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Original scientific paper

Calendula officinalis L. (Asteraceae) possess antioxidant properties on Fe^{2+}-initiated peroxidation of rat brain microsomes

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Abstract

In this study the effects of Calendula officinalis L. (Asteraceae) extract (CO) on the polyunsaturated fatty acid composition, chemiluminescence and unsaturation index of microsomes isolated from brain rat, are presented. After incubation of microsomes in an ascorbate (0.4 mM)-Fe^{2+} (2.15 µM) system (180 min at 37 °C) it was observed that the total cpm/mg protein originated from light emission:chemiluminescence was lower in brain microsomes obtained from CO group compared to the control group (without extract supplementation). Moreover, it was observed that the addition of the extract reduced chemiluminescence -measured as total cpm- in a concentration dependent manner. The fatty acid composition of brain microsomes from control group was profoundly modified when subjected to non-enzymatic lipoperoxidation with a considerable decrease of arachidonic acid C20:4ω6 and docosahexaenoic acid C22:6ω3. As a consequence, the unsaturation index, a parameter based on the maximal rate of oxidation of specific fatty acids, was higher in the CO group compared to controls. The simultaneous analysis of unsaturation index, chemiluminescence and fatty acid composition indicate that CO may act as an antioxidant protecting rat brain microsomes from peroxidative damage.

Keywords

Marigold; lipoperoxidation; chemiluminescence

Introduction

The polyunsaturated acids located in biological membranes are excellent targets for peroxidation with peroxides formation [1,2]. The consequence of peroxidation of unsaturated fatty acids membranes is severe, damage of membrane function, enzymatic inactivation, toxic effects on the cellular division, etc. [3-5]. Such alterations have been implicated in causation of several diseases such as liver cirrhosis [6], inflammation and atherosclerosis [7]. Duthie *et al*. have stated that microsomes may be considered as an interesting system for lipoperoxidation studies [8]. The microsome is a convenient experimental model for detailed studies of kinetic reaction and peroxidation mechanism, considering the injured microsome a reason of tissue alterations in many pathological processes [9]. The production of reactive oxygen species in the brain has been implicated as a common factor in the etiology of a number of neurodegenerative diseases [10]. The link between free radicals and disease processes has led to considerable research into

nontoxic drugs that can scavenge the free radicals. Several plant extracts and plant products have been shown to possess significant antioxidant potential [11,12]. *Calendula officinalis* L. (Asteraceae, "marigold") extract (CO) is rich in flavonoids, terpenoids and lutein and has both antioxidant and anti-inflammatory activities and has been linked to the reduction in the risk of chronic diseases such as macular degeneration, cancer, cardiovascular and neurodegenerative disorders [13]. Present study was designed to determine whether microsomes from rat brain could be a target for non-enzymatic lipoperoxidation as well as to establish the level of protection of such membranes incubated with an extract of *Calendula officinalis*. The degradative process was followed simultaneously by the determination of chemiluminescence and fatty acid composition of brain microsomes. The unsaturation index was used to evaluate the fatty acid alterations observed during the process [14].

Experimental

Female Wistar AH/HOK rats were obtained from Laboratory Animal Facility, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata. BSA (fraction V) was obtained from Wako Pure Chemical Industries Ltd, Japan. Standards of fatty acids methyl esters were kindly supplied by NU Check Prep. Inc, Elysian, MN, USA. L(+) ascorbic acid and boron trifluoride-methanol complex were from Merck Laboratories. All other reagents and chemicals were of analytical grade from Sigma-Aldrich.

