Identification of bioactive compound from *Citrus maxima* fruit against carbohydrate-hydrolysing enzymes

S. K. Reshmi¹, H. K. Manonmani², J. R. Manjunatha³ and M. N. Shashirekha¹,*

¹Department of Fruit and Vegetable Technology, ²Department of Food Protectants and Infestation Control, and ³Department of Spices and Flavor Science, CSIR-Central Food Technological Research Institute, Mysuru 570 020, India

The aim of this study was to identify the bioactive compound of *Citrus maxima* fruit that targets the enzymes responsible for hyperglycaemia. Preliminary *in vitro* screening of extracts of fruit parts revealed that juicy segments (ethanol, methanol and aqueous) showed the highest inhibition towards the carbohydrate-hydrolysing enzymes. Further, juicy segment extracts were subjected to preparative HPLC for separation and isolation of compounds. After a series of separations, *in vitro* analysis and characterization, naringin was identified to be the bioactive compound that is responsible for inhibiting carbohydrate-hydrolysing enzyme activities. Further application, such as developing food formulations from *C. maxima* will benefit diabetic populations.

Keywords: Bioactive compound, *Citrus maxima*, carbohydrate-hydrolysing enzymes, hyperglycaemia.

DIABETES is a chronic metabolic disorder which has now become an epidemic with a worldwide incidence of 5% in the general population. Reducing postprandial hyperglycaemia is one of the therapeutic approaches to treatment at the earlier stage of diabetes, by inhibiting the carbohydrate-hydrolysing enzymes. The inhibitor of these enzymes shows a reduction in glucose absorption and blunting the increase in postprandial plasma glucose. The synthetic anti-diabetic drugs (acarbose, sulphonylureas, metformin) have certain adverse effects. Thus, many herbal medicines have been recommended for the treatment of diabetes because of their low cost and effectiveness, besides lesser side effects. In traditional and herbal medicine, citrus fruits have been recommended as the source of diabetic medication. *Citrus maxima*, commonly known as pomelo, belongs to the family Rutaceae. It is the largest citrus fruit native to Southeast Asia and Malaysia. However, it ranks at the lowest position occupying only ~6% of world cultivation, whereas other citrus fruits like oranges (Mandarin or Santa; 62%), tangerines, mandarins and clementines (~17%) and lime/lemon (11%) are of commercial importance.

*C. maxima* has been found to have several medicinal properties such as antioxidant, anti-inflammatory, anti-diabetic, anti-glycation, anti-obesity and anti-tumour activity. In ancient and medieval literature, it is reported to act as an appetizer, cardiac stimulant, stomach tonic and also as a remedy for fever, insomnia and sore throat. Though several studies have already characterized the chemical components in fruits of *C. maxima*, the active component of the fruit responsible for anti-diabetic property has still not been elucidated. So in this study we identify the bioactive compound acting against carbohydrate-hydrolysing enzyme, which is one of the important strategies in the management of type-II diabetes. This study indeed promotes the production rate of *C. maxima* by preparing various products from the fruit for health benefits.

Materials and methods

Plant material

The fruit of *C. maxima* was obtained from a local market of Mysore, Karnataka, India during the month of February. The plant was identified and authenticated by the Botanical Survey of India, Tamil Nadu Agriculture University, Southern Regional Centre, Coimbatore (2015) (voucher no. BSI/SRC/5/23/2015/Tech.467) and has been deposited in the herbarium for future reference.

Sample preparation

The peel, albedo and juicy segments of *C. maxima* fruit were collected and freeze-dried. The freeze-dried samples were grounded, powdered and stored in a zipper bag until further analysis.

Extraction of the sample

The freeze-dried powder (peel, albedo and juicy segments) of *C. maxima* (10 g) was weighed and extracted

*For correspondence. (e-mail: shashirekham33@gmail.com)
with solvents (150 ml × three times) such as methanol, ethanol, water, ethyl acetate and hexane in a shaker at room temperature for 24 h. Subsequently, extracts of the fruit fractions were concentrated using rotary vacuum evaporator at 35°C. The dried extract was weighed and resuspended in dimethyl sulphoxide (DMSO) for further analysis.

**In vitro assays**

Analysis of in vitro enzyme inhibition assay of *C. maxima* fruit against α-glucosidase, α-amylase and amyloglucosidase were performed.

