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COMPARISON BETWEEN NORMAL COFFEE AND DECAFFEINATED COFFEE EFFECTS ON LYMPHOCYTES AND MACROPHAGES: ROLE OF THE ANTIOXIDANT ACTIVITY OF CAFFEINE

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ABSTRACT

*Coffee (*Coffea arabica *L.) presents antioxidant effects. Due to negative effects of caffeine (Ca), more people consume decaffeinated coffee. Reactive oxygen species are involved in immune cell physiology controlling proliferation, death and cellular metabolism. The aim of this study was to evaluate the influence of Ca in the effect of normal and decaffeinated coffee on normal mice lymphocytes and macrophages. It were assayed MTT and nitroblue tetrazolium reduction, proliferation by tritiated thymidine uptake and "per se" superoxide and catalase (CAT) activities both related to H_2O_2 modulation. The decaffeinated coffee induced a decrease in lymphocytes proliferation in all concentrations assayed; this effect was related to an increase of superoxide anion and with the absence of Ca, which by itself increased lymphocytes proliferation through a decrease in H_2O_2 level by CAT activity "per se." On macrophages, both extracts induced cell activation not related to the presence of Ca. In conclusion, caffeinated coffee could be better than decaffeinated coffee in the maintenance of the oxidative balance in lymphocytes cells.*

PRACTICAL APPLICATIONS

Coffee has become one of the most widely consumed psychoactive beverages. A major compound in coffee is caffeine (Ca). Due to the negative effects of Ca, more people consume decaffeinated coffee. Decaffeinated coffee would be able to exert negative properties on redox equilibrium in cells. The

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Ca in coffee could protect cells from death. Therefore, we propose that should be more favorable to drink normal coffee than decaffeinated coffee as antioxidant and for maintenance of normal redox equilibrium in cells. This work assists in the knowledge of the properties of coffee which can be used together with the background of previous results in order to select beverages with the least negative effects for human health.

INTRODUCTION

Coffee (*Coffea arabica* L.) was discovered around the sixth century by Abyssinian/Ethiopian shepherds in the province of Keffa (Ethiopia). Since then, coffee has become one of the most widely consumed psychoactive beverages. In recent years, due to the increasing interest in finding physiologically functional foodstuffs, especially as antioxidants (Del Castillo *et al*. 2002; Daglia *et al*. 2004; Yanagimoto *et al*. 2004; Yukawa *et al*. 2004), the relationship between coffee and health has been extensively studied (Higdon and Frei 2006). It is known that coffee possesses several compounds besides caffeine (Ca), for example, caffeic, ferulic and vanillic acids (Clifford 1985). Among the different phenolic compounds, the best example is chlorogenic acid (5-caffeoylquinic acid) (CGA) (Clifford 1999). Structurally, CGA is the ester formed between certain *trans* cinnamic acid and (L)-quinic acid (1L-1(OH),3,4/5-tetrahydroxycyclohexanecarboxylic acid) (Clifford *et al*. 2003). CGA has been long known as an antioxidant and might therefore contribute to the prevention of type 2 diabetes mellitus and cardiovascular disease (Morton *et al*. 2000; Johnston *et al*. 2003; Paynter *et al*. 2006). On the other hand, CGA is metabolized to ferulic acid (FA) which is reported to scavenge superoxide anions (Toda *et al*. 1991). Furthermore, another major compound in coffee is Ca, which may account for as much as 2.2% of the dry matter in coffee (Viani 1991). Ca is a bitter white crystalline xanthine alkaloid that acts as a psychoactive stimulant drug and a mild diuretic (speeds up urine production) in humans and other animals. Unlike CGA, Ca is rapidly and completely absorbed in humans and later metabolized by the liver to produce dimethylxanthines, 1-methyluric acid (1-U) and other related metabolites (Crews *et al*. 2001). Ca can be absorbed through the skin and is a potent central nervous system stimulant (Van De Sandt *et al*. 2004). The Ca-induced stimulation is followed by depression (Klein and Salzman 1975). Studies show that Ca has a profound effect on sleep (Bolton and Null 1981). Therefore, due to negative actions of Ca, more people consume decaffeinated coffee (Ramalakshmi *et al*. 2008). Two studies realized in Spain and in the U.S.A. reveled that the consumption of decaffeinated coffee is progressively increasing (Cerdeño 2007).

In accordance with said below, it is very important to have concise data about the safety of consumption of normal coffee (Nor) or decaffeinated coffee (Dec). Therefore, we propose in this work realize a comparative study about the effect of Nor, Dec and Ca on immune system cells such as, normal lymphocytes and macrophages, evaluating their effects on cell proliferation and on oxidation status.

MATERIALS AND METHODS

Coffee Samples

Decaffeinated and caffeinated roasted coffee beans were commercially obtained in "Café Martinez," Buenos Aires City, Argentina. The grains were ground by means of a mill (Arthus Thomas, Co). Coffee extracts, Dec and Nor (10% p/v) were obtained by adding 10 g of coffee to 100 mL of distilled water and kept for 45 min at 52C. After maceration for 72 h at 5C, the extracts were filtered (Anesini *et al*. 1999), sterilized through a 0.22-μm filter, lyophilized, aliquoted and stored at −20C until use (Davicino *et al*. 2008). Percentage yield: 35% (w/w).

High-Performance Liquid Chromatography Analysis

The amount of CGA and Ca present in the Dec and Nor was determined by high-performance liquid chromatography (HPLC). Lyophilized extracts, Ca and CGA content were analyzed according to Spanish Pharmacopoeia (2005), European Pharmacopoeia (2007) (Real Farmacopea Española 2005) and Filip *et al*. (1998). The HPLC analysis was performed in a Varian Pro Star instrument with ultraviolet (UV) photodiode array detector. The Ca quantitation was carried out using a reverse phase IB-SIL RP 18 (5 μm, 250 × 4.6 mm I.D.) Phenomenex Luna column. The mobile phase was A: Water: acetic acid (98:2) and B: Methanol: acetic acid (98:2); the gradient was from 17% B to 20% B in 10 min; 20% B (isocratic) for 5 min; 20% B to 23% B in 10 min and 23% B to 100% B in 5 min, flow 1.0 mL/min, room temperature. Detection: with UV 273 nm (Filip *et al*. 1998). CGA was quantified using the same column as Ca. Mobile phase: A: water: phosphoric acid (99.5: 0.5), B: acetonitrile: phosphoric acid (99.5: 0.5); the gradient was from 8% B to 25% B in 20 min, 25% B isocratic during 13 min, 25% B to 100% B in 2 min, 100% B to 8% B in 2 min, flow rate 1.2 mL/min at 40C. Detection with UV 330 nm (European Pharmacopoeia 2007). The samples were analyzed with a program provided by Varian S.A. Pure standards: CGA and Ca were obtained from Carl Roth (Karlsruhe, Germany).

