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Flavonoids content of 70% methanolic extract of *Erucaria pinnata (Viv.)* and its effect as anticancer and antiangiognic: *in vitro*, *in vivo* and docking study

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ABSTRACT

Erucaria pinnata (Viv.) is a wild annual plant growing in North-Western Coastal Region in Egypt. This study reports for the first time the cytotoxic activity of different extracts of *Erucaria pinnata* plant against HEP-G2 cell line. The 70% methanolic extract (E1) recorded the best potent cytotoxic activity (IC_{50}=13.6 μg/ml), so we analysis the flavonoids constituent of this extract using HPLC, which show that our extract is rich with important flavonoids compounds (rutin, quercetin, leutolin, etc.). We evaluated its antitumor activity against hepatocellular carcinoma (HCC) induced by diethylnitrosamine (DEN) (200mg/Kg. b.wt., i.p, single dose) after two weeks, animals received carbontetrachloride (CCl_4) (3ml/Kg. b.wt., SC, once a week for 6 weeks) and the experiment continued for 44 weeks in rats. After the experimental period, the administration of DEN/CCl_4 showed significant increase in the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin, gamma-glutamyl transferase (γGT) and significant decrease in the levels of total proteins and albumin content in the serum with reduction in the liver antioxidants, including superoxide dismutase (SOD) and catalase (CAT). This was accompanied by increases in serum specific tumor markers (AFP). The 70% methanolic plant extract (E1) was orally administrated (400mg/kg/day respectively) for the whole study period, and it showed a significant improvement at the different biological liver functions, remodeled the antioxidant enzymes activity and down-regulated the serum AFP. All these findings were confirmed by histopathological studies of the liver samples obtained from all groups.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a primary malignancy of the liver, occurs predominantly in patients with underlying chronic liver disease, and is the most common cause of death in people with cirrhosis (Satir, 2007). HCC is now the second leading cause of cancer deaths worldwide (Marquardt *et al.*, 2015). The occurrence of HCC is most elevated in Asia and Africa, where the endemic high commonness of hepatitis B and hepatitis C firmly inclined to the advanced of chronic liver disease and following the development of HCC (Chatterjee and Mitra,

2015).

Angiogenesis (spreading and formation of new blood vessels) plays a critical role in physiological and pathological human conditions (Kim and Byzova, 2014) and results from an imbalance between proangiogenic factors and inhibitors (Mekuria and Abdi, 2017). VEGF activates VEGFR, which will activate downstream pathways lead to stimulates the release of endothelium-derived nitric oxide (NO) from venous endothelial cells, and up-regulates the expression of nitric oxide synthase (NOS), which lead to vasodilation in physiological and pathological angiogenesis (Simons *et al.*, 2016).

Vascular endothelial growth factors (VEGF) and the related receptors seem to play a critical role in cancer development and metastasis (Sullivan and Brekken, 2010; Olsson *et al.*, 2006), and they are critical for HCC growth and development (Morse *et al.*, 2019).

In the course of the most recent decade, FDA was approved many anti-angiogenic agents targeting VEGF for the treatment of cancers, as bevacizumab, sorafenib, and pazopanib, etc. (Meadows and Hurwitz, 2012). The most common problems for these approved drugs that it's expensive, often show a poor biodistribution and pharmacokinetic profile, multidrug resistance and having severe side effects (e.g., hypertension, proteinuria, thrombosis, bleeding, etc.) (Ebos and Kerbel, 2011). Moreover, these inhibitors developed multidrug resistance and enhanced invasiveness during treatments that have limited effects on the overall survival, and authenticated prognostic biomarkers are unavailable for observing the response to a treatment (Ellis and Hicklin, 2008). Cancer is a complex disease that involves many pathways and biological mechanisms, in other hand phytochemicals (plant extracts) containing a lot of compounds which act on different biological mechanisms and pathways at the same time which make it suitable field for searching for anticancer treatment (Singh *et al.*, 2016).

Brassicaceae is one amongst the major spermatophyte families with around 338 genera and over 3709 species distributed worldwide (Al-Shehbaz *et al.*, 2006). Regarding fifty-three genera and 107 species distributed in Egypt (Boulos, 1999). It is the 4^{th} of 11 large families that are extensively spread in all phytogeographic regions within the Egyptian flora (Boulos, 1995). Historically, Plants of this family were usually utilized for their antidiabetic, antibacterial, antifungal, antirheumatic, and anticancer action (Rizk, 1986). The genus *Erucaria* is delineated by four species that grow in Egypt; *Erucaria crassifolia*, *Erucaria hispanica, Erucaria microcarpa*, and *Erucaria pinnata*.

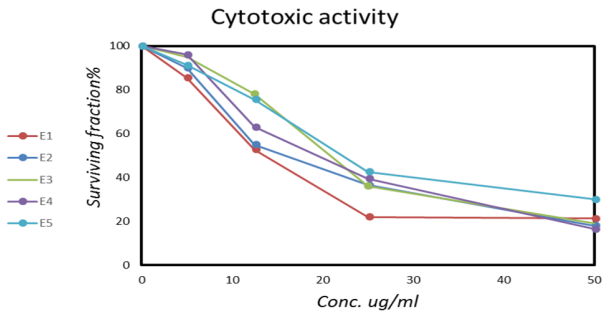

Figure 1: Cytotoxic activity for different extracts of *Erucaria pinnata* against (Hep-G2)

In the present study, we try to evaluate the antitumor activity of methanol extract of *Erucaria pinnata* plant against HCC with antiangiogenic effect targeting on VEGF/VEGFR-2 *in vitro* and *in vivo* studies, our promising result have pushed us to investigate the phytochemical constituent of the extract using HPLC and to try to understand the molecular biology mechanism behind this activity using docking study focusing in the anti-angeogenisis activity of this extract.

MATERIALS AND METHODS

Chemicals and Kits

Chemicals and reagents were high analytical grade, namely Aldrich-Sigma Chemical (St. Louis MO, USA) & ADWIC, Egypt. The VEGF-R_2/KDR ELISA kit was Biovendor (Brno, Czech Republic) and VEGF-A ELISA Kit EIAab (Shanghai, China).

