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Isolation of stigmasterol from hexane extract of leaves of Pisonia grandis R.Br, in vitro anti-diabetic and its molecular docking studies

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ABSTRACT

Pisonia grandis R.Br belonging to the family *Nyctaginaceae* is a widely distributed evergreen tree in India known for its medicinal uses. The study was aimed to investigate the anti-diabetic property in the leaves of *Pisonia grandis* R.Br. The isolation and purification were performed by the conventional column chromatography and the resultant yield was found to be a white crystalline powder, which was further subjected for characterisation through IR, ^{1}H NMR, ^{13}C NMR and mass spectroscopy. From the characterisation data, the isolated compound was identified as stigmasterol; it was first time isolated from the hexane extract of the leaves. The α-amylase inhibitory activity of stigmasterol from the hexane extract of the leaves of *Pisonia grandis* R.Br showed high potent activity with an IC_{50} value of 46 μg/mL. The anti-diabetic activity of the compound against α-amylase and four other diabetic enzymes- α-*glucosidase,* acid *phosphatase, endo-*β*-N-acetylglucosaminidase* and β-*glucuronidase* were further investigated by molecular docking studies and proved that stigmasterol can be a potential anti-diabetic agent.

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INTRODUCTION

Drugs from natural sources have proved to be effective in the treatment of human diseases. Throughout history, plant material has served as a reservoir of potential sources for new drugs. A great example is that of vincristine and vinblastine, which are used in the treatment of cancerous cells. Diabetes mellitus is a disease that has been affecting humanity globally. The number of casualties with diabetes is estimated to rise from 171 million in 2000 to around 366 million in 2030 (Wild *et al.*, 2004). According to the International Diabetic Federation, there was nearly 73 million diabetic's diagnosis in India in 2017. WHO has predicted that by 2030, diabetes will be the seventh among causes of death.

Pisonia grandis is an evergreen tree native to tropical islands in the Indian and Pacific Oceans, and adjoining coastal areas (CSIR. 1969). It belongs to the family of *Nyctaginaceae*. *Pisonia grandis* has been extensively used in Indian traditional medicine for the treatment of inflammatory diseases (Anbalagan *et al.*, 2002; Elumalai *et al.*, 2012a), ulcer (Mabberley, 1997), arthritis (Elumalai and Prakash, 2012),

rheumatic disorders (McClatchey, 1996) and also used in case of snake bites (Sripathi *et al.*, 2011; Mohankumar *et al.*, 2017). It is also reported to possess anti-diabetic (Poongothai and Sripathi, 2012), anti-pyretic (Elumalai *et al.*, 2012b), anti-fungal (Kirtikar and Basu, 2012; Rahman *et al.*, 2011) and hepatoprotective (Majumdar *et al.*, 2012; Mohankumar *et al.*, 2018) activities. Our study was focused on understanding the anti-diabetic property in the leaves of *Pisonia grandis* R.Br. In the process, stigmasterol was isolated for the first time from the hexane extract of the leaves. We also gave a good account of its anti-diabetic activity, first by proving its potent α-amylase inhibitory activity and then establishing it further through its molecular docking on some of the common diabetic enzymes.

EXPERIMENTAL

General

All the reagents used were of analytical grade. The melting point was recorded using the Veego Digital melting point apparatus. The ^{1}H and ^{13}C NMR spectra were recorded by a Bruker (500 MHz) Avance instrument using $CDCl_3$ as a solvent and the chemical shift (δ) was reported in ppm with respect to tetramethylsilane (TMS) as an internal standard. The mass spectrum was recorded on Shimadzu prominence Liquid Chromatography–Mass Spectrometry (LC-MS) 2020. The IR spectra were measured on a Perkin Elmer FT-IR spectrometer by the KBr pellet press method and the values were given in cm^{-1}. TLC chromatography was performed on pre-coated silica gel GF_{254} (E.merck) plates.

