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In vitro evaluation of the healing activity and antioxidant capacity of ethanolic propolis extract

Evaluación *in vitro* de la actividad cicatrizante y capacidad antioxidante del extracto etanólico de propóleo

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ABSTRACT

A wound is defined as a cut or rupture in the continuity of any tissue. The wound healing process can be compromised by infection, inadequate oxygen supply, malnutrition, and oxidative processes. In pursuit of alternatives to aid the healing process, we evaluated the healing and antioxidant activity of ethanolic extract of propolis (EEP). The propolis underwent processing, yielding EEP at different concentrations in µg/mL. The total polyphenol content, antioxidant capacity using various methods (ABTS, DPPH, and FRAP), reactive oxygen species (ROS) and nitric oxide (NO) levels, and healing activity using the Scratch Wound Healing technique, were assessed. At 1000 µg/mL, the EEP exhibited a concentration of 754.36 mgEAG/gbs of total polyphenols. The antioxidant capacity, measured by DPPH, ABTS, and FRAP methods at 5000 µg/mL, showed concentrations of 41.33 mgET/gbs, 60.53 mgET/gbs, and 280 µMET/gbs, respectively. The concentration of ROS and NO at 300 µg/mL exhibited inhibition percentages of 32.96 % and 39.93 %, respectively. The highest healing percentage was observed at 600 µg/mL. The polyphenols, along with the antioxidant activity of EEP, show promise, potentially linked to the observed acceleration of the healing process.

KEY WORDS: Healing, propolis, antioxidants, cellular repair.

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RESUMEN

Una herida se define como corte o ruptura en la continuidad de cualquier tejido. El proceso de curación de heridas puede verse comprometido por infección, suministro inadecuado de oxígeno, desnutrición y proceso oxidativo, en la búsqueda de alternativas que coadyuben los procesos de cicatrización, se evaluó la actividad cicatrizante y antioxidante del extracto etanólico de propóleo (EEP). El propóleo fue procesado y se obtuvo el EEP a diversas concentraciones en µg/mL, se cuantificó el contenido de polifenoles totales, la capacidad antioxidante por diferentes métodos (ABTS, DPPH y FRAP), las especies reactivas de oxígeno (ROS) y óxido nítrico (ON) y la actividad cicatrizante por la técnica Scratch Wound Healing. A 1000 µg/mL el EEP arrojo una concentración de 754.36 mgEAG/gbs de polifenoles totales, la capacidad antioxidante por los métodos de DPPH, ABTS y FRAP a 5000 µg/mL mostró concentraciones de 41.33 mgET/gbs, 60.53 mgET/gbs y 280 µMET/gbs respectivamente, la concentración de ROS y ON a 300 µg/mL mostró un porcentaje de inhibición de 32.96 y 39.93 %. El mayor % de cicatrización se observó a 600 µg/mL. Los polifenoles, así como la actividad antioxidante del EEP son prometedoras, lo cual puede estar ligado a la aceleración del proceso de cicatrización observado.

PALABRAS CLAVE: Cicatrización, propóleo, antioxidantes, reparación celular.

Introduction

The skin is a fibroelastic membrane, considered the “living envelope of the body” (Guarín-Corredor *et al.,* 2013). It is the most extensive organ of the human body, with an approximate surface area of 1.6 m^2 and a weight of about 4 kg, which is equivalent to 6 % of the total body weight (Tresguerres *et al.,* 2009). Its main function is to constitute a protective barrier against microorganisms, UV rays, fluid loss, and stress from mechanical forces. At the same time, it serves as the main sensitive organ for communication with the outside (Huether & McCance, 2015). Being the outermost layer, it is susceptible to mechanical processes that can cause tissue wounds.

A wound can be defined as a cut or rupture in the continuity of any tissue. It also includes a breakdown in the protective function of the skin, and loss of the epithelium continuity, with or without loss of connective tissue underlying the skin after injury (Gunde, 2018).

Following injury, a complex and dynamic process of restoration of cellular structures and tissue layers of the skin or other organs of the body is triggered. This process is known as scarring (Agyare *et al.,* 2014; Schencke *et al.,* 2016; Al-Waili, 2018). Normally, scarring is divided into four stages: Coagulation, inflammatory, proliferative, and re-epithelialization.

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The wound-healing process can be compromised by infections, inadequate oxygen supply, malnutrition, and oxidative processes (Al-Waili, 2018). An important consideration in physiological wound healing is the oxygen supply to the base of the wound (Darby *et al.,* 2014).

