Research Article

Antioxidant Effect of *Cannabis sativa* L. Resin Associated with *Larrea divaricata* Cav. Extract: Synergism, Additivity and Antagonism

Malén Saint Martin, Carla Marrassini, Fresia M. Silva Sofrás, Ignacio Peralta, Laura Cogoi, Catalina van Baren, María R. Alonso, Claudia Anesini*

Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Instituto de Química y Metabolismo del Fármaco (IQUIMEFA), Cátedra de Farmacognosia, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires. Cuidad Autónoma de Buenos Aires, Argentina.

***Corresponding author's E-mail:** canesini@yahoo.com.ar

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ABSTRACT

Cannabis sativa L. (Cannabaceae) is used as anticonvulsant in refractory epilepsy. Convulsions induce oxidative stress in the brain. Synergistic interactions between plants could improve the therapeutic and reduce adverse effects. *Larrea divaricata* Cav.'s (Zygophylliaceae) antioxidant activity is well-documented. The aim of this work was to evaluate the antioxidant activity of an ethanolic extract of *C. sativa* (CSR), study cannabidiol's (CBD) participation and evaluate the possible synergistic effect of CSR with an aqueous extract of *L. divaricata* (LE). Antioxidant activity was determined by the 1,1-diphenyl-2-picryhydrazyl (DPPH) and the lipid peroxidation assays. For the DPPH assay, the inhibitory concentration 50 (IC_{50}) were: CSR=19.82 ±1.47 µg/ml, LE=24.18±0.54µg/ml, CBD=12.49 ± 0.79µg/ml. The IC50 values for the combinations: CSR + LE 25 µg/ml=2.91±0.10µg/ml ($p<0.001$), CBD + LE 25 µg/ml=4.19±0.11µg/ml ($p<0.01$). Combination indexes (CI) were: 15 µg/ml CSR / 25 µg/ml LE=0.38 (significant synergism), 10 µg/ml CBD / 25 µg/ml LE=0.39 (significant synergism). IC_{50} values for the lipid peroxidation assay: CSR=30.50±3.00µg/ml, LE=630.95±63.00µg/ml, CBD=10.20±1.00µg/ml. The IC_{50} values of the best combinations: CSR + 500 µg/ml LE=2.39±0.10µg/ml ($p< 0.0001$), CBD + 500 µg/ml LE=2.19±0.18 µg/ml ($p<0.0001$); CI values: 10 µg/ml CSR /500 µg/ml LE=0.16 (strong synergism), 3 µg/ml CSR /500 µg/ml LE=0.36 (significant synergism). Conclusions: CBD is involved in CSR antioxidant activity. LE acted synergistically with CSR in both assays. CSR-LE association at optimal concentrations could be a good option to reach a significant synergism (CI<1). At low doses, the combination of CSR and LE could be used as co-adjuvant for the treatment of epilepsy to manage oxidative stress.

Keywords: *Cannabis sativa*, *Larrea divaricata*, antioxidant activity, combination index.

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INTRODUCTION

Cannabis sativa L. (marihuana) is a dioicous plant of the Cannabaceae family widely distributed worldwide that has been used as a psychoactive drug, as a folk medicine ingredient and as a source of textile fiber since ancient times [1].

Nowadays, *C. sativa* is used as a palliative therapy for several diseases or co-administered with the primary therapy [2]. It is used to treat chronic pain, fibromyalgia, depression, arthritis, neuropathy [3, 4, 5] and inflammatory bowel disease [6]. Moreover, medicinal *Cannabis* and its derivatives are used in the treatment of drug-resistant epilepsy, which occurs in around one-third of the patients with epilepsy, especially infants [7]. It is known that seizures caused by epilepsy induce the production of oxidative stress in the brain tissue causing inflammation, which in turn affects the central nervous system altering cognitive functioning. It is reported that the generation of oxidative and nitrosative stress are regarded as possible mechanisms in the pathogenesis of epilepsy [8]. Antioxidants and free radical scavengers could potentially be used as a neuroprotective co-adjuvant therapy in the treatment of epilepsy [9]. Plants are a good resource of efficacious antioxidant compounds, such as polyphenols, with low adverse effects.

C. sativa is characterized by a complex chemical composition, including phenolic compounds, terpenes, carbohydrates, fatty acids and their esters, amides, amines, phytosterols and the specific compounds of this plant, namely the cannabinoids, which are terpenophenolic compounds [1]. CBD, one of the major cannabinoids in *C. sativa*, has been found to have high antioxidant and anti-inflammatory activities, together with antibiotic, neuroprotective, anxiolytic and anticonvulsant properties. In fact, it has been recently approved for the treatment of some refractory syndromes, such as Dravet and Lennox-Gastaut syndromes despite the adverse effects that produces such as: vomiting, diarrhea, pyrexia, fatigue, somnolence, lethargy and abnormal results on liver-function tests [10, 11]. As well, CBD-rich extracts seem to present a better therapeutic profile than purified CBD in the population of patients with refractory epilepsy [12], although there is still a lack of controlled clinical trials to

confirm this. Moreover, a systematic review conducted by Pratt et al. states that adverse effects were reported in most reviews comparing cannabis with placebo, including short and long-term adverse effects such as sedation, dysphoria, memory impairment, depression, hallucinations, muscle relaxation and long-term immunosuppression [13]. Because of this, it would be advisable to lower the doses.

Taking into account the synergy and the mitigation of adverse effects that can be achieved with plants, it can be hypothesized that Cannabis could be combined with other extracts at low doses to improve its effects and mitigate adverse effects.

