

# Spectroscopic Characterisation of Bioactive Constituents Isolated from *Ludwigia Palustris* (L.) Elliott

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## ABSTRACT

*Ludwigia palustris* (L.) Elliott is an ethnomedicinal aquatic macrophyte with documented antioxidant and antibacterial activities in crude extracts, yet spectroscopically confirmed characterization of its individual phytoconstituents has remained absent from the literature. The present study reports, to the best of our knowledge, the first isolation and structural elucidation of bioactive compounds from the chloroform and ethanol extracts of this species. Successive solvent extraction followed by thin-layer chromatography (TLC)-guided column chromatography yielded four compounds in quantities adequate for complete spectroscopic characterization. Structural identities were established by FT-IR, LC-MS (ESI<sup>+</sup>/ESI<sup>-</sup>, ACCUCORE C18), <sup>1</sup>H NMR, and <sup>13</sup>C NMR (Bruker AVANCE NEO, 500/125 MHz), and purity was confirmed by melting point determination. The isolated compounds were identified as: rutin (quercetin-3-O-rutinoside, C<sub>27</sub>H<sub>30</sub>O<sub>16</sub>, mp 194–196°C), quercetin (3,3',4',5,7-pentahydroxyflavone, C<sub>15</sub>H<sub>10</sub>O<sub>7</sub>, mp 314–317°C), ethyl gallate (ethyl 3,4,5-trihydroxybenzoate, C<sub>9</sub>H<sub>10</sub>O<sub>5</sub>, mp 150–152°C), and oleanolic acid (3β-hydroxyolean-12-en-28-oic acid, C<sub>30</sub>H<sub>48</sub>O<sub>3</sub>, mp 308–310°C). Quercetin was identified as a compound common to both extracts, confirmed by co-TLC, FT-IR, and melting point comparison. A comprehensive literature search across SciFinder, PubMed, Reaxys, and Google Scholar confirmed no prior spectroscopic isolation reports for these compounds from *L. palustris*. DPPH radical scavenging activity of the pure isolated compounds followed the order: ethyl gallate (IC<sub>50</sub> 14.16 μg/mL) > quercetin (IC<sub>50</sub> 16.27 μg/mL) > rutin (IC<sub>50</sub> 19.10 μg/mL) > oleanolic acid (IC<sub>50</sub> 33.53 μg/mL), with ascorbic acid as positive control (IC<sub>50</sub> 12.94 μg/mL). The identification of flavonol glycosides, a phenolic ester, and an oleanane-type triterpenoid establishes the first chemotaxonomic profile for this species within the genus *Ludwigia* and provides a molecular basis for its previously documented bioactivities.

**KEYWORDS:** *Ludwigia palustris*; Rutin; Quercetin; Ethyl gallate; Oleanolic acid; Column chromatography; Onagraceae; Chemotaxonomy; Pentacyclic triterpenoid

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## 1.0 INTRODUCTION

In terms of the genus *Ludwigia*, which comprises over 82 species, the exploration of its phytochemistry and therapeutic potential has only been done in about 16 species. These findings suggest that flavonols and triterpenoids constitute conserved metabolic features within the genus *Ludwigia*, prompting the hypothesis that *L. palustris* may exhibit a comparable yet undocumented secondary metabolite pattern. However, only a limited number of species within the genus have been investigated at the compound and mechanistic levels <sup>1</sup>.

*Ludwigia palustris* (L.) Elliott is a marshy herb that grows in both emergent and submerged forms and has been traditionally used in folk medicine. While preliminary phytochemical screenings and biological

evaluations have indicated promising antioxidant and antibacterial activities of its crude extracts <sup>2</sup>, comprehensive isolation and structural characterization of individual constituents from this species remain scarce. Despite preliminary phytochemical screenings of *Ludwigia palustris*, the absence of isolated, spectroscopically confirmed marker compounds has prevented its meaningful placement within the chemotaxonomic framework of the genus *Ludwigia* and limited mechanistic interpretation of its reported bioactivities.

*Ludwigia* species are rich in flavonols, largely quercetin and its glycosides (Quercetin-3-O-arabinoside, -glucoside, and -rutinoside) reported from *L. adscendens*, *L. perennis* and *L. stolonifera*. These compounds exhibit strong antioxidant and anti-inflammatory effects and

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likely contribute significantly to the genus's pharmacological profile<sup>3,4</sup>. Phytosterols like  $\beta$ -sitosterol and its glucoside occur in *L. hyssopifolia* and *L. abyssinica* found to have synergistic effect and good antibacterial activities<sup>5, 6</sup>. Triterpenoids are also represented within the genus; lupane-type compounds, including betulin and betulinic acid, have been isolated from *L. adscendens*, while oleanane-type metabolites, specifically oleanolic acid and ursolic acid have been reported from *L. octovalvis* and *L. leptocarpa*<sup>7-9</sup>. However, no triterpenoid constituents have been documented for *L. palustris* to date.<sup>1</sup>

In this context, the present study was commenced to perform a systematic phytochemical investigation of *L. palustris* using successive solvent extraction, TLC-guided fractionation, and wet-column chromatographic isolation, followed by comprehensive structural elucidation using advanced FT-IR, LC-MS, <sup>1</sup>H-NMR, and <sup>13</sup>C-NMR techniques. The study aims to identify and characterize major phytoconstituents, provide chemotaxonomic insights into the species, and establish a molecular basis for the previously reported biological activities of *L. palustris*.

## 2.0 MATERIAL AND METHODS

### 2.1 Collection and authentication of Plant:

*Ludwigia palustris* (L.) Elliott was procured from Green Park Complex, Kuravilangad, Kerala, India. The plant material was authenticated by Dr. K. Singh, Taxonomist, Dev Sanskriti Vishwavidyalaya, Haridwar (Uttarakhand) based on exomorphic characters, chemical reactions, and review of the appropriate literature. A voucher specimen (No. DSVV/MPS/GEN/2023/03/D.O. 0039) was deposited in the Herbarium of the Department of Medicinal Plant Sciences, Dev Sanskriti Vishwavidyalaya, Rishikesh Road, Haridwar, Uttarakhand, India.

Plant collection was conducted in compliance with the Biological Diversity Act (2002), Government of India, and applicable state biodiversity regulations of Kerala. No protected or Schedule I species were collected. The plant material is an invasive aquatic macrophyte not listed under any threatened species convention.

### 2.2 Extraction of plant Material

300 grams of whole plant was pulverised into coarse powder and then successively extracted in soxhlet apparatus with petroleum ether, chloroform, ethyl acetate, acetone and ethanol (95%) respectively in increasing polarity order<sup>10</sup>. The extracts were collected, dried under reduced pressure using a Rota-vapor (Heidolph, Heizbad, Laborota 4001, Germany, 2000). Solvents were used from CDH-fine chemicals. The extracts were stored at 4 degrees Celsius for future use<sup>11</sup>.

### 2.3 Thin Layer chromatography of extracts

Thin-layer chromatography (TLC) analyses were performed using Merck precoated silica gel GF<sub>254</sub> aluminium plates. All solvents employed were of analytical grade (CDH-Fine Chemicals). For flavonoid

profiling, chromatograms were developed using two solvent systems, namely Chloroform: Acetone: Formic acid (60:30:10) and ethyl Acetate: Water: Formic acid (80:10:10). Phenolic constituents were resolved using Toluene: Ethyl acetate (60:40) and Toluene: Acetone (70:30) solvent systems, which yielded well-defined and distinct spots. The developed plates were visualized under visible light, short-wavelength UV (254 nm), and long-wavelength UV (365 nm), followed by derivatization with vanillin-sulfuric acid reagent. The number of spots was recorded, and retention factor (Rf) values were calculated using the standard formula<sup>12,13</sup>.

### 2.4 Isolation of Compounds using column chromatography

Silica gel 60-120 mesh was used as the stationary phase for column chromatography. Analytical-grade solvents were used throughout the study. Chromatographic monitoring was performed using precoated silica gel 60 F<sub>254</sub> TLC plates.

The dried plant material was successively extracted by Soxhlet extraction using petroleum ether, chloroform, ethyl acetate, acetone, and ethanol. Solvents were removed under reduced pressure to obtain the corresponding crude extracts. Based on TLC profiling and previously reported bioactivity, 10 g of the chloroform extract and 45 g of the ethanol extract were selected for further fractionation<sup>2</sup>.

Column chromatography was carried out on a glass column (25 mm × 60 cm) packed by the wet slurry method, with samples applied by dry loading. The chloroform extract was eluted using a gradient of hexane-ethyl acetate (95:5 to 50:50), while the ethanol extract was eluted with dichloromethane-methanol (95:5 to 50:50). Fractions were collected at intervals of 100-150 mL.

All fractions were analyzed by TLC, rendering the isolation process TLC-guided. Plates were observed under UV light (254 and 365 nm) and post-derivatized with vanillin-sulfuric acid reagent followed by heating. Fractions displaying similar TLC profiles were pooled and concentrated to yield isolated compounds, the isolated compounds were also recrystallized to increase the purity, then melting point was recorded for testing the purity of the isolated compounds.<sup>14-18</sup>

### 2.5 Spectral analysis of Isolated compounds:

Total four compounds were procured in significant amount to study the spectrometry, the samples were named S1, S2, S3 and S4, Compounds S1, S2, and S3 were isolated from the ethanol extract, while S2 and S4 were isolated from the chloroform extract. The identity of compound S2 as quercetin in both extracts was confirmed by co-TLC, Melting point and by direct comparison of IR spectral data, thereby demonstrating that quercetin is distributed across polarity fractions in *L. palustris*. Two additional fractions were obtained in quantities insufficient for complete spectroscopic characterization and were therefore excluded from structural analysis. All

four samples were then employed for following techniques:

- **FT-IR:** FT-IR spectra were recorded using a PerkinElmer Spectrum instrument (Version 10.03.05) over the range 4000-450  $\text{cm}^{-1}$  at a data interval of 1  $\text{cm}^{-1}$  (3551 data points per spectrum). All four samples were analysed by a single analyst. Spectra were collected in %T (percent transmittance) mode.
- **LC-MS:** LC-MS analysis was performed on an ACCUCORE C18 column (150×4.6×2.6  $\mu\text{m}$ ) using a water–acetonitrile gradient mobile phase. Electrospray ionisation in both positive (ESI<sup>+</sup>) and negative (ESI<sup>-</sup>) modes was acquired simultaneously on Waters Alliance e2695/HPLC-TQD Mass spectrometer. UV detection was performed in parallel. Data are reported as base peak ion (BPI) chromatograms with mass spectra extracted at the relevant retention times
- **NMR (<sup>1</sup>H and <sup>13</sup>C):** <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were recorded on a Bruker AVANCE NEO 500 MHz instrument (<sup>1</sup>H: 500 MHz; <sup>13</sup>C: 125 MHz) at 298 K. Solvents: S1 in CD<sub>3</sub>OD; S2 and S3 in DMSO-d<sub>6</sub>; S4 in CDCl<sub>3</sub>. Chemical shifts ( $\delta$ ) are reported in ppm relative to TMS; coupling constants (J) in Hz. <sup>13</sup>C NMR spectra were acquired using the zgpg30 pulse program (broadband proton-decoupled) with 1024 scans.

