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GC-MS Analysis of Major Compounds and Antihyperglycemic and Cytotoxicity Properties of *Ficus racemosa* L. fruit Extract: An *in-vitro* and *in-silico* Study

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Abstract

Ethnobotanical relevance: *Ficus racemosa* L. is one of Assam's best-known plants with rich ethnomedicinal values. The Bodo population of Assam consumes the fruit extract from the plant as a preventative measure against diabetes.

Aim of the study: The goal of this study was to investigate metal content, major phytocompounds, α-amylase and α-glucosidase inhibitory activity as well as cytotoxicity properties of *Ficus racemosa* fruit extracts.

Materials and methods: The GC-MS technology was used to analyze the main phytochemical content of the plant and heavy metal detection was performed by Atomic absorption spectrophotometer. Enzyme inhibition was studied by UV/VIS spectrophotometric methods.

Furthermore, the druglikeness and bioavailability properties of compounds were carried out using computer-aided tools, SwissADME and admetSAR. Docking and visualization were performed in AutoDock vina and Discovery studio. The latter research has been supported by the pharmacophore modeling and structure superimposition of studied compounds for lead molecules.

Results: GC-MS analysis showed six major compounds from the plant. However, the study found that the fruits of *Ficus racemosa* contain negligible amounts of toxic elements. Biochemical studies found that the fruit of *F. racemosa* possesses strong α-amylase and α-glucosidase inhibitory properties. All the six compounds identified were predicted to have druglikeness property with high cell membrane permeability and bioavailability. The Docking study showed strong binding affinities between compounds and enzymes. At the same time *in-vitro* cytotoxicity and apoptosis results on healthy PBMC and splenocytes showed negligible toxicity (5-12%) after 24 h of exposure. Pharmacophore features and structure superimposition revealed compound 3 as a pivot molecule

Conclusion: *Ficus racemosa* fruit extract inhibited α-amylase and α-glucosidase activity indicating its antihyperglycemic properties. Interestingly, negligible cytotoxic effect was seen in healthy PBMC and splenocytes.

1. Introduction

Type-2 Diabetes is one of the most prevalent metabolic disorders characterized by high blood glucose levels leading to serious complications to heart, blood vessels, eyes, kidneys, and nerves (Andrade-Cetto et al., 2008; Peter et al., 2019). Today, it is one of the most common non-communicable diseases in the world associated with the metabolism of carbohydrate and insulin function (Saini, 2010; Tiwari, 2015). Over the last few decades, there is an alarming increase in diabetes, mainly in economically poor countries. It is estimated that about 1.6 million deaths were caused by diabetes in 2016 making it the seventh leading cause of death globally (https://www.who.int/news-room/fact-sheets/detail/diabetes). Along with many other control measures, medication is one of the most common components of diabetes treatment. Several antidiabetic medicines such as biguanides, meglitinide, sulfonylureas, thiazolidinedione, sodium-glucose co-transporter inhibitors, α-glucosidase, etc. are available today. However, medications are known to cause several undesirable effects such as cardiovascular complications, weight gain, nausea, vomiting, dehydration, and many other gastrointestinal disturbances are reported from the use of antidiabetic medicines (Choudhury et al., 2017). α-Amylase and α-glucosidase are two of the most important enzymes responsible for the catabolism of starch, glycogen, and disaccharides. Inhibition of these enzymes controls the blood glucose level by inhibiting carbohydrate metabolism (Azad et al., 2017). Designing therapeutic drug

candidates that inhibit amylase and glucosidase activity is an important aspect in the field of antidiabetic medicines and controlling diabetes.

Natural products have been the source of medicines since ancient times and have played an invaluable role in the treatment of various diseases (Ivorra et al., 1989; Chukwuma et al., 2019). Since ancient times, several plants have been used in ethnomedicine system to cure many diseases, including diabetes. Phytochemicals or plant-derived medicines are considered to be safer to human consumption, less expensive, easily available, and more effective than synthetic drugs (Gilani, 2005). In the recent years, many plants have been reported to have the antidiabetic property by many researchers (Sachan et al., 2019; Magaji, 2020; Alarcon-Aguilara et al., 1998). *Ficus racemosa* L. belonging to the family Moraceae is a deciduous tree popularly known as cluster fig tree. It is a famous medicinal plant in India with several ethnomedicinal values. Traditionally, *F. racemosa* is used to cure cough, dysentery, leprosy, swelling, kidney and spleen problem, abscess, chronic wounds, cervical adenitis, piles, haemoptysis, toothache, cancer, and diabetes (Deepa et al., 2018). Several studies have reported the pharmacological properties of *F. racemosa* such as antimicrobial, antidiabetic, antipyretic, anti-inflammatory, hepatoprotective, and larvicidal activities (Ahmed and Urooj, 2010; Rai et al., 2013; Khan et al., 2017; Mandal et al., 2000). The stem bark extracts of the plant is found to have remarkable antidiabetic, hepatoprotective, and hypolipidemic properties (Chaware et al., 2020). A wide range of phytochemical compounds such as β-sitosterol, campesterol, glauanol acetate, hentriacontanol, kaempferol, tetra triterpene, racemosic acid, etc are reported from different parts of *F. racemosa* (Padma, 2009). Keshari et al. (2016) reported the antidiabetic activity of isolated flavonoids of *F. racemosa*. Our earlier studies have revealed that the fruit extracts of *F. racemosa* is used by the local people of Kokrajhar district of Assam to cure diabetes (Daimari et al., 2019). In southern Assam, the fruit extract of *F. racemosa* is used as antidiabetic medicine (Banik et al., 2010). Dhungana et al. (2018) also reported that the boiled fruit of *F. racemosa* is given as a medicine to a diabetic patient. Similarly, Sarma (2020) reported that the tribal communities of four districts of lower Assam vis. Nalbari, Barpeta, Udalguri, and Kamrup uses the fruit extract as antidiabetic medicine. Therefore, the present study has been designed to investigate the major phytochemicals present in the fruits of *F. racemosa*, and to test α-amylase and α-glucosidase inhibitory activity of plant extract and phytochemicals using *in-vitro* and *in-silico* methods respectively. Furthermore, the toxicity profiles of plant extract was also evaluated in normal PBMC and splenocyte cell lines using MTT and apoptosis assays.

2. Materials And Methods

2.1. Plant material

Fresh and unripe fruits of *Ficus racemosa* L. were collected from the Tinali area of Kokrajhar district of Assam with the help of local people. The plant sample was submitted to the Department of Botany, Bodoland University for identification (specimen voucher number BUBH2018077). The fruits were washed properly, cut into small pieces, and thoroughly dried in the hot-air oven at 40 °C.

2.2. Preparation of plant extract

The dried plant parts were ground into powder form using a mechanical grinder. Plant powders were soaked into 80% methanol for 72 hrs and filtered using whatman filter paper-1. The process was repeated three times and the filtrate obtained was evaporated in a rotary evaporator. After complete evaporation, dry and solid *F. racemosa* methanolic extract (FRME) obtained was kept at -20ºC till further use. The process of crude extract was carried out as per the method described in our earlier publication (Swargiary et al., 2016).

2.3. Heavy metal analysis

Seven elements such as lead (Pb), chromium (Cr) Nickel (Ni), cadmium (Cd), copper (Cu), zinc (Zn), and manganese (Mn) were analyzed using Atomic Absorption Spectroscopy (AAS, Shimadzu AA-7000) following the method of Zheljazkov and Nielson (1996) with little modification. Briefly, 1 g dry plant powder was digested with conc. HNO_3, at 90ºC for 45 minutes. The temperature is then increased up to 100ºC and boiled for 6–7 hours by addition of 5 ml HNO_3 till complete digestion of the plant. The process was continued until the extract is colourless. The solution was filtered by whatman filter No.1 and diluted to 100 ml of distilled water.

