Supplementary Information

Antibacterial, antifungal, antioxidant, and molecular docking studies of (E)-4-(1-(2-aminophenylimino)ethyl)benzene-1,3-diol

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2.0 EXPERIMENTAL SECTION

2.1 Materials

o-Phenylenediamine(OPD) (Loba Chemie Private Limited), 2',4'-dihydroxyacetophenone (2,4DA) (Loba Chemie Private Limited), methanol (Loba Chemie Private Limited)Mueller-Hilton agar (MHA) plates, bacterial culture (*E. coli*, MTCC- 452*),*Whatman No. 1 filter paper discs (5mm), dimethyl sulphoxide-DMSO (SRL Chem, Cat No. - 28580), Ciprofloxacin (SRL Chem- 78079), Sabouraud dextrose agar –SDA (SRL Chem, Cat no.- 19427) plates, fungal culture (*C. albicans,* MTCC 854), Amphotericin B - (Amphocare), APS, ABTSsolution, ascorbic acid (SD Fine- F13A/0413/1106/62),EDTA (HiMedia TC038M), deoxyribose (SRL, Cat No. - 84384), $FeCl_3$ (Fischer Scientific, Cat no. - 23585), H_2O_2 (Neurochem Laboratories- HP6520) (6%), water, phosphate buffer, ascorbic acid(- SD Fine, F13A/0413/1106/62), gallic acid (SRL-Cat no.-5995-86-8), 10% TCA (Fischer Scientific-Cat no.-28444) 1% TBA (HiMediaCat no.-RM1594), sodium nitroprusside (Fisher Scientific,Cat no. - 27864), distilled water, gallic acid (SRL, Cat no.-13142), Griess reagent,sodium phosphate buffer (Hetu Aakaar Biotech, Cat no. - 77381), riboflavin- (SRL Chem India, Cat no. - 77381),

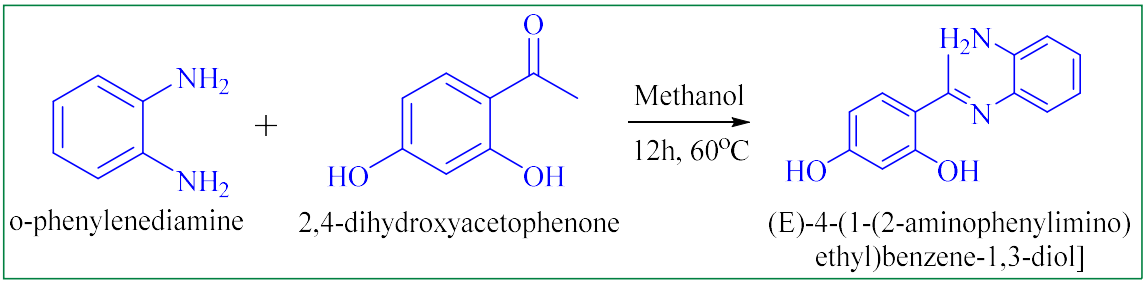
gallic acid (SRL Chem India, Cat no. - 43206), potassium pyrophosphate buffer, PMS (HIMEDIA, Cat no. - MB206), NBT (SRL Chem India,Cat no. - 11207), GSH, DPPH (SRL Chem, Cat no.– SR-29128), methanol (SD fine, Cat no.- 10930lC250), ascorbic acid (SD Fine, F13A/0413/1106/62), DMSO/methanol, $CuCl_2$ solution –(SD fine, Cat no. - 37834K05), ammonium acetate (NH_4Ac) (Fischer scientific, Cat no.- 11145) buffer at pH ¼ 7.0, neocuproine (Nc) (SRL chem, Cat no. – 93007), trolox (Ottokemi , Cat no. – T7723), ascorbic acid (SRL, Cat no. - 23006), sodium phosphate (Rankem, Cat no.- S0240), buffer (pH 6.6), 1% potassium ferricyanide [$K_3Fe(CN)_6$] solution (SRL, Cat no. - 15766), 10% trichloroacetic acid (SRL, Cat no.- 92390), deionized water, 0.1% ferric chloride (Fischer scientific, Cat no.- 23585)

