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INTEGRATED NETWORK PHARMACOLOGY, MICROARRAY ANALYSIS AND MOLECULAR DYNAMICS TO IDENTIFY A SUBSET OF POTENTIAL CAMEL MILK AND URINE BIOACTIVE COMPOUNDS IN TREATING AUTISM SPECTRUM DISORDER

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ABSTRACT

Camel milk and urine (CMU) are traditionally known for their medicinal properties, with camel milk has been reported to improve Autism Spectrum Disorder (ASD) symptoms. ASD is a complex neurodevelopmental disorder with no universally effective treatment. This study employs integrated network pharmacology, cheminformatics, microarray analysis and molecular modeling approaches to explore the potential therapeutic effects of a subset of camel CMU compounds for ASD. In our study, CMU compounds were analyzed collectively to explore their combined therapeutic potential in treating ASD, with the final impact assessed based on individual bioactive compounds. The study utilized a combination of active compounds collection and screening, potential target genes screening against autism, pathway and functional enrichment analysis, network construction, protein-protein interaction (PPI) network, docking and ADMET profiling. Twenty-two active compounds were identified in camel CMU, with 169 genes found to be common targets. Ten hub ASD-related genes, comprising ESRI, AKT1, CTNNB1, EGFR, CASP3, ERBB2, MMP9, PTGS2, JAK2, and GSK3β. Network analysis revealed a multi-target and multi-pathway interaction, suggesting a synergistic effect of CMU compounds on ASD. Molecular docking studies indicated strong binding affinities between selected compounds and key target proteins associated with ASD, such as AKT1 and GSK3β. ADMET profiling confirmed the pharmacokinetic viability of these compounds as therapeutic agents and molecular dynamics simulations confirmed the top compounds-GSK3β complexes stability. This study provides a foundation for understanding the therapeutic potential of CMU compounds against ASD, highlighting their multi-target and multi-pathway mechanisms. CMU compounds, particularly homogentisic acid, 4-hydroxyphenylacetic acid, 4-hydroxyphenylpyruvic acid, and phenylpyruvic acid, have the potential to modulate biological pathways implicated in ASD.

Keywords: Autism, Camel milk, Network Pharmacology, Enrichment Analysis, Molecular Modeling

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List of abbreviations: ASD - Autism Spectrum Disorder, CMU - Camel Milk and Urine, PPI - Protein-Protein Interaction, ADMET - Absorption, Distribution, Metabolism, Excretion, and Toxicity, AKT1 - AKT Serine/Threonine Kinase 1, CTNNB1 - Catenin Beta 1, EGFR - Epidermal Growth Factor Receptor, CASP3 - Caspase 3, ERBB2 - Erb-B2 Receptor Tyrosine Kinase 2, MMP9 - Matrix Metallopeptidase 9, PTGS2 - Prostaglandin-Endoperoxide Synthase 2, JAK2 - Janus Kinase 2, GSK3β - Glycogen Synthase Kinase 3 Beta, DL - Drug-Likeness, ADME - Absorption, Distribution, Metabolism, Excretion, DAVID - Database for Annotation, Visualization, and Integrated Discovery, GO - Gene Ontology, KEGG - Kyoto Encyclopedia of Genes and Genomes, STRING - Search Tool for the Retrieval of Interacting Genes/Proteins, DEGs - Differentially Expressed Genes, BBB - Blood-Brain Barrier, P-gp - P-glycoprotein, RMSD - Root Mean Square Deviation

INTRODUCTION

Autism is a complex neurological condition with a polygenic basis, affecting about 1 in 59 children (Rylaarsdam and Guemez-Gamboa 2019). The development of effective and affordable treatment options for ASD is critically required, as the disease's incidence rises worldwide (Mazzone *et al*. 2017). De novo drug discovery, however, is a costly and drawn-out procedure; it takes over a billion dollars and over ten years to create a new medication (Xue *et al*. 2018). Autism spectrum disorder (ASD) is not well-suited to the conventional drug development approach, which finds lead compounds with action against one or more specific therapeutic targets. ASD poses a lifelong threat and challenge to affected individuals (Hirvikoski *et al*. 2016). ASD patients are more likely to die younger than healthy controls, and the disorder has an impact on behavior and social skills. To treat ASD, it is therefore necessary to develop novel and efficient drug discovery techniques. Network pharmacology is a compelling approach to find possible ASD treatment medications (Gao *et al*. 2021). The authentic way of identification of ASD pathogenic genes, the disclosure of molecular pathways, and the identification of therapeutic targets that present opportunities for drug repositioning are all made achievable by the network-based computational approach.

The highly variable genetic structure and complicated clinical features of ASD are mostly due to gene interactions, as the disorder is polygenic (Bourgeron 2015). An efficient way to assess gene interactions is through network analysis, which can be applied to high-dimensional transcriptome data to create plausible gene interaction networks with related functions. These genes and their associated pathways could be new targets for ASD drugs (Parikshak *et al*. 2016). Furthermore, protein-protein interactions, drug–target interactions, and drug-disease modules can be arranged into a network using network-based analysis techniques to provide information on molecular mechanisms and possible therapeutic drugs (Gao *et al*. 2021, Park *et al*. 2017).

Camel milk shows promise as a therapeutic intervention for ASD, with studies indicating positive effects on behavioral characteristics and oxidative stress biomarkers in affected children. A randomized controlled trial evaluated camel milk's effects on 65 ASD-diagnosed children aged 2 to 12. Assessments using the Autism Rating Scale (CARS), Social Responsiveness Scale (SRS), and Autism Treatment Evaluation Checklist (ATEC) scales before and after two weeks of camel milk therapy revealed significant improvements in ASD symptoms compared to a placebo group (Al-Ayadhi *et al*. 2015). Furthermore, a recent study investigated the effects of camel milk consumption on oxidative stress biomarkers in autistic children. Plasma levels of various antioxidants were measured before and after two weeks of camel milk intake. Significant improvements were observed in all measured parameters after camel milk consumption, indicating its potential to reduce oxidative stress and improve antioxidant levels. The study also noted behavioral improvements, supported by enhanced CARS scores (Al-Ayadhi and Elamin 2013). A recent meta-analysis qualitative synthesis supported the benefits of camel milk in autism, albeit with statisitcal limitations (Kandeel *et al*., 2024).

CMU are increasingly recognized for their unique medicinal properties, offering a potent combination of bioactive components not found in other natural products. Camel milk is rich in immunoglobulins, lactoferrin, and lysozymes, which provide strong antimicrobial, antiviral, and anti-inflammatory benefits, making it suitable for individuals with lactose intolerance or dairy allergies (Al-Ayadhi and Elamin 2013, Alkattan *et al*. 2023). Additionally, camel urine has been traditionally used for its antimicrobial properties, providing a multifaceted approach to health that addresses a wide range of conditions from metabolic disorders to infectious diseases (Amina *et al*. 2024).

This study's goal is to utilize network pharmacology and computational tools to analyze and uncover new insights into the beneficial effects of CMU on ASD from an analytical and new discovery perspective. The use of network pharmacology to elucidate the therapeutic effects of camel milk on ASD represents a modern scientific approach aimed at uncovering the complex interactions between the bioactive components of camel milk and the biological mechanisms of ASD. This study could identify the molecular targets and signaling pathways influenced by CMU, shedding light on its multi-target and multi-pathway mechanisms. By integrating traditional knowledge with cutting-edge network analysis, this research holds the potential to validate camel milk's therapeutic use, discover novel ASD therapeutic targets, and contribute to the development of new treatment strategies. Moreover, this study offers a computational foundation and potential explanations for the mechanisms behind the observed enhancements in cases of ASD when patients are nourished with camel milk or compounds derived from camel urine.

MATERIALS AND METHODS

Active Compounds Collection and Screening: The study sourced data from bioactive chemicals present in CMU by reviewing scholarly documents and structures from databases such as Google Scholar, PubMed, PubChem and the previously published patent (Iglesias Pastrana *et al*. 2022, Tony 2010). This research undertook in silico analysis to evaluate the bioavailability and drug-likeness (DL) properties of a subset of

chemicals in CMU. To advance in the trial, a chemical had to meet particular ADME benchmarks, such as a minimum DL score of 0.18 and a bioavailability score (F) of at least 0.30. SwissADME and Molsoft were used to calculate F and DL scores for each bioactive molecule. Those that did not match these requirements were not investigated further.