2.4. GC-MS Analysis

The phytochemical component of the plant was analyzed by GC-MS system (TQ-8030 Shimadzu Corporation Kyoto, Japan) (Kalita et al., 2016). GC was run on an EB-5MS capillary column (30 m x 0.25 mm i.d.; 0.25 µm) at 57.4 kPa pressure with an initial temperature of 50 °C and maintained at the same temperature for 2.5 min. Next, the oven temperature was raised to 300 °C, at the rate of 15 °C/min, and maintained for 8 min. Injection port temperature was ensured at 300 °C and Helium flow rate as 1 ml/min. The ionization voltage was 70 eV. The plant sample was injected in split mode as 20:1. The Mass spectral scan range was set at 45–450 (m/z). Compound identification was carried out by comparing the spectra with the databases (NIST-11) using a probability-based algorithm.

2.5. Enzyme inhibition assays

2.5.1. Inhibition of α-amylase activity

The inhibition of α-amylase enzyme activity of FRME was carried out following Kwon et al. (2008) with little modification. The crude extract of *F. racemosa* was dissolved in 5% DMSO. Different concentrations of FRME and reference inhibitor (acarbose) were mixed with 200 µl of amylase enzyme (0.5 mg/ml). The assay mixture was incubated at 25ºC for 10 min. Next, 0.5 ml 1% starch solution added and re-incubated for another 20 min at 37ºC. After the incubation, 0.5 ml DNS reagent added to stop the reaction and the assay mixture boiled for 5 min. The reaction mixture was then diluted after adding 5 ml distilled water, and the absorbance (Abs) was measured at 540 nm in UV/VIS double beam spectrophotometer. The control samples were prepared without any plant extracts/compounds.

The percent inhibition of amylase activity was calculated using the following formula-

$$\text{Inhibition (\%)} = \frac{(\text{Abs}\ control - \text{Abs}\ sample)}{\text{Abs}\ control}\ x\ 100$$
.... (1)

Abs control means absorbance of assay mixture without extract and acarbose whereas; Abs sample means absorbance of assay mixture with extract or acarbose

2.5.2. Inhibition of α-glucosidase activity

α-Glucosidase inhibition assay was carried out following the method of Elya et al. (2012) Plant extract was dissolved in 5% DMSO and α-glucosidase in 100 mM sodium phosphate buffer, pH-6.9. Different concentrations of FRME and acarbose were mixed with 50 µl glucosidase (0.5 µg/ml) and incubated for 10 min at 37 °C. Next 100 µl of 5 mM *p*-Nitrophenyl-α-D-glucopyranoside added and incubated for another 20 min at 37 °C. The reaction was stopped by adding 2 ml of 0.1 M Na_2CO_3. The α-glucosidase activity was determined by measuring the absorbance at 405 nm using a UV/VIS spectrophotometer. Inhibition (%) of α-glucosidase activity was calculated using Eq. (1).

2.6. Cytotoxicity assay on normal cells

The *in-vitro* cell cytotoxicity or growth inhibitory effect of plant extract on normal cell i.e., peripheral blood nuclear cells (PBMC) and splenocytes was evaluated by colorimetric MTT assay (Mosmann, 1983). MTT assay is based on the ability of a reducing agent i.e, nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase enzymes to reduce the tetrazolium salt (MTT) to insoluble purple color formazan product. Thus, the purple color formation of final reaction mixture serves as a useful and convenient marker for the viable cells. However, it is also evident that the enzymatic reduction of MTT to MTT-formazan is also mediated by mitochondrial succinate dehydrogenase (SDH) enzyme. Hence, the MTT assay is also dependent on mitochondrial respiration and indirectly serves as an indicator to assess the cellular energy capacity of a cell. The test was performed in different concentrations (10, 25, 50, 100, and 200 mg/mL) of plant extract for 24 h in a 96-well plate(s), tissue culture grade, flat bottomed, sterile (ThermoFisher Scientific, Waltham, Massachusetts, USA) containing 1×10^6 cells in 100 µl then 10 µl of the MTT reagent (5 mg/ml in phosphate-buffered saline) was added into each well. The treated plate was incubated for four hours under 5% CO_2 and 95% air at 37ºC followed by addition of 100 µl of DMSO into each well and gently shaken. After 30 min extraction at room temperature, the absorbance of the final formazan product was recorded spectrophotometrically at 570 nm (Manufacturer: Rapid Diagnostics; SKU, LISA-R, India) (Verma and Singh, 2020).

2.7. Apoptosis assay on normal cells

Additionally, plant extract mediated apoptotic cell death was also determined by acridine orange and ethidium bromide (AO/EB) staining method (Squir and Cohen, 2001) in peripheral blood nuclear cells (PBMC) and splenocytes. Acridine orange fluorescent dye is permeable to both viable and apoptotic cells and emits green fluorescence after binding with DNA, whereas; ethidium bromide is taken up only by apoptotic cells due to compromised plasma membrane and stains the nucleus red. Control and treated cells were stained with acridine orange and ethidium bromide (100 µg/ml each in PBS, pH-7.4) for 5 min in dark, cold room (Prasad and Verma, 2013). The control and treated cells were examined in three replicates using fluorescence microscope and photographed (Medlab Solutions Lx400 FLR Fluorescence Microscope). About 1000 cells were counted per group, and the percentage of apoptotic nuclei (orange/red) was calculated based on differential staining patterns.

2.8. Analysis of druglikeness and ADMET profile

The phytochemicals of *F. racemosa* identified by GC-MS analysis were verified for the druglikeness properties using SwissADME (Daina et al., 2017) and PubChem database. The druglikeness property of compounds was evaluated based on the Lipinski rule which states that an active oral drug should qualify the following criteria: molecular weight in the range from 0 to 500 Dalton, LogP (lipophilicity) value < 5, Hydrogen bond donor should be 0–5, and hydrogen bond acceptor should be < 10 in numbers (Lipinski, 2000). Similarly, the absorption, distribution, metabolism, excretion, and toxicity (ADMET) profile of compounds were predicted using admetSAR (Yang et al., 2019).

2.9. Molecular docking

The significant enzyme inhibitory activity of plant extract prompted us to perform molecular docking study to understand the biochemical behaviour of compounds with the target proteins. The details methodologies are as follows:

2.9.1. Preparation of ligands and enzymes

Phytochemicals reported from *F. racemosa* by GC-MS were retrieved from the PubChem database. The SDF files were converted into *.pdb* format using OpenBabel (O'Boyle et al., 2011) and the *.pdb* file then converted to *.pdbqt* format using AutoDock Tools (version 1.5.6). Acarbose was used as a reference inhibitor. The crystal structure of α-amylase (PDB: 2QV4) and α-glucosidase (maltase) (PDB: 2QMJ) was downloaded from the PDB database (http://www.rcsb.org/pdb). PDB files were converted into *.pdbqt* format after removing the attached ligands and other hetatms using AutoDock Tools. The active site amino acids of both the enzymes were obtained from the ligand-interaction data from PDB database itself. The amino acids interacting with the major ligands of each enzyme is taken as the active sites and docking was performed with the identified compounds. The active site amino acids were Trp^{59}, Gln^{63}, Gly^{104}, Asn^{105}, Val^{106}, Val^{107}, Thr^{163}, Arg^{195}, Asp^{197}, His^{201}, Glu^{233}, His^{299}, and Asp^{300} for α-amylase and Asp^{203}, Asp^{327}, Arg^{526}, Asp^{542} and His^{600} for α-glucosidase.

2.9.2 Molecular interactions

After the ligands (plant compounds and acarbose), and the target enzymes were prepared, docking was performed in AutoDock Vina (Trott and Olson, 2010). The grid parameters were set as x, y, z size coordinate and grid box centre coordinate i.e. 10.749, 48.629, 21.111, and 56, 66, 60 for amylase and − 23.755, -5.19, -11.162, and 40, 50, 42, for glucosidase, respectively. The docking algorithm was carried out by keeping the default exhaustiveness i.e., 8. After docking, the best pose scoring the lowest binding energy (kcal/mol) was selected and visualize in Discovery Studio software.

