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Phytochemical Analysis of Tinospora Cordifolia by Gc-Ms and Evaluation of its Antiurolithiatic Potential by *In Siilico*

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Abstract

Urolithiasis is the third most common urinary tract disorder affecting both men and women with an estimated global frequency of approximately 14%. Ethylene glycol is a lithiatic agent which is a potent component for experimental induction of urolithiasis. The enzymes like glycolate oxidase, oxalate oxidase are employed in oxalate metabolism which involve in caliculi formation. CD44 is a receptor of the stone modulating protein, Osteopontin (OPN). *Tinospora cordifolia* is a medicinal plant distributed throughout the Indian subcontinent, and contains many phytochemicals used for various ailments. GC-MS and ADME analysis were done and active principles (ligands) were selected. The protein and ligand structures were prepared and molecular docking was done in Autodoc Vina. 2D and 3D structures were generated by Discovery Studio Visualizer. The molecular interaction revealed that the phytochemical Columbin has greater binding activity against all three proteins namely glycolate oxidase, oxalate oxidase and CD44. Khellin, Valerenic acid and Capsazepine showed moderate binding activity against these targets. Thus, *Tinospora cordifolia* is found to exhibit significant antiurolithiatic activity through molecular interaction by *in silico.*

Key words: *Tinospora cordifolia,* urolithiasis, *in silico*, glycolate oxidase, oxalate oxidase, CD44, GC-MS

Urolithiasis has a long history that starts before the emergence of humanity (Eknoyan, 2004). Urolithiasis is the third most common urinary tract disorder affecting both men and women (Hiatt *et al.,*1982), with an estimated global frequency of approximately 14. In animals urolithiasis is found in many species including cats, dogs, horses, pigs, sheep, birds, rabbits, guinea-pigs, turtles, cattle, goats, whales, deer etc., (Kopecny *et al.,* 2021; Robinson *et al.,* 2008). The most important constituent of kidney stones in human and animals were found to be calcium containing calculi mainly calcium oxalate (Curhan *et al*., 2007).The important risk factors of calcium oxalate urolithiasis include hypercalciuria, hyperoxaluria, hypocitraturia, low urine volume and hyperuricosuria (O'Kell *et al*., 2017).Calcium oxalate calculi formation is a multistep process involving nucleation, aggregation and growth (Hewagama and Hewawasam, 2022). Ethylene glycol is a lithiatic agent which is metabolized in the liver producing various intermediate metabolites like glycoaldehyde, glycolic acid, glyoxalic acid and oxalic acid by major enzymes likealcohol dehydrogenase, aldehyde dehydrogenase, glycolate oxidase, lactate dehydrogenase andalanine glyoxalate aminotransferase (Kruse, 2012). These metabo-

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lites are toxic than the ethylene glycol itself. In the tubular fluid in the presence of calcium ion, oxalate forms the poorly soluble calcium oxalate. The deposition of Calcium Oxalate Monohydrate crystals (COM) in kidney tissue produces renal tubular injury which leads to renal failure (McMartin, 2009). Modulators of stone formation include proteins and glycosaminoglycans like CD44, nucleolin, hyaluronan (HA), heat shock protein 90 (HSP90), Annexin II, and osteopontin (OPN) (Wang *et al*., 2021). Thecontemporary medical treatment of urolithiasis involves medical expulsive therapy and various lithotripsy techniques like Extracorporeal Shock Wave Lithotripsy (ESWL), percutaneous nephrolithotomy (PCNL) etc (Jiang *et al.,*2021). However the medical procedures pose a significant financial burden for numerous patients and are linked to the recurrence of kidney stones. Contrary to this, ancient herbal remedies have demonstrated effectiveness, along with being readily accessible and cost-effective (Kasote *et al.,*2017).

