Alpha-glucosidase inhibition and antioxidant activity of *Ensete superbum* (Roxb.) Cheesman seeds: GC-MS-based profiling of the active metabolites and molecular docking study

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The present study aims to uncover the alpha-glucosidase inhibition (AGI) and antioxidant activities of *Ensete superbum* seed extract. The AGI activity of crude extract (IC_{50} = 0.17 μg/ml) and silica gel column purified fraction (ESSFR4; IC_{50} = 0.02 μg/ml) was excellent when compared to acarbose (IC_{50} = 1720 μg/ml). Also, the free-radical scavenging ability of ESSFR4 was comparable to ascorbic acid. Among the five compounds identified from ESSFR4, pentasiloxane, dodecamethyl and 2,4-hexadienedioic acid, bis(trimethylsilyl) ester, had the best binding affinities against human and yeast AG enzymes respectively. However, ADME/toxicity evaluation of these five compounds revealed that they would require further structural scrutiny and *in vivo* studies before recommending them as an alternative to the present AGIs to treat type-2 diabetes.

Keywords: Alpha-glucosidase inhibition, antioxidants, diabetes, *Ensete superbum*, toxicity evaluation.

ENSETE SUPERBUM (Roxb.) Cheesman is a medicinal plant belonging to the banana family Musaceae. It is commonly known as wild banana and is distributed in the Anaimalai hills, Dindigul, Western Ghats, and some parts of peninsular, India[1]. The plant is documented for its culinary as well as medicinal purposes by local people and traditional health practitioners across India and Ethiopia. Different parts of the plant such as fruit, pseudo-stem, leaf, inflorescence, rhizome and seed have been utilized for day-to-day requirements by different communities across India as food, medicine in rituals and religious purposes[2]. Many communities in Ethiopia depend on the plant as a traditional source for expelling the placenta, healing bone fracture and controlling (viral, bacterial, nematode and fungal) infections[3].

In India, states like Karnataka, Kerala, Tamil Nadu, Maharashtra, Gujarat, Assam, Mizoram and Arunachal Pradesh have extensively relied on different parts of the plant for everyday requirements as food and medicines[4,5]. Every part of *E. superbum*, especially the raw seeds or its preparations have been extensively used in the treatment of stomach ache, kidney stones, semen depreciation, painful delivery, psychosomatic disorder, measles, dog bite, diabetes, venereal diseases, urinary infection, early abortion, leucorrhoea and fever with body pain by several communities in India[2,6]. The plant has also been widely used in the treatment of diabetes by different communities across India[2].

The present study focuses on the natural molecules to inhibit the alpha-glucosidase (AG) enzymes that are present on the brush border membrane of the small intestine. These enzymes take part in the hydrolysis of the oligosaccharides into monosaccharides. In the case of diabetic patients, blood glucose homeostasis will be altered whenever they consume starchy food due to the regular activity of AG enzymes. So, the concept of partial inhibition would reduce the regular action of the AG enzymes and lower the blood glucose level, which in turn helps in maintaining glucose homeostasis[7]. In the current scenario, there are three medications – acarbose, miglitol and voglibose – available in the market as alpha-glucosidase inhibitors (AGIs), which are of bacterial origin[8]. All three AGIs showed considerable adverse effects when the patients were administered with these medications[9]. Hence in recent decades, the natural sources has been focused to determine potent AGIs. However, none of them has been successful in finding effective AGIs which can be an alternative to the present inhibitors in the market. Among the different characterization techniques, GC-MS is one of the easy and reliable analytical techniques widely used in the identification of both primary and secondary metabolites[10]. Recently, GC-MS-based metabolite profiling of nutrients[11], antioxidants, antimicrobials[12], antifungal, antibacterial[13] and alpha-glucosidase inhibitor molecules[14] has been done using plants.

Even though earlier studies have reported the antioxidant and AGI properties of *E. superbum*[15], a systematic study relating *in vitro* and *in silico* analysis is lacking. In this context, the present study focuses on AGI properties,

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antioxidant activity, phytochemical analysis, identification of molecules from the active extract of *E. superbum* using GC-MS analysis, and their *in silico* analysis.