2.2 Instruments and Software's

The infrared spectroscopic analysis of the APEB was carried out by using Brucker Optics, Gm BH Germany spectrometer with the KBr pellet technique from 4000 - 400 cm^{-1}. The UV-Visible spectrum was determined by the UV-Visible spectrophotometer(UV-Vis, SL- 159, Elico, 149 India) instrument from 190nm to 1100 nm. NMR spectra for APEBwas recorded in dimethyl sulfoxide (DMSO) solution using Bruker's NMR spectrometer (^{1}HNMR at 400 MHz, ^{13}CNMR at 101 MHz). After incubation measured absorbance of the decolorization and turbidity reading taken by using a microplate reader (iMark, BioRad). Biological properties such as antimicrobial and antioxidant activities were also analyzed by the Aakar biotechnologies private limited Lucknow, up, India. Graph Pad Prism 6 software is utilized to calculate the $IC_{50.}$The docking was done using AutoDockTools – 1.5.7 and PyRx software. Structure visualization has done by Biovia Discovery Studio 2024 Client.

2.3 Synthesis of (E)-4-(1-(2-aminophenylimino)ethyl)benzene-1,3-diol (APEB)

To synthesize APEB, 1.08 g (10 mmol) of *o*-phenylenediamine (OPD) was dissolved in 15 mL of methanol, while another solution was prepared by dissolving 1.52 g (10 mmol) of 2,4-dihydroxyacetophenone (2,4DA) in 15 mL of methanol. Both solutions were mixed thoroughly and transferred to a reflux setup. The mixture was heated at 60°C with continuous stirring for 12 hours, during which a dark brown solution formed, suggesting the formation of the. Once the reaction was ended, the solution was cooled to ambient temperature and left undisturbed to allow the solvent to evaporate slowly. This process resulted in the gradual desired product precipitation of the product, which was collected by filtration. The precipitate was then washed to remove impurities and dried in the desiccator to ensure complete removal of residual solvent. The final product, APEB, was acquired and ready for additional examination (Pradhan et al. 2020; Shi et al. 2019; Tahir et al. 2024).



Scheme S1 Synthetic Route of APEB

2.4 Antimicrobial activity

The evaluation of antimicrobial activity using different methods provides a comprehensive understanding of potential of APEB. The MIC method determines the lowest concentration of a compound required to inhibit bacterial growth in a liquid medium, whereas the zone of inhibition method measures the extent of bacterial growth inhibition on a solid agar surface. Given the

distinct nature of these methods, it is essential to compare their results to assess the antimicrobial efficacy of APEB comprehensively. This study employs both methods to evaluate the antimicrobial activity of APEB.

2.4.1 Evaluation of Minimum Inhibitory Concentration (MIC)

By examining the discernible growth of microorganisms in the agar broth, MIC is utilised to calculate the bacterial growth inhibition induced by the generated compounds. Using the serial dilution approach, the MIC data were obtained in this manner, and the sample was incubated for a whole day. The minimum inhibitory concentration (MIC) of an antibacterial agent is defined as the concentration at which no visible bacterial growth occurs in the tubes. The MIC value is determined by observing the visible opacity of the tubes before and after incubation. E. Coli MTCC452 gram (-) bacterial strains were selected in order to study the antibacterial activity of the APEB. 0.5 McFarland Standard dilution of microbes to be used for the study. The micro centrifuge tube was filled with 100 µl of diluted log cultures of E. coli – MTCC452 bacteria. Additionally, 5 µl of prepared treatment dilutions at various concentrations (0, 0.1,1,10,100,500,1000, pc) were added to the designated tubes, and the mixture was incubated for a whole day. After Incubation all content was transferred to the 96 well plate and turbidity reading was taken by Elisa Plate Reader (iMarkBiorad) at 630 nm. Ciprofloxacin (10µg) was used as Positive Control 0.5 McFarland Standard dilution of microbes to be used for the study (**Figure 1, 2**) (Fowler et al. 2022; Shumi et al. 2024). 100 µl diluted log cultures of test organism (C. albicans– MTCC) was added to the micro centrifuge tube and added with 5 µl of prepared treatment dilutions of different concentrations (0. 0.1, 1, 10, 100, 500, 1000, pc) to the defined tubes and incubated for the 24 hours. After Incubation all content was transferred to the 96 well

plate and turbidity reading was taken by Elisa Plate Reader (iMarkBiorad) at 630 nm. Amphotericin B (50 µg) was used as Positive Control (**Figure 3, 4**) (Fowler et al. 2022).

