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# Bio-Assay Guided Fractionation and Isolation of α-Glucosidase Inhibitory Constituents from *Dillenia indica* L. Fruit Extracts

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***Abstract:*** *The plant Dillenia indica Linn. is used for the treatment of diabetes by the rural community of Dhemaji district of Assam, by the Khamptis "Chauya" traditional healers and Arunachal Himalaya, northeast, India. The plant is also reported to possess anti-diabetic and hypolipidemic activities in animal models. Based on the traditional uses and reported scientific contribution the plant was selected for the present investigation to study its α-glucosidase targeted action. The objective of the present study was to investigate the α-glucosidase inhibitory assay guided fractionation and isolation of phytoconstituents from D.Indicia fruit extracts. In the present investigation the methanolic fruit extract was prepared. The ethyl acetate fraction (EAF) from the methanol extract has shown a significant α-glucosidase enzyme inhibition activity ($IC_{50}$ 262.76 µg/ml). Total six constituents were isolated from different fractions and they were characterized and screened for α-glucosidase inhibition activity. Compound 2 (masilinic acid) was found to exert maximum $IC_{50}$ (272.634 µg/ml) followed by compound 6 ($IC_{50}$ 669.8 µg/ml). Docking studies have revealed that masilinic acid binds through H-bonds to amino acid residues of α- glucosidase. Enzyme kinetics of masilinic acid has shown the probable chance of competitive binding to α-glucosidase. From the data it was concluded that the masilinic acid has exhibited the maximum inhibitory activity against α-glucosidase and it was the first report about the α-glucosidase inhibitory activity of D.Indica fruits.*

***Keywords:*** *α- Glucosidase inhibition, Compound 6, Dillenia indica, Docking studies, Masilinic acid.*

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## I. Introduction

One therapeutic approach to treat diabetes is to retard the absorption of glucose via inhibition of enzymes like α-glucosidase in the digestive organs. [1] α-Glucosidase inhibitors constitute one of the major classes of anti-diabetic drugs used for the prevention of postprandial hyperglycemia and are used in combination with other drugs to increase the efficacy of the treatment. [2-3] The first clinically used drug under this class was a natural product (Acarbose) and other drugs used are also based on natural products, but these are not entirely free from side effects associated with GIT. Therefore, new leads with α-glucosidase inhibitory potential are required and plant extracts are known to possess many therapeutic properties including α-glucosidase inhibition. [4]

The fruits of plant *Dillenia indica* Linn (*D. indicia*) have been used for the treatment of diabetes by the rural communities of various parts of India. [5-6] *D.indica* (Family: *Dilleniaceae*) is commonly called "Elephant tree". For many years the juices of *D. indica* leaves, bark, and fruits were mixed and given orally for the treatment of cancer and diarrhea. [7] Fruits and leaf extracts of *D. indica* were found to have antioxidant activity, [8] CNS depressant activities, [9] and anti-inflammatory activity [10] in mice. Traditionally, the plant has been used for treatment of diabetes. [11] The methanolic leaves extract of the plant shows antidiabetic activity in alloxan and streptozotocin induced diabetes. [12] As there is no previous report on antidiabetic activity of fruits, the present study focuses on bio-assay guided fractionation and isolation of α-glucosidase inhibitory constituents from *D. indica L.* fruits.

## II. Materials And Methods

α-Glucosidase (Maltase, EC 3.2.1.20), and p-nitrophenylα-D-glucopyranoside were purchased from SRL Mumbai, India. $Na_2HPO_4$, $NaH_2PO_4$, DMSO, $Na_2CO_3$, Silica gel (Merck, 100-200 mesh) and other chemicals and solvents were of analytical grade and purchased from Merck, Mumbai, India. Milli-Q water (Milli-Q Academic with 0.22 µm Millipak(R) 40 was used for all the enzymatic assays. Acarbose was purchased from Sigma.

**2.1** ***Selection of plant***

Based on the traditional uses and reported scientific contributions the plant *D. indica* was selected for the present investigation.

