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Isolation, Characterization, and Utilization of Secondary Metabolites from *Ehretia acuminata*: Bioactive Compounds for Pharmaceutical and Industrial Applications

Amanpreet Kaur,[1,*] Man Vir Singh,[2,*] Abha Shukla,[3] Muneesh Sethi,[4] Pranjal Jayaswal,[5] Poonam Singhal,[6] Elzira Kyrbassova,[7] Nazgul Altybayeva,[8] Shubham Sharma[9] and Gulgaisha Baigaziyeva[10,*]

Abstract

Ehretia acuminata, a member of the *Boraginaceae* family, is a wild tree native to Asia, Africa, Australia, and North America, commonly referred to as Koda. The leaves and bark of *E. acuminata* have a rich history of utilization in traditional Chinese herbal medicine, where they have been employed to address various health concerns, including fever, oral sores, dysentery, and numerous other ailments. Column chromatography was employed to isolate phytoconstituents from this remarkable plant to uncover its pharmacological potential. Notably, the ethyl acetate extract derived from its leaves and the ethanol extract from its bark exhibited the most promising profiles regarding diverse biological activities. The isolated compounds were characterized through a comprehensive analysis involving ultraviolet-visible (UV), infrared (IR) spectroscopy, ^{1}H and ^{13}C nuclear magnetic resonance (NMR), and mass spectrometry (MS) techniques. The resulting spectral data unequivocally revealed the identities of the compounds as 3,4′,5,7-tetrahydroxyflavone (85mg), 3-Phenylprop-2-enoic acid (65mg), and 4-Hydroxybenzoic acid (62mg). Significantly, these phytoconstituents were isolated from the *Ehretia acuminata* plant for the first time. Among these compounds, 3,4′,5,7-tetrahydroxyflavone demonstrated outstanding antioxidant and antimicrobial properties, while 3-Phenylprop-2-enoic acid and 4-Hydroxybenzoic acid exhibited substantial potential in the realms of antioxidant, anti-diabetic, and antimicrobial activities. This groundbreaking research underscores the therapeutic potential of *Ehretia acuminata*, shedding light on its valuable contributions to traditional medicine and modern pharmacology.

Keywords: Ehretia acuminata, Bioactive compounds, 4-Hydroxybenzoic acid, Kaempferol biological activities.
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1. Introduction

Phytopharmaceutical preparations, another name for plant-based medications, are made entirely of whole plants or plant components.[1] Pharmaceutical formulations that have been refined or in their raw forms can be used to generate these medications. The market is flooded with herbal products, and usage of herbal medication was estimated to be between 10% and 75% in 2019. Traditional medicine is widely utilized in Indonesia to treat a wide range of ailments, with malignant tumors and cancer having the highest frequency at 14.4%.[2] Treatments for rheumatism and high cholesterol come next, with a prevalence of 11.3% for each. Traditional medicine is also commonly used to treat the following conditions: stroke (10%), diabetes (9%), kidney illnesses (9%), and liver problems (8%). It has a long history in China for pregnant women to take herbal remedies.[3] The percentage of people who use herbal remedies is relatively large, at 66% of the population. Of these, 56% keep using them after giving birth, and 6% use them the entire time they are pregnant.[4] Since secondary metabolites have therapeutic effects, they are important in herbal medicine. These substances are a great

[1] *Department of Chemistry, School of Sciences, IFTM University, Moradabad, Uttar Pradesh, 244102, India*
[2] *School of Applied and Life Sciences, UIT, Uttaranchal University, Dehradun-248007, Uttarakhand, India*
[3] *Department of Chemistry, Kanya Gurukul Campus, Gurukul Kangri Vishwavidyalaya, Haridwar, Uttarakhand, 249404, India*
[4] *Dean (Research & Development) COER University, Roorkee, Uttarakhand, 247667, India*
[5] *Department of Chemistry, Birla Institute of Technology & Science, Pilani Campus, Pilani, Rajasthan, 333031, India*
[6] *Dean School of Allied Sciences, Dev Bhoomi Uttarakhand University, Dehradun, Uttarakhand, 248007, India*

source of lead compounds for the pharmaceutical industry because of their diverse range of unique biological activities.[5] Given that some of these metabolites have additive or synergistic effects, their potential for use in the development of novel medications is constantly being investigated.[6] Many different plant species contain phenolic compounds, a class of secondary metabolites with potent biological activity.[6] Phenolic compounds show a range of functions, such as anti-inflammatory and antibacterial properties, and they may be useful as a treatment for diseases like diabetes, cancer, and obesity.[7] Antioxidants are essential for stopping the oxidation process, which is a chemical reaction that can result in the production of free radicals, which can seriously harm an organism's cells.[8] Phenolic chemicals can scavenge free radicals since they are antioxidants. A free radical can receive a hydrogen atom from the hydroxyl group on the phenolic ring, resulting in the formation of a phenoxy radical, a delocalized, stabilized unpaired electron across the phenolic ring.[9] The free radical chain reaction is essentially stopped by this stabilization, which is made possible by the aromatic nucleus's resonance effect.[10,11]

DNA damage can result from the oxidation of lipids and proteins by free radicals, reactive oxygen species (ROS), and reactive nitrogen species (RNS) such as superoxide, hydroxyl, and nitric oxide radicals within biological system cells.[11] Numerous organ systems can become dysfunctional due to oxidative stress, which is brought on by excessive exposure to ROS.[12] When ROS or other highly reactive molecules and antioxidants are out of balance, oxidative stress results, disrupting redox processes and causing molecular damage from low antioxidant activity.[13] Conditions like insulin resistance, hepatocyte dysfunction, heart hypertrophy and fibrosis, and neurotoxicity have all been linked to excessive ROS exposure.[14] Because ROS can cause DNA damage or mutations, change gene expression, start pro-oncogenic signaling pathways, and create genomic instability, a high quantity of ROS increases the likelihood of cell transformation. ROS/RNS can potentially promote the growth of cancer by drawing inflammatory cells to areas of tissue injury.[15,16] Driven by ROS/RNS, chronic inflammation results in oxidative and nitrosative stress in addition to inducing inflammatory cells and activating gene expression via a variety of pathways.[17] Cytokines, which are released by inflammatory cells, encourage the oxidation and nitration of proteins, carbohydrates, and fats. Furthermore, the equilibrium between pro- and anti-apoptotic settings is upset by transcription factors and cytokines, which ultimately triggers apoptosis.[18] This imbalance ultimately contributes to the development of cancer by causing both cell death and regeneration.[19] It is caused by gene alterations, mutations, proliferation, angiogenesis, and related mechanisms.

Natural antioxidants have anti-inflammatory, anti-aging, anti-atherosclerosis, anti-cancer, and antioxidant qualities. Examples of these substances are polyphenols and phenolic compounds, which are present in foods and medicinal plants.[20] The petals of Hibiscus rosa-sinensis were used to extract one such flavonoid, hibiscetin-3-glucoside. Hiscetin-3-glucoside exhibits more antioxidant activity than ascorbic acid, a common antioxidant. This substance can counteract free radicals, prevent the production of hazardous byproducts, and increase the shelf life of medications and food.[21] Antioxidant substances such as agathisflavone and a mixture of quercetin 3-O-rhamnoside and quercetin 3-O-rutinoside are present in the ethyl acetate fraction of leaves from *Anacardium occidentale* L. With an IC50 value of 0.96 ± 0.01 µg/mL in the DPPH assay, this combination—especially in a 2:1 ratio of quercetin 3-O-rutinoside to quercetin 3-O-rhamnoside—has shown notable efficiency in scavenging free radicals. Furthermore, in the ferric-reducing antioxidant power and total antioxidant capacity (TAC) tests, this mixture had the best activity.[22] It is a complicated procedure to turn bioactive molecules from natural items into pharmaceutical medications. It goes through several phases, such as the following: natural product screening, bioactive compound separation, characterization and optimization, mechanism of action identification, and pharmaceutical development.[23] One type of phenolic coumarin is scopoletin, which is also referred to as 7-hydroxy-6-methoxy coumarin. It has an oxo group, a methoxy group, a hydroxyl group, and two aromatic rings.

