International Journal of Applied Pharmaceutics

ISSN- 0975-7058 Vol 13, Issue 3, 2021

Original Article

# MOLECULAR DOCKING STUDIES OF ISOLATED COMPOUNDS ANDROGRAPHOLIDE AND BETULIN FROM METHANOLIC LEAVES EXTRACT OF *ANDROGRAPHIS ECHIOIDES* AS ALPHA-AMYLASE AND ALPHA-GLUCOSIDASE ACTIVATORS

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*Received: 04 Sep 2020, Revised and Accepted: 08 Mar 2021*

**ABSTRACT**

**Objective:** The purpose of this study is to isolate and characterize the andrographolide and betulin from methanolic leaves extract of *Andrographis echioides* and also used to evaluate the alpha-amylase and alpha-glucosidase inhibitory activity of isolated compounds using *in silico* docking studies.

**Methods:** The isolation was done using column chromatography using gradient mobile phase. Structural elucidation was carried out on the basis of spectral analysis. In this view, andrographolide and betulin were prepared for the docking evaluation. *In silico* docking studies were carried out using a recent version of Auto Dock 4.2, which has the basic principle of Lamarckian genetic algorithm.

**Results:** On the basis of the spectral data, the compounds have been established as andrographolide and betulin are being reported from this plant for the first time. The result showed that the andrographolide showed a binding affinity for amylase: (-7.9 kcal/mol) and for glucosidase (-7.2 kcal/mol) while betulin showed (-8.6 kcal/mol) and (-5.2 kcal/mol), respectively.

**Conclusion:** Therefore, it is suggested that isolated compounds andrographolide and betulin contributed excellent α-amylase and α-glucosidase inhibitory activity because of its structural parameters. Thus, these isolated compounds can be effectively used as drugs for treating diabetes which is predicted on the basis of docking scores.

**Keywords:** Andrographolide, Betulin, Binding energy, *Andrographis echioides,* Leaves


DOI: https://dx.doi.org/10.22159/ijap.2021v13i3.39641. Journal homepage: https://innovareacademics.in/journals/index.php/ijap

## INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder and it also affects the metabolism of carbohydrates, protein and fat. The main reason is the production of low amount of insulin by the pancreas [1]. Type I diabetes occur due to low amount of insulin production by **β**-cells, while type II diabetes occur due to **β**-cell dysfunction [2]. The enzymes alpha-glucosidase are responsible for the breakdown of oligo-and disaccharides to monosaccharides. α-amylase and α-glucosidase inhibitors is useful for lowering the process of glucose absorption and decreases glucose level in blood [3]. Diabetes mellitus patients suffer with high level of sugar in blood, unusual thirst, frequent urination, extreme hunger and loss of weight, blurred vision, nausea and vomiting, extreme weakness and irritability, tiredness and mood change [4]. Inhibitors of amylase and  glucosidase responsible for the high amount of glucose in the blood [5]. Now day's herbal medicines are more effective than synthetic medicines. There is no side effect while using herbal medicine [6]. *In silico* studies are computer-oriented programming analysis which predicts, with reasonable accuracy, the results that are expected from actual experimental measurements in less time and much lower cost. Such studies are used to enhance our understanding of biological processes and protein-ligand interactions. In recent years, computer-aided studies are widely used in pharmaceutical industries in the drug discovery exercise to identify or optimize lead compounds with significant activity against biological receptors.

*Andrographis echioides* is belonging to the family of Acanthaceae and its tamil name is Gopuramthangi [7]. It is an ayurvedic herb plant used in the treatment of many ailments such as anti-inflammatory, anti-arthritic, antimicrobial, anti-ulcer, anti-oxidant activity, hair problems, etc., [8]. Their phytochemical constituents like flavonoids, tannins, phenol, glycosides, terpenoids, saponins, steroids, etc., [9]. Some anti-diabetic plants have been reported to possess triterpenes as their bioactive principles [10, 11]. Terpenoid, andrographolide-lipoeic acid conjugate has demonstrated that hypoglycemic potentials [12]. The anti-hyperglycemic action of andrographolide was investigated in streptozotocin-induced diabetic rats. Oral treatment of andrographolide decreased the plasma glucose concentration of streptozotocin-diabetic rats in a dose-dependent manner. Similar treatment with andrographolide also decreased the plasma glucose in normal rats [13]. Numerous studies have demonstrated that the betulin is a lupane-type compound, characterised by isopropylidene group and five-membered ring and elicits a broad range of biological and pharmacological properties, including antifungal, antibacterial and antiviral activities [14]. The molecular docking methods were developed with a purpose of acquiring a large amount of compounds are docked against one target molecule and also used for the detection of new lead compounds or to reproduce an experimental conformation at elevated accuracy for the justification with experimental data [15]. Therefore, in the present study is to predict the *in silico* evaluation of α-amylase and α-glucosidase inhibitory activity and the stereochemistry binding of the isolated compound andrographolide and betulin on α-amylase and α-glucosidase has been carried out, which may helpful in the development of potent α-amylase and α-glucosidase inhibitors.

## MATERIALS AND METHODS

### Collection of plant material

The leaves of *Andrographis echioides* were collected in the month of May from the Mullipatti, Pudukkottai, Tamil Nadu, India. The plant was identified and leaves of *Andrographis echioides* were authenticated and confirmed from Dr. S. John Britto, Director, Rapinat herbarium, St. Joseph College, Tiruchirappalli, and Tamil Nadu for identifying the plants. The voucher specimen number SGP001 (7.06.2017).

