JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue
JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)
An International Scholarly Open Access, Peer-reviewed, Refereed Journal

Comprehensive Steps in Phytochemical Screening Pertaining to Extraction, Isolation, Purification, Characterization and Identification of Crude Drugs

Anirban Saha*, Sumaila Saifi

Department of Pharmacognosy and Phytochemistry, School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi, India.

Abstract:

Phytochemical screening is a crucial aspect of pharmacognosy and natural product research, enabling the identification of bioactive constituents from crude drugs of plant origin. The systematic approach to phytochemical investigation involves a series of interlinked steps — extraction, isolation, purification, characterization, and identification — each contributing to the accurate elucidation of plant-derived compounds. Extraction serves as the initial step, employing solvents of varying polarity to separate phytoconstituents from plant matrices. This is followed by isolation and purification, utilizing techniques such as chromatography and crystallization to obtain individual compounds in pure form. Subsequent characterization and identification are achieved through advanced spectroscopic methods, including UV-Vis, IR, NMR, and mass spectrometry, which provide structural insights into the isolated phytochemicals. Collectively, these steps form the foundation of natural drug discovery, offering a scientific basis for the development of novel therapeutics and validating traditional medicinal uses of plants. A comprehensive understanding of these processes enhances quality control, standardization, and reproducibility in herbal research and formulation.

Keywords:

Phytochemical screening, extraction, isolation, purification, characterization, identification, crude drugs, natural products, chromatography, spectroscopy.

Introduction:

Phytoconstituents (Greek: Phyton = plant; Constituents = chemical compounds) naturally present in the plants attributing to positive or negative health effects. Medicinal plants used in different diseases and ailments are the richest bio reservoirs of various phytochemicals. The medicinal properties of the plants are determined by the phytochemical constituents. Some of the important phytochemicals include alkaloids, flavonoids, phenolics,

tannins, saponins, steroids, glycosides, terpenes, etc. which are distributed in various parts of the plants. Nature is a unique source of structures of high phytochemical diversity representing phenolics (45%), terpenoids and steroids (27%) and alkaloids (18%) as major groups of phytochemicals . Although, these compounds seem to be non-essential to the plant producing them, they play a vital role in survival by mediation of ecological interactions with competitors, protect them from diseases, pollution, stress, UV rays and also contribute for colour, aroma and flavour with respect to the plant. The metabolites produced by the plants to protect themselves against biotic and abiotic stresses have turned into medicines that people can use to treat various diseases (1,2,3). Phytochemicals can be separated from the plant material by various extraction techniques. The most commonly used conventional methods include maceration, percolation, infusion, digestion, decoction, hot continuous extraction (Soxhlet extraction) etc., recently, eco-friendly techniques such as Ultrasound-Assisted Extraction (UAE), Microwave-Assisted Extraction (MAE), Supercritical Fluid Extractions (SFE) and Accelerated Solvent Extraction (ASE) have also been introduced. Different types of solvents viz. water, ethanol, methanol, acetone, ether, benzene, chloroform etc. are used in the extraction process. Extraction of phytochemicals from the plant materials is affected by pre-extraction factors (plant part used, its origin and particle size, moisture content, method of drying, degree of processing etc.) and extraction-related factors (extraction method adopted, solvent chosen, solvent to sample ratio, pH and temperature of the solvent, and length of extraction) (4). Previously, the plant parts were directly used as such for the treatment, but now-a-days, the active principles are identified and isolated in pure form and also synthetically produced with the help of advance techniques. In the development of new synthetic drugs, the chemical structures derived from these phytoconstituents can be utilized as models. Identification of phytoconstituents in the plant material helps to predict the potential pharmacological activity of that plant(5). Characterization and evaluation of plants and their phytoconstituents can explore the evidences to support therapeutic claims of those plants against various ailments . Advanced techniques like Gas Chromatography (GC), Liquid Chromatography (LC), High-Performance Liquid Chromatography (HPLC), High-Performance Thin Layer Chromatography (HPTLC) etc. are very helpful for detection of phytoconstituents both qualitatively as well as quantitatively. However, when these techniques are unavailable or unaffordable, the conventional phytochemical tests which are economic, easy and require fewer resources, remain the good choice for preliminary phytochemical screening(6). The present communication deals with the collection and compilation of maximum possible qualitative phytochemical tests from various published literatures. The preliminary qualitative phytochemical tests for the detection of different phytoconstituents have been summarized(7).

Reagents/Solutions		Composition
1	Dragendroff's reagent	Stock solution: 5.2gm Bismuth carbonate + 4gm sodium iodide + 50mL glacial acetic acid, boiled for few min., After 12 hr. precipitated sodium acetate crystals are filtered by sintered glass funnel; 40mL filtrate + 160mL ethyl acetate + 1mL distilled water, (stored in amber-coloured glass bottle). Working solution: 10mL stock solution + 20mL acetic acid + distilled water to make final volume 100mL
2	Hager's reagent	Saturated aqueous solution of picric acid

3	Mayer's reagent	Solution A : 1.358gm mercuric chloride + 60mL distilled water Solution B : 5gm potassium iodide + 10mL distilled water Working solution: solution A + solution B + distilled water to make final volume 100mL
4	Wagner's reagent	1.27gm iodine + 2gm potassium iodide + distilled water to make final volume 100mL
5	Barfoed's reagent	30.5gm copper acetate + 1.8mL glacial acetic acid
6	Seliwanoff's reagent	0.05 resorcinol + 100mL dilute HCl
7	Benedict's reagent	Solution A: 173gm sodium citrate + 100gm sodium carbonate + 800mL water, dissolve & boil to make solution clear Solution B: 17.3gm of copper sulphate dissolved in 100mL distilled water Working solution: Mix solution A and solution B
8	Fehling's solutions	Solution A: 34.66gm copper sulphate + distilled water to make final volume 100mL. Solution B: 173gm potassium sodium tartarate + 50gm NaOH + distilled water to make 100mL.
9	Baljet's reagent	95mL 1% picric acid + 5mL 10% NaOH
10	Millon's reagent	1gm mercury + 9mL fuming nitric acid + equal amount of distilled water (after completion of reaction)

Table 1: Reagent Preparation for Phytochemical Screening

Methods Used for Bioactive Compound Extraction, Isolation, and Purification:

- Extraction of Phenolic Compounds Using Solvents

Scientists have studied and analyzed the impact of different types of solvents, such as methanol, hexane, and ethyl alcohol, for the purpose of antioxidant extraction from various plants parts, such as leaves and seeds. In order to extract different phenolic compounds from plants with a high degree of accuracy, various solvents of differing polarities must be used. Moreover, scientists have discovered that highly polar solvents, such as methanol, have a high effectiveness as antioxidants(8). Anokwuru et al. reported that acetone and N,N dimethylformamide (DMF) are highly effective at extracting antioxidants, while Koffi et al. found that methanol was more effective in at a large amount of phenolic contents from walnut fruits when compared to ethanol. It has been reported that ethanolic extracts of Ivorian plants extracted higher concentrations/amount of phenolics compared to acetone, water, and methanol. Multiple solvents have been commonly used to extract phytochemicals, and scientists usually employed a dried powder of plants to extract bioactive compounds and eliminate the interference of water at the same time. Solvents used for the extraction of biomolecules from plants are chosen based on the polarity of the solute of interest(9,10). A solvent of similar polarity to the solute will properly dissolve the solute. Multiple solvents can be used sequentially in order to limit the amount of analogous compounds in the desired yield. The polarity, from least polar to most polar, of a few common solvents is as follows: Hexane < Chloroform < Ethylacetate < Acetone < Methanol < Water(11).

- Microwave-Assisted Extraction (MAE)

MAE has attracted the attention of researchers as a technique to extract bioactive compounds from a wide variety of plants and natural residues. Microwaves have electromagnectic radiation that occurs at frequencies between 300 MHz to 300 GHz, and wavelengths between 1 cm and 1 m. These electromagnetic waves consist of both an electrical field and a magnetic field. These are described as two perpendicular fields(12). The first application of microwaves was to heat up objects that can absorb a part of the electromagnetic energy and convert it into heat. Commercial microwave instruments commonly use the frequency 2450 MHz, which corresponds to an energy output of 600–700 Watts. Recently, advanced techniques have become available to

reduce the loss of bioactive compound without increasing the extraction time. Therefore, microwave-assisted extraction is demonstrated to be a good technique in multiple fields, especially in the medicinal plant area(13). Moreover, this technique reduced the losses of the biochemical compounds being extracted . Microwave-assisted extraction (MAE) has been used as an alternative to conventional techniques for the extraction of antioxidants because of its ability to reduce both time and extraction solvent volume. In fact, the main objective of using MAE is to heat the solvent and extract antioxidants from plants with a lesser amount of these solvents(14). Li et al. reported that conventional methods using various solvents presented less antioxidant activity and phenolic content than MAE. Therefore, the finding confirmed that MAE was more effective at increasing antioxidant activity by measuring ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC), and total phenolic content (TPC). The efficiency of the microwave extraction can be changed through some factors such as extraction temperature, solvent composition, and extraction time(15,16). The extraction temperature was usually studied more than other factors due to its ability to increase the efficiency of the microwave extraction. Tsubaki et al. reported that 170 ◦C was the most effective temperature for extracting phenolic compounds from Chinese tea. In addition, increasing the extraction temperature beyond this point resulted in a reduced extraction yield. Recently, Christophoridou et al. used a new microwave-assisted extraction (MAE) process, which converts energy to heat, thereby cooperating with solvents in order to extract a specific compound. Williams et al. showed many advantages of MAE, including lower solvent consumption, shorter extraction times, and higher sensitivity towards target molecules. A comparison of some antioxidant methods used has been provided in Table 2.

Table 2. Comparison of methods for assessing antioxidant capacity based upon mechanism, endpoint, quantitation method, and whether the assay is adaptable to measure lipophilic and hydrophilic antioxidants.

Antioxidant Assay	Mechanism	Endpoint	Quantification	Lipophilic and Hydrophilic AOC
ORAC	HAT	Fixed time	AUC	Yes
TRAP	HAT	Lag phase	IC50 lag time	No
FRAP	SET	Time varies	ΔOD fixed time	No
TEAC	SET	Time varies	ΔOD fixed time	Yes
DPPH	SET	IC50	ΔOD fixed time	No
LDL oxidation	SET	Lag phase	Lag time	No

- **Ultrasonic-Assisted Extraction:**

Ultrasound-assisted extraction (UAE) has been used in diverse applications of food-processing technology to extract bioactive compounds from plant materials. Ultrasound, with levels greater than 20 kHz, is used to disrupt plant cell walls, which helps improve the solvent's ability to penetrate the cells and obtain a higher extraction yield. UAE can use a low operating temperature through processing, maintaining a high extract quality for compounds. UAE is known to be one of the easiest extraction techniques because it uses common laboratory equipment such as an ultrasonic bath. In this technique, a smashed sample is mixed with the suitable solvent and placed into the ultrasonic bath, while temperature and extraction time are controlled. UAE of various organic and inorganic samples can use a wide range of solvents. Common equipment used in ultrasound-assisted extraction includes an ultrasonic bath and an ultrasonic probe system(17,18). Unfortunately, ultrasonic probe has two main negative properties mainly related to experimental repeatability and reproducibility. Tabaraki et al. noted that green technology is necessary to protect the environment from toxic substances. Therefore, extraction of phenolic compounds by ultrasound has grown during recent years due to its role in reducing the amount of solvent and energy used. Corrales et al. have shown that UAE can break down

plant tissue and work properly during the production process and release of active compounds in solvents with a high efficiency. Results showed an increase in antioxidant activity from 187.13 µmol TE g−1DM to 308 µmol TE g−1DM by using UAE as an effective method to extract antioxidants from different sources. Recently, Albu et al. studied and applied the use of ultrasound to extract phenolic compounds from rosemary. Multiple criteria have been compared including ultrasonic bath extractions, ultrasonic probe system, a shaking water bath at various temperatures, and different solvents to select the most efficient method. In all situations, the operation time was dramatically decreased by applying and using the ultrasonic bath and probe systems. Similar behavior was reported by Cho et al. when extracting resveratrol from grapes. In another study, Barbero et al. suggested the use of ultrasound in different industries because of its positive effects in the extraction of capsaicinoids of hot peppers (19,20). Moreover, the ultrasonic method had the ability to decrease the degradation of phenolics. Mulinacci et al. compared the extraction time of phenolic compounds from strawberries with other extraction methods such as solid–liquid, subcritical water, and microwave-assisted method. The results confirmed that UAE was the most effective method(21).