Tinospora cordifolia (Menispermaceae) is an ayurvedic medicinal plant distributed throughout the Indian subcontinent and China. It possess phytochemical compounds, which include glycosides, alkaloids, steroids, and diterpenoid lactones (Anjum *et al*., 2023). There are studies which describes the nephroprotective, diuretic and antiurolithiatic potential of *Tinospora cordifolia*. However, a comprehensive examination of its phytochemical constituents for therapeutic interventions in kidney stone management has not been adequately explored. This paper focuses on delving into the molecular interactions between enzymes and ligands (phytochemical constituents of *T. cordifolia*), revealing a potential mechanism of action for these compounds in the management of calcium oxalate stones.

Materials and Methods

Collection of *T. cordifolia* stem

The stems of *Tinospora cordifolia* (Menispermaceae) were collected from parts of Erode region of Tamil Nadu during the month of March to April 2023. It was identified and authenticated by Dr. S. Sankaranarayanan, Department of Medical Botany and Pharmacognosy of Government Siddha Medical College, Arumbakkam, Chennai-600106 (Voucher Specimen GSMC/MP/621).

Preparation of ethanolic extract of *T. cordifolia*

The stems of *Tinospora Cordifolia* were collected and shade dried for about 15 days. Dried stems were ground using a blender and sieved using 60 mesh sieves to get fine powder. To 100 gram of stem powder of *Tinospora Cordifolia*, 1:10 dilution of ethanol was added in a 2 litre conical flask and soaked for 3 days with occasional shaking and stirring using mechanical stirrer. The mixture was filtered using muslin cloth followed by filtration through a whatman filter paper no. 1. The residue was again soaked in all the three solvents for 2 days and the filtration is followed as above. The filtrate was concentrated using Rotary Evaporator (Heidolph, Germany) at 55°C and freeze dried using lyophilizer at -47°C for 48 hours. The prepared extracts were stored in an airtight container in a refrigerator until use. The yield of the extracts were determined with respect to the original weight of the stem powder.

$$\text{Yield (\%)} = \frac{\text{Weight of the lyophilized extract}}{\text{Weight of the stem powder}} \times 100$$

Gas Chromatography- Mass Spectrometry (GC-MS)

GC-MS analysis of the ethanolic extract of *Tinospora cordifolia* was performed at Sophisticated Analytical Instrument Facility, Indian Institute of Technology, Chennai 600 036. The analysis was performed using GC-MS system (Agilent 8890, Agilent Technologies Inc., Santa Rosa, CA, USA) equipped with Agilent column (30m x 250μm x0.25μm). The injection volume of sample was 1μL with the flow rate of 1.2 mL/min. The temperature of injection port was set at 75˚C with holding time of 30 seconds and the column temperature were set as follows: 180˚C for 3 minutes at a rate of 5˚C/minute, an increase in temperature up to 300˚C for 5 minutes at a rate of 5˚C/minute in split mode with the pressure of 11.367 psi and holdup time of 1.2376 minutes. The MS conditions included an EI ion source temperature of 230°C, an ionization energy of 70 eV, and a mass scan range of 50-600 m/z. The total run time of sample was 53.5 minutes. The separated constituents were tentatively identified by comparing their mass spectra with those in the NIST MS library (National Institute of Standard and Technology, Gaithersburg, MD, USA).

Insilico Molecular Interaction Studies

Ligand preparation

The various phytochemical constituents of *T. cordifolia* obtained by GC-MS method and also from IMPAAT database weredownloaded in SDF format in Pubchem database. These ligands were subjected to preliminary screening to determine its drug-likeness using SWISS-ADME software (http://www.swissadme.ch). The ligands were chosen based on their physiochemical properties, lipophilicity and drug likeness (Lipinski's rule of five). The SDF format of the ligands that pass ADME analysis were saved in PDB format using Biovia Discovery studio visualizer followed by converting PDB format to PDBQT formats using Autodock Vina (Trott and Olson, 2010). This process of file conversion makes the ligands flexible for molecular docking.

Protein preparation

In this study, we have chosen the proteins associated with urolithiasis namelyglycolate oxidase, oxalate oxidase and CD44 as a target of the potential lead molecules of *T. cordifolia* (Table I). The 3D structure of these targets were downloaded from the Protein Data Bank (https://www.rcsb.org) in PDB format and it is further converted to PDBQT formats using Autodock Vina.

