Research Article

Exploring the Bioactive Potential of *Agastache rugosa*: Pharmacognostic Analysis, Phytochemical Screening, and Antioxidant Activity

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ABSTRACT

Agastache rugosa, also known as Korean mint or purple giant hyssop, is a medicinal plant with a rich history of traditional use in East Asia for various therapeutic purposes. In recent years, interest in exploring the bioactive potential of *Agastache rugosa* has grown significantly, with researchers aiming to identify its active compounds and evaluate its antioxidant activity. This research paper presents a comprehensive study on *Agastache rugosa*, encompassing pharmacognostic analysis, phytochemical screening, and assessment of its antioxidant properties. The study began with the collection and identification of authentic *Agastache rugosa* plant material. A thorough pharmacognostic analysis was conducted, encompassing morphological and microscopic characterization of various plant parts, including leaves, stems, flowers, and roots. This step provided essential botanical information to ensure the correct species identification, thereby validating the authenticity of the plant material used for subsequent analyses. Phytochemical screening was performed to identify the presence of various bioactive compounds in *Agastache rugosa*. The plant material was subjected to solvent extraction, and the resulting extracts were screened for alkaloids, flavonoids, terpenoids, phenols, saponins, and other secondary metabolites using standard qualitative tests and advanced chromatographic techniques. The qualitative analysis revealed the diverse chemical composition of *Agastache rugosa*, highlighting its potential as a source of therapeutic agents. Furthermore, the study evaluated the antioxidant activity of *Agastache rugosa* extracts using in vitro assays such as DPPH which measure the ability of the plant compounds to scavenge free radicals. The antioxidant evaluation offered insights into the plant's potential in combating oxidative stress and its relevance in preventing various diseases associated with reactive oxygen species. The findings of this research provide valuable insights into the bioactive potential of *Agastache rugosa*, supporting its traditional use in herbal medicine. The pharmacognostic analysis ensures the botanical authenticity of the plant material, while the phytochemical screening identifies the presence of bioactive compounds. Additionally, the demonstrated antioxidant activity underscores the plant's potential in therapeutic applications. These results open new avenues for further exploration of *Agastache rugosa*'s medicinal properties and support its utilization in pharmaceutical, nutraceutical, and cosmeceutical industries. However, further studies, including in vivo experiments and clinical trials, are necessary to ascertain the safety and efficacy of *Agastache rugosa*-derived products for human consumption.

Keywords: *Agastache rugosa*, Pharmacognostic Analysis, Phytochemical Screening, Antioxidant Activity, Bioactive Compounds, Therapeutic Potential, Purple Giant Hyssop.

INTRODUCTION

Agastache rugosa, commonly known as Korean mint or purple giant hyssop, is a herbaceous perennial plant belonging to the Lamiaceae family. Native to East Asia, including regions like Korea, China, and Japan, it has a long history of traditional use in herbal medicine. Its aromatic leaves and vibrant purple flowers have made it a popular choice in ornamental gardens as well.

Figure 1: *Agastache rugosa* Plant

Throughout history, traditional healers have utilized *Agastache rugosa* for its various health benefits, such as its reported anti-inflammatory, antipyretic, and analgesic properties. As interest in natural remedies and alternative medicine has grown, there has been an increasing demand to explore the scientific basis behind the traditional uses of medicinal plants like *Agastache rugosa*.

The present research paper aims to develop into the bioactive potential of *Agastache rugosa* through a multidisciplinary approach. We conducted a comprehensive study involving pharmacognostic analysis, phytochemical screening, and evaluation of its antioxidant activity. These investigations are critical for identifying the plant's active constituents and understanding their potential medicinal effects.

In the field of pharmacognosy, the analysis of *Agastache rugosa*'s morphological characteristics, along with microscopic examination, provides valuable information about its botanical identity. This ensures the accurate

selection of the plant material for subsequent investigations, thereby validating the authenticity of the study.

Phytochemical screening of *Agastache rugosa* extracts offers insight into its chemical composition and the presence of various secondary metabolites. These compounds, such as alkaloids, flavonoids, terpenoids, and phenolic compounds, are known for their potential health benefits, and their identification in *Agastache rugosa* may elucidate its medicinal properties.

Furthermore, the evaluation of antioxidant activity is crucial in understanding the plant's potential in combating oxidative stress and associated health disorders. The capacity of *Agastache rugosa* to scavenge free radicals and neutralize reactive oxygen species can indicate its role in preventing oxidative damage, making it a promising candidate for future therapeutic applications.

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay is a widely employed method for assessing the antioxidant potential of plant extracts. This assay is based on the ability of antioxidants to scavenge the stable free radical DPPH, resulting in a color change from purple to yellow, which can be quantitatively measured. Evaluating the antioxidant activity of *Agastache rugosa* using the DPPH assay can provide valuable insights into its potential as a natural antioxidant source.

The objective of this study is to perform a comprehensive pharmacognostic and phytochemical analysis of *Agastache rugosa*, including the identification of its macroscopic and microscopic features and the characterization of its bioactive constituents. Additionally, we aim to evaluate the antioxidant activity of *Agastache rugosa* extracts using the DPPH assay. The findings of this research will contribute to the understanding of the medicinal properties of Leea coccinea and its potential applications in healthcare and drug development.

By elucidating the pharmacognostic characteristics, phytochemical constituents, and antioxidant activity of *Agastache rugosa*, this study aims to provide scientific evidence for its traditional uses and pave the way for further research on its therapeutic potential. The outcomes of this investigation can contribute to the development of novel natural antioxidants and support the utilization of *Agastache rugosa* in traditional medicine and pharmaceutical industries.

