

Article

Chemical Composition, Antioxidant, Analgesic, and Wound-Healing Effects of *Pinus pinaster* Aiton and *Pinus halepensis* Mill Needles: A Natural Approach to Pain and Oxidative Stress Management

Widad Tbatou ^{1,2} , Hassan Laaroussi ¹ , Beybeti Ishagh ¹, Karima El Yagoubi ³, Akissi Zachée Louis Evariste ⁴ , Bruno Eto ² , Badiaa Lyoussi ^{3,*}  and Zineb Benziane Ouaritini ¹

- ¹ Laboratory of Biotechnology, Conservation, and Valorization of Bioresources (BCVB), Research Unit: Api-Phytotherapy, Physiology, Environment, and Health, Department of Biology, Faculty of Sciences, Dhar Mehraz, Sidi Mohamed Ben Abdellah University, Fez 30000, Morocco; hassan.laaroussi@usmba.ac.ma (H.L.); beybeti.ishagh@usmba.ac.ma (B.I.)
- ² Laboratories TBC, Laboratory of Pharmacology, Pharmacokinetics and Clinical Pharmacy, Faculty of Pharmacy, University of Lille, 3, rue du Professeur Laguesse, B.P. 83, 59000 Lille, France
- ³ Laboratory of Natural Substances, Pharmacology, Environment, Modeling, Health, and Quality of Life (SNAMOPEQ), Department of Biology, Faculty of Sciences, Dhar Mehraz, Sidi Mohamed Ben Abdellah University, Fez 30000, Morocco
- ⁴ BioEcoAgro Joint Cross-Border Research Unit 1158, University Lille, 59650 Villeneuve d'Ascq, France
- * Correspondence: lyoussi@gmail.com; Tel.: +212-661354246

Abstract

Pine needles are traditional herbal remedies used for centuries to treat various ailments, including rheumatism, bronchitis, burns, inflammation, and infections. This study aimed to evaluate the antioxidant, analgesic (peripheral and central), and wound-healing activities of *Pinus pinaster* (PPN) and *Pinus halepensis* (PAN) needles while identifying the bioactive compounds responsible for these effects. Phytochemical analysis revealed several phenolic compounds, including p-coumaroylquinic acid, quercetin, narcissin, and myricetin-3-O-glucoside. Both extracts showed strong antioxidant activity, with high total phenolic content (TPC: 384.84 ± 0.84 and 524.46 mg GAE/g DM for PPN and PAN, respectively) and flavonoid content (TFC: 109.44 ± 0.62 and 111.64 ± 0.62 mg QE/g DM, respectively). Peripheral analgesic activity, assessed using the acetic acid-induced writhing test, revealed that PAN (300 mg/kg) significantly reduced pain by 72.3%, while central analgesic effects, evaluated by the tail immersion test, were comparable to the reference drug for both extracts. In vivo wound-healing tests showed accelerated wound contraction and complete closure by day 21, indicating strong regenerative potential. Overall, this study demonstrates that PPN and PAN needle extracts possess significant antioxidant, analgesic, and wound-healing activities, supporting their traditional use and highlighting their potential as natural therapeutic agents for managing oxidative stress, pain, and skin injuries.

Keywords: *Pinus pinaster*; *Pinus halepensis*; bioactive compounds; analgesic effect; antioxidant potential; wound-healing effect; oxidative stress



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1. Introduction

Pain is a complicated and diverse phenomenon that serves as an indicator of tissue damage. It has a substantial impact on the patients' quality of life and can be classified as acute or chronic. The International Association for the Study of Pain (IASP) defines pain as

an unpleasant sensory and emotional experience associated with possible tissue damage [1]. Chronic pain is often associated with inflammatory diseases, neuropathy, and oxidative stress, making its treatment a serious medical challenge [2]. Pain is not only a symptom but also triggers a physiological stress response that leads to further inflammation and cellular damage, which worsens the condition. Painful stimulation increases the production of free radicals and enhances lipoperoxidation, leading to oxidative stress [3].

In fact, oxidative stress is caused by an imbalance between the generation of reactive oxygen species (ROS) and the body's antioxidant defenses, contributing to a variety of pain-related symptoms [3]. Many chronic diseases, including diabetes, cardiovascular disease, and neurodegenerative disorders, are linked to oxidative stress and pain and arthritis [4]. There is an urgent need for the research and development of new, effective, and safer medicinal compounds with analgesic and antioxidant properties.

One of the most painful conditions is burns, which involve severe tissue damage, oxidative stress, and persistent inflammation [5]. For effective burn wound healing, it is important to protect the skin with antioxidants, relieve pain, and prevent infection with antimicrobial support, all of which help promote healthy tissue regeneration [6]. However, conventional treatments for pain relief and wound healing often depend on synthetic drugs, which can lead to a range of side effects when used over time.

Given these concerns, the search for natural products with analgesic, antioxidant, and wound-healing properties has emerged as a promising alternative. Plants used in traditional medicine, in particular, have drawn growing interest from researchers aiming to identify new treatments that are both effective and safe [7]. In fact, the use of medicinal plants for wound healing and the treatment of various ailments is deeply rooted in both historical practices and modern approaches [8].

The *Pinus* genus is a forest tree classified in the Pinaceae family, which includes many well-known and economically valuable conifers, including cedar, fir, hemlock, larch, pine, and spruce. They grow in temperate climates and can also be found in various environments, ranging from subarctic to tropical areas, throughout the Northern Hemisphere [9]. Several ethnobotanical studies have been done to investigate the traditional usage of various elements of the pine tree. Some of these studies have documented the use of different pine components to treat respiratory conditions, including infections, bronchitis, colds, and pneumonia. Their applications extend to wound care, prostate and urinary tract infections, gastrointestinal ulcers, inflammation, and infertility. In addition, pine trees are valued for their antiseptic properties and their ability to stimulate the adrenal glands [10,11]. Traditional medicine has been used to treat ailments in developing countries for centuries, although its application differs internationally [11]. In Morocco, pine forests are mainly distributed in the Mediterranean and montane regions, particularly in the Rif and Middle Atlas Mountains. These areas are characterized by favorable climatic and ecological conditions that support the natural growth of pine species. *Pinus pinaster* Aiton and *Pinus halepensis* Mill. are among the most widespread pine species in these regions, where they occur naturally and are traditionally used by local populations for medicinal and therapeutic purposes [12]. The Chaouen region, located in the Rif Mountains, is especially rich in these pine species, making it a suitable and relevant study area for investigating their phytochemical composition and pharmacological potential. Pine resin and turpentine oil are used to treat respiratory disorders such as coughs and colds [13,14]. Pine needle essential oil has antibacterial, diuretic, and rubefacient properties. According to [15], the charred pine wood known as “Kaalo” can serve as an antiseptic [15]. Pine resin with honey can treat wounds, abscesses, gonorrhea, and ulcers and provide relief from burning feelings [16]. Pine resin combined with onion paste can also heal wounds [17].

Pinus species contain phytoconstituents such as terpenes, polyphenols, flavonoids, tannins, and alkaloids, which have diverse biological activities [18]. *Pinus* species include bioactive compounds that address several health concerns, including metabolic diseases [19], pain management [20], and skin maladies [21]. *Pinus* species have significant industrial potential in addition to their therapeutic benefits. Oleoresin derived from *Pinus* species has applications in the cosmetics, food, paint, and pharmaceutical industries [18]. Pine needles have attracted increasing attention because of their rich mix of natural compounds, especially antioxidants and both micro- and macronutrients, that have shown anti-inflammatory, antimicrobial, and antioxidant effects [22,23]. These bioactive compounds show promising potential as natural analgesics and wound-healing agents, making them attractive candidates for therapeutic use [24]. Although pine leaves have been traditionally used for wound healing and pain relief, scientific evidence supporting these effects remains limited and fragmented. In particular, comprehensive studies combining detailed biochemical characterization of pine leaf extracts with experimental evaluation of their biological efficacy are still scarce. Moreover, the relationship between the chemical composition of pine leaf extracts and their therapeutic potential has not been clearly elucidated. The present study aimed to investigate the phytochemical composition of *Pinus pinaster* Aiton and *Pinus halepensis* Mill needle extracts and to evaluate their antioxidant, analgesic (peripheral and central), and wound-healing activities using in vivo experimental models. This work aims to provide scientific evidence supporting the traditional use of these compounds and to highlight their potential as natural therapeutic agents for managing oxidative stress, pain, and skin injuries.