Potential Target Genes Screening against Autism: The potential targets of the selected compounds from CMU were predicted using the SwissTargetPrediction (Gfeller *et al*. 2014) and STITCH databases (Szklarczyk *et al*. 2016). The compounds that were retrieved underwent further analysis in the STITCH database to pinpoint their potential targets, focusing specifically on those relevant to humans ('Homo sapiens'). Only targets that achieved a cumulative score of 0.7 or higher were chosen for additional investigations. To identify these targets, the online resource SwissTargetPrediction was employed, using a reverse pharmacophore matching approach based on the SMILES notation of each compound's ingredients.

The databases GeneCards (Safran *et al*. 2010) and Online Mendelian Inheritance in Man (OMIM) were accessed using the keywords 'Autism' and 'Autism spectrum disorder' to obtain genes associated with the disease. Following that, the process involved predicting the target genes for the compounds found in CMU, as well as those associated with Autism. Subsequently, a Venn diagram was constructed to illustrate the overlap of targets between these two categories, identifying the common genes.

Pathway and Functional Enrichment Analysis: The Database for Annotation, Visualization, and Integrated Discovery (DAVID) was utilized to conduct an in-depth functional annotation and enrichment analysis (Sherman *et al*. 2007). This approach allowed to examine the primary targets through DAVID's analytical framework, aiming to forecast their roles across three distinct dimensions: biological processes (BP), molecular functions (MF), and cellular components (CC). In the ambit of this study, a selection was made to focus on the top 20 Gene Ontology (GO) enrichments alongside the top 20 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that demonstrated significant numerical prominence. This refined selection was subjected to further analytical scrutiny, adhering to a rigorously adjusted p-value threshold of 0.05.

Construction of Network: A network analysis was conducted to uncover the role of CMU-derived compounds in Autism. The analysis involved creating and visualizing a network with Cytoscape 3.9.1, an open-source graphical interface used for importing, visually navigating, and analyzing networks of biomolecular interactions. Within this network, active compounds and target genes were depicted as nodes, with their relationships illustrated as connecting lines, or edges. The significance of compounds, target genes, and pathways within the network was determined by calculating their degree, a measure of connectivity importance, using the network analyzer tool. Furthermore, "key targets" were identified as those genes with the highest connectivity.

Protein–Protein Network (PPI) Construction: To find the functional relationships among important targets with a combined score greater than 0.4, the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) was employed (Mering *et al*. 2003). To examine the PPI network's main regulatory genes as well as key targets, the STRING-obtained PPI network was run using the CytoHubba plugin of Cytoscape.

Microarray Data Analysis: The microarray dataset 'GSE29691' was obtained from the NCBI GEO database (https://www.ncbi.nlm.nih.gov/geo/) on the GPL570 platform to corroborate the results of this research. The GEO database serves as a collection point for high throughput chip, gene expression, microarray, and hybridization array data. This particular dataset comprises 15 samples, with 13 serving as controls and 2 being affected, all derived from whole blood. The 'Limma' package in the R programming language was then employed to identify differentially expressed genes (DEGs) under the criteria of an adjusted p-value < 0.05 and an absolute value of log (fold change) ≥ 1 or ≤ -1. A volcano plot was created to display genes with significant and insignificant expression levels. Subsequently, DEGs and key genes were analyzed to isolate common genes for further examination.

Molecular Docking and ADMET Profiling: The selection and identification of candidate compounds and their potential targets were primarily based on the docking scores obtained between the target and the compounds. The precision of each docked complex was assessed by choosing the model with the greatest absolute binding energy value. Chimaera X-and Discovery Studio were utilized to visually represent the interactions between the active compounds and the protein targets. The ucsf chimera tool was used to accomplish the structural refining process. To prevent clashes and improper configurations, non-standard residues were also eliminated from the protein's receptors and energy minimization was carried out in 1000 decent steps.

Molecular dynamics simulation: Molecular Dynamics Simulation of the selected complexes was conducted at 100 ns using Desmond software as previously described (Kandeel and Suganuma 2022, Kandeel and El-Deeb 2022). Briefly, the complexes were preprocessed, optimized, and minimized with OPLS_2005 forcefield, incorporating TIP3P solvent model and 0.15 M NaCl to mimic physiological conditions.

RESULTS

3.1. Screening of Active Compounds and Their Targets: Following a search, using search filters, and elimination of duplicates, a total of twenty two potential compounds (4-hydroxyphenyl acetic acid, phenylpyruvic acid, phenylacetic acid, benzaldehyde, benzoic acid, hippuric acid, phenylbutyrate, homovanillic acid, 4-hydroxyphenylpyruvic acid, 3-hydroxybutyric acid, homogentisic acid, 4-hydroxy-4-methyltetrahydro-2H-pyran-2-one, 2-hydroxyphenylacetic acid, phenol, phenyl acetate, acetoacetic acid, methylmalonic acid, 2-methylcitric acid, creatinine, meglutol, methylsuccinic acid and ethylmalonic acid) were identified. These compounds were selected based on their F value of 0.30 and DL value of 0.18, as shown in Table 1.

In addition, the SwissTargetPrediction database revealed 352 possible target genes associated with 15 active components. Upon identification of the potential targets of drugs, a total of 4053 genes associated with autism were obtained from the GeneCards and OMIM databases. Subsequently, a Venn diagram was used to determine the shared targets between autism and the genes associated with the compounds targets (Figure 1). About 169 genes were identified as common between CMU compounds targets and autism disease putative genes and considered as key targets. CytoHubba was implemented to select the top 100 targets from these 169 genes employing the 11 cytoHubba algorithms.

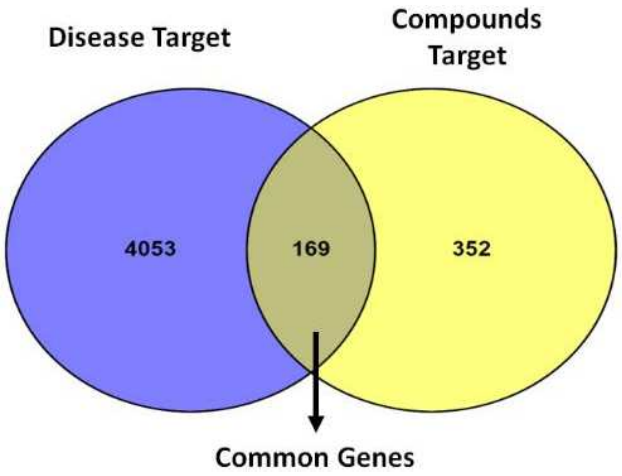

Figure 1. Venn diagram illustrating the overlap between disease-specific targets and compound targets. The figure represents the intersection between genes associated with ASD and genes targeted by various compounds. The blue circle on the left indicates the total number of genes implicated in ASD (4,053), while the yellow circle on the right represents the genes targeted by compounds under investigation (352). The overlapping area in light green shows the common genes (169) that are both associated with ASD and are targets of the compounds,

Table 1. The screening results show 22 CMU active compounds with their properties.

Serial No.	Molecule name	MW (g/mol)	DL	F	PubChem ID
01	4-Hydroxyphenylacetic acid	152.15	0.83	0.85	127
02	Phenylpyruvic acid	164.16	1.19	0.85	997
03	Phenylacetic Acid	136.15	1.32	0.85	999
04	Benzaldehyde	106.12	1.8	0.55	240
05	Benzoic Acid	122.12	1.0	0.85	243
06	Hippuric Acid	179.17	0.41	0.85	464
07	Phenyl butyrate	164.2	1.03	0.55	20354
08	Homovanillic Acid	168.15	0.46	0.85	1738
09	4-Hydroxyphenylpyruvic acid	180.16	0.59	0.85	979
10	3-Hydroxybutyric acid	104.1	1.05	0.85	441
11	Homogentisic acid	168.15	0.22	0.56	780
12	4-Hydroxy-4-methyltetrahydro-2H-pyran-2-one	130.139	0.99	0.55	10428
13	2-Hydroxyphenylacetic acid	152.15	0.96	0.85	11970
14	Phenol	94.11	1.07	0.55	996
15	Phenylacetate	136.15	1.24	0.55	31229
16	Acetoacetic acid	102.09	1.47	0.85	96
17	Methylmalonic acid	118.09	1.08	0.85	487
18	2-Methylcitric acid	206.15	0.18	0.56	515
19	Creatinine	113.12	0.96	0.55	588
20	Meglutol	162.14	0.46	0.56	1662
21	Methylsuccinic acid	132.11	0.84	0.85	0.83
22	Ethylmalonic acid	132.12	1.04	0.85	1.04

Compounds-Target Network Construction: A collection of the 22 active chemicals, along with 100 primary targets and their associated pathways, was chosen. These components were employed to construct a network diagram that illustrates the relationships between the active compounds and the targeted genes (referenced in Figure 2). Each active compound is connected to multiple targets, indicating a complex interaction network. This complexity suggests the possibility of a synergistic effect arising from the interaction of multiple targets when compounds found in CMU are utilized for anti-autism purposes. Such a complex interaction implies that the combined effect of these compounds on the targets could significantly enhance the therapeutic potential of camel milk in treating autism, highlighting the network of biological interactions that underpin its efficacy.

