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Antioxidant and AntiproliferativeEffects on Human Liver HePG2Epithelial Cells from Artichoke (*Cynara scolymus* L.) By-Products

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Abstract
Cynara scolymus L.is a medicinal plant frequently used in traditional medicine for liver diseases. This study aimed to evaluate the antioxidant and Anti-tumer effect in human Liver HePG2 epithelial cells of artichoke. The antioxidant activity of the free phenolic of bracts methanolic extract, as measured with the DPPH freeradical scavenging activity and iron chelating showed significant antioxidant activity ≥ 0.05 as well as yielded the largest quantity of phenolics and flavonoids compounds. A significant increase was observed in hepatocellular carcinoma cell line (HePG2) 78.3 % treated with free phenolic extract of bracts higher than that found in by the heart of artichoke 36.7%. This work showed that extracts with high antioxidant activity can be obtained from artichoke and artichoke by-products. Therefore, this study indicate that the free phenolic extract from bracts of *Cynara scolymus*L. might be of interest within the developing market of nutritional ingredients and is capable of yielding nutritional supplements with antioxidant and anticancer activities.
Key words: Artichoke by-products, antioxidant, antitumor, HepG2

1. Introduction

Artichoke (*Cynara scolymus* L.) represents an important component of the Mediterranean diet. Artichoke is a rich source of minerals, a low amount of lipids, dietary fibre and a high proportion of phenolics(Llorach et al., 2002&Fratianni*et al*., 2007).Nutritional and pharmaceutical properties of both artichoke bracts and heart are showed high levels of polyphenolic compounds and inulin(Luttanizio *et al*. 2009).In Egypt there is an annual production of nearly 202458 MT of artichoke (FAO, 2012).The manipulation of food processing wastes is now becoming a very serious environmental issue. Peels and leaves are often the waste part of various fruits. These wastes have not generally received much attention with a view to being used or recycled rather than discharged. This might be due to their unknown benefit of commercial application.
Recent studies on artichoke demonstrated their health-protective potential, especially their hepatoprotective(Gebhardt, 1997&Aktay *et al*., 2000)anticarcinogenic(Wang *et al*., 2003), and hypocholesterolemicactivities (Lupattelli *et al*., 2004), antimicrobial (Zhu *et al*., 2004).Artichoke leaf extracts (*Cynarascolymus*L.) has been shown to reduce symptom severity in a subset of patients with dyspepsia identified as suffering from irritable bowel syndrome(Walker *et al*., 2001).Also,Jimenez-Escrig*et al*., (2003)demonstrated a pronounced antioxidant potential by artichoke leaf extract. Aktay*et al*., (2000)have shown that artichoke extract is very effective as an antioxidant and its health-protective potential has been attributed to its antioxidant power. The artichoke (*Cynara scolymus* L.)canning industry generates large amounts of agricultural waste, represent (about 80–85% of the total biomass of the plant) consisting mainly of the leaves, stems and the external parts of the flowers (bracts) which are not suitable for human consumption and could be used as a source of inulin,phenolics, and should be considered as a raw material for the production of food additives and nutraceuticals. The aim of the present study is to evaluatethe potential role of artichoke and artichoke by-products as a source of health-promoting phenolics associated with their antioxidant, and anti-hepatocellular effect in human liver HePG2 epithelial cells**.**

2. Materials and Methods

2.1Sampling extraction of free and bound phenolic compounds
Phenolic compounds were extracted into free and bound phenolics according to the methods of Adom and Liu (2002) &Sosulski, *et al*. (1982),respectively, with a slight modification. Free phenolic compounds of flours (1 g) were extracted with 10 ml of 80% chilled ethanol for 20 min with continuous shaking. After centrifugation at 2500g for 10 min, the supernatant was collected. The residue was re-extracted twice with 10 ml of 80% chilled ethanol under the same conditions. All supernatants were combined and evaporated to dryness under reduced pressure. Then the concentrated slurry was dissolved with methanol to a final volume of 10 ml. The free phenolic compounds were then stored at – 40 °C until use.
The residue from the extraction of free phenolic compound was hydrolyzed directly with 20 ml of 2 N NaOH for 90 min with continuous shaking at 60 °C (Yeh, *et al*. 1980). The hydrolysate was acidified to pH 2 (6 N HCl) and

centrifuged to separate cloudy precipitate. The clear supernatant was extracted five times with hexane at a hexane to water phase ratio of 1:1 to remove free fatty acids and other lipid contaminants. The liberated phenolic acids were then extracted six times with ethyl acetate at a solvent to water phase ratio of 1:1. The ethyl acetate extracts were evaporated to dryness and then bound phenolic compounds were dissolved and filled up to 10 ml of methanol and stored at (– 40)°C until use.

