In-vitro Assessment of Cholinesterase Inhibitory and Thrombolytic Activity of Six Available Citrus Fruits in Bangladesh: Relevant for Treating Neurodegenerative Disorder

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Authors’ contributions

This work was carried out in collaboration between all authors. Author KB designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors SH and SA are mainly involved in finding “total flavonoids”, “total flavonols” and “total phenolics” whereas authors TS, FK and SD managed the analyses of “cholinesterase inhibitory” and “thrombolytic activity” of the study. Authors KB and SD are involved in literature searches. All authors read and approved the final manuscript.

Article Information

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ABSTRACT

 Aim: Citrus fruits are well known for its medicinal and food value. Aim of this study is to investigate acetylcholinesterase ((AChE)) inhibitory activity, butyrylcholinesterase (BuChE) inhibitory activity, total phenolics, flavonoids, flavonols content and thrombolytic activities of crude methanol extracts of 6 citrus fruits (Citrus limon, Citrus aurantifolia, Citrus bergamia, Citrus maxima, Citrus sinensis and Citrus macroptera).

Methods: The fruits were extracted by using methanol as solvent. Ellman’s colourimetric method was applied to determine both cholinesterase inhibitory activities, while folin-ciocalteau reagent

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1. INTRODUCTION

Citrus has long been regarded as food and medicinal plant. Due to their low cost and easy availability, Citrus fruits are offers significantly low-cost nutritional dietary supplement [1-3]. The genus Citrus belonging to the family “Rutaceae” comprises about 40 species widely distributed in the Bangladesh, India, China, Malaysia, Sri Lanka and Australia. It is one of the most important world fruit crops and consumed fresh as fresh or as juice because of its nutritional value and special flavor [4-6]. Citrus fruits and juices are an important source of bioactive compounds including antioxidants such as phenolic compounds, flavonoids, ascorbic acid and others. Flavonoids, flavones and flavonols are three common types of flavonoids which occur in Citrus fruit. The main flavonoids found in citrus species are hesperidin, narirutin, naringin and eriocitrin [7-10]. Epidemiological studies on dietary Citrus flavonoids improved a reduction in risk of coronary heart disease and are attracting more and more attention not only due to their antioxidant properties but also due to their anti-carcinogenic and anti-inflammatory agents because of their lipid anti-peroxidation effects [11-15]. The interest in these classes of compounds is due to their pharmacological activity as radical scavengers [16].

Neurodegenerative disorder (ND) is incurable condition due to the progressive dysfunction of the nervous system mainly caused by neuronal degeneration and loss of total nerve cells for reasons. The actual reasons have not yet been fully understood [17,18]. Today, a growing number of people worldwide are affected by ND, characterized by deterioration in emotional control, social behavior and social communication. ND exist in many forms, such as Multiple Sclerosis, Alzheimer’s, Parkinson’s, Huntington’s, Human prion and Motor neuron diseases [19-22]. To treat ND there are several hypotheses have been developed. Like antioxidant hypothesis, cholinergic hypothesis, tau hypothesis, Aβ hypothesis etc. Currently, there is no effective treatment for ND, and the marketed drugs are mainly symptom-oriented, albeit with many side effects, limited efficacy and partial capability to inhibit disease progression [23-28]. Therefore, to develop novel preventive strategies or co-adjuvant therapy for ND, within the past decades, a great number of natural medicinal plants have gained attention as potential neuroprotective agents [29]. Moreover, an increasing number of studies have suggested that dietary intake of vegetables and fruits can prevent or delay the onset of ND. These properties might be due to the presence of polyphenols, an important group of phytochemicals that are abundantly present in fruits, vegetables, cereals and beverages. As Citrus fruits are rich in antioxidants, polyphenols and flavonoids, these might be a good alternative for treating ND and lowering its effects [30-33].

