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Evaluation of antioxidant and hepatoprotective effects of white cabbage essential oil

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ABSTRACT

Context: There have been no reports of the extraction of essential oil (EO) from white cabbage [*Brassica oleracea* L. var. *capitata* (L.) Alef. f. *alba* DC. (Brassicaceae)] (Bocfal) or its chemical composition, antioxidant activity, or hepatoprotective effects.

Objective: To extract Bocfal EO, to identify and quantify its chemical components, to assess their antioxidant capacity, and to evaluate the hepatoprotective properties of Bocfal EO.

Materials and methods: Bocfal EO was obtained using hydrodistillation (200 mm Hg/58 °C). The chemical composition was analyzed using GC-MS and was quantified using GC-FID. The antioxidant activity of Bocfal EO and its main constituents was evaluated using TBARS in rat brain homogenates. A Bocfal EO hepatoprotective effect (192 mg/kg) on acute carbon tetrachloride (CT)-induced liver damage was determined in rats using biochemical markers and histological analysis. Diallyl disulphide (DADS) (1 mmol/kg) was used as a control for comparison.

Results: Bocfal EO contained organic polysulphides (OPSs), such as dimethyl trisulphide (DMTS) 65.43 ± 4.92% and dimethyl disulphide (DMDS) 19.29 ± 2.16% as major constituents. Bocfal EO and DMTS were found to be potent TBARS inhibitors with IC_{50} values of 0.51 and 3 mg/L, respectively. Bocfal EO demonstrated better hepatoprotective properties than did DADS ($p < 0.05$), although both slightly affected the hepatic parenchyma *per se*, as observed using histopathology.

Discussion and conclusion: The antioxidant properties of Bocfal EO and DMTS may be the mechanism of hepatoprotective action; the parenchymal disturbances by Bocfal EO or DADS alone may be related to the high doses used.

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Brassica oleracea var. *capitata* f. *alba*; organic polysulphides; hepatoprotection

Introduction

The liver is one of the largest internal organs in the human body, and it controls the flow and safety of substances absorbed from the digestive system into the systemic circulation. The main liver functions include the production of energy by protein, carbohydrate and lipid metabolism, the synthesis of bile salts, the storage of vitamins, and the production of lipoproteins, angiotensin and coagulation factors among others. Therefore, hepatic diseases such as fatty liver disease, hepatitis and cirrhosis highly impact the homeostasis of the body (Arias et al. 2010). Hepatopathies are caused by toxic chemicals, drugs, alcoholism, viral infections and glutathione exhaustion, among others; thus, numerous animal models of liver injury have been used to test the hepatoprotective potential of natural products (Jaeschke et al. 2013). The hepatotoxic carbon tetrachloride (CCl_4, CT) model is widely used for the experimental induction of acute or chronic liver damage; the metabolism of CT involves the homolytic cleavage of C–Cl bonds, which lead to the formation of free radicals ($CCl_3^{\cdot}$) that bind covalently to a series of molecular structures, in particular to membrane lipids, during the peroxidation process (Muriel 2009). In this regard, the genus *Brassica* (Brassicaceae) comprises diverse vegetable species that contain bioactive compounds such as glucosinolates and phenolic compounds. Many of these compounds are famous for their antioxidant activity, via preventing carcinogen bioactivation and increasing the detoxification of reactive oxygen species (ROS) (Wagner et al. 2013; Šamec et al. 2016).

The research on the aromas from cruciferous vegetables (i.e., cabbages and related vegetables, *Brassica oleracea* Linn. spp.) and, in consequence, their bioactive compounds, represents a real challenge because of the scarce yields of these aroma compounds, which have been described to contain isothiocyanates (ITCs) and organic polysulphides (OPSs) as major components (Stoewsand 1995). The OPSs or sulfane sulphur compounds contain a labile, highly reactive sulphur atom in a reduced oxidation state with a valence of 0 or −1, covalently bound to another sulphur atom ($R–S_n–R'$) (Iciek et al. 2009), and this chemical characteristic makes OPSs good antioxidants (Toohey & Cooper 2014). Moreover, cruciferous vegetables contain other antioxidant compounds; for instance, polyphenols from dry cabbage powder have exhibited moderate antioxidant activity in chemical assays.

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In addition, the anthocyanin-rich extract from red cabbage [*B. oleracea* L. var. *capitata* (L.) Alef. f. *rubra* DC.] has been shown to attenuate cardiac and hepatic oxidative stress in atherogenic rats (Sankhari et al. 2012). Likewise, the genus *Allium* (Amaryllidaceae) contains OPSs such as allyl sulphides (Calvo-Gómez et al. 2004). The biological properties of the diallyl disulphide (DADS) found in garlic (*Allium sativum* L.) have been widely studied, and they include modulation of cellular oxidative stress, involvement in signal transduction and post-translational modification of proteins by the formation of mixed disulphides (Iciek et al. 2009) and hepatoprotective effects, which were observed on the model of CT-induced liver damage (Zhu et al. 2014).

Currently, there are no published reports concerning the extraction, identification and quantification of the chemical constituents of white cabbage essential oil (*B. oleracea* var. *capitata* f. *alba,* Bocfal EO), its antioxidant capacity or its hepatoprotective activity. Therefore, the aim of this study was to obtain Bocfal EO via hydrodistillation (at low pressure/temperature), to identify and quantify its chemical constituents (OPSs content), to assess their antioxidant capacity through an *in vitro* assay, and to evaluate Bocfal EO hepatoprotective properties by comparing them with those of DADS in an acute, CT-induced liver damage rat model.

Materials and methods

Chemicals

All reagents were of analytical grade. Dichloromethane of chromatographic grade and diallyl disulphide (DADS) were purchased from Sigma-Aldrich (St Louis, MO). Dimethyl trisulphide (DMTS) and dimethyl disulphide (DMDS) (purity 98%) were supplied by Natural Advantage (Oakdale, LA).

Vegetable material

The aerial parts of fresh Bocfal were collected in parcels at the 'Ejido de San Vicente Chicoloapan, Estado de México' in August 2015. The Bocfal showed edible maturity and was harvested within 24 h period. The identity of the plant was confirmed by Dr N. Ivalú Cacho and a voucher specimen (1420213MEXU) was deposited at the Herbario Nacional de México (MEXU).

