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Original Article

Phytochemical Profiling, Antioxidant and Antimicrobial Activities of a Traditional Polyherbal Formulation Containing *Citrus limon, Bergera koenigii, Citrus medica* and *Andrographis paniculata*

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ABSTRACT

Background: Polyherbal formulations combining medicinal plants are widely studied for their enhanced therapeutic potential. **Materials and Methods:** This study evaluates a polyherbal formulation containing leaves of *Citrus limon, Bergera koenigii, Citrus medica*, and *Andrographis paniculata* for its antimicrobic and antioxidative properties. An ethanolic extract was examined to determine its phytochemical composition, which revealed a rich existence of bioactive substances like bioflavonoids, alkali-like compounds, phenolics, and Steroidal glycoalkaloids. **Results:** *In vitro* Total Antioxidant Capacity (TAC), including DPPH radical, FRAP and scavenging of nitric oxide, exhibited significant radical-neutralizing capacities comparable to standard antioxidants. The formulation also exhibited potent antibacterial action against strains of fungi and bacteria, both gram-positive and gram-negative, suggesting a synergistic enhancement of efficacy due to the combination of plant extracts. **Conclusion:** The outcomes highlight the prospect of this polyherbal extract as a natural therapeutic agent to treat microbial infections and oxidative stress. The future studies should focus upon *in vivo* evaluations and elucidating molecular mechanisms to validate these promising results.

Keywords: Polyherbal Formulation, Phytochemicals, Oxidative Stress, Natural Therapeutics.

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INTRODUCTION

The increasing interest in natural products, particularly from medicinal plants, has driven research into their therapeutic potential for treating a range of diseases. Bioactive substances present in these plants, including alkaloids, flavonoids, phenolics, and terpenoids, was demonstrated to exhibit a vast array of biological processes, including antimicrobial, anti- inflammatory, anticancer, and antioxidant effects.[1,2] Antioxidants play a crucial function in preventing oxidative stress, which is related to cardiovascular conditions and other chronic conditions, diabetes, neurodegenerative diseases, and cancer.[3,4]

An imbalance between the body's antioxidant defense systems and the generation of Reactive Oxygen Species (ROS) results in oxidative stress.[5] A significant tendency has emerged recently toward utilizing natural antioxidants derived from plants as safer and more sustainable alternatives to synthetic antioxidants.[6] These natural antioxidants are favored due to their lower toxicity, fewer side effects, and additional health-promoting benefits. The concept of polyherbal formulations has a broad therapeutic index which combines multiple medicinal plants, has gained attention for its synergistic effects, providing enhanced therapeutic properties. Polyherbal formulations are commonly preferred for treatment due to their proven safety and effectiveness. The application of such combinations in conventional medicine, where various plant species are used together to treat complex ailments, has been a longstanding practice.[7-9]

The current investigation aims to assess the antioxidant potential of a Polyherbal Formulation (PHE) composed of medicinal plants with a reputation for being bioactive. This formulation includes *Citrus limon, Bergera koenigii, Citrus medica*, and *Andrographis paniculata*, which individually have demonstrated significant Antimicrobial, anti-inflammatory, and antioxidant

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properties. The choice of these plants for the PHE preparation is based on their individual therapeutic properties, especially their antioxidant, anti-inflammatory, and antimicrobial effects. *Citrus limon* (lemon), *Bergera koenigii* (curry leaves), *Citrus medica* (bitter citron), and *Andrographis paniculata* (Kalmegh) each has a long history of being used medicinally and contain bioactive substances that support their therapeutic potential.[10,11] By combining these plants, the polyherbal formulation aims to harness the synergistic effects of their active constituents, potentially enhancing the overall pharmacological efficacy of the extract.[12]

Citrus limon (Lemon) is a widely recognized fruit renowned for having a lot of vitamin C, flavonoids, and phenolic compounds. Studies have reported that *C. limon* possess potent antibacterial, antifungal, anticancer as well as antioxidant qualities.[13] These bioactive components present in the potent antioxidants found in *C. limon* aid in scavenging free radicals and lowering oxidative stress.[14] Additionally, lemon has been traditionally utilized because of its antibacterial and anti-inflammatory qualities, making it a valuable component in the polyherbal formulation for combating oxidative damage and assisting with immunological function.[15] *Bergera koenigii* (Curry Leaves) are abundant in phenolic compounds, alkaloids, flavonoids, and essential oils that have antibacterial, anti-inflammatory, and antioxidant properties.[16] These substances aid in scavenging Reactive Oxygen Species (ROS) and lowering oxidative stress, both of which are important factors in chronic illnesses. Curry leaves also have hepatoprotective characteristics, making them a crucial plant for preserving general health characteristics, making them a crucial plant for preserving general health and preventing cellular damage from environmental toxins.[17] In a particular study *Citrus medica* (Bitter Citron) is recognized for its high levels of essential oils, flavonoids, and alkaloids.[18] It has strong antioxidant characteristics and has long been utilized in herbal therapy to relieve digestive issues, inflammation, and infections. The presence of these bioactive compounds in *Citrus medica* enhances the antioxidant capacity of the polyherbal formulation, particularly in combating oxidative stress and supporting cardiovascular health.[19] Recently Research have revealed that phytoconstituents present in *C. medica* has potential in reducing viral load of SARS-CoV-2.[20] Traditional medicine has made use of *Andrographis paniculata* because of its many health advantages, such as its antibacterial, anti-inflammatory, and antioxidant properties.[21] The active compounds such as andrographolide, are known for their potent free radical scavenging abilities as well as anti-inflammatory properties, therefore it is a crucial plant for reducing oxidative stress and supporting immune health in the polyherbal formulation.[22]

By combining these four plants, the Polyherbal Extract (PHE) harnesses the synergistic effects of their bioactive constituents, enhancing the overall therapeutic efficacy. The antioxidant activities of each plant complement one another, providing enhanced protection against oxidative damage and promoting overall health.[23,24] The polyherbal approach also helps minimize the side consequences linked to the usage of individual plants, offering a balanced and holistic remedy with multiple therapeutic effects.[25] The primary objective of this research was to assess the antioxidant potential of the ethanolic extract of PHE using a variety of *in vitro* tests, such as hydrogen peroxide scavenging, nitric oxide scavenging, superoxide anion scavenging, hydroxyl radical scavenging, Ferric Reducing Antioxidant Power (FRAP), and DPPH radical scavenging.

By determining the antioxidant capacity and chemical composition of this polyherbal extract. The goal of the study is to offer insightful information about its possible therapeutic uses in managing oxidative stress-related disorder. This research may also pave the way for further investigations into the synergistic effects of combined medicinal plants in polyherbal formulations.

MATERIALS AND METHODS

Materials

Fresh *Citrus limon, Bergera koenigii, Citrus medica*, and *Andrographis paniculata* leaves were collected from its natural habitat at their peak maturity from Coimbatore, Tamil Nadu, India. The Botanical Survey of India verified the authenticity of the plant samples. For future reference, a voucher specimen was kept in our lab. Every other chemical and reagent utilized was of the caliber of an analytical reagent.

Methods

Gathering and Preparing the Plant Material

The collected samples were thoroughly washed to remove any dirt, dust, or other contaminants. After washing, the plant materials were allowed to dry at room temperature in the shade thoroughly under controlled conditions to preserve their chemical composition and prevent degradation from direct sunlight. Once fully dried, a coarse powder was created by grinding the plant ingredients.

Extraction Procedure

To prepare the Polyherbal Extract (PHE) formulation, the coarse powders of the four plant samples were mixed in equal ratios (1:1:1:1). This uniform mixture of Plant material powder was then undergoing Soxhlet extraction in different solvents like Chloroform, Ethanol, Hydroethanol and Water to extract both lipophilic and hydrophilic compounds. After the extraction, In order to extract the solvent and use it for additional analysis, the solvents were evaporated using a rotary evaporator set to 40°C and lower pressure.