2.2Total phenolic content (TPC)

The total phenolic content (TPC) of free and bond phenolic extracts of different parts of artichoke was spectrophotometrically determined by FolinCiocalteu reagent assay using Gallic acid for the preparation of calibration curve (20 – 120 mg/l) according to Singleton *et al*., (1965).A suitable aliquot (1 ml) of each extract or standard solution was added to 25 ml volumetric flask, containing 9 ml of distilled water. One milliliter of Folin Ciocalteu`s phenol reagent was added to the mixture and shaken. After 5 min. 10 ml of 7 % Na_2CO_3 solution were added to the mixture. The solution was diluted to 25 ml with distilled water and mixed. After incubation for 90 min. at room temperature, the absorbance was determined at 750 nm with Spectrophotometer (Unicum UV 300) against prepared reagent as blank. Total phenolic contents in samples were expressed as mg Gallic acid equivalents (GAE)/g dry weight. All samples were analysed in triplicates.

2.3Total flavonoid content (TFC)

Total flavonoid content (TFC) of free and bond phenolic extracts of different parts of artichoke was spectrophotometrically determined by the aluminium chloride method using quercetin as a standard (Zhishen *et al*., 1999). One ml of extract or standard solution (quercetin, 20–120 mg/l) was added to 10 ml volumetric flask, containing 4 ml of distilled water. To the flask 0.3 ml 5 % $NaNO_2$ was added and after 5 min 0.3 ml 10 % Al Cl_3 was added. At 6th min, 2 ml 1M NaOH were added and the total volume was made up to 10 ml with distilled water. The solutions were mixed well and the absorbance was measured against prepared reagent blank at 510 nm by using spectrophotometer (Unicum UV 300). Total flavonoids in sample were expressed as mg quercetin equivalents (QE)/ g fresh weight.Samples were analysed in triplicates.

2.4 Determination of free radical scavenging activity

Determination of free radical scavenging activity was performed spectrophotometrically as described by Chu *et al*. (2000). An aliquot of 0.5 ml of 0.1 mM 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) radical (Sigma) was added to a test tube with 1 ml of artichokeextracts at different concentrations10,25,50,100µg/ml. Methanol was used as blank .The reaction mixture was shaken vigorously at room temperature. Butyl Hydroxytoluene (BHT, Sigma) was used as a positive control, and negative control contained the entire reaction reagent except the extracts. Then the absorbance was measured at 515 nm with spectrophotometeragainst blank (methanol pure).Lower absorbance of the reaction mixture indicated higher free radical scavenging activity.

The capacity to scavenge the $DPPH^{\cdot}$radical was calculated using the following equation:

$DPPH^{\cdot}$scavenging effect (Inhibition %) = [(Ac – A_S / Ac) × 100].

Where Ac was the absorbance of the control reaction and $A_s$as the absorbance in the presence of the artichoke extracts.

2.5 Determination of chelating effects on ferrous ions

Metal chelating effects on ferrous Ions was carried out as described by Hsu *et al*. (2003). One ml of artichoke extracts, and or EDTA solution as a positive control at different concentrations (10, 25, 50, 100 µg/ml) were mixed with 0.1 ml of 2 mM $FeCl_2$- 4H2O and 0.2 ml of 5 mM ferrozine solution and 3.7 ml methanol were mixed in a test tube and reacted for 10 min, at room temperature .The absorbance was then measured at 562 nm. Mixture without extract was used as the control. A lower absorbance indicates a higher ferrous ion chelating capacity. The percentage of ferrous ion chelating ability was calculated using the following equation: Iron chelating activity (Inhibition %) = [(Ac – AS / Ac) × 100]

Where Ac was the absorbance of the control reaction and $A_s$as the absorbance in the presence of the artichoke extracts.

2.6 Cytotoxic effect on human cell line (HePG2)

Cell viability was assessed by the mitochondrial dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan(Mosmann,1983).

