ^{13}C NMR (δ, $CDCl_3$, 100 MHz): 39.98, 42.90, 218.92, 46.74, 50.73, 83.20, 50.70, 72.64, 47.89, 144.44, 137.65, 171.64, 123.96, 113.93, 13.80. IR spectrum absorption: (ν_{max} KBr cm^{-1}): 3473 (–OH), 3084 (C=C–H), 2933 (–CH2), 1737 (C=O), 1644 (C=C) and 1444 (C—O). EI-Mass spectrum: *m/z* (% relative abundance): 262 (M^+) (19), 245(90), 227 (100), 199 (6) and 181 (10) (Yayli et al. 2006) are shown in supplementary data Figures S. (21–24).

Cynaropicrin (**7**) m.p. 130–133 °C ^{1}H NMR (δ, $CDCl_3$, 400 MHz): (2.88, m), (2.18, m), (1.77,dt), (4.50,dd), 2.88,m, (4.29, dd, *J* = 9.3, 9 Hz), (3.28,m), (5.35,m), (2.43, m),(2.72, m), (6.22, d, *J* = 3.4 Hz), (5.66, d, *J* = 3.0 Hz), (5.17bs), (4,83 bs), (5.41, bs. 5.38 Hz), 6.33bs, 5.99 bs, 4.64bs. ^{13}C NMR (δ, $CDCl_3$, 100 MHz): 47.06, 38.96, 72.72, 152.63, 48.06, 78.93, 47.85, 74.26, 36.30, 138.28, 140.50, 169.71, 121.07, 116.79, 111.38, 165.19, 142.63, 124.6, 62.5. IR spectrum: (ν_{max} KBr cm^{-1}): 3423 (–OH), 2928 (–CH2), 1762 (C=O), 1642 (C=C) and 1445 (C—O). EI-Mass spectrum: *m/z* (% relative abundance): 347 ($C_{19}H_{22}O_6$) (M^+ +1) (19), 329 ($M-H_2O$), 227 (100), 245 (90), 199 (6), 181(10) and 171(10) (Yayli et al. 2006) are shown in supplementary data Figures S. (25–28).

3.3. Cytotoxic activity of isolated components against Hep G2 cell line

The isolated compounds from petroleum ether soluble fraction were examined for their cytotoxicity against Hep G2 cell line. These compounds showed weak or no activity upon comparison with cisplatin and vinblastine, (IC_{50} of 26.8, 180, 111, 61.6 and 53.6 μg/mL for compounds 1–5, respectively), reflecting that the activity of total petroleum ether fraction is attributed to the synergistic effect of its components. On the other hand, the two compounds isolated from methylene chloride soluble fraction exhibited cytotoxic activities against Hep G2 cell line with IC_{50} values (7.49 and 13.9 μg/mL, for **6** and **7**, respectively). Cell viability percentages of different concentrations of isolated compounds from *C. cardunculus* relative to cisplatin and vinblastine standards against Hep G2 cell lines are shown in Fig. 4.

3.4. Mechanism of action of Hep G2 cytotoxic components based on molecular docking study

Caspasa-3 represents one of the main enzymes that initiate apoptosis in cancer cells including Hep G2 (Dong et al. 2018). Using molecular docking study, we aimed to postulate the mechanism by which *C. cardunculus* potent compounds can stimulate caspase-3 and consequently initiate apoptosis of Hep G2. The results showed that the docked compounds were able to form interactions with amino acids responsible for caspase-3 activity through hydrogen bond formation. Cynaropicrin was able to form hydrogen bonds with Thr62, Arg207 and Arg64 with bonding energy (-13.4211), while grosheimin formed only one hydrogen bond with Arg207 (Binding energy: −11.8934) (Fig. 5). These results came in agreement with previous studies on the role of cyanopicrin in the inhibition of cell proliferation throughout caspase-3-dependent apoptosis (De Cicco et al. 2021; Li et al. 2021).

4. Discussion

HCC is a major health concern that could threaten or even end the patient's life. Efforts are made to discover a treatment for HCC to decrease the high mortality rate of the available treatments. In the present study, the effect of *C. cardunculus* was investigated to determine if it could afford any antitumor effect when administered after HCC induction. The results demonstrated that treatment with *C. cardunculus* for four weeks provided a significant chemotherapeutic activity against HCC induced by DEN in rats.