Many plants, such as the roots of *Hypochaeris radicata, Lasianthus lucidus Blume*, and *Eupatorium laevigatum*, can be used to extract scopoletin.[24] An essential initial step in the biological characterization process is the isolation of secondary metabolites. This procedure becomes particularly important when dealing with substances that are difficult to synthesize or lack available commercial standards.[25,26] Various techniques are employed to isolate phenolic compounds with antioxidant properties, including column chromatography, high-performance liquid chromatography (HPLC), medium-pressure liquid chromatography (MPLC), centrifugal partition chromatography (CPC), and high-performance counter-current chromatography (HPCCC).[27,28] Through a process known as molecularly imprinted polymerization, which creates synthetic polymeric materials with homologous adsorption sites customized to a template

[7] *Department of Biology of the Institute of Natural Sciences, NPJSC, Kazakh Women's Teacher Training University, A05C9Y7, St. Gogol, Almaty 114 k1, 05000, Kazakhstan*

[8] *Faculty of biology and biotechnology, Al-Farabi Kazakh National University, Almaty, 050040, Kazakhstan*

[9] *Department of Chemistry, GLA University, 17km Stone, NH-19, Mathura-Delhi Road, Chaumuhan, Bharthia, Uttar Pradesh, India, 281406*

[10] *Department of Technology of bread products and processing industries, Faculty of Food Technology, Almaty Technological University, Almaty, Tole bi str.100, 050012, Kazakhstan*

**Email:* amanpreet2225@gmail.com (A. Kaur);
manvir24365@gmail.com (M. V. Singh);
bgulgaishailias@mail.ru (G. Baigaziyeva)

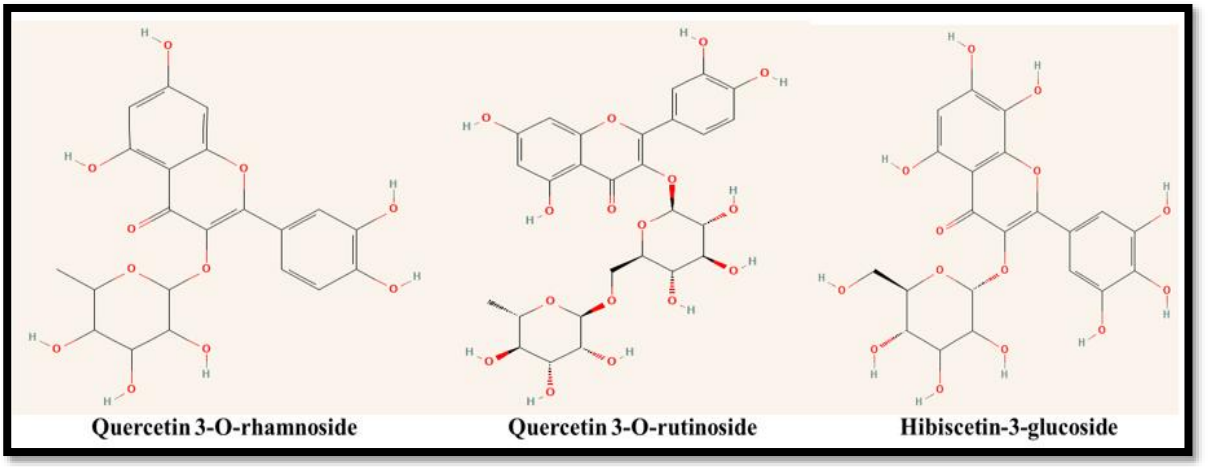

Fig. 1: Phenolic compounds responsible for antioxidant potential in plants.

molecule, phenolic compounds—which are recognized for their antioxidant qualities—can be extracted from extracts with selectivity. To separate phenolic chemicals from natural materials, this technique works especially well. The literature has studied the extraction of phenolic compounds having antioxidant activity from natural sources in great detail.[29,30]

The current study's objective was to separate the phytoconstituents from the leaves and bark of *Ehretia acuminata* R. Br. using column chromatography. These constituents were then identified using spectral techniques such as ultraviolet (UV), Fourier transform infrared spectroscopy (FTIR), ^{1}H, ^{13}C nuclear magnetic resonance (NMR), and mass spectroscopy (MS)and their biological activity was assessed by measuring antioxidant, antidiabetic, and antimicrobial activity.

2. Experimental

2.1 Materials

All solvents, including methanol, ethanol, dichloromethane, chloroform, carbon tetrachloride, n-hexane, acetone, and ethyl acetate, underwent distillation before usage to ensure their purity. For thin-layer chromatography (TLC), we employed silica gel containing 13% $CaSO_4$ as a binder, sourced from SRL in Mumbai. Column chromatography was conducted using SilicaGel-G (60–120 mesh) obtained from Merck in Mumbai. In our antioxidant, antidiabetic, and antimicrobial assays, we utilized the following standards: Quercetin (Merck), ascorbic acid (Merck), acarbose (Bayer India Limited), tetracycline (Sigma–Aldrich), and erythromycin (Sigma–Aldrich). The stable free radical 1,1'-Diphenyl-2-picryl hydrazyl (DPPH) was procured from Sigma. Additionally, the employed 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), Nitrophenyl-α-D-glucopyranoside (SRL Pvt., Ltd), Tris buffer (Merck), α-amylase ex porcine pancreas (SRL Pvt., Ltd), Dimethyl sulfoxide (DMSO) from Merck, 3,5-dinitrosalicylic acid (DNSA) (SRL Pvt., Ltd), α-glucosidase for biochemistry ex microorganism (SRL Pvt Ltd), and acarbose (Bayer India Limited). All chemicals used in our experiments met the highest analytical grade standards.

2.2 Methods

2.2.1 Extraction of plant material

Plant materials collected include leaves and bark from *Ehretia acuminata* in Pantnagar, Uttarakhand, India. The Botanical Survey of India (BSI) in Dehradun verified and authenticated the samples (Voucher specimen number 117138 05/2022). After washing, we dried the leaves and bark in the shade for 30 days, ground them into a powder, and stored them in an airtight container. We then used a Soxhlet apparatus to extract the dried powder with petroleum ether, chloroform, ethyl acetate, ethanol, and water in increasing order of solvent polarity (as per our previous work).[31] The resulting extracts were concentrated under reduced pressure using a rotary vacuum evaporator and kept refrigerated for later use. This process was repeated until we obtained the desired amount of extract.

2.2.2 Isolation of compound from *E. acuminata* leaves

The ethyl acetate extract of *Ehretia acuminata* leaves demonstrated significant potential in various biological activities, including antioxidant, anti-inflammatory, antihemolytic, and antimicrobial effects. As a result, this extract was selected for the isolation of active constituents. To initiate the process, 15 g of the ethyl acetate leaf extract were dissolved in a 20% hydroalcoholic solution and fractionated with chloroform to remove chlorophyll. The chloroform fraction, containing non-polar compounds along with chlorophyll, was set aside. Meanwhile, the hydroalcoholic fraction was concentrated to dryness using a rotary vacuum evaporator under reduced pressure. Now, ethyl acetate extract (hydroalcoholic fraction) of 12.56 gm free from Chlorophyll was subjected to silica gel (430 gm 100-200 mesh), column (100 cm × 4 cm in diameter) chromatography and eluted with petroleum ether: ethyl acetate mixture (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10) then with ethyl acetate: acetone mixture (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10). Each fraction of 100 mL was collected, concentrated and then checked for TLC profile. Developed TLC was first visualized under UV light (λ_{max} 254, 356nm), and then spots were

completely developed by iodine vapors or by spraying 10% sulfuric acid in ethanol, followed by heating. The column fractions (F) were combined based on their R_f value; fractions F_{115}–F_{138} and F_{160}-F_{207} were collected and evaporated under reduced pressure and collected in a vial. Further separation was carried out employing preparative TLC. Further separation was carried out through preparative TLC. The concentrated fractions were dissolved in methanol and loaded onto Silica gel-G preparative TLC plates. These plates were placed in a TLC chamber using different solvent systems (chloroform: methanol: water at 4:1:0.1 v/v for F_{115}–F_{138} and chloroform: methanol: water at 4:1:0.4 v/v for F_{160}-F_{207}). Bands with R_f values of 0.62 and 0.45 were carefully selected from their respective collected fractions and subsequently eluted with methanol.

