Chapter II

Isolation and purification of bioactive compounds from *Spondias pinnata* stem bark by solvent fractionation
2.1. Introduction

Medicinal plants are fine biochemical factories for the biosynthesis of both primary and secondary metabolites. Their ingenuity in creating diverse structures with generous sprinkling of functionalities and delicacies is thrilling. This is the outcome of evolution through millions of years during which they have been withstanding many tough tests of survival (Valiathan, 1998). The use of herbal medicine for the treatment of diseases and infections is as old as mankind. The world Health Organization supports the use of traditional medicine provided they are proven to be efficacious and safe (Government of India, 2001). Therefore, the need to use medicinal plants as alternatives to orthodox medicines in the provision of primary health care cannot be over-looked. India has a rich heritage of traditional herbal medicine since ancient times. These herbal medicines formed the basis of healthcare worldwide since earliest days of mankind. They are still used and have considerable importance in international trade. Additionally, herbal medicines have received much attention as sources of lead compounds since they are considered as time tested and relatively safe for both human use and environment friendly (Fazly-Bazzaz et al., 2005). They are also economic, easily available and affordable. Therefore, there is need to look inwards to search for herbal medicinal plants with the aim of validating the ethno medicinal use and subsequently an isolation and characterization of compounds which will be added to the potential lists of drugs (Agrawal & Talele, 2011).

A large number of bioactive molecules have been isolated from marine organisms (Donia et al., 2003). The crude extracts having biological activity are used as a source for isolation of active molecule(s) using different methods of purification. The extract is subjected to several rounds of fractionation to separate other molecules from bioactive molecule. Majority of the biologically active natural products have been isolated using bioactivity-guided fractionation (Pezzuto et al., 1997). In bioactivity-guided fractionation, the extract of an organism or a mixture of unknown molecules is fractionated and simultaneously biological activities of purified fractions are tested to determine the active fraction in each step of purification. In this process, extract of an organism having large number of molecules is initially separated into several parts based on their solubility/polarity in organic and aqueous solvents or a combination of organic and aqueous solvents. Then the bioactive sample is further purified into small fractions using chromatographic methods and HPLC, etc. Purified fractions in each step of purification are subjected to biological activity testing. This procedure is also useful to select and make changes in the process of purification to purify the active molecule without significant changes in its activity.

The first two phases in elucidation of the structure of molecules are identification of functional groups and carbon skeleton. The main objective of spectroscopic analysis is to identify functional groups in the molecule and molecular fragments. IR spectrum is important to reveal details of functional groups and also interaction between functional groups in the molecule. Mass spectroscopy is useful to calculate the accurate mass of small molecules, peptides and proteins etc. as well as to identify chemical entities in the structure of the molecule. In this, majority of structural correlations are obtained empirically by examining the spectra of known compounds. NMR is used to give information on detailed environment of the nucleus and its relationship to its neighbour. In this, chemical shift of a proton resonance is a reflection of magnetic environment of the nucleus. However, structural conclusions are drawn from various methods using complementary information regarding the structure of the
molecule. Use of two or more complementary methods of structural analysis provide valuable structural details necessary for definitive assessment of identity and to ensure a high degree of experimental confidence.

Previous work in our laboratory suggest that, crude extracts of *S. pinnata* bark acts as a very good antioxidant and free radical scavenger (Hazra et al., 2008) as well as a potent iron chelator both in vitro as well as in vivo (Hazra et al., 2013). Our recent findings also revealed that the bark of *S. pinnata* also possesses promising anticancer activity (Ghate et al., 2014). These findings further culminate our interest in isolating and characterizing the active principles from *S. pinnata* and investigated for their various pharmacological potentials like antioxidant, iron chelator and anticancer activity.

### 2.2. Materials and Methods

#### 2.2.1. Chemicals

1,1-diphenyl-2-picrylhydrazyl (DPPH), was obtained from MP Biomedicals, France. HEPES buffer was obtained from HiMedia Laboratories Pvt. Ltd, Mumbai, India. Dulbecco’s modified eagle’s medium (DMEM), Ham’s F-12 medium, antibiotics and Amphotericin-B were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Fetal bovine serum was purchased from HyClone Laboratories, Inc., Utah, USA. Cell Proliferation Reagent WST-1 was purchased from Roche diagnostics, Mannheim, Germany. All other commonly used chemicals and solvents are of minimum analytical grade and obtained from MERCK, India.

#### 2.2.2. Collection of Samples & Authenticity of Medicinal Plants

The barks of *Spondias pinnata* were collected from Jaipur forest of Bankura district of West Bengal, India. The plant was identified by the Central Research Institute (CRI) Ayurveda, Kolkata with a voucher specimen no: CRHS 111/08).

#### 2.2.3. Preparation of Extract

The stem bark of *S. pinnata* was dried at room temperature for 7 days, finely powdered and used for extraction. The powder (100 g) was mixed with 1000 ml methanol:water (7:3) using a magnetic stirrer for 15 hours, then the mixture was centrifuged at 2850 × g for 20 min and the supernatant was decanted. The pellet was mixed again with 1000 ml methanol-water and the entire extraction process was repeated. The supernatants collected from the two phases were mixed in a round bottom flask and concentrated in a rotary evaporator [250-200 mbar at 37°C]. The concentrated extract was then lyophilized. The residue was kept at -20°C until use.

#### 2.2.4. Solvent Partitioning of Methanolic Extract

1.05 kg of the freeze-dried extract was sequentially re-extracted with 2x10 lit of n-hexane, chloroform, ethyl acetate and water following the same procedure as extract preparation. All organic solvent fractions were evaporated under reduced pressure whereas water fraction was concentrated under reduced pressure and freeze-dried and stored in cold temperature leveling them as SPH (Hexane fraction), SPCH (Chloroform fraction), SPE (Ethyl acetate fraction), SPW (Water fraction).
2.2.5. Phytochemical Analysis of the Fractions

2.2.5.1. Qualitative Phytochemical Analysis

Qualitative analysis of SPH, SPCH, SPE and SPW was performed using standard qualitative methods as described earlier (Harborne & Baxter, 1995; Kokate et al., 2003).

