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Original Article

Evaluation of Bioactive Compounds from Various Citrus Fruits in Bangladesh and Evaluation of Their Effect as Antioxidant and on Glucose Metabolizing Enzymes *In Vitro*

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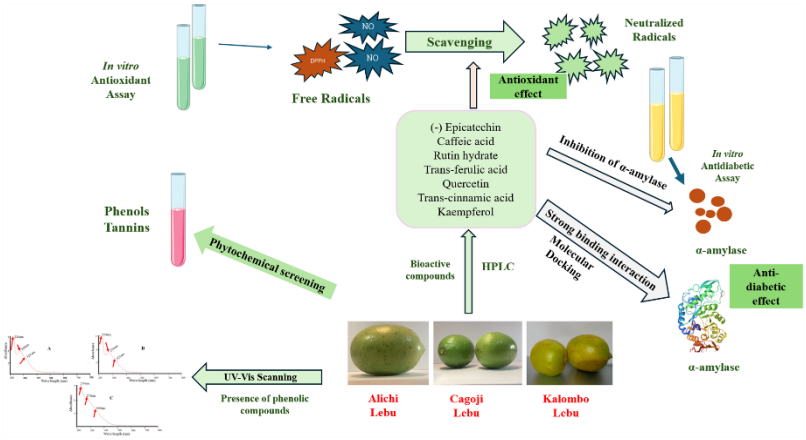
Abstract: Synthetic antioxidants are used against oxidative stress and nitrosative stress, which have many harmful effects and possess lower efficacies; hence, the interest of using natural antioxidants such as citrus fruits as therapeutic tools exist. This study set out to assess the flavonoids identified from various citrus fruits in Bangladesh (cagoji lebu, kalombo lebu, and alichi lebu) as antioxidants and their effect on α-amylase inhibitory activity in *in vitro* condition. Phytochemical screening, UV-vis spectra and HPLC-DAD analysis were performed to determine the class, nature and identity of bioactive components. Antioxidant potential was determined by determining the total phenol content, flavonoid content, ortho diphenol content, DPPH free radical scavenging assay and nitric oxide scavenging assay. α -Amylase inhibitory assay was done to evaluate the effect on glucose metabolizing enzymes. Molecular docking was done for alpha-amylase enzyme affinity. Phenols and tannins were found in 3 citrus extracts and all the extracts are composed of flavonoid compounds as they provided clear peaks at 280 nm and/or 330 nm in the UV-vis scan data. HPLC analysis showed the presence of (-) epicatechin, caffeic acid, rutin hydrate, *trans*-ferulic acid, quercetin, and kaempferol in all 3 extracts, and *trans*-cinnamic acid in cagoji and kalombo lebu. The three extracts show significant phenolic content, the amount for alichi, cagoji and kalombo lebu were 13.958±0.001, 13.380±0.0005, 11.773±0.001 mg GAE/ g DW respectively; flavonoid contents were 3.324±0.0005, 2.827±0, 7.589±0 mg QAE/ g DW respectively and ortho diphenol contents were 0.124±0, 0.185±0.118, 0.161±0 mg CAE/ g DW respectively. The extracts of three citrus fruits exhibited the comparable antioxidant activity for both DPPH and NO scavenging methods compared to ascorbic acid. In the *in vitro* α-amylase inhibitory activity assay, the IC_{50} values of alichi, cagoji and kolombo lebu were 56.45, 39.97 and 55.31µg/mL respectively whereas the IC_{50} value of acarbose was 25.53 µg/mL. This investigation revealed that these three citrus fruits in Bangladesh are potential sources of bioactive compounds which can be used as alternative supplements for natural antioxidants.

Keywords: Citrus; bioactive compounds; antioxidants; α-amylase



Graphical abstract: The phenolic compounds are strong scavengers of free radicles and inhibited the α-amylase activity.

1. Introduction

Reactive nitrogen species (RNS) and reactive oxygen species (ROS) are byproducts of regular cellular metabolism with a variety of physiological functions. Nonetheless, excessive ROS and RNS generation and a lack of both enzymatic and non-enzymatic antioxidant defense systems occur during oxidative stress, which eventually results in cellular malfunction and death [1]. In recent years, many diseases have appeared and are mainly due to "oxidative stress" and "nitrosative stress". Evidences that ROS accumulation in biological systems causes oxidative tissue damage and affects cellular integrity [2]. Lipid peroxides, singlet oxygen, superoxide anion, and hydroxyl radical are a few of them. Elevated ROS levels can harm lipids, proteins, DNA, and RNA among other components since they are highly reactive [3]. Oxidative damage caused by ROS has often been the origin of the pathogenesis of several diseases such as aging, arthritis, cancer, inflammation, diabetes and heart diseases [4]. Many antioxidants and ROS scavengers, both synthetic and natural, have been created and researched recently in order to protect biomolecules against the damage by ROS [5]. The most widely used synthetic antioxidants are butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate, and tert-butylhydroquinoneare; these substances are known to have the ability to inhibit free radicals in the human body, but they can also be harmful and pose a risk for liver damage, fatal hemorrhages and carcinogenesis in laboratory animals [6-8]. Moreover, despite their higher manufacturing costs, certain synthetic antioxidants have lower efficacies than natural antioxidants [9]. Therefore, finding naturally occurring antioxidants and studying their pharmacological effects through *in vitro* and *in vivo* studies is the most important and urgent research for the safety of human life.

