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ORIGINAL ARTICLE **OPEN ACCESS**

GC-MS analysis, *In silico* docking studies and diuretic activity of methanolic extract of *Citrus medica* l. Leaf in wistar albino rat model

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ABSTRACT
A diuretic is an agent that increases the rate of urination thereby decreasing body fluid, especially the extracellular fluid. Its role in situations of fluid overload, like acute and chronic renal failure, hypercalciuria, cirrhosis of liver and also act as an antihypertensive agent. Citrus medica Linn., commonly known as the Citron. Various parts of citrus species were known for different activities like antimicrobial, antidiabetic and hypoglycemic, antiulcer, fungitoxocity, estrogenic, antihyperglycemic and fruits were used for antilithiatic activity and antioxidant activity. The present study aimed to analyse in vivo diuretic activity by Lipschitz test model and molecular docking studies to establish mechanism of active constituents present in the plant. Preliminary resulted in the presence of alkaloids, flavonoids, saponins, carbohydrates, terpenoids, steroids and glycosides. Citrus medica methanolic extract had shown significant increase in urine parameters like urine volume, urine pH and urine excretion and showed effect on serum electrolyte parameters like sodium and potassium that has potent diuretic index and Lipschitz values which might be responsible for diuretic activity. Molecules identified from GC-MS were selected and insight of the binding mode of these compounds (ligand) into the binding sites of Phospholipase A2 inhibitor (PDF code: 5OW8) was provided by docking studies, performed with the help of Maestro Schrodinger docking software, out of all the constituents Dasycarpidan-1-methanol, acetate (ester) showed good binding score. This study provides proof of evidence for the prominent diuretic activity and specifically inhibiting Phospholipase A2 inhibitor by protein ligand interaction.
Keywords: *Lipschitz. extracellular fluid, citron, docking and diuretic index.*

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INTRODUCTION

A diuretic is an agent that increases the rate of urination thereby decreasing body fluid, especially the extracellular fluid. Diuretics play an important role in situations of fluid overload, like acute and chronic renal failure, hypercalciuria, cirrhosis of liver and as antihypertensive agent [1]. Besides, the World Health Organization has estimated that over 75% of the world's population still rely on plant-derived medicines, usually obtained from traditional healers for basic health-care needs [2]. The primary action of most diuretics is thedirect inhibition of sodium transport International at one or more of the four major anatomic sites along the nephron where sodium reabsorption takes place [3]. A number of diuretics like thiazides, furosemide, mannitol and ethacrinic acid are used in practice. Drug-induced diuresis is beneficial in many life-threatening disease conditions such as heart failure, cirrhosis, hypertension, renal failure and pregnancy toxaemia[4].*Citrus medica* Linn., commonly known as the Citron in English and bijapura [5]. Different parts of citrus species were screened against pathogenic organisms [6-10]. Various parts of citrus species were known for different activities like antimicrobial [11], antidiabetic, hypoglycemic [12], antiulcer [13], Fungitoxocity [14], estrogenic[15] and antihyperglycemic [16] and fruits were used for antilithiatic activity [18-19] and antioxidant activity. Molecular docking helps us inpredicting the intermolecular framework formed between a protein and ligand or a protein and protein and suggest the binding modes responsible for inhibition of the protein. To accurately carry out docking studies one requires homology-modelled structure with known/predicted binding site in the biomolecule docking studies was carried out computationally to get insight of the structural parameters leading to activity like schrodinger, autodock [24]. The aim of the study is to evaluate test extracts for diuretic activity and to perform docking with protein 5OW8 by Schrodinger software.

MATERIAL AND METHODS

Plant collection

The leaves of *Citrus medica* were collected from Bibipet mandal, kamareddy district. This material was identified and authenticated by pharmacognist in the month of December 2018and was identified by Professor & Head of Department of Botany of government degree college, kukatpally. The whole plant used simple distillation technique. Before the extraction process the leaf powder placed in round bottom flask and suspended insolvent chloroform, this was then equipped with a condenser. The flask was then heated; for about an hour to remove the chlorophyll pigments. Then filtered and filtrate discarded and hard mass dried under shade and used was cleaned, reduced to small fragments, dried under shade and pulverized in the laboratory.

