Uncovering the Role of Cow Urine as a Bioenhancer Investigated Towards Network Pharmacology

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Tilwani et al.: Role of Cow Urine as a Bioenhancer

Traditional Indian medicine practice (Ayurveda) emphasized the role of "panchgavya" five products from Bos indicus for human welfare. Ayurveda classics "Sushruta Samhita", "Ashtanga Sangrah" alluded to the therapeutic potential of pristine cow urine as drug or drug ingredients. Compelling evidence exhibits the innumerable medicinal properties of cow urine; accordingly, this elixir can directly treat complex ailments such as leprosy, tuberculosis and fever. Also, the classics narrated many formulations that have utilized cow urine for the preparation of drugs, supplemented to enhance the potency. This practice is more empirical, and only a few pieces of experimental evidence supporting the claim are known. The associated mechanisms are poorly understood and so render its appeal to the limited mass. The study aims to investigate the bio-enhancer-like properties of cow urine toward network pharmacology. For that, 25 medicines having antibacterial, anti-fungal, anti-viral, enzyme inhibitors, and anti-inflammatory actions were selected as a reference. Network analysis for twenty chemotypes found in cow urine was carried out. First, through enrichment analysis, the kyoto encyclopedia of genes and genomes and gene ontology terms were obtained. Second, we performed protein-protein interaction studies to screen more targets. Towards this, the drug-protein and cow urine-protein interaction networks are built separately and processed.

Key words: Ayurveda, cow urine, network analysis, molecular docking

Ayurveda, an Indian traditional medicine system, has adopted a holistic approach, nucleated towards balancing five life-ruling vital factors. The repertoire includes diet, lifestyle, thought modulation, and herbs' use. The combinatorial practice emphasized the progressive restoration of the body through balancing these factors. In Ayurveda, a cow is bestowed; the status of the medical dispensary is a source of boons, considered a boon to humanity. It provides cow dung, urine, milk, curd, and ghee. These are altogether referred to as "panchgavya" in Ayurveda. Cow Urine (CU), usually regarded as a nonessential by-product in Ayurveda, is used to prepare many herbal formulations. Has medicinal value, a whole or with other active ingredients, can treat ailments, including terminal illness. The benefits of supplemented CU are thoroughly reviewed in Ayurvedic classics Charaka Samhita and Sushruta Samhita (an early journal for surgical practices). In the present era, recent studies conducted at CU Treatment and Research Centre, Indore (India), show the positive association of CU (Gomutra) in the treatment of disorders like blood pressure, artery blockages, and cancer. First records cite its application around 4000 BC; though deep-rooted, its compatibility with the allopathic practice must be established to gain broad acceptance and mass appeal. Despite extensive therapeutic uses, the method is more empirical than experimental and poorly recognized in a region outside the Indian subcontinent. There is a dire need for re-investigation towards established gold-standard research design of biomedical sciences.

Recently, United States patent (No. 6896907 and

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and a few other laboratories are reportedly claiming bioenhancer properties of CU. Generally, a bioenhancer can be a natural or artificially synthesized substance/s that surges the efficacy of the drug when administered together. It increases the bioavailability of an orally administered pharmaceutical compound through several mechanisms. The bioenhancer often improves the solubility or adsorption of medicine, inhibits the action of drug-metabolizing enzymes and enhances the permeability of the cytoplasmic membrane for drug entry. It’s a non-exhaustive list, and a few other speculated ways of activities are presented in fig. 1.

In earlier attempts, the study of Lee et al. noted a steep rise in the anti-inflammatory activity of apigenin when applied with resveratrol. In another study, the potency of Rifampicin greatly improved when supplemented with the naturally occurring organic compound Piperidine; upon addition, the improvement in anti-tuberculosis activity was documented.