Due to geographical consideration, a wide variety of Citrus fruit grows in Bangladesh. Among all species, six species were used (Citrus limon, Citrus aurantifolia, Citrus bergamia, Citrus maxima, Citrus sinensis and Citrus macroptera) for the test. Their physical properties are given in Table 1. The objectives of this study were to investigate and comparison of (I) Cholinesterase enzymes inhibitory activity of 6 citrus species (II) determination of their phenol, flavonoids and flavonol contents (III) comparative thrombolytic activity and (IV) analysis of the correlation between them.

<table>
<thead>
<tr>
<th>Keywords: Citrus fruit; phenolics; flavonoids; flavonols; cholinesterase inhibition.</th>
</tr>
</thead>
</table>

(FCR) and aluminium chloride were used to quantify total phenolics, flavonoids, flavonol content of those fruits. Blood clot lysis method was applied for determining the thrombolytic activity of those fruits.

Results: All citrus fruits contain a good amount of phenolics, flavonoids and flavonols. C. maxima found more prominent in containing phenolics and flavonoids compare to other citrus fruits, with 414.06 ± 2.87 mg Gallic Acid Equivalent/gm and 12.94 ± 1.31 mg Catechin Equivalent/gm dried extract respectively. Citrus sinensis showed the highest content in flavonoids with 21.16± 1.37 mg Catechin 20 Equivalent /gm dried extract. Citrus fruits are also a quality source of cholinesterase inhibitors. All the examined citrus fruits were found capable of inhibiting both acetylcholinesterases (AChE) as well as butyrylcholinesterase (BuChE). C. bergamia was most effective in inhibiting AChE with IC50 of 27.18 µg/ml where C. macroptera was best in inhibiting BuChE (IC50 32.5 µg/ml). But none of the citrus fruits was found fit for thrombolytic activity.

Conclusion: Citrus fruits are found the sound in inhibiting AChE and BuChE as well as containing Phenolics, flavonoids and flavonols. But they lack in their thrombolytic activity.
Table 1. Physical characteristics of six different species of Citrus [34,35]

<table>
<thead>
<tr>
<th>Species</th>
<th>Common name</th>
<th>Color</th>
<th>Size (cm)</th>
<th>Shape</th>
<th>Taste</th>
<th>Texture</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. lemon</td>
<td>Common Lime</td>
<td>Greenish Yellow</td>
<td>7-10</td>
<td>Oval</td>
<td>Sour</td>
<td>Fibrous</td>
</tr>
<tr>
<td>C. aurantifolia</td>
<td>Key Lime</td>
<td>Greenish Yellow</td>
<td>2.5-5</td>
<td>Round</td>
<td>Sour</td>
<td>Smooth</td>
</tr>
<tr>
<td>C. bergamia</td>
<td>Bergamot</td>
<td>Yellow</td>
<td>5-10</td>
<td>Round</td>
<td>Sour</td>
<td>Smooth</td>
</tr>
<tr>
<td>C. maxima</td>
<td>Pomelo</td>
<td>Greenish Yellow</td>
<td>15-25</td>
<td>Round</td>
<td>Sour</td>
<td>Fibrous</td>
</tr>
<tr>
<td>C. sinensis</td>
<td>Orange</td>
<td>Orange</td>
<td>5-10</td>
<td>Round</td>
<td>Sweet</td>
<td>Smooth</td>
</tr>
<tr>
<td>C. macroptera</td>
<td>Satkora</td>
<td>Green</td>
<td>5-7</td>
<td>Round</td>
<td>Sour</td>
<td>Fibrous</td>
</tr>
</tbody>
</table>

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Acetylthiocholine iodide (ATCI), butyrylthiocholine iodide (BTCI), 5’dithio-bis-(2-nitro) benzoic acid (DTNB), acetylcholinesterase (AChE), butyrylcholinesterase (BuChE), eserine, galantamine, gallic acid, catechin and Streptokinase were purchased from Sigma-Aldrich (Japan). Tris.HCl buffer, sodium chloride, sodium carbonate, sodium acetate, sodium hydroxide, magnesium chloride, Triton X-100, folin-ciocalteau reagent (FCR), aluminium chloride, ammonium sulphate were collected from Wako Pure Chemical Company Ltd. (Japan). Analytical grade chemicals and solvents were used in this study.