Preparation of methanolic extract of *Citrus medica* leaves

The powdered plant material was successively extracted in methanol for further extraction process.

The plant material suspended in the round bottom flask containing the extraction solvent methanol. This was then equipped with a condenser. The flask was then heated; the extract goes in to the solvent. At the end of extraction process extract was filtered. The filtrate was evaporated. The extract were then kept in desiccators to remove remaining moisture, if present, finally stored in air tight containers for further use.

Preliminary Phytochemical Investigation of Plant

The plant is a biosynthetic laboratory, not only for chemical compounds such as carbohydrates, protein and lipids, but multitude of compounds like glycosides, alkaloids, volatile oils, tannins etc., that exert a physiological and therapeutic effect. Selected extracts were screened for the presence of various groups of compounds.

GC Condition and Identification of Compounds:

The sample was investigated through Gas Chromatography Mass Spectrometry/Mass Spectrometry Electron Ionization (GC-MS/EI) mode. The GC-MS/MS is a Scion 436- GC Bruker model coupled with a Triple quadruple mass spectrophotometer with fused silica capillary column BR-5MS (5% Diphenyl/95% Dimethyl polysiloxane) and Length: 30m; Internal diameter: 0.25 mm; Thickness: 0.25 µm. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1 ml/min and an injection volume of 2 µl was employed (split ratio of 10:1). The injector temperature was 250°C; ion-source temperature 280°C. The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10°C/min, to 200°C, then 5°C/min to 280°C, ending with a 9 min isothermal at 280°C and total GC running time was 41 min. This last increase was to clean the column from any residues. The mass spectrometer was operated in the positive electron ionization (EI) mode with ionization energy of 70eV. The solvent delay was 0-3.0 min. A scan interval of 0.5 seconds and fragments from m/z 50 to 500 Da was programmed. The inlet temperature was set at 280 °C, source temperature 250°C [19].

Animals

Inbred colony of adult wistar albino rats (150-200gm) are procured from Jeeva Life Sciences Laboratory and they are used for the present study. They were kept in polypropylene cages at 25 ± 2^0C, with relative humidity 45-55% under 12 hour light and dark cycles. All the animals were acclimatized to the to the laboratory conditions for a week before use. They werefed with standard animal feed and water *ad libitum.* All the pharmacological experimental protocols were approved by the Institutional Animal Ethical Committee (approval no: 1175/PO/Re/S/08/CPCSEA, 28/04/2017).

Acute toxicity testing

Studies were carried out in order to check the toxic effects of the extract. The study was performed as per organization for economic cooperation and development (OECD). The method is used to evaluate the acute oral toxicity is up and down procedure (OECD - 425).

Grouping and dosing of animals

The wistar albino rats of either sex were divided in to four groups for diuretic activity. Group I – negative control treated with vehicle (distilled water) with 1ml/100 gm bd.wt. *p.o.* Group - II Treated with *Citrus medica* methanolic extract 100 mg/kg (CMME – 100). Group III – Treated with *Citrus medica* methanolic extract 200 mg/kg (CMME – 200) *p.o*. Group IV – Standard positive control treated with furosemide 20 mg/kg, *p.o*.

The dose was determined based on the acute toxicity studies as per OECD – 425 guidelines. After the safety the CMME determined, $1/10^{th}$ of the maximum dose was considered as high dose (200 mg/kg), half of the high dose was selected and considered as low dose respectively. The test dose was prepared on the day of the experiment [20].

Assessment of diuretic activity

Rats were placed in metabolic cage prior to two hour commencement of the experiment for adaptation and fasted over night with access to water. Diuretic activity was carried with respect to lipschitz method. Animals pre-treated with (0.9 %) NaCl at 1 ml/100 g of bd.wt to improve a uniform water and salt load.

Then with respect to groups, administered with vehicle, CMME 100 mg/kg, CMME 200 mg/kg and furosemide 20 mg/kg.

