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ISOLATION, PHYTOCHEMICAL INVESTIGATION AND BIOLOGICAL SCREENING IN LEAVES OF *OLDENLANDIA AURICULARIA*

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Abstract: The study was aimed to isolate, phytochemical analysis, biological screening and *in-silico* antidiabetic activity of *Oldenlandia auricularia.* From the present work the chemical compounds from leaves of *Oldenlandia auricularia* has been extracted successfully. Methanol, ethanol and chloroform are used individually for extraction and to find extractive value. The higher extractive value is found in the chloroform solvent. This research work visualized successfully the application of column chromatography techniques for the isolation of biologically active secondary metabolites from the plant sample. After separating the fractions using the column chromatography, all the fractions were checked, using the TLC, and as well as subjected to FT-IR and Mass spectroscopy for the identification of compounds. The screening of antidiabetic activity was carried out by *in-vitro* method. *In-vitro* antidiabetic activity was carried out, which showed that more activity was present in chloroform extract of *Oldenlandia auricularia*. *In-silico* antidiabetic activity was also carried out by using software like Autodock and found the good binding affinity from the component present in the *Oldenlandia auricularia* leaves. Molecular docking was taken as first step in this research as it is a powerful tool for the ligand-based drug discovery. The investigation concludes that the ligand (Auricularine) was docked with the target alpha-amylase inhibitor (44w93 receptor) and, compared with the standard (Acarbose with 4w93) and also studied their ADMET properties by using SwissADME software.

Index Terms – *Oldenlandia auricularia*, Antidiabetic activity, *In-silico*, *In-vitro*, Alpha-amylase, Chemical constituents, Plant extract, Chloroform, Methanol, Auricularine, Spectroscopy, Ligand, Receptor, 4w93.

I. INTRODUCTION

Diabetes mellitus is a complex and a diverse group of disorders that disturbs the metabolism of carbohydrates, fat and proteins. Diabetes mellitus cases have been rising globally in recent years. According to a research by the World Health Organization, there were 171 million persons with diabetes worldwide in 2000, and by 2030, that number will be expected to rise to 366 million[1]. Thus, searching for a new class of compound is essential to overcome diabetes problems. There is continuous search of alternative drugs[2]. The treatments for diabetes are reduction of the demand for insulin, stimulation of insulin secretion, enhance the mode of action of insulin at the target tissues and inhibition of degradations of oligo- and disaccharides[3,4].

Oldenlandia auricularia is a therapeutic plant belongs to the Rubiaceae family which is begun from South India, Australia, Malaysia, Philippines, and Sri Lanka. It likewise grows in open woodland and bushes. *Oldenlandia auricularia* shows antihypertensive, antibacterial, antioxidant and anti-inflammatory features and data was gathered about the plant constituents. Many *invitro* and *in vivo* studies have been conducted to exhibit the plant properties[5].

Inhibitors of alpha-amylase delay the breaking down of carbohydrates in the small intestine and diminish the postprandial blood glucose excursion[6].

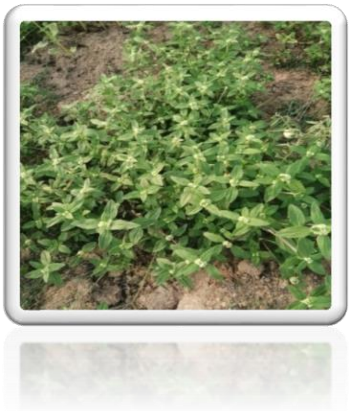


Figure no. 1: Leaf of *Oldenlandia auricularia*

TAXONOMICAL CLASSIFICATION

Table no. 1 Taxonomical classification

Domain	Eukaryota
Kingdom	Plantae
Subkingdom	Viridiplantae
Phylum/Division	Tracheophyta
Subdividion	Spermatophytina
Clade	Angiosperms
Class	Magnolipsida
Order	Gentianales
Family	Rubiaceae
Genus	*Oldenlandia*
Species	*Auricularia*

MATERIALS AND METHODS

PLANT MATERIAL IDENTIFICATION AND COLLECTION

Collection of *Oldenlandia auricularia leaves*.