Ayurveda treatment regime advocates the application of whole CU or CU distillate. CU is a rich source of bioactive components known to influence multiple biological pathways toward interacting with diverse biological targets. Viewing multi-target impact Network pharmacology (NP) is the best method to investigate as it deals with numerous networks and components systematically. Bioenhancer, a collective term given to the compounds that enhance the potency/efficiency or solubility of the drug when administered. To elucidate the role of CU as a bio-enhancer using NP, we first screened 20 chemical components from CU. Unique (limited to certain species) and conditions specific chemicals excluded. Next, we have chosen drugs from five distinct functional categories and such are antibacterial, anti-fungal, anti-viral, enzyme inhibitors and Anti-Inflammatory (AIF). First, a network for 38 common targets of drugs (25) and CU components (20) is visualized. Overlapping targets annotated through Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. Next, towards another strategy, we built a CU-AIF core target-Protein-Protein Interaction (PPI) network and functional modules obtained. Finally, we curtailed the six key targets showing the role of CU in drug metabolism and cell signaling pathways. Molecular docking and pharmacokinetic analysis studies elucidate the inhibition of the drug-metabolizing enzyme by CU components. Fig. 2 represents the overall experimental approach.

Fig. 1: Possible ways towards which bio-enhancer can enhance drug potency
MATERIALS AND METHODS

The addition of CU to the drug could augment the potency of the drug, to comprehend the impact, the study was conducted by taking two different approaches. First, the overlapping gene targets are fetched from CU and medicines (from all five classes). In the second attempt, we selected only AIF drugs and conducted the PPI and topological analysis to screen other related targets. The conventional data processing and preparation methods are typical for both approaches and are narrated in subsequent sections.

Chemotype processing and target prediction:

A few reports citing the chemical nature of CU have been published so far; patents, reported literature and ayurvedic records through keyword CU names of chemical compounds were obtained. Next, sorting the chemicals into specific chemical classes, their canonical SMILES were collected from PubChem (https://pubchem.ncbi.nlm.nih.gov/) sub sequently converted to SDF formats (Table 1). Through considering the mechanism of action and commercial applications, drugs from five categories, anti bacterial, anti fungal, anti-viral, AIF and enzyme inhibitors were chosen to investigate the bioenhancer properties of CU.

Putative gene targets for CU components and medicines were retrieved from STITCH (http://stitch.embl.de/) and Swiss Target Prediction (http://www.swisstargetprediction.ch/). Hits with a confidence score of 0.9 to 1 were considered and converted to their corresponding UniProtKB ID (https://www.uniprot.org/) for gene annotation. The list was re-organized and manually curated using published experimental records. Specifically, the gene targets involved in membrane transports, such as ABC transporters and cell proton pumps, were counted.

PPI networks and functional clusters:

Towards strategy II the network for CU and AIF, PPI was visualized. A PPI network built through Bisogenet, a plugin of Cytoscape. Bisogenet is a three tire application for investigating the bimolecular relationships. It combines the information from six central PPI databases viz., Molecular Interaction Database (MINT), IntAct Molecular Interaction Database (IntAct), Database of Interacting Proteins (DIP), Human Protein Reference Database (HPRD), Biomolecular Interaction Network Database (BIND), and Biological General Repository for Interaction Datasets (BioGRID). Input entities' protein identifiers for Homo sapiens were selected in Bisogenet, gene identifiers were uploaded, and the distance from the input node was adjusted to 1. In the output column, only protein identifiers have opted. Next, through an intersection, merged PPI networks of CU-AIF targets were visualized. Subsequently, we used CytoNCA, a plugin of Cytoscape, to identify the essential proteins from the merged network. To determine the node present at a critical location, six centrality measures were selected in CytoNCA. An analysis for significant centrality values such as Degree Centrality (DC), Closeness Centrality (CC), Network Centrality (NC), Betweenness Centrality (BC), Local Average Connectivity-based method (LAC), and Eigenvector Centrality (EC) was conducted.

