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Lipid profile, antiradical power and antimicrobial properties of *Syzygium aromaticum* oil

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RESUMEN

Perfil lipídico, poder antirradicalario y propiedades antimicrobianas del aceite de *Syzygium aromaticum*

Se ha estudiado el aceite de clavo (*Syzygium aromaticum*) obtenido mediante presión en frío (CO) y sus diferentes clases de lípidos, perfiles de ácidos grasos y contenido en tocoles. También se evaluó el potencial captador de radicales y las propiedades antimicrobianas del CO. Los niveles de lípidos neutros en CO fue mayoritario (aprox. 94,7% de lípidos totales), seguido de glicolípidos y fosfolípidos. Los principales ácidos grasos en CO fueron linoleico y oleico, que comprenden juntos el 80% de ácidos grasos totales. Los ácidos esteárico y palmítico fueron los principales ácidos grasos saturados. α- y γ-Tocoferol y δ-Tocotrienol fueron los principales tocoles. CO atrapó el 70% de los radicales DPPH• después de 1 h, mientras que el aceite de oliva virgen extra fue capaz de atrapar sólo el 45%. Las medidas de ESR también mostraron el mismo patrón, en el que CO inactivó 57% de radicales galvinoxil os y el aceite de olive desactivó aproximadamente el 38%. Los resultados de las propiedades antimicrobianas revelaron que el CO inhibió el crecimiento de todos los microorganismos ensayados. CO mostró un efecto drástico en la biosíntesis de proteínas y lípidos en las células de B. subtilis. En relación al potencial utilización, el conocimiento detallado de la composición y propiedades funcionales de CO es de gran importancia.

PALABRAS CLAVE: Actividad antimicrobiana – Actividad captadora de radicales – Clavo – Lípidos – Syzygium aromaticum.

SUMMARY

Lipid profile, antiradical power and antimicrobial properties of *Syzygium aromaticum* oil

In this investigation cold pressed clove (*Syzygium aromaticum*) oil (CO) was studied for its lipid classes, fatty acid profiles and tocol contents. The radical scavenging potential and antimicrobial properties of CO were also evaluated. The levels of neutral lipids in CO was the highest (*ca.* 94.7% of total lipids), followed by glycolipids and phospholipids. The main fatty acids in CO were linoleic and oleic, which comprise together *ca.* 80% of total fatty acids. Stearic and palmitic acids were the main saturated fatty acids. α- and γ-tocopherols and δ-tocotrienol were the main tocols. CO quenched 70% of DPPH• radicals after 1 h, while extra virgin olive oil was able to quench only 45%. ESR measurements also showed the same pattern, wherein CO quenched 57% of galvinoxyl radical and olive oil deactivated about 38%. The results of antimicrobial properties revealed that CO inhibited the growth of all tested microorganisms. CO had a drastic effect on the biosynthesis of protein and lipids in the cells of *B. subtilis.* In consideration of is tpotential utilization, detailed knowledge on the composition and functional properties of CO is of major importance.

KEY-WORDS: Antimicrobial – Clove – Lipids – Radical scavenging activity – Syzygium aromaticum.

1. INTRODUCTION

Spices are food adjuncts that have been utilized as flavoring, seasoning and preservatives. Many spices have been recognized to have medicinal characteristics and possess numerous health-promoting effects, such as antioxidant activity, digestive stimulant action, anti-inflammatory, antimicrobial, hypolipidemic, antimutagenic and anticarcinogenic (Pizzale *et al.*, 2002; Shan *et al.*, 2005).

Clove (*Syzygium aromaticum* L., family Myrtaceae) is the dried flower buds, from the tree which is widely cultivated in many tropical countries (Wenqiang *et al.*, 2007). Cloves are used in the food industry because of their special aroma and their beneficial health properties. Clove essential oil is obtained by distilling the flowers, stems and leaves of the clove tree (Mylonasa *et al.*, 2005; Gulcin *et al.*, 2012). Clove bud essential oil has many biological impacts, such as antimicrobial, insecticidal and antioxidant properties, and the oil is used traditionally as flavoring agents in foods (Huang *et al.*, 2002). Clove essential oil showed antilisteric activity in meat and cheese (Menon and Garg, 2001; Matan *et al.*, 2006). In addition, clove oil is used in many non-food applications such as antimicrobial agents against oral bacteria associated with dental caries and periodontal disease (Cai and Wu, 1996) and as a protective agent against tissue injuries in the lens and cardiac muscles. Moreover, clove treatment significantly reduced blood sugar increases and lipid

peroxidation in streptozotocin-induced diabetic rats by restoring the levels of antioxidant enzymes (Shukri *et al*., 2010).

Cloves contain a wide variety of potentially bioactive compounds such as sesquiterpenes, tannins and triterpenoids. The main aroma constituent of clove buds, eugenol (4-allyl-2-methoxyphenol), was reported to have antifungal activity (Lee and Shibamoto, 2002; Miyazawa and Hisama, 2003). As a food additive, eugenol was classified by the United States Food and Drug Administration (FDA) to be a substance that is generally regarded as safe (Gulcin *et al*., 2012). The high levels of eugenol found in clove essential oil give it strong biological and antimicrobial activities. Eugenol was reported to have antifungal activity and inhibited malonaldehyde formation from cod liver oil and the formation of hexanal (Gulcin *et al*., 2004). Clove oil has been listed as a "Generally Regarded as Safe" substance by the FDA when administered at levels not exceeding 1500 ppm in food categories. In addition, the World Health Organization (WHO) Expert Committee on Food Additives has established the acceptable daily human intake of clove oil at 2.5 mg/kg body weight for humans (Kildeaa *et al*., 2004).

Food professionals are continually searching for unique spice flavorings because of the rising global demand for authentic ethnic and cross-cultural cuisines. Consumers are also looking for natural foods and natural preservatives for healthier lifestyles. Thus, spices are being sought for their medicinal value as antioxidants and as antimicrobials (El-Ghorab *et al*., 2010). A need for identifying alternative natural and safe sources of antioxidants has been created and notably increased in recent years. Recently, *S. aromaticum* essential oil exhibited an antioxidant effect on retarding the lipid oxidation of hazelnut and poppy oils (Ramadan 2008, 2013; Ozcan and Arslan, 2011).

