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In-vitro Pharmacological investigation and Molecular Docking validation of *Cissus quadrangularis* against Bacterial and Helminthiasis infections

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ABSTRACT:
The present study's objective was to identify the phytoconstituentspresent in n-hexane extract of *Cissusquadrangularis*(*C.quadrangularis*)with a help of Gas chromatography-Mass Spectrometry (GC-MS). The extract was subjected for the antibacterial (*Escherichia coli*) and anthelmintic (adult Indian earthworms-*Pheretimaposthuma)in-vitro* activities followed by the validation of the same by subjecting the bioactive compounds obtained in GCMS analysis to molecular docking studies for anthelmintic and antibacterial protein targets.Threecompounds with molecular formula $C_{20}H_{42}O_2S$, $C_{14}H_{25}O_3N_2Cl$ and $C_{30}H_{50}O$ were identified in GCMS analysis. Different extract concentrations viz 25, 50, 75 and 100 mg/ml and25, 50, 75 and 100µg/mlfor anthelmintic activity and antibacterial activityrespectively.
For anthelmintic protein target, results of molecular docking (presented by 3D & 2D model) revealed that the identified compounds (binding energy -6.05 to -8.40 Kcal/mol) exhibited a comparable binding affinity as that of albendazole(binding energy -9.54 Kcal/mol) except C3 (binding energy +33.42 Kcal/mol) compound. Antibacterial protein target, compounds' binding affinities were found to be in the range -4.49 to -6.68 Kcal/mol, while Chlorobiocin was found to be -4.75 Kcal/mol. The extract exhibited dose-dependent *in-vitro*antihelmintic (75 and 100 mg/ml) and antibacterial activity (75 and 100 µg/ml). The minimum lethal amount of extract concentration for anthelmintic activity was found to be 100 µg/ml. At this concentration, *Pheretimaposthumadied in*31.09 ± 1.25min while with a standard dose of albendazole, the lethal time was found to be 38.54 ± 1.54 min.

Finally,concluded that the identified bioactive compounds from *C.quadrangularis*have the potentialantihelmintic and antibacterial activity as justified by *in-vitro* biological evaluations and molecular docking studies.

KEYWORDS: *Cissusquadrangularis,* GC-MS, Earthworm, *Escherichia coli,* Molecular Docking

INTRODUCTION

*Cissusquadrangularis*Linn. It has been grown in a number of countries around the world, including India, Sri Lanka, Malaya, Java, West Africa, and Thailand. It is an edible plant that belongs to the Vitaceae family. It must be grown in a warm climate. It is commonly referred to as a "bone setter," and it is also referred to as "Asthisamdhani" in Sanskrit and "Hadjod" in Hindi due to its ability to join bones. Gout, syphilis, venereal diseases, piles, tumours, haemorrhoids, peptic ulcers, and leucorrhoea are all treated with this plant[1-4].The patent for *C. quadrangularis* available in a database of health applications, either alone or incombination with other constituents. The majorities of patents are concerned with bone fracture healing, anti-osteoporotic activity, and blood glucose and lipid regulation. *C. quadrangularis*L. formulations are available on the market [5].*C. quadrangularis'* hypoglycemic activity is mediated by the regulation of carbohydrate metabolic enzyme activities [6]. Methanolic (95%) extracts have been shown to have strong proton pump inhibitory activity [7]. Anti-inflammatory activity of a mixture of methanolic root extract of *C. quadrangularis* L. and seed extract of *L. sativum* L. (LS) was studied in rats [8]. The antiepileptic activity of aqueous extract was assessed using maximal electroshock and the hot plate method for analgesic and smooth muscle relaxant activity using the rotarod method [9]. They looked into the effectiveness of *C. quadrangularis* in the treatment of haemorrhoids [10].Alpha amyrin, Beta amyrin, Beta sitosterol, Friedelin, Quercetin, Genistein, and Daidzein are the primary bioactive compounds found in plants. Chemical constituents found in the plant's stem parts include -amyrins, -sitosterol, ketosetosterol, phenols, tannins, vitamins, carotene, calcium oxalate, 31 methyl tritiacontanoic acid, taraxeryl acetate, taraxeroliso-pentadecanoic acid, calcium ions, and phosphorus [11]. GC-MS identified chemical constituents such as eugenol, n-hexadecanoic acid, 1, 2-benzenedicarboxylic acid, diisooctyl ester, phenol, 2, 4-bis(1-phenylethyl) [12]. HPTLC and HPLC analysis of Ayurvedic crude drugs revealed four important markers: *C. quadrangularis* L. [13]. Aqueous and methanolic extracts of C. quadrangularis stems were investigated by GC-MS [14].