The body temperature was measured for each group after administration of vehicle, test extract and standard compound at 0, 1, 2, 3 and 4 hour. Body temperature observed after administration of extract and checked whether they are maintained at normal temperature. Animals of each group placed in metabolic cage. Urine collected in each of the collecting flasks and measured using a graduated measuring cylinder for a 24 hour after dosing. A drop of concentrated Hydrochloric acid was added to urine and stored at 40˚C. Under anaesthesia blood was withdrawn from retro orbital sinus at 24 hour after dosing day and sample was centrifuged at 3000 rpm for 15 min. Serum obtained was analysed for sodium and potassium. Other parameters like urinary excretion, diuretic index and lipschitz value are measured using following formulae [21].

$$\text{Urinary excretion} = \frac{\text{Total urine output}}{\text{Total liquid administration}} * 100\%$$

$$\text{Diuretic Index} = \frac{\text{Urinary excretion of treatment group}}{\text{Urinary excretion of control group}} * 100\%$$

$$\text{Lipschitz value} = \frac{\text{Urinary excretion of test group}}{\text{Urinary excretion of standard group}} * 100\%$$

Molecular Docking Studies

Molecular docking is an attractive scaffold to understand drug bimolecular interactions for the rational drug design and discovery, as well as in the mechanistic study by placing a molecule (ligand) into the preferred binding site of the target specific region of the DNA/protein (receptor) mainly in a non-covalent fashion to form a stable complex of potential efficacy and more specificity. Steps in docking are protein preparation, active site generation, ligand preparation, ligand docking by schrodinger software [22-23].

Statistical analysis

Data results are expressed as the mean ± SEM. Data was analyzed by one-way analysis of variance (ANOVA) followed by Dunnetts multiple comparison tests. A value of $p < 0.05$ were considered statistically significant. We used graph pad prism for statistical analysis.

RESULTS:

The results of the evaluations were carried out and listed in following tables below followed by discussion.

Physical properties of CMME

Extraction with methanol using soxhlet apparatus yielded a semi solid greenish black residue. The percentage yield was found to be 9.6%w/w. (Table 1).

Table 1: Colour, texture and %yield of CMME

Plant part	Type of extract	COLOUR	TEXTURE	YIELD (%w/w)
Leaves of *Citrus medica*	Methanolic extract	Greenish black	Semisolid	9.6

Preliminary phytochemical analysis of CMME

The methanolic extracts of CMME revealed the presence of flavonoids, terpenoids, alkaloids, glycosides, carbohydrates, steroids and saponins. (Table 2)

Table 2: Phytochemical constituents in CMME

Phytochemical Constituents	Results of CMME
Carbohydrates	+
Alkaloids	+
Flavanoids	+
Terpenoids	+
Steroids	+
Saponins	+
Glycosides	+

Acute toxicity study

According to guideline OECD 425 the acute oral toxicity, the limit test was performed and mortality was not observed up to 2000 mg/kg.

GC MS of *Citrus medica* methanolic leaf extract:

Gas Chromatography and Mass spectroscopy analysis of compounds was carried out in methanolic leaf extract of *Citrus medica* L, shown in Table 3. In the GC-MS analysis, 16 bioactive phytochemical compounds were identified in the methanolic extract of *Citrus medica* L. The identification of phytochemical compounds is based on the peak area, retention time molecular weight and molecular formula. The composition and identification of the main components present in the leaves of *Citrus medica* L are shown (Table 3) [20].

Table 3: Phytochemical components of *Citrus medica.L* Leaf extract

S. No.	RT	Name of the compound	Molecular Formula	Molecular Weight	Peak Area%
1.	5.08	Tetradecane	$C_{14}H_{30}$	198	8.44
2.	9.05	Isopinocarveol	$C_{10}H_{16}O$	152	10.73
3.	10.88	1-Dodecanol, 3,7,11-trimethyl-	$C_{15}H_{32}O$	228	3.69
4.	11.20	2-Bromotetradecanoic acid	$C_{14}H_{27}BrO_2$	306	9.52
5.	12.89	1-Aminononadecane, N-trifluoroacetyl-	$C_{21}H_{40}F_3NO$	379	7.84
6.	13.21	1-Hexadecanol, 2-methyl-	$C_{17}H_{36}O$	256	1.34
7.	14.63	2-Myristynoyl pantetheine	$C_{25}H_{44}N_2O_5S$	484	8.05
8.	16.50	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436	5.01
9.	18.53	5β,7βH,10α-Eudesm-11-en-1α-ol	$C_{15}H_{26}O$	222	6.35
10.	22.43	trans-13-Octadecenoic acid	$C_{18}H_{34}O_2$	282	4.89
11.	22.58	Strychane, 1-acetyl-20α-hydroxy-16-methylene-	$C_{21}H_{26}N_2O_2$	338	2.30
12.	23.02	Propanamide, 2-(2,4-dichlorophenoxy)-N-(2,6-diethylphenyl)-	$C_{19}H_{21}Cl_2NO_2$	365	13.40
13.	24.24	Dasycarpidan-1-methanol, acetate (ester)	$C_{20}H_{26}N_2O_2$	326	5.58
14.	24.66	2-Methyl-E,E-3,13-octadecadien-1-ol	$C_{19}H_{36}O$	280	2.14

