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Isolation, Characterization and quantification of gallic acid from hydro alcoholic flower extract of *Woodfordia fruticosa* (L.) kurz along with fingerprinting analysis by HPTLC, HPLC, FT-IR, and NMR (^{1}H and ^{13}C) spectroscopy

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Abstract
Ethnopharmacological knowledge of the plant *Woodfordia fruticosa* (L.) Kurz. is very precious in traditional system of medicine. Gallic Acid (3,4,5-trihydroxybenzoic acid) is one of the most abundant phenolic acids of the flower extract of this plant. Gallic acid (GA) shows a broad range of beneficial effects in prevention and treatment of several diseases. The pharmacological activities of GA are antioxidant, anti-diabetic, antimicrobial, anti-inflammatory, cardioprotective, anticancer, neuroprotective and gastroprotective. The research deals with isolation and quantification of GA by using Column Chromatography and HPLC. The abundant of Gallic acid (GA) was come out 2.28µg/mg in the WFF extract. The FT-IR spectrum analysed the presence of carboxylic group (2700-3600 cm^{-1}), hydroxyl phenolic groups (3284, 3382 cm^{-1}), carbonyl group (1705 cm^{-1}) and an aromatic moiety (1541, 1618 cm^{-1}). ^{1}H-NMR and ^{13}C- NMR spectrum of Gallic Acid (GA) was successfully recorded. So the hydro alcholic extract of WFF confirm the presence of Gallic Acid.

Keywords: Gallic Acid, Isolation, HPTLC, HPLC, FT-IR, NMR Analysis

Introduction
Flower of *Woodfordia fruticosa* (L.) Kurz is frequently referred to as a *fire-flame bush* in English and as *Dhaiphul* in Bengali. The flower part of the plant is used extensively as herbal cure in Ayurvedic systems of medicine. Dysentery, sprue, intestinal problems, rheumatism, dysuria, hematuria, wounds, bleeding injuries, otorrhea, leucorrhea, and dysmenorrhea are among the traditional conditions that are treated using the plant's blossoms. Numerous bioactive compounds, including as flavonoids, anthraquinone glycosides, polyphenols, and tannins have been produced by this plant. Gallic Acid may be regarded as a possible starting molecule for the creation of novel drugs because of these activities. The flowers are in high demand in both domestic and international markets that are centered on the manufacturing of herbal remedies [1]. The objective of this study is to update knowledge about the occurrence, qualitative, quantitative, and methods that have been developed for the extraction, isolation and analysis of Gallic Acid (GA) from hydro alcoholic flower extract (WFF). The pharmaceutical industry frequently uses Gallic Acid due to the numerous *in vivo* and *in vitro* researches conducted on humans, animals, and cell cultures [2].

Materials and Methods
Collection
Flower of *Woodfordia fruticosa* (L.) Kurz was collected from the hill area of Sevok (26.8806°N 88.4689°E), Darjeeling, West Bengal in the month of March 2024. The plant was authenticated. The plant was identified and authenticated by the Scientist-E, Dr. K Karthigeyan, Botanical Survey of India, Central National Herbarium, Howrah - 711 103, India, bearing reference No. CNH/Tech.II/2021/13 [3].

Extraction

The fresh flower was dried by shade drying method at room temperature for 5 days. The dried flower was powdered by using grinder to get coarse and fine powder. 100 gm powder was taken for thimble preparation for continuous hot percolation extraction process by using Soxhlet apparatus (1000 ml) [4]. Methanol and water were taken at ratio 7: 3 for solvent (800ml) of the extraction process. The extraction was carried out for 72 hours until clear solvent came out. The menstrum was filtered and collected. It was evaporated by IKA Rotary pump evaporator at 45^{O} C at 25 rpm to semisolid extract [5]. The final powder extract (WFF) (3.73gm) was collected after 24 hours freeze drying.

