Research Square

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Comparative Analysis of Pinus halepensis, Pinus brutia, and Pinus pinea Extracts: Chemical Composition and in vitro Bioactivities

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Abstract

Purpose Traditionally, medicinal plants were frequently used to treat various diseases. In this regard, *Pinaceae* species (various parts, residues, extracts) is one of the potential traditional plants with health issues such as antibacterial, anti-cancer, and antioxidant activities. In the context of biomass valorization (forest residues) and the development of a circular bioeconomy, pine species generate large amounts of unvalorized cones. In this study, different solvents were used to extract bioactive compounds and evaluate bioactivities from dried and ground pinecones from *P. halepensis* PA, *P. brutia* PB and *P. pinea* PP.

Methods Petal P and heart C from pinecones were manually separated and were milled to investigate successive solvent extraction with increasing polarity: Cyclohexane 1SV, ethyl acetate 2SV and methanol 3SV at 20 °C. Spectrophotometry was used to quantify the total phenolic content TPC and to evaluate the antioxidant and anticancer activities. Gas chromatography with mass spectrometry GC-MS and High-performance liquid chromatography with diode-array HPLC-DAD were used to identify bioactive compounds.

Results The P-3SV extracts showed the highest TPC values and had a significant antioxidant capacity. The extracts of 1SV and 2SV had moderate anticancer activity. HPLC analysis allowed the identification of 38 compounds, twenty-seven of which were not previously detected in these species. Forty-six volatile compounds were identified using GC-MS, thirty-three of which were detected for the first time in this species.

Conclusions This study highlights the considerable potential of pinecones as a valuable reservoir of bioactive compounds and suggests that they can contribute to advances in health.

Statement of Novelty

The novelty of this work lies in the fact that the article provides an insight into the chemical composition, antioxidant and anticancer properties of the petals and hearts of *P. halepensis, P. brutia and P. pinea*. Previous studies have reported approaches to extract essential oils from the total cones. New molecules have been identified for the first time in these species.

Highlight

- Comparison of yield extraction and biochemical analysis versus species, fractions and solvents.
- Quantification of Total polyphenol and reducing sugar content using Folin Ciocalteu and DNS methods.
- DPPH and MTT tests were performed to evaluate the antioxidant and anticancer activities.
- HPLC and GC-MS were performed to the identification of bioactive compounds.

1. Introduction

Phytochemicals have been recognized and studied for their bioactive properties in biological and pharmacological contexts. Among the most representative phytochemicals are polyphenols. Polyphenols are currently the subject of extensive scientific research due to their bioactive properties [1], including their anticancer, anti-inflammatory, antioxidant, antimutagenic, antithrombotic, antiviral and antibacterial effects [2]. The antioxidant activity is the most interesting activity due to its potential health benefits [3].

The *Pinus genus*, comprising around 250 species, stands as the most extensive genus within the conifer family. It grows naturally in many regions of the northern hemisphere, particularly in the Mediterranean, the Caribbean, Asia, Europe and North and Central America [4]. Pine cone extracts have been extensively studied as a promising source of compounds with potential commercial applications in medicine, therapy and bioconservation [5]. The study of Villagomez et al. [6] examining the total phenolic content and antioxidant activity in various parts of the pine tree revealed a hierarchy in the following order, from highest to lowest: juvenile cones > needles > new cones > bark > old cones > wood. This means that cones, especially those from species with comparatively low antioxidant activity in their wood, could serve as a promising source of antioxidants.

To our knowledge, despite the existing research on Pinus species, there is a notable lack of studies conducting biochemical analyses of pinecones, particularly on the different fractions of the petals and hearts of *P. halepensis*, *P. brutia* and *P. pinea*. Our study fills this research gap through a careful methodology. A successive solvent extraction with increasing polarity serves as the basic step. Subsequent analyses include the quantification of reducing sugars and total polyphenols as well as the evaluation of antioxidant and anticancer activities. The final phase involves the identification of bioactive compounds using advanced chromatographic techniques such as HPLC and GC-MS.

2. Materials and Methods

2.1. Chemical

Cyclohexane (1SV), ethyl acetate (2SV), methanol (3SV), acetonitrile (ACN), acetic acid, dimethyl sulfoxide (DMSO), 3,5-dinitrosalicylic acid (DNS), sodium potassium tartrate, sodium hydroxide (NaOH), Folin–Ciocalteu reagent (2 N), sodium carbonate, 1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, potassium dihydrogen phosphate (KH2PO4), sodium dihydrogen phosphate (Na2HPO4), Dulbecco's modified eagle medium (DMEM), foetal bovine serum (FBS), non-essential amino acids, penicillin, streptomycin, gentamicin, thiazolyl blue tetrazolium bromide (MTT), and analytical standards were purchased from Sigma-Aldrich (France). The analytical standards used to identify the bioactive compounds present in the pinecone extracts were: baicalein; ethyl 3,5-dihydroxybenzoate; ethyl trans-2-hydroxycinnamate; kaempferol; 5,8-dihydroxy-1,4-naphthoquinone; ethyl 4-hydroxy-3-cinamate; 7-hydroxy-4-phenylcoumarin; 2-chloro-3-(4-hydroxy-phenylamino)-(1,4) naph-thoquinone; 3-chloro-7-hydroxy-4-methylcoumarin; 5-hydroxy-4'-methoxylflavone; chry-sin; trihydroxyethylrutin;

