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INTEGRATING PHYTOCHEMISTRY WITH THERAPEUTICS: FLAVONOIDS EXTRACTION: PURIFICATION METHODS AND HEALTH THERAPEUTIC BENEFITS

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Abstract

The diverse group of natural compounds known as flavonoids includes subclasses such as flavonols, flavones, flavanones, isoflavones, and anthocyanins, which exhibit extensive therapeutic attributes such as antibacterial, antioxidant, anti-inflammatory, antitumor, as well as neuroprotective effects. Novel flavonoids such as quercetin, kaempferol, naringenin, and genistein, isolated and purified from natural sources have received much notice due to their applications in pharmaceutical and health products. Although conventional extraction and purification methods use organic solvents which put both environment and human health at risk, The resulting need for green technologies that are developed for the extraction and purification of flavonoids has become a critical research objective. This inter-collaborative study between different institutes aimed to explore the application of green technologies for the isolation and purification of novel flavonoids and the determination of their therapeutic activity. Green methodologies utilize eco-friendly solvents and new selective extraction methods like supercritical fluid extraction, ultrasound-assisted extraction, and microwave-assisted extraction. These techniques present many advantages such as a reduction in solvent usage, a shortening the processing time and improving extraction efficiency. In addition, the therapeutic potential of the isolated flavonoids will be determined quantitatively by in vitro as well as in vivo assays such as antioxidant, antibacterial, cytotoxicity, anti-inflammatory and neuroprotective assays. Results from this study will contribute to our understanding of the pharmaceutical potential of novel flavonoids. Finally, this research attempts to set up a green and sustainable approach for the isolation and purification of flavonoids from natural sources. Their therapeutic activities may be valuable in the development of innovative drugs or natural health products. Further, the application of green technologies in the extraction process would provide ecological sustenance, as well as reduce health-related issues of traditional methods.

Keywords: *Flavonoids, Therapeutic agents, Quercetin, Kaempferol, Naringenin, Genistein, Modern extraction techniques, Flavonoid transport mechanisms, Environmental sustainability*

INTRODUCTION

Flavonoids are naturally occurring polyphenolic substances existing across a range of plant-derived goods, which includes, vegetables, flowers and fruits. They have important roles in plant physiology such as UV protection, pigmentation, and pathogen defense (1). As far as humans are concerned, flavonoids are celebrated for their plethora of range for bioactive characteristics include anti-inflammatory, antioxidants, antimicrobial, anticancer as well as neuroprotective outcomes. Flavonoids are known to be something of a focal point in the development of pharmaceuticals, nutraceuticals and functional foods as these attributes have become important (2). The potential of flavonoids, however, is limited by their effective and sustainable extraction and purification, making it a challenge in need of innovation.

Traditional flavonoid extraction as well often relies upon organic solvents like hexane, methanol and acetone that can be hazardous to both human beings and their surroundings. These solvents are sourced from nonrenewable petroleum sources, which contributes to resource depletion and environmental degradation. Improper disposal of solvent waste can contaminate soil and water, harming ecosystems and reducing biodiversity (3). Furthermore, many of these solvents are classed as volatile organic compounds (VOCs), which cause air pollution and increase greenhouse gas emissions, exacerbating climate change. Exposure to these toxic solvents can have serious health consequences for both workers and consumers. Hexane is known to be neurotoxic, whereas methanol exposure can induce systemic toxicity and organ damage, and acetone has been associated to respiratory and dermatological problems. Residual solvent traces in finished products risk consumer safety, especially in pharmaceuticals and nutraceuticals. Traditional methods are unsustainable and economically expensive because to their high energy requirements, wasteful solvent use, and insufficient specificity for target molecules. These limitations highlight the critical need for greener, safer, and more efficient extraction methods. (4).

