Original Article

Dermatophyte Growth and Enzyme Assays on Indigenously Formulated Low-Cost Plant Extract-Based Media

Allied Medical Research Journal
Vol:3 No.1: 31-41

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ABSTRACT

Background: The high cost of microbiological culture media is a significant economic burden for diagnostic laboratories. This study aims to evaluate the potential of plant-based extracts as low-cost alternatives to expensive conventional media for growing, isolating, and screening dermatophyte enzyme activity.

Methods: Extracts from indigenous medicinal/edible plants were used to prepare media for growing Trichophyton mentagrophytes and Microsporum gypseum. Growth and enzyme activity were assessed and compared with conventional Sabouraud's dextrose agar (SDA).

Results: Dermatophyte species showed reproducible growth on various concentrations of general plant extracts (GPx), with optimal results when sugarcane peel extract (SPx) was added as an additional carbon source. Maximum growth of T. mentagrophytes (85 mm) and M. gypseum (55 mm) occurred on GPx+SPx media within 7 days, compared to 14 days on SDA. Combinations of chicken feather extract (CHFx) or orange lentil extract (OLx) with GPx+SPx also enhanced fungal growth. No lipase, phospholipase, or gelatinase activity was observed on any media. However, gelatin liquefaction occurred when GPx was combined with SPx and OLx as the nitrogen source. Hemolysis was observed with M. gypseum at 37°C, whereas neither fungal species demonstrated enzymatic activity in conventional media.

Conclusion: Plant extract-based media offer a viable alternative to commercially available culture media for dermatophyte growth and screening, providing a cost-effective solution for diagnostic laboratories.

Keywords: Clinical laboratory, Culture media, Dermatophytes, Hemolysis, Microsporum, Trichophyton.

Received: April 19, 2024**; Revised:** September 23, 2024**; Accepted:** December 10, 2024

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DOI: https://doi.org/10.59564/amrj/03.01/008

INTRODUCTION

Dermatophytes are filamentous fungi classified into three types of genera i.e. Trichophyton, Microsporum, and Epidermophyton, that causes dermatophytosis worldwide[1]. Accurate diagnosis of these infections requires a combination of molecular technologies as well as traditional methodologies[2].

Despite advancements, culture-based techniques for isolating and identifying the causative agents from clinical specimens remain the gold standard in diagnostic settings[3-4], with continued reliance on traditional, expensive, dehydrated commercially available culture media for fungal identification. To date, no new media formulations for dermatophyte growth and enzyme assays have been introduced. This study highlights the need for innovative, cost-effective diagnostic tools to improve fungal identification.

Culture media provide a growth substrate that meets the nutritional requirements of fungi[5]. Dermatophytes can grow using various carbon sources, including glucose, fructose, mannose, galactose, trehalose, and cellobiose. Certain oligopeptides and amino acids, in addition to nitrogen sources, may also serve as carbon and energy sources for fungal growth. Macronutrients such as phosphorus, sulfur, potassium, and magnesium, as well as trace elements like zinc and iron, are essential for optimal growth[6]. Dermatophytes can be cultivated on both general-

purpose and selective-differential culture media. An example of general-purpose medium is Sabouraud's dextrose agar (SDA), whereas dermatophyte test medium (DTM) is a selective/differential medium[3,7]. Regardless of medium type, rising global costs are a major concern that hinders its availability and purchase. In Pakistan, the current price of Sabouraud's dextrose medium (500 grams) is approximately $8.46 (23,000 PKR). To prepare the medium, a significant amount (65 grams/liter) is required, making 50 petri plates (90 mm) cost 2,990 PKR ($10.79) for 50 plates, i.e., ~60 PKR ($0.22) per plate, exclusive of electricity, gas, and labor charges. This means that a 500-gram bottle of dehydrated medium can be used 7-8 times to prepare 1,000 mL of the medium[8]. It is important to note that dextrose and peptone are the main ingredients of Sabouraud's dextrose medium[9]. Even if these are combined to formulate the medium, the cost per plate increases. Currently, dextrose and peptone are available for $69.91 (PKR 19,500) and $12.55 (PKR 3,500), respectively, equivalent to the total cost of SDA. The cost of agar (78.87$/PKR 22,000) is not included in the estimate, further adding to the overall cost.

