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HR-LCMS BASED METABOLITES PROFILING, PHARMACOGNOSTIC STUDY, AND ANTIMYCOTIC ACTIVITY OF LEAVES OF RUELLIA ASPERULA

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ABSTRACT

Background: Fungal infections pose a global health challenge, exacerbated by rising drug resistance and immunocompromised populations. *Ruellia asperula*, a traditional medicinal plant, has garnered attention for its bioactive compounds, including flavonoids and alkaloids. This study aims to profile its metabolites using HR-LCMS and evaluate its antimycotic potential, contributing to the discovery of natural therapeutic agents. **Methodology:** Leaves and bark of *Ruellia asperula* were collected, authenticated, and processed for analysis. Physicochemical standards like moisture content, ash values, and extractive yields were determined. Preliminary phytochemical screening identified bioactive compounds. Ethanolic extracts were prepared via Soxhlet extraction, fractionated through column chromatography, and analyzed using HR-LCMS. In vitro, antimycotic assays were conducted against Alternaria and Macrophomia. **Results:** Physicochemical analysis revealed a total ash content of 2.87% w/w, water-soluble ash of 25.78% w/w, and alcohol-soluble extractive value of 9.53% w/w, indicating substantial secondary metabolites. Phytochemical screening identified alkaloids, flavonoids, and saponins. HR-LCMS analysis detected 18 compounds in the ethanolic fraction and 13 in the chloroform fraction. In vitro assays demonstrated significant inhibition of Alternaria and Macrophomia, with activity comparable to Itraconazole. **Discussion:** *Ruellia asperula* leaves demonstrated high phytochemical quality and are rich in flavonoids and alkaloids. HR-LCMS profiling identified bioactive metabolites, while in vitro tests confirmed significant antifungal activity. These findings underscore the plant's potential as a natural antimycotic agent. **Conclusion:** This study highlights the phytochemical richness of *Ruellia asperula*, confirmed by HR-LCMS and pharmacognostic analyses. Its potent antimycotic activity, comparable to Itraconazole, positions it as a promising candidate for natural fungal therapies

INTRODUCTION

Fungal infections pose a growing threat to public health worldwide [1]. These infections are caused by pathogenic fungi, which can invade and persist in human tissues and cause systemic diseases [2]. The prevalence of fungal infections has been rising, influenced by the increased use of

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immunosuppressive therapies, a growing population of immunocompromised individuals, and the spread of antibiotic resistance [3]. Globally, it is estimated that billions of people suffer from fungal skin infections, while more severe invasive fungal infections kill approximately 1.5 million people each year [4]. The distribution and impact of fungal diseases vary significantly across different geographical regions and populations, making them a complex and persistent global health challenge [5]. *Ruellia asperula* is a lesser-studied herbaceous plant belonging to the Acanthaceae family, commonly found in tropical and subtropical regions [6]. The plant is characterized by its rough-textured leaves and vibrant flowers (Figure 1), often thriving in diverse environmental conditions [7]. Traditionally, *Ruellia asperula* has been utilized in folk medicine for its supposed therapeutic properties, including treatments for various ailments such as inflammation, infections, and pain. The plant's traditional use reflects empirical knowledge, forming a foundation for its exploration in modern pharmacology. Phytochemical analyses indicate the presence of bioactive compounds like flavonoids, saponins, and alkaloids, which support its medicinal potential. [8]. Despite its traditional uses, the scientific community has only recently begun systematically studying and documenting the pharmacognostic aspects and the full spectrum of bioactive constituents in *Ruellia asperula* [9]. Bridging this gap requires advanced analytical methods to validate traditional knowledge and explore the plant's potential in addressing contemporary health challenges, including antifungal resistance. The genus *Ruellia* contains diverse bioactive metabolites, including phenolics, flavonoids, alkaloids, saponins, and triterpenoids, associated with its wide range of pharmacological activities [10].

Figure 1: *Ruellia asperula* Plant

High-resolution liquid chromatography-mass spectrometry (HR-LCMS) is a sophisticated analytical technique that combines the separation capabilities of high-performance liquid chromatography (HPLC) with the precise mass detection abilities of mass spectrometry (MS) [11]. This integration allows for the detailed analysis and identification of complex mixtures of compounds at very low concentrations, making HR-LCMS an invaluable tool in pharmacognosy, metabolomics, and biochemical profiling [12]. The technique's high resolution distinguishes molecules that differ by even the smallest mass differences, which is crucial for identifying minor metabolites in plant extracts and natural substances [13]. In this study, HR-LCMS will be utilized to profile the phytochemical composition of *Ruellia asperula* leaves, focusing on identifying metabolites with potential antifungal properties. By cataloguing the plant's secondary metabolites, HR-LCMS is expected to uncover key bioactive compounds and support the exploration of their pharmacological significance, particularly in antifungal applications. This scientific validation of Ruellia asperula's traditional uses links ethnomedicine and modern therapeutic advancements. This planned analysis will provide a strong foundation for understanding the therapeutic potential and chemical diversity of *Ruellia asperula* [14]. The present research seeks to harness the advanced capabilities of HR-LCMS to conduct a comprehensive metabolite profiling of *Ruellia asperula* leaves. Focusing on this particular plant, our study aims to fill a significant gap in the existing scientific literature regarding its pharmacognostic properties and the spectrum of its bioactive compounds. We intend to meticulously analyze the leaf extracts of *Ruellia asperula* to identify and quantify the phytochemical constituents, examining their potential antimycotic properties against a range of fungal pathogens. This detailed analysis will enhance our understanding of the plant's pharmacological potential and contribute to the broader field of natural product research, potentially leading to development new antimycotic agents derived from *Ruellia asperula*. The outcomes of this study are expected to provide valuable insights into the plant's medicinal value and pave the way for future pharmacological and therapeutic explorations.

MATERIALS AND METHODS

Collection, identification, and preparation of plant materials

Fresh leaves and barks of the plant Ruellia asperula were collected from the Hadapsar of Pune district, Maharashtra, India, and identified by the taxonomists of the, where a voucher specimen (Voucher specimen no- PVPV/Bot./2023-24/211) has been deposited for further reference. After authentication, plant materials were collected in bulk, washed, shade-dried, and pulverized in a mechanical grinder to obtain coarse powder. It was stored in a well-closed container and used for further studies.