2.10 Pharmacophore features of potent compounds

Pharmacophore features of all the compounds were determined by Ligandscout software which demonstrated Structural Activity Relationship (SAR) (Muchtaridi et al., 2017) and also suggest compound's descriptors necessary for optimal molecular interactions with biological targets. The optimized chemical structure (PDB) of all the compounds were loaded into Ligandscout working space and key pharmacophore features were determined including H–bond donor, H–bond acceptor, hydrophobic, aromatic, halogen bond donor, positively and negatively ionizable groups (Muchtaridi et al., 2017). All the aforesaid pharmacophore features mediates intermolecular interaction and contribute in binding energy with receptors.

2.11 Validation based on structural superimposition

The analysis of molecular shape similarity is based on the similarity of small molecules as an index for the determination of a minimum common structure in a set of compound libraries. As with pharmacophore theory, the molecular shape similarity index can effectively utilize the overall structural features of the integrated compounds (Schneidman-Duhovny et al., 2008). All the studied compounds along with reference drug in Mol2 file format were zipped and loaded into PharmaGist web server (Dror et al., 2009). The current study focuses more on obtaining potent compound(s) taking into account the rule that molecules with similar structures may have similar or similar biological activity (Martin et al., 2002).

2.12. Statistical analysis

All the results were expressed as means of three experiments ± standard deviation (SD). SPSS tool was used for the study of statistical difference between the extract and reference compound. Other statistical calculations and graphical presentations were prepared in Excel and OriginPro. The significance test was calculated at $P \leq 0.05$ level.

3. Results

3.1. Heavy metal analysis

The present study investigates the content of seven essential heavy metals - Cd, Mn, Cr, Zn, Pb, Ni, and Cu in the fruit extracts of *F. racemosa*. Two heavy metals, Cd and Pb were not detected in the fruits of *F. racemosa*. Among the heavy metals, Zn was found to be the highest content (0.3111 ppm) followed by Ni (0.1974 ppm), Cr (0.1806 ppm), Cu (0.0336 ppm), and Mn (0.023 ppm).

3.2. GC-MS Analysis

GC-MS analysis of methanolic crude extracts of the *F. racemosa* showed the presence of six major compounds such as Silane, dimethyl(2,3,5,6-tetrachlorophenoxy)octadecyloxy- (**C1**), Indole-3-carboxylic acid, 1-(2-acetylaminoethyl)-6-bromo-5-methoxy-2-methyl-, ethyl ester (**C2**), 1H-4-Oxabenzo(f)cyclobut(cd)inden-8-ol,1a-.alpha.,2,3,3a,8b-alpha,8c-alpha-hexahydro-1,1,3a-trimethyl-6-pentyl- (**C3**), Meclofenamic acid di-methyl derivative (**C4**), 2-(3,3-Diethoxypropyl)-6-methoxypyridine (**C5**), and beta.-Alanine, N-cyclohexylcarbonyl (**C6**). The GC-MS intensities of *F. racemosa* fruit extract is presented in Fig. 1. The retention time, m/z, peak area, and other GC-MS profiles of the identified compounds are presented in the Table 1.

3.3. Enzyme inhibition assays

The α-amylase and α-glucosidase inhibitory activity of FRME is presented in Fig. 2. The plant extract showed concentration-dependent inhibition in both the enzyme activities. In both the enzymes, FRME showed stronger inhibitory activity compared to reference inhibitor, acarbose. At a similar concentration (2 mg/ml), the inhibition of α-amylase was found to be 88.8 ± 3.98% and 59.80 ± 4.89% for FRME and acarbose, respectively (Fig. 3a,b). The IC_{50}s were found to be 0.875 mg/ml and 1.72 mg/ml for the plant extract and acarbose, respectively. Unlike α-amylase, the plant extract showed much stronger inhibitory activity against α-glucosidase enzyme (Fig. 3c,d). The enzyme inhibition was found to be significantly different between plant extract and acarbose ($P \leq 0.05$ level). At 10 μg/ml plant extract the enzyme inhibition was found to be 60.45 ± 2.86% while almost similar enzyme inhibition was seen at higher dose of acarbose (500 μg/ml). The IC_{50}s of enzyme inhibition were found to be 0.977 μg/ml and 304.14 μg/ml for FRME and acarbose, respectively.

3.4. Cytotoxicity assay on normal cells

Growth of the cells after plant extract treatment by MTT assay is quantitated by the ability of viable cells to reduce the dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-terazolium bromide (MTT) to a blue formazan product. The cytotoxicity activity of the plant extract carried out against peripheral blood nuclear cells (PBMC) and splenocytes cell line at different concentrations showed very low toxicity (5–12%). As compared to PBMC, the cytotoxicity induced in splenocytes was higher ($P \leq 0.05$) at different point of treatment (Fig. 3).

3.5. Apoptosis assay on normal cells

Acridine orange/ethidium bromide (AO/EB) assay has been used in this study to evaluate apoptotic and viable cells in control and treated groups. Treatment of PBMC and splenocytes cells with plant extract led to the development of apoptotic features including membrane blebbing, nuclear condensation and apoptotic bodies (Fig. 4). The apoptosis associated morphological changes in treated cells showed very low percentage (Fig. 5) of apoptotic cells (5–10%). As compared to PBMC, the apoptosis induced in splenocytes was higher ($P \leq 0.05$) at different point of treatment.

3.6. Analysis of druglikeness and ADMET profile

The druglikeness of the identified compounds of *F. racemosa* is shown in Table 2. According to Lipinski's rule, compound C3 and C4 violated the rule in one parameter (LogP) and C1 in two parameters (molecular weight and LogP). In contrast, the reference compound acarbose violated the rule in all the four parameters. The TPSA of the identified compounds is found to be less than 100. The *in-silico* pharmacokinetics of all the identified compounds of *F. racemosa* and their ADMET profile is represented in Fig. 6. ADMET study showed that the identified compounds have moderate oral bioavailability, while C4 and acarbose have high value. Similarly, the compounds were predicted to be easily absorbed by the gastrointestinal tract and have high permeability through the blood-brain barrier and thus distribute throughout the body. Acarbose, on the other hand, showed low permeability through the blood-brain barrier. In terms of toxicity risk, all the compounds showed a moderate toxicity level. The LD_{50} for acute toxicity were predicted to be 413.175 mg/kg, 1089.142 mg/kg, 1332.226 mg/kg, 967.882 mg/kg, 1438.699 mg/kg, and 1528.968 mg/kg for C1, C2, C3, C4, C5, and C6, respectively while the reference acarbose was found to be 5874.473 mg/kg.

3.7. Molecular docking

Computer-aided investigation of binding affinities of phytochemicals, and the amylase and glucosidase enzymes are shown in Table 3. Among all the six compounds, C3 showed the strongest binding affinity with − 8.5 kcal/mol and − 7.4 kcal/mol energy with respect to amylase and glucosidase enzyme, respectively. Reference chemical, acarbose showed slightly less affinity (amylase − 8.1 kcal/mol and glucosidase − 7.2 kcal/mol) compared to C3. Other phytochemicals, however, showed a lower binding affinity with the enzymes. The 2D display of binding sites and the amino acids involved between the enzymes and the C3 are presented in Fig. 7. Acarbose-amylase interactions involved six conventional H-bond with five amino acids (Gln^{63}, Asn^{105}, Thr^{163}, His^{299}, and Asp^{300}), and van der Waal's interactions with several other amino acids (Fig. 7a). Similarly, acarbose and glucosidase interactions showed four H-bonds with amino acids Tyr^{301}, Gln^{302}, Asn^{306}, and Met^{331}. The study also revealed three other interactions, van der Waal's interactions, C-H bond, and one unfavorable donor-donor bond (Fig. 7c). Amylase and C3 interactions showed one H-bond (Asp^{300}) and 12 other interactions, including van der Waals, Pi-sigma, and Pi-alkyl interactions (Fig. 7b). Similarly, glucosidase and C3 did not show any conventions H-bond interactions, while 13 other types of interactions such as van der Waal's, and Pi-alkyl interactions were found (Fig. 7d).