*Procedure***:** All the following procedures were done in a sterile area using a Laminar flow cabinet bio safety class II level(Baker, SG403INT, Sanford, ME, USA). Cells were suspended in RPMI 1640 medium for HePG2. The media are supplemented with 1% antibiotic-antimycotic mixture (10,000U/ml Potassium Penicillin, 10,000µg/ml Streptomycin Sulfate and 25µg/ml Amphotericin B), 1% L-glutamine and 10% fetal bovine serum and kept at 37 °C under 5% CO_2. Cells were batch cultured for 10 days, then seeded at concentration of 10x103 cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37 °C for 24 h under 5% CO_2 using a water jacketed Carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA). Media was aspirated, fresh medium (without serum) was added and cells were incubated either alone (negative control) or with different concentrations of sample to give a final concentration of (100 – 50 – 25–12.5–6.25–3.125–0.78 and

1.56 µg/ml). After 48 h of incubation, medium was aspirated, 40µl MTT salt (2.5µg/ml) were added to each well and incubated for further four hours at 37°C under 5% CO_2. To stop the reaction and dissolving the formed crystals, 200µl of 10% Sodium dodecyl sulphate (SDS) in deionised water was added to each well and incubated overnight at 37ºC. A positive control which composed of 100µg/ml was used as a known cytotoxic natural agent who gives 100% lethality under the same conditions (Thabrew, *et al*., 1997).The absorbance was then measured using a micro plate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at 595nm and a reference wavelength of 620nm. A statistical significance was tested between samples and negative control (cells with vehicle) using independent t-test by SPSS 11 program. DMSO is the vehicle used for dissolution of plant extracts and its final concentration on the cells was less than 0.2%. The percentage of change in viability was calculated according to the formula:

((Reading of extract / Reading of negative control) -1) x 100

A probit analysis was carried for IC_{50} and IC_{90} determination using SPSS 11 program.

2.7Statistical Analysis

The statistical analysis was performed according to Snedecor& Cochran (1989) for comparison between different mean values, LSD test at 5% level was used (Duncan 1995). Differences were considered significant when $p \leq 0.05$.

3. Results and Discussion

3.1Phenolicsand Flavonoids contents

The artichoke (*Cynara scolymus* L.) canning industry generates large amounts of agricultural waste, represent (about 80–85% of the total biomass of the plant) consisting mainly of the leaves, stems and the external parts of the flowers (bracts) which are not suitable for human consumption and could be used as a source of inulin, phenolics, and should be considered as a raw material for the production of food additives and nutraceuticals.

Data presented in (Table 1) indicated that theartichoke (bracts) showed a higher content of total free phenolic compounds FTPC (14.16 mg/gDW) followed by theartichoke (heart) which contained only 9.06 mg/gDW.On the other hand both inner and outer parts of artichoke showed the lower of the bound phenolic compounds (5.35 and 4.2 mg/gDW, respectively).

Previous studies showed that the artichoke (bracts) contained higher amount of phenolic compounds along with higher amount of minerals and dietary fibres. As a role of phenolics, the artichoke (bracts) should posses higher free radicals scavenging activity than the artichoke (heart), These resultsweresupported by the results of who found that nutritional and pharmaceuticalproperties of both heart and bracts are linked to their special chemical composition which includes high levels of polyphenoland inulin which possess potential antioxidant activity (Perez-Garcia *et al*., 2000).

Lattanzio *et al*., (2009) mentioned that the therapeutic properties have been ascribed to the cynarin (1,3-O dicaffeoylquinic acid)content of these extracts.in various pharmacological test systems,artichoke bracts extracts have exhibitedhepatoprotective,anticarcinogenic,antioxidative,antivacterial and ani-HIV activities.

Similar results found by Sallam *et al*., (2005) &Sallam *et al*.,(2008) who found that artichoke by-productcontained (8.1 mg Tannic acid/gDW). Lattanzio *et al*., (2009) found that by-product of artichoke are very rich in phenolic compounds and hence can regarded as a functional food.

In addition, the total flavonoidscontents (TFC) of free and bound phenolic extracts of artichoke bracts and heart are shown in (Table 1). The flavonoid content of the free phenolic extracts was higher than that the bound phenolic extracts.

The total flavonoids concentrations of free phenolic in artichoke bractsextracts was significantly higher (9.85 mg/g DW) when compared toartichoke heart (5.91 mg/gDW). In conclusion, among the twofractions ofartichoke the (bract and heart) was found to contain the highest content offlavonoidsand phenolic offreephenolic extract. Significantly different was observed in the total flavonoids of the different parts of artichoke.

Table 1.Total phenolic and total flavonoids content of free and bound methanolic extracts of different parts of artichoke.