In this work, DEN was used to induce HCC. DEN is an excellent experimental model of chemically-induced HCC (Verna et al. 1996). It is a hepatotoxin which is biotransformed by cytochrome P450 enzymes into alkylating metabolites that affect the DNA and

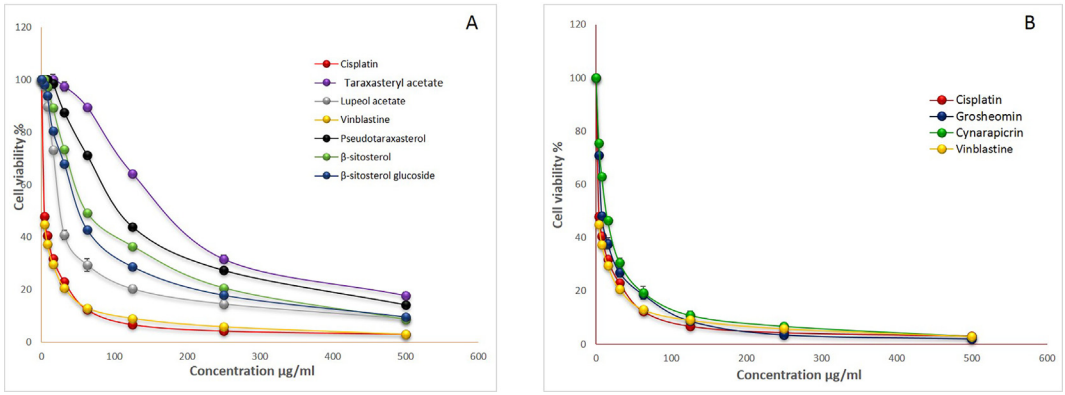


Fig. 4. Cell viability percentage of isolated compounds from *C. cardunculus* compared to cisplatin and vinblastine standards against Hep G2 cell lines. A: compounds from petroleum ether soluble fraction; B: compounds from methylene chloride soluble fraction.

