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Phytochemical Testing Methodologies and Principles for Preliminary Screening/ Qualitative Testing

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Authors' contributions

This work was carried out in collaboration among all authors. These authors contribute equally. All authors read and approved the final manuscript.

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Review Article

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ABSTRACT

Plants are basically biosynthetic factories that contain various pathways that produce phytochemicals with the help of different parts of the cell while specialized cells or tissues hoard and use them as needed. Human population has used this to their advantage for centuries.

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Aims: This review article aims to provide a comprehensive guide to qualitative phytochemical tests, focusing on their principles, procedures, and interpretation of results.
Scope of Review: This review covers qualitative tests for major phytochemical groups, including alkaloids, flavonoids, tannins, saponins, and phenols.
Methodology: Various qualitative tests are compiled and described, detailing the chemical principles underpinning each test and the expected outcomes for the identification of phytochemicals.
Summary of Findings: Qualitative phytochemical tests are highlighted for their simplicity, cost-effectiveness, and accessibility, making them valuable tools, particularly in resource-limited settings. These tests provide baseline information on phytochemical profiles and serve as initial screening tools that can lead to more detailed studies.
Conclusion: While advanced analytical techniques exist, qualitative methods remain essential for initial phytochemical screening due to their practical advantages. This review emphasizes the importance of these classical techniques in both research and practical applications.

Keywords: Phytochemicals; phytochemical screening; alkaloids, flavonoids; tannins; saponins; phenols.

1. INTRODUCTION

Phytochemicals, which translate to chemicals derived from plants, lack a universally accepted definition. In nutritional contexts, this term refers to bioactive compounds from plants that may offer health benefits [1]. Typically, phytochemicals are secondary metabolites in plants, produced by plant cells, but serving roles beyond the cell's basic needs, aiding the plant's overall survival. Most compounds have their natural pesticidal property, they can also add natural color or smell that acts as a signaling molecule and for interaction with other organisms. Some of these phytochemicals have pharmacological effects, others are very unpleasant in taste or highly toxic [2,3]. This would imply that the traditional knowledge of the phytochemical effects regarding enzymes, receptors, ion channels, and biological pathways is no longer tenable, as their broad pharmacological impact could not be reduced to an action on a single target. The activities of apparently unrelated proteins are modified by distinct phytochemicals, indicating there is one mechanism underlying the far-reaching effects of these compounds, which parallels their uses in traditional medicine [4].

The structure of plant cells, having cell walls, membranes, and chloroplasts, certainly supports phytochemical synthesis and storage. A number of plant tissues, such as parenchyma, collenchyma, and sclerenchyma, help in carrying out different functions and thus control the phytochemical distribution in the plant. It comes as no surprise that there exist main biosynthesis pathways for various phytochemicals to be generated, such as the shikimic acid pathway for phenolics, the mevalonate pathway for terpenes, and the flavonoid biosynthesis pathway. In contrast, the different plant parts—for example, leaves, stems, roots, flowers—each contribute to the overall phytochemical diversity and thus health-promoting potential. These include examples such as monoterpenes in the leaves of peppermint, oleoresin terpenes in conifers and polyketides in multicellular cavities of *Hypericum perforatum* [5].

Phytochemicals can be classified in various ways to better understand their diverse properties and potential health benefits. One common method is based on their chemical structure, dividing them into categories such as alkaloids, phenolics, terpenoids, glycosides, etc. Alternatively, phytochemicals can be classified by their biological activity, such as antioxidants, anti-inflammatory agents, antimicrobials, and anti-cancer agents, or based on the plants in which they are found, like cruciferous vegetables, citrus fruits, legumes, and spices [6,7]. Additionally, solubility offers another classification method, distinguishing between water-soluble and fat-soluble phytochemicals. Lastly, they can be grouped into functional groups, such as phenolic acids, flavonoids, and lignans [2].

The nature of the plant substance, type of solvent used, pH of the solvent, temperature conditions, the ratio of the volume of applied solvent to sample weight, and other factors are determinants in the medicinal plant extraction process. The intended use of the final product also leads through to the choice of extraction methodology. Other critical factors include accurate identification and authentication of the plant, timely collection of plant material, and pre-

extraction procedures, all of which impact the quality of the end product. Research indicates that storage conditions significantly affect the bioactivity of plant extracts, emphasizing the importance of proper storage post-extraction. The plants or plant parts, if necessary, undergo pre-treatment processes like air drying, microwave drying, or freeze drying before moving on to the actual extraction where maceration, infusion, digestion, decoction, percolation, Soxhlet extraction, microwave-assisted extraction, sonication, countercurrent extraction, and many other methods are applied according to the requirement. Subsequently, plant extracts are evaporated, and dry extracts are obtained to maintain the viability of the extract [8,9]. After the necessary material is obtained, one can proceed with the experiments, in this case, a phytochemical screening for detecting the presence of primary and secondary metabolites.

Phytochemical tests at the qualitative level are an important preliminary screening in a plant for the detection of bioactive compounds. They are cheap, fast, and easily carried out, hence making them worthwhile for preliminary studies as the major mode of detection will be color changes occurring (visible to the naked eye) due to the reactions among the constituents in the extract and added reagents. Such tests will be helpful in further studies, validation of traditional medicine, and basic quality control in drug development. This review aims to explore various qualitative chemical tests that can be employed to test for phytochemicals, dive into their principles, and understand the importance and effectiveness of such tests.

2. MAJOR CLASSES OF PHYTOCHEMICALS

2.1 Classification

Concerning the chemical structures, there are primary and secondary metabolites, which are normally used as methods to classify phytochemicals present in plants. The primary phytochemicals include carbohydrates, proteins/ amino acids, and fatty acids, all of which make up the structure and major functions of the plant. There are phytoconstituents that the plant does not necessarily need but produces to protect itself from foreign entities. They are often called secondary metabolites produced by several plants' pathways, which have many benefits for humankind. Secondary metabolites include alkaloids, polyphenols, terpenes, and sulfur-containing compounds taking into account that these are umbrella terms and there is no specific type of classification even with chemical structures [2,10].

2.2 Overview

Plants are natural factories for phytochemicals, which are compounds with bioactive properties. These compounds affect biochemical pathways to achieve specific results, such as interacting with biological membranes [4], boosting immunity, protecting against malignancies [11], and combating drug-resistant bacteria [12].

Plants produce secondary metabolites in response to external factors like infections or climate changes. These metabolites participate in the natural defense and confer color, aroma, and flavor to the plant. More than 4,000 phytochemicals have been identified, some of which have immunomodulatory activity. Research shows that antioxidants and phytochemicals can boost immunity in humans and animals. Many native plants studied for their immune-modulating abilities also have significant antioxidant properties. By reducing oxidative stress or increasing antioxidant levels, phytochemicals can help enhance immune development. Additionally, some phytochemicals activate immune cells, increase white blood cell count, and provide neuroprotection [3]. Some good examples of phytochemicals with proven specific defenses/effects include beta-carotene, which is chemopreventive; vitamins, which support immunity; morentenone and glutinol, which act as general analgesics; beta-sitosterol and stigmasterol, also for their analgesic properties; titanone and terpenoids, which are used for inflammatory disorders like arthritis; polyphenols, which are effective against Herpes simplex virus types 1 and 2; isoprenylflavones, which are effective against bacterial infections such as Strep; and tetrahydroxyflavones, which are effective against the bacteria Methicillin-resistant *Staphylococcus aureus* [13].

Nowadays, more and more consumers are resorting to herbal medicines and natural therapies because they have apprehensions. This is due to reactions against the side effects of modern medicines and the belief that herbal drugs are much safer, particularly as they have been in use for so many years. Fueling this further is an increased interest in alternative medicines among those who feel herbal medicines just may do what all other conventional therapies may have failed to do.

Another fact feeding this interest is the growing interest in preventive medicine on the rise, particularly with aging populations. This has led to a shift towards self-medication using herbal remedies. Consumers are also demanding higher quality, proof of efficacy, and assurance of safety in herbal remedies. The high cost of synthetic medicines is also another very significant contributory factor towards showing interest in herbal forms [14]. Patients undergoing conventional treatment for their various ailments become dependent on lifetime medications. Most of the medications have troublesome side effects and withdrawal symptoms when the medication is discontinued at a later stage [15].

Phytochemicals are of great variety and specific to the plant they come from. Sometimes, a phytochemical can singularly produce the desired effect, while most of the time, multiple compounds could be responsible for it, causing a synergistic effect [11]. Enhancing uptake of each other, synergistic boosting of antioxidant activity, targeting gut integrity, targeting signaling pathways, and targeting inflammatory markers are some proposed mechanisms of synergistic effect of phytochemical consumption. However, they may not always act this way. Antagonistic effects where one drug blocks or reduces the effect of another drug are also possible [1].

3. PHYTOCHEMICAL SCREENING

Preliminary phytochemical screening acts as a starting step for many applications. Pharmacognosy and drug discovery help identify bioactive compounds for new medicines [23,24]. In agriculture, it aids in developing natural biomaterials and improving crop productivity [25]. The food industry benefits by using natural antioxidants for preservation and enhancing nutritional value. Cosmetics utilize plant extracts for skincare and haircare products. Nutraceuticals develop dietary supplements and functional foods promoting health. Ethnobotany documents traditional medicinal knowledge and supports biodiversity conservation [13,26]. According to the WHO, more than 80% of the population in developing countries relies on traditional plant-based medicines, thereby merging traditional and conventional medicine into 'phytomedicines' [27].

For effective phytochemical research, accurately selecting and identifying plant samples is crucial to maintaining the study's integrity. Selection can be based on traditional uses, random or systematic collection in biodiverse areas, phylogenetically related species, or those with documented biological activities. Collected samples, ideally from the wild or herbariums, should be processed promptly to preserve secondary metabolites. Identification involves reviewing regional flora, field identification to the genus level, and verification by taxonomic experts. Post-collection, plants must be properly cleaned (washed, peeled, stripped) and dried immediately to prevent spoilage, ensuring the removal of water content for storage [8,27,28]. After identification, extraction and testing follows.

Table 1. Common phytochemical groups and their common roles

Phytochemicals	Role
Alkaloids	Antimicrobial and defense against herbivore attack, anti-inflammatory [8,16,17,18] Anticancer agents, glycosidase inhibitors, analgesic [2]
Phenolics, Tannins, Phlobatannins, Flavonoids	Antimicrobial, anti-inflammatory, antifungal, antiparasitic, antioxidant activity [2,4,16,17,18,19,20] anti-ulcer activity [21]
Carbohydrates, Glycosides	Primary metabolite; Plant development, Toxic to animals [2]
Amino acids/ Proteins	Primary metabolite; plant development [2]
Lipids, phytosterols	Primary metabolite [2], Suppresses different tumor cell lines through the induction of apoptosis and concomitant cell cycle arrest in the G1 phase [20]
Terpenes, saponins, carotenoids	Anti-tumour activity, enzyme activity, resistance to pathogens, antimicrobial, anti-inflammatory, and antiulcer agent [2,16,17,18,19,20,22]

3.1 Principles of Phytochemical Screening, Procedures and Results

3.1.1 Carbohydrates

3.1.1.1 Molisch test

Principle: The carbohydrate that is present in the plant undergoes a dehydration reaction when concentrated hydrochloric or sulphuric acid is added to it, forming an aldehyde. This undergoes a condensation reaction along with two phenol molecules (α-naphthol, resorcinol, or thymol), giving a reddish-purple colored ring [29].

