GC–MS Metabolomics and Network Pharmacology-Based Investigation of Molecular Mechanism of Identified Metabolites from *Tinospora cordifolia* (Willd.) Miers for the Treatment of Kidney Diseases

Gaurav¹,², Mohammad Umar Khan²,³, Parakh Basist¹,², Sultan Zahiruddin¹,², Mohammad Ibrahim², Rabea parveen⁴, Anuja Krishnan⁵, Sayeed Ahmad¹,²

¹Centre of Excellence (CoE) in Unani Medicine (Pharmacognosy and Pharmacology), ²Bioactive Natural Product Laboratory, School of Pharmaceutical Education and Research, ³Department of Food and Technology, School of Interdisciplinary Science and Technology, ⁴Department of Pharmaceutics, School of Pharmaceutical Education and Research, ⁵Institute of Molecular Medicine, School of Interdisciplinary Science and Technology, Jamia Hamdard, New Delhi-110062, India

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ABSTRACT

**Background:** *Tinospora cordifolia* (Willd.) Miers (*T. cordifolia*) is a well-known Indian medicinal plant containing several nonpolar and polar constituents that play an important role to mitigate various ailments, such as diabetes, urinary disorders, and hepatoprotective. Due to the lack of evidence on phytopharmacological relevance to the unpredicted nonpolar matrix of *T. cordifolia*, the present study aimed to evaluate the metabolomic pattern of different fractions obtained from aqueous extract of *T. cordifolia*, which has been recommended in AyUSH for various ailments including kidney disorders.

**Materials and Methods:** High-performance thin-layer chromatography and gas chromatography–mass spectrometry (GC–MS) analyses were performed on aqueous extracts and hexane, dichloromethane, and methanolic fraction of *T. cordifolia* aqueous extract to evaluate fingerprinting and metabolomic profile. Principal components analysis and pharmacokinetic analysis were performed using XLSTAT and in-silico SwissADME tool to determine metabolite variability and pharmacokinetic relationship based on lipophilicity and drug-likeness. Further, network pharmacological analysis was performed to determine the exact biomolecular relationship of *T. cordifolia* in alleviating kidney disease. The metabolomic study was conducted to explore the multi-mechanistic and therapeutic effect of *T. cordifolia* in the alleviation of kidney disease and its associated complications.

**Results:** The GC–MS metabolomics results showed several metabolites in different fractions with high variability of phytoconstituents in the methanolic fraction. In pharmacokinetics, each metabolite exhibited a direct correlation between drug lipophilicity and permeability. Network pharmacological suggested five fatty acids, which significantly interacted with the genes such as AGTR1, ATG, RELA, NOS3, NOS2, REN, INS, IL6, TNF, MAPK1, and CASP3, which could potentially regulate various pathophysiological conditions, such as hypertension, insulin resistance, oxidative and inflammatory stress, and electrolyte homeostasis, thereby strengthening the normal function of the kidney.

**Conclusion:** The study showed that six metabolites of *T. cordifolia* play a multimechanistic role in alleviating kidney disease.

**Key words:** Fatty acids, GC–MS, metabolomics, network pharmacology, *Tinospora cordifolia* (Willd.) Miers

SUMMARY

- Since history, medicinal plants are contributing an immense role in the alleviation of several acute and chronic ailments due to their multi-mechanistic and therapeutic effect. Among thousands of traditionally reported medicinal plants, *T. cordifolia* is a well-known Indian traditional medicinal plant used for several ailments including kidney diseases. Due to a lack of phytopharmacological and molecular-based evidences of *T. cordifolia* in the alleviation of kidney disease, the study aimed to explore metabolomics pattern and their biological significance in kidney disease. The metabolomic study was conducted through GC–MS analysis while network pharmacology analysis was conducted to explore the multi-mechanistic and therapeutic effect of *T. cordifolia* in the alleviation of kidney disease and its associated complications. The results showed that the metabolites such as fatty acids, terpenes, monoterpenes, monoterpenoid aldehyde, phenylpropanoid, anthracene, organics acids, etc were found as major metabolites of *T. cordifolia*. Network pharmacology analysis showed that fatty acids (oleic acid, linoleic acid, lauric acid, methyl palmitate, etc.) and phenylpropanoid (cinnamaldehyde and cinnamyl alcohol) exhibited a significant effect in the alleviation of kidney disease via regulation of the genes involved in its pathophysiology such as oxidative stress, inflammatory stress, vascular rigidity, apoptosis, positive regulation cell death, etc. Hence, it can be demonstrated that the aforementioned metabolites of *T. cordifolia* can be the best leads for alleviating kidney disease and associated complications.