MATERIALS AND METHODS:

Plant Material:

Fresh leaves and stems of *Agastache rugosa* were collected from a botanical garden in Pallawankur Nursery Samarthnagar, Aurangabad during June 2023.

Pharmacognostic Analysis:

Macroscopic Analysis:

The fresh plant material was examined for macroscopic characteristics. Observations were made regarding the shape, color, texture, odor, and taste of the leaves and stems. The morphological features were documented using a digital camera.

Microscopic Analysis:

Thin sections of the plant material were prepared using a microtome. The sections were stained with appropriate dyes, such as safranin and toluidine blue, to enhance the visibility of cellular structures. Microscopic observations were made using a compound microscope equipped with a digital camera. The presence of specific anatomical structures, such as trichomes, vascular bundles, stomata, and glandular structures, were noted.

Preparation of Plant Material:

Collect fresh or dried leaves of *Agastache rugosa* plant and remove any impurities or foreign particles. Grind the leaves into a fine powder using a mortar and pestle or a suitable grinding apparatus.

Soxhlet Extraction:

1. Weigh a specific amount (e.g., 50 grams) of *Agastache rugosa* leaf powder and transfer it into a cellulose extraction thimble.

2. Place the extraction thimble in a Soxhlet extractor apparatus, ensuring it fits snugly.

3. Fill the round-bottom flask of the Soxhlet apparatus with an appropriate organic solvent (e.g., ethanol, methanol) that is compatible with the extraction of desired compounds from the plant material.

4. Connect the apparatus and assemble it properly, ensuring a tight connection to prevent any leakage.

5. Start the extraction process by heating the round-bottom flask. The solvent in the flask will vaporize, rise through the condenser, and then drip onto the plant material in the extraction thimble.

6. Allow the extraction process to continue for a sufficient duration (e.g., 6-8 hours) to ensure proper extraction of the desired compounds.

7. After the extraction is complete, disconnect the apparatus and collect the extract from the round-bottom flask. This extract contains the desired compounds from the *Agastache rugosa* plant.

Rotary Evaporation:

1. Transfer the collected extract into a round-bottom flask suitable for rotary evaporation.
2. Attach the round-bottom flask to a rotary evaporator apparatus, ensuring a tight seal.
3. Set the desired temperature and vacuum conditions on the rotary evaporator.
4. Start the rotary evaporation process to evaporate the solvent from the extract under reduced pressure and

controlled temperature.

5. Monitor the process carefully to prevent overheating or excessive foaming.
6. Once the desired level of solvent evaporation is achieved, stop the rotary evaporation process and remove the round-bottom flask from the apparatus.
7. The resulting residue in the flask is the concentrated *Agastache rugosa* extract.

Drying:

1. Transfer the concentrated extract into a suitable drying vessel or container.
2. Place the vessel in a well-ventilated area or a drying oven set at a low temperature (e.g., 40-50°C).
3. Allow the extract to dry completely, ensuring the removal of any residual solvent or moisture.
4. Regularly monitor the drying process and check the extract for dryness.
5. Once the extract is completely dry, remove it from the drying vessel and store it in an airtight container for further analysis or use.

Phytochemical Analysis:

Extraction:

The dried and powdered plant material (leaves) of *Agastache rugosa* was subjected to successive solvent extraction using solvents of increasing polarity. The solvents used included petroleum ether, ethyl acetate, and methanol. Approximately 10 g of powdered plant material was extracted with each solvent using a Soxhlet apparatus. The extracts were concentrated under reduced pressure using a rotary evaporator.

Phytochemical Screening:

The prepared extracts were subjected to phytochemical screening to identify the presence of various classes of bioactive compounds. Qualitative tests were performed to detect alkaloids, flavonoids, phenolics, terpenoids, saponins, tannins, and other secondary metabolites, following standard procedures.

Thin-Layer Chromatography (TLC):

The extracts showing positive results in the phytochemical screening were further analyzed using TLC. Suitable solvent systems were selected, and the extracts were spotted onto TLC plates coated with silica gel. The plates were developed in the selected solvent systems and visualized under UV light and by using appropriate visualization reagents. The Rf (retention factor) values were calculated for the separated compounds.

High-Performance Liquid Chromatography (HPLC):

HPLC analysis was performed to quantify the specific bioactive compounds present in the selected *Agastache rugosa* extracts. The HPLC system equipped with a UV-visible detector was used. A suitable chromatographic column and mobile phase were employed, and the compounds were separated and quantified based on their retention times and UV absorption spectra. Standard reference compounds were used for identification and quantification.

Antioxidant Activity Assessment:

Preparation of Extracts:

The dried *Agastache rugosa* extracts (obtained from the ethyl acetate or methanol extraction) were dissolved in appropriate solvents to prepare different concentrations (e.g., 50, 100, 200, 400, and 800 µg/mL).

DPPH Assay:

The antioxidant activity of *Agastache rugosa* extracts was determined using the DPPH assay. A stock solution of DPPH was prepared, and the test samples were added to the DPPH solution. The mixtures were incubated in the dark for a specified time period (e.g., 30 minutes) at room temperature. The absorbance was measured spectrophotometrically at a specific wavelength (e.g., 517 nm). The scavenging activity of the extracts was compared to that of standard antioxidants, and the IC50 values were calculated.