Minimal Common Oncology Data Elements (MCODE), a cluster analysis algorithm in Cytoscape, detects the densely-connected area in PPI networks. MCODE effectively builds a molecular interaction network based on connectivity.
Functional annotation of hits of strategy I and strategy II:

In a first attempt, 38 overlapping hits were uploaded to Database for Annotation, Visualization, and Integrated Discovery (DAVID)\(^\text{[20]}\) to find out the enriched terms through KEGG\(^\text{[21]}\) and GO\(^\text{[22]}\) databases. Hits with a p-value of 0.005 were selected for achieving the list of significant targets. We used the ImageGP gene enrichment tool to visualize enriched GO and KEGG terms.

Second, the hits of PPI clusters were analyzed and information on associated biological pathways was obtained. KEGG and GO terms from the strategy I and II were screened for repetitive entries and repeated names were pooled for subsequent analysis.

Structure-based docking and activity predictions:

The overlapping GO and KEGG terms from both the strategies were screened and further investigated for their drug binding affinities. Frail interaction of a protein-ligand was weed out through performing molecular docking in the Autodock vina ver 4.1\(^\text{[23]}\). The X-ray crystal structures for selected targets; CYP1A1(PDB ID:6DWM), CYP1A2(PDB ID: 2HI4), UGT1A3(PDB ID: 1V4T), ABCB1(PDB ID: 6GDI), CYP3A4(PDB ID: 4I3Q), CYP2D6(PDB ID: 2F9Q), COX-1(PDB ID:6Y3C) and COX-2 (PDB ID:5KIR) were obtained from research collaboratory for structural bioinformatics protein data bank (www.rcsb.org). Ligand-protein chemical interactions scored through visualization and interaction analysis in PyMol 1.8\(^\text{[24]}\) and LigPlot v 4.5.3\(^\text{[25]}\). Using Prediction of Activity Spectra for Substances (PASS) software, we predicted the pharmacokinetic activities for CU and drugs. PASS allows us to predict the mechanism of action a ligand have on protein targets; results were re-confirmed with SwissADME (http://www.swissadme.ch/) pharmacokinetic analysis\(^\text{[26]}\).

RESULTS AND DISCUSSION

CU can act as bioenhancer. The present study investigates the hypothesis through NP tools. Towards it, through an extensive literature review, two ligand datasets were generated. One represents CU compounds containing 20 distinct chemotypes (dataset-1), and the other has the list of lead compounds of five commercially available drugs (dataset-2). Drugs having an analogous mechanism of action were scrutinized manually through published reports. From a class anti-viral, medicines Benazepril and Captopril inhibit the conversion of angiotensin I to angiotensin II. They share a similar protein target and are mainly taken to enrich the maximum genes from biological pathways associated with the various medicines of the same class. The selected categories of drugs are known to act on numerous macromolecules from bacteria, fungi, viruses and Homo sapiens. Our study limits human macromolecular targets; this reduces the noise in the analysis at the end.

A ligand-target network constructed through Cytoscape (https://cytoscape.org/;version 3.8.0)\(^\text{[27]}\) shows a reasonably extensive network.

### TABLE 1: CHEMOTYPES COMMONLY PRESENT IN COW URINE; CLASS-WISE SUMMARY

<table>
<thead>
<tr>
<th>Benzoids</th>
<th>Homogeneous non-metal compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoic acid</td>
<td>Chloride</td>
</tr>
<tr>
<td>p-Cresol</td>
<td>Copper</td>
</tr>
<tr>
<td>Phenol</td>
<td>Phosphorus Pentoxide</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>Organo heterocyclic compound</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>p-Phosphate</td>
</tr>
<tr>
<td>Organic oxygen compound</td>
<td>Sulfate</td>
</tr>
<tr>
<td>Lactose</td>
<td>Nitrite</td>
</tr>
<tr>
<td>Organic acid and derivatives</td>
<td>Phosphorus Pentoxide</td>
</tr>
<tr>
<td>Creatinine</td>
<td>Uric acid</td>
</tr>
<tr>
<td>Lipid and lipid-like molecule</td>
<td>Allantoin</td>
</tr>
<tr>
<td>Thymol</td>
<td>Nicotine</td>
</tr>
<tr>
<td></td>
<td>Phenylpropenoids and polyketides</td>
</tr>
</tbody>
</table>

