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Phytochemical, Antioxidant, Anti-Inflammatory, and Anti-Arthritic Evaluation of a Hexane Extract of *Papaver somniferum* L.

Evaluación fitoquímica, antioxidante, antiinflamatoria y antiartrítica de un extracto hexánico de *Papaver somniferum* L.

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RESUMEN

This study evaluated the phytochemical, antioxidant, anti-inflammatory, and anti-arthritic properties of a hexane extract from *Papaver somniferum* L. seeds. Qualitative and quantitative phytochemical analyses were performed, including spectrophotometric assays to determine the total phenolic, flavonoid, tannin, and anthocyanin content. Antioxidant activity was assessed using DPPH, ABTS, and FRAP methods, yielding values of 12.40 mg TE/g DW, 82.48 % inhibition, and 2.95 mg TE/g DW, respectively. *In vitro* models were applied to determine anti-inflammatory activity (erythrocyte membrane stabilization) and anti-arthritic activity (protein denaturation inhibition), with inhibition percentages above 80 %. FT-IR analysis confirmed the presence of functional groups characteristic of lipophilic metabolites. Despite the hexane limitations as a solvent for polar compounds, the results suggest that the hexane fraction retains significant bioactivity, likely due to lipophilic secondary metabolites. These findings support the therapeutic potential of non-polar extracts from *P. somniferum* and encourage further study using advanced characterization techniques.

PALABRAS CLAVE: *Papaver somniferum* L., hexane extracts, total phenols, antioxidant, anti-inflammatory, anti-arthritic.

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ABSTRACT

This study evaluated the phytochemical, antioxidant, anti-inflammatory, and anti-arthritic properties of a hexane extract from *Papaver somniferum* L. seeds. Qualitative and quantitative phytochemical analyses were performed, including spectrophotometric assays to determine the total phenolic, flavonoid, tannin, and anthocyanin content. Antioxidant activity was assessed using DPPH, ABTS, and FRAP methods, yielding values of 12.40 mg TE/g DW, 82.48 % inhibition, and 2.95 mg TE/g DW, respectively. *In vitro* models were applied to determine anti-inflammatory activity (erythrocyte membrane stabilization) and anti-arthritic activity (protein denaturation inhibition), with inhibition percentages above 80 %. FT-IR analysis confirmed the presence of functional groups characteristic of lipophilic metabolites. Despite the hexane limitations as a solvent for polar compounds, the results suggest that the hexane fraction retains significant bioactivity, likely due to lipophilic secondary metabolites. These findings support the therapeutic potential of non-polar extracts from *P. somniferum* and encourage further study using advanced characterization techniques.

KEY WORDS: *Papaver somniferum* L., hexane extracts, total phenols, antioxidant, anti-inflammatory, anti-arthritic.

Introduction

Papaver somniferum L., commonly known as opium poppy, is a plant native to Western Asia that has traditionally been used for its latex, rich in alkaloids such as morphine, codeine, and papaverine (Labanca *et al*., 2018). However, beyond its narcotic applications, the seeds of this species have attracted scientific interest due to their content of unsaturated lipids, phenolic compounds, and natural pigments with bioactive potential (Shahidi & Ambigaipalan, 2015; Butnariu *et al*., 2022).

P. somniferum seeds are legally employed in bakery and confectionery products owing to their nutritional profile and high oil content (Paniagua-Zambrana *et al*., 2020). In recent years, phytochemical studies have further revealed the presence of flavonoids, anthocyanins, and phenolic acids, all of which exhibit well-documented antioxidant and anti-inflammatory activities (Ambriz-Pérez *et al*., 2016; Ghasemzadeh *et al*., 2011).

In this context, the present study aimed to chemically characterize the hexane extract of *P. somniferum* seeds, quantitatively assessing its phenolic compound content as well as its antioxidant and anti-inflammatory potential. Although phenolic compounds are typically soluble in polar solvents, several studies have demonstrated that lipophilic fractions may contain nonpolar

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phenolics or acylated phenolics with high bioactivity (Arzola-Rodríguez *et al*., 2022). Therefore, this study explored the activity of this apolar fraction to expand current knowledge regarding the potential therapeutic and functional applications of opium poppy seeds.

Material and Methods

Plant Material Procurement

The seeds of *Papaver somniferum* L. used in this study were purchased from Hierbas Orgánicas México, a specialized supplier in food-grade and herbal ingredients. The commercial product specifies that the seeds are intended exclusively for culinary purposes, such as baking and infusions, and are not suitable for sowing; this was considered for the ethical and legal compliance of the present study. According to the supplier, the material originated from crops in the Netherlands, with the dried whole seed being the part used.

To ensure traceability and botanical authenticity, morphological and photographic inspection of the batch was carried out. A voucher specimen was also deposited in the IBUG Herbarium at the University of Guadalajara.

Extract Preparation

The plant material (300 g of *Papaver somniferum* L. seeds) was subjected to static maceration with 300 mL of hexane in amber glass flasks for 7 days at room temperature (27 ± 2 °C) and under dark conditions to preserve the stability of oxidation-sensitive compounds. Afterwards, the mixture was filtered through Whatman No. 1 paper to separate the extract from the plant residue. The filtrate was concentrated under reduced pressure using a rotary evaporator (Büchi R-210, Switzerland) at 40 °C, yielding a dry extract.

The dry extract was subsequently resuspended in absolute ethanol (≥99.9 %) or in an ethanol: water mixture (80:20 v/v), depending on the analysis to be performed, to ensure miscibility in aqueous systems, particularly for antioxidant assays. This strategy is supported by previous evidence recommending the use of polar-compatible solvents to facilitate the interaction of extracts with free radicals and chromogenic reagents in aqueous phase (Ghasemzadeh *et al*., 2011). The prepared solutions were stored in amber glass tubes at 4 °C until experimental use.

All assays were performed in triplicate, using specific positive controls: gallic acid (total phenols), quercetin (flavonoids), catechin (tannins), and Trolox (antioxidant capacity). Reagent blanks were also included for each assay. Spectrophotometric readings were conducted using a UV–Visible Multiskan SkyHigh Microplate Spectrophotometer (Thermo Scientific™, USA).