2. Materials and Methods

2.1. Plant Material and Extraction

Pinus pinaster Aiton and *Pinus halepensis* Mill needles were gathered from Chaouen in the northern part of Morocco (Latitude: 35°10'39" N, Longitude: 5°16'11" W) in February 2023. Only healthy, fully developed needles were selected for the study. Prof. Hamid Abdelhalim Khamar taxonomically authenticated the species at the Scientific Institute of Rabat, a major research center dedicated to the study of Moroccan flora. Voucher specimens were deposited at the National Herbarium of the Scientific Institute, Rabat, Morocco, under the reference numbers (RAB115029) for *Pinus pinaster* Aiton and (RABM5028) for *Pinus halepensis* Mill. Dried in an oven at 40 °C for 48 h until the pine needles of *Pinus pinaster* Aiton and *Pinus halepensis* Mill were finely powdered before extraction. The extraction was carried out using a hydroethanolic solvent (70% ethanol), selected for its efficiency in recovering phenolic compounds. Briefly, a known amount of plant powder was macerated with the solvent at room temperature (25 ± 2 °C) under continuous stirring at 300 rpm for 72 h. The mixture was then filtered using Whatman No. 1 filter paper, and the solvent was removed under reduced pressure using a rotary evaporator at 40 °C with a rotation speed of 120 rpm to obtain the crude extract. The obtained extracts were weighed to calculate the extraction yield and stored at 4 °C until further phytochemical, antioxidant, analgesic, and wound-healing analyses [25].

2.2. Identification of Phytochemicals

2.2.1. Chemical Analysis by Ultra-High-Performance Liquid Chromatography Coupled with Mass Spectrometry (UHPLC-MS)

UHPLC-MS/MS analysis was performed using the Acquity UPLC H-Class Waters® system (Guyancourt, France), equipped with two pumps, a controller, and a diode array detector (DAD) coupled to a QDa electrospray quadrupole mass spectrometer. Ionization was carried out in negative mode over a mass range of 50–1000 Da, with a cone voltage

of 15 V and capillary voltage of 0.8 kV. The column temperature was maintained at 30 °C, with a flow rate of 0.5 mL/min. The stationary phase was a 2.6 µm Uptisphere C18-AQ column (2.1 × 100 mm). The mobile phase consisted of ultrapure water + 0.1% formic acid and acetonitrile + 0.1% formic acid, with the following gradient: 5–60% B (0–7.59 min), 100% B (8.0–10.59 min), and 5% B (11–13 min) [26]. Wavelengths were monitored from 200 to 790 nm with 1.2 nm accuracy. 1 mg of extract was dissolved in 1000 µL of HPLC-grade methanol and centrifuged for 5 min. 2 µL of supernatant was injected for analysis [27].

2.2.2. Examination of Total Phenolic Content (TPC)

The TPC of all extracts was calculated using the procedure described in our earlier article [25]. After that, 100 µL of the extract was placed in a test tube along with 500 µL of Folin–Ciocalteu reagent and 400 µL of 7.5% sodium carbonate. The sample's absorbance was then measured at a wavelength of 765 nm using a Perkin Elmer Lambda 40 UV/Vis spectrophotometer after two hours at room temperature and in the dark. To generate the calibration curve, gallic acid samples ranging from 0 to 1 mg/mL were examined ($y = 1.6724x + 0.0054$; $R^2 = 0.99$). TPC was calculated and expressed as milligrams of gallic acid equivalent per gram of plant dry matter (mg GAE/g DM).

2.2.3. Examination of Total Flavonoid Content (TFC)

The total flavonoid content was measured by adding 500 µL of extract to 500 µL of a 20 mg/mL solution of $AlCl_3$, letting it sit in the dark for thirty minutes. The plot for the standard curve showed that quercetin's absorbance increased linearly from 0 to 1 mg/mL ($y = 2.2688x - 0.0697$; $R^2 = 0.99$). The optic density was determined at 510 nm [25]. Total flavonoids in a sample were described by comparing their weight to the amount of quercetin in the same amount of dried plant matter (mg QE/g DM) [25].

2.3. In Vitro Antioxidant Assays

2.3.1. 2,2-Diphenyl-1-Picrylhydrazyl Radical Assay (DPPH)

Samples of the extract were different in concentration, and all were mixed with a 150 µM DPPH solution, and then the absorbance was measured. Then, the researchers left the mixture at room temperature in the dark for half an hour [28]. When the incubation period ended, the absorbance at 515 nm was measured, and then a particular Equation (1) allowed us to find the antiradical activity (% inhibition). The graph of inhibition was used to calculate the IC_{50} for DPPH inhibition, and the results were written in milligrams of extract per milliliter (µg/mL). Ascorbic acid was used as the positive control in this experiment.

$$\text{Inhibition \%} = \frac{Ab - Aa}{Ab} \times 100 \quad (1)$$

Ab: The optical density value of the control (-).

Aa: The optical density value of the extract.

2.3.2. Azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) Radical Scavenging Activity (ABTS Assay)

Fifty microliters of each pine needle extract at different dilutions were mixed with 825 microliters of the ABTS radical cation solution. After 30 min in the dark, the Perkin Elmer Lambda 40 UV/Vis spectrophotometer was used to determine the amount of color created in the sample by measuring absorbance at 734 nm. The results obtained using Equation (1) were described as IC_{50} data in mg/mL [29].

2.3.3. Total Antioxidant Capacity (TAC)

The total antioxidant capacity (TAC) of the extracts was assessed using the phosphomolybdenum approach [29]. In brief, 1000 µL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was mixed with 50 µL of the extracts. Using a Perkin Elmer Lambda 40 UV/Vis spectrophotometer, the sample was incubated in a water bath for 90 min at 95 °C before its absorbance at 695 nm was measured against a blank. The results are expressed as the average of three replicates ± standard deviation (SD) in milligrams of ascorbic acid equivalents (AAE) per gram of dry plant (mg AAE/g DW).

2.4. Pharmacological Investigations

2.4.1. Animal Protocol

Male Swiss mice (25–30 g) ($n = 6$) and Wistar rats (200–230 g) ($n = 6$), and old, 6–8 weeks, were procured from the Faculty of Sciences' Animal Breeding Centre at Dhar El-Mahraz, USMBA in Fez, Morocco. The animals were housed in a controlled setting, with a temperature of 26 ± 2 °C and a 12-h light/dark cycle. They had unrestricted access to tap water and were fed regular rat food throughout the experiment. During the experience, no blinding was used. The research knows the group allocations. No euthanasia was performed in this study because no invasive sampling or organ harvesting was performed. Animals were treated and monitored throughout the experiment and returned to the animal facility in good condition at the end of the study. The ethical protocols used in this investigation were authorized by the institution's animal protection committee, following the guidelines described in the ethical approval registration number L.20. USMBA-SNAMOPEQ 2023-03.

2.4.2. Analgesic Effect of Pinus Needle Extracts

To evaluate the analgesic activity of *Pinus pinaster* Aiton (PPN) and *Pinus halepensis* Mill. (PAN) needle extracts, all animals were allowed to adapt to the experimental conditions for 7 days before the analgesic experiments, with free access to food and water under controlled temperature and light–dark cycles. The acetic acid-induced writhing test was performed according to [30] with minor modifications. Thirty-six male Swiss mice (25–30 g) were randomly assigned to six experimental groups ($n = 6$ per group) [26]:

- Control group—received distilled water (vehicle).
- Standard drug group—received diclofenac sodium (50 mg/kg b. wt.).
- PPN low dose (D1)—100 mg/kg b. wt.
- PPN high dose (D2)—300 mg/kg b. wt.
- PAN low dose (D1)—100 mg/kg b. wt.
- PAN high dose (D2)—300 mg/kg b. wt.