Preliminary phytochemical profiling

The prepared Polyherbal Extract (PHE) formulation in different extracts like chloroform, Utilizing various established techniques, ethanol, methanol, and aqueous extracts were screened for precursory phytochemicals like alkaloids, flavonoids, carbohydrates, proteins and amino acids, sterols, triterpenoids, tannins, phenolics, and saponins.[26]

In vitro Polyherbal Extract Antioxidant Assay

Ferric-Reducing Antioxidant Power (FRAP) Determination

The Fe^{2+}/ferricyanide complex is reduced to its ferrous state, forming a violet-colored solution, which is the basis for the Ferric Reducing Antioxidant Power (FRAP) assay. Stronger antioxidant activity is shown by a higher absorbance, which is directly correlated with the sample's reducing capacity. By measuring a substance's capacity to contribute electrons to neutralize free radicals, this technique gives an indication of its reducing power.[27]

The Poly Herbal Extract (PHE) FRAP was calculated in this investigation using the method described by.[28] Different quantities of the extract (50-250 mg/mL) were combined with 2.5 mL of 200 mM Phosphate buffer (pH 6.6) and 2.5 mL of 30 mM potassium ferricyanide to create the reaction mixture. The mixture was incubated for 20 min at 50°C. After that, 2.5 mL of 600 mM trichloroacetic acid was added. The mixture was centrifuged for 10 min at 3000 rpm, and 2.5 mL of the supernatant was combined with 0.5 mL of 6 mM ferric chloride and 2.5 mL of distilled water. A measurement of the resultant solution's absorbance was made at 700 nm. Ascorbic acid was utilized as a standard reference in the preparation of a blank sample, which did not contain the extract, for calibration. Every test was run three times.

DPPH Free Radical Scavenging Activity Determination

The DPPH scavenging activity in the PHE was measured using the technique.[29] This technique assesses a compound's antioxidant qualities by looking at how well it can neutralize free radicals or donate hydrogen atoms. The DPPH radical, which has a deep violet hue, is transformed into a pale yellow in this assay compound, 2,2-diphenyl-1-picrylhydrazine, upon interaction with antioxidants. The change in color corresponds to a reduction in absorbance, which is measured at 517 nm.

To perform the assay, the ethanolic extract of the polyherbal formulation was made with concentrations between 0.05 and 5 mg/mL. One milliliter of the extract, three milliliters of methanol, and half a milliliter of a 1 mM DPPH solution in methanol comprised the reaction mixture. Following complete mixing with a vortex, the solution was left to remain at room temperature for 5 min before the absorbance at 517 nm was measured. Methanol combined with DPPH served as the blank, while a standard solution of vitamin C had been employed as the positive control. Three duplicates of each measurement were made, and the following formula was used to determine the percentage of radical scavenging activity:

$$\%\ \text{DPPH Radical Scavenging activity} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100$$

Where command OD=the sample or blank optical density OD stands for optical density of the standard or extract.

Plotting various concentrations allowed us to calculate the percentage DPPH radical scavenging activity, and a graphic representation of the IC_{50} (half maximum Inhibitory Concentration) was produced.

Nitric Oxide Radical Scavenging Activity Determination

The experiment depends on Sodium Nitroprusside (SNP) producing Nitric Oxide (NO) in an aqueous media. By reacting with molecular oxygen, the released NO produces nitrite ions, which the Griess reagent can detect. The presence of nitric oxide scavengers in the sample reduces the production of nitrite ions by competing with molecular oxygen. The assay's foundation is this interaction.

A modified procedure based on was used to assess the Polyherbal Extract's (PHE) capacity to scavenge nitric oxide radicals.[30] 5 mM SNP in Phosphate-buffered saline (pH 7.4) and different amounts of PHE (250-2500 mg/mL) made in ethanol were added to a reaction mixture, which was then incubated for 30 min at 25°C. After incubation, 1.5 mL of Griess reagent was combined with 1.5 mL of the reaction mixture. 0.1% N-1-naphthyl Ethylenediamine Dihydrochloride (NED), 1% sulfanilamide, and 2% phosphoric acid made up the reagent. After reacting with sulfanilamide and NED, nitrite ions diazotized to produce a pink chromophore. At 546 nm, the absorbance of the resultant solution was determined using a blank that contained every element except the extract. The standard reference chemical used was quercetin. The following formula was used to determine the percentage of nitric oxide radical scavenging activity for each experiment, which was carried out in triplicate:

$$\%\ \text{Nitric oxide radical scavenging activity} = \frac{A_0 - A_1}{A_0} \times 100$$

Where A_1 is the extract/standard absorbance and A_0 is the blank control reaction absorbance.

Superoxide Radical Scavenging Activity Measurement

This experiment evaluates the extract's capacity to counteract superoxide radicals produced in a riboflavin-light-NBT system. Nitro Blue Tetrazolium (NBT) is converted to formmazan, a colorful substance, by superoxide radicals. By scavenging these radicals, the extract inhibits formazan formation, which can be quantified spectrophotometrically.[31]

A 3 mL reaction mixture including 50 mM sodium phosphate buffer (pH 7.6), 20 mg riboflavin,12 mM Ethylenediaminetetraacetic Acid (EDTA), 0.1 mg NBT, and varying doses of the Polyherbal Extract (PHE) or a standard substance from 50 to 250 mg/mL was prepared in order to conduct the experiment. The reaction mixture spent 90 sec under the light. The blank was a unilluminated combination devoid of PHE, while the negative control was a reactive mixture without the extract but exposed to illumination. The resultant formazan's absorbance was measured at 562 nm following illumination. Three duplicates of each experiment were carried out, with quercetin serving as the positive control. Using the following formula, the scavenging activity (%) was determined:

$$\% \text{ Superoxide radical scavenging activity} = \frac{A_0 - A_1}{A_0} \times 100$$

Where A_1 is the extract/standard absorbance and A_0 is the reaction control absorbance (blank).

Assessment of Hydroxyl Radical Scavenging Activity (-OH)

This test assesses the extract's capacity to stop the Fenton system-assisted reaction that breaks down deoxyribose due to hydroxyl radicals. The production of the hydroxyl radicals through the interaction of Fe^{3+}-EDTA, ascorbic acid, and hydrogen peroxide (H_2O_2), which initiate the degradation of deoxyribose. The assay is based on the extract's inhibitory action on this process.[32,33]

The reaction mixture was made by mixing 100 µL of 28 mM 2-deoxy-2-ribose, 20 mM potassium dihydrogen phosphate buffer (KH_2PO_4-KOH, pH 7.4), 200 µL of 200 µM $FeCl_3$- EDTA solution (1:1 v/v), 100 µL of 1 mM H_2O_2, 100 µL of 1 mM ascorbic acid, and different amounts of the Polyherbal Extract (PHE) ranging from 100 to 500 µg/mL. A final volume of 1 mL was set for the reaction mixture. To create a pink chromogen, 1 mL of 1% Thiobarbituric Acid (TBA) and 1 mL of 2.8% Trichloroacetic Acid (TCA) were added after an hour of incubation at 37°C. The mixture was then heated to 100°C for 20 min. After cooling, the absorbance of the solution was measured at 532 nm. Gallic acid was utilized as the positive control, and a blank solution comprising all the chemicals except the extract was utilized for comparison. Every experiment was carried out three times. The following formula was used to calculate the hydroxyl radical scavenging activity (%):

$$\% \text{ Hydroxyl radical scavenging activity} = \frac{A_0 - A_1}{A_0} \times 100$$

Where A_0 is the blank control absorbance and A_1 is the extract/standard absorbance.