Phytochemical Analysis

Qualitative phytochemical screening was performed according to the methodology of Colina

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Ramos, (2016), with minor modifications, to preliminarily detect the presence of secondary metabolites in the hexane extract of *Papaver somniferum* L. The following groups of bioactive compounds were evaluated: phenols, flavonoids, tannins, anthocyanins, alkaloids, saponins, triterpenes, and steroids.

Determination of Saponins

- **Foam Test**

The extract was vigorously shaken for 40 seconds, and the persistence of the generated foam was evaluated over a minimum period of 3 minutes. The assay was performed at room temperature (27 ± 2 °C).

Triterpenes and Steroids

- **Salkowski Reagent**

Three drops of the extract were placed in a test tube, followed by the sequential addition of 4 drops of chloroform, 4 drops of concentrated sulfuric acid, and 4 drops of acetic anhydride. The mixture was left to stand for 1 minute and then gently shaken to observe possible color changes. The test was carried out at room temperature (27 ± 2 °C).

- **Salkowski Reagent, Variant A**

Three drops of the extract were sequentially mixed with 5 drops of chloroform, 5 drops of acetic anhydride, and finally 2 drops of concentrated sulfuric acid, which were carefully added along the walls of the tube. The mixture was left to stand for 4 minutes inside a fume hood, after which color changes were evaluated. The assay was performed at room temperature (27 ± 2 °C).

- **Liebermann–Burchard Reagent**

Three drops of the extract were combined with 5 drops of acetic acid, 5 drops of acetic anhydride, and 2 drops of concentrated sulfuric acid, which were slowly added along the walls of the tube. After standing for 4 minutes, color development was observed. All reactions were conducted at room temperature (27 ± 2 °C).

Determination of Flavonoids

- **Sodium Hydroxide Reagent (20 % NaOH)**

Three drops of the extract were mixed with 5 drops of a 20 % NaOH solution, and the immediate appearance of a color change was evaluated. The assay was conducted at room temperature (27 ± 2 °C).

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- **Shinoda Test**

Three drops of the extract were combined with 0.01 g of magnesium chloride, followed by the addition of 3 drops of concentrated hydrochloric acid. The mixture was left to stand for 5 minutes in a fume hood, after which color changes were recorded. The test was performed at room temperature (27 ± 2 °C).

Determination of Tannins

- **Ferric Chloride Reagent**

Three drops of the extract were combined with 2 drops of a 10 % w/v ferric chloride solution, and the immediate color change was observed. The assay was carried out at room temperature (27 ± 2 °C).

Determination of Phenolic Compounds

- **Potassium Dichromate Reagent**

Three drops of the extract were combined with 5 drops of a 10 % w/v potassium dichromate ($K_2Cr_2O_7$) solution. The mixture was left to stand for 1 minute, after which color changes were observed. The assay was conducted at room temperature (27 ± 2 °C).

Determination of Anthocyanins and Betalains

- **Addition of Ammonium Hydroxide**

Five drops of ammonium hydroxide were mixed with 3 drops of the extract. The reaction was allowed to stand for 2 minutes in a fume hood, and color changes were evaluated. The assay was carried out at room temperature (27 ± 2 °C).

Determination of Alkaloids

- **Mayer’s Reagent**

Mayer’s reagent was freshly prepared by dissolving 0.2040 g of mercuric chloride in 9 mL of water and 0.7500 g of potassium iodide in 6 mL of water. For the test, 3 drops of the extract were mixed with 7 drops of 10 % HCl, followed by 5 drops of Mayer’s reagent. The presence of a color change was evaluated at room temperature (27 ± 2 °C).

- **Wagner’s Reagent**

Wagner’s reagent was prepared by dissolving 0.1905 g of freshly sublimated iodine and

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0.3000 g of potassium iodide in 15 mL of bidistilled water. For the assay, 3 drops of the extract were combined with 3 drops of 10 % HCl, followed by 5 drops of Wagner's reagent. The color change was observed at room temperature (27 ± 2 °C).

- **Dragendorff's Reagent**

Dragendorff's reagent was prepared by dissolving 1.20 g of bismuth nitrate pentahydrate in 3 mL of concentrated nitric acid and 4.0800 g of potassium iodide in 7.5 mL of bidistilled water. Both solutions were mixed and brought to 15 mL with bidistilled water. For the assay, 3 drops of the extract were mixed with 3 drops of 10 % HCl and 6 drops of Dragendorff's reagent. The color change was evaluated at room temperature (27 ± 2 °C).

Determination of Total Sugars

- **Molisch Test**

Molisch reagent was prepared by dissolving 2.25 g of α-naphthol in 15 mL of ethanol. For the assay, 3 drops of the extract were mixed with 3 drops of the reagent, followed by the slow addition of 5 drops of concentrated sulfuric acid along the walls of the test tube inside a fume hood. Color development was immediate. The test was performed at room temperature (27 ± 2 °C).

Determination of Reducing Sugars

- **Fehling's Test**

Fehling's reagent was prepared in two separate solutions: solution A consisted of 0.5190 g of copper sulfate and 0.0075 mL of sulfuric acid, brought to a final volume of 7.5 mL; solution B contained 2.5950 g of sodium–potassium tartrate and 0.7500 g of NaOH, also adjusted to 7.5 mL. Both solutions were mixed immediately prior to use. For the assay, 3 drops of the extract were combined with 5 drops of 50 % sulfuric acid and heated in a water bath at 90 ± 2 °C for 10 minutes. The mixture was then cooled to room temperature (27 ± 2 °C) and neutralized with 10 % NaOH. Finally, 5 drops of Fehling's reagent were added, the solution was gently shaken, and reheated in a water bath at 90 ± 2 °C for another 10 minutes, after which the resulting color change was observed.

- **Benedict's Test**

Benedict's reagent was prepared by dissolving 2.5950 g of sodium citrate and 1.5000 g of anhydrous sodium carbonate in 12.75 mL of water. Additionally, 0.2595 g of copper sulfate pentahydrate was dissolved in 1.5 mL of distilled water, and the two solutions were combined and adjusted to a final volume of 15 mL. For the assay, 3 drops of the extract were mixed with 5 drops of 50 % sulfuric acid and heated in a water bath at 90 ± 2 °C for 10 minutes. The mixture was then cooled, neutralized with 10 % NaOH, and 5 drops of Benedict's reagent were added. The solution was gently shaken and reheated in a water bath at 90 ± 2 °C for an additional 10 minutes, and the

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observed color change was recorded.