Reagent required:

1. Molisch reagent: 1 mL
2. Conc. sulfuric acid

Procedure: After adding 1 mL of Molisch reagent (α-naphthol in ethanol) to 2 mL of aqueous extract, a few drops of strong sulfuric acid are gradually dripped in, and the mixture is gently agitated.

Results: The presence of carbohydrates in the aqueous extracts is shown by a violet ring at the interface between the two liquids (Fig. 2).

3.1.1.2 Benedict's test

Principle: On heating, reducing sugar in the presence of an alkali gets converted to an enediol, which is a comparatively strong reducing agent. Hence, in the presence of reducing sugars in the analyte, the cupric ions Cu^{2+} of Benedict's reagent are reduced to cuprous ions Cu+ Copper (I) oxide formed by this reduction precipitates out as a brick-red colored compound [29].

Reagents: Benedict's reagent: 5 mL

Reagent preparation: Benedict's reagent requires two solutions. Solution A is made by dissolving 173 g of sodium citrate and 100 g of sodium carbonate in 800 mL of water, then boiling the solution until it becomes clear. Solution B is prepared by dissolving 17.3 g of copper sulfate in 100 mL of distilled water [30].

Procedure: 1 mL of aqueous extract is added followed by 5 mL of Benedict's reagent. The mixture is then boiled for 5 min.

Results: When the mixture boils, a red, yellow, or green precipitate is produced. The solution first turns green (Fig. 3).

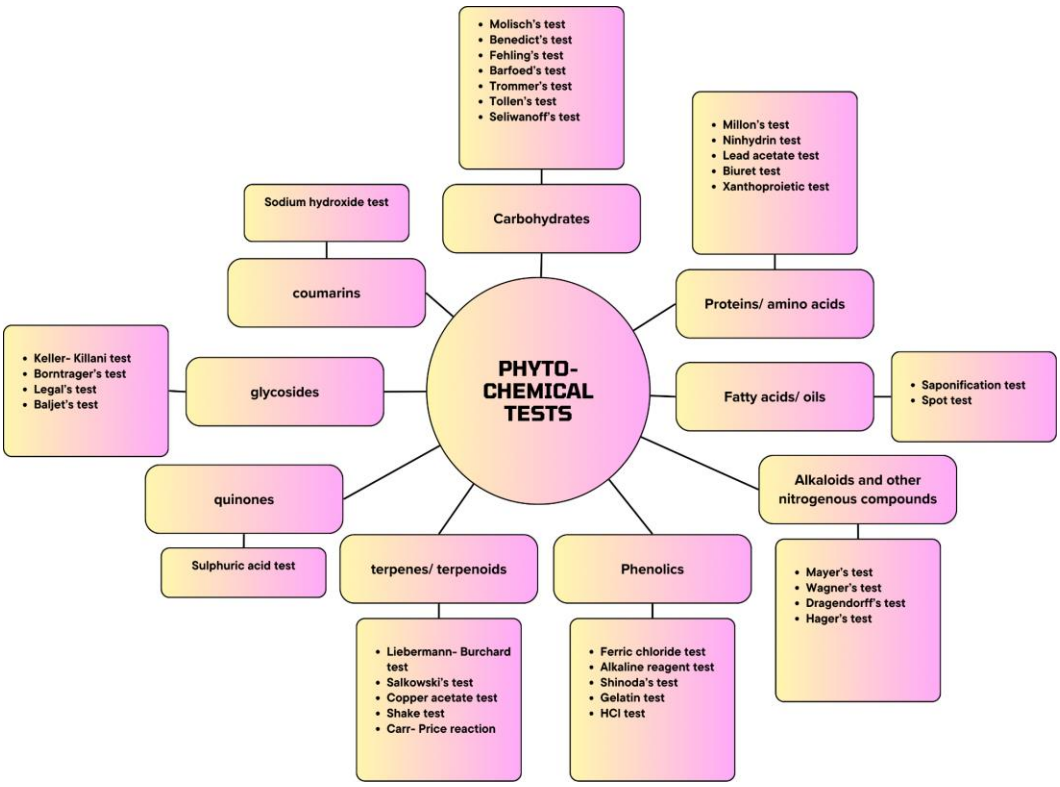


Fig. 1. Summary of all screening tests

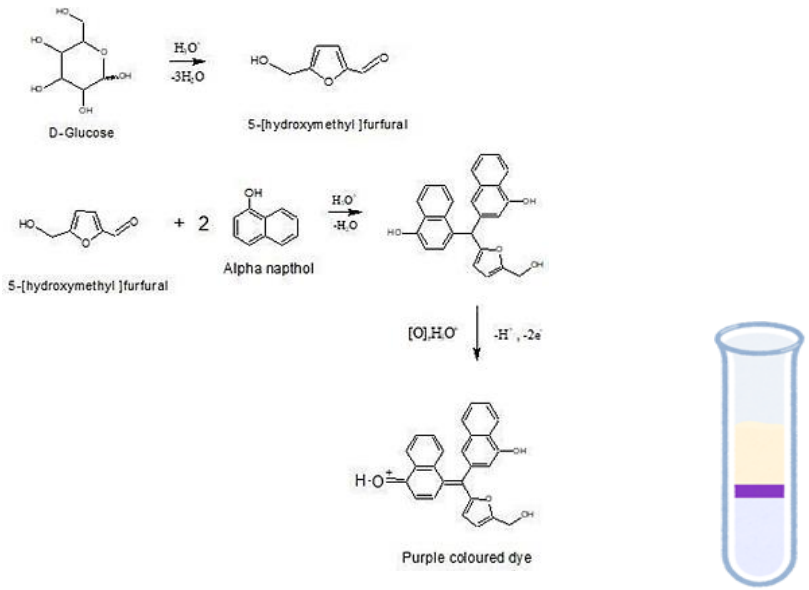


Fig. 2. Reaction and result - Molisch test

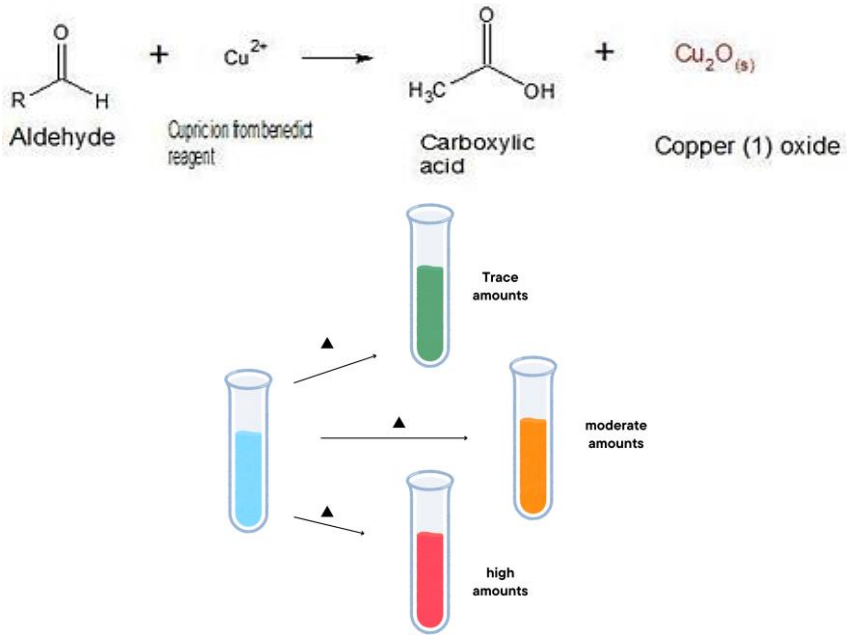


Fig. 3. Reaction and result - Benedict's test

3.1.1.3 Fehling's test

Principle: The Fehling's test essentially involves the reaction of the aldehyde group in a reducing sugar with Fehling's solution, comprising copper (II) sulfate, sodium hydroxide, and potassium sodium tartrate. In the presence of a reducing sugar, the aldehyde group is oxidized to a carboxylic acid, while the copper (II) ions in Fehling's solution are reduced to copper (I) oxide, a red-colored precipitate of Cu_2O [31].

Reagents:

1. Fehling A reagent
2. Fehling B Reagent

Reagent preparation: Fehling's reagent consists of two solutions. Solution A: Solution A contains

copper sulfate, 34.66 g in distilled water to a final volume of 100 mL. Solution B is prepared by mixing 173 g of potassium sodium tartrate and 50 g of sodium hydroxide in distilled water and adjusting the final volume to 100 mL [30].

Procedure: Procedure: Add 1 mL of Fehling's reagent, a mix of A and B, to 1 mL aliquot of test solution. These are shaken well, and the test tubes placed in a vigorously boiling water bath. Observe the formation of red preciptate of cuprous oxide indicating the presence of reducing sugars in the test solution.

Results: The positive response will be the development of a brown coloration (Fig. 4).

3.1.1.4 Barfoed test

Principle: The test detects reducing sugar by a series of chemical reactions. First, the cupric acetate is hydrolyzed into acetic acid and cupric hydroxide; then, this cupric hydroxide undergoes decomposition to form cupric oxide and water. In the presence of D-glucose, which is a reducing sugar, there will be an interaction with the cupric oxide. In this reaction, glucose is oxidized to gluconic acid (D-gluconic acid), while cupric oxide is reduced to cuprous oxide with the formation of a red precipitate. The appearance of this red precipitate indicates the presence of any reducing sugar in the given sample being tested [29,31].

Reagent: Barfoed's reagent: 3 mL

Reagent preparation: Barfoed's reagent is prepared by dissolving 30.5 g of copper acetate in 1.8 mL of glacial acetic acid [30].

Procedure: This test identifies the presence of reducing monosaccharides. To 1 mL aqueous extract add 3 mL Barfoed's reagent, boil for 2 min, and cool.

Results: A red precipitate is obtained(Fig. 5).

3.1.1.5 Trommer test

Principle: This test is based on the action of diluting alkalis on reducing sugars by NaOH. On heating, part of the copper hydroxide turns black due to the formation of copper oxide. On the addition of another portion $Cu(OH)_2$ to a little reducing sugar solution and heating to boil, a precipitate appears. The color varies from yellow to orange to red showing Cu_2O precipitate [29].

