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AN OVERVIEW ON EXTRACTION AND PURIFICATION OF FLAVONOIDS FROM MEDICINAL PLANTS

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ABSTRACT

Therapeutic plants gained attention in the current scenario due to their utilization in ethnomedicine for the treatment of a vast number of diseases. This review describes different strategies which have been employed for the isolation, extraction and purification of flavonoids obtained from different plant sources which includes *Cedar deodar, Citrus reticulate, Zingiber officinalis, Smilax China, Apium graveolens, Reaumria vermiculata, Pink lady apples, Nelumbo nucifera, Canarium album, Mentha piperita, Dracocephalum sp, Opuntia milpa, Alpinia zerumbet, Potamogeton crispus, Zizphus jujube, Momordica charanti, Citrullus colocynthis, Hiptage bengalensis, peach and pumpkin, E.neriifolia, Launaea procumbens, Orthosiphon stamineus, Bedelia ferruginea, Salvia officinalis L. and Salvia glutinosa L, L. pyrotechnica, Psidium guajava, Warburgia salutaris, Rhoicissus tridentate, Oroxylum indicum and Terminalia sericea. W. salutaris,Carica papaya, Cotinus coggygria, A.bilimbi, Bauhinia forficate.*

KEYWORDS: Ethnomedicine, Flavonoids, Extraction, Purification, Identification.

INTRODUCTION

Flavonoids are the polyphenol class of phytonutrients compounds that are categorised as per their chemical structure into significant groups, including anthocyanidins, flavanol, flavones, myricetin, flavanones and kaempferol.[1] The flavonoids have aroused considerable interest recently because of their remarkable scope of potential health beneficial effects on human

health. They have been reported to have antiviral, antiallergic, antiplatelet, antitumor, and antioxidant properties. They have been regarded as the most utilizable bioactive substances due to a basic skeleton of phenyl benzopyrone derivative and two aromatic rings linked by 3 carbons and a central pyrin oxygenated ring.[1] *Ramularia vermiculate* which grows in saline area possess large number of phenolic compounds and it is active against liver anticancer activity against liver (Huh-7), colorectal (HCT-116), breast (MCF-7) and prostate (PC-3) tumour cell lines.[2] Peel of pink lady apples was found effective against colon cancer cells.[3] Smilax china L-rhizome (SCR) showed anti cancerous property against HeLa cell lines including BEL-7402, HeLa 95-D, A375 and HL60 cells.[4,5] Aerial and rhizome part of *Zingiber officinalis* showed therapeutic effect against MCBZ and MDA-MB-231[6] Juice of *Citrus reticulate* containing benzo-r-pyrone structure was found active against anaplastic thyroid carcinoma (ATC) cell lines.[7] Pine needles of *Cedrus deodara* inhibits cell cycle of lung carcinoma A549, HepG2 Hepatocellular carcinoma cells and cervical carcinoma HeLa cells.[8] Lotus (*Nelumbo nucifera*), which is one of the perennial aquatic plants, its seeds and leaves have been used in folk medicine as inflammatory, anticancer, antiemetic, antiviral and antiobesity remedies. Its flowers also have a long history in Chinese folk medicine, where they are primarily used for the treatment of hematemesis, eczema and stomach troubles,[8] *Canarium album* belonging to Burseraceae family has antioxidant and free-radical scavenging activities.[9] Dracocephalum used as analgesic, antispasmodic, remedy for leukaemia and gastrointestinal cancer.[10] *Opuntia milpa* contains phenolic compounds such as flavanol 3-O-glycosides (quercetin, kaempferol, and isorhamnetin), flavonones, and flavanonols.[11] *Alpinia zerumbet* (Zingiberaceae) is an herbaceous perennial plant with wide use in Brazil. This flora is among the most cited in folk medicine. It is indicated to treat arterial hypertension.[12] *Potamogeton crispus* L. were reported to induce apoptosis in HeLa cells in vitro and has anti-tumor activities in human breast cancer and ovarian cancer cell lines ES-2.[13] *Mentha piperita* L., belonging to the family Lamiaceae, is a perennial herb peppermint oil have been used in antispasmodics, aromatics, antiseptics and even in the treatment of colds, cramps, indigestions, nausea, sore throat, toothache or even cancer.[14] The active component present in the extract of the Oroxylum indicum was found to have caused the inhibition of cell proliferation, survival, cell cycle and apoptosis of promyelotic leukemia cells (HL-60 cell line).[15] Garden sage (S. officinalis L.) is a valuable medicinal plant, which is used widely in traditional medicine. It is used in the prevention of menopausal symptoms and osteoporosis.[16] In present study, different extraction method of flavonoids from various