3.8 Pharmacophore modelling

The events of intermolecular recognition and binding are mediated through forces of attraction and repulsion between chemical descriptors present in the interacting molecules. Here, key pharmacophore features of all the compounds were determined including hydrophobic (H), H-bond donor (HBD), H-bond acceptor (HBA), Negative ionizable area (NI), Aromatic ring (AR) and halogen bond interaction (XBD) that might be responsible for biological activities. In addition, all of these compound pharmacophores contribute to the free binding energy and are therefore essential for biological action. The results showed that compound 1 possess HBA-2, XBD-4 and H-10; compound-2 has HBA-3, HBD- 1, XBD- 1, AR-1 and H-3; compound-3 has HBA-2, HBD-1, AR-1 and H-3; compound-4 has HBA-1, XBD- 2, AR-2 and H-5; compound-5 has HBA-4, AR-1 and H-3 and compound-6 possess HBA-3, HBD-1, NI-1 (Fig. 8a and b).

3.9 Validation based on structural superimposition

Structural superimposition plays a key role in determination of a structural template with a common minimum structure. In this study, the PharmaGist web server was used to align all of the aforementioned compounds in 3D orientation, followed by the determination of the pivot molecule(s) (Schneidman-Duhovny et al., 2008). The output file consists of a number of aligned structures with scores based on the descriptors determined with several versatile

ligand alignments (Dror et al., 2009). After superimposition, the obtained result showed that compound 3 served as a pivot molecule (aligned Score: 13.58) and shares most of the pharmacophore features of the submitted compounds (Fig. 9). Interestingly, compound 3 has shown strong intermolecular interactions with target proteins during docking simulation.

4. Discussion

Ficus racemosa is an important medicinal plant of Assam with a wide range of ethnomedicinal values. Many researchers have pharmacologically investigated several parts of this plant. The present study investigated the heavy metal content, phytochemical and amylase and glucosidase inhibitory property of the plant along with in-silico docking and toxicity profile. Metallic content and trace elements play an important role in the physiology of plants and animals (Ozcan and Akbulut, 2008). Nevertheless, some heavy metals such as Cd, Cr, Pb, etc. are known to have toxic effects beyond certain limits. Several health complications related to lung, liver, kidney, and heart are reported from high exposure of heavy metals (Barbee and Prince, 1999; Prashanth et al., 2015). The present study found that the fruits of *F. racemosa* contain negligible amount of toxic elements as per the WHO permissible level (WHO, 1996). Several chemicals and active ingredients are reported from different parts of *F. racemosa*. GC-MS analysis in the present study revealed six major compounds from the fruits of *F. racemosa*. However, we did not find any literature regarding the biological reports of identified compounds. Lipinski's rule of five is considered to be an important parameter that predicts the druglikeness of a compound. In the present in-silico druglikeness study, compounds C1, C3, and C4 were found to violate the rule in one or two parameters. LogP is a partition coefficient which determines the lipophilicity of a molecule, its absorption, distribution, and penetration in the body (Arnott and Planey, 2012). All the compounds identified from *F. racemosa* fruit extract were found to be lipophilic in nature while acarbose is strongly hydrophilic. Cell permeability of a drug candidate also depends on the topological polar surface area of the molecule. The increasing value of TPSA is associated with the non-permeability or non-bioavailability of the compounds (Palm et al., 1997). The upper limit of TPSA for a molecule to enter through cell membrane and brain is about 140$Å^2$ and 90$Å^2$, respectively (Pjouhesh and Lenz, 2005; Matsson and Kihlberg, 2017). The identified compounds from *F. racemosa* were found to have small TPSA value, and therefore highly permeable through the cell membrane.

The study of pharmacokinetics is an important prerequisite in the present-day drug discovery pipeline. The drug candidate needs to be easily absorbed and distributed throughout the body without any metabolism before reaching the target site. The lead compound needs to be less toxic and easy excretion from the body (Hodgson, 2019). The sum of the properties, as mentioned above, can be studied as ADMET profile. Computer-aided methods have been employed by many researchers to understand the ADMET profiles of compounds. By adopting the in-silico approaches, the cost and the time factor may be minimized compared to standard experimental approaches (Dimasi et al., 2003). The identified compounds from *F. racemosa* showed a moderate to high ADMET profile suggesting the possibility of a drug candidate. Because of its small size and lipophilic property, all the compounds are predicted to be easily absorbed by the human intestine, except C6. Organ toxicity such as hepatotoxicity, acute oral toxicity, and genotoxicity such as carcinogenicity, Ames mutagenesis also showed low to moderate levels of toxicological effects. The predicted LD_{50}s were found to be 413.175 mg/kg, 1089.142 mg/kg, 1332.226 mg/kg, 967.882 mg/kg, 1438.699 mg/kg, and 1528.968 mg/kg for C1, C2, C3, C4, C5, and C6, respectively. Reference acarbose showed the lowest LD_{50} 5874.473 mg/kg.

Amylase and glucosidase are two of the most important enzymes of chemotherapeutic drug targets in antidiabetic drug designing. By inhibiting these enzymes blood glucose level can be reduced significantly. Many research

findings have revealed the α-amylase and α-glucosidase inhibitory properties of several medicinal plants (Kazeem et al., 2013; Somtimuang et al., 2018). In the present study methanolic crude fruit extracts of *F. racemosa* showed strong α-amylase and α-glucosidase enzyme inhibition. Earlier study by Chaware et al. (2020) reported several pharmacological properties of *F. racemosa* including the antidiabetic property of bark extracts. Flavonoids such as Kaempherol, Quercetin, Naringenin, and Baicalein isolated from stem bark of *F. racemosa* were found to have considerable antidiabetic property (Keshari et al., 2016). Baicalein is also reported to have glucose metabolism improving property in insulin-resistant HepG2 cells (Yang et al., 2019). Similarly, Deep et al. (2013) revealed the antidiabetic properties of several other plant species belonging to genus *Ficus* such as *F. benghalensis, F. carica, F. glomerata, F. glumosa*, and *F. religiosa*. Molecular docking is another aspect of drug designing approach which is a widely used, relatively fast, and economical computational tool that predicts the binding affinities between ligands and proteins. Using in-silico method virtual screening of a large number of chemicals can be screened to select the lead compounds of probable drug candidates (Huang and Zou, 2010). In-silico molecular docking has been used by many researchers to verify the effectiveness of phytochemicals (Ghaedi et al., 2020; Surriya et al., 2013; Swargiary et al., 2020). Ligand–enzyme binding and interaction studies have shown that the compounds from *F. racemosa* showed better binding affinity in both the enzymes than acarbose. The binding energy of C3 was found to be stronger in both the enzymes. Thus, the molecular docking interactions reflect the results of *in-vitro* enzyme assays indicating the α-amylase and α-glucosidase inhibitory property of *Ficus racemosa*. One of the most commonly used *in-vitro* methods of screening healthy cells to observe the growth, reproduction and morphological effects of chemical agents or plant extracts is the cytotoxicity test (Nath et al., 2020). Cytotoxicity is preferred as a pilot project test and an important indicator for toxicity evaluation of chemical agents as it is simple, fast, has a high sensitivity and can save animals sacrifices from toxicity assays (Li et al., 2015). In the present study, we used PBMC and splenocytes to test toxicity profiles of plants extract on healthy cells and result revealed limited toxicity with 5–10% cell death by apoptosis.