Methanolic extract of stem has been analysed using various analytical techniques, which results in useful chemical profiling [15]. Thermodefragmentation of *C. quadrangularis* L. for characterization [16]. LC-MS analysis of extracts of this plant in different solvent systems revealed the presence of phytosterols in petroleum ether and ethanol extracts, but not in chloroform extracts [17]. Polyphenols in stem and leaf samples are quantitatively analysed using UHPLC-PDA-MS, LC-QToF, and HPTLC-analytical. *C. quadrangularis* can also be found in dietary supplements [18]. Antigout arthritic activities in silico were studied using stem extracts in ethanol and aqueous [19].Literature is available in the database, which is very useful in designing the current work. To the best of our knowledge, the outcome of the present is not available.

2. Experimental Work:

2.1 Plant Materials:

The Ethanolic extract of *C.quadrangularis*obtained gift sample (ECQ) Batch No-6124 from Himalaya drug company, Bangalore, India.The 10gm ofEthanolic extract of

*C.quadrangularis*wassuccessive fraction with n-hexane solvent in separating funnel, get the green colour crude mass of yield of 900mg.

2.2 Culture Media Bacteria:

*E.coli*microorganismprovided by Owaisi Hospital and Research Center, department of microbiology, Hyderabad, Mueller-Hinton agar (Himedia, Lot 0000333943, Code M173)NaCl, Beef Extract procured by Himedia Pvt. Ltd., India.

2.3GC-MS Analysis: Chromatographic data were obtained using Clarus 680 GC. A fused silica column packed with Elite-5MS (containing, 5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250µm df), separation of components was carried out using Helium gas with a constant flow rate of 1ml/minute. Whereas, the injector temperature was maintained at 260°C throughout the chromatographic. The volume of the extract injected into the instrument was 1 µL. The oven temperature was initially maintained at 60 °C for two minutes, followed by 300 °C at the rate of 10 °C min^{-1} for 6 minutes.Meanwhile, for the mass detector, transfer line temperature and ion source temperature was maintained at 240° C. Ionization mode was set with an electron impact of 70 eV with 0.2 seconds, scan time and 0.1 sec scan interval. Fragmentation was observed between 40 to 600 Da. The resultant spectra were compared with the reference spectrum using GC-MS NIST (2008) library.

2.4 Preparation of Extracts:

The *C.quadrangularis*n-hexane extract different concentrationwere prepared 25, 50,75 and 100 mg/ml for antihelmintic activity and 25, 50,75 and 100 µg/ml for anti-bacterial activity. Albendazole 20mg/kg bw and Chlorobiocn (20µg/ml)inapply as standard. In each Groupcontain3-4*Pheretimaposthuma*earthwormsapproximately equal size areindividually placed in each petridish fill with10-15 ml normal saline solution and desired concentration of drug and extracts.

2.5 Antibacterial testing by agar well diffusion method:

Assessment of antibacterial activity *C.quadrangularis*extracts was determined by agar well diffusion methoddescribed**[20,21].** Inoculums of *Escherichia coli* bacterial strains were plated using sterile swabs into Petri dishes containing approximately 25 ml of Nutrient agar media, where, 6 mm wells were made and filled with different concentration 25, 50, 75 and 100 µg/ml of extracts. The Petri dishes were pre-incubated for 3 h at room temperature, allowing the complete diffusion of the samples and then, incubated at 37±1°C for 24 h. Further antibacterial activity of the above samples was determined by measuring the zone of inhibitionmillimeters (mm) according to the standard clinical parameters.