Docking

Each ligand underwent individual docking with the abovementioned targets, and the corresponding binding affinity (kcal/mol) was documented. The binding efficiency was then evaluated to assess the adsorbing capacity of the targets against the phytochemicals (ligand). The interaction between these three target proteins and the phytochemicals, as well as the visualization of output structures, was analyzed using Biovia Discovery Studio Visualizer (Fig 2 & Fig 3).

Results and Discussion

The ethanolic extract of *T. cordifolia* was prepared and the yield of this extract was calculated to be 2.15 % w/w. This result correlates with the study by Mehta *et al.* (2011) in which the authors reported that the yield of ethanolic extract of this plant was 2.54% w/w. The GC-MS analysis results revealed the presence of various phytochemical compounds with 13 peaks (Fig. 1). The list of phytochemical compounds at various retention times are given in (Table II).

The major phytochemical component present in ethanolic extract of *T. cordifolia* with high probability percentage were Capsazepine, Khellin, Ethyl palmitate, Columbin,

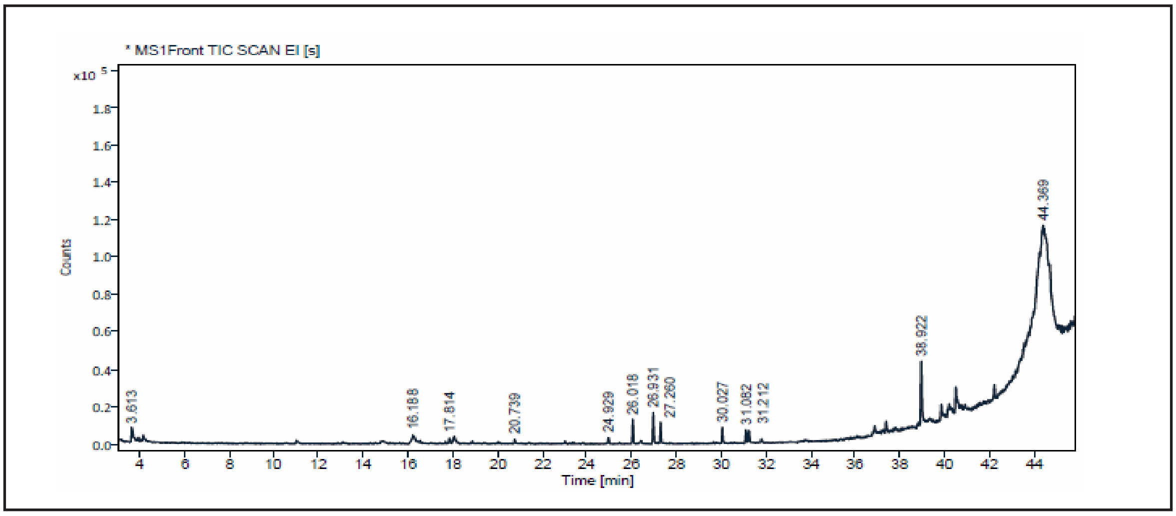

Fig 1. Chromatogram of GC-MS analysis of *T. cordifolia* ethanolic extract

Table I. Structures of the target proteins and their respective PDB Ids

S.No	Protein	PDB ID	Main function
1	**Glycolate oxidase** Three-dimensional structures of glycolate oxidase with bound active-site inhibitors	1AL7	It helps in the oxidation of glycolate to glyoxalate in oxalate pathway to produce kidney stones
2	**Oxalate oxidase** Crystal structure of Germin (Oxalate oxidase)	1FI2	It catalyzes the conversion of oxalate to carbon dioxide and hydrogen peroxide
3	**CD44** The hyaluronon binding domain of murine CD44	2JCP	It is a cell adhesion molecule that helps in adhesion of urinary crystals to renal epithelial cells

4-hydroxyvalsartan and Phytol with retention times of 16.188, 26.018, 27.260, 38.922, 20.74 and 30.027 (min) respectively. The compounds that showed highest peak area percentage shows the greatest pharmacological activity. These compounds were subjected to ADME analysis and 11 phytochemical compounds were selected for *insilico* docking purpose from 30 phytochemicals detected through GC-MS analysis (Table III).