Effect of CMME on body temperature

The body temperature was measured after administration of CMME *p.o.* The body temperature of wistar rats was maintained normal throughout the period *i.e.,* between 35˚C - 38˚C so it was allowed to continue the diuretic activity. Body temperature of control, CMME 100 mg/kg, CMME 200 mg/kg and furosemide 20 mg/kg group were given.

Effect of CMME on urine pH, urine volume and urine excretion for diuretic activity

The urine pH, total urine volume and urine excretion were increased in furosemide group compared to that of control group. CMME 200 treated group has high urine pH, urinevolume and urine excretion compared to CMME 100 group, which says that there is significant increase in urine parameters of furosemide and test groups compared to that of control group (Table 4) (figure 1).

Table 4: Effect of CMME on urine pH, urine volume and urine excretion in wistar albino rats.

Group	Urine pH (Mean±SEM)	Total volume intake (ml) (Mean±SEM)	Total urine volume (ml) (Mean±SEM)	Urine excretion (ml) (Mean±SEM)
Control	7±0.05	2.46±0.021	2.14±0.032	87.5±0.88
CMME (100 mg/kg)	8±0.06	2.4±0.08	2.52±0.07$^{**C\#}$	105.3±0.912$^{*A\#}$
CMME (200 mg/kg)	8.15±0.11	2.51±0.06	2.85±0.06$^{*b\#\#}$	113.28±0.90$^{*a\#}$
Furosemide (20 mg/kg)	8.2±0.03	2.66±0.06	3.23±0.07$^{* aB}$	121±0.57^{*aA}

The values are expressed as mean ± SEM (n=6) analysis was performed with one way ANNOVA followed by Dunnett's multiple comparison test against control (* = p=0.0001, ** p<0.001), against CMME - 100 (a = p=0.0001, b = p>.001) against CMME - 200 (A= p=0.0001, B = p<0.001, C = p>0.001) and against furosemide (# = p=0.0001, ## = p<0.001). CMME – *Citrus medica* methanolic extract, 100 200 indicates dose mg /kg.

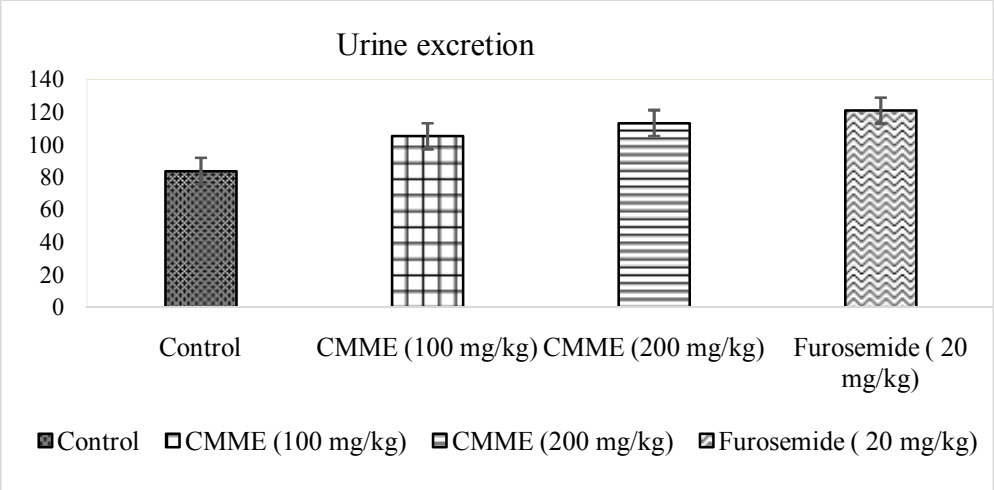

Fig 1: Significantly elevated urine excretion (ml in furosemide (20 mg/kg) group compared to control group. Treatment with CMME (200 mg/kg) showed significant increase in urine excretion compared to CMME (100 mg/kg).