Treatments were administered orally via gavage 30 min before the intraperitoneal injection of 1% acetic acid (10 mL/kg b. wt.) to induce nociceptive abdominal contractions (writhing). The number of writhings and stretches was counted for 10 min, starting 5 min after acetic acid injection. The percentage inhibition of writhing was then calculated using the following Equation (2):

$$PP \% = \frac{Mc - Mt}{100} \times 100 \quad (2)$$

Mc: The mean number in the control group.

Mt: The standard group or PPN, and PAN extract-treated group.

2.4.3. Tail Immersion Experiment with Rats

The approach given by [26] was employed to carry out this test. Male Wistar rats ($n = 6$ per group) were randomly assigned to six experimental groups. For the test, a 3 cm tail section was immersed in hot water at 55 ± 0.5 °C. Within a few minutes, the rats responded by removing their tails. The reaction time was measured with a stopwatch. The animals were given either PPN or PAN at two doses (100 mg/kg or 300 mg/kg), plain water, or a standard medication (diclofenac sodium at 50 mg/kg) 30 min before the tail was immersed. The reaction times were collected at 30 min, 60 min, and 120 min after delivering the various treatments [31].

2.4.4. Wound Healing Effect

Enrichment of Ointments with Pine Needle Extract

The enriched ointment was made at a 10% (w/w) ratio by combining 1 g of every extract from each plant with 9 g of Vaseline®. The dry extracts were progressively mixed with the Vaseline® in a beaker and heated to 50 °C while stirring until the mixture was completely homogenized. The resulting extract-based ointments were then placed in sterile opaque containers and refrigerated at 4 °C [27,31].

Burn Wound Induction

To induce the wound, we began by anesthetizing the rats with pentobarbital (63 mg/kg, intraperitoneally). Then we shaved the dorsal region of each rat and, using a 1.7 cm metal rod heated in hot water (50 °C) to induce the burn wound, placed it for 10 s. Afterwards, we divided the rats into four groups ($n = 6$, $N = 24$ rats). Group 1 acted as the negative control group, receiving Vaseline®. Group 2 received a therapeutic ointment called Madecassol® at a concentration of 1%. Rats in groups 3 and 4 applied two kinds of pine needle extract ointments (PPN and PAN). Ointment was coated on the wound every day, covering the entire site for three weeks [19]. We took pictures of the wounds with a digital camera and analyzed the wound sizes using the methods and software provided by Image J® software v.1.5. Then, each type of treatment was analyzed to determine its healing rate. The measurement of the reduction was calculated by comparing the size of the last wound to the size of the first wound based on Equation (3).

$$\% Wh = \frac{\text{Wound area on day 0} - \text{Wound area on day } n}{\text{wound area on day 0}} \times 100 \quad (3)$$

%Wh: percentage of wound healing, and n: number of days, with values of 5, 10, 15, and 21.

2.5. Statistical Analysis

All analyses of data were performed using GraphPad Prism version 8, one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons. Differences were considered statistically significant at $p < 0.05$. Experts regarded a p -value of less than 0.05 to be significant.

3. Results and Discussion

3.1. Phenolic Screening of PPN and PAN Using HPLC-MS

Experiments on HPLC-MS were carried out to find out what phenolic compounds are contained in the extract from PPN and PAN needles. To determine the phenolic compounds, experts analyzed their retention periods and checked the standard [M – H]– ions. Using this method, 15 types of phytochemicals were identified. The findings are presented in Table 1 and Figure 1, and the HPLC chromatograms display different peaks.

As we can see, nine flavonoid peaks from various subclasses were identified based on their retention times and mass spectral data. Four flavonol glycosides were characterized in the investigation of the two *Pinus* needle species. Regarding flavonoids, myricetin-3-O-glucoside (m/z 479.28) and quercetin-3-O-glucoside (m/z 463.33) were common to both species. Narcissin (m/z 623.25) was also detected in both *Pinus* species, while Laricitrin rutinoside (m/z 639.27) was only present in *P. halepensis*. In addition, among the detected p-O-Coumaroylquinic acid and its derivatives were found in both *Pinus* species, with a molecular ion at m/z 337.21. Protocatechuic acid (m/z 153.13) was exclusively present in *P. pinaster*. While the chromatographic profiles are qualitatively similar, it should be noted that the signal intensity in sample PAN is approximately 2.3 times higher than in sample PPN, as reflected by the different Y-axis scales in Figure 1. Several unidentified (N.I.) compounds were also detected, with molecular ions ranging from m/z 535.32 to 605.39. These results are consistent with previous studies. For instance, ref. [32] analyzed needle extracts from six pine species (*P. sylvestris*, *P. densiflora*, *P. pinaster*, *P. strobus*, *P. koraiensis*, and *P. pinea*) and reported similar phenolic profiles, including p-coumaric acid (4118.4 ± 25.7 $\mu\text{g/g}$), protocatechuic acid (97.7 ± 5.1 $\mu\text{g/g}$), quercetin galactoside (76.4 ± 2.9 $\mu\text{g/g}$), and kaempferol glucoside (1185.6 ± 33 $\mu\text{g/g}$) in *P. pinaster*. The presence of these compounds in both PPN and PAN may explain the observed antioxidant properties of the extracts, as flavonol glycosides are known for their radical scavenging and metal-chelating activities. Notably, the exclusive detection of laricitrin rutinoside in *P. halepensis* suggests species-specific differences that could contribute to variation in bioactivity.

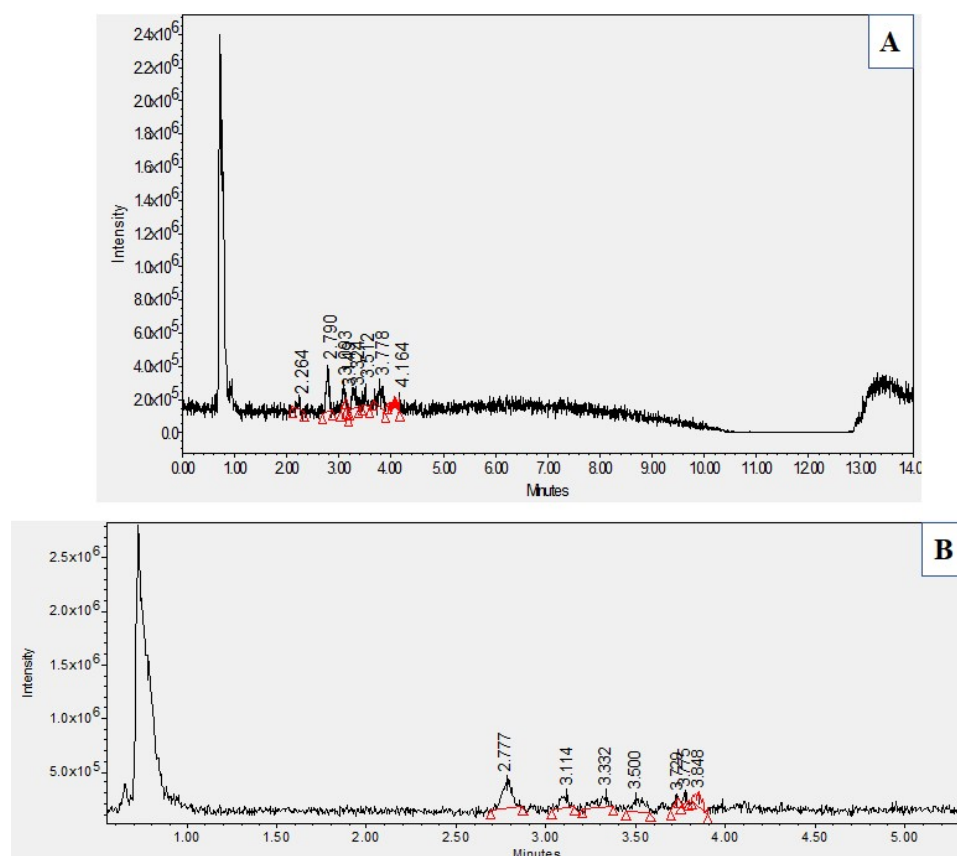


Figure 1. Representative HPLC-MS chromatograms of *Pinus pinaster* Aiton. (A), and *Pinus halepensis* Mill. (B). The compounds' identities, retention values, and MS data of all peaks are listed in Table 1. Red triangles indicate reference peaks corresponding to the compounds reported in Table 1. The Y-axis scales are set independently for each chromatogram to ensure the clear visualization of low-intensity peaks and to allow for an accurate qualitative comparison of retention times.

