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Bioprofiling of Polyherbal Mixture Towards Plant-Derived Pharmaceuticals

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Abstract

Herbs are the major sources that protect us from the attack of free radicals. The methanolic extract of polyherbal mixture comprising the leaves of *Ocimumtenuiflorum, Mentha piperita, Trigonella foenum-graecum, Plectranthusamboinicus* and *Acalypha indica* was taken for the study. The research was designed to evaluate phytonutrients, antioxidant and antimicrobial activities of polyphenolic compounds extracted from the polyherbal mixture. The results of the investigation indicated a high phenol content of 10.452± 0.010 mg/g along with flavonoids 6.976± 0.014mg/g and tannins 9.08± 0.012mg/g which are also polyphenols as compared to saponins0.250± 0.006mg/g, glycosides 0.058± 0.009mg/g and alkaloids 0.051± 0.005 mg/g in the polyherbal extract. The polyherbal formulation showed maximum yield of polyphenolic compounds in maceration extract which was 476.63µg GAE/mL compared to that of soxhlet method of 403.95µg GAE/mL. The phenolic Thin Layer Chromatography (TLC) profile of methanol:hexane exhibited 7 to 8 spots (3:1). The polyphenolic compounds exhibited higher antioxidant activity with EC50 values of 61.67±0.07 µg/mL and 33.95±0.10 µg/mL through ferric reducing antioxidant power (FRAP) assay and phosphomolybdenum analysis respectively and IC50 values of 73.34±0.12 µg/mL and 26.76±0.10 µg/mL through superoxide scavenging radical analysis and 2, 2 azino bis 3 ethylbenzothiazoline 6 sulfonic acid (ABTS) radical scavenging analysis respectively. Disk diffusion method revealed that they exhibited antimicrobial activities against oral pathogens such as *Streptococcus mutans, Candida albicans, Actnimyces viscosus* and *Staphylococcus aureus* with inhibition zones of 14.20±1.15 mm, 15.00±1.00 mm, 21.07±0.57 mm and 22.00±0.51 mm respectively. Finally, the research concluded that the polyherbal extract had antimicrobial and antioxidant properties, and that it could be a significant source of bioactive compounds with potential biological benefits.

Keywords: Polyherbal mixture, Polyphenols, Maceration and Soxhlet Extraction, TLC, Antioxidant Activity and Antimicrobial Activity.

Introduction

The role of plants as a significant source of drugs and therapeutic agents has been passed down through the generations and playsa crucial part in health care. India is the huge manufacturer of medicinal plants and is thus capably known as the agricultural garden of the globe (1). Polyherbal mixture has been utilized throughout the world due to its wide range of therapeutic properties and for reducing toxicity. Due to synergistic effect, polyherbal formulations confer more effectiveness in many diseases compared to single plant (2). These plants can thrive in temperatures between 20°C to 30°C, as they are very adaptable to a variety of conditions. These can be cultivated in farms, gardens, pots, or other containers, based on the resources and space that are available. Based on the similarity between environmentalconditions and the ease of cultivation process, these plants were selected for this study (3).

Mentha piperita (family- Lamiaceae) commonly known as Peppermint is a strongly aromatic herb and most widely used in

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medicinal preparations. Traditionally, the peppermint plant has many pharmacological effects such as anticarcinogenic, antitumorigenic, dental protective, antioxidant, antimicrobial, antinociceptive, antiallergic, anti-inflammatory and antiviral activities with high phenol, tannin and flavonoid content (4).

Ocimum tenuiflorum (family-Lamiaceae) commonly known as Holy basil is a traditional plant used in Ayurvedic medicine. The scientific studies indicated tulsi has antiviral, antistress, antifungal, antioxidant, hepatoprotective, antibacterial, immunomodulating, antipyretic, anti-inflammatory, antimalarial, antidiuretic, hypolipidemic and antidiabetic properties. Holy basil is being used in combination with other plants to show good healing properties with high polyphenolic content (5).

Acalypha indica (family-Euphorbiaceae) commonly known as Indian acalypha is an ethnomedicinal herb used in many other countries. Indian acalypha has various therapeutic uses such as anticancer, antiulcer, anti-inflammatory, antifungal, anthelmintic, antibacterial, antihyperlipidemic, antidiabetic, antiobesity, hepatoprotective, wound healing and antivenom properties with high polyphenol and flavonoid content (6).

Trigonella foenum- graecum (family-Fabaceae) commonly known as Fenugreek is used as a dietary supplement. Fenugreek is beneficial for the control of different complications through its antioxidant, antitumor, anti-inflammatory, anticancer, cardioprotective, hepatoprotective, neuroprotective, nephroprotective, antimicrobial and immunomodulatory activities with high polyphenol and flavonoid content with high polyphenol, tannin and flavonoid content (7).

Plectranthus amboinicus (family-Lamiaceae) commonly known as Indian borage is an herbal medicine used in the traditional system. Indian borage shows many biological properties such as antidiabetic, antimicrobial, anxiolytic, antifungal, analgesic, anti-inflammatory, antiplatelet aggregation, diuretic, skincare, antimalarial, antibiofilm, wound healing, and antineoplastic activities. *P. amboinicus* possesses increased effect if given in combination with different herbs (8).

In the latest periods, the quantitative analysis evaluates the total content of secondary metabolites such as phenols, flavonoids, tannins and alkaloids show the therapeutic importance of plant. This technique is active in a crucial role to analyze the secondary phytonutrients that provide health-promoting and disease-curing properties (9). The maceration and soxhlet extraction have the most important effect on phenolic compounds which act as antioxidant sources useful in the pharmaceutical and food industry (10). The thin layer chromatography (TLC) separates the bands in different solvent system to analyze the bioactive constituents (11).Ferric reducing power assay (FRAP), phosphomolybdenum assay, superoxide scavenging radical analysis and 2, 2 azino bis 3 ethylbenzothiazoline 6 sulfonic acid (ABTS) radical scavenging analysis are employed to assay the free radical eliminating activity in the prevention of numerous illness and promotion of health in plant species (12).Many disorders are treated using herbal combinations because of their pharmacological qualities. The majority of polyherbal mixtures are used in microbial therapy (13).

A detailed literature review for the individual plants of *M. piperita, O. tenuiflorum, A. indica, T. foenum-graecum* and *P. amboinicus*is available (3). But no published papers are available so far explaining the synergistic effect imposed by the combination of these herbs and the details regarding the quantitative analysis, free radical scavenging property and antimicrobial activity. Hence, the present work was framed to determine quantitative analysis, antioxidant activity and antimicrobial activity of the polyherbal mixture. The synergistic effect is so effective to emphasize that polyherbal mixture has significant applications. This further helps in the therapeutics against diseases such as dental caries, inflammatory diseases and cancer.

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Materials and Methods

Assemblage and Certification of Herbal Plants

Herbs such as *M. piperita, O.tenuiflorum, A. indica, T. foenum- graecum* and *P. samboinicus* were collected from Chennai, Tamilnadu and were authenticated by Dr. P. Sathiyarajeswaran, Assistant Director in charge and Dr. K. N. Sunil Kumar, Researcher and Pharmacognosy, Department Head, Central Council for Research in Siddha (CCRS), Madras (Authentication number: 252-06082101-05).

