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Flavonoid Composition and Antioxidant Efficacy of Citrus Peels: An Integrated *in vitro* and *in silico* Approach toward Potential Neuroprotective Agents

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The current study explored the therapeutic significance of different standardized citrus peel extracts as potential neuroprotective agents using integrated *in vitro* and *in silico* approaches. Hydroethanolic extract of five industrially important citrus fruit peels were subjected to HPLC-based quantification of estimating major flavonoids of nutraceutical importance. Pharmacological activities like antioxidant and anti-inflammatory activities of the extracts were determined by *in vitro* assays. Further, The identified bioactive metabolites were subjected to the Prediction of Activity Spectra for Substances program to get a prediction of their biological activity spectrum. Amongst various solvent combinations, 80% ethanol provided maximum (≥ 20% w/w) extract yield. Mandarin peels of *Citrus reticulata* showed the highest amount of polyphenolics (*Citrus reticulata* Blanco; 42.24 ± 0.57 mg gallic acid equivalent/g) and flavonoids (*Citrus reticulata c.v.*;13.08 ± 0.17 mg quercetin equivalent/g) content. The most abundant flavonoid compound present in all the citrus peel was hesperidin, except *Citrus reticulata* Blanco and *Citrus grandis*, which showed a considerably high amount of nobiletin and naringin, respectively. *Citrus reticulate c.v.* peel extract showed potent antioxidant [IC_{50} = 118.82 ± 1.97 µg/mL in 2,2-diphenyl-1-picrylhydrazyl assay and IC_{50} = 138.12 ± 2.67 µg/mL in 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid assays)], anti-inflammatory (IC_{50} = 50.61 ± 6.79 µg/mL), and acetylcholinesterase inhibitory ((IC_{50} = 130.61 ± 2.04µg/mL) activities compared to the other extracts. *In silico* assessment revealed a high (Pa > 0.7) activity score for free radical scavenging, lipid peroxidase inhibitory, membrane integrity agonistic, anti-inflammatory, antioxidant, and several other important biological activities of the identified flavonoids in the extracts, thus supported neuroprotective potential. Citrus flavonoids naringin, rutin, and tangeretin showed high activity scores for anti-inflammatory activity strengthening the results of *in vitro* assay. These potentials of citrus peels could be utilized in the development of functional foods and nutraceuticals for neurodegenerative conditions. Furthermore, such a practice will help citrus agro-industrial waste valorisation.

Keywords: *Citrus reticulata c.v.*, Hesperidin, Oxidative stress, Prediction of activity spectra, Reactive oxygen species

List of Abbreviations

ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
AChE: Acetylcholinesterase
ACTI: Acetylthiocholine iodide
BSA: Bovine serum albumin
CG: *Citrus grandis*
CL: *Citrus limetta*
CRB: *Citrus reticulata* Blanco
CRK: *Citrus reticulata c.v.* (Kinnow)
CS: *Citrus sinensis*
DPPH: 2,2-diphenyl-1-picrylhydrazyl
DTNB: 5,5'-dithio-bis-(2-nitrobenzoic acid)
GAE: Gallic acid equivalents
HIF: Hypoxia inducible factor
HPLC: High-performance liquid chromatography
IC_{50}: Half-maximal inhibitory concentration
Pa: Probable activity
PASS: Prediction of activity spectra for substances
Pi: Probable inactivity
TFC: Total flavonoid content
TPC: Total phenolic content

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Introduction

Neurodegenerative diseases are a major medical and public health concern around the world.[1] As the average human lifespan has increased with the progression in medical amenities, the prevalence of these conditions is also expected to increase in the near future.[2] There is extensive evidence suggesting the importance of flavonoids in age-related issues such as memory loss, cognitive decline, and learning disabilities. Different mechanisms involved in neurological functions can be influenced by supplementation of either single dietary flavonoid or flavonoid-rich preparations.[3] In a study performed by Gao *et al.*[4], it is observed that habitual intake of flavonoid-rich fruits such as tea, berry fruits, apples, red wine, and oranges reduced the risk of Parkinson's

disease in men. Flavonoids found in various fruits and vegetables have been shown to improve neural protection, support existing neural activities, and stimulate neuronal regeneration.[5]

Citrus fruits are an important source of dietary flavonoids. Mandarins, oranges, limes, tangerines, lemons, sour oranges, and grapefruits are among the common citrus fruits, with an annual production of 102 million tonnes globally.[6] Citrus fruits are widely known for their diversified phenolic constituents, which play a vital role in promoting human health when consumed directly as food or in the form of isolates. Peels of citrus fruits contain a significant amount of polymethoxylated flavones that are physiologically more active than their methylated equivalents found in pulp and are unique to these plants. Moreover, due to the presence of various phenolics, flavonoids, carotenoids, reducing sugars, and ascorbic acid, the peel is alleged to have a higher antioxidant capacity than the other fruit parts.[7] Interestingly, the main citrus flavonoids can also traverse the blood-brain barrier.[8]

Several scientific studies have confirmed the role of these bioactive compounds against inflammation, viral infections, oxidative stress, cancer, and other life-threatening conditions.[9] These activities can be attributed to the properties of polyphenols like free radical scavenging, pro-oxidant metal ions chelation, and capability to act as enzyme cofactors.[10] Moreover, as per Ayurveda, orange peel prevents health-related problems originating from the Kapha and the Pitta doshas imbalance.

