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Original Article

HR-LCMS Metabolite Profiling and *in silico* Evaluation of the Antidiabetic Activity of Methanolic Leaf Extract of *Chrozophora rottleri* (Geiseler) A. Juss. Ex Spreng.

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ABSTRACT

Background: *Chrozophora rottleri* (Geiseler) A. Juss. ex Spreng. is a well-known traditional medicinal plant with a complex biological profile, though inadequate research has been established on its possible anti-diabetic effects and active metabolite profiling. In the present work, Methanolic leaf extracts (MECR) was used to identify plant metabolites by HR-LCMS analysis, and *in silico* docking studies and screened to assess their hypoglycaemic potential. **Materials and Methods:** In the preliminary investigation, the druggable abilities of the compounds were examined using the Swiss ADME *in silico* tool. The phytochemicals were virtually evaluated to determine their binding efficacies against the following proteins: sodium-glucose co-transporter 1 (PDB ID: 5EQG), insulin receptor (PDB ID: 1IR3), glucose co-transporter 1 (PDB ID: 3DH4), and human dipeptidyl peptidase-IV (DPP-IV) (PDB ID: 4A5S). Besides, the impact of MECR on oxidative stress was evaluated by DPPH, H_2O_2, and FRAP assays. **Results:** In the HR-LCMS analysis, a total of 31 phytochemicals were found, including 14 flavonoids, 7 glycosides, 3 phenols, and a few carbohydrates, fatty acids, and alkaloids. Most phytochemicals showed favourable oral bioavailability and druggable characteristics, with 0 or 1 violation of Lipinski's criteria. In contrast to dapagliflozin, a flavonoid compounds remikiren (AA12) and aureusidin (AA11); and an isoflavone genistein 8-C-glucoside (AA6) demonstrated greater affinity for 5EQG in docking study. A phenolic compound, cryptochlorogenic acid (AA1), had a notably higher binding affinity for 4A5S in comparison to vildagliptin. **Conclusion:** The present work has undertaken the first-ever investigation of bioactive phytochemicals and *in silico* hypoglycaemic profiling of leaves of *C. rottleri*. It can provide insight into further research for the discovery of new potential anti-diabetic agents.

Keywords: *C. rottleri*, Antidiabetic activity, Antioxidant activity, Docking, HR-LCMS, *in silico* study.

INTRODUCTION

Diabetes Mellitus (DM) is one of the more widespread metabolic disorders, affecting people worldwide. Herbal remedies have long been regarded as a highly valued source of medicine; as a result, they are now a significant component of contemporary, advanced healthcare. In the general context, the antidiabetic therapeutic approach is to reduce glucose production in the bloodstream and its absorption by targeting different proteins like Insulin receptor IR3, Sodium-Glucose Cotransporter 1 (SGLT1), DPP-IV, and GLUT1, etc. When insulin binds to its receptor, it activates the receptor's tyrosine kinase domain, which then initiates a cascade of signalling events that lead to the uptake of glucose by cells and the inhibition of glucose production by the liver.[1] SGLT1 is a protein that helps to transport glucose from the small intestine into the bloodstream.[2] It is the primary transporter for glucose absorption in the gastrointestinal tract. In people with diabetes, SGLT1 is overactive, which can lead to high blood sugar levels. SGLT1 inhibitors are a class of drugs that work by blocking the action of SGLT1. It prevents glucose from being absorbed from the intestine, which lowers blood sugar levels. DPP-IV is an enzyme that breaks down incretin hormones, such as GLP-1 and GIP.[3] Incretin hormones are released by the gut in response to food intake and help regulate blood sugar levels. DPP-IV is responsible for breaking down these hormones, so it plays a role in the development of diabetes. GLUT1 is a protein that helps cells in the uptake of glucose from the bloodstream. It is expressed

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in many tissues throughout the body, including the brain, retina, kidneys, and muscles. Inhibition of α-amylase and α-glucosidase enzymes is also involved in the digestion of carbohydrates and can significantly decrease the post-prandial increase of blood glucose after a mixed carbohydrate diet and therefore can be an important strategy in the management of blood glucose.[4]

C. rottleri, commonly known as 'Cancer Bush,' is a medicinal plant widely distributed in various parts of the world. It has been traditionally used in folk medicine for the treatment of various diseases.[5] However, the chemical constituents responsible for its therapeutic effects and their underlying mechanisms remain largely unknown.[6] Despite considerable challenges, scientists have been able to address methodological approaches in characterizing plant metabolites from chemically divergent complex crude mixtures by continuing to understand the complex chemistry in plants. This was made possible by researchers who have recently pushed for the adoption of approaches for untargeted phytochemical profiling that combine Liquid Chromatography and Mass Spectrometry (LCMS).[7] High-Resolution (HR)-LCMS is a most reliable method to separate and identify the phytochemicals in plant extracts.[8] HR-LCMS is a powerful analytical tool which can provide in-depth analysis for characterization, identification, structural elucidation, quantification, biomarker discovery, metabolomics, proteomics and environmental analysis. Therefore, in the present study it was utilised for rapid analysis of phytocompounds in crude plant extracts. The findings from such approaches will contribute to understanding the chemical composition of a diverse range in the target plant *C. rottleri.*

In our earlier search, the methanolic leaf extract of *C. rottleri* (MECR) was proven to have potential antidiabetic properties in *in vitro* assays.[5] Therefore, an attempt was made to characterize the phytochemicals in leaf extract by the HR-LCMS technique. In this connection, qualitative phytochemical tests were undertaken and determined phenolic and flavonoid contents to explore the diversity of phytochemicals. Thereafter, phytoconstituents were separated and identified by HR-LCMS. In addition, the antioxidant profile of MECR was tested by familiar methods like DPPH, H_2O_2, and FRAP assays.[9] To explore the bioactive phytochemicals, preliminary screening was carried out using molecular docking studies against target proteins such as IR3, SGLT1, DPP-IV, and GLUT1.

