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ORIGINAL ARTICLE

Metabolomic analysis of *Pennisetum glaucum* seed extracts using advanced LC–MS/MS and Q-TOF technology

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Abstract

Pearl millet (*Pennisetum glaucum*) is a cereal widely cultivated and grown in Africa and the Indian subcontinent for centuries. The present investigation aims to use LC–MS/MS to analyze the secondary metabolites present in pearl millet seeds using different solvents such as methanol, hexane, chloroform, and ethyl acetate. METLIN software was used to identify the metabolites. The analysis revealed the presence of 650 metabolites, among which 145 were commonly found in all the solvent extracts. The major classes of identified metabolites are terpenoids, flavonoids, sterols, amino acids, fatty acids, glycoconjugates, and carbohydrates. 80% methanolic extract and ethyl acetate extract yielded the highest concentrations of terpenoid (23%) and flavonoid (17%). The enrichment analysis was performed to statistically examine and identify the metabolites present in the metabolomic library dataset. In the hexane extract, notable metabolites such as quercetin and rutin were identified, which possess potential for the management of Alzheimer's disease due to their neuroprotective effects ($p < 4e\text{-}35$). In the methanol extract, metabolites like gallic acid and caffeic acid were associated with uremia treatment due to their antioxidant activity ($p < 5e\text{-}37$). Overall, the present study provides an overview of the metabolites present in the pearl millet seeds and the nutritive as well as therapeutic potential of these millets in the management of human diseases.

Keywords Metabolic profiling · Pearl millet · LC–MS/MS · METLIN · Biological activities

Introduction

Metabolic profiling involves analyzing the quantities of metabolites present in biological sources (Wolfender et al. 2015). Plant-based metabolic profiling has gained significant popularity in recent decades due to its diverse array of compounds and significant biological impacts (Li and Lou 2018). Pearl millet (*Pennisetum glaucum*) is a vital cereal crop cultivated widely in arid and semi-arid regions, serving as a staple food for millions owing to its agronomical and nutritive benefits (Saxena et al. 2018). Beyond its role as a source of macronutrients, pearl millet is one of the richest sources of diverse classes of secondary metabolites (Nithiyanantham et al. 2019). Secondary metabolites including phenolics, flavonoids, and terpenoids play critical roles in plant defense mechanisms against pathogens, pests, and environmental stressors. Furthermore, these metabolites exhibit many medicinal properties including antioxidant, antimicrobial, and anti-inflammatory properties (Ojo et al. 2022).

Despite their importance, the secondary metabolites of pearl millet remain underexplored compared to other cereals. Understanding their biosynthesis, diversity, and bioactivity could unlock opportunities for enhancing the crop's nutritional and therapeutic potential. This research aims to investigate the secondary metabolites of pearl millet and explore

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their medicinal applications. Researchers frequently use hyphenated approaches to barcode various types of metabolites (Xiao et al. 2012). This study employed LC–MS/QTOF-based metabolomics to analyze the metabolites associated with pearl millet seeds. A variety of solvents were used in the analysis, leading to the identification of unique metabolite signatures. The METLIN software database was utilized to identify and examine previously unrecognized compounds. This study aims to catalog metabolites as a dietary management strategy for various diseases using pearl millet.

Materials and methods

Materials

LC–MS/MS grade Solvents: isopropanol, methanol, and acetonitrile. The additives, namely ammonium fluoride, acetic acid, ammonium formate, formic acid, and ammonium acetate were procured from Fluka (Honeywell International Inc., Germany). The mobile phase was prepared using MilliQ water (Millipore Elix 10 model, Darmstadt, Germany).

Collection of seed material and extraction procedure

Sampling

The pearl millet seeds have been obtained from the ICAR-AICRP Mysore research center, which is affiliated with the University of Mysore, India. The seeds were extracted using the maceration method, which involved the use of different solvents (Siddaiah et al. 2021). The LC–MS grade solvents were selected based on their polarity and ability to mix with secondary metabolites to obtain a wide range of metabolites.

