Pharmacogn J. 2020; 12(4): 798-804
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
www.phcogj.com

Original Article

Protection of Erythrocytes against Lipoperoxidation and Anti-inflammatory Effects of Ethanolic Extract of *Encelia canescens* Lam Leaves in Mice

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ABSTRACT

Background. *Encelia canescens* Lam is a plant traditionally used in Peru for medicinal purposes, and is attributed antioxidant properties, indicating that it could be used in the prevention of non-communicable diseases. **Objective:** This study aims to evaluate the protection of erythrocytes from lipoperoxidation and the anti-inflammatory effect of ethanolic extract of *E. canescens* leaves in mice. **Materials and methods:** Protection from lipoperoxidation was evaluated by inhibition of hemolysis and quantifying malondialdehyde (MDA) concentration against oxidative stress induced with hydrogen peroxide (H_2O_2) at 200, 150, 100, 50 and 25 µg/mL *E. canescens* concentrations. The 1% carrageenan-induced air pouch model was used for evaluated inflammation, where albumin, total proteins, MDA, number and leukocyte differentiation were determined in the exudate, and a histopathological evaluation was performed. The concentrations evaluated were 100, 250 and 500 mg/kg of *E. canescens* **Results:** All the concentrations evaluated protected protected erythrocytes from lipoperoxidation ($p<0.05$), being E.D. value 200 µg/mL. Regarding anti-inflammatory effect, the albumin, total proteins and MDA values of the treatment groups were lower than carrageenan 1% group ($p<0.05$), but, due to less leukocyte migration and presence of macrophages and the histopathological evaluation, the E.D value was 500 mg/kg. **Conclusion:** Ethanolic extracts of *E. canescens* leaves protect erythrocytes from lipoperoxidation and have dose-dependent anti-inflammatory effects maybe for presence of p-hydroxyacetophenone-derived, and these could be new safer anti-inflammatories.

Key words: *Encelia canescens Lam*, Hemolysis, Malondialdehyde, Lipoperoxidation, Anti-inflammatory, Carrageenan.

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History
- Submission Date: 03-05-2020;
- Review completed: 11-05-2020;
- Accepted Date: 20-05-2020.

DOI : 10.5530/pj.2020.12.114

Article Available online
http://www.phcogj.com/v12/i4

INTRODUCTION

Noncommunicable diseases (NCDs) are a health problem that have been the main causes of death for years in low and medium-income countries[1], generating a negative impact on social and economic development worldwide[1–3], being the inflammatory processes one of the ones that demand the highest costs worldwide[4], without having the treatment it can lead to much more serious problems.[5] The four main types of NCDs are cardiovascular, cancer, lung, and diabetes[6]; in such a way that one of the main causes of these diseases is oxidative stress[7], a process that shows an imbalance between oxidants and antioxidants and that is the cause of cellular damage.[7-9]

Peru is considered an area of high plant richness and mainly of the Asteraceae, with more than 1613 species[10] within them we have the species *Encelia canescens* Lam (*E. canescens*) known as "coronilla del fraile" or "hierba lingo" that is empirically used for its analgesic[11], galactophore properties, menstruation regulation[12], cancer treatment[13] and viral and bacterial diseases, against urine retention.[11] This species has triterpenoids[14], steroids, flavonoids, coumarins, and tannins[13], being flavonoids, metabolites with potential antioxidant and anti-inflammatory activity[15,16], but triterpenoids has same anti-inflammatory activities.[17,18]

Antioxidant capacity can be measured in lipoperoxidation processes and also by means of oxidative stress markers[6]; since lipid oxidation has been shown to be associated with damage at level of the cell membrane, this leads to apoptosis in a wide variety of cells.[19-21] Therefore, protection from lipoperoxidation through antioxidants application, particularly antioxidants derived from medicinal plants, may be a possible strategy to protect cellular damage generated by oxidative stress.[19-21]

This study therefore aimed at evaluating protection from lipoperoxidation in erythrocytes and the anti-inflammatory effect of *E. canescens* leaves ethanolic extract in mice.

MATERIALS AND METHODS

The study was conducted at the Pharmaceutical Research Center of the Faculty of Pharmacy and Biochemistry at Norbert Wiener University.