The leaves of *Oldenlandia auricularia* were collected from Kallakurichi, Tamilnadu, India. The leaves were identified Dr. Arjun Shetty, HOD, Department of Botany, Sharanabasava University, Kalaburgi, Karnataka.

Preparation of plant material

The required plant material was collected and washed with tap water. The cleaned leaves were shade dried at room temperature. The dried leaves were size reduced to coarse powder. The powder was then weighed and stored in an airtight container.

PREPARATION OF PLANT EXTRACTS

Cold extraction (Maceration)

10 g of leaves was taken and performed analysis called maceration that was nothing but determining about the percentage solubility of phytoconstituents in the selected solvents like methanol, chloroform and ethanol. Maceration process gave an information that mixture of plant material has maximum solubility in Chloroform. Hence, the selected solvent is used for further extraction process.

Hot extraction (Soxhlation)

10 g of coarse powder (leaves) was successfully extracted using a Soxhlet apparatus with chloroform. The extracts were then filtered through Whattmann No.1 filter paper and filtrate were then transferred to a weighed porcelain dish and extracts were concentrated to dryness by keeping filtrate for complete evaporation of solvent.

PHYSIOCHEMICAL ANALYSIS

Physicochemical constants such as the foreign matter, taste, odor, color and nature were calculated based upon standard procedures prescribed by Kokate as follows[7].

PRELIMINARY PHYTOCHEMICAL ANALYSIS OF PLANT EXTRACTS

Qualitative analysis of methanol, chloroform and ethanolic extracts of leaves of *Oldenlandia auricularia* for the identification of various classes of active chemical constituents like alkaloids, flavonoids, terpenoids, glycosides, etc. using different methods of Harborne[8] and using standard procedures[9-12].

1.Test for alkaloids:

a) Dragendroff 's test: 1 ml of 1% hydrochloric acid was added to 2 ml of plant extract and was heated in a water bath for 10 min., 1 ml this solution was taken and 6 drops of Dragendroff's reagent were added and mixed. Orange precipitate indicated the presence of alkaloids.

b) Mayer's Test: To 1 ml of the extract, 2 ml of Mayer's reagent was added, a dull white precipitate indicates the presence of alkaloids.

c) Wagner's Test: To 1 ml of the extract, 2 ml of Wagner's reagent was added. The appearance of a reddish-brown precipitate indicates the presence of alkaloids.

2.Test for tannins and phenolic compound:

a) Ferric chloride test: Few drops of 5% Ferric chloride solution was added to 2 to 3 ml of extract and observed for deep blue- black color.

b) Gelatin test: Gelatin solution (1%) containing 10% sodium chloride solution was added to 1 ml of extract and observed for formation of a precipitate.

3.Test for saponins:

a) Foam test: The extract (2 ml) mixed with 2 ml of distilled water was shaken vigorously and persistent foam was observed.

4.Test for flavonoids:

a) Aluminium solution test: A few drops of 1 % aluminium solution were added to 1 ml of the extract. A yellow color indicates the presence of flavonoids.

b) Lead acetate test: 1 ml of extract was mixed with 1 ml of 10% lead acetate solution and observed for yellow colored precipitate.

c) NaOH Test: 1 ml of extract was treated with aqueous NaOH and HCl, observed for the formation of yellow orange color which disappears on addition of HCl.

d) H2SO4 Test: 1 ml of extract was treated with concentrated H2SO4 and observed for the formation of orange color.

5.Test for terpenoids:

Salkowski's test: Chloroform (1 ml) was added to 1 ml of extract. Concentrated sulphuric acid (few drops) was added along the sides of test tubes and observed for the formation of the yellow-colored layer indicating the presence of terpenoids.