Determination of analytical standards

Analytical standards and physicochemical constants of the leaf were found to assess the drug's quality and purity. These included the moisture content, extractive values, sulphated ash value, water-insoluble ash value, acid-insoluble ash value, and total ash value [15]**.**

Total ash values

A tarred nickel crucible was placed in a muffle furnace for about 15 minutes at 450 °C, then cooled in a desiccator for about an hour before being weighed (W1). The nickel crucible was filled with 3.0g of the powdered material and heated slowly until all the moisture was removed and the plant material burned entirely (W2). The sample turned gray (white ash) when the heat was gradually increased until the carbon vaporized and the residue was carbon-free at 650°C. After being taken out using crucible tongs, the crucible was cooled in a desiccator and weighed again (W3). The relationship was used to calculate the percentage of ash content [16].

$$\%Ash = \frac{W3-W1}{W1} \times 100$$

W3= Final weight of crucible, W1= Initial weight of crucible

Water insoluble ash value

The ash contents of the crucible obtained from Total ash were transferred into a beaker, which was then filled with 25 ml of water and boiled for 5 minutes. After passing the mixture through ashless filter paper, the residue, and the filter paper were baked to dryness. After being compressed into the crucible and heated to 450 degrees Celsius, the residue-containing ashless filter paper was removed. After reweighing the crucible (W3), the variations were recorded using the formula [17].

$$\%\ \text{Water Insoluble Ash} = \frac{\text{Weight of sample and crucible (W2)} - \text{Initial weight of crucible (W1)}}{\text{Final weight of crucible (W3)}} \text{ x } 100$$

Acid insoluble ash

The ash from the total ash was transferred to a beaker containing 25 ml of dilute hydrochloric acid and boiled for 5 minutes. An ash-free filter paper and a sintered crucible were used to gather the insoluble material. Hot water was used to rinse the crucible and beaker through the filter paper several times until no more acid was present. The filter paper was put into a crucible and burned in a muffle furnace at 500°C until it was carbon-free. After being cooled in a desiccator, the crucible and its contents were weighed. The air-dried material was used to compute the percentage of acid-insoluble ash [18].

Sulphated ash value

A nickel crucible was ignited to a constant weight at 450ºC, then cooled and weighed. The weight was measured again after covering the crucible's bottom with 3.0 g of the dried material. After moistening the material with diluted sulfuric acid, it was burned at 450°C, increasing the heat gradually until all carbon was removed. After cooling in a desiccator, more diluted sulfuric acid was added to the crucible. After cooling down and reweighing periodically, the heating was kept up to about 800°C until a steady weight was achieved. The difference between two weights was used to calculate the % of sulphated ash value [19].

$$\%\ \text{Sulphated Ash} = \frac{\text{Final weight of sample} - \text{Initial weight of sample}}{\text{Initial weight of sample}} \text{x } 100$$

Determination of extractive yields [20]

Alcohol soluble extractive value

5.0 g of the material was precisely weighed and placed in a stoppered conical flask. After adding 100 ml of 90% alcohol, the conical flask's stopper was firmly replaced. After being mechanically shaken for roughly six hours, the flask's contents were allowed to be macerated for an additional eighteen hours before being filtered. After gathering and drying the filtrate, the residue was dried at 105°C to a consistent weight.

Water soluble extractive value

The material was precisely weighed to 5.0 g and then put into a stoppered conical flask. After adding 100 ml of chloroform water, the stopper was firmly replaced. After being mechanically shaken for six hours, the flask's contents were allowed to be macerated for an additional eighteen hours before being filtered. After gathering the filtrate and evaporating it until it was dry, the residue was dried at 105°C to a consistent weight.

Determination of moisture content

A porcelain crucible with a tarred interior that had been heated was weighed, and its weight with the lid was noted (W1). The dried sample was added to the crucible with a spatula full, and the weight was then measured again (W2). The sample was heated to 65° C in an oven for 12 hours, weighing it every 6, 3, 2, and 1 hour until it reached a constant temperature. The sample was weighed again after being allowed to cool in a desiccator.

Noted was the continuous weight, W3. The relationship was used to calculate the percentage of moisture.

$$\% \text{ moisture } = \frac{\text{Weight of sample in crucible (W2) – Constant weight (W3)}}{\text{Weight of sample in crucible (W2) – Weight of crucible (W1)}} \text{ x } 100$$

Where, W2–W1=weight of sample, W2-W3=weight of moisture

Phytochemical analysis [21]

Qualitative phytochemical analysis of the crude extract

Qualitative phytochemical tests to detect the presence of various secondary metabolites in the crude extract were carried out using standard procedures.

Test for carbohydrates (Molisch's test)

0.1 g of each sample was boiled and filtered in 2 ml of distilled water. In small drops, Molisch's reagent (α naphthol solution in ethanol) was added to the filtrates. Concentrated sulfuric acid was carefully poured down the test tube's side to create a lower layer. A purple interfacial ring indicates the presence of carbohydrates.

Test for glycosides (combined reducing sugars)

0.1 g of the powder and 5 ml of diluted sulfuric acid were combined in a test tube, brought to a boil over a water bath for 15 minutes, and then neutralized with a 20% KOH solution. After adding 10 mL and heating it for five minutes, equal parts Fehling solutions I and II were combined. A denser brick-red precipitate indicates the presence of glycosides.

Test for alkaloids [22]

A 0.5g powdered extract was stirred in 5 mL of 1% HCl on a steam bath for 5 minutes. Next, Whatman's No. 1 filter paper was employed to filter the mixture. One milliliter of the filtrate was mixed with two drops of Dragendoff's reagent. An orange-red color was seen, which suggests the presence of alkaloids.

Test for saponin.

2 g of the extract was combined with 20 ml of distilled water and heated to a boil for two minutes using a hot water bath. Following a hot filtering and cooling period, the mixture's filtrate was used for the subsequent tests:

- **Frothing test:** 5 ml of filtrate was mixed with 15 ml of distilled water and vigorously shaken for two minutes. A stable foam (froth) indicates the presence of saponins.
- **Emulsion test:** Two drops of olive oil were added to the frothing solution, and the mixture was vigorously shaken. The existence of an emulsion indicates the presence of saponins.

Test for tannins.

For the ensuing tests, 1g of the powdered substance was boiled with 20 ml of water, filtered, and utilized.

Ferric chloride test: A few drops of ferric chloride were added to 3 ml of the filtrate. A greenish-black precipitate indicates the presence of tannins.

Test for flavonoids

0.2 g of the powder was combined with 10 ml of ethyl acetate and heated for 3 minutes on a water bath. The mixture was then cooled and filtered, and the filtrate was used for the subsequent tests [22].

- **Ammonium hydroxide test:** 1 ml of diluted ammonia solution was shaken with 4 ml filtrate. After the layers were allowed to separate, the yellow color in the ammoniacal layer confirmed the presence of flavonoids.
- **1 % Aluminium chloride solution test:** A further 4 ml portion of the filtrate was shaken with 1 ml of 1% aluminium chloride solution. After the layers were separated, flavonoids were found in the yellow aluminium chloride layer.

Test for steroids.

A mixture of 0.5 g of the powdered sample, 2 mL H2S04, and 5 mL of acetic anhydride was added. When the color shifts from violet to blue, steroids are present.

