In Vitro and In Silico Antioxidant, Anti-Diabetic, Anti-HIV and Anti-Alzheimer Activity of Endophytic Fungi, Cladosporium uredinicola Phytochemicals

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Abstract. The present work was aimed to identify phytochemicals in C. uredinicola methanol extract from qualitative, TLC and GC-MS method and evaluated for antioxidant, anti-HIV, anti-diabetes, anti-cholinesterase activity in vitro and in silico. The C. uredinicola extract showed flavonoids, tannins, alkaloids, glycosides, phenols, terpenoids, and coumarins presence in qualitative method. From GC-MS analysis, identified seven different phytochemicals and out of seven, four (coumarin, coumarilic acid, hymecromone, alloisoimperatorin) are coumarins. The C. uredinicola extract has shown significant antioxidant activity in DPPH (73) and FRAP (1359) method. The HIV-1 RT (83.81 ± 2.14), gp 120 (80.24 ± 2.31), integrase (79.43 ± 3.14) and protease (77.63 ± 2.14), DPPIV, β-glucosidase and acetyl cholinesterase activity was significantly reduced by the extract. The 2-diphenylmethyleneamino methyl ester had shown significant interaction with oxidant and HIV-1 proteins whereas alloisoimperatorin have interacted with diabetes and cholinesterase proteins followed by hymecromone with high binding energy. These three phytochemicals are non-carcinogens, non-toxic, readily degradable and have drug likeliness properties. The C. uredinicola phytochemicals are responsible for management of diabetes, HIV-1 and Alzheimer. Further in vivo work is needed to justify our research.

Introduction

Plant drugs constitute 25% of the total drugs and have no or minimal side effects. If we use these plant-based drugs continuously we need plants in large quantity and they may vanish on the earth in the future. So, to save plants, exploiting the endophytes to obtain plant-based drugs is practicing nowadays. Endophytes are endosymbionts resides in plant tissues, they either bacteria or fungi but they are not causing any diseases to host. The endophytic fungi are able or capable to produce what host is producing, by using these endophytes we can produce large quantity of the drug within short period by applying biotechnological aspects to meet public demand.

Calophyllum tomentosum (Calophyllacace) is endemic plant commonly known as bintangur grows in Sri Lanka and Western Ghats regions of Karnataka, India. In Ayurveda, the extracts are being practiced to treat ulcers, snake bites and eye diseases. Xanthones and triterpenes were identified from bark of C. tomentosum [1] and flavonoids, saponins and terpenoids from leaf part [2] exhibited strong α-glucosidase inhibitory activity. The C. tomentosum shown alkaloid, flavonoid, terpenoid, tannin, glycoside, saponin [3] are responsible for inhibition of α-glucosidase activity. The literature survey indicates that no reports on endophytic fungal species from C. tomentosum plant. In our lab, we have isolated three fungal endophytes from (different parts of C. tomentosum), analysed their phytochemicals through GC-MS and identified by molecular level using 18S rRNA (unpublished data). The present research was aimed to identify phytochemicals using qualitative, TLC, GC-MS
Using methanol solvent extract of *C. uredinicola*, endophyte of *C. tomentosum*. The extracts were used to analyse antioxidant, anti-HIV, anti-diabetes and anti-Alzheimer activity *in vitro* and *in silico* experimental analysis.

**Materials and Methods**

**Collection, identification and mass culture of endophytic fungi, *Cladosporium uredinicola* from *Calophyllum tomentosum***

The Department of Biotechnology of Shridevi Institute of Engineering & Technology, Tumakuru, Karnataka, India had provided the *C. uredinicola* culture and was inoculated in potato dextrose broth to get high mycelia mass at 26±2 °C for a period of 15 days. The *C. uredinicola* was previously isolated from bark part of the *C. tomentosum*.

**Extract preparation and phytochemical analysis**

Methanol was used for extraction from *C. uredinicola* mycelia using microwave method two cycles of 10 min at 100 °C. The qualitative methods were employed to identify phytochemicals in *C. uredinicola* extract [4-5].

**TLC**

Chromatography was performed using endophytic fungal extracts [6].

**Analysis of GC-MS**

Separation of the phytochemicals was observed by employing GC-MS method in *C. uredinicola* extract [7] and identified each separated phytochemicals based on the parameters viz., molecular weight, structures of the component, total ionic chromatograms, retention time and ionization chromatograms.

**Assay of antioxidant activity in *C. uredinicola* extract**

Antioxidant properties of *C. uredinicola* methanol extract was carried out using “Ferric Ion Reducing Antioxidant Power (FRAP) and 2, 2-Di Phenyl-1-Picryl Hydrazyl (DPPH)” assays.

**DPPH activity**

The extract of *C. uredinicola* was used for DPPH activity [8]. The freshly prepared samples were dissolved in 24 mg DPPH in 100 ml ethanol and stored at –20 °C. The sample solution of 150 µl (10 µl of sample and 140 µl of distilled water) was mixed with 2850 µl of sample containing 190 µl of reagent and 2660 µl of distilled water and allowed 24 h for reaction each in dark condition. The reaction was measured at 515 nm. The standard ascorbic acid curve was range of 25 to 800 µM used to analyse test sample. To get absorbance of 1.1±0.02 units at 517 nm, the 45 ml of methanol was added to stock solution (10 ml) [9]. Triplicate was maintained to all the experiments. The percent inhibition of DPPH due to sample was measured and used the standard formula for calculation as mentioned below;

\[
\text{Inhibition (%) = } \frac{\text{AC} - \text{AS}}{\text{AC}} \times 100,
\]

where AC- absorbance of DPPH activity with ethanol, AS- absorbance of DPPH activity with sample or absorbance of DPPH activity with standard.