rutin; catechin; 3,4-dihydroxy-5-methoxybenzoic acid; quercetin 3-β-D-glucoside; poly-datin; 2,4-dihydroxycinnamic acid; ellagic acid; (±) synephrine; chlorogenic acid; gallic acid; (−)-epicatechin; gallocyanine; brilliant yellow; methyl 3,5-dihydroxybenzoate; 3-amino-4-hydroxybenzoic acid; 3,4-dihydroxycinnamic acid (caffeic Acid); sinapic acid; trans-3-hydroxycinnamic acid; p-coumaric acid; trans-ferulic acid; 4,7-dihydroxycouma-rin; 7-hydroxycoumarin-3-carboxylic acid N-succinimidyl ester; 7-hydroxycoumarin-3-carboxylic acid; methyl 4-hydroxybenzoate; myricetin; 6-hydroxycoumarin; coumarin; 7-hydroxy-4-methyl-3-coumarinylacetic acid; 3-cyanoumbelliferone; isopropyl 3,4,5-trihy-droxybenzoate; resveratrol; 4-hydroxy-3-methoxycinnamic acid; 2-hydroxycinnamic acid; quercetin; ethyl 3,4-dihydroxycinnamate; 7,8-dihydroxy-2,2-dimethylchromane-6-car-boxylic acid; trans-cinnamic acid; 3,4-dihydroxy-benzoic acid methyl ester; 4-ethyl-7-hy-droxy-8-methyl-2H-chromen-2-one; α-cyano-4-hydroxycinnamic acid; naringenin; trolox; (±)-taxifolin; 7,3'-dihydroxyflavone; 2,4-dihydroxy-3,6-dimethylbenzoic acid; 3-cyano-7-hydroxy-4-methylcoumarin; (±)-6-hydroxy-2,5,7,8-tetramethylchroumane-2-carboxylic acid; butyl gallate; 6-hydroxyflavone; warfarin (4-hydroxy-3-(3-oxo-1-phenylbutyl)coumarin); icariin; 3'-hydroxy-a-naphthoflavone; 3-tert-butyl-4-hydroxybenzoic acid; 5,7-dihydroxy-3',4',5'-trimethoxyfla-vone; 7-hydroxyflavone; β-carotene; lutein; 4-hydroxytamoxifen; 5,7-dihydroxy-4-propyl-coumarin; shikonin; 3'-hydroxy-6-methylflavone; 7-hydroxy-4-(trifluoromethyl) couma-rin; 5-hydroxyflavone; isobutyl 4-hydroxybenzoate; 3,3',4'_trimethoxyflavone; diethylstil-bestrol; butyl 4-hydroxybenzoate; 7-hydroxy-3',4',5'-trimethoxy-alpha-naphthoflavone; 3'-hydroxy-b-naphtoflavone; 3,3'-dimethoxyflavone; 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone; 3,6,3'-trimethoxyflavone; 3,7-dimethoxyflavone; 5-hydroxy-3'-methox-yflavone; xanthurenic acid; 4',5'-dimethoxy-2'-hydroxy-4-methylchalcone; rosmarinic acid; (z)-3-(3-ethoxy-4-hydroxy-phenyl)-2-phenyl-acrylic acid; 2-chloro-3-(3,5-di-tert-bu-tyl-4-hydroxy-phenyl)(1−4)naphtoquinone; hamamelitannin; 9-chloro-10-hydroxy-2,3-di-méthyl-anthracène-1,4-dione; 3,4-dihydroxy-5-methoxycinnamic acid; and 5-hydroxy-7-((3-methylbenzyl)oxy)-2-phenyl-4h-chromen-4-one.

2.2. Plant Material

In this study, the cones of *P. halepensis, P. brutia and P. pinea* (family Pinaceae) were sampled in Bizerte (altitude: 37°16'27", longitude: 9°52'26", northern Tunisia) in December 2016. The specimens were identified by Dr. Hamrouni Lamia and stored at the National Institute of Research on Rural Engineering, Water and Forests (INRGREF) in Ariana, Tunisia. The cones were selected at the right stage of maturity. The petals and hearts were then separated manually. Grinding was carried out in a standardized two-step process. In the first step, a SACEM hammer mill (model: G8042, power: 5.5 kW, frequency: 50 Hz, speed: 1450 rpm) with a 13 mm sieve was used. The grinding time for heart (C) and petals (P) was approximately 30 min. In the second step, a knife mill (FRITSCH Pulverisette 19) with five knives (frequency: 50–60 Hz and 2100 watts) was used. A 1 mm mesh was used and the comminution times were between 24 and 31 min in this step.

2.3. Extraction

The maceration method was applied to extract secondary metabolites from different pine species, including PA, PB and PP, which comprise both P and C. In this method, five grams of each sample were subjected to continuous extraction for two hours at 20°C, using moderate stirring and organic solvents with increasing polarity: Cyclohexane (1SV), ethyl acetate (2SV) and methanol (3SV), with a sample to solvent ratio of 1:10 (w/v) under ambient pressure and temperature. Subsequently, the filtered extracts were concentrated by distillation in a rotary evaporator (IKA, RV 10 auto V, Germany) under vacuum at reduced pressure and a temperature of 35°C.

The extraction yield was determined using the following formula:

$$\%Yield = 100\text{x}\left(\frac{m_{DW}}{M_{dm}}\right)$$

Where: m_{DW} represents the weight of dry extract (g) and M_{dm} denotes the weight of dry plant material (g).

2.4. Quantification of Reducing Sugar Content

Quantification of reducing sugars (RSC) in extracts 1SV, 2SV and 3SV of PA, PB and PP for both fractions P and C was performed according to the 3,5-dinitrosalicylic acid (DNS) method as described by Ayadi et al. [7], with minor modifications. 150 µL of each extract (350 mg/L) was mixed with 150 µL of DNS solution. After incubation at 100°C for 5 min with constant stirring, 750 µL of deionized water was added. Then, the absorbance of the mixture was measured at 530 nm. This measurement was performed with a reference blank containing solvent (sodium potassium tartrate in NaOH 2 M) instead of DNS and a negative control in which the extract was replaced by dimethyl sulfoxide (DMSO). The sugar content was determined in milligrams of glucose equivalent per gram of dry extract (mg GAE/g DW).

2.5. Total Phenolic Content (TPC)

The total phenolic content (TPC) of different obtained 1SV, 2SV and 3SV of PA, PB and PP for both fractions P and C, was estimated with a colorimetric assay using the Folin–Ciocalteu method as described by Ayadi et al. [7]. In a basic environment generated by the sodium carbonate (Na_2CO_3) and upon oxidation of the sample's phenols, the Folin–Ciocalteu reagent's phosphotungstic acid and phosphomolybdic acid were reduced to a blue-colored complex, which was proportional to the amount of phenolic compounds present. The blue color intensity was assessed with a microplate reader (Multiskan Go, F1-01620, Thermo Fisher Scientific, Vantaa, Finland) at 765 nm. TPC content was reported as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW) using the regression equation derived from the standard calibration curve of known gallic acid concentrations (0 to 115 mg/L).

2.6. Antioxidant activity

The free radical scavenging activity (DPPH) of the different extracts 1SV, 2SV and 3SV of PA, PB and PP for both fractions P and C was established by Ayadi et al. [7]. In a 96-well microplate (Micro Well; Thermo Fisher Scientific, lllkirch, France), 20 µL of each diluted extract (0.5 mg/mL) was combined with 180 µL of a methanolic DPPH solution (0.2 N). Afterward, the reaction mixture was incubated for 25 min at 25°C. A microplate reader (Multiskan Go F1-01620, Thermo Fisher Scientific, Vantaa, Finland) was then used to measure absorbance at 524 nm. In this test, the ascorbic acid was utilized at 1.5 and 5 µg/mL as references. The percentage inhibition of DPPH was determined as the following formula:

$$\%inhibition = 100\text{x}\left(\frac{A_{blank} - A_{sample}}{A_{blank}}\right)$$

The "A_{blank}" represents the absorbance of the solvent and DPPH radical when no samples are present. And the "A_{sample}" represents the absorbance of the sample and DPPH radical.