***2.2 Collection of plant material***

The frutis of *D. indica* were purchased from the local market and were authenticated by the Botanical Gardens, Howrah, India.

***2.3 Extraction and Fractionation***

The fruits were cut into small pieces and air-dried at room temperature (25-28$^0$C). The dried fruits (calyx) of *D. indica* (500g) were extracted with methanol for 72 hrs at room temperature. The whole extract was filtered and the solvent was evaporated under vacuum at 40-45$^o$C. The step was executed thrice to afford 185g crude (yield 37%) methanol extract (ME). 150g of ME was then suspended in water and partitioned successively with ethyl acetate and n-butanol. Some portion remains insoluble which was collected separately. Each fraction was evaporated under vacuum to yield the residues of ethyl acetate fraction (EAF) 42g, n-butanol fraction (NBF) 38g, aqueous (AF) 50g and insoluble fractions 20g respectively. The ME, EAF, NBF and AF were stored at 4$^o$C until further analysis.

***2.4 Isolation of Phytoconstituents***

A part of EAF (11 g) was subjected to column chromatography on silica gel (110 g) using a step gradient of petroleum ether (1 L, fr-1), petroleum ether:$CHCl_3$ (1:1 v/v, 1 L, fr-2), $CHCl_3$ (1 L, fr-3), and $CHCl_3$: MeOH (9:1, v/v, 1 L, fr-4; 8:2, v/v, 1 L, fr-5; ; 7.5:2.5 v/v, 1 L, fr-6; 7:3 v/v, 1 L, fr-7) to get a total of 7 fractions. Based on TLC, Fr-4 was further eluted with increasing polarity of solvents (petroleum ether, $CHCl_3$ and MeOH) to obtain a solid fraction. This fraction gave a bright reddish color spot on TLC with Liebermann Burchard reagent and was subjected to crystallization using $CHCl_3$ and MeOH to afford off white solid; supernatant on TLC analysis showed one minor spot. The supernatant part was further subjected to silica gel column chromatography to afford a white solid.

In a similar fashion a portion of NBF (11 g) was eluted with solvents of different polarities- petroleum ether (1 L, fr-1) petroleum ether: $CHCl_3$ (1:1 v/v, 1 L, fr-2), $CHCl_3$ (1 L, fr-3), and $CHCl_3$: MeOH (9:1, v/v, 1 L, fr-4; 8:2, v/v, 1 L, fr-5; ; 7.5:2.5 v/v, 1 L, fr-6; 7:3 v/v, 1 L, fr-7) to afford a total of 7 fractions. Fr-5 and fr-7 on further silica gel column chromatography resulted in isolation of compounds 6, 4 and 3 (figure1).

