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Cytotoxic activity, Molecular docking study and Phytochemical investigation on *Cichorium intybus* Herb

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Abstract

The purpose of this study is to evaluate the cytotoxic activity of *Cichorium intybus* herb as well as to identify the molecular mechanism of the cytotoxicity. In addition, it aims to investigate its phytoconstituents that are responsible for the bioavailability. The cytotoxic activity of *Cichorium intybus* herb was assayed by SRB **(**Sulforhodamine B) assay against ovarian cancer cell line (SKOV-3), liver cancer cell line (HepG2) and prostate cancer cell line (PC-3). The effect on tubulin polymerization was studied to identify the mechanism of cytotoxicity. The binding affinity to the target molecule was examined by docking study. In addition, two flavonoidal compounds were isolated and identified by different spectroscopic methods. The results showed that the methanol extract of *Cichorium intybus* herb as well as the isolated compounds (myricetin and pinobanksin) possessed a potent cytotoxicity against HepG2 (IC_{50} =0.95, 4.26 and 7.23 µg/mL), respectively, moderate cytotoxicity against PC-3 (IC_{50} =25.34, 36.24 and 42.53 µg/mL), respectively, and weak cytotoxicity against SKOV-3 (IC_{50} >100 µg/mL) for all tested samples. Molecular docking analysis confirmed that both of the isolated compounds showed high binding affinity to colchicine binding site of tubulin microtubules, supported the high cytotoxicity of these compounds.

Keywords: *Cichorium intybu,* cytotoxic activity, myricetin, phytoconstituents, pinobanksin

Introduction

Cancer is the disease which means uncontrolled cell division. It is considered a worldwide killer, although the enormous researches and rapid development in the last years. According to recent statistics, cancer accounts for about 23% of the total deaths in the USA and is the second most common cause of death after heart disease. Only (5–10%) of all cancer cases can be attributed to genetic defects, while the remaining (90–95%) have their roots in the environment and lifestyle **[1].**Tubulin microtubules destabilization is one of the most promising strategies for the treatment of cancer **[2].** The polymerization of microtubules is a highly dynamic process and is important for the cell division **[3].** Colchicine domain is one of the most important binding sites that most chemotherapeutic agents targeting microtubules destabilization bind to this site **[4].**

Natural plants have been used to prevent and treat various diseases, including cancer, over thousands of years due to the presence of bioactive constituents such as; flavonoids, alkaloids, saponins and tannins **[5].** *Cichorium intybus* L., (family Asteraceae), is known as "chicory". It showed hepato-protective **[6-9],** gastro-protective **[10],** cardiovascular **[11],** analgesic **[12],** anthelmintic **[13],** antimicrobial **[14, 15],** antidiabetic **[16],** anti-inflammatory **[17],** antioxidant**[18-20],** tonic **[21[,** wound healing abilities **[22],** anticancer **[23],** immunological **[24],** reproductive effects **[25],** as well as many other pharmacological applications **[14,26].** Moreover, many researches showed the anticancer potential of *Cichorium intybus* roots and leaves on different human cancer cell lines such as; melanoma, prostate, breast and colon cancer cell lines due to the presence of various biologically active compounds **[5, 27].** Many research showed that *Cichorium intybus* contains varieties of phytoconstituents that play a vital role in the biological activities of chicory such as; volatile compounds, terpenoids, flavonoids, alkaloids, caffeic acid derivatives, coumarins **[26],** bitter substances, resins and mucilages **[28].**

There has been no detailed research on the anticancer potential of *Cichorium intybus* herb grown in Egypt. Therefore, and in continuing to the

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investigation of the phytoconstituents of *Cichorium intybus* herb , the aim of this research is to evaluate the cytotoxcicity of *Cichorium intybus* herb as well as to identify the molecular mechanism of the cytotoxicity. In addition, the phytoconstituents of the plant were also investigated.

Materials and Methods

Materials for Phytochemical Study

Plant collection and Identification

The herbs of *Cichorium intybus* family Asteraceae were obtained from Medicinal and Aromatic Plant Research Department, Horticulture Research Institute, Agriculture Research Centre, Giza, Egypt. The plant was identified by Dr. M. El-Gebaly, the taxonomist at the Department of Botany, National Research Centre (NRC), Giza, Egypt.

Extraction procedure

The dried sample (1kg) of *Cichorium intybus* herb was powdered by using laboratory mill before extraction, then the plant was extracted with (methanol/distilled water) (70:30, v/v) several times at room temperature by maceration method. The extract was concentrated under reduced pressure to give 80 g dried extract.

Phytochemical Screening

Chemical tests were carried out on the methanolic extract using standard procedure to identify the constituents as described by **[29, 30].**

Total Phenolic Assay

The total phenolic content (TP) was determined applying the Folin–Ciocalteu colorimetric method using gallic acid as a standard **[31, 32],** TP was expressed as milligrams of gallic acid equivalents (GAE)/g of the dry plant materials.

Total Flavonoid Assay

Total flavonoid content (TFC) was measured using an aluminum chloride colorimetric assay **[31, 32].** A calibration curve was established using rutin as a standard. TFC was expressed as mg rutin equivalent (QE)/g of the dry plant materials

HPLC analysis for both flavonoids and phenolics of 70% methanol extract of (Cichorium intybus) herb.

