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Anti-Oxidant and Anti-Inflammatory Activities of Flavonoids from Fruits & Vegetables

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ABSTRACT

Flavonoid, a group of natural substances with variable phenolic structures, are found in fruits, vegetables, grains, bark, roots, stems, flowers, tea and wine. These natural products are well known for their beneficial effects on health and efforts are being made to isolate he ingredients so called flavonoid. Flavonoids are now considered as an indispensable component in a variety of nutraceutical, pharmaceutical, medicinal and cosmetic applications. The aim of study was to identify the most promising Citrus flavanones by a preliminary antioxidant and anti-inflammatory screening by in vitro cell-free assays, and then to mix the most powerful ones in equimolar ratio in order to investigate a potential synergistic activity. The obtained flavanones mix (FM) was then subjected to in vitro simulated digestion to evaluate the availability of the parent compounds at the intestinal level.

Keywords: Flavonoid:Structure and composition, Biological activity, Anti-oxidant, Anti-inflammation, glycosides, Fruit & Vegetables.

INTRODUCTION

Flavonoid are an important class of natural products; particularly, they belong to a class of plant secondary metabolites having a polyphenolic structure, widely found in fruits, vegetables and certain beverages. They have miscellaneous favourable biochemical and antioxidant effects associated with various diseases such as cancer, Alzheimer's disease (AD), atherosclerosis, etc.[1] Flavonoid are associated with a broad spectrum of health-promoting effects and are an indispensable component in a variety of nutraceutical, pharmaceutical, medicinal and cosmetic applications. This is because of their anti oxidative, anti-inflammatory, anti-mutagenic and anti-carcinogenic properties coupled with their capacity to modulate key cellular enzyme functions.[2] They are also known to be potent inhibitors for several enzymes, such as xanthine oxidase (XO), cyclo-oxygenase (COX), lipoxygenase and phosphoinositide 3-Kinase.

Flavonoid represent one of the most representative classes of plant secondary metabo-lites, exceeding 8000 compounds, including aglycons, glycosides, and polymers with Flavonoid, which represents one of the most diffused sub-classes in the Citrus genus. They show unquestionable antioxidant and free-radical scavenging, as well as anti-inflammatory properties.[3] Thus, they are considered very promising multi-target agents against a wide range of chronic disorders, such as cardiovascular and intestinal-nal bowel diseases, diabetes, and cancer Recently, several studies have focused their attention on Citrus flavanones and their spective glycosylated derivatives as anti- inflammatory agents, particularly in the context of inflammatory bowel diseases (IBDs). IBDs are characterized by chronic and uncontrolled pro-inflammatory states associated with deregulation of both adaptive and innate immunity of the gastrointestinal tract.[4] As a result, an increase of the vascular permeability and blood flow, as well as an increase of leukocyte mobilization and production of inflammatory mediators occur.

The exact cause of these pathologies is not fully known, although it is well-known that several factors, such as immune system disturbance, genetic predisposition, and environmental factors play a predominant role. To date, are solution's pharmacological treatment is not avail- able and therapeutic strategies are mainly focused on non-specific immunosuppression drugs.

Inflammation occurs when an organism combats invasion, either physically or via noxious chemical stimuli.[5] The inflammatory response is a mechanism that inactivates invading pathogens (Guzik et al., 2003). Since ROS are activators of inflammation, we proceeded to investi-gate the anti-inflammatory properties of the aforementionedsamples. Both nitric oxide (NO) and ROS modulate inflammation. Lipopolysaccharides (LPS), specific ligands to toll-like receptors and inducers of inflammation, are chemicalmoieties that are present in the outer membrane of gram-negative bacteria. In our study, we also investigated the anti-inflammatory properties of the samples by treating RAW 264.7 cells with LPS in order to determine NO production and expression of pro-inflammatory mediators (iNOS and COX-2) and cytokines (TNF-α, IL-1β, and IL-6) at the transcriptional level. Our results showed that additional processing of these fruit and vegetable extracts elevated their antioxidant and anti-inflammatory properties.

Classification

Flavonoid can be subdivided into different subgroups depending on the carbon of the C ring on which the B ring is attached and the degree of un saturation and oxidation of the C ring.[6] Flavonoid in which the B ring is linked in position 3 of the C ring are called isoflavones. Those in which the B ring is linked in position 4 are called neoflavonoids, while those in which the B ring is linked in position 2 can be further subdivided into several subgroups on the basis of the structural features of the C ring.[3] These subgroups are: flavones, flavonols, flavanones, flavanonols, flavanols or catechins, anthocyanins and chalcones.