MATERIALS AND METHODS

Materials

Gallic acid, quercetin, and ascorbic acid were purchased from Merck India (Mumbai, India). All the reagents used were analytical grade and purchased from Merck India (Mumbai, India). Solvents such as petroleum ether, ethyl acetate, and methanol used were analytical grade and obtained from Merck India (Mumbai, India).

Plant Material and Extraction

The arial parts of *C. rottleri* were collected from the dry lands of Telangana (Warangal, India), in April 2019. The authentication and traditional knowledge were registered by a botanist Prof. Madhav Shetty (Sri Venkateshwara University in Tirupati, Andhra Pradesh, India). The leaves were separated, washed and shade dried for seven days, and powdered. The powdered material was subjected to successive Soxhlet extraction, employing defatting with petroleum ether followed by ethyl acetate and methanol treatment. Thereafter, the collected solvent extracts were filtered and concentrated by heating at 50°C using vacuo.[10]

Phytochemical Analysis

A preliminary analysis of the phytochemicals was performed by following the standard qualitative tests to determine the occurrence of different classes of phytochemicals.[5]

Estimation of Total Phenolic Content (TPC)

TPC was determined by using the Folin-Ciocalteu (FC) method.[11]100 µL of the diluted extract was mixed with 500 µL of the FC reagent and 400 µL of 7.5% (w/v) saturated sodium carbonate (aq.); then the mixture was thoroughly homogenized and incubated for 30 min at 40°C. Following incubation, each sample absorbance was measured at 765 nm against blank (methanol). The TPC was calculated by calibration curve method using gallic acid as a reference standard. All experiments were run in triplicate and results were expressed as Gallic Acid Equivalents (GAE) in milligrams per gram of dry extract (mg GAE/g).

$$\text{TPC} = \text{Gallic acid equivalent (GAE) mg}/g \text{ of plant extract}$$

Estimation of Total Flavonoids Content (TFC)

The TFC of MECR was estimated using the Aluminum Chloride ($AlCl_3$) colorimetric method.[12] About 250 µL of plant extract was mixed with 1250 µL of distilled water and then with a 5%w/v sodium nitrite solution; the mixture was incubated at room temperature for 6 min. Later, 150 µL of 10% $AlCl_3$ solution was mixed and allowed to stand for 6 min. To this 275 µL of distilled water was mixed with 500 µL of 4% w/v sodium hydroxide solution. The contents were then thoroughly mixed and allowed to stand at room temperature for 30 min. The absorbance was measured at 510 nm in comparison to a reagent blank (methanol). Quercetin was used as a reference to calculate total flavonoids. All trials were carried out in triplicate. The results were expressed in grams of Quercetin Equivalents (QE) per milligram of dry extract (g QE/mg).

$$\text{Total flavonoid content} = \text{Quercetin equivalent mg}/g \text{ of plant extract}$$

Antioxidant Activity

The MECR was subjected to screen for its antioxidant activity by three methods namely DPPH, H_2O_2, and FRAP methods.

DPPH Assay

The radical scavenging activity of MECR was measured using the stable free DPPH radical.[13] DPPH methanolic solution (1 mL, 0.2 mM) was added to the leaf extracts (1 mL, 2.5-100 µg/mL). The mixture was thoroughly vortexed, and after 30 min of incubation in darkness (at room temperature) the absorbance was measured at 517 nm with a blank (DPPH in methanol). The positive control used was ascorbic acid, and the radical scavenging activity was calculated using the following equation:

DPPH radical scavenging activity (% inhibition) = [''A'' of blank – ''A'' of sample] / ''A'' of blank × 100

H_2O_2 Scavenging Assay

In this assay, H_2O_2 solution (43 mM) was prepared in phosphate buffer (0.1 M, pH 7.4).[14] The extract was added to 0.6 mL of H_2O_2 (43 mM) solution at various concentrations (1 mL, 2.5-100 µg/mL) in 3.4 mL of phosphate buffer (43 mM). At 230 nm, the absorbance of the reaction mixture was measured and calculated the potential of inhibition using following equation,

$$H_2O_2 \text{ scavenging activity (\% inhibition)} = (\text{"A" of blank} - \text{"A" of sample}) / \text{"A" of blank} \times 100$$

FRAP Assay

In this assay, 1 mL of test sample solution at different concentrations (2.5-100 µg/mL) was mixed with 2.5 mL of potassium ferricyanide (1% w/v), and 2.5 mL of phosphate buffer (0.2 M, pH 6.6).[15] Then the mixture was incubated for 20 min at 50°C, and 2.5 mL of trichloroacetic acid (10%) was added. After centrifugation of the mixture for 10 min at 3000 rpm, 2.5 mL solution was collected from the upper layer, and mixed with 2.5 mL of distilled water, and 0.5 mL of $FeCl_3$ (0.1%). Ascorbic acid (10 µg/mL) was used as a standard, and the absorbance was measured at 700 nm using a UV-vis spectrophotometer. All tests were performed in triplicate.

$$\text{Ferric reducing power (\% inhibition)} = (\text{"A" blank} - \text{"A" sample}) / \text{"A" blank} \times 100$$