6.Test for glycosides:

Liebermann's test: 1ml of extract was dissolved in 1 ml of chloroform and 1 ml of acetic acid. The solution was cooled well in ice and a few drops of sulphuric acid was added carefully. A colour change from violet to blue to green indicates the presence of steroidal nucleus (aglycone portion of glycoside).

6.8. ISOLATION OF ACTIVE CONSTITUENTS FROM THE EXTRACT:

6.8.1. Detection of mobile phase:

The ethyl acetate extract was introduced to activated TLC plates using a capillary tube at 1/2 inch besides the lower edge of the TLC plate, and thus the plate was left in a developing chamber bearing a proper solvent system for a specified time. Once the developing solvent reached the top of the top edge of the TLC plate, the plate has been removed from the chamber; the solvent front was marked with lead pencil and dried. Visual detection of compound bands/spots has been carried out on TLC chromatoplate which detected under UV light (254 nm) for the presence of specific compounds. The spots of the components in the TLC plate were marked and the Rf value of each spot was calculated by the formula:

$$Rf \text{ value} = \frac{\textit{Distance traveled by the solute}}{\textit{Distance traveled by the solvent}}$$

Column Chromatography:

Partial purification of ethyl acetate extract was carried out using silica gel column chromatography. The glass column was packed by the wet method. The adsorbent slurry (silica gel; 60–120) was prepared by stirring the adsorbent with the same mobile phase and used as a stationary phase. Then, it was dripped into the glass column (43 cm x 3.5 cm) (a sintered glass disc at the bottom) and allowed to remain and settle. The air entrapped was removed by tapping the column with a rubber tube. A small amount of sand and cotton was kept at the top of the column to provide the latter with a flat base. Excess solvent was run off once the mobile phase level dropped to 1 cm just above the upper edge of the sand and cotton layer. 6 g of ethyl acetate extract was mixed with 3 g of silica gel as the stationary phase loaded onto the column. The flow rate was set to 1 ml/min. The column was eluted with Hexane and Methanol (8:2).

Separation of bioactive constituents from the first mobile phase was carried out by eluting the column at a uniform interval (3 drops per minute), the eluents (each of five ml), which were collected in a test tube and the progress of separation was monitored

by thin layer chromatography (TLC) (silica gel G 60 F254 TLC plates of E. Merck, layer thickness 0.2 mm) using the same solvent system.

SPECTROSCOPY

IR SPECTROSCOPY

IR spectroscopy is used to establish whether a given sample of an organic substance is identical with another or not. This is because large number of absorption bands is observed in the IR spectra of organic molecules and the probability that any two compounds producing identical spectra is almost zero. So, if two compounds have identical IR spectra then both of them must be samples of the same substances[13].

MASS SPECTROSCOPY

In order to measure the characteristics of individual molecules, a mass spectrometer converts them to ions so that they can be moved about and manipulated by external electric field and magnetic fields. The three essential functions of a mass spectrometer, and the associated components

- **The Ion Source:** A small sample is individual molecules, usually to cations by loss of an electron.
- **The Mass Analyzer:** The ions are sorted and separated according to their mass and charge.
- **The detector:** The separated ions are then measured and the results displayed on a chart.

BIOLOGICAL STUDIES (ANTI-DIABETIC ACTIVITY)

The following anti-diabetic assay were performed on Chloroform extract compound.

***In-vitro* antidiabetic activity:**

Inhibition of alpha-amylase enzyme:

A starch solution (0.1% w/v) was obtained by stirring 0.1 g of potato starch in 100 ml of 16 mM of sodium acetate buffer. The enzyme solution was prepared by mixing 27.5 mg of alpha-amylase in 100 ml of distilled water. The colorimetric reagent is prepared by mixing sodium potassium tartarate solution and 3,5-dinitro salicylic acid solution (96 mM). Both control and plant extract were added with starch solution and left to react with alpha-amylase solution under alkaline conditions at 25℃. The reaction was measured over 3 min. The generation of maltose was quantified by the reduction of 3,5-dinitro salicylic acid to 3-amino-5-nitro salicylic acid. This reaction is detectable at 540 nm[14].

