European Journal of Medicinal Plants

22(3): 1-11, 2018; Article no.EJMP.39693
ISSN: 2231-0894, NLM ID: 101583475

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

Kushal Biswas[1*], Saiyara Hossain[1], Sanzida Alam[1], Taposhi Sultana[1], Farzana Khan[1] and Sanjay Dutta[2]

[1]Department of Pharmacy, East West University, Bangladesh.
[2]Department of Pharmacy, Khwaja Yunus Ali University, Bangladesh.

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

DOI: 10.9734/EJMP/2018/39693
Editor(s):
(1) Shanfa Lu, Professor, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College, China.
(2) Marcello Iriti, Professor, Plant Biology and Pathology, Department of Agricultural and Environmental Sciences, Milan State University, Italy.
Reviewers:
(1) Muhammad Ali, Kano University of Science and Technology, Nigeria.
(2) Yongchun Zhu, Shenyang Normal University, China.
(3) Francisco José Queiroz Monte, Universidade Federal do Ceará, Brasil.
Complete Peer review History: http://www.sciencedomain.org/review-history/23447

Original Research Article

Received 27th November 2017
Accepted 23rd February 2018
Published 5th March 2018

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 aurantifollia, 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

**Corresponding author: E-mail: kushal71@outlook.com, ksb@ewubd.edu;*

(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 flavonols 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.

Keywords: Citrus fruit; phenolics; flavonoids; flavonols; cholinesterase inhibition.

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. Flavonols, 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 as 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 conditions 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 aurantifollia, 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 1. Physical characteristics of six different species of Citrus [34,35]

*Citrus species	Common name	Color	Size (cm)	Shape	Taste	Texture
C. lemon	Common Lime	Greenish Yellow	7-10	Oval	Sour	Fibrous
C. aurantifollia	Key Lime	Greenish Yellow	2.5-5	Round	Sour	Smooth
C. bergamia	Bergamot	Yellow	5-10	Round	Sour	Smooth
C. maxima	Pomelo	Greenish Yellow	15-25	Round	Sour	Fibrous
C. sinensis	Orange	Orange	5-10	Round	Sweet	Smooth
C. macroptera	Satkora	Green	5-7	Round	Sour	Fibrous

**Citrus limon(C. lemon), Citrus aurantifollia (C. aurantifollia), Citrus bergamia (C. bergamia), Citrus maxima (C. maxima), Citrus sinensis (C. sinensis) and Citrus macroptera (C. macroptera)*

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. aurantifollia, 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 filtered 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 aluminum 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 Ellman'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, Ellman'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, 50mM 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 50µL 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 the above reaction mixture, to adjust final volume of 3 mL. To verify the result, all reading was repeated three times. 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:

> % of Clot lysis = (Weight of clot after release of fluid/Weight of clot before releasing of fluid) x 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

GAE/gm and 146.44 ± 1.55 mg Gallic acid equivalent per gram (GAE/gm) respectively. Next, to *C. maxima*, key lime and common lime contain greater phenolics with 377.45 ± 2.64 mg GAE/gm, 318.61 ± 2.23 mg GAE/gm of dry extract respectively. Orange and *C. bergamia* were found moderate in phenolics content with 268.81 ± 1.83mg GAE/gm, 221.13 ± 1.82 mg GAE/gm of dry extract respectively. According to our result, all Citrus fruit contain a significant amount of total which increases antioxidant and free radical scavenger in the daily human diet.

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 dried extracts. *C. maxima* were found a leader in holding flavonols 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. aurantifollia* 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.