Larrea divaricata Cav. (Zygophyllaceae) is a South American autochthonous plant used in popular medicine mainly for its anti-inflammatory properties [14]. This plant has been reported to have antioxidant activity. In previous studies, it was demonstrated that an aqueous extract of *L. divaricata* presents antioxidant [15, 16] anti-proliferative, immunomodulatory and anti-microbial activities [17, 18, 19, 20].

It is known that the combination of antioxidants can produce additivity, synergism or even antagonism; therefore, combinations must be carefully studied by at least two different methods [21].

This study assesses, for the first time, the interaction between an ethanolic extract of *C. sativa* (CSR) and an aqueous extract of *L. divaricata* (LE) on antioxidant activities such as the scavenger activity on the free radical DPPH, as well as the inhibition of lipid peroxidation. All these assays were performed with the final aim of determining the lowest antioxidant concentrations of *C. sativa* to be used in association with LE without exerting antagonism. This combination could be used in the future as a co-adjuvant therapy in refractory epilepsy to manage oxidative stress and convulsions.

MATERIALS AND METHODS

Plant material and extract preparation

Leaves of *Larrea divaricata* Cav. were collected in the province of Córdoba, Argentina (Río Dolores district, Aguas Azules neighborhood (Sector B), Capilla del Monte, Punilla Department, province of Córdoba, Argentina; land register data: Dep. 23- Ped. 01- Pueblo 06- Circ. 05- Secc. 02- Manzana 054- Parcela 003). The plant was collected in January and identified by morphological, anatomical and histochemical criteria by Dr. Gustavo Giberti † and Dr. Hernán Gerónimo Bach from the Museum of Pharmacobotany, School of Pharmacy and Biochemistry, University of Buenos Aires. One voucher specimen of *L. divaricata* was deposited at the Museum of Pharmacobotany (BAFC no. 38).

The extract was prepared as a decoction according to the Argentinean National Pharmacopoeia (FNA) VII edition as follows: fifty g of air-dried leaves and 1000 ml of distilled water were boiled for 20 min. The extracts were then filtered and lyophilized. The final yield was 22.86 ± 0.244% of plant material.

Cannabis sativa var. CBD Skunk Haze inflorescences were provided by a local private cutter. The inflorescences presented 1:1 CBD:THC ratio. The plant was identified by morphological, anatomical and histochemical criteria by Dr. Hernán Gerónimo Bach from the Museum of Pharmacobotany, School of Pharmacy and Biochemistry, University of Buenos Aires. One voucher specimen was deposited at the Museum of Pharmacobotany. The resin was obtained from the female flowers, dried at 20 °C and 55 % humidity for 8 days, final humidity of the flower 11 %, through extraction in absolute ethanol for 10 min at 0 °C. The extract was filtered and the solvent was evaporated on a rotary evaporator at 40 °C. The resin obtained was stored at -20 °C until use. The final yield was 8.61 ± 0.59% (w/w) of plant material.

Phytochemical studies

Determination of total polyphenol and total flavonoids

The total polyphenols content was determined by spectrophotometry according to the Folin-Ciocalteu's method using gallic acid as standard. Briefly, the lyophilized LE extract was weighted and dissolved in distilled water and the CSR extract was dissolved in absolute ethanol. Five ml of the Folin-Ciocalteu's reagent diluted at 10% (v/v) were added to 1.0 ml of the extracts. After 3 to 8 min, 4 ml of a 7.5% (w/v) sodium carbonate solution were added. Solutions were then allowed to stand at room temperature for 60 min and then the absorbance at 765 nm was measured using an UV-vis spectrophotometer. The concentration of polyphenols in samples was derived from a standard curve of gallic acid ranging from 10 to 50 µg/ml (Pearson's correlation coefficient: $r^2 = 0.9996$). Results were expressed as % w/w (ISO 14502-1, 2005) [22].

Total flavonoids were also determined on the LE and CSR extracts according to Chang et al. (2002) [23]. Quercetin was used as a standard. Briefly, 0.5 ml of the diluted extracts or standard were mixed with 1.5 ml of either ethanol or distilled water, 0.1 ml of 10% (w/v) aluminum chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of ethanol or distilled water. After incubation at room temperature for 30 min the absorbance at 415 nm was measured using an UV-vis spectrophotometer. Results were expressed as %w/w.

Identification and quantification of CBD in Cannabis sativa extract by HPLC-UV

The CBD content was determined in CSR. An HPLC Agilent Infinity 1260 apparatus equipped with a UV detector and a Kinetex XB-C18 100A column (250 mm × 4.6 mm and 5 µ pd) was used. Samples were eluted at 35°C with a gradient of A: H_2O with 0.085% phosphoric acid and B: acetonitrile with 0.085% phosphoric acid at a flow rate of 1.6 ml/min according to the method of Mandrioli et al. (2019) [24], with slight modifications. The identification of CBD was carried

out by comparing the retention time and areas obtained with those of a commercial standard (CBD ENECTA batch: 0-156, 99%).

Identification of main compounds in Larrea divaricata by HPLC UV and HPLC MS/MS

NDGA was identified and quantified in *L. divaricata* extract by HPLC, which was performed in a Varian Pro Star instrument equipped with a Rheodyne injection valve (20 µl) and a photodiode array detector set at 280 nm. A reversed-phase AgilentZorbax Eclipse XDB-C18 (250 mm x 4.6 mm and 5 µ pd) column was used. Samples were eluted with a gradient of A: water:acetic acid (98:2) and B: methanol:acetic acid (98:2) from 15% B to 40% B in 30 min, 40% B to 75% B in 10 min, 75% B to 85% B in 5 min and 100% B in 5 min. Solution B (100%) was run for 10 min and back to initial conditions. The flow rate was 1.2 ml/min and the separation was done at 30ºC. Data were analyzed with a Varian Star 5.5 program (USA). Lyophilized aqueous extracts (10 mg/ml) and the pure standard were dissolved in methanol:water (70:30). The water employed to prepare the working solution was of ultrapure quality (Milli-Q). Methanol (J.T. Baker) and acetic acid (Merck, Argentina) were HPLC grade. A pure standard of NDGA (Sigma, USA, lot #BCBP3728V, 97%) was employed [16].