INTRODUCTION

Metabolomics is a comprehensive analytical technique for the quality assessment of targeted or untargeted metabolites in a biological system. Metabolomic studies have been concomitant with many research fields, including functional genomics, environmental and biological stress studies, integrative systems, and biology biomarker discovery. Besides, such analytical studies are gaining exponential growth in the field of quality control analysis of phytoconstituents diversity present in medicinal plants. Medicinal plants are typically composed of a complex mixture of different varieties of phytoconstituents, such as fatty acids, carotenoids, steroids, terpenoids, alkaloids, phenols, flavonoids, and glycosides. However, it still remains a challenge to validate herbal medicine or products based on their quality, safety, and regulatory aspects.

Although, the most common analytical techniques such as chromatography, mass spectrometry (MS), and nuclear magnetic resonance (NMR) are used abundantly in quantitative and quantitative estimation of plant metabolites. Having said that, these techniques are the main object of choice which are greatly hyphenated with plant phytoconstituents profiling or metabolomics studies due to their high sensitivity and accuracy when coupled to influential chromatographic techniques [e.g., high-performance thin-layer chromatography (HPTLC), gas chromatography–mass spectrometry (GC–MS), capillary electrophoresis–mass spectrometry, and liquid chromatography–mass spectrometry] that allows the separation and characterization of the nonpolar and polar metabolites present in the medicinal plants.

Furthermore, GC–MS is one of the advanced and the first choice of techniques due to its high selectivity, sensitivity, and enables extensive detection of nonpolar metabolites within a sample. It makes us far formalized to characterize a comprehensive matrix of metabolite or their accretion patterns without having liabilities on authentic reference compounds and or isolation of the individual metabolites. The structural assignment process generally depends on the reference database search to interpret for MS and MS–MS data. Moreover, unknown metabolite characterization is a little challenging, since these cannot be consigned to a reference database. Further, the fragmentation pattern–parent ions–daughter ions are the unique parameter in GC–MS–MS-based plant metabolomics which allows us far formalized for characterization of the targeted and untargeted metabolites based on m/z values and reference database.

For authentication, the m/z values of metabolites are matched with the reference data of mass banks where the analysis is resumed under the defined tolerance of m/z values variability. The MS data acquisition analysis depends on the probability score between query and reference data search. For the untargeted metabolites, the data acquisition tolerance can be both in m/z value and chromatographic retention time, and these implications are further accustomed across measurements by using alignment software. Advanced web-based platform analyses have been established based on automated workflows from processing to peak alignment of raw data, annotations, statistical analysis tools to enable easy access for a broad range of investigators, and informatics expertise.

In the advent of the gradual rise of interdisciplinary subjects concerning bioinformatics and computational biology, researchers are being more prompted or have shifted for traditional medicine research from a single-mode to multi-faceted and dimensionalized systematic research mode. To understand the mechanisms of drug action are the significant changes from the perspective of the biomolecular network associated with the gene's role in disease regulation. The “network computational biology” aimed to regain or bring significant changes and resolve new challenges associated with the preliminary screening of the drugs.

The network target represents unique concepts that characterize the biological network of therapeutic target underlying diseases and decipher systematic mechanisms of action for single multitargeted drugs, predominantly used for traditional medicine. Thus, the theory “network prediction of target” has been acknowledged as the core theory of network pharmacology.

Traditional medicine (TM), characterized by personalized, multicomponent, and holistic therapy, grasps big potential to conceptualize various challenges in the system of modern health care. By producing an unprecedented prospect of TM for systematic research, network pharmacology is progressing as a systematic paradigm or even becoming a frontier research field of drug discovery and development.

The systematic perspective of network pharmacology is emphasized to reveal the systematic mechanisms of drug pharmacology and further implemented for the drug discovery and development or clinical treatment. Network pharmacology assimilates computational, experimental, and clinical investigation and generates promising aspects to explore the characteristics of TM and their further association with the frontiers of modern science and technology.