**Assay for FRAP**

FRAP solution was prepared by mixing 2,4,6-tris-(2-pyridyl)-S-triazine (TPTZ) solution (2.5 ml) (10mM in 40mM–1 HCl), acetate buffer (25ml) (300 mM, pH 3.6) and mixed the 2.5 ml FeCl3 (20mM) water. The *C. uredinicola* (150 µl and 0.5 mg/ml) was mixed with methanol later added 4.5 ml of FRAP, FRAP without sample was used as blank and reaction was observed at 593 nm [10-11]. The sample reaction was compared with standard ascorbic acid.
**In vitro anti-diabetic activity of C. uredinicola extract**

**Activity of inhibition of α-amylase**

The working solution contains 250 µL of 2% (w/v) starch (250 µL), α-amylase solution (250 µL of 1 U/mL) and C. uredinicola extract (250 mL of 500 µg/mL) was incubated for 3 min at 20 °C. To stop the reaction, dinitrosalycilic acid (500 µL) was added to the reaction mixer, subjected to boiled water immediately added α-amylase (250 µL) and heated the solution for 15 min. After heating, the reaction mixer was kept at 26±2 °C for 3 min. To get total volume of 6000 µl, 4500 µL of aqua dest was mixed and homogenized the mixer in vortex. At 540 nm, the activity of α-amylase was measured in with sample or standard or without sample with the help of spectrophotometer and triplicate was maintained for each experiment. Inhibition of α-amylase activity was calculated using standard equation [11].

**Inhibitory activity of α-glucosidase**

The reaction mixer contains phosphate buffer solution (36 µL), C. uredinicola solution (30 mL) at different concentrations of 10, 25, 50, 100 and 150 µg/ml and 4-nitrophenyl--α-D glycopyranoside (PNPG) substrate (17 µl) was allowed to reaction for 5 min at 37 °C. Added the α-glucosidase solution (17 µl of 0.15 U/mL) to each well to get 100 mL of volume after 5 min of incubation. The reaction solution allowed for 15 min and added sodium carbonate (100 µl of 200 mM). The reaction was observed at 405 nm in micro plate reader and repeated the each experiment thrice and calculated the reaction [11].

**Inhibitory activity of dipeptidyl peptidase IV**

Incubated the reaction mixture of 50 µL dipeptidyl peptidase (DPP-IV) was mixed with 25 µL C. uredinicola extract for 5 min at 37 °C. Added the 100 µL Gly-Pro-P-Nitroanilide (GPPN) (2 mM) to the reaction mixture and enzyme activity allowed for 15 min. The reaction was terminated by adding 25 µL of acetic acid glacial (25%) and reaction activity was measured at 405 nm [11].

**In vitro HIV-1 enzymes inhibition of C. uredinicola methanol extract**

**Inhibition of activity of HIV-1 reverse transcriptase (RT)**

Using 5mM MgCl2, 150 mM KCl, 0.05% NP-40, 5mM DTT, 0.5 mM EGTA, 0.3M Glutathione, 2.5 µg/ml BSA, 2.5µg/ml Poly(rA).p(dT), 20 µM dTTP, 0.5µCi (microcurie) of [3H]TTP, 50mM Tris (pH 7.8), the 100 µl of reaction mixture was prepared. To the reaction mixture added the 0.5 units RT enzyme and incubated for 3 h at 37 °C. By adding 0.1M EGTA (25µl) the enzyme activity was terminated later incubated the reaction mixer on ice for chilling. 100 µl of C. uredinicola was spotted on 2.5cm Whatman filter paper (circular) and was incubated for 15 min at 26±2° C to dry. By using 5% aqueous NaHPO4.7H2O, washed the filters four times later two times with double distilled water. The filters were subjected to dry and to scintillation counting. To analysis, used azidothymidine as positive control and without sample considered as negative control. The percentage of inhibition calculated as,

\[
\text{Per cent inhibition} = \frac{\text{Negative control} - \text{Test sample}}{\text{Negative control}} \times 100
\]

**Inhibitory activity of HIV-1 gp120 binding**

ELISA kit was used to study binding of CD4 with gp120 [12]. We have studied our extract could interfere with biding of gp120 with CD4. The 5mg/ml of extract was added to gp120 (25ng) at 50 µl of equal concentration of 50 µl and was subjected to incubation at 26±2°C. Then transformed the reaction mixture to CD4 ligand containing microtiter plate wells and was subjected to incubation at 26±2 °C incubated for 1h. By using buffer washed the reaction mixer three times. Through detector reagent, analysed the gp120 binding. For positive control, the heparin was considered as standard control and with sample used as negative control.
Calculated the percentage of inhibition as mentioned below,

\[
\text{Inhibition Percentage} = \left(\frac{\text{AC} - \text{AS}}{\text{AC}}\right) \times 100,
\]

A is optical density.

**HIV-1 protease inhibition assay**

The assay was performed based on standard procedure of Narayan and Rai [13]. Using buffer (50 mM of sodium acetate (pH 5.0), 1 mM ethylenediamine disodium (EDTA.2Na) and 2 mM 2- mercaptoethanol (2-ME), the HIV-1 PR solution was diluted and added the glycerol in the ratio 3:1. The Arg-ValNle-NH2 (substrate peptides) was diluted with 50 mM of sodium acetate (pH 5.0). Two µl of extract, *C. uredinicola* and HIV-1 PR (4µl) was mixed with substrate solution (2µl, 2 mg/ml) and 10 µl of reaction mixture was incubated for 1h at 37°C. Without endophytic extract was used as control and terminated the reaction by keeping the reaction mixture for 1 min at 90 °C. Later, added the 20 µl of sterile water and an aliquot of 10 µl was analyzed by HPLC using RP-18 column (4.6 mm X 150 mm). The reaction mixture of 10 µl was injected to the column and eluted gradient by using 15-40 % of acetonitrile and trifluoroacetic acid (TFA) (0.2%) in water with 1.0 ml/min flow rate. Monitored the elution profile at 280 nm. The HIV-1 PR inhibitory assay was analysed using following formula:

\[
\text{% inhibition} = \left(\frac{\text{AC} - \text{AS}}{\text{AC}}\right) \times 100,
\]

for positive control the acetyl pepstatin was used.