2.2.5.1.1. Test for Phenols - Ferric chloride Test

To 1 ml of alcoholic solution of sample, 2 ml of distilled water followed by a few drops of 10% aqueous ferric chloride solution were added. Formation of blue or green colour indicated the presence of phenols.

2.2.5.1.2. Test for Flavonoids - Alkaline Reagent Test

To 1 ml of the sample (1 mg/ml), a few drops of dilute sodium hydroxide were added. An intense yellow colour was produced, which become colourless on addition of a few drops of dilute acid indicates the presence of flavonoids.

2.2.5.1.3. Test for Carbohydrates - Molisch Test

To 1 ml of sample (1 mg/ml) 1% alcoholic α-naphthol and 2 ml concentrated H₂SO₄ were added. Appearance of brown ring at the junction of two liquids indicates the presence of carbohydrates.

2.2.5.1.4. Test for Tannins

To 5 ml of the sample a few drops of 1% lead acetate were added. A yellow precipitate was formed, indicates the presence of tannins.

2.2.5.1.5. Test for Alkaloids - Mayer’s Test

1.36 gm of mercuric chloride dissolved in 60 ml and 5 gm of potassium iodide were dissolved in ten millilitre (ml) of distilled water respectively. These two solvents were mixed and diluted to 100 ml using distilled water. To 1 ml of acidic aqueous solution of samples few drops of reagent was added. Formation of white or pale precipitate showed the presence of alkaloids.

2.2.5.1.6. Test for Terpenoids

100 mg of dried sample was dissolved in 2 ml of chloroform. Sulphuric acid was carefully added to form a lower layer. A reddish brown colour at the interface was an indicative of the presence of steroidal ring.

2.2.5.1.7. Test for Triterpenoids - Libermann Buchard Test

10 mg of the extract was dissolved in 1 ml of chloroform; 1 ml of acetic anhydride was added following the addition of 2 ml of concentrated H₂SO₄. Formation of reddish violet colour indicates the presence of triterpenoids.

2.2.5.1.8. Test for Anthraquinones

5 ml of the sample solution was hydrolysed with diluted H₂SO₄ extracted with benzene. One ml of dilute ammonia was added to it. Rose pink coloration suggested the positive response for anthraquinones.
2.2.5.1.9. Test for Saponins

A drop of sodium bicarbonate was added in a test tube containing about 5 ml of an aqueous extract of sample. The mixture was shaken vigorously and kept for 3 min. A honey comb like froth was formed and it showed the presence of saponins.

2.2.5.1.10. Test for Glycosides - Legal’s Test

The sample was hydrolysed with HCl for few hours on a water bath. To the hydrolysate, 1 ml of pyridine was added and a few drops of sodium nitroprusside solutions were added and then it was made alkaline with sodium hydroxide solution. Appearance of pink to red colour shows the presence of glycosides.

2.2.5.2. Quantitative Phytochemical Analysis

2.2.5.2.1. Total Phenolic Content

Quantification of total phenolic content was done using previously reported method with slight modifications (Singleton & Rossi, 1965). Briefly, 0.1 ml of extract was mixed with 0.75 ml of FC reagent (previously diluted 1000-fold with distilled water). The reaction mixture was incubated for 5 min at 22°C; then 0.06% Na$_2$CO$_3$ solution was added to the mixture. After incubation at 22°C for 90 min, the absorbance was measured at 725 nm. The phenolic content was evaluated from a gallic acid standard curve.

2.2.5.2.2. Total Flavonoid Content

Total flavonoid content was quantified according to a standard method using quercetin as a standard (Zhishen et al., 1999). The extract of 0.1 ml was added to 0.3 ml distilled water followed by 0.03 ml 5% NaNO$_2$. After 5 min of incubation at 25°C, 0.03-ml 10% AlCl$_3$ was added. After another 5 min, the reaction mixture was treated with 0.2 ml 1 mM NaOH. Finally the reaction mixture was diluted to 1 ml with water. Then the absorbance was measured at 510 nm. The flavonoid content was calculated from a quercetin standard curve.

2.2.5.2.3. Total Carbohydrate Content

Quantification of carbohydrate content was carried out using previously described method (Sadasivam, 1997). 100 mg of the extract was weighed into a test tube, hydrolysed by keeping it in a boiling water bath for 3 h with 5 ml of 2.5 N HCl and cooled to room temperature. The volume was made to 100 ml and centrifuged. 0.25 ml sample extract made up to 0.5 ml distilled water and mixed with anthrone reagent (4 ml) and was incubated at 95°C for 8 min. After incubation, cooled rapidly and absorbance was measured of green to dark green colour at 630 nm. The carbohydrate content was evaluated from a glucose standard curve.

2.2.5.2.4. Total Tannin Content

This was assayed as described earlier with a slight modification (Robert, 1971). 0.1 ml aliquots of extract (1 mg/ml) in water were mixed with the 0.5 ml vanillin hydrochloride reagent (Mix equal volumes of 8% hydrochloric acid in methanol and 4% vanillin in methanol. The solutions were mixed just before use, and avoid using even if it is slightly coloured). After incubation for 20 min at room temperature the absorbance was measured of magenta-pink colour at 500 nm. The tannin content was evaluated from the catechin standard graph.
2.2.5.2.5. Total Alkaloid Content

Quantification of alkaloid content was carried out using previously reported method (Singh et al., 2004). To the 1 ml of extract (1 mg/ml) in water 0.1 ml of FeCl₃ (2.5 mM FeCl₃ in 0.5 M HCl) was added followed by addition of 0.1 ml 1,10-phenanthroline. After incubation for 30 min at 70°C the absorbance was taken at 500 nm. The alkaloids content was evaluated from the reserpine standard graph.