### Preparation of methanolic extract

The methanol extracts were prepared by soaking 1.5 kg of the dried powder plant materials in 3 L of methanol by using a soxhlet extractor for 10 hr continuously. The extracts were filtered through Whatman filter paper No. 42 (125 mm). The filtered extract was

concentrated and dried by using a rotary evaporator under reduced pressure. The final dried samples (998 g) were stored in labeled sterile bottles and kept at-20 °C [16].

**Isolation of phytochemical compounds by column chromatography**

The condensed methanolic extract of leaves (998 g) of the sample was subjected to column chromatography over TLC grade silica gel. The preparation of solvent systems used to obtain andrographolide (154 mg/998g) ethyl acetate: methanol (70:30v\v) from fractions 15 and betulin (578 mg/998g) ethyl acetate: methanol (80:20v\v) from fractions 13. The isolated compounds were detected on TLC plates by spraying with Libermann-Burchard reagent and heated at 100 °C for 10 min [17].

**Purification of isolated compounds by High-performance liquid chromatography**

The analytical HPLC system (Shimadzu) was equipped with a diode array detector, a 20 µl loop, 200 x 4.6 mm C18 column, methanol (HPLC grade, 0.2 mm filtered) used as a mobile phase. The isolated Andrographolide compounds were separated using a mobile phase of chloroform: methanol (70:30 v/v) at a flow rate of 1.0 ml/min, column temperature 30 °C. Injection volume was 40 µl and detection was carried out at 346 nm. The isolated betulin compounds were separated using a mobile phase of n-hexane: ethyl acetate (8:2 v/v) [18].

**Structural elucidation study of isolated compound**

Different spectroscopic methods including UV, $^{1}H$ NMR, $^{13}C$ NMR were used to elucidate the structure of isolated compounds. The UV–visible spectrum of the isolated compounds in methanol was recorded using a Shimadzu 160A UV–visible spectrophotometer. $^{1}H$ and $^{13}C$ NMR spectra were acquired on Bruker WP 200 SY and AM 200 SY instruments ($^{1}H$, 200.13 MHz; $^{13}C$, 50.32 MHz) using TMS as internal standard and $CDCL_3$ as solvent. GC-MS analysis of the methanolic extract was performed using a Perkin-Elmer GC Clarus 500 system and Gas chromatograph interfaced to a Mass spectrometer (GC-MS) equipped with a Elite-I, fused silica capillary column (30 mmX0.25 mm 1D X 1 µMdf, composed of 100% Dimethyl polysiloxane) [19, 20].

**Molecular docking studies on α-amylase and α-glucosidase inhibitory activity with andrographolide and betulin**

To investigate the molecular association, three-dimensional structures of receptor and ligands have to be retrieved from the molecular graphics laboratory tools (MGL-1.5.6 version, Scripps Research Institute, Florida, FL, USA). In order to execute the docking, the protein and ligands were downloaded from the protein data bank and pubchem (http://www.rcsb.org/pdb). Preparation of protein and compounds were completed using dock prep in chimera (1.10.1 version UCSF Resources for biocomputing visualization and informatics, NIH, USA) and Compounds retrieved from pubchem database were optimized and converted to pdb format.

**Molecular docking**

Molecular docking analysis was carried out to predict the affinity between the two compounds, namely, betulin, andrographolide, against alpha-amylase and glucosidase using Auto Dock 4.2 program. Polar hydrogen atoms were individually added and merged to the protein structure. Kollman charges and solvation parameters were determined by default. For energy minimization, gasteigor charges were added along with rotatable and flexible bonds. Grid maps were assigned by co-crystallized ligands in the x, y and z-dimensions of 20 × 26 × 20 points were set to cover the active site of the protein. The Lamarckian Genetic Algorithm (LGA) was used to search for the lowest binding energy by implementing local minimization of the genetic algorithm to enable modification of the gene population. LGA parameters were set as follows: 100 search (docking) runs; population size of 150; 25,000,000 of energy evaluations; 27,000 numbers of generations; mutation rate of 0.02 and crossover rate of 0.8. Docking calculation was performed in the Auto Dock software 4.2 [21, 22].

**RESULTS AND DISCUSSION**

Structural elucidation of isolated compounds andrographolide and betulin from methanolic leaves extract of *Andrographis echioides*

**Structural Elucidation of isolated compound andrographolide from methanolic leaves extract of *Andrographis echioides***

The chemical name of andrographolide is 3*α*, 14, 15, 18-tetrahydroxy-5*β*, 9*β*H, 10*α*-labda-8, 12-dien-16-oic acid *γ*-lactone and its molecular formula and molecular weight are $C_{20}H_{30}O_5$ and 350.4 (C 68.54%, H 8.63%, and O 22.83%), respectively. The previous study suggested that the andrographolide is a major bioactive phytoconstituents found in various parts of *Andrographis paniculata* but particularly in the leaves [23].

The UV $\lambda_{max}$ value of compound andrographolide was 257 nm (fig. 1).

