https://doi.org/10.31925/farmacia.2025.6.8 **ORIGINAL ARTICLE**

ANTIFUNGAL ACTIVITY AND METABOLOMIC PROFILING OF DIFFERENT PARTS OF *HELMINTHOSTACHYS ZEYLANICA* (L.) USING UNTARGETED LC-HRMS ANALYSIS

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Abstract

Fungal infections caused by *Candida* species pose serious global health challenges due to increasing resistance to existing antifungal agents. *Helminthostachys zeylanica* (L.) is a traditionally used medicinal plant with underexplored antifungal potential. This study provides the first comprehensive antifungal metabolomic analysis of *H. zeylanica* using untargeted LC-HRMS. Antifungal activity against *C. albicans, C. glabrata* and *C. krusei* was evaluated using disc diffusion, minimal inhibitory concentration and minimal fungicidal concentration assays. The extract of the leaf had the best antifungal activity particularly against *C. krusei* and *C. albicans*. Metabolomic profiling revealed flavonoids (such as rutin, isosakuranetin and eriodictyol), loliolide, as the important bioactive metabolites. Multivariate analysis (PCA and PLS-DA) substantiated their association with antifungal activity. These results highlight the potential of *H. zeylanica*, particularly its leaves, as a source of natural antifungal agents, thereby advancing phytopharmaceutical development and efforts to address antifungal resistance. Further research should examine compound isolation, in vivo efficacy and formulation techniques.

Rezumat

Infecțiile fungice cauzate de speciile de *Candida* reprezintă provocări majore pentru sănătatea globală, din cauza rezistenței crescânde la agenții antifungici existenți. *Helminthostachys zeylanica* (L.) este o plantă medicinală utilizată tradițional, al cărei potențial antifungic a fost puțin investigat. Acest studiu oferă prima analiză metabolomică antifungică a plantei *H. zeylanica* folosind LC-MS nețintit. Activitatea antifungică împotriva *C. albicans, C. glabrata* și *C. krusei* a fost evaluată prin metoda disc-difuzimetrica, determinarea concentrației minime inhibirorii și a concentrației minime fungicide. Extractul de frunze a prezentat cele mai puternice efecte antifungice, în special împotriva *C. albicans* și *C. krusei*. Profilarea metabolomică a identificat flavonoide precum rutina, isosakuranetina și eriodictiolul, împreună cu loliolida, ca metaboliți bioactivi principali. Analizele multivariate (PCA și PLS-DA) au confirmat corelația acestora cu activitatea antifungică. Aceste rezultate evidențiază potențialul plantei *H. zeylanica*, în special al frunzelor, ca sursă de agenți antifungici naturali, susținând dezvoltarea fitofarmaceutică și combaterea rezistenței antifungice. Cercetări viitoare ar trebui să exploreze izolarea compușilor, eficacitatea *in vivo* și strategiile de formulare.

Keywords: *Helminthostachys zeylanica*, antifungal activity, metabolomics, LC-HRMS, *Candida* spp.

Introduction

Fungal infections seem to be a huge health issue in the global community as it impacts individuals and animals regardless of their age [1]. The infections might show up on the skin, under the skin, or all over the body. The systemic form is the most dangerous, especially for people whose immune systems aren't very strong [2]. Typical causal agents consist of *Candida* spp. and *Aspergillus* spp. [3]. The rise in fungal infections is due to factors like HIV, immunosuppressive therapies and climate change [4]. It can be hard to figure out what's wrong, and it usually involves looking at clinical, radiological and microbiological evidence [5].

Antifungal resistance is becoming more and more of a concern, especially when it comes to *Aspergillus* and *Candida* infections that fail to respond to azole drugs [6]. Azoles, echinocandins and polyenes are the main medicines that can be used to treat aspergillosis now. Voriconazole is still thought to be the best treatment among these [7]. However, the emergence of multi-drug-resistant strains necessitates the development of innovative antifungal agents and treatment methodologies. Current research focuses on the creation of novel antifungal agents and the application of immunotherapy techniques to address these challenges [7].

Plant secondary metabolites are increasingly being recognized as an alternative to the traditional antifungal

agents in the treatment of fungus in human beings. Natural compounds exist in large diversity of chemical structures and mechanisms of action against various fungal pathogens including *Candida albicans* [8, 9]. These compounds have the benefits of being low-toxic, widely available and capable of regulating the immune system [8]. The alkaloids, terpenoids and flavonoids are significant classes of the antifungal compounds derived from plants and fungal endophytes [10]. These naturally occurring substances could be used to make new antifungals to fight the growing problem of fungal infections and the fact that current treatments are not always effective.

Recent research has shown that medicinal plants could be useful as antifungal agents. Several plant extracts, including those from *Cinnamomum zeylanicum*, *Allium sativum* and *Mahonia bealei*, have demonstrated strong antifungal activity against various fungal pathogens [11]. Moreover, studies on various plant components have indicated the wide range of biological functions especially the ability to suppress fungal growth because of the existence of secondary metabolites. It has been established in numerous studies that the various sections of plants contain various secondary metabolites [12].

Helminthostachys zeylanica (L.), is a plant species from Indonesia that has many traditional medicinal uses [13, 14]. In Central Borneo, people also eat the plant as a vegetable because it is very nutritious, especially in terms of protein, carbohydrates and minerals [15]. Studies have shown that *H. zeylanica* may be able to help people with respiratory problems, especially asthma, by making the airways less sensitive and lowering the number of eosinophils [16]. The plant also shows that it can help with diabetes by stopping α-glucosidase [13]. Our previous study revealed that root extracts of dichloromethane exhibited antibacterial properties against various pathogens, with Minimum inhibitory concentrations (MIC) ranging from 125 - 500 µg/mL [17]. Although it is well known that *H. zeylanica* is utilized in traditional medicine, most of the scientific studies that have been carried out to date have concentrated on the antibacterial, antidiabetic and anti-inflammatory properties. Its effectiveness in terms of respiratory diseases and its inhibitory action in α-glucosidase has been reported in the previous studies; moreover, some studies have shown the antibacterial effect of its root extract. However, the availability of research that specifically looks at the antifungal efficacy of this species and against *Candida* species which are among the most therapeutically relevant fungal infections are notably lacking.

Moreover, no previous studies have employed a metabolomic method, especially untargeted LC-HRMS analysis, to study and relate the bioactive secondary metabolites of *H. zeylanica* with fungicidal activity. This means that there is a great gap in knowledge as the prevalence of fungal diseases in the world is increasing, and the antifungal resistance is evolving, thus it is necessary to find new and effective anti-fungal drugs. Medical use of plants can be therapeutic and given the established ethnomedicinal importance and chemical diversity *H. zeylanica* is a potential, but untapped, prospect.