HPLC analysis was carried out according to **[33]** using an Agilent 1260 series. The separation was carried out using Kromasil C18 column (4.6 mm x 250 mm i.d., 5 μm). The mobile phase consisted of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) at a flow rate 1 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (82% A); 0–5 min (80% A); 5-8 min (60% A); 8-12 min (60% A); 12-15 min (85% A) and 15-16 min (82% A). The multi-wavelength detector was monitored at 280 nm. The injection volume was 10 μl for each of the sample solutions. The column temperature was maintained at 35 °C. Peaks were identified by congruent retention times and UV spectra in comparison with those of the standards.

General experimental procedures

UV/VIS: Shimadzu UV-visible recording spectrophotometer model-UV 240 (NRC,Egypt). ^{1}H-NMR (Varian Unity Inova). Sephadex LH-20 (Pharmacia Fine Chemicals).Thin layer chromatography (TLC) F $_{254}$ plates. Solvent mixture, (20:80 methanol:chloroform).

Isolation and purification of flavonoids from 70% methanol extract of Cichorium intybus herbs

The 70% methanol extract of *Cichorium intybus* herbs (50g) was defatted with n-hexane and part of the defatted methanol extract (20 g) was developed on preparative TLC using Chloroform–methanol (80:20 v/v) as developing system. The plate was examined under the UV light at 254 and 366 nm, resp. Two major bands marked, scratched and extracted separately with MeOH–H2O (1:1) to afford two main fractions. Each fraction was purified separately on sephadex LH–20 column using methanol and different systems of methanol and distilled water (methnaol: distilled water, 1:1, 2:1, v/v). Each band gave one pure compound. The isolated compounds were co-chromatographed against the available authentic flavonoids for the confirmation of the isolated compounds at the same Rf values. The isolated compounds were identified by different spectral analyses (UV, H1-NMR).

Material for cytotoxic study

Human tumor cell lines: Liver cancer cell line (HepG2), Ovarian cancer cell line (SKOV-3) and Prostate cancer cell line (PC-3) were obtained from Nawah Scientific Inc., (Mokatam, Cairo, Egypt).

Chemicals

Doxorubicin, (Pharmacia, Sweden), was used as a reference anticancer agent, Sulphorhodamine B stain, from Sigma Co, Egypt and Tris EDTA buffer, from Sigma Co, Egypt.

Cell culture

Cells were maintained in RPMI media, supplemented with 100 mg/ml of streptomycin, 100 units/ml of penicillin and 10% of heat-inactivated fetal bovine serum in humidified, 5% (v/v) CO_2 atmosphere at 37 °C.

***Assay method for cytotoxic activity* [34, 35]**

Cell viability was assessed by SRB assay. Aliquots of 100µL cell suspension (5x10^3 cells) were in 96-well plates and incubated in complete media for 24 h. Cells were treated with another aliquot of 100µL media containing drugs at various concentrations ranging from (0.01,0.1,1,10,100ug/ml). After 72 h of drug exposure, cells were fixed by replacing media with 150µL of 10% TCA and were incubated at 4 °C for 1 h. The TCA solution was removed, and the cells were washed 5 times with distilled water. Aliquots of 70µL SRB solution (0.4% w/v) were added and were incubated in a dark place at room temperature for 10 min. Plates were washed 3 times with 1% acetic acid and allowed to air-dry overnight. Then, 150µL of TRIS (10mM) were added to dissolve protein-bound SRB stain; the absorbance was measured at 540 nm using a BMG LABTECH®- FluoStar Omega microplate reader (Ortenberg, Germany).

Molecular docking

Docking experiment was used to investigate the binding affinity of the active compounds to the binding residues of the active site of tubulin protein. The crystal structure of tubulin protein complexed with colchicine (code: 4O2B) was downloaded from protein data bank http: //www. pdb. org/ pdb/home/home.do. The phytochemical compounds, myricetin and pinobanksin were selected as ligands for docking with cancer target tubulin protein receptor. The chemical structures of myricetin and pinobanksin were drawn as 2D structures by using Biovia draw tool, and saving under MOL format. The MOL files of the compounds were later converted to PDB file, in 3D structures using Avogadro software. The target protein (4O2B), was subjected to Optimization and energy minimization. In optimization, unwanted atoms are removed from protein, followed by Energy Minimization of the Optimized target protein. This was done by using Swiss PDB-viewer. Once the protein was optimized, the target protein was ready for docking and analysis. In AutoDockTools-1.5.6, polar hydrogens were added to the protein structure and stored as PDBQT. Gridboxe was established relative to native ligand (colchicine) . AutoDock Vina was used for docking **[36].**

Result and Discussion

Cytotoxic activity

The cytotoxic activity of 70% methanol extract of *Cichorium intybus* herb as well as the isolated compounds (myricetin and pinobanksin) in *vitro* was evaluated against liver cancer cell line (HepG2), ovarian cancer cell line (SKOV-3) and prostate cancer cell line (PC-3). The results showed that by increasing the concentration of the tested samples, the viability % was decreased in SKOV-3 (figure 1& 2), PC-3 (figure 3& 4) and HepG2 (figure 5& 6) compared with Doxorubicin (a reference anticancer agent). These results indicated that 70% methanol extract of *Cichorium intybus* herb as well as the isolated compounds showed cytotoxic activity against all tested cell lines.