Reagents:

1. 2.5% copper sulphate
2. 2 mL of 5% sodium hydroxide

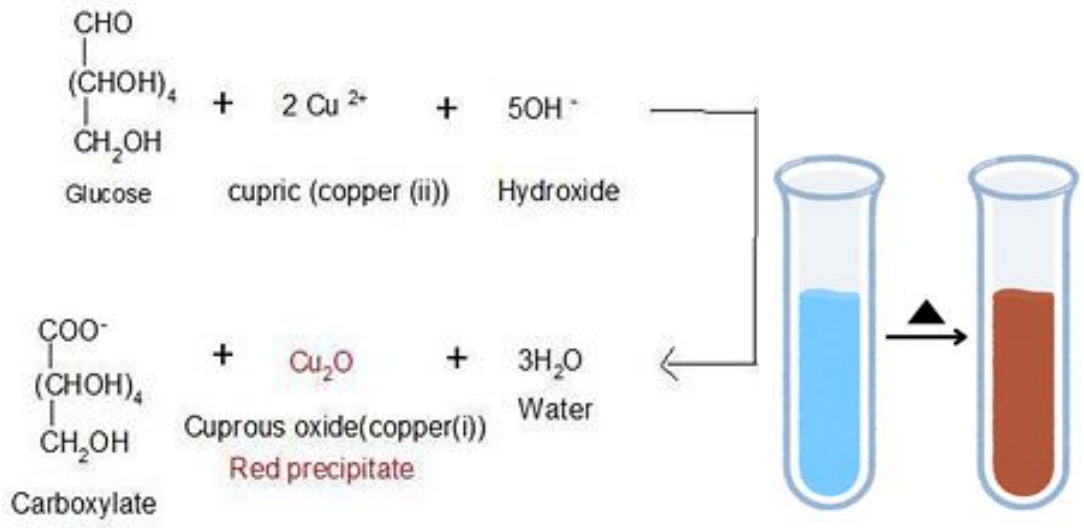
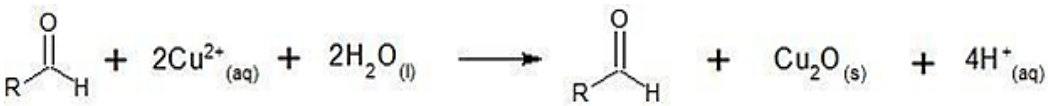


Fig. 4. Reaction and result - Fehling's test



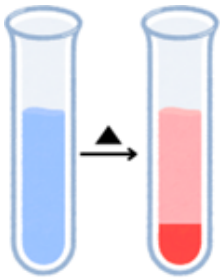


Fig. 5. Reaction and result - Barfoed's test

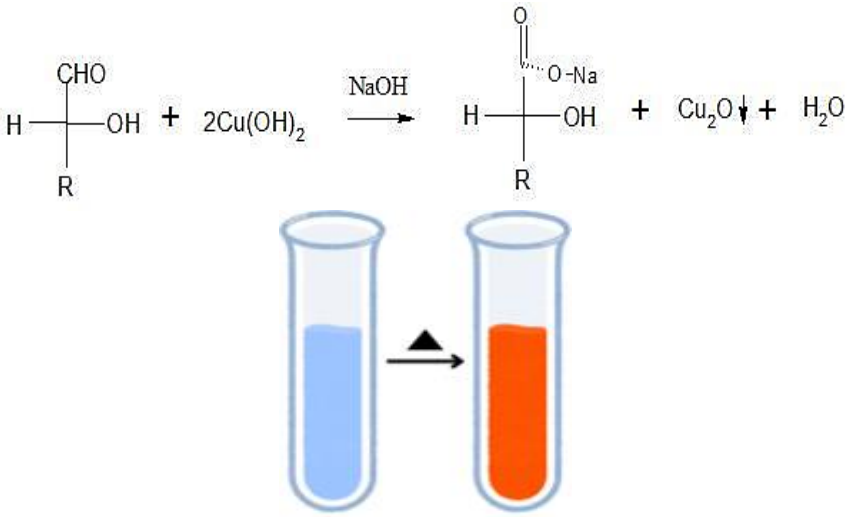


Fig. 6. Reaction and result - Trommer's test

Procedure: 3 mL of the aqueous extract, add one mL of 2.5 % copper sulphate and 2 mL of 5 % sodium hydroxide and boil for 3 min.

Results: First, there is the blue precipitate that becomes red when heated; hence, it would indicate the presence of reducing sugars (Fig. 6).

3.1.1.6 Tollen's test

Principle: Those reducing sugars which have at least one free aldehyde or ketone group reduce Tollens' reagent silver ions to metallic silver. As a result of this reduction in silver ions, a visible deposition in the form of a silver mirror or gray-black precipitate on the inner wall of the test tube occurs. The aldehyde group is oxidized to carboxylic acid, while the silver ions are reduced to metallic silver [32].

Reagents:

1. Dil. NH_4OH
2. 0.1 M silver nitrate

Procedure: Add a drop of dilute NH4OH to 4 mL of aqueous extract and add to the resulting solution of 0.1 M silver nitrate. Boil for 5-10 min.

Results: A silver mirror is formed (reduction of silver) (Fig. 7).

3.1.1.7 Seliwanoff's test

Principle: Keto sugars dehydrate and react with resorcinol under acidic conditions to form furfural derivatives that give rise to a red complex [31].

Reagents: Seliwanoff's reagent (resorcinol in hydrochloric acid).

Reagent preparation: Seliwanoff's reagent consists of 0.05 g of resorcinol dissolved in 100 mL of dilute hydrochloric acid [30].

Procedure: Add Seliwanoff's reagent to the sample solution. Heat in a boiling water bath.

Results: A red color indicates the presence of keto sugars, such as fructose (Fig. 8).

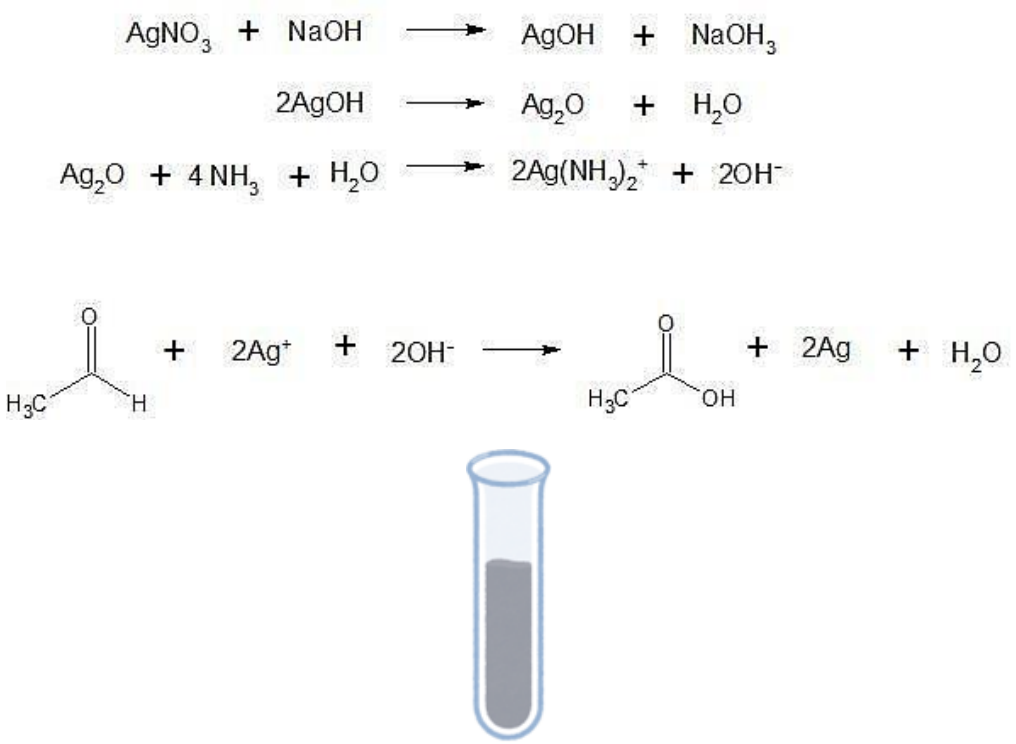


Fig. 7. Reaction and result - Tollen's test

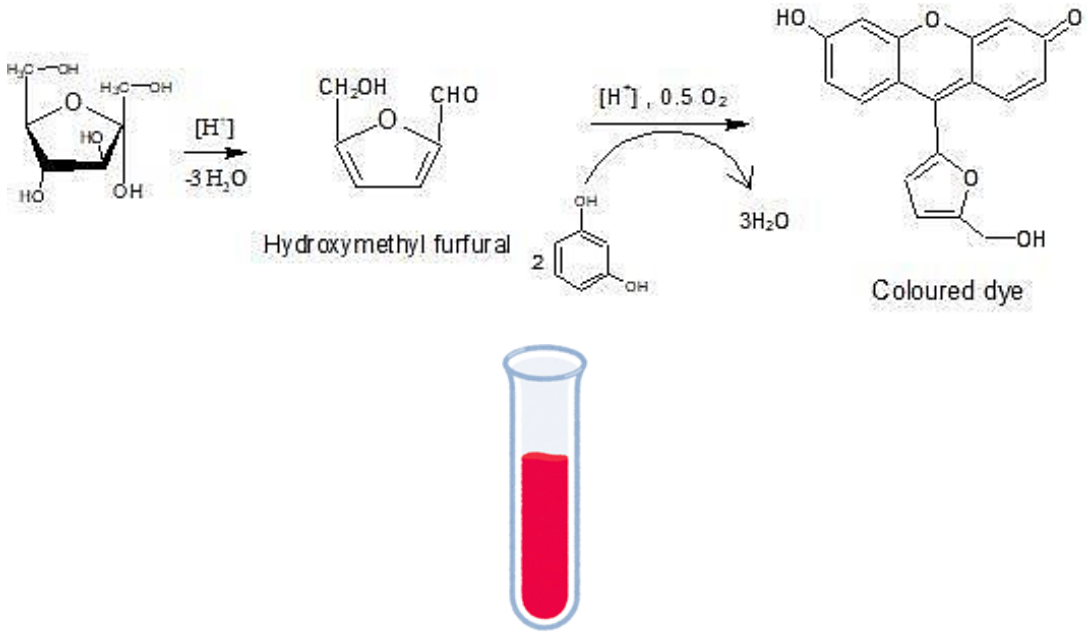


Fig. 8. Reaction and result - Seliwanoff's test

3.1.2 Amino acids and proteins

3.1.2.1 Millon's test

Principle: Millon's test is based on the principle of nitrating the phenol group of tyrosine and its subsequent complexing with heavy metals like mercury. Millon's reagent contains mercuric nitrate and mercurous nitrate dissolved in concentrated nitric acid. During the test, the phenol group on the tyrosine molecule gets nitrated because of the nitric acid present in the

reagent. The nitrated tyrosine reacts with the mercury ions in the solution, forming a red-colored precipitate or solution. The reaction of mercuric nitrate and proteins having tyrosine initially forms a white or yellow precipitate. Upon the addition of nitric acid and subsequent heating, this residue turns red. Both results constitute a positive test for tyrosine in the solution [29].

Reagents: Millon's reagent: A mixture of mercuric nitrate, mercurous nitrate, concentrated nitric acid, and distilled water.

Reagent preparation: 1 g of mercury is dissolved in 9 mL of fuming nitric acid. Equal quantity of distilled water is added to the reagent prepared after the reaction is complete [30].