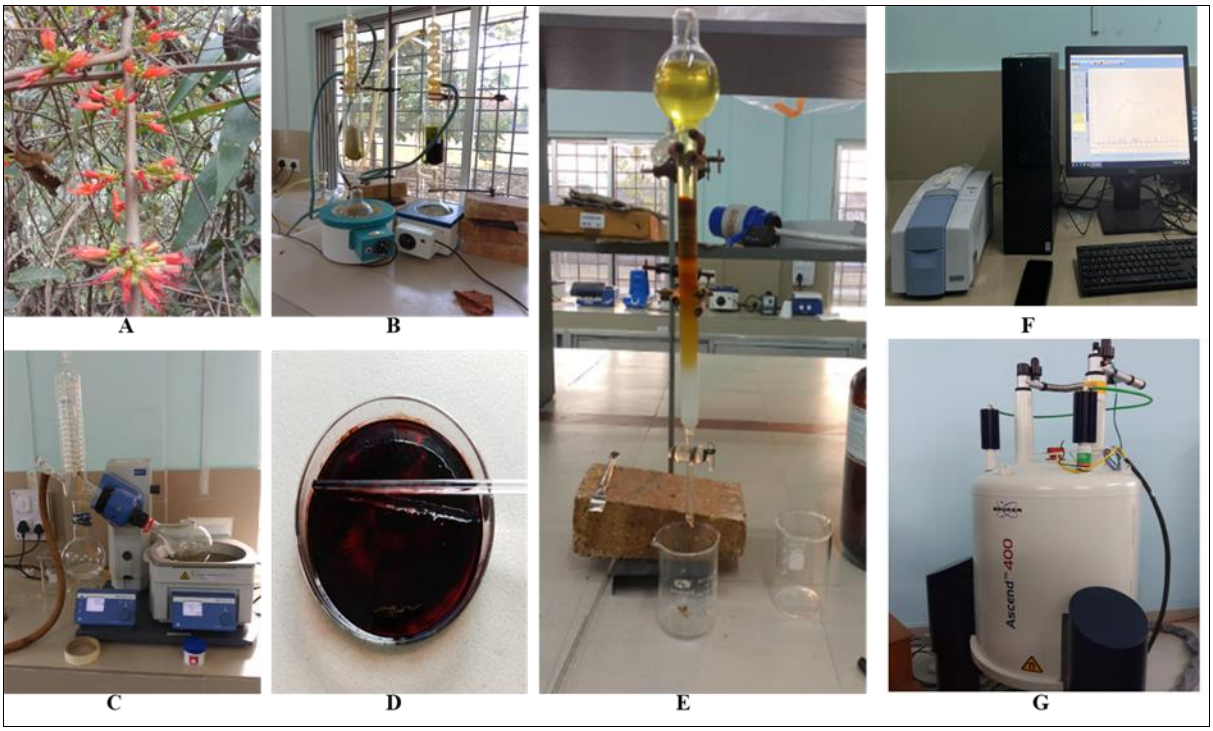


Fig 1: Fresh flower of *Woodfordia fruticosa* (L.) Kurz (A), Soxhlet Apparatus (B), Rotary Pump Evaporator (C), Dried Extract of WFF (D), Open Silica Column (E), FT-IR Instrument (F), and NMR (G).

Isolation of Gallic Acid by Open Silica Column

The open silica column was prepared by wet packing method using THOMAS BAKER silica gel (60-120 mesh). The hydro alcoholic flower extract (WFF) (3g) was loaded on open silica column. According to increasing polarity petroleum ether, toluene chloroform, and ethyl acetate are used as a mobile phase. The nine fractions were eluted and labelled F-1 to F-9. The fractions were tested by TLC method to ensure the presence of Gallic Acid bearing R_f 0.56 in solvent system toluene: ethylacetate: formic acid (6:6:0.1). The fraction F-5 was evaporated by IKA Rotary pump evaporator at 45^{O}C at 25 rpm to get white crystals. The white crystals of were purified by hot water re-crystallization with the help of activated charcoal. The yield of white crystalline F-5 was 37mg**.** It was purified by re-crystallization process with hot water [6].