Insilico docking of the above selected phytochemicals (ligand) against three targets namely glycolate oxidase, oxalate oxidase and CD44 revealed good binding affinity. The binding affinity of the protein-ligand interactions are given in the (Table IV). Among the chosen structures, Columbin showed greater binding affinity against all three targets. Khellin, Valerenic acid and Capsazepine revealed better binding affinity with CD44 compared to glycolate oxidase

Fig.2 3D structure of columbin and khellin interaction with target proteins

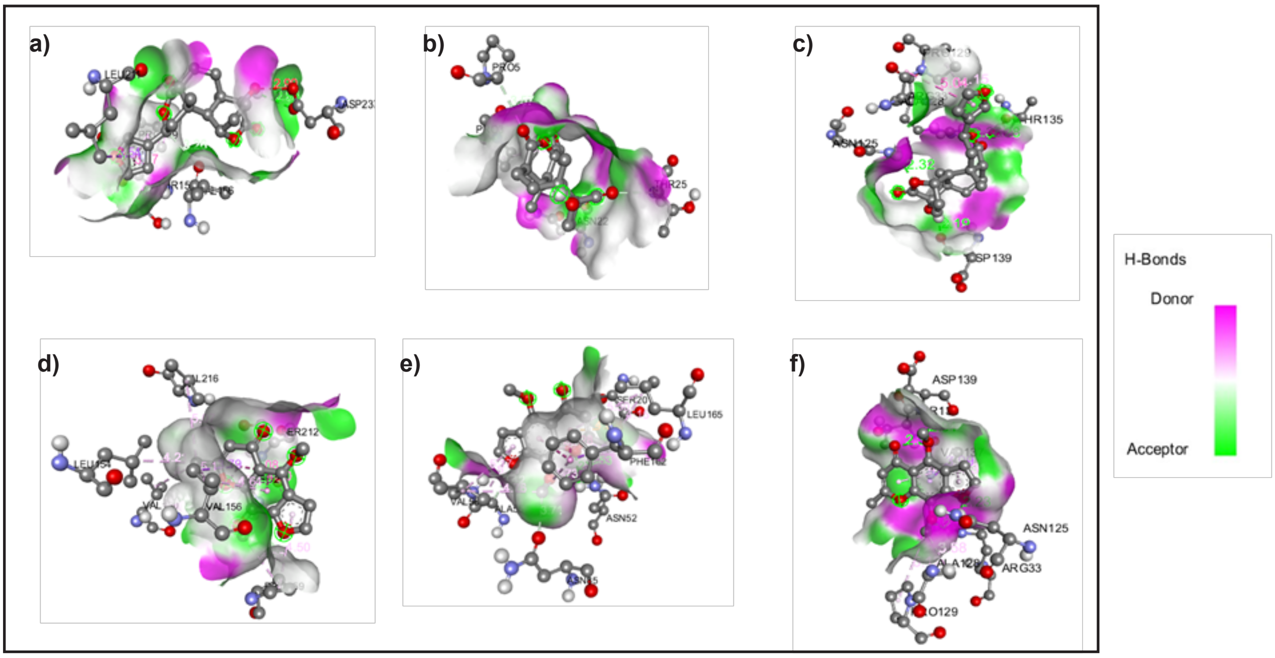

Fig.2a, 2b, 2c indicates 3D structure of Columbin with glycolate oxidase, oxalate oxidase and CD44 respectively. Fig d,e,f indicates 3D structure of Khellin with glycolate oxidase, oxalate oxidase and CD44 respectively

Table II: List of phytochemical compounds of T. cordifolia obtained by GC-MS analysis