2.7. Cytotoxic Activity (MTT)

The antiproliferative activity of the extracts 1SV, 2SV and 3SV of PA, PB and PP for both fractions P and C was estimated on a human liver cancer cell line (HepG2), and their cytotoxicity was also assessed in human embryonic kidney cells (HEK-293) purchased both from the American Type Culture Collection (ATCC, Manassas, VA, USA). HepG2 and HEK-293 cell lines were cultured in DMEM (Advanced DMEM, Thermo Fisher Scientific) and high-glucose DMEM (Dulbecco's Modified Eagle's Medium, France), respectively. Each growth medium was supplemented with 10% decomplemented fetal bovine serum, 1% non-essential amino acids, and antibiotics including penicillin, streptomycin, and gentamicin. Cell cultures were maintained in a humidified incubator at 37°C with 5% carbon dioxide (CO_2). Upon reaching 70–80% confluence, the cells were harvested and used for conducting cytotoxicity assays. Adherent cells were seeded at a density of 12,000 cells/well in a 96-well microplate for HepG2 and HEK 293. The microplate was subsequently incubated overnight at 37°C in a thoroughly humidified atmosphere with 5% CO_2. Following that, the cells were treated in triplicate with each diluted extract at 50 g/mL and incubated for 48 hours at 37°C. To evaluate cytotoxicity, we employed the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test as described by Ayadi et al. [7]. After removing the supernatant, cells received 50 µL of MTT solution and incubated at 37°C for 40 min. Following incubation, the MTT solution was removed, and the resulting dark-blue formazan crystals, generated by the reduction of the yellow soluble MTT through mitochondrial dehydrogenase enzymes in viable cells, were dissolved in 80 µL of DMSO. The absorbance at 605 nm was then measured using a microplate reader (Mullikan Go, F1-01620, Thermo Fisher Scientific, Vantaa, Finland). Tamoxifen at 1, 10 and 100 µM was used as a reference in this test.

2.8. High-Performance Liquid Chromatography (HPLC-DAD)

The analysis of the extracts 1SV, 2SV and 3SV of PA, PB and PP for both fractions P and C was performed using an HPLC-DAD system consisting of a Thermo Scientific Accela pump equipped with an Accela PDA detector as described by Ben Khadher et al. [8]. Compound detection was selected at 280 nm. The separation was conducted using an RP-C18 column (Phenomenex; Le Pecq, France) with dimensions of 25 cm × 4.6 mm and a particle size of 5 µm. Elution was carried out at a flow rate of 0.5 mL/min. The mobile phase consisted of acidified water (pH = 2.65) as solvent A and a mixture of acidified water/ACN (20:80 v/v) as solvent B. A linear gradient elution method was employed: the concentration of solvent B increased from 12 to 30% over 15 min, then further rose from 30 to 50% within 2 min, and finally reached 99.9% in 3 min. Subsequently, it was returned to 12% B in 7 min. The extracts were dissolved in a solution consisting of acidified water/ACN (20:80 v/v) at 10 mg/mL. Subsequently, they were filtered using (Sigma Aldrich; Millex-HA filter 0.2 µm; Saint-Quentin-Fallavier, France). Compound identification was based on the comparison of their retention times and lambda max values with established reference standards.

2.9. Gas Chromatography-Mass Spectrometry (GC-MS)

The volatile composition of the extracts 1SV, 2SV and 3SV of PA, PB and PP for both fractions P and C was analyzed according to the method described by Ben Khadher et al. [8] with modifications. The extracts obtained were dissolved in their respective extraction solvents at a concentration of 3 mg/mL. The analysis was conducted using a Saturn 2000 gas chromatograph (Les Ulis, France) equipped with a fused silica capillary DB-5MS column (5% phenylmethylpolysiloxane, 30 × 0.25 mm, with a film thickness of 0.25 µm). Hydrogen gas was employed as the carrier gas in this analytical procedure. The column oven temperature program followed this sequence: starting at 60°C, it was maintained for 1 min, then gradually increased at a rate of 10°C per min until it reached 150°C. It was held isothermally at 150°C for 1 min. Subsequently, another gradient was applied to reach 260°C at a rate of 12°C per min and then held at 260°C for 10 min. For mass spectrometry, each acquisition recorded data in full-scan mode within the range of 70 to 800 AMU. The ion source was maintained at 220°C, and the transfer line was heated to 240°C. An injection volume of 5 µL was used for each extract. Compound identification in the extracts was accomplished by comparing their mass spectra with those available in the NIST08 database (National Institute of Standards and Technology, https://www.nist.gov/, MS library version 2.4, build 25 March 2020).

Derivatization method: The derivatization procedure consisted of taking 290 µL of the samples prepared as described above and adding 60 µL of N, O bis(trimethylsilyl)trifluoroacetamide (BSTFA) reagent. This mixture was then incubated at 40°C for 30 min. Subsequent spectral analysis of each derivative solution followed the procedure described in the previous section.

2.10. Statistical Analysis

The experimental data were expressed as mean values with standard deviations, and each sample was measured in triplicate. The difference between the solvents used and the different pinecone species was evaluated using the Tukey test. In addition, principal component analysis (PCA) was performed using XLSTAT (version 2021.3.1, Addinsoft, Pearson edition, Waltham, MA, USA).

3. Result and Discussion

3.1. Extraction Yields

In this study, maceration was used to investigate the effects of (i) species (PA, PB and PP), (ii) fraction (P and C) and (iii) organic solvents with increasing polarity (1SV, 2SV and 3SV) on extraction yield, antioxidant and cytotoxic activities and chemical compositions. The results for the extraction yield are illustrated in Table 1. The yield of fraction P was higher than that of fraction C for all samples. The highest value was obtained for PA-P-3SV (12.52%), while PB-C-1SV had the lowest value (0.50%). In addition, the yield of the polar fractions is higher than that of the apolar fractions. These results indicate that the

pinecones of the studied species contain more polar than apolar compounds. The highest extraction yield was obtained with PA, followed by PB and finally PP for all extracts. These differences in yield between the different pine species and extraction solvents underline the importance of species selection and solvent choice for the optimization of extraction processes. Compared to the literature, our results demonstrated a higher extraction yield for P (12.52%) compared to the total cone (10.60% with 3SV) studied by Salim et al. [9], while the C extraction yield was lower. This highlights the potential benefits of focusing on individual fractions to optimize extraction protocols and increase overall yield. The same study showed a higher extraction yield with methanol (10.60%) compared to hexane (3.80%), indicating that a higher solvent polarity leads to a higher extraction yield. Another study by Dhibi et al. [10] using green PA cones with methanol showed a lower extraction yield (2%), which illustrates the influence of the degree of maturity of the cones on the extraction process.