Lipid Peroxidation Activity Determination

The amount of Malondialdehyde (MDA), a consequence of the oxidative breakdown of polyunsaturated fatty acids, generated is measured in this experiment to assess the inhibition of lipid peroxidation. The pink chromogen that results from the reaction of MDA and Thiobarbituric Acid (TBA) has the highest absorbance at 532 nm.[34]

The assay was carried out using, with minor adjustments,[35] methodology. The reaction mixture, which had a final volume of 1 mL, was made by combining different concentrations of the Polyherbal Extract (PHE) ranging from 50 to 250 µg/mL with 2 mL of the TCA-TBA-HCl reagent, which contains 15% (w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid, and 0.25 N hydrochloric acid. The mixture was centrifuged at 10,000 rpm for 10 min to extract precipitated proteins, cooled to room temperature, then heated in a water bath at 90°C for 10 min to speed up the reaction. The clear supernatant, which contained the MDA-TBA adduct and exhibited a light pink color, was compared to a blank solution using spectrophotometry at 532 nm. The reference standard was ascorbic acid. The following formula was used to determine the percentage of lipid peroxidation inhibition in each experiment, which was carried out in triplicate:

$$\% \text{ Lipid peroxidation} = \frac{A_0 - A_1}{A_0} \times 100$$

Where A_1 is the extract/standard absorbance and A_0 is the blank control absorbance.

Hydrogen Peroxide Radical Scavenging Activity Determination

This test determines if antioxidants can neutralize Hydrogen peroxide (H_2O_2) and cause a detectable drop in absorbance. The scavenging ability of the compounds is directly correlated with the lowering of H_2O_2.[36] The assay was performed using a modified method based on.[37] H_2O_2 was produced in a 40 mM solution in phosphate buffer (pH 7.4). The Polyherbal Extract (PHE), was combined with the hydrogen peroxide solution and let to sit at room temperature for 10 min at concentrations between 0.1 and 0.5 mg/mL. Using spectrophotometry, the absorbance drop was detected at 230 nm. Ascorbic acid was utilized as the positive reference standard, while a blank solution comprising a phosphate buffer devoid of H_2O_2 served as the control. The following formula was used to determine the percentage of hydrogen peroxide scavenging activity for each experiment, which was carried out in triplicate:

$$\% \text{ Hydrogen peroxide radical scavenging activity} = \frac{A_0 - A_1}{A_0} \times 100$$

Where, A1 is the extract/standard absorbance and A_0 is the blank control absorbance.

Analysis of Gas Chromatography-Mass Spectrometry

To identify the phytochemical compounds, present in the ethanolic PHE obtained from *Citrus limon, Bergera koenigii, Citrus medica*, and *Andrographis paniculata* leaves, GC-MS QP 2010 plus (Shimadzu) was employed. The oven was set to 70°C for 5 min, and then it was changed to between 100 and 250°C for 35 min. For this investigation, a carrier gas of high purity helium was

employed. The ionization voltage of the MS 220°C was regulated by the EI process at 70 eV, and the helium gas flow rate was set at 1.0 mL/min with a split proportion of 1:20 mode for the injection of 1µl of sample. The sample was injected into the GC column where it was separated based on the volatility and polarity of its constituents. The individual components were then detected by mass spectrometry, where each compound's molecular ion and fragment ions were recorded, enabling the chemicals to be identified using their mass spectra. To verify the compounds' identities, the phytochemical constituents were identified using information from the GC-MS analysis and compared with spectral databases [NIST 20M1 LIBRARY].

The antimicrobial property Preparation of the bacterial inoculum

MTCC737 was a stock culture of bacteria that included both Gram Positive (*Staphylococcus aureus*) and Gram Neg (*Escherichia coli*, MTCC1687, *Pseudomonas aeruginosa*, MTCC1688, and *Klebsiella pneumoniae*, MTCC530). Stock cultures were kept on blood and nutrient agar slopes at 4°C. A loopful of cells was transferred from stock cultures to test tubes with 50 mL of nutrient broth in order to create an active culture for the experiments. Additional bacterial cultures were cultured on a shaking incubator at 37°C for 24 hr. The fungal strain was cultured for three to five days at 25°C. The cultures of bacteria and fungi were further incubated for 24 hr at 37°C and 3-5 days at 25°C, respectively.

Minimum Level of Inhibition

The broth microdilution method was used in 5-well plates to calculate the MIC of the ethanolic PHE.[38] To get a concentration of 100 mg/mL, the extracts were dissolved in 20% Ethanol. They were then serially diluted to get concentrations between 100 and 5 mg/mL. In a microplate reader (T0), the Optical Density (OD) was measured at 660 nm prior to incubation. The 5-well plates were kept at 37°C for 24 hr on an incubator that was shaken regularly. The plates were discovered again after a 24-hr period (T24). The MIC is the lowest dose at which the organism being studied is totally inhibited from growing.

Antibacterial activity by well diffusion process

The well diffusion method was used to perform antibacterial and antifungal activity. Both Gram Positive (*Staphylococcus aureus*; MTCC737) and Gram Neg (*Escherichia coli*; MTCC1687, *Pseudomonas aeruginosa*; MTCC1688, and *Klebsiella pneumoniae*; MTCC530) Ethanolic extract of the PHE was dissolved in 10% Ethanol. The test organisms mentioned above were swabbed on the plates. Following different concentration of extract of PHE (25, 50, 75 and 100 µg) was poured in each well. diffused plates were incubated at 37°C, the diameter of the inhibitory zone was measured in millimeters. The negative control was autoclaved distilled water. For bacterial cultures, chloramphenicol was employed as the reference standard, whereas fluconazole was employed for fungal strains. Several times, the experiment was conducted in duplicate.[39]

Analysis of Statistics

In order to assess the statistical significance of the data, the results (mean±standard deviation) from this study were subjected to One-Way Analysis of Variance (ANOVA) using GraphPad v 9.0 (for Windows, GraphPad Software, San Diego, California, USA) and Graphpad-InStat version 3.05. Statistical significance was defined as a probability value of $p<0.05$.

RESULTS

Phytochemical analysis

The outcomes of the polyherbal extract's qualitative Phytochemical Analysis (PHE) prepared from the leaves of *Citrus limon, Bergera koenigii, Citrus medica*, and *Andrographis paniculata* shown in Table 1. Different extracts showed varied composition of phytoconstituents however ethanolic extract showed presence of almost all phytoconstituents like alkaloids,

Table 1: Qualitative Polyherbal Extract Phytochemical Screening.

Sl. No.	TEST	Chloroform	Ethanol	Methanol	Aqueous
1.	Alkaloids				
	Dragendorff's	+	+	+	-
	Wagner's	+	+	+	+
2.	Flavonoids				
	Alkaline reagent	-	+	+	+
	Zinc test	-	+	+	+
3.	Carbohydrates				
	Molisch test	+	+	+	+
	Fehling's test	+	+	-	-
	Benedicts test	+	+	+	-
4.	Amino acids and proteins				

Sl. No.	TEST	Chloroform	Ethanol	Methanol	Aqueous
	Biuret test	+	+	+	+
	Millon's test	+	+	+	-
	Ninhydrin test	+	+	+	-
	Glycosides	+	+	+	+
5.	Sterols and triterpenoids				
	Liebermann–Burchard test	+	+	+	+
	Salkowski's test	+	+	+	+
6.	Tannins and phenolics				
	Iodine test	-	+	+	+
	Nitric acid test	-	-	-	-
	Ferric chloride test	+	+	+	-
	Quininoes	+	+	+	-
7.	Saponins	+	+	-	+

(+)/(-) Sign indicates presence and absence of phytoconstituents respectively.

flavonoids and so on. Hence based on the primary screening for phytochemicals for additional examination, the ethanolic extract of PHE was employed. This comprehensive phytochemical profile underscores the potential bioactivity of the PHE.

