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Research Article

PHENOLIC ACID PROFILING IN THE LEAVES OF *TABERNAEMONTANA HEYNEANA* WALL. AN ENDEMIC PLANT OF THE WESTERN GHATS USING ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED WITH QUADRUPOLE-TIME-OF-FLIGHT

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ABSTRACT

Objectives: The study was conducted to identify the phenolic compounds and other possible bioactive compounds present in the leaf extracts of *Tabernaemontana heyneana* Wall.

Methods: Phenolic acid profiling was carried out using ultra-high-performance liquid chromatography coupled with quadrupole-time-of-flight (QTOF). An internal standard syringic acid was used for quantitation of phenolic acids and naringenin for quantitation of flavonoids.

Results: The leaf extracts analysis revealed the presence of 17 compounds consisting of 14 phenolic compounds and three terpenes. Among 17 compounds, eight were the major compounds, namely, coniferyaldehyde, resveratrol, sinapic alcohol, protocatechuic acid, 4-hydroxybenzaldehyde, chlorogenic acid, rutin, and protocatechuic aldehyde. This forms the first report on the identification of these pharmaceutically important compounds in *T. heyneana*.

Conclusion: These findings offer clear evidence and scientific support for further research on the leaf extract of *T. heyneana* plant for its therapeutic purpose.

Keywords: *Tabernaemontana heyneana* Wall, *Apocynaceae*, Phenolic acids, Terpenes, UHPLC.

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INTRODUCTION

Plant products have been regarded as a major part of traditional medicine on which about 80% of the people are dependent for their primary health care since long herbal medicines are used as remedies for different ailments. Nowadays, there is an increasing demand for plant products in drug development since they are effective, less toxic with no side effects. Plant secondary metabolites include phenolic compounds, alkaloids, tannins, saponins, carbohydrates, glycosides, flavonoids, and steroids. Out of these, phenolic compounds have been considered as good therapeutic agents possessing various biological activities. Development of an effective tool for chemical profiling of plant and estimation of chemical constituents is essential. Chromatographic techniques are efficient and economical ways to determine the bioactive principles in herbal drug formulation [1].

Apocynaceae, commonly known as dogbanes, is a family of shrubs, small trees which are rich in alkaloids and glycosides, especially in seeds and latex. Some species are valuable sources of medicine, insecticides, fibers, and rubber [2]. The family includes 4555 species, distributed in 415 genera [3]. The genus *Tabernaemontana* is included under this family that consists of shrubs or small trees. *Tabernaemontana heyneana* Wall. is a shrub that is distributed in the Western Ghats region of Karnataka, India. It is known for its traditional uses which possess curative properties against venereal diseases, gonorrhea, respiratory problems, nervous disorders, diabetes, chronic bronchitis, rheumatism, cardiotonic ailments, and snakebite [4]. Sukumaran and Raj [5] demonstrated that the flower juice (mixed along with coconut oil) has the therapeutic effect against burning sensation of eyes and improved vision.

Several bioactive compounds were previously identified in the roots, leaves, and flowers of *T. heyneana* [6]. The presence of alkaloids in *Apocynaceae* has been well-studied [7]. Sathishkumar *et al*. [8] identified the presence of flavonoids such as quercetin and rutin in leaves of *T. heyneana*. Not much work has been carried out on the identification of phenolic compounds of this plant.

In the present work, the profiling of phenolic acids present in *T. heyneana* was carried out with the help of advanced UHPLC techniques combined with QTOF and mass spectrometry (MS).

METHODS

Plant material

The leaves of *T. heyneana* were collected from the natural forests of Dakshina Kannada, (12.8158°N and 74.9241°E) Karnataka, India. The collected samples were authenticated and voucher specimen [MU/AB/DJM-01] was deposited in the herbarium collections of the Department of Applied Botany, Mangalore University. The samples were shade dried, ground into a fine powder using a domestic grinder and stored at 4°C until further use.