## 2.6 determination of DPPH free radical scavenging activity:

The DPPH radical scavenging activity of the isolated compounds was tested according to the technique described in the paper, with slight modifications. Stock solutions of each sample (S1–S4) were prepared in DMSO and then diluted with ethanol to obtain working concentrations of 10, 20, 30, 40, and 50  $\mu\text{g}/\text{mL}$ . Each concentration (0.5 mL) was combined with 1.5 mL of freshly prepared DPPH solution (1 mM in ethanol) in a 1.5 mL reaction container. Reaction mixtures were incubated in the dark at room temperature for 30 minutes, and absorbance was measured at 517 nm against a blank using a UV-Vis spectrophotometer. Ascorbic acid was utilised as the positive control at the same concentrations. All tests were performed in triplicate (n = 3), and findings are reported as mean  $\pm$  SD. The % radical scavenging activity was estimated using the formula <sup>19</sup>:

$$\% \text{ Scavenging} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

where A<sub>control</sub> is the absorbance of DPPH solution without sample and A<sub>sample</sub> is the absorbance of the reaction mixture containing the test compound. The IC<sub>50</sub> value (concentration required to scavenge 50% of DPPH radicals) was determined from the dose-response curve by linear regression analysis using GraphPad Prism.

## 3.0 RESULTS

**3.1 Extraction of the plant material:** Table 1 is arranged in decreasing yield of extracts.

Solvent Extract	Yield (%)	Weight (g)	Colour	Consistency
Ethanol (95%)	15.1	45.0	Dark Brown	Dry and dense
Acetone	7.3	21.9	Brownish green	Dry and non-sticky
Petroleum ether	4.6	13.8	Dark green	Sticky and fatty
Chloroform	3.3	10.0	Dark green	Sticky and dense
Ethyl acetate	3.0	9.0	Light green	Dry and non-sticky

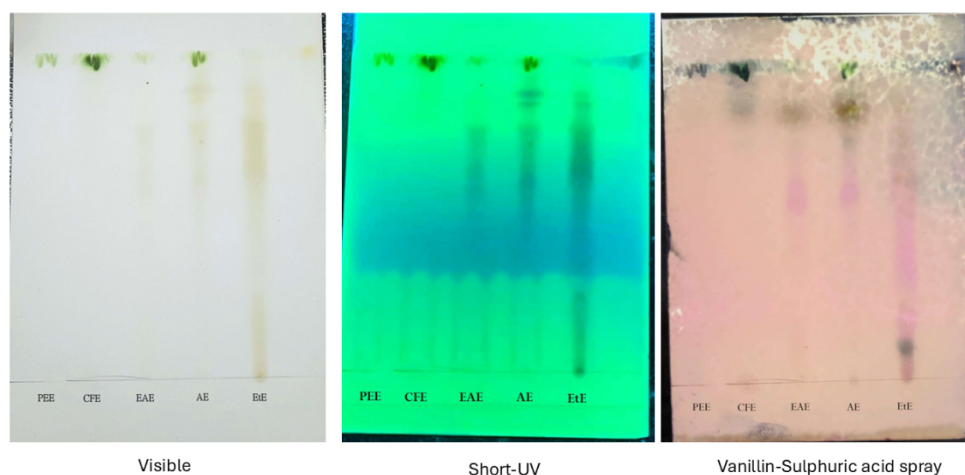
**Table 1:** Characteristics of Extracts

## 3.2 Thin Layer Chromatography (TLC) of extracts

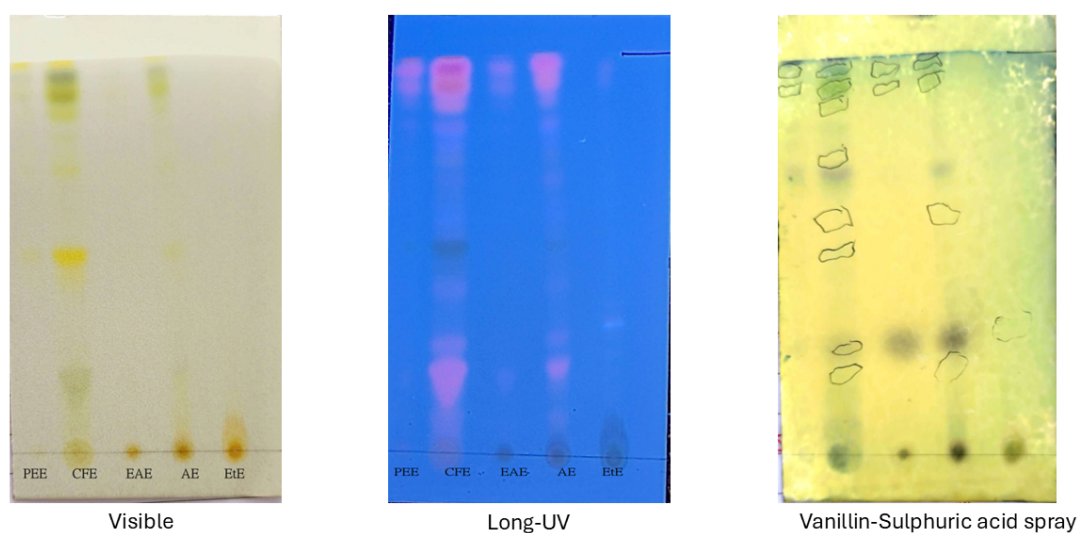
Thin-layer chromatography (TLC) analysis revealed the presence of multiple flavonoids and phenolic constituents in the extracts. Using Ethyl acetate: Water: Formic acid (80:10:10), six flavonoid spots were resolved, with spots sharing identical R<sub>f</sub> values considered common.

For phenolics and chalcones, the solvent system Toluene: Ethyl acetate (60:40) resolved thirteen distinct spots, while toluene: acetone (70:30) yielded ten spots.

The detected spots for flavonoids showed R<sub>f</sub> values ranging from approximately 0.10 to 0.88 across the different solvent systems, and phenolics showed R<sub>f</sub> values ranging from approximately 0.20 to 0.94 indicating the presence of phytoconstituents with varying polarity Figure 1 and 2 below shows the spots detected.



**Figure 1: TLC of Flavonoids in Ethyl acetate: Water: Formic acid (80:10:10)**



**Figure 2: TLC of Phenols/Chalcone in Toluene: Ethyl acetate (60:40)**

### 3.3 Isolation of compounds using column chromatography:

Column chromatographic separation produced a total of six distinct fractions. Two additional fractions (designated S5 and S6) were obtained in approximate quantities of 2 mg each, insufficient for complete spectroscopic characterization. Co-TLC analysis confirmed both fractions are distinct from S1–S4 in all solvent systems tested. Both gave positive ferric chloride tests confirming phenolic character; ninhydrin tests were negative. These fractions warrant future scale-up investigation.; Four compounds were isolated in quantities adequate for full FT-IR, LC-MS, and NMR

characterization and were code-named as S1, S2, S3, and S4.

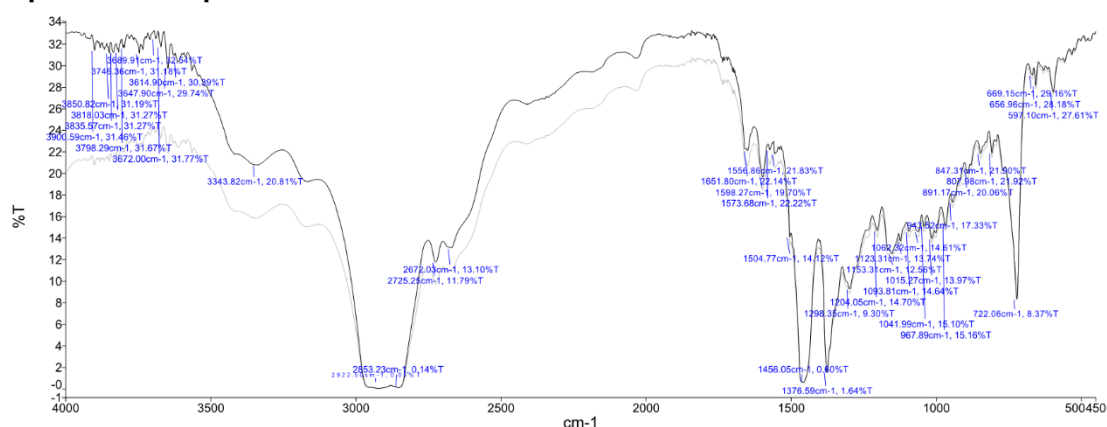
Fractions of 100 mL were collected and monitored by TLC. In the ethanol extract column, quercetin (S2) eluted earlier in the gradient, followed by rutin (S1) at higher methanol concentrations, and ethyl gallate (S3) at the most polar gradient compositions. In the chloroform extract column, oleanolic acid (S4) eluted in the early hexane-rich fractions, while quercetin (S2) required higher ethyl acetate proportions. The identity of quercetin across both extracts was confirmed by co-TLC, FT-IR, and melting point comparison, establishing it as a compound common to both polarity fractions. The physical characteristics and solubility profiles of the isolated compounds are summarized in Table 2:

S.n.	Compound code name	Colour	Consistency	Solubility	Weight (mg)	% Yield	Melting point
1.	S1	Yellowish Green	Solid Crystalline Powder, slightly lumpy	Polar	42	0.093%	194-196°C

2.	S2	Yellow	Solid Crystalline Powder	Mid-Polar	22	0.22%	314–317°C
3.	S3	White	Crystalline solid Powder	Mid-Polar	31	0.069%	150–152°C
4.	S4	Bright White	crystalline solid Powder	Non-Polar	17	0.17%	308–310°C

**Table 2:** characteristics of the isolated compounds**3.4 Spectrometric Analysis of Isolated compounds:****3.4.1 Interpretation of sample S1:****3.4.1.1 FT-IR**

A broad O–H band at  $3343\text{ cm}^{-1}$  indicated extensive hydrogen bonding from phenolic and glycosidic hydroxyl groups. The flavonol C-4 carbonyl appeared at  $1651\text{ cm}^{-1}$ , shifted  $\sim 45\text{ cm}^{-1}$  higher than the aglycone (S2), consistent with glycosidic electron withdrawal<sup>20,21</sup>. Aromatic C=C bands occurred at  $1598\text{--}1556\text{ cm}^{-1}$ <sup>22</sup>. Multiple absorptions at  $1204\text{--}1093\text{ cm}^{-1}$  confirmed glycosidic C–O–C linkages<sup>23</sup>.

**Spectrum Graph****Figure 3:** IR spectra of Sample S1**3.4.1.2 <sup>1</sup>H NMR (CD<sub>3</sub>OD, 500 MHz)**

The chelated 5-OH proton persisted at  $\delta$  12.32 ppm, confirming the C-5/C-4 intramolecular hydrogen bond characteristic of 5-hydroxyflavones<sup>24,25</sup>.

The B-ring showed an ABX system ( $\delta$  7.62–7.67, 6.88 ppm;  $J = 8.5, 2.0\text{ Hz}$ ) consistent with a 3',4'-dihydroxy pattern. A-ring meta-coupled doublets at  $\delta$  6.38 and 6.20 ppm ( $J = 2.0\text{ Hz}$ ) confirmed the 5,7-dioxygenated substitution<sup>26,27</sup>.

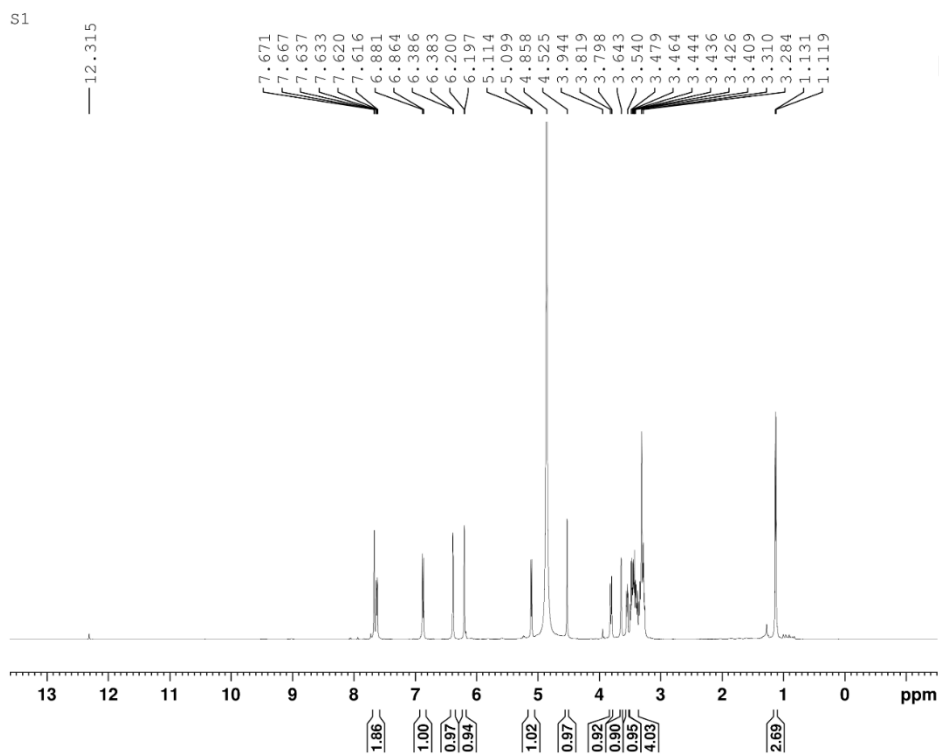
Sugar moieties were established by:

$\beta$ -glucose anomeric proton at  $\delta$  5.10 (d,  $J = 7.8\text{ Hz}$ )

$\alpha$ -rhamnose anomeric proton at  $\delta$  4.53–4.86 (d,  $J \approx 1.5\text{ Hz}$ )

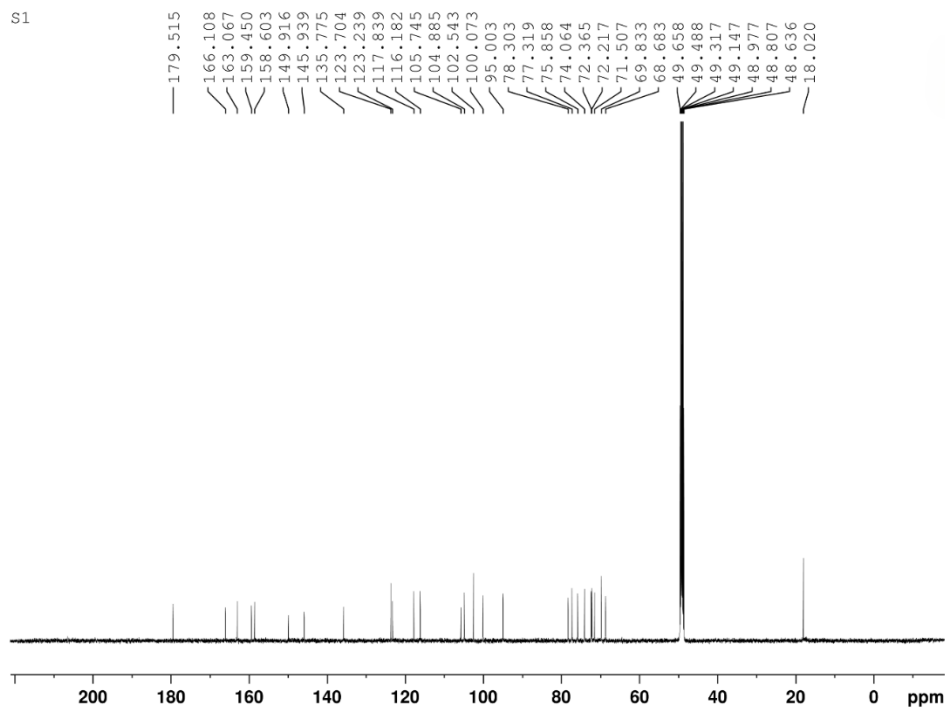
Diagnostic rhamnose methyl doublet at  $\delta$  1.12 (3H, d,  $J = 6.2\text{ Hz}$ )<sup>28–30</sup>

This methyl signal unambiguously differentiates rutin from monoglycosides

Figure 4:  $^1\text{H}$  NMR Spectra of S1

### 3.4.1.3 $^{13}\text{C}$ NMR ( $\text{CD}_3\text{OD}$ , 125 MHz)

The C-4 carbonyl resonated at  $\delta$  179.5 ppm. Two anomeric carbons appeared at  $\delta$  100.1 (C-1'') and 95.0 ppm (C-1''').<sup>28</sup> Sugar carbons were observed at  $\delta$  69–78 ppm, and the rhamnose methyl carbon at  $\delta$  18.0 ppm. All shifts matched reported data for rutin<sup>30–32</sup>.

Figure 5:  $^{13}\text{C}$  NMR Spectra of S1

### 3.4.1.4 LC-MS

RT 6.9–7.0 min.

ESI<sup>+</sup>:  $[\text{M}+\text{H}]^+$  m/z 611.1

ESI<sup>-</sup>: [M-H]<sup>-</sup> m/z 609.2

Fragmentation sequence: 611 → 465 (-146, rhamnose) → 303 (-162, glucose) → 273 (-CO).

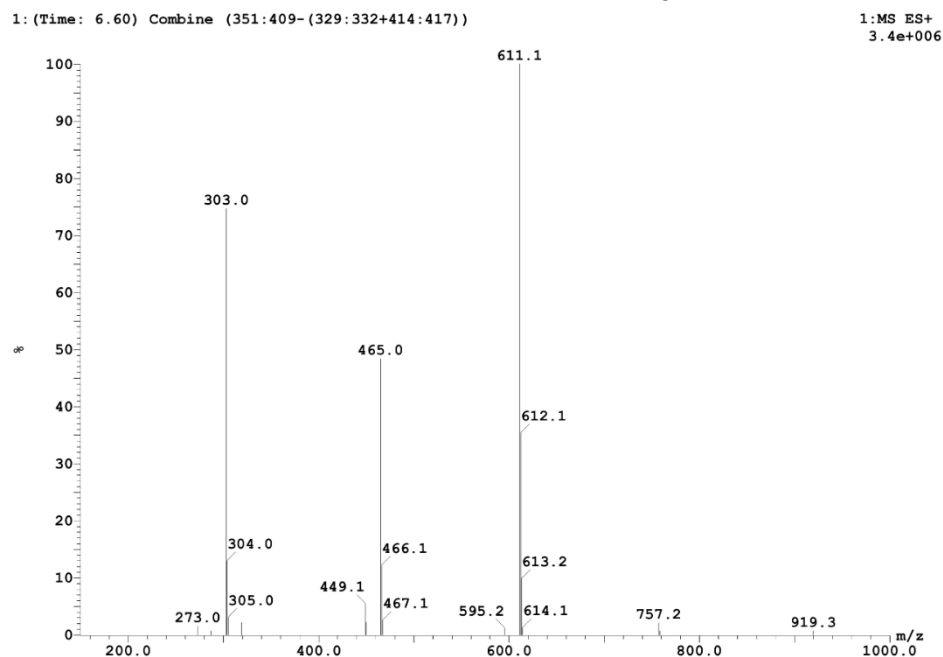
This sequential neutral loss confirms a rutinoside disaccharide attached at C-3<sup>33-35</sup>.