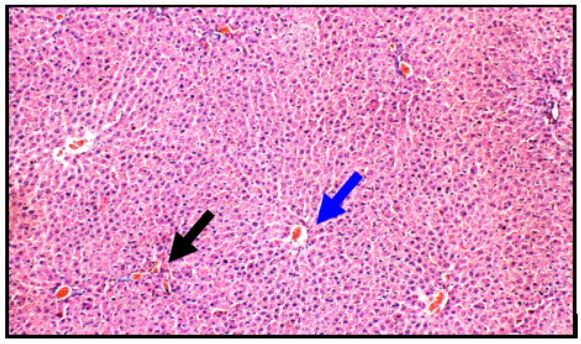

Figure 2: Photomicrograph of liver tissue of normal control group showing average central vein (blue arrow) and average portal tract (black arrow) (H&E X 200)

Plant collection and preparation of the extracts

The fresh aerial parts of *Erucaria Pinnata* (Viv.) were collected at its growth period of spring sea-

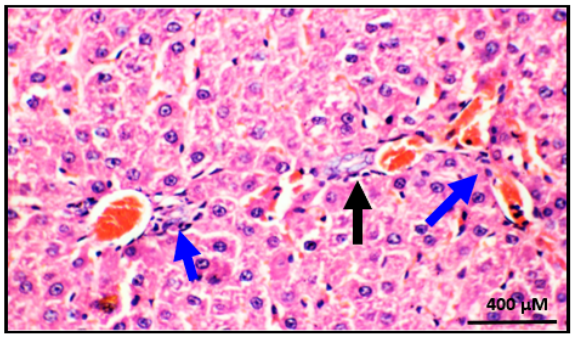

Figure 3: Photomicrograph of high power view of previous slide showing average portal tract (blue arrows) with average portal veins (PV) and average bile duct (black arrow)

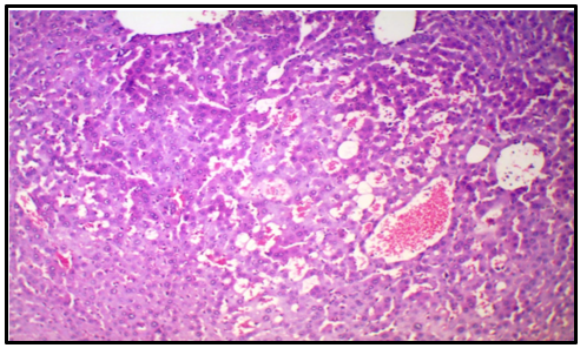

Figure 4: Photomicrograph of liver tissue of DEN+CCl_4 treated group showing average hepatocellular carcinoma (H&E X 20

son from the North-Western Coastal region in Egypt. The plant was air-dried at lab-temperature till constant weight, then ground to a fine powder and kept to be used for different plant analysis. Two kg of plant powder were extracted by percolation with a minimum amount of 70% methanol and purified according to (Graf *et al.*, 1970; Harborne, 1984). The slurry was allowed to stand for 24 h with occasional stirring and then filtered off. The residue was repeatedly extracted with an excess volume of 70% methanol. Collected filtrates were evaporated under reduced pressure using rotavapour apparatus at a temperature of 55 ºC. Then divided the residue into two parts the first part (E1), the second part was fractionated successively with petroleum ether (E2), chloroform (E3),ethyl acetate (E4) and methyl alcohol 96% (E5) using percolation, each fraction was evaporated under reduced pressure till dryness at 45°C to get sick mass. All the dried residues were stored in the refrigerator at 5 ºC until the use.

Qualitative and Quantitative Determination of Flavonoids Using HPLC

The determination of flavonoids was done using HPLC (Mattila *et al.*, 2000). HPLC Agilent 1100 series with the Quaternary pump was used. The flow rate was set at 1 ml/min. The temperature of the column compartment, degaser, and autosampler was adjusted at 35°C. The detector set at wavelength 330 nm for flavonoid compounds. Hypersil column (5μm, 250x4 mm) was used. The standard which used were purchased from El-Gomhoria-chemical company, Egypt, and the work was done in the Food Technology Research Institute, Agric. Res. Center, Giza, Egypt.

Mammalian cell lines

We used the human hepatocellular cancer cell line (HepG2 cells). These lines were obtained from the Pharmacological Unit in the tumor biology department in the National Cancer Institute, Cairo University, and propagated according to the standard protocol (Hay, 2000).

Cytotoxic activity by colorimetric sulphorhodamine-B (SRB) assay

Cell viability was measured according to the method reported by Skehan *et al.* (1990). Cells were seeded in 96 well microtiter plates at a concentration of

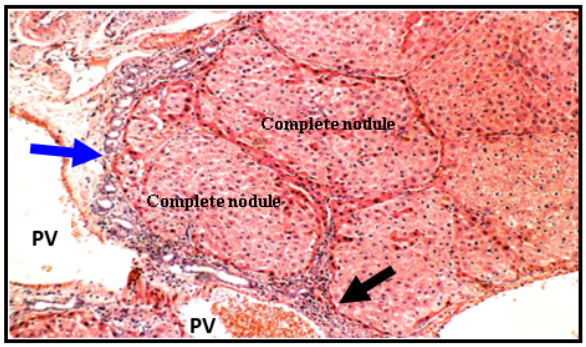

Figure 5: Photomicrograph of liver tissue of DEN+CCl_4 treated group (H&E X 200)

1000-2000 cells/well, 100μl/well. After 24 hr. Cells will be incubated for 72 hr. with various concentrations of plant extract (0, 5,12.5,25,50 μg/ml). Following 48 hr. The medium was discarded and fixed with 10% trichloroacetic acid 150 μl/well for 1hr. at 4ºC. Then Wells were stained for 10-30 minutes at room temperature with 0.4% SRB dissolved in 1% acetic acid 70μl/well, washed in acetic acid 1% to remove the unbounded dye. The plates were air-dried before the dye was solubilized with 150μl/well of 10mM Tris base (pH 7.4) for 5minutes on a shaker at 1600 r.p.m. The optical density (OD) of each well was measured spectrophotometrically at 540nm on ELISA microplate reader (ELX 800-*BioTek*-USA). The IC_{50} values were calcu-

lated using sigmoidal concentration-response curve fitting models (sigmaplot software).

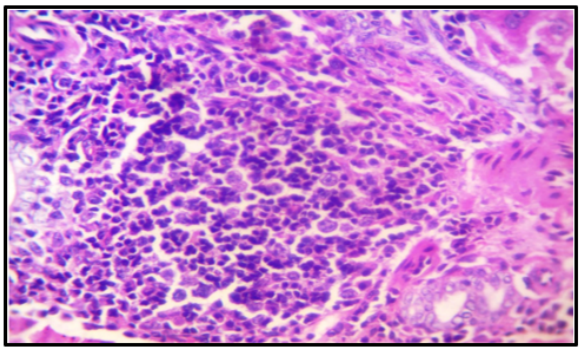

Figure 6: Photomicrograph of liver tissue of DEN+CCl_4 treated group showing Tumor cells

Detection of VEGFR-2 /KDR content in cell culture medium by ELISA

We have our Cells from American Type Culture Collection. Cells were cultured according to the standard protocol in a volume of 100μl complete growth medium + 100 ul of the plant extract per well in a 96-well plate for 18–24 hours, the supernatant was collected. VEGFR-2 in the supernatant was determined quantitatively using a human VEGFR-2 ELISA kit, according to the manufacturer's instructions.