Preparation of extract

Powdered leaf sample (20 g) was subjected to Soxhlet extraction with three different organic solvents (chloroform, methanol, and dichloromethane – 300 ml) for 36 h. 20 g of powdered leaf sample was mixed with 300 ml of water, kept in boiling water bath (70°C) for 36 h to get an aqueous extract. The obtained solution was filtered with Whatman filter paper No. 1 to obtain the filtrate. The filtrate was evaporated to dryness in a flash evaporator and the residue was stored in a refrigerator.

Phenolic acid analysis

Preparation of standard

The external standards used for the experiment are obtained from Sigma-Aldrich and are listed in Table 1. Standard stock solutions were prepared in methanol at a concentration of 1.0 mg/ml and stored in a refrigerator at −20°C until use. The standards were filtered (0.45 µm

Table 1: External standard mixture

S. No.	Reference standard	RT [min]	Exact mass	m/z positive (+H)	m/z negative (−H)	Conc. [µg/ml]
1	CUDA (internal standard)	15.53	340.273	341.28	339.265	0.1
2	Umbelliferone	4.08	162.032	163.039	161.024	1
3	Rutin	5.12	610.153	611.161	609.146	1
4	Naringin	6.26	580.179	581.187	579.172	1
5	Naringenin	8.43	272.069	273.076	271.061	1
6	Chrysin	12.08	254.058	255.065	253.051	1
7	Daidzin	4.3	416.111	417.118	415.104	1
8	Daidzein	7.11	254.058	255.065	253.051	1
9	18-b-Glycyrrhetinic acid	19.55	470.34	471.347	469.332	1
10	Glycitein	7.69	284.069	285.076	283.061	1
11	Glycitin	4.63	446.121	447.129	445.114	1
12	Genistin	5.36	432.106	433.113	431.098	1
13	Betulinic acid	21.55 negative	456.36	457.368	455.353	1
14	Genistein	8.62	270.053	271.06	269.046	1
15	6-Hydroxyflavone	10.86	238.063	239.07	237.056	1

CUDA: (12-[(cyclohexylamino) carbonyl] amino]-dodecanoic acid), RT: Retention time, Conc: Concentration

filters) and diluted whenever necessary with methanol. Further, these solutions were used for method development.

Preparation of sample for analysis

The extract (10 µg) was suspended in methanol (50 µl) containing 100 µg/ml of an internal standard CUDA. The samples were sonicated (5 min) and centrifuged (3 min at 14,000 rpm), subsequently transferred to amber vials and closed immediately.

Instrumentation

Agilent 1290 Infinity UHPLC coupled with Agilent 6530 Accurate Mass QTOF with the following specifications was used for the analysis.

- Waters Acquity UHPLC BEH C_{18} 1.7 µm, 2.1 × 100 mm column for sample analysis.
- The column temperature was 65°C.
- A binary solvent system was used A: Water with 0.1% acetic acid; B: 100% acetonitrile without a modifier.
- Flow rate 0.5 ml/min.
- An injection volume of 1 µl was used in both the polarity modes.
- A 15 min gradient was established with an elution starting from
  0–1 min 0% B
  1–6 min 30% B
  7–9.5 min 80% B
  9.5–10 min 99% B
  11 min hold
  12 min back to 0%.
- All solutions were filtered through a cellulose membrane with 0.45 µm aperture before injection.
- For mass spectroscopic analysis, phenolic compounds were run both in negative ionization mode (Electron spray ionization – ESI^-) and positive ionization mode (ESI^+).
- The autosampler was maintained at 4°C.
- The phenolic acids were detected over the range of mass to charge ratio (m/z) 100–1700.
- An internal standard syringic acid was used for quantitation of phenolic acids, whereas (±) naringenin was used for the quantitation of flavonoids.
- Sample aliquots were pooled for MSMS analysis from like treatments to ease analyte profiling.
- The injections were repeated 17 times to check the stability of the method for every 2 h.
- Tentative identification of phenolic compounds was done based on accurate mass and retention times.

Data analysis

Data acquisition on metabolic profiles was subjected to further processing adopting the liquid chromatography QTOF MS Mass Hunter Qualitative (for alignment and molecular feature extraction) and MassHunter Professional (statistical analysis) programs. All the graphs were plotted in Microsoft Excel, and the structure of the identified compounds was drawn using ChemSketch software.