Procedure:1 mL of the aqueous extract reacts with 5–6 drops of Millon's reagent. This should comprise a mixture of mercuric nitrate, mercurous nitrate, concentrated nitric acid and distilled water in known volumes.

Results: A white precipitate is obtained which turns red on heating (Fig. 9).

3.1.2.2 Ninhydrin test

Principle: Ninhydrin is applied during amino acid analysis as a strong oxidizer for the correct quantification of protein levels. Its major function is as a detector in liquid chromatography techniques, very often combined with post-column systems of derivatization using ninhydrin. The reaction of amino acids with ninhydrin causes decarboxylation and deamination of these compounds—formation of CO_2 and ammonia—and also formation of aldehyde, whose structure contains one carbon atom less. The ninhydrin is reduced to hydrindantin. The reduced ninhydrin condenses with ammonia and other non-reduced ninhydrin molecules to form a violet-blue condensation product called Ruhemann's purple. The absorbance of this complex will be read later on at 570 nm using the spectrophotometer [33].

Reagents: Ninhydrin (in acetone).

Procedure: Mix 3 mL of the extract with three drops of ninhydrin.

Results: A purple or blue coloration indicates a positive reaction (Fig. 10).

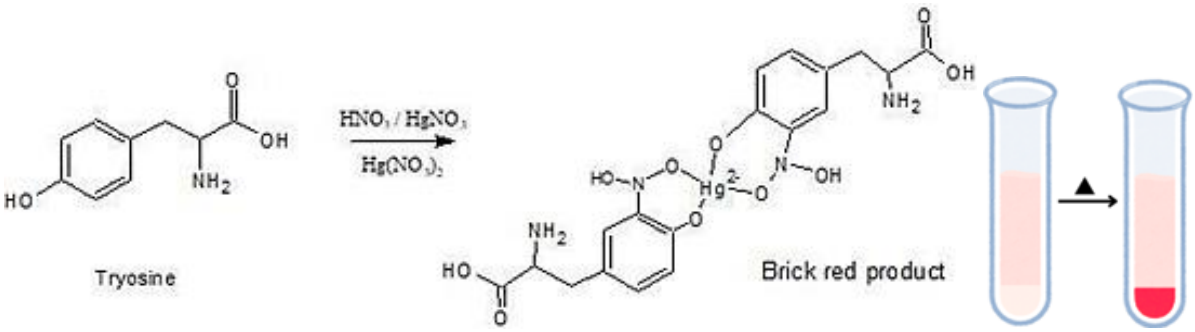


Fig. 9. Reaction and result - Millon's test

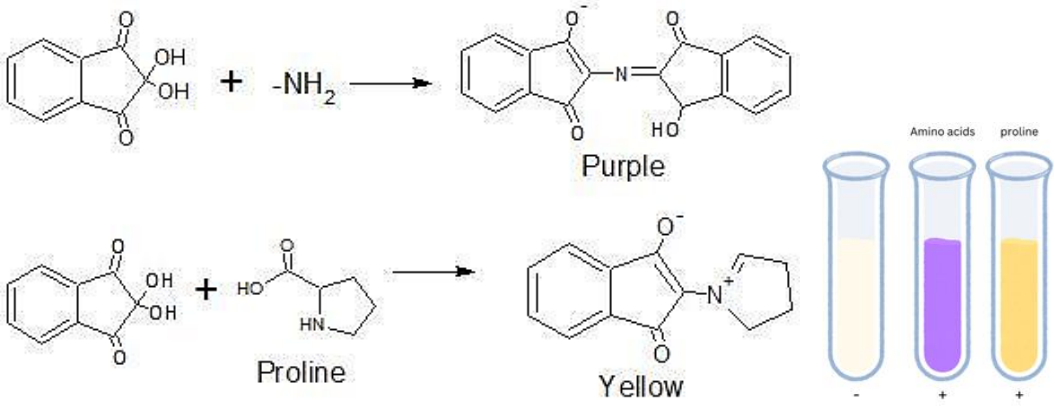


Fig. 10. Reaction and result - Ninhydrin test

3.1.2.3 Test for cysteine: Lead acetate test

Principle: The test detects the sulfur in a solution following the breaking of either S-H or S-S groups of amino acids under strongly alkaline conditions. Amino acids such as cysteine and cystine release sulfur when treated with strong alkalis at raised temperatures. The released sulfur reacts with the alkali, NaOH, to eventually form Na_2S. This sodium sulfide, Na_2S, reacts with the added lead acetate to form lead sulfide, which precipitates black [29].

Reagents:

1. 40% NaOH
2. 5% lead acetate

Procedure: Boil 5 mL of the aqueous extraction with a little 40% NaOH and add a few drops of 5% lead acetate solution.

Results: A black precipitate is formed(Fig. 11).

3.1.2.4 Biuret test

Principle: Biuret test is an example of a colorimetric reaction whose color changes from blue to purple or violet due to the action of an alkaline environment. Copper ions in the biuret reagent bind with the nitrogen atoms in the peptide bonds of proteins in the presence of an alkaline environment to yield a violet-colored copper coordination complex. The color purple indicates the presence of peptide bonds in the sample, and its intensity is related to their concentration in the solution [29].

Reagents:

1. 4% sodium hydroxide solution: 3 mL
2. 1% copper sulfate

Procedure: Add 3 mL of aqueous extract to 3 mL of 4% sodium hydroxide solution with a few drops of 1% copper sulfate.

Results: A purple solution(Fig. 12).

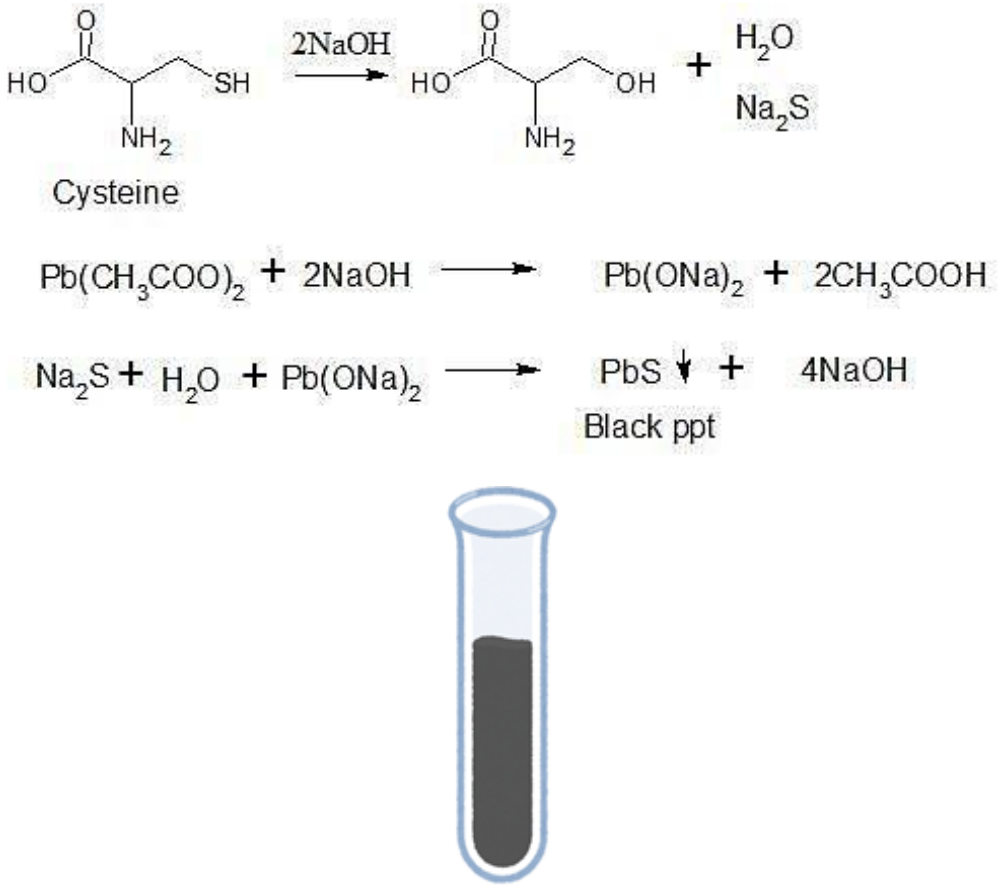


Fig. 11. Reaction and result - Lead acetate test

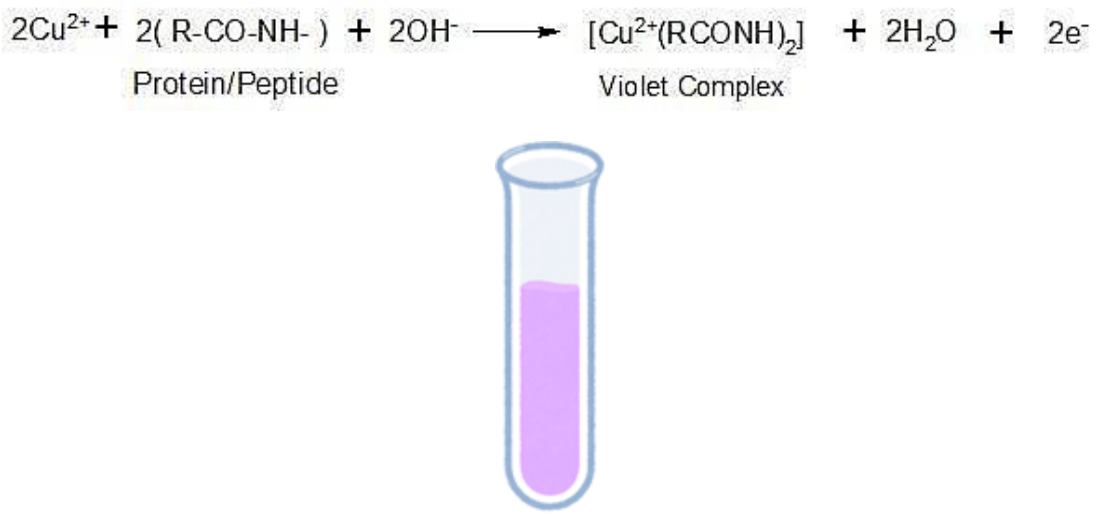


Fig. 12. Reaction and result - Biuret test

3.1.2.5 Xanthoproteic test

Principle: The Xanthoproteic test utilizes a nitration reaction in detecting the presence of proteins in a solution. On treatment with hot, concentrated nitric acid, the sample reacts with aromatic amino acids such as Phenylalanine, Tyrosine, and Tryptophan to form a yellow product called Xanthoprotein. The addition of a strong base, for instance NH_3 or NaOH, will turn this product further into deep orange coloring. Thus, this test can be used to demonstrate the presence of proteins containing aromatic amino acids in their side chains [29,33].

Reagents:

1. conc.H_2SO_4 – 1 mL
2. NH_4OH – 1 mL

Procedure: To 3 mL aqueous extract, 1 mL of concentrated H_2SO_4 is slowly dropped. A white precipitate appears that turns yellow upon boiling and orange after 1 mL of NH_4OH solution is added.

Result: A white precipitate is formed that turns yellow when the mixture is boiled and orange when 1 mL of NH_4OH solution is added (Fig. 13).