**HIV-1 integrase inhibition assay**

The biotinylated long term repeat donor DNA (LTR-D) sequence of 3'-GAAAATCAGTCACCTTTAGAGATCGTCA-5' (LTR-D2) and 5'-biotin-ACCCCTTTTAGTAGTGTGGAAATCTCTAGCAGT-3'(LTR-D1) and were unlabelled complements. These targets the digoxigenin-labelled DNA (TS1) and its 3'-labelled component were 5'-TGACCAAGGGCTAATTCACT-digoxigenin-ACTGGTTCCCATTAAGTGA-5' (TS2). 12 µl of integrase buffer containing 25% of glycerol, 500 µg/ml of bovine serum albumin, 5mM of dithiothritol (DTT), 75mM of MnCl2, 150 mM of 3-(N-morpholine) propane sulfonic acid, pH 7.2 (MOPS) and added 1µl of digoxigenin labelled target DNA (5pmol/mol) and sterilized water (32 µl) and these mixture were transferred to each well of a 96 well plate. Added the 1/5 dilution of integrase enzyme (9 µl) and sample solution (6 µl) to the reaction mixture and the reaction plates were subjected to incubation at 37° C for 80 min. Using PBS, washed the wells four times, incubated the reaction mixture at 37° C for 1 h after adding 100 µl of alkaline phosphatase (AP) labelled anti-digoxigenin antibody (500 mU/ml). Using washing buffer, the plates were washed four times. 150 µl of AP buffer [5mM MgCl2, 10mM of p-nitrophenyl phosphate, 100mM of NaCl, 100 mM of Tris-HCl (pH 9.5)] was added to each well containing reaction solution and subjected to incubation at 37°C for 1h. Using microplate reader, the reaction was measured at 405 nm. 50% of DMSO and an integrase containing negative solution was referred as negative, buffer-E containing 20 mM MOPS (7.2), 20% of glycerol, 0.1% of nonidet-P40, 4M of urea without the integrase enzyme, 400 mM of potassium glutamate and 1mM of ethylenediamine tetra acetate disodium salt (EDTA-2Na) was referred as blank. For positive control, the suramin (polyanionic HIV-1 integrase inhibitor) was used.

\[
\text{Inhibition percentage of integrase} = \text{ODcontrol} - \text{ODsample/ ODcontrol X 100}
\]

**Inhibition of protease enzyme**

In a 500µl of reaction mixture, 800µg haemoglobin, *C. uredinicola* extract and 50µg pepsin was incubated for 20 min at 37° C to allow proper mixing and to stop the reaction added the 5% of TCA. Centrifuged the reaction for 5 min at 14000 g and the supernatant OD was recorded at 280 nm. To compare the reaction effect of our sample, both negative (pepstatin A, a protease inhibitor) and positive controls (enzyme and standard substrate) were used. Triplicate was maintained for each sample.
In vitro anti-Alzheimer activity

Acetyl and butyryl cholinesterase inhibition assay

96-well microplate reader was used to carry out acetylcholinesterase assay. The ChE enzyme (10 mL volume, diluted 100 times in phosphate buffer, pH 7.4) was mixed with DTNB (5,5-dithiobis-(2-nitro-benzoic acid)) (104 M concentration), 70 mM of phosphate buffer (Na2HPO4/NaH2PO4, pH 7.4) and ATCh (1.35 X 10-4 M concentration) in plate wells and allowed for reaction at 37° C and reaction was measured for 5 min at 412 nm. For each experiment, three replicates were maintained. The enzyme inhibition percentage was calculated by comparing with negative and positive control [14].

In silico antioxidant, anti-HIV, anti-diabetic and anti-cholinesterase activity

Selection of proteins/ receptors/ enzymes

The oxidant proteins, 1cb4 (SOD), 1qqw (Catalase), 1spd (SOD), 2cag (Catalase), 2he3 (gpx), 2p31 (Glutathione peroxidase 7), 3mng (human oxidant enzyme), the HIV-1 proteins 1bi4 (integrase), 1c0u (RT), 1dmp (protease), 1eby (protease), 1ex4 (integrase), 1exq (integrase), 1gc1 (gp120), 1rev (RT), 1w5x (protease), 2bvu (capsid), 2m8n (capsid), 2nxy (gp120), 2ny7 (gp120), 3h47 (capsid), 3kk2 (RT), 3ndw (protease), 3p05 (capsid), the diabetic proteins 1ppi (amylase), 1uok (glucosidase), 2g5p (pppiv), 3q6e (human insulin), 3w37 (glucosodase), 4gqr (human pancreatic amylase), 4x9y (amilase) and acetyl and butyryl cholinesterase proteins, 1b41 (AChE), 1gqr (AChE), 1xlw (BChE), 2ace (AChE), 2j4c (BChE), 2x8b (AChE), 4a9q (BChE), 4bds (BChE), 4pqe (AChE) were used for the molecular interaction studies.

Preparation of ligands

All the seven phytochemicals of C. uredinicola structures were obtained from NCBI PubChem database and their canonical smiles were used to generate 3D structure from www.mn-am.com/online_demos/corina_demo. The pharmacokinetic properties such as carcinogenicity, toxicity, inhibitory properties and various other properties were screened using admet-SAR device. Sedate likeliness, Adsorption, Dissolution, Metabolism, Excretion profile, toxicity and adverse factors of the ligand was anticipated. The ADME incorporates rate of retention, metabolism, digestion system and excretion. The admet-SAR employs Caco-2-cell (human epithelial colorectal adenocarcinoma cell lines) and MDCK (Madin-Darby Canine Kidney) cell models for oral medication, retention, skin porousness and human intestinal absorption to demonstrate oral and transdermal medical assimilation. Pre-ADMET predicts poisonous quality in view of the ADMET parameters and Rat acute toxicity [15-16].