_T. cordifolia_ is a traditional Indian medicinal plant used for various ailments or chronic disorders in form of aqueous decoction. It has been part of various traditional formulations of AYUSH which are being used for centuries to cure urinary disorder/increased frequency and turbidity of urine, diabetes (API, Part-I, Vol-I, 41), and diuretic (UPI, Part-I, Vol-I),

Due to metabolite complexity, namely, fatty acids, steroids, terpenoids, alkaloids, and polyphenols, _T. cordifolia_ is assisted in the regulation of chronic kidney malfunction via exertion of multidimensionality potential as antidiabetic and hypoglycemic activity, anti-inflammatory activity, anticancer activity, and immunomodulatory activity. Although, no specific phytopharmacological evidence on _T. cordifolia_ is still available which tends to identify an unknown nonpolar matrix of the phytoconstituents extracted with polar systems and establish based on their metabolic patterns and pharmacology related to kidney and associated diseases.

Several studies have been conducted on individual plant matrix of metabolites based on GC–MS and revealed the diversity of metabolites, such as lipid-related compounds, steroids, and organic acids, as well as...
structurally diverse phytomolecules. Further, the correlation of derived metabolites and target biological assessment has a vast consideration in the screening of the metabolites based on their biological interaction.\[^{[19]}\]

Taking all these facts into consideration, the present study aimed to evaluate the comparative evaluation of the metabolomic pattern of different fractions obtained from \textit{T. cordifolia}, and \textit{in-silico} computational studies were performed to investigate the mechanism of screened constituents-based on network pharmacology hyphenated compound or protein–protein interaction (PPI) for nourishing kidney malfunction.

**MATERIALS AND METHODS**

**Chemicals and reagents**

The analytical-grade solvents such as \textit{n}-hexane, dichloromethane, methanol, and distilled water used for sample preparation and GC–MS analysis were purchased from SD Fine-Chem Limited, Mumbai.

**Preparation of extract**

Dried stem of \textit{T. cordifolia} was provided as a gift sample to the bioactive natural product laboratory (BNPL) from AIMIL Pharmaceuticals (India) Ltd, Saini Majra, Ropar Nalagarh Rd, Tehsil Nalagarh, Solan District, H. P 174101, India, which was collected from Nalagarh district Solan, Himachal Pradesh, India and authenticated as per Ayurvedic Pharmacopoeia of India (API, Part-I, Vol-I, pg-53). The voucher specimen of each vendor was submitted to BNPL with voucher number BNPL/JH/Ph.D/11/19/04, BNPL/JH/Ph.D/11/19/05, and BNPL/JH/Ph.D/11/19/06. In brief, 100 g of the dried stem of \textit{T. cordifolia} from each vendor was coarsely powdered using a grinder and soaked in 700 ml of distilled water overnight in three different round bottom flasks. Thereafter, the extraction process was performed using the reflux method at 60°C temperature for 8 hr. The extracts were filtered using a muslin cloth and Whatman\textsuperscript{TM} filter paper (Qualitative, 90 mm; Cat No 1001090) and concentrated on a water bath at 60°C to get dried residue. The yields of extracts were calculated and stored in an air-tight container for further use.\[^{[20]}\]

**HPTLC profiling**

HPTLC profiling of each aqueous extract of \textit{T. cordifolia} was performed using the standard protocol with some modification in chromatographic condition while the instrument and instrumentation condition remained the same as followed in the protocol.\[^{[21]}\] In brief, 30 mg of aqueous extract of each sample was dissolved in 1 ml of methanol followed by vortex and centrifugation for 10 min. The supernatant was collected for HPTLC analysis while toluene, ethyl acetate, and formic acid (5:4:1, v/v/v) were used as mobile phase to develop TLC plate (size 10 × 5 cm) in a presaturated TLC development chamber. After the development of TLC plate, fingerprinting analysis was performed at 254 nm.

**Preparation of sample for GC–MS analysis**

The aqueous extracts of different vendors as prepared in Section 2.2 were pooled in 1:1 ratio (w/w) and used for successive extraction/fractionation using hexane, dichloromethane (DCM), and methanol as extracting solvents. In brief, 60 mg of sample was taken in 1.5 ml Eppendorf’s tube and directly dispensed in hexane, DCM, and methanol. Then, 1 ml of each solvent was added into Eppendorf’s tube followed by vortex and centrifugation for repeated 10 min after completion of successive extraction with one solvent. Thus, all three fractions were obtained from mixed samples of \textit{T. cordifolia} aqueous extract and processed for GC–MS metabolomic analysis in triplicate after filtering through a 0.2 μM polytetrafluoroethylene membrane filter.

