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The Effect of *Cynara cornigera* L. in HepG2 Hepatocellular Carcinoma Cells

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Abstract: Amongst all cancer types, liver cancer is the fourth leading cause of cancer mortality. It is frequently stated as hepatocellular carcinoma (HCC) and occurs in hepatocytes. Genetic alterations of hepatocytes such as Wnt/β-catenin and JAK / STAT signaling pathways play a key role for the development of the HCC. Currently, there are a few available treatments for HCC; such treatments include transplantation, surgical resections and anti-cancer drugs. Most of the anti-cancer drugs target the signaling pathways for achieving an effective treatment. However, these treatments have some undesirable side effects. Thus, there is a need for discovering alternative anti-cancer agents with no or lesser side effects. Plant constituents are promising anti-cancer agents. *Cynara cornigera* L. contains plenty of phenolic compounds including quercetin, apigenin, etc. This study aimed to analyze the anti-cancer property of the fractionated methanol extract of the flowers of *C. cornigera*. All the fractions obtained were analyzed to determine the cytotoxic activity on HepG2 cells. Two of the fractions containing polyphenolic compounds had a significant cytotoxic activity related to non-canonical Wnt11 signaling pathways on HepG2 cells.

Keywords: *Cynara cornigera*; phenolic compounds; HepG2; cytotoxicity; Wnt11; liver cancer.

1. Introduction

The liver is the largest internal organ of the human body with multi-functional and complex properties [1]. Many diseases such as hepatitis -B, -C and cancer may affect the functioning of this

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fundamental organ. Cancer occurs when there is uncontrolled proliferation of the cells within the body and it is the leading cause of death worldwide after cardiovascular diseases [2,3]. Amongst all cancer types, liver cancer is the fourth most frequent cancer leading to death and the sixth most prevalent cancer type worldwide [4]. Liver cancer is frequently referred to as Hepatocellular Carcinoma (HCC) which occurs in the hepatocytes; a specific type of liver cell [5].

Untreated Hepatitis –B, -C infection may lead to cirrhosis and the development of HCC through years [6,7]. Liver diseases are a worldwide problem, but they occur particularly frequently in developing countries; such as China and sub-Saharan Africa [8,9]. HCC occurs with approximately 80% of all cases of cirrhosis. [10]. Other risk factors contributing to liver disease are tobacco and alcohol consumption, aflatoxin exposure, obesity, diabetes, non-fatty liver disease and diet [11]. In addition to these risk factors, regional differences play a significant role in developing HCC associated with liver diseases [7].

The process of developing HCC involves interactions between different cellular mechanisms such as hypoxia, oxidative stress, necroinflammation and changes in the microenvironment of a tumor [12]. HCC can occur as a consequence of both genetic and epigenetic alterations [13]. Transcription factors with genetic alterations such as the dysregulation of the Wingless-related integration site and β-catenin complex (Wnt/β-catenin), and Janus kinase-signal transducer and activator transcription factor (JAK / STAT) pathways have devastating effects on the pathogenesis of HCC [14]. Wnt/β-catenin pathway activation leads to increased cell proliferation, angiogenesis, decrease of apoptosis and formation of the extracellular matrix supporting the generation of the tumor [15-18]. Activation of Wnt3a which is a member of Wnt protein family leads to cell proliferation in HepG2 cells [19]. On the other hand, non-canonical Wnt11 inhibits cell proliferation and migration of hepatocellular carcinoma [20]. Moreover, β-catenin has a distinct role in the nucleus, affecting the Wnt/β-catenin signaling pathway which leads to cell proliferation and angiogenesis in HCC [18,21].

Another important transcription factor involving hepatocellular carcinoma is Stat3. Its function is to increase cell proliferation, differentiation and affect cell survival. Stat3 functions as an oncogene as well as a tumor supressor, that blocks cell apoptosis and suppresses tumor growth in HCC upon its inhibition [22,23]. Transcription factor Jak2 is also a frequently active agent for cancer progress [24] JAK/STAT pathway thus leads to dysregulation of genes which control metastasis, angiogenesis and cell survival in HCC [25]. Activation of JAK / STAT pathway occurs in tumor cells and it causes progression of cancer.

There are different treatment options for HCC, such as liver transplantation, surgical resections and particularly, when surgical sectioning is not possible, pharmaceuticals such as sorafenib, regorafenib, nivolumab cabozantinib, ramucirumab [12,13]. All drugs used to treat HCC have serious side effects; particularly stomach pain, loss of hair, decrease of white blood cell (WBC) count, diarrhea and fatigue [26]. Also, HCC is highly resistant to chemotherapy [12]. Therefore, researchers are in the search of a treatment to reduce or do away with negative effects of current treatment methods. In this context, compounds in plants with anti-cancer activity have great potential to find a treatment for cancer.

There are a large variety of compounds such as phenolic molecules which are produced by plants in nature as secondary metabolites. Phenolic compounds are an important group of phytochemicals biosynthesized by plants [27].

Polyphenols have pharmaceutical properties such as anti-inflammatory, anti-microbial, anti-cancer and anti-oxidant activities [28]. Flavonoids are also polyphenolic compounds naturally occurring in almost every fruit and vegetables [29]. In this sense, *Cynara cornige*ra L. is rich in polyphenolic compounds and also flavonoids [30,31].

C. cornigera (wild artichoke) belongs to the family *Asteraceae*. Ten *Cynara* species exist in nature and six of them (*Cynara syriaca, Cynara scolymus, Cynara makrisii, Cynara cyrenaica, Cynara cornigera, Cynara cardunculus*) grow in Cyprus. The plant is used in folk medicine for various liver diseases in Cyprus. Previous studies have shown that compounds of *C. cornigera* possess good treatment and hepatoprotective activity in animals with liver damage [30]. *C. cornigera* has plenty of flavonoids such as quercetin, apigenin, kaempferol etc. [31]. Quercetin, in particular, is an inhibitor of cell migration, apoptosis and invasion in hepatocellular carcinoma [32].