Green extraction technologies search for solvents which are environmentally benign, such as water, ethanol, its mixtures, and implementation with innovative procedures to reduce energy consumption and reduce the processing time (5). The methods used are supercritical fluid extraction (SFE), ultrasound assisted extraction, microwave assisted extraction (MAE), methods (UAE). These methods enhance the rate of flavonoid extraction while decreasing the environmental impact related to conventional methods. For example, MAE and UAE take advantage of energy efficient processes to enhance solvent penetration and promote flavonoid release from plant matrices, and SFE utilizes carbon dioxide at conditions that can provide flavonoid isolation with minimal residual (6). Green extraction becomes more important in light of growing global focus on environmental sustainability and human health. The application of such methods is consistent with the green chemistry principles of reducing hazardous substances, waste minimization, and energy conservation. In addition, green technologies are necessary to preserve flavonoids structural integrity and therapeutic efficacy, rendering flavonoids more suitable for pharmaceutical and health applications (7). The green technology for isolation and purification of novel flavonoids was investigated to improve the yield and purity while maintaining eco-friendly nature. The therapeutic effects of the extracted flavonoids are also evaluated using in vivo and in vitro assays for antioxidant, antimicrobial, cytotoxic as well as neuroprotective activities (8). The purpose of these evaluations is to understand the influence of flavonoids in optimizing health and wellness, as shown in Fig. 1.

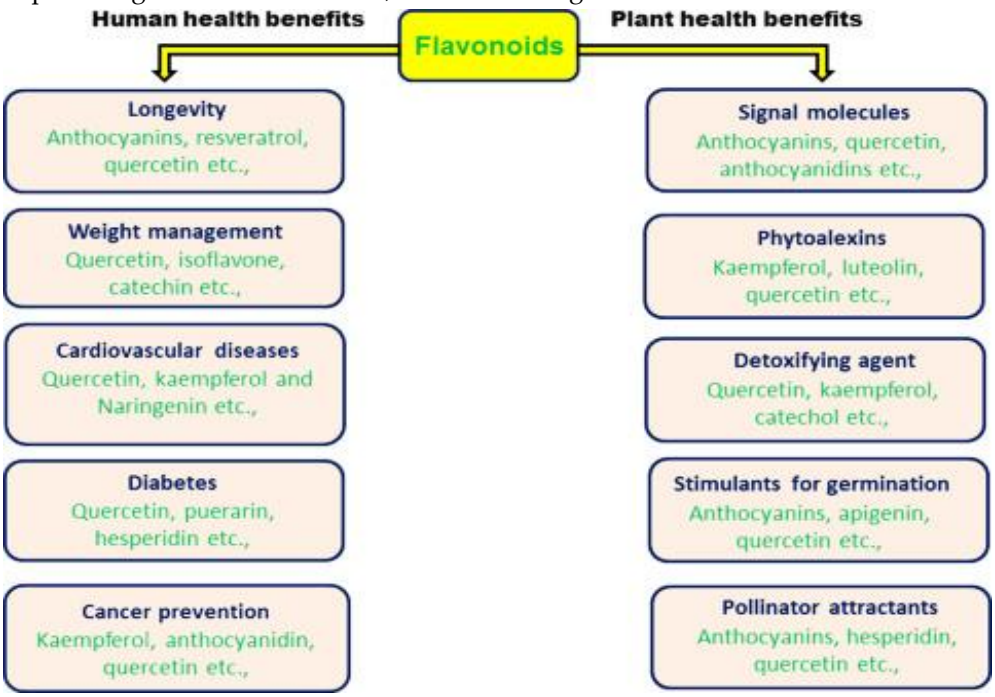

Fig. 1. The main benefits of various types of bioactive Flavonoids in plants and human beings

This study represents a contribution to the growing body of research on sustainable bioactive compound production, by addressing the challenges presented by conventional extraction methods. The

results are expected to pave the path for the development of innovative medicines and natural health products that benefit both human well-being and environmental sustainability (9). Importantly, this green approach not only improves the value of flavonoids, but also sets the standard for sustainability in natural product research and development.

Flavonoids may accumulate with specificity to the cell and tissue, and flavonoids have been demonstrated to exhibit inter- and intracellular mobility by means of transport proteins. Chemical Structure of flavonoids is shown in Fig. 2.

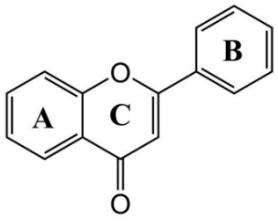

Fig. 2. Chemical structure of flavonoids

The enzymatic redox cyclic transformations that flavonoids can also scavenge free radicals by means of single electron oxidation reactions. Free radical quenching activity of flavonoids is due to hydroxyl groups in A, B-rings; methylation or glycosylation reduces the activity. Flavonoids also suppress the production of enzymes that produce radicals including, cyclooxygenase, xanthine oxidase, monooxygenase, lipoxygenase and GST. Moreover, flavonoids suppress the production of superoxide anion radical by the alteration of membrane bound NADPH oxidase activity.