2.6 Anthelmintic Activity:

The *in-vitro*anthelminticstudy was done as per the protocol described by **[22-24**], **Group 1:** Normal Control Group using normal saline 0.5% w/v,**Group 2:** Standard GroupAlbendazole 20 mg/ml kg bw,**Group 3:** *C.quadrangularis*n-hexane extract 25 mg/ml, **Group 4:** *C.quadrangularis*n-hexane extract 50 mg/ml, **Group 5:** *C.quadrangularis*n-hexane extract75 mg/ml, **Group 6:** *C.quadrangularis*n-hexane extract 100 mg/ml, *Pheretimaposthuma* adult Indian earthworms (n=3, or 4) were placed in each Petri dish. The worms were observed for the time of loss of movement (paralysis), and death. Average time for the loss of movement (paralysis) and complete loss of movement even upon aggressive shaking (death) was recorded. Albendazole was used as the reference standard to compare the activity of the extract.

2.7 Molecular docking studies:

Dock Thor was used to perform 3D molecular interactions of selected ligands with two different proteins (DT). The default values for the grid box centre and grid size in the x, y, and z axes, spacing between two consecutive grids, and genetic algorithm attributes such as number of evaluations, population size, and number of runs. The Dock Thor score, which is similar to the G score, represented the binding affinity and ranking of various ligand molecules.Protein structures were created as docking targets using Autodock Tools (version 1.5.6). Water molecules, metal atoms, co-crystallized ligands, and other non-covalently bonded molecules were removed from the protein structures. Gesteiger charges were added, and the target file was saved in the appropriate pdbqt format. When the structure is saved, the software automatically adds polar hydrogen and merges nonpolar hydrogens**[25-28].**

2.8 Statistical Analysis:

The IBM SPSS 25.0 software (SPSS Inc., Wacker Drive, Chicago, IL, USA) was used to compute the mean and standard deviation of three independent tests involving triplicate analyses for each sample. To test for significant differences between the mean values obtained from antioxidant activity measurements (at the 5% level), a post-hoc test (Tukey) was used.

3. RESULTS and DISCUSSION:

3.1 GC-MS Analysis: GC–MS chromatogram of extract *C.quadrangularis*clearly shows three peaks representing the presence of 3 bio active compounds. Bioactive compounds from the extracts were identified from the data obtained in terms of the peak area, retention time and the molecular formula (**Table 1**) and **Figure 1 (Chromatogram).** From the NIST library the GC-MS spectra compared three bioactive compoundsDi-N-Decylsulfone, 2-t-butyl-5-chloromethyl-3-methyl-4-oxoimidazolidine-1-carboxylic acid ester and URS-12-EN-28-OLwith standardalbendazole and Chlorobiocn.

3.2 Antibacterial testing by agar well diffusion method:

The antimicrobial activity of extracts *C.quadrangularis*were studied in different concentrations (25, 50,75 and 100 µg/ml) against Gram-negative *Escherichia coli* pathogenic bacterial strains. The extract shows that varying degree of antimicrobial activity 75 and 100 µg/ml as shown in**(Figure 2). On the basis of zone of inhibition the two concentrations are effective as shown in the result.**

3.3 Anthelmintic Activity:

The *C.quadrangularis*extractwas studied in different concentrations (25,50,75,100 mg/ml) anthelmintic activity on *Pheretimaposthuma*worms.Extractshows a significant activityin a dose dependent manner as shown in (Figure3).The anthelmintic activity *C.quadrangularis*extract in lower concentration not significant, in higher concentration extract 75,100 mg/ml exhibit significantly 31.15 ± 4.35for paralysis and 29.54 ± 3.21minute,in death time of the worms 37.28 ± 4.42 minute and 33.56 ± 2.15 minute. Albendazole (20 mg/ml) 22.75 ± 1.63 and 31.20 ± 1.25 minutes for paralysis and death respectively.Effectiveness of the extract*C.quadrangularis*was inversely proportional to the time for paralysis (vermifuge) and death (vermicidal) of the worms.

3.4 Molecular docking studies:

3.4.1 Antibacterial activity docking studies

PDB ID: 1KZN

Antibacterial activity is measured on by Schrodinger on a scale of-2 to +2 where -2 represents Inactive and +2 represents highly active .