Table 2 displays a classification of various chemical components, organizing them according to their degree of interaction. It specifically highlights the interaction levels of the top 15 chosen compounds. Among these, compounds with the highest degree scores were selected for further analysis through molecular docking. Notably, homogentisic acid demonstrated a connectivity degree value of 40, making it one of the most interconnected compounds. Close behind, 4-hydroxyphenylacetic acid showed a connectivity degree of 35. Additionally, 4-hydroxyphenylpyruvic acid and phenylpyruvic acid were also prominent, possessing connectivity degrees of 33 and 29, respectively. This systematic categorization and subsequent selection for molecular docking emphasize the significance of these compounds based on their potential interactions, laying the groundwork for more detailed biochemical investigations.

PPI Network Construction: To construct the PPI network, the overlapping set of 169 genes was uploaded to the STRING database. This step was instrumental in illustrating the complex interplay among various targets involved in the progression of the disease, as represented by the nodes and their connections within the PPI network (Figure 3A). Furthermore, the cytoHubba tool was applied to these PPI interactions involving the 169 targets, from which the top 100 genes were identified based on a comprehensive statistical analysis that included the number of nodes, edges, average number of neighbors, path length characteristics, clustering coefficient, and network density, which were found to be 10, 108, 21.64, 2.04, 0.29, and 0.10, respectively (Figures 3B and 3C).

In an additional step, the top 10 genes were pinpointed using the 11 algorithms provided by cytoHubba, highlighting the results from a further examination of the PPI network of overlapping genes with the help of a network analyzer tool. The genes with the most connections, indicating significant overlap, were ESRI, AKT1, CTNNB1, EGFR, CASP3, ERBB2, MMP9, PTGS2, JAK2, and GSK3B (Figure 4A). Moreover, the co-expression patterns of these central hub genes were analyzed (Figure 4B). This analysis suggests that these genes are critical targets for further investigation, as their high degree of connectivity within the network implies a potentially pivotal role in the biological processes under study (Table 3).

GO and KEGG Analysis: The functional annotation and enrichment analysis identified the possible biological roles of the targets of CMU compounds. Based on the GO functional analysis, the targets of CMU compounds were found to be associated with cellular response to chemical stress, response to oxidative stress, cellular response to organic cyclic compounds, and other related functions. A KEGG pathway analysis was carried out to determine the significant signaling pathways associated with the anti-autism action of CMU compounds. Significantly, the majority of the genes were implicated in the subsequent pathways: EGRF tyrosine kinase inhibitor resistance, prolactin signaling pathway, Endocrine resistance, IL-17 signaling pathways, TNF signaling pathway, and pathways in cancer. KEGG pathway analysis identified ESRI, AKT1, CTNNB1, EGFR, CASP3, ERBB2, MMP9, PTGS2, JAK2, and GSK3B as genes that showed high enrichment (Figure 5).

The compound-target networks and pathway-target networks were combined to illustrate the connections between candidate potential targets, bioactive chemical components, and disease processes (Figure 6). Every active component is connected to many targets that correspond to signaling pathways. This network serves as a roadmap for understanding the potential therapeutic effects and off-target impacts of the compounds, as well as their broader implications in disease pathology and treatment. The analysis identified key active chemicals (indicated by pink nodes) that interact with potential gene targets (green nodes), which in turn participate in critical biological pathways (blue triangular nodes). The network illustrates the multi-faceted nature of CMU compounds-target interactions and their extended influence on signaling pathways, providing a view of the potential mechanisms through which these compounds exert their effects.

Microarray Data Analysis: The microarray data was examined, leading to the identification of differentially expressed genes (DEGs). The genes retained the criteria of adj. p val < 0.05 and $|\log (FC)| \geq 1$, $|\log (FC)| \leq -1.0$ were considered as DEGs. Within the hub genes, only 1 gene named GSK3β was found. In the analyzed dataset, 500 upregulated and 115 downregulated genes were observed (Figure 7). The GDK3β was upregulated in this dataset. Further docking studies were based on AKT1 and

GSK3β, which were suggested by their interaction degree or overexpression in microarray data, respectively.

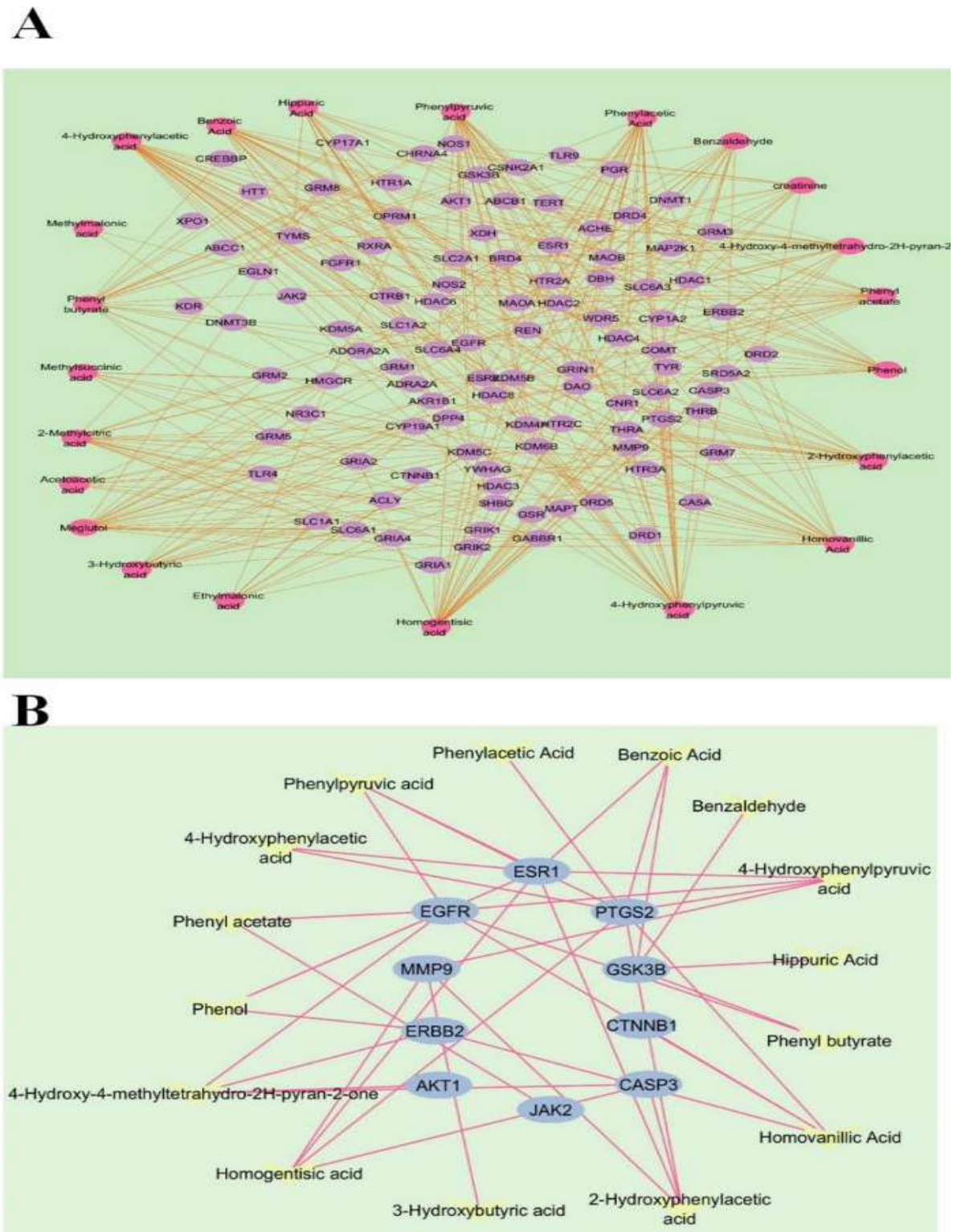

Figure 2. Compounds network interactions. A) Compound target network representation of CMU compounds and their target genes. The compounds are highlighted in purple, while the targets are in blue. The connections between these nodes are illustrated by lines. B) The interaction of the top 15 compounds with the top 10 hub genes.