Animals

The work was done in the Cancer Biology department, National Cancer Institute, Cairo University-Egypt. This study involved a total number of 60 adult male Sprague-Dawely albino rats of 100-130 g, body weight. They were housed in separate screen bottom cages under controlled environmental conditions (20-25 ºC, 55-60 % relative humidity and 12 hours light-dark cycle). Food and water were offered ad-libitum. Animals were kept up on stock diet in the form of pellets having the following composition: protein (18.8 % w/w), barley (37 % w/w), and corn (15 % w/w) were the main constituent (Lewi, 1981). The study was managed after obtaining Institutional Animal Ethical Committee (IAEC) clearance.

Experimental design

Animals were divided randomly into four groups. Each group contained fifteen animals.

Group 1 (Normal control)

This is healthy untreated rats eating standard food and drinking tab water ad-libitum.

Group 2 (diethylnitrosamine and carbon tetrachloride) (DEN+CCl_4)

Rats received a single intraperitoneal dose of DEN (200 mg/kg, body weight) to initiate hepatic carcinogenesis. Two weeks later, animals received subcutaneous injections of CCl_4 once a week in a dose of 3 ml/kg bodyweight for 6 weeks to stimulate liver cell proliferation and regeneration. The experiment continued for 44 weeks.

Group 3 (Plant extract (E1))

Rats treated daily with 70% methanol plant extract of *Erucariapinnata* alone by oral gavaging at a dose of 1 ml (400 mg/ kg b.wt.) for 44 weeks.

Group 4 (DEN+CCl_4+E1)

Rats treated as group 2 with daily orally administrated by a dose of 1 ml (400 mg/ kg b.wt.) of the 70% methanol extract (E) till the end of experimental.

Blood sampling for biochemical assays

After 44 weeks, the rats were sacrificed. Blood samples were collected, and the serum was then separated and divided into three aliquots and used fresh or kept frozen at -80ºC and thawed only once for demand.

Tissue Preparing

liver tissue was divided into two sections, the first section was kept at 10% neutral-buffered formalin for histopathological investigation, while the second one was homogenized to obtain tissue homogenate supernatant, which are used for estimation of our parameters.

Estimation of liver injury markers in blood serum

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured based on the principles described by Bergmeyer *et al.* (1978), the activity of alkaline phosphatase (ALP) was estimated according to the method described by King and Armstrong (1988), γ-Glutamyl transferase (γ-GT) was determined by the method of Persijn and and (1976), The total bilirubin level (TBL) was determined according to the method of Walter and Gerade (1970), Total protein and albumin concentrations were determined colorimetrically by the methods adopted by Gornall *et al.* (1949); Doumas *et al.* (1997) respectively and The serum globulin concentration was calculated by subtracting the value of serum albumin from the total serum protein

(Globulin conc. (g/dl)) = Total protein conc. (g/dl) - albumin conc. (g/dl)).

Liver antioxidant enzymes

The activity of superoxide dismutase (SOD) and Catalase (CAT) in the liver were measured by the

Table 1: IC_{50} of crude 70% methanol and its successive extracts of Erucaria pinnata against HEPG2 cell line

Extract	IC_{50} (μg / ml) HEPG2
Methyl alcohol 70 % (E1)	13.6
Petroleum ether (E2)	15.8
Chloroform (E3)	20.8
Ethyl acetate (E4)	19.4
Methyl alcohol 96% (E5)	23.3

Table 2: Quantitative estimations of flavonoid constituents (mg/100gm) in 70% methanolic extract of *E. pinnata*

Peak No.	Flavonoid name	RT	Concentration (mg/100gm)
1	Luteo.6-arabinose8-glucose	9.378	490.9
2	Luteo.6-glucose8-arabinose	10.489	25.038
3	Apig.6-arabinose8-glactose	11.478	38.76
4	Apig.6-rhamnose8-glucose	11.801	72.499
5	Apig.6-glucose8-rhamnose	11.981	198.69
6	Luteo.7-glucose	12.051	7.3636
7	Narengin	12.139	25.557
8	Rutin	12.279	2412
9	Hespiridin	12.441	75.066
10	Apig.7-o-neohespiroside	12.797	5.7446
11	Kamp.3,7-dirhamnoside	12.883	7.5999
12	Apig.7-glucose	13.135	2.1073
13	Quercetrin	13.236	34.823
14	Quercetin	14.704	49.671
15	Naringenin	17.984	2.4948
16	Hespirtin	15.194	5.9607
17	Kampferol	15.994	4.2185
18	Rhamnetin	16.060	0.8569
19	Apegnin	16.256	2.3036
20	Acacetin	18.443	19.11

Table 3: Effect of 70% methanolic extract of *E. pinnata* on VEGFR-2 in HEPG2 cell line

Test samples	VEGFR-2 residual conc. Pg/ml	VEGFR-2 % inhibition
Crude extract (E1)	1184	72.36%
Control HepG2	4283	

procedure described by Marklund and Marklund (1974); Cohen *et al.* (1970), respectively.

Tumor marker

Alpha-fetoprotein (AFP) measured quantitatively by solid-phase enzyme-linked immunosorbent assay Elisa according to the method of Belanger *et al.* (1973).

Quantitative Determination of Vascular Epidermal Growth Factor (VEGF-A)

Vascular endothelial growth factor (VEGF) concentration in the serum was depended on binding VEGF antigen to a specific immobilized antibody. The formed immune complex binds to avidin-peroxidase conjugate, and a color developed in proportion to the amount of VEGF bound, which was measured at 450 nm.

Histopathological Assessment

Tissue specimens were dehydrated, cleared,

Table 4: Serum levels of biomarkers of liver functions in different animals groups.