**α-Glucosidase inhibitory assay:** The effect of fruit extracts on α-glucosidase (*Saccharomyces cerevisiae* α-glucosidase) activity was determined using the modified method of Kim et al. The reaction mixture containing 50 mM phosphate buffer (pH 6.8), 10 μl α-glucosidase (10 U/mg; 3.2.1.20) and 20 μl of sample of varying concentrations (5–10 mg) was pre-incubated for 5 min at 37°C. Further, 20 μl of 1 mM *p*-nitrophenyl-glucopyranoside (PNPG) was added and incubated at 37°C for 25 min. The reaction was terminated by adding 0.5 ml of 0.1 M sodium carbonate solution. The release of the final product nitrophenol (yellow colored) from the substrate (PNPG) was detected at 405 nm. The absorbance of the sample was obtained after subtracting absorbance of the sample reaction (sample + buffer + enzyme + substrate) with the absorbance of the sample control (phosphate buffer + sample) in order to nullify the colour value. In addition, a control was also prepared using the same procedure (enzyme + substrate) by replacing the extract with distilled water. The percentage of inhibition was obtained using the formula

\[
\% \text{ Inhibition} = \frac{(abs_{control} - abs_{extracts})}{abs_{control}} \times 100.
\]  

(1)

**α-Amylase inhibitory assay:** This assay was carried out using a modified procedure of Kazeem et al. The reaction mixture consisted a total of 50 μl (10 mg concentration) of the extract and 250 μl of 0.02 M sodium phosphate buffer (pH 6.9) containing α-amylase solution (10 U/mg; 3.2.1.1). Further, 250 μl of starch solution (1%) was added to the above reaction mixture and incubated at 25°C for 15 min. The reaction was stopped by the addition of dinitrosalicylic acid (DNS) reagent. The tubes were then placed in boiling water for 5 min, cooled and absorbance was read at 540 nm using a spectrophotometer. Sample control (containing phosphate buffer with sample) was taken for all samples to nullify the colour value. A control was prepared by replacing the extract with distilled water. The percentage of inhibition of α-amylase was calculated using eq. (1).

**Amyloglucosidase inhibitory assay:** This assay was carried out with a modified procedure of Eynard et al. The reaction was prepared by the addition of 100 mM sodium acetate buffer (pH 4.5), 60 μl of diluted amyloglucosidase solution (EC-3.2.1.3), 20 μl of extract (10 mg concentration) and 1 mM PNPG substrate. The reaction was further incubated at 37°C for 20 min. Next 1% sodium bicarbonate was added in order to terminate the reaction and absorbance was read at 405 nm. Sample control was used for all samples to nullify the colour value. A control was prepared by replacing the extract with distilled water. The percentage of inhibition of amyloglucosidase was calculated using eq. (1).

**High performance liquid chromatography**

High-performance liquid chromatography (HPLC) analyses were carried out using a Shimadzu HPLC system equipped with two LC 10 AD Vp pumps, an SCL-10-Avp controller, a DGU-14A degasser, and SPD-M10 Avp UV detector. The separation in preparative HPLC was achieved using reverse-phase Supelco 516 C18 column (250 mm × 4 mm, 5 μm, Agilent Technologies). The mobile phase consisted of (A) 1% acetic acid: water (v/v), and (B) 1% acetic acid: acetonitrile (v/v). The linear gradient profile was as follows: 0 min, 5% B; 5 min, 12% B; 7 min, 22% B; 17 min, 35% B; 24 min, 55% B; 30 min, 100% B; 32 min, 5% B followed by 5 min of equilibration of the column. Flow rate was 10.0 ml/min and wavelength was recorded at 360 nm. Samples were injected and eluted in groups and checked for enzyme inhibition assays. The group which gave the highest inhibition was further subjected to preparative HPLC to separate individual compounds and determine the inhibition of the enzymes. Eventually, after a series of separations and enzyme assays, the purified compound with the highest inhibition was considered for further characterization.

**Determination of molecular weight of individual compounds**

The individual compounds separated by preparative HPLC were analysed using liquid chromatography mass spectrometry (LCMS; Alliance 2690 module from Waters, UK. equipped with an automatic injector) for determination of molecular weight of the compounds. The analysis was carried out in negative ion mode with direct injection of the sample (10 μl) and UV spectrum from m/z 200 to 1000 nm.