Calculation of 50% Inhibitory Concentration (IC_{50})

The concentration of the plant extracts required to scavenge 50% of the radicals (IC50) was calculated by using the percentage scavenging activities at five different concentrations of the extract. Percentage inhibition (I%) was calculated by[15]

I%=(AC-AS)/AC×10

Where, I% is the percentage inhibition,

AC is the absorbance of the control and

AS is the absorbance of the sample.

***In-silico* antidiabetic activity.**

The *in-silico* antidiabetic activity was performed for the screened receptor and the chemical compound present in *Oldenlandia auricularia*.

Active site prediction

The geometric and topological possessions of protein configuration, such as surface pockets, interior cavities and cross-channels, are of critical importance for proteins to accomplish their key functions. Discovery studio is a web server which actually provides online services for retrieving, outlining and quantifying these geometric and topological properties of protein structures[16].

Ligand preparation

Ligand was isolated from *Oldenlandia auricularia* and the compounds was sketched through Chemsketch and its 3D optimized and saved in sdf format. The ligand optimization was performed in Autodock.

Pharmacophore and ADMET prediction

The pharmacophore and ADMET prediction were carried out using SwissADME online server.

Molecular docking

Docking evaluation of these ligand was obtained from *Oldenlandia auricularia* leaves docked with selected target protein using Auto Dock Tools (ADT) 1.5.7, AutoDock 4.2, Auto Dock Vina, Open Babel. Subsequently, the output of the docking process was analyzed using Biovia Discovery studio 2021.

RESULTS AND DISCUSSION

PHYSICOCHEMICAL PARAMETERS:

The determination of physical and chemical parameters was important in the determination of adulterants and improper handling of drugs. The extract was analyzed for physicochemical characteristics. The observed parameters were recorded as shown in table no. 2.

Table no. 2: Physiochemical parameter for leaf extract of *Oldenlandia auricularia*

SL.NO	*TEST*	*OBSERVATION*
1	Nature	Coriaceous
2	Color	Upper Surface is shiny and dark green, lower surface is paler.
3	Odor	No characteristic odour
4	Taste	Slightly bitter
5	Foreign matters	Nil

EXTRACTIVE VALUES:

The extractive values obtained from *Oldenlandia auricularia* using different solvents like methanol, chloroform and ethanol were recorded and the values are shown in table no. 3. It is useful for evaluating a crude drug because it provides information about the nature of the chemical constituents present in it and allows for the estimation of chemical constituents soluble in the solvent used for extraction. Water soluble extractive values indicated the presence of sugar, acids, and inorganic compounds, and alcohol soluble extractive values indicated the presence of polar constituents like phenols, alkaloids, saponins, glycosides, flavonoids, and secondary metabolites present in the plant sample. The chloroform extract gives better extractive values.

Table no. 3: Extractive values

Solvent	Extract (mg)	Percentage (%)
Methanol	0.63	6.3
Chloroform	0.67	6.7
Ethanol	0.61	6.1

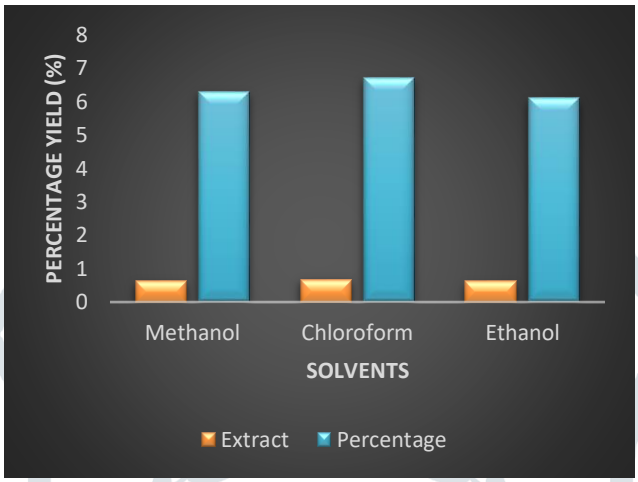


Figure no. 2: Extractive values

PHYTOCHEMICAL SCREENING:

The results of phytochemical screening of different extracts of *Oldenlandia auricularia* plant were reported in table no. 4. The phytochemical study revealed the presence of various Phyto-compounds in different solvent extracts.

Table no. 4: Phytochemical screening

SI.NO	Name of the phytoconstituent	Methanol	Chloroform	Ethanol
1	Alkaloid	+	+	+
2	Tannins and phenols	+	-	-
3	Saponins	-	-	-
4	Flavonoids	+	+	+
5	Terpenoids	-	-	+
6	Glycoside	-	+	-

(+) =Presence, (-) = Absence

Phytochemical screening of various leaf extracts *Oldenlandia auricularia* showed the presence of flavonoids, alkaloids, phenolic compounds, tannins, glycosides, while it gave negative results for saponins. In chloroform extract, except saponins and tannins and terpenoids, other compounds were found to be present. And in methanolic extract, alkaloids, tannins, phenolic compounds and flavonoids were tested positive.

ISOLATION OF ACTIVE CONSTITUENTS FROM THE EXTRACT:

Thin layer chromatography (TLC)

About 50 TLC plates were eluted using different solvents, in which the TLC plates show the number of bands (chemical compounds) for each fraction. These can be further isolated and purified using Column Chromatography (CC). Out of 50 TLC plates using different solvents (single solvent and in combination), the best TLC plates with good separation were selected for carrying out the Column Chromatography. The selected mobile phase with good separation compared to other mobile phases was found to be TLC plate Hexane: methanol in the ratio of 8:2.

$$R_F \textbf{ value} = \frac{\textit{Distance traveled by the solute}}{\textit{Distance traveled by the solvent}}$$

$$= \frac{4.6}{6.9}$$

= 0.6

Column chromatography

The same solvent system that was found by TLC chromatography, Hexane: methanol, was used for column chromatography. After running the column, the fractions were collected and checked for their R_f value by TLC.

IDENTIFICATION AND DETECTION OF SUBSTANCES WITH FT-IR SPECTROSCOPY:

IR spectroscopy is used to establish whether a given sample of an organic substance is identical to another or not. This is because a large number of absorption bands are observed in the IR spectra of organic molecules and the probability that any two compounds will produce identical spectra is almost zero. So, if two compounds have identical IR spectra, then both of them must be samples of the same substance.

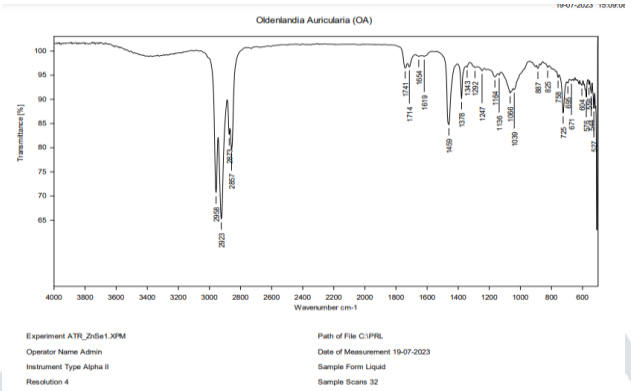


Figure no. 3: IR spectra of Chloroform extract of *Oldenlandia auricularia.*

Qualitative analysis of chloroform extracts of *Oldenlandia auricularia* leaves was performed for the identification of various classes of active chemical constituents like alkaloids, carbohydrates, glycosides, proteins, amino acids, steroids etc. IR Spectroscopy is used to establish whether a given sample of an organic substance is identical with another or not. This is because a large number of absorption bands is observed in the IR spectra of organic molecules and the probability that any two compounds if produce identical spectra is almost zero. So, if two compounds have identical IR spectra then both of them must be samples of the same substances.