Animals and Cell Culture Conditions

Seven-week-old male C3H (H-2d) inbred male mice were mainly provided by Dr Norberto San Juan (Dept. of Microbiology, UBA, Buenos Aires, Argentina). Animals were used according to the Guide for the Care and Use of Experimental Animals (DHEW Publication, NIH 80-23) and maintained on a standard laboratory diet and water ad libitum. [27]

Lymphoid cell suspensions from lymph nodes were obtained aseptically from nylon wool purification of T cells as described previously (Anesini *et al*. 1996). Cells, at a concentration of 1×10^6 cells/mL, were cultured in RPMI 1640 medium (Gibco, Rockville, MD) supplemented with 10% fetal calf serum (FCS) (Gibco), 2 mM glutamine (Sigma**,** St. Louis, MO) and antibiotics in 96-well flat-bottomed microtiter plates (Nunc, Naperville). Peritoneal cells (PC) were harvested by sterile lavage with 20 mL HBSS (Sigma) supplemented with 20 µg/mL gentamicin (Sigma) and heparin (Sigma) (50 U/mL) as previously (Rabinovich *et al*. 2000) and adjusted to 1×10^6 cell/mL. Macrophages (Mφ) were purified from PC by adherence onto 96-well flat-bottomed tissue culture plates in Dulbecco Modified Eagle Medium (DMED) (Sigma) with 20 µg/mL gentamicin and 5% heat-inactivated FCS. Nonadherent cells were removed after 2 h at 37C and complete medium was added. The adherent Mφ monolayers showed >90% of purity according to morphologic analysis or nonspecific esterase staining. [28] [29] [30] [31] [32]

Proliferation Assay

The effects of Dec and Nor at 1, 10, 100 and 1,000 µg/mL and Ca at 0.1, 1, 10, 100 and 1,000 µg/mL were evaluated in the absence or presence of concanavalin A (CONA) (Sigma) (0.0625 and 0.125 µg/mL) and 5×10^{-3} M of hydrogen peroxide (H_2O_2) (Sigma). Lymphocytes were settled at a final volume of 0.2 mL in 96-well flat-bottomed microtiter plates (Nunc) for microculture. Cells were cultured during 24 h and then pulsed with tritiated thymidine [^{3}H]TdR (20 Ci/mmol) for the last 6 h. The proliferation was evaluated by the uptake of [^{3}H]TdR as previously described (Anesini *et al*. 1996). CONA as positive control of proliferation was used. Results were expressed as cpm.

MTT Reduction

The assay was determined by the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma). Briefly, normal lymphocytes and Mφ (1×10^6 cells/mL) were incubated with Dec, Nor and Ca (1, 10, 100 and 1,000 µg/mL) during 1 and 24 h. After incubation, 10 µL of MTT (5 mg/mL) (Sigma) in 100 µL of culture medium were added, and after 4 h

incubation, the purple formazan formed was solubilized by the addition of acidic isopropanol. The absorbance was measured using a microplate reader (Microplate Reader Benchmark, Bio-Rad, Hercules, CA) at 570 nm. Untreated cells were used as control and results were expressed as % of reduction of MTT relative to control (Pervin *et al*. 2001).

Production of Superoxide

The method described by Schoff (Schopf *et al*. 1984) was employed. The O_2^- anion was evaluated by the reduction of nitroblue tetrazolium (NBT) (Sigma) to formazan. Briefly, normal (1×10^6 cells/mL) lymphocytes trated with Nor, Dec and Ca (1, 10, 100, 1,000 µg/mL) were incubated with 300 µL of NBT during 30 min. The reaction was stopped with 1N HCl. Formazan was extracted with dioxane and the absorbance was measured in a microplate reader at 525 nm (Microplate Reader Benchmark, Bio-Rad).

Catalase Activity of Ca

The samples, 50 µL of Ca (1, 5, 50, 250 and 500 µg/mL) or Nor (1, 10, 100, 1,000 µg/mL), were added to a sodium phosphate buffer (50 mM), pH: 7, then 100 mM (v/v) H_2O_2 were included. The absorbance was monitored for 5 min at 240 nm. The change in absorbance is proportional to the breakdown of H_2O_2; one unit of the enzyme activity was defined as the amount of the enzyme required for the breakdown of 1 µM H_2O_2 (Carrillo *et al*. 1991).

Superoxide Dismutase Activity of Ca, CGA and Nor

The superoxide dismutase (SOD) activity was used to detect the level of epinephrine autoxidation inhibition as a method to evaluate antioxidant activity. The samples, 50 µL of Ca (1, 5, 50, 250, 500, 750, 2,500, 5,000 and 10,000 µg/mL), CGA (1, 2.5, 5, 10, 25, 50, 500, 750, 2,500 µg/mL) and Nor (800 µg/mL), were treated with 910 µL of sodium phosphate buffer (0.05 M), pH: 10.7 and 1 mM epinephrine. Under these conditions, the epinephrine rapidly undergoes auto-oxidation to produce adrenochrome, which is a pink-colored product that can be measured at 480 nm using a UV/visible spectrophotometer (Shimadzu, UV-2101PC, Torrance, CA) in kinetic mode. Results are expressed as units (U) of SOD activity/milliliter. One U of SOD activity induces approximately a 50% inhibition of the auto-oxidation of adrenaline (Carrillo *et al*. 1991).

Reagents

RPMI 1640 medium and FCS were obtained from Gibco; glutamine, HBSS, DMED culture medium, CONA, tritiated thymidine, 3-(4,

5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Ca, hydrogen peroxide, epinephrine, gentamicin and heparin were obtained from Sigma; 96-well flat-bottomed microtiter plates were purchased in Nunc.

Statistical Analysis

Differences between group means were assessed using one-way analysis of variance followed by comparison by Dunnett's test. A $P \leq 0.05$ was considered statistically significant (Dunnett 1964).