2.2 Preparation of Fruit Sample

Fresh fruits of C. lemon, C. aurantifolia, C. bergamia, C. maxima, C. sinensis and C. macroptera at the mature commercial stage were harvested from several commercial orchards in the month of October-November, from Sylhet, Hobiganj, Mymensingh, Dhaka and Rajshahi, Bangladesh. Only healthy fruits were selected randomly for their uniformity in color and shape. Fruits were then washed thoroughly with distilled water and then dried in air. Then fruits were chopped into thin slices and dried under a shadow. Dried fruit slices were then grounded into finer powder using a powerful grinder. The ground sample was sieved to get uniform particle size and kept it into an air-tight container to prevent it from any photolytic degradation.

2.3 Extraction

Powdered fruits (500 g) were placed into an amber coated bottle and soaked into 1000 ml of methanol and contents were sealed into the bottle for ten days with occasionally stirred and shaken. After ten days, the whole mixtures were filtrated by Whitman No. 1 filter papers, and the filtrated solutions were concentrated under reduced pressure, heating below 50°C. Finally, near about 20 g of crude methanolic extracts (CMEs) of fruits were obtained.

2.3.1 Determination of total phenolics: [36]

The total content of phenolics in fruits was measured by using substrate FCR, where gallic acid used as a standard. In a reaction mixture 0.5 mL CME of fruits, 2.5 ml of FCR and 2 ml of sodium carbonate (7.5%) were added. The tubes were mixed and let to stand for 2 hours. At 760 nm absorbance was measured.

2.3.2 Estimation of total flavonoids: [37]

The total content of flavonoids was measured according to the method of Zhishen et al. [37]. Fruit extract was added with 0.5 mL in 0.15 mL of 5% sodium nitrite and well mixed. After 5 min of incubation, 0.3 mL of 10% aluminium chloride solution was added. After 6 min of the interval, one mL of 1M sodium hydroxide was added to the mixture, and the volume was made up to 10 mL with distilled water. The absorbance was taken at 510 nm with UV–vis spectrophotometer. The total content of flavonoids was calculated from a Catechin standard curve and expressed as mg Catechin equivalents/gm (mg CE/gm).

2.3.3 Determination of total flavonols: [38]

Total amount flavonol was determined by using aluminium chloride as a substrate and standard Gallic acid as a standard. 300μl/mL CME was placed in a 10 mL test tube & methanol was added up to 1 mL. Then, one mL of aluminium chloride solution (2%) is added to it. Finally, 1.5 mL of 5% w/v sodium acetate was added to the test tube which is then incubated at room temperature for two and half hours. Absorbances were taken at 440 nm. Total Flavonol amounts were expressed as Gallic acid equivalents/g (mg GAE/gm) dry matter. All samples were analyzed thrice and resulted averaged.


2.3.4 Determination of AChE inhibitory activity: [39]

Modified Eillman’s colourimetric method was applied to run In-vitro AChE inhibitory assay, and ATCI used as a substrate. AChE hydrolysis rate was monitored spectrophotometrically. Each fruit extract or standard (various concentrations) was mixed with 200 μL of enzyme solution (5.21 x 10⁻³ U) and incubated at 37°C for 30 min. After that, Eillman’s reaction mixture (400 μL of 0.35 mM ATCI, 200 μL of 0.7 mM DTNB) was placed in an extraction buffer saline (50 mM Tris.HCl buffer, 50 mM MgCl₂, 50 mM NaCl, 1% Triton X-100, pH 8.0) to adjust it 3 ml of final volume. Absorbance at 412 nm was taken after 30 min incubated this mixture at 37°C. The blank reaction was measured by substituting buffer saline for the enzyme. Eserine was used as a standard drug. Percentage of inhibition of AChE enzymes were determined by comparison of reaction rates of samples related to blank using the formula of (E-S)/E x 100, where E is the activity of enzyme without test sample, and S is the activity of the enzyme with the test sample.