Citrus is one of the world's major horticultural crops, with a global production of 100 million metric tons per year due to their multiple health benefits, refreshing scent, and flavor [10]. Citrus fruits have been known for sources of natural antioxidants Vitamin-C for many years. Recently, citrus flavonoids are considered as valuable bioactive compounds showed strong antioxidant and anti-inflammatory activities [11]. Phenolic chemicals, particularly flavonoids and phenolic acids, are abundant in the peels of citrus fruits. Studies on the effects of nutrition on living things have shown that citrus flavonoids are safe and harmless [12]. Naturally occurring flavonoids, which are mostly found in peel, are also abundant in citrus plants. Citrus fruits are rich in bioflavonoids, which are the most well-known agents that protect cancer. These natural substances include hesperidin, narirutin, naringin, neohesperidin, eriocitrin, neoeriocitrin, rutin, diosmin, neoponcirin, and nobiletin [13]. Because of their antioxidant action, natural polyphenols have a positive impact on health. They can eliminate free radicals, chelate metal catalysts, activate antioxidant enzymes, lower α-tocopherol radicals, and block oxidases [14]. Numerous pharmacological effects of these flavonoids include protection against coronary heart disease, suppression of important enzymes in mitochondrial respiration, and anti-spasmolytic, anti-inflammatory, antioxidative, vascular, estrogenic, cytotoxic, antitumor, and antibacterial actions [15]. Citrus flavonoids,

naringin and hesperidin showed protective effect in diabetes and in obesity [16]. A class of metabolic disorders known as diabetes mellitus is typified by persistently high blood sugar levels brought on by deficiencies in either insulin production, insulin action, or both. Furthermore, as mitochondrial ATP generation is required for hormone secretion, ROS may influence the long-term decline of insulin secretory capability at the islet β-cell level. Additionally, it seems that adipose, liver, and muscle tissue's sensitivity to insulin is significantly influenced by mitochondrial function. One of the most popular methods for lowering or postponing the intestinal absorption of glucose is to suppress enzymes that hydrolyze carbohydrates, like α-amylase [17, 18]. One of the most effective inhibitors of the enzymes that hydrolyze carbohydrates, acarbose, is commonly linked to adverse effects such acidity, diarrhea, bloating, fatigue, cramps, and stomach pain [19]. As strong antioxidants, these citrus flavonoids can protect against free radicles mediated damage to the tissues in diabetes and obesity condition. Moreover, citrus flavonoids may protect pancreatic beta cells from oxidative stress and restored the insulin production as well as reduced the insulin resistance in the muscles and peripheral organs [20]. However, their mechanism in glucose lowering effect in diabetes is still not explained properly. α-amylase and β-glucuronidase are important enzymes responsible for the breakdown of carbohydrate and make glucose available in plasma [21]. A vital digesting enzyme is pancreatic α-amylase. Majority of human starch digestion is caused by this calcium-based metalloenzyme, which functions as a catalyst and helps break down the α-1,4 glycosidic linkages of polysaccharide molecules like glycogen, amylose, amylopectin, and other maltodextrins [22]. In diabetes, inhibition of these enzymes may reduce the glucose production from carbohydrate digestion and reduces the plasma glucose concentration. This investigation will thus, evaluate the citrus fruits extract for the presence of flavonoids as antioxidants and their effect on α-amylase inhibitory activity in *in vitro* condition. Moreover, this investigation will also focus on the comparison of various citrus fruits extracts found in Bangladesh.

2. Materials and Methods

2.1 Collection of plant materials

Cagoji lebu, *Citrus aurantifolia*; kalombo lebu, *Citrus sinensis*; and alichi lebu, *Citrus limon* fruits were collected from the local market from Dhaka city, Bangladesh. The fruits were authenticated by the experts in National Herbarium, Mirpur, Dhaka, Bangladesh. The voucher specimen was preserved and accession numbers for these plants are DABC 99123, DABC 99125, and DABC 99126 respectively.

2.2 Extract Preparation from Citrus Peel Powder

By collecting the 3 different types of (Cagoji lebu, *Citrus aurantifolia;* Kalombo lebu, *Citrus sinensis;* and Alichi lebu, *Citrus limon*) citrus peels, it was cut into small pieces, washed with tap water properly, and dried in open air. The dried peels were then grinded into coarse powder. After that it was put into the jar and added enough ethanol (70% v/v) to make sure that all the peel powder was soaked in the solvent ethanol. After 10 days, it has been observed that the solvent color is changed. Then, the extract was separated from the remaining debris portion by using a funnel and cotton. The extract was then put into the rotary evaporator so that all the ethanol could be separated from the extract. After separation of ethanol from the extract, a dense sticky extract was found. Then it was collected in a petri dish for further test.

2.3 Quantitative and Qualitative Analysis Through HPLC

2.3.1 Chemicals

Gallic acid, 3,4-dihydroxybenzoic acid, catechin hydrate, catechol, (-) epicatechin, caffeic acid, vanillic acid, syringic acid, rutin hydrate, *p*-coumaric acid, trans-ferulic acid, rosmarinic acid, myricetin, quercetin, trans-cinnamic acid and kaempferol were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC), methanol (HPLC), acetic acid (HPLC), and ethanol was obtained from Merck (Darmstadt, Germany). α-amylase, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and naphthyl ethylenediamine dihydrochloride was obtained from Sigma-Aldrich Chemical Co. (USA). Starch soluble (extra pure) was obtained from J.T. Baker Inc., Phillipsburg, USA. Other chemicals and reagents used were of analytical grade.

2.3.2 HPLC Analysis

Detection and quantification of selected polyphenolic compounds in citrus peels extracts were determined by HPLC-DAD analysis as described by Ahmed et al. (2021) with some modifications [23]. HPLC analysis was performed on a LC-20A (Shimadzu, Kyoto, Japan) equipped with a binary solvent delivery pump (LC-20AT), an auto sampler (SIL-20A HT), column oven (CTO-20A) and a photodiode array detector (SPD-M20A) and controlled by the LC solution software (Lab Solution Separation was performed using Luna C18 (5µm) Phenomenex column (4.6 x 250 mm) at 33ºC. The mobile phase composed of A (1% acetic acid in acetonitrile) and B (1% acetic acid in water) with gradient elution: 0.01-20 min (5-25% A), 20-30 min (25-40% A), 30-35 min (40-60% A), 35-40 min (60-30% A), 40–45 min (30–5% A), and 45–50 min (5% A) was used in this study. The sample injection volume was 20µL, and the flow-rate was set at 0.5 mL/min. The UV detector was set at 270 nm and applied for validation of method and analysis. The mobile phase was filtered through a 0.45 µm nylon 6, 6 membrane filter (India) and degassed under vacuum. For the preparation of calibration curve, a standard stock solution was prepared in methanol containing Gallic acid (20 µg/ml); 3,4-dihydroxybenzoic acid (15 µg/ml); catechin hydrate (50 µg/ml); catechol, (-) epicatechin, rosmarinic acid (30 µg/ml each); caffeic acid, vanillic acid, syringic acid, rutin hydrate, *p*-coumaric acid, trans-ferulic acid, quercetin (10 µg/ml each); myricetin, kaempferol (8 µg/ml each); trans-cinnamic acid (4 µg/ml).