RESULTS

HPLC Analysis

First, the chemical composition of Nor and Dec was determined in order to analyze the Ca and GGA content, the majority compounds found in coffee. Nor and Dec contain polyphenolic compounds (CGA) and methyl xanthines such as Ca; these compounds were quantified in the extracts (Table 1). Nor extract contained significantly more Ca than Dec extract ($P \leq 0.05$), but there were no significant differences in CGA content between both extracts. Retention times (RT): Ca Standard: 23.834 min (Fig. 1A), Dec: 23.291 min (Fig. 1B); Nor: 23.608 min (Fig. 1C); CGA Standard: 10.872 min (Fig. 2A), Dec: 10.769 min (Fig. 2B), Nor: 10.872 min (Fig. 2C). 36

MTT Reduction

The results showed that the treatment of mouse lymphocytes with Dec, Nor or Ca during 1 h did not induce significantly modifications in MTT reduction 37

TABLE 1.
QUANTIFICATION OF CHLOROGENIC ACID AND CAFFEINE IN DECAFFEINATED COFFEE (DEC) AND NORMAL COFFEE (NOR) AQUEOUS EXTRACTS BY HPLC

Compounds	Dec	Nor
Chlorogenic acid	0.045 ± 0.0030	0.049 ± 0.003
Caffeine	0.011 ± 0.004*	0.15 ± 0.02

The values are expressed in g/% (w/v). The identification and quantification of the compounds were carried on by HPLC, confronting the retention time and UV spectrum obtained from diode array detector with the retention times of standards substances. Results represent the mean ± SEM of two experiments performed by triplicate. *$P \leq 0.05$ respect caffeine obtained in Nor.

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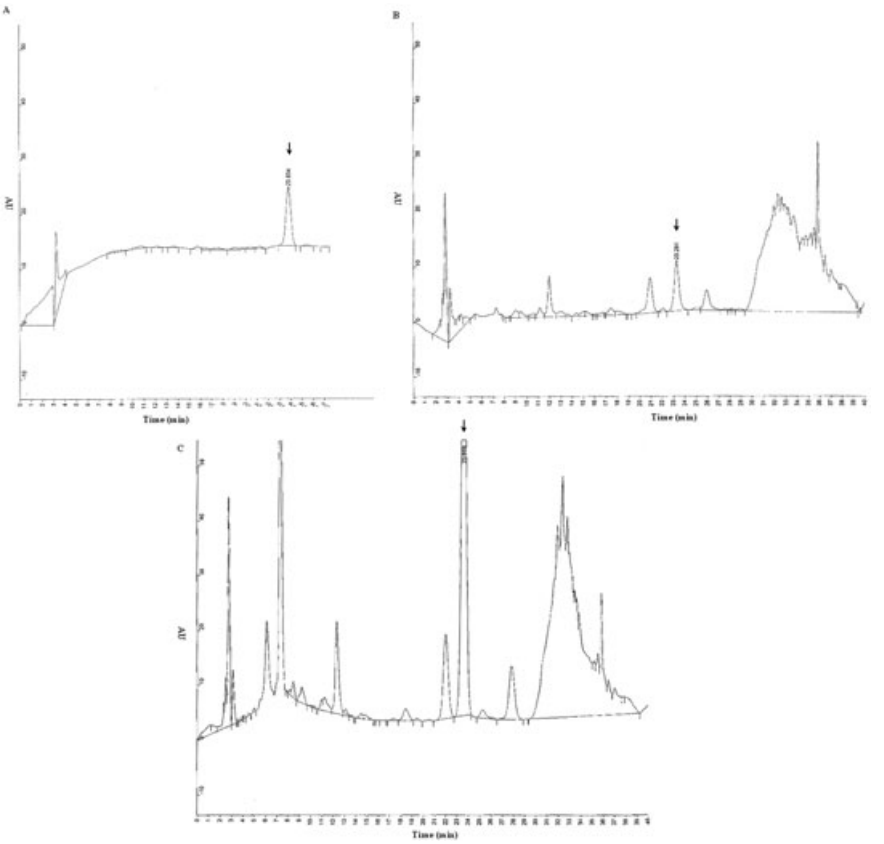

FIG. 1. QUANTIFICATION OF CA IN COFFEE EXTRACTS USING HPLC. (A) CHROMATOGRAM OF CA USED AS CONTROL. (B) CHROMATOGRAM OF THE DEC C) CHROMATOGRAM OF NOR. FOR SAMPLES AND THE CA STANDARD THE SAME RUNNING CONDITIONS WERE USED

with respect to control (Fig. 3A–C; Table 2). Dec and Nor at 1,000 µg/mL induced MTT reduction in Mφ after 1 h of treatment (Fig. 3D,E; Table 2) ($P \leq 0.05$). Ca did not modify the levels of MTT reduced with respect to control in Mφ (Fig. 3F; Table 2). After 24 h treatment, Nor induced significantly MTT reduction at 10, 100 and 1,000 µg/mL in lymphocytes ($P \leq 0.05$) (Fig. 4A; Table 2). MTT reduction was induced by Dec ($P \leq 0.05$) in all tested concentrations (Fig. 4B; Table 2). Dec produced significantly ($P \leq 0.05$) higher levels of reduced MTT than Nor (Fig. 4A,B; Table 2). Ca did not induce increase in reduced MTT level (Fig. 4C; Table 2). The treatment of Mφ with Nor, Dec and Ca during 24 h did not induce MTT reduction (Fig. 4D–F; Table 2).

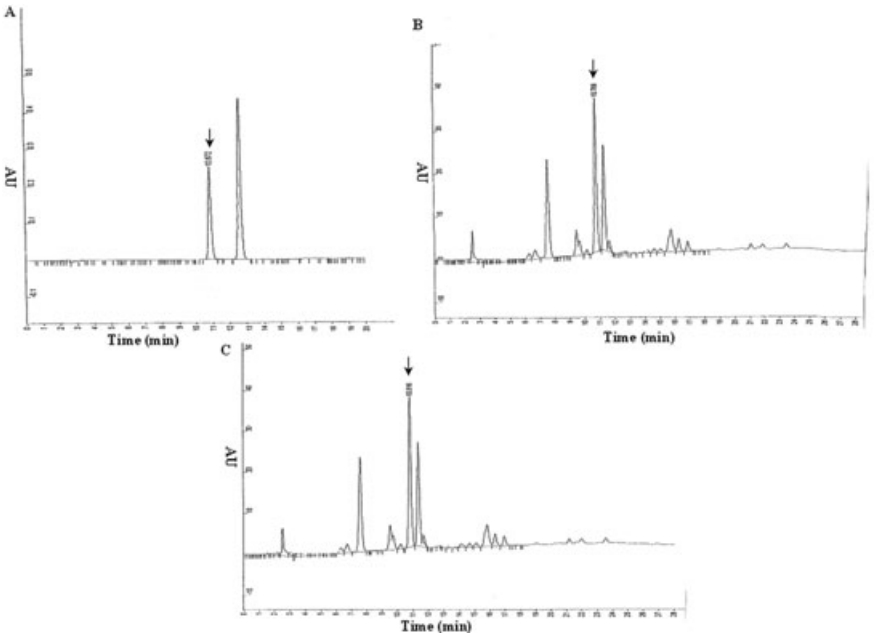

FIG. 2. QUANTIFICATION OF CGA IN COFFEE EXTRACTS USING HPLC. (A). CHROMATOGRAM OF CGA USED AS CONTROL. (B) CHROMATOGRAM OF THE DEC (C) CHROMATOGRAM OF NOR. THE CGA CONTENT REPRESENTS ≅ 0.047% W/V. FOR SAMPLES AND CGA STANDARD THE SAME RUNNING CONDITIONS WERE USED