Given this scenario, residual plant materials can be effectively processed into nutritionally rich aqueous extracts that may replace traditional media components. While previous studies have primarily assessed the potential of plant extracts for their antifungal properties, emerging evidence from experiments conducted in our laboratory suggests their ability to support fungal growth in culture media compositions[10]. The exact mechanisms in this context are yet to be explored; however, certain underlying factors may influence the outcome.

The effectiveness of plant extracts depends on the presence of several bioactive components or phytochemicals such as polyphenols, carotenoids, saponins, dietary fibers, phytosterols, flavonoids, glycosides, and specific polysaccharides[11]. These compounds exhibit varied affinities for different solvents[12]. For example, flavonoids, mostly occurring as glycosides[13], are highly water-soluble due to sugar moieties and hydroxyl (OH-) groups[14]. Water, being a universal and eco-friendly solvent, is commonly used in extraction procedures because of its non-selectiveness. Compounds such as phenolics, terpenoids, and carbohydrates can be efficiently extracted using water[12]. An appropriate extraction method is also crucial for the effective release of bioactive compounds from plant extracts[14]. Decoction, a water-based method involving boiling plant materials for a specific duration, facilitates the extraction of alkaloids, tannins, and soluble polysaccharides[12]. In addition to the phytochemical composition of extracts, their concentration also influences their activity.

Studies indicate that the efficacy of various water-based plant extracts against pathogenic fungi, particularly dermatophytes, significantly decreases at lower concentrations[15-18], and extracts with the lowest levels of phytochemicals exhibit less anti-dermatophytic activity[18]. This suggests that lower concentrations diminish the phytochemical content, potentially weakening their inhibitory effect and altering the properties of the plant extracts. Furthermore, some bioactive compounds may serve as nutrient sources, further affecting their inhibitory properties. Aqueous extracts of medicinal plant components are known to contain substantial concentrations of carbonaceous (54%) and nitrogenous compounds[19].

Dermatophytes, by utilizing these sources, produce various metabolites that integrate into energy-generating pathways, including glycolysis, the tricarboxylic acid (TCA) cycle, and amino acid degradation. Additionally, they possess pH-mediated systems for proteases, lipases, adhesins, and other enzymes essential for initiating and establishing infection in the host by triggering the de-repression of genes coding for these proteins. Therefore, in vitro dermatophyte studies focus on understanding their adaptation strategies within specific niches, nutrient availability, and response to environmental stress[2]. Building on these insights, this study aims to develop an indigenously formulated, low-cost plant extract-based medium for cultivating dermatophytes, with a particular focus on evaluating fungal growth and enzyme activity in species such as Trichophyton mentagrophytes and Microsporum gypseum. By addressing both the cost and efficacy of these alternative media,

this research seeks to contribute to the development of sustainable and eco-friendly solutions for dermatophyte cultivation in clinical and research settings.

METHODOLOGY

The experimental study design utilizes plant-based extracts at varying concentrations: 20% weight by volume (w/v), 15%, 10%, 5%, and 2.5% volume by volume (v/v). SDA or other conventional formulations were used as reference media/experimental controls. The 20% (w/v) concentration served as the stock extract, from which the other concentrations were prepared. This concentration was chosen to maximize bioactive compound extraction while maintaining an optimal ratio between plant materials and extractants.

Preparation of Stock Generalized Plant Extracts (GPx 20% w/v)

To prepare the GPx, plant components from sources such as bougainvillea branches, coconut, date palm, eucalyptus, neem bark, coconut fibers, eucalyptus leaves, and neem leaves were randomly collected from the campus of the University of Karachi during the clearing of vegetation between varying seasons. Sugarcane peels were obtained from fresh sugarcane vendors, and orange lentils were purchased from local supermarkets at the existing price of 175 PKR/kg. The materials were cut into 0.5 cm segments, rinsed with water, dried, and weighed at 20 g per 100 ml distilled water. Hot water extraction (decoction) was conducted at 100°C for 15-30 minutes, followed by filtration using Whatman No. 1 filter paper[20-21]. The filtrates were transferred into glass bottles with rubber caps, sterilized by autoclaving for 15 minutes at 121°C, and then either stored at room temperature in capped plastic containers or placed in the freezer for future use[20,21].