COMPREHENSIVE ANALYTICAL METHODOLOGIES

PREPARATIVE ISOLATION OF FLAVONOIDS FROM MULBERRY (*MORUS ALBA* L.) BY MACROPOROUS RESIN ADSORPTION

This inter-collaborative study persists from July to December 2023, in affiliated institutes with experimental and accessory roles. Mulberry leaf works as conventional oriental herbal remedy, has been used for several years as a strong invigorant for the blood pressure, liver and eyesight. Previous reports have shown that mulberry leaves have some kinds of medical elements, among which alkaloids and polysaccharides, particularly 1-deoxynojirimycin (DNJ), have a capacity of preventing and treating diabetes mellitus. Thus, we attempted to develop a simple and efficient process for preparing three different types of active ingredients separately from mulberry leaves for use in functional or therapeutic foods. Flavonoids are conventionally isolated through repeated column chromatography, however it only applies to small quantities of flavonoids that are required for analytical purposes. Moreover, our preceding investigation found that the polysaccharides could not be extracted if the ethanol–water solution of suitable proportion was applied as the extracting solvent, however, the DNJ and other total flavonoids as well as alkaloids could be coextracted efficiently.

To achieve complete development of the mulberry leaves, the resin can be chosen so that some exhibit stronger selective adsorption for flavonoids while not adsorbing for DNJ, essentially separating the flavonoids and DNJ into two products. As a result, in this study, static adsorption experiments were initially performed to determine the appropriate resin and desorption solvent. Dynamic adsorption and desorption studies were then carried out to optimize the process conditions. The mulberry leaves (M. Alba L.) were from Shijiazhuang Pharmaceutical Co., Ltd. (Shijiazhuang, China). A milling machine was used to finely grind the leaves, and the powder was then mesh with a 200 mm aperture size and kept in a desiccator until the experiment.

ANALYTICAL METHOD: DETERMINATION OF TOTAL FLAVONOIDS

Total flavonoids contents in mulberry leaf were quantified by UV spectrophotometry, the parent nucleus of flavonoids contains basic oxygen atoms which form red complex absorbance through aluminum ion under an alkaline condition. Rutin equivalents are reported for TFA content, with the reference material being rutin. To a 25 mL volumetric flask, a portion of the sample (1–6 mL) was added and a volume of 6 mL

was created by adding 80% ethanol and water. After which the mixture was left to stand at room temperature for 6 minutes, then the mixture was mixed with 1.0 mL of 5% NaNO2. The mixture was placed on a burner to warm it, and 10 mL of 5% KOH and 80% ethanol were combined to create the volume, 1.0 mL of Al(NO3)3, 10% was added and incubated for an additional 6 minutes. It was measured 15 min after incubation at optimum conditions for absorbance at 500 nm. A line of regression for the absorbance to flavonoids content (mg/mL): A ~ 12.81C - 0.001060 (n = 7). Rutin has high linearity of the first order (R = 0.9998) (n = 5 RSD 3.07%) (Hu, Wang et al. 2021), concentration ranged from 0.00214–0.0643 mg/mL with average recovery of 97.9%, RSD 1.33% (repeatability).

RUTIN AND ISOQUERCITRIN DETERMINATION

State College, Pennsylvania's Lab Alliance liquid chromatographic system, which included an Agilent TC-C18 column (250 4.60 mm i.e., Agilent Co., Santa Clara, California; 5 mm); a Series III Lab Alliance pump, a type of 500 UV detector, a Spectra Physics 4020 pump; and a Spectra Physics 870 Rheodyne injector. Acetonitrile, methanol, and aqueous phosphoric acid (100:The mobile phase used was (v/v/v/v) (10: 340: 0.3). The rate of flow was 1.0 mL/min, with 20 mL injection volume. There was no attempt to heat the column temperature, and the effluent was measured at 254 nm. All samples were first prepared in methanol and then pumped through 0.45 mm membranes (Chromatography Science and Technology Co., Tianjin, China) prior to HPLC analysis.