Investigation of cytotoxic effect of the flower part of *C. cornigera* methanol extract fractions on the HepG2 liver cancer cells and also identification of their anti-carcinogenic effect on Wnt11, Wnt3a, Stat3, Jak2 and β-catenin signaling pathways constituted the aims of this study.

2. Materials and Methods

2.1. Plant Materials

C. cornigera samples were collected in May 2018 from Akdoğan in North Cyprus. A voucher specimen is deposited in Near East University Herbarium with the code NEUN6893.

2.2. Extraction and Fractionation

The flowers of *C. cornigera* (300 g) were treated with 700 mL MeOH. The extract was evaporated till dryness by using a rotary evaporator (Buchi® (Model: R-210 / Water Bath: B-491 model / Vacuum Pump: V-700 model / Vacuum Pump Control Panel: V-850 model and Water Coolant: Thermo EZ Cool 80) to yield 18.7 g total extract (yield: 6.23%). The dried extract was partitioned with dichloromethane (DCM, Merck, 1.06009.2500) (100 mL) and distilled H_2O (3 × 250 mL). DCM (S_1) and H_2O (S_2) phases were separated and evaporated separately till dryness.

S_2 (8.4 g) was dissolved in 100 mL distilled H_2O and fractionated on a polyamide column (75 g [(Fluka Polyamides – Sigma-Aldrich, 63428-83-1) 5-10 µm] R=3cm h = 41cm). For the elution, H_2O and H_2O-MeOH mixtures with increasing proportion of MeOH were used. 38 obtained fractions were separately evaluated with Thin Layer Chromatography (TLC, TLC Silica Gel 60 F254, Merck, 1.05554.0001) profiles. The fractions were combined according to the TLC results into 6 combined fractions (Fr. A – F). The combined fractions were concentrated and dissolved in H_2O and frozen. The frozen fractions were lyophilized using freeze-dryer (Christ® - Model: Alpha 1-4 LD plus).

2.3. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Analysis

LC-MS/MS analysis was carried out using an Absciex 3200 Q trap MS/MS dedector. Experiments were performed with a Shimadzu 20A HPLC system coupled to an Applied Biosystems 3200 Q-Trap LC- MS/MS instrument equipped with an ESI source operating in negative ion mode. For the chromatographic separation, a GL Science Inertsil ODS - 3 250 × 4.6 mm, 5 µm particle size, analytical column operating at 40º C has been used. The solvent flow rate was maintained at 0.5 mL/min. Detection was carried out with PDA detector. The elution gradient consisted of mobile phases (A) methanol:water:formic acid (10:89:1, v/v/v) and (B) methanol:water:formic acid (89:10:1, v/v/v). The composition of B was increased from 10% to 100% in 40 min. LC-ESI-MS/MS data were collected and processed by Analyst 1.6 software.

2.4. Cell Line and Cell Culture

The human hepatocellular carcinoma cell line (HepG2, ATCC® HB-8065™) was used. HepG2 cells were cultured in RPMI-1640 (Capricorn Scientific, RPMI-A) medium supplemented with 10% heat inactivated fetal bovine serum (FBS, Capricorn Scientific, FBS-HI-11B), 1% penicillin-streptomycin (Capricorn Scientific, PS-B) and 1% L-glutamin (Capricorn Scientific, GLN-B) at 37 ˚C and in a 5% CO_2 containing humidified chamber. They were sub-cultured with 0.25% tripsin-EDTA solution (Capricorn Scientific, TRY-1B) when they reached at least %75 confluent situation.

2.5. Cell Viability and Cell Growth

Cytotoxicity and cell viability analysis was evaluated with MTT test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Glentham Life Sciences, 471OVO). Following trypsinization of HepG2 cells, they were seeded into 96 well plate and each well contained $5x10^3$ cells. After 24h incubation, five different concentrations (5, 10, 25, 50 and 100 µg/mL) of each fraction were prepared by diluting with the medium. Negative control group did not contain any fraction or cell, while positive

control group contained only HepG2 cells. HepG2 cells were incubated for 24, 48 and 72 hours for MTT analysis. Then, 10 µL MTT solution was added to each well and all the wells were incubated for 4 hours at 37 ˚C and in a 5% CO_2 containing humidified chamber. After incubation time, 50 µL Dimethyl Sulfoxide (DMSO, Biomatik, A2424-500ML) was added to each well to dissolve the formazan crystals and absorbance was measured with spectrophotometer (VersaMax, Molecular Device, Sunnyvale, USA) at 570 nm. All the experiments were repeated three times.

2.6. Reactives and Applications

Fractions which were dissolved as 100 mM concentration in Dimethyl Sulfoxide (DMSO, Biomatik, A2424-500ML) were diluted by the HepG2 culture medium. For all fractions, concentration of DMSO was less than 0.05%. Different concentrations (5, 10, 25, 50 and 100 µM) of fractions were separately treated to the cells for determining the active dose and cytotoxicity.

2.7. Immunocytochemistry

Cultured HepG2 cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, STBH3693) in phosphate buffer saline (PBS) at 4 ºC for 30 minutes. After the cells were washed with PBS for three times, % 0.1 Triton X-100 (Sigma, 9002-93-1) was added to the cell permeabilization and incubated for 15 minutes on ice. HepG2 cells were washed with PBS for three times and incubated with %3 hydrogen peroxide (H_20_2, Riedel-de Haën, 70570) to inhibit the activity of endogen peroxidase for 5 minutes at room temperature. After washing the cells with PBS three times for 5 minutes, a blocking solution (Histostain – Thermo Fisher Plus Kit HRP, 859043) was added and incubated for 1 hour. After discharging of the blocking solution without washing, the cells were incubated with primer antibodies which are against Wnt11 (Elabscience, E-AB-10836), Wnt3a (St John's Laboratory, STJ115662), Stat3 (St John's Laboratory, STJ27462), Jak2 (St John's Laboratory, STJ90945) and β-catenin (Proteintech, 51067-2-AP) for overnight at 4 ºC. After washing with PBS for 5 minutes three times, the cells were incubated with a biotinylated-secondary antibody (Protein Novex Life Technologies, 1666262A) for 30 minutes. After washing again with PBS, 100 µl Horseradish peroxidase (HRP) – Streptavidin complex (Protein Novex Life Technologies, 1666262A) was added and incubated at room temperature for 30 minutes. The cells were washed with PBS and they were incubated in diaminobenzidine (DAB, ScyTek Laboratories, ACK125) for 5 minutes in order to screen for the immunoreactivity. After washing the cells with distilled water, Mayer's Hematoxylin solution was applied to counterstain for 5 minutes. All the samples were mounted with the mounting medium (Merck Millipore, 107961, Germany) and examined with light microscope (Olympus CX31, CX31RTSF). The staining of Wnt11, Wnt3a, Stat3, Jak2 and β-catenin were determined semi-quantitatively by using Histologic-Score (H-Score). H-Score was calculated as; H-Score = Σл (i + 1) equation. In the equation, i is intensity of staining and value of i can be 1 (mild), 2 (moderate) and 3 (strong). Л is the percentage of cells which were dyed each intensity (values changing from 0% to 100%).

