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Isolation of Catechin and Gallic Acid from Colombian Bark of *Pinus patula*

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Abstract

Pine bark is a rich source of natural polyphenols, compounds which have attracted increasing attention in the fields of nutrition, health and medicine. Extractive components include large amounts of phenolic compounds. The ethanolic extract was obtained from *Pinus patula* bark grown in the forests of the Cauca Department in Colombia South America. The Gallic acid, catechin, epicatechin and procyanidin B2 contents in ethanolic extract were 3.12, 1.99, 0.80, and 0.71%, respectively, these compounds were determined using HPLC with UV-Vis detection. Ethanolic extract was divided using column chromatography to obtain an ethanol-soluble fraction with tannins and phlobaphenes as well as an ethanol-insoluble fraction mainly composed of Phenolic acids. Catechin and Gallic acid finally isolated were characterized using UV-Vis, IR, NMR, ^{13}C, ^{1}H, HSQC, HMBC and GC-MS spectrometry.

Keywords: Catechin; Gallic acid; Pinus; Phenolic compounds

Introduction

Pine bark is rich in phenolic compounds. The main phenolic compounds found in pine bark are catechin/epicatechin, epigallocatechin and epicatechin gallate [1]. Possible applications for pine bark are antiradical, antioxidant and anti-inflammatory properties and are marketed as food supplement and an herbal-based medication. Tannins from pine bark have been used to make wood adhesives and foams [2]. Pine bark is mainly composed of polysaccharides (cellulose and hemicellulose) and lignin, as well as minerals such as oxalates, phosphates, silicates, boron, copper, and manganese [3]. Extractive components are divided into lipophilic and hydrophilic substances. Lipophilic substances include fats, waxes, terpenes, terpenoids, and higher aliphatic alcohols. The hydrophilic fraction includes substances that are extractable by water or organic solvents and contains large amounts of phenolic constituents such as tannins, monomeric flavonoids such as quercetin and hydroquercetin, and a lesser quantity of soluble carbohydrates, proteins, and vitamins. Some pine species have produced aqueous extraction yields of 13.3% for *P. radiata* [4] and 4.1% for *P. contorta*, ethanol extraction yields of 11.6% for *P. echinata* [5], and acetone extraction yields ranging from 8.8% to 50% for *P. loblolly* [6]. It has also been reported that the use of 80% aqueous ethanol to obtain the extract of *P. durangensis* yielded 16.15%, lower than the 18.7% yield obtained using 70% aqueous acetone [7]. In studies by Pan, up to twenty-six phenolic compounds have been found in the bark of *Pinus sylvestris* from Sweden, with catechins and procyanidins being the most abundant [8]. Phenolic compounds extracted from pine bark include Gallic acid, ferulic and p-hydroxybenzoic acids, monomers such as catechin, epicatechin and taxifolin, and condensed flavonoids such as procyanidins [9]. The chemical composition of tannins has been determined thanks to the development of techniques such as gas chromatography, high performance liquid chromatography, thin layer chromatography, infrared spectroscopy, mass spectrometry, and nuclear magnetic resonance [10]. In this way, studies have been conducted on tannins extracted from the bark of species such as *Pinus radiata* [11], *Pinus pinaster* and *Pinus radiata* [12], *Pinus pinaster* and *Pinus massoniana* [13], maritime pine [1], *Acacia mangium* [14], and *Acacia confusa* [15]. The composition of tannins in *Pinus brutia* bark has been studied using MALDI-TOF MS and ^{13}C NMR spectroscopy [16]. It is well known that phenolic compounds present antifungal, antibiotic, antioxidant, and allelopathic effects, among others [17]. These compounds have earned a reputation for their activity in plants, in defense against insect attacks and in microbial diseases. It is believed that procyanidins can protect from cardiovascular diseases [18,19]. *Pinus bark* has shown to have antioxidant, anticarcinogenic, antimutagenic, antimicrobial, anti-inflammatory, and cardio-cerebrovascular protective effects, among others [9,20]. Phenolic compounds such as catechin have applications in the prevention of food oxidation, astringency, reactions with metals and proteins, and the synthesis of derivatives for pharmaceutical applications, as well as antibacterial activity and different medicinal benefits (cholesterol-lowering and antiallergic effects, etc.). Gallic acid is used in the production of antioxidants in the food industry, in brewing, in the pharmaceutical industry for the synthesis of mescaline and trimethoprim, in photography, and in papermaking, among others [17]. In the Cauca-Colombia department there are more than 14 thousand hectares planted with pine. Forest residues generated in the field can be used to obtain products with different applications. This study considers the identification of phenolic compounds present in *Pinus patula* bark and the isolation and spectroscopic identification of catechin and Gallic acid present in the ethanol extracts obtained.

Materials and Methods

Samples

Samples of *P. patula* bark were collected from Smurfit Carton de Colombia forest sub nucleus (Cabuyerita) located in the northwest of Popayan city in the Cauca department (Colombia), latitude 1°56´ north, longitude 77°10´ west, and altitude 1700 m. The trees´ diameters ranged from 25 to 30 cm and were 16 years old. The barks were air-dried and ground in a mill and sieve to select particles smaller than 1 mm.

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Chemicals

Procyanidin B2 (98%) Catechin (98%) and Epicatechin (98%) were supplied by Sigma. Ethyl acetate (99.8%), n-Hexane (96%) were supplied by Merck, EM Science. Absolute Ethanol (99.8%) was by ACS, Riedel-de Haen. Sephadex G-25 was by Pharmacia Biotech. Acetonitrile, HPLC, Purity 99.9% and acetone reactive grade were by Mallinckrodt. Deuterated-d_6 acetone (99.9% atom % D) was by Aldrich. Methanol (99%) was by Fisher Scientific.

Extraction and characterization of phenolic compounds

An ethanol-water solvent mixture (30:70) was used for the leaching extraction of phenolic compounds. The extract obtained was used to identify phenolic compounds and to separate the analytes of interest [21]. The hydroalcoholic extract obtained was roto evaporated at 55°C until the solvent was completely removed [22]. The composition of the hydroalcoholic extract was determined by high performance liquid chromatography (HPLC), and the phenolic compounds of interest were isolated by column chromatography [23].