The citrus fruit pulp is used to produce different products such as juices, jams, jellies, concentrates, *etc*. Global fruit juice and nectar consumption were reported to be 38.5 billion litres in 2015 by the European Fruit Juice Association.[11] Despite having vast ethnomedicinal importance, the citrus peel is either forwarded for dumping in open environments or used as animal feed. In 2009–2010, the global production of citrus fruits was estimated to be 82 million tons, of which orange production alone accounts for 50 million tons and yields about 44% peel as a by-product. The waste generated from orange peel was assessed to be approximately 3.8 million tons in 2014.[(12)] Considering the wealth of micronutrients and the plethora of reported health-promoting activities in traditional medicine, citrus peels can be used for therapeutic purposes. With its low cost and easy availability, citrus waste is proficient in offering substantial low-cost nutritional supplementation. The present study explored the therapeutic significance of different standardized citrus peel extracts as potential neuroprotective agents using integrated *in vitro* and *in silico* approaches. The peel of five industrially important citrus fruits, including CRB [*Citrus reticulata* Blanco (orange)], CG [*Citrus grandis* (Chakotra)], CRK [*Citrus reticulata c.v*. (Kinnow)], CL [*Citrus limetta* (Mausambi)] and CS [*Citrus sinensis* (Malta)] were selected and extracted using different solvent combinations. Most of these plants are harvested in the Punjab, India, and lower regions [300–600 MASL (meters above sea level)] of the Himachal Pradesh, India. In contrast, CRB is a native crop of Maharashtra, India. HPLC-based quantification of major flavonoids of nutraceutical importance was carried out in the extracts. The identified bioactive metabolites in the peel extracts were subjected to the PASS (prediction of activity spectra for substances) program to get a prediction of their biological activity spectrum. The activities supporting neuroprotection were validated using *in vitro* assays.

Material and Methods

Chemicals

Quercetin, apigenin, nobiletin, tangeretin, ACTI (acetylthiocholine iodide) and donepezil hydrochloride were purchased from Tokyo Chemical Industry. Hesperidin, naringin, AChE (acetylcholinesteras) type VI-S, DTNB [5,5'-dithio-bis-(2-nitrobenzoic acid)] and BSA (bovine serum albumin) were procured from Sigma Aldrich. Folin-Ciocalteu reagent, sodium carbonate, aluminium chloride, potassium persulfate and sodium hydroxide were purchased from Merck India. DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] were purchased from HiMedia Laboratories. HPLC grade acetonitrile, formic acid, and methanol were procured from Merck Life Sciences. Purified distilled water was obtained from Milli-Q® Integral Water Purification System. All the chemicals utilized were of analytical grade and were stored in the laboratory according to the standard storage conditions, as specified in the respective Safety Data Sheet.

Plant Material and Extraction

CG, CRK, CL, and CS were procured from Himachal Pradesh, India, whereas CRB was obtained from Nagpur, Maharashtra, India. The collected peels were cleaned and dried in a hot air oven at 40 ± 5°C. The dried peels were cut into small pieces, grounded

using a mixer grinder (Philips, India), and sieved to 500-micron particle size. Finally, the powder was stored in amber-colored glass bottles at ambient temperature until used.

The peel powder was subjected to a thermal-assisted overnight maceration extraction process. The process consisted of thermal treatment of the sample with a solvent system (1:10) at 80°C for 20 minutes and further cooling at room temperature. Different solvent combinations were used for extraction, including aqueous, 50%, 70%, 80% and 100% *v/v* ethanol. Maceration was carried out overnight, followed by filtration. The filtrate was concentrated under reduced pressure using a rotary evaporator (Rotavapor R-300 BUCHI, Switzerland) and dried using a lyophilizer (Labconco FreeZone 6Plus). The extraction yield of the lyophilized extracts was expressed as percentage of the weight of the crude extract to the raw material. Based on the results (described in the results section), the 80% ethanol extract was selected for further studies.

High-Performance Liquid Chromatography (HPLC) based Quantification

The quantification was performed on a Shimadzu HPLC system (Model LC-20AT pump, DGU-20A5 degasser) attached with an autosampler (SIL-20AC, set at 20 µL injection volume) and a PDA (Photodiode array) detector (SPDM20A, customized at a wavelength of 280 nm). The chromatographic separation was achieved on the RP-18 column (250 mm × 4.6 mm; 5 µm, Waters). The mobile phase was eluted with a gradient consisting of solvent A: water (0.1% formic acid) and solvent B: acetonitrile (0.05% formic acid). The gradient varied from 0 to 10% B in 5 min, 10 to 22% B in 20 min, 22 to 61% B in 45 min, and lastly 61 to 100% B in 50 min. The flow rate was set at 1 mL/min, and the column temperature was fixed at 30°C. The extracts were dissolved in HPLC grade methanol and filtered through the Whatman syringe filter of pore size 0.45µm before injection. Chromatographic data were recorded, and peak identification was made in comparison to the reference standards with corresponding retention time. The separated flavonoids were quantified using standard calibration curves (Rutin: y = 73223x + 20605, R2 = 0.999; Naringin: y = 216709x + 1905.2, R2 = 0.998; Hesperidin: y = 162484x + 142953, R2 = 1; Quercetin: y = 102581x – 213098, R2 = 0.99; Apigenin: y = 91488x – 13192, R2 = 0.99; Nobiletin: y = 159331x + 67128, R2 = 0.99 and; Tangeretin: y = 271611 x + 71614, R2 = 0.999).