S. No	RT (min)	Name of the compound	Probability %	Peak area %
1	3.613	Glycerin	18.72	3.44
		1,3-Dicyclohexylurea	4.9	
2	16.188	Capsazepine	96.62	5.75
3	17.814	Ethyl α-D-glucopyranoside	31.28	1.29
4	20.739	4-Hydroxyvalsartan	51.3	1.42
		7-Hydroxy-8-methylisoflavone	36.22	
5	24.929	Valerenic acid	6.62	1.79
		2,4-Dimethylethcathinone	6.11	
		3-Cyclopentene-1-octanoic acid, 2-(3-hydroxy-1-penten-1-yl)	3.94	
6	26.018	Khellin	86.8	7.27
7	26.931	Spiro[furan-3(2H),2’-[2H]inden]-2-one, decahydro-3’a,4’-dimethyl-4-methylene	38.2	9.15
		Dispiro[5.2.5.2]hexadecan-1-one		
8	27.260	Hexadecanoic acid, ethyl ester	70.45	5.95
		Heptadecanoic acid, ethyl ester	4.5	
		Pentadecanoic acid, ethyl ester	3.8	
9	30.027	Phytol	37.37	4.15
		Oxirane, decyl-	9.78	
		(R)-(-)-(Z)-14-Methyl-8-hexadecen-1-ol	6.9	
10	31.082	11-Dodecyn-1-ol acetate	9.21	3.14
		13-Tetradece-11-yn-1-ol	7.06	
		Bicyclo[10.1.0]tridec-1-ene	5.12	
11	31.212	2-Cyclododecenol	10.94	2.29
		(R)-(-)-14-Methyl-8-hexadecyn-1-ol	10.51	
		13-Oxabicyclo[10.1.0]tridecane	9.29	
12	38.922	Columbin	64.3	19.29
		1,4-Etheno-3H,7Hbenzo [1,2-c:3,4-c’]dipyran-3,7-dione, 9-(3	13.33	
		1,4-Methanoazulen-9-ol, decahydro-1,5,5,8atetramethyl-,[1	2.83	
13	44.369	9-Octadecenoic acid (Z)-,2-hydroxy-3-[(1-oxohexadecyl)oxy] propyl ester	36.71	35.07
		9-Octadecenoic acid (Z)-,2-hydroxy-1-(hydroxymethyl)ethyl ester	33.86	
		9-Octadecenoic acid (Z)-, oxiranylmethyl ester	14.4	

Table III. Chemical nature of phytochemical compounds obtained from *T. cordifolia*

S.No	Phytochemical compound	Pubchem ID	Molecular formula	Molecular weight (g/mol)	Lipophilicity (log *p*)	Hydrogen bond donors	Hydrogen bond acceptors	Topological Polar Surface Area (TPSA) (□^{2})	Lipinski rule
1	Glycerin	753	C3H8O3	92.09	-1.09	3	3	60.69	0
2	1,3-Dicyclohexylurea	4277	C13H24N2O	224.34	2.66	2	1	41.13	0
3	Capsazepine	2733484	C19H21ClN2O2S	376.90	3.39	3	2	87.82	0
4	Ethyl α-D-glucopyranoside	9815668	C8H16O6	208.21	-1.35	4	6	99.38	0
5	4-Hydroxyvalsartan	9911647	C24H29N5O4	451.52	2.90	3	7	132.30	0
6	7-Hydroxy-8-methylisoflavone	5408595	C16H12O3	252.26	3.02	1	3	50.44	0
7	Valerenic acid	4229818	C15H22O2	234.33	3.18	1	2	37.30	0
8	2,4-Dimethylethcathinone	82100531	C13H19NO	205.30	2.71	1	2	29.10	0
9	3-Cyclopentene-1-octanoic acid, 2-(3-hydroxy-1-penten-1-yl)	5373082	C18H28O3	292.41	4.05	1	3	54.37	0
10	Khellin	3828	C14H12O5	260.24	2.29	0	5	61.81	0
11	9-acetoxyfukinanolide	78385431	C17H24O4	292.37	2.87	0	4	52.60	0
12	Dispiro[5.2.5.2]hexadecan-1-one	616403	C16H26O	234.38	4.22	0	1	17.07	0
13	Hexadecanoic acid, ethyl Ester	12366	C18H36O2	284.48	5.90	0	2	26.30	1
14	Heptadecanoic acid, ethyl ester	26397	C19H38O2	298.50	6.34	0	2	26.30	1
15	Pentadecanoic acid, ethyl ester	38762	C17H34O2	270.45	5.58	0	2	26.30	1
16	Phytol	5280435	C20H40O	296.53	6.22	1	1	20.23	1
17	Oxirane, decyl-	17858	C12H24O	184.32	4.02	0	1	12.53	0
18	(R)-(-)-(Z)-14-Methyl-8-hexadecen-1-ol	12487634	C17H34O	254.45	5.37	1	1	20.23	1
19	11-Dodecyn-1-ol acetate	538082	C14H24O2	224.34	3.96	0	2	26.30	0
20	13-Tetradece-11-yn-1-ol	543337	C14H24O	208.34	4.12	1	1	20.23	0
21	Bicyclo[10.1.0]tridec-1-Ene	548879	C13H22	178.31	4.30	0	0	0.00	1
22	2-Cyclododecenol	53886827	C12H22O	182.30	3.15	1	1	20.23	0
23	(R)-(-)-14-Methyl-8-hexadecyn-1-ol	10944926	C17H32O	252.44	5.19	1	1	20.23	1
24	13-Oxabicyclo[10.1.0]tridecane	9248	C12H22O	182.30	3.54	0	1	12.53	0