RESULTS AND DISCUSSION

UHPLC detection in both positive $[M+H]^+$ and negative $[M-H]^-$ ionization modes revealed the presence of 17 compounds of which 14 are phenolic acids and three are terpenes. The phenolic acids included four flavonoids, two stilbenes, five cinnamic derivates, and four benzoic acid derivatives. Data concerning the identification of the compounds are shown in Tables 2 and 3 where the RT, m/z, molecular weight, molecular formula, and the class of metabolite for which they belong to, in positive $[M+H]^+$ and negative $[M-H]^-$ ion mode of all the detected compounds are reported. The reported structures of these phytocompounds are shown in Table 4.

Five phenolic acids and one terpene were identified in positive mode $[M+H]^+$ (Table 2), namely, 6-methoxyluteolin [m/z 316.26], coniferyaldehyde [m/z 178.18], sinapic alcohol [m/z 210.229], resveratrol [m/z 228.247], polydatin [m/z 390.384], and cauloside C, respectively. Ten phenolic acids and two terpenes were detected in negative mode (Table 3), namely, hesperidin [m/z 610.57], rutin [m/z 610.521], spiraeoside [m/z 464.379], syringaldehyde [m/z 182.17], protocatechuic acid [m/z 154.12], 4-hydroxybenzaldehyde [m/z 122.123], protocatechuic aldehyde [m/z 138.122], chlorogenic acid [m/z 354.31], esculetin [m/z 178.14], ferulic acid [194.18], asiatic acid [m/z 488.699], and medicagenic acid [m/z 502.692].

Identification of phenolic acids

Flavonoids

Phenolic acids are a group of secondary metabolites existing as a soluble ester or glucoside forms in plants. These compounds are mainly generated through phenylpropanoid pathway and are broadly classified into derivatives of the hydroxycinnamic acid such as ferulic acid and caffeic acid; derivatives of the hydroxybenzoic acid such as gallic acid and vanillic acid [9]. Similarly, flavonoids, as the main class of phenolic compounds, demonstrate a wide range of biochemical and pharmacological effect [10]. Sathishkumar and Baskar [11] revealed the presence of flavonoids which agrees with the results of the present investigation.

Altogether, four flavonoids were detected in both the ionization modes with a maximum of three in negative mode $[M-H]^-$. It has been well studied that the negative ionization mode $[M-H]^-$ gives better resolution for flavonoid, probably because of the loss of protons from the acidic hydroxyl groups and their enhanced ionization. However, the positive ionization $[M+H]^+$ mode may reveal more structural and fragmentation information about the flavonoid. Plenty of flavonoids synthesized in plants is linked to sugars (glycosides), despite being

Table 2: Phenolic acids in positive mode

S. No.	Annotations	Identifier	Class of metabolite	RT (Library)	RT (Sample)	$[M+H]^+$	Molecular weight	Molecular formula
1	6-Methoxy luteolin	4.53–317.06	Flavones	9.329	4.531	317.06	316.26	$C_{16}H_{12}O_7$
2	Coniferyaldehyde	4.11–179.07	Cinnamic acid derivative (Hydroxycinnamaldehyde)	6.081	4.107	179.07	178.18	$C_{10}H_{10}O_3$
3	Sinapic alcohol	3.86–211.10	Cinnamic acid derivative (Phenylpropionates)	6.474	3.864	211.098	210.229	$C_{11}H_{14}O_4$
4	Resveratrol	3.87–229.11	Stilbene	6.95	3.868	229.11	228.247	$C_{14}H_{12}O_3$
5	Polydatin	3.82–391.16	Stilbene	5.149	3.868	391.16	390.38	$C_{20}H_{22}O_8$
6	Cauloside C	10.99–767.53	Triterpene	19.241	10.991	767.53	766.959	$C_{41}H_{66}O_{13}$

$[M+H]^+$: Samples analyzed by UHPLC–ESI-MS in positive ion mode, RT: Retention time