Determination of Total Phenolic and Flavonoid Content

The total phenolic content (TPC) was determined using a spectrophotometric method with the Folin-Ciocalteu reagent. [13] The quantification was performed with gallic acid as a standard. The value of TPC in the extracts was expressed as gallic acid equivalent (GAE/g). The total flavonoid content (TFC) was also determined spectrophotometrically as described previously.[13] A calibration curve was prepared using quercetin (flavonoid standard), and the values were expressed in terms of standard equivalent (QE/g).

Antioxidant Activity

The free radical scavenging activity of the extracts was studied spectrophotometrically by using a standard DPPH assay. The activity was determined by recording the decrease in absorption when the color of the DPPH solution changes from purple to light yellow in the presence of the extract. The results were expressed as IC_{50} (half percent inhibition concentration) values and ascorbic acid was used as a reference standard.[13]

In another assay, ABTS was converted to its radical cation by adding potassium persulfate, and the scavenging activity of the extracts was studied spectrophotometrically. The IC_{50} values were calculated through extrapolation from regression analysis and ascorbic acid was used as a reference standard. [13]

AChE Inhibition Assay

AChE inhibition assay was conducted using a standard colorimetric method. Briefly, different extracts/standard donepezil (in pH 7.5 tris buffer; containing 0.5% DMSO) were added to the reaction mixture containing DTNB, tris buffer (pH 8.0), and AChE in a 96-well plate. The plate was incubated for 15 min at 25°C, and ATCI was added, and absorbance was measured at 412 nm (Synergy H1 Multi-Mode microplate reader). All assays were carried out in triplicate. The inhibitory percentage (%) of AChE was calculated using a formula E-C/E × 100 (E and C indicate the observation without and with the test agent, respectively). Based on the results, IC_{50} values were calculated.

Protein Denaturation Assay for Anti-Inflammatory Activity

In vitro anti-inflammatory activity of the extracts was evaluated using the bovine serum albumin protein denaturation method described by Williams *et al*.[14] with some modifications. Different extracts/standard diclofenac sodium concentrations in tris buffer containing 0.5% DMSO were mixed with 1% BSA solution (prepared in tris buffer pH 6.3). The mixture

was incubated for 15 min at 37°C, and heated in a water bath for 10 min at 72°C. After that, the mixture was cooled down to room temperature, and the absorbance was measured at 660 nm. All the reactions were performed in triplicate. The percentage inhibition was calculated using formula: C-S/C × 100 (C and S indicate the observation without and with the test agent, respectively). Based on the observations, IC_{50} values were calculated.

PASS Assisted *in silico* Analysis

The biological activity spectrum of each quantified flavonoid in the extracts was predicted by *in silico* analysis using PASS software (http://www.way2 drug.com/passonline/). The chemical structure of each flavonoid in MOL file format was subjected to the PASS program. The software assesses a compound's biological activity spectrum as probable activity (Pa) and likely inactivity (Pi). The PASS spectrum predictions are based on structure-activity relationship analysis of a training set that consists of more than 2 lakh compounds with around 6 thousand biological activities. Pa and Pi are considered as probabilities, and their value ranges from 0.000 to 1.000, and in general, Pa ≠ Pi = 1.

Data Analysis

The results were expressed as the mean ± standard deviation from replicates. The PASS predictions were interpreted as (i) activities with Pa > Pi were only considered possible for a given compound; (ii) if Pa > 0.7, the chance to find an activity experimentally was high; (iii) if 0.5 < Pa < 0.7, the chance to find an activity experimentally was less, but the compound of interest was probably not as similar to known pharmaceutical agents; (iv) if Pa < 0.5, the chance to find the activity experimentally is less, but the probability of finding a structurally new compound, *i.e.*, a new chemical entity is more. Based on the PASS prediction results, the predicted activities supporting neuroprotection for each flavonoid with a score of Pa > 0.7 were only considered.

Results and Discussion

Extraction yield

The extraction results showed that 80% ethanol, followed by 70% ethanol, yielded a higher amount of extract (w/w) compared to the other ratios. The 80% ethanol yielded 22.7%, 20.4%, 23.54%, 20% and 21.4% of dry extract from CRB, CG, CRK, CL and CS respectively. Therefore, the 80% ethanol extract was selected for further flavonoid quantification and *in vitro* studies. However, the minimum extraction yield was obtained with an aqueous solvent without ethanol. The extract yield with different solvent ratios from all the peels is shown in Fig. 1A.