Food-borne pathogens are major concerns of food safety. They are responsible for 76 million cases of food-borne illnesses, 325.000 hospitalizations and 5000 deaths in the US annually (Luther *et al*., 2007). The estimated annual cost related to the top five bacterial pathogens: *Campylobacter, Salmonella* (nontyphoidal serotypes only), *Listeria monocytogenes*, *Escherichia coli* 0157 and non-0157 STEC is 6.9 billion USD. The antimicrobial activity of natural extracts is closely linked with their polyphenolic content (Ahn *et al*., 2003). Therefore, plant extracts rich in phenolics and other bioactive compounds may serve as potential natural antimicrobial agents (Luther *et al*., 2007). Recently, clove essential oil exhibited antimicrobial activity against the growth of Gram-negative and Gram-positive bacteria (Goñi *et al*., 2009).

Over the last few years, increased interest in cold pressed oils has been observed as these oils have high nutritive properties. The cold pressing technique is becoming a promising substitute for conventional practices because of consumers' desire for natural food products (Lutterodt *et al*., 2010). Cold pressing is a method which involves no heat or chemical treatments. Cold pressing also involves no refining process and may contain a high level of lipophilic phytochemicals including natural antioxidants.

Many exotic oils have been introduced to the market recently, and therefore data on their composition, antioxidant potential and antimicrobial characteristics have not been reported. Such data are of importance for the evaluation of the nutritional and health impact of these oils. It is hard to find any data in the literature on cold-pressed clove oil (CO). As a continuation of the efforts in developing edible oils with health-beneficial characteristics, this study was carried out to: 1) determine the lipid classes, fatty acid and tocol profile of cold-pressed CO, 2) determine the total phenolic contents and *in vitro* radical scavenging activity of CO; and 3) estimate the antimicrobial properties of CO. The results from the present study will be used to develop novel edible oil products rich in bioactive molecules with a desirable shelf life.

2. MATERIALS AND METHODS

2.1. Materials and oils

Three different samples of cold-pressed CO and extra virgin olive oil were purchased from a local market in Zagazig (Egypt). Neutral lipid (NL) standards were from Sigma (St. Louis, MO, USA). Standards used for glycolipids (GL) identification; monogalactosyldiacylglycerol (MGD), digalactosyldiacylglycerol (DGD), cerebrosides (CER), steryl glucoside (SG) and esterified steryl glucoside (ESG) were of plant origin (plant species unknown) and purchased from Biotrend Chemikalien GmbH (Köln, Germany). Standards used for phospholipids (PL); phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI) from Bovine liver and phosphatidylcholine (PC) from Soybean were purchased from Sigma. Standards used for tocols were purchased from Merck (Darmstadt, Germany).

2.2. Methods

2.2.1. Column chromatography (CC) and thin-layer chromatography (TLC) of lipid classes

Fractionation of lipid classes and subclasses

Cold pressed CO was separated into different classes by elution with different solvents over a glass column (20 mm × 30 cm) packed with a slurry of activated silicic acid (70 to 230 mesh; Merck, Darmstadt, Germany) in chloroform (1:5, w/v). NL were eluted with 3-times the column volume of chloroform (Ramadan and Elsanhoty, 2012). The major portion of GL was eluted with 5-times the column volume of acetone and that of PL with 4-times the column volume of methanol. The amount of the lipid classes obtained was determined by gravimetry. By means of TLC on

Silica gel F_{254} plates (thickness = 0.25 mm; Merck, Darmstadt, Germany) a further characterization of the GL and PL subclasses was carried out with the following solvent system chloroform/methanol/25% ammonia solution (65:25:4, v/v/v). For the characterization of NL subclasses, TLC plates were developed in the solvent system *n*-hexane/diethyl ether/acetic acid (60:40:1, v/v/v). For the detection of the lipids, the TLC plates were sprayed with the following agents: for the marking of all lipids with sulphuric acid (40%), for the marking of GL with α-naphthol/sulphuric acid and for the marking of PL with the molybdate-blue reagent (Ramadan *et al*., 2006). Each spot was identified with lipid standards as well as their reported retention factor (R_f) values. Individual bands were visualized under ultraviolet light, scraped from the plate and recovered by extraction with chloroform/methanol (2:1, v/v). The fatty acid compositions of CO as well as NL, GL and PL were determined by GLC/FID as described below.

Quantitative determination of lipid subclasses

For the quantitative determination of NL subclasses individual bands were scraped from the plate and recovered by extraction with 10% methanol in diethyl ether, followed by diethyl ether. The data presented are the average of three gravimetrical determinations. For the quantitative estimation of GL subclasses, the acetone fraction obtained by CC was separated by TLC in the above given solvent system. The silica gel regions with the corresponding GL subclasses were scraped out followed by hexose measurement photometrically at 485 nm using the phenol/sulphuric acid in acid-hydrolysed lipids (Ramadan *et al*., 2006). The percent distribution of each component was obtained from the hexose values. From the extinction values the quantitative amount was determined and related to their portion of the GL fraction. The determined portion was set into relation with the amount of oil, which had been separated by CC into the main lipid fractions. For the determination of the PL, the methanol fraction from CC was also separated by TLC in the above given solvent system and after scraping out of the individual PL subclasses brought to reaction with the hydrazine sulphate/sodium molybdate reagent at 100 °C for 10 min and photometrically analyzed at 650 nm. From the obtained extinction values *via* a calibration chart for phosphorus the amount of PL was calculated. The individual values were put into relation to the PL fraction (methanol fraction from CC) and to the amount of oil.

2.2.2. Gas chromatography (GC) analysis of fatty acid methyl esters (FAME)

The fatty acids of CO and lipid classes were transesterified into FAME using N-trimethylsulfoniumhydroxide (Macherey-Nagel, Düren, Germany) according to the procedure reported by Arens *et al*. (1994). FAME were identified on a Shimadzu GC-14A equipped with a flame ionization detector (FID) and a C-R4AX chromatopac integrator (Kyoto, Japan). The flow rate of the carrier gas helium was 0.6 mL/min and the split value with a ratio of 1:40. A sample of 1 µL was injected onto a 30 m × 0.25 mm × 0.2 µm film thickness Supelco SPTM-2380 (Bellefonte, PA, USA) capillary column. The injector and FID temperature was set at 250 °C. The initial column temperature was 100 °C programmed by 5 °C min^{-1} until 175 °C and kept 10 min at 175 °C, then 8 °C min^{-1} until 220 °C and kept 10 min at 220 °C. A comparison between the retention times of the samples with those of an authentic standard mixture (Sigma, St. Louis, MO, USA; 99% purity specific for GLC), run on the same column under the same conditions, was made to facilitate identification.

