

Protective effect of *Calendula Officinalis* L. extract against non-enzymatic peroxidation of rat kidney microsomes and mitochondria.

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Abstract

Cellular metabolic changes during kidney disease may induce higher production of oxygen radicals that play a significant role in the progression of renal damage and in the onset of important comorbidities. In the study reported here the effect of *Calendula Officinalis* L. extract (CO) on chemiluminescence of mitochondria and microsomes isolated from kidney rat was analysed. After incubation of mitochondria and microsomes in an ascorbate (0.4 mM)-Fe⁺⁺ (2.15 μM) system (180 min at 37°C), non-enzymatic peroxidation, it was observed that the total cpm/mg protein originated from light emission: chemiluminescence was lower in kidney mitochondria and microsomes obtained from CO group than in the control group (without CO). Moreover, it was observed that the CO was reduced, concentration dependent (0.05, 0.25, 0.35 and 0.45 mg of extract), of chemiluminescence, measured as total cpm. The analyses of chemiluminescence indicate that CO may act as antioxidant protecting rat kidney mitochondria and microsomes from peroxidative damage.

Keywords: *Calendula officinalis* L. (Asteraceae), peroxidation, chemiluminescence, mitochondria, microsomes

Introduction

The biological membranes are excellent targets for peroxidation with peroxides formation (Miyamoto et al., 2006; Miyamoto et al., 2007). The consequence of peroxidation of membranes is severe, damage of membrane function, enzymatic inactivation, toxic effects on the cellular division, etc. (Porter, 2013; Lee et al., 2013; Niki, 2009). The microsomes or mitochondria as an interesting system for peroxidation studies (Anderson et al., 2012; Navarro and Boveris, 2009a; Navarro et al., 2009b). These organelles are a convenient experimental model for detailed studies of kinetic reaction and peroxidation mechanism, considering the injured is a motive of tissue alterations in many pathological processes (Du et al., 2012; Chan, 2012). The production of reactive oxygen species has been implicated as a common factor in the etiology of a number of diseases (Sanderson et al., 2013). *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 diseases (Guler et al., 2014). Present study was designed

to determine whether mitochondria and micromes from rat kidney could be a target for non-enzymatic peroxidation 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 (Dikalov and Harrison, 2014; Howard, 2013).

Material and methods

Female Wistar AH/HOK 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. L (+) ascorbic acid was from Merck Laboratories. All other reagents and chemicals were of analytical grade from Sigma.

CO extract preparation

Fifteen grams of CO dried flowers were milled until gross powder 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.) 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 fractionated 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 H₂SO₄); Fraction B for the investigation of the presence of steroids (acetic anhydride + concentrated H₂SO₄, 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) (Bruneton, 2001; Harborne, 1998).

Animals and preparation of microsomes and mitochondria

Female Wistar AH/HOK rats 7 weeks-old, weighing 120-137g were used. All rats were fed commercial rat chow and water *ad-libitum*. The rats were sacrificed by cervical dislocation and kidneys were 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-Elvehjem homogenizer. The homogenate was spun at 10,000 x g for 10 min. The supernatant (3ml) obtained was applied to Sepharose 4B column (1.6 x 12cm) equilibrated and eluted with 10mM Tris-HCl pH 7.4, 0.01% Na₃. The microsomal fraction appearing in the void volume (10-16 ml) was brought to 0.25M sucrose. All operations were performed at 4 °C and under dim light. The quality of this microsomal preparation is of similar composition with respect to concentrations and activities of certain microsomal enzymes to that obtained by ultracentrifugation (Tangen et al., 1973). Mitochondria were obtained by method described by Schneider and Hogeboom, 1958.

Peroxidation of rat kidney microsomes and mitochondria

Chemiluminescence and peroxidation were initiated by adding ascorbate to microsomes or mitochondria (Wright et al., 1979). The microsomes or mitochondria (1 mg of microsomal or 0.5mg mitochondrial protein) with addition of CO (0.05, 0.25, 0.35 and 0.45 mg of extract, CO group) 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 sufficient iron to provide the necessary ferrous or ferric iron (final concentration in the incubation mixture was 2.15 μM) for peroxidation (Tadolini and Hakim, 1996). Mitochondria or microsomal preparations, which lacked ascorbate, were carried out simultaneously. Membrane light emission was determined over a 180 min period, chemiluminescence was recorded as 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 a liquid scintillation

analyzer Packard 1900 TR equipment with a program for chemiluminescence.

Protein determination

Proteins were determined by the method of Lowry et al. (1951) using BSA as standard.