Group (4)	Group (3)	Group (2)	Group (1)	Dependent Variable
65.73 ± 2.49c -39.13%	47.67 ± 3.46 6.24 %	108.00 ±6.89b 140.69 %	44.87 ± 3.09	ALT (U/L)
176.5 ± 11.32c -40.43%	140.1 ± 3.77 0.76%	296.30± 3.07b 118.51%	135.60 ± 0.77	AST (U/L)
141.60 ± 0.94c -46.72%	86.40 ± 2.23 2.28%	265.8 ± 1.09b 224.15%	84.47 ± 0.37	ALP (U/L)
7.20 ± 0.27c - 51.58 %	3.55 ± 0.33 18.33 %	14.87 ± 0.27b 395.66%	3.00 ± 0.06	GGT (U/L)
0.46 ± 0.03c -48.89%	0.39 ± 3.46 5.41%	0.90 ± 0.02b 143.24%	0.37 ± 0.02	Total bilirubin (mg/dl)
6.61 ± 0.23c 22.63 %	7.90 ± 0.37 -4.13 %	5.39 ±0.09b -34.59 %	8.24 ± 0.67	T. Protein (g/dl)
3.44 ± 0.09c 54.95 %	4.53 ± 0.12 -2.58 %	2.22± 0.21b -52.26 %	4.65 ± 0.18	Albumin (g/dl)
3.16 ± 0.29 -0.32%	3.37 ± 0.40 6.31 %	3.17 ± 0.25a -11.69 %	3.59 ± 0.72	Globulin (g/dl)
1.10 ± 0.12c 54.93 %	1.36 ± 0.17 1.49 %	0.71 ± 0.12b -47.01 %	1.34 ± 0.27	A/G ratio

* Group (1): Normal control, Group (2): DEN+CCl_4, Group (3):70% methanolic plant extract (400 mg/kg b. wt.), Group (4): DEN+CCl_4+70%methanolic plant extract (400mg/kg b. wt.).

*All data are expressed as mean ± SEM. a: Significantly different from the normal control group ($P < 0.05$). b: Significantly different from the normal control group ($P < 0.001$). c: Significantly different from the HCC group($P < 0.001$).

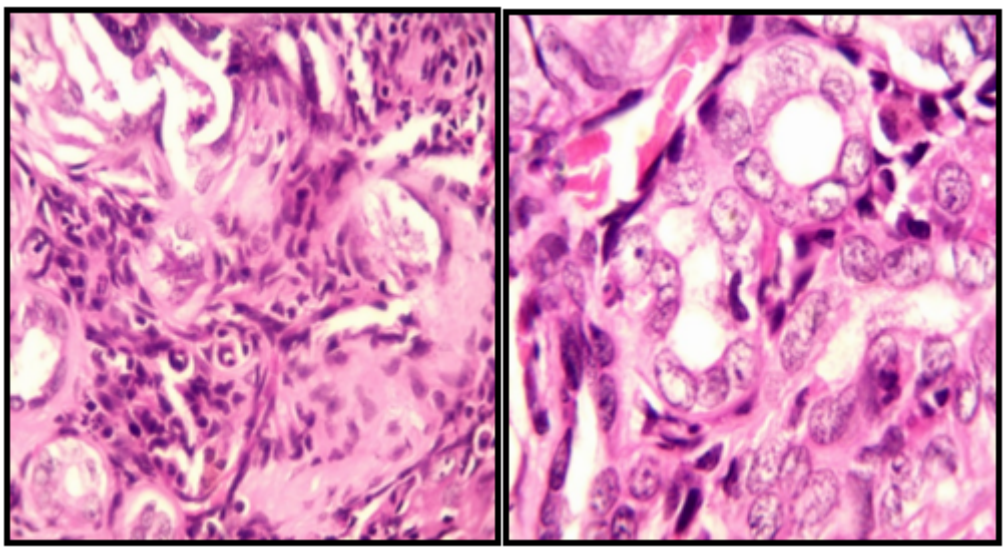

Figure 7: Photomicrograph of liver tissue of DEN+CCl_4 treated group(Gp 2) : high power view of previous slide (H&E X 400)

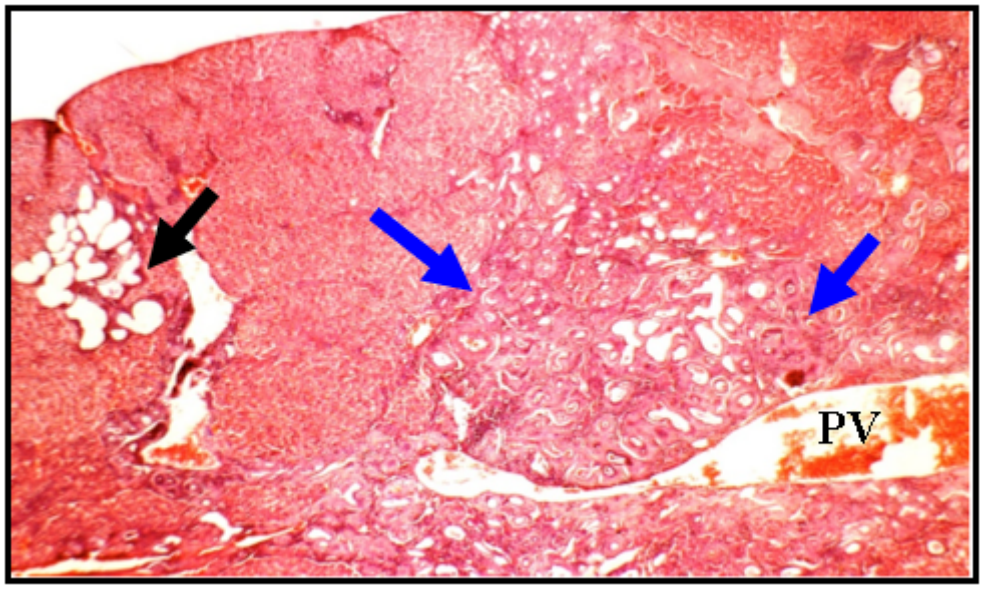

Figure 8: Photomicrograph of liver tissue of DEN+CCl_4 treated group showing markedly expanded portal tract with markedly dilated portal vein (PV) (H&E X 100)