Materials and methods

The standard chemicals and reagents were purchased and used for all the experiments. Different samples of *E. superbum* were collected and processed using standard protocols. Yeast alpha-glucosidase (YAG) inhibition assay was done using the 4-nitrophenyl-β-D-glucopyranoside method. The biochemical assays such as antioxidant assays, phytochemical assays and other characterizations were done using standard methods. The molecular docking and ADME/toxicity studies were done using authentic software. Statistical analysis was done using Excel/GraphPad prism. All the methods used are explained in detail in the Supplementary Materials.

Results and discussion

Extraction and purification

Figure 1 shows the plant, *E. superbum*, and its different parts (fruits and seeds) used for the study. Twenty grams of seed extract (aqueous) was collected using the cold-extraction method from 250 g of seed powder. Further, silica gel column purification of the seed extract gave six fractions which yielded a dry weight of 23, 42, 71, 750, 800 and 725 mg for the fractions ESSFR1, ESSFR2, ESSFR3, ESSFR4, ESSFR5 and ESSFR6 respectively. The dried powder obtained from the fractions along with the crude extract was analysed for *in vitro* AGI activity. In previous studies, seeds of *E. superbum* (Roxb.) Cheesman were extracted using ethanol, methanol, water and chloroform[1,15,16].

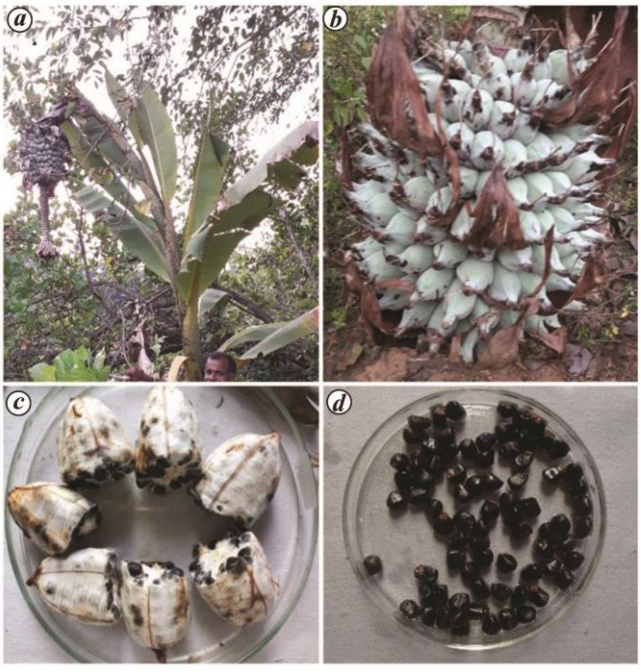

Figure 1. Plant and parts of *Ensete superbum*. ***a***, Whole plant. ***b***, Bunch of banana fruits. ***c***, Raw fruits along with seeds. ***d***, Separated seeds used for extraction.

YAG inhibition assay

Among the silica gel column purified fractions, fraction 4 (ESSFR4) showed the highest AGI activity with IC_{50} value of 0.02 ± 0.001 µg/ml, followed by ESSFR5 (IC_{50} = 0.03 ± 0.01 µg/ml) and ESSFR6 ($IC_{50} = 5.10 \pm 1.90$ µg/ml). The remaining fractions, viz. ESSFR1, ESSFR2 and ESSFR3 did not show any activity at the analysed concentration (Table 1). The AGI activity of crude extract ($IC_{50} = 0.17 \pm 0.02$ µg/ml) and all the purified fractions was far better than the activity of standard AGI, acarbose ($IC_{50} = 1720 \pm 60$ µg/ml). Inhibiting the AG enzyme is one of the therapeutic strategies to treat type-2 diabetes (T2D)[7]. Recently, several studies have focused on the inhibition activity of AG enzyme using plant extracts as a therapy to control the blood glucose level in T2D patients[17,18]. In a recent study, the methanol extract of *E. superbum* seeds showed inhibition activity against alpha-glucosidase (1.8 ± 0.1 mg/ml) and protective activity against pancreatic β-cells induced with hydrogen peroxide[15]. Except for the study by Habtemariam and Varghese[15], there have been no reports available on the AGI activity of *E. superbum* plant extracts. In the present study, the aqueous extract of seeds showed thousand-fold better AGI activity when compared to the previous study of Habtemariam and Varghese[15]. Further, we have studied the purified fraction of the extract in detail to determine the compounds responsible for AGI and antioxidant activity.