Procurement, Growth, and Maintenance of Dermatophyte Cultures

Cultures of Trichophyton mentagrophytes and Microsporum gypseum (clinical isolates) were obtained from the Department of Microbiology, University of Karachi. Following identification through standard methods based on cultural and morphological characteristics[4], the fungal cultures were maintained on SDA, which also served as the reference medium for comparing dermatophyte growth with other extract-based media combinations.

Media Preparation

Sabouraud's Dextrose Agar (SDA)

The medium was prepared according to the manufacturer's instructions (Oxoid). Briefly, glucose, mycological peptone, and agar (40, 10, and 15 grams, respectively) were transferred into an Erlenmeyer flask containing distilled water, and the volume was adjusted to 1 liter. The contents were mixed thoroughly, gently heated while stirring, and brought to a boil. The rest of the procedure, including sterilization, pouring, and storage, was conducted as detailed in the following section.

Extract Media Combinations

Chicken Feather Extract (Chfx 10% w/v)

Chicken feathers, procured from a local poultry farm, were washed thoroughly with mild detergent and water, then shade-dried. After being shredded into small fragments, the feathers were weighed to obtain a concentration of 10 g per 100 ml of distilled water (Table-1). The resulting extract served as the stock, which was then diluted to prepare a 5.0% (v/v) working extract.

Reference and Extract Media Combinations for Enzyme Assays

The media formulation for assessing enzyme and hemolytic activity of dermatophytes used GPx as the base medium, supplemented with sugarcane peel extract, SPx (stock 25% w/v) as the carbon source and orange lentil extract, OLx (stock 20% w/v) as the nitrogen source. SPx and OLx were prepared and stored following the previously described methods. Based on optimizing studies, the working concentrations for GPx, SPx and OLx were determined as 2.5, 5 and 10 % (v/v), respectively. Details of media preparation are mentioned in the table below:

Table-1 Procedure to Prepare Chfx Medium plus Combinations

Media and its combinations	Ingredient (s)	Concentration of stock % (w/v)	Volume (ml) / amount (g)	Final concentration extracts % (v/v)
CHFx	Chicken feather extract	10	500	5
	Distilled water	-----	500	----
	Agar	-----	15	----
GPx + CHFx	Generalized plant extract	20	125	2.5
	Chicken feather extract	10	500	5
	Distilled water	-----	375	----
	Agar	-----	15	----
GPx + CHFx + SPx	Generalized plant extract	20	125	2.5
	Chicken feather extract	10	500	5
	Sugar cane peel extract	35	286	10
	Distilled water	-----	89	----
	Agar	-----	15	----

Table-2 Procedure to Prepare Reference and Test Media

Specification	Media			
	Composition /liter	Reference	GPx + SPx + OLx	Description
		Amount (g) / Volume (ml)		
Lipase activity	Peptone	10	-	The media were autoclaved poured in 90 mm petri dishes and allowed to solidify. A 5mm plug of respective culture was cut with borer and placed in the center of plate. This was followed by the incubation of plates at 37°C plus ambient temperature for a week. Precipitation zone around colonies indicated positive result[22].
	NaCl	5	50	
	$CaCl_2$	0.1	0.1	
	Generalized plant extract (GPx)	-	125	
	Sugarcane peel extract (SPx)	-	200	
	Orange lentil Extract (OLx)	-	500	
	Agar	15	15	
	Tween 80	5	10	
	Distilled water	980	165	
Phospholipase activity	Dextrose	10	-	The media were sterilized by autoclaving and kept for cooling on 50°C. Eggs were disinfected by using 70% alcohol followed by the separation of egg yolks thereby transferring into the sterilized flask having glass beads.
	Peptone	10	-	
	NaCl	57.3	57.3	

	$CaCl_2$	0.1	0.1
	Generalized plant extract (GPx)	-	125
	Sugarcane peel extract (SPx)	-	200
	Orange lentil Extract (OLx)	-	500
	Agar	15	15
	Distilled water	900	175

Flask was vigorously shaken by thorough mixing of the egg yolks, 5% of which was transferred into the molten medium and poured in sterile plates[22]. The plates were inoculated, incubated and results were observed as described above