Table 1
Extraction yields of PA: P. halepensis, PB: P. brutia and PP: P. pinea petal (P) and heart (C) extracts (DW %) extracts (1SV: Cyclohexane, 2SV: Ethyl acetate, 3SV: Methanol).

Yields (%)			
Fractional Extraction	**1SV**	**2SV**	**3SV**
PAP	4.30	5.50	12.52
PA-C	2.30	1.90	3.87
PB-P	1.20	4.59	8.47
PB-C	0.50	2.54	2.84
PP-P	1.13	3.00	3.08
PP-C	0.70	1.60	1.18

Table 2
Reducing sugar content (mg GE/g DW) of different pinus PA: P. halepensis, PB: P. brutia and PP: P. pinea petal (P) and heart (C) extracts (DW %) extracts (1SV: Cyclohexane, 2SV: Ethyl acetate, 3SV: Methanol). GE: glucose equivalent. DW: dry weight. Results are mean ± SD (n = 3). Different letter on the table means a significant difference ($p \leq 0.05$).

Sugars (mg GE/g DW)			
Fractional Extraction	**1SV**	**2SV**	**3SV**
PAP	10.86 ± 2.09^{aC}	70.93 ± 2.37^{aB}	367.24 ± 1.48^{aA}
PA-C	21.84 ± 1.62^{aC}	82.59 ± 3.97^{aB}	594.17 ± 5.08^{aA}
PB-P	19.09 ± 2.72^{bC}	90.69 ± 9.70^{bB}	282.55 ± 9.31^{bA}
PB-C	25.34 ± 4.79^{bC}	87.02 ± 3.88^{bB}	144.18 ± 9.01^{bA}
PP-P	7.96 ± 1.14^{cC}	70.72 ± 3.85^{cB}	201.32 ± 8.93^{cA}
PP-C	21.77 ± 4.30^{cC}	80.87 ± 4.08^{cB}	231.37 ± 7.94^{cA}

3.2. Reducing Sugar Content

The determination of reducing sugars was carried out using the DNS method. The content of reducing sugars in PA, PB and PP for the two fractions P and C varied depending on the polarity of the solvents. A moderate RSC (less than 30 mg/g DW) was found in the 1SV extracts for all samples. However, the remaining extracts had high sugar contents ranging from 70.72 to 594.17 mg/g DW. For all samples except PB-3SV, C had a higher RSC value than P. The highest RSC was found for PA-C-3SV (594.17 mg/g DW), which accounted for 59.41% of the total original extract (1 g). PP-P-1SV had the lowest RSC (7.96 mg/g DW), which represented 0.79% of the total original extract (1 g). These results illustrate the richness of pinecones in sugars and the influence of the solvent used on their extraction. Compared to the literature, the study by Gamli et al. [11] showed that the cones of PB contain between 22.41 and 22.97% of the total sugars in the aqueous extract.

3.3. Total Polyphenol Content

Quantification of TPC in PA, PB and PP for both fractions P and C with 1SV, 2SV and 3SV extracts was performed using the Folin–Ciocalteu method and illustrated in Fig. 1. The data analysis showed that polar fractions have a significantly higher TPC concentration than apolar fractions. The 1SV extracts showed a low TPC for all species. The 2SV extracts showed a moderate TPC between 17.95 and 43.53 mg GAE/g DW. Statistically, the PA-3SV showed higher TPC values than PB and PP. Looking at the fractions, P demonstrated higher TPC values than C in all 3SV extracts. PA-P-3SV and PA-C-3SV showed the highest TPC values with 460.66 and 359.24 mg GAE/g DW, respectively. Compared to the literature, the TPC for the total cone of PB-3SV reported by Semercì et al [12] is the lowest at 91 mg GAE/g DW. This discrepancy can be attributed to the possible influence of the solid-liquid ratio used in the extraction. In another study conducted by Meziti et al. [13], the use of MeOH/H2O (80/20) as a solvent for the extraction of phenolic compounds from the total cone of PA yielded a lowest TPC value of 251.40 mg GAE/g DW). The addition of water to their solvent mixture may have reduced the extraction of polyphenolic compounds. In the study by Costa et al. [14] ultrasound was used for the extraction of phenolic compounds from P and C of PP. The results confirm that P has

a higher phenolic compound than C (601.8 and 360.6 mg GAE/g DW, respectively) for the ethanolic extract. These values are higher than our results, which may be due to the potential of ultrasound in extracting the total phenolic compounds compared to the maceration method.

3.4. Antioxidant Activity

The antioxidant potential of the extracts was determined using the DPPH assay. At 50 µg/mL, a significant difference was observed between the different extraction solvents. Only the methanolic extracts showed significant antioxidant activity against the DPPH radical. The PP-P represents the highest inhibition values (94.75%). These results indicate that polar compounds play a key role in the generation of antioxidant compounds. In the methanolic extracts of the different species, the P showed higher values for antioxidant activity values compared to the C, except for PA. Table 3 shows the values of inhibitory concentrations with 50% free radical inhibitory activity (IC_{50}) generated from the curves showing the dependence of inhibitory activity on the extract concentration of the methanolic extract. The lowest IC_{50} value was determined for the methanolic extract, indicating the highest free radical scavenging activity. However, the IC_{50} values for the extracts 1SV and 2SV were above 50 µg/mL. As for the methanolic extracts, the P showed higher antioxidant values compared to the C. The PP-P-3SV had the highest IC_{50} (10.54 µg/mL). These results have implications for possible applications of pine extracts as natural antioxidants. Compared to the literature, a study by Meziti et al. [13], which investigated the antioxidant potential of the total cone of PA using the DPPH assay, showed lower antioxidant activity for the MeOH/H_20 (80/20) extract, with an IC_{50} of 18.87 µg/mL. The study by Dhibi et al. [10] showed significantly lower antioxidant activity, with an IC_{50} value of 474 µg/mL for methanolic extracts of the green cone of PA. This discrepancy could be due to the different maturation stages of the pinecone. It's well known that the antioxidant properties of plant material can be influenced by factors such as growth stage and environmental conditions. A comparison of our results with those of Costa et al. [14] for PP, confirmed that the P has a higher antioxidant activity than the C when a polar solvent is used. However, it's noteworthy that the ethanolic extract obtained by ultrasound at 50°C, exhibited significantly lower antioxidant activity, with IC_{50} values for the P and C of 46.8 and 103.8 µg/mL respectively. Such a discrepancy is likely due to the effect of temperature, leading to the degradation of antioxidant molecules in the extracts. This highlights the crucial role that extraction conditions, especially temperature, play in influencing the overall antioxidant potential of the extracts.