Table 1. Identification of biomolecules using HPLC-MS-MS of *Pinus* needles extracts.

No.	Tr (min)	Compounds	Class	[M – H] – (m/z)	Molecular Formula	<i>Pinus halepensis</i>	<i>Pinus pinaster</i>
1	2.777	p-O-Coumaroylquinic acid	Hydroxycinnamic acid	337.21	C ₁₆ H ₁₈ O ₈	+	+
2	3.332	Narcissin	Flavonoid	623.25	C ₂₈ H ₃₂ O ₁₅	+	+
3	3.500	Myricetin-3-O-glucoside	Flavonol glycoside	479.28	C ₂₁ H ₂₀ O ₁₂	+	+
4	3.729	Laricitrin rutinoside	Flavonoid	363.28, 364.0, 639.27	C ₂₇ H ₃₀ O ₁₆	+	–
5	3.775	Quercetin-3-O-galactoside and N.d	Flavonol glycoside	463.29, 561.19	C ₂₁ H ₂₀ O ₁₁	+	+
6	3.848	Quercetin-3-O-glucoside and N.d	Flavonol glycoside	463.33, 493.26	C ₂₁ H ₂₀ O ₁₁	+	+
7	2.264	Protocatechuic acid	Phenolic acid	153.13	C ₇ H ₆ O ₄	–	+
8	2.790	p-O-Coumaroylquinic acid	Hydroxycinnamic acid	337.17	C ₁₆ H ₁₈ O ₈	+	+
9	3.149	p-3-O-Coumaroylquinic acid	Hydroxycinnamic acid	337.24	C ₁₆ H ₁₈ O ₈	+	+
10	3.324	2-[3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-2-[2-hydroxy-4-(3-hydroxypropyl)phenoxy]propoxy]-6-(hydroxymethyl)oxane-3,4,5-trio	Lignan	525.38	C ₂₅ H ₃₀ O ₁₂	–	+
11	3.512	Myricetin-3-O-glucoside	Flavonol glycoside	479.25	C ₂₁ H ₂₀ O ₁₂	+	+
12	3.778	Prunin, Quercetin-3-O-glucoside	Flavonoid	433.41, 463.29	C ₁₅ H ₁₂ O ₅ , C ₂₁ H ₂₀ O ₁₁	–	+

Tr (min): retention time (minutes).

3.2. Quantification of Total Flavonoid Content (TFC) and Total Polyphenol Content (TPC)

Table 2 shows that the *Pinus* needles extract in PPN and PAN had high total phenolic content (TPC), with values of 384.84 mg EAG/g DM and 524.46 mg EAG/g DM, respectively. Furthermore, the total flavonoid concentration (TFC) was very high, indicating a complex flavonoid composition, as validated by HPLC-MS analysis. The TFC concentration in the PPN extract was 109.44 mg QE/g DM, while in the PAN extract it was 111.64 mg QE/g DM. The PAN extract has a higher concentration of total polyphenols and flavonoids than the PPN extract. These phenolic and flavonoid compounds are well-known for their potent antioxidant activities, supporting the observed high TPC and TFC levels in both species [33]. Notably, *P. halepensis* exhibited the highest concentrations, which may contribute to enhanced antioxidant potential compared to *P. pinaster*. The HPLC-MS analysis revealed the presence of multiple flavonoids, including flavonol glycosides and hydroxycinnamic acids, providing a chemical basis for the antioxidant activity of these extracts. These findings are consistent with previous studies reporting that pine needle extracts are rich in bioactive phenolics and flavonoids, which are implicated in free radical scavenging and metal chelation mechanisms.

Table 2. Quantification of the Total polyphenol content (TPC) and flavonoid content (TFC) of *Pinus* needles extract.

	TPC (GAE mg/g DM)	TFC (QE mg/g DM)
PPN	384.84 ± 0.84	109.44 ± 0.62
PAN	524.46 ± 2.11 ***	111.64 ± 0.62 ***

PPN: *Pinus pinaster* needles; PAN: *Pinus halepensis* needles. GAE: Gallic Acid Equivalent; QE: Quercetin Equivalent; DM: Dry Matter; *** $p < 0.001$. TPC: Total phenolic content; TFC: Total flavonoid content; GAE: gallic acid equivalents; QE: quercetin equivalents.

3.3. Antioxidant Effect of *Pinus* Needles Extract

In Figure 2, the antioxidant capacity of the *Pinus* needles extract was presented, and it was analyzed by different and complementary tests (DPPH and ABTS scavenging assays) expressed as IC₅₀ value, along with the total antioxidant capacity (TAC) assay. The findings of these tests demonstrated a strong antiradical effect with an IC₅₀ equal to 0.47 ± 0.21 µg/mL in the DPPH radical test. In contrast, the activity of the PAN extract was lower with an IC₅₀ = 1.49 ± 0.01 µg/mL, as the same ABTS assess-

ment showed that the PPN displayed a significant radical scavenging ability, with an IC_{50} value of 2.69 ± 0.06 mg/mL, compared to the PAN extract, which displayed an $IC_{50} = 10.9 \pm 2.1$ mg/mL. For the TAC assay, PAN extract provides a high value, reaching 938.50 ± 23.23 mg EAA/g DM, whereas the PPN extract showed a lower TAC of 182.01 ± 10.10 mg EAA/g DM. These findings show that the PPN extract had higher free radical scavenging activity than the PAN extract in both the DPPH and ABTS assays, despite exhibiting a lower overall antioxidant capacity. The diversity in antioxidant activity could be due to changes in their individual antioxidant molecules as well as the potential interaction effects of bioactive chemicals found in each extract, particularly in their chemical interactions and ability to quench DPPH and ABTS free radicals. Flavonoids and phenolic acids primarily contributed to the antioxidant ability exhibited by the extract in various tests (TAC, DPPH, and ABTS), indicating that both PAN and PPN had strong powers to fight free radicals and provided significant antioxidant activity. This study reported higher values than the ones found by [33], who studied the substances and antioxidative effects present in the needles of *Pinus halepensis* Mill and *P. pinaster* Aiton. It was also found that a second study tested the wound healing, anti-inflammatory, and antioxidant properties, and the chemical content of *maritime pine* needle extract. The antioxidant research found that the DPPH and ABTS tests provided 171.12 and 163.45 μ g/mL values, respectively [34]. Ref. [31] assessed the antioxidant effects of pine needle extracts made using hot water, ethanol, hexane, HWH (a mix of hot water and hexane), and HWE (a mix of hot water and ethanol), using the DPPH radical method. The hot water extract showed better antioxidant activity than the other extracts, registering an IC_{50} of 0.27 ± 0.00 mg/mL.