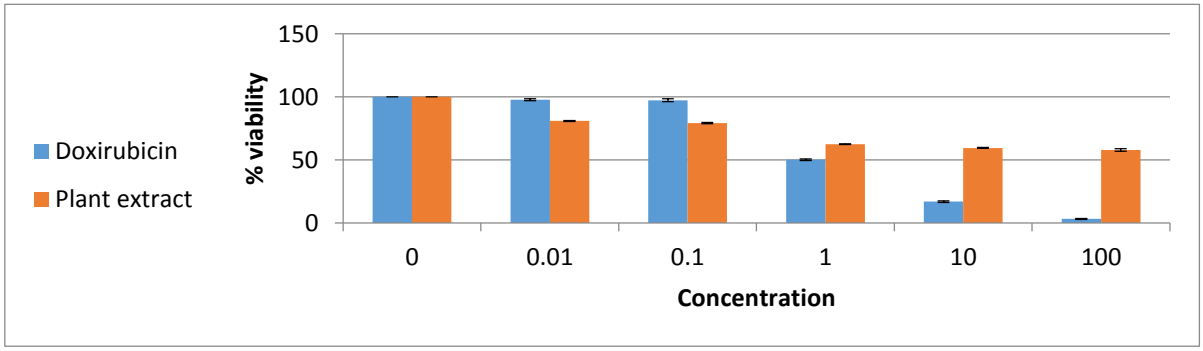

Fig. 1: Cytotoxic activity of 70% methanol extract of *Cichorium intybus* herb against SKOV-3 human cell line *in vitro*

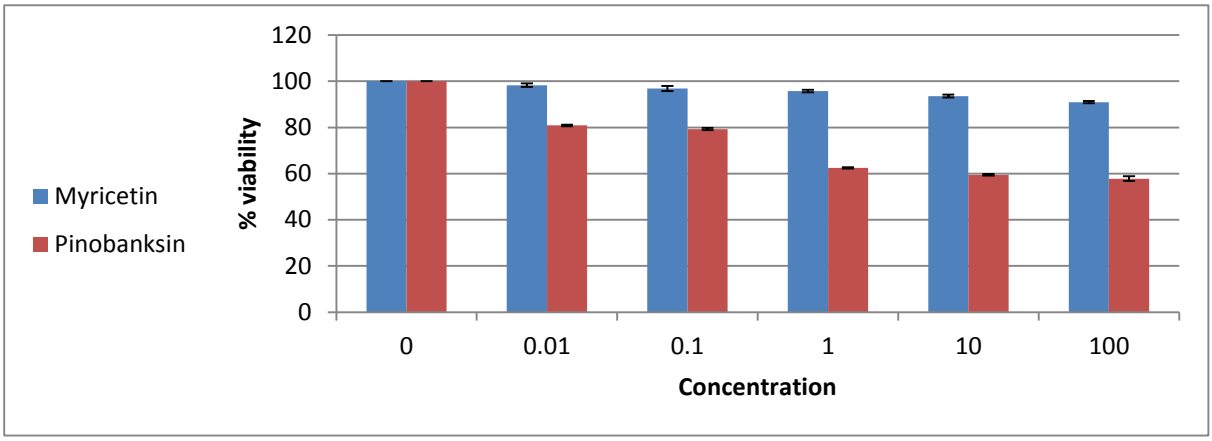

Fig. 2: Cytotoxic activity of the isolated compounds against SKOV-3human cell line *in vitro*

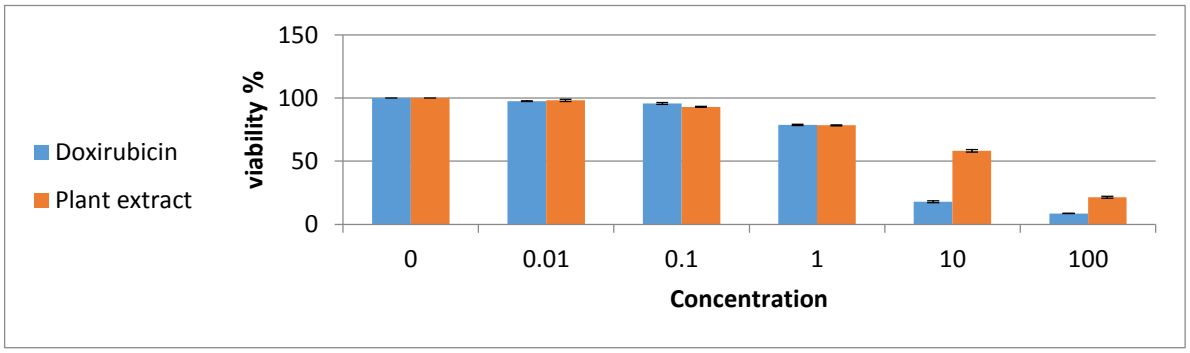

Fig. 3: Cytotoxic activity of 70% methanol extract of *Cichorium intybus* herb against PC-3 human cell line *in vitro*