HR-LCMS analysis

The HR-LCMS equipped with UHPLC-PDA detector-ESI-QTOP-MS (Agilent Technologies, USA) system was used for the analysis of the phytoconstituents in MECR. The chromatographic separation was achieved on the Hypersil GOLD C18 (2.1×100 mm 3-µ) column. The mobile phase comprised 0.1% formic acid in water (A) and 90 % acetonitrile+10% water+0.1% formic acid (B) at 0.3 mL/min flow rate. The separation was carried with a gradient system (A: B v/v): 95:5(0-1 min), 0:100(1-30 min), 95:5(30-31 min), and 100:0 (31-35 min) at 1,200 bar pressure. The injection volume was 5 µL. Mass data were recorded with an MS Q-Tof (Agilent) mass spectrometer equipped with dual AJS ESI operating in positive (+) and negative (–) ionization modes. MS was acquired over the m/z range of 100-1, 200 at a mass resolution of 22,000 full-widths half at maximum. The carrier gas temperature was maintained at 250°C. Phytochemicals were characterized with the aid of mass spectra and distinctive mass fragmentation patterns, retention time (Rt), m/z values, and NIST library hits.

In silico ADME profile

The pharmacokinetic profile (ADME) of the identified plant metabolites was evaluated virtually using SwissADME tools. The test was performed to identify the most promising compounds with minimal risk of drug attrition[16]. Metabolites with the most reliable ADME properties have been investigated for protein-ligand binding interactions. About 13 compounds got desired bioavailability scores with zero and one violation. [17]

Docking Study

The phytoconstituents with more reliable ADME properties were subjected to molecular docking using AutoDock vena (PyRx 0.8) to understand the metabolite interactions[18-19] with prospective protein targets, IR3, SGLT1, DPP-IV, and GLUT1. The crystal structure of the target proteins IR3 (PDB ID: 1IR3), SGLT1 (PDB ID: 3DH4), DPP-IV (PDBID: 4A5S), and GLUT1 (PDB ID: 5EQG) were retrieved from a protein data bank (rcsb.com/pdb database). The protein was refined by eliminating water molecules and adding polar hydrogens. The co-crystal ligand was extracted using the Discovery Studio Visualizer 2021 program and saved in pdb format. The protein structure was checked for any missing amino acid residues, and the Ramachandran plot was used to check for any structural problems. The created pdb file protein was converted to pdbqt format using the macromolecule option in the AutoDock tool of the PyRx virtual screening application 0.8. The target compounds 2D structures were drawn using ChemDraw and saved as pdb files and were subjected to energy minimization (force field-off), and then generated conformers (AutoDock pdbqt files) using the open babel tab in PyRx software. The macromolecule (protein pdbqt file) and ligands were chosen using Vina Wizard for docking (AutoDock pdbqt files) by drawing a grid box around the area where the co-crystal ligand exhibits iterations with amino acids, the active site of the protein was defined to dock ligands to proteins[20]. The potential compounds with a high binding affinity against the target proteins were identified as the ligands with the lowest binding energies. The binding interactions were visualized using the Discovery Studio Visualizer 2021 program.[4]

Statistical Analysis

All tests were carried out in triplicate, and data were expressed as mean Standard Deviation (SD). The plots were prepared using a Graph Pad Prism [version 5.0]. The comparison of means among groups was accomplished with a one-way Analysis of Variance (ANOVA) followed by the Tukey test. Results are expressed as Mean±SEM (n=3) and significance was expressed statistically as, **=p<0.01; ***=p<0.001.

RESULTS

Phytochemical Analysis

The qualitative tests of MECR presented with the occurrence of glycosides, saponins, flavonoids, tannins, alkaloids, sterols, and triterpenes.[5] Further, it was displayed with a significant amount of phenolic and flavonoid composition (Table 1).

Antioxidant Activity

The results demonstrated the concentration-dependent free radical scavenging at test concentrations (25-500 µg/mL) of MECR. The extract has showed similar pattern of effect to that of standard up to 500 µg/mL (Figure 1). The results indicated that MECR comprised with good number of antioxidants that were capable enough to have potent activity in neutralizing free radicals.

At the test concentration (25-500 µg/mL), the extracts exhibited varying degrees of inhibition in three assays. In the DPPH assay,