- **Techniques of Isolation and Purification of Bioactive Molecules from Plants:**

Purification and isolation of bioactive compounds from plants is a technique that has undergone new development in recent years(22). This modern technique offers the ability to parallel the development and availability of many advanced bioassays on the one hand, and provided precise techniques of isolation, separation, and purification on the other. The goal when searching for bioactive compounds is to find an appropriate method that can screen the source material for bioactivity such as antioxidant, antibacterial, or cytotoxicity, combined with simplicity, specificity, and speed(23). In vitro methods are usually more desirable than in vivo assays because animal experiments are expensive, take more time, and are prone to ethical controversies(24). There are some factors that make it impossible to find final procedures or protocols to isolate and characterize certain bioactive molecules. This could be due to different parts (tissues) in a plant, many of which will produce quite different compounds, in addition to the diverse chemical structures and physicochemical properties of the bioactive phytochemicals(25). Both the selection and the collection of plant materials are considered primary steps to isolate and characterize a bioactive phytochemical. The next step involves a retrieval of ethno-botanical information to discern possible bioactive molecules(26). Extracts can then be made with various solvents to isolate and purify the active compounds that are responsible for the bioactivity. Column chromatographic techniques can be used for the isolation and purification of the bioactive compounds. Developed instruments such as High Pressure Liquid Chromatography (HPLC) accelerate the process of purification of the bioactive molecule(27). Different varieties of spectroscopic techniques like UV-visible, Infrared (IR), Nuclear Magnetic Resonance (NMR), and mass spectroscopy can identify the purified compounds(28).

- **Purification of the Bioactive Molecule:**

Many bioactive molecules have been isolated and purified by using paper thin-layer and column chromatographic methods. Column chromatography and thin-layer chromatography (TLC) are still mostly used due to their convenience, economy, and availability in various stationary phases. Silica, alumina, cellulose, and polyamide exhibit the most value for separating the phytochemicals(29). Plant materials include high amounts of complex phytochemicals, which make a good separation difficult. Therefore, increasing polarity using multiple mobile phases is useful for highly valued separations. Thin-layer chromatography has always been used to analyze the fractions of compounds by column chromatography. Silica gel column chromatography and thin-layer chromatography (TLC) have been used for separation of bioactive molecules with some analytical tools(30) .

- **Structural Clarification of the Bioactive Molecules:**

Determination of the structure of certain molecules uses data from a wide range of spectroscopic techniques such as UV-visible, Infrared (IR), Nuclear Magnetic Resonance (NMR), and mass spectroscopy(31). The basic principle of spectroscopy is passing electromagnetic radiation through an organic molecule that absorbs some of the radiation, but not all. By measuring the amount of absorption of electromagnetic radiation, a spectrum can be produced. The spectra are specific to certain bonds in a molecule. Depending on these spectra, the structure of the organic molecule can be identified. Scientists mainly use spectra produced from either three or

four regions—Ultraviolet (UV), Visible, Infrared (IR), radio frequency, and electron beam —for structural clarification(32).

- **UV-Visible Spectroscopy:**

UV-visible spectroscopy can be performed for qualitative analysis and for identification of certain classes of compounds in both pure and biological mixtures(33). Preferentially, UV-visible spectroscopy can be used for quantitative analysis because aromatic molecules are powerful chromophores in the UV range. Natural compounds can be determined by using UV-visible spectroscopy(34). Phenolic compounds including anthocyanins, tannins, polymer dyes, and phenols form complexes with iron that have been detected by the ultraviolet/visible (UV-Vis) spectroscopy. Moreover, spectroscopic UV-Vis techniques were found to be less selective and give information on the composition of the total polyphenol content. The UV-Vis spectroscopy was used to determine the total phenolic extract (280 nm), flavones (320 nm), phenolic acids (360 nm), and the total anthokyanids (520 nm). This technique is not time-consuming, and presents reduced cost compared to other techniques(35).

- **Infrared Spectroscopy:**

Some of the frequencies will be absorbed when infrared light passes through a sample of an organic compound; however, some frequencies will be transmitted through the sample without any absorption occurring. Infrared absorption is related to the vibrational changes that happen inside a molecule when it is exposed to infrared radiation(36). Therefore, infrared spectroscopy can essentially be described as a vibrational spectroscopy. Different bonds (C–C, C=C, C≡C, C–O, C=O, O–H, and N–H) have diverse vibrational frequencies. If these kinds of bonds are present in an organic molecule, they can be identified by detecting the characteristic frequency absorption band in the infrared spectrum. Fourier Transform Infrared Spectroscopy (FTIR) is a high-resolution analytical tool to identify the chemical constituents and elucidate the structural compounds. FTIR offers a rapid and nondestructive investigation to fingerprint herbal extracts or powders(37).

- **Nuclear Magnetic Resonance Spectroscopy (NMR):**

NMR is primarily related to the magnetic properties of certain atomic nuclei; notably the nucleus of the hydrogen atom, the proton, the carbon, and an isotope of carbon. NMR spectroscopy has enabled many researchers to study molecules by recording the differences between the various magnetic nuclei, and thereby giving a clear picture of what the positions of these nuclei are in the molecule. Moreover, it will demonstrate which atoms are present in neighboring groups(38). Ultimately, it can conclude how many atoms are present in each of these environments. Several attempts have been made in the past by using preparative or semi preparative thin-layer chromatography, liquid chromatography, and column chromatography to isolate individual phenols, the structures of which are determined subsequently by NMR off-line(39).

- **Mass Spectrometry for Chemical Compounds Identification:**

Organic molecules are bombarded with either electrons or lasers in mass spectrometry and thereby converted to charged ions, which are highly energetic(40). A mass spectrum is a plot of the relative abundance of a fragmented ion against the ratio of mass/charge of these ions. Using mass spectrometry, relative molecular mass (molecular weight) can be determined with high accuracy and an exact molecular formula can be determined with a knowledge of places where the molecule has been fragmented(41). In previous work, bioactive molecules from pith were isolated and purified by bioactivity-guided solvent extraction, column chromatography, and HPLC. The techniques of UV-visible, IR, NMR, and mass spectroscopy were employed to characterize the structure of the bioactive molecule. Furthermore, molecules may be hydrolyzed and their derivatives characterized. Mass spectrometry provides abundant information for the structural elucidation of the compounds when tandem mass spectrometry (MS) is applied. Therefore, the combination of HPLC and MS facilitates rapid and accurate identification of chemical compounds in medicinal herbs, especially when a pure standard is unavailable. Recently, LC/MS has been extensively used for the analysis of phenolic compounds. Electrospray ionization (ESI) is a preferred source due to its high ionization efficiency for phenolic compounds(42).