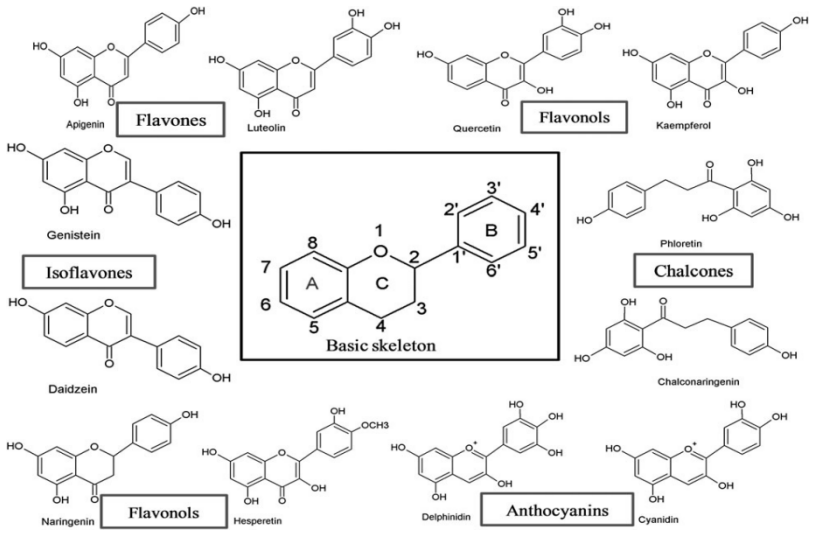


Fig 1: Classification of Flavonoids

Current research and trends on flavonoid

Serial no.	Flavonoid	Class	Dietary sources
1	Quercetin	Flavonols	Vegetables, fruits and beverages, spices, soups, fruit juices
2	Rutin	Flavonols	Green tea, grape seeds, red pepper, apple, citrus fruits, berries, peaches
3	Macluraxanthone	Xanthones	*Madura tinctoria* (Hedge apple), Dyer's mulberry
4	Genistein	Isoflavone	Fats, oils, beef, red clover, soyabeans, psoralea, lupin, fava beans, kudzu, psoralea
5	Scopoletin	Coumarin	Vinegar, dandelion coffee
6	Daidzein	Isoflavone	Soyabeans, tofu
7	Taxifolin	Flavanonol	Vinegar
8	Naringenin	Flavanone	Grapes
9	Abyssinones	Flavanone	French bean seeds
10	Rutin	Flavonol	Citrus fruits, apple, berries, peaches
11	Eriodictyol	Flavanone	Lemons, rosehips
12	Fisetin	Flavonol	Strawberries, apples, persimmons, onions, cucumbers
13	Theaflavin	Catechins	Tea leaves, black tea, oolong tea
14	Peonidin	Anthocyanidin	Cranberries, blueberries, plums, grapes, cherries, sweet potatoes
15	Diosmetin	Flavone	Vetch

Flavonoid mechanisms

Almost every group of flavonoid has a capacity to act as anti-oxidants. It has been reported that the flavones and catechism seem to be the most powerful flavonoid for protecting the body against reactive oxygen species. Body cells and tissues are continuously threatened by the damage caused by free radicals and reactive oxygen species, which are produced during normal oxygen metabolism or are induced by exogenous dam-age. The mechanism and these sequence of events by which free radicals interfere with cellular unctions are not fully understood, but one of the most important events seems to be lipid per oxidation, which result in cellular membrane damage. This cellular damage causes a shift in the net charge of the cell, changing the osmotic pressure, leading to swelling and eventually cell death.[7]

Functions and applications of flavonoids

Plants produce a vast and diverse assortment of organic compounds, the great majority of which do not appear to participate directly in growth and development. These substances, traditionally referred to as secondary metabolites (flavonoid), often are deferentially distributed among limited taxonomic groups within the plant kingdom. The flavonoid are categorized in different classes as alkaloids, terpenoids and phenolics. Flavonoid carry out a number of protective functions in the human body. Many flavonoid have evolved asbioactive compounds that interfere with nucleic acid orproteins and show antimicrobial or insecticidal and pharmacological properties.[8]

Flavonoids are therefore of interest in medicine as therapeutics and at the same instance in agriculture as pesticides. In vitro technology has given new insight to explore the potency of plant cell tissue culture to produce the same valuable chemical compounds as those of the parent plant.