Identification of Gallic Acid by HPTLC Analysis

A standard solution (GA) of Gallic Acid (1 mg/ml) was prepared by dissolving 2 mg of accurately weighed Gallic Acid in methanol and making up the volume to 2 ml with methanol. Accurately weigh 20 mg of hydro alcoholic extract (WFF) in 2 ml of methanol 10mg/ml). The standard (GA) and the sample (WFF) solutions were gone through sonication (20 min.) and centrifugation (1000 rpm for 10min). The supernatants were collected for the HPTLC analysis. TLC aluminium plates (10 × 10 cm) pre-coated with silica gel 60 F_{254} (Merck, Germany) were used as the stationary phase. The both samples were spotted in the form of bands, of 8 mm, with the help of a Hamilton glass syringe (100 µl) using a CAMAG Linomat V sample applicator [7]. The amount of standard and sample were injected 0.10µl and 0.20µl respectively. A constant application rate of 150 nL/ S was employed and the space between two bands was 11.4 mm. After that the mobile phase was prepared by using chloroform: ethyl acetate: formic acid: methanol (5:4:0.1 v/v/v). Then the twin trough glass chamber was saturated by the mobile phase (10 ml) at room temperature (25 ± 2ºC) for 30 minutes. The spotted plate was placed in the pre saturated mobile phase in twin trough glass chamber for the development of chromatogram up to 80mm. Then the developed HPTLC plate was dried at room temperature and scanned over the wavelength of 254, 366 and 416 nm. by using TLC Scanner 4 with Vison CATS software 4.0. The images of developed HPTLC plate were documented with the help of UV chamber [8, 9].

Quantification of Gallic Acid by HPLC Analysis

Separation of Gallic Acid was carried out by Shimadzu HPLC System (operated 271nm) and inject on valve with 20uL sample loop. The compound was separated on 271X4 nm. Compounds were separated on a 250 mm x 4.6 mm, i.d., 5-µm pore size RP C18 column protected by a guard column containing the same packing. Data were integrated by photo diode array (PDA) LC software and the results were obtained by comparison with standards.

Standard Preparation

Gallic Acid (10 mg) was weighed accurately and transferred to separate 100 mL volumetric flasks. The standard was

dissolved in 100 mL methanol to prepare standard stock solution of 100µg mL^{-1}.

Sample Preparation

The brown solid lyophilized residue of WFF was stored at -20°C and dissolved in methanol (10 mg/ml) for the analysis. The solutions were filtered through 0.45-µm Nylon membrane filter before the use.

Calibration Curve

The Gallic Acid (GA) standard with different concentrations was prepared by dilution of the standard stock solution [10]. The standard curve for Gallic Acid was a linear regression fitted to triplicate values obtained at each of six concentrations (5, 10, 20, 30, 40, 50 µg/mL). The corresponding peak areas were plotted against the concentration of the standard injected. Peak identification was achieved by comparison of the retention time for standard [11].

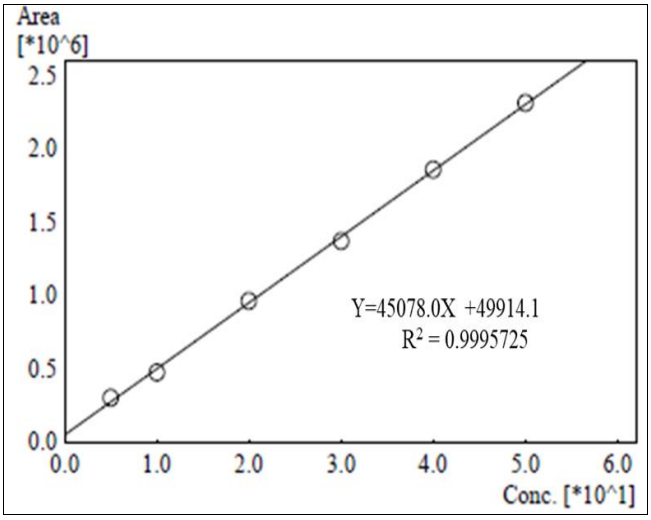


Fig 2: Calibration Curve of Gallic Acid

FT-IR Spectrum Analysis: FTIR spectra were acquired using an FT-IR Alpha-II BRUKER Spectrophotometer. Spectral analysis was conducted at a scan time of 24, at resolution 4 within the range of 4000 cm^{-1} to 400 cm^{-1}, using approximately 0.2 mg of sample [12, 13].

^{1}H and ^{13}C -NMR Spectrum Analysis

^{1}H NMR spectrum reveals the chemical shifts corresponding to various protons within the tested samples. The Nuclear Magnetic Resonance spectra ^{1}H-NMR, were recorded using a BRUKER Ascend™ Apparatus, at 400 MHz in deuterated dimethyl sulfoxide (DMSO-d_6). The displaced chemical signals present in the registered spectra, were represented using δ values in parts per million (ppm).