CO extract preparation

Fifteen grams of CO dried flowers were milled until plant material passed through a 2 mm screen and put in a flask together with 50 ml methanol for extraction under mechanical agitation at 870 rpm and dim light during 12 h. After extraction CO extract was submitted to vacuum filtration and then concentrated using a rotary evaporator (Senco Ltd, Zhong Shan Nan Yi Rd., Shanghai, China.) until total evaporation of the solvent. A residue of 3.15 g was obtained, which was re-suspended in 20 ml methanol. From the total methanolic extract 10 ml were used for the qualitative analysis of phytochemical constituents while the remaining volume was used to test the anti-oxidative properties. For the phytochemical analysis of the extract the original volume of 10 ml was fractioned in three aliquots using a 1-5 ml adjustable volume-pipette: Fraction A for testing the presence of flavonoids (zinc hydrochloride reduction test, Shinoda´s test), tannins (ferric chloride test), lipids (iodine reaction) and hydrocarbons (phenol 5 % + concentrated H_2SO_4); Fraction B for the investigation of the presence of steroids (acetic anhydride + concentrated H_2SO_4, Liebermann-Burchard reaction) and anthraquinones (sodium hydroxide test, Bornträger´s test); and Fraction C for the determination of alkaloids (potassium iodide-bismuth nitrated test, Dragendorff´s reagent), cardenolides (dinitrobenzoic acid + sodium hydroxide, Kedde´s reagent), steroids (Liebermann-Burchard reaction) and leucoanthocyanins (concentrated HCl + amyl alcohol, Rosenheim reaction) [15,16].

Animals and membrane preparation

Female Wistar AH/HOK rats, 7 weeks old, weighing 120-137 g were used. All rats were fed commercial rat chow and water *ad libitum*. The rats were euthanized by cervical dislocation and the brain was rapidly removed, cut into small pieces and extensively washed with 0.15 M NaCl. A 30 % (w/v) homogenate was prepared in a 0.25 M sucrose solution, 10 mM Tris-HCl pH 7.4 using a Potter-Elvejhem homogenizer. The homogenate was spun at 10,000 x g for 10 min. The supernatant (3 ml) was applied to a Sepharose 4B column (1.6 x 12 cm) equilibrated and eluted with 10 mM Tris-HCl pH 7.4, 0.01 % NaN_3. The microsomal fraction appearing in the void volume (10-16 ml) was brought to 0.25 M sucrose by adding solid sucrose. All operations were performed at 4 °C and under dim light. The quality of microsomal preparation is similar in composition as regards concentrations and activities of certain microsomal enzymes to that obtained by

ultracentrifugation [17].

Lipid peroxidation of rat brain microsomes

Chemiluminescence and lipid peroxidation were initiated by adding ascorbate to microsomes [18]. The microsomes (0.5 mg microsomes protein) with addition of CO total extract (0.1, 0.2, 0.3 and 0.4 mg) were incubated at 37 °C with 0.01 M phosphate buffer pH 7.4, 0.4 mM ascorbate, final vol. 2 ml. Phosphate buffer is contaminated with enough iron to provide the necessary ferrous or ferric iron (final concentration in the incubation mixture was 2.15 µM) for lipid peroxidation [19]. Microsome preparations, which lacked ascorbate, were carried out simultaneously. Membrane light emission was determined over a 180 min period, chemiluminescence was recorded as count per minute (cpm) every 10 min and the sum of the total chemiluminescence was used to calculate cpm/mg protein. Chemiluminescence was measured as counts per min in liquid scintillation analyzer Packard 1900 TR equipment with a program for chemiluminescence.

Fatty acid analysis

Microsomal lipids were extracted with chloroform/methanol (2:1 v/v) [20] from native or peroxidized membranes. Fatty acids were transmethylated with F_3B in methanol at 60 °C for 3 h. Fatty acid methyl esters were analyzed with a GC-14A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with packed column (1.80 m x 4 mm i.d.) GP 10 % DEGS-PS on 80/100 Supelcoport. Nitrogen was used as the carrier gas. The injector and detector temperatures were maintained at 250 °C, at the column temperature was held at 200 °C. Fatty acid methyl ester peaks were identified by comparison of the retention times with those of standards.

Protein determination

Proteins were determined by the method of Lowry *et al*. [21] using BSA as standard.