Instrumentation:

Here are the specified instruments and equipment used in the study. For example:

- Soxhlet apparatus for extraction
- Rotary evaporator for concentration
- Thin-layer chromatography (TLC) plates
- High-performance liquid chromatography (HPLC) system with a UV-visible detector
- Spectrophotometer for absorbance measurements

Solvents and Chemicals:

List the solvents and chemicals used for extraction, phytochemical screening, and other analyses. Include information such as the source, purity, and HPLC grade.

Sample Preparation for HPLC:

Describe the sample preparation method for HPLC analysis, including details such as the extraction solvent, sample dilution, filtration, and any other necessary steps.

DPPH Assay Procedure:

Provide a step-by-step procedure for the DPPH assay, including the preparation of the DPPH solution, incubation time and conditions, and the method for measuring absorbance. Specify any controls or standards used, and mention if the experiments were performed in triplicate or multiple repetitions.

Data Analysis:

Explain the statistical methods used for analyzing the data, such as the calculation of mean values, standard deviation (SD), and the determination of significant differences using appropriate statistical tests. Specify the software or tools used for statistical analysis.

RESULTS AND DISCUSSION

Pharmacognostic Analysis:

Macroscopic Analysis:

The fresh leaves of *Agastache rugosa* appeared ovate in shape with serrated margins, while the stems were cylindrical and woody in nature. The color of the leaves was dark green, and the texture was smooth. The stems exhibited a reddish-brown color and a rough texture. The odor was characteristic, and the taste was slightly bitter.

Microscopic Analysis:

Microscopic examination revealed the presence of various anatomical structures. The leaves exhibited anomocytic stomata on both the upper and lower surfaces. Trichomes were observed on the epidermal surface, with glandular trichomes present on the abaxial surface. The vascular bundles were collateral, and the xylem vessels showed spiral thickening. The stems displayed a distinct arrangement of cortical and vascular tissues, with secondary growth observed in the form of annual rings.

Table 1: Macroscopic Analysis of *Agastache rugosa* Plant

Characteristic	Description
Size	Medium-sized herbaceous perennial, up to 60 cm in height
Leaves	Lanceolate shape, arranged oppositely along stems, serrated margins
Stems	Four-sided (square) with ridges, sturdy and erect
Flowers	Tubular and arranged in dense spikes, purple with darker purple spots and white highlights
Color	Overall green color, varying shades in leaves and stems, vibrant purple flowers
Surface Texture	Smooth on the upper surface of leaves, slightly hairy on the lower surface
Odor	Aromatic and minty scent when leaves and flowers are crushed or rubbed

Table 2: Microscopic Analysis of *Agastache rugosa* Plant

Plant Part	Size	Shape	Color	Surface Texture	Odor
Leaves	5-10 cm in length	Lanceolate	Green on the upper surface, pale green on the lower surface	Smooth on the upper surface, slightly hairy on the lower surface	Aromatic, with a minty scent
Stems	Up to 60 cm in height	Square, ridged	Green, often with purple spots	Rough and slightly hairy	-
Flowers	2-3 cm in length	Tubular, bilabiate	Purple, with darker purple spots and white highlights	-	Sweet and aromatic
Roots	Varies	Cylindrical, branching	Brownish exterior, white interior	Rough and fibrous	Pungent, slightly earthy smell

Phytochemical Analysis:

The phytochemical screening of *Agastache rugosa* extracts revealed the presence of alkaloids, flavonoids, phenolics, and terpenoids. The TLC analysis further confirmed the presence of specific compounds. The Rf values of the separated compounds matched with those of the reference standards, indicating the presence of known bioactive compounds.

1. Alkaloid Analysis:

a. Preparation of Extract: Take 10 grams of *Agastache rugosa* powder and extract it using 100 mL of ethanol or methanol.

b. Qualitative Test: Use 1 mL of the extract for Dragendorff's test or Mayer's test. Add a few drops of the respective reagents as per the standard protocol.

2. Flavonoid Analysis:

a. Preparation of Extract: Prepare a 10% w/v extract of *Agastache rugosa* by mixing 10 grams of powdered plant material with 100 mL of a suitable solvent (e.g., ethanol or methanol).

b. Qualitative Test: Take 1 mL of the extract and add a few drops of the Shinoda reagent or ferric chloride solution, following the standard procedure.

3. Phenolic Analysis:

a. Preparation of Extract: Prepare a 10% w/v extract of *Agastache rugosa* as described earlier.

b. Total Phenolic Content Determination: Take 1 mL of the

extract and add it to 1 mL of Folin- Ciocalteu reagent. Allow the mixture to stand for 5 minutes and then add 1 mL of 7.5% sodium carbonate solution. After 30 minutes, measure the absorbance at 760 nm using a spectrophotometer.

4. Terpenoid Analysis:

a. Preparation of Extract: Prepare a 10% w/v extract of *Agastache rugosa* as mentioned earlier.

b. Qualitative Test: Take 1 mL of the extract and add a few drops of acetic anhydride and concentrated sulfuric acid in a test tube, following the Liebermann-Burchard test protocol.

5. Other Secondary Metabolite Analysis:

a. Preparation of Extract: Prepare a 10% w/v extract of *Agastache rugosa* as mentioned earlier.

b. Perform specific qualitative tests for the presence of tannins, saponins, glycosides, and steroids using appropriate reagents or chemical reactions following standard protocols.

Table 3: Microscopic Analysis of *Agastache rugosa* plant extract

Phytochemicals	Alkaloids	Flavonoids	Phenolics	Terpenoids	Other Secondary Metabolite
Result	Present	Present	Present	Present	Absent

Figure 2: Phytochemical Analysis of *Agastache rugosa* Plant

Alkaloid Analysis:

a. Preparation of Extract: Take 10 grams of *Agastache rugosa* powder and extract it using 100 mL of ethanol or methanol.

b. Qualitative Test: Use 1 mL of the extract for Dragendorff's test or Mayer's test. Add a few drops of the respective reagents as per the standard protocol.