Flavonoids and phenolic acids were identified in *L. divaricata* by HPLC-MS/MS analysis, which was performed in UltiMate 3000 coupled to a TSQ Quantum Access MAX Triple Quadrupole mass spectrometer with an ESI ionization source (Massachusetts, USA). Calibration conditions: spray voltage 3.5 kV, vaporizer temperature 233°C, capillary temperature 314°C, sheath gas pressure and auxiliary gas were set at 10 and 45 units, respectively. The method consists of two events, single ion monitoring (SIM) and selective reaction monitoring (SRM). The equipment was operated in a positive mode for flavonoid glycosides and in a negative mode for phenolic acids. A reversed-phase Hypersil C18 (150 mm x 4.6 mm and 5 µ pd) was used. Samples were eluted with a gradient of A: water: 0.1% formic acid and B: methanol: 0.1% formic acid from 15% B to 40% B in 25 min; solution B (40%) was run for 25 min, 40% B to 85% B in 10 min and 100% B in 5 min. Solution B (100%) was run for 10 min and back to initial conditions. The flow rate was 1.0 ml/min.

Antioxidant activity

DPPH free radical scavenger activity

The antioxidant activity measured through the scavenging capacity of the free radical diphenyl-2-picrylhydrazyl (DPPH) was performed according to the methodology described by Blois (1958) [25]. Briefly, the LE extract was diluted in Milli-Q water, CSR and CBD were diluted in absolute ethanol in order to obtain solutions with final concentrations in the reaction tube ranging from 0.001 to 1000 µg/ml. NDGA was diluted in ethanol:water. Sample blanks were prepared using Milli-Q water. Vitamin C was used as the antioxidant reference standard. One hundred µl of each sample solution were placed in a vial. Four hundred µl of 100 mM Tris-hydrochloric buffer and 500 µl of a 500 µM DPPH solution in absolute ethanol were added. Samples were incubated in the dark for 20 min and the absorbance at 517 nm was measured. A DPPH control was prepared in100 mM Tris-hydrochloric buffer.

The antioxidant activity was determined by comparing the absorbance obtained with the reference solutions or the sample solutions to that obtained with the DPPH control. Results were expressed as percentage with respect to the control according to the following equation:

Radical Scavenger Act.: [(Control Abs.- Sample Abs.) / Control Abs.] X 100

Inhibition of lipid peroxidation: TBARS determination of egg yolk

The antioxidant activity was determined in an egg yolk phospholipid peroxidation model described by Dissanayake et al. (2009) [26], with modifications. Briefly, 25 µl of diluted extracts, with final concentrations in the reaction tube ranging from 0.1 to 1000 (CSR) to 2000 (LE) or to 100 (CBD) µg/ml, were mixed with 100 µl of distilled water and 125 µl of a 10% (v/v) egg yolk solution prepared in 1.15% (w/v) potassium chloride. A volume of 375 µl of a 20% solution of acetic acid and 375 µl of 1% (w/v) thiobarbituric acid (TBA) in 1.1% (w/v) sodium laureth sulfate (SD) solution were added. This mixture was kept in a water bath at 95°C for 90 min. A volume of 1.250 ml of butanol was added to each tube and vortexed for 10 sec. After centrifuging at 3000 rpm for 10 min, the absorbance of the butanol layer was measured at 532 nm in a UV-vis spectrophotometer. Butylhydroxytoluene (BHT) was used as the antioxidant reference standard. The percentage inhibition of peroxidation was calculated with the following equation:

$$\% \text{ Inhibition: } [(A_0 - A_s) / A_0] \times 100$$

where A_0 was the absorbance of the oxidation control and A_s was the sample absorbance.

The concentration of the extract able to inhibit 50% of the initial DPPH or phospholipid peroxidation (IC_{50}) was calculated in both methods from concentration-response curves using a mathematical method based upon the principles of a right-angled triangle:

$$IC_{50} = D - [(A - 50\% \text{ max response}). X] / Y$$

where A is the immediately higher response of 50% maximum response; B is the immediately lower response of 50% maximum response; D= log concentration corresponding to A response; C= log concentration corresponding to B response; X= D-C; Y= A-B [27].

Determination of combination index

To investigate the possible interaction between different extracts, an isobolographic analysis based on the median-effect principle was performed [28]. The combination index (CI) based on the classic isobologram equation was used for data analysis of a two-way combination:

$$CI = D_1/(DX)_1 + D_2/(DX)_2$$

where $(D)_1$ and $(D)_2$ are the IC_{50} values of extracts or CBD in the combination system, and $(Dx)_1$ and $(Dx)_2$ are the IC_{50} values of the extracts or CBD alone. CI>1, CI=1 and CI<1 represent antagonism, additivity and synergism, respectively.

Statistical analysis

The statistical analysis was done with three or more independent experiments performed in triplicate. In all cases, the mean and the standard error of the mean (SEM) were determined. The significance between the means was analyzed by a one-way ANOVA and the Dunnett's multiple comparison test, one-way ANOVA + Tukey's multiple comparison test or Student´s *t* test, as required. In all cases, the differences were considered significant when $p<0.05$.