Extraction

Dried plant materials of 500 g each in the ratio 1:1:1:1:1 were mixed and extracted with methanol by using an orbital shaker for 72 hours. The polyherbal combinations were filtered by grade one chromatography sheet. An extract was subjected for evaporation in front of a stream of air at 37^{o} C which yielded a polyherbal semisolid mass of 9.18 g of methanol extract and then the polyherbal mixture was stored in an airtight container for further analysis. Applying the formula, the percentage (%) yield was determined by calculating the dry weight of polyherbal mixture (14).

Percentage yield = Amount of the wet polyherbal methanolic extract/ Amount of the wet polyherbal mixture material * 100

Quantitative Analysis of Phytonutrients

Quantitative Assessment of Alkaloid Content

About 5 ml of phosphate buffer solution, equal volume of bromocresol green reagent and small amount of test substance were mixed. The resultant combination was thoroughly dissolved in 4 ml of chloroform. Chloroform was utilized to dilute the polyherbal mixture. At 470 nm, the complex's absorbance in chloroform was evaluated in comparison to a control that was made identically but without polyherbal extract. Atropine equivalents in mg/g were used to report the results, with atropine acting as the reference.(15, 16).

Quantitative Assessment of Flavonoid Content

Quercetin was used as a standard in the aluminum chloride procedure to calculate the total flavonoid concentration. To a 10 mL volumetric flask, 1 mL of the polyherbal test solution and 4 mL of water were mixed. Aluminum trichloride and sodium nitrate were mixed shortly after 5 min. The resulting mixture was mixed with 2 ml of 1M sodium hydroxide after it had been kept at appropriate temperature for the experiment. The final volume was quickly increased to using water. Using a blank spectrometer, the absorbing capacity of the resultant was determined at 510 nm. The findings were given as mg QE/g dry weight, or quercetin equivalents (15,17).

Quantitative Assessment of Saponin Content

After diluting the polyherbal test sample in 80% methanol, 2 ml of vanilin in ethanol was mixed. Next, 2 ml of a mixture of 72% sulfuric acid was thoroughly mixed before being heated on a water bath for 10 min at 60°C. At 544 nm, the absorbance was measured in relation to a reagent blank. The data was represented as mg/g equivalent of diosgenin, which acts as a standard (18, 19).

Quantitative Assessment of Phenol Content

Using Folin-Ciocalteu's reagent, the polyherbal test sample's total phenolic content was measured with minor modification. In short, 0.4 ml of diluted folin's solution was mixed to polyherbal test solution. 4 mL of sodium carbonate reagent was mixed after 5 min. Add 10 mL distilled water to the tubes to reach a final capacity; they were left to remain at ambient temperature for 90 minutes. Using a spectrophotometer, the absorbing capacity of the polyphenolic extract was determined at 750 nm compared to the blank. Gallic acid serves as the standard in the construction of a calibration curve, and the extract's total amount of polyherbal phenolic compounds was reported in gallic acid (mg) per gram of wet mass (15, 18).

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Quantitative Assessment of Tannin Content

By combining equal amount of polyherbal extract and distilled water and agitating for 20 min, the entire tannin quantity of the polyherbal formulation was measured with slight modification. About 0.5 mL of 0.008M potassium ferrocyanide solution and 1 mL of 0.1M ferric chloride in HCL solution was added. At normal room temperature, the tubes were mixed for 5 min. Using a spectrophotometer, the formulation's absorbing capacity was calculated at 120 nm. Using tannic acid as a reference, a curve for calibration was created. In mg of tannic acid per gram of dehydrated extracts, total tannins were determined (17, 20).

Quantitative Assessment of Glycosides Content

The modified method to calculate the total amount of glycosides in the polyherbal extract, the polyherbal extract was mixed with distilled water, then agitated for 10 min. To the above polyherbal mixture, 1 mL of acetic acid and 0.1 mL of 5% ferric chloride was mixed and let undisturbed for 90 min. In comparison to the blank reagent, the absorbance was determined at 568 nm. The standard utilized to generate the curve for calibration was digoxin. Digoxin mg/g of dried polyherbal extracts was used for calculating total glycoside content (21, 22). All the quantitative assays were carried out in triplicates, and the mean ± SD was used to represent the results.

Extraction Methods

Maceration Method

A conical flask containing about 25 g of polyherbal substance was mixed with 75 mL of methyl alcohol and left for 72 hours. Polyherbal extracts were obtained by filtering and collecting the supernatant. A modified method of using 70% instead of 80% methyl alcohol was followed to obtain maximum yield.The optimal solvent concentration had an impact based on the solubility, selectivity, density, interactions and mass transfer of the target compounds (23, 24).

Soxhlet Method

The modified method of the polyherbal substance was ground up with a mechanical blender, around 20 g of polyherbal fine powder was poured into a 12 x 3 cm thimble made of sturdy filter paper, which was then put within the Soxhlet extractor's thimble chamber. 300 mL of 70% methyl alcohol solvent were placed in a round-bottomed flask and connected to a Soxhlet extractor; the extractor was then used when combined with the condenser. The water circulation device was attached to the condenser's input and outflow knobs for water movement (cooling). In this configuration a round-bottomed flask was put on the heating mantle. Upon heated through the heating mantle, the solvent that was used started to evaporate and passed through both the Soxhlet chamber and condenser. The compounds disintegrated and emerged from the substance when the condensate then dropped down into the thimble container. The process was restarted after the solvent level achieved its maximum level and was taken off *via* the siphon arm and emptied into the round-bottom flask. For the production of gummy extract, the polyherbal supernatant in the round-bottomed flask was then condensed at 50°C (23, 25).

Thin layer chromatography

The modified method using a capillary tube, the polyherbal phenolic sample was spotted on the TLC paper approximately 0.5 cm above the bottom. The developing phenolic solvent was poured into the chromatographic beaker and left to run until it reached three quarters of the TLC sheet (the spot shouldn't come into contact with the solvent). After the plates being removed, the solvent front was marked by a pencil and let to evaporate. Next, the TLC plate was seen in the presence of ultraviolet (UV) light. Iodine pellets were put into the plates in order to see the distinct bands (26).

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R_f value = Solute's travel distance/ Solvent's travel distance

Free Radical Scavenging Assays

Ferric ion reducing antioxidant power test

The modified ferric ion reducing antioxidant power method employs a redox-linked colorimetric procedure with antioxidants serving as reductants (27). The varying doses of 20-120µg/mL polyherbal extracts were mixed with 1 mL of a 0.2M buffered phosphate solution at pH 6.6 and 1 mL of 1% potassium ferricyanide solution. To the resulting mixture, 1 mL of 10% trichloroacetic acid and iron chloride (0.1%) was mixed and incubated at room temperature. The resultant solution's measured at 700 nm was estimated. The positive control used was ascorbic acid. The assays were conducted in triplicates, and the mean ± SD was used to represent the results.