2.2.3. HPLC analysis of tocols

For the tocol analysis, a solution of 250 mg of oil in 25 mL *n*-heptane was directly used for the HPLC (Ramadan, 2013). The HPLC analysis was conducted using a Merck Hitachi low-pressure gradient system, fitted with an L-6000 pump, a Merck-Hitachi F-1000 Fluorescence Spectrophotometer (The detector wavelength was set at 295 nm for excitation, and at 330 nm for emission) and a D-2500 integration system; 20 µL of the samples were injected by a Merck 655-A40 Autosampler onto a Diol phase HPLC column 25 cm 9 4.6 mm ID (Merck, Darmstadt, Germany) using a flow rate of 1.3 mL min^{-1}. The mobile phase used was n-heptane/tert-butyl methyl ether (99:1, v/v).

2.2.4. Extraction and quantification of phenolic compounds

Aliquots of cold-pressed CO and extra virgin olive oil (1 g) were dissolved in *n*-hexane (5 mL) and mixed with 10 mL methanol-water (80:20, v/v) in a glass tube for two min in a vortex (Ramadan *et al*., 2010). After centrifugation at 3000 rpm for 10 min, the hydroalcoholic extracts were separated from the lipid phase by using a Pasteur pipette then combined and concentrated *in vacuo* at 30 °C until a syrupy consistency was reached. The lipidic residue was re-dissolved in 10 mL methanol-water (80:20, v/v) and the extraction was repeated twice. Hydroalcoholic extracts were re-dissolved in acetonitrile (15 mL) and the mixture was washed three times with *n*-hexane (15 mL each). Purified phenols in acetonitrile were concentrated *in vacuo* at 30 °C then dissolved in methanol for further analysis. Aliquots of phenolic extracts were evaporated to dryness under nitrogen. The residue was re-dissolved in 0.2 mL water and diluted (1:30) then Folin-Ciocalteu's phenol reagent (1 mL) was added. After 3 min, 7.5% sodium carbonate

(0.8 mL) was added. After 30 min, the absorbance was measured at 765 nm using a UV-260 visible recording spectrophotometer (Shimadzu, Kyoto, Japan). Gallic acid was used for the calibration and the results of triplicate analyses are expressed as parts per million of gallic acid.

2.2.5. Radical scavenging activity (RSA) of CO and olive oil toward DPPH•

RSA of CO and olive oil was assayed with DPPH• radical dissolved in toluene (Ramadan, 2012). A toluenic solution of DPPH• radicals was freshly prepared at a concentration of 10^{-4} M. The radical, in the absence of antioxidant compounds, was stable for more than 2 h of normal kinetic assay. For evaluation, 10 mg of CO or extra virgin olive oil (in 100 µL toluene) was mixed with a 390 µL toluenic solution of DPPH• radicals and the mixture was vortexed for 20 s at ambient temperature. Against a blank of pure toluene without DPPH•, the decrease in absorption at 515 nm was measured in 1-cm quartz cells after 1, 30 and 60 min of mixing using a UV-260 visible recording spectrophotometer (Shimadzu, Kyoto, Japan). RSA towards DPPH• radicals was estimated from the differences in absorbance of the toluenic DPPH• solution with or without the sample (control) and the inhibition percent was calculated from the following equation:

$$\%\ inhibition = [(absorbance\ of\ control - absorbance\ of\ test\ sample)/absorbance\ of\ control] \times 100.$$

2.2.6. RSA of CO and olive oil towards galvinoxyl radical

A miniscope MS 100 ESR spectrometer (Magnettech GmbH; Berlin, Germany) was used in this analysis (Ramadan *et al*., 2010). Experimental conditions were as follows: measurement at room temperature; microwave power, 6 db; centerfield, 3397 G, sweep width 83 G, receiver gain 10 and modulation amplitude 2000 mG. Ten mg of oil (in 100 µL toluene) were allowed to react with 100 µL of a toluenic solution of galvioxyl (0.125 mM). The mixture was stirred in a vortex stirrer for 20 s then transferred into a 50 µL micro pipette (Hirschmann Laborgeräte GmbH, Ederstadt, Germany) and the amount of galvinoxyl radical inhibited was measured exactly after a total incubation time of 60 min after the addition of the galvinoxyl radical solution. The galvinoxyl signal intensities were evaluated by the peak height of signals against a control. A quantitative estimation of the radical concentration was obtained by evaluating the decrease in the ESR signals in arbitrary units after 60 min incubation using the KinetikShow 1.06 Software program (Magnettech GmbH; Berlin, Germany). The reproducibility of the measurements was 5% as usual for kinetic parameters.

2.2.7. Antimicrobial activity

The antimicrobial activities were carried out according to the conventional agar diffusion test using cultures of *Bacillus subtilis* NRRL B-94, *Escherichia coli* NRRL B-3703, *Pseudomonas aeruginosa* NRRL, *Staphylococcus aureus* NRRL, *Aspergillus niger* NRRL313, *Aspergillus flavus* NRC, *Saccharomyces cerevisiae* NRC and *Candida albicans* NRRL477 (Ramadan *et al*., 2012). The bacterial strains were cultured in a nutrient medium, while the fungi and yeast strains were cultured in a malt medium and yeast medium, respectively. Broth media included the same contents of nutrient medium except for the agar. For bacteria and yeast, the broth media were incubated for 24 h. As for moulds, the broth media were incubated for approximately 48 h, with subsequent filtering of the culture through a thin layer of sterile sintered Glass G2 to remove mycelia fragments before the solution containing the spores was used for inoculation. For plate preparation, 1.0 mL Tween 20 and 0.5 mL of inocula were added to 50 mL of agar media (50 °C) and mixed by simple inversion. The agar was poured into 120 mm Petri dishes and allowed to cool to room temperature. Wells (4 mm in diameter) were cut in the agar plates using sterile paper tubes. The wells were filled to the surface of the agar with the oils to be tested (50 µL $well^{-1}$). The microbial growth inhibition zones, clear microbial free inhibition zones, were measured after incubation at 30 ºC, beginning within 24 h for yeast, 24-48 h for bacteria and 48-72 h for fungi. Antimicrobial activities were calculated as a mean of three repetitions.