Table 2. The degree and class of chemical components in the interaction analysis of chemicals.

Sr. No.	Compounds name	Class	Degree
01	Homogentisic acid	Phenolic	40
02	4-Hydroxyphenylacetic acid	Benzoid	35
03	4-Hydroxyphenylpyruvic acid	Alpha-keto acid	33
04	Phenylpyruvic acid	Alpha-keto acid	29
05	Phenylacetic Acid	Carboxylic acid	24
06	Homovanillic Acid	Phenolic	22
07	Phenyl butyrate	Aromatic fatty acid	20
08	Benzoic Acid	Carboxylic acid	20
09	4-Hydroxy-4-methyltetrahydro-2H-pyran-2-one	Lactones	19
10	2-Hydroxyphenylacetic acid	Phenolic	19
11	Phenylacetate	Aromatic	19
12	Phenol	Phenols	16
13	Hippuric Acid	Benzamides	15
14	Benzaldehyde	Aromatic aldehyde	15
15	3-Hydroxybutyric acid	Alkanoic acid	13

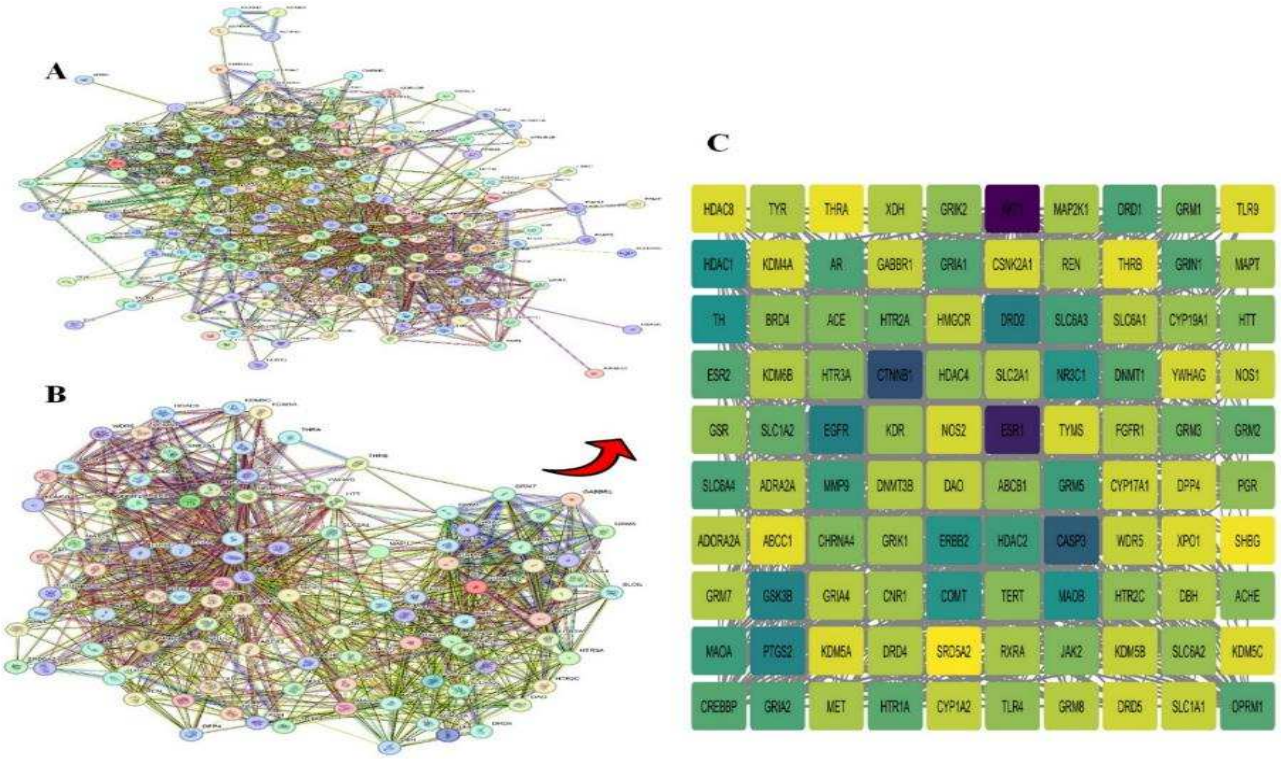

Figure 3. Protein-Protein Interaction (PPI) Network Analysis of Potential Targets: The PPI network was constructed using the STRING database, providing a visualization of the web of protein interactions within the studied biological system. Each node in the network represents a protein, while the edges denote evidence-based interactions derived from experimental data, databases, and computational predictions. Panel A illustrates the entire network, showing the complexity and interconnectedness of protein interplay. Panel B focuses on the top 100 genes based on their connectivity, highlighting these highly interactive hubs as critical components within the network. This selection emphasizes the most connected proteins, which play central roles in the biological pathways under investigation. Panel C presents an optimized PPI network generated with Cytoscape software, where nodes are color-coded by interaction intensity, with blue indicating the highest interaction scales. This enhanced visualization aids in identifying key proteins with significant interaction densities, thereby elucidating the hierarchical structure and functional modules within the network.

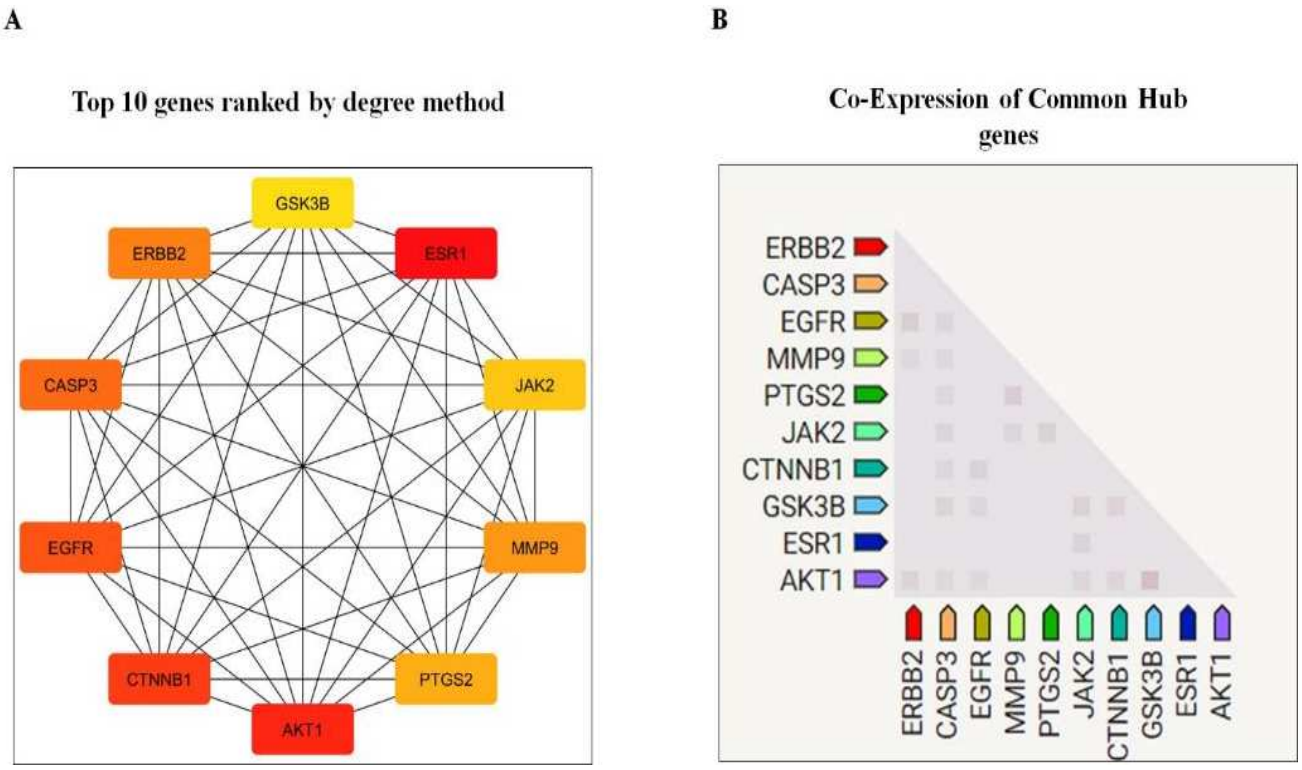

Figure 4. Top 10 hub genes with a higher degree of connectivity and their Co-Expression. Network representation of the top 10 genes ranked by the degree. Nodes represent individual genes. The nodes are color-coded: red indicates the gene with the highest degree of connectivity, and orange represents the other genes. Lines between the nodes denote the presence of a functional interaction or relationship between the genes. B. Heatmap displaying the co-expression levels of common hub genes. Each row and column correspond to one of the top 10 genes identified in the network analysis. The color intensity in the heatmap indicates the strength of co-expression, with darker shades representing higher levels of co-expression between the gene pairs.