Extraction

The extraction process was performed following the protocol described by Theodoridis et al. (2012), with a few minor modifications, for instance, the quantity of the solvent. The seeds were pulverized, and 10 g of the resulting powder was dissolved in 50 ml of each solvent, including 80% methanol, hexane, ethyl acetate, and a mixture of methanol, chloroform, and water in a ratio of 40:60:20, respectively. The powdered samples were immersed in solvents for 20 min, then vigorously mixed for 10 min, and uniformly agitated for 15 min at room temperature. Further, the samples were subjected to centrifugation at 1000 g for 10 min, and the filtrate was collected for further analysis. In the case of a combined solvent system, two distinct phases were employed, which were collected based on their polarity, wherein the lower phase was of chloroform and the upper phase was of methanol. These solvent phases were separately collected and dried using a rotary flash evaporator IKA RV 10 and then analyzed using ultra-performance liquid chromatography-time of flight-mass spectrometry (UPLC-TOF–MS).

Fraction collection

The dried extract obtained from solvent organic phases was dissolved as per the fractionation method described by Siddaiah et al. (2021), which includes 200 µL of mobile phases A and B in the ratio 50:50 and 30:70, respectively. The vials were subjected to sonication, and HPLC was performed by injecting the mixtures into an Agilent 1 (Santa Clara, CA, USA) 260 Infinity analytical purification system equipped with a 1 mL manual FL-injection valve (p/n: 5067-4191). The individual fractions were collected in 45 different wells of a 90-well plate and subjected to drying in a speed vac.

Metabolite profiling using hyphenated techniques

The Waters Ultra Performance Liquid Chromatography System (UPLC-XEVO-G2XS-ESI-QTOF) analyzed the samples in positive ionization mode. The system was equipped with an ACQUITY UPLC HSS T3 column (2.1 × 100 mm, particle size 1.8 µm), provided by Waters, USA. The Xevo-G2-XS instrument (Waters, Milford, MA, USA) analyzed the metabolites separated by UPLC using both mass spectrometry (MS) and tandem mass spectrometry (MS/MS) fragmentation. For lipid fraction analysis, a gradient method was employed. Mobile phase A consisted of ultra-pure water with 0.1% formic acid, while mobile phase B was methanol with 0.1% formic acid. The flow rate for each phase was set at 0.3 mL/min. The gradient began at 0 min with 85% mobile phase A and maintained this composition until 2 min. At 6 min, the gradient transitioned to 20% mobile phase A, followed by 10% at 10 min, 5% at 15 min, and 1% at 25 min. Finally, at 31 min, the mobile phase returned to 85% A, which was sustained until the end of the run at 35 min. In positive mode, the capillary voltage was set at 2.0 kV, and the cone voltage was set at 40 V. The flow rate of nitrogen gas (N_2) used for desolvation was maintained at 800 L/h at a temperature of 350 °C. The cone gas flow rate was set to 50 L/h, and the source temperature was fixed at 120 °C. Data acquisition utilized a multiplexed MS/MS method known as MSE, which alternated between low- and high-energy acquisition. This approach covered a mass-to-charge ratio (m/z) range of 50 to 2000 and operated in centroid mode. MS/MS tests were performed using ultrapure argon (Ar) as the collision gas, with varying collision energies from 15 to 40 V. For lock mass calibration, all acquisitions used leucine encephalin (Leu-Enk, m/z 554.262, [M-H]-). Data acquisition was performed using Mass Lynx 4.1 SCN 9.16

software from Waters, and the Progenesis QI 2.3 program was employed for the analysis of raw data and identification of metabolites.

Data analysis

The metabolite identification was carried out using METLIN software as per the protocol described by Siddaiah et al. (2021). The Agilent MassHunter Qualitative Analysis software version B.06.00 SP1 was employed to process MS and AutoMS/MS data obtained through LC/Q-TOF. To omit the molecular features data acquired from each fraction, background subtraction was performed using blank data in MPP to eliminate background features. The putative metabolite identification was carried out using ID browser software searching against the METLIN database (MassHunter PCDL Manager version B.04.00), which has an in-built database for 64,092 metabolites. Spectral information was matched with the Agilent Fiehn library and cross-referenced with the NIST 11 and Wiley 9 mass spectral libraries. Compounds with library match scores exceeding 70% were considered, and their therapeutic significance was explored through a literature review.

Results

Metabolite profiling of pearl millet seeds using different solvents

In the present study, 650 metabolites were identified by obtaining their closest match using METLIN software. The METLIN software facilitates the interpretation of spectra and aids in the characterization of metabolites. The study identified several chromatograms that correspond to separate metabolites, as shown in Supplementary File 1. Different solvent systems of various ranges of polarity (from hydrophobic to hydrophilic) such as ethyl acetate, hexane, methanol, and a mixture of chloroform and methanol were used for the extraction of metabolites. A total of 145 metabolites were consistently recovered from all the examined solvents (Fig. 1) and comprehensive information about the same has been provided in Table 1.