2.2.8. Minimal inhibitory concentration (MIC)

A concentration of 0.5% (v/v) Tween 20 was incorporated into the agar after autoclaving to enhance oil solubility. The culture medium (25 mL) was poured into Petri dishes (9 cm in diameter) and maintained at 45 °C until the samples were incorporated into the agar. The samples were added as 15, 25, 50, 75, 100, 125, 150, 175, 200 and 250 µL 25 mL^{-1} agar media using an automatic micropipette while constantly stirring to assure a uniform distribution. The different microbial strains were layered by using an automatic micropipette to place 30 µL over the surface of the solidified culture medium containing a sample. After the microorganisms were absorbed into the agar, the plates were incubated at 30 °C for 24-48 h. MIC was determined as the lowest concentration of oil inhibiting the visible growth of each organism on the agar plate (Ramadan and Asker, 2009).

2.2.9. Mode of action

The effects of different concentrations of the cold-pressed CO on some biochemical activities were studied. Immediately after incubating the flasks with *B. subtilis*, the cells were harvested

during the middle logarithmic growth phase and CO was applied in concentrations of 1/8, 1/4 and 1/2 MIC. Each test was repeated three times. Subsequently, the flasks were shaken using a rotary shaker of 150 rpm at 30 °C. Samples were withdrawn at the onset of the experiment and after incubation periods of 20, 40, 60, 80, 100 and 120 min. The bacterial cells were subjected to the following determinations: acid soluble phosphorus compounds, total lipids, soluble protein and nucleic acids (Ramadan and Asker, 2009).

2.2.9.1. Extraction of intercellular components of bacterial cells

2.2.9.1.1. Acid soluble phosphorous compounds

The cells were collected by centrifugation, washed twice with ice cold saline and extracted twice with 5% ice cold trichloroacetic acid (TCA). The suspensions were finally centrifuged at 5000 rpm. The extract (1 mL) was added to a 4 mL reagent (40 mL of 6N H_2SO_4, 80 mL distilled water, 40 mL ammonium molybdate solution and 40 mL ascorbic acid), mixed and incubated at 37 °C for 2h, then cooled at room temperature. The absorbance was measured at 680 nm.

2.2.9.1.2. Total lipids

The residue after removing of the acid soluble compounds was extracted three times with a mixture of chloroform-methanol (2:1, v/v). A volume of 0.1 mL of extract was added to 5 mL of concentrated H_2SO_4. The mixture was heated for 10 min in a boiling water bath, cooled and a 0.4 mL aliquot was placed in a dry test tube. Six mL of phosphor-vanillin reagent (0.6 g vanillin dissolved in 10 mL ethanol before diluting to 100 mL with distilled water were mixed with 400 mL of concentrated orthophosphoric acid) were then added to each test tube. The mixture was set in the dark for 45 min and the absorbance was measured at 525 nm.

2.2.9.1.3. Soluble protein

The delipidated cells were solubilized in 1 N KOH at 37 °C for 20 h. The protein in the extract was determined at 595 nm.

2.2.9.1.4. RNA extraction

The remaining portions of the sample after hydrolysis by 1N KOH were subjected to extraction of RNA fractions. 6N HCl was added to each sample and the solution was completed with the same volume of 10% TCA. After concentration the residue was washed with 5% TCA. One mL of RNA fraction was added to 3 mL of reagent (135 mg of ferric ammonium sulphate and 0.2 g orcinol were dissolved in 15 mL distilled water, then 85 mL concentrated HCl were added), mixed and heated for 20 min a boiling water bath. The tubes were cooled at room temperature and measured at 670 nm.

2.2.9.1.5. DNA extraction

The residue after the extraction of RNA was hydrolyzed by 5% TCA and the supernatants were heated at 90 °C for 30 min, cooled and centrifuged at 6000 rpm. The residue was washed once with the least amount of 5% TCA. One mL of DNA extract was added to 2.5 mL of the diphenylamine reagent (1 g of diphenylamine was dissolved in 98 mL glacial acetic acid, 2 mL of H_2SO_4 were then added), the mixture was heated for 5 min in a boiling water bath. The sample was cooled and absorbance was measured at 540 nm.

All work was carried out under subdued light conditions. All experimental procedures were performed in triplicate if the variation was routinely less than 5% and the mean values (± standard deviation) were determined.

3. RESULTS AND DISCUSSION

3.1. Levels of lipid classes and subclasses

The proportion of lipid classes and subclasses presented in CO as well as R_f values of these subclasses are shown in Table 1 and Figure 1. The level of NL was the highest (*ca*. 94.7%), followed by GL (0.76%) and PL (0.39%). These data are in agreement with Suzuki *et al*. (2000) who analyzed the lipid classes of clove lipidic extracts. The subclasses of NL contained triacylglycerol (TG), free fatty acids (FFA), diacylglycerol (DG), esterified sterols (STE) and monoacylglycerol (MG) in decreasing order. A significant amount of TG was found (*ca*. 96.6% of total NL) followed by a relatively low level of FFA (*ca*. 1.4% of total NL), while DG and STE were recovered in lower levels. The subclasses of GL found in CO were sulphoquinovosyldiacylglycerol (SQD), digalactosyldiglycerides (DGD), cerebrosides (CER), sterylglycosides (SG), monogalactosyldiglycerides (MGD) and esterified sterylglycosides (ESG) as presented in Table 1. The proportion of each component was estimated by the lipid-carbohydrate determination. CER, SG and ESG were the prevalent components and made up about 92% of the total GL. The PL subclasses in CO were separated into four major fractions *via* TLC. The TLC fractions of PL revealed that the predominant PL subclasses were PC followed by PE, PI and PS, respectively (Table 1). About half of the total PL was in PC and a quarter was in PE, while PI and PS were isolated in smaller quantities.