2.8. Statistical Analysis

Data were stated as mean standard deviation. Results were evaluated by using Graphpad Prism 8 and SPSS 20.0. For comparison of continuous data between multiple groups, One Way Analysis of Varience, ANOVA Analysis was used. When they were statistically significant, Tukey test was used as a post-hoc test for paired comparisons. IC_{50} values and graphics were determined by using Graphpad Prism 8.

3. Results and Discussion

3.1. Extraction and Fractionation

DCM and H_2O phases of the methanolic extract (18.7 g) were evaporated till dryness yielding 10.3 g DCM extract (S_1, 55.08%) and 8.4 g H_2O extract (S_2, 44.92%). The methanol extraction yielded 6.23g extract per 100g of dried flowers of *C. cornigera*.

S_2 (8.4 g) was dissolved in distilled water and loaded on a polyamide column. 38 fractions (fraction volume: 50 mL) were collected from the fractionation of S_2. First eight fractions (total volume, 400 mL) were combined according to TLC results, but the combination of first eight fractions were not evaporate till dryness. Also, they did not freeze due to possibility of sugar. Therefore, mass of solid sample of first eight fractions ingredients was not determined.

Remaining thirty fractions were combined into six fractions (Frs. A – F) according to the TLC results. The combined fractions were evaporated till dryness and frozen. The frozen fractions were freeze-dried and solid samples were weighted. Amounts of the fractions are shown below (Table 1). S_1 was not fractionated due to chlorophyll or chlorophyll residues.

Table 1. Amount of freeze-dried fractions

Fraction	Amount
Fr A	469.9 mg
Fr B	195.3 mg
Fr C	269 mg
Fr D	132.4 mg
Fr E	208.6 mg
Fr F	393.1 mg
Total	1668.3 mg

3.2. Compound Identification of Fractions with LC-MS/MS

A total of twenty two compounds were identified with LC-MS/MS analysis from all fractions (Table 2). The diagnostic fragment ion based identification method was used to identify the compounds. “Hierarchical key for identification of chlorogenic acid derivatives” of Clifford [34] was used for the identification of chlorogenic acid derivatives. On the otherhand, flavonoid derivative identification was completed by using previously published LC-MS/MS analysis of several *Cynara* species, refer to Table 2. Flavonoid derivatives as flavone or flavonol derivatives and quinic acid esters were identified. All of these compounds were previously determined in several *Cynara* species.

Flavone or flavonol derivatives identified with LC-MS/MS were not precisely identified due to their similar chemical compositions and molecular weight. For this reason, these compounds were given together at the same retention time in Table 2. It was avoided to use a precise identification of these compounds such as kaempferol and luteolin. Because it is not possible to precisely identify a compound without using standard reference compounds with LC-MS analysis. But according to previous studies, *Cynara* species contain luteolin derivatives rather than kaempferol derivatives.

Apigenin and apigenin derivatives were identified from the fragment ion peak at m/z 269 $[M-H]^-$ arising from apigenin aglycone. Additionally, the characteristic fragments resulting from the cleavage of the interglycosidic linkages at m/z 309, 162, and 176 arising from the rhamnopyranosyl-glucopyranosyl (= rutinosyl) and sugar units (glucopyranosyl and glucuronopyranosyl), respectively.

Methoxyflavonoid derivaties are thought to be identified , such as, unknown 300 molecular weight methoxyflavonoid rutinoside and unknown polymethoxyflavonoid contain methoxy groups due to their moleculer weights and fragmentations.

Table 2. LC – MS/MS analysis results of fractions A, B, C, D, E, F

RT	*m/z* [M-H$^-$]$^-$	Fragments	Identified as	Fraction	Reference
11.9	353	191, 179, 135	3-*O*-Caffeoylquinic acid	A,B	[34, 35]
12.1	353	191, 179, 135	5-*O*-Caffeoylquinic acid	A,B,C,D, E	[34, 36]
13.7	515	353, 335, 179	1,3-Dicaffeoylquinic acid	D,E	[34, 35]
15.3	337	191, 173, 163	5-*p*-Coumaroylquinic acid	A	[34, 37]
16.6	367	191, 179, 135	3-*O*-Feruloylquinic acid	A	[34, 36]
17.3	623	447, 285	Luteolin 7-glucoside 3'-glucuronide / Kaempferol-glucoside-glucuronide	E	[36]
18.9	463	301	Quercetin-3-glucoside	E	[38]
20.2	515	353,335, 191,179	1,5-dicaffeoylquinic acid	D,E,F	[34, 36]
20.6	593	285, 151	Luteolin-7-rutinoside / Kaempferol-3-rutinoside	A,B,C,D	[36]
20.9	615	515,453,353, 191	Dicaffeoyl-succinoylquinic acid	E,F	[39]
21.3	433	271, 177, 151	Naringenin glucoside	A	[38]
21.3	609	314, 299, 285, 271	Similar to Rhamnetin hexosyl pentoside / isorhamnetin hexosyl pentoside	A	
22.3	461	285	Luteolin glucuronide / Kaempferol glucuronide	E,F	[36]
22.7	577	269	Apigenin rutinoside	A	[36]
23.1	607	299, 284	methoxyflavonoid (like Diosmetin/kaempferide/hispidulin)	A	
23.3	431	268, 117	Apigenin glucoside	B,C,D	[36]
23.9	461	327,313,297,283, 269,254	Unknown polymethoxy flavonoid	B	
24.3	477	314, 299, 285	Isorhamnetin / rhamnetin hexoside	C,D	[40]
24.5	445	269, 113	Apigenin glucuronide	D,E,F	[36]
28.2	459	311,269	Apigenin derivative	E	
29.2	285	151, 133	Luteolin or Kaempferol	F	[36]
31.7	269	117	Apigenin	B,C,D,E	[36]