In vitro antioxidant assay

The Polyherbal Extract (PHE) *in vitro* antioxidant tests demonstrated significant radical scavenging activities for all the methods tested including assays for hydrogen peroxide, DPPH radical scavenging, nitric oxide, superoxide, hydroxyl, and lipid peroxidation. The IC_{50} values indicated strong antioxidant potential.

Ferric reducing antioxidant capacity

The ethanolic extract of the Polyherbal (PHE) formulation Ferric Reducing Antioxidant Power (FRAP) is presented in Figure 1. The results showed concentration dependent increase in the ferric reductive activity, The range is 50-250 µg/mL. The ethanolic extract's activity at all concentrations was equivalent to that of regular ascorbic acid, with slightly lower values at higher concentrations. These findings demonstrate the strong ferric reducing potential of the ethanolic extract, highlighting its effective antioxidant capacity.

Activity of DPPH radical scavenging

The Figure 2 displays the Polyherbal Formulation's (PHE) ethanolic extract's DPPH radical scavenging efficacy. PHE demonstrated impressive *in vitro* DPPH radical scavenging activity in a dose-dependent pattern between doses of 0.5 and 3 mg/mL. The PHE ethanolic extract's percentage DPPH radical scavenging activities demonstrated 19±1.5% inhibition at 0.5 mg/mL, but they progressively increased and peaked at 89±3.5% at 3 mg/mL. The results of the vitamin C standard were similar to those of the PHE extract, with an inhibition of 93±5.6% at 3 mg/mL.

Radical Scavenging Activity of Nitric Oxide

Figure 3 shows the Polyherbal Formulation's (PHE) ethanolic extract's capacity to scavenge nitric oxide radicals. PHE's nitric oxide scavenging activity was superior to that of the quercetin standard under investigation. PHE was investigated at a range of concentrations, from 100 to 500 µg/mL. The results demonstrated inhibition of 23±1.8% for 100 µg/mL however it gradually increased to 82±5.6% for 500 µg/mL which is comparable to quercetin standard which showed inhibition of 85±4.1% at 500 µg/mL.

Activity of Superoxide Radical Scavenging

The Polyherbal formulation (PHE) ethanolic extract's capacity to scavenge superoxide radicals is demonstrated in Figure 4. Studies have shown that the presence of superoxide anion can cause damage to the biomolecules by formation of hydrogen peroxide, singlet oxyget or peroxy nitrite and so on.[40] As the quantity of PHE grew from 50 to 250 µg/mL, its superoxide scavenging activity improved significantly. The results demonstrated inhibition of just 19±2.4% for 50 µg/mL however it gradually increased to 83±2.4% for 250 µg/mL which is comparable to quercetin standard which showed inhibition of 86±2.6% at 250 µg/mL.

Hydroxyl Radical ($^{-}$OH) Scavenging Activity

The Polyherbal Formulation's (PHE) ethanolic extract's hydroxyl radical scavenging efficacy shown in Figure 5. A number of hydroperoxides combine with transition metal ions to produce hydroxyl radicals, which are extremely reactive.[40] The PHE's ability to scavenge hydroxyl radicals rose significantly as its concentration grew from 100 to 500 µg/mL. The results

demonstrated inhibition of 24±1.6% for 100 µg/mL however it gradually increased to 85±5.2% for 500 µg/mL which is comparable to Gallic acid standard which showed inhibition of 88±5.3% at 500 µg/mL.

Activity of Lipid Peroxidation

The ethanolic extract capacity to peroxide lipids of the Polyherbal Formulation (PHE) is presented in Figure 6. PHE showed superior lipid peroxidation activity as that of the Ascorbic acid standard studied. PHE was investigated at a range of concentrations, from 50 to 250 µg/mL. The results demonstrated inhibition of 19±2.3% for 50 µg/mL however it gradually increased to 84±3.4% for 250 µg/mL which is comparable to Ascorbic acid standard which showed inhibition of 86±4.2% at 250 µg/mL.

Activity of Hydrogen Peroxide Radical Scavenging

The Polyherbal Formulation's (PHE) ethanolic extract's hydrogen peroxide radical scavenging ability is demonstrated in Figure 7. PHE showed superior lipid peroxidation activity as that of the Ascorbic acid standard studied. PHE was studied with a different range of concentrations 0.1-0.5 mg/mL. The results demonstrated inhibition of 12±1.4% for 0.1 mg/mL however it gradually

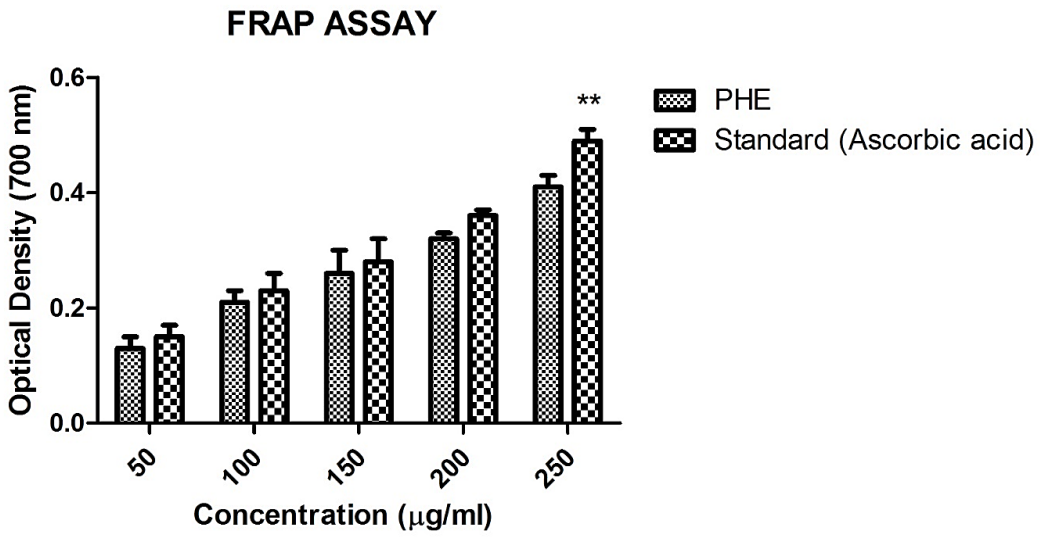

Figure 1: *In vitro* ferric reducing antioxidant power of ethanolic PHE extract comprising *Citrus limon, Bergerakoenigii, Citrus medica*, and *Andrographis paniculata* leaves. Bar graphs denotes the optical density values for the PHE and the ascorbic acid standard studied. Bar graphs with asterisks (**) within the same concentration are significant (p<0.01).