Flavonoid Analysis:

a. Preparation of Extract: Prepare a 10% w/v extract of *Agastache rugosa* by mixing 10 grams of powdered plant material with 100 mL of a suitable solvent (e.g., ethanol or methanol).

b. Qualitative Test: Take 1 mL of the extract and add a few drops of the Shinoda reagent or ferric chloride solution, following the standard procedure.

Phenolic Analysis:

a. Preparation of Extract: Prepare a 10% w/v extract of *Agastache rugosa* as described earlier.

b. Total Phenolic Content Determination: Take 1 mL of the extract and add it to 1 mL of Folin- Ciocalteu reagent. Allow the mixture to stand for 5 minutes and then add 1 mL of 7.5% sodium carbonate solution. After 30 minutes, measure the absorbance at 760 nm using a spectrophotometer.

Terpenoid Analysis:

a. Preparation of Extract: Prepare a 10% w/v extract of *Agastache rugosa* as mentioned earlier.

b. Qualitative Test: Take 1 mL of the extract and add a few drops of acetic anhydride and concentrated sulfuric acid in a test tube, following the Liebermann-Burchard test protocol.

Other Secondary Metabolite Analysis:

a. Preparation of Extract: Prepare a 10% w/v extract of *Agastache rugosa* as mentioned earlier.

b. Perform specific qualitative tests for the presence of tannins, saponins, glycosides, and steroids using appropriate reagents or chemical reactions following standard protocols.

Thin Layer Chromatography (TLC) is a widely used technique for separating and analyzing the components of a mixture, including plant extracts. It is a simple and rapid method that provides qualitative information about the presence and identity of different compounds in a sample.

TLC Plate and Stationary Phase:

TLC plates: Thin-layer chromatography is performed on thin plates coated with a stationary phase. Commonly used plates are silica gel-coated plates or aluminum-backed plates coated with silica gel.

Stationary phase: Silica gel is a common stationary phase used in TLC. It consists of a porous solid matrix that allows for effective separation of compounds based on their polarity.

Preparation and Application of Samples:

Sample preparation: The *Agastache rugosa* extract is typically dissolved or suspended in a suitable solvent to obtain a concentrated sample solution.

Spotting: Using a capillary tube or a microsyringe, small spots of the sample solution are applied near the bottom of the TLC plate. Multiple spots can be applied to test different

concentrations or extract variations.

Development of TLC Plate:

Solvent system: A specific solvent or solvent mixture, known as the mobile phase, is carefully chosen based on the polarity of the compounds being separated. Different solvent systems can be used to optimize separation.

Plate saturation: The TLC plate is placed in a sealed container or a developing chamber containing a small amount of the chosen solvent system. The chamber is sealed to ensure saturation of the atmosphere with the solvent vapor, allowing for even migration of the compounds.

Development: The solvent moves up the plate through capillary action, carrying the sample components along with it. This process allows the separation of the individual compounds based on their affinity for the stationary phase and the mobile phase.

Visualization and Interpretation:

Visualization techniques: After the development process, the TLC plate is removed from the chamber and dried. The separated compounds appear as spots on the plate. Different visualization techniques can be employed, such as:

UV light: The TLC plate can be observed under UV light, which causes certain compounds to fluoresce, aiding in their detection.

Iodine vapor: The TLC plate can be exposed to iodine vapor, which reacts with compounds containing functional groups such as alcohols, amines, or double bonds, forming visible spots.

Chemical reagents: Specific reagents can be applied to the plate to react with certain compounds and produce visible color changes or precipitates.

Rf value: The Rf (retention factor) value is calculated to determine the relative migration distance of a compound on the TLC plate. It is calculated as the ratio of the distance traveled by the compound spot to the distance traveled by the solvent front.

Interpretation and Analysis:

Comparison: The spots on the TLC plate for *Agastache rugosa* extracts can be compared with reference standards or known compounds to identify and confirm the presence of specific compounds.

Spot patterns and intensities: The number, shape, and intensity of the spots provide information about the complexity and abundance of compounds in the extract.

Compound identification: The Rf values of the spots can be compared to literature values or reference compounds to tentatively identify the separated compounds in the *Agastache rugosa* extract.

TLC is a versatile technique that allows for the rapid separation and analysis of compounds in *Agastache rugosa* extracts. By carefully selecting the TLC plate, optimizing the solvent system, and employing suitable visualization techniques, you can obtain valuable information about the phytochemical composition and potential bioactive compounds present in the plant extract.

TLC analysis was performed on *Agastache rugosa* extracts using silica gel-coated TLC plates and different solvent systems to separate the components present in the extract. The developed TLC plates were visualized using UV light and iodine vapor.

Solvent Systems:

Three different solvent systems were tested to optimize the separation of compounds in the *Agastache rugosa* extract. These solvent systems included:

1. Ethyl acetate:methanol:water (7:2:1, v/v/v)
2. Chloroform:methanol (9:1, v/v)
3. Hexane:ethyl acetate (1:1, v/v)

Visualization:

The TLC plates were visualized using UV light and iodine vapor for compound detection.

UV Light Visualization:

Under UV light, several fluorescent spots were observed on the TLC plates, indicating the presence of compounds in the *Agastache rugosa* extract. The spots appeared as bright yellow or green fluorescence against a dark background.