Phosphomolybdenum assay

The reduction test method, which is predicated on the creation of green phosphomolybdenum complex, was used to assess the antioxidant capacity with small modifications (27). 1 mL of phosphomolybdenum solution was mixed with extracts at different concentrations of 20–120 µg/mL. After being sealed, the tubes were left to incubate for 30 minutes at 95°C in a water bath. After bringing the sample down to a normal room temperature, the antioxidant activity of the polyphenolic extracts was measured at 695 nm using the positive control ascorbic acid. The assays were conducted in triplicates, and the mean ± SD was used to represent the results. The percentage of polyphenolic extract reduction for both the assays was calculated by

% of reduction = [(Abs (sample) – Abs (control)/Abs (sample)] x 100

Superoxide radical scavenging activity

This method was used to scavenge radicals containing superoxide with minor modifications (28). Different amounts of methanol extract, 50 mM phosphate buffer solution at pH 7.8, 1.5 mM the B vitamin riboflavin, 12 mM edetic acid and 50 mM Nitrotetrazolium Blue chloride were added to the resulting mixture in that sequence. The process was initiated by subjecting the resultant mixture to UV light for 90 sec. The absorbing capacity at 590 nm was calculated shortly after illumination, and the IC50 was determined. Every test was carried out in triplicates, and the outcomes were reported as mean ± SD. The positive control used was ascorbic acid.

$ABTS^+$ Radical cation scavenging assay

The ABTS free radical cation scavenging capability was analyzed to determine the antioxidant properties with small modifications (29). The reaction mixture of persulfate of potassium with ABTS reagent and allowed to stand at room temperature in a dark place for 12–16 hours prior being utilized. After being stable for two days, the ABTS solution was diluted to an absorbance of 730 nm using 5 mM phosphate-buffered saline solution at pH 7.4. The absorbance was determined 10 minutes by adding 10–60 µg/mL various doses of polyphenolic solution to diluted ABTS solution and incubated at 25°C. The positive control was ascorbic acid. Every test was carried out in triplicates, and the outcomes were reported as mean ± SD.

The percentage of polyphenolic extract inhibition for both the assays was calculated by:

$$\%\ \text{of inhibition} = \frac{\text{Absorbance}_{\text{Control}} - \text{Absorbance}_{\text{Sample}}}{\text{Absorbance}_{\text{Control}}} \times 100$$

Evaluation of Antimicrobial Activity

Bactericidal Assay

Using the agar disc diffusion method, the polyphenolic extract of bacteriostatic capability of *S. mutans*, *S.aureus* and *A.viscosus* was investigated. After being cascaded into an aseptic cell culture dish, Mueller-Hinton agar was densified. Mueller-Hinton agar was inoculated with thick peptidoglycan gram-positive *S. aureus, S.*

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mutans, and A. viscosus using aseptic cotton buds and nutritional liquid broth medium. A cork auger that was aseptic was used to make pentaholes in agar for the samples, standard, and control (0.8cm width). Each individual hole was filled with various concentration of polyphenolic extract against a positive control tetracycline. The cell culture dish was then kept at 98.6°F for the duration of the night. The outcomes were shown as mean ± SD, with each assay carried out in triplicate. The zone of inhibition in mm surrounding the hole was used to measure the antibacterial activity (30,31).

Fungicidal Assay

Densification was achieved by cascading potato dextrose agar into an aseptic cell culture dish. C. albicans fungus swabbed and left in a cell culture dish for 48 hours. Next, using a cork auger, the sample of polyphenolic extract was poured into the wells in various amounts. After filling each individual hole with various concentrations against a positive control fluconazole, the cell culture dish was kept at 98.6°F for 48 hours. The outcomes were shown as mean ± SD, with each assay carried out in triplicates. The zone of inhibition in mm surrounding the hole was used to assess the fungicidal capability (32, 33). Elevated humidity has the ability to modify the agar medium's diffusion characteristics and hydration level, which could impact the antibacterial agents' rates of diffusion. The size and form of inhibitory zones may vary as a result of this. When handling and preparing agar plates, weather factors like heat or high humidity might raise the risk of contamination. This may result in lower antibacterial agent concentrations in the agar, which could compromise the assay's accuracy. The assays were repeated to overcome this problem.

Results and Discussion

Polyherbal Methanolic Fractionation

Polyherbal mixture upon extraction with methanol solvent resulted in the extract with the yield of 1.836%. Some of the key elements influencing extract yields and the plant materials' subsequent antioxidant activity are the type of extracting solvent used. The activity varies depending on the different chemical properties and polarity of the phytoconstituents.

Quantitative Analysis of Phytonutrients

Quantitative Assessment of Alkaloid Content

The total alkaloid content in the polyherbal methanol extract were found to be 0.051 ± 0.005 mg/g of polyherbal extract (y=0.2292 x+0.1658, R^2 =0.9931) (Figure 1). Earlier studies have indicated the total

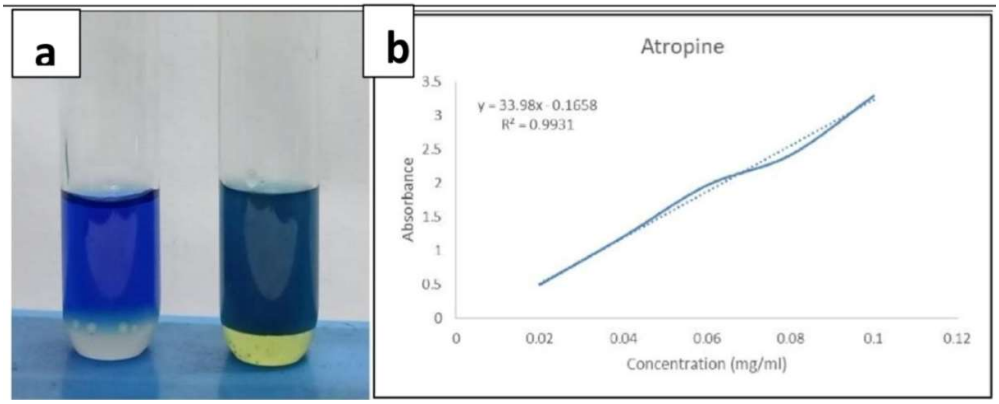

Figure 1: Quantitative Assessment of Alkaloid Content (a) Alkaloid formation at 20mg/ml (b) Standard graph and estimation of alkaloid in the sample

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alkaloid content was found to be 0.00313mg/g in ocimum leaf and 5.92% in *Moringa concanensis.*Alkaloids are employed for their antimicrobial and hypoglycemic qualities. Due to the presence of numerous components that are essential for maintaining good health, the phytochemical constituents appeared to have the potential to both improve their health and serve as a source of helpful pharmaceuticals (34, 35).

Quantitative Assessment of Flavonoid Content

The total flavonoid content in the polyherbal methanol extract was found to be 6.976± 0.014mg/g (y=0.2292x+0.2441, R^2 =0.9989) which was more significant than earlier work of polyherbal extract (Figure 2). In a study, the total flavanoid content of calotropis leaf was found to be 2.285mg/g (36). Earlier work on polyherbal formulations such as DBC (34 multiple herbs) and DMV (22 multiple herbs) showed total flavanoid content of 2.30 and 1.78mg/g respectively (37). The nature of these chemical components, which are in charge of the intended therapeutic characteristics and specific physiological consequences, dictated the pharmacological activity of the polyherbal extract of the herbal formulation. Strong positive linear correlation (r), which is near to +1, was displayed in all of the calibration graphs. According to the graphs, absorbance values rise in tandem with concentration levels. The polarity of the solvents utilized in the extraction process affects the quantity of flavonoids present in plant extracts. With the aid of several calibration curves, the sample values were examined, and various graphs were used to produce the various concentration values. The highest concentration of flavonoids is produced by a polyherbal mixture of *Cassia alata, Wedeliatrilobata, and Hugonia syntactic*. (38).