Wound healing is a crucial process for skin regeneration and recovery of damaged tissues. In addition to conventional medical treatments, some natural therapies such as propolis can help accelerate this process (Arcos *et al.,* 2019), therefore the strategies to implement are varied.

They have a large part of their biological and therapeutic activity due to the flavonoids presence in their composition, (Darby *et al.,* 2014; Delgado Aceves *et al.,* 2015; Chaa *et al.,* 2019) which varies depending on the geographical origin (Farrér *et al.,* 2004; Somerville, 2008; Eyng *et al.,* 2015; Chaa *et al.,* 2019; Elkhenany *et al.,* 2019).

The role of antioxidant properties in wound healing has been studied. The addition of these antioxidants has been evaluated in dressings and hydrogels (Frykberg & Jaminelli, 2015; Fu *et al.,* 2020). Reactive oxygen species (ROS) are crucial regulators of several phases of wound healing. Indeed, low levels of ROS are required to fight against external damage (Dunnill *et al.,* 2017). However, excessive oxidative stress in tissues and decreased antioxidant capacity result in a redox imbalance, which is one of the main causes of non-healing wounds (Sanchez *et al.,* 2018). Therefore, this oxidative stress must be reversed with increased use of antioxidant compounds (Frykberg & Jaminelli, 2015; Fu *et al.,* 2020).

In this research, the use of propolis is proposed since it has been known for many years for its use in wound healing (Barron & Wynn, 2011; Asgharpour *et al.,* 2019; Baygar, 2020; Belvedere *et al.,* 2020). Similarly, it presents antitumor and antioxidant properties (Harnaj *et al.,* 1978; Agyare *et al.,* 2014) and several effects on bacteria, fungi, parasites, and viruses (Castillo, 2018; Carter & Skilbeck, 2014).

Indeed, it is precisely this aspect that makes this study particularly interesting, since propolis contains attributes that can help in wound healing processes. For this reason, the present study aimed to evaluate the wound healing activity and antioxidant capacity of the ethanolic extract of propolis.

Material and Methods

The processing of propolis to obtain the ethanol extract, the quantification of total polyphenols, and the determinations of antioxidant capacity by the three methods described below were conducted in the Laboratorio de Farmacia y Productos Naturales of the Facultad de Ciencias Químicas, at Gómez Palacio campus, within the Durango state, Mexico, under the auspices of the Universidad Juárez del Estado de Durango from January to July 2020. The ROS and NO concentration, as well as *in vitro* healing using the Scratch Wound Healing Assay, was carried out in the Biochemistry Department of the Biomedical Research Center at the Universidad Autónoma de Coahuila, Torreon campus, during the period from August to December 2020.

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Propolis

Propolis was obtained from the Universidad Autónoma Agraria Antonio Narro (UAAAN) in the Torreón municipality, Coahuila, Mexico, in January 2020, under the conditions established by the Biology Department, UAAAN Laguna Regional campus.

Fibroblasts

The cell lines, named Primary Dermal Fibroblast Normal; Human, Neonatal (HDFn), and 3T3 mouse fibroblasts, were obtained from the Laboratory of Molecular Biology of Cancer of the Faculty of Biological Sciences of the Universidad Autónoma de Nuevo León.

Elaboration of ethanolic extract of propolis

The extract was prepared by combining 25 g of propolis with 250 mL of absolute ethanol as the solvent, maintaining it at room temperature in a dry, cool place, and protected from light. After 24 hours on an orbital shaker (Heathrow Scientific, Sea Star Model 1.11) at 150 rpm, it underwent filtration using a Buchner funnel with thick pore filter paper (Whatman No. 1) attached to a Kitasato flask, assisted by a vacuum pump (AutoScience, Model AP-9950), until the resulting residue was as clear as possible. Subsequently, it underwent rotary evaporation (Buchi, Model R-210) at 100 rpm and 50 °C for 30 minutes until the propolis attained a semisolid consistency. This semisolid fluid was then divided into three Petri dishes, and placed in a vacuum oven (Napco, Model 5831) under conditions of 40 °C and 20 atm, purged every hour, for 24 hours. Upon obtaining completely dried material, the dishes were weighed using an analytical balance (Adventurer Ohaus, Model AR2140) to quantify the yield of the ethanolic extract of propolis.

Yields of ethanolic extract of propolis

The following equation was used to establish the yield of propolis extracts (Equation 1):

Equation 1. Yield of propolis extracts.

$$Yield\ (\%) = (Px100)/m$$

P = Weight of dry extract (g).

m = Dry weight of sample (g).