3.4 Acetylcholinesterase Inhibitory Activity

Acetylcholinesterase (AChE), a hydrolase, plays a crucial role in cholinergic transmission by catalyzing acetylcholine (ACh), the vital neurotransmitter for cognition. In several neurodegenerative disorder the expression of AChE increases enormously, causes breakdown of ACh to a greater extent, which leads to a deficit in cognitive function. Beside this in several types of dementia and Alzheimer's disease the number of neuron decreases. This gets more worsen when limited neuron released neurotransmitter (especially ACh) breaks apart with AChE. Inhibiting AChE found beneficial for these patients [52-56]. Inhibitory activities of the fruits are demonstrated in Fig. 1. All most all citrus fruit that we were used in our experiment found active against inhibiting AChE and *C. bergamia* found most active with the IC50 of 27.18 µg/ml. Next to *C. bergamia*; *C. limon, C. sinensis* and *C. aurantifollia* found similar in inhibiting AChE with IC50 of 38.21 µg/ml, 35.92 µg/ml and 40.52 µg/ml respectively. *C. maxima* and *C. macroptera* have moderate activity in inhibiting AChE with IC50 of 59.16 & 100.62 µg/ml respectively. IC50 of these fruits may be higher than the standard, but as a dietary supplement, it can be a potential source of AChE inhibitor.

Table 2. Total phenolics, flavonoids and flavonol content of citrus fruits

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

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. aurantifollia*) gave moderate activity. (Fig. 2)

From all six citrus fruit *C. limon, C. aurantifollia, C. bergamia* and *C. maxima* found more prominent in inhibiting AChE 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. aurantifollia* 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-