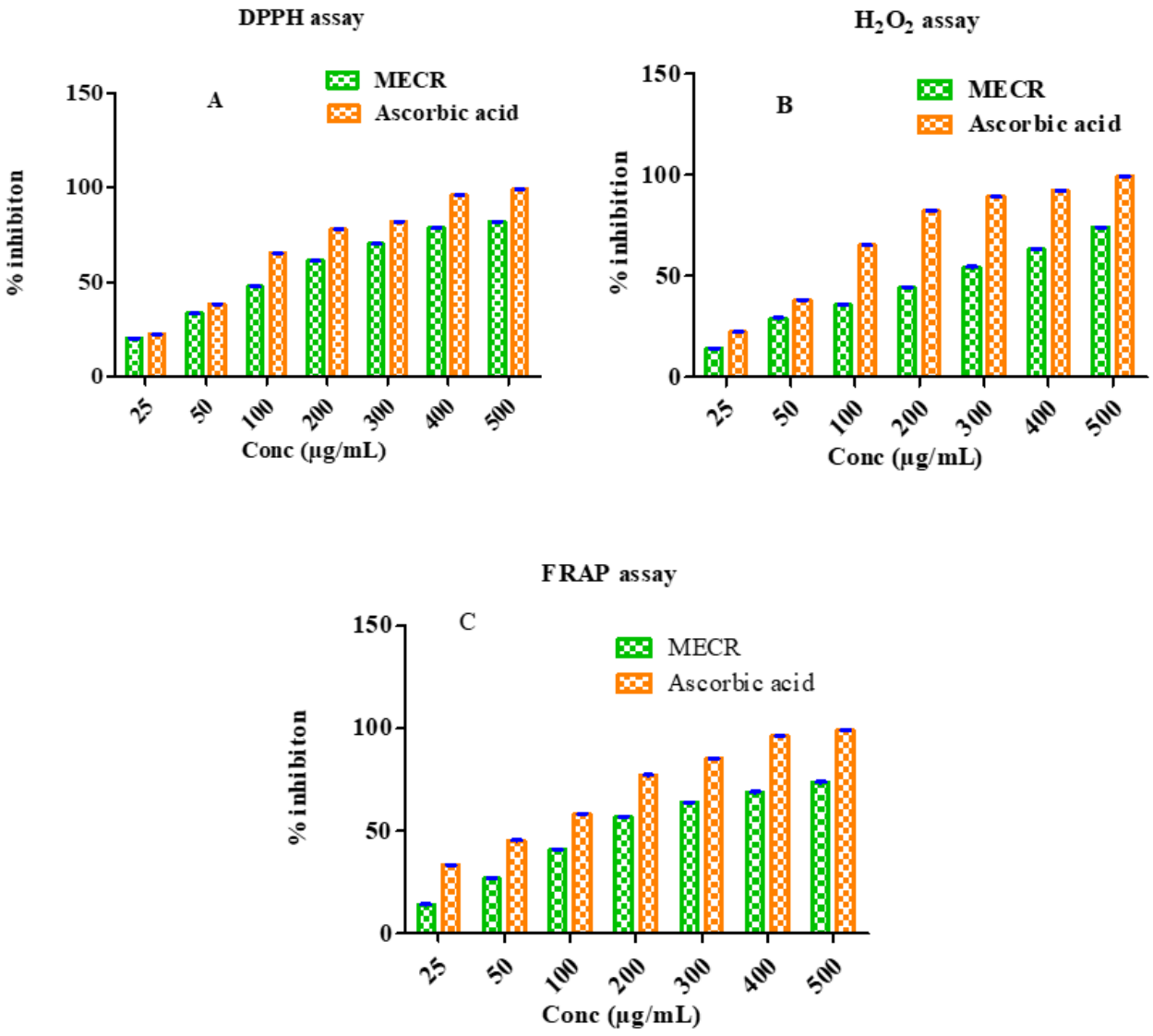

Figure 1: % inhibition of DPPH (A), H_2O_2, (B) and FRAP (C) radical activity by MECR and ascorbic acid. Results are expressed as Mean±SEM., n=3; statistically significant **p<0.01 ***p<0.001 .

it has showed 81.92 % inhibition of radical activity. It consistently demonstrated the highest level of free radical inhibition (74.08%) of H_2O_2 at 500 µg/mL. However, the antioxidant ability was markedly lesser when compared to the DPPH radical scavenging. Concentration dependent manner of radical inhibition was observed at the test doses. At 500 µg/mL, the extracts displayed moderate inhibition (73.62%) in FRAP assay.

It could be assumed that the variation in % free radical inhibition could be due to the occurrence of a variety of plant metabolites. Inhibition of free radical activity proportionally improved with increasing test concentration of extracts primarily indicating its ability to have antioxidant ion property.

HR-LCMS Analysis

A total of 31 compounds with molecular weights ranging from 164 to 624 g/mol were identified (Table 2) based on their spectral data obtained through HR-LCMS analysis. Each compound was described with their common name or identifier, chemical formula, Rt and known or reported biological uses based on scientific studies and literature.

The HR-LCMS analysis of MECR revealed a diverse range of compounds, including phenolics, glycosides, flavonoids, isoflavones, fatty acids, carbohydrates, etc., Some of the notable compounds detected include flavonoids like aureusidin,[21] 3,6-diglucopyranosyl-5,7- dihydroxy-4'-methoxyflavone,[22] lespenefril,[23] hesperetin 3'-O-glucuronide,[24]glycosides like isoacteoside, cartormin, quercetin 3,7-dirhamnoside, rutin,[25] phenolic glycosides like α-hydrojuglone 4-O-b-D- glucoside, polyphenol like ellagic acid, etc., These compounds have been shown to possess a wide range of biological activities, including antioxidant, anti-inflammatory, anti-cancer, and anti-microbial properties.

Table 1: Total phenolic and flavonoid content of MECR.

Extract	TPC (mg GAE/g dried extract)	TFC (mg QE/g dried extract)
MECR	91.95±0.17	18.92±0.17

Note: MECR- Methanol extract of *C. rottleri.*

ADME profile

In general context, *in silico* studies for therapeutics have two fundamental approaches - firstly, ligand-protein interaction and secondly, Absorption, Distribution, Metabolism, and Excretion (ADME) exploration.[26] The therapeutic profile of the principal biologically modulated components in the MECR was determined using Lipinski's rule of five. Absorption of any therapeutic component depends on its five properties including H-Bond Donor (HBD), H-Bond Acceptor (HBA) or acceptance potential, molecular weight, and their logP.[27]All the compounds identified in the HR-LCMS analysis were studied for their ADME properties virtually. In this study, the Swiss ADME has explored that about 13 phytochemicals out of 31 showed 0 or 1 violation of Lipinski's rule of five. Also, these compounds possess excellent ADME profiles including gastrointestinal absorption, BBB permeability, and bioavailability score, etc., (Table 3). Among these few compounds such as glycitin, furcatin, ellagic acid, genistein 8-C-glucoside, γ-sitosterol, and ethyl iso-allocholate are known to be as bioactive substances as per the reports.