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Total polyphenol content in ethanolic extract of propolis

Total polyphenols were quantified using the Folin-Ciocalteu Method (Cortés-Chitala *et al.,* 2021). Extracts were diluted to 5000, 1000, 620, and 320 µg/mL, then the reaction was performed by mixing 0.5 mL of 0.67 N Folin reagent and 0.5 mL of Na_2CO_3 1.9 M. After 1 h of rest, the samples were read at 760 nm in a UV/Visible spectrophotometer using gallic acid (GA) as a reference standard. The following equation was used to establish the total polyphenol content (Equation 2):

Equation 2. Total polyphenol content expressed in milligram gallic acid equivalents (mgEAG).

$$Total\ polyphenol\ content\ (\frac{mgEAG}{gdb}) = ppm\ \frac{Extract\ (L)}{Sample\ (g)}$$

ppm= concentration in mg/mL,

L= volume in liters of solution.

g= grams of the dry sample of the extract.

Antioxidant activity of ethanolic extract of propolis

2,2′-azino-bis-3- ethylbenzothiazolin-6-sulfonic acid (ABTS)

The free radical scavenging capacity using the ABTS method was conducted following the procedure described by Re *et al*. (1999), with modifications.

The ABTS+ radical was generated by combining 7 mM ABTS with 2.25 mM potassium persulfate. This mixture was allowed to react for 12-16 hours in the dark at room temperature. Subsequently, the radical was diluted with a mixture of ethanol and water in a ratio of 75:25. For the assay, samples were prepared by mixing 10 µL of the ethanolic extract of propolis (at concentrations of 5000, 1000, 620, and 320 µg/mL) with 190 µL of the ABTS+ solution, and the absorbance was measured after 30 minutes at 740 nm. The results were expressed as the antioxidant capacity equivalent to percent inhibition. The inhibition percentage of the ABTS+ radical was calculated using Equation 3.

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Equation 3. Percentage of ABTS+ radical inhibition.

$$Inhibition\ \% = \frac{(\text{ABS Control} - \text{ABS Sample})}{\text{ABS Control}} x\,100$$

ABS= Absorbance.

1,1-Diphenyl-2-picrylhydrazyl (DPPH)

The antioxidant capacity of the extracts was determined using visible spectrophotometry in the presence of the 1,1-diphenyl-2-picrylhydrazine (DPPH) radical at 518 nm in a UV/Visible spectrophotometer. For each sample, 2 mL of 80 % methanol (blank) and 2 mL of the diluted extracts at various concentrations (5000, 1000, 620, and 320 µg/mL) were prepared. then, 2 mL of freshly prepared DPPH solution (2.5 mM) was added. The absorbance of both the blank and samples was measured after 30 minutes. The inhibition percentage was calculated according to the equation described by Cortés-Chítala *et al*. (2021) (Equation 4).

Equation 4. Inhibition (%) for the DPPH test considers the absorbance (Abs) of both the control and sample.

$$Inhibition\ \% = 1 - \frac{\text{ABS Sample}}{\text{ABS Control}} x\,100$$

ABS= Absorbance.

Ferric reducing antioxidant potential (FRAP)

The FRAP assay was conducted following the procedure Serra and Lacalle (2010) outlined. The 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) reagent was prepared by mixing 2.5 mL of TPTZ (0.01 M), 2.5 mL of $FeCl_3$ *$6H_2O$ (0.02 M), and 25 mL of buffer solution (pH 3.6).

For the samples, 10 µL of each concentration of ethanol extract of propolis (5000, 1000, 620, and 320 µg/mL) was taken, and 190 µL of the FRAP reagent was added. Subsequently, the mixture was incubated for 30 minutes, and its absorbance was measured at 595 nm. A Trolox curve was prepared in vials protected from light, and the necessary dilutions were made to obtain concentrations of 0, 25, 50, 100, 200, and 300 ppm. Finally, the results were expressed as µmol Trolox equivalents/grams of dry base using the following equation (Equation 5):

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Equation 5. µmol Trolox equivalents/grams dry basis.

$$\frac{\mu molTE}{gdb} = \frac{(ppm)(L)(DF)}{\text{propolis } (g)}$$

ppm = Parts per million.

L = Liters.

DF = Dilution factor.

g = grams.