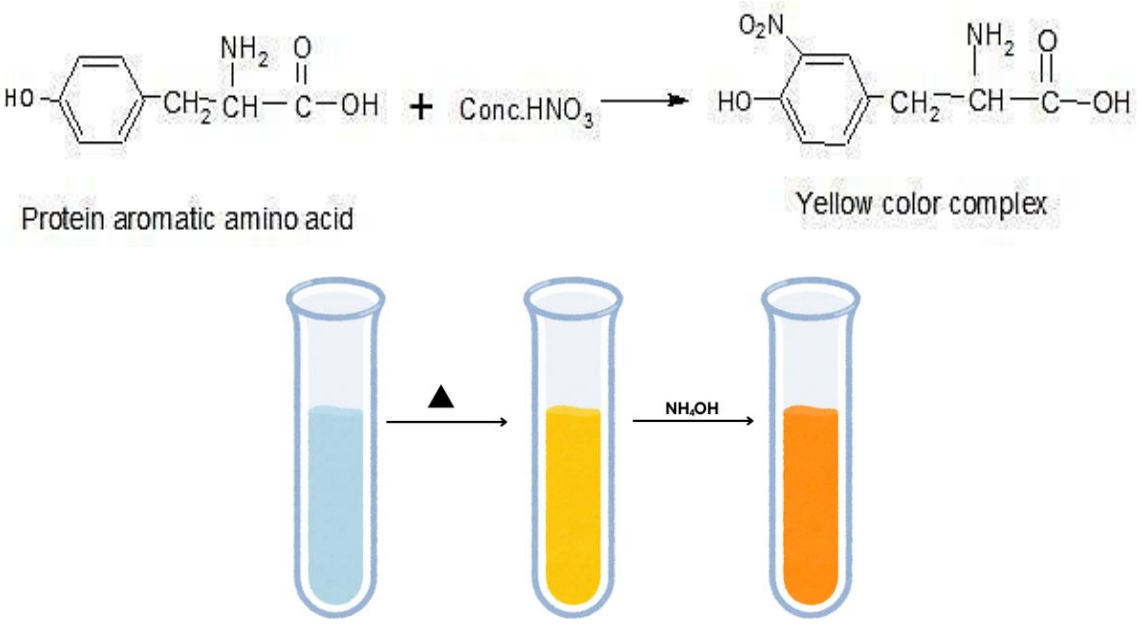


Fig. 13. Reaction and result - Xanthoproteic test

3.1.3 Glycosides

3.1.3.1 Keller-Killiani test: cardiac glycosides

Principle: Digitoxose transforms into furfurol in acids, which gives a blue color to acetic acid. The brown ring on the surface of the two phases is the reaction of the terpene skeleton [34]. The Keller- Kiliani test is based on the following reaction; the ferric ions (Fe^{3+}) and the deoxy sugars in cardiac glycosides, forming a colored complex. The presence of glacial acetic acid helps to stabilize the glycoside, while the addition of sulfuric acid causes the reaction to produce distinct color changes at the interface and in the upper layer, indicating a positive result for cardiac glycosides.

Reagents:

1. Glacial acetic acid
2. 5% $FeCl_3$
3. Concentrated H_2SO_4

Procedure: Add 5 mL of the aqueous extract, 2 mL of glacial acetic acid, one drop of $FeCl_3$ solution, and 1 mL of concentrated H_2SO_4.

Results: a brown ring forms; often a purple ring forms below the brown ring (Fig. 14).

3.1.3.2 Borntrager test: anthraquinonic glycosides / anthraquinones

Principle: The anthraquinone glycosides are hydrolyzed in an acidic solution (such as HCl). This breaks the glycosidic bond and releases the aglycone (anthraquinone) and the sugar moiety. The released anthraquinones are extracted into an organic solvent (such as benzene or chloroform). The organic layer containing anthraquinones is then treated with an alkaline solution (like NaOH or NH_3). This reaction converts the anthraquinones into their corresponding anthraquinone salts, which are soluble in the aqueous phase [35].

Reagents:

1. H_2SO_4
2. Chloroform
3. Ammonia

Procedure: Add 2 mL H_2SO_4 to 2 mL of the aqueous extract and boil. Filter the solution. Add an equal volume of chloroform and shake vigorously. Two layers can be seen. Separate the organic layer and add ammonia.

Result: pinkish-red color develops on top aqueous layer if anthraquinonic glycosides are present (Fig. 15).

3.1.3.3 Legal's Test: cardiac glycosides

Principle: Legal's test for glycosides involves the hydrolysis of glycosides to release the aglycone part, which often contains an unsaturated lactone ring in the genin portion. This aglycone, once freed from the sugar moiety, reacts with sodium nitroprusside in an alkaline medium to form a colored complex. The unsaturated lactone ring in the genin portion of the aglycone is crucial for this reaction, as it participates in the formation of the colored product. The reaction results in a red or pink color, indicating the presence of glycosides in the sample, particularly cardenolides [36].

Reagents:

1. Pyridine
2. Sodium nitroprusside
3. Sodium hydroxide (NaOH)

Procedure: Prepare a sample solution by dissolving in pyridine. Add a few drops of sodium nitroprusside solution to the prepared sample solution. Make the solution alkaline by adding a few drops of NaOH solution.

Results: The presence of glycosides is indicated by the appearance of pink or red color (Fig. 16).

3.1.3.4 Baljet Test: cardiac glycosides

Principle: The Baljet test detects the presence of cardiac glycosides, in that these are able to develop colored complexes upon reaction with alkaline reagents. In carrying out the test, a sample containing the glycoside is added together with a reagent made of sodium picrate and sodium hydroxide. Cardiac glycosides containing a lactone ring system will merge with picrate solution in alkaline conditions to form an orange or reddish-orange complex. Color change occurs due to the interaction of alkaline conditions and picrate ions with the aglycone part of the glycoside. There is increased visibility of the reaction, which indicates the presence of cardiac glycosides [37].

Reagents:

1. Sodium picrate solution
2. Sodium hydroxide (NaOH) solution

Reagent preparation: Baljet's reagent is made by 95 mL of 1% picric acid mixed with 5 mL of 10% sodium hydroxide [30].

Procedure: Prepare the Baljet reagent by mixing equal portions of sodium picrate and sodium hydroxide. Add a few drops of the prepared Baljet reagent to the sample solution containing glycosides. Mix the solution well.

Results: Orange or reddish orange color indicates the presence of cardiac glycosides (Fig. 17).

Fig. 14. Result - Keller- Killani test

$$\text{Anthraquinone Glycoside} + HCl \rightarrow \text{Anthraquinone} + \text{Sugar}$$

$$\text{Anthraquinone} + NaOH \rightarrow \text{Anthraquinone Salt}$$

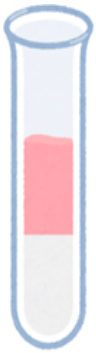


Fig. 15. Reaction and result - Borntrager's test

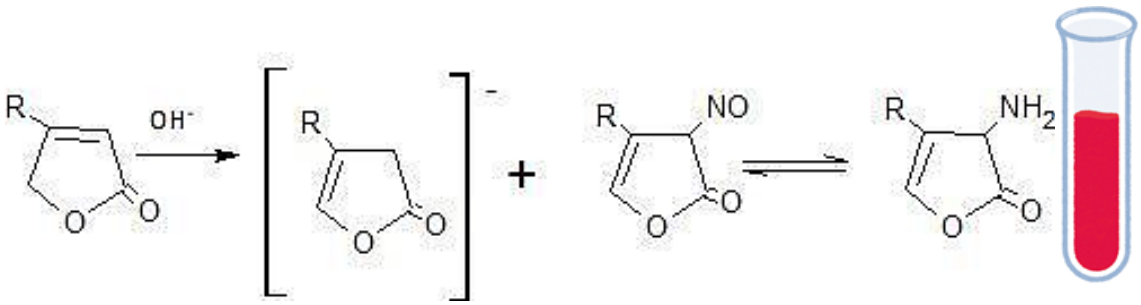


Fig.16. Reaction and result - Legal's test

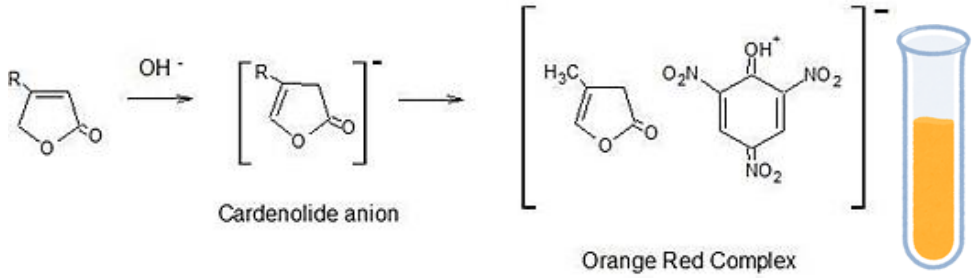


Fig. 17. Reaction and result - Baljet's test

3.1.4 Alkaloids

3.1.4.1 Mayer's test

Principle: A complex is formed between alkaloid and Mayer's reagent. Facilitation results from the fact that the nitrogen atom in the alkaloids has a lone pair of electrons which facilitates the formation of a coordinate covalent bond with a metal ion. In this case, it is the nitrogen atom of the alkaloid that forms a coordinate-covalent bond with the potassium ion of the Mayer's reagent, also known as potassium tetraiodomercurate. A brownish-yellow complex is formed which appears as precipitates in form. The formation of the precipitate confirms the presence of the alkaloid in the sample [38].

Reagents: Mayer's reagent

Reagent preparation: Mayer's reagent is a solution composed of two solutions: A and B. Solution A: 1.358 g of mercuric chloride is dissolved in 60 mL of distilled water. Solution B: 5 g of potassium iodide is dissolved in 10 mL of distilled water. Working solution: Mix Solution A and B and make up the volume to 100 mL with distilled water [30].

Procedure: Add 1-2 drops of Mayer's reagent to a few mL of the filtrate.

Results: A brownish-yellow complex that in the form of precipitates is formed. The formation of precipitates indicates the presence of alkaloids in the sample (Fig. 18).

3.1.4.2 Wagner's test

Principle: It will form a covalent coordinate with the nitrogen of the alkaloid, hence resulting in a complex precipitate of the potassium salt of an alkaloid [38].

Reagents: Wagner's reagent

Reagent preparation: In the preparation of Wagner's reagent, 1.27 g of iodine and 2 g of potassium iodide are dissolved in distilled water and the volume adjusted to 100 mL [30].

Procedure: Add reagent to the plant extract.

Results: Reddish brown precipitate (Fig. 19).

3.1.4.3 Dragendorff test

Principle: The hydrolysis reaction regulates the presence of Bi^{3+} ions in the solution. To achieve this, an acidic compound should be added to the solution. Additionally, the Bi^{3+} ions of bismuth nitrate can form a dark brownish precipitate of Bismuth (III) iodide with potassium iodide, The formed precipitate in excess potassium iodide dissolves to form potassium tetraiodobismuthate . Nitrogen forms a covalent coordination bond with the K+ ion [38].