2.4 DPPH Radical Scavenging Assay

In HPLC grade methanol, a DPPH solution (0.004% w/v) was prepared [24]. To make the stock solution (500 µg/mL), the crude extracts were combined individually with milliQ water. One mL extracts were taken to the test tubes to make serial dilution of different concentrations (12.5 µg/mL to 500 µg/mL). The freshly made 1 mL DPPH solution (0.004% w/v) were added to each test tube. 10 minutes of incubation period was given and all test tubes were placed in a dark place. The absorbance was measured at 515 nm using a spectrophotometer (HACH 4000 DU UV-visible spectrophotometer). As a reference standard, ascorbic acid was dissolved in milliQ water to create a stock solution with the same strength (500 mg/mL). Percent scavenging of the DPPH free radical activity was measured by using the following equation:

% of inhibition = [(absorbance of the control – absorbance of the test sample) / absorbance of the control] X 100

Lower absorbance of the reaction mixture indicates higher free radical-scavenging activity. All the tests were performed as triplicates.

2.5 Nitric Oxide (No) Radical Scavenging Assay

For NO radical scavenging assay, a previously described method was used [25]. At a physiological pH, sodium nitro-prusside solution produced nitric oxide (NO) radicals. In phosphate buffer, 1 ml of sodium nitroprusside (10 mM) was combined with 1 ml of ethanolic extracts of various concentrations (12.5 - 150 g/ml) (pH 7.4). 150 minutes were spent incubating the mixture at 25°C. Griess' reagent (1% sulphanilamide, 2% o-phosphoric acid, and 0.1% naphthyl ethylene diamine dihydrochloride) was added to 1ml of the incubated solution. The absorbance was measured at 546 nm, and the formula for % inhibition was applied are mentioned below:

% of inhibition = [(absorbance of the control – absorbance of the test sample) / absorbance of the control] X 100

2.6 Alpha Amylase Inhibitory Assay

Sodium phosphate buffer (0.02 M), 250 µl was added with citrus extracts with alpha amylase solution (0.5 mg/mL). Before phosphate buffer was added mixer was pre-incubated for 10 minutes at 25 °C with addition of 1% starch. To stop the reaction, dinitrosalicylic acid was added. After that it was kept in boiling water for 5 minutes which was then cooled to room temperature. The absorbance was taken using a spectrophotometer at 540 nm. Acarbose was used as standard reference.

% Inhibition = [Absorbance of control – Absorbance of extract/Absorbance of control] ×100

The IC50 or 50% inhibition of enzyme activity was determined.

2.7 Molecular Docking

2.7.1 Receptor Preparation

The receptors, alpha-amylase (PDB ID: 1BLI), and beta-glucosidase (PDB ID: 3TA9) as PDB format was downloaded from Protein Data Bank. (https://www.rcsb.org/). By using PyMol, water molecules and original ligands were deleted [24, 26]. Autodock tools 1.5.7 was used to prepare the protein; Addition of polar hydrogen, and Kollman charge. The receptors were saved as PDBQT format [27, 28].

2.7.2 Ligand preparation

The 3D structure of ligands, caffeic acid (Pubchem ID: 689043), epicatechin (PubchemID:72276), kaempferol (Pubchem ID: 5280863), quercetin (Pubchem ID: 5280343), rutin hydrate (Pubchem ID: 16218542), trans-cinnamic acid (Pubchem ID: 444539), trans-ferulic acid (Pubchem ID: 445858), gallic acid (Pubchem ID: 370), hesperidin (Pubchem ID: 10621), naringin (Pubchem ID: 442428), nobiletin (Pubchem ID:72344) were downloaded from Pubchem as SDF format (https://pubchem.ncbi.nlm.nih.gov/). PDB format is required for using Autodock tools, therefore using PyMol, SDF files were converted into pdb format. By using Autodock tools 1.5.7, the ligands were prepared for docking and saved as PDBQT file. [28, 29].

2.7.3 Grid preparation

The grid menu of Autodock tools was used to calculate the autogrid parameters [29]. The receptor, alpha-amylase (PDB ID: 1BLI) was uploaded as pdbqt format and from the grid menu, grid box was selected and a box appeared. The default centers X, Y, Z -36.510, 35.680, -8.028 respectively, the grid dimension 40 X 40 X 40, and the spacing 0.375 Å were taken under consideration. The grid files were saved as gpf format [28].

2.7.4 Docking

AutoDock Vina is a complete computational docking method based on a quick conformational search and a basic scoring system [29]. The default techniques in AutoDock and AutoDock Vina have been extensively utilized for applications like virtual screening since they are quite efficient for typical drug-like ligands [30]. AutoDock Vina 1.5.7 was run by using command prompt [31] , and the docked file was saved as pdbqt format [30].

2.7.5 Visualization:

For visualizing 2D and 3D structure of docked protein and ligand Bovia Discovery studio client was used 2021 [29].

3. Results

3.1 Qualitative analysis

3.1.1 Phenol and tannins presence in citrus fruits peel extracts

The following **Table 1** showed the presence of phenolic compounds in several citrus extracts using chemical class test. All samples showed the presence of phenols and tannins.

Table 1: Presence of phenol and tannins in all the three citrus fruits peel extracts.