plant parts has been reviewed. Identification of flavonoids, total flavonoid content, antioxidant and antitumor activity of the different extracts is also reported.

MECHANISM OF ACTION

Our daily dietary foods which consists up of fruits and vegetables have an array of flavonoids with diverse bioactivities action against a large number of diseases. Flavonoids blocks and suppresses the effect of cancerous cells by biosynthesis of several CYPs and also involved in the regulation of enzymes of phase-II responsible for xenobiotic biotransformation and colon microflora. The role of flavonoids also includes the inhibition of activation of pro-carcinogens, selective death of cancer cells by apoptosis, inhibition of metastasis and angiogenesis and activation of immune response against cancer cells. Also, bioactivity of flavonoid extracts or monomers have been utilized to prevent neurodegeneration by means of antioxidant functionality.[17] The 32 plants that we have reviewed are listed below and their action against cancer lines were observed as follows, Zingier officinal is (Syedpuri rhizomes) at an elevated CO_2 concentration has free radical scavenging activity motivating apoptic cell death via peroxidising activity.[6] S. china L. rhizome containing 7-0-beta-d-glucoside cleaves DNA at inter nucleosomal linkers, inhibits the cell cycle progression and activity of transcriptional factors causing the cell death against HeLa cell lines.[4] The apple peel containing cruder component which inhibits cancer cell attachment from the basement membrane. The flavonoid myricetin was found active against breast carcinoma cells MCF-7, lung carcinoma and colorectal carcinoma cells (HCT-116).[6] Citrus reticulate inhibits the progression of cell cycle from G2/M phase and it blocks the migration of anaplastic thyroid cancer cells.[6] The TFPNCD of Cedrus deodara inhibits the growth of HepG2 Hepatocellular carcinoma cells, which inhibits the proliferation of cervical carcinoma cells, lung carcinoma cells A549 and retard HepG2 cells at G0/G1 phase.[8]

PURIFICATION OF FLAVONOID

The various primary and secondary metabolites obtained from plants provide a unique opportunity for the development of new drugs. The rapidly increasing demand for the isolation and purification of bioactive compounds from crude plant extracts leads to the development and advancement in separation techniques. Plant extract usually occurs in a combination of different classes of compounds or phytochemicals with different solubility rate, hence they are isolated, extracted and purified from different natural plant sources by using various solvent systems and chromatographic techniques. Since the separation of each

desired class of compound of crude plant extract is a difficult task for their identification purification. Therefore, it is essential to have efficient chemical and biological screening systems for rapid investigations of the plant extracts. Practically most of them must be purified by the combination of several chromatographic techniques. Several sophisticated separation techniques have been used in the past, such as Thin Layer chromatography (TLC), Column chromatography and High-Performance Liquid Chromatography (HPLC) to obtain the pure compound from mixture of compounds in the plant extract. In this review paper, we have compiled 35 research papers outcomes, focusing on therapeutic flavonoids purification strategies adopted by various researchers.