with 208 nodes and 234 edges (fig. 3a). Post abandoning non-overlapping hits, 38 common-hits were curtained and presented in fig. 3b, describing 32 hub nodes and 123 edges. The hub nodes having edges five or above were manually scrutinized and selected for further analysis. The thirty-eight annotated targets were associated with 76 biological processes, 25 molecular functions, 15 cellular components and 27 KEGG pathways. The results indicate that the CU-associated targets are mainly located on the plasma membrane and involved in the metabolism and transport of small molecules, evident from gene enrichment plots. We only considered the hits with Q-value ≤ 0.05 (fig. 4a and fig. 4b). CU components are generally enriched in the following KEGG pathways: hsa00140: Steroid hormone biosynthesis, hsa00980: Metabolism of xenobiotics by cytochrome P450 hsa00982: cytochrome P450 drug-metabolism pathways. The following genes, UGT1A3, CYP1A1, CYP2D6, and CYP1A2, are acting for both CU and drugs in the pathways mentioned above.

Cellular processes are linked and accomplished by the coordinated action of functional modules. It encompasses the group of proteins involved in catering to similar functions. Towards strategy-II, the PPI networks of CU and AIF-associated proteins developed through Bisogenet (fig. 5a and fig. 5b). The same nodes and edges from the two separate PPI networks were selected to obtain intersection, a merged network containing 2016 hub nodes and 42347 edges (fig. 5c), which is still complex to interpret. Post-screening through six-criterion-based topological analysis in CytoNCA, 200 significant nodes were obtained (fig. 5d). Next, the MCODE clustering analysis gave seven significant clusters (fig. 5e). Proteins from the modules I-V and VII are enriched in cell signaling.

In contrast, the proteins from module VI are associated with the process of drug metabolisms. Overall, the results suggest that the majority hits enriched to two cellular processes; cell signaling and drug metabolism (Table 2). Similar observations strike out from the analysis conducted to the strategy I; Hence a merged list of targets from both approaches curtailed contains CYP2D6, UGT1A3, CYP3A4, ABCB1, CYP1A1, and CYP1A2.

Fig. 3: Compound-drug targets network and CU-Drugs shared target network, sorted to degree centrality (red color) and confidence score, shows ranked order for 32 hub nodes.
Fig. 4: Functional enrichment analysis of the 32 overlapping hits; (a) it represents a graded list of enriched pathways; (b) enriched GO terms (biological processes); The color scale correlates to the significant Q-values, and the sizes of the dots represent the number of genes corresponding to each node.

Fig. 5: CU-AIF PPI network; (a) CU-related targets PPI network (b) AIF-related targets PPI network. (c) Intersection of PPI networks. (d) PPI network by the screening criteria of DC≥4.0, EC≥10, LAC≥4.0, BC≥100, CC≥4 and NC≥5.0 and (e) Clusters of core-target PPI network.