Analysis of reactive oxygen species (ROS) and nitric oxide (NO) concentration in mouse macrophages

Macrophage induction was conducted via peritoneal lavage in two 8-week-old male Swiss mice injected with 1 mL of thioglycollate [17.5 ppm] for 72 hours. Following abdominal massage, samples (1 mL) were collected and centrifuged at 1,500 rpm for 10 minutes. Cell viability was assessed by resuspending the sample pellets in RPMI-1640. A Neubauer chamber was used for total cell count after adding 10 µL of cells and 90 µL of trypan blue. The macrophages were cultured in triplicate and exposed to 20 µL of NBT reagent, followed by incubation for 18 hours at 37 °C and 5 % CO_2. A 500 µL solution of RPMI medium with 20 µL NBT was used as a control, as well as a positive control of cells with the addition of LPS, while as a negative control without LPS, a concentration of 300 µg/mL was additionally used as a control for EEP treatment. Subsequently, 92 µL of DMSO and 108 µL of KOH were added, and the mixture was centrifuged at 10,000 rpm for 3 minutes before reading the absorbance at 620 nm.

For nitric oxide determination, a NO_2 curve [100 µgM] was prepared at concentrations of 0, 1.5, 3.12, 6.25, 12.5, 25, 50, and 100 µL, kept at 4 °C under light-protected conditions and mixed before colorimetric readings. Mouse samples (25 µL aliquots) were used, and 500 µL of concentrated Griess solution was added to all tubes. After incubation for 10 minutes at room temperature in the dark, 500 µL of diluted Griess solution was added, followed by another incubation and reading at an absorbance of 540 nm.

In vitro healing was assessed through the Scratch Wound Healing Assay

The cell cultures were maintained for about 15 days before performing the experiments in which the adaptation of the cells was monitored daily through the formation of the monolayer, changing the medium every 3 days.

In vitro healing was evaluated through the Scratch Wound Assay, which assesses the factors that alter the motility and/or growth of the cells, which can increase or decrease the rate of “healing” of the break (Lampugnani, 1999).

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To determine *in vitro* healing, human and mouse fibroblasts were used: $1x10^6$ cells were seeded in 96-well culture plates in triplicate until 90 % confluence was reached. After reaching the appropriate confluence, a scratch was made in the center of the plate with a yellow automatic pipette tip with a diameter of 0.9 mm. The four treatments (0, 300, 450, and 600µg/ml) of propolis extract in DMEM medium supplemented with 2 % FBS were then applied.

DMEM medium alone was used as a negative control and DMEM medium supplemented with 10 % FBS as a positive control. After the exposure, photographs were taken at 0, 6, 12, 24, and 48 h. Data analysis was performed with an ImageJ analyzer.

Experimental design

For the *in vitro* experimentation, a 4 x 4 experimental design with three replicates was used, corresponding to the four tests established for antioxidant capacity: ABTS, DPPH, FRAP, and total polyphenol content (4) by the concentrations of ethanolic extract of propolis (4).

For ROS and NO determination in mouse macrophages, a concentration of 300 µg/mL EEP was used with a 1x2 design, with three replicates.

Human and mouse fibroblasts were used for the *in vitro* healing experimentation within a 3 x 5 experimental design with three replicates, corresponding to the concentrations of the ethanolic extract of propolis established (3) by the monitoring time (5).

Data analysis

Values were expressed as percentages to determine extract yield and free radical inhibition in antioxidant capacity evaluation, and measures of central tendency (means) were used for determining polyphenol concentration, antioxidant capacity, ROS, and NO levels. ImageJ and GraphPad Prism 9 software were employed for analyzing cellular monitoring images (% healing) and comparing means of healing levels of human and mouse fibroblast cell lines at different concentration intervals and exposure times. Two-way ANOVA with Dunnett's post-hoc test was conducted, with $p < 0.001$ considered statistically significant.

Results and Discussion

Table 1 shows the initial and final weight, as well as, the yield percentage of the EEP after being exposed to a vacuum oven at 40 °C. The data obtained generated a yield of 47.51 %, according to the formula used for the percentage yield of ethanolic extract of propolis. Although NOM-003-SAG/GAN-2017 for the production and specifications in the processing of propolis does not have an established parameter for this result, a high percentage yield may indicate a greater possibility of obtaining a greater amount of active components such as polyphenols to which antioxidant properties are attributed.

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Rodriguez *et al*. (2020) found similarities in EEP from Michoacan with a 43 % yield. This association contradicts what is established in the literature, since even though both propolis have different origins, flora, and surrounding fauna, among other factors, the difference in yields is minimal. Similarly, it is important to highlight that the bioactive compounds proportions such as polyphenols, terpenoids, steroids, and amino acids may differ for this reason. In another study conducted by Delgado Aceves *et al*. (2015) in Jalisco, Mexico, a yield of 33 % was found. Similarly, the propolis in this study showed a better percentage compared to another whose origin is very different. The percentage yield is a crucial indicator since the quality of the raw material determines the quality of the raw material; the higher the value of this fraction, the better the final product, since the compounds of interest are present therein. Therefore, it is evident that all of the above suggests a synergistic biological activity of the different compounds.