RT: Retention time

3.3. Cell Viability and Cytotoxicity

HepG2 cells were treated with freeze-dried fractions with concentrations ranging from 5, 10, 25, 50 100 µg/mL of *C. cornigera* extracts. MTT assay was used to determine the cell viability and cytotoxic effect of fractions on HepG2 cells for 24h and 48h. Two of the *C. cornigera* extracts (Fr B and Fr E) showed a decrease of HepG2 cell proliferation and a toxic effect in a dose and time-dependent manner (Table 3).

Table 3. Percentage of cell viability results of freeze-dried fractions of the methanolic extract of *C. cornigera* flowers treated in HepG2 cells, in dose and time-dependent manner.

Fraction	5 µg/mL		10 µg/mL		25 µg/mL		50 µg/mL		100 µg/mL	
	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
Fr A	90.82	92.56	95,20	83,92	96,47	85,13	91,43	70,34	84,47	59,24
Fr B	104.42	84.10	115,51	79,58	90,21	69,40	113,72	72,23	76,55	54,37
Fr C	106.37	99.38	108,15	100,20	121,78	90,18	121,95	95,25	115,49	83,06
Fr D	111.97	92.46	101,04	95,46	98,50	81,63	94,54	80,73	77,14	52,06
Fr E	93.40	91.53	99,19	89,14	101,73	83,31	82,76	80,74	84,22	55,06
Fr F	82.35	96.47	99,70	84,07	84,45	75,89	87,27	72,91	76,91	64,76

Percentage of cell viability was calculated based on the absorbance of the control group of related fraction as 100. Percentage of cell viability was determined by using the [(Absorbance of Fraction / Absorbance of Control Group) x 100] formula.

Cell viability of cells treated with Fr A, C, D and F did not decrease significantly. On the other hand, HepG2 cells treated with Fr B and Fr E showed a significant decrease of cell viability. This result shows that Fr B and Fr E have significant cytotoxic effect.

Fr A is the previous fraction of Fr B and Fr C is the next fraction of Fr B. Fr A and Fr C do not have a significant cytotoxic effect on HepG2 cells. Compounds of Fr B were compared with compounds of Fr A and Fr C. Unknown polymethoxy flavonoid was only determined in Fr A.

Fr D is the previous fraction of Fr E and Fr F is the next fraction of Fr E. For this reason, LC-MS/MS results of Fr E were compared with Fr D and Fr F LC-MS/MS results to determine the differences among them.

Fr B and Fr E which showed significant cytotoxic effect in HepG2 cells, were treated with the concentration range from 25, 50, 100, 200, 400 µg/mL for 24h, 48h and 72h.

Table 4. Percentage of cell viability of Fr B and Fr E compounds for 24h, 48h and 72h at the concentration ranging from 25, 50, 100, 200 and 400 µg/mL.

Fraction	**25 µg/mL**			**50 µg/mL**			**100 µg/mL**			**200 µg/mL**			**400 µg/mL**		
	24h	**48h**	**72h**	**24h**	**48h**	**72h**	**24h**	**48h**	**72h**	**24h**	**48h**	**72h**	**24h**	**48h**	**72h**
Fr B	102.83	96.39	91.76	110.31	83.75	81.88	87.16	7.32	63.28	65.75	26.95	11.21	9.07	4.59	3.74
Fr E	106.70	99.78	97.43	96.84	91.45	81.78	115.74	76.11	63.54	67.59	11.25	5.00	11.62	5.88	4.74

Fr A, B, C, D, E and F were performed at 5, 10, 25, 50, 100 µg/mL concentration range in MTT assay. IC_{50} values were calculated for all fractions for 24h and 48h by using Graphpad Prism 8 program. However, cell viability tests of Fr B and Fr E were repeated at 25, 50, 100, 200 and 400 µg/mL for 24h, 48h and 72h due to having significant cytotoxicity.

Table 5. 24h, 48h and 72h IC_{50} values for freeze-dried fractions

Fraction	24h IC_{50} Value (µg/mL)	48h IC_{50} Value (µg/mL)	72h IC_{50} Value (µg/mL)
Fr A	236.6	143.0	-
Fr B*	207.2	134.7	110.2
Fr C	39872	642.2	-
Fr D	255.0	99.0	-
Fr E*	220.4	128.5	97.87
Fr F	334.9	156.9	-

* Fraction which has significant cytotoxic effect.

Fr C showed the highest IC_{50} value and Fr B had the lowest IC_{50} value for 24h (Table 5). Fr C has the highest IC_{50} value and Fr D has the lowest IC_{50} value for 48h (Table 5). For 72h, IC_{50} value of Fr B was higher than Fr C (Table 5). IC_{50} values of Fr B and Fr E graphics are shown in Figure 1.