Essential oil (EO) extraction

Distillation is the traditional method for EO extraction (Amorati et al. 2013), but hydrodistillation is the most used because it allows the application of low-pressure conditions (200–150 mm Hg) and low temperatures (55–58 °C) to avoid chemical changes that are induced by thermal shock in the EO at 95 °C (Kim et al. 2011). Bocfal EO was extracted by hydrodistillation as reported previously (Calvo-Gómez et al. 2004); in brief, 12 kg of vegetable was ground with 4 L of water for 20 min in an industrial blender (Santos model Miss Blend, Mexico). Then, the plant material was introduced into a distillation apparatus and heated for 12 h in a double boiler until the temperature reached 58 °C. The pressure was then slowly reduced from 200 to 150 mm Hg. The Bocfal EO was stored in Pyrex glass flasks at −20 °C.

EO chemical composition by gas chromatography – mass spectrometry (GC-MS) analysis and gas chromatography – flame ionization detector (GC-FID) quantification

GC-MS analyses of the EO samples was performed using an AutoSystem XL-GC equipped with a polar capillary column coated with free fatty acid phase (HP-FFAP, 30 m × 0.32 mm ID ×0.25 µm film thickness) and coupled to a selective quadrupole TurboMass-MS detector with an electron impact ionization system at 70 eV and 215 °C (Perkin-Elmer, Norwalk, CT). The oven temperature started at 50 °C and was gradually increased up to 130 °C at a rate of 6 °C/min, where it was maintained for 3 min. A second program was used, where the temperature was increased to 200 °C at a rate of 8 °C/min, where it was maintained for 8 min. The injector temperature was 180 °C, and the flow rate of helium was 1.0 mL/min at 8 psi. The EO samples were diluted (1.0 µL) in dichloromethane 1:25 v/v, and they were then manually injected in split mode.

The linear retention indices (RI) of the volatile compounds were calculated with a series of *n*-alkanes (C_{10}–C_{26}), and DMDS, DMTS and allyl isothiocyanate (AITC) as standards. The identification of each detected compound was accomplished by comparing its retention time and mass spectra with those of authentic injected compounds as well as with information available from a computerized spectral database (NIST MS Search 1.7) and from published literature. Relative percentage amounts of the separated compounds in the Bocfal EO were expressed as percentages by peak area normalization of the total ion chromatogram (TIC) from the average value for each of three GC-MS iterations ($n = 9$).

The quantification of the major EO constituents was performed using a GC-FID on an Agilent Technologies 6890 plus GC (Agilent Technologies, Santa Clara, CA) equipped with a single injector, the same HP-FFAP polar capillary column was used. The flow of the carrier gas (N_2) was 0.8 mL/min. The initial column temperature was 60 °C, and it programmed as in the GC-MS equipment. Split injection was conducted with a split ratio of 1:100. A volume of 1 µL of EO and standard samples diluted with dichloromethane was injected. The amounts of DMTS, DMDS and AITC, the three most abundant compounds in the Bocfal EO, were calculated using the linearity of the responses tested and the preparation of standard curves for each pure control compound in the following volume ranges (µL): DMTS 0.04–0.01; DMDS 0.015–0.005; AITC 0.005–0.002. The final diluted sample of Bocfal EO was 0.04 µL.

Animals

Male Wistar rats weighing approximately 180–200 g were housed in standard plastic cages at a temperature of 22–24 °C, under a 12 h light–dark cycle. They had free access to food (standard Purina chow diet, USA) and purified water. All the animals received humane care according to the respective institutional guidelines, the Mexican Official Norm (NOM-062-ZOO-999) regarding technical specifications for the production, care and use of laboratory animals, and the criteria outlined in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health 1985).

Effect of Bocfal EO on lipid peroxidation inhibition in rat brain homogenates

The capacity to inhibit the products of lipid peroxidation was determined using thiobarbituric acid reactive substance (TBARS) quantification in homogenized rat brain samples according to the procedure described in the literature, with minor modifications (Ohkawa et al. 1979). In brief, the rats were sacrificed by CO_2, and their brains were rapidly dissected and homogenized in cold phosphate-buffered saline (9.5 mM) at pH 7.4, 1:10 (w/v).

The homogenate was centrifuged at 3400 rpm for 10 min, the supernatant was recovered, and its protein content was measured and adjusted at 2.66 mg of protein/mL in PBS according to the method described by Bradford (1976). Then, in an ice bath, 375 µL of supernatant was added to 50 µL of 20 µM EDTA, and this mixture was incubated at 37 °C for 30 min in the absence or presence of 25 µL aliquots of the different dilutions of each test sample; the screened concentration ranges were the following: Bocfal EO 0.15–4.7; DMTS 1.40–14.06; DMDS 1.05–10.56; AITC 1.12–6.34 and DADS 17.78–1000 mg/L.

Lipid peroxidation was initiated by the addition of 50 µL of 100 µM $FeSO_4$ (final concentration 10 µM). This blend was incubated at 37 °C for 1 h, and 0.5 mL of TBA reagent (0.5% TBA in 0.05 N NaOH and 30% TCA, 1:1 v/v) was added. The mixture was cooled by ice for 10 min, centrifuged at 10,000 rpm for 5 min, and heated at 75 °C for 30 min. After the mixture had cooled to room temperature, the absorbance was measured at 540 nm using a Bio-Tek model ELx808 microplate reader. Butylated hydroxytoluene (BHT) and α-tocopherol were used as synthetic controls. TBARS content was calculated using 1,1,3,3-tetramethoxypropane (TMP) for the standard curve. The lipoperoxidation inhibition percentage was calculated as the inhibition ratio (IR) as follows:

IR (%) = $[(C–E)/C] \times 100$, where C is the absorbance of the control sample, and E is the absorbance of the test sample.