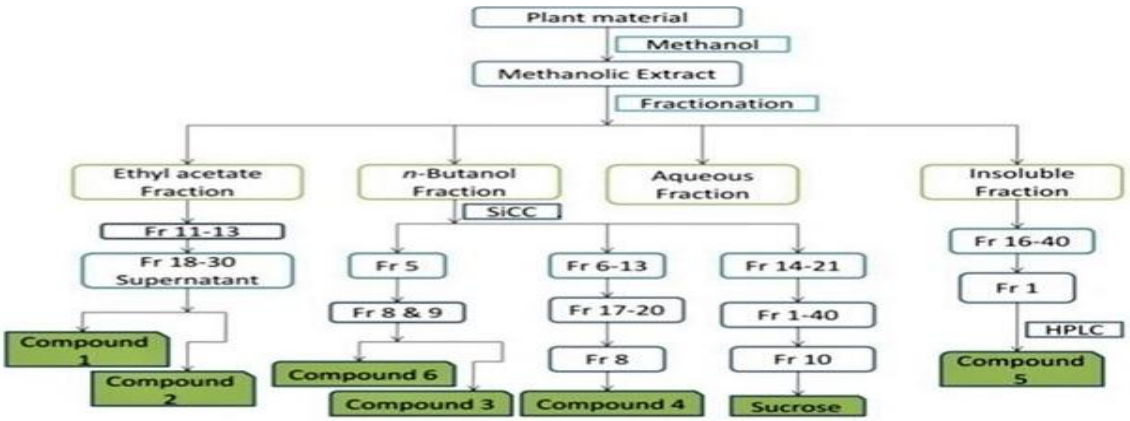


**Figure 1: Flow chart of extraction and isolation of compounds from *D. indica* fruits**

***2.5 Spectral data***

The isolated constituents were subjected for $^3$C-NMR, $^1$H-NMR and ESI-MS spectral analysis

Compound 1 (Betulinic acid) (3β)-3-Hydroxy-lup-20(29)-en-28-oic acid; +ve ESI-MS ion at m/z: 479.52 [M+Na]+ ( $C_{30}H_{48}O_3$)

$^{13}$C-NMR($CDCl_3$): δc 179.6 (s, -COOH) δc 78.9 (d, -$CH_2$OH) δc 110.8 (t, exocyclic double bond) (fig 2.1); $^1$H-NMR(CDCl3): δH 0.80, 0.91, 0.99, 1.04, 1.21 and 1.77(3H each, s). δH 4.93(Hα ,s, H-29), δH 4.75(Hβ,s, H-29), 3.44(1H, t, J = 8.1 Hz). Comparing the data with those reported earlier the compound was characterized as betulinic acid (2-α hydroxy oleanolic acid) [13-14] (figure 2)

Compound 2 (masilinic acid) (2α,3β)-2,3-Dihydroxyolean-12-en-28-oic acid; +ve ESI-MS ion at m/z: 495.41 $[M+Na]^+$ ( $C_{30}H_{48}O_3$)

$^{13}$C-NMR($CDCl_3$): 4.10 (m, H-2), 3.44(H-3 d, J = 9 Hz) 5.49 (brs , H-12) (Fig. 5.2), $^{13}$C-NMR ; 181.3 (s, -COOH), at δc 122.7 and 145.1(m, Ar), δc 84.0 (s,-$CH_2$OH), 68.9(s,-$CH_2$OH) ; $^1$H-NMR: 0.92-1.36(m, 7 -$CH_3$),

3.44 (1H, d, J = 9 Hz, H-3) ,4.10 (1H, m, H-2), 5.49 (1H, brs, H-12) (figure 3) . Comparing the data with those reported earlier the compound was characterized as masilinic acid (2-α hydroxy oleanolic acid) [15].
Compound 6; +ve ESI-MS ion at m/z: 321.04 $[M+Na]^+$. (fig 4) The $^{13}$C-NMR($CDCl_3$): $\delta_c$ 179.6-108.44(m, Ar), $\delta_c$ 86.1(hydroxylated carbon), 65.9( aliphatic carbon)(figure 4). The complete structure was not elucidated due to lack of compound.