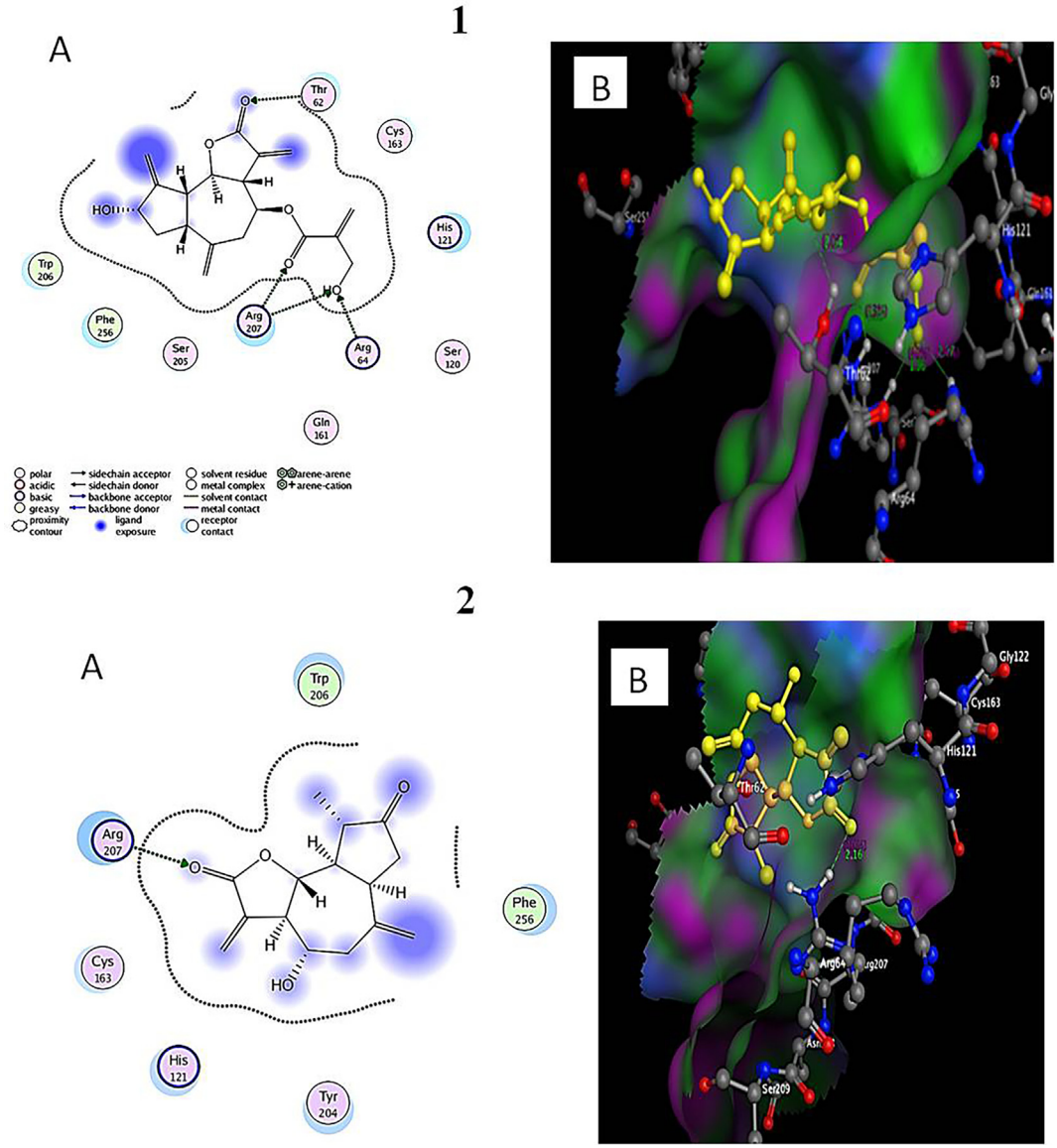


Fig. 5. Molecular docking of active compounds to caspase-3 using MOE, 1: cynaropicrin; 2: grosheimin; A: two-dimensional; B: three-dimensional.

start the onset of the toxic effects (Tolba et al. 2015). DEN is known to induce oxidative stress which produces liver injury and damages the hepatocytes (Qi et al. 2008) as demonstrated in the current study by the elevation in plasma ALT and AST levels. Moreover, in the present work, DEN long term administration significantly increased the tumor serum AFP which supports the hepatocarcinogenic effects of DEN as an experimental model for HCC. These results are in agreement with other studies regarding the hepatocarcinogenic effect of the used DEN dose in rats (Ahmed et al. 2013). DEN administration causes a decrease in the final body weight and a significant increase in liver index, an effect that could be attributed to the hepatotoxic and hepatocarcinogenic effects of DEN (Sayed-Ahmed et al. 2010).

The effect of *C. cardunculus* extract on the aforementioned trajectory was counter-intuitive. ALT, AST and AFP, widely used in animal studies to diagnose and monitor the development of HCC (Liu et al. 2006). The increase in serum levels of ALT, AST and AFP indicated that DEN may induce acute liver injury. As seen from the results, treatment with *C. cardunculus* extract significantly reduced serum ALT, AST levels compared to DEN-treated rats, which suggests that *C. cardunculus* extract could trigger a promising protective and therapeutic effect against the DEN-induced liver toxicity. Moreover, AFP, has long been used as a reference tumor biomarker to validate diagnosis and monitor HCC in human (Song et al. 2013). Treatment with *C. cardunculus* extract significantly reduced serum AFP level suggesting that it might decrease injury of the liver and delay the DEN induced HCC in rats. These effects may be attributed to the powerful antioxidant and hepatoprotective effects previously reported for the plant (Moglia et al. 2008).

DEN-induced histopathological changes is in harmony with previous reports (Pascale et al. 1993). *C. cardunculus* extract administration induced obvious improvement in the histopathological status of DEN-induced HCC and confirmed its anticarcinogenic effect. The efficacy of the extract may be explained on bases of the antioxidant, antiproliferative and hepatoprotective effects of its active constituents. The plant contains a variety of dicaffeoylquinic acids and many kinds of flavonoids (Lombardo et al. 2022) that possess antioxidant, anti-inflammatory, and anticancer effects (Wider et al. 2013). *C. cardunculus,* bioguided fractionation and isolation showed that petroleum ether and methylene chloride fractions were potent inhibitors against Hep G2 cell lines. Moreover, the methylene chloride components namely, grosheimin and cynaropicrin were of significant cytotoxic activity against Hep G2. Molecular docking of Hep G2 cytotoxic constituents to caspase-3 indicated their ability to bind with the enzyme active site via hydrogen bonds and consequently start apoptosis process of cancer cells.

Author contributions

R.H. performed the extraction and isolation. R.A. carried out chemical characterization of the isolated compounds. Y.M. performed the *in vivo* antitumor activity. The molecular docking was performed by I.M. R.H., R.A., O.I.F and Y.M. interpreted the results and wrote the paper. A.A conceived and designed the project. All authors revised the manuscript and approved it for publication.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Figure S1-S28: EI-MS, IR and NMR Spectra of isolated compounds **1**-**7**. Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsps.2022.11.011.

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