RESULTS

Phytochemical composition

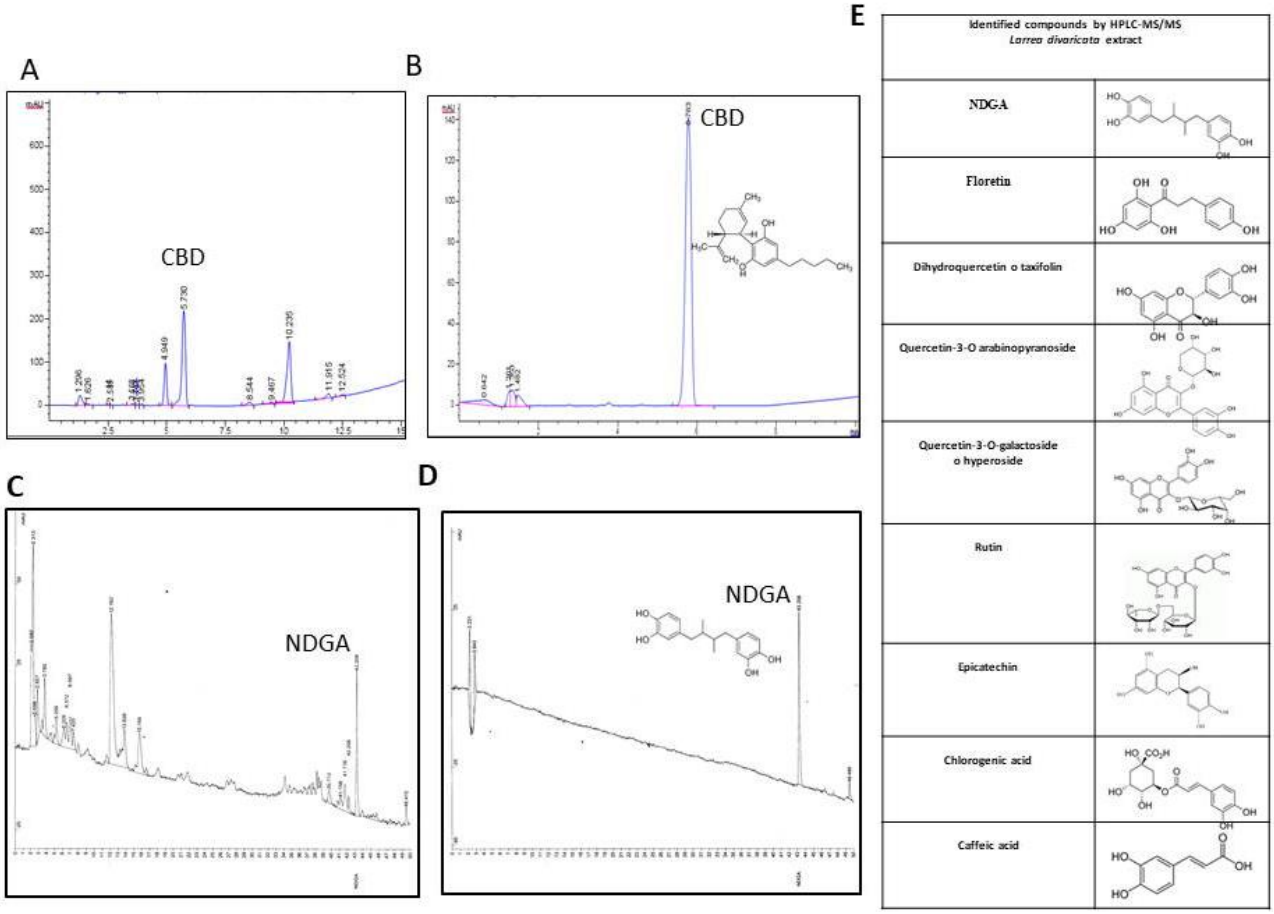
The CSR was subjected to HPLC to identify and quantify CBD. CBD was present at 23.13±0.01g% resin (Figure 1A and B). The amount of NDGA was determined in LE by HPLC-UV (Figure 1C and D). By HPLC-MS/MS, NDGA and other polyphenols were identified in LE, such as rutin, epicatechin, quercetin-3-O-arabinopyranoside and quercetin-3-O-galactoside, and the phenolic acids such as caffeic acid and chlorogenic acid (Figure 1E). Polyphenols and flavonoids were also quantified in the extracts (Table 1).

Table 1: Quantification of total polyphenols, flavonoids, CBD and NDGA in the extracts

Extracts	Total polyphenols (g%w/w)	Total flavonoids (g%w/w)	CBD (g%w/w)	NDGA (g%w/w)
Larrea divaricata	17.09 ± 0.28[a]	2.03 ± 0.05[a]	____	1.56 ± 0.08
Cannabis sativa	12.54 ± 0.79[b]	0.44 ± 0.02[b]	23.13 ±0.01	____