In silico Docking Study

The docking study was performed through AutoDock vina software using standard protocols.[28] Since Insulin Receptor (1IR3), SGLT1 (3DH4), DPP-IV (4A5S), and GLUT1 (5EQG) are the key proteins that facilitate the release of glucose into the bloodstream, they were selected as targets for *in silico* docking screening. Different chemical classes of compounds with dependable ADME profiles were chosen for predicting protein-ligand binding interactions. In this study, well-known antidiabetic drugs such as dapagliflozin, glibenclamide, metformin, and vildagliptin were taken as reference ligands.[29-30]

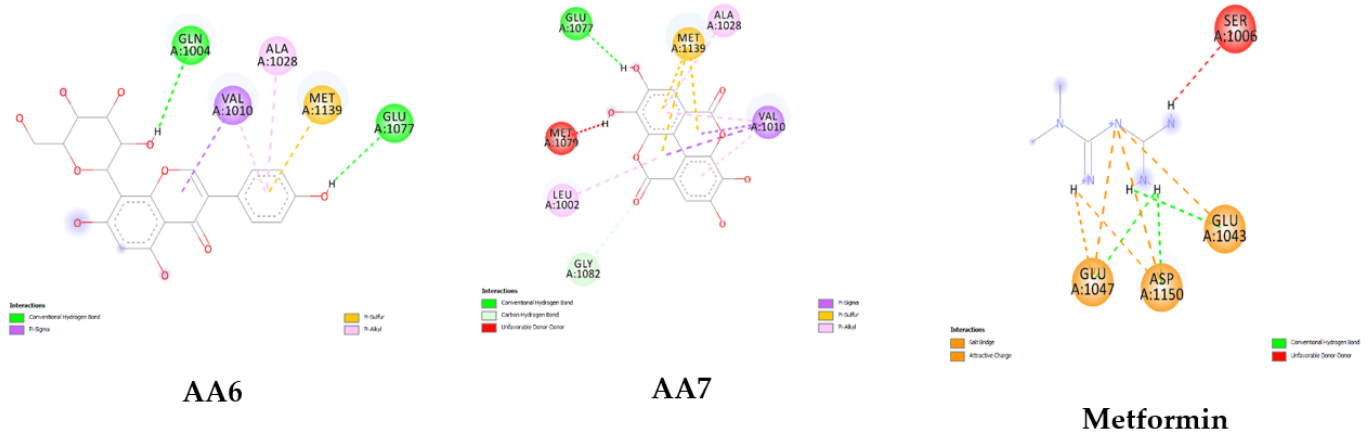

Figure 2a: 2D interaction of AA6, AA7 and Metformin against 1IR3.

Table 2: List of phytochemicals identified in MECR by HR-LCMS technique.

Sl. No.	R_t	Mass	Compound name	Formula	Category
1	4.47	354.0944	Cryptochlorogenic acid	$C_{16}H_{18}O_9$	Phenolic acid
2	4.72	610.1523	Rutin	$C_{27}H_{30}O_{16}$	Glycoside
3	4.77	458.1425	7-Hydroxy-4-methyl phthalide O-[arabinosyl -(1->6)- glucoside]	$C_{20}H_{26}O_{12}$	Glycoside
4	5.11	594.1574	Astragalin 7-rhamnoside	$C_{27}H_{30}O_{15}$	Glycoside
5	5.27	564.1474	Kaempferol 3-rhamnoside 7- xyloside	$C_{26}H_{28}O_{14}$	Flavonol glucoside
6	5.29	580.1426	Kaempferol 3- xylosylglucoside	$C_{26}H_{28}O_{15}$	Flavonol glucoside
7	5.76	448.1013	Luteolin 4'-O-glucoside	$C_{21}H_{20}O_{11}$	Glycoside
8	5.79	448.1017	Kaempferol 7-O-glucoside	$C_{21}H_{20}O_{11}$	Flavonol glucoside
9	5.85	462.1168	Swertiajaponin	$C_{22}H_{22}O_{11}$	Flavonoid
10	5.89	594.1587	Quercetin 3,7-dirhamnoside	$C_{27}H_{30}O_{15}$	Glycoside
11	6.03	164.0468	Furcatin	$C_9H_8O_3$	Glycoside
12	6.05	575.1644	Cartormin	$C_{27}H_{29}NO_{13}$	Glycoside
13	6.22	498.0928	Methyl 4,6-di-O-galloyl-β-D-glucopyranoside	$C_{21}H_{22}O_{14}$	Tannins
14	6.43	578.1643	Daidzein 4',7-diglucoside	$C_{27}H_{30}O_{14}$	Isoflavonoid o-glycosides
15	6.49	624.2058	Isoacteoside	$C_{29}H_{36}O_{15}$	Glycoside
16	6.70	578.1635	Lespenefril	$C_{27}H_{30}O_{14}$	Flavonoid
17	6.81	476.1328	Hesperetin 3'-O-glucuronide	$C_{23}H_{24}O_{11}$	Flavonoid
18	7.53	194.0571	Isoferulic acid	$C_{10}H_{10}O_4$	Phenolic acid
19	8.03	270.0533	Remikiren	$C_{15}H_{10}O_5$	Isoflavone
20	19.29	278.224	α-Linolenic Acid	$C_{18}H_{30}O_2$	Omega-3 fatty acid
21	1.05	342.1159	Isomaltulose	$C_{12}H_{22}O_{11}$	Carbohydrate
22	5.16	494.0877	11-O-Demethyl-7-methoxypradinone II	$C_{25}H_{18}O_{11}$	Tetracenes
23	5.23	338.0998	α-Hydrojuglone 4-O-b-D- glucoside	$C_{16}H_{18}O_8$	Phenolic glycosides
24	5.69	168.0423	Vanillic acid	$C_8H_8O_4$	Flavouring agent
25	6.19	432.1064	Genistein 8-C-glucoside	$C_{21}H_{20}O_{10}$	Isoflavones
26	6.34	302.0064	Ellagic acid	$C_{14}H_6O_8$	Polyphenol
27	6.91	446.1224	Glycitin	$C_{22}H_{22}O_{10}$	Isoflavone
28	8.03	270.0533	Genistein	$C_{15}H_{10}O_5$	Isoflavone
29	8.77	286.0476	Aureusidin	$C_{15}H_{10}O_6$	Flavonoid
30	17.45	509.3016	2-[4,6-Bis(2,4-dimethy lphenyl)-1,3,5-triazin- 2-yl]-5-(octyloxy) phenol	$C_{33}H_{39}N_3O_2$	Phenolic compound
31	17.67	518.2549	Geranylfarnesyl diphosphate	$C_{25}H_{44}O_7P_2$	Sesterterpenoids
32	19.55	596.2958	Salannin	$C_{34}H_{44}O_9$	Alkaloid