Production of Superoxide

Taking into account that the extracts induced MTT reduction and that this increase can be related to the superoxide anion production, the effect of the extracts and Ca was studied on O_2^- production. The results showed that after 24 h of treatment, Nor induced significantly superoxide production at 10, 100 and 1,000 µg/mL in lymphocytes ($P \leq 0.05$) (Fig. 5A; Table 2). Dec induced superoxide production ($P \leq 0.05$) in all tested concentrations starting from 1 µg/mL (Fig. 5B; Table 2). Dec produced significantly ($P \leq 0.05$) higher levels of superoxide than Nor (Fig. 5A,B; Table 2). Ca did not induce increase of superoxide production (Fig. 5C; Table 2).

Proliferation Assay

On cell proliferation, our results showed that Nor decreased significantly ($P \leq 0.05$) proliferation on lymphocytes at 1 and 1,000 µg/mL (Fig. 6A). Dec

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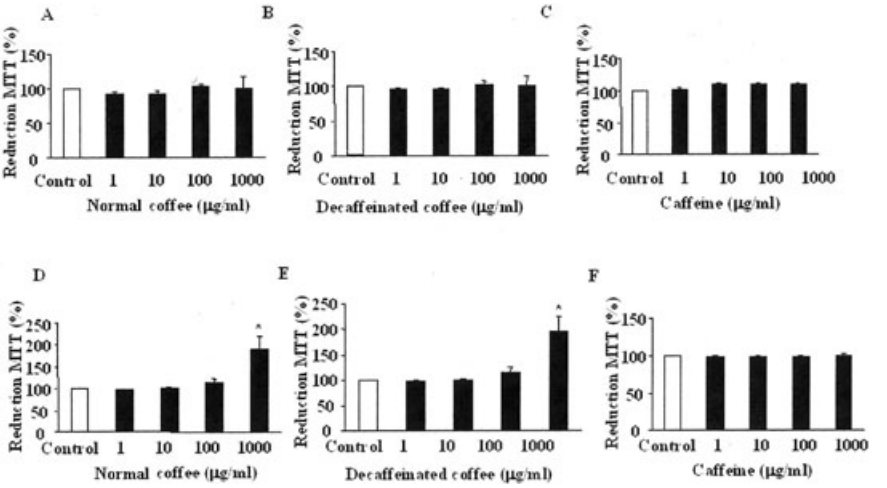

FIG. 3. EFFECT OF NOR, DEC AND CA ON REDUCTION OF MTT. NORMAL MOUSE LYMPHOCYTES (A–C) AND PERITONEAL MACROPHAGES (D–F) WERE INCUBATED 1 H WITH NOR (A,D), DEC (B,E) AND CA (C,F). RESULTS ARE EXPRESSED AS % OF REDUCED MTT WITH RESPECT TO THE CONTROL (□). RESULTS REPRESENTED THE MEAN ± SEM OF THREE EXPERIMENTS PERFORMED IN TRIPLICATE. *$P < 0.05$ SIGNIFICANTLY DIFFERENCES BETWEEN BASAL VALUES AND TREATMENT ACCORDINGLY TO ANOVA PLUS DUNNETT'S TEST

decreased ($P \leq 0.05$) proliferation with respect control in all used concentrations (Fig. 6B). Ca treatment enhanced proliferation ($P \leq 0.05$) on lymphocytes at all concentrations analyzed (Fig. 6C). Dec only decreased significantly cell proliferation with respect Nor at 10, 100 and 1,000 µg/mL ($P \leq 0.05$) (Fig. 6A,B). Dec decreased proliferation with respect to Ca in all used concentrations ($P \leq 0.05$) (Fig. 6B,C). Since cell proliferation was higher increased by 1 and 10 µg/mL of Ca, we assayed the effect of this compound at concentrations of 0.1, 1 and 10 µg/mL on proliferation of lymphocytes treated with CONA at 0.0652 and 0.125 µg/mL. The treatment with Ca and CONA at 0.0652 µg/mL increased significantly ($P \leq 0.05$) the proliferation starting at 1 µg/mL, while the treatment with Ca and 0.125 µg/mL of CONA increased proliferation at all used concentrations ($P \leq 0.05$) (Fig. 6D). Taking into account the stimulatory effect of Ca on cell proliferation and with the aim to relate this effect with an antioxidant action, we assayed the effect of Ca at 1.5 µg/mL and Nor at 10 and 100 µg/mL in a model of stress state induced in lymphocytes by H_2O_2. It can be seen that H_2O_2 decreased cell proliferation, but Ca and Nor reverted the effect of H_2O_2 ($P \leq 0.05$) (Figs. 6 E and 7).

TABLE 2.
EFFECT OF NOR, DEC AND CA ON REDUCTION OF MTT AND NBT

A

Reduction of MTT (%)

1 h

	C	Nor (μg/mL)				Dec (μg/mL)				Ca (μg/mL)			
Mφ		1	10	100	1,000	1	10	100	1,000	1	10	100	1,000
	99.9 ± 0.07	97.5 ± 1.1	99.8 ± 1.96	112 ± 8.07	189.4* ± 29.0	98.1 ± 1.57	101 ± 1.71	114 ± 10.0	195.1*± 30.0	98.5 ± 1.00	97.8 ± 1.21	98.2 ± 1.40	100.6 ± 1.64
L	99.9 ± 0.07	92.1 ± 2.5	92.3 ± 3.4	102 ± 4.4	100.1 ± 16.9	95.8 ± 2.08	96.5 ± 1.5	102 ± 5.57	101.6 ± 13.6	101 ± 2.6	109 ± 2.6	110.8 ± 0.919	109.8 ± 1.25

Reduction of MTT (%)