Fig 2: Experiment of Diuretic activity by using metabolic chamber.

Effect of CMME on serum electrolytes – sodium and potassium for diuretic activity

The electrolytes sodium and potassium concentration in serum were measured. The furosemide group, CMME 100 and CMME 200 groups have low serum sodium and potassium values compared to that of control group (Table 5). The low sodium and potassium levels in CMME and furosemide indicated that it showed diuretic activity (Figure 3).

Table 5: Effect of CMME on sodium and potassium electrolytes in serum of wistar albino rats.

Group	Sodium (mEq/L) (Mean±SEM)	Potassium (mEq/L) (Mean±SEM)
Control	141.45±0.64	5.48±0.17
CMME (100 mg/kg)	132.36±0.66	8.3±0.23
CMME (200 mg/kg)	127±0.89	6.1±0.12
Furosemide (20 mg/kg)	109.83±0.60	5.35±0.13

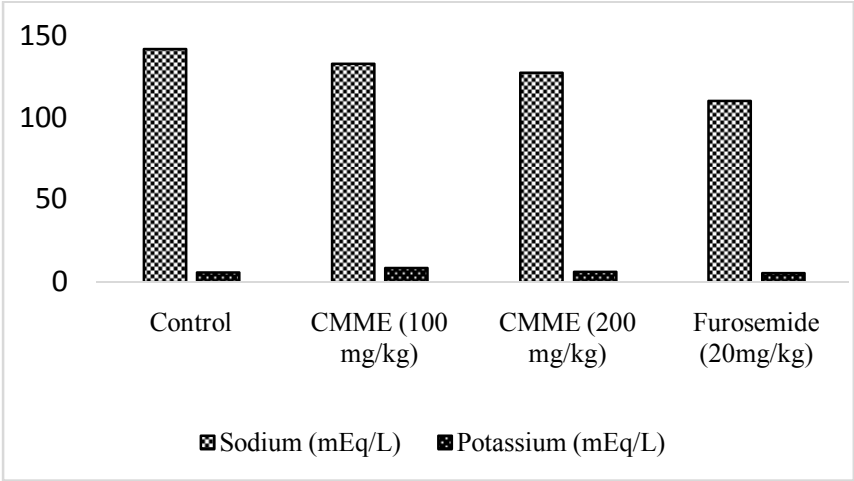

Fig 3: Significantly decreased sodium and potassium (mEq/L) in furosemide (20 mg/kg) group compared to control group. Treatment with CMME (200 mg/kg) showed significant decrease in serum sodium and potassium levels compared to CMME (100 mg/kg).

Effect of CMMEon diuretic index and Lipschitz value

By considering respective formulae, diuretic index and lipschitz values were obtained, there it resulted that there is significant increase in diuretic index from (control)1 to 1.22, 1.29, 1.38 (CMME 100, CMME 200 and furosemide 20). Lipschitz value was significantly increased to 1 (Furosemide group) from 0.72, 0.87 and 0.93 (control, CMME 100 and CMME 200) (Table 6). Comparative estimation of diuretic index and Lipschitz value were given (Figure 4).

Table 6: Diuretic activity of *Citrus medica* extract by using lipschitz test method.

Group	Diuretic index	Lipschitz value
Control	1	0.72
CMME (100 mg/kg)	1.22	0.87
CMME (200 mg/kg)	1.29	0.93
Furosemide (20 mg/kg)	1.38	1