All determinations were performed in triplicate for each test, using positive controls when available. Qualitative results were recorded according to response intensity (absent, weak, moderate, or strong).

Spectrophotometric Quantification

- **Total Phenolic Content (TPC)**

The determination of total phenolic compounds was performed by UV–Visible spectrophotometry, based on the redox reaction of the Folin–Ciocalteu reagent, following the method of Lillo *et al*., (2016) with minor modifications. A volume of 100 µL of the hexane extract, previously redissolved in ethanol:water (80:20 v/v), was mixed with 2 mL of distilled water, 250 µL of Folin–Ciocalteu reagent, and 1 mL of 10 % w/v Na_2CO_3. The mixture was adjusted to 5 mL with distilled water and incubated for 30 minutes at room temperature in the dark. Subsequently, the samples were centrifuged at 3000 rpm for 10 minutes to remove insoluble residues, and absorbance was measured at 765 nm using a UV–Visible Multiskan SkyHigh Microplate Spectrophotometer (Thermo Scientific™, USA). Gallic acid (0–1000 mg/L) was used to construct the calibration curve. Results were expressed as mg gallic acid equivalents per g of dry weight (mg GAE·g^{-1} DW).

- **Total Flavonoid Content (TFC)**

The total flavonoid content was quantified using the aluminum chloride ($AlCl_3$) colorimetric method, as described by Cimpoiu *et al*., (2011), with modifications. A volume of 100 µL of the extract resuspended in ethanol:water (80:20 v/v) was mixed with 100 µL of 5 % $AlCl_3$ (prepared in ethanol) and 100 µL of 1 M sodium acetate. The mixture was brought to a final volume of 5 mL with absolute ethanol and incubated for 30 minutes at room temperature in the dark. Absorbance was measured at 425 nm using a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Scientific™, USA). A quercetin calibration curve (0–1000 µg/mL) was constructed, and results were expressed as mg quercetin equivalents per g of dry weight (mg QE·g^{-1} DW). All determinations were performed in triplicate, and solutions were filtered through 0.45 µm PVDF membranes prior to spectrophotometric analysis.

Although flavonoids are predominantly polar compounds, certain subgroups, such as methylated flavanones, lipophilic aglycones, or glycosylated flavonoids with nonpolar side chains, may show partial solubility in apolar solvents. However, the use of hexane as the primary extraction solvent may limit the representativeness of the full flavonoid profile. Therefore, the dry extract was reconstituted in a hydroalcoholic phase compatible with the $AlCl_3$ assay, which allowed indirect evaluation of the potential presence of partially soluble flavonoids or those associated with nonpolar molecules. This analysis was considered exploratory, and future studies are recommended to complement it with polar solvent fractions to ensure broader recovery of these bioactive compounds.

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- **Phenolic Acids**

The procedure described by Lillo *et al*., (2016) was followed with minor adjustments. A volume of 100 µL of the hexane extract was successively diluted in ethanol: water (80:20 v/v) to ensure miscibility. To each aliquot, 1 mL of 0.5 M HCl, 1 mL of Arnow's reagent, and 1 mL of 1 M NaOH were added sequentially, and the volume was adjusted to 5 mL with distilled water. The mixture was incubated for 30 minutes in the dark, and absorbance was measured at 520 nm using a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Scientific™, USA). A calibration curve was constructed with caffeic acid (0–1000 mg/L), and results were expressed as mg caffeic acid equivalents per g of dry weight (mg CAE·g^{-1} DW).

- **Proanthocyanidins**

The assay was carried out following the protocol of Lillo *et al*., (2016), with modifications. A volume of 200 µL of extract was mixed with 1200 µL of 4 % w/v vanillin in butanol and 600 µL of concentrated HCl. The mixture was incubated in the dark for 30 minutes, and absorbance was measured at 500 nm. A calibration curve was constructed with (+)-catechin (0–1000 mg/L), and results were expressed as mg catechin equivalents per g of dry weight (mg CE·g^{-1} DW). All assays were performed in triplicate.

- **Anthocyanins**

The modified differential pH method of Lillo *et al*., (2016) was employed. Buffers were prepared at pH 1.0 (0.1 M KCl) and pH 4.5 (0.1 M sodium acetate–acetic acid). Four aliquots of 100 µL of extract were mixed with 900 µL of the respective buffer. Absorbance was measured at 520 and 700 nm, and anthocyanin concentration was calculated using Equation 1:

$$\textbf{Ecuation 1: } Total\ anthocyanins = \frac{A \times MW \times 1000}{\varepsilon \times l}$$

Where:

$A = (Abs_{520} - Abs_{700})_{pH1} - (Abs_{520} - Abs_{700})_{pH4.5,}$

MW= Molecular weight of cyanidin 3-glucoside (449.2 g/mol),

DF= Dilution factor,

l= Path length of the cell (cm),

ε= Molar extinction coefficient for cyanidin 3-glucoside (26,900 L/cm*mg),

1000= Conversion factor (g to mg).

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The anthocyanin concentration in the extract was expressed as mg cyanidin-3-glucoside equivalents per g of dry weight (mg C3G·g^{-1} DW).

- **Tannins**

The protein precipitation method with bovine serum albumin (BSA) was employed according to Ricco *et al*., (2011). A volume of 1 mL of extract was mixed with 1 mL of BSA solution (0.17 M NaCl, 0.2 M sodium acetate, pH 5.0, 1 mg/mL fraction V). After 15 minutes at room temperature, 50 µL of the supernatant was collected for the determination of total phenols (as precipitated tannins) using the Folin–Ciocalteu method. Although this assay was performed with the hexane extract, it should be noted that tannins are polar compounds with poor solubility in hexane, which may lead to underestimation. This limitation is acknowledged, and future studies are recommended to use polar solvent fractions for more accurate quantification. The analysis was performed in triplicate.

Antioxidant Activity Evaluation

- **ABTS$^{\bullet+}$ Radical Scavenging Capacity**

The antioxidant capacity of the hexane extract of *Papaver somniferum* L. was determined according to the method described by Re *et al*., (1999), with minor modifications. To generate the ABTS$^{\bullet+}$ radical, 25 mL of a 7 mM ABTS solution was prepared, and 12.8 mg of ammonium persulfate (2.45 mM) was added, allowing the mixture to stand at room temperature in the dark for 30 minutes under constant agitation. This stock solution was then diluted with absolute ethanol to adjust the absorbance to 0.700 ± 0.002 at 750 nm. Although the original protocol specifies measurement at 734 nm, a spectral scan was conducted, and it was determined that the hexane extract displayed a maximum absorption peak at 750 nm; thus, this wavelength was selected for the assay.