Quantitative Assessment of Saponin content

The total saponin contents in the polyherbal methanol extract was found to be 0.250 ± 0.006mg/g of polyherbal extract (y=3.3753 x+0.0179, R^2 =0.9971) (Figure 3). Earlier studies have indicated total saponin content of ocimum leaf as 0.016mg/g (34). In intracellular histochemistry labeling, saponins act as mild detergents and are utilized to enable antibodies to interact with proteins within the cell. Saponins are involved in weight loss, hyperglycemia and hypercholesterolemia with antioxidant, anticancer and anti-inflammatory properties (35). As a natural defense mechanism, saponins bitter flavor has assisted in keeping plants safe from soil microbes, insects, and mammalian herbivores. Numerous studies

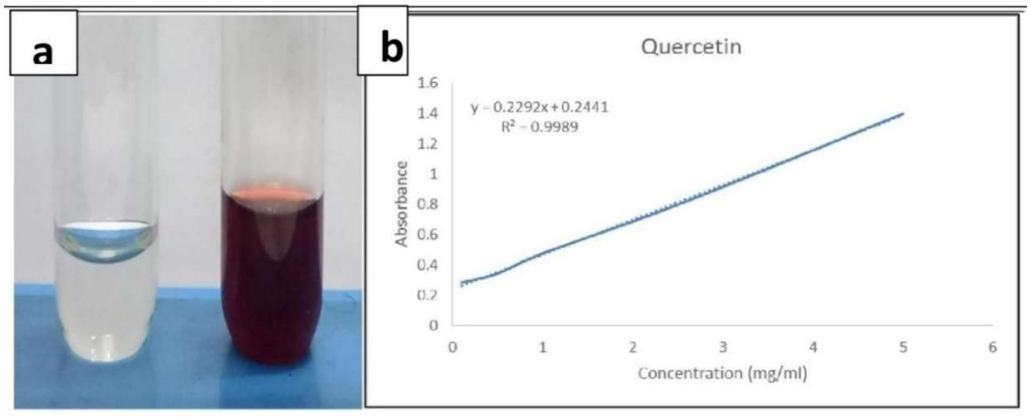

Figure 2: Quantitative Assessment of Flavonoid Content (a) Flavonoid formation at 20mg/ml (b) Standard graph and estimation of flavonoid in the sample

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have documented a broad spectrum of pharmaceutical and therapeutic properties of saponins, including minimal oral toxicity in humans. *Crinum zeylanicums* total saponin content was found to be 37% through quantitative research. The findings showed that the medicinal plant is beneficial to health and is thought to be a versatile herb that can treat a wide range of ailments (39).

Quantitative Assessment of Phenol content

The total phenol contents in the polyherbal methanol extract were found to be 10.452 ± 0.010mg/g of polyherbal extract (y=0.2107 x+0.1887, R2 =0.8608) which was more significant than that found in earlier works of polyherbal extracts (Figure 4). In a study, the total phenol content of mentha leaf was found to be 7.96 mg/g (34). Earlier work on polyherbal formulations such as DBC (34 multiple herbs) and DMV (22 multiple herbs) showed total phenol content of 4.503 and 5.21mg/g respectively (37). The highest standard by which other bactericides that is evaluated is still phenols and phenolic compounds, which are widely employed in disinfections (33). The leaves of *Psidium*

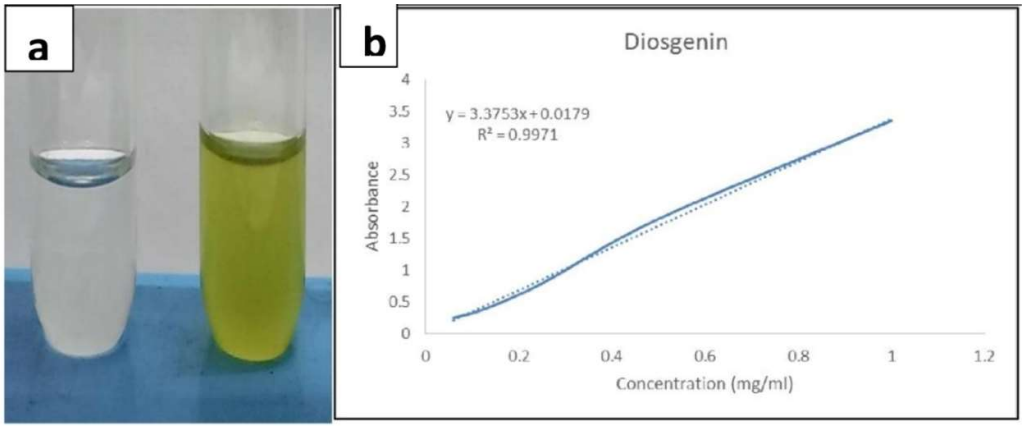

Figure 3: Quantitative Assessment of Saponin Content (a) Saponin formation at 20mg/ml (b) Standard graph and estimation of saponin in the sample

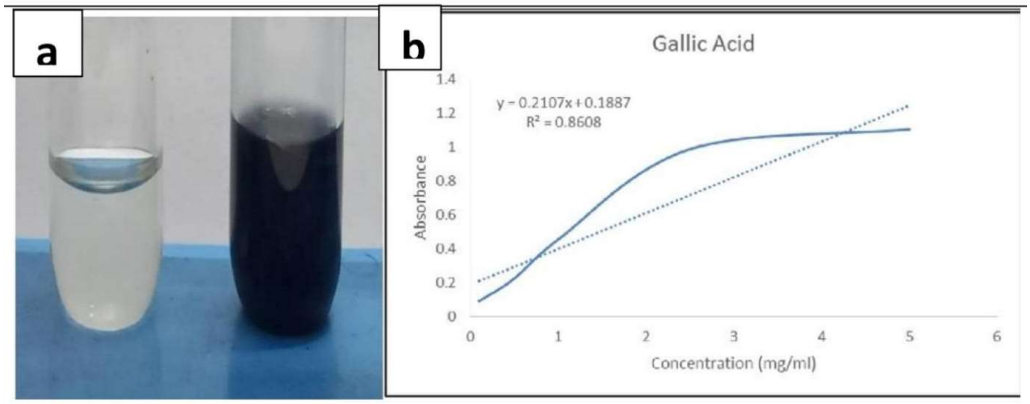

Figure 4: Quantitative Assessment of Phenol Content (a) Phenol formation at 20mg/ml (b) Standard graph and estimation of phenol in the sample

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guajava methanol extract showed 2.6% total phenol content. Polyphenolic substances have a well-established antibacterial action in addition to their substantial antiphlogistic, free radical-scavenging and carcinoma-preventive effects. Phenolic substances exhibit many bactericidal modes of action; including preventing the synthesis of nucleic acid and neutralizing the outer layer of cells. Research also revealed that polyphenols may have anti-fungal properties through blocking glucosamine, a development signal found only in the cells of fungi of specific genera, and ergosterol, an essential component of the microbial cell wall (41).