Hemolytic Activity

Columbia blood agar base was sterilized by autoclaving, cooled to 50°C, and added 5% (v/v) sheep blood. The contents were thoroughly mixed and poured into sterilized plates. Inoculation and incubation were performed as previously described, with hemolysis around the colony indicating a positive result[22]. The test medium hemolytic activity used the same ingredients as those for lipase/ phospholipase activity (GPx, SPx, OLx and NaCl), with 5% human blood replacing sheep blood. Media preparation followed the above-described method. Positive and negative controls included β-hemolytic Streptococcus pyogenes and Enterococcus faecalis, respectively.

Gelatin Plate Assay for Protease Activity

Gelatin plate assay for protease activity followed the method of Nwofor et al.[23] with slight modifications. Peptone, beef extract, and NaCl were replaced with Sabouraud's dextrose medium (Oxoid). The medium was prepared per the manufacturer's instructions by adding 1% gelatin. Plates were inoculated and incubated as previously described. Zones of inhibition were measured using mercuric chloride (0.1% w/v). The same ingredients used in the hemolytic activity assessment were employed for the test medium, substituting human blood with 1% gelatin.

Gelatin Liquefaction Test

The reference medium containing peptone, beef extract and gelatin (5, 3, and 120 g/l) was dispensed into test tubes, autoclaved, and cooled upright. Organisms were inoculated by stabbing, and the results were compared with the controls. Staphylococcus aureus was used as a positive control, and un-inoculated tubes were used as a negative controls. After incubation at 25°C for one week, the tubes were refrigerated at 4°C for 10 minutes and examined for gelatin liquefaction[24]. The same method was applied to prepare the test medium using the ingredients listed to assess hemolysis and gelatinase activity.

Determination of pH, Protein, and Sugar Content of Stock Extracts

The pH of the stock extracts was measured using a pH meter (CCMD.510 WPA, pH conductivity meter, UK). Protein content was assessed using the method described by Pokhrel et al.[25]. A standard curve was prepared with bovine serum albumin concentrations of 2,000, 1,000, 500, 250, and 125 µg/ml. Protein values were determined at 590 nm, and a standard curve was applied to calculate the protein content of the test (Table-5). Reducing sugar content was assessed using a dinitrosalicylic acid (DNS) reagent[26]. Tubes containing 1 ml of the sample were mixed with 3 ml of DNS, then heated for 5 minutes in a boiling water bath and cooled under tap water. Blanks of various extract media served as controls. Absorbance was recorded at 530 nm, and a standard curve with glucose concentrations of 10, 8, 6, 4, and 2 mg/ml was used to calculate the reducing sugar content.

RESULTS

Dermatophytes' Growth on SDA and Combinations of Extract Media Plant-extract media used in our study supported the growth of dermatophytes, Microsporum gypseum and Trichophyton mentagrophytes. The growth pattern and its extent were comparable to that of the reference medium, SDA. GPx + SPx demonstrated growth within 7 days of incubation, compared to the 14-day incubation period required on SDA. Results also showed that fungal growth was not well supported by CHFx and CHFx + GPx (Table-3, Figure 1B, D, I, and K).

However, the amendment of SPx and CHFx + GPx resulted in reproducible fungal growth (Table-3, Figures 1E and L).

Enzyme Assays

The enzyme producing ability of dermatophyte species was tested by supplementing plant extract media with specific substrates.

Enzyme assays for lipases, phospholipases, gelatinase and hemolysin production were performed at 37°C and ambient temperatures.

Lipase and Phospholipase Production

The lipase and phospholipase activity of T. mentagrophytes and M. gypseum was not observed in reference or extract media combinations (Table-4).