2.2.3 Isolation of compound from *E. acuminata* bark

Extracts obtained from ethanol in the bark of *Ehretia acuminata* were found to be most promising towards many biological activities, such as antioxidant.[32] Thus, the same was selected for active constituent separation. Ethanol extract (13 gm) of bark was dissolved in 35% hydroalcoholic solution and fractionated with hexane: chloroform (5:5 v/v) to remove some part of non-polar compounds. The hydroalcoholic fraction was concentrated to dryness in a rotary vacuum evaporator at reduced pressure. Now, 11.24 gm of ethanol extract (hydroalcoholic fraction) was subjected to silica gel (430 gm 100-200 mesh), column (100 cm × 4 cm in diameter) chromatography and eluted with petroleum ether: ethyl acetate mixture (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10) then with ethyl acetate: acetone mixture (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10). Each fraction of 100 ml was collected, concentrated and then checked for TLC profile. Developed TLC was first visualized under UV light (λ_{max} 254, 356 nm), and then spots were completely developed by iodine vapors or by spraying 10 % sulphuric acid in ethanol, followed by heating. The column fractions (F) F_{140}–F_{184} were pooled together based on their R_f value, evaporated under reduced pressure, and collected in a vial. Further separation was carried out utilizing preparative TLC. The concentrated fraction was dissolved in methanol and loaded on Silica gel-G preparatory TLC. Place preparatory TLC in a TLC chamber with hexane/methanol/water (4:1:0.2 v/v) solvent system. Some bands were observed, a band with 0.40 R_f was selected and scraped, then finally eluted with methanol.

2.3 Spectral analysis

Compounds obtained from both the leaves and bark of *E. acuminata* were subjected to a comprehensive analysis using various advanced spectral techniques. These included UV (Ultraviolet) spectroscopy, IR (Infrared) spectroscopy, 1^11H-NMR (Proton Nuclear Magnetic Resonance), 13^{13}13C-NMR (Carbon-13 Nuclear Magnetic Resonance), and Mass Spectroscopy. The UV spectrum was carefully recorded using a Systronic 2203 spectrophotometer, while the FTIR spectrum was accurately measured with a Perkin Elmer - Spectrum RX-FTIR spectrophotometer. For NMR used a state-of-the-art Bruker Avance II 400 MHz NMR spectrophotometer was used to ensure high-resolution spectral data. Melting points (Mps) were ascertained with utmost accuracy utilizing a Kofler hot-stage apparatus. Mass spectra, crucial for molecular identification, were diligently captured using a Waters Q-Tof Micromass (ESI-MS) spectrophotometer, further enhancing our comprehensive analysis. Data of RX-IFTIR, ESI-MS, FTIR, UV, and NMR are shown in the supporting information.

2.4 Spectral data for isolated compounds from leaves

2.4.1 Compound 1

UV, $\lambda_{max}(CH_3OH)$ nm: 371.04, 289.6 and 222.6; IR(KBr) ν_{max} cm^{-1}: 3427.1, 2813.4, 2746.4, 1661.1, 1612.4, 1506.9, 1442.0, 1382.4, 1314.9, 1253.0, 1225.8, 1176.2, 975.0 and 4116; ^{13}C-NMR (DMSO, D_2O, 400.140 MHz) δ ppm:176.38, 164.37, 161.18, 159.66, 159.65, 147.28, 136.13, 129.97, 122.14, 115.91, 103.51, 98.67 and 93.95; ^{1}H-NMR (DMSO, 400 MHz)δ ppm { multiplicity: Singlet(s), doublet(d), triplet(t), multiplet(m), doublet- doublet(d-d)}: 2.508, 3.350, 6.198 (1H, d), 6.445 (1H, d), 6.941 (1H, d-d), 8.058, (1H, d), 9.410, 10.113, 10.789 (1H, s), 12.485 (1H, s); MS m/z:287.04$[M+H^+]^+$ and 285.02$[M- H^+]^-$.

2.4.2 Compound 2

UV, $\lambda_{max}(CH_3OH)$ nm: 299.86, 228.64 and 201.72; IR(KBr) ν_{max} cm^{-1}: 3447.1, 3025.1, 2967.0, 2836.3, 2594.4, 1683.4, 1630.3, 1577.5 1493.3, 1448.9, 1421.2, 1314.0, 1286.0, 1224.0 980.3, 706.6, 542.7, 505.7 and 481.4; ^{13}C-NMR (DMSO, D_2O, 400.140 MHz) δ ppm:167.53, 143.88, 134.19, 130.18, 128.87, 128.16 and 119.20; ^{1}H-NMR (DMSO, 400 MHz)δ ppm { multiplicity: Singlet(s), doublet(d), triplet(t), multiplet(m), doublet- doublet(d-d)}: 2.5318, 3.3515, 6.5187, 6.5588(1H,d), 7.4147, 7.5813, 7.7017 (1H,d-d), 12.4085(1H, s); MS m/z:149.03$[M+H^+]^+$and 147.05$[M- H^+]^-$.

2.4.3 Compound 3

UV, $\lambda_{max}(CH_3OH)$ nm: 289.65, 240.52 and 220.47; IR(KBr) ν_{max} cm^{-1}: 3386.9, 3201.9, 2956.6, 2826.8, 2662.8, 2545.8, 2052.9, 1925.9, 1785.3, 1675.3, 1607.5, 1594.0, 1509.6 1447.2, 1421.7, 1364.0, 1317.1, 1243.0, 930.1, 546.3, 505.7 and 437.4; ^{13}C-NMR (DMSO, 400 MHz) δ ppm: 167.13, 161.56, 131.49, 121.31 and 115.08; ^{1}H-NMR (DMSO, 400MHz) δ ppm { multiplicity: Singlet(s), doublet(d), triplet(t), multiplet(m), doublet- doublet(d-d)}: 2.5098, 3.4250, 6.8484(1H,d), 7.8188(1H,t), 10.2198(1H, d), 12.4245(1H, s); MS m/z:139.02 $[M+H^+]^+$ and 137.02 $[M- H^+]^-$.

2.5 Biological activities

2.5.1 Antioxidant activity 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay

The ABTS method was employed to evaluate antioxidant activity.[33] To generate the ABTS radical cation, an aqueous ABTS solution containing a stable radical was first incubated

in the dark with 2.4 mM potassium persulfate for 12 to 16 hours. Before testing, the ABTS solution was diluted in ethanol at a ratio of 1:89 (v/v) to achieve an absorbance of 0.700 ± 0.02 at 734 nm. For the experiment, 1 mL of the diluted ABTS solution was combined with three identical 10-µL samples, each containing 1 mg/mL of the corresponding organic solvents and ascorbic acid. The reaction mixture was prepared and incubated for exactly thirty minutes at a controlled temperature of 30 °C. After incubation, the absorbance of the mixture was measured at 734 nm, with ethanol used as the blank reference. The radical scavenging activity was calculated as a percentage of inhibition using Equation (1). This method can assess and quantify the antioxidant properties of the substances being tested.

$$\text{Radical scavenging activity (\%)} = \frac{OD\ of\ blank - OD\ of\ sample}{OD\ of\ blank} \times 100 \quad (1)$$

2.5.2 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) assay

The methodology utilized to evaluate the radical scavenging activity of isolated compounds was modified from Kaur *et al.*[34] Test tubes containing ascorbic acid and different concentrations of the separated compounds (5, 10, 20, and 30 µL) were filled with methanol (MeOH) to bring the total capacity of each tube to 100 µL. Subsequently, each tube was filled with 3 mL of a 0.1 mM methanol solution containing DPPH (2,2-diphenyl-1-picrylhydrazyl), and the solutions were shaken rapidly. After that, the tubes were incubated for precisely thirty minutes at a steady temperature of 27 °C. There was also a control sample that was made in the same manner without the tested ingredient. Following the incubation time, 512 nm was used to precisely measure the samples' changes in absorbance. This method made it possible to assess the separated compounds' capacity to scavenge radicals efficiently.

