Section A- Research paper

Anti-alpha-amylase property of *Avicennia marina*, mangrove plant from Indian Sundarban

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Declaration
The authors have declared that no competing interests exist.

Section A- Research paper

ABSTRACT

Oral administration of aqueous extract of *Avicennia marina* leaves (ALE) is known to reduce serum blood glucose level and improve organ functions in diabetic models of rats. The anti α-amylase property of crude ALEagainst human salivary amylase (HSA) has been investigated in this work as it can be the possible reason behind the abovesaid hypoglycemic effect. TheALE was found to inhibit ~90% of HSA, comparable to standard blockers while the inhibition was only ~45% against fungal diastase (FD). Known α-amylase blocker quercetin, found in ALE through GCMS, inhibits HSA and FD 91% and 42% respectively. Blind molecular docking showed that quercetin binds to HSA in the same pocket as miglitol whereas it interacts differently with FD. The atomic contact energy of the interactions also support the fact. Hence, quercetin or derivatives present in ALE has a definite role in lowering of blood sugar concentration by inhibiting □-amylases in our alimentary canal.

Keywords: *Avicennia marina*, quercetin, anti □-amylase activity, anti fungal diastase activities, molecular docking

KEY WORDS : *Avicennia marina,* alpha-amylase inhibition, Mangrove plants, Secondary metabolites

Section A- Research paper

INTRODUCTION

The Sundarbans delta, and the Sundarbans mangrove ecosystem encompassing India and Bangladesh, is a rich reservoir of flora and fauna due to the presence of extremely dynamic physico-chemical factors [Naskar and Guha Bakshi**,** 1987]. There are more than 46 true mangrove species belonging to 14 families and 22 genera, which includes 42 species and 4 natural hybrids in Indian Sundarbans [Pandisamy, Saxena, Jayaraj and Mohan, 2016]. Mangrove plants have been used in folklore medicines and extracts from mangrove species have proven inhibitory activity against human, animal, and plant pathogens [Sahoo, Mulla,. Ansari, and Mohandass, 2012]. Tribal people of Sundarbans use crude extracts of different parts of specific mangrove plants to treat diseases like cancer, rheumatism, inflammation, painful arthritis, asthma, in control of blood sugar level, for hepatoprotection etc. [Bandaranayake, 1998; Rangasamy, Kumaravel, and Ranganathan, 2019; Sundaram and Murugesan, 2011]. The current study is to evaluate the role of *A.marina* leaves on the antidiabetic property as the incidence of diabetes mellitus is on the rise all over the world and it is a chronic disorder which needs to be controlled through medication [Global report on diabetes, WHO 2016]. Along with insulin injections and medicines, another way to control blood glucose concentration is by inhibiting the activity of □-amylase in the mouth and in the gut [Hamdan., Afifi and Taha, 2004]. This enzyme α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) hydrolyses α-D-(1–4) glucosidic linkages in starch and other polysaccharides andincrease the availability of free sugar for absorption through GI tract.

Some specific mangrove plant extracts are known to possess anti α-amylase and α-glucosidase activity [Ranjana and Jadhav, 2019]. After screening of many mangrove plants from Patharpratima, South 24 parganas, West Bengal, for anti α-amylase activity, *Avicennia marina* (Avicenniaceae) was selected as previous reports show that the leaf extracts of this grey mangrove have hypoglycemic effects in diabetes models of rat [Ali et al, 2020; Okla et al., 2019]. However, there was no report on its α-amylase inhibition activity. The available α-amylase blockers like acarbose, miglitol, voglibose etc. have considerable side effects on human health, so the current focus is on plant products or derivatives having anti α-amylase properties with minimal harmful effects. In this paper, phytochemicals of *A.marina* first evaluated through its secondary metabolite contents then specifically identified through GC-MS analysis.

MATERIALS AND METHODS

Chemicals and reagents

Glucose, tris, soluble starch, hydrochloric acid, sodium hydroxide, sodium carbonate, dinitro salicylic acid, potassium-sodium tartrate, 1,1-diphenyl-2-picrylhydrazyl,ethanol, methanol, acetone, n-hexane were obtained from Hi-Media. Human salivary alpha amylase, fungal diastase, quercetin, miglitol were obtained from Sigma-Aldrich.