Plant material and extract

Plant species was collected in the province of Huaral, North of Lima to 250 m.s.n.m. The taxonomic

Cite this article: Fernández-Flores N, Rojas-Cardenas NF, Vásquez-Quispe AD, Chávez-Flores Juana E, Justil-Guerrero Hugo J, Parreño-Tipian JM, *et al.* Protection of Erythrocytes against Lipoperoxidation and Anti-inflammatory Effects of Ethanolic Extract of *Encelia canescens* Lam Leaves in Mice. Pharmacogn J. 2020;12(4):798-804.

classification of the species was carried out at the Museo de Historia Natural of Universidad Nacional Mayor de San Marcos (N° 198-USM-2018). The process of obtaining the ethanolic extract from the *E. canescens* leaves was carried out in accordance with the stipulations of Lock O. (2016).[22]

Phytochemistry analysis

The process of phytochemistry analysis of ethanolic extract from the *E. canescens* leaves was carried out in accordance with coloring and precipitation techniques of Lock O. (2016).[22]

Biological material

For the evaluation of oxidative stress protection, a 2 mL sample of erythrocytes was obtained from rats of the Holtzman strain of 12 weeks of age weighting 220 ± 10 g from Instituto Nacional de Salud (INS) and to evaluate the anti-inflammatory effect, male mice 25 g each, strain Balb/C53/CNPB was used. This study was approved by the Norbert Wiener University Ethics Committee under opinion N° 005-08-2019 FB/UPNW.

Hemolysis and malondialdehyde quantification

Erythrocytes protection from lipoperoxidation induced by H_2O_2 was evaluated by determining hemolysis and quantifying malondialdehyde (MDA) following the methods of Fátima *et al.* (2013) and Esterbauer and Cheeseman (1990) evaluating concentrations of 200, 100, 50 and 25 µg/mL of *E. canescens*.[23,24]

Anti-inflammatory effect

1% carrageenan-induced air pouch model described by Duarte *et al.* (2016) was used, the inhibition of inflammation was measured with albumin, total protein and MDA parameters at concentrations of 100, 250 and 500 mg / kg of *E. canescens*.[25] Number and differentiation of leukocytes obtained from the exudate was determined by Wright staining as indicated by Mahat *et al.* (2010) with some modifications.[26]

Histopathology

Histopathological evaluation was performed as described by Fronza *et al.* (2016). The skin sections were fixed in Bouin's solution for 24 hours. Samples were dehydrated through graduated alcohol, clarified with xylene, and embedded in paraffin. 6 µm thick sections were made and stained with hematoxylin-eosin (H&E).[27]

RESULTS

E. canescens ethanolic extract was soluble in polar solvents and insoluble in apolar solvents. In addition, the presence of secondary metabolites such as flavonoids, phenolic compounds, and alkaloids was identified by qualitative analysis.

In the evaluation of erythrocyte protection against induced oxidative stress via H_2O_2, inhibition of hemolysis and release of MDA content depend on the concentration of the extracts, so concentration of 200 µg/mL presented highest protection (Tables 1 and 2 and Figure 1).

Albumin, total proteins and MDA decreased significantly compared to the Control group ($p<0.05$) (Table 3) in the evaluation of biochemical parameters in air pouch exudate after carrageenan administration. Dexamethasone and *E. canescens* 500 mg/kg significantly decreased migration of polymorphonuclear leukocytes, monocytes, and lymphocytes compared to the control group (Table 4). All the treated groups showed a decrease in leukocyte migration, presence of fibroblasts and macrophages in histopathological evaluation, *E. canescens* 500 mg/kg group standing out.

DISCUSSION

Secondary metabolites such as flavonoids, alkaloids, tannins, steroids and/or triterpenes were identified in this study. These metabolites have also been identified in Chile by Cayún *et al.* (2015), except for alkaloids; it seems that the climate and altitudinal floors in Peru are favorable for the development of these secondary metabolites.[13,28] According to the reviewed literature, flavonoids, tannins, coumarins, steroids and/or

Table 1: Phytochemical analysis of the ethanolic extract of E. canescens leaves.