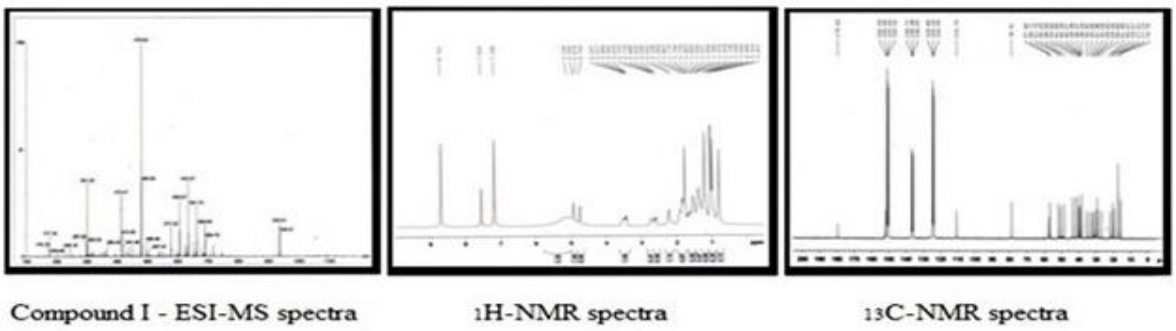


**Figure 2: Spectral data of Compound 1 (Betulinic acid)**

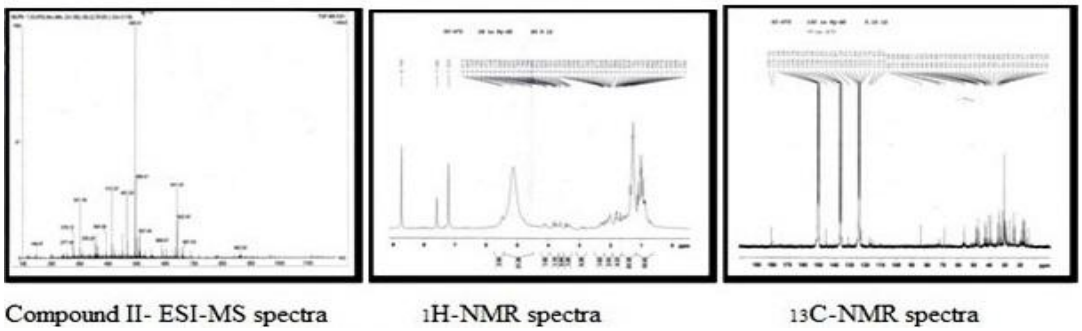


**Figure 3: Spectral data of Compound 2 (Masilinic acid)**

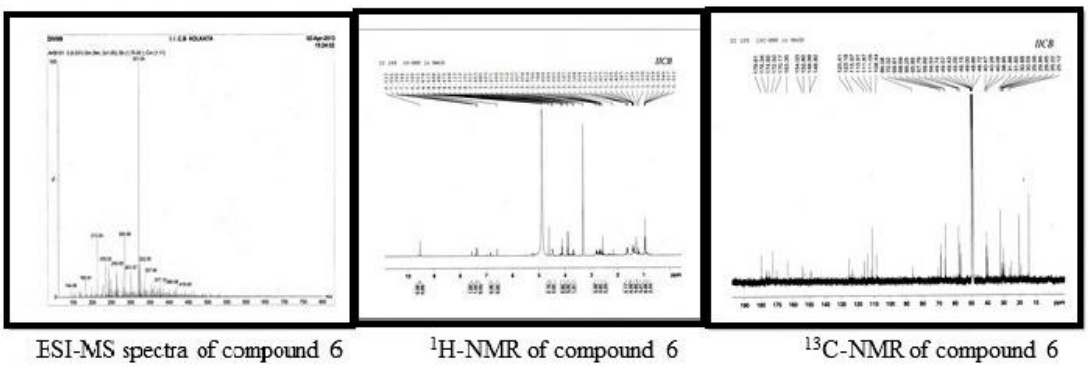


**Figure 4: Spectral data of Compound 6**

### *2.6 α-Glucosidase Inhibition Assay*

The α-glucosidase inhibitory assay was performed in a 96 well plate as reported earlier [16-17]. 25 µL of enzyme solution containing 0.5 U/ml was taken and incubated at 37 ±1ºC for 30 min, and then the reaction was terminated by the addition of 100 µL of 0.2 M sodium carbonate solution. The absorbance of the solution was measured at 405nm. Acarbose was used as standard. The uninhibited enzyme was taken as control, an appropriate DMSO control was used wherever applicable. Percentage inhibition of the enzyme was calculated compared to control. $IC_{50}$ values were calculated from concentration v/s percentage inhibition curves.
Percentage of α-glucosidase inhibition is calculated by

$$\text{The percentage inhibition} = \frac{(\text{OD of control} - \text{OD of Sample}) \times 100}{\text{OD of Control}}$$

***Statistical analysis*:** All the results were represented as mean ± standard error of mean (SEM).
***Docking studies:*** Docking was performed on windows 2007 using MOE 2008.10 version.