Docking studies/Virtual screening

Molecular docking is the study utilized to predict the binding interaction of a molecule (ligands/drug candidates) to target proteins/receptors to discern the fitness of the ligand in the active site of the receptor. Consequently, the knowledge about the affinity and activity of the ligand can be determined. The 3D structure of all the seven ligands obtained from GC-MS study were developed using the CORINA tool (http://mn-am.com/online-demos/corina-demo), by entering the chemical structures as SMILES strings. CORINA is a fast and powerful tool which generates single, high-quality and low energy 3D structures of drug like molecules used for in silico profiling. The receptors were prepared by eliminating the water molecules from the PDB structures. Docking was carried out using iGEMDOCKv2.1. It is a tool used to study the interactions of pharmacologically important drugs. It provides basic idea on interactive or binding sites of receptor or proteins. After docking is completed a protein-ligand complex is generated along with the interaction profile which is used to rank the ligands based on the pharmacological energy. We have used two different methods to evaluate the docking scores, which accounts for biasing by dividing by molecular weight and non-hydrogen atoms [17].
AdmetSAR test

The ADMET (Absorption Distribution Metabolism Excretion Toxicity) profiling describes the disposition of pharmaceutical compound within an organism. The inhibitory properties of phytochemicals were studied through ADMET profile by submitting the canonical smiles downloaded from PubChem in admetSAR (admetSAR@LMMD). Human intestinal absorption, human oral bioavailability, penetration of blood–brain barrier, binding of plasma protein, volume of distribution, cytochrome P450 substrate, inhibitor, inducer, activator, half time (t1/2), renal clearance, drug induced toxicity, genomic toxicity, aquatic and terrestrial toxicity, reproductive toxicity, environmental factor – biodegradability.

Drug-likeliness studies

Drug likeliness properties of each ligand were studied using Molsoft L.L.C. online portal (www.molsoft.com) [18].

Results and Discussion

The methanol extract of *Cladosporium uridinicola* shows important phytochemicals viz., flavonoids, tannins, alkaloids, glycosides, phenols and coumarins but it not shown the saponin, anthraquinones (Table 1). The *Cladosporium uridinicola* reported as endophytic fungi from *Tinospora cordifolia* [19], *Psidium guajava* [20], Guava fruit [21]. Similar kinds of phytochemicals were noticed from endophytic fungi, *Neurospora crassa* [22] and *Penicillium* species [23].

Table 1. Phytoconstituents present in methanol extract of *Cladosporium uridinicola*

| Phytochemicals     | *Cladosporium uridinicola* extract |
|--------------------|-----------------------------------|
| Anthraquinones     | -                                 |
| Flavonoids         | +                                 |
| Saponins           | -                                 |
| Tannins            | +                                 |
| Alkaloids          | +                                 |
| Glycosides         | +                                 |
| Phenols            | +                                 |
| Terpenoids         | +                                 |
| Coumarins          | +                                 |

*Repeated the each experiment thrice, + =Presence and - = Absence

The obtained pure culture (Fig. 1A) was mass cultured in PDB (Fig. 1B) and the TLC have shown well separated many compounds in the extract (Fig. 1C). The GC-MS results have exhibited seven different phytochemicals they are 2H-1-Benzopyran-2-one (1), 3-Benzofurancarboxylic acid (2), Hymecromone (3), 16-Octadecanoic acid, methyl ester (4), 4-Hydroxy-9-(3-methyl-2-butenyl)furo(3,2-g)chromen-7-one (5), Z,E-2-Methyl-3,13-octadecadien-1-ol (6), [Z]-Cinnamic acid, 2-diphenylmethyleneamino-, methyl ester (7) and identified based on retention time using phytochemical library (Fig. 1D and Table 2).
Figure 1. A. Pure culture of *Cladosporium uredinicola* on PDA media, B. *C. uredinicola* grown in liquid media, C. TLC shows separated phytochemicals, and D. methanol extract of *C. uredinicola* showing 7 biologically important phytochemicals in GC-MS. 1-2H-1-Benzopyran-2-one, 2-3-Benzofurancarboxylic acid, 3- Hymecromone, 4-16-Octadecanoic acid, methyl ester, 4-4-Hydroxy-9-(3-methyl-2-butenyl)furo(3,2-g)chromen-7-one, 5-4-Hydroxy-9-(3-methyl-2-butenyl)furo(3,2-g)chromen-7-one, 6- Z,E-2-Methyl-3,13-octadecadien-1-ol, 7-[Z]-Cinnamic acid, 2-diphenylmethyleneamino-, methyl ester

Table 2. Bioactive compounds of methanol fraction of *Cladosporium* species BCt of *Calophyllum tomentosum*

| Peak No | RT (min) | Identified compound Name | Structure | Synonyms |
|---------|----------|--------------------------|-----------|----------|
| 1       | 16.05    | 2H-1-Benzopyran-2-one    | ![2H-1-Benzopyran-2-one](image) | 1) Coumarin  
2) 2H-1-Benzopyran-2-one  
3) 2H-Chromen-2-one  
4) 2H-Chromen-2-one  
5) 2H-Chromen-2-one  
6) Chromen-2-one |
| 2       | 17.22    | 3-Benzofurancarboxylic acid | ![3-Benzofurancarboxylic acid](image) | 1) Coumarilic acid,  
2) Coumarone-2-carboxylic acid,  
3) 2-Benzofurancarboxylic acid  
4) 1-benzofuran-3-carboxylic acid |
| 3       | 17.75    | Hymecromone              | ![Hymecromone](image) | 1) 2H-1-Benzopyran-2-one, 7-hydroxy-4-methyl-  
2) Coumarin, 7-hydroxy-4-methyl-  
3) β-Methylumbelliferone  
4) Bilcolic |
| No. | Value  | Chemical Name                                                                 | Formula                                                                 |
|-----|--------|--------------------------------------------------------------------------------|------------------------------------------------------------------------|
| 4   | 18.93  | 16-Octadecanoic acid, methyl ester                                            | 1) 16-Octadecenoic acid, methyl ester                                  |
|     |        |                                                                                | 2) Methyl (E)-octadec-16-enoate                                         |
| 5   | 21.12  | 4-Hydroxy-9-(3-methyl-2-butenyl)furo(3,2-g)chromen-7-one                      | 1) 4-Hydroxy-9-(3-methyl-2-butenyl)-7H-furo[3,2-g]chromen-7-one,         |
|     |        |                                                                                | 2) 4-Hydroxy-9-(3-methyl-2-butenyl)furo(3,2-g)chromen-7-one,             |
|     |        |                                                                                | 3) 4-hydroxy-9-(3-methylbut-2-enyl)furo(3,2-g)chromen-7-one,             |
|     |        |                                                                                | 4) Alloisoimperatorin                                                    |
| 6   | 25.47  | Z,E-2-Methyl-3,13-octadecadien-1-ol                                            | 1) 3E,13E)-2-Methyl-3,13-octade dién-1-ol                               |
|     |        |                                                                                | 2) 3,13-Octadecadien-1-ol, 2-methyl-, (3E,13E)                           |
| 7   | 27.95  | [Z]-Cinnamic acid, 2-diphenylmethylene amino-, methyl ester                   | 1) (E)-Cinnamic acid-alpha-diphenylmethylene amino-, methyl ester       |
|     |        |                                                                                | 2) Methyl (2Z) -2- [(diphenyl methylene) amino]-3-phenyl-2-propenoate    |
|     |        |                                                                                | 3) methyl (Z)-2- di (phenyl) methylidene amino -3-phenylprop-2-enoate    |
|     |        |                                                                                | 4) (Z)-2- di (phenyl) methylene amino] -3-phenyl- acrylic acid methyl ester |