Pharmacophore modeling is a computationally effective and pragmatic technique for the discovery and optimisation of biologically active compounds (Wolber et al., 2008) and the study of intermolecular interactions *in silico* (Mortier et al., 2017). In current drug discovery field, pharmacophore features is most commonly used drug designing tool that led to establishment of more stable and targeted drugs with no or limited side effect in the host, as they are supposed to more precisely targeted (Bredel and Jacoby, 2004). The process of identifying a target, synthesizing bio-active compound with desired pharmacological action like minimal toxicity, high bioavailability, cost-effective synthesis, etc., and finally developing it to introduce in the market is a time-consuming, extremely complex and risky endeavour (Atanasov et al., 2015). To overcome these challenges, LigandScout, a standalone platform that address all the problems related to pharmacological action of drugs and it has been used in the present study to generate essential pharmacophore features of synthesized compounds (Wolber et al., 2008). The obtained pharmacophore features of aforesaid compounds revealed their potential role during intermolecular interaction with target proteins (Fig. 8a and b). For a set of molecules sharing a similar biological response, a ligand-based pharmacophore model can be derived by superposing them and determining the maximum number of overlapping chemical features (Wolber et al., 2008). The structure superimposition result showed that compound 3 possess most of the chemical descriptors common with other aligned compounds and hence bind more strongly with target proteins (Fig. 9 and Fig. 7). Further study is underway to isolate and characterize the bioactive compound of the plant and also to investigate the *in vivo* effect of isolated compounds of the plant.

5. Conclusion

Diabetes and its treatment by plant-derived compounds have gained momentum in recent years. *Ficus racemosa* has been an invaluable plant since ancient times because of the rich ethnomedicinal values. The present study highlights the α-amylase and α-glucosidase inhibition by the crude extract of the plant. Similarly, docking studies with major phytochemicals showed similar results, thereby suggesting the possibility of the antidiabetic drug candidates. The docking findings are further corroborated by pharmacopore features and superposition parameters of the compounds structure. Moreover, the cytotoxicity and apoptosis result of the plant extract revealed very low cell death (5–12%) on normal PBMC and splenocytes. However, further investigation needs to be carried out to isolate and purify the plant's active compounds so that the exact mode of action can be understood.

Declarations

Ethics approval and consent to participate: Not applicable

Manuscripts reporting studies involving human participants, human data or human tissue must: *Include a statement on ethics approval and consent (even where the need for approval was waived) *Include the name of the ethics committee that approved the study and the committee's reference number if appropriate. Studies involving animals must include a statement on ethics approval. If your manuscript does not report on or involve the use of any animal or human data or tissue, this section is not applicable to your submission. Please state "Not applicable" in this section.

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Availability of data and materials: No datasets were generated during the current study.

All authors must include an "Availability of Data and Materials" section in their manuscript detailing where the data supporting their findings can be found. Authors who do not wish to share their data must state that data will not be shared, and give the reason. If this is not applicable, please write: "Data sharing not applicable to this article as no datasets were generated or analysed during the current study. If you do not wish to publicly share your data, please write: "Please contact author for data requests."

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Competing interests: Nil

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include the statement “The authors declare that they have no competing interests.” If you are unsure whether you or any of your co-authors have a competing interest please contact the editorial office.

Authors' contributions

The individual contributions of authors to the manuscript should be specified in this section. We suggest the following kind of format (please use initials to refer to each author's contribution):

AS did conceptualization, provided resources, funding acquisition, supervision and project administration. **AS** was also involved in plants extraction, GCMS data analysis and performed the molecular docking and data analysis. **MD** collected the material, prepared the extract, and tested enzymatic activity. **AKV** performed cytotoxicity and apoptosis study in normal cell lines (PBMC and splenocytes), performed statistical analysis of the data. The first draft of the manuscript was written by all authors. All authors read and approved the final manuscript.

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Conflict of Interest

Authors declare no conflict of interest.

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Tables

Table 1

GC-MS profiles of the compounds identified from Ficus racemosa.

Sl. Nos.	Name of the compounds	Retention time	Base m/z	Area (%)	Height (%)	Mol. Weight (g/mol)	Mol. formula
1	Silane, dimethyl(2,3,5,6-tetrachlorophenoxy)octadecyloxy	3.980	55.00	12.67	18.41	558.5	$C_{26}H_{44}Cl_4O_2Si$
2	Indole-3-carboxylic acid, 1-(2-acetylaminoethyl)-6-bromo-5-methoxy-2-methyl-, ethyl ester	6.275	123.00	10.66	15.58	397.3	$C_{17}H_{21}BrN_2O_4$
3	1H-4-Oxabenzo(f) cyclobut(cd)inden-8-ol, 1a-.alpha.,2,3,3a,8b-alpha,8c-alpha-hexahydro-1,1,3a-trimethyl-6-pentyl	15.485	230.00	11.68	15.60	314.5	$C_{21}H_{30}O_2$
4	Meclofenamic acid di-methyl derivative	20.310	317.00	29.76	14.59	324.2	$C_{16}H_{15}Cl_2NO_2$
5	2-(3,3-Diethoxypropyl)-6-methoxypyridine	21.580	194.00	23.62	18.13	239.31	$C_{13}H_{21}NO_3$
6	beta.-Alanine, N-cyclohexylcarbonyl	22.065	521.00	11.60	17.70	199.25	$C_{10}H_{17}NO_3$

Table 2
Lipinski's druglikeness properties of phytochemicals of Ficus racemosa.

Compounds	PubChem CID	Mol. weight (< 500 Da) (g/mol)	LogP (< 5)	H-bond donor (< 5)	H-bond acceptor (< 10)	TPSA (Å^2)	Lipinski violation
Acarbose	41774	645.60	-8.5	14	19	321	4
C1	91745980	558.50	14.2	0	2	18.5	2
C2	633903	397.30	2.5	1	4	69.6	0
C3	30607	314.50	6	1	2	29.5	1
C4	626169	324.20	5.1	0	3	29.5	1
C5	606554	239.31	2.4	0	4	40.6	0
C6	11983471	199.25	1.1	2	3	66.4	0

Table 3
Docking score of amylase and compounds from Ficus racemosa.

Compounds	α-amylase (kcal/mol)	α-glucosidase (kcal/mol)
Acarbose	-8.1	-7.2
C1	-6.1	-5.0
C2	-6.5	-6.3
C3	-8.5	-7.4
C4	-6.7	-5.9
C5	-5.7	-4.9
C6	-6.2	-6.7

Figures

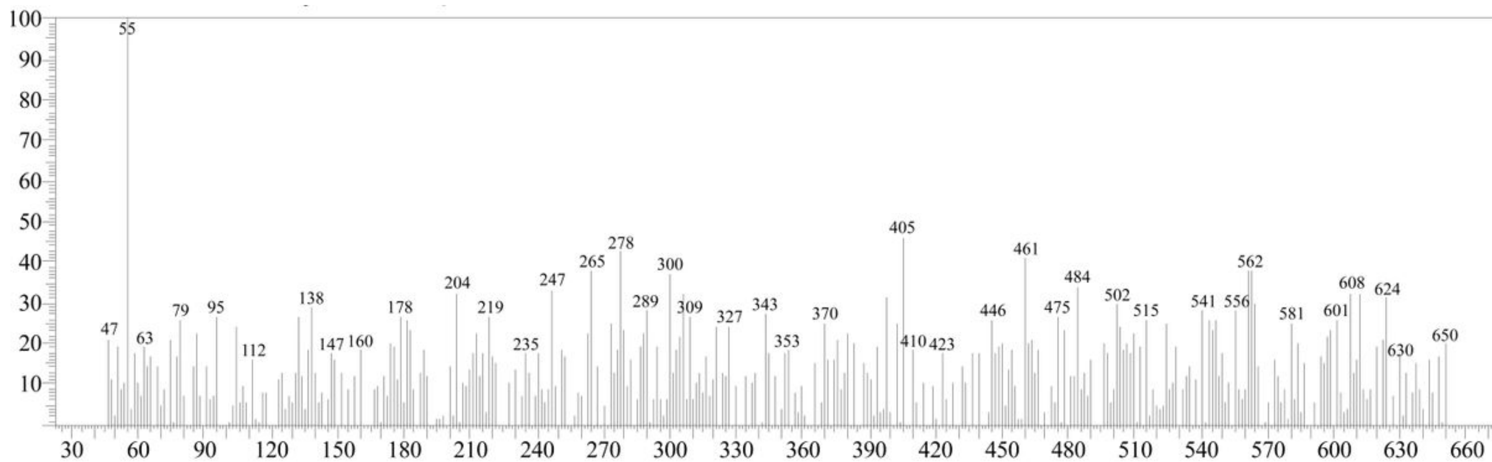


Figure 1

GC-MS chromatogram of methanolic fruit extract of Ficus racemosa.