## III. Results And Discussion

The chemical structures of isolated compounds 1 and compound 2 were established after the interpretation of spectral data. The thin layer chromatography (TLC) study was performed for these two compounds. Chemical structures of compound 1 (betulinic acid) and compound 2 (maslinic acid) were established as shown in figure 5.

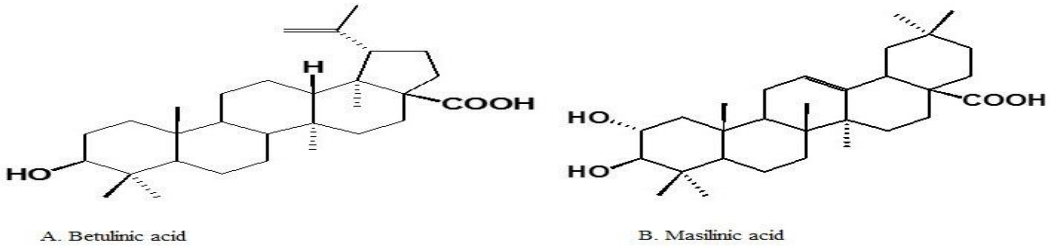


**Figure 5: A. Structure of Betulinic acid B. Structure of Masilinic acid**

### *3.1 α- Glucosidase Inhibition Assay*

In the present investigation, the fruit extract was evaluated for its potential to inhibit the enzyme α-glucosidase. The α-glucosidase inhibitory activity of leaf extracts of *D.indica* was previously reported [18] but this was the first report on fruit extracts. The fruit extract has shown significant enzyme inhibition with $IC_{50}$ 696.48 µg/mL (table 1). Subsequently, all the fractions were evaluated against the enzyme where EAF was found to exert maximum inhibition with $IC_{50}$ 262.76 µg/mL and n-Butanol fraction has shown inhibition of the enzyme with $IC_{50}$ 328.12 µg/mL. The aqueous fraction was found to exert non significant inhibition while activity testing was not performed for an insoluble fraction because of a solubility issue in the buffer. Thereafter the isolated compounds were tested for enzyme inhibitory potential and all the results were summarized in the table 1. Among these, compound 2 which is characterized as hydroxy oleanolic acid was found to exert maximum inhibition with $IC_{50}$ 272.634 µg/mL followed by compound 6 ($IC_{50}$ 669.8 µg/mL) while the major compound betulinic acid and other compounds were inactive against this enzyme. % Inhibition of all the test samples was compared in figure 6.

***"Table 1:** Inhibitory effect ($IC_{50}$) of Dillenia indica Linn fruit extract, fractions and isolated compounds on enzyme α-glucosidase"*

| Sample | $IC_{50}$ , µg/mL |
|---|---|
| Methanolic extract | 696.48±2.23 |
| Ethyl acetate fraction | 262.76 ±1.07 |
| n-Butanol Fraction | 328.12±0.76 |
| Aqueous Fraction | NI |
| Insoluble Fraction | NI |
| Compound 1 | NI |
| Compound 2 | 304.84±1.09 |
| Compound 3 | NI |
| Compound 4 | NI |
| Compound 5 | NI |
| Compound 6 | 669.8±0.98 |
| Acarbose | 9.67±0.82 |