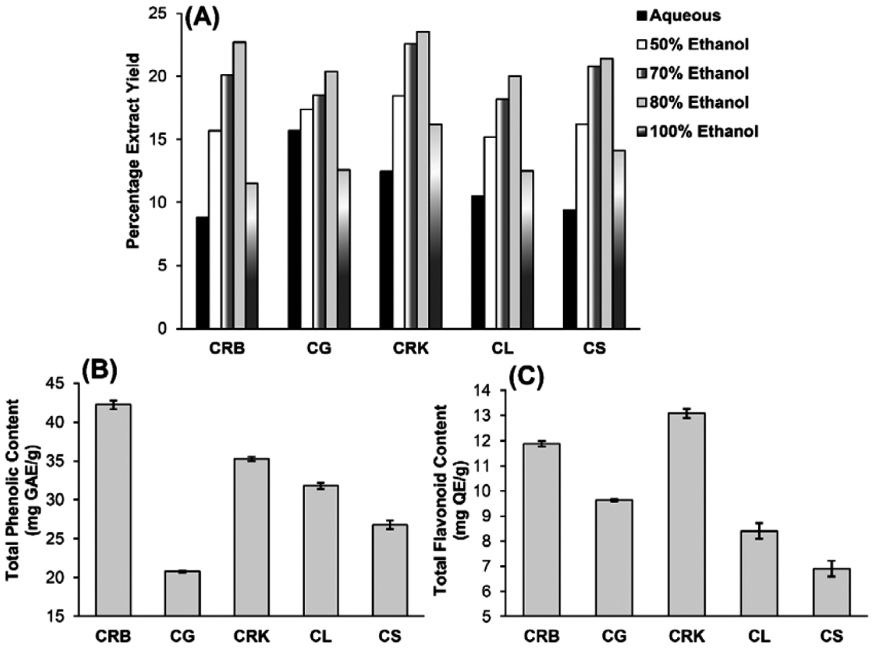


Fig. 1 — Hydroethanolic extraction of dry citrus peel: (A) Percentage yield of different citrus peels with varied solvent combinations, (B) Total polyphenol content (TPC) represented in mg GAE/g, (C) Total Flavonoid content (TFC) represented in mg QE/g; Values of TPC and TPC expressed as mean ± standard deviation (n = 3)

It is known that various factors could influence the effectiveness and yield of extraction, such as technique employed, solvent used, particle size, sample to solvent ratio, temperature and time, *etc*. Thermal-assisted maceration is one of the economic and desirable methods for extraction. Where plant tissue disintegration is enhanced by introducing thermal treatment. In our study, 80% ethanol gave the maximum extraction yield, which is in accordance with the previously conducted studies. Likewise, in a study performed by Safdar *et al.*[15] highest extraction yield of *Citrus mandarin* (Kinnow*)* peel was achieved with 80% ethanol compared to other hydroethanolic ratios.

Quantification of Flavonoids

Hesperidin came out to be the major flavonoid in most of the selected peel extracts and was highest in CRK (555.75 ± 4.3 µg/100 g), followed by CRB (396.45 ± 1.15 µg/100 g) peel. However, it was not detected in CG extract that was found to be rich in naringin (613.27 ± 0.63 µg/100g). Naringin was also detected in CRK and CRB extracts in relatively low concentrations. Quercetin was present in all the peels, with the highest concentration found in CRK peel (140.66 ± 0.526 µg/ 100g). CRB peel showed the maximum amount of rutin (219.081 ± 4.75 µg/100g) as compared to other peels. Apigenin was the other flavonoid which showed highest presence in CRK peel (20.03 ± 4. 205 µg/100g).The concentration of nobiletin and tangeretin came out to be higher in CRB extract (438.9 ± 1.38 µg/100g and 174.94 ± 1.81 µg/100g) followed by CRK extract (336.39 ± 17.07 µg/ 100g and 84.027 ± 3.43 µg/100g), while both these flavonoids were present in little amount in all other peels (Table 1).

Thus, qualitative analysis using HPLC system confirmed that the peels of citrus fruits contain a mixture of different flavonoids in various ratios. The most prominent flavonoid in the citrus peel was hesperidin. While, rutin, naringin, nobiletin, quercetin, apigenin, and tangeretin were among the other flavonoids found. Earlier, Safdar *et al.*[15] also reported that hesperidin is the major flavonoid present in Kinnow peel to the extent of 92.94 ± 1.23 µg/g in an ethanol extract. After studying the composition of citrus peels, *In vitro* assays were performed in order to evaluate the neuroprotective profile of different peels.

TPC and TFC Content

Among all the tested extracts, the highest TPC was determined in CRB (42.24 ± 0.57 mg GAE/g), followed by CRK (35.27 ± 0.26 mg GAE/g). Phenolic content in *Citrus reticulata* species was followed by CL, CS, and CG with 31.81 ± 0.39 mg GAE/g, 26.79 ± 0.53 mg GAE/g, and 20.76 ± 0.13 mg GAE/g, respectively (Fig. 1B).

The biological activities of citrus fruits have primarily been attributed to their polyphenolic constituents. Phenolic compounds are the bioactive molecules exhibiting aromatic rings along with one or many hydroxyl groups. Total phenolic content comprises both flavonoids as well as phenolic acids. The TFC content was found to be maximum in CRK (13.08 ± 0.1 mg QE/g), followed by CRB (11.87 ± 0.11 mg QE/g). The TFC values for CG and CL were 9.63 ± 0.04 mg QE/g and 8.41 ± 0.31 mg QE/g, respectively. Whereas, least flavonoid content (6.90 ± 0.31 mg QE/g) was observed in CS (Fig. 1C).