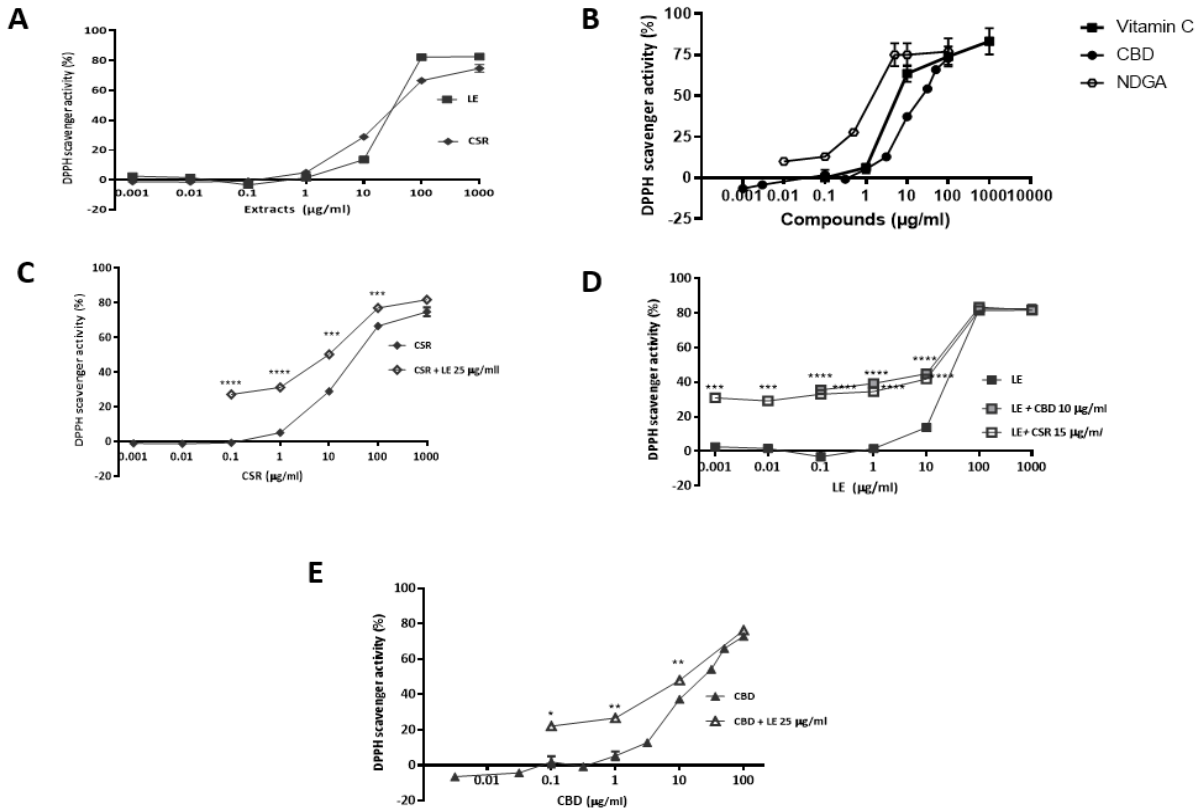
Results are expressed as mean ± SEM of three determinations. a,b,c significantly different (one way ANOVA+ Tukey's test)

Figure 1: HPLC-UV Chromatogram



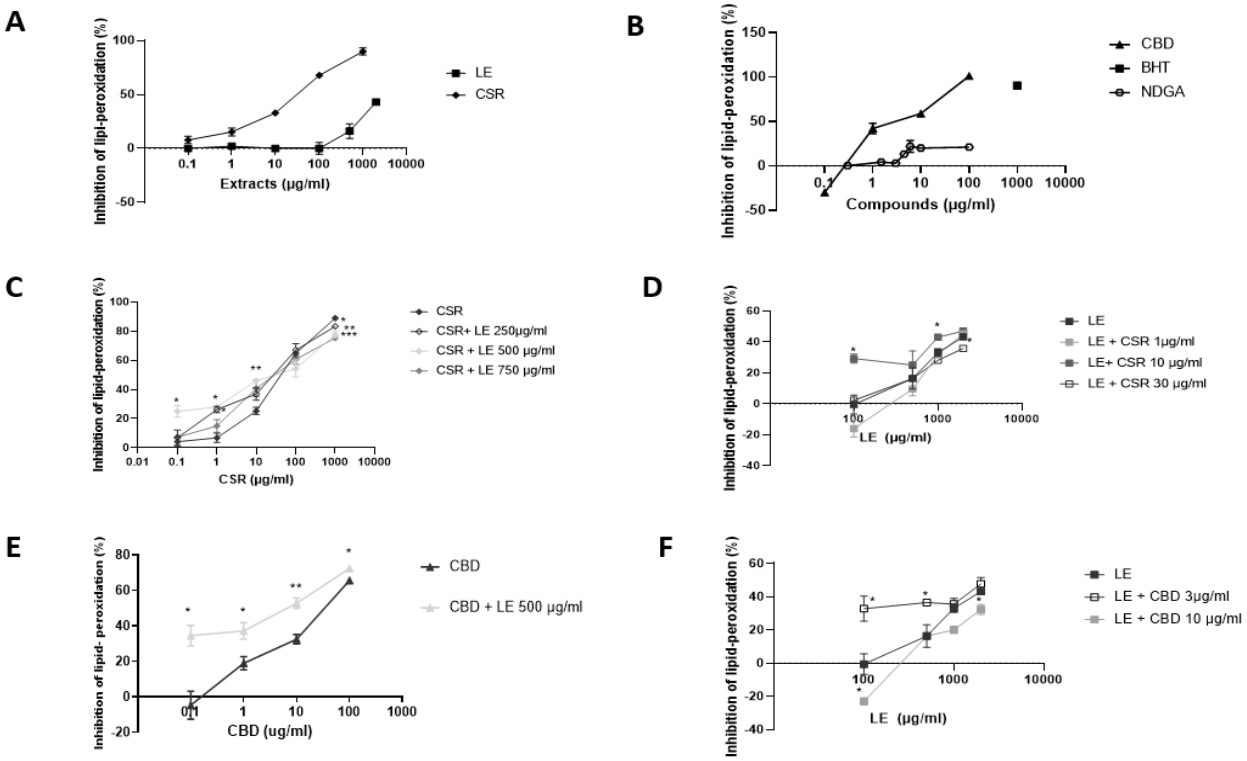
A: *C. sativa,* **B**: CBD, **C**: *L. divaricata* **D**: NDGA. **E**: *L. divaricata* compounds identified by HPLC MS/MS. A representative chromatogram of three determinations is shown.