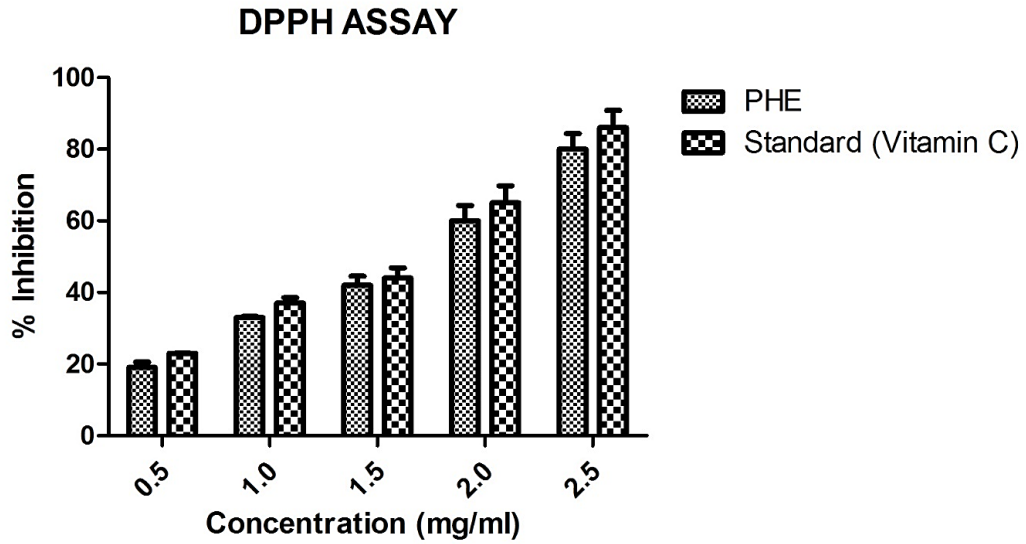

Figure 2: DPPH radical scavenging activity of ethanolic PHE extract comprising *Citrus limon, Bergerakoenigii, Citrus medica*, and *Andrographis paniculata* leaves. Bar graphs denotes the percentage inhibition values for the PHE and the Vitamin C standard studied.

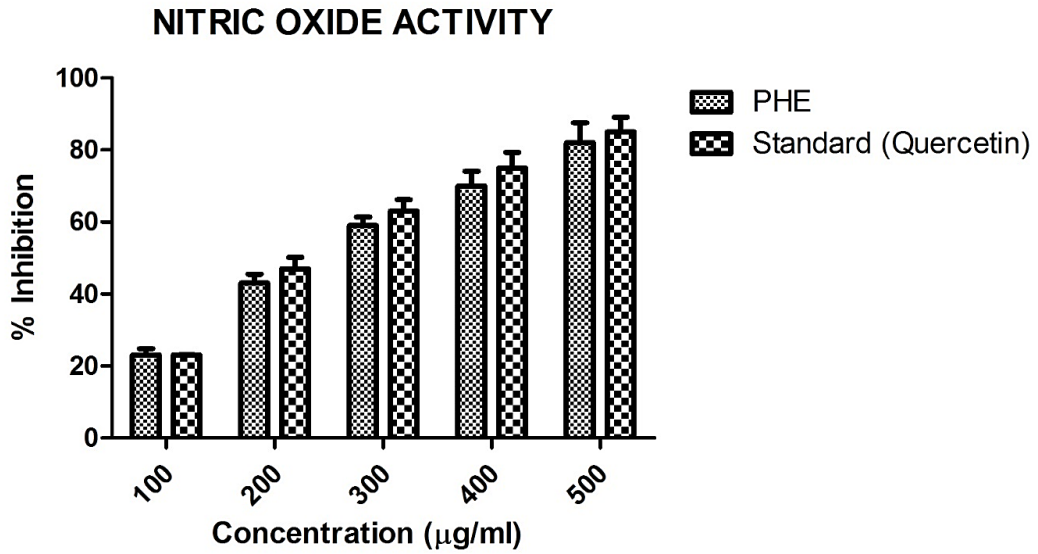

Figure 3: Nitric Oxide Radical Scavenging activity of ethanolic PHE extract comprising *Citrus limon, Bergerakoenigii, Citrus medica*, and *Andrographis paniculata* leaves. Bar graphs denotes the percentage inhibition values for the PHE and the quercetin standard studied.

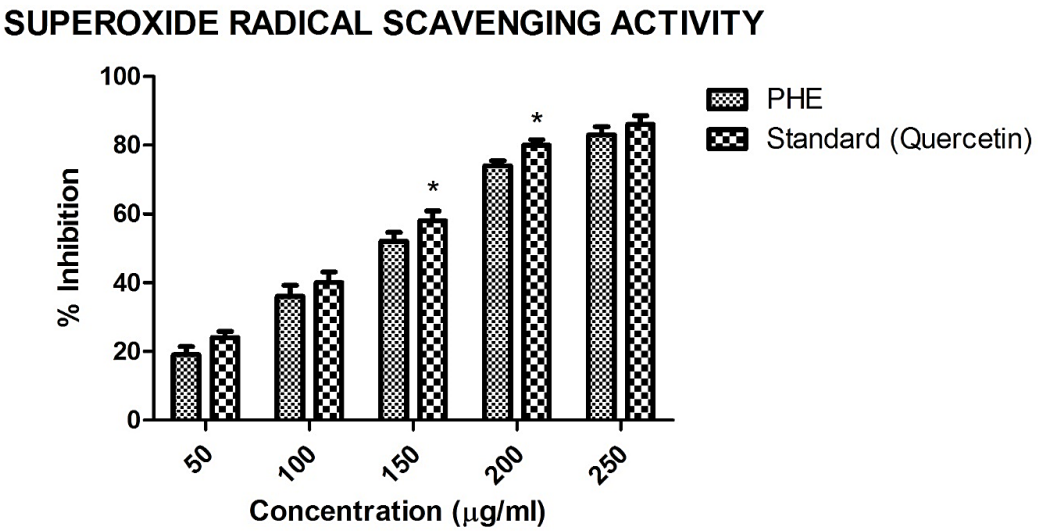

Figure 4: Superoxide Radical Scavenging activity of ethanolic PHE extract comprising *Citrus limon, Bergerakoenigii, Citrus medica*, and *Andrographis paniculata* leaves. Bar graphs denotes the percentage inhibition values for the PHE and the quercetin standard studied. Bar graphs with asterisks (*) within the same concentration are significant ($p<0.05$).

increased to 80±5.9% for 0.5 mg/mL which is comparable to Ascorbic acid standard which showed inhibition of 89±6% at 0.5 mg/mL. The Polyherbal Extract (PHE) antioxidant properties were evaluated using multiple *in vitro* assays, including DPPH, Nitric Oxide (NO), superoxide, Hydroxyl (OH), lipid peroxidation and hydrogen peroxide scavenging methods, as shown in Table 2. The IC_{50} values for the PHE were 1.471 mg/mL for DPPH, 229 µg/mL for NO, 127.8 µg/mL for superoxide, 250.8 µg/mL for OH, 122.4 µg/mL for hydrogen peroxide scavenging tests, and 0.28 mg/mL for lipid peroxidation tests. The DPPH and hydrogen peroxide scavenging capabilities of these were similar to the standards, ascorbic acid and quercetin, demonstrating significant free radical neutralizing potential. The lipid peroxidation and superoxide scavenging assays also indicated notable antioxidant activity with IC_{50} values close to the respective reference standards. The comprehensive antioxidant profile of the PHE suggests its potential efficacy in mitigating oxidative stress through multiple pathways.

Analysis of GC-MS

Analysis of GC-MS with ethanolic PHE led to discovery of fifty constituents. The main constituents of the PHE were

n-Hexadecanoic acid (16.55%), 7-Tetredecanal (11.70%), Phytol (6.78%) and Benzyl Benzoate (5.96%), Phytol, Stevioside, Andrograpanin, Nonacosane, Tocopherol and additional trace elements have been found as shown in Figure 8 and Table 3.

Antimicrobial activity

The ethanolic extract of the PHE comprising *Citrus limon, Bergera koenigii, Citrus medica*, and *Andrographis paniculata* leaves were tested for the antimicrobial activity. PHE shown variable levels of inhibitory activity against the tested bacteria, which included a fungal strain (*Candida albicans*) as well as Gram Positive (*Staphylococcus aureus*) and Gram Neg (*Escherichia coli, Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*) bacteria (Table 4). The growth-inhibition zone diameter was used to illustrate the findings. The findings unequivocally show that the PHE extract was effective against the tested microorganisms. The results demonstrated that the diameter inhibition zone showed high activity against *E. coli* (9.33±1.1 mm), *S. aureus* (10.63±0.9 mm), *P. aeruginosa* (8.0±1.2 mm) and *C. albicans* (8.83±0.4mm) however moderate activity against *K. pneumoniae* (5.5±0.6 mm). The results obtained were compared to the standard chloramphenicol for bacteria and Fluconazole for fungal strains studied.