24 h

	C	Nor (μg/mL)				Dec (μg/mL)				Ca (μg/mL)			
Mφ		1	10	100	1,000	1	10	100	1000	1	10	100	1000
	99.5 ± 0.07	98 ± 1.1	93 ± 1.05	81 ± 2.91	101 ± 3.8	99.2 ± 0.5	97 ± 3.2	98.0 ± 1.3	101.6 ± 3.3	98.4 ± 1.00	95.5 ± 3.7	97.7 ± 1.06	101.1 ± 2.02
L	99.5 ± 0.07	110 ± 5	122* ± 4.6	145* ± 5.2	158* ± 5.1	120*,**,***± 4.83	133*,**,*** ± 12.7	170.4*,**,*** ± 18.3	177*,**,*** ± 5.8	102 ± 2.8	110 ± 2.5	110.3 ± 0.7	110.8 ± 1.7

B

NBT red/10^6 cells

24 h

	C	Nor (μg/mL)				Dec (μg/mL)				Ca (μg/mL)			
L	22.9 ± 0.1	1	10	100	1,000	1	10	100	1,000	1	10	100	1,000
		24.2 ± 1.45	25.8* ± 0.87	27.3* ± 1.65	34.2* ± 1.6	27.3*,**,*** ± 1.12	30.3*,**,*** ± 2.89	38.8*,**,*** ± 4.10	40.2*,**,*** ± 1.31	23.4 ± 0.63	24.2 ± 1.82	25.3 ± 0.16	25.4 ± 0.40

Results represent the mean ± SEM of two experiments performed by triplicate.
* $P < 0.05$ significantly differences between control values and treatment accordingly to ANOVA plus Dunnett's test.
** $P < 0.05$ significantly differences between Dec and Nor values accordingly to ANOVA plus Dunnett's test.
*** $P < 0.05$ significantly differences between Dec and Ca values accordingly to ANOVA plus Dunnett's test.
C, control; Ca, caffeine; Dec, decaffeinated coffee; L, lymphocytes; Mφ, macrophages; Nor, normal coffee.

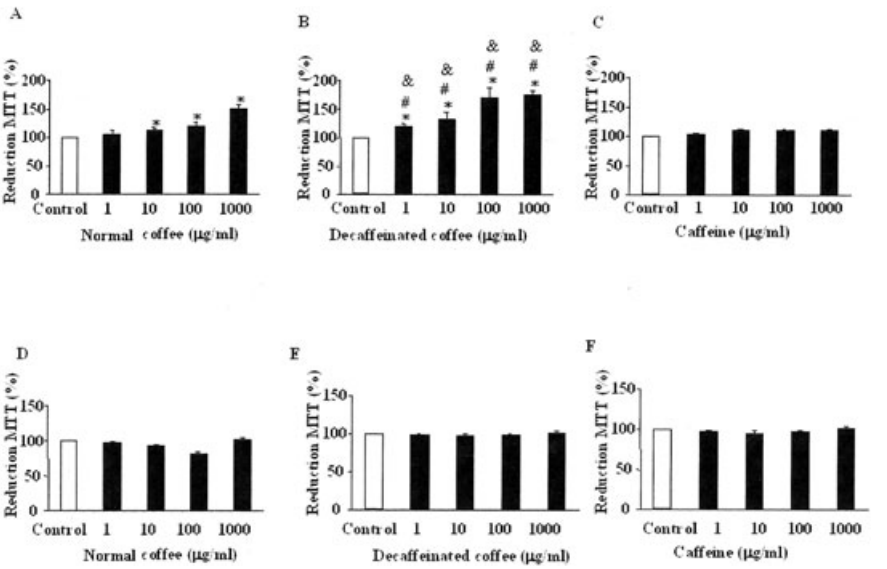

FIG. 4. EFFECT OF NOR, DEC AND CA ON REDUCTION OF MTT. NORMAL MOUSE LYMPHOCYTES (A–C) AND PERITONEAL MACROPHAGES (D–F) WERE INCUBATED 24 H WITH NOR (A,D), DEC (B,E) AND CA (C,F). RESULTS ARE EXPRESSED AS % OF REDUCED MTT WITH RESPECT TO THE CONTROL (□). RESULTS REPRESENTED THE MEAN ± SEM OF THREE EXPERIMENTS PERFORMED IN TRIPLICATE. *$P < 0.05$ SIGNIFICANTLY DIFFERENCES BETWEEN CONTROL VALUES AND TREATMENT ACCORDINGLY TO ANOVA PLUS DUNNETT'S TEST. #$P < 0.05$ SIGNIFICANTLY DIFFERENCES BETWEEN DEC AND NOR VALUES ACCORDINGLY TO ANOVA PLUS DUNNETT'S TEST. & $P < 0.05$ SIGNIFICANTLY DIFFERENCES BETWEEN DEC AND CA VALUES ACCORDINGLY TO ANOVA PLUS DUNNETT'S TEST

Catalase and SOD Activity

Bearing in mind that Ca did not modify the level of reduced MTT and NBT in lymphocytes, and that it reverted the antiproliferative effect of H_2O_2, we suspect that Ca could have an antioxidant activity. Therefore, the antioxidant effect of Ca related to the modulation of O_2^- and H_2O_2 was studied, determining the catalase (CAT) and SOD activities. Ca exhibited SOD activity starting at 750 µg/mL with a maximum value at 10,000 µg/mL (Fig. 8A). The concentrations of Ca in Nor at 1, 10, 100 and 1,000 µg/mL were 0.015, 0.15, 1.5 and 15 µg/mL. We assume that in the presence of other compounds, the effect of Ca may be observed at lower concentrations by a synergistic action. For this reason, we assayed the SOD activity of CGA (1–2,500 µg/mL). CGA showed SOD activity starting at 5 µg/m (Fig. 9A). In used concentrations of Nor (1, 10, 100, 1,000 µg/mL), we founded 0.0045, 0.045, 0.45 and 4.5 µg/mL