Figure 6: LC-MS Spectra of S1

S1 was identified as Rutin

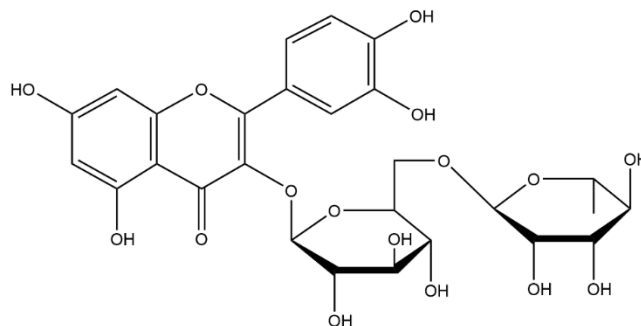
3,3',4',5,7-pentahydroxyflavone 3-O-[ $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 6)- $\beta$ -D-glucopyranoside]; C<sub>27</sub>H<sub>30</sub>O<sub>16</sub>; MW = 610.52 g/mol

Figure 7: Chemical Structure of Rutin

### 3.4.2 Interpretation of sample S2:

#### 3.4.2.1 IR

Broad O-H stretch at 3334 cm<sup>-1</sup>. The C-4 carbonyl appeared at 1606 cm<sup>-1</sup>, ~45 cm<sup>-1</sup> lower than S1, reflecting enhanced conjugation in the free aglycone<sup>20</sup>. Absence of glycosidic C-O-C bands confirmed lack of sugar moiety<sup>23</sup>.

## Spectrum Graph

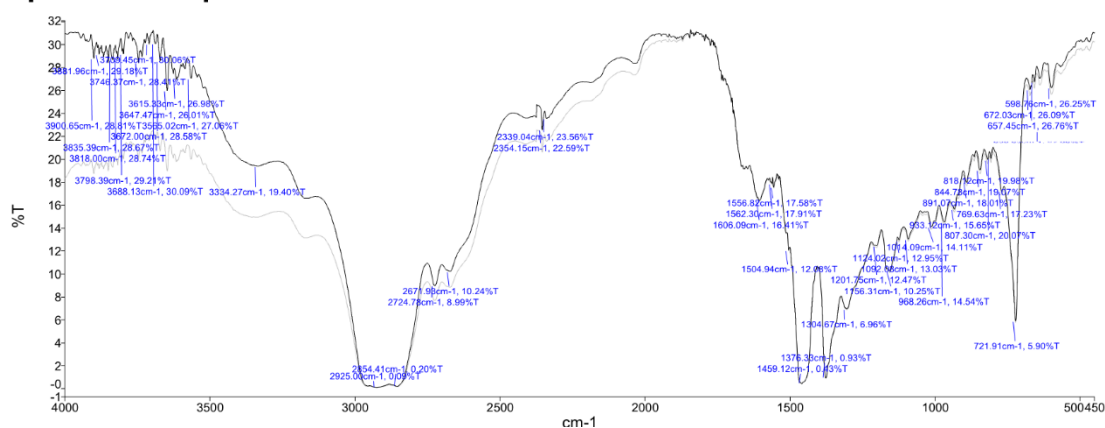


Figure 8: IR spectra of S2

3.4.2.2 <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 500 MHz)

All five phenolic OH protons were observed:

5-OH at  $\delta$  12.59 ppm

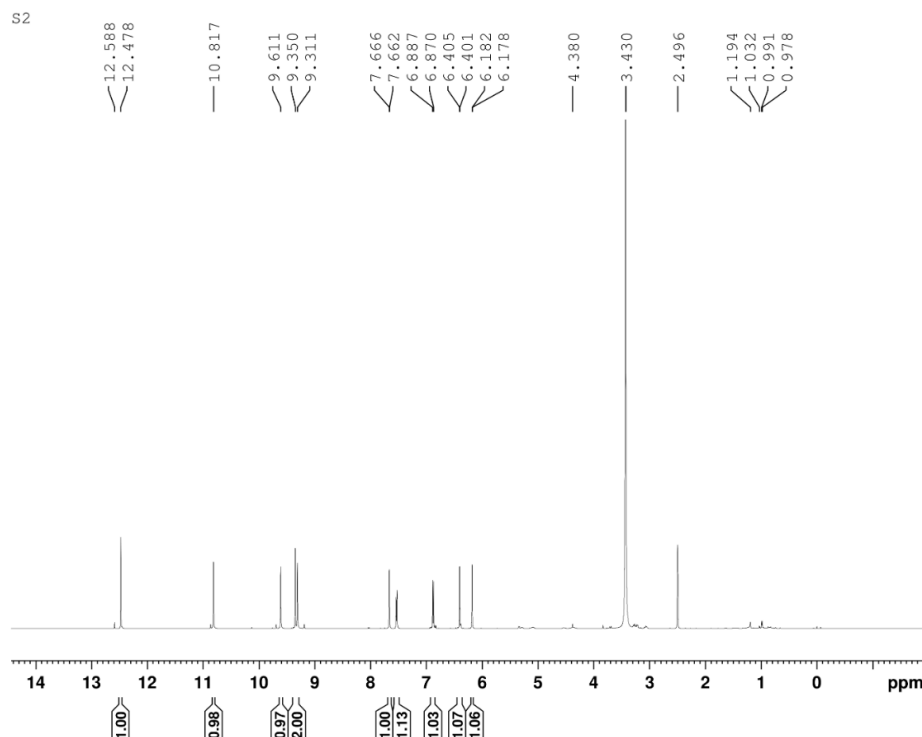
4'-OH at  $\delta$  10.82 ppm

3-OH at  $\delta$  9.61 ppm

7-OH and 3'-OH at  $\delta$  9.35–9.31 ppm<sup>26, 36</sup>

The aromatic ABX system ( $\delta$  7.67, 6.89 ppm) confirmed the catechol B-ring. Meta-coupled A-ring protons appeared at  $\delta$  6.40 and 6.18 ppm<sup>26</sup>.

Absence of anomeric or methyl sugar signals clearly established the aglycone structure.

Figure 9: <sup>1</sup>H-NMR spectrum of S23.4.2.3 <sup>13</sup>C NMR (DMSO-d<sub>6</sub>, 125 MHz)

Fifteen carbons were observed. C-4 resonated at  $\delta$  175.9 ppm. Oxygenated aromatic carbons appeared at  $\delta$  145–164 ppm. A-ring methines at  $\delta$  98.3 and 93.4 ppm; B-ring methines at  $\delta$  115–122 ppm, fully consistent with quercetin<sup>31, 37</sup>.

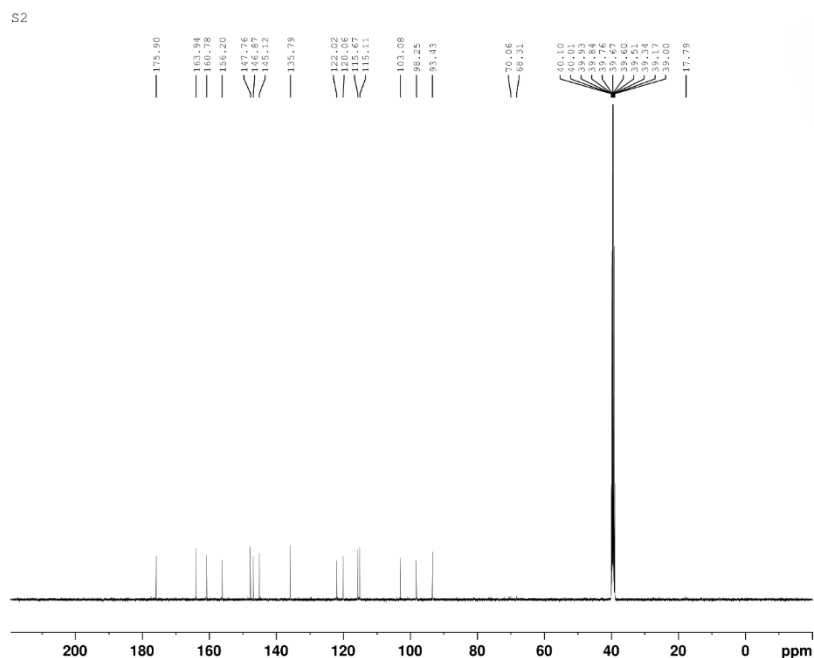


Figure 10: <sup>13</sup>C-NMR Spectra of S2

### 3.4.2.4 LC-MS

RT 11.0–11.1 min (later than S1, consistent with higher lipophilicity).

ESI<sup>+</sup>: m/z 303.0

ESI<sup>-</sup>: m/z 301.0 (stronger signal, typical for polyphenols)<sup>34, 38</sup>

RDA fragments at m/z 179 and 151 confirmed a 3',4'-dihydroxy B-ring<sup>35</sup>.