2.4.2 Zone Inhibition Test (ZIT)

The Zone Inhibition Method (also known as the Kirby-Bauer method) was used to investigate the antibacterial property. After distributing 100 µl of bacterial culture (E. coli) adjusted to 0.5 McFarland Unit - Approx cell density (1.5 X 108 CFU/mL), the MHA plates were infected. Next, discs containing 10 µl of various concentrations (0 to 100 mg/ml) were placed. To get the appropriate amount to be loaded on the disc, 10% of the sample was collected and serially diluted. Ciprofloxacin discs (10µg) were taken as the positive control, while one disc in each plate was filled with solvent alone to act as the vehicle control. The E. Coli plates were incubated for 24 hours at 37 °C. Measurements and records were made in the clear zone that was formed around the disc. In APEB, the clear zones were not observed with different concentration in antibacterial activity with respect to positive control (**Figure 5, 7**). The Antifungal activity was examined by following Zone Inhibition Method (Kirby-Bauer method). The SDA plates were inoculated by spreading with 100 µl of Fungal culture (*C. albicans*) (adjusted to 0.5 McFarland Unit - Approx cell density (1.5 X 108 CFU/mL) and followed by placing the discs containing 10 µl of different concentration (0 to 100 mg/ml). One disc in each plate was loaded with solvent alone which served as vehicle control and Amphotericin B disc (50 µg) were taken as positive control. The plates of *C. albicans* were incubated (Basil Scientific Corp. India Incubator) at 37 °C for 24 hrs. The clear zones created around the disc were measured and recorded. In APEB, after treatment the clear zones were observed at size of 4 mm at 250 µg/disc, 7 mm at 500µg/disc and 8.33mm at 1000µg/disc with respect to positive control in antifungal activity (**Figure 6, 8**) (Kirby 2009; Christenson et al. 2018).

2.5 Antioxidant activity

DPPH method, ABTS method, CUPRAC method, FRAP method, SOARSA Method, RNOSA and HFRSA were used to assess the antioxidant capabilities of APEB.

2.5.1 DPPH Assay

0.1 ml of 0.1 mM DPPH solution was added to 5µl of various APEB stock (0, 10, 50, 100, 125, 250, 500, 1000) on a 96-well plate. The experiment was conducted in triplicate, and blanks with 0.2 ml DMSO/Methanol and 5 µl of variously concentrated compounds (0, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50 (µg/ml)) were generated. The plate was left in the dark for thirty minutes. Using a micro plate reader (iMark, BioRad), the decolorisation was measured at 495 nm at the conclusion of the incubation. The control was a reaction mixture that contained 20µl of deionised water. The graph (**Figure 9, 10**) depicts the figures. The scavenging activity in relation to the control was shown as "% inhibition." Software Graph Pad Prism 6 was used to compute the IC_{50} (Abdulrasheed-Adeleke and Musa Bola 2020; Imam et al. 2011; Sarfraz et al. 2024; Mahadik et al. 2024; Lalsangpuii et al. 2022).

2.5.2 ABTS Assay

By combining APS (2.45 mM) and ABTS (7 mM) solution, which was diluted 100X to make ABTS free radical reagent, ABTS (SRL-Chem-Cat no.-28042) radicals were created. Add 10µl of different stock of the standard (Ascorbic Acid -SD Fine- F13A/0413/1106/62, (0, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50) and APEB (1, 2, 3, 4, 5, 6, 7, 8) to the 200µl of ABTS free radical reagent in 96 well plate and incubated at RT for 10 min in dark. Using a microplate reader (iMark, BioRad), measure the absorbance of the decolorization at 750 nm after incubation. A

graph is used to display the data (**Figure 11, 12**). Results pertaining to the negative control were displayed. The program Graph Pad Prism 6 is used to determine the IC_{50} value (Lalsangpuii et al. 2022; Islam et al. 2024; Deveci et al. 2024; Cao and Prior 1998; Kambayashi et al. 2009; Gupta et al. 2009).