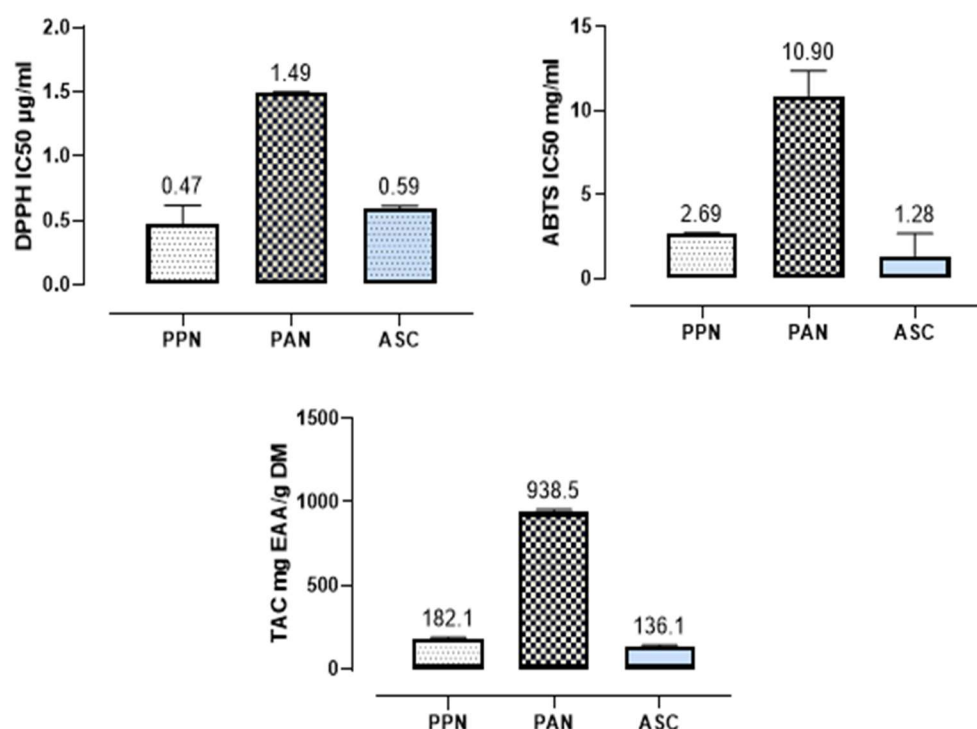


Figure 2. Antioxidant activity of different *Pinus* needle extracts. IC_{50} : Inhibition concentration 50; EAA: Equivalent Ascorbic acid; DM: Dry Matter; PPN: *Pinus pinaster* needles; PAN: *Pinus halepensis* needles. Each value is reported as mean $\pm$ SD.

The potent antioxidant capacity of PAN and PPN plays a crucial role in reducing pain sensation through an analgesic effect and enhancing wound healing potential, as observed in the *in vivo* assessment. One of the primary causes of inflammation and pain perception is oxidative stress. Research has demonstrated that enhanced formation of reactive oxygen

species (ROS) and other free radicals causes major cell structure and function changes, including DNA damage, protein oxidation, and lipid peroxidation. Specific indications, such as lipid peroxidation products, include 4-hydroxy-2-nonenal (HNE), an extremely cytotoxic aldehyde formed by polyunsaturated fatty acid oxidation, which aids in the detection of excessive ROS levels. Since it forms Michael adducts when it reacts with the amino acids cysteine, histidine, and lysine, HNE analysis can detect lipid peroxidation and protein oxidation [35]. Recently, it was shown that HNE can enhance nociceptor excitability and lead to pain hypersensitivity in response to tissue injury [36].

3.4. Pharmacological Investigations

3.4.1. Analgesic Effect of Pine Needle Extract

After producing pain with 1% acetic acid, we counted the number of abdominal contractions and determined the percentage of protective effect for each treatment group. The findings are summarized in Table 3 and Figure 3. The extracts of *Pinus* needles (PPN and PAN) with two different doses (100 and 300 mg/kg b.wt.) showed a substantial decrease in abdominal contractions due to pain when compared to the control group ($p < 0.05$), indicating a dose-dependent response. PAN, which showed a high amount of TPC and TFC, exhibited the most notable impact in reducing writhing and stretching, achieving a high percentage of protection at the lower dose (46.4%) and the higher dose (72.3%), which demonstrates its most significant potential. Diclofenac sodium (the reference medicine) demonstrates a strong protective impact, with a protection percentage of 29.6%; however, this is still lower than our extract.

Table 3. Protective effect (%) of *Pinus* needle extracts against acetic acid-induced pain.

Treatments	Dose (mg/kg b. wt.)	Protection %
Control	0	0%
Diclofenac Sodium	50	29.5% ****
PPN D1	100	28.6% ****
PPN D2	300	39.3% ****, ###
PAN D1	100	46.4% ****, ####
PAN D2	300	72.3% ****, ####

PPN D1: *P. pinaster* needles dose 1; PPN D2: *P. pinaster* needles dose 2; PAN D1: *P. halepensis* needles dose 1; PAN D2: *P. halepensis* needles dose 2; b. wt: Body weight. ****: $p < 0.0001$. ###: $p < 0.001$, ####: $p < 0.0001$.

The analgesic activity of *Pinus* extracts can be explained by their modulation of both peripheral and central pain mechanisms. The brain and spinal cord significantly influence central pain processes. The dorsal horn of the spinal cord contains numerous neurotransmitters and receptors, such as substance P, somatostatin, neuropeptide Y, inhibitory amino acids, nitric oxide (NO), endogenous opioids, and monoamines. It is also a major source of pain and inflammation [37,38]. The acetic acid-induced writhing model simulates pain sensation by inducing a local inflammatory reaction [26]. This painful stimulus releases free arachidonic acid from tissue phospholipids [39]. The writhing reaction caused by acetic acid is a sensitive test for testing peripherally acting analgesics. The reaction seems to be mediated by acid-sensing ion channels, peritoneal mast cells [40], and the prostaglandin pathway [41]. The potent analgesic effect of PPN and PAN extracts is likely linked to their high content of polyphenols and flavonoids, which possess anti-inflammatory and antioxidant properties. By scavenging reactive oxygen species and modulating inflammatory mediators, these bioactive compounds reduce nociceptor sensitization and inflammatory pain, as reflected in the significant decrease in writhing and stretching behaviors. Overall, PAN demonstrated superior analgesic efficacy, consistent with its higher TPC and TFC levels.

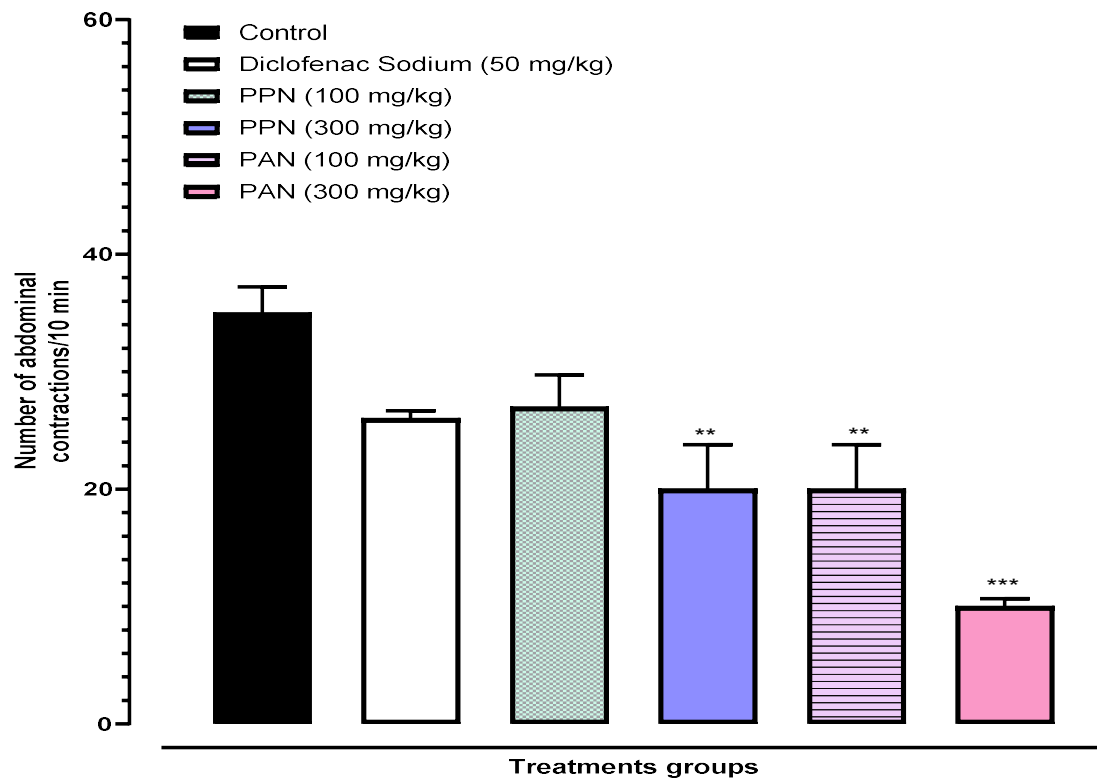


Figure 3. Analgesic effect of Pinus needles extracts at two doses (100 and 300 mg/kg b.wt.) and the standard drug diclofenac. Data are expressed as means $\pm$ SEM ($n = 6$). PPN: *P. pinaster* needles; PPN: *P. pinaster* needles; PAN: *P. halepensis* needles; PAN: *P. halepensis* needles. ** $p < 0.001$; *** $p < 0.0001$.