For the evaluation, the hexane extract was resuspended in absolute ethanol to ensure compatibility with the reaction medium and adequate interaction with the ABTS$^{\bullet+}$ radical. This step was necessary since the assay system is hydroalcoholic and not directly miscible with apolar solvents such as hexane. A Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standard solution was prepared at 800 µM by dissolving 2 mg in 10 mL of ethanol, and a calibration curve was constructed in the range of 0–400 µM. Results were expressed as µmol Trolox equivalents per gram of dry weight (µmol TE·g^{-1} DW).

It is recognized that the ABTS$^{\bullet+}$ assay is more suitable for extracts obtained with polar solvents, as antioxidants with higher radical scavenging capacity typically exhibit hydrophilic characteristics. In this study, the assay was considered exploratory to detect potential nonpolar compounds with antioxidant capacity in the hexane fraction. The percentage inhibition of ABTS$^{\bullet+}$ radical scavenging was calculated using Equation 2:

$$\textbf{Equation 2: } \%\ Inhibition = \frac{Abs_{ABTS^{\bullet+}i} - Abs_{ABTS^{\bullet+}sample}}{Abs_{ABTS^{\bullet+}i}} \times 100$$

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Where:

$Abs_{ABTS^{\bullet+}i}$ = absorbance of the *ABTS*$^{\bullet+}$ solution,

$Abs_{ABTS^{\bullet}sample}$ = absorbance of the remaining radicals after reduction reached steady state (Salazar *et al*., 2018).

- **DPPH$^{\bullet}$ Radical Scavenging Capacity (2,2-Diphenyl-1-Picrylhydrazyl)**

The methodology was based on the procedure described by Brand-Williams *et al*., (1995), with modifications. To prepare 100 mL of DPPH$^{\bullet}$ solution, 7.1 mg of reagent were dissolved and brought to volume with 80 % methanol, yielding a final concentration of 180 µM.

For the standard curve, 4 mg of Trolox were dissolved in 10 mL of 80 % methanol to obtain a 1600 µM stock solution, from which dilutions ranging from 0 to 1280 µM were prepared. Then, 20 µL of the sample solution was added into a 96-well microplate, followed by 260 µL of the DPPH• solution. The mixture was gently shaken and incubated at room temperature in the dark for 30 minutes. Absorbance was measured at 510 nm using a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Scientific™, USA).

Although the standard wavelength for DPPH$^{\bullet}$ measurement is 517 nm, in this study 510 nm was used due to the technical specifications of the microplate reader, which lacked a 517 nm filter or monochromator but included 510 nm. Similar adjustments have been reported in previous studies (Gulcin & Alwasel, 2023; Kedare, 2011). These works indicate that such minor deviations do not significantly affect method sensitivity, provided consistency is maintained across experimental and control measurements.

Results were expressed as percentage inhibition of the DPPH$^{\bullet}$ radical, and the half maximal inhibitory concentration (IC_{50}) was calculated by nonlinear sigmoidal regression analysis using GraphPad Prism® v9.0.

The percentage inhibition was calculated using Equation 3:

$$\textbf{Ecuación 3: } \%\ Inhibition = \frac{Abs_{DPPH^{\bullet}i} - Abs_{DPPH^{\bullet}sample}}{Abs_{DPPH^{\bullet}i}} \times 100$$

Where:

$Abs_{DPPH^{\bullet}i}$ = absorbance of the *ABTS*$^{\bullet+}$ solution,

$Abs_{DPPH^{\bullet}sample}$ = absorbance of the remaining radicals after reduction reached steady state (Salazar *et al*., 2018).

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- **Ferric Reducing Antioxidant Power (FRAP)**

The ferric reducing antioxidant power of the extract was determined following the procedure of Benzie & Strain (1996), with modifications proposed by Salazar *et al*., (2018).

A 40 mM HCl solution was prepared by diluting 123 µL of concentrated HCl (37 %) to 100 mL with distilled water. A 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) solution was prepared by dissolving 78.08 mg in 40 mM HCl and adjusting to 25 mL. In parallel, a 20 mM ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) solution was prepared by dissolving 135.18 mg in 25 mL of distilled water. The sodium acetate buffer was prepared at 0.3 M, adjusted to pH 3.6 with 40 mM HCl, by dissolving 2.4612 g of CH_3COONa in distilled water to a final volume of 100 mL.

The FRAP working solution was freshly prepared by mixing 2.5 mL of 10 mM TPTZ, 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$, and 25 mL of 0.3 M sodium acetate buffer. For the standard curve, a 1000 µM Trolox stock solution was prepared by dissolving 2.5 mg in 10 mL of sodium acetate buffer:ethanol (50:50 v/v). Dilutions in the range of 0–400 µM were prepared.

Absorbance was measured at 593 nm, and results were expressed as µmol Trolox equivalents per gram of dry weight (µmol TE·g^{-1} DW).

Anti-Inflammatory Activity

- **Evaluation of Anti-Inflammatory Activity by the Erythrocyte Membrane Stabilization Method**

Anti-inflammatory activity was assessed following the methodology described by López *et al*., (2018), with minor modifications. For this assay, a sample of 5 mL of human blood was obtained from a healthy donor who provided written informed consent for the exclusive use of the sample for research purposes. Since the protocol did not involve clinical procedures or diagnostic testing of the donor, and the sample was used solely for *in vitro* evaluation, approval from an ethics committee was not required according to institutional regulations. The blood was collected in an EDTA anticoagulant tube to preserve erythrocyte integrity. The sample was centrifuged at 3000 rpm for 10 minutes to obtain a red blood cell (RBC) pellet, which was then used to prepare a 10 % v/v suspension in isotonic saline solution.

- **Hypotonic Solution-Induced Hemolysis**

The reaction mixture consisted of 0.5 mL of 10 % RBC suspension, 1 mL of phosphate-buffered saline (PBS, pH 7.4), 1 mL of hypotonic saline solution (0.3 % w/v), and 1 mL of the extract under evaluation.