Table 1. Alpha-glucosidase inhibition (AGI) activity of crude extract and column-purified fractions of *Ensete superbum* seeds. The crude extract was purified using silica gel column chromatography with chloroform (100%, 200 ml), chloroform : methanol (1 : 1; 200 ml), methanol (100%; 200 ml) and methanol : water (8 : 2; 100 ml). AGI activity was represented as IC_{50} values

Sample	AGI activity (IC_{50}, µg/ml)
Crude extract	0.17 ± 0.02
ESSFR1	–
ESSFR2	–
ESSFR3	–
ESSFR4	0.02 ± 0.001
ESSFR5	0.03 ± 0.01
ESSFR6	5.10 ± 1.90
Acarbose	1720 ± 60

–, No activity; ESSFR, *E. superbum* seed fraction. The IC_{50} values are mean of triplicates ($n = 3$) with standard deviation (SD ± 3). Each value is statistically significant at $P < 0.05$.

Antioxidant activity

DPPH and ABTS assay: The antioxidants have a role in several cellular mechanisms which include the breaking of chains by donating electron or hydrogen atoms to the free radicals, thereby making them more stable and decomposing lipid peroxides that convert them into stable final products[19]. Based on the highest AGI activity, the column purified fraction ESSFR4 was analysed for its antioxidant activity (Figure 2). It exhibited 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity with IC_{50} value of 21.91 ± 1.07 µg/ml (Figure 2 *a*). The 2,2′-azino-bis(3-ehtylbenzothiazoline-6-sufonic acid (ABTS) free radical scavenging activity of ESSFR4 was also significant, with IC_{50} value of 9.14 ± 0.63 µg/ml (Figure 2 *b*). The DPPH and ABTS free radical scavenging activities of ESSFR4 were comparable to the standard antioxidant ascorbic acid (IC_{50} for DPPH = 4.87 ± 0.03 µg/ml and ABTS = 3.81 ± 0.05 µg/ml). In an earlier study, seed extract of *E. superbum* prepared in methanol showed DPPH free radical scavenging effect with IC_{50} of 6.2 ± 0.3 µg/ml (ref. 15). Both DPPH and ABTS assays have been used to measure the antioxidant properties of plant extracts in different studies[20,21]. Hence, in the present study, the ABTS free radical scavenging assay was used along with the DPPH assay for assessment of free radical scavenging activity of *E. superbum* seed extract.

Phytochemical analysis

Total phenolic and total flavonoid contents: Phenolics have been known for their different biological activities mainly as anti-bacterial, anti-viral, anticancer, antioxidant and anti-diabetic, among others[22]. In the present study, the total phenolic content (TPC) of ESSFR4 purified from *E. superbum* seed extract was found to be 48.57 ± 0.20 mg gallic acid equivalent (GAE)/g of the sample (Supplementary Table 1). The TPC quantified in the fraction was slightly better than the previous study of *E. superbum* seed extract prepared in methanol (38.2 ± 1.7 mg GAE/g of the extract)[15]. However, the present study did not show total flavonoid content (TFC) in ESSFR4 at 1 mg/ml concentration. The absence of TFC and variation in TPC in the present study may be because the recovery of phenolics was greatly influenced by several factors such as geographic location, variation in environmental factors, solvents used for the extraction, etc.[23].