Sample name	Name of the Chemical Class	
	Phenol	**Tannins**
Alichi lebu	+	+
Cagoji lebu	+	+
Kalombo lebu	+	+

3.1.2 UV-Vis Spectra

The UV-vis spectra provide initial information about the nature of the compounds found in different citrus fruits. This preliminary data serves as a foundation for further analysis and helps researchers understand the types of molecules present in the plant. The information obtained from the UV-vis spectra is then used to identify specific wavelengths that are suitable for monitoring through HPLC. Different compounds absorb light at different wavelengths, and selecting the right wavelengths enhances the accuracy and sensitivity of the HPLC analysis. As anticipated, citrus fruits contain different types of polyphenolic compounds. All the extracts possess flavonoid compounds provided a clear peak at 280nm and/or 330nm in the UV-vis scan data which are the universal γ_{max} for flavonoids. To avoid repetition, we explained only alichi lebu extract scan data. The UV-vis spectrum of the uninfected sample is described in detail. In acidic solution, it exhibits a major band at 330 nm with shoulders at 204, and 259 nm. The band at 330 nm is possibly due to the presence of flavones and/or hydroxycinnamic acid derivatives. On the other hand, in neutral solution, a major band at 330 nm with shoulders at 258, and 280 nm was visible in alichi lebu ethanol extract. The alkali solution showed a very clear peak at 229 and 321 nm with shoulders at 210, and 285nm which indicated the presence of phenolic compounds.

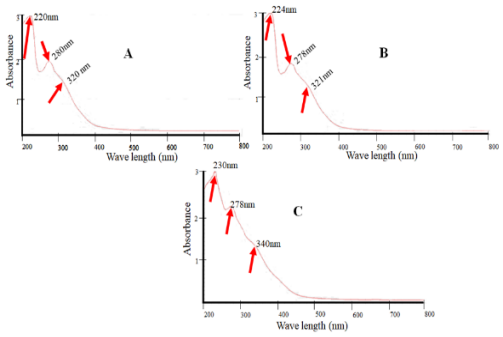


Figure 1. UV-Visible scan data of alichi lebu extract in neutral solution (Fig A) showed very clear peak at 220, 280 and a shoulder at 320 nm; in acidic solution (Fig B) peak at 224, 278 nm and a shoulder at 321 nm and in alkali solution (Fig C) showed very clear peak at 230, 278, and a shoulder at 340nm which indicated the presence of phenolic compounds.

3.2 Quantitative analysis for the determination of total phenol, flavonoids content and ortho-diphenol content

3.2.1 Determination of total phenol content

The total phenolic content present in the three citrus extracts are represented in the **Table 2**. These extracts possess considerable phenolic contents and are presented as gallic acid equivalent.

Table 2. Table for the determination of total phenolic content for Alichi lebu, Cagoji lebu and Kalombo lebu extract

Sample Name	mg GAE/ g DW
Alichi lebu	13.958±0.001
Cagoji lebu	13.380±0.0005
Kalombo lebu	11.773±0.001

3.2.2 Determination of total flavonoids content

The total flavonoids content present in the three citrus extracts are represented in the **Table 3**. These extracts possess considerable amount of flavonoids contents and are presented as quercetin equivalent.

Table 3. Determination of total flavonoids content for alichi lebu, cagoji lebu and kalombo lebu extract

Sample Name	mg QAE/ g DW
Alichi lebu	3.324±0.0005
Cagoji lebu	2.827±0
Kalombo lebu	7.589±0

3.2.3 Determination of ortho-diphenol content

The total ortho-diphenol content present in the three citrus extracts are represented in the **Table 4**. These extracts possess considerable amount of flavonoids contents and are presented as caffeic acid equivalent.

Table 4. Determination of total ortho-diphenol content for alichi lebu, cagoji lebu and kalombo lebu extract

Sample Name	mg CAE/ g DW
Alichi lebu	0.124±0
Cagoji lebu	0.185±0.118
Kalombo lebu	0.161±0

3.3 Qualitative and quantitative analysis using HPLC

The **Figure 2** represented the HPL chromatogram of sixteen phenolic compounds. The quantitative analysis of different phenolic antioxidants present in the citrus fruit peel extracts are presented in the **Figure 3**, **Figure 4** and **Figure 5**. Gallic acid, 3,4-dihydroxybenzoic acid, catechin hydrate, catechol, (-) epicatechin and caffeic acid are found in most of the extracts through HPLC-DAD analysis. The amount of these phenolic compounds present in the citrus extracts are shown in the **Table 5**.

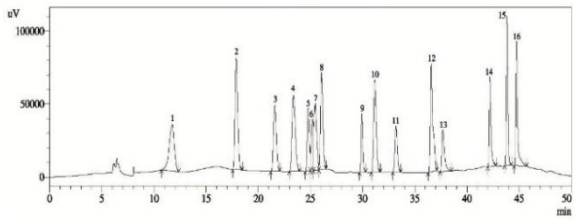


Figure 2: HPLC chromatogram of standard phenolic compounds presented chronologically- Gallic acid, 3,4-dihydroxybenzoic acid, catechin hydrate, catechol, (-) epicatechin, caffeic acid, vanillic acid, syringic acid, rutin hydrate, *p*-coumaric acid, trans-ferulic acid, rosmarinic acid, myricetin, quercetin, trans-cinnamic acid, kaempferol.

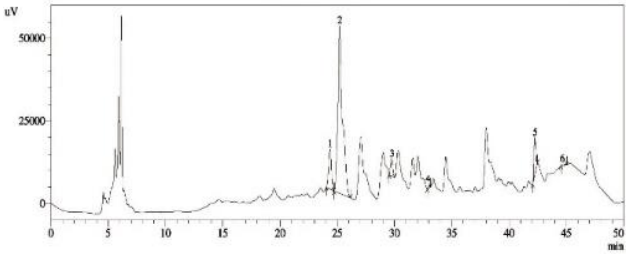


Figure 3: HPLC chromatogram of ethanolic extract of **alichi lebu extract**. Peaks: 1, (-) epicatechin; 2, caffeic acid; 3, Rutin hydrate; 4, Trans-ferulic acid; 5, Quercetin; 6, Kaempferol.