Table 3. Top 10 genes ranked by connection degree. The top 10 genes were ranked by their degree of connectivity within a PPI network, as identified using cytoHubba algorithms. The degree indicates the number of connections to other genes, highlighting their significance in biological processes.

Sr. No.	Gene name	Degree
01	AKT1	65
02	ESR1	58
03	CTNNB1	49
04	CASP3	46
05	EGFR	39
06	GSK3B	38
07	PTGS2	38
08	ERBB2	34
09	MMP9	31
10	JAK2	22

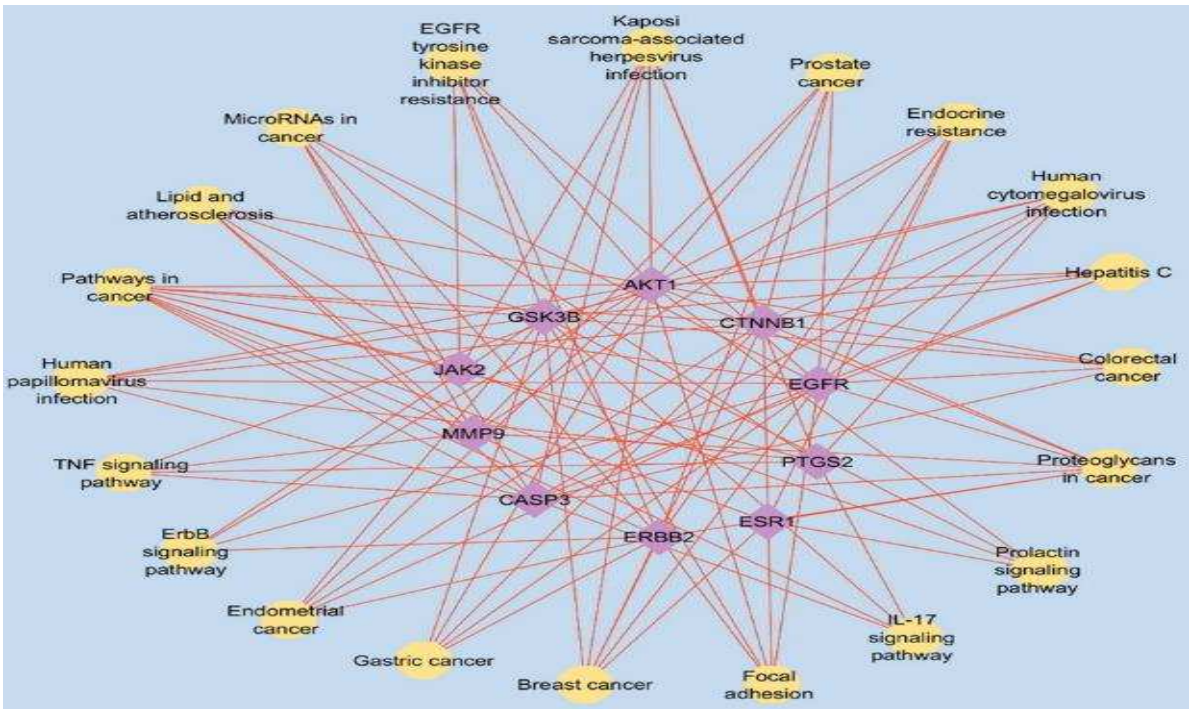

Figure 5. Targeted genes and pathway interaction. This network diagram visualizes the interactions between key genes (central nodes) and their associated biological pathways (peripheral nodes). Genes are shown as central hubs with multiple connecting lines, emphasizing their involvement in several pathways and potential as therapeutic targets.

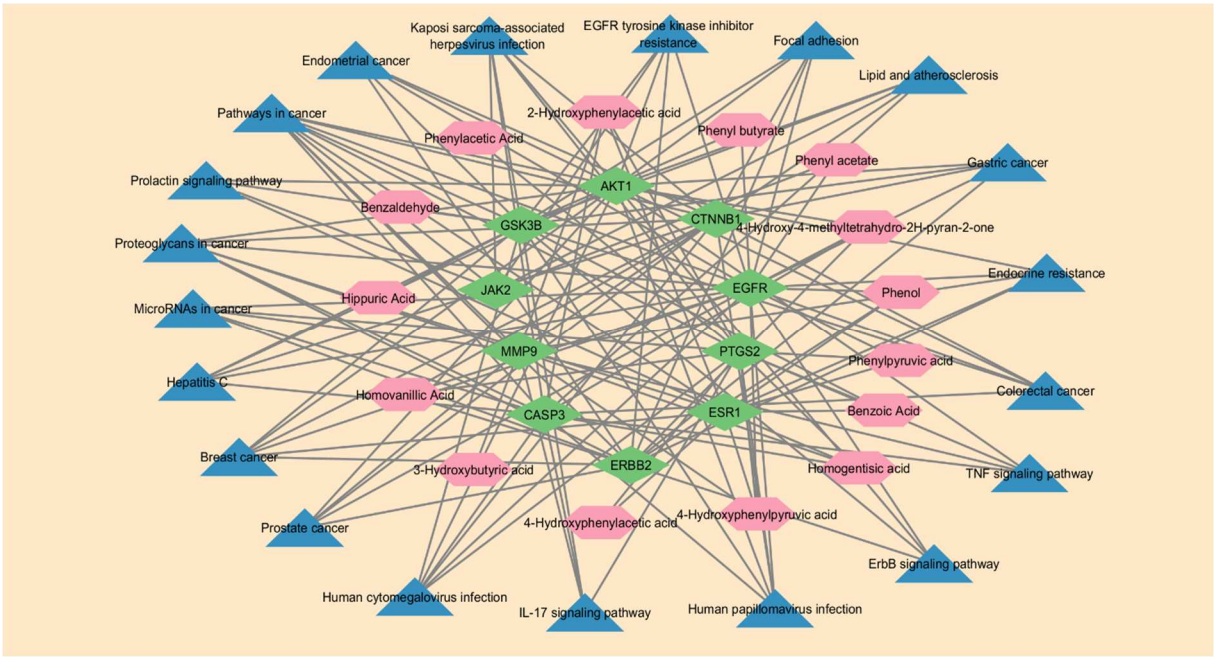

Figure 6. Network Visualization of Compound-Target-Pathway Interactions. The figure illustrates the complex interactions between bioactive compounds, their target genes, and associated biological pathways. The network was constructed using Cytoscape to provide insights into the molecular mechanisms of action. Pink nodes represent active compounds identified through chemical analysis. Green nodes denote potential target genes that these compounds may influence, highlighting their roles in cellular processes. Blue triangular nodes correspond to signaling pathways that are implicated by the interactions between compounds and genes.