Samples		TPC mg/g	TFC mg/g
Heart	Free phenolic extract	$9.06^{c} \pm 0.06$	$5.91^{c} \pm 0.12$
	Bound phenols extract	$5.35^{b} \pm 0.08$	$4.17^{b} \pm 0.15$
Bract	Free phenols extract	$14.16^{d} \pm 0.08$	$9.85^{d} \pm 0.12$
	Bound phenols extract	$4.20^{a} \pm 0.07$	$2.06^{a} \pm 0.11$
LSD at 0.05		0.14	0.23

All values are the mean of three replicates ± SD. All values with the same letters are not significantly different at $p \geq 0.05$

3.2 Antioxidant activity

3.2.1 DPPH radical scavenging of free and bound methanolic extracts

The scavenging of the stable DPPH radical was widely used to evaluate antioxidant activity of phenolic compounds extracted from fruit, vegetable, cereal grain, wine, etc. (Jimenez-Escrig, *et al*.,2000). It is based on the measurement of the reducing ability of antioxidants toward DPPH (Huang, *et al*., 2005).The results showed that the methanolic extract of artichoke bracts and heart (free phenolics) demonstrated the lowest IC_{50} (55.68 µg/ml and 76.62 µg/ml respectively) in comparison with BHT (30.93 µg/ml).

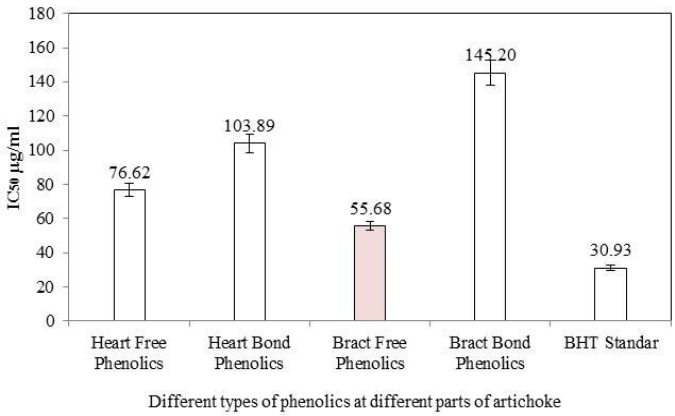

Figure 1.DPPH scavenging activity of free and bound methanolic extracts of different parts of artichoke

The IC_{50} of free phenolic extractofartichoke bracts and heart exhibited higher scavenging capacity of DPPH radical than the bound phenolic extracts. Thus, the larger total phenolic compounds in the outer parts enhanced the antioxidant capacity (Liyana-Pathirana&Shahidi, 2006).

In addition, IC_{50} for methanolic extracts of artichoke heart and bracts (bound phenolics were 103.89 µg/ml and 145.20µg/ml compared toBHT (IC_{50} 55.68) whichexhibited the highest scavenging activity (30.93 µg/ml).Artichoke (bracts) free phenolics appeared to be more active as compared to the hearts parts in scavenging activity of the free radicals. Both extracts showed lower activities than BHT due to the significantly higher IC_{50} values required to inhibit the free radicals (Figure 1). It has been reported that the strong free radical scavenging in innerparts and outer parts of artichoke may be attributed to the presence of phenolics(Wang *et al*., 2003). As reported previously by Wang *et al*., (2003) who found that antioxidant activity of inner and outer parts of artichoke extracts was correlated to their total phenolic compounds. Artichoke is a good source of polyphenol oxidase that catalyze the oxidation of phenolics to quinons that induce secondaryproducts (Espin *et al*., 1997).Also Jimenez Escrig *et al*. (2003) found that artichoke extract is very effective as an antioxidant and its health protective potential has been attributed to its antioxidant activity due to the decreaseof ROS production (ZapolskaDownar *et al*., 2002).Also Varmanu *et al*., (2011)showed that the ethanol extract of artichoke showed highest antioxidant as well as yielded the largest quantity of polyphenol compound.

3.2.2 Ferrous ion chelating activity

Many plant phenolic compounds have been described as antioxidants due to their chelating ability to iron ions. As shown in (Table 2) the artichoke extracts displayed the Fe^{2+}-chelating effect in a concentration dependent manner. The percentages of metal chelating capacity at 100 µg/ml of tested methanol extracts of free phenolicswere found to be 41.34% ± 0.43and48.13% ± 0.41in artichoke bracts and heart parts of artichokerespectively.