Unsaturation Index (UI)

UI was calculated as the sum of the percentage of each fatty acid x the number of olefinic bonds, divided by the sum of the percentage of saturated fatty acids.

Statistical analysis

Results were expressed as mean ±S.D. of six independent determinations. Data were statistically evaluated by one-way analysis of variance (ANOVA) and Tukey's test. The statistical criterion for significance was selected at different p-values, which was indicated in each case.

Results and Discussion

Phytochemical analysis of CO extract

Table 1 shows the results of the phytochemical analysis of the CO extract. Qualitative chemical determinations were performed in order to assess the presence of those compounds that exert anti-oxidative properties, as mentioned in CO extract preparation.

Light emission of rat brain microsomes during lipid peroxidation

The incubation of rat brain microsomes in the presence of ascorbate-Fe^{2+} resulted in the peroxidation of membranes as evidenced by emission of light (chemiluminescence). After incubation of microsomes in an ascorbate-Fe^{2+} system at 37 °C for 180 minutes, the cpm originated from light emission was lower in the CO group than in the control group in a concentration dependent manner (Fig. 1) Figure 2 shows the total light

emission obtained from CO group and control group. The values were from 2,476.853 ± 312.987 cpm in the control group to 1,462.044 ± 142.044 cpm with the addition of 0.4 mg extract/mg protein.

Table 1. Qualitative analysis of CO extract.

Determination	Fraction A	Fraction B	Fraction C
Shinoda	+		
Ferric chloride	+		
Iodine	+		
Phenol 5% + conc. H_2SO_4	+		
Liebermann-Burchard		+	+
Bornträger		-	
Dragendorff			-
Kedde			-
Rosenheim			+

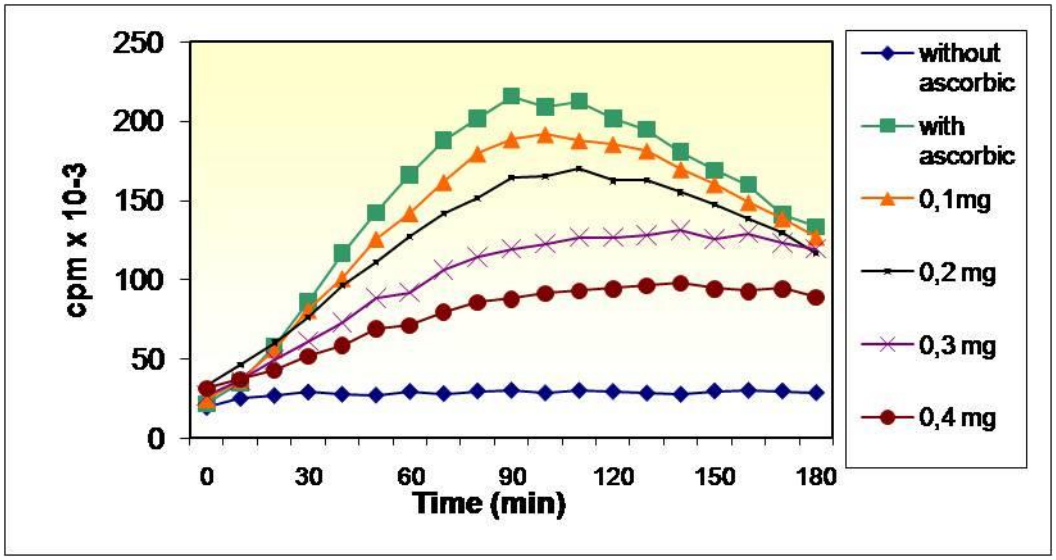

Figure 1. Lipid peroxidation of rat brain microsomes with different concentrations of CO extract.