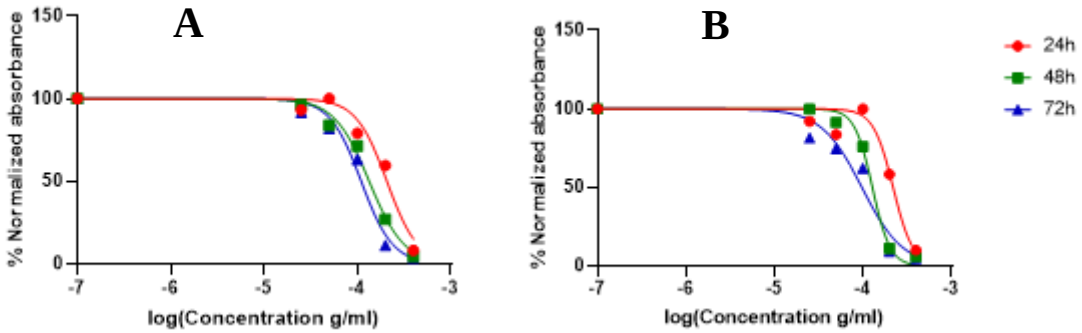


Figure 1. IC_{50} graphics of Fr A and Fr B for 24h, 48h and 72h on HepG2 cells

3.4. Cell Morphology

HepG2 cells are epithelial-like cells which grow epithelial colony morphology after 48-hour in culture. The morphology of HepG2 cells changed and the number of cells decreased after the cells were treated with Fr B and Fr E (Figure 2). Although we were unable to determine the precise concentration of each compound in a fraction, we can however speculate that the morphological occurred due to a synergetic effect.

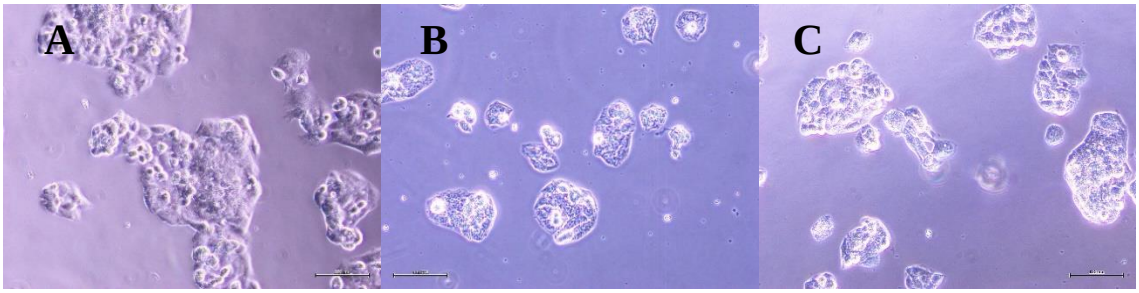


Figure 2. Microscope images of control (A), Fr B treated (B) and Fr E treated (C) HepG2 cells. Scale Bars = 100 µm

3.5. Immunocytochemical Evaluation

For immunocytochemical evaluation, 100 µg/mL of Fr B and Fr E were used separately to treat HepG2 cells for 48h. Concentration which was used for immunocytochemical investigation was determined from IC_{50} calculation.

While the immunostaining intensity of Wnt11 was weak in the HepG2 cell control group (Figure 3, A), immunoreactivity was moderate in both Fr B (Figure 3, B) and Fr E (Figure 3, C) treated HepG2 cells. Wnt11 intensity was higher in the cells which were treated with Fr B and Fr E than control group (Figure 3) and it was statistically significant ($p < 0.05$, Table 6). Thus, Wnt11 pathway may be activated by Fr B and Fr E and thus cell proliferation decreased. Presence of Wnt11 inhibits the stabilization of β-catenin and related signaling pathway. Consequently, the presence of Wnt11 inhibits hepatocellular carcinoma cell proliferation and migration [20]. In this study, non-canonical Wnt11 pathway could be activated by fractions B and E. Non-canonical Wnt11 related inhibition of proliferation is depend on β-catenin. Because of this reason, β-catenin level is important. Previous studies showed that activation of β-catenin pathway induces cell proliferation and inhibits apoptosis in many cancer types. Immunostaining intensity was strong in HepG2 cells which were treated with Fr B (Figure 3, O) and was moderate in HepG2 cells which were treated with Fr E (Figure 3, N). Immunostaining intensity of the control group (Figure 3, M) was weaker than Fr B and Fr E treated cells. Fr B and Fr E intensities were statistically higher and significant according to control group. ($p<0.05$, Table 6). In this research, β-catenin inhibition was expected but β-catenin pathway was not inhibited by Fr B and Fr E.

Other important pathway for cancer development is the canonical pathway and it is related with Wnt3a [41]. Immunostaining intensities of Wnt3a was strong in the control group (Figure 3, D), Fr B (Figure 3, E) and Fr E (Figure 3, F) treated cells. Therefore, Wnt3a intensity was strong in Fr B and Fr E and was not statistically significant in Fr B and Fr E treated cells when compared with the control group cells ($p>0.05$, Table 6). It previously has been shown that canonical activation of the Wnt3a pathway causes cell proliferation in HepG2 cells [19]. In this context, according to the strong staining intensity of Wnt3a in control group Fr B and Fr E were not effective for the inactivation of cell proliferation.