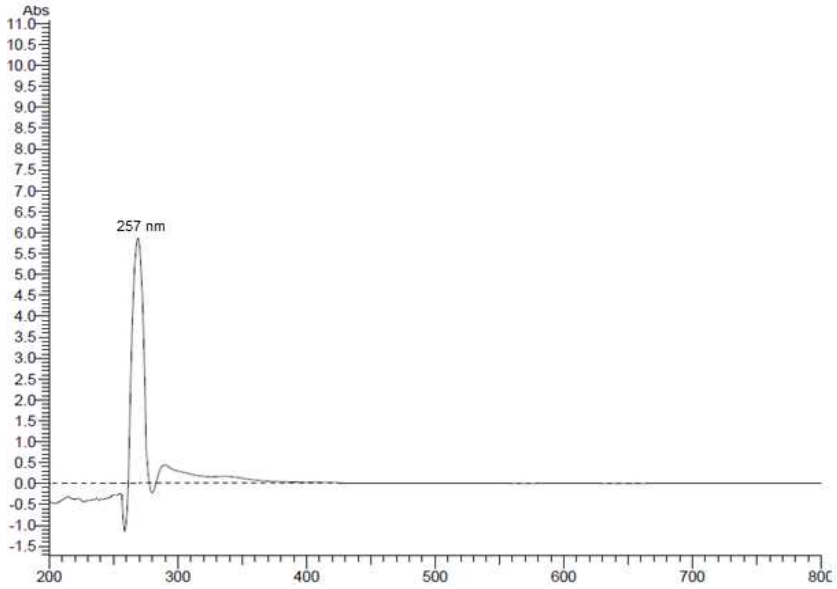


**Fig. 1: UV spectra for isolated compound andrographolide,**

**In the proton $^{1}H$ NMR spectra of andrographolide (fig. 2) showed δ 6.62 (1H, t, C12), 4.90 (1H, d, C14), 4-15-4.40 (2H, m, C-15), 2.492-2.499 (5H, m, C2, C7, C9), 0.65 (2X$CH_3$, s, C18, C20)**

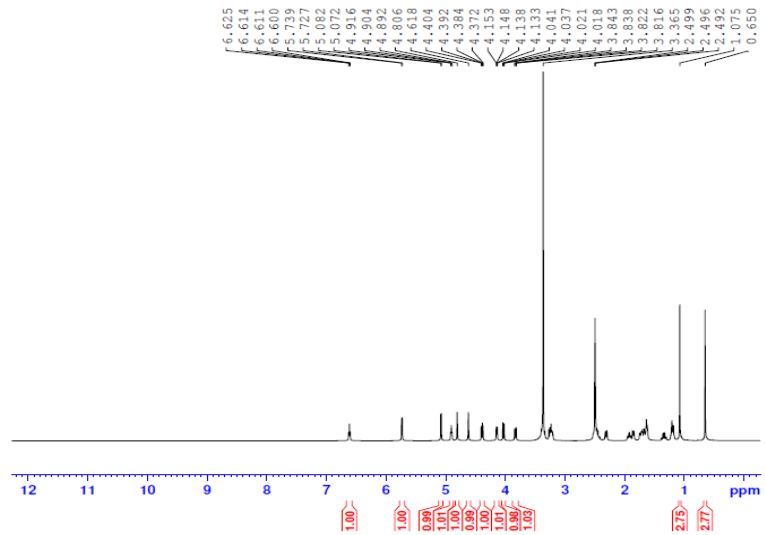


**Fig. 2: [1]H-NMR spectra for isolated compound andrographolide,**

**In the [13]C NMR spectra of andrographolide (fig. 3) showed 39.6 (C1), 28.3 (C2), 78.8 (C3), 40.2 (C4), 55.9 (C5), 24.42 (C6), 39.60 (C7), 148.0 (C8), 55.93 (C9), 39.60 (C10), 24.4 (C11), 146.8 (C12), 129.4 (C13), 64.9 (C14), 74.8 (C15), 170.4 (C16), 108.7 (C17), 23.5 (C18), 64.9 (C19), 15.2 (C20)**