(a)
Amylase inhibition (%)
Concentration in mg/ml

(c)
Glucosidase inhibition (%)
Concentration in μg/ml

(b)
Amylase inhibition (%)
Concentration in mg/ml

(d)
Glucosidase inhibition (%)
Concentration in μg/ml

Figure 2

Activities of amylase and glucosidase in response to plant extracts and acarbose treatment. (a) α-amylase and acarbose; (b) α-amylase and Ficus racemosa; (c) α-glucosidase and acarbose and (d) α-glucosidase and F. racemosa.

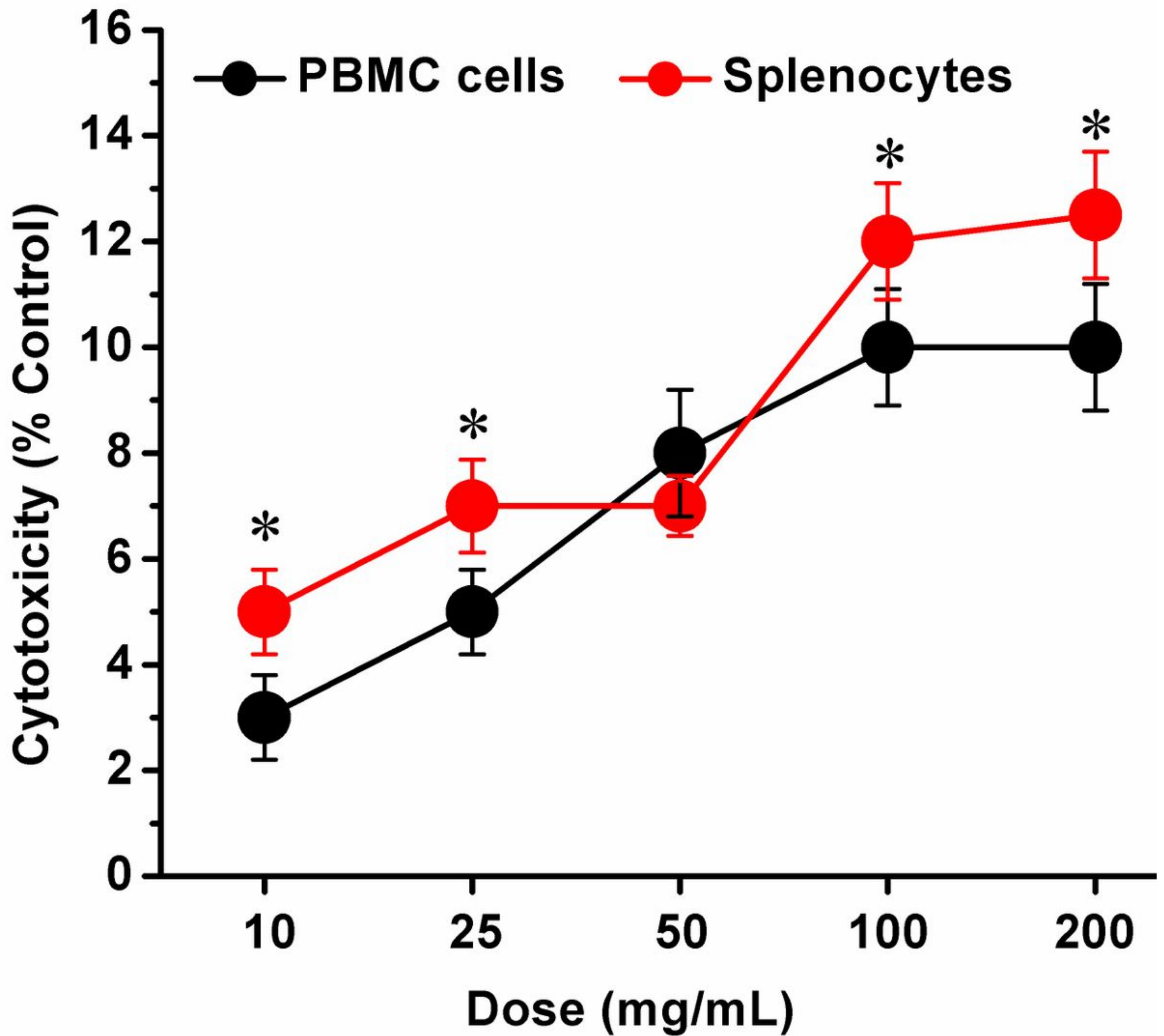


Figure 3

the cytotoxicity induced in splenocytes was higher (P≤0.05) at different point of treatment

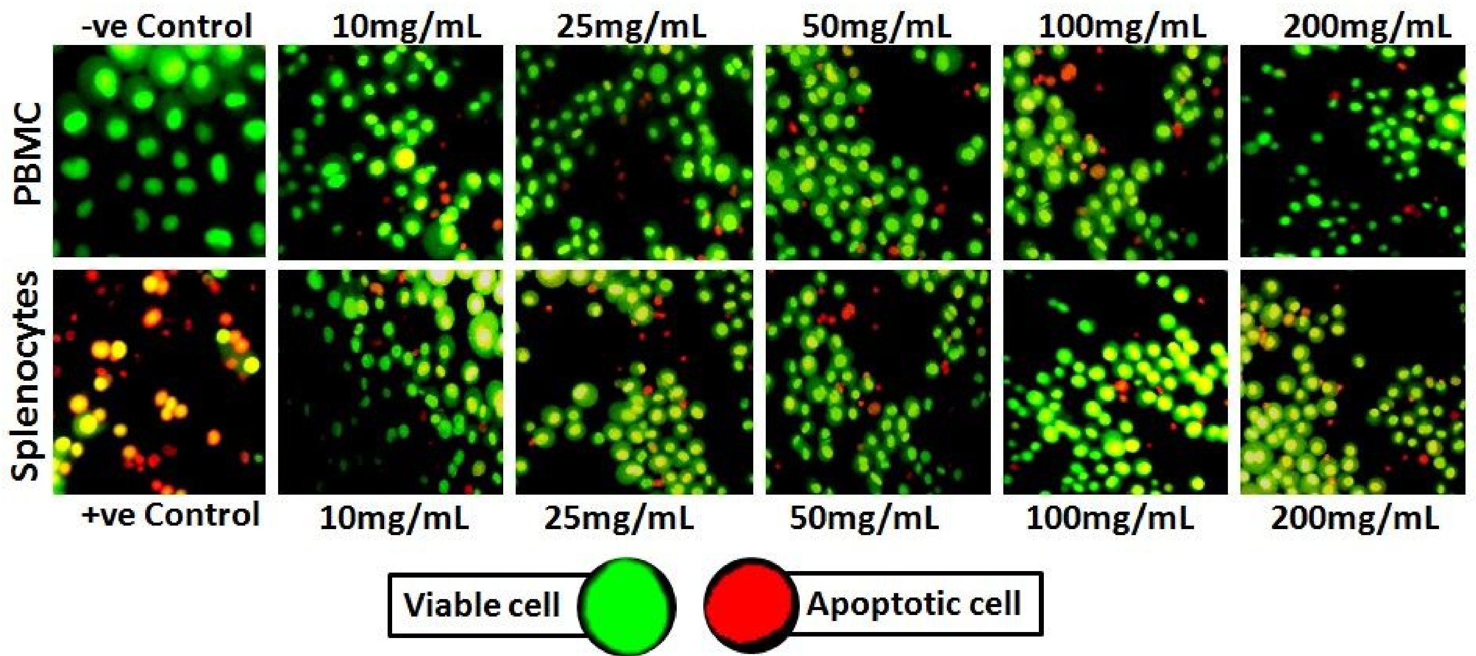


Figure 4

Morphological features of apoptotic and viable cells observed under fluorescence microscope after treatment in PBMC and splenocytes cells. Cisplatin was used as a positive control, treatment with plant extract showed apoptotic cells (red/orange nuclei), negative control cells showed viable cells (green nucleus).