- **Plants as a Source of Antioxidants:**

Antioxidants can be defined as bioactive compounds that inhibit or delay the oxidation of molecules. Antioxidants are categorized as natural or synthetic antioxidants. Some synthetic antioxidants commonly used are: BHT, BHA, propyl gallate, and tertbutyl hydro-quinine. Many scientists have concerns about safety because synthetic antioxidants have recently been shown to cause health problems such as liver damage, due to their toxicity and carcinogenicity(43). Therefore, the development of safer antioxidants from natural sources has increased, and plants have been used as a good source of traditional medicines to treat different diseases. Many of these medicinal plants are indeed good sources of phytochemicals that possess antioxidant activities. Some typical examples of common ingredients that have been used in ethnic foods are tamarind, cardamom, lemon grass, and galangal basil. These spices or herbs have been shown to contain antioxidants. Deterioration of food due to either bacterial or fungal infection has always been a major concern, causing huge losses to food industries and societies throughout the world(44). Moreover, the spread of food pathogens has become a major public health concern. With an increasing awareness of the negative effects of synthetic preservatives, there has been increased demand for the use of nontoxic, natural preservatives, many of which are likely to have either antioxidant or antimicrobial activities. Herbs have always been used for flavour and fragrance in the food industry, and some of them have been found to exhibit antimicrobial properties. Therefore, the call for screening and using plant materials for their antioxidant and antimicrobial properties has increased. Approximately 20% of all plant species have been tested in both pharmacological and biological applications to confirm their safety and advantages. A summary of the types of compounds, plant species, plant parts from which compounds were extracted(46).

- **Presence of Antioxidant in Red Algae:**

Red algae are aquatic plant species considered one of the oldest groups of eukaryotic algae(47). The antioxidant activity of a red alga, Palmaria palmate, has been studied. The results reported that 9.68 µg of ascorbic acid and 10.3 µg of total polyphenol can equally reduce activity in 1 mg of dulse extracts. The reducing activity was correlated with aqueous/alcohol soluble compounds due to the presence of functional groups such as hydroxyl, carbonyl, etc., which lead to reduced or inhibited oxidation(48).

- **Antioxidants from Monocots:**

Ashawat et al. studied the antioxidant properties of ethanolic extracts of Areca catechu and showed that Areca catechu had the highest antioxidant activity when compared to other eudicots like *Centella asiatica*, *Punica granatum*, and *Glycyrrhiza glabra*. Londonkar and kamble studied *Pandanus odoratissimus L*. in order to determine its antioxidant activity. Zahin et al. screened Acorus calamus to estimate antioxidant activity and total phenolic contents. The observations confirmed that there was a significant correlation between the phenolic content and antioxidant activity. Another monocot, *O. sanctum*, showed that the inhibition of lipid peroxidation in vivo and in vitro increased proportionally with an increase in the concentration of the extract(49).

- **Antioxidants from Vegetables:**

Consumption of vegetables has been linked to a reduction in the risk of many diseases, such as cancer and cardiovascular disease, when studied in epidemiological studies. Numerous studies have attempted to screen vegetables for antioxidant activity by using different oxidation systems. These vegetables include carrots, potatoes, sweet potatoes, red beets, cabbage, Brussels sprouts, broccoli, lettuce, spinach, onions, and tomatoes. In addition to the concise studies, which have used different methodologies to release bioactive compounds, it is becoming increasingly difficult to ignore advanced extraction methods, which have paved the way to extract bioactive compounds rapidly. Despite scientists' successes in showing the activity of vegetables' bioactive compounds, there is little known about the activity of the antioxidant components that have been isolated from these vegetables. Researchers have tended to focus on advanced methods to isolate and measure the activity of antioxidant compounds such as flavonoids, phenolic acids, tocopherols, carotenoids, and ascorbic acid(9).

- **Antioxidants from Fruits:**

Fruit consumption has also been linked to a reduction in the risk of many diseases. Peaches (*Prunus persica L.*) are an economically important fruit in many countries. Studies have shown that phenolic compounds found