25	Columbin	188289	$C_{20}H_{22}O_6$	358.39	2.11	1	6	85.97	0
26	1,4-Etheno-3H,7Hbenzo[1,2-c:3,4-c’]dipyran-3,7-dione, 9-(3	56678199	$C_{20}H_{20}O_6$	356.37	2.09	1	6	85.97	0
27	1,4-Methanoazulen-9-ol, decahydro-1,5,5,8atetramethyl-, [1	534588	$C_{15}H_{26}O$	222.37	3.60	1	1	20.23	0
28	9-Octadecenoic acid (Z)-, 2-hydroxy-3-[(1-oxohexadecyl)oxy]propyl ester	5363257	$C_{37}H_{70}O_5$	594.95	10.62	1	5	72.83	2
29	9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester	5319879	$C_{21}H_{40}O_4$	356.54	5.07	2	4	66.76	0
30	9-Octadecenoic acid (Z)-, oxiranylmethyl ester	5354568	$C_{21}H_{38}O_3$	338.52	5.89	0	3	38.83	0

Table IV. Binding affinity of phytochemicals against target proteins

S. No	**Ligand**	**Binding energy (Kcal /mol)**		
		Glycolate oxidase	**Oxalate oxidase**	**CD44**
1	Columbin	**-7.6±0.25**	**-7.5±0.53**	**-8.2±0.32**
2	Khellin	-6.5±0.35	-5.5±0.21	-7.7±0.65
3	Valerenic acid	-6.4±0.27	-6.4±0.50	-7.4±0.84
4	Capsazepine	-6.2±0.35	-6.2±0.22	-6.7±0.47
5	1,4-Methanoazulen-7-ol, decahydro-1,5,5,8a-tetramethyl-,[1s-(1.alpha.,3a.beta.,4.alpha.,7.beta.,8a.beta.)]-	-6.2±0.32	-6.0±0.57	-6.6±0.22
6	Cyclododec-2-en-1-ol	-6.2±0.46	-5.5±0.27	-6.6±0.51
7	9-Acetoxyfukinanolide	-6.2±0.29	-5.8±0.21	-7.7±0.51
8	Bicyclo[10.1.0]tridec-1-ene	-5.9±0.23	-5.5±0.22	-6.6±0.44
9	Epoxycyclododecane	-5.7±0.32	-4.8±0.16	-6.4±0.37
10	Decyloxirane	-4.7±0.28	-3.3±0.13	-4.6±0.18
11	13-Tetradecen-11-yn-1-ol	-4.6±0.30	-3.7±0.24	-4.5±0.33