2.3.5 Determination of BuChE inhibitory activity: [39]

BuChE inhibitory assay was performed by modified Ellman’s colourimetric method, where BTCI acts as a substrate. BuChE hydrolysis rate was spectrophotometrically examined to run this test. Each fruit extract or standard (various concentrations) was mixed with 50 μL enzyme solution (4.16 x 10⁻³ U) and incubated at 37°C for 30 min. After adding Ellman’s reaction mixture (400 μL of 0.35 mM BTCI, 200 μL of 0.7 mM DTNB) in a buffer saline (50 mM of Tris.HCl buffer, 50 mM of MgCl₂, 50 mM of NaCl and 1% Triton X-100, pH 8.0) to adjust it 3 ml of final volume. After adding Eillman’s reaction mixture (400 μL of 0.35 mM BTCI, 200 μL of 0.7 mM DTNB) in a buffer saline (50 mM of Tris.HCl buffer, 50 mM of MgCl₂, 50 mM of NaCl and 1% Triton X-100, pH 8.0) to the above reaction mixture (400 μL of 0.35 mM BTCI, 200 μL of 0.7 mM DTNB) the mixture at 37°C. The blank reaction was measured by substituting buffer saline for the enzyme. Galantamine was used as a reference standard. Percentage of inhibition of BuChE enzymes was determined by comparison of reaction rates of samples related to blank using the formula of (E-S)/E x 100, where E is the activity of enzyme without test sample, and S is the activity of the enzyme with the test sample.

2.3.6 Thrombolytic activity test: [40]

For thrombolytic activity test for the fruits, human blood was used. Blood was withdrawn from healthy human volunteers (n=10) having no history of blood-related disorder, oral contraceptive pills administration or ongoing anticoagulant therapy. 1.0 ml of venous blood from each volunteer was transferred to the sterilized eppendorf tubes (volume 1.5 ml) and incubated for 45 min at 37°C and was allowed to form a clot. Fruits extracts (100 mg) were suspended in 10 ml of distilled water. After clot formation, the serum was completely removed from eppendorf tube. The blood clot was again weighed to determine the weight of clot. For each eppendorf tube with the pre-weighed clot, 100 μL aqueous solution of the crude extract was added separately. 100 μL of SK (30,000 IU) was added to the positive control and 100 μL distilled water were added to negative control tubes, respectively. All tubes were then again incubated for 90 min at 37°C to observe clot lysis. Then, the released fluid was removed, and tubes were again weighed. The difference obtained in weight taken before and after clot lysis by the extract, positive control and negative control, was expressed as a percentage of clot lysis and the equation is shown below:

\[ \% \text{ of Clot lysis} = \frac{(\text{Weight of clot after release of fluid} - \text{Weight of clot before releasing of fluid})}{\text{Weight of clot before releasing of fluid}} \times 100\% \]

2.4 Statistical Analysis

Values in this experiment are expressed as the mean of triplicate determination ± Standard Deviation. All data used are subjected to one-way analysis of variance (ANOVA) and the significant difference between means was determined by Dancan’s Multiple Test (P<0.05) using Statistical Package for the social science version 13.0 (SPSS Inc., Chicago, IL, USA).

3. RESULTS AND DISCUSSION

3.1 Total Phenolic Content

Phenolic compounds, like secondary metabolites, are excellent antioxidant due to their ability to donate electron or hydrogen from phenolic hydroxyl groups, which possesses ideal structure for scavenging free radicals generated in the body. These are a major class of bioactive molecules. So, regular consumption of these chemicals from a dietary supplement can be beneficial by inhibiting carcinogenesis and mutagenesis [41-43]. Among all Citrus fruit C. maxima contain the maximum amount of phenolics compare to other citrus fruits whereas C. macroptera contains least, 414.06 ± 2.87 mg
3.2 Total Flavonoids Content