PRE-PURIFICATION STRATEGIES

Extraction

For extraction, suitable solvent is chosen for each of flavonoid. For example, chloroform, dichloromethane, diethyl ether and ethyl acetate. Flavonoid glycoside which are more polar are extracted with alcohol water mixture in a Soxhlet apparatus. Manel Karker et al., extracted flavonoid from the shoot tip of Reaumaria vermiculata by using methanol.[2] Shufang Yang et al., extracted flavonoids from Pink Lady apples by cutting1mm of fresh peels, removed the core and then weighed 10 g of peel and flesh and mashed it respectively. The two samples were placed in 80% ethyl alcohol in a dark room for two hours at room temperature conditions with an ultrasonic extractor. Then vacuum filtration was conducted, and the crude products of peel flavonoids were stored in a refrigerator at 4°C.[3]

Shahedur Rahman et al., for extracted of Kaempferol from Zingiber officinalis. they dried aerial parts of rhizome, powdered it and extracted using methanol by continuous stirring for 1 hour using orbital shaker, the extracts obtained were suctioned, evaporated and crude extract was stored at -25°C.[5] Xiaofeng Shi et al., ground the powder (40 mesh) of pine needles of Cedrus deodara (30 g) and extracted it two times (2 h and 1 h) with 40 % ethanol (25 times volume) and then filtered it. The obtained TFNPCD from the filtrate was then evaporated under reduced pressure by using a rotary evaporator to obtain the crude extract solution, with the concentration of 2.85 mg/ml.[7] Wenshui Xia et al., extracted kaempferol from the fruits of Canarium album by grounding it into small particles using a household flour mill, then the mashed fruit was extracted with a 1L of an ethanol: water solution (70:30; v:v) at 30°C with intermittent stirring. The extraction solution was then filtered, centrifuged and the supernatant

was concentrated with a rotary evaporator under reduced pressure in a water bath at 40°C to eliminate the ethanol solvent.[8]

Xing Feng Guo et al., pulverised petals of N. nucifera (500 g) and refluxed three times in 80% ethanol (3 h each time). The extracts were combined and concentrated under reduced pressure at 60ºC until ethanol had been removed. The residue was diluted with 2 L water and extracted five times with water-saturated light petroleum (b.p. 60-90ºC) and ethyl acetate successively; a total of 13.7 g of light petroleum extract and 8.7 g of ethyl acetate extract was obtained.[17] Ghazaal Moghaddam et al. extracted flavonoids from the aerial parts of D.kotschyi 50g of it was powdered and extracted overnight with 400 ml of ethyl acetate using Soxhlet method, the extract was dried at 40°C, dissolved in chloroform, filtered through Whatman filter paper, the aqueous phase was treated with ammonia solution (pH11) and pH lowered to 2 using concentrated HCl, the aqueous phase was then extracted using ethyl acetate, it was evaporated to dryness and stored at -20°C.[9] Steenkamp V. et al collected, dried and ground the plant material to a fine powder using an Ika Analytical Mill. The ground material of the different parts of these plants was used to prepare crude methanol and water extracts. They are then used for the determination of their total flavonoid and phenolic acid contents as well as for the assessment of their ability to scavenge the ABTS. ABTS radical is needed to determine the antioxidant activity. The ground material of these plants was also used to prepare free and bound phenolic acid extracts followed by their identification with gas chromatography-mass spectrometry. It was found that T. sericea contained the highest flavonoid and phenolic content, whereas for methanol extracts, the bark of W. salutaris contained the highest total phenolic content.[5]

Weirong Cai et al., peeled of the cladodes of O. milpa and cut it into small pieces of thickness of about 3-4 mm, dried and then they ventilated it in an oven at 60°C, the peel fragments were ground for a few minutes in a domestic coffee grinder and the samples were sieved and kept in a big Soxhlet extractor and refluxed with ether at 60°C for 8 h to remove oil and chlorophyll, and then it was air dried for 12 hours. After all ether was evaporated, the peel fragments were ground and separated by sieves of different sizes, the particles obtained between the 20-mesh (0.84 mm) and 28-mesh (0.6 mm) was selected and stored in vacuum packaged in polyethylene pouches at –20°C.[10] Yuanda Du et al., powdered, and extracted Potamogeton crispus with ethanol under heating reflux three times, for 90 min per extraction. The ethanol extract was then suspended in water before partitioning with petroleum ether