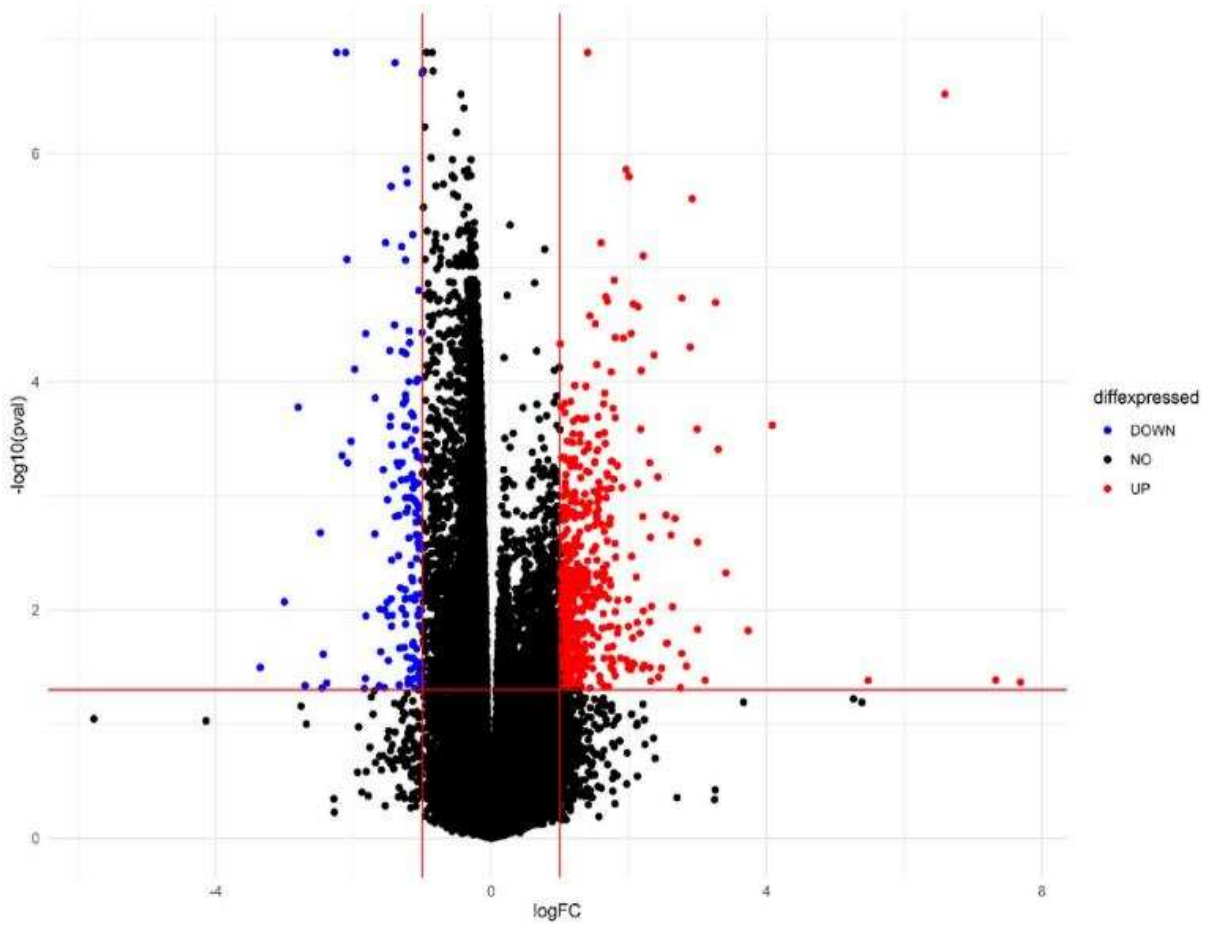

Figure 7. Graphical representation of DEGs through volcano plot with red and blue dots indicating the upregulated and downregulated genes respectively. Non-significant genes are represented using black color.

Molecular Docking: The top four compounds homogenestic acid, 4-hydroxyphenylacetic acid, 4-hydroxyphenylpyruvic acid, and phenolpyruvic acid were identified for molecular docking after a systematic network pharmacology analysis of the PPI network and CMU compounds. Target proteins AKT1 (PDB id: 3O96) and GSK3B (PDB id: 1UV5) were found in the PDB database and their crystal structures were retrieved. Using molecular docking, potential targets of compounds that could reduce the incidence of autism were filtered out. Strong binding affinities between the compounds and the binding pockets of two target proteins were predicted by docking analysis. For compound screening, binding energy was used as an important parameter (Figure 8A). Clusters with the highest conformation and a significant absolute binding energy value were identified. The highest binding energies were demonstrated by phenylpyruvic acid and 4-hydroxyphenylpyruvic acid with AKT1, as well as with the target protein GSK3B. Nevertheless, the maximum binding energy of phenylpyruvic acid with AKT1 was -6.80 kcal/mole, while the maximum energy of 4-hydroxyphenylpyruvic acid with GSK3B protein was -6.10 kcal/mole. These findings might suggest that the active ingredients in camel milk served as an autism repressor by forming a stable binding with two target proteins.

Molecular docking studies have revealed that compounds such as homogentisic acid, 4-hydroxyphenylacetic acid, 4-hydroxyphenylpyruvic acid, and phenolpyruvic acid exhibit strong binding affinities with the target proteins. This evidence supports the relevance of the study and highlights the necessity of fully understanding the interactions between these compounds and their target proteins to elucidate how they may counteract autism. Specifically, the interactions involving hydrogen bonds, pi-pi stacking, and van der Waals forces between the drug candidates and the receptor proteins AKT1 and GSK3B are depicted through dotted lines in Figures 8B and 8C, respectively. Taken together, our findings add to a growing body of evidence suggesting that these proteins could be promising targets for the development of small molecule compounds for autism, offering new avenues for therapeutic intervention.