Collection of samples

Fresh twigs of *A.marina* were collected from Patharpratima, Sundarban area, West Bengal with prior permission from forest department in the month of March. Twigs of each sample were preserved for herbarium sample and identified and authenticated by experts from Indian

Botanical Garden, Shibpur, Kolkata. Leaves from twigs of each sample were collected, washed, sun-dried and kept in clean sterilized glass container in powdered form at 25^0C.

Preparation of crude extract

The crude extracts of the sample in different solvents for each experiment were obtained by using powdered sample of same dry weight with different solvents in same volumes. In each case, 5mg of dried leaf powder was soaked in 1ml of solvent overnight, centrifuged at 20000 rpm, supernatant collected and filtered with the help of Whatman no. 1 filter paper. The filtrate was diluted and used as the crude sample extract.

Alpha-amylase inhibition assay

An α-amylase inhibition assaywas performed according to the chromogenic DNSA method [Miller, 1959].Enzyme (0.05mg/ml) and ALE (1mg/ml) were mixed in 1:1 ratio and incubated at 25^0C for 10 minutes followed by the addition of the same volume of starch (0.2mg/ml) and incubated for 5 minutes. DNSA was added in double volume and incubated in a water bath at 90^0C for 5 minutes. The reaction was stopped by the addition of sodium potassium tartrate solution and OD was taken at 540nm after cooling. The amount of glucose formed was determined from the glucose standard curve. Miglitol, a standard α-amylase drug was taken as the positive control. The percentage of inhibition was measured by the following formula:

$$Percentageinhibition = \frac{Absorbancewithoutinhibitor - Absorbancewithinhibitor}{Absorbancewithoutinhibitor}$$

Estimation of secondary metabolites

Total phenol

The content of total phenol was measured by the Folin-Ciocalteu method [Ainsworth and Gillespie, Nature Protocol]. 1g of dry sample was ground with a mortar and pestle in 5ml of 80% ethanol. The supernatant was evaporated and dissolved in 5ml of distilled water. 1ml of this was treated with 0.5ml of Folin-Ciocalteau reagent (reagent diluted at 1:1 ratio), and 20% sodium carbonate solution and boiled for 1 minute. The colour intensity was measured at 765nm against a reagent blank and content was expressed as mg/g gallic acid equivalent.

Total flavonoids

The amount of total flavonoid was measured by aluminium chloride assay [Kamtekar, Keer and Patil, 2014]. 1ml of ALE was diluted to 5ml and 0.3ml of 5% sodium nitrite was added to it. After 5mins of incubation, 0.3ml of 10% aluminium chloride was added and mixed properly. After 1min incubation, 2ml of 1M sodium hydroxide was added to the mixture. Immediately, the mixture was diluted by the addition of 3.3ml distilled water and mixed thoroughly. The absorbance was determined at 510nm versus a blank. Quercetin was used as standard for the calibration curve. Total flavonoids content of the extract was expressed as mg quercetin equivalents per 100gm of dry mass.

Total flavonols

Total flavonols were estimated according to the method of Kumaran and Karunakaran [Kumaran and Karunakaran, 2006]. 1gm leaf was crushed in 10ml of methanol and centrifuged. 1ml of extract was mixed with 1ml of 2% $AlCl_3$ ethanol and 3ml sodium acetate solution (50g/L) and incubated for 2.5hours at 20^0C. Absorbance were taken in 440nm and the content of flavonol was expressed as mg equivalent quercetin/gm of dry leaves.

Tannin

0.5g of dried powdered materials were boiled for 30minutes in 75ml water and volume made up to 75ml. 1 ml of this extract was diluted up to the volume 75ml by adding more water. 5ml of Folin-Denis reagent and 10ml of sodium carbonate solution were added to the diluted extractions and incubated for 30minutes in 25^0C. Colour intensity of the reaction mixture were read at 700nm and expressed as mg equivalent of tannic acid. [Saeed, Khan and Shabbir, 2012]

Determination of IC50 values

The IC50 values of ALE of *A. marina* was found out and calculated by logarithmic regression analysis. To eliminate the absorbance produced by the compound, appropriate controls were included. Six different concentrations (0.5mg/ml, 1mg/ml, 2mg/ml, 3mg/ml, 4mg/ml, 5mg/ml) were used for *A. marina* leaf extracts and well-known amylase and glucosidase blockers, acarbose and miglitol in DNSA inhibition assay, keeping other parameters constant. All the experiments were triplicated and measured against a suitable control every time.