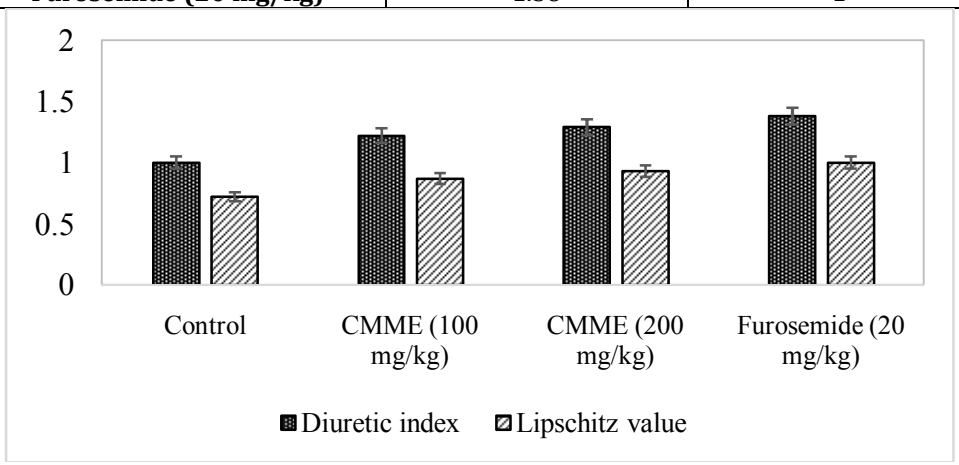

Fig 4: Comparative estimation of diuretic index and lipschitz value of control, CMME (100 mg/kg), CMME (200 mg/kg) and Furosemide group (20 mg/ kg), there is significant increase in diuretic index and lipschitz value in furosemide 20, CMME 100 and CMME 200 compared to control group.

Molecular Docking Studies

a. Structure based drug design

Initially the protein was downloaded from PDB was prepared by removing chain B. Water molecules present in both the chains are removed. Energy minimization was done. Molecules identified from GCMS were selected. Later molecules drawn using chemdraw were converted to mol format and ligprep was created. Grid generation was done and the structures were docked against 5OW8 protein.

b. Schrodinger XP-docking results

XP docking indicated that some of our compounds have good binding ability with phospholipase A2 inhibitor protein (PDB ID: 5OW8) were given [Figure 5]. Docking Results with glide score, lipophilicity, XP H Bond and similarity (Table 7).

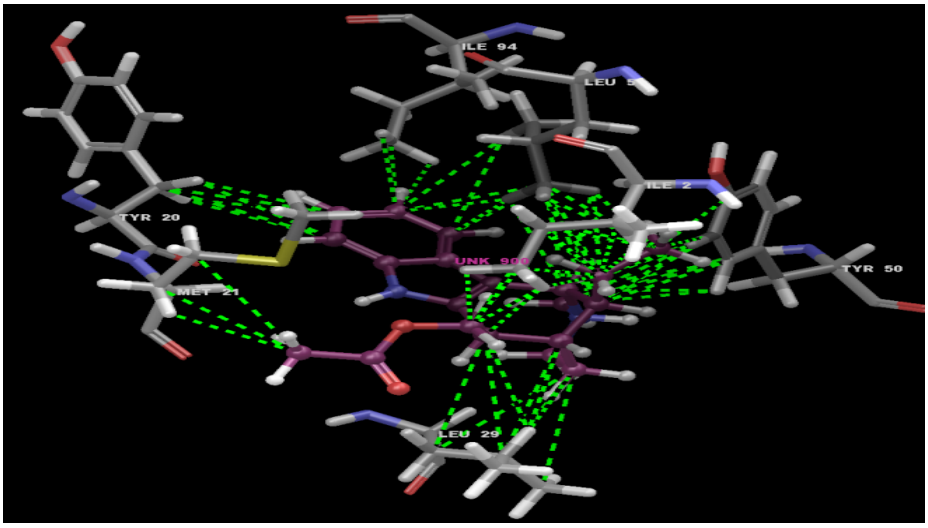

Fig 5: Hydrogen bonding interactions of Dasycarpidan-1-methanol, acetate (ester) with PDB ID: 5OW8

Dasycarpidan-1-methanol, acetate (ester) (total score -7.53) demonstrated hydrophobic interactions with LEU 29,LEU 5, ILE 2, ILE 94, TYR 20, MET 21, TYR 20.