Results and Discussion

Identification of Gallic Acid by HPTLC

The mobile phase chloroform: ethyl acetate: formic acid (5:4:0.1v/v/v) presented a good resolution with R_f = 0.55 and 0.56 for Gallic Acid (GA) and 4Th peak of hydroalcoholic extract (WFF) respectively at 254nm. Seven distinct plant peaks were subjected to hydroalcoholic extract (WFF) bearing R_f 0.02, 0.103, 0.37, 0.56, 0.74, 0.87, 0.98. Thus, the R_f = 0.56 value of the plant extract was compared with the standard. In addition of result R_f = 0.55 and 0.56 for Gallic Acid (GA) and 3rd peak of hydro alcoholic extract (WFF) respectively at 416nm. Four distinct plant peaks were subjected to hydro alcoholic extract (WFF) bearing R_f 0.10, 0.37, 0.56, 0.98. Thus, the R_f = 0.56 value of the plant extract was compared with the standard. The Gallic Acid does not show any fluorescence in long UV (366nm) that's why there is no peak of Gallic Acid is recorded during the scanning of long UV (366nm). Seven distinct peaks were subjected to hydro alcoholic extract (WFF) analysis during scanning of short UV (254nm). The third peak of the sample chromatogram match with the standard Gallic Acid in Figure 01.