3.2. Fatty acid profile of cold-pressed CO and lipid classes

The fatty acid profiles of CO and lipid classes (NL, GL and PL) are presented in Table 2. According

Table 1
Levels of lipid classes (g kg^{-1}TL) in cold pressed CO

Neutral lipid Subclass	R_f values × 100[a]	g kg^{-1} TL	Glycolipid Subclass	R_f values × 100[b]	g kg^{-1} TL	Phospholipid Subclass	R_f values × 100[b]	g kg^{-1} TL
MG	14	4.97 ± 0.07	SQD	6	0.09 ± 0.03	PS	4.7	0.77 ± 0.02
DG	39	7.97 ± 0.13	DGD	17	0.37 ± 0.05	PI	11	0.57± 0.03
FFA	56	13.5 ± 0.15	CER	29-35	3.22 ± 0.08	PC	20	1.88 ± 0.07
TG	79	915 ± 2.09	SG	41	2.38 ± 0.08	PE	30	0.95 ± 0.06
STE	95	5.56 ± 0.09	MGD	64	0.11 ± 0.04			
			ESG	76	1.39 ± 0.07			

[a] Solvent system used in TLC development: *n*-hexane/diethyl ether/acetic acid (60:40:1, v/v/v).
[b] Solvent system used in TLC development: chloroform/methanol/ammonia solution 25% (65:25:4, v/v/v).
Results are given as the average of triplicate determinations ± standard deviation.
Abbreviations: MAG, monoacylglycerols; DAG, diacylglycerols; TAG, triacylglycerols; FFA, free fatty acids; STE, sterol esters. SQD, sulphoquinovosyldiacylglycerol; DGD, digalactosyldiacylglycerol; CER, cerebrosides; SG, steryl glucoside; MGD, monogalactosyldiacylglycerol; ESG, esterified steryl glucoside; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

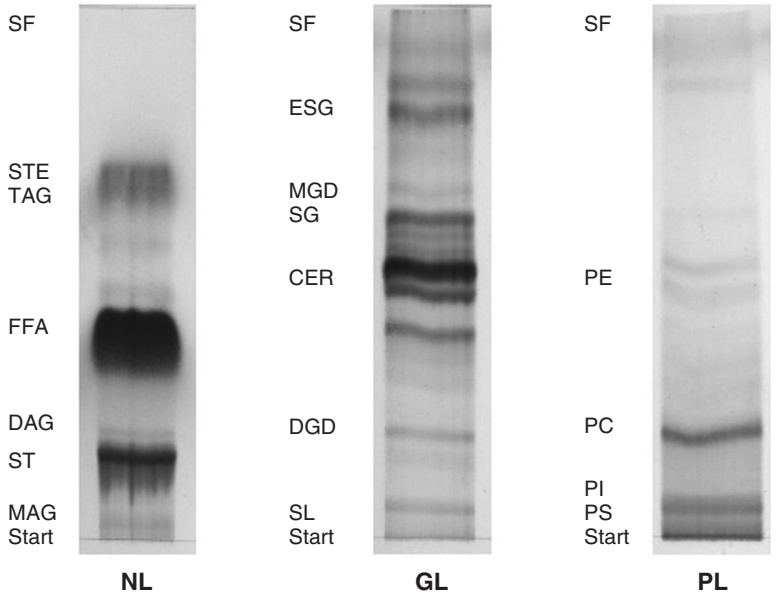

Figure 1
Thin layer chromatography profile of CO lipid classes
Abbreviations: MAG, monoacylglycerols; **ST,** sterols; **DAG**, diacylglycerols; **TAG**, triacylglycerols; **FFA**, free fatty acids; **STE**, sterol esters. **SQD**, sulphoquinovosyldiacylglycerol; **DGD**, digalactosyldiacylglycerol; **CER**, cerebrosides; **SG**, steryl glucoside; **MGD,** monogalactosyldiacylglycerol; **ESG**, esterified steryl glucoside; **PS,** phosphatidylserine; **PI,** phosphatidylinositol; **PC,** phosphatidylcholine; **PE,** phosphatidylethanolamine; **SF,** solvent front.

to the results, nine fatty acids were identified in CO. Linoleic and oleic acids were the main fatty acids in CO. Both fatty acids comprise together about 80% of the total FAME. Cold-pressed CO contained significant levels of monounsaturated fatty acids (MUFA, 39.7 g 100 g^{-1} total fatty acids) which is comparable to the cold pressed hemp, cranberry, blueberry, onion and milk thistle seed oils but was much lower than that of 81 and 82% in the carrot and parsley cold pressed seed oils (Parker *et al*., 2003; Parry *et al*., 2005). Cold-pressed CO had a polyunsaturated fatty acid (PUFA) content of 42.1 g 100 g^{-1} of total fatty acids (Table 2). This PUFA content was lower than that in the cranberry (67.6 g 100 g^{-1}), onion (64-65 g 100 g^{-1}), milk thistle (61 g 100 g^{-1}) and blueberry (69 g 100 g^{-1}) cold pressed seed oils (Parry *et al*., 2006).

Palmitic and stearic were the major saturated fatty acids (SFA), comprising together about 15% of total identified FAME. Cold-pressed CO contained about 18 g of SFA per 100 g of total fatty acids, which is much lower than that of 30.8 g 100 g^{-1}of total fatty acids in the cold pressed cardamom seed oil and comparable to that of 13.8 and 15.9 g 100 g^{-1}

Table 2
Fatty acid composition (relative content, %) of cold pressed CO and its lipid classes

	CO	Neutral lipids	Glycolipids	Phospholipids
C 10:0	2.76 ± 0.09	2.85 ± 0.09	2.89 ± 0.09	2.95 ± 0.09
C 12:0	0.07 ± 0.01	0.15 ± 0.01	0.27 ± 0.01	0.32 ± 0.01
C 14:0	0.06 ± 0.01	0.12 ± 0.01	0.16 ± 0.01	0.39 ± 0.01
C 16:0	8.57 ± 0.24	8.59 ± 0.24	8.59 ± 0.24	8.87 ± 0.24
C 16:1	0.36 ± 0.05	0.33 ± 0.05	0.32 ± 0.05	0.31 ± 0.05
C 18:0	6.59 ± 0.19	6.67 ± 0.19	6.65 ± 0.19	6.75 ± 0.19
C 18:1n-9	39.4 ± 1.53	39.3 ± 1.53	39.2 ± 1.53	38.8 ± 1.53
C 18:2n-6	40.2 ± 2.10	40.1 ± 2.10	40.1 ± 2.10	39.8 ± 2.10
C 18:3	1.99 ± 0.05	1.89 ± 0.05	1.82 ± 0.05	1.81 ± 0.05
ΣSFA	18.05	18.38	18.56	19.28
ΣMUFA	39.76	39.63	39.52	39.11
ΣPUFA	42.19	41.99	41.92	41.61

Results are given as the average of triplicate determinations ± standard deviation.
[a] N.D. = not detected.

of total fatty acids found in the cold pressed milk thistle and roasted pumpkin seed oils, respectively (Parry *et al*., 2006). The SFA levels were higher than those of 7.4-9.7 g 100 g^{-1} of total fatty acids in the cold pressed parsley, onion, hemp, mullein and cranberry seed oils (Parker *et al*., 2003).