QPPCaco (Gut-blood barrier) <25-Poor >500-Great

QPPMDCK (Blood-brain barrier) <25-Poor >500-Great

All the 3 molecules under investigation along with the standard clorobiocin were docked on the protein with PDB ID: 1 KZN using Schrodinger suite. The ADME toxicity predictions of drug-candidates were done using Qikprop of the Schrodinger suite. In the ADME data mentioned the CNS activity represents predicted central nervous system activity on a –2 (inactive) to +2 (active) scales. QPlogKhas represents the prediction of the molecule binding to human serum albumin and the recommended values lie between -1.5 to 1.5. The lipinski's rule of values represents deviation from the mentioned rule and the maximum allowed deviations are 4. Caco-2 cell permeability represents predicted apparent Caco-2 cell permeability in nm/sec. Caco2 cells are a model for the gut-blood barrier. These predictions are for only active transport and values<25nm/sec represent poor and >500nm/sec great permeability. MDCK cell permeability represent predicted apparent MDCK cell permeability in nm/sec. MDCK cells are considered to be a good mimic for the blood brain barrier. These predictions are also for active transport only and a value of <25nm/sec represent low permeability whereas a value >500nm/sec represent great permeability. These values are taken from Qikprop manual.

*3.4.2***Anthelminthic activity *docking studies:***

PDB ID:4KRH

All the 3 molecules under investigation along with the standard albendazole were docked on the protein with PDB ID: 1KZN using Schordinger suite. The ADME toxicity predictions of drug-candidates were done using Qikprop of the schrodinger suite. In the ADME data mentioned the CNS activity represents predicted central nervous system activity on a –2 (inactive) to +2 (active) scale. QPlogKhsarepresents the prediction of the molecule binding to human serum albumin and the recommended values lie between -1.5 to 1.5. The last column represents number of likely metabolic reactions

4. Conclusion: The dose dependent effect of*C.quadrangularis*herbal extract have studied and the results have shown effectiveness in concentration of 75 and 100 mg/ml for anti-helminthic and, 75 and 100 µg/ml for anti-oxidant activity. Further theses in-vitro results have been validated by performing molecular docking with respective receptor against the standard drug AlbendazoleandChlorobiocn for anti-helminthic and anti-oxidant activity respectively.

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ONFLICT OF INTEREST

We have no conflict of interest to declare.

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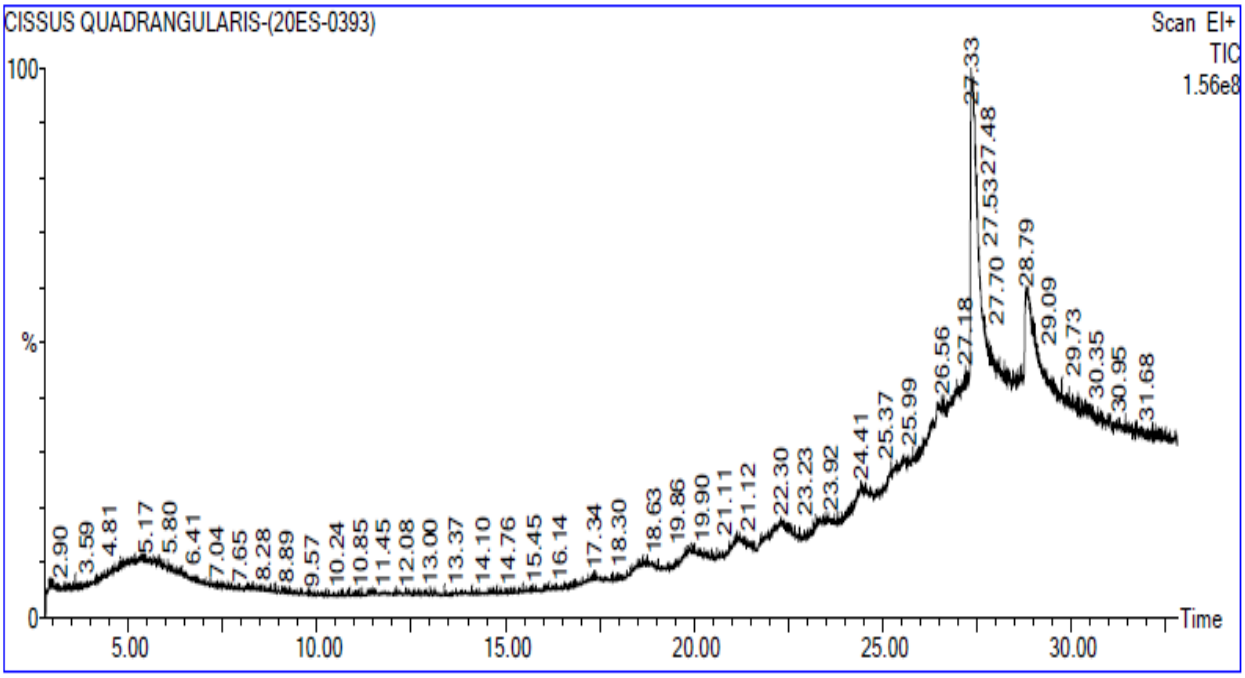