Statistical analysis

Results are expressed as means ± 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

Phytochemical analysis of CO extract

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

Table 1. Qualitative analysis of CO extract.

Determination	Fraction A	Fraction B	Fraction C
Shinoda	+		
Ferric chloride test	+		
Iodine	+		
Phenol 5% + concentrated H ₂ SO ₄	+		
Liebermann-Burchard		+	+
Bornträger		-	
Dragendorff			-
Kedde			-
Rosenheim			+

Chemical reactions were used as follows: Shinoda for flavonoids,

Ferric chloride test for tannins and phenolic OH, Iodine for lipids,

Phenol 5% + concentrated H₂SO₄ for carbohydrates, Liebermann-Burchard for steroids,

Bornträger for anthraquinones, Dragendorff for alkaloids,

Kedde for cardenolides and Rosenheim for leucoanthocyanins.

Light emission of rat kidney mitochondria during peroxidation.

The incubation of rat kidney mitochondria in the presence of ascorbate-Fe⁺⁺ resulted in the peroxidation of membranes as evidenced by emission of light (chemiluminescence). After incubation of mitochondria in an ascorbate-Fe⁺⁺ system at 37°C for 180 minutes, the cpm originated from light emission was lower (concentration dependent) in the CO group than in the control group. Figure 1 show the light emission obtained from CO group and control group. The values were from 2119,2 ± 355,7 in the control group to 311,6 ± 4,5 cpm with the addition of 0,45 mg of CO.

Light emission of rat kidney microsomes during peroxidation.

The incubation of rat kidney microsomes in the presence

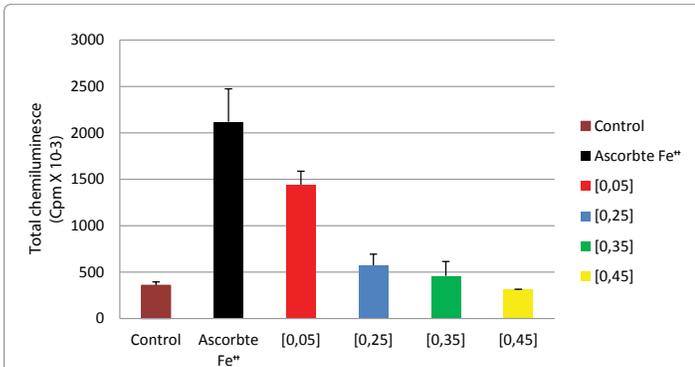


Figure 1: Light emission of rat kidney mitochondria during peroxidation with different concentration of CO. Data are given as the mean ± SD of six experiments.

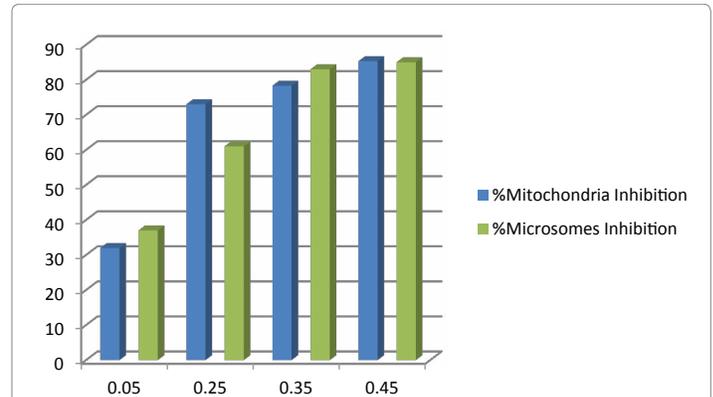


Figure 3: Percentage of light emission inhibition in microsomes and mitochondria of rat kidney by CO

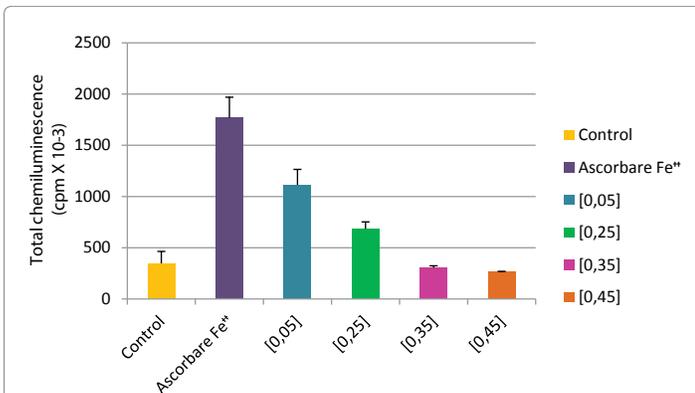


Figure 2: Light emission of rat kidney microsomes during peroxidation with different concentration of CO. Data are given as the mean ± SD of six experiments.