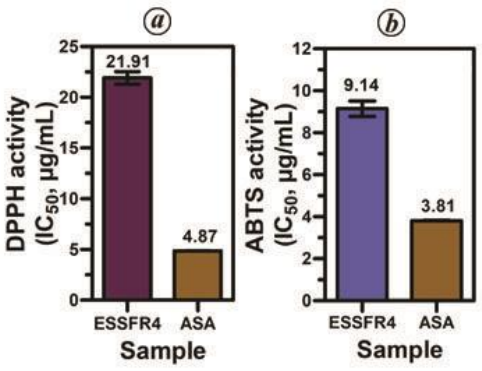

Figure 2. Antioxidant activity of the purified fraction (ESSFR4). ***a***, DPPH free-radical scavenging activity. ***b***, ABTS free-radical scavenging activity. The free-radical scavenging activities of the fraction are represented as IC_{50} values. Values are mean of triplicates (n = 3) with standard deviation (SD). All the values are statistically significant at $P < 0.05$ plotted using GraphPad Prism-5. ESSFR4, *Ensete superbum* seed fraction number-4 and ASA, Ascorbic acid.

GC-MS analysis

The GC-MS is considered as one of the versatile techniques in the field of metabolomics due to its robustness, outstanding separation capacity, sensitivity, high selectivity and reproducibility[24]. The GC-MS-based profiling of AGIs has been documented from different plants, including *Tetracera scandens*[14], *Cosmos caudatusi*[25], etc. In the present study, the GC-MS chromatogram of ESSFR4 revealed five major peaks at different retention times. These peaks correspond to the five different compounds (Figure 3 and Supplementary Table 2). There are minor peaks that have not been identified due to low abundance. The compounds identified from ESSFR4 were pentasiloxane, dodecamethyl-(compound-**1**), phthalic acid, butyl tetradecyl ester (compound-**2**), inositol, 1,2,3,4,5,6-hexakis-*O*-(trimethylsilyl)-, epi-(compound-**3**), 2,4-hexadienedioic acid, bis(trimethylsilyl) ester, (E,E)-(compound-**4**) and alpha-D-glucopyranuronic acid, 1,2,3,4-tetrakis-*O*-(trimethylsilyl)-, trimethylsilyl ester (compound-**5**). Eleven compounds were identified in *E. superbum* seeds which was extracted in 95% methanol using the GC-MS technique[26]. Also, compounds such as phytic acid[27], 4-(4-hydroxy-3-methyl-hex-5-enyl)-chroman-2,7-diol[28] and delphinidin-3-rutinoside and cyanidin-3-rutinoside[29] have been identified from different parts of *E. superbum* (Roxb.) Cheesman in earlier studies. All these compounds were from a crude extract. However, in the present study, a semi-purified fraction was analysed using GC-MS and five compounds have been identified from *E. superbum* seeds.

An organosilicon compound, compound-**1**, was identified in *Helleborus vesicarius* plant, where the extract showed antibacterial activity when tested against different Gram-positive and Gram-negative bacteria[30]. GC-MS-based phytochemical screening of *Hibiscus asper* leaves revealed the presence of compound-**1** in a study conducted by Olivia *et al.*[31]. Compound-**2** was identified in *Euphorbia hirta* L. leaves, where the study predicted that the compound could be responsible for the anti-inflammatory property of the plant[32]. Compound-**3** was identified in the rhizomes of *Curcuma aeruginosa* Roxb. in an earlier study[33]. Also, the GC-MS-based detection of compound-**3**