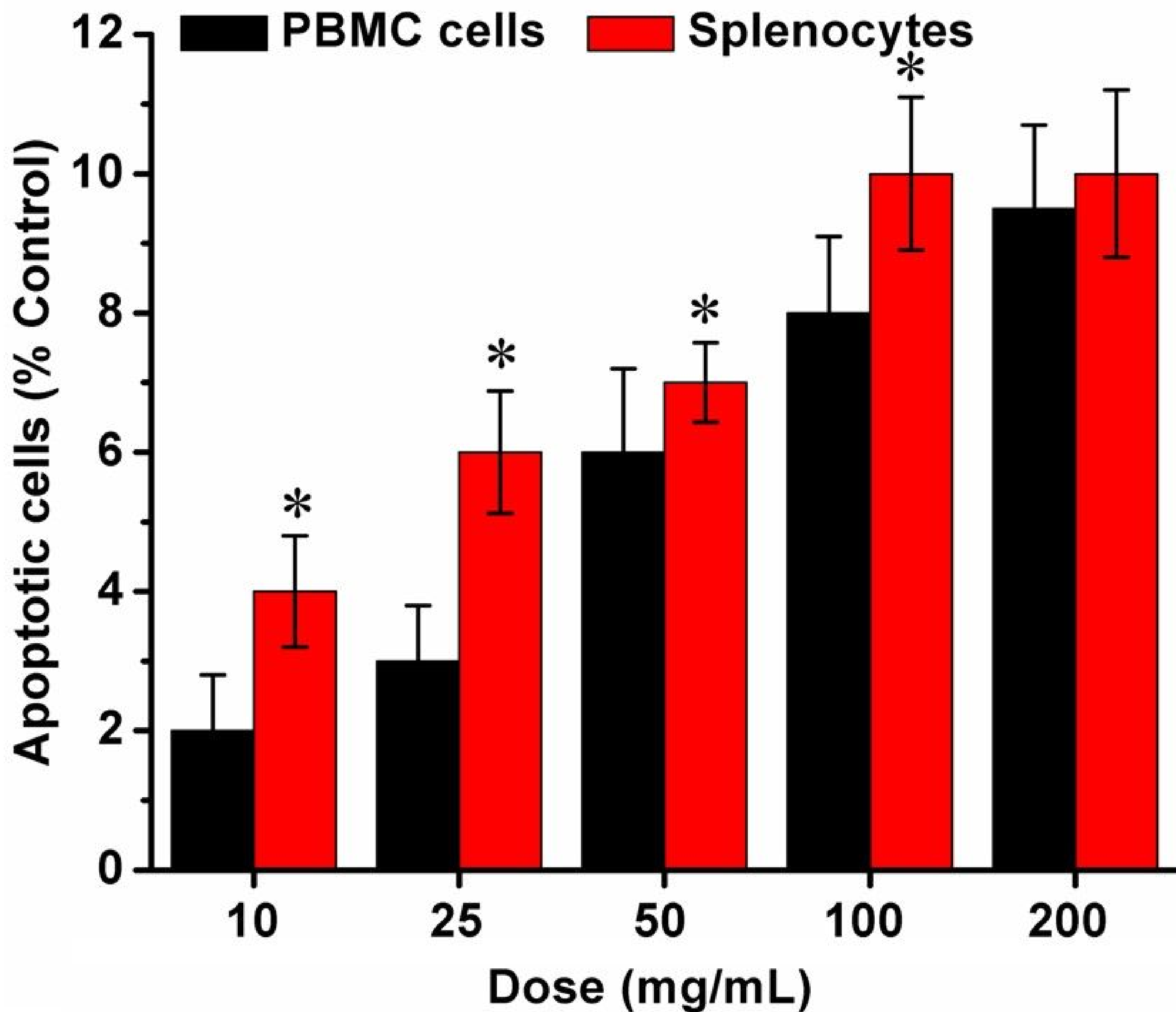


Figure 5

Percentage apoptotic cells after treatment with plant extract. Data are mean ± S.D., n=3, one way ANOVA, *P≤0.05 (as compared to PBMC).