Table 3: Phenolic acids in negative mode

S. No.	Annotations	Identifier	Class of metabolite	RT (Library)	RT (sample)	$[M-H]^-$	Molecular weight	Molecular formula
1	Hesperidin	6.29–609.18	Flavanone	7.886	6.288	609.181	610.57	$C_{28}H_{34}O_{15}$
2	Rutin	4.10–609.15	Flavone	5.201	4.1	609.147	610.521	$C_{27}H_{30}O_{16}$
3	Spiraeoside	4.22–463.09	Flavonol	6.747	4.222	463.089	464.379	$C_{21}H_{20}O_{12}$
4	Syringaldehyde	3.99–181.05	Benzoic derivative-Hydroxybenzaldehyde	4.779	3.987	181.05	182.17	$C_9H_{10}O_4$
5	Protocatechuic acid	1.20–153.02	Benzoic derivative-Hydroxybenzoic acids	1.57	1.204	153.02	154.12	$C_7H_6O_4$
6	4-Hydroxybenzaldehyde	2.82–121.03	Benzoic derivative-Hydroxybenzaldehyde	2.871	2.823	121.03	122.123	$C_7H_6O_2$
7	Protocatechuic aldehyde	1.82–137.02	Benzoic derivative- Catechols	2.101	1.816	137.02	138.122	$C_7H_6O_3$
8	Chlorogenic acid	1.31–353.09	Cinnamic acid derivative-Cinnamates	2.375	1.31	353.089	354.31	$C_{16}H_{18}O_9$
9	Esculetin	2.52–177.02	Cinnamic acid derivative-Hydroxycoumarins	2.752	2.5217	177.021	178.14	$C_9H_6O_4$
10	Ferulic acid	4.04–193.05	Cinnamic acid derivative-Hydroxycinnamic acids	4.92	4.041	193.051	194.18	$C_{10}H_{10}O_4$
11	Asiatic acid	8.32–487.34	Terpenes-pentacyclic triterpenes	18.737	8.782	487.34	488.699	$C_{30}H_{48}O_5$
12	Medicagenic acid	7.85–501.32	Terpenes-Triterpenes	20.21	7.848	501.369	502.692	$C_{30}H_{46}O_6$

$[M-H]^-$: Samples analyzed by UHPLC-ESI-MS in negative ion mode, RT: Retention time

found as aglycones. During the analysis, three flavonoids were detected as $[M-H]^-$ ions such as flavones (Rutin), flavanone (Hesperidin), and flavonol (Spiraeoside) whereas 6-methoxyluteolin (flavone) as $[M+H]^+$ ion which is in accordance with Sanchez-Rabaneda *et al*. [12] who could detect flavonoids such as rutin, 6-methoxy luteolin, and quercetin in $[ESI]^-$ mode. Previous studies reported the presence of rutin in *T. heyneana* [8] and *Tabernaemontana catharinensis* [13]. El-Gayed *et al*. [14] also witnessed the identification of hesperidin in *Tabernaemontana coronaria.* Therapeutic effects of this plant might be attributed to the presence of these flavonoids. Among the identified flavonoids rutin was found to be the major flavonoid in the leaf extracts of *T. heyneana* (Fig. 1). The presence of 6-methoxyluteolin and hesperidin in *T. heyneana* is being reported for the first time.

Benzoic acid derivatives

Benzoic acid derivatives are simple phenolic acids ubiquitously present in plants which usually occur in conjugated or esterified forms. The simpler type of benzoic acid derivatives identified in the leaf extracts of *T. heyneana* includes syringaldehyde (m/z 182.05), protocatechuic acid (m/z 154.12), 4-hydroxybenzaldehyde (m/z 122.123), and protocatechuic aldehyde (m/z 138.122). The identification of these compounds was facilitated by the analysis of fragmentation pathways of $[M-H]^-$/$[M+H]^+$ ions in the negative ion $[M-H]^-$ modes. Some of the benzoic acid derivatives were detected as negative ions in the fruits of *Melicoccus bijugatus* Jacq. [15] which is in accordance with the result of the present study. Sun *et al*. [16] detected the benzoic acid derivatives in $[M+H]^+$ mode which is contrary to the present report. Protocatechuic acid is widely distributed and present in most of the edible plants which are used in folk medicine.