Test for terpenoids (Salkowski test)

5 mL of the crude extract was carefully mixed with 2 mL of chloroform before gradually adding 3 mL of concentrated H_2S0_4 solution. There are terpenoids present because a reddish-brown color forms at the interface.

Preparation of Plant Extract

A hundred grams of *Ruellia asperula* Each weighed packet of powdered leaves was placed into the Soxhlet extractor's thimble. A measuring cylinder measured 300 milliliters of the solvent (ethanol) and then poured it into the Soxhlet extractor's still pot. The apparatus was then coupled, and the condenser unit was connected to an overhead water tank to cool the rising solvent vapor. A Bunsen burner at 68°C served as the heat source [23]. The solvent condensed at the Soxhlet extractor's condenser unit

after evaporating via the expansion adapter, thimble, and distillation path. The sample was inside the thimble when the condensed vapor returned there in the form of liquid droplets. The entire contents of the thimble and siphon were emptied back into the still pot of the Soxhlet extractor when the solvent in the thimble reached the level of the siphon top. After roughly nine refluxes over three hours, the extraction procedure was finished. A thermometer was used to control the temperature [24].

Fractionation by Column Chromatography

A 15 g of RAET (Ethanolic extract) was subjected to column chromatography using a glass column (6 cm × 110 cm) and silica as an adsorbent. The column was packed by wet packing in pet ether. The column was eluted with an increment of Ethanol. Once 100% Ethanol was reached, an increment of chloroform was performed. The column was eluted with 1000 mL of mobile phase or till complete elution of the compound. Each fraction was monitored with TLC. Fractions with similar TLC profiles were mixed. A total of 2 fractions were collected [25].

High-Resolution Liquid Chromatography-Mass Spectroscopy (HR-LCMS)

The Agilent 6550 Q-TOF MS instrument from Agilent Technologies in Santa Clara, California, was used as an HR-LCMS to analyze the metabolomics data. MassHunter LC/MS Data Acquisition software version B.06.01 was used to control the apparatus and collect the data. MassHunter Qualitative and Quantitative Analysis software (version B.07.00) was used to evaluate the data. Every sample was run through a 0.2 µm-pore nylon membrane filter before being injected. A Zorbax Eclipse C18 column with a 2.1 ¼ 150 mm diameter and a 5-micron thickness was used for the chromatographic separation. A gradient solvent system comprised 10% water and 0.1% formic acid in acetonitrile and water. A 95% separated in 2–20 minutes, B 5% in 5 minutes, A 5% in 20–25 minutes, B 5% in 5 minutes, and A 95% and 5% in 26–30 minutes. 0.2 milliliters per minute of flow were maintained at a constant pressure of 1,200 bar [25]. The mass spectral data was obtained by positive mode electrospraying. The capillary voltage, source cone voltage, and extraction cone voltage were all maintained at 3.25 kV, 30 V, and 4 V, respectively, indicating the presence of the positive mode. Nitrogen was used as the desolvation gas in this investigation, and 900 liters of nitrogen were provided per hour [26]. The source temperature was maintained at 120°C, and the desolvation temperature was maintained at 550°C. Using the mass-to-charge ratio (m/z) range of 100–1,200 and a mass resolution of 22,000 FWHM (full-width half), mass spectra were obtained [27].

Identification of compounds

The Metlin library database was used to interpret the mass spectrum after the components were identified through retention index analysis. The collection contains about 62,000 records of recognized chemicals. The standard mass spectra of recognized constituents found in the Metlin library were compared with the spectra of the unidentified constituents of the *Gardenia gummifera* fraction [25].

***In-Vitro* antimycotic activity**

Alternaria and *Macrophomia* were grown on sabouraud dextrose agar and incubated overnight. The yeast culture cultured overnight was introduced into a normal saline solution (4.85% concentration). The saline that had been infected was mixed vigorously using a vortex mixer and then adjusted to a concentration of 0.5 McFarland standards (equivalent to 1–5 ¼ 106 cells/ml) by comparing it to white paper with black lines [28]. A micropipette was used to pipette a 100 µl suspension of *Alternaria* and *Macrophomia*. This suspension was then placed onto the surface of sabouraud dextrose agar [29]. The agar was then swabbed at a 60° rotation to ensure an even distribution of yeast across the media surface. A cotton swab was then used for this purpose [30]. The swabbed sabouraud dextrose agar was allowed to stand for 15 minutes to facilitate the adherence of yeast to the media. Subsequently, the sterilized cork borer with a diameter of 6 mm was employed to puncture the swabbed media, resulting in the formation of wells with a diameter of 6 mm. The determination of the fraction concentration for the experiment was derived from a prior investigation conducted on the plant. Fractions at 200 and 100 mg/ml concentrations were introduced into the wells. To promote the diffusion of fractions in the media, the Petri dishes inoculated were refrigerated at a temperature of 4C for 2 hours. Next, the Petri plates were incubated at 37°C for 24 hours. The diameter of the inhibitory zone was measured using a ruler in millimeters after a 24-hour incubation period and thereafter documented. Three replicates were conducted for the experiment [31].

Statistical analysis

The results of all experiments were statistically analysed with GraphPad Prism and Microsoft Office Excel 2016. The means

were computed, and a one-way analysis of variance (ANOVA) test was conducted to compare all the mean values across different instances. The mean differences were calculated using the standard deviation (SD) at a significance level 0.05.

RESULTS AND DISCUSSION

Results of physicochemical parameter

Table 1: Results of physicochemical parameter

Parameters	***Ruellia asperula Leaves***
Ash value	
Total ash % w/w	2.87±0.34
Acid insoluble ash % w/w	0.362±0.76
Water soluble ash % w/w	25.78±0.687
Extractive values	
Alcohol soluble extractive % w/w	9.53±0.087
Hydro-alcoholic extractives (% w/w)	11.64±0.0984
Water soluble extractive	12.764±0.762
Loss on drying	5.74±0.087
Foreign organic matter	2.77±0.098
Crude Fiber content	1.8±0.8
Foaming index	<100
pH of 10% aqueous solution	7.6±0.87

Values are expressed in mean±SD, (n=3)

Table 2: Results of Phytochemical Screening of *Ruellia asperula Leaves*

Phytochemicals	**Test/reagent**	***Ruellia asperula***
Alkaloids	Dragendorff's test	+
	Mayer's test	+
	Hager's test	+
	Wagner's test	+
Carbohydrates	Molisch's test	+
	Fehling's test	+
	Benedict's test	+
Glycosides	Legal's test	+
	Keller-Killiani test	+
Steroids	Libermann-Burchard test	-
	Salkowski test	-
Flavonoids	Shinoda's test	+
Saponins	foam test	+
Tannins and phenolic compounds	Lead acetate test	+
	Ferric chloride test	+
	Potassium dichromate test	+
Triterpenes	Sulphuric acid test	+

+present, -absent

Identification of compounds using the Metlin library

The HR-LCMS data obtained from the metabolite screening were compared to the Metlin library for compound identification, a comprehensive database of known metabolites. The compounds were identified based on accurate mass measurements and fragmentation patterns. In the HR-LCMS report, compounds with higher abundance were selected from *Ruellia asperula*, where 18 compounds were selected from the ethanol fraction and 13 compounds from the Chloroform fraction.

