Artichoke Waste as a Source of Health Promoting Hepatoprotective Phenolics

A.A. Seida*, N.D. El Tanbouly*, W.T. Islam*, H.H. Eid*, S.A. EL-Maraghy** and A.S. El Senousy*

* Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Giza, Egypt
** Department of Biochemistry, Faculty of Pharmacy, Cairo University, Giza, Egypt

Abstract
The hepatoprotective and antioxidant activities of the hydroalcoholic extract of artichoke (Cynara scolymus L.) waste (AE), collected from the agro-food industry, in Egypt were evaluated compared to a standardized extract (NE), using CCl4-induced oxidative stress and hepatic damage in rats. The hepatoprotective activity was assessed via measuring the level of serum markers such as alanine aminotransferase, aspartate aminotransferase, total protein and albumin. The antioxidant effect was evaluated by measuring hepatic malondialdehyde, reduced glutathione, protein thiol, nitrite/nitrate levels, glutathione-peroxidase and glutathione-S-transferase activities. Pretreatment with AE revealed significant hepatoprotective and antioxidant activities, compared to NE. Fractionation of (AE), using VLC, followed by sephadex and reversed phase chromatography, afforded three major dicaffeoylquinic acids viz., 3,5-di-O-caffeoyl quinic acid (1), 1,5-di-O-caffeoylquinic acid (2) and, 3,4-di-O-caffeoylquinic acid (3). The isolated compounds were identified, using spectral data. AE was standardized, using a validated HPLC method, where 1,5-Di-O-caffeoylquinic acid (2.42 g%) was the major compound, followed by chlorogenic acid (1.22 g%). The three major di-O-caffeoylquinic acids (1,2 and 3) revealed a total content of 4.55 g%, calculated as 1,5-di-O-caffeoylquinic acid. Moreover, the nutritive value of artichoke waste was investigated. This study showed interesting promising results for the reuse of agro-food waste as source of health promoting phenolics in nutraceuticals

Keywords
Antioxidant; artichoke waste; caffeoylquinic acids; hepatoprotective, HPLC

INTRODUCTION

Egypt is considered as one of the main producers of artichokes in the Mediterranean basin. Artichoke processing generates waste, consisting mainly of the outer and inner bracts, representing ≈70% of the weight of its head.

Utilization of artichoke processing by-products has been taken into consideration, worldwide. Studies investigated the suitability of these by-products as animal feed (Sallam et al., 2008), for fiber and inulin production (Femenia et al., 1998; Lopez-Molina et al., 2005) and for recovering high amounts of phenolics with antioxidant properties (Mabeau et al., 2007).

Several reports demonstrated the antioxidant activity of artichoke by-products, collected abroad, using different in vitro assays (Peschel et al., 2006; Mabeau et al., 2007). However, to our knowledge, the extraction of phenolic compounds as hepatoprotective from artichoke by-products has not yet been studied.

The aim of this work is to assess the phenolic constituents of the artichoke by-product and their hepatoprotective activity, as well as the nutritive value of the waste, in order to evaluate its potential use as a cheap nutraceutical for commercial use.

METHODS

Plant material

Finzelberg standardized artichoke extract (NE) was supplied by Finzelberg Company, Germany. Artichoke (*Cynara cardunculus* L. var. scolymus (L.) Hayek (formerly *Cynara scolymus* L.) by-product was supplied by Ismailia National Company for Food Industries (FOODICO) (7^{th} km Ismailia-Portsaid Road, Egypt).

Preaparation of artichoke by-product extract

Two kg of shade-dried artichoke by-product (outer and inner bracts) were extracted by boiling ethanol (70%) till exhaustion, then concentrated under vacuum and lyophilized (-80ºC), to obtain the artichoke by-product extract (AE) (7.6 gm % on dry weight basis, 2.65 gm % on fresh weight basis), which was stored at -20ºC until use. For biological study, aliquot of this extract was suspended in 0.5% carboxy methyl cellulose (CMC) in distilled water, at the appropriate concentration.