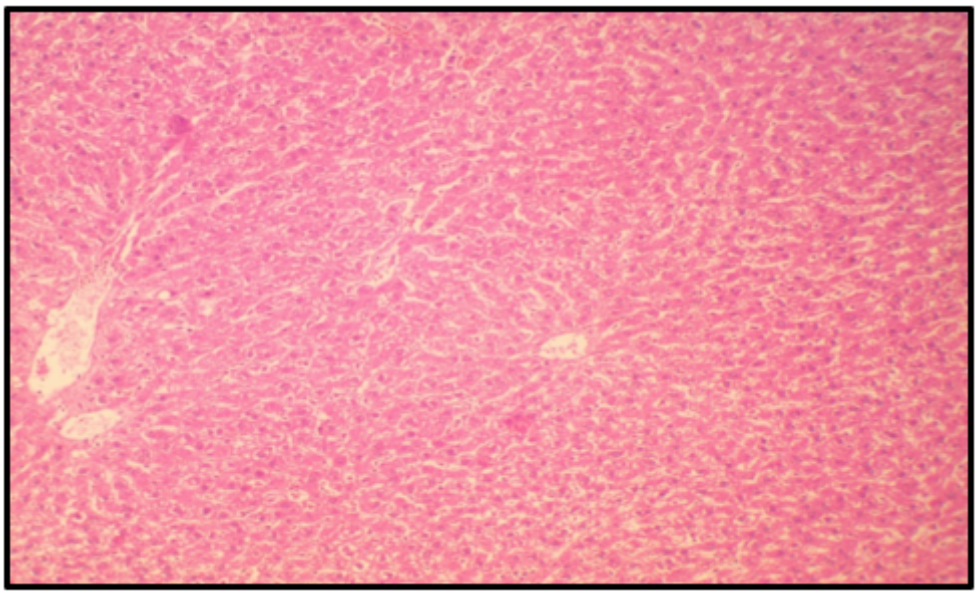

Figure 9: Photomicrograph of liver tissue of E1 treated group showing normal liver architecture (H&EX 200)

immersed, and sectioned (5 μm) using ethanol series, xylene, Paraplast wax, and microtome, respectively. Liver sections were mounted on a glass slide then the tissue sections were deparaffinized and stained by haematoxylin and eosin.

Molecular docking

To study the possibility of our extract to act as anti-angiogenesis by inhibited the activation of VEGFR-2 kinase, a docking study was performed by maestro 11.4 to determine the ability of flavonoid aglycones with a higher concentration in our extract (HPLC result) to bind with and inhibit VEGFR-2 tyrosine kinase. (PDB entry: 3VID) used for our study. Ligprep have been used to prepare our compounds for docking. Protein preparation have been done using the protein preparation wizard. Validation of our docking study was done by docking of the co-crystallized ligand (4TT), the result indicates that our protocol is valid with rmsd= 0.3561 and the same amino acid interactions were recognized (one hydrogen bond with Cys 919, one hydrogen bond

with Glu 917 and one aromatic hydrogen bond with Lys 920). The ligand-binding area was determine using the co-crystallized ligand in 3VID, to avoid any false positive result and to make the docking study highly accurate two type of constraints have been used the first one is the positional constraint where, we define spherical regions of indole ring of the co-crystalized ligand should be occupied by particular ligand atom during docking, the second one is the H-bond constrain where we pick Cys 919, Glu 917 and Lys 920 should participate in hydrogen bond (acceptor or donor) interaction during docking also extra precision (XP) setting have been used during docking.

Statistical analysis

All results were expressed as the mean ± standard errors of mean (SEM). Statistical analysis was performed using statistical packages for the Social Science for Windows (SPSS, Version 11.0, Chicago, IL, USA). The data were analyzed by Student's t-test. The P-value of less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Cytotoxic potential assessment against liver cancer cell (HepG2) cell line

Serial dilution of crude methanol 70% (E1) and its successive extracts (petroleum ether (E2), chloroform (E3), ethyl acetate (E4), 96% methyl alcohol (E5)) of *E. Pinnata* (0, 5, 12.5, 25 and 50 μg/ml) were examined for their cytotoxicity potency against hepatic carcinoma (HEP-G2) using sulforhodamine B stain (SRB) assay. It was observed from the obtained results in (Table 1) and (Figure 1) that, all extracts of *E. Pinnata* achieved cytotoxic effect against HEPG2 While the crude methanol 70% recorded the best potent cytotoxicity at low concentration (IC_{50}=13.6 μg/ ml) followed by the petroleum ether extract (IC_{50}= 15.8 ug/ml) While the 96% methyl alcohol extract recorded the lowest cytotoxic effect (IC_{50} =23.3 μg / ml).

Flavonoids Constituent of 70% methanolic extract by HPLC

Quantitative and qualitative estimation for the flavonoid (Table 2) compounds of the 70% methanolic extract of *E. Pinnata* were achieved by using HPLC, where, each compound was separated. Identification was done using authentic pattern. We found that rutin was the highest concentration of flavonoids compounds by 2412 mg/100mg, and rhamnetin was the lowest concentration of 0.8569 mg/100mg in the plant.

VEGFR-2 /KDR content in cell culture medium

It was clear that in Table 3, the crude methanol 70% inhibited the VEGFR-2 in HepG2 cell line by (72.36%).

Effect of 70% methanolic extract of *Erucaria pinnata* on liver markers in blood serum

From our study, its clear that Group 2 (DEN+CCl_4) developed hepatocellular carcinoma while in Group 4 (DEN+CCl_4+E1), the carcinogenesis process was prevented or at least showed (hepatoprotective activity) as its clear from the level of hepatic serum parameters. While in Group 3 (E1) recorded no significance change comparing to Group 1 (normal control group) (Table 4).

Antioxidant and oxidative stress determination

Results arranged in Table 5, showed that administration of DEN+CCl_4 in rats produced highly significant decreases in the level of SOD and CAT at ($p < 0.001$) by (-74.58% & -41.71%) respectively when compared with HCC group, while rats treated with extract alone showed highly significant increase ($p < 0.001$) in the levels of antioxidants including SOD and Catalase in the liver, when compared to the control group by (54.99% & 17.26%) respectively. Post-treatment with Plant Extract (400 mg/kg body weight daily for 44 weeks) caused significant increases ($p < 0.001$) in the hepatic levels of SOD and Catalase by (239.63% & 33.42%) respectively as compared to untreated HCC rats.

Effect of 70% methanolic extract of *Erucaria pinnata* on serum AFP and VEGF-A level

Alpha-fetoprotein (AFP) and Vasculoendothelial growth factor (VEGF) were found to be significantly increased in DEN+CCl_4 treated animals ($P<0.001$) when compared to control group by (5148.57% & 142.14%) respectively. While rats treated with *(E1)* alone recorded no significant change comparing with normal control and Post-treatment with 70% methanolic extract of *E. Pinnata* at the tested dose level significantly reduces the elevated levels of tumor-specific markers AFP and VEGF ($P<0.001$) when compared to DEN+CCl_4 treated animals group by (-95.65% & -41.54%) respectively, all values are presented in (Table 6).