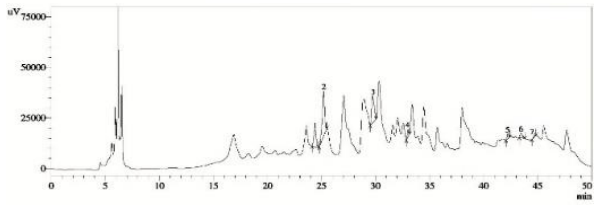


Figure 4: HPLC chromatogram of ethanolic extract of cagoji lebu extract. Peaks: Peaks: 1, (-) epicatechin; 2, caffeic acid; 3, Rutin hydrate; 4, Trans-ferulic acid; 5, Quercetin; 6, Trans-cinnamic acid; 7, Kaempferol.

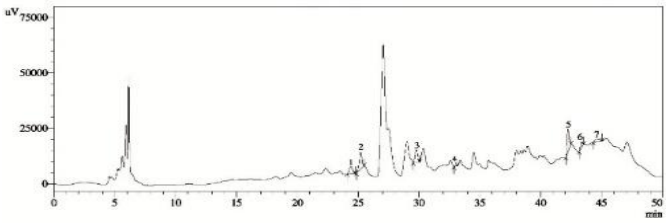


Figure 5: HPLC chromatogram of ethanolic extract of kalombo lebu extract. Peaks: 1, (-) epicatechin; 2, caffeic acid; 3, Rutin hydrate; 4, Trans-ferulic acid; 5, Quercetin; 6, Trans-cinnamic acid; 7, Kaempferol.

Table 5. HPLC quantification of phenolic compounds present in alichi lebu, cagoji lebu and kalombo lebu peel extracts

Serial no.	Name of the standard compound	Al (mg/100 g dry extract)	Cl (mg/100 g dry extract)	Kal (mg/100 g dry extract)
1	Gallic acid	-	-	-
2	3,4-dihydroxybenzoic acid	-	-	-
3	Catechin hydrate	-	-	-
4	Catechol	-	-	-
5	(-) epicatechin	85.95 ± 0.40	25.19 ± 0.84	60.85 ± 0.24
6	Caffeic acid	271.68 ± 1.46	24.94 ± 0.77	69.62 ± 0.19
7	Vanillic acid	-	-	-
8	Syringic acid	-	-	-
9	Rutin hydrate	15.25 ± 0.14	18.68 ± 0.73	45.89 ± 0.27
10	P-coumaric acid			
11	Trans-ferulic acid	1.08 ± 0.03	1.49 ± 0.44	6.57 ± 0.01
12	Rosmarinic acid	-	-	-
13	Myricetin	-	-	-
14	Quercetin	15.92 ± 0.17	15.21 ± 0.31	3.08 ± 0.04
15	Trans-cinnamic acid	-	1.25 ± 0.12	1.09 ± 0.26
16	Kaempferol	0.50 ± 0.04	2.58 ± 0.36	0.68 0.03

3.4 Free radical scavenging and enzyme inhibition assays

Free radicle scavenging activity are represented in **Figure 6**. All the extracts showed considerable free radicle activities in DPPH and NO scavenging assay system (**Figure 6A** and **Figure B**). The DPPH scavenging system showed that the ascorbic acid is a strong scavenger of DPPH free radicles with IC_{50} value 15.40 µg/mL, whereas the alichi lebu, cagoji lebu and kalombo lebu extracts showed comparable DPPH free radicles with IC_{50}

value 32.32, 31.69, and 24.65 µg/mL respectively (**Table 6**). Similar trend was also seen in NO scavenging assay system (**Figure 6B**). The ascorbic acid was the strongest scavenger of NO compared to the alichi lebu, cagoji lebu and kalombo lebu extracts. However, the IC50 values for all the extracts were also considerably close to the ascorbic acid (**Table 6**). All citrus peel extracts also showed strong inhibitory activity in α-amylase inhibition assay compared to acarbose (**Figure 6C**). The acarbose IC50 value for α-amylase inhibition assay was 25.53 µg/mL, whereas the alichi lebu, cagoji lebu and kalombo lebu extracts showed considerable IC_{50} values (56.45, 39.97 and 55.31 µg/mL respectively) (**Table 6**).

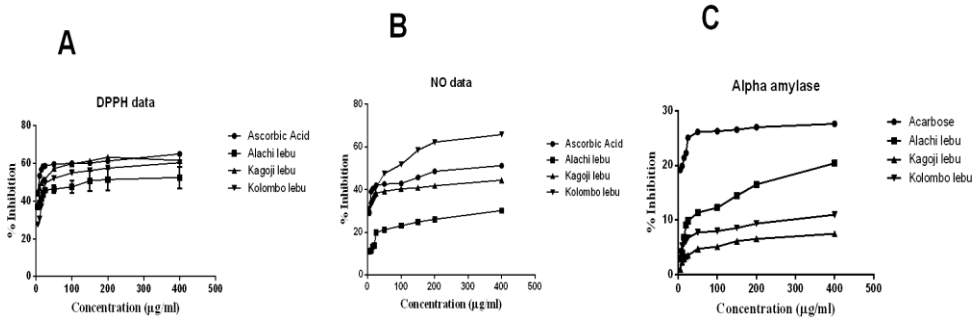


Figure 6: Effect of DPPH scavenging, NO scavenging and alpha amylase inhibitory assays in alichi lebu, cagoji lebu and kalombo lebu extract.

Table 6: The IC_{50} values of alichi lebu, cagoji lebu and kalombo lebu in different assay systems

Assay name	Ethanolic extracts of	IC_{50} value (µg/ mL)
DPPH Scavenging Assay	Alichi lebu	32.32
	Cagoji lebu	31.69
	Kolombo lebu	24.65
	Ascorbic acid	15.40
NO Scavenging Assay	Alichi lebu	56.20
	Cagoji lebu	27.98
	Kolombo lebu	60.41
	Ascorbic acid	22.31
Alpha Amylase Inhibitory Assay	Alichi lebu	56.45
	Cagoji lebu	39.97
	Kolombo lebu	55.31
	Acarbose	25.53

3.5 The docking study for the phenolic compound present in the extracts of alichi lebu, cagoji lebu and kalombo lebu peel.