The ethyl acetate extract exhibited the presence of terpenoids (23%), amino acids (16%), fatty acids (6%), carbohydrates (5%), sterols, and flavonoids (3%) (Fig. 2a). Additionally, the presence of sphing-4-enine-1-phosphate and sphingosine-1-phosphate as well as vitamins and cofactors were noted, suggesting possible nutritional benefits. The hexane extract revealed the presence of terpenoids (20%), amino acids (19%), fatty acids (16%), sterols (6%), flavonoids (5%), carbohydrates (4%), and other identified metabolites included are 2-methyl-4-amino-5-(formylaminomethyl) pyrimidine, tropinone, and l-alpha-diamino-beta-dithiolactic acid were detected in the hexane extract (Fig. 2b). The 80% methanol extract of pearl millet seeds revealed the availability of amino acids (19%), fatty acids (16%), terpenoids (16%), flavonoids (7%), carbohydrates (6%), sterol (3%), and alkaloids (3%) such as N-acetyl-5-methoxytryptamine, nicotinamide adenine dinucleotide (NAD), xanthine, and tropinone (Fig. 2c). The extraction was also performed using the combination of chloroform and methanol in which methanol formed an upper layer. The methanolic layer yielded amino acids (16%), fatty acids (16%), terpenoids (14%), carbohydrates (7%) flavonoid-related metabolites (4%), sterols (4%), and alkaloids (2%) (Fig. 2d). The dot plot of these metabolites derived from different solvents based on *p*-value is cited in Fig. 3.

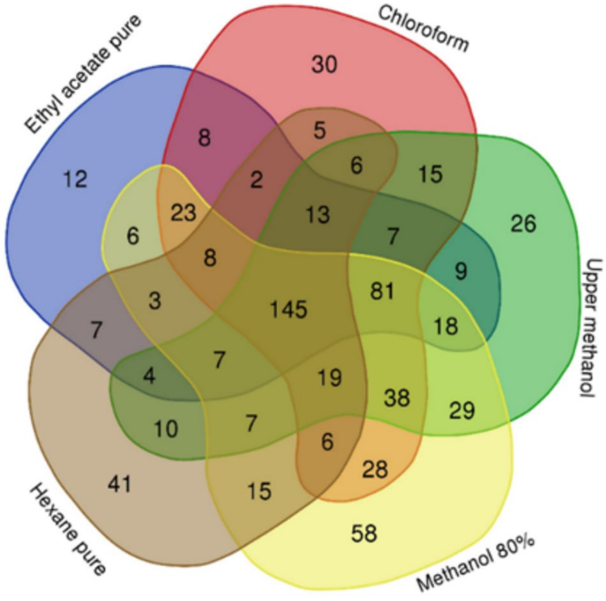

Fig. 1 Distribution of pearl millet seed metabolites across various solvents in the METLIN library

Biological activities of major metabolites from pearl millet seeds

The effectiveness of certain metabolites in managing human diseases was evaluated using enrichment ratios and *p*-values. A summary of the metabolites derived from the hexane extract indicated that there were between 70 and 79 metabolites with biological activity ($p < 4\text{e-}35$) (see Fig. 4b). Figure 4a illustrates the enrichment ratio of the metabolites identified in the ethyl acetate extract which showed between 86 to 90 metabolites with significant biological activity ($p < 3\text{e}{-}33$). Additionally, a modest number of metabolites (22) are reported to have distinct biological functions and could be used in the treatment