Fig. 3 2D structure of columbin and khellin interaction with target proteins

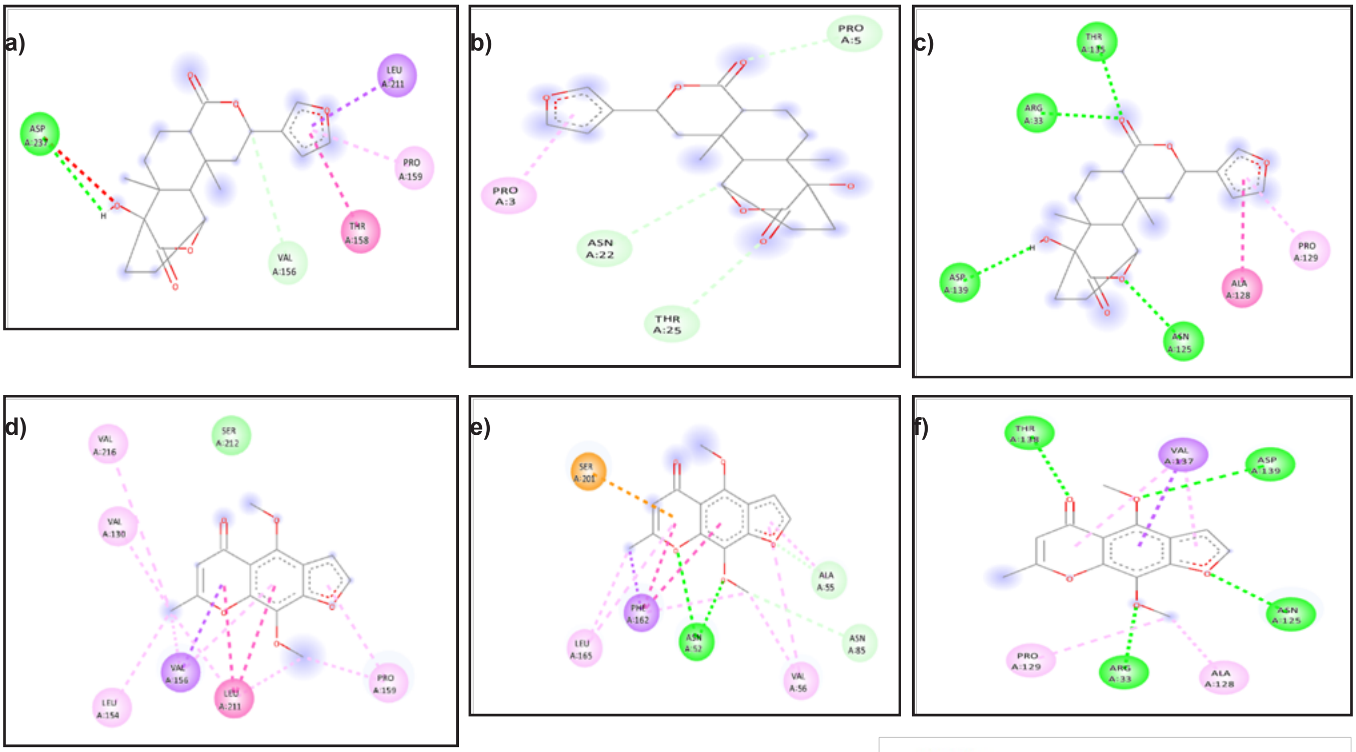

Fig. 3a, 3b, 3c indicates 2D structure of Columbin with glycolate oxidase, oxalate oxidase and CD44 respectively. Fig 3d, 3e, 3f indicates 2D structure of Khellin with glycolate oxidase, oxalate oxidase and CD44 respectively

Interactions

■	Conventional Hydrogen Bond	■	Pi-Sigma
■	Carbon Hydrogen Bond	■	Amide-Pi Stacked
■	Unfavorable Acceptor-Acceptor	■	Pi-Alkyl
■	van der Waals	■	Alkyl
■	Pi-Anion	■	Pi-Pi Stacked