As a negative control, the extract was replaced with 1 mL of isotonic saline solution. As a

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pharmacological control, 1 mL of indomethacin dissolved in isotonic saline at a concentration of 3.33 mg/mL was used.

All tubes were incubated at 37 °C for 30 minutes and subsequently centrifuged at 3000 rpm for 20 minutes. The absorbance of the supernatant was measured in triplicate at 560 nm using a Thermo Scientific Multiskan SkyHigh UV–Vis Microplate Spectrophotometer (USA) to determine the degree of hemolysis, as an indicator of erythrocyte membrane stability.

- **Heat-Induced Hemolysis**

For this assay, the same reaction mixture as described above was used. To each tube, 1 mL of *Papaver somniferum* L. hexane extract was added at concentrations of 100, 250, and 500 µg/mL. Prior to use, the extract was resuspended in 10 % v/v ethanol in water to ensure adequate miscibility with the aqueous phase.

The negative control consisted solely of isotonic saline solution. The pharmacological control included 1 mL of indomethacin at 3.33 mg/mL. All determinations were performed in triplicate. Samples were incubated at 57 °C for 30 minutes and subsequently centrifuged at 2500 rpm for 5 minutes.

Absorbance readings were taken at 560 nm using a Multiskan SkyHigh UV–Vis Microplate Spectrophotometer (Thermo Scientific™, USA) to calculate hemoglobin release as an indicator of hemolysis.

The percentage inhibition of hemolysis was calculated using Equation 4:

$$\textbf{Equation 4: } \%Stability = 100 - \left(\frac{Abs_{sample} - Abs_{control}}{Abs_{control}}\right) \times 100$$

Anti-Arthritic Activity Evaluation

- **Bovine Serum Albumin (BSA) Method**

This assay began with the preparation of a 0.5 % bovine serum albumin (BSA) solution by dissolving 500 mg of BSA in 100 mL of distilled water. A phosphate-buffered saline (PBS) solution at pH 6.3 was then prepared by dissolving 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 800 mL of distilled water. The pH was adjusted to 6.3 with 1 N HCl, and the volume was completed to 1 L with distilled water.

A mixture was prepared consisting of 0.05 mL of the hexane extract resuspended in 10 % v/v ethanol in distilled water, 0.05 mL of indomethacin (reference drug, 3.33 mg/mL), and 0.45 mL of 0.5 % BSA solution. Samples were incubated at 37 °C for 20 minutes and subsequently heated to 57 °C for 3 minutes to induce protein denaturation. After cooling, 2.50 mL of PBS buffer was added to each tube.

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Absorbance was measured at 255 nm using a Multiskan SkyHigh UV–Visible Microplate Spectrophotometer (Thermo Scientific™, USA). The control consisted of BSA solution without extract or drug, representing 100 % protein denaturation.

The results were expressed as the percentage inhibition of protein denaturation, calculated by comparing the absorbance of each sample with that of the negative control according to Equation 5:

$$\textbf{Equation 5: } \%\ Inhibition = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100$$

Where:

$A_{control}$= absorbance of the *control,*

A_{sample}= absorbance of the sample

Egg Albumin Method

The percentage inhibition of protein denaturation was calculated using Equation 6:

$$\textbf{Equation 6: } \%\ Inhibition = 100 \times \left(\frac{V_t}{V_c} - 1\right)$$

Where:

V_t= absorbance of the *sample,*

V_c= absorbance of the control

The reaction mixture (5.00 mL) consisted of 0.20 mL of egg albumin, 2.80 mL of PBS solution (pH 6.4), and 2.00 mL of the hexane extract previously resuspended in an ethanol:water mixture (10:90 v/v) to ensure compatibility with the aqueous medium. An equal volume of bidistilled water was used as the control.

The mixtures were incubated at 37 ± 2 °C for 15 minutes and then heated to 70 °C for 5 minutes to induce protein denaturation. After cooling, absorbance was measured at 660 nm using the appropriate vehicle as a blank. Indomethacin at 3.33 mg/mL was employed as the reference drug under the same conditions as the extracts.

Fourier Transform Infrared Spectroscopy (FT-IR)

To identify the functional groups present in the hexane extract of *Papaver somniferum*

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L., Fourier transform infrared (FT-IR) spectroscopy was performed using a Nicolet iS50 spectrophotometer (Thermo Scientific™, USA) equipped with an attenuated total reflectance (ATR) accessory. Spectra were recorded in the range of 4000–500 cm^{-1}, with a resolution of 4 cm^{-1} and an average of 30 scans per sample.

The extract was analyzed directly without additional preparation by placing a small amount on the ATR crystal. Spectra were interpreted based on characteristic bands of functional groups, supported by specialized literature.

Statistical Analysis

All experiments were performed in independent triplicates, and results are expressed as mean ± standard deviation (SD). To determine significant differences among antioxidant capacity evaluation methods, one-way analysis of variance (ANOVA) was applied, followed by Tukey's multiple comparison test. Differences were considered statistically significant at $p < 0.05$. Statistical analysis was conducted using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA).

Results and Discussion

After the maceration and rotary evaporation concentration process, a residual volume of 25.00 mL of concentrated extract corresponding to the hexane fraction was obtained. This volume was used for the experimental determinations. It should be noted that this was not a completely dried extract, since no full drying or gravimetric quantification was performed; therefore, subsequent results are expressed based on relative concentrations of the extract in solution.

The qualitative phytochemical analyses of the hexane extract (Table 1) evidenced the presence of secondary metabolites such as flavonoids, tannins, and phenolic compounds (Díaz *et al*., 2015; Lu *et al*., 2017). The detection of these molecules, traditionally considered polar in nature, may be explained either by the partial resuspension of the hexane extract in a polar hydroalcoholic vehicle prior to analysis, or by the concentration of lipophilic forms (or aglycones) with affinity for apolar solvents Díaz *et al*., (2015).

Additionally, a positive signal was observed in the sugar test; however, this was not supported by a quantitative method nor reported in the methodological section, and thus should be interpreted with caution.

Regarding the assays in which certain metabolites were not detected, this does not necessarily imply their complete absence in the extract. Rather, it may reflect limitations of the experimental conditions, such as pH, solvent polarity, sensitivity of the colorimetric tests, or the concentration used Lu *et al*., (2017).