(PE), ethyl acetate (EtOAc), and n-butanol sequentially; these were concentrated under a vacuum to give a PE extract, a EtOAc extract, and a n-butanol separation. The EtOAc extract was chromatographed on an MCI gel column, followed by Sephadex LH-20 column chromatography, the two main compounds were then prepared using high-performance liquid chromatography (HPLC). The two compounds were identified as Luteolin-3-O-β-d-glucopyranoside (LU3´O-GP) and Flavone-6-C-β-D-glucopyranoside (FL6C-GP) by HPLC, nuclear magnetic resonance (NMR) and high-resolution electrospray ionization mass spectrometry.[18]

Xin Liu et al., dried M. piperita stems and leaves (1000.0 g) were ground in a rotary mill and sieved (10 mesh) to obtain a fine powder as pre-treated samples. They were then extracted with 90% ethanol at 80 °C for 2 h × 3 to defat and remove coloured materials and fat-soluble small molecule materials. The pre-treated dried samples were extracted with distilled water, with the temperature of water bath kept at a constant temperature within ±1.0 °C, and an electric mixing paddle was used for a given time during the entire extraction process.[13] Huan xia et al., collected Jujube samples and seed hulls were removed and dried at 50°C, it was ground and washed. The powder was extracted with 95%ethanol taken for120r/min. The resultant slurries were centrifuged for 5 min at 1400rpm.The supernatant were collected and evaporated, water was removed, the freeze-dried extract to obtain phenolic compound containing flavonoid.[19] Sing Pei Tan et al., dried one of the variety of bitter guard i.e., Moonlight variety to compare the extraction of flavonoids using five solvents: methanol, ethanol, n-butanol, acetone and water. Moonlight powder samples (1g) were added to 100 mL of each solvent and incubated for 1 h. The extraction temperatures were set below the boiling point of each solvent: 80˚C for ethanol, n-butanol and water and 50˚C and 60˚C for acetone and methanol, respectively. After extraction, the samples were cooled down and settled for 10 min on ice. The extracts were then centrifuged at 4350 × g for 10 min at 10˚C using a Beckman J2-MC Centrifuge and a JA-20 rotor and the supernatant from each sample was filtered through a 0.45µm syringe filter.[20]

Shweta yadav and Padma Kumar separated the aerial part of Hiptage benghalensis, shade dried, powdered, weighed and stored separately for extraction. It was extracted with 80% methanol for 24hrs and filtered. The filtrate obtained from each sample was extracted in petroleum ether, diethyl ether and ethyl acetate. The ether fraction was used for finding the free flavonoids whereas ethyl acetate fraction for bound flavonoids.[22] Burt et al., collected

samples of Alpinia zerumbet then the plant material was dried for 3 days and macerated in 70% ethanol or distilled water, in the same proportion of 1 g dried leaves/20 mL solvent (10%w/v). He did four extraction methods from dried leaves: maceration in a shaker at 100 rpm, ultrasonic bath (40 kHz), microwaved it and stirred it. In the microwave extraction, the suspensions were irradiated under microwaves in pre-setting procedures for three times to the desired temperature about 60 and 70ºC. The temperature was measured using a thermometer after turning off the microwave and extracts were poured into a beaker. For the ultrasonic extraction, the 30mL flask containing 1g of dried leaves and 20 mL of one of extracting solvents was partially immersed into the ultrasonic bath and temperature was controlled. Aqueous extracts were frozen, then lyophilize and the hydro alcoholic extracts were evaporated to dry under reduced pressure at 60ºC.[11]