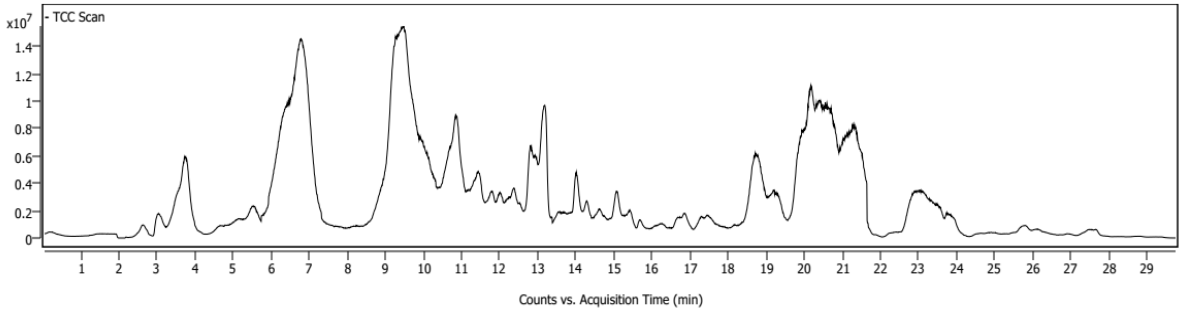


Figure 2: HR-LCMS report of ethanolic fraction of *Ruellia Asperula*

Table 3: List of the compounds identified in the ethanolic fraction of *Ruellia asperula.*

S. No.	Compound Name	Retention Time	Mass	Molecular Weight	Score
1	*Isoorientin 2''-O-rhamnoside*	11.001	$C_{27}H_{30}O_{15}$	594.1586	90.40
2	*Rustoside*	11.135	$C_{26}H_{28}O_{15}$	580.1434	95.95
3	*Vitexin*	14.955	$C_{21}H_{20}O_{10}$	432.3773	92.62
4	*Suberic acid*	15.481	$C_8H_{14}O_4$	174.2010	92.62
5	*Isofraxidin*	11.001	$C_{11}H_{10}O_5$	222.0534	93.98
6	*Eujambolin*	10.373	$C_{24}H_{24}O_{13}$	520.1226	94.91
7	*Biotripyrrin-b*	Unknown	$C_{25}H_{27}N_3O_6$	461.1986	94.91
8	*9,10-Dihydroxy-12,13-epoxyoctadecanoate*	19.392	$C_{18}H_{34}O_5$	330.2411	94.19
9	*9S,12S,13S-trihydroxy-10E-octadecenoic acid*	19.392	$C_{18}H_{34}O_5$	330.2411	94.19
10	*9,10,13-trihydroxy-11-octadecenoic acid*	19.392	$C_{18}H_{34}O_5$	330.2411	94.19
11	*2-methoxy-6Z-hexadecenoic acid*	19.392	$C_{17}H_{32}O_3$	284.2357	94.06
12	*12-(2,3-Dihydroxycyclopentyl)-2-dodecanone*	19.392	$C_{17}H_{32}O_3$	284.2357	94.06
13	*Muricatacin*	19.392	$C_{17}H_{32}O_3$	284.2357	94.06
14	*16-Oxo-palmitate*	19.392	$C_{16}H_{30}O_3$	270.2200	94.02
15	*Quercetin 3-O-glucuronide*	13.432	$C_{21}H_{18}O_{13}$	478.1243	97.04
16	*Kaempferol*	17.231	$C_{15}H_{10}O_6$	286.0483	96.23
17	*Luteolin*	17.231	$C_{15}H_{10}O_6$	286.0483	96.23
18	*Apigenin 6-C-glucoside 8-C-arabinoside*	10.549	$C_{26}H_{28}O_{14}$	564.1486	97.08

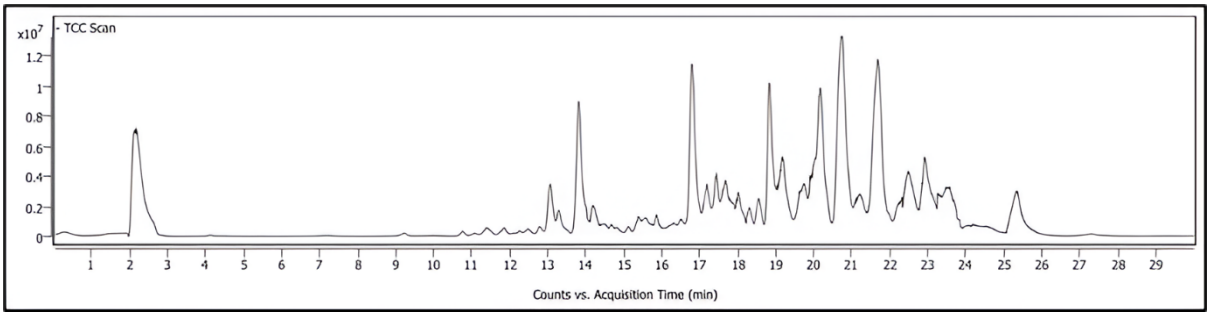
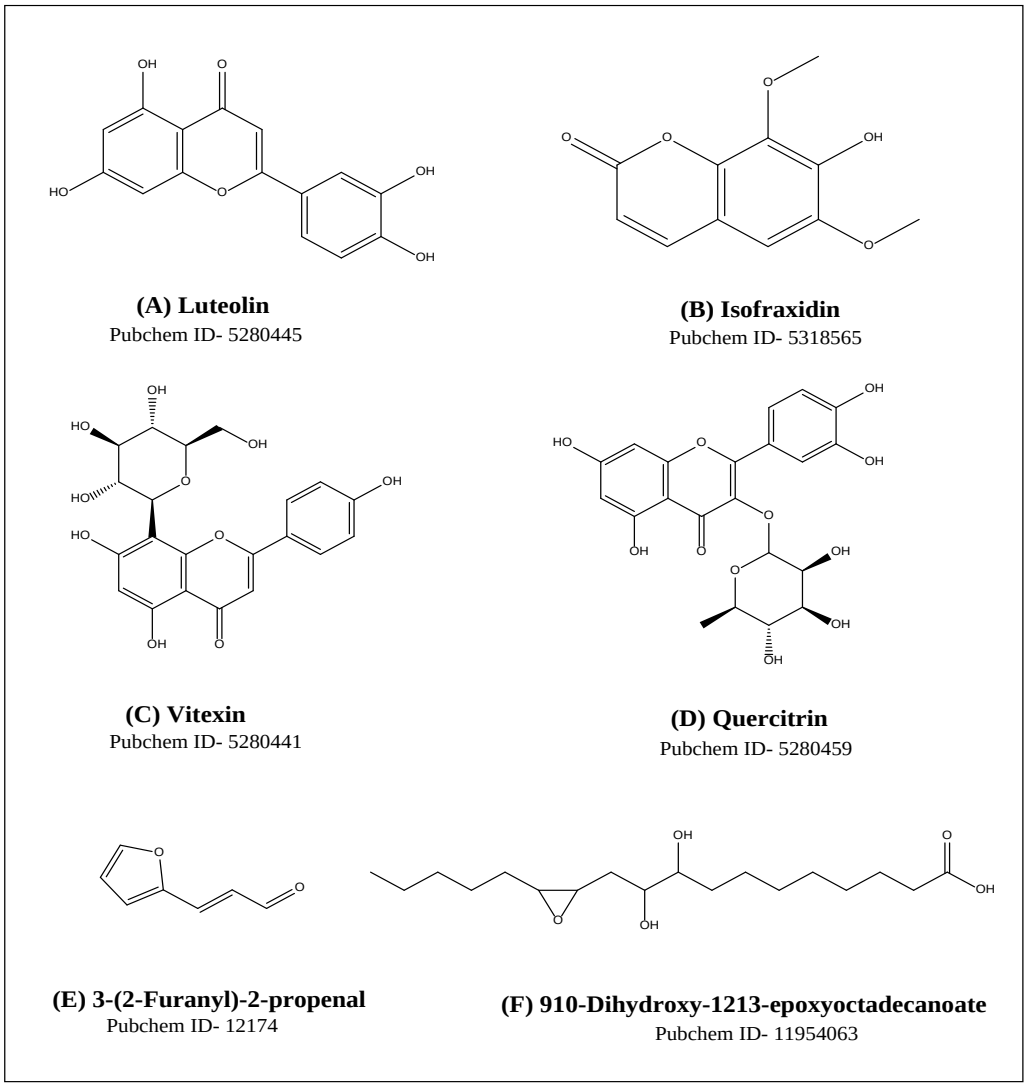