Flavonoids have an elementary C6-C3-C6 structure (two aromatic rings and a heterocyclic ring with one oxygen atom). They are synthesized through the phenylpropanoid pathway with phenylalanine as a precursor.[16] Flavonoids are also known to operate as singlet oxygen quenchers and chelators of transition metals like copper and iron, which act as food pro-oxidants. Extended conjugation, the quantity and

Table 1 — HPLC-PDA-based quantification of flavonoids in different citrus extracts

S. No.	Extract	Content (µg/100 mg of extract)						
		Rutin	Naringin	Hesperidin	Quercetin	Apigenin	Nobiletin	Tangeretin
1	CRB	219.08 ± 4.75	4.22 ± 0.14	396.45 ± 1.15	24.73 ± 0.98	11.61 ± 0.53	438.9 ± 1.38	174.94 ± 1.81
2	CG	15.29 ± 0.73	613.27 ± 0.63	ND	38.45 ± 2.14	14.07 ± 0.37	5.35 ± 1.4	1. 24 ± 0.17
3	CRK	72.6 ± 7.89	11.47 ± 0.16	555.75 ± 4.3	140.66 ± 0.526	20.03 ± 4.2	336.39 ± 17.07	84.02 ± 3.43
4	CL	28.57 ± 0.02	ND	102.83 ± 3.41	33.22 ± 0.1	ND	8.78 ± 0.17	0.59 ± 0.03
5	CS	47.92 ± 0.17	ND	112.40 ± 1.07	32.2 ± 0.9	ND	7.76± 0.05	0.98± 0.02

Each value expressed as Mean ± SD (n = 3) and; ND: Not detected

rearrangement of phenolic substituents, and molecular weight are the major factors that affect the capacity of monomeric phenolics to behave as antioxidants.[17]

Free Radical Scavenging Activity

DPPH free radical scavenging assay revealed that CRK followed by CG exhibited the lowest IC_{50} (118.82 ± 1.9 µg/mL and 119.42 ± 3.9 µg/mL respectively) value in comparison to the other peel extracts. IC_{50} for CRB and CS was 136.1 ± 2.3 µg/mL and 139.33 ± 3.0 µg/m respectively. However, CL showed the least antioxidant activity against DPPH radical with an IC_{50} value of 218.44 ± 3.2 µg/mL. The IC_{50} value for standard ascorbic acid was 3.17 ± 0.004 µg/mL (Fig. 2A). In ABTS assay, IC_{50} values of *Citrus reticulata* species (CRK: 138.12 ± 2.6 µg/mL and CRB: 148.77 ± 1.33 µg/mL) were found to be lowest, followed by CG: 198.54 ± 0.8 µg/mL, CL: 220.56 ± 1.9 µg/mL and CS: 310.61 ± 4.8 µg/mL consecutively (Fig. 2B). The results were expressed in comparison to ascorbic acid (IC_{50}: 2.88 ± 0.005 µg/mL), used as a reference standard.

Most of the neurological diseases and neurodegenerative processes share common neuroinflammatory pathways. Neuroinflammation can occur as a cause as well as a consequence to free radical generation. This ultimately leads to neuronal degeneration and apoptotic cell death resulting in cognitive impairment.[18] All the commercially available treatments for neurological problems are also focused on this mechanism. Hence, in the current study the effect of citrus flavonoids on these parameters was studied. It was observed that citrus flavonoids can exert protective effect at one or more steps in this cascade. DPPH and ABTS assays verified the free radical scavenging and antioxidant potential of various flavonoids. Among all the peels tested CRK extract exhibited higher antioxidant activity with the lowest IC_{50} values followed by CG.

Effect on AChE Activity

CRK had the lowest IC_{50} value (130.6 ± 2.04 µg/mL) of all the citrus peels examined, followed by CL (312.27 ± 15.92 µg/mL), CS (367.20 ± 28.72µg/mL), CRB (441.34 ± 33.75 µg/mL), and CG (475.18 ± 49.71 µg/mL). The IC_{50} value for standard drug donepezil was (0.68 ± 0.009 µg/mL) (Fig. 3A).

There is an ongoing interest in discovering effective inhibitors of enzymes involved in ageing, inflammation, and degenerative processes. Acetylcholinesterase is one such enzyme which causes rapid hydrolysis of acetylcholine leading to termination of impulse transmission in cholinergic pathways. Acetylcholine is the major neurotransmitter of the parasympathetic nervous system. Attention, learning, and short-term