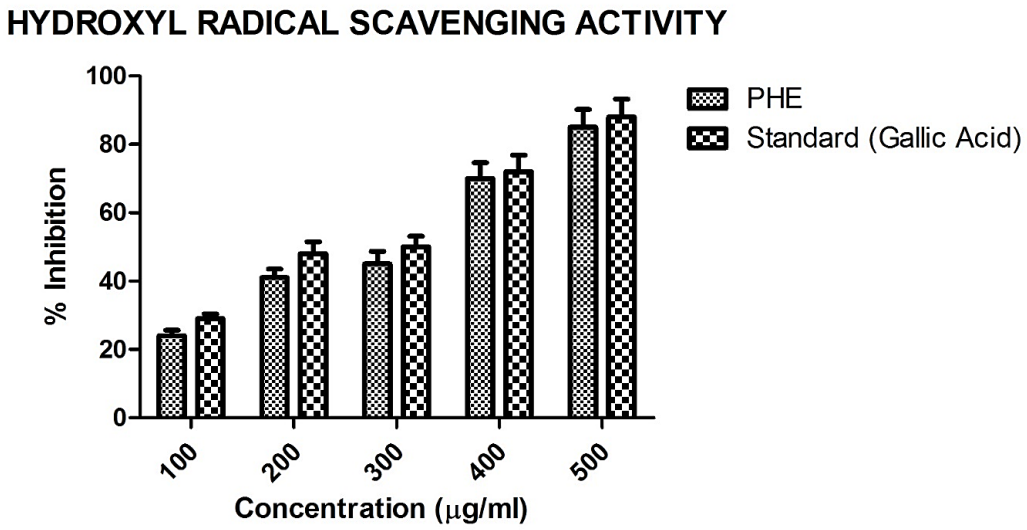

Figure 5: Hydroxyl Radical Scavenging Activity of ethanolic PHE extract comprising *Citrus limon, Bergerakoenigii, Citrus medica*, and *Andrographis paniculata* leaves. Bar graphs denotes the percentage inhibition values for the PHE and the Gallic acid standard studied.

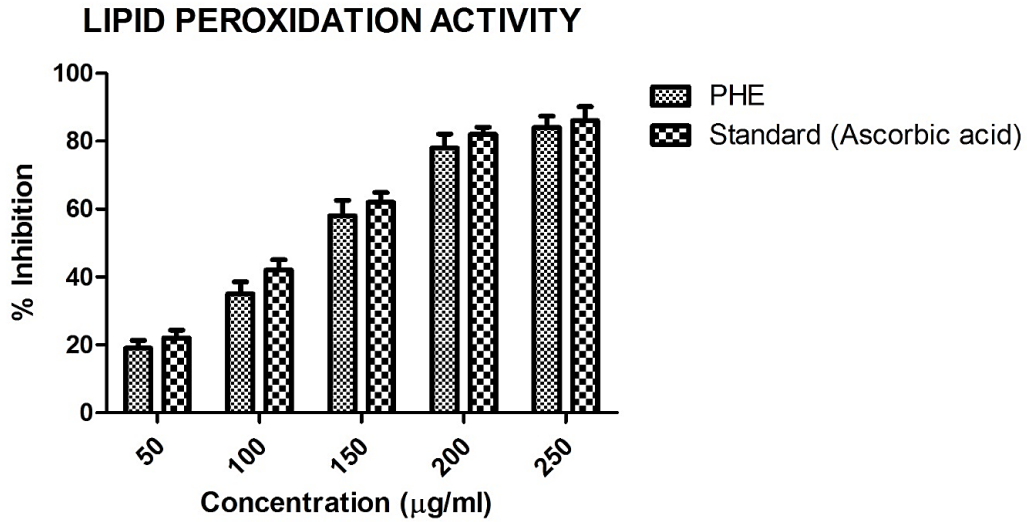

Figure 6: Lipid Peroxidation activity of ethanolic PHE extract comprising *Citrus limon, Bergerakoenigii, Citrus medica*, and *Andrographis paniculata* leaves. Bar graphs denotes the percentage inhibition values for the PHE and the Ascorbic acid standard studied.

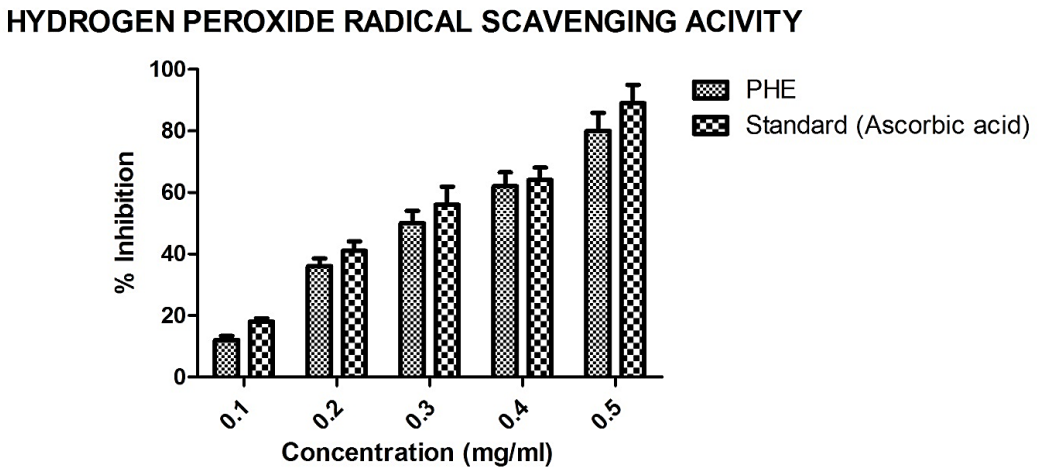

Figure 7: Hydrogen Peroxide Radical Scavenging activity of ethanolic PHE extract comprising *Citrus limon, Bergerakoenigii, Citrus medica*, and *Andrographis paniculata* leaves. Bar graphs denotes the percentage inhibition values for the PHE and the Ascorbic acid standard stdied.

Minimum Inhibitory Concentrations (MIC)

The MIC was the lowest concentration at which 100% growth inhibition was observed. The results of the MIC of ethanolic PHE for the bacterial and fungal strains under investigation are displayed in Table 5. The MIC (Minimum Inhibitory Concentration) results for PHE against the tested microorganisms indicate varying activity. The lowest concentration inhibiting growth for *E. coli* and *C. albicans* is 25µg/mL, while *S. aureus* shows inhibition at 25 mg/mL. Increased doses of 50 mg/mL and 75 mg/mL, respectively, are found to inhibit *P. aeruginosa* and *K. pneumoniae*. This demonstrates the compound's differential antimicrobial potential across microbial species.

DISCUSSION

In this investigation, we assessed the phytochemical composition, antimicrobial and antioxidant activities of a polyherbal formulation comprising the leaves of *Citrus limon, Bergera koenigii, Citrus medica*, and *Andrographis paniculata*. The results demonstrate promising antioxidant and antimicrobial activities, which align with these plants historical application in traditional medicine.