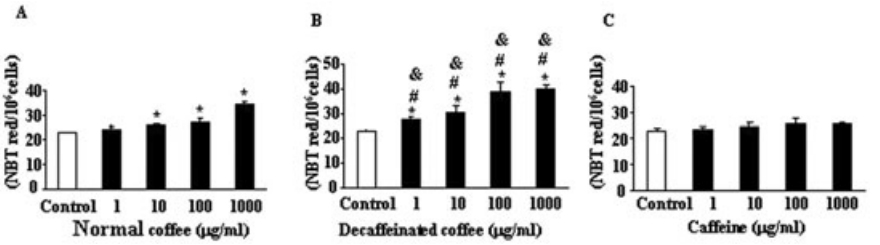

FIG. 5. EFFECT OF NOR, DEC AND CA ON THE SUPEROXIDE PRODUCTION BY LYMPHOCYTES. LYMPHOCYTES OF MICE WERE INCUBATED FOR 24 H AT 37C WITH NOR (A), DEC (B) AND CA (C). AS CONTROLS, UNTREATED CELLS WERE USED. THE PRODUCTION OF SUPEROXIDE ANION WAS ASSESSED BY THE REDUCTION OF NITRO BLUE TETRAZOLIUM (NBT) TO THE INSOLUBLE FORMAZAN. EXPERIMENTS WERE PERFORMED BY TRIPLICATE AND ONE REPRESENTATIVE IS SHOWN. *$P < 0.05$ SIGNIFICANTLY DIFFERENCES BETWEEN CONTROL VALUES AND TREATMENT ACCORDINGLY TO ANOVA PLUS DUNNETT'S TEST. #$P < 0.05$ SIGNIFICANTLY DIFFERENCES BETWEEN DEC AND NOR VALUES ACCORDINGLY TO ANOVA PLUS DUNNETT'S TEST. & $P < 0.05$ SIGNIFICANTLY DIFFERENCES BETWEEN DEC AND CA VALUES ACCORDINGLY TO ANOVA PLUS DUNNETT'S TEST

of CGA, respectively, by which, this compound alone did not possess SOD activity, but could potentiate the effect of Ca or other compounds. In this sense, we assayed the SOD activity of Nor in a selected concentration (800 µg/mL). We observed that Nor showed an elevated SOD activity (Fig. 9B). We selected 800 µg/mL of Nor because is a medium concentration between 100 and 1,000 µg/mL. As shown in Fig. 8B, Ca possessed CAT activity starting at 1 µg/mL with the highest effect at 500 µg/mL. Nor showed CAT activity in all assayed concentrations (Fig. 10).

DISCUSSION

In this work, the protective effect of Ca on cells related to the immune system such as lymphocytes and macrophages was demonstrated. In order to determine the difference in Ca amount present in both coffee extract, HPLC analysis of Nor and Dec was done. Nor extract presented 14 times more Ca than Dec extract (Table 1, Fig. 1), but no difference in chlorogenic content was found (Table 1, Fig. 2). These results are in accordance with those presented in the literature which showed that Dec extracts possess Ca and CGA in similar quantities that was founded by us (Blauch and Tarka 2006; Fujioka and Shibamoto 2008).

In the first approach, the effect of Nor and Dec on cell viability was evaluated in comparison with Ca. Cell viability was studied on lymphocytes

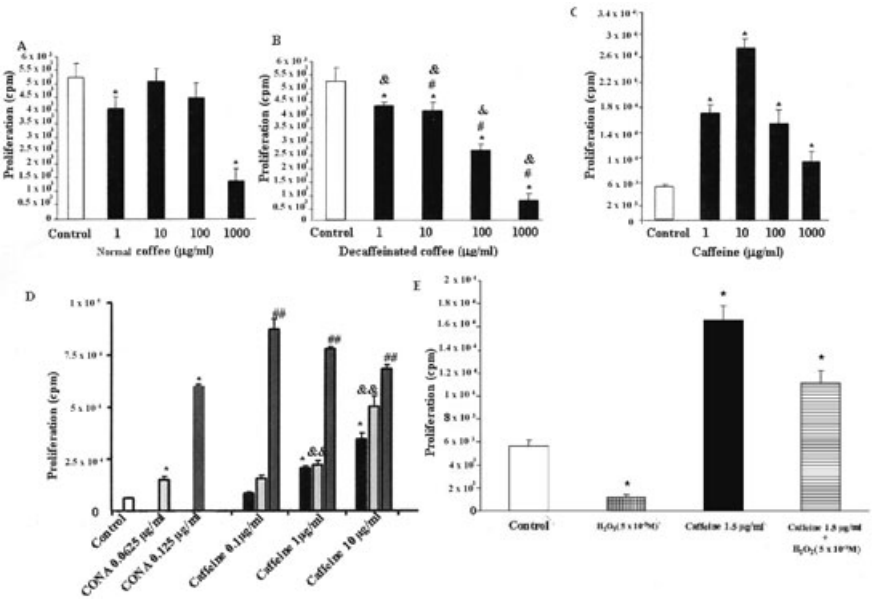

FIG. 6. EFFECT OF DIFFERENT CONCENTRATIONS OF DEC, NOR AND CA ON CELL PROLIFERATION AFTER 24 H INCUBATION. NORMAL LYMPHOCYTES (□) WERE TREATED WITH NOR (A), DEC (B) AND CA (C). IN D, CELLS WERE INCUBATED WITH CA IN PRESENCE OR ABSENCE OF CONA. TO DETERMINATE THE EFFECT OF CA ON PROLIFERATION, A MODEL OF STRESS INDUCED WITH H_2O_2 WAS USED (E). LYMPHOCYTES (□) WERE TREATED DURING 24 H WITH H_2O_2 (5×10^{-3} M) (▦), CA (1.5 µg/mL) (■) AND CA PLUS H_2O_2 (5×10^{-3} M) (▤). RESULTS WERE EXPRESSED AS MEAN ± SEM OF THREE EXPERIMENTS MADE BY TRIPLICATE. * $P < 0.05$ SIGNIFICANTLY DIFFERENCES RESPECT TO NORMAL BASAL VALUE. # $P < 0.05$ SIGNIFICANTLY DIFFERENCES BETWEEN DEC VALUE AND NOR TREATMENT. & $P < 0.05$ SIGNIFICANTLY DIFFERENCES BETWEEN DEC AND CA VALUES. ## $P < 0.05$ SIGNIFICANTLY DIFFERENCES BETWEEN CONA (0.125 MG/ML) VALUES AND CONA (0.125 MG/ML) PLUS CA. $^{\&\,\&}$ $P < 0.05$ SIGNIFICANTLY DIFFERENCES BETWEEN CONA (0.0625 MG/ML) VALUES AND CONA (0.0625 MG/ML) PLUS CA

and on macrophages. Neither of the samples assayed decreased cell viability, but an increase in MTT reduction was registered in lymphocytes and in macrophages (Figs. 3 and 4; Table 2). Since MTT is a membrane-permeable tetrazolium salt (Knight and Dancis 2006), which is reduced to formazan by superoxide anion in mitochondria or intracellular vesicles (Madesh and Balasubramanian 1997; Bernhard *et al*. 2003), it can be supposed that the amount of reduced MTT could be related to an increase of superoxide anion level. Therefore, it could be suggested that Ca was related with the differences in MTT reduction observed in lymphocytes, but exerting the effect associated with other compounds present in the extract. This last action was in order to