The aim of this study is to examine the antifungal efficacy of various parts (roots, stems and leaves) of *H. zeylanica* against clinically significant *Candida* species, and to identify the bioactive metabolites responsible for this activity through untargeted LC-HRMS-based metabolomic profiling. We also assume that these metabolites, including flavonoids and other secondary metabolites, are also a part of the antifungal activity of the plant.

Besides identifying the antifungal activity and the metabolomic characteristics of *H. zeylanica*, the project will also contribute to the design of new plant-based antifungal drugs due to the increasing antifungal resistance in the world. This paper seeks to present scientific support of the customary utilisation of *H. zeylanica* and highlight its possible assets as an alternative treatment method by combining the results of untargeted metabolomic analysis in antifungal screening. Furthermore, the findings may enhance future investigations of unexamined medicinal plants and foster a synergistic relationship between ethno-botanical knowledge and contemporary pharmaceutical research.

Materials and Methods

Sample collection

The *H. Zeylanica* (L.) Hook was collected from Sungai Kampar, located in the Riau Province of Indonesia at coordinates 0°09'23.6"N 101°21'54.0"E. The specimen bearing the identifier 67/UN19.5.1.1.3/2019 was identified as *H. Zeylanica* (L.) Hook by Prof. Fitmawati. The specimen was later stored at the Biology Department of Universitas Riau. Since *H. zeylanica* is neither designated as endangered nor protected, specific ethical approval for its collection was unnecessary.

Extraction

The leaves, stems and roots of *H. Zeylanica* (100 g each) were finely chopped and pulverized, and then subjected to methanol extraction (500 mL) for a total of 3 cycles, with each cycle lasting 24 hours. Methanol was selected as the solvent because of its extensive polarity range, facilitating the effective extraction of both polar and semi-polar secondary metabolites, such as flavonoids, alkaloids and terpenoids, all of which are associated with antifungal activity. The obtained isolates were then filtered using Whatman filter paper after which the filtrates were evaporated under low pressure to remove all forms of solvents and thereby produce solid extracts [18, 19].

In vitro antifungal activity

Preparation of media

The fungal specimen was cultured using Sabouraud Dextrose Agar (SDA) and Sabouraud Dextrose Broth (SDB). Preparation of the media was as *per* the guidelines that were provided beforehand.

Test organisms

Antifungal studies were performed with reference strains obtained by way of already existing microbial culture repositories (ATCC), and no human or animal subjects were used in the study, institutional ethical approval was not considered necessary. The cultures of *Candida albicans* ATCC 10231, *C. glabrata* ATCC 15126 and *C. krusei* ATCC 14243 were grown on SDA slant agar using a cross-streak method. The cultures were placed in an incubator at around 37°C for a period of 1 - 2 days to promote their growth and proliferation.

Kirby Bauer disc diffusion method

The Sabouraud Dextrose Agar (SDA) was carefully dispensed into sterile petri dishes post-sterilization, and the mixture was permitted to solidify. An optical density of approximately 0.05 at 530 nm was uniformly distributed across the surface of the SDA with fungi. This was accomplished with the aid of a sterile cotton swab. Blank disc papers were intentionally placed on the surface of the fungi-infested SDA to evaluate its antifungal properties. The test extract was administered dropwise onto the blank discs utilizing a micropipette containing 10 µL of the extract solution, achieving a final concentration of 1000 µg/disc, alongside ketoconazole at a concentration of 30 µg/mL. A similar procedure was employed for the negative control discs. Following an extended duration of exposure to the solutions on the discs, they were allowed to dry naturally.

The incubation of the petri dishes was done at a constant temperature of 37°C between 18 and 24 hours after the preparation process was done. At this stage, fungal culture and the substances given were able to interact. The petri dishes were also analysed at the end of the incubation with clear layers around the discs, meaning that the fungal growth was inhibited. These clear zones were measured in terms of their critiques and documented as concerns the antifungal activity. In order to ensure consistency and reliability of the findings, the test was performed three times on each fungal strain, and the samples proved to exhibit different inhibition areas, which showed that the test is antifungal [20].

Minimum inhibitory concentration (MIC)

The microdilution technique and a 96-well microplate were employed to ascertain the minimum inhibitory concentration (MIC). The optical density of *C. albicans, C. glabrata* and *C. krusei* cultures was adjusted to approximately 0.05 at 530 nm. All detail experiments of the assay based on Hendra *et al*. (2024) and Agustha *et al*. [21, 22].

Minimum fungicidal concentration (MFC)

Furthermore, to determine the MFC, SDA plates were employed to identify the MFC based on Hendra *et al*. (2024) and Agustha *et al*. [21, 22].

Identification of metabolites by UHPLC-Q-Orbitrap HRMS

An ultrasonic extraction procedure was employed to obtain approximately 50 mg of dried extract for metabolite profiling. The extraction was conducted for 15 minutes at 30°C utilizing 1.5 mL of methanol. The filtrates were gathered in vials after the extraction being filtered through a 0.22 µm nylon syringe filter. The analysis utilized Thermo Scientific™ Orbitrap™ Exploris 240 HRMS in Full MS/dd-MS2 acquisition mode. The positive polarity was employed and a Full MS resolution of 90,000 FWHM and scan range of 70 - 800 m/z was applied. The injection time was maximized to 100 ms and intensity threshold was set to 5000 and mass tolerance was 5 ppm. The dd-MS2 resolution used was 22,500 FWHM, and the normalized collision gas energies of 30, 50 and 70 and nitrogen was used as the collision gas. The ion source used was the Optamax™ NG Heated Electro-spray Ionizations (H-ESI) which was used at a spray voltage of 3500 V in positive mode and 2500 V in negative mode. The sheath gas flow rate was 35 arbitrary units (AU), the auxiliary gas flow rate was 7 AU and the sweep gas flow rate was 1 AU. The ion transfer tube temperature was established at 300°C, while the vaporiser temperature was set at 320°C. The mobile phase comprised of MS grade water containing 0.1% formic acid (A) and MS grade acetonitrile with 0.1% formic acid (B). The flow rate was 0.3 mL/min, and the total duration was 25 minutes. The gradient run was initiated at 5 percent B, which slowly increased to 90 percent for 16 minutes, was slowly kept at 90 percent for 4 minutes, and then again to 5 percent B until the 25-minute point. The volume of injection was 5 µL and the temperature of the column maintained at 40°C.

Data analysis

The statistical analysis of the mean ± standard deviation (SD) of antibacterial test results was conducted utilizing Minitab version 21. Prior to conducting one-way analysis of variance (ANOVA), data were tested for normality using Shapiro-Wilk test and for homogeneity of variance using Levene's test to ensure the assumption of ANOVA were met. The means were analysed using one-way analysis of variance (ANOVA) in conjunction with Tukey's test. Differentials were considered statistically significant when the p-value was below 0.05.