Figure 3: HR-LCMS report of chloroform fraction of *Ruellia asperula*

Table 4: List of the compounds identified in the chloroform fraction of *Ruellia asperula.*

S No.	Compound Name	Retention Time	Formula	Mass	Score
1	*3-(2-Furanyl)-2-propenal*	11.504	$C_7H_6O_2$	122.0367	99.80
2	*Quercitrin*	11.766	$C_{21}H_{20}O_{11}$	448.1014	97.71
3	*3-oxo-dodecanoic acid*	12.080	$C_{12}H_{22}O_3$	214.1574	98.60
4	*trans-4-Hydroxycyclohexanecarboxylate*	12.144	$C_7H_{12}O_3$	144.0789	96.88
5	*Suberic acid*	12.425	$C_8H_{14}O_4$	174.0891	94.31
6	*Quercetin 3-O-glucuronide*	12.633	$C_{21}H_{18}O_{13}$	478.0754	97.51
7	*Quercetin 3-galactoside*	12.646	$C_{21}H_{20}O_{12}$	464.0962	97.18
8	*910-Dihydroxy-1213-epoxyoctadecanoate*	16.257	$C_{18}H_{34}O_5$	330.2411	98.68
9	*5-Nonyltetrahydro-2-oxo-3-furancarboxylic acid*	16.592	$C_{14}H_{24}O_4$	256.1681	95.13
10	*(±)-14-Nonanediol diacetate*	16.658	$C_{13}H_{24}O_4$	244.1677	99.63
11	*Ethyl(EZ)-decadienoate*	16.802	$C_{12}H_{20}O_2$	196.1470	96.70
12	*91013-Trihydroxystearic acid*	18.842	$C_{18}H_{36}O_5$	332.2570	98.87
13	*Hexadecanedioic acid*	19.862	$C_{16}H_{30}O_4$	286.2146	98.90



(A) Luteolin
Pubchem ID- 5280445

(B) Isofraxidin
Pubchem ID- 5318565

(C) Vitexin
Pubchem ID- 5280441

(D) Quercitrin
Pubchem ID- 5280459

(E) 3-(2-Furanyl)-2-propenal
Pubchem ID- 12174

(F) 910-Dihydroxy-1213-epoxyoctadecanoate
Pubchem ID- 11954063

Figure 4: Structure of compounds having potent antimycotic activity identified in fraction of JKET and JKCH (A) Luteolin, (B) Isofraxidin, (C) Vitexin, (D) Quercitrin, (E) 3-(2-Furanyl)-2-propenal, (F) 910-Dihydroxy-1213-epoxyoctadecanoate.

Table 5: Results of Antimycotic activity of fractions of *Ruellia asperula*

Compound	Code No.	Concentration (mg/ml)	Zone of Inhibition (mm)	
			Alternaria	***Macrophomia***
JKET	F1	100	10.7±0.53	12.6±0.65
	F2	200	18.8±0.64	19.3±0.43
	F3	300	22.1±0.87	24.4±0.72
JKCH	F1	100	11.5±0.64	14.9±0.42
	F2	200	19.2±0.72	21.4±0.54
	F3	300	23.5±0.89	25.1±0.83
MS (Itraconazole)	F1	100	12.8±0.53	13.2±0.87
	F2	200	17.4±0.67	19.6±0.88
	F3	300	24.8±0.77	26.7±0.47

Values are expressed in mean±SD (n=3)

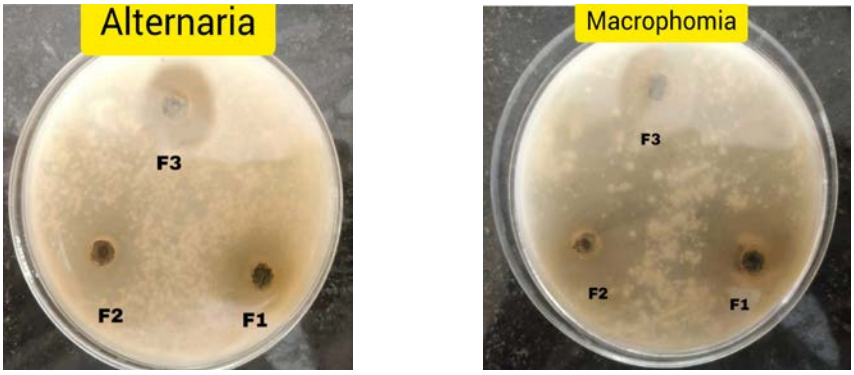


Figure 5: Antimycotic activity of fraction JKET with *Alternaria* and *Macrophomia*