2.2.5.2.6. Ascorbic Acid Content

This quantification was carried out according to the previously described method (Roe, 1961). 1 ml aliquots of extract (1 mg/ml) in water were mixed with 1 ml of ‘2,4-dinitrophenylhydrazine reagent’ (2% 2,4-dinitrophenylhydrazine and 4% thiourea in 9 (N) H₂SO₄) and was incubated at 95°C water bath for 15 min. After incubation 5 ml of 85% H₂SO₄ was added drop-wise in an ice bath. Then the mixture was stood for 30 min, the absorbance was measured at 520 nm. The ascorbic acid content was evaluated from L-ascorbic acid standard curve.

2.2.5.2.7. Total Glycoside content

The quantification of glycoside content was carried out according to the previous method (Williams et al., 1995). 1 ml aliquots of extracts (1 mg/ml) in water were mixed with freshly prepared 1 ml of ‘Baljet’s reagent’ (95 ml 1% picric acid + 5 ml 10% NaOH) and allow to stand for 1 h in room temperature. After incubation the mixture were diluted to 1:1 with water and mixed well. Read the intensity of the colour obtained against the blank provide, at 495 nm using a suitable spectrophotometer. The glycoside content was evaluated from the rutin standard graph.

2.2.5.3. High Performance Liquid Chromatography Analysis

For HPLC analysis, stock solutions (10 µg/ml) were prepared in the mobile phase for the sample and different standard phytocompounds. Samples were then filtered through 0.45 µm polytetrafluoroethylene (PTFE) filter (Millipore) to remove any particulate matter. Analysis was performed using a HPLC-Prominence System RF10AXL (Shimadzu Corp.) equipped with degasser (DGU-20A3), quaternary pump (LC-20AT), auto-sampler (SIL-20A) and detectors of Reflective Index (RID-10A), Fluorescence (RF-10AXL) and Diode Array (SPD-M20A). The injection volume used was 20 µl, and the sample and standards were analysed in triplicates. Gradient elution consecutive mobile phases of acetonitrile and 0.5 mM ammonium acetate in water, at a flow rate of 1 ml/min for 80 min through the column (ZIC®-HILIC) that was maintained at 25°C. The detection was carried out at 254 nm.

2.2.6. Isolation of Phytochemicals from the Fractions

2.2.6.1. Thin Layer Chromatography (TLC)

Thin layer chromatography is used to separate the solutes based on their differential partition between the stationary and mobile phase. Aluminium sheet with silica gel layer (60F₂₅₄, Merck) was used as a chromatographic plate and samples were spotted from the bottom of TLC plate using capillary tubes with a distance of 1.5 cm. The bottom of TLC plate was placed in a chamber pre-saturated with solvent system (different mixture of hexane, ethyl acetate, dichloromethane and methanol) to a depth of 1.5 cm and the solvent front was run till it reaches to the other end of TLC plate. The sports in the TLC plates were visualized generally by short (254nm) or long wave (366nm) of UV, otherwise with the help of iodine chamber and KMnO₄ solution.
2.2.6.2. Silica gel Column Purification

The silica gel column was used to separate the molecules based on their polarity from the solvent fractions of the crude extract. The silica column (different size) was prepared using silica gel (Mesh size: 100-200/230-400) suspended in Hexane prepare gel slurry. The gel slurry was loaded into the column having glass sintered at the bottom. The column was loaded with the mixture of compounds with minimum bead volume. The column was eluted with different mixture of solvents like hexane, ethyl acetate, dichloromethane and methanol to obtain gradient of increasing polarity. The eluents were collected in test tubes and checked the purity with the help of TLC. Similar type of sports were pulled together and evaporated to get the dry compounds. Some of them were re-purified with repeated column chromatography and solvent washing to get the most pure isolated compounds.

2.2.6.3. Reverse Phase High Performance Liquid Chromatography

Reverse phase high performance liquid chromatography (RP-HPLC) was used to analyse the purity of bioactive fraction. The ZIC®-HILIC column was used in RP-HPLC and was degassed to eliminate the formation of bubbles and was calibrated with 93 0.1% Trifluoroacetic acid solvent to ensure the absence of detectable signals in UV-VIS absorption spectrum. Then a 20 µl of sample was injected into the sample loop of HPLC and sample was run into the column with gradient elution consecutive mobile phases of acetonitrile and 0.5 mM ammonium acetate in water, the flow rate of 1 ml/min. After the elution of void volume, presence of analytes in the column was recorded by detecting a change in the absorption spectrum at a set wavelength of 254 nm.

2.2.7. Bio activity screening of the Fractions/Isolated Compounds

2.2.7.1. Screening for In Vitro Antioxidant Activity

The antioxidant activity of the test samples were evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) using a standard method (Mahakunakorn et al., 2004). Briefly the reaction mixture containing 0.05 ml of 1 mM DPPH solution, 0.5 ml of 99% ethanol and 0.45 ml of samples and standard ascorbic acid at different concentrations (0-100 µg/ml). The solution was rapidly mixed and the reduction of DPPH was measured by reading the decrease in absorbance at 517nm. All tests were performed six times. Ascorbic acid was used as a reference compound. The percentage radical scavenging activity was calculated from the following formula:

\[
\text{% scavenging [DPPH]} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100
\]

Where \( A_0 \) was the absorbance of the control and \( A_1 \) was the absorbance in the presence of the samples and standard.

2.2.7.2. Screening for Iron Chelation Activity

The chelating activity of the fractions and the isolated compounds for ferrous ion was evaluated by a previously described method (Haro-Vicente et al., 2006). Briefly, in a Hepes buffer (20 mM, pH 7.2) medium, test samples (0-120 µg/ml) was added to ferrous sulfate solution (12.5 µM) and the reaction was started by the addition of ferrozine (75 µM). The mixture was shaken vigorously and left standing for 20 min at room temperature. The absorbance was then taken at 562 nm. All tests were performed six times. EDTA was used as a positive control.
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2.2.7.3. Screening for In Vitro Cytotoxic Activity against different Cancer Cell Lines

2.2.7.3.1 Cell Lines and Culture

Human lung adenocarcinoma (A549), human glioblastoma (U87), human cervical carcinoma cell line (HeLa) and human lung fibroblast (WI-38) cell lines were National Centre for Cell Science, India. A549 cells were grown in Ham’s F-12 medium whereas U87, HeLa and WI-38 cells were grown in DMEM. All the cells were maintained in the laboratory at 37°C in a humidified atmosphere containing 5% CO₂ in CO₂ incubator. Both the media were supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml Penicillin G, 50 µg/ml Gentamycin sulfate, 100 µg/ml Streptomycin and 2.5 µg/ml Amphotericin B.