Ammar Altemimi et al., harvested fresh Peaches (Red Haven) and Pumpkins and they used an ultrasonic device with heated water bath which was set at 37kHz for their study. User adjustable controls were heated bath temperature and power setting as a percentage of full power (30–100 %). The standard ultrasonic mode was used. The P30 had a proprietary algorithm to adjust power based on the impedance of the system, resulting in the effective power rating. For a specific power setting, samples experienced the same degree of cavitation regardless of the load in the tank. For all the treatments, they kept the1.7L of water in the water bath before the treatment containers were added.[22] Veena Sharma and Prajeta janmeda shade dried the leaves and powdered (250 g) of E. neriifolia and extracted it with 70% (v/v) ethanol and vaccum they concentrated it to dryness under reduced pressure at 60 ±1°C. After drying in hot air oven (40-45°C), they stored it in an airtight container and refrigerated it at 5 °C. The residue was designated as hydroethanolic extract of E. neriifolia (HEEN). Dried leaves of it (250 g) were extracted successively with ether, benzene, chloroform, ethyl acetate, and ethanol and finally macerated with distilled water (non-polar to polar) to get respective extracts.[23]

Yahaya Mobmi Musa defatted around 150g of sample with 250ml of n-Hexane in batches with 30g in each batch using a Soxhlet extractor. The extraction was carried out for about six hours at a temperature range between 65°C and 75°C. After the extraction, he dried out all the thimbles in an oven at 50°C. He further extracted the extracted Marc with 250 ml of Methanol solvent and then the extract was evaporated using a water bath to remove the solvent completely. The Methanol extract was adjusted with water to give 90% and then it

was partitioned with t-Butyl methyl ether and n-hexane (9:1) mixture and then it was shaken very well till the methanol portion was separated out and evaporated.[24]

Gang Wang et al., dried roots and stems of Cotinus coggygria and ground it into powders, and then extracted it with 95% ethanol by a liquid-liquid extraction method. They took 100g of crushed powder of the roots and stems and extracted it 2000ml of 95% ethanol for 3h. The extraction was boiled under reflux. Then, the alcohol extract obtained was decolorized on a macroporous resin column (maximum absorption quantity was 1.15g of herbs per mL of macroporous resin) and eluted with 95% ethanol (elution volumes, 10–20BV), followed by the purification of the phenolic rich extract obtained using a polyamide column (diameter: 2cm, length: 50cm), the extraction was evaporated under a vacuum. The identity and content of the flavonoid rich compounds were confirmed by comparing the elution time of pure controls by HPLC spectrometry. They dissolved the samples obtained in deionized water. The content of each flavonoid sample so obtained was determined by the absorbance at 365nm using HPLC.[25]

Maya S. Nair et al., dried the leaves of A. bilimbi in shade and crushed it into powder and were extracted successively with petroleum ether, chloroform, ethyl acetate, 80% ethanol and water in a Soxhlet apparatus. They cut the fruits into pieces and dried in a hot air oven at 40ºC. The dried fruits were ground into fine powder. A freshly prepared crude extracts from the fruit and leaves of A. bilimbi were tested for the presence of various chemical constituents qualitatively. The fruit extract showed the presence of tannins, phlobatannins, saponins, flavonoids, terpenoids, cardiac glycosides and anthocyannins. The leaf extract contains flavonoid, alkaloids and phenols. Flavonoid content of the hydromethanolic extract of the fruit and 80% ethanolic leaf extracts were estimated by MTT assay. The fruit extract showed a higher content of flavonoid. The total flavonoid content in crude hydromethanolic fruit extract of A. bilimbi was found to be 358 ± 0.7 µg-1 plant extract (in Rutin equivalent).[26]

Claudia R.F. Souza et al., dried and powdered leaves of Bauhinia forficata, and stored in dark bags to protect them from the humidity and from the light. They extracted it in Ethyl acetate, methanol, acetone, aluminium chloride, ethanol, chloridric acid (Synth-Brazil), hexametilenetetramine (Vetec-Brazil), Samples of the vegetal drug (5g) obtained were maintained in an oven at 102±1°C until constant mass was obtained. Solutions 1:100 grams of the vegetal material (dry basis) in distilled water and in the water: ethanol (70 % v/v) were

heated until boiling being filtered after cooling. Five samples of 20.0 g of the filtrate were withdrawn and put in oven to 102±1°C until constant mass was obtained. The extractive matter was calculated by the percentage ratio between the dry residues to the vegetal material masses. They determined the total flavonoid content by spectrophotometry.[27]

PURIFICATION

Chromatographic Techniques

Thin Layer Chromatography (TLC)