within various peach genotypes are a major source of potential antioxidants. Interestingly, peaches have shown a great inhibition of low density lipoprotein (LDL) oxidation with a percentage of antioxidant activity of 56–87%. This antioxidant activity can be attributed to its essential compound content including hydroxycinnamic acids, chlorogenic, and neochlorogenic acids, but not to carotenoids such as b-carotene and b-cryptoxanthin. Moreover, low antioxidant activity was found in peach peel. In contrast, Plumb et al. pointed out that hydroxycinnamic acids do not contribute to the inhibition of lipid peroxidation of the liver using plums and peaches because hydroxycinnamic acids had weak ability to scavenge hydroxyl radicals. Grape (*Vitis vinifera L.*) is a fruit crop grown throughout the world. Grapes and its juices have been recently studied by. Phenolic compounds were high in both fresh grapes and commercial grape juices. The percentage of inhibition LDL oxidation was about 22% to 60% for fresh grapes, while it was approximately 68% to 75% for commercial grape juices, when standardized at 10 mg gallic acid equivalents (GAE). According to, both grapes and its juices exhibited high oxygen radical absorbance capacity (ORAC), and the anthocyanin pigment malvidin-3,5-diglucoside was a major compound isolated in grapes. Anthocyanins with malvidin nucleus malvidin 3-O-(6-O-p-coumaroylglucosido)-5-glucoside and phenolics were common compounds isolated from wild grapes (*Vitis coignetiae*). Wangensteen et al. tested the activity of many bioactive compounds by releasing them from grape pomace, and demonstrated that bioactive compounds have the ability to significantly inhibit LDL oxidation in the human body. Grape seeds are an amazing source of polyphenol compounds including monomerics such as catechin, epicatechin, and gallic acid, and polymerics such as procyanidins. Both polyphenols and carotenoids are the major phenolic compounds of apples (*Malus domestica L.*) including caffeic, quinic, and p-coumaric acids. These polyphenols can act as antioxidants. Flavanol monomers and oligomers, as well as quercetin, contribute to the beneficial health aspects of fruits and vegetables. Apple pomace has mainly been used as a major source of polyphenols such as chlorogenic acid. In addition phenolics like caffeic, p-coumaroyl quinic, arbutin, p-coumaric acids, and especially flavonol procyanidins have been mentioned as constituents of apple pomace. The ability of procyanidins to work as oxygen radical scavengers, superoxides, and hydroxyl radicals was estimated. Despite the low content in total phenols in apples obtained by using acetone 70%, it has shown strong antioxidant activities towards oxidation of linoleic acid. In this case, the major bioactive compounds obtained were chlorogenic acid and phloretin glycosides; however, Vitamin C was a minor fraction in apple juice. Antioxidant and antibacterial activities of various solvent (ethyl acetate, acetone, methanol, and water) extracts of Punica granatum peel were examined by applying the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method. The results obtained showed a significantly higher decreasing power in the methanol extracts and a significantly higher antibacterial activity in the acetone extracts. Soong and Barlow investigated the antioxidant activity and phenolic content of various fruit seeds. Petroleum ether was used to get rid of the excess fat from the seeds and extraction has been carried out with methanol. The 2,2-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS), DPPH, and the ferric reducing ability of plasma (FRAP) methods were used to investigate the antioxidant activity. Abdille et al. examined the antioxidant activity of *Dillenia indica* fruit using different kinds of solvents using DPPH, phospho-molybdenum, and β carotene bleaching methods . The methanol extracts showed the highest antioxidant activity, followed by the ethyl acetate and water extracts. Antioxidant activity of *Syzygium cumini* fruit in vitro has been investigated. Antioxidant activity was measured by DPPH, superoxide, lipid peroxidation, and hydroxyl radical scavenging activity methods. The results brought to light a significant correlation between the concentration of the extract and the percentage of inhibition of free radicals. The antioxidant property of the fruit might be from the presence of antioxidant vitamins, anthocyanins, phenolics, and tannins. It has been reported that blackberry (*Rubus fruticosus L.*) fruit extracts produced in varying climatic regions showed that antioxidant activity depended on the genotype, rather than the climate or season. Juntachote and Berghofer measured the stability of the antioxidant activity of ethanolic extracts for Holy basil and galangal using DPPH, superoxide, β carotene bleaching, reducing power, and iron chelation methods. They found higher antioxidant activity at neutral pH compared to an acidic pH. Holy basil and galangal extracts provided strong iron chelation activity, superoxide anion scavenging activity, and reducing power proportional to the concentration of the extracts. Liyana-Pathirana et al. investigated the antioxidant activity of cherry laurel fruit (*Laurocerasus officinalis Roem*) and its concentrated juice (Pekmez) using in vitro methods such as superoxide, DPPH scavenging activity, and inhibition of LDL oxidation [73]. The results confirmed the presence of a significantly higher antioxidant activity in pekmez compared to the cherry laurel fruit. Employing in vitro methods such as DPPH and superoxide scavenging activity, Orhan et al. measured the antioxidant activity of Arnebia *densiflora Ledeb* and observed that polar extracts had a higher

antioxidant activity compared to non-polar extracts. Rathee et al. studied the antioxidant activity of Mammea longifolia buds extracted in both methanol and aqueous ethanol. The results found a significant antioxidant activity, and the activity of aqueous ethanol was higher than methanol. The antioxidant activity of leaf extracts of Annona species in vitro reveals that *Annona muricata* possessed a higher antioxidant activity compared to *Annona squomosa(11,17,32)*.

- **Herbs as an Important Source of Antioxidants**:

The antioxidant activity of 32 herbs belonging to 21 different families has been screened. The finding confirmed that there was a positive correlation between the total antioxidant activity and total phenolic content. Lu and Yeap Foo studied *Salvia officinalis L.* for its antioxidant activity and polyphenol content and reported that rosmarinic acid and various catechols were responsible for the radical scavenging activity and caffeic acid was responsible for the xanthine oxidase inhibition. Zhao et al. investigated the antioxidant activity of Salvia miltiorrhiza and Panax notogensing. The results showed that Salvia miltiorrhiza had a higher reducing power and scavenging activities against free radicals, including superoxide and hydroxyl radicals, although it showed weak hydrogen peroxide scavenging. Furthermore, Javanmardi et al. tested the *Iranian Ocimum sp*. accessions to determine the antioxidant activities and total phenolic contents and demonstrated that the antioxidant activity increased in parallel with the total phenolic content. Evaluation of the pomegranate peel extracts to discover its antioxidant and antimutagenic activities using different solvents such as ethyl acetate, acetone, methanol and water has been carried out. Dried extracts were examined by using the Ames test and the phosphorus-molybdenum method to test both anti-mutagenic and antioxidant activities. The results showed the highest anti-mutagenic and the lowest antioxidant activity in the water extract. Moreover, the phenolic content and antioxidant activity of parsley (*Petroselinum crispum*) and cilantro (*Coriandrum sativum*) have been tested. The total phenolic content was observed to be different between parsley and cilantro leaves and stems, as well as methanol and water extracts. The methanol leaf extracts exhibited significant antioxidant activity towards both lipid- and water-soluble radicals. The works also investigated the antioxidant activity of aqueous plant extracts using in vitro methods such as DPPH scavenging activity and FRAP. The results revealed a strong correlation between total antioxidant activity and phenolic content and a weak correlation between cupric ion chelators and polyphenols. The antioxidant activity and lipid peroxidation inhibition of *Satureja montana L.* subsp. Kitaibelii extracts were tested using hydroxyl radical scavenging. The results obtained showed that there was a significant correlation with total phenolic content(33,39).

- **Antioxidant from Legumes**:

Antioxidant property of methanol extracts of Mucuna pruriens L. (Fabaceae) seed extracts has been investigated in vitro using the DPPH radical scavenging method. The results obtained showed a positive correlation between the antioxidant activity and the total phenolic compounds. Siddhuraju and Manian studied horsegram (*Macrotyloma uniflorum Lam.*) seeds to measure the antioxidant and free radical scavenging activity. Acetone extracts had a higher activity of about 70%. Samak et al. studied Wagatea sp. to measure its scavenging activities of superoxide and hydroxyl radicals and showed a high oxidation inhibition because it was rich in both phenolic and flavonoid contents. The authors also reported that bark and leaf extracts of Wagatea sp. exhibited high scavenging action against super radicals(42).