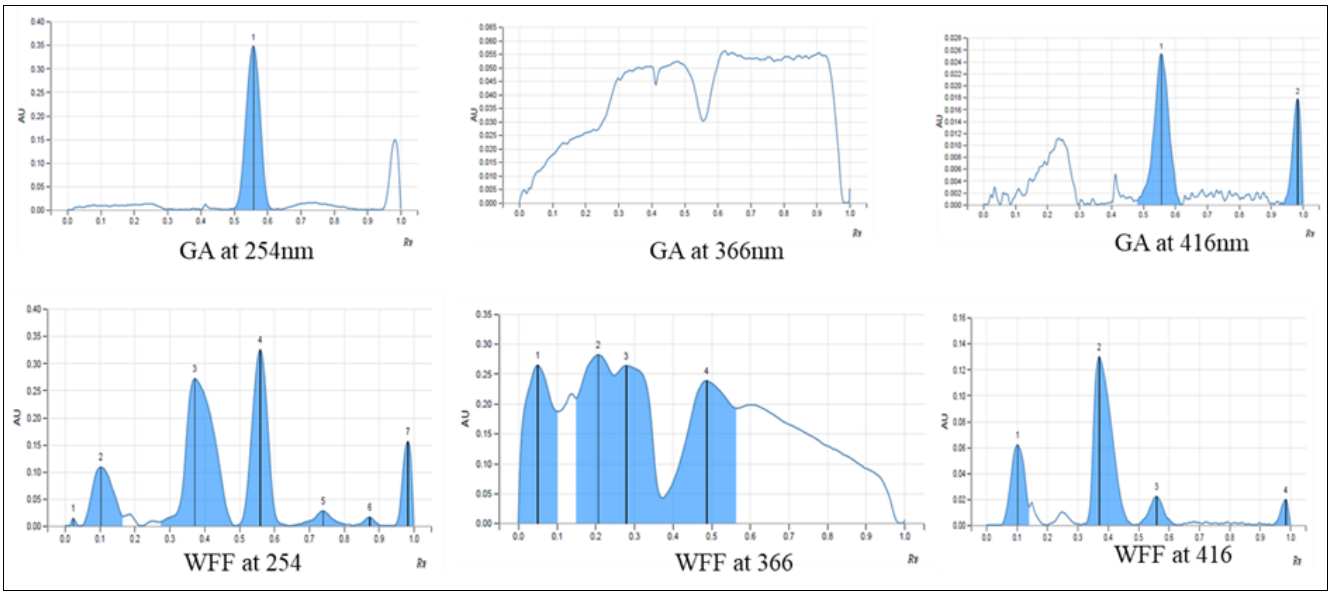


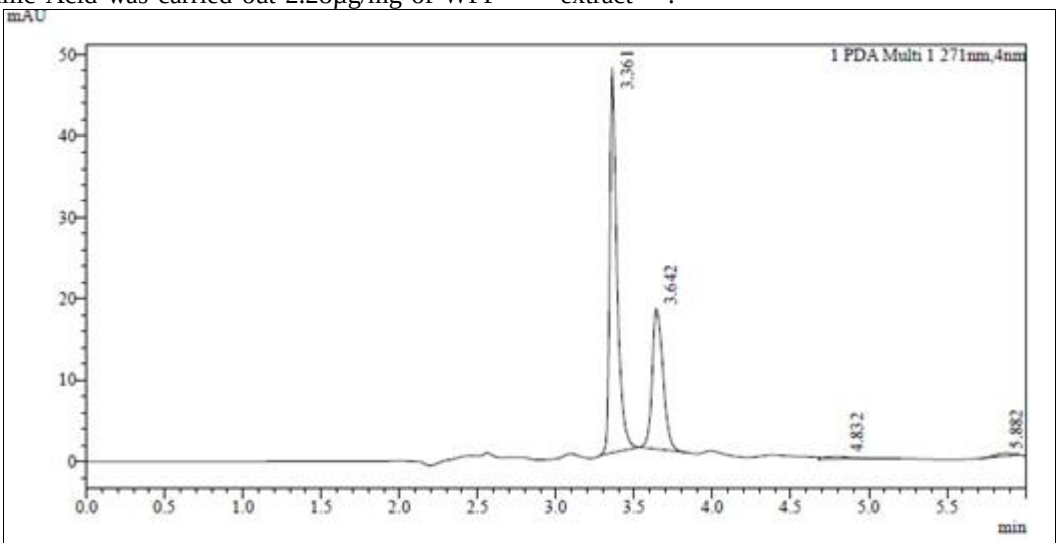
Fig 3: Identification of Gallic Acid by HPTLC Analysis

Table 1: Identification of Gallic Acid by HPTLC Analysis

Name of Sample	254nm		366nm		416nm	
	Peaks	R_f	peaks	R_f	peaks	R_f
Gallic Acid	01	0.558	0	0	01	0.557
WFF	07	0.560	4	0.487	04	0.560

Quantification of Gallic Acid by HPLC: The content of the Gallic Acid was quantified using calibration curve (Figure 1) of Gallic Acid. The concentration of Gallic Acid of WFF extract was successfully quantified. The retention time for Gallic Acid was 3.361 min. and the area (152705) of the peak was plotted at the calibration curve of Gallic Acid. The

abundant of Gallic Acid was carried out 2.28µg/mg of WFF extract [14].



Peak	Retention Time	Area Under Curve	Height
01	3.361	152705	47292
02	3.642	83413	17206
03	4.382	3994	261
04	5.882	3162	358

Fig 4: Quantification of Gallic Acid by HPLC Analysis

FT-IR Spectrum Analysis of Gallic Acid and WFF Extract

Gallic Acid (GA) is confirmed by the presence of bands observed at 1385 and 1309 cm^{-1}. The band at 3083 cm^{-1} is characteristic of the stretching vibration of the C-H bond in the aromatic ring. In the ranges of 2957-2853 cm^{-1} and 1626-1549 cm^{-1}, bands appear due to stretching vibrations of -CH_3 groups and the >C=C< bond, respectively. Bending vibrations of the aromatic CH bond and the -CH_3 group occur at 1453 cm^{-1} and 1349 cm^{-1}, respectively. Bands in the ranges of 1247-1040 $cm^{-1}$and 558-496 $cm^{-1}$are attributed to in-plane and out-of-plane deformation vibrations of the aromatic ring.