Fragmentation patterns (MS and MS/MS) were discerned in data analysed by UHPLC-Q-Orbitrap HRMS (molecular weight values) utilizing Compound Discoverer™ Software (Thermo Scientific™, Waltham, MA, USA). To enable the qualitative analysis of the data later, Raw mass spectrometry data contained in

the form of a file with the extension of *.raw were converted to the form of a file with the extension of *.abf by means of Abf Converter version 4.0.0. MS FileReader 3.0 and MSDIAL version 4.9.221218 were employed for the analysis of compounds, specifically for peak discrimination, filtering and alignment. The identification was conducted utilizing internal and MSP databases, an adduct type of $[M + H]^+$, and reference blank alignment with an 80% threshold. The criteria for compound identification involved mass error values of 10 ppm. MS-FINDER 3.52 was employed to predict the structures, fragments and formulas of unknown compounds. The software utilized subsequent databases: PlantCyc, ChEBI, Natural Products Atlas (NPA), NANPDB, COCONUT, KNApSAcK, PubChem and UNPD.

Multivariate data analysis (MVDA) was conducted using MetaboAnalyst 5.0 (www.metaboanalyst.ca). The antibacterial test clear zone data and peak area data for all compounds identified as the adduct type $[M + H]^+$ were compiled in *.csv format. Principal Component Analysis was employed to produce patterns of sample clustering, as well as to identify similarities and differences among plant parts through multivariate analysis (PCA). The partial least squares (PLS) method was employed to investigate the relationship between chemical compounds and antibacterial activity in *H. Zeylanica* samples. Furthermore, the heatmap and hierarchical cluster dendrogram analyses were conducted to depict the results of identifying the suspected compound in extracts derived from different components of *H. Zeylanica*.

Results and Discussion

Antifungal activities

The antifungal efficacy of different components of *H. Zeylanica* is encapsulated in Table I, which presents the inhibition zones of the roots, stems and leaves against three *Candida* species assessed at a concentration of 1000 µg *per* disc. According to widely recognized classifications of antifungal efficacy, inhibition zones measuring less than 7 mm are deemed weak, those ranging from 7 to 14 mm are categorized as moderate, and zones exceeding 14 mm are classified as strong [23, 24]. The antifungal activity of the root extract with *C. krusei* (10.33 ± 0.15 mm) was much greater in comparison with its activity with *C. glabrata* (8.37 ± 0.42 mm, $p < 0.05$). However, the inhibition areas show a moderate activity compared to the leaf extract. The antifungal activity of stem extract was superior to the root extract due to the fact that the inhibition zone of stem extract against *C. albicans* (13.37 ± 0.31 mm) was greater compared to the antifungal activity of root extract (8.93 ± 0.32 mm, $p < 0.01$). The extract of leaves proved to be the most effective against *C. albicans* (14.43 ± 0.32 mm), which is significantly high when compared to the same of root and stem extracts. Ketoconazole is a positive control, and it showed significantly bigger inhibition areas in all three species, ranging between 25.02 ± 0.32 mm with *C. albicans* and 28.28 ± 0.28 mm with *C. krusei*.

The results were statistically evaluated using Tukey's test to determine the significance of the differences between the inhibition zones of the plant extracts and ketoconazole. The statistical results indicate that the antifungal efficacy of *H. Zeylanica* varies markedly among its components, with the leaves consistently exhibiting the most potent antifungal activity, especially against *C. albicans* and *C. krusei*, as demonstrated by the statistically significant differences in inhibition zones. An analysis of the differences between plant parts did not reveal a statistically significant variation in *C. glabrata*, suggesting that the species may exhibit reduced sensitivity to the antifungal properties of *H. Zeylanica*.

Table I

Antifungal activities of various parts of *H. Zeylanica* at concentration 1000 µg/disc

Sample	Inhibition zone (mm)		
	C. albicans	***C. krusei***	***C. glabrata***
Roots	8.93 ± 0.32^a	10.33 ± 0.15^a	8.37 ± 0.42^a
Stem	13.37 ± 0.31^b	12.70 ± 0.20^b	8.93 ± 0.25^a
Leaves	14.43 ± 0.32^b	14.58 ± 0.20^c	9.05 ± 0.22^a
Ketoconazole	25.02 ± 0.32^c	28.28 ± 0.28^d	25.78 ± 0.10^b

a-d Mean ± SD in the same row with different superscript letters indicates a statistically significant difference based on Tukey's test (α = 5%)

In this study, the disc diffusion technique was used as an initial evaluation in determining the antifungal activity of the different constituents of *H. zeylanica*. The method provided interesting initial information, yet, it required additional in-depth research to determine the antifungal properties of the various plant extracts [25]. To do this, the minimum inhibitory concentration (MIC) values were determined using serial dilution, an important parameter in antimicrobial studies to define the lowest concentration of an antifungal agent with the ability to inhibit fungal species growth. It was a systematic dilution process that enabled more precise evaluation of each extract's efficacy against *Candida* species and offered deeper insights than the one obtained after using disc diffusion technique. By ascertaining the MIC values, the initial results were corroborated, and a clearer comprehension of the therapeutic potential of the plant extracts was attained [26].

Table II displays the Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC) values for various parts of *H. Zeylanica* against *Candida* species. The root extract had a minimum inhibitory concentration of 250 µg/mL against *C. albicans* which was the same as that of the leaf extract, which was also 250 µg/mL across the entire species that were tested. Root extract had lesser activity against *C. glabrata* as well as *C. krusei* with MIC values of 500 µg/mL indicating lesser potency when compared to the leaf and stem extracts. The stem extract has a greater minimum inhibitory concentration (MIC) with *C. albicans* (500 µg/mL) and a smaller MIC with *C. glabrata* and *C. krusei* (250 µg/mL and 250 µg/mL respectively). The minimum fungicidal concentration (MFC) of *C. albicans* was 250 µg/mL, but the MFC of *C. glabrata* and *C. krusei* was higher implying moderate fungicidal efficacy. The leaf extracts had the strongest antifungal activity with the lowest MIC value of 250 µg/mL across all the tested species. The lowest fungicidal concentration (MFC) of *C. albicans* was 500 µg/mL, as compared to 1000 µg/mL of *C. glabrata* and *C. krusei*, which was the best compared with the stems and roots. The use of ketoconazole as a control revealed significant reduction in MIC and MFC (25 - 50 µg/mL and 50 µg/mL, respectively) which confirmed its improved antifungal activity.