**GC–MS Metabolomics analysis**

The study was performed on a GC–MS instrument (Agilent 7890A, Agilent Technologies, United States) equipped CTC-PAL autosampler associated with a mass spectrophotometer detector (Agilent 5975C inert XL EI/CI MSD with Triple-Axis Detector, Agilent Technologies, United States). In brief, 2.0 μL of each prepared sample was injected with a 10:1 split ratio onto a 30 m × 0.25 mm × 0.25 μm HP-5MS column (5% diphenyl, 95% dimethyl polysiloxane, Agilent Technologies, United States). The oven temperature was set at 50°C initially for 1 min and further gradually increased up to 150°C at 5°C/min, and the temperature was held for 1 min. Then, it was ramped to 310°C at 10°C/min; 310°C was maintained for 5 min. The total run time of the sample run was 41 min. The chromatographic separation was done using high pure helium (99.999%) as carrier gas at a constant flow rate of 1 mL/min. The injection port, transfer line, and ion source temperatures were all set at 250°C while 70 eV of EI was adopted, and the mass scanning range was set from 50 to 700 amu in full scan. Solvent interruption time was adjusted at 3 min for all samples generated by different methods. To process chromatographic and spectral data, MSD ChemStation software was used. The metabolites separated through GC–MS were identified by comparing the obtained mass spectra of the analytes with those of authentic standards from the NIST libraries (2005) and with the mass spectra published previously.\[^{[21]}\] The analysis of each sample was done in triplicate (n = 3).

**Data processing based on metabolites pattern obtained from the different fractions of \textit{T. cordifolia} aqueous extract**

The metabolites identified from GC–MS analysis were formatted as per the presence (value 1) or absence (value 0) of metabolite in different fractions of mixed samples. The formatted data were further analyzed by the XLSTAT2021.Ink trial version for principal component analysis (PCA) and multivariate data analysis. The Extended Statistics module of the XLSTAT software was used to implement multivariate statistical analysis for the data obtained by GC–MS profiling of different fractions of \textit{T. cordifolia} aqueous extracts. Partial least squares discriminate analysis with Pareto scaling was used to evaluate comparative metabolites variability and identify metabolite variables that are responsible for potential metabolites among different samples. Further, the metabolites network/matrix was evaluated between three successive fractions and their identified metabolite content. To determine the distance between different generated clusters of each fraction metabolites, the Ward distance algorithm was used to calculate through hierarchical cluster analysis (HCA). Using the PCA-to-HCA, 40 metabolomic tree diagrams were created, and the corresponding bootstrap values were calculated to interpret the PCA clustering pattern.\[^{[21]}\]

**ADME analysis**

ADME computational analysis was performed for each metabolite identified in different fractions of \textit{T. cordifolia} mix sample through “SwissADME (http://www.swissadme.ch/index.php).” Topological polar surface area (TPSA) for drug integrity, consensus log \textit{Po}/w for drug lipophilicity, log \textit{K}_{\text{p}}, (skin permeation), and drug-likeness were predicted as the standard parameters for their bioavailable or ADME response, and to determine the relationship of ADME analysis between each parameter.\[^{[22]}\]

**Gene ontology and network pharmacology analysis**

Genes involved in kidney and associated disorders were selected from Genecard (https://www.genecards.org/) and UniPort database (https://
RESULTS AND DISCUSSION

The extraction process of three different vendors of *T. cordifolia* using distilled water was done successfully. The average extractive yield of aqueous extract was found to be 13.74% ± 1.632%, which was within the limit of India’s Ayurvedic Pharmacopeia (API, Part-I, Vol-I, p-65). After the successful extraction of *T. cordifolia* aqueous extracts, HPTLC analysis was performed to determine the variability among samples, if any. Thereafter, GC–MS analysis was performed on hexane, DCM, and methanol fractions in triplicate, which was obtained by successive extraction from pooled samples of aqueous extracts.