Figure 2: DPPH scavenger activity



A: LE, CSR. **B:** Vitamin C (antioxidant control), NDGA and CBD. **C:** CSR, CSR+ 25 µg/ml LE. **D**: LE, LE+ 15 µg/ml CSR, LE + 10 µg/ml CBD **E:** CBD, CBD + 25 µg/ml LE. Results are expressed as mean ± SEM of three experiments performed in triplicate. LE: *L. divaricata* extract; CSR: *C. sativa* resin, CBD: cannabidiol; NDGA: nordihydroguayaretic acid. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001: significant differences between each concentration of: CSR alone and CSR + LE, LE alone and LE + 15 µg/ml CSR or LE + 10 µg/ml CBD, and CBD alone and CBD + 25 µg/ml LE (Student´s *t* test).

Figure 3: Inhibition of lipid peroxidation



A: LE, CSR. **B:** CBD, NDGA, BHT (antioxidant control). **C:** CSR, CSR+ 250 µg/ml LE, CSR + 500µg/ml LE, CSR + 750µg/ml LE. **D:** LE, LE+ 30 µg/ml CSR, LE+ 10 µg/ml CSR, LE+ 1 µg/ml CSR. **E:** CBD, CBD+ 500 µg/ml LE. **F:** LE, LE+ 10 µg/ml CBD, LE+ 3 µg/ml CBD. Results are expressed as mean ± SEM of three experiments performed in triplicate.*p<0.05; **p< 0.01, ***p< 0.001: significant differences with respect to CSR or LE alone (one-way ANOVA + Dunnett's test) or CBD alone (Student´s *t* test). LE: *L. divaricata* extract, CSR: *C. sativa* resin, CBD: cannabidiol, NDGA: nordihidroguaiaretic acid.

DPPH scavenger activity of CSR and LE and determination of the combination index

The extracts, CBD and NDGA exerted DPPH scavenger activity in a concentration-response relationship, reaching the same maximum effect and similar to that observed with vitamin C (antioxidant control) ($p>0.05$) (Figure 2A and B), but NDGA presented the lowest IC_{50} value. Moreover, CBD exerted a pro-oxidant effect at low concentrations (Figure 2B). Then, the antioxidant activity of CSR was analyzed in the absence and presence of LE. When LE was added at 25 µg/ml, a concentration lower than the IC_{50} (25 µg/ml LE produced an inhibition of 26.39 ± 1.19%), a significant shift of the inhibitory curve to the left was observed ($p<0.001$, $p<0.0001$) (Figure 2C). The effect of CSR and CBD added to LE was also studied to determine the type of interaction between them. When 15 µg/ml CSR and 10 µg/ml CBD were added to LE, at concentrations lower than their IC_{50} (15 µg/ml CRS exerted an inhibition of 36.45 ± 0.10% and CBD exerted an inhibition of 35.07±1.84%), a parallel shift to the left of the *LE* concentration-response curve was observed, with no decrease of the maximum response ($p< 0.001$, $p< 0.0001$) (Figure 2D). A significant shift to the left was also observed when CBD was incubated in presence of 25 µg/ml LE ($p<0.05$, $p<0.01$) (Figure 2E). The IC_{50} values were calculated and are shown in Table 2A. The combination index is shown in Table 2B.

Table 2: Inhibitory concentration 50 (IC_{50}) of each extract and their combinations and combination index (CI) of each combination on DPPH scavenging activity

A

Extract	IC_{50} (µg/ml)
LE	24.18 ± 0.54[a]
LE + 15 µg/ml CSR	5.76 ± 1.19 ***
LE + 10 µg/ml CBD	1.36 ± 0.31 ***
CBD	12.49 ± 0.79[b]
CBD + 25 µg/ml LE	4.19 ± 0.11 **
CSR	19.82 ± 1.47[c]
CSR + 25 µg/ml LE	2.91 ± 0.10 ***
NDGA	0.80±0.05[d]

B

Extracts	Combination index (CI)	Type of interaction
10 µg/ml CBD + 25 µg/ml LE	0.39	Significant synergism
15 µg/ml CSR + 25 µg/ml LE	0.38	Significant synergism

Results are expressed as mean ± SEM of three experiments performed in duplicate. (*p<0.05, **p<0.01, ***p<0.001 respect to extracts or CBD alone (Student's *t* test or one way ANOVA + Dunnett's test). a,b,c significantly different (one way ANOVA+ Tukey's test). IC<1, IC=1, IC>1 represent synergism, additivity or antagonism, respectively.

Inhibition of lipid-peroxidation by CSR and LE and determination of the combination index

CSR and LE presented inhibitory activity on lipid peroxidation, following a concentration-response relationship (Figure 3A), while NDGA exerted a very weak effect. CBD also exerted an inhibitory effect but showed pro-oxidant activity at low concentrations (Figure 3B). BHT was used as control at a concentration usually used to exert antioxidant activity. Then, the effect of CSR was studied in the presence of LE at three different concentrations, 250µg/ml, 500µg/ml and 750 µg/ml, two lower and one higher than the IC_{50}. Only with 250µg/ml and 500 µg/ml LE there was a significant shift to the left of the concentration-response curve of CSR, mainly at low concentrations of CSR ($p<0.05$, $p<0.01$), but also a light but significant decrease ($p<0.05$, $p<0.01$ and $p<0.001$) of the relative maximum response was observed with all concentrations of LE (Figure 3C). When LE was incubated in the presence of CSR at 1µg/ml, 10µg/ml and 30 µg/ml, concentrations lower than or equal to the IC_{50}, a significant shift to the left was observed with 10 µg/ml of CSR ($p< 0.05$), while with 1µg/ml and 30 µg/ml CSR a shift to the right was observed. However, significant differences were only observed with high concentrations of LE in the presence of 30 µg/ml CSR ($p<0.05$) (Figure 3D).