CUPRAC Assay

10µl of different concentration of the APEB / Trolox (0, 1, 10, 50, 100, 250, 500, 1000) / (0, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50) were added in defined wells of 96 well plate. Then 200µl of reagent mixture was added in a 96 well plate. Reaction mixture in triplicate form and blank in duplicate form was prepared containing 200µl Methanol and 10µl of compound of different concentrations for APEB and standard (Trolox – Ottokemi - Cat no.–T7723) and incubated for 30 minutes in dark. At the end of the incubation, absorbance of the decolorization was taken at 490 nm using a microplate reader (iMark, BioRad). Reaction mixture containing 20µl of deionized water in the place of APEB/standard was served as Control. Data are presented in the form of graph (**Figure 13, 14**). The percentage inhibition of scavenging activity in relation to the control was displayed. Using Graph Pad Prism 6 software, the IC_{50} was determined (Deveci et al. 2024; Rubio et al. 2016; Apak et al. 2007).

2.5.3 FRAP Assay

10µl of different stock of the APEB (0, 1, 10, 50, 100, 250, 500, 1000) and standard (Ascorbic Acid – (SRL, Cat no- 23006) (0.00, 0.78, 1.56, 3.13, 6.25, 12.50, 25.00, 50.00) was added to 0.04 ml of 0.2 M sodium phosphate (Rankem, Cat no.- S0240) buffer (pH 6.6) and 0.05 ml of 1% potassium ferricyanide [K**3**Fe(CN)6] (SRL, Cat no- 15766) solution. The reaction mixture was vortexed well and then incubated at 50°C for 20 min using vortexed. At the end of the incubation, 0.5 ml of 10% trichloroacetic acid (SRL, Cat no-92390) was added to the mixture.

Then 50µl of deionized water and 50µl of 0.1% ferric chloride (Fischer scientific – Cat no. 23585) were added. The colored solution was read at 750 nm against the blank using microplate reader (iMark, BioRad). Data are presented in the form of graph (**Figure 15, 16**) IC_{50} was calculated by using software Graph Pad prism 6 (Mahadik et al. 2024; Deveci et al. 2024; Rao et al. 2013).

2.5.4 Super Oxide Anion Radical Scavenging Method

After adding various APEB concentrations to riboflavin solution, the combination was allowed to sit in light at room temperature for 30 minutes. Reaction mixture was added to the mixture that had been incubated above and well mixed after incubation. Then, at 560 nm, absorbance was measured using an Elisa plate reader (iMark, Biorad, USA). Software Graph Pad Prism 6 was used to determine the IC_{50} (Imam et al. 2011; Lalsangpuii et al. 2022; Islam et al. 2024; Deveci et al. 2024). The following are the graphic representations (**Figure 17, 18**).

2.5.5 Reactive Nitrogen Oxide Scavenging Method

A reaction mixture was formed containing 50 µL of 10 mM sodium nitroprusside (Fisher Scientific, Cat no.-27864), 40 µL of distilled water and 10 µL APEB/ standard (Gallic Acid – SRL, Cat no.- 13142)/ blank. The mixture was pre-incubated for 15 minutes under light at room temperature. After incubation 100 µL of Griess reagent was added in the test and control wells. And again, incubated for 5-10 mins at room temperature for chromophore development and stabilization. Absorbance was measured at 540 nm and 660 nm using a microplate reader (iMark, BioRad). Data are represented in the graph (**Figure 19, 20**). The IC_{50} value was determined using GraphPad Prism 6 software (Rao et al. 2013; Apak et al. 2016).

2.5.6 Hydroxyl Free Radical Scavenging Assay

66µl Reagent Mixture (10µl EDTA (HiMedia TC038M) (0.5M), 24.14 mg Deoxyribose (SRL-84384), 88µl $FeCl_3$ (Fischer Scientific-Cat no.-23585) (10mg/ml), 28 µl H_2O_2 (Neurochem Laboratories- HP6520) (6%), water up to 33 ml), 10µl APEB (0, 10, 25, 50, 100, 250, 500, 1000), 24µl of phosphate buffer (50 mM, pH 7.4) and 10µl of ascorbic acid(-SD Fine- F13A/0413/1106/62) were added in the wells of 96 well plate in sequence and the mixture was incubated at 37°C for 1hr. Gallic Acid (SRL-Cat no.-5995-86-8) (0,0.78, 1.56, 3.125, 6.25, 12.5, 25, 50) was used as standard. After incubation 50µl of 10% TCA (Fischer Scientific-Cat no.-28444) and 50µl of 1% TBA (HiMedia-Cat no.-RM1594) were added to each well. The result was a pink chromogen. After that absorbance was taken at 540 nm wavelength using microplate reader (iMark, BioRad). Data are represented by the graphs (**Figure 21, 22**). Formula to calculate scavenging activity as following (Islam et al. 2024; Deveci et al. 2024; Rahman et al. 2015; Hazra et al. 2008).