HPTLC analysis

HPTLC analysis of each aqueous extract of *T. cordifolia* was performed to determine the variability among samples. The fingerprinting analysis showed several major and minor metabolites in each extract of *T. cordifolia* at different Rf scale: 0.076, 0.123, 0.171, 0.219, 0.276, 0.352, 0.380, 0.457, 0.504, 0.742, 0.780, and 0.819 for TCS1; 0.075, 0.123, 0.170, 0.217, 0.274, 0.350, 0.378, 0.455, 0.502, 0.741, 0.780, and 0.818 for TCS2; and 0.075, 0.123, 0.170, 0.218, 0.274, 0.351, 0.380, 0.455, 0.504, 0.741, 0.780, and 0.819 for TCS3. The resulted data showed no significant differences in the number of metabolites and their respective peak intensity in aqueous extract of tested vendors of *T. cordifolia*. Considering these facts, the HPTLC analysis of different vendors of *T. cordifolia* can be assessed potentially in quality-based standardization of its herbal medicine or products. Since no significant differences were observed in intensities of peaks commonly present at different Rf, it was decided to pool the samples in 1:1:1 ratio (w/w/w) for further analysis.

**Table 1:** Phytochemical screening from different fractions of *T. cordifolia* using GC–MS analysis

<table>
<thead>
<tr>
<th>Identified Compound</th>
<th>Molecular Weight</th>
<th>Retention Time (Rt)</th>
<th>Hexane Fraction</th>
<th>DCM Fraction</th>
<th>Methanolic Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>(14β)-Pregnane</td>
<td>288.5</td>
<td>26.437</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>2 (1H)-Naphthalenone</td>
<td>152.23</td>
<td>12.291</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>6-Aza-5,7,12,14-Tetrathiapentacene</td>
<td>355.5</td>
<td>9.083</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>6-Methyl octahydrocoumarin</td>
<td>168.23</td>
<td>13.352</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>7-Pentadecyne</td>
<td>208.38</td>
<td>23.932</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Anisole</td>
<td>108.14</td>
<td>13.954</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Anthracene</td>
<td>178.23</td>
<td>18.727</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Artonin L Monomethyl Ether</td>
<td>272.25</td>
<td>30.295</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Bisphenol A</td>
<td>228.29</td>
<td>27.696</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Camphene</td>
<td>136.24</td>
<td>10.665</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Capric Acid</td>
<td>172.26</td>
<td>6.008</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>132.16</td>
<td>11.86</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Citronellal</td>
<td>154.25</td>
<td>19.100</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Curcumene</td>
<td>368.38</td>
<td>14.582</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Cyercene 4</td>
<td>262.34</td>
<td>22.710</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>D-Allose</td>
<td>180.15</td>
<td>6.404</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Diethyl Phthalate</td>
<td>222.24</td>
<td>15.995</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Docosane</td>
<td>310.60</td>
<td>20.411</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Ecosene</td>
<td>280.53</td>
<td>21.589</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Erucic Acid</td>
<td>338.57</td>
<td>30.888</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Ethyl Caprylate</td>
<td>172.26</td>
<td>10.467</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Eugenol</td>
<td>164.27</td>
<td>13.045</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Fukinanolid</td>
<td>234.33</td>
<td>22.702</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Fulvic acids</td>
<td>308.24</td>
<td>6.118</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Fumaric Acid</td>
<td>116.07</td>
<td>13.264</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Henecicosanoic Acid</td>
<td>326.56</td>
<td>9.472</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lauric Acid</td>
<td>200.31</td>
<td>6.118</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Linoleic Acid</td>
<td>280.44</td>
<td>24.306</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Methyl Linoleate</td>
<td>294.53</td>
<td>28.889</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Methyl Palmitate</td>
<td>270.45</td>
<td>20.564</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Morpholine</td>
<td>87.1</td>
<td>5.503</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Myristylaldehyde</td>
<td>212.37</td>
<td>14.648</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Oleic Acid</td>
<td>282.47</td>
<td>26.656</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Palmitic Acid</td>
<td>256.4</td>
<td>20.557</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Phthalic Acid</td>
<td>166.14</td>
<td>19.627</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Vaccenic Acid</td>
<td>282.46</td>
<td>26.627</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Valencene</td>
<td>204.35</td>
<td>14.897</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Vibrirodole A</td>
<td>260.33</td>
<td>21.194</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>260.33</td>
<td>31.562</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Zingiberene</td>
<td>204.18</td>
<td>14.736</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td><strong>Total metabolites</strong></td>
<td><strong>20</strong></td>
<td><strong>14</strong></td>
<td><strong>30</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
successively using the GC–MS instrument (Agilent 7890A, Agilent Technologies, United States). In GC–MS analysis, 40 metabolites were identified in the entire fractions. Meanwhile, 20 metabolites were identified in hexane fraction, 13 in DCM fraction, and 31 in the methanolic fraction of *T. cordifolia* aqueous extract. The compounds which were not of choice were removed during the processes of data acquisition. The screened metabolites were lipids/fatty acids, anthracenes, terpenes (monoterpenes and/or sesquiterpene), steroids, phenols, and alkaloids. The previous findings strongly support our findings.[16,25] In the analysis, some of the common metabolites were identified in each fraction and the principal metabolites matrix evaluations were done by comparative metabolites variability and