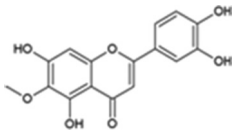
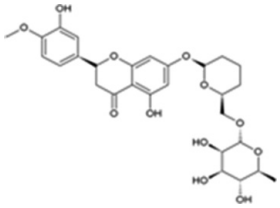
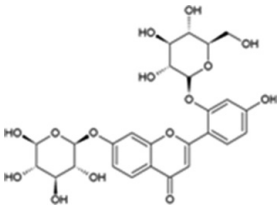
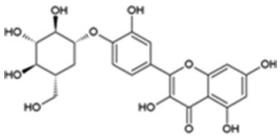
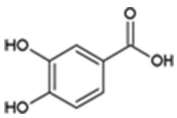
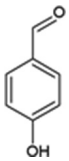
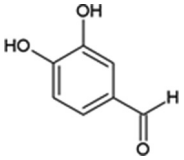
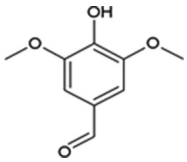
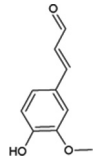
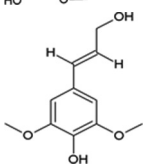
Cinnamic acid derivatives

Cinnamic acid derivatives are widespread in occurrence usually exist in a bound form such as esters. In the current study, five cinnamic acid derivatives were identified which includes coniferyaldehyde $[M+H]^+$ at m/z 178.18, sinapic alcohol $[M+H]^+$ at m/z 210.229, chlorogenic acid $[M-H]^-$ at m/z 354.31, esculetin $[M-H]^-$ at m/z 178.14, and ferulic acid $[M-H]^-$ at m/z 194.18. In the present study, cinnamic acid derivatives were detected in both the modes of ionization which is in agreement with the previously published data [17]. Chlorogenic acid was earlier reported in *Tabernaemontana catharinensis* by Piana *et al*. [13] and ferulic acid in *Tabernaemontana coronaria* by El-Gayed *et al*. [14]. Sinapyl alcohol was documented earlier in cell suspension cultures of *Tabernaemontana divaricata* by Dagnino *et al*. [18]. Earlier reports are available on the identification of coniferaldehyde and esculetin in other medicinal plants [19]. This is the first study reporting these cinnamic acid derivatives in the leaf extracts of *T. heyneana* which may be associated with medicinal uses exhibited by this plant.

Stilbenes

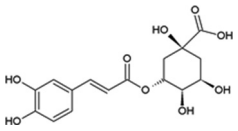
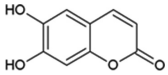
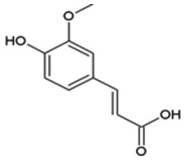
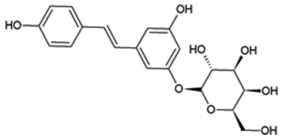
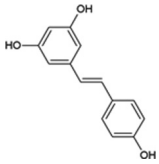
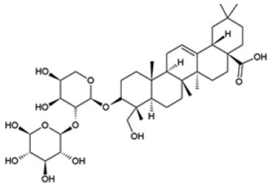
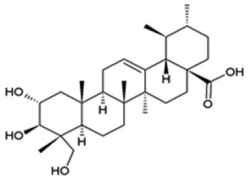
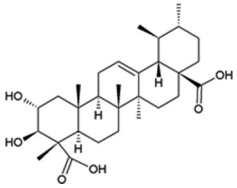
Stilbenes such as resveratrol $[M+H]^+$ at m/z 228.247 and polydatin $[M+H]^+$ at m/z 390.38 were detected in the leaf extracts of *T. heyneana* in the present study. Stilbenes are simple secondary metabolites derived from phenylpropanoid pathway having a number of implications on human health and plant defense mechanism. Kimura and Okuda [20] reported polydatin in *Polygonum cuspidatum*. Resveratrol exists in plants as its glycoside form, polydatin [21] which exerts a wide variety of pharmacological properties. Even stilbenes are being reported for the first time in *T. heyneana*.

Table 4: Structure of the identified compounds in the leaf extracts of *Tabernaemontana heyneana* Wall

Name of the compound	Structure of the compound
Flavonoid	
1. 6-Methoxy luteolin	
2. Hesperidin	
3. Rutin	
4. Spiraeoside	
Benzoic derivatives	
1. Protocatechuic acid	
2. 4-Hydroxy benzaldehyde	
3. Protocatechuic aldehyde	
4. Syringaldehyde	
Cinnamic derivatives	
1. Coniferyaldehyde	
2. Sinapic alcohol	

(contd...)