Table II

Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC) of various part of *H. Zeylanica*

Sample	*C. albicans*		*C. glabrata*		*C. krusei*	
	MIC (µg/mL)	**MFC (µg/mL)**	**MIC (µg/mL)**	**MFC (µg/mL)**	**MIC (µg/mL)**	**MFC (µg/mL)**
Roots	250	> 1000	500	> 1000	500	> 1000
Stem	500	250	250	1000	250	500
Leaves	250	500	250	1000	250	500
Ketoconazole	25	50	50	50	50	50

According to the classification system established by Pankey and Sabath, samples are deemed fungicidal if the MFC/MIC ratio is less than 4 and fungistatic if it exceeds 4 [27]. The extracts were categorized according to their efficacy against the *Candida* species. The root extract demonstrated fungistatic properties against all examined *Candida* species, indicating that while it inhibits fungal growth, it does not effectively eradicate the fungi. Furthermore, the stem extract exhibited a dual profile, demonstrating fungicidal activity against *C. albicans* and *C. krusei*, while being fungistatic against *C. glabrata*. This indicates that the stem extract can kill *C. albicans* and *C. krusei* and only prevent the proliferation of *C. glabrata*. The leaf extract was found to have fungicidal and fungistatic activity against *C. albicans* and *C. krusei* and *C. glabrata* respectively. Consequently, the leaves demonstrated significant potential as antifungal agents, particularly against *C. albicans* and *C. krusei*.

The antifungal activity against the *Candida* spp. of the distinct components of the *H. Zeylanica* is different under the results as illustrated in Table I and Table II. The leaves extracts showed the strongest antifungal action in terms of large inhibition zones and evident fungicidal effects on the pathogen, *C. albicans* and *C. krusei*. The stem extracts also showed great potency in antifungal activity as it achieved fungicidal properties against the same species of *Candida*. Furthermore, to our knowledge, the antifungal properties of various parts of *H. zeylanica* against pathogenic fungi, especially *Candida* species, have not been documented previously. This study represents the initial comprehensive examination of the antifungal properties of the roots, stems and leaves of *H. zeylanica*, with a specific focus on *Candida* spp. In addition, it is found that the leaf and stem have high potential as sources of further studies and development into natural antifungal agents. However, the effectiveness of the root extract needs to be improved by future research to fully evaluate the therapeutic benefits of the plant.

These findings are supported by the MIC and MFC data that were received because of the disc diffusion assay. Extracts with larger zone of inhibition like the leaf extract against the *C. albicans* and the extract against the *C. krusei* had lower MIC and MFC values, so confirming their better antifungal performance. The consistency of the disc diffusion results is proved by the correlation between zone diameter and the concentration of the inhibitory material. The leaf extract produced both the largest areas of inhibition and also exhibited fungicidal effects at significantly low levels thus confirming the initial qualitative data with quantitative data. On the other hand, extracts with marginal zones of inhibition like extracts of the roots had high values of MIC and MFC values and were mostly fungistatic, as well as these results were in agreement with their disc diffusion profiles. Taken together, our results are consistent in a variety of antifungal tests and contribute to the general conclusion on the topic of the variable activity of the plant parts of the genus *H. zeylanica* against the species of the genus *Candida*.

Identification of metabolites

Separated metabolites in various tissues of *H. zeylanica* were analysed by UHPLC-Q-Orbitrap-HRMS (high-resolution mass spectrometry), providing discrete metabolites profiles of the respective extracts, as fully detailed in the supplementary information (https://bit.ly/

SuppInfantifungalHzeylanica). The identification process, conducted using Compound Discoverer 2.2, encompassed spectrum selection, retention time alignment and the detection of both known and unknown compounds. Further validation of the identified compounds was accomplished *via* MS2 spectra fragmentation analysis. Each extract contained approximately one hundred validated compounds (supplementary information, Table S1), satisfying the criteria of a mcCloud Best Match score greater than 80.00 and a DeltaMass of less than 10 ppm [28, 29].

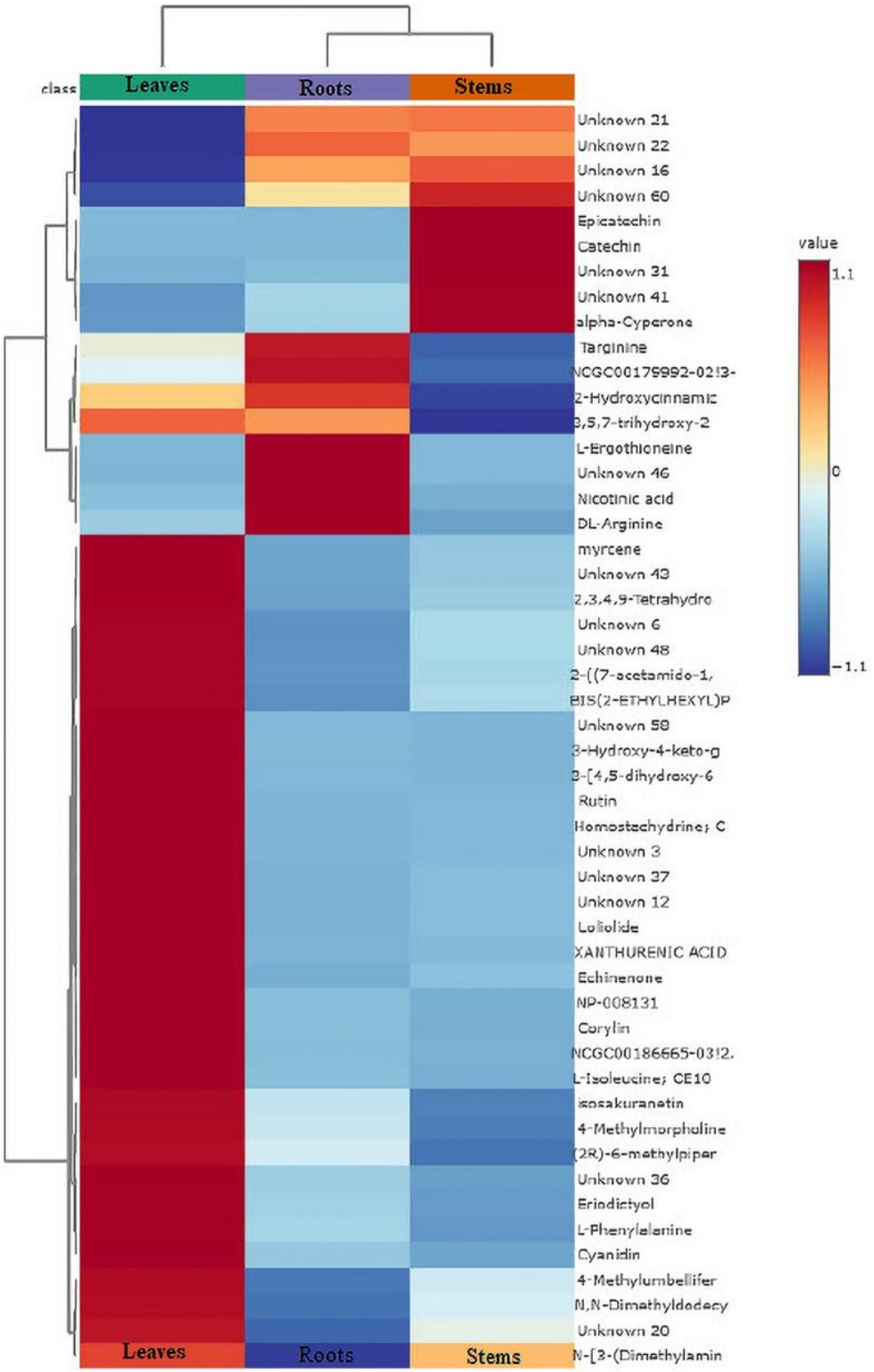


Figure 1.