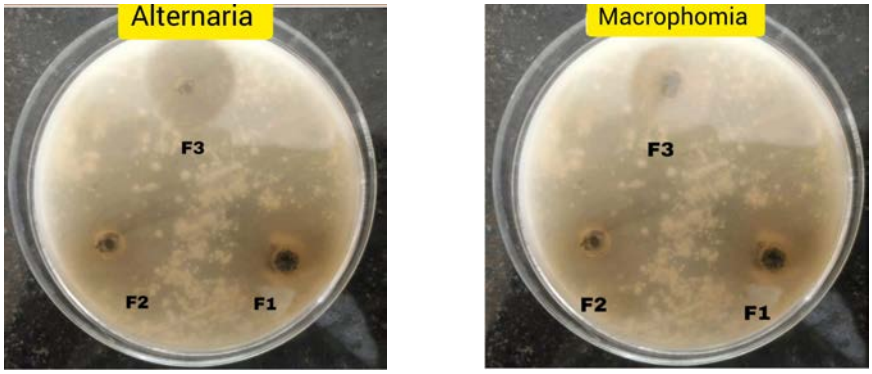


Figure 6: Antimycotic activity of fraction JKCH with *Alternaria* and *Macrophomia*

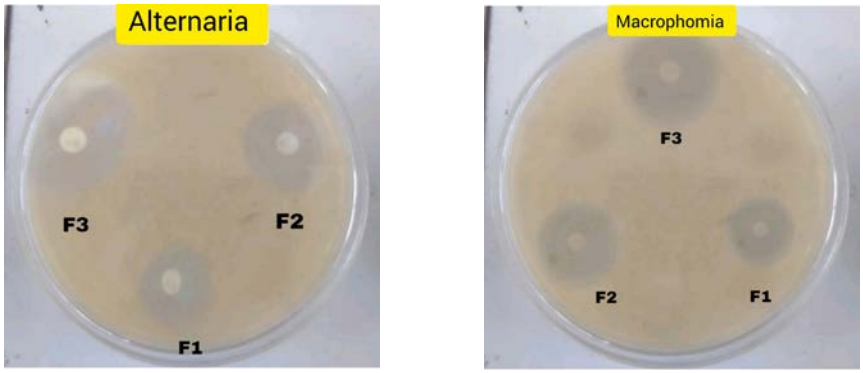


Figure 7: Antimycotic activity of MS (Marketed standard- Itraconazole) with *Alternaria* and *Macrophomia*

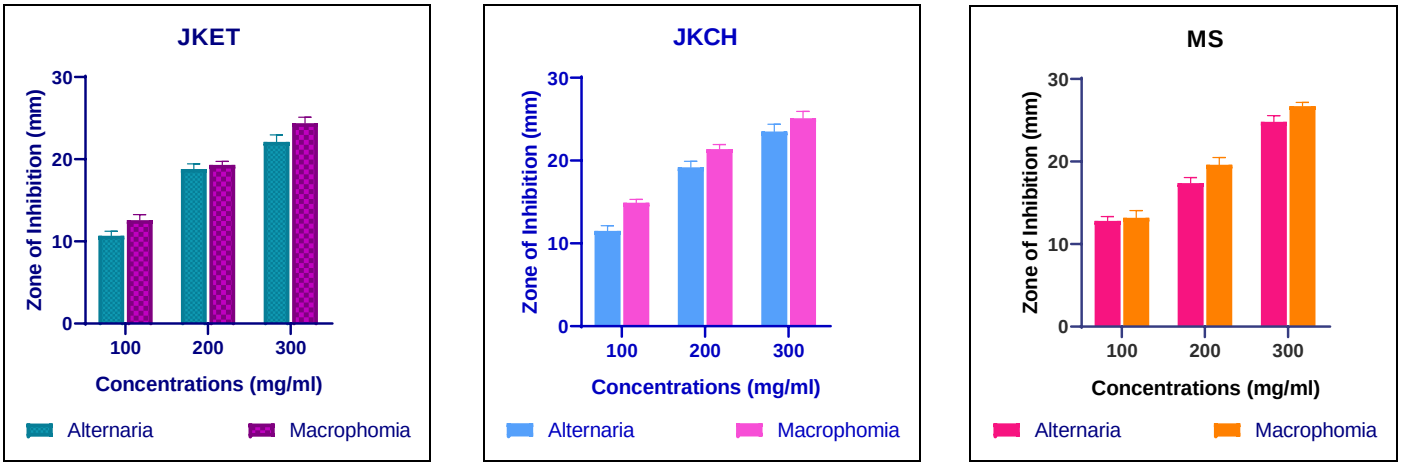


Figure 8: Graphical representation of *In-Vitro* antimycotic activity of fraction JKET (Ethanolic fraction), JKCH (Chloroform fraction) and MS (Itraconazole)

DISCUSSION

The physico-chemical analysis of *Ruellia asperula* leaves, as detailed in Table 1, provides insightful data on their composition and potential medicinal applications. The total ash content, at 2.87%±0.34, and particularly the high water-soluble ash percentage of 25.78%±0.687, indicate a significant presence of inorganic constituents and good solubility in water, which may correlate with the bioavailability of mineral nutrients and basic salts. This high water-soluble ash value suggests that the plant's essential minerals and bioactive compounds are readily available for absorption, enhancing its pharmacological effectiveness in therapeutic applications. The relatively low acid insoluble ash value of 0.362%±0.76 suggests minimal contamination with silica and other acid-insoluble materials, supporting the purity of the leaf material. Additionally, the extractive values such as the alcohol soluble extractive (9.53%±0.087) and hydro-alcoholic extractive (11.64%±0.0984) provide evidence of the leaves' substantial secondary metabolite content, which are crucial for pharmacological activities. The elevated water-soluble extractive value (12.764%±0.762) highlights the plant's potential to efficiently release bioactive compounds in aqueous formulations, enhancing their accessibility and activity in biological systems. This is further supported by the water-soluble extractive value of 12.764%±0.762, underscoring the potential efficacy of aqueous extracts in herbal formulations. The loss on drying value recorded at 5.74%±0.087 implies a relatively low moisture content, which is beneficial for the stability and storage of the plant material. The foreign organic matter content is also minimal (2.77%±0.098), indicating good harvesting and post-harvest handling practices. The low crude fiber content, at 1.8%±0.8, contributes to the bulk and can aid in extracting active ingredients during preparation. The pH of the 10% aqueous solution is 7.6±0.87, suggesting a nearly neutral nature often preferred in phyto-preparations to avoid irritation and promote compatibility with the human body. This neutral pH not only supports the stability of active compounds but also ensures better compatibility with physiological conditions, further enhancing the pharmacological relevance of the plant. The foaming index, less than 100, indicates low saponin content, which might limit use in applications requiring high foaming properties but could also mean less potential for adverse saponin-related effects. These physicochemical characteristics collectively support using *Ruellia asperula* leaves in traditional medicine, reflecting the good quality and potential efficacy as indicated by the extractive values and ash content. The data emphasize the plant's ability to deliver bioactive compounds effectively, enhancing its therapeutic potential in antifungal and other medicinal applications. The phytochemical screening of *Ruellia asperula* leaves, as reported in Table 2, reveals a diverse array of bioactive compounds, suggesting multiple therapeutic applications. The positive results across all tests for alkaloids (Dragendorff's, Mayer's, Hager's, and Wagner's) indicate a robust presence of these nitrogen-containing compounds, known for their broad spectrum of pharmacological activities, including anti-malarial, analgesic, and anti-inflammatory properties. Similarly, as confirmed by Molisch's, Fehling's, and Benedict's tests, the presence of carbohydrates underscores their role in providing energy and potentially modulating immune system responses. The detection of glycosides via Legal's and Keller-Killiani tests further suggests the presence of heart-active