The similar types of phytochemicals were also observed from different endophytes 2H-1-Benzopyran-2-one [24], 3-Benzo[turan]carboxylic acid [25], Hymecromone [26], 16-Octadecanoic acid, methyl ester [27], alloisoimperatorin [28], Z,E-2-Methyl-3,13-octadecadien-1-ol [29], [Z]-Cinnamic acid, 2-diphenylmethylene amino-, methyl ester [30].
These endophytic fungal phytochemicals are responsible for *in vitro* antioxidant activity [31-32]. The *in vitro* antioxidant method, DPPH is universally considered to screen the antioxidant compounds and it is causing any effect on enzyme inhibition and metal [33]. The methanol extract of *C. uredinicola* was studied free radical scavenging activity using DPPH method and standard ascorbic acid was used. The *C. uredinicola* extract had shown significant antioxidant activity and it was concentration dependent. Fig. 2 represents the antioxidant activity of endophytic activity was compared with ascorbic acid. The phytochemicals present in extract of *C. uredinicola* are responsible for antioxidant properties. The endophytic fungal phytochemicals donate electrons to DPPH and they are responsible for reduction of purple coloured DPPH to colourless solution [34]. Our findings are agreement with results of Manjunath et al. [35], Hulikere et al. [31]. The methanol extract of *C. uredinicola* phytochemicals reduced the Fe3+ TPTZ complex to Fe2+- tripyridytriazine (blue coloured complex) by donating electron at low pH and the reaction was observed and measured in absorbance at 593 nm. The *C. uredinicola* phytochemicals are acts strong as antioxidants agents in reducing power potential (Fig. 3) [36-38].

**Figure 2.** DPPH scavenging activities of *Cladosporium uredinicola* methanol extract compared the values with standard ascorbic acid

**Figure 3.** Total antioxidant activity of *Cladosporium uredinicola* methanol extract compared with standard ascorbic acid
The *C. uredinicola* methanol extract significantly reduced the activity of α-amylase, α-glucosidase and dipeptidyl peptidase IV activity *in vitro* condition (Fig. 4). The result confirms that inhibitory activities of diabetic enzymes are dependent on concentration of the sample. The *C. uredinicola* phytochemicals strongly inhibited the activity of α-amylase and it was significant when compared to positive control standard drug acarbose. Our results are confirmation with the findings of endophytic fungal α-glucosidase inhibitory activity [39-40]. The *C. uredinicola* extract inhibited the α-glucosidase activity at maximum level. The activity of DPP-IV significantly inhibited by *C. uredinicola* extract and it was compared with standard drug diprotin. The literature survey indicates that no results were found in dipeptidyl peptidase IV inhibitory activity using fungal extracts. The obtained results proving significant inhibition of dipeptidyl peptidase IV by *C. uredinicola* extract and are confirmation with the results of Kumar et al. [41]. The plant extracts also exhibited the dipeptidyl peptidase IV inhibitory activity [41-42].

![Figure 4. Per cent inhibition of diabetic enzymes by *C. uredinicola* phytochemicals](image)

The *C. uredinicola* extract had shown inhibition of HIV-1 proteins viz., protease, RT, protease and gp120. The *C. uredinicola* was inhibited RT activity strongly (83.81±2.14) and it was high compared to standard AZT (74.36±1.89) (Table 3) [27, 43]. Similarly, the gp120 (80.24±2.4) (Table 4), protease (77.63±2.14) (Table 5), integrase (79.43±2.14) (Table 6) proteins activity was decreased due to *C. uredinicola* extract. The HIV-1 proteins activity was inhibited due to potent phytochemicals of *C. uredinicola* and the activity may in combination of all the phytochemicals or any single potent phytochemical [41-42].

### Table 3. Per cent inhibition of RT by *Cladosporium uredinicola* methanol extract compared with respective standard drugs

| Treated agents               | Per cent inhibition of RT (mean ± SD) |
|------------------------------|----------------------------------------|
| *Cladosporium uredinicola* extract | 83.81±2.14                             |
| AZT (0.0016 mg/ml)           | 74.36±1.89                             |

Note: inhibition ≥ 50% is considered as significant

### Table 4. Inhibition percentage of gp120 by *Cladosporium uredinicola* methanol extract compared with respective standard drugs

| Treated agents               | Per cent inhibition of gp120 (mean ± SD) |
|------------------------------|-----------------------------------------|
| *Cladosporium uredinicola* extract | 80.24±2.31                              |
| Heparin (12.5 units)         | 76.48±1.69                              |

Note: inhibition ≥ 50% is considered as significant
Table 5. Inhibition percentage of protease by *Cladosporium uredinicola* methanol extract compared with respective standard drugs

| Treating agents                  | IC₅₀ (µg/ml)±SD |
|----------------------------------|-----------------|
| *Cladosporium uredinicola* extract | 77.63±2.14     |
| Acetyl pepstatin (positive control for protease) | 82.91±2.14     |

Table 6. Inhibition percentage of integrase by *Cladosporium uredinicola* methanol extract compared with respective standard drugs

| Treating agents                  | IC₅₀ (µg/ml)±SD |
|----------------------------------|-----------------|
| *Cladosporium uredinicola* extract | 79.43±2.14     |
| Suramin (positive control for integrase) | 81.97±2.14     |

The *C. uredinicola* methanol extract significantly inhibited the AChE and BChE activity *in vitro*. The study explains that AChE (33.47±2.8) was strongly inhibited due to the endophytic fungi extract compared with BChE (56.52±2.8) and these results were compared with standard drug Galantamine (Table 7). The inhibition of these enzymes may be due to the *C. uredinicola* phytochemicals may adsorbed or interact with AChE or BChE protein [44-45].