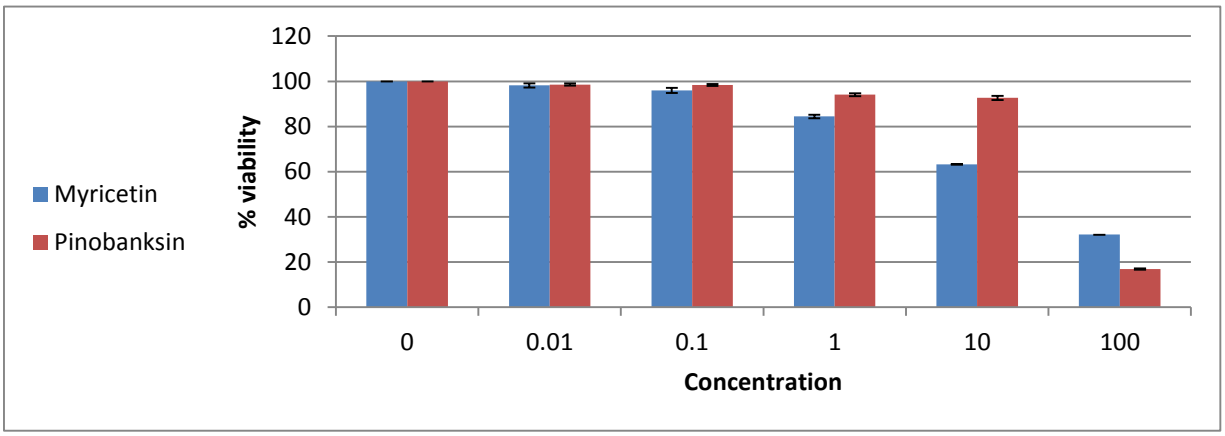

Fig. 4: Cytotoxic activity of the isolated compounds against PC-3 human cell line *in vitro*

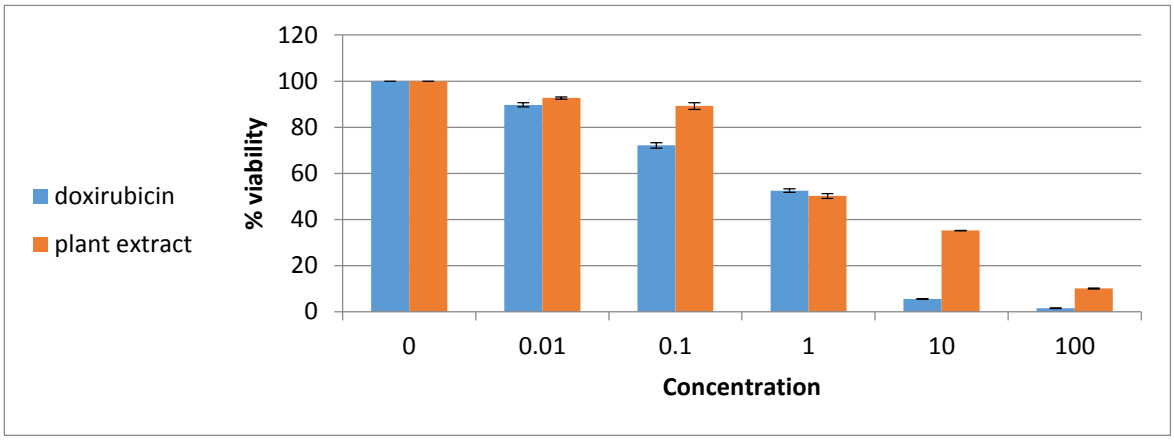

Fig. 5: Cytotoxic activity of 70% methanol extract of *Cichorium intybus* herb against HEPG-2 human cell line *in vitro*

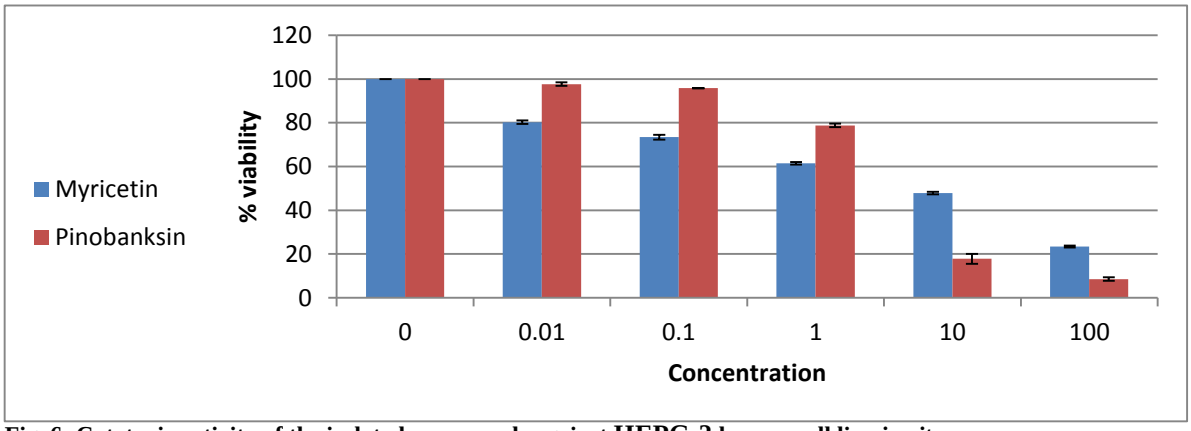

Fig. 6: Cytotoxic activity of the isolated compounds against HEPG-2 human cell line *in vitro*

TABLE 1: IC_{50} values (µg/ml) of 70% methanol extract of *Cichorium intybus* herb and the isolated compounds in *vitro* on different human cell line

Type of cell line	IC_{50} µg/ml			
	70% methanol extract of *Cichorium intybus* herb	Myricetin	Pinobanksin	Doxorubicin
SKOV-3	>100	>100	>100	0.97
PC-3	25.34	36.24	42.53	5.37
HEPG-2	0.95	4.26	7.23	0.65