The binding parameters of various polyphenolic compounds are presented in the **Table 7** and **Table 8** for the alpha-amylase and beta-glucosidase enzymes. Best rank poses of interaction of α-amylase and beta-glucosidase enzymes with various polyphenolic compounds are presented in **Figure 7, Figure 8** and **Figure 9**.

Table 7. Binding parameters of citrus components with the alpha-amylase

Receptor	Ligand	Affinity Kcal/mol	Number of Hydrogen bonds	Residue	Amino Acid	Distance (Å)
Alpha-amylase (1BLI)	Caffeic acid *Pubchem ID: 689043*	-6.4	3	A: 138	TRP	2.49
				A: 163	THR	2.44
				A: 164	ASP	2.44
	Epicatechin *Pubchem ID: 72276*	-7.5	2	A: 164	ASP	2.13
				A: 106	LYS	2.89
	Kaempferol *Pubchem ID: 5280863*	-7.6	4	A: 231	ASP	2.63
				A: 263	TRP	1.91
				A: 261	GLU	2.46
				A: 334	SER	2.15
	Quercetin *Pubchem ID: 5280343*	-7.6	1	A: 261	GLU	2.1
	Rutin hydrate *Pubchem ID: 16218542*	-9.1	6	A: 4	ASN	2.01
				A: 5	GLY	2.73
				A: 98	TYR	2.18
				A: 317	PRO	2.33
				A: 318	LYS	2.29
				A: 319	LEU	2.3
	Transcinnamic acid *Pubchem ID:* 444539	-5.9	2	A: 108	GLY	2.81
				A: 109	ALA	2.05
	Transferulic acid *Pubchem ID:* 445858	-6.5	3	A:109	ALA	2.07
				A: 109	ALA	2.31
				A: 138	TRP	2.28

Note: ALA: Alanine, ASP: Aspartic acid, ASN: Asparagine, GLN: Glutamine, GLU: Glutamic acid, GLY: Glycine, LEU: Leucine, LYS: Lysine, PRO: Proline, SER: Serine, THR: Threonine, TRP: Tryptophan, TYR: Tyrosine.

Table 8. Binding parameters of citrus components with the alpha-amylase and beta glucosidase

Receptor	Ligand	Affinity Kcal/mol	Number of Hydrogen bonds	Residue	Amino Acid	Distance (Å)
Alpha-amylase (1BLI)	Gallic acid *Pubchem ID: 370*	-6.2	3	A: 163	THR	2.21
				A:163	THR	2.21
				A:138	TRP	2.29
	Hesperidin *(Pubchem ID: 10621)*	-9.7	8	A:53	ASP	2.42
				A:193	TYR	3.02
				A:232	ALA	2.90
				A:261	GLU	2.14
				A:261	GLU	2.23
				A:334	SER	2.54
				A:334	SER	2.98
				A:334	SER	3.19
	Naringin *(Pubchem ID: 442428)*	-8.9	3	A:94	ASP	2.32
				A:98	TYR	3.19
				A:320	SER	2.25
	Nobiletin *(Pubchem ID:72344)*	-7.2	2	A:98	TYR	3.03
				A:357	GLY	3.35
Beta-glucosidase (3TA9)	Gallic acid Pubchem ID: 370	-6.3	6	A:129	GLN	2.40
				A:129	GLN	3.10
				A:131	LYS	2.37
				B:135	THR	2.77
				B:184	THR	3.22
				B:189	THR	3.00
	Hesperidin *(Pubchem ID: 10621)*	-12.4	8	A:132	GLY	2.31
				A:135	THR	2.58
				A:129	GLN	3.14
				A:183	GLY	2.11
				B:41	THR	3.25
				B:129	GLN	3.00
				B:189	THR	2.70
				A:41	THR	3.07

	Naringin *(Pubchem ID: 442428)*	-11.8	7	A:129	GLN	2.46
				A:132	GLY	2.69
				A:185	LYS	3.18
				A:185	LYS	3.09
				B:126	GLN	2.90
				B:182	PRO	2.42
	Nobiletin *(Pubchem ID:72344*	-8.9	5	A:129	GLN	3.06
				A:129	GLN	3.27
				B:129	GLN	2.89
				B:129	GLN	3.23
				B:44	LYS	3.13

Note: ALA: Alanine, ASP: Aspartic acid, ASN: Asparagine, GLN: Glutamine, GLU: Glutamic acid, GLY: Glycine, LEU: Leucine, LYS: Lysine, PRO: Proline, SER: Serine, THR: Threonine, TRP: Tryptophan, TYR: Tyrosine.

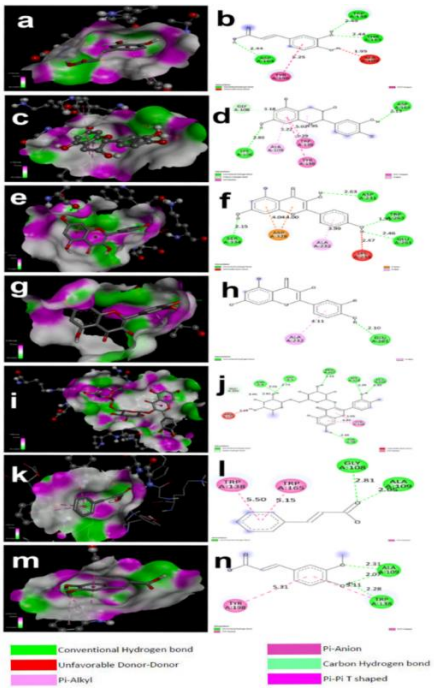


Figure 7. Best rank poses of interaction of α-amylase and caffeic acid 3D (a) and 2D (b); α-amylase and epicatechin 3D (c) and 2D (d); α-amylase and kaempferol 3D (e) and 2D (f); α-amylase and quercetin 3D (g) and 2D (h), α-amylase and rutin hydrate 3D (i) and 2D (j), α-amylase and trans-cinnamic acid 3D (k) and 2D (l), α-Amylase and trans-ferulic acid 3D (m) and 2D (n).