Table 7. Anticholinesterase activity of *Cladosporium uredinicola* methanol extract compared with respective standard drugs

| Samples                      | AChE assay IC₅₀ (mg/ml) | BChE assay IC₅₀ (mg/ml) |
|------------------------------|-------------------------|-------------------------|
| *Cladosporium uredinicola* extract | 33.47±2.8               | 56.52±1.08              |
| Galantamine                  | 39.81±2.8               | 2.6±1.1                 |

IC₅₀ values represent the means ± standard deviation of three parallel measurements

The molecular interaction studies were carried out between *C. uredinicola* phytochemicals with oxidant, HIV-1, diabetes and acetyl-cholinesterase proteins. The seven different phytochemicals have shown different binding energy with different oxidant proteins. The [Z]-Cinnamic acid, 2-diphenylmethyleneamino methyl ester had shown highest interaction with all the oxidant proteins followed by alloisoimperatorin and hymecromone. The 2-diphenylmethyleneamino methyl ester have interacted with val165 of 2p31 of 2p31 (-97.3) followed by 2cag (-96.14), 2he2 (-90.02), 1cb4 (-88.38), 1qqw (-88.25). The alloisoimperatorin ester had shown highest interaction with 2cag proteins (-97.56) at ser336, tyr337 followed by 1spd (-86.41), 3mng (-84.75), 1qqw (-83.18) (Fig. 5) (Table 8). No reports on 2-diphenylmethyleneamino methyl ester antioxidant activity but alloisoimperatorin [46] and hymecromone [47] had shown strong antioxidant activity *in vitro* condition but no reports on *in silico* antioxidant activity.
The diphenylmethyleneamino methyl ester had shown strong interaction with HIV-1 protease (1w5x: -103.41; 1eb: -102.58; 3ndw: -100), HIV-1 gp120 (2ny7: -108.47; 2nxy: -92.57; 1gc1: -84.24), HIV-1 RT (3kk2: -98.69; 1rev: -92.88; 1c0u: -88.41) and HIV-1 integrase (1ex4: -80.66; 1exq: -80.45; 1bi4: -79.23) followed by hymecromone and alloisoimperatorin (Table 9) (Fig. 5) with high binding energy. The diphenylmethyleneamino methyl has exhibited strong in vitro anti-HIV activity [48]. There is no report on in vitro and in silico anti-HIV activity of alloisoimperatorin and hymecromone. The diphenylmethyleneamino methyl ester had ability to interact with HIV-1 integrase, protease, RT, gp120 proteins with high binding energy leads to confirmation changes to inhibit their functions.
Table 9. Molecular interaction study between *C. uredinicola* phytochemicals with HIV-1 proteins

| PDB number | Alloisoimperatorin | [Z]-Cinnamic acid, 2-diphenylmethyleneamino methyl ester | Hymecromone |
|------------|---------------------|--------------------------------------------------------|-------------|
|            | 1                   | 2                                                      | 3           |
|            | 1                   | 2                                                      | 3           |
| HIV-1 integrase |                     |                                                        |             |
| 1bi4       | -77.59              | -61.05                                                 | -16.54      |
| 1ex4       | -79.08              | -69.7                                                  | -9.38       |
| 1exq       | -84.39              | -78.92                                                 | -5.47       |
| HIV-1 protease |                   |                                                        |             |
| 1dmp       | -86.81              | -75.21                                                 | -11.6       |
| 1ebv       | -85.19              | -72.1                                                  | -13.09      |
| 1w5x       | -85.38              | -83.61                                                 | -1.77       |
| 3dw        | -80.74              | -62.48                                                 | -18.26      |
| HIV-1 gp120 |                     |                                                        |             |
| 1gc1       | -86.57              | -60.57                                                 | -26.08      |
| 2nxy       | -91.24              | -75.79                                                 | -15.46      |
| 2ny7       | -105.91             | -97.41                                                 | -8.49       |
| HIV-1 RT   |                       |                                                        |             |
| 1c0u       | -77.3               | -73.8                                                  | -3.5        |
| 1rev       | -89.9               | -86.79                                                 | -3.11       |
| 3kk2       | -94.51              | -88.64                                                 | -5.87       |

1- Binding energy (kJ/mol), 2-VDW, 3-H-bond

The alloisoimperatorin was shown to interact highest with all the three diabetic enzymes (α-amylase, β-glucosidase and DPPIV) by showing highest binding energy compared to diphenylmethyleneamino methyl ester and hymecromone (Table 10) (Fig. 6). The alloisoimperatorin have firmly interacted with α-amylase and DPPIV followed by β-glucosidase. The alloisoimperatorin had interacted with val234, glu233 of 4x9y and showed highest binding energy. No reports are available on these compounds as antidiabetic activity from *in vitro* and *in silico* assays.

Table 10. Molecular interaction study between *C. uredinicola* phytochemicals with diabetic proteins

| PDB number | Alloisoimperatorin | [Z]-Cinnamic acid, 2-diphenylmethyleneamino methyl ester | Hymecromone |
|------------|---------------------|--------------------------------------------------------|-------------|
|            | 1                   | 2                                                      | 3           |
|            | 1                   | 2                                                      | 3           |
| α-amylase  |                     |                                                        |             |
| 1ppi       | -83.35              | -65.71                                                 | -17.64      |
| 4x9y       | -98.81              | -82.91                                                 | -15.89      |
| 4gr        | -92.71              | -76.41                                                 | -16.3       |
| β-glucosidase |                   |                                                        |             |
| 1uok       | -80.96              | -70.93                                                 | -10.04      |
| 3w37       | -80.73              | -74.73                                                 | -6          |
| DPPIV      |                     |                                                        |             |
| 2g5p       | -83.58              | -68.64                                                 | -14.94      |
| 3c45       | -92.44              | -73.55                                                 | -18.89      |

1- Binding energy (kJ/mol), 2-VDW, 3-H-bond
The alloisoimperatorin had shown strong interaction with AChE and BChE proteins with biding energy and results shows that the compounds have firm interaction with AChE. The alloisoimperatorin have ability to bind with tyr124, aer125, thr83, gly82, tyr337 of 4pqe. No reports on in vitro and in silico acetylcholinesterase activity of alloisoimperatorin (Fig. 6) (Table 11). From in silico results, we have discussed only three best phytochemicals possessing antioxidant, anti-diabetic, anti-HIV and anti-acetylcholinesterase activity. Out of seven phytochemicals of C. uredinicola, the diphenylmethyleneamino methyl ester showed strong anti-oxidant and anti-HIV
activity and alloisoimperatorin shows potent anti-diabetic and anti-cholinesterase activity in both in vitro and in silico experimental analysis.