2.2.7.3.2 WST-1 Cytotoxicity Assay

Cell proliferation and cell viability were quantified using the WST-1 Cell Proliferation Reagent, Roche Diagnostics. Cells (1x10⁴ cells/well) were treated with fractions/isolated compounds (0-200 µg/ml) and incubated for 48 h in 96-well culture plate. After treatment, cells were incubated with WST-1 Cell Proliferation Reagent and cell viability was quantified by measuring absorbance at 460 nm using a microplate ELISA reader.

2.2.8. Structural Determination Experiments

Structures of the bioactive compounds were analysed using different spectroscopic methods such as EIMS (JEOL JMS-700, Germany), LCMS (Agilent Technologies-6400, USA), FT-IR spectra recorded in KBr pellet (Perkin Elmer, USA), different nuclear magnetic resonance (NMR) experiments including 1H, 13C, DEPT-135, COSY, HMBC and HSQC with a Bruker-500 MHz NMR Spectrometer (Germany) and XRD with a Bruker APEX-II CCD.

2.2.9. Statistical Analysis

All data are reported as the mean±SD of six measurements. Statistical analysis was performed using KyPlot version 2.0 beta 15 (32 bit). Comparisons among groups were made according to 'pair t-test'. The results with a value of p<0.05 were considered significant.

2.3. Results

2.3.1. Extract Preparation and Solvent Partitioning of the Extract

The extraction process was carried out with 5.22 kg powder of S. pinnata bark and 1.059 kg 70% methanol extract was yielded. This methanol extract was again re extracted by different solvents based on their polarity to separate the similar type of compounds into one group. The schematic diagram of fraction preparation as well as the yield for each of the fractions is shown in Figure 2.1.

2.3.2. Bio activity Screening of the Fractions

2.3.2.1. In Vitro antioxidant and Iron Chelation Potential

The fractions were tested for their antioxidant and iron chelation potentials. The Figure 2.2A depicted that except hexane fraction (SPH) all the other fractions possessed DPPH scavenging ability whereas in case of iron chelation potential among the fractions SPCH failed to chelate iron effectively (Figure 2.2B).
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Figure 2.1  Schematic Diagram of Fraction Preparation

70% methanolic extract of *Spondias pinnata* stem bark (1.05 kg)

2x10 lit n-hexane

n-hexane Fraction (5.76 g) Remaining residue (1.043 kg)

2x10 lit Chloroform

Chloroform Fraction (2.86 g) Remaining residue (1.038 kg)

2x10 lit Ethyl acetate

Ethyl acetate Fraction (27.12 g) Remaining residue (1.004 kg)

Aqueous Fraction (1.003 kg) 2x10 lit Water

Figure 2.2  DPPH scavenging and Iron Chelation Potential of the Fractions

(A) DPPH radical scavenging activities, (B) Iron chelation capacity of SPH, SPCH, SPE and SPW. The results are mean±S.D. of six parallel measurements. ***p<0.001 vs 0 μg/ml.
2.3.2.2. In Vitro Cytotoxic Activity against different Cancer Cell Lines

Cytotoxic activity of the fractions was tested on three different cancer cells (A549, HeLa and U87) as well as normal fibroblast cells (WI-38). According to the Figure 2.3 it was found that SPH is not at all toxic towards all the cancer cells as well as normal cells. In contrary, both SPCH and SPE is toxic towards all the cancer cells specially U87 and A549 cells. SPW showed its selective toxicity on A549 cells only. Results from toxicity of the fractions on WI-38 cells suggested that, except SPCH all the other fractions are totally non-toxic, although the toxicity of SPCH is much less than its toxicity towards cancer cells.

![Figure 2.3 Cytotoxicity of theFractions against A549, HeLa, U87 and WI-38 Cells](image)

Cytotoxicity of the fractions was tested against (A) A549, (B) HeLa, (C) U87 and (D) WI-38 cells. The results are mean±S.D. of six parallel measurements. *p<0.05, **p<0.01 and ***p<0.001 vs 0 μg/ml.

2.3.3. Phytochemical Analysis of the Fractions

2.3.3.1. Qualitative and Quantitative Phytochemical Analysis of the Fractions

The phytochemical screenings of chemical constituents related to biological activity of the fractions are phenolics, flavonoids, alkaloids, flavonoids, carbohydrates, glycosides, tannins, terpenoids etc. (Table 2.1). The quantitative phytochemical estimation indicates that with the increasing polarity of the extracting solvents the quantity of different phytochemicals also increase (Table 2.2). Both qualitative and quantitative analysis suggest that phenolic and
flavonoids were present in all the fractions. The alkaloids content of both SPE and SPW is much higher than the other phytochemicals tested.