Essays	Secondary metabolite	Ethanolic extract
$FeCl_3$	Phenolic compounds	-
$AlCl_3$	Flavonoids	+
Shinoda	Flavonoids	+
NaOH/gelatin	Tannins	+
Bertrand	Alkaloids	+
Dragendorff	Alkaloids	+
Mayer	Alkaloids	+
Popoff	Alkaloids	+
Wagner	Alkaloids	+
Liebermann -Burchard	Steroids and or triterpenes	+
Salkowski	Steroids	-

Legend: Presence (+), absence (-).

Table 2: E. canescens protection in erythrocytes against H2O2-induced hemolysis at 30, 60 and 90 minutes.

Treatment	Hemolysis inhibition (%)		
	30 min	60 min	90 min
H_2O_2 200 mmol	-	-	-
Dexamethasone 200 µg/mL	18.92[a]	17.49[a]	16.72[a]
E. canescens 200 µg/mL	97.74[b]	97.56[b]	89.02[b]
E. canescens 150 µg/mL	85.39[c]	23.21[c]	22.43[c]
E. canescens 100 µg/mL	86.68[d]	23.02[d]	21.87[c]
E. canescens 50 µg/mL	46.11[e]	20.05[d]	21.58[c]
E. canescens 25 µg/mL	3.97[f]	3.91[e]	7.35[d]

The results in each period (n=3); different letters in each column indicate significant difference ($p<0.05$), equal letters indicate that there is no difference between groups.

Table 3: Effect of E. canescens on biochemical parameters in air pouch exudate after administration of carrageenan at 1%.

Group (n)	Albumin (g/dL)	Total proteins (g/dL)	MDA (nmol/mL)
Observer	0.26[a]	2.13[a]	1.12[a]
Control	1.84	5.31	1.67
Dexamethasone 2 mg/kg	1.21[a]	3.57[a]	1.44[a]
E. canescens 100 mg/kg	1.65[a]	4.58[a]	1.57[a]
E. canescens 250 mg/kg	1.48[a]	4.15[a]	1.45[a]
E. canescens 500 mg/kg	1.01[a]	3.67[a]	1.24[a]

In each column (n = 6), a: indicates a significant difference ($p<0.05$) compared to the control group (carrageenan at 1%).

Table 4: Effect of E. canescens on leukocyte infiltration in air pouch exudate after administration of carrageenan at 1%.

Treatment	Leukocytes x mm³	Leukocyte differentiation		
		Polymorphonuclear x 10^3 / mm³	Monocytes x 10^3 / mm³	Lymphocytes x 10^3 / mm³
Observer	0	0	0	0
Control	24.16	15.56	2.72	5.88
Dexamethasone 2 mg/kg	4.15[a]	2.81[a]	0.46[a]	0.89[a]
E. canescens 500 mg/kg	4.57[a]	2.80[a]	0.84	0.93[a]
E. canescens 250 mg/kg	6.98	4.08	1.27	1.62
E. canescens 100 mg/kg	8.38	4.14	1.80	2.44

In each column (n = 6), a: indicates a significant difference ($p<0.05$) compared to the control group (carrageenan at 1%).