Table 3: *In silico* ADME profile of phytochemicals.

Sl. No.	Comp. Name	RB	HA	HD	TPSA	C.Log.p	GI A	BBB	Pgp Sub.	log Kp (cm/s)	LV	BA S
1	Cryptochlorogenic acid	5	9	6	164.75	-0.32	Low	No	No	-8.76	1	0.11
2	Isofrulenic acid	8	10	6	158.3	-0.6	Low	No	No	-9.58	1	0.55
3	α-Linolenic acid	3	4	2	66.76	1.39	High	Yes	No	-6.41	0	0.85
4	α-Hydrojuglone 4-O-b-D-glucoside	18	7	5	169.86	3.38	Low	No	Yes	-8.07	1	0.55
5	Vanillic acid	13	2	1	37.3	5.09	High	Yes	No	-3.41	1	0.85
6	Genistein 8-C-glucoside	3	8	6	139.84	-0.37	Low	No	No	-8.69	1	0.55
7	Ellagic acid	2	4	2	66.76	1.08	High	No	No	-6.31	0	0.85
8	Glycitin	3	10	7	181.05	0.01	Low	No	No	-8.79	1	0.55
9	Furcatin	0	8	4	141.34	1	High	No	No	-7.36	0	0.55
10	Genistein	5	10	5	159.05	0.5	Low	No	No	-8.57	0	0.55
11	Aureusidin	1	5	3	90.9	2.04	High	No	No	-6.05	0	0.55
12	Remikiren	1	6	4	107.22	1.72	High	No	No	-5.95	0	0.55
13	Salannin	9	9	0	110.5	4.3	Low	No	Yes	-7.15	1	0.55

Note: RB: Rotatable Bonds, HA: Hydrogen bond Acceptors, HD: Hydrogen bond Donors, CLogP: Consensus Log P, GIA: Gastrointestinal Absorption, BBB: BBB permeant, LV: Lipinski Violations, BA S: Bioavailability Score.

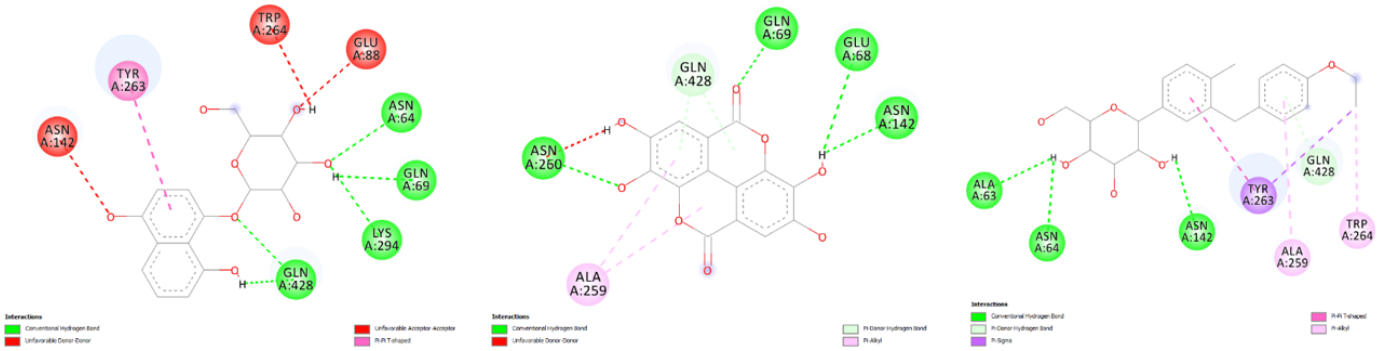

Figure 2b: 2D interaction of AA4, AA7 and Dapagliflozin against 3DH4.