A number of studies have been carried out on properties of antioxidant in relation to different flavonoid and these studies emphasized that the flavonoids can be used as potential drugs to prevent oxidative stresses. Antioxidants are com-pounds that protect the cells against the oxidative effect of reactive oxygen species, and the impaired balance between these reactive oxygen species and antioxidants results in oxidative stress. The oxidative stress may lead to cellular damage which is related to various health ailments such as diabetes, cancer, CVD, neurodegenerative disorders and ageing. Oxidative stress can also damage. [9]

MATERIALS AND METHODS

Chemicals

Neoeriocitrin (eriodictyol-7-O-neohesperidoside), eriocitrin (eriodictyol-7-O-rutinoside), hesperetin (30,5,7-trihydroxy-40-methoxyflavanone), hesperidin (hesperetin-7-O-rutinoside), neohesperidin (hesperetin-7-O-neohesperidoside), diosmin (diosmetin-7-O-oside) neodiosmin (diosmetin-7-O-neohesperidoside), naringin (naringenin- 7-O-neohesperidoside) and tangeretin (40,5,6,7,8-pentamethoxyflavone) analytical standards (HPLC grade, pu-rity ≥ 98%) were purchased by Extra syntheses (Genay, France). Sodium chloride (NaCl), potassium chloride (KCl), calcium chloride (CaCl2), urea, cholesterol, sodium phosphate monobasic (NaH2PO4), zinc sulfate heptahydrate (ZnSO4·7H2O), α-amylase from human saliva type XI (A1031-1KU), egg-phosphatidylcholine (PC, 840051P), pepsin from porcine gastric mucosa (P6887), α-chymotrypsin type II from bovine pancreas (C4129), trypsin type IX-S from porcine pancreas (T0303), lipase type VI-S from porcine pancreas (L0382), colipase from porcine pancreas (C3028), α-amylase type VI-B from porcine pancreas, sodium glycodeoxycholate (G9910), taurocholic acid sodium salt hydrate (T4009) as well as analytical and HPLC-grade solvents were purchased from Merck KGaA (Darmstadt, Germany).

Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were obtained from WelGENE (South Korea). Streptomycin and penicillin were obtained from Lonza (MD, USA). TRIzol reagent was sourced from In-vitrogen (Carlsbad, CA, USA).

Sample preparation

Malus domestica (apple), Pyrus communis L. (pear), Daucus carota L. (carrot), Brassica oleracea var.(broccoli), Brassica oleracea var. capitata (cabbage), and Raphanussativusl. (radish) samples were agricultural products grown in South Korea and locally purchased from a traditional market. The samples were washed, cut into uniform shapes of 0.5 cm × 0.5 cm × 0.5 cm, freeze-dried, sealed dry to keep moisture away, and stored in -70°C. Raw samples were prepared by extracting the samples at 60°C for 2 h.To obtain processed samples, previously freeze-dried sam-ples were heat-treated under pressurized conditions (10 kg/cm2) using a heating apparatus (Jusco, Seoul, South Korea).Samples were placed in the inner compartment and wate rwas added to the outer compartment of the container. The apparatus was heated according to predetermined temperature and time values (140~150°C for 6 h) to prevent carbonization of the samples from direct heat. Finally, samples were weighed for further experiments.

In Vitro Cell-Free Antioxidant and Anti-Inflammatory Screening[10]

In order to select the most promising flavonoid, a preliminary antioxidant and anti-inflammatory screening by colorimetric in vitro cell-free assays was carried out on the most representative flavanones in the Citrus genus.[4] Unless otherwise specified, the absorbance data were recorded by an UV-VIS Spectrophotometer (Shimadzu UV-1601, Kyoto, Japan). Results were expressed as half-maximal inhibitory concentration (IC50, µg/mL) with confident limits (C.L.) at 95% calculated by Litchfield and Wilcoxon test, using the PHARM/PCS software version 4 (MCS Consulting, Wynnewood, PA, USA). Data represent the mean ± standard deviation (S.D.) of three independent experiments in triplicate (n=3). The concentration ranges reported below refer to the final concentrations of the samples or reference compounds in the reaction mixture.