Histopathological study

Microscopically liver of control untreated rat normal architecture of hepatic strands around the central veins, normal portal tract (PT) with normal portal veins (PV) and normal bile duct (BD), The liver showed intact hepatocytes with normal sinusoids in between. The hepatic cells are polygonal in shape with one or two rounded nuclei (Figure 2 &Figure 3). Meanwhile, the liver of rat treated with (DEN+ CCl_4) showed the clear-cell variant of hepatocellular car-

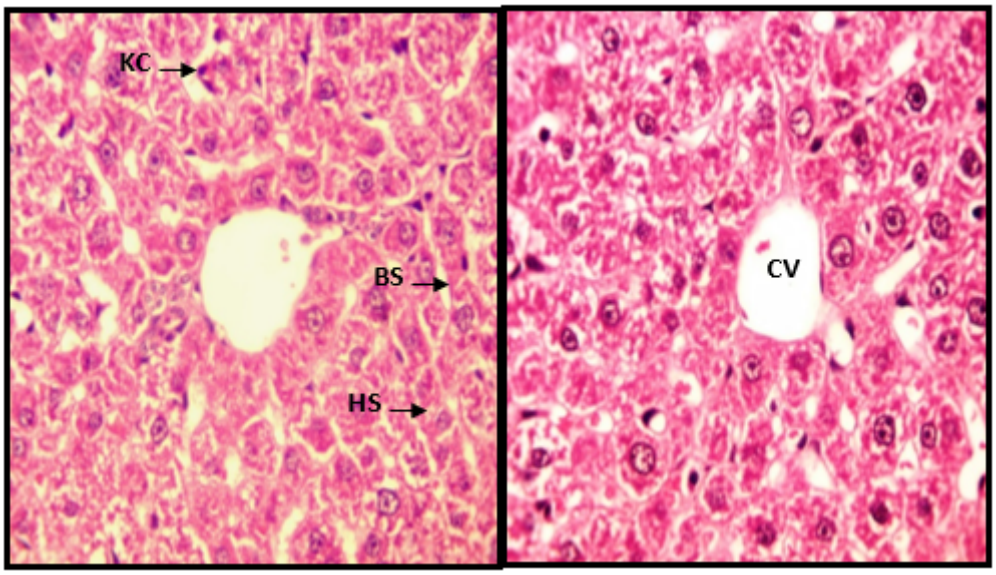

Figure 10: Photomicrograph of liver tissue of E1 treated group, high view of previous slide showing no histopathological effect of hepatocytes (H&E X 200)

Table 5: Tissue Antioxidant Enzymes levels in different animal groups

Group (4)	Group (3)	Group (2)	Group (1)	Dependent Variable
77.98 ± 5.51c 239.63 %	140.00 ± 4.63b 54.99 %	22.96 ± 2.09b -74.58 %	90.33 ± 2.11	SOD (U/gm tissue)
468.30 ± 49.29c 33.42 %	759.00 ± 31.61b 17.26 %	351.00 ± 27.91b -41.71 %	647.30 ± 27.91	CAT (U/gm tissue)

*Concerning the groups as those of (Table 5)
*All data are expressed as mean ± SEM. b: Significantly different from the normalcontrol group (P < 0.001). c: Significantly different from the HCC group(P < 0.001)

Table 6: Serum levels of AFP and VEGF in different animal groups

Group (4)	Group (3)	Group (2)	Group (1)	Dependent Variable
0.80 ± 0.05c -95.65 %	0.39 ± 0.05 11.43 %	18.37 ± 0.77b 5148.57 %	0.35 ± 0.02	AFP (ng/ml)
211.6 ± 6.60c -41.54 %	157.30 ± 6.99 5.22 %	362.00 ± 9.66b 142.14 %	149.50 ± 9.39	VEGF-A (pg/ml)

*Concerning the groups as those of (Table 5)
*All data are expressed as mean ± SEM. b: Significantly different from the normal control group (P <0.001). c: Significantly different from the HCC group(P < 0.001).

Table 7: *In-silico* docking analysis of flavonoids from *Erucaria Pinnata*

Compound name	Amino acids	Interacting group	Type of interaction	Docking score
Quercetin	Glu 917	OH	H-bond (donner)	-7.274
	Cys 919	OH	H-bond (acceptor)	
	Lys 920	OH	H-bond (donner)	
Luteolin	Glu 917	OH	H-bond (donner)	-6.921
	Cys 919	OH	H-bond (acceptor)	
	Lys 920	OH	H-bond (donner)	

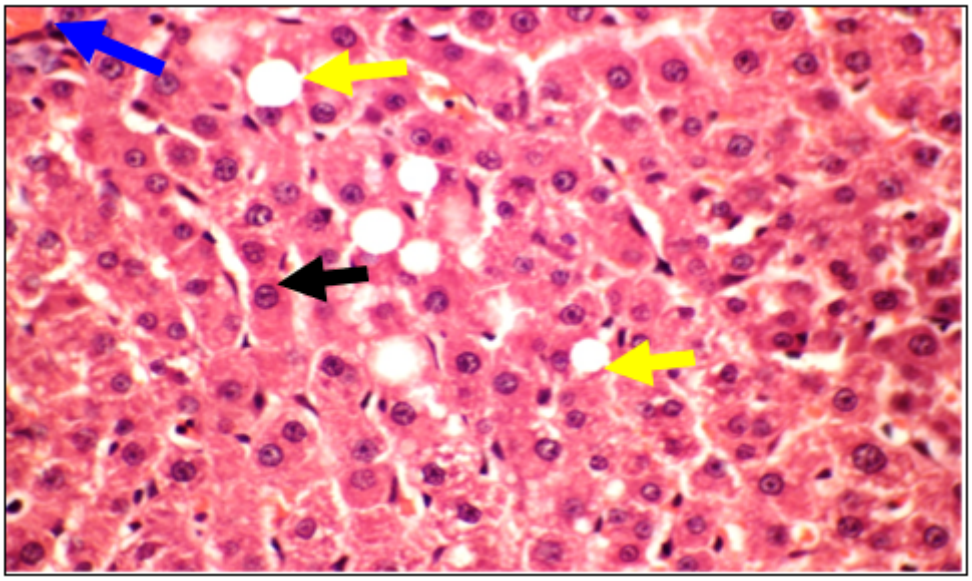

Figure 11: Photomicrograph of liver tissue of DEN + CCl_4 + E1 treated group (H&E X 400)