Table 1 represents IC_{50} values (µg/ml) of 70% methanol extract of *Cichorium intybus* herb as well as the isolated compounds in *vitro* against the tested human cell lines**,** the results revealed that 70% methanol extract of *Cichorium intybus* herb as well as the isolated compounds (myricetin and pinobanksin) showed cytotoxic activity against HepG2, SKOV-3 and PC-3 cell lines with different IC_{50} values**,** by comparing these results with that of doxorubicin, it was found that the methanol extract, myricetin and pinobanksin possessed a potent cytotoxicity against HepG2 (IC_{50} =0.95,4.26 and 7.23 µg/mL), respectively, moderate cytotoxicity against PC-3 (IC_{50} =25.34,36.24 and 42.53 µg/mL), respectively, and weak cytotoxicity against SKOV-3 (IC_{50} >100 µg/mL) for all tested samples. Moreover, the effect of methanol extract *Cichorium intybus* herb as cytotoxic against the tested cell lines more than that of the isolated compounds**.** To explain this result **,**we can point to the synergistic effect of the phytoconstituents in the methanol extract which play an important role in the biological activities of the plant extract. In addition, myricetin showed more potent activity as cytotoxic than that of pinobanksin. A previous research revealed that myricetin showed cytotoxic towards a number of human cancer cell lines, including colon, pancreatic, skin and hepatic cancer cells. Moreover, it inhibits the enzymes involved in cancer initiation and progression **[37].**

Although methanol extract showed a potent cytotoxicity against HepG2, a previous research stated that both doxorubicin and *Cichorium intybus* extract were safe against the normal cell line **[38].**
In addition, *Cichorium intybus* was considered a safe drug and during the administration of designated therapeutic dosages of *Cichorium intybus* extract no side effects or health hazards were reported **[26].**
Cichorium intybus herb contains different phytoconstituents that play an important role in the bioactivity of plant extract. This is in agreement with what was reported by Alnajjar and Elsiesy which revealed that the phytocompounds showed a potent activity against hepatocellular carcinoma **[39].** Previous researches revealed that *Cichorium intybus* roots, seeds and leaves showed a potent cytotoxic activity against different cell lines including human leukemia HL-60, amelanotic melanoma C32 , human prostate cancer PC-3, human breast carcinoma T47D and colon cancer RKO cell **[40- 42, 27].**

Molecular modeling

The isolated compounds (myricetin and pinobanksin) showed a potent activity against human liver HepG-2 cancer cell line. Molecular docking was performed to investigate if these compounds have a similar mechanism as tubulin protein inhibitors. Docking experiment was used to investigate the binding affinity of the active compounds to the binding residue of the active site of tubulin protein. Molecular docking analysis confirmed that the isolated compounds (myricetin and pinobanksin) strongly interact with active site of tubulin protein with highest binding energies supported that the high cytotoxicity of these compounds against human hepatocellular cancer. As shown in the figures 7,8 and 9, the isolated compounds as well as native ligand (colchicine) were docked inside the receptor binding site of tubulin protein. The binding energy score of myricetin, pinobanksin and colchicine were -10.3, -9.2 and -10.4 kcal/mol, respectively. From this analysis, myricetin showed higher negative binding energy than pinobanksin compared with native ligand.

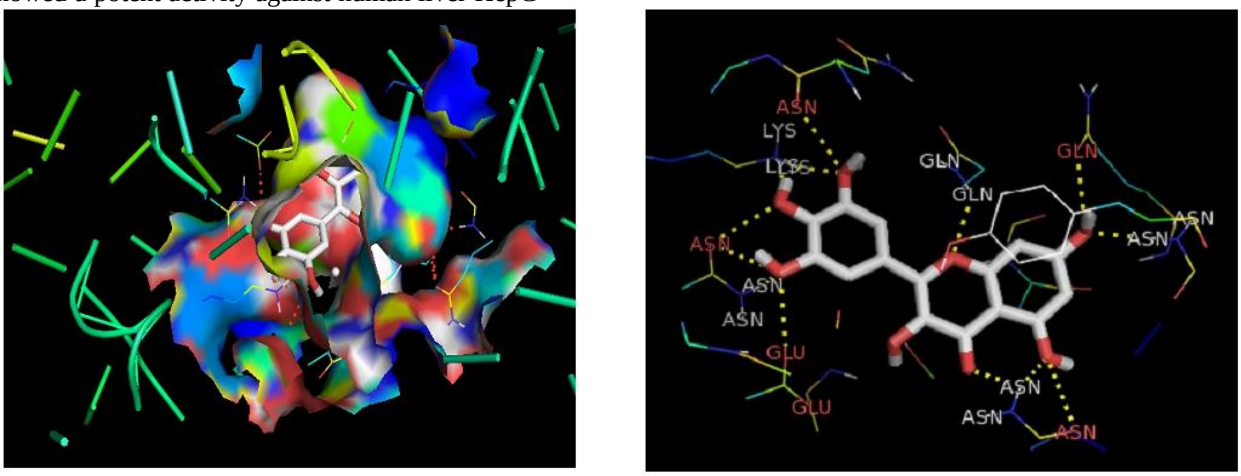

Fig. 7: Binding disposition and ligand-receptor interactions of myricetin inside tubulin protein binding site