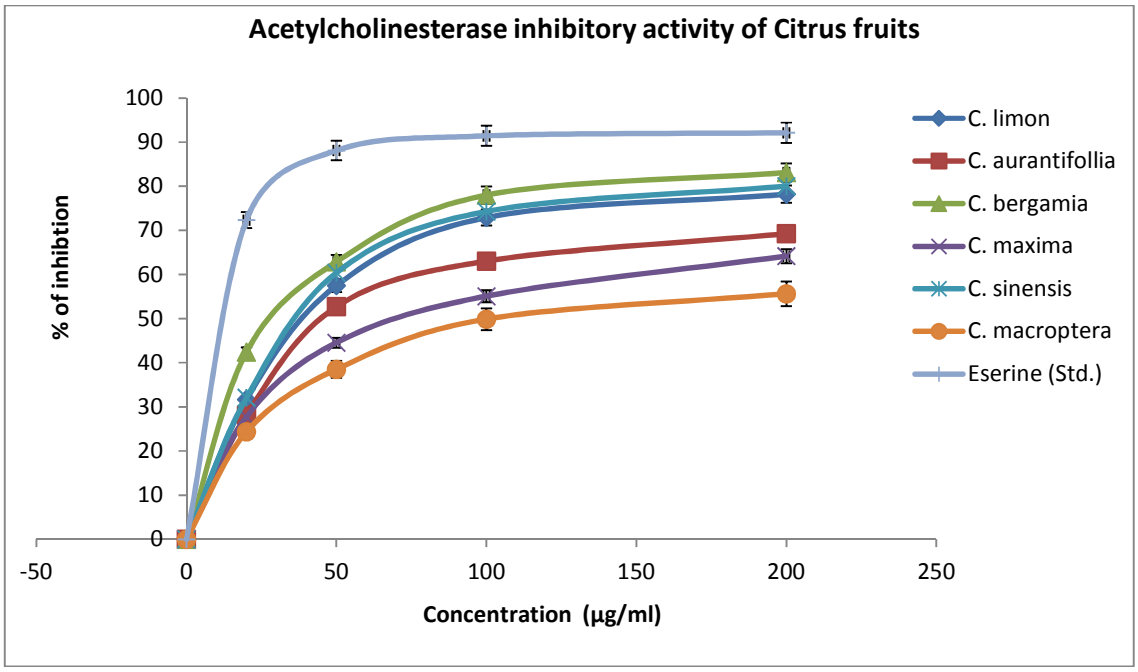

Fig. 1. Acetylcholinesterase inhibitory activity of Citrus fruits

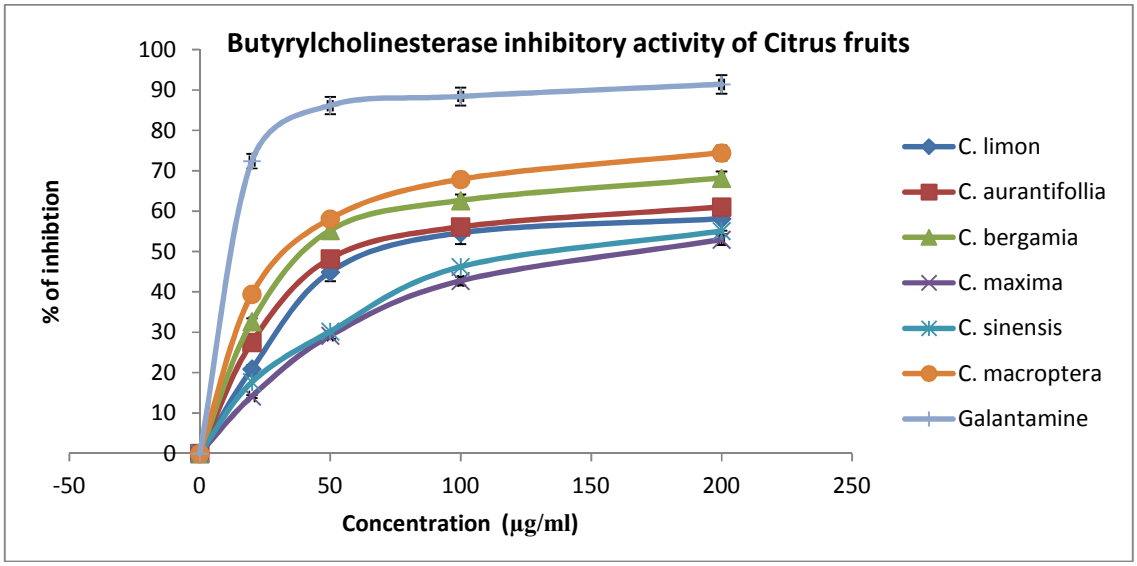

Fig. 2. Butyrylcholinesterase inhibitory activity of Citrus fruits

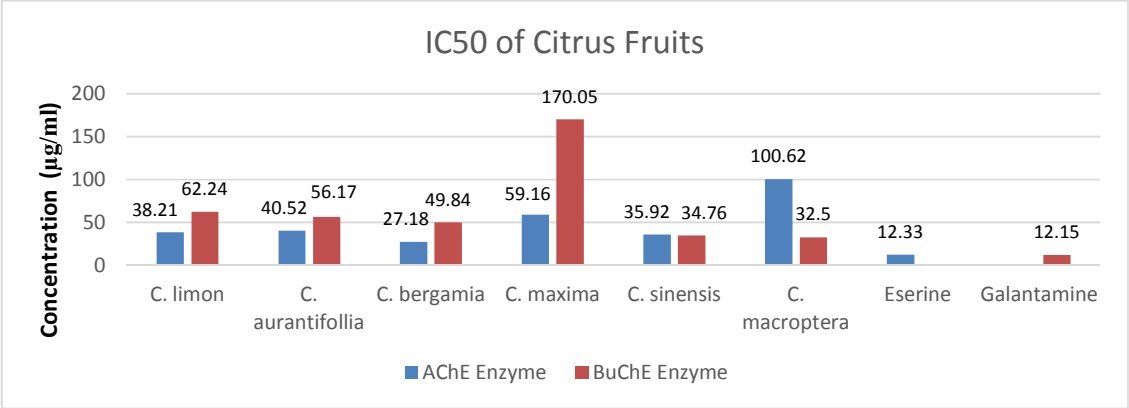

Fig. 3. IC50 of Citrus fruits extracts for both AChE and BuChE

Table 3. Thrombolytic activity of Citrus fruits

Citrus species	% of clot lysis (Con. 100 µg/ml)
C. limon	0.369 ± 0.148
C. aurantifollia	5.453 ± 0.896
C. bergamia	5.942 ± 1.179
C. maxima	1.785 ± 0. 478
C. sinensis	4.798 ± 0.806
C. macroptera	6.908± 1.702
Streptokinase (Std.)	87.016 ± 2.253

Values are means of triplicate determination ± Standard Deviation

platelet bonding [67-69]. This binding can also trap other blood cells which accelerate the process of plaque development and progression. 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

ACh. 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.