- **Antioxidants from Trees:**

Antioxidants from trees have been also measured. Phenolics from almond hulls (*Prunus amygdalus L.*) and pine sawdust (*Pinus pinaster L.)* have been extracted employing various methods in order to determine the gram fresh yield of polyphenol compounds and antioxidant activity. The antioxidant activity was measured by the DPPH radical scavenging method. The results showed that ethanol was most appropriate either for phenolics or any bioactive compounds, while methanol was more selective for extracting polyphenolics. The antioxidant activity of juniper (*Juniperus communes*) fruit extracts has been investigated in vitro. The results confirmed that both water and ethanol extract showed strong antioxidant activity. The concentration of 60 µg/mL of water and ethanol extracts exhibited 84% and 92% inhibition, respectively, on the peroxidation of linoleic acid. Ibrahim et al. studied the antioxidant activity of Cupressus sempervirens L., and set up goals to isolate quercetin, rutin, cupress flavone, caffeic acid, and para-coumaric acid. The results showed higher antioxidant activity related to quench DPPH and identified these active compounds successively. Higher values

of antioxidant activity have been obtained by using a methanolic solvent to extract the bioactive compounds from Anacardium occidentale, while other solvents like ethyl acetate gave lower values of antioxidant activity. Kaur et al. studied the *Chickrassy Chukrasia tabularis A.* Juss leaves to confirm its ability to inhibit lipid peroxidation and showed that there was a large inhibition considering its high content of phenolic compounds. Finally, *Acacia nilotica L.* antioxidant activity has been measured using ethyl acetate as a solvent to extract phenolic compounds. The results exhibited the highest antioxidant activity when the concentration of extracts was relatively high(2,7,9).

- **Antioxidant from Shrubs:**

Many shrubs have been shown to contain antioxidant activity. Singh et al. tested several plants to measure the antioxidant activity from different extracts. The antioxidant activity was determined by using peroxide value, thiobarbituric acid, DPPH radical scavenging activity, and reducing power. The results showed that the antioxidant activity of *Coriandrum sativum L.* and *Sarcolobus globosus L.* exhibited high activity by using acetone solvent, and its activity was similar to synthetic antioxidants. Eleven Algerian medicinal plants have been measured for phenolic compound content and antioxidant activity using the ABTS method. The tested plants showed antioxidant activity. Artemisia campestris L. had better antioxidant activity than caffeic acid and tocopherol. Moreover, HPLC analyses exhibited a good correlation between the antioxidant activity and hydroxycinnamic derivative content. Evaluation of Vitex negundo Linn seed antioxidant activity using different methods such as superoxide, hydroxyl, and DPPH scavenging activity has been carried out. The highest antioxidant activity was in both raw and dry heated seed extracts, while lower antioxidant activity was observed in the hydrothermally processed samples(44).

- **Characterization of Antioxidants from Other Eudicots**:

The nitric oxide and superoxide scavenging activity of green tea have been studied by Nakagawa and Yokozawa, who concluded that certain tannins had the ability to exhibit excellent antioxidant activity. Zin et al. estimated the antioxidant activity of the extracts from various parts of Mengkudu (*Morinda citrifolia L.*), including the leaves, fruits, and roots, using different solvents such as methanol and ethyl acetate. Ferric thiocyanate and thiobarbituric acid were used as models to observe and evaluate the antioxidant activity. The results exhibited a higher antioxidant activity in the methanol extract of Mengkudu root, although it was not significantly different from tocopherol and BHT extracts. The methanol extracts of the fruits and leaves showed unassuming activity. According to these scientists, the antioxidant activity in the roots resulted from polar and non-polar compounds, but the antioxidant activity in leaves and fruits was only due to non-polar compounds. Increase of the antioxidant activity of fennel (*Foeniculum vulgare*) seed extracts in vitro has been shown to be proportional to the increase in the concentration of extract. Nine other extracts of Bolivian plants have been measured for radical scavenging and antioxidant activity using the DPPH and β carotene bleaching methods. It was found that the ethyl acetate fractions had higher radical scavenging and antioxidant activity compared to the other extracts. It has been reported that the bioactive compounds of Rhodiola rosea extracted in methanol showed a significant yield of phenolics, about (153 ± 2 mg/g). Wangensteen et al. investigated the antioxidant activity of Ss globosus using DPPH scavenging and inhibition of lipoxygenase. Coriander had a high capacity to inhibit oxidation. There was also a positive correlation between total phenolics and antioxidant activity. Moreover, it was observed that the leaves of the coriander had higher antioxidant activity than the seeds. Antioxidant activity of Phyllanthus niruri was estimated using methanol and water as a solvent. The extracts of leaves and fruits exhibited high antioxidant activity by using the inhibition of lipid peroxidation and DPPH scavenging. The results also noticed a higher superoxide scavenging activity in the aqueous extract compared to the methanol extract. Moreover, the antioxidant and free radical scavenging activity of Phyllantus species from India in an aqueous extract has been also evaluated. The antioxidant activity was estimated using DPPH, β carotene, superoxide, nitric oxide scavenging, and reducing power methods. The extract of Coleus aromaticus exhibited a moderate inhibition on DPPH and nitric oxide scavenging activity(44). Panax exhibited strong iron chelating and weak superoxide scavenging. Ajila et al. carried out bioactive compounds and antioxidant activity of mango peel extract. The results showed a higher concentration of anthocyanins and carotenoids in the ripe peel compared to the raw peel, while the raw peel exhibited higher polyphenol content. The range of IC50 values of lipid peroxidation and DPPH were 1.39–5.24 µg of gallic acid equivalent. Chen and Yen investigated the antioxidant activity and free radical scavenging capacity of dried guava leaves and fruit. The results