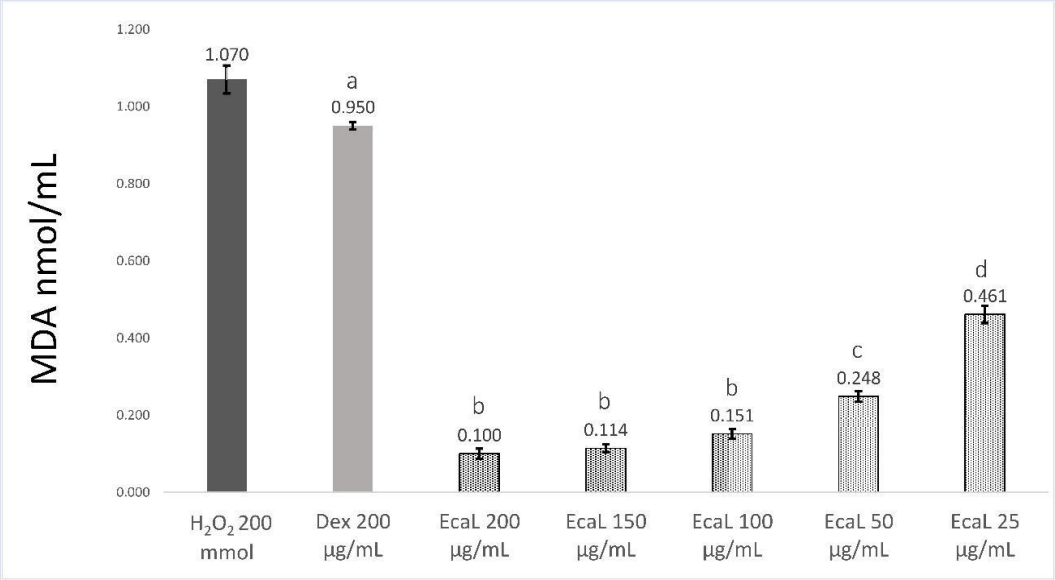

Figure 1: Results (n=3) of MDA concentration expressed in nmol / mL of erythrocytes subjected to oxidative stress with H2O2; letters a, b, c, d indicate significant difference ($p<0.05$) compared to the H2O2 group; equal letters indicate that there is no difference between groups. EcaL: E. canescens.

triterpenes are metabolites that have demonstrated anti-inflammatory and antioxidant effects, contributing to the prevention of chronic diseases of higher prevalence.[29,30]

In Table 2, *E. canescens* 200 µg/mL presented greater protection against hemolysis compared to the H_2O_2 control group ($p<0.05$) at the three-time marks (30, 60 and 90 min). Radical H_2O_2 oxidizes the ferrous ion of hemoglobin to ferric, producing methemoglobin in the Fenton reaction, generating hydroxyl ion (•OH)[31], a reactive species capable of damaging DNA, lipids, and cell proteins, among others.[32] Secondary metabolites with antioxidant properties transfer electrons to the hydroxyl ion causing reduction of the ferric ion and stabilizing hemoglobin, preventing hemolysis and restoring the balance of the plasma membrane in erythrocytes.[33]

In Figure 1, the concentration of MDA in erythrocytes is shown after having been exposed to oxidative stress with H_2O_2, concentration-dependent MDA decrease of *E. canescens* extracts is evidenced, showing a significant difference with the H_2O_2 group, the one in which the concentration of 200 µg/mL stands out. Lipoperoxidation occurs in the erythrocyte membrane and MDA is an indicator of this phenomenon, which is mediated by inflammatory processes that will damage cellular components as well as increased levels of hydroxyl radical (•OH), superoxide (O^{2-}), radicals derived from nitrogen (NO) which destabilize the cell membrane.[34-36] In this sense, *E. canescens* extract stabilizes the radicals that will cause lipoperoxidation as it is an electron donating compound.

Table 3 shows that albumin, total proteins and MDA differ significantly from carrageenan 1% ($p<0.05$). This is because flavonoids act by