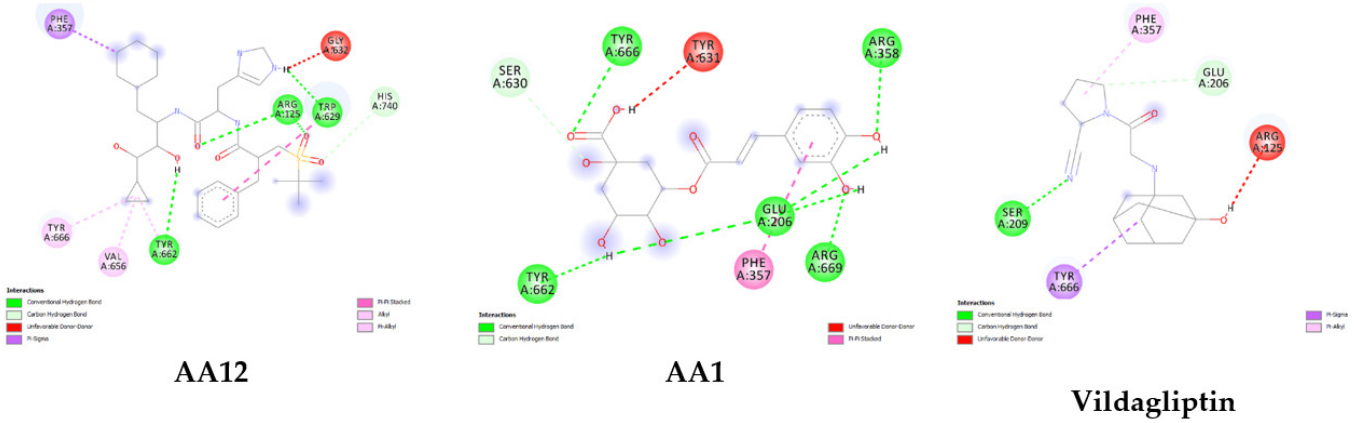

Figure 2c: 2D interaction of AA12, AA1 and Vildagliptin against 4A5S.

A flavonoid compound: remikiren (AA12) (-10.0 Kcal) and aureusidin (AA11) (-8.8 Kcal) showed good binding affinity towards 5EQG; an isoflavone, genistein 8-C-glucoside (AA6) has shown good binding affinity (-9.1Kcal) towards 5EQG to that of dapagliflozin (-9.1Kcal). A phenolic compound: cryptochlorogenic acid (AA1) had a significant binding affinity (-8.6 Kcal) for 4A5S compared to vildagliptin (-8.1 Kcal). Moderate binding interactions were observed towards 1IR3 for all the phytochemicals. The binding interactions of the potential ligands at active site were indicated with 2D pictures in Figure 2a-2d (Table 4).

DISCUSSION

In this investigation, methanolic extracts showed a notable flavonoid content in a preliminary phytochemical test. At a 500 µg/mL concentration, MECR reduced the free radical activity of DPPH by 81.92%. In the H_2O_2 and FRAP assay, it was demonstrated 74.08% and 73.62% inhibition of free radical activity, respectively. At 500 µg/mL, the ferric ion (Fe^{3+}) was reduced by 73.62% in the FRAP assay. These findings suggest that leaf extract had considerable antioxidant potential in comparison to the ascorbic acid. The HR-LCMS technique has several advantages for determining metabolic profiling, including high resolution, quick analysis, sensitivity, and effectiveness for

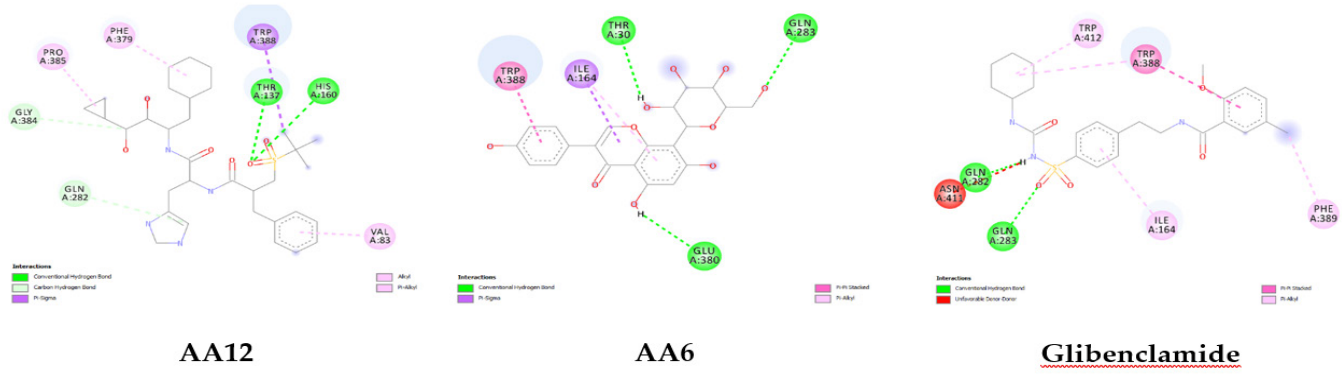

Figure 2d: 2D interaction of AA12, AA6 and Glibenclamide against 5EQG.

Table 4: Docking energy of phytochemicals and standards against target proteins.

Sl. No.	Compound name (ID)	Binding energy (Kcal/Mol)			
		1IR3	3DH4	4A5S	5EQG
1	Cryptochlorogenic acid (AA1)	-7.5	-8.8	-8.6	-8.3
2	Isoferulic acid (AA2)	-5.8	-6.7	-6.3	-6.6
3	α-Linolenic acid (AA3)	-5.4	-7	-5.8	-6.2
4	α-Hydrojuglone 4-O-b-D glucoside (AA4)	-7.8	-9.2	-7.8	-9
5	Vanillic acid (AA5)	-5.4	-6.5	-5.7	-5.9
6	Genistein 8-C-glucoside (AA6)	-8.9	-4.2	-8.1	-9.8
7	Ellagic acid (AA7)	-8.3	-8.8	-8.4	-9.3
8	Glycitin (AA8)	-8	-3.1	-8.3	-9.7
9	Furcatin (AA9)	-7.9	-8.8	-8.4	-8.9
10	Genistein (AA10)	-7.9	-7.9	-8.2	-9.1
11	Aureusidin (AA11)	-7.9	-7.7	-8.5	-8.8
12	Remikiren (AA12)	-7.5	-1.9	-9	-10
13	Salannin (AA13)	-6.4	5.1	-8.1	-6.9
14	Stand. 1	-	-7.9	-	-9.1
15	Stand. 2	-	-7.7	-	-10
16	Stand. 3	-4.3	-	-	-
17	Stand. 4	-	-	-8.1	-