3.4.2. Tail Immersion Experiment with Rats

We used the tail immersion assay to assess the analgesic effects of our extracts. This commonly employed method measures acute thermal pain by immersing the last 3 cm of the tail in water heated to 55 °C [42]. The tail immersion test is a widely used method for quantitatively and consistently assessing acute thermal pain across various treatment groups. The latency of the tail withdrawal reflex was recorded at 30 min, 60 min, and 120 min. The data in Table 4 demonstrate the effectiveness of different doses (100 mg/kg and 300 mg/kg) of Pinus needle extracts (PPN and PAN) in alleviating this pain condition.

Table 4. Protective effect of Pinus needles extract (PPN, PAN) on the tail withdrawal response triggered by tail immersion.

Treatment	30 min	60 min	120 min
Control	2.86 $\pm$ 0.56	97.31 $\pm$ 88.5 ⁺⁺⁺⁺	101.1 $\pm$ 90.6
Diclofenac Sodium	127.94 $\pm$ 81.38 ^{****}	150 $\pm$ 78 ^{****, +++}	157.7 $\pm$ 87.6 ^{****}
PPN D1	3.73 $\pm$ 1.55 ^{*, #####}	181.67 $\pm$ 23.1 ^{****, ###, +++++}	186.9 $\pm$ 22 ^{****, #####}
PPN D2	9.33 $\pm$ 2.45 ^{***, #####}	236.33 $\pm$ 43.9 ^{****, #####, +++++}	257.2 $\pm$ 43.9 ^{****, #####}
PAN D1	10.58 $\pm$ 3.43 ^{***, #####}	189.63 $\pm$ 154.2 ^{****, #####, +++++}	198 $\pm$ 162.7 ^{****, #####}
PAN D2	28.69 $\pm$ 15.82 ^{***, #####}	289.67 $\pm$ 133.6 ^{****, #####, +++++}	289.3 $\pm$ 127.8 ^{****, #####}

Reaction time in seconds (s). Results are expressed as mean $\pm$ SEM. PPN D1: *P. pinaster* needles dose 1; PPN D2: *P. pinaster* needles dose 2; PAN D1: *P. halepensis* needles dose 1; PAN D2: *P. halepensis* needles dose 2; b. wt: Body weight, *: As compared to the control group. * $p < 0.1$, *** $p < 0.0001$, **** $p < 0.00001$, ##### $p < 0.00001$, +++ $p < 0.0001$, +++++ $p < 0.00001$.

As reported, we can see in Table 4 that this is the case within the first 30 min. All groups did not show any notable variations compared to the control group. However, after 60 and 120 min, PAN reduced the pain sensation over a prolonged time in hot water before the tail withdrawal reflex at both tested doses. Diclofenac Sodium, a typical analgesic, exhibited a strong effect, particularly within the first 30 min, with a withdrawal latency of 127.94 ± 81.38 s. The impact increased over time. This result supports the analgesic effect of Pinus needle extract in pain management. Notably, the high dose of *Pinus halepensis* needle extract (PAN D2) has the most pronounced effect, with a withdrawal latency of 289.3 ± 127.8 s in 120 min, comparable to the other treatment groups. The current result supports the findings presented in Table 4.

The tail immersion assay is a selective method for studying compounds interacting with opioid receptors [42]. The findings of this study support the analgesic effect of Pinus needle extract in pain management. Specifically, the high dose of PAN D2 of *Pinus halepensis* needle extract yielded a withdrawal latency of 289.3 ± 127.8 s during a 120 min observation period. The ability of rats to endure pain caused by hot water (55 °C) significantly increased over time, indicating a centrally mediated analgesic mechanism, which may be due to an activation of endogenous central inhibitory systems, potentially via interaction with opioid receptors, monoaminergic systems, or other neuromodulator pathways, leading to a suppression of pain perception. This model, which is known as the tail immersion test, as we mentioned before, is susceptible to centrally acting analgesic agents, such as opioids. The increased latency reflects the tested substance's capacity to interfere with the central processing of nociceptive information [42]. Central analgesia affects pain signaling at both spinal and supraspinal levels by adhering to μ -opioid receptors in key areas of the nervous system, such as the periaqueductal gray, rostral ventromedial medulla, dorsal horn of the spinal cord, and thalamus [37]. When engaged, these receptors inhibit adenylate cyclase function via Gi/o proteins, resulting in lower intracellular cAMP levels. This activation inhibits voltage-gated Ca^{2+} channels, causing presynaptic inhibition, and opens K^{+} channels, resulting in postsynaptic hyperpolarization [43]. Overall, these actions suppress the release of glutamate and substance P and slow down the activity of neurons [44]. Besides the opioid system, central analgesia can stimulate downward blocking pathways. The PAG, part of the brainstem, and the larger area known as the RVM act as the source of these systems. From that point, nerve signals reach the spinal cord and stimulate it to release serotonin (5-HT) and norepinephrine (NA). As a result, these neurotransmitters activate certain interneurons that keep the brain from feeling pain. α 2-adrenergic, 5-HT1A, and GABAergic receptors take part in this process by reducing the electrical activity sent by nerve cells involved in pain [44].

Possibly, its ability to neutralize free radicals is why pine needle extract can decrease the generation of inflammatory substances, including prostaglandins and cytokines. Yen and his team investigated the ability of pine needles to protect against LDL oxidation and have anti-inflammatory effects via regulating the quantity of nitric oxide (iNOS) and cyclooxygenase 2 (COX-2) in lipopolysaccharide (LPS)-stimulated macrophages [45]. The effects observed may result from the ability of pine needle extract to neutralize free radicals, which helps reduce the production of inflammatory mediators such as prostaglandins and cytokines. These findings indicate that pine needle extract can effectively prevent the oxidation of LDL and decrease nitric oxide production in vitro. Its antioxidant properties may play a key role in protecting LDL from oxidative damage. Furthermore, downregulation of iNOS and COX-2 at the protein and mRNA levels inhibited nitric oxide production in LPS-stimulated cells [45]. Moreover, the flavonoid content may increase the amount of endogenous serotonin or act upon 5-HT2A and 5-HT3 receptors, which may be involved in the central analgesic activity mechanism [46]. HPLC analysis of PPN and PAN extracts revealed

the presence of various polyphenols such as quercetin (flavonol), epicatechin (flavanol), and p-coumaric acid (hydroxycinnamic acid), which inhibited LPS-induced production of inflammatory mediators nitric oxide (NO) and cytokines in RAW 264.7 macrophages [33]. Inhibition of NO and cytokine production is particularly relevant to our study, as over-expression of these mediators plays a key role in central sensitization and inflammatory responses [33]. NO enhances nociceptive signaling in the spinal cord, while cytokines such as TNF- α can increase neuronal excitability in the dorsal horn. This suggests that the bioactive compounds in our pine needle extracts may contribute to the centrally mediated antinociceptive effects observed in the tail immersion test, likely through the suppression of neuroinflammatory pathways [47].

In addition, some studies reported that p-coumaric acid was effective in delaying LDL oxidation, as reflected by an increase in the lag time required for linoleic acid hydroperoxide and 7-ketocholesterol formation and consumption of cholesterol-1-linoleic acid [48]. Regarding the bioactive effects of epicatechin, Ref. [49] found that epicatechin inhibited LDL oxidation and prevented damage by transporting oxidized LDL to endothelial cells [49]. Purin compounds identified in our pine needles extract were examined for their analgesic effect using in vivo models (writhing syndrome and formalin tests) [50]. This mechanism could explain the significant analgesic effects observed, with pine needles showing a higher analgesic effect, possibly due to their higher flavonoid content, interacting with both opioid and non-opioid pain pathways.