Biological study

Animals and experimental design: Male Wistar albino rats (170-200 g), fed standard chow diet, provided with water *ad libitum*, were left for an initial adaptation period of 2 weeks before any experimental manipulation. Animals were divided into 4 groups (7 rats/group). Group A (normal control group) was orally given 0.5% CMC. Group B was given CCl_4 (1.5 ml/kg b.w., i.p., 5 times/week**)** for 2 weeks. Groups C and D were pretreated respectively with a daily oral dose of 200 and 250 mg/ kg b.w. of artichoke by-product extract (AE) and the standard extract (NE), for 2 weeks before CCl_4 administration and concurrently for another 2 weeks with CCl_4 administration. Thereafter, the animals were sacrificed by decapitation and blood samples were collected for separation of serum. The liver from each rat was rapidly isolated, washed with ice cold saline and blotted dry.

Biochemical analysis. The separated serum was used for the estimation of aspartate aminotransferase (AST), alanine aminotransferase (ALT) (Reitman and Frankel, 1957), as well as, serum total albumin (Doumas et al., 1971) and serum total protein (Weichselbaum, 1946). Results are presented in Figs. (1 and 2)

The liver tissue was used for the determination of hepatic malondialdehyde (MDA) (Mihara and Ushiyama, 1978), hepatic nitric oxide (NO) (Miranda et al., 2001) and protein thiol (PSH) (Koster et al., 1986). Reduced liver glutathione (GSH) (Beutler et al., 1963), glutathione peroxidase (GSH-Px) (Paglia and Valentine, 1967) and glutathione-S-transferase (GST) (Gawai and Pawar, 1984) were also evaluated. The protein content was measured by the method of Lowry et al.,1951. Results are presented in Tables (1 and 2).

Statistical analysis. Values are given as mean ± S.E.M. The level of statistical significance was taken at $p < 0.05$, using one-way analysis of variance (ANOVA), followed by Tukey-Kramer's multiple comparison

Standardization of artichoke by-product extract by HPLC analysis

Chemicals. Chlorogenic (>97 %) and caffeic (> 95%) acids were obtained from Roth (Germany), 1,5 dicaffeoylquinic acid (≥98%) was supplied by Chengdu Biopurify Phytochemicals Ltd.(China).

*Sample preparation***.** AE ext. (one g) was introduced into a 50 ml volumetric flask, then 30 ml of 50% aqueous methanol (v/v) was added, the sample was sonicated till complete dissolution (10

min.) and the flask was filled up to the mark with 50% aqueous methanol (v/v). Aliquot of 0.5 ml of the prepared extract was purified using C18-reversed phase cartridges, eluted with 50% aqueous methanol (2×1 ml), followed by 60% aqueous methanol (2×1 ml). The eluates were transferred quantitatively to a 5 ml volumetric flask and made up to volume with 60% aqueous methanol. The standard solutions were prepared by dissolving in methanol. All solutions were filtered through 0.45 μm membrane filter into sample vials and degassed, before HPLC analysis.

Quantitative determination of hydroxycinnamic acid derivatives by HPLC. An Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA), equipped with a quaternary pump, degasser G1322A, series 1200 Agilent, UV detector and ChemStations software were employed. A Lichrospere C18 column (250 × 4mm, 5μm) (Merck, Germany), guarded by a guard column (4 × 10 mm id, 5μm) was used. The mobile phase consisted of methanol (solvent A) and 0.3% H_3PO_4 in water (solvent B). The gradient elution program was 25-60% A (0-20 min.), followed by a 5 min. (60-25% A) equilibrium period. The flow rate was 1.2 ml min^{-1}. Injection volume was 20 μL. Analysis was carried out at room temperature and the detection wavelength was set at 325 nm.

Validation of the quantification method. Linearity, precision, repeatability and recovery were determined. (Jiang et al., 2005; Liu et al., 2008).

General Procedures

Silica gel Rp-18 (70-230 mesh) (Fluka, Germany), sephadex LH-20 (Pharmacia) were used for column chromatography, silica gel H (Merck) for vacuum liquid chromatography (VLC). Thin layer chromatography (TLC) was performed on silica gel 60 F_{254} (Merck). UV spectra were determined in methanol on a Hewlett Packard 8452A diode array spectrophotometer in the region of 200-500 nm. ^{1}H, ^{13}C-NMR and ^{1}H-^{1}H COSY spectra were recorded in DMSO, on a Jeol TMS Route instrument (^{1}H, 300 MHz, ^{13}C, 75 MHz, Japan).