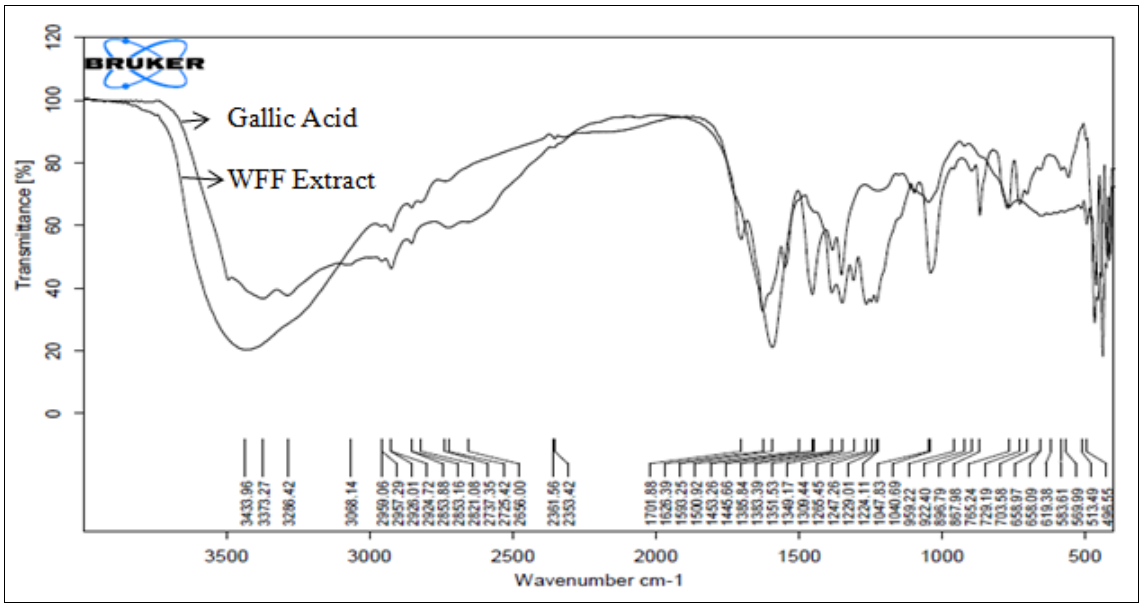


Fig 5: FT-IR Spectrum Analysis of Gallic Acid and WFF Extract

NMR Spectrum Analysis of WFF Extract

The ^{1}H-NMR spectrum of Gallic Acid portrays different distinctive sharp and intense peak at δ = 9.17 ppm, 8.82 ppm and 6.91 ppm representing the protons H2, H3 and H1 respectively. ^{13}C- NMR spectrum of Gallic Acid represented chemical shifts at δ = 169.42 ppm, 145.35 ppm, 139.82 ppm, 121.27 ppm and 109.55 ppm which could be assigned to carbon atom of C=O group at C-7 position, C-3, C-4, C-1 and C-2, respectively [15, 16].