Identification of the phenolic compounds present in the hydro alcoholic extract of *Pinus patula* bark by HPLC

Using standard solutions, identification and quantification of gallic acid, catechin, epicatechin, and procyanidin B2 was carried out in *Pinus patula* bark extracts using a liquid chromatograph Water 1515 with UV detection [24]. The chromatographic conditions used allowed the identification of phenolic compounds. A 150 x 3.9 mm µ-Bondapak C_{18} column was used, with a flow of 1.0 mL/min, isocratic elution, and a mobile phase composed of 0.5% methanol in 0.01 M Acetic acid-acetonitrile (96.5%-3.5%). UV detection at 280 nm and room temperature were used. Validation of the chromatographic method was performed to determine parameters such as linearity, precision, accuracy, and sensitivity. Solid-phase extraction was used prior to HPLC [25]. The Robards method [26] was used to eliminate matrix interference using solid-phase extraction. The percent recovery of these compounds (gallic acid, catechin, epicatechin and procyanidin B2) was determined after solid phase extraction (SPE).

Column separation of phenolic compounds

The hydroalcoholic extract was divided into an ethanol-soluble fraction and an ethanol-insoluble fraction [22]. Column chromatography was performed (Silica, Sephadex and C_{18}) for these fractions with methanol, acetone and hexane, collecting 5 mL volumes in different tubes. This process was followed by TLC on silica gel using, as eluent, toluene-acetone-formic acid (3:6:1), and the plates were developed with UV light (254 nm and 365 nm), vanillin, iron chloride and an iodine chamber to establish the composition of the fractions. In TLC, standards of catechin and Gallic acid were also used (gallic acid, Rf 0.88; catechin, Rf 0.86) [27].

Obtention of ethanol-soluble fraction

The hydroalcoholic fraction was extracted with water and further separated into two fractions: a soluble fraction (crude tannin, fraction T_1) and an insoluble fraction (crude phlobaphenes, fraction F_1). Crude tannin was washed successively with ethyl ether a number of times, sufficient to obtain spots only near the origin of the chromatogram using two-dimensional paper chromatography. [Paper chromatography (Whatman 3, 37 cm × 47 cm); eluent in the first direction: n-butanol-Acetic acid-water (3:1:1); eluent in the second direction: 15% (w/v) aqueous Acetic acid; developers: iron chloride, ultraviolet light, and iodine vapor]. Crude tannin (T_1) was washed with ether and ethyl acetate (fraction T_2) and passed through a Sephadex G-25 column (30 cm × 2 cm), eluting with methanol followed by methanol-ethyl acetate 1:1 (v/v). TLC was performed on the fractions obtained with toluene-acetone-formic acid (3:6:1), and the plates were developed with ferric chloride, a UV lamp, vanillin, and iodine vapor. Crude phlobaphenes (fraction F_1) were also washed with ethyl ether and ethyl acetate. The fraction was roto evaporated, and an infrared spectrum was taken from this fraction [22].

Obtention of ethanol-insoluble fraction

The ethanol-insoluble fraction was extracted with a 1% (w/v) aqueous solution of NaOH at reflux temperature for 4 hours. The soluble fraction mainly corresponds to an aqueous solution of the sodium salt of Phenolic acids, which were then separated by precipitation with a concentrated HCl solution (fraction AF_1) [22]. The insoluble fraction of the NaOH extraction corresponds to lignocellulosic material (Fraction LC). This material, after being washed with 5% Acetic acid and water, was treated with 72% sulfuric acid for 2 hours at 20°C, diluted with water to a total volume of 575 mL, and then refluxed for 4 hours. The solid was decanted and filtered on a weighed Gooch crucible, washed thoroughly with water, and vacuum dried at 55°C to constant weight. The melting point and solubility in solvents such as water, acetonitrile, dichloromethane, acetone, and methanol were determined for all the compounds obtained from the different fractions.

Spectrometric characterization of gallic acid and catechin

The separated compounds were characterized by IR, UV-VIS, and NMR spectroscopy using an Infrared FT-IR 8400 (Shimadzu), spectrophotometer UV-Vis (Spectronic 21) and NMR, Brucker Ultra shield Advance II 400 MHz respectively. Correlation spectroscopy of nuclear magnetic resonance (NMR) such as heteronuclear single-quantum coherence spectroscopy (HSQC) and heteronuclear multiple-bond coherence spectroscopy (HMBC) also were used. Mass spectrometer GC-MS (Shimadzu QP 2010) was also used for structure elucidation.

Results and Discussion

Extraction of phenolic compounds

The extraction conditions for the extraction of phenolic compounds from *Pinus patula* bark were applied for 6 h, with a particle size of less than 1.18 mm, a temperature of 60°C, a 30:70% (water: ethanol) solvent ratio, a ratio of bark to solvent of 1-10 g-mL, and stirring at 250 rpm [25]. Studies conducted in Cuba have reported bark extract yields of 8.29% for *P. caribaea* and of 10.19% for *P. cubensis* [28]. The use of ethanol (50%) to do extraction of phenolic compounds yielded 6.66% for *P. engelmannii* and 19.4% for *P. ayacahuite*. These observations indicate that the extraction performance obtained with 50% ethanol is double or more than that obtained with water [29,30].

Separation and identification of phenolic compounds by chromatography

The identification of gallic acid, catechin, epicatechin, and procyanidin B2 was performed according to the method by Romani et al. [24]. The most widely used analytical technique for the identification of flavonoids is reverse phase HPLC. However, the resolution of condensed phenolic compounds is possible up to the tetramer level [31]. Generally, phenolic compounds are chromatographed on C_{18} sorbents [32]. C_{18} columns are generally used in the analysis of pine extracts, wine, and

grape tissue because lower retention times are obtained; on the other hand, C_6 columns are typically used for the analysis of apple tissue, juice, and cider. Isocratic and gradient separations were performed; gradients usually start with a large percentage of the aqueous phase (usually 80-100%), and methanol, acetonitrile, and Acetic acid are commonly used as the organic component of the eluents. The eluent should contain an acid to suppress the ionization of Phenolic acids, and methanol or acetonitrile are commonly used as the organic component of the eluent. Tetrahydrofuran is used for the analysis of hawthorn procyanidins [33]. Figure 1 shows the chromatogram of a mixture of gallic acid, catechin, epicatechin, and procyanidin B2 standards under the established optimum chromatographic conditions.