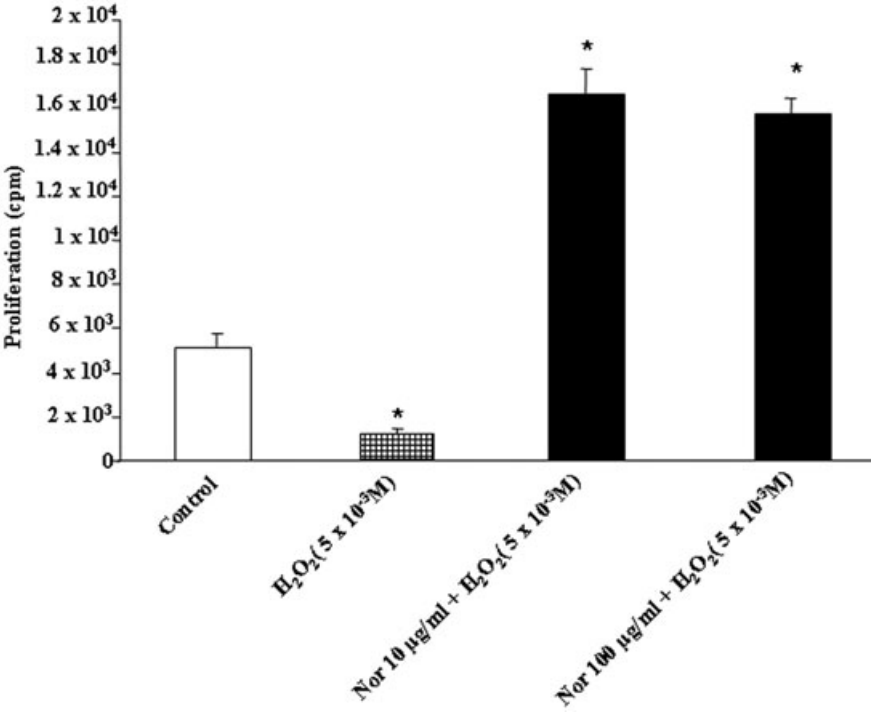

FIG. 7. EFFECT OF DIFFERENT CONCENTRATIONS OF NOR AND H_2O_2 ON CELL PROLIFERATION AFTER 24 H INCUBATION. LYMPHOCYTES (□) WERE TREATED DURING 24 H WITH H_2O_2 (5×10^{-3} M) (⊞) AND NOR (■) AT 10 AND 100 µg/mL. RESULTS WERE EXPRESSED AS MEAN ± SEM OF THREE EXPERIMENTS MADE BY TRIPLICATE. *$P < 0.05$ SIGNIFICANTLY DIFFERENCES RESPECT TO NORMAL BASAL VALUE

eliminate the superoxide anion. Contrarily, MTT reduction in Mφ could be independent of Ca. In accord with the obtained results, the different effects observed in lymphocytes and Mφ would be due to the action of different compounds in the extracts.

In order to confirm that the increase in MTT was related to the superoxide production, this anion was measured in the presence of Nor, Dec and Ca in lymphocytes. Taking into account the obtained results (Fig. 5, Table 2), it can be suggested that Ca could protect the cells of superoxide effect. The protective effect of Ca was also observed on lymphocytes proliferation (Fig. 6). The difference observed between Nor and Dec in the antiproliferative actions could be due to the difference in Ca content; in Nor extract, when concentration was 1 µg/mL, the Ca amount was very low (0.015 µg) and this compound did not affect the proliferation. When the extract concentration was increased (10,

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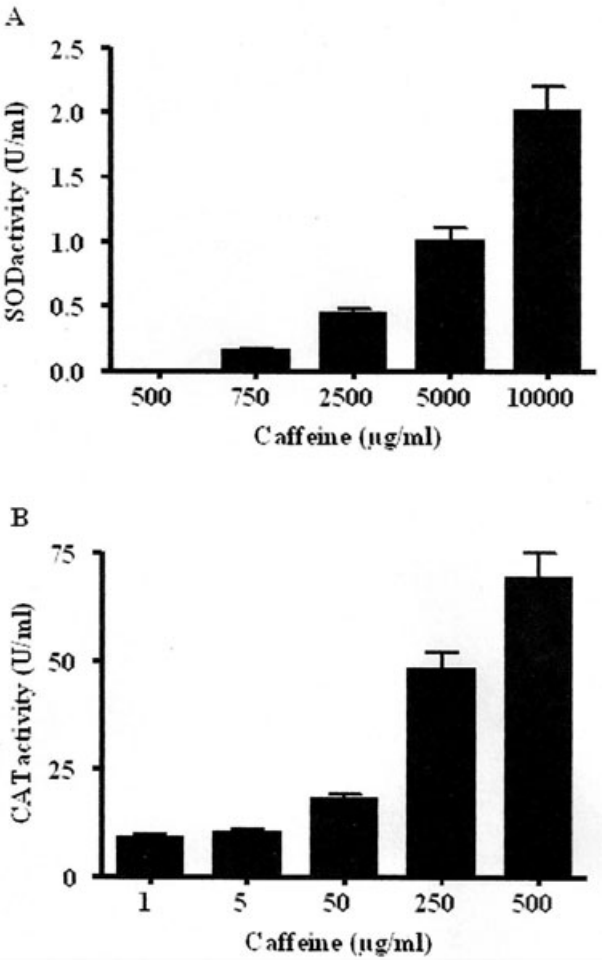

FIG. 8. CAT AND SOD ACTIVITY OF CA WERE EVALUATED. SOD AND CAT ACTIVITY WERE DETERMINED ON CA (A,B). RESULTS REPRESENT THE MEAN ± SEM OF THREE DETERMINATIONS PERFORMED BY TRIPLICATE

100 µg/mL), Ca concentration increased, too (0.15–1.5 µg), and exerted the proliferative effect. Meanwhile, in Dec extract, the lowest concentration of Ca allowed the others antiproliferative compounds to exert its action. It is important to note that, the results obtained in stimulated lymphocytes stimulated and unstimulated with CONA (Fig. 6D) confirmed that Ca increased proliferation as polyclonal mitogen.