Figure 1: Chromatogram of *C.quadrangularis GC MS analysis*

Table 1: GC–MS analysis of extract *C.quadrangularis*

S. no	Peak name	Molecular formula	Molecular weight	Retention time	% Area
1	Di-N-Decylsulfone	$C_{20}H_{42}O_2S$	346	26.563	3.983
2	2-t-butyl-5-chloromethyl-3-methyl-4-oxoimidazolidine-1-carboxylic acid ester	$C_{14}H_{25}O_3N_2Cl$	304	27.333	62.011
3	URS-12-EN-28-OL	$C_{30}H_{50}O$	426	28.844	34.006

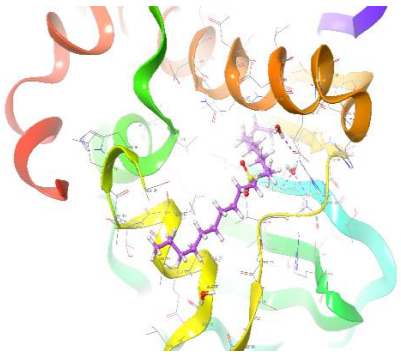
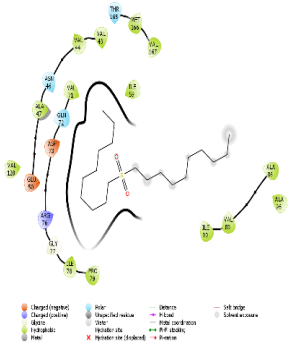
Table 2: Molceur docking Antibacterial activity

Compound	Docking Score (ligand binding	CNS activity	Prediction of binding to human serum	Number of violations of	Apparent Caco-2 cell permeability (nm/sec)	Predicted apparent MDCK cell permeability

	free energy kCal)		albumin (QPlogKhsa)	Lipinksi's rule of five	(Gut-blood barrier, Non-active transport)	(nm/sec) (Blood-brain barrier, Non-active transport) QPPMDCK
Di-N-Decylsulfone	-3.716	-2 (Inactive)	0.962000	1	2011.272000	1056.789000
2-t-butyl-5-chloromethyl-3-methyl-4-oxoimidazolidine-1-carboxylic acid ester	-3.897	1 (Active)	-0.362000	0	1506.316000	3008.047000
URS-12-EN-28-OL	-2.294	1(Active)	1.971000	1	4093.716000	2269.743000
Clorobiocin (Standard)	-4.849	-1(Slightly active)	-1.147000	0	17.172000	16.684000

CNS activity is measured on by Schrodinger on a scale of-2 to +2 where -2 represents Inactive and +2 represents highly active .QPPCaco (Gut-blood barrier) <25-Poor >500-Great, QPPMDCK (Blood-brain barrier) <25-Poor >500-Great

Figure 2: 2D and 3D Antibacterial activity:

S.N	3D-Interaction diagram	2D-Interaction diagram
1	 Di-N-Decylsulphone	 Di-N-Decylsulphone