Table 3
IC50 of Antioxidant activity of methanolic extract (3SV) from P. halepensis, P. brutia and P. pinea (P: petal; C: heart). Mean values ± SD (n = 3). Different letter on the table means a significant difference ($p \leq 0.05$).

Fractional Extraction						
3SV Extracts						
	PA-P	PA-C	PB-P	PB-C	PP-P	PP-C
DPPH IC_{50} (µg/mL)	14.16 ± 2.25^{b}	16.63 ± 0.67^{b}	26.57 ± 0.95^{d}	50.01 ± 3.74^{e}	10.54 ± 0.24^{a}	22.46 ± 1.82^{c}

3.5. Cytotoxic Activity

The MTT assay was used to analyze the anticytotoxic effects of the extracts obtained at 50 µg/ml on a human liver cancer cell line (HepG2) and their cytotoxicity in human embryonic kidney cells (HEK-293) following a 48 h treatment. Tamoxifen was used as a control at a concentration of 1, 10 and 100 µM. HepG2 represents a commonly utilized hepatic cell line in scientific investigations, extensively applied in studies ranging from oncogenesis to assessing the impact of different substances on liver cells [15]. A moderate cytotoxic activity was observed with the 1SV and 2SV extracts. No inhibition was detected with the 3SV extracts for all species, except for PA-P with 41.11%. The highest inhibition value was observed in the petal of P. *halepensis* with 57.59% with the ethyl acetate extract. There are not significant differences between P and C for studied species. Compared to the literature, it's worth noting the scarcity of similar studies on the species under investigation. However, a study conducted by Li et al. [16], although it focused on the cones of Pinus *yunnanensis*. Their findings revealed a notable inhibition rate of 73% against the HepG2 cell line. This underscores the species-dependent nature of the anticancer potential. Regarding the HEK-293 cells, it's worth noting that HEK-293 cells are a transformed cell line originally derived from human embryonic kidney cells through the introduction of sheared adenovirus type 5 DNA fragments [17]. These cells are classified as immortalized, meaning they possess the capability for indefinite cell division. This attribute renders them exceptionally valuable and extensively employed in biomedical research [18]. All the extracts tested at 50 µg/mL, a low percentage of HEK-293 cell viability, ranging from 0.72 to 11.05 µg/mL. This observation is consistent with our results, as all our extracts showed no negative effects on the viability of healthy normal cells. These findings emphasize the safety and non-toxic characteristics of pinecone extracts from *P. halepensis*, *P. brutia*, and *P. pinea*. They estimate that these extracts hold promise for further investigation and potential applications in diverse areas, including pharmaceuticals, nutraceuticals, and functional foods.

3.6. Chromatographic Analysis

3.6.1. Identification of compounds using HPLC-DAD

The compounds found in the both fractions P and C of the extracts of PA, PB and PP were identified using HPLC-DAD technique. To determine the composition of the extracts, the retention time (RT) and maximum wavelength (λ_{max}) of each peak were compared with those of reference compounds. These reference standards were introduced into the system under the same experimental conditions as the *Pinus* extracts.

Phytochemical analysis of extracts resulted in the identification of 38 compounds. Twenty-seven compounds have not been previously identified in these species (Table 4). The dominance of these compounds was detected in the petal fractions of all species. Catechin and chlorogenic acid were exclusively detected in the PA-P-3SV, measuring 59.87 and 56.70 mAUmin respectively, two compounds known for their antioxidant and anticancer properties, as confirmed by Santos et al. [19] and Myo et al. [20], which confirms the results of this extract against DPPH and HepG2. Gallic acid, a major antioxidant and anticytotoxic commercial standard [19], was identified only in PA-C-3SV and was 131.61 mAUmin. This result highlights the specificity of this extract

compared to the C fraction of the other species studied towards DPPH. Trans-cinnamic acid, 5-hydroxy-4'-methoxyflavone, and 4-hydroxy-3-(3-oxo-1-phenylbutyl)coumarin, three bioactive molecules found exclusively in PP-P-3SV, are known for their strong antioxidant properties [21]. These compounds provide information on the lowest IC_{50} value (10.54 µg/mL) of this extract against DPPH. The 3-amino-4-hydroxybenzoic acid and caffeic acid were detected in 1SV and 2SV of all three species. This indicates that these compounds play an important role in the chemical properties of these species. In addition, beta-carotene was found specifically in PB and PP extracts. These results indicate a significant difference in the phytochemical composition of these two species compared to PA. Various compounds, including 3'-hydroxy-6-methylflavone, 5-hydroxyflavone, 3,3',4'-trimethoxyflavone, 3,3'-dimethoxyflavone, 3,7-dimethoxyflavone, 5-hydroxy-3'-methoxyflavone, xanthurenic acid, 4',5'-dimethoxy-2'-hydroxy-4-methylchalcone, and (z)-3-(3-ethoxy-4-hydroxy-phenyl)-2-phenyl-acrylic acid, were detected in the majority of the extracts. This common chemical composition suggests common characteristics between these species.

Table 4
Identification of compounds by HPLC-DAD of petal (P) and heart (C) extracts from P. halepensis, P. brutia and P. pinea. Cyclol