1: (Time: 6.41) Combine (340:398-(324:327+421:424))

1: MS ES+  
5.5e+005

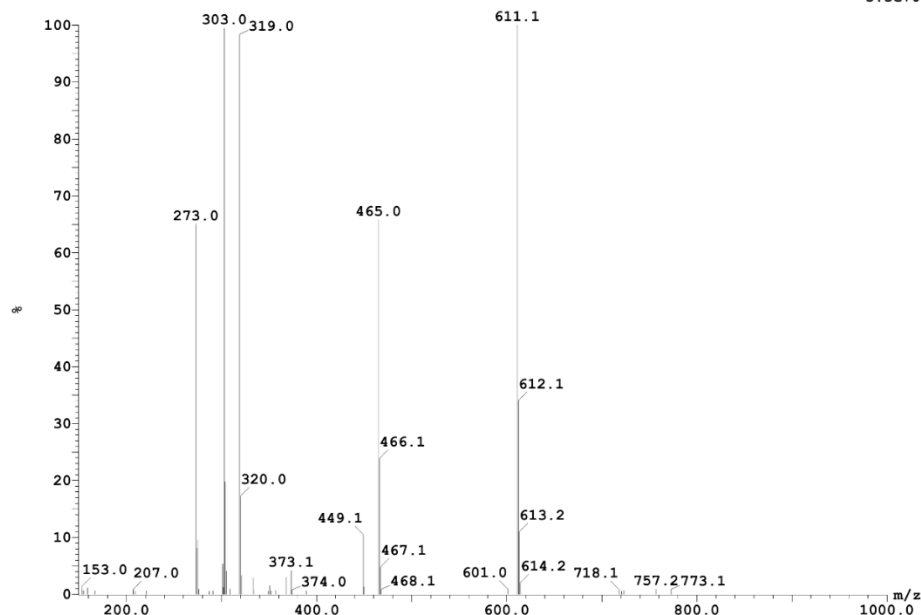
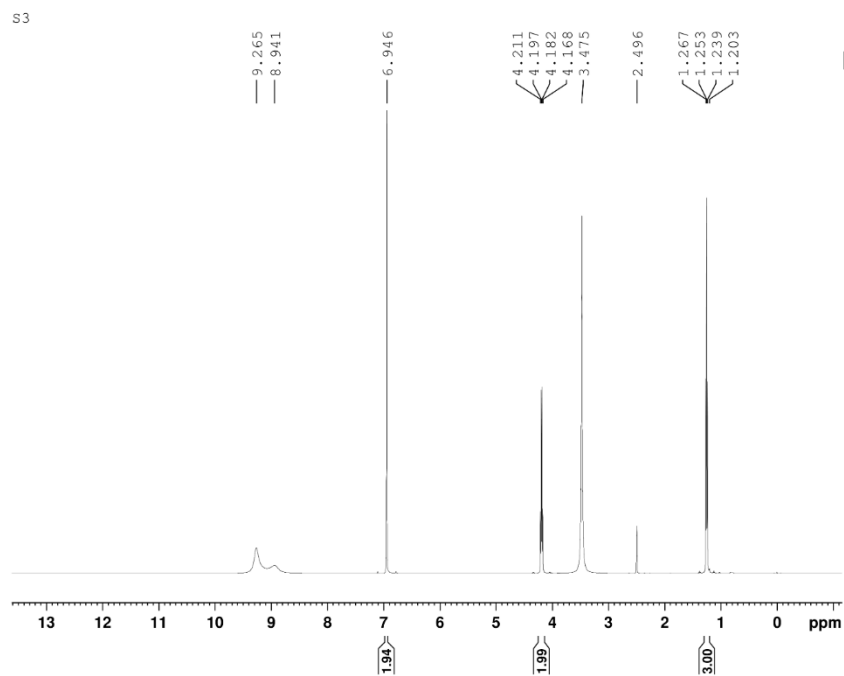


Figure 11: LC-MS spectra of S2

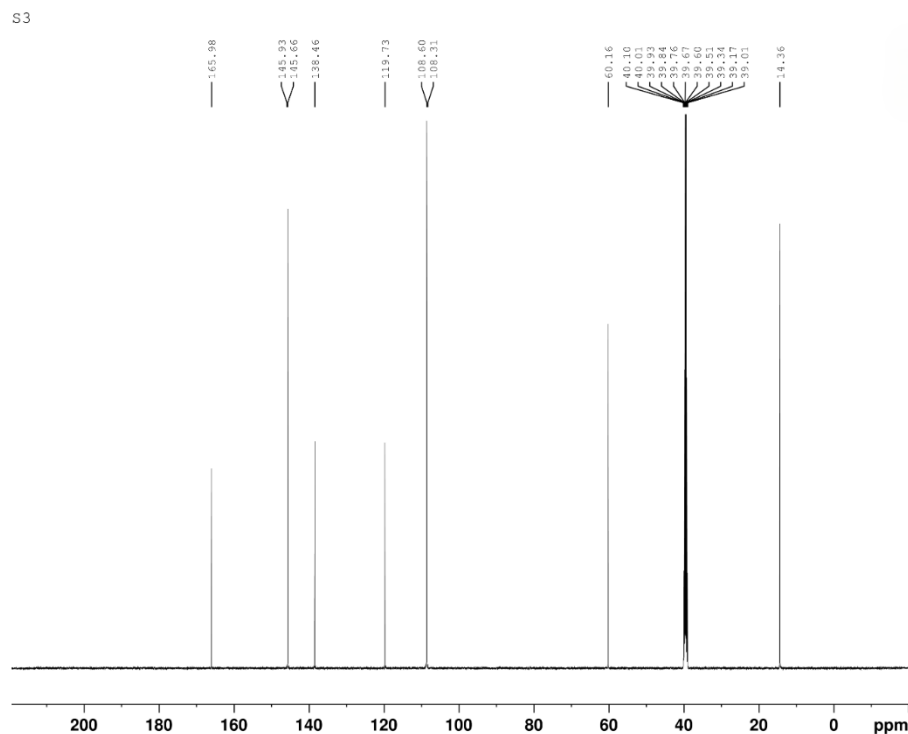
Sample S2 was identified as quercetin (3,3',4',5,7-pentahydroxyflavone; C<sub>15</sub>H<sub>10</sub>O<sub>7</sub>; MW = 302.24 g/mol)



Figure 14:  $^1\text{H-NMR}$  spectra of S3

### 3.4.3.3 $^{13}\text{C NMR}$ (DMSO- $d_6$ , 125 MHz)

Ester carbonyl at  $\delta$  165.98 ppm. Aromatic carbons at  $\delta$  145–146 ppm. Ethyl carbons at  $\delta$  60.16 ( $\text{CH}_2$ ) and 14.36 ppm ( $\text{CH}_3$ )

Figure 15:  $^{13}\text{C-NMR}$  Spectra of S3

### 3.4.3.4 LC-MS

RT 6.96 min.

ESI<sup>-</sup> dominant:  $[\text{M-H}]^-$   $m/z$  197.0<sup>40,43</sup>.

Fragments consistent with galloyl ester decarboxylation.

Sample Report (continued):

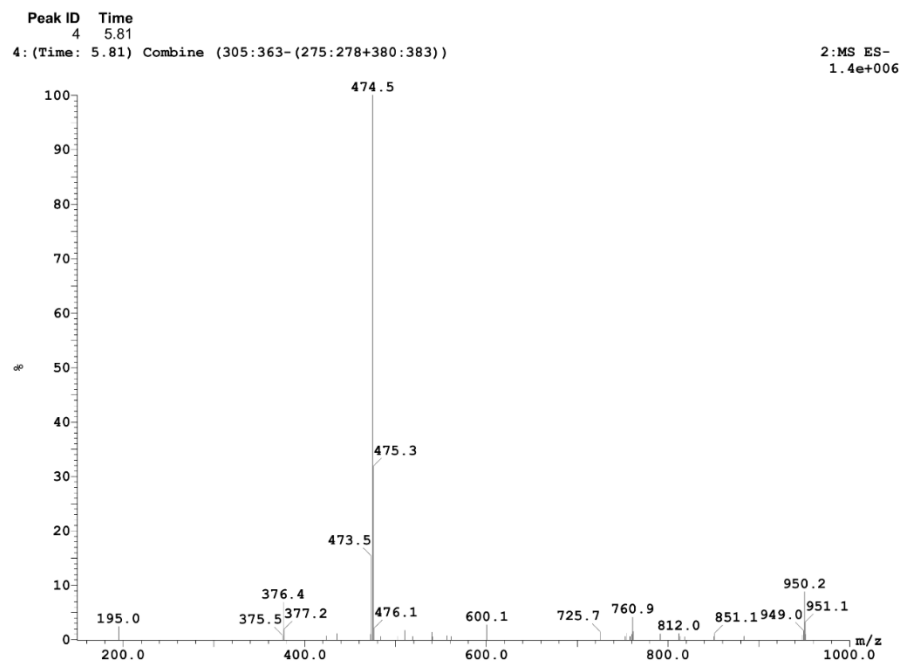


Figure 16: LC-MS Spectra of S3

S3 was identified as ethyl gallate (ethyl 3,4,5-trihydroxybenzoate;  $C_9H_{10}O_5$ ; MW = 198.17 g/mol)

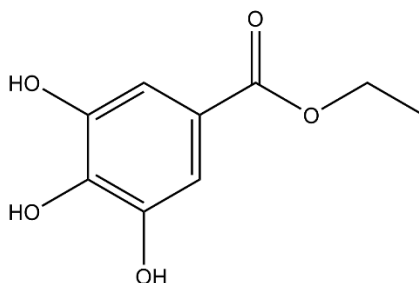


Figure 17: Chemical Structure of Ethyl Gallate

### 3.4.4 Structure determination of Sample S4

#### 3.4.4.1 FT-IR

Strong aliphatic C-H stretch at  $2903\text{ cm}^{-1}$  (30,31). Carboxylic acid C=O at  $1694\text{ cm}^{-1}$  and  $\Delta^{12}$  olefinic C=C at  $1619\text{ cm}^{-1}$  confirmed an oleanane-type triterpenoid<sup>44, 45</sup>.