CHEMICALS AND MATERIALS

Fresh Bhagwa pomegranates (Punica granatum L.) can be bought in Sahakari Bhandar, Mumbai, Maharashtra, India.They have cleaned and sorted fouths. After the arils were removed, the fruits were dried at 40 °C in a hot air oven till the weight remains constant. The dried peels were processed into a finely ground powder in a lab.A powdered material was filtered through a sieve sized at 40 microns. Before being used, this homogeneously sized pomegranate peel powder was stored in a dry, sealed plastic bottle at -20°C in the freezer. Methanol, Na2CO3, AlCl3, potassium acetate, NaCl, HCl, and NaOHI came from SD Fine Chemicals, Mumbai, India. The Triton X-100, Triton 114, Tergitol NP-12 (Nonylphenol Ethoxylate), gallic acid, quercetin, Folin-Ciocalteu's reagent, DPPH, and ABTS were obtained from Sigma Aldrich Chemicals Co (St. Louis, MO, USA). The distilled liquid was made utilizing a Sartorius arium advance series water purification equipment (Mumbai, India). The test was performed with every other substance used, all of which were of analytical quality.

CLOUD POINT EXTRACTION (CPE) OF TOTAL PHENOLS AND FLAVONOIDS POMEGRANATE PEEL POWDER.

Polyphenol and flavonoid CPE was carried out following a few minor modifications of the Katsoyannos et al. method (Fig. 3). The mixture's pH was changed with surfactant (Triton X-114%, v/v), mixed with pomegranate peel powder (0.5g) and distilled water, after one minute of vortexing, it was centrifuged for ten minutes at 10,000 RPM. Other tubes were filled with the supernatant. Salt (NaCl) was added to the sample solution to raise the density of the aqueous water phase and decrease the cloud point temperature (CPT) through the salting-out action, which facilitated the smooth phase separation. After that, the mixture was kept for half an hour in a water bath with a thermostat. After centrifuging the sample for 10 minutes at 8000 rpm, the volumes of the aqueous and surfactant phases were determined, and the responses were computed using these volumes. After that, a syringe was used to separate the aqueous bottom phases in these two. The residual phase of surfactant settled down at the bottom of the tube because of its high viscosities. The samples were examined six times, and each experimental trial was run in triplicate. The response values are means of the result of each test.

OPTIMIZATION OF EXTRACTION PROCESS WITH EXPERIMENTAL DESIGN

For the RCCD, the surfactant concentration (%, v/v), pH, temperature (°C), and salt concentration (%, w/v) have been varied. Each of four parameters was coded from resultant report acquired from CPT

with the lower (-1) and further upper (+1) limits, so as to establish them in coded form. The experimental plan called for 30 experimental runs which were performed which comprised of 16 factor points (level ±1), 8 axis points (±α = ±2 level) and 6 repetition runs at 0 or middle points. The total phenolic and total flavonoid content was evaluated for each experimental run and four such responses i.e., recovery (Y1, %R), partition coefficient (Y2, Ks/a) and loss during extraction (Y3, %L) and concentration fraction (Y4, fc) of total phenolic and total flavonoids were measured.

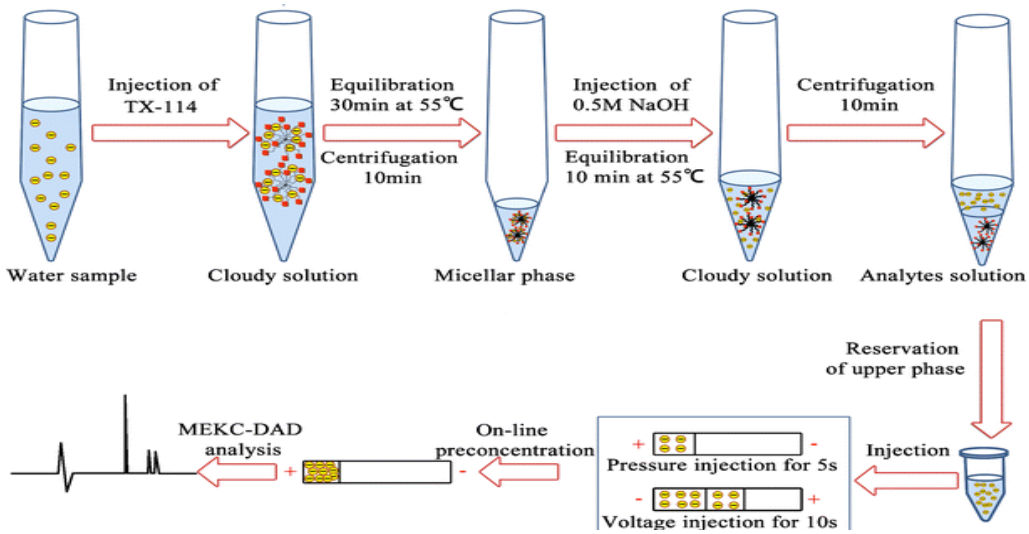

Fig. 3. Cloud point extraction (CPE)