It can be observed that Gallic acid elutes first, followed by catechin, a monomeric flavonoid. It is observed that procyanidin B2 elutes before epicatechin, although procyanidin B2 is a dimeric flavonoid and epicatechin a monomeric one; this is not the case when using polar stationary phases such as silica (normal phase), where the phenolic compounds are eluted in order of increasing molecular weight. The retention times of these compounds are determined largely by the substitution on the phenyl group attached to the benzopyrone ring and the overall polarity of the molecule. The (-)-epicatechin and its oligomers elute later than those derived from (+)-catechin [34]. The elution order of the dimeric and trimeric procyanidins (C_1 and C_2) remains surprisingly constant, regardless of the acid eluent and stationary phase used. On the other hand, the retention times of procyanidins B1, B4, and C1 are the most affected by changes in the chromatographic system compared to the rest of the procyanidins [34]. Similarly, the chromatographic behavior of procyanidins is also affected by the concentration of the injected sample. When high amounts of the procyanidin fraction were added to a C_{18} column, their peaks in the elution profile shifted to lower retention times. This effect, caused by high concentrations, is reversible and may be due to the formation of polar complexes of small oligomers between themselves or with high molecular weight compounds [2]. Retention time of each of the compounds in the mixture of standards was determined and their percent relative standard deviation (%RSD) was calculated. Retention times (in minutes, with %RSD) were determined to be 3.231 (0.552%), 10.057 (0.984%), 17.653 (0.727%), and 19.828 (0.646%) for Gallic acid, catechin, procyanidin B2 and epicatechin, respectively. Under the established chromatographic conditions, a curve from 10 to 100 ppm was prepared for each compound, which showed coefficients of determination of 0.999, 0.993, 0.997, and 0.995 for gallic acid, catechin, epicatechin, and procyanidin B2, respectively. To determine the limits of detection and quantification, curves from 1 to 10 ppm were generated. Detection and quantification limits of 0.065 and 0.217, 0.071 and 0.236, 0.114 and 0.381, and 0.090 and 0.299 ppm was obtained for gallic acid, catechin, epicatechin and procyanidin B2, respectively. Detection limits of 0.66 and 0.60 ppm have been obtained for catechin and gallic acid, respectively, using reverse phase liquid chromatography with a mixture of acetonitrile-water-phosphoric acid as the mobile phase [35]. Quantification limits of 1.3 and 1.4 ppm for epicatechin and procyanidin B2, respectively, have been obtained using a methanol-water-Acetic acid mixture [36]. The method was repeated for the four phenolic compounds by measuring each concentration in triplicate using the calibration curves. The coefficient of variation ranged between 0.53 and 1.45% for the four compounds, values which are below 5%, as suggested by other authors for the analysis of these compounds [35]. Reproducibility was evaluated by determining the coefficient of variation for areas throughout the working range at concentrations of 10, 50, and 100 ppm, in which coefficients of variation were observed in the range of 1.21 to 1.78% for the four phenolic compounds. The coefficients of variation were lower than 5%, showing good reproducibility of the method. The accuracy of the system was determined in the working curves, looking at the recovery areas in each of the working curves. Accuracy was defined as t_{obt} being less than t_{table}. The t_{table} value was determined taking into account the total number of areas; thus, t_{table} for eight degrees of freedom and a 95% confidence level is 2.306. It was found that for gallic acid, catechin, epicatechin, and procyanidin B2, t_{obt} is less than t_{table}, with no significant differences between mean recovery time and 100. Overall, percent recovery data correspond to the ratio of the value of the concentration giving the signal and the concentration of the standard prepared. Validation data of the chromatographic method for the determination of phenolic compounds in *Pinus patula* bark were within the limits established by Gutierrez et al. [37] for linearity (r^2=0.99), repeatability (%RSD<5%), reproducibility (%RSD<5%), and accuracy with $t_{obt}<t_{table}$=2.306 for n=9 and 95% confidence. To eliminate interference, solid phase extraction (SPE) was used for phenolic compounds [26]. After SPE, the sample is concentrated using a nitrogen and water bath. Three milliliters of mobile phase are then added, and the chromatographic analysis is carried out. SPE accuracy was determined using the Student's *t*-test in

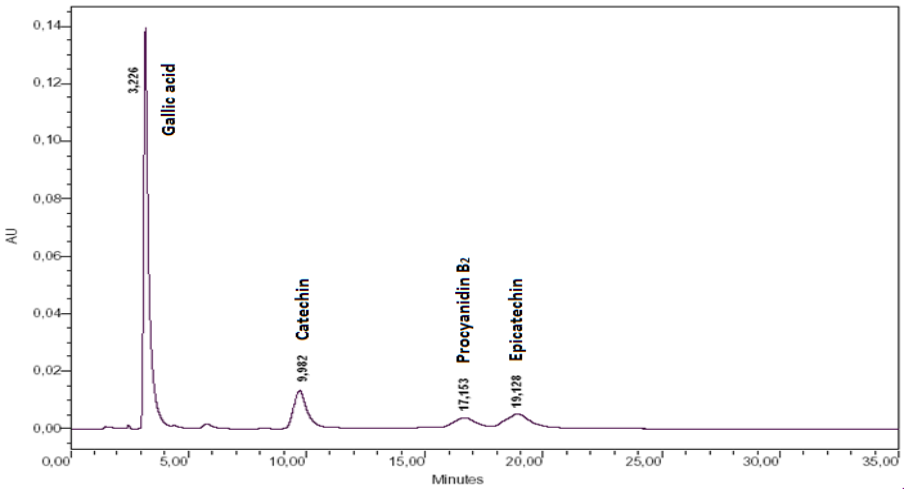

Figure 1: Chromatogram of the mixture of gallic acid, catechin, epicatechin, and procyanidin B under optimum working conditions.

the chromatographic range and using standards of gallic acid, catechin, epicatechin, and procyanidin B2. Mixtures of these standards at 10, 50, and 100 ppm were used, performing SPE in triplicate for each. Table 1 shows the average percent recovery and t_{obt} for gallic acid, catechin, epicatechin, and procyanidin B2 to be 101.58 and 0.82, 101.59 and 1.21, 96.21 and 2.21, and 98.17 and 1.31, respectively.