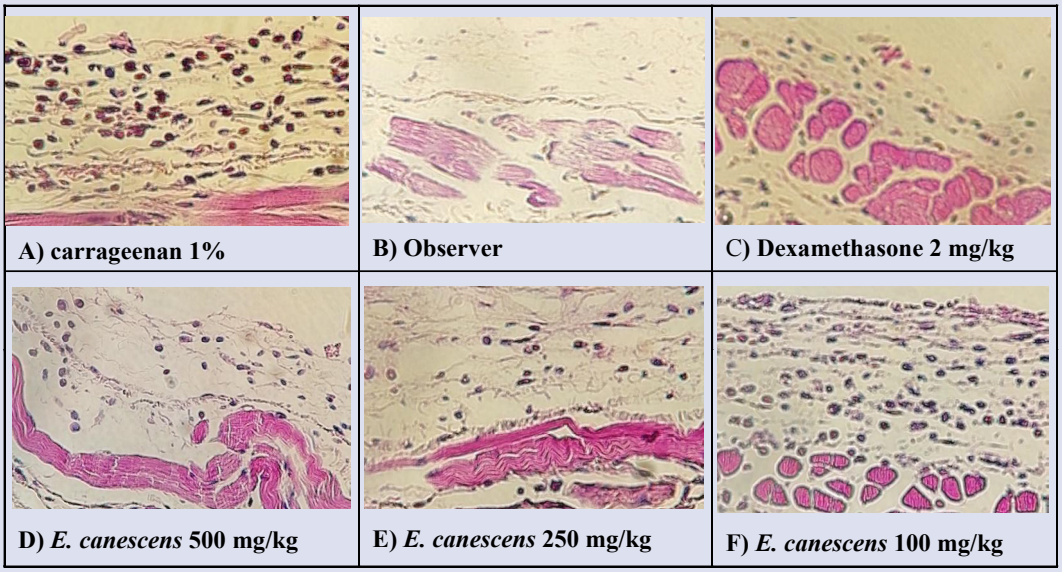

Figure 2: Effect of dexamethasone and E. canescens on leukocyte migration, presence of macrophages in the hypodermis in the air pouch after administering carrageenan 1% (photomicrographs increased by 40x, staining: H-E). Where: A: Dense and organized layer of macrophages, leukocytes and fibroblast cells; B: Presence of fibroblasts, macrophages in minimal quantity; C: Presence of leukocytes, fibroblasts and macrophages in lower density than in carrageenan 1%; D and E: Presence of leukocytes, fibroblasts and macrophages in lower density than in carrageenan 1% and similar to Dexamethasone 2 mg/kg; and F: Presence of leukocytes, fibroblasts and macrophages in lower density than carrageenan 1%, but higher density than Dexamethasone 2 mg / kg, E. canescens 500 mg/kg and E. canescens 250 mg/kg.

inhibiting the lipoxygenase pathway 5 which prevents the inflammation cascade from forming and prevents the release of inflammation mediators.[37,38] Furthermore, steroids participate by inhibiting the inflammatory process, since they stabilize the lysosome membrane, intracellular particles that contain proteases and hydrolytic enzymes which form inflammation chemical mediators, preventing the disintegration of said lysosomes that are produced in inflammatory processes and are responsible for damage and other inflammatory phenomena.[39] When evaluating the biochemical parameters, *E. canescens* 500 mg/kg presented better results, showing that the higher the concentration, greater anti-inflammatory effect. Cochachi and Fernández (2015), had similar results when evaluating the ethanolic extract of the stems of *E. canescens* where they found a total proteins and albumin decrease, being attenuated after the administration of the treatment groups, being attenuated after the administration of the treatment groups, being most activity concentration was 400 mg/kg with similar activity to silymarin 300 mg/kg but, in this study was not evaluated anti-inflammatory activity.[40] Finally, Chilquillo and Cervantes (2017), demonstrated that the hydroalcoholic extract from the leaves of *Senecio canescens* (Humb. & Bonpl.) Cuatrec "vira-vira" present favorable results of MDA in blood, noting a significant decrease in MDA values in the groups to which the treatment was administered compared to the stress group[41], this is according with the results obtained in the present research work.

Table 4 shows leukocyte migration evaluation in the exudate, *E. canescens* 500 mg/kg showed decreased migration of lymphocytes and polymorphonuclear cells. They participate in phagocytosis by releasing superoxide and hydrogen peroxide free radicals to produce a hydroxyl radical, which attacks leukocytes and leads to premature cell death.[25] Likewise, an increase in lymphocytes and monocytes was observed, being greater in the *E. canescens* 100 mg/kg treatment, which means that these leukocytes would be responsible for phagocytosis or macrocytosis of apoptotic neutrophils. Leukocyte migration to the area of injury causes leukocytes to engulf pathogens, destroy bacteria and microorganisms, and degrade necrotic tissue, but can also prolong tissue injury by releasing enzymes, chemical mediators, and reactive species from the oxygen (oxygen free radicals, RLO).[42]

The anti-inflammatory effect of *E. canescens* was examined histologically (Figure 2), in the normal tissue (observer) inflammation was not evidenced where a layer of flattened cells superimposed on loose vascular connective tissue is observed. In carrageenan 1% group, macrophages and fibroblasts increased in the lining layer of flattened cells in the groups, observing inflammatory changes such as leukocyte infiltration and edema (evidenced by tissue thickening). *E. canescens* 500 mg/kg significantly reduced inflammatory changes with less edema and cell infiltration. The anti-inflammatory potency of the extract 500 mg/kg was related to the lower leukocyte migration in the exudate and the lower concentration of MDA.