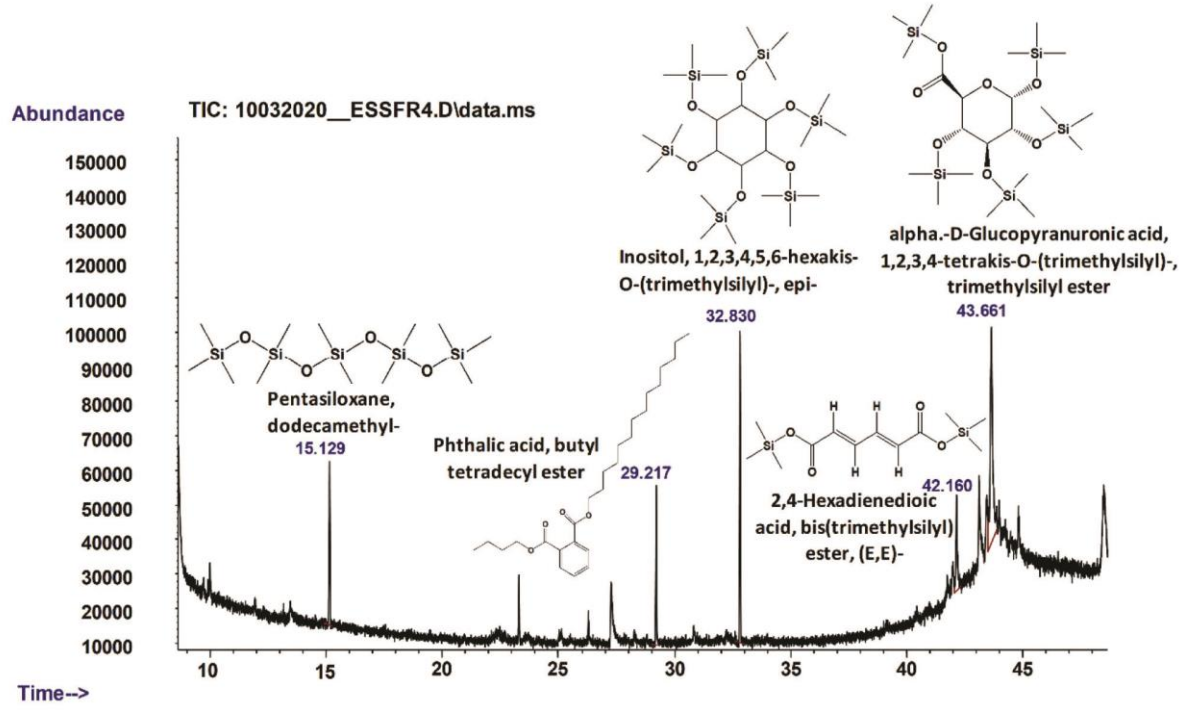

Figure 3. GC-MS chromatogram and structure of the identified compounds from the purified fraction (ESSFR4). Five peaks with the highest peak area at different retention times were annotated for identification of the compounds. The compounds corresponding to the five peaks were identified using the National Instrument of Standard Technology library attached with the instrument. The structures of the compounds were drawn using ChemDraw.

was made in the non-toxic extract of *Mimosa tenuiflora* (Willd.) Poiret plant, where the extract showed antioxidant activity and antinociceptive effect[34]. However, to the best of our knowledge, compound-**4** and compound-**5** have not been reported earlier in any of the plant sources.

In the present study, four (compounds **1**, **3**, **4** and **5**) out the five compounds identified have silane moiety in their structure. Interestingly, the compounds with silane moieties were identified in the different plant extracts reported in earlier studies, where some of the extracts have shown different biological activities[34,35]. However, to the best of our knowledge, none of the compounds identified in the present study has been reported for its AGI activity so far.

FTIR analysis

FTIR analysis of ESSFR4 revealed several bands which correspond to the various functional groups of the compounds (Supplementary Figure 1). The band at 3394 cm^{-1} may be due to stretching vibrations of O–H and N–H groups of phenols. The band at 2928 cm^{-1} could be due to the O–H stretching of intramolecular bonded alcohols. The bending vibrations of C=C and N–H groups of esters appeared at 1608 cm^{-1}. The band that appeared at 1447 cm^{-1} could be attributed to the presence of an aromatic C=C bond. The band at 1724 cm^{-1} may be due to the existence of a C=O bond. The bending of CH_3 groups could be attributed to the band at 1370 cm^{-1}. The band at 1246 cm^{-1} could be attributed to the C–O stretching vibration of esters and acids. Interestingly, few of the detected bands could be related to the silane groups of the compounds identified in the present study. For example, the band at 1145 cm^{-1} could be associated with the Si–CH_3 moiety. The bending vibration in Si–O–C might be related to a band at 769 cm^{-1}. It is also possible that the bands at 769 and 833 cm^{-1} could be related to Si–CH of the silane compounds[36–38].

Molecular docking analysis

Docking studies of different compounds against the homology model of YAG were recently conducted to understand the interactions between the compounds and enzymes[39]. The present study revealed that among the five compounds identified from ESSFR4, compounds **4** and **1** had the highest binding energy of –3.799 and –3.448 kcal/mol against YAG and Ct-MGAM enzymes respectively (Supplementary Table 3). Figure 4 shows the interactions between the compounds with high binding energies and active-site residues of the enzymes.