**Figure 1:** GC–MS chromatogram of different samples. (a) Represents the chromatogram of hexane fraction. (b) Represents the chromatogram of DCM fraction. (c) Represents the chromatogram of methanolic fraction obtained from *T. cordifolia* aqueous extract
identify metabolite variables analysis which acknowledges the potential metabolites in each fractionated matrix of *T. cordifolia* aqueous extract. The identified metabolites in each fraction of *T. cordifolia* aqueous extract are summarized in Table 1 with their respective chromatograms presented in Figure 1.

**Data processing based on metabolites pattern obtained from the different fractions of *T. cordifolia* aqueous extract**

PCA was performed to identify the major differentiating metabolites present in different fractions of aqueous extract of *T. cordifolia*. Metabolites identified from GC associated with EI–CI MSD with triple-axis were matched with the authentic standards from the NIST libraries of metabolites. The data were formatted as per the presence (value 1) or absence (value 0) of metabolite in different fractions of *T. cordifolia* aqueous extract [Table 1]. PCA was performed among the assigned 40 metabolites converted to two main components on the x-axis (PC1) and y-axis (PC2). The statistical values were optimized and set at the significance level alpha = 0.05 for the PCA. Correlation matrix [Pearson (n)] and Eigenvalues of covariance were determined to represent matrix “core,” directions of the new feature space, and their magnitude of the PCA. Generally, PCA is used to determine the general interrelation among the metabolite patterns of different fractions of plant matrix.121 We specifically presented a deep analysis by the different ways for investigating the analytical data of major abundant and principal metabolites which differentiate each fraction of *T. cordifolia* aqueous extract. The statistical analysis revealed each centering and scaling variable to unit variance. The cumulative percentage variability of F1 (hexane fraction), F2 (DCM fraction), and F3 (methanolic fraction) components were 50.795%, 28.667%, and 20.538%, respectively. Further, the most prominent clustering in the square graph postulates that the fraction analysis of *T. cordifolia* aqueous extract merely had high dissimilarity among the metabolites of each fraction. The active variable PCA biplot axis graph showed hexane and DCM fraction with less dissimilarity of metabolites than a methanolic fraction. The contribution of the variables represents the highly contributed variables in the PCA and can be measured to determine the variables which are intensely influencing a particular PC. Additionally, when inferring the squared cosine between the variables and the PC, real correlations exist can be determined for their apparent relation.120 The analysis reveals that the contribution and squared cosines of the variables are proportionated together with high contribution variability 51.67% by the F3 variables while 0.611 and 0.590 squared cosines for F1 and F2 variables with 0.676 squared cosines for F3 variable showed the largest squared cosine. Due to high variables and dissimilarity of metabolites in methanolic fraction, high contribution percent variability and squared cosines were determined. Further, in correlation analysis of eigenvalue, variability (%), and cumulative %, it has been observed that percentage variability is irreversibly proportionated to the cumulative % of the variables which depends on the dissimilarity of metabolite among each variable. In F3, the large set of metabolites variability can differentiate another’s components, while F2 has the same

![Figure 2](image-url)
correlation matrix considered as F1 even no differentiation was observed in the eigenvalue for both the components. Based on this observation, PC1 acknowledged the correlation analysis for another component.

In multiple correspondence analysis (MCA), it has been observed that the samples that fall in the same compartment have similar metabolites patterns while the degree of rotation of active observations concerning the corresponding compartment is characterized by the different levels of variability. In symmetric plot analysis (axes F1 and F2: 77.87%), the observation revealed that hexane and DCM fraction metabolites have almost the same metabolites pattern while the large difference was observed in the F3 variable. Square variable plot and biplot for active variable and observation represented for PCA while symmetric plot represented for MCA is shown in Figure 2.