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The qualitative phytochemical analysis of the hexane extract revealed a prominent presence of phenolic compounds, such as flavones, tannins, and anthocyanins, with reaction intensities ranging from (+++) to (++++), suggesting a complex metabolic profile with bioactive potential (Table 1). These findings are particularly relevant, as the mentioned compounds are often associated with pharmacological and antioxidant properties.

Although these metabolites are typically soluble in polar solvents, their detection in the hexane extract may be explained by the intermediate polarity of certain seed-derived compounds, as well as by possible co-extraction of residual polar traces during maceration. Previous studies have shown that although hexane extracts generally exhibit a lower polyphenol content compared with solvents such as ethanol or methanol, some less polar phenolic compounds can indeed be recovered in this fraction (Ng *et al*., 2020; Kulshreshtha & Ranawat, 2022).

Table 1. Results of Phytochemical analysis where: (++++) very abundant, (+++) abundant, (++) moderately abundant, (+) slightly abundant, and (-) not observed.

Metabolite	Phytochemical test	**Result**
Saponins	Foam test	+ ++
	Salkowski	++
	Liebermann-Burchard	
Flavones	Shinoda	+++
Phenols	Potasium dichromate	+++
Tannins	Ferric chloride	+++
Anthocyanins	20 % NaOH	++++
Quinones	5 % NaOH	+
	Borntränger	+
Alkaloids	Mayer	+
	Wagner	+
	Dragendorff	+
Sugars	Molisch	+++
Reducing sugars	Fehling	+++
	Benedict	+++

Furthermore, it was observed that the diluted extract allowed for better detection of metabolites compared with its concentrated counterpart. This behavior may be attributed to reduced matrix interference in diluted samples, which facilitates specific interactions between

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metabolites and chromogenic reagents, thereby enhancing the sensitivity of qualitative assays. This phenomenon has been previously described in phytochemical tests, where appropriate extract dilution improves the visibility of colorimetric reactions (Khoddami *et al*., 2013).

With regard to the detection of sugars and reducing sugars, although their presence in a hexane extract is unexpected due to their polar nature, the possibility of trace amounts or analytical interferences cannot be completely excluded. Factors such as incomplete removal of aqueous solvent or the interaction of non-sugar compounds with Molisch, Fehling, and Benedict reagents could have generated positive results. In this context, future evaluations using polar fractions and negative controls are recommended to confirm the nature of the observed signals.

Overall, these results underscore the importance of optimizing extraction and analytical procedures, particularly when working with complex plant matrices. The implementation of quantitative spectrophotometric techniques, such as Folin–Ciocalteu for total phenols, aluminum chloride for flavonoids, and Folin–Denis for tannins, will contribute to validating and complementing the findings obtained through qualitative methods. Moreover, it is recommended to standardize the extraction process, clearly specifying the phases used and their concentrations, to ensure reproducibility and comparability of results.

Table 2. Quantification results of secondary metabolites.

	Total Phenols [A]	Flavones and Flavonols [B]	Phenolic Acids [C]	Proanthocyanidins [D]	Anthocyanins [E]	Tannins [A]
Extracto	0.3510 ± 0.05	0.2081 ± 0.011	0.1332 ± 0.010	0.0193 ± 0.013	2.1457 ± 0.005	0.0247 ± 0.004

[A] mg GAE g^{-1} DW; [B] mg EQ g^{-1} DW; [C] mg CAE g^{-1}; [D] mg CE g^{-1} DW; [E] mg C3G g^{-1}DW: Values represent mean ± standard derivation.

Comparative Analysis of Antioxidant Activity by $ABTS^{\bullet+}$, $DPPH^{\bullet}$, and FRAP Assays

$ABTS^{\bullet+}$ Assay Results

The direct extract reached its highest antioxidant capacity at 60 minutes of reaction, at which point the maximum inhibition percentage was recorded. The value obtained for this assay was 82.48 % inhibition.

$DPPH^{\bullet}$ Assay Results

A significant antioxidant activity of the hexane extract was observed, reaching 79 %

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inhibition, which stabilized at 85 minutes after the start of the reaction. In addition, a content of 12.40 mg TE·g^{-1} DW was quantified in terms of Trolox equivalents.

FRAP Assay Results

The extract exhibited a reducing capacity corresponding to 2.95 mg TE·g^{-1} DW, indicating a moderate antioxidant activity.

The results obtained demonstrate a significant antioxidant activity of the hexane extract, as evidenced in the ABTS$^{\bullet+}$, DPPH$^{\bullet}$, and FRAP assays, although this type of extract is generally associated with a lower presence of antioxidant metabolites due to its low polarity. The inhibition values of 79 % in the DPPH• assay and 82.48 % in the ABTS$^{\bullet+}$ assay indicate the presence of compounds with electron- or proton-donating capacity, possibly less polar phenolic compounds, carotenoids, or other lipophilic metabolites extracted with hexane.

In the case of the FRAP assay, the value obtained (2.95 mg TE/g DW) was moderate compared to extracts obtained with more polar solvents, which may be due to the limited extraction of strongly reducing polyphenols in nonpolar solvents. However, the detection of activity in this assay complements the previous results and suggests that the extract contains at least some compounds capable of reducing ferric ions, reinforcing its antioxidant potential.

The statistical analysis performed by one-way ANOVA revealed statistically significant differences in the antioxidant capacity values of the hexane extract of *Papaver somniferum* L. obtained with the ABTS, DPPH, and FRAP methods ($F = 316.77$; $p = 8.26 \times 10^{-7}$). The mean values observed were 4.4965 ± 0.012 mg TE/g DW (ABTS), 12.4039 ± 0.011 mg TE/g DW (DPPH), and 2.9487 ± 0.80 mg TE/g DW (FRAP), evidencing significant variability attributable to the analytical methodology employed. Tukey's test confirmed that at least one of the methods (DPPH) behaved significantly differently from the others, particularly by showing a higher antioxidant capacity expressed in mg TE/g DW.

Moreover, the kinetics observed in the ABTS$^{\bullet+}$ and DPPH$^{\bullet}$ assays showed that antioxidant activity stabilized after approximately one hour, suggesting that the interaction between extract compounds and free radicals occurs progressively, possibly due to sustained release or differences in the affinity of metabolites for each type of radical.