REFERENCES

1. Khan MK, Dangles O. A comprehensive review on flavanones, the major citrus polyphenols. Journal of Food Composition and Analysis. 2014;33(1):85-104.
2. Ma G, Zhang L, Yungyuen W, Tsukamoto I, Iijima N, Oikawa M, Kato M. Expression and functional analysis of citrus carotene hydroxylases: Unravelling the xanthophyll biosynthesis in citrus fruits. BMC Plant Biology. 2016;16:148.
3. Lv X, Zhao S, Ning Z, Zeng H, Shu Y, Tao O, Liu Y. *Citrus*fruits as a treasure trove of active natural metabolites that potentially provide benefits for human health. Chemistry Central Journal. 2015;9: 68.
4. Wang S, Tu H, Wan J, Chen W, Liu X, Luo J, Zhang H. Spatio-temporal distribution and natural variation of metabolites in citrus fruits. Food Chemistry. 2016:199:8-17.
5. Hijaz F, Nehela Y, Killiny N. Possible role of plant volatiles in tolerance against huanglongbing in citrus. Plant Signaling & Behavior. 2016;11(3):e1138193.
6. Vingeliene S, Chan DS, Aune D, Vieira AR, Polemiti E, Stevens C, Norat T. An update of the WCRF/AICR systematic literature review on esophageal and gastric cancers and citrus fruits intake. Cancer Causes & Control. 2016;27(7):837-851.
7. Zou Z, Xi W, Hu Y, Nie C, Zhou Z. Antioxidant activity of Citrus fruits. Food chemistry. 2016;196:885-896.
8. Sun Y, Shen Y, Liu D, Ye X. Effects of drying methods on phytochemical compounds and antioxidant activity of physiologically dropped un-matured citrus fruits. LWT-Food Science and Technology, 2015;60(2):1269-1275.
9. Tareen H, Mengal F, Masood Z, Mengal R, Ahmed S, Bibi S, Farman N. Determination of Vitamin C content in Citrus fruits and in non-citrus fruits by titrimetric method, with special reference to their nutritional importance in human diet. In Biological Forum. Research Trend. July, 2015;7(2): 367.
10. Díaz L, Del Río JA, Pérez-Gilabert M, Ortuño A. Involvement of an extracellular fungus laccase in the flavonoid metabolism in Citrus fruits inoculated with Alternaria alternata. Plant Physiology and Biochemistry. 2015;89:11-17.
11. Mulvihill EE, Burke AC, Huff MW. Citrus flavonoids as regulators of lipoprotein metabolism and atherosclerosis. Annual review of nutrition. 2016;36:275-299.
12. Suzawa M, Guo L, Pan MH, Ho CT, Li S. *I-vivo* anti-carcinogenic property of a formulated citrus peel extract. Functional Foods in Health and Disease. 2014; 4(3):120-129.
13. Parhiz H, Roohbakhsh A, Soltani F, Rezaee R, Iranshahi M. Antioxidant and anti-inflammatory properties of the citrus flavonoids hesperidin and hesperetin: An updated review of their molecular mechanisms and experimental models. *Phytotherapy Research.* 2015; *29*(3):323-331.
14. Singh J, Sood S, Muthuraman A. *In-vitro* evaluation of bioactive compounds, anti-oxidant, lipid peroxidation and lipoxygenase inhibitory potential of Citrus karna L. peel extract. Journal of Food Science and Technology. 2014;51(1):67-74.
15. Benavente-Garcia O, Castillo J(). Update on uses and properties of citrus flavonoids: New findings in anticancer, cardiovascular, and anti-inflammatory activity. Journal of agricultural and food chemistry. 2008; 56(15):6185-6205.
16. Rauf A, Uddin G, Ali J. Phytochemical analysis and radical scavenging profile of juices of Citrus sinensis, Citrus anrantifolia, and Citrus limonum. Organic and Medicinal Chemistry Letters. 2014;4(1):1-3.
17. Iranzo A, Fernández-Arcos A, Tolosa E, Serradell M, Molinuevo JL, Valldeoriola F, Gaig C. Neurodegenerative disorder risk in idiopathic REM sleep behavior disorder:

Study in 174 patients. PLoS One, 2014;9(2):e89741.
18. Paulsen JS, Nance M, Kim JI, Carlozzi NE, Panegyres PK, Erwin C, Williams JK. A review of quality of life after predictive testing for and earlier identification of neurodegenerative diseases. Progress in neurobiology. 2013;110:2-28.
19. Novarino G, Fenstermaker AG, Zaki MS, Hofree M, Silhavy JL, Heiberg AD, Masri A. Exome sequencing links corticospinal motor neuron disease to common neurodegenerative disorders Science. 2014;343(6170):506-511.
20. Asante EA, Smidak M, Grimshaw A, Houghton R, Tomlinson A, Jeelani A, Brandner S. A naturally occurring variant of the human prion protein completely prevents prion disease. Nature. 2015; 522(7557):478-481.
21. Wingerchuk DM, Carter JL. Multiple sclerosis: Current and emerging disease-modifying therapies and treatment strategies. In Mayo Clinic Proceedings. Elsevier. 2014;89(2):225-240.
22. Henderson VW. Alzheimer's disease: Review of hormone therapy trials and implications for treatment and prevention after menopause. The Journal of steroid biochemistry and molecular biology. 2014; 142:99-106.
23. Castello MA, Soriano S. On the origin of Alzheimer's disease. Trials and tribulations of the amyloid hypothesis. Ageing Research Reviews. 2014;13:10-12.
24. Kumar A, Singh A. A review on Alzheimer's disease pathophysiology and its management: An update. Pharmacological Reports. 2015;67(2):195-203.
25. Lim D, Ronco V, Grolla AA, Verkhratsky A, Genazzani AA. Glial calcium signaling in Alzheimer's disease. In Reviews of Physiology, Biochemistry and Pharmacology. Springer International Publishing. 2014;167:45-65.
26. Padurariu M, Ciobica A, Lefter R, Lacramioara Serban I, Stefanescu C, Chirita R. The oxidative stress hypothesis in Alzheimer's disease. Psychiatria Danubina. 2013;25(4):0-409.
27. Birks JS, Grimley Evans J. Rivastigmine for Alzheimer's disease. The Cochrane Library; 2015.
28. Montero-Odasso M, Muir-Hunter SW, Oteng-Amoako A, Gopaul K, Islam A, Borrie M, Speechley M. Donepezil improves gait performance in older adults with mild Alzheimer's disease: A phase II clinical trial. Journal of Alzheimer's Disease. 2015;43(1):193-199.
29. Karunaweera N, Raju R, Gyengesi E, Münch G. Plant polyphenols as inhibitors of NF-κB induced cytokine production—A potential anti-inflammatory treatment for Alzheimer's disease? Frontiers in Molecular Neuroscience. 2015;8.
30. Harasym J, Oledzki R. Effect of fruit and vegetable antioxidants on total antioxidant capacity of blood plasma. Nutrition. 2014;30(5):511-517.
31. Aguilera Y, Martin-Cabrejas MA, de Mejia EG. Phenolic compounds in fruits and beverages consumed as part of the mediterranean diet: their role in prevention of chronic diseases. Phytochemistry Reviews. 2016;15(3):405-423.
32. Lima GPP, Vianello F, Corrêa CR, da Silva Campos RA, Borguini MG. Polyphenols in fruits and vegetables and its effect on human health. Food and Nutrition sciences; 2014.
33. Giacoppo S, Galuppo M, Calabrò RS, D'Aleo G, Marra A, Sessa E, Mazzon E. Heavy metals and neurodegenerative diseases: an observational study. Biological Trace Element Research. 2014; 161(2):151-160.
34. Ladanyia M, Ladaniya M. Citrus fruit: Biology, technology and evaluation. Academic press; 2010.
35. Wu QS, Zou YN, He XH. Contributions of arbuscular mycorrhizal fungi to growth, photosynthesis, root morphology and ionic balance of citrus seedlings under salt stress. Acta Physiologiae Plantarum. 2010;32(2):297-304.
36. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and Viticulture. 1965;16(3):144-158.
37. Zhishen J, Mengcheng T, Jianming W. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food chemistry. 1999;64(4):555-559.
38. Wang H, Helliwell K. Determination of flavonols in green and black tea leaves and green tea infusions by high-performance liquid chromatography. Food Research International. 2001;34(2):223-227.
39. Ellman GL. A colorimetric method for determining low concentrations of