Table 3: Inhibitory concentration 50 (IC_{50}) of each extract and their combinations on inhibition of lipid peroxidation

	IC_{50} µg/ml	Relative maximum response (%)
CSR	30.50 ± 3.00	89.00±1.10
CSR + 250 µg/ml LE	14.10±0.79****	83.40± 1.70*
CSR + 500 µg/ml LE	2.39± 0.10****	77.68± 2.00**
CSR + 750 µg/ml LE	7.89±0.49****	75.39± 0.60***
LE	630.95 ± 63.00	43.00± 5.00
LE + 1 µg/ml CSR	758.59±50.00	45.28± 4.00
LE + 10 µg/ml CSR	56.19± 4.00****	47.00± 5.00
LE + 30 µg/ml CSR	540.00±60.00	35.79± 4.00 *
LE + 10 µg/ml CBD	478.59±40.00	32.39± 4.00*
LE + 3 µg/ml CBD	< 100	47.78± 5.00
CBD	10.20 ±1.00	65.70 ±5.00
CBD + 500 µg/ml LE	2.19± 0.18****	72.40±6.00*

Results are expressed as mean ± SEM of three experiments performed in duplicate. *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001 between extracts or CBD alone and their combinations (one way ANOVA+ Dunnett's test or Student's *t* test)

Table 4: Combination Index of each combination on inhibition of lipid peroxidation

	Combination index (CI)	Type of interaction
30 µg/ml CSR + 250 µg/ml LE	1.31	Antagonism
30 µg/ml CSR + 500 µg/ml LE	0.93	Weak synergism
30 µg/ml CSR+ 750 µg/ml LE	1.11	Antagonism
10 µg/ml CSR + 250 µg/ml LE	0.55	Significant synergism
10 µg/ml CSR +500 µg/ml LE	0.16	Strong synergism
10 µg/ml CSR + 750 µg/ml LE	0.35	Significant synergism
1 µg/ml CSR + 250 µg/ml LE	1.65	Antagonism
1 µg/ml CSR + 500 µg/ml LE	1.28	Antagonism
1 µg/ml CSR + 750 µg/ml LE	1.46	Antagonism
10 µg/ml CBD + 500 µg/ml LE	0.96	Weak synergism
3 µg/ml CBD + 500 µg/ml LE	0.36	Significant synergism

IC<1, IC=1, IC>1 represent synergism, additivity or antagonism, respectively.

In addition, the incubation of CBD with LE produced a significant shift to the left only with 500 µg/ml of LE ($p<0.05$ and $p<0.01$) (Figure 3 E). LE was also incubated with 3 and 10 µg/ml CBD, concentrations that are lower than or equal to the IC_{50}. In this case, a significant shift of the concentration-response curve was observed in the presence of 3 µg/ml CBD ($p<0.05$) (Figure 3 F). With 10 µg/ml CBD, a shift to the right was observed, but in this case, the values were only significant for the lowest and the highest concentrations of LE ($p<0.05$). The IC_{50} values and the combination indexes are shown in Table 3 and Table 4, respectively.

DISCUSSION

In this work it was demonstrated the synergism of the antioxidant activity of *C. sativa* ethanol extract (resin) with an extract of *L. divaricata* in two different systems. The *C. sativa* extract presented DPPH scavenger activity and was able to inhibit egg phospholipid-peroxidation. When associated with *L. divaricata*, a decrease of the IC_{50} values of *C. sativa* extract in both activities was observed; however, this effect was not observed for all combinations. The participation of CBD in the effect exerted by *C. sativa* was proved, though other compounds with polyphenolic structure could also be involved.

Depending on their mechanism of action, antioxidants can be classified into primary and secondary ones. Primary antioxidants prevent oxidation by acting as chain breakers, reacting directly with lipid radicals, ROS or RNS, and converting them into more stable products, this is the reason why they inhibit the start of the redox chain and break its propagation. On the other hand, secondary or preventive antioxidants act indirectly by limiting lipid oxidation through several mechanisms, including the chelation of transition metals, singlet-oxygen quenching (in photo-oxidation), oxygen scavenging and the activation or inhibition of enzymes involved in ROS metabolism or synthesis, respectively [21].

In the present work, the DPPH assay was used to evaluate the anti-radical activity by evaluating the electron donating capacity, since the DPPH radical is a relatively stable reactive compound.

The extracts and compounds analyzed exerted DPPH scavenger activity but with a different potency (NDGA > CBD and *C. sativa* > *L. divaricata*). When LE was added to CSR, a decrease of 85% of the IC_{50} value was observed. The addition of CSR and CBD to LE decreased the IC_{50} value by around 76% and 94%, respectively.

The inhibition of lipid peroxidation is generally employed to determine the capacity of a substance of preventing the oxidation of lipids such as cell membrane phospholipids and the appearance of illnesses related to oxidative stress[29].

All the drugs presented inhibitory activity of lipid peroxidation, but with different potencies (CBD > NDGA and *C. sativa* > *L. divaricata*). The addition of LE caused a decrease of the IC_{50} values of both CSR and CBD between 54%-92% in the case of CSR and 79% in the case of CBD.

It is noteworthy that the extracts presented higher potency on the DPPH scavenger model than on the inhibition of lipid peroxidation assay, the activity was 26 and 1.55 times higher for the DPPH scavenger activity for LE and CSR, respectively. This could be explained if the differences between the two methods used are considered, for example, the DPPH scavenger activity assay is more sensitive, as compounds react rapidly with the DPPH, while in the inhibition of lipid peroxidation, compounds react more slowly with the peroxide radicals formed. Consequently, there is not always a good correlation between both methods[30]. In general, to achieve a protective effect, e.g. through the inhibition of lipid peroxidation, it is necessary to have concentrations that exert a free radical neutralization close to 100%. As a result, the concentrations needed to scavenge the DPPH free radical were lower than those needed for the inhibition of lipid peroxidation.