N°	Compound	RT	Aera mAU*min										
		min	PA-C			PA-P			PB-C			PB-P		
			1SV	2SV	3SV	1SV	2SV	3SV	1SV	2SV	3SV	1SV	2SV	3
1	Catechin	0.90						59.88						
2	Chlorogenic acid	1.19						56.70						
3	Gallic acid	2.05			131.61									
4	Gallocyanin	2.34				1.11		216.16						1
5	Methyl 3,5-dihydroxybenzoate	2.52		2.65	184.98	11.97	34.88							
6	3-Amino-4-hydroxybenzoic acid	2.97	18.89				69.26			38.31		27.80		
7	Caffeic acid	3.15				34.76	71.50			33.54			29.94	
8	Sinapic acid	3.33					79.53			38.61	42.02		24.34	
9	Trans-3-hydroxycinnamic acid	3.65			99.40						46.89			
10	p-coumaric acid	3.85									46.70			
11	Trans-ferulic acid	4.29									76.24			
12	Trans-cinnamic acid	10.94												
13	5-Hydroxy-4'-methoxylflavone	18.70												
14	Chrysin	18.92										1.58		
15	4-Hydroxy-3-(3-oxo-1-phenylbutyl)coumarine	19.10												
16	3'-Hydroxy-a-naphthoflavone	19.37		21.98								90.72		
17	7-Hydroxyflavone	19.70					17.59							
18	Beta carotene	19.79							33.59			5.13		1
19	Lutein	19.81					3.87			218.07				
20	4-Hydroxytamoxifen	20.28			2.20	11.27	21.10			355.27	1.11		7.74	
21	5,7-Dihydroxy-4-propylcoumarine	20.62							5.98		199.33			
22	3'-hydroxy-6-methylflavone	20.75		17.37	28.50	19.14	57.41				258.76	101.82	8.43	6
23	5-hydroxyflavone	21.00												1
24	3,3',4'_trimethoxyflavone	21.07			1.91	118.44			111.57		3.59	105.41		
25	Butyl 4-hydroxybenzoate	21.12		179.66						102.08		53.40	491.80	
26	Cardamonin	21.26												
27	Caffeic acid 1,1-dimethylallyl ester	21.34		220.98								2.45		
28	Benzyl 4-hydroxybenzoate	21.50					20.31		73.51					
29	7-Hydroxy-3',4',5'-trimethoxy-alpha-naphthoflavone	21.50		8.14	8.67						82.97			
30	3,3'-Dimethoxyflavone	21.60			13.07	90.63			154.03					
31	3,6,3'-Trimethoxyflavone	21.79	90.08				17.67	3.93				92.96		
32	3,7-Dimethoxyflavone	21.83					212.04						12.71	

N°	Compound	RT	Aera mAU*min										
		min	PA-C			PA-P			PB-C			PB-P		
			1SV	2SV	3SV	1SV	2SV	3SV	1SV	2SV	3SV	1SV	2SV	3
33	5-Hydroxy-3'-methoxyflavone	22.01				86.86	256.58		125.17		1.91	81.65		
34	Xanthurenic acid	22.12		17.22	2.35	101.45			54.68	686.13			141.72	
35	4',5'-Dimethoxy-2'-hydroxy-4-methylchalcone	22.60	8.46	108.94	13.74	192.08	187.48		19.19		2.44	243.18		
36	(z)-3-(3-Ethoxy-4-hydroxy-phenyl)-2-phenyl-acrylic acid	23.40	50.13			10.98	170.16			246.22	1.61		14.01	
37	Hamamelitannin	24.06										7.95		
38	3,4-Dihydroxy-5-methoxycinnamic acid	25.04			5.27	64.18								

3.6.2. Identification of Volatile Compounds by GC-MS

The volatile composition of the different extracts was evaluated by GC-MS analysis. Tables 5 and 6 list the different compounds detected in the extracts before and after derivatization respectively. The application of GC-MS allowed the identification of 45 compounds, both before and after derivatization. Thirty-three of the identified volatile compounds were identified for the first time in *Pinus*. Nine phenolic compounds were identified: 2,4-di-tert-butylphenol, phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-], catechol, vanillin, vanillic acid, protocatechuic acid, p-coumaric acid, and caffeic acid. It's worth mentioning that p-coumaric acid and caffeic acid were also identified in this study by HPLC. The minority identification of compounds in the methanolic extracts by GC-MS analysis raises important questions about the chemical nature of the molecules present. GC-MS is known for its ability to analyze volatile or semi-volatile compounds, but it can pose limitations when dealing with larger or highly polar molecules. The exclusive presence of compounds such as isobornyl formate, 17-pentatriacontene, dehydroabietin, 3-hydroxybutyric acid, vanillin, D-(-)-ribofuranose, vanillic acid, p-coumaric acid, and caffeic acid in PA-1SV and PA-2SV extracts, and their absence in 1SV and 2SV of PB and PP, provides interesting insights into the differential bioactivity of these extracts. This observation could confirm the superior anticancer activity observed in this species compared to the other species studied. Among the numerous compounds identified, verbenone, carveol, 2,4-di-tert-butylphenol, oleamide, propylglycol, cyclohexanol, caproic acid, myrtenic acid, and palmitic acid were most frequently found in all extracts. Their presence in these extracts could provide valuable insights into the chemistry of these plant species and their potential applications in various fields. In the literature, the research conducted by Fekih et al. [30] resulted in the identification of 49 volatile compounds through GC-MS analysis in various parts of *P. halepensis*, including needles, twigs, and buds. Notably, their analysis revealed the presence of caryophyllene oxide and bornyl acetate. As highlighted by Dhibi et al [10], their investigation into *Pinus halepensis* seed and cone compositions led to the identification of palmitic acid and stearic acid. In a study conducted by Pasqualini et al. [31], a comprehensive analysis of the volatile compounds in *Pinus halepensis* Mill. needles revealed the quantification of various compounds, including protocatechuic acid, vanillic acid, p-coumaric acid, 4-hydroxybenzoic acid, vanillin, syringic acid, and gallic acid.

Table 5

Identification of volatile compounds by GC-MS of P. halepensis, P. brutia and P. pinea petal and heart Extracts (before derivatization. Cyclohexane: (1SV); Ethyl
X: detected. RT: retention time.

N°	Compound	RT	Aera mAU*min															
		min	PA-C			PA-P			PB-C			PB-P			PP-C			PP
			1SV	2SV	3SV	1SV	2SV	3SV	1SV	2SV	3SV	1SV	2SV	3SV	1SV	2SV	3SV	1S
1	Isopinocarveol	9.04				X			X	X			X					
2	(+)-Cis-verbenyl, acetate	10.84				X								X				
3	Isobornyl formate	11.39	X				X											
4	p-Cymen-8-ol	11.82		X									X					
5	Carveol	12.27	X	X			X							X	X	X		X
6	Verbenone	12.58	X	X	X	X	X	X	X	X		X	X	X	X			X
7	Isobornyl acetate	13.27	X	X		X												X
8	Bicyclohexyl	13.43	X			X			X			X			X			X
9	Tetradecane	13.87										X						
10	Bicyclo[2.2.1]heptane-2,5-diol, 1,7,7-trimethyl-, (2-endo,5-exo)-	14.19								X								
11	Limonene glycol	14.49						X						X	X	X		X
12	2,4-Di-tert-butylphenol	16.33		X	X		X	X		X			X	X		X	X	
13	Caryophyllene oxide	17.62	X															X
14	Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-	17.92					X									X		
15	18-Norabieta-8,11,13-triene	21.86	X	X		X	X						X					X
16	Sclareol	21.97				X						X	X		X			X
17	Dehydroabietan	22.99		X		X	X						X					X
18	17-Pentatriacontene	23.23					X											
19	7-Isopropyl-1,4a-dimethyl-1,2,3,4,4a,9,10,10a-octahydro-1-phenanthrenol (isomer 1)	25.11		X		X	X		X	X		X	X					
20	Dehydroabietal	27.98				X	X											
21	Oleamide	28.26	X		X		X	X		X		X					X	
22	Methyl dehydroabietate	29.23	X			X												

Table 6
Identification of volatile compounds by GC-MS of P. halepensis, P. brutia and P. pinea petal and heart Extracts (after derivatization). Cyclohexane: (1SV); Ethyl
(3SV). X: detected. RT: retention time.