Antioxidant Assays

Ferric Reducing Antioxidant Power (FRAP). The FRAP assay was carried out according to Smeriglio et al.. Fifty microliters of each flavanone (3.13–3200 µM) or trolox as reference compound (5.0–40 µM) were added to 1.5 mL of fresh working FRAP reagent consisting of 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tris(2-pyridyl)-S-triazine (TPTZ) solution in 40 mM HCl and 20 mM FeCl3·6H2O

solution (10:1:1 v/v/v), pre-warmed at 37 ◦C. The reaction mixture was incubated for 4 min at room temperature (RT) and the absorbance recorded at 593 nm.[10]

DPPH (2,2-Diphenyl-1-Picrylhydrazyl) Assay

The radical scavenging activity of samples against DPPH was evaluated according to Smeriglio et al.. Briefly, 37.5 µL of each flavonoid (3.13–6400 µM) or trolox as reference compound (2.5–20 µM) were added to 1.5 mL of fresh 10−4 M DPPH methanol solution,mixed for 10 s and incubated for 20 min in the dark at RT. The absorbance was recorded at.517 nm.

Anti-Inflammatory Assays

Albumin Denaturation Assay (ADA) This assay evaluates the inhibitory activity of sample on heat-induced denaturation of bovine serum albumin (BSA)[10]. Briefly, 80 µL of each flavonoid (100–6400 µM) or diclofenac sodium as reference compound (4.0–32 µM) were added to 100 µL of 0.4% fatty free BSA solution and 20 µL of phosphate buffer saline (PBS, pH 5.3) into a 96 well-plate. The absorbance was recorded at 595 nm at the starting time (t = 0) and after 30 min (t = 30) at 70 ◦C by a microplate reader (Multiskan GO, Thermo Scientific, Waltham, MA, USA). A blank consisting of PBS instead of sample was used as negative control. Percentage of denaturation (ID %) was calculated as follows

$$\% \text{ ID} = ((1 - (A - B))/(C - B)) \times 100 \qquad (1)$$

Where, A = sample absorbance (t = 30); B = blank absorbance at (t = 0); C = blank absorbance at (t = 30).

Flavanones Mix Preparation[10]

Based on the results of the preliminary antioxidant and anti-inflammatory screening (see Section 2.2), the most powerful flavanones (neohesperidin, hesperidin, neoeriocitrin, eriocitrin, and hesperetin) were selected in order to prepare a flavanones mix (FM), which was, at first instance, investigated by the same in vitro colorimetric assays (see Section 2.2) in order to evaluate its potency with respect to the single flavanones and to elucidate po- tential synergistic mechanisms. The following final concentration range of FM were used: FRAP (2.50–20 µM), ORAC (0.08–0.60 µM), TEAC (0.31–2.50 µM), DPPH (1.25–10 µM), ADA (5.0–40 µM), and APA (2.50–20 µM). After that, being the results of the preliminary antioxidant and anti-inflammatory screening of FM very promising, an appropriate con- centration was chosen to proceed with the subsequent analyses. In particular, the FM was prepared on the assumption that digested sample, which would then be applied to the Caco- 2 cells for the anti-inflammatory experiments, had to contain an equimolar concentration of the five most powerful flavanones (neohesperidin, hesperidin, neoeriocitrin, eriocitrin, and hesperetin) equal to 10 µM,[4] which represents the mean efficacy concentration taking into account the IC50 values obtained in the preliminary antioxidant and anti-inflammatory screening of the FM. At this purpose, stock solutions (14 mM) of flavanones in DMSO were mixed and diluted 10-fold with Milli-Q water to obtain the 1.4 mM FM, which was used to carried out the in vitro simulated human digestion.

In Vitro Simulated Human Digestion

In vitro gastric and duodenal digestion of FM was carried out according to Trombetta et al.[4] with some modifications. FM solution (1.5 mL) (see Section 2.3), has been dis-solved in 7.5 mL of simulated gastric solution containing 0.127 mM egg-phosphatidylcholineand adjusting the pH to 2.5, by 1 M HCl. Gastric pepsin and lipase (9000 U/mL and 60 U/mL, respectively) were added to the mixture starting the gastric digestion phase which was protracted for 2 h at 37 ◦C, incubating under continuous stirring (170 rpm) by an Innova 4000 Benchtop Incubator Shaker (New Brunswick Scientific, Edison, NJ, USA). The gastric phase was stopped by the addition of 1M NaOH in order to reach a pH of 7.5, and 6 mL of the previous solution passed to the duodenal digestion phase.[10]