compounds, which could be valuable in treating cardiac conditions. Conversely, the absence of steroids, indicated by negative results in both Libermann-Burchard and Salkowski tests, might limit the leaves' utility in therapies targeting inflammation and immune modulation, where steroids are often beneficial. However, the presence of flavonoids, as determined by the positive Shinoda test, aligns with potential antioxidant and anti-cancer benefits, supported by their ability to scavenge harmful free radicals. Additionally, the detection of saponins via the foam test suggests usefulness in formulations for their antimicrobial and immune-boosting properties. The positive tests for tannins and phenolic compounds (Lead acetate, Ferric chloride, and Potassium dichromate tests) further emphasize the antioxidant capacity of the leaves, which could contribute to reducing the risk of chronic diseases. Lastly, triterpenes, identified by the Sulphuric acid test, hint at possible anti-inflammatory and anti-viral activities. Collectively, these results highlight *Ruellia asperula* leaves as a potential source of natural compounds for medicinal applications, leveraging their rich phytochemical profile.

The high-resolution liquid chromatography-mass spectrometry (HR-LCMS) analysis of *Ruellia asperula*, detailed in Figure 2 and Table 3, effectively illustrates the plant's rich phytochemical composition. Utilizing the Metlin database for comparison, a total of 18 compounds were identified in the ethanolic fraction, indicating a diverse array of bioactive molecules. Prominent among these are flavonoids like Isoorientin 2"-O-rhamnoside, Rustoside, and Apigenin 6-C-glucoside 8-C-arabinoside, with high identification scores (90.40, 95.95, and 97.08 respectively), pointing to their significant presence and potential antioxidant, anti-inflammatory, and anticancer properties. Flavonoids such as Quercetin 3-O-glucuronide and Kaempferol, known for their potent bioactivities, further confirm the ethanolic extract's utility in therapeutic applications. Several unique fatty acids and their derivatives were identified, such as 9,10-Dihydroxy-12,13-epoxyoctadecanoate and 9S,12S,13S-trihydroxy-10E-octadecenoic acid, each with a score of 94.19. These compounds are less commonly observed in plant extracts and could indicate specific biosynthetic pathways active in *Ruellia asperula*. Their structural complexity and high scores suggest high reliability in their identification and potential roles in anti-inflammatory and other metabolic regulatory functions. Despite its unknown retention time, the presence of unique compounds such as Biotripyrrin-b, with a high identification score (94.91), underscores the extract's potential for yielding novel bioactive substances. This comprehensive identification validates the plant's phytochemical richness and enhances the scope for further pharmacological explorations based on these compounds.

The HR-LCMS analysis of the chloroform fraction of *Ruellia asperula*, as depicted in Figure 3 and elaborated in Table 4, has successfully identified 13 compounds, emphasizing the chemical diversity and potential pharmacological significance of this plant extract. The ethanolic fraction (JKET) identified 18 compounds, including flavonoids such as Kaempferol, Isoorientin 2"-O-rhamnoside, and Vitexin, known for their antioxidant and antimicrobial properties. In contrast, the chloroform fraction (JKCH) contained 13 compounds, including unique metabolites like 3-(2-Furanyl)-2-propenal and Hexadecanedioic acid, contributing to its potential for disrupting fungal cell membranes. Notably, the analysis highlighted the presence of several potent flavonoids, such as Quercitrin, Quercetin 3-O-glucuronide, and Quercetin 3-galactoside, with high identification scores of 97.71, 97.51, and 97.18, respectively. These compounds are well-recognized for their antioxidant, anti-inflammatory, and cardioprotective properties, which could substantiate the traditional uses of *Ruellia asperula* and suggest promising avenues for therapeutic development. The identification of unique fatty acids and derivatives, like 3-oxo-dodecanoic acid and 9,10-Dihydroxy-12,13-epoxyoctadecanoate, with scores of 98.60 and 98.68, respectively, reveals the presence of specialized metabolites that may contribute to the plant's bioactivity. These compounds could play roles in cell signaling pathways and have potential anti-inflammatory or antimicrobial effects. The presence of unusual compounds such as (±)-14-Nonanediol diacetate and Hexadecanedioic acid, both with high scores (99.63 and 98.90), highlights the extract's complexity and the precision of the HR-LCMS method in identifying components with significant structural diversity. Flavonoids exert antifungal activity by disrupting fungal cell membranes, inhibiting ergosterol synthesis, or generating reactive oxygen species (ROS) that damage fungal cells. Similarly, fatty acids like 9,10-Dihydroxy-12,13-epoxyoctadecanoate may integrate into fungal membranes, altering their structure and permeability and leading to cell lysis. Unlike Itraconazole, which specifically targets fungal ergosterol biosynthesis by inhibiting lanosterol 14α-demethylase, these compounds may offer broader activity