Table 2.Iron chelating activity of free and bound methanolic extracts of different parts of artichoke

Extract		Inhibition %			
		10 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml
Heart	Free phenolic extract	7.56 ^{b} ± 0.54	12.87 ^{c} ± 0.34	21.05 ^{c} ± 0.23	41.34 ^{c} ± 0.43
	Bound phenols extract	5.04 ^{a} ± 0.21	8.95 ^{b} ± 0.21	16.91 ^{b} ± 0.21	35.72 ^{b} ± 0.34
Bract	Free phenols extract	10.39 ^{c} ± 0.49	17.05 ^{d} ± 0.28	28.83 ^{d} ± 0.41	48.13 ^{d} ± 0.41
	Bound phenols extract	4.72 ^{a} ± 0.49	8.23 ^{a} ± 0.36	15.70 ^{a} ± 0.55	31.85 ^{a} ± 0.49
EDTA Standard		23.62 ^{d} ± 0.49	34.55 ^{e} ± 0.49	46.74 ^{e} ± 0.55	71.48 ^{e} ± 0.28
LSD at 0.05		0.83	0.64	0.76	0.72

All values are the mean of three replicates ± SD. All values with the same letters are not significantly different at $p \geq 0.05$

Contrary the bound phenolics extracts showed less values 35.72% ± 0.34 and 31.85 %± 0.49. While EDTA (standard) exhibited the highest ferric chelating ability due to their chemical structure properties (71.48± 0.28).Iron and copper are essential transition metal elements in the human body for the activity of a large range of enzymes and for some proteins involved in cellular respiration, O_2 transport and redox reactions. But, because they are transition metals, they contain one or more unpaired electrons that enable them to contribute one-electron transfer reactions. Hence, they are powerful catalysts of autoxidation reactions, such as participation in the conversion of H_2O_2 to $OH^{\cdot}$ to the highly reactive alkoxyl and hydroxyl radicals (Perez Garcia *et al*., 2000). Due to this property, transition metal chelating to form low redox potential complexes is an important antioxidant property (Halliwell *et al*., 1995) and measuring chelating of iron (II) is one method for assessing this property.

4- Correlation coefficient

The Correlation coefficient of different type of phenolicsextracts from different parts of artichoke with different antioxidant assay was linear and strong to moderate, depending upon the assay system used. The correlation coefficient between IC_{50} and different types of phenolics wasR^2= 0.8332 and between different types of flavonoids and $IC_{50}$was R^2=0.8996 (Fig.2).Strong correlation ofdifferent types ofphenolicsand flavonoids with Fe^{2+}-chelating (R^2= 0.9992) and (R^2=0.9664), respectively, indicatesthatphenolics are key contributors to antioxidant activity in free and bound phenolicextracts (Fig. 3).

The results revealed that phenolics and, flavonoids from different types of phenolicsmethanolic extracts (free and bond) and different parts of artichoke were strongly contributed to the antioxidant scavenging activity.

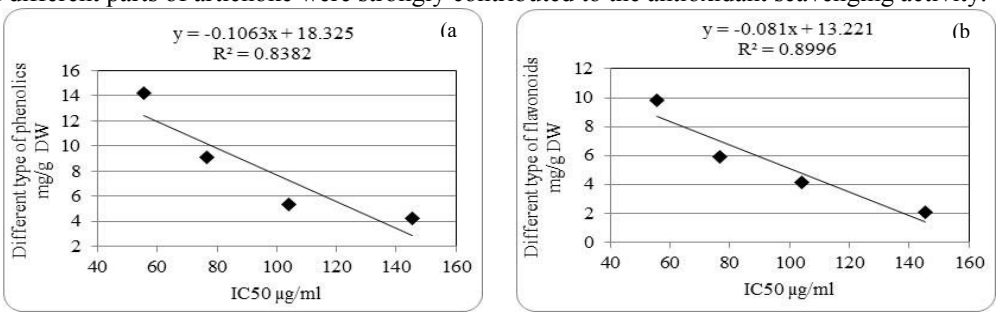

Figure2.Correlation between $IC_{50}$and different type of phenolics (a) and flavonoid (b) of differentparts of artichoke