of ascorbate-Fe⁺⁺ resulted in the peroxidation of membranes as evidenced by emission of light (chemiluminescence). After incubation of microsomes in an ascorbate-Fe⁺⁺ system at 37°C for 180 minutes, the cpm originated from light emission was lower (concentration dependent) in the CO group than in the control group. Figure 2 show the light emission obtained from CO group and control group. The values were from 1768,3 ± 201,2 in the control group to 263,3 ± 6,1 cpm with the addition of 50 µg of SM / mg prot.

Comparative percentage of light emission inhibition in microsomes and mitochondria of rat kidney

After incubation of microsomes and mitochondria in an ascorbate-Fe⁺⁺ system (180min at 37°C) it was observed that the percentage cpm/mg of protein originated from light emission (chemiluminescence) was lower in kidney microsomes and mitochondria with addition of CO extract. Thus, the percentage of peroxidation inhibition produced by CO was 85.3% in mitochondria and 85% in micromes with 0,45 mg of CO, Figure 3.

Discussion

The aim of our study was evaluate the capacity of CO

extract to protect kidney microsomes or mitochondria against peroxidation. Rat brain mitochondria incubated with CO extract were protected against lipid peroxidation when compared to similar membranes from control group, as demonstrated by the results from chemiluminescence. Peroxidation studies *in vitro* are useful for the elucidation of possible mechanism of peroxide formation *in vivo* (Rauchová et al., 1993). These results are in concordance with previous reports of Braga et al. (2009). *In vitro* lipid peroxidation studies are useful for the elucidation of possible mechanisms of peroxide formation *in vivo* (Brand et al., 2014), since the composition of membranes causes susceptibility to peroxidative degradation (Shichiri, 2014). Although considerable research has already been performed to characterize the changes in structure, composition and physical properties of membranes subjected to oxidation (Ayala et al., 2014; Vyssokikh et al., 2015; Omotayo et al., 2014; Naserzadeh et al., 2015), 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 (Babae et al., 2013). New data indicates that CO has a potent antioxidant activity (Hamzawy et al., 2013), 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 consistent with those found in previous works, which also presented the chemical identity of such compounds (Arora et al., 2013; Muley et al., 2009). 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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References