Table 1 List of metabolites present in extracts of different solvents

	Ethyl acetate	Hexane	Methanol (80%)	Chloroform and methanol
Terpenoids	Farnesal, gibberellin A8, kaurenol, myrcene, plastoquinol A, abscisic acid, 6-deoxocathasterone, 6-deoxotyphasterol, phytofluene, solanesyl pyrophosphate, etc	8-hydroxy geraniol, gibberellin A8, 6-geranylgeranyl-2-methylbenzene-1,4-diol, plastoquinol A, etc	Teasterone, phytoene, geranyl diphosphate	Gibberellin A8, gamma-tocotrienol, subaphyllin, 3β,20-dihydroxylupane, 10,16-dihydroxyhexadecanoic acid, docosahexaenoic acid, gibberellin A12 aldehyde, 3β-hydroxyergosta-7, 24(24(1)).—dien-4alpha-carboxylate, gibberellin A9, 22-hydroxydocosanoic acid, phytosphingosine, phytosterols, etc
Amino acids	Lysine, uridine, L-arginine, pyruvic acid, histidine, etc	Lysine, L-tryptophan, ornithine, glycine, L-histidine, etc	Lysine, L-homocysteine, L-selenocysteine, 2-(4'-methylthio) butylmalate, ornithine, L-tyrosine, phenylalanine, D-pantothenoyl-L-cysteine, citrulline, etc	Lysine, uridine, L-arginine, pyruvic acid, histidine, L-homocysteine, L-argininosuccinic acid, etc.,
Carbohydrates and glycoconjugates	Glucoiberverin, UDP-6-sulfoquinovose	UDP-4-keto-6-deoxy-D-glucose, GDP-4-oxo-6-deoxy-D-mannose, UDP-rhamnose, 1-alpha-D-Glucopyranosyl-2-β-D-fructofuranoside, stachyose, sorbitol, etc	D-ribose, 3-β-D-galactosyl-sn-glycerol, xylitol, saccharopine, UDP-D-glucose, 6-phosphosucrose, sorbitol, D-ribose, UDP-D-glucose, etc	Glucoiberverin, UDP-6-sulfoquinovose, CMP-2-trimethyl aminoethyl phosphonate, UDP-D-glucose, UDP-glucuronate, D-ribose, L-fucopyranose, xylitol, L-menthone, etc
Sterols	Ergosterol, stigmasterol, β-sitosterol and campesterol	episterol, β-sitosterol, castasterone	Episterol, spinacene, β-sitosterol, 6-deoxocastasterone	Ergosterol, stigmasterol, β-sitosterol, etc
Flavonoids and related metabolites	Apigenin, delphinidin, pelargonidin, quercetin, and kaempferol	Myricetin, cyanidin 3-glucoside, leucopelargonidin, pelargonin, garbanzol, etc	3,7,4'-trihydroxy flavanone, homoeriodictyol chalcone, 4',7-dihydroxy flavanone, kaempferol, cyanidin 3-O-glucoside, and pelargonidin 3,5-di-β-D-glucoside	Homoeriodictyol chalcone, phoenicoxanthin, 3'-hydroxyechinenone, 4-(3-hydroxy-1-propenyl)-2-methoxy phenol, 3'-hydroxyechinenone, 4-(3-hydroxy-1-propenyl)-2-methoxy phenol, kaempferol
Vitamins and cofactors	Thiamine, riboflavin, and biotin			
Lipids	Sphing-4-enine-1-phosphate and sphingosine-1-phosphate			
Fatty acids	–	Linoleic acid, oleic acid, palmitic acid, arachidic acid etc	Arachidic acid, palmitoleic acid, docosahexaenoic acid, linoleic acid, stearic acid, etc	Arachidic acid, palmitoleic acid, 9-oxononanoic acid, linoleic acid, stearic acid