Note: Stand.1-Dapagliflozin; Stand. 2- Glibenclamide; Stand. 3-Metformin; Stand. 4-Vildagliptin.

understanding phytocomponent structure. Numerous secondary metabolites, including flavonoids, phenolics, alkaloids, and terpenoids, were found in MECR. In general scenario, such Phyto substances usually possess a variety of pharmacological properties including antioxidant and antidiabetic effects. In the overall study, a comprehensive knowledge of the chemical composition of MECR was obtained through metabolite profiling using the HRLC-MS technique. Alkaloids, phenolics, flavonoids, and terpenoids were few of among the identified metabolites that may have antioxidant and anti-diabetic properties.

The antidiabetic potential of *C. rottleri* leaf extract was further supported by the *in silico* docking results. The findings of molecular docking studies confirmed that the plant constituents of the extract have a good affinity for molecular targets related to glucose metabolism, including the insulin receptor (PDB ID: 1IR3), glucose cotransporter 1 (PDB ID: 3DH4), human dipeptidyl peptidase-IV (DPP-IV) (PDB ID: 4A5S), and sodium-glucose cotransporter 1 (PDB ID: 5EQG). These results imply that *C. rottleri* could serve as a promising antidiabetic candidate.

In silico ADME examination is a standard approach for a more reliable assessment of possible bioactive compounds. In ADME analysis, most of the identified phytochemicals accomplished Lipinski's rule of five. Identify possible therapeutic targets, ADME profile was taken into consideration. The results of the docking study indicated that AA6 (genistein 8-C-glucoside) and AA7 (ellagic acid) had the best binding affinities towards 1IR3. α-hydrojuglone 4-O-b-D-glucoside (AA4), cryptochlorogenic acid (AA1), ellagic acid (AA7), and furcatin (AA9) were exhibited the greater affinity for 3DH4. Conversely, compounds AA1, aureusidin (AA11), AA7, and remikiren (AA12) demonstrated greater specificity towards 4A5S. Furthermore, AA6, AA8 (glycitin), and AA12 showed binding interactions with 5EQG. AA12 had the strongest binding affinity against both 4A5S and 5EQG out of all the test compounds. Docking studies revealed that an isoflavone (AA12) and a phenolic compound (AA1) possess significant binding affinities towards potential drug targets 4A5S and 5EQG to that of vildagliptin and glibenclamide.

CONCLUSION

Insights into the molecular pathways involved in the etiology of DM and anti-diabetic actions may be further gained using advanced molecular techniques. In the present study, plant metabolite profiling by HR-LCMS technique *in silico* assessment of anti-diabetic activity of the *C. rottleri* leaf extract was performed. The methanolic leaf extract demonstrated noteworthy antioxidant activity, as evidenced by its ability to scavenge DPPH, H_2O_2, and FRAP methods. The antioxidant activity of the extract is of immense importance, as it can protect against oxidative stress-induced damage and reduce the risk of diabetic complications. The present study found that MECR contains a variety of phytochemicals, including flavonoids, glycosides, phenols, carbohydrates, fatty acids, and alkaloids. Most of these phytochemicals showed favourable oral bioavailability and druggable characteristics. *In silico* docking studies showed that few of the phytochemicals, such as remikiren, genistein 8-C-glucoside, aureusidin, and cryptochlorogenic acid, have a greater affinity for potential drug targets involved in glucose regulation compared with standard drugs. These findings suggest that *C. rottleri* may be a promising source of potential anti-diabetic agents.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

A: Absorbance; **GC-MS:** Gas chromatography-mass spectrometry; **HR-LCMS:** High Resolution Liquid Chromatograph Mass Spectrometer; **SGLT1:** Sodium-glucose cotransporter 1; **IR:** Insulin receptor; **GLUT1:** Glucose cotransporter 1; **DPP-IV:** Human dipeptidyl peptidase-IV; **GLP-1:** Glucagon-like peptide-1; **GIP:** Glucose-dependent insulinotropic polypeptide.

SUMMARY

The present study was undertaken for the investigation of phytochemicals by HR-LCMS technique and *in silico* screening of the anti-diabetic potential of methanolic leaf extracts of *C. rottleri*. The crude extract possesses a significant range of antioxidant properties. The results indicated that about 31 phytochemicals were comprised in the methanolic leaf extracts including flavonoids, glycosides, phenols, carbohydrates, fatty acids, and alkaloids. The majority of identified phytochemicals showed favourable oral bioavailability and druggable properties *in silico* screening. Docking studies showed that the flavonoid compound remikiren, the isoflavone genistein 8-C-glucoside, and aureusidin demonstrated a greater affinity for sodium-glucose co-transporter 1 (5EQG). In addition, a phenolic compound cryptochlorogenic acid had a higher binding affinity for human dipeptidyl peptidase-IV (4A5S). It is necessary to conduct further research to elucidate the underlying mechanism of action and evaluate the extract's therapeutic potential in *in vivo* models. In conclusion, these outcomes advise that *C. rottleri* leaves may be a potential source for the discovery of novel anti-diabetic drug candidates.

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