*NI – No Inhibition. Values are expressed in mean±SEM

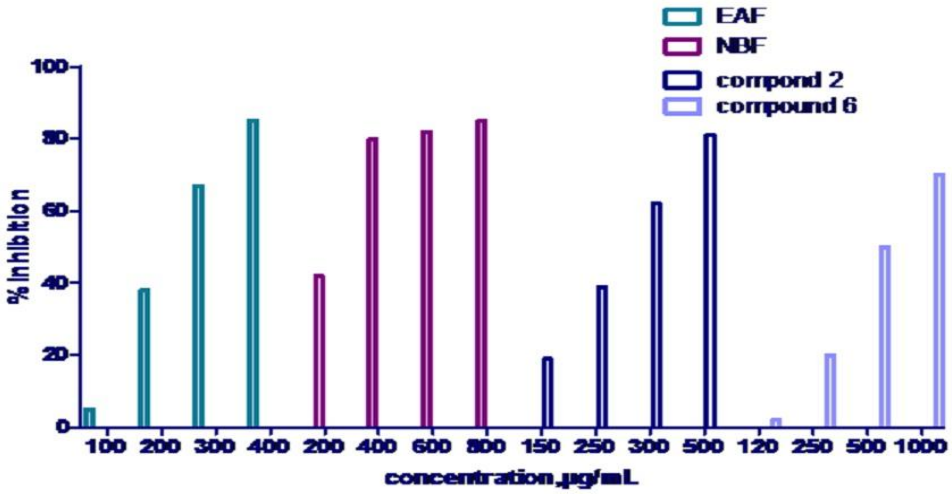


**Figure 6:** % Inhibition of Dillenia indica Linn fruit extract, fractions and isolated compounds on enzyme α-glucosidase

***3.2 Docking studies***

In the current research two isolated and structurally elucidated phytochemicals namely masilinic acid and betulinic acid were docked on the active site of α-glucosidase (*Saccharomyces cerevisiae*). Docking was performed on windows 2007 using MOE 2008.10 version. Molecular docking study helped us to predict the interaction between the inhibitors of α-glucosidase (*Saccharomyces cerevisiae*, PDB: 4J57) by considering interaction of phytochemicals with specific active site amino acids; they were also involved interaction with known standard like acarbose. This interaction study gave insight in to the binding mode, pattern of binding and different amino acids involved in the interaction and type of binding involved. Masilinic acid interacted strongly with four amino acids namely Glu 158, Leu 159, Lys 277 and Glu 2 with a percentage of 52.1, 49.4, 22.5 and 14.2% binding. This study revealed that masilinic acid hydroxyl group interacted with Glu 158 at a distance of 1.66Å with a hydrogen donor type of interaction whereas hydroxyl group of masilinic acid interacted with Leu 159 at a distance of 1.71 Å with a hydrogen donor type and one more hydroxyl group of masilinic acid interacted with Lys 277 at a distance of 3.05 Å with hydrogen acceptor type and carboxylic carbonyl carbon of masilinic acid interacted with Glu 2 at a distance of 3.00 Å with a hydrogen donor type of binding (Figure 7). Betulinic acid hydroxyl group interacted with Lys 510 at a distance of 2.77Å with a hydrogen acceptor type of interaction whereas carboxylic acid hydroxyl group of betulinic acid interacted with Glu 158 at a distance of 1.28 Å with a hydrogen donor type of bonding (figure 8). This study well correlates with the experimental α-glucosidase inhibition. Masilinic acid has strongly inhibited α-glucosidase with $IC_{50}$ 262.76 µg/ml and betulinic acid does not show any significant binding.