Identification of secondary metabolites by Gas chromatography and Mass spectrometry (GCMS)

Gas chromatography and mass spectrometry of the methanolic extract of the leaves of *A. marina* was done to identify the phytochemicals present in them. [Ghose, Brahmachary, Ray and Kumar, 2019]

Study of interaction between the enzymes and ligands

The cannonical smiles and 3D structures of the flavonoids, acarbose, voglibose and miglitol were obtained in SDF format from PubChem. Two enzymes, fungal diastase (PDB ID: 3A4A) and human salivary amylase (PDB ID: 1SMD) were chosen for docking purposes. The selected ligands were docked with the enzymes separately using PATCHDOCK and the

results were selected based on both DOCKING SCORE and ATOMIC CONTACT ENERGY (ACE) [Schneidman-Duhovny et al., 2005]. The pockets in the enzyme where ligands bind was studied using DOGSITE SCORER and the best suited pocket with the amino acid residues interacting with the enzyme was determined [DoGSiteScorer, 2012]. The interactions were compared with that of standards. The docked complexes were placed in PDBSUM to identify the molecular interaction between ligands and enzymes [Laskowskiand Swindells, 2011]. Docking results were verified by Autodock using PYRX [Dallakyan and Olson, 2015].

Statistical analysis

Each experiment was carried out 5 times with each set-in triplicate. Results are presented as mean±SD. The best-fit values were achieved by applying either linear fit or non-linear least square regression using the regression calculator. Differences were considered statistically significant for $p < 0.05$.

RESULTS

α-amylase inhibition by the crude extract

The ALE of *A.marina* was found to inhibit HSA to the maximal of 90% though only 45% of inhibition was seen for FD at a conc. of 5mg dry leaves/ml of water. The positive control, acarbose, voglibose and miglitol was found to inhibit HSA ~92%, 88% and ~89% respectively at the same concentration, whereas they inhibit FD ~87%, ~89%, ~82%

respectively (Figure 1 and Fig 2). The inhibition ability of the leaves was also checked in other solvents like methanol, ethanol, hexane, acetone and found to be comparable with the aqueous extract. Hence, all the experiments were carried out with the aqueous extract of the leaves.

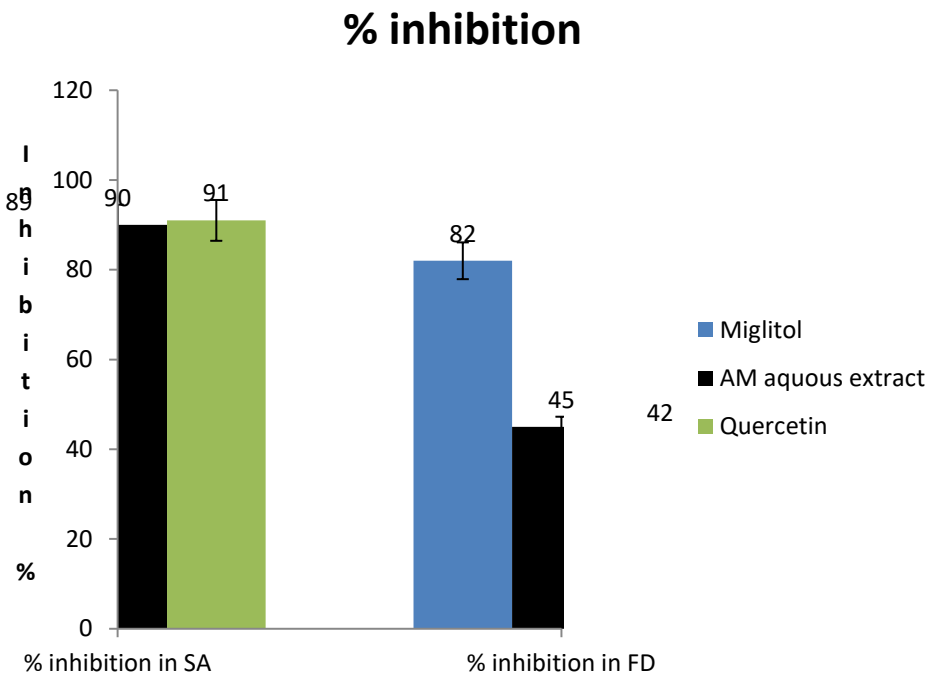


Figure 1: Percentage inhibition of HSA by aqueous extract of plant leaves of *Avicennia marina*, standard amylase blocker miglitol and Quercetin.