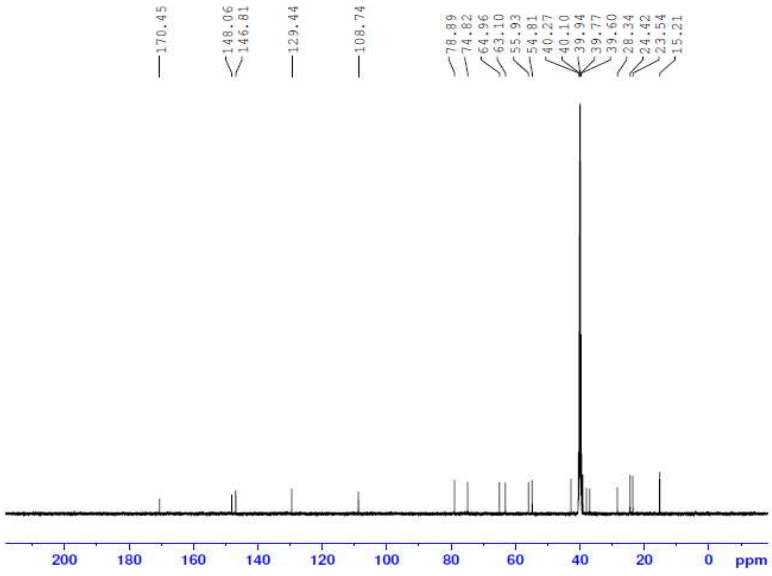


**Fig. 3: [13] C-NMR spectra for isolated compound andrographolide**

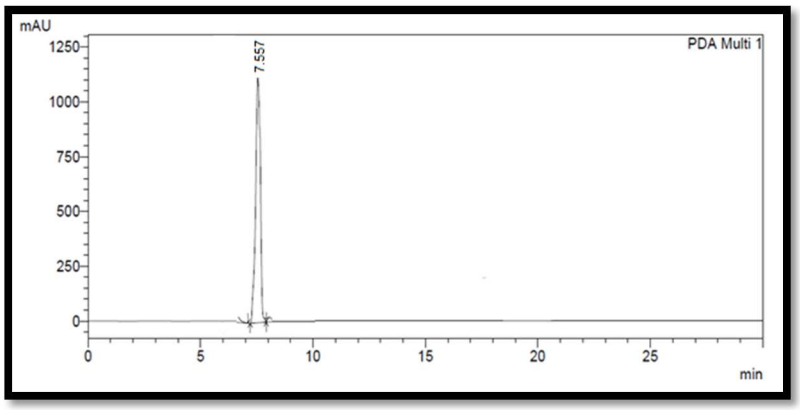


**Fig. 4: HPLC spectra for isolated compound andrographolide**

The isolated compound obtained is colourless solid with melting point range of 228 °C-238 °C. This was further supported by $^{13}C$ NMR spectral analysis, which displayed 20 signals for all carbon atoms in the molecule, including one carboxyl, four methyl's, eight non-protonated carbons, two methylene's, three methane's, one cyclic alkene, and one benzylic carbon atom. This corresponds to a similar report by (Koteswara Rao *et al.*, 2004) [24], suggesting the isomer to be similar to andrographolide ($C_{20}H_{30}O_5$). However, the 1H NMR spectrum had several signals comparable to that of 13C NMR of the isolated compound Viz are methylene signalling at (δ 6.62, t), cyclic alkene (δ 4.90, s) and carboxyl (δ 4.40, s). Thus, the strong similarities to andrographolide as described by (Du *et al.*, 2003) [25].

**Purification of an isolated compound by HPLC**

The Retension time of andrographolide isolated from the methanolic Leaves extract of *Andrographis echioides* was about 7.557 was shown by HPLC peak (fig. 4).

Mass spectrum of isolated compound andrographolide showed parent molecular ion [$M^+$] peak at mlz 350.4 g/mol, which corresponds to the molecular formula $C_{20}H_{30}O_5$. The GCMS spectra of these isolated compounds revealed the characteristic fragments m/z with % abundance 350.65, 301.17, 283.30, 272.19, 256.27, 254.27, 240.09, 229.95, 214.00, 211.98, 199.99, 186.05, 171.98, 159.91, 145.86, 133.80, 117.72, 105.65, 91.56, 81.5, 79.51, 71.49, 67.41. The molecular weight and fragmentation pattern indicate that the compounds presenting andrographolide, respectively (fig. 5).