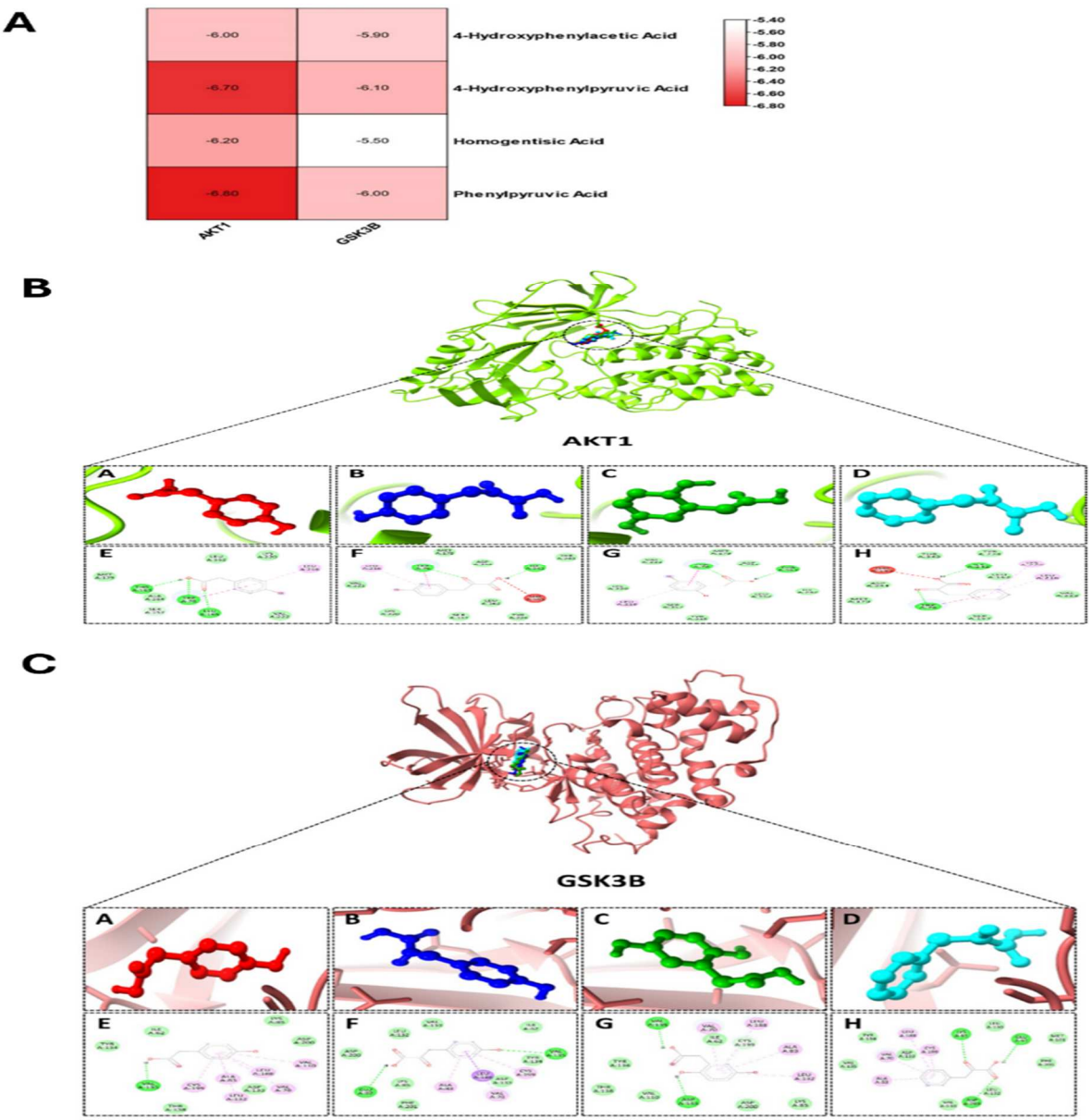

Figure 8. Docking studies. A) Comparison of binding affinity of active compounds with AKT1 and GSK3B proteins. B) 2D and 3D visualization of docking complexes of AKT1 protein. The central image shows the three-dimensional crystal structure of the AKT1 protein, with its active site highlighted. Panels (A-D) depict the 3D conformations of four different ligand molecules docked into the active site of AKT1. These ligands are color-coded: red for 4-hydroxyphenylacetic acid, blue for 4-hydroxyphenylpyruvic acid, green for homogenestic acid, and cyan for phenolpyruvic acid. Panels (E-H) provide the corresponding two-dimensional interaction diagrams between AKT1 and each ligand, detailing the specific amino acid residues within AKT1 that interact with the ligands via hydrogen bonds (green dashed lines) and stacking interactions in orange dashed lines. C) 2D and 3D visualization of docking complexes of GSK3B protein. The central image shows the three-dimensional crystal structure of the GSK3B protein, with its active site highlighted. Panels (A-D) depict the 3D conformations of four different ligand molecules docked into the active site of GSK3B. These ligands are color-coded: red for 4-hydroxyphenylacetic acid, blue for 4-hydroxyphenylpyruvic acid, green for homogenestic acid, and cyan for phenolpyruvic acid. Panels (E-H) provide the corresponding two-dimensional interaction diagrams between GSK3B and each ligand, detailing the specific amino acid residues within GSK3B that interact with the ligands via hydrogen bonds (green dashed lines) and stacking interactions in orange dashed lines.

ADMET Profiling: ADMET analysis plays a crucial role in the process of drug discovery. For this purpose, the SwissADME database was employed to illustrate that certain drugs exhibit favorable pharmacokinetic properties. The ADMET evaluation of the top-selected compounds revealed no negative impacts on the pharmacokinetic parameters of any potential drugs, as shown in Table 4. The ADMET characteristics of these compounds, such as being P-glycoprotein substrates, their ability to penetrate the blood-brain barrier (BBB), and their gastrointestinal absorption, presented promising outcomes. These findings suggest the compounds' viability as therapeutic agents.

Molecular dynamics simulation: Molecular dynamics simulations were employed to investigate the dynamic behavior and interactions of GSK3β. The two complexes under scrutiny are identified as follows: Complex 1 (1UV5-GS3KB-Hydroxyphenyl) for 4-hydroxyphenylacetic acid and complex 2 (1UV5-GS3KB-Phenylpyruvic) for 4-hydroxyphenylpyruvic acid. The experiment was done to check the complex stability over 100 ns simulation Fig. 9.

Protein RMSD values did not show significant fluctuations throughout the entire 100 ns simulation, indicating a stable overall protein structure with minor fluctuations. The results indicate the extreme stability of these complexes and support the other findings in this study.

Table 4. ADMET profiling of the top compounds.

	Homogentisic acid	4-Hydroxyphenylacetic acid	4-Hydroxyphenylpyruvic acid	Phenylpyruvic acid	Phenylacetic Acid
GI absorption	High	High	High	High	High
BBB permeant	No	Yes	No	Yes	Yes
P-gp substrate	No	No	No	No	No
CYP1A2 inhibitor	No	No	No	No	No
CYP2C19 inhibitor	No	No	No	No	No
CYP2C9 inhibitor	No	No	No	No	No
CYP2D6 inhibitor	No	No	No	No	No

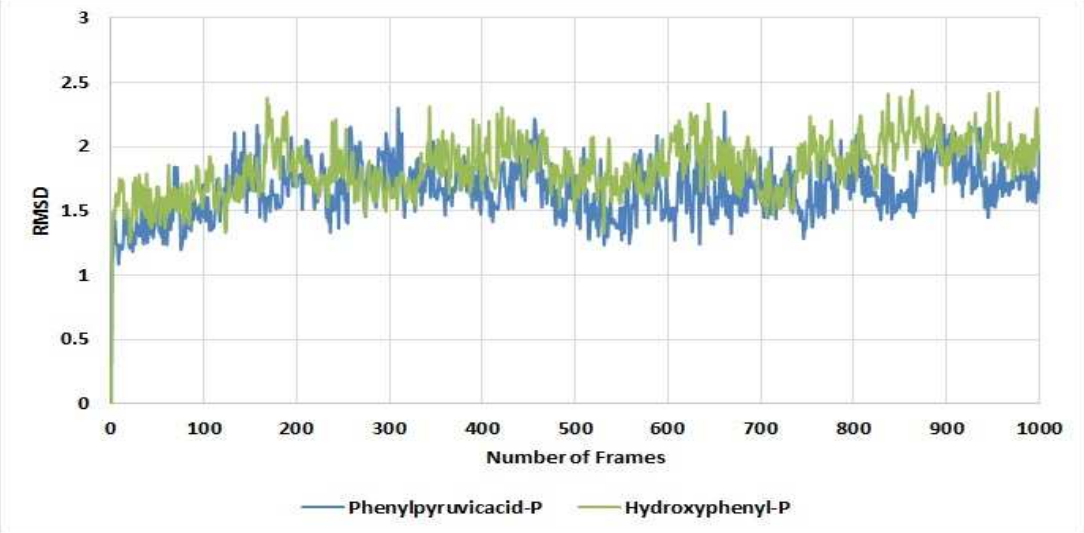

Figure 9. RMSD of the interaction of Complex 1 (1UV5-GS3KB-Hydroxyphenyl) for 4-hydroxyphenylacetic acid and complex 2 (1UV5-GS3KB-Phenylpyruvic) for 4-hydroxyphenylpyruvic acid after 100 ns of simulation.

Exploring residue interactions within protein dynamics, complex 1 (1UV5-GS3KB-Hydroxyphenyl) and complex 2 (1UV5-GS3KB-Phenylpyruvic) displayed hydrogen bonding interactions involving Lys 85 (Figure 10).

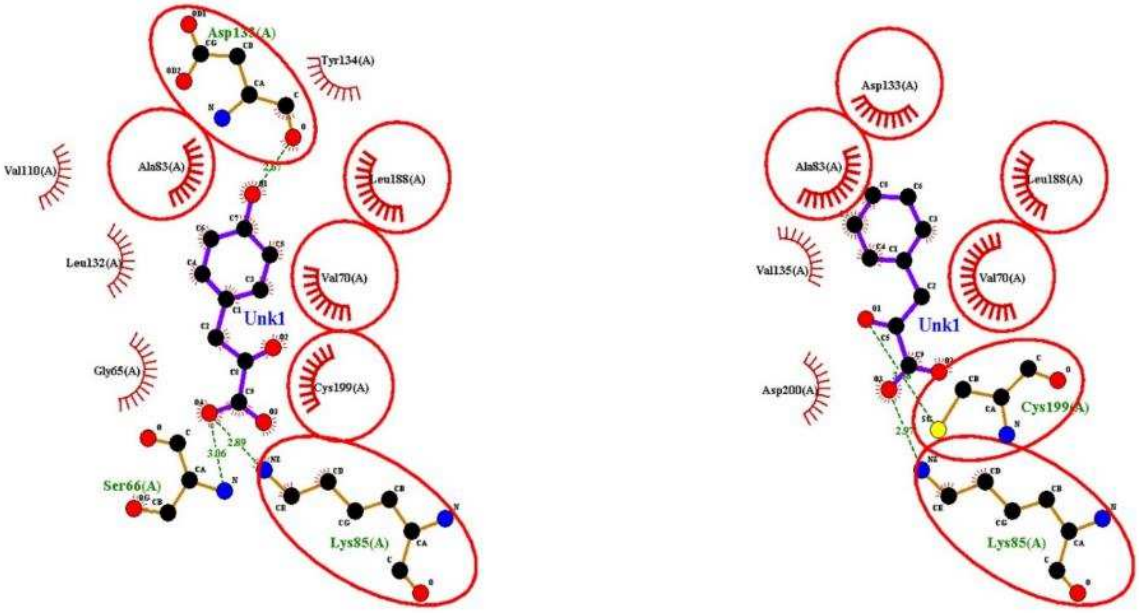

Figure 10. The ligand interactions of the interaction of Complex 1 (1UV5-GS3KB-Hydroxyphenyl) for 4-hydroxyphenylacetic acid and complex 2 (1UV5-GS3KB-Phenylpyruvic) for 4-hydroxyphenylpyruvic acid.