With SPE, percent recoveries of 97.86 and 96.40% have been obtained for Gallic acid and catechin, respectively [35]. For epicatechin and procyanidin B2, percent recoveries of 96.0 and 92.6% have been obtained, respectively, using Superspher® 100 RP-18 (4 µm) cartridges (Merck) [38]. The Student's t-test was applied to nine samples (n=9), with a t_{table} of 2.306 for eight degrees of freedom and 95% confidence. In all four phenolic compounds, $t_{obt}<t_{table}$, showing the relationship between percent recovery at the concentration giving the signal and the extracted external standard. When performing the extraction, a blank was prepared for the cartridge and solvents used for extraction. The hydroalcoholic extract of *Pinus patula* bark was submitted to the SPE process, and identification and quantification of gallic acid, catechin, epicatechin, and procyanidin B2 in the sample was performed. *Pinus patula* bark extract was found to have contents of gallic acid, catechin, epicatechin, and procyanidin B2 of 3.12, 1.99, 0.80 and 0.71%, respectively. There have been reports of catechin and epicatechin contents of 8.1 and 10.4%, respectively, in *Pinus pinaster* bark [39]. In the bark of *Pinus sylvestris* from Sweden, twenty-six phenolic compounds were found, with catechins and procyanidins being the major constituents [8]. In grape seed extract, a percentage of phenolic compounds of low molecular weight has also been found, which include gallic acid, catechin, epicatechin, and procyanidin B1, B2, and C estimated at between 5.5 and 12.2% (w/w) [40]. In extracts of pine bark (*Pinus maritima L.*), the following contents have been reported: 24.4 mg/g of gallic acid; 74.2 mg/g of catechin, epicatechin, and gallocatechin; and 285.3 mg/g of procyanidin and gallocatechin dimers [41]. In wine, the following contents have been reported: 320, 145, 128, and 30 mg of gallic acid, catechin, epicatechin, and procyanidin B2, respectively [42]. Total catechins, procyanidin dimers, trimers and tetramers in directly peeled peanut skin were 16.1, 111.3, 221.3 and 296.1 mg/100 g, respectively [43]. In studies carried out by Yesil-Celiktas et al. [44], the ethyl acetate phase of the extract obtained from four types of barks from species from Turkey were analyzed by HPLC (LC-MS). The authors found that the extract from *P. brutia* bark contained 18.6% taxifolin and 7% catechin (percent of each compound in the respective pine bark extract). The GC-MS chromatogram of the ethyl acetate extract showed high amounts of taxifolin (≥80% of the total peak area) with small amounts of taxifolin isomer (2%). Additionally, small amounts of catechin (2%) and quercetin (0.4%) and other low amounts of ferulic acid (<0.1%), glycerol (2.5%), p-hydroxybenzoic acid (0.5%), and 3,4-dihydroxybenzoic acid (2.5%) were identified. According to several studies, approximately 15 to 30% of *Pinus brutia* bark extract is composed of monomeric flavonoids, with taxifolin being the predominant compound [44,45]. Figure 2 shows the chromatogram of the hydroalcoholic extract of *Pinus patula* bark. In Figure 2, the compounds labeled 1, 4, 6, and 7 correspond to gallic acid, catechin, procyanidin B2, and epicatechin, respectively. Given that the process of solid-phase extraction is specific for phenolic compounds, it could be thought that chromatogram peaks correspond to phenolic compounds. It is worth mentioning that pine bark has other phenolic compounds, such as caffeic and ferulic acids, catechin and epicatechin dimers, and other constituents including trimers and tetramers [46].

According to the chromatogram and taking into account the studies conducted by Escribano-Bailon et al. [47], compounds 2 and 3 could correspond to other Phenolic acids and peak 5 could correspond to quercetin, as it is a very common compound in the bark of pine grown in Colombia [48]. The peaks numbered 8, 9, and 10 may correspond to condensed flavonoids such as catechin (procyanidins) or hydrolysable tannins (gallotannins) [49]. The Gebert method [22], indicated for the separation of flavanols and Phenolic acids, was used to isolate catechin and gallic acid. The process started with 1000 g of pine bark, previously degreased with hexane, and then, the extraction of phenolic compounds was carried out under the best extraction conditions. After roto evaporating the mixture at 55°C, 75.0245 g of extract was obtained. An amount of 7.5102 g of hydroalcoholic extract was taken and divided into two fractions, one soluble in ethanol (2.9785 g) and the other insoluble in ethanol (4.2931 g). The hydroalcoholic fraction obtained was extracted with water and separated into two fractions, a soluble one (1.5677 g) (crude tannin, fraction T_1) and an insoluble fraction (3.8513 g) (crude phlobaphenes, fraction F_1). Crude phlobaphenes were washed three times with ethyl ether, and the insoluble fraction was extracted three times with ethyl acetate. The soluble fraction was discarded and not subjected to further analysis. The insoluble fraction in ethyl acetate was rot evaporated at 55°C, yielding 3.6234 g of a red colored solid. The infrared spectrum of phlobaphenes showed bands at 3382, 871 and 756 cm^{-1} corresponding to aliphatic and aromatic -OH elongations. Bands for vibrations of C=C aromatic rings were observed at 2362, 1614 and 1532 cm^{-1}. A band for C=O elongations non-conjugated to an aromatic

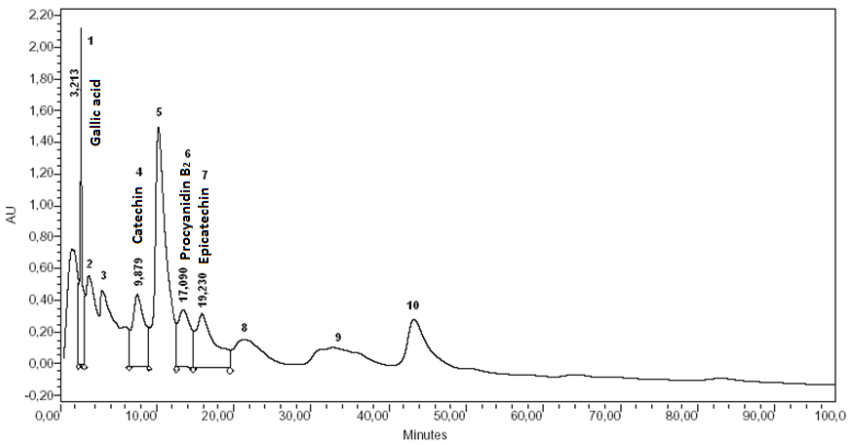

Figure 2: Chromatogram of the hydro alcoholic extract of *Pinus patula* bark.

ring was observed at 1713 cm^{-1}. A siringil ring vibration is observed at 1327 cm^{-1}. Characteristic bands for C-O bonds and vibration appeared at 1204 and 586 cm^{-1}, respectively; and bands for C-H deformations were shown at 1449, 1087 and 1029 cm^{-1} [50]. Crude tannins were washed successively with ethyl ether, a number of times sufficient to obtain spots only near the origin of the chromatogram using two-dimensional paper chromatography, excluding the presence of other compounds such as Phenolic acids. To this end, paper chromatography (Whatman 3) was used (37 cm × 47 cm) with n-butanol-Acetic acid-water (3:1:1) as the eluent in the first direction and 15% (w/v) aqueous Acetic acid in the second direction. The developers used were iron chloride, ultraviolet light, and iodine vapor. Subsequently, this fraction was injected into the liquid chromatograph to ensure the presence of catechin Cf_1. Figure 3 shows the chromatogram of the crude tannin fraction. The chromatogram in Figure 3 shows the presence of catechin at a retention time of 9.910 minutes. Other compounds are also present in the chromatogram: the peaks at 17.110 minutes (9) and 19.210 minutes (10) may correspond to procyanidin B2 and epicatechin, respectively. Peaks 1, 3, 4, 5, 6, 7, and 8 correspond to other possible monomeric phenolic compounds present in this fraction.