Milled dried artichoke by-product was analyzed for moisture, crude protein, crude fat, ash and total carbohydrates contents. Amino acid analysis was carried out according to AOAC, 2005, using Amino Acid Analyzer Biochrome 30 (England). All amino acids were determined, except tyrosine and tryptophan. The contents of vitamin C (ascorbic acid), vitamins B_1(thiamine) and B_2 (riboflavin) (Nielsen, 1998), as well as, vitamin A (β-carotene) (Holden, 1981) and vitamin E (α-tocopherol) (Bourgeois,1992) were determined.

Isolation Procedures.

AE extract of artichoke by-product (100 g) was fractionated using VLC column chromatography, eluted with different solvents of increasing polarity. Fractions were collected and monitored by TLC using solvent systems EtOAc-MeOH-H_2O (100:16:12) and $CHCl_3$-MeOH (9:1). The spots were detected under UV light before & after spraying with Naturstoff's reagent. Subfractions **7** and **8** revealed spots, corresponding to caffeoylquinic acids. Subfraction **7** (9.36 g), eluted with 24-35% MeOH in EtOAc, was defatted with CH_2Cl_2, then dissolved in 50% MeOH-H_2O to afford a residue (5.53 g), which was purified on Sephadex columns using MeOH, followed by reversed phase C-18 column chromatography, using gradient elution with H_2O and MeOH to yield **compounds 1** (18 mg, eluted at 3% MeOH/H_2O) and **2** (42 mg, eluted at 1% MeOH / H_2O). Subfraction **8** (4.93 g) eluted with 37.5-40% MeOH/EtOAc was similarly treated as subfraction **7** to afford a residue of 3.65 gm. This residue was further purified as subfraction **7** to give **compound 3** (30 mg, eluted at 24-28% MeOH/H_2O).

RESULTS AND DISCUSSION

Hepatoprotective and antioxidant activities.

The enhanced activities of ALT and AST observed in CCl_4-treated rats corresponded to the extensive liver damage (Fig.1) induced by CCl_4. The tendency of these enzymes to return towards a near normal level in the tested extract (AE) and the standard extract (NE) groups, demonstrated the cytoprotective activity of both. The lowered levels of serum total protein and albumin in CCl_4-treated rats, revealing the severity of hepatopathy, were significantly increased on pretreatment with AE and NE, resulting in a subsequent recovery towards normalization (Fig.2).

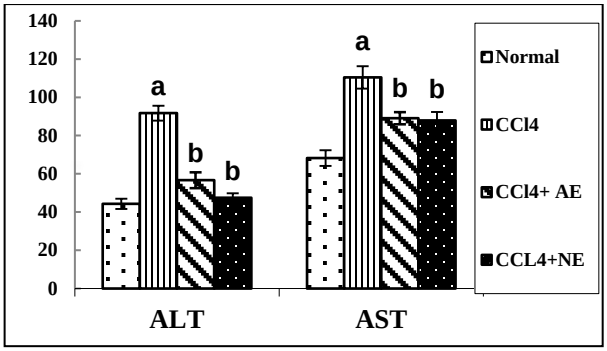


Figure 1. Effect of the artichoke by-product extract (AE) on serum ALT and AST activities in CCl_4-induced hepatic damage in rats. Values expressed as mean ± S.E.M. (U/L); n = 7 rats per group; [a] $P < 0.05$ Vs normal control group; [b] $P < 0.05$ Vs CCl_4 group

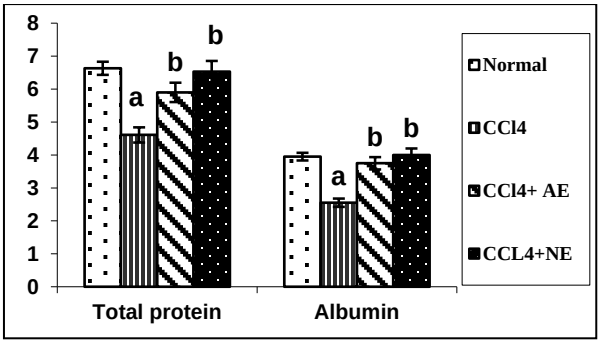


Figure 2. Effect of the artichoke by-product extract (AE) on serum total protein and albumin in CCl_4-induced hepatic damage in rats. Values expressed as mean ± S.E.M. (g/dl), n = 7 rats per group; [a] $P < 0.05$ Vs normal control group; [b] $P < 0.05$ Vs CCl_4 group