N°	Compound	RT	Aera mAU*min																
		min	PA-C			PA-P			PB-C			PB-P			PP-C			PP-P	
			1SV	2SV	3SV	1SV	2SV	3SV	1SV	2SV	3SV	1SV	2SV	3SV	1SV	2SV	3SV	1SV	2S
1	Glycol	7.13		X			X			X			X						
2	Propyl glycol	7.29	X	X		X	X		X			X	X		X			X	
3	Cyclohexanol	7.86	X			X			X			X			X			X	X
4	Lactic acid	8.74		X			X			X			X			X			
5	Caproic acid	8.84	X	X		X	X		X	X			X			X			X
6	3-Hydroxybutyric acid	10.06					X												
7	(+)-Cis-verbenol	11.55		X			X											X	
8	Glycerol	11.7		X			X									X			
9	Caprylic acid	12.05	X				X											X	
10	(-)-Myrtenol	12.84							X			X							
11	Succinic acid	13.06					X						X						
12	Pelargonic acid	13.57	X			X	X											X	
13	Citric acid	14.01								X									X
14	Cicrotoic acid	14.17				X			X										
15	Myrtenoic acid	14.89	X			X	X		X	X		X	X					X	
16	Vanillin	16.93		X															
17	D-(-)-Ribofuranose	17.22		X			X												
18	Vanillic Acid	18.82					X												
19	Protocatechuic acid	19.1		X			X			X									X
20	p-Coumaric acid	20.48		X															
21	Palmitic Acid	20.94	X	X					X	X		X	X		X	X		X	X
22	Caffeic acid	21.96	X																
23	Stearic acid	23.35		X					X				X						X

3.7. Principal Component Analysis (PCA)

The investigation involved analyzing the correlation among total polyphenol content (TPC), antioxidant capacity (measured by DPPH assay), and anticancer activity (against HepG2 cells). Table 7 presents the data, revealing both positive and negative correlations. Specifically, a noteworthy correlation coefficient of 0.86 was observed between TPC and DPPH. The correlation between polyphenol content and antioxidant activity in plant extracts has been explored in various studies. In this regard, the study of Ait Atmane et al. [40] found a substantial correlation ($R^2 = 0.95$) between TPC and DPPH in *P. halepensis* seeds. Additionally, we can observe that there is a good correlation between TPC, reducing sugar and DPPH, which suggests that the antioxidant activities in pinecone extracts may be attributed to the presence of phenolic compounds and reducing sugars. Conversely, significant negative correlations were identified for TPC, DPPH, reducing sugar, and HepG2.

Table 7
Correlation coefficients of (DPPH: Antioxidant activity) (TPC: total phenolic content) and HepG2: cytotoxic activity) from P. halepensis, P. brutia and P. pinea (P: petal; C: heart) extracts.

Variables	TPC	% inhibition DPPH	% inhibition HepG2	Reducing sugar
TPC	**1.00**			
% inhibition DPPH	0.86	**1.00**		
% inhibition HepG2	-0.55	-0.74	**1.00**	
Sucre	0.92	0.83	-0.64	**1.00**

Based on their polyphenol content, reducing sugar, antioxidant properties and anticancer activities, the extracts are depicted in Fig. 2. Pinecone extracts exhibit clear segregation into three principal groups (A, B, and C). Group A comprises two extracts (PA-P-3SV and PA-C-3SV), group B includes (PP-P-3SV, PP-C-3SV, PB-P-3SV, and PB-C-3SV), and the third group C encompasses the remaining extracts. Notably, extracts in group A are distinguished by elevated polyphenol content, reducing sugars, and DPPH radical scavenging activity. Furthermore, the positive correlation among TPC, reducing sugars, and DPPH is prominently represented in group A. These two extracts are the methanolic extracts of petal and heart fractions of *P. halepensis*, confirming its superiority compared to other studied species. Group B represents the remaining methanolic extracts of *P. brutia and P. pinea*, characterized by high TPC, reducing sugars, and antioxidant activity but with less significance compared to group A. Group C encompasses extracts in cyclohexane and ethyl acetate from all species, demonstrating moderate activity against the HepG2 cell line. This grouping highlights the varying bioactivity profiles of the extracts from different pine species and fractions, with *P. halepensis* showing superior characteristics.

4. Conclusion

A comprehensive analysis of petal and heart extracts from three different Pinus species: *P. halepensis, P. brutia and P. pinea*. Chemical analysis by HPLC-DAD led to the identification of 38 compounds. 46 volatile compounds were detected by GC-MS. In terms of bioactivity, our results showed that the methanolic extracts exhibited significant antioxidant potential. Cytotoxicity assays revealed moderate inhibitory activities, mainly in the cyclohexane and ethyl acetate extracts, with inhibition percent between 27.07 and 57.59% against HepG2 and low inhibition percent against HEK293. These results are of great importance for research in the pharmaceutical and food industries and open new avenues for the application of the extracts. Scientific perspectives will be essential to identify and isolate the bioactive molecules responsible for the observed activity.

Declarations

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Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by AC, LF, MR and JB. The first draft of the manuscript was written by AC and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability

All raw and processed data / materials will be made available by the authors on request.

Code Availability

Not applicable

Conflict of Interest

The authors declare that they have no conflicts of interest.