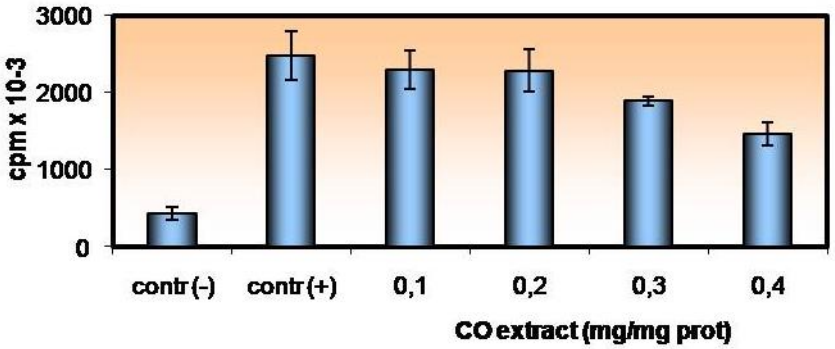

Figure 2. Total chemiluminescence of rat brain microsomes induced by ascorbate Fe^{2+} system.

Fatty acid composition of brain microsomes and unsaturation index

Table 2 shows the fatty acid composition of brain microsomes from control group, native and peroxidized in ascorbate Fe^{2+} system. The lipid peroxidation of brain microsomes showed significant changes in the polyunsaturated fatty acid composition, significant decreases of arachidonic acid C20:4ω6 and docosahexaenoic acid C22:6ω3 were observed. There were marked differences when the unsaturation

index of peroxidized control group and native control group brain microsomes were compared.

Table 2. Fatty acid composition (area %) of rat brain native and peroxidized microsomes without CO extract.[a]

Fatty acid	Brain microsomes native	Brain microsomes peroxidized
C16:0	21.941 ± 1.018	28.006 ± 1.804
C18:0	16.496 ± 1.192	19.579 ± 1.032
C18:1ω9	30.991 ± 2.589	37.191 ± 2.879
C18:2ω6	0.717 ± 0.040	0.337 ± 0.053
C18:3ω3	2.404± 1.062	3.270 ± 1.527
C20:4ω6	10.370 ± 0.526 [1]	3.457 ± 0.225 [1,b,c]
C22:6ω3	13.194 ± 0.487 [2]	0.173 ± 0.040 [2, d, e]
Saturated	38.346 ± 1.730	47.586 ± 2.094
Monounsaturated	30.991 ± 2.589	37.171 ± 2.201
Polyunsaturated	29.317 ± 1.991 [4]	8.297 ± 1.003 [4]
Total unsat./sat.	60.309 ± 1.114	45.468 ± 2.205
Saturated/unsaturated	0.637 ± 0.039	1.115 ± 0.531
Unsaturation Index	173.445 ± 7.002 [3]	67.657 ± 2.391 [3]

[a]Data are given as the mean ±SD of six experiments. Statistically significant differences in fatty acids concentrations and UI of brain microsomes native and peroxidized are indicated by [1,4] $p < 0.01$, [2] $p < 0.004$, [3] $p < 0.03$

The fatty acid composition from isolated brain microsomes after addition of CO extract to the ascorbate Fe^{2+} system is presented in Table 3. When peroxidized control group (Table 2) is compared to CO group after lipid peroxidation it is observed that arachidonic acid C20:4ω6 and docosahexaenoic acid C22:6ω3 increased in the CO group (concentration dependent). Polyunsaturated fatty acids ranged from 29.32 % in native microsomes, 8.3 % in peroxidized and 24.58 % in peroxidized + 0.4 mg extrac /mg protein. Significant changes in the unsaturation index were observed when compared peroxidized control group against CO group. The changes were less pronounced in membranes from CO group. As a result, the unsaturation index of peroxidized membranes in the CO group was significantly higher than in the control group.