2.5.3 Antidiabetic activity by α-amylase method

With slight modifications, the α-amylase enzyme inhibition protocol was carried out following the reported methodology.[35] The activity of α-amylase was determined by measuring the amount of reducing groups produced when isolated pancreatic α-amylase hydrolyzed soluble starch. This activity was monitored photometrically by measuring the absorbance at 540 nm, which corresponds to the color change resulting from the reduction of 3,5-dinitrosalicylic acid to nitroaminosalicylic acid. When an α-amylase inhibitor is used to inhibit starch hydrolysis, the absorbance at 540 nm is lower compared to the control. Acarbose (AC) was used as a standard inhibitor for α-amylase. The percentage inhibition of α-amylase was expressed using the half-maximal inhibitory concentration (IC_{50}). The inhibition percentage can be calculated using Equation (2):

$$\%\ \text{INHIBITION} = \frac{Absorbance\ of\ control - (absorbance\ of\ extract)}{Absorbance\ of\ control} \times 100 \quad (2)$$

2.5.4 Inhibition assay of α-glucosidase enzyme

With a few minor modifications, the α-glucosidase enzyme inhibition protocol was conducted following the methodology outlined by Shukla *et al*. in 2021.[36] This assay is based on the hydrolysis of p-nitrophenyl-α-D-glucopyranoside (p-NPG) by α-glucosidase, resulting in the production of D-glucose and p-nitrophenol. Acarbose (AC) was used as the standard reference drug for the α-glucosidase inhibition experiment. The absorbance of the released p-nitrophenol was measured at 410 nm. Each experiment was performed in triplicate, and a parallel control without the test sample was included. The percentage of enzyme inhibition, except for α-glucosidase, was expressed using the half-maximal inhibitory concentration (IC_{50}). The percentage of inhibition can be calculated using the previously mentioned formula.

2.5.5 Antimicrobial bioassay

The disc diffusion method was employed to assess the antibacterial activity of the isolated compounds, as stated by Kaur *et al*. (2024). Microorganisms were kept at -80 °C after being taken from the Microbial Type Culture Collection (MTCC). The bacteria were planted onto nutrient agar (Microxpress Ltd.) in Petri dishes for purity tests before the bioassay. Both the isolated compounds and the conventional medications were produced as dimethylsulfoxide (DMSO) solutions. Inoculated plates (70 mm) were covered with sterile discs that had been saturated with 20 µL of the sample apiece. The test samples (20 µL per disc) were then injected onto the surface of nutrient agar plates after the specific bacterial strains had been spread out on them. For twenty-four hours, the plates were kept in a bacteriological incubator at 37 °C. The zones of inhibition were measured in millimeters in order to assess the antibacterial activity.[37]

2.5.6 Statistical analyses

All experiments were conducted in triplicate, and the results were expressed as Mean ± SD. The data were statistically analyzed using one-way ANOVA followed by Duncan's test. Mean values were considered statistically significant when $p > 0.05$.

3. Results and discussion

3.1 Compound 1

Isolated compound 1 from leaves of *E. acuminata* is a yellow amorphous powder. UV spectrum of isolated compound, which indicates characteristic absorption maximum at 370.04 nm for n→π* transition of C=O group; 289.6 nm represents π→π* transition for aromatic C=C, and 222.6 nm exists due to n→σ* transition for OH group.[38] The IR spectrum of the isolated compound reveals the presence of the OH group having a stretching frequency at 3427.1 cm^{-1}, a broad absorption band representing intermolecular hydrogen

bonding.[39] Whereas the band at 2813.4 cm^{-1} shows asymmetric stretching of the aromatic moiety, and the band at 2746.4 cm^{-1} is due to C-H stretching of the alkene.[40] The absorption peaks positioned at 1661.1 cm^{-1} represent C=O ketonic stretching.[41] The band at 1612.4 cm^{-1} was due to the C-C aromatic ring stretching, the 1506.9 cm^{-1} band represents C=O aromatic stretching, and the 1442.0 cm^{-1} band depicts C=C aromatic stretching. Bending vibrations of phenolic OH were observed at 1382.4 cm^{-1}.[42] The absorption bands at 1314.9 cm^{-1} and the bands at the lower frequencies between 975.0 cm^{-1} and 411.6 cm^{-1} were depicted as the C-H bending vibrations of aromatic hydrocarbons. Bands at 1253.0 cm^{-1} and 1225.8 cm^{-1} represent C-O stretching vibrations of aryl ether and phenols, respectively.[40]

The absorption band positioned at 1176.2 cm^{-1} depicts C-CO-C stretching and bending vibrations of ketones. Fig. S1 represents the FTIR spectrum of isolated compound 1 of *E. acuminata* leaves. The ^{13}C-NMR spectrum showed the presence of fifteen carbon atoms. Carbonyl (C-4) group shows signal at 176.38ppm and aromatic carbon exist at 164.37, 161.18, 159.66, 159.65, 147.28, 136.13, 129.97, 122.14, 115.91, 103.51, 98.67, and 93.95 ppm for C-7, C-9, C-5, C-4', C-2, C-3, C-3',5', C-1', C-2', 6', C-10, C-6 and C-8 respectively.[43] Fig. S3 gives the information of the ^{13}C-NMR spectrum of isolated compound 1 of *E. acuminata* leaves. The ^{1}H-NMR spectrum shows solvent peaks at δ 2.508 and 3.350 ppm. Isolated compound peaks observed at δ 12.485, 10.783, 10.113, and 9.410 revealed the presence of phenolic proton at C3, C5, C7, and C4'.[42] Aromatic protons are present at C3',5', C2',6', C8', and C6, giving peaks at chemical shift (δ) 8.058, 6.941, 6.445, and 6.198 δ respectively. Fig. S2 shows the ^{1}H-NMR spectrum of isolated compound 1 of *E. acuminata* leaves.[43] The base peak in positive mode is observed by the mass spectrum to be 287.04, and the base peak in negative mode is 285.02, confirming that the isolated compound has an approximate 286 molecular weight.[44] Fig. S4 depicts the mass spectrum of isolated compound 1 of *E. acuminata* leaves. The results obtained from UV, FTIR, ^{1}H-NMR, ^{13}C-NMR & MASS spectrum showed resemblance with the pure 3,4′,5,7-tetrahydroxyflavone (Fig. 2).

The UV spectrum of compound 1 shows λ_{max} at 370.40 nm. The typical UV-vis spectra of flavonoids include the same absorbance band in the range of 385-220 nm.[38] Thus, the compound shows that the compound may belong to the category of flavonoids. IR spectrum exhibit peaks at 3421 cm^{-1} (OH), 1661.1 cm^{-1} (C=O ketonic stretching of alkene), 1612.4 cm^{-1} (C-C aromatic ring stretching), 1506.9 cm^{-1} (C=O aromatic stretching), 1253 cm^{-1} (C-O stretching of aryl ether), 1176.2 cm^{-1} (C-CO-C of ketone) are important stretching show the presence of flavonoid.[45] ^{13}C-NMR depicts 13 signals of isolated compounds and the position of carbons. Carbon 4 has the highest chemical shift due to the presence of the ketonic group. Peakes observed at 164.37, 159.66, 156.65, and 136.13 cm^{-1} showed the presence of phenolic groups attached at C7, C5, C4 C3. Carbon 3',5' has a higher chemical shift (129.97 ppm) than carbon at 2',6' (115.91 ppm) due to the presence of one –OH group attached at the C4' position.[43] The ^{1}H-NMR spectrum of the isolated compound was recorded to depict eight signals and the positions of protons. Peaks at 6.198 and 6.445 ppm represent the presence of aromatic protons at carbon 6 and carbon 8, respectively. From the ^{1}H-NMR spectrum, the presence of the phenolic substitution was indicated by the peaks observed at 12.485, 10.783, 10.113, and 9.41 ppm at positions 3, 5, 7, and 4'.[44] C3 carbon has the highest chemical shift due to the presence of one phenolic group and one ketonic group (electron-withdrawing group) present at C4. Doublet peaks present at 8.058 and 6.941 ppm representing the proton at carbon 3',5', and 2',6'.[45]

Further evidence in favour of the above structure accumulated from the mass spectrum, the base peak observed at positive and negative modes confirms that the isolated compound has approximately 286 molecular weight.[46] The melting point of the compound was 275 °C. Thus, an isolated

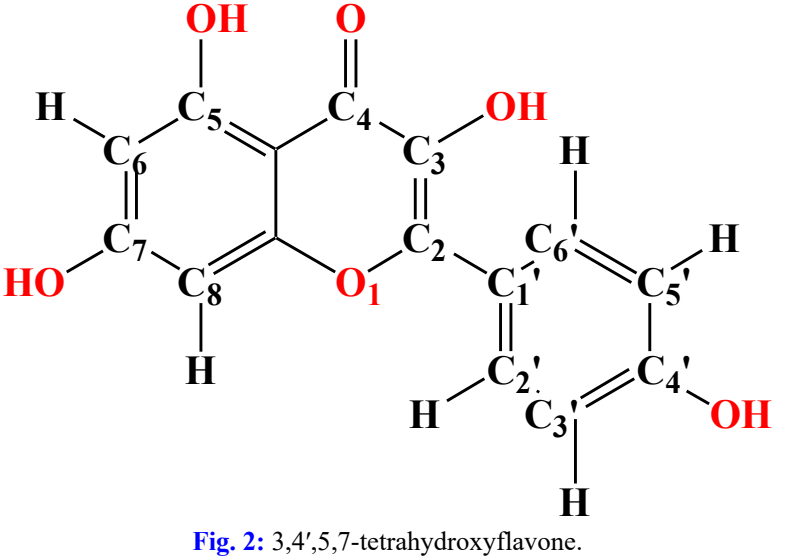

Fig. 2: 3,4′,5,7-tetrahydroxyflavone.