Thin layer chromatography was performed with extracts obtained from E.neriifolia using standard methods. Small quantities of samples (2 mg/ml) were dissolved in their respective solvents. Quercetin (1 mg) standard was dissolved in methanol. Different mobile phases with varying concentrations were employed in the screening programme and selected the one in which separation of flavonoid was clear: n-butanol: acetic acid: water (2:2:6). All plates were visualized directly after drying and with the help of UV at 254 nm and 366 nm in UV TLC viewer. The Rf value of the different spots that were observed were calculated. The presence of flavonoids was confirmed by screening tests, which gave a positive test for flavonoids.[23]

High Performance Liquid Chromatography (HPLC)

The phenolic compounds in the dichloromethane fraction of Reaumuria vermiculata shoot was performed via an HPLC apparatus. The separation was carried out on a reverse phase C18 analytical column of 4.6 x 100 mm and 3.5 µm particle size. The flow-rate was adjusted to 400 µl/min while the injected sample volume was 2 µl and the temperature was kept at 25 °C. The mobile phase consisted of methanol (solvent A) and 0.1 % formic acid (solvent B). Total flavonoid contents, in R. vermiculata shoots were obtained in the methanol extract (29.9 mg CE/g) closely followed by dichloromethane (25.1 mg CE/g), then water and finally hexane extracts (18.9 and 10.6 mg CE/g, respectively).[2] A.M. Y. Moustafa et al. collected fresh aerial parts (leaves, flowers and stems) of L. pyrotechnica during the flowering stage. These parts were then air-dried and ground together as a fine powder. This powder was defatted with petroleum ether and was then percolated with methanol until exhaustion. The methanol extract was evaporated and was extracted using ethyl acetate. About 4 g of the total ethyl acetate extract was applied on LPLC using 95% methanol/water as eluent. This was followed by Preparative Thin Layer Chromatography (PTLC) and was subjected to further purification on Sephadex LH-20 column chromatography. The isolation of these compounds was also carried out using Sephadex LH-20 low pressure liquid chromatography (LPLC),

preparative paper chromatography (PPC), and high-performance liquid chromatography (HPLC). The LC_{50} values were 11.89 and 84.14 ppm in the total alcoholic and total flavonoid extracts, respectively.[28]

Cedrus deodara (15 mL; adjusted to pH 4.0) was loaded in glass columns which were wet-packed with HPD722 macroporous resin (10 g) at room temperature. The feed rate was set at 2 bed volume (BV)·h^{-1}. The adsorbate-laden column was washed with 4 BV of deionized water, and then eluted with 2 BV of aqueous solution (70 % ethanol) at 2 BV·h−1. The eluting solution was concentrated in the rotary evaporation apparatus and dried under vacuum before further analysis.[17] The SCR ethyl acetate fraction containing kaempferol from Smilax china was fractioned on a silica gel column by elution with chloroform/methanol and six fractions of flavonoids and glycoside was obtained. The n-butanol fraction was applied onto polyamide column chromatography eluted with water and 95% ethanol, to yield ethanol soluble portions which were abundant in flavonoid and flavonoid glycoside name n2,rechromatography was done on water soluble portion on diaion and eluted with water and ethanol by turns, the ethanol soluble portion was collected which was rich in saponins.[4]

The aerial parts and rhizomes of Zingiber officinales were extracted (0.25g) with 60% aqueous methanol, 0.6M HCl (5ml) was added to each extract of give a 25ml solution of 1.2M HCl in 50% aqueous methanol, it was refluxed at 90°C for 2 hours and it was filtered through a 0.45 micrometre filter.[6] Takuya Suetsugu et al., dried 0.1 g of plant material Citrus junos and extracted it with 50 % of methanol solution flavonoid extracts were analysed using an HPLC LC-10AD gradient system, equipped with Diode Array the mobile phase consisted of solvent A, 0.1 % acetic acid in water, and solvent B, 0.1 % acetic acid in acetonitrile (acetonitrile/water= 75/25, v/v). The flow rate was 1.0 mL/min. Peaks were measured at a wavelength of 285 nm to quantify flavonoids.[29]