Heatmap of identified metabolites, with their distribution and relative abundance ranging from high (+2) to low (-2), as indicated by the colour gradient box. The group averages simplify the visualization of the distribution

As shown in Figure 1, the heatmaps generated from the post hoc ANOVA analysis illustrate the distribution and relative abundance of identified metabolites across the roots, stems and leaves of *H. zeylanica*. LC-HRMS analysis indicated that the metabolites identified in the extracts from different parts of the plant predominantly belonged to the groups of phenolics, terpenoids and steroids. The given analysis highlights the chemical heterogeneity of the *H. zeylanica* and demonstrates the successfulness of the techniques applied to the discovery of the common and unique metabolites in different parts of the plant. The hierarchical clustering classifies the metabolites according to their patterns of distribution at the section of the parts of the plants and brings insights into the metabolic activity of the tissues.

Epicatechin and catechin, which are both recognized for their antioxidant activity and structural defence [30], are more abundant in the stem than in the leaves, contrary to initial expectations. Figure 1 has a very strong red coloration that indicates that the stems might have a more active contribution to the mechanisms of structural support and defence of this species. Also, the stems have similar concentration levels of various unknown metabolites, such as Unknown 21, Unknown 22 and Unknown 60, compared to other parts of the plant, indicating that this part of the plant is more metabolically active.

The heatmap examination demonstrates that a particular metabolic pattern in the leaves is to be observed, with a high concentration of different flavonoids, rutin, isosakuranetin, eriodictyol, corylin and spiraeoside. The compounds have been recognized to play significant roles in plant defence particularly in protecting leaves against oxidative stress and other environmental hazards such as UV radiation effects, pathogens and herbivory [30, 31]. Rutin exhibits great antioxidant activity, which contributes to the elimination of reactive oxygen species (ROS) and the protection of the photosynthetic system against oxidative stress, which plays an important role in the protection of leaves [32].

Despite their antioxidant effects, the flavonoids have significant antifungal effects. Tha isosakuranetin and eriodictyol are also found to have antimicrobial activity that enhances the resistance of the plant to fungi infections [33, 34]. Isosakuranetin is a methylated flavonoid that is known to have anti-inflammatory and antifungal effects, which protect against fungal infections [35]. Similarly, such antioxidant and photo-protective compound as eriodictyol also contributes to antifungal defence, which contributes to the increased protection of the plant against the threat of pathogens [36]. The discovery of corylin and spiraeoside improves the metabolic characteristic of the leaves. Corylin is one that has known antioxidants, anti-inflammatory and anti-fungal effects, which are needed in increasing the capability of the plant against fungal pathogens [37]. Spiraeoside is a flavonoid glycoside, known to have antioxidant and cytoprotective effects, which increases the multifunctional role of leaves in over-coming the primary oxidative stress of the metabolism, and antifungal protection [38]. This metabolic profile indicates the leaf tissue's active role in synthesizing protective compounds to ensure plant survival and adaptability in adverse environmental conditions.

The heatmap analysis of the roots reveals a distinctive metabolic profile, characterized by an elevated concentration of many undiscovered metabolites alongside known molecules such as L-ergothioneine, nicotinic acid and other flavonoid-related substances. The roots exhibit heightened levels of Unknown 21, Unknown 22 and Unknown 60. Besides the metabolites detected, the roots of *H. zeylanica* are also reported to harbour other Ugonin derivatives, a family of the prenylated flavonoids, which have been known to exhibit high biological activities [13]. However, these chemicals were not detected in the current analysis. This omission may be attributed to several factors, including constraints in the LC-HRMS database utilized or the low concentration of these chemicals. The sensitivity of the analytical method, limitations in ionization efficiency and variations in sample extraction or fragmentation behaviour may also contribute to this non-detection. It has been noted that the historical significance of Ugonin compounds is important, but the discourse about the topic has been restrained to limit speculative claims without supporting facts. Their presence, as well as to further question their potential pharmacological importance, should be confirmed by additional specific metabolomic studies with the use of real Ugonin standards and optimized analytical procedures.

Taking into consideration the high antifungal effects of the leaf and root extracts, it is necessary to suggest the possible action mechanisms of the main identified metabolites. Flavonoid rutin and eriodictyol are also assumed to act antifungally by various different mechanisms including cell membrane disruption, ergosterol biosynthesis inhibition and oxidative stress generation by means of reactive oxygen species (ROS) generation [39]. Monoterpenoid lactone loliolide has been implicated in the activity as an antibacterial agent by affecting fungal metabolic pathways and respiration. These suggested mechanisms are in line with the findings of other studies and are in tandem with the bioactivity profile of the current research. However, more molecular docking and in vitro mechanistic experiments are required to support exact mechanisms of action of such bioactive compounds.

Multivariate analysis

Recent studies have investigated the application of untargeted metabolomics and multivariate analysis in LC-HRMS for the identification of antifungal compounds in plant extracts. This has been applied to several plant species including *Orchidaceae*, *Artemisia annua* and *Cistus monspeliensis* [40]. Researchers have

been able to discover putative antifungal metabolites that are active against human pathogens and especially, the plant pathogen, *Fusarium oxysporum* [41, 42]. The datasets that have been effective in the identification of bioactive compounds include chemical composition and antifungal activity [42]. Metabolite profiling has progressed more with the use of advanced computational methods that include deconvolution algorithms, retention time prediction models and in silico fragmentation studies [43]. This methodology has been utilized to investigate the antimicrobial properties of food by-products such as cocoa bean shells to examine plant defence mechanisms against pathogens [44].

Principle component analysis (PCA) is a statistical method that condenses intricate data sets into principal components to elucidate correlations among samples. A PCA score plot is shown in Figure 2 in which every point represents an individual sample; clusters of closely placed points give similar metabolite profiles. The clear patterns of clusters that were seen in root, stem and leaf extracts suggest that these botanical constituents have a different chemical composition. These two main components PC1 (75.4%) and PC2 (24.6%), which represent 100% of the total variance, show that the model is quite competent in distinguishing the metabolite composition of the parts of the *H. zeylanica*. The variance explained is more than the standard minimum suggested by Boritnaban *et al*., at 70% of the total variance, demonstrating a high accuracy of the PCA model at explaining the variance in the sample [45].