compound from *E. acuminata* leaves was identified as a flavonoid 3,4′,5,7-tetrahydroxyflavone, commonly known as Kaempferol. Flavonoids are a common large group of secondary metabolites widespread in higher plants and contain 15 carbons in their structure.[40] An important number has been reported from natural and synthetic sources due to their several applications in the pharmaceutical and diet industries.[47] In this work, the first isolated compound from leaves of *E. acuminata* is Kaempferol, which is an important flavonoid and has given significant results of antioxidant and antimicrobial activities with sustainable inhibition.

3.2 Compound 2

The isolated compound from the leaves of *E. acuminata* is a white crystalline powder. UV spectrum of isolated compound, which indicates characteristic absorption maximum at 299.86 nm for n→π* transition of C=O group; 228.64 nm represents π→π* transition for aromatic C=C, and 201.72 nm exists due to n→σ* transition for OH group.[48] The IR spectrum of the isolated compound reveals the presence of the OH group having a stretching frequency at 3447.1 cm^{-1}, a broad absorption band representing intermolecular hydrogen bonding.[49] Whereas the band at 2836.3 cm^{-1} shows asymmetric stretching of the aromatic moiety and the band at 2967.1 cm^{-1} was due to C-H stretching of the alkene. The absorption peaks positioned at 1683.4 cm^{-1} represent C=O acidic stretching. The band at 1630.3 cm^{-1} was due to the C-C aromatic ring stretching, the 1577.5 cm^{-1} band represents C=O aromatic stretching, and the 1493.3 cm^{-1} band depicts C=C aromatic stretching.[50] Bending vibrations of phenolic OH were observed at 1421.2 cm^{-1}.[51] The absorption bands at 1314.0 cm^{-1} and the bands at the lower frequencies between 980.3 and 481.4 cm^{-1} were depicted as the C-H bending vibrations of aromatic hydrocarbons.[49] Fig. S5 represents FTIR spectrum of isolated compound 2 of *E. acuminata* leaves. The ^{13}C-NMR spectrum showed the presence of nine carbon atoms. The carbonyl group shows the signal at 167.53 ppm, and aromatic carbon exists at 143.88, 134.19, 130.18, 128.87, and 128.16 ppm for C1, C2, C3, C4, C5, and C6, respectively.[51] The peak present at 119.20 ppm shows the presence of the alkene (C=C) group.[52] Fig. S7 represents the ^{13}C-NMR spectrum of isolated compound 2 of *E. acuminata* leaves.

The ^{1}H-NMR spectrum shows solvent peaks at δ 2.5098 and 3.3515. The peak observed at δ 12.4085 revealed the presence of an acidic proton.[53] Aromatic protons are present at δ 7.7017, 7.5813, 7.4147, 6.5588, and δ 6.5187.[50] A single peak present at 3.3515 ppm indicates the presence of a proton at carbon, attached to the oxygen.[53] Fig. S6 shows ^{1}H-NMR spectrum of isolated compound 2 of *E. acuminata* leaves. The base peak at positive mode was observed by mass spectrum to be 149.03, and the base peak at negative mode is 147.05, confirming that the isolated compound has around 148 molecular weights.[54] Fig. S8 gives the information of mass spectrum of isolated compound 2 of *E. acuminata* leaves. Concluding all the data observed by different spectral techniques, it is analysed that the studied plant *E. acuminata* leaves contain 3-Phenylprop-2-enoic acid (Fig. 3).

The UV spectrum of the compound shows λ_{max} at 299.86 nm. The UV spectra of cinnamic acid include n→ π*, π→π* & n→ σ* transitions due to the presence of C=O, C=C & OH groups in the structure of cinnamic acid.[55] IR spectrum of isolated compound show peaks at 3441 cm^{-1} (OH), 2967.1 cm^{-1} (C-H stretching of alkene), 1683.4 cm^{-1} (C=O acidic stretching), 1630.3 cm^{-1} (C-C aromatic ring stretching), 1577.5 cm^{-1} (C=O aromatic stretching), 1493.3 cm^{-1} (C=C aromatic stretching) and 1421.2 cm^{-1} (phenolic OH stretching) are important stretching peaks found in cinnamic acid.[56] The ^{13}C-NMR spectrum of compound 2 depicts 7 signals and the position of the carbon. Carbon 3 has the highest chemical shift due to one phenyl group attached to C3, and C2 has the lowest chemical shift due to the presence of one acidic group at the C1 position.

Peaks observed at 134, 128.16, 130, and 128.87 are present at the aromatic ring at C4, C5,5', C6,6', and C7 positions. The 1H-NMR spectrum of the isolated compound was recorded to depict six signals and the positions of protons. Triplets at 6.5187 and 6.5588 ppm represent the presence of aromatic proton at carbon 2 and carbon 6, 6' respectively.[57] From the ^{1}H-NMR spectrum, the presence of the acidic substitution at 1

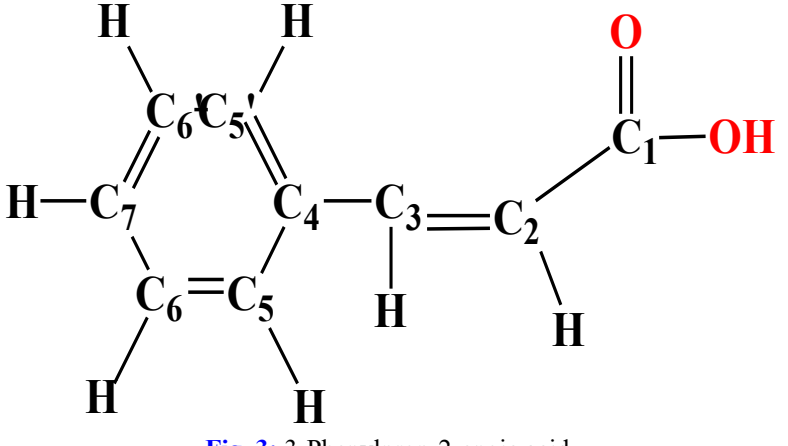

Fig. 3: 3-Phenylprop-2-enoic acid.

was indicated by the presence of one signal doublet at 12.4085 ppm and a peak present at 7.7017 ppm, also representing the proton at carbon 3. Proton present at carbon 3 has the lowest chemical shift (δ) value due to the presence of one electron-withdrawing (-COOH) group at carbon 1 and a phenyl ring attached to carbon 3 which increases the electron density at carbon 2 and shielded the proton attached to the carbon 2 and decrease the chemical shift at carbon 2. Another signal, as a doublet at 7.5813 ppm, represents a proton at carbon 5 and 5'. One signal at 7.4147 (triplicate) represents the chemical shift of the proton present at C6 and C6'. Further evidence in favour of the above structure accumulated from the mass spectrum, the base peak observed at positive and negative mode confirms that the isolated compound has approximately 148 molecular weights.[58] The melting point of the compound was 115 °C. Thus, an isolated compound from *E. acuminata* leaves was identified as a 3-Phenylprop-2-enoic acid, commonly known as Cinnamic acid.[59]

Plant derivatives contain various types of acids, including cinnamic acid and benzoic acid. Cinnamic acid is an important biochemical intermediary in the phenylpropanoid pathway, acting as a precursor to flavonoids and the structural plant material lignin. Cinnamic acid is widely found in plants and is known to be a significant pharmacologically active substance. Since phenolic chemicals have a high redox potential, they are well-known antioxidants. They serve as metal-chelating agents, hydrogen donors, reducing agents, and singlet oxygen quenchers.[24] Hydroxycinnamic acids stand out among them due to their potent antioxidant action as well as a host of other biological benefits. They show a noticeable variety of biological activities and are often used as promising starting compounds for the manufacturing of new, highly effective drugs. Cinnamic acid derivatives are widespread in food sources.[60] Because of their many uses, cinnamic acid and its derivatives are frequently found in cosmetics. Both manmade and naturally occurring materials are included in this group.[61] The Cosmetic Ingredient Database and existing research indicate that cinnamic acid and its byproducts are widely used in cosmetics throughout the world. Cinnamic acid has anti-allergic and photoallergic effects that are well-known. Furthermore, in cosmetic compositions, its derivatives are important for UV protection.[47] Numerous beneficial and significant biological effects, such as those that are anti-inflammatory, anti-microbial, anti-cancerous, and antidiabetic, are demonstrated by these substances.[62] The research findings indicate that cinnamic acid is a phytoconstituent of great importance, exhibiting noteworthy antioxidant, antidiabetic, and antimicrobial actions with long-lasting inhibition.