Fatty acids in NL and polar lipids did not differ significantly from each other, wherein linoleic and oleic acids were the main fatty acids. The ratio of unsaturated fatty acids to SFA, however, was not significantly higher in neutral fractions than in the corresponding polar fractions (GL and PL).

A striking feature of the CO was the relatively high level of MUFA and PUFA. γ-linolenic acid (GLA, C18:3*n*-6) was also estimated in relatively lower amounts. From a health point of view, MUFA have been shown to lower "bad" LDL (low density lipoproteins) cholesterol and retain "good" HDL (high density lipoproteins) cholesterol. This is in fact the major benefit of olive oil over the highly polyunsaturated oils, wherein PUFA reduce both the "bad" as well as the "good" serum cholesterol levels in our blood (Ramadan *et al*., 2010). A rapidly growing literature illustrates the benefits of PUFA, in alleviating cardiovascular, inflammatory, heart diseases, atherosclerosis, autoimmune disorder, diabetes and other diseases. The fatty acid profile of CO evinces the lipids as a good source of the nutritionally essential fatty acids. The fatty acid composition and high amounts of MUFA and PUFA makes the CO a special component for nutritional applications.

3.3. Tocols composition

Cold pressed CO was characterized by high levels of unsaponifiables (25.3 g kg^{-1}). The levels of α-, β-, γ- and δ- tocopherols in CO were 14890, 56, 4184 and 186 µg kg^{-1} oil, respectively. In addition, the amounts of α-, β-, γ- and δ- tocotrienols were 1110, 55, 85 and 9498 µg kg^{-1} oil, respectively. All tocol isomers were present in cold pressed CO, wherein α-tocopherol constituted *ca*. 49.5% of the total tocols followed by δ- tocotrienol (31.6% of the total tocols) and γ- tocopherol (13.9% of the total tocols). Other tocols were measured at lower levels. α- and γ-tocopherols proved to be the major tocopherols in vegetable oils and fats. γ-tocopherol occurred in highest concentrations in camelina, linseed, cold pressed rapeseed and corn oil (Schwarz *et al*., 2008). α-tocopherol is the most efficient antioxidant of the tocopherol isomers, while β-tocopherol has 25-50% of the antioxidative activity of α-tocopherol, and the γ-isomer 10-35%. The levels of tocopherols detected in cold pressed CO may contribute to the stability of the oil toward oxidation.

3.4. RSA of CO in comparison with extra virgin olive oil

The model of scavenging stable free radicals is widely used to evaluate the antioxidant properties in a relatively short time, as compared to other methods. A simple experiment using the toluene to dissolve fat or oil samples and the free radicals was developed (Ramadan and Moersel, 2007).

The oxidative stability of oils and fats depends on the fatty acid composition, the presence of minor fat-soluble bioactives and the initial amount of hydroperoxides. The RSA of antioxidants may be influenced by the radical system and other testing conditions. Two or more radical systems are needed to better study a selected antioxidant for its RSA. The antiradical properties of the cold pressed CO and extra virgin olive oil (as a standard crude oil with respective high levels of nutritive antioxidants and bioactives) were compared using

stable DPPH• and galvinoxyl radicals. Figure 2 shows that CO had higher RSA than olive oil. After 60 min of incubation with DPPH• radicals, 70% of DPPH• radicals were quenched by CO, while olive oil was able to quench only 45% (Figure 2A). ESR measurements showed the same pattern, wherein CO quenched 57% of galvinoxyl radical and olive oil deactivated about 38% after 1 h of reaction (Figure 2B). Ramadan and Moersel (2006) compared the antiradical performance of different edible oils against DPPH• radical. The order of effectiveness of oils in inhibiting free radicals was as follows: coriander > black cumin > cottonseed > peanut > sunflower > walnut > hemp > linseed >olive > niger.

Regarding the composition of CO and olive oil, they have different patterns of fatty acid and lipid-soluble bioactives. According to our experiments, CO was characterized by higher levels of phenolics (4.6 mg/g, respectively) than extra virgin olive oil (3.4 mg/g). Thus, the CO examined may be used in different food applications to provide nutrition and health benefits. Phenolic compounds have been reported to be present in vegetable oils, which is very important for the oxidative stability of the PUFA of these oils. Additionally, edible oils rich in natural antioxidants may play a role in reducing the risk of chronic diseases. Phenolic compounds in plant materials are closely associated with their antioxidant activity. The antioxidant effect of phenolic compounds is mainly due to their redox properties and is the result of various mechanisms: free-radical scavenging activity, transition-metal-chelating activity, and/or singlet-oxygen-quenching capacity (Bettaieb *et al*., 2010). The total phenolic values of CO was higher than that of 1.73-2.0 mg GAE g^{-1} oil for the cold-pressed red raspberry, blueberry and boysenberry seed oils, and that of 1.8-3.4 mg GAE g^{-1} oil for the cold-pressed parsley, onion, cardamom, mullein and milk thistle seed oils (Parry *et al*., 2006, 2005). On the other hand, tocopherol levels in oils may have a great impact on their RSA. Increasing the ring methyl substitution led to an increase in scavenging activity against the DPPH radical, and also to a decrease in oxygen radical absorbance capacity (Müller *et al*., 2010).