### Table 2.1. Qualitative Analysis of the Phytochemicals from SPH, SPCH, SPE and SPW

<table>
<thead>
<tr>
<th>Name of the Assay</th>
<th>SPH</th>
<th>SPCH</th>
<th>SPE</th>
<th>SPW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenols</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Triterpenoids</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Anthraquinones</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Glycosides</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*+* Represents presence of the phytoconstituent; ‘—’ represents absence of the phytoconstituent

### Table 2.2. Quantitative Analysis of the Phytochemicals from SPH, SPCH, SPE and SPW

<table>
<thead>
<tr>
<th>Name of the Assay</th>
<th>SPH</th>
<th>SPCH</th>
<th>SPE</th>
<th>SPW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolic content (mg/100 mg extract gallic acid equivalent)</td>
<td>17.23±0.41</td>
<td>17.90±0.63</td>
<td>34.73 ± 0.61</td>
<td>39.73±0.60</td>
</tr>
<tr>
<td>Flavonoid content (mg/100 mg extract quercetin equivalent)</td>
<td>3.39±0.09</td>
<td>11.93±0.36</td>
<td>15.40 ± 0.24</td>
<td>13.47±0.24</td>
</tr>
<tr>
<td>Carbohydrate content (mg/100 mg extract glucose equivalent)</td>
<td>-</td>
<td>-</td>
<td>6.77 ± 0.11</td>
<td>78.21±0.79</td>
</tr>
<tr>
<td>Tannin content (mg/100 mg extract catechin equivalent)</td>
<td>-</td>
<td>-</td>
<td>13.38 ± 0.05</td>
<td>28.74±0.44</td>
</tr>
<tr>
<td>Alkaloid content (mg/100 mg extract reserpine equivalent)</td>
<td>-</td>
<td>-</td>
<td>199.59 ± 0.80</td>
<td>216.65±5.24</td>
</tr>
<tr>
<td>Ascorbic acid content (mg/100 mg extract L-ascorbic acid equivalent)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.74±0.05</td>
</tr>
<tr>
<td>Glycoside content (mg/100 mg extract Rutin equivalent)</td>
<td>-</td>
<td>-</td>
<td>6.26 ± 0.08</td>
<td>21.28 ± 0.08</td>
</tr>
</tbody>
</table>

Values are mean ± SD (n=6)
2.3.3.2. HPLC Analysis of the Fractions

HPLC analysis was then performed to identify the presence of bioactive compounds by comparing the retention time of reference compounds under the same condition. Different peaks having retention time 3.17, 3.21, 3.82, 10.61, 15.08, 25.48, 26.58 and 68.63 min appeared on the HPLC chromatogram that was found to correspond to catechin, tannic acid, reserpine, ellagic acid, methyl gallate, ascorbic acid, gallic acid and rutin respectively (Figure 2.4). As evident from the phytochemical screening and HPLC standardization of the samples, the likely presence of bioactive compounds also justifies the procedure for extraction as well as the activity of the fractions. The bioactivity of SPH is less than the other fractions as it contained less number of bioactive compounds present in it whereas several numbers of compounds are present in other fractions in adequate amount as evident from the phytochemical analysis.

![HPLC Analyses of the Fractions](image)

**Figure 2.4  HPLC Analyses of the Fractions**

HPLC chromatogram of (A) SPH, (B) SPCH, (C) SPE and (D) SPW. Peaks marked signify the retention peak of fractions matched with the retention time of the known bioactive compounds in the same condition.

2.3.4. Isolation of Phytochemicals from the Bioactive Fractions

Form the bio activity evaluation and phytochemical analysis it was found that hexane fraction was less active than the other fractions. So, hexane fraction was omitted from phytochemicals isolation process.
2.3.4.1. Isolation from Chloroform Fraction

47 gm crude fraction was subjected to silica gel (Mesh size 100-200) column chromatography (12×17 cm) eluting with Hexane and Ethyl acetate gradient system to separate one major compounds (SPCH2=750 mg) with several impurities. This fraction was purified by silica gel chromatography.

2.3.4.1.1. Re-purification of SPCH2

SPCH2 (700 mg) was further purified through silica gel (Mesh size 230-400) column chromatography eluting with hexane and ethyl acetate gradient system. At 15% ethyl acetate in hexane the major spot was eluted as a single spot in TLC. After concentration the fractions 50 mg pure SPCH2 was obtained.

2.3.4.2. Isolation from Ethyl acetate Fraction

The crude ethyl acetate fraction (27.12 g) was subjected to silica gel (Mesh size 100-200) column chromatography (14×17 cm) eluting with dichloromethane (DCM) and methanol gradient system to separate four major compounds, SPE1 (500 mg), SPE2 (1.25 g), SPE3 (5.15 g) and SPE4 (1.6 mg) with several impurities. These fractions were further purified by silica gel chromatography.

2.3.4.2.1. Re-purification of SPE1

SPE1 (450 mg) was further purified through silica gel (Mesh size 230-400) column chromatography eluting with hexane and ethyl acetate gradient system. At 10% ethyl acetate in hexane the major spot was eluted as a single spot in TLC in KMNO₄ staining solution. After concentration the fractions 300 mg pure SPE1 was obtained.

2.3.4.2.2. Re-purification of SPE2

The column (dia-3.18 cm) was packed up to 11 cm by 230-400 mesh size silica gel and 1.05 g of SPE2 was again subjected to column purification. The column was eluted with different percentage of ethyl acetate in hexane. At the ratio of 1:3 ethyl acetate and hexane mixture, SPE2 was eluted and the spot was detected along with a faint spot. For more purification, the eluted fraction was dissolved in dichloromethane (DCM) and crystallized by drop wise addition of hexane. Finally, 80 mg of purified (99.15%) SPE2 was yielded that was confirmed by HPLC analysis. The compound was eluted at 31.8 min in acetonitrile and 0.1% TFA in water gradient system from reverse phase ZIC®-HILIC column (Figure 2.5A).