Effect of Bocfal EO on acute, CT-induced liver damage in vivo

Experimental groups, treatment and doses

To assess the hepatoprotective properties of Bocfal EO on CT-induced liver damage, the rats were divided into six groups with $n = 7$ animals in each group. The first group was the normal control group (VE + VCT) that received two p.o. doses that were 12 h apart of 1 mL of olive oil as the vehicle (VE) for the Bocfal EO or DADS, which was used as a positive control because its hepatoprotective properties have been previously demonstrated and because it is a sulfane sulphur, or OPS, compound. In addition, 2 h after the first administration of VE, the rats were orally given 1 mL of mineral oil as the vehicle for CT (VCT). All the remaining groups underwent the same administration schedule. The second (Bocfal EO + VCT) and third (DADS + VCT) groups received Bocfal EO and DADS, respectively, twice orally dissolved in their vehicles and also received the VTC p.o.; thus, both were control groups intended to show any adverse effects of Bocfal EO or DADS *per se* on normal rats. The fourth group was the damaged control (VE + CT), wherein rats received the VE twice orally and a single dose of 4 g/kg CT dissolved in its vehicle (1:1 v/v). Finally, the fifth (Bocfal EO + CT) and sixth (DADS + CT) groups were administered their respective substances and were injured by CT.

Regarding the Bocfal EO dose, it was considered an amount of EO that contained 1 mmol/kg of DMTS (192 mg/kg of EO), which was later determined to be its main constituent at 65.43 ± 4.92%, as well as 1 mmol/kg of DADS (146 mg/kg). These doses were based on a previous report wherein this moderate dose of pure OPSs, including DADS, was used in Wistar rats (Siess et al. 1997).

All the animals were sacrificed by exsanguination under light ether anaesthesia 24 h after CT administration; hence, blood was collected by cardiac puncture using a syringe containing sodium heparin as an anticoagulant. The liver was rapidly removed and rinsed in saline. All the samples were either kept on ice for immediate use or frozen at −70 °C until analyzed.

Plasma enzyme activities and bilirubin determinations

Plasma was obtained for the determination of the canalicular membrane enzyme activities of cholestasis markers, such as alkaline phosphatase (AP) and γ-glutamyl transpeptidase (GGTP), and for the cytosolic activity of the necrosis indicator alanine aminotransferase (ALT) (Fernández-Martínez et al. 2006) as well as for the quantification of the total bilirubin (TB) concentration (TECO Diagnostics kit, CA).

Glycogen determination

Small liver pieces (0.5 g) were separated for glycogen measurement using anthrone–sulphuric acid reagent (Seifter, et al. 1950).

Assessment of lipid peroxidation

The extent of lipid peroxidation (LP) was estimated in the liver homogenates by measuring malondialdehyde (MDA) formation using the thiobarbituric acid method (Ohkawa et al. 1979). Protein was determined according to the method described by Bradford using bovine serum albumin as a standard (Bradford 1976).

Catalase as an oxidative stress indicator

For hepatic catalase (CAT) activity, H_2O_2 consumption was measured at 480 nm (Cohen et al. 1970). In brief, 5 mL of cold 6 mM H_2O_2 was added to 0.5 mL aliquots of the 10% liver homogenate. After 3 min, the reaction was stopped with 1.0 mL of 6 N H_2SO_4. The H_2O_2 reacted with a standard excess of 0.01 N $KMnO_4$, and the residual $KMnO_4$ was measured at 480 nm. CAT activity was calculated as the first-order reaction rate constant of the H_2O_2 decomposition ($k \times 10^2$/min).

Histology

Liver samples were taken from all the animals and fixed with 10% formaldehyde in phosphate-buffered-saline for 24 h. Then, they were washed with tap water, dehydrated in alcohols and embedded in paraffin. Sections of 6–7 µm were mounted on glass slides covered with silane; the paraffin was previously eliminated. They consequently were used for haematoxylin/eosin staining for histological examinations using light microscopy.

Statistical analysis

TBARS data underwent a one-way variance analysis (ANOVA), and the significant differences were obtained using Dunnett's test. For the hepatoprotective assessment, an ANOVA with the Student–Newman–Keuls test was used to compare groups. The resulting data are expressed as the means ± SEM and were analyzed using Sigma Stat software version 3.1 (Systat Software Inc., San Jose, CA). In all cases, a difference was considered significant when $p < 0.05$.

Table 1. Major components of *B. oleracea* var. capitata F. *alba* essential oil (Bocfal EO) obtained by hydrodistillation at low pressure (200–150 mmHg) and low temperature (55–58 °C) conditions.

Peak (#)	RT (Min)	RI	Compound	Content (%)
			Organopolysulphanes (OPS)	
(1)	2.26	1224	Dimethyl disulphide (DMDS)	19.29 ± 2.16
(11)	6.24	1544	Dimethyl trisulphide (DMTS)	65.43 ± 4.92
(13)	7.12	1595	Methyl pentyl disulphide	0.19 ± 0.04
(17)	10.07	1750	Allyl methyl trisulphide	0.22 ± 0.07
(19)	11.39	1818	Methyl methylthiomethyl disulphide	1.39 ± 0.24
(20)	12.89	1893	Dimethyl tetrasulphide	1.50 ± 1.01
			Isothiocyanates and indoles	
(10)	5.94	1527	Allyl isothiocyanate (AITC)	4.31 ± 0.70
(14)	7.59	1621	3-Butenyl isothiocyanate	0.85 ± 0.16
(22)	17.64	2109	Indole	0.10 ± 0.01
(23)	18.04	2125	1-Methoxyindole	0.01 ± 0.00
(24)	19.38	2181	3-(Methylthio)propyl isothiocyanate	0.47 ± 0.13
(26)	25.62	2511	Butyl isothiocyanate	0.19 ± 0.05
(28)	27.67	2731	Phenylethyl isothiocyanate	0.61 ± 0.15
			Nitriles	
(3)	3.21	1336	3-Butenenitrile	1.50 ± 0.23
(6)	4.03	1415	4-Methylpentanenitrile	0.16 ± 0.03
(7)	4.48	1441	2-Pentenenitrile	0.41 ± 0.06
(8)	4.83	1462	Hexanenitrile	0.14 ± 0.01
(25)	22.04	2352	Benzene;propanenitrile	0.08 ± 0.02
			Alcohols	
(2)	2.94	1306	Penten-3-ol	0.51 ± 0.07
(5)	3.84	1404	2-Pentenol	0.23 ± 0.04
(9)	5.76	1516	1-Hexanol	1.32 ± 0.34
(12)	6.73	1556	2-Hexen1-ol	0.39 ± 0.10
			Aldehydes	
(4)	3.67	1388	2-Hexenal	0.30 ± 0.05
(18)	10.71	1783	Heptanal	0.07 ± 0.01
(21)	14.43	1970	2,4-Decadienal	0.05 ± 0.02
			Miscellaneous	
(15)	8.00	1642	3,3-Dimethylallylbromide	0.23 ± 0.03
(16)	8.60	1673	Camphor	0.04 ± 0.01
(27)	26.93	2601	Eugenol	0.03 ± 0.02

RI: retention index; RT: retention time.