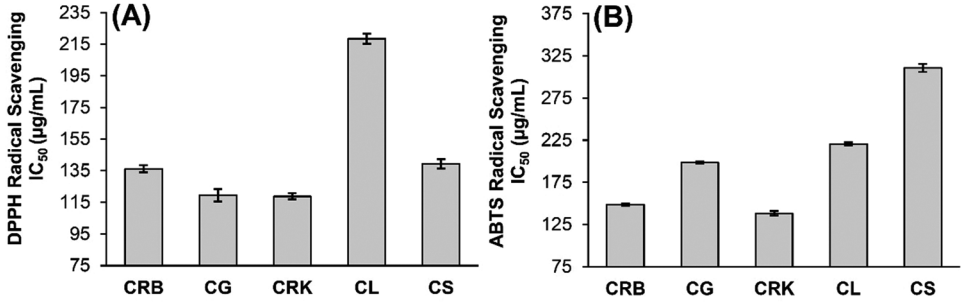


Fig. 2 — Inhibitory concentration 50 (IC_{50}) of various citrus extracts in (A) DPPH and (B) ABTS antioxidant assays; Values expressed as a mean ± standard deviation (n = 3)

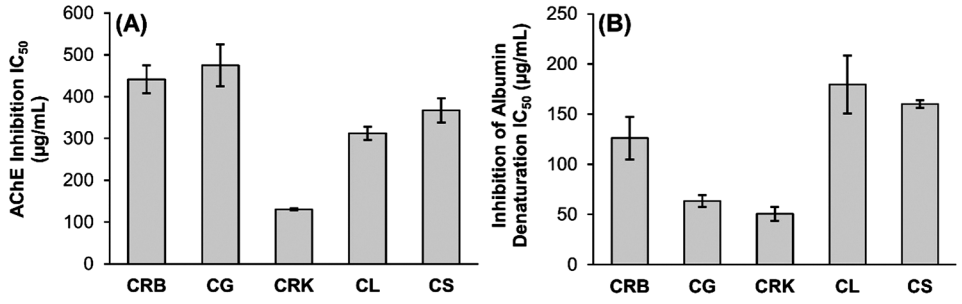


Fig. 3 — Inhibitory concentration 50 (IC_{50}) of various citrus extracts in (A) AChE and (B) protein denaturation assays; Values expressed as a mean ± standard deviation (n = 3)

memory have all been implicated to it. It's been reported that, both the cholinergic receptors (nicotinic and muscarinic) are engaged in the encoding of new memories.[19] The shortage of acetylcholine is related with conditions such as learning and memory impairments as well as chronic disorders like myasthenia gravis, dementia, Alzheimer's and Parkinson's disease etc. Pro-cholinergic drugs constitute the first line of treatment for such disorders. It was revealed in the present study that citrus extract can effectively inhibit the acetylcholinesterase enzyme which is responsible for the depletion of acetylcholine in the brain.

Effect on Protein Denaturation

According to the *in vitro* anti-inflammatory experiment, CRK and CG were the peels with the strongest anti-inflammatory potential having IC_{50} value of 50.61 ± 6.79 µg/mL and 63.43 ± 6.17 µg/mL respectively. IC_{50} values for other peels were CRB (126.17 ± 21.34 µg/mL), CS (160.15 ± 3.94 µg/mL) and CL (179.4 ± 28.87 µg/mL) respectively. Anti-inflammatory drug diclofenac was used as a standard in this assay which showed an IC_{50} value of 78.39 ± 0.45 µg/mL (Fig. 3B).

Anti-inflammatory potential was ascertained by *in vitro* anti-inflammatory assay, which demonstrated positive results with CRK peel having the lowest IC_{50} value. Promising results in anti-inflammatory and anti-oxidant assays strengthen the beneficial role of citrus peel in several neurological problems associated with high ROS generation like Alzheimer's, Parkinsons and amyotrophic lateral sclerosis *etc*.

***In silico* Assessment**

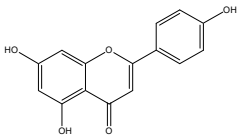
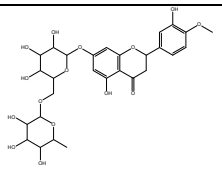
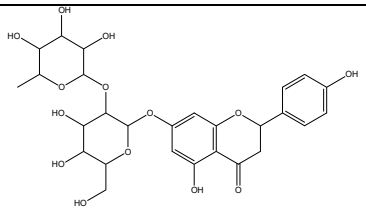
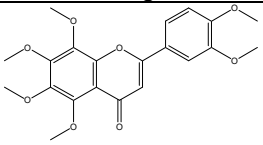
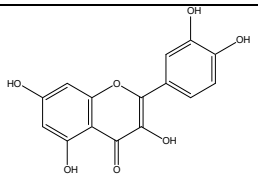
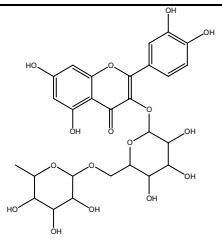
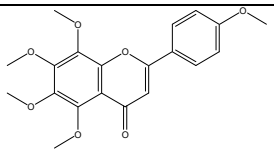
The *in vitro* analysis carried out so far suggested a strong neuroprotective potential of the citrus flavonoids *via* acting as antioxidant, anti-inflammatory and acetylcholinesterase inhibitory molecules. In order to verify the other molecular targets *in silico* PASS analysis was carried out.