confirmed that guava leaf and guava tea extracts had the ability to inhibit oxidation by 94–96% at a concentration of 100 µg/mL. Fruit extracts exhibited less activity compared to leaf extracts, while the scavenging effect increased with an increase in the concentration. Also, there was a correlation between antioxidant activity and phenolic compounds. Dastmalchi et al. investigated the chemical composition and antioxidant activity of water-soluble Moldavian balm (*Dracocephalum moldavica*) in vitro by using DPPH, ABTS, and superoxide activity. The finding confirmed that polar compounds such as caffeic acid and rosmaric acid were responsible for the antioxidant activity observed. Mulberry leaves were investigated to determine the antioxidant activity using different solvents. The procedure used DPPH and inhibition of lipid peroxidation methods to evaluate its activity. The results showed that the methanolic extract exhibited the highest yield of total phenolics, and it was the most essential antioxidant in all the methods used. The antioxidant activity of kale (*Brassica obraceae L.*) has been screened after removing a fat fraction from the samples. The extraction process used methanol to investigate its antioxidant activity while using DPPH scavenging activity as tested method. The works successfully isolated nine phenolic acids using HPLC and MS, and confirmed that the total phenolic content was correlated with DPPH scavenging activity. In another study, ethanol has been used to estimate the antioxidant activity of sun-dried cashew nuts (*Anacardium occidentale L.*) skin. First, bioactive compounds were extracted with a protocol including lipid peroxidation, ABTS, and DPPH methods to measure the capability to inhibit oxidation. The results found that epicatechin was the major polyphenol in the extract, which was responsible for antioxidant activity. Kaviarasan et al. measured the antioxidant and antiradical activity of fenugreek (Trigonella foenum ssp. graecum) seeds in vitro; the results showed that there was a positive relationship between the antiradical activity and phenolic compound content in the extract. Hexane and methanol were used to extract the bioactive compounds and measured the antioxidant activity of Pueraria tuberosa by using ABTS, lipid peroxidation, and superoxide and hydroxyl scavenging activity. An independent study has shown an inhibition of the lipid peroxidation. The rhizome of the lotus (*Nelumbo nucifera Gaertn.*) has been measured for its antioxidant activity in various solvent extracts using β Carotene bleaching and DPPH methods. Methanol extraction had a higher DPPH scavenging activity than acetone. Helichrysum pedunculatum has been tested to determine the antioxidant activity, and total phenolic and flavonoid content. The results demonstrated that whenever the amount of phenolic content and flavonoid content was increased, higher antioxidant activity was obtained. Meot-Duros and Magn screened the leaves of *Crithmum maritmum* to show if there was any correlation between the antioxidant activity and phenolic content and found a significant correlation between antioxidant activity and phenolic content when methanol was used as the solvent. Another dicot, *Tricholepis glaberrima L.* (Asteraceae), has been investigated for antioxidant activity using different kinds of extracts. Higher antioxidant activity was found by methanol, and a lower antioxidant activity in both chloroform and aqueous extracts. Sakat et al. investigated Oxalis corniculata L. in order to measure the antioxidant and anti-inflammatory activity employing methanol as a solvent. The IC 50 values of DPPH and nitric acid were about 93 and 73.07 µg/mL, respectively. Jain et al. studied Tabernaemontana divaricata L. to determine the phytochemical and free radical scavenging activities in vitro. The results indicated that the antioxidant activity was the same in both ethanol and water extracts, but less in petroleum ether. It has been reported that Ascleipiadaceae and Periplocoideae presented high antioxidant activity, with the presence of a strong correlation between antioxidant activity and phenolic content. Laitonjam and Kongbrailatpam studied the chemical composition and antioxidant activities of Smilax lanceafolia by isolating the flavonol glycoside and steroidal saponin, which showed high antioxidant activity. Spinach (*Spinacea olerace L.*) is among the most popular vegetables in the world. It was domesticated and first cultivated in West Asia. According to analytical chemistry, spinach is a source of violaxanthin and neoxanthin antioxidants that cannot be commercially produced. Although they may be present, pigments such as carotenoids can be masked by chlorophyll in greenish vegetables such as spinach. B-carotene, lutein, violaxanthin, and neoxanthin are the major carotenoids in raw spinach . Pumpkins belong to the family Cucurbitaceae. This family is classified depending on the texture and shape of stems, such as in Cucurbita pepo, Cucurbita moschata, Cucurbita maxima, and Cucurbita mixta. Nowadays, the market offers a wide variety of vegetables, with pumpkin being one of them because of its many applications for nutrition or decoration(31,37,43).

Test	Procedure	End Point
Detection of alkaloids		
Dragendroff's/ Kraut's test	Few mL filtrate + 1-2 mL Dragendorff's reagents	A reddish-brown precipitate
Hager's test	Few mL filtratea + 1-2 mL Hager's reagents	A creamy white precipitate
Mayer's/ Bertrand's/ Valser's test	Few mL filtratea + 1-2 drops of Mayer's reagent (Along the sides of test tube)	A creamy white/yellow precipitate
Wagner's test	Few mL filtratea + 1-2 drops of Wagner's reagent (Along the sides of test tube)	A brown/reddish precipitate
Picric acid test	Few mL filtratea + 3-4 drops of 2% picric acid solution	An orange colour
Iodine Test	3mL extract solution + few drops of iodine solution	A blue colour, which disappears on boiling and reappears on cooling
Bouchardat's test	6mL plant extract, evaporated completely + 6mL ethanol (60 °C) + few drops of Bouchardat's reagent (dilute iodine solution)	A reddish brown colour
Tannic acid test	Acidified extract + 10% tannic acid solution	A buff colour precipitate

Test	Procedure	End Point
Detection of Carbohydrates		
Barfoed's test	1mL filtrateb + 1mL Barfoed's reagent + Heated for 2 min.	A red precipitate {monosaccharides}
Molish's test	2mL filtrateb + 2 drops of alcoholic α–naphthol + 1mL conc. H_2SO_4 (along the sides of test tube)	A violet ring
Seliwanoff's Test	1mL extract solution + 3mL seliwanoff's reagent + heated on water bath for 1 min.	A rose red colour {ketoses}
Resorcinol test	2mL aq. extract solution + few crystals of resorcinol + equal volume of conc. HCl + heated	A rose colour {ketones}
Test for pentoses	2mL conc. HCl + little amount of phloroglucinol + equal amount of aqueous extract solution + heated over flame	A red colour
Test for starch	Aqueous extract + 5mL 5% KOH solution	A cinary colouration