The stronger RSA of CO compared to olive oil may be due to (i) the differences in content and

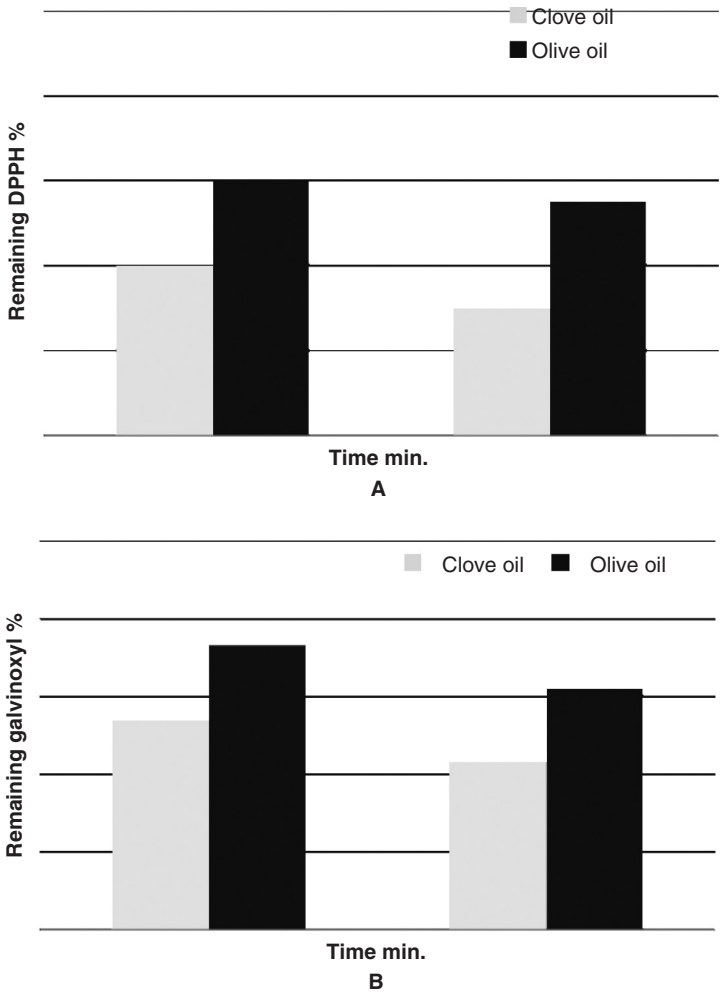

Figure 2
Scavenging effect at different incubation times of cold-pressed CO and olive oil on (A) DPPH radical as measured by changes in absorbance values at 515 nm and on (B) galvinoxyl radical as recorded by ESR.

composition of unsaponifiable materials (ii) the diversity in structural characteristics of potential phenolic antioxidants present, (iii) a synergism of phenolic antioxidants with other active components, and (iv) different kinetic behaviors of potential antioxidants. From the results we can suggest that cold-pressed CO may serve as a dietary source of phenolic substances, which may act as antioxidants for disease prevention and/or general health promotion through improved nutrition.

3.5. Antimicrobial activity of CO

The exploration of naturally occurring antimicrobials for food preservation receives increasing attention due to consumer awareness of natural food products and a growing concern for microbial resistance towards conventional preservatives (Omidbeygi *et al*., 2007). The antimicrobial properties of spices have been recognized since ancient times for food preservation. Biologically active molecules from food are of interest to prevent the deleterious effects of free radicals and also to prevent the deterioration of foods due to lipid oxidation and microbial spoilage. Spices are an integral part of the human diet to impart flavor, taste and color to the food. Food borne illness caused by the consumption of contaminated foods with pathogenic bacteria and/or their toxins has been of great concern to public health. Controlling pathogenic microorganisms would reduce food borne outbreaks and assure consumers a safe, wholesome, and nutritious food supply. Spices are generally applied to food, which is a nutrient rich environment for most bacteria. Extracts from botanicals have shown antimicrobial activity against various pathogenic microorganisms (Luther *et al*., 2007; Allahghdri *et al*., 2010).

Different microbial species, including Gram negative bacteria, Gram positive bacteria, moulds (yeasts and fungi) were used to screen the possible antimicrobial activity of cold pressed CO. The examination of antimicrobial activity of the cold pressed CO by the agar diffusion method revealed that CO inhibited the growth of all microorganisms tested (Table 3). Cold pressed CO was very active against bacteria and yeasts and produced great zones of inhibition. The maximum inhibition zone obtained with CO was that against *A. flavus, A. niger* and *C. albicans* (31 mm, 29 and 27 mm, respectively). On the other hand, CO led to the high inhibition of bacteria, wherein the inhibition zones were between 17 mm and 26 mm. Recently, the antimicrobial activity of the vapor generated by a combination of cinnamon and clove essential oils against the growth of four Gram-negative (*Escherichia coli, Yersinia enterocolitica, Pseudomonas aeruginosa and Salmonella choleraesuis*) and four Gram-positive bacteria (*Staphylococcus aureus, Listeria monocytogenes, Bacillus cereus and Enterococcus faecalis*) was assessed by means of the fractional inhibitory concentration index (Goñi *et al*., 2009).

The MIC of CO toward Gram negative bacteria, Gram positive bacteria, yeasts and fungi was examined and the results are also summarized in Table 3. CO exhibited strong antimicrobial action against Gram negative bacteria, Gram positive bacteria and moulds. It has broad-spectra activity against Gram negative bacteria, Gram positive bacteria and moulds with MIC ranging from 0.6 to 1.0 mL L^{-1}. The variation in the effectiveness of CO against different strains may depend on the differences in the permeability of the cells of those microbes (Dorman and Deans, 2004).

The impact of different concentrations of CO on the biosynthesis of acid soluble phosphorus compounds, total lipids, protein and nucleic acids (DNA and RNA) in the cells of *B. subtilis* was studied and the data are presented in Figure 3. It was found that CO had a drastic effect on the biosynthesis of protein and total lipids in the cells of *B. subtilis* (Figures 3A and 3B). This effect increased with increasing the concentration (1/8-1/2 MIC) and incubation period. On the other hand, CO had a slight impact on the biosynthesis of acid soluble phosphorus (Figure 3C). The cold pressed CO had a drastic effect on nucleic acids (DNA and RNA) in the cells of *B. subtilis* (Figure 3D and 3E). This effect increased by increasing the concentration (1/8-1/2 MIC) and incubation period. These results indicate that CO greatly affects the biosynthesis of proteins by inhibiting some steps in the complex process of translation. The most important antibiotics with the same action are the tetracyclines. On the other hand, some chemotherapeutic agents affect the synthesis of DNA or RNA, or can bind to DNA or RNA so that their messages cannot be read. The majority of these drugs is unselective and affects animal and bacterial cells alike, thus these drugs have no therapeutic applications (Shuichi *et al*.,