Table 1. Difference for the yield of the ethanolic extract of propolis in grams of dry base (gdb).

Initial weight (g)	*Final weight (g)*	*Yield %*
25	11.879	47.51

Total polyphenol content of ethanolic extract of propolis

The polyphenolic compounds presence in an extract is directly related to antioxidant capacity as they constitute the main classes of plant secondary metabolites associated with this property (Rodriguez Perez *et al.,* 2020).

The values of the total polyphenol content of EEP between 5000 and 320 µg/mL are shown in Table 2, where the results of the concentrations ranged from 3771.8 to 241.39 mgEAG/gbs.

Table 2. Content in milligram equivalents of gallic acid (mgEGA)/ grams of dry basis (gdb) of the concentrations of the ethanolic extract of propolis (EEP).

Concentration [µg/mL]	*mgEGA/gdb*
5000	3771.8
1000	754.36
620	467.70
320	241.39

Karapetsas *et al*. (2019) reported that Greek ethanolic extract of propolis (EEP) at concentrations equivalent to 5000 and 100 µg/mL contained polyphenol content ranging from 4114 to 107.73 mgEAG/gbs. Despite similarities in factors affecting these components, such as collection method, storage, solvent, and extraction method, other factors, notably geographical origin and collection season, could significantly influence the observed results. For instance, the

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Greek propolis was collected and stored in spring, whereas the propolis in this study was obtained in winter. Temperature variations, as documented in the literature, can have a significant impact on the total polyphenol content and consequently on antioxidant capacity. However, despite potential environmental differences, both propolis samples exhibited similar antioxidant capacities.

In another study conducted by Talla *et al*. (2014) with propolis from Cameroon, Africa, comparisons were made between different solvents, revealing a concentration of 4510 mgEAG/gbs in ethanolic extracts. Although ethanol was not the solvent yielding the highest polyphenolic content in the research, these findings align with the scientific literature, highlighting the significant influence of both the solvent choice and the extraction method on the total polyphenol content determination.

Antioxidant activity of ethanolic extract of propolis

DPPH (2,2-diphenyl-1-picrylhydrazyl)

For the IC_{50} in the DPPH radical by monitoring its reduction by an antioxidant with the decrease in absorbance and change in coloration from purple to yellow, the result was 4020 µg/mL (Table 3). This result was obtained based on Equation 4. A correlation between the determination of total polyphenols and the antioxidant activity in the DPPH and ABTS methods is also observed, as well as the inhibition percentage of both methods increases as the concentration of propolis extract increases.

Table 3. Content in percentage (%) and milligrams are equivalent to Trolox (mgET)/grams dry base (gdb) of the concentrations of the ethanolic extract of propolis (EEP).

***Concentration* [µg/mL]**	***Inhibition (%)***	***mgET/gdb***
5000	62.13	41.33
1000	12.42	8.26
620	7.70	5.12
320	3.97	2.64

In a comparative study of Mexican propolis, Rodriguez *et al*. (2020) conducted research in Veracruz state, where they observed an average inhibitory concentration of 950 µg/mL. The diverse geographical characteristics of both areas, being considered as geographical extremes of the country, including variations in vegetation, climate, and type of forager bee species, would be reasons to attribute the different results.

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ABTS (2,2'-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid)

The decolorization of the ABTS radical by the presence of antioxidant compounds in the extract was performed using the formula for the inhibition percentage and mgET/gbs (Table 4). The IC_{50} was reported at 4800 µg/mL. The percentage inhibition was evaluated by Equation 3.

The results of the ethanolic extract of propolis for ABTS are shown in Table 4, where concentrations from 5000 to 320 µg/mL ranged from 60.53 to 3.87 mgET/gbs.

In this study, a proportional relationship between the total content of polyphenols and the DPPH assay was observed, which was consistent with the ABTS method. However, when comparing the obtained results with other studies conducted in Latin America, significant differences were noted, particularly in the ABTS method. For instance, Benavidez (2017) reported an IC_{50} of 1069 µg/mL for Peruvian propolis, while Yang *et al*. (2011) found an IC_{50} of 42 µg/mL for Chinese EEP. This noticeable diversity is not unique to Latin American propolis but is also observed in propolis from other regions.

Table 4. Content in the percentage of inhibition (%) and milligrams were equivalent to Trolox (mgET)/grams of dry base (gdb) of the concentrations of the ethanolic extract of propolis (EEP).