Table-3 Comparison of Cultural Characteristics and Growth Patterns of on Different Media

Media	Microsporum Gypseum		Grading of Growth	Incubation Period (Days)	Trichophyton Mentagrophytes		Grading of growth	Incubation period (days)
	Cultural characteristics	Reverse			Cultural characteristics	Reverse		
SDA	White, cottony thick growth	Straw	++	14	White, cotton like thick growth	Straw	++	14
GPx	Cotton like white fluffy growth	Brown	++	14	Colourless margins with central cotton like growth	Brown	++	14
GPx + SPx	White fluffy dense cotton like growth	Straw	++++	7	White fluffy cotton like dense growth	Straw	++++	7
CHFx	Straw with occasional white cottony growth	Straw	++	14	White cotton like margins with central white and occasional brown growth	Straw with brown spots	+++	14
GPx + CHFx	Very light brown with occasional white growth	Brown	++	14	--do--	Brown	++	14
GPx + CHFx + SPx	White fluffy cotton like growth	Brown	+++	14	White thin cotton like growth	Brown	+++	14

Hemolytic and Gelatinase Assay

T. mentagrophytes and M. gpseum did not produce any hemolysis on reference media (Table 4). However, M. gypseum exhibited hemolysis on extract media combinations when incubated at 37°C (Figure-1S). In contrast, no hemolysis was observed for T. mentagrophytes in extract media combinations at varying incubation temperatures (Table-4). Protease (gelatinase) enzyme activity was not produced by dermatophyte species on any reference or extract media combinations during plate assays. Gelatin liquefaction was not observed on conventional medium. However, liquefication was observed at 37°C with GPx in combination with SPx and OLx, indicating gelatinase activity of T. mentagrophytes and M. gypseum (Table-4, Figure 1P, and Q). The culture of S. aureus served as a positive control during the enzyme assay (Figure-1O).

pH, Protein and Sugar Content of Stock Extracts

The pH of stock extracts was within the acidic limit: 5.11-6.50. The protein content in GPx was found to be greater (492±0.75µg/ml) followed by CHFx (317±two µg/ml), whereas minimum protein content was present in OLx (50±0.03 µg/ml) and negligible in SPx. However, it had the highest concentration of reducing sugar, 6.23±0.14 mg/ml, followed by GPx and OLx 1.525 ± 0.2, 0.53±0.02 mg/ml, respectively (Table-5).

Table-4 Determination of pH, Protein and Sugar Content of Stock Extracts

Parameter /activity	Dermatophytes							
	Trichophyton Mentagrophytes				Microsporum Gypseum			
Media	Reference		GPx+SPx+OLx		Reference		GPx+SPx+OLx	
Temperature	Ambient	37°C	Ambient	37°C	Ambient	37°C	Ambient	37°C
Lipase assay	-	-	-	-	-	-	-	-
Phospholipase assay	-	-	-	-	-	-	-	-
Hemolysis (β)	-	-	-	-	-	-	-	+
Gelatinase assay	-	-	-	-	-	-	-	-
Gelatin liquefaction (tube method)	-	+	-	-	-	-	-	+