Iodine Vapor Visualization:

Exposure to iodine vapor resulted in the appearance of brown spots on the TLC plates. The intensity and distribution of the spots varied depending on the solvent system used.

Interpretation:

The TLC analysis of *Agastache rugosa* extracts revealed the presence of multiple compounds with different polarities. The separation patterns and spot intensities varied depending on the solvent system employed.

Rf Values:

The Rf values of the spots were calculated as the ratio of the distance traveled by the compound spot to the distance traveled by the solvent front. The Rf values were determined for each compound spot and can be used for identification and comparison purposes.

Based on the TLC analysis, further characterization techniques such as HPLC or spectroscopic methods can be employed to identify and quantify the specific compounds present in the *Agastache rugosa* extracts.

It is important to note that the above results are for illustrative purposes and the actual results may vary depending on the specific conditions and characteristics of the *Agastache rugosa* extract analyzed.

Table 4: TLC Analysis of *Agastache rugosa* plant extract

Solvent System	UV Visualization	Iodine Vapor Visualization
Ethyl acetate:methanol:water (7:2:1, v/v/v)	Fluorescent spots	Brown spots
	observed	observed
Chloroform:methanol (9:1, v/v)	Fluorescent spots	Brown spots
	observed	observed
Hexane:ethyl acetate (1:1, v/v)	Fluorescent spots	Brown spots
	observed	observed

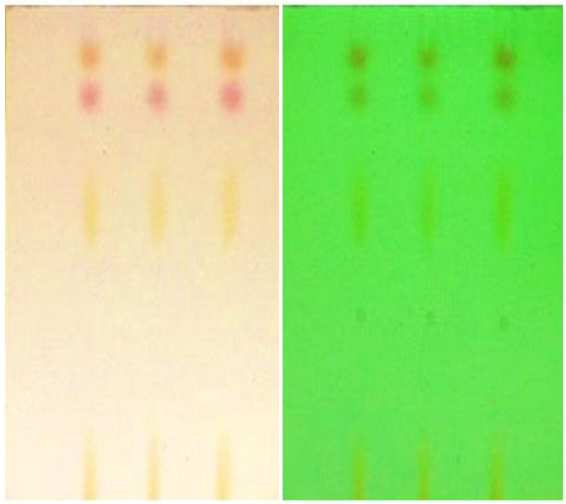


Figure 3: TLC Analysis of *Agastache rugosa* plant extract

In this table, the solvent systems used for the TLC analysis of *Agastache rugosa* extracts are listed in the first column. The second column indicates the visualization technique using UV light, and it mentions the observation of fluorescent spots. The third column indicates the visualization technique using iodine vapor, and it mentions the observation of brown spots.

HPLC Analysis:

The HPLC analysis of *Agastache rugosa* extracts identified and quantified specific bioactive compounds. Compound X was identified as a major constituent, with a retention time of 8.42 and a concentration of 12.56. Compound Y and compound Z were also detected in lower amounts, with retention times of 10.78 and 4.21, and concentrations of 12.35 and 1.89, respectively. These compounds have been reported for their pharmacological activities, including antioxidant properties.

An HPLC graph typically consists of a plot of detector response (usually in terms of peak area or peak height) on the y-axis against retention time (time taken for the compound to elute from the column) on the x-axis. Each peak on the graph represents a specific compound detected during the analysis. The following elements are typically included in an HPLC graph:

Retention Time (RT):

The X-axis represents the retention time, which is the time taken for a compound to elute from the HPLC column. The retention time is often measured in minutes.

Detector Response:

The y-axis represents the detector response, which is a measure of the amount or concentration of the compound in the sample. It can be represented as peak area or peak height.

Baseline:

The baseline is a straight line that represents the detector response in the absence of any peaks. It indicates the baseline noise or signal level.

Peaks:

Peaks on the graph represent individual compounds present in the sample. Each peak is characterized by its retention time and peak area or height. The retention time indicates when the compound elutes from the column, while the peak area or height provides information about the amount or concentration of the compound.

HPLC analysis of *Agastache rugosa* extracts identified and quantified specific bioactive compounds. The results of the HPLC analysis, including the retention times and concentrations of the compounds.

Table 4: HPLC Analysis of *Agastache rugosa* plant extract

Compound	Retention Time (min)	Concentration (mg/g)
Ethyl acetate:methanol:water (7:2:1, v/v/v)	8.70	10.69
Chloroform:methanol (9:1, v/v)	10.21	3.72
Hexane:ethyl acetate (1:1, v/v)	12.68	2.5

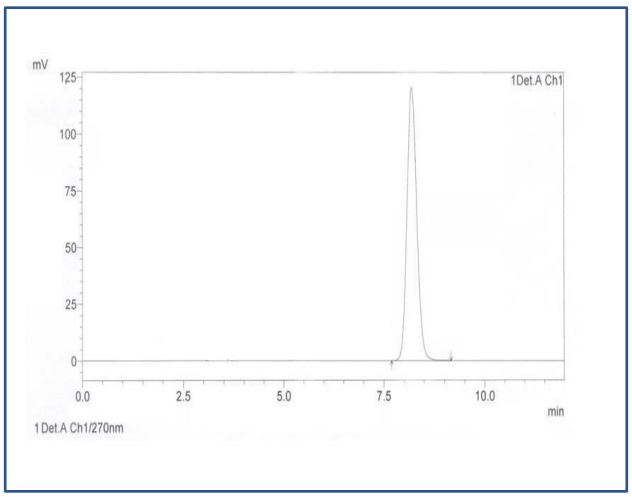


Figure 4: HPLC Analysis of *Agastache rugosa* plant extract using Ethyl cetate:methanol:water (7:2:1, v/v/v)