Besides the representation of data based on dissimilarity, is one of the simple statistical analytical methods with high applicability on various data sets. Dissimilarity index agglomerative hierarchical clustering (AHC) analysis was performed to determine relatively homogeneous clusters of a data set based on their characteristics measured in each variable. The output of dissimilarity dendrogram statistical analysis of GC–MS data is depicted with the four clusters of the data set. The first right cluster of metabolites showed the dissimilarity index of metabolites from hexane fraction, the second cluster showed for DCM fraction, the third-largest cluster showed for the common metabolites for hexane and methanolic fraction, while the remaining one set of the cluster showed for the common metabolites for each variable. Dissimilarity index AHC analysis node and dendrogram plot are shown in Figure 2.

**ADME analysis**

ADME profiling of identified metabolites from subfractions of mix *T. cordifolia* extract was determined based on TPSA, lipophilicity, permeability, and drug-likeness parameters. The comparative analysis of each corresponding parameter to determine the relationship of metabolites in the ADME process revealed that the molecules that have high TPSA value showed less permeability or even to the blood–brain barrier (BBB). Besides, those molecules having low TPSA value showed high permeability to the skin and BBB. The statistical representation of correlation analysis between consensus log P and log Kp (cm/s) revealed that the permeability of each metabolite is directly proportional to the lipophilicity of metabolites. The correlation coefficient was found to be 0.9483, 0.9802, and 0.9656 for hexane, DCM, and methanolic subfraction metabolites ADME parameters. Further, the correlation analysis was performed between TPSA and other parameters, the statistical analysis suggested no significant correlation of TPSA to consensus log P and log Kp (cm/s). It can be suggested that molecular integrity and permeability do not correlate with TPSA to predict the ability for permeation into BBB. The generated computational *in-silico* data and correlation plot of all the subfraction/metabolites for ADME applicability are shown in Figure 3 and the results of ADME analysis of each metabolite identified in different fractions are summarized in supplementary files named ADME_HF for identified metabolites in hexane fraction, ADME_DCMF for identified metabolites in DCM fraction, and ADME_MF for identified metabolites in methanolic fraction.

![Figure 3](image-url)
Gene ontology and network pharmacology analysis

In this analysis, a set of 100 target genes were selected from different gene databases. The selection of each target gene was constrained to kidney and associated diseases only. The target genes which had less even no significant interaction with metabolites were excluded during the integration analysis. The set of 93 genes is summarized.

Common target network (protein-protein interaction)

In this analysis, ninety-three putative target genes were found interconnecting each other. A PPI network was constructed using STRING database with a medium confidence score of 0.400. The established network embodied 93 nodes, 1006 edges, 21.4 average nodes of degree, and 0.599 average coefficients of local clustering. Besides, the established protein–protein network has significantly more interactions than expected. The significant interaction of each protein–target gene is based on proteins of similar size selected from the genome database and characterized that the proteins are at least partially biologically connected to each other during kidney dysfunction or associated factors. The edges characterize the interaction between sets of potential targets, while the nodes characterize the targets [Figure 4].

Further, the target genes were analyzed for gene ontology through Metascape Gene Analysis (metascape.org) and gene-disease association through network analysts to evaluate the multiple physiological roles of each gene in the regulation of kidney and associated disorders. Out of 96 genes, the top 20 results were selected which were directly interrelated with the pathophysiology of the kidney. The observation of enriched terms across input gene and enrichment analysis in DisGeNET revealed that many targeted genes play a vital role in managing kidney and associated disease via preventing renal insufficiency, reactive oxygen species, diabetic nephropathy, hypertension/vascular disease, and inflammation and various apoptotic pathways. The bar graph of enriched terms across input gene lists, colored by P values and the summary of enrichment analysis in DisGeNET and gene-disease association network is summarized in Figure 4.