The flavonoids are one of the most prominent groups of secondary metabolites in Citrus fruit with enormous biological activity like anti-microbial, anti-inflammatory, anti-oxidant and anti-carcinogen. They are also strong free radical scavengers [44-46]. Total flavonoids content of Citrus fruits is expressed as mg catechin equivalent/gm. Our research shows that these citrus fruits are different in their flavonoid content. C. sinensis was the most prominent in flavonoid content with 21.16 ± 1.37 mg CE/gm compared to other citrus fruits. C. maxima and C. macroptera comes next with 18.40 ± 1.61 mg CE/gm and 17.44 ± 1.18 mg CE/gm respectively. The remaining three citruses contain flavonoids between 11 to 15 mg CE/gm.

3.3 Total Flavonol Contents

Flavonols are one of the classes of flavonoids containing 3-hydroxy-2-phenylchromen-4-one ring in it. Biologically they play an important role in neuroprotection, as they can re-establish the redox regulation of proteins, transcription factors and signaling cascades that are otherwise inhibited by elevated oxidative stress. The final survival or death of the neuron depends on flavonol concentrations, time of exposure as well as metabolic and oxidative neuronal circumstances [47-51]. Citrus fruits are always a very eminent source of flavonols. In our study, we can see those Citrus fruits contain an almost similar amount of flavonols, ranges between 8-13 mg Catechin equivalent/gm of dry extracts. C. maxima were found a leader in holding flavonol with 12.94 ± 1.31 mg CE/gm of dehydrated extracts beating C. macroptera (12.52 ± 1.35 mg CE/gm) and C. sinensis (10.86 ± 1.82 mg CE/gm). C. aurantifolli was least in containing this bioactive molecule, with 8.16 ± 0.74 mg CE/gm. Other Citrus fruits C. limon and C. bergamia contain 10.68 ± 1.78 mg CE/gm and 9.60 ± 1.06 mg CE/gm respectively.

<table>
<thead>
<tr>
<th>Plant Name</th>
<th>Phenolics*</th>
<th>Flavonoids**</th>
<th>Flavonols**</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. limon</td>
<td>318.61 ± 2.23</td>
<td>14.98 ± 1.67</td>
<td>10.68 ± 1.78</td>
</tr>
<tr>
<td>C. aurantifolli</td>
<td>377.45 ± 2.64</td>
<td>11.44 ± 1.49</td>
<td>8.16 ± 0.74</td>
</tr>
<tr>
<td>C. bergamia</td>
<td>221.13 ± 1.82</td>
<td>13.31 ± 1.02</td>
<td>9.60 ± 1.06</td>
</tr>
<tr>
<td>C. maxima</td>
<td>414.06 ± 2.87</td>
<td>18.40 ± 0.61</td>
<td>12.94 ± 1.31</td>
</tr>
<tr>
<td>C. sinensis</td>
<td>268.81 ± 1.83</td>
<td>21.16 ± 1.37</td>
<td>10.86 ± 1.82</td>
</tr>
<tr>
<td>C. macroptera</td>
<td>146.44 ± 1.55</td>
<td>17.44 ± 1.18</td>
<td>12.52 ± 1.35</td>
</tr>
</tbody>
</table>

Values are means of triplicate determination ± Standard Deviation.

* mg GAE/gm of dried sample
** mg CE/gm of dried sample
3.5 Butyrylcholinesterase Inhibitory Activity

Butyrylcholinesterase is a nonspecific cholinesterase enzyme that hydrolyses many different choline-based esters. It not only breakdown both cholinesterases (Ach and BuCh), but also synergists function of AChE enzyme. In neurodegenerative disorder, like the AD, the expression of BuChE also increases enormously. So, inhibiting this enzyme can also be found very effective in the AD and other types of Dementia [57-62]. BuChE have structural similarity with AChE, so sometimes AChE inhibitors can inhibit BuChE. Citrus fruit is capable of inhibiting this enzyme, more or less. In our study, we found that C. macroptera and C. bergamia were most effective fruits that inhibit BuChE at a lower concentration with IC50 of 32.50 and 34.74 µg/ml compared to the rest. C. maxima had shown least activity against this enzyme. Remaining fruits (C. limon, C. sinensis and C. aurantifolia) gave moderate activity. (Fig 2)

From all six citrus fruit C. limon, C. aurantifolia, C. bergamia and C. maxima found more prominent in inhibiting BuChE enzyme compare to their BuChE inhibitory activity. But C. sinensis and C. macroptera had shown their potentiality in inhibiting BuChE than AChE.