Table 4: *(Continued)*

Name of the compound	Structure of the compound
3. Chlorogenic acid	
4. Esculetin	
5. Ferulic acid	
Stilbenes	
1. Polydatin	
2. Resveratrol	
Terpenes	
1. Cauloside	
2. Asiatic acid	
3. Medicagenic acid	

Identification of terpenes

Terpenes are the large and diverse group of phenolic compounds built up from five-carbon isoprene units linked together in a head-to-tail arrangement which possesses a large variety of physical, chemical**,** and biological activities. The study revealed the presence of three terpenes in *T. heyneana***,** namely, asiatic acid $[M-H]^-$ at m/z 488.699, cauloside C $[M+H]^+$ at m/z 766.959**,** and medicagenic acid $[M-H]^-$ at m/z 502.692. This is the pioneering report of these terpenes in *T. heyneana*. Rastogi and Dhar [22] reported asiatic acid, cauloside C in *Centella asiatica*.

Effect of solvents on the extraction of phenolic acids

Results of this work demonstrated a remarkable variability in the amounts of phenolic compounds in different solvents (Figs. 1 and 2). The high content of coniferyaldehyde, resveratrol,

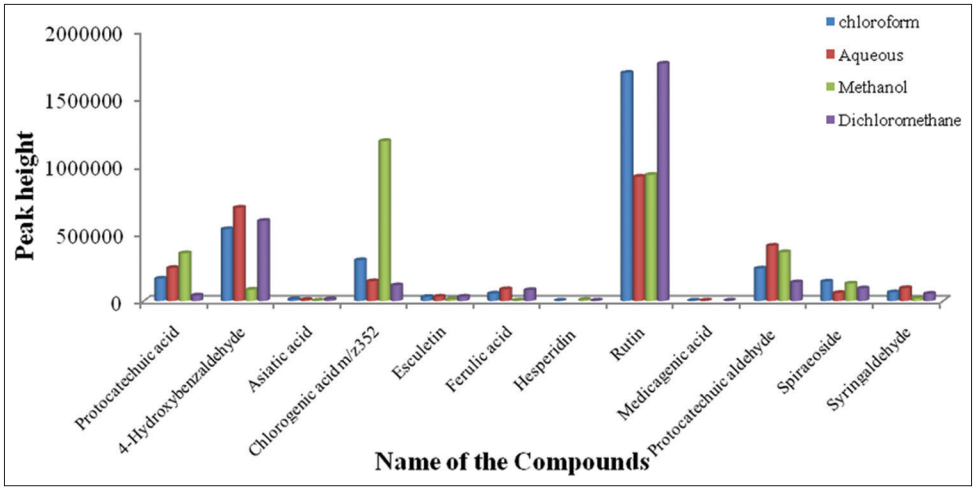


Fig. 1: Phenolic acids in different leaf extracts of *Tabernaemontana heyneana* (negative mode)

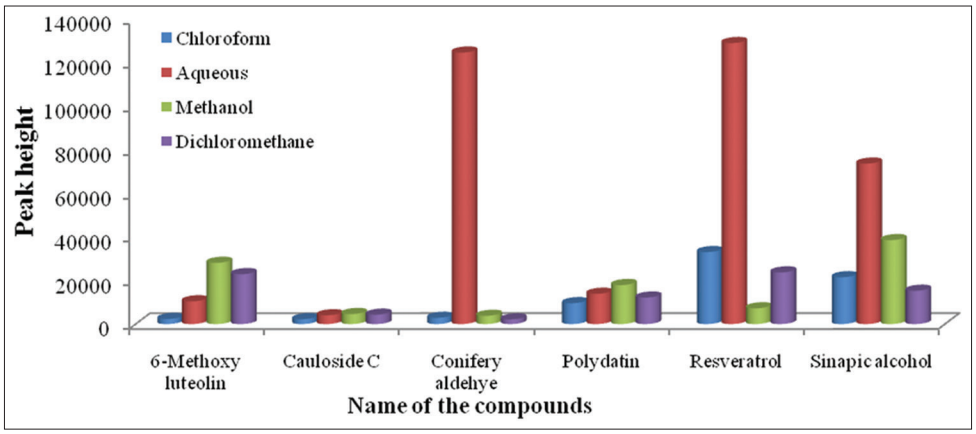


Fig. 2: Phenolic acids in different leaf extracts of *Tabernaemontana heyneana* (Positive mode)