mercaptans. Archives of biochemistry and Biophysics. 1958;74(2):443-450.
40. Ramjan A, Hossain M, Runa JF, Md H, Mahmodul I. Evaluation of thrombolytic potential of three medicinal plants available in Bangladesh, as a potent source of thrombolytic compounds. Avicenna Journal of Phytomedicine. 2014;4(6):430.
41. Lagha-Benamrouche S, Madani K. Phenolic contents and antioxidant activity of orange varieties (*Citrus sinensis L. and Citrus aurantium L.*) cultivated in Algeria: Peels and leaves. Industrial Crops and Products. 2013;50:723-730.
42. Park YS, Cvikrová M, Martincová O, Ham KS, Kang SG, Park YK, Gorinstein S. *In-vitro* antioxidative and binding properties of phenolics in traditional, citrus and exotic fruits. Food Research International. 2015;74:37-47.
43. Ross KA. Concepts important in understanding the health benefits of phenolics in fruits and vegetables: Extractable & non-extractable phenolics and the influence of cell wall polysaccharides on bioaccessibility & bioavailability. Research in Health and Nutrition. 2014;2:29-43.
44. Qiao L, Sun Y, Chen R, Fu Y, Zhang W, Li X, Ye X. Sonochemical effects on 14 flavonoids common in citrus: Relation to stability. PLoS ONE. 2014;9(2):e87766.
45. Assini JM, Mulvihill EE, Huff MW. Citrus flavonoids and lipid metabolism. Current opinion in lipidology. 2013;24(1):34-40.
46. Kozlowska A, Szostak-Wegierek D. Flavonoids-food sources and health benefits. Roczniki Państwowego Zakładu Higieny. 2014;65(2).
47. Harborne JB. The flavonoids: Advances in research since 1980. Springer; 2013.
48. Kozlowska A, Szostak-Wegierek D. Flavonoids-food sources and health benefits. Roczniki Państwowego Zakładu Higieny. 2014;65(2).
49. Xie Y, Huang S, Su Y. Dietary flavonols intake and risk of esophageal and gastric cancer: A meta-analysis of epidemiological studies. Nutrients. 2016;*8*(2):91.
50. Durand-Hulak M, Dugrand A, Duval T, Bidel LPR, Jay-Allemand C, Froelicher Y, Fanciullino AL. Mapping the genetic and tissular diversity of 64 phenolic compounds in Citrus species using a UPLC–MS approach. Annals of Botany. 2015;115(5): 861–877.
51. Hui C, Qi X, Qianyong Z, Xiaoli P, Jundong Z, Mantian M. Flavonoids, flavonoid subclasses and breast cancer risk: A meta-analysis of epidemiologic studies. *PLoS ONE*. 2013;*8*(1):e54318.
52. Leinonen A, Koponen M, Hartikainen S. (). Systematic review: representativeness of participants in RCTs of acetylcholinesterase inhibitors.*PloS One.* 2015;*10*(5):e0124500.
53. Biswas K, Islam MA, Sharmin T, Biswas PK. *In-vitro* cholinesterase inhibitory activity of dry fruit extract of Phyllanthus emblica relevant to the treatment of Alzheimer's disease; 2015.
54. Biswas K, Azad AK, Sultana T, Khan F, Hossain S, Alam S, Khatun Y. Assessment of *In-vitro* cholinesterase inhibitory and thrombolytic potential of bark and seed extracts of *Tamarindus indica* (L.) relevant to the treatment of Alzheimer's disease and. Journal of Intercultural Ethnopharmacology. 2017;6(1):115-120.
55. Uddin MJ, Alam MN, Biswas K, Rahman MA. *In-vitro* antioxidative and cholinesterase inhibitory properties of *Thunbergia grandiflora* leaf extract. Cogent Food & Agriculture. 2016;2(1):1256929.
56. Nasrullah M, Haque A, Yasmin Z, Uddin MA, Biswas K, Islam MS. Phytochemical screening, antioxidant and anticholinesterase effects of *Alangium salvifolium* (LF) Wang root extracts. Journal of Medicinal Plants Research. 2015;9(42):1060-1069.
57. Mehndiratta MM, Pandey S, Kuntzer T. Acetylcholinesterase inhibitor treatment for myasthenia gravis. The Cochrane Library; 2014.
58. Uddin MJ, Abdullah-Al-Mamun M, Biswas K, Asaduzzaman M, Rahman MM. Assessment of anticholinesterase activities and antioxidant potentials of *Anisomeles indica* relevant to the treatment of Alzheimer's disease. Oriental Pharmacy and Experimental Medicine. 2016;16(2): 113-121.
59. Biswas K, Begum MM, Sarker A, Huq TB, Sarwar A. Anticholinesterase and antioxidant potentials of a medicinal plant abroma augusta: Implications for the alternative treatment therapy of cognitive deficits in alzheimer's disease. Clin Pharmacol Biopharm. 2015;4(148):2.
60. Lockridge O. Review of human butyrylcholinesterase structure, function, genetic variants, history of use in the clinic,