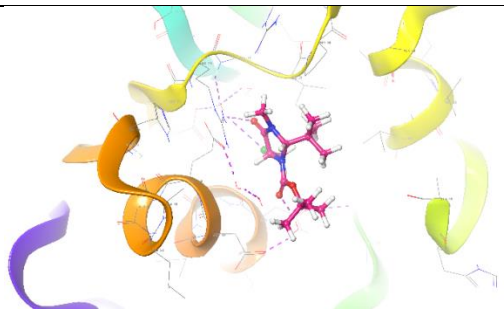
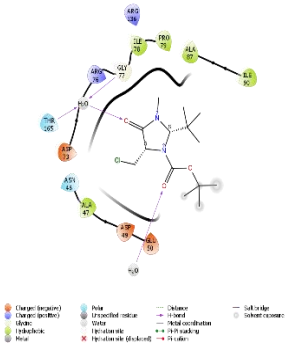
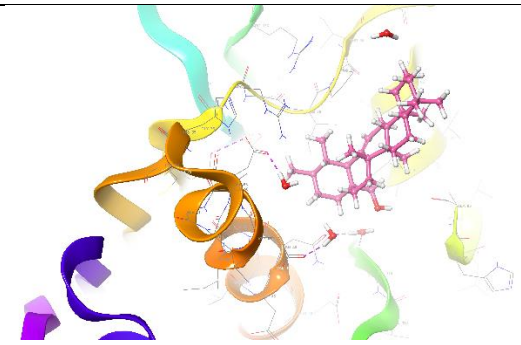
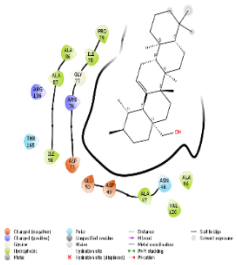
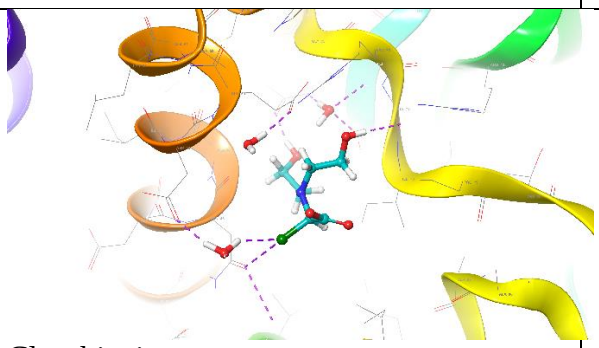
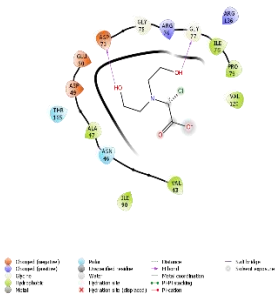
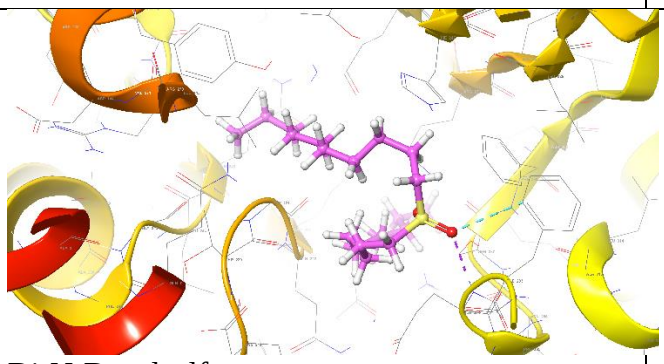
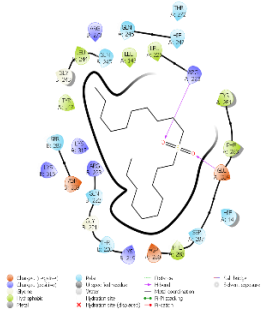
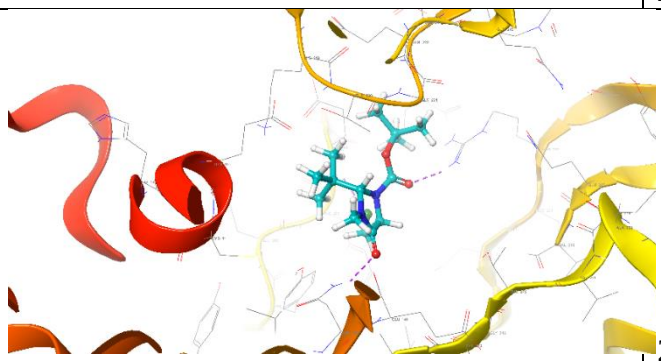
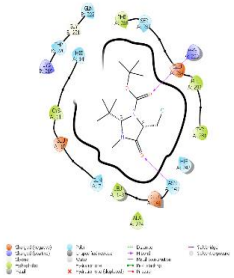
2	 2-t-butyl-5-chloromethyl-3-methyl-4-oxoimidazolidine-1-carboxylic acid ester	 2-t-butyl-5-chloromethyl-3-methyl-4-oxoimidazolidine-1-carboxylic acid ester
3	 URS-12-EN-28-OL	 URS-12-EN-28-OL
4	 Clorobiocin	 Clorobiocin