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Figures

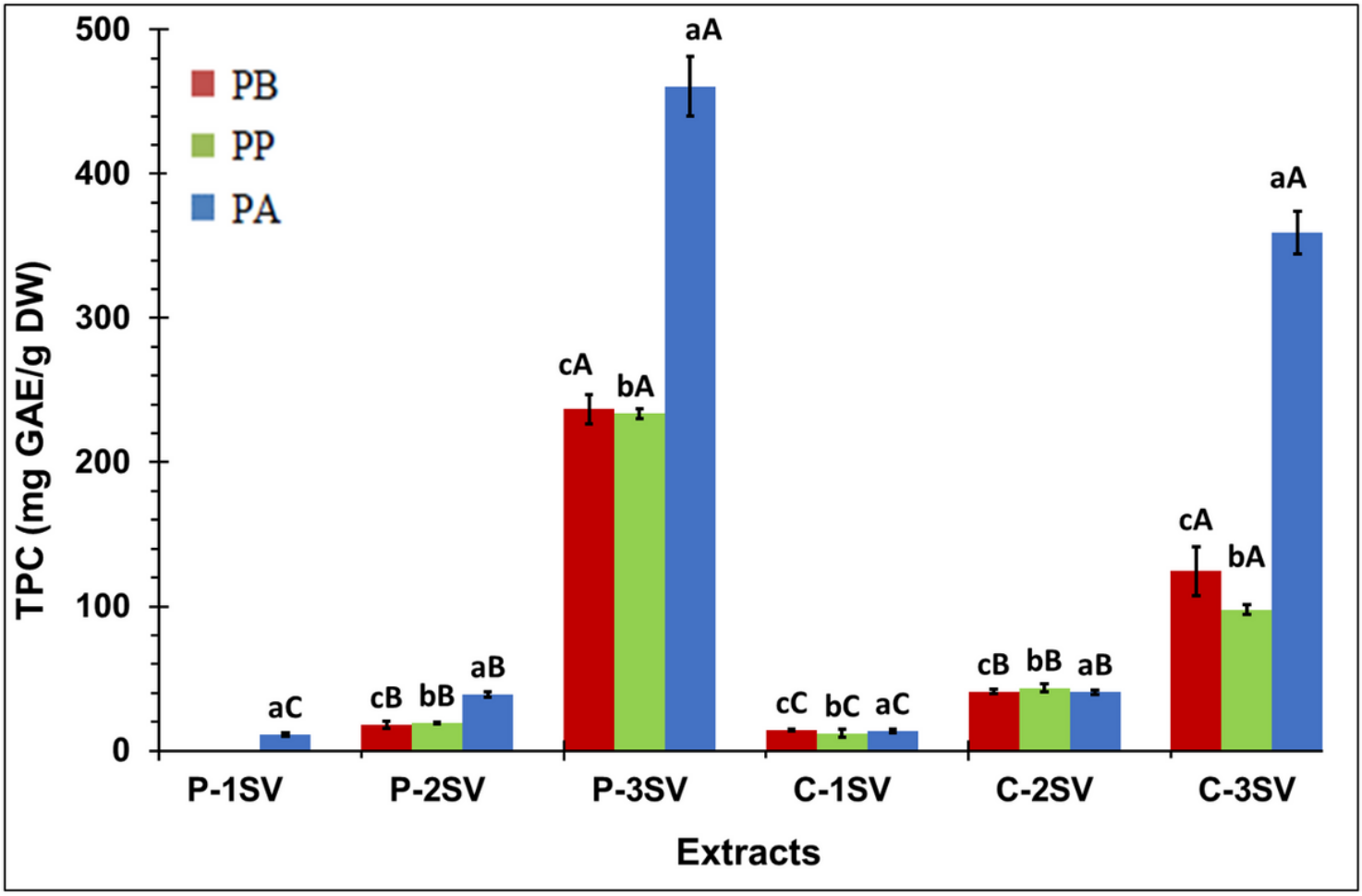

Figure 1

Total phenolic content (TPC) of P. halepensis, P. brutia and P. pinea (P: petal ; C: heart) extracts 1SV: cyclohexane, 2SV: ethyl acetate, 3SV: methanol. GAE: Gallic Acid. DW: dry weight. Mean values ± SD (n = 3). Different letter on the histograms means a significant difference ($p \leq 0.05$).

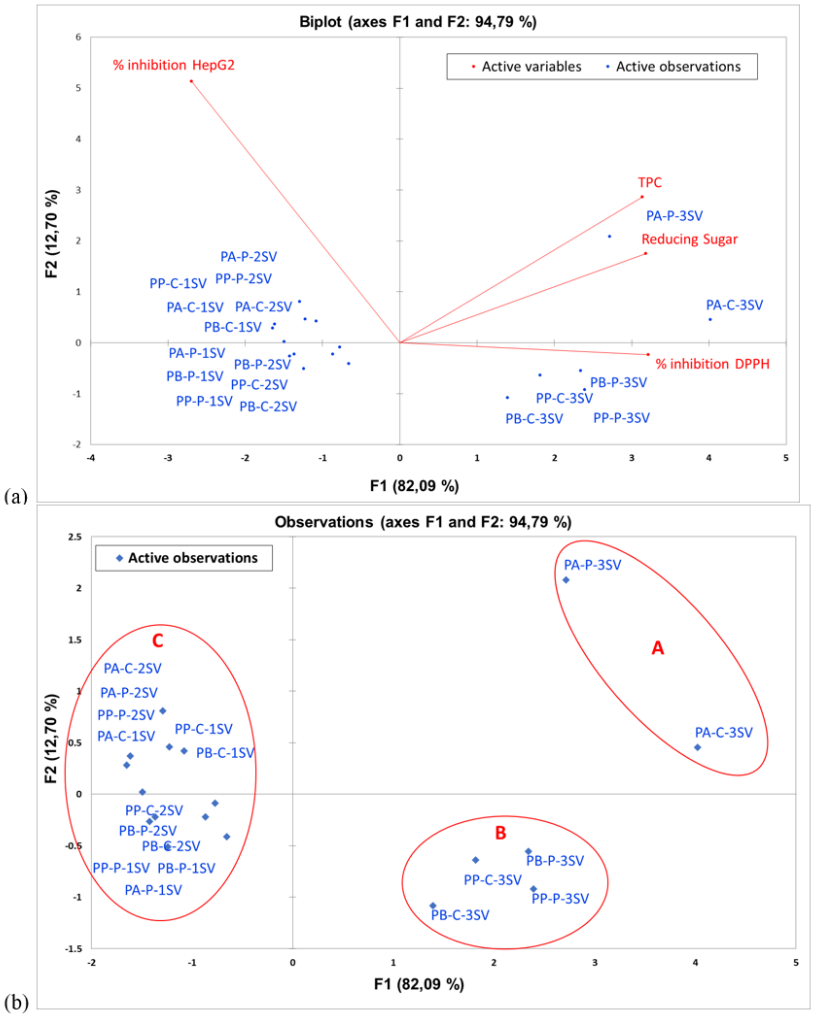

Figure 2

Principal Components analysis : (a) Biplot and (b) score plot of (DPPH: Antioxidant activity) (TPC: total polyphenol content) and HepG2: cytotoxic activity) of P. halepensis, P. brutia and P. pinea (P: petal and C: heart) extracts. 1SV : Cyclohexane, 2SV : ethyl acetate and 3SV : methanol.

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- GraphicalAbstract.png