Quantitative Assessment of Tannin content

The total tannin contents in the polyherbal methanol extract were found to be 9.08 ± 0.012mg/g of polyherbal extract (y=0.1069 x+0.0555, R^2 =0.9934) which was more significant than that in earlier works of polyherbal extracts (Figure 5). In a study, the total tannin content of mentha leaf was found to be 2.15 mg/g (40). Earlier work on MAT20 polyherbal formulations of total tannin content showed 0.3453mg/g (41). The total tannin content of the methanol extract of *Psidium guajava* leaves in a study was 3.1%. Strong antibacterial properties are exhibited by tannins, which are soluble in water, chemical substances that are extensively distributed in the kingdom of plants. Tannins prevent the development of microbes through a variety of mechanisms, such as iron chelation process, deprivation of vital compounds that drive microbial development, disruption of microbe activity in metabolism through inhibition of oxidative phosphorylation, and inhibition of enzymes that necessary for the outside of cell membrane. Through processes like the blocking of enzymes outside the cell, deprivation of substrate, and suppression of the process of oxidative phosphorylation, tannins function as antibacterial agents. The polyphenolic molecule is a bio preservative that finds application in both the food and medicinal industries. (32).

Quantitative Assessment of Glycosides content

The total glycosides contents in the polyherbal methanol extract were found to be 0.058 ± 0.009mg/gof polyherbal extract (y=0.0174 x+0.0319, R^2 =0.9881) (Figure 6). In a study, the total glycosides content of different Nigerian samples was found to be 3 to 7.3 mg/ g. Congestive heart failure has been treated with cardiac glycosides because of their direct action, which increases the contraction of the myocardium. Cardiac glycosides directly affect smooth muscles within the circulatory system. They have an indirect impact on cardiac electrical activity, vascular resistance, and capacitive in addition to their impact on brain tissues. he glycosides found in *Cordia millenii, Tetrapleura tetraptera, Afzeliabipindensis, Moringa species* and *Combretodendron macrocarpum* were

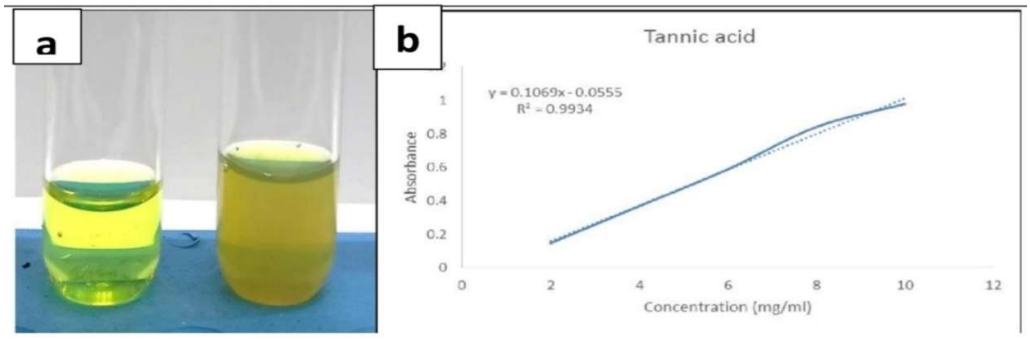

Figure 5: Quantitative Assessment of Tannin Content (a) Tannin formation at 20mg/ml (b) Standard graph and estimation of tannin in the sample

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found to have potential medical uses. According to the findings, these softwood grains from Nigeria may be a source for the phytonutrients that are useful to the complementary and alternative healthcare sectors (43).

The high phenol content along with flavonoids and tannins which are also polyphenols were indicative of application of the polyherbal mixture in the treatment of inflammation and cancer (44-46).

Maceration and Soxhlet Extraction method

The average of polyherbal polyphenolic compound yields of the maceration and Soxhlet extraction method is presented in Figure 7. The result showed that the phenol content yield of maceration extraction method is 476.63µg GAE/mL. The tannin content yield of maceration extraction method is 623.86µg GAE/mL. The flavonoid content yield of maceration extraction method is 27.21µg GAE/mL. The result showed that the phenol content yield of Soxhlet extraction method is 403.95µg GAE/mL. The tannin content yield of Soxhlet extraction method is 565.70µg GAE/mL. The flavonoid content yield of Soxhlet extraction method is 26.85µg GAE/mL

On the whole, maceration extraction method has maximum yield of extraction compared to soxhlet extraction method. Methanol extracted more phytochemicals than chloroform and hexane, according to research on five distinct Sudanese medicinal plants that are rich in phytochemicals, particularly phenolic compounds. In comparison to soxhlet extraction, the maceration extract has a higher percent yield. The methanolic maceration technique has better antioxidant activity against standard propyl gallate (47).

The maceration extraction of wali seeds has more total phenolic content about 4.9mgGAE/100g sample compared to soxhlet extraction with about 3.8mg GAE/100g sample and has higher antioxidant activity reported in maceration extraction (48).

The macerated southern bugle subspecies Pseudoiva extracts was rich in polyphenols with about 25.26 µg gallic acid equivalents per mg of extract, flavonoids about 821.43 µg quercetin per mg of phenolic solution, and tannins about 95.58 µg catechin equivalents per mg of phenolic solution. The macerated methanol extract has the ability to scavenge free radicals (49).

Thin layer chromatography (TLC)

The results of methanolic polyphenol TLC profile are shown in Fig 8. In the solvent system, methanol and hexane taken in the ratio 3:1 respectively exhibited the presence

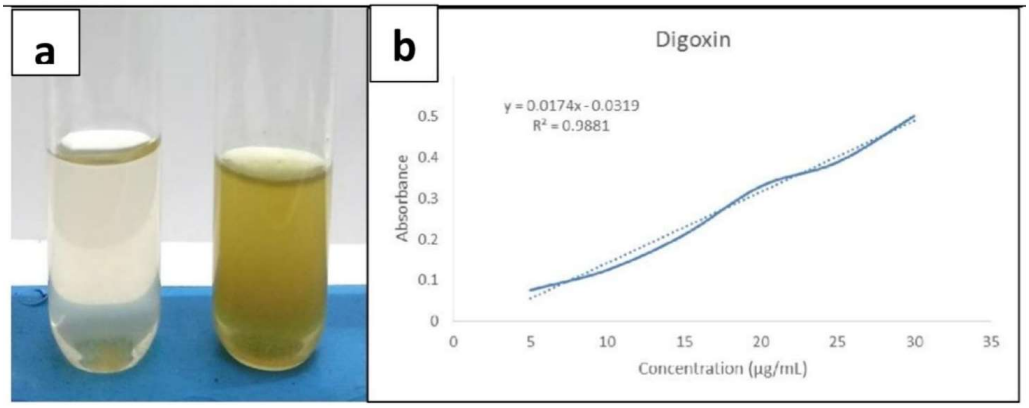

Figure 6: Quantitative Assessment of Glycoside Content (a) Glycosides formation at 20mg/ml (b) Standard graph and estimation of glycosides in the sample

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of phenol (Rf 0.56). Flavonoid was present in 3:1 ratio of toluene and chloroform solvent system respectively (Rf 0.34). In the solvent system, methanol and hexane taken in the ratio 3:1 respectively exhibited the presence of tannin (Rf 0.45).