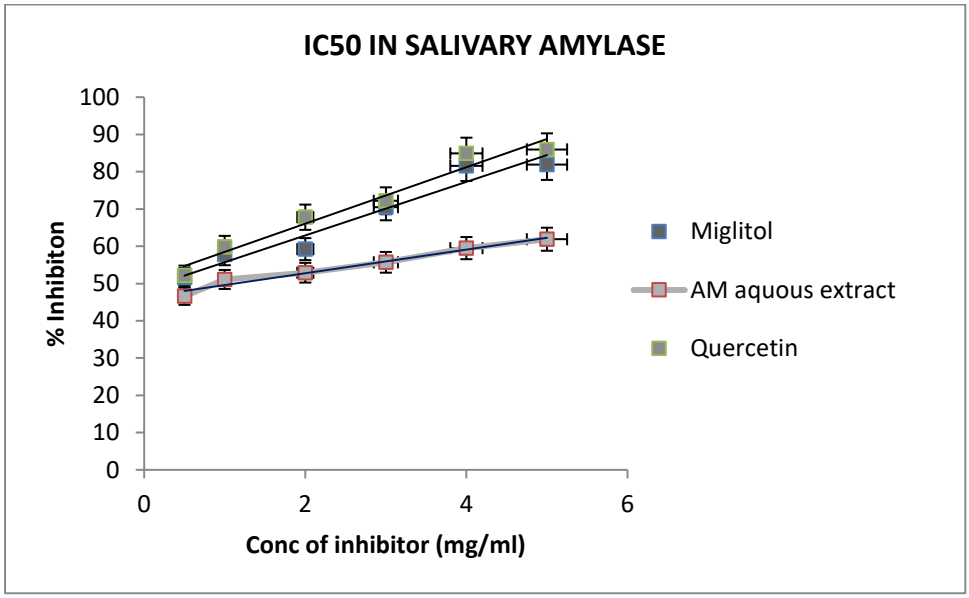


Figure 2: IC50 values of aqueous extract of *A. marina* and standards like miglitol and quercetin against HSA.

IC50 values

The IC50 values of ALE of *A.marina* against HSA and FD were found to be 0.61mg/ml and 1mg/ml respectively. The IC50 values of miglitol, voglibose and acarbose were 0.5mg/ml, 0.59mg/ml and 0.22mg/ml & 0.79mg/ml, 0.39mg/ml and 0.31 mg/ml against HSA and FD respectively (Table 1).

Sample	%Inhibition in FD	%Inhibition in HSA	IC50 against FD (mg/ml)	IC50 against HSA (mg/ml)	Nature of inhibition against alpha amylase
Acarbose	87	92	0.31±0.005	0.22±0.005	Competitive
Miglitol	82	89	0.76±0.005	0.50±0.005	Competitive
Voglibose	89	88	0.39±0.005	0.59±0.005	Mixed
Crude *A. marina* leaves aqueous extract	45	90	1.00±0.005	0.61±0.005	Competitive
Ethanol extract of *A. marina* leaves	51	89	0.89±0.005	0.55±0.005	Mixed

Quercetin	42	91	0.97±0.005	0.30±0.005	Competitive

Table 1: Percent inhibition and IC50 values of standard and aqueous and ethanolic extract of *A. marina*