Duodenal solution was prepared by adding 2.10 mL of simulated bile solution (6.5 mM phosphatidylcholine,4 mM cholesterol, 12.5 mM sodium taurocholate, and 12.5 mM sodium glycodeoxycholate) to 5.9 mL of simulated pancreatic juice containing pancreatic lipase (590 U/mL), colipase (3.2 µg/mL), trypsin (11 U/mL), α-chymotrypsin (24 U/mL), and α-amylase (300 U/mL).The duodenal mixture was incubated for 4 h under continuous stirring at 37 ◦C as described above. At the end, both gastric and duodenal digesta were immediately stored at −80 ◦C until the subsequent analyses. [11]

The HPLC method was validated according to the current international guidelines, regarding selectivity, linearity, precision, robustness, limit of detection (LOD), limit of quantitation (LOQ) and recovery. LOD and LOQ values were calculated following the approach based on the standard deviation of the response and the slope of the calibration Curves.

Anti-Inflammatory Activity on In Vitro Cell-Based Model

Cell Model

Experiments were carried out on transwell models (CacoReadyTM, Readycell, Barcelona, Spain) consisting of Caco-2 cells (8.5 × 104 cells/cm2 , passage number 41–58) seeded on m polyester micro porous filters in 24-well HTS plates (6.5 mm diameter, 0.33 cm2 area and 0.4 µm of pore size) (Corning Incorporated, Corning, NY, USA). Completed Dulbecco's Modified Eagle Medium (DMEM), prepared according to Denaro et al. , was added on the apical (0.3 mL) and baso lateral side (0.9 mL) of the Transwell® insert. After 21 days of culture, Caco-2 cells were completely differentiated and polarized, such that their phenotype resembled the morphological and functional features of mature enterocytes lining the small intestine.

Anti-Inflammatory Assay

Before starting with the anti-inflammatory experiments, the mono layer integrity was checked by measuring the trans-epithelial electrical resistance (TEER) with a Millicell® ERS-2 V/ohmmeter (Merck Millipore, Darmstadt, Germany) equipped with STX 100C electrode (World Precision

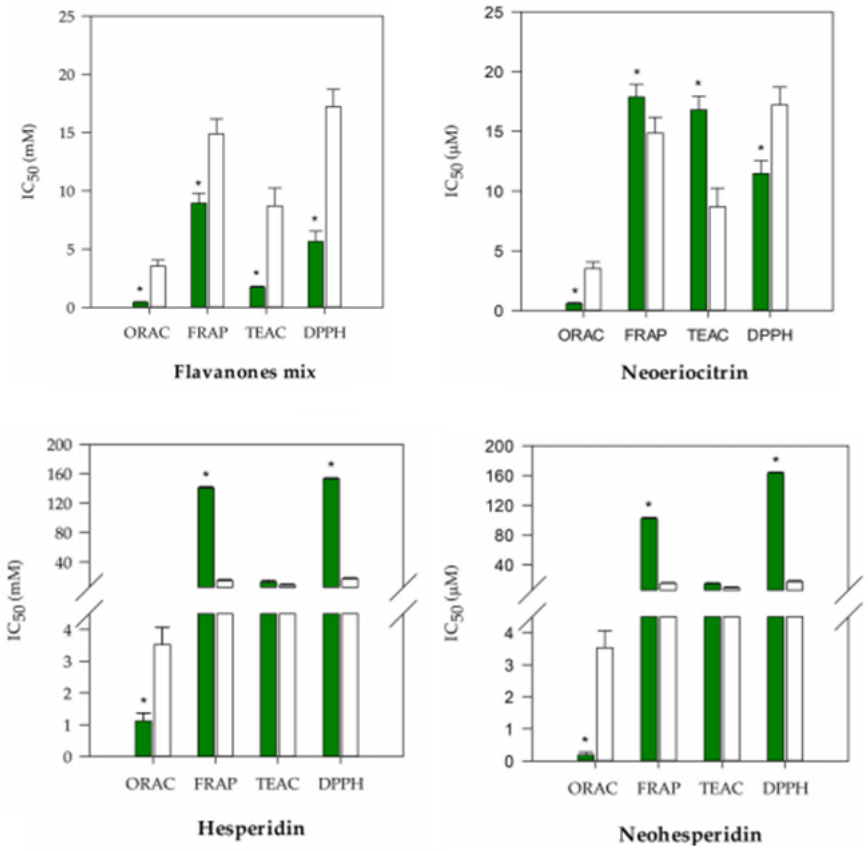
Instruments, Sarasota, FL, USA). Caco-2-plated filters with epithelial resistance ≥800 Ω/cm2 were used. Samples were collected and stored at −80 ◦C until the subsequent analyses. Post-quality control assays, such as TEER, as well as the apparent permeability coefficient (Papp) and paracellular flux (Pf) of Lucifer yellow (LY) were assessed in order to evaluate the Caco-2 monolayer integrity.