Reagents: Dragendorff reagent

Reagent preparation: The stock solution is prepared by mixing 5.2 g of bismuth carbonate, 4 g of sodium iodide, and glacial acetic acid 50 mL. The solution is boiled for a few minutes. After 12 hours the precipitate crystals of sodium Acetate are filtered using a sintered glass funnel. The filtrate of 40 mL is added to 16 mL of ethyl acetate and 1 mL of distilled water and kept in an amber-colored glass bottle. A working solution can be prepared by mixing 10 mL of stock solution with 20 mL of acetic acid and enough distilled water added to make 100 mL [30].

Procedure: Add 1 mL of reagent to 2 mL of plant extract.

Results: A positive result is the formation of a brownish or yellowish precipitate, which is a complex compound of potassium alkaloid (Fig. 20).

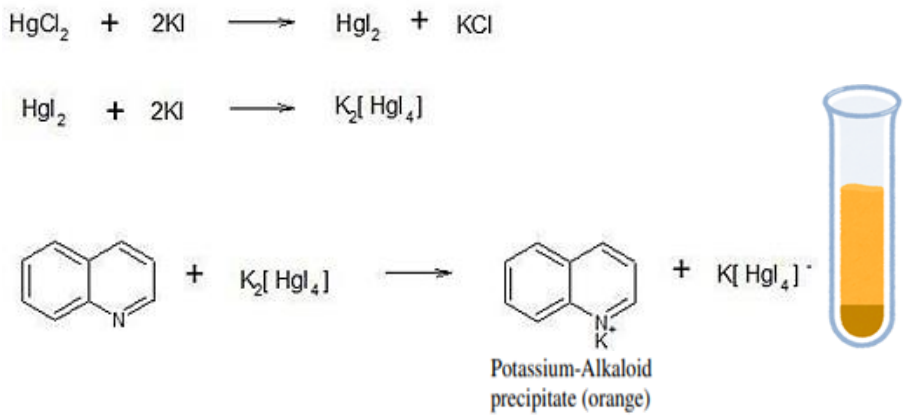


Fig.18. Reaction and result - Mayer's test

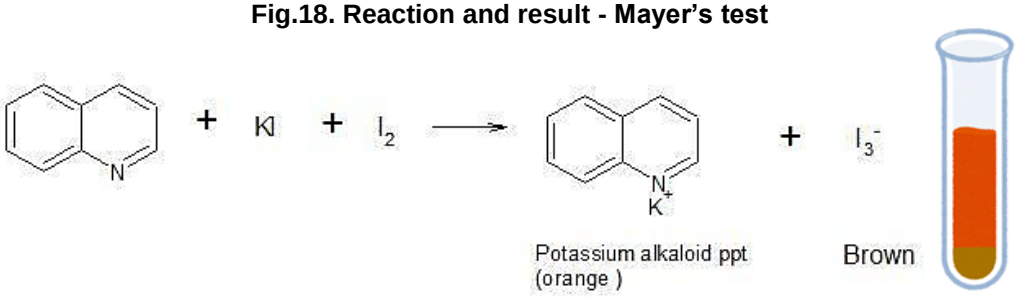


Fig. 19. Reaction and result - Wagner's test

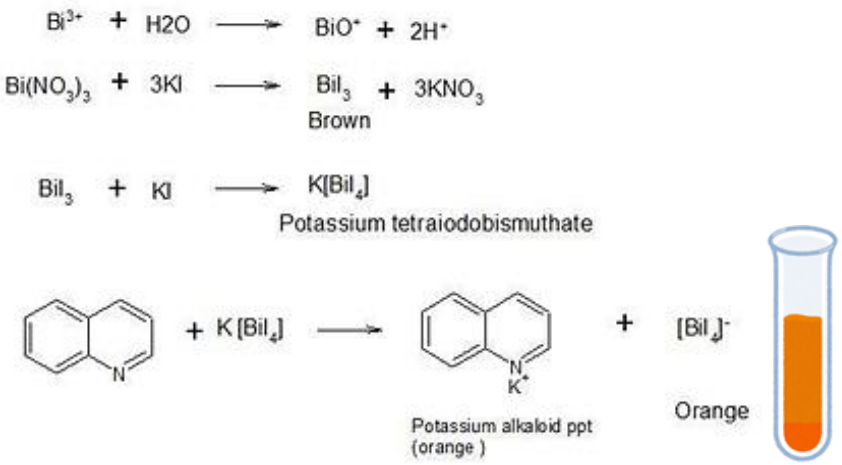


Fig. 20. Reaction and result - Dragendorff's test

3.1.4.4 Hager's test

Principle: formation precipitates when alkaloids react with a reagent known as, which is a solution of saturated picric acid [39].

Reagents: Hager's reagent

Reagent preparation: Reagent is a saturated aqueous solution of picric acid [30]

Procedure: When the reagent is added, the picric acid reacts with the alkaloids to form a precipitate of yellow or yellow-orange.

Results: yellow or yellow-orange precipitate (Fig. 21).

3.1.5 Phenolics and related phytochemicals

3.1.5.1 Ferric chloride test

Principle: Formation of a colored complex between ferric ions (Fe^{3+}) and phenolic hydroxyl

groups. The phenolic hydroxyl group reacts with ferric chloride to form a complex that exhibits a characteristic color change, depending on the specific phenol present in the sample [32,40].

Reagents: 5% Ferric chloride solution ($FeCl_3$).

Procedure: To 1 mL of the plant extract add 1-2 drops of 5% ferric chloride. Mix the contents of the test tube gently.

Results: The appearance of a color change, such as blue, green, purple, or red, indicates the presence of phenolic compounds like tannins (Fig. 22).

3.1.5.2 Alkaline reagent test

Principle: The test is related to the weakly acidic nature of the phenolic hydroxyl group. When phenols are treated with aqueous sodium hydroxide (NaOH) solution, they deprotonate to form phenoxide ions, resulting in a noticeable color change due to the formation of a more soluble and stable phenoxide compound. To confirm the presence of phenols, an acid such as hydrochloric acid (HCl) is added to the mixture. The added acid neutralizes the sodium hydroxide, converting the phenoxide ions back to phenol, which typically results in a color reversion or a different color change. This reformation of phenol from phenoxide ions solidifies the initial observation, ensuring that the color change observed was indeed due to the phenolic compound [36].

Reagents:

1. Sodium hydroxide (NaOH) solution
2. Diluted HCl

Procedure: Add NaOH solution followed by a few drops of diluted HCl to the sample.

Results: The indication of a deep yellow color is the presence of flavonoids. In addition to an acid, it turns colorless (Fig. 23).

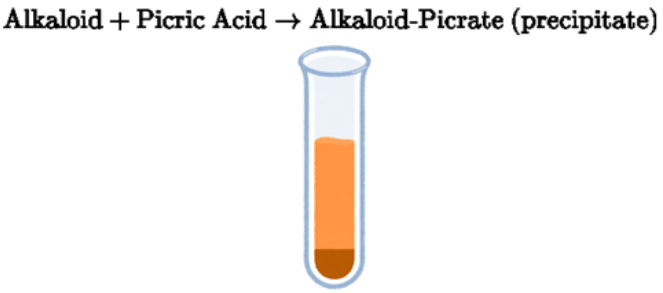


Fig. 21. Reaction and result - Hager's test

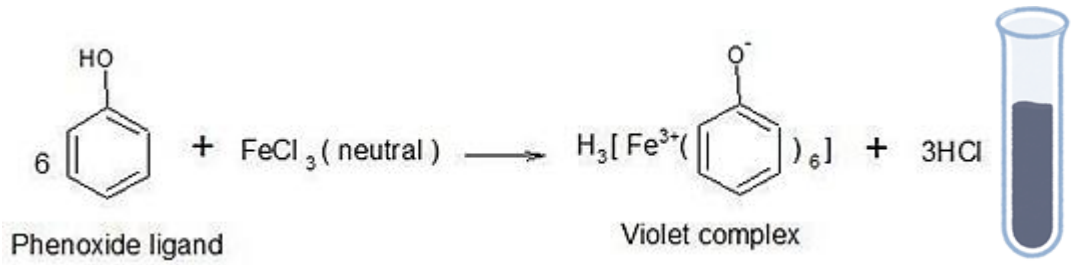


Fig. 22. Reaction and result - Ferric chloride test

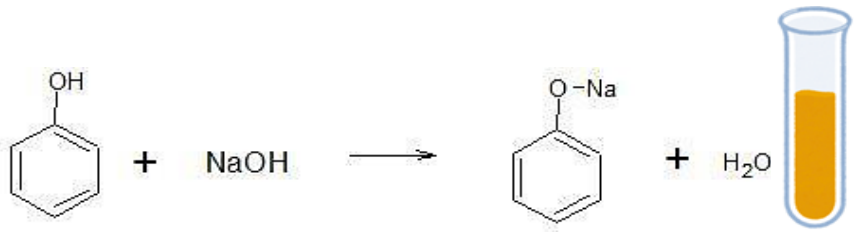


Fig. 23. Reaction and result - Alkaline reagent test

3.1.5.3 Shinoda's Test

Principle: The Shinoda test for flavonoids is based on reducing flavonoids by metallic magnesium in the presence of hydrochloric acid, resulting in the formation of colored complexes. When magnesium is introduced to a solution containing flavonoids and concentrated hydrochloric acid is added, the flavonoid compounds undergo a reduction reaction. This reduction converts the flavonoids into their corresponding aglycone forms (non-glycosylated flavonoids). The interaction between the reduced flavonoids, magnesium, and hydrochloric acid leads to the development of distinctive colors, typically ranging from pink to red, that indicate the presence of flavonoids in the tested sample. This is similar to Clemmenson's reduction [41].

Reagents:

1. Magnesium ribbon
2. Concentrated hydrochloric acid (HCl)

Procedure: Add a piece of magnesium ribbon to the sample. Add concentrated HCl.
Results: A positive result is a pink or red color (Fig. 24).

3.1.5.4 Gelatin test

Principle: Tannins are polyphenolic compounds that can form complexes with proteins like gelatin through hydrogen bonding and hydrophobic interactions. The formation of a precipitate or turbidity in the test tube indicates that tannins are present and have formed complexes with the gelatin. Adding NaCl facilitates precipitation [42,43].

Reagent:

1. Gelatin Solution: Prepare a 1-2% gelatin solution in distilled water.
2. NaCl

Procedure: Add a small quantity of the plant extract to a test tube and then add an equal volume of gelatin solution to it and add NaCl as well. Mix the contents gently. Allow it to stand for a few minutes.

Results: positive results are indicated by the formation of a precipitate or turbidity (Fig. 25).