**Table 11.** Molecular interaction study between *C. uredinicola* phytochemicals with cholinesterase proteins

| PDB number | Alloisoimperatorin  | [Z]-Cinnamic acid, 2-diphenylmethylenemethyloamino methyl ester | Hymecromone |
|------------|---------------------|---------------------------------------------------------------------|-------------|
|             | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| **AChE**    |   |   |   |   |   |   |   |   |   |
| 1b41        | -90.52 | -75.54 | -14.98 | -84.25 | -83.06 | -1.18 | -81.33 | -68.44 | -12.88 |
| 1gqr        | -101.5 | -92.53 | -8.98  | -94.87 | -92.37 | 2.5   | -84.18 | -70.34 | -13.83 |
| 2ace        | -103.69 | -97.77 | -5.92  | -84.73 | -84.73 | 0     | -71.08 | -52.14 | -18.94 |
| 2x8b        | -99.13 | -88.17 | -10.97 | -94.46 | -80.51 | -13.94 | -82.46 | -66.1  | -16.36 |
| 4pqe        | -103.95 | -93.87 | -10.08 | -90.89 | -84.77 | -6.12  | -81.68 | -69.69 | -11.99 |
| **BChE**    |   |   |   |   |   |   |   |   |   |
| 1x1w        | -92.42 | -79.56 | -12.86 | -87.03 | -87.03 | 0     | -72.73 | -60.13 | -12.6  |
| 2j4c        | -85.41 | -72.21 | -13.2  | -83.64 | -83.64 | 0     | -78.19 | -64.88 | -13.31 |
| 4aqd        | -93.6 | -75.1 | -18.5 | -84.69 | -82.19 | -2.5 | -76.46 | -72.96 | -3.5   |
| 4bds        | -91.51 | -78.23 | -13.27 | -87.16 | -87.16 | 0     | -75.9 | -65.42 | -10.48 |

1- Binding energy (kJ/mol), 2-VDW, 3-H-bond

The admet-SAR results confirm that these three promising compounds are non-AMES toxic, non-carcinogens, readily biodegradables (Table 12A-B). They also showed drug likeliness property in Molsoft (Table 13 and Fig. 7). Further, in vivo work will be carried out in future.
Table 12A. ADMET Predicted profile of the potent phytochemicals of *Cladosporium uredinicola* species