Concentration [µg/mL]	*Inhibition* (%)	*mgET/gdb*
5000	51.98	60.53
1000	10.39	12.10
620	6.44	7.50
320	3.32	3.87

FRAP (Ferric Reducing/Antioxidant Power)

In the case of the FRAP method, observing the change from colorless to an intense greenish-blue in the presence of antioxidants, an IC_{50} was found at 3190 µg/mL (Table 5), inferring a higher presence of non-enzymatic antioxidants.

Table 5. Content in milligrams equivalent to Trolox (mgET)/grams of dry base (gdb) of the concentrations of the ethanolic extract of propolis (EEP).

Concentration [µg/mL]	*µMET/ gdb*
5000	280
1000	56
620	34
320	17

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Touzani *et al*. (2019) with a hydroalcoholic extract of propolis from Morocco, Africa, reported an IC_{50} of 1080 µg/mL, while Yang *et al*. (2011) found an IC_{50} of 5 µg/mL for EEP. Despite the differences in obtained data, both studies share a commonality: they represent results obtained with lower amounts of propolis. This suggests a higher presence of non-enzymatic components capable of reducing ferric iron to its ferrous form, thereby exhibiting antioxidant activity. Remarkably, propolis from various locations tends to produce good results in this widely used technique.

Analysis of reactive oxygen species (ROS) and nitric oxide (NO) concentration in mouse macrophages

ROS and nitric oxide concentrations, when treated with EEP at a concentration of 300 µg/mL, exhibited an inhibition percentage of 32.96 % and 39.93 %, respectively, as illustrated in Figures 1 and 2.

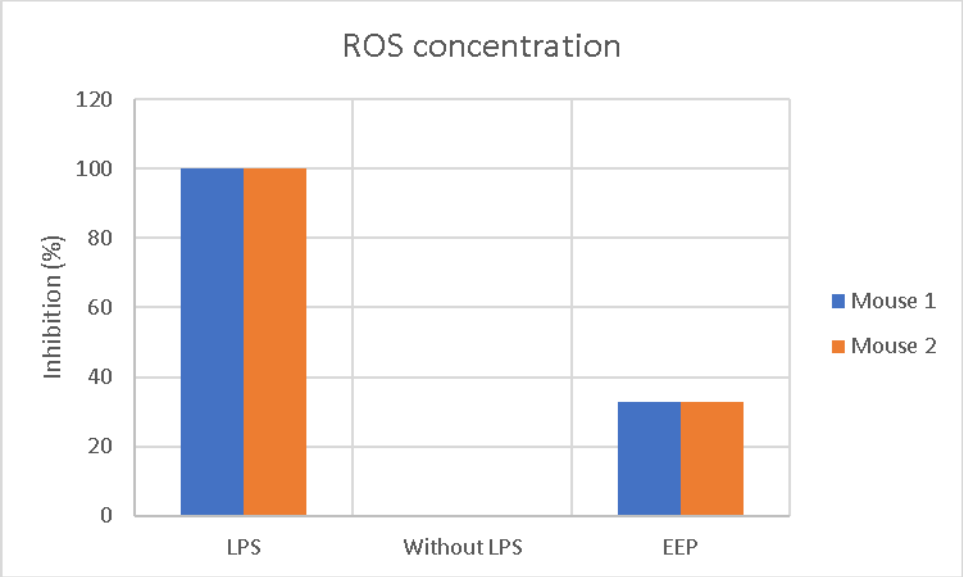

Figure 1. Samples treated with positive control (LPS), negative control (without LPS), and the ethanolic extract of propolis (EEP) in which 32.96 % inhibition was shown.

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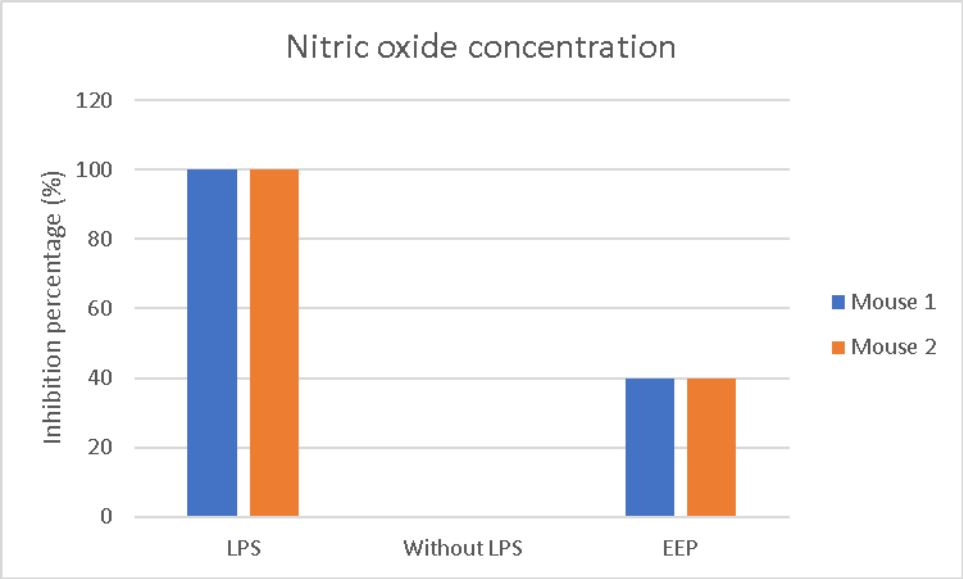