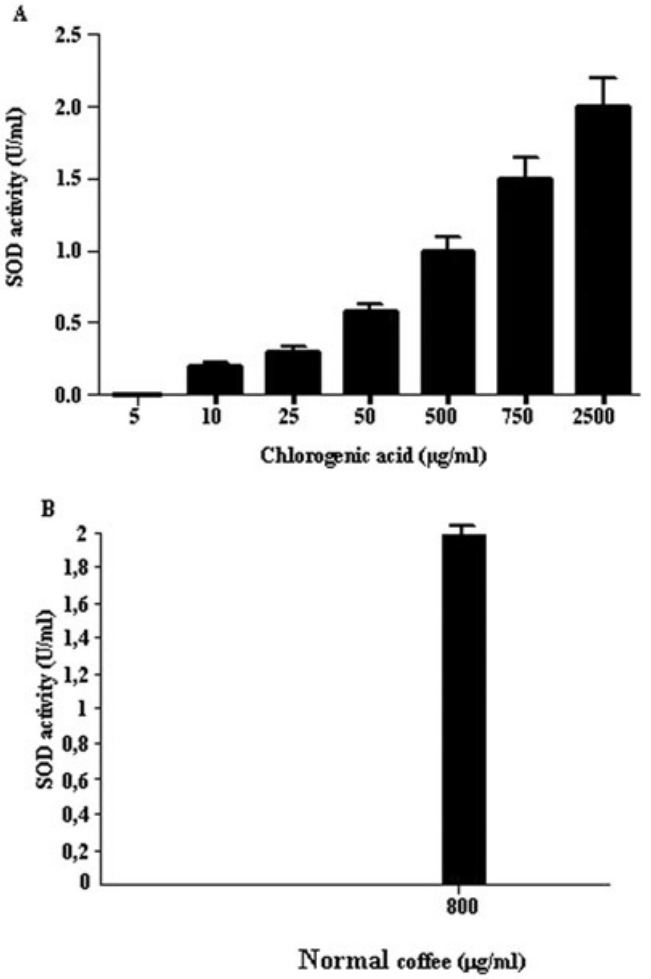

FIG. 9. SOD ACTIVITY OF CGA AND NOR WAS EVALUATED. SOD ACTIVITY WAS DETERMINED ON CGA (1, 2.5, 5, 10, 25, 50, 500, 750, 2,500 µg/mL) (A) AND NOR (800 µg/mL) (B). RESULTS REPRESENT THE MEAN ± SEM OF THREE DETERMINATIONS PERFORMED BY TRIPLICATE

In the other hand, Ca alone has a proliferative effect in both H_2O_2-treated or -untreated lymphocytes ($P \leq 0.05$) (Fig. 6E), suggesting an antioxidant action related to H_2O_2 elimination. Also, Nor presented a protective effect on H_2O_2 stressed cells, suggesting the participation of Ca in this response (Fig. 7). The protective action on stressed cells was related to the elimination of H_2O_2 through CAT activity "per se," exerted by both Ca and Nor. Nevertheless, other

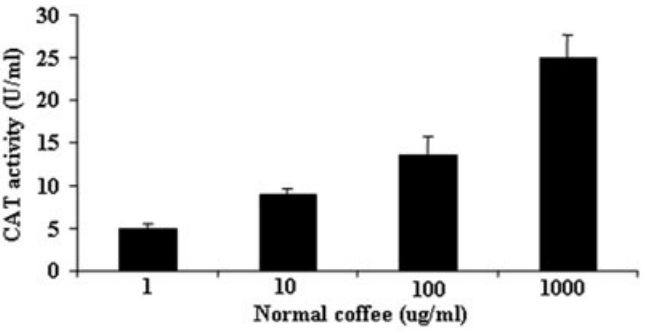

FIG. 10. CAT ACTIVITY OF NOR WERE EVALUATED. RESULTS REPRESENT THE MEAN ± SEM OF THREE DETERMINATIONS PERFORMED BY TRIPLICATE

compounds different from Ca were involved in the effect of low concentrations of Nor, but at high concentrations, Ca appeared to be involved in this action. At low concentrations of Nor, low concentrations of Ca were found without CAT activity.

The fact that Nor and Ca possessed CAT activity contributed to the maintenance of cell proliferation and also to the increase in cell proliferation exerted by Ca, as it is described that H_2O_2 is associated with DNA damage, mutations and genetic instability (Park *et al*. 2005). In addition, H_2O_2 is considered to be an efficient inductor of apoptosis (Hipara *et al*. 2004)

n some other way, Ca presented SOD activity (related to superoxide elimination) only at higher concentrations. These concentrations are not present in Nor, in our assay conditions (Fig. 8A). Besides, Nor showed elevated SOD activity (Fig. 9B). This mean that Ca probably needs to be associated with others compounds to exert antioxidant effects at low concentrations. So, again, this demonstrated that, the presence of Ca is important to protect the cells but in combination with other antioxidant compounds. One of the implicated compounds would be CGA (≅0.049 g % w/v) (Table 1); this compound showed important antioxidant effects (Kono *et al*. 1997), although in our assays did not possess SOD activity in the concentration present in the extract (Fig. 9A). It could be able to associate with Ca to exert its effects. The experiments to confirm this observation will be assayed at soon.

Superoxide and hydroxyl radicals are the two most representative reactive oxygen species (ROS). ROS have harmful and destructive effects on cell membranes and inhibit cell proliferation (Fridovich 1978; McCord 1985; Halliwell and Guttridge 1989). On the other hand, some authors showed that the activation of T lymphocytes cells is redox dependent, and recent studies have shown that its activation induces rapid production of ROS. The activation of T lymphocytes is related with apoptosis in a normal and physiological

process. The controlled deletion of activated T cells that occurs upon stimulation is one mechanism for maintaining T cell equilibrium during an immune response and is termed activation-induced cell death. Besides, it is known that antioxidants inhibit both "life" (activation) and "death" of stimulated T cells. Thus, manipulating the concentration of oxygen and, consequently, the production of oxygen-derived radicals can affect T cell survival and/or differentiation (Williams and Kwon 2004). According to the obtained results (Fig. 5; Table 2), we can suggest that some compounds in Dec could be activating the T lymphocytes. This activation would be able to produce a disorder in the delicate redox equilibrium and lead cells to death. The compounds responsible for these effects could be acting as a polyclonal stimulant. It activates cells through interaction with membrane glycoproteins including those involved in the immunological synapse (Toscano *et al*. 2006).

In conclusion, in relation to the obtained results upon MTT and NBT reduction, proliferative effects and CAT activity of Ca, we can suggest an important antioxidant action of this compound. Ca presented SOD activity at concentrations not present in Nor in our assay conditions. As well as, Nor showed elevated SOD activity also exerted CAT activities. Therefore, Ca could act as eliminating hydrogen peroxide alone or in combination with other compound or compounds, protecting cell from death. Then, although Ca presents some negative properties (Klein and Salzman 1975; Bolton and Null 1981; Van De Sandt *et al*. 2004), according to our results, it could be more favorable to drink Nor than Dec for maintenance of normal or improved redox equilibrium in cells.

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