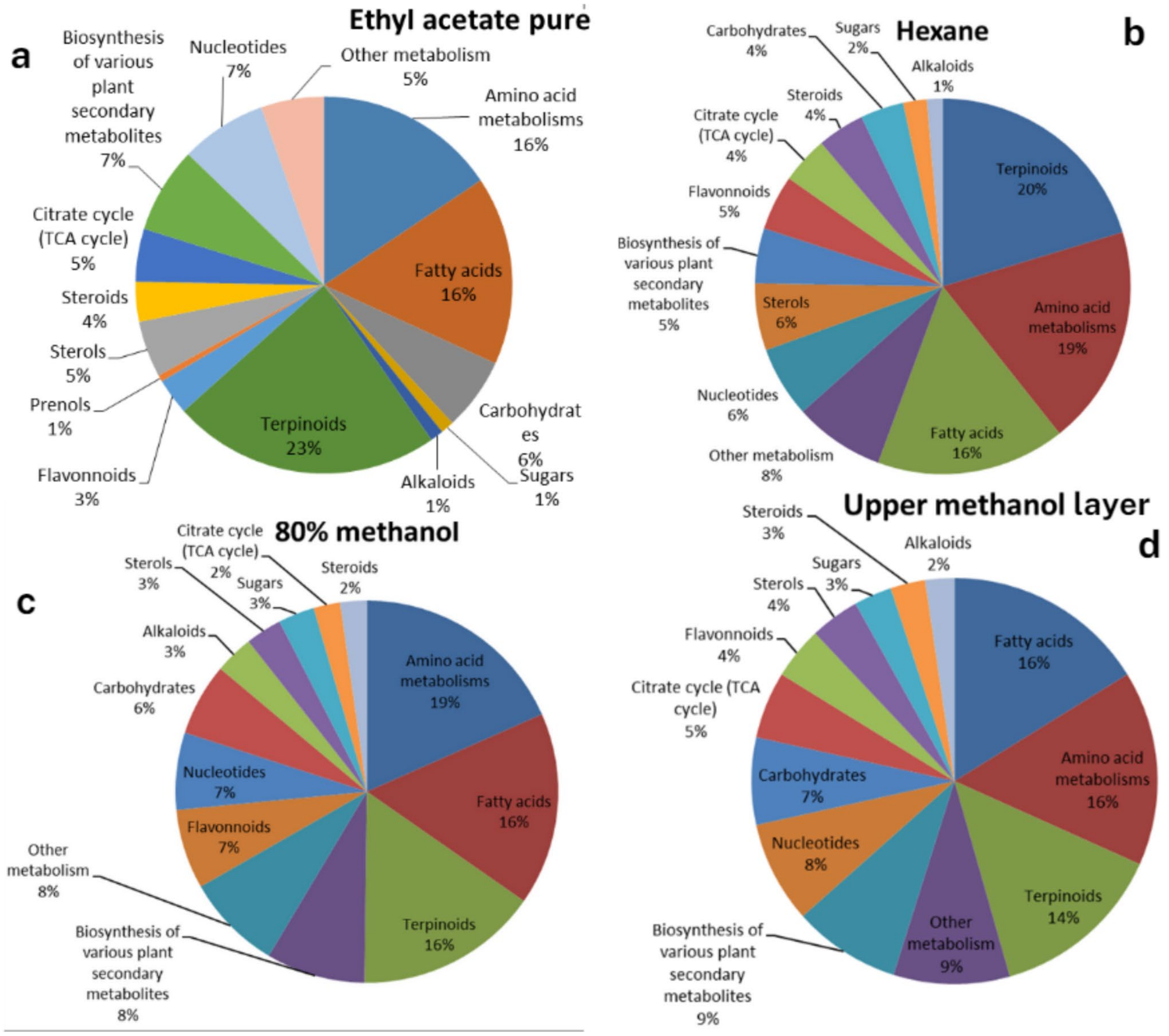

Fig. 2 Distribution of metabolites derived from different solvents: **a** ethyl acetate **b** hexane **c** 80% methanol **d** upper methanol extracts of pearl millet seeds

of disorders such as cirrhosis, N-acetylglutamate synthetase insufficiency, and lipoyltransferase 1 deficiency ($p < 3e-05$). The enrichment ratio of the metabolites from the 80% methanol extract, as shown in Fig. 4c, resulted in the presence of approximately 100 to 105 metabolites. These metabolites are reported to have different biological activities in schizophrenia, epilepsy, uremia, isovaleric acidemia, Canavan disease, etc. ($p < 4e-42$). Similarly, a moderate number of metabolites (approximately 21) are reported to be used in the management of diseases like gestational diabetes, essential hypertension, pancreatic cancer, etc. ($p < 7e-06$), The upper methanol layer contains approximately 80 metabolites with an enrichment ratio of 10 and ($p < 5e-37$) (Fig. 4d).

Discussion

The phytochemical analysis of pearl millet revealed a diverse profile of bioactive compounds, highlighting its nutritional significance. Our study identified a total of 650 metabolites across all the solvents examined, as illustrated in Fig. 2. There are different groups for these metabolites, such as terpenoids, prenol lipids, carboxylic acids, and organooxygen compounds. These findings align with previous studies, highlighting the diverse range of metabolites present in pearl millet (Yadav et al. 2021). In our study, ethyl acetate extract showed the highest percentage of terpenoids (23%) encompassing compounds such as farnesal, gibberellin A8, kaurenol, and myrcene. In the earlier study on phytochemical