#### Spectrum Graph

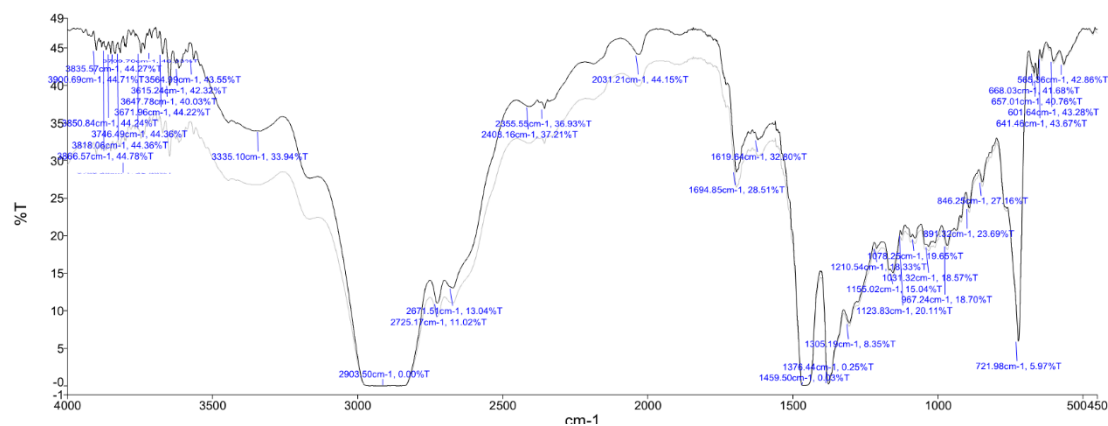
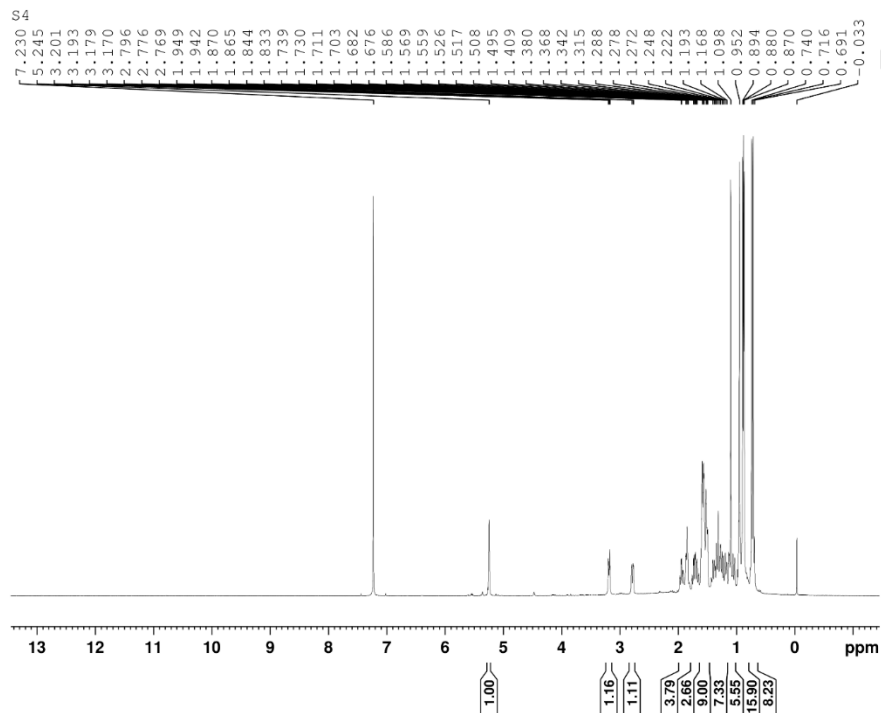


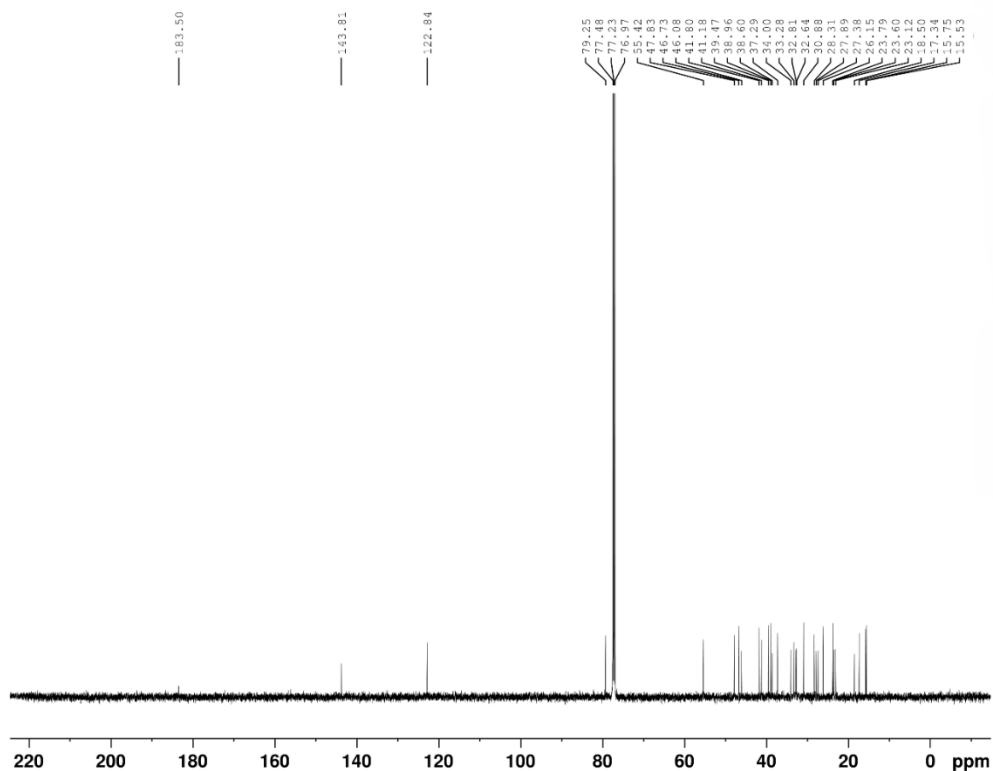
Figure 18: FT-IR Spectra of S4

**3.4.4.2  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz)**

Key diagnostic signals:

Olefinic H-12 at  $\delta$  5.245 ppm $3\beta$ -oxymethine H-3 at  $\delta$  3.17–3.20 ppm (dd)H-18 at  $\delta$  2.77–2.80 ppm (dd) <sup>(46–48)</sup>Six tertiary methyl singlets ( $\delta$  0.74–1.14 ppm) confirmed the pentacyclic triterpene skeleton.**Figure 19:  $^1\text{H}$ -NMR Spectra of S4****3.4.4.3  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 125 MHz)**Carboxyl carbon at  $\delta$  183.5 ppm.Olefinic carbons at  $\delta$  143.8 (C-13) and 122.8 ppm (C-12).C-3 hydroxyl carbon at  $\delta$  79.2 ppm.These shifts distinguish oleanolic acid from its ursane isomer <sup>46, 48</sup>.

S4

Figure 20:  $^{13}\text{C}$ -NMR Spectra of S4**3.4.4.4 LC-MS**

RT 24–25 min (most lipophilic compound).

ESI<sup>+</sup>:  $[\text{M}+\text{H}]^+$  m/z 457.4;  $[\text{M}+\text{Na}]^+$  m/z 479.4ESI<sup>-</sup>:  $[\text{M}-\text{H}]^-$  m/z 455.4MS cannot differentiate oleanolic vs ursolic acid; NMR data confirmed the oleanane skeleton<sup>47, 49</sup>.

7: (Time: 13.65) Combine (756:814–(742:745+824:827))

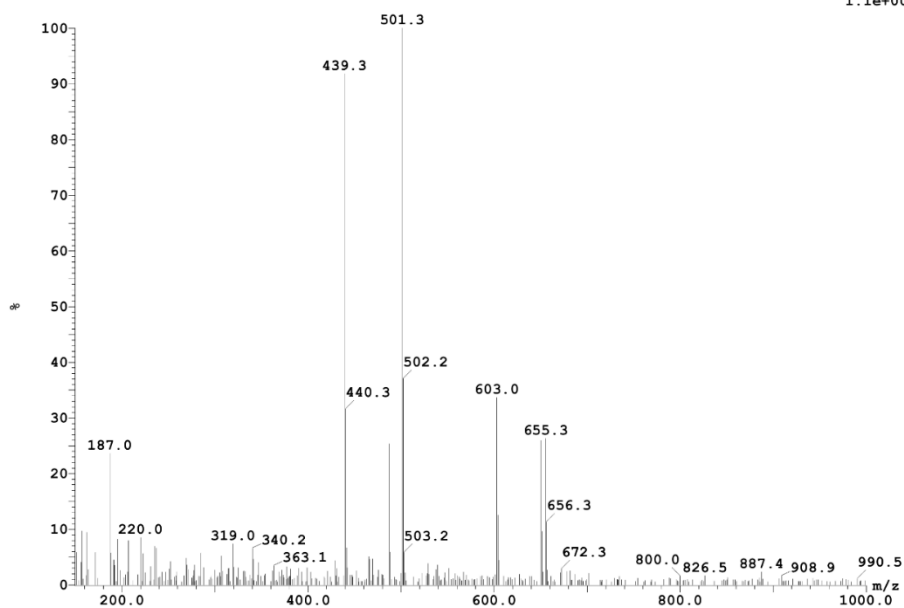
1: MS ES+  
1.1e+005

Figure 21: LC-MS Spectra of S4

S4 was identified as oleanolic acid ( $3\beta$ -hydroxyolean-12-en-28-oic acid;  $\text{C}_{30}\text{H}_{48}\text{O}_3$ ; MW = 456.70 g/mol)

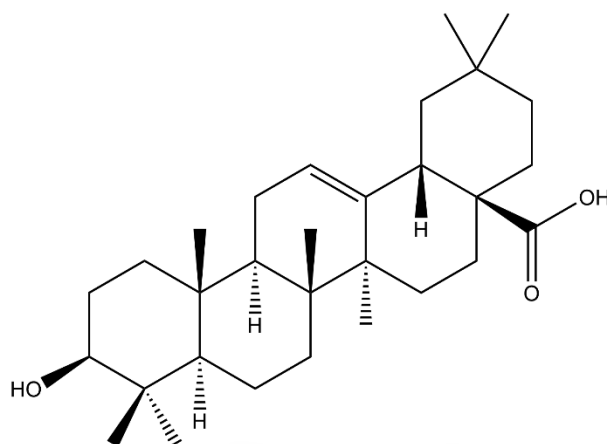


Figure 22: Chemical structure of Oleanolic acid

**Spectroscopic Summary:** Table 3 below shows the spectroscopic summary of the interpretation that was done by various spectrometric techniques on the compounds isolated.