| Property                        | Cinnamic acid, 2-diphenylmethene | 2H-1 Benzopyran-2-one ND | 3-Benzofurancarboxyclic acid ND | 4 Hydroxy-9 (3-methyl-2-butenyl) |
|---------------------------------|----------------------------------|--------------------------|---------------------------------|---------------------------------|
|                                 | Value                            | Value                    | Value                           | Value                           |
| Blood Brain Barrier             | BBB+                             | BBB+                     | BBB+                            | BBB+                            |
| Human intestinal absorption     | HIA+                             | HIA+                     | HIA+                            | HIA+                            |
| Caco-2-permeable                | CaCo2-                           | CaCo2+                   | CaCo2+                          | CaCo2+                          |
| P-glycoprotein-substrate        | Substrate                        | Non-substrate            | Non-substrate                   | Substrate                       |
| P-glycoprotein-inhibitor I      | Non-inhibitor                    | Non-inhibitor            | Non-inhibitor                   | Non-inhibitor                   |
| Renal organic transporter       | Non-inhibitor                    | Non-inhibitor            | Non-inhibitor                   | Non-inhibitor                   |
|                                 | Plasma membrane                  | Plasma membrane          | Plasma membrane                 | Mitochondria                    |
| Metabolism                      | CYP450 2C9 Substrate             | Non-substrate            | Non-substrate                   | Non-substrate                   |
|                                 | CYP450 2D6 Substrate             | Non-substrate            | Non-substrate                   | Non-substrate                   |
|                                 | CYP450 3A4 Substrate             | Non-substrate            | Non-substrate                   | Non-substrate                   |
|                                 | CYP450 1A2 Substrate             | Non-substrate            | Non-substrate                   | Non-substrate                   |
|                                 | CYP450 2C9 Inhibitor             | Non-substrate            | Non-substrate                   | Non-substrate                   |
|                                 | CYP450 2D6 Inhibitor             | Non-substrate            | Non-substrate                   | Non-substrate                   |
|                                 | CYP450 2C19 Inhibitor            | Non-substrate            | Non-substrate                   | Non-substrate                   |
|                                 | CYP450 3A4 Inhibitor             | Non-substrate            | Non-substrate                   | Non-substrate                   |
|                                 | CYP Inhibitory Promiscuity       | Low CYP inhibitory       | Low CYP inhibitory              | Low CYP inhibitory              |
|                                 | Human Ether-ago-Related Gene     | Weak inhibitor           | Weak inhibitor                  | Weak inhibitor                  |
|                                 | AMES Test                        | Non-AMES toxic          | Non-AMES toxic                  | Non-AMES toxic                  |
|                                 | Carcinogens                      | Non-carcinogens          | Non-carcinogens                  | Non-carcinogens                  |
|                                 | Fish Toxicity                    | High FHMT                | High FHMT                        | High FHMT                        |
| Property                        | 16-octadecanoic acid, methyl ester | ND | 16-hymercromone | ND | Z-3,13-octadecadine-1-01 | ND |
|--------------------------------|------------------------------------|----|----------------|----|-------------------------|----|
| Value                          | Probability                        |    | Value          | Probability | Value                      | Probability |
| Blood Brain Barrier            | BBB+                               | 0.9848 | BBB+           | 0.9488 | BBB+                     | 0.9488       |
| Human Intestinal absorption    | HIA+                               | 0.9881 | HIA+           | 0.9888 | HIA+                     | 0.9888       |
| Caco-2-permeable               | CaCo2+                             | 0.8141 | CaCo2+         | 0.8326 | CaCo2+                   | 0.8326       |
| P-glycoprotein-substrate       | Non-Substrate                      | 0.7061 | Non-substrate  | 0.6321 | Non-substrate            | 0.6321       |
| P-glycoprotein-inhibitor I     | Non-inhibitor                      | 0.8951 | Non-inhibitor  | 0.9598 | Non-inhibitor            | 0.9598       |
| Non-Inhibitor                  | 0.7988                             | Non-inhibitor | 0.9277 | Non-inhibitor            | 0.9277       |
| Renal organic cation transporter| Non-inhibitor                      | 0.8908 | Non-inhibitor  | 0.9266 | Non-inhibitor            | 0.9266       |
| Subcellular localization       | Mitochondria                       | 0.4276 | Mitochondria   | 0.5152 | Mitochondria             | 0.5152       |
| CYP450 Substrate               | 2C9                                | 0.8648 | Non-substrate  | 0.8786 | Non-substrate            | 0.7886       |
| CYP450 Substrate               | 2D6                                | 0.8885 | Non-substrate  | 0.7886 | Non-substrate            | 0.8956       |
| CYP450 Substrate               | 3A4                                | 0.6454 | Non-substrate  | 0.8956 | Non-substrate            | 0.6982       |
| CYP450 Substrate               | 1A2                                | 0.5548 | Non-inhibitor  | 0.6982 | Non-inhibitor            | 0.8326       |
| CYP450 Inhibitor               | 2C9                                | 0.9329 | Non-inhibitor  | 0.8326 | Non-inhibitor            | 0.8808       |
| CYP450 Inhibitor               | 2D6                                | 0.9502 | Non-inhibitor  | 0.8808 | Non-inhibitor            | 0.9554       |
| CYP450 Inhibitor               | 2C19                               | 0.9524 | Non-inhibitor  | 0.9554 | Non-inhibitor            | 0.9578       |
| CYP450 Inhibitor               | 3A4                                | 0.9773 | Non-inhibitor  | 0.9484 | Non-inhibitor            | 0.9484       |
| Phytochemicals                        | MF          | MW   | HBA | HBD | LogP | LogS | PSA  | MV  | NSC |
|--------------------------------------|-------------|------|-----|-----|------|------|------|-----|-----|
| 2H-1-Benzopyran-2-one                | C9H6O3      | 162.03 | 3   | 1   | 2.27 | 0.320 | 36.94 | 141.66 | 0   |
| 3-Benzofurancarboxylic acid          | C15H15NO3   | 257.11 | 3   | 1   | 1.93 | -3.09 | 43.86 | 262.08 | 0   |
| Hymecromone                          | C16H32O2    | 256.24 | 2   | 1   | 6.65 | -5.66 | 28.89 | 305.93 | 0   |
| 16-Octadecanoic acid, methyl ester   | C19H38O2    | 298.29 | 2   | 0   | 7.97 | -6.78 | 21.09 | 364.74 | 0   |
| 4-Hydroxy-9-(3-methyl-2-butenyl)fluoro(3,2-g)chromen-7-one | C16H14O4 | 270.09 | 4   | 1   | 3.55 | -4.93 | 44.24 | 296.33 | 0   |
| Z,E-2-Methyl-3,13-octadecadien-1-ol  | C19H38O2    | 298.29 | 2   | 1   | 7.93 | -6.64 | 28.92 | 356.87 | 1   |
| [Z]-Cinnamic acid, 2-diphenylmethyleamino-, methyl ester | C9H8O2 | 148.05 | 2   | 1   | 2.33 | -2.61 | 28.62 | 151.60 | 0   |

MF: Molecular Formula, MW-Molecular Weight, HBA-Hydrogen Bond Acceptor, HBD-Hydrogen Bond Donor, MolLogP-MolLogS-, MolPSA-, MolVol-, NSC-Number of Stereo Centers

Table 13. Physiochemical properties of *Cladosporium uridinicola* phytochemicals
The C. uredinicola extract biologically phytochemicals are responsible for strong antioxidant, anti-HIV, anti-diabetes and anti-cholinesterase activity. The in silico experiment clearly understands that the three phytochemicals (diphenylmethyleneamino methyl ester, alloisoimperatorin, hymecromone) have the ability to interact with oxidant, HIV-1, diabetic and cholinesterase proteins with highest binding energy. The admetSAR and Molsoft proven that these three compounds are non-toxic, non-carcinogens, easily biodegradable and having drug likeliness properties.

The oxidant proteins SOD, catalase, gp, glutathione peroxidase 7, human oxidant enzyme are inhibited by endophytic fungal extract. All these enzymes break down potentially harmful molecules in cells and these oxygen molecules play a role in disease or cell damage.

The diabetic proteins, α-amylase, β-glucosidase and DPP-IV are strongly inhibited by the C. uredinicola extract. The α-amylase and β-glucosidase are involved in digestion of carbohydrates lead to increase of blood glucose level in diabetes -2. The DPP-IV increases glucagon and blood glucose level. These enzymes play vital role progression of diabetes-2. HIV-1 RT (essential step in retroviral replication), protease (play crucial role HIV life cycle and it cleaves the newly synthesized polyproteins to obtain mature components of protein of an infective HIV), integrase (requires for multidomain enzyme essential for the viral DNA in the host genome), gp120 (essential for virus entry into cells and helps in attachment to specific cell surface receptors) are greatly inhibited by the endophytic fungal extract. The AChE and BChE are pathogenesis of Alzheimer disease and progression.
Conclusion

Based on outcomes of our in vitro and in silico research clearly indicates that the endophytic fungi C. uredinicola have shown biologically important phytochemicals in methanol extract and these are responsible for antioxidant, anti-HIV, anti-diabetes and anticholinesterase activity and suggested their possible role. Further in vivo work is needed to justify our research.

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Conflicts of Interest

The authors declare no conflict of interest.

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