The interaction between YAG and compound-**4** revealed that the residue ARG439 formed a hydrogen bond with the oxygen atom of that compound. Also, ARG312

which is one of the active-site residues and constitutes the side chain of the enzyme was involved in hydrogen bond formation with the oxygen atom of compound-**4**. Further, the hydrophobic interactions of PHE157, PHE158, PHE177, PHE300, PHE311 and TYR313 could facilitate the stacking of compound-**4** in the active-site pocket of the enzyme (Figure 4 *a* and *b*). No hydrogen bond interaction was observed between YAG and compounds **1**, **2** and **5**. Compound-**3** did not show any interactions with the active-site residues of the YAG enzyme. However, the presence of compounds **1**, **2** and **5** in the active site could be due to the strong hydrophobic interactions with the aromatic residues which border the active-site pocket of YAG (Supplementary Figure 2). Interestingly, the hydrophobic interactions of the compounds with PHE177 and TYR71 are considered to be crucial because both the residues play a vital role in the recognition of the terminal ring of the substrates[40]. However, similar interactions were observed in previous studies, where ARG312 and ARG439 formed hydrogen bonds with different compounds which acted as inhibitors of the AG enzyme[41]. Also, both ARG312 and ARG439 along with the other active-site residues were involved in the interaction of standard AGI, acarbose[42], indicating that compound-**4** could occupy the active site of the YAG enzyme and hinder substrate activity similar to that of acarbose.

The interaction of Ct-MGAM and compound-**1** revealed that there was no hydrogen bond formation. Nonetheless, the aromatic residues TYR1251, TRP1355, TRP1369, PHE1427, PHE1559 and PHE1560 surrounding the active site could be stabilizing compound-**1** in the active site of Ct-MGAM (Figure 4 *c* and *d*). Also, both compounds **2** and **4** interacted through similar residues like compound-**1**. Additionally, compound-**4** interacted through hydrogen bonds with LYS1460 and ARG1510 (Supplementary Figure 3). Among the interacting aromatic residues, TRP1369 may contribute to the stabilization of the third ring of acarbose in the +2 subsite of Ct-MGAM. Also, it is noteworthy that TYR1251 is one of the important active-site residues that plays a vital role in the cleavage of α-1,4 glucosidic linked substrates along with other residues[7]. It is noteworthy that LYS1460, ARG1510, TYR1251 and TRP1369 along with other residues are involved in the interaction between acarbose and Ct-MGAM (Supplementary Figure 4). Hence, due to the interactions of TRP1369 and TYR1251 along with the high binding energy, compound **1** could be an effective candidate that can inhibit the Ct-MGAM enzyme; however, this needs further detailed studies.