3.6 A Thrombolytic Activity of Citrus Fruits

Thrombus formation in the blood vessels can obstruct blood flow through the circulatory system leading hypertension, stroke to the heart, anoxia, and so on [63-65]. If it occurs in the brain, it can also lead to neurodegeneration [66]. Thrombolytic drugs are mainly prescribed for controlling thrombosis patients. According to our test, we found Citrus fruits are not very potential for clot dissolving manners. They reported very minor thrombolytic activity ranges from 0.3 to 7% in total. C. macroptera can dissolve 6.908 ± 1.702% of a total blood clot, which was the highest among all citrus fruits. C. aurantifolia and C. bergamia have almost similar types of thrombolytic property with 5.453 ± 0.896 % and 5.942 ± 1.179% clot lysis. Similar to other citrus fruits, rest of the fruit extracts are also not so good in dissolving blood clots. As C. sinensis can clot 4.798 ± 0.806% clot, C. maxima can break 1.785 ± 0.478% clot and C. lemon can lysis only 0.369 ± 0.148% clot. So treating thrombus and clotting disorder by using citrus fruit is found impossible. (Table 3) Platelets play a significant role in blood clotting by the development of thrombosis atherothrombosis, which in mainly initiated from the damage the regions of an endothelial surface by reactive oxygen species (ROS). The stimulated platelets enhance platelet-

![Acetylcholinesterase inhibitory activity of Citrus fruits](image-url)

Fig. 1. Acetylcholinesterase inhibitory activity of Citrus fruits
Fig. 2. Butyrylcholinesterase inhibitory activity of Citrus fruits

![Butyrylcholinesterase inhibitory activity of Citrus fruits](image)

Fig. 3. IC50 of Citrus fruits extracts for both AChE and BuChE

![IC50 of Citrus Fruits](image)

**Table 3. Thrombolytic activity of Citrus fruits**

<table>
<thead>
<tr>
<th>Citrus species</th>
<th>% of clot lysis (Con. 100 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. limon</td>
<td>0.369 ± 0.148</td>
</tr>
<tr>
<td>C. aurantifolia</td>
<td>5.453 ± 0.896</td>
</tr>
<tr>
<td>C. bergamia</td>
<td>5.942 ± 1.179</td>
</tr>
<tr>
<td>C. maxima</td>
<td>4.798 ± 0.478</td>
</tr>
<tr>
<td>C. sinensis</td>
<td>3.25 ± 0.806</td>
</tr>
<tr>
<td>C. macroptera</td>
<td>12.33 ± 1.702</td>
</tr>
</tbody>
</table>

Values are means of triplicate determination ± Standard Deviation

As citrus fruits are highly effective against ROS by scavenging them, they lack in the thrombolytic property [70,71].

4. CONCLUSION

All six citrus fruits are rich in phenolics, flavonoids and flavonols, while C. maxima contain maximum. In enzyme inhibitory capabilities, C. bergamia was found most capable of inhibiting AChE (IC50 27.18 µg/ml), and C. macroptera was most active in inhibition of BuChE (IC50 32.50 µg/ml). According to the study, citrus fruit is not that much suitable for thrombolysis. So, by cholinesterase inhibitory activity and chemical contents, this study provides information that, citrus fruit can improve platelet bonding [67-69]. This binding can also trap other blood cells which accelerate the process of plaque development and progression.
ACl. Further study is needed to find an actual molecule that is responsible for their specific action.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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