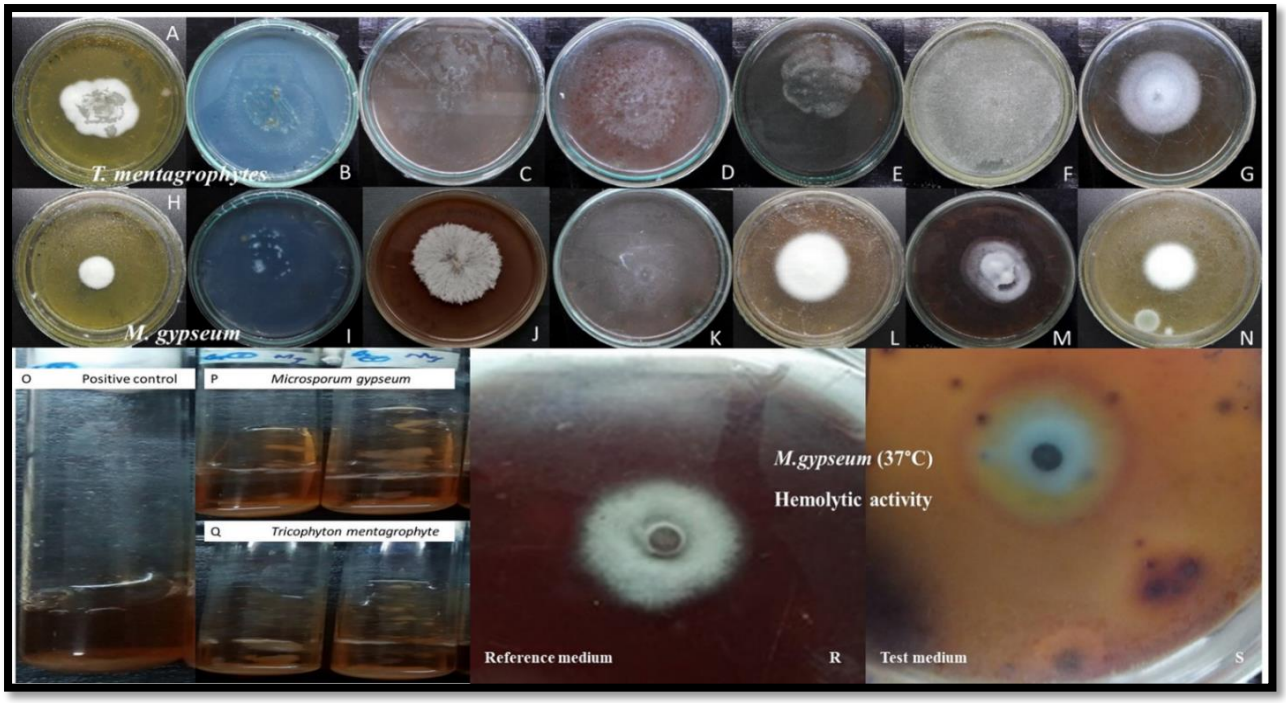

Figure 1-A, B, C, D, E, F, G Growth of Trichophyton mentagrophytes on SDA, CHFx, GPx, GPx+CHFx, GPx+CHFx+SPx, GPx+SPx, GPx+SPx+Olx
H, I, J, K, L, M, N Growth of Microsporum gypseum on SDA, CHFx, GPx, GPx+CHFx, GPx+CHFx+SPx, GPx+SPx, GPx+SPx+Olx
O, P, Q Gelatin liquefaction test; positive control (S.aureus), liquified gelatin by M. gypseum and T. mentagrophytes
R, S Haemolytic activity; M. gypseum at 37°C (negative on reference and positive on test medium)

Table-5 Determination of Ph, Protein and Sugar Content of Stock Extracts

S.No	Name of the extract	Concentration	pH	Protein (µg/ml)	Sugar concentration (mg/ml)
1.	GPx	20 % (w/v)	5.11±0.01	492.0±0.75	1.525±0.2
2.	CHFx	10 % (v/v)	6.50±0.01	317.0±2	Negligible
3.	SPx	25% (v/v)	5.59±0.03	Negligible	6.23±0.14
4.	OLx	20 % (w/v)	6.23±0.03	50.0±0.03	0.53±0.02

DISCUSSION

The results of this study demonstrate the potential of plant-extract media as viable, low-cost alternatives to conventional media for dermatophyte growth and enzyme assays. The formulation of media using plant-based extracts like GPx, SPx, and OLx successfully supported the growth of Microsporum gypseum and Trichophyton mentagrophytes, showing comparable fungal growth patterns to the traditional SDA. These findings suggest that plant-derived media, despite having fewer added nutrients, can provide sufficient resources to promote fungal growth. This aligns with previous studies, which have shown that plant extracts, such as those from neem, eucalyptus, and other botanicals, contain bioactive compounds, including polysaccharides, amino acids, and polyphenols, that support fungal development[12].

The growth of dermatophytes in GPx was particularly notable because it provided essential bioactive compounds, such as carbohydrates, polysaccharides, and proteins, which allowed for robust fungal growth. The combination of GPx with SPx provided additional carbon sources, particularly sucrose from sugarcane peels, which facilitated better growth of the dermatophyte species in the media. These findings support the hypothesis that plant-extract media could balance the C: N ratio necessary for fungal development, despite having lower concentrations of sugars compared to conventional media like SDA[9]. It is also important to note that, while SPx had a significantly lower concentration of reducing sugars (2.49±0.27 mg/ml), dermatophytes still adapted and utilized the available nutrients efficiently, further indicating their metabolic flexibility in nutrient-limited environments. The inclusion of CHFx in media formulations aimed to introduce keratin as a nitrogen source. However, as noted, the lack of fungal growth in CHFx-based media may be attributed to the poor solubility of keratin and the difficulty in extracting its bioactive components. This observation highlights the limitations of the decoction method for keratin extraction, which may not be effective in breaking down the complex structure of keratin. Previous studies[28-29] have indicated that specialized extraction techniques such as oxidation, reduction, or hydrolysis are needed to release the smaller peptides and amino acids from keratin, which could otherwise serve as a nitrogen source for dermatophytes. Future work could optimize extraction methods to improve the efficacy of CHFx in fungal growth.