2.3.4.2.3. Re-purification of SPE3

The column (dia-4.5 cm) was packed up to 15 cm by 230-400 mesh size silica gel and 5.05 g of SPE3 was again subjected to column purification. The column was eluted with different percentage of methanol in DCM. At 3.5% methanol in DCM mixture, SPE3 was eluted and the spot was detected along with very slight impurity. The eluted fraction was washed with DCM with 4-5 drop of methanol for three times to get more purified product and 1.8 g purified (96.9%) SPE3 was yielded that was confirmed by HPLC analysis. The compound was eluted at 21.670 min in acetonitrile and 5mM Ammonium acetate in water gradient system from reverse phase ZIC®-HILIC Column (Figure 2.5B).
2.3.4.2.4. Re-purification of SPE4

The column (dia-3.18 cm) was packed up to 10 cm by 230-400 mesh size silica gel and 1.5 g of SPE4 was again subjected to column purification. The column was eluted with different percentage of methanol in DCM. At 0.6% methanol in DCM mixture, SPE4 was eluted and the spot was detected along with very slight impurity. The eluted fraction was washed with DCM for three times to get more purified product and 180 mg purified (98.6%) SPE4 was yielded that was confirmed by HPLC analysis. The compound was eluted at 7.33 min in acetonitrile and 5mM Ammonium acetate in water gradient system from reverse phase ZIC®-HILIC column (Figure 2.5C).

Figure 2.5  HPLC Analyses of the Fractions

HPLC chromatogram of (A) SPE2, (B) SPE3 and (C) SPE4. The purity of the compounds were determined by the percentage of area of the respective peaks.
2.3.4.3. Isolation from Water Fraction

The polarity based fractionation by different solvents revealed that the water fraction yielded more than 90% of the total extract, indicating the fact that the activity of the crude extract was mainly due to water soluble polar compounds. The crude water fraction was checked in TLC to separate components but nothings were separated. Further to regroup the probable compounds in water fraction, it was acetylated to convert the polar acid groups into non-polar acetyl group. The water fraction again fractionated by acetylation (2 ml of pyridine and 2 ml of acetic anhydride was stirred with 500 mg of water fraction at 40°C for 6 hours) followed by silica gel column chromatography purification (major spot) and deacetylation (150 mg of sodium methoxide was stirred with 500 mg of acetylated product dissolved in 50% methanol in dichloromethane at room temperature for 6 hours) to get the fraction namely SPW1.

2.3.5. Bio activity Screening of the Isolated Compounds

2.3.5.1. In Vitro Antioxidant and Iron Chelation Potential

The isolated compounds were tested for their antioxidant and iron chelation potentials. From the Figure 2.6A it was found that the DPPH radical scavenging potential is as follows SPE4>SPE3>SPW1>SPE2 whereas SPCH2 and SPE1 failed to show any activity. Iron chelation activity of the isolated compounds was checked and the results were as depicted in Figure 2.6B. Except SPW1, SPE4 and SPE3 other compounds failed to chelate iron significantly.

Figure 2.6  DPPH Scavenging and Iron Chelation Potential of the Isolated Compounds

(A) DPPH radical scavenging activities, (B) Iron chelation capacity of SPCH2, SPE1, SPE2, SPE3, SPE4 and SPW1. The results are mean±S.D. of six parallel measurements. *p< 0.05, **p< 0.01 and ***p< 0.001 vs 0 μg/ml.

2.3.5.2. In Vitro Cytotoxic Potentials of the Isolated Compounds

Cytotoxic activity of the isolated compounds was tested on three different cancer cells (A549, HeLa and U87) as well as normal fibroblast cells (WI-38). According to the result it was found that SPE2, SPE3 and SPE4 showed cytotoxic potential against all the cancer cell line. On the other hand, SPW1 is toxic towards HeLa cells and SPCH2 is toxic against U87 cells only and SPE1 failed to show any activity against the tested cancer cell lines (Figure 2.7).
Figure 2.7  Cytotoxicity of the Isolated Compounds against A549, HeLa, U87 and WI-38 Cells

Cytotoxicity of the isolated compounds was tested against (A) A549, (B) HeLa, (C) U87 and (D) WI-38 cells. The results are mean±S.D. of six parallel measurements. *p<0.05, **p<0.01 and ***p<0.001 vs 0 μg/ml.

2.3.6. Structure Elucidation of the Isolated Compounds

Among the isolated compounds the structure of SPE2, SPE3, SPE4 and SPW1 was evaluated because the others failed to show any activity in both the antioxidant and anticancer property.

2.3.6.1. Structure Elucidation of SPE2

The LCMS spectra of the compounds depicted that the compound is 98.5% pure and EIMS data showed the parent mass (m/z):192 and the fragments are 177, 123, 105 (Figure 2.8A). FTIR spectra of the compound was recorded in KBR pellet and the characteristic peaks showed the presence of hydroxyl group at 3340 (Strong), ester carbonyl group at 1702 and aromatic unsaturation at 1608, 1566, 1510, 1435 (Figure 2.8B). Melting point of this compound is 206-210°C. Different experiments in NMR such as 1H 1D NMR (Figure 2.8C), 13C 1D NMR (Figure 2.8D), DEPT 135 1D NMR (Figure 2.8E), COSY 2D NMR (Figure 2.8F), HMBC 2D NMR (Figure 2.8G), HSQC 2D NMR (Figure 2.8H) were performed.
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The structure was determined using the following experiments (A) LCMS, (B) FTIR, (C) 1H 1D NMR, 
(D) 13C 1D NMR, (E) DEPT-135 1D NMR, (F) COSY 2D NMR, (G) HMBC 2D NMR and (H) HSQC 2D NMR.

The final structure was confirmed by X-ray crystallography that suggests the following 
structure (Figure 2.9). Compound data bank search suggest that SPE2 is a novel quinonyl type of 
compound. This compound is yet to be patented so the exact structure was not shown.

(i) NMR data:
1H NMR (500MHz, DMSO d6)-δ: 10.29 (s, 1-OH), 7.90 (1H, d, J=9.5, Ar-3), 7.2 (1H, s, Ar-8), 6.77 
(1H, s, Ar-5), 6.21 (1H, d, J=9.5, Ar-4), 3.80 (3H, s, Ar-6-COCH3).

13C NMR (500MHz, DMSO-d6)-δ: 161.13 (C-2), 151.59 (C-7), 149.96 (C-6), 145.70 (C-9), 144.95 
(C-4), 112.15 (C-10), 111.00 (C-3), 110.05 (C-5), 103.22 (C-8), 56.46 (C-11).