Increased oxidative stress was observed in CCl_4-treated rats, as evidenced by the remarkable increase in hepatic MDA levels, with significant depletion of hepatic GSH and PSH levels, as well as, the concomitant decrease in the activities of hepatic GSH-Px and GST (Tables 1and 2). Pretreatment with the tested extract (AE) and the standard extract (NE) significantly decreased hepatic MDA, recovered hepatic GSH and PSH levels, restored the GSH-Px activity and significantly increased the activity of GST as compared to CCl_4-treated rats. Moreover, a significant increase in the hepatic NO levels (Table 2) in CCL_4-group, indicated hepatic damage. Under high local concentrations of NO, it can react with the superoxide anion to form a potent and versatile oxidant, peroxynitrite. Pretreatment with the tested extract and reference, significantly inhibited CCL_4-induced elevation of hepatic NO levels.

Table 1. Effect of the hydroalcoholic extract of artichoke by-product (AE) on hepatic MDA, PSH and GSH levels in CCl_4-induced hepatic damage in rats.

Groups	MDA (nmole/mg protein)	PSH (nmole/mg protein)	GSH (mg/gm tissue)
Normal control	1.8±0.12	138.48±7.55	1.75±0.11
CCl_4 (1.5 ml/kg)	4.45±0.5 [a]	93.75±5.74 [a]	0.62±0.05 [a]
CCl_4+AE (200mg/ kg)	2.63±0.18 [b]	141.16±10.7 [b]	1.38±0.12 [b]
CCl_4+NE (250mg/ kg)	2.1±0.17 [b]	152.8±5.84 [b]	1.59±0.07 [b]

NE: standard extract (Finzelberg standardized extract); Values expressed as mean ± S.E.M.; n = 7 rats per group
[a]: significant difference from normal control group at $p < 0.05$; [b]: significant difference from CCl_4 group at $p < 0.05$.

According to these results it can be suggested that hepatoprotective action of AE may be due to its membrane stabilizing effect on hepatic cells by the antioxidant effect of the extract. Thus, it was concluded that the hydroalcoholic extract of artichoke by-product (AE) possessed significant antioxidant and hepatoprotective effects, which were comparable to Finzelberg standardized artichoke extract (NE), using CCl_4-induced oxidative stress and hepatic damage in rats.

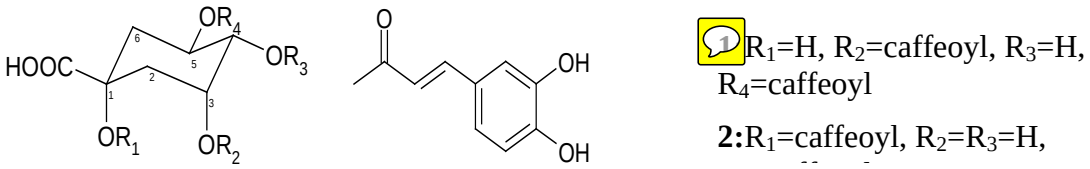
Table 2. Effect of the hydroalcoholic extract of artichoke by-product (AE) on hepatic NO level, GSH-Px and GST activities in CCl_4-induced hepatic damage in rats.

Groups	GST (U/mg protein)	GSH-Px (U/mg protein)	NO (nmole/mg protein
Normal control	543±12.04	302.16±20.42	2.15±0.138
CCl_4 (1.5ml/ kg)	440.28±7.45 [a]	227.16±7.53 [a]	3.33±0.266 [a]
CCl_4+AE (200mg/ kg)	475.38±6.44[a,b]	295.33±10.47 [b]	1.8±0.09 [b]
CCl_4+NE (250mg/ kg)	496.5±10.6[a,b]	304.16±10.05 [b]	2.06±0.147 [b]

NE: standard extract (Finzelberg standardized extract); Values expressed as mean ± S.E.M.; n = 7 rats per group
[a]:significant difference from normal control group at $p < 0.05$; [b]: significant difference from CCl_4 group at $p < 0.05$.

Isolation of caffeoylquinic acids from artichoke by-product

Three major dicaffeoyl quinic acids viz., 3,5-di-O-caffeoyl quinic acid (**1**), 1,5-di-O-caffeoyl quinic acid (**2**) and 3,4-di-O-caffeoyl quinic acid (**3**) were isolated from the active hydroalcoholic extract of artichoke by-product. These compounds were identified, based on their UV, ^1H,^{13}C-NMR and ^1H-^1H COSY spectra and its comparison with literature data (Basnet et al.,1996; Carnat et al., 2000; Satake et al., 2007). The protons of the quinic acid moiety were assigned, using ^1H-^1H COSY.