$$\text{Scavenging activity} = \frac{A(\text{control}) - A(\text{sample})}{A(\text{control})} \times 100$$

A (control): Absorbance of the control and

A (Sample): Absorbance of the extracts/standard

2.6 Molecular docking

Molecular docking is a computational technique employed to predict the potential conformations of a complex that forms between a ligand and its target, providing insight into their interactions. (El-Shalakany et al. 2024) Blind docking refers to the process in which a ligand is docked across the entire surface of a protein without prior knowledge of its binding pocket. This molecular

modelling approach enables the prediction of interactions between proteins (such as enzymes) and small molecules (ligands), as well as the behaviour of these ligands within the binding pockets of target proteins (Borrego-Muñoz et al. 2022). Molecular interactions between biological receptors and substrates play key roles in various biological processes. These interactions typically lead to the formation of stable target-substrate complexes that carry out important biological functions. Understanding the structure and binding sites of a biologically active receptor is crucial for identifying the different binding modes and affinities between interacting molecules. The complexity and high cost of experimentally determining the structures of these complexes have driven researchers to rely on molecular docking simulations as a primary method for gaining insights into target-substrate interactions (El-Shalakany et al. 2024). We have performed molecular docking of APEB with two distinct PDB IDs (1AI6 and 5AEZ) using two different docking software (AutoDockTools – 1.5.7 and PyRx).

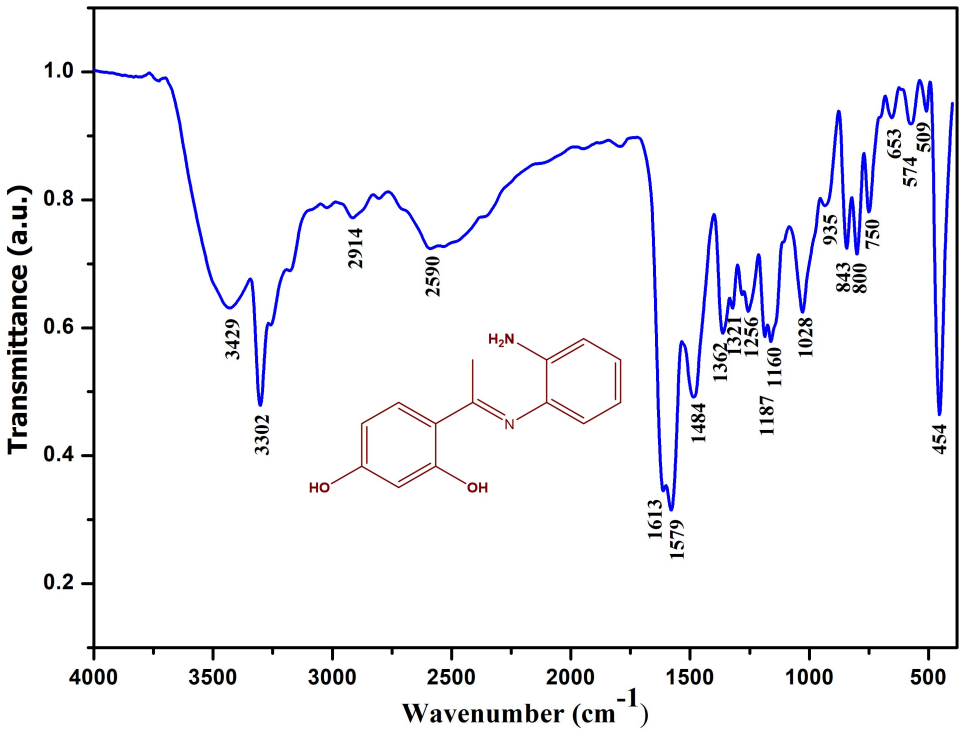


Figure S1 The IR spectra data of Schiff base (APEB)