3.3 Compound 3

Isolated compound from the bark of *E. acuminata* is a white amorphous powder. UV spectrum of isolated compound, which indicates characteristic absorption maximum at 289.65 nm for n→π* transition of C=O group; 240.52 nm represents π→π* transition for aromatic C=C, and 220.47 nm exists due to n→σ* transition for OH group.[63] The IR spectrum of the isolated compound confirms the presence of the OH group, having a stretching frequency at 3386.9 cm^{-1}, a broad absorption band representing intermolecular hydrogen bonding. Whereas the band at 2956.6 cm^{-1} shows asymmetric stretching of the aromatic moiety and the band at 2826.8 cm^{-1} was due to C-H stretching of the alkene.[64] Fig. S9 represents the FTIR spectrum of isolated compound 3 of *E. acuminata* bark. The absorption peaks positioned at 1675.3 cm^{-1} represent C=O acidic and 1607.5 for phenolic OH stretching. The band at 1594.0 cm^{-1} was due to the C-C aromatic ring stretching, the 1509.6 cm^{-1} band represents C=O aromatic stretching, and the 1447.3 cm^{-1} band depicts C=C aromatic stretching. Bending vibrations of phenolic OH were observed at 1364.0 cm^{-1}. The absorption bands at 1317.1 cm^{-1} and the bands at the lower frequencies between 930.1 cm^{-1} and 546.3 cm^{-1} were depicted as the C-H bending vibrations of aromatic hydrocarbons. The band at 1243.0 cm^{-1} represents the C-O stretching vibrations of phenols.[65] The ^{13}C-NMR spectrum showed the presence of seven carbon atoms. The Carbonyl group shows a signal at 167.13 ppm, and aromatic carbon exists at 161.56, 131.49, 121.11, and 115.08 ppm for C1, C2, C3, C4, C5, and C6, respectively.[66] Fig. S11 represents the ^{13}C-NMR spectrum of isolated compound 3 of *E. acuminata* bark.

The ^{1}H-NMR spectrum gave the peaks of the acidic proton at δ 12.4245 and the phenolic proton at δ 10.2198. Peakes present at δ7.8188, 7.7840, 6.8484, and 6.8133 showed the presence of an aromatic ring. Fig. S10 shows the ^{1}H-NMR spectrum of isolated compound 3 of *E. acuminata* bark. A single peak present at δ3.4250 indicates the presence of a proton at carbon, attached to the oxygen. Spectra showed a solvent peak observed at δ 2.5098, 3.425. The base peak observed at positive mode by mass spectrum is 139.02, and the base peak at negative mode is 137.02, confirming that the isolated compound has approximately 138 molecular weights. Fig. S12 shows the mass spectrum of isolated compound 3 of *E. acuminata* bark. Concluding all the data observed by different spectral techniques, it is analyzed that the studied plant *E. acuminata* bark contains 4-Hydroxybenzoic acid (Fig. 4).

The UV spectrum of compound 3 shows λ_{max} at 289.65 nm. Classic UV-vis spectra of phenolic acid include n→ π*, π→π* & n→ σ* transition due to the presence of C=O, C=C and OH groups. UV-vis spectrum of isolated compound 3 is also in good agreement with published UV spectra of phenolic acids.[31] IR spectrum exhibits peaks at 3386.9 cm^{-1} (OH) 2826.8 cm^{-1} (C-H stretching of aklene), 1675.3 cm^{-1} (C=O stretching), 1607.5 cm^{-1} (phenolic OH), 1509.6 cm^{-1} (C=O aromatic stretching), 1447.3 cm^{-1} (C=C aromatic stretching), 1364 cm^{-1} (bending vibration of OH) and 1240 cm^{-1} (C-O stretching vibration of phenol) reveals the functional groups and nature of bonding of isolated compound is similar to phenolic acid.[67] 13C-NMR spectrum gives 5 signals and the position of carbons of Para-hydroxy benzoic acid, isolated from the bark of *E. acuminata*. The peak observed at 167.13

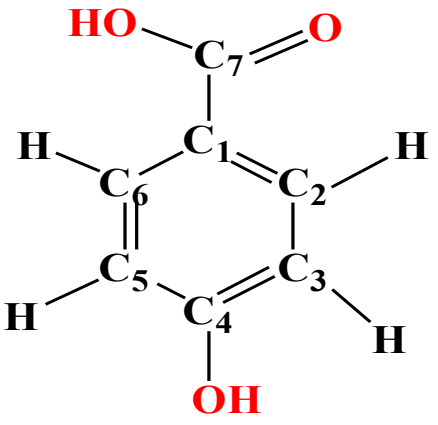

Fig. 4: Chemical structure of 4-Hydroxybenzoic acid.

showed the presence of an acidic group attached to C5. The peak observed at 161.56 positions showed the presence of a phenolic group at C4.[67] The ^{1}H-NMR spectrum of the isolated compound was recorded to depict four signals and the positions of protons. Doublets at 6.8484 ppm represent the presence of an aromatic proton at carbon 2. From the ^{1}H-NMR spectrum, the presence of the hydroxyl substitution at 4 was clearly indicated by the presence of one signal triplet at 10.2198 ppm. Another signal, as a doublet at 7.8188 ppm, represents a proton at carbon 3. The ^{1}H-NMR spectrum of the isolated compound showed the presence of an acidic proton at 12.314 ppm at carbon 7. More evidence in favour of the above structure accumulated from the mass spectrum.[68] The base peak observed at positive and negative modes confirms that the isolated compound has approximately 138 molecular weights.[51] The melting point of the compound was 215 °C.

Thus, an isolated compound from *E. acuminata* bark was identified as 4-Hydroxybenzoic acid, commonly known as p-hydroxybenzoic acid. Phenolic acids are a large class of structurally diverse compounds found in plants with known antioxidant, anti-inflammatory, antidiabetic, and antimicrobial properties.[69] These phytochemicals are common nutrients in the diet and are divided into different subclasses like phenolic acids, derivatives of benzoic acid, and cinnamic acid.[70] White and crystalline, benzoic acid finds application in a variety of industries, such as food preservation, cosmetics, and pharmaceuticals.[56] It is found in many plants naturally and serves as a transitional chemical in the synthesis of several secondary metabolites.[57] Derivatives of benzoic acid are frequently used as food preservatives, and benzoic acid itself is an essential building block for the synthesis of numerous other chemical compounds. Our study explored that p-hydroxybenzoic acid is an important phytoconstituent and has given significant results of antioxidant, antidiabetic, and antimicrobial activities with sustainable inhibition.

3.3 Biological and antioxidant activity

We conducted antioxidant assays using two distinct in-vitro methods: the ABTS assay and the DPPH assay.

3.3.1 ABTS assay

The $ABTS^{\cdot+}$ scavenging assay serves as a widely recognized method for quantifying total antioxidant activity. The outcomes of this assay are expressed as the percentage of inhibition in µg/mL. Notably, all three compounds isolated from E. acuminata, particularly compound 1, demonstrated significant results when compared to the standard antioxidants ascorbic acid and quercetin. However, it is worth highlighting that among the isolated compounds, Kaempferol, sourced from the leaves, exhibited the highest ABTS radical cation scavenging activity, surpassing the performance of other isolated compounds. Ascorbic acid and quercetin, in comparison, displayed IC_{50} values within the range of 25 and 8.01 µg/mL. Detailed results can be found in Fig. 5.