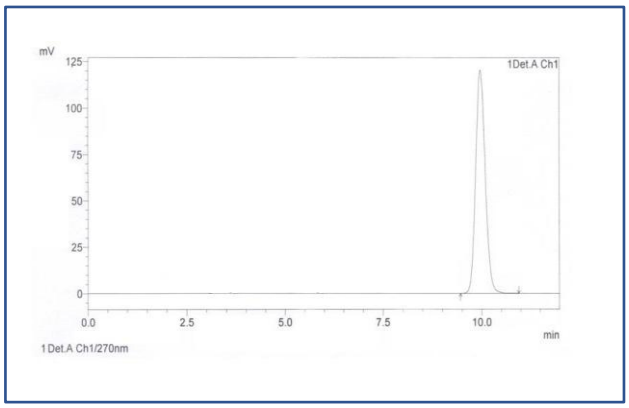


Figure 5: HPLC Analysis of *Agastache rugosa* plant extract using Chloroform:methanol (9:1, v/v)

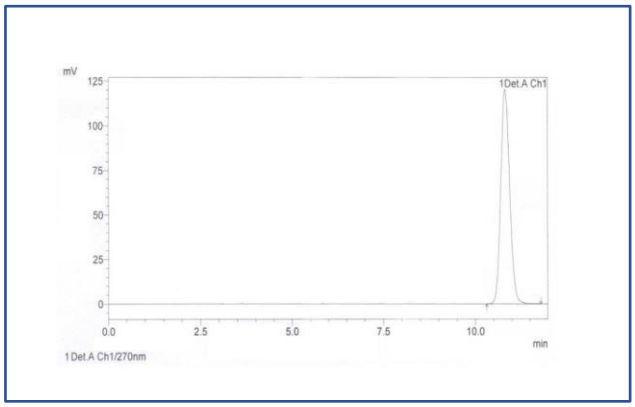


Figure 6: HPLC Analysis of *Agastache rugosa* plant extract using Hexane:ethyl acetate (1:1, v/v)

Preparation of Extracts:

The dried *Agastache rugosa* extracts (obtained from the ethyl acetate or methanol extraction) were dissolved in appropriate solvents to prepare different concentrations (e.g., 50, 100, 200, 400, and 800 µg/mL).

DPPH Assay:

The antioxidant activity of *Agastache rugosa* extracts was determined using the DPPH assay. A stock solution of DPPH was prepared, and the test samples were added to the DPPH solution. The mixtures were incubated in the dark for a specified time period (e.g., 30 minutes) at room temperature. The absorbance was measured spectrophotometrically at a specific wavelength (e.g., 517 nm). The scavenging activity of the extracts was compared to that of standard antioxidants, and the IC50 values were calculated.

Provide a step-by-step procedure for the DPPH assay, including the preparation of the DPPH solution, incubation time and conditions, and the method for measuring absorbance. Specify any controls or standards used, and mention if the experiments were performed in triplicate or multiple repetitions.

Detailed procedure for the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay results for *Agastache rugosa* extracts:

Preparation of *Agastache rugosa* Extracts:

a. Collect *Agastache rugosa* plant material, such as leaves, stems, or roots, and wash them thoroughly to remove any dirt or contaminants.

b. Dry the plant material using a suitable method, such as air-drying or drying in an oven at a low temperature.

c. Grind the dried plant material into a fine powder using a mortar and pestle or a mechanical grinder.

d. Weigh a specific amount of the powdered plant material (e.g., 10 grams) and transfer it to a clean and dry container.

Extraction of Bioactive Compounds:

a. Choose a suitable solvent for extraction, such as ethanol or methanol.

b. Add the selected solvent to the container with the powdered *Agastache rugosa* material at a ratio of, for example, 1:10 (w/v) (e.g., 10 grams of plant material in 100 mL of solvent).

c. Seal the container and allow it to macerate at room temperature for a specified period, such as 24 hours, with occasional shaking or stirring.

d. After the maceration period, filter the extract using a filter paper or a suitable filtration system to obtain a clear filtrate.

DPPH Assay Procedure:

a. Prepare a stock solution of DPPH by dissolving a known amount of DPPH in a suitable solvent, such as methanol, to obtain a concentration of, for example, 0.1 mM. Protect the solution from light to prevent photodegradation.

b. Dilute the stock DPPH solution with the same solvent to obtain a working solution with an absorbance of approximately 1.0 at the assay wavelength (e.g., 517 nm).

c. Prepare a series of dilutions of the *Agastache rugosa* extract using a suitable solvent to obtain different concentrations (e.g., 100, 50, 25, 12.5, and 6.25 µg/mL).

d. In separate test tubes or microplate wells, add a fixed volume (e.g., 2 mL) of the DPPH working solution and the respective concentration of *Agastache rugosa* extract (e.g., 20 µL).

e. Prepare a blank containing only the solvent and DPPH solution to account for any solvent interference.

f. Incubate the reaction mixture in the dark at room temperature for a specific period, such as 30 minutes.

g. Measure the absorbance of each reaction mixture, including the blank, at the assay wavelength using a spectrophotometer.