3.4.3. Wound Healing Effect

In this study, a second-degree burn wound model was employed to evaluate the healing potential of *Pinus pinaster* (PPN) and *Pinus halepensis* (PAN) needle extracts formulated at 10% in Vaseline, with Madecassol serving as a reference treatment. The diameter of the excision wound was measured every five days, with pictures taken on days one, five, ten, fifteen, and twenty-one.

The data from this investigation revealed that by day 5, the diameter of the wound was notably reduced ($p < 0.01$) in the groups administered PPN and PAN compared to the control group (Figure 4). The percentage of wound healing was 35% for PPN and 48% for PAN (Table 5). By day 15, the wounds were nearly closed, demonstrating rapid epithelialization, with remarkable percentages of wound healing of 86% for PPN and 85% for PAN. The diameter of the scars had significantly decreased, with a $p < 0.001$, indicating a significant acceleration in healing compared to both Madecassol and the control group. On day 21, as shown in Figure 5, the wounds were completely healed, with a 100% healing percentage for both PPN and PAN. In contrast, the Madecassol treatment resulted in a 71% closure rate by day 21. While Madecassol improved wound closure relative to the control group, it did so at a slower rate than PAN and PPN therapies, and the difference was not statistically significant.

Table 5. The wound healing % of *Pinus* needles extract.

Days	Control	Madecassol 1%	PPN 10%	PAN 10%
Percentage (%) of Wound Healing Effect				
Day 5	4	6	35	48
Day 10	16 ****	36	56 ****	61 ****
Day 15	34 ****, #####	57 ****, #####	86 ****, #####	85 ****, #####
Day 21	45 ****, #####	71 ****, #####	100 ****, #####	100 ****, #####

Control (Group 1), Madecassol 1% standard drug (Group 2), PPN: *P. pinaster* Needles extract 10% (Group 3), *P. halepensis* needles extract PAN 10% (Group 4). **** $p < 0.00001$, ##### $p < 0.00001$.

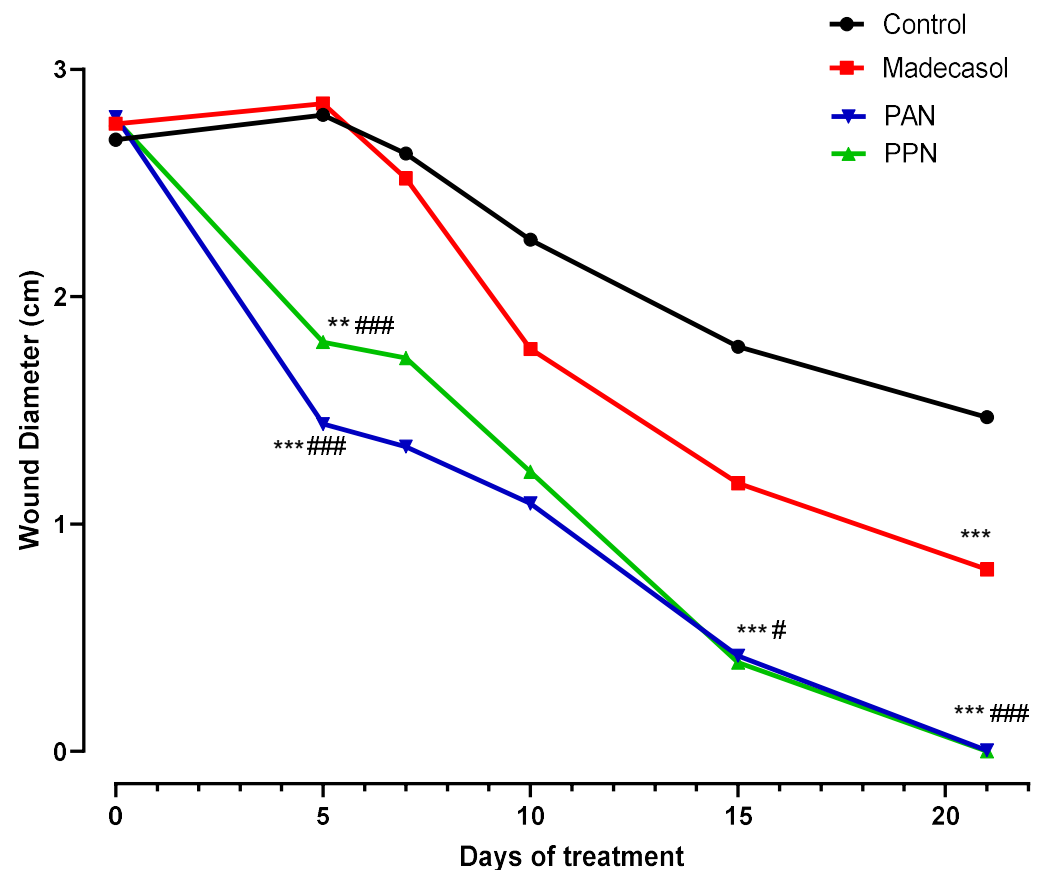


Figure 4. The diameter of the wound area (cm) was measured at 0, 5, 10, 15, and 21 days. Results are presented as mean $\pm$ SD (n = 6). Control (Group 1), Madecassol 1% standard drug (Group 2), PPN: *P. pinaster* needles extract 10% (Group 3), *P. halepensis* needles extract PAN 10% (Group 4). ns: no significant difference between PPN and PAN groups. ** $p < 0.001$; *** $p < 0.0001$, # $p < 0.01$, ### $p < 0.0001$.

Wounds impair the functional continuity of cells and tissues around the injury site. Physical, chemical, microbiological, or immunological processes can all contribute to their occurrence. Both humans and animals possess the inherent ability to heal wounds through continuous tissue repair and regeneration [37]. Curing acute and chronic wounds involves several key phases: hemostasis, inflammation, proliferation, fibroplasia, collagen deposition, epithelialization, contraction, remodeling, and maturation [51]. During the wound healing process, a series of events that aid repair primarily through the activities of activated platelets, neutrophils, and macrophages [52]. This process leads to increased vascular permeability and angiogenesis, which result from various cellular and cytokine-mediated events. Activated cells secrete soluble factors that upregulate the actions of endothelial cells. These factors include fibroblast growth, transforming growth, epidermal, and vascular endothelial growth factors [53]. Substances from the vascular wall also activate the platelets; key activators such as fibronectin, fibrillar collagen, and other matrix proteins initiate the process [53].