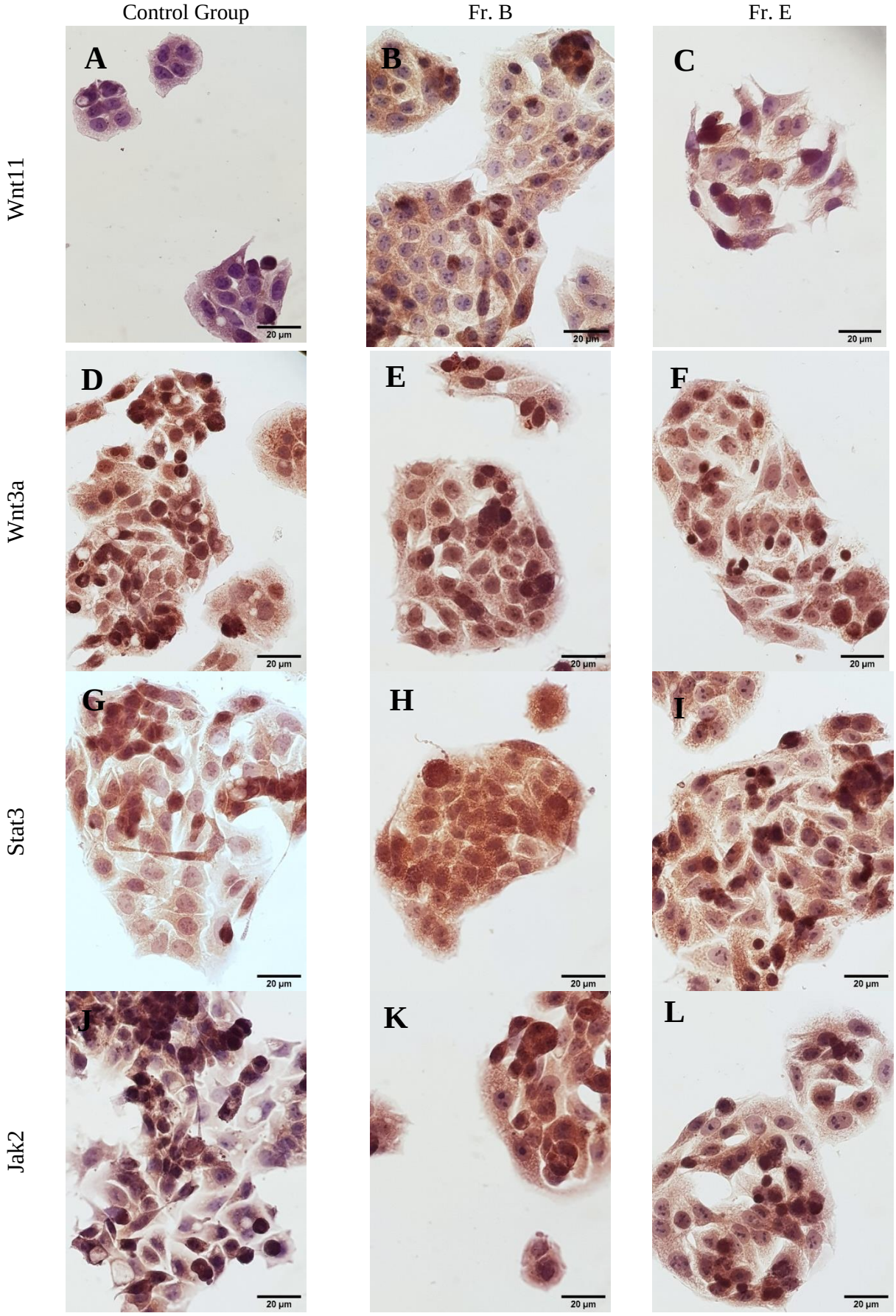
Immunostaining intensity of JAK2 was weak in the control group (Figure 3, J). JAK2 intensity was high for Fr H (Figure 3, K) and Fr K (Figure 3, L). JAK2 intensity was determined statically and significantly higher in HepG2 cells which were treated with Fr B and Fr E ($p<0.05$, Table 6). Immunostaining intensities of STAT3 was strong in the control group (Figure 3, G), Fr B (Figure 3, H) and Fr E (Figure 3, I) treated cells. As a result, STAT3 intensity was not statistically significant in Fr B and Fr E treated cells according to control group ($p>0.05$, Table 6). STAT3 pathway inhibition induces apoptosis of cancer cells [23]. However, according to the current study results, the STAT3 pathway wasn't inhibited in Fr B and Fr E treated cells. Accordingly, apoptosis was not induced by Fr B and Fr E.

Table 6. The H-Score of Wnt11, Wnt3a, Stat3, Jak2 and β-catenin in HepG2 cells which treated with Fraction B, and Fraction E at 100 µg/mL concentration for 48 hours

	Wnt11	**Wnt3a**	**Stat3**	**Jak2**	**β-catenin**
Control group	117.91 ± 9.83	285.41 ± 80.53	266.48 ± 81.15	151.90 ± 66.90	224.65 ± 71.10
Fr. B	283.37 ± 33.92[a]	299.46 ± 82.87	284.70 ± 35.58	268.31 ± 54.06[c]	299.39 ± 23.50[e]
Fr. E	153.84 ± 20.34[b]	304.92 ± 19.74	303.29 ± 35.45	250.90 ± 40.04[d]	328.44 ± 11.02[f]

Data is expressed as means ± standard deviation. One way Anova Test was used to determine the comparisons. [a,b,c,d,e,f] The data was significant when compared with the control group ($p<0.05$).

Biactivity of *Cynara cornigera* L.



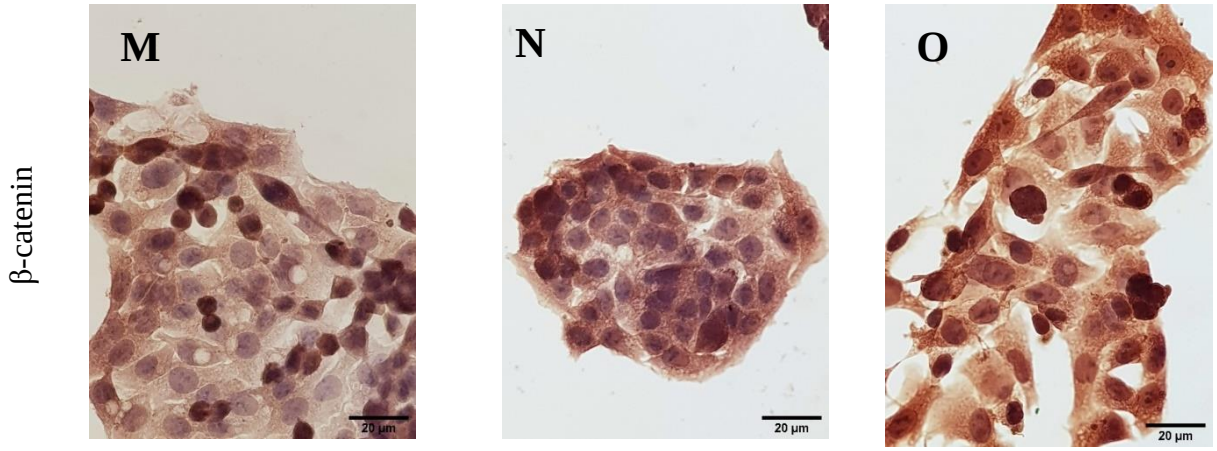


Figure 3. Light microscope images of HepG2 cells which show immunoreactivity of Wnt11 (A-C), Wnt3a (D-F), Stat3 (G-I), Jak2 (J-L) and β-catenin (M-O). Cells were treated without (A, D, G, J, M) or with 100 µg/mL concentration of Fr B (B, E, H, K, N) and Fr E (C, F, I, L, O) for 48 hours. Scale Bars = 20 µm