Results

Extraction yields, constituents and quantification of Bocfal essential oil (EO)

The Bocfal EO extraction yield was 37.56 ± 3.71 mg/kg (33.33 ± 3.29 µL/kg) ($n = 3$). The extraction process was repeated until the amount of EO was enough to carry out the biological tests. The GC-MS permitted the identification of 28 volatile compounds in the Bocfal EO, accounting for approximately 99% of the total amount (Table 1). They were categorized into six chemical families, which are listed according to their relative abundance. The first family includes OPS compounds (R-S_n-R, $n = 1$–4 and R = CH_3) at 88.02 ± 5.11% as follows: DMTS (65.43 ± 4.92%) is the most abundant OPS, and DMDS (19.29 ± 2.16%) is the second most abundant compound. The second family includes the ITCs and indoles at 6.53 ± 1.09%, including AITC (4.31 ± 0.70%), 3-butenyl isothiocyanate (0.85 ± 0.16%), and phenylethyl isothiocyanate (0.61 ± 0.15%), among others. The third family is the nitriles family (2.29 ± 0.33%); five compounds were identified in this study, with 3-butenenitrile (1.50 ± 0.23%) being the most abundant, followed by 2-pentenenitrile (0.41 ± 0.06%) and three more compounds. The fourth family is the alcohol family (1.76 ± 0.53%) with 1-hexanol (1.32 ± 0.34%), 2-hexen1-ol (0.39 ± 0.10%), and some others. Aldehydes (0.57 ± 0.11%) form the fifth family, including 2-hexenal (0.30 ± 0.05%) and others. Finally, miscellaneous compounds constitute the sixth family at 0.30 ± 0.04%.

Table 2. Antioxidant activity by TBARS assay.

Tested compound	TBARS – IC_{50} (mg/L)
Bocfal EO	0.51 ± 0.03
DMTS	3.00 ± 0.2
DMDS	No activity
AITC	3.16 ± 0.13
DADS	444.64 ± 49.57[a]
BHT	0.27 ± 0.10
α-Tocopherol	2.92 ± 0.93

Results are expressed as the mean value ± SEM of experiments performed in duplicate with samples of rat brain homogenates from at least five animals.
[a]Different from Bocfal EO, $p < 0.05$, ANOVA, Dunnett's test.

The quantifications of DMTS, DMDS and AITC amounts, the three most abundant compounds in the Bocfal EO, were calculated using the linearity of the responses. This was conducted by preparing standard curves in a concentration range of pure volatile controls for comparison and using the equation from the linear regression obtained from each curve with the GC-FID equipment. The results indicate that each 1000 µL of Bocfal EO contains 575 ± 125 µL of DMTS, 483 ± 88 µL of DMDS and 50 ± 39 µL of AITC. The correlation coefficients of the three standard curves were values higher than 0.95.

In vitro *antioxidant activity by TBARS assay*

Table 2 presents the antioxidant activity of Bocfal EO through a TBARS assay with rat brain homogenates; which showed an IC_{50} value of 0.51 ± 0.03 mg/L. Bocfal EO showed a very similar antioxidant capacity when compared with that of the synthetic controls, BHT and α-tocopherol (0.27 ± 0.01 and 2.92 ± 0.93 mg/L, respectively). Additionally, in this assay, the pure compounds, DMTS and AITC exhibited IC_{50} values almost equal to that of α-tocopherol. In contrast, DMDS seemed to be inactive, resembling DADS; its IC_{50} was 444.64 ± 49.57 mg/L, the highest value in this test. Indeed, the TBARS assay seems to be more suitable to quantify the antioxidant capacity of OPSs, because Bocfal EO, DMTS and DMDS were ineffective as antioxidants in the usual *in vitro* tests with DPPH and FRAP (data not shown), wherein polyphenolic compounds are very active.

Effect of Bocfal EO on acute CT-induced liver damage in vivo

Figure 1 depicts the plasma enzyme activities of AP and GGTP as well as TB concentration. Due to CT-provoked injury, the AP activity was increased twofold in the damaged group (VE + CT) when compared with that of the normal control group (VE + VCT). In addition, the administration of Bocfal EO and DADS elevated that marker, though neither resulted in a significant difference in contrast with VE + VCT. However, the treatment with Bocfal EO significantly decreased the enzyme activity ($p < 0.05$) during the liver injury compared with VE + CT, which shows an anticholestatic effect. In addition, DADS did not modify the CT-induced AP elevation.