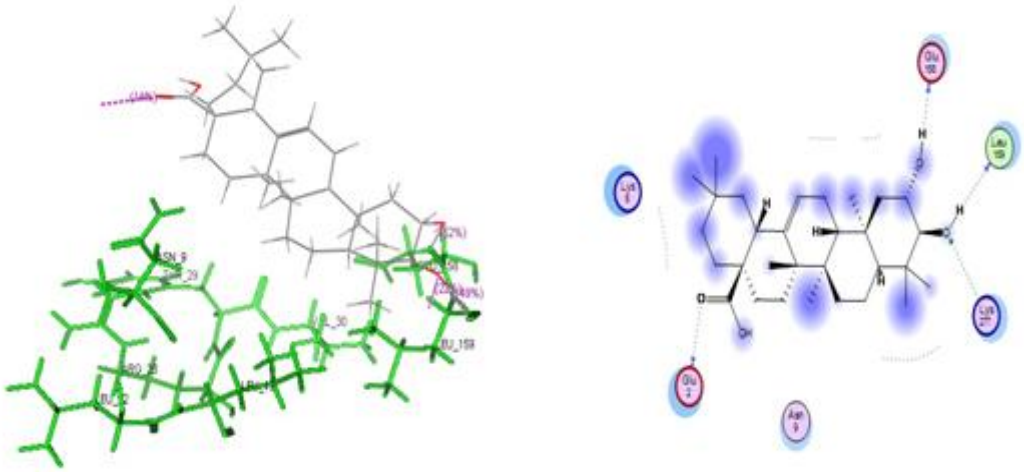

**Figure 7:** Docking studies of Masilinic acid

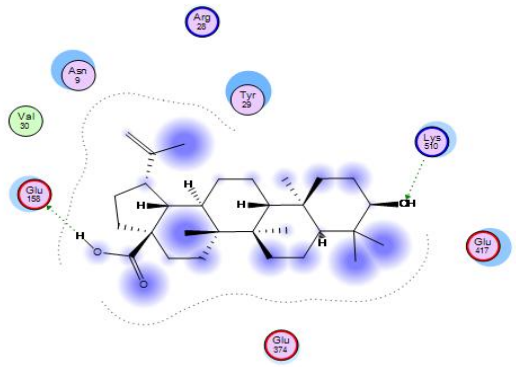


**Figure 8:** Docking studies of Betulinic acid

### *3.3 Enzyme kinetics*

Masilinic acid displayed sigmoidal respose curve (figure 9) suggests the interaction between the inhibitor and the enzyme. Unaffected Vmax (78.1465 ± 4.861), increased Km value (405.52 ± 32.63) and low hill coefficient value (2.35583 ±0.2901) represents competitive inhibition (figure 10). Low hill coefficient value indicates ability of the enzyme inhibitor to compete with substrate for the enzyme. Increase in Km value of the enzyme in the presence of inhibitor implies there is a specific interaction between masilinic acid and enzyme active site by reaching to proximity. Masilinic acid does not affect velocity of the reaction (Vmax) but increases Km value implies probably masilinic acid inhibit α-glucosidase by competitive mechanism.

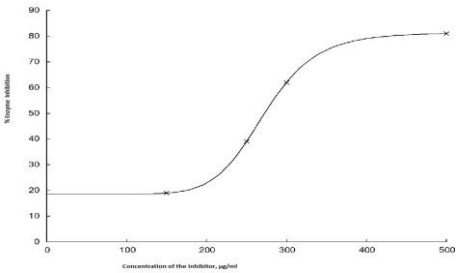

**Figure 9: Sigmoid curve of masilinic acid**

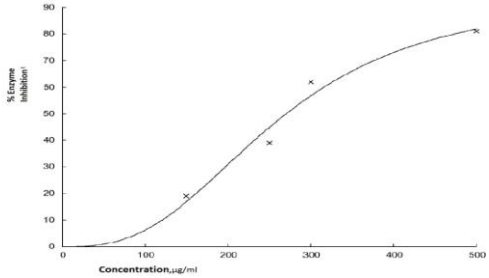


**Figure 10: Exponential curve of masilinic acid**

## IV. Conclusion

Present investigation was the first attempt to isolate and identify the constituents from *D.indica* fruits, which were responsible for inhibition of the enzyme α-glucosidase. This approach led to isolation of six constituents, including triterpene and polyphenolic compounds. Among these, compound 2 (masilinic acid) which belongs to oleanane class of triterpene showed greatest activity, while the major compound 1 (betulinic acid) was inactive; suggested that the lupine class of triterpenes does not have potential to inhibit α-glucosidase. Docking studies and enzyme kinetics supported the enzyme inhibitory activity of masilinic acid. In conclusion, results of this study have revealed that the plant has exhibited potential anti-diabetic effect by inhibiting α-glucosidase enzyme. As this fruit is edible, further *in vivo* studies were recommended to promote as a functional food.

**CONFLICT OF INTEREST:** No conflict of interest

## Acknowledgement

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