(ii) Crystallographic data:

Experimental

Chemical formula - C10H9NO3                      Cell volume (V)=418.22(6) Å³
Chemical formula weight-191.06                       Cell formula units (Z)=2
Crystal type- Triclinic, P1                          Cell measurement temperature (T)=293(2) K
Cell length-                                        Crystal size=0.16×0.14×0.12 mm
a=6.7548(6) Å                      Absorption coefficient (µ)=0.113 mm⁻¹
b=7.6045(6) Å                      Crystal colour=off Green
c=8.7945(7) Å
Cell angle-
α=109.9530(10)°
β=90.989(2)°
γ=98.977(2)°

Data collection

Diffractometer : Bruker APEX-II CCD          3979 measured reflections
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Absorption correction: multi-scan, (SADABS; Sheldrick, 2004)

Tmin = 0.982, Tmax = 0.987

Refinement

R[F2 > 2σ(F2)] = 0.077
wR factor = wR(F2) = 0.2015
Restrained S = 1.060

Number reflections -1462
129 parameters

Δρmax = 0.692 e Å⁻³

Δρmin = -0.316 e Å⁻³

(iii) Data collection: APEX2 (Bruker, 2004); cell refinement: SAINT-Plus (Bruker); data reduction: SAINT-Plus; program(s) used to solve structure: SHELXS 97; program(s) used to refine structure: Mercury, SHELXL-97; software used to prepare material for publication: SHELXL-97.

Figure 2.9 Structure of SPE2

The structure of SPE2. The number was indicating the Carbone number as depicted in 13C NMR data. R1, R2, R3 are different functional groups which were not shown.

2.3.6.2. Structure Elucidation of SPE3

EIMS spectra showed the parent mass (m/z)-170 and the fragments are -153, 125, 85 (Figure 2.10A). FTIR spectra was recorded in KBR pellet and the characteristic peaks showed the presence of broad hydroxyl group at 3402 (Strong), ester carbonyl group at 1693, aromatic unsaturation at 1617 (Figure 2.10B). Melting Point-220-223°C. Different experiments in NMR such as 1H 1D NMR (Figure 2. 10C), 13C 1D NMR (Figure 2.10D) were performed. Form the different spectroscopic data it was found that SPE4 is 3,4,5-trihydroxybenzoic acid, commonly known as Gallic Acid (Figure 2.11).

(i) NMR data:

1H NMR (500MHz, DMSO d6)-δ: 6.71 (2H, s, Ar-H-2, H-6), 8.64 (s, Ar-4,1-OH), 8.99 (s,Ar-3,5 2-OH), 12.02 (s, -COOH)

13C NMR (500MHz, DMSO d6) δ : 109.17 (C-2, C-6), 120.88 (C-1), 138.44 (C-4), 145.85 (C-3, C-5), 167.93 (C7)
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2.3.6.3. Structure Elucidation of SPE4

The LCMS spectra of the compounds depicted that the compound is 99.94% pure and EIMS spectra showed the parent mass (m/z)-184 and the fragments are 153, 126, 115 (Figure
2.12A). IR spectra was recorded in KBR pellet and the characteristic peaks showed the presence of broad hydroxyl group at 3435 (Strong), ester carbonyl group at 1698, aromatic unsaturation at 1618 and the presence of alkanes at 1374 represent the CH3 (Figure 2.12B). Melting point is 180-200°C.

The structure was determined using the following experiments (A) LCMS, (B) FTIR, (C) 1H 1D NMR, (D) 13C 1D NMR and (E) DEPT-135 1D NMR.

Different experiments in NMR such as 1H 1D NMR (Figure 2.12C), 13C 1D NMR (Figure 2.12D) and DEPT-135 1D NMR (Figure 2.12E) were performed. Form the different spectroscopic data it was found that Methyl 3,4,5-trihydroxybenzoate, commonly known as Methyl Gallate (Figure 2.13).
(i) NMR data:

**1H NMR** (500MHz, DMSO d6)-δ: 3.73 (3H, s, -OCH3), 6.93 (2H, s, Ar-H-2, H-6), 8.93 (s, 1-OH), 9.26(s, 2-OH).

**13C NMR** (500MHz, DMSO d6) δ: 52.05 (C-8), 108.96 (C-2, C-6), 119.75 (C-1), 138.87 (C-4), 146.04 (C-3, C-5), 166.79 (C7).

![Figure 2.13 Structure of SPE4 (Methyl Gallate)](image)

The structure of SPE4. The number was indicating the Carbone number as depicted in 13C NMR data.

### 2.3.6.4. Chemical Characterization of SPW1

The acetylation process facilitated the separation of the similar group of compounds through silica gel chromatography and further deacetylation process remove the acetyl groups to revert the native free carboxylic acid groups of the compounds in that fraction. This was confirmed by phytochemical analysis (Table 2.3) and HPLC analysis (Figure 2.14).

#### Table 2.3. Qualitative and Quantitative Phytochemical Analysis of SPW1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tests</th>
<th>Phytochemicals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Qualitative</td>
<td><strong>Phenol</strong></td>
</tr>
<tr>
<td>SPW1</td>
<td>Quantitative</td>
<td>44.23 ± 1.08</td>
</tr>
</tbody>
</table>

Carb-Carbohydrate, Anth-Anthraquinones, Sap-Saponin, Ter-Terpenoids, Triter-Triterpenoids. '+' Represents presence of the phytoconstituent; '-' represents absence of the phytoconstituent. "ND" represents Not Determined. Total phenolics (mg/100 mg extract gallic acid equivalent), Total flavonoids (mg/100 mg extract quercetin equivalent), Total tannin (mg/100 mg extract catechin equivalent), Total carbohydrate (mg/100 mg extract glucose equivalent), Total glycoside (mg/100 mg extract rutin equivalent).