and oxalate oxidase. Swaminathan *et al.,* 1989 described the structure of Columbin isolated from *T. cordifolia* that it contains two terpene rings, two δ-lactones, two methyl groups, a tertiary hydroxyl group and a β-substituted furan ring are present in the structure. The hydroxyl group is involved in intramolecular hydrogen bonding. Columbin acts against neurodegenerative diseases by inhibiting acetylcholinesterase (AChE) (Onoja *et al*., 2021), anti-tumor activity (Kohno *et al*., 2002) and anti-inflammatory activity (Abdelwahab *et al*., 2012). This study has established the molecular interaction between columbin, a diterpenoid furanolactone, and proteins implicated in urolithiasis namely glycolate oxidase, oxalate oxidase and CD44. The human liver serves as the primary origin of endogenous oxalate, with glyoxylate acting as the key intermediate precursor for oxalate synthesis by glycolate oxidase enzyme (Baker *et al*., 2004). Other than Columbin (-7.6 kcal/mol), Khellin, Valerenic acid, Capsazepine, 1,4-Methanoazulen-7-ol, decahydro-1,5,5,8a-tetramethyl-, [1s-(1α,3aß,4α,8aß.)]-, cyclododec-2-en-1-ol and 9-acetoxyfukinanolideshowed moderate binding energy with glycolate oxidase. Oxalate oxidase catalyzes the conversion of oxalate and dioxygen to hydrogen peroxide and carbon dioxide (Opaleye *et al*., 2006). Oxalate oxidase binds with Columbin with the binding energy of -7.5 kcal/mol followed by moderate interaction with Valerenic acid (-6.4 kcal/mol) and Capsazepine (-6.2 kcal/mol). The CD44 family of surface receptors regulates adhesion, movement, and activation of normal and neoplastic cells. CD44 acts as a receptor for Osteopontin (OPN) (Weber *et al* 1996). The retention of crystals in the human kidney might be influenced by the expression of damaged distal tubular epithelium, characterized by CD44, osteopontin (OPN), and a hyaluronic acid (HA)-rich cell coat. Vanachayangkul *et al*., 2011 reported that the extract of Ammi visnaga L. and its constituents khellin and visnagin were found to prevent renal crystal deposition in hyperoxaluric rats. Khellin(-7.7 kcal/mol) and 9-Acetoxyfukinanolide (7.7 kcal/mol) showed higher binding energy against CD44 followed by Columbin (-8.2 kcal/mol).

There are few reports regarding the antiurolithiatic potential of *Tinospora cordifolia*. *Invivo* evaluation of *Tinospora cordifolia* by Rai and Gupta (1967) reported that the water extract of *Tinospora cordifolia* demonstrated the ability to dissolve urinary calculi.Kumar *et al*.(2011) investigated the effect of ethanolic extract of *Tinospora cordifolia* on calcium oxalate crystallization *in vitro* and concluded that concluded that *T. cordifolia* stem extract remarkably inhibited the calcium oxalate crystal formation because of its antiurolithiatic potential.

This *in silico* study of the multipurpose plant *T. cordifolia* against proteins involved in urolithiasis revealed that the phytochemicals of this plant have significant interaction in the urolithiasis pathway. The result has to be further validated by *in vitro* and *in vivo* studies to prove the antiurolithiatic potential of *T. cordifolia* for use of mankind.

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A Case Study on Medicinal Plants for Swine Dermatophytosis

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Abstract

The present work is a case study that was conducted at the Livestock Farm Complex, Veterinary College and Research Institute; Orathanadu (Tamil Nadu) to assess the efficacy of topical herbal plant mixtures on swine dermatophytosis. Dermatophytosis is a contagious fungal disease affecting various animal species and is a high economic burden with limited antifungal agents. Skin disease in swine can significantly impact production, carcass value and reputation; diagnosing and treating these diseases can be challenging. Eight adult sows in the farm exhibiting moist, reddish brown colour irregular foci on different parts of the body with itching were selected for the study. A mixture of three medicinal plants were selected and combined with common salt in the ratio of 5:2:2:1, comprising *Acalypha indica and Azadirachta indica* leaves, *Curcuma longa* rhizome and salt, respectively. The herbal mixture was well grounded fresh every time to make a topical paste, which was applied over the affected area three times a day for five days. The response to the treatment was assessed based on the successful reduction of the severity of the cases. The current study's findings suggest that the plants chosen for the study have the potential to

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