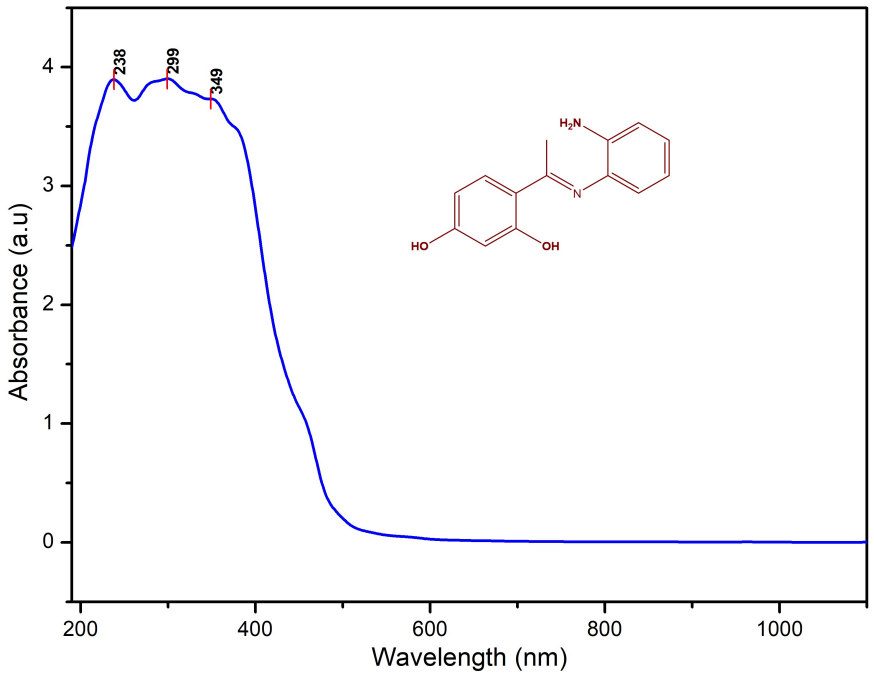


Figure S2 UV-Vis spectrum APEB

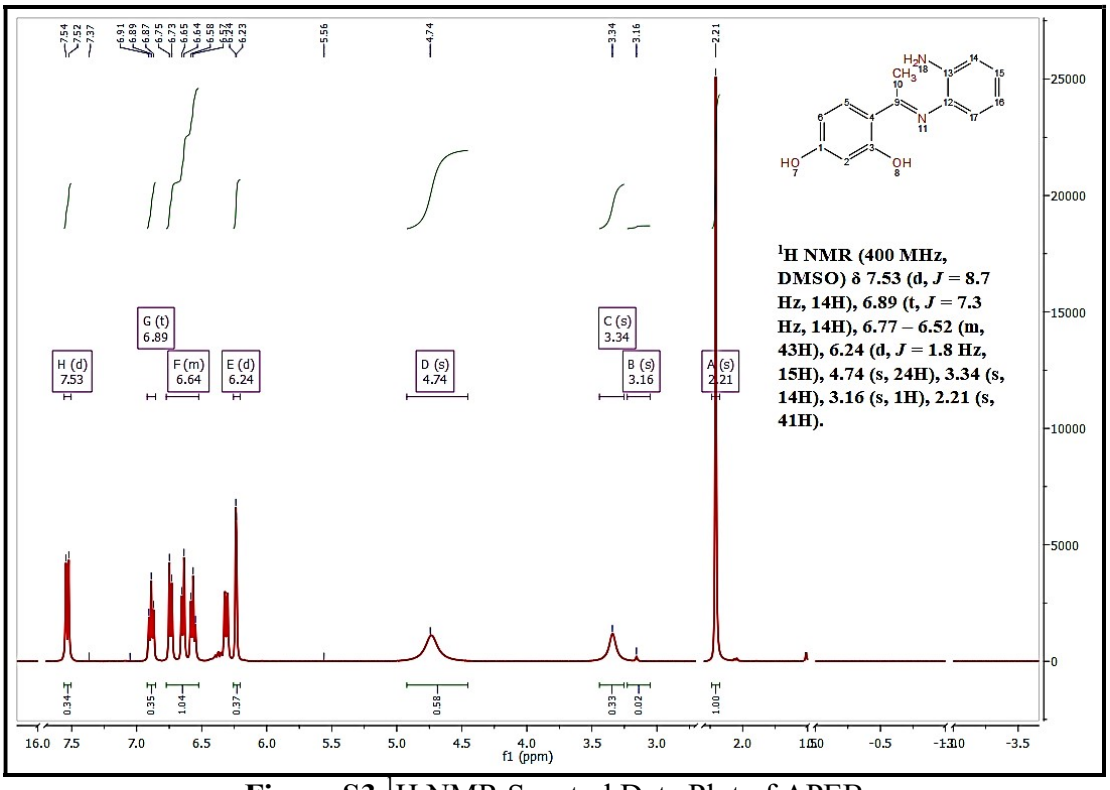