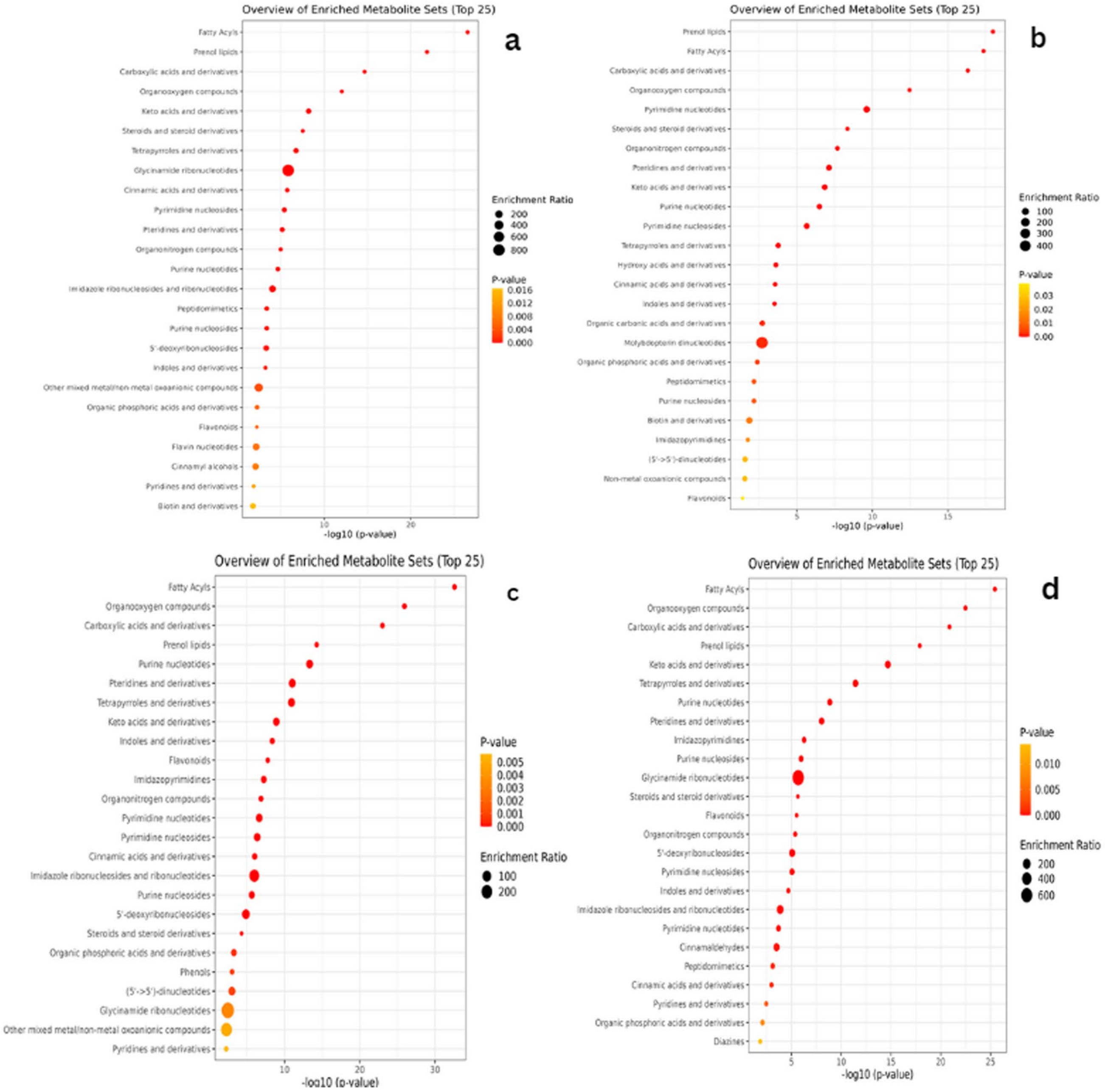

Fig. 3 Dot plot of metabolites from pearl millet seeds extracted with different solvents based on *p*-value: **a** ethyl acetate **b** hexane **c** 80% methanol **d** upper layer of methanol

screening of pearl millet by Ndiku & Mutuku (2015) have identified the presence of terpenoids, but the quantification of these metabolites has not been reported. Our findings are consistent with the previous reports on the phytochemical composition of other millet variants including foxtail millet (*Setaria italica*), where terpenoid-rich extracts have been linked to antioxidant and anti-inflammatory properties (Suma and Urooj 2012). As per the reports of Pujari and Hoskeri (2022), the solvent extracts of Little millet and Proso millet have also shown the presence of similar terpenoids, albeit in varying quantities. Our study also demonstrated that pearl millet possesses a unique amino acid composition which accounts for about 16%. The amino acid profile of pearl millet was found to be similar to that of foxtail millet, both being rich in essential amino acids; however, the specific concentrations and types of amino acids vary between the two (Wang et al. 2024). Finger millet is known for its higher lysine content, rendering it a significant protein source (Bangar et al. 2022). In contrast, sorghum generally has reduced lysine content, indicating that pearl