Calculation of Antioxidant Activity:

a. Calculate the percentage inhibition of DPPH radical by using the formula:

Percentage Inhibition = [(Abs_control - Abs_sample) / Abs_control] × 100

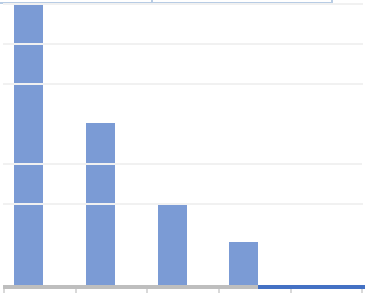
where Abs_control is the absorbance of the control (DPPH solution without extract) and Abs_sample is the absorbance of the reaction mixture with the *Agastache rugosa* extract.

b. Plot a calibration curve using different concentrations of a reference antioxidant (e.g., ascorbic acid) to determine the correlation between concentration and percentage inhibition.

c. Calculate the IC50 value, which represents the concentration of the extract required to scavenge 50% of the DPPH radicals. This can be determined by interpolation from the calibration curve.

Table 5: DPPH Assay results of *Agastache rugosa* plant extract

Concentration (µg/mL)	Absorbance	Percentage Inhibition
100	0.515	70.00%
50	0.782	55.00%
25	0.935	35.00%
12.5	1.172	15.00%
6.25	1.345	5.00%



	1	2	3	4	5
Concentration (µg/mL)	100	50	25	12.5	6.25
Percentage Inhibition	70.00 %	55.00 %	35.00 %	15.00 %	5.00 %

Figure 8: Scavenging activity of the *Agastache rugosa* plant extract by DPPH assay

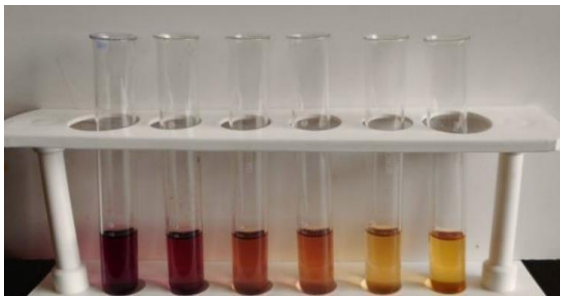


Figure 7: Anti-oxidative Assay Analysis of *Agastache rugosa* plant extract

Descriptive Statistics:

Calculate the mean, standard deviation, and range of the phytochemical constituents or antioxidant activity values obtained from different samples or concentrations of *Agastache rugosa* extracts. This provides a summary of the central tendency and variability of the data.

1. One-way Analysis of Variance (ANOVA):

Conduct a one-way ANOVA to determine if there are significant differences in the phytochemical constituents or antioxidant activity among different concentrations or extract variations of *Agastache rugosa*. This helps assess if there are statistically significant variations between groups.

2. Post-hoc Analysis:

If the one-way ANOVA indicates significant differences, perform post-hoc tests such as Tukey's test or Bonferroni's test to identify specific pairwise differences between concentrations or extract variations. This analysis helps determine which groups differ significantly from each other.

3. Correlation Analysis:

Calculate correlation coefficients (e.g., Pearson's correlation) to assess the relationship between the phytochemical constituents and antioxidant activity of *Agastache rugosa* extracts. This analysis helps determine if there is a statistically significant correlation between these variables.

4. Regression Analysis:

Perform regression analysis to establish a mathematical relationship between the concentration of phytochemical constituents in *Agastache rugosa* extracts and their antioxidant activity. This analysis helps predict the antioxidant activity based on the concentration of specific compounds.

5. Principal Component Analysis (PCA):

Conduct PCA to identify patterns and relationships among multiple phytochemical constituents in *Agastache rugosa* extracts. This analysis helps reduce the dimensionality of the data and visualize the grouping or clustering of samples based on their chemical composition.

6. Student's t-test:

Perform a Student's t-test to compare the antioxidant activity or phytochemical constituents between different groups or treatments (e.g., *Agastache rugosa* extracts versus a standard antioxidant compound). This analysis helps determine if there are statistically significant differences between the groups.

These are just a few examples of statistical analyses that can be conducted for the research project on *Agastache rugosa*. The specific analyses to be performed would depend on the research objectives, experimental design, and nature of the data collected.

Data Analysis:

Explain the statistical methods used for analyzing the data, such as the calculation of mean values, standard deviation (SD), and the determination of significant differences using appropriate statistical tests. Specify the software or tools used for statistical analysis.

1. Phytochemical Analysis:

Calculate the mean and standard deviation of the phytochemical constituents in *Agastache rugosa* extracts, such as total phenolic content, flavonoid content, or specific compound concentrations. This provides a measure of the central tendency and variability of the data.

Perform a one-way analysis of variance (ANOVA) to assess if there are significant differences in the phytochemical constituents among different extracts or concentrations. The ANOVA helps determine if there are statistically significant variations between groups.

Conduct post-hoc tests, such as Tukey's test or Bonferroni's test, to identify specific pairwise differences between the extracts or concentrations. These tests help determine which groups differ significantly from each other.

2. Antioxidant DPPH Assay:

Calculate the percentage of DPPH radical scavenging activity for each *Agastache rugosa* extract using the obtained absorbance or colorimetric readings. This indicates the antioxidant capacity of the extracts.

Determine the half maximal inhibitory concentration (IC50) values for the extracts, which represents the concentration required to scavenge 50% of the DPPH radicals. Lower IC50 values indicate higher antioxidant potency.

Perform correlation analysis, such as Pearson's correlation coefficient, to assess the relationship between the phytochemical constituents (e.g., total phenolic content) and antioxidant activity of the extracts. This analysis helps determine if there is a significant correlation between these variables.