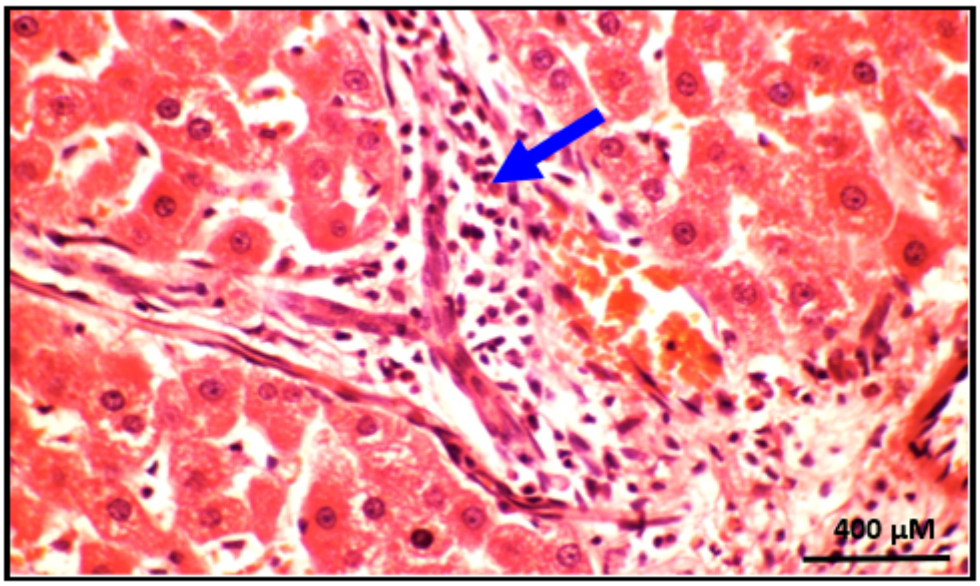

Figure 12: Photomicrograph of another view of previousslide showing expanded portal tract with mild inflammatory cellular infiltrate (blue arrow)

cinoma is usually arranged in a trabecular pattern and is characterized by a clear cytoplasm that contains glycogen and a variable amount of fat vesicles as (Figure 4).

Moreover, the examined section revealed that liver tissue showing expanded portal tract and showing dilated portal veins (PV) with bile duct proliferation (blue arrow) and inflammatory infiltrate (black arrow) with complete nodules as (Figure 5). Tumor cells are large, eosinophilic with prominent granular eosinophilic cytoplasm, vesicular nuclei, and conspicuous nucleoli or dense lymphoid infiltrate in a portal tract (nodular cirrhosis) this was obvious in (Figure 6), and hepatocellular carcinoma were identified, with pseudo glandular and infestation of blood cells were observed, tumor cells are surrounded by hyaline fibrous bands pale bodies may be observed in the tumor cells (Figure 7). While HCC was identified with the chronic cholangitis, peircholangiolar fibrosis which indicates the malignancy happened and proliferating bile ducts (black arrow) with an area of bile duct cystification (blue arrow) (Figure 8).

Histopathological liver of rat treated with (E1)

revealed normal hepatic architecture (Figure 9 & Figure 10). Histopathological Liver of the DEN+CCl_4-rats treated with (E1) revealed significant improvement compared to DEN+CCl_4 treated group no evidence of HCC and showed normal central vein (blue arrow) surrounded by hepatocytes arranged in single-cell cords (black arrow) with scattered mild steatosis (yellow arrows) (Figure 11), bi-nucleation cell and mild perivascular lymphocytes infiltrate (Figure 12) this mean that the extract of *Erucaria pinnata* showed a sign of protection.

Docking study

This study revealed that our extract contains flavonoid aglycones (quercetin and luteolin) that could stably bind to the ATP-binding pocket of

VEGFR-2 with almost the same pattern like co-crystallized ligand (Table 7, Figure 13). Previous studies demonstrated that a variety of natural angiogenesis inhibitors, such as ellagic acid prevented VEGFR-2 phosphorylation via competing with cellular ATP binding to the ATP-binding sites.

Natural products which originated from the plant are affluent source for many small molecules which causing tumor cell kill by interacting with a predefined target(s) present on malignant cells and exhibited low toxicity to normal tissues.

In the present study, the cytotoxic activity of different extracts of *Erucaria pinnata* plant against HEPG2 cell line was evaluated, the 70% methanolic extract (E1) recorded the best potent cytotoxicity at low concentration so we analysis the flavonoids constituent of this extract using HPLC which show that our extract is rich with important flavonoids compounds (rutin, quercetin, leutolin, etc.) so we evaluated it's antitumor activity against hepatocellular carcinoma (HCC) induced by (DEN+CCl_4) in rats and evaluated its anti-angeogenic activity (*in vitro*, *in vivo* and docking study). Our study showed that the prolonged administration of (E1) a no significant change in liver function tests, AFP, and VEGF but was showed an extremely significant increase in SOD and CAT in comparison with a normal control group.

It is known that diethylnitrosamine (DEN) is an effective environment carcinogen used as an initiator for hepatic cancer in an animal model with another factor like CCl_4 as a promoter of carcinogenesis (Subramanian *et al.*, 2007). A reactive ethyl diazonium ion is produced from bioconversion of DEN by cytochrome P450 within the rat liver, and it reacts with DNA creating adducts that's identified as the initial step in DEN-evoked carcinogenesis (Verna *et al.*, 1996). Moreover, free radicals were produced due to the metabolization of CCl_4 by liver microsomal cytochrome P450, which exhaust hepatic antioxidants and harm macromolecules, leading to advance tumor formation (Weber *et al.*, 2003).

So, (DEN+CCl_4) stimulate oxidative stress which induces liver tissue injury and caused a significant increase in the activities of liver enzymes, the liver completely lose its functions and significant decrease the antioxidant enzyme activity (SOD and CAT which are involved in scavenging superoxide anion and hence reducing the toxic effect caused by these radicals). In this study, it was observed that the administration of our extract to (DEN+CCl_4) treated rats showed highly significant decrease in the biochemical liver marker enzyme activities and highly significant increase in total protein, albumin, SOD and CAT activities This showed that 70% methanolic extract of *Erucaria pinnata* can reduce reactive free radicals, thereby reducing oxidative damage to the tissues besides improving the activity of hepatic antioxidant enzymes.

We also interested in our study by another factor which has high specificity for HCC, the diagnosis of tumor response to therapy and is detected with elevated levels is AFP (Afzal *et al.*, 2012). There are many reports which show that AFP plays an important role in the regulation of tumor growth and cell differentiation (Li *et al.*, 2002). In the present study, serum AFP level of DEN+CCl_4 treated rats showed a significant increase in comparison to that of the control group, confirming the occurrence of premalignant liver changes in DEN+ CCl_4 treated rats. Treatment with (E1) significantly reduced serum AFP levels.