Using TLC to screen out *Cyperus rotundus*, the Rf values were ascertained. TLC is a separation method used to determine many bioactive components. Excellent pharmacological qualities and great selectivity, efficacy, and peak parameters are all provided by the chromatographic condition (50).

A high concentration of flavonoids and tannins, which are phenolic components, was found in the methanol extract of Armenian herbs. As mobile phases in TLC study, various solvent systems were employed to draw conclusions about the characteristics of active antibacterial agents. The development of TLC plates using the solvent systems glacial acetic acid, methanol, butanol, and water demonstrated the presence of polyphenolic compounds as separated bands (51).

Antioxidant Assays

FRAP Test

The method is frequently employed to assess the antioxidant potential of polyphenols. The polyherbal mixture exhibited a stronger reducing property, as

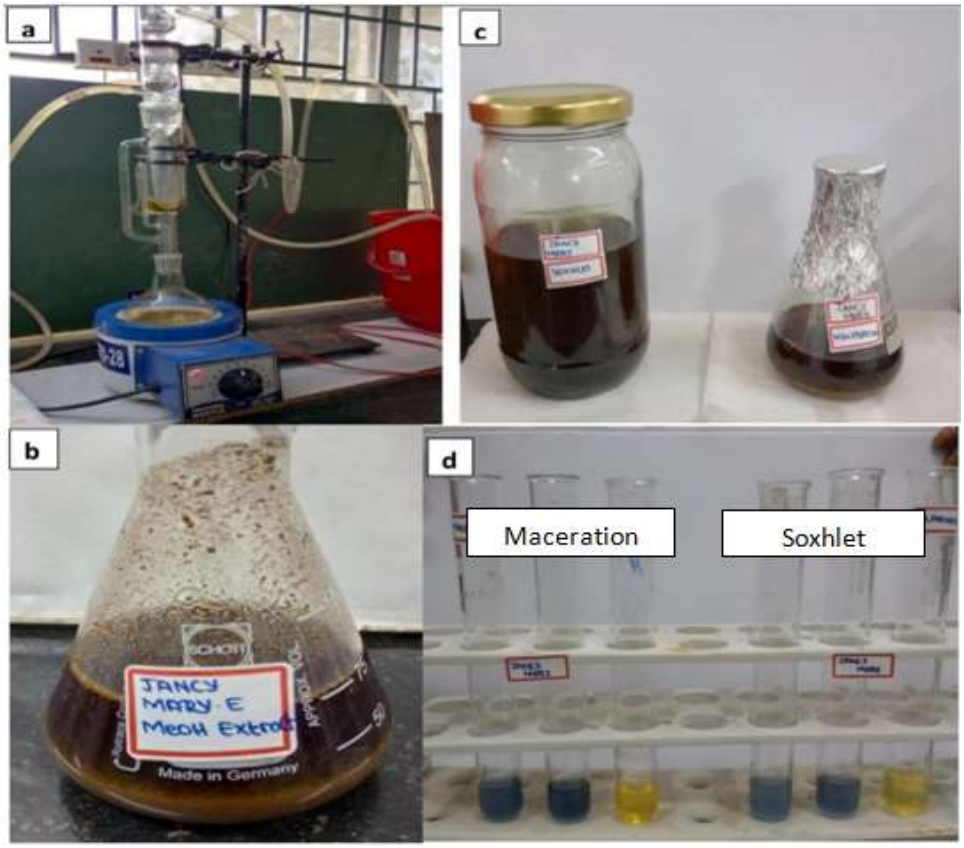

Figure 7: Maceration and Soxhlet Extraction Methods (a) Soxhlet Extraction (b) Maceration Extraction (c) Polyphenolic Extract (d) Phyto polyphenol

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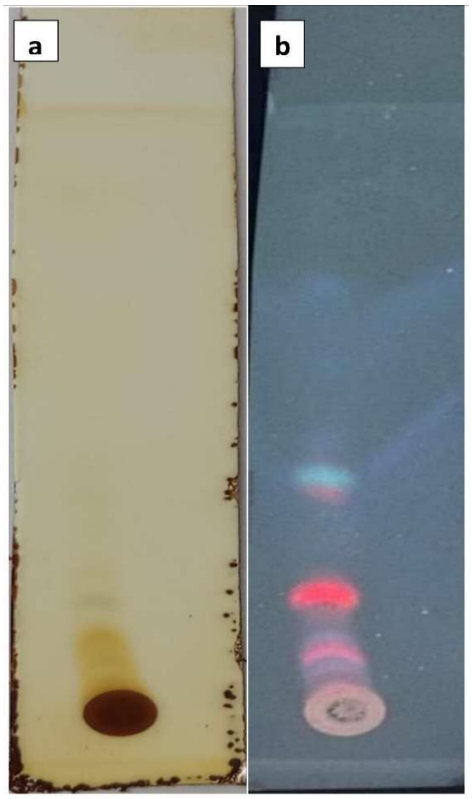

Figure 8: Thin Layer Chromatography (a) TLC Profile (b) TLC with UV light

evidenced by the percentage inhibition of the polyphenolic extracts. Results as in Figure 9 depicted that the polyphenolic extracts were stronger in the FRAP analysis with a concentration at 50% of reduction (EC50) value as 61.67±0.07 µg/mL. The reducing property improved with an increase in polyphenol concentration and was dosage dependent. An earlier study indicated concentration at 50% of inhibition (IC50) value of 79.92mg/mL by the polyherbal extract of Dasamoolarishtam through FRAP analysis (52).

Using the FRAP assay to measure maximum antioxidant activity, a strong synergism was demonstrated when *Syzygium aromaticum* and *Rosmarinus officinalis* (methanol extracts) were combined. Excellent free radical scavenging activities are demonstrated by the combination of *Mentha piperita* and *Thymus vulgaris* methanol extracts by FRAP tests. Because of their synergistic interactions, using extracts in different combinations produced an optimal antioxidant effect even at lower doses than when using the extracts alone (53).

Phosphomolybdenum Assay

The method is employed to determine the antioxidant potential of plant extracts. The polyherbal mixture showed a significant reducing effect, as evidenced by the percentage inhibition of the polyphenolic extracts. Results as in Figure 10 depicted that the polyphenolic methanol extracts have greater potency in the phosphomolybdenum test with a concentration at 50% of reduction (EC50) value is33.95±0.10 µg/mL. The reducing property improved with an increase indosage dependent and polyphenolic concentration. An earlier study indicated EC50 value of 80mg/mL by the polyherbal extract of Dasamoolarishtam through phosphomolybdenum activity (52)

The phosphomolybdenum complex was used to test the polyherbal DhanwantaramKashayam decoction, and the results showed that it had a significant impact on the antioxidant properties, with an inhibitory concentration (IC50) of 50.4µg/ml. The phytochemicals in the polyherbal kashayam indicate its potent antioxidant properties, and it reduces molybdenum radicals to treat gynecological illness. The potent antioxidant polyherbal decoction activity may be the cause for its positive effects in body's system as reported in Ayurvedic medical system. (54).