1: R_1=H, R_2=caffeoyl, R_3=H, R_4=caffeoyl

2: R_1=caffeoyl, R_2=R_3=H,

caffeoyl moeity

Figure 3. Isolated caffeoylquinic acids from the extract of artichoke by-product (AE)

HPLC analysis

Opimization of the extraction condition. In this study, solid-phase extraction was employed for further purification of the crude extract, using different solvent compositions (water-water/methanol-100 % methanol). Best results were obtained with 50% aqueous methanol (v/v), followed by 60% aqueous methanol (v/v) to ensure the complete elution of phenolic compounds.

Validation of the quantification method. The linearity of the plot of concentration (x, µg/ml) for each standard versus peak area (y) was investigated. The calibration curves indicated good linearity ($r^2 > 0.999$), within the tested range. Results are illustrated in Table (3). The limit of detection (LOD) was determined to be 0.4, 0.08 and 0.12 µg/ml for chlorogenic, caffeic and 1,5 dicaffeoylquinic acids, respectively. The analytical precision from the results of inter-day (five times a day) and intra-day (over three consecutive days) determinations, is indicated by the relative

standard deviations (RSDs), which were both less than 5% for the five quantified compounds. The results shown in Table (4), indicated that the method was precise for simultaneous determination of the five components. The RSDs of the repeatability tests were less than 4% for all determined compounds (Table 5). The mean recovery percentage (Mean±SD) of 1,5-di-O-caffeoylquinic acid was 104.06±0.35 and the RSD was 0.34%. This study demonstrated the high extraction recovery of the applied method.

Table 3. Regression equations and respective correlation coefficients for the quantitative determination of hydroxycinnamate derivatives, using the developed HPLC method.

Standard	Linear range (μg/ml)	Regression equation	correlation coefficient (r^2)
Chlorogenic acid	12-300	Y= 69.513x-74.485	0.9995
Caffeic acid	1.2-24	Y= 102.66x-4.518	0.9993
1,5-di-O-caffeoyl quinic acid	2.4-120	Y=74.053x+68.266	0.9998

Y= peak area and x= corresponding concentration (μg/ml)

Table 4. Intra-day and Inter-day precision of the developed HPLC method for the determination of five hydroxycinnamate derivatives in the alcoholic extract of artichoke by-product (AE)

Compound	Intra-day[a]		Inter-day[b]	
	Content*	RSD (%)	Content*	RSD (%)
chlorogenic acid	12262.7±240.05	1.96	12315.9 ±41.91	0.34
Caffeic acid	701.4 ± 13.16	1.88	700.97 ± 31.8	4.54
3,5-di-O-caffeoylquinic acid	9544.5 ± 171.38	1.80	9607.2 ± 59.52	0.62
1,5-di-O-caffeoylquinic acid	24436.2 ± 466.81	1.91	24589.0 ± 260.94	1.06
3,4-di-O-caffeoylquinic acid	11898.4 ± 281.73	2.37	12088.9 ± 102.06	0.84

[a] Sample analyzed five times during one day, (n=5)
[b] Sample analyzed each day over three consecutive days, (n=3); * Mean±SD (mg/Kg alc. extract)

Table 5. Repeatability of the developed HPLC method for the determination of five hydroxycinnamate derivatives in the alcoholic extract of artichoke by-product (AE)

Compound	Content*	RSD (%)
chlorogenic acid	12158.5 ± 209.07	1.72
Caffeic acid	697.3 ± 21.92	3.14
3,5-di-O-caffeoyl quinic acid	9458.9 ± 210.37	2.22
1,5-di-O-caffeoyl quinic acid	24164.0 ± 405.07	1.68
3,4-di-O-caffeoyl quinic acid	11846.2 ± 257.22	2.17

* Mean±SD (mg/Kg alc. extract); (n=3)