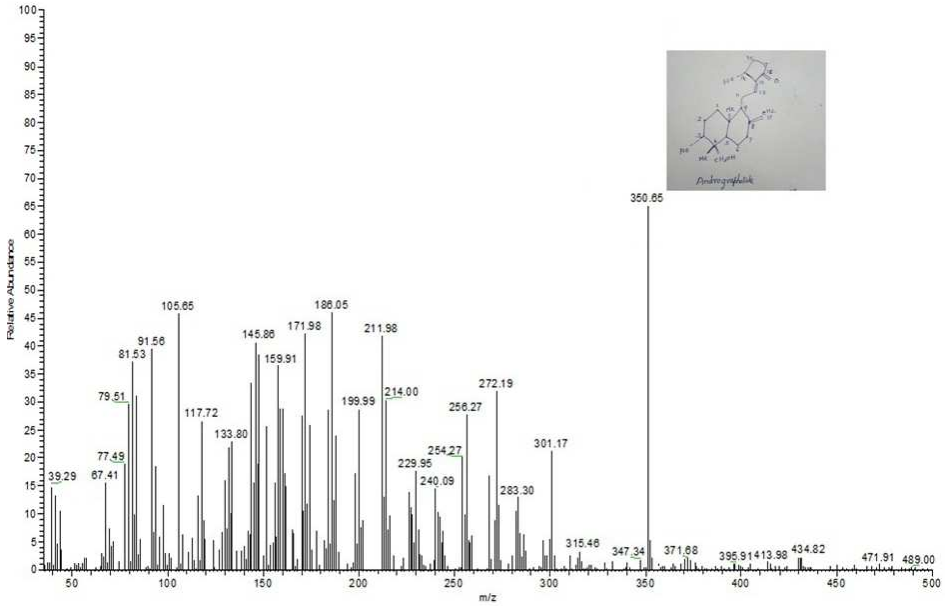


**Fig. 5: Mass spectra for the isolated compound andrographolide**

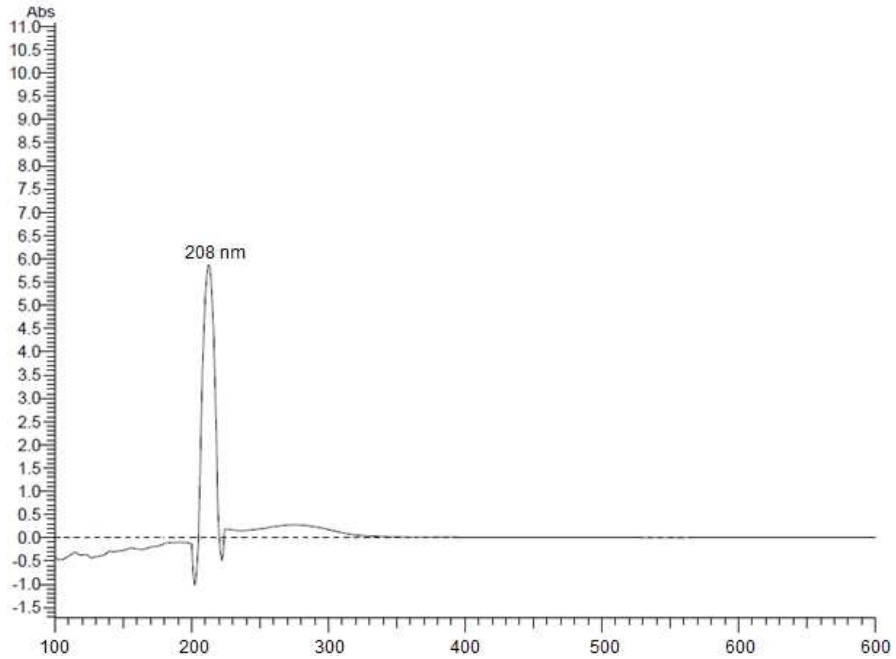


**Fig. 6: UV spectra for isolated compound betulin,**

**In the proton $^1H$ NMR spectra of betulin (fig. 7) showed *δ* 4.70 (1H, d, H-29b), 4.58 (1H,d, H-29a), 3.79 (1H, d, *J* = 10.8, H-28b), 3.33 (1H, d, *J* = 10.8, H-28a), 3.18 (1H, dd, *J* =5.3, H-3*α*), 1.67 (3H, s, H-30), 0.99 (3H, s, H-27), 0.97 (3H, s, H-26), 0.96 (3H, s, H-23),0.80 (3H, s, H-25), 0.75 (3H, s, H-24)**

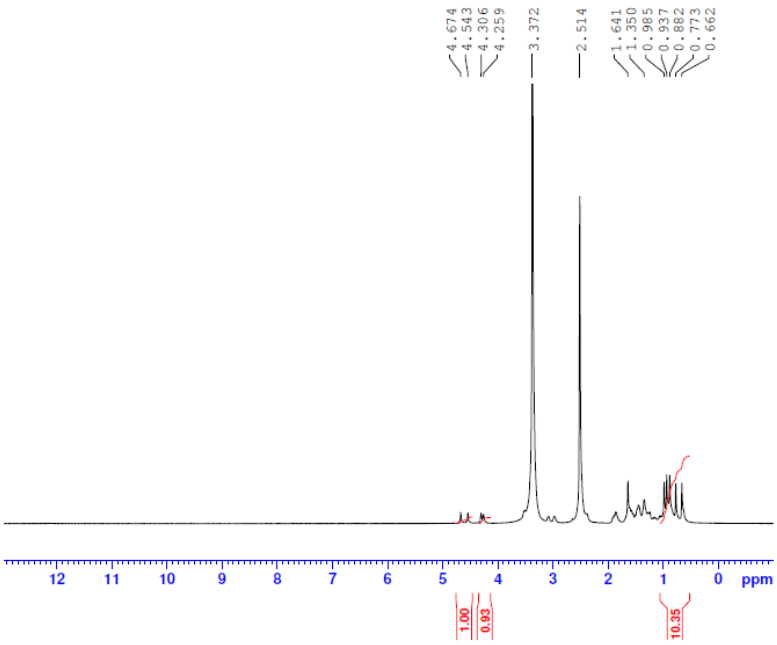


**Fig. 7: $^{1}$H-NMR for isolated compound betulin,**

**In the $^{13}$C NMR spectrum of betulin (fig. 8) showed $\delta$ 150.25 (C-20),109.42 (C-29), 79.02 (C-3), 76.60 (C-28), 55.29 (C-5), 50.43 (C-9), 48.29 (C-19), 48.29 (C-17),48.00 (C-18), 42.84 (C-14), 40.83 (C-8), 40.01 (C-1), 38.70 (C-4), 38.04 (C-10), 37.17 (C-13),35.56 (C-7), 34.29 (C-22), 29.83 (C-21), 29.83 (C-16), 27.99 (C-23), 27.99 (C-2), 27.44 (C-15),25.10 (C-12), 20.93 (C-11), 19.31 (C-30), 18.32 (C-6), 16.13 (C-25), 15.98 (C-26), 15.38 (C-24),14.55 (C-27)**