Plant Material

The leaves of the plant were collected inside SRM University, Kattankulathur campus and authenticated by Dr. P. Jayaraman, Director, Plant Anatomy Research Centre, Medicinal Plant Research Unit, Tambaram, Chennai.

Extraction and Isolation

The leaves of *Pisonia grandis* (1 kg) were shade dried and coarsely powdered. The extraction procedure used was the cold maceration method using hexane as an extracting solvent. The crude extract was filtered through Whatman filter paper No. 1 and concentrated with a rotary evaporator under reduced pressure. The extract was then fractionated using silica gel column chromatography and analysed by thin-layer chromatography. The fractions collected were subjected to concentrate on a boiling water bath and the residue was further dissolved using methanol and further concentrated using Rotavac. The compound obtained was characterised using IR, nuclear magnetic resonance (NMR) and mass spectroscopic analysis.

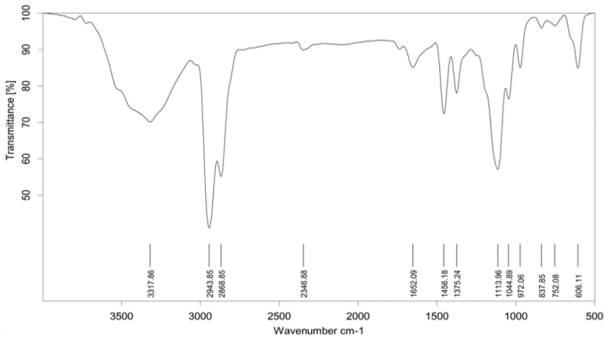

Figure 1: IR spectrum of the isolated compound

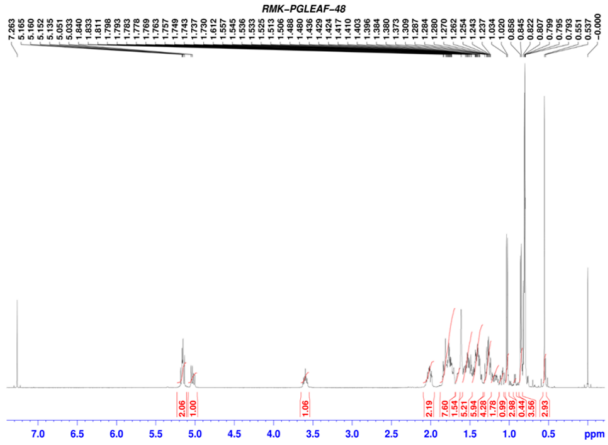

Figure 2: ^{1}H NMR spectrum of the isolated compound

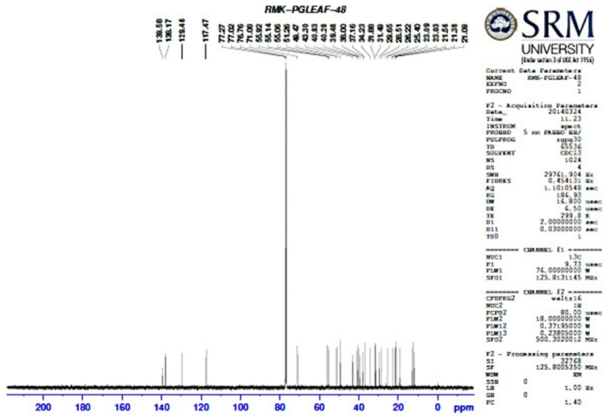

Figure 3: ^{13}C NMR spectrum of the isolated compound

In Vitro α-amylase Inhibitory Assay

The alpha-amylase inhibitory activity (Lee *et al.*, 2017) of the isolated compound was carried out at different concentrations using the method described in Sigma-Aldrich (EC 3.2.1.1) with slight modifications. The assay was determined based on