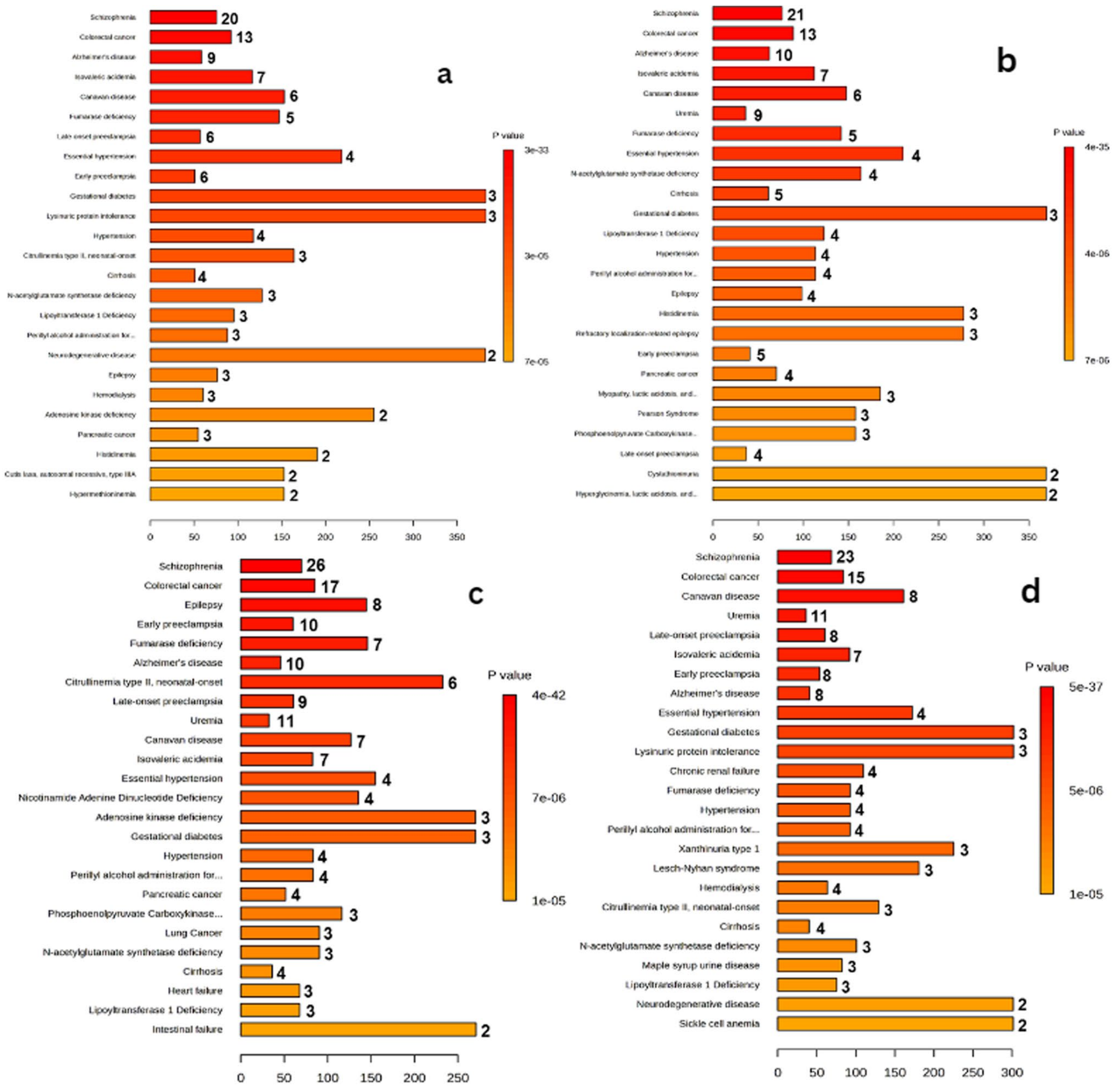

Fig. 4 Biological activity of pearl millet seeds metabolites extracted with: **a** ethyl acetate **b** hexane **c** 80% methanol **d** upper layer of methanol extract