Figure 2. Samples treated with the positive control (LPS), negative control (without LPS), and the ethanolic extract of propolis (EEP) in which 39.93 % inhibition was shown.

The ethanolic extract of Iranian Propolis demonstrated that mouse macrophages treated at concentrations of 0.15 µg/mL significantly reduced LPS-induced ROS production (>50 %). However, at higher concentrations (1.5 and 15 µg/mL), they were unable to inhibit these reactive species (Asgharpour *et al.,* 2019), indicating greater inhibition at lower concentrations compared to our findings. However, although in the present study, only one concentration of the extract was exposed, according to the cytotoxicity tests, no toxic effects were observed at higher concentrations. Thus, it could be assumed that employing a higher or lower dose could achieve observed or not such results obtained with Iranian propolis, in which the dose-response effect had an important impact. On the contrary, Eyng *et al*. (2015) added Brazilian EEP in concentrations up to 5000 ppm in the diet of chickens without finding any effect on macrophages or erythrocytes.

In vitro healing through Scratch Wound Healing Assay

The healing percentages of EEP at employed concentrations are shown at different monitoring times in human fibroblasts (Figure 3) and mouse fibroblasts (Figure 4).

In both cell lines, a significant difference ($p < 0.0001$) was observed at 12h between the concentrations of 450 and 600 µg/mL, with the highest percentage of healing observed at 600 µg/mL. Specifically, in human fibroblasts, the healing percentage was 37.23 %, while in

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mouse fibroblasts, it reached 57.45 % at 48h. However, none of the administered concentrations achieved complete healing within the determined monitoring time.

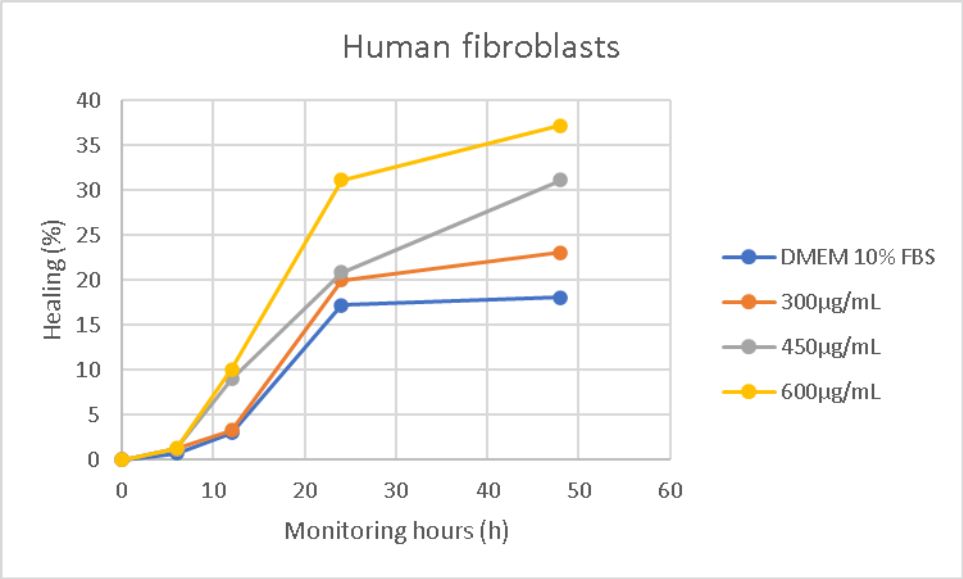

Figure 3. Healing percentages at different EEP concentrations at different time intervals in human fibroblasts.