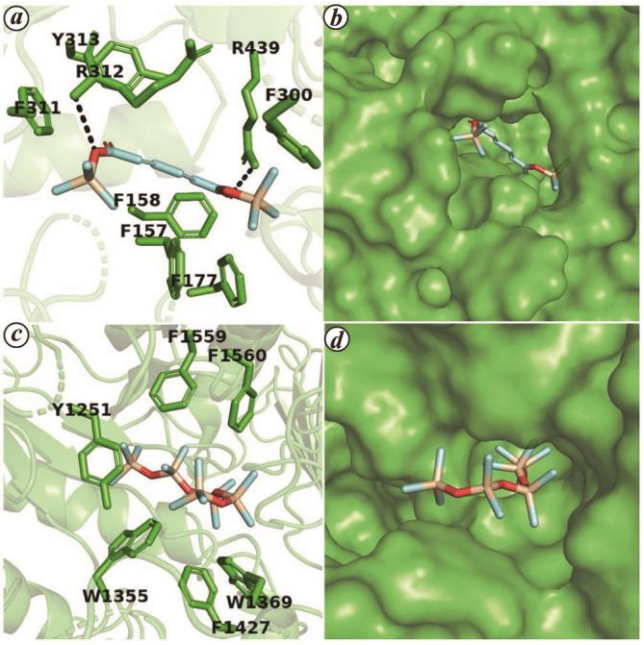

Figure 4. Docking study of the identified compounds from ESSFR4. ***a***, Interaction of compound-**4** with the active-site residues of YAG represented in a stick view. ***b***, Active-site of YAG represented in a surface view along with compound-**4**. ***c***, Interaction of compound-**1** with the active site residues of Ct-MGAM represented in a stick view. ***d***, Active site of Ct-MGAM represented in a surface view along with compound-**1**. The 3D images were drawn using Pymol.

ADME/toxicity evaluation

The main intention of the absorption, distribution, metabolism, excretion (ADME), and toxicity evaluations is to determine the pharmacological and pharmacodynamics properties of compounds within the biological system in order to develop them as appropriate candidates for drug molecules[43]. The physico-chemical properties of all the compounds are provided in the Supplementary Table 4. During the evaluation of drug-like properties, the compound-**4** had passed all the rules without any violations with a bioavailability score of 0.55. Compound-**1** had only one violation for Muegge rules, with a bioavailability score of 0.55. Compounds **2**, **3** and **5** had one or more violations for Lipinski, Ghose, Veber, Egan and Muegge rules (Supplementary Table 5). The lipophilicity and water solubility of the compounds are some of the important factors that affect the absorption and distribution of drugs in the body[44]. The lipophilicity (consensus log $P_{O/W}$) of the compounds ranged from 2.50 to 6.83. Also, among the five compounds, compounds **1** and **4** showed water solubility under different water-solubility classes (Supplementary Table 6). Analysis of the radar plot revealed that compound-**4** obeyed all the properties, whereas compound-**1** violated only one property (LIPO). Additionally, compounds **2**, **3** and **5** violated more than one property of the radar plot (Supplementary Figure 5). Compounds **1** and **4** showed properties of blood–brain barrier (BBB) permeation, whereas compound-**1** acted as a substrate for P-glycoprotein (PGP+). The remaining compounds were the non-substrate for P-glycoprotein (PGP–). Compounds **1**, **4** and **5** exhibited gastrointestinal (GI) absorption. In the case of cytochrome-P450 enzymes, CYP3A4 was inhibited by compound-**2** while compound-**4** acted as an inhibitor for

CYP2C19 and CYP2C9 enzymes (Supplementary Table 7 and Supplementary Figure 6). Also, all the compounds obeyed the PAINS alerts without any violations, whereas one or more violations were observed by all the compounds for Brenk and lead likeness alerts in the medicinal property category (Supplementary Table 8).

Salmonella typhimurium reverse mutation assay (AMES) toxicity is one of the preliminary drug-screening tests which predicts the mutagenic properties of the analysed compounds in the DNA of *S. typhimurium*[43]. Interestingly, none of the analysed compounds was positive for the AMES toxicity test. Compounds **1** and **4** have been predicted to have carcinogenic property and all the compounds belong to class III of acute oral toxicity, except compound-**2**. The highest rat acute toxicity was observed for compound-**2** (LD_{50} = 1.02 mol/kg) and the lowest, value was predicted for compound-**5** (LD_{50} = 2.61 mol/kg) (Supplementary Table 9). However, *in vitro* analysis of the fraction containing these compounds was effective against the AG enzyme. Thus, though the ADME/toxicity evaluations of the identified compounds are not encouraging, future *in vitro*/*in vivo* studies on these compounds would provide more details, which is beyond the scope of the present study.

Conclusion

The aqueous extract of *E. superbum* (Roxb.) Cheesman seeds and its purified fraction (ESSFR4) showed remarkable AGI activity during *in vitro* analysis. The detailed characterization of ESSFR4 revealed the presence of five compounds in this study. The molecular docking analysis of compounds **1** and **4** showed comparatively similar interactions when compared to acarbose against AG enzymes. This indicates that both compounds could act as effective inhibitors like acarbose. However, before considering them as therapeutics for the successful management of T2D, further detailed *in vitro* and *in vivo* studies are necessary.

Conflict of interest: None.

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