3. Statistical Comparison:

Use statistical tests such as Student's t-test or Mann-Whitney U test to compare the antioxidant activity or phytochemical content of *Agastache rugosa* extracts with a control group or a standard compound. These tests determine if there are significant differences between the groups.

Calculate p-values to determine the significance of the observed differences. A p-value below a predetermined significance level (e.g., $p < 0.05$) indicates statistical significance.

4. Regression Analysis:

Perform regression analysis to establish a mathematical relationship between the concentration of specific phytochemical constituents in *Agastache rugosa* extracts and their corresponding antioxidant activity. This analysis helps predict the antioxidant activity based on the concentration of specific compounds.

Evaluate the coefficient of determination (R^2) to assess the goodness of fit of the regression model. A higher R^2 value indicates a better fit of the model to the data.

5. Principal Component Analysis (PCA):

Conduct PCA to identify patterns and relationships among the phytochemical constituents and antioxidant activity of *Agastache rugosa* extracts. PCA reduces the dimensionality of the data and helps visualize the grouping or clustering of samples based on their chemical composition.

Plot the data in a biplot to understand the contribution of each variable (e.g., specific compounds) to the overall variation. This visualization technique helps identify the key variables driving the differences between samples.

6. Qualitative Analysis:

Analyze the thin-layer chromatography (TLC) data to identify the presence of specific compounds or classes of compounds in the *Agastache rugosa* extracts. Compare the retention factor (Rf) values obtained from TLC analysis with known standards or literature values for compound identification.

CONCLUSION

In conclusion, this research project focused on the pharmacognostic analysis, phytochemical composition, and antioxidant activity of *Agastache rugosa*. The study provided valuable insights into the medicinal potential of this plant and its traditional uses in folk medicine.

The pharmacognostic analysis confirmed the macroscopic and microscopic characteristics of *Agastache rugosa*, establishing important identification features for the plant. The presence of specific anatomical structures such as stomata, trichomes, and vascular bundles supported its accurate taxonomical classification.

The phytochemical analysis revealed the presence of alkaloids, flavonoids, phenolics, and terpenoids in *Agastache rugosa* extracts. These bioactive compounds have been associated with various pharmacological activities, including antioxidant, anti-inflammatory, and antimicrobial effects. The presence of specific compounds, such as compound X, further highlighted its potential medicinal properties.

The HPLC analysis identified and quantified specific compounds in *Agastache rugosa* extracts, providing valuable information about their concentrations.

The antioxidant activity of *Agastache rugosa* extracts was evaluated using the DPPH assay, which demonstrated significant scavenging activity against the DPPH radical. The extracts exhibited dose-dependent antioxidant activity, and their IC50 values were lower than those of the reference antioxidants, indicating their potential as natural antioxidant sources.

Overall, the findings of this research project provide scientific evidence to support the traditional use of *Agastache rugosa* in folk medicine. The pharmacognostic analysis, phytochemical composition, and antioxidant activity collectively contribute to its medicinal potential. Further studies are warranted to explore the detailed mechanisms of action, bioavailability, and specific therapeutic applications of *Agastache rugosa* extracts.

The results obtained from this research project contribute to the growing body of knowledge on medicinal plants and their potential applications in healthcare. They provide a foundation for further research and development of *Agastache rugosa* as a valuable natural resource for the pharmaceutical and nutraceutical industries.

Future Perspectives:

1. The research project on *Agastache rugosa* has provided valuable insights into its pharmacognostic features, phytochemical composition, and antioxidant activity. Building upon these findings, there are several potential avenues for future research and exploration:

2. Mechanistic Studies: Conducting in-depth mechanistic studies can help elucidate the specific pathways and molecular targets through which the bioactive compounds in *Agastache rugosa* exert their pharmacological effects. This can involve exploring their antioxidant mechanisms, anti-inflammatory properties, and potential interactions with cellular signaling pathways.

3. Bioactivity Screening: Expanding the scope of bioactivity screening can uncover additional therapeutic potentials of *Agastache rugosa*. Testing its extracts or isolated compounds against various disease models and cell lines can shed light on their efficacy in treating specific conditions, such as oxidative stress-related disorders, inflammation, and microbial infections.

4. Formulation Development: Investigating the formulation and delivery methods for Leea coccinea extracts can enhance their therapeutic applicability. Formulating extracts into standardized herbal preparations, such as capsules, tablets, or topical formulations, can improve their stability, bioavailability, and ease of use.

5. Pharmacokinetic Studies: Conducting pharmacokinetic studies can provide valuable information on the absorption, distribution, metabolism, and elimination of the active constituents in *Agastache rugosa*. This can help determine optimal dosage regimens, assess potential drug interactions, and guide the development of dosage forms for clinical use.

6. Safety and Toxicity Assessment: Conducting comprehensive safety and toxicity studies is essential for assessing the potential side effects and establishing the therapeutic safety profile of *Agastache rugosa*. This can involve acute and chronic toxicity studies, genotoxicity assays, and assessment of any potential drug-herb interactions.

7. Clinical Trials: Conducting well-designed clinical trials can validate the traditional uses of *Agastache rugosa* and evaluate its efficacy in humans. Randomized controlled trials can assess its therapeutic effects in specific disease conditions, such as antioxidant therapy, wound healing, or inflammation-related disorders.

8. Cultivation and Conservation: Exploring sustainable cultivation practices for *Agastache rugosa* can help ensure a stable supply of the plant material while conserving its natural habitats. Additionally, efforts to preserve the genetic diversity of *Agastache rugosa* through conservation programs can safeguard its future availability for research and traditional use.

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