Determination of secondary metabolites in *A.marina*

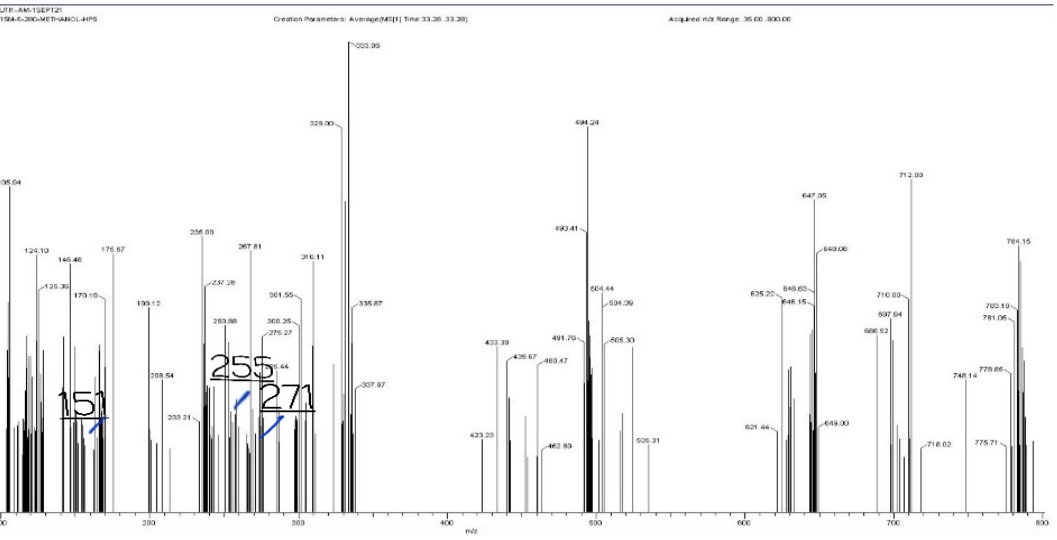
The leaves of *A.marina* were rich in secondary metabolites with total polyphenol content of 104.4±6.2mg/gm of dry leaves, total tannin content of 84.5±5.4mg tannic acid equivalent/gm, total flavonoid and flavonol content of 52.4±4.1 and 14.5±2.3mg quercetin equivalents/gm respectively (Table 2).

Polyphenol (mg/gm dry leaves)	Tannin (mg tannic acid/gm)	Flavonoid (mg quercetin equivalents/gm)	Flavonol (mg quercetin equivalent/gm)
104.4±6.2	84.5±5.4	52.4±4.1	14.5±2.3

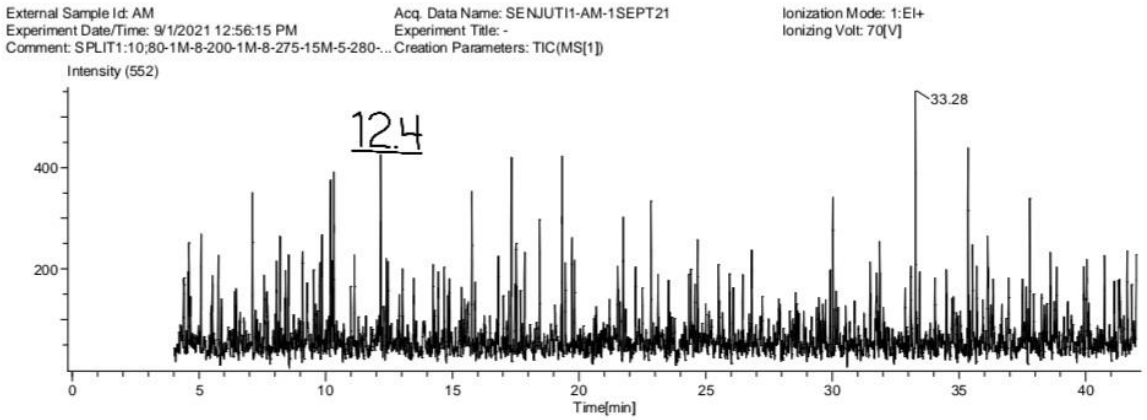
Table 2: Quantity of secondary metabolites found in the aqueous extract of *A. marina* leaves.