Phenolic compounds such as catechin, epicatechin, and procyanidin B2 and B3 have been determined in crude tannins of grape seed [51]. Crude tannins (T_1) were washed with ether and ethyl acetate (fraction T_2) and passed through a Sephadex G-25 column (30 cm × 2 cm), eluting with methanol followed by a methanol-ethyl acetate mixture 1:1 (v/v). Sixteen fractions were obtained, which underwent TLC with toluene-acetone-formic acid (3:6:1). The plates were then developed with ferric chloride, a UV lamp, vanillin, and iodine vapor. Fractions 9-16 were discarded, as no phenolic compounds were present. Fractions 1-8 underwent a second round of TLC, and developers indicated the same compound, presenting an Rf of 0.86, with some type of interference from other compounds (Rf 0.8 and 0.74). The eight fractions were pooled, and roto evaporated to concentrate the solution, which was again separated on a silica column (20 cm × 2 cm). The fractions were eluted with a mixture of chloroform-methanol, gradually increasing the proportion of methanol to 100%. A total of 18 fractions were obtained, out of which the top 10 had the same compound at Rf (0.86). These fractions were pooled, roto evaporated, and finally purified on a C_{18} column (20 cm × 2 cm) using acetonitrile-ethyl acetate 3:1 (v/v). After rot evaporation, a light-yellow precipitate was obtained (200 mg) Cf_1. Methanol recrystallization was performed to obtain a final precipitate of 96 mg [3]. The ethanol-insoluble fraction was extracted with a 1% (w/v) NaOH aqueous solution and heated at 80°C for 4 hours. The soluble fraction mainly corresponds to an aqueous solution of the sodium salt of Phenolic acids, which subsequently were separated by precipitation with a concentrated HCl solution (fraction AF_1). The phenolic acid fraction was injected into the liquid chromatograph to determine the presence of Gallic acid Cf_2. Figure 4 shows the chromatogram of the phenolic acid fraction. The chromatogram in Figure 4 shows the presence of Gallic acid at 3.210 minutes.

Gallic acid (Cf_2) showed an intense band in the UV-visible spectrum in methanol at 212.4 nm and a medium intensity band at 260.6 nm, characteristic of flavonoids. The infrared spectrum of Gallic acid (Cf_2) showed a stretching vibration of -OH at 3464.88 cm^{-1} and a band at 1705.92 cm^{-1} corresponding to a C=O bond tension. There was a C-O stretching band at 1246.90 cm^{-1}. Two O-H bending bands can be observed at 1448.44 cm^{-1} and 868.87 cm^{-1}. A C=C stretching vibration can be observed at 1526.55 cm^{-1}. A band can be observed at 821.62 cm^{-1} corresponding to a vibration of the O-H deformation. Mass spectrum (EIMS 70 eV) analysis of Cf_2 presents the molecular ion at m/z 170, coincident with the molecular formula of a phenolic acid such as Gallic acid ($C_7H_6O_5$), with a relative intensity of 100%, which also corresponds to the base peak, characteristic of phenols whose molecular ion peak is also its base [52-62]. The presence of the main ions at m/z values of 153, 135, 125, and 107 are due to loss of acid and hydroxyl functional groups. Ions at m/z values of 96, 79, 68, 51, and 39 correspond to characteristic aromatic ring breakdowns. Tables 2 and 3 shows the Cf_2 mass spectrum data. Figures 5-7 shows the mass spectrum of Gallic acid.

Conclusions

The extraction of phenolic compounds from *P. patula* bark was successfully performed. The ethanolic extract was divided into different fractions to isolate Gallic acid and catechin. The content of phenolic compounds in the *Pinus patula* bark extract was determined by HPLC with 3.120% of gallic acid, 1.990% of catechin, 0.801% of epicatechin, and 0.706% of procyanidin B2. Solid-phase extraction and HPLC made it possible to properly clean and isolate the phenolic compounds present in the *Pinus patula* bark. The phytochemical study of *Pinus patula* bark resulted in the identification of two phenolic compounds (Gallic acid and catechin). These compounds were isolated using different laboratory techniques, including percolation, TLC, CC (Sephadex, silica and C_{18}), and recrystallization for purification. Structural elucidation was performed using UV-V is, IR, ^1H and ^{13}C NMR spectroscopic techniques to ascertain the chemical structure of

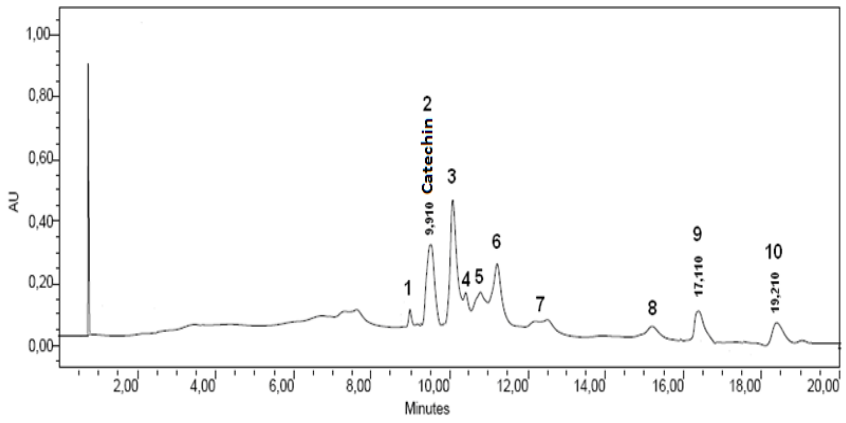

Figure 3: Chromatogram of crude tannins.