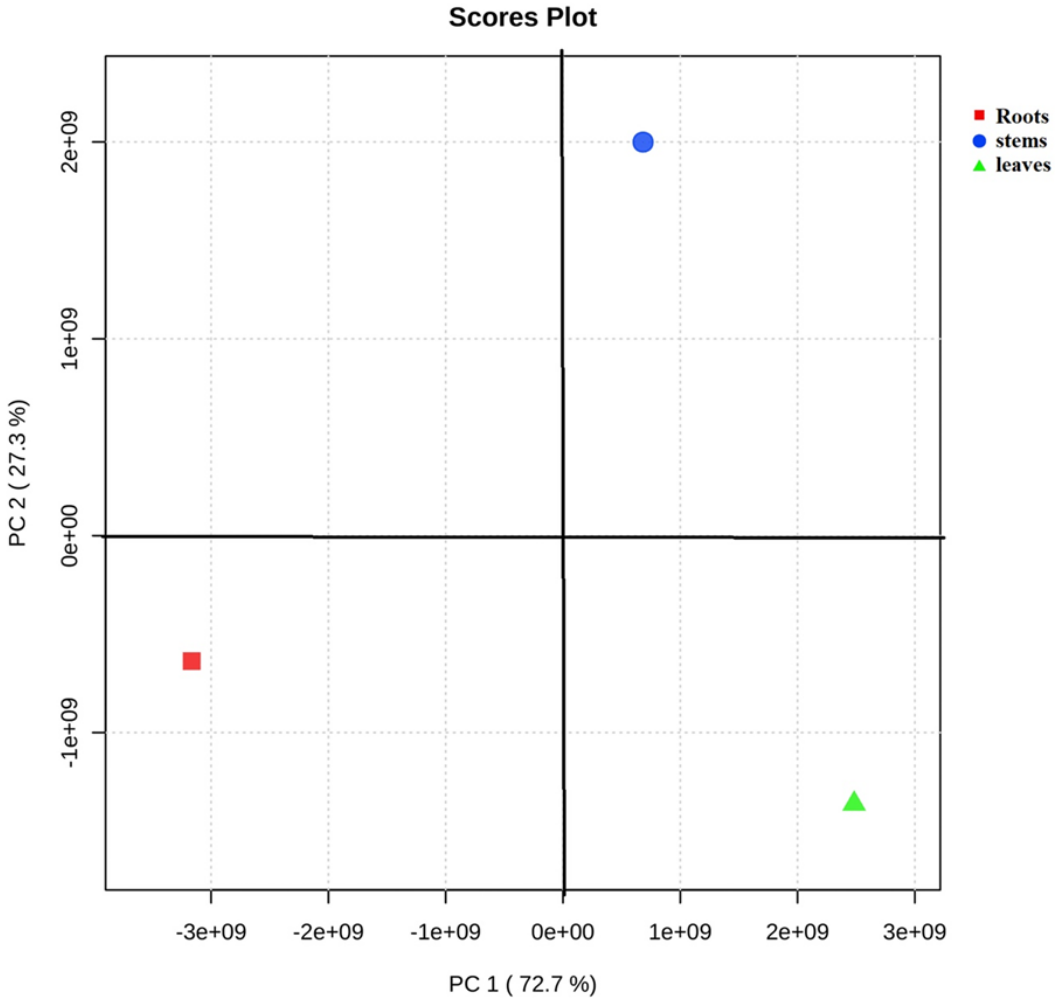


Figure 2.
The PCA score plot derived from LC-HRMS data of methanol extracts from the roots, stems and leaves of *H. zeylanica*

The score plot shows that the root extract lies on the bottom left corner, and it has negative loadings on PC1 and PC2 which indicates a different metabolite composition compared to that of the stems and the leaves. The stem extract is in the upper right quadrant, positively influencing both PC1 and PC2, indicating that the stem possesses distinctive metabolites that differentiate it from other parts. The leaf extract in the lower right quadrant exhibits a positive contribution to PC1 and a marginally negative contribution to PC2.

The leaf extract shows some similarities with the stem extract on PC1, but the general metabolite pattern of the leaf extract is different, which means that they are different in compositions.

The clear stratification of the extracts in the score plot demonstrates the specific metabolite composition of each of the parts of the plant. The largest component of variance (75.4%) in the form of PC1 shows that the main variables affecting the separation are the compounds that are specific to each part of the plant. Might be due to secondary differences that PC2 (24.6%) captures which can only be due to differences in other metabolites which appear in smaller amounts. This clustering pattern highlights the possibility of different groups of *H. zeylanica* to exhibit distinct biological activities and especially antifungal activity.

Moreover, the present study used partial least squares discriminant analysis (PLS-DA). This analysis offers a more comprehensive examination of the relationship between antifungal activity and the presence of metabolites. The study employed this methodology as PLS-DA effectively correlated biological activity with metabolites and generated predictive models. The analysis will be performed under the model that determines the linear relationship between independent variable set X (peak area of the detected metabolites) and the dependent variable set Y (antifungal activity) to predict the metabolites [29].