Although the relationship between chemical composition and activity has not been established, some reported compounds may be responsible for the activity. Bohlmann et al (1982) reported presence aromatic compounds like p-hydroxyacetophenone derivatives, p-hydroxyacetophenone-derived compounds probably be related to anti-inflammatory activity by being closely related to cyclooxygenase inhibitors structure.[14,43] Anti-inflammatory activity by others acetophenones derivative had be reported.[44-46]

CONCLUSION

Under experimental conditions, the ethanolic extract of Encelia canescens Lam leaves protects erythrocytes from lipoperoxidation induced via H_2O_2 and presents an anti-inflammatory effect at dose-dependent in carrageenan 1%-induced airbag model, maybe for presence of p-hydroxyacetophenone-derived. These could be new safer anti-inflammatories drugs but it's necessary more studies to support the effectiveness and safety of the use of extracts of this plant.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest in this study.

ETHICAL APPROVAL

This study was approved by the Norbert Wiener University Ethics Committee under opinion N° 005-08-2019 FB/UPNW.

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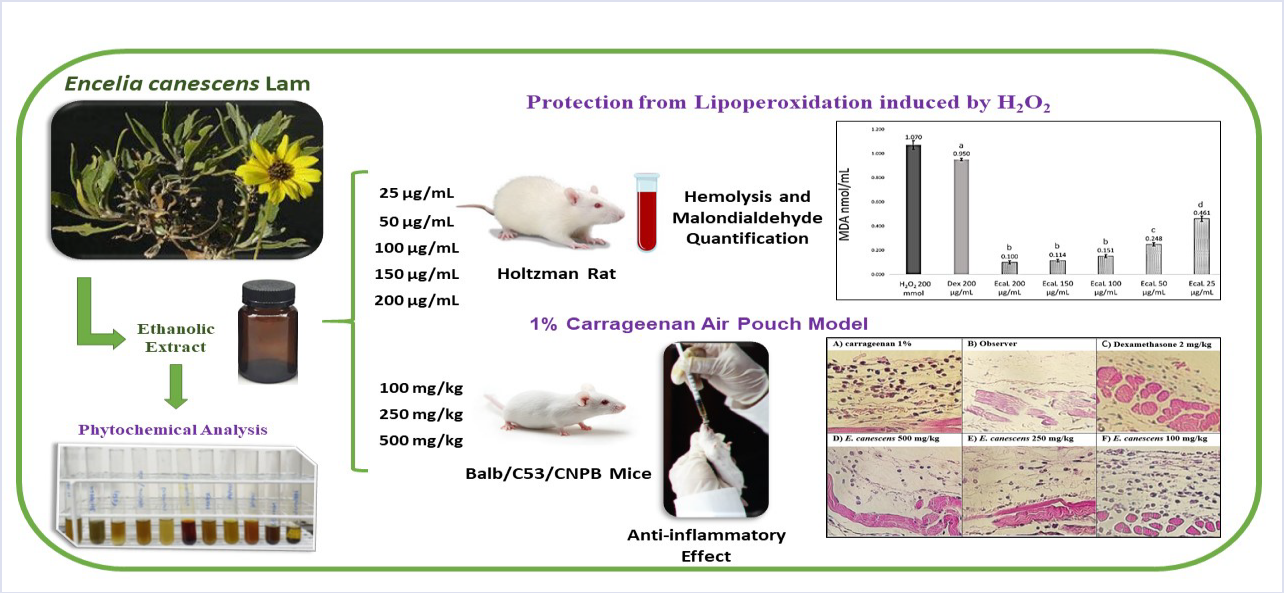
GRAPHICAL ABSTRACT



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Cite this article: Fernández-Flores N, Rojas-Cardenas NF, Vásquez-Quispe AD, Chávez-Flores Juana E, Justil-Guerrero Hugo J, Parreño-Tipian JM, *et al*. Protection of Erythrocytes against Lipoperoxidation and Anti-inflammatory Effects of Ethanolic Extract of *Encelia canescens* Lam Leaves in Mice. Pharmacogn J. 2020;12(4):798-804.