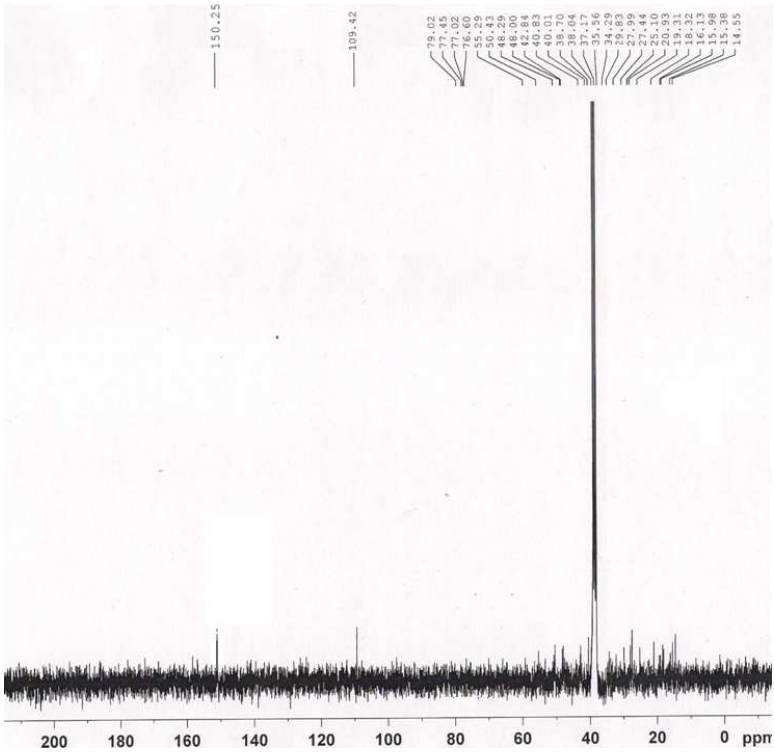


**Fig. 8: $^{13}$C-NMR spectra for isolated compound betulin**

The 1H NMR shows the presence of a doublet of doublets was present at $\delta$ 3.372 ppm, which is characteristic of an $\alpha$-oriented hydrogen at C-3 of a 3$\beta$-hydroxy triterpene. Doublets for geminal protons at $\delta$ 4.674 and 4.543 ppm, along with the methyl group at $\delta$ 1.641 ppm, suggests that 1 was a lupeol-type triterpene derivative. Another pair of doublets at $\delta$ 3.37 and 2.514 ppm, rather than a seventh methyl singlet around $\delta$ 0.882 ppm, confirms the presence of a second hydroxyl group at C-28. The $^{13}$C NMR spectrum further established 1 as a lupeol-type triterpene derivative. The characteristic pair of sp2 carbons comprising the double bond of betulin was observed as shifts at $\delta$ 150.25 and 109.42 ppm [27]. Oxygenated carbon shifts for C-3 and C-28 were observed at $\delta$ 79.02

and 55.29 ppm, respectively. In all, the spectra revealed a compound with six methyl groups, thirtycarbon atoms (which is equivalent to the total number of carbon atoms in triterpenoid), a lupene-type triterpenoidal nucleus with two hydroxyl groups at C-3 and C-28 (a lupeoltype triterpene). Consequently, the compound was determined to be the known structure, 20(29)-lupene-3, 28-diol, more commonly known as betulin. Experimental NMR data was compared to that reported in the literature [28].

**Purification of an isolated compound by HPLC**

The Retension time of betulin isolated from the methanolic leaves extract of *Andrographis echioides* was about 10.293 was shown by HPLC peak (fig. 9).

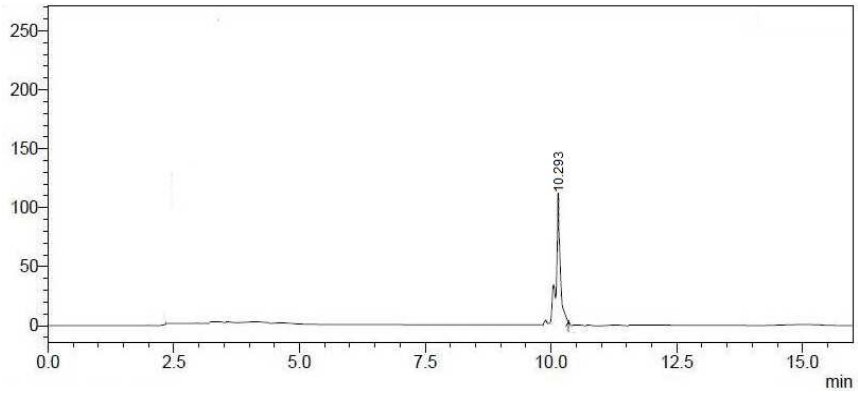


**Fig. 9: HPLC spectra for isolated betulin**

Mass spectrum of isolated compound betulin showed parent molecular ion [M+] peak at mlz 442.72 g/mol which corresponds to the molecular formula $C_{30}H_{50}O_2$. The GCMS spectra of these isolated compounds revealed the characteristic fragments m/z with % abundance 441.96, 406.16, 401.32, 83.35, 358.28, 341.96, 327.96, 316.07, 281.86, 257.80, 255.87, 253.78, 231.84, 21.77, 209.83, 207.66, 191.55, 173.70, 161.64, 159.64, 147.58, 135.55, 119.51, 105.47, 91.42, 7.36, 47.23, 45.23. The molecular weight and fragmentation pattern indicate that the compounds presenting betulin respectively (fig. 10).