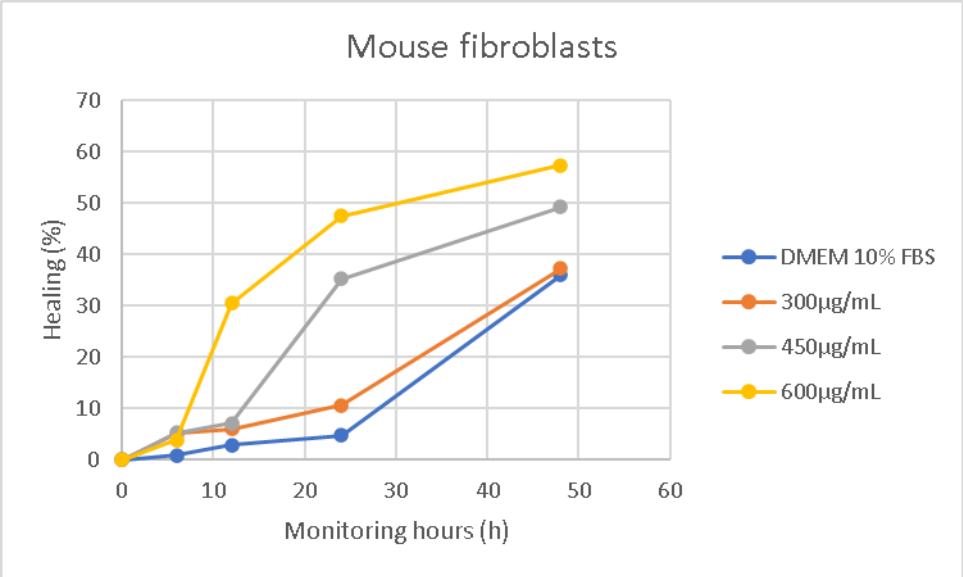

Figure 4. Healing percentages at different EEP concentrations at different time intervals in mouse fibroblasts.

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Jacob *et al*. (2015) investigated the effects of Malaysian propolis on human fibroblasts, at a 250µg/mL concentration, a rapid migration rate was observed, while the maximum proliferation occurred at 500 µg/mL. Significant differences were observed between these two concentrations after 12 hours of exposure, and after 48 hours, the maximum dose used showed the best effect. However, none of the concentrations resulted in complete monolayer formation, indicating that a total scar formation did not occur by the end of the test.

In contrast, Elkhenany *et al*. (2019) found that nearly 85 % of wound closure was achieved by the fourth day of exposure to propolis. They suggest that due to the conditions of cell cultures, a 48-hour monitoring period may not typically yield results of 100 % monolayer restorations. Nevertheless, these findings demonstrate the effectiveness of propolis in promoting wound healing in human fibroblasts. It's emphasized that the overall composition of propolis, regardless of its origin, plays a crucial role in its impact on wound closure by *in vitro* simulations.

Conclusion

In conclusion, the levels of polyphenols and antioxidant activity observed in the analyzed propolis are promising and likely contribute to the accelerated healing process observed. The extract also demonstrated inhibition of reactive oxygen species (ROS) and nitric oxide (NO) in mouse macrophages, further confirming its antioxidant properties. These findings support the potential therapeutic use of the ethanolic extract of propolis for wound healing. Future research should include characterization of the extract and *in vivo* studies to validate its efficacy.

Authors contribution

Conceptualization: Téllez-López M.A., García-Lujan C., Martínez-Rojas P.S., Avalos-Soto J. Methodology development: Martínez-Rojas P.S., Pedroza-Escobar D., Castillo-Maldonado I. Software management: Martínez-Rojas P.S., Hernández-González S.I., Olivas-Calderón H.E. Experimental validation and toxicology: Téllez-López M.A., Pedroza-Escobar D., Castillo-Maldonado I. Analysis of results: Martínez-Rojas P.S., Hernández-González S.I., Olivas-Calderón H.E., Téllez-López M.A. Data management: García-Lujan C., Martínez-Rojas P.S., Avalos-Soto J. Manuscript writing and preparation: Téllez-López M.A., García-Lujan C., Martínez-Rojas P.S. Writing, revising, and editing: Martínez-Rojas P.S., Pedroza-Escobar D., Olivas-Calderón H.E., Avalos-Soto J., García-Lujan C., Hernández-González S.I., Castillo-Maldonado I., Téllez-López M.Á. Project manager: Téllez-López M.A.Fund acquisition: Téllez-López M.A., García-Lujan C., Avalos-Soto J.

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Ethical Statements

The authors declare that they have complied with current national and international regulations regarding the ethical handling of animals.

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Conflict of Interest:

The authors declare no conflict of interest.

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