GGTP enzyme activity was increased almost fivefold by CT administration ($p < 0.05$) with respect to that in VE + VTC control group; conversely, the Bocfal EO or DADS administrations slightly reduced the normal levels, but not in a significant manner. Regarding the CT-injured groups treated with the respective

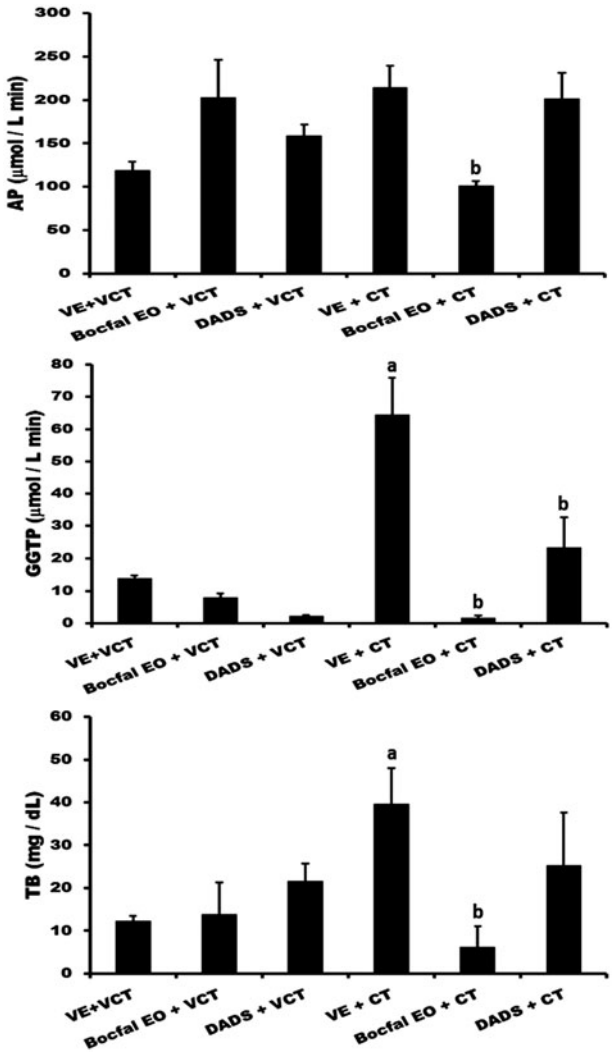

Figure 1. Plasma cholestasis markers: enzyme activities of alkaline phosphatase (AP) and γ-glutamyl transpeptidase (GGTP); total bilirubin determined in the plasma from control rats treated with their respective vehicles (VE + VTC), with Bocfal EO (Bocfal EO + VCT) or with DADS (DADS + VCT) as well as those administered the hepatotoxicant CT (VE + CT) but treated with the same compound, respectively (Bocfal EO + CT and DADS + CT). Each bar represents the mean value of experiments performed in duplicate with samples from at least four animals ± SEM. (a) Difference from the VE + VCT group and (b) from the VE + CT group, $p < 0.05$, Student–Newman–Keuls test.

compounds, their GGTP activity increases were completely prevented; again, Bocfal EO was more effective as an anticholestatic agent than was DADS.

TB in the experimental groups showed a pattern very similar to that of AP because CT-induced liver damage increased the TB concentration, but in a statistically significant way; furthermore, the Bocfal EO and DADS control groups also exhibited a small degree of elevated TB concentration. However, although DADS lowered this marker, this change was not statistically significant. In contrast, Bocfal EO completely lowered the TB level ($p < 0.05$).

Figure 2 shows the markers of liver injury: ALT, glycogen, LP and CAT. ALT is a cytoplasmic enzyme marker of necrosis; its activity was slightly diminished by Bocfal EO but significantly lowered by DADS administration. As expected, CT induced a notable increment in ALT activity ($p < 0.05$), while treatments with both Bocfal EO and DADS abolished such elevation of activity, indicating their antinecrotic properties during oxidative damage, particularly for Bocfal EO.

The Bocfal EO + VCT and DADS + VCT groups showed normal levels of glycogen with respect to the control group, while the CT-damaged group exhibited depleted glycogen content ($p < 0.05$). However, Bocfal EO treatment completely prevented the depletion of glycogen; DADS did as well but to a much lesser extent.

LP represents the oxidative stress of cellular membranes. This hepatic indicator was augmented several-fold through CT administration in contrast with the VE + VCT and the control groups of Bocfal EO and DADS, which did not alter the normal level of LP *per se*. In contrast, Bocfal EO treatment partially prevented the CT-induced oxidative injury by lowering the LP level approximately 70%; in addition, DADS maintained this marker at normal levels.

The normal liver CAT activity was augmented by Bocfal EO administration; conversely, DADS administration lowered it, and both of these results were significant ($p < 0.05$). In addition, treatment with either Bocfal EO or DADS completely prevented the CT-induced CAT activity elevation in a similar way.

Liver damage was also evaluated by a histological approach through haematoxylin/eosin staining, which stains nuclei a black/dark blue colour and parenchymal hepatocyte cytoplasm a pink/magenta colour (Figure 3). A normal hepatic cell population and tissue homogeneity are shown in a representative liver sample from the VE + VCT normal control group (Figure 3(a)). In contrast, type CT-injured liver sample showed important damage zones, diffuse ballooning necrosis, pyknotic nuclei and high hepatic steatosis with hepatocyte vacuolization (Figure 3(b)). Bocfal EO administration provoked diffuse hepatocyte ballooning by itself (Figure 3(c)); thus, when Bocfal EO was administered concomitantly with CT, the liver presented an increased diffuse ballooning degeneration (Figure 3(d)). The treatment with the OPS standard resulted in a similar case, as the DADS + VCT group demonstrated focal swollen hepatocytes (Figure 3(e)). Furthermore, liver samples from animals damaged by CT and treated with DADS showed individual and zonal necrosis accompanied by high neutrophil infiltration (Figure 3(f)). Moreover, despite the apparent hepatoprotective properties, the mortality in groups treated with the substances was higher (up to 30%) than the regular mortality observed for the acute CT model was (less than 10%); thus, perhaps hepatoprotection was achieved in the stronger rats. Additionally, the administration of Bocfal EO or DADS caused weight loss (data not shown), piloerection and diminished activity in the rats.