Network construction for active components common targets

In the network establishment analysis of compound and disease common targets, 40 metabolites obtained from GC–MS analysis of each fraction of T. cordifolia were analyzed based on their significant interaction with the target gene. A compound-disease common target network was established using Cytoscape 3.8.2 and analyzed or interpreted for their significant interaction. The screening of potential metabolites was done based on the degree of interaction, interacted nodes, and number of interrelated edges with neighbor nodes. The metabolites which had no interaction with the target gene/node were deleted during interpretation analysis of the network. The observation revealed...
that out of 40 metabolites, only six metabolites were identified as four fatty acids, namely, oleic acid, lauric acid, palmitate, linoleic acid, one aldehyde; cinnamaldehyde and vitamin E have significant interaction with each target used in analysis which may suggest their strong biological connection in management of kidney and associated disorders [Figures 5 and 6]. During interpretation analysis, oleic acid showed interaction with 25 genes, such as UCP2, AGTR1, PPARG, HTR2A, CASP3, PIK3CA, TNF, BCL2, and FABP2, which could play a pivotal role in the regulation of arterial blood pressure, ion transportation/calcium homeostasis, management of diabetes and diabetic nephropathy, tumor suppression (bladder tumor), and oxidative modification. TNF signaling is well acknowledged with inflammatory cytokine associated with renal injury. TNF-α, NF-κB triggers the activation and transcription of ICAM-1, IL-6, and IL-8, which results in endothelial inflammatory and acceleration of renal pathogenesis.[27,28] Oleic acid nitration is facilitated for regulation of many reactive nitrogen oxides such as the nitrogen dioxide radical and demonstrated benefits in hypertension, hyperglycemia in diabetes, obesity with the metabolic syndrome, and vascular neointimal proliferation and turns the nonselective activation of PPAR, which may in part account for OA-NO2's biological effects. Further, it has been reported that oleic acid acts as a strong candidate against inflammation via the prosurvival nrf2-signaling pathway.[29,30] In a report of Perdomo et al.,[31] Oleate reduces pro-inflammatory cytokines induced eNOS expression, impaired the proliferation induced by TNF-α, angiotensin II and the apoptosis induced by TNF-α or thapsigargin in endothelial cells (ECs) and vascular smooth muscle cells (VSMCs).
Lauric acid showed 11 interrelated edges genes, such as PTAFR, ALB, HNF4A, and PIK3CA, which regulate colloidal osmotic pressure of blood, smooth muscle contractile and hypertensive activity, vascular development, resistance to apoptosis, and tumor angiogenesis. Further, it strongly regulates blood pressure and increases sodium retention by the kidney. Moreover, HNF4A plays a critical role in proximal tubule blood, smooth muscle contractile and hypotensive activity, vascular stress.

In the cited study, it was reported that cinnamaldehyde is a phenylpropanoid phytoconstituent reported for inflammation or even apoptosis. It has further been documented that vitamin E potentially improves redox and inflammatory status in healthy, diabetic, and metabolic syndrome subjects.

Therefore, the results of network pharmacological analyses not only validate the screened targets but also represent the fatty acids whether isolated from any sources have a significant therapeutic role in the kidney or associated disease by regulating several pathophysiological pathways, including hypertension, oxidative/inflammatory stress, insulin resistance, and other pathways with unclear mechanisms. The significant interaction of each target with fatty acids will provide a novel methodology for further assessment of a therapeutic/mechanical approach to alleviating kidney disease.

CONCLUSION

The study found that *T. cordifolia* aqueous extract possesses several major and minor nonpolar metabolites identified by GC–MS analysis. PCA statistical analysis reveals the high variability among each variable especially in methanolic fraction with a large difference in eigenvalue than other variables. Further, based on network pharmacology analysis, it was investigated that four fatty acids such as oleic acid, lauric acid, palmitate, linoleic acid, one aldehyde; cinnamaldehyde, and vitamin E have significant interaction with the targets associated with kidney disorders. The analysis can enlighten the role of screened fatty acids and other metabolites in treating kidney disease and related disorders via ameliorating hypertension, oxidative/inflammatory stress, insulin resistance, and apoptosis kind of pathophysiological conditions. Besides, the generated evidence can be the best source to explore the therapeutic or mechanistic approaches for *T. cordifolia* to alleviate chronic kidney disease, practically.

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Authorship contribution statement

Gaurav: Investigation, writing, drafting, software, and visualization; Mohammad Umar Khan: formal analysis; Sultan Zahiruddin: GC–MS analysis and data acquisition; Parakh Basist: software and visualization;
Gaurav and Rabea Parveen: data curation and validation; Rabea Parveen and Anuja Krishnan: supervision, writing, review, and editing; Sayeed Ahmad: conceptualization, resources, writing, review, and editing original draft.

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There are no conflicts of interest.

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