Parameter	S1 Rutin	S2 Quercetin	S3 Ethyl Gallate	S4 Oleanolic Acid
<b>Molecular Identity</b>				
<b>Molecular formula</b>	C <sub>27</sub> H <sub>30</sub> O <sub>16</sub>	C <sub>15</sub> H <sub>10</sub> O <sub>7</sub>	C <sub>9</sub> H <sub>10</sub> O <sub>5</sub>	C <sub>30</sub> H <sub>48</sub> O <sub>3</sub>
<b>MW (g/mol)</b>	610.52	302.24	198.17	456.70
<b>Compound class</b>	Flavonol glycoside	Flavonol aglycone	Galloyl ester	Pentacyclic triterpene
<b>Source extract</b>	Ethanol	Ethanol/Chloroform	Ethanol	Chloroform
<b>FT-IR Key Bands (cm<sup>-1</sup>) PerkinElmer Spectrum, 4000–450 cm<sup>-1</sup></b>				
<b>O–H stretch</b>	3343.82 (broad)	3334.27 (broad)	~3000-3600 (unresolved)	3335.10
<b>Carbonyl C=O</b>	1651.80 (flavone)	1606.09 (flavone)	1729.68 (ester)	1694.85 (–COOH)
<b>Olefinic C=C</b>	-	-	-	1619.64 (Δ <sup>12</sup> )
<b>Aromatic C=C</b>	1598.27, 1573.68	1556.82	1607.48	-
<b>C–O–C stretches</b>	1093-1204 (glycosidic + aryl ether)	1092-1201 (aryl ether only)	1093-1304 (ester C–O–C)	1078-1210 (COOH/C–OH)
<b>gem-Dimethyl CH<sub>3</sub></b>	—	—	1376.19 (ester CH <sub>3</sub> )	1376.44 (6× CH <sub>3</sub> )
<b><sup>1</sup>H NMR Key Signals (δ ppm), 500 MHz, Bruker AVANCE NEO</b>				
<b>Solvent</b>	CD <sub>3</sub> OD	DMSO-d <sub>6</sub>	DMSO-d <sub>6</sub>	CDCl <sub>3</sub>
<b>Exchangeable –OH</b>	5-OH: 12.32 (chelated; others exchanged)	5× OH: 12.59, 10.82, 9.61, 9.35, 9.31	3× OH: 9.27 (1H), 8.94 (2H)	No OH visible (exchangeable)
<b>Aromatic/olefinic H</b>	H-2'+H-6': 7.62-7.67 (2H); H-5': 6.88; H-8: 6.38; H-6: 6.20	H-2': 7.67; H-6': 6.89; H-8: 6.41; H-6: 6.18	H-2+H-6: 6.95 (s, 2H)	H-12: 5.245 (br s, 1H)
<b>Most diagnostic <sup>1</sup>H signal</b>	Rha 6-CH <sub>3</sub> : δ 1.12 (d, J=6 Hz, 3H) - distinguishes from other Quercetins	5 phenolic –OH fully resolved; no anomeric/sugar H - confirms aglycone	Single aromatic singlet 2H + quartet/triplet ethyl system (J=7.0 Hz)	H-3: 3.17–3.20 (dd); H-18: 2.78 (dd); 6× CH <sub>3</sub> singlets δ 0.74-1.14
<b>Anomeric H</b>	Glc H-1'': 5.10 (d, J≈7.8 Hz, β); Rha H-1''': 4.53 (d, J≈1.5 Hz, α)	None (aglycone confirmed)	None	None
<b><sup>13</sup>C NMR Key Signals (δ ppm), 125 MHz, Bruker AVANCE NEO</b>				
<b>Solvent</b>	CD <sub>3</sub> OD	DMSO-d <sub>6</sub>	DMSO-d <sub>6</sub>	CDCl <sub>3</sub>
<b>Carbonyl carbon</b>	C-4: 179.515	C-4: 175.90	C-1 ester: 165.98	C-28 COOH: 183.50
<b>Olefinic carbons</b>	-	-	-	C-13: 143.81 (quat); C-12: 122.84 (CH)

<b>Aromatic C–O</b>	C-2: 159.45; C-4': 149.92; C-3': 145.94; C-7: 166.11; C-5: 163.07	C-4': 147.76; C-3': 146.87; C-9: 145.12; C-7: 163.94; C-5: 160.78	C-3/C-5: 145.93, 145.66; C-4: 138.46	C-3 oxymethine: 79.25 (CH-O)
<b>Key aliphatic/sugar</b>	Anomeric: 100.07 (Glc), 95.00 (Rha); Sugar C-O: 68-78 ppm (8 carbons)	None (no aliphatic carbons)	-OCH <sub>2</sub> –: 60.16; -CH <sub>3</sub> : 14.36	6× CH <sub>3</sub> : 15.53-28.31; total 30 carbons assigned
<b>Solvent artifact</b>	CD <sub>2</sub> HOD septet at δ 49.0 ppm	DMSO-d <sub>6</sub> septet at δ 39.52 ppm	DMSO-d <sub>6</sub> septet at δ 39.52 ppm	H: CDCl <sub>3</sub> residual at δ 7.26 ppm (t) <sup>13</sup> C: CDCl <sub>3</sub> triplet at δ 77.16 ppm
<b>Impurity signals</b>	None	δ 70.06, 68.31, 17.79 (trace rutin co-impurity)	Minor δ 1.203 (trace)	None
<b>LC-MS, ACCUCORE C18 150×4.6×2.6 μm, Water–Acetonitrile Gradient</b>				
<b>Retention time</b>	6.92–7.04 min	11.02–11.13 min	6.96 min	24.16–24.97 min
<b>[M+H]<sup>+</sup> (ES<sup>+</sup>)</b>	611.1	303.0	199.0 (weak)	457.4
<b>[M–H]<sup>–</sup> (ES<sup>–</sup>)</b>	609.2	301.0	197.0 (dominant)	455.4
<b>Dominant mode</b>	ES <sup>+</sup> ≈ ES <sup>–</sup>	ES <sup>–</sup> (1.7× stronger)	ES <sup>–</sup> (14× stronger)	ES <sup>–</sup> > ES <sup>+</sup>
<b>Key fragment ions</b>	465.0 (-rha, 146 Da); 303.0 (-rutinose, 308 Da); 273.0 (-CO)	301.0 [M–H] <sup>–</sup> ; 179.0 (catechol B-ring); 151.0 (A-ring)	153.0 [M+H–H <sub>2</sub> O–CO] <sup>+</sup> ; 197.0 [M–H] <sup>–</sup> ; 153.0 [M–H–CO] <sup>–</sup>	439.4 [M+H–H <sub>2</sub> O] <sup>+</sup> ; 411.4 [M+H–H <sub>2</sub> O–CO] <sup>+</sup> ; 479.4 [M+Na] <sup>+</sup>
<b>Structural confirmation</b>	Sequential rhamnose (-146) then glucose (-162) loss-diagnostic rutinose fragmentation	m/z 303 = same as S1 aglycone fragment; confirms shared quercetin core; B-ring frag (m/z 179) confirms catechol-OH	ES <sup>–</sup> dominance (galloyl ester signature); [M–H] <sup>–</sup>	[M+Na] <sup>+</sup> dominant (typical triterpene); MS cannot distinguish ursolic acid (confirmed by NMR)

**Table 3:** Spectroscopic Summary of interpretation

### 3.5 DPPH Free radical scavenging activity of the isolated compounds

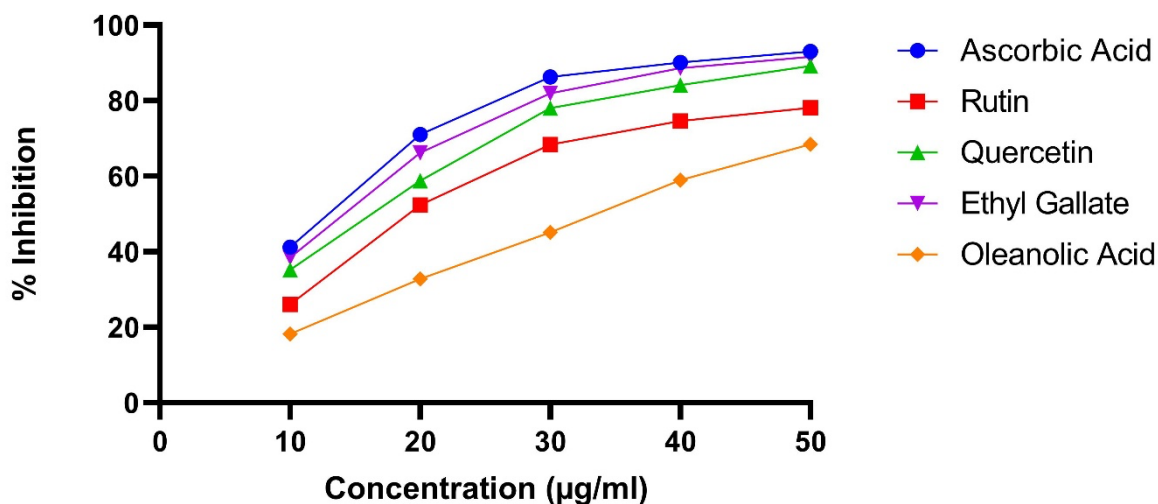
All four samples were employed for the antioxidant activity, table 4 shows the values of standard and samples, including IC<sub>50</sub> value.