This improvement in the biochemical parameters were accompanied with improvement in the histopathological abnormalities. All this finding agrees with the phenolics and flavonoids' rich nature of this extract, which were identified by HPLC and many compounds have been reported to possess antioxidant and free radical scavenging ability, which could possibly be accounted for the antitumor property of this plant (Kandaswami *et al.*, 2005; Hay, 2000).

The Fifth Hallmark of Cancer "Sustained Angiogenesis" is an important target in cancer thereby due to the role of angiogenesis in cancer development and metastasis is critical and the main problem of anti-angeogenisis medication is the development of resistance (most of them is working only in single step in the angeogenic pathway), expensive cost and toxicity (most of them is protein in nature). So, it is important to distinguish new and effective anti-angiogenic molecules. Several studies have indi-

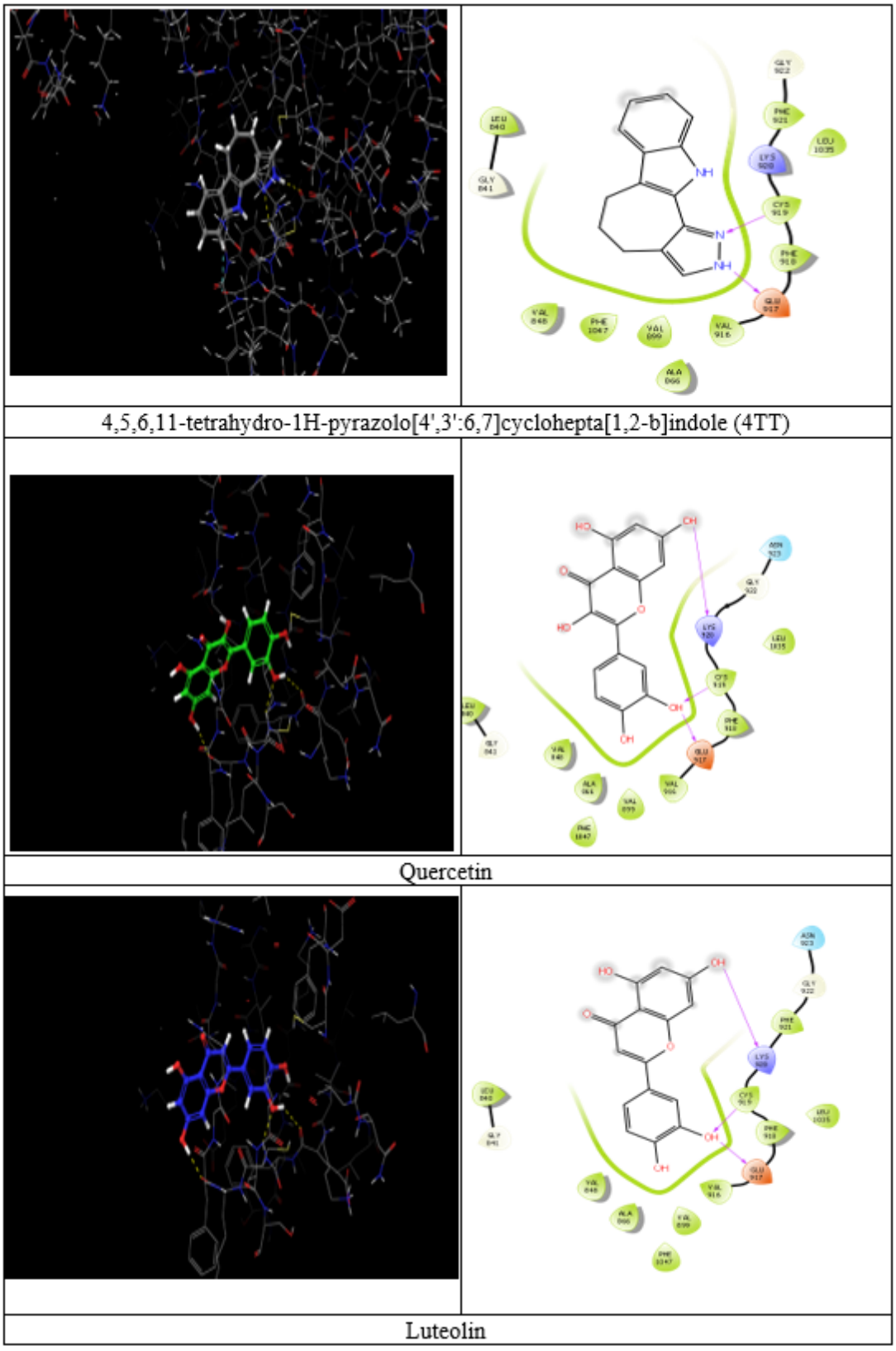

Figure 13: 3D and 2D photo of the ligands

cated that natural plant products can meet these criteria (Lu *et al.*, 2016).

In this study, we tried to determine the anti-angeogenic potential of our extract. The extract effectively inhibited VEGFR-2 expression *in vitro* and significantly inhibited the concentration of VEGF-A in cancer-bearing animals. This antiangiogenic activity of our plant extract may be due to the presence of high contents of flavonoids in the extract. Numerous flavonoids, for example, rutin, genistein, luteolin, kaempferol, quercetin, naringin, and apigenin have shown strong inhibition to cell proliferation and VEGF expression (Luo *et al.*, 2008; Oak *et al.*, 2005).

Our results were confirmed by docking study revealed that our extract contain flavonoid aglycones (Quercetin and Luteolin) that could stably bind to the ATP-binding pocket of VEGFR-2 with almost the same pattern like co-crystallized ligand (which mean the ability of our extract to inhibit the kinase enzyme activity of the receptor domain) also its previously reported that flavonoids can prevent overproduction of NO, all that proves that our extract have anti-angeogenic activity through working in different steps in the angeogenic pathway which will decrease the chance of resistance to its anti-angeogenic activity.

From another view, flavonoids could reduce any side effect associated with angiogenesis target therapy as flavonoids exerts vasodilator, antiplatelet and antiproliferative effects and reduces blood pressure, oxidative status and end-organ damage in hypertensive humans and animal models (Perez *et al.*, 2014).

CONCLUSIONS

This is the first study showed that 70% methanolic *Erucaria pinnata* extract possesses strong anticancer activity against DEN-induced HCC due to its antioxidant activity and antiangeogenic activity through working in different steps in the angeogeniesis pathway in the same time due to its flavonoids phytochemical constituents which give our extract the advantage of being strong antiangeogenesis medication with less toxic effect. These findings provide useful documents for further study on our plant to identify its effect in another hallmarks of cancer.

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