The screening of phytochemicals identified several bioactive substances, such as terpenoids, alkaloids, phenolic compounds, and flavonoids. Their antibacterial and antioxidant qualities are well-known.[41,42] The identification of these bioactive molecules supports the therapeutic potential of the polyherbal formulation. Earlier research has also documented the existence of such substances in *Citrus limon* (*C. limon*), *Andrographis paniculata*, and *Bergera koenigii*.[43] For example, *C. limon* is rich in flavonoids such as quercetin and rutin, which have demonstrated potent antioxidant qualities. The observed antioxidant and antimicrobial activities of the polyherbal formulation corroborate prior findings reported by Klimek-Szczykutowicz *et al.*, (2020), who documented

Table 2: IC_{50} **Concentration of the Poly Herbal Extract (PHE).**

Antioxidant Assay	Sample	IC_{50} of Sample
DPPH Free Radical Scavenging Activity	PHE	1.471 mg/mL
	Standard (Vitamin C)	1.324 mg/mL
Nitric Oxide Radical Scavenging Activity	PHE	229 µg/mL
	Standard (Quercetin)	210.5 µg/mL
Superoxide Radical Scavenging Activity	PHE	127.8 µg/mL
	Standard (Quercetin)	112.1 µg/mL
Hydroxyl Radical ($^{\cdot}OH$) Scavenging Activity	PHE	250.8 µg/mL
	Standard (Gallic acid)	214.2 µg/mL
Lipid Peroxidation Activity	PHE	122.4 µg/mL
	Standard (Ascorbic Acid)	108.8 µg/mL
The Radical Scavenging Activity of Hydrogen Peroxide	PHE	0.28 mg/mL
	Standard (Ascorbic Acid)	0.24 mg/mL

the pharmacological efficacy of *Citrus limon*, attributing its bioactivity to a high content of flavonoids, phenolics, and essential oils. Notably, the present study advances this understanding by demonstrating that the integration of *Citrus limon* with *Bergera koenigii, Citrus medica*, and *Andrographis paniculata* yields a synergistic enhancement in bioactivity, as evidenced by superior radical scavenging efficiencies and broad-spectrum antimicrobial effects, thereby underscoring the formulation's potential as a multifunctional therapeutic candidate.[44] Similarly, *Andrographis paniculata* contains andrographolides, known for their anti-inflammatory and antimicrobial effects.[45]

Table 3: Identification of active phytoconstituents using GC-MS analysis of Poly Herbal Extract (PHE).

Peak#	R.Time	Peak Area	Peak Area%	Peak Height	Peak Height%	Name
1	3.042	14038113	2.18	2740571	1.53	1-Ethoxypropan-2-yl acetate
2	3.172	18757676	2.91	2909249	1.62	Propane, 1,1-diethoxy-2-methyl-
3	6.418	1890915	0.29	950610	0.53	Propane, 1,1,3-triethoxy-
4	9.163	1783893	0.28	580809	0.32	2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-
5	11.686	3892437	0.60	1779492	0.99	Bicyclo[7.2.0]undec-4-ene,
6	12.659	5970831	0.93	2440857	1.36	Phenol
7	13.382	7251811	1.12	2915943	1.62	Diethyl Phthalate
8	13.565	5003251	0.78	2050444	1.14	(-)-Spathulenol
9	13.633	7474487	1.16	3709105	2.07	trans-Z-.alpha.-Bisabolene epoxide
10	14.238	5386172	0.84	1558729	0.87	Tricyclo[5.1.0.0(2,4)]octane-5-carboxylic
11	14.465	16767334	2.60	1428405	0.80	Stevioside
12	15.428	38420265	5.96	11973403	6.67	Benzyl Benzoate
13	15.599	4669161	0.72	1941542	1.08	n-Hexadecanoic acid
14	16.094	2800640	0.43	1109492	0.62	Octadecanoic acid, 2-oxo-, methyl ester
15	16.444	2413285	0.37	1160655	0.65	2-Undecanone, 6,10-dimethyl-
16	16.496	11153260	1.73	5999093	3.34	Neophytadiene
17	16.802	1829987	0.28	776346	0.43	n-Heptadecanol-1
18	16.925	2967218	0.46	1638659	0.91	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
19	17.557	3913622	0.61	1651680	0.92	Isophytol
20	17.754	106726823	16.55	15263378	8.50	n-Hexadecanoic acid
21	17.916	5687351	0.88	2623323	1.46	Ethyl tridecanoate
22	18.387	5583788	0.87	1317009	0.73	6-Methoxyfuro[2,3-h]chromen-2-one
23	18.602	3972022	0.62	1045188	0.58	Heptadecanoic acid
24	18.795	3647995	0.57	1832664	1.02	n-Heptadecanol-1
25	19.024	7185462	1.11	3644594	2.03	6-Methyl-4,6-bis(4-methylpent-3-en-1yl)
26	19.109	43729819	6.78	16825578	9.37	Phytol
27	19.348	75433292	11.70	11348459	6.32	7-Tetradecenal, (Z)-
28	19.450	2030812	0.31	822799	0.46	1-Octadecyne
29	19.559	19521702	3.03	6632784	3.70	Octadecanoic acid
30	19.779	2150556	0.33	608857	0.34	Octadecanoic acid, ethyl ester
31	19.954	6831386	1.06	1638247	0.91	7H-Furo[3,2-g][1]benzopyran-7-one,
32	22.449	3352881	0.52	1756911	0.98	Heptacosane
33	22.565	25941360	4.02	11727911	6.53	Bis(2-ethylhexyl) phthalate
34	23.217	7504720	1.16	4137345	2.31	Hexacosane
35	23.892	1854899	0.29	390180	0.22	Heptadecyl trifluoroacetate
36	24.053	15232901	2.36	6891847	3.84	Nonacosane
37	24.316	4151305	0.64	1774398	0.99	(E)-3,7-Dimethylocta-2
38	24.919	7865546	1.22	2033228	1.13	Andrograpanin
39	25.010	18027705	2.80	6402117	3.57	Nonacosane
40	25.159	5078183	0.79	2075243	1.16	Supraene
41	25.408	2116503	0.33	633891	0.35	alpha.-Tocospiro A

Peak#	R.Time	Peak Area	Peak Area%	Peak Height	Peak Height%	Name
42	25.679	3556238	0.55	775564	0.43	alpha.-Tocospiro A
43	25.954	4453042	0.69	1520957	0.85	Heneicosyl heptafluorobutyrate
44	26.131	23238374	3.60	7345719	4.09	Nonacosane
45	26.192	2571249	0.40	1205172	0.67	(9Z,12Z,15Z)-(E)-3,7-Dimethylocta-2,
46	27.127	22389889	3.47	4046449	2.25	Mahanimbine
47	27.455	9042270	1.40	2758544	1.54	Hexacosane
48	27.936	7487844	1.16	1229402	0.68	Mahanimbine
49	29.077	19105179	2.96	5030270	2.80	Nonacosane
50	29.236	23117526	3.58	4838924	2.70	dl-.alpha.-Tocopherol
		644972980	100.00	179492036	100.00	