Fig. no.3 The IR peaks obtained for the extract N-H (stretching) = 2923 cm^{-1}, C-H (bending) = 1714 cm^{-1}, C-N (stretching) = 1066 cm^{-1} and C=C (bending) = 887 cm^{-1}.

MASS SPECTRUM

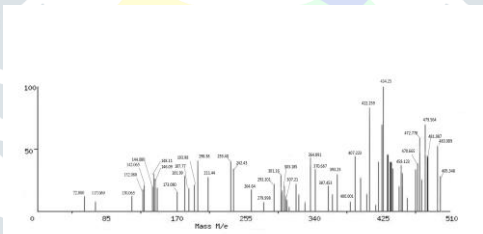


Figure no. 4: Mass spectrum of sample

The Mass spectrum peak obtained here is Molecular ion peak = 483.089, Base peak = 434.25, 2nd Highest peak = 422.259, 3rd Highest peak =479.564.

In the above spectrum different fragments were observed in that for the m/z ratio 493.33, got the fragment with molecular formula $C_{33}H_{40}N_4$, for 436.27, got the fragment with formula $C_{28}H_{32}N_2^+$, for 434.25, got the fragment with formula $C_{32}H_{39}N_3$, 422.26 got the fragment with formula $C_{29}H_{35}N_3$, for 393.23 , got the fragment with formula $C_{29}H_{42}N_2$, for 301.16, got the fragment with formula $C_{21}H_{22}N_2$, for 173.10, got the fragment with formula $C_{11}H_{12}N_2$, for 144.08, got the fragment with formula $C_{10}H_{11}N$, for 72.08, got the fragment with formula C_4H_9N and for 58.06, got the fragment with formula $C_3H_8N_7$.

BIOLOGICAL SCREENING

***In-vitro* antidiabetic activity**

Inhibition of alpha-amylase enzyme

Using different concentrations of alpha-amylase solution such as 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml and 500 µg/ml which are prepared by using different proportions of alpha amylase enzyme such as 0.1 mg, 0.2 mg, 0.3 mg, 0.4 mg and 0.5 mg, respectively to get a different concentration. The same way is done for standard *i.e.*, Acarbose.

The chloroform extract of *oldenlandia auricularia* leaves revealed a significant inhibitory action of alpha-amylase enzyme. Similar study conducted without plant extract as control. Acarbose is taken as standard. There was a dose-dependent increase in percentage inhibitory activity against α-amylase enzyme. At a concentration 100µg/ml of extract showed a percentage inhibition 42.47 and for 500 µg/ml it was 77.86 and at concentration 400 µg/ml, percentage of inhibition is 69.54%. The extract gave an IC_{50} value of 62.74 ± 7.16 µg/ml. The IC_{50} value of standard drug acarbose was found to be 69.53 ± 7.31 µg/ml (Table no. 6)