Column Chromatography

Yahaya Mobmi Musa chromatographed around 6g of methanolic extract over a Silica gel column (200g) in 60-200 mesh and eluded with a solvent mixture of $CH_2Cl_2/CH_3OH/H_2O$ (70:30:1V/V) 350ml, 150ml, and 5ml of water respectively. He further screened for the presence of flavonoids from the leaves of Carica papaya using the methods adopted and a yield of 0.23% was obtained.[24]

Reverse Phase-High Performance Liquid Chromatography (RP-HPLC)

The crude sample obtained i.e., Dracocephalum kotschyi from liquid-liquid extraction was subjected to reversed-phase HPLC using an isocratic solvent system consisting of 55% HCl 0.01 M, 25% acetonitrile, 19% methanol and 1% water. The flow rate was 7mL/min and the column were maintained at a controlled temperature. The injection volume was 1.7mL and the detector was set at 226nm. Data acquisition was carried out using an Advantec PCI1716 data acquisition card and an in-house developed software. Fractions were collected and pooled from different runs and each fraction was weighed after drying in a vacuum. The isolated flavonoids were subjected to analytical HPLC. The mobile phase (55% HCl 0.01 M 25% acetonitrile, 19% methanol and 1% water) was pumped at 1mL/min and the detector was set at 220nm.[9]

High-Speed Counter Current Chromatography (HSCCC)

The ethyl acetate extract of Nelumbo nucifera containing Syringetin-3-O-β-D-glucoside; quercetin-3-O-β-D-glucoside; isorhamnetin-3-O-β-D-glucoside; kaempferol-3- O-β-D-glucoside was further subjected to polyamide chromatography by stepwise elution with aqueous ethanol (0, 30, 60 and 90%, v/v) and four fractions were obtained. After TLC and HPLC analysis, the second fraction (30% aqueous ethanol) was evaporated to dryness under reduced pressure and stored for subsequent HSCCC separation.[17]

Preparative Thin Layer Chromatography (PTLC)

Moustafa et al. Collected fresh aerial parts (leaves, flowers and stems) of L. pyrotechnica during the flowering stage. These parts were then air-dried and ground together as a fine powder. This powder was defatted with petroleum ether and was then percolated with methanol until exhaustion. The methanol extract was evaporated in vacuum and was extracted using ethyl acetate. About 4 g of the total ethyl acetate extract was applied on LPLC using 95% methanol/water as eluent. This was followed by Preparative Thin Layer Chromatography (PTLC) and was subjected to further purification on Sephadex LH-20 column chromatography. The isolation of these compounds was also carried out using Sephadex LH-20 low pressure liquid chromatography (LPLC), preparative paper chromatography (PPC), and high-performance liquid chromatography (HPLC).[28]

M. C. Meena and V. Patni dried samples of Citrullus colocynthis (stem, leaf, fruit and root) were extracted in 80% methanol on a water bath for 24 hours.[31] The extracts were concentrated in petroleum ether (Fraction I), ethyl ether (Fraction II) and ethyl acetate

(Fraction III) and the steps were repeated for complete extraction. Fraction I was rejected. Fraction 11 of ethyl ether containing flavonoid was analyzed and fraction III containing ethyl acetate and crude sample were hydrolyzed and filtered. The residues were taken in small volumes of ethanol and were analyzed further by means of Thin Layer Chromatography (TLC) and Preparative Thin Layer Chromatography (PTLC). The Rf value of isolated quercetin and standard quercetin was calculated. The purified material was subjected to its IR spectra and HPLC.[30]

