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Extraction methods, qualitative and quantitative techniques for screening of phytochemicals from plants

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Abstract
Phytochemicals are secondary metabolites which have different health benefits and with respect to plants, they possess color, aroma and flavor. There are different extraction methods of phytochemicals which have been used from the past and which are novel. Those novel techniques are very efficient and they will enable to extract large yields from small amount of plant material. Further, there are some techniques which can be used for both qualitative and quantitative measurements. Gas chromatography, liquid chromatography, high performance liquid chromatography and high performance thin layer chromatographyare some advanced techniques which can be used for quantitative analysis of phytochemicals. The aim of this study is to elaborate different extraction methods and different qualitative and quantitative techniques for screening phytochemicals from plant materials.

Keywords: Phytochemicals, qualitative, quantitative, analysis, chromatography

1. Introduction
Phytochemicals are naturally occurring substances found in plants which provides health benefits. These are known as secondary metabolites and may often be created by modified synthetic pathways from primary metabolite or share substrates of primary metabolite origin [5]. Alkaloids, flavonoids, tannins, phenolics, saponin, steroids, glycoside, terpenes and etc. They protect plants from disease and contribute for plant's color, aroma and flavor. Further, they have a role in protection of human health when their dietary intake is significant. Dietary phytochemicals are found in fruits, vegetables, legumes, whole grains, nuts, seeds, fungi, herbs and spices [13]. They have antioxidant, anti-inflammatory, anti-cancer and anti-bacterial properties [7]. The extraction procedures are vital important in analysis of phytochemicals. There are some traditional extraction methods and novel extraction methods. Maceration, percolation and soxhlet extraction methods are prominently used in phytochemical screening studies. But there are some advanced methods such as supercritical fluid extraction (SFE), microwave assisted (MAE), ultrasound-assisted extraction (UAE) and accelerated solvent extraction [2, 12].

2. Extraction methods
2.1 Maceration
The whole powdered material is allowed to contact with the solvent which is in a stoppered container for a particular time period with frequent agitation [13]. At the end of the process the solvent is drained off and the remaining miscella is removed from the plant material through pressing or centrifuging. Maceration is not an advanced technique since active ingredients cannot be totally extracted [12].

2.2 Percolation
A percolator which has a narrow cone shaped vessel open at both ends is used for this technique [7]. The plant material is moistened with the solvent and allowed to place in a percolation chamber. Then the plant material is rinsed with the solvent for several times until the active ingredient is extracted. The solvent can be used until its point of saturation [12].

2.3 Soxhlet extraction
This method is widely used when the desired compound has a limited solubility in the particular solvent and impurities are less soluble in the solvent.

The finely ground sample is placed ina porous bag or "thimble" which made out of filter paper or cellulose. The solvent which the desired compounds are going to extracted is kept in the round bottom flask [2].

2.4 Supercritical fluid extraction

Supercritical gases such as carbon dioxide, nitrogen, methane, ethane, ethylene, nitrous oxide, sulfur dioxide, propane, propylene, ammonia and sulfur hexafluoride are used to extract active ingredients. The plant material is kept in a vessel which filled with a gas under controlled conditions such as temperature and pressure. The active ingredients which dissolved in the gas separate when both temperature and pressure are lower [11]. The important factor of this technique is the mass transfer of the solute in the supercritical solvent. Generally, temperature and pressure has the biggest influence. However the effect of the pressure is more direct. As the pressure increases, higher densities are achieved by the supercritical fluid. Thus the density of the medium increases and the solubility of the solute will be increased. In order to get higher yields the process has to be optimized. Using response surface methodology the optimum parameters can be found [10].

2.5 Microwave assisted extraction

In this method microwave energy facilitate the separation of active ingredients from the plant material into the solvent. Microwaves possess electric and magnetic fields which are perpendicular to each other. The electric filed generates heat via dipolar rotation and ionic conduction. As high as the dielectric constant of the solvent, the resulting heating is fast. Unlike the classical methods, microwave assisted extraction heats the whole sample simultaneously. During the extraction, heat disrupts weak hydrogen bonds due to dipole rotation of molecules and the migration of dissolved ions increases the penetration of solvent in to the sample or matrix [6].