Superoxide Radical Scavenging Activity

The method is used to scavenge superoxide radicals and enhance the nutritional value of food. This method of antioxidants is the most important inhibition mechanism. Results as in Figure 11 depicted that the polyphenolic extracts have good effect in the superoxide radical scavenging activity with a concentration at 50% of

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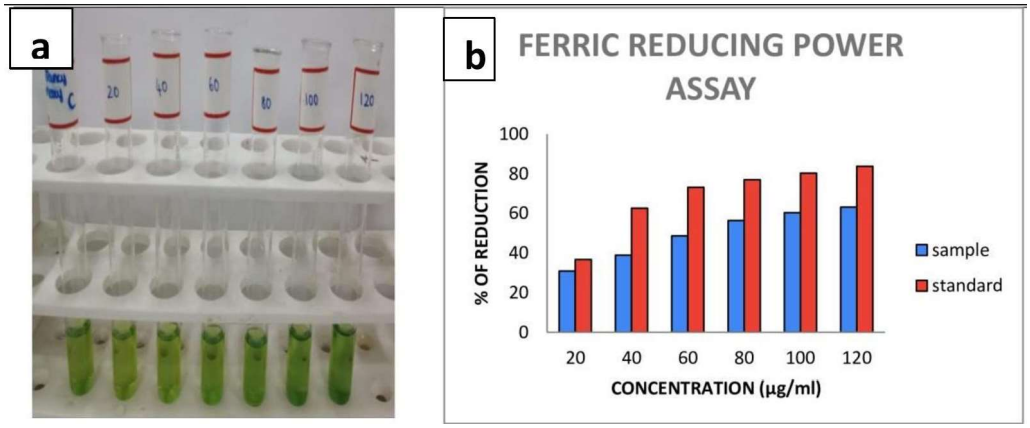

Fig 9: Ferric Ion Reducing Antioxidant Power Test (a) Experimental analysis (b) Graphical representation of reducing power

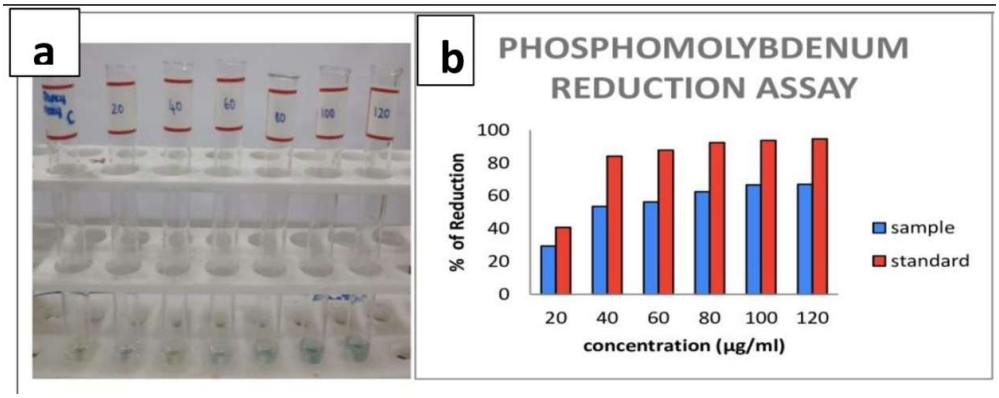

Fig 10: Phosphomolybdenum Assay (a) Experimental analysis (b) Graphical representation of reducing power

inhibition (IC_{50}) value as 73.34±0.12 µg/mL.The ability to scavenge radicals was dose-dependent and had polyphenolic concentration. An earlier study indicated the IC50 value of 0.22mg/mL by the polyherbal extract of Dasamoolarishtam through superoxide radical scavenging activity (52).

The polyherbal methanolic extract of thirikaduguchooranam from zingiber and piper species showed good strong effect with IC50 concentration of 2230 µg/ml. The methanolic extract of polyherbal parangipattaichooranam from smilax and ocimum species exhibited notable radical elimination action with IC50 concentration of 2860 µg/ml. The strong antioxidant qualities of the polyherbal mixture may be attributed to phenols and their derivatives. The removal of fungus and bacteria in polyherbal formulations may be due to the presence of several secondary phytoconstituents (55).

$ABTS^{+}$Radical Cation Scavenging Assay

The procedure is technically simple and has been frequently used for screening and routine measurement of natural extracts. Results as in Figure 12 depicted that the polyphenolic methanol extracts

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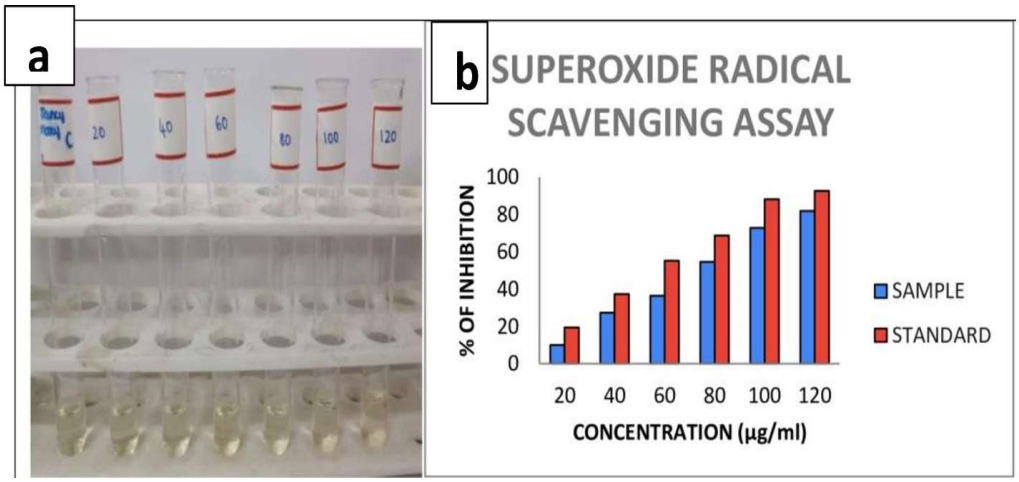

Figure11: Superoxide Radical Scavenging Activity (a) Experimental analysis (b) Graphical representation of radical scavenging activity

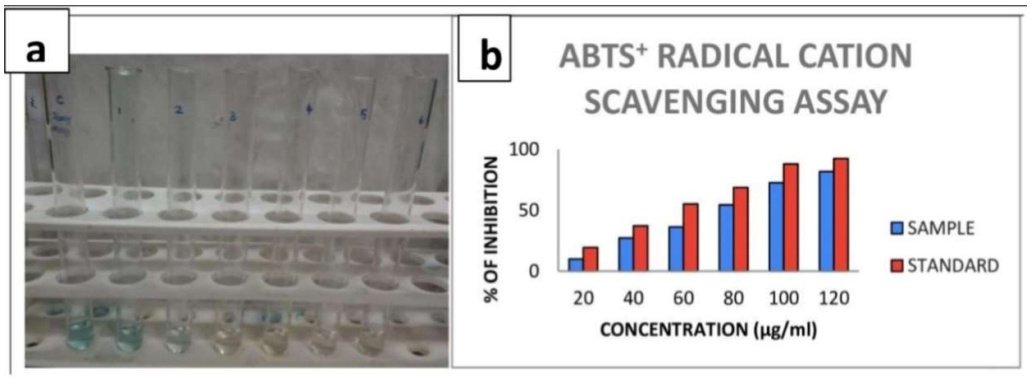

Figure 12: $ABTS^+$ Radical Cation Scavenging Assay (a) Experimental analysis (b) Graphical representation of radical scavenging activity

exhibited $ABTS^+$ radical scavenging activity with a concentration at 50% of inhibition (IC_{50}) value of 26.76±0.10 µg/mL.The capacity to scavenge radical cation was based on the polyphenolic concentration. This could have explained the extract's potent antioxidant action against free radicals as well as its numerous pharmacological qualities. An earlier study indicated the IC50 value of 0.0278µg/mL by the polyherbal extract of Dasamoolarishtam through $ABTS^+$ radical scavenging activity (52)

The polyherbal combination of vati from Withania, Cucurma, Zingiber, Boswellia, Commiphora, Aloe, Hemidesmus and Berberis species showed highest ABTS scavenging activity (96%) and demonstrated potential as a source of anti-inflammatory agents that could be useful in treating a variety of human ailments. The study also demonstrated the significance of OH group location and number in phenolics for antioxidant action. The synergistic interaction between the herbs may aid to increase the antioxidant activity. (56).