millet may serve as a superior option for mitigating protein malnutrition (Hegde et al. 2025). The present study indicates that pearl millet contains 5% flavonoids. Our findings are consistent with the flavonoid content found in other millets, such as foxtail millet and barnyard millet, which have been reported to contain flavonoids like quercetin derivatives, kaempferol, apigenin, and dihydroquercetin (Ofosu et al. 2020). Finger millet contains flavonoids such as quercetin, found in the seed coat, which exhibit multifunctional properties and possess antioxidant activity (Devi et al. 2014). Flavonoids significantly enhance the antioxidant capacity of millets, facilitating the reduction of oxidative stress and inflammation (Goudar et al. 2023). Flavones like luteolin and tangeritin, commonly found in diverse dietary sources, are also present in millets, with their specific categorization differing according to the type of millet and its distinct composition (Sadh et al. 2024). These findings establish millet as an important dietary element with notable health benefits. Numerous polyphenols were identified, including protocatechuic acid, vanillic acid, syringic acid, and coumaric acid.

Polyphenols have been identified in various millets (Sadh et al. 2024) and exhibit promising antimicrobial and antioxidant properties (Eelager et al. 2023), along with potential anticancer and neuroprotective effects. Millets are acknowledged for their potential health benefits, including antidiabetic (Alzahrani et al. 2022), anticancer (Hajri et al. 2024), antioxidant, anti-inflammatory, and antiobesity properties, attributed to their high content of bioactive compounds (Sadh et al. 2024). This study highlights the carbohydrate content in pearl millet, which is comparable to other millet varieties. Finger millet is rich in complex carbohydrates, dietary fiber, and resistant starch, making it a suitable diet for diabetic patients due to its low glycaemic index (Devi et al. 2014). Foxtail millet offers high levels of polysaccharides and slowly digestible starches for sustained energy. Barnyard millet is noted for its high dietary fiber, which aids digestion and weight management, while Proso millet provides a balance of simple and complex carbohydrates as a reliable energy source (Nanje Gowda et al. 2024). Pearl millet, however, has a unique carbohydrate composition that includes specific sugars and phosphorylated compounds, suggesting potential benefits for energy metabolism, and making it a valuable part of a nutritious diet. The extraction of these metabolites using various solvents demonstrates a significant diversity of metabolites and potential applications across multiple fields, including medicine, agriculture, and nutrition. Numerous flavonoids identified in the ethyl acetate extract were reported to exhibit significant biological activities. For instance, apigenin is noted for its anti-inflammatory, anticancer, and neuroprotective properties, as well as its role in managing ulcerative colitis through modifications of the intestinal microbiota (Fu et al. 2022). Delphinidin and pelargonidin are known for their anticancer properties (Sharma et al. 2021). Quercetin has been demonstrated for its antimicrobial and anti-inflammatory properties (Yang et al. 2020), while kaempferol also exhibited many types of pharmacological activities. The presence of various phytochemicals indicates potential antioxidant and anti-inflammatory effects. Compounds like sphing-4-enine-1-phosphate and sphingosine-1-phosphate are reported to modulate cell signaling (Chen et al. 2022). The presence of phytosterols, including beta-sitosterol and campesterol, suggests potential benefits for lowering cholesterol levels (Yuan et al. 2019). The identification of various vitamins and cofactors, such as thiamine, riboflavin, and biotin, indicates potential nutritional benefits.

Conclusion

The analysis of secondary metabolites derived from pearl millet seeds revealed a diverse array of compounds, with ethyl acetate and 80% methanol extracts demonstrating the highest concentrations of terpenoids and flavonoids, respectively. The METLIN database was utilized to ascertain the metabolites obtained from various solvents. Enrichment analysis identified significant connections with several diseases, including schizophrenia, colorectal cancer, Alzheimer's disease, pancreatic cancer, hypertension, and lung cancer. The metabolites present in the elevated methanol extract suggest a probable association with conditions such as uremia, schizophrenia, and early preeclampsia. The findings enhance our comprehension of the therapeutic properties of pearl millet, hence expanding potential applications in dietary management and pharmacology.

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Code availability Not applicable.

Declarations

Conflict of interest The authors declare no conflict of interest.

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Consent to participate Not applicable.

Consent for publication Not applicable.

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