sinapic alcohol, 4-hydroxybenzaldehyde, ferulic acid, syringaldehyde, and protocatechuic aldehyde was obtained from the aqueous extract. 6-methoxyluteolin, cauloside C, polydatin, protocatechuic acid, and chlorogenic acid were found significantly in higher quantity in methanol extract. Our findings are in agreement with the previous investigation by Ertas *et al*. [23] wherein abundant phenolic acids were found in methanol extract. In the present study, water and methanol were proved to be the most efficient solvents for extraction of phenolic compounds compared to chloroform and dichloromethane indicating the presence of bioactive compounds which are polar in nature. In the context of these observations, it should be noted that the phenolic compounds are often associated with other biomolecules (polysaccharides, proteins, terpenes, chlorophyll, and inorganic compounds) and a solvent suitable for the extraction of particular classes of compound must be used based on the structural features and related level of aqueous solubility of a particular target molecule [24] and polarity index of the solvent. Rutin is the most abundant compound found in nonpolar solvents such as chloroform and dichloromethane (Fig. 1). Chloroform and dichloromethane gave the lowest recovery of phenolic compounds because of their lower efficiency of solvation since these are proton acceptors while methanol and water are proton donors. It was observed that a diverse group of phenolic compounds was extracted from methanol in *Bucida bucera* L. and *Phoradendron Californicum* [25] which is in tune with the present study. In contrast, water and polar solvents seemed to be less effective in extracting phenolics in *Beijing propolis* extracts [26].

Among the identified phenolic acids cauloside C, asiatic acid, esculetin, hesperidin, and medicagenic acid were found to be present in lower amounts. The leaf extracts of *T. heyneana* showed higher contents of coniferyaldehyde, resveratrol, sinapic alcohol, protocatechuic acid, 4-hydroxybenzaldehyde, chlorogenic acid, rutin, and protocatechuic aldehyde as observed in walnut leaves [27].

The identified phenolic compounds were responsible for numerous biological activities such as antioxidant, antimicrobial, anti-inflammatory, antimutagenic, and anticarcinogenic properties [28]. They also contribute to apoptosis by arresting the cell cycle, regulating carcinogen metabolism, ontogenesis expression, inhibiting deoxyribose nucleic acid binding and cell adhesion, migration, proliferation or differentiation, and blocking signaling pathways [29,30]. All the phenolic acids identified are thought to be potent therapeutic agents. Hence, this study is the most comprehensive profiling of phenolic constituents of *T. heyneana* to date, encompassing not only flavonoids but also hydroxybenzoic and hydroxycinnamic acids. Thus, the UHPLC profiling of phenolic acids and flavonoids is likely to give a relatively realistic representation of the phytochemical contents available in *T. heyneana*.

CONCLUSION

Except for rutin, all the phenolic compounds detected in *T. heyneana* are being reported for the first time, which can serve as the source for these chemical constituents. Four flavonoids, two stilbenes, five cinnamic derivatives, four benzoic acid derivatives, and three terpenes were reported in *T. heyneana.* The current pioneering study suggests that these medicinally important phytocompounds are potent therapeutic agents. It sets ease for the development of numerous treatment system based on this plant extract. In the present study, water and methanol

were proved to be the most efficient solvents for the extraction of phenolic acids and flavonoids. Further research is needed to purify the bioactive compounds responsible for its therapeutic action.

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AUTHORS' CONTRIBUTIONS

The experimental part of the research was done by the first author (Manasa DJ). The preparation of the manuscript draft and revising it critically for the intellectual content was done by Prof. Chandrashekar KR.

CONFLICTS OF INTEREST

We declare that we do not have any conflicts of interest.

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