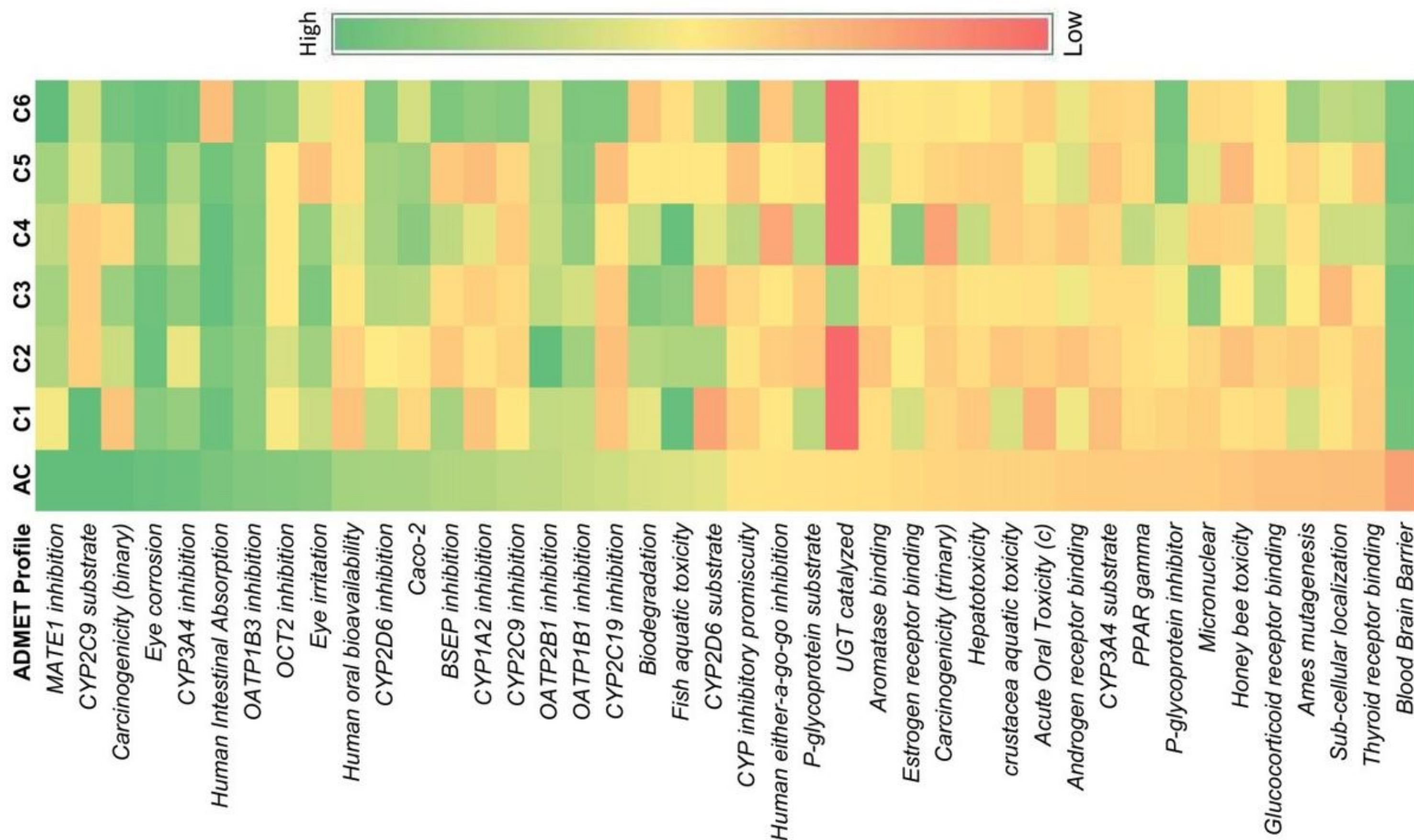


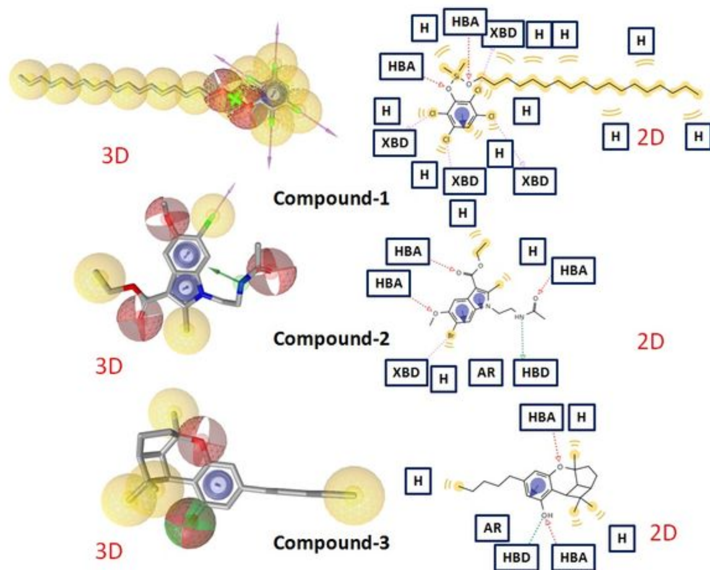
Figure 6

Heat map of ADMET profile of Ficus racemosa phytochemicals and acarbose (AC).

Van der Waals
Conventional H- bond
Carbon-Hydrogen bond
Unfavorable donor-donor
Pi-Sigma
Alkyl
Pi-Anion
Pi-Sulfur

Figure 7

Docking interactions between enzymes and phytochemicals of Ficus racemosa. (a) α-amylase and acarbose, (b) α-amylase and C3, (c) α-glucosidase and acarbose, and (d) α-glucosidase and C3.



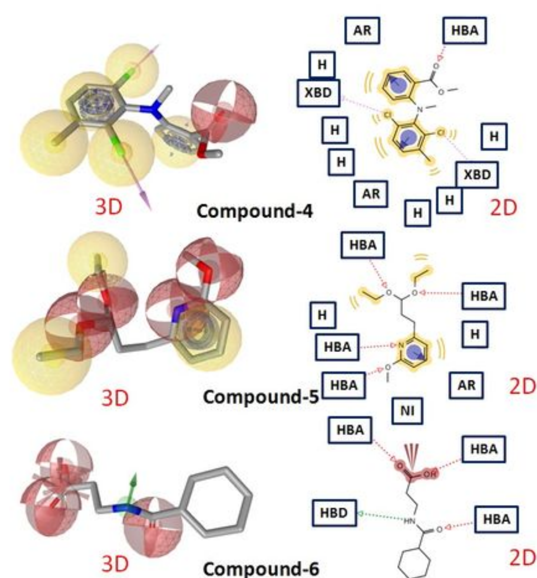


Fig 8a

Fig 8b

Figure 8

Pharmacophore features (2D and 3D) of all the compounds predicted using Ligandscout software. a) compound 1-3 and b) compound 4-6. The pharmacophore color code is yellow for hydrophobic regions, red for hydrogen acceptors and green for hydrogen donors. 2D Pharmacophore features represent hydrophobic (H), H-bond donor (HBD), H-bond acceptor (HBA), Negative ionizable area (NI), Aromatic ring (AR) and halogen bond interaction (XBD).

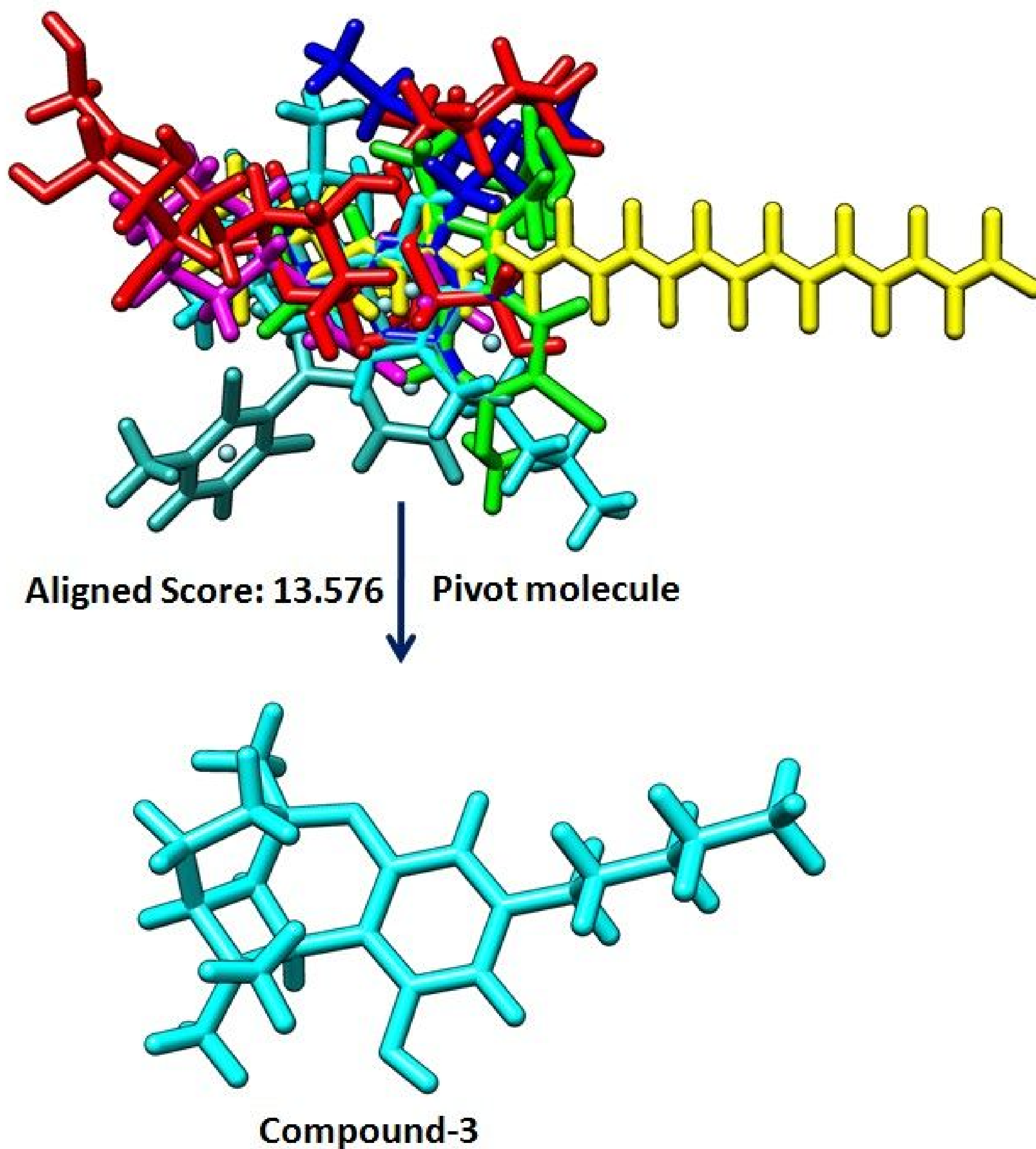


Figure 9

Pharmagist based compounds superimposition with reference drug and determination of pivot molecule (key structure). Compound-3 showed structural fragment similarity with high aligns score and hence may be able to bind with target proteins more efficiently than others.