Test	Procedure	End Point
Detection of Reducing sugars		
Benedict's test	0.5mL filtrateb + 0.5mL Benedict's reagent + Boiled for 2 min.	Green/yellow/red colour
Fehling's test	1mL each of Fehling's solution A & B + 1mL filtrateb + boiled in water bath	A red precipitate

Test	Procedure	End Point
Detection of Glycosides		
Borntrager's test	2mL filtrated hydrolysatec + 3mL Chloroform + shaken well + chloroform layer is separated + 10% Ammonia solution	A pink coloured solution
Modified Borntrager's test	Plant extract + ferric chloride solution + boil for 5min. + cooled + equal volome of benzene + benzene layer is separated + Ammonia solution	A rose-pink to blood red coloured solution
Legal's test	Dissolve 50gm plant extract in pyridine + Sodium nitroprusside + 10% Sodium hydroxide	A pink coloured solution
10% NaOH test	1mL dil. H2SO4 + 0.2mL extract + boiled for 15min. + allowed cooling + neutralize with 10% NaOH + 0.2mL Fehling's solution A & B	A brick red precipitate
Aqueous NaOH test	Alcoholic extract + dissolved in 1mL of water + few drops of aqueous NaOH solution	A yellow colour
Concentrate H2SO4 test	5ml plant extract + 2mL glacial acetic acid + a drop of 5% FeCL3 + conc. H2SO4	A brown ring
Raymond's test	Extract solution + dinitrobenzene in hot methanolic alkali	A violet colour

Test	Procedure	End Point
Detection of Cardiac Glycosides		
Keller-Killani test	1mL filtrate + 1.5mL glacial acetic acid + 1 drop of 5% ferric chloride + conc. H2SO4 (along the side of test tube)	A blue coloured solution (in acetic acid layer)
Kedee's test	4mL extract evaporated to dryness + 1-2 mL methanol + 1-2 mL alcoholic KOH + 3-4 drops of 1% alcoholic 3,5-dinitrobenzene + heated	A disappearing violet colour {Cardenolides}
Test for Cardenolides	Extract + pyridine + Sodium nitroprusside + 20% NaOH	A red colour, fades to brownish yellow
Bromine water test	Plant extract + few mL of bromine water	A yellow precipitate
Baljet test	2mL extract + a drop of Baljet's reagent	A yellow-orange colour

Test	Procedure	End Point
Detection of Proteins and Amino acids		
Biuret test	2mL filtrate + 1 drop of 2% copper sulphate sol. + 1mL of 95% ethanol + KOH pellets	A pink coloured sol. (in ethanolic layer)
Millon's test	2mL filtrate + few drops of Millon's reagent	A white precipitate
Ninhydrin test	2mL filtrate + 2 drops of Ninhydrin solution (10mg ninhydrin + 200mL acetone)	A purple coloured sol. {Amino acids}
Xanthoproteic test	Plant extract + Few drops of conc. Nitric acid	A yellow coloured sol.

Test	Procedure	End Point
Detection of Flavonoids		
Alkaline reagent test	1mL extract + 2mL of 2% NaOH solution (+ few drops dil. HCl)	An intense yellow colour, becomes colourless on addition of diluted acid
	Plant extract + 10% ammonium hydroxide sol.	A yellow fluorescence
Lead acetate test	1mL plant extract + few drops of 10% lead acetate solution	A yellow precipitate
Shinoda's test/ Mg hydrochloride reduction test	Plant extract is dissolved in 5mL alcohol + Fragments of magnesium ribbon + few drops of conc. HCl	A pink to crimson coloured solution {flavonal glycosides}
Shibata's reaction/ Cyanidin	1gm Aq. extract + dissolved in 1-2 mL 50% methanol by	A red colour {flavonols},

test	heating + metal magnesium + 5-6 drops of conc. HCl	orange colour {flavones}
Ferric chloride test	Extract aqueous solution + few drops 10% ferric chloride solution	A green precipitate
Pew's test	Few mL aqueous extract solution + 0.1gm metallic zinc + 8mL conc. H2SO4	A red colour {flavonols}
Zinc-hydrochloride reduction test	Plant extract + pinch of zinc dust + conc. HCl along the side of test tube	Magenta colour
Ammonia test	Filtrate + 5mL dil. Ammonia solution + conc. H2SO4	A yellow colour
Conc. H2SO4 test	Plant extract + conc. H2SO4	An orange colour

Test	Procedure	End Point
Detection of Phenolic compounds		
Iodine test	1mL extract + few drops of dil. Iodine sol	A transient red colour
Ferric chloride test	Extract aqueous solution + few drops 5% ferric chloride sol.	Dark green/bluish black colour
Gelatin test	Plant extract is dissolved in 5mL distilled water + 1% gelatin solution + 10% NaCl	A white precipitate
Lead acetate test	Plant extract is dissolved in 5mL distilled water + 3mL of 10% lead acetate sol.	A white precipitate
Ellagic Acid Test	Plant extract aqueous solution + 5% glacial acetic acid + 5% sodium nitrite solution	Solution turns muddy / Niger brown precipitate
Potassium dichromate test	Plant extract + few drops of potassium dichromate solution	A dark colour
Hot water test	Warm water in beaker + mature plant part is dipped + warmed for a min.	Black or brown colour ring at the junction of dipping
Test for Cartenoids	(1gm extract + 10mLchloroform, vigorously shaken and filtered). Filtrate + conc. H2SO4	A blue colour at the interface

Test	Procedure	End Point
Detection of Tannins		
Gelatin test	Plant extract is dissolved in 5mL distilled water + 1% gelatin solution + 10% NaCl	A white precipitate
Braymer's test	1mL filtrated + 3mL distilled water + 3 drops 10% Ferric chloride solution	Blue-green colour
10% NaOH test	0.4mL plant extract + 4mL 10% NaOH + shaken well	Formation of emulsion {Hydrolysable tannins}
Bromine water test	10 ml of bromine water + 0.5gm plant extract	Decoloration of bromine
Lead sub acetate test	1mL filtratee + 3 drops of lead sub acetate solution	A creamy gelatinous precipitate
Phenazone test	(5mL aq. extract + 0.5 g of sodium acid phosphate, heated, allowed to cool + filtered); filtrate + 2% solution of phenazone	Precipitation
Mitchell's test	Extract solution + iron + sodium tartarate (+ ammonium acetate solution)	A water-soluble iron-tannin complex, which is insoluble in solution of ammonium acetate

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