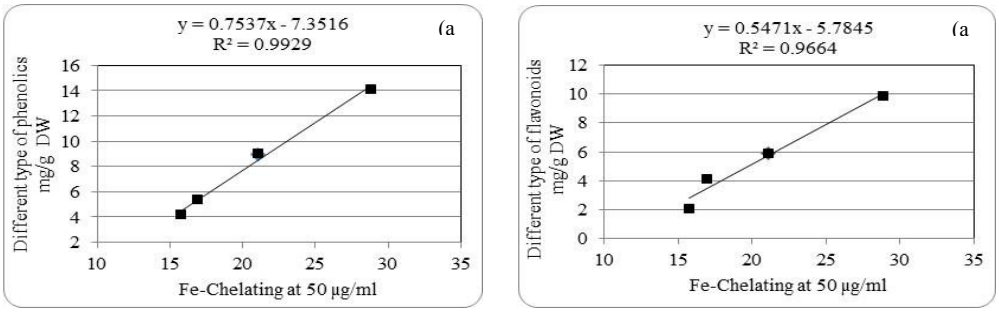

Figure3.Correlation between Fe^{2+}-Chelating at (50µg/ml) and different type of phenolics(a) and flavonoid (b) of different parts of artichoke

These findings supported the positive relationship between phenolics, flavonoids and antioxidant activity of different plant species.Ghasemzadeh *et al*., (2012) reported that strong positive relationship between phenolics and antioxidant activity which appears to be the trend in many plant species. Recently, it was found that phenolic compounds are the candidate compounds responsible for antioxidant activity. Flavonol glycosides (quercetin and kaempferol), sinapic and caffeoyl-quininc acid were found to be the major phenolic compounds found in artichoke (Wang *et al*., 2003).

5. Cytotoxicity Activity

The effects of free and bound methanolic extract of heart and bract of artichoke on human cancer cell lines: hepatocellular carcinoma cell line (HePG2) is present in Table (3).The methanolic extract of different parts of artichoke exhibited cytotoxic effects on cancer human cells in a dose dependent manner assessed by MTT assay. Treatment with free phenolic methanolic extract of artichoke(bracts) for 24 h resulted in a significant differences

at p>0.05 in cell viability for hepatocellular carcinoma cell line (HepG2). Remarkable differences between the responses of hepatocellular carcinoma cell line (HepG2) for treatment with free and bound phenolicextracts for artichoke (bracts) and (heart) were detected. A significant increase was observed in hepatocellular carcinoma cell line (HePG2)75.3% treated with bracts free phenolic extract higher two fold than that found in free phenolic extract of artichoke (heart) (36.7%). Cancer is a global health problem with high morbidity and mortality and poses both economic and psychological challenges (Moyad& Carroll, 2004,Dossus& Kaaks 2008).It is well known that different cell lines might exhibit different sensitivities towards an anti-proliferative compound, so the use of more than one cell line is therefore considered necessary in the detection of antiproliferative compounds.
These data suggest a possible mechanism of cytotoxicity in cancer cell line, at least in part, through the regulation of apoptosis-related proteins and/or cell cycle deregulation. Artichoke wastes are rich sources of functional components such as phenolics and flavonoids which have antioxidant and radical scavenging activities (Llorach *et al*. 2002).Our results are in agreement with them and with Sperocni *et al*., (2003) who mentioned that methanolicartichoke extract could inhibit cancer cell growth or enhance the molecular mechanism of the chemopreventive effects on cancer. In addition, morphological characterization of treated cells revealed that the mode of action of cell death induced by methanolic artichoke extract was mediated through apoptosis.Thenutraceutical benefits of artichoke leaves are confirmed and believed to their antioxidant properties related to, phenolics and flavonoids (Juzyszyn *et al*., 2010).

Table 3.Cytotoxic effect of free and bound phenolicsmethanolic extractsagainst hepatocellular carcinoma cell line (HePG2) in different parts of artichoke

Extracts		IC_{50} (µg/ml)	IC_{90} (µg/ml)	Remarks
Heart	Free phenolic extract	-	-	36.7% at 100 ppm
	Bound phenols extract	-	-	27.2% at 100 ppm
Bract	Free phenols extract	69.8	116.8	75.3% at 100 ppm
	Bound phenols extract	-	-	14% at 100 ppm
DMSO		-	-	1% at 100 ppm
Negative control		-	-	0 %

6. CONCLUSION

The results obtained from this study indicated thatthe presence of various phenolics and flavonoids, together with other metabolites that are probably involved in the antioxidant and cytotoxic activities,supported the traditional medicinal use of artichoke by-products, and provide grounds for further establishing its use as a functional food.The extract ofbracts free phenolic methanol extract showed a potential activity to inhibit hepatocellular cell cancer growth indicates the potential value of artichoke by-products (bracts free phenolic extract) as antioxidant, anticancer and alternative source of therapeutic agents which requires further investigation.

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