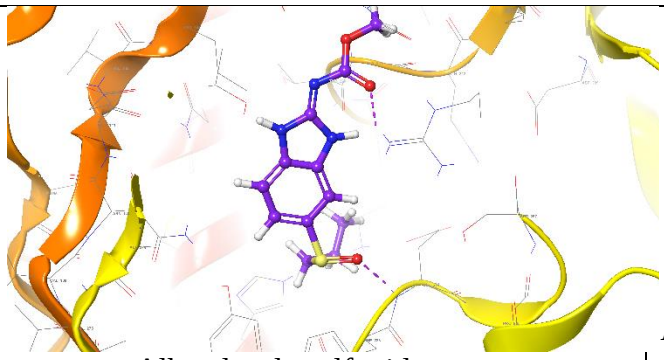
Table 3: molecular docking Anthelminthic activity

Compound	Docking score (ligand binding free energy kCal)	CNS activity	Prediction of binding to human serum albumin (QPlogKhsa)	Metabolic reactions
Di-N-Decylsulfone	-3.836	-2 (Inactive)	0.962000	0
2-t-butyl-5-chloromethyl-3-methyl-4-oxoimidazolidine-1-carboxylic acid ester	-4.187	1 (Active)	-0.362000	1

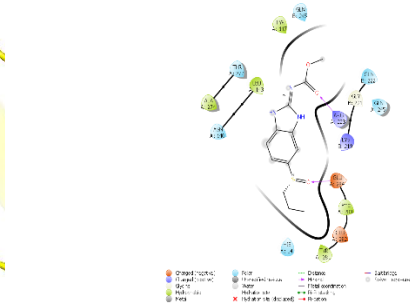
URS-12-EN-28-OL	Not applicable	1(Active)	1.971000	3
Albendazole sulfoxide (Standard)	-3.352	-2(Inactive)	-0.238	1

Figure 3: 2D and 3D Anthelminthic activity

S. N	3D-Interaction diagram	2D-Interaction diagram
	 Di-N-Decylsulfone	 Di-N-Decylsulfone
	 2-t-butyl-5-chloromethyl-3-methyl-4-oxoimidazolidine-1-carboxylic acid ester	 2-t-butyl-5-chloromethyl-3-methyl-4-oxoimidazolidine-1-carboxylic acid ester

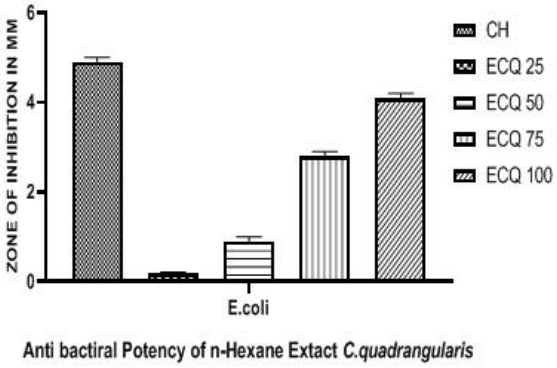


Albendazole sulfoxide



Albendazole sulfoxide

Figure 5.showing the anti bacterial effect of *C.quadrangularis*differentconcentrationsagainst*E. Coli*



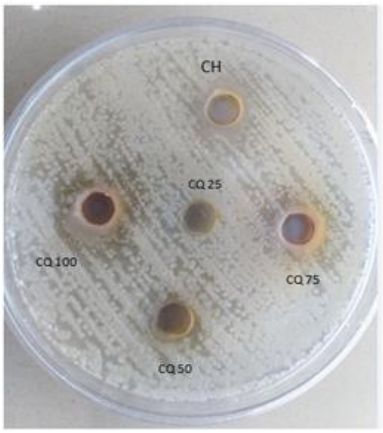
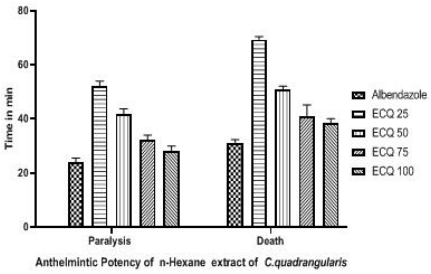


Figure 6.Showing anthelmintic activity*C.quadrangularis*



Anthelmintic Potency of n-Hexane extract of *C.quadrangularis*