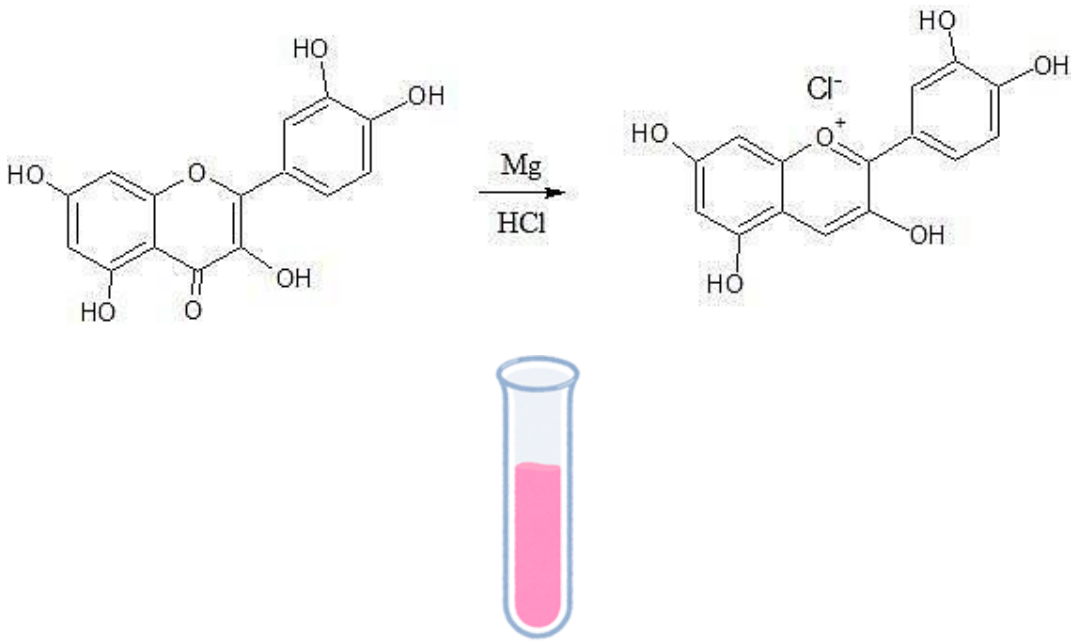


Fig. 24. Reaction and result - Shinoda's test

$$\text{Tannins} + \text{Gelatin} + \text{NaCl} \rightarrow \text{Tannin-Gelatin Complex (precipitate)}$$

Fig. 25. Reaction and result - Gelatin test

3.1.5.5 HCl test

Principle: Phlobatannins are an insoluble type of tannins in water, diluting acid but soluble in alkalis. When HCl is added to a solution containing phlobatannins, the phlobatannins react with the acid to form a red precipitate due to the presence of phenolic hydroxyl groups in the phlobatannins [44].

Reagents: Dilute Hydrochloric Acid (HCl).

Procedure: Add a small quantity of the plant extract in a test tube and then add a few drops of dilute hydrochloric acid (HCl) to the plant extract. Observe the color change or formation of a precipitate.

Results: The positive results indicate a red precipitate or a color change to red (Fig. 26).

3.1.6 Terpenoids, sterols and related phytochemicals:

3.1.6.1 Liebermann - Burchard test

Principle: Based on the reaction between sterols and acetic anhydride in the presence of sulfuric acid, which produces a series of color changes. The sterols undergo sulfonation and subsequent formation of conjugated dienes and trienes, resulting in characteristic color changes. The final deep green color is a positive indication of the presence of sterols or terpenes [45,46].

Reagents

1. Acetic anhydride
2. Concentrated sulfuric acid (H_2SO_4)
3. Chloroform

Procedure: To 1 mL of the chloroform solution of the sample add 2 mL of acetic anhydride and then carefully add (H_2SO_4) 1-2 drops of concentrated sulfuric acid to the mixture. Mix the contents of the test tube gently.

Results: A series of color changes will occur, typically starting from pink and progressing to blue, green, and then to deep green, indicating the presence of sterols such as cholesterol (Fig. 27).

3.1.6.2 Salkowski's test

Principle: Based on the reaction between sterols and concentrated sulfuric acid. When cholesterol or other sterols are treated with concentrated sulfuric acid, they undergo sulfonation, forming a colored product [47].

Reagents:

1. Concentrated sulfuric acid (H_2SO_4)
2. Chloroform

Procedure: To a dry test tube add about 2 mL of the chloroform solution of the sample and then Carefully add (H_2SO_4) 2 mL of concentrated sulfuric acid through the wall of the test tube to form a separate layer beneath the chloroform. Do not mix the contents; allow the layers to separate.

Results: At the interface, a red or reddish-brown color indicates the presence of sterols. The chloroform layer may also develop a green fluorescence (Fig. 28).

$$\text{Phlobatannins} + HCl \rightarrow \text{Polymerized phlobatannins (Red precipitate)}$$

Fig. 26. Reaction and result - HCl test

1. **Reaction with Acetic Anhydride:**

$$\text{Cholesterol} + \text{Acetic Anhydride} \rightarrow \text{Cholesterol Acetate (Intermediate)}$$

2. **Formation of Colored Complex:**

$$\text{Cholesterol Acetate} + H_2SO_4 \rightarrow \text{Colored Sulfonic Acid Derivative}$$

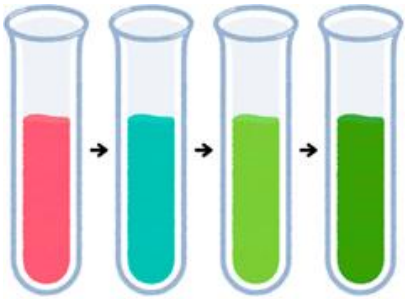


Fig. 27. Reaction and result - Liebermann- Burchard test

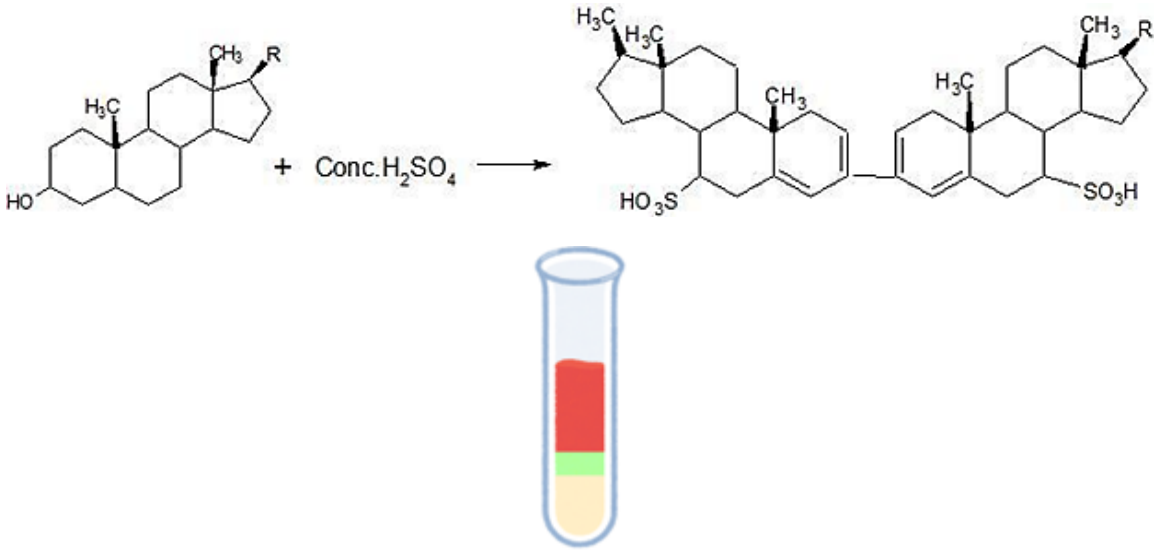


Fig.28. Reaction and result - Salkowski's test

3.1.6.3 Copper Acetate Test

Principle: The copper acetate test for detecting diterpenes is based on the formation of colored complexes between diterpenes and copper ions. Diterpenes are a class of chemical compounds composed of four isoprene units, and they readily react with copper ions to form stable complexes. In the test, when copper acetate solution is added to a sample containing diterpenes, the copper ions (Cu^{2+}) interact with the diterpene molecules. This interaction leads to the formation of a blue-green complex. The color change is due to the coordination between the copper ions and the diterpenes, which alters the electronic structure of the copper, resulting in a visible color indicative of the presence of diterpenes in the sample [29].

Reagents: Copper acetate solution.

Procedure: Add copper acetate solution to the sample in the test tube.

Results: A blue-green color indicates a positive result (Fig. 29).

3.1.6.4 Shake test

Principle: Saponins consist of polar groups, glycosyls, and steroids/triterpenoids as non-polar groups. Compounds containing both polar and nonpolar groups are surface-active compounds. If these compounds are shaken vigorously with water, saponins will form miscellanea. In the miscellanea, polar groups face out and non-polar groups face in. This phenomenon is known as foam [38].

Reagents:

1. Distilled water
2. Dry extract

Procedure: the extract will be reconstituted in 20 mL of distilled water. The graduated cylinder will be well-shaken for 15 minutes.

Results: The presence of saponinis indicated by a 2 cm layer offoam (Fig. 30).

3.1.6.5 Carr-Price Reaction

Principle: The Carr-Price reaction for carotenoids is based on forming a colored complex between carotenoids and antimony trichloride ($SbCl_3$) in an organic solvent like chloroform. Carotenoids, which possess highly conjugated double-bond systems, interact with $SbCl_3$ to form a stable complex. This interaction drastically changes the electronic structure of the carotenoids and usually ends up forming a blue or blue-green color. the color intensity formed will be with the concentration of carotenoids present in the sample, allowing for their qualitative and, to some extent, quantitative detection [48,49].

Reagents Needed: Antimony trichloride ($SbCl_3$) solution in chloroform.

Procedure: Add antimony trichloride solution to the sample.

Results: The formation of blue color gives out positive results (Fig. 31).

3.1.7 Coumarins

3.1.7.1 Sodium hydroxide test

Principle: The chemical principle behind the Sodium Hydroxide test for coumarins involves the alkaline hydrolysis of coumarins to form a yellow-colored product. Coumarins contain a lactone ring that is susceptible to nucleophilic attack by hydroxide ions (OH^-) in the presence of a strong base like sodium hydroxide (NaOH). This hydrolysis reaction breaks the lactone ring, forming a carboxylate anion and an aldehyde or ketone. The yellow color observed in the test is likely due to the forming of a conjugated system in the product, which absorbs light in the visible range and appears yellow [50].

Reagents: Sodium hydroxide (NaOH) solution.

Procedure: Add a small quantity of the sample suspected to contain coumarin in a test tube and then add several drops of sodium hydroxide solution to the test tube. Heat the mixture gently. Observe any color change under UV light.