### TABLE 2: LIST OF SIGNIFICANT PATHWAYS AND GENES OBTAINED THROUGH CLUSTERING AND ENRICHMENT ANALYSIS

<table>
<thead>
<tr>
<th>Cluster classification</th>
<th>p-value</th>
<th>Gene targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Signaling pathways</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa04020: Calcium signaling pathway</td>
<td>2.00×10-3</td>
<td>GNA15, LTB4R2, ADRA1A</td>
</tr>
<tr>
<td>hsa04080: Neuroactive ligand-receptor interaction</td>
<td>4.70×10-3</td>
<td>P2RY2, LTB4R2, ADRA1A</td>
</tr>
<tr>
<td>hsa04062: Chemokine signaling pathway</td>
<td>2.10×10-3</td>
<td>CCL13, CXCL5, CXCL6</td>
</tr>
<tr>
<td>hsa04060: Cytokine-cytokine receptor interaction</td>
<td>3.60×10-3</td>
<td>CCL13, CXCL5, CXCL6</td>
</tr>
<tr>
<td>hsa04010: MAPK signaling pathway</td>
<td>4.90×10-5</td>
<td>PTPN7, DUSP4, DUSP2, DUSP6</td>
</tr>
<tr>
<td>hsa00140: Steroid hormone biosynthesis</td>
<td>1.70×10-2</td>
<td>UGT1A3, AKR1C1</td>
</tr>
<tr>
<td>II Metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa00980: Metabolism of xenobiotics by cytochrome P450</td>
<td>1.10×10-4</td>
<td>UGT1A3, CYP2D6, AKR1C1</td>
</tr>
<tr>
<td>hsa00982: Drug metabolism - cytochrome P450</td>
<td>2.00×10-3</td>
<td>UGT1A3, CYP2D6</td>
</tr>
</tbody>
</table>
From 4342 CU targets, performing ligand-target docking weak interaction was weed out. Anti-inflammatory drugs relieve pain and inflammation by inhibiting the cellular cyclooxygenase (COX1 and COX2) activity; here, we selected Celecoxib, diclofenac, and Aspirin as reference drugs. Post docking, the ligands were classified according to the binding affinities shown in fig 6. The lowest binding free energy and the number of H-bonds accounted for the analysis. The binding energy of Uric acid (-7.2 kcal/mol) is compatible with the standard drug diclofenac (-6.4 kcal/mol). For target CYP1A2, diclofenac and Uric acid use the same binding pocket evident from shared interaction with following amino acid residues; Ser279, Phe275, Gly206, His208, and Asp282 (fig. 7A and fig. 7B). This result also suggests that uric acid is a better candidate for the target CYP1A2 than diclofenac. For the prime drug target COX-1 the binding affinity of uric acid (-6.6 kcal/mol) is identical to diclofenac (-6.7 kcal/mol), and again, a similar active site pocket where Gln144, Lys468, and Arg469 are interacting with ligands and showing similar H-bond profiles (fig. 7c and fig. 7d). For another drug target, COX-2, the binding affinities of uric acid (-6.9 kcal/mol) are more significant than diclofenac (-6.3 kcal/mol), having the following common residues at binding pockets Gln372, Phe371, Ser121, His122 (fig. 7e and fig. 7f).

Pharmacokinetic analysis of screened targets presents a promising role of CU in drug formulations. It suggests that the CU component, gallic acid downregulates the expression of CYP3A4 and thereby delays the drug metabolism\[28,29\] that further increasing the drug retention time in the body, eventually may result in increases in gastrointestinal absorption of a drug. Another Cu component, p-cresol, and Thymol inhibits the activity of CYP1A2 and CYP2D6\[30,31\] and exerts similar activity to gallic acid. p-Cresol in CU inhibits the membrane p-gp efflux by facilitating the optimum drug delivery in the cell interior\[32,33\]. Benzoic acid inhibits the activity of CYP1A1, and Uric acid regulates the activity of UGT1A3 in drug metabolism\[34,35\].
Fig. 7: Molecular docking analysis; (a) Receptor-ligand interaction binding pose of uric acid against CYP1A; (b) Diclofenac-CYP1A2; (c) Uric acid-COX-1; (d) Diclofenac-COX-1; (e) Uric acid-COX-2 and (f) Diclofenac-COX-2
In a grim scenario of the evolution of drug resistance and drug insensitivity, there is a need for quick and affordable solutions\(^{[36,37]}\). The microbes acquire resistance through metabolizing the drug that poses a severe threat to existing therapeutics\(^{[38]}\). Discovering a novel drug entirely to combat this unforeseen change in the behavior of bacteria or cells is an economically challenging and lengthy process\(^{[39]}\). The science of drug designing is perturbed by the rapid emergence of drug resistance towards established antibiotics. The discipline demands an urgent need for repositioning or reformulating the current drugs. Researcher across the globe is testing various strategies such as the inclusion of metabolic inhibitors\(^{[40]}\) to single-gene deletion\(^{[41,42]}\). Adding bioenhancer may be one more alternative that may affect the drug metabolism\(^{[43-45]}\). During the literature analysis for Ayurveda practice, we observed the practice of inclusion of CU distillate in many Ayurveda formulation, has numerous application and exert its impact through modulating the fundamental processes\(^{[46]}\).