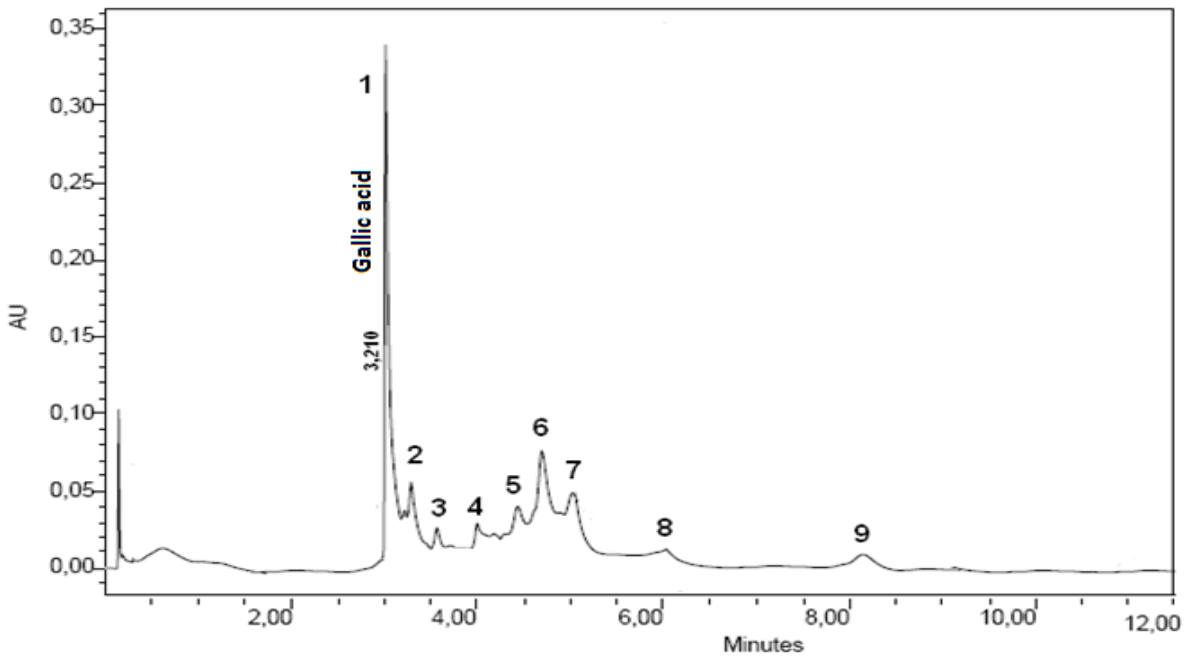

Figure 4: Chromatogram of the phenolic acid fraction.

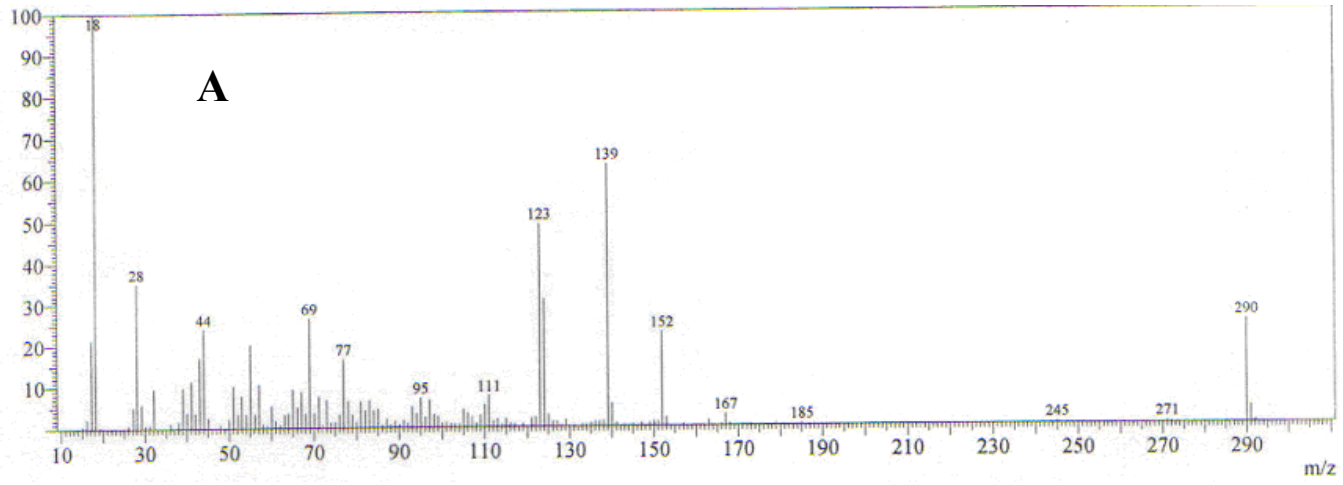

Figure 5: Mass spectra of (A) Catechin (Cf_1) and (B) Gallic acid (Cf_2).

Phenolic compound	Percent of average recovery	Standard deviation	Coefficient of variation	Value of t_{obt}
Gallic acid	101.58	2.11	2.1	0.82
Catechin	101.59	4.01	3.95	1.21
Epicatechin	96.21	4.96	5.15	2.21
Procyanidin B2	98.17	4.13	4.2	1.31

T_{table}=2.306 for n=9 and 95% confidence

Table 1: SPE accuracy data for the extraction of phenolic compounds.

Phenolic compound Cf_1*		Phenolic compound Cf_2*	
Experimental, m/z	**Reported, m/z**	**Experimental, m/z**	**Reported, m/z**
290.00 (25.25) (M^+)	291.00 (4.1)	169.95 (100.00) (M^+, B.P.)	170.00 (100.00) (B.P.)
152.00 (22.98)	152.00 (47.1)	154.00 (6.77)	154.00 (5.5)
139.05 (63.34)	139.00 (100) (B.P.)	153.00 (85.84)	153.00 (69.00)
124.05 (31.12)	124.0 (20.6)	135.00 (8.32)	135.00 (7.30)
123.05 (49.19)	123.0 (51.8)	125.95 (4.41)	126.00 (15.80)
111.05 (7.67)	111.0 (2.3)	125.00 (51.18)	125.00 (15.00)
95.05 (7.23)	95.0 (2.6)	113.00 (17.85)	113.00 (4.50)
77.05 (16.65)	77.0 (9.7)	107.00 (20.73)	107.00 (5.30)
69.00 (26.67)	69.0 (9.5)	96.00 (20.65)	96.00 (3.80)
55.05 (20.29)	55.0 (7.9)	79.00 (81.12)	79.00 (14.50)
44.05 (24.13)	44.0 (2.3)	71.00 (27.15)	71.00 (3.50)
28.10 (35.26)	28.0 (7.3)	69.00 (20.32)	69.00 (2.60)
18.20 (100) (B.P.)	18.0 (28.7)	68.00 (29.45)	68.00 (4.80)
17.20 (21.45)	17.0 (4.7)	53.05 (53.30)	53.00 (8.70)
-	-	51.05 (74.90)	51.00 (12.90)
-	-	50.05 (42.93)	50.00 (6.70)
-	-	39.10 (75.24)	39.00 (11.00)
-	-	38.10 (31.94)	38.00 (4.80)
-	-	29.10 (23.65)	29.00 (3.90)
-	-	18.20 (29.98)	18.00 (3.20)