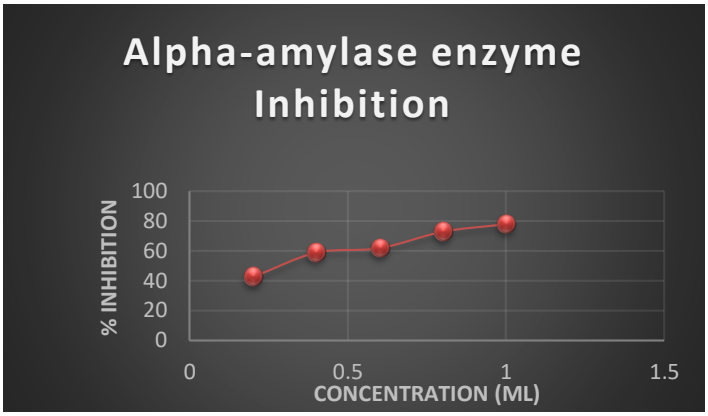


Figure no. 5: Inhibition of alpha-amylase enzyme

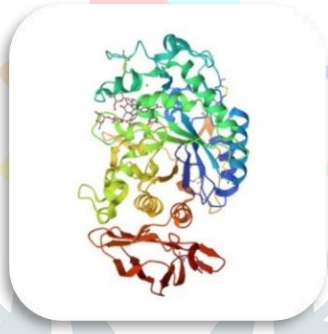
Table no. 6: *In-vitro* alpha-amylase inhibitory activity.

SL.NO	CONCENTRATION (µg/ml)	% Inhibition	
1	100	42.47	Standard (Acarbose) 69.53%
2	200	58.79	
3	300	61.77	
4	400	72.81	
5	500	77.86	

***In-silico* antidiabetic activity:**

The following *in-silico* antidiabetic activity performed for the selected alpha-amylase inhibitor (Figure no.6) protein and the chemical component present in *Oldenlandia auricularia.*

Targeted protein receptor



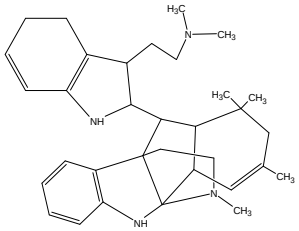
Alpha-amylase

Figure no. 6: Targeted protein receptor

Alpha amylase is a crucial target in antidiabetic activity due to its role in carbohydrate digestion. Inhibiting alpha amylase reduces the breakdown of complex carbohydrates into glucose, leading to lower post-meal blood sugar spikes. This mechanism helps manage diabetes by controlling glucose levels, making alpha amylase an important focus in the development of antidiabetic drugs.

Ligands isolated from *Oldenlandia auricularia.*

The following ligand isolated from the *Oldenlandia auricularia,* i.e., Auricularine (Figure no.7)



Auricularine

Figure no. 7: Ligands

Ligands can be retrieved from several databases such as ZINC, PubChem or can be sketched applying Chemsketch tool. Then it's optimized from 2D to 3D structure. While picking out the ligand, the LIPINSKI'S RULE OF 5 should be utilized. Lipinski's rule of 5 assists in discerning amongst non-drug like and drug like candidates. It promises high chance of success or failure due to drug likeness for molecules abiding by with 2 or more than of the complying rules. For choice of a ligand allowing to the LIPINSKI'S RULE:

LIPINSKI'S RULE

1. Less than five hydrogen bond donors
2. Less than ten hydrogen bond acceptors
3. Molecular mass less than 500 Da
4. High lipophilicity (expected as LogP not over 5)
5. Molar refractivity should be between 40-130.

Table no. 7: LIPINSKI'S RULE

Formula	C33H42N4
MLogP	>4.15
MW	≤500
N or O	≤10
NH or OH	≤5

MOLECULAR DOCKING

LIGAND PREPARATION

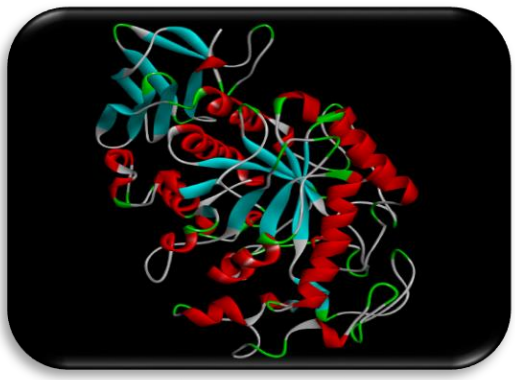
The ligand which is isolated from the *Oldenlandia auricularia* was downloaded from PubChem in sdf format and converted to pdb format using OpenBabelGUI. The following chemical compound is given in figure no.8.