2.6 Ultrasound assisted extraction

This is an advanced technique which has the capability of extracting large amount of bioactive compounds within shorter extraction time. The main advantage of this technique is the increasing the penetration of solvent into the matrix due to disruption of cell walls produced by acoustical cavitations. And also this achieves at low temperatures and hence this is more suitable for extraction of thermally unstable compounds [8].

2.7 Accelerated solvent extraction

In accelerated solvent extraction technique, solvents are used at elevated temperatures and pressures to keep the solvent in liquid form during the extraction process. Due to elevated temperature the capacity of the solvent to solubilize the analytes increases and thus the diffusion rate increases. A further, higher temperature reduces the viscosity and the solvent can easily penetrate the pores of the matrix. The pressurized solvent enables more close contact with the analytes and solvent. However, this method uses less time and less amount of solvent for the extraction of active ingredients. The advantages of this method are extractions for sample sizes 1-100g in minutes, dramatic solvent reduction and wide range of applications and handling of acidic and alkaline matrices [9].

3. Qualitative techniques for the determination of phytochemicals

3.1 Alkaloids

Mayer's test

Two drops of Mayer's reagent are added along the sides of test tube in to few amount of plant extract. The presence of alkaloids is indicated by a white creamy precipitate [3].

Wagner's test

A few drops of Wagner's reagent are added to a few amount of plant extract and a reddish brown precipitate depicts the presence of alkaloids [3].

Dragendroff's test

The addition of few drops of Dragendroff's reagent into the extract gives red precipitate if alkaloids are present in the sample [14].

Hager's test

A small amount of Hager's reagent is added to the extract. The formation of yellow precipitate indicates the presence of alkaloids [14].

3.2 Carbohydrates

The 100 mg of extract is dissolved in 5 ml of distilled water and filtered [12].

Molish's test

Two drops of alcoholic solution of α–naphthol are added to 2 ml of filtrate and 1 ml of concentrated sulpuric acid is added slowly along the sides of test tube. A violet ring indicates the presence of carbohydrates.

Fehling's test

An equal volume of Fehling solution A and B are added to and equal volume of filtrate and it should boil in a water bath. The formation of red precipitate indicates the presence of sugar.

Barfoed's test

An equal volumes of filtrate and Barfoed's reagent are mixed and heat in a water bath. A red precipitate confirms the presence of sugar.

Benedict's test

A mixture of plant extract and the Benedict reagent is heated on water bath for 2 minutes and a characteristic colored precipitate indicates the presence of sugar.

For detection of glycosides, the plant extract is hydrolyzed with concentrated hydrochloric acid and the filtrate should be subjected to following tests.

Borntrager's test

A 2 ml of filtrate is mixed with 3 ml of chloroform and 10% ammonia is added to that. A pink color solution indicates the presence of glycosides.

Legal's test

The plant extract is dissolved in pyridine and sodium nitroprusside is added to that. Then the solution is made alkaline using 10% sodium hydroxide and pink color solution proves the presence of glycoside.

3.3 Saponins
The plant extract (50 mg) is diluted with distilled water up to 20 ml and this is shaken for 15 minutes in a graduated cylinder. The formation of 2 cm thick foam indicates the presence of saponins [3].

3.4 Proteins and amino acids
The plant extract is dissolved in 10 ml of distilled water and the filtrate is used for the following tests [12].

Millon's test
A few drops of Millon's reagent is added to 2 ml of filtrate. The white precipitate proves the presence of proteins.

Biuret test
One drop of 2% copper sulphate solution is added to 2 ml of filtrate. Then 1 ml of 95% ethanol is added following by excess of potassium hydroxide pellets. Pink color in ethanolic layer indicates the presence of proteins

Ninhydrin test
Two drops of ninhydrin solution are added to 2 ml of the filtrate and purple color proves the presence of amino acids.