Cell Viability

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to Kenzaoui and co-workers..

Statistical Analysis

Three independent experiments in triplicate (n = 3) were carried out for each in vitro cell-free and cell-based assay. Results were expressed as mean ± standard deviation S.D.). Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test by SigmaPlot12 (Systat Software, Inc., San Jose, CA, USA). Results were statistically significant.



All data is presented as mean ± SEM. One-way ANOVA and Dunnett's test were applied for statistical evaluation of the data. Statistical analyses with $P < 0.001$ were considered to be significant.

RESULTS

Antioxidant and Anti-Inflammatory Screening of Flavanones Mix

With the aim of identifying the most promising flavanones from an antioxidant and Anti-inflammatory point of view, nine of the most representative compounds of the Citrus - genus were chosen: neoeriocitrin, eriocitrin, hesperetin, hesperidin, neohesperidin, dios min, neodiosmin, naringin, and tangeretin. Among these, after a preliminary antioxidant and anti-inflammatory screening by in vitro cell-free assays, the five most promising ones were selected and suitably mixed in equine molar ratio (flavanones mix) in order to elucidate possible synergistic effects.

Pre- and Post-Digestion Analysis of Flavanones Mix

The quail-quantitative determination of flavanones in FM and gastric and duodenal DFM, was carried out by a LC-DAD-ESI-MS/MS analysis. The analytical method was validated and,as observed from the data, reported in, the method resulted as sensitive, precise, accurate, and repeatable, with recovery values ≥90.36%. Moreover, according to the current international guidelines, the robustness of the method was evaluated, taking into account the following parameters: pH variation of mobile phase, variations in mobile phase composition, different lots, and/or suppliers of column, temperature, and flow rate. Maintaining the conditions reported in Section, the analytical method results robust, because it is reliable, with respect to deliberate variations in the method parameters mentioned above (data not shown).[5]

DISCUSSION

In our study, we selected six commonly available domestic fruits and vegetables, which included Malus domestica (Vane et al.), Pyrus communis L. (pear), Daucus carota L. (carrot), Brassica oleracea var. (broccoli), Brassica oleracea

var.capitata (cabbage), and Raphanus sativus L. (radish), and investigated them for their antioxidant and anti-inflammatory activities. Furthermore, raw and processed samples were compared to determine whether additional heat treatment on the raw samples improved their bioactive properties. ROS are produced during normal metabolic and physiological reactions such as signal transduction, gene expression, and mitochondrial electron transport (Bayr, 2005). ROS generated from these reactions affect cellular components like DNA, proteins, and lipids, resulting in oxidative DNA damage.[12]

Hence, there is a need to reduce ROS quantities via the intake of food and nutrients exhibiting high antioxidant activity. DPPH, which has an absorption band of 517 nm and diminishes due to reduction in the presence of dietary antiradical compounds, was used here to investigate the antioxidant activity of compounds and extracts.[13]

DPPH assay results indicated that the processed forms of apple, carrot, pear, broccoli, and cabbage showed increased radical scavenging activities, indicating that processed samples exhibited better antioxidant activities when compared to unprocessed samples. Among the samples, processed pear showed potent antioxidant activity similar to ascorbic acid (positive control). In the ABTS assay, apple, carrot, broccoli, cabbage, and radish samples showed similar radical scavenging activities via both raw and processed samples.

CONCLUSION

Flovonoids is an essential bioactive compounds found in our daily life in the form of fruits and vegetable. It possesses wound healing properties. Flavonoids are a sudfivision of polyphenols, a versatile class of natural compounds that represents secondary metabolites from higher plants and are abundant in human diet. Various protective effects of flavonoids have been reported including antimicrobial and antifungal activities. These substance are more commonly used in the developing countries. Therapeutic use of new compounds must be validated using specific biochemical tests. With the use of genetic modifications, it is now possible to produce flovonoids at large scale. Further achievements will provide newer insights and will certainly lead to a new era of flvonoid based pharmaceutical agents for the treatment of many infectious and degenerative diseases.

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