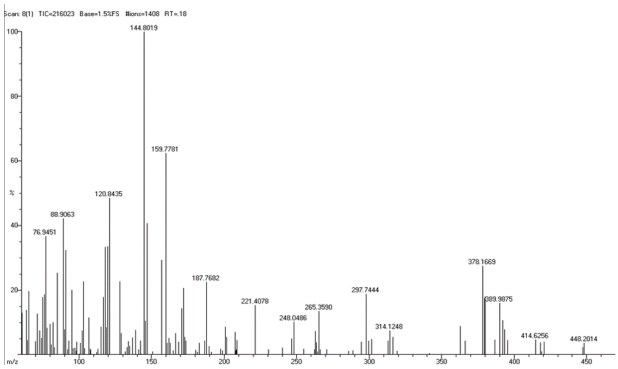

Figure 4: Mass spectral data of the isolated compound

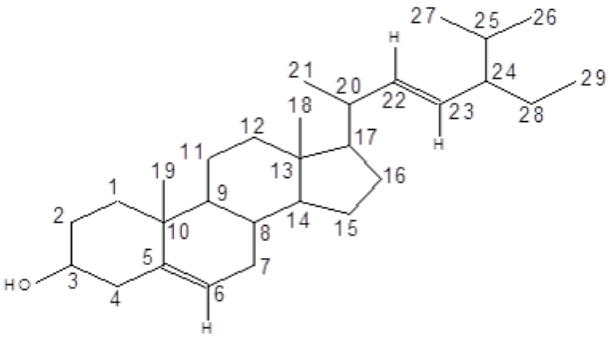

Figure 5: Structure of the isolated compound, stigmasterol

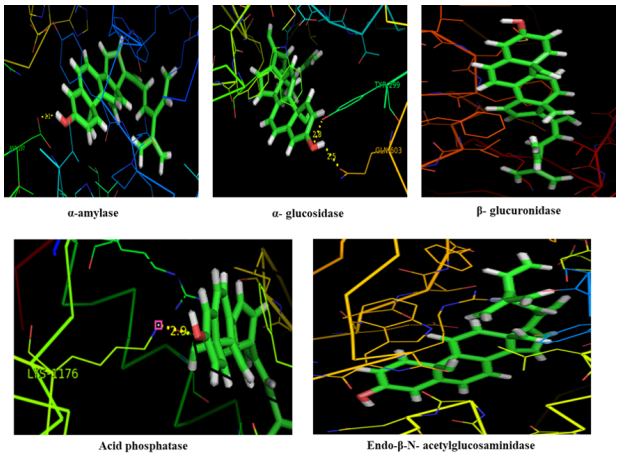

Figure 6: PyMol visualization of the enzymes

a colorimetric method using acarbose as the reference compound. The starch solution (0.5% w/v) was obtained by stirring and boiling 0.25 g of potato starch in 50 mL distilled water for 15 minutes. The enzyme solution (0.5 units/mL) was prepared by mixing 0.001 g of alpha-amylase in 100 mL of 20 mM phosphate buffer (pH-6.9) containing 6.7 mM sodium chloride. Phenol (stock solution 1 mg/mL) was dissolved and made up with DMSO and from the solution, different concentrations (two-fold dilutions) 50, 100, 200, 400 and 800 μg/mL were prepared. The indicator prepared was comprised of 96 mM 3, 5-dinitro salicylic acid (20 mL), 5.31 mM potassium tartrate in 2M sodium hydroxide (8 mL) and distilled water (12 mL). 1 mL of phenol and 1 mL enzyme solution were mixed and incubated at 25 ^{o}C for 30 minutes. To this, 1 mL of the coloring reagent was added and closed, which was further placed into a water bath maintained at 85 °C. 15 minutes later, the reaction mixture was removed from the water bath, cooled and diluted with 9 mL distilled water. The generation of maltose was quantified by the reduction of 3, 5-dinitro salicylic acid to 3-amino-5-nitro salicylic acid and detected at an absorbance of 540 nm. The readings were compared with the control (acarbose), containing buffer instead of the sample. The percentage inhibition of alpha-amylase was assessed by the given formula below,

Percentage Inhibition

$$= \left[\frac{Absorbance\ of\ control - Absorbance\ of\ test}{Absorbance\ of\ control}\right] \times 100$$

Molecular Docking Studies

Molecular docking study (Eissa *et al.*, 2009) was carried out to study the binding mechanism of the isolated compound in the active site of α-amylase.