Figure S3 ^{1}H NMR Spectral Data Plot of APEB

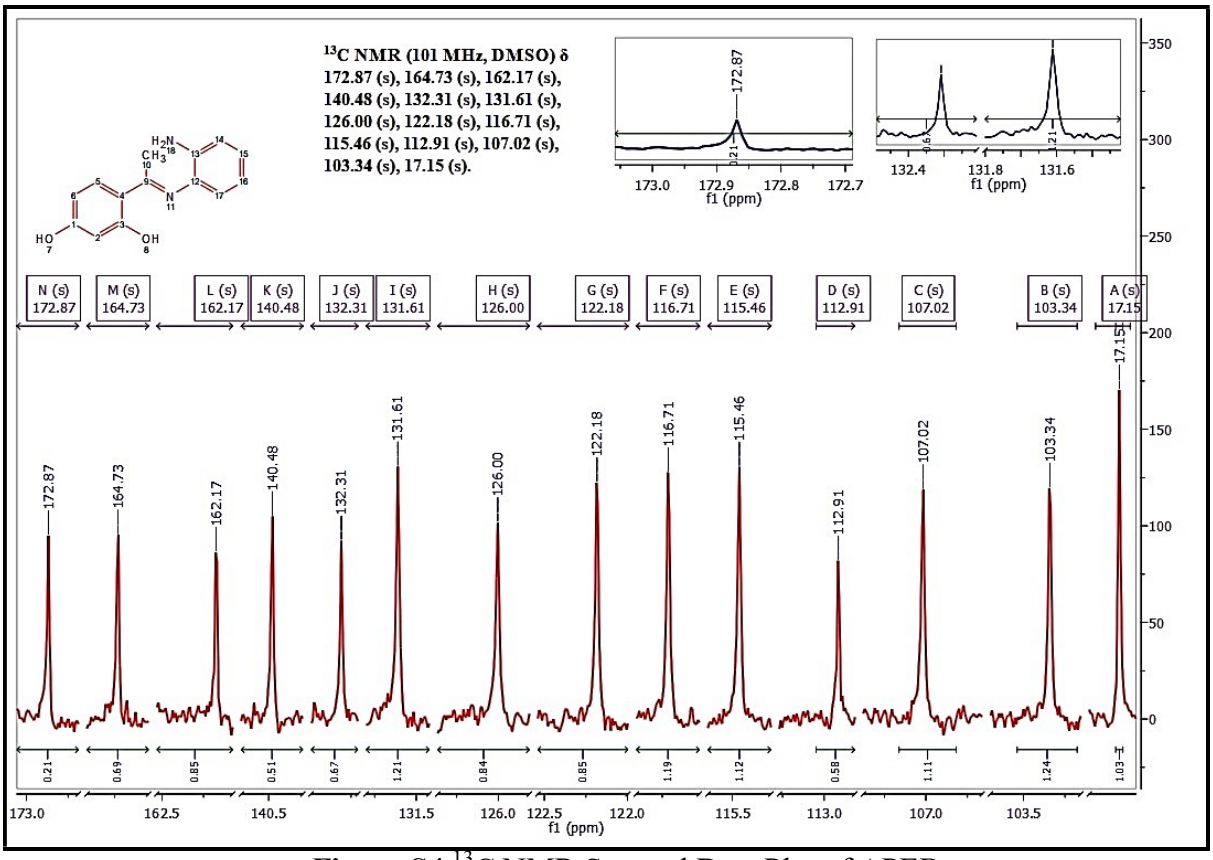


Figure S4 ^{13}C NMR Spectral Data Plot of APEB