Xanthoproteic test
The plant extract is treated with few drops of conc. Nitric acid. The formation of yellow color indicates the presence of proteins [14].

3.5 Flavonoids
Alkaline reagent test
A small amount of the extract is treated with few drops of sodium hydroxide and if the intense yellow color solution becomes colorless on addition of dilute acid proves the presence of flavonoids [13].

Lead acetate test
Extract is treated with few drops of lead acetate solution and the formation of yellow color solution indicates the presence of flavonoids [14].

Magnesium and hydrochloric acid reduction
A small amount of extract (50 mg) is dissolved in 5 ml of alcohol and few fragments of magnesium ribbon and few drops of concentrated hydrochloric acid is added. Any pink to crimson color development indicates the presence of flavanol glycosides [12].

3.6 Phytosterols
Libermann-Burchard's test
A small amount of the extract (50 mg) is dissolved in 2 ml of acetic anhydride and few drops of concentrated sulphuric acid is added. An array of color change shows the presence of phytosterols [12].

Salkowski's test
Extract is treated with chloroform and the filtrate of that is treated with few drops of acetic anhydride. Then the solution is boiled and cooled and the formation of brown ring at the junction indicates the presence of phytosterols [14].

3.7 Detection of phenols
The plant extract is treated with few drops of ferric chloride solution and the formation of bluish black color proves the presence of phenols [14].

3.8 Tannins
A few drops of 1% gelatin solution containing sodium chloride is added to the plant extract. The formation of white precipitate indicates the presence of tannins [14].

3.9 Diterpenes
Copper acetate test
The plant extract is dissolved in water and 3-4 drops of copper acetate solution. Formation of emerald green color indicates the presence of diterpenes [14].

4. Quantitative techniques
Chromatography techniques can be used for both qualitative as well as quantitative analysis. Gas chromatography, liquid chromatography, high performance liquid chromatography and high performance thin layer chromatography can be used for quantitative analysis.

4.1 Gas Chromatography (GC)
A mixture of volatile substances which are vaporizable without decomposition can be used in this technique. Gas chromatography coupled with mass spectrometry can be used for both qualitative and quantitative measurements [12]. The plant extract can be dissolved in methanol and filter with polymeric solid phase extraction column before analyzing for different components [11].

4.2 Liquid Chromatography (LC)
A low viscosity liquid is used as the mobile phase. The stationary phase bed may be comprised of an immiscible liquid coated onto a porous support and a thin film of liquid phase bonded to the surface of a sorbent or a sorbent of controlled pore size. There are several types of liquid chromatography such as reverse phase, high performance and size exclusion liquid chromatography [12].

4.3 High Performance Liquid Chromatography (HPLC)
The compounds or active ingredients are separated on the basis of their interaction with the solid particles of a tightly packed column and the solvent of the mobile. HPLC is useful for compounds that cannot be vaporized or that decompose under high temperatures. This provides both qualitative and quantitative measurements in a single operation. HPLC coupled with a UV photodiode array detector and mass spectrometer provides more structural information on the compounds [4].

4.4 High Performance Thin Layer Chromatography (HPTLC)
This is a planer chromatography where separation of the sample components is achieved on high performance layers with detection and data acquisition using an advanced work station. This is robust, rapid and efficient tool in quantitative analysis of compounds. Even though this is based on TLC, this comprises with several enhancements which intend to increase the resolution of the compounds to be separated and to allow quantitative analysis of the compounds. In this technique high quality TLC plates with finer particle sizes in the stationary phase which allow better solution. The separation of compounds can be improved by repeated development of the plate using a multiple development device [1].

5. Conclusion
Phytochemicals can be screened using different qualitative

techniques. But some advanced methods can be used to discover them qualitatively as well as quantitatively at once without performing several individual tests.

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