and potential therapeutic uses. Pharmacology & Therapeutics. 2015;148: 34-46.
61. Nordberg A, Ballard C, Bullock R, Darreh-Shori T, Somogyi M. A review of butyrylcholinesterase as a therapeutic target in the treatment of Alzheimer's disease. Prim Care Companion CNS Disord. 2013;15(2).
62. Anand P, Singh B. A review on cholinesterase inhibitors for Alzheimer's disease. Archives of Pharmacal Research. 2013;36(4):375-399.
63. Bentzon JF, Otsuka F, Virmani R, Falk E. Mechanisms of plaque formation and rupture. Circulation Research. 2014; 114(12):1852-1866.
64. Holy EW, Besler C, Reiner MF, Camici GG, Manz J, Beer JH, Tanner FC. High-density lipoprotein from patients with coronary heart disease loses anti-thrombotic effects on endothelial cells: Impact on arterial thrombus formation. Thrombosis and haemostasis. 2014;112(5):1024-1035.
65. Matsuura Y, Yamashita A, Iwakiri T, Sugita C, Okuyama N, Kitamura K, Asada Y. Vascular wall hypoxia promotes arterial thrombus formation via augmentation of vascular thrombogenicity. Thrombosis and haemostasis. 2015;114(1):158-172.
66. Schuhmann MK, Kraft P, Stoll G, Lorenz K, Meuth SG, Wiendl H, Kleinschnitz C. CD28 superagonist-mediated boost of regulatory T cells increases thrombo-inflammation and ischemic neurodegeneration during the acute phase of experimental stroke. Journal of Cerebral Blood Flow & Metabolism. 2015;35(1):6-10.
67. Walsh TG, Berndt MC, Carrim N, Cowman J, Kenny D, Metharom P. The role of Nox1 and Nox2 in GPVI-dependent platelet activation and thrombus formation. Redox Biology. 2014;2:178-186.
68. De Witt SM, Swieringa F, Cavill R, Lamers MM, Van Kruchten R, Mastenbroek T, Scharrer I. Identification of platelet function defects by multi-parameter assessment of thrombus formation. Nature communica-tions. 2014;5.
69. Aizawa K, Takahari Y, Higashijima N, Serizawa K, Yogo K, Ishizuka N, Ishida H. Nicorandil prevents sirolimus-induced production of reactive oxygen species, endothelial dysfunction, and thrombus formation. Journal of Pharmacological Sciences. 2015;127(3):284-291.
70. Molassiotis A, Job D, Ziogas V, Tanou G. Citrus plants: A model system for unlocking the secrets of NO and ROS-inspired priming against salinity and drought. Frontiers in Plant Science. 2016;7.
71. Riaz A, Khan RA, Mirza T, Mustansir T, Ahmed M. *In-vitro*/*In-vivo* effect of *Citrus limon* (L. Burm. f.) juice on blood parameters, coagulation and anticoagulation factors in rabbits. Pak. J. Pharm. Sci. 2014;27(4):907-915.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/23447