DISCUSSION

Autism is a syndrome that affects neuropsychological function, leading to difficulties in communication, social interaction, and behavior (Hodges *et al*. 2020). It is a neuro-developmental disorder that impairs the ability to interact and communicate with other people. Autism can cause a decrease in the quality of life, gastrointestinal symptoms, sleep problems, and social problems (Chen *et al*. 2022).

In our study, CMU compounds were analyzed collectively rather than as separate entities to explore their combined therapeutic potential and complex interactions in ASD. However, the combined action was applied during data processing and handling, while the final impact was assessed based on individual compounds that can be derived from either camel milk or urine. This approach aims to highlight the synergistic effects of multiple compounds in CMU, providing a comprehensive understanding of their complex interactions with ASD-related molecular pathways and targets.

The appeal of natural products and their derivatives lies in their structural variety, ability to target multiple pathways, and minimal toxic side effects, marking them as a focal point of recent research trends and a promising source for the development of targeted medications (Atanasov *et al*. 2021). Remarkably, natural products and their derivatives constitute approximately half of the pharmaceuticals currently in clinical use (Clark 1996). Recent years have seen the recommendation of high-throughput screening methods as an efficient way to evaluate the pharmacological potential of chemical compounds in the drug discovery process (Mishra *et al*. 2008). This era is characterized by the identification of potentially bioactive substances capable of halting the pathophysiology of various diseases and conditions.

Our screening identifies key bioactive compounds in CMU, such as homogentisic acid, 4-hydroxyphenylacetic acid, 4-hydroxyphenylpyruvic acid, and phenylpyruvic acid, which may influence autism development through their interactions with the AKT1, ESR1, CTNNB1, EGFR, GSK3B, and PTGS2 genes. Molecular docking further corroborates our findings, demonstrating stable interactions between the core compounds and the top targets. Employing a 'compounds-targets-pathways' model, our analysis reveals that homogentisic acid and 4-hydroxyphenylacetic acid are particularly linked to autism characteristics due to their significant network connections with the AKT1 gene.

GO functional analysis revealed pathways related to responses to chemical and oxidative stress, as well as cellular response to organic cyclic compounds.

KEGG pathway analysis highlighted significant signaling pathways linked to the anti-autism effects of CMU compounds, including EGRF tyrosine kinase inhibitor resistance, prolactin signaling pathway, Endocrine resistance, IL-17 signaling pathways, and TNF signaling pathway.

Based on the 'chemicals-targets network', we conducted a docking experiment using four compounds and two targets with high degree scores and connected pathways. Moreover, the docking results have confirmed our findings and demonstrated that homogentisic acid, 4-hydroxyphenylacetic acid, 4-hydroxyphenylpyruvic acid, and phenylpyruvic acid form stable bonds with the active pockets of the target genes. This highlights the potential of these compounds to modulate the actions of AKT1 and GSK3B genes. Interestingly, different studies reported the role of these genes in autism. For instance, AKT1 is known to have a significant impact on regulating cell growth, cancer cell metastasis, and programmed cell death. Disruptions in the PI3K/AKT/mTOR signaling pathway are associated with impairments in the synthesis of synaptic proteins and the development of ASD (Sharma and Mehan 2021). Current research suggests that programmed cell death can be regulated and maintain cellular function by modulating proteins that are targeted by AKT1 (Sharma and Mehan 2021, Fu *et al*. 2019). The selection of AKT1 and GSK3β as primary targets for our molecular docking studies is particularly significant given their central roles in the PI3K/AKT/mTOR and Wnt/β-catenin signaling pathways, both of which are critical to the neurodevelopmental processes implicated in ASD. The high connectivity of these proteins within the ASD-related protein-protein interaction network further underscores their potential as key therapeutic targets. By demonstrating stable interactions between these proteins and bioactive compounds from CMU, our study provides a strong rationale for their continued exploration in the development of novel ASD treatments.

The activation or enhancement of the PI3K-AKT/mTOR signaling pathway is associated with neurological abnormalities in humans, including ASD (Sharma and Mehan 2021). AKT inhibitors are emerging as a promising avenue in the treatment of autism by focusing on the PI3K-AKT/mTOR signaling pathway, which plays a pivotal role in learning and memory formation. Targeting this pathway can be highly beneficial for autism therapy, as evidenced by the restoration of social behaviors in Cntnap2-deficient mice, a model that is widely recognized for its relevance in ASD drug discovery (Xing *et al*. 2019).

GSK3β could potentially raise the risk of ASD through its regulatory effects on genes and pathways associated with high risk, including the Wnt signaling/β-catenin pathway and the mTOR pathway (Rizk *et al*. 2021). These pathways are critical for various cellular processes, and their dysregulation may contribute to the development of ASD. GSK3's involvement in modulating these pathways highlights its significant role in influencing ASD. Recently, tideglusib, a novel inhibitor of GSK3β, has demonstrated potential benefits in a phase-2 randomized controlled trial for individuals with ASD (Baribeau and Anagnostou 2022). This trial observed promising trends towards the alleviation of core ASD symptoms, such as social withdrawal and repetitive behaviors. In addition, GSK3β is linked to oxidative stress and inflammation in the brain, which inhibits the hippocampal neural network's ability to form new connections, reduces dendritic spine density and plasticity, and results in antisocial behavior (Jorge-Torres *et al*. 2018). Thus, GSK3β plays a role in autism, which can be modulated by using CMU-related compounds.

The comprehensive computational analysis undertaken in this study has illuminated the potential of CMU compounds as therapeutic agents against ASD. By integrating network pharmacology, microarray analysis, and molecular modeling, we have identified a subset of twenty-two bioactive compounds in CMU that exhibit strong interactions with 169 key targets associated with ASD. The construction of compound-target networks and pathway analyses underscore the complex, multi-target, and multi-pathway mechanisms through which these compounds may exert their therapeutic effects.

While this study provides promising insights into the therapeutic potential of CMU compounds for treating ASD, it is not without limitations. Primarily, our findings are based on in silico analyses, including network pharmacology, cheminformatics, and molecular modeling, which, while robust, require experimental validation to confirm the predicted interactions and efficacy. The study also pooled compounds from both camel milk and urine for initial interaction analyses, which, although effective for identifying high-efficiency compounds, may overlook individual nuances and potential synergistic effects unique to each source. Additionally, the bioavailability and pharmacokinetics of these compounds in human subjects need further exploration through in vivo studies. Finally, the used compounds were only a subset of compounds in CMU and not a complete array of compounds present in milk or urine. Future research should focus on experimental validation of the identified compounds, understanding their mechanisms of action, and developing targeted CMU-based therapeutic products. Furthermore, large-scale compounds library will deliver deeper insights into treatment and its mechanisms in ASD and other diseases.

Conclusions: This study highlights the potential of CMU compounds as therapeutic agents for ASD through an integrative approach combining network pharmacology, microarray analysis, and molecular modeling. Our comprehensive analysis identified 22 bioactive

compounds, revealing their significant interactions with ASD-related genes and pathways. Key compounds such as homogentisic acid, 4-hydroxyphenylacetic acid, 4-hydroxyphenylpyruvic acid, and phenylpyruvic acid demonstrated strong binding affinities with crucial ASD-related proteins like AKT1 and GSK3β, suggesting their therapeutic potential. By pooling compounds from both camel milk and urine for initial interaction analysis, we were able to identify the most promising bioactive agents and assess their synergistic effects. The results indicate that these CMU compounds can modulate biological pathways implicated in ASD, providing a multifaceted approach to treatment. Future research should focus on validating these results through in vivo studies, exploring the specific mechanisms of action, and developing targeted CMU-based therapeutic products. Additionally, future study of large compounds subsets based on complete milk and urine analysis will give insights into more potent candidates and treatment mechanisms. This study lays the groundwork for the development of novel, natural treatments for ASD, emphasizing the importance of integrating traditional knowledge with modern scientific approaches in drug discovery and development.

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