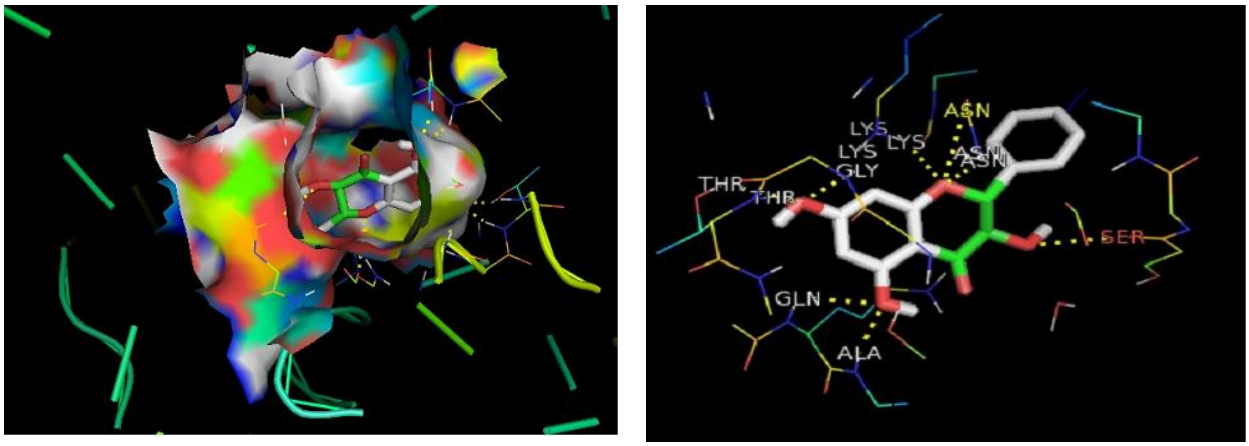

Fig. 8: Binding disposition and ligand-receptor interactions of Pinobanksin inside tubulin protein binding site

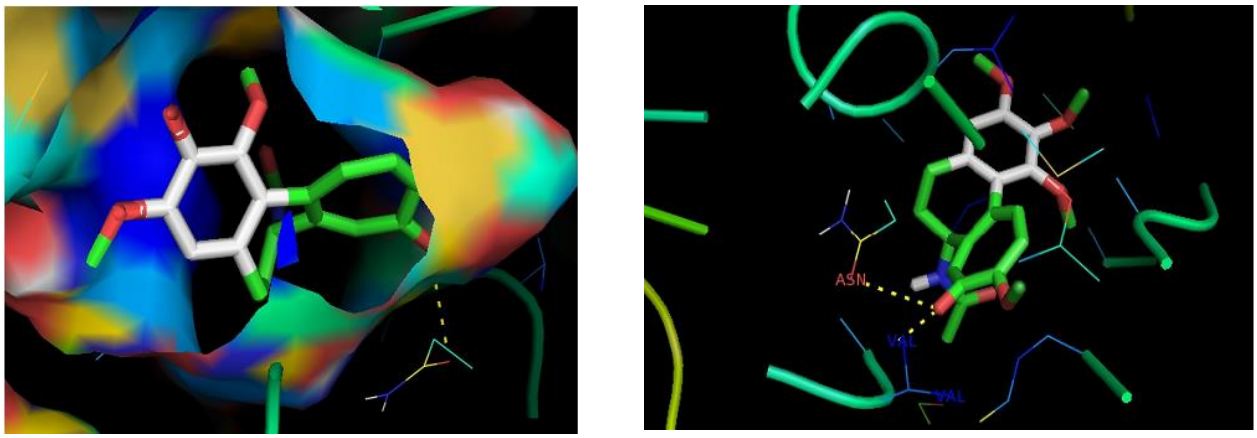

Fig. 9: Binding disposition and ligand-receptor interactions of native ligand (colchicine) inside tubulin protein binding site.

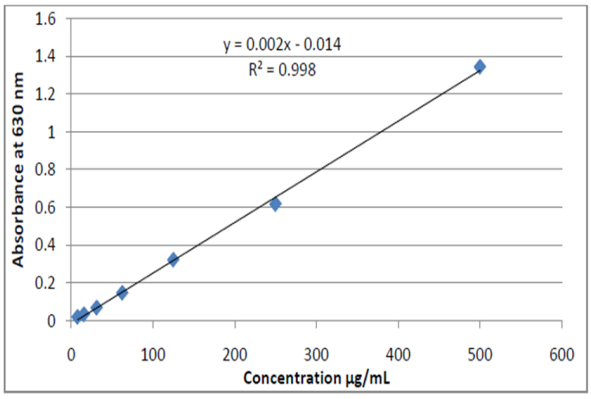

Fig. 10: Calibration curve of gallic acid standards absorbance

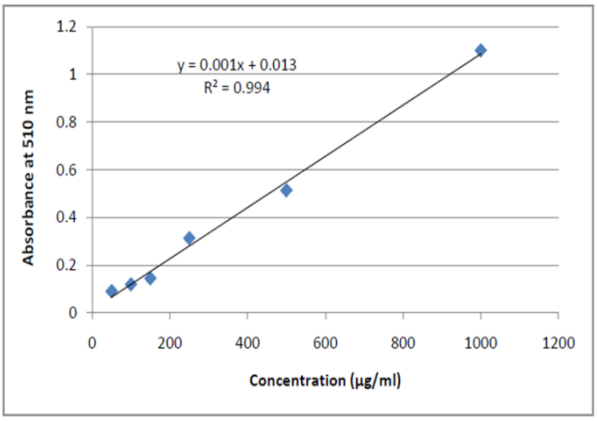

Fig.11: Calibration curve of rutin standards absorbance

Phytochemical study

Phytochemical screening

The results revealed the presence of diversity of phytoconstituents in 70% methanol extract of *Cichorium intybus* herb such as; flavonoids, tannins, carbohydrates and/or glycosides, sterols and/or triterpenes, alkaloids and/or nitrogenous compounds ,saponins and coumarins. These phytoconstituents showed different biological activities **[5, 43].**

Total Phenolics

Different absorbance of Gallic acid got in the different concentrations. The average of the readings of the 6 replicates was taken to illustrate the calibration curve. Average Absorbance at 630 = 1.2996 (Standard Deviation = 0.053). By substitution in the linear regression equation, the total phenolics was 656.8 /ml (Standard Deviation = 26.7).
-Total phenolics (Gallic Acid Equivalent) = 101.05 mg / g extract (Standard Deviation = 4.1).
This result was shown in table 2 and figure 10.