Result: Positive results indicated by the formation of a yellow color fluorescence (Fig. 32).

$$C_{20}H_{32} + Cu(C_2H_3O_2)_2 \rightarrow [\text{Diterpene-Cu}]^{2+} + \text{Blue-green color}$$

Fig. 29. Reaction and result - copper acetate test

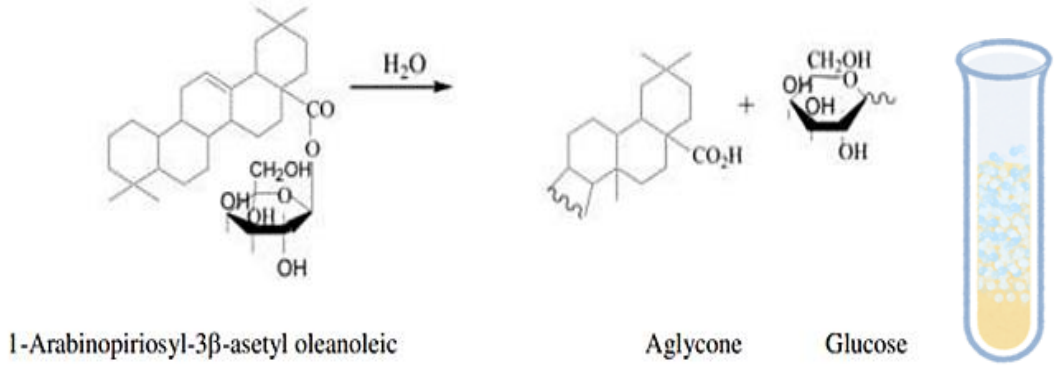


Fig. 30. Reaction and result - Shake test

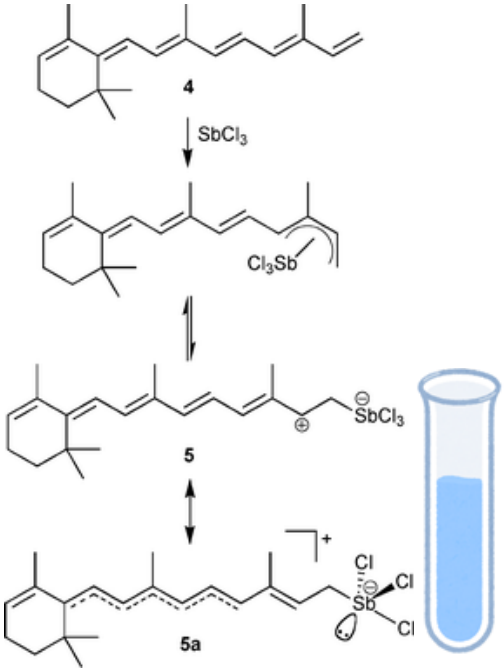


Fig. 31. Reaction and result - Carr-Price reaction

$$\text{Coumarin}(C_9H_6O_2) + NaOH \rightarrow \text{Carboxylate anion}(C_9H_5O^-) + \text{Aldehyde}(CH_2O)$$

Fig. 32. Reaction and result - Sodium hydroxide test

$$\text{Quinone} + H_2SO_4 \rightarrow \text{Protonated quinone} \rightarrow \text{Colored product}$$

Fig. 33. Reaction and result - Sulphuric acid test

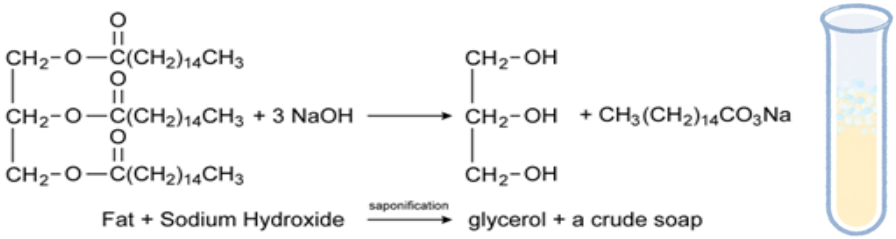


Fig. 34. Reaction and result - Saponification test

3.1.8 Quinones

3.1.8.1 Sulphuric acid test

Principle: The sulfuric acid test for quinones is based on their reaction with concentrated sulfuric acid, which results in the formation of colored products. When quinones are treated with sulfuric acid, they typically undergo protonation, leading to changes in their electronic structure and resulting in a color change. This color change, which can range from red to orange to brown depending on the specific quinone, serves as a qualitative indicator of their presence in a sample [51].

Reagents: Concentrated sulfuric acid (H_2SO_4).

Procedure: Add several drops of the sample solution to a clean, dry test tube and then add an equal volume of concentrated sulfuric acid cautiously to the tube. Mix gently the contents of the test tube either by swirling or with the aid of a glass stirrer. Observe any immediate color change in the solution.

Results: The color can range from red to orange to brown (Fig. 33).

3.1.9 Fixed oils

3.1.9.1 Saponification test

Principle: Reaction of fatty acids with alkalis (in this case, KOH) to form soap [29].

Reagents:

1. Alcoholic potassium hydroxide (KOH) solution
2. Suitable indicator (e.g., phenolphthalein)

Procedure: Dissolve the extract in a known volume of alcoholic KOH solution. Add an indicator and wait for two hours.
Result: Soap formation or partial neutralization of alkali (Fig.34).

3.1.9.2 Spot test

Principle: The principle behind the spot test for fixed oils on filter paper is based on the solubility of the oils in the solvent and their ability to leave a visible residue on the paper. Fixed oils are non-volatile oils that are liquid at room temperature and are generally non-drying. When a plant extract containing fixed oils is applied to the filter paper and dried the oils will be retained as visible spots [29].

Reagents/ materials: Filter paper

Procedure: Apply a small amount of the plant extract to a spot on the filter paper. Allow the spot to dry completely. Hold the filter paper up to the light and observe for any translucent or oily spots. Optionally, you can touch the spot gently with a clean, dry finger to feel for any oiliness.

Result: The presence of an oily or translucent spot indicates the presence of fixed oils.

4. ADVANCED TECHNIQUES IN PHYTOCHEMICAL SCREENING

The quantitative analysis of phytochemicals in the plant identifies and quantifies the phytochemicals present in the plant. Starting from the determination of total flavonoid content and total phenolic content to chromatographic analysis like high-performance liquid chromatography (HPLC), gas chromatography, and thin layer chromatography (TLC), there are varieties of methods that can be used to separate the compounds and quantify them, while spectroscopy methods such as ultraviolet (UV), infrared (IR), mass spectroscopy, nuclear magnetic resonance and X-ray crystallography can be used to detect and analyze the constituents [28,52].

Extensive research and careful usage would place compounds with leads that possibly can be in the preclinical screening model. In other words, extensive research and judicious use of preclinical screening models are capable of providing compounds with leads that will probably prove efficacious. The usage of mouse models for in vivo studies is the most available option [53]. If the drug passes these series of models and eventually ends as a developed drug, it must be tested with appropriate clinical trials with patients, which is tricky since most phytochemical compounds have barely passed due to several factors such as the time duration of the trial or lack of control groups. This makes the bioactivity of the compound a little questionable and probably not ready to be released. Repeated attempts at in vitro and in vivo experiments that reveal the essential molecular patterns and mechanisms are appreciated and will eventually support the quality of the compound [11].

5. DISCUSSION: CHALLENGES OF PHYTOCHEMICAL SCREENING

A significant limitation of the phytochemical analysis is that its accuracy in evaluating the medicinal utilities of a plant and identifying active compounds responsible for biological activities is highly dependent on the solvent used for extraction and the tests applied; thus, multiple tests are necessary to obtain more precise results [30]. Preliminary phytochemical screening has several limitations, since the solvent used predominantly determines the efficiency of extraction, and different solvents may lead to incomplete or biased results. Most probably, different tests can yield completely different results for the presence or absence of various phytochemicals, and several tests have to be carried out to reconfirm the results. Preliminary screenings are mostly qualitative and rarely enable the determination of exact concentrations of active compounds. Besides, some screening methods are not specific, thus resulting in false positive or negative results. Interference from a plant matrix can be an obstacle to the detection and identification of certain phytochemicals. Preliminary screenings only give an extent; hence they miss all putative phytochemicals, more so those of low concentrations, which require more complex analytical methods for determination. Lastly, results obtained from preliminary screenings are subjected to be challenging without further detailed studies and advanced analytical methods.

Table 2. Advanced techniques used in determination of phytochemicals

Method	Principle	Result
Phytochemical analysis (eg. TPC, TFC)	Colorimetric methods are based on the reaction of desired compounds with specific reagents to form a colored complex. The colored complex formed is then measured spectrophotometrically. The colored complex formed will have an intensity directly proportional to the concentration of these compounds in the sample [54].	Concentrations of such compounds in the sample, generally in standard equivalents.
HPLC	It is a separation technique of compounds based on their interaction with a stationary phase and liquid mobile phase under high pressure [28].	A chromatogram with peaks that correspond to different compounds in the sample. The area under each peak can be used in quantifying the compounds, and retention time helps in identifying them.
Gas Chromatography	Separation of the volatile compounds is based on their distribution between the stationary phase and the gas-like mobile phase [28].	A chromatogram containing peaks of volatile compounds in a sample. As is the case with HPLC, quantification and identification are based on peak area and retention time.
TLC	Compounds are separated based on their movement over a stationary phase layer (usually silica), under the influence of sparingly diffusing solvents [28].	Spots on a TLC plate corresponding to different compounds. Their Rf value, which is the distance each travels compared to the solvent front, serves for their identification qualitatively. Sometimes visualized under UV light or with chemical reagents.
UV Spectroscopy	It is the measure of a sample's ability to absorb UV light, correlating to the concentration of certain compounds, based on Beer-Lambert law [28].	An absorption spectrum showing the amount of UV light that is absorbed by the sample at a range of different wavelengths. The absorbance will allow quantification of compounds at specific wavelengths based on Beer-Lambert's law.
IR Spectroscopy	Molecular vibrations are identified by measuring the absorption of infrared light, owing information about the functional groups present in the sample [28].	IR spectrum of absorption bands due to different molecular vibrations. Such absorption bands indicate the presence of specific functional groups in a molecule.
Mass Spectroscopy	It involves ionization of the compounds into charged particles and the subsequent determination of their mass-to-charge ratio for purposes of identification and quantification [28].	Mass spectrum corresponding to ions differing in mass-to-charge ratios (m/z). The pattern formed by peaks is indicative of the molecular weight and structure of a compound.
Nuclear magnetic resonance	It is a spectroscopic technique applied for the detection of magnetic properties exhibited by some atomic nuclei, like hydrogen, within a magnetic field. This provides very fine details of the molecular structure [28].	An NMR spectrum showing signals, or peaks, that correspond to different environments of nuclei in a molecule (usually hydrogen or carbon). Detailed structural information is based on the chemical shift, splitting patterns, and integration of the signals.
X-Ray Crystallography	It's used for the determination of atomic structures of materials by measuring the diffraction pattern of X-rays passing through a crystallized sample [28].	A diffraction pattern from which a 3D model of the atomic structure of a crystallized compound is generated, providing all the atomic positions within the molecule.

6. CONCLUSION

Phytochemical screening is important in understanding the therapeutic potential against plant-based compounds. This identification of bioactive compounds is characterized through various qualitative and quantitative analyses that permit exploration into new pharmaceuticals and health supplements. Findings from this review not only reinforce the importance of traditional medicine but also lead to further research into the pharmacological properties and mechanisms of action of the phytochemicals. As we go on our journey through this natural world, one of the foundational tools for the discovery and development of novel therapeutic agents is screening plants.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Authors hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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