ANALYTICAL METHODS: PHENOLICS CONTENT DETERMINATION

Phenolics content of the pomegranate peel extract gained from CPE was measured through slightly modifying the Folin-Ciocalteu assay. The standard, gallic acid solution (10 mg mL-1) in 80% methanol, was used in order to provide acceptable concentrations (0.1–1 mg mL-1) for the standard curve. 0.1 mL of standard gallic acid solution, 0.1 mL 80% methanol, 0.7 mL Na2CO3, 2 mL Eppendorf, and Folin-Ciocalteu reagent were analyzed by putting them into 2 mL Eppendorf's. Samples were immediately vortexed, incubated for 20 minutes at normal temperature, in the dark. Following incubation, each sample was centrifuged at 8000 RPM x 8 minutes x 4 °C. The absorbance of the supernatant of each sample was recorded in 1 mL quartz cuvettes using a UV-Vis spectrophotometer (Jasco, V-730) at 735 nm. We expressed the results in milligrammes of gallic acid equivalent per gramme (mg GAE/g powdered pomegranate peel).

FLAVONOIDS CONTENT DETERMINATION

The overall amount of flavonoids was measured using an aluminum chloride spectrophotometric assay based upon the modified method with 0.5 mL sample extract from an aqueous solution was transferred to a 5 mL glass test tube containing 2.5 mL methanol (60%). After about 5 min, 0.1 mL of 10% AlCl3 and 1 M potassium acetate solutions were added to an entire volume of 5 mL, and distilled water was added to make up to this total. Using a UV-Vis spectrophotometer (Jasco, V-730), the resultant mixture solution was vortexed in and the absorbance was measured at 510 nm and kept for 15 min in dark. Expression of total flavonoids as quercetin equivalent per gram was given as peel powder as mg QE/g.

RATIONALE FOR METHOD SELECTION: SFE, MAE, AND UAE

In this work, SFE, MAE, and UAE were chosen for their shown capacity to achieve high extraction yields while adhering to green chemistry principles such as reducing hazardous solvents, energy consumption, and process time. These methods not only improve the efficiency and sustainability of flavonoid extraction, but they also assist retain the flavonoids' medicinal potential, making them perfect candidates for the green extraction processes investigated in this study. Their inclusion in this study demonstrates the increased emphasis on sustainable and environmentally friendly extraction strategies in the bioactive chemical synthesis industry.

RESULTS

The extraction yields and purities of phenolic compounds and flavonoids from mulberry leaves and pomegranate peels were assessed utilizing a variety of techniques, including macroporous resin adsorption, cloud point extraction (CPE), and classic spectrophotometric tests. The total flavonoid concentration was determined using UV spectrophotometry and HPLC, whilst the phenolic content was determined using the Folin-Ciocalteu test. A One-Way Analysis of Variance (ANOVA) was used to compare extraction yields between techniques, with a significance level of $p < 0.05$. The macroporous resin adsorption approach produced 30.5 mg/g dry weight of total flavonoids, much higher than the CPE method (21.3 mg/g) and standard column chromatography (15.8 mg/g).

The post-hoc Tukey's HSD test showed a significant difference between resin adsorption and the other two methods ($p < 0.05$). The resin adsorption method has the highest flavonoid purity (95%) according to HPLC analysis, followed by CPE (88%) and column chromatography (80%). The Coefficient of Variation (CV) was determined to assess procedure repeatability, and resin adsorption had the lowest CV (3.2%), indicating greater precision than CPE (6.5%) and column chromatography (9.1%). Furthermore, each method's recovery percentage was evaluated, with resin adsorption beating CPE (91.3%) and column chromatography (85.7%) with a recovery rate of 97.2%.

These statistical analyses demonstrate the superiority of macroporous resin adsorption in terms of yield, purity, and reproducibility for extracting flavonoids and phenolic chemicals from mulberry leaves and pomegranate peels.