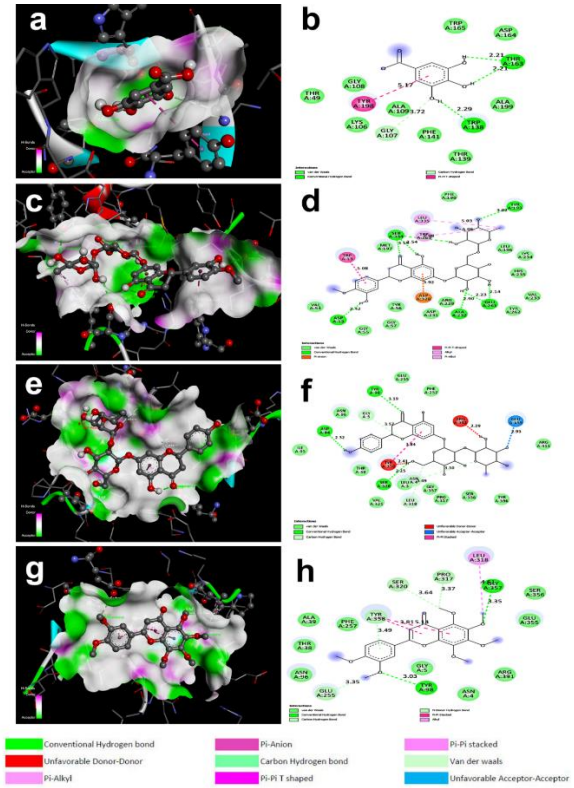


Figure 8. Best rank poses of interaction of α-amylase and gallic acid 3D (a) and 2D (b); α-amylase and hesperidin 3D (c) and 2D (d); α-amylase and naringin 3D (e) and 2D (f); α-amylase nobiletin 3D (g) and 2D (h).

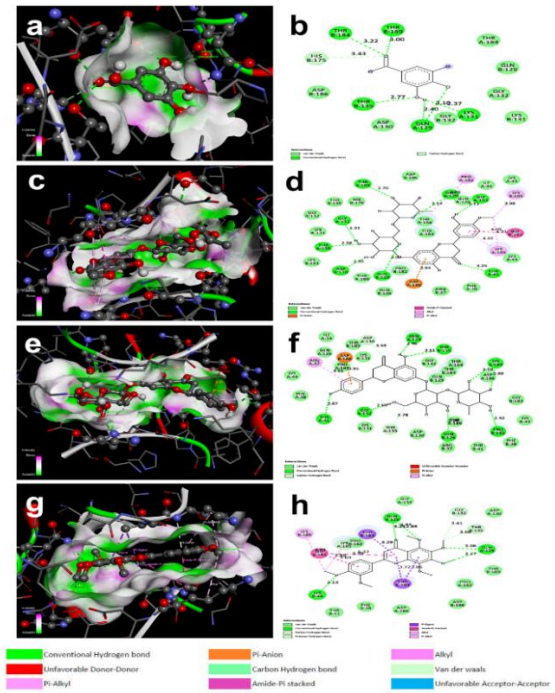


Figure 9. Best rank poses of interaction of beta-glucosidase and gallic acid 3D (a) and 2D (b); beta-glucosidase and hesperidin 3D (c) and 2D (d); beta-glucosidase and naringin 3D (e) and 2D (f); beta-glucosidase and nobiletin 3D (g) and 2D (h).

4. Discussion

Phytochemicals such as phenols, are found in plants that work with nutrients, contribute to flavor and color, dietary fibers to protect human against diseases through antioxidant activity and reduce the risk of many diseases [32]. Various citrus fruits, including, alichi, cagoji and kalombo lebu extract screening to detect the presence of phenolic components. The chemical class test revealed the presence of phenolic compounds such as phenols and their water-soluble naturally synthesized derivatives tannins in the citrus fruit extracts as anticipated from their color. The results are predictable and also justified from the previous articles [33]. The UV-scan data has followed the chemical group test results and provided peak and shoulder at 280 and 330 nm and near which were the universal wavelength for phenolic compounds [34]. Though, the chemical test and UV scan data are very tentative but we got the preliminary idea about the chemical constituents of the extracts. Phenolic compounds, such as flavonoids, hydroxylated polyphenolic compounds, perform crucial roles in plants, such as attracting pollinating insects, combating environmental stresses like microbial infection, and regulating cell growth. Among the six major subclasses of flavonoids—anthocyanidins, flavan-3-ols, flavanols, flavanones, flavones, and isoflavones—flavanols are the most prevalent in the human diet [35]. It has been suggested that consuming up to 1 g of polyphenolic compounds daily may have inhibitory effects on human carcinogenesis and mutagenesis because of their capacity to neutralize free radicals [36]. Phenolic compounds, such flavonoids and phenolic acids, attach covalently to alpha-amylase and change its activity because they can react with nucleophilic groups on the enzyme molecule to produce quinones or lactones [37].