The fact that the extracts and CBD exerted DPPH scavenger activity and, at the same time, inhibited lipid peroxidation, suggested that they could be acting by neutralizing free radicals, behaving like a primary antioxidant. Nevertheless, these assays cannot rule out the presence of another mechanism of action. For example, *Cannabis* resin

demonstrated to have metal chelating action (data not shown).

To determine the type of interaction and to select the best combination to be used in future experiments, the combination index was calculated. For the DPPH scavenger activity, the CI was lower than 1, indicating the existence of synergism. The combination index for the association of 25 µg/ml LE with 15 µg/ml CSR or 25 µg/ml LE and 10 µg/ml CBD produced a significant synergism.

As for the inhibition of lipid peroxidation, the association of CSR with LE showed strong, significant or weak synergism and even antagonism depending on the concentrations employed. For example, the combination of 1 µg/ml CSR with 250µg/ml, 500 µg/ml or 750 µg/ml LE produced antagonism. In addition, 30 µg/ml CSR was antagonized by 250µg/ml and 750 µg/ml LE, but a weak synergism quasi additivity (CI near 1) was observed with 500 µg/ml LE. On the contrary, 10 µg/ml CSR was potentiated by LE at all concentrations, with the best association being 10 µg/ml CSR and 500 µg/ml LE, which showed a strong synergism with a lower CI. The combination of 3 µg/ml CBD with 500 µg/ml LE was also suitable, exerting a significant synergism.

The antagonism between the extracts could be produced through several mechanisms, for example, regeneration of the less effective antioxidant by the more effective antioxidant [31], oxidation of the more effective antioxidant by the radicals generated by the less effective antioxidant, competition between formation of antioxidant radical adducts and regeneration of the antioxidant [32], and alteration of microenvironment of one antioxidant by another antioxidant. On the other hand, synergism could be produced by the regeneration of one antioxidant by the other, in this case the stronger antioxidant or primary antioxidant (CSR, with a lower IC_{50}) is regenerated by the less active antioxidant (LE), the latter acting as co-antioxidant. It has been observed that the regeneration of primary antioxidants contributes to a higher net interactive antioxidant effect than the simple sum of individual effects [21].

It could also be possible that the less effective antioxidant traps alkyl or alkyl peroxy radicals, resulting in a greater degree of protection of the effective antioxidant from the oxidation due to antioxidant action [21]. Moreover, as it has been shown that an aqueous extract of *L. divaricata* at 7.5% w/v exerts other antioxidant activities such as peroxidase, superoxide and catalase-like activities related to free radical scavenging and inhibition of free radical synthesis[33, 34], it could be possible that the synergism exerted by LE could be achieved through other antioxidant activities. In this sense, it is known that two or more antioxidants with different antioxidant mechanisms can exert synergism, as it has been shown with a combination of metal chelators and free radical scavengers[21].

The antioxidant activity of *C. sativa* has been previously described in cell-free assays such as DPPH, ABTS and ORAC[35]. Moreover, it has been shown that an essential oil, obtained from *C. sativa* can scavenge the free radical DPPH, inhibits linoleic acid peroxidation and presents ferric power activity [36]. In another report, it was demonstrated that six different *C. sativa* extracts containing various proportions of CBD and Delta-9-*tetrahydrocannabinol* (Δ^9-THC) can eliminate DPPH radicals and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). For both activities the participation of CBD and THC was verified [37].

Both extracts presented polyphenols and flavonoids in their composition. The DPPH scavenger activity of CSR appeared to be related not only to CBD but also to polyphenols, as the IC_{50} value of CSR for this activity was 19.8 µg/ml, a concentration that is equivalent to 5.4 µg of CBD. In turn, the latter compound had an IC_{50} of 12.5 µg/ml. A similar phenomenon occurred with LE, whose DPPH scavenger activity appeared to be partially related to the presence of NDGA, as the IC_{50} value of LE was 24.18 µg/ml, which was equivalent to 0.38 µg/ml of NDGA. This compound presented an IC_{50} of 0.80 µg/ml, indicating that polyphenols other than NDGA were involved in the effect. It has previously been demonstrated that CBD exerts DPPH scavenger activity when added to different oils; however, according to the results presented herein, at low concentrations, this compound could act as pro-oxidant [38].

The inhibition of lipid peroxidation exerted by CSR appeared to be strongly related to CBD, as the amount of CBD present in the extract was near its IC_{50} value (10 µg/ml) and similar to the IC_{50} of CSR (7.05 µg/ml). On the contrary, the effect of LE was not related to the presence of NDGA, which proved to exert a weak effect when tested alone, thus indicating that other polyphenols could be involved in the effects observed.

It is important to note that the compounds identified in *L. divaricata* extract could also contribute to the anticonvulsant activity of *Cannabis*, for example: the flavonoid rutin possesses anticonvulsant activity reported without any adverse effects [39]. By other way, chlorogenic and caffeic acids are known to exert neuroprotective action in glutamate-induced cortical neurons injury by exerting antioxidant activities [40].

CONCLUSION

The results obtained in the present work support the association of CSR with LE at certain concentrations, which can be used in future *in vitro* and *in vivo* studies.

In this preliminary study the combination of CSR with the LE extract would allow reducing the CSR doses. Nevertheless ¨in vitro¨ experiments with cells will be performed to confirm this results and to support this strategy that could be used in the future as co-adjuvant for the treatment of resistant epilepsy through a reduction of oxidative stress.

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