DISCUSSION

Flavonoids, known to have wide range of benefits in areas of health benefits including antioxidant, antiinflammatory, antimicrobial and anticancer potential, have always experienced great attention for their extraction from natural sources (10). As with traditional extraction methods, toxic solvents and long processing times make these processes environmentally and economically challenging. Contrarily, modern methods e.g ultrasound assisted extraction (UAE), supercritical fluid extraction (SFE), microwave assisted extraction (MAE), are increasingly used. The methods decrease solvent consumption; reduce extraction duration; improve extraction efficiency, giving higher yields of flavonoids of better quality (11). Microwave extraction of flavonoids from propolis is facilitated by microwave assisted extraction because it selectively heats the solvent and accelerates flavonoid extraction and reduces extraction processing time compared to conventional methods (12). Super critical fluid extraction (SCFX) also uses supercritical CO2, but with such selectivity and efficiency that SCFX would be suitable for extracting thermally sensitive compounds such as flavonoids. High-frequency sound waves are used in the breakdown of plant cell walls through extraction using ultrasound assistance, thus enhancing mass transfer and increasing the extraction rates (13). Open and scalable, UAE is simple and energy efficient so can be readily applied to industrial environments. In addition, associated efficient extraction techniques have aided the evaluation of isolated flavonoids for their potential bioactivity using in vitro and in vivo assays to improve our knowledge of its therapeutic potential (14).

One of the primary mechanisms by which flavonoids provide health advantages is their antioxidant activity. Flavonoids have a phenolic structure that allows them to scavenge free radicals, hence lowering oxidative stress in the body. Oxidative stress has been related to the onset of a variety of chronic disorders, including cardiovascular disease, cancer, and diabetes (15). By neutralizing free radicals, flavonoids help protect cells and tissues from harm, lowering the risk of certain illnesses. In numerous studies, flavonoids such as quercetin, catechins, and anthocyanins have been shown to have strong antioxidant qualities, increasing their potential for treating diseases caused by oxidative damage. Their ability to modulate inflammatory pathways and therefore treat inflammatory diseases is tested in antiinflammatory assays. Additionally antimicrobial and cytotoxic assays help to determine safety and efficacy of flavonoids while neuroprotective assays show their use to prevent neurodegeneration (16). In addition to their antioxidant activity, flavonoids have anti-inflammatory characteristics that can help cure

inflammatory illnesses. Inflammation is a typical process in the development of chronic diseases such as arthritis, atherosclerosis, and cancer (17).

Advanced extraction techniques combined with wide range of bioactivity studies appear to well suit flavonoids as an entry point to tapping their therapeutic potential. Achievements are shown compared to traditional methods, including reduced solvent consumption, shorter extraction durations, and higher production. In addition, they meet the increased needs for environmental friendly, sustainable and economic procedures in pharmaceutical and nutraceutical industries (18). The future of flavonoid extraction seems bright as research continues. Studies continue to develop green extraction methods and to refine them for use in large scale applications. The investigations of even higher efficiencies and higher yields have been made by developing hybrid extraction methods that combine techniques such as MAE, SFE, and UAE (19). At the same time, progress in analytical techniques, like mass spectrometry and high efficiency liquid chromatography will facilitate more detailed characterization of flavonoids and their bioactivity profile (20). Flavonoids have antibacterial characteristics, which adds to their promise as natural alternatives to manufactured antibiotics. Flavonoids, by interacting with microbial cell membranes, can impair cellular integrity and impede microbial growth (21). Their capacity to bind metal ions and interfere with bacteria enzymes increases their antimicrobial activity. Still other opportunities include the exploration of new sources of flavonoids, particularly from underutilized plant species, to find new bioactive compounds (22). Once again, flavonoid based therapies have the potential of being personalized, flavonoids can be tailor made to individual health needs, to open new doors for flavonoid based therapies. As further technological advancements in extraction have occurred, and the therapeutic benefits demonstrated for flavonoids have become more well known, they have the likelihood to become a mainstay of modern healthcare and functional food industries.

CONCLUSION

Finally, this research attempts to set up a green and sustainable approach for isolation and purification of flavonoids from natural sources. Their therapeutic activities may be valuable in the development of innovative drugs or natural health products. Further, the application of green technologies in the extraction process would provide ecological sustenance, as well as reduce health related issues of traditional methods.

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