These findings are consistent with studies such as Ng *et al*., (2020) and Shah & Patel, (2021) who reported that although hexane extracts tend to exhibit lower total phenolic content compared with methanolic or aqueous extracts, they can still display moderate to high antioxidant activity depending on the plant matrix and the types of compounds present.

Taken together, the results provide evidence that the hexane extract of *Papaver somniferum* L. contains metabolites with potential antioxidant activity, the detection of which is influenced by the chemical nature of the radicals employed in each assay and the inherent sensitivity of the methods. The greater response observed in the DPPH assay suggests the presence of compounds with

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strong electron- or hydrogen-donating ability, whereas the FRAP method, which assesses ferric reducing power, exhibited lower sensitivity to this lipophilic matrix. These differences, supported by statistically significant variation among methods ($p < 0.001$), highlight the importance of employing complementary methodologies for a more comprehensive characterization of antioxidant potential. Furthermore, it is recommended to complement these findings with specific quantitative analyses (e.g., HPLC or Folin–Ciocalteu) to identify the bioactive compounds responsible for such activity.

Table 3. Antioxidant capacity and mg TE/g dry weigth of the hexane extract of *Papaver somniferum* L. By different methodologies.

Sample	Result (mgTE g^{-1} DW)	% Inhibition
($ABTS^{\bullet+}$)	4.4965 ± 0.012	82.4756
($DPPH^{\bullet}$)	12.4039± 0.011	79.6251
(FRAP)	2.9487 ± 0.80	N/A

Results of Anti-Inflammatory and Anti-Arthritic Activity

Anti-Inflammatory Activity

The hexane extract exhibited a significant capacity to inhibit induced hemolysis, reflecting a membrane-stabilizing effect. An inhibition rate of 82.21 % was obtained in the hypotonic solution-induced hemolysis (HISH) method and 85.13 % in the heat-induced hemolysis (HIC) method. These values are comparable to the pharmacological control (indomethacin at 5.33 mg/mL), which showed 95.15 % inhibition. Although slightly lower, the percentages observed for the extract suggest relevant anti-inflammatory activity, particularly considering the nonpolar nature of the solvent used for its preparation.

Anti-Arthritic Activity

In the *in vitro* models for the evaluation of anti-arthritic activity, the extract demonstrated an inhibitory effect on protein denaturation. Inhibition of 82.0 % was recorded with the bovine serum albumin (BSA) method and 87.04 % with the egg albumin (EA) method. These values support the extract's ability to stabilize proteins under denaturing conditions, a mechanism closely associated with inflammatory and arthritic processes.

One-way analysis of variance (ANOVA) applied to the inhibition percentage data revealed statistically significant differences ($F = 10{,}209.11$; $p = 5.28 \times 10^{-18}$). Post hoc analysis using Tukey's multiple comparison test confirmed that the hexane extract differed significantly from the positive control (indomethacin at 3.33 mg/mL), although it maintained inhibition within a biologically relevant

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range. Among the models evaluated, the egg albumin (EA) method showed the highest inhibition percentage, followed by the heat-induced hemolysis (HIC) model and, to a lesser extent, the hypotonic solution-induced hemolysis (HISH) model. These differences suggest that the extract's response may depend on the specific mechanism of cellular destabilization in each model, being more effective under conditions of protein or thermal denaturation. Overall, the statistical results support the anti-inflammatory and anti-arthritic potential of the hexane extract, with variations attributable to the particular sensitivity of each *in vitro* model.

The findings from the anti-inflammatory and anti-arthritic assays reinforce the potential of the hexane extract as a bioactive agent. Although the inhibition values were slightly lower than those of the standard drug, they remain within a biologically relevant range. These effects may be attributed to the presence of lipophilic secondary metabolites such as alkaloids, terpenoids, or nonpolar flavonoids. Previous studies have reported that lipophilic extracts of stinging nettle exhibited stronger anti-inflammatory effects than polar extracts (Johnson *et al*., 2013), and that dichloromethane extracts of purslane inhibited COX while promoting IL-10 (Ahmed *et al*., 2022).

Furthermore, natural compounds derived from plants and marine sources, including alkaloids, terpenoids, and flavonoids, have been documented to modulate inflammatory pathways in macrophages and other immune cells (Merecz-Sadowska *et al*., 2020; Mohammed *et al*., 2014; Saudagar & Saokar, 2019; Verma *et al*., 2016). Talhouk *et al*. (2007) and Justo *et al*., (2015) also noted that the vehicle of administration can influence the observed efficacy, with DMSO being one of the most effective solvents in revealing *in vitro* anti-inflammatory activity.

Taken together, these findings suggest that the lipophilic metabolites present in the hexane extract may exert a stabilizing effect on membranes and structural proteins, acting as anti-inflammatory agents through physical protection mechanisms or early molecular modulation. Despite the limited polarity of hexane, its ability to extract bioactive compounds with relevant effects justifies its exploratory use.

It is recommended that these findings be complemented with phytochemical characterization studies using HPLC or other advanced techniques to identify the responsible compounds and to evaluate their *in vivo* bioactivity.

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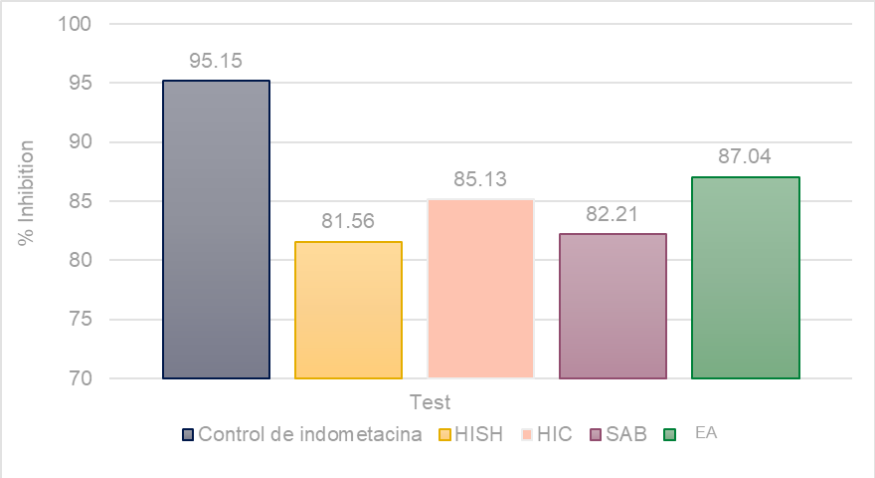

Figure 1. Graph of % inhibition of the hexane extract of *Papaver somniferum* L. compared with a commercial drug (indomethacin).