B.P.=Base Peak, M^+=Molecular Ion, *=Relative Intensity

Table 2: Electron Impact Mass Spectrum (EIMS) of Cf_1 and Cf_2.

^{1}H NMR (ppm)	^{1}H NMR (ppm)*	^{13}C NMR (ppm)	^{13}C NMR (ppm)*	#	^a_C	δ^a_H (J in Hz)	Integral	HSQC	HMBC
Experimental	**Reported**	**Experimental**	**Reported**						
acetone-d6	**acetone-d6**	**acetone-d6**	**acetone-d6**						
Catechin Cf1									
2.55 (dd, 1H, H-4[a])	2.56 (dd, 1H, H-4A)	27.96 (C-4)	28.66 (C-4)	2	81.78	4.58, d	1	H-2	H4B, H-8
2.91 (dd, 1H, H-4B)	2.93 (dd, 1H, H-4B)	67.44 (C-3)	68.33 (C-3)	3	67.44	4.010, m	1	H-3	H4A, H4B, H2
3.20 (s, OH-3)	3.46 (s, OH-3)	81.78 (C-2)	82.56 (C-2)	4	27.96	2.55, dd; 2.91, dd	1; 1	H-4[a], H-4B	
4.010 (m, 1H, H-3)	4.04 (m, 1H, H-3)	94.48 (C-8)	95.45 (C-8)	5	156.36				
4.58 (d, 1H, H-2)	4.59 (d, 1H, H-2)	95.30 (C-6)	96.17 (C-6)	6	95.3	6.03, d, 2.3	1	H-6	
5.89 (d, 1H, H-8)	5.90 (d, 1H, H-6)	99.69 (C-10 [4[a]])	100.60 (C-10 [4[a]])	7	156.86				
6.03 (d, 1 H, H-6)	6.04 (d, 1H, H-8)	114.37 (C-2')	115.20 (C-2')	8	94.48	5.89, d, 2.3	1	H-8	H-6
6.75 (dd, 1H, H-6')	6.77 (dd, 1H, H-6')	114.80 (C-5')	115.33 (C-5')	9	155.95				
6.79 (d, 1H, H-5')	6.81 (d, 1H, H-5')	119.12 (C-6')	120.04 (C-6')	10	99.69				H4A,H4B, H6
6.90 (d, 1H, H-2')	6.91 (d, 1H, H-2')	131.16 (C-1')	132.09 (C-1')	1'	131.16				H6'
7.94 (d, OH's, 3', 4')	8.01 (4 OH)	144.81 (C-3')	145.58 (C-3')	2'	114.37	6.90, d, 2.00	1	H-2'	H6', H2
8.29 (d, OH's, 5, 7)		144.87 (C-4')	145.64 (C-4')	3'	144.81				
-	-	155.95 (C-9 [4[a]])	156.82 (C-9 [8[a]])	4'	144.87				H6',H5'H2'
-	-	156.36 (C-5)	157.14 (C-5)	5'	114.8	6.79, d, 8.55	1	H-5'	
-	-	156.86 (C-7)	157.63 (C-7)	6'	119.12	6.75, dd, 8.55, 2.00	1	H-6'	H-2'
-	-	-	-	-	-	-	-	-	-
Gallic acid Cf2									
7.16 (s, H-2, H-6)	7.10 (s, H-2, H-6)	109.22 (C-2, C-6)	110.52 (C-2, C-6)	1	121.09				H2, H6
		121.09 (C-1)	121.38 (C-1)	2	109.22	7.16, s	1	H-2	H6
		137.81 (C-4)	135.93 (C-4)	3	145.1				H2

		145.10 (C-3, C-5)	144.94 (C-3, C-5)	4	137.81				H6,H2
		167.10 (C-7)	170.76 (C-7)	5	145.1				H6
				6	109.22	7.16, s	1	H-6	H2
				7	167.1				H2, H6

[a]=displacement in ppm

Table 3: NMR, ^{13}C NMR, ^{1}H, HSQC, and HMBC spectroscopy data of Cf_1 and Cf_2.