Discussion

The Bocfal EO extraction yield was limited (37.56 ± 3.71 mg/kg), considering that there are few studies on the extraction of volatile compounds from *Brassica* spp. using a Likens–Nickerson-type apparatus for hydrodistillation (Hifnawy et al. 2013). Since yield values of 2 mg/kg for broccoli, cabbage and cauliflower have been described (Buttery et al. 1976), this work reports an unprecedented yield of volatile compounds in Bocfal EO, in comparison with those other *Brassica* vegetables. It has been strongly suggested that variations in type, presence and abundance of compounds within EO from the same cultivars depend on the genetic and ecological growth factors (Charron et al. 2005) as well as the extraction method and temperature (Kim et al. 2011). The main reason for the limited amount of data about concentrated extracts of the volatile compounds present in *Brassica* is that ITCs and OPSs have glucosinolates and *S*-methyl-L-cysteine sulphoxide as precursors, which exist in low concentrations in

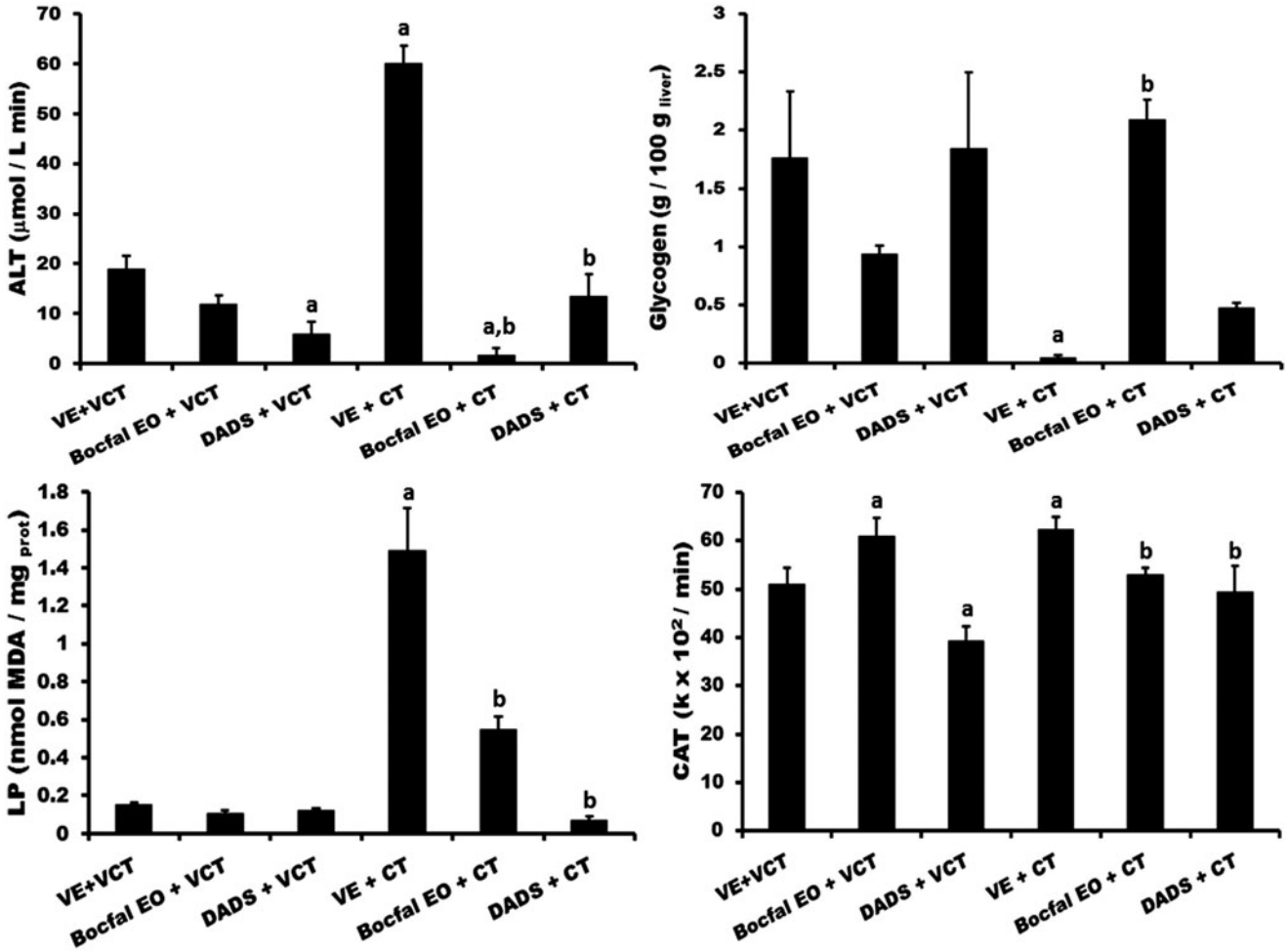

Figure 2. Plasma and liver markers of necrosis, functionality and oxidative stress: alanine aminotransferase enzyme activity (ALT) determined in the plasma, hepatic levels of lipid peroxidation (LP) and glycogen, and catalase activity (CAT) quantified in the livers of control rats treated with their respective vehicles (VE + VTC), with Bocfal EO (Bocfal EO + VCT) or with DADS (DADS + VCT) as well as those administered the hepatotoxicant CT (VE + CT) but treated with the same compound, respectively (Bocfal EO + CT and DADS + CT). Each bar represents the mean value of experiments performed in duplicate with samples from at least four animals ± SEM. (a) Difference from the VE + VCT group and (b) from the VE + CT group, $p < 0.05$, Student–Newman–Keuls test.

the vegetable tissues (Macleod & Macleod 1990; Stoewsand 1995).

The volatile compounds in Bocfal EO were identified and classified into six chemical families, listed according to their relative abundance. DMTS (65.43%) was the most abundant OPS, in agreement with many works on *Brassica* species (Maruyama 1970; Hifnawy et al. 2013). DMDS (19.29%) was the second most abundant OPS, and it is more frequently reported than DMTS in *B. oleracea* (Valette et al. 2003; Fernandes et al. 2009). Variations in composition and amount may be due to the extraction method used (Valette et al. 2003). The ITC and indole family includes AITC, which was the third most abundant compound and has been described in some publications as the major volatile constituent in the varieties of *B. oleracea* spp. (Stoewsand 1995). Five nitriles were identified and have previously been described in *Brassica*; they are considered ITC subproducts, which are generated under higher temperatures and more aggressive distillation methods than those used in this work (Valette et al. 2003). Additionally, all the alcohols identified herein have been reported previously (Macleod & Macleod 1990; Valette et al. 2003). Many aldehydes have been described for Brussels sprouts (Van Langenhove et al. 1991), and these compounds are considered high-temperature reaction markers, which are usually observed in extractions at 95 °C (Buttery et al. 1976); in this case, only three aldehydes were identified in EO, which may be due to the low extraction temperature applied. The sixth family included miscellaneous compounds in trace amounts.