The PASS spectra of the identified flavonoids exhibited high predicted scores for free radical scavenging, HIF1A (hypoxia-inducible factor) expression inhibitor, and cell membrane integrity agonist activity. Hesperidin, apigenin, naringin, quercetin, and rutin came out to be strong antioxidants. Activity scores of naringin, rutin, and tangeretin were remarkable for anti-inflammatory activity. Flavonoids, notably hesperidin, naringin, nobiletin, quercetin, and rutin, were found to be effective lipid peroxidase inhibitors. It was also revealed that quercetin, apigenin, nobiletin, and tangeretin have peroxidase inhibitory properties, while apigenin, nobiletin, quercetin, and rutin are effective NADPH oxidase inhibitors. Although, all of the flavonoids investigated presented strong levels of free radical scavenging activity strengthening the findings of our *in vitro* analysis. Apart from this, citrus flavonoids possess high PASS activity scores for other important activities which also reinforced their neuroprotective potential (Table 2).

Each flavonoid is having a unique pharmacological profile, which was confirmed by their pass activity scores. According to earlier studies, when a compound is tested for its PASS suggested pharmacological activity, there are very high chances to get the efficacious results in wet lab too. The oxidative stress generated due to excessive production of free radicals plays an important role in the underlying pathology of many neurological disorders. Citrus flavonoids confirmed the inhibition of oxidative enzymes such as peroxidase, NADPH oxidase, and lipid peroxidase with high activity scores. Peroxidase inhibitory action was demonstrated by quercetin, apigenin, nobiletin and tangeretin. NADPH oxidase is a family of enzymes that produces superoxide radicals by using NADPH as an electron donor. According to McCann *et al.*[20] blocking particular NADPH oxidase can aid in the treatment of ischemic stroke. As suggested by PASS analysis apigenin, Nobiletin, Quercetin, and Rutin are good NADPH oxidase inhibitors. Correspondingly, it was observed that citrus flavonoids specifically hesperidin, naringin, nobiletin, quercetin and rutin can act as better lipid peroxidase inhibitor. Lipid peroxidation is a complex chain reaction comprising oxidative degradation of membrane lipids, producing highly reactive electrophilic aldehydes that damage cells.[21] High activity scores of flavonoids against these oxidative enzymes indicates their ability to protect the neuronal damage caused due to excessive free radical generation.

Another activity presented evidently was cell membrane integrity agonist, as all the flavonoids showed high activity scores. This activity is very important for cell survival, function and resistance against injury. Changes in cell integrity can disturb cell homeostasis and cause a variety of pathological changes. Increased axolemmal membrane permeability has been witnessed in *in vitro* and *in vivo* models of traumatic brain damage and spinal cord injury, and has been reported as one of the initial biophysical events following traumatic brain injury.[22] All of the flavonoids examined had substantial membrane permeability inhibitor activity scores.

Table 2 — PASS predicted activities of the quantified flavonoids supporting neuroprotection

S. No.	Compound	Activity	Pa	Pi
1	 Apigenin	Membrane integrity agonist	0.967	0.002
		Membrane permeability inhibitor	0. 946	0.002
		Peroxidase inhibitor	0.924	0.002
		HIF1A expression inhibitor	0.911	0.005
		NADPH oxidase inhibitor	0.764	0,002
		Antioxidant	0.732	0.004
		Free radical scavenger	0.719	0.004
2	 Hesperidin	Lipid peroxidase inhibitor	0.991	0.001
		Free radical scavenger	0.989	0.001
		Membrane permeability inhibitor	0.976	0.001
		Membrane integrity agonist	0.960	0.003
		Caspase 3 stimulant	0.953	0.003
		Antioxidant	0.846	0.003
		Caspase 8 stimulant	0.764	0.002
		HIF 1A expression inhibitor	0.721	0.018
3	 Naringin	Membrane permeability inhibitor	0.944	0.002
		Free radical scavenger	0.981	0.001
		Lipid peroxidase inhibitor	0.978	0.002
		Membrane integrity agonist	0.970	0.002
		Caspase 3 stimulant	0.896	0.004
		Antioxidant	0.851	0.003
		HIF 1A expression inhibitor	0.730	0.017
		Caspase 8 stimulant	0.730	0.003
		Anti-inflammatory	0.700	0.016
4	 Nobiletin	HIF 1A expression inhibitor	0.946	0.004
		Membrane permeability inhibitor	0.913	0.007
		Peroxidase inhibitor	0.745	0.008
		NADPH oxidase inhibitor	0.737	0.003
		Membrane integrity agonist	0.919	0.007
		Free radical scavenger	0.742	0.003
		Lipid peroxidase inhibitor	0.702	0.005
5	 Quercetin	Peroxidase inhibitor	0.962	0.001
		NADPH oxidase inhibitor	0.928	0.002
		Antioxidant	0.872	0.003
		Lipid peroxidase inhibitor	0.778	0.004
		Membrane integrity agonist	0.973	0.002
		Membrane permeability inhibitor	0.938	0.003
		Free radical scavenger	0.811	0.003
		HIF 1A expression inhibitor	0.969	0.002
6	 Rutin	Lipid peroxidase inhibitor	0.987	0.001
		Antioxidant	0.923	0.003
		NADPH oxidase inhibitor	0.850	0.002
		Caspase 3 stimulant	0.839	0.004
		Membrane permeability inhibitor	0.990	0.000
		Free radical scavenger	0.988	0.001
		Membrane integrity agonist	0.984	0.001
		HIF 1A expression inhibitor	0.842	0.009
		Anti-inflammatory	0.728	0.013
7	 Tangeretin	Membrane integrity agonist	0.937	0.004
		HIF1A expression inhibitor	0.942	0.004
		Membrane permeability inhibitor	0.887	0.004
		Peroxidase inhibitor	0.737	0.009
		Free radical scavenger	0.715	0.004
		Anti-inflammatory	0.705	0.015