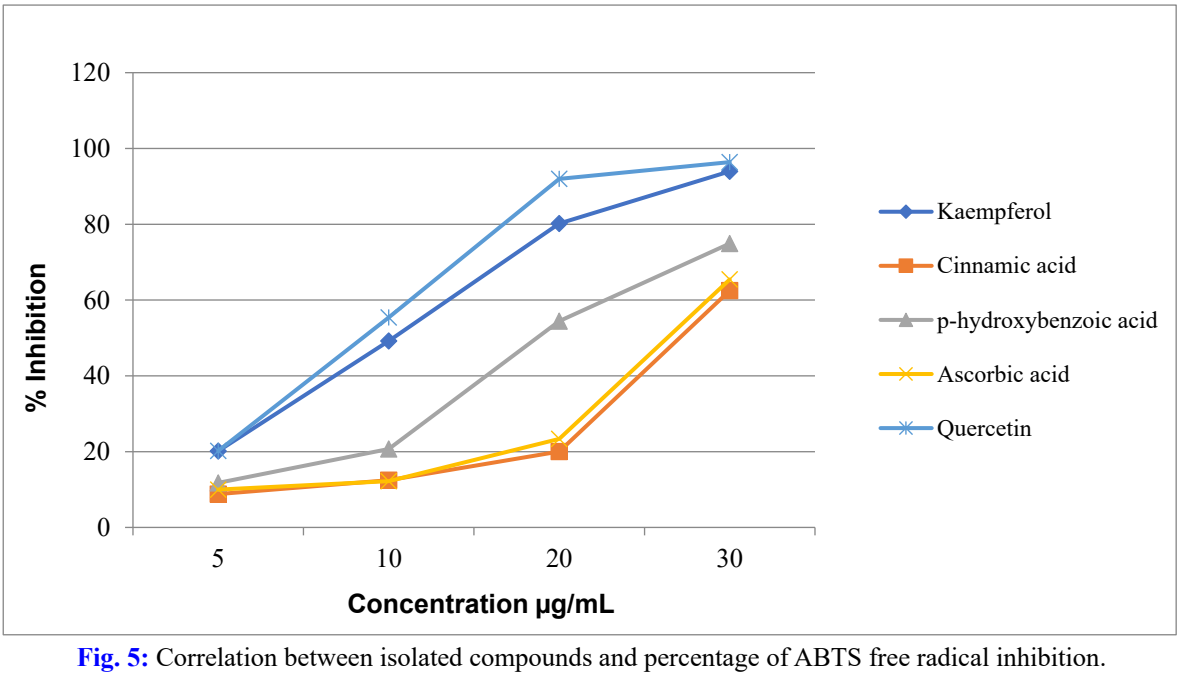

Fig. 5: Correlation between isolated compounds and percentage of ABTS free radical inhibition.

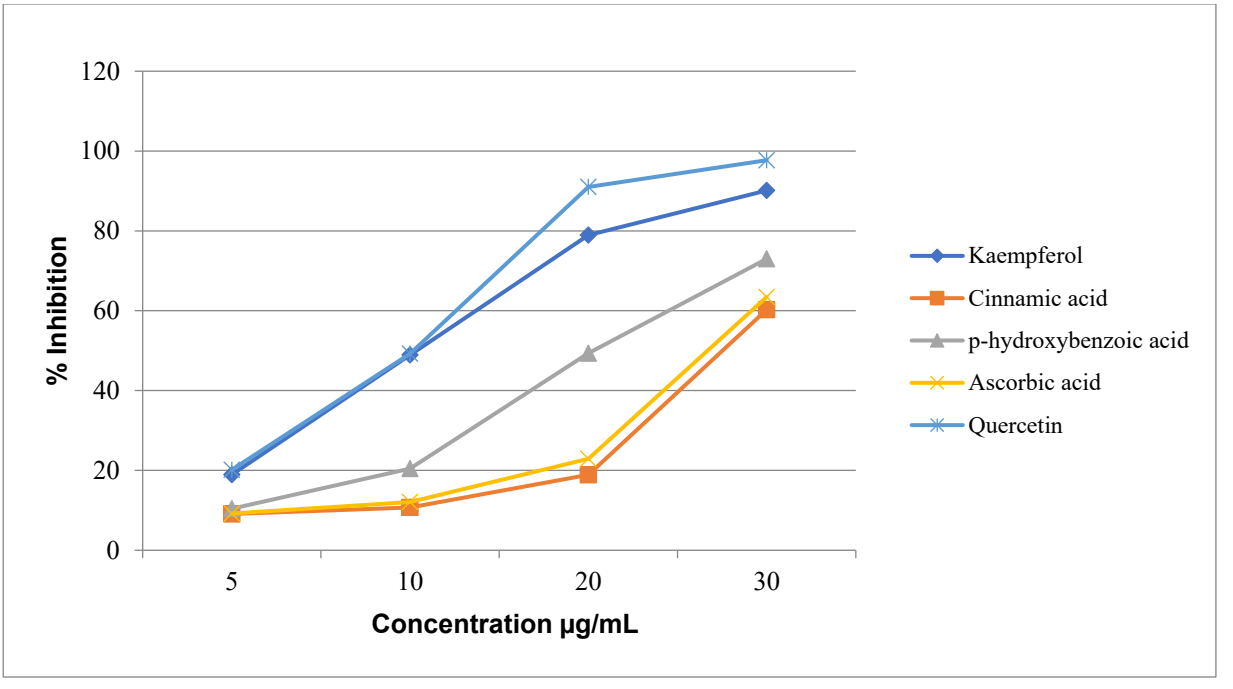

Fig. 6: Correlation between isolated compounds and percentage of DPPH free radical inhibition.

3.3.2 DPPH assay

The DPPH radical scavenging activity of the compounds isolated from various parts of E. acuminata, alongside the commercial antioxidants ascorbic acid and quercetin, was meticulously assessed, as illustrated in Fig. 6. Notably, when DPPH encounters proton radical scavengers, the initially purple solution swiftly transitions to a vibrant yellow hue. For all the isolated compounds, the assessment was conducted within a concentration range of 5-30 µg/mL, revealing a clear dose-dependent relationship in their activity. Notably, Kaempferol exhibited the most impressive performance, boasting the lowest IC_{50} value recorded at a mere 11.41 µg/mL.

3.3.3 Antidiabetic activity

In the α-amylase inhibitory assay, a standard curve for acarbose was meticulously constructed by employing a range of concentrations of this reference compound (refer to Fig. 7). Subsequently, all the isolated compounds were subjected to testing at varying concentrations of 25, 50, 100, and 250 µg/mL to assess their inhibitory potential against α-amylase. Remarkably, cinnamic acid and p-hydroxybenzoic acid demonstrated substantial inhibition, whereas Kaempferol