The antioxidant activity of Bocfal EO assessed using a TBARS assay showed an antioxidant capacity very similar to that of the synthetic controls, BHT and α-tocopherol. Similarly, DMTS and AITC exhibited IC_{50} values almost equal to that of α-tocopherol. Conversely, DMDS seemed to be inactive, resembling DADS inactivity. This could be because lipids in brain are good targets for OPSs, which inhibit TBARS formation as products of brain lipid peroxidation; in addition, the number of sulphur atoms contained in a molecule has been directly related to its antioxidant capacity (Wang et al. 2010).

DADS, diallyl sulphide (DAS), and diallyl trisulphide (DATS) are the principal constituents of garlic EO (Calvo-Gómez et al. 2004) and have been demonstrated to be antioxidants as well as hepatoprotective agents in a CT model (Hosono-Fukao et al. 2009; Lee et al. 2014; Zhu et al. 2014). On this rationale, DADS was used as a control OPS because it possesses anticholestatic, antinecrotic and antioxidant effects, which were corroborated in this study. Furthermore, the dose-dependent protective effects of DADS and DATS against CT-induced hepatotoxicity possibly involve mechanisms of inducing antioxidizing or detoxifying enzymes by activating nuclear factor E2-related factor 2 (Nrf2)

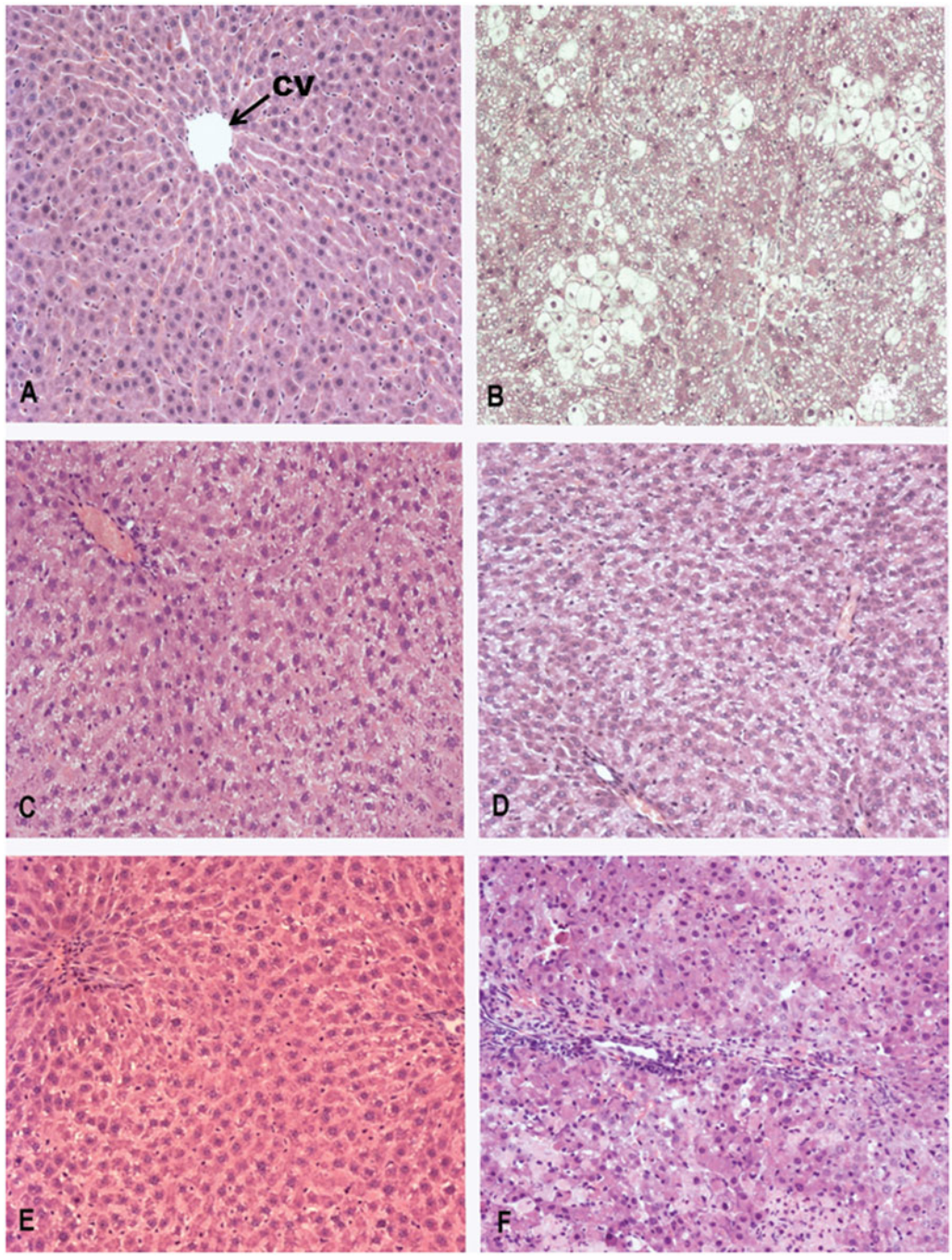

Figure 3. Haematoxylin/eosin staining of representative liver sections from: (A) normal control rats administered vehicle (VE + VCT); (B) damaged control rats administered CT (VE + CT); (C) control rats administered Bocfal EO (Bocfal EO + VCT); (D) damaged rats administered Bocfal EO (Bocfal EO + CT); (E) control rats administered DADS (DADS + VCT) and (F) damaged rats administered DADS (DADS + CT). Central vein (CV), original magnification: 10×.

and blocking the metabolic activation of CCl_4 by suppressing CYP2E1 (Hosono-Fukao et al. 2009). Many authors have achieved significant liver damage amelioration through the use of doses of DADS (with 50 and 100 mg/kg, and even 100 µmol/kg) lower than the dose administered in this study.