4. Conclusion

In conclusion, two of all obtained fractions from the methanol extracts of *C. cornigera* flowers containing phenolic compounds showed significant cytotoxic activity in HepG2 cells. These two fractions were investigated for their effects on the carcinogenesis mechanism of hepatocellular carcinoma in HepG2 cells. Significant effects for the inhibition of cell proliferation or inducing apoptosis were not determined on Wnt3a, Stat3, Jak2 and β-catenin signaling pathways. Compounds of B and E fractions of *C. cornigera* methanol extracts individually or coordinately have a significant role in the inhibition of proliferation on noncanonical Wnt11 signaling pathway. The remaining parts of the *C. cornigera* plant, such as the leaves and roots, are also expected to contain polyphenolic compounds and may have potential for further HCC anti-cancer drug studies. Consequently, cytotoxic effects of both fractions were not determined by a specific compound solely or a group of compounds, so cytotoxicity occurred by synergic effect of all compounds in the fractions.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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References

[1] I. Arias, H. Alter, J. Boyer, D. Cohen, D. Shafritz, S. Thorgeirsson and W. Allan (2020). The liver : Biology and pathobiology. Wiley, Blackwell UK.

[2] M.A. Feitelson, A. Arzumanyan, R.J. Kulathinal, S.W. Blain, R.F. Holcombe, J. Mahajna, M. Marino, M.L. Martinez-Chantar, , R. Nawroth, and I. Sanchez-Garcia et.al. (2015). Sustained proliferation in cancer: Mechanisms and novel therapeutic targets, *Semin. Cancer Biol.* **35**, 25–54.

[3] Ma, Xiaomei and Herbert Yu. (2006). Global burden of cancer, *Yale J. Biol. Med*. **79**, 85-94.

[4] GLOBOCAN 2018: cancer incidence and mortality, version 1.1. IARC Cancer Base ed. Lyon: IARC https://gco.iarc.fr/ Access date: 30/05/2022

[5] S. Thorgeirsson and J. Grisham (2002). Molecular pathogenesis of human hepatocellular carcinoma, *Nat. Genet*. **31**, 339–346.

[6] H. Kim and H.B. El-Serag (2019). The epidemiology of hepatocellular carcinoma in the USA, *Curr. Gastroenterol. Rep*. **21**, 17.

[7] World Health Organization. Regional Office for South-East Asia. (2011). Frequently asked questions on viral hepatitis. WHO Regional Office for South-East Asia. https://apps.who.int/iris/handle/10665/205127 Access date : 30/05/2022

[8] F. Wang, J. Fan, Z. Zhang, B. Gao and H. Wang (2014). The global burden of liver disease: The major impact of China, *Hepatology* **60**, 2099–2108.

[9] M.C Kew (2013). Epidemiology of hepatocellular carcinoma in sub-Saharan Africa, *Ann. Hepatol*. **12**, 173-182.

[10] J. Rowe, Y. Ghouri and I. Mian (2017). Review of hepatocellular carcinoma: epidemiology, etiology, and carcinogenesis, *J. Carcinog*. **16**, 1 doi:10.4103/jcar.JCar_9_16. eCollection 2017.

[11] H. Alotaibi, N. Atabey, K. Diril, E. Erdal and M. Ozturk (2016). Molecular mechanisms of hepatocellular carcinoma. In: Hepatocellular Carcinoma. *Current Clinical Oncology*, *ed:* B. Carr, Springer International, pp.43-63

[12] A. Bishayee (2014). The role of inflammation in liver cancer. In: Book inflammation and cancer, advances in experimental medicine and biology, *ed*: B. B. Aggarwal, B. Sung, S. C. Gupta, Springer, Basel, Switzerland, pp.401–435.

[13] M. Liu, L. Jiang and X.Y. Guan (2014). The genetic and epigenetic alterations in human hepatocellular carcinoma: A recent update, *Protein Cell*. **5**, 673–691.

[14] M. Dimri, A. Manali and A. Satyanarayana (2020). Molecular signaling pathways and therapeutic targets in hepatocellular carcinoma, *Cancers* **12**, 491.

[15] M. Nishikawa, S. Nishiguchi, S. Shiomi, A. Tamori, N. Koh, T. Takeda, S. Kubo, K. Hirohashi, H. Kinoshita, E. Sato and M. Inoue (2001). Somatic mutation of mitochondrial DNA in cancerous and noncancerous liver tissue in individuals with hepatocellular carcinoma, *Cancer Res*. **61**, 1843–1845

[16] W.Y. Lau, P.B. Lai, M.F. Leung, B.C. Leung, N. Wong, G. Chen, T.W. Leung and C. T. Liew (2000). Differential gene expression of hepatocellular carcinoma using cDNA microarray analysis, *Oncol. Res.* **12**, 59–69.

[17] M. Honda, S. Kaneko, H. Kawai, Y. Shirota, and K. Kobayashi (2001). Differential gene expression between chronic hepatitis B and C hepatic lesion, *Gastroenterology* **120**, 955–966

[18] Y. Takigawa, and M.A. Brown (2008). Wnt signaling in liver cancer, *Current Drug Targets*. **9(11)**, 1013–1024.

[19] C. Lu, Y. He, J. Duan, Y. Yang, C. Zhong, J. Zhang, W. Liao, X. Huang, R. Zhu, and M. Li (2017). Expression of Wnt3a in hepatocellular carcinoma and its effects on cell cycle and metastasis, *Int. J. Oncol*. **51**, 1135–1145.

[20] T. Toyama, H.C. Lee, H. Koga, J.R. Wands, and M. Kim (2010). Noncanonical Wnt11 inhibits hepatocellular carcinoma cell proliferation and migration, *Mol. Cancer Res*. **8**, 254–265.