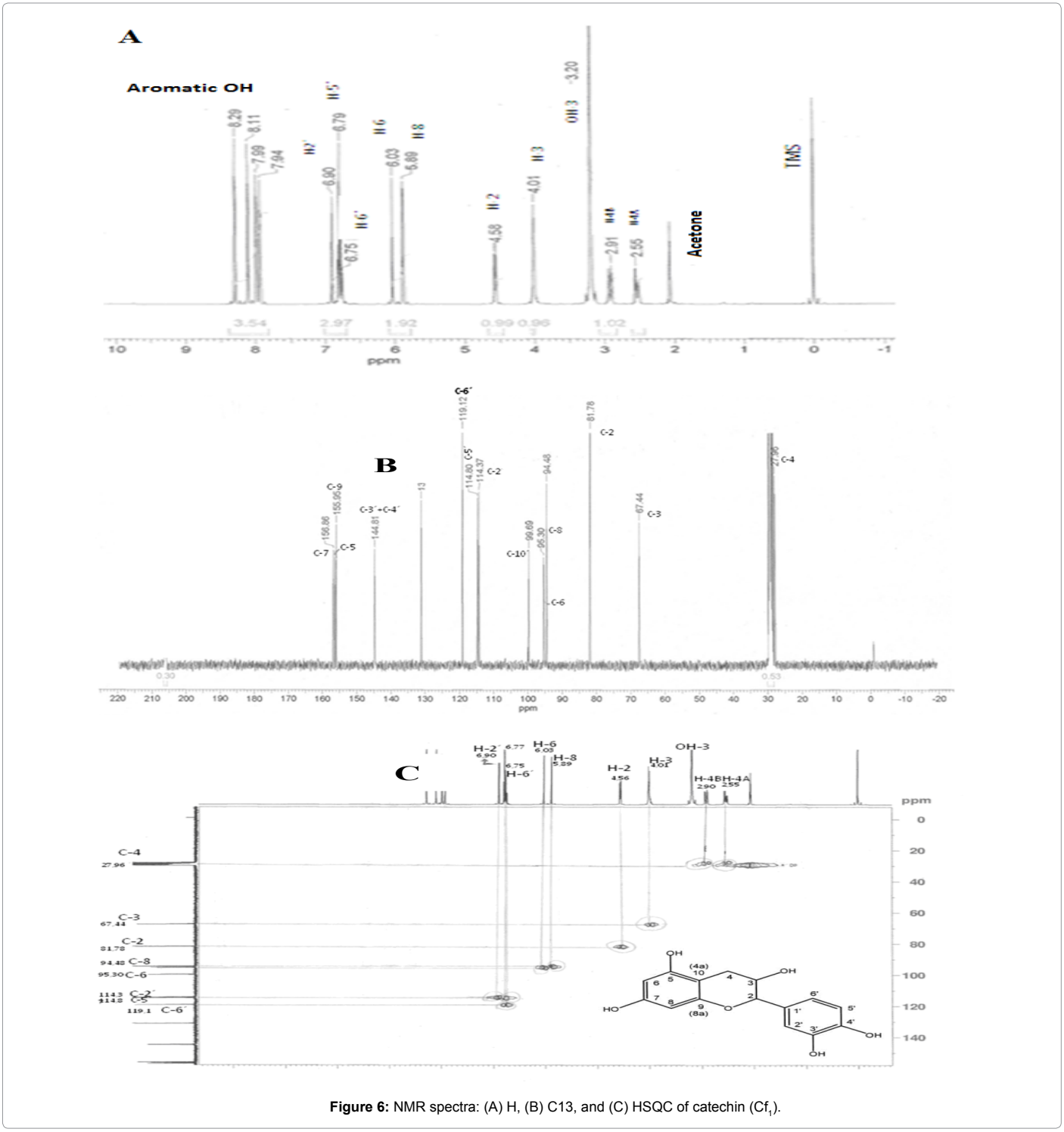

Figure 6: NMR spectra: (A) H, (B) C13, and (C) HSQC of catechin (Cf_1).

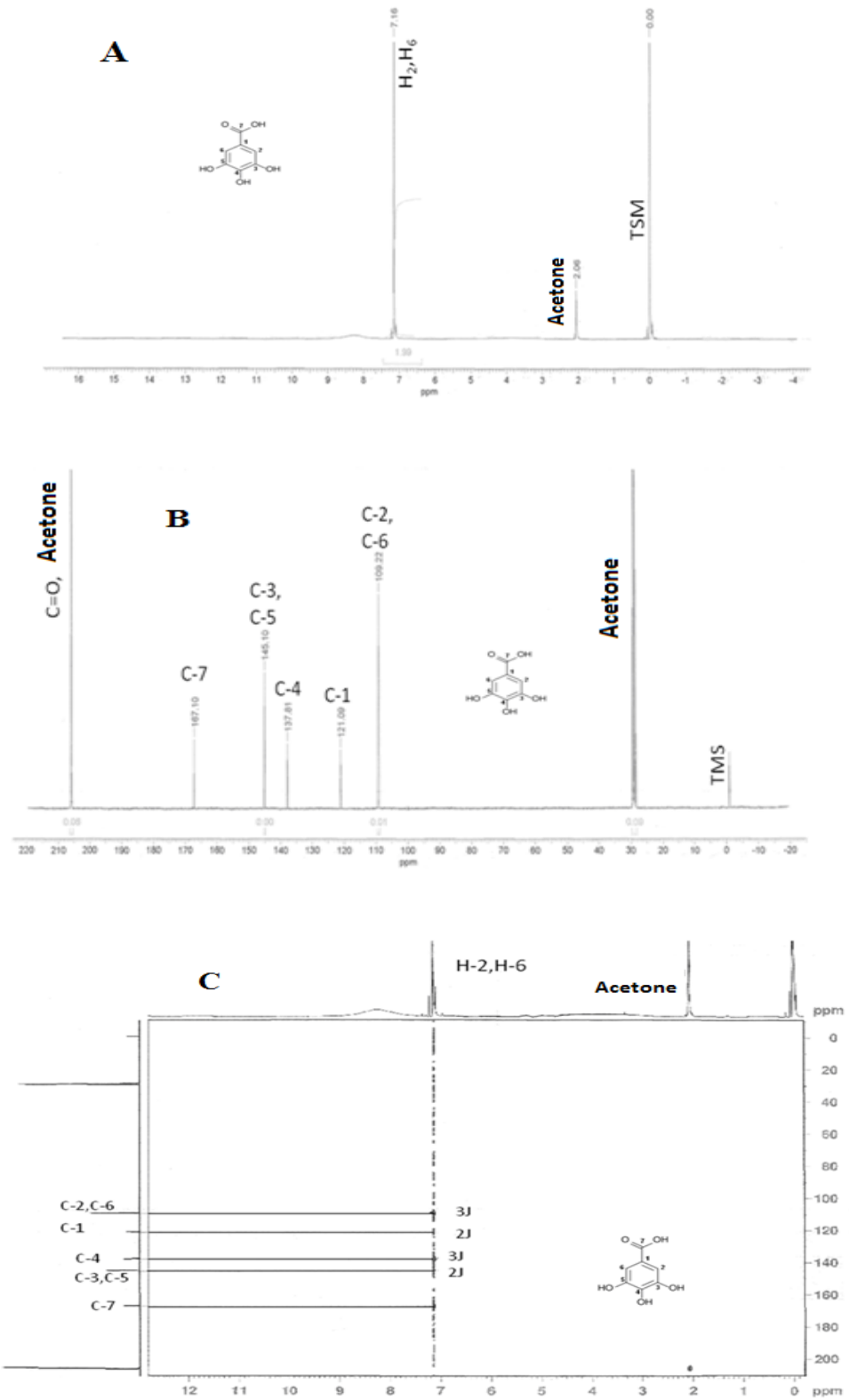

Figure 7: NMR spectra: (A) H, (B) C13 and (C) HMBC of gallic acid (Cf_2).

catechin and Gallic acid isolated from *Pinus patula* bark.

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