The DPPH assay is widely used to evaluate an antioxidant product's or molecule's capacity to scavenge free radicals. It is regarded as one of the most common simple colorimetric techniques for assessing the antioxidant qualities of both natural and purified substances [38]. A stable free radical with the capacity to take an electron, DPPH gives solutions a pink or purple color. DPPH changes from pink/purple to yellow when an antioxidant gives it an electron, making it a stable diamagnetic molecule [39]. Spectrophotometric monitoring of the color shift is used to determine the characteristics associated with antioxidant capabilities. Various extracts of citrus demonstrated dose-dependent free radical scavenging activity in contrast to ascorbic acid (used as standard) in the DPPH free radical scavenging assay. The figure illustrates how an increase in ascorbic acid and citrus extract concentration in the DPPH causes an increase in the percentage of inhibition. In this investigation, the standard ascorbic acid exhibited an IC_{50} value of 15.40 µg/ml, whereas the ethanolic extract of three distinct citrus extracts, alichi lebu, cagoji lebu, and kolombo lebu, demonstrated significant free radical scavenging activity with IC_{50} values of 32.32 µg/ml, 31.69 µg/ml, and 24.65 µg/ml, respectively. The kolombo lebu extract has a lower IC_{50} value than the cagoji and alichi lebu. The lower IC_{50} of the kolombo lebu extract may be due to the higher concentration of total flavonoids content which was mentioned in **Table 2.2**. The sequence in which these three citrus fruits scavenged the DPPH free radicles was as follows: kolombo lebu>cagoji lebu>alichi lebu. Rest of the two citrus extracts have strong antioxidant capacity, as indicated by their IC_{50} values, though they are not significantly higher than ascorbic acid.

Nitric oxide modulates a variety of physiological processes, including neural signaling, smooth muscle relaxation, platelet aggregation inhibition, and cell-mediated toxicity regulation [40]. Different molecules generated from nitric oxide, such as nitroxyl anion, nitrosonium cation, higher oxides of nitrogen, S-nitrosothiols, and dinitrosyl iron complexes, are referred to as reactive nitrogen species (RNS). Increased RNS has been linked to nitrosative stress, which can cause cell damage and death [41]. The production of reactive peroxynitrite ($^{\bullet}ONOO^{-}$) by $NO^{\bullet}$ reactions intensify its toxicity and damage, and it can generate major toxic interactions with biomolecules. Scavenging NO helps to stop a series of harmful reactions that are brought on by excessive NO production [42]. Three extracts of citrus reduced the amount of nitrite generated from sodium nitroprusside and the scavenging potential was found to increase in concentration dependent manner. In this investigation, the standard ascorbic acid exhibited an IC_{50} value of 22.31 µg/ml for NO scavenging assay. Except the ethanolic extract of cagoji lebu, other two citruses, alichi lebu, and kolombo lebu, demonstrated high IC_{50} value for NO scavenging activity, (IC_{50} values of 56.20 µg/ml, and 60.41 µg/ml, respectively). Cagoji lebu has lower IC_{50} which indicates strong NO scavenging activity. Though, alichi lebu and kolombo lebu possess high IC_{50} value

than ascorbic acid, they have also strong NO scavenging activity and antioxidant capacity. So, this study suggests, these three citrus extracts have strong NO scavenging activity and strong antioxidant effect compared with ascorbic acid. The sequence of scavenging activity was as follows: cagoji lebu>alichi lebu>kolombo lebu.

High blood sugar levels, which can lead to major issues with the kidneys, eyes, and cardiovascular system, are a hallmark of diabetes. Thus, lowering blood sugar swings and ameliorating the physiological problems are the major goals of diabetes treatment [40]. The enzyme α-amylase makes glucose in the blood more bioavailable. Postprandial glucose levels are raised when α-amylases hydrolyze complex polysaccharides into oligosaccharides and disaccharides. α-glycosidase then hydrolyzes these precursors into monosaccharides, which are absorbed by the small intestine and into the hepatic portal vein [43]. According to reports, inhibiting α-amylase lowers glucose's bioavailability, which may help individuals with non-insulin dependent diabetes mellitus (NIDDM) improve their defective glucose metabolism and minimize postprandial hyperglycemia without increasing insulin secretion [44]. One commercially available enzyme inhibitor for type II diabetes is called acarbose. However, a number of secondary effects, including flatulence, diarrhea, and stomach distention, have been recorded. There is growing interest in the search for safe and efficient inhibitors derived from natural sources [37]. The α-amylase inhibitory activity of three extracts and acarbose were shown to rise in a concentration-dependent way. The IC_{50} value of acarbose for α-amylase inhibition was 25.53 µg/mL. The IC_{50} value of the extracts of alichi lebu, cagoji lebu, and kolombo lebu were followed the similar pattern as acarbose for α-amylase inhibition. The result suggested that all the selected citrus extracts assumed to be potential for α-amylase inhibitory capability. The presence of various phenolics and flavonoids may be the cause of the inhibitory potentials against the target enzymes.

Furthermore, the docking results showed that the phenolic compounds which were identified and quantified using HPLC technique had a great affinity to the α-amylase enzyme binding. Other common citrus components were taken under the consideration of docking to identify their affinity towards α-amylase and β-glucosidase. In computational study, caffeic acid, epicatechin, kaempferol, quercetin, rutin hydrate, trans-cinnamic acid, trans-ferulic acid, bind strongly with α-amylase (1BLI) by forming hydrogen bonds, rmsd was 0. Among the mentioned components, rutin hydrate showed the highest binding affinity (-9.1 Kcal/mol) towards α-amylase and formed 6 hydrogen bonds with α-amylase. Other citrus components like gallic acid, hesperidin, naringin, nobiletin also bind with α-amylase and β-glucosidase (3TA9) strongly by forming strong hydrogen bonds. Among these four components hesperidin binds with α-amylase (9BLI) with the highest binding affinity (-9.1 Kcal/mol) and eight hydrogen bonds; and hesperidin binds with the binding affinity (-12.4 Kcal/mol) and forms eight hydrogen bonds with β-glucosidase.

5. Conclusions

The escalating costs of prescribed drugs aimed at preserving health and well-being have sparked a renewed interest in traditional medicines within healthcare system. This underscores the enduring importance of plant-based traditional medicine in human healthcare for the future. Presently, a large proportion of the global population relies on herbal remedies as an integral part of their medical regimen. Additionally, medicines derived from plants offer potential advantages such as reduced costs, lower toxicity levels, or even complete absence of toxicity, facilitated by bioprospecting. The results showed that the peels extract of various citrus fruits are a good source of antioxidant phenolic compounds which also possess considerable enzyme inhibitory activities related to carbohydrate digestion. Thus, these extracts could be used as potential anti-diabetic food supplements.

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