The adequate amounts of phenolic and glycosides in SPW1 revealed that the fractionation procedure mainly concentrated the glycosidic phenolics and flavonoids from the water fraction. This observation was also confirmed by the HPLC analysis, where only two compounds were identified, one of them is tannic acid (phenolic) and other is rutin (glycoside) and the major peak at 50.12 min is probably the combined peak of the other glycoside compounds.
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2.4. Discussion

Many plants have been known to synthesize active secondary metabolite include peptides, unsaturated long chain fatty acids, alkaloids, flavonoids, phenolic etc., which have potential therapeutic applications. However only crude plant extract cannot show us the path of their therapeutics. Thus finger printing and bioactive marker compound analysis by chemical and validated chromatographic techniques are gaining importance for use in standardizing herbal medicinal formulations. Most of the drugs used in current medical practice are derivatives of natural products. The natural products are purified and identified from their sources using sequential purification strategies such as chromatographic methods and bioactivity-guided fractionation etc. The bioactivity-guided fractionation is a commonly used process to identify bioactive natural products. Several novel chemical structures having antioxidant, iron chelating anti-cancer property, etc. was identified from the medicinal plants using this method (Pezzuto et al., 1997). This particular approach has the advantage over other methods of isolation in that detected bioactivity of the natural product is sequentially screened with purified fractions in each step of purification process to identify the bioactive agent.

Exhaustive literature survey revealed that some phytocompounds have been isolated from the fruit and leaf of this plant. While quite a less attention have been paid towards the isolation of phytocompounds from the stem bark, which is considered as a rich source of phytoconstituents. Therefore, the present work was undertaken to explore the bioactive compounds in S. pinnata bark extract, which has the potency as antioxidants, iron chelator and anticancer agent, was purified by bioactivity-guided fractionation.

To isolate the bioactive compounds in adequate amount from S. pinnata bark a large quantity of the bark was collected form the localities. Previous study suggested that 70% methanol extract possessed excellent bioactivity. So, the dried and ground powder was extracted with 70% methanol to get the crude extract. To concentrate the similar polar
compounds the crude extract was again sequentially re-extracted with different solvents based on their polarity such as hexane, chloroform, ethyl acetate and water.

The obtained fractions then tested for their bioactivity to identify the most bioactive fraction where the chance of obtaining the bioactive compounds is more than the other fractions. The DPPH scavenging, iron chelation and cytotoxicity study on different cancer cells suggested that SPCH, SPE and SPW are more biologically active than SPH. This observation also supported by the phytochemical analysis as well as HPLC study, where less number of phytocompounds are present in SPH compared to the other fractions. So hexane fraction (SPH) was omitted from further compound purification process.

The bioactive fractions were subjected to isolation of phytocompounds through silica gel column chromatography. First crude fractions of SPCH and SPE were loaded in silica column with lower mesh size (mesh size 100-200) to separate the major compounds in the mixture. Then the major fractions with impurities were further purified through silica gel column loaded with much higher mesh size (mesh size 230-400). Total five compounds were isolated; one from chloroform fraction (SPCH2) and four compounds from ethyl acetate fraction namely SPE1, SPE2, SPE3, SPE4. From TLC analysis it was found that SPCH2 and SPE1 were single compounds and HPLC analysis of SPE2, SPE3, SPE4 suggest that they are more than 95% pure.

The polarity based fractionation by different solvents revealed that the water fraction yielded more than 90% of the total extract, indicating the fact that the activity of the crude extract was mainly due to water soluble polar compounds. So, it was difficult to separate the phytocompounds by silica TLC or by silica gel column chromatography. Further to separate the probable compounds in water fraction, it was acetylated to convert the polar acid groups into non-polar acetyl group. This process would facilitate the separation of the similar group of compounds through silica gel chromatography. The major spot, among the other several minor spots, was isolated by silica gel column chromatography and further deacetylated to remove the acetyl groups to revert the native free carboxylic acid groups of the compounds in that fraction. This process separates a compound, namely SPW1 from water fraction.

Now the isolated compounds were again tested for their bioactivity to determine the bioactivity of the compounds and the results suggested that SPCH2 is cytotoxic towards U87 cells in higher concentration but totally inactive towards other cells as well as failed to possess antioxidant and iron chelation potency. On the other hand, SPE1 did not possess any bioactivity but the other four compounds SPE2, SPE3, SPE4 and SPW1 is displayed excellent bioactivity. So SPCH2 and SPE1 were left out from structure elucidation.

The structures of the four bioactive compounds were elucidated with the help of different spectral analysis such as mass (EIMS/LCMS), FTIR, different 1D and 2D NMR experiments. It was found to be difficult to determine the structure of SPE2 with the help of EIMS, FTIR, and 1D NMR experimental spectra. So, different 2D NMR experiments were performed but there was still there were no similarities with the reported compounds or known structure. Finally X-ray crystallography report revealed the exact structure of the compounds and compound library search disclose that SPE2 is a novel quinolin type of compound. In case of SPE3 and SPE4, the preliminary spectral data confirmed that the compound is actually Gallic acid and Methyl gallate respectively.

When the only compound isolated from water fraction (SPW1) was tested for its purity, it was found that it was not a totally purified compound. So, qualitative as well as quantitative
phytochemical analysis was done to unfold the type of compound present in this fraction. From the result can be concluded that the isolation procedure actually concentrated the glycosidic phenolics and flavonoids from the water fraction which was further confirmed by comparing the HPLC chromatogram with the standard compounds. The unidentified peak at 50.12 min is just before the known glycosidic compound rutin which confirmed SPW1 is glycoside rich group of compounds from water fraction.

Bioactivity guided isolation of the phytocompounds from the crude extract of *S. pinnata* stem bark revealed that among the fractions mid polar to high polar fractions such as chloroform ethyl acetate and water fraction possessed better bioactivity than nonpolar hexane fraction. Among the isolated compounds, SPCH2, SPE1 failed to show any activity so the structure was determined for the rest of the compounds. Among them SPE2 is a novel quinolin compound, SPW1 is a glycoside rich fraction and remaining are previously known Gallic acid and Methyl gallate. The mechanism for the bioactivities will be studies with these compounds in detail in the next chapters.