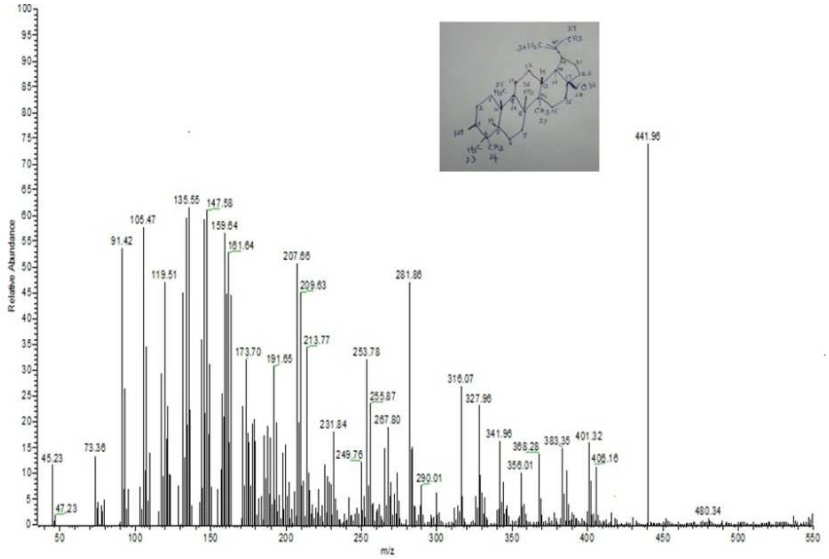


**Fig. 10: Mass spectra for the isolated compound betulin**

**Molecular docking analysis of isolated compounds andrographolide and betulin on alpha-amylase and alpha-glucosidase**

Docking of protein and small molecules is a method to distinguish the importance of atoms exchange. Major forces in molecular modeling and docking are hydrogen, hydrophobic and Vander waals interactions. In the primary analysis, active site or binding site identification is significant and challenging task. Basically, two methods are preferred in identifying active site; sequence similarity and co-crystallization. In recent era, there are many algorithms developed for probing active sites in particular protein structure and sequence. In this study, co-crystallized ligands were chosen to explore binding site.

In fig. 11, docked pose of α-amylase enzyme with the andrographolide clearly demonstrated the binding positions of the ligand with the enzyme. The potential binding sites of the andrographolide (fig. 11a) was found to be TRP 58, GLN 62, THR 162, LEU 164, HIS 100, TYR 61, HIS 298, LEU 161, ASP 196, ALA 197, GLU 232, ARG 194, ASN 297, ASP 299, ILE 234. The potential binding sites of the betulin (fig. 11b) were found that TRY 150, THR 162, HIS 200, TRP 58, TYR 61, TRP 57, ASP 196, ARG 194, GLU 22, ALA 306, LYS 199. It proves that the ability of inhibiting the α-amylase enzyme by the selected ligands.

In the (fig. 12) docked pose of α-glucosidase enzyme with the andrographolide clearly demonstrated the binding positions of the ligand with the enzyme. The potential binding sites of the

andrographolide (fig. 12a) was found to be LEU 678, PHE 649, ASP 616, HIS 674, TRP 61, ILE 441, LEU 405, MET 519, SER 676. The potential binding sites of the betulin (fig. 12b) was found that LEU 671, SER 676, TRP 376, PHE 649, TRP 481, PHE 525, LEU 678, ARG 411. It proves that the ability to inhibit the α-glucosidase enzyme by the selected ligands.

Binding energy of the individual compounds was calculated by using the following formula, Binding energy = A+B+C-D where A denotes final intermolecular energy+van der Walls energy (vdW)+hydrogen bonds+desolvation energy+electrostatic energy (kcal/mol), B denotes final total internal energy (kcal/mol), C denotes torsional free energy (kcal/mol), D denotes unbound system's energy (kcal/mol).