Additionally, four other enzymes associated with diabetes mellitus, namely α-*glucosidase*, acid phosphatase, endo-β-N-acetylglucosaminidase and β-*glucuronidase* were selected to assess the binding efficacy and inhibitory effects of the isolated compound against the active enzymes in the *in silico* method. The protein sequences were taken from NCBI (National Center for Biotechnology Information) and were converted into FASTA format. The sequences were then allowed into the BLAST (Basic Local Alignment Search Tool) database. The protein structure files were taken from Protein Data Bank. Water molecules and other heterogeneous atoms were removed and hydrogen atoms were added to the structure. PyMOL software was used to view the structure and calculate the length of the hydrogen bond. Docking was performed using Argus Lab.exe. The ligand molecules (standard) were obtained from PubChem and generated by using Chem Sketch ACD Lab.

RESULTS AND DISCUSSION

Characterisation of the isolated compound

The column chromatography of the hexane extract of *Pisonia grandis* leaves over silica gel led to the iolation of a white crystalline compound (48 mg). The compound was recovered and subjected to thin-layer chromatography for identification. R_f value of

Table 1: NMR spectral data of isolated compound

Atom	Carbon, δ, ppm	Hydrogen, δ, ppm
1	31.88	1.02 – 1.61 (m, 2H)
2	37.16	1.02 – 1.61 (m, 2H)
3	71.08	3.59 (m, 1H)
4	43.80	1.73 – 2.05 (m, 2H)
5	139.58	——
6	117.47	5.03 (m, 1H)
7	31.49	1.73 – 2.05 (m, 2H)
8	38.00	1.02 – 1.61 (m, 1H)
9	49.47	1.02 – 1.61 (m, 1H)
10	39.48	——
11	25.40	1.02 – 1.61 (m, 2H)
12	40.28	1.02 – 1.61 (m, 2H)
13	55.06	—–
14	55.92	1.02 – 1.61 (m, 1H)
15	29.65	1.02 – 1.61 (m, 2H)
16	29.65	1.02 – 1.61 (m, 2H)
17	55.14	1.02 – 1.61 (m, 1H)
18	19.83	0.53 – 1.03 (s, 3H)
19	26.22	0.53 – 1.03 (s, 3H)
20	40.83	1.73 – 2.05 (m, 2H)
21	19.01	0.53 – 1.03 (s, 3H)
22	129.46	5.15 (m, 1H)
23	138.17	5.18 (m, 1H)
24	51.26	1.73 – 2.05 (m, 1H)
25	34.23	1.73 – 2.05 (m, 1H)
26	21.56	0.53 – 1.03 (s, 3H)
27	21.38	0.53 – 1.03 (s, 3H)
28	28.51	1.02 – 1.61 (m, 2H)
29	13.05	0.53 – 1.03 (s, 3H)

Table 2: α-amylase inhibitory activity

S/No	Concentration (μg/mL)	% Relative Enzyme Inhibitory activity (R.E.I.)	%Inhibitory Activity (100-R.E.I.)	IC_{50} (μg/mL)
1	50	68.98	31.02	46
2	100	18.76	81.24	
3	150	18.12	81.88	
4	200	12.60	87.40	

Table 3: Molecular docking scores

S/No	Enzyme	Docking Score	Amino Acid	Hydrogen Bond Length
1	Endo-β-N-acetylglucosaminidase	-12.6655 kcal/mol	-	-
2	α- glucosidase	-13.1196 kcal/mol	Glutamine-603 Tyrosine-299	2.5 2.8
3	α-amylase	-13.6192 kcal/mol	Asparagine-197	2.1
4	Acid phosphatase	-14.1174 kcal/mol	Lysine-1176	2.9
5	β- glucuronidase	-16.1866 kcal/mol	-	-

0.26 was found using the mobile phase system, i.e., hexane: ethyl acetate (4:1).