GCMS analyses of the methanolic leaf extract of *A.marina*

The identification ofchemical constituents was based on comparison of theirrelative retention times and mass spectra with those obtained from authentic sample and/or the NIST/NBS andWiley libraries spectra.GCMS analysis of the methanolic leaf extract of *A.marina* showed the presence of quinoline, isoquinoline derivatives, thiazole, imidazole, oxazolone, cyclohexanone, steroids, anilines flavonoids etc. Two well-known flavonoids, naringenin and quercetin derivatives were observed (Figure 3). Figure 3A presents the MS data showing m/z peak of quercetin, figure 3B presents GC data showing retention time of quercetin (12.4 min), and figure 3C showing the measured area percentage from peak obtained from GC-graph.



A



B

Name of the compound	Molecular formula	Molecular weight	% area	m/z	Retention time (min)
4H-1-Benzopyran-4-one,2-(3,4 dihydroxyphenyl)-3,5,7-trihydroxy- Quercetin (pubchem cid: 5280343)	$C_{15}H_{10}O_7$	302.236 g/mol	40	170.19,255	12.400

C

Figure 3: GCMS data showing A. MS data showing m/z peak of quercetin B. GC data showing retention time of quercetin (12.4 min), C. measured area percentage from peak obtained from GC-graph.

Analysis of Drug Likeliness and Studies on Interactions using Molecular docking

Fungal diastase (3A4A) and human salivary amylase (1SMD) were chosen for docking port as macromolecules from PDB. Blind docking was performed using patchdock and results with the lowest ACE and highest docking scores were chosen and saved as PDB files. These files were observed on CHIMERA and the active sites were identified using Dogsite scorer protein plus software. The molecular interactions between the protein and the ligand were studied using PDBsum. The amino acid composition in the binding pocket (pocket 1) of HSA

for quercetin, naringenin and miglitol are almost similar. Only acarbose binds to pocket 1 of domain A of HSA. The -OH group attached to 7th carbon of quercetin and the -OH group attached to 13th carbon of naringenin make H-bond with Tyr 2 of HSA (2.39 A^0 and 2.35 A^0respectively) (Table 3). In the case of miglitol, a H-bond length of 3.65A^0 was seen between the hydroxyl group attached to carbon 4 of miglitol and Tyr 2(A). In case of voglibose, H-bonds were seen with proline 228(A) and glycine 249(A) of HSA with bond length 4.92 A^0 and 6.30A^0 respectively. All the other amino acid residues Leu 211, 214, Asn 216, Lys 227, Ileu 230 in the pockets are same (Table 3). The atomic contact energy (ACE) value of quercetin, naringenin, miglitol and voglibose with HSA were found to be -204.5, -200 and -116.7 and -110.78 Kcal/Mol respectively suggesting strong binding interactions between enzyme and ligands.

In the case of interaction with FD, the 3D binding poses are different to some extent among the ligands. Miglitol, voglibose and quercetin bind to the same pocket (pocket 0) involving amino acid residues from domain A and C though miglitol forms a H-bond with His 423 which was missing in case of quercetin. Naringenin binds to a different pocket (pocket 2) in FD, which is like acarbose binding site. The ACE values for the interactions of quercetin, naringenin, acarbose, voglibose and miglitol with FD were -59, -110.89, -109.78, -111.22 and -32.21Kcal/Mol respectively. (Table 3)

Ligands	Mol. Interaction with HSA	Mol. Interaction with FD	Pocket involved in FD	Pocket involved in HAS

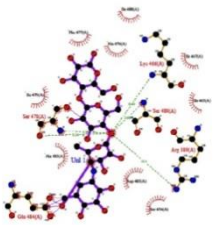
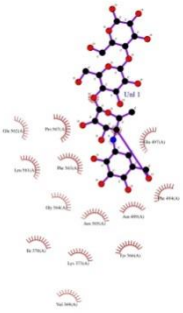
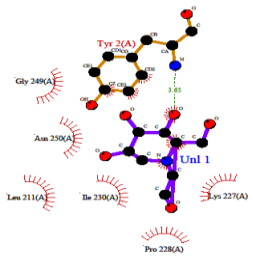
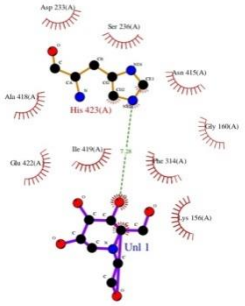
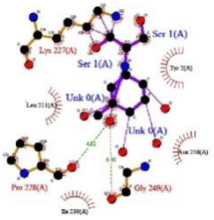
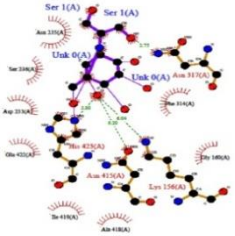
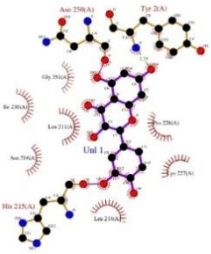
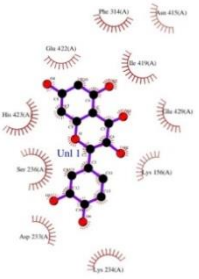
Acarbose			Pocket 2	Pocket 3
Miglitol			Pocket 0	Pocket 1
Voglibose			Pocket 0	Pocket 1
Quercetin			Pocket 0	Pocket 1