**Fourier transform infrared spectroscopic analysis**

The Fourier transform infrared spectroscopic (FTIR) analysis was carried out with a Shimadzu FTIR spectrometer.
Two milligrams of the sample was mixed with 100 mg KBr (FTIR grade) and then compressed to prepare a salt disc (3 mm diameter). The disc was placed in the sample holder and absorption spectra of FTIR were recorded in the range 4000–500 cm$^{-1}$.

Nuclear magnetic resonance experimental parameters

The nuclear magnetic resonance (NMR) spectral studies were carried out using a Bruker Avance AQS 500 MHz NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 11.74 T and equipped with a BBO probe. The $^1$H spectra were recorded at 500 MHz with the spectral width of 7500 Hz over 65 K data points. The sample was dissolved in 600 μl deuterated methanol, and the solvent moisture peak was suppressed using presaturation technique with low power (53 dB) radiofrequency irradiation. The relaxation delay was maintained at 4 s. The data processing was carried out by applying exponential window function with a line broadening of 0.3 Hz, followed by Fourier transformation (32 K data points) using TOPSPIN version 1.3 (Bruker Biospin GmbH, Germany). The spectrum was calibrated by referring methanol D4 signal to 3.30 ppm.

The $^{13}$C NMR spectra were acquired at 125 MHz with a spectral width of 28,985 Hz over 16 K data points; the number of scans was 2 K.

The 2D HSQC ($^1$H,$^{13}$C correlation) was obtained using hsqctgpp pulse program with F2 ($^1$H) and F1 ($^{13}$C) data points set at 2 K and 256 respectively. The 2D spectrum was acquired with a spectral width of 7500 Hz and 25,000 Hz for $^1$H and $^{13}$C nuclei respectively.

The COSY spectrum ($^1$H–$^1$H correlation) was acquired using cosygqpf pulse program with F2 ($^1$H) and F1 ($^1$H) data points set at 2K and 256 respectively. The spectral width for both the frequency axes was 7500 Hz. The data were processed by applying sine window function with a line broadening of 1.0 and 0.3 Hz before Fourier transformation.

The HMBC (heteronuclear multiple bond correlation) spectra were recorded using hmbcgplpndqf pulse program with the following parameters: number of scans 32; size of FID, F2 (2K for $^1$H) and F1 (256 for $^{13}$C); spectral width, 7500 Hz (for $^1$H) and 28,930 Hz (for $^{13}$C). The data processing was carried out by multiplying squared sine function followed by Fourier transformation.

Statistical analysis

Values are expressed as mean ± standard deviation. The samples in triplicate were taken for all the three inhibitory enzyme assays independently. The data were subjected to one-way analysis of variance (ANOVA) and the significant difference between the groups ($\alpha$-glucosidase, $\alpha$-amylase and amyloglucosidase) was determined by Duncan’s multiple test ($P < 0.05$) using GraphPad Prism software version 4.03 for Windows (San Diego, CA, USA).

Results and discussion

In vitro inhibitory assays

In this study, the inhibitory effects of the extracts from three fractions (peel, albedo, juicy segments; 3 × 5 solvents) of C. maxima fruit against $\alpha$-glucosidase, $\alpha$-amylase and amyloglucosidase enzymes at 10 mg/ml concentration have been examined. The fruit fractions exhibited potential enzyme inhibition towards all the enzymes. Juicy segment ethanol (72%, 33.69%), methanol (82.85%, 42.25%) and water (71.80%, 33.22%) extracts showed highest inhibition against $\alpha$-glucosidase and amyloglucosidase enzymes compared to the extracts of other fractions (Figure 1a–c). The results indicate that the inhibitory activity of C. maxima is maximum against $\alpha$-glucosidase in all fruit fractions followed by amyloglucosidase and $\alpha$-amylase with least inhibitory activity. Our results are in accordance with those of Pinto et al.17, who found that foods or botanical supplements which have moderate $\alpha$-amylase inhibition can efficiently target the dietary management of hyperglycaemia. Several other reports also suggest that lower percentage of inhibition of $\alpha$-amylase compared to $\alpha$-glucosidase is preferred for the management of diabetes to prevent some of the side effects produced by synthetic drugs15,18.

Based on the data obtained from preliminary screening, juicy segment extracted with ethanol, methanol and water showed the highest percentage of inhibition towards the three enzymes compared to the other two fruit parts, i.e. peel and albedo. Thus, the selected three extracts from juicy segments were analysed for their IC$_{50}$ values (Table 1) and considered for further identification of individual bioactive compounds by preparative HPLC analysis.