Auricularine

Figure no. 8: Prepared ligand

PROTEIN (RECEPTOR)

The Proteins or the target receptors are downloaded from the Protein data bank (PDB) and it's visualized in Molegro molecular viewer (MMV) its then processed by removing the water molecules, co-factors and native ligands. The processed proteins are visualized in BIOVIA-Discovery studio visualizer (Figure no. 9)



4w93

Figure no. 9: Processed protein

PROTEIN-LIGAND INTERACTIONS:

ALPHA AMYLASE

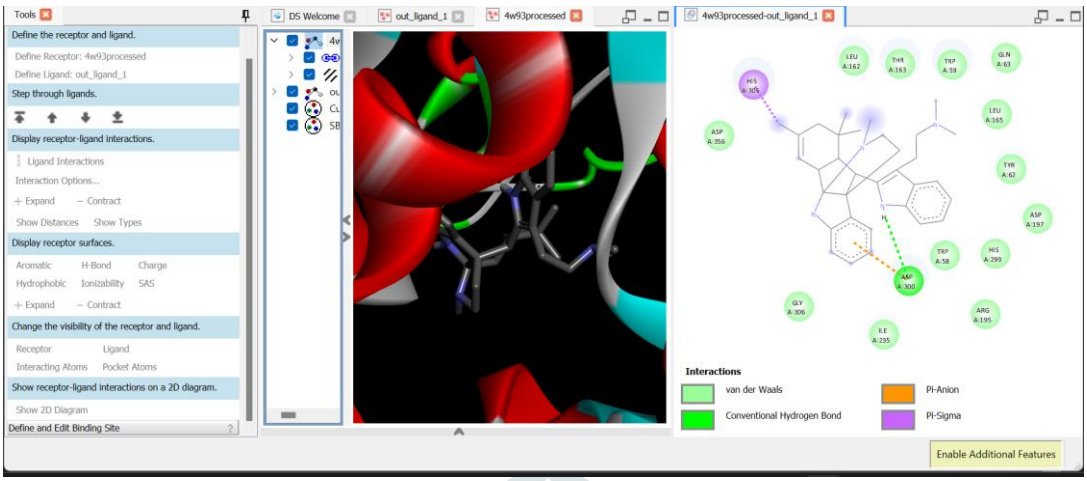


Fig. no. 10. Alpha amylase binding energy with amino-acid interactions

Table no. 8: Alpha amylase binding energy with amino-acid interactions

Type	Protein	Ligand	Binding energy	Amino acid interactions
Sample	Alpha amylase (4w93)	Auricularine	-9.2	ILE A:235, HIS A:305, LEU A:162, LEU A:165, ASP A:197, TYR A:62, ASP A:300, GLU A:233
Standard	Alpha amylase (4w93)	Acarbose	-7.1	ARG A:195, GLN A:63, TRP A:59, HIS A:305, ASP A:300.

According to Autodock results (binding scores) obtained, Acarbose shows the score -7.1, whereas the score of Auricularine was to be -9.2. Compared to standard drug, the plant constituent (Auricularine) showed better binding with the target.

CONCLUSION

This research visualized successful application of chromatography techniques for the isolation of biologically active secondary metabolites from plant sample, it was thoroughly, investigated for its physiochemical characters and phytochemical investigation of *Oldenlandia auricularia* leaves. The screening of antidiabetic activity was carried out by using *in-vitro* showed the activity present in the chloroform extract of *Oldenlandia auricularia* leaves. & *In-silico* shows the good binding affinity of the auricularine present in *Oldenlandia auricularia* leaves. It can be concluded that the leaves contain ligand, which was having antidiabetic activity, the auricularine was showing better binding affinity towards the target receptor.

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