Sample	Concentration (μg/ml)	%inhibition (mean SD)	IC <sub>50</sub> (μg/ml)
Ascorbic acid	10	41.24± 1.32	12.94
	20	71.02 ±1.86	
	30	86.28± 0.61	
	40	90.11± 0.41	
	50	93.08 ±0.11	
Rutin	10	26.05 ± 1.20	19.10
	20	52.37 ± 1.70	
	30	68.34 ± 0.55	
	40	74.59 ± 0.40	
	50	78.12 ± 0.10	
Quercetin	10	35.17± 0.25	16.27
	20	58.81± 0.19	
	30	78.03 ±0.15	
	40	84.14 ±0.34	
	50	89.24± 0.20	
Ethyl Gallate	10	38.45 ± 0.55	14.16
	20	66.23 ± 0.41	
	30	81.96 ± 0.42	
	40	88.61 ± 0.26	
	50	91.61 ± 0.45	
Oleanolic acid	10	18.24 ± 0.61	33.53

20	32.78 ± 0.74
30	45.11 ± 0.63
40	58.96 ± 0.52
50	68.42 ± 0.41

**Table 4: DPPH antioxidant activity of isolated compounds**

the antioxidant activity was determined by IC<sub>50</sub> value and following sequence can be deduced by the table, figure 23 shows the graph of the activity:

**Figure 23: Graph of DPPH antioxidant activity of the isolated compounds**

Ascorbic acid > Ethyl Gallate > Quercetin > Rutin > Oleanolic acid

#### 4.0 Discussion:

Successive extraction of *L. palustris* employing solvents of increasing polarity generated extracts with varied physicochemical features, with the largest yield obtained from the ethanol extract, showing a predominance of polar phytoconstituents such as flavonoids and phenolic compounds. TLC analysis of the extracts revealed many well-resolved spots corresponding to flavonoids, phenolics, and chalcone-type compounds, particularly in chloroform and ethanol extracts, validating their selection for further fractionation.

Column chromatographic fractionation of both extracts yielded a total of six distinct fractions, of which four (S1–S4) were recovered in quantities sufficient for complete spectroscopic analysis. Two additional fractions were obtained in insufficient quantities for full characterization; both displayed positive ferric chloride tests indicative of phenolic character and exhibited mid-polar TLC profiles distinct from S1–S4 upon co-spotting analysis and represent candidates for future scale-up investigation. The recovery of quercetin (S2) from both the chloroform and ethanol extracts, confirmed by co-TLC, FT-IR, and melting point comparison, reflects its relative abundance and broad polarity distribution within the plant matrix.

Compound (S1) was identified as rutin (quercetin-3-O-rutinoside) based on its characteristic LC-MS fragmentation sequence ( $m/z$  611 → 465 → 303, corresponding to sequential loss of rhamnose and glucose), the diagnostic rhamnose methyl doublet at  $\delta$

1.12 ppm in <sup>1</sup>H NMR, two anomeric carbon signals at  $\delta$  100.1 and 95.0 ppm in <sup>13</sup>C NMR, and a melting point of 194–196°C consistent with the literature value. Rutin is well represented in the genus *Ludwigia* and quercetin-3-O-rutinoside has been reported from *L. adscendens* and *L. stolonifera*, suggesting rutinoside conjugation is a conserved biosynthetic feature within the genus. In the DPPH assay, rutin demonstrated an IC<sub>50</sub> of 19.10 µg/mL, consistent with the well-established antioxidant activity attributed to its catechol B-ring and 3-O-glycosidic substitution pattern.

Quercetin (S2) was confirmed by its molecular ion at  $m/z$  303, characteristic retro-Diels-Alder fragments at  $m/z$  179 and 151, five fully resolved phenolic hydroxyl signals in <sup>1</sup>H NMR, and a melting point of 314–317°C. Its presence in both polarity fractions reflects its intermediate polarity and abundance in the plant. In the DPPH assay, quercetin showed an IC<sub>50</sub> of 16.27 µg/mL, stronger than rutin under the conditions employed. The conservation of quercetin as a free aglycone alongside its rutinoside conjugate confirms an active flavonol biosynthetic pathway in *L. palustris*.

Ethyl gallate (S3) was established by its symmetric galloyl aromatic singlet (2H,  $\delta$  6.95 ppm), ethyl ester coupling pattern ( $J$  = 7.0 Hz), ester carbonyl at  $\delta$  165.98 ppm, dominant ESI<sup>-</sup> ion at  $m/z$  197, and melting point of 150–152°C. Notably, ethyl gallate demonstrated the strongest DPPH radical scavenging activity among the isolated compounds (IC<sub>50</sub> 14.16 µg/mL), approaching that of ascorbic acid (IC<sub>50</sub> 12.94 µg/mL). This potency is

consistent with the three hydroxyl groups on its electron-rich galloyl ring, which facilitate efficient hydrogen atom transfer to DPPH radicals. Ethyl gallate represents a comparatively rare galloyl ester within the genus *Ludwigia*, where gallic acid derivatives have not been prominently reported. Its occurrence rather than free gallic acid may reflect partial transesterification during ethanol Soxhlet extraction of a galloyl precursor, or genuine plant biosynthesis — a distinction deserving future investigation with fresh-plant aqueous extraction. Oleanolic acid (S4) was unambiguously assigned by its <sup>1</sup>H NMR signature (olefinic H-12 at δ 5.245 ppm, 3β-oxy methine H-3 at δ 3.17-3.20 ppm, six tertiary methyl singlets at δ 0.74–1.14 ppm), <sup>13</sup>C NMR resonances (C-28 carboxyl at δ 183.5, C-13 at δ 143.8, C-12 at δ 122.8 ppm), and melting point of 308-310°C. The mass spectrum could not distinguish oleanolic acid from its ursane isomer ursolic acid, but NMR data decidedly confirmed the oleanane skeleton. In the DPPH assay, oleanolic acid showed the lowest antioxidant activity (IC<sub>50</sub> 33.53 µg/mL), consistent with the established understanding that pentacyclic triterpenoids scavenge DPPH primarily through weak C-3 hydroxyl hydrogen donation rather than through the electron-delocalisation mechanisms available to phenolic compounds. Chemotaxonomically, the isolation of an oleanane-type triterpenoid from *L. palustris* is significant: Lupane-type compounds (betulin, betulinic acid) predominate in *L. adscendens*, while oleanolic acid characterizes *L. octovalvis* and *L. leptocarpa*. The presence of oleanolic acid in *L. palustris* therefore places this species within the oleanane-producing subgroup of the genus, a distinction that may reflect deeper phylogenetic divergence within Onagraceae.

The DPPH antioxidant activity profile of the isolated compounds collectively provides a compound-level resolution of the antioxidant activity previously reported for *L. palustris* crude extracts. Ethyl gallate, rutin, and quercetin form a mechanistically coherent antioxidant ensemble: galloyl hydroxyl groups, flavonol catechol B-rings, and 3-hydroxyl chelation sites collectively generate multiple hydrogen-donating and electron-delocalising mechanisms. The substantially weaker activity of oleanolic acid relative to the phenolic compounds reinforces that the antioxidant capacity of the plant extracts is predominantly attributable to its flavonol and galloyl fraction rather than the triterpenoid component. These findings establish a direct molecular bridge between the isolated constituents and the crude extract bioactivity, and provide a foundation for targeted mechanistic and *in vivo* pharmacological investigations of *L. palustris*.

## 5.0 Conclusion:

The present study reports the first isolation and spectroscopic characterization of bioactive constituents from *Ludwigia palustris* (L.) Elliott at the individual compound level. TLC-guided column chromatographic fractionation of the chloroform and ethanol extracts, combined with FT-IR, LC-MS, <sup>1</sup>H NMR, and <sup>13</sup>C NMR

characterization and melting point purity confirmation, yielded four structurally confirmed compounds: rutin, quercetin, ethyl gallate, and oleanolic acid. To the best of our knowledge, this constitutes the first spectroscopically validated report of these compounds from *L. palustris*, establishing the inaugural chemotaxonomic profile of this species.

DPPH radical scavenging evaluation of the pure isolated compounds revealed a clear activity gradient: ethyl gallate (IC<sub>50</sub> 14.16 µg/mL) ~ ascorbic acid (IC<sub>50</sub> 12.94 µg/mL) > quercetin (16.27 µg/mL) > rutin (19.10 µg/mL) > oleanolic acid (33.53 µg/mL). This profile provides compound-level resolution for the antioxidant activity previously documented in *L. palustris* crude extracts and identifies the galloyl and flavonol fraction as the primary antioxidant contributors.

The co-occurrence of rutin and quercetin confirms conservation of a flavonol-dominant biosynthetic pathway in this species, consistent with the genus *Ludwigia*. Ethyl gallate introduces a galloyl ester constituent not previously reported in the genus. The identification of oleanolic acid, rather than the lupane-type triterpenoids found in certain other *Ludwigia* species, provides a chemotaxonomically informative distinction that aligns *L. palustris* with the oleanane-producing members of the genus. These findings provide a validated chemical and biological foundation for future bioactivity-guided fractionation, quantitative metabolite profiling, mechanistic pharmacological studies, and *in vivo* validation.

## Abbreviations:

LC-MS = Liquid chromatography-Mass spectroscopy, NMR = Nuclear Magnetic Resonance. FT-IR = Fourier Transform- InfraRed, PEE = Petroleum Ether Extract, CFE = Chloroform Extract, EAE = Ethyl Acetate Extract, AE = Acetone Extract, EtE = Ethanol Extract, TLC = Thin Layer Chromatography

## Author contributions:

Vaibhav Kumar Rathi: Conceptualization, Methodology, Investigation, Formal analysis, Writing original draft.

Mukesh Singh Sikarwar: Supervision, Resources, Writing review & editing.

Pushpendra Kumar Shukla: Supervision, Validation, Writing review & editing.

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