Table 3. Fatty acid composition of rat brain microsomes with different concentrations of CO extract[a]

Fatty acid	CO extract concentration			
	0.1 mg	0.2 mg	0.3 mg	0.4 mg
C16:0	25.285 ± 2.072	26.406 ± 2.325	28.333 ± 2.734	23.109 ± 3.109
C18:0	23.067 ± 1.439	24.579 ± 1.973	22.947 ± 1.995	20.926 ± 2.505
C18:1ω9	34.601 ± 2.231	36.869 ± 2.163	34.429 ± 3.029	31.389 ± 2.942
C18:2ω6	3.019 ± 0.178	2.514 ± 0.710	0.645 ± 0.225	4.343 ± 0.982

C18:3ω3	8.715 ± 1.322	6.447 ± 1.721	4.438 ± 1.045	7.450 ± 1.227
C20:4ω6	5.313 ± 0.621	3.184 ± 0.109	5.685 ±0.538 [b]	7.682 ± 0.301 [c]
C22:6ω3	0.324 ± 0.057 [1]	0.435 ± 0.093 [2]	3.530 ± 0.611 [d]	5.102 ± 0.471 [1,2, e]
Saturated	48.352 ± 2.425	50.985 ± 3.094	51.280 ± 3.723	44.035 ± 3.429
Monounsaturated	34.601 ± 1.327	36.689 ± 2.629	34.429 ± 2.408	31.389 ± 2.929
Polyunsaturated	17.047 ± 1.005	12.145 ± 1.005	14.298 ± 1.300	24.577 ± 1.853
Total unsat./sat.	51.640 ± 3.109	49.014 ± 2.116	48.727 ± 3.513	55.966 ± 4.003
Saturated/unsaturated	0.936 ± 0.127	1.040 ± 0.057	1.052 ± 0.281	0.786 ± 0.217
Unsaturation Index	88.036±4.076 [3]	73.974 ±3.226 [4]	92.957 ± 4.007	123.765±4.216 [3,4]

[a]Data are given as the mean ±SD of six experiments. Statistically significant differences in fatty acids concentrations and UI of brain microsomes native and peroxidized are indicated by [1,2] $p < 0.008$, [3,4] $p < 0.05$. Statistically significant differences between Tables 1 and 2 are indicated by [b, c] $p < 0.05$, [d] $p < 0.009$, [e] $p < 0.006$

Conclusions

Rat brain microsomes incubated with CO extract were protected against lipid peroxidation when compared to similar membranes from control group, as demonstrated by the results from chemiluminescence, polyunsaturated fatty acids composition and unsaturation index. As a result of the analysis of UI of peroxidized microsomes membranes, it was observed that CO group was successful against lipid peroxidation compared to control group. These results are in concordance with previous reports of Braga *et al*. [22]. *In vitro* lipid peroxidation studies are useful for the elucidation of possible mechanisms of peroxide formation *in vivo* [23], since the high concentration of polyunsaturated fatty acid membranes causes susceptibility to lipid peroxidative degradation [24]. In our assay it was found that arachidonic acid C20:4ω6 and docosahexaenoic acid C22:6ω3 from controls were more peroxidized than those from CO group. This predisposition to peroxidation of arachidonic acid C20:4ω6 and docosahexaenoic acid C22:6ω3 was demonstrated in previous works [25]. Although considerable research has already been performed to characterize the changes in structure, composition and physical properties of membranes subjected to oxidation [26,27], it is important to know how biological compounds with antioxidant properties contribute to the protection of specialized membranes against deleterious effects produced by reactive oxygen species and other free radicals. Evidence leading to the recognition of the anticarcinogenic activity of *Calendula officinalis* L. (Asteraceae) has been reviewed [28]. Hamzawy *et al*. [29] also reported the anticancer effects of *C. officinalis* extract due to chemoprotector properties as well as radical scavenging activity because of the presence of phenolic compounds. New data indicates that CO has a potent antioxidant activity [29], being marigold extracts able to improve biochemical parameters and decrease the formation of inflammatory cytokines, thus preventing the oxidative stress. The phytochemical constituents present in our CO extract were similar to those found in previous works, which also presented the chemical identity of such compounds [30,31]. In conclusion, our results are consistent with the hypothesis that *Calendula officinalis* L. (Asteraceae) extract may act as a physiological antioxidant in cell membranes.

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