TABLE 2: Gallic acid standards absorbance

Concentration (µg/ml)	Absorbance
500	1.343
250	0.618
125	0.323
62.5	0.148
31.2	0.07
15.6	0.034
7.8	0.021

Total flavonoid

Different absorbance of rutin got in the different concentrations. The average of the readings of the 6 replicates was taken to illustrate the calibration curve. Average absorbance at 510 = 0.4306 (Standard Deviation = 0.012). By substitution in the linear regression equation, the total flavonoids were 417.6 µg/ml (Standard Deviation = 11.8)
-Total flavonoids (Rutin Equivalent) = 64.25 mg / g Extract (Standard Deviation = 1.82)
This result was shown in the table 3 and figure 11.

TABLE 3: Rutin standards absorbance

Concentration (µg/ml)	Absorbance
1000	1.0995
500	0.5124
250	0.3125
150	0.1436
100	0.1186
50	0.09

From these results, it was found that70% methanol extract of *Cichorium intybus* herb is a rich source of polyphenolic compounds which play a vital role in the plant bioactivities. This result is similar to what was reported by Gerber which indicated that the very common phytocompounds are phenolic acids, which include chlorogenic acids, and flavonoids (anthocyanins, flavanols, flavanone, and flavan-3-ols) , these compounds have extensive biological activities such as antioxidant, and anticancer activities **[43].**

HPLC analysis of polyphenolic compounds

The result of HPLC analysis of polyphenolic compounds showed that thirteen polyphenolic compounds were identified representing 85.2% of the total area, the major compound was caffeic acid **(**15.9%) followed by methyl gallate (13.6%), catechin (12.1%) and rutin (10.1%). This result was shown in table 4 and figure 12. The previous research on chicory (*Cichorium intybus*) roots revealed the identification of lactucin as a major compound by HPLC **[44].** Previous study recorded the extraction of phenolic acids and flavonoids from several types of *Cichorium intybus* and the characterization of the compounds using high-performance liquid chromatography electrospray ionization /mass spectrometry. Sixty-four compounds were detected, which include several hydroxyl cinnamic acid derivatives comprising eight mono- and dicaffeoylquinic acids, three tartaric acid derivatives, thirty-one flavanol and two flavone glycosides, and ten anthocyanins as well as several isomers of caffeic acid derivatives **[45].** Recently, polyphenolic compounds showed different biological activities **[46, 47].**

Structure elucidation and Identification of the isolated compounds

Compound (1): 8 mg, yellow powder. EI-MS: *m/z* 318, UV spectral data are similar to those of **[48] .** It was obtained as yellow green spot and gave a bright yellow color when spraying with AlCl3 and by comparing the spectral data of compound 1 with the published data **[48],** compound 1 was identified as myricetin.

Compound (2): 12 mg, white crystals with *Rf* values 0.97 cm (BAW) and 0.1cm (15% HOAc) on PC. EI-MS *m/z*:272 (calculated for $C_{15}H_{12}O_5$), *m/z* 78 (C_6H_6), indicating that ring B is free of hydroxylation ^{1}H-NMR spectral data are similar to those of **[49].** It appeared as dark spot under UV light changing to yellowish green by spraying with aluminium chloride reagent, and by comparing the spectral data of compound 2 with the published data **[49],** compound 2 is identified as pinobanksin.

Figures 13 & 14 showed 2Dand 3D images of the isolated compounds.

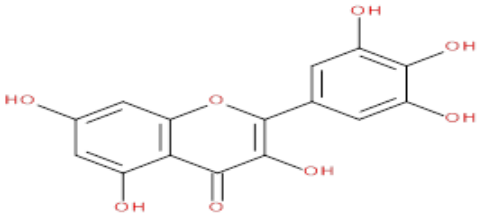

Fig. 13: A. 2D image of Myricetin

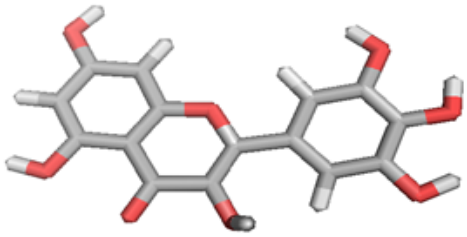

Fig. 13 B. 3D image of Myricitin

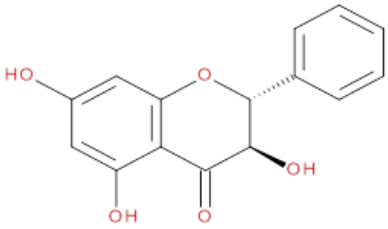

Fig. 14: A. 2D image of Pinobanksin

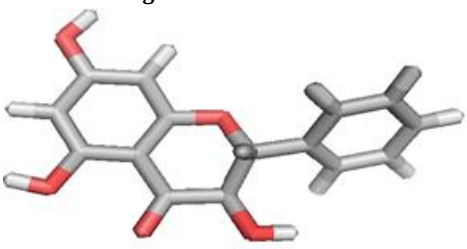

Fig. 14: B. 3D image of Pinobanksin
Different colors represent different atom types; Oxygen (red), and Carbon-Hydrogen skeleton (Grey-white).