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The polyphenolic methanol extract showing excellent scavenging activity implied its antioxidant potential towards inhibition of inflammation and carcinogen activation (45, 46).

The results of all the antioxidant assays indicated significant antioxidant activity of the polyherbal extract under study over the earlier works.

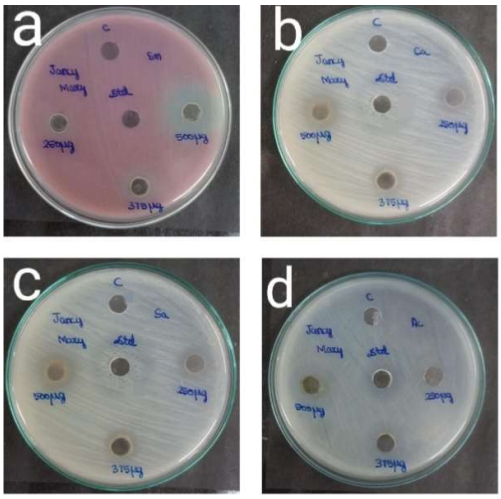

Figure 13: Antimicrobial Activity of Polyphenolic extract against a-*S. mutans,* b- *C. albicans,* c- *S. aureus and* d-*A. viscosus a*nd standard- Tetracycline (a, c, and d), Fluconazole (b)

Evaluation of Antimicrobial Activity

By assessing the zone of inhibition, the methanol extract of the polyphenols had the highest inhibitory property against oral cavity-producing organisms like *S. mutans, C. albicans, A. viscosus*and *S. aureus*. The polyphenolic extract exhibited the largest zone of inhibition and maximum antibacterial activity against *S. aureus* at a dose of 500 µg/mL and *S. mutans* activity appeared to be the lowest. The outcomes were contrasted with the typical tetracycline. When compared to the other bacteria under investigation, *S. aureus* was the target of more activity at all concentrations. There is an increase in both the concentration and the zone of inhibition. Comparing the fungicidal action with fluconazole, the highest effect was observed against *C. albicans*. *S. aureus* > *A. viscosus*> *C. albicans* > *S. mutans* was the overall order of the polyherbal extract's antibacterial effectiveness against the germs (Figure 13 and Table 1). The results showed that the extracts' arithmetic average ± standard deviation was statistically significant at **p<0.01 when compared to different concentrations. This approach may

Table 1: Zone of inhibition against dental pathogens

Organisms	Extract Concentrations (µg/mL)	Polyphenolic Extract
S. mutans	250	11.53± 0.57**
	375	12.00± 1.00**
	500	14.20± 1.15**
	Standard (500)	16.35 ± 1.50**
S. aureus	250	18.30± 1.00**
	375	20.10± 1.00**
	500	22.00± 0.57**
	Standard (500)	20.50 ± 0.57**
A. viscosus	250	18.00± 1.00**
	375	19.50± 1.00**
	500	21.07± 0.57**
	Standard (500)	20.63 ± 0.57**
C. albicans	250	10.37± 1.52**
	375	12. 07± 1.52**
	500	15. 00± 1.00**
	Standard (500)	12.83 ± 0.57**

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frequently produce more beneficial results in the rainy season than it does during the summer season because of the ideal environmental conditions and decreased danger of contamination (57).

When examined against the standard vitamin C by DPPH levels evaluation, the polyherbal mixture extract from *Phyllanthus* species, *Camellia sinensis*, *Khaya senegalensis*, *Nauclealati folia* and *Zingiber officinale* displayed a substantial antioxidant capacity than to its component extracts (58).In comparison to cavity germs, the polyherbal mixture of *Salvia officinalis, Thymus serpyllum, Mentha arvensis, Cinnamomum zeylanicum* and *Rosemaryinus officinalis* demonstrated important microbicidal properties that inhibited the development of oral pathogens, reduced symptoms of gum infection and periodontal disorders, prevented bacteria from sticking to surfaces, and strengthened the gingivae in the dental cavity (59). *Streptococcus mutans*, *Lactobacillus* and *Actinomyces viscosus* as a polyherbal mixture of kantahkari and mastic shown strong microbicidal activities, suppressed the cariogenic microbial flora and might be utilized for decay inhibition in the form of dentifrices and buccal region cleanses (60). The amount of phenolic and flavonoid compounds demonstrated antioxidant and antimicrobial activities that might be of significance in novel therapeutic and pharmaceutical applications (61). The potential mechanism of polyherbal formulation through synergistic interactions between various compounds, such as phenols and flavonoids, which have the dual abilities of scavenging free radicals and inhibiting microbial growth, may be responsible for the antioxidant and antimicrobial effects of the polyherbal methanol extract. Polyherbal formulation should be further analysed for cytotoxic effect to check the toxicity. The manifestation of a large number of diseases may be due to variations and defects in antioxidant and antimicrobial mechanisms. Compounds with antioxidant and antimicrobial properties may provide solutions to such diseases. Due to their wide range of health benefits, phytonutrients such as polyphenols are used in many different areas of healthcare including cardiovascular protection, brain support, gut health promotion and anti-inflammatory responses.

Conclusion

The appearance of distinct polyphenolic nutrientsin methanolic extract of polyherbal formulation containing leaves of *M. piperita, O. tenuiflorum, A. indica, T. foenum- graecum* and *P. amboinicus* showed higher phenolic concentration than other constituents, including flavonoids and tannin. When compared to soxhlet extraction techniques, the macerated methanolic phenol compound exhibited an excellent extraction rate. Referential data from the investigation would be used to accurately identify the bioactive chemicals and select the best solvent system for separating them from the phenolic TLC profile. The results of the research indicated that the polyphenolic extract had a higher radical scavenging, metal ion reducing potential and microbicidal effect. Polyherbal phytonutrients, antioxidant and antimicrobial are related to their applications in traditional medicine. The study concluded that the polyherbal mixture's numerous phytonutrients with strong antibacterial and antioxidant properties make it useful for its cytoprotective role against number of diseases. Because of thesynergistic interactions of potential bioactive compounds prevailing in the polyherbal mixture, the research helps to develop safe, effective, and evidence-based herbal treatments for the prevention of a variety of health issues through the discovery of novel herbal drugs.

Conflict of Interest

The authors declare no conflict of interest.

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