- Anderson EJ, Katunga LA, Willis MS. (2012). Mitochondria as a source and target of lipid peroxidation products in healthy and diseased heart. *Clin Exp Pharmacol Physiol*; 39: 179–93.
- Arora D, Rani A, Sharma A. (2013). A review on phytochemistry and ethnopharmacological aspects of genus *Calendula*. *Pharmacogn Rev*; 7: 179-87.
- Ayala A, Muñoz MF, Argüelles S. (2014). Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal. *Oxid Med Cell Longev*; 2014:360438. doi: 10.1155/2014/360438.
- Babae N, Moslemi D, Khalilpour M. (2013). Antioxidant capacity of calendula officinalis flowers extract and prevention of radiation induced oropharyngeal mucositis in patients with head and neck cancers: a randomized controlled clinical study. *Daru*; 21: 18-21.
- Braga PC, Dal Sasso M, Culici M. (2009). Antioxidant activity of *Calendula officinalis* extract: inhibitory effects on chemiluminescence of human neutrophil bursts and electron paramagnetic resonance spectroscopy. *Pharmacology*; 83: 348-55.
- Brand MD, Affourtit C, Esteves TC. (2004). Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. *Free Radic Biol Med*; 37: 755-67.
- Bruneton J. (2001). *Farmacognosia*. 2a. Ed. Editorial Acribia. Zaragoza, España. Pp. 1099.
- Chan DC. (2012). Fusion and Fission: Interlinked Processes Critical for Mitochondrial Health. *Annu Rev Genet*; 46: 265-87.
- Dikalov SI, Harrison DG. (2014). Methods for Detection of Mitochondrial and Cellular Reactive Oxygen Species. *Antioxid Redox Signal*; 20: 372-82.
- Du H, Guo L, Yan SS. (2012). Synaptic Mitochondrial Pathology in Alzheimer's Disease. *Antioxid Redox Signal*; 16: 1467-75.
- Guler E, Barlas FB, Yavuz M. (2014). Bio-active nanoemulsions enriched with gold nanoparticle, marigold extracts and lipoic acid: In vitro investigations. *Colloids Surf B Biointerfaces*; 121: 299-06.
- Hamzawy MA, El-Denshary ES, Hassan NS. (2013). Dietary Supplementation of *Calendula officinalis* Counteracts the Oxidative Stress and Liver Damage Resulted from Aflatoxin. *ISRN Nutr*; 2013:538427. doi: 10.5402/2013/538427.
- Harborne JB. (1998). *Phytochemical methods*, 3rd Ed., London: Chapman and Hall.
- Howard GS, Mansi K, Genter MB. (2013). Dietary whey protein stimulates mitochondrial activity and decreases oxidative stress in mouse female brain. *Neurosci Lett*; 548: 159-64.
- Lee LK, Shahar S, Rajab N. (2013). The role of long chain omega-3 polyunsaturated fatty acids in reducing lipid peroxidation among elderly patients with mild cognitive impairment: a case-control study. *JNB*; 24: 803–08.
- Lowry OH, Rosebrough NJ, Farr AL. (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem*; 193: 265-75.
- Miyamoto S, Martinez GR, Rettori D. (2006). Linoleic acid hydroperoxide reacts with hypochlorous acid, generating peroxy radical intermediates and singlet molecular oxygen. *Proc Natl Acad Sci USA*; 103: 293-98.
- Miyamoto S, Ronsein GE, Prado FM. (2007). Biological hydroperoxides and singlet molecular oxygen generation. *IUBMB Life*; 59: 322–31.
- Muley BP, Khadabadi SS, Banarase NB. (2009). Phytochemical constituents and pharmacological activities of *Calendula officinalis* Linn (Asteraceae): A review. *Trop J Pharm Res*; 8: 455-65.
- Naserzadeh P, Hosseini MJ, Arbabi S. (2015). A comparison of toxicity mechanisms of cigarette smoke on isolated mitochondria obtained from rat liver and skin. *Iran J Pharm Res*; 14: 271-77.
- Navarro A, Boveris A. (2009a). Brain mitochondrial dysfunction and oxidative damage in Parkinson's disease. *J Bioenerg Biomembr*; 41: 517-21.
- Navarro A, Boveris A, Bández MJ. (2009b). Human brain cortex: mitochondrial oxidative damage and adaptive response in Parkinson's disease and in dementia with Lewy bodies. *Free Radic Biol Med*; 46: 1574-80.
- Niki E. (2009). Lipid peroxidation: Physiological levels and dual biological effects. *Free Radic Biol Med*; 47: 469–84.
- Omotayo TI, Akinyemi GS, Omololu PA. (2014). Possible involvement of membrane lipids peroxidation and oxidation of catalytically essential thiols of the cerebral transmembrane sodium pump as component mechanisms of iron-mediated oxidative stress-linked dysfunction of the pump's activity. *Redox Biol*; 4: 234-41.
- Porter NA. (2013). A Perspective on Free Radical Autoxidation: The Physical Organic Chemistry of Polyunsaturated Fatty Acid and Sterol Peroxidation. *J Org Chem*; 78: 3511–24.
- Rauchová H, Kalous M, Drahotka Z, Koudelová J, Mourek J. (1993). Lipid peroxidation in isolated membranes of cerebral cortex, heart and kidney. *Physiol Res*; 42:323-327.
- Sanderson TH, Reynolds CA, Kumar R. (2013). Molecular mechanisms of ischemia-reperfusion injury in brain: pivotal role of the mitochondrial membrane potential in reactive oxygen species generation. *Mol Neurobiol*; 47: 9-23.
- Shichiri M. (2014). The role of lipid peroxidation in neurological disorders. *J Clin Biochem Nutr*; 54: 151-60.
- Schneider WC, Hogeboom GH. (1951). Cytochemical studies of mammalian tissues; the isolation of cell components by differential centrifugation: a review. *Cancer Res*; 11:1-22.
- Tadolini B, Hakim G. (1996). The Mechanism of Iron (III) Stimulation of Lipid Peroxidation. *Free Radic Res*; 25: 221-27.
- Tangen O, Jonsson J, Orrenius S. (1973). Isolation of rat liver microsomes by gel filtration. *Anal Biochem*; 54:597-603.
- Vyssokikh MY, Antonenko YN, Lyamzaev KG. (2015). Methodology for use of mitochondria-targeted cations in the field of oxidative stress-related research. *Methods Mol Biol*; 1265: 149-59.
- Wright JR, Rumbaugh RC, Colby HD. (1979). The relationship between chemiluminescence and lipid peroxidation in rat hepatic microsomes. *Arch Biochem Biophys*; 192: 344–51.