Table 3: **Comparative molecular interactions of standards and quercetin with HSA and FD.**

Determination of a-amylase inhibition and IC50 value of quercetin

Quercetin was found to have considerable anti HSA activity of 91% whereas it showed only 42% of inhibition against FD. The IC50 values were 0.30±0.005mg/ml and 0.97±0.005mg/ml against HSA and FD respectively (Table 1, Figure 2).

DISCUSSION

Secondary metabolites like polyphenols and flavonoids serve as excellent sources of various therapeutic agents. There are compounds like quercetin 3-(6-malonylglucoside), rutin, isoquercetin, epigallocatechin gallate, caffeic acid, p-coumaric acid, curcumin derivatives, diosgenin which are known to reduce blood sugar concentration [Zhan, Liu, Li and Yun, 2016]. Some plant extracts like *Phylanthus amerus* are known to inhibit α-amylase and α-glucosidase activity due to presence of those compounds in it [Iniyan et al., 2010; Mauldina, Sauriasari and Elya, 2017; Jhong et al., 2015]. *A.marina* leaf extracts are known to have many properties like antioxidant, antihyperglycemic, antiapoptotic etc. The extract acts as an antihyperglycemic agent when given orally to mice with streptozotocin induced diabetes [Ali et al, 2020]. It also showed neurobehavioral effects on autoimmune diabetic mice [Okla et al., 2019]. In the present study, the potential anti-α-amylase inhibitory activity (~90%) of ALE of *A. marina* against HSA was evaluated. The IC50 values of crude ALE was quite low, 0.61mg/ml against HSA. However, the extract didn't show same level of activity against FD and the IC50 value was 1mg/ml. Total polyphenol, flavonoid and flavonol content in the *A.marina* leaves were quite high and GCMS analysis confirmed the presence of flavonoids like naringenin and quercetin derivative which are known antioxidants and α-amylase blockers. Molecular docking interaction showed that quercetin binds to HSA in a similar

pattern to voglibose and miglitol with much lower ACE value while it interacts differently with FD. ACE value of quercetin, with HSA were found to be -204.5Kcal/Mol suggesting strong binding interactions between the enzyme and ligand which corresponds to the 90% inhibition of HSA by the ALE. The ACE values for the interactions of quercetin with FD were -59Kcal/Mol and quercetin interacts with FD differently than standard α-amylase blockers. The higher ACE value for binding of quercetin to FD (-59Kcal/Mol and 42% of inhibition) justifies 45% inhibition of FD by ALE. Hence, the presence of quercetin and their derivatives are the main reason behind the antiα-amylase activity of the aqueous leaf extract of *Avicennia marina*.

CONCLUSION

The leaves of the mangrove plant, *Avicennia marina*, are rich in polyphenols specially in flavonoids including quercetin. This molecule is a potent antioxidant and is known to inhibit α-amylase. Hence, the observations received earlier of lowering blood glucose concentrations by oral administration of *A.marina* extracts, possibly because of the inhibition of salivary and pancreatic α-amylases, which results in less absorption of sugar through the gut wall. Though people of Sundarbans use the mangrove plant extracts for many years, more research on *A.marina* should be carried on to characterize all the phytochemicals present in it for their roles on human health.

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CONFLICTS OF INTEREST

The authors confirm that there is no conflict of interests.

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