Preparative HPLC

Initially, the methanol, ethanol and water extracts of juicy segments were subjected to preparative HPLC, and the compounds were eluted in groups and further tested for enzyme inhibition assays against all the three enzymes (Table 2). From the results, it is evident that group 2 of juicy segment water, ethanol and methanol extracts shows higher inhibitory activity against the three enzymes compared to the other five groups tested. Group 2 of ethanol extract showed the highest percentage of inhibition of about 95.80, 25.01 and 6.81 for $\alpha$-glucosidase, $\alpha$-amylase and amyloglucosidase respectively, which is higher than the water and methanol extracts of the same group. Hence, group 2 of juicy segment ethanol extract was further subjected to preparative HPLC to separate individual bioactive compounds.
Determination of molecular weight of the compounds

Six individual compounds from group 2 ethanol extract were obtained using modified chromatographic conditions. All six separated compounds were subjected to LCMS to determine the molecular weight of the compounds. Compounds 1–3 were found to have a similar molecular weight of 390 and could not be identified further. Compounds 4–6 were identified using the available literature\textsuperscript{11,15} as neoeorictinin, naringin and acetyl naringin (Table 3). Though the presence of these compounds in \textit{C. maxima} has been reported, their synergistic action towards the inhibition of key enzymes responsible for postprandial hyperglycaemia has not been reported hitherto.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure1.png}
\caption{Preliminary screening of inhibition activity of the extracts against carbohydrate-hydrolysing enzymes: \textit{a}, juicy segments; \textit{b}, Albedo; \textit{c}, peel. Note: Differences in the assays are indicated by different symbols with significant difference level of *$P<0.05$; **$P<0.01$; \textsuperscript{*}$P<0.001$ for $\alpha$-glucosidase; \textsuperscript{*}$P<0.05$, \textsuperscript{*}$P<0.01$, \textsuperscript{*}$P<0.001$ for $\alpha$-amylase; \textsuperscript{*}$P<0.05$, \textsuperscript{*}$P<0.01$, \textsuperscript{*}$P<0.001$ for amyloglucosidase between the fractions; ns, Not significant. Data were assessed statistically using ANOVA followed by Tukey’s post hoc test.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure2.png}
\caption{Enzyme inhibition assays for six individual compounds from group 2 juicy segment ethanol extract. Note: Different letters indicate significant difference level of $P<0.001$ between the respective samples that were assessed statistically using ANOVA followed by Tukey’s post hoc test.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure3.png}
\caption{Comparative chart for the activity of compound 5 with other compounds.}
\end{figure}
Table 1. Determination of IC₅₀ value in samples showing highest inhibition in preliminary screening

<table>
<thead>
<tr>
<th>Sample</th>
<th>α-Glucosidase (mg/ml)</th>
<th>α-Amylase (mg/ml)</th>
<th>Amyloglucosidase (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Juice segments aqueous extract</td>
<td>3.53</td>
<td>62.50</td>
<td>18.88</td>
</tr>
<tr>
<td>Juice segments ethanol extract</td>
<td>3.44</td>
<td>14.28</td>
<td>13.94</td>
</tr>
<tr>
<td>Juice segments methanol extract</td>
<td>3.73</td>
<td>47.87</td>
<td>18.76</td>
</tr>
</tbody>
</table>

Table 2. Enzyme inhibition (%) for fractions collected from preparative HPLC (at 1 mg concentration) in three assays

<table>
<thead>
<tr>
<th>Groups collected (HPLC)</th>
<th>Segment water</th>
<th>Segment ethanol</th>
<th>Segment methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>1</td>
<td>1.24 ± 1.32a</td>
<td>0.00 ± 1.29a</td>
<td>0.00 ± 0.52a</td>
</tr>
<tr>
<td>2</td>
<td>48.95 ± 1.03b</td>
<td>1.38 ± 0.86b</td>
<td>25.88 ± 0.65b</td>
</tr>
<tr>
<td>3</td>
<td>27.88 ± 0.79a</td>
<td>0.00 ± 1.35a</td>
<td>3.81 ± 1.23a</td>
</tr>
<tr>
<td>4</td>
<td>24.66 ± 0.99d</td>
<td>0.00 ± 0.95a</td>
<td>11.36 ± 1.54c</td>
</tr>
<tr>
<td>5</td>
<td>30.27 ± 1.10f</td>
<td>0.00 ± 0.60a</td>
<td>17.34 ± 0.71c</td>
</tr>
<tr>
<td>6</td>
<td>0.00 ± 0.89a</td>
<td>0.00 ± 1.08a</td>
<td>13.49 ± 1.02a</td>
</tr>
</tbody>
</table>