A prediction score of Pa > 0.7 was only considered

Also, HIF1A expression inhibitor activity was shown by all the citrus flavonoids. HIF is a transcriptional factor that plays a key role in the cellular adaptive response to hypoxia. HIF stabilisation and activation promotes neovascularization, cell respiration, apoptosis, glucose metabolism, and embryogenesis in hypoxic cells. In a study performed by Kunimi *et al.*[23] it was observed that administration of HIF1A inhibitor topotecan in eight weeks old male mice induces protective response against diminution of retinal thickness, ganglion cell number and impairment of visual function in retinal ischemia/reperfusion model. In addition to this, hesperidin and naringin showed caspase 3 as well as caspase 8 stimulating activity. Rutin also had high activity scores for caspase 3 stimulant activity. Apart from being the central regulator of programmed cell death, caspase 8 is reported to involved in many nonapoptotic and few antiapoptotic activities as well.[24] Leonard *et al*.[25] found that caspase 3 defective animals have a number of severe neurodevelopmental phenotypes. Caspase-3 possibly acts as key regulating molecule in processes like physiological neuron death during neurodevelopment, and neurological illnesses, synapse pruning at the time of differentiation, synapse degeneration, synaptic plasticity as well as synaptic pathology seen in neurodegenerative disease.[26]

The biological activities of the citrus peels can be attributed to the synergistic effect of various flavonoids present, such as hesperidin naringin, rutin, quercetin, nobiletin, *etc.* Recently, Meneguzzo *et al.*[27] claimed that hesperidin can help in the management of COVID-19 by effectively binding with three of the major cellular receptors of SARS- CoV-2 virus.CRK peel extract showed supremacy over other peels by showing maximum flavonoid content and exhibiting the lowest IC_{50} values in antioxidant and anti-inflammatory assays. CRB peel can also be considered as a promising candidate for further utilization as it showed a good amount of phenolics and flavonoids. CG is also one of the vastly cultivated citrus fruits, which generates a considerable amount of waste due to large and thick epicarp. However, the peel displays an exceptional antioxidant effect and can be exploited in the future. Apart from the antioxidant effect, CG peel also exhibits promising antimicrobial activities.[28] Although CL and CS peel showed a lesser flavonoid content and antioxidant activity than the other peels evaluated, their health-benefiting abilities cannot be neglected. Apart from polyphenols, citrus fruit peel is also rich in constituents like carbohydrates, proteins, organic acids, volatile oils, lutein, and pectin. Many of these compounds possess health-promoting properties and are not naturally synthesised in the human body.[29] Considering the high amount of micronutrients, phenolic compounds and antioxidant activity, citrus peel can be utilized for therapeutic as well as value addition purposes of food products.[30] The ethnomedicinal properties of citrus peel can be further validated by conducting planed *in vivo* experiments which can lay a foundation for the better transformation of this underrated citrus waste in the future.

Conclusions

The study concluded that citrus peel, specially Mandarian species (CRB and CRK) contains a significant amount of polyphenolic compounds with antioxidant potential. It was observed that among aqueous and various hydroethanolic ratios, maximum yield was obtained from 80% ethanol. When the further screening of 80% ethanolic extract was carried out, peels belonging to *Citrus reticulata* species showed the highest amount of phenolics and flavonoid content. CRK also exhibited higher antioxidant activity with the lowest IC_{50} values against DPPH and ABTS radicals. Anti-inflammatory and acetylcholinesterase inhibiting activities were observed in citrus peels with CRK having the lowest IC_{50} value. The presence of hesperidin, naringin, rutin and nobiletin, as well as other key flavonoids, was confirmed by HPLC quantification experiments. Finally, when the citrus flavonoids were subjected to *in silico* PASS analysis, the flavonoids showed high activity scores for various neuroprotective properties like antioxidant, anti-inflammatory, peroxidase inhibitory activities, *etc*.

Altogether, the present study showed that the citrus peels bioactive flavonoids interact with different cellular processes involved in neuroprotection. Based on the results, it can be suggested that development of nutraceutical formulation from citrus peel will lead to value addition to agro-industrial waste along with effective management of neurological disorders. Furthermore, such a practice will help citrus agro-industrial waste valorisation.

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Research Fellowship vide File no: 5/3/8/38/ITR-F/2018-ITR and 3/1/2/82/2018-Nut, respectively. Shubham Nilkanth Rahmatkar is thankful to CSIR, India for providing Senior research fellowship (31/GATE/54(06)/2019-EMR-I]. The institute publication number for the manuscript is 4804.

Conflict of interest

The authors have no conflict of interest to declare.

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