HISH: Hypotonic Solution-Induced Hemolysis, HIC: Heat-Induced Hemolysis, BSA: Bovine Serum Albumin, EA: Egg Albumin.

Fourier Transform Infrared Spectroscopy (FT-IR) Analysis

The hexane seed extract of *Papaver somniferum* L. was analyzed by Fourier transform infrared (FT-IR) spectroscopy to identify the main functional groups present.

The spectrum obtained (Figure 2) revealed several characteristic bands that allow inference of the extract's general chemical composition.

A broad band at 3000 cm^{-1} was observed, attributable to the stretching vibrations of C=C double bonds present in olefins. Additional bands at 2930 cm^{-1} and 2850 cm^{-1} were associated with the asymmetric and symmetric stretching vibrations of methylene groups ($–CH_2–$), typical of aliphatic hydrocarbons.

An intense signal at 1750 cm^{-1} suggested the presence of ester groups, attributable to C=O stretching. A band at 1680 cm^{-1} may be related to conjugated C=C bonds or substituted aromatic compounds rather than isolated olefins, and should therefore be interpreted with caution.

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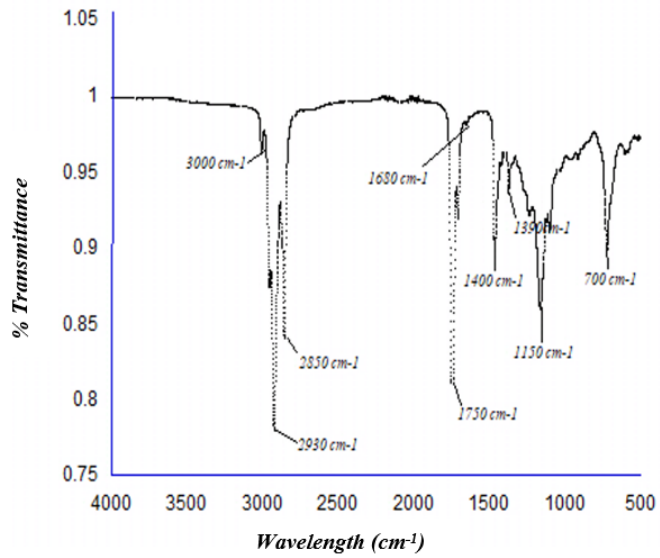

Figure 2. FT-IR spectrum of the hexane extract of *Papaver somniferum* L. seed.

Bands at 1400 cm^{-1} and 1390 cm^{-1} could be assigned to methyl group ($–CH_3$) deformations or aromatic ring vibrations, although they are not diagnostic by themselves for the presence of alkenes.

The signal at 1150 cm^{-1} corresponded to C–O–C stretching, characteristic of ester groups, supporting the previous observation at 1750 cm^{-1}. Finally, the band at 700 cm^{-1} may be associated with out-of-plane vibrations of aromatic rings, although its intensity and shape suggest caution in interpretation.

The signals observed in the FT-IR spectrum are consistent with the presence of lipophilic compounds such as fatty acids, esters, aliphatic hydrocarbons, and terpenes, which are commonly extracted with nonpolar solvents such as hexane. The identification of ester groups is particularly relevant, as these compounds may be associated with bioactive secondary metabolites, including certain triterpenes or esterified fatty acids with antioxidant and anti-inflammatory potential. FT-IR spectroscopy has been successfully employed to detect lipids, fatty acids, and terpenes in extracts obtained with nonpolar solvents (Felix-Sagaste *et al*., 2024; Shekhawat, Deora, & Sarswati, 2025). The signals observed in the spectrum are consistent with active lipophilic components and corroborate the efficacy of hexane as a solvent for isolating nonpolar anti-inflammatory metabolites (Hari & Nair, 2018; Forfang *et al*., 2017).

FT-IR characterization provides an initial qualitative approach to the chemical composition of the extract, which should be complemented with more specific techniques such as chromatography or mass spectrometry for the definitive identification of the compounds present.

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Study Limitations

Although the findings provide evidence of the antioxidant, anti-inflammatory, and anti-arthritic potential of the hexane extract of *Papaver somniferum* L., several limitations should be considered. First, the biological assays were performed with a limited number of replications, which restricts the possibility of applying robust statistical analyses in some models, such as the anti-inflammatory assays. Second, the study did not include a toxicological evaluation of the extract, limiting the inferences regarding its safety and therapeutic applicability. Furthermore, an exhaustive phytochemical characterization to identify the metabolites responsible for the observed activities was not conducted; thus, the application of specific analytical techniques (e.g., HPLC, GC-MS, or LC-MS/MS) will be required in future studies. Finally, although a qualitative relationship between phenolic content and bioactivity was observed, no statistical correlation analyses were performed to establish a quantitative association between these variables. These limitations should be addressed in subsequent research to consolidate the pharmacological value of the extract.

Conclusion

The results obtained demonstrate that the hexane extract of *Papaver somniferum* L. seeds exhibits notable antioxidant, anti-inflammatory, and anti-arthritic activity, possibly attributable to the presence of lipophilic compounds such as terpenoids, alkaloids, and nonpolar flavonoids. Spectroscopic characterization by FT-IR supported the identification of these metabolites. Although hexane is not the optimal solvent for polar compounds, the phytochemical profile and observed biological activity indicate that this fraction may contain relevant active principles with potential therapeutic applications. The findings highlight the need to complement the analysis with techniques such as HPLC-MS or GC-MS, as well as *in vivo* assays, to validate the pharmacological potential of this extract in clinical or nutraceutical contexts. This study contributes to the revaluation of nonpolar extracts in the research of bioactive natural products.

Author Contributions

Conceptualization of the study, V.R.S.F.; Methodology development, M.G.M.A., V.R.S.F., R.A.A.C., G.S.C.E.; Data analysis, V.R.S.F., R.A.A.C., G.S.C.E.; Data management, V.L.J.M., Z.O.A.; Writing—original draft preparation, M.G.M.A., V.R.S.F., R.A.A.C.; Writing—review and editing, V.R.S.F., R.A.A.C.

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Conflict of Interest

The authors declare no conflict of interest.

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