Cholestasis is defined as the mechanical or functional stoppage of bile flow in the intrahepatic or extrahepatic bile ducts, with bile components passing into the blood (Kuntz & Kuntz 2006). AP and GGTP are indicators of cholestasis, because they leak from damaged hepatocellular membranes by lipid peroxidation. Bilirubin is a breakdown product of haem catabolism; indeed, its increase in plasma may be a sign of impaired excretory hepatic functions such as cholestasis (Kuntz & Kuntz 2006). Bocfal EO administration consistently showed a better anticholestatic effect than did DADS administration; these results are in agreement with various reports of anticholestatic effects by the administration of *Brassica* extracts in diverse liver damage animal models. Although polar extracts rich in flavonoids were used in those studies (John 2011; Rajamurugan et al. 2012), Bocfal EO may have similar cholagogue effects, perhaps through activating canalicular membrane transporters to improve the excretion of bile products.

CT induced a notable increase of the necrosis marker ALT (Hosono-Fukao et al. 2009; Zhu et al. 2014); however, Bocfal EO and DADS showed antinecrotic properties during the oxidative damage. These results are in accordance with those of various

authors who prevented the ALT increase using *Brassica* extracts and DADS as antioxidant treatments (John 2011; Rajamurugan et al. 2012; Lee et al. 2014). Additionally, the inhibition on the nuclear factor NF-κB, with the activation of Nrf2, seems to provoke immunomodulatory effects by diminishing the proinflammatory cytokine TNF-α (Wagner et al. 2013). In this regard, hepatic glycogen is perhaps the main source of energy in an organism and is indicative of metabolism and functionality. Glycogen synthesis and glycogenolysis are affected by TNF-α; therefore, this marker is very sensitive to liver stress (Fernández-Martínez et al. 2006). Interestingly, Bocfal EO treatment completely prevented the CT-induced depletion of stored glycogen, even better than did by DADS.

Several hepatotoxic chemicals cause liver damage by inducing free radicals that, in turn, provoke oxidative stress, which is defined as a strong imbalance between antioxidant defences and an excessive production of oxidative species. The liver is an important source of free radicals, as these reactive species are metabolic products of exogenous or endogenous antioxidants that involve a variety of enzymatic and non-enzymatic mechanisms, which induce oxidation and electron uncoupling (Zhu et al. 2012). Bocfal EO and DADS prevented the CT-induced LP; this antioxidant effect was associated with the OPS content in the Bocfal EO as well as to the recognized antioxidant activity of DADS (Lee et al. 2014). Nevertheless, those results in the liver did not match those of the *in vitro* TBARS assay in the brain, where DADS was not as effective as the mixture with Bocfal EO. These results suggest an influence of the *in vivo* experiment or the tissue-type assessed.

CAT is an endogenous antioxidant enzyme involved in the elimination of ROS, particularly hydrogen peroxide. CAT activity was significantly augmented by CT and Bocfal EO, while it was lowered by DADS; nevertheless, the treatment with either Bocfal EO or DADS during the CT-induced injury diminished CAT activity elevation. In contrast, most reports have found a CT-induced decrease in the activity of CAT that reflects an impaired antioxidant defence system, whereas the administration of DADS or *Brassica* extracts promotes its recovery and inhibits the LP (Sankhari et al. 2012; Lee et al. 2014). This contradictory result may be explained by a liver that is still functional enough to respond by elevating antioxidant enzymes during oxidative damage, where the whole antioxidant system is not exhausted. In addition, DADS has been reported to significantly inhibit CAT activity (Truong et al. 2009), as was detected in the DADS control group.

A histopathological analysis was performed; despite the hepatoprotective properties demonstrated by the biochemical markers for Bocfal EO and DADS, both substances produced hepatic parenchymal damage. These deleterious effects may be related to the 1 mmol/kg dose of Bocfal EO or DADS, which is higher than the majority of pharmacological doses used by others. The LC_{50} of DADS is 1 mM in hepatocytes. Additionally, garlic fed to rats, cats, dogs and sheep causes haemolytic anaemia along with liver and lung toxicity (Truong et al. 2009). Thounaojam et al. (2011) evaluated the effect of high doses of red cabbage (*B. oleracea* L.) ethanolic extract in mice and found that it caused an elevation of AP and TB, as well as weight loss, in a manner similar to that observed herein. Concerning the adverse effects of *Brassica* in humans, there is only one report of liver toxicity due to excessive broccoli juice drinking (Ekiz et al. 2010). It has been assumed that garlic and many other antioxidants (such as OPSs) might act as potent prooxidants when used in high concentrations and, consequently, cause harmful effects (Iciek et al. 2009).

Bocfal EO or DADS administration resulted in inhibitory effects on the enzyme activities of liver damage markers, particularly ALT, and ALT activity in rats treated with Bocfal EO was even lower during CT-induced injury; the effects on GGTP activity exhibited a similar pattern. These inhibitory properties on the enzymatic liver damage markers may mask adverse hepatic outcomes and mislead the true biochemical diagnosis. OPSs are perhaps capable of either inhibiting the intrinsic enzyme activity or the *de novo* synthesis, considering that diverse OPSs suppress CYP2E1 activity in the rat liver (Melega et al. 2013). Because GGTP is indispensable for the resynthesis of the most important non-enzymatic endogenous antioxidant in the cell, reduced glutathione (GSH), it is considered an antioxidant enzyme; however, it can also be classified as a prooxidant enzyme, due to the product of GSH hydrolysis, Cys-Gly. For this reason, the inhibition of GGTP activity may lead to a cysteine deficit in the cell and a consequent decline in the level of GSH, as well as to the elimination of a physiological source of oxidative stress (Kwiecien et al. 2012).

Conclusions

This is the first report regarding the extraction, chemical constituent analysis, and evaluation of the antioxidant and hepatoprotective activities of the EO obtained from Bocfal. Thus, at the right dose, the edible OPSs in white cabbage are suggested as hydrogen sulphide donors that may serve as useful tools for the research of a variety of diseases. In addition, they might be economical and promising drug candidates for restoring endogenous organic sulphur compounds.

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Disclosure statement

The authors declare no conflicts of interest.

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