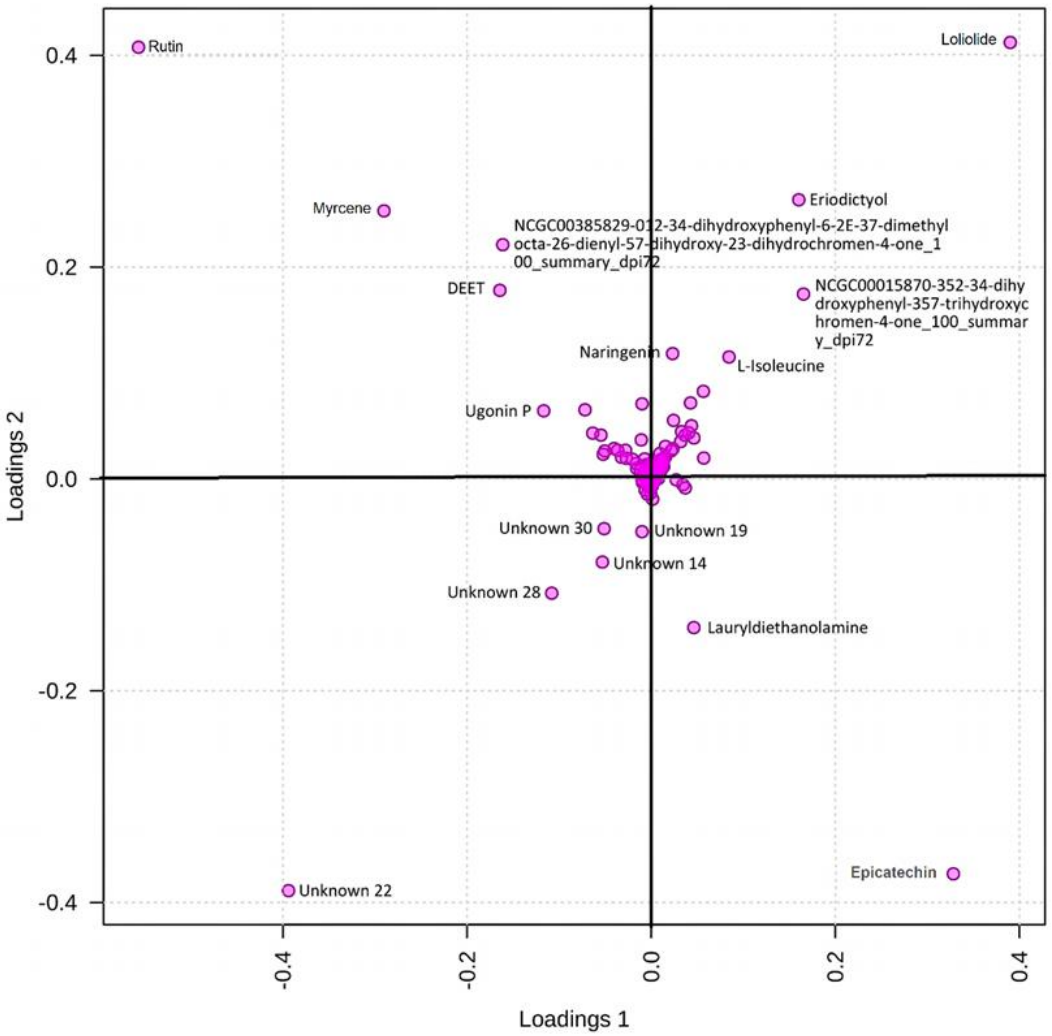


Figure 3.
The relationship between metabolites and their possible contribution to antifungal activity is demonstrated by PLS-DA loading plot

Figure 3 displays the PLS-DA loading plot, which illustrates the association between specific metabolites and antifungal activity. PLS-DA is a supervised statistical technique which determines metabolites that strongly modulate the changes in antifungal activity. Figure 3 displays the effect of a metabolite through the distance between the metabolite and the origin in the loading plot. Loliolide and rutin are far apart the centre, which is a good indication that they are strongly correlated with the antifungal properties. The metabolites, located in different quadrants of the plot, are expected to enhance the observed activity. Conversely, specific

unidentified metabolites, including Unknown 22 and Unknown 28, are located in the negative loading regions, signifying a minimal or adverse effect on antifungal activity. The metabolites such as naringenin, L-isoleucine and some unknown metabolites that were accumulated around the source may have little to play with the antifungal activity or their roles may involve other complex ways and a need to investigate further.

The PLS-DA results may also be placed into context by comparing it with the PCA results of the *H. zeylanica* metabolite profiles. The PCA was useful to separate the extracts of the root, stem and leaves with the two main components (PC1 and PC2) explaining all the variance (Figure 2). These plant components were separated in the PCA score plot, and this corresponds to the separation of the metabolites in the PLS-DA loading plot. The comparison of root and stem extracts in both models shows that each constituent has different metabolites, which enhance antifungal activity. The fact that metabolites like loliolide, rutin, myrcene, were identified by PLS-DA as possessing a large portion of the antifungal effect could relate to the specific clustering of the PCA, particularly in the root and stem extracts. All these analyses lead to a conclusion that a difference in the metabolite composition of various parts of *H. zeylanica* correlates with a difference in biological activity, in terms of antifungal activity.

Figure 4 shows the VIP (variable importance in projection) scores of metabolites that indicate their importance in differences of antifungal activity across the samples that were tested. The study conducted a correlation analysis to determine the association between the identified chemicals and their activities using the variable importance in projection (VIP). Metabolites are ranked based on their VIP scores, with values greater than 1 considered significant for sample differentiation. These metabolites are deemed crucial for distinguishing the biological activity present in the sample group [28]. The VIP plot emphasizes metabolites based on their significance in differentiating samples with diverse antifungal efficacy. The compounds that have a high VIP score (*e.g.*, loliolide, Unknown 22) are the major contributors to the reported antifungal properties. It was shown in this study that the most significant VIP rating was metabolites like Unknown 22, loliolide and epicatechin which showed that the metabolites played major roles in the antifungal activity of the plant extracts. The metabolites mostly were found to be linked to the samples procured on roots and leaves tested against *C. albicans* and *C. glabrata*.

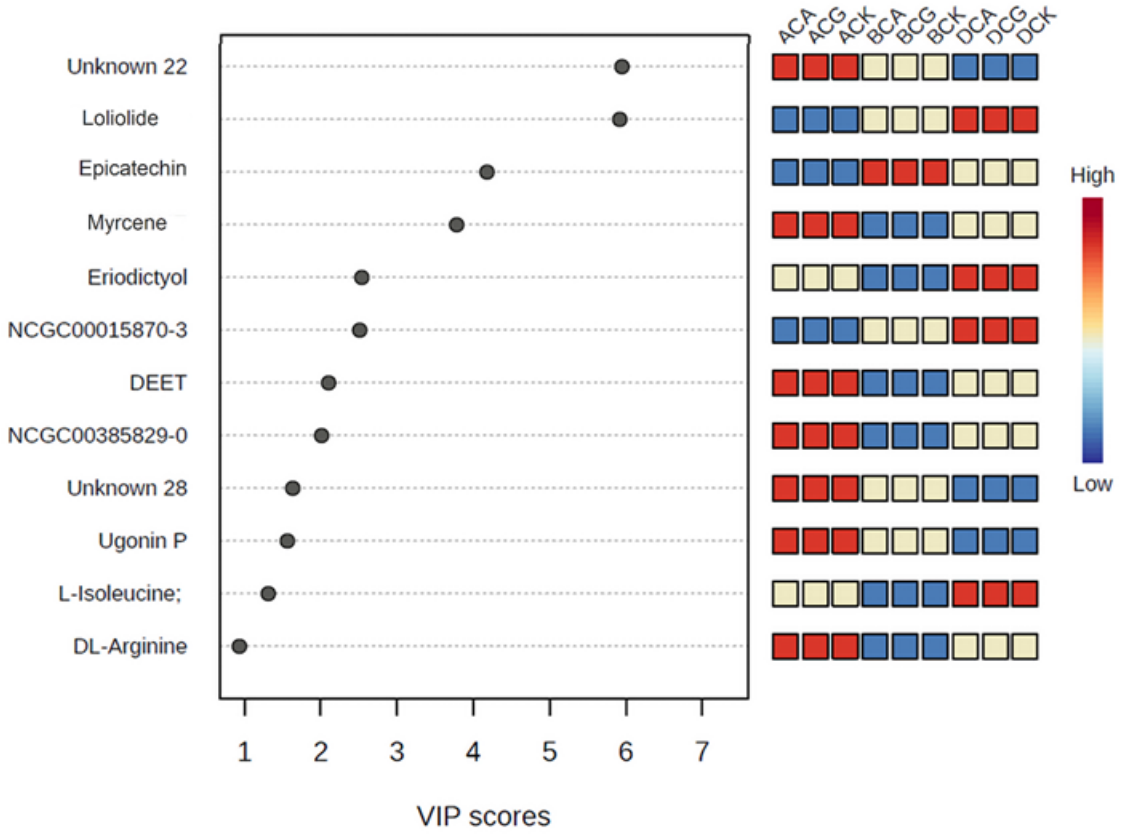
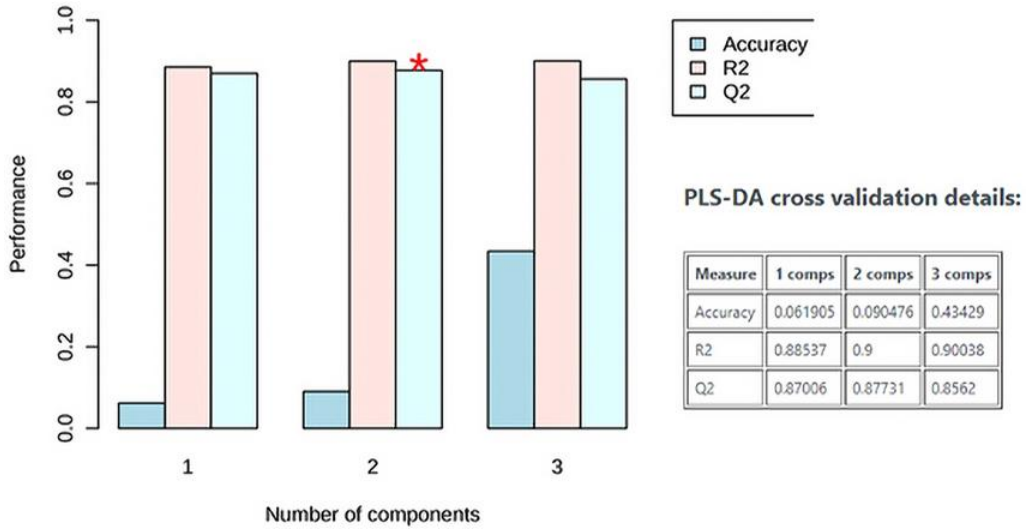