Content of five hydroxycinnamate derivatives in the extract of artichoke by-product (*AE*) *by HPLC analysis.* As shown in Fig. (4), caffeoylquinic acids represented the main peaks (> 5% of total peak area), displayed in the HPLC chromatogram of the extract (AE)**,** based on comparison with isolated compounds and available standards. The following compounds were identified and quantified: 3,5-di-O-caffeoylquinic acid (**1**); 1,5-di-O-caffeoylquinic acid (**2**); 3,4-di-O-caffeoylquinic acid (**3**); 5-O-caffeoylquinic acid (chlorogenic acid) (**4**) and caffeic acid (**5**). The principal compound was 1,5-

di-O-caffeoylquinic acid (**2**) (24164.0 mg/Kg ext.), followed by chlorogenic acid (**4**) (12158.5 mg/Kg ext.), 3,4-di-O-caffeoylquinic acid (**3**) (11846.2 mg/Kg ext.) and 3,5-di-O-caffeoylquinic acid (**1**) (9458.9 mg/Kg ext.). Thus three major dicaffeoylquinic acids **(1, 2 and 3)** were found in artichoke waste extract, in approximately the following ratio: 0.4:1:0.5, respectively, with a total content of 45469.1 mg/Kg ext (4.55 g%), calculated as 1,5-di-O-caffeoylquinic acid. In addition, low amount of caffeic acid (697.3 mg/Kg ext.), was also detected.

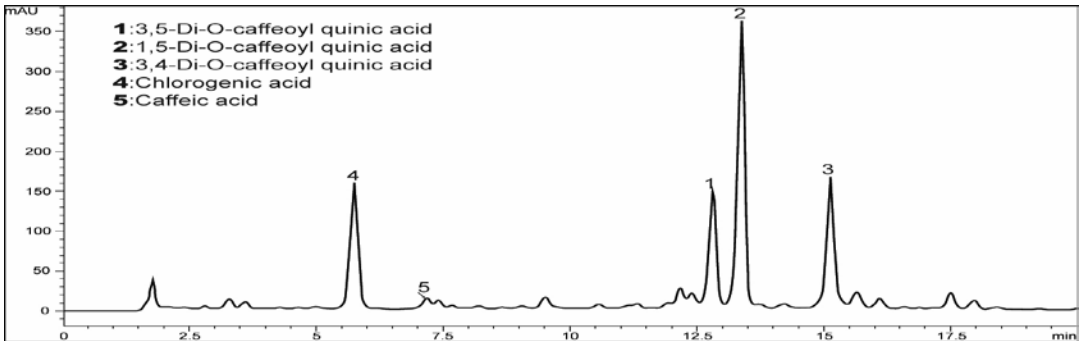


Figure 4. HPLC chromatogram of the extract of artichoke by-product (AE)

Nutritive value of artichoke by-product

The percentage of moisture, crude protein, crude fibre, crude fat, ash and total carbohydrates were 5.6, 13.7, 10.5, 0.88, 8.59 and 60.73 %, respectively on dry wt basis. The amino acid composition revealed 16 amino acids, with aspartic acid (3.45%), being the major, followed by glutamic acid (1.08%) and proline (1.03%), whereas, cysteine (0.17%) and methionine (0.11%) were the minor identified amino acids. Elemental analysis of the ash of artichoke waste, revealed the presence of Mg, Si, P, S, Cl, K, Na, Ca and Fe. K (34.85%), Ca (7.96%) and P (3.04%) were the major identified minerals. Investigation of the vitamin profile revealed the presence of vitamins C (25.2 mg%), B_1(1.53 mg%) and B_2 (0.39 mg%), in addition to traces of vitamin E (19.11 µg%) and vitamin A (66.19 µg%), on dry wt. basis.

According to our findings, AE possessed hepatoprotective and antioxidant activities which are mainly attributed to the presence of caffeoylquinic acids (Basnet et al., 1996; Gebhardt and Fausel, 1997). The AE extract contained 8.82 g% caffeoylquinic acids, calculated as chlorogenic acid, which was comparable to that in commercial artichoke extract products (3-15% total caffeoylquinic acids, calculated as chlorogenic acid). The artichoke by product is rich as well with minerals, vitamins and amino acids.

CONCLUSIONS

Based on this study artichocke by-products could be potential for production of phenolics and use as antioxidant and hepatoprotective nutraceutical because both of their low cost and ease of availability. Likewise, techno-economical studies are required to determine the feasibility of utilizing this agro-food residue for commercial production. Much work has still to be done in order to increase the yield of the extract and optimize the process of extraction and make it economically feasible.

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