[21] E. Kim, A. Lisby, C. Ma, N. Lo, U. Ehmer, K. E. Hayer, E. E. Furth and P. Viatour (2019). Promotion of growth factor signaling as a critical function of β-catenin during HCC progression, *Nat. Commun.* **10**, 1909.

[22] A. Shodeinde, K. Ginjupalli, H. D. Lewis, S. Riaz and B. E. Barton (2013). STAT3 inhibition induces apoptosis in cancer cells independent of STAT1 or STAT2, *J. Mol. Biochem*. **2**, 18–26.

[23] J. F. Bromberg, M. H. Wrzeszczynska, G. Devgan, Y. Zhao, R. G. Pestell, C. Albanese and J.E. Darnell Jr. (1999) Stat3 as an oncogene, *Cell* **98**, 295–303.

[24] R.L. Levine, A. Pardanani, A. Tefferi and D. G. Gilliland (2007). Role of JAK2 in the pathogenesis and therapy of myeloproliferative disorders, *Nat. Rev. Cancer*. **7**, 673–83.

[25] J.J. Hin Tang, D. K. Hao Thng, J. J. Lim and T. B. Toh (2020). JAK/STAT signaling in hepatocellular carcinoma, *Hepat. Oncol.* **7**, 18.

[26] Y. Li, Z. H. Gao and X. J. Qu (2015). The adverse effects of sorafenib in patients with advanced cancers, *Basic Clin. Pharmacol. Toxicol*. **116**, 216-221.

[27] G. Velderrain-Rodríguez, H. Palafox-Carlos, A. Wall-Medrano, J. Ayala-zavala, C.O. Chen, M. Robles-Sánchez, H. Astiazaran-García, E. Alvarez-Parrilla, G. A.González-Aguilar (2014). Phenolic compounds: their journey after intake, *Food Funct*. **5**, 189-197.

[28] R. Tsao (2010). Chemistry and biochemistry of dietary polyphenols, *J.. Nutr*. **2**, 1231–1246.

[29] G. Di Carlo, N. Mascolo, A. A. Izzo and F. Capasso (1999). Flavonoids: old and new aspects of a class of natural therapeutic drugs, *Life Sci*. **65**, 337-353.

[30] S. M. El Sohafy, A. M. Metwally, A. A. Omar, M. E. Amer, M. M. Radwan, M. S. Abdel-Kader, S. A. El Toumy and M. A. ElSohly (2016). Cornigerin, a new sesqui-lignan from the hepatoprotective fractions of *Cynara cornigera* L., *Fitoterapia* **115**, 101–105.

[31] A. Hegazy, S. Ezzat, I. Qasem, M. Ali-Shtayeh, M. Basalah, H. Ali and A. Hatamleh (2015). Diversity of active constituents in *Cichorium endivia* and *Cynara cornigera* extracts, *Acta. Biol. Hung*. **66**, 103–118.

[32] L. Wu, J. Li, T. Liu, S. Li, J. Feng, Q. Yu, J. Zhang, J. Chen, Y. Zhou, J. Ji, K. Chen, Y. Mao, F. Wang, W. Dai, X. Fan, J. Wu and C. Guo (2019). Quercetin shows anti-tumor effect in hepatocellular carcinoma LM3 cells by abrogating JAK2/STAT3 signaling pathway, *Cancer Med*. **8**, 4806–4820.

[33] M. N. Clifford, K. L. Johnston, S. Knight and N. Kuhnert (2003). Hierarchical scheme for LC-MS n identification of chlorogenic acids, *J. Agric. Food Chem.* **51**, 2900–2911.

[34] Q. Shen, Z. Dai and Y. Lu (2010). Rapid determination of caffeoylquinic acid derivatives in *Cynara scolymus* L. by ultra-fast liquid chromatography/tandem mass spectrometry based on a fused core C18 column, *J. Sep. Sci.* **33**, 3152–3158.

[35] I. M. Abu-Reidah, D. Arráez-Román, A. Segura-Carreteroed and A. Fernández-Gutiérrez (2013). Extensive characterisation of bioactive phenolic constituents from globe artichoke (*Cynara scolymus* L.) by HPLC–DAD-ESI-QTOF-MS, *Food Chem.* **141**, 2269–2277.

[36] M. Pistón, I. Machado, C. S. Branco, V. Cesio, H. Heinzen, D. Ribeiro, E. Fernandes, R. C. Chisté and M. Freitas (2014). Infusion, decoction and hydroalcoholic extracts of leaves from artichoke (*Cynara cardunculus* L. subsp. cardunculus) are effective scavengers of physiologically relevant ROS and RNS, *Food Res. Int.* **64**, 150–156.

[37] F. Sánchez-Rabaneda, O. Jáuregui, R. M. Lamuela-Raventós, J. Bastida, F. Viladomat and C. Codina (2003). Identification of phenolic compounds in artichoke waste by high-performance liquid chromatography–tandem mass spectrometry, *J. Chromatogr. A.* **1008**, 57–72.

[38] L. M. da Silva, A. Allemand, D. A. G. B. Mendes, A. C. dos Santos, E. André, L. M. de Souza, T. R. Cipriani, N. Dartora, M. C. A. Marques, C. H. Baggio and M. F. Werner (2013). Ethanolic extract of roots from *Arctium lappa* L. accelerates the healing of acetic acid-induced gastric ulcer in rats: Involvement of the antioxidant system, *Food Chem. Toxicol.* **51**, 179–187.

[39] F. Chen, X. Long, Z. Liu, H. Shao and L. Liu (2014). Analysis of phenolic acids of jerusalem artichoke (*Helianthus tuberosus* L.) responding to salt-stress by liquid chromatography/tandem mass spectrometry, *Sci. World J.* **2014**, 1-8

[40] S. He, Y. Lu, X. Liu, X. Huang, E.T. Keller, C. Qian and J. Zhang (2015). Wnt3a: functions and implications in cancer, *Chin. J. Cancer.* **34**, 50.