Drug effectiveness depends on several biological processes, including membrane transport/binding to blood plasma proteins/vasodilation of human intestinal tight junctions/endocytosis\(^{[47]}\). These processes mainly exalt drug absorption and distribution in the human body. The pharmacokinetics of any drug is related to many such biological processes. The results of studies conducted for the strategy I and II show that drug metabolism, one-carbon metabolism, response to the drug, cellular transports, and cell signaling pathways are shared among all the selected drugs associated with CU components. Though it is \textit{in silico} studies, it firmly establishes the interaction of CU with key gene targets that are often essential for drug activity. Hence, CU could be introduced as a bio enhancer. Laboratory validation studies are imperative to prove this claim further.

The elaborated results of docking and pharmacokinetic analysis display the strong association of CU with gene targets CYP2D6, UGT1A3, CYP3A4, ABCB1, CYP1A1, and CYP1A2. Typically, their gene products metabolize the drugs and facilitate their excretion for the human body. Cytochromes P450 (CYPs) are a superfamily of monooxygenase enzymes that oxidize steroids, xenobiotics, and drugs and play a role in its clearance\(^{[48-50]}\). Activation or inhibition of CYP1A1, CYP2D6 CYP3A4, and CYP1A2 isoforms usually activate or prevent the detoxification of numerous xenobiotics. It is one mechanism that regulates the bioavailability of drugs. The CU components interact with these isoforms, and few are involved in its suppression, which reduces the rate of drug metabolism. The reduced rate of drug metabolism increases drug absorption and sometimes drug toxicity.

Uridine diphosphate glucuronosyltransferase 1A3 (UGT1A3) belongs to the uridine diphosphate glucuronosyltransferase superfamily. In a mammal liver, the enzymes predominantly detoxify the endobiotic and xenobiotics and transform them into more polar and water-soluble glucuronides\(^{[51,52]}\). These altered metabolites are biologically inactive and easily extracted from the body. Drugs or herbs that inhibit some members of this family may lead to the accumulation of such molecules in the body, and even sometimes, clinical toxicity is observed. Though the inclusion of bio-enhancer in a drug formulation is a wise choice, it needs optimization to a certain extent; however, the \textit{in silico} studies exhibit the multilevel impact of CU on drug targets.

The use of CU in folklore date back to 4000 BC; however, limited knowledge of pharmacologically active compounds renders its application. It's a prima facie report that supports the ages-old Ayurveda practice that uses CU to prepare many herbal formulations. For target exploration and bioactive mining, NP currently relies on various databases. Despite being curated, databases may show discrepancies due to multiple sources of information, theoretical data, and experimental results. Though NP can accurately predict the genome-wide association of a selected compound, further laboratory investigations are required to elucidate the activity of CU's component towards the drug-metabolizing enzymes.

Furthermore, cows' diet affects the chemical composition of urine, so the chemical constituents must be validated before use. In some cases, the issue can be resolved by fixing the diet of bovines whose urine will be used.
for medicinal purposes. In the future, detailed pharmacokinetic and pharmacodynamic studies could be designed based on observations of the present manuscript.

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Conflict of interests:

The authors declared no conflict of interests.

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