Table 7: Schrodinger XP docking scores

Compound	Glide score	Lipophilic EdW	XP HBond	Hydrophobic interactions
Dasycarpidan-1-methanol, acetate (ester)	-7.53	-3.24	0.00	LEU 29,LEU 5, ILE 2, ILE 94, TYR 20, MET 21, TYR 20.
Isopinocarveol	-6.65	-2.93	-0.78	LEU 29,LEU 5, ILE 2, ILE 94, TYR 20, MET 21, TYR 20
1-Dodecanol, 3,7,11-trimethyl-	-5.79	-3.07	-0.17	TYR 50, LEU 5, ALA 6, MET 21, VAL 9, TYR 20.
2-Methyl-E,E-3,13-octadecadien-1-ol	-5.14	-3.07	-0.17	ILR 2, LEU 29, LEU 5, VAL 9, TYR 20, CYS 27, CYS 43, ILE 94
Tetradecane	-4.72	-2.04	0.00	LEU 5, TYR 20, PRO 17, VAL 9
2-Bromotetradecanoic acid	-4.33	-1.90	0.00	PRO 17, MET 21, TYR 20, LEU 5, LEU 29
Propanamide	-3.71	-1.56	-0.12	MET 21, LEU 29, TYR 50, TYR 20, ILE 94, LEU 5, ILE 2
Ethyl iso-allocholate	-2.31	-3.00	-0.70	LEU 29, ILE 2, LEU 5, TYR 20, CYS 27, HIE 46

The more negative the Glidescore the more favourable the binding.

G score = glidescore, Lipophilic EvdW = Lipophilic term derived from hydrophobic grid potential, H bond = Hydrogen bonding term, Sim = Similarity.

Dasycarpidan-1-methanol, acetate (ester) is having the highest glide score -7.53 when compared to other compounds. The docked Dasycarpidan-1-methanol, acetate (ester) and other compounds were found to occupy the same binding site and form hydrogen bonding interactions.

DISCUSSION

Diuresis has two components; increase in urine volume and a net loss of solutes in the urine. The processes result from suppression of renal tubular reabsorption of water and electrolytes into the blood stream [21]. Body temperature was measured at the start of study after administration of CMME and furosemide to make sure that there is maintenance of normal body temperature.

As the concentration of urea in glomerular filtrate increases, reabsorption of a proportional amount of water is prevented, resulting in increases in rate and volume of urine flow. Urine pH, urine volume and urine excretion were increased in co-treatment of CMME 200 and furosemide, CMME 100 has less effect compared to CMME 200. Diuretic and Lipschitz value was found to be raised increasing order in following groups CMME 100, CMME 200 and furosemide. Furosemide is the loop diuretic act by blocking reabsorption of sodium and potassium and increase urine flow. Loss of sodium and potassium in urine is more in CMME 100 compared to CMME 200.CMME and furosemide show decreased sodium and potassium levels in serum compared to control group.

The compounds isolated from GCMS, they are docked with protein 5OW8. Dasycarpidan-1-methanol, acetate (ester), Isopinocarveol, 1-Dodecanol, 3,7,11-trimethyl, Tetradecane, 2-Bromotetradecanoic acid, 2-Methyl-E,E-3,13-octadecadien-1-ol, Ethyl iso-allocholate and propanamide shown good docking score. Dasycarpidan-1-methanol, acetate (ester) is having the highest glide score -7.53 when compared to other compounds. The docked Dasycarpidan-1-methanol, acetate (ester) and other compounds were found to occupy the samebinding site and form hydrogen bonding interactions. In the present study the superposition of Dasycarpidan-1-methanol, acetate (ester) structure from both crystal structure and docking in addition to the similar interactions found in cytosolic PLA_2 binding site have validated the accuracy of our docking study [23].

CONCLUSION

Citrus medica was extracted using soxhlet apparatus and percentage yield was found to be 9.6% w/w. CMME revealed the presence of phytochemical constituents like flavonoids, terpenoids, alkaloids, glycosides, carbohydrates, steroids and saponins. The body temperature of wistar rats was maintained normal throughout the period *i.e.,* between 35˚C - 38˚C so it was allowed for the diuretic activity *Citrus medica* methanolic extract had shown significant increase in urine parameters like urine volume, urine pH and urine excretion. It also showed effect on serum electrolyte parameters like sodium and potassium. CMME has potent diuretic index and Lipschitz values. The compounds that are obtained from GC-MS, docking is performed which elicited good binding score, all the information provided the basis for conclusion that CMME endowed with diuretic activity.

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