A. α-Glucosidase assay; B. α-Amylase assay; C. Amyloglucosidase assay. Mean of three replicates ± SD. Values in a column followed by different letters indicate the statistical difference at P < 0.05 using ANOVA followed by Tukey’s post hoc test.

Table 3. Liquid chromatography mass spectrometry analysis for molecular weight of separated compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular weight m/z</th>
<th>Identification of compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>390.86</td>
<td>Similar unidentified compound</td>
</tr>
<tr>
<td>2</td>
<td>390.84</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>390.90</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>594.95</td>
<td>Neoeeriocitrin</td>
</tr>
<tr>
<td>5</td>
<td>578.72</td>
<td>Naringin</td>
</tr>
<tr>
<td>6</td>
<td>620.99</td>
<td>Acetyl naringin</td>
</tr>
</tbody>
</table>

Identification of bioactive compound by in vitro assay

Six individual identified and unidentified compounds were subjected to enzyme inhibition assay against all three enzymes to determine the bioactive compound. Compound 5 gave the highest inhibition compared to other isolated individual compounds with IC₅₀ value of 200 μg/ml. Since compound 5 was predicted to be naringin by its molecular weight, the activity was compared with that of standard naringin (Figure 2). The activity was on par with that of standard naringin. However, the activity of isolated naringin (compound 5) was less when compared to positive control acarbose (a synthetic drug), which showed 97% inhibition. As naringin can be converted to naringenin when taken into the biological system, it can exhibit enhanced activity similar to acarbose in the biological system (Figure 2). Compound 5 showed 27.65% inhibition individually, whereas the remaining five individual compounds contributed on an average only 37.13% inhibition even in combined form (Figure 3). This shows that compound 5 could exhibit 50% of activity individually at 100 μg/ml concentration.
So, compound 5 which exhibited the highest activity against enzymes was taken for characterization by FTIR and NMR analysis.

**FTIR analysis**

Figure 4 shows the FTIR spectra of compound 5 and standard naringin. Both spectra exhibit similar functional groups with the characteristic peaks of −OH groups at about 3500–3200 cm⁻¹, the aliphatic C–H stretching vibration at about 2980 and 2850 cm⁻¹, C–O–C at 1715–1641 cm⁻¹, benzene ring at about 1514 and 1515 cm⁻¹, C–O stretch at 1320–1000 cm⁻¹ and aromatic hoop pattern at 900–600 cm⁻¹. The above spectral characteristics indicate with high probability that the compound is naringin.

**Identification of isolated compound**

The ¹H (¹H and ¹³C) and ²D NMR (HSQC, COSY and HMBC) experiments that were carried out, revealed the presence of rhamnose, glucose and 2-phenylbenzopyran-4-one. Thus, by comparing NMR spectral data of compound 5 with that of standard naringin it was confirmed that compound 5 to be Naringin (Figure 5). Table 4 presents the NMR spectral characterization data of naringin. The hydroxyl proton signals were not detected as water pre-saturation pulse sequence was employed to collect the ¹H NMR spectrum (Supplementary Figures 1–4).

**Conclusion**

The bioactive compound in the fruit segments of *C. maxima* which acts against carbohydrate-hydrolysing enzymes has been identified as naringin, by subjecting the purified bioactive compound to spectral analyses and *in vitro* assays. Further animal model studies are required to elucidate the pharmacological activities and its role in the treatment of diabetes. Besides naringin, other isolated compounds might also help in providing beneficial effect when considered *in situ* (fruit). Thus the present study indicates the possibility of developing products from the fruit segments of *C. maxima* for reducing postprandial hyperglycaemia.

**Conflict of interest.** Conflict of interest declared none.

---


ACKNOWLEDGEMENTS. We thank Prof. Ram Rajasekharan, Director, CSIR-CFTRI, Mysuru for encouragement and the Department of Biotechnology, Government of India, for their financial support (BT/PR5994/FNS/20/563/2012).