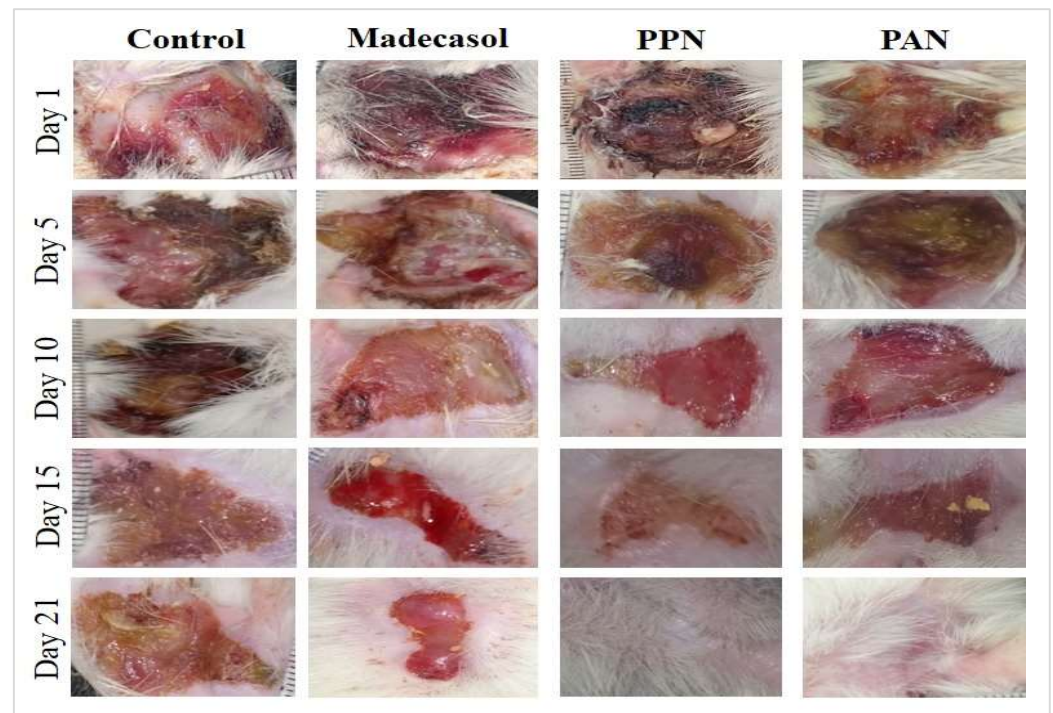


Figure 5. Photographic representation of the wound healing process in a burn wound model. Control (Group 1), Madecassol 1% standard drug (Group 2), PPN: *P. pinaster* needles extract 10% (Group 3), *P. halepensis* needles extract PAN 10% (Group 4) on 1, 5, 10, 15, and 21 days post-wounding.

Effective healing of burn wounds requires antioxidant protection, pain relief, and anti-inflammatory measures to promote tissue regeneration [54]. The wound-healing effects observed in this study were clearly illustrated in photos taken on different days. Notably, on day 21, the percentage of wound regeneration reached 100% with both enriched PPN and PAN ointments, which is a more significant result than the group treated by the standard ointment (Madecassol). The enhanced contraction rate and faster epithelialization observed with both extracts can be attributed to their anti-inflammatory, antimicrobial, and antioxidant capacities. The enriched PPN and PAN ointments contain Vaseline, which serves as a base in the ointment formulation and is crucial in enhancing the delivery and effectiveness of the active compounds present in the *Pinus* needle extract, due to each semi-solid hydrophobic vehicle aspect being widely used in topical applications due to its excellent occlusive and emollient properties, which create a protective film over the skin. More importantly, Vaseline's lipophilic nature facilitates the percutaneous absorption of bioactive molecules, particularly those with lipid-soluble characteristics, by enabling their diffusion through the stratum corneum [55]. In the present study, although the hydroethanolic extract contains various hydrophilic phenolic compounds, Vaseline's prolonged contact time and occlusive properties may still enhance passive diffusion into the dermal layers, especially in the context of damaged or compromised skin barriers. Therefore, using Vaseline supports the topical bioavailability of the enriched extract, ensuring sustained exposure at the wound site without exerting its direct therapeutic effects [55]. The bioactive flavonoids, namely quercetin and catechins, are well known for stimulating fibroblast proliferation, collagen deposition, and angiogenesis, which are necessary for wound healing (Figure 6) [56]. In addition, specific phenolic acids, such as protocatechuic acid, were cited as a topical antimicrobial for surgical skin antisepsis [57], which might help prevent infection, thus hastening the healing process.

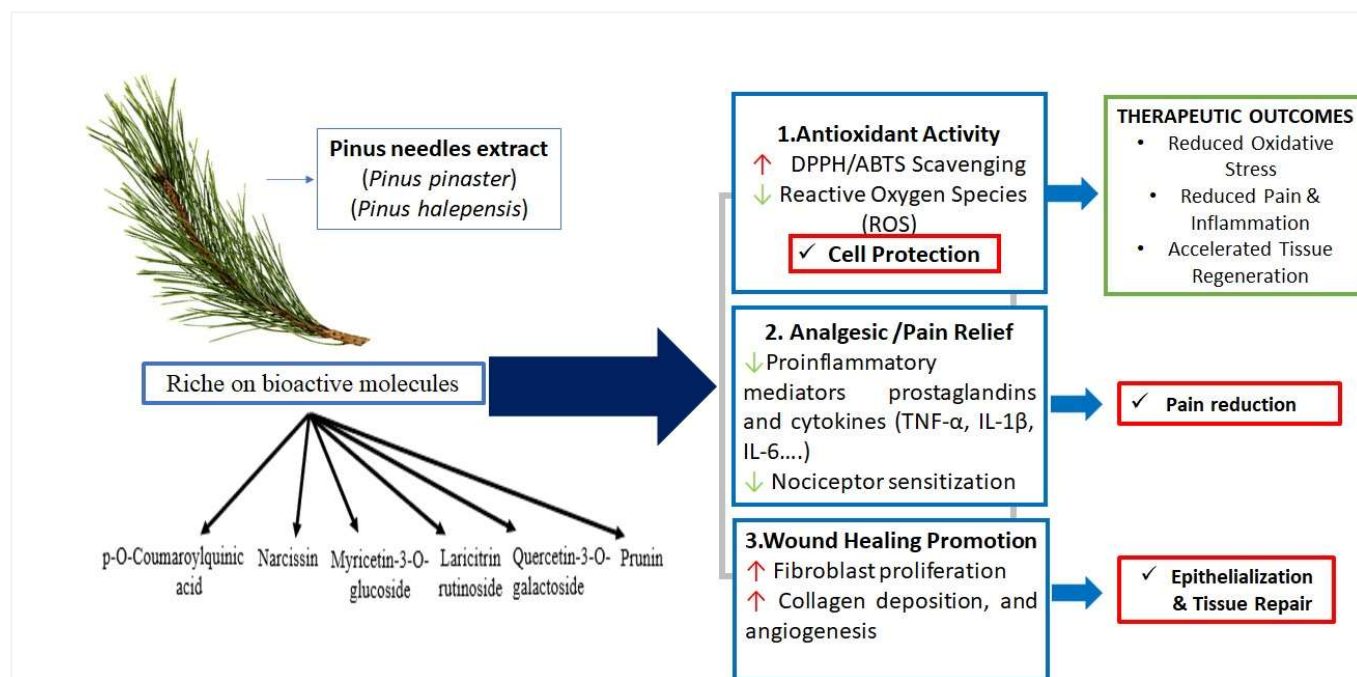


Figure 6. Schematic diagram illustrating the possible mechanisms by which pine needle extract reduces oxidative stress and provides analgesic and wound-healing effects.

4. Conclusions

Understanding the effects of the major phenolic and flavonoid compounds identified in *Pinus pinaster* and *Pinus halepensis* needle extracts, such as catechin, quercetin, and gallic acid, helps explain their observed antioxidant, analgesic, and wound-healing activities as detected by UHPLC-MS. Extracts from *Pinus halepensis* exhibited superior bioactivity, likely due to higher concentrations of these bioactive molecules. While only phenolic and flavonoid compounds were quantified in this study, other bioactive metabolites, including alkaloids and terpenoids, may also contribute to the observed effects. No correlation or association analysis was performed; therefore, direct links between total phenolic content and biological activity remain indicative rather than definitive. Nevertheless, the findings highlight the therapeutic potential of pine needle extracts for managing oxidative stress, pain, and tissue regeneration. Among the two species tested, *Pinus halepensis* exhibited stronger antioxidant and wound-healing activities compared with *Pinus pinaster*. Further research, including histopathological analysis, bioavailability studies, identification of additional bioactive compounds, and clinical evaluations, is warranted to fully elucidate the mechanisms and optimize the use of these extracts in pharmaceutical and cosmeceutical applications.

Author Contributions: W.T. was involved in conceptualization, methodology, investigation, formal analysis, and data curation, and wrote the original draft. H.L. was involved in reviewing the manuscript. B.I. was involved in methodology and investigation. K.E.Y. investigated the study. A.Z.L.E. performed formal analysis and data curation. B.E. supervised, conceptualized, reviewed, and edited the manuscript. B.L. was involved in conceptualization and methodology and supervised the study. Z.B.O. conceptualized, reviewed, and supervised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

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