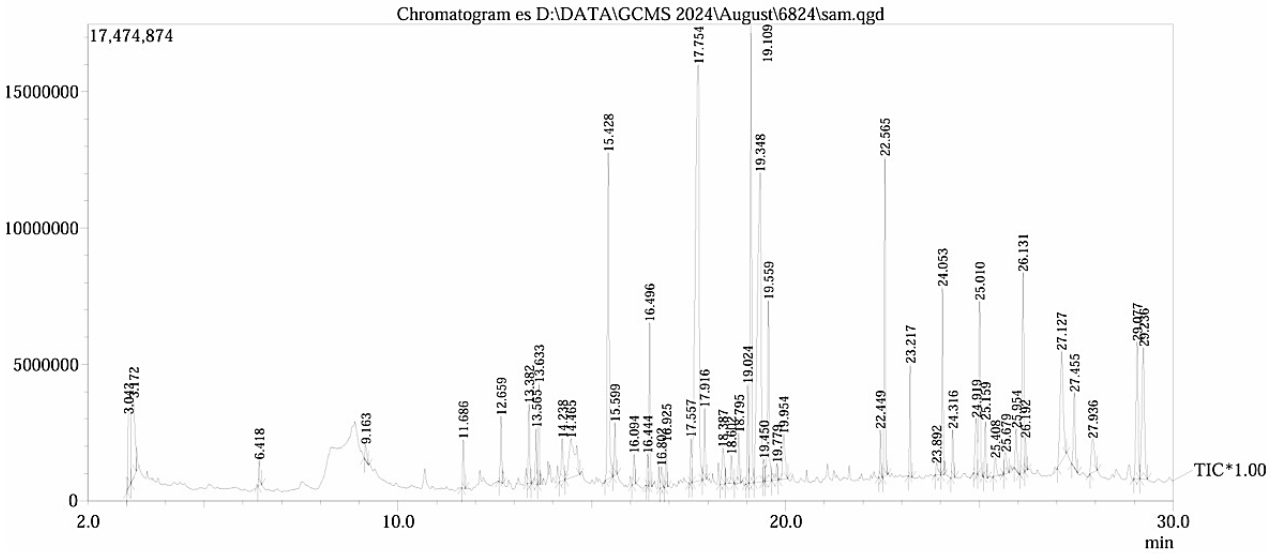

Figure 8: GC-MS chromatogram of ethanolic PHE extract showing major identified compounds.

The antioxidant capacity of the polyherbal formulation was evaluated with conventional tests, including DPPH, ABTS, and FRAP. Our results demonstrated a significant free-radical scavenging ability, which corroborates with findings from earlier studies. *Citrus limon* and *Citrus medica* are both recognized for their high antioxidant capacity due to the existence of ascorbic acid, flavonoids, and phenolic acids.[46] Moreover, *Andrographis paniculata* has been shown to have potent antioxidant properties because of its diterpenoid concentration.[47] The combined impact of these antioxidant-rich chemicals may be responsible for the synergistic effect seen in our formulation, which work together to scavenge free radicals more effectively than individual plant extracts. In line with our findings, several studies have shown that polyherbal formulations often exhibit enhanced antioxidant activities compared to their individual components.[48] This suggests that combining multiple plants with complementary antioxidant properties could result in a more potent therapeutic formulation. A broad range of strains of bacteria and fungi were used to test the polyherbal formulation's antimicrobial efficacy. The formulation exhibited strong inhibitory effects, particularly against Gram-positive bacteria, which aligns with earlier reporting on the antimicrobial potential of the individual plant species. *Bergera koenigii* has demonstrated antimicrobial characteristics that protect against a variety of infections, such as *Escherichia coli* and *Staphylococcus aureus*.[49] Similarly, *Citrus limon* and *Citrus medica* have been found to have antimicrobial properties due to their essential oils and flavonoids.[50,51] *Andrographis paniculata* has also been found to exhibit broad-spectrum antibacterial action, especially against microorganisms that are resistant to drugs.[52] The observed antibacterial properties of our polyherbal formulation might be ascribed to the synergistic effect of the bioactive compounds present in each plant, as many plant-based phytochemicals exert antimicrobial effects by many processes, like breaking down the cell wall or preventing the creation of proteins.[53] The findings of this study align with previous reports on polyherbal formulations, such as those by Inavally *et al.*, (2024) and Jilakara *et al.*, (2025), which demonstrated significant antioxidant, antimicrobial, and hypoglycemic effects mediated

Table 4: Antibacterial and antifungal activity of ethanolic Polyherbal Extract (PHE).

PHE Concentration	*E. coli*	*S. aureus*	*P. aeruginosa*	*K. pneumoniae*	*C. albicans*
25 µg	4.33±0.5 mm	5.66±0.5 mm	5.0±1.0 mm	3.83±0.6 mm	4.66±1.1 mm
50 µg	6.33±0.3 mm	5.83±0.2 mm	5.16±0.7 mm	4.33±0.4 mm	5.5±0.7 mm
75 µg	7.66±0.5 mm	8.83±0.2 mm	6.33±0.3 mm	5.16±0.3 mm	6.50±0.4 mm
100 µg	9.33±1.1 mm	10.63±0.9 mm	8.0±1.2 mm	5.5±0.6 mm	8.83±0.4mm
Standard used for Bacteria (Chloramphenicol) Fungus (Fluconazole)	12.2±1.8 mm	14.60±1.2 mm	10.6±1.9 mm	8.4±0.6 mm	8.6±0.3 mm

Table 5: The Minimum Inhibitory Concentration (MIC; mg/mL) of ethanolic PHE is a measure of antimicrobial activity.

Isolates	MIC of Ethanolic PHE (mg/mL)
E. coli	24.0±1.2
S. aureus	25.3±1.5
P. aeruginosa	24.2±2.7
K. pneumoniae	26.2±1.3
C. albicans	21.8±1.8

by synergistic phytoconstituent interactions. Similar to Mentone, which exhibited notable DPPH scavenging and antimicrobial activities due to triterpenoids like asiatic acid and glycyrrhizin, our formulation displayed potent free radical scavenging ability and broad-spectrum antimicrobial efficacy, highlighting comparable pharmacological profiles. Notably, our study extends these observations by incorporating a novel herbal combination involving *Citrus limon, Bergera koenigii*, and *Andrographis paniculata*, revealing enhanced bioactivities and suggesting potential therapeutic superiority through multi-targeted mechanisms.[54-56]

CONCLUSION

In conclusion, the polyherbal formulation containing *Citrus limon, Bergera koenigii, Citrus medica*, and *Andrographis paniculata* demonstrates significant antioxidant and antimicrobial activities. These outcomes imply that the formulation may be used as a natural substitute for treating microbial infections and oxidative stress. The lack of *in vivo* testing prevents us from making conclusive claims regarding the therapeutic efficacy of the formulation Furthermore, studies investigating the method in which the bioactive substances function could provide a deeper understanding of how these compounds exert their effects.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

TAC: Total Antioxidant Capacity; **DPPH:** 2,2-diphenyl-1-picrylhydrazine; **FRAP:** Ferric Reducing Antioxidant Power; **ROS:** Reactive Oxygen Species; **PHE:** Polyherbal Formulation; **OD:** Optical Density; **IC_{50}:** Half Maximum Inhibitory Concentration; **SNP:** Sodium Nitroprusside; **NO:** Nitric Oxide; **NED:** N-1-naphthyl ethylenediamine dihydrochloride; **NBT:** Nitro blue tetrazolium; **EDTA:** Ethylenediaminetetraacetic acid; **-OH**: Hydroxyl Radical; **H_2O_2:** hydrogen peroxide; **TBA:** Thiobarbituric acid; **TCA:** Trichloroacetic acid; **MDA:** Malondialdehyde; **GC-MS:** Gas Chromatography-Mass Spectrometery; **MTCC:** Microbial Type Culture Collection; **ANOVA:** Analysis of Variance; **MIC:** Minimum Inhibitory Concentration.

DATA AVAILABILITY

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICAL STATEMENT

There were no studies involving humans and animals in this study and therefore approval by ethics committee is not required.

SUMMARY

The study evaluates a polyherbal formulation composed of *Citrus limon, Bergera koenigii, Citrus medica*, and *Andrographis paniculata* for its phytochemical composition, antioxidant, and antimicrobial properties. The ethanolic extract showed a rich presence of bioactive compounds, including flavonoids, phenolics, and alkaloids, contributing to its significant radical-scavenging activity in various *in vitro* antioxidant assays. Antimicrobial tests demonstrated strong inhibitory effects against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Candida albicans*, with varying degrees of minimum inhibitory concentrations. GC-MS

analysis identified key phytoconstituents responsible for the formulation's bioactivity. These findings suggest the polyherbal extract as a promising natural therapeutic agent for combating oxidative stress and microbial infections, warranting further *in vivo* studies.

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