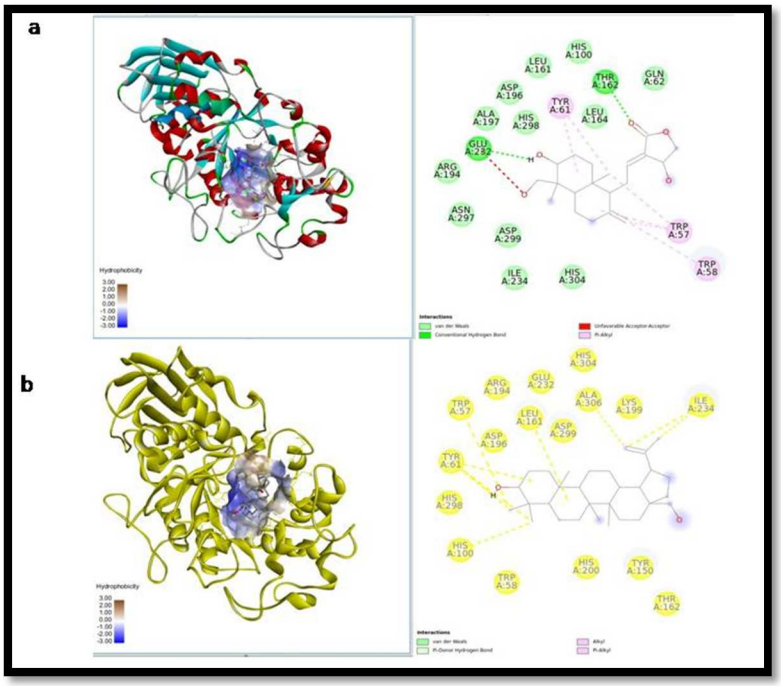


**Fig. 11: 3D-schematic interactions pose of amylase with (a) andrographolide and (b) betulin**

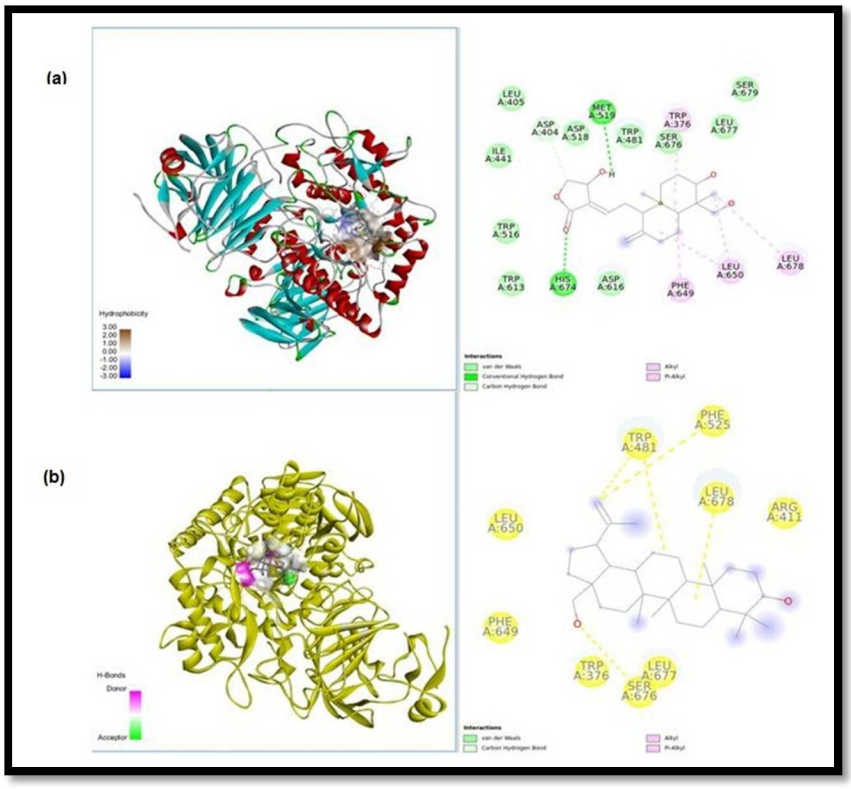


**Fig. 12: 3D-schematic interactions pose of glucosidase with (a) andrographolide and (b) betulin**

**Table 1: Alpha-amylase and alpha-glucosidase protein with betulin and andrographolide were docked for predicting binding affinity by autodock in MGL tools**

| Protein-ligand | Compounds | Binding energies of the compounds based on their rank (kcal/mol) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Amylase-andrographolide | Andrographolide | -7.9 | -7.9 | -7.8 | -7.5 | -7.5 | -7.5 | -7.4 | -7.1 |
| Amylase-betulin | Betulin | -8.6 | -8.2 | -7.7 | -7.7 | -7.6 | -7.5 | -7.4 | 7.3 |
| Glucosidase-andrographolide | Andrographolide | -7.2 | -6.6 | -6.2 | -6 | -6 | -6 | -5.8 | -5.7 |
| Glucosidase-betulin | Betulin | -5.2 | -5.1 | -4.8 | -4.6 | -4.4 | -4.1 | -3.9 | -3.8 |

Docking and prediction of binding affinity is presented in table 1. Affinity values predicted was clearly describing the best fit among alpha-amylase and glucosidase. Andrographolide has been showing a binding affinity for amylase: -7.9 and glucosidase e: -7.2 while betulin was showing 8.6 and 5.2, respectively. Binding affinity among the complex structures was showing the competitive and noncompetitive inhibition method. Mode of interactions is auspicious that hydrogen donor and acceptor playing a vital role and key aspect in molecular rendering. Specific amino acids in the catalytic site are clearly explaining the bonded and non-bonded interaction. Based on the *in silico* evaluation and stereochemistry binding of the isolated compounds, andrographolide possess potential α-amylase and α-glucosidase inhibitory excellent binding sites when compared to that of the betulin. This may be attributed due to the differences in the position of the functional groups in the compounds [29, 30].

## CONCLUSION

Andrographolide and betulin isolated and characterized from the methanolic leaves extract by using column chromatography and purification were confirmed by high-performance liquid chromatography for the first time reported in this study. In conclusion, the results of the present study clearly demonstrated that Isolated compounds andrographolide and betulin have excellent binding sites and interactions with α-amylase and α-glucosiade. As a result, it can be concluded that the Isolated Compounds have inhibitory activity against α-amylase and α-glucosidase enzymes and this therapeutic potentiality could be exploited in the management of postprandial hyperglycemia in the treatment of Type-II diabetes mellitus.

## ACKNOWLEDGEMENT

S. G acknowledges Dr. S. John Britto, Director, Rapinat herbarium, St. Joseph College, Tiruchirappalli, Tamil Nadu for identifying the plants. S. G acknowledges Assistant Professor, DR. L. Cathrine of Holy Cross College, Tiruchirappalli, Tamil Nadu for constant support for this research.

## FUNDING

Nil

## AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

## CONFLICTS OF INTERESTS

The authors declare that they have no conflict of interest. It has not been published elsewhere. That it has not been simultaneously submitted for publication elsewhere. All authors agree to the submission to the journal.

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