Figure 4.

The VIP (Variable Importance in Projection) of PLS-DA metabolites on antifungal activity. The relative concentration of metabolites is indicated by coloured boxes (red colour indicates high levels and blue colour indicates low levels). A = Root, B = Stem, D = Leaf, CA = *Candida albicans*, CG = *C. glabrata*, CK = *C. krusei*

The heatmap (Figure 1) and the VIP scores represent how these metabolites are found in various parts of the plant. To illustrate, Unknown 22 exhibited greater concentration of roots and leaves compared to *C. albicans* and *C. glabrata* (Figure 4), which suggests that the organism has the potential to act as an anti-fungal to the said two species. Loliolide was also significantly present in the same sample groups and particularly in roots and leaves, which further supports its contribution towards antifungal activity against *C. albicans*. Other metabolites, such as epicatechin, myrcene and eriodictyol, also had important roles, but their more consistent distribution across samples suggests that they have a broader range of importance to antifungal activity in a variety of plant parts and *Candida* spp.

The reliability and predictive efficiency of PLS-DA model were considered using the cross-validation, and the critical performance indices, including Accuracy, R^2 and Q^2 (Figure 5). The results indicate a gradual enhancement in model performance with the incorporation of components. The accuracy, which is an indicator of the models aptitude in the correct classification of samples, rose to 0.0619 at one component to 0.4343 at three components, hence, resolving to better classification performance at the expense of more components, even though the accuracy was relatively low when compared to the R^2 and Q^2 measures. The values of R^2, which indicate the calibration performance, were always high (0.8854, 0.9000 and 0.9004) indicating that the model captures a large percentage of the variation in the data used in calibration. However, the fact that the change was only slightly positive and the difference between components two and three indicates a decreasing calibration accuracy. Similarly, the predictive ability of the Q^2 of the data. The decrease in Q^2 having three components shows there is the possibility of overfitting meaning that two component is the best balance, calibration and strong predictive capability.



Measure	1 comps	2 comps	3 comps
Accuracy	0.061905	0.090476	0.43429
R2	0.88537	0.9	0.90038
Q2	0.87006	0.87731	0.8562

Figure 5.
Cross-validation PLS-DA

Collectively, the PCA and PLS-DA models demonstrate that the bioactive compounds of plant parts are different. The leaf and root extracts, enriched in metabolites like loliolide and rutin, show stronger antifungal activity. These statistical models confirm the link between metabolite profiles and biological efficacy, thus guiding the identification of key antifungal agents from H. zeylanica.

The study demonstrates the effectiveness of untargeted metabolomics and multivariate analysis including the use of PCA and PLS-DA in the detection of anti-fungal compounds in *H. zeylanica*. The metabolite profiling, along with antifungal activity data, underscores the unique composition of the plant's roots, stems and leaves, which demonstrate varying bioactivity, especially against fungal pathogens. Metabolites including loliolide, rutin, epicatechin, myrcene and eriodictyol were recognized as significant contributors to antifungal efficacy, as evidenced by their elevated VIP scores.

The strong performance of the PCA and PLS-DA models highlights the correlation between metabolite composition and biological activity, with a two-component PLS-DA model providing the optimal balance of accuracy and predictive capability. However, even amidst an extensive metabolomic studies, there are a set of unknown metabolites whose role in anti-fungal activity is still not clearly understood and which should be the objects of additional examination. One of the methods, the principal component analysis (PCA) with the partial least squares discriminant analysis (PLS-DA), in combination with each other, shows that different plant sections contain unique bioactive compounds. The leaf and root extracts, loaded with metabolites such as loliolide and rutin, exhibit enhanced

antifungal activity. These statistical models validate the correlation between metabolite profiles and biological efficacy, thereby facilitating the identification of essential antifungal drugs from *H. zeylanica*.

Conclusions

This study offers the first thorough antifungal metabolomic analysis of *H. zeylanica* by untargeted LC-HRMS, highlighting its potential as a valuable source of antifungal drugs. The leaf extract demonstrated the most potent antifungal activity among the assessed plant components, namely against *C. albicans* and *C. krusei*, as validated by disc diffusion, MIC and MFC assays. Significant bioactive metabolites, including rutin, isosakuranetin, eriodictyol and loliolide, were identified and they were also found associated with antifungal activity through the PCA and PLS-DA analysis. These findings underscore the medicinal significance of *H. zeylanica*, particularly in combating antifungal resistance. The identified metabolites have the potential of now being developed into antifungal preparations or being used as drug discovery candidates. The focus of future research ought to be on isolation of active molecules, in vivo efficacy verification and formulation methodology. Further testing of unidentified metabolites, in addition, can show new compounds of pharmacological interest.

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Supplementary materials are available at https://my.unri.ac.id/SuppInfantifungalHzeylanica.

Conflict of interest

The authors declare no conflict of interest.

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