against resistant fungal strains and a reduced likelihood of side effects. These findings support the notion that the chloroform fraction of *Ruellia asperula* is rich in bioactive compounds, which could be pivotal in developing novel pharmaceutical agents. Identifying compounds with potent antimycotic activity in the JKET and JKCH fractions of *Ruellia asperula*, as presented in Figure 4, highlights a range of structurally diverse bioactive molecules. This collection includes flavonoids known for their broad spectrum of biological activities: (A) Luteolin is a flavonoid extensively studied for its antioxidant, anti-inflammatory, and antimicrobial properties. Its effectiveness against fungal pathogens contributes to its potential as a natural preservative or therapeutic agent in treating fungal infections. Luteolin likely exerts its antifungal effects by disrupting fungal cell membranes and generating reactive oxygen species (ROS), leading to cellular damage. (B) Isofraxidin is another noteworthy compound recognized not just for its antimycotic properties but also for its roles in anti-inflammatory and anticancer activities. Its inclusion underscores the pharmacological versatility of the compounds present in *Ruellia asperula*. Vitexin disrupts fungal membrane integrity and interferes with metabolic processes essential for fungal growth. **(**C) Vitexin, a flavone glycoside, has shown considerable bioactivity against various microbial pathogens, including fungi. Its presence enhances the extract's utility in potentially developing antimycotic therapies or supplements. (D) Quercitrin, a quercetin derivative, is well-documented for its strong antioxidant and antimicrobial effects. Its efficacy against fungal species makes it a valuable component for pharmaceutical research focusing on plant-based antimycotic agents. Quercitrin chelates metal ions required for fungal enzyme activity, impairing fungal metabolism and growth. (E) 3-(2-Furanyl)-2-propenal stands out for its unique structure among the identified compounds. Known for its antimicrobial properties, this compound could be particularly effective against certain strains of fungi, contributing to the antimycotic profile of the extract. 3-(2-Furanyl)-2-propenal likely disrupts fungal protein synthesis and metabolic pathways, leading to cellular dysfunction. (F) 9,10-Dihydroxy-12,13-epoxyoctadecanoate is a fatty acid derivative, which may contribute to the overall antimycotic activity by disrupting fungal cell membranes or inhibiting essential metabolic processes. This compound integrates into fungal membranes, altering their structure and permeability, ultimately leading to cell lysis. Together, these compounds provide a substantial basis for the antimycotic efficacy of the fractions from *Ruellia asperula*. Their structural diversity and potent bioactivity highlight the therapeutic potential of this plant, particularly in the development of treatments or preventative measures against fungal infections.

The phytochemical screening of *Ruellia asperula* leaves, as reported in Table 2, indicates the absence of steroids based on negative results from the Libermann-Burchard and Salkowski tests. The lack of steroids may limit the plant's use in therapies targeting inflammation and immune modulation, as steroids are often associated with potent anti-inflammatory and immunosuppressive effects. However, this absence does not diminish the therapeutic potential of *Ruellia asperula*; instead, it underscores its safety profile by reducing the likelihood of steroid-associated side effects, such as hormonal imbalances or long-term immunosuppression. Furthermore, alternative bioactive compounds, including flavonoids, alkaloids, and saponins, provide comparable or even broader pharmacological activities, such as antioxidant, antimicrobial, and immune-boosting effects. This diversity of bioactive constituents compensates for the absence of steroids and enhances the plant’s applicability in natural and safe therapeutic formulations.

Altitude, soil type, climate, and seasonal variations can significantly affect the biosynthesis of secondary metabolites, resulting in variations in their concentration and diversity. For example, plants grown in nutrient-rich soils may exhibit higher levels of flavonoids and alkaloids. In contrast, those exposed to stress conditions, such as drought or high altitudes, may produce increased amounts of stress-induced metabolites like tannins and phenolic compounds. In the case of *Ruellia asperula*, geographical variations in the growth region could explain the diversity of bioactive compounds identified in this study, such as flavonoids and fatty acids, which are known to be influenced by environmental factors. Further comparative studies on plants collected from different regions may provide additional insights into the extent of this variability and its impact on pharmacological properties. The *in-vitro* antimycotic activity results of *Ruellia asperula* fractions, as summarized in Table 5 and depicted in Figures 5 through 8, show a promising antimycotic profile against Alternaria and Macrophomia fungi. The JKET (ethanolic fraction) and JKCH (chloroform fraction) display significant zones of inhibition, which increase with the concentration of the extract, indicating a dose-dependent response. While both fractions showed dose-dependent

antifungal activity, the chloroform fraction (JKCH) exhibited slightly higher efficacy, with inhibition zones of 23.5±0.89 mm and 25.1±0.83 mm against *Alternaria* and *Macrophomia* at 300 mg/mL, compared to 22.1±0.87 mm and 24.4±0.72 mm for the ethanolic fraction (JKET). This difference may be attributed to fatty acids and specific flavonoids in JKCH that enhance fungal inhibition.

The standard antimycotic agent, Itraconazole (MS), at the same highest concentration, showed inhibition zones of 24.8±0.77 mm for *Alternaria* and 26.7±0.47 mm for *Macrophomia*. This comparison highlights that the antimycotic activity of *Ruellia asperula* fractions is very close to that of the standard drug, particularly at higher concentrations. The comparable antifungal activity of the *Ruellia asperula* fractions (JKET and JKCH) to the standard drug Itraconazole has significant practical implications. While Itraconazole remains a widely used synthetic antifungal, it is often associated with limitations such as drug resistance, adverse side effects, and high cost. In contrast, the natural fractions of *Ruellia asperula* offer a safer and potentially cost-effective alternative for antifungal therapy, particularly in resource-limited settings. Additionally, diverse bioactive compounds like flavonoids and fatty acids in these fractions may provide complementary mechanisms of action, such as disrupting fungal cell membranes and generating reactive oxygen species, which could address resistant fungal strains more effectively. Figures 6 and 7 illustrate these effects graphically, showing the gradual increase in antimycotic activity with higher concentrations across all samples. This promising result suggests that the *Ruellia asperula* extracts could be potential sources of natural antimycotic agents and might be particularly useful in cases where synthetic agents prove ineffective or cause undesirable side effects. The identified potent antimycotic compounds correlate with the observed biological activity, supporting further research into their mechanisms and therapeutic potential.

CONCLUSION

The comprehensive study on *Ruellia asperula*, encapsulating HR-LCMS based metabolite profiling, pharmacognostic analysis, and evaluation of antimycotic activity, has significantly elucidated this plant's phytochemical richness and potential medicinal applications. Metabolite profiling confirmed the presence of diverse bioactive compounds, including flavonoids and unique fatty acids, which align with the pharmacological properties observed. The pharmacognostic evaluation revealed the high quality and purity of the leaf material, further supported by substantial extractive values and favorable physicochemical properties. Notably, the antimycotic assays demonstrated potent activity comparable to the standard antimycotic agent, Itraconazole, establishing the extracts as promising candidates for natural antimycotic therapies. This study underscores the potential of *Ruellia asperula* as a source of natural bioactive compounds with significant therapeutic applications, paving the way for future research and development in phytotherapy.

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ABBREVIATIONS

HR-LCMS: High-Resolution Liquid Chromatography-Mass Spectroscopy; RAET: Ethanolic fraction of *Ruellia asperula*; WHO: World Health Organization; MS: Marketed Standard

FINANCIAL ASSISTANCE

NIL

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Patel A.P. contributed significantly to the conceptualization, experimental design, data acquisition, and interpretation of results. Patel A.P. also played a key role in drafting and revising the manuscript. Kudnar J.R. was actively involved in conducting the experimental work, optimizing methodologies, and assisting in data analysis. Jadhav R.S. contributed to literature review, statistical analysis, and manuscript preparation. Ghuge B. provided technical support, assisted in data collection, and contributed to manuscript formatting. All authors have read and approved the final manuscript.

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