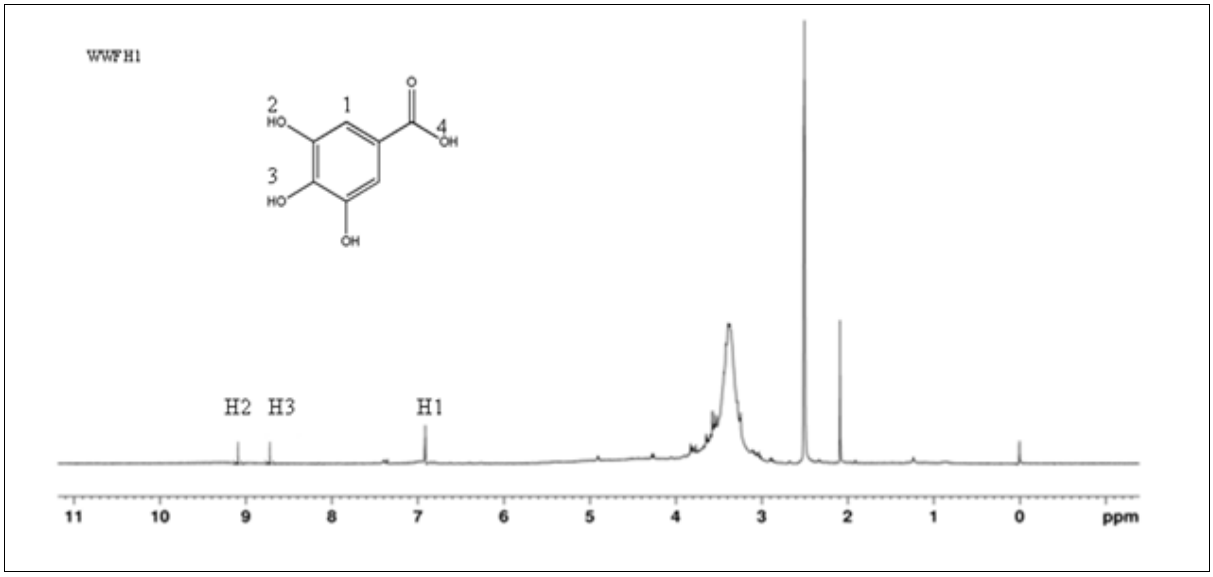


Fig 6: ^{1}H-NMR Spectrum Analysis of WFF Extract

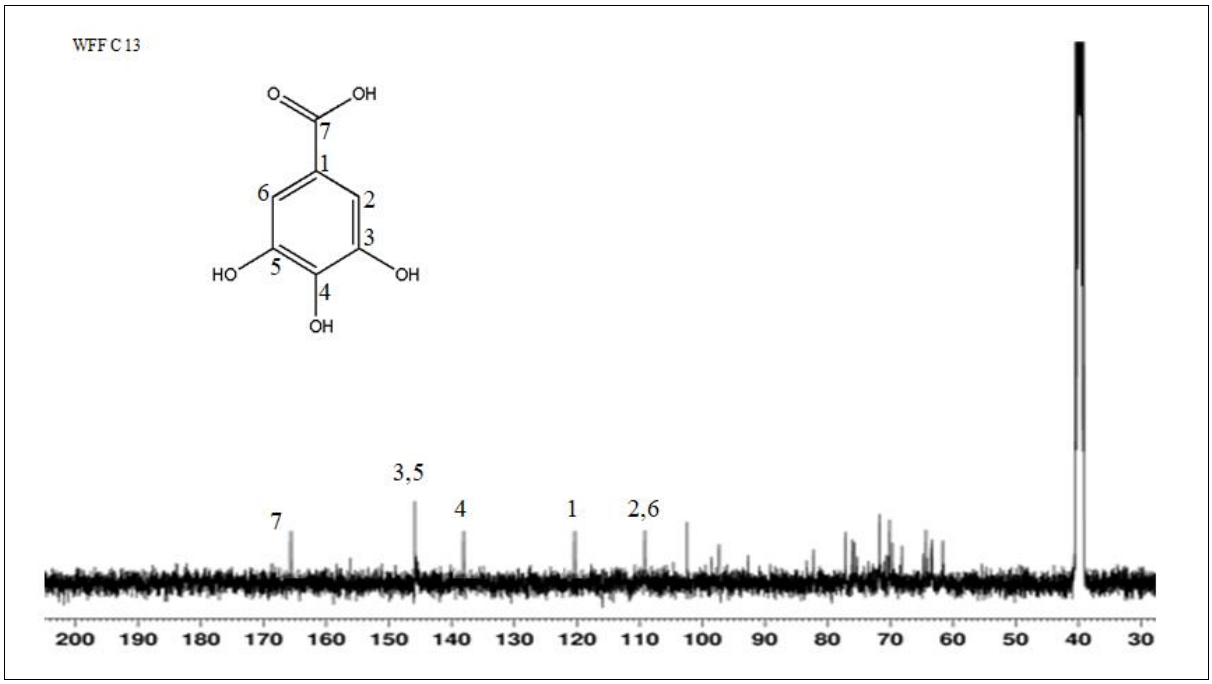


Fig 7: ^{13}C- NMR Spectrum Analysis of WFF Extract

Conclusion

For survival, growth, development, and defence against a variety of bacterial, fungal, and viral diseases, plants produce a vast array of metabolites. Since these substances are found in large quantities in leaves, flowers, fruits of plants in nature, the human community uses them either directly or indirectly as food ingredients. One such prominent phenolic acid is Gallic Acid. More reliable research on the potential medical benefits of Gallic Acid must be encouraged. It is regarded as a promising lead molecule for the creation of novel medications.

The present work described the isolation, characterization and quantification of Gallic Acid from hydro alcoholic flower extract of *Woodfordia fruticosa* (L.) Kurz. The gallic Acid was isolated from hydro alcoholic flower extract of WFF by using open silica column. The characterization of Gallic Acid was performed by Fingerprinting HPTLC, HPLC analysis. The structure of Gallic Acid was determined by using data obtained from FT-IR, ^{1}H and ^{13}C NMR spectra. Gallic Acid has been connected to a variety of biological characteristics, such as antibacterial, anticancer, anti-inflammatory, and antioxidant. Numerous studies have demonstrated the safety and effectiveness of Gallic Acid. But its pharmacokinetic profiles include low absorption, poor bioavailability, and rapid elimination, restricts its clinical application. In order to help human treatment or recovery, it implies that applying nanotechnology in medicine may increase therapeutic efficacy and generate the intended pharmacological response.

Competing Interests: The authors declare no conflicts of interest.

Authors' Contributions: All three authors contributed equally.

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