Interestingly, combining CHFx with GPx + SPx demonstrated improved growth, which might be explained by the presence of sucrose in the sugarcane extract. The sucrose likely hydrolyzed into glucose and fructose during the extraction process, providing an additional carbon source. This combination likely facilitated better growth than CHFx alone, showing that nutrient availability, especially carbon sources, plays a crucial role in fungal development. The presence of glucose, though a non-reducing disaccharide, could trigger metabolic pathways in dermatophytes, leading to increased enzyme production, as seen in other studies[30]. Our results also revealed that the pH of the media was slightly acidic (pH 5.5-6.9), which is within the optimal range for dermatophyte growth. Dermatophytes thrive in slightly acidic environments, which also enhances the activity of enzymes such as phosphatases, lipases, and proteases, critical for tissue penetration and host infection[2]. The pH of our plant-extract media likely contributed to supporting enzymatic activity, as evidenced by the gelatinase activity observed with dermatophyte growth on 1% gelatin media. The acidity of the extracts may have triggered specific metabolic pathways in the fungi, helping them adapt to nutrient limitations and promote enzyme production. Regarding the enzyme assays, the lipase activity was not detected in the dermatophytes grown in either plant-extract or reference media, aligning with some studies that report variable lipase activity in dermatophytes[36]. This lack of lipase production may suggest that these particular species of dermatophytes do not rely on lipases during initial infection stages, or that the environmental conditions in our study were not conducive to lipase production. Similarly, despite phospholipase activity being important in the early stages of infection[1], no significant phospholipase production was observed in our study, which could be due to factors such as substrate specificity or the nature of the medium. These findings suggest that enzyme production in dermatophytes is influenced by a variety of

factors, including nutrient availability, species-specific metabolic pathways, and the presence of specific substrates. The gelatinase (protease) assay, however, provided promising results, with dermatophytes being able to liquefy gelatin in the media at 37°C. This aligns with previous research suggesting that gelatinase production is correlated with dermatophyte growth, particularly under optimal nutrient conditions[24]. It is likely that the 1% gelatin in the media was sufficient to induce protease activity, supporting the idea that dermatophytes produce proteases for host tissue degradation. However, further investigations are needed to explore how other factors, such as carbon source availability, affect protease production in dermatophytes. Hemolytic activity was observed in M. gypseum but not in T. mentagrophytes. Hemolysin production by dermatophytes is considered a survival strategy, as it helps release iron from red blood cells, which is critical for fungal growth[1]. In our study, M. gypseum showed hemolytic activity under nutrient stress, particularly under low iron conditions. This result suggests that M. gypseum may rely on hemolysin production as part of its virulence strategy during infections, a finding that warrants further exploration to understand the pathogen's role in disease progression.

CONCLUSION

Plant-based extracts offer a viable, low-cost alternative to traditional media for the cultivation and study of dermatophytes. The GPx + SPx + OLx formulation provided a suitable environment for fungal growth and enzyme production, demonstrating the potential of plant-extract media as sustainable substitutes in dermatophyte isolation and diagnostics. While there are limitations related to extract variability and the need for more refined extraction methods, this study lays the foundation for future research to optimize plant-extract media for industrial-scale applications and fungal pathogen studies.

Acknowledgments
None.

Author Contributions
Sadia Saleem contributed to the conceptualization, methodology, data collection, analysis, and manuscript writing. **Warda Hakeem** was responsible for the literature review, data interpretation, manuscript editing, and final approval of the manuscript.

Ethical Approval
This study received approval from the Institutional Ethical Review Committee (ASRB/File No. 01027/Sc.) of University of Karachi.

Conflict of Interest
None.

Grant Support and Funding Disclosure
None.

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