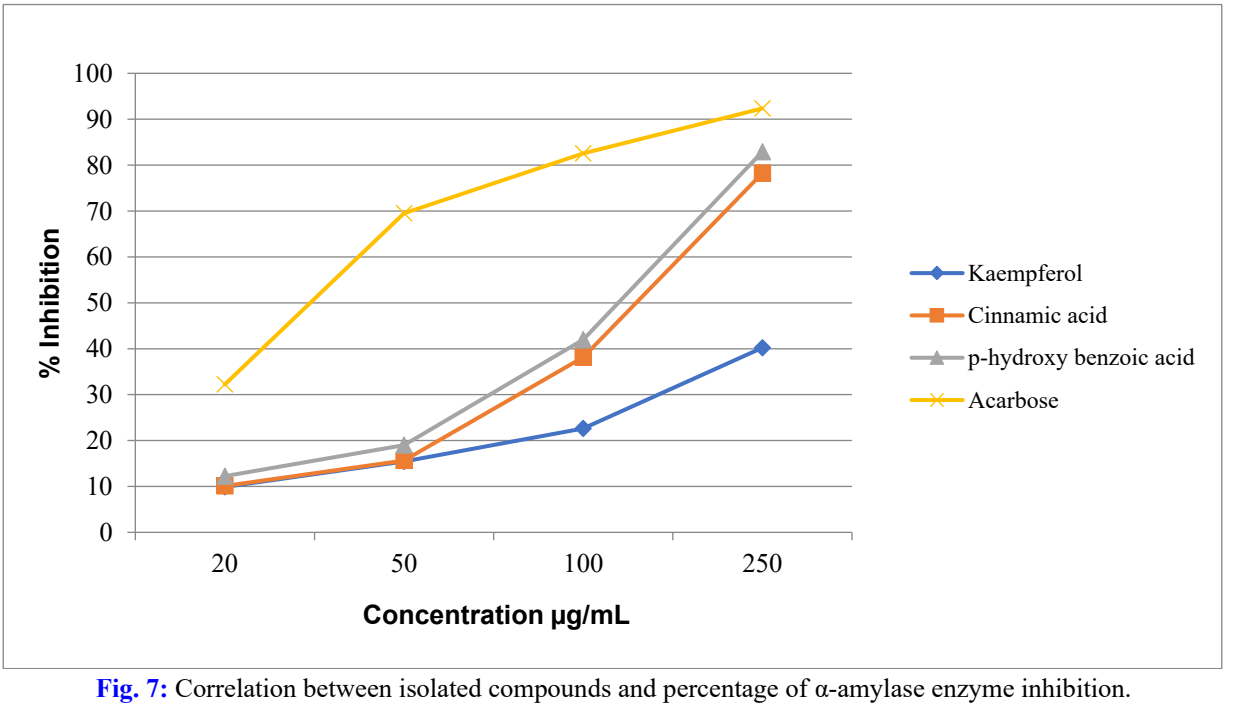

Fig. 7: Correlation between isolated compounds and percentage of α-amylase enzyme inhibition.

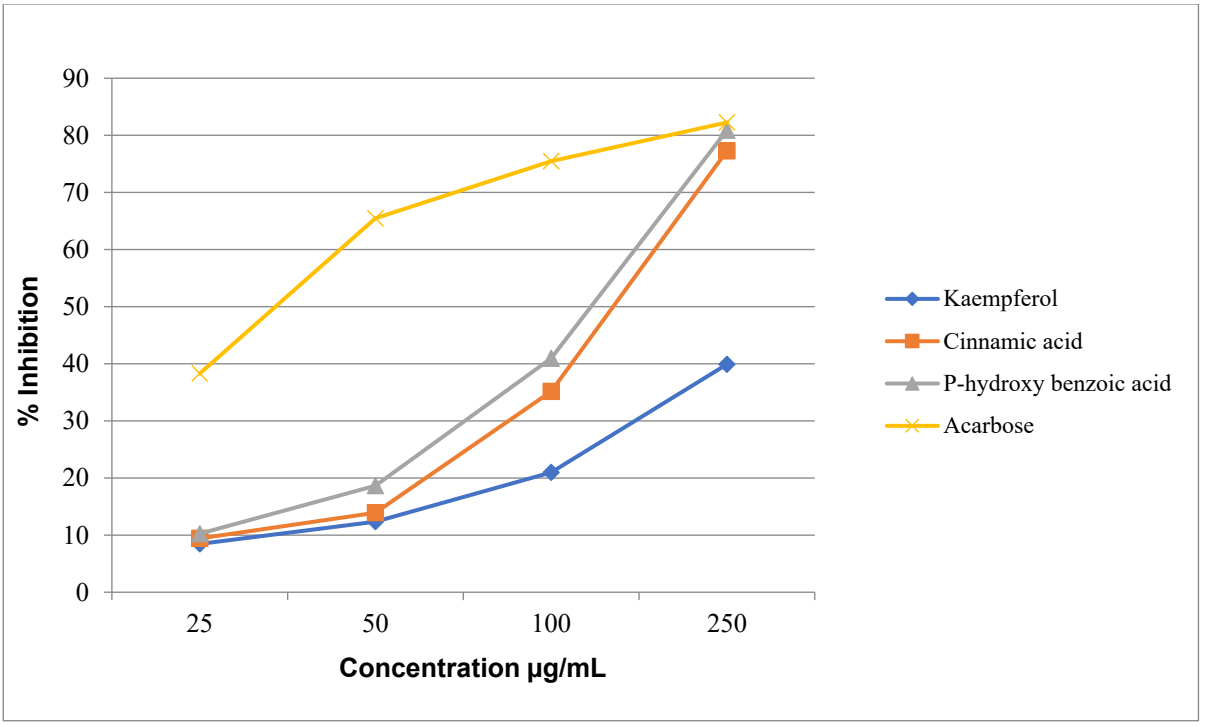

Fig. 8: Correlation between isolated compounds concentration and percentage of α-glucosidase enzyme inhibition.

exhibited the weakest inhibitory effect among the three compounds, with the highest IC_{50} value recorded at 275.40 µg/mL. Notably, the positive control, acarbose, exhibited a significantly lower IC_{50} value, indicating a robust inhibitory effect at 40.24%.

Fig. 8 visually depicts the in vitro α-glucosidase inhibitory activity of the compounds isolated from *E. acuminata* in comparison to acarbose. This illustration showcases the effect of varying compound concentrations on α-glucosidase inhibition, providing valuable insights into the IC_{50} values for each isolated compound and the standard, acarbose. Notably, the compound exhibiting the most robust α-glucosidase inhibitory activity was p-hydroxybenzoic acid, boasting an IC_{50} value of 120.2 µg/mL, followed closely by cinnamic acid with an IC_{50} of 135.38 µg/mL. Particularly impressive is the proximity of p-hydroxy benzoic acid's IC_{50} to that of acarbose (38.44 µg/mL), underscoring its remarkable potency. Consequently, these phytoconstituents hold promise as potential antidiabetic agents, offering an alternative to synthetic drugs fraught with side effects such as abdominal discomfort, bloating, flatulence, and diarrhea.[30]

Pytoconstituents's antibacterial potential is examined by disrupting bacterial cell penetrability and infiltration inside the bacterial cell. Once in interaction with the cytoplasm, secondary metabolites can disrupt the mitochondria's initial function, resulting in the generation of ROS (Reactive Oxygen Species). ROS causes a wider spectrum of irreversible cell damage. These hydroxyl radicals (HO_2, OH, and O^{2-}) penetrate the cell membrane and damage cells.[71] The antimicrobial efficacy of the isolated compounds was assessed against a range of foodborne pathogens, including Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Listeria monocytogenes, Salmonella typhi, Listeria candida, and the fungus Candida albicans. Norfloxacin served as the positive control in this evaluation. Remarkably, all the compounds demonstrated potent antibacterial activity against both gram-negative bacteria (E. *coli, P. aeruginosa, L. monocytogenes, S. typhi*) and gram-positive bacteria (*S. aureus*), as well as the pathogenic fungus *Candida albicans*. Among the tested samples, Kaempferol exhibited the highest antibacterial activity, producing an impressive 22 mm inhibition zone against *Escherichia coli*. These findings underscore the substantial antimicrobial potential of Kaempferol, as well as the reference antibiotic Norfloxacin. The observed antimicrobial effects of these compounds can likely be attributed to their flavonoid and phenolic acid constituents. Extensive literature reports highlight that plants containing flavonoids and phenolic acids often exhibit antimicrobial properties. [72-74] Notably, Cushnie and Lamb have conducted a comprehensive review of the antimicrobial activity associated with flavonoids. Consequently, the antibacterial activity observed across all compounds may be attributed to the presence of diverse phenolic groups inherent in the plant.

4. Conclusion

This present study represents the inaugural revelation that compound 1 stands as the flavonoid 3,4′,5,7-tetrahydroxyflavone, more commonly known as Kaempferol, while compound 2 is identified as 3-Phenylprop-2-enoic acid, also recognized as cinnamic acid. These discoveries were extracted from the leaves of E. acuminata. Furthermore, compound 3 emerges as 4-hydroxybenzoic acid or p-hydroxybenzoic acid, isolated from the bark of E. acuminata.

The pharmacological efficacy of medicinal plant-based drugs hinges upon the presence of vital phytoconstituents such as flavonoids and phenolic acids. Therefore, the isolation and meticulous characterization of these bioactive phytoconstituents assume paramount significance in the realm of alternative medicine. Kaempferol has exhibited a plethora of remarkable antioxidant and antimicrobial activities, while both cinnamic acid and p-hydroxybenzoic acid have demonstrated distinct antioxidant, antidiabetic, and antimicrobial properties. These findings not only expand upon prior research but also furnish invaluable scientific insights into this traditional plant, setting the stage for further investigations. Presently, ongoing studies are being conducted to evaluate the in vivo biological activities and clinical trials of these isolated compounds, propelling us closer to harnessing their full potential.

Acknowledgments

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Conflict of Interest

There is no conflict of interest.

Supporting Information

Applicable.

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