The ^{1}H and ^{13}C NMR spectrum of the isolated product (Table 1) indicates that a multiplet at 3.59 ppm (Figure 2) is due to the CH bond connected to oxygen (C-O) in proton NMR and at 71.08 ppm (Figure 3) in carbon NMR. This is confirmed by the IR spectrum stretching appearing at 1375 cm^{-1}(Figure 1). The signal which appeared in the range of 0.53-2.05 ppm (Figure 2) was assigned to CH_3, CH_2 and CH groups and in carbon NMR signals, which appeared in the range of 13.05 ppm to 55.92 ppm (Figure 3). This is further confirmed by IR stretching at 2943, 2868 cm^{-1}(Figure 1). A peak at 5.03 to 5.18 ppm (Figure 2) in proton and 117.47 and 138.17 ppm (Figure 3) in carbon NMR, corresponds to –C=CH- group. The peak at 3317 cm^{-1} (Figure 1) in the IR spectrum corresponds to the hydroxyl (OH) group. The actual molecular weight of the compound is 412 and in the mass spectrum, it shows a peak at 448 $(M+2NH_4)^+$, as seen in Figure 4. All the above spectral data confirm the structure of the compound to be stigmasterol (Poongothai and Sripathi, 2018) (Figure 5). Salkowski and Liebermann-Burchard tests were carried out (Njoku and Obi, 2009; Kandati *et al.*, 2012) and stigmasterol tested positive for both confirming the presence of steroidal moiety (Rambeloson *et al.*, 2014; Pierre and Moses, 2015; Kirtikar and Basu, 2012).

In Vitro α-amylase Inhibitory Assay

The IC_{50} value of stigmasterol was found to be 46 μg/mL (Table 2). The plant claimed to have anti-diabetic activity as the leaves contain stigmasterol, which mimics the action of acarbose.

Molecular Docking Studies

The PyMol visualization of the interaction between stigmasterol and the enzymes is given in Figure 6. At the end of the docking studies, it was found that α-amylase and *α-glucosidase* gave good docking scores of -13.6192 kcal/mol and -13.1196 kcal/mol, respectively (Table 3). *α-amylase* bonded with isoleucine and lysine. A bond length of 2.1 with asparagine signifies a close interaction between *α-amylase* and stigmasterol (ligand). *α-glucosidase* showed hydrogen bond lengths of 2.5 and 2.8, respectively, with glutamine and tyrosine, which also signifies a close interaction with the ligand. The interaction could mean an inhibitory effect. Acid phosphatase gave a bond length of 2.9 with lysine. *Endo-β-N-acetylglucosaminidase* and *β-glucuronidase* did not show any bonding with stigmasterol. These enzymes are associated with diabetes mellitus.

CONCLUSION

The study aimed to isolate, characterise, screen and perform molecular docking studies of the isolated phytosterol present in the hexane extract of the leaves of *Pisonia grandis* R.Br. The isolated compound was physically characterised and was found to have a melting point in the range of 164-166 ^{o}C. The characterised compound was structurally confirmed to be stigmasterol by spectroscopic methods like IR, ^{1}H NMR, ^{13}C NMR and mass spectroscopy. The compound, when subjected to biological screening test (*alpha-amylase* inhibitory activity), gave an IC_{50} value of 46 μg/mL. This signifies that stigmasterol has an inhibitory activity on alpha-amylase enzyme and can be of use in the treatment of diabetes mellitus. Finally, molecular docking studies were carried out with stigmasterol and enzymes associated with diabetes mellitus which further established that stigmasterol can be a promising anti-diabetic agent. The furtherance of this research work will involve the semi-synthetic modification of this ligand using the computer-aided drug designing tools and subsequently evaluating its in-vitro activity for anti-diabetic activity.

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