Table 3
Antimicrobial activity and Minimum inhibitory concentration (MIC) of cold-pressed CO[a,b]

	Diameter of inhibition (mm)							
	Bacteria				moulds			
	E. coli	*P. aeruginosa*	*St. aureus*	*B. subtilis*	*A. niger*	*A. flavus*	*C. albicans*	*S. cerevisiae*
Diameter of inhibition (mm)	17.0±0.12	25.0±0.09	22.0±0.09	26.0±0.20	29.0±0.04	31.0±0.25	29.0±0.09	27.0±0.04
MIC (mL L^{-1} media)	0.6	0.6	1.0	1.0	< 0.6	< 0.6	< 0.6	< 0.6

[a] Each value represents mean of sample ± SD for $n = 3$.
[b] Diameter of inhibition zone was measured as the clear area centered on the agar well containing the sample.

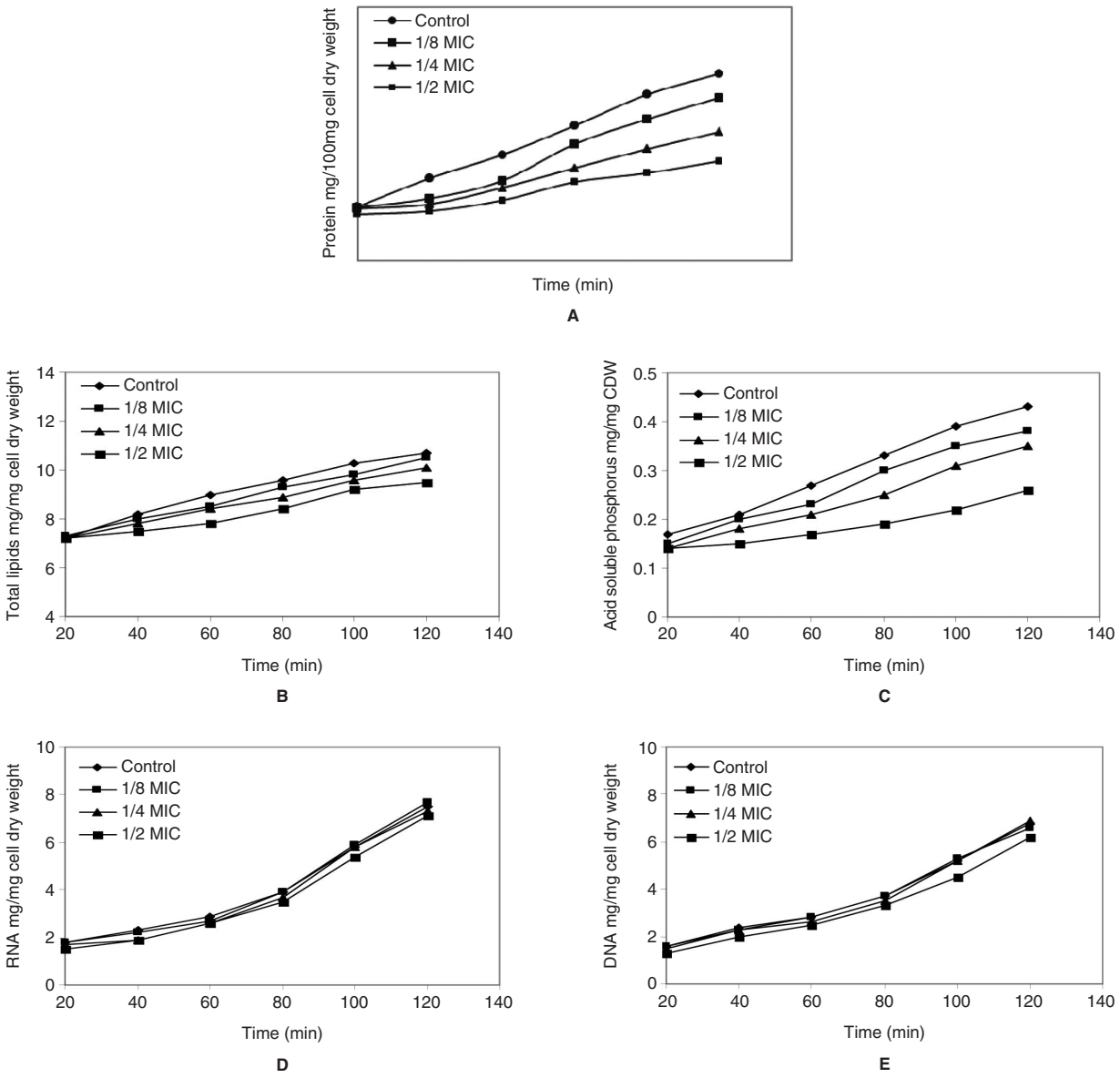

Figure 3
Effects of different concentrations of cold-pressed CO on (A) the biosynthesis of proteins, (B) the biosynthesis of total lipids, (C) the biosynthesis of acid soluble phosphorus, (D) the biosynthesis of RNA, and (E) the biosynthesis of DNA in the cells of *B. subtilis* NRRL B-94.

2000). These data suggest that CO has potential applications as natural food preservatives to inhibit microbial growth.

4. CONCLUSIONS

The characterization of the bioactive molecules in novel foods and agricultural products is required to improve the quality and nutritional value of the human diet. These are also important to improve the utilization of food and pharmaceutical products. The present study was designed to investigate the composition and functional properties of cold pressed CO. No such previous studies have yet been conducted on cold pressed CO. Knowledge on the composition and functional properties of cold pressed CO would assist in efforts for the industrial, nutritional and medical application of these plants. This report might serve as a milestone toward the development of healthy oils with high nutritional value. It could be concluded that CO is a good source of essential fatty acids and lipid-soluble bioactives. The high linoleic and oleic content makes CO nutritionally valuable. The present study demonstrated that CO contained significant levels of natural antioxidants. Tocols and phenolics at the levels estimated may be of nutritional importance as natural antioxidants and might directly react with and quench free radicals and prevent lipid peroxidation. Cold pressed CO could be nutritionally considered as a non-conventional supply for pharmaceutical industries, edible purposes and provide health benefits to consumers.

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