Mishra et al., took 10gm of plant powder from Launaea procumbens and added 100 ml of the solvent to it. The solution was then heated at 55°C on a water bath for about 5min and then sealed with the glass stopper and kept on the rotary shaker for 24hrs. After 24hrs, the solution was concentrated at 45°C. For the screening of flavonoids 0.5 ml of the extract was mixed with 2 ml of Conc. H2SO4 and few magnesium turnings were also added, after which they carried out Thin Layer Chromatography, which confirmed the presence of flavonoids by revealing the fluorescent bands which on further derivatization gave yellow fluorescence on the long wavelength (360nm) and forms yellow coloured zone when heated at 100°C for 5-10 min.[31] S. Mahmood cleaned the leaves of Celery plants and dried them. The dried leaves were then powdered. The dried powder was extracted with hexane in Soxhlet apparatus and was filtered. The crude extract was suspended in ethanol. After removing solvents in rotary, the extract was dissolved in 5% NaOH in a separation funnel, into which chloroform was added adequately. TLC serpents and three mobile phases were used for the analysis of the flavonoid exudates. Flavonoid reaction products were identified by RF values and chromatography with authentic substances on TLC in different solvent system.[32]

The crude extracts of Opuntia milpa were filtered, and ethanol was subsequently evaporated. The solution was poured onto a column (400 × 2.5 cm id.) packed with pre-treated AB-8 resin. After complete absorption of the solution, the column was washed with enough distilled water to remove carbohydrates, and further washed with 65% ethanol to elute flavonoids. The eluate abundant in flavonoids was collected and then concentrated at 40°C with a Laboratory 4000 rotary evaporator until the sediment was formed. It was then collected and vacuum-dried at 40°C, the sediment thus obtained represented the solid-state product of flavonoids, which was then separated and collected per their retention time (RT). The results of LC-MS and flavone diagnostic reagent indicated that the main components of

the extracted flavonoids were isorhamnetin 3-O-(2,6-dirhamnosyl) glucoside and isorhamnetin 3-O-d-rutinoside.[10]

Donates Beer Owe and Nonaka Urawa dried samples of Bedelliid ferruginea it was milled and ground into powder (912 g) the powdered plant sample (500g) was packed into a Soxhlet apparatus (2L) and extracted exhaustively with 1000 ml ethanol for 24 hours. The ethanol extract was concentrated using a rotary evaporator at room temperature and pigment was obtained. The column was packed with silica gel and the green pigment was placed on the top and eluted with methanol, chloroform and petroleum ether in the ratio (20:30:50) to afford a yellow orange powder (0.80g). The yellow, orange powder was re-crystallized from hexane. Compounds isolated as a yellow crystal solid was identified as 4l propane dihydroxy-7-hydroxyl anthocynidines.[33] Amzad etal., dried the leaves of the plant Orthosiphon stamineus (1kg) milled into powder and then extracted it with direct methanol (10 L) in a Soxhlet extractor for 36 h. The extract was evaporated in a rotatory evaporator they dried it using a vacuum pump. The methanolic extract (50 g) was suspended in water and extracted successively with n-hexane, chloroform, ethyl acetate, and n-butanol to yield n-hexane (5.5 g), chloroform (11.5 g), ethyl acetate (9.3 g) and n-BuOHsoluble (5.2 g) fractions, respectively. Chloroform soluble fraction (10 g) was subjected to chromatography on silica gel.[34]

Yindi Zhu et al. collected leaves of Psidium gujava dried, ground and ultrasonically extracted the plant leaves using ethyl acetate. The extract was filtered and evaporated and dried. The dried extract was then prepared for subsequent HSCCC isolation and purification. A suitable amount of crude sample was added into a series of pre-equilibrated two-phase solvent systems, and the solution was then shaken fully. The partition coefficients were determined by HPLC as follows. The peak fractions were collected manually according to the elution profile in HSCCC and evaporated under reduced pressure, and the residues were dissolved in methanol for subsequent purity analysis was done by using the HPLC. The purity was obtained by HPLC peak area calculation.[35]

CONCLUSION

The isolation, extraction, purification and the analysis of flavonoids is highly dependent on the matrix characteristics and the complexity of techniques. Many techniques offer the real possibility of preparation of the sample and assess the flavonoid content, but none the less

there is still no standardised procedure available for sample preparation and extraction. Current review provides conclusive evidence for the purification of value added flavonoids.

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