TABLE 4: HPLC analysis of polyphenolic compounds in 70% methanol extract of *Cichorium intybus* herb

No	1	2	3	4	5	6	7	8	9	10	11	12	13
Polyphenol	Gallic acid	Chlorogenic acid	Catechin	Methyl gallate	Caffeic acid	Rutin	Ellagic acid	Coumaric acid	Vanillin	Ferulic acid	Naringenin	Taxifolin	Kaempferol
Area %	1.3	6.9	12.1	13.6	15.9	10.1	1.7	9.6	3.9	4.7	2.1	0.4	0.2

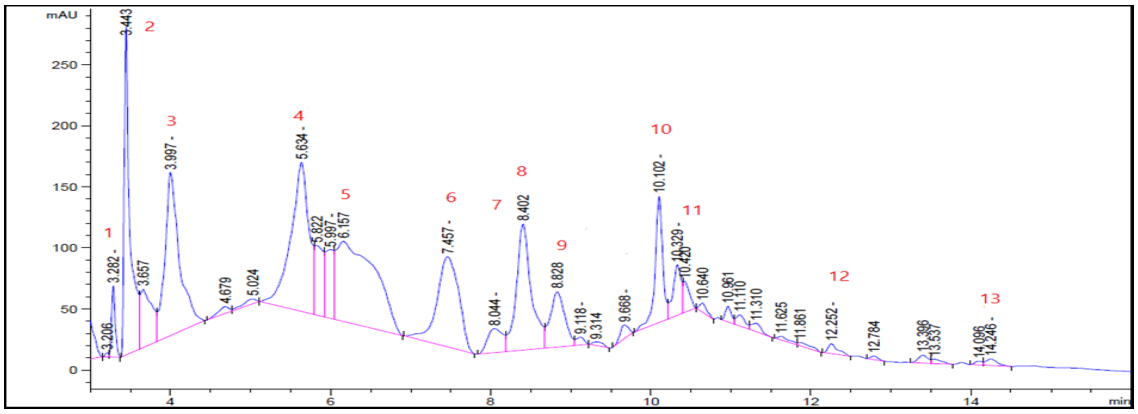

Fig. 12: HPLC analysis of polyphenolic compounds in 70% methanol extract of *Cichorium intybus* herb

Conclusion

The current research showed that the methanol extract of *Cichorium intybus* as well as the isolated compounds (myricetin and pinobanksin) had potent cytotoxic activities against HepG2 cell line and moderate activities against prostate cell line. Moreover, the methanol extract showed a promising cytotoxic effect more than that of the isolated compounds .The activity of methanol extract of *Cichorium intybus* may be due to its chemical constituents that were found in different concentrations and were confirmed by HPLC analysis. Molecular docking analysis confirmed that the isolated compounds (myricetin and pinobanksin) strongly interact with active site of tubulin protein with highest binding energies. Further researches are needed to perform clinical trials aiming to enter the field of drug discovery.

Conflict of interests

There are no conflicts of interest.

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النشاط السام للخلايا ودراسة الالتحام الجزيئى والفحص الفيتوكيميائى على نبات الشيكوريا

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الغرض من هذه الدراسة هو تقييم النشاط السام للخلايا لعشب الشيكوريا (*Cichorium intybus*) وكذلك التعرف على الآلية الجزيئية للسمية الخلوية. بالإضافة إلى ذلك ، فإنه يهدف إلى فحص المكونات النباتية المسؤولة عن النشاط البيولوجي. تم تقييم النشاط السام للخلايا لعشب *Cichorium intybus* بواسطة SRB(Sulforhodamine B) ضد خلايا سرطان المبيض (SKOV-3) و خلايا سرطان الكبد (HepG2) و خلايا سرطان البروستاتا (PC-3). تمت دراسة التأثير على بلمرة توبولين للتعرف على آلية السمية الخلوية. تم فحص اليةالارتباط بالجزيء المستهدف من خلال دراسة الالتحام. بالإضافة إلى ذلك ، تم فصل مركبين من مركبات الفلافونويد وتم التعرف عليهما بطرق طيفية مختلفة. أظهرت النتائج أن مستخلص الميثانول لعشب *Cichorium intybus* وكذلك المركبات المفصولة (myricetin و pinobanksin) لهم سمية خلوية قوية ضد خلايا سرطان الكبد (HepG2) (IC_{50}= 0.95 و 4.26 و 7.23 ميكروغرام / مل) ، على التوالي ، سمية متوسطة للخلايا ضد خلايا سرطان البروستاتا (PC-3) (IC_{50} = 25.34، 36.24 و 42.53 ميكروغرام / مل) ، على التوالي ، وسمية خلوية ضعيفة ضد خلايا سرطان المبيض (SKOV-3) (IC_{50}> 100 ميكروغرام / مل) لجميع العينات المختبرة. أكد تحليل الالتحام الجزيئي أن كلا المركبين المعزولين أظهرا تقاربًا عاليًا للارتباط مع موقع ربط الكولشيسين لأنابيب التوبولين الدقيقة ، مما يدعم السمية الخلوية العالية لهذه المركبات.